



POLYTECHNIC UNIVERSITY OF MADRID
SCHOOL OF AGRICULTURAL, FOOD AND
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***PhD program in Project Planning for Rural Development &
Sustainable Management***

**RHIZOCULTURE MODEL TO MITIGATE ENVIRONMENTAL
IMPACTS OF GREENHOUSE GASES (GHG) AND DIFFUSE
CONTAMINATION: FOCUS ON CARBON, PHOSPHORUS AND
NITROGEN IN SOILS OF FORESTS AND AGRO-ECOSYSTEMS**

LUIS GONZAGA GARCÍA MONTERO

Directors: M^a Inmaculada Valverde Asenjo & Antonio Damián García Abril

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INDEX

a. ABSTRACT/RESUMEN	a-1
a.1. Abstract	a-1
a.2. Resumen	a-2
a.3. Abbreviations and acronyms used in the thesis	a-3
 b GENERAL INTRODUCTION	 b-1
b.1. Relevant aspects of foreknowledge	b-1
b.1.1. Importance of the soil and the rhizosphere	b-1
b.1.2. Eight main threats to soil on a global scale	b-2
b.1.3. Erosion and loss of organic matter in agricultural and forest soils	b-3
b.1.4. Biodiversity loss in agricultural and forest soils	b-4
b.1.5. Fertility and organic matter content in agricultural and forest soils	b-7
b.1.6. Storing carbon in agricultural and forest soils	b-8
b.1.7. Difficulties in developing international strategies for agricultural and forest soils protection	b-10
b.1.8. Foundations for understanding the role of biology in soil quality	b-11
 b.2. Research interest	 b-15
b.2.1. Need and opportunity	b-15
b.2.2. New approaches on sustainable agricultural and forest soils management based on the ecological intensification of the rhizosphere	b-16
 b.3. Objectives and hypothesis	 b-19
 c. METHODOLOGY	 c-1
c.1. Study areas of natural habitats of truffles	c-1
c.2. Study areas of cultures of truffles	c-1
c.3. Study of the productivity and truffle brûlés characteristics	c-2
c.4. Soil analysis	c-3
c.5. Study of <i>Tuber</i> spp. mycorrhizas	c-3
c.6. Taxonomy studies	c-5
c.7. Soil microarthropod extraction	c-5
c.8. Statistical analysis	c-5
c.9. Bibliographic review on rhizoculture topic, and on evolutionary and ecological dynamics of ectomycorrhizas and lichens	c-5

d. COLLECTION OF PUBLICATIONS AND CONTRIBUTIONS	d-1
d.1. <u>L.G. García-Montero</u> , J.L. Manjón, C. Pascual, <u>A. García-Abril</u> (2007). <i>Ecological patterns of Tuber melanosporum and different Quercus Mediterranean forests: quantitative production of truffles, burn sizes and soil studies</i> . FOREST ECOLOGY and MANAGEMENT 242: 288-296. JCR: Q1 in Forestry Area (6 th from 39 journals); Impact Factor in 2007 = 1.579	288-296
d.2. <u>L.G. García-Montero</u> , P. Díaz, S. Martín-Fernández, M.A. Casermeiro (2008b). <i>Soil factors that favour the production of Tuber melanosporum carpophores over other truffle species: a multivariate statistical approach</i> . ACTA AGRICULTURAE SCANDINAVICA SECTION B-SOIL and PLANT SCIENCE 58: 322-329. JCR: Q4 in Agronomy Area (41 th from 49 journals); Impact Factor 2008 = 0.41	322-329
d.3. <u>L.G. García-Montero</u> , G. Di Massimo, J.L. Manjón and <u>A. García-Abril</u> (2008a). <i>New data on ectomycorrhizae and soils of the Chinese truffles Tuber pseudoexcavatum and Tuber indicum, and their impact on truffle cultivation</i> . MYCORRHIZA 19: 7-14. JCR: Q2 in Mycology Area (7 th from 19 journals); Impact Factor SCI 2008 = 2.205	7-14
d.4. <u>L. G. García-Montero</u> , <u>I. Valverde-Asenjo</u> , P. Díaz, C. Pascual (2009b). <i>Statistical patterns of carbonates and total organic carbon on soils of Tuber rufum and T. melanosporum (black truffle) brûlés</i> . AUSTRALIAN JOURNAL OF SOIL RESEARCH 47: 206-212. JCR: Q3 in Soil Science Area (21 th from 31 journals); Impact Factor SCI 2009 = 1.007	206-212
d.5. <u>I. Valverde-Asenjo</u> , <u>L.G. García-Montero</u> , A. Quintana, J. Velázquez (2009). <i>Calcareous amendments to soils to eradicate Tuber brumale from T. melanosporum cultivations: a multivariate statistical approach</i> . MYCORRHIZA 19: 159–165. JCR: Q2 in Mycology Area (5 th from 19 journals); Impact Factor SCI 2009 = 2.650	159-165
d.6. <u>L.G. García-Montero</u> , A. Quintana, <u>I. Valverde-Asenjo</u> , P.Díaz. (2009a). <i>Calcareous amendments in truffle culture: a soil nutrition hypothesis</i> . SOIL BIOLOGY and BIOCHEMISTRY 41: 1227–1232. JCR: Q1 in Soil Science Area (1 st from 31 journals); Impact Factor SCI 2009 = 2.978	1227-1232
d.7. <u>L.G. García-Montero</u> , <u>I. Valverde-Asenjo</u> , P. Díaz, C. Pascual, C. Menta (2010). <i>New data on impact of earthworms activity on black truffle soils</i> . In: D. Donnini, B. Falini, B. Mattia, G. di Massimo (Eds.). <i>Atti dell 3º Congresso Internazionale di Spoleto sul Tartufo</i> . Comunità Montana dei Monti Martani, Spoleto. Pg.: 723 (COMMUNICATION IN CONGRESS)	723
d.8. <u>L.G. García-Montero</u> (2012-2015). SEMINAR CYCLE in the Dpt. Crop & Soil Science of the Oregon State University, The North American Truffling Society and the Cascade Mycological Society in Oregon (EEUU) (2012); in the University of Parma (2013); in the University of Montpellier SupAgro (2013); and in the Institute of Ecology (Soil Sci. and Protection) of the Technische Universität Berlin (2015), titled: “ <i>Research on the ecology of black truffles in Central Spain: could ectomycorrhizae be liming host plant roots?</i> ” or “ <i>Model on soil-nutrient management through fungus-plant interaction</i> .”	19, 23, 21
d.9. <u>L.G. García-Montero</u> , <u>I. Valverde-Asenjo</u> , D. Moreno, P. Díaz, I. Hernando, C. Menta, K. Tarasconi (2012). <i>Influence of edaphic factors on edible ectomycorrhizal mushrooms: new hypotheses on soil nutrition and C sinks associated to ectomycorrhizae and soil fauna using the Tuber brûlé model</i> . In: A. Zambonelli, G. Bonito (Eds.). <i>Edible Ectomycorrhizal Mushrooms</i> . Springer-Verlag, Berlin. Pp. 83-104 (ABSTRACT of a BOOK CHAPTER; see the complete chapter is in the Annexes g.1)	83-104

- d.10.** L.G. García-Montero, I. Valverde-Asenjo, M.A. Grande-Ortíz, C. Menta, I. Hernando (2013). *Impact of earthworm casts on soil pH and calcium carbonate in black truffle burns*. AGROFORESTRY SYSTEMS 87: 815-826. **JCR: Q2** in Forestry Area (32th from 79 journals); Impact Factor 2013 = 1.240 815-826
- d.11.** F. Arredondo, L.G. García-Montero, I. Valverde-Asenjo, C. Menta (2014). *Soil quality indicators for forest management*. In: E. Martínez-Falero, S. Martín-Fernández, A. García-Abril (Eds.). *Quantitative Techniques in Participatory Forest Management*. CRC press, Boca Raton. pp 179-240 (ABSTRACT of a BOOK CHAPTER; see the complete chapter is in the Annexes g.2) 179-240
- d.12.** C. Menta, L.G. García-Montero, S. Pinto, F.D. Conti, G. Baronía, M. Maresi (2014). *Does the natural "microcosm" created by Tuber aestivum affect soil microarthropods? A new hypothesis based on Collembola in truffle culture*. APPLIED SOIL ECOLOGY 84: 31-37. **JCR: Q1** in Soil Science Area (8th from 34 journals); Impact Factor 2014 = 2.644 31-37
- d.13.** L.G. García-Montero, F. García-Robredo, S. Ortuño, E. Ayuga, L. Pinto, P. Pita, C. Pascual, A. Álvarez, L. Benito-Matías, M.P. Andrés (2015a). *Basis on rhizoculture: management of "underground agroforestry systems" to improve their climate change resilience*. In: E. Tielkes (ed.) *Book of Abstracts of Tropentag 2015*. Cuvillier, Gottingen. Pg. 330 (COMMUNICATION IN CONGRESS) 330
- d.14.** Luis G. García-Montero, V. Monleon, D. Myrold, T. Kuyper, J. Trappe, J. Baham, I. Valverde-Asenjo, X. Parladé, A. Álvarez, L.F. Benito, J.R. Quintana, A. Quintana, P. Andrés, M. Morcillo, C. Menta, S. Pinto, P. Díaz, C. Lefevre, D. Luoma, C. Turkmen, J.A. Domínguez, A. Santiago, L. Pinto, P. Pita, F. García-Robredo, S. Ortuño, J. Pera, E. Verrecchia (2015b). *Could mycorrhizae perform "ecosystem engineering" on the host plants? Basis on rhizoculture of agroforestry systems to improve their resilience*. In: P. Grenni and A. Bevivino (Eds.). *Soil Biological Communities and Aboveground Resilience. Proceedings of the 3rd Annual Meeting*. Cost Action FP1305 "Biolink-Linking belowground biodiversity and ecosystem function in European forests", Rome. Pg. 26 (COMMUNICATION IN CONGRESS) 26
- d.15.** L.G. García-Montero (2017) Coordinator of RHIZOFARMING: a Horizon 2020 proposal to research in rhizoculture (ABSTRACT; see the "first stage of the project" complete in the Section e.4. *Future research lines*) H2020-1
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- d.17.** L.G. García-Montero (2018) Coordinator of RHIZOINTENSE: a Horizon 2020-PRIMA proposal to research in rhizoculture (ABSTRACT; see Section e.4. *Future research lines*) H2020-2

e. GENERAL DISCUSSION	
e.1. Analysis of the results and bibliographic review on ectomycorrhizal fungi and <i>Tuber brûlé</i> model	e-1
e.1.1. Introduction	e-1
e.1.2. Ecological comparison between the lichenic model and mycorrhizal symbioses	e-2
e.1.3. Limitations of the current state of knowledge of ectomycorrhizal fungal symbiosis	e-4
e.1.4. Ectomycorrhizal fungi in disturbed sites: a new hypothesis on nutrition dynamics for the symbiosis between plants and <i>Tuber</i> (truffles)	e-5
e.1.5. A new global-scale calcium-induced-chlorosis hypothesis for ectomycorrhiza-host plant symbioses based on the ability of ectomycorrhizas to mobilize calcium	e-6
e.1.6. Additional aspects of the calcium[pH]-induced-chlorosis hypothesis: modifying soil pH in the rhizosphere	e-7
e.1.7. Evolutionary dynamics and stability of mycorrhizal associations	e-10
e.1.8. Ecological dynamics and stability of mycorrhizal associations	e-12
e.1.9. A new conceptual ectomycorrhizal fungal framework for stable mycorrhizal associations: controlled parasitism?	e-14
e.1.10. The oxalate-calcium carbonate pathway in the interactions between fungi and oxalotrophic bacteria	e-15
e.1.11. Interactions between soil fauna and mycorrhiza-host plant systems associated to the “paradox of calcium”	e-16
e.1.12. Liming effects on arbuscular mycorrhizal fungi in agriculture	e-18
e.1.13. Liming effects on nitrogen-fixing bacteria in agriculture	e-21
e.2. Integrated description of the solution: “rhizoculture model to mitigate environmental impacts of GHG and diffuse contamination, focusing on carbon, phosphorus and nitrogen in soils of forests and agro-ecosystems”	e-23
e.2.1. Food production and forest resources and needs at the global level.....	e-23
e.2.2. Soil biology and resilience of ecosystems to climate change	e-23
e.2.3. Rhizoculture approach for the intensification of roots and rhizosphere activity to reduce fertilization and improve resilience in agriculture and forestry	e-25
e.2.4. Strategic keys to implement rhizoculture models to optimize its impact on the mitigation of greenhouse gas emissions, pollution associated to fertilizers use and food security	e-27
e.2.5. Basis to integrating the rhizoculture and the soil organic matter management by livestock, biomass and bioenergy uses	e-30
e.2.6. Basis to integrating the rhizoculture with alternative fertilizers based on waste recycling and aquaculture	e-35
e.2.7. Expected impacts of rhizoculture on agro-ecosystems and forests	e-36
e.3. Conclusions	e-39

e.4. Future research lines	e-41
e.4.1. RHIZOFARMING (2017): a Horizon 2020 proposal to research in rhizoculture (first stage of the project, complete)	e-43
e.4.2. RHIZOINTENSE (2018): a Horizon 2020-PRIMA proposal to research in rhizoculture (abstract of the first stage of the project)	e-53
f. BIBLIOGRAPHY	f-1
g. ANNEXES	g-1
g.1. <u>L.G. García-Montero, I. Valverde-Asenjo, D. Moreno, P. Díaz, I. Hernando, C. Menta, K. Tarasconi (2012). <i>Influence of edaphic factors on edible ectomycorrhizal mushrooms: new hypotheses on soil nutrition and C sinks associated to ectomycorrhizae and soil fauna using the Tuber brûlé model</i>. In: A. Zambonelli, G. Bonito (Eds.). <i>Edible Ectomycorrhizal Mushrooms</i>. Springer-Verlag, Berlin. Pp. 83-104 (BOOK CHAPTER COMPLETE)</u>	83-104
g.2. F. Arredondo, <u>L.G. García-Montero, I. Valverde-Asenjo, C. Menta (2014). <i>Soil quality indicators for forest management</i>. In: E. Martínez-Falero, S. Martín-Fernández, A. García-Abril (Eds.). <i>Quantitative Techniques in Participatory Forest Management</i>. CRC press, Boca Raton. pp 179-240 (BOOK CHAPTER COMPLETE)</u>	179-240
g.3. <u>L.G. García-Montero, P. Manzano, D. Alwanney, I. Valverde-Asenjo, A. Álvarez-Lafuente, L.F. Benito-Matías, X. Parladé, S. Ortuño, M. Morcillo, A. Gascó, C. Calderón-Guerrero, F. Mauro, M. Méndez, A. Sánchez-Medina, M.P. Andrés, J.R. Quintana, C. Menta, S. Pinto, L. Pinto, P. Pita, C. Turkmen, C. Pascual, E. Ayuga, F. Torrent, J.C. Robredo, P. Martín-Ortega, J. Pera, L. Gómez, G. Almendros, C. Colinas, E.P. Verrecchia (2017). <i>Towards integrated understanding of the rhizosphere phenomenon as ecological driver: can Rhizoculture improve agricultural and forestry systems?</i> In: M. Lukac, P. Grenni and M. Gamboni (Eds.). <i>Soil biological communities and ecosystem resilience</i>. Springer International Publishing, Cham, Switzerland. Pp 43-75 (BOOK CHAPTER COMPLETE)</u>	43-75

a. ABSTRACT/RESUMEN

a.1. Abstract. Agriculture and forestry traditionally focus on improving plant growth traits based on a plant-centric point of view. This paradigm has led to global problems associated to soil overexploitation such as soil losses, reductions of the C stock in soils, and the generalized use of fertilizers (increasing production costs and diffuse contamination). This view may also have limited our understanding of mutualistic symbioses of plants assuming that the main role of non-photosynthetic symbionts is to mobilize the nutrients, and being plants the dominant agents of the symbiotic relationship. In response to these issues, this thesis offers an alternative approach taking advantage of the “rhizo-centric” point of view, where non-photosynthetic partners are the main protagonists in play; and secondly, it builds a multidisciplinary body of knowledge that could be called “rhizoculture”, which includes techniques focussing on the ecological intensification of the roots and rhizosphere in agro-ecosystems and forests. After review the evolutionary and ecological dynamics of ectomycorrhizas and lichens, this rhizo-centric approach can be illustrated using the lichenic model, because some authors proposes that the lichenic fungi rather 'farm' the photobionts under 'controlled parasitism'. In a similar way, this thesis proposes to explore a ground-breaking hypothesis suggesting that some ectomycorrhizal fungi, such as truffles, could modify the soil environment to 'farm their host plants' under a controlled parasitic relationship. After monitored truffle cultures and their natural habitats, this thesis confirms that the truffle brûlés -productive spots of *Tuber melanosporum* and *T. aestivum*- show an ecological intensification of the rhizosphere of the host plants. This ecological engineering process would be related with (i) *the “paradox of calcium” (i.e., Ca^{2+} and its salts are simultaneously nutrients and stressors for the plants) that would explain a dominance of mycorrhizal fungi over plants based on inducing a Ca[pH]-mediated chlorosis to the host plants; and (ii) decreased soil contents of the total carbonate (through ECM fungal rock-eating activity) and total organic carbon (presumably aimed to modify the ratios of symbiotic/saprophytic soil communities). Soil fauna, such as earthworms and microarthropods, also participate in the ecological engineering processes inside the brûlé. Comparable patterns could be performed by other ectomycorrhizal fungi, such as *Tuber brumale*, *T. oregonense*, *T. pseudoexcatum*, *T. indicum* and mat-fungi species of *Piloderma*, *Hysterangium* and *Ramaria*. The brûlé model would provide the conceptual bases to introducing the rhizoculture approach, aimed to the ecological intensification of roots and rhizosphere in agriculture and forestry, that would allow to manage N-P-C-water loops in the soil, through: *the handling of Ca^{2+} salts (by liming), mycorrhizal rock-eating activity, soil organic matter (to increase the ratio symbiotic/saprophytes microorganisms through livestock and biomass control), N-P-water availability and recycling (by smart use of aquaculture and organic waste), C sequestration and soil physical properties (through liming, and soil microorganisms and fauna)*. The rhizoculture could significantly decrease the high cost and associated diffuse pollution of the application of fertilizers; as well as it could increase soil C stocks, improve the resilience of agricultural and forest systems to environmental disturbances, such as climate change, and enhance food production and security. The results of this thesis have been published or disseminated through 17 contributions: 8 articles (2007-2014) in journals indexed in the JCR list (3 in Q1, 3 in Q2, 1 in Q3 and 1 in Q4, quartiles); **3 chapters** of international books (2012; 2014; 2017); **3 communications** in international congresses (2010; 2015a; 2015b); **1 seminars cycle (2012-2015)** presented in OSU and 2 institutions in Oregon, University of Parma, University of Montpellier SupAgro, and University TU-Berlin; and **2 proposals for research H2020 projects (2017; 2018)** have been led by the UPM, which have been presented in separate European calls for Horizon 2020.*

a.2. Resumen. La agricultura y silvicultura tradicionalmente se han centrado en mejorar las características del crecimiento de las plantas, desde un punto de vista 'planta-céntrico'. Este paradigma ha llevado a problemas globales asociados con la sobreexplotación del suelo, tales como la erosión, la reducción del almacenamiento de C en los suelos, y el uso generalizado de fertilizantes (que han aumentado los costes de producción y la contaminación difusa). Esta visión también ha limitado nuestra comprensión de las simbiosis mutualistas de las plantas, al suponer que la función principal de los simbiontes no fotosintéticos es movilizar los nutrientes, siendo las plantas los agentes dominantes de la relación simbiótica. En respuesta a estos problemas, esta tesis ofrece un enfoque alternativo que aprovecha el punto de vista 'rizo-céntrico', donde los socios no fotosintéticos serían los principales protagonistas en la simbiosis, y en segundo lugar, propone un conjunto de conocimientos multidisciplinarios que podría denominarse 'rizocultura', que incluye técnicas centradas en la intensificación ecológica de las raíces y la rizosfera en los agroecosistemas y bosques. Después de revisar las dinámicas evolutivas y ecológicas de las ectomicorrizas (ECM) y los líquenes, este enfoque rizo-céntrico puede ilustrarse utilizando el modelo liquénico, porque algunos autores proponen que los hongos de los líquenes 'cultivan sus algas simbióticas (fotobiontes) bajo un parasitismo controlado'. De manera similar, esta tesis propone explorar una hipótesis pionera que sugiere que algunos hongos ECM, como las trufas, podrían modificar el ambiente del suelo para 'cultivar a sus plantas hospedadoras' bajo una relación de parasitismo controlado. Después de monitorizar cultivos de trufas y sus hábitats naturales, esta tesis ha confirmado que en los 'quemados o brúls' de las trufas (puntos productivos de *Tuber melanosporum* y *T. aestivum*), se observa una intensificación ecológica de la rizosfera de las plantas hospedadoras. Este proceso de ingeniería ecológica estaría relacionado con (i) la 'paradoja del calcio' (es decir, el Ca^{2+} y sus sales son simultáneamente nutrientes y factores estresantes para las plantas), que explicaría un predominio de los hongos micorrízicos sobre las plantas hospedadoras a través de un control mediado por una 'clorosis- $\text{Ca}[\text{pH}]$ -inducida'; y (ii) la disminución en el suelo del contenido de carbonato total (a través de la actividad de 'rock-eating' de los hongos ECM), y del carbono orgánico total (probablemente asociado a un cambio en la relación simbiosis/saprófitos de las comunidades de suelo). La fauna del suelo, como las lombrices de tierra y los microartrópodos, también participarían en estos procesos de ingeniería ecológica dentro del brúlé. Se han observado patrones comparables en otros hongos ECM, como *Tuber brumale*, *T. oregonense*, *T. pseudoexcavatum*, *T. indicum* y especies de hongos-mat de *Piloderma*, *Hysterangium* y *Ramaria*. El modelo del brúlé proporcionaría las bases conceptuales para introducir el enfoque de la rizocultura, dirigido a la intensificación ecológica de las raíces y la rizosfera en la agricultura y silvicultura, lo que permitiría gestionar los ciclos del N-P-C-agua en el suelo, a través de: el manejo de la sales de Ca^{2+} (mediante enmiendas calcáreas), la actividad 'rock-eating' de las micorrizas, la gestión de la materia orgánica del suelo (para aumentar la proporción de microorganismos simbióticos/saprófitos a través del control del ganado y la biomasa), la disponibilidad de N-P-agua en el suelo y su reciclaje (mediante el uso inteligente de la acuicultura y los residuos orgánicos), el secuestro de carbono y la gestión de las propiedades físicas del suelo (a través de las enmiendas calcáreas, los microorganismos y la fauna del suelo). La rizocultura podría disminuir significativamente el elevado coste y la contaminación difusa, que están asociados a la aplicación actual de fertilizantes, y además podría incrementar el secuestro del C en el suelo, mejorar la resiliencia de los sistemas agrícolas y forestales a las perturbaciones ambientales, como el cambio climático, y aumentar la producción y seguridad de los alimentos. Los resultados de esta tesis han sido publicados o diseminados a través de 17 contribuciones: 8 artículos (2007-2014) en revistas indexadas en la lista del JCR (cuartiles: 3 en Q1, 3 en Q2, 1 en Q3 y 1 en Q4); 3 capítulos de libros internacionales (2012; 2014; 2017); 3 comunicaciones en congresos internacionales (2010; 2015a; 2015b); 1 ciclo de seminarios (2012-2015) presentado en la Universidad OSU y 2 instituciones de Oregón, en la Universidad de Parma, la Universidad de Montpellier SupAgro y la Universidad TU-Berlin; y 2 propuestas de proyectos H2020 de investigación (2017; 2018), lideradas por la UPM, que han sido presentadas en sendas convocatorias europeas Horizonte 2020.

a.3. Abbreviations and acronyms used in the thesis

AAS = Atomic absorption spectroscopy	JCR list = Journal citation report list
AFSs = Agroforestry systems	LCA = Life Cycle Assessment
AM = Arbuscular mycorrhizas	MBR = Membrane bioreactors
Brûlés = Productive spots of truffles	MN = Mycorrhizal Network
CA = Conventional agriculture	MSP = Membrane separation processes
CAP = Common Agricultural Policy	NF = Nanofiltration
CAP = Common Agricultural Policy	NfB = N-fixing bacteria
CBD = The Convention on Biological Diversity	NM = Non mycorrhizal plants
CC = Climate change	N-P-C = Nitrogen, phosphorous, carbon
CEC = Soil cationic exchangeable capacity	NPK = Nitrogen, phosphorous and potassium
CGIAR = Research centers consortium of the World Bank	OA = Organic agriculture
DO = Direct one	OSU = Oregon State University
EAP = Environmental Action Program	pCO ₂ = partial pressure of carbon dioxide
EC = European Commission	PES = Payments for ecosystem services
ECM = Ecto mycorrhizas	R+D+i = Research and development and innovation
EI = Ecological intensification	RO = Reverse osmosis
EU = European Union	SIC = Soil inorganic carbon
EU-CC = European Union-Climate Change	SMEs = Small and medium-sized enterprises
FAO = Food and Agriculture Organization of the United Nations	SOC = Soil organic carbon
GHG = Greenhouse gas	SOM = Soil organic matter
IGME = Geology and mining institute of Spain	TOC = Total organic carbon
IMTA = Integrated Multi-Trophic Aquaculture	TU-Berlin = Technische Universität Berlin
IPCC = Intergovernmental Panel on Climate Change	UNCTAD = United Nations Conference on Trade and Development
ISRIC = International Soil Reference and Information Center	UPM = Polytechnic University of Madrid
ISSS = International Society of Soil Science	V% = Soil exchangeable cation saturation percentage
	WFD -EU = Water Framework Directive

b. GENERAL INTRODUCTION

b.1. Relevant aspects of foreknowledge

b.1.1. Importance of the soil and the rhizosphere

Soil is one of the most fundamental components for supporting life on Earth. Jeffery *et al.* (2010) and Menta (2012) point out that the processes occurring within soil (most of which are driven by its living organisms) perform ecosystem and global functions that help maintain life above ground. Moreover, soil delivers numerous services, ranging from providing the food we eat, to filtering and cleaning the water we drink. It is used as a platform for building, it provides vital products such as antibiotics, and it contains an archive of our cultural heritage in the form of archeological sites. However, life within the soil is hidden and often suffers from being 'out of sight and out of mind' (Blum, 1993; Ebel, 2007).

Soils may be characterized in terms of the properties they inherit from the underlying rock (the parent material) and the properties resulting from alteration of the original parent material by soil-forming or 'pedogenic' processes, namely climate, vegetation, time and human activity. Pedogenic processes operate mainly in the surface and subsurface horizons normally found in the upper 2 m (Blum, 1993; Ebel, 2007).

From the point of view of both forests and agro-ecosystems, another essential aspect of the functioning of the soil, vegetation and ecosystem is the importance of the rhizosphere (Akeem, 2012). The term rhizosphere is used in a broader sense to refer to the portion of soil surrounding roots in which the soil organisms are influenced by their presence (Killham *et al.*, 1994). The rhizosphere can be distinguished from the greater part of the soil on the basis of its chemical, physical and biological characteristics. As the roots penetrate into the ground, they act on clay minerals and the particles of soil surrounding them, leading to the formation of an area in which the water pathway and the movement of nutrients and microflora is more heavily channeled than in the rest of the soil. For the same reasons, the organic matter released by the roots accumulates nearby (Arredondo *et al.*, 2014).

The chemical nature of the rhizosphere is significantly different from the rest of the soil; this is largely the result of the fact that the roots release carbon and selectively capture ions in solution in the groundwater. The plants act as carbon pumps, fixing what is available in the atmosphere in the root exudates, which in turn are quickly captured by soil organisms; for this reason the level of carbon available around the roots is never very high. Instead, the selective absorption of ions causes some to be depleted in the rhizosphere, and the accumulation of others which are not absorbed by the roots (Arredondo *et al.*, 2014).

The relationship between the roots and microflora can be very close, and leads to (i) bacteria or fungi becoming an integral part of the roots as in mycorrhizal symbiosis; and (ii) the association of bacteria and legumes. The peculiar characteristics of the rhizosphere are also reflected in the selectivity of the animal element. The interaction between soil animals and the plant roots can take a variety of forms that either produce benefits or repress plant growth, and often involve interactions with the microbial populations in the soil (Akeem, 2012).

However, despite its importance, soils are affected by human activity which often results in their degradation and the loss of their functions. Growing pressure from an ever increasing global population, as well as threats such as climate change and soil erosion, are placing increasing stresses on the ability of soil to sustain its important role in the planet's survival. Evidence suggests that while the increased use of monocultures, intensive agriculture and forestry has led to a decline in soil quality and biodiversity in many areas, the precise consequences of this loss are not always clear. Jeffery *et al.* (2010) and Menta (2012) indicate that too rarely do we pause to reflect on the fact that soil is the foundation upon which society is sustained and evolves, and that it is a vital component of ecological processes and cycles, as well as the basis on which our infrastructure rests. These authors insist that more importance should be given to the fact that soil quality and its protection contributes significantly to preserving the quality of life, and that the nutrition and health of humans and animals cannot be separated from the quality of the soil.

Only by understanding soil in all its complexity, while maintaining its functionality and quality through actions designed to protect its properties, and acknowledging its importance in the quality of life worldwide, can we embark on a truly sustainable use of soil perceived as a resource, and build a proper human/soil relationship to be passed on to future generations.

b.1.2. Eight main threats to soil on a global scale

The soil is under an increasing number of threats. These threats are complex and although unevenly spread, their dimension is global and they are frequently interlinked. When numerous threats occur simultaneously, their combined effects tend to compound the problem. Therefore, soil disturbances linked to natural forces and human activities alter the physical, chemical and biological properties of soil, which in turn can impact its long-term productivity (Gupta and Malik, 1996).

Human activities are increasingly polluting soils and groundwater through applied agrochemicals, deposition of atmospheric pollutants and the spreading of sewage sludge and manures, and these can have adverse impacts on the ability of soil to perform its vital functions (Blum, 1993; Ebel, 2007). Consequently, numerous soils around the world have lost their fertility or their capacity to perform their functions due to processes that are accelerated or triggered directly by human activities and which often act in synergy with each other, amplifying the negative effect (Arredondo *et al.*, 2014).

Among these processes, the most widespread at the worldwide level are erosion, organic matter loss, compaction, salinization, flooding and landslide phenomena, contamination, and the reduction in biodiversity (Menta, 2012). In this regard, the communication of the European Commission to the Council and the European Parliament, entitled "*Towards a Thematic Strategy for Soil Protection*" defined the following eight main soil threats (European Commission, 2005):

- (i) Soil sealing occurs mainly through the development of technical, social and economic infrastructures, especially in urban areas;

- (ii) Erosion is mainly due to the inadequate use of soil by agriculture and forestry, but also through building development and uncontrolled water runoff from roads and other sealed surfaces;
- (iii) Loss of organic matter is mainly due to intensive use of the land by agriculture, especially when organic residues are not sufficiently produced or recycled back into the soil. Agronomists consider soil with less than 1.7% organic matter to be in the pre-desertification stage;
- (iv) The decline in biodiversity is linked to the loss of organic matter, as biodiversity depends on organic matter, meaning that all soil biota live on the basis of organic matter;
- (v) Contamination can be either diffuse (widespread) or localized, and may be caused by many human activities such as industrial production, traffic, etc., and predominantly through the use of fossil material such as ores, oil, coal, salts and others, or through agricultural activities;
- (vi) Compaction of soil is a rather new phenomenon caused mainly by high pressures exerted on soil through heavily loaded vehicles used in agriculture and forestry. An estimated 4% of soil throughout Europe suffers from compaction;
- (vii) Hydro-geological risks are complex phenomena that result in floods and landslides deriving partly from uncontrolled soil and land uses (e.g. sealing, compaction and other adverse impacts), as well as from unregulated mining activities;
- (viii) Salinization is mainly a regional problem, but in the areas where it occurs -such as the Mediterranean basin and in Hungary-, it severely endangers agriculture, forestry and the sustainable use of water resources.

Of these eight main threats to soil, and insofar as forests and agro-ecosystems management is concerned, the impact implied by the loss of organic matter, erosion and loss of biodiversity in the soils of agricultural and forest systems should be highlighted on a global scale.

b.1.3. Erosion and loss of organic matter in agricultural and forest soils

Erosion is regarded as one of the most widespread forms of soil degradation, and as such, poses potentially severe limitations on sustainable land use. Topsoil is the most productive soil layer, and the most dominant effect of erosion is the loss of topsoil, which may not be conspicuous but is nevertheless potentially very damaging. Erosion literature commonly identifies 'tolerable' rates of soil erosion, but these usually exceed the rates that can be balanced by the natural weathering of parent materials to form new soil particles. Soil loss in some places may be considered acceptable from an economic standpoint but some modern agricultural and forest production methods are causing overall erosion rates that are becoming increasingly unacceptable from a long-term point of view (Arredondo *et al.*, 2014).

Soil erosion is a physical phenomenon involving the removal of soil and rock particles by water, wind, ice and gravity. Therefore climate, topography and soil characteristics are

important physical factors affecting the amount of erosion. The most effective strategy for reducing erosion is to increase the cover of vegetation or litter, and preferably both. Plants - especially woody plants with strong, deep roots- greatly increase soil strength by providing a stabilizing effect on slopes, in addition to protecting the soil with their canopy and adding litter to the soil surface. In some cases the plants also transpire significant quantities of water from the slope, thus reducing the weight that contributes to mass movements (European Commission, 2005; Mansourian, 2005).

Loss of organic matter is another of the most widespread forms of soil degradation. Following the unprecedented expansion and intensification of agricultural and forest production during the 20th century, there is clear evidence of a consequent decline in the soil organic carbon (SOC) contents of many soils. This decline in SOC contents has important implications for agricultural and forest production systems, as well as for ecosystems, as SOC is a major component of soil organic matter (SOM) (Ebel, 2007).

A wide range of agricultural and forest management practices influence the abundance of organic matter, biomass and diversity of soil biota and litter. These management practices include variations in tillage, treatment of pasture and crop residues, crop rotation, applications of pesticides, fertilizers, manure, sewage and ameliorants such as clay and lime, drainage and irrigation, and vehicle traffic (Baker, 1998).

b.1.4. Biodiversity loss in agricultural and forest soils

Humans have caused a widespread reduction in biodiversity on a global scale. The Convention on Biological Diversity (CBD) was the first global agreement aimed at the conservation and sustainable use of biological diversity (Secretariat of the Convention on Biological Diversity, 2000). The CBD lies at the heart of biodiversity conservation initiatives. It offers opportunities to address global issues at a national level through locally grown solutions and measures. One important requirement is the development of national biodiversity strategies and action plans channeled into relevant sectors and programs, as a primary means of implementing the Convention at the national level. The recent Conference of the Parties of the CBD (May 2008, Bonn) demonstrated that there is unanimous acknowledgement of the need for action to protect biodiversity. Biodiversity conservation is essential both for ethical reasons, and especially for the ecosystem services provided for current and future generations by the complex of living organisms. These ecosystem services are essential for the functioning of our planet (Menta, 2012).

Changes in biodiversity alter ecosystem processes and affect their resilience to environmental change. Human activities are estimated to have increased the rates of extinction 100-1,000 times (Lawton and May, 1995). In the absence of major changes in policy and human behavior, our effect on the environment will continue to alter biodiversity. Land use is considered to be the main element of global change for the near future, and land use change is projected to have the greatest impact on biodiversity by the year 2100. In a review on changing biodiversity, Chapin *et al.*(2000) consider that land use will be the main cause of change in biodiversity for tropical, Mediterranean and grassland ecosystems.

Forests, tropical or temperate, generally represent the biomes with the largest soil biodiversity. Consequently any land use change resulting in the removal of perennial tree vegetation will produce a reduction in soil biodiversity. In some cases pasture or perennial grasslands succeed forests, while in others, arable land replaces formerly wooded areas. The change in soil biodiversity will therefore be influenced by the subsequent use of the land after the forest. However, reduction of soil biodiversity as a result of urbanization can be even more severe. The urbanization process leads to the conversion of indigenous habitat to various forms of anthropogenic land use, the fragmentation and isolation of areas of indigenous habitat, and an increase in local human population density. The urbanization process has been identified, for example, as one of the leading causes of declines in soil arthropod diversity and abundance in some areas (Menta, 2012). These reductions in soil biodiversity result in artificial ecosystems that require constant human intervention and extra running costs, whereas natural ecosystems are regulated by communities of plant, animal and soil organisms through flows of energy and nutrients, a form of control which is being progressively lost with agricultural intensification.

Furthermore, differences in agricultural and forest production systems, such as integrated, organic or conventional systems, have been demonstrated to affect soil fauna in terms of numbers and composition (Hansen *et al.*, 2001; Cortet *et al.*, 2002). The impact of soil tillage operations on organic matter and soil organisms is highly variable, depending on the tillage system adopted and on the soil characteristics. Conventional tillage by plowing inverts and breaks up the soil, destroys soil structure and buries crop residues, thereby causing the highest impact on soil fauna; the intensity of these impacts is generally correlated to soil tillage depth (Menta, 2012). Minimum tillage systems may be characterized by a reduced tillage area (i.e. strip tillage) and/or reduced depth (i.e. rotary tiller, harrow, hoe); crop residues are generally incorporated into the soil instead of being buried. The negative impact of these conservation practices on soil fauna is reduced in comparison with conventional tillage. Under no-tillage crop production, the soil remains relatively undisturbed and plant litter decomposes at the soil surface, much like in natural soil ecosystems (Arredondo *et al.*, 2014).

Taking as an example the communities of soil micro-arthropods, observations on the impacts of different forms of agricultural and forest management on these communities showed that the high input of intensively managed systems tends to promote low diversity, while lower input systems conserve diversity (Menta, 2012). The influence on soil organism populations is expected to be most evident when conservation practices such as no-till are implemented on previously conventionally tilled areas, as the relocation of crop residues to the surface in no-till systems will affect the soil decomposer communities (Beare *et al.*, 1992). No-till (Hendrix, 1986) and minimum tillage generally lead to an increase in micro-arthropod numbers (Menta, 2012). Conventional tillage caused a reduction in micro-arthropod numbers as a result of exposure to desiccation, destruction of habitat and disruption of access to food sources (House and Del Rosario, 1989). The influence of these impacts on the abundance of soil organisms will either be moderated or intensified depending on their spatial location; that is, in-row where plants are growing, near the row where residues accumulate or between rows where they are subjected to possible compaction from mechanized traffic (Fox *et al.*, 1999).

Moreover, the observed impact of different forms of agricultural and forest management on bacterial communities showed similar patterns to micro-arthropod communities (Bardgett and Cook, 1998; Siepel and Bund, 1988). In fact, it is also evident that high-input systems favor bacterial pathways of decomposition, dominated by labile substrates and opportunistic bacterial-feeding fauna. In contrast, low input systems favor fungal pathways with a more heterogeneous habitat, leading to domination by more persistent fungal feeding fauna (Bardgett and Cook, 1998)

The effects of fertilizers on soil invertebrates and microbial communities are a consequence of both their impact on the vegetation and directly on the organisms themselves. Increases in the quantity and quality of the food supplied by vegetation are frequently reflected in greater fecundity, faster development and increased production and turnover of invertebrate herbivores (Curry, 1994). The effects of organic and inorganic fertilizers in terms of nutrient enrichment may be comparable, but these two types of fertilizers differ in that organic forms provide additional food material for the decomposer community. Ryan (1999) concluded that the total soil microbial biomass and the biomass of many specific groups of soil organisms will reflect the level of SOM inputs.

Hence organic or traditional farming practices that include regular inputs of organic matter in their rotation determine larger soil communities than conventional farming practices (Ryan, 1999). Generally the responses of soil fauna to organic manure will depend on the characteristics of the manure and the rates and frequency of application. Herbivore dung, a rich source of energy and nutrients, is exploited initially by a few species of coprophagous dung flies and beetles, and subsequently by an increasingly complex community comprising many general litter-dwelling species (Curry, 1994).

Pfotzer and Schuler (1997) also reported that the soil microbial and faunal feeding activity responded to the application of compost with higher activity rates than with mineral fertilization. Studies related to compost from sewage sludge application on agricultural and forest soils showed an increase in the abundance of *Collembola* (Lüben, 1989), *Carabidae* (Larsen, 1986), *Oligochaeta* (Cuendet and Ducommun, 1990), soil nematodes (Bruce *et al.*, 1999) and *Arachnidae* (Bruce *et al.*, 1999). In some cases, the application of sewage sludge to agricultural and forest systems can input toxic substances that accumulate in the soil and reach potentially toxic levels for soil fauna (Bruce *et al.*, 1999). The knowledge gained in relation to the effects of sewage sludge showed that species that are more sensitive to the toxic substances contained in sewage sludge may disappear, while other more tolerant species may dramatically increase (Menta, 2012).

Organic wastes could also become an easily available and cheap source of organic matter after composting processes. The use of compost obtained from organic waste in agricultural and forest activity enables waste materials to be converted into a useful resource. This has led national authorities in recent years to promote both the use of compost to reduce soil fertility loss and the research aimed at assessing its effects on both agricultural and forest production and soil environment (Pinamonti *et al.*, 1997; Bazzoffi *et al.* 1998; Allievi *et al.*, 1993). However, the possible negative effects on soil fauna deriving from the use of organic waste include the accumulation of trace metals in the soil (Tranvik *et al.*, 1993).

Pesticide application to the soil can affect the soil fauna by influencing the performance of individuals and modifying ecological interactions between species. When pesticides impact one or more ecosystem components, they also affect micro-arthropod communities in terms of number and composition. Pesticide toxicity on soil fauna is determined by various factors, such as the pesticide's chemical and physical characteristics, the species' sensitivity and the soil type. In fact among soil micro-arthropods, different taxa showed a variety of responses (Arredondo *et al.*, 2014).

The physical and chemical characteristics of the soil, such as its texture, structure, pH, organic matter content and quality and the nature of the clay minerals, are important factors determining the toxic effects of pesticides and other xenobiotics. A study carried out by Joy and Chakravorty (1991) showed reduced toxic effects, as a function of soil type, in the following order: sand > sandy-loam > clay > organic soil. Often, the toxicity of pesticides can be directly related to SOM content (Van Gestel and Van Straelen, 1994). However pesticide application does not always cause negative impacts on the entire soil micro-arthropod community. For example, for certain types of soil there is evidence that some taxa can obtain a competitive advantage from the application of certain specific pesticides (Menta, 2012).

Finally, in biodiversity recovery processes using agroforestry restoration, it must be considered that after stimulating the natural regeneration processes that establish forest species, it is necessary to manage and direct succession processes toward the desired objectives. It is important to promote continued development of the vegetation to conserve soil, nutrients, and organic resources; to restore fully functional hydrological processes, nutrient cycling and energy flow processes; and to create self-repairing landscapes that provide the goods and services necessary for biophysical, biochemical and socioeconomic sustainability (Arredondo *et al.*, 2014).

Different stages of agroforestry soil degradation call for management actions that focus on different processes. Severely degraded sites require early repair of hydrological, nutrient-cycling and energy capture and transfer processes. As the agroforestry vegetation increases in biomass and stature, it reduces the abiotic limitations of the site by improving soil and micro-environmental conditions. Directing natural processes toward land-use goals requires an understanding of the processes driving succession. The rate and direction of succession is influenced by the availability of species, the availability of suitable sites, and by differential species performance (Mansourian, 2005).

b.1.5. Fertility and organic matter content in agricultural and forest soils

The soil functions that determine vegetation composition/structure are of particular importance, as they serve as a medium for root development, and provide moisture and nutrients for plant growth (Minnesota Forest Resources Council, 1999). Natural soil fertility derives from the combination of many of these soil properties, including organic matter content, clay content and mineralogy, the presence of weatherable minerals, pH, base saturation, and biological activity, mainly in the rhizosphere.

In association with the concept of soil fertility, it is worth noting the importance of SOM and total organic carbon (TOC). Living soil organic matter represents only a small percentage of soil TOC, and includes soil micro-, meso- and macro-organisms. In particular, soil microbial biomass is regarded as the most active and dynamic pool of SOM, and plays an important role in driving soil mineralization processes. Three main aspects of soil microbial biomass are usually considered, due to their effects on soil functions: pool size, activity and diversity. The determination of the total amount of carbon immobilized within microbial cells allows soil microbial biomass to be determined as a pool of SOM. Since this pool is responsible for the decomposition of plant and animal residues and the immobilization and mineralization of plant nutrients, it is ultimately responsible for maintaining soil fertility (Brookes, 2000). Thus the concept of microbial biomass has developed to serve as an “early warning” of changing soil conditions and as an indicator of the direction of change.

Carbon mineralization activity, a key process of the soil's C cycle, determines the speed of the SOM degradation process in soil. Numerous studies have recently been carried out in order to verify -directly or indirectly- the potential for increasing carbon storage in soil by manipulating C inputs with a view to minimizing the rate of carbon mineralization (Jans-Hammermeister *et al.*, 1997). The carbon mineralization process has a low sensitivity to changes in soil management, as small microbial populations in degraded soil can mineralize organic matter to the same extent and at the same rate as large microbial populations in undegraded soils (Brookes, 1994). A combination of the two measurements, relating to both the size and activity of the microbial biomass, is more sensitive to soil management changes, and more helpful as a soil quality indicator (Akeem, 2012).

b.1.6. Storing carbon in agricultural and forest soils

Among the soil functions cited, and with regard to the soil's capacity to act as an environmental buffer, the last decade saw significant interest in forests and agroforestry systems -and especially in soils- as a means of storing carbon dioxide from the atmosphere and providing a mechanism to combat the increase in atmospheric CO₂ (EC, 2005). Forest soils generally accumulate carbon. Estimates range from 17 to 39 million tons of carbon per year, with an average of 26 million tons per year in 1990, and an average of as much as 38 million tons per year in 2005 (EC, 2002).

The atmospheric concentration of carbon dioxide (CO₂) has increased by over one third since the Industrial Revolution. This increase is primarily attributed to fossil fuel combustion, and also significantly to changes in land cover and use (e.g., forest degradation or conversion of forests to agriculture). In order to curb atmospheric CO₂ concentrations it is essential to reduce human dependence on fossil fuels and impose legally binding targets for reduced CO₂ emissions, and this should be the central focus of any policy program. However, in order to stabilize atmospheric CO₂ concentrations, the international community must also reduce the rate of destruction of natural ecosystems that serve as important stocks and sinks of carbon. In addition to slowing the rate of land conversion, other mitigation tools considered to stabilize the

burgeoning concentration of CO₂ in the atmosphere include increasing the land coverage of carbon-absorbing vegetation, and soil carbon sinks.

The theory behind land-based carbon trading is that governments or institutions that wish or are required to reduce their fossil fuel emissions can offset some of these emissions by investing in afforestation and reforestation activities to sequester carbon. Indeed, in some cases private companies are voluntarily electing to offset some of their fossil fuel CO₂ emissions through the purchase of carbon credits from land-based carbon sequestration projects (Mansourian, 2005). Recent studies under various agroforestry systems (AFSs) in a range of ecological conditions showed that the extent of C sequestered in AFSs largely depends on environmental conditions and system management. Trading of sequestered C is a viable opportunity for bringing economic benefit to agroforestry practitioners, who are mostly resource-poor farmers in developing countries (Ramachandran *et al.*, 2010; EC, 2002). For example, in contrast with industrial plantation approaches, the Scolel Té project for rural livelihood and carbon management (Mexico) aims to demonstrate how carbon finance can allow low-income rural farmers to invest in forest conservation, sustainable land-use systems and improvements in their livelihood that would otherwise be inaccessible to them (Mansourian, 2005). However, more rigorous research results are required for AFSs to be used in global agendas for C sequestration (Ramachandran *et al.*, 2010; EC, 2002).

However, there is still limited knowledge concerning the long-term impact of an increase in global average temperatures, and the real role that trees and SOM could play in absorbing carbon, in addition to the costs and benefits involved in using restoration as a mechanism to offset carbon emissions. For these reasons, carbon knowledge projects have been proposed as a way of testing these parameters in the context of landscape-based forest restoration activities (Mansourian, 2005).

The concept of carbon sinks is based on the natural ability of trees, plants and soil organisms to take up CO₂ from the atmosphere and store the organic and inorganic carbon forms in wood, roots, leaves, and the soil. Trueman and Gonzalez-Meler (2005) explain that the interplay between plant C intake, soil properties, community structure of the soil organisms and climate agents will determine the biosphere's organic terrestrial C sequestration strength at future atmospheric CO₂ concentrations. They studied tree growth and stabilization of SOM in an ecosystem exposed to elevated CO₂ concentrations, and reviewed other works, concluding that the inability to retain C in soils exposed to elevated CO₂ may stem from the lack of stabilization of SOM, allowing its rapid decomposition by soil heterotrophs. They explain that increases in heterotrophic respiration in response to elevated CO₂ may result in soils becoming a net source of CO₂ into the atmosphere if input of new C does not keep pace, and indicate that these facts make it impossible to forecast SOC sequestration potential.

In addition, it is important to analyze soil inorganic C sinks and biological models for the study of soil reaction and the decarbonation-recarbonation soil processes associated with fungal and soil organism communities, vegetation and climate, in order to evaluate in-soil C cycling and C sequestration.

b.1.7. Difficulties in developing international strategies for agricultural and forest soils protection

The realization that all of the soil degradation processes described are an environmental problem of global significance, and the recognition of the importance of protecting our soils, have led to an increase in international initiatives (Akeem 2012). Protection of the soil and the preservation of its biological health and overall quality has therefore become a key international goal.

In Europe, for example, the threat to soil health was reported and its biological roots were recognized almost three decades ago (Filip, 1973; Kovda, 1975). Nevertheless, there was a strong belief in soil's capacity for self-remediation. In the 1980s, the first moves towards the development of well-targeted soil protection initiatives were made in Germany and the Netherlands, and later also in the European Community (Barth and L'Heremite, 1987; Howard, 1993). Finally, on 1 March 1999 a Federal Soil Protection Act came into force in Germany. Its practical application, however, requires the availability of properly justified standards and reliable monitoring methods (Filip *et al.*, 2002). Moreover, Howard (1993) indicated that variations in the approaches adopted by different countries reflect discrepancies in the nature and perceived seriousness of soil problems.

Continuing with the example of the European Union's (EU) soil policies, Howard (1993) and Gzyl (1999) reported that European countries considered the "extension of urbanization, pollution by heavy metals, organic contaminants (including pesticides), acidification, over-fertilizing and artificial radio nucleids, groundwater nitrates, loss of organic matter, deteriorating soil structure, soil compaction and water and wind erosion" to be major threats to their soils. However, soil biodiversity is only indirectly addressed in a few European countries through specific legislation on soil protection, or regulations promoting environmentally-friendly farming practices (Turbé *et al.*, 2010; Jeffery *et al.*, 2010).

Given the differences between belowground and aboveground biodiversity, policies aimed at aboveground biodiversity may not be very effective for the protection of soil biodiversity. In contrast, the management of soil communities could form the basis for the conservation of many endangered plants and animals, as soil biota steer plant diversity and many of the regulating ecosystem services. No legislation or regulation exists to date that is specifically targeted at soil biodiversity, whether at the international, national or regional level. This reflects the lack of awareness of soil biodiversity and its value, as well as the complexity of the subject (Akeem 2012; Menta, 2012). Therefore, due to the complexity of these topics, there is so far no instrument that specifically addresses the protection of soil (CE, 2005) on any significant scale, such as at the level of the EU.

In connection to the issue of biodiversity loss, for example, there are several policy areas that directly affect and could address soil biodiversity, including policies for soil, water, climate, agriculture and nature. This aspect could be taken into account or highlighted in future biodiversity policies and initiatives such as the new European strategy for biodiversity protection post-2010 (Turbé *et al.*, 2010; Jeffery *et al.*, 2010). Previously in this area, among the priorities set by the Sixth Environment Action Program of the European Union (EC, 2002) for the

conservation of biodiversity and natural resources, it assumed the commitment of addressing soil alongside water and air as an environmental medium, and as a non-renewable resource to be preserved, hence undertaking to develop a thematic strategy for the protection of the soil (CE, 2005).

Johnston and Crossley (2002) highlight the study and protection of soil ecology as an essential component of ecosystems recovery and protection. They indicate that for many years there was a lack of concern for soil conservation: Brauns (1955) cautioned that it would be impossible to continue ignoring soil biology when resolving land management problems, and urged more consideration for the improvement of soil biological status. Similarly, Wells (1984), reflecting upon the increasing demands for shorter rotations and faster-growing trees, concluded that it would not be sufficient to maintain the existing fertility of forest and agroforestry soils. Bloem *et al.* (2003) and Brussard *et al.* (1998) explain that cryptobiota -hidden soil life- plays a key role in life-support functions, but is not part of any recognized list of endangered species. It is questionable whether a species-based approach is sufficient to attain a sustainable use of ecosystems inside -and especially outside- protected areas. Therefore research networks have been established to monitor large areas, including agricultural and forest soils (Bloem, 2003).

Johnston and Crossley (2002) also remarked that the chemical and biological conditions of forest and agroforestry soils are not routinely monitored and studied, as they should be in view of the priority of improved regional soil fertility within the over-arching goal of ecosystem management. They posed two questions:

- (i) *Can land managers continue to manage ecosystems without striving to improve soil fertility over the long-term?*
- (ii) *Does the field of soil ecology have little to offer to the practice of land managing?*

b.1.8. Foundations for understanding the role of biology in soil quality

Soil communities participate in most key soil functions, driving fundamental nutrient cycling processes, regulating plant communities, degrading pollutants and helping to stabilize soil structure. The ecosystem services provided by soil biota are thus one of the most powerful arguments for the conservation of edaphic biodiversity. Moreover, food chain interactions among the soil biota (including plant roots) have major effects on the quality of crops (affecting human and animal nutrition and other aspects of life on earth), the incidence of soil-borne plant and animal pests and diseases (affecting production levels), and beneficial organisms such as edible mushrooms (Brussaard *et al.*, 2007).

Some researchers have defined the soil biota as a “super organism” that assumes a crucial significance due to the chemical-physical and biological processes that are rooted in the soil. A rapid survey of invertebrate and vertebrate groups reveals that at least a quarter of the living species described are strictly soil or litter dwelling. These large soil communities play an essential role in soil functions as they are involved in processes such as the decomposition of organic matter, the formation of humus and the nutrient cycling of many elements (nitrogen, sulfur, carbon). Moreover, edaphic communities affect soil porosity and aeration as well as the

infiltration and distribution of organic matter within its horizons (Maharning *et al.*, 2008; Menta, 2012). It is therefore necessary to understand the role of biodiversity in soil functions as the foundation for identifying soil management strategies, and practices, based on soil biology.

With regard to biodiversity and soil function interactions, it must be noted that decomposition of organic matter by soil organisms is crucial for the functioning of an ecosystem, due to its substantial role in providing ecosystem services for plant growth and primary productivity (Menta, 2012). For example, soil fauna performs a mainly mechanical action, whereas fungi and bacteria, both free and intestinal symbionts of other organisms, essentially perform chemical degradation; furthermore, during digestion, organic substances are enriched by enzymes that are dispersed in the soil along with the feces, contributing to humification (Maharning *et al.*, 2008; Menta, 2012). However, at present we are not sure of the extent of the importance of this biodiversity in the sustainability of a soil's functions, and it is extremely rare for the biological relationships between soil organisms to consist of a simple and clear interaction. Current ecological conditions are the result of many complex interactions that typically involve multiple participants in soil life such as plants, microbes, fungi and animals (Killham *et al.*, 1994).

Thus the relationships between biodiversity and its functions are complex and somewhat poorly understood, even in aboveground situations. The exceptional complexity of belowground communities further confounds our understanding of soil systems. Four important mechanisms underlying relationships between biodiversity and function are responsible for:

- (i) *Driving fundamental nutrient cycling processes;*
- (ii) *Regulating plant communities;*
- (iii) *Degrading pollutants;*
- (iv) *Helping to stabilize soil structure.*

Some of the most significant functions -and the main biotic groups that carry them out- are:

- (i) *Primary production [plants, cyanobacteria, algae];*
- (ii) *Secondary production [herbivores];*
- (iii) *Primary decomposition [bacteria, archaea, fungi, some fauna];*
- (iv) *Secondary decomposition [some microbes, protozoa, nematodes, worms, insects, arachnids, mollusks];*
- (v) *Soil structural dynamics [bacteria, fungi, cyanobacteria, algae, worms, insects, mammals];*
- (vi) *Suppression of pests and diseases [bacteria, actinomycetes, fungi, protozoa, nematodes, insects];*
- (vii) *Symbioses [bacteria, actinomycetes, fungi -notably mycorrhizas];*
- (viii) *SOM formation, stabilization and C sequestration [virtually all groups, directly or indirectly];*
- (ix) *Atmospheric gas dynamics, including generation and sequestration of greenhouse gases [bacteria for nitrous oxides, methane, all biota for CO₂];*
- (x) *Soil formation [bacteria, fungi] (European Commission, 2005).*

Rough estimates of soil biodiversity indicate several thousand invertebrate species per site, as well as relatively unknown levels of microbial and protozoan diversity. Soil ecosystems generally contain a large variety of animals including nematodes and micro-arthropods such as mites and collembolans, symphylans, chilopodans, pauropodans, enchytraeids and earthworms. In addition, a large number of meso- and macro fauna species (mainly arthropods such as beetles, spiders, diplopods, chilopods and pseudoscorpions, as well as snails) live in the uppermost soil layers, the soil surface and the litter layer. Despite several decades of soil biological studies it is still very difficult to provide average abundance and biomass values for soil invertebrates. This is partly due to their high variability in both time and space, as well as to differences in the sampling methods used (Jeffery *et al.*, 2010). In addition, most of the work has been conducted in the forest soils of temperate regions, while other eco-regions such as the tropics, or other land uses such as agriculture, have been seriously neglected (Jeffery *et al.*, 2010).

The presence of roots is generally associated with a greater density of micro-organisms in the nearby soil (rhizosphere) compared with soil devoid of roots (Killham *et al.*, 1994). The interaction between soil animals and plant roots, and their rhizosphere, may take a variety of forms that lead to benefits or repress the growth of the plant, and often involve interactions with the microbial population of the soil. The dispersion of the inocula of mycorrhizal fungi by soil animals can have beneficial effects for the plants; this dispersion is particularly favored by burrowing organisms belonging to the mega- and meso fauna categories.

The hyphae of mycorrhizal fungi may comprise a significant proportion of the total microbial biomass in some soils, and may become one of the most important sources of food for fungus-grazing animals such as springtails. Numerous soil animals feed directly on the roots of plants; these include a large number of springtail and myriapod species. It is still not entirely clear how much of the damage inflicted on plants can be attributed to the direct action caused by grazing on the roots, or to the subsequent vulnerability of the roots to pathogens in the soil, especially fungi (Menta, 2012).

Among the width range of natural communities that can be exploited to enhance biological interactions in sustainable food production, arbuscular mycorrhizas fungi (AM) is among the most ubiquitous and abundant symbiosis in the world (Yang *et al.*, 2016). In fact, AM play a role in the diversity and functioning of natural ecosystems (Van der Heijden *et al.*, 1998). This group of fungi encompasses a range of fundamental processes such as organic matter decomposition, and pathogenicity (Thorn *et al.*, 1997; Kowalchuk *et al.*, 2004). In addition, AM readily colonize the roots of virtually all plant species that occupy upland grasslands (Sparling and Tinker, 1978); providing their host plants with increased amounts of nutrients, particularly P (Smith and Read, 1997). AM enhance nutrient translocation and uptake, through an extensive network of external mycelium that acts as an extension of the root absorption system (Mosse, 1981). Furthermore, AM induce changes in secondary metabolism which improve nutraceutical compounds and interfere with the phytohormone balance of host plants (Sánchez and Salinas, 1981; Mäder *et al.*, 2002; Rouphael *et al.*, 2015). Guo *et al.* (2012) have speculated that the ability to form AM associations may be important for crop species adaptable to low-input systems. AM can also alleviate stresses such as drought and root pathogen attack, improve soil structure, and confer

tolerance to metal in plants (Clark and Zeto, 1996; Smith and Read, 1997; Augé, 2001). AM have been confirmed to enhance plant tolerance to environmental pollution in many previous studies (Luo *et al.*, 2016a).

In temperate (and some tropical) forests and agroforestry areas, ectomycorrhizal (ECM) fungi mobilize soil nutrients, improve soil quality, and are important in the remediation of soil loss (Poma *et al.*, 2006; Lian *et al.*, 2007) mainly. Moreover, Wang and Hall (2004) indicate that the most highly-prized edible mushrooms belong to the ECM group. Among the numerous interactions between ECM fungal metabolism and soils, there are three which may be relevant to soil processes on a significant scale:

- (i) *The soil reaction (as measured by pH);*
- (ii) *The amount and balance of soil carbonates;*
- (iii) *The interaction of ECM rock-eating processes in the nutrition of their host trees (Van Schöll *et al.*, 2008).*

ECM fungi can strongly acidify their immediate soil environment (hyphosphere), and their mycelia can provoke a significant change in soil properties and soil-ECM-plant interactions. Cooke and Whipps (1993) report that fungal mycelia excrete H^+ as a result of the cation metabolism. This H^+ excretion is linked to K^+ capture, which occurs by means of active transport, and exceeds concentration gradients of 5000:1. One of the immediate consequences on the soil acidification process is the change in the equilibrium of soil carbonates and inorganic C sinks.

In Mediterranean areas, truffles (species of *Tuber Micheli* ex Wiggers) are one of the ECM group that yield the highest economic benefits. Moreover, they constitute a group of fungi that shows one of the most complex and interesting soil ecologies (Moreno *et al.*, 1986; Oria 1989, 1991; Montecchi and Sarasini 2000; Rioussel *et al.*, 2001). The biology of truffles, and ECM fungi in general, have been fully described. However, there are still gaps in our knowledge of ECM fungi biology, and truffles in particular, and there is insufficient knowledge of the ecological relationships between ECM fungi and soil properties, processes and organisms.

b.2. Research interest

b.2.1. Need and opportunity

Since the 1930s, the Green revolution has replaced traditional agriculture with conventional agriculture (CA) by the application of high inputs of chemical fertilizers and power derived from fossil fuels. These technologies saved over a billion people from starvation, but nowadays entail serious sustainability issues due to the limitation of fertilizers supply from current sources, greenhouse gas (GHG) emissions, and diffuse pollution (Farmer, 1986; Te Pas and Rees, 2014). Moreover, worldwide farmers only collect 50% of the yield they would obtain under optimal conditions, with 60-70% of the losses attributable to abiotic factors such as nutritional deficiencies or drought. The remaining 30-40% losses are attributed to biotic factors connected to the rhizosphere (Peleg *et al.*, 2011). While organic agriculture (OA) promotes organic fertilization and outperforms CA in environmental and social aspects, critical questions arise on whether OA benefits compensate for its lower crop yield or if it will preserve soil fertility over time (Andersen *et al.*, 2015; Hansen *et al.*, 2001; Meier *et al.*, 2015).

New solutions and policies are urgently needed to improve availability of fertilizers, OA and CA yields, and meet the “greening” requirements set by, for example, the Common Agricultural Policy (CAP) (Eurostat, 2015). In this regard, European societal challenge analyses indicate that new nature-based solutions considering the multi-functional use of ecosystems are required to perform the transition towards a circular economic eco-innovative management system changing the traditional model based on production and consumption towards a green society.

Powers *et al.* (1998) and Page-Dumroese *et al.* (2000) pointed out also that soil productivity is a key factor for maintaining ecosystem functions in forest and agricultural soils. Agricultural and forestry management on a sustainable basis has consistently included the maintenance or enhancement of soil quality as a criterion of sustainability (Burger and Kelting, 1999; Schoenholtz *et al.*, 2000; Arredondo *et al.*, 2014). Furthermore, the overall goal of soil protection involves the conservation of good soil quality and requires certain soil functions to be fulfilled.

However, although considerable activity is currently aimed at the development and evaluation of sustainable management systems for agricultural and forest soils, these efforts have been hampered by the lack of agreement as to what constitutes credible measures of sustainability. The means to evaluate soil sustainability both in terms of design and performance have yet to be fully determined (Larson and Pierce, 1991; Pierce and Larson, 1993; Doran, 1994), but in recent decades several new institutional, technical and scientific approaches are being and have been developed.

As an example of the evolution of institutional approaches for sustainable soil management, the European Commission in its Sustainable Development Strategy published in 2001 (EC, 2001) noted that soil loss and declining fertility were eroding the viability of agricultural land (COM, 2001). In 2002, the European Parliament and Council established the Sixth Environmental Action Program (Sixth EAP), which covers a period of ten years (Decision 1600/2002). This program addresses the Community's key environmental objectives and priorities to be met through a range of measures, including legislation and strategic approaches. In Article 6, 'Objectives and

priority areas for action on nature and biodiversity', the Sixth EAP foresees the development of a thematic strategy on soil protection, addressing the prevention of pollution, erosion, desertification, land degradation, and hydrogeological risks, taking account of regional diversity, and including the specificities of mountain and arid areas (Bloem, 2003).

As an example of the evolution of technical approaches for sustainable agricultural and forest soil management, several authors indicate that the current aims of these approaches are to maintain good crop yields with minimal impact on the environment, while at least avoiding deterioration in soil fertility and providing essential nutrients for plant growth. Furthermore, sustainable agricultural and forest systems and agriculture should support a diverse and active community of soil organisms, exhibit a good soil structure and allow for undisturbed decomposition. Thus current sustainable agricultural and forest practices are adjusting to integrate organic -or more extensive- management. The main principles involve restricting stocking densities, avoiding synthetic pesticides and mineral fertilizers and using organic manure. This will ultimately result in an increased role of soil organisms, e.g. decomposers, nitrogen fixers and mycorrhizas, in plant nutrition and disease suppression.

In this regard, European Union addressed the need of transition toward an optimal and renewable use of biological resources and towards sustainable primary production and processing systems (Tilman *et al.*, 2002). These systems must go along with the global rising of food and fiber demands, which is expected to reach some 3 billion tones by 2050, up from 2009's nearly 2.1 billion tones (FAO, 2009). Current sustainable agriculture and forestry should therefore seek to conserve and to intensify natural resources based on a concept of productivity which is closely linked to maintaining a system aimed at saving energy and resources in the mid to long term, through optimizing recycling and enhancing biodiversity, and through biological synergy (Hansen *et al.*, 2001; Máder *et al.*, 2002; Bloem, 2003).

b.2.2. New approaches on sustainable agricultural and forest soils management based on the ecological intensification of the rhizosphere

There are many gaps in our knowledge of both ECM and AM fungal soil ecology. One of the reasons is because the ecological role of mycorrhizal fungi have mostly been demonstrated under simplified, artificial experimental conditions (Smith and Read 2008), because of the difficulties in controlling mycorrhizas in nature (Nara 2008), and we cannot conclude that mycorrhizas would perform these same functions in the nature.

To minimize research difficulties of mycorrhizas field studies, one potential approach may be to study disturbed sites in which there are simpler mycorrhizal and host plants communities established on relatively homogeneous substrates (Nara 2008). Plants infected with ECM fungi sometimes show a zone around their trunk where the growth of other plants is inhibited either permanently or for part of the year, and where the soil biodiversity decreases. Some fungi that produce those clearings, or brûlés, are *Tuber melanosporum* Vittad. (black truffle), *T. aestivum* Vittad. (summer truffle) and *Scleroderma* Pers. In the Mediterranean region, where *Tuber melanosporum* inhabits, their mycelia produces multiple substances that adversely affect young

plants and seed germination in their brûlés, and fungal infection in the roots of weeds results in severe necrosis in the root cortices (Plattner and Hall 1995).

In the *Tuber melanosporum* brûlés, it has been observed that this ECM fungus can modify soil properties, such as CaCO_3 fractions (*increasing the carbonated fractions below 50 μm in diameter; called active carbonate*). In this regard, García-Montero (2000) and García-Montero *et al.* (2006) suggested an interesting hypothesis about the activities of "rock eating" of *T. melanosporum* in his brûlés. However, they also explained that this ability of *T. melanosporum* to increase soil active carbonate in connection with his rock-eating activity may explain the mortality of some host plants, such as rockroses (*Cistus* L.). Therefore, it is necessary to consider the possibility that the ECM rock-eating activity could cause both positive or negative effects on the host plants.

The observations about the ability of ECM fungi to modify the soil properties, is not limited to the brûlés of truffles. Another structural pattern of ECM fungi also showing a significant alteration of soil biogeochemistry has been reported in acidic soils of *Pseudotsuga menziesii* (Mirb.) Franco forests in the the North America Pacific Northwest. That is, in the organic soil horizons of coniferous forests, there are distinct rhizomorphic mats formed by ECM species of *Piloderma* Jülich and *Hysterangium* Vittad. covering up to 40% of the soil surface, and *Ramaria* spp. in mineral soils. These fungal mats have physical and biochemical properties that distinguish them from the surrounding non-mat soils. In particular, in the organic horizon, the rhizomorphic mats had 2.7 times more oxalate than the non-mats and significantly lower pH. In the mineral horizon, hydrophobic mats had 40 times more oxalate, significantly lower pH and greater microbial biomass C than non-mat mineral soils (Cromack *et al.*, 1979; Kluber *et al.*, 2010).

Therefore, it would be of great interest to explore whether the abilities of ECM fungi, such as truffles, to modify the soil and rock properties could provide the support for a new model of intensification of rhizosphere that could be developed in crops, rangelands, agroforestry systems and forests.

b.3. Objectives and hypothesis

The **main objective and hypothesis** of the present thesis would be to explore a ground-breaking approach on "*ecological intensification of the rhizosphere*" in crops, rangelands, agroforestry systems and forests -i.e. farming the rhizosphere-, focusing on two kinds of solutions to increase the abundance, biomass, depth and activity of roots and their beneficial soil microorganisms, that would be through:

- (i) *Elaborate some basis to propose new models on practices and technologies on agriculture and forestry;*
- (ii) *Elaborate some basis to modelling new productive prototypes applied to the agro-ecosystems, forests and agroforestry systems.*

With this framework, the **first general objective** is to deep in the hypothesis that *Tuber* mycelia and their rock-eating activities could explain some processes of "*ecological engineering*" inside the brûlé, that could intensify the rhizosphere interactions with the host plants.

The **second general objective** is to evaluate if the "*ecological engineering model of the Tuber brûlé*" could be extrapolated to hypothetical managing-models on "*ecological intensification of roots and rhizosphere*" in crops, rangelands, agroforestry systems and forests.

Aiming to understand the importance of soil pH, Ca salts, SOM, biodiversity and nutrients availability for the hypothetical "*ecological intensification of the rhizosphere*" inside the *Tuber* brûlé; the **first general objective** has been developed through **7 specific objectives (S.O.)**, that have been explained through 8 articles (2007-2014) in journals indexed in the JCR list (3 in Q1, 3 in Q2, 1 in Q3 and 1 in Q4, quartiles), 1 international book chapter (2012), 1 communication in international congresses (2010) and 1 seminar cycle in EEUU (2012):

First, "What is it happening in the brûlés?"

- (i) S.O.1.- *Analyze the ecological patterns of Tuber melanosporum brûlés in different natural habitats:* García-Montero et al. (2007) [Section d.1];
- (ii) S.O.2.- *Regarding other truffles species, look for comparable patterns to the brûlé of Tuber melanosporum:* García-Montero et al. (2008a; 2008b) [Sections d.2 and d.3].

Second, "Why is it happening in the brûlés?"

- (iii) S.O.3.- *Compare the soil properties inside versus outside of the Tuber melanosporum brûlé:* García-Montero et al. (2009b) [Section d.4];
- (iv) S.O.4.- *Experimentation with calcareous amendments in truffle culture:* Valverde et al. (2009) and García-Montero et al. (2009a) [Sections d.5 and d.6].

Third, "How is it happening in the brûlés?"

- (v) S.O.5.- *Analyze the interactions of soil fauna on the ecological intensification of the rhizosphere inside the Tuber brûlés:* García-Montero et al. (2010; 2013) and Menta et al. (2014) [Sections d.7, d.10 and d.12];
- (vi) S.O.6.- *Review bibliography on evolutionary and ecological dynamics of ECM and AM*

fungus, and NfB, interactions and comparable associations to the Tuber brûlé, such as the lichen structure: General Discussion [Sections e.1.1 to e.1.3; e.1.7 to e.1.10; e.1.12 and e.1.13]

- (vii) *S.O.7.- Propose a synthetic model explaining the ecological intensification of the rhizosphere in the Tuber brûlés: General Discussion [Sections e.1.4 to e.1.6; and e.1.11], García-Montero (2012), and García-Montero et al. (2012) [Sections d.8, d.9 and g.1].*

The **second general objective** has been focused on to explore hypothetical new models on "ecological intensification of the rhizosphere" in crops, rangelands, agroforestry systems and forests based on the *Tuber brûlé* model. This second objective has been developed through other **3 specific objectives (S.O.)** that have been developed through 2 international book chapters (2014, 2017), 2 communications in international congresses (2015a; 2015b), and 2 research project proposals led by UPM (2017; 2018), in separated Horizon 2020 calls:

- (viii) *S.O.8.- Review bibliography related to the soil quality and its indicators in forest and agricultural soils: General Introduction [Sections b.1 and b.2] and Arredondo et al. (2014) [Sections d.11 and g.2];*
- (ix) *S.O.9.- Discuss and integrated the obtained results about the Tuber brûlé model to give support to a new framework called "rhizoculture", that would allow to explore new practices and prototypes focused on "ecological intensification of the rhizosphere" in crops, rangelands, agroforestry systems and forests: General Discussion [Section e.2] and García-Montero et al. (2015a, 2015b, 2017) [Sections d.13, d.14, d.16 and g.3];*
- (x) *S.O.10.- Design and write research projects to submit in the European calls Horizon 2020. The aim of these proposals would be to develop research, if possible, on the "rhizoculture model": Future Research Lines and García-Montero as coordinator (2017; 2018) [Sections e.4, e.4.1 and e.4.2]*

c. METHODOLOGY

c.1. Study areas of natural habitats of truffles

In García Montero (2012), García Montero et al. (2007; 2008b; 2009b) and Menta et al. (2014), it have been performed ecological studies in natural habitats of *Tuber melanosporum*, *T. aestivum*, *T. mesentericum* Vittad. and *T. rufum* Pico ex Fries var. *rufum* form *rufum* Montecchi et Lazzari, in the Natural Park of “Alto Tajo” in Central Spain (province of Guadalajara and Cuenca). In this location, the study area covers a 10 km radius between the municipal areas of Peralejos de las Truchas (Guadalajara) and Belvalle (Beteta, Cuenca). This study area is situated in a mountainous region at an altitude of more than 1,000 m, in a supra-Mediterranean bioclimatic belt with a sub-humid, shady climate. Average annual precipitation is 797 mm, with low average annual temperatures (9.7 8C) and very cold winters.

In this region, *Tuber* spp. are associated with *Quercus faginea* Lam., *Q. ilex* L. subsp. *ballota* (Desf.) Samp., *Tilia platyphyllos* Scop., *Corylus avellana* L., *Pinus nigra* Arnold subsp. *salzmannii* (Dunal) Franco and *P. sylvestris* L in different wood types. The lithology of the area is dominated by Jurassic and Cretaceous limestone and dolomites, and soils are frequently lithic and rendzic leptosols with marked surface stoniness and high peaks. The vegetation of the study area fell within the Mediterranean Ibero-Levantina Province Castellano Maestrazgo-Manchega Subprovince geobotanical classification type (Peinado and Martínez, 1985; Rivas-Martínez, 1987; IGME, 1989; Rivas-Martínez and Loidi, 1999).

c.2. Study areas of cultures of truffles

In García Montero (2012), García Montero et al. (2009a; 2010; 2013) and Valverde et al. (2009), it have been performed ecological studies in cultures of *Tuber melanosporum* in different regions of Spain.

In 2007, in the province of Alava (northern/central Spain), it has been carried out two calcareous amendments of finely divided carbonate (2,500 kg ha⁻¹) in two *Tuber melanosporum* cultures contaminated by *T. brumale* Vittad. And two control *T. melanosporum* cultures (which were uncontaminated by *T. brumale* and had no calcareous amendments). All cultures had comparable features, i.e., more than 14 years old, with a brûlé production higher than 300 g year⁻¹ of fruit bodies of these truffles associated to *Q. ilex* subsp. *ballota* or *Corylus avellana*. The studied cultures and wood located in Alava were in a region with natural vegetation of oak forests (*Spiraeo hispanicae–Querceto rotundifoliae sigmetum* geobotanical classification type), in supra-Mediterranean bioclimatic belts. After liming, it has been monitored the fruit body production in the treated cultures, and it was compared with the nearby control cultures. It has been also analysed soil samples from the four cultures in Alava, comparing amended *T. brumale* brûlés, amended *T. melanosporum* brûlés, and control brûlés (unamended *T. melanosporum* brûlés). It was consulted the data on average monthly precipitation and temperature provided by the National Spanish Meteorological Agency (AEMGS, 2008) in the study area in Alava.

Moreover, it has been performed soil and ecological studies from *Tuber brumale* and *T. melanosporum* brûlés from other two cultures of *T. melanosporum*, that were compared as control, with natural brûlés in two nearly woods, with and without a natural presence of *T. brumale*. One of the cultures was located in Sarrión (Teruel) –that was 15 years old and has 50% productive *T. melanosporum* brûlés and 20% contaminated brûlés that produce *T. brumale* carpophores–; the second culture was located in Campezo (Alava) –that was 14 years old and has 60% productive *T. melanosporum* brûlés and 20% contaminated brûlés that produce *T. brumale* carpophores. The two cultures and the two control natural woods were located in mountainous regions of Central Spain with predominantly Jurassic and Cretaceous limestones and dolomites, and the soils lithic and rendzic leptosols.

Finally, in other culture of *Tuber melanosporum* located in Sarrión (Teruel) was performed an study about the impact of earthworms on the *Tuber melanosporum* brûlés. [100 g]. This culture was 16 years old, and has 60 % productive *T. melanosporum* brûlés. Earthworm cast samples were collected from *T. melanosporum* brûlés from this culture, that showed high earthworm activity. A sample of ten earthworms was collected and sent for classification to the University of Parma and University of Siena. It was selected this truffle culture because all the brûlés showed a high abundance of earthworm casts (at least, 7 casts little piles per brûlé). The cultures located in Sarrión (Teruel) were located in supra-Mediterranean bio-climatic belts, with natural vegetation consists of open oak forests (*Junipero thuriferae–Querceto rotundifoliae sigmetum*) alternating with dryland mosaic farming.

c.3. Study of the productivity and truffle brûlés characteristics

In García Montero (2012), García Montero et al. (2007, 2008b; 2009a; 2009b; 2010; 2013), Valverde et al. (2009) and Menta et al. (2014), it have been performed ecological studies about the natural brûlés in woods located in the Natural Park of “Alto Tajo”.

In this region, *Tuber melanosporum* has been harvested since the end of the 1960s. The studied brûlés have been regularly harvested by professional collectors; and have been monitored systematically during the field research of this thesis. Harvesting is done according to a system established by the corresponding local councils in order to restrict the number of collectors. Carpophores were harvested with the help of trained dogs. This study was based on the collaboration with six truffle collectors who shared their knowledge and collection data, including the location of several harvesting points. During many expeditions undertaken between 1994 and 2012, and with these collectors, 238 brûlés of *T. melanosporum* –in addition to 25 brûlés of *T. aestivum* and *T. mesentericum*– were selected and monitored, based on their regular production

In each brûlé, of these *Tuber* species, it has been determined the host plant, size (m²) of the brûlé and carpophore production. In natural woods, harvesting data supplied by gatherers who exploited these brûlés regularly were used. The maximum annual production of *Tuber* carpophores was calculated as the greatest quantity of fresh carpophores, in grams, picked during a harvest season, over a period of time between 5 and 15 years. The maximum annual production (instead of the average yield) was chosen to standardize and compare the optimum

yields of brùlés (in the most favorable year for each site) located in different areas. The reliability of the information provided by the collectors was confirmed independently in systematic field trips made with the collectors at the time of harvesting throughout the studied periods. The exact geographic coordinates of these brùlés were not given, in order to maintain the secrecy of the truffle locations.

c.4. Soil analysis

In García Montero (2012), García Montero et al. (2007; 2008a; 2008b; 2009a; 2009b; 2010; 2013), Valverde et al. (2009) and Menta et al. (2014), it have been performed the analyses of 257 soil samples associated with 7 truffle species –*Tuber melanosporum*, *T. aestivum*, *T. mesentericum*, *T. brumale*, *T. rufum*, *T. pseudoexcavatum* and *T. indicum*–, that were studied in Spain. In addition, it has been analyzed 42 earthworm cast samples, located inside the truffle brùlés. The soil studies were related both with mycorrhized plants produced in greenhouses, and productive truffle brùlés in cultures or in natural woods.

In the truffle brùlés, soil samples were selected inside and/or outside the brùlés according to the studied dependent variables (brùlé size, truffle species, etc.). Inside the brùlés, the soil samples were taken from the bare soil in the brùlé, at those points where the greatest number of carpophores had been extracted in recent years. Reference samples were taken from patches of bare soil without production situated between 1 and 50 m from the brùlés. Only the first 30 cm of each soil profile was studied because *T. melanosporum* usually bears fruit in this range (Verlhac et al., 1990). The soils were sampled always using the same spade (0.20m blade) and a tape measure. These samples included a mixture of 1,000g of the soil surface horizons that have not been differentiated because *T. melanosporum* natural brùlés contain a constant mixture of horizons, due to the continuous digging that the harvesters, their dogs and wildlife engage in to extract the carpophores. Sampling was done according to the FAO (1990).

The following soil determinations were made: pH, total organic carbon (TOC), total calcium carbonate (equivalent carbonate), granulometric analysis, cationic exchangeable capacity (CEC) and the exchangeable cation saturation percentage (V%), following the methods of the ISRIC (1995); the textures were classified according to the International Society of Soil Science (ISSS); the total nitrogen was analysed using the methodical variant of Bouat and Crouzet (1965); and the active carbonate (carbonate extractable with ammonium oxalate) was determined according to AFNOR (1982). The exchangeable cations of Ca^{2+} and Mg^{2+} were determined by AAS (Atomic absorption spectroscopy), and K^{+} and Na^{+} with a flame photometer.

c.5. Study of *Tuber spp. mycorrhizas*

In García Montero (2012) and García Montero et al. (2007; 2008a), it has been produced mycorrhized plants with different *Tuber spp.* under controled conditions. The mycorrhizal synthesis of *Tuber pseudoexcavatum* Wang, G. Moreno, L. G. Rioussset, J. L. Manjón, and G. Rioussset and *T. indicum* Cooke and Massee have been performed with *Quercus ilex* subsp.

ballota as host plants. While the mycorrhizal synthesis of *Tuber melanosporum* have been performed with *Quercus faginea* as host plants.

The mycorrhizal synthesis of *Tuber* species were done by applying a method of intensive mycorrhization on *Quercus* plants, respectively, as described by Bencivenga (1982), but modified according to Manjón and García-Montero (1996). The *Quercus* acorns and truffle carpophores were carefully washed, sterilized on the surface, and conserved in a fridge at a temperature of +4°C. The acorns were sown in semi-rigid plastic containers with a volume of 1,000 cc. The substrate used for seeding was a mixture of calcareous soils from natural *T. melanosporum* brûlés, mixed with vermiculite and perlite, which was sterilized in an autoclave at 120°C for 4 h, in order to eliminate any mycorrhizal contamination. At the moment of sowing, acorns were inoculated with a water suspension of ascospores obtained from finely-chopped carpophores. An acorn and a suspension containing 2 g of fruit body were placed in each container. The plants were kept under controlled environmental conditions in a greenhouse with an average daily temperature of 20 to 25°C; relative humidity of 60 to 70%; watered by micro-sprinkler between one and three times a day for 1-3 min depending on the time of year; and under natural light conditions, with no artificial lighting. These parameters were controlled automatically using a computer. The plants were not fertilized. We obtained batches of well-lined, disease-free plants, with diameter at the base of the plant of 2 cm.

The degree of mycorrhization of each plant was expressed as the number of *Tuber* mycorrhizae of the total number of apices counted, according to Bencivenga et al. (1987). The ectomycorrhizae were identified with a stereoscopic microscope (photo-Leica WildMZ8) and a microscope (photoLeica LeitzDMRB) following the terms, descriptions, and recommendations of Agerer (1987–2002), Bencivenga et al. (1995) and Granetti (1995). The ectomycorrhizal color was described following Munsell (1976) standard soil color charts.

In García-Montero et al (2008a), it has also been verified the existence of natural contamination by *Tuber aestivum* and *T. mesentericum* Vittad. mycorrhizae in the natural brûlés of *T. melanosporum*. This was done by sampling roots in the interior of productive brûlé sites of *T. melanosporum*. Sampling was done in accordance with the procedures of Di Massimo et al. (1996). The mycorrhizae were identified with both the stereoscopic microscope and the microscope following Verlhac et al. (1990) and Granetti's (1995) descriptions and recommendations.

However, in the natural study area, harvesting was undertaken according to a regime established by the corresponding local councils. Therefore, in the natural brûlés we were unable to carry out studies on the abundance of the *Tuber melanosporum* and *T. aestivum* mycorrhizae, as this type of study requires damaging the truffle burns in order to extract the root samples which would enable us to analyse this aspect. We have not been authorized by the corresponding local councils to perform these kinds of studies.

c.6. Taxonomy studies

Samples of the studies truffle species were conserved in the herbariums of the University of Alcalá and the Polytechnical University of Madrid (ETSI Montes). The taxonomy of the truffle species were identified with a stereoscopic microscope (Leica WildMZ8) and a microscope (Leica LeitzDMRB) following the descriptions of Montecchi and Sarasini (2000) and Rioussel et al. (2001). Macroscopic features of the carpophores and microscopic studies of the morphological characteristics of the ascospores permitted a clear identification of the studied truffles.

c.7. Soil microarthropod extraction

In Menta et al. (2014), it have been performed a microarthropod community analysis in 9 natural brûlés of *Tuber aestivum*, located in the Natural Park of “Alto Tajo”. Eighteen soil samples were taken inside each brûlé (9 more or less centrally) and outside it (9 at least 5 m from the brûlé edge). Each soil sample measured 100 cm² and was taken at a depth of 10 cm. Microarthropods were extracted using a Berlese–Tüllgren funnel. The specimens were placed in a preserving solution (75% ethyl alcohol and 25% glycerol by volume) and identified to different taxonomic levels by researchers of the University of Parma.

c.8. Statistical analysis

Statistical treatments were performed using both Statistica Program v. 6 (StatSoft, Inc., Tulsa, Ok, 1999) and Statistica Release seven Program (StatSoft, Inc. 1984–2006). It has been applied ANOVA, ANCOVA, MANOVA tests, Correlation Matrix, Regression function models, and Principal Components Analysis (*in order to analyse the statistical patterns of the overall soil properties regarding the analyzed independent variables*); moreover, when necessary it was used using a Monte Carlo permutation test. When the data did not follow a normal distribution, non-parametric tests were applied to these variables, such as the Mann-Whitney U test.

Before proceeding with the analysis, the necessary transformations were made to adjust the distributions of the variables to the prerequisites required for statistical analysis. These transformations were selected using the Box and Cox (1964) test. Normality were checked using the Shapiro-Wilks test. In addition, for the ANOVA test, the homogeneity of variances was checked using the Levene test.

c.9. Bibliographic review on rhizoculture topic, and on evolutionary and ecological dynamics of ectomycorrhizas and lichens

This bibliographic research has been done using the references provided by the international bibliographic managing systems and databases of Web of Science, Science Direct, Scopus and Ingenio. These databases provide a significant sample of the literature in relevant scientific journals and publications.

d. COLLECTION OF PUBLICATIONS AND CONTRIBUTIONS

- d.1. L.G. García-Montero, J.L. Manjón, C. Pascual, A. García-Abril (2007). *Ecological patterns of Tuber melanosporum and different Quercus Mediterranean forests: quantitative production of truffles, burn sizes and soil studies*. FOREST ECOLOGY and MANAGEMENT 242: 288-296. JCR: Q1 in Forestry Area (6th from 39 journals); Impact Factor 2007 = 1.579
- d.2. L.G. García-Montero, P. Díaz, S. Martín-Fernández, M.A. Casermeiro (2008b). *Soil factors that favour the production of Tuber melanosporum carpophores over other truffle species: a multivariate statistical approach*. ACTA AGRICULTURAE SCANDINAVICA SECTION B-SOIL and PLANT SCIENCE 58: 322-329. JCR: Q4 in Agronomy Area (41th from 49 journals); Impact Factor 2008 = 0.41
- d.3. L.G. García-Montero, G. Di Massimo, J.L. Manjón and A. García-Abril (2008a). *New data on ectomycorrhizae and soils of the Chinese truffles Tuber pseudoexcavatum and Tuber indicum, and their impact on truffle cultivation*. MYCORRHIZA 19: 7-14. JCR: Q2 in Mycology Area (7th from 19 journals); Impact Factor 2008 = 2.205
- d.4. L.G. García-Montero, I. Valverde-Asenjo, P. Díaz, C. Pascual (2009b). *Statistical patterns of carbonates and total organic carbon on soils of Tuber rufum and T. melanosporum (black truffle) brûlés*. AUSTRALIAN JOURNAL OF SOIL RESEARCH 47: 206-212. JCR: Q3 in Soil Science Area (21th from 31 journals); Impact Factor 2009 = 1.007
- d.5. I. Valverde-Asenjo, L.G. García-Montero, A. Quintana, J. Velázquez (2009). *Calcareous amendments to soils to eradicate Tuber brumale from T. melanosporum cultivations: a multivariate statistical approach*. MYCORRHIZA 19: 159-165. JCR: Q2 in Mycology Area (5th from 19 journals); Impact Factor 2009 = 2.650
- d.6. L.G. García-Montero, A. Quintana, I. Valverde-Asenjo, P. Díaz (2009a). *Calcareous amendments in truffle culture: a soil nutrition hypothesis*. SOIL BIOLOGY and BIOCHEMISTRY 41: 1227-1232. JCR: Q1 in Soil Science Area (1st from 31 journals); Impact Factor 2009 = 2.978
- d.7. L.G. García-Montero, I. Valverde-Asenjo, P. Díaz, C. Pascual, C. Menta (2010). *New data on impact of earthworms activity on black truffle soils*. In: D. Donnini, B. Falini, B. Mattia, G. di Massimo (Eds.). *Atti dell 3º Congresso Internazionale di Spoleto sul Tartufo*. Comunità Montana dei Monti Martani, Spoleto. Pg.: 723 (COMMUNICATION IN CONGRESS)
- d.8. L.G. García-Montero (2012-2015). SEMINAR CYCLE in the Dpt. Crop & Soil Science of the Oregon State University, The North American Truffling Society and the Cascade Mycological Society in Oregon (EEUU) (2012); in the University of Parma (2013); in the University of Montpellier SupAgro (2013); and in the Institute of Ecology (Soil Sci. and Protection) of the Technische Universität Berlin (2015); titled: "Research on the ecology of black truffles in Central Spain: could ectomycorrhizae be liming host plant roots?" or "Model on soil-nutrient management through fungus-plant interaction.
- d.9. L.G. García-Montero, I. Valverde-Asenjo, D. Moreno, P. Díaz, I. Hernando, C. Menta, K. Tarasconi (2012). *Influence of edaphic factors on edible ectomycorrhizal mushrooms: new hypotheses on soil nutrition and C sinks associated to ectomycorrhizae and soil fauna using the Tuber brûlé model*. In: A. Zambonelli, G. Bonito (Eds.). *Edible Ectomycorrhizal Mushrooms*. Springer-Verlag, Berlin. Pp. 83-104 (ABSTRACT of a BOOK CHAPTER; see the complete chapter is in the Annexes g.1)

- d.10.** L.G. García-Montero, I. Valverde-Asenjo, M.A. Grande-Ortíz, C. Menta, I. Hernando **(2013)**. *Impact of earthworm casts on soil pH and calcium carbonate in black truffle burns*. AGROFORESTRY SYSTEMS 87: 815-826. **JCR: Q2** in Forestry Area (32th from 79 journals); Impact Factor 2013 = 1.240
- d.11.** F. Arredondo, L.G. García-Montero, I. Valverde-Asenjo, C. Menta **(2014)**. *Soil quality indicators for forest management*. In: E. Martínez-Falero, S. Martín-Fernández, A. García-Abril (Eds.). *Quantitative Techniques in Participatory Forest Management*. CRC press, Boca Raton. pp 179-240 (ABSTRACT of a BOOK CHAPTER; see the complete chapter is in the Annexes **g.12**)
- d.12.** C. Menta, L.G. García-Montero, S. Pinto, F.D. Conti, G. Baronía, M. Maresi **(2014)**. *Does the natural "microcosm" created by Tuber aestivum affect soil microarthropods? A new hypothesis based on Collembola in truffle culture*. APPLIED SOIL ECOLOGY 84: 31-37. **JCR: Q1** in Soil Science Area (8th from 34 journals); Impact Factor 2014 = 2.644
- d.13.** L.G. García-Montero, F. García-Robredo, S. Ortuño, E. Ayuga, L. Pinto, P. Pita, C. Pascual, A. Alvarez, L. Benito-Matías, M.P. Andrés **(2015a)**. *Basis on rhizoculture: management of "underground agroforestry systems" to improve their climate change resilience*. In: E. Tielkes (ed.) *Book of Abstracts of Tropentag 2015*. Cu villier, Gottingen. Pg. 330 (COMMUNICATION IN CONGRESS)
- d.14.** L.G. García-Montero, V. Monleon, D. Myrold, T. Kuyper, J. Trappe, J. Baham, I. Valverde-Asenjo, X. Parladé, A. Álvarez, L.F. Benito, J.R. Quintana, A. Quintana, P. Andrés, M. Morcillo, C. Menta, S. Pinto, P. Díaz, C. Lefevre, D. Luoma, C. Turkmen, J.A. Domínguez, A. Santiago, L. Pinto, P. Pita, F. García-Robredo, S. Ortuño, J. Pera, E. Verrecchia **(2015b)**. *Could mycorrhizae perform "ecosystem engineering" on the host plants? Basis on rhizoculture of agroforestry systems to improve their resilience*. In: P. Grenni and A. Bevivino (Eds.). *Soil Biological Communities and Aboveground Resilience. Proceedings of the 3rd Annual Meeting*. Cost Action FP1305 "Biolink-Linking belowground biodiversity and ecosystem function in European forests", Rome. Pg. 26 (COMMUNICATION IN CONGRESS)
- d.15.** L.G. García-Montero **(2017)** Coordinator of RHIZOFARMING: a Horizon 2020 proposal to research in rhizoculture (ABSTRACT; see the "first stage of the project" complete in the Section **e.4. Future research lines**)
- d.16.** L.G. García-Montero, P. Manzano, D. Alwanney, I. Valverde-Asenjo, A. Álvarez-Lafuente, L.F. Benito-Matías, X. Parladé, S. Ortuño, M. Morcillo, A. Gascó, C. Calderón-Guerrero, F. Mauro, M. Méndez, A. Sánchez-Medina, M.P. Andrés, J.R. Quintana, C. Menta, S. Pinto, L. Pinto, P. Pita, C. Turkmen, C. Pascual, E. Ayuga, F. Torrent, J.C. Robredo, P. Martín-Ortega, J. Pera, L. Gómez, G. Almendros, C. Colinas, E.P. Verrecchia **(2017)**. *Towards integrated understanding of the rhizosphere phenomenon as ecological driver: can Rhizoculture improve agricultural and forestry systems?* In: M. Lukac, P. Grenni and M. Gamboni (Eds.). *Soil biological communities and ecosystem resilience*. Springer International Publishing, Cham, Switzerland. Pp 43-75 (ABSTRACT of a BOOK CHAPTER; the complete chapter is in the Annexes **g.3**)
- d.17.** L.G. García-Montero **(2018)** Coordinator of RHIZOINTENSE: a Horizon 2020-PRIMA proposal to research in rhizoculture (ABSTRACT; see Section **e.4. Future research lines**)

Ecological patterns of *Tuber melanosporum* and different *Quercus* Mediterranean forests: Quantitative production of truffles, burn sizes and soil studies

Luis G. García-Montero^{a,*}, José L. Manjón^b, Cristina Pascual^c, Antonio García-Abril^d

^a Department of Forestry Engineering, Technical University of Madrid (UPM), E.T.S.I. Montes, Ciudad Universitaria s/n, Madrid 28040, Spain

^b Department of Plant Biology, University of Alcalá, Crtra. Barcelona Km 33, Alcalá de Henares 28870, Spain

^c Department of Environmental and Chemistry Technology, University of Rey Juan Carlos, C/Tulipán s/n, Móstoles 28933, Madrid, Spain

^d Department of Projects and Rural Planning, Technical University of Madrid (UPM), E.T.S. Ingenieros de Montes, Ciudad Universitaria s/n, Madrid 28040, Spain

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Abstract

Most studies on *Tuber melanosporum* fail to supply statistical data on carpophore production and other quantitative data associated with truffle ecology, such as the development of its burns (truffle production sites). During 7 years, we studied 208 burns associated with *Quercus ilex* subsp. *ballota*, *Q. faginea*, *Corylus avellana* and *Tilia platyphyllos* in the centre of the Iberian Peninsula. The results showed that burn size is a very significant factor as it accounts for 38% of the variance in carpophore production. However, no significant difference in the size of burns was observed between five types of wood, although significant variability in carpophore production existed between these wood types. Furthermore, statistical analysis shows that a high concentration of active carbonate in the soil accounts for up to 51% of the variance in *T. melanosporum* burn size. The ecological conditions of burns cause elevated concentrations of active carbonate and exchangeable Ca^{2+} , and *T. melanosporum* activity and burn size are simultaneously favoured by a high concentration of both factors, which suggests a feedback process. These results may indicate a possible application for the use of calcareous amendments in natural burns and truffle cultivation. Moreover, we also determined the considerable truffle-producing ability and mycorrhizing capacity of *Quercus faginea*. Although *Q. faginea* is a very little-known species in truffle culture, the results provide support for its implementation.

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Keywords: Active carbonate; *Quercus faginea*; *Tuber melanosporum*; Truffle burn; Truffle production; Truffle soil

1. Introduction

The production of fungi provides higher economic returns than any other forest product in many Mediterranean woods. In these areas, truffles (*Tuber Micheli* ex Wiggers) and mushrooms of *Boletus pinophilus* Pilát and Dermek, *B. edulis* Bull: Fr. and *Lactarius deliciosus* L.: Fr. are the fungi that yield the highest economic benefits. Among the *Tuber* species, the black Périgord truffle (*T. melanosporum* Vittad.) is especially appreciated for its culinary value due to its intense aroma

(economic value more than €1000 kg⁻¹). In addition, *T. melanosporum* is of great ecological and agricultural value because it inhabits carbonated soil in impoverished mountain ecosystems within the Mediterranean continental climate (Moreno et al., 1986; Oria, 1989, 1991; Montecchi and Sarasini, 2000; Rioussel et al., 2001).

The biology of *Tuber melanosporum* has been fully studied and described in extensive monographs. However, there are gaps in our knowledge of *T. melanosporum* biological cycle, such as its adaptive responses to the physical environment, factors inducing fruiting, soil influence in truffle development and interactions with host plants and other fungi, etc. According to Bencivenga (1999) and Chevalier (1999), production of *T. melanosporum* has barely advanced, and current interests in truffle production are focusing more on ecological approaches. Also, most studies of *T. melanosporum* fail to supply statistical

* Corresponding author at: U.D. Operaciones Básicas, Department of Forestry Engineering, E.T.S. Ingenieros de Montes, The Technical University of Madrid (UPM), Ciudad Universitaria s/n, Madrid 28040, Spain.
Tel.: +34 91 3365565; fax: +34 91 5439557.

E-mail address: luisgonzaga.garcia@upm.es (L.G. García-Montero).

data on carpophore production and other quantitative data associated with the ecology of truffles, such as the development of burns (Bencivenga and Granetti, 1990; Pacioni and Marra, 1993; Callot, 1999; Courvoisier et al., 1999; García-Montero, 2000; Montecchi and Sarasini, 2000; Rioussset et al., 2001; Ricard, 2003).

The production points of *T. melanosporum* are known as burns. The term “burn” is used to describe the spots where *T. melanosporum* grows and refers to its phytotoxic capacity and its ability to create clearings in the vegetation where its carpophores bear fruit (Fasolo-Bonfante et al., 1971; Montacchini et al., 1972; Papa, 1992). The environmental conditions created by burns are very favourable for the development, competition and fruition of this truffle species. *T. melanosporum* burns generally present a circular shape around the symbiotic log, and the surface area spreads outwards at a speed of 10–15 cm per year. It must be remembered that the burn does not appear if there are no *T. melanosporum* mycorrhizas present. The burn formation occurs when the mycorrhized roots come close to the soil surface (5–10 cm deep). The growth of the burn follows the radial development of the surface roots carried by *T. melanosporum*'s mycorrhizas, sometimes reaching areas several dozen meters in diameter. Carpophore production takes place inside the burns, mainly behind the burn line, positioned according to climatic conditions. Sourzat (2004), Granetti et al. (2005) and other authors have explained the production and development of the burns, nevertheless, Callot et al. (1999) and Ricard (2003) indicate that the ecological conditions relating to the development of burns have yet to be clarified.

In view of the need to expand current knowledge of the ecology of *T. melanosporum*, and with the aim of increasing production of this valuable forest resource, we have carried out a study on the quantitative production of carpophores, the size of *T. melanosporum* burns and their soil properties in different woods situated close together in the Alto Tajo Nature Reserve, in the central region of Spain. This region is a suitable area for the study of *T. melanosporum* since it is a continental elevated region with particular substrates and plants whose relationships with *T. melanosporum* are unknown. In this region, *T. melanosporum* is associated with *Quercus faginea* Lam., *Q. ilex* L. subsp. *ballota* (Desf.) Samp., *Tilia platyphyllos* Scop. and *Corylus avellana* L., in different wood types. Data published by García-Montero et al. (2005) substantiates these observations, and these authors indicate that this study area has a maximum total production of 500 kg of *T. melanosporum* carpophores per year.

The primary objective of the present work was to study the carpophore production and size of 208 burns of *T. melanosporum* in order to analyze whether significant differences existed between five types of woods. The second objective was to explain more fully the relationship between carpophore productivity and burn size, and to analyse the soil interactions with burn development through a quantitative statistical estimation of the influence of the conventional surface horizon properties (granulometric texture, carbonate fractions, organic carbon, total nitrogen, exchangeable cations).

Many authors studied the relationships between *T. melanosporum* and *Quercus ilex* or *C. avellana*, and their use in truffle culture. However, the ecology and the symbiosis between *Q. faginea* and *T. melanosporum* are relatively unknown. For this reason, the last objectives of the present work were to study the truffle production and soil features in carpophore-producing *Q. faginea* formations in the study area, and to determine the ability of this plant to establish mycorrhizas with *T. melanosporum* in laboratory conditions.

Q. faginea is a gall oak species (known as “quejigo”) with a western Mediterranean distribution, whose populations are present in the majority of the regions of the Iberian Peninsula, northwest Africa and the Balearic Islands. This plant has arboreal and shrub-like forms, and inhabits areas under a non-extreme continental Mediterranean or sub-Mediterranean climate. The nature of the substratum is unimportant, but it prefers calcareous or clay-calcareous soils and it is more xerophytic than oaks (*Q. robur* L. and *Q. petraea* (Mattuschka) Liebl., etc.). It has been observed in the altitudinal range between 500 and 1500 m (1900 m) (Castroviejo, 1990).

2. Materials and methods

2.1. Case study area

The study area covers a 10 km radius between the municipal areas of Peralejos de las Truchas (Guadalajara) and Belvalle (Beteta, Cuenca) (Geographic coordinates: 40°35'49.65"N; 1°54'12.78"W) (Fig. 1). This area is situated in a mountainous region at an altitude of more than 1000 m, in a supra-Mediterranean bio-climatic belt with a sub-humid, shady climate. Average annual precipitation is 797 mm, with low average annual temperatures (9.7 °C) and very cold winters. The lithology of the area is dominated by Jurassic and Cretaceous limestone and dolomites, and soils are frequently lithic and rendzic leptosols with marked surface stoniness and high peaks. The vegetation of the study area fell within the Mediterranean Ibero-Levantina Province Castellano Maestrazgo-Manchega Subprovince geobotanical classification type

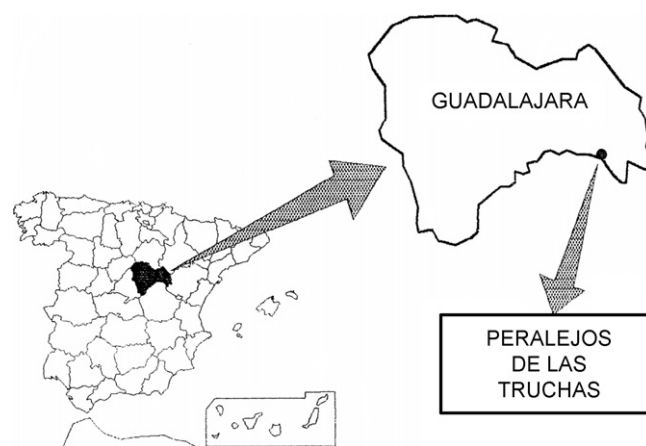


Fig. 1. Location of the study area.

(Peinado and Martínez, 1985; Rivas-Martínez, 1987; ITGME, 1989; Rivas-Martínez and Loidi, 1999).

2.2. Wood types

García-Montero et al. (2005) described the characteristics of five wood types, defined as a function of the vegetation, symbiotic plants, physiography and stoniness. These five wood types are summarized as follows.

2.2.1. Wood I

Woods of *Q. faginea* with *T. melanosporum* burns located in mono-species *Q. faginea* formations of the geobotanical vegetation series *Cephalantho longifoliae–Querceto fagineae* S. The physiography of this wood type was variable but in line with the general topography of the upper Tajo Basin. Truffle sites occurred on steep slopes with a mean gradient of 19%. Moreover, 24% of the burns appeared on slopes of 30–70%. Surface stoniness was high and accounted for 73% of the cover, on average, with 45% of the burns located in a region with stones in a proportion of as high as 90–100%. Inventories were established for 83 burns.

2.2.2. Wood II

Mixed woods of *Q. faginea* and *Q. ilex* subsp. *ballota*. These mixed woods were small and, compared to Wood I, the soil was more rocky and clayey and often more exposed to the sun. The oaks of these permanent communities were related to more xeric lithologies and occurred in an isolated manner in the domain of the association between *C. longifoliae–Q. fagineae* S. The physiography of this wood was variable, with areas of mild topography alternating with more abrupt zones. The burns occurred on steep slopes, with a mean slope for the area of 19%. Eighteen percent of sites had slopes in the 35–70% range. Surface stoniness was high, representing 76% of the cover, on average, and 41% of the burns were located in an area with a 90–100% proportion of stones. Inventories were established for 38 burns in this wood type.

2.2.3. Wood III

Mixed woods of *C. avellana*, *T. platyphyllos* and *Q. faginea*. These woods contained riparian formations hosting *T. melanosporum* in areas of abrupt topography and ravines. This wood type is composed of mixed formations of the geoseries *Astrantio-Coryleto avellanae* S. Physiography was varied, but generally abrupt. The burns occupied steep slopes with an average gradient of 22%, at 17% incidence on slopes of 40–70%. Surface stoniness was substantial and occupied a mean area of 60%, with 25% of burns located in regions containing 100% stones. Inventories were established for 27 burns.

2.2.4. Wood IV

Mixed woods of *C. avellana* and *Q. faginea*. These woods were formed by hazel trees and oaks related to riparian geoseries of *A.-C. avellanae* S. and *C. longifoliae–Q. fagineae* S. The physiography of this wood type was mild and its truffle burns appeared on practically flat areas with a 5% average

slope. Surface stoniness was low, with an average cover of 33%. Inventories were established for 25 burns.

2.2.5. Wood V

Woods of *Q. ilex* subsp. *ballota*. This wood type was composed of trees of the series *Junipero thuriferae–Querceto rotundifoliae* S. The vegetation in these areas was clearly more xeric than in the previous woods. These oak woods appeared on gentle slopes or on practically flat areas with a mean slope of <10%, with intermediate stoniness, occupying a mean area of <60%. Inventories were established for 35 burns.

2.3. Study of the productivity and burn size of *T. melanosporum*

T. melanosporum has been harvested in these woods since the end of the 1960s. Harvesting is done according to a system established by the corresponding local councils in order to restrict the number of collectors. Carpophores are harvested with the help of trained dogs. This study was based on the collaboration with six truffle collectors who shared their knowledge and collection data, including the location of several harvesting points. During many expeditions undertaken between 1994 and 2000 with these collectors, 208 of the collectors' favourite burns were selected. In this sample of 208 *T. melanosporum* burns, the size of the burns (m²), the carpophore production, and the plant composition were determined.

The maximum annual production of *T. melanosporum* carpophores was calculated as an expression of the greatest quantity of fresh carpophores, in grams, picked each year over a period of 15 years. Harvesting data supplied by six gatherers who exploited these 208 burns regularly were used. The reliability of the information provided by the collectors was confirmed independently in systematic field trips made with the collectors at the time of harvesting throughout the period between 1994 and 2000. The exact geographic coordinates of these burns were not given, in order to maintain the secrecy of the *T. melanosporum* locations.

2.4. Soil analysis

Twenty soils were selected according to the *T. melanosporum* burn size: six samples were taken from the interior of bigger burns (10–28 m²) and eight samples from smaller burns (4–9 m²). The soil samples were taken from the bare soil in the burn, at those points inside the burn where the greatest number of carpophores had been extracted in recent years. Six reference samples were taken from patches of bare soil without production situated at least 50 m from the burns. Only the first 30 cm of each soil profile was studied because *T. melanosporum* usually bears fruit in this range (Verlhac et al., 1990). These samples include soil surface horizons that have not been differentiated because *T. melanosporum* natural burns contain a constant mixture of horizons, due to the continuous digging that the harvesters, their dogs and wildlife engage in to extract the carpophores.

Sampling was done according to the FAO (1990). The gradient of each plot was measured with a clinometer. Surface stoniness

and litter were estimated as percentages. The following soil determinations were made: pH, total organic carbon (T.O.C.), total calcium carbonate (equivalent carbonate), granulometric analysis, cationic exchangeable capacity (C.E.C.) and the exchangeable cation saturation percentage (%V), following the methods of the ISRIC (1995); the textures were classified according to the International Society of Soil Science (I.S.S.S.); the total nitrogen was analysed using the methodical variant of Bouat and Crouzet (1965); and the active carbonate (carbonate extractable with ammonium oxalate) was determined according to AFNOR (1982). The exchangeable cations of Ca^{2+} and Mg^{2+} were determined by AAS (Philips UP9100x), and K^{+} and Na^{+} with a flame photometer (Sherwood 410).

2.5. Statistical analysis

Based on the size and production of 208 burns of *T. melanosporum*, a statistical analysis was conducted to determine whether significant differences in production and burn size existed between the different wood types. We also analysed the soil interactions with burn development through a quantitative statistical estimation of the influence of the surface horizon properties. Statistical treatment was performed using the Statistica Program v. 6 (StatSoft, Inc., Tulsa, Ok, 1999). Before proceeding with the analysis, the necessary transformations were made to adjust the distributions of the variables to the prerequisites required for statistical analysis. Normality was checked using the Shapiro–Wilks test. In addition, for the ANOVA test, the homogeneity of variances was checked using the Levene test.

2.6. Study of *T. melanosporum* mycorrhizas

The mycorrhizal synthesis of *T. melanosporum* with *Q. faginea* was determined by applying a method of intensive mycorrhization of *T. melanosporum* on 20 *Q. faginea* plants, as described by Bencivenga (1982), but modified according to Manjón and García Montero (1996). Acorns and carpophores from the study area were grown in forestry containers 20 cm deep and with a surface area of 5 cm × 5 cm. The substrate used for seeding was a mixture of limestone from the soil in the study area mixed with vermiculite and perlite and sterilized in an autoclave at 120 °C, in order to eliminate any mycorrhizal contamination. The plants were kept for 15 months under controlled environmental conditions in the Juan Carlos I Royal Botanical Garden at the University of Alcalá (Madrid, Spain). The mycorrhizas were identified with a stereoscopic microscope (Leica WildMZ8) and an optical microscope (Leica LeitzDMRB) following the descriptions and recommendations of Verlhac et al. (1990), Bencivenga et al. (1995), Donnini and Bencivenga (1995) and Granetti (1995). The degree of mycorrhization was expressed as the percentage of radicular mycorrhized apexes, according to the procedure of Bencivenga et al. (1987).

3. Results

From the 208 burns located in the study area, a scatter plot of “ L_n (production)” with “ L_n (area)” was produced. A linear

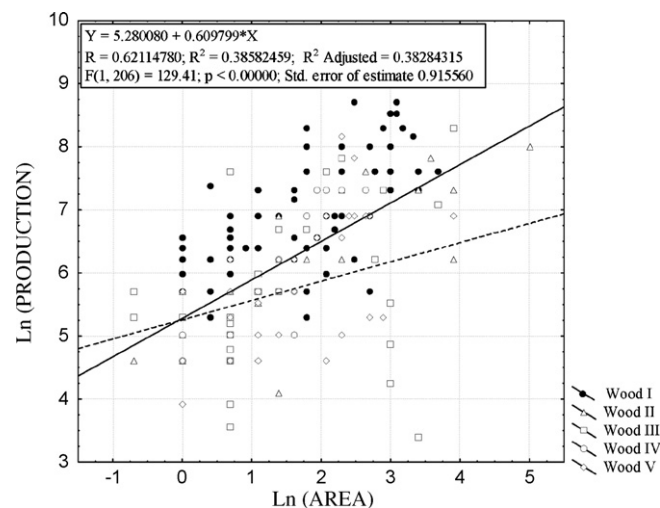


Fig. 2. Scatterplot of linear regression of “ L_n (PRODUCTION)” with “ L_n (AREA)”: truffle yields and sizes of 208 burns found in five wood types. The continuous line represents the regression function for the 208 burns. The dotted line represents the regression model for the 27 burns found in Wood type III, with these results: $y = 5.258391 + 0.305631x$; $R = 0.3021$; $R^2 = 0.0913$; R^2 adjusted = 0.0550 (p -level = 0.1256 no significative).

Table 1
ANOVA of “ L_n (annual production)”; factor: wood type

Source	SS	d.f.	MS	F	p-Level
Model	52.14885	4	13.03721	11.55535	0.000000
Residuals	229.0329	203	1.128241	–	–

regression function was estimated relating L_n (production) and L_n (area) for each of the 208 burns (Fig. 2). It was observed that truffle production increased with the burn size ($r = 0.621$; $n = 208$; $p < 0.001$), and that size accounted for 38% of the variance in carpophore production (R^2 adjusted = 0.3828).

An ANOVA test was performed to determine whether production varied among the five wood types. A very significant difference ($F_{4,203} = 11.55$; $p < 0.001$) was confirmed (Table 1). The average carpophore production values determined in each wood type are illustrated in Table 2. Some wood types demonstrated higher production values. A ‘post hoc’ (Tukey) test was conducted to determine which averages were different (HDS Tukey test, probabilities for post hoc tests; wood I versus wood II: $p = 0.000818$; wood I versus wood III: $p = 0.000051$; wood I versus wood IV: $p = 0.000041$; wood I versus wood V: $p = 0.000028$. The other probabilities for post hoc tests were not significant). The results showed that production was significantly higher in Wood I (mono-species *Q. faginea* formations of the geobotanical vegetation series *C. longifoliae*–*Q. fagineae* S.) (Table 2).

A second ANOVA was conducted to determine whether the surfaces of the burns showed any changes among the five wood types. The statistical test confirmed that no significant differences existed in the size of the burns ($F_{4,203} = 1.14$; $p = 0.339$) (Table 3). Table 2 illustrates a summary of burn sizes in the five wood types in which *T. melanosporum* were found. In another regression model established between the production

Table 2

Truffle yields and sizes of 208 burns found in five wood types

Wood features	Wood I	Wood II	Wood III	Wood IV	Wood V	Total
Vegetation	Woods of <i>Q. faginea</i>	Mixed woods of <i>Q. faginea</i> and <i>Q. ilex</i>	Mixed woods of <i>T. platyphyllos</i> , <i>C. avellana</i> and <i>Q. faginea</i>	Mixed woods of <i>C. avellana</i> and <i>Q. faginea</i>	Woods of <i>Q. ilex</i> with <i>Juniperus thurifera</i>	–
Geobotanical classification	<i>C. longifoliae</i> – <i>Q. fagineae</i> S.	<i>C. longifoliae</i> – <i>Q. fagineae</i> S.	A.– <i>C. avellanae</i> S.	A.– <i>C. avellanae</i> S. and <i>C. longifoliae</i> – <i>Q. fagineae</i> S.	<i>J. thuriferae</i> – <i>Q. rotundifoliae</i> S.	–
Slope mean	19%	19%	22%	5%	<10%	–
Stoniness mean	73%	76%	60%	33%	<60%	–
No. burns	83	38	27	25	35	208
Mean yield (g/burn)	1351	743	686	534	577	925
S.D.	1322	825	951	586	712	1084
Minimum yield	100	60	30	100	50	30
Maximum yield	6000	3000	4000	1500	3500	6000
Mean burn area (m ²)	8	19	10	4	8	10
S.D.	8	36	13	4	9	18
Minimum area	1	0.5	1	1	1	0.5
Maximum area	40	150	50	14	50	150

Table 3

ANOVA of “ L_n (area)””; factor: wood type

Source	SS	d.f.	MS	F	p-Level
Model	6.402963	4	1.600741	1.138808	0.339361
Residuals	285.3425	203	1.405628	–	–

and the size of the 27 burns in the least productive wood (Wood type III) a non-significant function was obtained (p -level = 0.1256; Fig. 2).

Tables 4–6 show the conventional parameters and characteristics of the surface horizons in the study. Many of them are situated on steep inclines with considerable surface stoniness and

moderately abundant fine sand and clay, which produce a texture that tends to be sandy clay loam soil. Most have a moderately basic pH and a low percentage of total carbonate, but an elevated concentration of active carbonate. The levels of organic carbon are moderate and the C/N ratio is close to 10, which indicates good humidification. They have high exchangeable cation complex values and 100% saturated exchangeable cations, with a high proportion of exchangeable Ca^{2+} , Mg^{2+} of abundant but relatively heterogeneous exchange, K^+ in large and relatively homogeneous concentrations, and scarce and variable Na^+ . They are well-structured, with a granular tendency and abundant pores, characteristics that are closely related to texture and levels of organic carbon and calcium carbonate.

Table 4

Description of the localization, vegetation, stoniness and topography of the soils studied

No.	Burn size (m ²)	Vegetation	Altitude	Orientation	Topography	Slope (%)	%surface stoniness	%below ground stoniness
1	28	Open wood of <i>Q. faginea</i>	1090	W	Slope	20	90	70
2	22	Open pine wood with <i>Q. faginea</i>	1250	E-NE	Flat on slope	30	60	20
3	20	Open wood of <i>Q. faginea</i>	1200	S	Slope	15	60	50
4	14	Thicket with <i>Q. ilex</i> and <i>Q. faginea</i>	1290	E-SE	Slope	70	20	15
5	13	Open wood of <i>Q. ilex</i>	1140	S-SW	Slope	20	100	80
6	10	Thicket with <i>Q. ilex</i>	1235	S	Almost flat	5	25	25
7	9	Open wood of <i>T. platyphyllos</i> with <i>Q. faginea</i>	1080	W-NW	Slope	40	70	40
8	8	Open wood of <i>Q. faginea</i>	1215	E-NE	Slope	30	50	30
9	6	Thicket with <i>Q. faginea</i>	1155	E-SE	Low undulate hills	5	20	10
10	6	Open wood of <i>Q. faginea</i>	1185	S-SW	Hilly	15	100	60
11	5	Mixed wood of <i>C. avellana</i> and <i>Q. faginea</i>	1000	1000	Almost flat	0	30	0
12	4	Thicket with <i>Q. faginea</i>	1000	S-SW	Hilly	10	20	20
13	4	Open wood of <i>Q. faginea</i>	1095	SW	Slope	40	90	75
14	4	Wood of <i>C. avellana</i> , <i>T. platyphyllos</i> and <i>Q. faginea</i>	>1100	S	Steep craggy slope	70	85	40
15	0	Thicket with <i>Q. faginea</i>	1150	SW	Low undulate hills	5	20	10
16	0	Wood of <i>Q. faginea</i>	1285	E	Hilly	50	0	50
17	0	Thicket with <i>Q. faginea</i>	1185	N	Low undulate hills	5	5	10
18	0	Pine wood with <i>Q. faginea</i>	1100	NE	Slope	30	50	20
19	0	Wood of <i>C. avellana</i>	>1100	N	Moderate sloped hills	5	40	0
20	0	Wood of <i>C. avellana</i>	>1100	NE	Slightly hilly	3	5	5

Table 5

Structure and texture of soils of *T. melanosporum* burns and reference soils

No.	Burn size (m ²)	%coarse sand	%fine sand	%total sand	%silt	%clay	Texture
1	28	6	22	28	59	13	Silt loam
2	22	8	36	44	19	37	Coarse clay
3	20	38	28	66	22	12	Sandy loam
4	14	18	47	64	13	23	Sandy clay loam
5	13	26	19	45	32	23	Coarse clay
6	10	23	32	55	10	35	Sandy clay
7	9	35	47	82	8	11	Sandy loam
8	8	29	47	77	9	15	Sandy clay loam
9	6	47	21	68	13	19	Sandy clay loam
10	6	14	26	41	14	46	Fine clay
11	5	6	53	59	7	34	Sandy clay
12	4	42	40	82	3	14	Sandy loam
13	4	14	33	47	19	34	Coarse clay
14	4	22	14	37	38	25	Clay loam
15	0	43	39	83	6	12	Sandy loam
16	0	13	39	52	40	8	Loam
17	0	25	40	64	12	24	Sandy clay loam
18	0	7	7	14	17	69	Fine clay
19	0	19	42	62	17	21	Sandy clay loam
20	0	36	28	64	10	26	Sandy clay

Simple correlations were obtained between the quantitative variables of the surface soil horizons and the burn sizes (Table 7). The results show that active carbonate has a positive and very significant correlation with burn size ($R = 0.7310$; $p < 0.001$). A forward stepwise regression of these soil variables was done in order to distinguish the soil variables potentially associated with burn size. The aim of the multiple regression analysis was to quantify the overall influence of the soil variables on the development of *T. melanosporum* burns. The multiple regression model obtained without intercept (Table 8) incorporates the active carbonate as a single independent variable to explain the size of the burns. The

ANOVA for the model (Table 9) indicates that it is very significant ($p < 0.001$). Finally, the model shows that the percentage of active carbonate accounts for 51% of the variance in burn size (R^2 adjusted = 0.5058). Residual analysis confirmed the assumptions of normality and homocedasticity of the sample, and no atypical values were detected.

To determine whether a relationship existed between *Q. faginea* and *T. melanosporum*, we examined the ability of *T. melanosporum* to form mycorrhizas with *Q. faginea*. The seed plants from the greenhouse located at the University of Alcalá demonstrated that 60–80% of the root tips formed mycorrhizal fungi with *T. melanosporum*. The microscopic study of the

Table 6

Analytical results of the soils of *T. melanosporum* burns and reference soils

No.	pH _{H2O}	%CaCO ₃ total	%CaCO ₃ active	T.O.C.	N	C/N	Ca ²⁺	Mg ²⁺	Na ⁺	K ⁺	C.E.C.	%V
1	7.80	7.19	2.88	7.52	0.56	13.43	46.16	3.15	0.00	0.73	50.04	Saturated
2	7.90	3.12	2.25	3.40	0.25	13.60	13.19	7.92	2.13	1.03	24.27	Saturated
3	7.88	7.88	7.01	3.56	0.27	13.19	21.14	0.74	0.45	1.10	23.44	Saturated
4	8.15	4.18	2.63	1.16	0.18	6.44	11.19	4.89	0.08	0.29	16.46	Saturated
5	7.90	11.50	8.14	3.61	0.23	15.70	18.87	0.41	0.00	0.22	19.49	Saturated
6	8.15	4.53	2.38	1.08	0.09	12.00	15.49	1.85	0.27	0.51	18.12	Saturated
7	7.90	10.21	3.38	2.55	0.27	9.44	27.49	1.48	0.00	0.29	29.26	Saturated
8	8.07	6.28	0.38	0.53	0.00	–	5.16	6.34	0.08	0.22	11.80	Saturated
9	7.10	14.56	1.50	0.10	0.00	–	9.65	1.79	0.00	0.22	11.66	Saturated
10	7.50	12.79	1.63	2.30	0.25	9.20	20.17	0.60	0.08	1.47	22.32	Saturated
11	7.92	4.38	1.57	1.96	0.18	10.89	12.7	2.13	0.08	0.07	14.99	Saturated
12	7.90	9.18	0.38	3.15	0.29	10.86	5.23	4.98	0.08	0.37	10.66	Saturated
13	7.95	7.81	2.88	3.11	0.29	10.72	12.04	2.63	0.00	0.66	15.33	Saturated
14	8.17	13.38	0.21	2.86	0.38	7.53	18.43	0.78	0.08	1.03	20.33	Saturated
15	7.70	14.02	0.38	0.06	0.00	–	4.77	3.43	0.00	0.29	8.50	Saturated
16	7.70	9.03	0.63	7.34	0.51	14.39	30.71	6.31	0.08	1.47	38.57	Saturated
17	8.17	9.66	1.50	0.74	0.06	12.33	10.13	3.62	0.08	0.29	14.13	Saturated
18	7.92	16.35	0.18	5.31	0.36	14.75	24.89	8.35	0.08	1.99	35.32	Saturated
19	7.85	0.21	0.00	9.17	0.74	12.39	23.49	6.50	0.00	0.66	30.65	Saturated
20	7.00	18.44	0.00	4.32	0.38	11.37	16.01	1.54	0.00	1.10	18.66	Saturated

T.O.C. and N expressed in g kg⁻¹; C.E.C. and exchangeable cations expressed in cmol kg⁻¹.

Table 7
Simple correlations between size of *T. melanosporum* burns and soil variables

Variables	Correlation with the square root of the burn size		
	<i>r</i>	<i>p</i>	<i>n</i>
%CaCO ₃ total	−0.3864	0.092	20
<i>L_n</i> (<i>x</i> + 1) %CaCO ₃ active	0.7310	<0.001**	20
%T.O.C.	−0.1218	0.609	20
%N	−0.1141	0.632	20
% Course sand	−0.1341	0.573	20
%Fine sand	−0.0318	0.894	20
<i>L_n</i> (%silt)	0.2624	0.264	20
<i>L_n</i> (%clay)	−0.0592	0.804	20
C.E.C.	0.1435	0.546	20
Ca ²⁺ exchangeable	0.2057	0.384	20
Mg ²⁺ exchangeable	−0.2423	0.303	20
<i>L_n</i> (K ⁺ exchangeable)	−0.1323	0.578	20

** very significant.

Table 8
Forwarded stepwise regression summary for dependent variable: squared (burn size)

Variable	<i>L_n</i> (<i>x</i> + 1) %CaCO ₃ active
BETA	0.903756
Standard error BETA	0.098201
B	2.294488
Standard error B	0.249316
<i>t</i>	9.203133
<i>p</i> -Level	<0.0000

morphological characteristics of the roots permitted clear identification of *T. melanosporum* mycorrhiza by its spinulae (particularly short hyphae growing out of the mantle and differing from external mycelium) and sheath ornamentation (Palenzona, 1969).

4. Discussion

The results demonstrated that carpophore production of *T. melanosporum* increased in pace with the increase in burn size, and we have quantified this relationship as: “burn size explains 38% of the variance in production”. Nevertheless, there was no significant difference in burn size among the five wood types tested in the study area. In summary, the size of burns was a very significant feature in the study and production of *T. melanosporum*, but the variability in the size of burns was not associated with the variables used to define the five wood types (vegetation, symbiotic plants, physiography and stoniness).

Table 9
ANOVA of the regression model dependent variable: squared (burn size)

Source	SS	d.f.	MS	<i>F</i>	<i>p</i> -Level
Model	28.4875	1	28.4875	19.30772	<0.001
Residuals	28.03342	19	1.475443	–	–

Multiple *R* = 0.7099; multiple *R*² = 0.5040; adjusted *R*² = 0.5058; *F*(1, 19) = 19.3077; *p* < 0.001; standard error of the model = 1.4754.

In order to understand the processes related to burn development, the next step in our study was to study the statistical relationship between burn size and conventional soil features. The simple statistical correlations and multiple regression analysis show that *T. melanosporum* burn size is related to the percentage of active carbonate present in the soil surface horizons, and we have quantified this relationship as: “active carbonate explains 51% of the variance in burn size”. Nevertheless, the collective influence of the other soil features studied is not significant to the development of *T. melanosporum* burns.

Active carbonate is a finely divided fraction of calcareous rock which measures less than 50 μm, susceptible to rapid mobilization and very chemically active. Continuous formation of active carbonate maintains high Ca²⁺ levels in the soil solution and the exchangeable cation complex, as it counterbalances losses from leaching and other processes. Ricard (2003) indicated that it is difficult to judge the impact of active carbonate on truffle development because several factors must be considered. However, this author suggests that the lack of studies on active carbonate in *T. melanosporum* production is an oversight. García-Montero (2000) and García-Montero et al. (2006) found that active carbonate and exchangeable Ca²⁺ account for up to 40% of *T. melanosporum* carpophore production. The presence of active carbonate and exchangeable Ca²⁺ is very important to *T. melanosporum* for various reasons, including the capacity to regulate soil pH and its participation in the flocculation of the colloidal fraction (clays and humus), which contributes to the organization and maintenance of the soil structure. Both these factors also play a part in the truffle's nutrition.

The availability of active carbonate in the soil depends on complex pedologic, climatic and biological factors. The dissolution, transport and accumulation of carbonates in the soil depend on the relationship between water dissolution and gaseous CO₂, soluble H₂CO₃ and HCO₃[−], and solid CaCO₃ phases. The solubilization and leaching of carbonates increases with pluviosity, respiration and metabolism of roots and mycelia, and other factors that increase the partial pressure of CO₂ (pCO₂) in the soil. Bicarbonate precipitates in the form of carbonate when it is concentrated in capillary water (evaporated or absorbed by the roots). This also occurs when the pCO₂ diminishes due to aeration caused by macro fauna and the disappearance of biological respiration of the roots at depth, among other factors (Poitou, 1988; Bencivenga and Granetti, 1989; Coli et al., 1990; López and López, 1990; Wild, 1992; Marañés et al., 1994; Breemen and Buurman, 1998; Callot, 1999; Ourzik, 1999; Ricard, 2003). *T. melanosporum* eliminate plants from their burns, and the disappearance of roots in the burns favours the accumulation of active carbonate. In summary, the development of the burn encourages the formation of a large amount of active carbonate and exchangeable Ca²⁺. *T. melanosporum* activity and burn size are simultaneously favoured by a high concentration of both factors, which suggests a feedback process (García-Montero, 2000; García-Montero et al., 2006).

The five wood types of *T. melanosporum* showed a significant difference in the amount of productive carpophores.

The mono-species *Q. faginea* formations of Wood I showed much greater productivity, with an average of 1351 g per burn per production year (in the most productive years). Some burns with *Q. faginea* reached a production of 6000 g of carpophores in a single year. However, the formations of *Q. faginea* in Wood I did not show a significantly larger size of burns than in other wood types. Other factors must be therefore be considered in order to explain the production results obtained, and more research is required into the symbiotic relationship with *Q. faginea* and the ecology of the *Q. faginea* formations in Wood I, as their ecological characteristics proved to be very suitable for the production of *T. melanosporum*. We also confirmed that *Q. faginea* mycorrhized easily with *T. melanosporum*.

Q. faginea is relatively little known in truffle production. Martínez and Grigelmo (1991), Reyna (2000), García-Montero et al. (1999) and Granetti et al. (2005) have pointed out that *Q. faginea* is a species of interest for truffle symbiosis. However, Reyna (1992, 2000) emphasizes that not all *Q. faginea* woods present favourable ecological conditions for *T. melanosporum*. This author describes various Spanish *Q. faginea* woods which produce low amounts of carpophores because they exist in a region with a high density of trees and are located on clay soils with flat shady plots and/or northerly orientations. These features enable *Q. faginea* to grow on Mediterranean soils in areas with greater humidity, but this ecology is detrimental to *T. melanosporum*.

Nevertheless the results of the present study show that *Q. faginea* has a higher carpophore production than *Q. ilex* subsp. *ballota* and *C. avellana*, under certain conditions described in the study area. This territory is fairly high, continental and cold, with a sub humid pluviometry, abrupt topography with high slopes and abundant surface stoniness, soils with very suitable humification, structure and porosity for *T. melanosporum*; these features favour water permeability and aeration. The texture of these soils is loam with clay balanced with sand. These properties guarantee an adequate level of soil humidity for the growth of *Q. faginea* (above all in periods of summer drought). However, the high slopes and the soil texture, structure and porosity of these woods prevent soils from becoming too humid for the development of *T. melanosporum*.

5. Conclusions

The size of burns is a variable of considerable significance that accounted for 38% of the variance in *T. melanosporum* production. However, no significant difference in the size of burns was observed among different wood types, although significant variability in carpophore production existed among the wood types examined within a relatively small territory. An elevated concentration of active carbonate accounted for up to 51% of the variance in *T. melanosporum* burn size. The ecological conditions of burns cause elevated concentrations of active carbonate and exchangeable Ca^{2+} , and a high concentration of both these factors simultaneously favours *T. melanosporum* activity and burn size, suggesting a feedback process. These results may indicate a possible application for the use of calcareous amendments in natural burns and truffle

culture. Moreover, the considerable ability and facility of *Q. faginea* to form mycorrhizas with *T. melanosporum* and to produce carpophores was clearly demonstrated. Although *Q. faginea* is a very little-known species in truffle culture, the results support its implementation under the appropriate climatic and soil conditions required for its growth, and the development of *T. melanosporum*.

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ORIGINAL ARTICLE

Soil factors that favour the production of *Tuber melanosporum* carpophores over other truffle species: a multivariate statistical approach

L. G. GARCÍA-MONTERO¹, P. DÍAZ², S. MARTÍN-FERNÁNDEZ³ & M. A. CASERMEIRO⁴

¹Department of Forestry Engineering, E.T.S.I. Montes, Technical University of Madrid (UPM), Madrid, Spain,

²Department of Industrial Chemistry and Polymers, E.U.I.T. Industrial, UPM, Madrid, Spain, ³Department of Economy and Forest Management, E.T.S.I. Montes, UPM, Madrid, Spain, ⁴Department of Soil Science, University Complutense of Madrid, Madrid, Spain

Abstract

The natural harvesting and cultivation of *Tuber melanosporum* (black truffle of Périgord) has spread throughout many countries in recent decades. Other black truffles such as *T. aestivum*, *T. brumale*, and *T. mesentericum* cause damage to *T. melanosporum* exploitation. These truffle species have been found to be very much present in the mycorrhizal contamination of natural *T. melanosporum* burns. Nevertheless, different studies have been unable to determine whether the aggressiveness of *T. melanosporum* and its resistance to this contamination pressure are qualities of the environment or of the soil. Moreover, the biological and physical-chemical conditions causing *T. melanosporum* burns have yet to be clarified. The aim of this study is therefore to establish the relationship between the carpophore presence of *Tuber* species (*T. melanosporum* as compared with *T. aestivum* and *T. mesentericum*) and the concentration of soil variables. Principal component analysis (PCA) and analyses of variance (ANOVAs) show that active carbonate content is the variable that accounts for the greatest percentage of occupancy in the *T. melanosporum* habitat. A high concentration of active carbonate accounts for up to 43% of *T. melanosporum* carpophore production and 51% of the variance in the size of *T. melanosporum* burns. We propose that active carbonate is a major factor in the fruiting and aggressiveness of *T. melanosporum* versus *T. aestivum* and *T. mesentericum*. Moreover, the development of the *T. melanosporum* burn increases the formation of active carbonate, suggesting a feedback process. The results obtained could have applications in natural harvesting and truffle culture.

Keywords: Active carbonate, truffle culture, *Tuber aestivum*, *Tuber melanosporum*, *Tuber mesentericum*.

Introduction

Many researchers and technicians have substantial empirical and unpublished knowledge on biology of truffles. Nevertheless, numerous studies and specialized books have failed to supply statistical data and numerical demonstrations on carpophore production and other quantitative data associated with the ecology of truffles. These circumstances have caused some gaps in the knowledge of truffles' biological cycle, including their adaptive responses to physical environment, factors inducing fruiting, and interactions with host plants and fungi, etc. Thus production of truffle culture has barely advanced since its beginnings, and furthermore current interests in

truffle production are focusing more on ecological approaches (Bencivenga, 1999; Chevalier, 1999).

Tuber melanosporum Vittad. (black truffle of Périgord) has greater culinary and economic value than other black truffle species (with an economic value of over 1000 € kg⁻¹), such as *T. brumale* Vittad., *T. aestivum* Vittad. form *meridionalis* Chevalier & Rioussset, and *T. mesentericum* Vittad. *Tuber melanosporum* is present in different Mediterranean regions (at latitudes of 40–47° N), and its distribution overlaps with the natural regions for *T. aestivum* and *T. mesentericum*.

These species of truffles compete in the same habitats and produce their carpophores on the same

Correspondence: L. G. García-Montero, U.D. Operaciones Básicas, Department of Forestry Engineering, E.T.S. Ingenieros de Montes, Technical University of Madrid (UPM), Ciudad Universitaria s/n, Madrid 28040, Spain. Tel.: +34-91-3365565. Fax: +34-91-5439557. E-mail: luisgonzaga.garcia@upm.es

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productive points. Frequently, *Tuber aestivum*, *T. brumale*, and occasionally *T. mesentericum*, contaminate and cause damage to *T. melanosporum* cultures and reduce their production (Callot, 1999; Rioussset et al., 2001; Ricard, 2003; Sourzat, 2005). Some authors have studied the soil properties which have the greatest impact on truffle interactions (Bencivenga et al., 1998; Lulli et al., 1999; Castignanò et al., 2000; Boumaza et al., 2002). However, Callot et al. (1999), Ricard (2003), and Granetti et al. (2005) pointed out that there is a lack of knowledge as to how the physical-chemical properties of soil influence natural truffle development and truffle cultivation.

In truffle culture, the terms 'burn' or 'brûlé' are used to describe spots where truffles grow and refers to truffles' phytotoxic capacity and their ability to create clearings in the vegetation where their carpophores bear fruit (Fasolo-Bonfante et al., 1971; Montacchini et al., 1972; Papa, 1992; Plattner & Hall, 1995; Ricard, 2003; Granetti et al., 2005). The environmental conditions created by the burns of *T. melanosporum* are very favourable for its development, mycorrhization, and fruition. García-Montero et al. (2007b) indicated that in 208 burns analysed in central Spain, the annual carpophore production of *T. melanosporum* increased with burn size, accounting for 38% of the variance in the productivity. Moreover, Falini and Granetti (1998) show that the number of *T. melanosporum* mycorrhizae is higher on plants surrounded by burns (75.4%) than on those without burns (46.3%), and Callot (1999) and Granetti et al. (2005) reported that the greatest carpophore production of *T. melanosporum* occurred inside the burns.

Ricard (2003) indicated that *Tuber melanosporum* burn development triggers a process within the mycorrhizal population that favours *T. melanosporum* development over that of other fungi. Interactions therefore take place between *T. melanosporum*, *T. aestivum*, *T. brumale*, *T. mesentericum*, and other fungi inside the burn. Therefore, these interactions of the species in time and space, associated with the processes involved in the burn evolution, generate a fungal succession. However, Callot et al. (1999), Ricard (2003) and Granetti et al. (2005) indicated that the biological and physical-chemical conditions related with burn development have yet to be clarified.

Sourzat and Dubiau (1999) and Sourzat et al. (1999) concluded that *Tuber melanosporum* burns can be sterile for different reasons. After noting that *T. aestivum* and *T. brumale* are very much present in mycorrhizal contamination, they proposed that environmental modifications or an internal loss of *T. melanosporum* virulence may lead to its replacement by *T. aestivum* and *T. brumale*. Sourzat (2005)

also indicated that the natural burns of *T. melanosporum* show that among their contaminant fungi, *T. aestivum* and *T. brumale* predominate but are dormant or never fruit. The author explained that the contamination pressure of *T. aestivum* and *T. brumale* has no negative effect on *T. melanosporum* production if this species is aggressive against grass and brush on its burns. Nevertheless, the author could not say whether the aggressiveness of *T. melanosporum* and its resistance to the pressure of fungal contamination are qualities of the environment or of the soil.

The aim of the present work is, using knowledge from previous studies by García-Montero et al. (2006, 2007a,b), to explain more fully the impact of soil on the interactions between truffle species inside the *Tuber melanosporum* burns, as compared with the *T. aestivum* and *T. mesentericum* burns in the same area. These soil studies have been done by means of statistical analysis using the conventional soil properties of the surface horizons to determine how these effects affect the development and fruiting of these truffles.

Materials and methods

Case study area

The study area is located in the Alto Tajo Nature reserve in central Spain. Twenty-four burns were selected close to the village of Peralejos de las Truchas (Guadalajara, Spain; geographic coordinates: latitude 40° 35' 49.65" N; longitude 1° 54' 12.78" W). These burns are situated close together in forests sharing similar topographic and mesoclimatic conditions (10 km radius). The study area is located in a mountainous area at an altitude of more than 1000 m in a supra-Mediterranean bioclimatic belt with a sub-humid, shady climate. Average annual precipitation is 797 mm, with low yearly average temperatures (9.7 °C). Its lithology is dominated by Jurassic and Cretaceous limestone and dolomites, and soils are frequently lithic and rendzic leptosols with considerable surface stoniness and high peaks. Its vegetation comprises forests of *Quercus faginea* Lam. (*Cephalanthero longifoliae*–*Querceto fagineae* S. geobotanical classification type), river forests (*Astrantio-Coryleto avellanae* S.) and oak forests (*Junipero thuriferae*–*Querceto rotundifoliae* S.) (García-Montero et al., 2007b).

Soil analysis

Twenty-four soils were studied. Eight soils were selected for their high production of *Tuber melanosporum* carpophores (600 to 6000 g/year) and the

absence of other truffle carpophores. The soil samples were taken from the bare soil in the interior of the burn, at those points inside the burn where the greatest number of carpophores had been extracted in recent years. Another 16 samples were then taken from the burns of other truffle species, 8 with only *T. aestivum* carpophore presence and 8 with only *T. mesentericum* carpophore presence. In each soil, the first 30 cm of the profile was studied, since *T. melanosporum* usually bears fruit in this range (Verlhac et al., 1990). These samples include soil surface horizons which have not been differentiated, as the horizons of natural truffle burns are constantly mixed as a consequence of the continuous digging by the harvesters, their dogs, and wildlife to extract the carpophores.

Sampling was done according to FAO (1990) procedures. In 24 soil samples, the following determinations were made: pH, total organic carbon (TOC), total calcium carbonate (equivalent carbonate), granulometric analysis, cation-exchange capacity (CEC), and saturation percentage of exchangeable cations (% V), following the methods of the ISRIC (1995); the textures were classified according to the International Society of Soil Science (ISSS); the total nitrogen was analysed following a variant of Bouat and Crouzet (1965); The determination of the exchange cations of Ca^{2+} and Mg^{2+} was done by atomic absorption spectroscopy (AAS) (Philips UP9100x), and K^{+} with a flame photometer (Sherwood 410). In the 24 soil samples, the active carbonate (carbonate extractable with ammonium oxalate) was determined according to AFNOR (1982).

Study of the truffle harvest

Tuber melanosporum, *T. aestivum*, and *T. mesentericum* have been harvested in the study area since the late 1960s. The local county councils regulate harvesting and restrict the number of collectors. Truffles are harvested with the help of trained dogs. This study was based on the collaboration of five truffle collectors, who offered their knowledge, including the location of several harvesting points, truffle samples, and their collection data. The reliability of the information provided by the collectors was confirmed independently in many field trips made with the collectors at the time of harvesting over the period 1994–2006. The exact geographic coordinates of the burns are not given in order to maintain the secrecy of the truffle locations (García-Montero et al., 2007b).

Taxonomy studies

The truffles obtained on the burns in the study area were conserved in the herbarium of the Technical University of Madrid (ETSI Montes). The truffle species were identified with a stereoscopic microscope (Leica WildMZ8) and a microscope (Leica LeitzDMRB) following the descriptions of Montecchi and Sarasini (2000) and Rioussset et al. (2001).

Mycorrhizae studies

We verified the existence of natural contamination by *Tuber aestivum* and *T. mesentericum* mycorrhizae in the natural burns of *T. melanosporum*. This was done by sampling roots in the interior of productive burn sites of *T. melanosporum*. Sampling was done in accordance with the procedures of Di Massimo et al. (1996). The mycorrhizae were identified with both the stereoscopic microscope and the microscope following Verlhac et al. (1990) and Granetti's (1995) descriptions and recommendations. However, in our natural study area, harvesting was undertaken according to a regime established by the corresponding local councils. Therefore, we were unable to carry out studies on the quantitative abundance of the *Tuber* spp. mycorrhizae, as this type of study requires damaging the truffle burns in order to extract the root samples which would enable us to analyse this aspect. We have not been authorized by the corresponding local councils to perform these kinds of studies.

Statistical analysis

The statistical treatment was performed with the Statistica Program v. 6 (StatSoft, Inc., Tulsa, OK, 1999). When necessary, variables were log-transformed to meet the requirements of parametric tests. Normality was checked using the Shapiro-Wilk test. The homogeneity of variance of the ANOVA tests was also checked by using the Levene test. A principal components analysis (PCA) was done with 12 soil variables of 24 soil samples (% CaCO_3 total; $\text{Log}(x+1)$ % CaCO_3 active; % TOC; % N; % coarse sand; % fine sand; Log % silt; Log % clay; square-root (SQRT) exchangeable Ca^{2+} ; $\text{Log}(x+1)$ exchangeable Mg^{2+} ; exchangeable K^{+} ; CEC), with the aim of synthesizing the variation patterns of those elements potentially associated with the presence of *Tuber melanosporum* carpophores as opposed to *T. aestivum* and *T. mesentericum*. We also used ANOVA tests to analyse the relationships between the carpophore presence of *Tuber* species (*Tuber melanosporum* compared with *T. aestivum* and

T. mesentericum) and the 12 soil variables of 24 soil samples.

Results

Table I shows the characteristics of the surface horizons in the study. Many of the soils have a texture that tends towards sandy clay loam soil. Most have a moderately basic pH, a low percentage of total carbonate, and a variable concentration of active carbonate. Levels of organic carbon are moderate and the C/N ratio is close to 10. They have high values of exchangeable cation complex. They display good structure with a granular tendency and abundant pores. The analysis of the roots extracted from the *Tuber melanosporum* burns confirmed the presence of numerous mycorrhizae of *T. melanosporum*, and mycorrhizal contamination by *T. aestivum* and *T. mesentericum*.

A PCA was done with the chemical soil variables studied in order to analyse the overall impact of the surface horizons on the presence of *Tuber melanosporum* carpophores as compared with *T. aestivum* and *T. mesentericum*. The first three factors (PC₁, PC₂, and PC₃) accounted for 73.32% of the variance contained in the original matrix (Figure 1).

PC₁ and PC₂ are the components that best explain the interactions between the variables. PC₁ accounts

for 40.07% of the variance and emphasizes the differences between soils with a greater capacity for cation exchange and a higher amount of exchangeable Ca²⁺, K, organic carbon, nitrogen, and silt and soils rich in coarse sand (Table II). The second factor (PC₂) represents 19.29% of the variance, and highlights the differences between soils with a greater quantity of total carbonate and clay, and soils rich in the active carbonate and fine sand (Table II). Figure 2 shows different soils depending on PC₁ and PC₂. This graph shows *T. melanosporum* soils have an important correspondence with lower semi-axis PC₂ related to high active carbonate and fine sand content.

The factor (*Tuber* species) of the ANOVA test of the variable Log (*x*+1) % CaCO₃ active shows that the mean concentrations of active carbonate differ very significantly ($F_{2, 21} = 16.294$; p -value = 0.000 053) depending on the carpophore presence of *Tuber* species present in the soil. The average active carbonate values determined in the burns of each *Tuber* species are illustrated in Figure 3. Some *Tuber* species burns demonstrated higher active carbonate values. A 'post hoc' (Tukey) test was conducted to determine which averages were different (HDS Tukey test, probabilities for post-hoc tests; *T. melanosporum* vs. *T. aestivum*: $p = 0.000 407$; *T. melanosporum* vs. *T. mesentericum*: $p = 0.000 258$. The other

Table I. Texture and analytical results of the soils studied.

No.	<i>Tuber species</i>	Coarse sand	Fine sand	Silt	Clay	pH _{H2O}	CaCO ₃ total	CaCO ₃ active	TOC	N	Ca ²⁺	Mg ²⁺	K ⁺	Na ⁺	CEC
1	<i>T. melanosporum</i>	77.0	359.7	192.5	370.8	79.0	31.2	22.5	3.40	0.25	13.19	7.92	1.03	2.13	24.27
2	<i>T. melanosporum</i>	378.0	283.0	219.0	120.0	78.8	78.8	70.1	3.56	0.27	21.14	0.74	1.10	0.45	23.44
3	<i>T. melanosporum</i>	144.0	263.1	136.0	456.8	75.0	127.9	16.3	2.30	0.25	20.17	0.60	1.47	0.08	22.32
4	<i>T. melanosporum</i>	58.0	223.0	589.0	130.0	78.0	71.9	28.8	7.52	0.56	46.16	3.15	0.73	0.00	50.04
5	<i>T. melanosporum</i>	175.8	465.3	132.1	226.8	81.5	41.8	26.3	1.16	0.18	11.20	4.89	0.29	0.08	16.46
6	<i>T. melanosporum</i>	262.0	188.0	321.0	229.0	79.0	115.0	81.4	3.61	0.23	18.86	0.41	0.22	0.00	19.49
7	<i>T. melanosporum</i>	231.8	318.9	101.1	348.2	81.5	45.3	23.8	1.08	0.09	15.49	1.85	0.51	0.27	18.12
8	<i>T. melanosporum</i>	349.6	468.5	76.5	105.4	79.0	102.1	33.8	2.55	0.27	27.49	1.48	0.29	0.00	29.26
9	<i>T. aestivum</i>	489.7	272.3	70.5	167.5	80.6	156.3	1.5	1.33	0.33	11.87	1.89	0.07	0.00	13.83
10	<i>T. aestivum</i>	273.0	295.8	89.3	342.0	75.2	179.2	0.0	4.97	0.36	20.14	4.39	0.96	0.00	25.49
11	<i>T. aestivum</i>	69.2	67.9	172.6	690.3	79.2	163.5	1.8	5.31	0.36	24.90	8.35	1.99	0.08	35.32
12	<i>T. aestivum</i>	139.2	139.0	131.9	575.7	77.2	170.7	8.5	5.10	0.36	18.63	7.03	1.63	4.61	31.90
13	<i>T. aestivum</i>	387.7	270.1	79.1	260.0	78.3	166.1	19.2	2.88	0.34	14.89	3.15	0.50	0.71	19.25
14	<i>T. aestivum</i>	304.8	226.5	91.3	364.7	77.7	168.6	0.0	3.62	0.34	15.96	4.44	0.87	2.20	23.47
15	<i>T. aestivum</i>	434.0	393.8	60.0	110.0	77.0	140.2	22.3	0.06	0.00	4.77	3.43	0.29	0.00	8.50
16	<i>T. aestivum</i>	131.0	389.0	400.0	80.0	77.0	90.3	10.2	7.34	0.51	30.71	6.31	1.47	0.08	38.57
17	<i>T. mesentericum</i>	223.6	141.9	383.4	251.1	81.7	133.8	2.1	2.86	0.38	18.43	0.78	1.03	0.08	20.33
18	<i>T. mesentericum</i>	194.3	421.6	169.4	214.7	78.5	2.1	0.0	9.17	0.74	23.49	6.50	0.66	0.00	30.65
19	<i>T. mesentericum</i>	361.8	281.4	99.5	257.4	70.0	184.4	0.0	4.32	0.38	16.02	1.54	1.10	0.00	18.66
20	<i>T. mesentericum</i>	247.5	245.5	152.9	244.6	79.0	65.7	7.4	5.42	0.45	19.05	1.15	0.94	0.00	21.14
21	<i>T. mesentericum</i>	250.9	223.1	175.6	247.9	80.8	88.9	7.2	4.98	0.42	18.52	1.07	0.98	0.00	20.57
22	<i>T. mesentericum</i>	252.1	214.7	194.6	249.1	81.4	100.8	10.1	4.67	0.41	18.33	1.03	1.00	0.00	20.36
23	<i>T. mesentericum</i>	294.1	472.2	90.0	150.0	80.7	62.8	7.4	0.53	0.00	5.16	6.34	0.22	0.08	11.80
24	<i>T. mesentericum</i>	55.7	531.0	70.0	340.0	79.2	43.8	11.6	1.96	0.18	12.71	2.13	0.07	0.08	14.99

Coarse sand, Fine sand, Silt and Clay expressed in g kg⁻¹. TOC (total organic carbon) and N expressed in g kg⁻¹; CEC (cation-exchange capacity) and exchangeable cations expressed in cmol kg⁻¹.

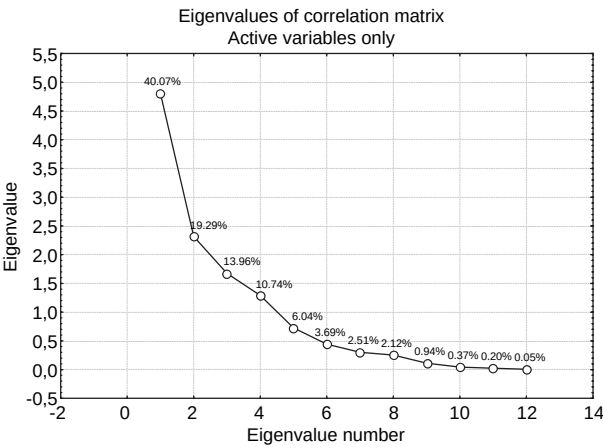


Figure 1. Scree plot of principal component analysis.

probabilities for post-hoc tests were not significant). The results showed that the abundance of active carbonate is significantly higher in burns that produce only *T. melanosporum* than in those that produce *T. aestivum* and *T. mesentericum*. Nevertheless, the ANOVAs of the other 11 soil variables indicate that mean concentrations are statistically the same in soils inhabited by *T. melanosporum* as in soils with *T. aestivum* and *T. mesentericum*.

Discussion

In the study area, the soil properties are suitable for correct development of *Tuber melanosporum* and other truffle species. The results suggest that active carbonate could be a major factor involved in the greater fruiting and aggressiveness of *T. melanosporum* as compared with *T. aestivum* and *T. mesentericum*. The abundance of active carbonate is significantly higher in burns that produce only *T. melanosporum* than in those that produce *T. aestivum* and *T. mesentericum* in the study area (Figure 3). García-Montero et al. (2006; 2007a; 2007b) also

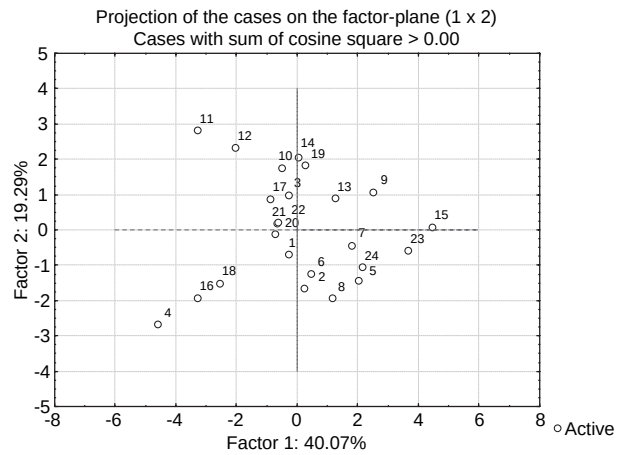


Figure 2. Relationship between carpophore production of truffles in different soils and PCA factors. Samples coded between 1 and 8 are *Tuber melanosporum* soils; samples numbered 9–16 are *T. aestivum*, and samples numbered 17–24 are *T. mesentericum* soils.

indicate that a high concentration of active carbonate accounts for up to 43% of *T. melanosporum* carpophore production and 51% of the variance in the size of *T. melanosporum* burns.

Active carbonate is a finely divided fraction of calcareous rock. It is smaller than 50 μm in size, very chemically active, and constitutes an important reserve of exchangeable Ca^{2+} . Active carbonate and exchangeable Ca^{2+} regulate soil pH and participate in the organization and maintenance of the soil structure. Ca^{2+} is also indispensable in the growth of the mycelium of *T. melanosporum* (Poitou, 1988; Bencivenga & Granetti, 1989; Coli et al., 1990; López & López, 1990; Wild, 1992; Marañés et al., 1994; Breemen & Buurman, 1998). Ricard (2003) indicated that it is difficult to judge the impact of active carbonate on truffle culture because several factors must be considered; he also considered the lack of studies on active carbonate in truffle culture to be a serious omission.

Table II. Principal components analysis (PCA): chemical soil variables studied in order to analyse the overall impact of the surface horizons on the presence of *Tuber melanosporum* carpophores as compared with *T. aestivum* and *T. mesentericum*. Factor loadings.

Variables	Factor 1 (PC_1)	Factor 2 (PC_2)	Factor 3 (PC_3)
% CaCO_3 total	−0.003	0.778	0.403
Log (x+1) % CaCO_3 active	0.191	−0.625	0.371
TOC	−0.898	−0.108	−0.062
N	−0.821	−0.037	0.049
% Coarse sand	0.600	0.160	0.512
% Fine sand	0.512	−0.582	−0.487
Log (x) % silt	−0.735	−0.372	0.261
Log (x) % clay	−0.179	0.694	−0.292
SQRT (x) Ca^{2+} exchangeable	−0.866	−0.252	0.243
Log (x+1) Mg^{2+} exchangeable	−0.190	0.120	−0.809
K^+ exchangeable	−0.708	0.454	−0.009
CEC	−0.900	−0.193	−0.051

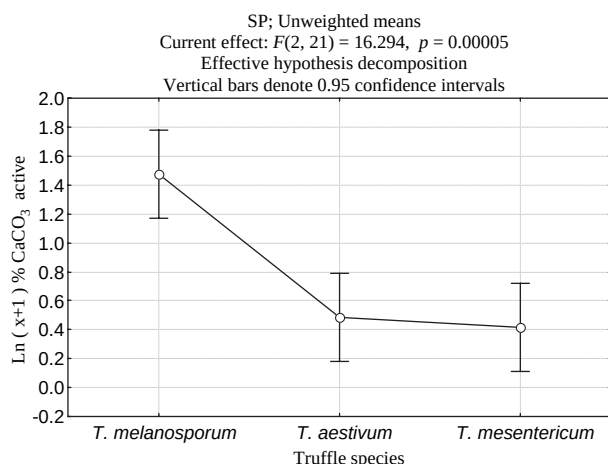


Figure 3. Average active carbonate values for comparison of burns of the 3 truffle species.

Tuber melanosporum inhabits Mediterranean dry soils; this dryness is further encouraged by this fungus as it eliminates plants from their burns. The soil water evaporation, soil aeration, and the disappearance of roots in the burns favour surface accumulation of active carbonate (Poitou, 1988; Callot, 1999; Lulli et al., 1999; Ourzik, 1999; Ricard, 2003). García-Montero et al. (2007a; 2007b) described these processes, and they indicated that the development of the *T. melanosporum* burn encourages the formation of a large amount of active carbonate and exchangeable Ca^{2+} . *Tuber melanosporum* fruition and burn size are simultaneously favoured by a high concentration of both factors, which suggests a feedback process. Finally,



Figure 4. The development of the truffle burn encourages the formation of active carbonate and exchangeable Ca^{2+} . *Tuber melanosporum* fruition and burn size are simultaneously favoured by a high concentration of both factors, which suggests a feedback process. Our results suggest that active carbonate is a major factor in the fruiting and aggressiveness of *T. melanosporum* over its competitors, *T. aestivum* and *T. mesentericum*.

our results show that this feedback process provides *T. melanosporum* an additional advantage over its competitors, *T. aestivum* and *T. mesentericum*, when active carbonate reaches high soil concentration in the *T. melanosporum* burns (Figure 4).

In conclusion, active carbonate is a major factor in the fruiting and aggressiveness of *Tuber melanosporum* versus *T. aestivum* and *T. mesentericum*. New studies are needed into cultivation practices that increase active carbonate, and especially on the use of calcareous amendments in truffle culture and natural burns, under strict ecological control, which would favour the presence of *T. melanosporum* over other truffles. Additional research is required into the cause-and-effect relationship between the burn size, active carbonate formation, and carpophore production of *T. melanosporum*.

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New data on ectomycorrhizae and soils of the Chinese truffles *Tuber pseudoexcavatum* and *Tuber indicum*, and their impact on truffle cultivation

Luis G. García-Montero · Gabriella Di Massimo ·
José L. Manjón · Antonio García-Abril

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Abstract Chinese truffles serve as a good complement to the market for *Tuber melanosporum* (Périgord black truffle). However, Chinese truffles could be introduced accidentally or fraudulently into the plantations of Mediterranean truffles, and they could have a negative effect on truffle production and natural ecosystems. The study of *Tuber* species from China which are commercialized in Europe began 14 years ago. *Tuber pseudoexcavatum* was proposed as a new species, and this has been validated by some authors based on molecular and phylogenetic studies. We synthesize their ectomycorrhizae using samples from the type collection, and we compare *T. pseudoexcavatum* and *Tuber indicum* ectomycorrhizae. The ectomycorrhizae of these species have a morphology which is related to the ectomycorrhizae of *T. melanosporum*. We provide useful

information for the rapid screening of the above-mentioned Chinese truffles ectomycorrhizae, for the quality control of commercial plants mycorrhized with *Tuber*. Moreover, we analyze the soil tolerance and the host plant affinity of *T. pseudoexcavatum* and *T. indicum*, in order to assess the capacity of both Chinese truffles to penetrate *T. melanosporum* plantations and habitats.

Keywords Chinese truffle · Ectomycorrhizae · Truffle cultivation · Truffle ecology · Truffle yield

Introduction

Many *Tuber melanosporum* Vittad. (Périgord black truffle) crops have a high carpophore production, but uncontrolled factors still induce a great deal of variability in production from year to year. Chevalier and Frochot (1997) and Lefevre and Hall (2001) indicate that contamination of truffières (truffle production sites) by other competing ectomycorrhizal fungi poses formidable problems for researchers working to optimize production of truffle species. Several authors have expressed their concern over the risk that commercial Chinese truffles (*Tuber* species) might be introduced accidentally or fraudulently into the plantations of Mediterranean truffles. Murat et al. (2008) have confirmed the appearance of *T. indicum* DNA in root tips and soil samples from a *T. melanosporum* plantation in Piedmont (Italy).

The international truffle market has seen regular shipments of Chinese truffles between China and Europe, Japan, United States, and Australia, for the last 14 years (Rey 2001; Wang and Hall 2001; Yamanaka et al. 2001; Yang 2001; García-Montero et al. 2005). Chinese truffles act as complements to the market for *T. melanosporum* because they are

L. G. García-Montero (✉)
Department of Forestry Engineering, U.D. Operaciones Básicas,,
Technical University of Madrid (UPM),
E.T.S. Ingenieros de Montes, Ciudad Universitaria s/n,
Madrid 28040, Spain
e-mail: luisgonzaga.garcia@upm.es

G. Di Massimo
Department di Biologia Vegetale e Biotecnologie Agroambientali,
University of Perugia,
Borgo XX Giugno 74,
Perugia 06121, Italy

J. L. Manjón
Department Plant Biology, University of Alcalá,
Crtra. Barcelona Km 33,
Alcalá de Henares 28870, Spain

A. García-Abril
Department of Projects and Rural Planning,
Technical University of Madrid (UPM),
E.T.S. Ingenieros de Montes, Ciudad Universitaria s/n,
Madrid 28040, Spain

cheaper, have a milder aroma and the carpophores are of good quality for culinary purposes (Rey 2001).

New Chinese truffle species have been discovered in European markets, such as *Tuber pseudoexcavatum* Wang, G. Moreno, L. G. Rioussset, J. L. Manjón, and G. Rioussset (Wang et al. 1998). From the taxonomical point of view, *T. pseudoexcavatum* species were confirmed by several studies on phylogeny and taxonomy based on morphological characteristics, and DNA methods (Rioussset et al. 2001; Zhang et al. 2005; Wang et al. 2006b). In the University of Alcalá, we synthesized *T. pseudoexcavatum* ectomycorrhizae using samples of fresh carpophores from the type collections. We observed that the ectomycorrhizae of this species had a similar morphology to the ectomycorrhizae of *T. melanosporum* (Manjón et al. 1998). However, we have not yet published a description of *T. pseudoexcavatum* mycorrhizae, as we are awaiting further information which would enable us to determine the taxonomy of the vast quantity of Chinese truffles that are being discovered and marketed.

Di Massimo et al. (1996), Zambonelli et al. (1996, 1997) and Comandini and Pacioni (1997) synthesized *Tuber indicum* Cooke and Massee ectomycorrhizae. They showed that these ectomycorrhizae also have a considerable affinity with *T. melanosporum* ectomycorrhizae. These authors, as well as García-Montero et al. (1997a, b, 2005) and Ferrara and Palenzona (2001), indicated the need to increase quality controls of mycorrhized plants in truffle culture, and warned that the Chinese truffles could displace truffles with a greater economic value and penetrate the Mediterranean *Tuber* ecosystems. In this regard, the latest trends for distinguishing the various *Tuber* ectomycorrhizae are based simultaneously on morphological taxonomical characters and a range of biochemical and DNA-based methods (Ferrara and Palenzona 2001; Mabru et al. 2001; Douet et al. 2004; Iotti and Zambonelli 2006).

It is, therefore, necessary to increase the current knowledge of the Chinese truffles ectomycorrhizae and to determine their ecological requirements and restrictions. We report the morphological characterization of *T. pseudoexcavatum* ectomycorrhizae, and we provide useful information for the rapid morphological screening of *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae versus *T. melanosporum* ectomycorrhizae. We also determine whether *T. pseudoexcavatum* and *T. indicum* could be easily introduced into Mediterranean habitats of *T. melanosporum*, depending on the characteristics of the soils and host plants common to *T. melanosporum*. We did this by analyzing samples of soils inhabited by *T. melanosporum* in natural Spanish woods, and we used these soil samples to synthesize *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae with the principal host plant of *T. melanosporum* in many Mediterranean areas, *Quercus ilex* L.

Materials and methods

Synthesis and study of Chinese truffles ectomycorrhizae

We synthesized *T. indicum* ectomycorrhizae using samples of fresh carpophores, and *T. pseudoexcavatum* ectomycorrhizae using samples of fresh carpophores from the same commercial batches as the type collections (*isotypus* AH-18383 and AH-18384). The taxonomical identifications are presented in Manjón et al. (1995) and Wang et al. (1998). The mycorrhizal synthesis of these Chinese species was done by applying a method of intensive mycorrhization on six *Quercus ilex* subsp. *ballota* (Desf.) Samp. plants, respectively, as described by Bencivenga (1982), but modified according to Manjón and García-Montero (1996). We also mycorrhized *T. melanosporum* with six plants as a comparative control test.

The *Quercus ilex* acorns and truffle carpophores were carefully washed, sterilized on the surface, and conserved in a fridge at a temperature of +4°C. The acorns were sown in semi-rigid plastic containers with a volume of 1,000 cc. The substrate used for seeding was a mixture of two calcareous soils from a natural *T. melanosporum* truffière, mixed with vermiculite and perlite, which was sterilized in an autoclave at 120°C for 4 h. At the moment of sowing, they were inoculated with a water suspension of ascospores obtained from finely-chopped carpophores. An acorn and a suspension containing 2 g of fruit body were placed in each container. The plants were kept under controlled environmental conditions in the Juan Carlos I Royal Botanical Garden at the University of Alcalá (Madrid, Spain). They were housed in a greenhouse with an average daily temperature of 20 to 25°C; relative humidity of 60 to 70%; watered by micro-sprinkler between one and three times a day for 1–3 min depending on the time of year; and under natural light conditions, with no artificial lighting. These parameters were controlled automatically using a computer. The plants were not fertilized. We obtained a batch of 18 well-lined, disease-free plants, with an average height of 17 cm and a diameter at the base of the plant of 2 cm.

The degree of mycorrhization of each plant was expressed as the number of *Tuber* mycorrhizae of the total number of apices counted, according to Bencivenga et al. (1987). The ectomycorrhizae were identified with a stereoscopic microscope (photo-Leica WildMZ8) and a microscope (photo-Leica LeitzDMRB) following the terms, descriptions, and recommendations of Agerer (1987–2002), Bencivenga et al. (1995) and Granetti (1995). This study was done with the criterion of selecting the macroscopic and microscopic characteristics of these ectomycorrhizae which would be most useful in the control procedures for mycorrhized plants in truffle culture, according to Bencivenga et al. (1987) and Granetti (1995). We, therefore, observed the morphological

Table 1 Morphological characters of *Tuber melanosporum*, *T. indicum* and *T. pseudoecavatum* ectomycorrhizae

Species	<i>T. melanosporum</i>	<i>T. melanosporum</i>	<i>T. indicum</i>	<i>T. indicum</i>	<i>T. pseudoecavatum</i>
Synthesis source	Granetti 1995; Granetti et al. 2005; Zambonelli et al. 1993	Present study	Comandini and Pacioni 1997; Zambonelli et al. 1997	Present study	Present study
Geographical location	Mediterranean Europe	Spain	China	China	China
Host plant	<i>Quercus pubescens</i> , <i>Corylus avellana</i> , <i>Pinus sylvestris</i>	<i>Quercus ilex</i> subsp. <i>ballota</i>	<i>Quercus pubescens</i> and <i>Q. cerris</i>	<i>Quercus ilex</i> subsp. <i>ballota</i>	<i>Quercus ilex</i> subsp. <i>ballota</i>
Mode of ramification	Simple with clavate shape and round apex, or with monopodial-pinnate ramifications	Idem	May be simple, monopodial-pinnate, or dichotomic branched	Idem	From slightly club-shaped to almost cylindrical, without ramifications in very young forms, or with monopodial-pinnate ramifications
Length of unramified ends	From 200 to 4,000 μm	From 250 to 2,800 μm	From 150 to 1,620 μm	From 500 to 2,700 μm	Up to 2,500 μm
Diameter of unramified ends	From 230 to 450 μm	From 200 to 350 μm	From 120 to 540 μm	From 500 to 680 μm	Up to 240 μm
Structure of the surface	Smooth with long cystidia especially on the tip.	Idem	Smooth; long loose cystidia especially on the tip. Older specimens seem to lose cystidia	Idem	Smooth with long cystidia especially on the tip.
Color of unramified ends	Dark amber to ochre	Idem	7.5 YR 6/6 ochreous-amber	Idem	7.5 YR 6/6 dark brown

Comparisons include ectomycorrhizae synthesized in the present and previous studies

characters of the ectomycorrhizae, such as shape, size, type of ramifications, color, and mantle surface. The ectomycorrhizal color was described following Munsell (1976) standard soil color charts. For the analysis of the anatomical characters, we chose the median parts of adult ectomycorrhizae, as indicated by Agerer (1987–2002) and we observed the outer mantle surface and the mycelium. We measured the different characters, and maximum and minimum ranges of values were established by taking the necessary measurements until regularity was attained in the values observed, with a minimum of 30 measurements.

The morphological characteristics of *T. indicum*, and *T. melanosporum* ectomycorrhizae were described within the ranges and descriptions proposed by Granetti (1995), Comandini and Pacioni (1997), Zambonelli et al. (1997) and Granetti et al. (2005).

Soil analysis

In the mycorrhizal synthesis of the Chinese truffles, we used soil samples from natural Spanish woods inhabited by *T. melanosporum*. We took the soil samples from around a

T. melanosporum truffière associated to *Quercus ilex* subsp. *ballota* and *Q. faginea* Lam., with a maximum carpophore production of up to 5,000 g/year. A more detailed description of the study area, the soils and the truffle habitats can be found in García-Montero et al. (2006).

We analyzed two soil samples. One soil sample was taken from the bare soil in the interior of this truffière, and the other at a distance of 5 m from the truffière. Only the first 30 cm of soil profile were taken and studied, as *T. melanosporum* usually bears fruit in this range. Sampling was done according to the FAO (1990). The following soil determinations were made: pH, total organic carbon (T.O. C.), total calcium carbonate (equivalent calcium carbonate), granulometric analysis, cation exchange capacity (C.E.C.) and the exchangeable cation saturation percentage (% V), following the methods of the ISRIC (1995); the textures are classified according to the International Society of Soil Science (I.S.S.S.); the total nitrogen was analyzed with the variant of Bouat and Crouzet (1965); and the active carbonate (calcium carbonate extractable with ammonium oxalate) was determined according to AFNOR (1982). The exchangeable cations of Ca^{2+} and Mg^{2+} were determined

Table 2 Anatomical characters of *Tuber melanosporum*, *Tuber indicum* and *Tuber pseudoexcavatum* ectomycorrhizae

Species	<i>T. melanosporum</i>	<i>T. melanosporum</i>	<i>T. indicum</i>	<i>T. indicum</i>	<i>T. pseudoexcavatum</i>
Synthesis source	Granetti 1995; Granetti et al. 2005; Zambonelli et al. 1993	Present study	Comandini and Pacioni 1997; Zambonelli et al. 1997	Present study	Present study
Host plant	<i>Quercus pubescens</i> , <i>Corylus avellana</i> , <i>Pinus sylvestris</i>	<i>Quercus ilex</i> subsp. <i>ballota</i>	<i>Quercus pubescens</i> and <i>Q. cerris</i>	<i>Quercus ilex</i> subsp. <i>ballota</i>	<i>Quercus ilex</i> subsp. <i>ballota</i>
Outer structure of the mantle	Pseudoparenchymatous. Hyphal pattern is formed by roundish cells, with well-defined irregular lobes, which give a puzzle-like appearance	Idem	Pseudoparenchymatous, very heterogeneous, formed by slightly roundish regular pseudocells, with puzzle-like appearance. More polygonal pseudocells which are smaller on average and less lobed than <i>Tuber melanosporum</i>	Idem	Pseudoparenchymatous, homogeneous, formed by pseudocells with a sinuous form. The general feature of the surface is its regular puzzle-like appearance.
Outer dimensions of hyphal cells	Mean length 10.6 (±2.4) µm, mean width 4.6 (±1) µm	Length between (10) 15–20 µm, width between 5–10 mm	Length between (8) 10–16 (24) µm, width between (4) 5–6 (10) µm	Length between 10–15 µm, width between 5–8 mm	Length between (8) 10–25 (26) µm, width between (5) 6–16 (17) µm
Cystidia form	Length up to 300 µm; straight; frequent right angle-like and 45° ramifications	Idem	Length up to 300 µm, with frequent right angle-like ramifications	Idem	Very long, sinuous to straight cystidia, sectioned, 15% right angle-like ramifications
Cystidia color	Hyaline	Idem	Pale yellow	Idem	Yellowish
Cystidia diameter	Plinth diameter mean (2.8) 3.5 (4.4) µm,	Plinth diameter mean 2 µm	Plinth diameter from 2 to 4 (8) µm, tip diameter from 2 to 3 (6) µm	Plinth diameter mean 2 µm	Plinth diameter from 2 to 4 µm
Septa distance	–	10–26	25–35 µm	12–29 µm	From 25 to 35 µm

Comparisons include ectomycorrhizae synthesized in the present and previous studies

using AAS (Philips UP9100x), and K⁺ and Na⁺ with a flame photometer (Sherwood 410).

Results

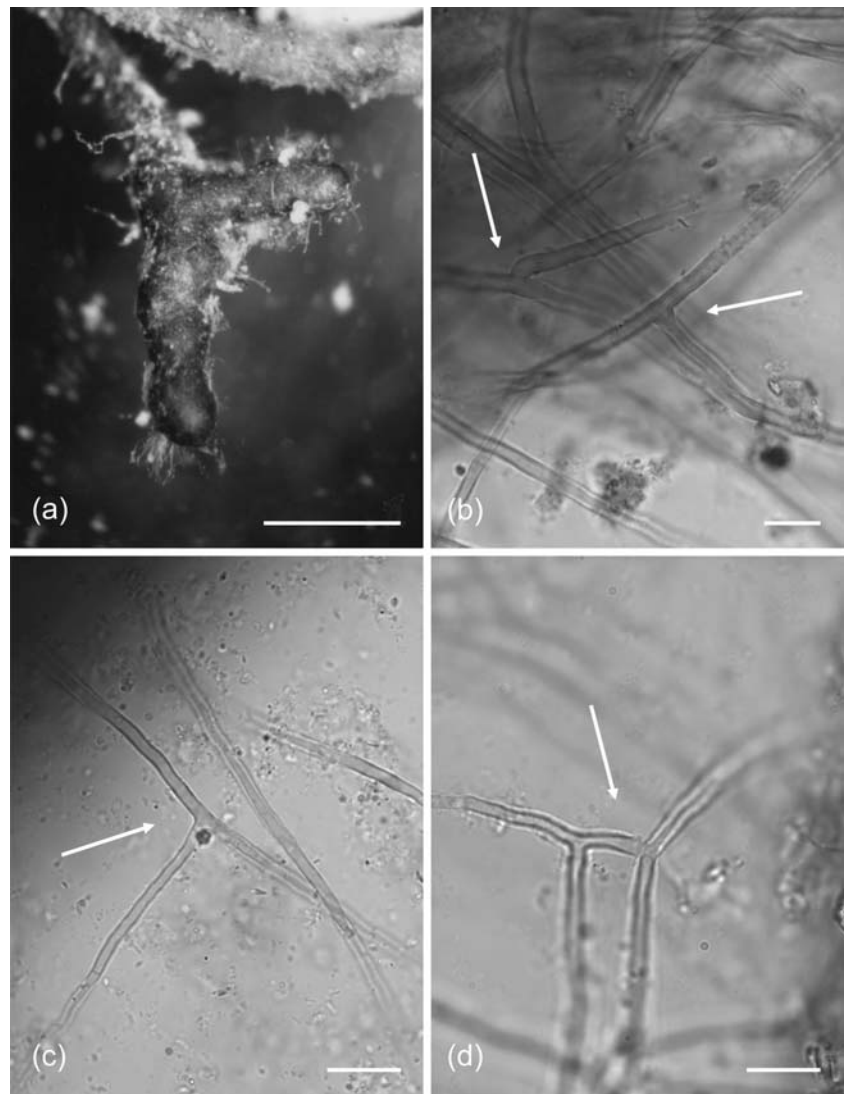
The 18 seed plants of *Quercus ilex* subsp. *ballota* showed that the roots had between 40 to 60% of their tips mycorrhized with *T. pseudoexcavatum*, *T. indicum*, and *T. melanosporum*, respectively, without contaminating ectomycorrhizae of other fungi. Tables 1 and 2 show the morphological characteristics of these ectomycorrhizae, to assist in their rapid identification in the routine control procedures for mycorrhized plants in truffle culture.

The ectomycorrhizae of *T. pseudoexcavatum* and *T. indicum* have a considerable morphological similarity with the ectomycorrhizae of *T. melanosporum*, due to the presence of cystidia with right angle-like ramifications and outer mantle pseudocells with a sinuous puzzle-like form, which up to now have been considered the most

distinctive characteristics of *T. melanosporum* ectomycorrhizae (Palenzona 1969; Zambonelli et al. 1993; Granetti 1995; Granetti et al. 2005). However, some morphological characteristics of *T. pseudoexcavatum* ectomycorrhizae make it possible to differentiate this species from *T. indicum*, and *T. melanosporum*. Specifically, the length of the unramified ends of the ectomycorrhizae in *T. pseudoexcavatum* is much greater than in the other Chinese truffles ectomycorrhizae, and the *T. pseudoexcavatum* ectomycorrhizae are darker in color than *T. indicum* and *T. melanosporum* ectomycorrhizae. Moreover, the outer mantle pseudocells and cystidia diameter of *T. pseudoexcavatum* are larger than the pseudocells and cystidia of *T. indicum* and *T. melanosporum* (Tables 1 and 2; Figs. 1 and 2).

We obtained the *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae using two soil samples with the following properties (Table 3): a relative abundance of sand and clay and moderate level of silt, which generates a texture tending towards sandy clay loam. These soils have a moderately basic pH, low levels of total carbonates but an

Fig. 1 **a** Macroscopic appearance of *T. pseudoexcavatum* mycorrhizae (50×) (*bar*=1000 μm); **b** detail of *T. pseudoexcavatum* cystidia with right angle-like ramifications (630×; *bar*=10 μm); **c** detail of *T. indicum* cystidia with right angle-like ramifications (630×; *bar*=10 μm); **d** detail of *T. melanosporum* cystidia with right angle-like ramifications (630×; *bar*=10 μm)



elevated level of active carbonate. Levels of organic carbon and total nitrogen are moderate and the C/N ratio is 13 to 15. They have high values of exchangeable cation complex and the degree of saturation of exchangeable cations is 100%, with a good proportion of exchangeable Ca^{2+} and Mg^{2+} , K^{+} in relatively large concentrations, and scarce Na^{+} . These soils have a good structure with a granular tendency and an abundance of pores.

Discussion

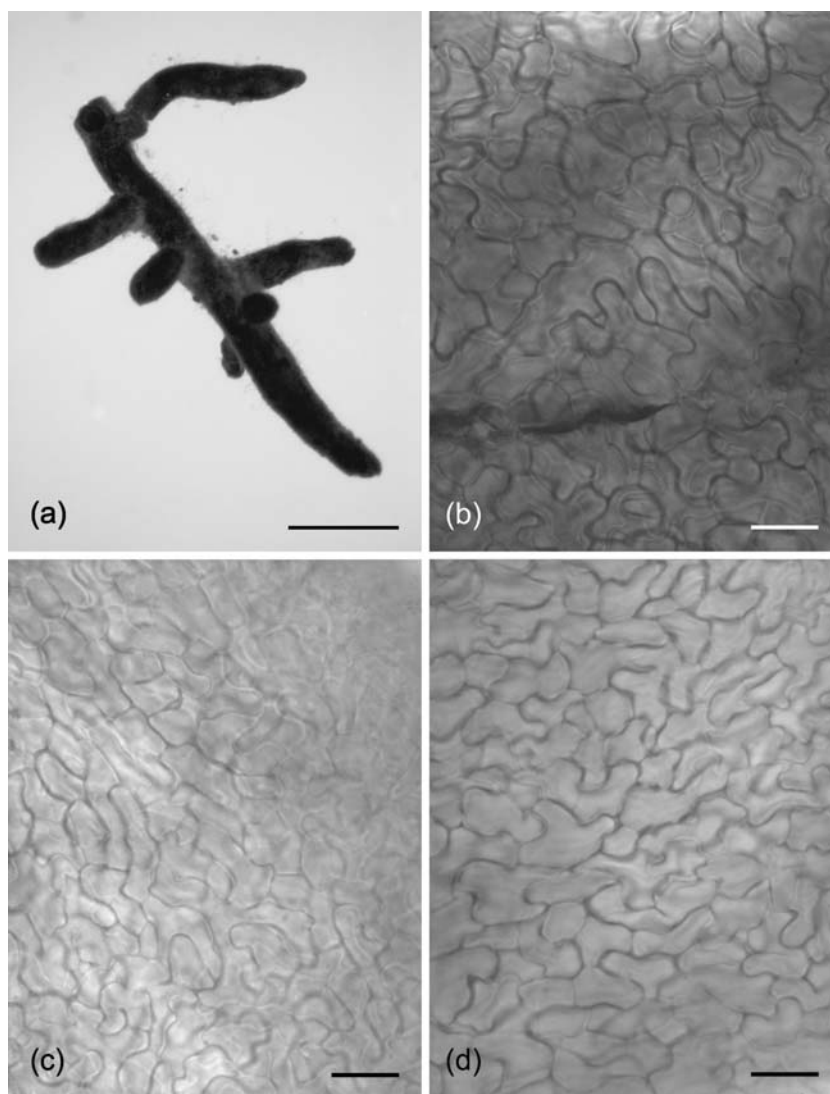
Wang and Hall (2001) confirm that, in Europe, some of the Chinese truffles that were similar to *T. melanosporum* were originally identified as *T. indicum*. However, these authors and Wang and He (2002) indicated that most of the truffles that were being exported from southwest China to Europe were *T. sinense* Tao and Liu and *T. pseudoexcavatum*, in

hundreds of tons a year. *T. pseudoexcavatum* has also been detected in Japanese truffle markets (Yamanaka et al. 2001).

Our results show that the morphological characteristics of *T. pseudoexcavatum* ectomycorrhizae enable this species to be differentiated from *T. indicum* ectomycorrhizae. We also show that the ectomycorrhizae of *T. pseudoexcavatum* have a similar morphology to the ectomycorrhizae of *T. melanosporum*, although there are some morphological characteristics which make it possible to differentiate both species. In any case, it is necessary to use molecular tools to positively separate these species. It would, therefore, be very useful to have a specific primer for *T. pseudoexcavatum* to be used in conjunction with the other black truffle specific primers already designed by Paolocci et al. (1999) for use in multiplex PCRs.

From the soil point of view, very few studies have been done so far on Chinese truffles. Most Chinese truffles have been found in calcareous soils. *Tuber furfuraceum* Hu and

Fig. 2 **a** Macroscopic appearance of *T. indicum* mycorrhizae (50×; bar=2,000 μm); **b** hyphal pseudocells of the outer surface of the *T. indicum* mantle with a sinuous form (630×; bar=10 μm); **c** hyphal pseudocells of the outer surface of the *T. melanosporum* mantle with a sinuous form (630×; bar=10 μm); **d** hyphal pseudocells of the outer surface of the *T. pseudoexcavatum* mantle with a sinuous form (630×; bar=10 μm)



Y. Wang and *T. pseudoexcavatum* live in calcareous soils (Wang and Li 1991; Rioussset et al. 2001; Wang and Hall 2001; Hu and Wang 2005). *Tuber sinense* carpophores have been found in the 0–30 cm layers of calcareous, high pH, and clay soils (Zhang and Wang 1990; Wang and Hall 2001). *T. indicum* var. *yunnanense* Yamanaka carpophores have been found at a depth of 2–12 cm, also in very poor, calcareous, and purple soils, with a pH of 6.5–7.4 (Yamanaka et al. 2000, 2001). *Tuber formosanum* Hu is found only in calcareous soils with a wide range of soil

parameters (pH, total nitrogen, carbon, sulfur, and available nutrients), suggesting that *T. formosanum* can adapt to a wide range of soil conditions (Hu et al. 2005).

Rioussset et al. (2001) report that *T. indicum* develops at a depth of 30–40 cm under a mulch of pine needles in mountain pinewoods, in soils free from calcium carbonate and with a moderate pH, rich in organic matter and with a high C/N ratio. However, other authors indicate that *T. indicum* inhabits calcareous soils. Fourré et al. (1996) report that *T. indicum* occurs on calcareous plateaus at

Table 3 Analytical results of the two soil samples from *Tuber melanosporum* burns (C.E.C. cation exchange capacity, %V exchangeable cation saturation percentage; Sand, clay, silt, total carbonate,

active carbonate, T.O.C., and N expressed in g kg⁻¹; C.E.C. and exchangeable cations expressed in cmol kg⁻¹)

No	Sand	Silt	Clay	pH _{H2O}	Total carbonate	Active carbonate	T.O.C.	N	Ca ²⁺	Mg ²⁺	K ⁺	Na ⁺	C.E.C.	%V
1	600	130	270	7.92	69.10	56.30	42.60	2.70	23.59	1.20	0.88	0.45	25.94	100
2	660	220	120	7.88	78.80	70.10	35.60	2.70	21.15	0.74	1.10	0.27	23.44	100

2,000–3,000 m. Granetti et al. (2005) indicate that *T. indicum* inhabits calcareous substrates with a pH which varies between 5.5 (due to organic matter) and 8.5.

Our results confirm that *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae develop well in calcareous substrates which are rich in active carbonate, whose properties fulfill the necessary values for the development of *T. melanosporum* according to Callot (1999), Ricard (2003), and García-Montero et al. (2006) (Table 3). Active carbonate is a finely divided fraction of calcareous rock, smaller than 50 µm in size, very chemically active, and constitutes an important reserve of exchangeable Ca²⁺. The presence of both soil components is very important to *T. melanosporum* due to the action of several factors (García-Montero et al. 2007a, b). These results contrast with the soils described by Rioussset et al. (2001) for *T. indicum*; these are devoid of calcium carbonate, have a moderate pH and are rich in organic matter. Therefore, these results and the studies of Wang (2006a) have confirmed that *T. indicum* appears to adapt to a wide range of soil conditions.

Regarding host plants, some authors have reported that *T. pseudoexcavatum* is associated with pines, whereas *T. indicum* is associated with pines and Asian *Quercus* (Cooke and Massee 1892; Zhang and Minter 1988; Rioussset et al. 2001; Wang and Hall 2001; Granetti et al. 2005). We have established that *T. pseudoexcavatum* and *T. indicum* showed a high capacity for mycorrhization with *Quercus ilex* subsp. *ballota*. Comadini and Pacioni (1997), Zambonelli et al. (1997) and Di Massimo et al. (1998) also mycorrhized *T. indicum* with *Quercus ilex* L. subsp. *ilex*, *Q. pubescens* Willd., and *Q. cerris* L. These four *Quercus* taxa are the principal host plants of *T. melanosporum* in European Mediterranean areas. Ferrara and Palenzona (2001) also indicate that the spores of *T. indicum* have a high germination capacity, and form young ectomycorrhizae and abundant pre-trophic mycelia 3 months after inoculation of spores in *Quercus pubescens* plants. In this regard, not only *T. indicum* but also *T. melanosporum* is able to form mycorrhizae as soon as 2.5 months after inoculation.

We conclude that *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae grow well in calcareous substrates rich in active carbonate inhabited by *T. melanosporum*; they also mycorrhize with the principal *T. melanosporum* host plants. Therefore, both Chinese truffle species have the potential capacity to penetrate numerous *T. melanosporum* plantations and Mediterranean ecosystems. The ectomycorrhizae of these Chinese truffles are morphologically similar to *T. melanosporum* ectomycorrhizae, and it is, therefore, important to develop new protocols, based on morphological and genetic analyzes, for the quality control of commercial plants mycorrhized with *Tuber*.

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Statistical patterns of carbonates and total organic carbon on soils of *Tuber rufum* and *T. melanosporum* (black truffle) brûlés

L. G. García-Montero^{A,D}, I. Valverde-Asenjo^B, P. Díaz^C, and C. Pascual^A

^ADepartment of Forest Engineering, E.T.S.I. Montes, Technical University of Madrid (UPM), Ciudad Universitaria s/n E-28040, Madrid, Spain.

^BDepartment of Soil Science, Universidad Complutense de Madrid, Facultad de Farmacia, Ciudad Universitaria s/n E-28040, Madrid, Spain.

^CDepartment of Industrial Chemistry and Polymers, E.U.I.T. Industrial, Technical University of Madrid (UPM), C/ Ronda de Valencia 3, Madrid 28012, Spain.

^DCorresponding author. Email: luisgonzaga.garcia@upm.es

Abstract. The ascocarps of several truffle species, such as *Tuber rufum*, are harvested in *T. melanosporum* brûlés; these species reduce *T. melanosporum* production. Some authors argue that this competition might be due to the evolution of organic matter in the soil. However, soil conditions in brûlés have yet to be clarified, and most studies on *T. melanosporum* fail to supply statistical data. We propose a study of 40 soils to compare the values for total organic carbon (TOC), pH, and carbonates inside the brûlés with the values for the outer edges of the same brûlés, where *T. rufum* ascocarps are collected. A principal component analysis relates the soils from the inner brûlés with high active carbonate content and soils outside the brûlés with high TOC and total carbonate content. ANOVA analyses indicate that the mean concentration of active carbonate, total CO₃²⁻, and % active/total carbonate differ significantly depending on soil location, but there are no significant differences for pH and TOC. These results lead us to propose a new hypothesis: *T. melanosporum* mycelia may solubilise active carbonate (<50 µm) and the other carbonate fractions (>50 µm) inside the brûlés; however the environmental conditions of the brûlés could favour a secondary carbonate precipitation with a net increase in active carbonate. This increase would counterbalance carbonate losses from leaching, which would in turn favour *T. melanosporum* mycelia, suggesting a feedback process.

Additional keywords: *Tuber melanosporum*, *Tuber rufum*, truffle soil, total carbonate, active carbonate, truffle culture.

Introduction

The properties of carbonated soils limit the development of many mycorrhizal mushrooms. Truffles (*Tuber Micheli* ex Wiggers) are nevertheless an exception to this rule, since the calcareous and well drained soils of Mediterranean forests afford the required habitat for many *Tuber* species. *Tuber melanosporum* Vittad. (black truffle of Périgord) has greater culinary and economic value (up to €1000/kg) than other truffle species, such as *T. brumale* Vittad., *T. aestivum* Vittad. form *meridionalis* Chevalier et Rioussset, *T. mesentericum* Vittad., and *T. rufum* Pico ex Fries var. *rufum* form *rufum* Montecchi et Lazzari. *Tuber melanosporum* cultivation has spread throughout Europe, New Zealand, Australia, the United States, Israel, Chile, and to other countries in recent years.

In truffle culture, the terms ‘burn’ or ‘brûlé’ are used to describe spots where truffles grow and refer to the phytotoxic capacity of the *Tuber* mycelia and to their ability to create clearings in the vegetation where the mycelia bear fruit (Papa 1992). The simultaneous production of ascocarps in various truffle species can be observed within the same brûlé; these

truffles are distributed within the space of the brûlé in a clearly defined manner. *Tuber brumale*, *T. rufum*, *T. aestivum*, and *T. mesentericum* ascocarps are frequently collected outside *T. melanosporum* brûlés, or in their innermost part. Inside the brûlés, a time succession of the various truffle species can also be observed; *T. rufum* ascocarps are the first to be collected, then *T. melanosporum*, and finally *T. brumale* (Montacchini *et al.* 1972; Falini and Granetti 1998; Callot 1999; Rioussset *et al.* 2001; Ricard 2003; Granetti *et al.* 2005).

García-Montero *et al.* (2006) review the soil requirements of these truffle species to determine which soil properties have the greatest effect on truffle interactions. However, the soils inhabited by *Tuber rufum* have not been extensively studied. Rioussset *et al.* (2001) indicate that *T. rufum* lives in chalky soils in identical habitats to those occupied by *T. melanosporum*, *T. brumale*, *T. aestivum*, *T. uncinatum* Chatin, and *T. mesentericum*. In many areas of Spain, collectors call the ascocarps of *T. rufum* ‘bordes’ (‘edges’); this common name refers to the way in which the ascocarps of this species are

collected on the outer edges of the *T. melanosporum* brùlés. *Tuber rufum* may also enter natural and cultivated brùlés when the production of *T. melanosporum* begins to decline (García-Montero 2000; Rioussset *et al.* 2001). Recently, Iotti *et al.* (2007) indicated that *T. rufum* constitutes a threat to commercial nurseries producing plants mycorrhized with truffles, and proposed techniques of molecular biology to detect their mycorrhizae.

Regarding the soil properties of *Tuber melanosporum* brùlés, Callot (1999), Ricard (2003), and Granetti *et al.* (2005) suggest that there is a lower quantity of total organic carbon (TOC) content inside the brùlés than outside, and than in the productive locations of other truffle species such as *T. brumale* and *T. rufum*. Those authors propose that *T. rufum* occurs outside the brùlés, or in the innermost part next to the symbiont plant, because in both these places there is a greater coverage of herbaceous plants and a greater accumulation of organic matter.

In any case, these authors indicate that the biological and physical-chemical conditions relating to the development of the brùlés should be further clarified. Moreover, most studies on truffles fail to supply any statistical analysis of the quantitative data associated with truffle ecology, such as the relationship between soils and brùlé development. García-Montero *et al.* (2006, 2007a, 2007b) report that the annual ascocarp production of *Tuber melanosporum* increases with the size of the brùlé, which explains 38% of the variance in its productivity; they also indicate that a high content of active carbonate (calcium carbonate extractable with ammonium oxalate) accounts for up to 51% of the variance in *T. melanosporum* brùlé sizes. The authors describe that a high content of active carbonate also explains 43% of the variance in *T. melanosporum* ascocarp production, and 42% of the variance in exchangeable Ca^{2+} concentration in the brùlés.

Active carbonate is a finely divided fraction of calcareous rock, <50 µm in size, very chemically active, which constitutes an important reserve of calcium. The presence of both soil components is very important to *Tuber melanosporum* due to the action of several factors. In this regard, Ricard (2003) and Jaillard *et al.* (2007) suggest that the lack of studies on active carbonate in truffle culture is a significant omission.

García-Montero *et al.* (2007b) propose that development of the brùlé may encourage the formation of a large amount of active carbonate, and *Tuber melanosporum* activity and brùlé size could be simultaneously favoured by a high concentration of active carbonate, which would suggest a feedback process. However, these hypotheses must be confirmed.

The general objectives of the present work are to increase our knowledge of the carbonate soil dynamics inside *Tuber melanosporum* brùlés, and to study the statistical relationships between soil properties and the presence of *T. melanosporum* ascocarps, and *T. rufum* ascocarps outside the brùlés. With these aims, we carry out a statistical study on the differences in pH, total carbonate, active carbonate, and TOC in soils inside and outside the brùlés. In order to achieve this, we have used some additional soil data from a previous work (García-Montero *et al.* 2006).

Materials and methods

Case study area

The study area covers a 7-km radius between the municipal areas of Peralejos de las Truchas (Guadalajara) and Belvalle (Beteta, Cuenca) (40°35'49.65"N; 1°54'12.78"W) (García-Montero *et al.* 2006, 2007b). Forty plots were selected associated to wild *Tuber melanosporum* orchards. The study area is a mountainous region at an altitude of <1000 m in a supra-Mediterranean bio-climatic belt with a sub-humid, shady climate. Average annual precipitation is 797 mm, with low yearly average temperatures (9.7°C). Its lithology comprises predominantly Jurassic and Cretaceous limestones and dolomites, and soils are frequently lithic and rendzic leptosols with considerable surface stoniness and high peaks. Its vegetation consists of forests of *Quercus faginea* Lam. (*Cephalanthero longifoliae*–*Querceto fagineae* S. geobotanical classification type), river forests (*Astrantio*–*Coryleto avellanae* S.), and holm oak forests (*Junipero thuriferae*–*Querceto rotundifoliae* S.).

Soil analysis

Forty soils were selected according to their position in *Tuber melanosporum* brùlés. Twenty soil samples were taken from inside the brùlés, at points with significant production of *T. melanosporum* (Table 1), as calculated by García-Montero *et al.* (2007b). Twenty samples were taken from outside these brùlés, situated at least 1 m from the brùlé borders, in points without *T. melanosporum* production. In these external soil samples, 13 samples were taken in *T. rufum* productive

Table 1. Analytical results of 20 soils located inside brùlés with *Tuber melanosporum* production
Sample code: I, internal brùlé soil

Sample code	pH _{H₂O}	Total CO ₃ ²⁻ (g/100 g)	Active CaCO ₃ (g/100 g)	Active/total CO ₃ ²⁻ (%)	TOC (g/1000 g)
1-I	8.35	2.50	1.95	78.13	4.89
2-I	7.73	42.37	2.75	6.49	125.60
3-I	7.54	39.93	6.50	16.28	116.90
4-I	7.90	62.82	1.13	1.79	68.10
5-I	7.90	66.23	1.06	1.60	79.00
6-I	8.03	68.42	0.69	1.00	39.70
7-I	8.08	50.16	0.00	0.00	38.50
8-I	7.97	1.46	1.19	81.28	16.40
9-I	7.90	3.12	2.25	72.12	34.03
10-I	7.88	7.88	7.01	88.96	35.62
11-I	7.50	12.79	1.63	12.74	23.02
12-I	7.90	9.18	0.38	4.14	31.52
13-I	7.80	7.19	2.88	40.06	75.24
14-I	8.15	4.18	2.63	62.92	11.58
15-I	7.90	11.50	8.14	70.78	36.07
16-I	8.15	4.53	2.38	52.54	10.80
17-I	7.90	10.21	3.38	33.10	25.51
18-I	8.07	6.28	0.38	6.05	5.25
19-I	7.95	7.81	2.88	36.88	31.10
20-I	7.10	14.56	1.50	10.30	1.00
Mean	7.89	21.66	2.54	33.86	40.49
s.d.	0.27	23.52	2.24	31.89	35.31
CV%	3.42	108.59	88.19	94.18	87.21

points (Table 2). Fifteen of the 40 soil samples were previously studied by García-Montero *et al.* (2006). Only the first 0.30 m of each soil profile was studied because *T. melanosporum* usually bears fruit in this range (Verlhac *et al.* 1990). The soils were sampled always using the same spade (0.20 m blade) and a tape measure. These samples included a mixture of 1000 g of the soil surface horizons that have not been differentiated, as natural *T. melanosporum* brûlés contain a constant mixture of horizons due to the continuous digging that the harvesters, their dogs, and wildlife engage in to extract the ascocarps.

Sampling was done according to the FAO (1990). The following soil determinations were made: pH in water; TOC; total carbonate (equivalent calcium carbonate; Maraños *et al.* 1994; ISRIC 1995), following the methods of the ISRIC (1995); active carbonate (calcium carbonate extractable with ammonium oxalate) determined according to AFNOR (1982).

Taxonomy studies

The truffles obtained in the brûlés in the study area were conserved in the herbarium of the Department of Forest Engineering (ETSI Montes; Technical University of Madrid). The carpophores, ascospores and mycorrhizas were identified with a stereoscopic microscope (Leica WildMZ8) and a microscope (Leica LeitzDMRB) following the descriptions and recommendations proposed by Agerer (1987–2002), Montecchi and Sarasini (2000) and Rioussset *et al.* (2001).

Statistical analyses

A principal component analysis (PCA) was done with the soil variables studied in order to analyse the statistical patterns of the overall soil properties regarding the presence of *Tuber melanosporum* and *T. rufum* ascocarps. An ANOVA test was performed to determine whether there were any significant differences between the variables for the soils inside and outside the brûlés. Statistical treatment was performed with the STATISTICA Program v. 6. Before proceeding to the analysis, the distributions of the variables were adjusted to comply with the prerequisites of the statistical analysis. These transformations were selected using the Box and Cox (1964) test. Normality was checked using the Shapiro–Wilks and Kolmogorov–Smirnov tests, and homogeneity of variances was verified by the Levene test.

Results

Macroscopic studies of the characteristics of the ascocarps, and microscopic studies of the morphological characteristics of the ascospores and mycorrhizas, permitted a clear identification of *Tuber* species located in the brûlés studied.

Tables 1 and 2 show physical-chemical properties of soil samples collected inside and outside the brûlés. Many of the soil samples had a moderately alkaline pH, and a very variable percentage of total carbonate and active carbonate concentration. Levels of TOC were moderate.

Table 2. Analytical results of 20 soils without *Tuber melanosporum* production located outside its brûlés
Sample code: E, external brûlé soil

Sample code	Ascocarp production	pH _{H₂O}	Total CO ₃ ²⁻ (g/100 g)	Active CaCO ₃	Active/total CO ₃ ²⁻ (%)	TOC (g/100 g)
1-E	Null	8.17	9.66	1.50	15.53	0.74
2-E	<i>Tuber rufum</i>	7.87	77.00	0.89	1.16	5.12
3-E	<i>Tuber rufum</i>	7.81	75.00	0.00	0.00	7.09
4-E	<i>Tuber rufum</i>	7.90	61.00	1.23	2.02	3.23
5-E	<i>Tuber rufum</i>	7.98	70.50	0.73	1.03	7.22
6-E	<i>Tuber rufum</i>	7.61	69.50	0.02	0.02	11.12
7-E	<i>Tuber rufum</i>	8.07	45.50	0.00	0.00	5.61
8-E	<i>Tuber rufum</i>	8.16	14.00	1.79	12.80	1.69
9-E	<i>Tuber rufum</i>	7.95	20.00	0.23	1.15	7.01
10-E	Null	7.92	6.91	5.63	81.36	4.26
11-E	Null	7.70	14.02	0.38	2.68	0.06
12-E	<i>Tuber rufum</i>	7.82	37.50	0.35	0.93	7.15
13-E	<i>Tuber rufum</i>	8.13	23.38	2.75	11.76	3.06
14-E	Null	7.70	9.03	0.63	6.92	7.34
15-E	<i>Tuber rufum</i>	8.09	34.00	4.43	13.03	2.83
16-E	<i>Tuber rufum</i>	7.91	21.50	1.60	7.46	5.04
17-E	Null	7.00	18.44	0.00	0.00	4.32
18-E	<i>Tuber rufum</i>	8.10	40.00	0.00	0.00	8.32
19-E	Null	8.16	9.93	1.00	10.07	4.59
20-E	Null	8.06	15.63	0.15	0.96	1.33
<i>Tuber rufum</i> mean	–	7.95	45.30	1.08	3.95	57.31
s.d.	–	0.16	22.80	1.32	5.27	26.22
CV%	–	2.01	50.33	122.22	133.42	45.75
External soil mean	–	7.91	33.63	1.17	8.44	48.37
s.d.	–	0.27	24.50	1.53	17.96	28.29
CV%	–	3.41	72.85	130.77	212.80	58.49

The first 3 factors of PCA with the soil variables (PC_1 , PC_2 , and PC_3) accounted for 91% of the variance contained in the original matrix (Fig. 1).

PC_1 and PC_2 are the components that best explain the interactions between the variables. PC_1 accounted for 45% of the variance. It highlights the differences between soils with a greater quantity of total carbonate and TOC, and soils rich in

active carbonate (Table 3). The second factor (PC_2) represented 24% of the variance. PC_2 indicates that it opposed pH and the other variables studied (Table 3). Figure 2 shows different soils according to PC_1 and PC_2 ; soils from inside the *T. melanosporum* brûlés have an important correspondence with right semi-axis PC_1 , related to high active carbonate content, and soils from outside the brûlé, including *T. rufum* soils, have a significant correspondence with left semi-axis PC_1 , related to high TOC and total carbonate content.

The results of the ANOVA indicate that the mean concentrations of $\log(x+1)$ % active $CaCO_3$ ($F_{1,38} = 7.344$; P value 0.010), $\log$ % total CO_3^{2-} ($F_{1,38} = 5.987$; P value 0.019), and $\log(x+1)$ % active/total carbonate ($F_{1,38} = 11.896$; P value 0.001) differ significantly depending on the location of the soil, whether inside or outside the brûlés.

The results show that in the soils from inside the *T. melanosporum* brûlés, the content of active carbonate is

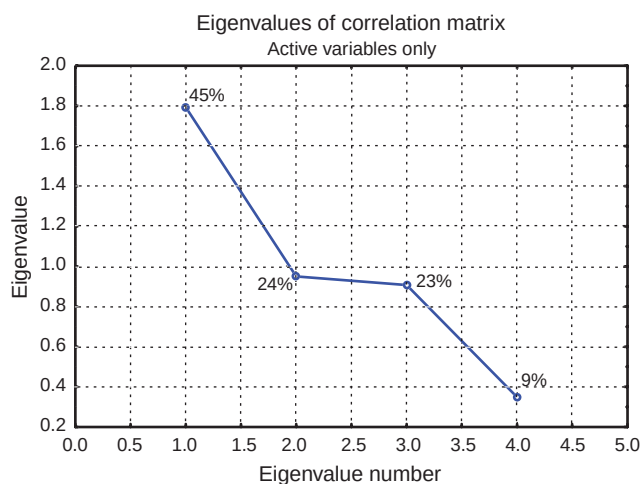


Fig. 1. Scree plot of principal component analysis.

Table 3. Principal components analysis: factor loadings
Total and active carbonates, g/100 g; TOC, g/1000 g

Variables	Factor 1 (PC_1)	Factor 2 (PC_2)	Factor 3 (PC_3)
pH	0.3973	0.5707	-0.7186
Log % total CO_3^{2-}	-0.8833	0.0823	-0.1722
Log $(x+1)$ % active carbonate	0.5097	-0.7326	-0.4076
Log $(x+1)$ % TOC	-0.7716	-0.2843	-0.4421

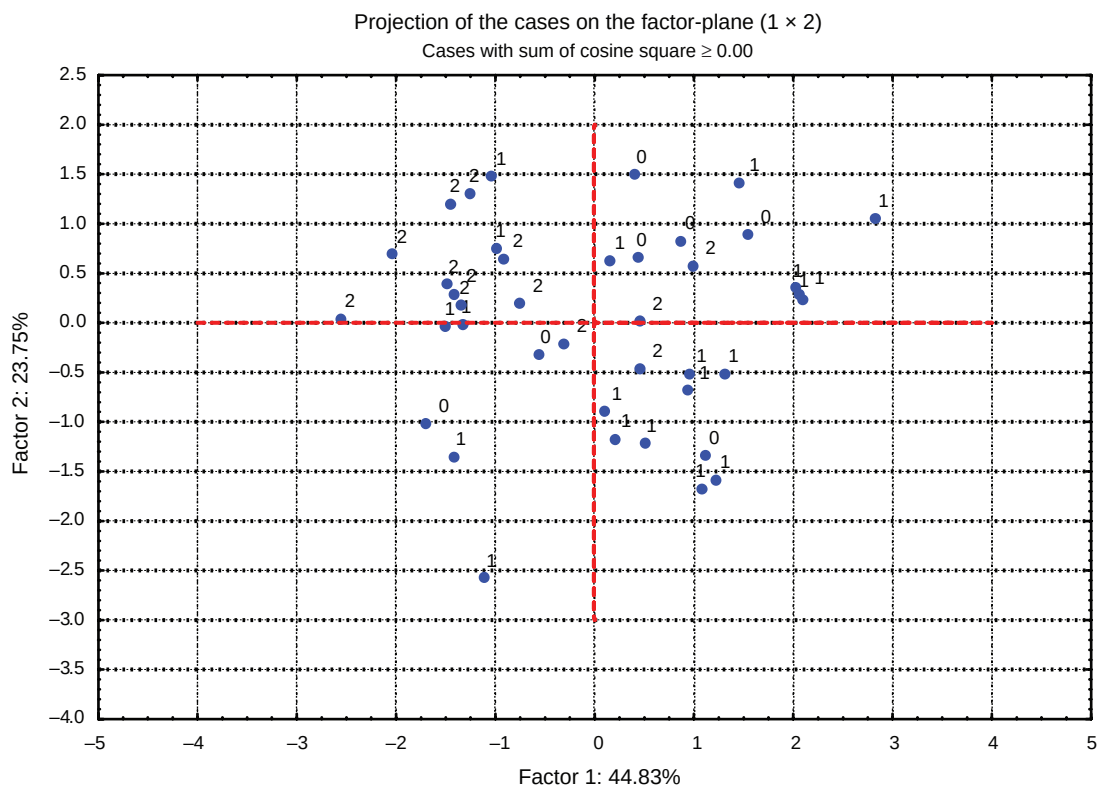


Fig. 2. Relationship between the presence/absence of *Tuber melanosporum* and *T. rufum* in the soils inside and outside the brûlés, and PCA factors. Samples coded with 1 are soil samples taken at points with production of *T. melanosporum* ascocarps inside the brûlés; samples coded with 2 are soil samples taken in production points, with production of *T. rufum* ascocarps taken outside the brûlés; samples coded with 0 are soil samples from the outside of the brûlés without any ascocarp production.

significantly higher, and the abundance of total carbonate is significantly lower, than in external soils (with *T. rufum* production). These results are more evident when the percentage of active/total carbonate inside and outside the brûlés is compared (Fig. 3). Nevertheless, the ANOVAs of pH and TOC indicate that there are no significant differences in the average values of these variables between the soils inside the *T. melanosporum* brûlés and the soils outside (with *T. rufum* production).

Discussion

The disappearance of grasses in the brûlé causes modifications in soil surface layers which affect soil organic matter (Lulli *et al.* 1999; Castrignano *et al.* 2000). Callot (1999), Ricard (2003), and Granetti *et al.* (2005) propose that the time succession of *Tuber rufum* and other truffle species in *T. melanosporum* brûlés may be due to the evolution of both the quantity and quality of the organic matter. Those authors report that the soils of *T. melanosporum* contain little coarse fraction organic matter, and a high proportion of fine fraction stable organic matter, in comparison with non-productive soils, and that the C/N ratio does not provide significant information to enable prediction of the productive capacity of the soil in the brûlé.

The PCA shows that in the study area, *T. rufum* ascocarps have been collected in soils with a greater concentration of TOC and total carbonate than *T. melanosporum* soils (Fig. 2). However, the ANOVA indicates that the differences in the TOC content between the soils outside and inside the brûlés is not statistically significant (Fig. 3). Therefore, new studies are necessary; for example, on the competition between different *Tuber* species and their location in regard to the brûlé, and according to the proposals of Callot (1999) and Ricard (2003),

on the characteristics and properties of soil organic matter in relation with the development, production, and competition of *T. melanosporum* with other *Tuber* species.

Our statistical results (PCA and ANOVA) show that the abundance of active carbonate is significantly higher inside *Tuber melanosporum* brûlés than outside, where *T. rufum* is collected. These results agree with a previous multivariate statistical study using 12 conventional soil properties (granulometric texture, pH, carbonate fractions, TOC, total N, exchangeable cations) in 24 brûlés, which showed that active carbonate content is the variable that best accounts for the degree of success by *T. melanosporum* in occupying the habitat, compared with *T. aestivum* and *T. mesentericum* (García-Montero *et al.* 2008).

PCA and ANOVA also show that the concentration of total carbonate is significantly higher outside the brûlés, while the active/total carbonate ratio is significantly higher inside the brûlés. These statistical patterns lead us to propose as a hypothesis that in the field *T. melanosporum* mycelia could be acting directly on the carbonated soil fractions (<2 mm). This hypothesis is related to the experimental results of Poitou (1988), which showed that, *in vitro*, *T. melanosporum* mycelia acidify their environment and dissolve calcium carbonate, and supply carbonates, bicarbonates, and other salts.

Fungal metabolism has an enormous capacity for transforming the environment. Callot (1999) reports the doctoral works of Pottier (1986), who carried out an experiment on the growth conditions of the *Tuber melanosporum* mycelia with populations of associated bacteria. This was done by cultivation in liquid media and agar substrates, with different initial values of pH and Ca^{2+} concentration, and demonstrated that, regardless of the initial conditions, pH values of 5.5–6 were reached in all the cultures. It was concluded that the *T. melanosporum* mycelium (associated

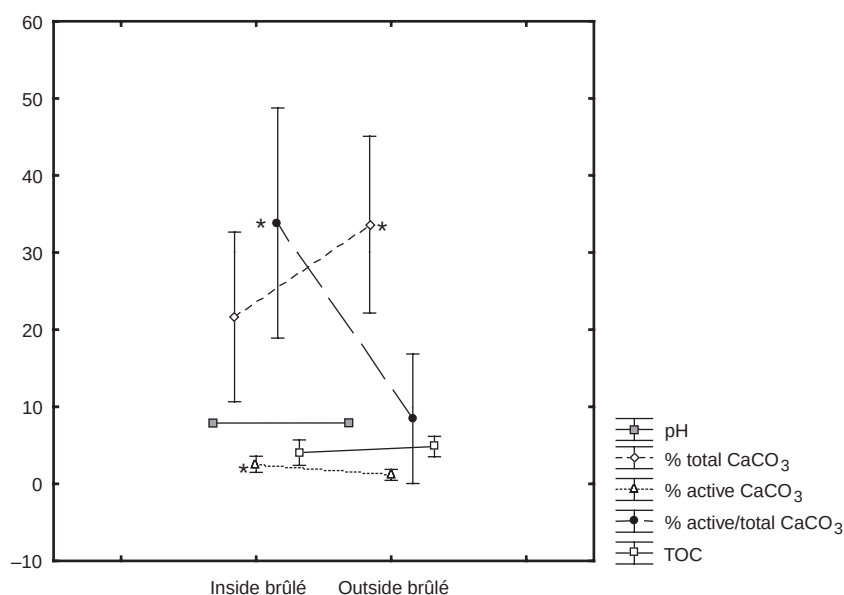


Fig. 3. Average of soil variables values for comparison of soils inside/outside of the brûlés (* $P < 0.05$). Whisker, mean $\pm$ 0.95 confidence interval.

to bacterial populations) strongly acidifies its environment. This experiment also confirms that Ca^{2+} favours the development of the *T. melanosporum* mycelium.

Cooke and Whipps (1993) describe that the mycelia of fungi excrete H^+ in relation to the metabolism of the cations. This excretion of H^+ is therefore linked to the capture of K^+ by the fungi, which occurs by means of active transport, and exceeds concentration gradients of 5000:1. This metabolic effort shows the importance of K^+ in the development of fungi. In the case of *T. melanosporum*, its relation with K^+ has been confirmed by Bencivenga and Granetti (1989), Coli *et al.* (1990), and Verlhac *et al.* (1990), who indicate that K^+ is the most abundant cation in its ascocarps. Regarding the affinity of fungi for K^+ , Cooke and Whipps (1993) explain that cation translocation involves mechanisms which permit ion movement, such as the H^+-K^+ or Na^+-K^+ exchangers linked to ATP. This increases levels of K^+ in the plasma membrane and simultaneously decreases the cell concentration of H^+ or Na^+ , excreting them to the outside environment.

Therefore, we propose that the metabolism of *T. melanosporum* (and its associated bacteria) may acidify its immediate soil environment. An increase in H^+ in the hyphosphere micro-environment could provoke an alteration in the chemical balance between the CO_3^{2-} solid phase, the water dissolution of H_2CO_3 and HCO_3^- , and the CO_2 of the soil atmosphere, and in consequence, the dissolution of the carbonate in the hyphosphere soil. Callot (1999) reports that the roots and the microflora associated with limy soils create microsites of acidified soils which enable the dissolution of calcareous rock.

Our statistical results lead us to confirm this hypothesis regarding the dynamic of carbonates in *T. melanosporum* brûlés. First, *T. melanosporum* mycelia may solubilise active carbonate ($<50\text{ }\mu\text{m}$) and larger carbonate fractions ($>50\text{ }\mu\text{m}$ and $<2\text{ mm}$), which would then dissolve in the water of the hyphosphere micro-environment. This carbonate-enriched water would allow the mobilisation and redistribution of carbonates throughout the brûlé soil. Part of the carbonate-enriched water would be incorporated into the processes of leaching and loss of soil carbonate, above all in periods of rain. This first part of the hypothesis is related to the significant decrease in total carbonates observed in the interior of the brûlés compared with the exterior (Tables 1 and 2, Fig. 3). The problem of the leaching of carbonates in *T. melanosporum* brûlés has been pointed out by Callot (1999) and Ricard (2003).

The second phase of our proposed hypothesis is that another part of the carbonate-enriched water from the hyphosphere may be redistributed throughout the soil brûlé and participate in the secondary accumulation of CaCO_3 in the brûlé surface horizons. This formation of new active carbonate counterbalances carbonate losses from leaching. This second phase of the hypothesis is linked to the net increase in active carbonate observed in the interior of the brûlés compared with the exterior (Tables 1 and 2, Fig. 3). This is more evident when comparing the percentages of active/total carbonate inside and outside brûlés. This proposal agrees with Callot (1999), Ricard (2003), and García-Montero *et al.* (2006, 2007a), who indicated that *Tuber melanosporum*

requires a secondary accumulation of CaCO_3 in the brûlé surface horizons.

Jaillard *et al.* (2007) also indicates that it is well known that the water dynamic in the soil is a major factor in the carbonate dynamic in *T. melanosporum* brûlés. However, the relationship between active and total carbonates in soils also depends on complex mechanisms related with soil-forming factors such as relief, vegetation, human and natural disturbances, time, and climate (Jenny 1941; Wild 1992; Breemen and Buurman 1998). These complex processes may explain the differences observed when comparing the balances of carbonates inside and outside the brûlés (Tables 1 and 2): the average difference for active carbonate is $\sim 1.4\text{ g}/100\text{ g}$ soil, whereas for total carbonate this difference is $\sim 12\text{ g}/100\text{ g}$ soil. This imbalance opens up new lines of research to design experiments which enable the balances of carbonate dissolution, leaching, decarbonation, and recarbonation in the brûlés to be analysed.

In summary, we propose the following new hypothesis, based on own analyses and on the works of various authors: *Tuber melanosporum* mycelia may solubilise active carbonate ($<50\text{ }\mu\text{m}$) and other carbonate fractions ($>50\text{ }\mu\text{m}$) inside the brûlés. The environmental conditions of the soils in the brûlés could favour a secondary carbonate precipitation with a net increase in active carbonate. This increase would counterbalance carbonate losses from leaching, which would in turn favour *T. melanosporum* mycelia, suggesting a feedback process. Moreover, this carbonate dynamic hypothesis provides a better explanation for the behaviour of *T. rufum* compared with *T. melanosporum* than the evolution of the TOC in the brûlés. However, new research is required into the cause-effect relationship between brûlé development, active and total carbonate dynamics, soil alkalinity, and metabolism of different *Tuber* species.

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Calcareous amendments to soils to eradicate *Tuber brumale* from *T. melanosporum* cultivations: a multivariate statistical approach

Inmaculada Valverde-Asenjo ·
Luis G. García-Montero · Asunción Quintana ·
Javier Velázquez

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Abstract Calcareous amendments are being used in *Tuber melanosporum* truffle plantations in attempts to eradicate *Tuber brumale*. However, there are no studies available which provide soil analysis and statistical data on this topic. We studied 77 soil samples to compare the values for carbonates, pH and total organic carbon in *T. brumale* truffières with the values for *T. melanosporum* truffières on contaminated farms and in natural areas. Statistical analyses indicate that the concentrations of active carbonate and total carbonate in the soil are significantly higher in *T. brumale* truffières than in *T. melanosporum* truffières, but that there are no significant differences in pH and total organic carbon. We conclude that liming would not suppress *T. brumale* ectomycorrhizas in contaminated *T. melanosporum*

farms, and calcareous amendments do not therefore seem to be a means of eradicating *T. brumale* in these farms.

Keywords Ectomycorrhizae · Liming · Truffle culture · *Tuber brumale* · *Tuber melanosporum*

Introduction

Truffles are a highly profitable cash crop growing in the forests of many Mediterranean regions, and the cultivation of *Tuber melanosporum* Vittad. (Périgord black truffle) has spread throughout many countries in recent decades. The ectomycorrhizas of other species such as *Tuber brumale* Vittad. contaminate *T. melanosporum* brûlés (zones around the host trees free of vegetation) reduce their carpophore production and pose formidable problems for those trying to optimise *T. melanosporum* cultivation (Chevalier and Frochot 1997; Callot 1999; Lefevre and Hall 2001; Rioussset et al. 2001; Olivier et al. 2002; Ricard 2003; Sourzat 2005).

It is not known how *T. melanosporum* and *T. brumale* interact with each other in *T. melanosporum* brûlés and the biological and physical–chemical properties of soil. Experiments have been proposed to study this in planted truffières (the truffle-producing area; Mamoun and Olivier 1993a, b) using molecular techniques to detect their mycorrhizas (Rubini et al. 1998; Paolocci et al. 1999; Giomaro et al. 2002; Douet et al. 2004). Other researchers have underlined the importance of conducting studies on the *T. melanosporum* brûlé to explain hydric behaviour and the dissolution and precipitation processes of carbonates (Callot 1999; Ricard 2003; Granetti et al. 2005; Jaillard et al. 2007).

A simultaneous production of carpophores in various *Tuber* species can be observed within the same truffière.

I. Valverde-Asenjo
Department Soil Science, University Complutense of Madrid,
Ciudad Universitaria s/n,
Madrid 28040, Spain

L. G. García-Montero (✉)
Department of Forest Engineering, U.D. Operaciones Básicas,
Technical University of Madrid (UPM),
E.T.S. Ingenieros de Montes,
Ciudad Universitaria s/n,
Madrid 28040, Spain
e-mail: luisgonzaga.garcia@upm.es

A. Quintana
Agriculture, Research and Technology IKT S.A.,
Granja Modelo s/n,
01192 Arkaute-Alava, Spain

J. Velázquez
Department of Projects and Rural Planning, Technical University
of Madrid (UPM), E.T.S. Ingenieros de Montes,
Ciudad Universitaria s/n,
Madrid 28040, Spain

Callot (1999), Ricard (2003) and Granetti et al. (2005) indicate that there is a lack of knowledge as to how the biological and physical–chemical properties of soil influence the development of *T. melanosporum* ectomycorrhizas and their interactions with other *Tuber* species.

These authors and Jaillard et al. (2007) explain that *T. melanosporum* is strictly calcicolous and that their ectomycorrhizas are closely linked to calcium carbonate. They underlined the importance of conducting an overall study of soil brûlé profiles to explain hydric behaviour and the dissolution and precipitation processes of carbonates. Some statistical studies have also shown that a high concentration of active carbonate (calcium carbonate extractable with ammonium oxalate; smaller than 50 µm in size) is responsible for up to 43% of the variance in *T. melanosporum* carpophore production; this accounts for up to 51% of the variance in brûlé sizes and is a major factor in the fruiting and aggressiveness of *T. melanosporum* versus *Tuber aestivum* Vittad., *T. mesentericum* Vittad. and *Tuber rufum* Pico ex Fries (García-Montero et al. 2007a, b, 2008a, b).

RiOUSset et al. (2001) and García-Montero et al. (submitted) outlined experiments in France and Spain where calcium carbonate was applied to *T. melanosporum* soils in attempts to favour *T. melanosporum* and deter *T. brumale*, but reported that these were unsuccessful. Consequently, we carried out a study to determine if there are differences in pH, carbonate fractions and abundance of

total organic carbon (TOC) in soils occupied only by *T. brumale* or *T. melanosporum* or where the two species were present together and from this infer whether calcareous amendments to soils to eradicate *T. brumale* had a sound scientific basis.

Materials and methods

Case study areas

The study areas were located in the mountainous regions of Central Spain in supra-Mediterranean bioclimatic belts, with predominantly Jurassic and Cretaceous limestones and dolomites, and the soils lithic and rendzic leptosols. Seventy-seven soil samples were collected from *T. brumale* and *T. melanosporum* truffières from two planted truffières and two natural truffières in regions with and without a natural presence of *T. brumale*.

Table 1 summarises the environmental characteristics of study areas. In Sarrión (Teruel), *T. melanosporum* plantation is 15 years old and has 50% productive *T. melanosporum* truffières (>500 g/year) and 20% contaminated truffières that produce more than 500 g/year of *T. brumale* carpophores. In Campezo (Alava), *T. melanosporum* plantation is 14 years old and has 60% productive *T. melanosporum* truffières (>500 g/year) and 20% contami-

Table 1 Study area characteristics

Species	Truffière type	Soil samples	Symbiotic plant	Village	Province	Altitude	P	T	Vegetation
<i>Tuber brumale</i>	Natural	17	<i>Quercus ilex</i> L. subsp. <i>ballota</i> (Desf.) Samp.	Iruña de Oca	Alava	498	700	10	<i>Spiraeo hispanicae-Querceto rotundifoliae sigmetum</i>
<i>Tuber melanosporum</i>	Natural	20	<i>Quercus faginea</i> Lam. and <i>Quercus ilex</i> L. subsp. <i>ballota</i> (Desf.) Samp.	Peralejos de las Truchas	Guadalajara	1,150	797	10	<i>Cephalanthero longifoliae-Querceto fagineae sigmetum</i> and <i>Junipero thuriferae-Querceto rotundifoliae sigmetum</i>
<i>Tuber melanosporum</i>	Planted	15	<i>Quercus ilex</i> L. subsp. <i>ballota</i> (Desf.) Samp.	Sarrión	Teruel	991	500	11	<i>Junipero thuriferae-Querceto rotundifoliae sigmetum</i>
<i>Tuber brumale</i>	Contaminated	15	<i>Quercus ilex</i> L. subsp. <i>ballota</i> (Desf.) Samp.	Sarrión	Teruel	991	500	11	<i>Junipero thuriferae-Querceto rotundifoliae sigmetum</i>
<i>Tuber melanosporum</i>	Planted	5	<i>Quercus ilex</i> L. subsp. <i>ballota</i> (Desf.) Samp.	Campezo	Alava	578	732	12	<i>Spiraeo hispanicae-Querceto rotundifoliae sigmetum</i>
<i>Tuber brumale</i>	Contaminated	5	<i>Quercus ilex</i> L. subsp. <i>ballota</i> (Desf.) Samp.	Campezo	Alava	578	732	12	<i>Spiraeo hispanicae-Querceto rotundifoliae sigmetum</i>

Altitude in metres. Vegetation = geobotanical classification type according to Rivas-Martínez (1987)

P average annual precipitation (in mm), T average annual temperature (in °C)

nated truffières that produce more than 300 g/year of *T. brumale* carpophores.

Soil analysis

The soil samples were taken according to FAO (1990) recommendations from inside the holes that the collectors made to extract the carpophores in truffières with a significant production (>300 g/year). Only the first 30 cm of each soil profile was studied because *T. melanosporum* usually bears fruit in this range (Verlhac et al. 1990). The soils were sampled always using the same spade (20-cm blade) and a rule. These samples included a mixture of 1,000 g of the soil surface horizons that have not been differentiated, as truffières contain a constant mixture of horizons due to the continuous digging that the harvesters, their dogs and wildlife engage in to extract the carpophores.

The following soil determinations were made: pH in water, total organic carbon and total carbonate (equivalent calcium carbonate: Maraños et al. 1994; ISRIC 1995) following the methods of the ISRIC (1995); active carbonate (calcium carbonate extractable with ammonium oxalate) was determined according to AFNOR (1982).

We have also used some additional data from 20 *T. melanosporum* soil samples from two previous works (García-Montero et al. 2006, 2008a).

Statistical analysis

Statistical analysis of the data was carried out using the Statistica Program v. 6 (StatSoft, Tulsa, OK, USA, 1999). Before the analysis, the distributions of the variables were adjusted to comply with the prerequisites of the parametric statistical analysis. These transformations were selected using the Box and Cox (1964) tests. Normality was checked using the Shapiro–Wilks and Kolmogorov–Smirnov tests, and homogeneity of variances was verified by the Levene test. The soil data obtained in natural areas did not follow a normal distribution, and therefore, non-parametric tests were applied to these variables.

Principal components analyses (PCA) were done with the soil variables studied in contaminated plantations in order to analyse the statistical patterns of the overall soil properties regarding the fruiting of *T. brumale* versus *T. melanosporum* carpophores in truffières of contaminated plantations. Analysis of variance (ANOVA) test was performed to determine whether there were any significant differences between the variables for the soils of *T. brumale* versus *T. melanosporum* in truffières of contaminated plantations. The non-parametric Mann–Whitney *U* test was applied to analyse the statistical patterns of the soil properties of *T. brumale* truffières versus *T. melanosporum* truffières in natural areas.

Table 2 Analytical results of 20 soils located inside burns with *T. brumale* production in contaminated *T. melanosporum* plantations (active carbonate, total carbonate and TOC expressed in g kg⁻¹)

No.	Province	pH _{H2O}	Active carbonate	Total carbonate	TOC
1	Teruel	8.80	134.53	342.50	20.66
2	Teruel	8.14	182.66	502.50	18.28
3	Teruel	8.23	92.66	227.50	23.87
4	Teruel	8.23	141.67	447.50	19.72
5	Teruel	8.24	128.54	472.50	16.45
6	Teruel	8.32	127.66	307.50	24.03
7	Teruel	8.24	173.91	442.50	21.19
8	Teruel	8.21	172.66	422.50	18.83
9	Teruel	8.31	158.91	397.50	20.00
10	Teruel	7.85	162.50	412.59	23.80
11	Teruel	7.87	173.13	457.19	17.30
12	Teruel	7.93	150.63	393.10	22.08
13	Teruel	8.18	155.63	395.57	21.87
14	Teruel	8.17	137.50	371.13	19.04
15	Teruel	7.90	78.75	273.27	33.08
16	Alava	8.06	160.16	422.50	19.97
17	Alava	7.92	126.22	472.50	19.60
18	Alava	8.16	87.16	312.50	20.37
19	Alava	8.15	133.91	537.50	8.39
20	Alava	8.24	132.66	492.50	10.54

Table 3 Analytical results of 20 soils located inside burns with *T. melanosporum* production in contaminated *T. melanosporum* plantations (active carbonate, total carbonate and TOC expressed in g kg⁻¹)

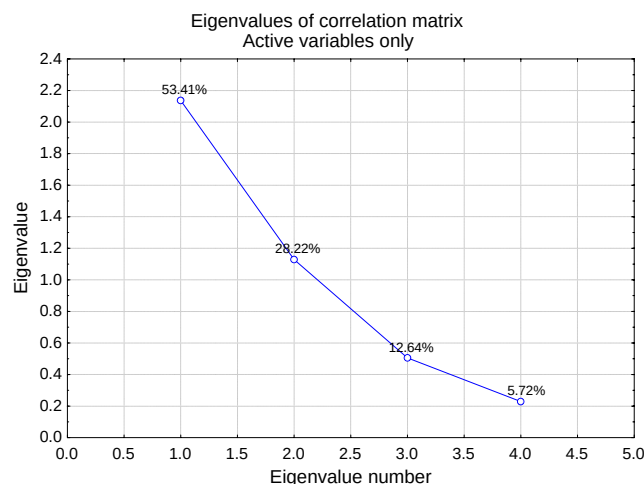
No.	Province	pH _{H2O}	Active carbonate	Total carbonate	TOC
21	Teruel	8.34	126.41	362.50	21.83
22	Teruel	8.18	156.41	492.50	21.20
23	Teruel	7.99	153.75	311.07	21.46
24	Teruel	7.85	68.75	226.33	33.99
25	Teruel	7.98	78.75	207.76	16.29
26	Teruel	8.06	65.00	241.43	14.83
27	Teruel	8.10	66.88	251.38	14.96
28	Teruel	8.09	47.50	55.31	15.11
29	Teruel	8.05	105.00	162.63	15.14
30	Teruel	8.12	135.00	320.04	22.07
31	Teruel	8.01	123.13	323.78	28.39
32	Teruel	8.12	150.63	391.49	25.93
33	Teruel	8.05	140.00	453.83	24.68
34	Teruel	7.69	61.25	253.00	37.63
35	Teruel	7.71	68.13	266.16	40.41
36	Alava	8.26	118.54	427.50	3.46
37	Alava	8.31	123.90	422.50	11.14
38	Alava	8.08	91.40	357.50	16.93
39	Alava	8.28	112.00	340.00	12.46
40	Alava	8.32	90.20	437.50	10.84

Table 4 Analytical results of 17 soils located inside burns with *T. brumale* production in natural areas (active carbonate, total carbonate and TOC expressed in g kg⁻¹)

No.	Province	pH _{H2O}	Active carbonate	Total carbonate	TOC
41	Alava	7.79	129.38	637.22	17.33
42	Alava	8.20	148.96	705.33	14.97
43	Alava	7.73	134.38	629.84	17.99
44	Alava	8.00	131.88	616.37	13.18
45	Alava	7.74	131.88	756.63	21.23
46	Alava	7.70	121.88	660.37	20.79
47	Alava	7.73	128.75	682.93	17.42
48	Alava	7.75	134.38	656.69	19.03
49	Alava	7.86	101.88	674.24	14.89
50	Alava	7.63	125.63	651.02	23.61
51	Alava	7.95	139.38	676.76	14.40
52	Alava	7.62	130.00	664.29	18.19
53	Alava	8.18	113.91	530.00	21.54
54	Alava	8.22	115.78	472.50	21.74
55	Alava	8.19	107.16	537.50	17.73
56	Alava	8.17	100.91	457.50	19.32
57	Alava	8.16	107.66	497.50	19.31

Table 5 Analytical results of 20 soils located inside burns with *T. melanosporum* production in natural areas (active carbonate, total carbonate and TOC expressed in g kg⁻¹; García-Montero et al. 2006, 2008a)

No.	Province	pH _{H2O}	Active carbonate	Total carbonate	TOC
58	Guadalajara	8.35	19.53	25.00	4.89
59	Guadalajara	7.73	27.50	423.69	125.60
60	Guadalajara	7.54	65.00	399.34	116.90
61	Guadalajara	7.90	11.25	628.23	68.10
62	Guadalajara	7.90	10.63	662.32	79.00
63	Guadalajara	8.03	6.88	684.24	39.70
64	Guadalajara	8.08	0.00	501.61	38.50
65	Guadalajara	7.97	11.88	14.61	16.40
66	Guadalajara	7.90	22.50	31.17	34.03
67	Guadalajara	7.88	70.10	78.80	35.62
68	Guadalajara	7.50	16.25	127.88	23.02
69	Guadalajara	7.90	3.75	91.82	31.52
70	Guadalajara	7.80	28.75	71.90	75.24
71	Guadalajara	8.15	26.25	41.76	11.58
72	Guadalajara	7.90	81.40	115.00	36.07
73	Guadalajara	8.15	23.75	45.27	10.80
74	Guadalajara	7.90	33.75	102.09	25.51
75	Guadalajara	8.07	3.75	62.77	5.25
76	Guadalajara	7.95	28.75	78.14	31.10
77	Guadalajara	7.10	15.00	145.63	1.00

**Fig. 1** Scree plot of principal components analysis

Identification of the harvested truffles

Macroscopic features of the carpophores and microscopic studies of the morphological characteristics of the ascospores permitted a clear identification of the harvested *T. brumale* and *T. melanosporum* truffles following the descriptions and indications proposed by Rioussset et al. (2001).

Results

Tables 2, 3, 4 and 5 show the physical–chemical properties of soil samples collected in the natural areas and contaminated *T. melanosporum* plantations. Many of the soil samples have a moderately basic pH and a very variable percentage of total carbonate and active carbonate concentration. Levels of TOC are moderate.

In the contaminated *T. melanosporum* plantations, the PCA shows that the first three factors (PC₁, PC₂ and PC₃) accounted for 94.27% of the variance contained in the original matrix (Fig. 1). PC₁ and PC₂ are the components that best explain the interactions between the variables. PC₁ accounts for 53.41% of the variance. This highlights the differences between soils with a greater quantity of total carbonate and active carbonate versus TOC (Table 6). The

Table 6 Principal components analysis of 40 soils located inside burns with *T. brumale* and *T. melanosporum* production in contaminated *T. melanosporum* plantations: factor loadings

Variables	Factor 1 (PC ₁)	Factor 2 (PC ₂)	Factor 3 (PC ₃)
% Active carbonate	−0.788498	−0.508883	0.131959
% Total carbonate	−0.827594	−0.426789	−0.161942
pH	−0.654151	0.564755	0.496169
% TOC	0.634011	−0.607285	0.464654

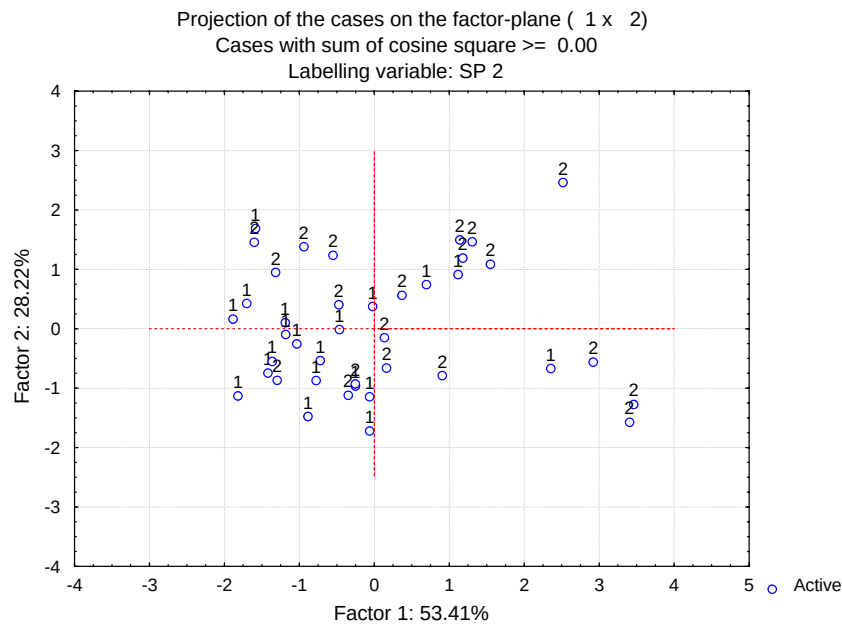


Fig. 2 Relationship between the presence/absence of *T. brumale* and *T. melanosporum* production in contaminated *T. melanosporum* plantations and PCA factors. Samples coded with 1 are soil samples

taken at points with production of *T. brumale* ascocarps inside the burns, and samples coded with 2 are soil samples taken at points with production of *T. melanosporum* ascocarps inside the burns

second factor (PC₂) represents 28.22% of the variance. PC₂ indicates that it opposes pH and the other variables studied (Table 6). Figure 2 shows different soils according to PC₁ and PC₂. This graph shows how soils from inside the *T. brumale* truffières have an important correspondence with left semi-axis PC₁, related to high active carbonate and total carbonate contents, and soils from inside the *T. melanosporum* truffières have a significant correspondence with right semi-axis PC₁, related to low active carbonate and total carbonate contents.

The ANOVAs indicate that the mean concentration of active carbonate ($F_{1,38}=13.02$; $p<0.001$) and total carbonate ($F_{1,38}=8.81$; $p=0.005$) differ significantly depending on the location of the soil. In the soils from *T. brumale* truffières, the content of active and total carbonates is

significantly higher than in *T. melanosporum* truffières (Table 7). Nevertheless, the ANOVAs of the pH and TOC indicate that there are no significant differences in the average values of these variables between the soils inside *T. brumale* truffières versus *T. melanosporum* truffières in contaminated plantations (Table 7).

The Mann–Whitney *U* test shows that significantly higher active carbonate content ($p<0.001$) is found in soils from *T. brumale* truffières than in *T. melanosporum* truffières (Table 7). The abundance of total carbonate ($p<0.001$) shows the same pattern (Table 7); however, the data distribution of total carbonate does not support the assumption of homocedasticity. There are no significant differences in the values of the pH and TOC between the soils inside *T. brumale* truffières versus *T. melanosporum* truffières.

Table 7 Analytical results of soils located inside brûlés with *T. melanosporum* or *T. brumale* carpophore production in three contaminated plantations without and with calcareous amendments (2,500 kg/ha) and three uncontaminated control plantations

N°	Data Table	Province	Study area type	Carpophore production	pH _{H2O}	Active carbonate	Total carbonate	TOC
Mean	Table 2	Alava/Teruel	Contaminated Plantations	<i>Tuber brumale</i>	8.16	140.57	405.14	19.95
SD					0.21	29.15	80.36	5.01
Mean	Table 3	Alava/Teruel	Contaminated Plantations	<i>Tuber melanosporum</i>	8.08	104.13	315.21	20.44
SD					0.18	34.51	109.15	9.39
Mean	Table 4	Alava	Natural areas	<i>Tuber brumale</i>	7.92	123.75	618.04	18.39
SD					0.23	13.77	86.74	2.89
Mean	Table 5	Guadalajara	Natural areas	<i>Tuber melanosporum</i>	7.89	25.33	216.56	40.49
SD					0.27	22.44	235.17	35.31

We have monitored the carpophore production of these 4 plantations. After 1 year, we analysed 46 soil samples from *T. melanosporum* and *T. brumale* brûlés (active carbonate, total carbonate and TOC expressed in g kg⁻¹)

Discussion

Several authors report that the simultaneous production of the carpophores of various truffle species is distributed within the space of the truffières in a clearly defined manner: *T. brumale*, *T. rufum*, *T. aestivum* and *T. mesentericum* carpophores are frequently collected outside *T. melanosporum* truffières or in their innermost part. Inside the truffières, a time succession of the various truffle species can also be observed: *T. rufum* carpophores are the first to be collected, then *T. melanosporum* and finally *T. brumale* (Montacchini et al. 1972; Falini and Granetti 1998; Callot 1999; Rioussset et al. 2001; Ricard 2003; Granetti et al. 2005).

The disappearance of grasses in the brûlés causes modifications in soil surface layers which affect soil organic matter (Lulli et al. 1999; Castrignano et al. 2000). Callot (1999), Ricard (2003) and Granetti et al. (2005) propose that the time succession of *T. brumale* in *T. melanosporum* truffières may be due to the evolution of both the quantity and quality of the organic matter. Our results indicate that in contaminated plantations and natural areas, the soils of *T. brumale* truffières show lower concentrations of TOC than *T. melanosporum* soils (Table 7). However, the statistical tests indicate that these differences in the TOC content are not statistically significant. Therefore, as proposed by Callot (1999) and Ricard (2003), new studies are required to determine the characteristics and properties of soil organic matter in relation with the competition of *T. melanosporum* and *T. brumale* ectomycorrhizas.

The statistical analyses show that the abundance of active carbonate and total carbonate are significantly higher in soils from *T. brumale* truffières compared to *T. melanosporum* both in contaminated plantations and natural areas (Figs. 3 and 4). From these results, it could be deduced that liming would not negatively affect the *T. brumale* ectomycorrhizas in contaminated *T. melanosporum* cultivations.

Calcareous amendments could otherwise play a significant role in boosting production of both *Tuber* species. This proposal is in agreement with the first results of an experimental design that we are developing in two *T. melanosporum* cultures contaminated by *T. brumale* over a period of 4 years. We are administering calcareous amendments, monitoring the production of carpophores and comparing them with two nearby control cultivations which are uncontaminated by *T. brumale* and were given no calcareous amendments. The results obtained in the first year show that *T. melanosporum* production increased by 30–32% and *T. brumale* production increased by 69–274% in the two cultures with calcareous amendments. However, the two control cultivations show that *T. melanosporum* production decreased by 18–26% due to the climatic conditions (García-Montero et al., submitted).

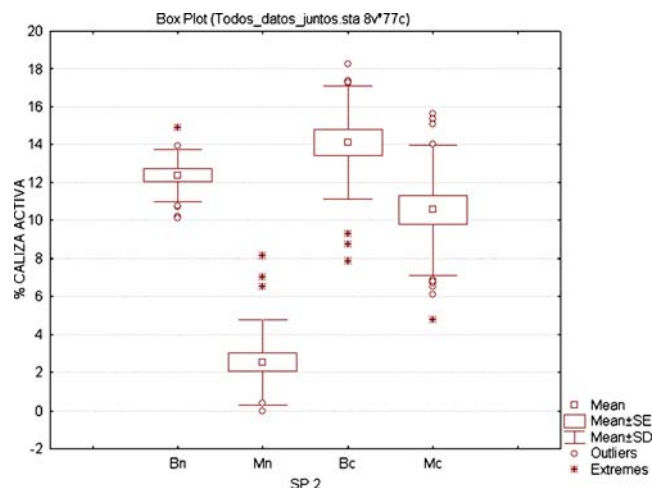


Fig. 3 Average of active carbonate (%) values for comparison of *T. brumale*/*T. melanosporum* soils from burns in natural areas and contaminated *T. melanosporum* plantations. Samples coded with Bn are soil samples taken at points with production of *T. brumale* ascocarps in natural areas; samples coded with Mn are soil samples taken at points with production of *T. melanosporum* ascocarps in natural areas; samples coded with Bc are soil samples taken at points with production of *T. brumale* ascocarps in contaminated plantations; samples coded with Mc are soil samples taken at points with production of *T. melanosporum* ascocarps in contaminated plantations

In summary, the statistical patterns of soil carbonates obtained in the study areas show that *T. brumale* ectomycorrhizas appear to be closely linked to calcium carbonate, as is *T. melanosporum*. Therefore, there is no scientific basis to

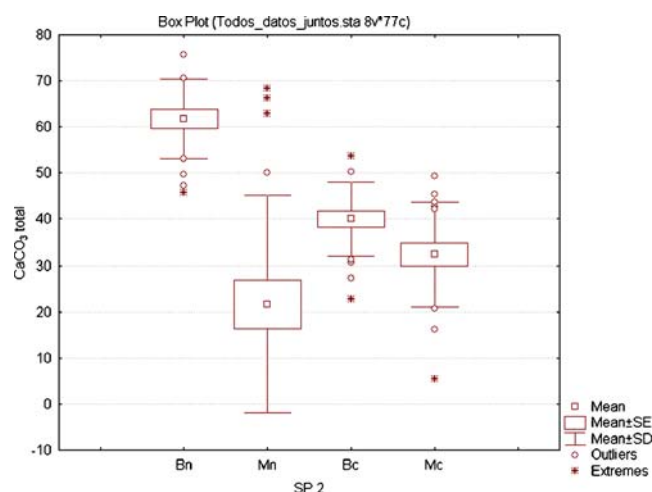


Fig. 4 Average of total carbonate (%) values for comparison of *T. brumale*/*T. melanosporum* soils from burns in natural areas and contaminated *T. melanosporum* plantations. Samples coded with Bn are soil samples taken at points with production of *T. brumale* ascocarps in natural areas; samples coded with Mn are soil samples taken at points with production of *T. melanosporum* ascocarps in natural areas; samples coded with Bc are soil samples taken at points with production of *T. brumale* ascocarps in contaminated plantations; samples coded with Mc are soil samples taken at points with production of *T. melanosporum* ascocarps in contaminated plantations

propose calcareous amendments as a means of eradicating *T. brumale* in contaminated *T. melanosporum* orchards.

However, calcareous amendments could increase the production of *T. brumale* and *T. melanosporum* carpophores, thereby increasing the profitability of contaminated orchards. Ricard (2003) also suggests the use of calcareous amendments of fine limestone in truffle culture, although he recommends that they should be used with care and in moderation. New studies are required to look into the effects of fine limestone on the biology of *T. brumale* and *T. melanosporum* ectomycorrhizas.

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Calcareous amendments in truffle culture: A soil nutrition hypothesis

Luis G. García-Montero^{a,*}, Asunción Quintana^b, Inmaculada Valverde-Asenjo^c, Paloma Díaz^d

^a Department Forest Engineering, U.D. Operaciones Básicas, E.T.S.I. de Montes, Technical University of Madrid (UPM), Ciudad Universitaria s/n, 28040 Madrid, Spain

^b Agriculture, Research and Technology IKT S.A., Granja Modelo s/n, 01192 Arkaute-Alava, Spain

^c Department Soil Science, University Complutense of Madrid, Ciudad Universitaria s/n, E-28040 Madrid, Spain

^d Department Industrial Chemistry and Polymers, Technical University of Madrid (UPM), C/Ronda de Valencia 3, Madrid 28012, Spain

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ABSTRACT

Several statistical studies have indicated that a high concentration of active carbonate in the soil favours *Tuber melanosporum* fruit body production. The amount of active carbonate is significantly higher and the total carbonate is significantly lower inside than outside the *T. melanosporum* brûlés. These statistical patterns and other works show that *T. melanosporum* mycelia may acidify their immediate soil environment and solubilise carbonated fractions. Subsequently, the particular environmental conditions of brûlé soils may favour a secondary carbonate precipitation with a net increase in active carbonate which would in turn favour *T. melanosporum* mycelia. The greater brûlé size and fruiting are simultaneously both related to how well the fungus is growing, suggesting that the model which best explains the cause–effect of all these observations is a feedback process. Other statistical studies suggest that active carbonate favours increased fruiting of *T. melanosporum* as compared with *Tuber aestivum*, *Tuber mesentericum* and *Tuber rufum*, which has led to the use of calcareous amendments in truffle culture. We have studied the carbonated fractions of 46 soil samples from *T. melanosporum* plantations contaminated and uncontaminated with *Tuber brumale* (a serious problem in truffle culture), and it appears that the use of calcareous amendments does not serve to eradicate *T. brumale* from these plantations. However, we have observed the positive effect of calcareous amendments on the production of fruit bodies of both species. The similar response by *T. melanosporum* and *T. brumale* to soil active carbonate may be related to the close phylogenetic relationships between both species. Based on these findings, we propose a new soil nutrition hypothesis as a guide for research procedures in truffle soil ecology. Soils which are high in active carbonate lead to host plant chlorosis, which is a symptom of an underlying nutrient deficiency. This nutrient deficiency could lead to greater colonisation of *T. melanosporum* ectomycorrhizas and favour the growth of mycelia, fruit body production and brûlé development, which in turn encourages the formation of new amounts of active carbonate on the basis of the proposed feedback model.

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1. Introduction

Tuber melanosporum Vittad. (black truffle) produces its fruit bodies in points called “brûlés”, where it competes with other *Tuber* species which have a lower economic value. The term “brûlé” refers to *T. melanosporum*'s ability to create clearings in the vegetation. This vegetation-free area occurs because its mycelium and fruit bodies produce multiple substances that adversely affect young plants and seed germination, such as 2-methylpropanal, 2-methylbutanal, 2-methyl-1-butanol and others.

Callot (1999) and Ricard (2003) indicate that the biological and physical–chemical soil conditions relating to the brûlés need to be

further clarified. Moreover, most studies on *T. melanosporum* fail to supply statistical analysis on fruit body production and other quantitative data associated with truffle soil ecology.

In recent years we have conducted research into the statistical patterns of soil factors which influence the fruiting of *T. melanosporum* in natural areas. Our first results showed that the collective influence of soil features (i.e. granulometric texture, pH, and calcareous fractions) explains only 27% of the variance in *T. melanosporum* productivity. However, the active carbonate (calcium carbonate extractable with ammonium oxalate; smaller than 50 µm in size) has a significant positive effect on *T. melanosporum* development. Active carbonate has shown a positive correlation with *T. melanosporum* fruit body production and explains up to 43% of its variance (Table 1) (García-Montero et al., 2006, 2007a).

We have also compared the carbonated fractions inside natural brûlés of *T. melanosporum* with the values of soils outside the brûlés.

* Corresponding author. Tel.: +34 913365565; fax: +34 91336386.

E-mail address: luisgonzaga.garcia@upm.es (L.G. García-Montero).

Table 1Statistical patterns in soil carbonates and *Tuber melanosporum* ecology which support the proposal of new hypotheses on this topic.

References	Work aims	Results	Treatments ^a	N	Statistics	P
GM, 2006, 2007a	Soil factors which influence <i>T.m.</i> fruit body production	Collective influence explains 27% of production variance	(PC), SC	20	$R = 0.521$	0.018
		Active carbonate explains 43% of production variance	SC	20	$R = 0.655$	0.002
		Ca ²⁺ exchangeable explains 20% of production variance	SC	20	$R = 0.452$	0.046
		Active carbonate explains 42% of Ca ²⁺ variance	SC	20	$R = 0.645$	0.002
GM, 2007c	Relationship among soil factors and <i>T.m.</i> brùlé size	Active carbonate explains 51% of brùlé size variance	(SC), R	20	$R^2 = 0.506$	<0.001
		Brùlé size explains 38% of <i>T.m.</i> production variance	R	208	$R^2 = 0.383$	<0.001
GM, 2009	Soil carbonate abundance inside brùlés versus outside brùlés	Active carbonate inside brùlés is significantly higher	An	40	$F = 7.344$	0.010
		Total carbonate inside brùlés is significantly lower	An	40	$F = 5.987$	0.019
GM, 2007b	Relationship among soil factors, <i>T.m.</i> production, C.I. and other symbiont plants	<i>T.m.</i> production with C.I. is 73% lower than other plants	(Ac), An, Tp	40	$F = 5.850$	0.006
		Active carbonate is 92% lower in brùlés with C.I. plants	An, Tp	20	$F = 12.80$	<0.001
GM, 2008, 2009	Relationship among soil factors and <i>T.m.</i> , <i>T.a.</i> , <i>T.ms.</i> and <i>T.r.</i> interactions	Active carbonate is higher in <i>T.m.</i> brùlés than in <i>T.a.</i> , <i>T.ms.</i> and <i>T.r.</i> brùlés	(PC), An, Tp	24	$F = 16.29$	<0.001
VA, 2009	Relationship among soil factors, <i>T.m.</i> and <i>T.b.</i> in cultures and natural areas	Active carbonate is higher in <i>T.b.</i> brùlés than in <i>T.m.</i> , brùlés	(PC), An, (Mw)	77	$F = 13.02$	<0.001

GM = García-Montero et al.; VA = Valverde-Asenjo et al.; *T.m.* = *Tuber melanosporum*; *T.a.* = *Tuber aestivum*; *T.ms.* = *Tuber mesentericum*; *T.r.* = *Tuber rufum*; *T.b.* = *Tuber brumale*; C.I. = *Cistus laurifolius*.

^a PC = principal component analysis; SC = simple correlation; R = regression; Ac = Ancova; An = Anova; Tp = Tukey poshoc test; Mw = Mann–Whitney U test.

The results indicate that the mean concentration of active carbonate inside the brùlés is significantly higher (2.17 times greater on average) and the abundance of total carbonate is significantly lower than in soils outside the brùlés (0.64 times lower on average). From these patterns, and from the works of some authors, it can be deduced that *T. melanosporum* mycelia may acidify their immediate soil environment and solubilise different carbonated fractions inside the brùlés. Subsequently, the particular soil environmental conditions associated to the brùlé (i.e., disappearance of roots, decrease in soil pCO₂, soil microclimate change, etc.) may favour a secondary carbonate precipitation with a net increase in active carbonate content (Table 1) (García-Montero et al., 2009).

In this regard, active carbonate has shown a positive correlation with brùlé size, which explains up to 51% of its variance. This fact would counterbalance carbonate losses from leaching, thereby favouring *T. melanosporum* mycelia, suggesting a feedback process as the best model to account for the cause–effect of all these observations. The proposed feedback model could therefore explain the fact that *T. melanosporum* growth and fruit body production increase with the size of the brùlé, which accounts for up to 38% of the variance in its productivity, and simultaneously, that the size of the brùlé is related to how well the fungus is growing (Table 1) (García-Montero et al., 2007c).

Active carbonate content could also be a major factor in *T. melanosporum* fruiting versus other *Tuber* species. The abundance of active carbonate is significantly higher in soils that produce only *T. melanosporum* fruit bodies than in soils that produce *Tuber aestivum* Vittad. or *Tuber mesentericum* Vittad. in the same study area. The soils outside *T. melanosporum* brùlés frequently present *Tuber rufum* Pico ex Fries fruit body production. We have compared the carbonated fractions inside the *T. melanosporum* brùlés with the values of the soils outside brùlés with *T. rufum* production in natural areas, and have confirmed that the active carbonate in the inner soils is on average 2.35 times greater than in the soils outside brùlés with *T. rufum* production (García-Montero et al., 2008, 2009).

Other statistical analyses have shown the effect of soil active carbonate on the capacity of the rockrose *Cistus laurifolius* L. to produce *T. melanosporum* fruit bodies. The average production of *T. melanosporum* brùlés associated with *C. laurifolius* is 73% lower on average than the production of *T. melanosporum* brùlés associated with *Corylus avellana* L. or *Quercus ilex* L. in the same study area. *C. laurifolius* develops small brùlés, which has a significant effect on its fruit body production. The soil active carbonate of *C. laurifolius* brùlés is 92% lower on average than *C. avellana* and *Q. ilex* brùlés. The low amounts of active carbonate encourage growth of *C. laurifolius*, which requires decarbonated soils, but impair *T. melanosporum* production (Table 1) (García-Montero et al., 2007b).

Active carbonate constitutes an important reserve of exchangeable Ca²⁺, and the presence of both soil components is very important to *T. melanosporum*. We have shown that active carbonate has a positive correlation with exchangeable Ca²⁺ concentration and explains up to 42% of its variance (Table 1) (García-Montero et al., 2007a).

On this point, Ricard (2003) and Jaillard et al. (2007) suggest that the lack of studies on active carbonate in truffle culture is a significant omission. All the statistical patterns for active carbonate that we have described confirm that active carbonate may be a major factor in *T. melanosporum* soil ecology; it is therefore necessary to propose new hypotheses for an in-depth study of these statistical results, and to propose new experimental designs around these topics.

In connection with soil carbonate studies, Rioussset et al. (2001) indicate that calcareous amendments are being used in truffle culture to eradicate *Tuber brumale* Vittad. from *T. melanosporum* cultivations (a serious problem in French truffle culture). However, these calcareous amendments have not produced the expected effects, and there are no data available which quantify their effects on contaminated cultures. *T. brumale* has also begun to contaminate Spanish *T. melanosporum* cultures in the provinces of Alava and Teruel (Valverde-Asenjo et al., 2009).

With the aim of increasing current knowledge of the effect of calcareous amendment on *T. melanosporum* plantations, and to study further the cause–effect relationship between active carbonate and development of *T. melanosporum* and *T. brumale* over time, we have begun a succession of controlled calcareous amendments to eradicate *T. brumale* from *T. melanosporum* cultivations. We have also initiated a succession of soil studies in unamended plantations to compare the evolution of soil from the brûlés of both species. Based on the first results of this experimental design and on the studies of the statistical patterns of active carbonate mentioned above, we present a new soil nutrition hypothesis as a guide for research procedures in truffle soil ecology.

2. Materials and methods

In May 2007, in the province of Alava (northern/central Spain), we carried out two calcareous amendments of finely divided carbonate (2500 kg ha⁻¹) in two *T. melanosporum* cultures with comparable features contaminated by *T. brumale* (coded Alava-1 and Alava-2). We have been monitoring the fruit body production in both cultures since December 2007, and comparing the results with nearby control *T. melanosporum* cultures which were uncontaminated by *T. brumale* and had no calcareous amendments (Control-1 and Control-2, respectively). After the first year, we analysed a total of 32 soil samples from these four cultures in Alava: 10 samples from amended *T. brumale* brûlés, 10 from amended *T. melanosporum* brûlés, and 12 from Control-1 and Control-2 extracted from unamended *T. melanosporum* brûlés (Table 2).

We have consulted the data on average monthly precipitation and temperature provided by the National Spanish Meteorological Agency (AEMGS, 2008) for March to November 2007 in the study area in Alava.

The contaminated *T. melanosporum* plantation coded Alava-1 is 14 years old, and has 60% productive *T. melanosporum* brûlés and 20% *T. brumale* brûlés. There is a very similar plantation 1 km away, coded Control-1. The brûlés studied in both plantations produce more than 300 g year⁻¹ of fruit bodies of these species associated to *Q. ilex* L. subsp. *ballota* (Desf.) Samp. Both plantations are close to the village of Campezo (Alava). The natural vegetation consists of oak forests (*Spiraeo hispanicae–Querceto rotundifoliae sigmetum* geobotanical classification type).

The contaminated *T. melanosporum* plantation coded Alava-2 is 15 years old, and has 10% productive *T. melanosporum* brûlés and 50% *T. brumale* brûlés. There is a very similar plantation 2.1 km away, coded Control-2. The brûlés studied in both plantations produce more than 300 g year⁻¹ of fruit bodies of these species associated to *C. avellana* and *Q. ilex* subsp. *ballota*. Both plantations are close to the village of Iruña de Oca (Alava). The natural

vegetation consists of oak forests (*Spiraeo hispanicae–Querceto rotundifoliae sigmetum*).

In the province of Teruel (eastern/central Spain), we have initiated another soil study in plantations which have not received calcareous amendments, with the aim of analysing the evolution of the soils in brûlés of both *Tuber* species over time. We have selected two comparable and nearby *T. melanosporum* cultures, with and without *T. brumale* contamination (coded Teruel-3 and Control-3, respectively). After the first year, we analysed a total of 14 soil samples from these cultures located in Alava: 9 samples from unamended *T. brumale* brûlés, and 5 from unamended *T. melanosporum* brûlés (Table 2).

The contaminated plantation coded Teruel-3 is 15 years old, and has 50% productive *T. melanosporum* brûlés and 20% *T. brumale* brûlés. There is a very similar plantation 0.7 km away, coded Control-3. The brûlés studied in both plantations produce more than 400 g year⁻¹ of fruit bodies of these species associated to *Q. ilex* subsp. *ballota*. Both plantations are close to the village of Sarrión (Teruel). The natural vegetation consists of oak forests (*Junipero thuriferae–Querceto rotundifoliae sigmetum*).

The fruit bodies were obtained in the brûlés using trained dogs, and identified with a stereoscopic microscope (Leica WildMZ8) and a microscope (Leica LeitzDMRB), following the descriptions and indications proposed by Rioussset et al. (2001). The soils were sampled always using the same spade (20 cm blade) and a tape measure. These samples included a mixture of 1 kg of the soil surface horizons that have not been differentiated, as *T. melanosporum* brûlés contain a constant mixture of horizons due to the continuous digging that the harvesters and their dogs engage in to extract the fruit bodies. The following soil determinations were made in the 46 selected soil samples (Table 3): pH in water, total organic carbon (TOC) and % total CaCO₃ (equivalent calcium carbonate), following the methods of ISRIC (2002) and % active carbonate according to AFNOR (1982).

Statistical treatment was performed with the Statistica Program v. 6 (StatSoft, Inc., Tulsa, OK, 1999). Normality was checked using the Shapiro–Wilks and Kolmogorov–Smirnov tests, and homogeneity of variances was verified by the Levene test.

3. Results

Table 3 shows the physical–chemical properties of soil samples collected in the *T. melanosporum* and *T. brumale* brûlés corresponding to each of the 6 plantations (4 from Alava and 2 from Teruel). Many of the soil samples show a moderately basic pH and a significant percentage of total carbonate and active carbonate. Levels of TOC are moderate.

Table 4 shows the mean value of variables for soils with calcareous amendments versus nearby soils without calcareous

Table 2
Study area characteristics.

Province	Village	Culture code	<i>Tuber</i> species	Amended ^a	Soil samples	Symbiotic plant	p ^b	T ^c	Altitude ^d	Geographic coordinates
Alava	Campezo	Alava-1	<i>T.m.</i>	+	5	<i>Q.i.</i>	732	12	619	42° 41' 12.12" N/2° 21' 46.80" W
		Alava-1	<i>T.b.</i>	+	5	<i>Q.i.</i>				
Alava	Campezo	Control-1	<i>T.m.</i>	–	8	<i>Q.i.</i>	732	12	641	42° 41' 44.28" N/2° 21' 20.20" W
Alava	Iruña de Oca	Alava-2	<i>T.m.</i>	+	5	<i>C.a./Q.i.</i>	700	10	605	42° 49' 14.49" N/2° 51' 44.08" W
		Alava-2	<i>T.b.</i>	+	5	<i>C.a./Q.i.</i>				
Alava	Iruña de Oca	Control-2	<i>T.m.</i>	–	4	<i>C.a./Q.i.</i>	700	10	554	42° 49' 7.79" N/2° 53' 18.47" W
Teruel	Sarrión	Teruel-3	<i>T.b.</i>	–	9	<i>Q.i.</i>	500	11	1031	40° 8' 32.59" N/0° 50' 22.32" W
Teruel	Sarrión	Control-3	<i>T.m.</i>	–	5	<i>Q.i.</i>	500	11	1041	40° 8' 7.33" N/0° 50' 22.84" W

T.m. = *Tuber melanosporum*; *T.b.* = *Tuber brumale*; *Q.i.* = *Quercus ilex* subsp. *ballota*; *C.a.* = *Corylus avellana*.

^a + = amended; – = unamended.

^b P = average annual precipitation in mm.

^c T = average annual temperature in °C.

^d Altitude in m.

Table 3

Analytical results of 46 soil samples located inside brùlés with *Tuber melanosporum* or *Tuber brumale* fruit body production. Cultures Alava-1 and Alava-2 were contaminated by *T. brumale*, had calcareous amendments, and were compared with nearby cultures Control-1 and Control-2 which were uncontaminated and had no calcareous amendments. Culture Teruel-3 was contaminated by *T. brumale*, had no calcareous amendments, and was compared with nearby culture Control-3 which was uncontaminated and had no calcareous amendments.

Province	Culture code	Samples	<i>Tuber</i> species ^a	Amended ^b	Brùlé production (g year ⁻¹) ^c	pH _{H₂O}	% Total CaCO ₃	% Active CaCO ₃	% Active/tot. CaCO ₃	% TOC
Alava	Alava-1	5	<i>T.m.</i>	+	>300	8.25 ± 0.10	42.05 ± 3.77	11.21 ± 2.05	26.61 ± 3.74	1.07 ± 0.48
	Alava-1	5	<i>T.b.</i>	+	>300	8.11 ± 0.12	44.75 ± 8.60	12.80 ± 2.63	28.87 ± 5.16	1.58 ± 0.58
Alava	Control-1	8	<i>T.m.</i>	–	>300	8.45 ± 0.09	43.11 ± 6.41	10.15 ± 2.02	23.60 ± 3.68	0.75 ± 0.49
Alava	Alava-2	5	<i>T.m.</i>	+	>300	7.95 ± 0.04	39.30 ± 6.85	11.87 ± 1.39	30.56 ± 3.33	1.85 ± 0.40
	Alava-2	5	<i>T.b.</i>	+	>300	8.18 ± 0.02	49.90 ± 3.49	10.91 ± 0.59	21.93 ± 1.65	1.99 ± 0.17
Alava	Control-2	4	<i>T.m.</i>	–	>300	8.20 ± 0.10	32.60 ± 8.29	5.51 ± 2.25	18.37 ± 9.09	1.09 ± 0.26
Teruel	Teruel-3	9	<i>T.b.</i>	–	>400	8.30 ± 0.19	39.58 ± 8.79	14.59 ± 2.88	37.43 ± 4.90	2.03 ± 0.25
Teruel	Control-3	5	<i>T.m.</i>	–	>400	8.29 ± 0.10	30.05 ± 12.58	10.78 ± 3.27	37.13 ± 3.92	1.75 ± 0.37

Soil analytical results = mean ± SD.

^a See Table 2.

^b See Table 2.

^c Brùlé production = g of fruit bodies collected year⁻¹.

amendments. ANOVA-1 indicates that the mean value of pH, active carbonate and TOC differs significantly depending on the liming treatment of the brùlés. Moreover, total carbonate differs significantly in a marginal way.

Table 5 shows the mean value of soil variables for the *T. brumale* brùlés with calcareous amendments versus nearby *T. melanosporum* brùlés with calcareous amendments. ANOVA-2 indicates that the mean value of total carbonate differs significantly depending on the *Tuber* species. Therefore, *T. brumale* brùlé soils show that mean total carbonate is 16% higher than in nearby *T. melanosporum* brùlé soils.

Table 6 shows the mean value of soil variables for the *T. brumale* brùlés without calcareous amendments versus nearby *T. melanosporum* brùlés without calcareous amendments. ANOVA-3 indicates that the mean value of active carbonate differs significantly depending on the *Tuber* species. Moreover, the total carbonate differs significantly in a marginal way. Therefore, in the unamended plantations, *T. brumale* brùlé soils also show that mean total carbonate is 32% higher and mean active carbonate is 35% higher than in nearby *T. melanosporum* brùlé soils.

Regarding fruit body production in the Alava cultures with calcareous amendments (Alava-1 and Alava-2), the results obtained in the first year show that *T. melanosporum* production increased by 30–32%, and *T. brumale* production increased by 69–274%. In the same period, the results obtained in the control cultures show that *T. melanosporum* production decreased by 18–26% due certainly to climatic conditions (Control-1 and Control-2). With regards precipitation, June and September 2007 were “dry” months (20 mm and 17 mm average precipitation respectively; both were 60% less than expected), and July was “very dry” (9 mm average precipitation; this was 80% less than expected). With regards temperature, May 2007 was a “warm” month (19 °C average temperature; this was 50% higher than expected), and July was “very hot” (34 °C average temperature; this was 80% higher than

expected). This pattern of rain and temperature has a negative effect on *T. melanosporum* production according to Callot (1999) and Ricard (2003).

4. Discussion

The application of calcareous amendments in *T. melanosporum* plantations has significantly modified the carbonated fraction of the soil brùlés from the first year of application. The results show that in brùlés with calcareous amendments, the mean content of active carbonate is 36% higher and the mean total carbonate is 11% higher than in nearby soil brùlés without calcareous amendments (Table 4). These amendments will be continued during several years to evaluate the evolution of the carbonated fraction in the brùlé soils in the study.

Regarding the use of calcareous amendments to eradicate *T. brumale* from *T. melanosporum* plantations, the results show that *T. brumale* production has increased considerably in the amended plantations since the first year. Moreover, from the results it can be deduced that liming does not negatively affect the *T. brumale* ectomycorrhizas in contaminated *T. melanosporum* cultivations. Valverde-Asenjo et al. (2009) have confirmed this result using 29 of the studied soils samples, which were subsequently integrated in another work focussing on a territorial design. These authors proposed a multivariate statistical analysis applied on 77 soil samples, to compare *T. brumale* and *T. melanosporum* brùlés from plantations versus *T. brumale* and *T. melanosporum* brùlés from natural areas (Table 1).

The results of the present work and all cited observations indicate that active carbonate could play a significant role in boosting production of both truffle species, thereby increasing the profitability of contaminated cultures. We therefore propose the

Table 4

Soil variable means on brùlés with different liming treatment in 4 plantations in Alava corresponding to ANOVA-1 = 20 soil samples located in 2 contaminated plantations (from Alava-1 and Alava-2) with calcareous amendments (2500 kg ha⁻¹) versus 12 soil samples located in 2 control plantations (from Control-1 and Control-2) without calcareous amendments.

Anova-1 Factors	Amended	Unamended	<i>F</i> _{1, 30}	<i>P</i> value
pH _{H₂O}	8.12 ± 0.14	8.36 ± 0.15	21.17	<0.001*
% Total CaCO ₃	44.00 ± 6.86	39.61 ± 8.47	2.58	0.12
% Active CaCO ₃	11.70 ± 1.84	8.60 ± 3.03	13.05	0.001*
% TOC	1.62 ± 0.54	0.87 ± 0.45	16.92	<0.001*

Soil analytical results = mean ± SD.

*Significant (*P* < 0.05).

Table 5

Soil variable means on brùlés with calcareous amendments (2500 kg ha⁻¹) and different *Tuber* species in 2 plantations in Alava corresponding to ANOVA-2 = 10 soil samples located inside *T. brumale* brùlés (from Alava-1 and Alava-2) with calcareous amendments versus 10 soil samples located inside *T. melanosporum* brùlés (from Alava-1 and Alava 2) with calcareous amendments.

Anova-2 factors	<i>T.b.</i> brùlés ^a	<i>T.m.</i> brùlés ^a	<i>F</i> _{1, 18}	<i>P</i> value
pH _{H₂O}	8.15 ± 0.09	8.10 ± 0.18	0.51	0.48
% Total CaCO ₃	47.33 ± 6.76	40.68 ± 5.41	5.90	0.026*
% Active CaCO ₃	11.86 ± 2.05	11.54 ± 1.69	0.14	0.71
% TOC	1.79 ± 0.46	1.46 ± 0.58	1.89	0.19

Soil analytical results = mean ± SD.

*Significant (*P* < 0.05).

^a See Table 2.

Table 6

Soil variable means on brûlés without calcareous amendments and different *Tuber* species in 2 plantations in Teruel corresponding to ANOVA-3 = 9 soil samples located inside *T. brumale* brûlés without calcareous amendments (from Teruel-3) versus 5 soil samples located inside *T. melanosporum* brûlés without calcareous amendments (from Control-3).

Anova-3 factors	<i>T.b.</i> brûlés ^a	<i>T.m.</i> brûlés ^a	$F_{1,12}$	<i>P</i> value
pH _{H₂O}	8.30 ± 0.19	8.29 ± 0.10	0.03	0.87
% Total CaCO ₃	39.58 ± 8.79	30.05 ± 12.58	2.80	0.12
% Active CaCO ₃	14.59 ± 2.88	10.78 ± 3.27	5.14	0.043*
% TOC	2.03 ± 0.25	1.75 ± 0.37	3.02	0.11

Soil analytical results = mean ± SD.

*Significant ($P < 0.05$).

^a See Table 2.

following new hypothesis as a guide for research on the role of active carbonate and calcareous amendments in truffle soil ecology, namely that *T. melanosporum* and *T. brumale* may favour or benefit from soils which are rich in active carbonate, to promote their ectomycorrhization in plants with nutritional deficiencies by lime-induced chlorosis.

Little is known about the physiological relationship between *Tuber* species and their symbiont plants (Ricard, 2003). On this point, our hypothesis on lime-induced chlorosis and *Tuber* ectomycorrhization is related with the experiments of Dupré et al. (1982) on the ectomycorrhization of *T. melanosporum* in substrates watered with nutrient solutions, which showed that the lack of phosphorous favours ectomycorrhization of *Quercus pubescens* Willd; and with the experiments of Gelpe and Timbal (1986) on lime-induced chlorosis in *Quercus rubra* L. provoked by a high active carbonate content.

The abundance of active carbonate generates a basic pH and increases HCO₃⁻ and exchangeable Ca²⁺ in the soil, which insolubilises the P, B, Fe and Mn (Gaucher, 1971; Follet et al., 1981; Loué, 1986; Douchafor and Souchier, 1979; Wild, 1992; Callot, 1999). Reactions between Ca²⁺ and P can occur in the soil solution, soil colloids or precipitated calcium carbonate. The degree of adsorption of P on the precipitated calcium carbonate is inversely related to particle size (Follet et al., 1981). An overabundance of Ca²⁺ and HCO₃⁻ leads to a deficiency in Mg²⁺ for the plants (Follet et al., 1981) and generates secondary calcites which immobilise the Mg²⁺ (magnesites) (Douchafor and Souchier, 1979).

pH significantly affects the absorption of microelements by plants. An increase in pH reduces the assimilability of Al, Co, Cu, Fe, Zn and Mn (Loué, 1986). A pH of 7.9–8.4, typical of *T. melanosporum* soils, leads to increasing deficiencies of Co, Cu, Mn and Zn in plants (Gaucher, 1971; Follet et al., 1981; Wild, 1992). When the pH is raised, the Mg²⁺ may be transformed into a non-exchangeable cation with no satisfactory explanation (Wild, 1992), leading to the formation of insoluble calcium borates (Follet et al., 1981). Numerous researchers have studied these effects of pH and lime-induced chlorosis on the growth of pines, firs, apples, grapevines, peanuts, groundnuts, pear rootstocks, chickpeas, bush beans, runner beans, soybeans, oilseed rapes, lupines and other plants.

Moreover, several studies on liming and tree ectomycorrhizas (ECM) reinforce our hypothesis. Püttsepp et al. (2004) indicated that local soil nutrient status influences ECM colonisation, and low P and K availability favours ECM colonisation. Liming stimulates fine root development (Schneider and Zech, 1990; Huettl and Zoetl, 1993; Kreutzer, 1995; Bakker et al., 2000), modifies the ectomycorrhizal community structure (Jonsson et al., 1999), increases the relative frequency of certain ECM morphotypes (Erland and Soderstrom, 1991; Antibus and Linkins, 1992), decreases the relative proportion of smooth in favour of hairy type ECM and significantly increases the total number of ECM tips (Bakker et al., 2000).

The lime-induced chlorosis and *Tuber* ectomycorrhization hypothesis represents an interesting approach for analysing the statistical patterns observed between *T. melanosporum* and soil active carbonate. The results introduce another novelty, namely that active carbonate could also play a significant role in *T. brumale* soil ecology. A similar response by both species to soil active carbonate may be related with the close phylogenetic relationships between *T. melanosporum* and *T. brumale* (Roux et al., 1999; Wang et al., 2006). However, *T. brumale* does not show the same capacity to form patent brûlés as *T. melanosporum* (Callot, 1999; Ricard, 2003). Therefore, *T. brumale* could benefit from the high amount of active carbonate which originates inside *T. melanosporum* brûlés (García-Montero et al., 2009). It is necessary to verify whether *T. brumale* brûlés also favour the formation of active carbonate.

In summary, active carbonate could boost *T. melanosporum* and *T. brumale* production, thereby increasing the profitability of contaminated cultures. We have proposed as a hypothesis that soils which are high in active carbonate lead to host plant chlorosis, which is a symptom of an underlying nutrient deficiency. This nutrient deficiency could lead to greater colonisation of *T. melanosporum* and *T. brumale* ectomycorrhizas and favours the growth of mycelia and fruit body production. The development of *T. melanosporum* brûlés could simultaneously encourage the formation of new amounts of active carbonate based on a feedback model proposed as the best way of explaining the cause–effect of all these observations. The statistical patterns of active carbonate and our proposed soil nutrition hypothesis constitute useful tools for gaining further knowledge of *Tuber* soil ecology, and for designing new experiments into calcareous amendments in truffle culture. In this line of research, the modern techniques of soil DNA extraction and monitoring of *Tuber* populations by using real time PCR could add significant information on these topics.

Acknowledgments

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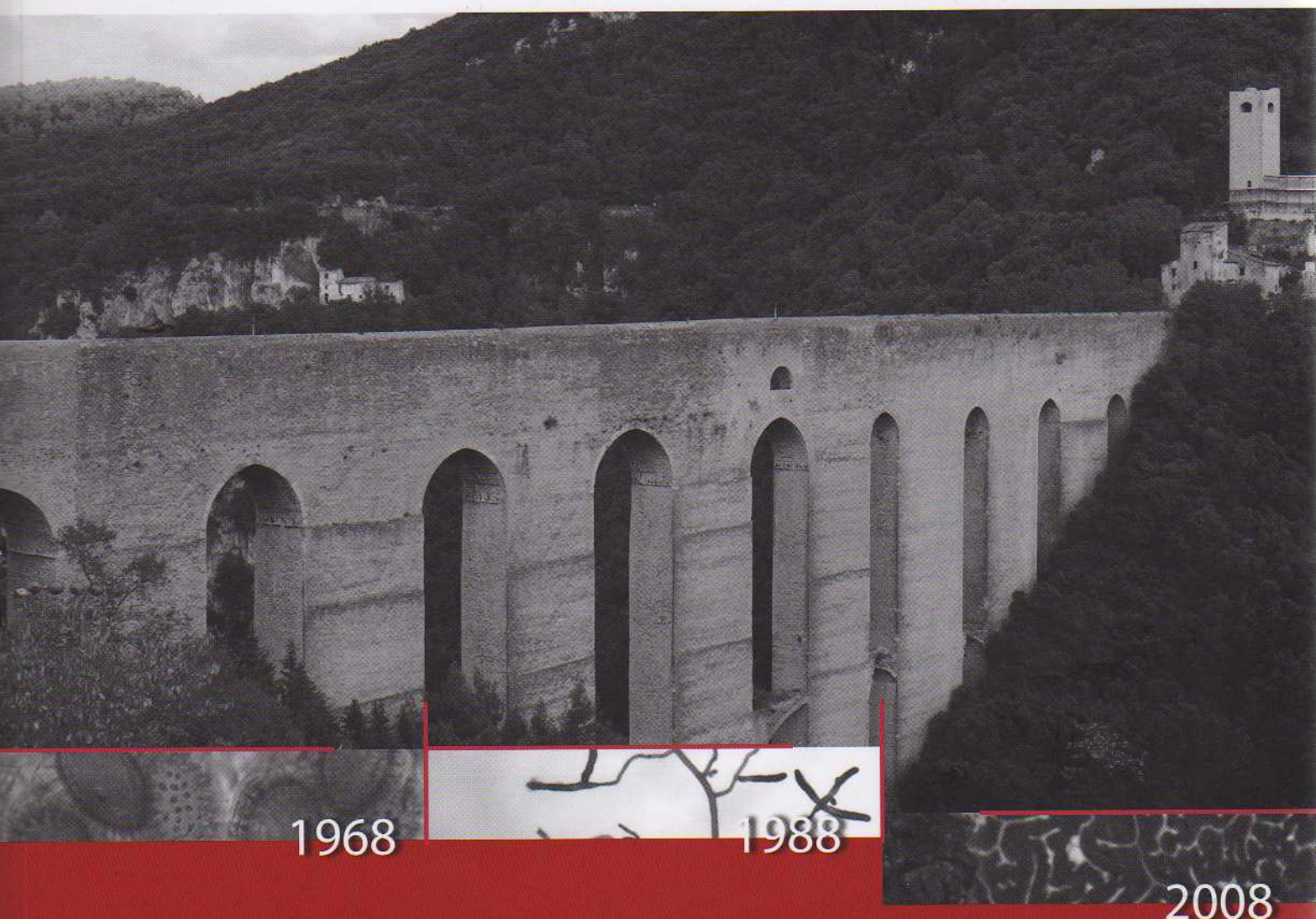
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NEW DATA ON IMPACT OF EARTHWORMS ACTIVITY ON BLACK TRUFFLE SOILS

García-Montero Luis Gonzaga¹, Valverde-Asenjo I.², Díaz P.³, Pascual C.¹, Menta C.⁴

¹Department of Forest Engineering, Technical University of Madrid (UPM)

e-mail: luisgonzaga.garcia@upm.es;

²Department of Soil Science, University Complutense of Madrid;

³Department of Industrial Chemistry and Polymers, Technical University of Madrid (UPM);

⁴Department of Evolutionary and Functional Biology. Natural History Museum of Parma

Abstract

Among soil invertebrates, the earthworms (Anellida, Oligochaeta) are unique in their ability to integrate physical, chemical and biological processes in the soil ecosystem (Edwards and Bohlen, 1996; Shuster *et al.*, 2002). Several authors have outstanding the research on earthworm feeding ecology and its implications for nutrient cycling and soil fertility. However Curry and Schmitd (2007) remarked that many aspects of the interactions between earthworms and soil mineral constituents are still poorly understood.

The amount of carbonates in soils depends on soil forming factors such as relief, biological factors, human and natural disturbances, time and climate (Jenny, 1941; Breemen & Buurman, 1998). Chan (2003) explained that soil carbonate mobility is related with the direct action of earthworms and other soil fauna, and demonstrated the earthworm ability to incorporate lime into subsoil in the calcareous amendments applying to ameliorated acid soils where the incorporation by tillage is not feasible.

On truffle research, many authors pointed that black truffle (*Tuber melanosporum* Vittad.) production is closely linked to calcium carbonate soil availability and mobility on overall soil profile. Callot (1999) empathises the currently study on the relationship of cause and effect among earthworms activity, pH of the soil and development of black truffle. Lulli *et al.*, (1999) and Castrignanò *et al.*, (1999) also point out that black truffle require considerable soil porosity originated by biological activity, above all, by earthworms and ants.

The objective of the present study is to analyse the pH, carbonate fractions and abundance of total organic carbon (TOC) in a representative number of soil samples from *Tuber melanosporum* burns and earthworm cast samples, to establish statistical patterns on these soils parameters and to analyse the impact of earthworms activity on black truffle soils.

Key words: Truffle ecology, lime movement, earthworm casts, truffle culture.



Department of Crop and Soil Science
Soils Division
Oregon State University, 3017 Agriculture and Life Sciences Building
Corvallis, OR 97331-7306
Office: (541) 737-2441; Fax: (541) 737-1589

December 12, 2012

David Myrold, Professor
Faculty of Soil Science
Agriculture and Life Sciences Building 4017
Oregon State University, Corvallis, OR 97331

CERTIFICATE

I am pleased to certificate that Luis G. Garcia-Montero as a courtesy faculty member in the Department of Forest Ecosystems and Society (College of Forestry), has developed his sabbatical and collaborative activities staying and researching in the Soil Biology and Microbiology Lab and the Greenhouses of the Soil Division of Crop and Soil Science Department (College of Agricultural Sciences) since June 1, 2012 to December 14, 2012, working with Dr. David Myrold and Dr. Daniel Luoma (Dpt. Forest Ecosystems and Society) on forest ectomycorrhizas and forest soil ecology.

During this time period, Luis G. Garcia-Montero has attended 22 seminars and a course (as listener) on Soil Biology. In addition, this professor has imparted two seminars, in our department, entitled:

"Ecology of truffles in Central Spain. A possible explanation for increased accumulation of soil carbonates associated to ectomycorrhizas" (October 16, 2012)"

"Could ectomycorrhizae be "liming" host plant roots? A possible explanation for increased accumulation of carbonates and oxalates around trees" (October 23, 2012)"

Sincerely,

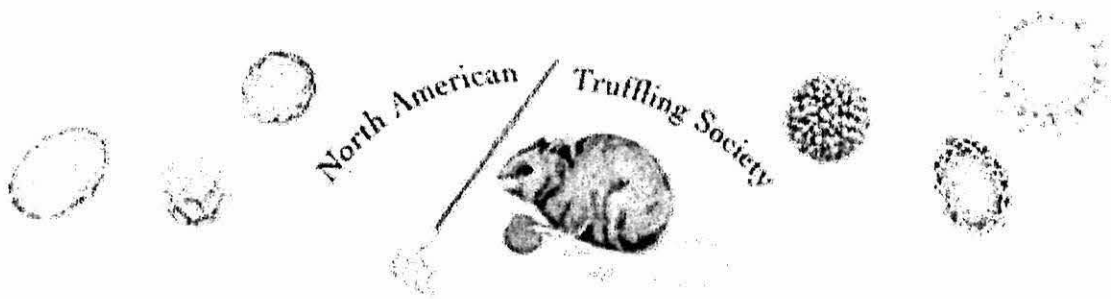
A handwritten signature in black ink, appearing to read "David Myrold".

David Myrold
Professor of Soil Biology, Oregon State University

A handwritten signature in black ink, appearing to read "Jay Noller".

Jay Noller, Associate Professor
Head of Soil Division of Crop and Soil Science Department
Oregon State University

1. A-6-19



December 2012

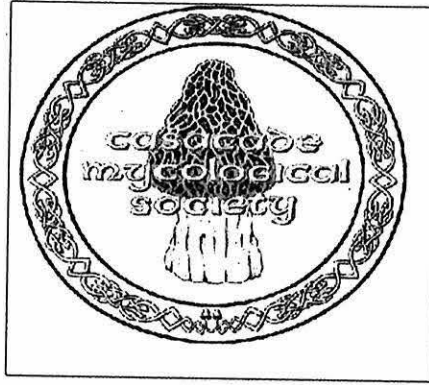
Dear Dr. Luis Garcia-Montero,

Thank you so much for presenting your talk "Research on the ecology of black truffles in central Spain" to our November NATS meeting. Our members were very interested in your research, and your presentation was just right for the audience. I hope that you will return to Corvallis in the future to share the results of the research you have done here.

With appreciation,

Joyce Eberhart
Vice President
The North American Truffling Society

1.A.6-23



Cascade Mycological Society

Eugene, Oregon

www.cascademyco.org

Luis Gonzaga
Visiting Professor, Oregon State University
University of Spain, Barcelona, Spain

Dear Dr. Luis Gonzaga,

Cascade Mycological Society was honored to have you visit Eugene, Oregon, on December 13, 2012, and speak to our membership and members of the public. Your presentation, **STUDIES ON BLACK TRUFFLE ECOLOGY WHICH ALLOWED FOR A NEW HYPOTHESIS: ECTOMYCORRHIZAE COULD BE "LIMING" HOST PLANT ROOTS**, was appreciated by all in attendance.

Your visit has increased the interest into the scientific research of culinary truffle cultivation.

Thank you.

Sincerely,

Chris Melotti.
Invited Speaker Series Chairperson 2012-2013
Cascade Mycological Society
P.O. Box 110
Eugene, OR 97440

1.A.6-21



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FIRMA Luis G. García Montero.....

1A6-25
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CERTIFICATE

I, Didier PILLOT, coordinator of the Agris mundus master of science, hereby certify that Mr Luis Gonzaga, from Universidad Politecnica de Madrid, has taught class (Impact of Mycorrhizal Management on Soil Fertility and Mushroom Production in Smallholder Systems) for an hour during the orientation week 2013.

Done at Montpellier, on December 4, 2014

A handwritten signature in blue ink, appearing to read 'D. Pillot', is written over a circular blue stamp.A circular blue stamp with the text 'MINISTÈRE CHARGÉ DE L'AGRICULTURE' around the top edge and 'Montpellier SupAgro' around the bottom edge. In the center, it reads 'Centre international d'études supérieures en sciences agronomiques'.

Didier PILLOT
Coordinator Agris mundus,
Master of Sciences on sustainable development in agriculture

TU Berlin | Institut für Ökologie | Bodenkunde
Sekretariat BH 10-1 | Ernst-Reuter-Platz 1. | 10587 Berlin

Fakultät VI | Bauen Planen Umwelt
Institut für Ökologie

Bodenkunde

Fachgebietsleiter
Prof. Dr. rer. nat.
Martin Kaupenjohann

Sekretariat 10-1 Raum BH-N 1009
Ernst-Reuter-Platz 1
10587 Berlin

Telefon +49 (0)30 314-73530
Telefax +49 (0)30 314-73548
martin.kaupenjohann@tu-berlin.de

Berlin, den 04.11.2015

To whom it my concern

On November 4th, Luis G. García Montero (Passport AAF0384871ESP6506135M2202130A0260780200), Associate Professor at the Department of Forest Engineering, Technical University of Madrid, gave a two-hour lecture for my students in the class of Advanced Soil Ecology (Soil Ecology II). The author presented his unique model on soil-nutrient management through fungus-plant interactions, which was intensively discussed by the students. A appreciate this valuable contribution to my teaching and I am very grateful to my colleague Luis Garcia Montero.

Technische Universität Berlin
Fakultät VI - Institut für Ökologie
Prof. Dr. Martin Kaupenjohann
FG Bodenkunde - BH 10-1
Ernst-Reuter-Platz 1, 10587 Berlin
Tel. 314 73 530

Berlin, November 4th 2015

(Prof. Dr. Martin Kaupenjohann)

> Seite 1/1 | Thema des Schriftstücks Blindtext



To whom it may concern

I am pleased and grateful to certify that Luis G. García Montero (Passport AAF0384871ESP6506135M2202130A0260780200), Associate Professor at the Department of Forest Engineering, Technical University of Madrid, gave an invited oral presentation in the frame of the institutional Geo-Ecological colloquium at the Technische Universität Berlin, Institute of Ecology, Chairs of Soil Science and Soil Protection. His 45 minutes talk on November 3rd 2015 was entitled *Seeking new models of agroforestry in Europe focusing on "Greening"*. The following in-depth discussion with researchers and students took another 45 minutes.

Berlin, November 4th 2015

Technische Universität Berlin
Fakultät 6 - Institut für Ökologie
Prof. Dr. Martin Kaupenjohann
FG Bodenkunde - BH 10-1
Ernst-Reuter-Platz 1, 10587 Berlin
Tel. 314 73 530



(Prof. Dr. Martin Kaupenjohann)

Soil Biology 34

Alessandra Zambonelli · Gregory M Bonito *Editors*

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Edible Ectomycorrhizal Mushrooms

Soil Biology

Alessandra Zambonelli
Gregory M Bonito *Editors*

Edible Ectomycorrhizal Mushrooms

Current Knowledge and Future
Prospects

 Springer

Chapter 6

Influence of Edaphic Factors on Edible Ectomycorrhizal Mushrooms: New Hypotheses on Soil Nutrition and C Sinks Associated to Ectomycorrhizae and Soil Fauna Using the *Tuber Brûlé* Model

Luis G. García-Montero, Inmaculada Valverde-Asenjo, Domingo Moreno, Paloma Díaz, Isabel Hernando, Cristina Menta, and Katia Tarasconi

6.1 Introduction

6.1.1 *A Global Perspective on Soil Organisms and Inorganic C Sinks*

Soil is a complex system where basic processes for terrestrial communities take place (Ruf et al. 2003). Soil represents an important substrate for a large part of the earth's biodiversity. Soil properties determine ecosystem function and vegetation structure, and they serve as a medium for root development and provide moisture and nutrients for plant growth (Minnesota Forest Resources Council 1999). Food chain interactions among the soil biota (including plant roots) have major effects on the quality of crops (affecting human and animal nutrition and other aspects of life on earth), the incidence of soil-borne plant and animal pests and diseases (affecting production

L.G. García-Montero (✉)

Department of Forest Engineering, E.T.S. Ingenieros de Montes, Technical University of Madrid (UPM), Ciudad Universitaria s/n, Madrid 28040, Spain
e-mail: luisgonzaga.garcia@upm.es

I. Valverde-Asenjo • I. Hernando

Department of Soil Science, University Complutense of Madrid, Madrid, Spain

D. Moreno

Association of Truffle Collectors, Peralejos de las Truchas, Spain

P. Díaz

Department of Industrial Chemistry and Polymers, Technical University of Madrid (UPM), Madrid, Spain

C. Menta • K. Tarasconi

Department of Evolutionary and Functional Biology, Section Natural History Museum, University of Parma, Parma, Italy

Impact of earthworm casts on soil pH and calcium carbonate in black truffle burns

Luis G. García-Montero · Inmaculada Valverde-Asenjo ·
María A. Grande-Ortíz · Cristina Menta ·
Isabel Hernando

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Abstract Granules of calcium carbonate are known to be produced by the calciferous glands of earthworms, and may have a measurable impact on the soil; however, little is known of their dynamics. Earthworms are often found in *Tuber melanosporum* burns, and this truffle is closely linked to calcium carbonate and soil pH. The present work shows that in weakly calcareous soil burns with high earthworm (*Proselodrilus* sp.) activity, earthworm casts showed a significant increase of 0.2 units of pH and 66 % more total calcium carbonate than soils in the burns, although the casts were produced from these same soils. Analysis of

the soil carbonate fractions (active carbonate versus calcium carbonate greater than 50 μm in size) showed that the origin of the increase in the total calcium carbonate content of the *Proselodrilus* sp. casts cannot be explained by the levels of original carbonate existing in the soils in the study, although it can be explained by the synthesis of calcite granules by earthworms. These results obtained in the field with *Proselodrilus* sp. confirm Canti's (2009) experiments in the laboratory using $\delta^{13}\text{C}$ concerning the synthesis of calcite granules by *Lumbricus terrestris*. Both these results and other works indicate that production of calcite granules by *Proselodrilus* sp., *L. terrestris*, *Aporrectodea longa*, *A. trapezoides* and *Anisochaetae* sp. has the ability to increase soil pH and calcium carbonate; additionally Lambkin et al. (2011) have found that higher soil pH and carbonates can cause an increase in the production of calcite granules by *L. terrestris*. All these results, therefore, point to a feedback process, scope of which is a function of the different soil types and earthworm species. In summary, different earthworm species may have a significant and positive impact on soil pH and net recarbonation of *T. melanosporum* burns, and the importance of both soil properties justifies the development of further experiments aimed at incorporating vermiculture into the truffle cultivations.

L. G. García-Montero (✉)
Department of Forest Engineering, Operaciones Básicas,
E.T.S.I. Montes, Technical University of Madrid (UPM),
Ciudad Universitaria s/n, 28040 Madrid, Spain
e-mail: luisgonzaga.garcia@upm.es

I. Valverde-Asenjo · I. Hernando
Department of Soil Science, Facultad de Farmacia,
Universidad Complutense de Madrid, Ciudad
Universitaria s/n, 28040 Madrid, Spain

M. A. Grande-Ortíz
Department of Applied Physics and Mechanics, E.T.S.I.
Montes, Technical University of Madrid (UPM),
Ciudad Universitaria s/n, 28040 Madrid, Spain

C. Menta
Department Evolutionary and Functional Biology,
University of Parma, Via Farini 90, 43100 Parma, Italy

Keywords Earthworm casts · Soil calcium carbonate · Soil pH · Truffle culture · *Tuber melanosporum* · Vermiculture

Introduction

The production of mycorrhizal fungi provides higher economic returns than any other forest product in many Mediterranean woods. In these areas, *T. melanosporum* (black truffle) has greater culinary and economic value than other edible fungi species, such as mushrooms of *Boletus* spp. and *Lactarius* spp.

Tuber melanosporum grows in the calcareous and well-drained soils of Mediterranean forests, its metabolism has an enormous capacity for transforming the environment, and its phytotoxic activity provokes clearings in the vegetation, called burns or brûlés, where the mycelia bear fruit. Callot (1999), Ricard (2003), Jaillard et al. (2007), García-Montero et al. (2009a) explain that *T. melanosporum* is closely linked to the soil reaction (as measured by pH) and to the availability of calcium carbonate in the soil, and highlight the interesting decarbonation-recarbonation processes observed in the soils of *T. melanosporum* burns. These authors have underlined the importance of an overall study of the soil profile in *T. melanosporum* brûlés to explain hydric behaviour, soil pH dynamics and the dissolution and precipitation processes of carbonates.

Elsewhere, Callot (1999), Ricard (2003) and Pargney et al. (2008) indicate that earthworms are often found around the truffle carpophores in the soil burns. These soils usually have low levels of organic matter, and earthworms therefore search near the carpophores for organic matter contained in the bacterial populations and droppings of microarthropods and macrofauna. These authors point out that earthworms benefit *T. melanosporum* development, and Lulli et al. (1999) and Castrignano et al. (2000) indicate that this truffle requires considerable soil porosity originated above all by earthworms and ants. Moreover, García-Montero et al. (2008) indicate that earthworm casts could have a great impact on the soil in the *T. melanosporum* burns. Callot (1999) stresses that cultural practices could reduce the activity of earthworms in the truffières (truffle producing areas). However, there are no quantitative data on the relationship between earthworm activity and cast production with soil pH dynamics and decarbonation-recarbonation processes in the soils of *T. melanosporum* burns.

Fraser et al. (2011) emphasize the role of earthworms to explain the dynamics of soil profiles and

describe earthworms as ‘ecosystem engineers’ due to their important contribution to soil porosity, breakdown of organic matter, and incorporation of organic matter into the soil. However, they point out that it is less well known that many species of earthworms secrete millimetric granules of calcium carbonate.

Spherical soil concretions composed of calcite crystals have been found in earthworm casts and soils containing earthworms. These calcite granules range from single calcite crystals to aggregations up to 2.5 mm in diameter and are produced by the calciferous glands of all species of earthworms of the *Lumbricidae* family (Wiecek and Messenger 1972; Canti and Pearce 2003; Briones et al. 2008a; Gago-Duport et al. 2008; Lee et al. 2008). Coleman et al. (2004), Briones et al. (2008b) indicate that the calciferous glands of earthworms could provide a mechanism for regulating CO₂ in their blood and tissues.

Jongmans et al. (2003) found earthworm calcite granules up to a soil depth of 50 cm, and Canti and Pearce (2003) indicate that these granules are regularly found in soil profiles, although they confirm that little is known of their origins and dynamics. Lee et al. (2008) report that earthworms produce calcite granules in sufficient volumes to have a measurable impact on soil C cycling, although they indicate that the volume and role of this earthworm biomineralisation process are little known.

In laboratory studies, Lambkin et al. (2011) estimated an average rate of granule production of $8 \times 10^{-3} \text{ mmol}_{\text{CaCO}_3} \text{ day}^{-1}$ per *Lumbricus terrestris* in a range of soils. Briones et al. (2008b) suggest that 10–20 mature *L. terrestris* per m² is a realistic value for the density of this species in neutral and high pH soils. In the case of this species and for these earthworm densities, the minimum and maximum granule production rates posited by Lambkin et al. (2011) suggest that annual granule production lies in the range of 18–3,139 $\text{mol}_{\text{CaCO}_3} \text{ ha}^{-1} \text{ year}^{-1}$, with an average value of 438 $\text{mol}_{\text{CaCO}_3} \text{ ha}^{-1} \text{ year}^{-1}$. These authors point out that their results are higher than the findings of Wiecek and Messenger (1972), who calculated that excreted CaCO₃ could contribute up to 11 $\text{mol}_{\text{calcite}} \text{ ha}^{-1} \text{ year}^{-1}$ in forests with acidic soils (on the basis of field measurements); also higher than Canti’s (2007) results, who estimated production rates of more than 0.02 $\text{mol}_{\text{calcite}} \text{ day}^{-1}$ per *L. terrestris*.

However, Lambkin et al. (2011) indicated that there is significant variation in the mass of granules produced by different earthworms exposed to the same soil, and explain that a large proportion of this variation must reflect biological variations between individuals. Furthermore, Canti and Pearce (2003) found different production rates when comparing the granules of eight species of earthworms.

Elsewhere, in laboratory studies, Canti (2009) analyses whether the C of the calcite granule is extracted from the earthworm's dietary intake (litter), atmospheric CO₂, soil organic matter (SOM) or calcium carbonate in the soil, using $\delta^{13}\text{C}$ levels traced from the substrate through to the granules produced from it by *L. terrestris*. The substrates for the worms were made up of 87.5 % mineral material, 10 % soil organic matter and 2.5 % calcium carbonate to correspond approximately with weakly calcareous organic-rich topsoil. His results show that the C in the calcite granule comes from dietary intake (litter), but may also be coming partially from atmospheric CO₂, and partially from old soil C SOM; however, very little appears to come from the calcium carbonate in the soil. Therefore, Canti (2009) shows that earthworm casts produce a net increase in soil total carbonate, and Fraser et al. (2011) confirm the finding that the earthworms are truly synthesizing calcium carbonate and not merely recycling ingested material.

In summary, earthworm activity and production of casts and calcite granules could have an impact on the soil properties of *T. melanosporum* burns and favour recarbonation of soils in truffières. However, the production of calcite granules by earthworms is a complex process that depends on inter- and intraspecific variability and soil typology, among other factors. It is therefore necessary to conduct further quantitative studies to reveal the interactions between these variables and their possible impact on the net recarbonation of the soils in *T. melanosporum* burns.

The objective of the present work is to carry out a statistical study in truffière soils to determine the carbonate, pH and organic carbon imbalances in earthworm casts compared to the soils from which the casts were produced. This statistical study aims to confirm—in the field and with other species of earthworms—Canti's (2009) proposals regarding sources of carbon in the synthesis of calcite granules by *L. terrestris*, which he established in a laboratory experiment. Based on Canti's (2009) experimental

design, we compare differences in pH, carbonate fractions and total organic carbon (TOC) contents between earthworm casts and the soils from which the casts were produced. This work was conducted in moderately basic and weakly calcareous topsoils of *T. melanosporum* burns (with high truffle production), similar to Canti's (2009) substrates. Our hypothesis is that if the mean carbon sources in calcite granule synthesis derive from the soil TOC and atmospheric CO₂ (instead of from the soil carbonates) associated to the earthworm inputs, the earthworm casts would be significantly more basic and carbonated than the weakly calcareous soils due to the granules produced from them; therefore, the earthworm granules would provoke a net recarbonation of these soils.

Materials and methods

Case study area

The truffières in the study are located near the village of Sarrión in the province of Teruel (eastern/central Spain). This area is located in supra-Mediterranean bio-climatic belts, with predominantly Jurassic and Cretaceous limestones and dolomites. The natural vegetation consists of open oak forests (*Junipero thuriferae-Querceto rotundifoliae* sigmetum) alternating with dryland mosaic farming. Table 1 summarises the environmental characteristics of the study area based on Rivas-Martínez (1987), Valverde-Aseñjo et al. (2009). More detailed characteristics of the truffières (irrigation, detailed production, tree density and others) are not given to maintain the confidentiality of the management techniques applied by their owners.

In March 2008, 30 soil samples and 42 earthworm cast samples were collected from *T. melanosporum* burns from one planted truffière (Truffière-1, Table 1, Fig. 1) showing high earthworm activity. We selected this truffière because all the burns showed a high abundance of earthworm casts (>7 casts little piles per burn, Fig. 2). In our study, 2–3 samples of earthworm casts were collected from each burn. We mixed several neighbouring casts located on the topsoils of the same burn to form each casts little piles sample (>100 g). The *T. melanosporum* truffière is 16 years old, and has 60 % productive *T. melanosporum* burns

Table 1 Study area characteristics

Province	Village	Truffière code	Tuber species	Cast abundance	Soil samples	Symbiotic plant	P	T	Altitude (m)	Geographic coordinates
Teruel	Sarrión	Truffière-1 with worms	<i>T.m.</i>	+	30	<i>Q.i.</i>	500	11	883	40°10'33.17"N/0°51'6.51"W
Teruel	Sarrión	Control truffière	<i>T.m.</i>	–	15	<i>Q.i.</i>	500	11	991	40°8'32.59"N/0°50'22.32"W
Teruel	Sarrión	Control truffière	<i>T.m.</i>	–	5	<i>Q.i.</i>	500	11	1,041	40°8'7.33"N/0°50'22.84"W

T.m., *Tuber melanosporum*; *Q.i.*, *Quercus ilex* subsp. *ballota*; +, high abundance; –, low abundance; P, average annual precipitation (mm) in municipal district; T, average annual temperature in (°C) in municipal district



Fig. 1 Harvester and their dog digging to extract *Tuber melanosporum* fruit bodies in the studied truffière with high earthworm cast abundance (M. Doñate, Sarrión, Teruel)



Fig. 2 Casts little pile in the studied truffière with high earthworm cast abundance (Sarrión, Teruel)

(>500 g/year) associated with *Quercus ilex* L. subsp. *ballota* (Desf.) Samp. and *Q. coccifera* L.

A sample of ten earthworms was collected and sent for classification to the University of Parma and University of Siena. The earthworms belong to the Franco-Iberian genus *Proselodrilus* (Oligochaeta: Lumbricidae), for which numerous species have been described, all very similar to each other (Prof. E. Rota, personal communication). In the study area, we and the truffle harvesters observed a high level of activity of these earthworm populations in the upper portions of the soil.

We also used some additional data from 20 soil samples as a control of the soil characteristics in two truffières in the same study area. In both control truffières, we and the truffle harvesters observed low

earthworm activity and a low abundance of casts (≤ 1 casts little pile per burn). The control soil samples (coded Control-2, Table 1) were collected from two comparable and nearby (with a 4 km radius) planted truffières (Table 1) that were analysed in two previous works (García-Montero et al. 2009a; Valverde-Asenjo et al. 2009). Both control truffières are 15 years old, and have 50 % productive *T. melanosporum* burns (>400 g/year) associated with *Quercus ilex* subsp. *ballota*.

Soil analysis

Soil samples were taken according to FAO (1990) recommendations from inside the holes made by the collectors to extract the carpophores and near the earthworm casts, always using the same spade (20 cm blade) and a tape measure. Only the first 30 cm of each soil profile was studied because *T. melanosporum* usually bears fruit in this range (Verlhac et al. 1990), and the earthworm casts were observed in the upper soil horizons; moreover, it was verified that there are no strongly calcareous substrates at depths of several metres in the soils studied. The samples included a mixture of 1 kg of undifferentiated soil surface from different depths, as truffières contain a constant mixture of horizons due to the continuous digging by the harvesters and their dogs to extract the fruit bodies (Fig. 1).

The following soil determinations were made: pH in water, total carbonate (equivalent calcium carbonate) and TOC following the ISRIC (2002) methods; active carbonate (calcium carbonate extractable with ammonium oxalate; smaller than 50 μm in size) was determined according to AFNOR (1982).

Statistical analysis

Statistical analysis of the data was carried out using the Statistica Release seven Program (StatSoft, Inc. 1984–2006). All variables were examined and, when necessary, log-transformed to conform to the assumptions of parametric statistics. Normality was checked using the Shapiro-Wilks and Kolmogorov–Smirnov tests, and homogeneity of variances was verified by the Levene test.

We used analysis of variance (ANOVA) to assess differences in quantitative variables between earthworm casts versus soil samples (the soils from which the

casts were produced), and analysis of co-variance (ANCOVA) to test for differences in total carbonate contents between cast and soil samples while controlling the effects of active carbonate contents. Before conducting the ANOVAs, we performed a correlation matrix and MANOVA to test for overall differences between cast and soil samples while taking into account the correlations between the four soil variables.

Levene's test showed that the percentage of total carbonate and the variable - [% total carbonate – % active carbonate] data does not maintain the assumption of homogeneity of variances (data transformations were not able to correct the heterogeneity of variance of these variables). Moreover, in the soil data obtained from burns from different truffières (with a high and low abundance of casts), only the pH supports the assumption of homogeneity of variances. Hsu (1938), Box (1954a, b) and Lindman (1974) indicate that in the ANOVA/MANOVA assumptions the F statistic is quite robust against violations of the assumption of homogeneity of variances. However, we have assessed the differences in total carbonate between cast samples and soils using a Monte Carlo permutation test (nominal alpha of 0.05; six groups with six individual data; variances 1:1:4.4:9:9; $\alpha = 0.071$; randomising 100 times) which provides information about sampling distributions when the exact theory for the sampling distribution is not available (after many replications, the stored results mimic the sampling distribution of the statistic).

The soil data obtained in the *T. melanosporum* burns from different truffières (with a high and low abundance of casts) did not follow a normal distribution, and therefore non-parametric tests were applied to these variables. The non-parametric Mann–Whitney U test was applied to analyses of the statistical patterns of the soil properties of burns with a high abundance of casts versus burns from truffières with a low abundance (control truffières).

Results

Tables 2, 3 and 4 show the analytical results of earthworm cast and soil samples from the truffière with high abundance of casts. In this truffière, the surface soil horizons of *T. melanosporum* burns present a moderately basic pH and very low content of total carbonate, active carbonate and TOC. Tables 2 and 5

Table 2 Average results of 92 soil and cast samples located inside burns with *Tuber melanosporum* fruit body production

Truffière code	Sample	Cast abundance ^a	<i>T.m.</i> burn production ^b	N	pH _{H₂O}	% Total CaCO ₃	% Active CaCO ₃	% (Total – active) CaCO ₃	% TOC
Truffière-1	Soil samples	+	>500	30	8.29 ± 0.17	2.81 ± 1.16	1.00 ± 0.33	1.86 ± 1.12	0.68 ± 0.16
Truffière-1	Cast samples	+	>500	42	8.44 ± 0.15	4.67 ± 3.52	0.84 ± 0.37	3.83 ± 3.26	0.71 ± 0.12
Control-2	Soil samples	–	>400	20	8.09 ± 0.19	29.11 ± 11.18	10.43 ± 3.70	18.68 ± 8.36	2.21 ± 0.79

Topsoils from a truffière with a high abundance of earthworm casts (>7 casts little piles per burn) are compared with nearby topsoils from two control truffières with low cast abundance (≤1 casts little pile per burn). Soil analytical results = mean ± SD

T.m., *Tuber melanosporum*

^a See Table 1

^b Burn production, g of fruit bodies collected year⁻¹

show the analytical results of soil samples from two control truffières (with a low abundance of casts). In both truffières, the surface soil horizons of *T. melanosporum* burns show a significant percentage of total carbonate and active carbonate, as well as a moderately basic pH and low levels of TOC.

Simple correlations were obtained between the variables of the cast samples and surface soil horizons (Table 6). The results show that active carbonate, organic matter and pH were intercorrelated in our combined sample of earthworm casts and soils. Total carbonate was (not unexpectedly) correlated with active carbonate, but was unrelated to either pH or TOC.

Results from the ANOVA/MANOVA indicate that earthworm cast samples had a higher percentage of total carbonate levels ($F_{1, 70} = 4.67$; p value = 0.034) and higher pH ($F_{1, 70} = 15.24$; p value < 0.001) than surface soils (Wilks lambda = 0.504; $F_{4, 67} = 16.4$; p value < 0.001) (Fig. 3, Table 2). However, Levene's test indicates that the data for percentage of total carbonate does not maintain the assumption of homogeneity of variances ($F_{1, 70} = 18.58$; p value < 0.001). The Monte Carlo test provided five out of 100 simulations (p value = 0.05) with F-values larger than the empirical value ($F_{1, 70} = 4.67$), therefore, the Monte Carlo test validated the finding that cast samples have a significantly higher percentage of total carbonate levels than surface soils.

The ANOVA accounted for only 6.26 % of variance in the percentage of total carbonate contents in our combined sample of earthworm casts and soils (Fig. 3). However, the results of an ANCOVA with [log- % total carbonate] as the dependent variable,

sample type (cast versus soil samples) as the factor, and [log- % active carbonate] as the covariate accounted for 28.05 % of the variance in the percentage of total carbonate contents in the combined sample of earthworm casts and soils (Fig. 4).

Another ANOVA of [log-(% total carbonate–% active carbonate)] indicates that earthworm cast samples had a higher percentage of carbonate when over 50 µm in size ($F_{1, 70} = 7.03$; p value 0.009) than soils from which the casts were produced (Table 2).

Results from the Mann–Whitney U test indicate that soils from *T. melanosporum* burns from the truffière with a high abundance of casts had a higher pH (p value < 0.001) and lower percentage of total carbonate (p value < 0.001), percentage of active carbonate (p value < 0.001) and TOC levels (p value < 0.001) than soil burns from the control truffières with a low abundance of casts (Table 2).

Discussion

Tuber melanosporum burn is an interesting model for studying the soil pH dynamics and processes of decarbonation-recarbonation associated with soil biology and their interactions with vegetation. In this regard, Callot (1999) reports that *T. melanosporum* mycelia (associated with bacterial populations) strongly acidify their immediate soil environment (pH values of 5.5–6 were reached in the immediate environment of the mycelia). This increase in H⁺ in the *T. melanosporum* hyphosphere microenvironment solubilises the carbonate fractions of the soil. In contrast, García-Montero et al. (2009a, b) indicate that

the particular environmental conditions of burn soils favour higher bicarbonate concentration in capillary water and lower $p\text{CO}_2$, which provokes a secondary carbonate precipitation with a net increase in active carbonate (calcium carbonate extractable with ammonium oxalate; smaller than 50 μm in size). They quantified these processes and indicated that the percentage of active carbonate inside the *T. melanosporum* burns is 2.17 times greater on average and the percentage of total carbonate is 0.64 times lower than in soils outside the burns. García-Montero et al. (2009a, b) have also shown a positive correlation between active carbonate and *T. melanosporum* fruit body production and burn size.

Callot (1999), Ricard (2003), García-Montero et al. (2008) highlight the relationships between earthworm activity, $p\text{CO}_2$, soil carbonates and soil pH in *T. melanosporum* burns. They propose that in calcareous, poorly aerated and badly-drained soils the episodes of rain and dryness increase the pH to over 8.4 (maybe even reaching ten locally), while in well-aerated and drained soils with high earthworm activity, the pH evolves to below 8.4. They explain that the $p\text{CO}_2$ increase is reflected in a lower pH that falls below seven when $p\text{CO}_2$ reaches 0.1 atmospheres (often achieved in calcareous soils and rhizospheres). They conclude that $p\text{CO}_2$ in the soil atmosphere not only depends on the respiration of roots and microorganisms, but also on soil aeration which is strongly conditioned by earthworm activity (generating up to 5,000 km of galleries per hectare in grassland soils). Our results show that burns with high earthworm activity present a lower pH than 8.4 (8.29 on average, Table 2) as proposed by these authors. However, these burns have a significantly higher pH than comparable control truffières with low earthworm activity (8.09 on average, Table 2). This suggests that in addition to $p\text{CO}_2$, other factors such as the properties of the casts could influence soil pH levels in soils with high earthworm activity.

Our results show that *Proselodrilus* sp. casts had a significantly greater pH, 0.2 units higher on average, than the burn soils from which the casts were produced (Table 2); the casts may therefore affect the soil pH. This result agrees Chan (2003), who shows that the pH of the casts is significantly higher ($\text{pH} > 6$) than that of the bulk acid soil material ($\text{pH} 4.0$) used in a laboratory experiment with *A. longa*, *A. trapezoides* and *Anisochaetae* sp. Wiecek and Messenger (1972)

Table 3 Analytical results of 30 topsoil samples located inside burns of *Tuber melanosporum* of Truffière-1 (with a high abundance of earthworm casts: >7 casts little piles per burn)

N°	pH _{H₂O}	% Total CaCO ₃	% Active CaCO ₃	% TOC
1	8.45	3.42	0.88	0.47
2	8.48	4.22	0.56	0.46
3	8.48	2.91	0.50	0.45
4	8.35	1.78	1.25	0.59
5	8.37	2.75	1.71	0.59
6	8.37	3.69	1.31	0.60
7	8.41	3.81	0.75	0.65
8	8.46	3.57	1.00	0.60
9	8.51	2.41	1.58	0.58
10	8.33	2.73	0.69	0.56
11	8.34	2.38	1.00	0.62
12	8.38	2.47	0.94	0.55
13	8.40	4.82	1.06	0.49
14	8.39	2.13	0.69	0.51
15	8.37	2.90	0.69	0.54
16	8.13	4.27	0.75	0.85
17	8.30	4.93	1.19	0.79
18	8.30	2.80	1.00	0.65
19	8.19	0.56	1.06	0.95
20	8.19	3.94	1.25	0.76
21	8.20	3.17	1.13	0.87
22	8.27	3.57	1.56	0.68
23	8.30	1.52	1.50	0.74
24	8.21	2.95	0.56	0.90
25	8.05	1.84	1.33	1.07
26	7.64	2.16	1.06	0.79
27	8.21	2.75	0.88	0.74
28	8.28	2.98	0.75	0.64
29	8.18	1.00	0.56	0.75
30	8.19	0.00	0.81	0.92

also indicate that *L. terrestris* casts are less acid than the ingested food, and propose that the weathering of earthworm calcite granules is partly responsible for pH values in excess of 7.0 in the A₁ horizons of acid soils under forest cover in some American woods.

Elsewhere, Lambkin et al. (2011) indicate that a laboratory experiment with different types of soils has shown that the increased production of granules by *L. terrestris* is only correlated with increased soil pH, and no significant correlations were observed for the

Table 4 Analytical results of 42 cast samples located inside burns of *Tuber melanosporum* of Truffière-1 (with a high abundance of earthworm casts: >7 casts little piles per burn)

N°	pH _{H₂O}	% Total CaCO ₃	% Active CaCO ₃	% TOC
31	8.17	1.41	1.06	0.86
32	8.31	1.89	0.37	0.89
33	8.21	1.65	0.75	0.86
34	8.25	1.19	0.81	0.84
35	8.21	9.34	0.75	0.82
36	8.27	3.02	0.94	0.91
37	8.28	6.34	1.00	0.76
38	8.33	1.91	0.81	0.80
39	8.20	3.64	1.06	0.74
40	8.32	1.82	0.94	0.74
41	8.62	5.84	0.59	0.61
42	8.60	4.70	0.47	0.62
43	8.64	4.90	0.41	0.61
44	8.62	3.81	0.59	0.45
45	8.62	1.14	0.47	0.57
46	8.58	1.14	0.41	0.54
47	8.58	1.73	0.47	0.55
48	8.63	1.73	0.59	0.58
49	8.57	2.03	0.66	0.55
50	8.53	1.93	0.66	0.57
51	8.51	6.68	0.91	0.73
52	8.51	7.97	0.78	0.64
53	8.56	8.07	0.97	0.66
54	8.54	7.87	0.84	0.71
55	8.55	1.68	0.66	0.61
56	8.55	4.11	0.66	0.62
57	8.54	2.03	0.47	0.63
58	8.53	1.68	0.84	0.59
59	8.49	2.08	0.53	0.67
60	8.49	1.44	0.59	0.65
61	8.53	1.19	0.66	0.71
62	8.53	1.49	0.59	0.77
63	8.26	7.44	1.66	0.83
64	8.28	8.35	1.04	0.68
65	8.28	8.25	1.10	0.89
66	8.36	13.47	1.73	0.85
67	8.42	9.83	1.54	0.91
68	8.47	9.68	1.66	0.76
69	8.37	4.48	0.79	0.83
70	8.38	3.88	0.66	0.74
71	8.31	12.33	1.41	0.68
72	8.30	11.11	1.54	0.79

other properties studied, including bulk and exchangeable Ca. Lambkin et al. (2011) describe also other studies confirming that this species and *L. rubellus*

increase production of granules in response to a higher soil pH, an abundance of calcareous substrate and various sources of calcium.

Table 5 Analytical results of 20 soil samples located inside burns of *Tuber melanosporum* of two control truffières with a low abundance of earthworm casts (≤ 1 casts little pile per

burn), based on García-Montero et al. (2009a) and Valverde-Asenjo et al. (2009)

N°	Truffière code	pH _{H₂O}	% Total CaCO ₃	% Active CaCO ₃	% TOC
73	Control-2 truffière	8.34	36.25	12.64	2.18
74	Control-2 truffière	8.18	49.25	15.64	2.12
75	Control-2 truffière	7.99	31.11	15.38	2.15
76	Control-2 truffière	7.85	22.63	6.88	3.40
77	Control-2 truffière	7.98	20.78	7.88	1.63
78	Control-2 truffière	8.06	24.14	6.50	1.48
79	Control-2 truffière	8.10	25.14	6.69	1.50
80	Control-2 truffière	8.09	5.53	4.75	1.51
81	Control-2 truffière	8.05	16.26	10.50	1.51
82	Control-2 truffière	8.12	32.00	13.50	2.21
83	Control-2 truffière	8.01	32.38	12.31	2.84
84	Control-2 truffière	8.12	39.15	15.06	2.59
85	Control-2 truffière	8.05	45.38	14.00	2.47
86	Control-2 truffière	7.69	25.30	6.13	3.76
87	Control-2 truffière	7.71	26.62	6.81	4.04
88	Control-3 truffière	8.44	20.75	8.14	1.50
89	Control-3 truffière	8.24	24.25	9.20	1.41
90	Control-3 truffière	8.23	19.75	8.27	1.53
91	Control-3 truffière	8.34	36.25	12.64	2.18
92	Control-3 truffière	8.18	49.25	15.64	2.12

Table 6 Simple correlation matrix of variables in our combined sample of soil and earthworm casts

Variables	% Total CaCO ₃	pH _{H₂O}	% TOC
% Active CaCO ₃	0.3946**	−0.4044***	0.3285*
% Total CaCO ₃	—	0.0702	0.0430
pH _{H₂O}	—	—	−0.6204***

* $0.05 > p \geq 0.005$; ** $0.005 > p \geq 0.001$; *** < 0.001 ; $n = 72$

Therefore, production and weathering of calcite granules by *Proselodrilus* sp., *L. terrestris*, *A. longa*, *A. trapezoides* and *Anisochaetae* sp. have the ability to increase soil pH; and increased soil pH can cause increased production of calcite granules by *L. terrestris*. Hence, all these results point to a feedback process, scope of which is a function of the different soil types and earthworm species.

Earthworm activity around *T. melanosporum* carpophores might affect also the soil carbonate dynamics.

Callot (1999) proposes that earthworms might provoke a local soil decarbonation in the burns as the calcite granules are coated with mucus (not carbonated) consisting of clay and polysaccharide during the intestinal transit through the earthworm. On the other hand, Jongmans et al. (2003) indicate that in soils without earthworm activity there are decalcification processes that could be ascribed to the production of organic acids in the litter layer and the absence of soil homogenisation by earthworms.

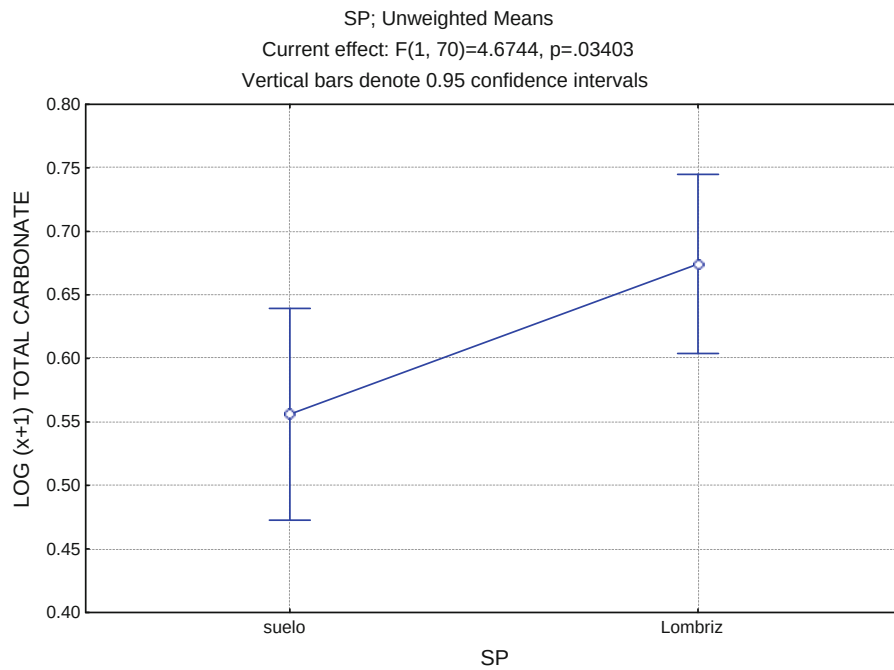


Fig. 3 Average of total carbonate (%) values for comparison of earthworm casts versus burn soil samples from which the casts were produced (ANOVA) (suelo = soil; lombriz = earthworm)

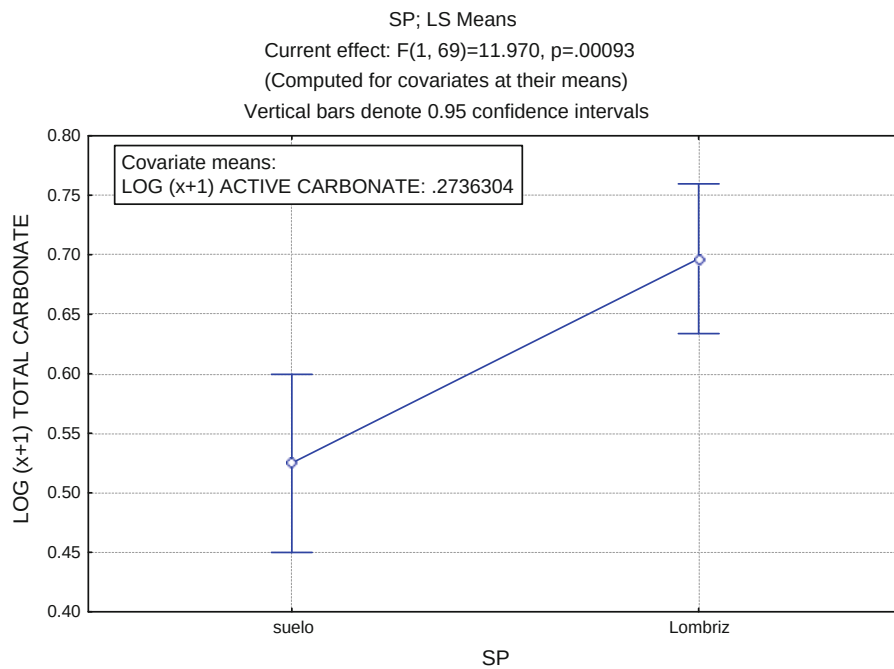


Fig. 4 Average of total carbonate (%) values for comparison of earthworm casts versus burn soil samples from which the casts were produced controlling the effects of active carbonate contents (ANCOVA) (suelo = soil; lombriz = earthworm)

Our results indicate that the *Proselodrilus* sp. casts had a significant average increase of 66 % in the percentage of total carbonate as compared to the soils from which the casts were produced (Table 2); however, there were no significant differences in the percentage of TOC between casts versus soils. Therefore, earthworm populations could have a significant impact on the soil recarbonation processes associated to the burns. To evaluate this impact, it is necessary to quantify the carbonate fraction balances associated to the casts versus burn soils and to consider the sources of C for the synthesis of calcite granules by earthworms.

Regarding the carbonate fractions associated to earthworm casts, Canti and Pearce (2003) indicate that species of *Lumbricus*, *Aporrectodea*, *Octolasion* and *Allolobophora* produce calcite granules larger than 0.125 mm. Thus the casts of these species contain calcium carbonate granules belonging to a carbonate fraction that is greater than the fraction of active carbonate (<50 µm in size). Our results show that *Proselodrilus* sp. casts had a significant average decrease of 16 % in percentage of active carbonate (under 50 µm in size) than the burn soils from which they were produced (possibly associated to the earthworm's intestinal digestion processes) (Table 2). However, ANCOVA/ANOVAs highlight the fact that earthworm casts have a significant average increase of 106 % in the carbonate fraction over 50 µm in size (% total carbonate – % active carbonate) compared to the burn soils (Table 2). Both results suggest that the origin of the 66 % increase in the percentage of total carbonate contents (Table 2) in the *Proselodrilus* sp. casts versus the soils is associated to the calcite granules produced by the earthworm's calciferous glands, instead of to the original carbonate fractions of the burn soils.

These field results with *Proselodrilus* sp. are therefore in agreement with the conclusions of Canti's isotopic study (2009) in the laboratory. He indicates that carbon from the soil calcium carbonate appears to be only slightly implicated in the formation of *L. terrestris* granules. This author indicates that earthworm calciferous glands provoke long-term soil carbonate net fixation in the form of calcite crystals that might have a long-term impact on soil pH and total carbonate contents in the soils.

In summary, *Proselodrilus* sp. and other earthworm species may increase pH and favour long-term net recarbonation through the increase in total

carbonate contents in truffière soils. Additionally, an increased soil pH may cause a greater production of calcite granules by earthworms, suggesting a feedback process. New experiments must be conducted to ascertain the scope of this feedback relationship between increasing pH and the production of calcite granules, taking into account the different earthworm species and different types of soils. The results obtained in this study point to the need for new experiments designed to increase our understanding of the intra- and inter-specific variability in granule production by earthworms, and to minimize the uncertainties associated with field studies in natural soils. (For example, additional experiments should be devised to include field monitoring of the vertical and horizontal movement of the various earthworm species in the soil profile). In conclusion, earthworm activity shows a positive impact on the soil properties of *T. melanosporum* burns, and it is thus necessary to develop further experiments aimed at incorporating vermiculture into the truffle cultivations.

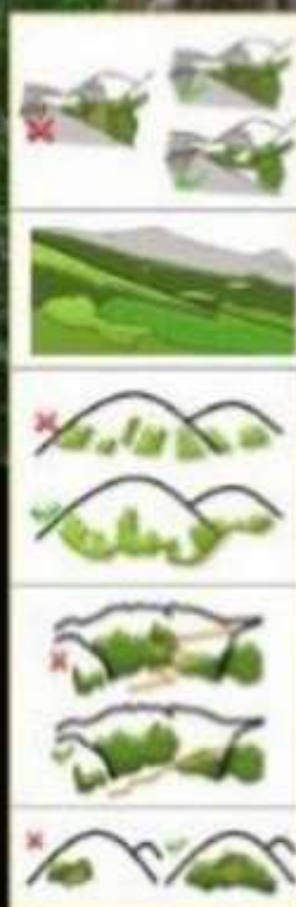
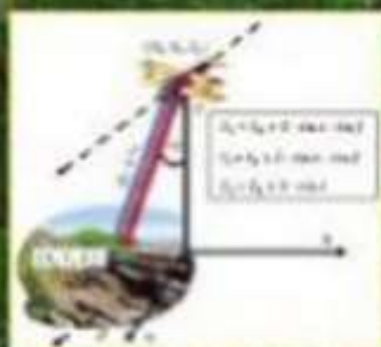
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Quantitative Techniques in Participatory Forest Management



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Eugenio Martínez-Falero
Susana Martín-Fernández
Antonio García-Abril

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4 Soil-Quality Indicators for Forest Management

*Fernando Arredondo-Ruiz, Luis García-Montero,
Inmaculada Valverde -Asenjo, and Cristina Menta*

CONTENTS

4.1	Introduction to Forest-Soil Quality	180
4.1.1	Importance of the Soil and the Rhizosphere	180
4.1.2	Foundations for Using Soil Properties as Indicators of Forest-Soil Quality	181
4.1.3	Fertility and Organic Matter Content in Forest Soils	182
4.1.4	Storing Carbon Dioxide in Forest Soils	183
4.1.5	Biodiversity and Forest Soils	184
4.2	Threats to Forest-Soil Quality	185
4.2.1	Eight Main Threats to Soil on a Global Scale	185
4.2.2	Erosion and Loss of Organic Matter in Forest and Agricultural Soils	186
4.2.3	Biodiversity Loss in Agricultural and Forest Soils	187
4.2.4	Difficulties in Developing International Strategies for Forest-Soil Protection	191
4.3	Soil-Quality Indicators in Sustainable Forest Management	193
4.3.1	Sustainable Forest Management and Soil Quality	193
4.3.2	Soil-Quality Indicators	195
4.3.3	Assessment of Soil-Quality Indicator Usage in Forestry	198
4.4	Biological, Chemical, and Physical Soil-Quality Indicators	202
4.4.1	Foundations for Understanding the Role of Biology in Soil-Quality Assessment	202
4.4.2	Biological Indicators to Assess Forest-Soil Quality	205
4.4.3	Microbiological, Biochemical, and Molecular Indicators to Assess Forest-Soil Quality	208
4.4.4	Chemical Indicators to Assess Forest-Soil Quality	212
4.4.5	Physical Indicators to Assess Forest-Soil Quality	214
4.5	Multiparametric Indices to Assess Soil Quality	215
4.5.1	Multiparametric Indices Including Physical, Chemical, and Biological Parameters	216
4.5.2	Physical–Chemical Indices to Assess Soil Quality	217
4.5.3	Multiparametric Indices Based on Mathematical Models to Assess Soil Quality	218





4.6 Practical Cases: Models for the Integration of Soil-Quality Indicators in Landscape Planning Projects	220
4.6.1 Model of Integration of Qualitative and Quantitative Soil and Environmental Indicators: An Example of the Assessment of the Environmental Quality of a Whole Country (Spain)	220
4.6.1.1 Introduction	220
4.6.1.2 Methodological Basis	221
4.6.2 Model of Integration of Quantitative Soil-Quality Indicators: An Example of the Generation of a Soil Fertility Index in Caribbean Agricultural and Forest Areas	223
4.6.2.1 Introduction	223
4.6.2.2 Methodological Basis	224
4.6.2.3 Model Steps and Logical Operations Applied	225
4.7 Conclusions	228
References	229

4.1 INTRODUCTION TO FOREST-SOIL QUALITY

4.1.1 ■ IMPORTANCE OF THE SOIL AND THE RHIZOSPHERE

Soil is one of the most fundamental components for supporting life on Earth. Jeffery et al. (2010) and Menta (2012) point out that the processes occurring within soil (most of which are driven by its living organisms) perform ecosystem and global functions that help maintain life aboveground. Moreover, soil delivers numerous services, ranging from providing the food we eat to filtering and cleaning the water we drink. It is used as a platform for building, it provides vital products such as antibiotics, and it contains an archive of our cultural heritage in the form of archeological sites. However, life within the soil is hidden and often suffers from being “out of sight and out of mind” (Blum, 1993; Ebel and Davitashvili, 2007).

Soils may be characterized in terms of the properties they inherit from the underlying rock (the parent material) and the properties resulting from alteration of the original parent material by soil-forming or “pedogenic” processes, namely, climate, vegetation, time, and human activity. Pedogenic processes operate mainly in the surface and subsurface horizons normally found in the upper 2 m (Blum, 1993; Ebel and Davitashvili, 2007).

AQ1 From the point of view of both agriculture and forestry, another essential aspect of the functioning of the soil, vegetation, and ecosystem is the importance of the rhizosphere (Akeem, 2012). The presence of roots is generally associated with greater microorganism density and soil fauna in the nearby soil when compared to soil devoid of roots; the term rhizosphere is used in a broader sense to refer to the portion of soil surrounding roots in which the soil organisms are influenced by their presence (Killham, 1994). The rhizosphere can be distinguished from the greater part of the soil on the basis of its chemical, physical, and biological characteristics. As the roots penetrate into the ground, they act on clay minerals and the particles of soil surrounding them, leading to the formation of an area in which the water pathway and the movement of nutrients and microflora are more heavily channeled than in the





Does the natural “microcosm” created by *Tuber aestivum* affect soil microarthropods? A new hypothesis based on Collembola in truffle culture



Cristina Menta^{a,*}, Luis Gonzaga García-Montero^b, Stefania Pinto^a,
Federica Delia Conti^a, Giampietro Baroni^a, Mattia Maresi^a

^a Department of Life Sciences, University of Parma, Via Farini 90, Parma 43121, Italy

^b Department of Forest Engineering, Operaciones Básicas, E.T.S.I. Montes, Technical University of Madrid (UPM), Ciudad Universitaria s/n, Madrid 28040, Spain

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ABSTRACT

The interactions between fungi and soil fauna are not well known. Some studies suggest that soil microarthropods play an important role in fungi dispersion, but little is still known about the interaction between truffle and soil microarthropods. The aim of this study was to investigate the ability of the truffle *Tuber aestivum* to modify soil biogeochemistry (i.e. create a zone of scarce vegetation around the host plant, called a burn or brûlé) and to highlight the effects of the brûlé on the soil fauna community. We compared soil microarthropod communities found in the soil inside versus outside the *T. aestivum* brûlé with the chemistry of soil collected inside versus outside the brûlé. The study was carried out in three Mediterranean areas, two in Italy and one in Spain. The results confirmed the ability of *T. aestivum* to modify soil biogeochemistry in the brûlé: pH was higher and total organic carbon tended to be lower inside the brûlé compared to outside. Soil fauna communities showed some interesting differences. Some groups, such as Symphyla and Pauropoda, adapted well to the soil; some Collembolan families, and biodiversity and soil quality indices were generally higher outside the brûlé. *Folsomia* sp. showed higher abundance in the soil of the brûlé compared to outside. The results suggest that some Collembola groups may be attracted by the fungal metabolites produced by *T. aestivum*, while other Collembola and other microarthropods may find an unfavourable environment in the soil of the brûlé. The next steps will be to confirm this hypothesis and to extend the study to other keys groups such as nematodes and earthworms and to link fluctuations of soil communities with the biological phases of truffle growth.

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1. Introduction

Fungi are widespread within the soil and represent a considerable potential food resource for soil fauna such as nematodes, springtails, earthworms, flies and beetles, many of which graze on fungal mycelia and can substantially alter fungal morphology and physiology (Anderson, 2001; Harold et al., 2005; Pacioni et al., 1991; Rotheray et al., 2011). The interaction between soil microarthropods and fungi deserves special attention because both groups represent major trophic and functional groups in the soil food web (Böhlmann et al., 2010).

Soil fauna could have a positive or negative effect on fungal growth, dispersion and fruit body production. Such effects are likely to alter fungal fitness, and therefore their combativeness in

interaction with other soil microorganisms, including fungi (Rotheray et al., 2011). Various authors have described some mechanisms explaining the impact of soil microarthropods on fungal communities. For instance, Hanlon and Anderson (1979) indicated that microarthropod feeding activities can exert a strong differential effect on fungal and bacterial populations, and some reviews on arbuscular mycorrhizae and soil fauna interactions suggest that Collembola have the potential to restrict mycorrhizal functioning in the field (Fitter and Garbaye, 1994; Fitter and Sanders, 1992).

It is less known, however, that certain communities of ectomycorrhizal (ECM) fungi can alter forest soil biogeochemistry, which could cause changes in microarthropod communities. Two important examples appear to be the truffle (*Tuber Micheli* ex Wiggers) brûlés and the dense hyphal mats formed by ectomycorrhizal (ECM) fungi.

Various ECM fungal species have been found to form dense aggregations of hyphae, known as mats, which are prominent

* Corresponding author. Tel.: +39 0521 903407; fax: +39 0521 347002.
E-mail address: cristina.menta@unipr.it (C. Menta).

features in Douglas-fir forest ecosystems. These soils, densely colonized by the ECM fungi, create a soil environment with different microbial activities when compared to non-mat forest soils. Cromack et al. (1988) showed increased TOC (total organic carbon) and N values in ECM-mat soils compared to non-mat soils. Kluber et al. (2010) indicate that ECM-mat soils show greater microbial biomass C (in mineral horizon) and enzyme profile differences (greater chitinase, phosphatase and phenoloxidase activity) compared to non-mat soils, and from 2.7 to 40 times more oxalate and lower pH than non-mat soils. Cromack et al. (1988) studied the interactions between soil fauna and some ECM mats and demonstrated that mites and springtails (and others, such as nematodes) are more abundant within ECM-mat-colonized soil compared to non-mat forest soils. These authors concluded that soil invertebrates, such as microarthropods, could utilize fungal-ECM-mat resources on an opportunistic basis, which could explain the observed dynamics of microarthropod communities in ECM-mat soils.

Trees infected with the ECM fungi sometimes have an area around the trunk where the growth of other plants is inhibited either permanently or for part of the year. Some ECM fungi that produce these clearings, or brûlés (burns), are *Tuber aestivum* Vittad. (summer truffle), *Tuber melanosporum* Vittad. (black truffle) and *Scleroderma* Pers. The *Tuber* mycelium produces multiple substances that adversely affect young trees and seed germination, and fungal infection in the roots of weeds results in severe necrosis in the root cortices (Plattner and Hall, 1995). *Tuber* mycorrhizal activity, mycelium growth and carpophore production take place inside the brûlé, mainly behind the brûlé line (Callot, 1999). Some authors have explained the production and development of brûlés (Granetti et al., 2005; Sourzat, 2004) but others have indicated that the ecological conditions relating to brûlés are yet to be clarified (Callot et al., 1999; García-Montero et al., 2009, 2014a,b; Ricard, 2003). There is virtually no reference to the possible effects of *Tuber* species on soil fauna.

As with ECM mats, García-Montero et al. (2009) indicated that *Tuber* brûlés can also alter soil biogeochemistry and some authors described how *T. melanosporum* brûlés decrease TOC in internal soils in relation to external soils (Callot, 1999; García-Montero et al., 2014a; Granetti et al., 2005; Ricard, 2003). In calcareous Mediterranean areas, García-Montero et al. (2014b) indicated that *T. aestivum* significantly increases soil pH and significantly decreases total carbonate in the internal soils of brûlés compared to external soils. Moreover, in Mediterranean climates where *Tuber* spp. thrives, Mello et al. (2013) indicated that the lack of vegetation

that occurs in their brûlés could cause significant changes in light exposure and soil moisture level, leading in turn to water stress in the brûlé soil, especially in the summer months.

On the basis of these considerations, the aims of this study were: (1) to increase knowledge of the ability of *Tuber aestivum* brûlés to modify soil biogeochemistry; (2) to characterize soil microarthropod communities in soils affected by the presence of *T. aestivum* brûlés. We therefore compared the soil found inside a *T. aestivum* brûlé with that observed outside the brûlé (control) in terms of physical and chemical characteristics, biodiversity and abundance of soil microarthropods.

With regard to the latter indicator, we hypothesize that some groups may be affected negatively by the almost total absence of vegetation in the brûlé, while others, especially fungivorous Collembola taxa, may find better conditions in the brûlé in terms of habitat and/or trophic source. We conducted the study in three different areas, one in Spain and two in Italy, to verify if the trend of soil chemistry and soil fauna would be confirmed in different areas. We assumed that the two conditions, inside and outside the brûlé, were characterized by the same climate, annual rainfall and tree presence, but had different grass and plant cover due to the direct action of the *T. aestivum* mycelium.

2. Materials and methods

2.1. Study areas

The study was carried out in three Mediterranean areas, two in Italy and one in Spain. Table 1 summarises the environmental characteristics of the study areas. The municipalities and exact geographic coordinates of the studied brûlés were not revealed, in order to maintain the secrecy of the *Tuber* brûlé locations. A total of nine brûlés characterized by the presence of *T. aestivum* were identified in each area. Soil samples were collected during *T. aestivum* fructification, June in Italy and May in Spain.

2.2. Soil analysis

The samples for soil analysis were taken according to FAO recommendations (FAO, 1990) from inside the holes made by the collectors to extract the carpophores in brûlés with a significant production. Only the first 30 cm of each soil profile was studied because most *Tuber* form mycorrhizas and bear fruit in this range (Verlhac et al., 1990). We collected 9 soil samples inside and 9 outside each brûlé for chemical analyses. The following soil

Table 1
Main characteristics of the two Italian and one Spanish areas where the study was conducted and soil samples were collected, both for chemical analysis and microarthropods characterization, to characterize *Tuber aestivum* brûlé, and soil sampling period.

	Area I	Area II	Area III
Country	Italy	Italy	Spain
Province	Teramo	Piacenza	Guadalajara
Altitude	630 m a.s.l.	721 m a.s.l.	1000 m a.s.l.
Average annual rainfall	937 mm	810 mm	797 mm
Medium temperature (°C)	14.9	10.6	9.7
Geological substratum	Cover detritus–eluvial of the Holocene origin	Lutetian limestones and marly limestones alternating marls and calcareous marls	Jurassic and Cretaceous limestone and dolomites
Soil	Mollisol ^a	Calcaric cambisol ^b	Lithic and rendzic leptosols ^c
Plant	<i>Quercus pubescens</i>	<i>Quercus pubescens</i>	<i>Quercus faginea</i>
Soil sampling period	Spring 2012	Spring 2013	Spring 2012

^a Soil is characterized by dark coloration, high content of organic matter and exchangeable bases, high content of calcareous material (total calcareous comprised between 9.6 and 14.3%, active calcareous between 2 and 14%), pH comprised between 7.2 and 8.3 (detected in H₂O) and high content of superficial stones.

^b Soil with at least the beginnings of horizon differentiation in the subsoil evident from changes in structure, colour, clay content or carbonate content. Medium and fine-textured. Materials derived from a wide range of rocks.

^c Soil is characterized by brown colour, high content of exchangeable bases, organic matter comprised between 3% and 7%. They are characterized by the high content of calcareous material (total calcium carbonate comprised between 7 and 8%, active calcium between 3 and 7%), pH comprised between 7.2 and 8.3 (detected in H₂O).

analyses were conducted: pH (in water), using the *ISRIC* (1995) method; total organic carbon (TOC) in the Spanish samples, using the *ISRIC* (1995) method; TOC in the Italian samples, using Ball's method (Ball, 1964) (using loss of weight on ignition and the Van Bemmelen conversion factor); and moisture content (%), using the gravimetric method.

2.3. Soil microarthropod extraction

Soil samples were taken inside each brûlé (more or less centrally) and outside it (at least 5 m from the brûlé edge). A total of 54 soil samples were collected (18 in each area, 9 inside the brûlé and 9 outside). Each soil sample measured 100 cm² and was taken at a depth of 10 cm. Microarthropods were extracted using a Berlese–Tüllgren funnel. The specimens were placed in a preserving solution (75% ethyl alcohol and 25% glycerol by volume) and identified to different taxonomic levels (class for Myriapoda, order for Hexapoda, Chelicerata and Crustacea, and family for Collembola). The organisms belonging to each taxon were counted in order to estimate their density at the sampling depth (10 cm), extrapolating the number of individuals to 1 m² (ind/m²).

2.4. Soil biodiversity indices

The biodiversity of soil animal communities was evaluated using the number of observed taxa (NT), the Shannon diversity index (H') and Pielou's evenness index (J). The latter two indices were calculated using the number of specimens observed in each sample for each taxonomic level.

Soil biological quality was estimated by applying the Acari-to-Collembola ratio (A/C, *Bachelier*, 1986), the QBS-ar index (*Parisi et al.*, 2005) and the QBS-c index (*Parisi and Menta*, 2008). The A/C index represents the biodynamic conditions of the soil. Under good stable conditions, the percentage of mites is higher than that of springtails, while it tends to decrease in favour of springtails in degraded situations. The QBS-ar index was developed in order to combine two important aspects regarding soil microarthropods: (1) their presence in the soil, intended as biodiversity; (2) the level of microarthropod adaptation to the soil, intended as vulnerability. This index is based on the idea that a high number of microarthropod groups (biodiversity) well adapted to soil habitats (vulnerable to soil perturbation) indicates a good soil biological quality. The QBS-ar index is applied to the soil microarthropod community, divided in accordance with the biological form approach (*sensu Sacchi and Testard*, 1971) in order to: (1) evaluate the microarthropods' level of adaptation to life in the soil environment (*Parisi*, 1974), and (2) overcome the well-known difficulties of taxonomic analysis to species level for soil mesofauna. Edaphic microarthropods are known to display morphological characteristics that give evidence of adaptation to soil environments, such as reduction or loss of pigmentation and visual apparatus, streamlined body form, with reduced and more compact appendages (hairs, antennae and legs), reduction or loss of flying, jumping or running adaptations and a reduced water-retention capacity, e.g. thinner cuticle and a lack of hydrophobic compounds on the outer surface (*Parisi*, 1974). By focusing on the presence of these characteristics, and not requiring complex taxonomic identification to the species level, QBS-ar analysis can also be used by non-specialists. The main phases for obtaining the QBS-ar values are: (1) soil sampling; (2) microarthropod extraction; (3) preservation of the collected specimens; (4) determination of biological forms and assignment of the ecological-morphological index (EMI); and (5) calculation of the QBS-ar index as the sum of the EMI values (*Parisi et al.*, 2005). The EMI is a value assigned at each taxonomic group and ranges from 1 to 20, from the lowest to the greatest adaptation to the soil. For more

details see *Gardi et al.* (2008); *Tabaglio et al.* (2009); *Menta et al.* (2011); *Blasi et al.* (2013) and *Menta et al.* (2014).

Because of the important role played by springtails in the litter turnover, an index based exclusively on the Collembola community was applied. This index, named QBS-c, is calculated using the biological form approach (*Parisi and Menta*, 2008). Like the QBS-ar, the QBS-c is based on the principle of the greater vulnerability of organisms that are well adapted to the soil conditions. After soil extraction, Collembola were separated and classified at a family level. For each family, the biological forms with different EMI values (ranging in relation to the level of adaptation to the soil, from 0 = no adaptation to 40 = maximum adaptation) were recorded. The sum of all the EMI values associated with the biological forms identified in the samples gives the QBS-c value (*Parisi and Menta*, 2008). Organisms belonging to the *Folsomia* (Isotomidae) genus were classified separately because they displayed a different trend compared to their family.

2.5. Statistical analysis

The Wilcoxon test was conducted to highlight differences in physical and chemical parameters between inside and outside the brûlé.

To characterize soil fauna community in the three areas, comparisons of both densities of organisms and indices were carried out. Taxon densities were transformed into $\sqrt{(X+0.5)}$ to avoid the problem of zeroes. The two-way ANOVA were applied, (area vs. burn) to highlight differences among the density groups; the influence of the study area and the position inside or outside the brûlé was investigated. Tukey's pairwise for the comparison of significant values (p -value < 0.05) was carried out for post-hoc analysis. Index data were analysed untransformed and compared using the Wilcoxon test for paired samples to highlight differences between soil fauna inside and outside the brûlé. The same test was applied to highlight differences in the Collembola densities inside and outside the brûlé. All the statistical analyses were performed using R 3.0.1 statistical software (*R Development Core Team*, 2013).

3. Results

3.1. Soil characterisation

Table 2 shows the physical and chemical properties of the soil samples collected inside and outside the *T. aestivum* brûlés. Many of the soil samples had a moderately alkaline pH. Levels of TOC were moderate.

The results of the Wilcoxon test indicated that TOC, organic matter and pH differed significantly according to the location of the soil, namely whether it was inside or outside the brûlés. Organic matter and TOC content were significantly lower (considering $p < 0.1$) in the soils collected inside the brûlés, in areas I and III. In area II the difference between the two environments was not significant. pH was significantly higher inside compared to external soils in the three areas, while water content was higher outside in area III.

3.2. Microarthropod community

A total of 9329 specimens belonging to 19 taxonomic groups of soil microarthropods (distributed between Chelicerata, Crustacea, Myriapoda and Hexapoda) were identified from the 54 soil samples collected. **Table 3** shows the taxa found and the average and standard errors of taxa abundances (expressed as ind/m²) for the three areas. When considering the entire soil microarthropod community, Acari and Collembola were the most abundant groups. Coleoptera, Diptera and Hymenoptera, all adults, showed

Table 2

Average and standard error of chemical and physical parameters detected in the soils inside and outside the *Tuber aestivum* brûlé of the two Italian (areas I and II) and one Spanish (area III) areas studied; *p*-value: statistical significance of the differences between inside and outside of the brûlé.

	Area I			Area II			Area III		
	Inside	Outside	<i>p</i> -value	Inside	Outside	<i>p</i> -value	Inside	Outside	<i>p</i> -value
pH	7.82 ± 0.03	7.6 ± 0.05	***	8.14 ± 0.03	7.97 ± 0.03	***	7.85 ± 0.06	7.58 ± 0.05	*
Moisture (%)	8.28 ± 1.38	9.65 ± 1.78	–	8.10 ± 0.73	8.73 ± 1.21	–	17.60 ± 1.06	20.16 ± 1.19	**
Organic matter (%)	9.60 ± 0.43	12.10 ± 1.30	0.065	5.04 ± 0.50	5.73 ± 0.40	–	5.70 ± 0.70	7.14 ± 0.88	0.055
Organic carbon (%)	5.58 ± 0.25	7.03 ± 0.75	0.065	2.93 ± 0.29	3.33 ± 0.23	–	3.31 ± 0.41	4.51 ± 0.51	0.055

* *p* < 0.05.

** *p* < 0.01.

*** *p* < 0.001.

abundance, in some cases even higher than 1000 ind/m². Pauropoda, Symphyla, Protura, Chilopoda, Hemiptera, Coleoptera and Diptera, the last two larvae, showed lower abundances. Pseudoscorpiones, Araneae, Isopoda, Diplopoda and Diplura were absent in several soil samples. Microcoryphia and Psocoptera were only sporadically present.

The two-way ANOVA showed significant differences between the three study areas for Pseudoscorpiones (*p*: 0.036), Acari (*p*: 0.033), Pauropoda (*p*: 0.006), Symphyla (*p*: 0.059), Chilopoda (*p*: 0.004), Protura (*p*: 4.7×10^{-8}), Collembola (*p*: 0.0002) and Coleoptera larvae (*p*: 0.036), and between inside and outside the brûlé for Acari (*p*: 0.040), Pauropoda (*p*: 0.077), Symphyla (*p*: 0.021) and Hemiptera (*p*: 0.028). These differences were investigated using Tukey's test, the results of which are set out below.

3.2.1. Area I

In this area we found lower densities of microarthropods inside compared to outside the brûlés (12,429 ind/m² inside and 25,861 ind/m² outside), and 15 taxonomic groups inside and 17

outside. The difference between the numbers of groups (NT) found inside and outside was statistically significant (Table 4). Symphyla and Hemiptera abundances were significantly lower inside the brûlés compared to outside (Table 3). The other microarthropod groups showed no significant trends, although in some cases (Coleoptera adults, Hymenoptera, and Lepidoptera larvae) abundances were higher inside the *T. aestivum* brûlés compared to outside. On the other hand, the abundances were on average higher outside for the other groups, but the numbers of specimens belonging to these groups were too low, and the dispersion of the data was too high, to highlight any significant differences.

An analysis of the Collembolan community showed significant differences between the densities of these organisms found outside and inside the burns (Table 5). Isotomidae was the most widely represented family, both inside and outside the burns, followed by the Hypogastruridae community collected outside the burns and the Tullbergidae family. The Wilcoxon test for paired samples highlights some significant differences for the Hypogastruridae, Isotomidae, Sminthuridae families, which show higher

Table 3

Average and standard error of microarthropod abundances (ind/m²) detected in the soil collected inside and outside the *Tuber aestivum* brûlés. Areas I and II located in Italy and area III in Spain; *p*-value: differences between inside and outside of the brûlés.

Taxa	Area I			Area II			Area III		
	Inside	Outside	<i>p</i> -value	Inside	Outside	<i>p</i> -value	Inside	Outside	<i>p</i> -value
Pseudoscorpiones	7 ± 7	38 ± 19	–	–	7 ± 7	–	–	–	–
Araneida	19 ± 19	64 ± 50	–	14 ± 9	57 ± 42	–	–	–	–
Acari	4223 ± 804	7892 ± 1892	–	2194 ± 412	3602 ± 610	–	5364 ± 949	5895 ± 1246	–
Isopoda	19 ± 10	25 ± 14	–	–	–	–	–	–	–
Diplopoda	7 ± 7	108 ± 50	–	7 ± 7	50 ± 35	–	–	–	–
Pauropoda	–	7 ± 7	–	21 ± 21	106 ± 38	***	50 ± 30	35 ± 15	–
Symphyla	96 ± 33	516 ± 168	*	50 ± 30	114 ± 54	–	120 ± 78	191 ± 69	–
Chilopoda	76 ± 34	172 ± 46	–	21 ± 15	14 ± 9	–	28 ± 11	42 ± 18	–
Protura	–	25 ± 17	–	219 ± 83	531 ± 214	–	28 ± 28	7 ± 7	–
Diplura	25 ± 14	83 ± 29	–	–	50 ± 26	–	28 ± 15	21 ± 15	–
Collembola	5285 ± 2169	13210 ± 7015	–	4465 ± 1434	2272 ± 524	–	709 ± 194	814 ± 230	–
Microcoryphia	–	–	–	–	–	–	14 ± 14	–	–
Dermoptera	–	–	–	–	–	–	–	–	–
Orthoptera	–	–	–	–	–	–	–	–	–
Psocoptera	–	–	–	35 ± 22	78 ± 35	–	–	–	–
Hemiptera	7 ± 7	446 ± 192	**	35 ± 28	28 ± 22	–	21 ± 11	64 ± 42	–
Thysanoptera	13 ± 8	45 ± 19	–	–	–	–	–	14 ± 9	–
Coleoptera	662 ± 167	634 ± 144	–	21 ± 11	14 ± 9	–	–	7 ± 7	–
	Adults	662 ± 167	634 ± 144	21 ± 11	14 ± 9	–	–	7 ± 7	–
	Larvae	38 ± 22	89 ± 22	64 ± 24	134 ± 70	–	71 ± 71	35 ± 19	–
Diptera	Adults	675 ± 179	1268 ± 333	–	–	–	21 ± 21	14 ± 14	–
	Larvae	70 ± 26	159 ± 59	21 ± 15	21 ± 11	–	–	21 ± 15	–
Hymenoptera	Adults	1134 ± 317	943 ± 118	396 ± 109	3843 ± 2920	–	446 ± 132	1111 ± 428	–
	Larvae	76 ± 31	140 ± 34	–	–	–	–	–	–
Lepidoptera	Larvae	13 ± 13	7 ± 7	–	–	–	7 ± 7	14 ± 9	–
Others	13 ± 8	25 ± 14	–	–	–	–	–	–	–
TOT	12429 ± 3296	25861 ± 6891	–	7594 ± 2082	10955 ± 4624	–	6885 ± 1540	8273 ± 2142	–

* *p* < 0.05.

** *p* < 0.01.

*** *p* < 0.001.

Table 4

Average and standard error of number of taxa (NT), microarthropod biodiversity indices (H': Shannon diversity index and J: Pielou's evenness index) and soil quality indices (A/C: Acari/Collembola ratio, QBS-ar and QBS-c based on microarthropods and Collembola respectively) detected in the soils collected inside and outside the *Tuber aestivum* brûlés in the two Italian (areas I and II) and one Spanish (area III) areas; p-value: differences between inside and outside of the brûlés.

Index	Area I			Area II			Area III		
	Inside	Outside	p-value	Inside	Outside	p-value	Inside	Outside	p-value
NT	9 ± 1	13 ± 1	***	6 ± 1	8 ± 1	*	5 ± 1	7 ± 1	–
H'	1.38 ± 0.04	1.42 ± 0.11	–	0.99 ± 0.80	1.31 ± 0.12	**	0.73 ± 0.13	0.89 ± 0.05	–
J	0.64 ± 0.04	0.57 ± 0.05	–	0.57 ± 0.05	0.63 ± 0.04	–	0.43 ± 0.05	0.50 ± 0.05	–
A/C	2.11 ± 0.80	0.93 ± 0.24	–	1.12 ± 0.52	1.80 ± 0.23	–	12.75 ± 3.36	13.76 ± 5.21	–
QBS-ar	111 ± 7	164 ± 10	***	83 ± 10	102 ± 13	0.097	76 ± 8	90 ± 8	–
QBS-c	182 ± 19	205 ± 12	–	143 ± 8	152 ± 25	–	95 ± 12	105 ± 18	–

* $p < 0.05$.** $p < 0.01$.*** $p < 0.001$.

values outside the burns, and for the *Folsomia* genus, which displayed the opposite trend.

QBS-ar was the only index that was significantly lower inside the *T. aestivum* brûlés (Table 4).

3.2.2. Area II

We found a density of 7594 ind/m² inside the brûlés and 10,955 ind/m² outside (Table 3). In this area we found 13 taxonomic groups inside the brûlés and 15 outside, and the number of groups was significantly lower inside than outside (Table 4). Pauropoda abundance was significantly lower inside *T. aestivum* brûlés compared to outside. Acari abundance was lower inside the brûlés, showing relative frequencies ranging from 28% inside to 33% outside the brûlés, and Hymenoptera adults showed highly variable relative abundance, ranging from 5% inside to 35% outside the brûlés, but the differences were not significant. Coleoptera larvae and Symphyla showed relative abundance higher than 1%, but only outside the brûlés. All the other groups found had relative abundance of less than 1%. Unlike area I, Protura reached high relative abundance in this area, ranging from 2.88% inside to 4.85% outside the brûlés, but the differences were not significant. Chilopoda, Hemiptera and Coleoptera adults were not significantly higher inside the *T. aestivum* brûlés compared to outside. Unlike area I, Collembola in this area reached a relative abundance of over 50% inside and 20% outside the brûlés (difference not significant). An analysis of the families belonging to the latter group revealed a trend similar to that highlighted in area I (Table 5), although the statistical results have not been confirmed. Almost all families showed higher values associated with samples collected outside the burns, but, in contrast with what we found in area I, the most abundant family, Isotomidae, showed higher values inside the burns. *Folsomia* genus showed

higher densities inside the brûlé, confirming what was observed in area I.

All the indices calculated (Table 4) showed higher values outside the brûlé, but the difference was significant only for the H' index.

3.2.3. Area III

We found an average density of 6886 ind/m² inside the *T. aestivum* brûlés and 8273 ind/m² outside. As in area II, Acari was the most representative group, reaching 74% inside and 71% outside the *T. aestivum* brûlés, while Collembola was present with lower relative abundances (10% inside and 9% outside). The differences between inside and outside were not significant for either group. As in areas I and II, Hymenoptera was, after Acari and Collembola, the group that reached the highest abundance, 1019 ind/m² and 3057 ind/m² inside and outside the brûlé, respectively. Symphyla showed a relative abundance of 1.7% inside and 2.3% outside the brûlé, while Coleoptera (larvae) and Lepidoptera (larvae) showed a relative abundance of about 1% both inside and outside the brûlé. None of the other groups reached 1%. In all cases, the differences were not significant.

The analysis of the Collembola community failed to show any significant differences between the families identified. Nevertheless, some of them revealed the same trends as highlighted in areas I and II. In particular, Isotomidae showed higher values outside the burns, as in area I, while organisms belonging to the *Folsomia* genus showed higher values inside the burns, as in both the other areas. The indices H', J, A/C, QBS-ar and QBS-c were higher outside the brûlés, but the differences were not significant (Table 4).

3.2.4. Differences between areas

Tukey's test highlights significant differences between the two Italian areas outside the brûlé for Pauropoda ($p: 0.0024$), Chilopoda

Table 5

Average and standard error of collembolans abundances (ind/m²) detected in the soil collected inside and outside the *Tuber aestivum* brûlés in the two Italian (areas I and II) and one Spanish (area III) areas; p-value: differences between inside and outside of the brûlés.

Families	Genus	Area I			Area II			Area III		
		Inside	Outside	p-value	Inside	Outside	p-value	Inside	Outside	p-value
Entomobryidae		217 ± 93	350 ± 61	–	120 ± 31	212 ± 55	–	85 ± 28	128 ± 70	–
Hypogastruridae		170 ± 109	4787 ± 3443	***	92 ± 70	170 ± 74	–	78 ± 35	78 ± 31	–
Isotomidae		2325 ± 940	6212 ± 1820	**	2520 ± 854	863 ± 144	–	354 ± 183	439 ± 201	–
	<i>Folsomia</i> sp.	771 ± 457	19 ± 14	*	1604 ± 467	510 ± 325	0.098	64 ± 24	28 ± 15	–
Onychiuridae		529 ± 346	535 ± 230	–	85 ± 26	184 ± 86	–	71 ± 44	99 ± 46	–
Tullbergidae		1248 ± 620	1179 ± 616	–	7 ± 7	50 ± 28	–	43 ± 28	42 ± 18	–
Sminthuridae		25 ± 27	128 ± 47	*	35 ± 22	283 ± 157	–	7 ± 7	–	–
TOT		5285 ± 2169	13210 ± 7015	0.053	4465 ± 1434	2272 ± 733	–	702 ± 194	814 ± 230	–

* $p < 0.05$.** $p < 0.01$.*** $p < 0.001$.

(p : 0.0027), Protura (p : 0.0001) and Hemiptera (p : 0.026). Pseudoscorpiones (p : 0.058), Chilopoda (p : 0.053) and Collembola (p : 0.005) showed significant differences between the Italian central area outside the burn and the same Spanish area. Protura showed significant differences between the north Italian area and the Spanish one, both inside and outside the burn (p : 0.024 and p : 0.00004, respectively), and between the two Italian areas inside (p : 0.012). Lastly, we found significant differences for Collembola between the north Italian area and the Spanish area inside the burns (p : 0.005).

4. Discussion

This study aimed to highlight the differences in soil fauna between inside and outside the brûlé, as a consequence of the different environment created by *T. aestivum*. Our results and other studies (García-Montero et al., 2014b) confirmed the ability of *T. aestivum* to modify soil biogeochemistry in the brûlé: pH was higher and total organic carbon tended to be lower inside the brûlé compared to outside in the three areas investigated. The TOC outcome confirms for *T. aestivum* the results of previous studies on *T. melanosporum* brûlés (Callot, 1999; Granetti et al., 2005; García-Montero et al., 2014a; Ricard 2003). The decrease in soil TOC inside both *Tuber* brûlés can be explained by the ability of both *Tuber* spp. to minimize the C inputs and turnover, by removing vegetation and other fungus populations from their brûlés (Benucci et al., 2011; García-Montero et al., 2014a; Mello et al., 2011, 2013; Napoli et al., 2010). Gryndler et al. (2013) found that the concentration of *T. aestivum* mycelia in soil is high compared to other ectomycorrhizal fungi, but the concentration of extraradical mycelium in the soil of *T. aestivum* (Gryndler et al., 2011, 2013) and *T. melanosporum* (Parladé et al., 2013) brûlés cannot be compared with the soil densely colonised by ECM-mat fungi (covering up to 40% of the soil surface), showing increased TOC (total organic carbon) and N values in mat soils compared to non-mat soils (Cromack et al., 1988; Kluber et al., 2010). Therefore, a decrease in soil organic matter content and increase in soil pH inside *T. aestivum* brûlés, together with other factors associated with the reduction of plant cover (such as variations in soil moisture content, temperature and luminosity), could negatively affect the soil fauna in the brûlés. The findings showed that, like the Collembola families, Hypogastruridae, Sminthurididae and Isotomidae in area I, Symphyla and Pauropoda were more present (in terms of abundance and frequency) outside the *T. aestivum* brûlé, but this trend was not confirmed in the other two areas. Within the Isotomidae family, *Folsomia* genus showed higher abundance inside the brûlé in area I and this trend seems to be present in the other two areas. This result suggests that the conditions created by *T. aestivum* inside the brûlé do not have a negative impact on *Folsomia* genus. This could be due to the group's well-known ability to graze on fungi and hyphae, and *T. aestivum* brûlés could contain a good trophic resource for this genus. A study pointed out that the presence of spores and extraradical mycelium of arbuscular mycorrhizal hyphae was documented (Hodge, 2000) in the gut contents of *Folsomia candida*. Rotheray et al. (2011) reported that Collembola play a role in determining fungal species combativeness during interactions, with a potential effect on species assemblages and diversity. They reported that the fungal response to Collembolan grazing during interspecific interactions appears to vary according to the combination of grazer species and the identity of the interacting fungi. Hanlon (1981) demonstrated that grazing by Collembola in cultures grown in low-nutrient media produced a significant decline in fungal activity in comparison with controls. Grazing by animals in cultures grown in high-nutrient concentrations did stimulate fungal respiration, however. As suggested by Crowther and A'Bear (2012) in soil microcosm studies, the grazing

impact of woodlice (*Oniscus asellus*), Collembola (*Folsomia candida*) and millipedes (*Blaniulus guttulatus*) on the foraging and distribution of two saprotrophic cord-forming basidiomycetes was not only taxon-specific, and within taxa, species-specific but also density-dependent. Larger populations of the three invertebrates limited mycelial development to a greater extent. In another study Gange (2000) showed that some mycorrhizal hyphae are not stimulated to grow at low Collembolan densities, but grazing is detrimental at high densities. At intermediate densities, the preferential removal of small-diameter mycorrhizal hyphae towards the exterior of the mycelium may result in proliferation, and consequently an increase in benefit for the host plant through mineral uptake. In a recent paper, A'Bear et al. (2014) proposed that the increased grazing pressure can counteract, or even amplify, warming-induced stimulation of mycelia growth. In addition, other authors indicate that mites are an important variable in studies of fungal growth during grain drying and storage (Armitage and George, 1986; Sinha and Mills, 1968). It is therefore important to investigate not only grazing pressure due to the Collembola but also the effects of other taxa, which are generally less abundant but play an important role in the soil microflora regulation.

Therefore, our results only partially confirmed the initial hypothesis that *T. aestivum*, by altering soil biogeochemistry and vegetation cover, causes differences in soil microarthropod community, as happens with microarthropod communities in ECM-mat soils (Cromack et al., 1988). We did not in fact observe a univocal trend in the *T. aestivum* brûlés since some microarthropod groups were less abundant in brûlés and others more abundant. We can suppose that some microarthropod groups, in particular *Folsomia* genus, may be attracted by the fungal metabolites produced by the mycelium of *T. aestivum*, while others may find an unfavourable environment in the soil of the brûlé. *Tuber* spp. are symbiotic fungi that depend closely on other organisms to complete their lifecycle. *T. aestivum* emits volatile organic compounds (VOCs) to attract animal vectors for spore dispersal and possibly other functions (Pacioni et al., 1991; Splivallo et al., 2011). Bellina-Agostinone et al. (1987) found that thiobismethane or dimethyl sulphide is the main VOC compound in *T. aestivum*. There are strong indications that thiobismethane emissions are recognized by insects and mammals (Culleréa et al., 2010; Díaz, 2002). It is possible that the VOCs play an important role in the microarthropods abundance regulation in the brûlé.

Moreover, our study contributed to highlight differences, in the microarthropod community, not only between inside and outside the brûlés but also comparing the three study areas. Further studies will therefore be required to analyse the biological role play by the different microarthropods, the vegetation phenology (outside the brûlés) and the different climatic conditions in the three study areas.

In conclusion, this study confirmed that the soil in *T. aestivum* brûlés differs partially in terms of pH, TOC, organic matter content and soil microarthropod community, from the soil around it. The three taxa showing differences between these two “environments” were Pauropoda, Symphyla and Hemiptera, suggesting that the conditions in the brûlé negatively affected these groups. Among springtails, Hypogastruridae, Isotomidae and Sminthurididae were significantly lower inside the brûlés compared to outside, while *Folsomia* genus seems to find favourable conditions in the brûlé, in spite of reduced vegetation cover. We hypothesize that *Folsomia* genus may be attracted by the fungal metabolites produced by *T. aestivum*, and, in relation to their high abundance in the brûlé, could be of interest in *Folsomia* ecology and *T. aestivum* ecology and truffle culture. It would be interesting to understand the relationship between *Folsomia* abundance and *T. aestivum* fructification and body fruit production and determine whether there is a range of *Folsomia* abundance within which *T. aestivum* can be

favoured in its fructification and above which fructification can be depressed, as reported for other fungi with regard to fungus-colonised soil.

Lastly, several questions regarding the relationship between soil fauna and *T. aestivum* brûlé are still open. The next stages of this study would be to determine whether there are fluctuations in terms of presence and abundance of soil microarthropods (especially the less-studied groups, such as Protura and Symphyla) directly or indirectly correlated to the different development and fructification of *T. aestivum*, and extend the research to other truffle species that cause brûlé formation, such as *T. melanosporum*. Another aspect of interest would be to compare different soil fauna groups, not only arthropods, but also nematodes and earthworms. This would enable us to understand whether *Tuber* is able to create a natural “microcosm”, i.e. the brûlé, as part of a larger macrocosm, the soil around it in terms of soil biodiversity and soil functionality.

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“Management of land use systems for enhanced food security:
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Basis on Rhizoculture: Management of “Underground Agroforestry Systems” to Improve their Climate Change Resilience

LUIS GONZAGA GARCÍA-MONTERO¹, FERNANDO GARCÍA-ROBREDO¹, SIGFREDO ORTUÑO¹,
ESPERANZA AYUGA¹, LILIANA PINTO¹, PILAR PITA¹, CRISTINA PASCUAL¹, AMAYA ÁLVAREZ²,
LUIS BENITO-MATÍAS³, MARÍA P. ANDRÉS¹, THOM KUYPER⁴

¹Technical University of Madrid (UPM), Dept. of Forest Engineering, Spain

²Centro Nacional de Recursos Genéticos Forestales El Serranillo, Dpto. of Mycorrhizae, Spain

³National Forest Genetic Resources Centre El Serranillo, Dpto. of Mycorrhizae, Spain

⁴Wageningen University, Soil Quality, The Netherlands

Abstract

Agroforestry has traditionally been focused on the most economically valuable elements of plants. However, Horizon 2020 Societal Challenge analyses indicate that new nature-based solutions that include the multiple functions of ecosystems are needed for the transition towards a circular economic eco-innovation system to change production and consumption patterns towards a green society. In agroforestry, a better understanding of soil and organism interactions with host plants would allow an intensification on the development and activity of their roots leading to increased resistance of plants to climate change and soil C sequestration and to decreased fertilisation dependence. The development of this multidisciplinary body of knowledge could be called “rhizoculture”. The present work propose a review and a research project to develop a new rhizoculture approach in Agroforestry connecting the root, rhizosphere, agroforestry uses, fertiliser managements and landscape planning, all supported by four research approaches: (1) agrotechnological support to root development, (2) biological and biogeochemical support to “soft” fertilisation and rhizosphere management, and (3) soil organism management by soil organic matter control (by extensive livestock and biomass uses) and other tools, (4) landscape planning using territorial analysis tools, as i.e., Openforis Collect Earth. Seeking to change production and consumption patterns towards a sustainable, green economy and society, the benefits associated to this type of research on rhizoculture may increase the biodiversity and C stock in the soil, moreover, the development of the roots in agroforestry systems would increase the plant’s ability to cope with environmental and climatic changes. This approach would also improve the health and performance of plants and decrease the high costs and pollution associated to fertilisers and phytochemicals.

Keywords: Biodiversity, fertiliser management, resilience to climate change, rhizosphere, root management, soil biodiversity, soil C stock, soil carbon

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COST Action FP1305 BioLink
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5-OP

**Could mycorrhizae perform “ecosystem engineering” on the host plants?
Basis on rhizoculture of agroforestry systems to improve their resilience**

Luis G. García-Montero¹ (Selected Abstract), V. Monleon², D. Myrold³, T. Kuyper⁴, J. Trappe², J. Baham³, I. Valverde⁵, X. Parladé⁶, A. Álvarez Lafuente⁷, L.F. Benito-Matías⁷, J.R. Quintana⁵, A. Quintana¹, P. Andrés¹, M. Morcillo⁸, C. Menta⁹, S. Pinto⁹, P. Díaz¹⁰, C. Lefevre³, D. Luoma³, C. Turkmen¹¹, J.A. Domínguez¹⁰, A. Santiago⁵, L. Pinto¹, P. Pita¹⁰, F. García-Robredo¹, S. Ortuño¹, J. Pera⁶, E. Verrecchia¹²

¹Dept. of Forest Engineering, E.T.S.I. Montes, Technical University of Madrid (UPM),
Ciudad Universitaria s/n, Madrid 28040, Spain;

²USDA Forest Service, USA;

³Oregon State University, USA

⁴Wageningen University, The Netherlands;

⁵University Complutense of Madrid, Spain;

⁶IRTA, Spain;

⁷National Forest Genetic Resources Centre El Serranillo, Spain;

⁸Micofora, Spain;

⁹University of Parma, Italy;

¹⁰Technical University of Madrid (UPM), Spain;

¹¹University of Çanakkale, Turkey;

¹²University of Lausanne, Switzerland

luisgonzaga.garcia@upm.es

Agroforestry has traditionally been focused on plant growth following plant-centric and anthropocentric points of view. Both paradigms have caused global problems such as soil loss, declining biodiversity and the stock of C in the soil, and the high cost and pollution associated to fertilizers. Both paradigms may have also limited the understanding of mutualistic symbioses of plants assuming that the role of non-photosynthetic symbionts is to mobilize the nutrients necessary for the plants which appear to be the dominant members of the symbiosis. We propose an alternative approach introducing a new ‘rhizo-centric’ point of view (where non-photosynthetic partners could be the dominant members). This rhizo-centric approach could be modelled by a comparison with the lichens, because (1) their fungal partner is in most cases the dominant member of the symbiosis and (2) some authors proposes that lichenic fungi ‘farm’ the photobionts in a controlled parasitism. Thus, the present hypothesis proposes to explore a ground-breaking hypothesis suggesting that many mycorrhizal fungi could farm their host plants in a controlled parasitism similarly lichenic model. Within this conceptual framework, the main objective of this hypothesis is to analyze whether ‘Ca paradox’ (which means that Ca acts simultaneously as nutrient and as stressor of plants) could explain the hypothesized dominance of mycorrhizal fungi on plants on a global scale. This Ca paradox could be provoked by an accumulation of Ca salts (and increased pH) in the rhizosphere associated to mycorrhizal rock-eating activity (and also encouraged by soil fauna). In the event that this hypothetical approach bears fruit it would provide the necessary basis to start to change our agroforestry technology by introducing the ‘rhizoculture’ based on soil microbiology management (following lichenic model), soil Ca management (using liming), mycorrhizal rock-eating capacity, and soil fauna and livestock management.

Keywords: *ectomycorrhizae; rhizosphere; calcium; rock-eating activity; agroforestry*

Rhizoculture technology to manage the closing loops on N, P and C cycling, in European and global agro-ecosystems

Acronym: RHIZOFARMING

TOPIC: H2020 SFS-30-2017; **BUDGET:** 6,999,663.00 Euros

CODE	Profile	Participant organization name	Acronym	Country
1	(Coordinator) Research	Universidad Politécnica de Madrid	UPM	Spain
2	Research	Consejo Superior de Investigaciones Científicas	CSIC	Spain
3	Research	Institut de Recerca i Tecnologia Agroalimentaries	IRTA	Spain
4	Research	Consiglio Nazionale delle Ricerche	CNR	Italy
5	Research	Czech University of Life Sciences	CZU	Czech Republic
6	Research	University of Lausanne	UL	Switzerland
7	Research	Technische Universität Darmstadt	TUD	Germany
8	Research	University of Copenhagen	UCPH	Denmark
9	Research	Natural Resources Institute of Finland	LUKE	Finland
10	Research	International Center for agroforestry	ICRAF	Kenya
11	International organism	Food & Agriculture Organization of United Nations	FAO	Italy
12	International organism	European Forest Institute	EFI	Finland
13	Farmers association	Asociación Agroforestal Española	AAE	Spain
14	Farmers association	Association Française d'Agroforesterie	AFAF	France
15	Farmers association	Český Spolek pro Agrolesnictví	CSA	Czech Republic
16	Companies association	European Feed Manufacturers' Federation	FEFAC	Belgium
17	Company	Micologia Forestal & Aplicada	MFA	Spain
18	Company	Fertinagro Nutrientes, S.L.	FN	Spain
19	Company	Sakata Vegetables Europe	SVE	France
20	Company	İSKO Plastik A.Ş.	IPAS	Turkey

Table of Contents	Page
1. EXCELLENCE	1
1.1 Objectives of RHIZOFARMING.....	1
1.2 Rhizoculture relation to the work program	3
1.3 Concept and methodology in RHIZOFARMING.....	4
1.3.(a) Rhizoculture concept.....	4
1.3.(b) Methodology in RHIZOFARMING.....	5
1.4 Ambition of the rhizoculture.....	6
2. IMPACT	9
2.1 Expected impacts of RHIZOFARMING	9

ABSTRACT

Increasing cost of fertilization and progressively limiting reserves of mineable mineral, together with impacts of agriculture on climate and world's ecosystems are casting serious doubts about the sustainability of the current agricultural model. Whilst unable to abandon intensive agriculture in the short run, we urgently need reduce its reliance on man-made soil amendments and thus to improve its sustainability. One of the ways to achieve this is to design a rhizosphere-centered approach entailing a suite of easy-to-apply solutions aiming to increase recycling of soil nutrients. RHIZOFARMING addresses an existing bottleneck constituted by the root/rhizosphere microbiome exchange of N, P and C cycling in agro-ecosystems. The project builds on the latest understanding of the role of soil beneficial organisms as a determinant of (i) the productivity of a rich array of crops at the farm level (ii) pollution and GHG at the farming and landscape levels, and (iii) waste management in intensive plant and animal production (including aquaculture) systems. The project will develop innovative and economically sound agricultural practices designed to maintain productivity whilst contributing to sustainable management of farm-to-landscape scale of N, P and C cycles. A carefully selected partnership of academic and industry partners will promote market-oriented innovation ready for commercial application. Farmer associations will provide a framework for a participatory-joint research, creating an environment designed to generate viable prototypes & technologies, such as new fertilizer & biofertilizer lines, screening rhizoculture-fit plant varieties aimed at future breeding programs, or innovative fodder designs featuring enzymatic complexes that link animal production to the management of nutrient loops in a circular economy. Finally, the support of key actors in European and global advocacy for agricultural policy provides a platform for effective communication-dissemination.

Sustainability in Plant and Crop Protection

Martin Lukac
Paola Grenni
Mauro Gamboni *Editors*

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Chapter 4

Towards Integrated Understanding of the Rhizosphere Phenomenon as Ecological Driver: Can Rhizoculture Improve Agricultural and Forestry Systems?

Luis G. García-Montero, Pablo Manzano, Deaa Alwanney, Inmaculada Valverde-Asenjo, Amaya Álvarez-Lafuente, Luis F. Benito-Matías, Xavier Parladé, Sigfredo Ortuño, Marcos Morcillo, Antonio Gascó, Carlos Calderón-Guerrero, Francisco Mauro, Mercedes Méndez, Alvaro Sánchez-Medina, María P. Andrés, José R. Quintana, Cristina Menta, Stefania Pinto, Liliana Pinto, Pilar Pita, Cafer Turkmen, Cristina Pascual, Esperanza Ayuga, Fernando Torrent, José C. Robredo, Pablo Martín-Ortega, Joan Pera, Luis Gómez, Gonzalo Almendros, Carlos Colinas, and Eric P. Verrecchia

Abstract Agriculture and forestry traditionally focus on improving plant growth traits based on an anthropocentric point of view. This paradigm has led to global problems associated to soil overexploitation such as soil losses, reductions of the C stock in soils, and the generalized use of fertilizers, which particularly increases the costs of production and pollution treatment. This view may also have limited our understanding of mutualistic symbioses of plants and microorganisms assuming

L.G. García-Montero (✉) • D. Alwanney • S. Ortuño • A. Gascó • C. Calderón-Guerrero
F. Mauro • M. Méndez • A. Sánchez-Medina • M.P. Andrés • L. Pinto • P. Pita • C. Pascual
E. Ayuga • F. Torrent • J.C. Robredo • P. Martín-Ortega • L. Gómez
Technical University of Madrid, ETSI Montes, Madrid, Spain

University of Lleida, Lleida, Spain

University of Lausanne, Lausanne, Switzerland
e-mail: luisgonzaga.garcia@upm.es

P. Manzano
IUCN Commission for Ecosystem Management, Gland, Switzerland

I. Valverde-Asenjo • J.R. Quintana
Complutense University of Madrid, Madrid, Spain

A. Álvarez-Lafuente • L.F. Benito-Matías
National Forest Genetic Resources Centre El Serranillo, Madrid, Spain

X. Parladé • J. Pera
IRTA, Sustainable Plant Protection Centre de Cabrils, Cabrils, Spain

that the main role of non-photosynthetic symbionts is to mobilize the nutrients that are necessary for plant growth and development, and being plants the dominant agents of the symbiotic relationship. In response to these issues, this chapter offers an alternative approach taking advantage of the “rhizo-centric” point of view, where non-photosynthetic partners are the main protagonists in play; and secondly, it builds a multidisciplinary body of knowledge that could be called “rhizoculture”, which includes techniques focussing on the intensification of the development and activity of roots, mycorrhizae, and other symbiotic and free living rhizosphere organisms. In short, rhizoculture may lead to decrease plant production dependence on fertilization and provides other benefits to agriculture, forestry, and the environment. Within this conceptual framework, the first objective of this book chapter is to explore whether there is a “paradox of calcium salts” (i.e., Ca^{2+} and its salts are simultaneously nutrients, promoters, and stressors for the host plants) that would explain a dominance of mycorrhizal fungi over plants based on inducing a $\text{Ca}(\text{pH})$ -mediated chlorosis to the host plants. If this paradigm shifting hypothesis were finally fully verified, it would provide conceptual bases to reconsider our current technologies in agriculture and forestry by introducing the “rhizocultural” approach, based on the management of roots (introducing alternative cultural practices), Ca^{2+} salts (using liming and other techniques), rock-eating mycorrhizae, organic matter, and the soil microbiome (increasing the presence of symbiotic microorganisms against saprophytes), N and P contents (by aquaculture and smart recycling of organic waste), and the physical properties of the soil (by the activity of soil symbiotic microorganisms and soil fauna, such as ants, termites and earthworms). The development of such new technological approaches in rhizoculture would significantly decrease the high cost and associated pollution of the application of fertilizers and phytochemicals; as well as it would increase soil C stocks, improve the resilience of agricultural and forest systems to environmental disturbances, such as climate change, and enhance food production and security.

Keywords Rhizoculture • Rhizosphere • Mycorrhizae • N-fixing bacteria • Soil fauna • Agriculture • Forestry

M. Morcillo

Micología Forestal Aplicada – Micofora, Sant Antoni De Vilamajor, Barcelona, Spain

C. Menta • S. Pinto

Evolutionary and Functional Biology Department, University of Parma, Parma, Italy

C. Turkmen

Canakkale Onsekiz Mart University, Canakkale 17020, Turkey

G. Almendros

Spanish National Research Council (CSIC), Madrid, Spain

C. Colinas

University of Lleida, Lleida, Spain

E.P. Verrecchia

University of Lausanne, Lausanne, Switzerland

PART I

Title of Proposal
Ecological intensification of the rhizosphere for Mediterranean sustainable agricultural development
Acronym: RIZHOINTENSE

1. Administrative data of participant organisations

- List of participants :

Participant No *	PI name	Organisation	Country
1 (Coordinator)	Luis Gonzaga García Montero	Universidad Politécnica de Madrid (UPM)	Spain
2 Partner 1	Salvador Lladó	Acondicionamento Tasrrasense (LEITAT)	Spain
3 Partner 2	Amghar Fateh	Université M'hamed Bougara Bumerdes (UMBB)	Algeria
4 Partner 3	Ahmed Nasr-Allah	World Fish CGIAR (WF)	Egypt
5 Partner 4	Nouran El Said	Agrimatic Farms (AgriF)	Egypt
6 Partner 5	Teodoro Calles	Food and Agriculture Organization of the United Nations (FAO)	Italy
7 Partner 6	Paolo Carnemolla	Federazione Italiana Agricoltura Biologica e Biodinamica (FederBIO)	Italy
8 Partner 7	Hussam Hawwa	DIFAF s.a.l (DIFAF)	Lebanon
9 Partner 8	Cafer Turkmen	University of Çanakkale Onsekiz Mart (UCK)	Turkey
10 Partner 9	Engin Yilmaz	YOLDA initiative (YOLDA)	Turkey

2. General information of the pre-proposal

Section:	Section 1. Farming Systems 2018
Call:	PRIMA
Topic:	Improving the sustainability of Mediterranean agro-ecosystems
Type of action:	RIA Research and Innovation action
Duration in months:	36 months
Free keywords	<i>ecological intensification, biofertilizers, biochar; rizhoculture, rhizosphere, roots, agroforestry, livestock, aquaculture, food quality</i>
Abstract:	<i>The aim of RHIZOINTENSE is to develop cropping systems with low-input and low environmental impact in the Mediterranean basin. The project will develop an alternative to current agriculture practices based on a novel concept called "rhizoculture": farming roots and rhizosphere. Rhizoculture proposes a set of Ecological Intensification (EI) strategies and tools to integrate ecological processes into land management, thus enhancing ecosystem services delivery by roots and rhizospheric organisms to produce food in a sustainable manner. This changes the traditional plant-centric point of view and focuses on a rhizo-centric approach, where the rhizosphere could perform the dominant role. RHIZOINTENSE will enhance the smart use of ecosystem services provided by soil, plants, soil organisms livestock and aquaculture for the cultivation of underexploited local cereal, legume and pasture varieties more adapted to the particular climatic conditions of the Mediterranean area under different agricultural systems. To intensify the activity of roots and rhizospheric organisms will allow increasing sustainability, water use efficiency, food production stability and food quality and security. RHIZOINTENSE will address the challenge of the topic by</i>

	<i>encouraging Mediterranean farmers to introduce innovative tailor-made strategies based on the ground-breaking combination of i) EI strategies and tools including the integration of agriculture with livestock and/or aquaculture, ii) diversification of agroecosystems including organic agriculture and agroforestry, iii) local crop varieties. An interdisciplinary experienced consortium (including industry, farmer's associations, RTOs and SMEs), will closely collaborate with the stakeholder's board to achieve this goal by means of a co-decision and multi-actor approach.</i>

Has this proposal (or a very similar one) been submitted in the past 2 years in response to a call for proposals under Horizon 2020 or any other EU programme(s)?

Yes

☐

No

☒

Declarations

1) The coordinator declares to have the explicit consent of all applicants on their participation and on the content of this proposal.	X
2) The information contained in this proposal is correct and complete.	X
3) This proposal complies with ethical principles (including the highest standards of research integrity — as set out, for instance, in the European Code of Conduct for Research Integrity — and including, in particular, avoiding fabrication, falsification, plagiarism or other research misconduct).	X

4) The coordinator confirms:

- to have carried out the self-check of the financial capacity of the organisation on http://ec.europa.eu/research/participants/portal/desktop/en/organisations/lfv.html or to be covered by a financial viability check in an EU project for the last closed financial year. Where the result was “weak” or “insufficient”, the coordinator confirms being aware of the measures that may be imposed in accordance with the H2020 Grants Manual (Chapter on Financial capacity check); or	X
- is exempt from the financial capacity check being a public body including international organisations, higher or secondary education establishment or a legal entity, whose viability is guaranteed by a Member State or associated country, as defined in the H2020 Grants Manual (Chapter on Financial capacity check); or	X
- as sole participant in the proposal is exempt from the financial capacity check.	X

5) The coordinator hereby declares that each applicant has confirmed:

- they are fully eligible in accordance with the criteria set out in the specific call for proposals; and	X
- they have the financial and operational capacity to carry out the proposed action.	X
The coordinator is only responsible for the correctness of the information relating to his/her own organisation. Each applicant remains responsible for the correctness of the information related to him/her and declared above. Where the proposal to be retained for EU funding, the coordinator and each beneficiary applicant will be required to present a formal declaration in this respect.	

According to Article 131 of the Financial Regulation of 25 October 2012 on the financial rules applicable to the general budget of the Union (Official Journal L 298 of 26.10.2012, p. 1) and Article 145 of its Rules of Application (Official Journal L 362, 31.12.2012, p.1) applicants found guilty of misrepresentation may be subject to administrative and financial penalties under certain conditions.

Personal data protection

The assessment of your grant application will involve the collection and processing of personal data (such as your name, address and CV) on the French National Research Agency evaluation and submission system. The PRIMA Foundation informs that in accordance with the French law "Informatique et Libertés" of the 6 of January 1978 as amended, applicants have the right to access and rectify their personal information. As such, they can access their user profile and rectify themselves some information about them. In addition, they have the right to exercise their rights by seizing the "Correspondant informatique et libertés" of the ANR. Unless indicated otherwise, your replies to the questions in this form and any personal data requested are required to assess your grant application in accordance with the specifications of the call for proposals and will be processed solely for that purpose.

3. General budget

Total amount requested to PRIMA / €:

2,000,000€

The PRIMA programme is an Art. 185 initiative supported and funded under Horizon 2020, the European Union's Framework Programme for Research and Innovation'



e. GENERAL DISCUSSION

e.1. Analysis of the results and bibliographic review on ectomycorrhizal fungi and *Tuber brûlé* model

e.1.1. Introduction

Since the development of agriculture in the early Neolithic, the models of agriculture and forestry performance have primarily been focused on improving plant growth (especially focused on the development of parts of plants that were economically valuable) following anthropocentric and economical points of view. This approach may have neglected the understanding of plants' interactions with their mutualistic symbionts, such as ectomycorrhizal (ECM) fungi, arbuscular mycorrhizal (AM) fungi and N-fixing bacteria (NfBs), and commensal organisms, such as earthworms (García-Montero *et al.*, 2015a; 2015b; 2017).

Therefore, the problem under study is assessing whether mycorrhizas and other symbiotic organisms affect agriculture and forestry in a different way to the one assumed from a “phyto-centric” point of view, which is based on the traditional approach viz.: *“Symbiotic organisms are related to plants following ecosystem models, but assuming that the role of non-photosynthetic symbionts is to mobilize the nutrients necessary for the primary production of plants, which appear to be the dominant actors of the symbiotic relationship. This conceptualization may result from phyto-centric and anthropocentric points of view which have led to the current agricultural and forestry models”* (García-Montero *et al.*, 2015a; 2015b; 2017). This traditional approach has led to high-intensity agricultural and forestry overproduction models, which are leading to soil degradation and loss, declining biodiversity and of soil C to the atmosphere.

In 1989, a new “rhizocentric model” was proposed as a promising tool for agriculture (Payne, 1989). Since 2000, this approach was renewed as the “rhizocentric view”, which considers that root-microbial interactions may be key processes in controlling plant productivity (Fitter *et al.*, 2000; Drigo *et al.*, 2008; Philips, 2007). Recently, it was observed that numerous fungi and bacteria modify rhizospheric environment (i.e., through pH and Ca levels), leading to intensifying rhizospheric activity through increasing root development, mycorrhization (leading to P inputs) and nodulation of N-fixing bacteria (leading to N inputs), which has a positive effect on host plant productivity (leading to C inputs) (Verrecchia *et al.*, 2006; García-Montero *et al.*, 2009a; 2017; Menta *et al.*, 2014; Monfort-Salvador *et al.*, 2015).

In response to these issues, this Section delves into this alternative hypothesis, improving on the phyto-centric point of view, where plants behave as dominant agents in the symbiosis, with a rhizo-centric point of view, where non-photosynthetic partners could play a predominant role.

This rhizo-centric hypothesis could be modelled in comparison with the model for lichens because their fungal partner is, in most cases, the dominant actor of the lichenic association. In this regard, there is some debate about the exact nature of the lichenic symbiotic association, which has raised the following question: *Are lichenic fungi farming the photobionts in a model that could be called controlled parasitic relationship?* (TBLS, 2016).

The present Section proposes to explore a ground-breaking hypothesis suggesting that mycorrhizal fungi (both ecto and arbuscular mycorrhizal) could modify soil environment to farm plants in a similar way of the controlled parasitic relationship that has been described for the lichenic model. In this review, ericoid, arbutoid, monotropoid and orchid mycorrhizas have not been included due to their lesser impact on agriculture and forestry (in economic terms).

For this purpose, this Section propose to integrate the following three lines of analyses focused on the hypothesis of the 'rhizo-centric' point of view:

- (i) Ecological comparison between lichenic model and mycorrhizal symbioses.
- (ii) Modelling a hypothetical ectomycorrhizal farming activity (regarding their host plants) in connection to the 'Ca paradox'.
- (iii) Exploring new abiotic and symbiotic interactions based on the Ca paradox, affecting the activity of mycorrhizas, NfBs, host plants, and soil fauna.

Within this conceptual framework, the first objective of this Section is revising in the literature whether the "paradox of calcium" (Ca^{2+} and its salts simultaneously act as nutrients, promoters and stressors of plants, in the framework of the symbiotic organism-host plant relationship) could contribute to explain the postulated dominance of some mycorrhizal fungi over host plants. As far as this hypothesis would be corroborated, the second objective of this Section would portray a suitable scenario to improve our technologies in agriculture and forestry by introducing "rhizoculture" (García-Montero *et al.*, 2009a; 2009b; 2012; 2015a; 2015b; 2017).

e.1.2. Ecological comparison between the lichenic model and mycorrhizal symbioses

From an ecological point of view, both lichenic and mycorrhizal mutualistic associations show remarkable functional asymmetries between partners, where participant organisms display complementary adaptations to living on land (Grube and Hawksworth, 2007; Selosse and Le Tacon, 1998; van Schöll *et al.*, 2008):

- (i) Single-cell or multi-cell photobionts are well adapted to gas exchange and photon collection, which enables them for the exploitation of atmospheric resources.
- (ii) Fungal mycelia is well-adapted to 3-dimensional substrate exploration being able to modify soil environmental conditions, that is, some fungi weather rocks ('rock eating'), providing host plants access to mineral nutrients (Blum *et al.*, 2002; Koele *et al.*, 2014).

However, a close evolutionary scrutiny of mutualistic interactions have revealed that heterogeneous selection pressures may alter the strength and net fitness effect of mutualistic associations, causing their interactions to become parasitic or, at least, cheating (Thrall *et al.*, 2007).

In the lichenic model, there are good arguments to propose that lichenic fungi could rather farm photobionts under "controlled parasitism" (TBLS, 2016), that is:

- (i) Up to half the C fixed by algae is immediately converted to fungal sugars that are inaccessible to the alga itself.

- (ii) Some lichens that can form stable associations with their 'usual' host algae form parasitic-type interactions with non-host algae when grown in the lab.
- (iii) There are many lineages of lichen fungi that are parasitic on other lichenicolous lichens. In some cases, non-lichen fungi have evolved from lichenized forms. These may be specialized opportunistic parasites or saprophytes, or even symbionts, competing for nutrients with other fungi in the lichen talus.

Would it thus be possible to extrapolate this lichenic behaviour pattern of controlled parasite mutualism to the mycorrhizal model? In order to explore this comparative hypothesis, a first good step would be to clarify the term 'lichen' itself. Grube and Hawksworth (2007) precisely indicate that this has been the cause of much debate. However, they adopt a lichen definition which includes the concepts of 'ecologically obligate' and 'stable mutualism' between an ex-habitant fungal partner and an inhabitant population of photobionts.

A second step would be noting that the majority of mycorrhizal fungal species are 'ecologically obligate', representing 'stable mutualisms' between ex-habitant fungal partners (which extend widespread the rhizosphere environment) and inhabitant photobionts (considering the tissues of roots surrounded or penetrated by fungal hyphae).

Therefore, from a theoretical point of view, it is possible to draw parallelisms between the lichenic model and the mycorrhizal model. A third step would be to highlight other interesting parallelisms between both models, such as (Simard *et al.*, 2012; Thrall *et al.*, 2007):

- (i) The mycorrhizal model proposes a very specific interface among soil, host plant and a diverse community of rhizosphere micro-organisms, such as some types of *bacteria* that can inhibit or stimulate each other (comparable with the structure of lichens) (Tarkka and Deveau, 2016).
- (ii) The production of mycorrhizal fungal auxins and metabolic signals affecting plant development (also comparable with the functional performance of lichens) (Streiblová *et al.*, 2012).
- (iii) There is experimental evidence that mycorrhizal fungal mycelia can link plants together in a Mycorrhizal Network (MN) that appears to be ubiquitous in nature, and can facilitate fungal colonization and/or interplant transference of compounds (comparable with the structure and functioning of lichen) (Heijden, 2016).

However, in order to model at large-scale, the ecological significance and impact of this type of parallelisms between mycorrhizal and lichenic models on agricultural and forest systems, a fourth step would be finding general and shared keys allowing the correct comparative interpretation of both models, such as:

- (i) Key 1. 'Evolutionary connection'. This key is stated by the following facts:
 - a. fungi existed as lichens in terrestrial paleoecosystems prior to the appearance of land plants (Schoch *et al.*, 2009)
 - b. primitive lichenicolous fungi facilitated further terrestrial fungi evolution (Lutzoni *et al.*, 2001)

- c. plant land colonization processes required mutualistic associations such as mycorrhizas development (Grube and Hawksworth, 2007)

(ii) Key 2: 'The Ca paradox' (see above). In this regard, García-Montero (2000; 2012) García-Montero *et al.* (2007, 2008a, 2008b, 2009a; 2009b; 2012; 2013), Valverde-Asenjo *et al.* (2009) and Menta *et al.* (2014) have studied the ecosystems of truffles (*Tuber* spp.) and other ectomycorrhizal fungal species, whose carpophore production were focused on the development of the root systems of host trees, along the last two decades. This route allowed these authors to propose that Ca, and its salts, are major factors explaining these symbiotic interactions. In a parallel way, since 1825, the presence of Ca oxalate salt has been reported as well-known in lichens, and can today be considered as a frequent, eclectic, cheap material in the economy of a lichen (Giordani *et al.*, 2003; Syers *et al.*, 1967); as well as Ca carbonate and calcium is still more abundant in some species of lichens (Asta, 1992; Garty, 2001).

In the following Sections, it will be explored whether Ca and its salts could contribute to explain the hypothesized dominance of mycorrhizal fungi over host plants as explained in a 'rhizo-centric' model similar to the well-known lichenic model.

e.1.3. Limitations of the current state of knowledge of ectomycorrhizal fungal symbiosis

There is still insufficient knowledge of ectomycorrhizal functional structure and its precise role in ecosystem processes and biogeochemical cycling. Numerous *in situ* studies on the ecological function of ectomycorrhizas (ECMs) have been hindered by the difficulty of controlling ECM in nature, and limited research has already been conducted on the successional processes of ECM communities (Courty *et al.*, 2010; Nara, 2008; Smith and Read, 2008), although ECM communities are extremely species-rich in most forest ecosystems (Nara, 2008). Particularly, the number of ECM species may often exceed 100 even at a small landscape scale (Nara, 2008), and a single tree species may form symbiotic associations with several hundreds of fungal species. However, only a small fraction of known ectomycorrhizas has physiologically been characterized up to date (Read and Pérez-Moreno, 2003), and the role of ECMs have mostly been demonstrated under simplified, artificial experimental conditions. Considering the variable environmental conditions and complex interorganismal interactions in the field, it cannot be assumed that ECMs would perform these same functions in nature, so the processes described under simple experimental conditions need to be scaled up to regional or global dimensions (Nara, 2008).

On the other hand, applying the contemporary "niche theory", Peay (2016) recently showed how ectomycorrhizal symbioses expand environmental ranges (requirement niche) and influence resource use (impact niche) for both plants and fungi. This author also highlighted that, although DNA sequencing has revealed the ubiquity of beneficial microbial symbioses, the role of mutualisms in shaping species niches is not yet broadly recognized.

In summary, the high taxonomical and functional diversity of ECM communities, and the lack of appropriate research methods, have limited current existent knowledge of the role of ECMs

in ecosystems. Therefore, studying ECM metabolism and functional performance diversity is the key to understand their role at the ecosystem level and determine their contribution to sustainable agriculture and forest management and conservation (Courty *et al.*, 2010). For this reason, the first objective of this Section would be to identify primary determinants controlling the development of ECM symbiosis in order to understand their full ecological significance (Varma, 2008).

e.1.4. Ectomycorrhizal fungi in disturbed sites: a new hypothesis on nutrition dynamics for the symbiosis between plants and Tuber (truffles)

As already mentioned in the *Introduction*, in order to minimize the research difficulty of ECM fungal field studies, a potential valid approach would be to study disturbed sites with simple ECM fungus/host-plant communities developed on relatively homogeneous substrates (Nara, 2008), such as truffle brûlés. *Tuber melanosporum* brûlé occurs because its mycelium and carpophores release multiple compounds that adversely affect seed germination and young plants development; as well as producing necrosis in grass roots (Plattner and Hall, 1995).

García-Montero (2012) and García-Montero *et al.* (2009a; 2009b; 2013) have performed soil comparisons between carbonated fractions and TOC inside and outside *Tuber melanosporum* brûlés. They have indicated that the concentration of total CaCO_3 inside the mature brûlé is significantly lower than in the outside soil (an average 0.64 times lower), which provides evidence of the “rock-eating” activity of *T. melanosporum*. However, the concentration of active CaCO_3 (extractable with ammonium oxalate; smaller than 50 mm in size) inside mature brûlés is significantly higher (2.17 times greater on average) due to the soil environmental conditions that are associated to the existence of a mature brûlé (clearance of roots, decrease in soil pCO_2 , and soil microclimate changes), thus favouring a secondary carbonate precipitation producing a net increase of active CaCO_3 and exchangeable Ca contents in the soil. On the other hand, the active CaCO_3 concentration inside *T. melanosporum* brûlés shows a positive correlation with the size of the brûlé (accounting for up to 51% of its variance), *T. melanosporum* fruit body production (accounting for up to 43% of its variance), and the concentration of exchangeable Ca (accounting for up to 42% of its variance). In addition, the concentration of exchangeable Ca inside the brûlé is significantly higher than that in soils outside the brûlés (estimated 16.26 cmol/kg of soil greater, i.e., 5.94 times higher).

On the other hand, in preliminary studies, García-Montero (2012) and have also observed that the content of TOC inside the *Tuber melanosporum* brûlé is significantly lower than in soils outside the brûlés: estimated 0.21 times lower inside the brûlé. In this regard, there are evidences that indicate that decreased TOC contents in the soil increase the ratios of soil symbiotic/saprophytic communities (García-Montero *et al.*, 2017).

Based on these findings, García-Montero *et al.* (2009a) have proposed a soil nutrition hypothesis considering the activity of *Tuber melanosporum* and *T. brumale* Vittad.: “Brûlé soils with high active CaCO_3 and exchangeable Ca concentration in the rhizosphere lead to host plant chlorosis (even on vegetation adapted to high levels of Ca), which is a symptom of an underlying nutrient deficiency. This nutrient deficiency could lead to greater colonization of *T.*

melanosporum and *T. brumale* ectomycorrhizas and favor the growth of mycelia, fruit body production and br  le development, which in turn encourages the formation of new amounts of active CaCO_3 on the basis of a br  le feedback model".

Therefore, in first place, this Ca-induced-chlorosis hypothesis is based on the fact that plants suffering nutritional stress have greater ability to form mycorrhizas, as proposed by Marx *et al.* (1977), Gelpe and Timbal (1986), Gogan *et al.* (2009) and Vaario *et al.* (2009), for example. In addition, this hypothesis is also based on the fact that an increase of soil Ca availability in the soil appears to have stressful and detrimental effects on the growing vegetation above.

An extreme example of the impact of the Ca-induced-chlorosis on host plants associated to *Tuber melanosporum* br  les has been observed in populations of rockroses (*Cistus* L.). Most *Cistus* species grow on siliceous or decarbonated soils, but some of them also thrive as symbiotic hosts of *Tuber melanosporum*. For example, a wild population of *Cistus laurifolius* L. from central Spain is able to produce *Tuber melanosporum* carpophores, but many host plants will nevertheless die in br  les showing a significant ECM activity, as measured by annual carpophore production and the secondary accumulation of biologically active CaCO_3 and exchangeable Ca (Garc  a-Montero, 2000). *Cistus incanus* L. mortality has also been reported significant in an experimental cultivation with *Tuber melanosporum* where plant colonisation rates were high (78% of the plants had developed br  les) (Manna, 1992). All these observations have led Professor Salvador Rivas-Mart  nez to propose the term '**calcidesert**' to describe black truffle br  les from a geobotanical point of view (pers. comm., 2014).

CaCO_3 -rich soils cover about the 30% of the world's surface land, being calcareous soils well-known to depress plant growth and increase iron deficiency. Because an increase in CaCO_3 soil content would lead to an increase in soil pH that limits the solubility of NPK and increases iron deficiency (Ylivainio, 2010). An increased soil Ca availability may address detrimental or stressful effects on plant nutrition, which can be very significant in the case of calcifuge plants. In addition, Gelpe and Timbal, (1986) showed that substrates with only a 1% of CaCO_3 content can cause the death of the 68% of calcifuge species seedlings, such as *Quercus rubra* L. Moreover, Wilmot *et al.* (1996) highlighted that Ca has also been identified as a hindering element in some forest stands, as well as it has been involved in producing dieback and physiological dysfunction to sugar maple on acid soils; and Str  m *et al.* (2005) indicated that calcareous soils are frequently characterized by their low bioavailability of plant nutrients. Finally, Sabir *et al.* (2010) reported that Turkish calcareous soils show a reduced iron availability and expose plants to severe chlorosis.

e.1.5. A new global-scale calcium-induced-chlorosis hypothesis for ectomycorrhiza-host plant symbioses based on the ability of ectomycorrhizas to mobilize calcium

The described soil nutrition affection hypothesis associated to Ca-induced-chlorosis could serve to a wider scope than generating br  les of *Tuber melanosporum*, and may be extended to other ECM fungal communities. In this regard, Garc  a-Montero *et al.* (2008a) confirmed that the Chinese truffles *Tuber pseudoexcavatum* Wang, G. Moreno, L. G. Rioussset, J. L. Manj  n, and G. Rioussset and *T. indicum* Cooke and Massee showed good ectomycorrhizal development in soils

with a moderately basic pH, low levels of total carbonates but an elevated level of active carbonate and a good proportion of exchangeable Ca^{2+} .

Some authors have reported comparable ECM soil activity patterns resulting in a significant accumulation of Ca, as calcium oxalate salt (CaC_2O_4), associated to the ECM mycelium presence in different soils. Oxalate and siderophore secretion are considered as the main mineral weathering agents being produced by ECMs (Rineau and Garbaye, 2010; Rineau *et al.*, 2010a). The ability to exude oxalate by some ECMs has been widely documented, for example as the mean to acquire P thanks to the induction of mineral weathering (Kluber *et al.*, 2010). Laccase is another ECM's exudate that, in the presence of oxalate, oxidizes Mn ion, initializing a set of reactions leading to the production of H_2O_2 , which further initiates or supports peroxidase reactions (Baldrian, 2006; Rineau *et al.*, 2010a) contributing to degrade litter phenolic compounds (Rineau *et al.*, 2010b).

A relevant example of this behaviour pattern of ECM accumulating calcium oxalate has particularly been described to occur in the soil horizons of coniferous forests of the American Pacific Northwest, where distinct rhizomorphic mats are formed by ECM species of *Piloderma* spp. and *Hysterangium* spp. covering up to 40% of the soil surface, whereas *Ramaria* spp. is found in the mineral soil. These fungal mats have physical and biochemical properties that distinguish them from the surrounding unmatted soil. In particular, matted mineral soil holds up to 40 times more calcium oxalate ($p < 0.01$) than the unmatted one. As a result, unmatted soil shows a decreased pH value (0.61 units inside matted mineral soil; $p = 0.02$) in comparison to unmatted one (Kluber *et al.*, 2010). García-Montero (2012) confirmed that this accumulation of calcium oxalate in fungus-matted soils effects a direct and significant impact increasing the soil exchangeable Ca content (4.68 cmol/kg of soil greater inside the fungal mat, in average; $p = 0.027$).

In American Northeastern mixed conifer-hardwood forests, the ECM "rock-eating" activity seems to be particularly important, increasing Ca availability in acidic soils. In these forests, there is a significant ectomycorrhizal weathering activity on apatite (calcium phosphate), and ECM mycelia absorb and translocate P and Ca directly to the host plant, bypassing the soil solution. In other American and European forest ecosystems, this "rock-eating" effect seems to be particularly important increasing Ca availability in acidic soils affected by acid deposition, decreasing the depletion of Ca and reducing the phytotoxicity of Al^{3+} (Blum *et al.*, 2002). Therefore, it is necessary to seriously consider the possibility of assuming that the ECM rock-eating activity, and its impact on soil Ca availability, could be responsible of both positive and negative effects on the host plants.

e.1.6. Additional aspects of the calcium [pH]-induced-chlorosis hypothesis: modifying soil pH in the rhizosphere

The impact of ECMs in the properties of the soil and rhizosphere bearing the host plants can also be mediated by other processes besides increasing soil Ca content (and its salts), as it has already been demonstrated in *Tuber aestivum* brûlés. The geographical distribution of *T. aestivum* brûlés overlaps with that of *T. melanosporum* ones in Mediterranean calcareous areas. However, soil

inside *T. aestivum* brûlés has not shown a significant amount of active CaCO_3 , nor exchangeable Ca (García-Montero *et al.*, 2008b). It has been confirmed the ability of *T. aestivum* to modify the soil biogeochemical status in the brûlé. In preliminary studies, García-Montero (2012) have observed that the soil content of TOC inside the *T. aestivum* brûlé is significantly lower than outside the brûlé (estimated 0.53 times lower). Menta *et al.* (2014) also indicate that the TOC and water contents of soil tended to be significantly (considering $p < 0.1$) lower inside the brûlé in comparison to its outside (estimated 0.21 to 0.27 times lower TOC inside the brûlé). Mello *et al.* (2013) indicated that the lack of vegetation that appears in the brûlés could cause significant changes in light exposure and soil moisture level, leading, in turn, to water stress in the brûlé soil, especially in the summer. Finally, inside *T. aestivum* brûlés, the pH of the soil was significantly higher (0.22 to 0.28 units on average) than outside it.

In a similar way, García-Montero (2012) also described as *Tuber oregonense* Trappe, Bonito and Rawlinson significantly increases soil pH (0.83 units in average) when comparing soils inside versus outside of his productive points, in the acidic soils of *Pseudotsuga menziesii* forests in the North America Pacific Northwest.

Based on the above findings, this Section upgrade a soil-plant nutrition hypothesis proposed above (García-Montero *et al.*, 2009a) associated to some ECM fungus-host plant mutualisms: “some ECM fungal communities could use different metabolic strategies to promote two biogeochemical changes, (i) increase soil Ca, and its salts, availability and/or (ii) increase soil pH in the rhizosphere of their host plants. Both strategies have been observed in carbonated soils as well as in acid soils. This ECM fungal activity could be a case of “ecosystem engineering” because increased pH and Ca^{2+} concentration in the rhizosphere may lead to host plant (i) chlorosis (via the paradox of calcium) and (ii) modification of root-growth patterns (i.e., a significant root tips generation increase). Both, the stimulation to produce root tips and the induced nutrient deficiency, could lead to a greater ectomycorrhizal colonization activity and promote further growth of ECM fungal mycelia, which in turn induces the formation of further content of Ca^{2+} or increase soil pH in the rhizosphere on the basis of a feedback model”.

The impact of liming and soil pH change on tree growth patterns reinforce this soil-plant nutrition hypothesis, that is, liming and/or increasing soil pH stimulate fine root development (Kreutzer, 1995). Tree root systems react to liming improving their ability to explore soil and build an absorption-efficient soil-root interface with extensive fine root development and a greater number of mycorrhizal tips. As a consequence of these effects after liming, it can be understood because (i) limed oak stands show improved mineral nutrition and better growth than untreated control stands (Bakker *et al.*, 2000); and (ii) a single application of dolomitic lime had shown long term effects on tree growth and vigour, remaining evident after 23 years (Long *et al.*, 2011).

Several studies of Ca-induced-chlorosis and modified soil pH impact on plant nutrition (what it has herein been called Ca[pH]-induced-chlorosis) have already reinforced the just proposed new soil nutrition hypothesis. The abundance of exchangeable Ca and active carbonate, and the increased pH level and HCO_3^- presence in the soil insolubilises P, B, Fe, and Mn (Douchafor and Souchier, 1979; Follet *et al.*, 1981; Gaucher, 1971; Loue, 1986; Wild, 1992). Reactions between Ca^{2+} and P may further occur in the soil solution, soil colloids or precipitated calcium carbonate.

In addition, lime has been reported to increase the mineralization of soil organic P in a good number of other studies, and it is thought to largely depend on the relative size of easily mineralisable pools of C and P within soil. However, the mechanisms by which lime affects the chemical reactions of P in the soil are still unclear (Higgins *et al.*, 2012). Furthermore, an overabundance of Ca^{2+} and HCO_3^- leads to a deficiency in Mg^{2+} availability for the plants (Follet *et al.*, 1981) and generates secondary calcites, which further immobilize Mg^{2+} (magnesites) (Douchafour and Souchier, 1979). Moreover, an increased soil pH significantly affects the absorption of microelements by plants. An increase in the soil pH also reduces the assimilability of Al, Co, Cu, Fe, Zn, and Mn (Loué, 1986). When the pH is raised in the soil, Mg^{2+} may be transformed into a non-exchangeable cation (Wild, 1992), leading to the formation of insoluble calcium borates (Follet *et al.*, 1981). The effects of pH and lime-induced chlorosis on the growth of pines, firs, apples, grapevines, peanuts, groundnuts, pear rootstocks, chickpeas, bush beans, runner beans, soybeans, oilseed rapes, lupines, and other plants have been reviewed elsewhere (García-Montero *et al.* 2009a; 2012).

Therefore, the processes associated with Ca[pH]-induced-chlorosis have a negative effect on the host plant because they reduce the plant's capacity to perform photosynthesis, which limits its growth and ability to further explore the terrain searching the limiting nutrients. As a consequence, the plant loses its ability to compete and survive. However, these dynamics would be counteracted if there were an increase in the mycorrhization ratio of the plant suffering chlorosis due to the fact that the mycorrhizal hyphae have a great ability to capture and exchange nutrients.

Monfort-Salvador *et al.* (2015) reviewed the impact of calcareous amendments on the soil-ECM fungal-host plant system. They assessed: (i) the influence of Ca^{2+} salts of biological origin on ECM fungal communities; (ii) the impact of Ca^{2+} on the growth patterns of the host tree roots; (iii) the importance of rock-eating processes associated to the presence of ECM fungi for the host plants; and (iv) the impact of the soil Ca^{2+} cycle (associated to litterfall) on ECM fungal activity. Tree root systems react to liming improving their ability to explore the soil through an enhanced absorption-efficient soil-root interface with extensive fine root development and a greater number of mycorrhizal tips.

Local soil nutrient status influences ECM colonisation, that is, low P and K availability favours ECM colonisation (Püttsepp *et al.*, 2004). In addition, liming modifies the ectomycorrhizal community structure (Jonsson *et al.*, 1999), increasing the relative frequency of certain ECM morphotypes (Erland and Soderstrom 1991) and significantly incrementing the total number of ECM tips (Bakkera *et al.*, 2000); but decreasing the relative proportion of smooth in favour to hairy types of ECM. The ECM species that are present in limed plots are generalist, or at least well-known as good competitors, thus replacing acidophilic and stressed ECM species. Moreover, the modification of soil chemical properties (pH, and Ca-Mg contents versus total free Al and Fe concentrations) has been shown as a stronger factor for ECM community structuring than for the host tree performance (Rineau *et al.*, 2010a). On the other hand, ECM mantles of *Lactarius subdulcis* ECM have addressed significantly higher Ca, Mg, Mn, K, Si, Al and Fe contents in limed plots. Furthermore, the storage role of ECM mantles can be interpreted in two different ways: i) as a detoxification pathway for Al or heavy metals, and ii) as a potential

very rich nutrient resource for the fungus, which can ultimately benefit the host tree (Rineau *et al.*, 2010b). In short, the potential capacity of nutrient uptake by forest trees is a function of site fertility, fine roots density, and the presence of mycorrhizal symbionts (Bakker *et al.*, 2000). In this regard, the overall effect of liming is complex: it directly acts modifying soil physical and chemical properties, as well as indirectly by modifying root morphology, symbiotic status, and absorption efficiency (Blaise and Garbaye, 1983).

Monfort-Salvador *et al.* (2015) highlighted that a greater knowledge of the cause-effect relationships between Ca and the soil-ECM-plant system in Mediterranean and temperate forests could have positive repercussions on reforestation projects, actions associated to soil management, the production of ECM edible fungi, and, ultimately, the overall economy of different rural zones in these forest areas.

In summary, ECM fungal populations and communities could be acting as “ecosystem engineering” agents at a global scale, changing soil properties in the rhizosphere of the host trees in order to cause them stress (e.g., via the paradox of calcium); therefore, modifying tree growth patterns and driving mycorrhization processes and nutrient exchange mechanisms. This, in turn, would make the host plants more dependent on the fungi to obtain nutrients. However, simultaneously, host plants will be more resistant to biological and environmental stresses (including climate change), and in the case of agriculture, host plants will be less dependent on fertilizers and irrigation.

The proposed soil nutrition hypothesis associated to truffle culture in calcareous Mediterranean areas (García-Montero *et al.*, 2009a) could be expanded, renovated, and/or reworded in a 'new approach or idea' that could be summarized as: “ECM fungi populations act as farmers growing trees”, which is applicable to boreal, temperate, and Mediterranean climate areas. Based on these findings, it is necessary to develop further research on a “new conceptual ECM framework for stable mycorrhizal associations” based on: (1) the evolutionary dynamics and the stability of mycorrhizal associations; and (2) the ecological dynamics and the stability of mycorrhizal associations (García-Montero, 2012).

e.1.7. Evolutionary dynamics and stability of mycorrhizal associations

Mutualistic partners generally share an evolutionary interest in protecting their mutualism from potential “cheaters”. Each one might evolve highly specific, and sometimes exaggerated characters to protect the interaction (e.g. the complex recognition signals exchanged by legumes and *Rhizobia* before infection). Moreover, provided the demonstrated variability of the effectiveness of mutualistic symbioses, and the problematic proliferation of cheating symbionts within mutualisms, mutualistic partners are expected to evolve to a greater specificity in response to an increasing diversity within their mutualists (Bever, 2002; Thrall *et al.*, 2007).

In the evolution of mycorrhizal associations, the emergence of new defensive properties has been reported to occur (= symbiogenetics), causing interesting interactions. Fungi may have facilitated the emergence of thick parenchymatous tissues in plants, whereas their charophycean ancestors had simple tissue morphology. Induced hyperplasia of organs may

enhance protection and increase opportunities for new adaptations. Particularly, the abundance of parenchymatous tissue may be related to increasing the storage capacity of photosynthates, nutrients and water; as well as to promote the refilling mechanism of the water transport pipeline, thus reducing hydraulic conductance losses (Taiz and Zeiger, 2015). In addition, the production of systemically-induced defensive compounds could have represented a significant advantage for primitive land symbiotic associations, which were presumably slow-growing, and maybe less tolerant to pathogens attacks and livestock grazing when pathogens and grazers appeared later in evolution. Furthermore, the stimulation of the metabolism of phenylpropanoid may have given evolutionary opportunities for the synthesis of lignin, a novelty of major consequences for vascular plants (Selosse and Le Tacon, 1998). On the other hand, there have not been described parallel mechanisms protecting fungi from their exploitation by host plants (Hibbet *et al.*, 2000).

Mycobiont-photobiont specificity and diversity asymmetries, the exceptions to the generalized mycorrhization of terrestrial *Plantae* “rule”, and the emergence of defensive properties along mycorrhizas evolution, are consistent with the premise of mutualisms ‘best viewed as a reciprocal exploitation that nonetheless provides net benefits to each partner’ (Wilkinson, 2001). Moreover, ectomycorrhizal transitions appear to have taken place between mutualists and free-living forms, rather than between mutualists and parasites. Wilkinson (2001) indicates that the repeated gains and losses of mycorrhizal states reveal a dynamic evolutionary situation, rather than mycorrhizal associations being the stable result of the evolution of a reduced virulence in ancient parasitic relationships. This author highlights that the mycorrhizal-mutualism evolution seems to be more complex and dynamic than what many authors have suggested. In this concern, current analyses attempt to explore the question formulated by Wilkinson (2001): *How can mycorrhizal mutualisms ever persist?*

Hibbet *et al.* (2000) compared the mycorrhizal and free-living basidiomycete phylogeny to determine the evolutionary stability of mycorrhizas, concluding that mycorrhizal symbionts with diverse plant hosts have repeatedly evolved from saprotrophic precursors, also stressing that multiple reversals to free-living conditions happened (suggesting 15 to 16 transformations in average = 9.0 gains of ectomycorrhizal condition + 6.3 losses of this state in basidiomycetes). These findings confirm that mycorrhizas are unstable and evolutionarily dynamic associations. These authors also explained that several factors are thought to promote the stability of mutualisms, including compensation mechanisms that limit exploitation of one partner by the other; strictly vertical transmissions of symbionts between host generations; asexual reproduction of symbionts; and one-to-one correspondence of host and symbiont species. Based on these criteria, Hibbet *et al.* (2000) explain that the apparent instability of ectomycorrhizas is not surprising.

Different authors have proposed some answers to questions on mycorrhizal evolutionary stability. Hibbet *et al.* (2000), for example, highlighted the importance of sexually-reproduced mycobionts and dispersing spores independently to plant propagules, meaning that most ectomycorrhizal plants draw partners from a pool of potential symbionts. These authors also remark that a factor that might promote stability to ectomycorrhizal associations is the putative dependence of the fungus on the photosynthates produced by the host. This fact could explain

why some ECM fungi have retained genes coding enzymes that are used to degrade the plant cell wall, which may have facilitated repeated evolutionary reversals to saprotrophy. In addition, Wilkinson and Sherratt (2001) further proposed including retaliation against exploitation by the partners, the local symbiont dispersal as pseudovertical transmission, or the partner choice for compatible symbionts within a “biological market”. Finally, using contemporary niche theory, Peay (2016) has recently indicated that a niche-based view of mutualism may also help explaining the stability of mutualisms, even in the absence of clear benefits.

e.1.8. Ecological dynamics and stability of mycorrhizal associations

Symbiotic mutualistic and parasitic associations are very important as drivers of ecophysiological and evolutionary processes. Both contrasting association types often exhibit similar features, including asymmetries (e.g. in body size), deep partner phylogenetic separation, and high degrees of genetic specialization. These elements are indicative of the intimate nature of both types of association, where the interacting organisms are not generally injured or even killed immediately, but for which the benefits of “cheating” or evolving heightened virulence leads to the selection of adaptations in the affected partner to resist, defend itself, or impose sanctions to the other one.

As described above from an ecological point of view, mycorrhizal (and lichenical) mutualistic associations show a remarkable functional asymmetry, where interacting partners display complementary adaptations to live on land.

On the other hand, there is evidence that mycorrhizal mycelia can link plants together in a Mycorrhizal Network (MN) that appears to be ubiquitous in nature, and facilitate fungal colonization and interplant transference of compounds. Simard *et al.* (2012) reviewed current knowledge on mycorrhizal networks and concluded that:

- (i) The facilitation of mycorrhizal colonization and growth of autotrophic plants by MNs have been demonstrated in a wide range of ecosystems (forests, woodlands, and grasslands). MNs facilitate the transference of C, nutrients, water, defence signals and allelochemicals, but it is not well understood how they affect autotrophic plant growth and health.
- (ii) MNs have been shown to influence plant interactions (facilitation and competition), forest regeneration, and plant dominance; however, their net effects on ecosystem diversity, productivity or stability are poorly understood. In some ecosystems, MNs appear to play important roles in facilitation and competition processes that contribute to stabilize them; but, in other ones, MNs can provide pathways for positive feedback that may destabilize ecosystems.
- (iii) MNs also appear to affect the composition, interactions, and diversity of ECM communities.
- (iv) From a global perspective, it is even suggested that MNs play an important role in ecosystem functioning as complex adaptive systems, being potentially able to cause global change feedback.

Increasing our knowledge on MN structure and function may lead to deeper understanding of the stability and evolution of ecosystems; thus providing new alternative theoretical approaches that may lead to improve conservation, management, and exploitation of the ecosystems (Simard *et al.*, 2012; Thrall *et al.*, 2007). The consideration of this approach and Wilkinson's (2001) question "How can mycorrhizal mutualisms ever persist?" lead to the necessity of evaluating mycobiont-phototroph interactions taking into account the environmental quality and biotic complexity of ecosystems, aiming to explain current ECM domination in temperate, boreal, and Mediterranean areas.

As described above, heterogeneous selection pressures can alter the strength and net fitness effect of mutualistic associations, causing their interactions to become parasitic, 'controlled parasitic', or at least cheating, under certain conditions (Thrall *et al.*, 2007). Therefore, the successful spread of mycorrhizas (and mutualists in general) can shift the environment from low to high productive, thus attracting other parasites and cheaters. Moreover, an enhanced competitiveness of mutualistic hosts might also lead to decrease community diversity. Both processes should favour exploiters or more-virulent host-specific pathogens, which might, in turn, self-limit the spread of the mutualist and its partner. Furthermore, in high-quality productive environments favouring antagonistic interactions, some authors have predicted a shift in the relative abundance of provider mutualisms (providing some essential, reduced, or missing components) favouring protector mutualisms (against natural enemies); whereas the opposite phenomenon is expected in low-quality environments (Hochberg and van Baalen, 2000; Thrall *et al.*, 2007).

In this regard, there is good support for the expectation that the provider in mutualisms, such as mycorrhizas, decline with increasing environmental quality. For example, N fertilization leads to the proliferation of mycorrhizal (and rhizobial) strains that are less beneficial to plants (Corkidi *et al.*, 2002; Johnson, 1993; Thrall *et al.*, 2007). These predictions are also supported by analytical models, which show that beneficial symbionts are favoured when host productivity is low, whereas more virulent symbionts, such as parasites, tend to dominate when the host productivity is high (Hochberg *et al.*, 2000; Thrall *et al.*, 2007).

Therefore, Thrall *et al.* (2007) proposed the integration of these ideas suggesting a synthetic theory of the evolution of host-symbiont interactions as a function of environmental quality and biotic complexity. This synthesis provides the basis for developing testable hypotheses and possible key characteristics regarding the evolution of ECM symbiotic partners. From a more applied perspective, the understanding of (1) ECM (and other symbiotic) communities' successional dynamics, and (2) the interactions among environmental quality, ecological, and co-evolutionary processes, will be significant steps towards addressing restoration ecology, rebuilding ecosystem biodiversity, and promoting sustainable forest management.

e.1.9. A new conceptual ectomycorrhizal fungal framework for stable mycorrhizal associations: controlled parasitism?

To the light of the described evolutionary framework regarding mycorrhization, it is possible to conclude that (Grube and Hawksworth, 2007; Selosse and Le Tacon, 1998; van Schöll *et al.*, 2008):

- (i) Recent molecular studies suggest a complicated phenotypic evolution in lichenized fungi and ancestral lichenization reconstruction that addresses the following question: “What is the role of lichenization and mycorrhization in the evolution of fungi and land *Plantae*?”
- (ii) Lichen definition includes concepts of “ecologically obligate” and stable mutualisms between an ex-habitant fungal partner and inhabitant photobiont populations emphasizing the mycobiont-photobiont 'extracellular relationship'.
- (iii) The diversity of lichenization “*sensu lato*” and mycophycobiosis processes, the older fossil plant records, the generalized current pattern of land plant mycorrhization, and the significant evolutionary patterns of non mycorrhizal (NM) plants show that fungi and land *Plantae* life-style definitions may be “flexible in relation to evolutionary pathways”. In this regard, the review of evolutionary asymmetries between mycobiont-photobiont patterns of specificity, dependence, and diversity are key elements in understanding ecology, distribution, and diversity of mutualistic associations; and allows the proposal of “*some parallel patterns between lichens and ECMs*”.
- (iv) The advantages of associating with specialist mycorrhizal fungi are unclear for the host plants, because the cost of associating with a specialist partner is greater than that of associating with a generalist. From the plant’s perspective, photobiont specialisation may lead to a decreased functional compatibility with the mycobiont (Den Bakker *et al.*, 2004). Therefore, it is possible to assume that the evolution of some nutrient-uptake mechanisms of photobionts provided a selective advantage due to the increased nutritional efficiency related to the mycobiont’s association cost. However, the analysis of the costs and benefits of root nutrient-uptake mechanisms is complex because mycorrhizal plants remain dominant in most habitats, whereas most NM plants are marginalized to wet, saline, dry, disturbed, cold habitats, or extremely infertile soils, where plant productivity is low and the inoculum of mycorrhizal fungi would be scarce.
- (v) Mutualistic partners generally share an evolutionary interest in protecting their mutualism from cheaters or other partners showing an evolved heightened virulence (with a tendency to parasitic or pathogenic interactions). Moreover, provided the demonstrated variability in the effectiveness of mutualistic symbioses, and the problematic proliferation of cheating symbionts within mutualisms, it has been expected that hosts would evolve a greater specificity in response to the increasing diversity within their potential mutualists. The emergence of defensive properties in the ECM host plants are consistent with considering that mutualisms are ‘*best viewed as a reciprocal exploitation that nonetheless provides net benefits to each partner*’. Therefore, previous

analyses attempt to explore the question addressed by Wilkinson (2001): How can these mycorrhizal mutualisms ever persist?

(vi) Several factors are thought to promote the stability of mutualisms; however, following these stability criteria, Hibbet *et al.* (2000) highlighted the apparent instability of ectomycorrhizas.

(vii) Plants under mycorrhizal symbiosis may not obtain a significant improvement from a particular situation yet, the symbiosis appears stable in evolutionary terms because mycorrhizas represent a 'life insurance' in the sense that mycorrhizal plants tolerate better any environmental stress they may experience.

(viii) Mutualistic associations are very important as ecological and evolutionary process drivers. In this concern, there is evidence that ECM networks that appear to be ubiquitous in nature play an important role in ecosystems functioning as complex adaptive systems, potentially able to cause global change feedback. Therefore, it is necessary to unravel the understanding of mycobiont-photobiont interactions considering environmental quality and biotic complexity in temperate, boreal, and Mediterranean ecosystem areas.

(ix) Therefore, it is necessary to “*search testable hypotheses and possible key-concepts*” regarding the evolution of host-symbiont interactions as a function of environmental quality and biotic complexity. These models would provide the basis for further developing the understanding of ECM ecological and co-evolutionary processes, as well as to reinforce the protection of ecosystems and sustainable forest management policies.

In summary, the present thesis proposes a “new conceptual ECM framework for stable mycorrhizal associations”, where ECMs change the soil environment to stress phototrophs (*i.e.*, via the 'Ca paradox', 'calcium[pH]-induced chlorosis' and the 'control of the soil TOC' content) acting in a similar way as lichenic 'controlled parasitism' in order to benefit themselves. These ground-breaking ideas can be summarized as follows: “ECM, and even AM, fungi act as farmers growing plants”, which leads to important practical implications for ecology, agriculture, and forest management at the global scale.

e.1.10. The oxalate-calcium carbonate pathway in the interactions between fungi and oxalotrophic bacteria

Verrecchia *et al.* (2006) analysed the interactions between fungi and oxalotrophic bacteria, remarking the biogeochemical impact of fungi on many elemental cycles. Fungi are not only biologically important as saprophytes for the recycling of organic matter, but also play a geological role by excreting notable amounts of organic acid, among which oxalic acid is particularly important, and can be a source of secondary calcium carbonate in the soil. In contrast, calcium oxalate crystals precipitate and may constitute almost the 25% of soil hyphae and rhizomorphs dry weight in some ecosystems (Cromack *et al.*, 1977). Verrecchia *et al.* (2006) demonstrated that oxalotrophic *bacteria* could effectively use these abundant oxalate crystals as C, electron, and energy sources by oxidizing them into calcium carbonate. This secondary

CaCO₃ may then accumulate modifying soil conditions and increasing pH in the soil solution. Therefore, when associated with plant biomineralisation, fungi and bacteria contribute to long-term C storage by transforming half of the organic C from oxalate (or low-molecular-weight organic acids) into carbonate, with longer residence time in the soil than organic substrates. The other half is released into the atmosphere as CO₂. In conclusion, because oxalate salts are of organic origin, the oxalate-carbonate pathway represents a potentially major C sink, and probably acts as a regulator of atmospheric pCO₂.

Moreover, the oxalate-calcium carbonate pathway associated to these interactions probably also acts as a regulator of soil Ca²⁺ content and pH level in the rhizosphere environment of mycorrhizal-plant systems. This could have a major impact at the global scale. Therefore, it may be necessary to further develop another new hypothesis on mutualistic symbiotic relationships between mycorrhizas and oxalotrophic *bacteria* associated to the “paradox of calcium” because the interaction of both soil microorganisms could increase their capacity to control host plants (based on the described Ca[pH]-induced-chlorosis).

On the other hand, calcium oxalate content showed a significant negative relationship with agricultural yield and crop quality attributes, emphasizing that increasing the levels of oxalate in the soil had a detrimental effect on soil microbial variables (Laxminarayana, 2016). In summary, oxalotrophic *bacteria* may have positive impacts on agricultural systems, because they reduce the level of soil calcium oxalate, which benefits agriculture; and simultaneously increase the content of calcium carbonate, which stimulates the mycorrhization of plants. Therefore, this particular deserves conducting further research.

e.1.11. Interactions between soil fauna and mycorrhiza-host plant systems associated to the “paradox of calcium”

Other relevant patterns of atmospheric CO₂ sequestration as soil carbonate (SIC) could be led by earthworms’ activity (i) producing calcite granules and (ii) the soil aeration generated by their tunnels. Spherical soil concretions up to the millimetric diametric size and composed of calcite crystals are found in soils containing earthworms. These calcite granules ranging from single crystals to aggregations up to 2.5 mm in diameter are produced by the calciferous glands present in all the species of the *Lumbricidae* family (Briones *et al.*, 2008a; Canti and Pearce, 2003; Lee *et al.*, 2008; Versteegh *et al.*, 2014; Wiecek and Messenger, 1972). Jongmans *et al.* (2001) found earthworm calcite granules down to 50 cm deep in the soil profile. Briones *et al.* (2008a, 2008b), Gago-Duport *et al.* (2008), and Lee *et al.* (2008) described the microstructural transformations involved in CaCO₃ precipitation from the calciferous glands of earthworms. Furthermore, these biomineral granules are stable. In particular, Canti (2009) reported that earthworm calcite granules have been found in archaeological soils and ancient sediments.

Coleman *et al.* (2004), Briones *et al.* (2008b) and Versteegh *et al.* (2014) indicated that the calciferous glands of earthworms could provide a mechanism for regulating CO₂ and Ca²⁺ in their blood and tissues. Canti (2009) showed that the C present in calcite granules mainly came from dietary inputs (litter), old soil C (soil OM), and from atmospheric CO₂ (although not in quantities larger than about one third of the total C in the granule), but very little quantity seemed to come

from soil CaCO_3 . Therefore, earthworms could lead to the promotion of significant amounts of secondary precipitation of CaCO_3 in the soil, whose C comes from the atmospheric CO_2 that is fixed by plant photosynthesis (in the same way as the fungal-bacteria oxalate-carbonate pathway). In any case, the use of fresh OM, soil OM, and atmospheric CO_2 to fix C in the earthworms' calciferous glands as granules formed from calcite crystals would result in a net soil C fixing (as SIC) and the recarbonation of soil. In addition, Fraser *et al.* (2011) conclude that earthworms truly synthesize CaCO_3 and they do not merely recycle ingested material.,

García-Montero *et al.* (2010; 2013) confirmed Canti's (2009) laboratory results in truffle orchards. They found a significant increase of carbonate contents (and a higher pH value) in earthworm casts associated to calcite granules production that cannot be just explained by the existing levels of original carbonate in weakly calcareous soils. Chan (2003) showed that the pH of casts is significantly higher (pH >6) than that of the bulk acid soil material (pH=4.0) that was used in laboratory experiments. Wiecek Messenger (1972) also indicated that *L. terrestris* casts are less acid than the ingested food, and proposed that the weathering of earthworm calcite granules is partly responsible for pH values greater than 7.0 in the A₁ horizons of acid soils under forests covering some American woodlands. Elsewhere, Lambkin *et al.* (2011) reported from a laboratory experiment that an increased soil pH could promote a higher production of calcite granules by *L. terrestris*. In consequence, García-Montero *et al.* (2013) pointed to a feedback loop of varying intensity with the different types of soils and earthworms.

Versteegh *et al.* (2014) addressed that, the observed increase of CaCO_3 production rate with temperature can be explained by an increase in their metabolic activity. Lee *et al.* (2008) highlighted that earthworms produce calcite granules in sufficient volume to have a measurable impact on soil C cycling (as SIC). Lambkin *et al.* (2011) estimated that annual granule production by *L. terrestris* lies in the range of 18-3,139 mol- CaCO_3 ha⁻¹ yr⁻¹, with an average value of 438 mol- CaCO_3 ha⁻¹ yr⁻¹. These authors pointed out that their values are higher than those reported by Wiecek Messenger (1972), who calculated that excreted CaCO_3 could contribute up to 11 mol-calcite ha⁻¹ yr⁻¹ in forests on acid soils; which are also higher than the results addressed in Canti (2007), who estimated *L. terrestris* production rates of more than 0.02 mol-calcite day⁻¹. However, Lambkin *et al.* (2011) indicated that there is significant variation in the mass of granules produced by different earthworms exposed to the same soil, and explain that a large proportion of this variation should reflect biological variations between individuals. Recently, Versteegh *et al.* (2014) reported that *L. terrestris* is a major CaCO_3 producing species in temperate soils. Production rates range from 0.8 to 2.9 mg earthworm⁻¹ day⁻¹. Considering 1.9-61.8 individuals m⁻², this equates to precipitating 2-261 kg C ha⁻¹ yr⁻¹, which represents a potentially significant contribution to carbon sequestration. These authors also highlighted the on-going debate on whether earthworms increase soil greenhouse-gas emissions or contribute to carbon sequestration, as well as on the timescale and nature of the experiments that would be required to determine this particular (Zhang *et al.*, 2013). Particularly, Versteegh *et al.* (2014) showed that it is likely that, at higher temperatures and atmospheric $[\text{CO}_2]$, CaCO_3 earthworm synthesis will increase. As granules can last in soil for >300,000 years, the potential sequestration of C in the form of CaCO_3 is on a longer-time scale than roots and soil organic matter, for example. On the other hand, the precipitation of soil CaCO_3 increases when the partial pressure

of CO₂ decreases in soil due to the aeration that is generated by the activity of earthworms themselves. In this regard, Callot *et al.* (1999) concluded that besides the contribution made by the respiration of roots and microorganisms, pCO₂ in the soil atmosphere depends on soil aeration, which is strongly conditioned by earthworms.

On the other hand, Verrecchia *et al.* (2006) showed that the accumulation of oxalate crystals by fungi may also produce unexpected consequences associated to soil fauna, because calcium oxalate can be disseminated inside soil by the action of oribatid mites. Such mites feed on calcium oxalate crystals produced by fungi, and then re-precipitate the mineral in their hardened cuticles (Norton and Behan-Pelletier, 1991). In fact, they are thought to process a significant portion of the Ca²⁺ pool in some ecosystems (Gist and Crossley, 1975). Therefore, it would be necessary to draw additional models on a mutualistic symbiosis existing between mycorrhizas and soil fauna (such as earthworms and mites) associated to the “paradox of calcium” because the interaction of these organisms could increase the control over host plants based on the described Ca[pH]-induced-chlorosis.

Menta *et al.* (2014) furthermore indicated that soil fauna could have an impact on fungal growth, dispersion and fruit body production, altering fungal fitness, and therefore, their combativeness in interaction with other soil microorganisms (Rotheray *et al.*, 2011). Several authors have described some mechanisms explaining the impact of soil microarthropods on fungal communities. For instance, Hanlon and Anderson (1979) reported that microarthropod’s feeding activity can exert a strong differential effect on fungal and bacterial populations; as well as some reviews on AM fungal and soil fauna interactions suggest that *Collembola* have the potential to restrict mycorrhizal functioning in the field (Fitter and Garbaye, 1994; Fitter and Sanders, 1992). On the other hand, Menta *et al.* (2014) addressed that some ECM fungi, such as *Tuber aestivum*, may modify soil fauna communities; that is, *Folsomia* sp. showed higher abundance inside the soil of the *T. aestivum* brûlé than in the outside.

e.1.12. Liming effects on arbuscular mycorrhizal fungi in agriculture

In order to provide a sustainable, safe and secure food supply chain, agriculture depends more and more on ecosystem services provided by different organisms. Among the wide range of natural communities that can be exploited to enhance biological interactions in sustainable food production, arbuscular mycorrhizal (AM) fungi are the more relevant (Yang *et al.*, 2016). In order to magnify the benefits derived from the AM fungal symbiosis relationship, ameliorating soil conditions looking for a better fitting of these fungi is necessary. Among agricultural practices, calcareous amendments are currently being used more frequently especially in acidic lands. For this reason, García-Montero (2012) and García-Montero *et al.* (2017) reviewed the impact of liming on AM fungal populations, thus opening the floor to future studies aiming to (1) better know the cause-effect relationship between liming and the composition of AM fungal communities, (2) address the suitable lime dose, and (3) clarifying the overlapped interaction with other soil organisms, such as N₂ fixation *bacteria*, and its role in the nitrogen cycle.

Microbial responses to lime may vary with soil type, including the kind and amount of organo-mineral colloids (Kennedy *et al.*, 2004). First, symbiotic organisms, including AM fungi, are

sensitive to short-term liming (Johnson *et al.*, 2005a; 2005b). While fungi population is reduced after liming (Ivarson, 1977), although Gomes *et al.* (2015) found an increment in the frequency of endophytic *Ascomycota* and other non-AM fungi in the limed soil. In addition, fungal fatty acids may increase by a 39% in a limed pasture according to Bardgett *et al.* (1996).

In general, AM fungi are sensitive to addition of fertilizers as well. They might experience shifts in their community composition (Egerton-Warburton and Allen, 2000) and stimulation of external mycelial development (Eom *et al.*, 1999); but their diversity will probably decline when the intensity of agricultural inputs increases (Bhadalung *et al.*, 2005; Oehl *et al.*, 2010; Troeh and Loynachan, 2009). The increase of the abundance of AM fungi mediated by fertilisation might be attributed to the increased P supplied by these treatments (2005a; 2005b). Particularly, fertility management including Ca sources appeared to improve root fresh weight colonized by AM fungi (Johnson *et al.*, 2005a; 2005b). This author also found that AM fungal colonization reached the 37% of the root length when lime and nitrogen fertilizers were applied together. On the other hand, Bradley *et al.* (2006) observed a depletion of AM fungi after N fertilising treatment.

Several studies confirmed the negative impact of lime on AM fungal community richness (Gomes *et al.*, 2015), species diversity (Guo *et al.*, 2012), and phylotypes presence (Guo *et al.*, 2012), especially when applied in long-term trials (Guo *et al.*, 2012; Gomes *et al.*, 2015). To the best of our knowledge, very few authors have reported the absence of liming effects on fungal communities (e.g., Da Mota *et al.*, 2008).

AMF seem to show different reactions to lime amendments. All three possible effects - positive, negative and neutral- were observed. Beginning with negative impacts, Raznikiewicz *et al.* (1994) demonstrated the reduction of AM fungal spores production in a Swedish limed grassland. In addition, high levels of limestone application can also depress root colonization by *G. mosseae* and *G. margarita* (Yawney *et al.*, 1982; Siqueira *et al.*, 1984); and Gomes *et al.* (2015) found that the richness of the AM fungal operational taxonomic units was higher in unlimed soil. Furthermore, AM fungal phylotypes were shown lower under limed treatment than in bulk unlimed soil. Apparently, long-term application of lime might influence the colonization of AM fungi and, thus, the sustainability of farming systems on acid soils (Guo *et al.*, 2012).

The effects of lime on AM fungi performance mainly depend on the variation among AM fungal species and isolates (Bhadalung *et al.*, 2005; Dumbrell *et al.*, 2010; Göransson *et al.*, 2008; Postma *et al.*, 2007; van Aarle *et al.*, 2003). However, some authors observed a neutral or a non-significant impact of lime on AM fungi. Shah *et al.* (1990), for example, showed (using two different methods) that no significant change was induced in the fungal population either after short- or long-term liming application. In addition, Sano *et al.* (2002) reported that different AM fungi equally responded and similarly reproduced when CaCO_3 was applied to the soil.

Regardless of the few studies that observed the little or no influence of liming on AM fungal colonization of crop plants (Robson and Abbott, 1989; Wang *et al.*, 1993), many authors have reported decisive beneficial effects. For example, liming has been reported to increase the AM fungal colonisation of barley (*Hordeum vulgare* L.) roots (Hamel *et al.*, 1996). Furthermore, the addition of 0.16 to 0.33 g of CaCO_3 per kg of soil encouraged the rate and extent of *Acaulospora*

laevis colonisation (Sano *et al.*, 2002). In addition, AM fungi presence was recommended by Ortas *et al.* (2011) to counteract Zn and P deficiency in clay and lime soils; and Read and Birch (1988) reported the rapid AM fungal colonisation of *Plantago lanceolata* calcareous grasslands. In particular, the addition of 75 mg L⁻¹ of Ca in a nutrient film resulted in a better corn root colonisation by AM fungi than when higher concentrations were applied (Hewitt, 1981). Moreover, the germination of *G. mosseae* was improved by 58% in a limed soil (Siqueira *et al.*, 1984). Dolomitic lime improved the mycorrhizas formation of *G. mosseae* and *G. margarita* (Siqueira *et al.*, 1984). Similarly, Guo *et al.* (2012) reported that lime application increased the intensity and abundance of mycorrhizal colonisation, but significant differences were noticed among plant species. In general, hyphal length and AM fungal colonisation extent were improved by lime application, which may be vital for conservation management systems, such as no-till farming (Schneider *et al.*, 2011). In other studies reporting beneficial effects, the configuration of AM fungal populations and the number of propagules were positively shifted in limed soil according (Sano *et al.*, 2002); as well as AM fungal infection was extended in members of the *Cyperaceae* and *Juncaceae* families in limestone grasslands (Read *et al.*, 1976). Furthermore, significantly greater amounts of roots were found to be colonised by AM fungi in limed plots (Johnson *et al.*, 2005a; 2005b). In fact, most AM fungi are well-adapted to the higher soil pH level induced by liming (Bradley *et al.*, 2006), which may explain the mycorrhizal colonisation enhancement after lime application. It can therefore be concluded that, in most cases, lime tends to improve AM fungal spore germination, colonisation, and overall performance.

In summary, when calcareous amendment has been applied to crops, negative and positive effects to the growth and development of their associated arbuscular mycorrhizas have been reported. However, this review allows concluding that, in the majority of cases, liming improves the colonisation of AM fungi, although their diversity (understood as species richness) may be reduced.

The simplest way in which calcareous amendments might interfere with the symbiotic relationship between plants and AM fungi is by the manipulation of the availability of nutrients. In general, adding high doses of CaCO₃ decreases the availability of nutrients, especially phosphorus, although this is not always the case. Abbott and Robson (1985) found that lime might decrease plant growth inducing the inhibition of the growth of AM fungal *hyphae* in alkaline soil and, thus, mycorrhizal root length. In addition, lime can reduce root weight when AM fungi have not been introduced in the system but, at the same time, it might increase the shoot:root ratio (Guo *et al.*, 2010). This authors also reported a reduction in the leaf:stem ratio under liming treatments, and an increment in the Ca concentration of the shoot tip when AM fungi were applied. In fact, a higher ionic content (Ca²⁺, particularly) in the cells might prevent water losses by osmoregulation (Taiz and Zeiger, 2015) and limiting the expression of aquaporins (e.g. Gerbeau *et al.*, 2002), which hinders the cell to cell water transport. In addition, N concentration in Lucerne (*Medicago sativa* L.) leaves was higher growing on limed soil not holding AM fungi, while P concentration was lower on limed soil bearing AM fungal inoculation (Guo *et al.*, 2010). These findings are consistent with those of Sano *et al.* (2002), who found that the inoculation of *A. laevis* and *Glomus invermaium*, in addition to CaCO₃ application, decreased

the dry weight of both shoots and roots of host plants; and the shoot dry weight of subterranean clover (*Trifolium subterraneum* L. cv Seaton Park) was gradually decreased along the increasing application of lime regardless the associated fungal species. Furthermore, liming increased the AM fungal colonisation of corn, *Brachiaria decumbes* (Siqueira *et al.*, 1984; 1990) and *Stylosanthes guianensis* (Lambais and Cardoso, 1990), and, moreover, increased the ability of AM fungi to enhance the efficiency of P use by soybean (Maddox and Soileau, 1991) and *Stylosanthes guianensis* (Lambais and Cardoso, 1993). In fact, lime can influence the development of mycorrhizal associations both, by decreasing fungal propagules growth, and by decreasing the fungal colonisation extent of root tissue (Siqueira *et al.*, 1984).

Therefore, developing further research is required to understand how soil CaCO₃ availability and liming practices could interfere with AM fungal symbiotic relationships hypothetically exerting nutrient stress; as well as to establish analogies and differences with the model based on Ca(pH)-inducing chlorosis to the host plants that has been hypothesized before for ECM-plant systems.

e.1.13. Liming effects on nitrogen-fixing bacteria in agriculture

The development of an indigenous AM fungal community stimulates other microbial communities (van Aarle *et al.*, 2003). In particular, Villegas and Fortin (2002) showed that such interactions may increase P solubility. In fact, the optimum growth of leguminous plants is usually dependent on symbiotic relationships with AM fungi and N₂-fixing bacteria (NfBs) (Xavier and Germida, 2003). In addition to AM fungal stimulation of nutrient uptake and water supply to the host plant, these relationships promote nodulation and N₂ fixation as well (Redecker *et al.*, 1997). Likewise, some *bacteria* species enhance AM fungal formation (*mycorrhiza*- helper *bacteria*) and are a key to be considered in future research on AM fungi (Mediavilla *et al.*, 2016).

The mixed inoculation of both plant-growth-promoting rhizobacteria and AM fungus improved grain quality, plant vitality, and nutrient uptake; as well as it showed a dramatic increase in the grain yield of wheat (Khan and Zaidi, 2007). Total *Rhizobium* nodule weight was a 146% higher when the AM fungus was introduced, leading to a 53% increase in the growth of aerial plant parts. However, the infection rate with AM fungal mycelium was not affected by the inoculation with *Rhizobium* or the addition of lime (Guo *et al.*, 2010).

Legumes face ineffective nodulation in low pH soils (Munns, 1986), although Guo *et al.* (2010) suggested that liming together with the double inoculation of plants with AM fungi and *Rhizobium* may solve this problem. For these reasons, García-Montero *et al.* (2017) also reviewed the impact of liming on N-fixing bacteria (NfBs) in agricultural systems. Lime increased N₂ fixation from 19 kg N ha⁻¹ produced by the unlimed legume field up to 35 kg N ha⁻¹ when limed (Peoples *et al.*, 1995). In addition, lime application also affects the symbiotic relationship between legumes and *rhizobia*, resulting in an increased nitrogen fixation (Wakelin *et al.*, 2009). Furthermore, liming has been reported to increase nodulation of *Rhizobium* spp. and *Trifolium repens* (Newbould and Rangeley, 1984); and the same effects were noticed on *Frankia* spp. and *Alnus rubra* (Crannell *et al.*, 1994).

Apart from symbiotic organisms, Ivarson (1977) demonstrated that with more liming, the numbers of total *Actinomycetes* remarkably increased in the media; whereas the fungal population decreased. Furthermore, lime was reported to increase the rate of bacterial activity recovery after 16 days from its application (Rajapaksha *et al.*, 2004). These findings strengthen the need to holistically assess the effects of lime application, not only on AM fungi, but rather on other free-living and symbiotic organisms as well. In conclusion, NfBs *bacteria* seem to be affected by lime application in a similar way to and show the same synergistic effect than AM fungi. Therefore, further research should help understanding how soil CaCO_3 availability and liming practices could interfere with symbiotic relationships of plants with soil organisms considering the proposed new soil nutrient hypothesis of the Ca[pH]-induced-chlorosis of the host plants.

e.2. Integrated description of the solution: “*rhizoculture model to mitigate environmental impacts of GHG and diffuse contamination, focusing on carbon, phosphorus and nitrogen in soils of forests and agro-ecosystems*”

e.2.1. Food production and forest resources and needs at the global level

The Earth sums up 1,600 M ha of cropland, 3,900 M ha of pastures and 3,900 M ha of forests, holding more than 1.400 M of farmers. These figures highlight the actual importance of the rural sector to the world economy. It is predicted that 50% more food will be needed in 2050 to feed a population of 9,000 M inhabitants, that is, 900 M tons more of cereals, 200 M tons more of meat, and 70 M ha more of cropland. In addition, it cannot be forgotten that there are currently 800 M people poorly nourished in the world. Even though they were almost 1,000 M in 2000, there is a lot to improve in this matter of concern (FAO, 2017b). Moreover, climate change may reduce up to a 25% of the yield of current crops in many areas of the planet. Therefore, there is still a big challenge for sustainability to overcome by investing and innovating in agricultural development.

Within the agricultural sector, cereals production is remarkable, covering 60% of the cropland worldwide, along with oleaginous crops and fruit trees; whereas bovine, including cow milk production, is the most important within the livestock sector together with porcine. Considering the forest sector, its industrial activity is remarkable taking into account cellulose and paper, wood boards, round wood, and bioenergy production. In fact, forest production has experienced an important concentration within forestry plantations and crops, so they nowadays account for the 35% of the total forest outcome, although it is predicted to grow up to 75% in 2050. As a result, FAO (2017) estimates that wood demand will be increased by a 30% in 2050, and forestry crops will be responsible to cover this increment. Nowadays, 400 M m³ of industrial wood are being produced by forestry plantations, and they are estimated to be 900 M m³ in 2050. Correspondingly, forestry plantations cover is estimated to grow by approximately 80-90 M ha. Non-timber forest products (NTFPs) are also of great relevance within the forest sector, and they are under a strong growth; although statistical data are partial and incomplete, so global statistics cannot be herein provided.

Within this global framework, new solutions and policies are urgently needed targeting current challenges for agriculture and forestry, such as growing welfare, changing diets, climate change mitigation, and food security of a growing global population needing 50% more food in 2050, as proposed by FAO (2017).

e.2.2. Soil biology and resilience of ecosystems to climate change

Current Earth system models display a relatively low predictability of soil C stocks, which limits our ability to estimate future climate conditions. In fact, the twenty-five per cent of the carbon emitted by humans is captured on land by locations still known as the 'missing sink'. This large uncertainty on soil carbon-climate feedback predictions has already been attributed to different

factors, such as, (i) an underestimation of arboreal masses in certain regions, such as dryland areas (Bastin *et al.*, 2017); and (ii) the microbial carbon-use efficiency and an incorrect parameterization of the decomposition temperature sensitivity. Therefore, some authors highlight that a more explicitly incorporate soil microbial mechanisms, and in particular mycorrhizas, in global carbon cycle models is necessary if we wish to accurately forecast ecosystem responses and feedbacks (including resilience) to climate change (Popkin, 2015; Tang and Riley, 2015; Treseder, 2016).

Soil contains approximately 2,344 Gt of organic C (SOC) globally, and is the largest terrestrial pool of organic C (Stockmann *et al.*, 2013). However, global soil C reservoirs are composed of both organic and inorganic C. In contrast to SOC, soil inorganic C (SIC) is poorly quantified and understood. Global SIC estimates are of various ranges up to 1,738 Pg in the top first meter of soil depth (Mikhailova *et al.*, 2006; 2013).

In the context of environmental change, SIC has the potential to fix atmospheric CO₂ as pedogenic carbonate and other forms. Carbonate minerals (calcite, aragonite, dolomite, and siderite) present in soils have generally been formed as the result of certain lithogenic and pedogenic processes, or a combination of the two. While lithogenic carbonate results from lithoclasts of carbonate parent material and thus has a negligible impact on the net C budget, pedogenic carbonate represents, in some conditions, a pathway by which atmospheric CO₂ can be partly sequestered by carbonate precipitation associated to the soil availability of Ca²⁺ and Mg²⁺ (Mikhailova *et al.*, 2013).

Carbonate precipitation in soils depends on complex pedological, climatic, and biological factors. The dissolution, transport, and accumulation of pedogenic carbonate depend on the relationship between gaseous CO₂ and dissolved CO₂, H₂CO₃, HCO₃³⁻ and CO₃²⁻, as well as solid CaCO₃ phases. Solubilisation and leaching of CaCO₃ is considered to increase with rainfall, respiration and metabolism of roots and mycelia, and any factor increasing the partial pressure of soil CO₂ (pCO₂). Carbonate ions precipitate as CaCO₃ when it concentrates in capillary water (evaporated or absorbed by the roots). Precipitation can also occur when the pCO₂ decreases, e.g. due to degassing generated by macrofauna or to the disappearance of biological respiration at depth, among other factors (Breemen and Buurman, 1998; Wild, 1992)

Thorley *et al.* (2015) states that carbonate rock weathering caused by soil biological activity can contribute to the sequestration of CO₂ and increase the land-ocean alkalinity flux, thus counteracting the ocean acidification process that has recently been described. They propose that direct fungal channelling in rock grains (as a result from a combination of physical, chemical, and enzymatic mechanisms) is a major factor explaining continental mineral weathering, which nevertheless varies depending on different tree and mycorrhizal functional groups. Soil pH is a central factor in determining carbonate dissolution rates, which are relevant to enhance carbonate weathering, CO₂ sequestration and potential amelioration of surface ocean acidification. These authors suggest also that strategic planting of suitable tree-mycorrhizal plant functional groups might offer a possibility for utilizing weathering as a natural ecosystem service to accelerate the described effects.

However, Verrecchia *et al.* (2006) indicate that, supported by plant photosynthesis, soil fungi (mycorrhizal as well as saprophytic) produce high amounts of Ca oxalate polymorphs (and other organic acids) that are converted into CaCO_3 when oxidized by oxalotrophic bacteria, which are using them as a source of carbon and energy. As a consequence, the oxalate-carbonate pathway leads to high amounts of secondary precipitation CaCO_3 in soils due to a pH increase in the soil solution. The C source proceeds from atmospheric CO_2 fixed by plants during photosynthesis, a process able of 'turning sunlight into stone' (Cailleau *et al.*, 2011). Verrecchia *et al.* (2006) and Cailleau *et al.* (2011) estimate that fungi and bacteria potentially transform half of the organic C from oxalate and other low-molecular-weight organic acids into CaCO_3 , which has a much longer residence time in soils than organic substrates. The other half is released as CO_2 into the atmosphere.

In summary, ecosystem models should consider the dynamic interactions among sorptive mineral surfaces, substrate weathering, and microbial and rhizosphere processes, to address more accurately the resilience of ecosystems to climate change and to improve simulations predicting carbon storage by large-scale Earth systems (Tang and Riley, 2015; Thorley *et al.*, 2015).

e.2.3. Rhizoculture approach for the intensification of roots and rhizosphere activity to reduce fertilization and improve resilience in agriculture and forestry

Agriculture and forestry have traditionally been focused on yielding the most economically valuable elements of plants. However, European societal challenge analyses indicate that new nature-based solutions considering the multi-functional use of ecosystems are required to perform the transition towards a circular economic eco-innovative management system changing the traditional model based on production and consumption towards a greener society.

To include the multiple functions of ecosystems in both OA and CA, the rhizosphere is essential to soil, vegetation and ecosystem functioning (Akeem, 2012). Rhizosphere is the main bottleneck for closing loops on N, P and C-cycling in ecosystems because roots allow the integration of plant photosynthesis (leading to C inputs) and the activity of symbiotic organisms, such as mycorrhizas and N-fixing bacteria (leading to P and N inputs, respectively). Therefore, mutualistic plant-microbe interactions offer a novel approach to enhance agricultural productivity while reducing dependence on fertilization and environmental costs.

In this regard, García-Montero *et al.* (2015a; 2015b; 2017) proposed the development of a multidisciplinary body of knowledge called “rhizoculture” (i.e. farming the rhizosphere), which could be defined as a set of techniques focused on intensifying the abundance, biomass, depth and activity of roots, symbiotic and free-living organisms associated to the rhizosphere. Therefore, the so-defined rhizoculture can lead to decrease fertilization and diffuse pollution, and increase soil C storage, N and P availability -from farms to a wider scale agro-ecosystems-, plant resilience, and agricultural and forest production through innovative, easy-to-adopt, economically sound new practices based on the management of (Figure 1):



Figure 1. R+D+i in the multidisciplinary body of knowledge called “rhizoculture”, that proposes to change our technologies in agro-ecosystems and forests through innovative, easy-to-adopt, economically sound new practices based on the management of: (i) *ecological intensification of roots and rhizosphere through new cultural practices, liming and mycorrhizal rock-eating capacity*; (ii) *soil C pools, organic matter content and the ratio of symbiotic/saprophytic communities (through livestock and biomass management)*; (iii) *N-P-water loops and recycling (including smart use of aquaculture and organic waste)*; (iv) *physical soil properties (through liming and soil symbiotic microorganisms and fauna)*; (v) *policies aiming to incorporate the socioeconomic value of the positive externalities associated to rhizoculture*

- (i) ecological intensification of roots and multiple symbioses among soil organisms and the host plants -through new cultural practices and soil Ca pathways, including soft liming-;*
- (ii) roots and rhizosphere in connection with biogeochemical processes -including the rock-eating capacity of mycorrhizae-;*
- (iii) soil organic matter content to increase the ratios of symbiotic to saprophytic communities in the soil -through livestock and biomass management-;*
- (iv) roots and rhizosphere impacts on soil diversity and C pools;*
- (v) N and P recycling- by aquaculture and smart recycling of organic waste-*
- (vi) water availability from soil and recycling (i.e., by aquaculture)*
- (vii) root and rhizosphere development to improve the ability of plants to cope with environmental disturbances such as climate change;*
- (viii) physical soil properties -through soil symbiotic microorganisms and fauna, such as ants, termites and earthworms-;*
- (ix) policies aiming to incorporate the socioeconomic value of the positive externalities associated to rhizoculture.*

Through the two projects on rhizoculture submitted to the European Horizon 2020 calls - RHIZOFARMING in 2017 and RIZHOINTENSE in 2018 (see *Section e.4. Future research lines*)-, it has been proposed some combinations of management techniques in agriculture, livestock, agroforestry and forestry systems (e.g., *enhanced crop rotation, polyculture, permanent rangelands, and convertible husbandry crop planting*), which would be developed through different scenarios and prototypes in agro-ecosystems and forests (Figure 1; Tables 1, 2 and 3).

Assuming the challenge of shifting production and consumption management patterns in agriculture and forestry towards a sustainable green economy and society, the value-added provided by this type of rhizoculture might include a reduction of the high costs and pollution associated to current fertilizers and phytochemicals inputs, which should result into improving both “environmental health” and the performance of agricultural and forestry systems.

e.2.4. Strategic keys to implement rhizoculture models to optimize its impact on the mitigation of greenhouse gas emissions, pollution associated to fertilizers use and food security

Considering this background, the main objectives of developing rhizoculture technology, both in OA and CA, would be to reduce fertilizer use, and to increase crop resilience, C sequestration, and even agricultural and forestry production, and N and P recycling, to be applied from the farm scale to much larger geographical units. These objectives would be directly related to the principles of organic agriculture and permaculture (Mollison and Reney-Mia, 1991; FAO, 2017b), and therefore, rhizoculture would have to propose models for closing N, P and C cycling loops in agro ecosystems, which rely on sustainable ecosystem management rather than on external agricultural inputs.

Rhizocultural technology could be based on a set of experimental studies at the laboratory scale aiming to unravel the biological mechanisms (including molecular, biochemical and physiological ones) that are involved in root and rhizosphere intensification. On the other hand, and both at farm and landscape scales across different biogeographic regions (such as, Boreal, Atlantic, Continental and Mediterranean) and different continents (such as, Europe, Africa, Asia and Latin America), comparable pilot scenarios could be selected to implement rhizoculture by: (i) developing new applications of conventional OA techniques; (ii) designing new agricultural practices based on the observation of rhizosphere patterns of performance (such as liming and the control of organic matter and water inputs into the soil); and (iii) monitoring the closure of loops of N, P and C cycling, as well as diffuse contamination, GHG emissions and C sequestration in agricultural ecosystems. Moreover, rhizoculture could be integrated with (a) livestock management and feed, and aquaculture; (b) new technologies to enhance their implementation (i.e., recycling of agricultural residues); (c) socioeconomic and environmental assessment models, aiming to designing new prototypes on agro-ecosystems, and disseminating them via agriculture extension programs (García-Montero *et al.*, 2015a; 2015b; 2017).

Therefore, it is necessary to develop research projects unfolding strategic steps to implement rhizoculture in potential designing prototypes of sustainable agro-ecological systems. The main objectives in the design of these experiments could be:

- (i) To develop agricultural, both organic and conventional, technologies to intensify root development and rhizosphere activity within both agricultural and forest management practices.
- (ii) To design rhizoculture strategies based on OM management models (e.g. livestock management and others) aiming to increase the ratio of symbiotic/saprophytic soil organisms in order to achieve these objectives: *(a) to intensify biological NPK fertilisation (increasing NfBs and mycorrhization, fungal rock-eating activity...); (b) to reduce CO₂ and GHG emissions and increase C stocks; (c) to increase plant resilience to climate change.*
- (iii) To develop rhizocultural strategies increasing the presence levels of Ca, and its salts, in the soil by liming and the management of biological activity (earthworms, oxalotrophic bacteria, etc.) in order to intensify the symbiosis of plants with mycorrhizas and NfBs (using the *Ca-induced-chlorosis* model).
- (iv) To implement rhizocultural strategies based on the management of fauna and organic waste recycling (including aquiculture, livestock, wild herbivorous, social insects, earthworm culture, and others) aiming to increase soil nutrients availability (by organic amendment, biological fertilisation, etc.) and the quality of soil properties (soil structure and aeration, cation exchange capacity, etc.).
- (v) To design strategies integrating the use of chemical fertilisers and pesticides with biological fertilisation and crop protection based on rhizoculture.
- (vi) To develop new information technologies that would favour the implementation of rhizoculture and its monitoring in agricultural and forestry systems. Some of these new technological developments may be: *(a) technological applications aiming to increase symbiotic activities among plants, mycorrhizas, and bacteria; (b) technological*

applications to understand and intensify calcium roles in the symbiotic processes between plants, mycorrhizas and bacteria; (c) molecular monitoring of biomass and diversity of soil organisms; (d) remote sensing technologies monitoring fungal and soil organism activity.

(vii) To develop new models integrating rhizoculture in agriculture and forestry with consumer behaviour, food security, resources use and green economy.

In order to generate a set of management models on rhizoculture on different scales, Table 1 shows a set of hypothetical scenarios of high agricultural and forest value at both the global and European scales, based on 15 crop types (*alfalfa, apple, barley, dry beans, green peas, hazelnut, maize, millet, olive, potato, rice, soybean, tomato, walnut, wheat*) and 15 agroforestry systems including 3 livestock species (*cattle, pig, sheep*) and 3 aquaculture systems (*intensive and extensive-rice fishing- aquaculture, and aquaponics*) which have been selected because:

(i) they represent 71.73% of the EU and 63.85% of the global crop areas, and 71.51% of EU and 61.88% of global meat yield in basis to FAOSTAT (FAO, 2017a) (Tables 4-6)

(ii) a bibliographic review shows that these crops have valuable root systems and a good response to calcareous amendments (Tables 2 and 3)

(iii) prototypes of rhizoculture based on this set of crops would allow comparisons of “*cover vs. row crops, annual vs. perennial, tall vs. short crops, deep vs. shallow roots, N-fixing and catch crops, species suited for companion planting and polyculture (e.g., beans, corn, peas, potato, tomato, alfalfa, walnut)*” (Table 1).

(iv) other prototypes of rhizoculture based on a set of agricultural, forestry and other land uses would allow the analysis of arable land vs. adjacent forests, field margins vs. hedges and trees, tree species with N fixation capacity, and others scenarios such as buffer strips and mountain farming (Table 1).

Table 4. Data from the global agricultural sector (FAO, 2017a)

Product	Cultivated area (Mha)	Production (Mtm)	Main countries (Mtm)	Economic value (M€)
Wheat	218.0	740.0	EU (145.0), China (125.0), India (90.0)	120,000.0
Barley	51.0	150.0	EU (60.0), Rusia (20.0), Australia (10.0)	-
Corn	175.0	1,050.0	USA (385.0), China (215.0), Brasil (80.0)	150,000.0
Rape	35.0	70.0	EU (20.0), Canada (18.0), China (13.0)	-
Sunflower	22.0	45.0	Ucrania (13.0), Rusia (10.0)	-
Soy	91.0	325.0	USA (118.0), Brasil (102.0), Argentina (57.0)	90,000.0
Grain legumes	-	42.0	India (12.0)	-
Fruit trees	123.0	790.0	-	150,000.0
Perennials	Olive (11.0), vine (7.5), almond (1.8), walnut (0.8)	-	-	-

Table 5. Data from the global livestock sector (FAO, 2017a)

Sector	Production (Mtm)	Main countries (Mtm)	Economic value (M€)
Beef	60.0	USA (11.0), Brasil (10.0), EU (7.5)	190,000.0
Milk	770.0	EU (145.0), India (140.0), USA (90.0)	270,000.0
Pork meat	110.0	China (50.0), EU (23.0), USA (10.0)	150,000.0

Table 6. Data from the global forestry sector (FAO, 2017a)

Product	Production	Main countries	Economic value (M€)
Sawn timber	421.0 Mm ³	China (86.0), USA (82.0), EU (80.0)	135,000.0
Round wood	1,850.0 Mm ³	USA (360.0), EU (243.0), Rusia (185.0)	145,000.0
Chips	26.0 Mtm (pellet)	EU (13.5), USA (5.0)	4,500.0
Pulp	175.0 Mtm	USA (40.0), China (17.0)	60,000.0
Wood boards	390.0 Mm ³	China (190.0), USA (36.0)	85,000.0
Paper	400.0 Mtm	China (105.0), USA (70.0)	115,000.0
Edible fungi (cultivated)	12.0 Mtm	-	20,000.0

e.2.5. Basis to integrating the rhizoculture and the soil organic matter management by livestock, biomass and bioenergy uses

Livestock constitutes a powerful management tool at the landscape level. Apart from its proven capacity to influence biodiversity (Teillard *et al.*, 2016), the high proportion of biomass consumed by livestock on grasslands (up to 85% according to estimates by Haynes and Willams (1993) and the dominance of domestic livestock in terrestrial grazed ecosystems (Smith *et al.*, 2015) show how determinant this component can be. While livestock funnels a large proportion of the grassland biomass, most of the ingested components are excreted in an organic form and only mineralized by soil microorganisms once buried (Haynes and Williams, 1993; Rufino *et al.*, 2006). The interdependence of the soil ecosystem and the grazing ecosystem becomes therefore very relevant, especially in grazed ecosystems that represent more than half of the continental lands (Manzano, 2015). It is then unsurprising that large mammalian herbivores influence the presence and activity of soil nematodes (Veen *et al.*, 2010) or mycorrhizas (Murray *et al.*, 2010).

At the interface between the grazing and the soil ecosystems, dung-burying invertebrates become critical in putting systems together and closing the nutrient cycle. While earthworms have been considered determinant in temperate humid climates, as well as dung beetles in semiarid Mediterranean systems (Lumaret *et al.*, 1992), the influence of the latter seems to be restricted to the most humid months both in temperate and tropical semi-arid and arid areas. Harvester ants have been observed to be much more relevant in terms of dung burial during dry seasons in the Mediterranean area (Manzano *et al.*, 2010), with similar roles attributed, in principle, to termites in the tropics (Freyman *et al.*, 2008; West, 1991). While such studies give a hint on the potential importance of social insects in dung burial, very little is known on which factors favour or limit the process or how it affects the soil community and the processes therein, such as carbon fixation or CaCO₃ production. Such research is particularly crucial when considering that a very large proportion of the land consists of drylands (up to 41.3% according to the Millennium Ecosystem Assessment), which have particularly been disregarded by research.

Table 1. Set of hypothetical scenarios for designing prototypes of sustainable agro-ecological systems based on rhizoculture

Regions	Countries	Annual crops to combining in some rotations: alternating crops and rhizoculture			Tree crops to be integrated with annual-rotations to stimulate rhizoculture	Intensive farming to integrate with N, P and C cycling in crops and rhizoculture	Agroforestry to integrate with N, P and C cycling in crops and with rhizoculture (farm and landscape levels)		Sources of P and N to intensify (circular economy)	C sinks to intensify (green economy)		
		Crop type	Deeper roots	Shallower roots			Livestock and crops, and natural grasslands	Small forest, dehesa (savannah) and crop mosaics				
Boreal EU	Finland	Cover	Wheat; millet	Barley	Apple	Cow; pig	Extensive cow cattle	Pinus sylvestris; Populus spp.		Rhizoculture and soil C sinks		
		Rows	Tomato	Potato				Alnus glutinosa				
		N-fixers	Alfalfa	Green peas								
Atlantic EU	Germany France	Cover	Wheat; maize; millet	Barley	Apple; walnut		Extensive cow, sheep cattle	P. sylvestris; Quercus robur ; Populus spp;		Rhizoculture and ocean C sinks		
		Rows	Tomato	Potato				Alnus glutinosa				
		N-fixers	Alfalfa	Green peas; soybeans				Improving soil N and P availability and C sinks				
Continental EU	Germany Czech Republic	Cover	Wheat; millet	Barley	Apple	Cow; pig; aquaculture		Aquaculture waste treatments (high- tech) and aquaponics		Rhizoculture and ocean C sinks		
		Rows	Tomato	Potato								
		N-fixers	Alfalfa	Green peas								
Mediterranean EU	Spain; Italy	Cover	Wheat; maize; millet	Barley	Apple; walnut; hazelnut; olive		Extensive cow, sheep, pig cattle; wild herbivores;	Q. ilex; P. sylvestris; Populus spp.; truffle culture; A. glutinosa; Ceratonia siliqua; soil social insects		Livestock and soil C sinks		
		Rows	Tomato	Potato				Rice-fishing				
		N-fixers	Alfalfa; dry beans	Green peas; soybeans								
Crops with global impact on N, P and C cycling and international trades	Argentina	Wetlands	Rice (paddy)							Aquaculture and soil C sinks		
		Cover	Maize; millet	Barley								
		Rows	Tomato	Potato								
	Brazil	N-fixers	Dry beans	Soybean								
		Cover	Maize; millet	Barley								
		Rows	Tomato	Potato								
	Kenya	N-fixers	Dry beans	Green peas								
		Rows	Tomato	Potato								
		N-fixers	Dry beans	Green peas								
	Bangladesh	N-fixers	L. sativus; S. rostrata		Aquaculture	Rice-fishing		Improving soil N and P availability and C sinks	Aquaculture waste treatments (soft- tech) and aquaponics	Aquaculture and ocean C sinks		
	Philippines	Wetlands	Rice (paddy)									

Table 2. Root patterns and positive effects of liming on 15 representative crops that have been selected for designing comparative experiments on rhizoculture

15 selected crops	Root pattern in the soil	Liming effect	Impact	AM species	References
Wheat ¹	Both spring and winter wheat are annuals with deeply penetrating, widely spreading, and profusely branching, fine, fibrous roots; the roots of the spring varieties are less extensive ³ . Effective root zone ⁴ = 50-100 cm; 61-68% of their roots in < 30 cm; 50% >15 cm (avg. 16.8); 95% <103.8 cm; max depth = 150.4 cm ⁵ . 2-year-old crested wheat grass plant possessed over 500 m of roots occupying about 2.5 m ³ of soil ¹³	Liming increased the efficiency of P use by wheat because of biological causes. Liming caused a significant increase in grain and straw yield of wheat (26.8 - 18.6%, respectively) over the no lime treatment, irrespective of the sources and levels of lime.	+	-	Wild (1992); Bhat <i>et al.</i> (2007)
Barley ²	Barley roots often occur nearer the surface than those of wheat; when grown in rich deep soil, has a root habit very similar to that of spring wheat; the fineness of the roots, degree of branching, and lateral spread often being intermediate. Effective root zone ⁴ = 50-100 cm; 67-76% of their roots in < 30 cm; 50% <13 cm (avg. 11.5); 95% <99.6 cm; max depth = 146.1 cm ⁵	Small positive effect on mycorrhizal colonization of barley and on propagule numbers.	≈	-	Hamel <i>et al.</i> (1996)
		AM inoculation increased the yield when pH is intermediate.	+	-	Wang <i>et al.</i> (1993)
		AM increases the productivity of limed maize	+	<i>Glomus etunicatum</i>	Sylvia <i>et al.</i> (1993)
		Liming favoured specific AM species and many non-AM fungi. Higher richness of AM in non-limed soil	+	<i>Funneliformis</i>	Gomes <i>et al.</i> (2015)
Maize ¹	Corn has a remarkably widely spreading, deeply penetrating, and profusely branching root system ³ . Effective root zone ⁴ = 50-100 cm; 50% of their roots <14.4 cm in average; 95% <88.9 cm; max depth = 118.3 cm ⁵ . In prairie soils, corn roots regularly penetrate to 2 m ¹³	AM improved maize growth in limed soil and higher AM infection rates were achieved	+	<i>G. margarita</i>	Higo <i>et al.</i> (2010)
		Increase plant height in limed soil. Liming and fertilizers (NPKS) increased maize yields as compared to the control plots which received NPK only.	+	-	Da Mota <i>et al.</i> (2008); Sirikare <i>et al.</i> (2015)
		Liming increased the AM fungal colonisation of corn. Lower AM fungal germination in non-limed and weak correlation with shoot dry weight	+	<i>G. mosseae</i>	Siqueira <i>et al.</i> (1984,1990); Sharif <i>et al.</i> (2011)
Olive ²	While other trees send their roots deep into the ground, olive trees feature shallow root systems. This allows olive roots to collect water from soil that typically dries fast, ensuring the tree gets enough moisture to stay hydrated	P and liming increased the leaf-P levels. Liming to many Australian soils, will improve the health, growth and crop of olive trees			Jordao <i>et al.</i> (1993); Olive Ag. Info. Serv. (2018)
Potato ²	Potatoes have more superficial roots than many crops such as corn, winter wheat and most legumes. After extending horizontally 30-60 cm, majority of roots penetrate < 60 cm (exceptionally < 115 cm)	Liming had positive effects on yield, protein content, ash, starch, and Ca ²⁺ of potato. However, Zn, Cu, Fe and P decreased with increased application of lime. AM inoculation increased yield when pH is intermediate	+	-	Wang <i>et al.</i> (1993); Lalljee and Facknath (2002)
Apple ¹	Apple trees roots consist of a deep taproot (analogous to the carrot) and lateral fibrous roots. Lateral fibrous roots can extent to more than twice the spread of the canopy. Fine roots develop from the fibrous roots. During the third year, the maximum lateral spread reached 9 m and the maximum depth reached was 5 m. This greatly exceeded the lateral spread of three-year-old tops, which was about 2 m, and the height of the trees, which was 2 to 2,4 m ⁶ . Roots of 18-year-old apple trees penetrated to a depth of at least 10 m and fully occupied the soil between the rows, which were about 10 m apart ¹³	Tree growth and apple fruit yield were significantly increased. Moreover, apple tree nutrition was benefited from liming.			Kotze and Joubert (1981); Neilsen <i>et al.</i> (1991)
Rice ²	Rice has a relatively shallow and compact (short and thick) root system compared with maize and wheat. Rarely grow deeper than 40 cm; about 90% of the total root system is restricted to the top 20 cm of the soil layer. Mean of root length of no fertilizer plants is 23.44 shorter than NPK fertilizer plants ^{7, 8, 9}	Upland rice dry matter and grain yields increased up to 32 and 19%, respectively, with lime addition	+	-	Fageria <i>et al.</i> (1991)

Table 3. Root patterns and positive effects of liming on 15 representative crops that have been selected for designing comparative experiments on rhizoculture

15 selected crops	Root pattern in the soil	Liming effect	Impact	AM species	References
Soybean ²	67-76% of their roots in < 30 cm; 50% of total root amount <13 cm (avg. 10.9); max depth = 111.3 cm ⁵	Liming increased the ability of AM fungi to enhance the efficiency of P use by soybean	+	-	Maddox and Soileau (1991)
Tomato ¹	Mature plants have a wonderfully extensive root system. Usually 15 to 20 major branches spread widely (maximum, 168 cm), have very numerous large branches, which usually penetrate deeply, and finally, turning downward, extend into the 90, 120, and sometimes the 150 cm of soil. All are so profusely furnished with masses of much rebranched rootlets that the absorbing area is extremely intricate and extensive even beyond the working level of 106 cm ¹⁰ .	Tomato fruit yield, plant growth and dry matter were significantly increased by liming at a pH between 3.3 to 5.2 while at a pH of 5.7 and above liming had little effects. In a soil of pH 4.2-5.1, liming 2 tones ha ⁻¹ of dolomite, and fertilizer (N-P-K-S-Zn), were optimum for better fruit yield tomato	-	-	Asiegbu and Uzo (1983); Koesrini and Nursyamsi (2014); Nazrul and Khan (2016)
Green peas ²	Plants 1.5 months old have a root depth of 60 cm. The soil at a depth of 5 to 20 cm is well filled with a network of nearly horizontal roots and their laterals to a distance of 46 cm on all sides of the plant. But in the deeper soil little absorbing area occurs ¹⁰ . Effective root zone ⁴ = 60-70 cm; 61-68% of their roots in < 30 cm; 50% of total root amount >15 cm (avg. 18.2); max depth = 150.4 cm ⁵	The number of rhizobia that occurred naturally in non-inoculated plots increased rapidly in high-lime plots	+	-	Fettell <i>et al.</i> (2007)
Beans (dry) ¹	The kidney bean rapidly develops a deeply penetrating taproot. When the plants are nearly mature, the soil to 60 cm on all sides is well ramified to a working level of 90 cm feet and numerous roots extend 120 cm deeper. Thus the general root habit of the kidney bean is not greatly unlike that of the pea although the lateral spread and depth of penetration are somewhat greater and the deeper soil, just beneath the plant, somewhat more thoroughly occupied ¹⁰ .	Growth parameters and yield of <i>P. vulgaris</i> were significantly increased with liming (40% and 45%, respectively) or growing in vermicompost amended soil. Nodulating were significantly related with liming, largely due to changes in the predominance of the rhizobial species groups	+	-	Fageria <i>et al.</i> (1991); Andrade <i>et al.</i> (2002); Valdez-Pérez <i>et al.</i> (2011); Kassa <i>et al.</i> (2014)
Hazelnut ²	Hazelnuts are more shallow-rooted than most fruit trees, growing well in the marginal locations for walnuts ¹¹ . Most of a hazelnut tree's roots are found in the first 0.6 m of soil; suitable soils allow trees to develop active root systems to depths of 1.8-3 m ^{11, 12}	Hazelnut uptake of N, K, Mg and Ca increase with liming, which also reduces Al and Mn uptake, but that liming is probably only cost effective up to pH 5.6 or 5.8. Liming increased very significantly the number of root tips	+	-	Baron <i>et al.</i> (1975); Silberman (1983); García-Montero <i>et al.</i> (2012); Olsen (2016)
Walnut ¹	Walnut has an intricate extensive root system with the taproot penetrating to 3.7 m deep and many lateral roots which are mainly located in the horizon B at depth 60-80 cm, and sometimes extended to a distance exceeding the tree height and/or width. These trees excrete toxins to keep other plants with deep roots systems from growing ¹³	Liming improved the yield of walnut very significantly, and improved also soil porosity, increased leave nitrogen content and leave total potassium	+	-	Ouertatani <i>et al.</i> (2015)
Alfalfa ¹	Alfalfa is a long-lived, very deeply rooted perennial ³ . Effective root zone ⁴ = 120 cm ; 61-68% of their roots in < 30 cm; 50% of total root amount >15 cm (avg. 19.8); max depth = 176.8 cm ⁵ . In prairie soils, alfalfa roots have been found at depths of 10 m ¹³	Higher rhizobium nodule numbers and total nodule weight. Higher shoots' N concentration without AM, while lower P concentration with AM	+	<i>Glomus intraradices</i>	Guo <i>et al.</i> (2010)
Pearl millet	Root system development is characterized by a fast growing primary root that quickly colonizes deeper soil horizons (> 2 m)	Liming provided increments in the dry biomass production and in the accumulation of nutrients (N, P, K, Ca, Mg and S) by millet plants	+	-	Santana and Nascente, 2014
Mushrooms	Liming produces positive effects in many mycorrhizal fungi and truffles with agroforestry interest; see reviews of García-Montero <i>et al.</i> (2009a; 2012), Monfort-Salvador <i>et al.</i> (2015)				

The management of grazing lands has also a very strong influence on their land degradation and biodiversity status, which in turn has a direct effect on C storage potential. Studies on rangeland governance have shown that security in land tenure is very determinant for keeping soils in grazing lands under a satisfactory conservation status (Herrera *et al.*, 2014). Grazing management as a tool, including adequate land tenure systems, therefore seems a cheap and far-reaching management tool to promote carbon-fixing processes in the soil, provided that concluding research on how the different regimes influence it would be enabled.

Therefore, livestock and biomass use management can be major factors to develop soil organic matter management strategies aiming to increase the ratio of symbiotic/saprophytic soil organisms in order to achieve the main objectives of rhizoculture (described above) (García-Montero *et al.*, 2015a; 2015b; 2017).

In this regard, the need to mitigate climate change effects reducing GHG emissions, the growing interest on reducing crude oil dependence, and several other beneficial effects are major motivations for increasing the use of biomass-derived bioenergy (Long *et al.*, 2015; UNTACD, 2016). Biomass use has undergone a significant transformation along the last decade, that is, biomass is not only used for energy generation, but also for biomaterial production (food, feed, and fibre) thanks to the application of more innovative biotechnological processes, leading to develop what it is currently known as the bioeconomy (UNCTAD, 2016). In 2014, the 10.1% of the world's Total Primary Energy Supply (TPES = 1,384 Mtoe = 57.8 EJ) was produced from biomass (solid and liquid biofuels; IEA, 2016). Biofuel production in 2015 was 74.85 Mtoe, whereas 239.4 thousand million tonnes of oil were produced from geological reserves. The three largest biofuel producers were USA, Brazil and Germany, accounting for the 41.5%, 23.6% and 4.2% of the total worldwide outcome, respectively (BP, 2016). The bulk of the production of the USA and Brazil is ethanol from corn and sugarcane, respectively; whereas the major part of the German production is biodiesel from rapeseed (BP, 2016; Long *et al.*, 2015). In the USA, a 30% of the average annual production of corn grain from 2006 to 2010 was used for ethanol production. Three oil crops supply the vast majority of biodiesel: soybean (7.0 Mt), oil palm (6.3 Mt) and rapeseed/canola (6.0 Mt). Most soybean biodiesel is produced and consumed in the major centres of soybean cultivation, which are Brazil, USA and Argentina (Long *et al.*, 2015). Bioenergy and biofuels produced from biomass have been considered carbon neutral in most Life Cycle Assessment (LCA) studies of biomass systems. It is assumed that the carbon released during the combustion of biomass is sequestered back into equivalent growing biomass. This convention is currently being discussed because the timing difference between the release and sequestration of carbon from forest biomass leads to a situation where part of the carbon remains in the atmosphere until it is fully integrated back into the growing forest. This would result in a warming impact if sequestration lags emission. Thus, carbon neutrality over a forest rotation period is not equal to climate change neutrality (Cherubini *et al.*, 2011; Helin *et al.*, 2013).

In biomass production (crop and harvest stages), other GHG emissions (i.e. N₂O and CH₄) should also be taken into account; and special attention should be paid to the role of fertilizers (Sastre *et al.*, 2016a; 2016b). Net GHG savings are really highly uncertain and depend, among others, on the N-fertilization level and assumed N₂O emissions factors (Erisman *et al.*, 2010).

Recent studies point out that typical fertilization doses for bioenergy crops could produce nitrogen deficits in soil stocks, compromising soil sustainability and future crop fertility. However, raising nitrogen fertilization above the typical dosage aiming to compensate soil nitrogen deficit could compromise the achievement of GHG savings, as well as it could generate excessive effects in other relevant impact categories (i.e. eutrophication) (Sastre *et al.*, 2016a; 2016b). In connection with rhizoculture, different crop management techniques are suggested to overcome this problem: (i) crop rotation with legumes; (ii) no-tillage farming; (iii) optimization of the crop collection time and/or the use of low N content species; (iv) introducing *gluconacetobacter* bacteria; and/or (v) using slow release fertilizers (Erisman *et al.*, 2010; Sastre *et al.*, 2016a; 2016b).

The cultivation of bioenergy crops is viewed as controversial because of this uncertainty with respect to net GHG-savings and its potential competition with biodiversity and food production for land use (Erisman *et al.*, 2010). Research is currently focused on a second-generation biofuels (i.e. produced from lignocellulosic biomass). Typical resources for these fuels are short rotation forestry crops (poplar, willow and eucalyptus), perennial grasses (*Miscanthus* spp., switch grass and reed canary grass), and residues from the wood industry, forestry, and agriculture. Most of these second-generation biofuels are in their early stages of pilot production phase and commercialization (UNCTAD, 2016).

e.2.6. Basis to integrating the rhizoculture with alternative fertilizers based on waste recycling and aquaculture

In 2012 and 2013, the average world's fertilizers consumption was about 120 kg/ha of cropland a year. Just considering developed countries with intensive agriculture, these figures turn to exceed 170 kg/ha yearly (i.e., the EU addressed an average of 177.026 and 176.419 kg/ha, respectively). These figures include N, P and K fertilizing fertilising products, but traditional organic compost and manure from plant and animal origins are not included (FAO, 2016; World Bank, 2016). The cost and environmental issues associated to these high levels of fertilizer application are promoting new alternative N and P sources, based on waste recycling, aquaculture and rhizoculture.

The world's production of aquatic animals from aquaculture summed up to 73.8 million tons in 2014, and 27.3 million tons of aquatic plants were also cultivated, accounting for an estimated first-sale value of €101,000 million, even overpassing the world fisheries captures of 93.2 million tons (FAO, 2016). The total European aquaculture production reached 2,350,278 tonnes in 2015. Cold-water marine species now represent the 71.4% of the total production, fresh water species sum up to the 15.1%, and marine Mediterranean species address the rest 13.5%. In 2015, the 94% of bred species were salmon, trout, seabream, seabass, and carp.

Within the aquaculture sector, Integrated Multi-Trophic Aquaculture (IMTA) is a multi-culture system where several species from different trophic levels are bred in proximity. While the theoretical potential for the growth of biomass in the ocean is evidently very large, the focus on this kind of solution has been surprisingly low. As long as it is performed within ecologically sustainable limits, IMTA offers potential solutions to an increased and profitable production of

local, resource-efficient, and climate-friendly food and biomass for energy purposes whilst capturing CO₂. This system is similar to a natural ecosystem, so the waste or excess of nutrients produced from fish being farmed (e.g. faeces and waste metabolites, and uneaten feed) become nourishment resources for lower trophic species, such as shellfish or seaweeds that are housed within the same system or environment.

This combination contributes to an ecosystem-based production that, in addition to recycling nutrients, naturally binds CO₂. Particularly within these systems, both “aquaponics and smart use of water from aquaculture, rich in nutrients, to irrigate crops” are innovative and sustainable food production systems integrating aquaculture with hydroponic vegetal crops and conventional crops, respectively. Both “aquaculture-agriculture integrate systems” may hold a key role to play in food provision and tackling global challenges such as water scarcity, food security, urbanization, and reductions in energy use and food miles.

On the other hand, membrane technologies, or membrane separation processes (MSP), are considered as highly efficient alternatives for the removal of nitrates and phosphates from water, in addition to other technological advantages such as the significant reduction of equipment size, energy savings, minimized environmental impact, and its easy staggering and automation (Drioli *et al.*, 2011). Thus, some researchers have addressed this issue by applying different types of membrane and other separation processes, showing promising results (removal of nitrates and phosphates > 99%), such as electrocoagulation (Bektaş *et al.*, 2004), microfiltration (MF) (Wasik *et al.*, 2001), membrane bioreactors (MBR) (Luo *et al.*, 2016b), nanofiltration (NF), or reverse osmosis (RO) or direct one (DO) (van Voorthuizen *et al.*, 2005). In many of these studies, different combinations of processes are also proposed, i.e. MBR+RO (Luo *et al.*, 2017), MBR+NF (Kangmin Jaeweon, 2012) or an integrated MF-NF-RO system, depending on the requirements of final water quality or the components to be removed. Nowadays, several companies are developing the application of these processes to the extraction of N and P from waste and agricultural waste recycling to produce alternative fertilizers, which is on the base of a circular economy compatible with rhizoculture.

e.2.7. Expected impacts of rhizoculture on agro-ecosystems and forests

Estimates of overall efficiency of fertilizer are lower than 50% for N and less than 10% for P (Baligar *et al.*, 2001). A major objective of the rhizoculture is to promote the natural ability of roots and beneficial soil organisms to close N, P and C loops in crops, rangelands, agroforestry systems and forests. In concert with other novel agronomic technologies and management practices, plant-microbial mutualisms can help increasing crop production from 115% and up to 300%, and reduce yield losses by improving resistance and/or resilience to edaphic, biologic, and climatic variability from both bottom-up and top-down perspectives (Adesemoye and Kloepper, 2009; Baum *et al.*, 2015; Hamilton *et al.*, 2016). Thus, relevant problems regarding N fixation costs, P fertilization and C-mediated climate change will be addressed.

Investigating the application of the rhizoculture model to extensive livestock introduces a new functionality to rangelands, which can use grazing animals for managing soil biology on wide scales (see H2020 project proposals in *Section e.4. Future research lines*). The same applies

to convertible husbandry crops, which can alternate grazing with other uses. Rhizoculture provides easy-to-adopt tools bearing an economic benefit to farmers, as the improved efficiency of N, P and C use –decreasing over 25% current inputs from fertilizers (Smil, 1999)– should translate into higher yields, lower costs, and healthier and more resilient crops.

The tools derived from the rhizoculture are intended to be readily up-scalable. The interests of international organisms such as the FAO, the CGIAR Consortium of the World Bank, and some of the most important agricultural associations in the EU (see H2020 project proposals in *Section e.4. Future research lines*) should greatly help in forwarding such tools to stakeholders and other potential beneficiaries.

Improved nutrient capture by plants, mediated by adequate rhizosphere manipulation, would reduce the amount of fertilizer inputs to maintain crop productivity. An enhanced circular economy in agro-ecosystems means reduced economic costs and extended lives for non-renewable resources, such as P or the amount of fossil fuel necessary to capture N from air. More importantly, a robust framework that promotes cost-saving measures guarantees a wide adoption of the practices promoted by the rhizoculture. Such goals are obviously attractive for the private sector-fertilizer companies and others. The most costly steps in fertilizer production are associated with N production or P mining, so innovative research that promotes nutrient capture by rhizospheres will be an attractive investment for industry. Identifying plant varieties more suited to rhizoculture is also a promising and innovative goal.

Increasing resource use efficiency through rhizosphere manipulation is expected to improve environmental sustainability. In the case of N fertilization, for example, beneficial impacts will range from energy saving, to reduced GHG emissions or higher C fixation –related to the Greening areas designated by the CAP (EC, 2018) that might balance C losses in conventional croplands– or the diversion of C into the deep ocean, through fungal rock eating (Thorley *et al.*, 2015). The increased efficiency promoted by the rhizoculture will also reduce P mining and P and N leaching into waterways and aquifers.

Aquaculture, the fastest-growing animal production sector worldwide, has experienced serious constraints in many countries because of its high impact on surface and groundwater quality, mainly due to high N pollution. Once again, the efficiency in N use sought by the integration of rhizoculture and aquaculture (see H2020 project proposals in *Section e.4. Future research lines*) will translate into reduced discharges and enhanced water quality. The interest on rhizoculture by organizations such as FAO, with active participation in forums such as UNF3C on climate change or UNECE on surface waters, should help extend the project's conclusions to a global scale.

The two projects on rhizoculture submitted to the European Horizon 2020 calls – RHIZOFARMING in 2017, and RHIZOINTENSE in 2018 (see *Section e.4. Future research lines*)– propose enhanced crop rotation, polyculture, permanent rangelands, and convertible husbandry crop planting. These agricultural practices fit into current CAP guidelines on Greening, and the same is true for reducing discharges from plant fertilizers and increasing C fixation. CAP Greening targets (EC, 2018) of 5% of ecological focus areas and 5% of grasslands in Europe's agricultural lands is a realistic goal with the efficiency measures addressed by the

rhizoculture. The environmental gains promoted by the project should also have an impact on European food security, especially in the context of CA.

Similarly, the existing strategies to limit water pollution by nutrients and decrease leakage and hence eutrophication will help fulfilling the WFD -EU Water Framework Directive (CE, 2000)- in terms of the chemical and biological quality of both surface and ground waters.

The innovations proposed by the rhizoculture also have great potential in positively influencing the EU National Action Plans for pesticides. Strengthening the beneficial action of the rhizosphere has proven to increase the resilience of crops to pests and diseases, reducing the need for pesticide use and reinforcing the use of Integrated Pest Management.

The integration of such solutions into European policies can provide excellent outcomes for increased food security and environmental health. Finally, improving agriculture efficiency follows the EU-CC (European Union-Climate Change) objectives for reducing net GHG and increase energy efficiency. The proposal includes some fodder crops such as maize or soybean, which are major protein and energy crops. In this sense, improving their culture will result in more sustainable and cheaper provision of proteins and biofuels, which in turn contribute to meet the EU-CC objectives.

e.3. Conclusions

Agricultural and forestry systems must support the global increasing demands of food and fibre, facing at the same time critical and outstanding issues as fertilizers availability, diffuse contamination, C sequestration, sustainable development, ocean acidification and climate change. Therefore, new nature-based solutions are required to perform the transition towards a circular economic system and a green society.

In response to these issues, after review the evolutionary and ecological dynamics of ECM fungi and lichens, this thesis offers an alternative approach taking advantage of the “rhizo-centric” point of view, where non-photosynthetic partners could be the dominant agents in the symbiosis ECM fungi-plants, how happens in the “lichen model” - *where fungi rather 'farm' the photobionts under 'controlled parasitism'*.-.

The rhizoculture approach -understood as rhizosphere farming- could lead to decrease fertilization and his diffuse contamination; and increase soil C storage, resilience of plants to climate change and other perturbations, and even agricultural and forest production. The rhizoculture proposes a set of easy-to-apply techniques focused on intensifying the abundance, biomass, depth, and activity of roots and the symbiotic microorganisms and fauna associated to the rhizosphere. This ecological intensification would allow to increase N, P and water availability, and C sinks, in soils of crops, rangelands, agroforestry systems and forests.

The hypotheses supporting the “ecological intensification” of the rhizosphere in crops and forests are based on imitating the promising possibilities of the behaviour of some ECM fungi and soil fauna. The *Tuber brûlé* is an interesting biological model for studying the ecological engineering processes associated with the ECM fungal community, soil fauna, calcareous amendments, and the rhizosphere of the host plants. After monitoring natural habitats and cultures of truffles for more than 10 years, this thesis has showed that an increased concentration of active carbonate and exchangeable Ca^{2+} , and decreased contents of TOC and total carbonate (through ECM fungal rock-eating activity) in the soils inside the brûlé, are major factors associated with ecological engineering processes of *T. melanosporum*. In a similar way, *T. aestivum* have showed the capacity to increase soil pH and decrease contents of TOC and total carbonate in the soils inside *versus* outside their brûlés.

In the present study, the ecological engineering strategies associated to the *Tuber brûlés* have been explained through the proposal of two hypotheses, aimed to modify the patterns of soil-plant nutrition, degree of plant mycorrhization, and activity of roots and rizosphere. These hypotheses are: (1) *the “calcium[pH]-induced chlorosis” associated to the “ Ca^{2+} paradox”*; and (2) *the “decrease of the soil TOC content” to modify the ratios of symbiotic/saprophytic communities in the soil*. To reinforce this approach, (i) it has been monitored calcareous amendments in truffle cultivations; and (ii) it has been reviewed bibliography both, on (a) liming impact on ECM and AM mycorrhizal fungi and N-fixing bacteria in crops and forests, and (b) evolutionary and ecological dynamics of ECM fungi.

Preliminary results of this thesis have also confirmed that comparable ecological engineering processes, including rock-eating, could be performed by other ECM fungi, such as *Tuber*

brumale, *T. oregonense*, *T. pseudoexcavatum*, *T. indicum* and mat-fungi species of *Piloderma*, *Hysterangium* and *Ramaria*.

Furthermore, field results obtained in *Tuber* brûlés and bibliographic review show that the synthesis of calcite granules by earthworms is based on C sources that are mainly extracted from the earthworm's dietary intake, SOC and atmospheric CO₂. Therefore the earthworm's calciferous glands could cause a net soil recarbonation, that would also have an impact on (i) the ecological engineering processes in the *Tuber* brûlés, (ii) on the soil carbon sequestration, and (iii) on the rhizoculture strategies.

Soil microarthropod community also responses to the the ability of the *Tuber* brûlé to change soil biogeochemistry. The results show as the modified conditions in *T. aestivum* brûlés negatively affects to some microarthropod groups, while some Collembola groups may be favored or attracted by the *T. aestivum* mycelia activity. In this regard, soil microarthropods could have a positive or negative effect on mycorrhizal fungal fitness and, therefore, in the rhizospheric activity that it is necessary to investigate.

As consequence, a deeper understanding of the ecological intensification of rhizosphere by mycorrhizal fungi and soil fauna in the brûlé, has allowed developing new strategies and technologies -including molecular tools- on rhizoculture.

Therefore, the rhizoculture approach proposes to change our technologies in agro-ecosystems and forests through innovative, easy-to-adopt, economically sound new practices based on the management of: (i) *ecological intensification of roots and rhizosphere through new cultural practices, soil Ca pathways (including liming) and mycorrhizal rock-eating capacity*; (ii) *soil C pools, organic matter content and the ratio of symbiotic/saprophytic communities (through biomass management, and using grazing animals for managing soil biology on wide scales)*; (iii) *N-P-water loops and recycling (including smart use of aquaculture and organic waste)*; (iv) *physical soil properties (trough liming and soil symbiotic microorganisms and fauna)*; (v) *policies aiming to incorporate the socioeconomic value of the externalities associated to rhizoculture.*

In order to generate a set of explanatory-management models on rhizoculture on different scales, it has been proposed a preliminary approach on rhizoculture focusing on 15 crop types (including 4 tree spp.), 15 agroforestry systems (including 3 livestock spp.) and 3 aquaculture systems, because together representing 71.73% of European and 63.85% of global crop areas, and 71.51% of European and 61.88% of global meat yield.

To develop future studies in rhizoculture, 2 proposals of research projects have been submitted to the European Horizon 2020 calls (2017 and 2018). Both proposals have been led by the Polytechnic University of Madrid and have integrated, respectively, 20 and 10 partners of 15 countries. In both proposals, the FAO and two African research centers belonging to the CGIAR Consortium of the World Bank -ICRAF and World Fish- have participated as partners. In summary, the results of this thesis have been published or disseminated through 17 contributions: 8 articles (2007-2014) of journals indexed in the JCR list (3 in Q1, 3 in Q2, 1 in Q3 and 1 in Q4, quartiles); **3 chapters** of international books (2012; 2014; 2017); **3 communications** of international congresses (2010; 2015a; 2015b); **1 seminars cycle (2012)** presented in 6 institutions in EEUU and UE, and the described **2 proposals of H2020 projects (2017; 2018).**

e.4. Future research lines

To develop future studies in rhizoculture, two proposals of research projects -first stage- have been submitted to the European Horizon 2020 calls -RHIZOFARMING in 2017 and RHIZOINTENSE in 2018-. Both proposals have been led by the Polytechnic University of Madrid and have integrated, respectively, 20 and 10 partners of 15 countries (*Spain, Italy, Czech Republic, Switzerland, Germany, Denmark, Finland, Kenya, France, Czech Republic, Belgium, Turkey, Algeria, Egypt and Lebanon*). In both proposals, the FAO (*Food and Agriculture Organization of the United Nations*) and two African research centers belonging to the CGIAR Consortium of the World Bank -ICRAF and World Fish- have participated as partners.

Strengthening trans-disciplinary research for long-lasting implementation of the results obtained, RHIZOFARMING proposal involved an international collaboration of 20 organizations under the 'multi-actor approach' concept, including R+D centers (50%), companies (including SME) (20%), professional associations (farmers and feed producers) (20%) and international organisms (10%), distributed through a pan-European area. This partnership was based on a trans-disciplinary approach including researchers and other actors working on different-scale subjects associated to rhizoculture. These range from the molecular to the landscape level. Additionally, the project included private companies, farmer associations and stakeholders that guarantee the field testing of the project's expected innovations. Applicability pervades all the proposed tasks. The presence of farmers ensured the applicability of participatory action research, where farmers in the field could inform about shortcomings of the proposed innovations, as well as the selection of the best technologies. Additionally the private companies involved in the project would support a market-oriented research that pursues both commercial viability and economic sustainability. Finally, the participation of top-level international organizations with proven influence would warrant an impact on agricultural policies.

RHIZOINTENSE proposal involved an international collaboration of 10 organizations and his design followed the same trans-disciplinary research pattern and 'multi-actor approach' concept.

e.4.1. RHIZOFARMING (2017): a Horizon 2020 proposal to research in rhizoculture

Topic: Closing loops at farm and regional levels to mitigate GHG emissions and environmental contamination: focus on carbon, nitrogen and phosphorus cycling in agro-ecosystems
SFS-30-2017

Rhizoculture technology to manage the closing loops on N, P and C cycling, in European and global agro-ecosystems

Acronym: RHIZOFARMING

TOPIC: H2020 SFS-30-2017; **BUDGET:** 6,999,663.00 Euros

CODE	Profile	Participant organization name	Acronym	Country
1	(Coordinator) Research	Universidad Politécnica de Madrid	UPM	Spain
2	Research	Consejo Superior de Investigaciones Científicas	CSIC	Spain
3	Research	Institut de Recerca i Tecnologia Agroalimentaries	IRTA	Spain
4	Research	Consiglio Nazionale delle Ricerche	CNR	Italy
5	Research	Czech University of Life Sciences	CZU	Czech Republic
6	Research	University of Lausanne	UL	Switzerland
7	Research	Technische Universität Darmstadt	TUD	Germany
8	Research	University of Copenhagen	UCPH	Denmark
9	Research	Natural Resources Institute of Finland	LUKE	Finland
10	Research	International Center for agroforestry	ICRAF	Kenya
11	International organism	Food & Agriculture Organization of United Nations	FAO	Italy
12	International organism	European Forest Institute	EFI	Finland
13	Farmers association	Asociación Agroforestal Española	AAE	Spain
14	Farmers association	Association Française d'Agroforesterie	AFAF	France
15	Farmers association	Český Spolek pro Agrolesnictví	CSA	Czech Republic
16	Companies association	European Feed Manufacturers' Federation	FEFAC	Belgium
17	Company	Micología Forestal & Aplicada	MFA	Spain
18	Company	Fertinagro Nutrientes, S.L.	FN	Spain
19	Company	Sakata Vegetables Europe	SVE	France
20	Company	İSKO Plastik A.Ş.	IPAS	Turkey

Table of Contents

	Page
1. EXCELLENCE	1
1.1 Objectives of RHIZOFARMING	1
1.2 Rhizoculture relation to the work program	3
1.3 Concept and methodology in RHIZOFARMING	4
1.3.(a) Rhizoculture concept	4
1.3.(b) Methodology in RHIZOFARMING	5
1.4 Ambition of the rhizoculture	6
2. IMPACT	9
2.1 Expected impacts of RHIZOFARMING	9

ABSTRACT

Increasing cost of fertilization and progressively limiting reserves of mineable mineral, together with impacts of agriculture on climate and world's ecosystems are casting serious doubts about the sustainability of the current agricultural model. Whilst unable to abandon intensive agriculture in the short run, we urgently need reduce its reliance on man-made soil amendments and thus to improve its sustainability. One of the ways to achieve this is to design a rhizosphere-centered approach entailing a suite of easy-to-apply solutions aiming to increase recycling of soil nutrients. RHIZOFARMING addresses an existing bottleneck constituted by the root/rhizosphere microbiome exchange of N, P and C cycling in agro-ecosystems. The project builds on the latest understanding of the role of soil beneficial organisms as a determinant of (i) the productivity of a rich array of crops at the farm level (ii) pollution and GHG at the farming and landscape levels, and (iii) waste management in intensive plant and animal production (including aquaculture) systems. The project will develop innovative and economically sound agricultural practices designed to maintain productivity whilst contributing to sustainable management of farm-to-landscape scale of N, P and C cycles. A carefully selected partnership of academic and industry partners will promote market-oriented innovation ready for commercial application. Farmer associations will provide a framework for a participatory-joint research, creating an environment designed to generate viable prototypes & technologies, such as new fertilizer & biofertilizer lines, screening rhizoculture-fit plant varieties aimed at future breeding programs, or innovative fodder designs featuring enzymatic complexes that link animal production to the management of nutrient loops in a circular economy. Finally, the support of key actors in European and global advocacy for agricultural policy provides a platform for effective communication-dissemination.

1. Excellence

1.1 Objectives

Vision: *A world applying circular economy to achieve a more efficient, less costly and less pollutant food production.*

Mission: *To promote rhizoculture in Europe as a nature-based solution to improve quality food production, reduce fertilizer use and tackle climate change from the farm to the global scale.*

Need and opportunity

Since the 1930s, the **Green revolution** has replaced traditional agriculture with conventional agriculture (CA) through high inputs of chemical fertilizers and new methods of intensive cultivation. These technologies saved over a billion people from starvation, but nowadays entail serious sustainability issues due to the supply limitation of fertilizers from current sources, greenhouse gas (GHG) emissions and diffuse pollution^{1,2}. Moreover, farmers around the world only collect 50% of the yield they would obtain under optimal conditions³, with 60-70% of the **yield losses** attributable to abiotic factors such as nutritional deficiencies or drought. The remaining 30-40% loss is attributed due to biotic factors connected to the rhizosphere³. While **organic agriculture (OA)** promotes organic fertilizers and outperforms CA in most environmental and social well-being aspects, critical questions arise on whether OA benefits compensate for its lower crop yields or if it maintains soil fertility levels over time^{4,5,6}.

New solutions and **accompanying policies** are urgently **needed** to improve OA and CA yields and meet the “**Greening**” requirements established by the **Common Agricultural Policy (CAP)**, and targeting **current challenges** for agriculture, such as growing welfare, changing diets, food security of a growing global population (needing 50% more food in 2050⁷), and climate change mitigation. **Horizon 2020 Societal Challenge** analyses indicate that **new nature-based solutions**, which include the multiple functions of agro-ecosystems, are needed for the transition towards a **circular economic** and **ecological innovation** system contributing to a **green society**.

RHIZOFARMING thus develops a ground-breaking concept of “**rhizoculture**” (i.e. *farming the rhizosphere*), through two main solutions (i) new **practices & technologies**, and (ii) new **Prototypes** applied to agro-ecosystems, by increasing the abundance, biomass, depth and activity of roots and their beneficial soil microorganisms in crops & rangelands. **Rhizosphere** is the main **bottleneck for closing loops on N, P & C cycling**, as the roots allow the integration of plant photosynthesis, and the activity of their associated organisms (leading to interlocking of C, P and N inputs)^{8,9}. Within this framework, **RHIZOFARMING** also proposes **policies** that **integrate rhizoculture** to crop and animal production, climate change mitigation, socioeconomics, consumers and markets.

Project overview, logical framework and specific objectives

Beneficial soil organisms targeted by **rhizoculture** can **increase crop yields** from **115%** up to **300%**^{10,11}, with stronger effects in stressful settings such as those **by climate change** & others¹², and have a great potential both in organic and conventional agro-ecosystems. **Rhizoculture** can **increase shoot** and **root N and P content** in a reduced fertilizer input context in CA¹¹; while in OA, rhizoculture can potentially **decrease leaching of P and N** caused by increased nutrient mobility in soils¹³. Together with an improvement and integration of production and efficiency of **animal feeding**, this approach will allow for an improvement of the **management of closing loops on N, P and C cycling** in agro-ecosystems. Rhizoculture will therefore have direct positive impacts on the **environment, food and energy security** in international **trade**, and on **consumer behavior**. The major **C sinks** in terrestrial ecosystems including those devoted to agriculture are to be found in soils¹⁴, highlighting the immense potential of rhizoculture to increase **C storage** with adequate management. Rhizoculture would allow increasing the **input of inorganic C into the seas** through river water flows, reducing **ocean acidification**³¹. **Table 1** draws up the **6 specific objectives** and **10 WPs** designed to efficiently deliver on them.

Table 1. Specific objectives & logical framework of RHIZOFARMING (timeline in months; partner acronyms in bottom of Table-2; TRL = Technology Readiness Level)

Action area (AA)	OBJECTIVES <i>WHERE TO?</i>	Achievements <i>WHY?</i>	Work packages <i>HOW?</i>	Deliverables List <i>WHAT?</i>	TRLs		Timeline <i>WHEN?</i>	Partners <i>WHO?</i>
					from	to		
AA.1- Scientific breakthroughs	Obj. 1- Promote root development and rhizosphere activity at lab & farm scales	Increased knowledge on rhizosphere mechanisms that intensify roots & soil symbiotic micro-organisms	WP1- <i>Molecular and physiological analysis of mechanisms involved in intensifying roots & rhizosphere</i>	Decision support systems (web based) addressing: (1) information on needs of farmers; (2) protocols on PES ¹ C markets valuation at farms	3	4	01-48	CNR , LUKE, CSIC, IRTA, UPM
		Intensification of rhizosphere at farm scale (by <i>OA-CA practices, liming & ratios of symbiotic/saprophytic soil communities</i>)	WP2- <i>Intensify roots & rhizosphere, and biogeochemical loops of N, P & C by rhizoculture at farm scale</i>		3	5	13-48	CSIC , IRTA, CZU, UL, LUKE, UPM
	Obj. 2- Promote root development and rhizosphere activity at landscape scale	Assessment of impact of rhizosphere on <i>N & P availability & C fixation in landscape</i>	WP3- <i>Biogeochemical of N, P, C loops, GHG & pollutants at landscape scale</i>	Decision support systems (web based) addressing: (1) information for stakeholders on CC ³ mitigation; (2) protocols on PES ¹ & C markets valuation at landscape ⁴	3	5	01-48	CZU , UL, UPM
		Intensification of rhizosphere & <i>C fixation in landscape (by management of extensive livestock, social insects & worms)</i>	WP4- <i>Impact analysis of extensive livestock management & interactions with soil invertebrates</i>		3	5	01-48	ICRAF , UPM
	Obj. 3- Improve the role of intensive animal production, including aquaculture, in the circular economy of fertilizers	Access to new sources of N and P (including <i>aquaculture</i>)	WP5- <i>Nutrient cycling among farms, agricultural-animal wastes, fertilizers, fodder in circular economy</i>	Innovations on (bio) fertilizers; propagules & microorganism-plant systems; N & P extraction from wastes systems; animal feeds screening for future breeding on rhizoculture	3	5	13-48	UCPH , UPM, TUD
		Indirect intensification of rhizosphere in farms (by <i>intensive livestock & fodder</i>)						
AA.2- Technological innovation in practice and application	Obj. 4- Farmers and companies will increase know-how to manage closing N,C,P loops in a sustainable way	Increased production in crops (linked to <i>N & P bio-availability</i>); decreased levels of fertilizers & GHG & pollution; increased levels of C sequestration & crops resilience (to <i>climate change & pests</i>)	WP6- <i>Apply technological tools associated to rhizoculture</i>		3-4	6	06-42	SVE , IRTA, FN, MFA, IPAS
			WP7- <i>Implement Prototypes of rhizoculture through participatory action research</i>	Prototypes (web based) for lower fertilizers & higher production	3	7	06-42	AFAF , AAE, CSA ² , ICRAF
AA.3- Policies for non-technical and social innovations	Obj. 5- Modeling socioeconomic impacts of improved N, P and C management through rhizoculture	Assessment of the potential impact of rhizoculture's innovations	WP8- <i>Application of trade, environmental, nutritional & food security models (farm to CAPRI³ model) to assessing the rhizoculture</i>	Models (web based) on environmental & socioeconomic impact assessment	4-5	8	13-48	UPM , TUD, UCPH
	Obj. 6- Achieve a positive political environment for successful application of rhizoculture	Rhizoculture outcomes will translate into sound, clear policy recommendations (by <i>agrarian extension programs; dissemination in society by eco-labeling products "from sustainable N, P & C management", enabling to responsible consumer choice</i>)	WP9- <i>Integration of rhizoculture in socioeconomic & agricultural policies & extension (dissemination-communication)</i>	Writing products; courses & events; specific delivers (i.e., <i>eco-labels</i>); innovative communication strategies	7	8	31-50	FAO , ICRAF, CNR, EFI, AFAF, AAE, CSA ² , FEFAC
			WP10- <i>Coordination, project management & Advisory board</i>	Intermediate & final reports	7	8	01-50	UPM , CNR, FAO, ICRAF, EFI, FEFAC, AFAF, AAE, CSA ²

¹[PES = mushrooms and truffles production]; ²[CSA is currently supporting but not involved in the project]; ³[CC = climate change]; ⁴[landscape level]

1.2 Relation to the work programme

Regarding the specific challenge

Specific Challenge.- Agriculture contributes significantly to N, P & C losses through land use change, soil C losses, animal production, fertilizer use, GHG emissions and increasing discharge in water.

RHIZOFARMING will assess the impact of agricultural disturbances on N, P, C losses, through remote sensing, flow monitoring & *in-situ* analysis in representative scenarios at different scales, promoting change of practices.

Specific Challenge.- Mitigation solutions need to be based on a thorough understanding of the cycling of N, P and C at various levels, to achieve lower emissions and pollution, and higher C sequestration.

RHIZOFARMING will use rhizosphere analysis and management at multiple scales to (i) better understand the N, P and C cycling; (ii) reduce GHG & pollution; (iii) increase C sequestration; and (iv) inform related policies.

Specific Challenge.- An integrated approach is needed to identify factors and mechanisms which govern those cycles ranging from agricultural management to consumption patterns.

RHIZOFARMING will integrate rhizoculture & new Prototypes of agro-ecosystems into innovative practices with (i) inputs & outputs in crops, livestock, aquaculture, and their waste recycling; and (ii) consumption patterns.

Regarding the scope

Scope.- Proposals will provide a comprehensive analysis of N, P and C flows and cycling on farms and within landscapes, with different types of production systems and impacts of land use intensification.

RHIZOFARMING will design models on rhizoculture & Prototypes of agro-ecosystems, from farms to landscape levels, optimizing N, P & C in OA, CA, agroforestry and animal farming, including aquaculture.

Scope.- Work shall consider trade-offs and synergies between the different impacts of N, P and C cycles (on climate, water quality, air, soil) as well as with farm productivity and the quality of agricultural products.

RHIZOCULTURE will balance N, P, C inputs & outputs among farms, livestock & aquaculture, promoting sustainable-labeled products with improved protein/carbohydrate ratios, enabling to responsible consumer choice.

Scope.- Proposals will seek for efficiencies and loops, including at the interfaces (i) between plants and animals, (ii) among plant crops; and (ii) within the animal sector.

RHIZOCULTURE will integrate (i) polyculture of “plants-trees-agroforestry-silvopastoralism” & soil organisms; with (ii) inputs & outputs of N, P & C associated to livestock & aquaculture feeds, and waste recycling

Scope.- Prototypes of sustainable agro-ecology systems, including OA, will be developed at various scales from farm level to landscape/territorial level in order to reduce GHG emissions and nutrients intensity.

RHIZOFARMING will show case efficient Prototypes of agro-ecosystems, from farms to landscape levels, decreasing GHG and fertilizer use in different biogeographic regions and guiding practice and policy change.

Scope.- Proposals will also tackle consumption patterns and establish how demand-side management can be paired with efforts to lower emissions and optimize C, N and P loops.

RHIZOFARMING will internalize rhizoculture as a key component of the green economy by (i) optimizing value chains from farms to markets; and (ii) labeling products "from sustainable N, P & C management".

Scope.- Looking at international trade in relation to EU supply with energy and proteins as well as land use changes and C sequestration in soils.

RHIZOFARMING will apply and further develop existing models for assessing the impact of rhizoculture on (i) food & energy security management & international trade; (ii) environment and changes in land use and C sinks.

Scope.- International collaboration & multi-actor approach.

RHIZOFARMING will boost knowledge transfer and participatory action research among scientists, technicians, farmers, consumers, companies and international organizations, promoting policy change.

1.3 Concept and methodology

Overall concept underpinning the project: **RHIZOFARMING** aims to solve the current scarcity and high economic and environmental cost of fertilizer use through agricultural practices and technologies that promote N and P recycling, as well as C fixation, through rhizoculture. A multi-actor approach will imply (i) basic & applied research on rhizosphere management, from small to global scales; (ii) innovations of companies on new sources of N & P and nutrient loops focused on a circular economy; and (iii) environmental/socioeconomic models for impact assessment and active advocacy to guide adequate policies for efficient management of roots & rhizosphere.

Main ideas, models or assumptions involved: **RHIZOFARMING** proposes an smart use of ecosystem services provided by roots & soil organisms, through rhizoculture, meaning: (i) low intensity liming (e.g. by earthworms); and (ii) controlling organic matter (OM) input at large scales by extensive livestock or agricultural practices to reduce soil saprophytes and promote symbionts (*because both techniques mimic natural processes that induce several mycorrhizal fungi such as truffles & mat-fungi, as a kind of natural rhizoculture*⁹). This will be combined with well-known OA & permaculture techniques that have been usually directed towards stimulating aboveground growth but that, in this new context, will intensify root development & rhizosphere activity. The final aims of **RHIZOFARMING** are (i) to design new agricultural models (named Prototypes of rhizoculture, both at farm & landscape scales) that not only cultivate plants, but also beneficial soil microorganisms to improve N, P & C loops; and (ii) to integrate technological innovations that reinforce the effectiveness of these Prototypes.

Identify inter-disciplinary considerations and, where relevant, use of stakeholder knowledge: from a feasible number of experimental combinations of crops, livestock and agroforestry types, **RHIZOFARMING** will integrate inter-disciplinary teams working on: crop, soil, soil fauna & livestock studies; rhizosphere studies on closing N, P & C loops; soil respiration and GHG emissions; biogeochemical studies on closing N, P & C loops; plant & soil monitoring by remote sensing and control of the chemical parameters of the rivers; plant & soil studies with molecular tools; recovering N & P in agricultural, livestock & aquaculture wastes; and C cycling in the latter.

RHIZOFARMING is structured through international collaboration of 20 organizations under a multi-actor approach that includes 10 research partners, 4 private companies (including SME), 1 feed industry association, 3 farmers' associations and 2 international organisms (FAO & EFI; acronyms in Table-2). Partners are located in 8 EU countries of 4 bioregions (Boreal, Atlantic, Continental & Mediterranean) and 3 non-EU countries (Kenya, Switzerland & Turkey). Such a setting guarantees a wide representation of farmers, promoting a Participatory Action Research; companies, for a Market-Driven Research; and stakeholders active in the policy advocacy & agricultural extension field. The geographic extent of partners and the relevance of the studied agro-systems set the scene for a pan-European applicability of rhizoculture, but also knowledge and good practice transfer to developing countries & South-North learning, in line with the UN Sustainable Development Goals.

Positioning of the project in the TRL scale (Table 1): while several goals are addressed simultaneously, **RHIZOFARMING** can be considered to cover activities from TRL3 (experimental proof-of-concept) to TRL6 (technology demonstrated in the relevant environment), with a specific work package to tackle TRL7 (system prototype demonstration in operational environment) and 2 specific work packages that will reach TRL8 (system complete & qualified) by mainstreaming direct policy recommendations and large scale applications (e.g. CAPRI²² model) in agricultural extension services, as relevant actors –FAO, ICRAF, EFI– are part of the project.

International and National initiatives linked to RHIZOFARMING: (i) regarding rhizoculture improvement: SoilACE (EC-ID727247) provides solutions for improving agro-ecosystem efficiency for water & nutrients; AM-NITRIFICATION (FP7- ID300298) analyzes the impact of arbuscular mycorrhizae on nitrification; ERASMUS-MUNDUS-PhD provides models on Payments for Ecosystem Services (PES) using product bundles (water conservation, biodiversity & soil C sequestration) to improve forestation & socioeconomic needs of farmers; PLAN NACIONAL studies mycorrhizal networks impact on plant recruitment. (ii) regarding nutrient management: QUINCY (H2020, ID 647204) provides the role of N & P cycles impact on C sinks & water fluxes; PLABIOF (FP7, ID 301443) explains land use change impacts on soil C allocation & biogeochemical cycles; NIT FOR (FP7, ID 908880) checks N impact on assimilated C in forests. COST ACTION FP1305 BIOLINK links belowground biodiversity & forest ecosystems. (iii) regarding innovation: PINBAC (H2020-ID705808) investigates impact of bacterias on N & S plant uptake & decreasing fertilizers; ROOTPOWER (FP7-ID289365) uses a tomato mapping to produce physiological/genetic information on rootstock-mediated performance under abiotic stresses; RHIZO (FP7-ID235879, 2010-12) explains rhizospheric biofilms in symbiotic associations; ROOTS (FP7-ID326202, 2013-15) analyses how rhizosphere interacts with aboveground armament for defense against abiotic stressors. ENV-2011-ECO-INNOVATION ECOZEO develops a pool of novel and eco-efficient applications of zeolite for agriculture.

METHODOLOGY. Tables 1 & 2 & Fig.1 summarize WPs, expected achievements & deliveries, stages of the methodology & the timing and flow charts of the following 10 WPs (see partner acronyms in bottom of Table-2):

WP-1 led by CNR: *Molecular & physiological analysis of mechanisms involved in intensifying roots & rhizosphere.*

RHIZOFARMING builds on the bottleneck constituted by root/rhizosphere exchanges of N, P & C (lab & plot scales), in order to reduce **fertilizers** use. WP1 will focus on the ground-breaking soil nutrition approach that posits that rhizosphere control host plants through varying signalling (*i.e.*, the *paradox of Ca*⁹). WP1 will provide the bases for viability of Prototypes of rhizoculture (WPs 2 & 7), through (i) the compatibility & reinforcing of rhizosphere associated to crop rotations & polycultures; (ii) increasing density and length of roots.

WP-2 led by CSIC: *Intensify roots & rhizosphere, and biogeochemical loops of N, P & C by rhizoculture at farm scale.* In a parallel to WPs 1 & 7, WP2 will carry out a monitoring of soil microorganisms (*by high-throughput molecular technologies focusing on comparing symbiotic/saprotrophic ratios*) & associated fauna (*by QBS²² and other indexes*) in the rhizosphere of plots at the farm scale. **N, P & C cycles** (including **GHG & C sinks**) will be monitored & related to the rhizoculture practices focused on intensifying biomass, depth & activity of roots & rhizosphere (*including new resources of N & P in deep soils*), and technologies implemented in WPs 5 & 6.

WP-3 led by CZU: *Biogeochemical analysis of N, P, C loops & GHG emissions & pollutants at landscape scale.* In connection with WPs 2, 4 & 7, WP3 will monitor **N, P, C loops, GHG & pollutants & C sinks** at landscape level, by temporal & spatial series of scenarios. The aim will be comparing scenarios with different land uses (*i.e.*, *CA* vs. *OA*) in order to explain the rhizoculture approach. Monitoring will be based on (i) remote sensing; (ii) soils analysis in stratified transects (*interpolated with geospatial statistics*); and (iii) water analyses at river and groundwaters.

WP-4 led by ICRAF: *Impact analysis of extensive livestock management & interactions with invertebrates.* In connection with WPs 2, 3 & 7, WP4 will monitor **extensive livestock** impact on **N, P, C loops, GHG & C sinks** at landscape scale. The ground-breaking approach is that livestock is a large-scale tool for managing soil biology, because modifies OM, N & P inputs in soils, which in turn modify the ratio symbiotic/saprotrophic communities.

WP-5 led by UCPH: *Nutrient cycling among farms, agricultural-animal wastes, fertilizers and fodder, in a circular economy.* In addition to the access to N & P resources in deep soils (WP2), WP5 will provide **N & P resources** from **wastes** (from agriculture, aquaculture & livestock), through integrating the “smart managing” of (i) farming processes & (ii) **innovations** on N & P recycling (*i.e.*, *by membrane & polymer technologies*). Regarding the ground-breaking approach of WP4 on livestock, WP5 will research on **innovative fodders** (*including agricultural wastes & enzymatic complexes*) that link **animal production** with the nutrient loops, based in a **circular economy**.

WP-6 led by SVE: *Apply technological tools associated to rhizoculture.* Starting from WPs 1, 2, & 5, an academic & industry partnership will promote **market-oriented research** focused on commercial viable innovations on fertilizers, new sources of N, fodder designs & screening rhizoculture-fit plant varieties (see deliveries list).

WP-7 led by AFAF: *Implement Prototypes of rhizoculture through participatory action research.* WP7 will develop innovative Prototypes of rhizoculture through economically sound agricultural practices designed to maintain productivity whilst contributing to sustainable management of farm-to-landscape scale of N, P & C cycles. WPs 6 & 7 will integrate **industry (Market Oriented Research) & farmer associations (Participatory Action Research)** within a participatory-joint research focused on viable rhizoculture Prototypes. **Table 2** shows the **basis to design Prototypes** of rhizoculture based on a set of crops selected in basis to FAOSTAT productivity **data** & a bibliographic review⁹ (*in order to compare crops, i.e., cover vs. row crops*). **Table 2 also includes** a set of agroforestry & land uses (*i.e., buffer strips*), with the aim to expanding rhizoculture through crop related scenarios, such as *arable land vs. adjacent forests; field margins vs. hedges-trees; N-fixation trees*.

WP-8 led by UPM: *Application of trade, environmental, consumer, nutritional & food security models (from farm to CAPRI model) based on rhizoculture.* Information from the previous WPs, will feed & integrate agricultural, environmental & socioeconomic **models** at multiple scales (*from farm to European CAPRI models*), that allow to **measure** the potential **impact** of **RHIZOFARMING**'s innovations.

WP-9 led by FAO: *Integration of rhizoculture in socioeconomic & agricultural policies & extension programs (dissemination-communication).* Previous WPs outcomes will translate into sound **policy recommendations, agrarian extension & dissemination-communication.** **RHIZOFARMING** counts on partners that are decisive for **agricultural policies**, *i.e.* **FAO, EFI, ICRAF** (CGIAR consortium), **EC, AFAF, CNR, CSA, AGFE & FEFAC** as members of the EU civil dialogue groups & the European Network for Rural Development. Dissemination-communication & policy advocacy activities will take the project results to the policy application stage.

WP-10 led by UPM: *Coordination & project management including an advisory board.* This WP will use managing project tools, *i.e.* **GA** (decision-making body) or steering committee (decision-implementing body). Moreover, WP10

will provide an **Advisory Board (M3AB)** with the aim of guaranteeing that the project follows the principles of **multi-actor approach, Market Oriented Research & Participatory Action Research**, to assuring that **innovations** will be effectively demand-driven through the project. M3AB will meet at least once a year for coordination meetings, & its members will be invited to workshops & consulted electronically as required.

Deliverables list: RHIZOFARMING will deliver (I) structured information coupled to decision support systems on rhizoculture (web based) addressing: [(i) information and advisory needs of farmers; (ii) protocols of valuation of C market products & PES (focused on new CAP policies) in farms (WP1 & 2), rangelands & landscape level (WP3 & 4)]; **(II) innovative knowledge with/without patent** protection on: [(i): fertilizers & biofertilizers, (ii) propagules of symbiotic microorganism & mycorrhizal/inoculated plants, (iii) systems of N & P extraction from wastes, (iv) animal feeds (WP5 & 6)]; **(III) screening of** new germplasm and elite lines as rootstocks aimed at future tailored breeding programs on rhizoculture (WP6); **(IV) establishment of Prototypes of rhizoculture (web based)** focused in designing of agroecosystems, at farm & landscape levels, relying on lower inputs of fertilizers & high agricultural production with lower costs (WP7); **(V) establishment of models (web based) of environmental & socioeconomic impact assessment on rhizoculture** (i.e., about value chains from farms to markets; or European approaches by CAPRI model) (WP8); **(VI)** outputs will be delivered appropriately for each stakeholder, including: [(i) writing products (i.e. open-access research papers, EIP-AGRI practice abstracts, articles in farmers' press, patents or policy briefs); (ii) courses & events; (iii) specific delivers, such as eco-labeling of products with sustainable management of N, P & C (aimed to allow responsible choice of consumers); (iv) innovative communication strategies (web-based, videos & social media). RHIZOFARMING aims to establish an international on-line platform (*beyond the project life*) for communication, dissemination & learning across countries and stakeholders, in order to foster novel knowledge and innovation (WP9). **(VII) WP10** will deliver intermediate & final reports.

Plan for communication, dissemination and exploitation of results: RHIZOFARMING will promote multi-directional communication allowing for continuous rhizoculture input & feedback as well as ensuring project impact beyond the consortium partners. Wider discussion and dissemination of the innovative results and scientific papers will target a broad range of stakeholders, from the general public to the scientific community and policy makers, and all along the agro-forestry production and value chain, from farmers' communities, extension agents, advisors, and agricultural schools/faculties to the industry, making wide use of existing communication channels. The overall integration of **RHIZOFARMING** scientific contributions and learning from each work package will be transformed into actionable information for the wide range of stakeholders in WP9.

Risk plan: The current proposal will incorporate a risk plan which will detail the risks that may occur during the project and a list of risk-mitigation measures. Roughly, risks will comprise: (i) project management (i.e. *delay in outputs from precedent WPs, deliverables, etc.*); (ii) unforeseen episodes on experiments (i.e. *extreme weather, lab accidents*); (iii) innovation transference (i.e. *low farmer participation, unwillingness to implement results/outputs*).

Gender dimension & rRRI: This proposal will follow the “requirements of the Responsible Research Innovation” (rRRI), such as Gender equality in the research teams, farmers and technicians involved. The RRI and gender requirements will also remain in the future dissemination, communication and policy processes of this project.

1.4 Ambition

Utilisation of the mutualistic **plant–microbe interactions** in the **rhizosphere** offer a novel approach to enhancing **agricultural productivity** while reducing **environmental costs**, given that these mutualisms can help **increase crop** production & reduce yield losses by improving **resilience** to edaphic, biologic & climatic variability¹⁵. The main ambition of **RHIZOFARMING** is to develop an **alternative approach** to current agriculture practices, where the rhizosphere is put in the spotlight of agricultural interventions.

Better understanding of mechanisms leading to rhizosphere intensification (WP1): RHIZOFARMING is proposing an alternative approach to the traditional **plant-centric point of view** (where the plant's photosynthesis is theoretically the dominant agent in the symbioses), focusing on a **rhizo-centric approach**, where the rhizosphere (roots & symbiotic partners) could perform the dominant role⁹. In 1989, a new “**rhizocentric model**” was proposed as a promising tool for agriculture¹⁶. Since 2000, this approach was renewed as the “**rhizocentric view**”, which considers that **root-microbial** interactions may be **key processes** in controlling plant productivity^{17,18,19}. Recently, it was observed that numerous **fungi & bacteria** modify rhizospheric **environment** (i.e., *through pH & Ca levels*), leading to intensifying rhizospheric activity through **increasing** root development, **mycorrhization** (leading to **P inputs**) & nodulation of **N-fixing bacteria** (leading to **N inputs**), which has a positive effect on host **plant productivity** (leading to **C inputs**)^{9,20,21,22,23}. **RHIZOFARMING** would allow a building on this novel approach based on microorganism control of **rhizospheric interactions**, to optimizing crop **yields**

Table 2. Basis to designing Prototypes of rhizoculture focused on N, P & C loops management through practices and technologies on crops & animal production, at different scales

Countries	Institutions ⁷ working on modeling technology & dissemination	Institutions ⁷ working on Agro-ecosystem Prototypes	Agro-ecosystem Prototypes based on rhizoculture	Annual crops to combining in rotations & polyculture into rhizoculture Prototypes			Tree crops to integrating in rotations & polyculture into rhizoculture Prototypes	Intensive farming to integrating with N, P & C cycling into rhizoculture Prototypes	agroforestry to integrating with N, P & C cycling into rhizoculture Prototypes		Alternative sources of P & N to intensifying (in a circular economy)	C sinks to intensifying (in a green economy)
				Crop type	Deeper roots	Shallower roots			Livestock and crops, and natural grasslands	Small forest, dehesa & crop mosaics		
Finland	EFI	LUKE	Boreal Prototypes	Cover	Wheat; millet ⁶	Barley	Apple ¹	Cow; pig	Extensive cattle	<i>Pinus sylvestris</i> ; <i>Populus</i> spp.; <i>Alnus glutinosa</i> ³	Deep soil resources through roots	
				Rows	Tomato	Potato						
				N-fixers	Alfalfa	Green peas						
France; Denmark ⁵ Belgium	SVE; UCPH; FEFAC	AFAF	Atlantic Prototypes	Cover	Wheat; maize; millet ⁶	Barley	Apple ¹ ; walnut ¹					
				Rows	Tomato	Potato						
				N-fixers	Alfalfa	Green peas; soybeans			Extensive cattle & sheep	<i>P. sylvestris</i> ; <i>Quercus robur</i> ; <i>Populus</i> spp; <i>A. glutinosa</i> ³	Agriculture waste treatments (high-tech)	Rhizoculture and soil C sinks
Germany; Switzerland; Czech Republic	UL; TUD; CZU	CSA	Continental Prototypes	Cover	Wheat; millet ⁶	Barley	Apple ¹	Cow; pig; aquaculture				
				Rows	Tomato	Potato						
				N-fixers	Alfalfa	Green peas						
Spain; Italy; Turkey	CNR; CSIC; MFA; FN; IPAS	AAE; UPM; IRTA	Mediterran. Prototypes	Cover	Wheat, maize; millet ⁶	Barley	Apple ¹ ; walnut ¹ ; hazelnut ² ; olive ²		Extensive cattle, sheep & pigs; wild herbivores; rice-fishing	<i>Q. ilex</i> ; <i>P. sylvestris</i> ; <i>Populus</i> spp.; truffles ⁴ ; <i>A. glutinosa</i> ³ ; <i>Ceratonia siliqua</i> ³ ; soil fauna; social insects	Aquaculture waste treatments (high-tech) and aquaponics	Livestock and soil C sinks
				Rows	Tomato	Potato						
				N-fixers	Alfalfa; dry beans	Green peas; soybeans						
				Wetlands	Rice (paddy) ⁵							
Argentina				Cover	Maize; millet ⁶	Barley			Extensive cattle & sheep	-		Aquaculture and soil C sinks
				Rows	Tomato	Potato						
				N-fixers	Dry beans	Soybean						
Kenya	FAO	ICRAF	Tropical Prototypes	Cover	Maize; millet ⁶	Barley	-		Extensive cattle & sheep; wild herbivores	Soil fauna; social insects	Aquaculture waste treatments (soft-tech) and aquaponics	Aquaculture and ocean C sinks
				Rows	Tomato	Potato						
				N-fixers	Dry beans	Green peas						
				N-fixers	<i>Lathyrus sativus</i> ; <i>Sesbania rostrata</i>							
Philippines				Wetlands	Rice (paddy) ⁵			Aquaculture	Rice-fishing	-		

1[Deeper roots]; 2[Shallower roots]; 3[N-fixing roots]; 4[Truffles = mushrooms and truffles production]; 5[Including N-fixer plants as *L. sativus* and *S. rostrata*]; 6[Catch crops]; 7[INSTITUTIONS: (EFI = European Forest Institute); (LUKE = Natural Resources Institute of Finland); (SVE = ; Sakata Vegetables Europe); (UCPH=University of Copenhagen); (FEFAC = European Feed Manufacturers' Federation); (AFAF = Association Française d'Agroforesterie); (UL = University of Lausanne); (TUD = Technische Universität Darmstadt); (CZU = Czech University of Life Sciences); (CSA = Český Spolek pro Agrolesnictví is currently supporting but not involved in the project); (CNR = Consiglio Nazionale delle Ricerche); (CSIC = Consejo Superior de Investigaciones Científicas); (MFA = Micología Forestal & Aplicada); (FN = Fertinagro Nutrientes, S.L.); (IPAS = ÍSKO Plastik A.Š.); (AAE = Asociación Agroforestal Española); (UPM = technological University of Madrid); (IRTA = Institut de Recerca i Tecnologia Agroalimentaries); (FAO = Food & Agriculture Organization of United Nations); (ICRAF = International Center for agroforestry)]

Influence of the rhizosphere in global biogeochemical cycles (WP2 & 3): a rhizocentric model can have a wide impact in nutrient cycles. RHIZOFARMING proposes to change our technologies in agriculture using the 'rhizoculture' approach with innovative, easy-to-adopt, economically sound new practices based on the management of: (i) soil microorganisms (increasing symbiotic against saprophytes) and OM (by livestock & aquaculture); (ii) roots (through new cultural practices); (iii) soil Ca pathways (by soft liming); (iv) rock-eating capacity of mycorrhizae; (v) physical soil properties (with symbiotic microorganisms & fauna, such as ants, termites & earthworms); aimed at having a decisive influence on the C fixation & N, P availability from farms to a wider scale agro-ecosystems. In order to generate a set of explanatory & management models on rhizoculture on different scales, 15 crop types (including 4 tree spp.) and 15 agroforestry systems (including 3 livestock spp.) have been selected (Table 2), together representing 71.73% of European and 63.85% of global crop areas.

Managing soil communities (symbiotic/saprophytic) by controlling organic matter inputs in rangelands (WP4): while rangelands occupy more than 50% of lands²⁴ and almost all drylands, livestock has a decisive role in processing rangeland primary productivity (up to 85%)²⁵. It has been shown that improvements in cellulose digestibility (sustainable intensification) has potential of CH₄-derived emission reductions and poverty reduction through increased productivity²⁶. The processes in the digestive system are key, as many soils host reduced microbial activity, so invertebrates have a decisive potential role in managing OM (ants incorporate large quantities of manure to the soil²⁷ and termites support similar functions²⁸ occupying 68% of land & process 28% of terrestrial net primary productivity²⁹). RHIZOFARMING will study 3 livestock species representing 71.51% of European and 61.88% of global meat yield, evaluating the influence of livestock management models and deeping in the role of invertebrates in soils, with particular attention to social insects and earthworms.

Reduce N, P & C loss in intensive animal production including aquaculture (WP5): large proportion of nutrients leach out due to uptake inefficiency in agro-ecosystems. RHIZOFARMING will promote the circular economy by better connecting production systems in crops, intensive livestock and aquaculture.

Implement field trials in association with the private sector to apply project innovations (WP6 & 7): to avoid that research innovations do not reach producers, RHIZOFARMING will use Participatory Action Research where crop farmers will be learning from the novel approaches and will influence results through their views. At the same time, the participation of private companies will facilitate innovation transfers and will support the application and validity of proposed Prototypes as well as their adequacy to markets (Market Oriented Research). Innovations found by the project will generate the ambition in the participating companies to register fodder and other patents that will generate benefits and stimulate further development of rhizoculture in Europe.

Promote the transposition of rhizoculture innovations into socioeconomic impact modelling and into agricultural policies (WP8 & 9): Faced with climate change & food production challenges, comprehensive socioeconomic models i.e. CAPRI will be used to build possible future scenarios, driven by different agricultural policies. At the same time, research results seldomly translate into transformative agricultural policies. RHIZOFARMING includes the major global actor at elaborating agricultural policy guidelines, i.e. the FAO, among its partners, as well as several important international organizations with a significant experience in policy influence or research transfer (ICRAF). The choice of crops & livestock species with multiregional importance guarantees the interest of the project's results, both for agricultural policies and for the multiple actors involved.

2. Impact

2.1 Expected impacts

Effective solutions for N, P and C efficient agro-ecosystems: A major objective of this project is to promote the natural ability of roots and beneficial soil organisms to close N, P & C loops. Thus, relevant problems regarding N fixation costs, P fertilization and C-mediated climate change will be addressed. Estimates of overall efficiency of fertilizer are lower than 50% for N and less than 10% for P³³. Investigating the application of the RHIZOFARMING model to extensive livestock introduces a new functionality to rangelands, which can use grazing animals for managing soil biology on wide scales. The same applies to convertible husbandry crops, which can alternate grazing with other uses. RHIZOFARMING provides easy-to-adopt tools bearing an economic benefit to farmers, as the improved efficiency of N, P & C use (decreasing over 25% current inputs from fertilizers³⁴) should translate into higher yields, lower costs, and healthier and more resilient crops. The tools derived from this project are intended to be readily up-scalable. The participation of partners such as the FAO or some of the most important agricultural associations in the EU should greatly help in forwarding such tools to stakeholders and other potential beneficiaries.

Improved overall sustainability and innovation capacity of the farming systems: Improved nutrient capture by plants, mediated by adequate rhizosphere manipulation, will reduce the amount of fertilizer inputs to maintain crop productivity. An enhanced circular economy in agro-ecosystems means reduced economic costs and extended lives for non-renewable resources, such as P or the amount of fossil fuel necessary to capture N from air. More importantly, a robust framework that promotes cost-saving measures guarantees a wide adoption of the practices promoted by the project. Such goals are obviously attractive for the private sector –fertilizer companies and others. The most costly steps in fertilizer production are associated with N production or P mining, so innovative research that promotes nutrient capture by rhizospheres will be an attractive investment for industry. Identifying plant varieties more suited to rhizoculture is also a promising and innovative goal.

Reduction of impacts on environmental issues: reducing GHG emissions, improvement of ground and surface water quality: Increasing resource use efficiency through rhizosphere manipulation is expected to improve environmental sustainability. In the case of N fertilization, for example, beneficial impacts will range from energy saving, to reduced GHG emissions or higher C fixation– related to the Greening areas designated by the CAP that might balance C losses in conventional croplands – or the diversion of C into the deep ocean (through fungal rock eating). The increased efficiency promoted by RHIZOFARMING will also reduce P mining and P & N leaching into waterways and aquifers. Aquaculture, the fastest-growing animal production sector worldwide, has experienced serious constraints in many countries because of its high impact on surface & groundwater quality, mainly due to high N pollution. Once again, the efficiency in N use sought by the project will translate into reduced discharges and enhanced water quality. Our partnership with organizations such as FAO, with active participation in forums such as UNF3C on climate change or UNECE on surface waters, should help extend the project's conclusions to a global scale.

Integrated scientific support to relevant EU policies (CAP, WFD, sustainable use of pesticides, CC objectives): Proposing enhanced crop rotation, polyculture, permanent rangelands, and convertible husbandry crop planting, fits into current CAP guidelines on Greening, and the same is true for reducing discharges from plant fertilizers and increasing C fixation. CAP Greening targets³⁰ of 5% of ecological focus areas and 5% of grasslands in Europe's agricultural lands is a realistic goal with the efficiency measures addressed by RHIZOFARMING. The environmental gains promoted by the project should also have an impact on European food security, especially in the context of CA. Similarly, the existing strategies to limit water pollution by nutrients and decrease leakage and hence eutrophication will help fulfilling the WFD in terms of the chemical and biological quality of both surface and ground waters. The innovations proposed by RHIZOFARMING also have great potential in positively influencing the National Action Plans for pesticides. Strengthening the beneficial action of the rhizosphere has proven to increase the resilience of crops to pests and diseases, reducing the need for pesticide use and reinforcing the use of Integrated Pest Management. The integration of such solutions into European policies can provide excellent outcomes for increased food security and environmental health. Finally, improving agriculture efficiency follows the EU CC objectives for reducing net GHG & increase energy efficiency. The proposal includes some fodder crops such as maize or soybean, which are major protein & energy crops. In this sense, improving their culture will result in more sustainable and cheaper provision of proteins & biofuels, which in turn contribute to meet the EU CC objectives.

Strengthening trans-disciplinary research for long-lasting implementation of the results obtained: RHIZOFARMING involves an international collaboration of 20 organizations under the 'multi-actor approach' concept, including R+D centers (50%), companies (including SME) (20%), professional associations (farmers & feed producers) (20%) and international organisms (10%), distributed through a pan-European area [CSA supports the project, but is not directly involved]. This partnership is based on a trans-disciplinary approach including researchers and other actors working on different-scale subjects associated to rhizoculture. These range from the molecular to the landscape level. Additionally, the project includes private companies, farmer associations and stakeholders that guarantee the field testing of the project's expected innovations. Applicability pervades all the proposed tasks. The presence of farmers ensures the application of participatory action research, where farmers in the field can inform about shortcomings of the proposed innovations, as well as the selection of the best technologies. Additionally the private companies involved in the project will support a market-oriented research that pursues both commercial viability and economic sustainability. Finally, the participation of top-level international organizations with proven influence will warrant an impact on agricultural policies.

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e.4.2. RHIZOINTENSE (2018): a Horizon 2020-PRIMA proposal to research in rhizoculture

Title of Proposal
Ecological intensification of the rhizosphere for Mediterranean sustainable agricultural development
Acronym: RIZHOINTENSE

1. Administrative data of participant organisations

Participant No *	PI name	Organisation	Country
1 (Coordinator)	Luis G. García Montero	Universidad Politécnica de Madrid (UPM)	Spain
2 Partner 1	Salvador Lladó	Acondicionamento Tasrrasense (LEITAT)	Spain
3 Partner 2	Amghar Fateh	Université M'hamed Bougara Boumerdes (UMBB)	Algeria
4 Partner 3	Ahmed Nasr-Allah	World Fish CGIAR (WF)	Egypt
5 Partner 4	Nouran El Said	Agrimatic Farms (AgriF)	Egypt
6 Partner 5	Teodardo Calles	Food and Agriculture Organization of the United Nations (FAO)	Italy
7 Partner 6	Paolo Carnemolla	Federazione Italiana Agricoltura Biologica e Biodinamica (FederBIO)	Italy
8 Partner 7	Hussam Hawwa	DIFAF s.a.l (DIFAF)	Lebanon
9 Partner 8	Cafer Turkmen	University of Çanakkale Onsekiz Mart (UÇK)	Turkey
10 Partner 9	Engin Yilmaz	YOLDA initiative (YOLDA)	Turkey

2. General information of the pre-proposal

Section:	Section 1. Farming Systems 2018
Call:	PRIMA (HORIZON 2020)
Topic:	Improving the sustainability of Mediterranean agro-ecosystems
Type of action:	RIA Research and Innovation action
Duration in months:	36 months
Free keywords	<i>ecological intensification, biofertilizers, biochar; rizhoculture, rhizosphere, roots, agroforestry, livestock, aquaculture, food quality</i>
Abstract:	<i>The aim of RHIZOINTENSE is to develop cropping systems with low-input and low environmental impact in the Mediterranean basin. The project will develop an alternative to current agriculture practices based on a novel concept called "rhizoculture": farming roots and rhizosphere. Rhizoculture proposes a set of Ecological Intensification (EI) strategies and tools to integrate ecological processes into land management, thus enhancing ecosystem services delivery by roots and rhizospheric organisms to produce food in a sustainable manner. This changes the traditional plant-centric point of view and focuses on a rhizo-centric approach, where the rhizosphere could perform the dominant role. RHIZOINTENSE will enhance the smart use of ecosystem services provided by soil, plants, soil organisms livestock and aquaculture for the cultivation of underexploited local cereal, legume and pasture varieties more adapted to the particular climatic conditions of the Mediterranean area under different agricultural systems. To intensify the activity of roots and rhizospheric organisms will allow increasing sustainability, water use efficiency, food production stability and food quality and security. RHIZOINTENSE will address the challenge of the topic by encouraging Mediterranean farmers to introduce innovative tailor-made strategies based on the ground-breaking combination of i) EI strategies and tools including the integration of agriculture with livestock and/or aquaculture, ii) diversification of agroecosystems including organic agriculture and agroforestry, iii) local crop varieties. An interdisciplinary experienced consortium (including industry, farmer's associations, RTOs and SMEs), will closely collaborate with the stakeholder's board to achieve this goal by means of a co-decision and multi-actor approach.</i>

3. General budget

Total amount requested to PRIMA / €: **2,000,000 €**

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g. ANNEXES

g.1. L.G. García-Montero, I. Valverde-Asenjo, D. Moreno, P. Díaz, I. Hernando, C. Menta, K. Tarasconi **(2012)**. *Influence of edaphic factors on edible ectomycorrhizal mushrooms: new hypotheses on soil nutrition and C sinks associated to ectomycorrhizae and soil fauna using the Tuber brûlé model*. In: A. Zambonelli, G. Bonito (Eds.). *Edible Ectomycorrhizal Mushrooms*. Springer-Verlag, Berlin. Pp. 83-104 (BOOK CHAPTER COMPLETE)

g.2. F. Arredondo, L.G. García-Montero, I. Valverde-Asenjo, C. Menta **(2014)**. *Soil quality indicators for forest management*. In: E. Martínez-Falero, S. Martín-Fernández, A. García-Abril (Eds.). *Quantitative Techniques in Participatory Forest Management*. CRC press, Boca Raton. pp 179-240 (BOOK CHAPTER COMPLETE)

g.3. L.G. García-Montero, P. Manzano, D. Alwanney, I. Valverde-Asenjo, A. Álvarez-Lafuente, L.F. Benito-Matías, X. Parladé, S. Ortuño, M. Morcillo, A. Gascó, C. Calderón-Guerrero, F. Mauro, M. Méndez, A. Sánchez-Medina, M.P. Andrés, J.R. Quintana, C. Menta, S. Pinto, L. Pinto, P. Pita, C. Turkmen, C. Pascual, E. Ayuga, F. Torrent, J.C. Robredo, P. Martín-Ortega, J. Pera, L. Gómez, G. Almendros, C. Colinas, E.P. Verrecchia **(2017)**. *Towards integrated understanding of the rhizosphere phenomenon as ecological driver: can Rhizoculture improve agricultural and forestry systems?* In: M. Lukac, P. Grenni and M. Gamboni (Eds.). *Soil biological communities and ecosystem resilience*. Springer International Publishing, Cham, Switzerland. Pp 43-75 (BOOK CHAPTER COMPLETE)

Soil Biology 34

Alessandra Zambonelli · Gregory M Bonito *Editors*

Edible Ectomycorrhizal Mushrooms

Current Knowledge and Future Prospects

Edible ectomycorrhizal mushrooms (EEMMs) comprise more than 1000 species and are an important food and forest resource. In this volume of Soil Biology, internationally recognized scientists offer their most recent research findings on these beguiling fungi.

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Contents

Part I Biology and Ecology of Edible Ectomycorrhizal Mushrooms

1 Laying the Foundations	3
Ian R. Hall and Alessandra Zambonelli	
2 Systematics and Ecology of Edible Ectomycorrhizal Mushrooms	17
Matthew E. Smith and Gregory M. Bonito	
3 Edible Ectomycorrhizal Mushroom Molecular Response to Heavy Metals	41
Sabrina Zeppa, Antonella Amicucci, Roberta Saltarelli, Giovanna Giomaro, and Vilberto Stocchi	
4 Genomics of <i>Tuber melanosporum</i>: New Knowledge Concerning Reproductive Biology, Symbiosis, and Aroma Production	57
Andrea Rubini, Beatrice Belfiori, Claudia Riccioni, and Francesco Paolocci	
5 State of the Art of the Research on <i>Boletus edulis</i>	73
Antonietta Mello	
6 Influence of Edaphic Factors on Edible Ectomycorrhizal Mushrooms: New Hypotheses on Soil Nutrition and C Sinks Associated to Ectomycorrhizae and Soil Fauna Using the <i>Tuber Brûlé</i> Model	83
Luis G. García-Montero, Inmaculada Valverde-Asenjo, Domingo Moreno, Paloma Díaz, Isabel Hernando, Cristina Menta, and Katia Tarasconi	
7 Ectomycorrhizal Fungal Communities of Edible Ectomycorrhizal Mushrooms	105
Alessandra Zambonelli, Mirco Iotti, Siham Boutahir, Enrico Lancellotti, Claudia Perini, and Giovanni Pacioni	

8	Ectomycorrhizal Helper Bacteria: The Third Partner in the Symbiosis	125
	Elena Barbieri, Paola Ceccaroli, Francesco Palma, Deborah Agostini, and Vilberto Stocchi	
 Part II Cultivation of Edible Ectomycorrhizal Mushrooms (with Main Focus on Truffles) and Case Studies		
9	Techniques for Host Plant Inoculation with Truffles and Other Edible Ectomycorrhizal Mushrooms	145
	Mirco Iotti, Federica Piattoni, and Alessandra Zambonelli	
10	Soils and Techniques for Cultivating <i>Tuber melanosporum</i> and <i>Tuber aestivum</i> in Europe	163
	G�rard Chevalier and Pierre Sourzat	
11	Truffle Cultivation in the Southern Hemisphere	191
	Ian R. Hall and Wayne Haslam	
12	Native and Cultivated Truffles of North America	209
	Charles Lefevre	
13	Truffle Cultivation in China	227
	Xianghua Wang	
14	<i>Terfezia</i> Cultivation in Arid and Semiarid Soils	241
	Asunci�n Morte, Alberto Andrino, Mario Honrubia, and Alfonso Navarro-R�denas	
15	Truffles, Timber, Food, and Fuel: Sustainable Approaches for Multi-cropping Truffles and Economically Important Plants	265
	Gian Maria Niccol� Benucci, Gregory Bonito, Leonardo Baciarelli Falini, Mattia Bencivenga, and Domizia Donnini	
16	Cultivation of Basidiomycete Edible Ectomycorrhizal Mushrooms: <i>Tricholoma</i>, <i>Lactarius</i>, and <i>Rhizopogon</i>	281
	Yun Wang, Nicholas Cummings, and Alexis Guerin-Laguette	
 Part III Wild Collected Edible Ectomycorrhizal Mushrooms: Economics, Conservation, Management		
17	Local Communities and Edible Ectomycorrhizal Mushrooms	307
	Eric Boa	
18	Medicinal Aspects of Edible Ectomycorrhizal Mushrooms	317
	Susanna Badalyan	

19	Insects Parasitizing Edible Ectomycorrhizal Mushrooms	335
	Nicola Sitta and Luciano Süss	
20	Edible Ectomycorrhizal Mushrooms: International Markets and Regulations	355
	Nicola Sitta and Paolo Davoli	
 Part IV The Edible Ectomycorrhizal Mushroom Industry in the Age of “-omics”		
21	Ten Years of Genomics for Ectomycorrhizal Fungi: What Have We Achieved and Where Are We Heading?	383
	Francis Martin and Gregory Bonito	
	Index	403

Chapter 6

Influence of Edaphic Factors on Edible Ectomycorrhizal Mushrooms: New Hypotheses on Soil Nutrition and C Sinks Associated to Ectomycorrhizae and Soil Fauna Using the *Tuber Brûlé* Model

Luis G. García-Montero, Inmaculada Valverde-Asenjo, Domingo Moreno, Paloma Díaz, Isabel Hernando, Cristina Menta, and Katia Tarasconi

6.1 Introduction

6.1.1 *A Global Perspective on Soil Organisms and Inorganic C Sinks*

Soil is a complex system where basic processes for terrestrial communities take place (Ruf et al. 2003). Soil represents an important substrate for a large part of the earth's biodiversity. Soil properties determine ecosystem function and vegetation structure, and they serve as a medium for root development and provide moisture and nutrients for plant growth (Minnesota Forest Resources Council 1999). Food chain interactions among the soil biota (including plant roots) have major effects on the quality of crops (affecting human and animal nutrition and other aspects of life on earth), the incidence of soil-borne plant and animal pests and diseases (affecting production

L.G. García-Montero (✉)

Department of Forest Engineering, E.T.S. Ingenieros de Montes, Technical University of Madrid (UPM), Ciudad Universitaria s/n, Madrid 28040, Spain
e-mail: luisgonzaga.garcia@upm.es

I. Valverde-Asenjo • I. Hernando

Department of Soil Science, University Complutense of Madrid, Madrid, Spain

D. Moreno

Association of Truffle Collectors, Peralejos de las Truchas, Spain

P. Díaz

Department of Industrial Chemistry and Polymers, Technical University of Madrid (UPM), Madrid, Spain

C. Menta • K. Tarasconi

Department of Evolutionary and Functional Biology, Section Natural History Museum, University of Parma, Parma, Italy

levels), and beneficial organisms such as edible ectomycorrhizal mushrooms (EEMM) (Brussaard et al. 2007).

Trueman and Gonzalez-Meler (2005) explain that the interplay between plant C intake, soil properties, community structure of the soil organisms and climate agents will determine the biosphere's organic terrestrial C sequestration strength at future atmospheric CO₂ concentrations. They studied tree growth and stabilization of SOM (soil organic matter) in an ecosystem exposed to elevated CO₂ concentrations and reviewed other works, concluding that the inability to retain C in soils exposed to elevated CO₂ may stem from the lack of stabilization of SOM, allowing its rapid decomposition by soil heterotrophs. They explain that increases in heterotrophic respiration in response to elevated CO₂ may result in soils becoming a net source of CO₂ into the atmosphere if input of new C does not keep pace and indicate that these facts make it impossible to forecast soil organic C sequestration potential.

In this scenario, it is important to have soil inorganic C sinks and biological models for the study of soil reaction and the decarbonation–recarbonation soil processes associated with fungal and soil organism communities, vegetation, and climate, in order to evaluate in-soil C cycling and C sequestration.

6.1.2 A Global Perspective on EEMM, Soil Nutrients, and Inorganic C Sinks

Ectomycorrhizal fungi (EM) mobilize soil nutrients, improve soil quality, and are important in the remediation of soil loss (Poma et al. 2006; Lian et al. 2007). Moreover, Wang and Hall (2004) indicate that the most highly prized edible mushrooms belong to the ectomycorrhizal group (EEMM). The total market for these fungi is measured in billions of US dollars, and the production of EEMM provides higher economic returns than any other forest product in many Mediterranean woods. In these areas, truffles (species of *Tuber* Micheli ex Wiggers), *Boletus pinophilus* Pilát and Dermek, *B. edulis* Bull.: Fr., and *Lactarius deliciosus* L.: Fr. are the fungi that yield the highest economic benefits (Moreno et al. 1986; Oria 1989, 1991; Montecchi and Sarasini 2000; Rioussset et al. 2001).

The biology of EEMM has been fully described. However, there are still gaps in our knowledge of EEMM biology, and there is insufficient knowledge of the ecological relationships between EEMM and soil properties, processes and organisms. Among the numerous interactions between soils and EM-EEMM populations, there are two which may be relevant to soil processes on a significant scale: fungal metabolism could have greater impact than is generally estimated on soil reaction (as measured by pH) and on the amount of soil carbonates.

Cooke and Whipps (1993) report that fungal mycelia excrete H⁺ as a result of the cation metabolism. This H⁺ excretion is linked to K⁺ capture, which occurs by

means of active transport, and exceeds concentration gradients of 5,000:1. Therefore, fungi could strongly acidify their immediate soil environment (hyphosphere), and their mycelia could provoke a significant change in soil properties and soil–EM–plant interactions. One of the immediate consequences on the soil acidification process is the change in the equilibrium of soil carbonates and inorganic C sinks.

6.1.3 *The Tuber Brûlé: A Model to Study EM-EEMM Soil Ecology*

The properties of carbonated soils limit the development of many EM-EEMM species. Truffles are nevertheless an exception to this rule, since the calcareous soils of Mediterranean forests afford the habitat required by many *Tuber* species.

Tuber melanosporum Vittad. has great economic value and grows in well-drained calcareous soils. Its phytotoxic activity provokes clearings in the vegetation (where the growth of other plants is inhibited either permanently or for part of the year), called burns or brûlés, where the mycelia bear fruit. The environmental conditions created by the brûlé are very favorable for *T. melanosporum* development, mycorrhization, and fruiting (Papa 1980; Plattner and Hall 1995; Dessolas et al. 2008; García-Montero et al. 2009a; Chevalier and Palenzona 2011).

Callot (1999), Ricard (2003), Jaillard et al. (2007), and Chevalier and Palenzona (2011) explain that *T. melanosporum* is closely linked to soil pH and to calcium carbonate availability and highlight the interesting decarbonation–recarbonation processes observed in *T. melanosporum* brûlés. García-Montero et al. (2009a) describe the *T. melanosporum* brûlé as an interesting biological model to study the soil reaction and the decarbonation–recarbonation soil processes associated with the EM-EEMM community, vegetation, and climate.

In the past 10 years we have provided new evidence of the influence of soil reactions and carbonate availability on *Tuber* ecology (in particular) and on EM-EEMM ecology (in general). The objective of this chapter is to review the results obtained during these 10 years of research into the influence of soil carbonate on truffles and soil organisms. As a result, we propose new hypotheses on soil chemical reactions, development of EM-EEMM populations and their interactions with soil inorganic C sinks.

Moreover, in this chapter, we will provide new evidence linking the presence and activity of soil fauna with *T. melanosporum*. Thus, we evaluate (1) earthworm impact on soil reaction and carbonate availability on *T. melanosporum* brûlés and (2) the impact of *T. melanosporum*'s ectomycorrhizal activity on biodiversity and soil quality.

6.2 Interactions Between Carbonates and Soil pH with EM

6.2.1 *Impact of EM Populations on the Amount of Carbonates in Soils*

The amount of soil carbonates depends on biological soil-forming factors such as relief, time, and climate (Breemen and Buurman 1998). Some important aspects include the processes of dissolution, transport, and accumulation of soil carbonates that depend on the relationships among water dissolution, gaseous CO_2 , soluble H_2CO_3 , HCO_3^- , and solid CaCO_3 phases.

An increase in H^+ in the microenvironment of the EM-EEMM hyphosphere could therefore provoke an alteration in these chemical balances and the dissolution of soil carbonates. This hypothesis is consistent with Poitou (1988), who showed that, in vitro, *T. melanosporum* mycelia acidify their environment and dissolve calcium carbonate. Callot (1999) reports on the doctoral thesis of Pottier (1986), who carried out an experiment on the growth conditions of the *T. melanosporum* mycelia with populations of associated bacteria. It was demonstrated that, regardless of the initial conditions, pH values of 5.5–6 were reached in all the cultures. The conclusion is that the *T. melanosporum* mycelium associated with bacterial populations strongly acidifies its environment. This experiment additionally confirms that Ca^{2+} favors the development of the *T. melanosporum* mycelium. Callot (1999) also reports that the roots and the microbiota associated with limestone soils create microsites of acidified soil, which in turn enables the dissolution of calcareous rock.

García-Montero et al. (2009b) studied the differences in pH and carbonate fractions in soils inside and outside the *T. melanosporum* brûlés (the study of exchangeable Ca^{2+} was difficult because the soil exchange complex is saturated in exchangeable Ca^{2+}). They indicated that there are no significant pH differences between the soils inside the brûlés and those outside brûlés. Nevertheless, the total carbonate is significantly lower inside the brûlés than outside. *T. melanosporum* mycelia may solubilize all the carbonate fractions, which would then dissolve in the water of the hyphosphere (this could buffer the increase in H^+ produced by the mycelia). The carbonate-enriched water would allow the mobilization and redistribution of carbonates throughout the brûlé soil. Part of the carbonate-enriched water would be leached out and contribute to soil carbonation losses, especially during periods of rain. The problem of carbonate leaching in *T. melanosporum* brûlés has been pointed out by Callot (1999) and Ricard (2003). Further studies are needed to confirm these experimental observations.

6.2.2 *Impact of Soil Carbonates on EM Populations*

Decarbonation–recarbonation soil processes could condition the dynamics of many EM-EEMM populations. Several experimental studies on liming and tree ectomycorrhizae reinforce this hypothesis:

1. Liming stimulates fine root development (Schneider and Zech 1990; Huettl and Zoetl 1993; Kreutzer 1995; Bakker et al. 2000).
2. Liming modifies the EEMM community structure (Jonsson et al. 1999), increases the relative frequency of certain EM morphotypes (Erland and Soderstrom 1991; Antibus and Linkins 1992), decreases the relative proportion of some smooth-type EM in favor of hairy-type EM and increases the total number of EM tips (Bakker et al. 2000). Even a moderate level of liming has long-lasting effects on ectomycorrhizal community composition (Kjøller and Clemmensen 2009).

The amounts of soil carbonates and the decarbonation–recarbonation processes also have an impact on truffle culture. *T. melanosporum* is present in different Mediterranean regions, and its distribution overlaps the natural regions for *Tuber aestivum* Vittad., *Tuber brumale* Vittad., *Tuber rufum* Pico and *Tuber mesentericum* Vittad. These truffle species compete in the same habitats (truffières) and frequently contaminate and cause damage to *T. melanosporum* truffières and reduce their production.

Simultaneous fruiting body production of various truffle species can be observed within the same brûlé. Ricard (2003) indicates that the development of the *T. melanosporum* brûlé triggers a process within the mycorrhizal population that favors *T. melanosporum* development over other fungi. The interactions of the species observed in time and space, associated with the processes involved in the brûlé development, generate a fungal succession (Falini and Granetti 1998; Callot 1999; Rioussset et al. 2001; Ricard 2003; Granetti et al. 2005; Sourzat 2005):

1. *Tuber brumale*, *T. rufum*, *T. aestivum*, and *T. mesentericum* fruiting bodies are frequently collected outside *T. melanosporum* brûlés or just inside the edge of the brûlé.
2. Inside the brûlés, a temporal succession of the various truffle species can also be observed: *T. rufum* fruit bodies are the first to be collected, followed by *T. melanosporum*, and finally *T. brumale*. Interactions therefore take place between *T. melanosporum*, *T. aestivum*, *T. brumale*, *T. mesentericum*, and other fungi.
3. *T. rufum* may also enter natural and cultivated brûlés when the production of *T. melanosporum* begins to decline (García-Montero 2000; Rioussset et al. 2001). In many areas of Spain, collectors call the fruiting bodies of *T. rufum* “bordes” (edges); this common name refers to the way in which the fruiting bodies of this species are collected on the outer edges of the *T. melanosporum* brûlés.

Sourzat and Dubiau (2001) and Sourzat et al. (2001) conclude that *T. melanosporum* brûlés could be unproductive for truffles due to different reasons. After noting that *T. aestivum* and *T. brumale* are present as mycorrhizal contaminants, they propose that environmental modifications or a loss of vigor in *T. melanosporum* might lead to its replacement by *T. aestivum* and *T. brumale*. Sourzat (2005) also indicate that natural *T. melanosporum* brûlés often contain *T. aestivum* and *T. brumale* as predominant competitor fungi but these species never fruit. The author explains that the contamination pressure of these other two *Tuber* species did not have a

negative effect on *T. melanosporum* production when grass and brush were limited on the brûlés. Nevertheless, the author was unable to say whether the aggressiveness of *T. melanosporum* and its resistance to the pressure of fungal contamination are qualities of the environment or of the soil.

García-Montero et al. (2008a) carried out a study to further explain the impact of soil on the interactions between truffle species inside the *T. melanosporum* brûlés, as compared to the *T. aestivum* and *T. mesentericum* brûlés in the same study area. These studies were done using ten conventional soil properties of surface horizons to determine how these properties affect the development and fruiting of these truffles. The results showed that the abundance of active carbonate is significantly higher in brûlés that produce only *T. melanosporum* than in those that produce *T. aestivum* and *T. mesentericum*.

RiOUSset et al. (2001) indicate that *T. rufum* lives in chalky soils in identical habitats to those occupied by *T. melanosporum*. García-Montero et al. (2009b) studied the relationships among pH, soil carbonates, and the presence of *T. melanosporum* versus *T. rufum* fruiting bodies outside the brûlés. The results showed that *T. rufum* fruiting bodies were collected in soils (outside the brûlés) with a greater concentration of total carbonate than *T. melanosporum* soils (inside the brûlés). The results also showed significantly higher active carbonate content inside the brûlés than in external soils (with *T. rufum*).

Therefore, the results described, in addition to Valverde-Asenjo et al. (2009), suggest that active carbonate, and its associated exchangeable Ca^{2+} , could be a major factor in the greater fruiting and aggressiveness of *T. melanosporum* and *T. brumale* when compared to *T. aestivum*, *T. mesentericum*, and *T. rufum*. These authors highlight that this similar response by *T. melanosporum* and *T. brumale* to soil carbonates may be related to their close phylogenetic relationship (Roux et al. 1999; Wang et al. 2006).

6.2.3 Impact of Calcareous Amendments on Truffle Culture

New studies are needed to understand the cultivation practices that increase total carbonate, active carbonate, and exchangeable Ca^{2+} . Particular attention should be given to the use of calcareous amendments in truffle culture, under strict ecological control, which would favor *T. melanosporum* production and its presence over other truffle species. Chevalier and Palenzona (2011) explain that the idea of using calcareous amendments in truffle culture goes back a long way. They describe the works of various authors on *T. melanosporum* and *Tuber uncinatum* Chatin (= *T. aestivum*) plantations on acid soils that had been treated with calcareous amendments in the United States, France, New Zealand, and Australia. Ricard (2003) also suggests adding fine limestone calcareous amendments in truffle culture, although he recommends using them carefully and in moderation. This assertion agrees with Callot (1999), who highlights the importance of high concentrations of surface carbonate in truffle culture. Moreover, RiOUSset et al.

(2001) report that calcareous amendments are being used in truffle culture to eradicate *T. brumale* from *T. melanosporum* cultivations. However, Valverde-Asenjo et al. (2009) explain that the calcareous amendments have not produced the expected effects of eradicating *T. brumale* from *T. melanosporum* cultivations.

García-Montero et al. (2009a) embarked on a succession of controlled calcareous amendments to eradicate *T. brumale* from *T. melanosporum* cultivations in Spain in order to expand the current knowledge of the effect of calcareous amendments on *T. melanosporum* plantations. These authors explain that calcareous amendments in *T. melanosporum* plantations significantly modified the soil's carbonated fraction in the brûlés one year after their application (mean content of active carbonate and total carbonate was higher than in nearby soil brûlés without calcareous amendments). These amendments will be continued in order to evaluate the development of the carbonated fraction in the monitored brûlé soils.

García-Montero et al. (2009a) also indicate that *T. melanosporum* and *T. brumale* production increased considerably in the amended plantations after 1 year. Therefore, liming does not negatively affect the *T. brumale* ectomycorrhizae in contaminated *T. melanosporum* truffières. These results and all cited observations indicate that active carbonate and its associated exchangeable Ca^{2+} could play a significant role in boosting production of both truffle species, thereby increasing the profitability of contaminated truffières.

6.3 Active Carbonate, Exchangeable Ca^{2+} , and the *Tuber* Brûlé Model: New Hypotheses on Soil Nutrition and C Sinks

There are still gaps in our understanding of *Tuber* ecology. In particular, Ricard (2003) points out that there is insufficient knowledge as to how physical–chemical soil properties influence truffle development. Most studies have failed to supply statistical data associated with the ecology and development of brûlés (García-Montero et al. 2007b). Recently, Chevalier and Palenzona (2011) have pointed out that current truffle culture techniques have not advanced much since the Second International Truffle Congress in Spoleto (1988).

However, new techniques are now being proposed thanks to fungal ecology studies: these include indirect control of the rootlet network by tree pruning and root cutting, root growth management, soil loosening, stimulation of burn formation, and organic fertilization. These authors also indicate that future research will benefit from a better knowledge of crucial points such as intraspecific variability, saprophytic nutrition habits, burn formation mechanisms, factors affecting fruiting body formation, influence of drought stress, or soil eutrophication by nitrogen. *Tuber* cultivation practices should benefit greatly from breakthroughs in understanding these key areas.

6.3.1 *Influence of Active Carbonate and Exchangeable Ca^{2+} on the Fruiting of Tuber melanosporum*

Various authors have examined soil properties to determine those with the greatest impact on *T. melanosporum* development. They indicate that *T. melanosporum* grows only in calcareous soils and prefers a C/N ratio close to 10, in which the soil texture tends to be balanced. Lulli et al. (1999), Castrignano et al. (2000), Callot et al. (2001), Ricard (2003), and Chevalier and Palenzona (2011) report that the development of *T. melanosporum* increases in deep, well-drained, and aerated soils with a reservoir of available water during dry periods. The most suitable texture for *T. melanosporum* is variable and depends on the stoniness of the soil. Both texture and stoniness should promote good aeration and water drainage. Organic matter content must be low to avoid pH modification, and considerable porosity is required, originating from biological activity.

Sourzat (2001) indicates that the soil parameters tolerated by *T. melanosporum* are highly variable. Callot (1999) and Ricard (2003) propose that the physicochemical properties of topsoil have a limited impact on truffle culture. Chevalier and Palenzona (2011) and others have emphasized the importance of conducting a thorough analysis of soil properties, including porosity, permeability and aeration, water balance, soil flora and fauna, exchangeable Ca^{2+} and recarbonation throughout the entire profile, as these factors determine the growth and production of *T. melanosporum* (Callot 1999; Bragato et al. 2001; Callot et al. 2001; Ricard 2003). Callot et al. (2001), Ricard (2003), and Granetti et al. (2005) also highlight the lack of knowledge as to how the physicochemical properties of fine soil influence truffle development.

García-Montero (2000) and García-Montero et al. (2006, 2007a) studied the relationship between *T. melanosporum* productivity and soil properties. Multivariate statistical analysis showed that the productivity of *T. melanosporum* is only slightly (and positively) influenced by the overall action of active carbonate, stoniness, organic carbon, clay content, and exchangeable cations present in the soil surface horizons. However, simple, positive, and very significant correlations were found among the percentage of active carbonate, percentage of exchangeable Ca^{2+} and the production of carpophores (Fig. 6.1). These authors explain that active carbonate is a fine fraction of calcareous rock measuring $<50\text{ }\mu\text{m}$, which is susceptible to rapid mobilization and is highly chemically active. Active carbonate indicates the extent and reactivity of carbonate surfaces and constitutes an important reserve of exchangeable Ca^{2+} . Continuous active carbonate formation preserves high levels of Ca^{2+} in the soil solution, regulates soil pH, and maintains the exchange complex, as it counteracts losses from leaching and other processes (Breemen and Buurman 1998).

The availability of active carbonate in soil depends on complex soil, climatic, and biological factors that are favored by soil stoniness. García-Montero et al. (2007a) showed that the percentage of surface stoniness is directly correlated to active carbonate concentration and *T. melanosporum* production. Surface stoniness regulates soil temperature and favors soil condensation and humidity, whereas

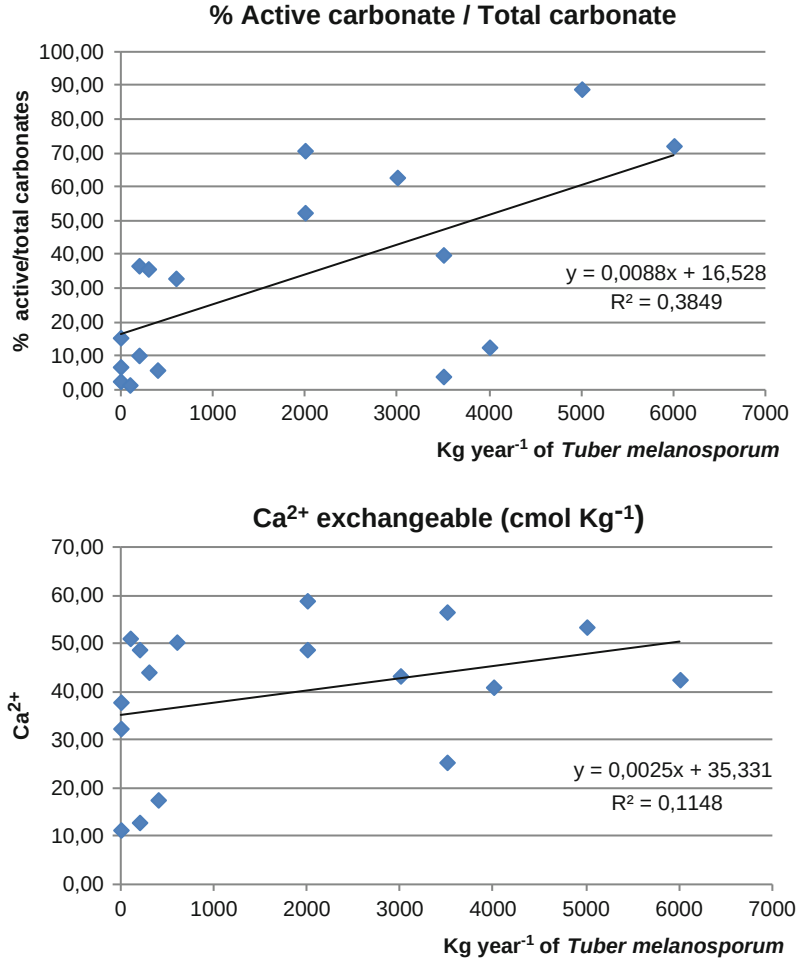


Fig. 6.1 Simple, positive and significant correlations among the percentage of active carbonate, percentage of exchangeable Ca²⁺ and the production of *Tuber melanosporum* carpophores (García-Montero 2000)

belowground stoniness lowers the fine earth volume and thereby encourages moisture. Certain types of rocky calcareous fragments are highly porous and retain and release significant amounts of water. These factors cause microenvironments to form on the lower side of rocks, which encourages the development of soil fauna, roots, microorganisms, and fungal mycelium. All of these factors modify the balance, dissolution, and precipitation of CaCO₃ and favor surface accumulation of secondary CaCO₃. Furthermore, rainwater flows over the rocks and accumulates in the lower parts, producing precipitations of carbonates in air spaces under the rocks when the water evaporates (Breemen and Buurman 1998; Callot 1999; Bragato et al. 2001; Callot et al. 2001; Ricard 2003).

Ricard (2003) suggests that the lack of studies on active carbonate in truffle culture is an oversight, and Jaillard et al. (2007) indicate that it is difficult to judge its impact. Ourzik (2001) points out that the knowledge of *T. melanosporum* biology is insufficient to establish a direct relationship between active carbonate and this truffle. These authors' objections have a rationale, since this carbonated fraction is highly variable and dynamic. In summary, Poitou (1988), García-Montero (2000), Ourzik (2001), and García-Montero et al. (2006, 2007a) emphasize the significance of active carbonate and exchangeable Ca^{2+} in the growth of *T. melanosporum* mycelium, and Chevalier and Palenzona (2011) conclude that exchangeable Ca^{2+} is the single most important element for truffle production. New studies are necessary on the interactions among active carbonate, exchangeable Ca^{2+} and *T. melanosporum*, linked to cultivation practices and soil physical and chemical properties.

6.3.2 *The Tuber Brûlé Model: A Feedback Process and New Hypotheses on Soil Nutrition and C Sinks*

Chevalier and Palenzona (2011) highlight the need to study the mycelium and the means by which the fruiting body acquires nutrients, as this is a key factor in its cultivation. The nutritional mechanism of the *T. melanosporum* fruiting body is still under discussion, and the theory of fruiting body independence has been called into question. The implications for truffle fertilization are important. Chevalier and Palenzona (2011) and Dessolas et al. (2008) indicate that the mechanism of brûlé formation is poorly known: they only know that truffles "prepare their nest" by emitting volatile herbicidal substances which strongly degrade minerals and soil organic matter.

Sourzat (2004), Granetti et al. (2005), and other authors have explained the production and development of brûlés. Chevalier and Palenzona (2011) suggested that truffle production may be correlated to tree vigor and brûlé growth. They explain that the idea of *T. melanosporum* aggressiveness proposed by Sourzat (2001) is a reflection of appropriate ecological conditions for the truffle and that its vigor may be assessed by measuring the ratio of the tree canopy radius to the brûlé's radius and its annual growth rate. In any case, Callot et al. (2001), Ricard (2003), Granetti et al. (2005), and Chevalier and Palenzona (2011) indicate that the biological and physiochemical conditions governing brûlé development have yet to be clarified.

García-Montero et al. (2007b) indicate that in different types of woodland, *T. melanosporum* showed a significant difference in the amount of productive carpophores. They also point out that the larger the size of the brûlés, the greater the carpophore production of *T. melanosporum*. Nevertheless, there were no significant differences in brûlé size among the different types of woodland tested. In summary, brûlé size was a significant feature in the production of *T. melanosporum*. To understand the processes related to brûlé development, these authors studied the relationship between brûlé size and soil features. The statistical

analysis revealed that *T. melanosporum* brûlé size is related to the percentage of active carbonate present in the soil surface horizons and that the collective influence of the other soil features studied is not significant to the development of *T. melanosporum* brûlés.

Soil carbonate solubilization and leaching increases with rainfall, respiration, and metabolism of roots and mycelia and other factors that increase the partial pressure of CO₂ (pCO₂) in the soil. Bicarbonate precipitates as carbonate when it is concentrated in capillary water (evaporated or absorbed by the roots). This also occurs when the pCO₂ diminishes due to aeration caused by macrofauna and the disappearance of biological respiration of the roots at depth, among other factors (Poitou 1988; Bencivenga and Granetti 1989; Coli et al. 1990; López and López 1990; Wild 1992; Marañés et al. 1994; Breemen and Buurman 1998; Callot 1999; Ourzik 2001; Ricard 2003). *T. melanosporum* eliminates plants from their brûlés, and the disappearance of their roots favors the accumulation of active carbonate. In summary, the development of the brûlé encourages the formation of large amounts of active carbonate and exchangeable Ca²⁺. *T. melanosporum* activity and brûlé size are simultaneously favored by a high concentration of both factors, which suggests a feedback process. These conclusions agree with Chevalier and Palenzona (2011) who also conclude that the single most important element for truffle production is exchangeable Ca²⁺.

García-Montero et al. (2009a) propose the following hypothesis as a guide for research into the role of active carbonate and exchangeable Ca²⁺ and the feedback processes associated with the brûlé. They hypothesize that *T. melanosporum* and *T. brumale* may favor or benefit from soils that are rich in active carbonate and exchangeable Ca²⁺ to promote their ectomycorrhization in plants with nutritional deficiencies due to lime-induced chlorosis.

Little is known about the physiological relationship between *Tuber* species and their symbiont plants (Ricard 2003). The proposed hypothesis on lime-induced chlorosis and *Tuber* mycorrhization is related to the experiments of Dupré et al. (1982) on the mycorrhization of *T. melanosporum* in substrates watered with nutrient solutions. These experiments showed that the lack of phosphorous favors the ectomycorrhization of *Quercus pubescens* Willd. The experiments of Gelpe and Timbal (1986) also demonstrate that a high active carbonate content induces chlorosis in *Quercus rubra* L. Moreover, Püttsepp et al. (2004) indicate that local soil nutrient status influences EM colonization, and low P and K availability favors EM colonization.

The abundance of active carbonate in soils raises the pH and increases HCO₃⁻ and exchangeable Ca²⁺, which immobilizes P, B, Fe, and Mn (Gaucher 1971; Follet et al. 1981; Loué 1986; Douchafour and Souchier 1979; Wild 1992; Callot 1999). Moreover, a significant rise in pH affects the absorption of microelements by plants. An increase in pH reduces the assimilability of Al, Co, Cu, Fe, Zn, and Mn (Loué 1986). A pH of 7.9–8.4, typical of *T. melanosporum* soils, leads to increasing deficiencies of Co, Cu, Mn, and Zn in plants (Gaucher 1971; Follet et al. 1981; Wild 1992). When the pH is raised, Mg²⁺ may be transformed into a non-exchangeable cation with no satisfactory explanation (Wild 1992), leading to the formation of insoluble calcium borates (Follet et al. 1981).

Reactions between Ca^{2+} and P can occur in the soil solution, soil colloids, or precipitated calcium carbonate. The degree of P adsorption on precipitated calcium carbonate is inversely related to particle size (Follet et al. 1981). An overabundance of Ca^{2+} and HCO_3^- immobilizes Mg^{2+} or leads to its deficiency in plants (Douchafour and Souchier 1979; Follet et al. 1981).

In summary, our soil nutrition hypothesis proposes that soils with high active carbonate and exchangeable Ca^{2+} produce host plant chlorosis, which is a symptom of an underlying nutrient deficiency. This nutrient deficiency could lead to greater colonization of *T. melanosporum* and *T. brumale* ectomycorrhizae and favor the growth of mycelia and fruiting body production. The development of *T. melanosporum* brûlés could simultaneously encourage the formation of new amounts of active carbonate and exchangeable Ca^{2+} based on a feedback model proposed as the best way of explaining the cause–effect of all these observations.

This brûlé model is essential for EM-EEMM fungi species from an evolutionary and ecological viewpoint, as it assumes that *T. melanosporum* and *T. brumale* have the ability to modify soil pH, soil carbonate, and exchangeable Ca^{2+} availability and may thus have a direct impact on the structure and dynamics of the mycorrhizal community. This brûlé model also has an added value in the form of a study of the C sequestration/loss process, as inorganic and organic carbon, in relation to the soil–plant–*Tuber* system.

6.4 Soil Fauna and the *Tuber* Brûlé Model: New Hypotheses on C Sinks

The diversity of soil fauna includes a quarter of described living species, the majority of which are insects and arachnids (Decaëns et al. 2006). Soil fauna play an essential role in agroecosystems by maintaining their functionality and productivity (Wardle et al. 1995), and it is also important in decomposition, nutrient recycling and the maintenance of physical and chemical soil properties and structure (Davidson and Grieve 2006). Just like plants, most soil animal taxa are directly influenced by soil parameters, since their mobility is limited.

Various soil invertebrate taxa, including Nematoda, Collembola, and Oligochaeta, are known to graze on fungal mycelia and can substantially alter fungal morphology and physiology (Harold et al. 2005; Bobby and Jones 2008). Such effects are likely to alter fungal fitness and therefore their combativeness in interaction with other soil microorganisms, including fungi (Rotheray et al. 2011).

Since fungi are an attractive food source for soil arthropods, certain fungi may have developed strategies to avoid grazing. The deleterious effect of toxins on the development of coleopterans has been studied intensively (Mier et al. 1996). Böllmann et al. (2010) hypothesized that crystals on the hyphal surface as well as toxic or bitter-tasting secondary metabolites may serve as feeding protection and will have a strong impact on the feeding preference of Collembola.

6.4.1 Soil Fauna and EM Populations

There is still much to learn about ectomycorrhizal ecology, and very little in particular is known about interactions between the EM–plant complex and soil fauna. In contrast, more is known about the interactions between arbuscular mycorrhizae and soil fauna (Hoffmann 2011).

The relationship between Collembola and ectomycorrhizae is beginning to receive increased attention because Collembola can affect plant growth and mycorrhizal infection (Kaneda and Kaneko 2004). Ectomycorrhizae are capable of “connecting” with nearby trees, even if they belong to different species, thus creating networks that establish a flow of nutrient and photosynthetic products among all the interconnected plants. The ecological role of these networks is crucial and is currently the subject of study. In addition to nutrient and water transport among plants (which sometimes only favors one of them), it has been demonstrated that there are other positive effects on plants, particularly on those aspects related to the capacity of new land colonization and the degree of forest evolution and maturity (Domínguez et al. 2006). These EM-plants are conditioned by several factors inherent in their soil habitat, and Collembola populations may condition network size or even interrupt them. Therefore, the relationship between Collembola preferences and mycorrhizal activities is important for understanding the potential effects of Collembola on plant mycorrhizal symbiosis, especially since a highly active mycorrhizal mycelium may be more effective than a senescent one in plant nutrient acquisition (Kaneda and Kaneko 2004; Menta et al. 2011; Tarasconi et al. 2011).

In summary, from a global perspective, soil fauna is an important component of soil systems. For this reason, there is growing interest in studying the interactions between soil fauna and soil properties (including soil pH and carbonate balances) and in determining their impact on EM–plant systems and soil C sinks.

6.4.2 Soil Mesarthropods and the Tuber Brûlé Model: Preliminary Studies

Callot (1999), Lulli et al. (1999), and Castrignano et al. (2000) highlight that the *T. melanosporum* brûlé requires considerable soil porosity and that much of this porosity is due to the activities of earthworms and ants. Chevalier and Palenzona (2011) propose the use of cultural practices that favor soil fauna and emphasize the importance of avoiding cultural practices that may damage soil life (such as pesticides). They also propose the experimental introduction of earthworms into truffle culture. However, there is little information about the relationships between *T. melanosporum* and soil fauna.

The mesofauna composition of the soil (from 200 μm to 2 mm) is highly influenced by humidity, pH, organic matter, and humus type (Hagvar 1982; Kuznestova 2002). It

is also necessary to determine how these factors affecting soil fauna are correlated to the type of vegetation and the existing EM–plant systems. Plants modify the physical and chemical properties of the soil (Materna 2004), which in turn indirectly influences soil fauna composition (Wild 1992). The *T. melanosporum* brûlé creates a particularly hostile environment that induces low in-soil humidity, modifies its temperature regime, and changes soil properties inside the brûlé.

Recently, Menta et al. (2011) and Tarasconi et al. (2011) have begun to study the interactions between mesoarthropods and *T. melanosporum* in a series of comparative studies between different Mediterranean areas in Italy and Spain. For this purpose, they used the QBS-ar biological quality index proposed by Parisi et al. (2005). In the Italian plots a total of 12 soil fauna taxonomic groups were found. Many of these are typical of forest environments: pseudoscorpions (Pseudoscorpionida), arachnids (Araneida), acarid (Acari), isopods (Isopoda), diplopods (Diplopoda), symphylans (Symphyla), pauropodans (Pauropoda), collembolans (Collembola), hemipterans (Hemiptera), hymenopterans (Hymenoptera), coleopterans (adults and larvae) (Coleoptera), and dipterans (adults and larvae) (Diptera). These studies found that there were distinct differences between the mesoarthropod communities inside and outside the *T. melanosporum* brûlé and that the communities outside the brûlés had a higher diversity. The Spanish plots showed a smaller number of groups (10) than the Italian plots: arachnids, acharina, diplopods, symphylans, collembolans, hemipterans, dermapterans, hymenopterans, coleopterans (adults and larvae), and dipterans (adults and larvae).

Soil mesoarthropod communities were partially different in the brûlé (1) the density of acharina and wasps and the number of biological forms outside the brûlé were higher; (2) the number of mesoarthropod taxa was lower inside the brûlés; and (3) some important groups, such as mites and ants, were negatively affected by the brûlé. However, collembolans do not seem to be affected by the brûlé. It is necessary to determine whether these soil fauna differences are a consequence of the direct action of the compounds produced by the *T. melanosporum* mycelium or are caused as an indirect effect of the truffle on vegetation and chemical and physical soil characteristics.

6.4.3 *Earthworms and the Tuber Brûlé Model: Cast Impacts on Soil Recarbonation and C Cycling*

6.4.3.1 Earthworms and Truffle Culture

Callot (1999), Ricard (2003), and Pargney et al. (2008) indicate that earthworms are often found around the truffle fruiting bodies in the brûlé soils. These soils usually have low levels of organic matter, and earthworms search near the carpophores for the bacterial populations and droppings of micro-arthropods and macrofauna. Lulli et al. (1999) and Castrignano et al. (2000) point out that the action of earthworms in

the soil benefits *T. melanosporum*. Callot (1999) stresses that the impact of cultural practices greatly reduces earthworm activity.

Callot (1999) and Ricard (2003) highlight the relationships between earthworm activity, $p\text{CO}_2$, and soil pH in *T. melanosporum* brûlés. They propose that in calcareous, poorly aerated, and badly drained soils, the episodes of rain and dryness increase the pH to over 8.4 (and local areas may sometimes reach pH 10), while in well-aerated and drained soils with high earthworm activity, the pH is generally maintained below 8.4. They explain that the $p\text{CO}_2$ increase is reflected in a lower pH that falls below 7 when $p\text{CO}_2$ reaches 0.1 atmospheres (often achieved in calcareous soils and rhizospheres). They conclude that $p\text{CO}_2$ in the soil atmosphere depends not only on the respiration of roots and microorganisms but also on soil aeration which is strongly conditioned by earthworm activity (generating up to 5,000 km of galleries per hectare in grassland soils) (Callot 1999).

6.4.3.2 Earthworm Calcite Granules

Earthworm activity around *T. melanosporum* carpophores may affect the soil carbonate dynamics associated to the brûlés. In this regard, Callot (1999) proposes that earthworms could provoke local soil decarbonation, as the calcite granules are coated with mucus (uncarbonated), consisting of clay and polysaccharide, during the intestinal transit through the earthworm.

Spherical soil concretions composed of calcite crystals have been found in soils containing earthworms. These calcite granules range from single calcite crystals to aggregations up to 2.5 mm in diameter and are produced by the calciferous glands of all species of earthworm of the family *Lumbricidae*. Wiecek and Messenger (1972) proposed a preliminary estimation on the abundance of earthworm calcite granules in some deeply leached forest soils in North America. They estimated a contribution of 1.1 kg/ha per year of calcite granules in the upper portions of the horizons (0–50 mm). However, they did not estimate the calcite granules associated with earthworm casts. Jongmans et al. (2003) found earthworm calcite granules down to a soil depth of 50 cm, and Canti and Pearce (2003) indicate that these granules are regularly found in soil profiles, although they confirm that little is known about their origins and dynamics.

Gago-Duport et al. (2008) explain that earthworm calcite granules do not appear to have any biological function. Coleman et al. (2004) and Briones et al. (2008) indicate that calciferous glands provide a mechanism for regulating CO_2 in blood and tissue. Briones et al. (2008) explain that both environmental and metabolic CO_2 can be fixed in this way and show that ^{13}C -labeled air rapidly enters the calciferous gland and can be detected in both the gland and the granules after 4 days. Based on laboratory studies, Canti (2009) indicates that the C calcite granule is extracted from the earthworm's dietary intake (litter), atmospheric CO_2 , and SOM; however, very little appears to come from soil calcium carbonate. Canti's conclusions (2009) may be of great value regarding soil C cycling and carbonate dynamics.

Lee et al. (2008) report that earthworms produce calcite granules in sufficient volume to have a measurable impact on soil C cycling, although they indicate that the volume and role of this earthworm bio-mineralization process is yet unknown. Jongmans et al. (2003) found that soils without earthworm activity undergo decalcification processes that could be ascribed to the production of organic acids in the litter layer and to the absence of soil homogenization by earthworms.

6.4.3.3 Earthworm Casts and the *Tuber Brûlé* Model: A New Hypothesis on Soil C Sinks

García-Montero et al. (2011) proposed the *T. melanosporum* brûlé model to analyze the interactions between earthworm calcite granules and decarbonation–recarbonation soil processes. They conducted studies in the topsoils of brûlés to determine the balance of carbonate and organic carbon associated to earthworm casts, as compared to the soils from which the casts were produced. These studies sought to demonstrate in the field Canti's (2009) laboratory proposals regarding the sources of C for the synthesis of calcite granules by earthworms.

García-Montero et al. (2011) indicate that earthworm cast samples had a significantly higher percentage of total carbonate levels and higher pH than the surface soils from which the casts were produced; however, there were no significant differences in the percentage of SOM. They also indicate that soils from *T. melanosporum* brûlés from a truffière with an abundance of casts had a significantly higher pH and a lower percentage of total carbonate, percentage of active carbonate, and SOM levels than nearby brûlés with low cast levels. These results agree with Chan (2003), who showed that cast pH is significantly higher than that of the bulk acid soil material used in a laboratory experiment. Wiecek and Messenger (1972) also indicate that earthworm casts are less acid than the ingested food and propose that the weathering of earthworm calcite granules is partly responsible for high pH values in the top horizons of acid soils under forest cover in some North American forests.

These field results obtained in *T. melanosporum* brûlés confirm Canti's (2009) laboratory results: there is a significant increase in carbonate contents in the earthworm casts (presumably due to the calcite granules) that cannot be explained by existing levels of carbonate in the original soils from which the casts were produced. This confirms that, in the field, the C in the calcite granule is mainly extracted from the earthworm's dietary intake (litter), SOM, and atmospheric CO₂. This was demonstrated by Canti (2009) using artificial substrates with marked differences in $\delta^{13}\text{C}$ levels.

Therefore, the calciferous glands of earthworms may be of great importance in evaluating C cycling and C sequestration in the soil, as they could fix different forms of C into bio-mineral granules formed by calcite crystals, and promote long-term soil C fixing. Lee et al. (2008) indicate that these biomineral granules are stable, and Canti (2009) reports that earthworm calcite granules have been found in archaeological soils and sediments after many years.

Earthworm calciferous glands may also have a major impact on soil reaction and recarbonation processes associated to *T. melanosporum* brûlés and other EM-EEMM populations. It is therefore advisable to further our knowledge of the role played by earthworm casts and their calciferous glands on a local and global scale.

6.5 Conclusions

Fungi can strongly acidify their immediate soil environment, and their mycelia can provoke a significant change in soil properties and soil–EM–plant interactions. One of the immediate consequences of the process of soil acidification is the change in the equilibrium of soil carbonates.

The *T. melanosporum* brûlé is an interesting biological model for studying soil reaction and the decarbonation–recarbonation soil processes associated with the EM-EEMM community, soil fauna (earthworms, mesoarthropods, and others), and vegetation. Several studies have indicated that a high concentration of active carbonate and exchangeable Ca^{2+} in the soil are major factors that favor *T. melanosporum* fruiting body production and increases in brûlé size; this points to the interest of studying the use of calcareous amendments in truffle culture. The amount of active carbonate is significantly higher, and total carbonate is significantly lower inside than outside the *T. melanosporum* brûlés. These patterns and other works confirm that *T. melanosporum* mycelia acidify their hyphosphere and solubilize carbonated fractions. Subsequently, the particular environmental conditions of brûlé soils may favor a secondary carbonate precipitation with a net increase in active carbonate, which preserves high levels of exchangeable Ca^{2+} .

Furthermore, field results obtained in *T. melanosporum* brûlés and recent experimental studies show that the synthesis of calcite granules by earthworms is based on C sources that are mainly extracted from the earthworm's dietary intake, soil organic matter, and atmospheric CO_2 . Therefore, the earthworm's calciferous glands could cause brûlé soil recarbonation based on long-term fixing of C in the form of calcite.

Based on these findings, we propose new hypotheses on EM-EEMM ecology, soil biology, and inorganic C soil sinks, suggesting that (1) the model which best explains the cause–effect of all brûlé observations is a feedback process; (2) this model assumes that the ability of *T. melanosporum* to modify soil properties may have a direct impact on soil–plant nutrition and the degree of plant mycorrhization, and this hypothesis could have a major impact on EM-EEMM from the evolutionary standpoint; and (3) the integrated action of *T. melanosporum* and/or other EEMM populations, in combination with earthworms, could be of great importance in the cycling and sequestration of inorganic C in the soil.

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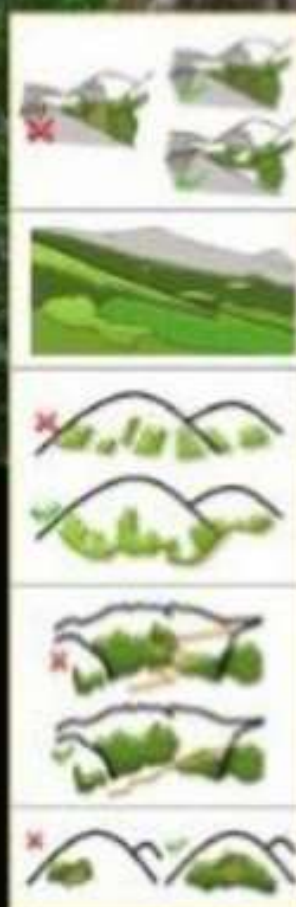
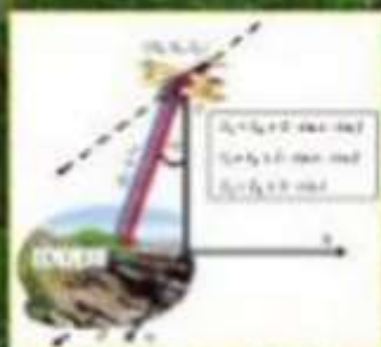
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Quantitative Techniques in Participatory Forest Management



Edited by
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Susana Martín-Fernández
Antonio García-Abril

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Contents

Preface.....	ix
Acknowledgment	xv
Editors	xvii
Contributors	xix
Chapter 1 Forest Sustainability and Public Participation	1
<i>Fernando García-Robredo, Eugenio Martínez-Falero, Sigfredo Ortuño, and Rosario Tejera</i>	
Chapter 2 Inventory Techniques in Participatory Forest Management	53
<i>Cristina Pascual, Francisco Mauro, Ana Hernando, and Susana Martín-Fernández</i>	
Chapter 3 Criteria and Indicators for Sustainable Forest Management.....	135
<i>M. Victoria Núñez, Rosario Tejera, Antonio García-Abril, Esperanza Ayuga-Téllez, and Eugenio Martínez-Falero</i>	
Chapter 4 Soil-Quality Indicators for Forest Management	179
<i>Fernando Arredondo-Ruiz, Luis García-Montero, Inmaculada Valverde-Asenjo, and Cristina Menta</i>	
Chapter 5 Functionality Indicators for Sustainable Management	241
<i>José Antonio Manzanera, Susana Martín-Fernández, and Antonio García-Abril</i>	
Chapter 6 Landscape Indicators for Sustainable Forest Management.....	263
<i>Antonio García-Abril, M. Victoria Núñez, M. Angeles Grande, M. Dolores Velarde, Patricia Martínez-Obispo, and Roberto Rodríguez-Solano</i>	
Chapter 7 Assessment of Sustainability Based on Individual Preferences.....	367
<i>Eugenio Martínez-Falero, Susana Martín-Fernández, and Antonio Orol</i>	

Chapter 8	Optimization Methods to Identify the Best Management Plan.....	421
	<i>Susana Martín-Fernández, Eugenio Martínez-Falero, and Miguel Valentín-Gamazo</i>	
Chapter 9	Multiparticipant Decision-Making.....	499
	<i>Esperanza Ayuga-Téllez, Concepción González-García, and Eugenio Martínez-Falero</i>	
Chapter 10	A Computer-Based Decision-Making Support System to Incorporate Personal Preferences in Forest Management.....	557
	<i>Eugenio Martínez-Falero, Antonio García-Abril, Carlos García-Angulo, and Susana Martín-Fernández</i>	



4 Soil-Quality Indicators for Forest Management

*Fernando Arredondo-Ruiz, Luis García-Montero,
Inmaculada Valverde -Asenjo, and Cristina Menta*

CONTENTS

4.1	Introduction to Forest-Soil Quality	180
4.1.1	Importance of the Soil and the Rhizosphere	180
4.1.2	Foundations for Using Soil Properties as Indicators of Forest-Soil Quality	181
4.1.3	Fertility and Organic Matter Content in Forest Soils	182
4.1.4	Storing Carbon Dioxide in Forest Soils	183
4.1.5	Biodiversity and Forest Soils	184
4.2	Threats to Forest-Soil Quality	185
4.2.1	Eight Main Threats to Soil on a Global Scale	185
4.2.2	Erosion and Loss of Organic Matter in Forest and Agricultural Soils	186
4.2.3	Biodiversity Loss in Agricultural and Forest Soils	187
4.2.4	Difficulties in Developing International Strategies for Forest-Soil Protection	191
4.3	Soil-Quality Indicators in Sustainable Forest Management	193
4.3.1	Sustainable Forest Management and Soil Quality	193
4.3.2	Soil-Quality Indicators	195
4.3.3	Assessment of Soil-Quality Indicator Usage in Forestry	198
4.4	Biological, Chemical, and Physical Soil-Quality Indicators	202
4.4.1	Foundations for Understanding the Role of Biology in Soil-Quality Assessment	202
4.4.2	Biological Indicators to Assess Forest-Soil Quality	205
4.4.3	Microbiological, Biochemical, and Molecular Indicators to Assess Forest-Soil Quality	208
4.4.4	Chemical Indicators to Assess Forest-Soil Quality	212
4.4.5	Physical Indicators to Assess Forest-Soil Quality	214
4.5	Multiparametric Indices to Assess Soil Quality	215
4.5.1	Multiparametric Indices Including Physical, Chemical, and Biological Parameters	216
4.5.2	Physical–Chemical Indices to Assess Soil Quality	217
4.5.3	Multiparametric Indices Based on Mathematical Models to Assess Soil Quality	218

179





4.6 Practical Cases: Models for the Integration of Soil-Quality Indicators in Landscape Planning Projects	220
4.6.1 Model of Integration of Qualitative and Quantitative Soil and Environmental Indicators: An Example of the Assessment of the Environmental Quality of a Whole Country (Spain)	220
4.6.1.1 Introduction	220
4.6.1.2 Methodological Basis	221
4.6.2 Model of Integration of Quantitative Soil-Quality Indicators: An Example of the Generation of a Soil Fertility Index in Caribbean Agricultural and Forest Areas	223
4.6.2.1 Introduction	223
4.6.2.2 Methodological Basis	224
4.6.2.3 Model Steps and Logical Operations Applied	225
4.7 Conclusions	228
References	229

4.1 INTRODUCTION TO FOREST-SOIL QUALITY

4.1.1 ■ IMPORTANCE OF THE SOIL AND THE RHIZOSPHERE

Soil is one of the most fundamental components for supporting life on Earth. Jeffery et al. (2010) and Menta (2012) point out that the processes occurring within soil (most of which are driven by its living organisms) perform ecosystem and global functions that help maintain life aboveground. Moreover, soil delivers numerous services, ranging from providing the food we eat to filtering and cleaning the water we drink. It is used as a platform for building, it provides vital products such as antibiotics, and it contains an archive of our cultural heritage in the form of archeological sites. However, life within the soil is hidden and often suffers from being “out of sight and out of mind” (Blum, 1993; Ebel and Davitashvili, 2007).

Soils may be characterized in terms of the properties they inherit from the underlying rock (the parent material) and the properties resulting from alteration of the original parent material by soil-forming or “pedogenic” processes, namely, climate, vegetation, time, and human activity. Pedogenic processes operate mainly in the surface and subsurface horizons normally found in the upper 2 m (Blum, 1993; Ebel and Davitashvili, 2007).

AQ1 From the point of view of both agriculture and forestry, another essential aspect of the functioning of the soil, vegetation, and ecosystem is the importance of the rhizosphere (Akeem, 2012). The presence of roots is generally associated with greater microorganism density and soil fauna in the nearby soil when compared to soil devoid of roots; the term rhizosphere is used in a broader sense to refer to the portion of soil surrounding roots in which the soil organisms are influenced by their presence (Killham, 1994). The rhizosphere can be distinguished from the greater part of the soil on the basis of its chemical, physical, and biological characteristics. As the roots penetrate into the ground, they act on clay minerals and the particles of soil surrounding them, leading to the formation of an area in which the water pathway and the movement of nutrients and microflora are more heavily channeled than in the



rest of the soil. For the same reasons, the organic matter (OM) released by the roots accumulates nearby. The chemical nature of the rhizosphere is significantly different from the rest of the soil; this is largely the result of the fact that the roots release carbon (C) and selectively capture ions in solution in the groundwater. The plants act as C pumps, fixing what is available in the atmosphere in the root exudates, which in turn are quickly captured by soil organisms; for this reason, the level of C available around the roots is never very high. Instead, the selective absorption of ions causes some to be depleted in the rhizosphere and the accumulation of others, which are not absorbed by the roots. The relationship between the roots and microflora can be very close and leads to (1) bacteria or fungi becoming an integral part of the roots as in mycorrhizal symbiosis and (2) the association of bacteria and legumes. The peculiar characteristics of the rhizosphere are also reflected in the selectivity of the animal element. The interaction between soil animals and the plant roots can take a variety of forms that either produce benefits or repress plant growth and often involve interactions with the microbial populations in the soil (Akeem, 2012).

As a result of all these factors and soil functions, soil health indicates the capacity of soil to function as a vital living system to sustain biological productivity, promote environmental quality, and maintain plant and animal health. However, soils are affected by human activity, which often results in their degradation and the loss of their functions. Growing pressure from an ever-increasing global population, as well as threats such as climate change and soil erosion, is placing increasing stresses on the ability of soil to sustain its important role in the planet's survival. Evidence suggests that while the increased use of monocultures, intensive agriculture, and forestry has led to a decline in soil quality and biodiversity in many areas, the precise consequences of this loss are not always clear. Jeffery et al. (2010) and Menta (2012) indicate that too rarely do we pause to reflect on the fact that soil is the foundation upon which society is sustained and evolves and that it is a vital component of ecological processes and cycles, as well as the basis on which our infrastructure rests. These authors insist that more importance should be given to the fact that soil quality and its protection contribute significantly to preserving the quality of life and that the nutrition and health of humans and animals cannot be separated from the quality of the soil.

Only by understanding soil in all its complexity while maintaining its functionality and quality through actions designed to protect its properties and acknowledging its importance in the quality of life worldwide can we embark on a truly sustainable use of soil perceived as a resource and build a proper human/soil relationship to be passed on to future generations.

4.1.2 FOUNDATIONS FOR USING SOIL PROPERTIES AS INDICATORS OF FOREST-SOIL QUALITY

Monitoring ecosystem components is essential for acquiring basic data to assess the impact of land-management systems and to plan resource conservation. For this reason, soil scientists have always sought to link soil type and soil variables to potentials or limitations of land use (Knoepp et al., 2000). Doran (1994) proposed three

main functions of soil in view of this goal: (1) to act as a medium for plant growth, (2) to regulate and partition water flow, and (3) to serve as an environmental buffer. Knoepp et al. (2000) described these approaches and remarked that this is borne out by the estimates of forest and agricultural productivity, as well as by their uses for recreation, wildlife, construction, and other functions listed in county soil surveys.

During the last decade, numerous authors have highlighted a fourth soil function, indicating that soil represents one of the leading reservoirs of biodiversity, as reported by Menta (2012). All these authors relate these soil functions to the need to identify soil-quality indicators (SQI). The increasing pressure on available land and the debate as to its proper use have brought about a parallel escalation in the movement to identify and set quality standards for both agricultural and forest soils.

The Soil Science Society of America officially defines soil quality as “the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation” (Karlen et al., 1997). Likewise, environmental indicators are “quantitative assessments informing of the state of conservation and health of the environment.” Some environmental indicators have a broad scope: for instance greenhouse gas emissions, protection areas, biodiversity indices, proportion of forest land area, net annual forest growing stock volume, average condition of health and vigor, etc. All are useful to characterize the current status and track or predict significant changes in the ecosystem (Blum, 1993; Ebel and Davitashvili, 2007).

Soil quality affects forestry, agricultural sustainability, and environmental quality and consequently plant, animal, and human health (Bloem, 2003; Menta, 2012). A common criterion for evaluating the long-term sustainability of ecosystems is to assess the fluctuations in soil quality (Schoenholtz et al., 2000). Soil reflects ecosystem metabolism; within soils, all the biogeochemical processes in the different ecosystem components are combined (Dylis, 1964). Maintaining soil quality is of the utmost importance for the preservation of biodiversity and for the sustainable management of renewable resources (Menta, 2012). Based on the concept of soil quality, Andrews et al. (2004) and Garrigues et al. (2012) provide a more detailed description of mean soil functions, including water flow and retention, solute transport and retention, physical stability and support, retention and cycling of nutrients, buffering and filtering of potentially toxic materials, and maintenance of biodiversity and habitat.

In summary, protecting the quality, biodiversity, and productive capacity of soil is a paramount goal for sustainable forest management. To support these goals, it is necessary to control or restrict agricultural and forestry activities that could reduce on-site agricultural and forest productivity and environmental quality in order to prevent soil degradation and restore its potential. Prerequisites for this objective include reliable soil data and the use of SQI, as well as an input for the design of soil usage systems and soil management practices for a truly sustainable forest management (FAO, 2009; Miller et al., 2010).

4.1.3 FERTILITY AND ORGANIC MATTER CONTENT IN FOREST SOILS

The soil functions that determine vegetation composition/structure are of particular importance, as they serve as a medium for root development and provide moisture

and nutrients for plant growth (Minnesota Forest Resources Council, 1999). Natural soil fertility derives from the combination of many of these soil properties, including OM content, clay content and mineralogy, the presence of weatherable minerals, pH, base saturation, and biological activity, mainly in the rhizosphere.

For reference purposes, soil groups in Europe can be classified into three levels of natural soil fertility as follows: (1) low (arenosols, planosols, acrisols, podzols), (2) moderate (regosols, andosols, calcisols, umbrisols, luvisols, albeluvisols, histosols), and (3) high (fluvisols, gleysols, vertisols, kastanozems, chernozems, phaeozems) (Soil Atlas of Europe).

In association with the concept of soil fertility, it is worth noting the importance of soil organic matter (SOM) and total organic carbon (TOC). Living SOM represents only a small percentage of soil TOC and includes soil micro-, meso-, and macroorganisms. In particular, soil microbial biomass is regarded as the most active and dynamic pool of SOM and plays an important role in driving soil mineralization processes. Three main aspects of soil microbial biomass are usually considered, due to their effects on soil functions: pool size, activity, and diversity. The determination of the total amount of C immobilized within microbial cells allows soil microbial biomass to be determined as a pool of SOM. Since this pool is responsible for the decomposition of plant and animal residues and the immobilization and mineralization of plant nutrients, it is ultimately responsible for maintaining soil fertility (Brookes, 2000). Thus, the concept of microbial biomass has developed to serve as an “early warning” of changing soil conditions and as an indicator of the direction of change.

C mineralization activity, a key process of the soil’s C cycle, determines the speed of the SOM degradation process in soil. Numerous studies have recently been carried out in order to verify—directly or indirectly—the potential for increasing C storage in soil by manipulating C inputs with a view to minimizing the rate of C mineralization (Jans-Hammermeister, 1997). The C mineralization process has a low sensitivity to changes in soil management, as small microbial populations in degraded soil can mineralize OM to the same extent and at the same rate as large microbial populations in undegraded soils (Brookes, 1994).

A combination of the two measurements, relating to both the size and activity of the microbial biomass, is more sensitive to soil management changes and more helpful as a SQI. C mineralization activity/unit of biomass (biomass-specific respiration) and the mineralization coefficient (respired C/TOC) indicate efficiency in C utilization and energy demand. Finally, soil microbial diversity measurements have assumed increasing importance as indicators of community stability and the impact of stress on that community (Akeem, 2012).

4.1.4 STORING CARBON DIOXIDE IN FOREST SOILS

Among the soil functions cited and with regard to the soil’s capacity to act as an environmental buffer, the last decade saw significant interest in forests—and especially in forest soil—as a means of storing carbon dioxide (CO_2) from the atmosphere and providing a mechanism to combat the increase in atmospheric CO_2 (Soil Atlas of Europe). Forest soils generally accumulate C. Estimates range from 17 to 39 million

AQ2 tons of C per year, with an average of 26 million tons per year in 1990 and an average of as much as 38 million tons per year in 2005 (EC 2002 Policy Makers).

However, there is still limited knowledge concerning the long-term impact of an increase in global average temperatures and the real role that trees and SOM could play in absorbing C, in addition to the costs and benefits involved in using restoration as a mechanism to offset C emissions. For these reasons, C knowledge projects have been proposed as a way of testing these parameters in the context of landscape-based forest restoration activities (Mansourian et al., 2005).

The atmospheric concentration of CO₂ has increased by over one-third since the Industrial Revolution. This increase is primarily attributed to fossil fuel combustion and also significantly to changes in land cover and use (e.g., forest degradation or conversion of forests to agriculture). In order to curb atmospheric CO₂ concentrations, it is essential to reduce human dependence on fossil fuels and impose legally binding targets for reduced CO₂ emissions, and this should be the central focus of any policy program. However, in order to stabilize atmospheric CO₂ concentrations, the international community must also reduce the rate of destruction of natural ecosystems that serve as important stocks and sinks of C. In addition to slowing the rate of land conversion, other mitigation tools considered to stabilize the burgeoning concentration of CO₂ in the atmosphere include increasing the land coverage of C-absorbing vegetation and soil C sinks.

The concept of C sinks is based on the natural ability of trees, plants, and soil organisms to take up CO₂ from the atmosphere and store the organic and inorganic C forms in wood, roots, leaves, and soil. The theory behind land-based C trading is that governments or institutions that wish or are required to reduce their fossil fuel emissions can offset some of these emissions by investing in afforestation and reforestation activities to sequester C. Indeed, in some cases, private companies are voluntarily electing to offset some of their fossil fuel CO₂ emissions through the purchase of C credits from land-based C sequestration projects (Mansourian et al., 2005).

Recent studies under various agroforestry systems (AFSs) in a range of ecological conditions showed that the extent of C sequestered in AFSs largely depends on environmental conditions and system management. Trading of sequestered C is a viable opportunity for bringing economic benefit to agroforestry practitioners, who are mostly resource-poor farmers in developing countries (EC 2002 Policy Makers; Ramachandran et al., 2010). For example, in contrast with industrial plantation approaches, the Scolel Té project for rural livelihood and C management (Mexico) aims to demonstrate how C finance can allow low-income rural farmers to invest in forest conservation, sustainable land-use systems, and improvements in their livelihood that would otherwise be inaccessible to them (Mansourian et al., 2005). However, more rigorous research results are required for AFSs to be used in global agendas for C sequestration (EU 2002 Policy Makers; Ramachandran et al., 2010).

4.1.5 BIODIVERSITY AND FOREST SOILS

Although global biodiversity was one of the focal points of the Rio Conference, in the 1990s, virtually no attention was paid to activities for the conservation of soil



communities. However, with the new millennium, the conservation of soil biodiversity has become an important aim in international environmental policies, as highlighted in the European Union (EU) Soil Thematic Strategy (2006), the Biodiversity Action Plan for Agriculture (EU 2001), the Kiev Resolution on Biodiversity (EU/ECE 2003), and subsequently in the Message from Malahide (EU, 2004) that lays down the goals of the Countdown 2010.

Historically, the study of soil biodiversity started with a mapping of soil food webs, perhaps because the most fundamental integrating feature of soil communities is the feeding relationships between organisms (EC, 2002). Nature's biodiversity reflects all ecosystem metabolisms, since all the biogeochemical processes of the different ecosystem components are combined within it; therefore, soil-quality fluctuations are considered to be a suitable criterion for evaluating the long-term sustainability of ecosystems. Within the complex structure of soil, biotic and abiotic components interact closely in controlling the organic degradation of matter and nutrient recycling processes.

Moreover, maintaining a high soil biodiversity may be of vital importance in structurally diverse ecosystems, as soil biodiversity may promote ecological stability (Grime, 1997; Van Bruggen and Semenov, 2000). Because soil is an environment that is constantly subjected to fluctuations, the establishment of an unfavorable environmental condition can result in the inhibition of some populations that perform essential functions. In highly diverse communities, however, there is a higher probability of the occurrence of quiescent microorganisms that could perform the same functional role but that have different physical, chemical, and biological demands (van Bruggen and Semenov, 2000; Chaer, 2008).

Therefore, soil communities, aside from being an important reservoir of global biodiversity, also play an essential role in these ecosystem functions and for this reason are often used to provide SQI (Menta, 2012).

4.2 THREATS TO FOREST-SOIL QUALITY

4.2.1 EIGHT MAIN THREATS TO SOIL ON A GLOBAL SCALE

Maintaining soil condition is essential for ensuring the sustainability of society and biodiversity. However, the soil is under an increasing number of threats. These threats are complex, and although unevenly spread, their dimension is global and they are frequently interlinked. When numerous threats occur simultaneously, their combined effects tend to compound the problem. Therefore, soil disturbances linked to natural forces and human activities alter the physical, chemical, and biological properties of soil, which in turn can impact its long-term productivity (Gupta and Malik, 1996).

Human activities are increasingly polluting soils and groundwater through applied agrochemicals, deposition of atmospheric pollutants, and spreading of sewage sludge and manures, and these can have adverse impacts on the ability of soil to perform its vital functions (Blum, 1993; Ebel and Davitashvili, 2007). Consequently, numerous soils around the world have lost their fertility or their capacity to perform





their functions due to processes that are accelerated or triggered directly by human activities and that often act in synergy with each other, amplifying the negative effect.

Among these processes, the most widespread at the worldwide level are erosion, OM loss, compaction, salinization, flooding and landslide phenomena, contamination, and reduction in biodiversity (Menta, 2012). In this regard, the communication of the European Commission to the Council and the European Parliament, entitled “Towards a Thematic Strategy for Soil Protection,” defined the following eight main soil threats (Soil Atlas of Europe):

1. Soil sealing occurs mainly through the development of technical, social, and economic infrastructures, especially in urban areas.
2. Erosion is mainly due to the inadequate use of soil by agriculture and forestry but also through building development and uncontrolled water runoff from roads and other sealed surfaces.
3. Loss of OM is mainly due to intensive use of the land by agriculture, especially when organic residues are not sufficiently produced or recycled back into the soil. Agronomists consider soil with less than 1.7% OM to be in the pre-desertification stage.
4. The decline in biodiversity is linked to the loss of OM, as biodiversity depends on OM, meaning that all soil biota live on the basis of OM.
5. Contamination can be either diffuse (widespread) or localized and may be caused by many human activities such as industrial production and traffic and predominantly through the use of fossil material such as ores, oils, coals, and salts or through agricultural activities.
6. Compaction of soil is a rather new phenomenon caused mainly by high pressures exerted on soil through heavily loaded vehicles used in agriculture and forestry. An estimated 4% of soil throughout Europe suffers from compaction.
7. Hydrogeological risks are complex phenomena that result in floods and landslides deriving partly from uncontrolled soil and land uses (e.g., sealing, compaction, and other adverse impacts), as well as from unregulated mining activities.
8. Salinization is mainly a regional problem, but in the areas where it occurs—such as the Mediterranean basin and in Hungary—it severely endangers agriculture, forestry, and the sustainable use of water resources.

Of these eight main threats to soil and insofar as forest management is concerned, the impact implied by the loss of OM, erosion, and loss of biodiversity in the soils of agricultural and forest systems should be highlighted on a global scale.

4.2.2 EROSION AND LOSS OF ORGANIC MATTER IN FOREST AND AGRICULTURAL SOILS

Soil degradation processes constitute a major worldwide problem with significant environmental, social, and economic consequences. As the world population increases, so does the need to protect soil as a vital resource for the production of





food and natural resources. Growing awareness by the international community of the need for global responses has led to an increasing number of international initiatives.

In fact, erosion is regarded as one of the most widespread forms of soil degradation and, as such, poses potentially severe limitations on sustainable land use. Topsoil is the most productive soil layer, and the most dominant effect of erosion is the loss of topsoil, which may not be conspicuous but is nevertheless potentially very damaging. Erosion literature commonly identifies “tolerable” rates of soil erosion, but these usually exceed the rates that can be balanced by the natural weathering of parent materials to form new soil particles. Soil loss in some places may be considered acceptable from an economic standpoint, but some modern agricultural and forest production methods are causing overall erosion rates that are becoming increasingly unacceptable from a long-term point of view.

Soil erosion is a physical phenomenon involving the removal of soil and rock particles by water, wind, ice, and gravity. Therefore, climate, topography, and soil characteristics are important physical factors affecting the amount of erosion. The most effective strategy for reducing erosion is to increase the cover of vegetation or litter and preferably both. Plants—especially woody plants with strong, deep roots—greatly increase soil strength by providing a stabilizing effect on slopes, in addition to protecting the soil with their canopy and adding litter to the soil surface. In some cases, the plants also transpire significant quantities of water from the slope, thus reducing the weight that contributes to mass movements (Mansourian et al., 2005; Soil Atlas of Europe, 2005).

Loss of OM is another of the most widespread forms of soil degradation. Following the unprecedented expansion and intensification of agricultural and forest production during the twentieth century, there is clear evidence of a consequent decline in the OC contents of many soils. This decline in OC contents has important implications for agricultural and forest production systems, as well as for ecosystems, as OC is a major component of SOM (Ebel and Davitashvili, 2007). A wide range of agricultural and forest management practices influence the abundance of OM, biomass, and diversity of soil biota and litter. These management practices include variations in tillage, treatment of pasture and crop residues, crop rotation, and applications of pesticides, fertilizers, manure, sewage, and ameliorants such as clay and lime, drainage and irrigation, and vehicle traffic (Baker, 1998).

4.2.3 BIODIVERSITY LOSS IN AGRICULTURAL AND FOREST SOILS

Humans have caused a widespread reduction in biodiversity on a global scale. The Convention on Biological Diversity (CBD) was the first global agreement aimed at the conservation and sustainable use of biological diversity (Secretariat of the Convention on Biological Diversity, 2000). The CBD lies at the heart of biodiversity conservation initiatives. It offers opportunities to address global issues at a national level through locally grown solutions and measures. One important requirement is the development of national biodiversity strategies and action plans channeled into relevant sectors and programs, as a primary means of implementing the Convention at the national level (United Nations 1992). The recent Conference of the Parties of the CBD (May 2008, Bonn) demonstrated that there is unanimous acknowledgement





of the need for action to protect biodiversity. Biodiversity conservation is essential both for ethical reasons and especially for the ecosystem services provided for current and future generations by the complex of living organisms. These ecosystem services are essential for the functioning of our planet (Menta, 2012).

Changes in biodiversity alter ecosystem processes and affect their resilience to environmental change. Human activities are estimated to have increased the rates of extinction 100–1000 times (Lawton and May, 1995). In the absence of major changes in policy and human behavior, our effect on the environment will continue to alter biodiversity.

Land use is considered to be the main element of global change for the near future, and land-use change is projected to have the greatest impact on biodiversity by the year 2100. In a review on changing biodiversity, Chapin et al. (2000) consider that land use will be the main cause of change in biodiversity for tropical, Mediterranean, and grassland ecosystems.

Forests, tropical or temperate, generally represent the biomes with the largest soil biodiversity. Consequently, any land-use change resulting in the removal of perennial tree vegetation will produce a reduction in soil biodiversity. In some cases, pasture or perennial grasslands succeed forests, while in others, arable land replaces formerly wooded areas. The change in soil biodiversity will therefore be influenced by the subsequent use of the land after the forest. However, reduction of soil biodiversity as a result of urbanization can be even more severe. The urbanization process leads to the conversion of indigenous habitat to various forms of anthropogenic land use, the fragmentation and isolation of areas of indigenous habitat, and an increase in local human population density. The urbanization process has been identified, for example, as one of the leading causes of declines in soil arthropod diversity and abundance in some areas (Menta, 2012). These reductions in soil biodiversity result in artificial ecosystems that require constant human intervention and extra running costs, whereas natural ecosystems are regulated by communities of plant, animal, and soil organisms through flows of energy and nutrients, a form of control that is being progressively lost with agricultural intensification.

Furthermore, differences in agricultural and forest production systems, such as integrated, organic, or conventional systems, have been demonstrated to affect soil fauna in terms of numbers and composition (Hansen et al., 2001; Cortet et al., 2002). The impact of soil tillage operations on OM and soil organisms is highly variable, depending on the tillage system adopted and on the soil characteristics. Conventional tillage by plowing inverts and breaks up the soil, destroys soil structure, and buries crop residues, thereby causing the highest impact on soil fauna; the intensity of these impacts is generally correlated to soil tillage depth (Menta, 2012). Minimum tillage systems may be characterized by a reduced tillage area (i.e., strip tillage) and/or reduced depth (i.e., rotary tiller, harrow, hoe); crop residues are generally incorporated into the soil instead of being buried. The negative impact of these conservation practices on soil fauna is reduced in comparison with conventional tillage. Under no-tillage crop production, the soil remains relatively undisturbed, and plant litter decomposes at the soil surface, much like in natural soil ecosystems.

Taking as an example the communities of soil microarthropods, observations on the impacts of different forms of agricultural and forest management on these



communities showed that the high input of intensively managed systems tends to promote low diversity, while lower input systems conserve diversity (Menta, 2012). The influence on soil organism populations is expected to be most evident when conservation practices such as no-till are implemented on previously conventionally tilled areas, as the relocation of crop residues to the surface in no-till systems will affect the soil decomposer communities (Beare et al., 1992). No-till (Hendrix et al., 1986) and minimum tillage generally lead to an increase in microarthropod numbers (Menta, 2012). Akeem (2012) also observed that higher values of mite density were associated with a decrease in tillage impact. Similarly, Cortet et al. (2002) reported that the mite community—and in particular oribatids—was more abundant in no-tillage as compared to conventional tillage. However, the differences were found only at certain periods of the year. Conventional tillage caused a reduction in microarthropod numbers as a result of exposure to desiccation, destruction of habitat, and disruption of access to food sources (House and Del Rosario, 1989). The influence of these impacts on the abundance of soil organisms will either be moderated or intensified depending on their spatial location, that is, in row where plants are growing, near the row where residues accumulate, or between rows where they are subjected to possible compaction from mechanized traffic (Fox et al., 1999).

Moreover, the observed impact of different forms of agricultural and forest management on bacterial communities showed similar patterns to microarthropod communities (Siepel and Bund, 1988; Bardgett and Cook, 1998). In fact, it is also evident that high-input systems favor bacterial pathways of decomposition, dominated by labile substrates and opportunistic bacterial-feeding fauna. In contrast, low-input systems favor fungal pathways with a more heterogeneous habitat, leading to domination by more persistent fungal-feeding fauna (Bardgett and Cook, 1998).

The effects of fertilizers on soil invertebrates and microbial communities are a consequence of their impact both on the vegetation and directly on the organisms themselves. Increases in the quantity and quality of the food supplied by vegetation are frequently reflected in greater fecundity, faster development, and increased production and turnover of invertebrate herbivores (Curry, 1994). The effects of organic and inorganic fertilizers in terms of nutrient enrichment may be comparable, but these two types of fertilizers differ in that organic forms provide additional food material for the decomposer community. Ryan (1999) concluded that the total soil microbial biomass and the biomass of many specific groups of soil organisms will reflect the level of SOM inputs.

Hence, organic or traditional farming practices that include regular inputs of OM in their rotation determine larger soil communities than conventional farming practices (Ryan, 1999). Generally, the responses of soil fauna to organic manure will depend on the characteristics of the manure and the rates and frequency of application. Herbivore dung, a rich source of energy and nutrients, is exploited initially by a few species of coprophagous dung flies and beetles and subsequently by an increasingly complex community comprising many general litter-dwelling species (Curry, 1994).

Pfotzer and Schuler (1997) also reported that the soil microbial and faunal feeding activity responded to the application of compost with higher activity rates than with mineral fertilization. Studies related to compost from sewage sludge application on agricultural and forest soils showed an increase in the abundance of

Collembola (Lüben, 1989), *Carabidae* (Larsen et al., 1986), *Oligochaeta* (Cuendet and Ducommun, 1990), soil nematodes (Bruce et al., 1999), and *Arachnida* (Bruce et al., 1999). In some cases, the application of sewage sludge to agricultural and forest systems can input toxic substances that accumulate in the soil and reach potentially toxic levels for soil fauna (Bruce et al., 1999). Field studies have suggested that metals contained in sewage sludge do not reduce the abundance of euedaphic (Lüben, 1989) and epigeic collembolans (Bruce et al., 1997) but may alter their population structure. Bruce et al. (1997) reported negative effects on collembolan communities in soil treated with sewage sludge, and these effects can be attributed to anaerobic conditions and high ammoniacal levels. Indeed, the knowledge gained in relation to the effects of sewage sludge showed that species that are more sensitive to the toxic substances contained in sewage sludge may disappear, while other more tolerant species may dramatically increase (Menta, 2012).

Organic wastes could also become an easily available and cheap source of OM after composting processes. The use of compost obtained from organic waste in agricultural and forest activity enables waste materials to be converted into a useful resource. This has led national authorities in recent years to promote both the use of compost to reduce soil fertility loss and the research aimed at assessing its effects on both agricultural and forest production and soil environment (Allievi et al., 1993; Pinamonti et al., 1997; Bazzoffi et al. 1998). However, the possible negative effects on soil fauna deriving from the use of organic waste include the accumulation of trace metals in the soil (Tranvik et al., 1993).

Pesticide application to the soil can affect the soil fauna by influencing the performance of individuals and modifying ecological interactions between species. When pesticides impact one or more ecosystem components, they also affect microarthropod communities in terms of number and composition. Pesticide toxicity on soil fauna is determined by various factors, such as the pesticide's chemical and physical characteristics, the species' sensitivity, and the soil type. In fact, among soil microarthropods, different taxa showed a variety of responses.

The physical and chemical characteristics of the soil, such as its texture, structure, pH, OM content and quality, and the nature of the clay minerals, are important factors in determining the toxic effects of pesticides and other xenobiotics. A study carried out by Joy and Chakravorty (1991) showed reduced toxic effects, as a function of soil type, in the following order: sand > sandy loam > clay > organic soil. Often, the toxicity of pesticides can be directly related to SOM content (Van Gestel and Van Straelen, 1994). However, pesticide application does not always cause negative impacts on the entire soil microarthropod community. For example, for certain types of soil, there is evidence that some taxa can obtain a competitive advantage from the application of certain specific pesticides (Menta, 2012).

Finally, in biodiversity recovery processes using forest restoration, it must be considered that after stimulating the natural regeneration processes that establish forest species, it is necessary to manage and direct succession processes toward the desired objectives. It is important to promote continued development of the vegetation to conserve soil, nutrients, and organic resources; to restore fully functional hydrological processes, nutrient-cycling processes, and energy flow processes; and



to create self-repairing landscapes that provide the goods and services necessary for biophysical, biochemical, and socioeconomic sustainability.

Different stages of forest-soil degradation call for management actions that focus on different processes. Severely degraded sites require early repair of hydrological, nutrient-cycling, and energy capture and transfer processes. As the forest vegetation increases in biomass and stature, it reduces the abiotic limitations of the site by improving soil and microenvironmental conditions. Directing natural processes toward land-use goals requires an understanding of the processes driving succession. The rate and direction of succession are influenced by the availability of species and the availability of suitable sites and by differential species performance (Mansourian et al., 2005).

4.2.4 DIFFICULTIES IN DEVELOPING INTERNATIONAL STRATEGIES FOR FOREST-SOIL PROTECTION

The realization that all of the soil degradation processes described are an environmental problem of global significance and the recognition of the importance of protecting our soils have led to an increase in international initiatives (Akeem, 2012). The protection of the soil and the preservation of its biological health and overall quality have therefore become a key international goal.

In Europe, for example, the threat to soil health was reported, and its biological roots were recognized almost three decades ago (Filip, 1973; Kovda, 1975). Nevertheless, there was a strong belief in soil's capacity for self-remediation. In the 1980s, the first moves toward the development of well-targeted soil protection initiatives were made in Germany and the Netherlands and later also in the European Community (Barth and L'Heremite, 1987; Howard, 1993). Finally, on March 1, 1999, a Federal Soil Protection Act came into force in Germany. Its practical application, however, requires the availability of properly justified standards and reliable monitoring methods (Filip, 2002).

The legislative interest in soil protection must therefore be accompanied by a concerted effort to seek a common methodology for the estimation of soil quality, in which biological and biochemical soil properties will play a very important role due to their high sensitivity to distorting agents (Filip, 2002). However, Howard (1993) indicated that variations in the approaches adopted by different countries reflect discrepancies in the nature and perceived seriousness of soil problems.

Continuing with the example of the EU's soil policies, Howard (1993) and Gzyl (1999) reported that European countries considered the "extension of urbanization, pollution by heavy metals, organic contaminants (including pesticides), acidification, over-fertilizing and artificial radio nucleids, groundwater nitrates, loss of organic matter, deteriorating soil structure, soil compaction and water and wind erosion" to be major threats to their soils. However, soil biodiversity is only indirectly addressed in a few European countries through specific legislation on soil protection or regulations promoting environmentally friendly farming practices (Jeffery et al., 2010; Turbé et al., 2010).

Given the differences between belowground and aboveground biodiversity, policies aimed at aboveground biodiversity may not be very effective for the



protection of soil biodiversity. In contrast, the management of soil communities could form the basis for the conservation of many endangered plants and animals, as soil biota steer plant diversity and many of the regulating ecosystem services. No legislation or regulation exists to date that is specifically targeted at soil biodiversity, whether at the international, national, or regional level. This reflects the lack of awareness of soil biodiversity and its value, as well as the complexity of the subject (Akeem, 2012; Menta, 2012).

Due to the complexity of these topics, there is so far no instrument that specifically addresses the protection of soil (UE, 2005) on any significant scale, such as at the level of the EU.

In connection to the issue of biodiversity loss, for example, there are several policy areas that directly affect and could address soil biodiversity, including policies for soil, water, climate, agriculture, and nature. This aspect could be taken into account or highlighted in future biodiversity policies and initiatives such as the new European strategy for biodiversity protection post-2010 (Jeffery et al., 2010; Turbé et al., 2010). Previously in this area, among the priorities set by the Sixth Environment Action Program of the EU (EC, 2002) for the conservation of biodiversity and natural resources, it assumed the commitment of addressing soil alongside water and air as an environmental medium and as a nonrenewable resource to be preserved, hence undertaking to develop a thematic strategy for the protection of the soil (UE, 2005).

Johnston and Crossley (2002) highlight the study and protection of soil ecology as an essential component of forest ecosystem recovery and protection. They indicate that for many years, there was a lack of concern for forest-soil conservation: Brauns (1955) cautioned that it would be impossible to continue ignoring soil biology when resolving forest management problems and urged more consideration for the improvement of soil biological status. Similarly, Wells (1984), reflecting upon the increasing demands for shorter rotations and faster-growing trees, concluded that it would not be sufficient to maintain the existing fertility of forest soils. Bloem et al. (2003) and Brussard et al. (1998) explain that cryptobiota—hidden soil life—plays a key role in life-support functions but is not part of any recognized list of endangered species. It is questionable whether a species-based approach is sufficient to attain a sustainable use of ecosystems inside—and especially outside—protected areas. Therefore, research networks have been established to monitor large areas, including agricultural and forest soils (Bloem, 2003).

Johnston and Crossley (2002) also remarked that the chemical and biological conditions of forest soils are not routinely monitored and studied, as they should be in view of the priority of improved regional soil fertility within the overarching goal of ecosystem management. They posed two questions: Can forest managers continue to manage forest ecosystems without striving to improve soil fertility over the long term? Does the field of soil ecology have little to offer to the practice of managing forest ecosystems, including forests that produce timber products?

In view of all these questions, it is necessary to conduct a review of the application of SQI in forest-soil conservation and protection and of the protocols that allow the integration of these factors in participatory forest management.

4.3 SOIL-QUALITY INDICATORS IN SUSTAINABLE FOREST MANAGEMENT

4.3.1 SUSTAINABLE FOREST MANAGEMENT AND SOIL QUALITY

Given the numerous terms and definitions for the concept of sustainable forest, Powers et al. (1998) and Page-Dumroese et al. (2000) pointed out that soil productivity is a key factor for maintaining ecosystem function. Forestry management on a sustainable basis has consistently included the maintenance or enhancement of forest-soil quality as a criterion of sustainability (Burger and Kelting, 1999; Schoenholtz et al., 2000). Furthermore, the overall goal of soil protection involves the conservation of good soil quality and requires certain soil functions to be fulfilled.

Schoenholtz et al. (2000) also reported that international and national calls for management of forestry on a sustainable basis have consistently included maintenance or enhancement of forest-soil quality as a criterion of sustainability. However, the choice of a standard set of specific soil chemical, physical, and biological properties as indicators of soil quality can be complex and will vary among forest systems and management objectives. Moreover, despite the dramatic changes in soil properties and processes detected during cropping and forest regeneration, no attempts have been made to develop SQI to assist in the assessment of soil conditions during such changes (Akeem, 2012; Bautista et al., 2012).

However, although considerable activity is currently aimed at the development and evaluation of sustainable management systems for agricultural and forest soils, these efforts have been hampered by the lack of agreement as to what constitutes credible measures of sustainability. The means to evaluate soil sustainability in terms of both design and performance have yet to be fully determined (Larson and Pierce, 1991; Pierce and Larson, 1993; Doran, 1994), but in recent decades, several new institutional, technical, and scientific approaches are being and have been developed.

As an example of the evolution of institutional approaches for sustainable soil management, the European Commission in its Sustainable Development Strategy published in 2001 noted that soil loss and declining fertility were eroding the viability of agricultural land (COM 2001, 264). In 2002, the European Parliament and Council established the Sixth Environmental Action Program (Sixth EAP), which covers a period of 10 years (Decision 1600/2002). This program addresses the community's key environmental objectives and priorities to be met through a range of measures, including legislation and strategic approaches. In Article 6, "Objectives and priority areas for action on nature and biodiversity," the Sixth EAP foresees the development of a thematic strategy on soil protection, addressing the prevention of pollution, erosion, desertification, land degradation, and hydrogeological risks, taking account of regional diversity, and including the specificities of mountain and arid areas (Bloem, 2003).

As an example of the evolution of technical approaches for sustainable agricultural and forest-soil management, several authors indicate that the current aims of these approaches are to maintain good crop yields with minimal impact on the environment while at least avoiding deterioration in soil fertility and providing essential nutrients for plant growth. Furthermore, sustainable agricultural and forest systems

and agriculture should support a diverse and active community of soil organisms, exhibit a good soil structure, and allow for undisturbed decomposition. Thus, current sustainable agricultural and forest practices are adjusting to integrate organic—or more extensive—management. The main principles involve restricting stocking densities, avoiding synthetic pesticides and mineral fertilizers, and using organic manure. This will ultimately result in an increased role of soil organisms, for example, decomposers, nitrogen fixers, and mycorrhizae, in plant nutrition and disease suppression.

Current sustainable agriculture and forestry should therefore seek to conserve natural resources based on a concept of productivity, which is closely linked to maintaining a system aimed at saving energy and resources in the mid to long term, through optimizing recycling and enhancing biodiversity and through biological synergy (Hansen et al., 2001; Máder et al., 2002; Bloem, 2003).

As examples of the evolution of technical approaches for sustainable forest-soil management, the two most common approaches for monitoring soil quality in forestry in the last decade may be (1) direct comparisons of biomass production in successive rotations and (2) soil-quality indices based on direct measurements of soil properties such as rooting volume, soil strength, and OM or through some integrated measure using multiple soil properties (Henderson et al., 1990; Powers et al., 1998; Fox et al. 2000).

Regarding the evolution of scientific approaches for sustainable agricultural and forest-soil management, Bloem (2003) highlights the need for standards for evaluating management systems that allow an assessment of their sustainability. In recent decades, two different approaches have frequently been employed to evaluate sustainable management systems: (1) comparative assessment and (2) dynamic assessment.

In the comparative approach (1), the performance of a system is determined in relation to alternatives. The characteristics and biotic and abiotic soil attributes of alternative systems are compared at a particular time, and the decision with regard to the relative sustainability of each system is based on the magnitude of the measured parameters. The main limitation of the comparative approach is that if only outputs are measured, it provides little information about the process that created the condition measured.

In contrast, in the dynamic approach, (2) a management system is assessed in terms of its performance determined over a period of time. The main disadvantage of this approach is that it needs measurements of indicators for at least two points in time and consequently does not provide an immediate assessment of soil quality. Moreover, it can be misleading in the case of soils that are functioning at their highest attainable level and cannot be improved or when they are functioning at their lowest attainable level and cannot deteriorate further. Both these cases would show a static trend—indicating sustaining systems—but would have a completely different quality. These two approaches to assessment are complementary, since they allow different scales of evaluation. While monitoring trends is more useful for evaluation at the farm level, comparative assessment appears to be more suited to a broader scale of evaluation (on a regional scale) (Seybold et al., 1997; Bloem, 2003).

Continuing with the topic of the evolution of scientific approaches for sustainable agricultural and forest-soil management, Bastida et al. (2008) point out that given



the complex nature of soils, it is important to select adequate indicators depending on the task. In order to obtain a complete picture of soil quality, a range of different parameters (physical, chemical, and biological) must be included (Frankenberger and Dick, 1983; Nannipieri et al., 1990; Dick, 1994; Gelsomino et al., 2006). The reason for the need to include a wider range of indicators is that certain factors may affect some indicators but not others, and this can affect the accuracy of the overall picture of soil quality.

However, Bastida et al. (2008) explain that despite the wide diversity of indices, they have never been used on large scales, nor even in similar climatological or agronomic conditions. They attribute this lack of applicability of soil-quality indices to the deficient standardization of certain methodologies, to the fact that some methods are beyond reach in some parts of the world, to spatial scale problems (soil heterogeneity), to poor definition of soil natural conditions (climate and vegetation), and to poor definition of the soil function to be tested for soil quality. They report that the most straightforward index used in the literature is a biparametric index (metabolic quotient qCO_2 or respiration-to-microbial biomass ratio) that has been widely used to evaluate ecosystem development, disturbance, and system maturity. However, they conclude that indices that integrate only two parameters did not provide enough information on soil quality or degradation.

Lately, as opposed to biparametric indices, there has been widespread development of multiparametric indices, which clearly establish differences between (1) management systems, (2) soil contamination or density, and (3) type of vegetation. These indices integrate different parameters, of which the most important are biological and chemical parameters such as pH, OM, microbial biomass C, respiration, and enzyme activities. The majority of multiparametric indices have been established on the basis of either expert opinion (subjective) or using mathematical–statistical methods (objective).

4.3.2 SOIL-QUALITY INDICATORS

Soil-quality assessment is of the utmost importance for determining the sustainability of land-management systems in the near and distant future. SQI/indices are needed to identify problem production areas, to make realistic estimates of food and natural resource production, to monitor changes in sustainability and environmental quality as related to agricultural and forest management, and to assist national and state or regional agencies in formulating and evaluating sustainable agricultural and forest land-use policies (Granatstein and Bezdicek, 1992; Doran, 1994).

The concepts of soil quality and SQI have evolved in recent decades. Soil scientists have always sought to link soil quality and soil variables to land use. Agronomists and farmers most commonly define soil quality as the suitability of a soil to function under different uses, illustrating a broader concept that highlights the fact that agriculture has traditionally been more focused on soil interaction than forestry. Foresters, by comparison, have traditionally linked soil quality to the measurement of soil productivity using tree growth or wood yield; they usually define soil productivity as the ability of a soil to produce biomass per area and per time units (Ford, 1983; Warkentin, 1995; Schoenholtz et al., 2000).



Among the definitions for the concept of general soil quality that have been suggested in the past, Doran (1994) highlights that soil quality is “The capacity of the soil to interact with the ecosystem in order to maintain biological productivity, environmental quality and to promote animal and plant health.” This definition is similar to the three essential criteria for soil quality that were identified by the Rodale Institute in 1991: (1) Productivity is the soil’s capacity to increase plant biological productivity; (2) environmental quality is the soil’s capacity to attenuate environmental contamination, pathogens, and external damage; and (3) the health of living organisms is the interrelation between soil quality and animal, plant, and human health (Benedetti and Dilly, 2003).

Schoenholtz et al. (2000) reviewed the concept of soil quality and defined it as a site’s quality for forest productivity. They explained that soil quality is a value-based concept related to the objectives of ecosystem management and hence it will be management and ecosystem dependent. Soil quality may be broadly defined to include water retention capacity, C sequestration, plant productivity, waste remediation, and other functions, or it may be defined more narrowly. For example, a forest plantation manager may define soil quality as the capacity of a soil to produce biomass.

Bastida et al. (2008) reported that the concept of soil quality arouses greater controversy than the concept of water or air quality. These authors point out that the maintenance of soil quality is critical for ensuring the sustainability of both the environment and the biosphere; for this reason, they reviewed SQI as well as the parameters comprising them and highlighted the lack of consensus concerning the use of these indicators.

Heightened concerns regarding the sustainability of agricultural and forestry practices and the influence of soil conditions on environmental sustainability have led to considerable research efforts into SQI and indices (Doran, 1994; Hailu and Chambers, 2012).

Basic SQI are useful for comparing quality among soil types and before and after certain management practices are imposed on a soil type. SQI may be simple state variables with a measurable unit or a complex construct of several soil variables known as “soil-quality indices” that may include a time or rate dimension, which makes them dynamic (Burger and Kelting, 1999; Schoenholtz et al., 2000). Whatever the case, SQI should give some measure of the capacity of a soil to function in terms of plant and biological productivity and environmental quality (Seybold et al., 1997).

Indicators of soil quality should also be as follows: (1) sensitive to long-term change in soil management and climate but sufficiently robust not to alter as a consequence of short-term variations in weather conditions, (2) well correlated with beneficial soil functions, (3) useful for understanding why a soil will or will not function as desired, (4) comprehensible and useful to land managers, (5) easy and inexpensive to measure, and (6), where possible, should also be components of existing soil databases.

Furthermore, the suitability of SQI depends on the kind of land, land use, and scale of assessment. Different land uses may require different soil properties, and in consequence, some SQI in a given situation can be more helpful than others for the purpose of the assessment (Karlen et al., 2001; Bloem, 2003).

In summary, an SQI is a measurable surrogate of a soil attribute that determines how well a soil functions (Burger and Kelting, 1999; Schoenholtz et al., 2000). Soil quality can be evaluated using a large number of indicators (chemical, physical, biological) depending on the scale and objective of the evaluation.

A review of SQI showed that there is heavy reliance on a few appraisals: (1) SOM among chemical indicators (Liebig and Doran, 1999; Bowman et al., 2000; Brejda et al., 2000a; Kettler et al., 2000; Gilley et al., 2001; Li et al., 2001), (2) bulk density (Liebig and Doran, 1999; Kettler et al., 2000; Gilley et al., 2001; Li et al., 2001), and (3) aggregate stability (Bowman et al., 2000; Six et al., 2000) among physical indicators, which were the most frequently used. In contrast, there were very few examples of biological indicators of soil quality (Pankhurst, 1997; Liebig and Doran, 1999; Gilley et al., 2001).

However, biological monitoring is necessary to correctly assess soil degradation and its correlated risks (Turco et al., 1994). In particular, there is an urgent need to identify indicator systems with the capacity to express soil-quality criteria so they can be used as benchmarks in environmental remediation, as well as to assess and monitor soil quality in soils subjected to risk of degradation (van Straalen and Krivolutsky, 1996; ANPA, 2002).

Due to the complexity of the soil system, a specific soil management system cannot be assessed using a single indicator of soil quality but requires the selection of a minimum data set (MDS) of attributes regarding the physical, chemical, and biological properties of the soil (Karlen et al., 2001; Bloem, 2003). Hailu and Chambers (2012) summarized the scientifically sound procedures for building soil-quality indices that combine diverse soil-quality attributes or indicators into summary measures in order to enhance the evaluation of land-management strategies. Over the last decade and a half, several procedures for building soil-quality indices have been proposed and implemented in the soil-science literature. They describe an overview of the index construction procedures employed in soil-science research provided by Andrews et al. (2002, 2004), Karlen et al. (2003), and Bremer and Ellert (2004).

The standard approach is to generate the index using the following three steps. The first step (1) involves choosing an MDS of physical, chemical, and biological soil-quality variables (Larson and Pierce, 1991), based on either expert opinion or statistical data reduction methods such as principal component analysis (PCA). In the second step (2), the variables in the MDS are transformed into 0–1 scores using either linear or nonlinear transformation functions. In the final step (3), the indicator scores are combined into a soil-quality index using different integration techniques including simple addition (Andrews and Carroll, 2001a), weighted addition (Harris et al., 1996), or decision support systems employing min-max objective functions (Yakowitz et al. 1993). Both the second and third steps are ad hoc. The transformation of individual soil-quality variables or indicators into 0–1 scores does not recognize that the impact of a soil-quality variable on outcomes (e.g., crop yield) might depend on the level of other soil variables or production inputs (Hailu and Chambers, 2012).

In summary, variation-sensitive soil indicators in forest management are needed to compare the effects of a management practice on soil over time (Schoenholtz et al., 2000). The assessment of soil quality and the identification of key soil properties that serve as indicators of soil function are complicated by the many issues defining



quality and the multiplicity of physical, chemical, and biological factors that control biogeochemical processes and their variation in time, space, and intensity. The practical assessment of soil quality therefore requires the consideration of these functions and their variations in time and space (Larson and Pierce, 1991; Doran, 1994).

4.3.3 ASSESSMENT OF SOIL-QUALITY INDICATOR USAGE IN FORESTRY

The assessment of sustainable forestry systems, which allow the combination of production targets and environmentally friendly management practices while protecting both soil and biodiversity, is essential in order to prevent the decline of forest landscapes (Menta, 2012). Despite these warnings, it does not currently appear that many forest soils are being managed and protected actively in the way this author suggests.

The process of forest sustainability assessment requires evaluators to be reasonably informed about the practical appraisal and interpretation of soil quality using soil indicators in order to increase their chances of achieving correct joint decision making (E. Martínez-Falero, pers. com.). Thus, to improve the evaluators' understanding of the current sustainable forest management status and the use of SQI in forestry, this chapter and the following describe the main scientific outcomes of SQI and their current applications in forest sustainability assessment.

Regarding forest soils, foresters have always relied on the knowledge of soil chemical and physical properties to assess the capacity of sites to support productive forests. However, Schoenholtz et al. (2000) pointed out that in the last few years the need to assess soil properties has expanded due to the growing public interest in understanding the consequences of management practices on the quality of soil relative to the sustainability of forest-soil functions (water flow, biodiversity conservation, and as an environmental buffer), in addition to plant productivity. Moreover, soil-quality assessment is fundamental for determining the sustainability of land-management systems in the near and distant future. The challenge for the future is to develop sustainable management systems that are at the vanguard of soil health; SQI are merely a means toward this end.

In this regard, with the aim of assessing the current usage of soil-quality indices in forestry, we analyzed the available scientific bibliography in all experimental areas related to the concepts of "soil quality and its indicators" and "SQI in forestry," by conducting a systematic study of all the available databases on the Web of Knowledge (Thomson Reuters, 2011). This analysis was done up until 2011, which was the last complete year.

This bibliographical study is structured into successive stages of analysis. The first stage involves consulting the ISI Web of Knowledge base using the keywords "soil quality + indicators," and in the second stage, we consulted the ISI Web of Knowledge base using the keywords "soil quality + forestry" to determine the temporal evolution and research contents of the scientific works associated to these keywords. The third stage revises the bibliographical information contents on the selected articles, such as soil indicator typologies and what they were used for in relation to the sustainable forestry approach.

The field of soil science has historically been concerned with soil quality; however, the results of our bibliographical analysis show that it was not until 1991 when



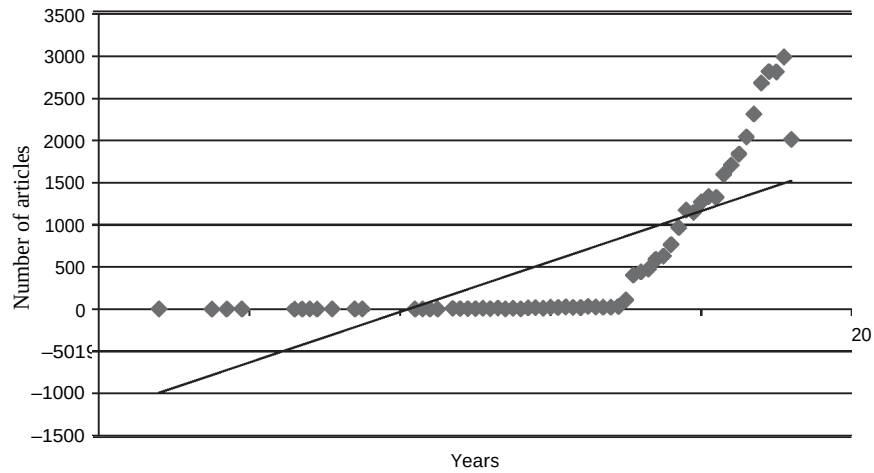


FIGURE 4.1 The number of articles studying soil quality has been rapidly increasing since 1990 to the present day (ISI WEB of Knowledge database. Keyword: “soil quality”).

AQ4

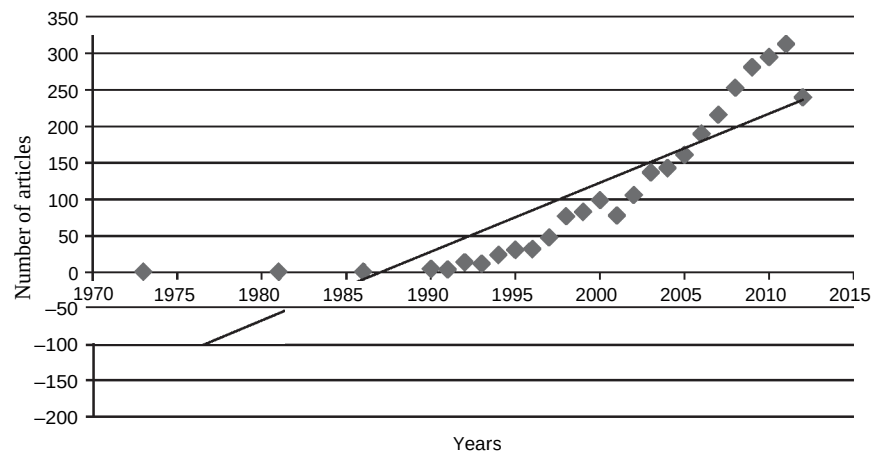


FIGURE 4.2 The number of articles using indicators to study soil quality has been rapidly increasing since 1990 to the present day (ISI WEB of Knowledge database. Keyword “soil-quality indicators”).

significant research into “soil quality” and “SQI” began to appear. Figure 4.1 shows the trends for the articles incorporating “soil-quality” concepts, and Figure 4.2 shows the trends for the articles incorporating “SQI.”

However, this interest in soil quality and its indicators has not been homogeneous across the various research and management areas. Figure 4.3 shows that out of the total articles incorporating the concept of “soil quality,” only 4% refer to forestry, in contrast with 52% to agriculture and 32% to environmental sciences.

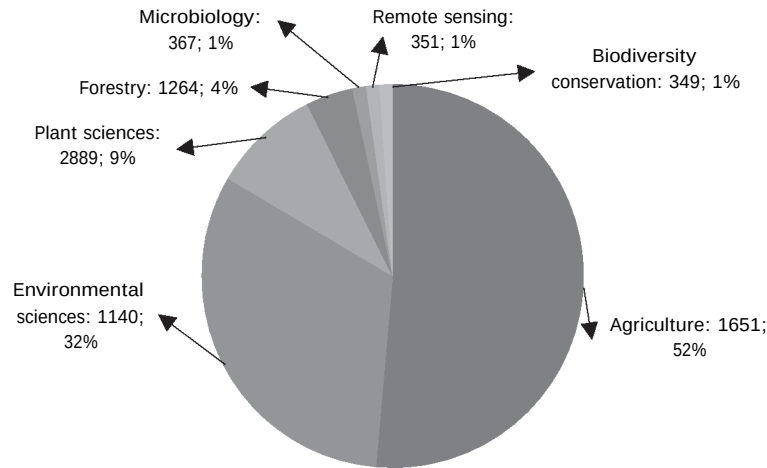


FIGURE 4.3 Article distribution by research and management areas studying soil quality (ISI WEB of Knowledge database: keyword “soil quality”).

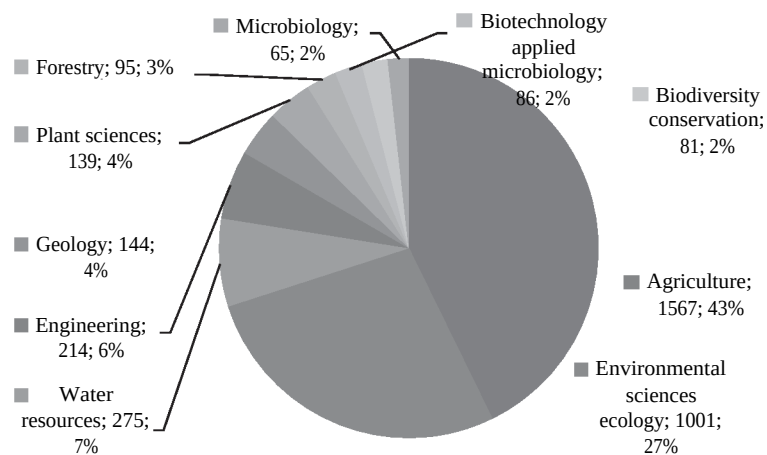


FIGURE 4.4 Article distribution by research and management areas using indicators of soil quality (ISI WEB of Knowledge database: keywords “soil-quality indicators”).

Moreover, Figure 4.4 shows that out of the total articles incorporating “SQI,” only 3% refer to forestry, as opposed to 43% to agriculture and 27% to environmental sciences.

Regarding forest production, soil fertility and soil quality determine vegetation composition, structure, and growth, as both serve as a medium for root development and provide moisture and nutrients for plant development. Both soil qualities would thus explain the reason that 55.1% of the forestry articles incorporating SQI correspond to research in applied forestry (51.7% articles on forestry production and 3.4% articles on forest management).

However, there has been a progressive decline in the number of articles incorporating SQI in the study of forest production and forest management in recent years. The study and protection of soil ecology are essential components of forest ecosystem conservation and recovery (Johnston and Crossley, 2002). However, our bibliographical analysis shows that only 25.2% of the forestry articles analyzed include SQI associated to edaphological and forest ecology studies (10.3% articles related to forest edaphology, 9.2% articles related to forest ecology, and 5.7% articles related to tree morphology and physiology). Moreover, only 6.4% of the forestry studies analyzed incorporate SQI relating forestry research to environmental issues. However, there has been a progressive increase in the number of articles incorporating SQI in the study of forest ecology in recent years.

The interest and use of SQI in forestry have not been homogeneous across all forest types. Our bibliographical analysis shows that 61.3% of the forestry articles using SQI are related to coniferous woods, which are usually associated to soils with limited fertility and limited biological productive capacity (29.3% articles related to *Pinus* spp. woods and 12.0% articles related to *Picea* spp. woods). In contrast, 28.0% of this kind of articles is associated to deciduous woods, and only 4.0% to tropical forests.

In the area of forest-soil elements and properties assessed using SQI, our bibliographical analysis showed that 72.1% of the articles are related to the study of soil fertility and soil biological production capacity (34.9% associated to soil nutrition, cationic exchangeable complex (CEC), N, C/N, P, and fertility; 16.3% associated to soil moisture and soil temperature; 14.5% associated to soil C, OM, and humus; and 6.4% associated to microbiology and soil biology). This kind of articles has increased over time.

In contrast, 25.0% of the forestry studies using SQI are related to soil profile characteristics and elements (8.1% are associated to geomorphology and soil depth, 4.7% are associated to pH, 4.7% are associated to texture and structure, 4.7% are associated to porosity and aeration, and 2.9% are associated to penetration resistance and bulk density). However, this kind of articles has increased over time. Finally, only 1.7% of forest SQI studies are related to soil degradation (salinity and contamination).

This last aspect is an important omission, as forest-soil degradation processes are an environmental problem of global significance with immediate consequences at both the economic and social level; recognition of the importance of protecting against this phenomenon has led to an increase in international initiatives (Akeem, 2012).

Foresters have always relied on the knowledge of the chemical and physical properties of soils to assess a site's capacity to support productive forests (Schoenholtz et al., 2000). Our bibliographical analysis shows that most of the soil-quality indices/indicator types used in forestry studies are either chemical (49.0%) or physical (43.5%). Moreover, both kinds of articles have increased over time.

However, in the field of forestry, SQI that integrate both physical and chemical variables have been poorly applied (2.0%), as have biological SQI (4.1%). Finally, only 12.6% of the forestry articles applying SQI have used statistical analysis and numerical models.

All these bibliographical analyses lead to the conclusion that there is a need for new procedures that integrate physical, chemical, biological, and other unforeseen

AQ5

elements into indices and indicators so they can perform seamlessly under any given forest-soil circumstance worldwide (Bastida et al., 2008). Current knowledge of forest-soil indicators has not benefited from the wealth of tools such as numerical models—as indicated by Hailu and Chambers (2012)—which incorporate the Luenberger indicator (currently available in economics), for example, into forest-soil-quality assessment.

4.4 BIOLOGICAL, CHEMICAL, AND PHYSICAL SOIL-QUALITY INDICATORS

4.4.1 FOUNDATIONS FOR UNDERSTANDING THE ROLE OF BIOLOGY IN SOIL-QUALITY ASSESSMENT

Some researchers have defined the soil biota as a “superorganism” that assumes a crucial significance due to the chemical–physical and biological processes that are rooted in the soil. A rapid survey of invertebrate and vertebrate groups reveals that at least a quarter of the living species described are strictly soil or litter dwelling. These large soil communities play an essential role in soil functions as they are involved in processes such as the decomposition of OM, the formation of humus, and the nutrient cycling of many elements (nitrogen, sulfur, C). Moreover, edaphic communities affect soil porosity and aeration as well as the infiltration and distribution of OM within its horizons (Maharning et al., 2008; Menta, 2012). It is therefore necessary to understand the role of biodiversity in soil functions as the foundation for identifying SQI based on soil biology.

With regard to biodiversity and soil function interactions, it must be noted that decomposition of OM by soil organisms is crucial for the functioning of an ecosystem, due to its substantial role in providing ecosystem services for plant growth and primary productivity (Menta, 2012). For example, soil fauna performs a mainly mechanical action, whereas fungi and bacteria, both free and intestinal symbionts of other organisms, essentially perform chemical degradation; furthermore, during digestion, organic substances are enriched by enzymes that are dispersed in the soil along with the feces, contributing to humification (Maharning et al., 2008; Menta, 2012).

However, at present, we are not sure of the extent of the importance of this biodiversity in the sustainability of a soil’s functions, and it is extremely rare for the biological relationships between soil organisms to consist of a simple and clear interaction. Current ecological conditions are the result of many complex interactions that typically involve multiple participants in soil life such as plants, microbes, fungi, and animals (Killham, 1994).

Thus, the relationships between biodiversity and its functions are complex and somewhat poorly understood, even in aboveground situations. The exceptional complexity of belowground communities further confounds our understanding of soil systems. Four important mechanisms underlying relationships between biodiversity and function are responsible for (1) driving fundamental nutrient-cycling processes, (2) regulating plant communities, (3) degrading pollutants, and (4) helping to stabilize soil structure.

Some of the most significant functions—and the main biotic groups that carry them out—are (1) primary production [plants, cyanobacteria, algae]; (2) secondary production [herbivores]; (3) primary decomposition [bacteria, archaea, fungi, some fauna]; (4) secondary decomposition [some microbes, protozoa, nematodes, worms, insects, arachnids, mollusks]; (5) soil structural dynamics [bacteria, fungi, cyanobacteria, algae, worms, insects, mammals]; (6) suppression of pests and diseases [bacteria, actinomycetes, fungi, protozoa, nematodes, insects]; (7) symbioses [bacteria, actinomycetes, fungi—notably mycorrhizae]; (8) SOM formation, stabilization, and C sequestration [virtually all groups, directly or indirectly]; (9) atmospheric gas dynamics, including generation and sequestration of greenhouse gases [bacteria for nitrous oxides, methane, all biota for CO₂]; and (10) soil formation [bacteria, fungi] (Soil Atlas of Europe).

Rough estimates of soil biodiversity indicate several thousand invertebrate species per site, as well as relatively unknown levels of microbial and protozoan diversity. Soil ecosystems generally contain a large variety of animals including nematodes and microarthropods such as mites and collembolans, symphylans, chilopodans, pauropodans, enchytraeids, and earthworms. In addition, a large number of meso- and macrofauna species (mainly arthropods such as beetles, spiders, diplopods, chilopods, and pseudoscorpions, as well as snails) live in the uppermost soil layers, the soil surface, and the litter layer. Despite several decades of soil biological studies, it is still very difficult to provide average abundance and biomass values for soil invertebrates. This is partly due to their high variability in both time and space, as well as to differences in the sampling methods used (Jeffery et al., 2010).

In addition, most of the work has been conducted in the forest soils of temperate regions, while other ecoregions such as the tropics, or other land uses such as agriculture, have been seriously neglected (Jeffery et al., 2010). Soil fauna is very variable, and most is also highly adaptable regarding feeding strategies, ranging from herbivores to omnivores and including carnivores. Depending on the available food sources, many soil faunas are able to change their feeding strategies to a greater or lesser extent, and many carnivorous species are able to feed on dead OM at times of low food availability. The interactions between soil fauna are numerous, complex, and varied. As well as in the predator/prey relationships and in some instances parasitism, commensalism also occurs (Menta, 2012).

The degree of interaction between soil organisms and the soil itself can be highly variable among taxa and dependent on the part of the life cycle that is spent in the soil (Wallwork, 1970). Specifically—and in combination with the morphological adaptations and the ecological functions of organisms—soil fauna can be classified into four main groups: (1) temporarily inactive geophiles, (2) temporarily active geophiles, (3) periodical geophiles, and (4) geobionts. It should be noted that these groupings do not have any taxonomical significance but are useful when studying the life strategies of soil invertebrates. These different types of relationships between soil organisms and the soil environment determine a differentiated level of vulnerability among various groups in response to possible impact on the soil environment. For instance, if soil contamination occurs, any impact will be higher on geobionts (as they cannot leave the soil and must spend all their life there) and lower on temporarily inactive geophiles (Menta, 2012).

Due to the absence of light, which makes photosynthesis unfeasible, there are very few photoautotrophic organisms and very few real phytophages among the organisms populating the soil. Regarding soil heterotrophic organisms, the activity of animals (typically protozoa, nematodes, rotifers, certain springtails, and mites) that feed on microflora—namely bacteria, actinomycetes, and fungi (both hyphae and spores)—is of crucial importance for both diffusing and regulating the density of these microorganisms. For example, through their feces, springtails—which feed on fungi—can spread fungal spores that are still viable to areas as far as a few meters away from their point of origin (Wallwork, 1970; Menta, 2012).

Therefore, the detritus food chain assumes an essential role within the soil, as it becomes the basis of the hypogean food web; in fact, numerous organisms such as isopods, certain myriapods, earthworms, springtails, many species of mites, and the larvae and adults of many insects feed on the vegetable and animal detritus that is deposited on the soil. For example, in the soil of a temperate forest—in which the contribution of litter each year can amount to 400 g/m^2 —about 250 g/m^2 is ingested by earthworms and enchytraeids, $30\text{--}40 \text{ g/m}^2$ by mites, and $50\text{--}60 \text{ g/m}^2$ by springtails.

There are many extremely old groups of microarthropods in soils—including collembolans and mites—dating from the Devonian period (more than 350 million years ago). They are very useful as indicators of soil quality, as their biodiversity and density are influenced by numerous soil factors, particularly OM and water content, but also other factors such as pollution (Menta, 2012).

Earthworms are among the most important organisms in many of the soils of the world. The activity of earthworms produces a significant effect, not just on the structure, but also on the chemical composition of the soil, since a large part of the OM ingested by earthworms is returned to the soil in a form easily used by plants. While they are feeding, earthworms also ingest large quantities of mineral substances (minimally so in the case of the epigeic), which are then mixed with the OM they ingest and, after having been cemented with a little mucous protein, are expelled in small piles known as worm casts. In addition to being rich in nitrogen and other nutritive substances such as calcium, magnesium, and potassium, worm casts also contain a large quantity of nondigested bacteria that proliferate easily in this substratum and contribute to the humification and mineralization of OM (Zanella et al., 2001). In addition to their worm casts, earthworms contribute to increasing the amount of nitrogen present in the ground through the excretion of ammonia and urea, forms that are directly useable by plants; furthermore, a sizable quantity of nitrogen is returned to the soil on the death of animals, which have a 72% protein content (Dindal, 1990).

The continual burrowing activity of earthworms and other soil organisms contributes to the creation of spaces within the soil with a resulting rise in its porosity; the increase in the pores between the particles in turn enhances aerobic bacterial activity and the consequent demolition speed of organic substances. This bioturbation also has positive effects on water retention, percolation processes, and the development of the rhizosphere. The burrowing activity enables the soil to be mixed, thereby incorporating OM from the surface layers into the lower layers, while mineral substances are brought toward the surface (Menta, 2012).

The anthill is one of the most interesting and elaborate examples of the modification of the soil by cunicular burrowing organisms. It consists of a complex of chambers, generally constructed on several levels and linked together by tunnels and corridors. Some nests can reach a depth of more than 5 m and contain over 2000 chambers, some of which are set aside for the cultivation of fungi. The presence of channels and tunnels increases the porosity of the soil, aiding the penetration of air and water. In addition, a consequence of the movement of the fine material toward the surface by ants during the course of the construction and maintenance of the anthill is the creation of a surface layer with a fine particle size, which is more mineral than organic in nature (Bachelier et al., 1971).

Soil fauna—particularly mollusks and earthworms—affects the soil through the secretion of cutaneous mucous, which has a cementing effect on the particles in the ground, contributing to the stability and structure of the soil and making it less vulnerable to erosion processes. The animals' mucous secretions, feces (especially those of earthworms), and their own bodies (when they die) considerably influence the concentration of nutrients present in the soil—particularly potassium, phosphorous, and nitrogen—reducing the C/N ratio of the litter and facilitating decomposition (Menta, 2012).

The presence of roots is generally associated with a greater density of microorganisms in the nearby soil compared with soil devoid of roots; the term rhizosphere is used in a broader sense to refer to the portion of soil surrounding roots in which the microorganisms are influenced by their presence (Killham, 1994). The interaction between soil animals and plant roots may take a variety of forms that lead to benefits or repress the growth of the plant and often involve interactions with the microbial population of the soil. The dispersion of the inocula of mycorrhizal fungi by soil animals can have beneficial effects for the plants; this dispersion is particularly favored by burrowing organisms belonging to the mega- and mesofauna categories.

The hyphae of mycorrhizal fungi may comprise a significant proportion of the total microbial biomass in some soils and may become one of the most important sources of food for fungus-grazing animals such as springtails. Numerous soil animals feed directly on the roots of plants; these include a large number of springtail and myriapod species. It is still not entirely clear how much of the damage inflicted on plants can be attributed to the direct action caused by grazing on the roots or to the subsequent vulnerability of the roots to pathogens in the soil, especially fungi (Menta, 2012).

In summary, soil communities participate in most key soil functions, driving fundamental nutrient-cycling processes, regulating plant communities, degrading pollutants, and helping to stabilize soil structure. The ecosystem services provided by soil biota are thus one of the most powerful arguments for the conservation of edaphic biodiversity.

4.4.2 BIOLOGICAL INDICATORS TO ASSESS FOREST-SOIL QUALITY

The growing recognition of the problems deriving from soil degradation has led to the identification of soil fauna research as a priority for soil-quality assessment (Bongers, 1990, 1999; Pankhurst, 1997; Pankhurst et al., 1997; van Straalen, 1997).



Measurement of soil organisms meets many of the criteria for useful indicators of sustainable land management: (1) They respond sensitively to anthropogenic disturbances; (2) the area covered during their life cycle is representative of the site under examination; (3) their life histories permit insights into the soil ecological condition; (4) their abundance and diversity are well correlated with beneficial soil functions; (5) they are useful for elucidating ecosystem processes.

Recently, various authors have proposed new methods for soil-quality assessment based on soil fauna, using three levels of analysis: The simplest one is (1) the bio-marker, which measures an individual's biochemical and physiological variability and its excretions; (2) bioindicators are organisms with specific ecological requirements, which serve as indicators of environmental change; and (3) the last type is community analysis, which has the greatest level of complexity and allows the totality of the information on zoocenosis and the relationships that characterize it to be assembled into one.

The obvious adaptations of soil fauna make edaphic organisms unable to leave the soil. This incapacity defines these organisms as being more sensitive to the variation in the physical and chemical parameters that can occur in the soil after tillage and other human activities. Moreover, the intricate relationships of soil invertebrates with their ecological niches in the soil, and the fact that many soil organisms live a rather sedentary life, serve as a good starting point for bioindicator changes in soil properties and the impact of human activities.

To be able to evaluate their role and function, it is important to use methodologies that highlight either the number of species present or the processes and roles they play in the soil environment. The key features for obtaining an assessment of soil disturbance are (1) species richness and diversity, (2) the relationship between species (rare vs. dominant species), (3) distribution of body sizes in the species analyzed, (4) classification of species' life-cycle attributes, (5) classification according to the ecophysiological preferences of the species analyzed, and (6) food chain structure.

The sample of the groups of organisms that can be used to assess soil ecosystems at the community level should meet the following requirements: (1) Their taxonomy must be well known and stable; (2) the main aspects of their natural history must be known; (3) their presence/absence should be easy to test and identify; (4) samples must contain individuals from a higher taxon level (genus, family, order) with a widespread diffusion both in geographic terms and in number of habitats; (5) they must contain individuals of lower taxa (species, subspecies) that are highly specialized and sensitive to environmental changes; (6) they must contain taxa with high ecological significance; (7) they must represent biodiversity models that reflect and represent other taxa, whether or not they are neighbors (Menta, 2012).

Three soil animal classes have been used as bioindicators: microfauna, mesofauna, and macrofauna. A selection of bioindicator and bioindex systems using these soil invertebrate groups to evaluate forest-soil quality (van Straalen, 2004) included

1. Soil microfauna (smaller than 0.02 mm): Comprised primarily free-living protozoa and Nematoda. In forest soils, the "nematode maturity index" (Bungers, 1990; Yeates and Bongers, 1999) uses nematodes classified on



a “colonizer–persister” scale to measure general response to stress (metals, acidification, eutrophication). The main problems associated with these indices are (a) systematic uncertainty, (b) spatial uncertainty due to the lack of definition of accurate models for the distribution of these organisms in the soil, (c) difficulty in achieving robust development indices without having a complete understanding of the systematic groups (species uncertainty), (d) the models and the data used that are processed in different countries (geographic uncertainty), and (e) the need for a reference site (control) in each study for a conclusive interpretation (Bongers, 1980; Wodarz et al., 1992; Ettema and Bongers, 1993; Korthals et al., 1996; Gupta and Yeats, 1997; Pankhurst et al., 1997).

2. Soil mesofauna (from 0.02 to 4 mm): Springtails (Acarida) are the most common soil arthropods, and other groups such as beetles (Carabidae), hymenoptera, and enchytraeids are present. Some of the most common biological indices are the “Acari/Collembola ratio,” “oribatea/other Acari ratio,” “biodiversity of oribatid Acari,” and “QBS-ar and QBS Collembola” (Aoki et al., 1977; Bernini et al., 1995; van Straalen, 1997; Behan Pelletier, 1999; Knoepp et al., 2000; Parisi and Menta, 2008). Other mesofauna biological indices are

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- a. In forest soils, the “predatory mite maturity index” has been applied (Ruf, 1998) using mesostigmatid mites classified according to an r-K score to measure soil properties related to humus.
- b. The “arthropod acidity index” (Van Straalen and Verhoef, 1997; Van Straalen, 1998) using a classification of collembolans, oribatids, and isopods according to pH preference in order to estimate quantitative pH measures from the invertebrate community structure.
- c. The “oribatid mite life-history strategies” (Siepel, 1994, 1996) use classification of mites according to their reproductive and dispersal strategies to indicate the intensity of anthropogenic influence and successional events.
- d. The “life forms of Collembola” (Van Straalen et al., 1985; Faber, 1991) using a classification of collembolans according to morphological types reflecting their position in the soil profile to indicate profile buildup and ecological soil processes.
- e. The “dominance distribution of microarthropods” (Hagvar, 1994) uses lognormal distribution of numbers over species to obtain a general diagnostic of disturbance associated to heavy metals and acid rain in forest and grassland soils.
- f. The “biological index of soil quality (BQS)” (Parisi, 2001; Gardi, 2002) involves scoring systems that assign scores to groups of soil microarthropods to provide an indication of their biodiversity status.

Some of these methods are based on the general evaluation of microarthropods (Parisi, 2001), while others are based on the evaluation of a single taxon (Bernini et al., 1995; Iturrondobeitia et al., 1997; Paoletti, 1999; Paoletti and Hassal, 1999; Parisi, 2001).

The simplest approach is to select a single species as an indicator. Single species bioindicators will have reasonable specificity as long as the indicator species reacts in a specific way but will have a low resolution due to unavoidable fluctuations in density. Using single species could obscure interspecies relationships, while considering all the species in a system will not reveal effects on rare species, which may be more affected than abundant species (Cortet et al., 1999).

The application of these biodiversity or biological indicators is often limited by the difficulties in classifying microarthropods. The introduction of a simplified ecomorphological index, which does not require the classification of organisms to species level, allows these methodologies to be more widely applied:

3. Soil macrofauna (larger than 4 mm): Snails and slugs are very good bioindicators as they have a wide range, are easy to sample, and have a high tolerance and bioaccumulation capacity; earthworms are also a good bioindicator, as they migrate over short distances and are both resistant and sensitive. Other macrofauna biological indices are
 - a. “Earthworm life-history strategies” have been used in forest soils (Bouché, 1977; Paoletti, 1999). Earthworms are classified according to the position they hold in the soil profile to measure aspects such as humus type, pH, and cultivation plowing.
 - b. “Real model for earthworms” (Bouché, 1996) is based on an integrated database of various aspects related to the ecological and agronomic role of earthworms and has a very wide application.
 - c. “Ant functional groups” (Andersen, 1995) are based on a classification of ants according to groups reflecting susceptibility to stress, with a wide application for evaluating nature restoration.
 - d. “Diptera feeding groups” (Frouz, 1999) are based on a classification of dipteran larvae to measure the different types of organic materials in soils.

4.4.3 MICROBIOLOGICAL, BIOCHEMICAL, AND MOLECULAR INDICATORS TO ASSESS FOREST-SOIL QUALITY

While most of the chemical and physical property variables that are relevant to soil quality are well understood, measures of soil biological properties have so far been much more difficult to use as decision support tools for monitoring soil quality.

Descriptions of soil biological properties can range from single-parameter variables such as microbial biomass or respiration to multiparametric data describing biochemical profiles, measurements of enzyme activities, and molecular analyses of microbial communities. However, in contrast to the extreme complexity of microbial communities in the soil, the ideal soil microbiological and biochemical indicators for the determination of soil quality must be simple to measure, work equally well in all environments, and reliably reveal which problems exist and where.

It is unlikely that a sole ideal indicator can be defined with a single measure, due to the multitude of microbiological components and biochemical pathways. Therefore, an MDS is frequently applied (Carter et al., 1997). The basic indicators

and the number of measures needed are still under discussion and depend on the aims of each investigation.

Bastida et al. (2008) indicate that given the complex nature of soils, it is important to select adequate indicators depending on the task; they also note the need to standardize indicator measurements. Further development of these tools should also help soil scientists to identify novel relationships and devise research to explore linkages between the biological, chemical, and physical properties of soils (Mele et al., 2008).

The star indicator for soil quality is SOM. In particular, since OM—or more specifically OC and the C cycle as a whole—can have an important effect on soil functioning, all the attributes linked to the soil C cycle are usually recommended as components in any MDS for soil-quality evaluation (Akeem, 2012). In addition, the visual output of the SOM analysis provides a rapid and intuitive means to examine covariance between variables, and with minimal training, it could be useful for assisting land managers to interpret multiparametric soil analyses.

According to a simplified scheme, SOM can be divided into two pools: nonliving and living. Nonliving SOM includes materials of different age and origin, which can be further divided into pools or fractions as a function of their turnover characteristics. For example, the humified fraction is more resistant to decay. The stability and longevity of this pool are a consequence of chemical structure and organomineral association. This pool of SOM influences different aspects of soil quality such as the fate of ionic and nonionic compounds, the increase in soil cation exchange capacity, and the long-term stability of microaggregates (Herrick and Wander, 1997). The interpretation guideline for the purpose of soil-quality assessment is that the higher the humified fraction of SOM, the higher its contribution to soil quality (Akeem, 2012).

The regulation of greenhouse gases is a largely overlooked function of soil quality in agroecosystems and can be calculated by using indicators such as gas flow or C sequestration (Liebig et al., 2001). This omission is surprising, especially in today's world where the soil is regarded as an important C sink and particularly since plants are CO₂ capturers, at a time when the greenhouse effect has become an issue of public concern (Bastida et al., 2008).

Regarding the living SOM pool, soil microbial communities are responsible for important physiological and metabolic processes of paramount interest for soil quality (Sessitsch et al., 2006). Soil microorganisms are mainly related to nutrient cycles, mineralization, humification, physical structure formation, degradation of contaminants, and soil fertility (Roldán et al., 1994; García et al., 2002).

Among all the SOM fractions, the most widely used indicator is microbial biomass C; another important microbial activity index for estimating soil quality is microbial respiration (Bastida et al., 2008). For this reason, national and international programs for monitoring soil quality currently include microbe biomass and respiration measurements but also extend to determining nitrogen mineralization, microbial diversity, and functional groups of soil fauna (Bloem, 2003). However, the structure, function, and relationships among soil microbial communities still represent a substantially uncharted territory that is intimately related to soil community and function.

From a biochemical soil approach, soil management practices can be monitored with other highly sensitive indicators such as enzymatic activity. The most widely used is dehydrogenase activity, an indicator of microbial activity linked to

the oxidation of organic compounds and electron transport during microbial cell energy generation. Other commonly used enzymatic activities are related to C, P, N, and S cycles. However, only a few indices use enzymes for assessing soil quality, despite the fact that enzymes have been proposed as sensitive soil change indicators (Nannipieri et al., 1990; Bastida et al., 2008).

The first enzymatic indices may be the enzymatic activity number (EAN) proposed by Beck (1984), the biological index of fertility (BIF) of Stefanic et al. (1984), and the biochemical index of soil fertility (Koper and Piotrowska, 2003). As an example, Saviozzi et al. (2001) also used these indices and observed lower values for cultivated soils than for forest and native grasslands. Other examples were reported by Riffaldi et al. (2002) and indicated an increase in EAN and BIF in untilled management systems (natural grassland and orange groves) when compared to tilled systems in southeastern Sicily (Italy); Koper and Piotrowska (2003) established a biochemical soil fertility index for comparing the effect of organic and mineral fertilization. The biochemical index of soil fertility is

$$\text{BISF} = C_{\text{org}} + N_{\text{total}} + \text{DH} + \text{P} + \text{PR} + \text{AM} \quad (4.1)$$

where

C_{org} is the OC content expressed as a percentage

N_{total} is the total nitrogen content expressed as a percentage

DH is the dehydrogenase activity, $\text{cm}^3 \text{H}_2 \text{kg}^{-1} 24 \text{h}^{-1}$

P is the phosphatase activity, $\mu\text{mol p-nitrophenylphosphate g}^{-1} \text{h}^{-1}$

PR is the protease activity, $\mu\text{mol NH}_4\text{N kg}^{-1} \text{h}^{-1}$

AM is the amylase activity, $\text{mg of decomposed starch h}^{-1}$

Until recently, one of the mean indices to take into account microbial diversity parameters was phospholipid fatty acids (PLFAs) devised by Puglisi et al. (2005), who established a soil alteration index. This methodology allows a quick and simple analysis and provides a profile of numerous fatty acids by gas chromatography, together with information on the abundance of microbial groups (fungi, bacteria Gram+ and Gram- and actinomycetes), but it provides little information on the alteration (and thus the quality or degradation) of a given soil, due to the scant number of parameters (only PLFAs) used, and one single indicator is not sufficient to evaluate the state of a soil.

However, PLFA as a sole technique is not sufficient to explain the processes and high-resolution microbial diversity of a soil. Subsequently, Puglisi et al. (2006) proposed three soil alteration indices, using various soil enzymatic activities to establish a soil degradation index for the assessment of agricultural practices, including crop density and the application of organic fertilizers in different locations:

1. The first index (AI 1) is expressed as a function of seven enzyme activities (arylsulfatase, β -glucosidase, phosphatase, urease, invertase, dehydrogenase, and phenoloxidase).
2. The second index (AI 2) uses β -glucosidase, phosphatase, urease, and invertase activities for its calculation.



3. The third index (AI 3) combines only three enzyme activities (β -glucosidase, phosphatase, and urease) and was established and validated from values obtained from the literature. This validation process could be considered as a valid option for endowing an index with greater spatial significance, although one problem is that the methods used must be identical, as minor methodological changes could lead to widely varying results.

Armas et al. (2007) determined a biological quality index for volcanic andisols and aridisols. This index presented the relationship among total C, different enzyme activities, and hot water-soluble C. The ratio between the predicted values for this model and the real total C values could be considered as a biological soil-quality index (Bastida et al., 2008):

$$\text{Total C} = -2.924 + 0.037 \text{ extC} - 0.096 \text{ cellulose} + 0.081 \text{ dehydrogenase} + 0.009 \text{ respiration} \quad (4.2)$$

where

extC (hot water-soluble C) is expressed in g C m^{-1}

Cellulose is expressed in $\mu\text{mol glucose m}^{-2} \text{ h}^{-1}$

Dehydrogenase is expressed in $\mu\text{mol INTF m}^{-2}$

Respiration is expressed in $\text{mg CO}_2\text{-C m}^{-2} \text{ h}^{-1}$

More or less sensitivity is needed depending on the objectives. In fact, some microbial activity parameters may suffer from too much sensitivity, such as the case of ATP. The adenosine triphosphate molecule stores cellular energy and can suffer climate and temperature-dependent variations (Jorgensen and Raubuch, 2003; Bastida et al., 2006).

Some authors have proposed biological soil-quality indices for polluted soils affected by different contaminants. Bécaert et al. (2006) proposed a similar index, entitled the relative soil stability index (RSSI), to evaluate the capacity of a soil to recover after contamination as a function of enzymatic activity. In this case, they included an overlooked variable: time (Bastida et al., 2008).

New methodologies based on rapid and clean nucleic acids and protein extraction from soil open the door to the use of these molecular approaches for soil-quality assessment (Persoh et al., 2008). Although there are an increasing number of publications based on molecular biology, only a few indices that exploit molecular methodologies (genomic, transcriptomic, proteomic, and the so-called omics) have been applied in attempts to evaluate soil quality (Roldán et al., 1994; García et al., 2002).

However, the development of genomic, transcriptomic, or proteomic methodologies could have an importance for the evaluation of soil quality, not only from the standpoint of diversity, but also from a functional aspect. These methods can provide information on the role of specific microorganisms and their enzymes in key processes related to soil functionality (Bastida et al., 2012). These new techniques in molecular ecology therefore further the information concerning microbial diversity and function.



The analysis of metagenomic libraries offers the possibility of determining the gene content of noncultivable bacteria that could be the key drivers of processes related to soil quality, thus providing a better understanding of global microbial ecology (Schmeisser et al., 2007).

In the same vein, microbial diagnostic microarrays also represent a powerful tool for the parallel identification of many microorganisms (Sessitsch et al., 2006). Functional microarrays target genes that encode specific functions and can offer valuable information about functional soil characteristics. For example, functional gene arrays target genes that encode enzymes, which play a key role in various ecological processes such as nitrification, denitrification, nitrogen fixation, methane oxidation, sulfate reduction, and degradation of pollutants (Sessitsch et al., 2006).

Since the incorporation of the labeled substrate into microbial DNA or RNA implies that the populations are active under the tested conditions (Radajewski et al., 2003), the subsequent analysis of labeled biomarkers of subpopulations with stable-isotope probing (SIP) (DNA-SIP, RNA-SIP, PLFA-SIP) reveals related phylogenetic and functional information about the soil organisms that metabolize specific compounds (Neufeld et al., 2007).

Microarrays, real-time PCR, SIP linked to nucleic acids, and other molecular methods offer several advantages but also several limitations that have been widely developed in the review by Saleh-Lakha et al. (2005).

In summary, these methodologies attempt to analyze and study genes or transcripts in order to detect potential functions of microorganisms in the soil under different conditions. However, where does the soil microbial function reside?

Proteomics could provide new insights in this direction. Proteins provide more straightforward information about microbial activity in soils than real-time PCR functional genes or even their RNA transcripts (Wilmes and Bond, 2006; Benndorf et al., 2007).

Linking SIP with metaproteomic analysis could provide additional information, not only on proteins or enzymes that disappear due to adverse phenomena in the soil, but also on the precise role of proteins in specific soil processes and on the origin of these proteins. The use of SIP binding methods and metaproteomics could help define and quantify soil quality but in a more specific sense, taking into account the processes involving microorganisms and their proteins and their ecosystem function (Bastida et al., 2012).

4.4.4 CHEMICAL INDICATORS TO ASSESS FOREST-SOIL QUALITY

It is often difficult to clearly separate soil functions into chemical, physical, and biological processes due to the dynamic and interactive nature of these processes (Schoenholtz et al., 2000). This interconnection is particularly significant between chemical and biological indicators of soil quality. It is therefore necessary to identify a set of attributes that soils must possess in order to perform their functions and then translate these attributes into first- or second-level measurable soil properties or processes.

Consequently, there is rarely a one-to-one relationship between function and indicator; more likely, a given function is supported by a number of soil attributes,

while any given soil property or process may be relevant to several soil attributes and/or soil functions simultaneously (Harris et al., 1996; Burger and Kelting, 1999; Schoenholtz et al., 2000). In this regard, Bastida et al. (2008) report that physical and chemical indicators have been extensively used for the design of soil-quality indices.

Although several soil chemical indicators are similar for agricultural and forest soils, there are nevertheless significant differences between agriculture and forestry as far as their use and assessment are concerned. As pointed out by Powers et al. (1998) and Schoenholtz et al. (2000), many analytical soil-testing methods frequently used in agriculture have proven to be only marginally useful in predicting forest growth.

The following is a summary of the mean soil chemical indicators used to characterize the soil quality in grassland and forest soils (Schoenholtz et al., 2000):

1. One of the oldest indicators that must be included is pH, as a basic indicator of soil quality and soil chemical “health.” Of the chemical parameters of nutrient availability, pH has specific scoring functions that can be used for plant productivity and/or environmental components of soil quality, assessing pedotransfer functions for rooting depth and soil productivity attributes and assessing aggregate stability to evaluate a soil’s resistance to erosion (Kiniry et al., 1983; Gale et al., 1991; Doran, 1994; Karlen and Stott, 1994; Larson and Pierce, 1994; Reganold and Palmer, 1995; Harris et al., 1996; Aune and Lal, 1997).
2. Soil OC status (Doran, 1994; Reganold and Palmer, 1995; Harris et al., 1996) is an important characteristic proposed as a first-order chemical indicator. It is also used as one of the chemical parameters of nutrient availability with specific scoring functions for plant productivity and/or environmental components of biological soil quality.
3. Regarding the indicators associated to nitrogen nutrient availability, the various soil N measures such as “total N,” “organic N,” and “mineral N” or “mineralizable N” (Doran, 1994; Reganold and Palmer, 1995; Powers et al., 1998) are soil chemical features that can be included as basic indicators of soil quality and have been proposed as good indices for the soil’s nutrient-supplying capacity. Moreover, “extractable NH_4 ” and “ $\text{NO}_3\text{-N}$ ” are chemical parameters of nutrient availability with specific scoring functions used for plant productivity and/or environmental components of soil quality (Harris et al., 1996).
4. Regarding the indicators associated to phosphorous nutrient availability such as “extractable P” (Burger et al., 1994; Reganold and Palmer, 1995) and “Bray P” (Harris et al., 1996; Aune and Lal, 1997), these include some nutrient-availability chemical parameters with specific scoring functions that can be used for plant productivity and/or environmental components of soil quality.
5. The CEC has also been suggested as a first-order chemical indicator and is calculated through the pedotransfer function using OC and clay content (Karlen and Stott, 1994; Larson and Pierce, 1994; Reganold and Palmer, 1995).

6. “Exchangeable K” and “extractable K, Ca, Mg” are soil chemical characteristics that can be included as basic indicators of soil quality, and the K measure is one of the nutrient-availability chemical parameters with specific scoring functions to be used for plant productivity and/or environmental components of soil quality (Aune and Lal, 1997; Harris et al., 1996; Reganold and Palmer, 1995).
7. “Salinity” could also be suggested as a first-order chemical indicator for its inclusion as part of the MDS for agricultural and forest soils and as a basic indicator of soil quality. It is used in the pedotransfer function for soil productivity attributes, to account for the salinity that reduces the productive capacity of soils (Kiniry et al., 1983; Burger et al., 1994; Doran, 1994; Karlen and Stott, 1994; Larson and Pierce, 1994).

Regarding the soil chemical indicators used in forest-soil-quality assessment, the productive indices (PI) should contain some measure of nutrient status, with the exact chemical parameter depending on the external stressor or anthropogenic impact. Possible critical soil chemical indicators may be OM, available N, soil P, and soil acidity (pH, base depletion, Al toxicity) (Henderson et al., 1990; Schoenholtz et al., 2000).

In connection with the soil biological indicators described, the inclusion of SOM as a soil chemical indicator can measure several soil functions simultaneously (Burger and Kelting, 1999), including OM decomposition and N mineralization as functional components of soil productivity. Moreover, potentially mineralizable N (anaerobic incubations) has also been proposed as a viable indicator of soil nutrient supply based on the positive correlation with site index and foliar N and with total organic C and N and its use as an index for microbial biomass (Powers et al., 1998).

4.4.5 PHYSICAL INDICATORS TO ASSESS FOREST-SOIL QUALITY

Regarding the soil’s physical properties as indicators of forest-soil quality, productive forest soils have attributes that are, in part, a function of soil physical properties and processes, as they (1) promote root growth; (2) accept, hold, and supply water; (3) hold, supply, and recycle mineral nutrients; (4) promote optimum gas exchange; (5) promote biological activity; and (6) accept, hold, and release C (Burger and Kelting, 1999). All of these factors will determine the extent to which each soil physical property or process is useful for measuring soil quality and monitoring the maintenance of soil quality over time.

Some soil physical properties are static in time, and some are dynamic over varying time scales. Some are resistant to change by forest management practices, while some can be easily changed in positive and negative ways. If changed, some properties and processes will recover at varying rates, while others are irreversible (Schoenholtz et al., 2000). A summary of the mean soil physical properties used to characterize soil quality in grassland and forest soils (Schoenholtz et al., 2000) included the following:

1. “Soil texture” measures the percentage of sand, silt, and clay to evaluate the soil’s capacity for water and nutrient retention and transport (Doran, 1994).

2. "Soil depth" uses the measure of the soil's thickness (cm) to evaluate total nutrient, water, and oxygen availability (Larson and Pierce, 1991; Arshad and Coen, 1992; Doran, 1994).
3. "Soil bulk density" is a measure of core sampling density to assess root growth and water's rate of movement in the soil (Larson and Pierce, 1991; Arshad and Coen, 1992; Doran, 1994; Kay and Grant, 1996).
4. "Available water-holding capacity (WHC)" is used for the evaluation of water availability for plants and erosivity (Larson and Pierce, 1991; Arshad and Coen, 1992; Doran, 1994; Kay and Grant, 1996).
5. "Saturated hydraulic conductivity" uses the measure of water flow in a soil column to assess the water/air balance and soil hydrology regulation (Larson and Pierce, 1991; Arshad and Coen, 1992).
6. "Soil strength" measures the soil's resistance to penetration in order to evaluate root growth (Powers et al., 1998; Burger and Kelting, 1999).
7. "Porosity" uses the percentage of in-soil air volume to assess the water/air balance, water retention, and root growth (Powers et al., 1998).
8. "Aggregate stability and size distribution" uses a wet-sieving method to evaluate root growth and air/water balance (Arshad and Coen, 1992; Kay and Grant, 1996).
9. "Least limiting water range" uses water retention curves and the penetration resistance measure to assess water/air balance and root growth (Arshad and Coen, 1992; da Silva et al., 1994; Kay and Grant, 1996; Burger and Kelting, 1999).
10. "Leaching potential" uses a model to evaluate the transport, transformation, and attenuation of applied chemicals (Petach et al., 1991; Wagenet and Hutson, 1997).
11. "Erosion potential" uses models such as water erosion prediction project (WEPP) (Nearing et al., 1989) and SEP (Timlin et al., 1986) to assess the available soil, water, nutrient, root growth, and environmental concerns associated to soil erosion (Wagenet and Hutson, 1997).

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4.5 MULTIPARAMETRIC INDICES TO ASSESS SOIL QUALITY

With the aim of developing practical measures for soil quality, integrative approaches are now being explored to sift out the interrelationships between several types of variables (Mele et al., 2008). In order to obtain a complete picture of soil quality, it is necessary to include different kinds of parameters (physical, chemical, and biological) (Frankenberger and Dick, 1983; Nannipieri et al., 1990; Dick, 1994; Gelsomino et al., 2006; Bastida et al., 2008). The reason behind the need to include a wider range of indicators is that some factors may affect some indicators but not others, and this may influence the accuracy of the picture of the soil quality.

The construction of soil-quality indices has been the subject of a large number of studies in the soil-science literature. However, the procedure currently used, which involves combining individual soil-quality scores into a soil-quality index, is the most controversial (Andrews et al., 2004), and no generally accepted criteria have been established for translating a set of individual soil attributes into simple



soil-quality indices (Hailu and Chambers, 2012). The standard approach to generating an index to assess soil quality consists of the following three steps:

1. The first step is to choose an MDS of physical, chemical, and biological soil-quality variables (Larson and Pierce, 1991), based on either expert opinion or statistical data reduction methods such as PCA.
2. In the second step, the variables in the MDS are transformed into 0–1 scores, using either linear or nonlinear transformation functions.
3. In the final step, the indicator scores are combined into a soil-quality index using different integration techniques, including simple addition (Andrews and Carroll, 2001b), weighted addition (Harris et al., 1996), or decision support systems employing min-max objective functions (Yakowitz et al., 1993).

Both the second and the third steps are ad hoc. The transformation of individual soil-quality variables or indicators into 0–1 scores does not recognize that the impact of a soil-quality variable on outcomes (e.g., crop yield) might depend on the level of other soil variables or other production inputs.

4.5.1 MULTIPARAMETRIC INDICES INCLUDING PHYSICAL, CHEMICAL, AND BIOLOGICAL PARAMETERS

Bastida et al. (2008) proposed a description of the past, present, and future of soil-quality indices, which provides a very appropriate perspective for understanding the evolution of these important instruments and for selecting some of the most relevant. They suggest that the first multiparametric index for soil quality was probably established by Karlen et al. (1994a), who based this index on a framework established by Karlen and Stott (1994). The main drawback of their index is the fact that the weighting is subjective and does not depend on mathematical or statistical methods. The formula of the index uses selected soil parameters that are then weighted and integrated according to the following expression:

$$\text{Soil Quality} = q_{\text{we}} (\text{wt}) + q_{\text{wma}} (\text{wt}) + q_{\text{rd}} (\text{wt}) + q_{\text{fqp}} (\text{wt}) \quad (4.3)$$

where

q_{we} is the rating for the soil's ability to accommodate water entry

q_{wma} is the rating for the soil's ability to facilitate water transfer and absorption

q_{rd} is the rating for the soil's ability to resist degradation

q_{fqp} is the rating for the soil's ability to sustain plant growth

wt is the numerical weight for each soil function

Burguer and Kelting (1999) presented a soil-quality index for pinewoods using various soil functions and a method similar to that of Karlen et al. (1994a, b). The resulting index was suitable for the evaluation of forest-soil sustainability when subjected to different management systems. These authors proposed a boundary above which certain practices are sustainable and also discussed several spatial scale considerations of importance for obtaining a larger-scale functioning model, despite the fact



that this factor has not been taken into account in many indices. The index was later applied by Kelting et al. (1999) to reflect the effect of different forest management practices. The information rendered was then used to help choose the most suitable index for sustainable forest-soil development in South Carolina (United States) (Bastida et al., 2008).

Andrews et al. (2002b) proposed a method that has been widely used by many authors (Sharma et al., 2005; Rezaei et al., 2006; Bastida et al., 2008). For the Andrews et al. (2002b) index, the indicators selected by factor analysis were bulk density, Zn, water-stable aggregates, pH, electrical conductivity, and SOM content; electrical conductivity and OM were the indicators assigned the highest importance in SQI. The equation proposed is

$$SQ = \frac{\sum_{i=1}^n (S_i \times W_i)}{\sum_{i=1}^n W_i} \quad (4.4)$$

where S is the score for the subscripted variable and the coefficients are the weighting factors derived from the PCA.

4.5.2 PHYSICAL-CHEMICAL INDICES TO ASSESS SOIL QUALITY

Foresters often integrate the analysis and knowledge of physical-chemical properties of soils to assess the capacity of sites to support productive forests, such as

1. The proposal by Pang et al. (2006) for the determination of an integrated fertility index (IFI) based on chemical, physical, and physical-chemical parameters for the study of soil-quality variations in Chinese forests.
2. Mohanty et al. (2007) applied multiple regressions and established a physical-chemical soil-quality index based on four parameters: (1) real density, (2) OM content, (3) resistance to root penetration, and (4) aggregate density.
3. Another example regarding future physical-chemical indices for soil quality is the use of spectroscopy as an inexpensive, noninvasive methodology for assessing soil quality. Shepherd and Walsh (2002) established a scheme for the development and use of soil spectral libraries for the rapid and non-destructive estimation of soil properties based on the analysis of diffuse reflectance spectroscopy and proposed r^2 regressions to validate several chemical and physical-chemical parameters. These authors concluded that the spectral library approach opens up new possibilities for soil-quality modeling.
4. Vagen et al. (2006) tested the potential of near-infrared spectroscopy as a tool for predicting and mapping soil physical-chemical properties in the highlands of Madagascar. Calibration models were developed for soil OC, total nitrogen, electrical conductivity, and clay contents in an area with a range of land uses and landscape forms.

5. Awiti et al. (2008) established a soil condition classification (good, average, poor) using infrared spectroscopy along a tropical-cropland chronosequence in sub-Saharan Africa. The study concludes that reflectance spectroscopy is rapid and offers the possibility for major efficiency and cost saving, allowing spectral case definition to define poor or degraded soils and enabling better targeting of management interventions.

4.5.3 MULTIPARAMETRIC INDICES BASED ON MATHEMATICAL MODELS TO ASSESS SOIL QUALITY

Andrews et al. (2004) established a computer-based mathematical model (SMAF, soil management assessment framework). SMAF represents an additive nonlinear standardization tool for evaluating a soil's quality from its functions: (1) microorganism biodiversity and habitat, (2) filtration of contaminants, (3) nutrient cycling, (4) physical stability, (5) resistance to degradation and resilience, and (6) water relations (Bastida et al., 2008).

The SMAF tool was subsequently used by Cambardella et al. (2004) and Wienhold et al. (2006) and is based on food production and nutrient cycling as quality functions to objectively evaluate the effect of agricultural management practices on soil quality.

Erkossa et al. (2007) used the Andrews et al. (2004) methodology and various scoring functions in their attempt to evaluate soil quality in terms of different parameters. The purpose of this work was to compare the effects of four land preparation methods and their influence on soil quality: (1) broad bed and furrows, (2) ridge and furrow, (3) green manure, and (4) reduced tillage. However, they did not observe any significant differences in soil-quality values among the different treatments.

A trigonometric approach based on three subindices (nutritional, microbiological, and crop related) was used by Kang et al. (2005) to establish a sustainability index in soils under wheat amended with manures in Punjab (India), noting that the quality increased with amendment. Bastida et al. (2008) point out that this index used a wide variety of chemical, physical, and biological parameters.

Agricultural multiparametric quality indices outnumber nonagricultural ones. Trasar-Cepeda et al. (1998) chose a series of soils covered by a climax vegetation in Galicia (Spain) and established an equation to define total nitrogen from several microbial parameters (microbial biomass C, mineralized N, phosphatase, β -glucosidase, and urease). The resulting equation led them to report the presence of a balance between total N and several biochemical parameters. The equations are as follows:

$$\begin{aligned} \text{Total N} = & \left(\frac{\text{Microbial biomass C}}{100} \right) + \left(\frac{\text{Mineralized N}}{100} \right) + \left(\frac{\text{Phosphatase}}{100} \right) \\ & + \left(\frac{\beta\text{-glucosidase}}{100} \right) + \left(\frac{\text{Urease}}{100} \right) \end{aligned} \quad (4.5)$$

where

Total N is expressed as a percentage

Microbial biomass C and mineralized N are expressed in (mg kg^{-1})

The enzyme activities are expressed in $\mu\text{mol of liberated product g}^{-1} \text{ h}^{-1}$

The model aims to approach the ideal state of soil quality in climax soils. However, in most of the developed world, these climax soils no longer exist. The validity and applicability of this ratio for soils contaminated by heavy metals, mine soils, and arable soils were demonstrated by Leirós et al. (1999).

Among the different statistical tools applied in the design of multiparametric indices for soil quality, an increasing number of studies have used artificial neural networks (ANNs) to probe complex data sets. As an example of how ANN can be used, we provide an example of the analysis of soils from two different regions of Southeast Australia using (1) Kohonen self-organizing maps, (2) data sets containing biochemical signatures of microbial communities determined by PLFA analysis, (3) genetic signatures obtained by terminal restriction fragment length polymorphisms (TRFLP), and (4) a range of single-parameter soil chemical, physical, and biological variables (Mele et al., 2008).

However, some of these approaches have limitations; for example, Gil-Sotres et al. (2003) criticized the work of De la Paz-Jiménez et al. (2002), who obtained an equation by multiple regression analysis relating OC to different enzymatic activities in La Paz (Argentina). The critique was based on the consideration that the combination of properties and experimental design was not adequate to design a soil-quality index for the purpose of evaluating the influence of soil use and management.

Regarding other multiparametric indices for soil quality based on statistical tools, Bastida et al. (2008) also highlight the design of Zornoza et al. (2007), which establishes two multilinear regression equation systems with the idea of evaluating soil environmental quality under natural vegetation with minimum human disturbance, working in semiarid conditions. The first established the affinity between nitrogen and different enzyme activities and physical–chemical indicators and was validated for mollisols, while the second defined soil organic C using similar indicators and was validated for entisols. The equations are as follows:

$$S = \frac{N_{Kjeldahl}}{P_{available}} + \frac{WHC}{\text{Phosphatase activity}} + \frac{\text{Microbial biomass C}}{\text{Urease activity}} + \frac{\beta\text{-glucosidase}}{\text{SOC (soil organic carbon)}} \quad (4.6)$$

$$S = \frac{N_{Kjeldahl}}{P_{available}} + \frac{WHC}{\text{Phosphatase activity}} + \frac{\text{Microbial biomass C}}{\text{Urease activity}} + \frac{\beta\text{-glucosidase}}{\text{SOC (soil organic carbon)}} \quad (4.7)$$

where

$N_{Kjeldahl}$ is expressed in g kg^{-1}

$P_{available}$ is expressed in mg kg^{-1}

WHC is expressed in %

Phosphatase activity is expressed in $\mu\text{mol } p\text{-nitrophenylphosphate g}^{-1} \text{ h}^{-1}$

Microbial biomass C is expressed in mg kg^{-1}

Urease activity is expressed in $\mu\text{mol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1}$

β -glucosidase is expressed in $\mu\text{mol } p\text{-nitrophenylphosphate g}^{-1} \text{ h}^{-1}$

SOC (soil organic carbon) is expressed in g kg^{-1}

Finally, Hailu and Chambers (2012) proposed a multiparametric index based on economic models to assess soil quality. They used the Luenberger productivity indicator, which is defined by differences in the values of the directional distance function. They introduced a distance function defined on the technology set, T , where

$$T = \{(x, y) \mid x \in R_+^N, \text{ a produce output } y \in R_+^M\} \quad (4.8)$$

and

$$D^T(x, y; x', y') = \sup_{\beta} \{x - \beta x' + \beta y'\} = T \quad (4.9)$$

is the directional distance function defined on T for the direction vector (g_x, g_y) .

Defining this function for time period t , we obtain

$$D^T(x_t, y_t; x_{t+1}, y_{t+1}) = \sup_{\beta} \{x_t - \beta x_{t+1} + \beta y_{t+1}\} = T \quad (4.10)$$

Chambers and Pope (1996) and Chambers et al. (1996) previously defined the Luenberger productivity indicator and provided the formula.

This indicator is the arithmetic average of the change in productivity measured by the technology at time $t + 1$ (the first two terms) and the change in productivity measured by the technology at time t (the last two terms).

4.6 PRACTICAL CASES: MODELS FOR THE INTEGRATION OF SOIL-QUALITY INDICATORS IN LANDSCAPE PLANNING PROJECTS

4.6.1 MODEL OF INTEGRATION OF QUALITATIVE AND QUANTITATIVE SOIL AND ENVIRONMENTAL INDICATORS: AN EXAMPLE OF THE ASSESSMENT OF THE ENVIRONMENTAL QUALITY OF A WHOLE COUNTRY (SPAIN)

4.6.1.1 Introduction

García-Montero et al. (2010a,b) proposed a geographic information systems (GIS) model using a qualitative approach to integrate SQI with other environmental indicators into a final map of environmental quality of Spain (1:500,000). This model was described in detail in an article published in the journal *Environmental Impact Assessment Review*. The map of the environmental quality of Spain was used in two strategic environmental assessments (SEAs) for two national transport infrastructure plans.

In this model, one vector—from a total of 102,240 different vectors with 12 components of environmental quality—was assigned to each one of the 50 million 1 ha grid squares (pixels) for Spain. Each vector was obtained by integrating qualitative and normalized values associated to four SQI and eight environmental indicators. The final classification of the 102,240 different vectors (based on the vector modulus) provided a raster map of Spain with five classes of territorial environmental quality. In this model, the GIS raster operations were able to deal with enormous amounts of information with no difficulty at all.



Finally, a check against the environmental quality map showed that these results had a good fit with reality (in relation to the scale and level of detail used). This checking process was based on a statistical analysis that showed that there was a very high frequency of 1 ha grid squares (pixels) with high environmental quality values associated to Spain's protected natural areas and that this was significantly greater than in the rest of the Spanish territory ($p < 0.0001$).

In summary, the GIS raster model developed to integrate SQI with other environmental indicators proved to be a simple and effective tool, which provided a useful environmental quality assessment for a large territory, based on panels of experts and on objective GIS calculations, and focused on SEA and landscape planning procedures.

4.6.1.2 Methodological Basis

The proposed model was used to compare territorial units (Ramos, 1979) and was combined with a multicriteria method to assess the quality of these territorial units (Norris and Farrar, 2001).

The map projections, GIS and mathematical software, and scale and level of detail used are described in García-Montero et al. (2010a,b). We analyzed enormous amounts of information, which made it impossible to apply vector-type GIS operations, and we thus chose to use a GIS raster-type model. The GIS models were based on logical operations of reclassifying and combining the raster layers, and the numerical vector operations were based on the vector module. Martínez-Falero and González (1995) and Tran et al. (2006) reported that the vector module is a suitable method for integrating multiple indicators into a single index for practical operating reasons in planning procedures.

We selected as the main valuation criteria “the conservation of biodiversity and the preservation of the environment,” and we used Ramos' methodology (1979) and a GIS model proposed by Mancebo et al. (2005) and García-Montero et al. (2010b) (large territory integrated environmental (LATINO) model). These methodologies are based on models that compare the territorial units in relation to each other, based on the attributes or natural variables in each one.

The precision threshold was established at 100 m root-mean-square (RMS) (equivalent to a scale of 1:500,000).

Step 1: Valuation of SQI and environmental indicators (12 variables)

We looked for different evaluation criteria to assess SQI and environmental indicators, based on five existing Spanish digital maps on a national scale. Moreover, we consulted some panels of experts in order to obtain a complementary set of valuation qualities. Finally, we generated—either directly or by deduction—a set of 12 environmental quality indicators (including four SQI) representing 12 raster layers.

Regarding the four SQI, we assessed the productive capacity, biodiversity, naturalness, and uniqueness of the soils in Spain, using the information associated to the soil map (FAO, 2000), using the FAO's hierarchical classifications of soil taxonomy, and with a panel of five soil-science experts from Madrid.

We considered that with 12 variables, it was possible to assess the environmental quality of the Spanish territory. However, this model gave us an open system that allowed continuous incorporation of new quality scores.



Step 2: Objective assessment of territorial singularity

In order to safeguard biodiversity, we assessed the territorial singularity of the different categories or classes in the habitats, Corine Land Cover, landscape, and soil maps. This was done using an objective classification of their units calculated with the GIS. The following index of singularity was applied (Ramos, 1979; MMA, 2000):

$$S_i = \frac{\frac{a_i}{a} - \frac{\min}{\max}}{\frac{\max}{\min} - 1} \quad (4.11)$$

where

S is the territorial singularity index

$\max$ is the ha of the map's largest category

$\min$ is the ha of the map's smallest category

x is the ha of the map category being evaluated

Singularity was assessed on a logarithmic scale. This transformation made it possible to maximize the value of the categories with smaller areas and also to obtain a scale with fewer units. Thus, the category with the greatest surface area was awarded the lowest singularity value (0), and the category with the least surface area was awarded the maximum value (4.62). This continuous scale was then transformed into a discrete or qualitative scale of five classes, which were obtained by rounding each decimal value up to the next whole number. We thus obtained a higher singularity value for the least represented classes in the territory in order to safeguard biodiversity.

Step 3: Normalization of the 12 variables

The 12 variables were then normalized to avoid overlapping during their subsequent integration into the model. Normalization consisted of changing the original valuation scale for each variable, which was transformed into a common final continuous scale from 0 to 1 for all the variables. This was done by means of an equation applied to the original discrete or qualitative scales and another equation applied to the original continuous scales. The following formula was used to convert the discrete or qualitative scales into a continuous scale from 0 to 1:

$$n_i = \frac{x_i - \min}{\max - \min} \quad (4.12)$$

The following equation was used to transform a continuous scale into another normalized continuous scale from 0 to 1:

$$n_i = \frac{x_i - \min}{\max - \min} \quad (4.13)$$

Step 4: Integration of the 12 variables into the model

The 12 normalized raster variables were integrated using GIS merging operations. Each pixel of 1 ha of territory was assigned a vector with the 12 natural



variables valued. We obtained n vectors distributed among the 50 million 1 ha pixels in Spain.

The next step was to order the n vectors using the modulus or Euclidean distance, to assign a synthetic value of theoretical biodiversity and environmental quality. The vector was used to order the n vectors obtained based on their components, and

$$v = \sqrt{v_1^2 + v_2^2 + \dots + v_n^2} \quad (4.14)$$

where

v is the vector modulus

i is a vector component

A total of 102,240 different vectors were obtained with 12 components, assigned to each of the 50 million 1 ha grid squares for Spain. Then the values obtained for each of the n Euclidean distances were normalized into five equivalent classes, corresponding to the five types of theoretical biodiversity and environmental quality (1 to 5). This normalized classification was obtained by applying the following formula:

$$\text{environmental quality class} = \left(\frac{V - V_{\min}}{V_{\max} - V_{\min}} \right) \cdot 5 \quad (4.15)$$

where

V is the vector modulus of each of the n vectors obtained

$V_{\min}$ is the minimum vector modulus obtained

$V_{\max}$ is the maximum vector modulus obtained

The very low biodiversity and environmental quality class is obtained when $0.5 \sim 1.5$

Low-quality class is obtained when $1.5 \sim 2.5$

Moderate-quality class is obtained when $2.5 \sim 3.5$

High-quality class is obtained when $3.5 \sim 4.5$

Very-high-quality class is obtained when $4.5 \sim 5.5$

García-Montero et al. (2010b) proposed 100 classes of environmental quality in the Spanish territory based on the LATINO model. However, in the study described, the use of five classes of biodiversity and environmental quality was sufficient for this classification, as it clearly distinguishes extreme cases of high and low environmental quality in a territory on a nation scale.

4.6.2 MODEL OF INTEGRATION OF QUANTITATIVE SOIL-QUALITY

INDICATORS: AN EXAMPLE OF THE GENERATION OF A SOIL FERTILITY

INDEX IN CARIBBEAN AGRICULTURAL AND FOREST AREAS

4.6.2.1 Introduction

Alexis et al. (2010) proposed a soil fertility index model based on a GIS tool, which integrated statistical multivariate methods and soil parameters, including heavy metal content, into models of land planning for agricultural and forestry development in



a rural Caribbean area. This quantitative model was described in detail in an article published in the journal *Agroforestry Systems*.

In the Jaragua-Bahoruco-Enriquillo Biosphere Reserve, located on the southern border between the Dominican Republic and Haiti, there are depressed rural areas with soils with a high content of cadmium and other heavy metals that originate naturally in the geological substrate. Soil data and an inventory of coffee and kidney bean plantations were used to design a GIS tool to generate a soil fertility index in the study area.

This GIS tool was based on open-source raster models, using combination and reclassification operations based on the maps, geostatistical methods (kriging), statistical analyses external to the GIS, and a cartography limiting and excluding particular factors for crops (including heavy metal soil content). The GIS tool discriminated extreme soil fertility situations associated to sustainable agricultural and forest planning in contaminated rural areas of the Caribbean.

The soil and its fertility and contamination constitute highly significant elements for sustainable agricultural and forest planning processes. It is therefore advisable to incorporate fertility indices into agricultural and forest planning models. Vagen et al. (2006) state that when developing fertility indices, the challenge is generally to integrate a series of soil characteristics within a single-value index. Many studies apply a points system based on agronomic values of reference for a range of soil characteristics, and in some cases, certain soil characteristics are qualified with more weight than others (Karlen et al., 1998; Lal, 1998). However, one of the greatest problems posed by tropical soils is that there are no robust reference values and the valuation of the weight of soil characteristics or the assignment of weighting values is not submitted to any scientific criterion (Sánchez et al., 2003).

The region in the study has some extremely poor rural areas. Hernández et al. (2007) and Alexis (2008) described the characteristics of the study area. We selected the agroecosystems that were home to two of the most representative crops in the study area: coffee, which has considerable importance in the region's overall economy, and kidney beans (*Phaseolus vulgaris* L.), grown by families as a subsistence crop for their nutritional qualities.

4.6.2.2 Methodological Basis

The map projections, GIS and mathematical software, and scale and level of detail used are described in Alexis et al. (2010).

The soil maps and GIS models were based on the prior study of 80 samples of the surface soil layer (0–20 cm in depth) analyzed by Hernández et al. (2007), randomly selected within each of the ecosystems studied. These authors analyzed pH in water, OM, the main elements related to fertility (N, P₂O₅, K), and available levels of Ca, Mg, Mn, Fe, and Zn. Total levels of Cd were also determined. An inventory was made of the crops and geographic features in the study area. The precision threshold was established at 100 m RMS (RMS error), and the minimum cartographic unit or pixel selected was 1 ha. A pixel with a resolution of 100 m made it possible to generate a GIS soil tool of moderate accuracy, which would enable the knowledge acquired through a limited number of soil analyses to be integrated in the land planning procedures in real time.



One of the advantages of using GIS raster models is that GIS processes can be created using mathematical operations with numerical matrices, without the need to visualize each of the intermediate steps on the maps. Moreover, the scale of the raster layers always remains associated to the pixel size, so the GIS zoom display tool can be used without modifying the scale or distorting the level of detail in the maps. This means that the maps and models can be visualized and any corrections applied throughout the process, as described by García-Montero et al. (2008; 2010a,b). These authors propose GIS models based on successive combination and reclassifying operations applied to raster maps.

4.6.2.3 Model Steps and Logical Operations Applied

The statistical treatment associated to the GIS models was performed with the Statistica v.6 program (StatSoft, Inc., Tulsa, OK, 1999). When necessary, the variables used were transformed to comply with the requirements of the parametric statistical treatments. The normality of the data was checked using the Lilliefors and Kolmogorov–Smirnov tests, and the homogeneity of the variances was verified using the Levene test. The statistical treatments were integrated in the GIS raster models. The Excel spreadsheet program with dBase IV format was used as an intermediate step in the integration of the data sets.

The methodology included the use of GIS raster operations to integrate the soil study into a procedure, which classified the pixels relating to land with the greatest capacity for cultivation of coffee and kidney beans, and to distinguish them from pixels with a lesser capacity and from pixels relating to protected areas and/or with unacceptable levels of heavy metal content for agriculture.

The methodology was based on the procedures of Ramos (1979), MMA (2000), and García-Montero et al. (2008), which incorporate the notions of “limiting and excluding capacity factors” into land planning procedures. These methodologies use models that compare the land units according to the attributes or biotic and abiotic variables present in each one. These authors recommend that planning models should “dedicate their greatest efforts to the most significant problems.” This philosophy should be part of both the environmental inventory stage and the design of the models. A GIS tool was devised to integrate the soil study into the kidney bean and coffee cultivation planning in the study area, using a model with five methodological stages, as described in the following.

This first stage consisted of making a digital inventory of a representative sample of the kidney bean and coffee cultivation existing in the study area. This was done by georeferencing 32 bean cultivation sites covering a total of 311 ha and 44 coffee cultivation areas with a total of 413 ha.

The second part of the digital inventory consisted of applying geostatistical treatments with the ArcGIS program to generate themed soil maps. This was done by using the data analyzed from the 80 soil samples described and then applying a random selection procedure to obtain 40 soil samples. Ordinary kriging interpolation treatments were applied to each of the variables in these 40 soils in order to produce the raster layers corresponding to pH; OM; N; C/N; P_2O_5 ; available levels of K, Mg, Ca, Fe, Mn, and Zn; and total levels of Cd, following the procedures of Moncayo et al. (2006) and Rodríguez et al. (2006). The resulting raster maps were



subsequently validated using the data from the remaining 40 soil samples that were not used in the kriging interpolation.

Several authors have demonstrated that statistical procedures applied to soil constitute a valuable tool in the planning procedures for certain crops, which are valid in particular study areas (Trangmar et al., 1985; Williams et al., 2008). The use of multivariate statistics can empirically simulate a probable cartography of fertility, which for practical operational reasons could be incorporated into the planning models.

The next stage of our model was to apply a multivariate statistical treatment to the data from the 80 soil samples described. A PCA was used to generate synthetic patterns of variation in soil variables, which could be potentially associated with the production of beans and coffee. With this in mind, variables more closely relating to fertility were used in the PCA: OM, N, P₂O₅, and available levels of K, Mg, Ca, Fe, Mn, and Zn. The principal factors or components obtained in the PCA were then examined, and one factor (PCx) was selected using the criterion of “greatest variance contained in the original matrix.”

The synthetic variable PCx was incorporated into the GIS by means of the creation of a new raster layer PCx, which was considered the synthetic expression of the integrated variability of the raster layers of pH, OM, N, C/N, P₂O₅, and available levels of K, Mg, Ca, Fe, Mn, and Zn. This was done with the original layers for these variables, which were integrated into the GIS function “map calculator” using the mathematical equation, which groups the correlations between the original soil variables and the factor PCx. Thus, the value of the factor PCx was calculated for each pixel, based on the values presented by the soil variables in a pixel. With this procedure, we obtained a new raster layer PCx, which empirically simulates a fertility map in the study area.

The third stage of the model begins with the integration of the raster map for fertility PCx and the digital inventories of bean and coffee crops (the remaining classes in the vegetation and uses map were also integrated). The data obtained from the GIS integration of these raster maps have been used as numerical values for input in other statistical treatments. The objective was to estimate whether the soil fertility calculated for each pixel using the synthetic variable PCx was associated in any significant way with the 76 coffee and bean crops in the inventory. Thus, the planning models for coffee and beans (and other crops) incorporated the soil values PCx when they were statistically significant for these crops. The synthetic variable PCx for fertility will therefore have a predictive character in relation to soils in the corresponding land planning models.

The raster map for fertility (PCx) was then integrated with the digital inventory for beans (311 pixels or ha) and coffee (413 pixels or ha), as well as the remaining classes in the map of vegetation and uses, using the GIS procedures for combination and reclassification. The results obtained for each pixel were exported using Excel sheets in the dBase IV format, which were incorporated into the statistical program. Using ANOVA and post hoc Tukey tests, we studied the significant relationships between PCx and the crop inventory, vegetation types, and land uses. In cases where the ANOVA and the post hoc test indicated that there was a statistically significant relationship between the PCx value and the type of crop or use—for example, coffee crops—new GIS combination and reclassification operations were applied to create a new raster layer, which we called “map of soil compatibility for coffee cultivation.”

In this new raster layer (compatibility of soil fertility with coffee cultivation), the value 2 was assigned to all the pixels in the territory with a PCx value between the average PCx value (calculated for the 413 coffee cultivation pixels) $\pm$ standard deviation (SD). The pixels with value 2 were called “soil compatible with coffee.” The remaining pixels in the territory, with different PCx values from the average PCx range $\pm$ SD, were assigned the value 1 and were designated pixels with “soil not compatible with coffee.”

Therefore, the predictive nature of the variable “soil compatibility with coffee” was due to the fact that the pixels with value 2 consisted of 66% of the values taken by PCx in the 413 coffee inventory pixels, corresponding to 66% of the most frequent fertility values for coffee, as its PCx values are around the average value of PCx established by the ANOVA and the post hoc test as a statistically significant value in the 413 coffee pixels. This compatibility model for soil fertility for coffee and bean cultivation could be repeated for each of the crops and vegetation classes it is wished to integrate into the planning process, provided that the ANOVA and post hoc test have assigned an average, statistically significant, PCx value for each class.

When the concepts of soil fertility and agrological capacity are applied in land planning processes, it is necessary to consider the appearance of “limiting factors,” which are intrinsic to the territory, as proposed by Ramos (1979), Van Groenigen et al. (2000), and Rodríguez et al. (2006), who propose quantifying the different soil factors and limiting environmental characteristics in each study area in order to classify the sites according to their suitability for agricultural use.

Thus, in the fourth stage of the model, for each type of crop (beans or coffee), a set of raster maps of limiting factors was generated for each variable analyzed in the study area. These maps were made by using the reclassification of the raster maps for mean annual temperature, mean annual rainfall, slope of the terrain, and the following soil variables: pH, OM, N, P₂O₅, and available levels of K, Ca, Mg, Mn, Fe, and Zn. To establish the threshold limiting values for each variable, we used the bibliographical information available for bean and coffee crops in the Dominican Republic and comparable tropical countries. The threshold values relating to the behavior of each variable as a limiting factor for a crop were classified in each of the new raster maps, using the following valuables: value 1 for pixels designated “not apt” for cultivation, value 2 for pixels designated “apt” for cultivation, and value 3 for pixels designated “optimal” for cultivation.

The raster maps for limiting factors for each crop type were integrated with the corresponding soil compatibility maps obtained previously for this crop. The result was one raster layer for the agrological soil capacity for beans and another for coffee, in the area studied.

The final stage of the methodology consisted of the GIS integration of the agrological soil capacity maps for coffee and beans with new raster layers for various associated features or features that are “intrinsic” to the planning process. Within this global perspective, the fifth stage of this GIS tool involved a primary integration of the agrological soil capacity maps with two factors, which are intrinsic to the planning process: a raster layer that represents soil heavy metal content and another representing protected spaces in the study area. The integration of these two new elements was based on restrictive criteria that introduced excluding factors (intrinsic to



the planning process) for beans and coffee. These layers were integrated using GIS outlining tools and combining and reclassification operations, which applied these excluding criteria.

The exclusion of areas for bean and coffee cultivation based on soil heavy metal content was exemplified with total Cd levels in the soil. We used the value of 3 mg/kg proposed by Cala et al. (1985) and Rodríguez et al. (2006) as the excluding criterion. This value agrees with the preliminary results obtained by Alexis (2008) in a series of bioassays on the impact of heavy metals on the roots and leaves of beans, coffee, sorghum (*Sorghum* sp.), and maize (*Zea mays* L.).

Areas for beans and coffee were then excluded by integrating the maps of protected natural spaces in the study area. The criterion for exclusion was the boundaries of the two national parks, which represent three quarters of Jaragua-Bahoruco-Enriquillo: the Jaragua National Park with 1651 km² and the Bahoruco National Park with 1125 km².

In this last stage, the soil study was integrated into the basic land planning process. The GIS tool for integrating the soil into the proposed plans was an open model that allowed the permanent incorporation of new environmental information on the soil (contaminants, soil processes, natural phenomena, etc.), as well as new information relating to other planning factors. In summary, the GIS tool developed discriminated extreme situations in sustainable agricultural and forest planning in contaminated rural areas of the Caribbean.



4.7 CONCLUSIONS



The process of sustainable forestry assessment requires evaluators to be reasonably informed about soil forest functions and SQI in order to increase their chances of achieving adequate joint decision making. Soil is one of the underlying matrices holding together many of the life-supporting processes and cycles on the planet.

The soil promotes water flow and retention, solute transport and retention, physical stability and support, retention and cycling of nutrients, buffering and filtering of potentially toxic materials, and maintenance of biodiversity and habitat. A healthy soil is capable of carrying out all the earlier functions. For this reason, soil quality is defined as the capacity of the soil to interact with the ecosystem in order to maintain biological productivity and environmental quality and promote animal and plant health.

Thus, the fundamental goals of protecting the quality, biodiversity, and productive capacity of soil for sustainable forest management can no longer be ignored. To support these goals, it is necessary to control or restrict agricultural and forestry activities that could reduce on-site agricultural and forest productivity and environmental quality in order to prevent soil degradation and restore its potential. Forest-soil-quality studies aimed at forest productivity are giving way to other kinds of studies based on forest ecological, edaphological, and physiological studies due to the increasing worldwide interest in sustainability.

Currently, there are different sets of SQI available for the purpose of identifying problem production areas, making realistic estimates of food and natural resource production, monitoring changes in sustainability and environmental quality in relation





to forestry management, and assisting national and state or regional agencies to formulate and evaluate sustainable land-use policies. More research areas are starting to study soil quality due to the growing interest in this issue. However, a bibliographical review shows that there is insufficient incorporation of soil-quality concepts and soil indicators in forestry management, which could compromise the objectives of reaching widespread sustainable forest management.

The development of soil-quality indices should be sensitive to long-term alterations in soil management and climate but sufficiently robust not to vary as a consequence of short-term changes in weather conditions. They must correlate well to beneficial soil functions. Soil-quality indices have to be useful for understanding why a soil will or will not function as desired. They have to be comprehensible and useful to land managers, easy, and inexpensive to measure, and where possible, they should also be components of existing soil databases. For these reasons, we must design new procedures that integrate physical, chemical, biological, and other unforeseen elements into indicators and indices that can perform seamlessly in any given soil conditions worldwide in order to maintain all life functions and cycles constant on this planet. In this regard, the current knowledge of SQI has not reaped sufficient benefits from the wealth of tools available from numerical and quantitative models.

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Martin Lukac
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Mauro Gamboni *Editors*

Soil Biological Communities and Ecosystem Resilience

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Editors

Martin Lukac
School of Agriculture, Policy and
Development
University of Reading
Reading, UK

Faculty of Forestry and Wood Sciences
Czech University of Life Sciences
Prague, Czech Republic

Mauro Gamboni
Department of Biology, Agriculture and
Food Sciences
CNR
Rome, Italy

Paola Grenni
National Research Council, Water Research
Institute
CNR-IRSA
Rome, Italy

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Contents

1	Introduction: The Role of Soil Biodiversity in Ecosystem Productivity and Resilience	1
	Martin Lukac	
2	Ecosystem Services Provided By Soil Microorganisms	9
	Maria Ludovica Saccá, Anna Barra Caracciolo, Martina Di Lenola, and Paola Grenni	
3	Comparison of Two Molecular Methods to Assess Soil Microbial Diversity	25
	Martina Di Lenola, Paola Grenni, Diogo N. Proença, Paula V. Morais, and Anna Barra Caracciolo	
4	Towards Integrated Understanding of the Rhizosphere Phenomenon as Ecological Driver: Can Rhizoculture Improve Agricultural and Forestry Systems?	43
	Luis G. García-Montero, Pablo Manzano, Deaa Alwanney, Inmaculada Valverde-Asenjo, Amaya Álvarez-Lafuente, Luis F. Benito-Matías, Xavier Parladé, Sigfredo Ortuño, Marcos Morcillo, Antonio Gascó, Carlos Calderón-Guerrero, Francisco Mauro, Mercedes Méndez, Alvaro Sánchez-Medina, María P. Andrés, José R. Quintana, Cristina Menta, Stefania Pinto, Liliana Pinto, Pilar Pita, Cafer Turkmen, Cristina Pascual, Esperanza Ayuga, Fernando Torrent, José C. Robredo, Pablo Martín-Ortega, Joan Pera, Luis Gómez, Gonzalo Almendros, Carlos Colinas, and Eric P. Verrecchia	
5	Impact of Agricultural Land Management on Soil Bacterial Community: A Case Study in the Mediterranean Area	77
	Annamaria Bevivino and Claudia Dalmastri	

6	A Metagenomic Study on the Effect of Aboveground Plant Cover on Soil Bacterial Diversity	97
	Mariantonietta Colagiero, Isabella Pentimone, Laura Cristina Rosso, and Aurelio Ciancio	
7	What Lies Beneath: Root-Associated Bacteria to Improve the Growth and Health of Olive Trees	107
	David Ruano-Rosa, Antonio Valverde-Corredor, Carmen Gómez-Lama Cabanás, Rafael Sesmero, and Jesús Mercado-Blanco	
8	Norway Spruce Fine Roots and Fungal Hyphae Grow Deeper in Forest Soils After Extended Drought	123
	Isabella Børja, Douglas L. Godbold, Jan Světlík, Nina E. Nagy, Roman Gebauer, Josef Urban, Daniel Volařík, Holger Lange, Paal Krokene, Petr Čermák, and Toril D. Eldhuset	
9	Ectomycorrhizal Diversity in Beech Dominated Stands in Central Europe	143
	Christoph Rosinger, Hans Sandén, and Douglas L. Godbold	
10	Arbuscular Mycorrhizal Fungal Communities Pushed Over the Edge – Lessons from Extreme Ecosystems	157
	Irena Maček	
11	An Intact Soil Core Bioassay for Cultivating Forest Ectomycorrhizal Fungal Communities	173
	Peter G. Avis, Ina C. Meier, and Richard P. Phillips	
12	Potential Role of Beneficial Soil Microorganisms in Plant Tolerance to Abiotic Stress Factors	191
	Raffaella Balestrini, Walter Chitarra, Vasileios Fotopoulos, and Michelina Ruocco	
13	Microbial Communities, Functional Genes, and Nitrogen Cycling Processes as Affected by Tree Species	209
	Relena R. Ribbons, Morag A. McDonald, and Lars Vesterdal	
14	Ectomycorrhizal Fungal Responses to Forest Liming and Wood Ash Addition: Review and Meta-analysis	223
	Rasmus Kjøller, Carla Cruz-Paredes, and Karina E. Clemmensen	
15	β-Glucosidase Activity of Forest Soil as an Indicator of Soil Carbon Accumulation	253
	Ewa Błońska and Jarosław Lasota	
16	Linking Ecosystem Variability and Carbon Sequestration: Estimation of Sequestered Carbon in Natural Forests and Perennial Crops	265
	Jelena Milovanović, Uroš Radojević, and Mirjana Šijačić-Nikolić	

17	Bioactive Peptaibols of Forest-Derived <i>Trichoderma</i> Isolates from Section <i>Longibrachiatum</i>	277
	Tamás Marik, András Szekeres, Maria A. Andersson, Mirja Salkinoja-Salonen, Chetna Tyagi, Balázs Leitgeb, Csaba Vágvölgyi, Irina S. Druzhinina, and László Kredics	
18	Plant-Assisted Bioremediation: An Ecological Approach for Recovering Multi-contaminated Areas	291
	Valeria Ancona, Paola Grenni, Anna Barra Caracciolo, Claudia Campanale, Martina Di Lenola, Ida Rascio, Vito Felice Uricchio, and Angelo Massacci	
19	Bioavailability of Polycyclic Aromatic Hydrocarbons in Soil as Affected by Microorganisms and Plants.	305
	Jose Julio Ortega-Calvo, Rosa Posada-Baquero, José Luis Garcia, and Manuel Cantos	
20	Soil Biodiversity and Tree Crops Resilience.	321
	Aurelio Ciancio and Mauro Gamboni	
	Index.	345

Chapter 4

Towards Integrated Understanding of the Rhizosphere Phenomenon as Ecological Driver: Can Rhizoculture Improve Agricultural and Forestry Systems?

Luis G. García-Montero, Pablo Manzano, Deaa Alwanney, Inmaculada Valverde-Asenjo, Amaya Álvarez-Lafuente, Luis F. Benito-Matías, Xavier Parladé, Sigfredo Ortuño, Marcos Morcillo, Antonio Gascó, Carlos Calderón-Guerrero, Francisco Mauro, Mercedes Méndez, Alvaro Sánchez-Medina, María P. Andrés, José R. Quintana, Cristina Menta, Stefania Pinto, Liliana Pinto, Pilar Pita, Cafer Turkmen, Cristina Pascual, Esperanza Ayuga, Fernando Torrent, José C. Robredo, Pablo Martín-Ortega, Joan Pera, Luis Gómez, Gonzalo Almendros, Carlos Colinas, and Eric P. Verrecchia

Abstract Agriculture and forestry traditionally focus on improving plant growth traits based on an anthropocentric point of view. This paradigm has led to global problems associated to soil overexploitation such as soil losses, reductions of the C stock in soils, and the generalized use of fertilizers, which particularly increases the costs of production and pollution treatment. This view may also have limited our understanding of mutualistic symbioses of plants and microorganisms assuming

L.G. García-Montero (✉) • D. Alwanney • S. Ortuño • A. Gascó • C. Calderón-Guerrero
F. Mauro • M. Méndez • A. Sánchez-Medina • M.P. Andrés • L. Pinto • P. Pita • C. Pascual
E. Ayuga • F. Torrent • J.C. Robredo • P. Martín-Ortega • L. Gómez
Technical University of Madrid, ETSI Montes, Madrid, Spain

University of Lleida, Lleida, Spain

University of Lausanne, Lausanne, Switzerland
e-mail: luisgonzaga.garcia@upm.es

P. Manzano
IUCN Commission for Ecosystem Management, Gland, Switzerland

I. Valverde-Asenjo • J.R. Quintana
Complutense University of Madrid, Madrid, Spain

A. Álvarez-Lafuente • L.F. Benito-Matías
National Forest Genetic Resources Centre El Serranillo, Madrid, Spain

X. Parladé • J. Pera
IRTA, Sustainable Plant Protection Centre de Cabrils, Cabrils, Spain

that the main role of non-photosynthetic symbionts is to mobilize the nutrients that are necessary for plant growth and development, and being plants the dominant agents of the symbiotic relationship. In response to these issues, this chapter offers an alternative approach taking advantage of the “rhizo-centric” point of view, where non-photosynthetic partners are the main protagonists in play; and secondly, it builds a multidisciplinary body of knowledge that could be called “rhizoculture”, which includes techniques focussing on the intensification of the development and activity of roots, mycorrhizae, and other symbiotic and free living rhizosphere organisms. In short, rhizoculture may lead to decrease plant production dependence on fertilization and provides other benefits to agriculture, forestry, and the environment. Within this conceptual framework, the first objective of this book chapter is to explore whether there is a “paradox of calcium salts” (i.e., Ca^{2+} and its salts are simultaneously nutrients, promoters, and stressors for the host plants) that would explain a dominance of mycorrhizal fungi over plants based on inducing a $\text{Ca}(\text{pH})$ -mediated chlorosis to the host plants. If this paradigm shifting hypothesis were finally fully verified, it would provide conceptual bases to reconsider our current technologies in agriculture and forestry by introducing the “rhizocultural” approach, based on the management of roots (introducing alternative cultural practices), Ca^{2+} salts (using liming and other techniques), rock-eating mycorrhizae, organic matter, and the soil microbiome (increasing the presence of symbiotic microorganisms against saprophytes), N and P contents (by aquaculture and smart recycling of organic waste), and the physical properties of the soil (by the activity of soil symbiotic microorganisms and soil fauna, such as ants, termites and earthworms). The development of such new technological approaches in rhizoculture would significantly decrease the high cost and associated pollution of the application of fertilizers and phytochemicals; as well as it would increase soil C stocks, improve the resilience of agricultural and forest systems to environmental disturbances, such as climate change, and enhance food production and security.

Keywords Rhizoculture • Rhizosphere • Mycorrhizae • N-fixing bacteria • Soil fauna • Agriculture • Forestry

M. Morcillo

Micología Forestal Aplicada – Micofora, Sant Antoni De Vilamajor, Barcelona, Spain

C. Menta • S. Pinto

Evolutionary and Functional Biology Department, University of Parma, Parma, Italy

C. Turkmen

Canakkale Onsekiz Mart University, Canakkale 17020, Turkey

G. Almendros

Spanish National Research Council (CSIC), Madrid, Spain

C. Colinas

University of Lleida, Lleida, Spain

E.P. Verrecchia

University of Lausanne, Lausanne, Switzerland

4.1 Introduction

Since the 1930s, the Green revolution has replaced traditional agriculture with conventional agriculture (CA) by the application of high inputs of chemical fertilizers and power derived from fossil fuels. These technologies saved over a billion people from starvation, but nowadays entail serious sustainability issues due to the limitation of fertilizers supply from current sources, greenhouse gas (GHG) emissions, and diffuse pollution (Farmer 1986; Te Pas and Rees 2014). Moreover, worldwide farmers only collect 50% of the yield they would obtain under optimal conditions, with 60–70% of the losses attributable to abiotic factors such as nutritional deficiencies or drought. The remaining 30–40% losses are attributed to biotic factors connected to the rhizosphere (Peleg et al. 2011). While organic agriculture (OA) promotes organic fertilization and outperforms CA in environmental and social aspects, critical questions arise on whether OA benefits compensate for its lower crop yield or if it will preserve soil fertility over time (Andersen et al. 2015; Hansen et al. 2001; Meier et al. 2015).

New solutions and policies are urgently needed to improve availability of fertilizers, OA and CA yields, and meet the “greening” requirements set by, for example, the Common Agricultural Policy (CAP) (Eurostat 2015). In this regard, European societal challenge analyses indicate that new nature-based solutions considering the multi-functional use of ecosystems are required to perform the transition towards a circular economic eco-innovative management system changing the traditional model based on production and consumption towards a green society.

4.1.1 *Rhizoculture: Strategies for the Intensification of Roots and Rhizosphere Activity to Reduce Fertilization and Improve Resilience in Agriculture and Forestry*

To include the multiple functions of ecosystems in both OA and CA, the rhizosphere is essential to soil, vegetation and ecosystem functioning (Akeem 2012). The term rhizosphere refers to the portion of soil surrounding roots where soil organisms are influenced by their presence (Killham 1994). Rhizosphere is the main bottleneck for closing loops on N, P and C-cycling in ecosystems because roots allow the integration of plant photosynthesis (leading to C inputs) and the activity of symbiotic organisms, such as mycorrhizae and N-fixing bacteria (leading to P and N inputs, respectively). Therefore, mutualistic plant-microbe interactions offer a novel approach to enhance agricultural productivity while reducing dependence on fertilization and environmental costs. In concert with other novel agronomic technologies and management practices, plant-microbial mutualisms can help increasing crop production from 115% and up to 300%, and reduce yield losses by improving resistance and/or resilience to edaphic, biologic, and climatic variability from both bottom-up and top-down perspectives (Adesemoye and Kloepper 2009; Baum et al. 2015; Hamilton et al. 2016).

In this regard, García-Montero et al. (2015a, b) proposed the development of a multidisciplinary body of knowledge called “rhizoculture” (i.e. *farming the rhizosphere*), which could be defined as a set of techniques focused on intensifying the abundance, biomass, depth and activity of roots, symbiotic and free-living organisms associated to the rhizosphere. Therefore, the so-defined rhizoculture can lead to decrease fertilization and diffuse pollution, and increase soil C storage, plant resilience, and agricultural and forest production by: (i) intensifying multiple symbioses among soil organisms and the host plants; (ii) managing roots and rhizosphere in connection with biogeochemical processes; (iii) rising soil organic matter content to increase the ratios of symbiotic to saprophytic communities in the soil; (iv) managing roots and rhizosphere impacts on soil diversity and C pools; (v) enhancing root and rhizosphere development to improve the ability of plants to cope with environmental disturbances such as climate change; and (vi) orienting policies aiming to incorporate the socioeconomic value of the positive externalities associated to rhizoculture.

Assuming the challenge of shifting production and consumption management patterns in agriculture and forestry towards a sustainable green economy and society, the value-added provided by this type of rhizoculture might include a reduction of the high costs and pollution associated to current fertilizers and phytochemicals inputs, which should result into improving both “environmental health” and the performance of agricultural and forestry systems.

In this way, developing new rhizoculture techniques connecting root and fertilizing management practices would be beneficial to agricultural and forestry sustainability, both supported by four R D areas: (1) agrotechnological support to root development, (2) “soft” fertilization application, (3) the management of soil organisms, and (4) and active management of soil water availability. The first RD area would incorporate a variety of small-medium industries as beneficiaries, and the other three areas would strengthen green agricultural and forestry policies, such as CAP.

4.2 Bases and Hypotheses for Developing Rhizoculture in Agricultural and Forestry Systems

Since the development of agriculture in the early Neolithic, the models of agriculture and forestry performance have primarily been focused on improving plant growth (especially focused on the development of parts of plants that were economically valuable) following anthropocentric and economical points of view. This approach may have neglected the understanding of plants’ interactions with their mutualistic symbionts, such as *ectomycorrhizal fungi* (ECMFs), arbuscular *mycorrhizal fungi* (AMFs) and N-fixing *bacteria* (NfBs), and commensal organisms, such as earthworms (García-Montero et al. 2015a, b).

Therefore, the problem under study is assessing whether *mycorrhizae* and other symbiotic organisms affect agriculture and forestry in a different way to the one assumed from a “phyto-centric” point of view, which is based on the traditional approach *viz.*: “Symbiotic organisms are related to plants following ecosystem models, but assuming that the role of non-photosynthetic symbionts is to mobilize the nutrients necessary for the primary production of plants, which appear to be the dominant actors of the symbiotic relationship. This conceptualization may result from phyto-centric and anthropocentric points of view which have led to the current agricultural and forestry models” (García-Montero et al. 2015a, b).

This traditional approach has led to high-intensity agricultural and forestry over-production models, which are leading to soil degradation and loss, declining biodiversity and of soil C to the atmosphere. In response to these issues, this chapter addresses an alternative hypothesis, improving on the phyto-centric point of view, where plants behave as dominant agents in the symbiosis, with a “rhizo-centric” (Phillips 2007) point of view, where non-photosynthetic partners could play a pre-dominant role.

Within this conceptual framework, the first objective of this chapter is revising in the literature whether the “paradox of Ca^{2+} salts” (Ca^{2+} and its salts simultaneously act as nutrients, promoters and stressors of plants, in the framework of the symbiotic organism-host plant relationship) could contribute to explain the postulated dominance of some mycorrhizal fungi over host plants (García-Montero et al. 2009; 2015a, b). As far as this hypothesis would be corroborated, the second objective of this chapter would portray a suitable scenario to improve our technologies in agriculture and forestry by introducing “rhizoculture”.

4.2.1 A Calcium (pH)-Induced Chlorosis Hypothesis in Some Ecmf-Host Plant Symbiosis Based on the Ability of ECMF to Mobilize Calcium Salts

There is still insufficient knowledge of ECMF functional structure, on its precise role in ecosystem processes and on its biogeochemical cycling. Numerous *in situ* studies on the ecological function of ECMFs have been hindered by the difficulty of monitoring ECMFs in nature, and limited research has already been conducted on the successional processes of ECMF communities (Courty et al. 2010; García-Montero et al. 2012; Nara 2008; Smith and Read 2008). In order to minimize the research difficulty of performing ECMF field studies, a potential valid approach would be to study disturbed sites with simple ECMF/host-plant communities developed on relatively homogeneous substrates (Nara 2008), such as truffle brûlés.

Plants that have been infected by some ECMF species exhibit a zone around their trunk where the growth of other plants is inhibited, either permanently or periodically. Some examples of fungi producing these vegetation clearings on the soil (brûlés) are *Tuber melanosporum* Vittad. (black truffle), *T. aestivum* Vittad.

(summer truffle) and several species of *Scleroderma* Pers. In particular, *Tuber melanosporum* brûlé occurs because its mycelium and fruit bodies release multiple compounds that adversely affect seed germination and young plants development; as well as producing necrosis in grass roots (Plattner and Hall 1995).

García-Montero et al. (2009, 2012, 2013) have monitored natural *Tuber melanosporum* brûlés in Mediterranean calcimorphic soils for over 15 years. A principal component analysis of 40 soil samples relates the soils inside the brûlés with high active carbonate content and lower total organic carbon (TOC) and total carbonate contents than in soils outside the brûlés. The disappearance of grasses in the brûlé causes modifications in soil surface layers affecting soil organic matter content (Castrignano et al. 2000). Callot et al. (1999) and Ricard et al. (2003) propose that the succession over time of different mycorrhizal fungi and microorganisms in *T. melanosporum* brûlés may be the result of the evolution of both the quantity and quality of the organic matter. Those authors report that the soils bearing *T. melanosporum* contain a small amount of coarse-fraction organic matter, and a high proportion of fine-fraction stable organic matter, in comparison with non-productive soils.

Statistical comparisons between carbonated fractions inside and outside these brûlés have indicated that the concentration of total CaCO_3 inside the mature brûlé is significantly lower than in the outside soil (an average 0.64 times lower), which provides evidence of the “rock-eating” activity of *T. melanosporum*. However, the concentration of active CaCO_3 (smaller than 50 μm in size) inside the brûlés is significantly higher (2.17 times greater on average) due to the soil physical, chemical and ecophysiological conditions that are associated to the existence of a mature brûlé, thus favouring secondary carbonate precipitation that produces a net increase of active CaCO_3 and exchangeable Ca^{2+} contents in the soil. The active CaCO_3 concentration inside the brûlés shows a positive correlation with the concentration of exchangeable Ca^{2+} (accounting for up to 42% of its variance). All these observations have led Prof. S. Rivas-Martínez to propose the term “Calcideserta” (calcic wilderness) for describing the brûlé of *T. melanosporum* from a geobotanical point of view (personal communication 2014).

Menta et al. (2014) have confirmed the ability of *T. aestivum* to modify the soil biogeochemical status in the brûlé. The TOC and water contents of soil tended to be significantly lower inside the brûlé in comparison to its outside. Mello et al. (2013) indicated that the lack of vegetation that appears in the brûlés could cause significant changes in light exposure and soil moisture level, leading, in turn, to water stress in the brûlé soil, especially in the summer. Finally, inside *T. aestivum* brûlés, the pH of the soil was significantly higher (0.22 to 0.28 units on average) than outside it.

On the other hand, some authors have reported comparable ECMF soil activity patterns resulting into significant accumulation of Ca, as calcium oxalate (CaC_2O_4), associated to ECMF mycelium presence on different soils. Oxalate and siderophore secretion are considered as the main mineral weathering means of ECMFs (Rineau and Garbaye 2010; Rineau et al. 2010). The ability to exude oxalate by some ECMFs has been widely documented, for instance as the means to acquire P by weathering mineral substrates (Kluber et al. 2010).

Based on the above findings, we upgrade a soil-plant nutrition hypothesis proposed by García-Montero et al. (2009) associated to some ECMF-host plant mutualisms: “*some ECMF communities could use different metabolic strategies to promote two biogeochemical changes, (i) increase soil Ca, and its salts, availability and/or (ii) increase soil pH in the rhizosphere of their host plants. Both strategies have been observed in carbonated soils as well as in acid soils. This ECMF activity could be a case of “ecosystem engineering” because increased pH and Ca^{2+} concentration in the rhizosphere may lead to host plant (i) chlorosis (via the paradox of Ca^{2+} salts) and (ii) modification of root-growth patterns (i.e., a significant root tips generation increase). Both, the stimulation to produce root tips and the induced nutrient deficiency, could lead to a greater ectomycorrhizal colonization activity and promote further growth of ECMF mycelia, which in turn induces the formation of further content of Ca^{2+} or increase soil pH in the rhizosphere on the basis of a feedback model*”.

The impact of liming and soil pH change on tree growth patterns reinforce this soil-plant nutrition hypothesis. Monfort-Salvador et al. (2015) reviewed the impact of calcareous amendments on the soil-ECMF-host plant system. They assessed: (i) the influence of Ca^{2+} salts of biological origin on ECMF communities; (ii) the impact of Ca^{2+} on the growth patterns of the host tree roots; (iii) the importance of rock-eating processes associated to the presence of ECMFs for the host plants; and (iv) the impact of the soil Ca^{2+} cycle (associated to litterfall) on ECMFs activity. Tree root systems react to liming improving their ability to explore the soil through an enhanced absorption-efficient soil-root interface with extensive fine root development and a greater number of mycorrhizal tips.

In summary, ECMF populations and communities could be acting as “ecosystem engineering” agents at a global scale, changing soil properties in the rhizosphere of the host trees in order to cause them stress (via the paradox of Ca^{2+} salts); therefore, modifying tree growth patterns and driving mycorrhization processes and nutrient exchange mechanisms. This, in turn, would make the host plants more dependent on the fungi to obtain nutrients. However, simultaneously, host plants will be more resistant to biological and environmental stresses (including climate change), and in the case of agriculture, host plants will be less dependent on fertilizers and irrigation.

4.2.2 The Oxalate-Calcium Carbonate Pathway in the Interactions Between Fungi and Oxalotrophic Bacteria

Verrecchia et al. (2006) analysed the interactions between fungi and oxalotrophic bacteria, remarking the biogeochemical impact of fungi on many elemental cycles. Fungi are not only biologically important as saprophytes for the recycling of organic matter, but also play a geological role by excreting notable amounts of organic acid,

among which oxalic acid is particularly important, and can be a source of secondary calcium carbonate in the soil. In contrast, calcium oxalate crystals precipitate and may constitute almost the 25% of soil hyphae and rhizomorphs dry weight in some ecosystems (Cromack et al. 1977). Verrecchia et al. (2006) demonstrated that oxalotrophic *bacteria* could effectively use these abundant oxalate crystals as C, electron, and energy sources by oxidizing them into calcium carbonate. This secondary CaCO_3 may then accumulate modifying soil conditions and increasing pH in the soil solution. Therefore, when associated with plant biomineralisation, fungi and bacteria contribute to long-term C storage by transforming half of the organic C from oxalate (or low-molecular-weight organic acids) into carbonate, with longer residence time in the soil than organic substrates. The other half is released into the atmosphere as CO_2 . In conclusion, because oxalate salts are of organic origin, the oxalate-carbonate pathway represents a potentially major C sink, and probably acts as a regulator of atmospheric pCO_2 .

Moreover, the oxalate-calcium carbonate pathway associated to these interactions probably also acts as a regulator of soil Ca^{2+} content and pH level in the rhizosphere environment of mycorrhizal-plant systems. This could have a major impact at the global scale. Therefore, it may be necessary to further develop another new hypothesis on mutualistic symbiotic relationships between *mycorrhizae* and oxalotrophic *bacteria* associated to the “paradox of Ca^{2+} salts” because the interaction of both soil microorganisms could increase their capacity to control host plants (based on the described Ca(pH)-induced-chlorosis).

On the other hand, calcium oxalate content showed a significant negative relationship with agricultural yield and crop quality attributes, emphasizing that increasing the levels of oxalate in the soil had a detrimental effect on soil microbial variables (Laxminarayana 2016). In summary, oxalotrophic *bacteria* may have positive impacts on agricultural systems, because they reduce the level of soil calcium oxalate, which benefits agriculture; and simultaneously increase the content of calcium carbonate, which stimulates the mycorrhization of plants. Therefore, this particular deserves conducting further research.

4.2.3 Interactions Between Soil Fauna and Mycorrhizae-Host Plant Systems Associated to the “paradox of Ca^{2+} salts”

Other relevant patterns of atmospheric CO_2 sequestration as soil carbonate (SIC) could be led by earthworms' activity (i) producing calcite granules and (ii) the soil aeration generated by their tunnels. Spherical soil concretions up to the millimetric diametric size and composed of calcite crystals are found in soils containing earthworms. These calcite granules ranging from single crystals to aggregations up to 2.5 mm in diameter are produced by the calciferous glands present in all the species of the *Lumbricidae* family (Briones et al. 2008a; Canti and Pearce 2003; Lee et al. 2008; Versteegh et al. 2014; Wiecek and Messenger 1972). Jongmans et al. (2001)

found earthworm calcite granules down to 50 cm deep in the soil profile. Briones et al. (2008a, b), Gago-Duport et al. (2008), and Lee et al. (2008) described the microstructural transformations involved in CaCO_3 precipitation from the calciferous glands of earthworms. Furthermore, these biomineral granules are stable. In particular, Canti (2009) reported that earthworm calcite granules have been found in archaeological soils and ancient sediments.

Coleman et al. (2004), Briones et al. (2008b) and Versteegh et al. (2014) indicated that the calciferous glands of earthworms could provide a mechanism for regulating CO_2 and Ca^{2+} in their blood and tissues. Canti (2009) showed that the C present in calcite granules mainly came from dietary inputs (litter), old soil C (soil OM), and from atmospheric CO_2 (although not in quantities larger than about one third of the total C in the granule), but very little quantity seemed to come from soil CaCO_3 . Therefore, earthworms could lead to the promotion of significant amounts of secondary precipitation of CaCO_3 in the soil, whose C comes from the atmospheric CO_2 that is fixed by plant photosynthesis (in the same way as the fungal-bacteria oxalate-carbonate pathway). In any case, the use of fresh OM, soil OM, and atmospheric CO_2 to fix C in the earthworms' calciferous glands as granules formed from calcite crystals would result in a net soil C fixing (as SIC) and the recarbonation of soil. In addition, Fraser et al. (2011) conclude that earthworms truly synthesize CaCO_3 and they do not merely recycle ingested material.

García-Montero et al. (2008, 2013) confirmed Canti's (2009) laboratory results in truffle orchards. They found a significant increase of carbonate contents (and a higher pH value) in earthworm casts associated to calcite granules production that cannot be just explained by the existing levels of original carbonate in weakly calcareous soils. Chan (2003) showed that the pH of casts is significantly higher (pH >6) than that of the bulk acid soil material (pH = 4.0) that was used in laboratory experiments. Wiecek and Messenger (1972) also indicated that *L. terrestris* casts are less acid than the ingested food, and proposed that the weathering of earthworm calcite granules is partly responsible for pH values greater than 7.0 in the A_1 horizons of acid soils under forests covering some American woodlands. Elsewhere, Lambkin et al. (2011) reported from a laboratory experiment that an increased soil pH could promote a higher production of calcite granules by *L. terrestris*. In consequence, García-Montero et al. (2013) pointed to a feedback loop of varying intensity with the different types of soils and earthworms.

Versteegh et al. (2014) addressed that, the observed increase of CaCO_3 production rate with temperature can be explained by an increase in their metabolic activity. Lee et al. (2008) highlighted that earthworms produce calcite granules in sufficient volume to have a measurable impact on soil C cycling (as SIC). Lambkin et al. (2011) estimated that annual granule production by *L. terrestris* lies in the range of 18-3,139 mol- CaCO_3 ha⁻¹ year⁻¹, with an average value of 438 mol- CaCO_3 ha⁻¹ year⁻¹. These authors pointed out that their values are higher than those reported by Wiecek and Messenger (1972), who calculated that excreted CaCO_3 could contribute up to 11 mol-calcite ha⁻¹ year⁻¹ in forests on acid soils; which are also higher than the results addressed in Canti (2007), who estimated *L. terrestris* production rates of more than 0.02 mol-calcite day⁻¹. However, Lambkin et al. (2011) indicated

that there is significant variation in the mass of granules produced by different earthworms exposed to the same soil, and explain that a large proportion of this variation should reflect biological variations between individuals. Recently, Versteegh et al. (2014) reported that *L. terrestris* is a major CaCO_3 producing species in temperate soils. Production rates range from 0.8 to 2.9 mg earthworm⁻¹ day⁻¹. Considering 1.9–61.8 individuals m⁻², this equates to precipitating 2–261 kg C ha⁻¹ year⁻¹, which represents a potentially significant contribution to carbon sequestration. These authors also highlighted the on-going debate on whether earthworms increase soil greenhouse-gas emissions or contribute to carbon sequestration, as well as on the timescale and nature of the experiments that would be required to determine this particular (Zhang et al. 2013). Particularly, Versteegh et al. (2014) showed that it is likely that, at higher temperatures and atmospheric $[\text{CO}_2]$, CaCO_3 earthworm synthesis will increase. As granules can last in soil for >300,000 years, the potential sequestration of C in the form of CaCO_3 is on a longer-time scale than roots and soil organic matter, for example. On the other hand, the precipitation of soil CaCO_3 increases when the partial pressure of CO_2 decreases in soil due to the aeration that is generated by the activity of earthworms themselves. In this regard, Callot et al. (1999) concluded that besides the contribution made by the respiration of roots and microorganisms, pCO_2 in the soil atmosphere depends on soil aeration, which is strongly conditioned by earthworms.

On the other hand, Verrecchia et al. (2006) showed that the accumulation of oxalate crystals by fungi may also produce unexpected consequences associated to soil fauna, because calcium oxalate can be disseminated inside soil by the action of oribatid mites. Such mites feed on calcium oxalate crystals produced by fungi, and then re-precipitate the mineral in their hardened cuticles (Norton and Behan-Pelletier 1991). In fact, they are thought to process a significant portion of the Ca^{2+} pool in some ecosystems (Gist and Crossley 1975). Therefore, it would be necessary to draw additional models on a mutualistic symbiosis existing between *mycorrhizae* and soil fauna (such as earthworms and mites) associated to the “paradox of Ca^{2+} salts” because the interaction of these organisms could increase the control over host plants based on the described Ca(pH)–induced-chlorosis. Menta et al. (2014) furthermore indicated that soil fauna could have an impact on fungal growth, dispersion and fruit body production, altering fungal fitness, and therefore, their combativeness in interaction with other soil microorganisms (Rotheray et al. 2011). Several authors have described some mechanisms explaining the impact of soil microarthropods on fungal communities. For instance, Hanlon and Anderson (1979) reported that microarthropod’s feeding activity can exert a strong differential effect on fungal and bacterial populations; as well as some reviews on AMFs and soil fauna interactions suggest that *Collembola* have the potential to restrict mycorrhizal functioning in the field (Fitter and Garbaye 1994; Fitter and Sanders 1992). On the other hand, Menta et al. (2014) addressed that some ECMFs, such as *Tuber aestivum*, may modify soil fauna communities; that is, *Folsomia* sp. showed higher abundance inside the soil of the *T. aestivum* brûlé than in the outside.

4.2.4 *The Liming Effect on AMFs in Agriculture*

AMFs are among the most ubiquitous and abundant symbiotic organisms, and colonise roots of almost all plant species spreading over upland grasslands (Sparling and Tinker 1978; Yang et al. 2016). AMFs enhance nutrient translocation and uptake by developing an extensive network of external mycelium acting as an extension of the root absorption system (Mosse 1981). Moreover, Guo et al. (2012) have hypothesized that the ability to form AMF associations may be important to crop species adaptation to low-input systems. The AMFs can also alleviate stresses, improve soil structure, and provide plants with tolerance to heavy metals (Augé 2001; Clark and Zeto 1996; Smith and Read 2008).

We have reviewed the impact of liming on AMFs populations, thus opening the field to future studies to (i) assess possible cause-effect relationships between liming and the composition of AMF communities, (ii) addressing the suitable lime dose, and (iii) establishing their interactions with other soil organisms, such as diazotrophs (i.e. NfBs), and the whole nitrogen cycle. AMFs seem to show different responses to lime amendments. They indicated that all three possible effects –positive, negative and neutral– were observed:

- (i) *Negative impact of liming on AMFs*: associated to the reduction of AMF spores, depressing root colonization by some AMF species and negative impacts on AMF community richness and diversity. In short, apparently, long-term application of lime might influence the colonization of AMF and, thus, the sustainability of farming systems on acid soils (Guo et al. 2012).
- (ii) *Non-significant impact of liming on AMFs*: very few authors have reported the absence impacts on fungal communities, i.e., Shah et al. (1990).
- (iii) *Positive impact of liming on AMFs*: in general, liming increases the intensity and abundance of mycorrhizal colonisation (but with significant differences among plant species), which may be crucial for conservation management systems, such as no-till farming (Guo et al. 2012; Johnson et al. 2005; Schneider et al. 2011). Liming increase the AMF colonisation of barley, corn, *Brachiaria decumbes*, and *Stylosanthes guianensis* and, moreover, increased the ability of AMFs to enhance the efficiency of P use by soybean and *Stylosanthes guianensis*. These authors also explained that liming increases the number of propagules, germination, and colonisation of several AMFs.

In summary, when calcareous amendment has been applied to crops, negative and positive effects on AMFs have been reported. However, our review allows concluding that, in the majority of cases, liming improves AMFs spore germination, the colonisation of AMFs, and their overall performance, although their diversity may be reduced.

In addition, liming can promote nodulation in N_2 -fixing *bacteria* (NfBs), and N_2 fixation itself as well (Redecker et al. 1997). Legumes experiment ineffective nodulation in low pH soils (Munns 1986); however, liming and the inoculation of plants with AMFs and *Rhizobium* may solve this problem (Guo et al. 2010). For these

reasons, we also reviewed the impact of liming on N-fixing *bacteria* (NfBs) in agricultural systems. Liming affects the interactions between legumes and *rhizobia*, resulting in an increased nitrogen fixation, which can duplicate N₂ fixation in some legume fields; i.e., liming can increase nodulation of *Trifolium repens* and *Alnus rubra* by *Rhizobium* spp. (Newbould and Rangeley 1984) and *Frankia* spp. (Crannell et al. 1994), respectively. Therefore, further research should help understanding how liming practices could impact on the whole system of symbioses of plants with soil organisms; as well as to establish analogies and differences with the model based on Ca(pH)–inducing chlorosis to the host plants that has been hypothesized above for ECMFs-plant systems.

4.3 Basis and Applications of Rhizoculture in Some Strategic Approaches

4.3.1 Food Production and Forest Resources at the Global Level

The Earth sums up 1,600 M ha of cropland, 3900 M ha of pastures and 3,900 M ha of forests, holding more than 1.400 M of farmers. These figures highlight the actual importance of the rural sector to the world economy. It is predicted that 50% more food will be needed in 2050 to feed a population of 9,000 M inhabitants, that is, 900 M tons more of cereals, 200 M tons more of meat, and 70 M ha more of cropland. In addition, it cannot be forgotten that there are currently 800 M people poorly nourished in the world. Even though they were almost 1,000 M in 2000, there is a lot to improve in this matter of concern (FAO 2017). Moreover, climate change may reduce up to a 25% of the yield of current crops in many areas of the planet. Therefore, there is still a big challenge for sustainability to overcome by investing and innovating in agricultural development. Within the agricultural sector, cereals production is remarkable, covering 60% of the cropland worldwide, along with oleaginous crops and fruit trees; whereas bovine, including cow milk production, is the most important within the livestock sector together with porcine. Considering the forest sector, its industrial activity is remarkable taking into account cellulose and paper, wood boards, round wood, and bioenergy production. In fact, forest production has experienced an important concentration within forestry plantations and crops, so they nowadays account for the 35% of the total forest outcome, although it is predicted to grow up to 75% in 2050. As a result, FAO (2017) estimates that wood demand will be increased by a 30% in 2050, and forestry crops will be responsible to cover this increment. Nowadays, 400 M m³ of industrial wood are being produced by forestry plantations, and they are estimated to be 900 M m³ in 2050. Correspondingly, forestry plantations cover is estimated to grow by approximately 80–90 M ha. Non-timber forest products (NTFPs) are also of great relevance within the forest sector, and they are under a strong growth; although statistical data are

partial and incomplete, so global statistics cannot be herein provided. Within this global framework, new solutions and policies are urgently needed targeting current challenges for agriculture and forestry, such as growing welfare, changing diets, climate change mitigation, and food security of a growing global population needing 50% more food in 2050, as proposed by FAO (2017).

4.3.2 Strategic Keys to Implement Rhizoculture Models to Optimize Its Impact on the Mitigation of Greenhouse Gas Emissions, Pollution Associated to Fertilizers Use and Food Security

Considering this background, the main objectives of developing rhizoculture technology, both in OA and CA, would be to reduce fertilizer use, and to increase crop resilience, C sequestration, and even agricultural and forestry production, and N and P recycling, to be applied from the farm scale to much larger geographical units. These objectives would be directly related to the principles of organic agriculture and permaculture (Mollison and Reney-Mia 1991; FAO 2017), and therefore, rhizoculture would have to propose models for closing N, P and C cycling loops in agro ecosystems, which rely on sustainable ecosystem management rather than on external agricultural inputs.

Rhizocultural technology could be based on a set of experimental studies at the laboratory scale aiming to unravel the biological mechanisms (including molecular, biochemical and physiological ones) that are involved in root and rhizosphere intensification. On the other hand, and both at farm and landscape scales across different biogeographic regions (such as, Boreal, Atlantic, Continental and Mediterranean) and different continents (such as, Europe, Africa, Asia and Latin America), comparable pilot scenarios could be selected to implement rhizoculture by: (i) developing new applications of conventional OA techniques; (ii) designing new agricultural practices based on the observation of rhizosphere patterns of performance (such as liming and the control of organic matter and water inputs into the soil); and (iii) monitoring the closure of loops of N, P and C cycling, as well as diffuse contamination, GHG emissions and C sequestration in agricultural ecosystems. Moreover, rhizoculture could be integrated with (a) livestock management and feed, and aquaculture; (b) new technologies to enhance their implementation (i.e., recycling of agricultural residues); (c) socioeconomic and environmental assessment models, aiming to designing new prototypes on agro-ecosystems, and disseminating them via agriculture extension programs (García-Montero et al. 2015a, b).

Therefore, it is necessary to develop research projects unfolding strategic steps to implement rhizoculture in potential designing prototypes of sustainable agro-ecological systems. The main objectives in the design of these experiments could be:

- (I) To develop agricultural, both organic and conventional, technologies to intensify root development and rhizosphere activity within both agricultural and forest management practices.
- (II) To design rhizoculture strategies based on OM management models (e.g. livestock management and others) aiming to increase the ratio of symbiotic/saprophytic soil organisms in order to achieve these objectives:
 - (a) To intensify biological NPK fertilisation (increasing NfBs and mycorrhization, fungal rock-eating activity, etc.).
 - (b) To reduce CO₂ and GHG emissions and increase C stocks.
 - (c) To increase plant resilience to climate change.
- (III) To develop rhizocultural strategies increasing the presence levels of Ca, and its salts, in the soil by liming and the management of biological activity (earthworms, oxalotrophic bacteria, etc.) in order to intensify the symbiosis of plants with mycorrhizas and NfBs (using the *Ca-induced-chlorosis* model).
- (IV) To implement rhizocultural strategies based on the management of fauna and organic waste recycling (including aquiculture, livestock, wild herbivorous, social insects, earthworm culture, and others) aiming to increase soil nutrients availability (by organic amendment, biological fertilisation, etc.) and the quality of soil properties (soil structure and aeration, cation exchange capacity, etc.).
- (V) To design strategies integrating the use of chemical fertilisers and pesticides with biological fertilisation and crop protection based on rhizoculture.
- (VI) To develop new information technologies that would favour the implementation of rhizoculture and its monitoring in agricultural and forestry systems. Some of these new technological developments may be:
 - (a) Technological applications aiming to increase symbiotic activities among plants, mycorrhizas, and bacteria.
 - (b) Technological applications to understand and intensify calcium roles in the symbiotic processes between plants, mycorrhizas and bacteria.
 - (c) Molecular monitoring of biomass and diversity of soil organisms.
 - (d) Remote sensing technologies monitoring fungal and soil organism activity.
- (VII) To develop new models integrating rhizoculture in agriculture and forestry with consumer behaviour, food security, resources use and green economy.

In order to generate a set of explanatory and management models on rhizoculture on different scales, Table 4.1 shows a set of hypothetical scenarios of high agricultural and forest value at both the global and European scales, based on 15 crop types (including 4 tree species) and 15 agroforestry systems (including 3 livestock species), which have been selected because: (i) they together represent 71.73% of the European and 63.85% of the global crop areas, in basis to FAOSTAT productivity data (FAO 2017); (ii) a bibliographic review shows that these crops have valuable root systems and a good response to calcareous amendments (Tables 4.2 and 4.3); (iii) prototypes of rhizoculture based on this set of crops would allow comparisons

Table 4.2 Root patterns and positive effects of liming on 15 representative crops that have been selected for designing comparative experiments on rhizoculture

15 selected crops	Root pattern in the soil	Liming effect	Impact	AM species
Wheat ¹	Both spring and winter wheat are annuals with deeply penetrating, widely spreading, and profusely branching, fine, fibrous roots; the roots of the spring varieties are less extensive ³ . Effective root zone ⁴ = 50–100 cm; 61–68% of their roots in < 30 cm; 50% >15 cm (avg. 16.8); 95% <103.8 cm; max depth = 150.4 cm ⁵ . 2-year-old crested wheat grass plant possessed over 500 m of roots occupying about 2.5 m ³ of soil ¹³	Liming increased the efficiency of P use by wheat because of biological causes. Liming caused a significant increase in grain and straw yield of wheat (26.8 – 18.6%, respectively) over the no lime treatment, irrespective of the sources and levels of lime.	+	–
Barley ²	Barley roots often occur nearer the surface than those of wheat; when grown in rich deep soil, has a root habit very similar to that of spring wheat; the fineness of the roots, degree of branching, and lateral spread often being intermediate. Effective root zone ⁴ = 50–100 cm ; 67–76% of their roots in < 30 cm; 50% <13 cm (avg. 11.5); 95% <99.6 cm; max depth = 146.1 cm ⁵	Small positive effect on mycorrhizal colonization of barley and on propagule numbers.	≈	–

Maize ¹	Corn has a remarkably widely spreading, deeply penetrating, and profusely branching root system ³ . Effective root zone ⁴ = 50–100 cm; 50% of their roots <14.4 cm in average; 95% <88.9 cm; max depth = 118.3 cm ⁵ . In prairie soils, corn roots regularly penetrate to 2 m ¹³	AM inoculation increased the yield when pH is intermediate.	+	–	
		AM increases the productivity of limed maize	+		<i>Glomus etunicatum</i>
		Liming favoured specific AM species and many non-AM fungi. Higher richness of AM in non-limed soil	+		<i>Funnelformis</i>
		AM improved maize growth in limed soil and higher AM infection rates were achieved	+		<i>G. margarita</i>
		Increase plant height in limed soil. Liming and fertilizers (NPKS) increased maize yields as compared to the control plots which received NPK only.	+	–	
		Liming increased the AMF colonisation of corn. Lower AM germination in non-limed and weak correlation with shoot dry weight	+		<i>G. mosseae</i>
Olive ²	While other trees send their roots deep into the ground, olive trees feature shallow root systems. This allows olive roots to collect water from soil that typically dries fast, ensuring the tree gets enough moisture to stay hydrated	P and liming increased the leaf-P levels. Liming to many Australian soils, will improve the health, growth and crop of olive trees			
Potato ²⁻	Potatoes have more superficial roots than many crops such as corn, winter wheat and most legumes. After extending horizontally 30–60 cm, majority of roots penetrate < 60 cm (exceptionally < 115 cm)	Liming had positive effects on yield, protein content, ash, starch, and Ca ²⁺ of potato. However, Zn, Cu, Fe and P decreased with increased application of lime. AM inoculation increased yield when pH is intermediate	+	–	
(continued)					

Table 4.2 (continued)

15 selected crops	Root pattern in the soil	Liming effect	Impact	AM species
Apple ¹	Apple trees roots consist of a deep taproot (analogous to the carrot) and lateral fibrous roots. Lateral fibrous roots can extend to more than twice the spread of the canopy. Fine roots develop from the fibrous roots. During the third year, the maximum lateral spread reached 9 m and the maximum depth reached was 5 m. This greatly exceeded the lateral spread of three-year-old tops, which was about 2 m, and the height of the trees, which was 2 to 2,4 m ⁶ . Roots of 18-year-old apple trees penetrated to a depth of at least 10 m and fully occupied the soil between the rows, which were about 10 m apart ¹³	Tree growth and apple fruit yield were significantly increased. Moreover, apple tree nutrition was benefited from liming.		
Rice ²	Rice has a relatively shallow and compact (short and thick) root system compared with maize and wheat. Rarely grow deeper than 40 cm; about 90% of the total root system is restricted to the top 20 cm of the soil layer. Mean of root length of no fertilizer plants is 23.44 shorter than NPK fertilizer plants ^{7,8,9}	Upland rice dry matter and grain yields increased up to 32 and 19%, respectively, with lime addition	+	–

¹[For annual crops¹: deeper rooting depth than other crops, or with 95% of roots < 100–138 cm, and max depth of 146–172 cm; or Effective Root Zone⁴ = 50–100 cm / For tree crops: deeper root systems than other trees]; ²[For annual crops: shallowest rooting depth than other crops, with 95% of roots 64–85 cm, and max depth of 64–85 cm / For tree crops: shallower root systems than other trees]; ³[Weaver 1926]; ⁴[Effective root zone is the depth within which most crop roots are concentrated;]; ⁵[Fan et al. 2016]; ⁶[Yocum 1937]; ⁷[Kirk 1994]; ⁸[Morita and Keisuke 1995]; ⁹[Jeon ,2006]; ¹⁰[Weaver and Bruner, 1927]; ¹¹[Baron 1985]; ¹²[Olsen 2013]; ¹³[Kramer and Boyer 1995]

Table 4.3 Root patterns and positive effects of liming on 15 representative crops that have been selected for designing comparative experiments on rhizoculture

15 selected crops	Root pattern in the soil	Liming effect	Impact	AM species	References
Soybean ²	67–76% of their roots in < 30 cm; 50% of total root amount < 13 cm (avg. 10.9); max depth = 111.3 cm ⁵	Liming increased the ability of AMFs to enhance the efficiency of P use by soybean	+	–	
Tomato ¹	Mature plants have a wonderfully extensive root system. Usually 15 to 20 major branches spread widely (maximum, 168 cm), have very numerous large branches, which usually penetrate deeply, and finally, turning downward, extend into the 90, 120, and sometimes the 150 cm of soil. All are so profusely furnished with masses of much rebranched rootlets that the absorbing area is extremely intricate and extensive even beyond the working level of 106 cm ¹⁰ .	Tomato fruit yield, plant growth and dry matter were significantly increased by liming at a pH between 3.3 to 5.2 while at a pH of 5.7 and above liming had little effects. In a soil of pH 4.2–5.1, liming 2 tones ha ⁻¹ of dolomite, and fertilizer (N–P–K–S–Zn), were optimum for better fruit yield tomato	–	–	
Green peas ²	Plants 1.5 months old have a root depth of 60 cm. The soil at a depth of 5 to 20 cm is well filled with a network of nearly horizontal roots and their laterals to a distance of 46 cm on all sides of the plant. But in the deeper soil little absorbing area occurs ¹⁰ . Effective root zone ⁴ = 60–70 cm; 61–68% of their roots in < 30 cm; 50% of total root amount > 15 cm (avg. 18.2); max depth = 150.4 cm ⁵	The number of rhizobia that occurred naturally in non-inoculated plots increased rapidly in high–lime plots	+	–	
Beans (dry) ¹	The kidney bean rapidly develops a deeply penetrating taproot. When the plants are nearly mature, the soil to 60 cm on all sides is well ramified to a working level of 90 cm feet and numerous roots extend 120 cm deeper. Thus the general root habit of the kidney bean is not greatly unlike that of the pea although the lateral spread and depth of penetration are somewhat greater and the deeper soil, just beneath the plant, somewhat more thoroughly occupied ¹⁰ .	Growth parameters and yield of <i>P. vulgaris</i> were significantly increased with liming (40% and 45%, respectively) or growing in vermicompost amended soil. Nodulating were significantly related with liming, largely due to changes in the predominance of the rhizobial species groups	+	–	

(continued)

Table 4.3 (continued)

15 selected crops	Root pattern in the soil	Liming effect	Impact	AM species	References
Hazelnut ²	Hazelnuts are more shallow-rooted than most fruit trees, growing well in the marginal locations for walnuts ¹¹ . Most of a hazelnut tree's roots are found in the first 0.6 m of soil; suitable soils allow trees to develop active root systems to depths of 1.8–3 m ^{11, 12}	Hazelnut uptake of N, K, Mg and Ca increase with liming, which also reduces Al and Mn uptake, but that liming is probably only cost effective up to pH 5.6 or 5.8. Liming increased very significantly the number of root tips	+	–	García-Montero et al. (García-Montero et al. 2012)
Walnut ¹	Walnut has an intricate extensive root system with the taproot penetrating to 3.7 m deep and many lateral roots which are mainly located in the horizon B at depth 60–80 cm, and sometimes extended to a distance exceeding the tree height and/or width. These trees excrete toxins to keep other plants with deep roots systems from growing ¹³	Liming improved the yield of walnut very significantly, and improved also soil porosity, increased leave nitrogen content and leave total potassium	+	–	
Alfalfa ¹	Alfalfa is a long-lived, very deeply rooted perennial ³ . Effective root zone ⁴ = 120 cm ; 61–68% of their roots in < 30 cm; 50% of total root amount >15 cm (avg. 19.8); max depth = 176.8 cm ⁵ . In prairie soils, alfalfa roots have been found at depths of 10 m ¹³	Higher rhizobium nodule numbers and total nodule weight. Higher shoots' N concentration without AM, while lower P concentration with AM	+	<i>Glomus intraradices</i>	Guo et al. (Guo et al. 2010)
Pearl millet	Root system development is characterized by a fast growing primary root that quickly colonizes deeper soil horizons (> 2 m)	Liming provided increments in the dry biomass production and in the accumulation of nutrients (N, P, K, Ca, Mg and S) by millet plants	+	–	
Mushrooms	Liming produces positive effects in many mycorrhizal fungi and truffles with agroforestry interest; see reviews of García-Montero et al. (García-Montero et al. 2009, García-Montero et al. 2012), Monfort-Salvador et al. 2015)				

¹[For annual crops¹: deeper rooting depth than other crops, or with 95% of roots <100–138 cm, and max depth of 146–172 cm; or Effective Root Zone⁴ = 50–100 cm/For tree crops: deeper root systems than other trees]; ²[For annual crops: shallowest rooting depth than other crops, with 95% of roots 64–85 cm, and max depth of 64–85 cm/For tree crops: shallower root systems than other trees]; ³[Weaver 1926]; ⁴[Effective root zone is the depth within which most crop roots are concentrated;]; ⁵[Fan et al. 2016]; ⁶[Yocum 1937]; ⁷[Kirk 1994]; ⁸[Morita and Keisuke 1995]; ⁹[Jeon Jeon 2006]; ¹⁰[Weaver and Bruner 1927]; ¹¹[Baron 1985]; ¹²[Olsen 2013]; ¹³[Kramer and Boyer 1995]

of “cover vs. row crops, annual vs. perennial, tall vs. short crops, deep vs. shallow roots, N-fixing and catch crops, species suited for companion planting and polyculture (i.e. beans, corn, peas, potato, tomato, alfalfa walnut)”; and (iv) other prototypes of rhizoculture based on a set of agricultural, forestry and other land uses would allow the analysis of arable land vs. adjacent forests, field margins vs. hedges and trees, tree species with N fixation capacity, and others scenarios such as buffer strips and mountain farming.

4.3.3 Basis to Integrating the Rhizoculture and the Soil Organic Matter Management by Livestock, Biomass and Bioenergy Uses

Livestock constitutes a powerful management tool at the landscape level. Apart from its proven capacity to influence biodiversity (Teillard et al. 2015), the high proportion of biomass consumed by livestock on grasslands (up to 85% according to estimates by Haynes and Williams (1993) and the dominance of domestic livestock in terrestrial grazed ecosystems (Smith et al. 2015) show how determinant this component can be. While livestock funnels a large proportion of the grassland biomass, most of the ingested components are excreted in an organic form and only mineralized by soil microorganisms once buried (Haynes and Williams 1993; Rufino et al. 2006). The interdependence of the soil ecosystem and the grazing ecosystem becomes therefore very relevant, especially in grazed ecosystems that represent more than half of the continental lands (Manzano 2015). It is then unsurprising that large mammalian herbivores influence the presence and activity of soil nematodes (Veen et al. 2010) or mycorrhizae (Murray et al. 2010).

At the interface between the grazing and the soil ecosystems, dung-burying invertebrates become critical in putting systems together and closing the nutrient cycle. While earthworms have been considered determinant in temperate humid climates, as well as dung beetles in semiarid Mediterranean systems (Lumaret et al. 1992), the influence of the latter seems to be restricted to the most humid months both in temperate and tropical semi-arid and arid areas. Harvester ants have been observed to be much more relevant in terms of dung burial during dry seasons in the Mediterranean area (Manzano et al. 2010), with similar roles attributed, in principle, to termites in the tropics (Freyman et al. 2008; West 1991). While such studies give a hint on the potential importance of social insects in dung burial, very little is known on which factors favour or limit the process or how it affects the soil community and the processes therein, such as carbon fixation or CaCO_3 production. Such research is particularly crucial when considering that a very large proportion of the continental land consists of drylands (up to 41.3% according to the Millennium Ecosystem Assessment), which have particularly been disregarded by research.

The management of grazing lands has also a very strong influence on their land degradation and biodiversity status, which in turn has a direct effect on C storage

potential., Studies on rangeland governance have shown that security in land tenure is very determinant for keeping soils in grazing lands under a satisfactory conservation status (Herrera et al. 2014). Grazing management as a tool, including adequate land tenure systems, therefore seems a cheap and far-reaching management tool to promote carbon-fixing processes in the soil, provided that concluding research on how the different regimes influence it would be enabled.

Therefore, livestock and biomass use management can be major factors to develop soil organic matter management strategies aiming to increase the ratio of symbiotic/saprophytic soil organisms in order to achieve the main objectives of rhizoculture (described above) (García-Montero et al. 2015a, b).

In this regard, the need to mitigate climate change effects reducing GHG emissions, the growing interest on reducing crude oil dependence, and several other beneficial effects are major motivations for increasing the use of biomass-derived bioenergy (Long et al. 2015; UNTACD 2016). Biomass use has undergone a significant transformation along the last decade, that is, biomass is not only used for energy generation, but also for biomaterial production (food, feed, and fibre) thanks to the application of more innovative biotechnological processes, leading to develop what it is currently known as the bioeconomy (UNCTAD 2016). In 2014, the 10.1% of the world's Total Primary Energy Supply (TPES = 1,384 Mtoe = 57.8 EJ) was produced from biomass (solid and liquid biofuels; IEA 2016). Biofuel production in 2015 was 74.85 Mtoe, whereas 239.4 thousand million tonnes of oil were produced from geological reserves. The three largest biofuel producers were USA, Brazil and Germany, accounting for the 41.5%, 23.6% and 4.2% of the total worldwide outcome, respectively (BP 2016). The bulk of the production of the USA and Brazil is ethanol from corn and sugarcane, respectively; whereas the major part of the German production is biodiesel from rapeseed (BP 2016; Long et al. 2015). In the USA, a 30% of the average annual production of corn grain from 2006 to 2010 was used for ethanol production. Three oil crops supply the vast majority of biodiesel: soybean (7.0 Mt), oil palm (6.3 Mt) and rapeseed/canola (6.0 Mt). Most soybean biodiesel is produced and consumed in the major centres of soybean cultivation, which are Brazil, USA and Argentina (Long et al. 2015). Bioenergy and biofuels produced from biomass have been considered carbon neutral in most Life Cycle Assessment (LCA) studies of biomass systems. It is assumed that the carbon released during the combustion of biomass is sequestered back into equivalent growing biomass. This convention is currently being discussed because the timing difference between the release and sequestration of carbon from forest biomass leads to a situation where part of the carbon remains in the atmosphere until it is fully integrated back into the growing forest. This would result in a warming impact if sequestration lags emission. Thus, carbon neutrality over a forest rotation period is not equal to climate change neutrality (Cherubini et al. 2011; Helin et al. 2013).

In biomass production (crop and harvest stages), other GHG emissions (i.e. N₂O and CH₄) should also be taken into account; and special attention should be paid to the role of fertilizers (Sastre et al. 2016). Net GHG savings are really highly uncertain and depend, among others, on the N-fertilization level and assumed N₂O emissions factors (Erisman et al. 2010). Recent studies point out that typical fertilization

doses for bioenergy crops could produce nitrogen deficits in soil stocks, compromising soil sustainability and future crop fertility. However, raising nitrogen fertilization above the typical dosage aiming to compensate soil nitrogen deficit could compromise the achievement of GHG savings, as well as it could generate excessive effects in other relevant impact categories (i.e. eutrophication) (Sastre et al. 2016). In connection with rhizoculture, different crop management techniques are suggested to overcome this problem: (i) crop rotation with legumes; (ii) no-tillage farming; (iii) optimization of the crop collection time and/or the use of low N content species; (iv) introducing *gluconacetobacter* bacteria; and/or (v) using slow release fertilizers (Erisman et al. 2010; Sastre et al. 2016).

The cultivation of bioenergy crops is viewed as controversial because of this uncertainty with respect to net GHG-savings and its potential competition with biodiversity and food production for land use (Erisman et al. 2010). Research is currently focused on a second-generation biofuels (i.e. produced from lignocellulosic biomass). Typical resources for these fuels are short rotation forestry crops (poplar, willow and eucalyptus), perennial grasses (*Miscanthus* spp., switch grass and reed canary grass), and residues from the wood industry, forestry, and agriculture. Most of these second-generation biofuels are in their early stages of pilot production phase and commercialization (UNCTAD 2016).

4.3.4 Basis to Integrating the Rhizoculture with Alternative Fertilizers Based on Waste Recycling and Aquaculture

In 2012 and 2013, the average world's fertilizers consumption was about 120 kg/ha of cropland a year. Just considering developed countries with intensive agriculture, these figures turn to exceed 170 kg/ha yearly (i.e., the UE addressed an average of 177.026 and 176.419 kg/ha, respectively). These figures include N, P and K fertilizing fertilising products, but traditional organic compost and manure from plant and animal origins are not included (FAO 2016; World Bank 2016). The cost and environmental issues associated to these high levels of fertilizer application are promoting new alternative N and P sources, based on waste recycling, aquaculture and rhizoculture.

The world's production of aquatic animals from aquaculture summed up to 73.8 million tons in 2014, and 27.3 million tons of aquatic plants were also cultivated, accounting for an estimated first-sale value of €101,000 million, even overpassing the world fisheries captures of 93.2 million tons (FAO 2016). The total European aquaculture production reached 2,350,278 tonnes in 2015.

Cold-water marine species now represent the 71.4% of the total production, fresh water species sum up to the 15.1%, and marine Mediterranean species address the rest 13.5%. In 2015, the 94% of bred species were salmon, trout, seabream, seabass, and carp. Within the aquaculture sector, Integrated Multi-Trophic Aquaculture (IMTA) is a multi-culture system where several species from different trophic levels

are bred in proximity. While the theoretical potential for the growth of biomass in the ocean is evidently very large, the focus on this kind of solution has been surprisingly low. As long as it is performed within ecologically sustainable limits, IMTA offers potential solutions to an increased and profitable production of local, resource-efficient, and climate-friendly food and biomass for energy purposes whilst capturing CO₂. This system is similar to a natural ecosystem, so the waste or excess of nutrients produced from fish being farmed (e.g. faeces and waste metabolites, and uneaten feed) become nourishment resources for lower trophic species, such as shellfish or seaweeds that are housed within the same system or environment.

This combination contributes to an ecosystem-based production that, in addition to recycling nutrients, naturally binds CO₂. Particularly within these systems, “aquaponics” is an innovative and sustainable food production system integrating aquaculture with hydroponic vegetal crops. Aquaponics may hold a key role to play in food provision and tackling global challenges such as water scarcity, food security, urbanization, and reductions in energy use and food miles.

On the other hand, membrane technologies, or membrane separation processes (MSP), are considered as highly efficient alternatives for the removal of nitrates and phosphates from water, in addition to other technological advantages such as the significant reduction of equipment size, energy savings, minimized environmental impact, and its easy staggering and automation (Drioli et al. 2011). Thus, some researchers have addressed this issue by applying different types of membrane and other separation processes, showing promising results (removal of nitrates and phosphates > 99%), such as electrocoagulation (Bektaş et al. 2004), microfiltration (MF) (Wasik et al. Wąsik et al. 2001), membrane bioreactors (MBR) (Wenhai et al. Wenhai et al. 2017), nanofiltration (NF), or reverse osmosis (RO) or direct one (DO) (Van Voorthuizen et al. 2005). In many of these studies, different combinations of processes are also proposed, i.e. MBR+RO (Wenhai et al. Wenhai et al. 2017), MBR+NF (Kangmin and Jaeweon 2012) or an integrated MF-NF-RO system, depending on the requirements of final water quality or the components to be removed. Nowadays, several companies are developing the application of these processes to the extraction of N and P from waste and agricultural waste recycling to produce alternative fertilizers, which is on the base of a circular economy compatible with rhizoculture.

4.3.5 Basis for Integrating Rhizoculture and Liming in Agriculture and Forestry: Impact of Mycorrhizae and Calcium on the Physiology of Plants

Calcium availability in the soil is crucial to many biological processes, so Ca²⁺ scarcity, whether caused by natural causes or human activity, may block certain plant physiological processes demanding leaf exchangeable Ca. In forest ecosystems, forest decay has partially been related to low soil Ca²⁺ contents (Borer et al. 2005;

Ting-Wu et al. 2011). Correspondingly, tree species generally react in a positive way to an increment of soil Ca^{2+} availability. Forestry extractions and acid deposition may cause substantial Ca^{2+} depletion in forest soils if rock weathering, atmospheric deposition or fertilization application do not compensate the losses (Huntington 2005), thus addressing a negative effect on the health of forests (Halman et al. 2014). Consequently, those forest species growing on soils with a higher soil Ca^{2+} availability show higher tolerance to root diseases, better leaf cold tolerance (Halman et al. 2008), higher leaf growth and biomass (Littke and Zabowski 2007), and increased leaf nutrients contents, as well as tree tops vitality is enhanced, stress metabolic indicators are lower (Minocha et al. 2010), and it promotes a higher relative growth of the basal area, in particular, and the overall plant growth, in general (Moore and Ouimet 2010; Huggett et al. 2007).

From a plant physiological and nutritional point of view, calcium is considered as an immobile essential element for plants, that is, it is an intrinsic component of their structure and metabolism; thus, its deficit may cause severe disorders to plant growth and development; as well as it limits the ability of plants to adaptively respond to environmental changes (Borer et al. 2005; Taiz and Zeiger 2015). Furthermore, attending to its relative abundance within plant tissue, it is considered as a macronutrient. In particular, the average content of Ca^{2+} in plant tissue is about the 0.5% (Epstein 1972). In addition, Ca^{2+} is considered as a nutrient that remains in its ionic form according to its biochemical functions (Mengel and Kirkby 2001), which comprises being a constituent of the middle lamella of cell walls, its participation as a cofactor of some enzymes of ATP hydrolysis and phospholipids, as well as it performs second messenger roles in metabolic regulation and serve to regulate the osmotic potential. Furthermore, being a divalent cation provides Ca^{2+} the ability to modify the permeability of plant membranes, which may serve, as well as cytosolic acidic pH values induced by flooding (Tournaire-Roux et al. 2003), to reduce the expression of aquaporins (e.g. Gerbeau et al. 2002), thus reducing the cell-to-cell water transport through roots tissues and, ultimately, water losses. Finally, the structural function of calcium on cell-walls is a major determinant of the xylem vulnerability to cavitation (Herbette and Cochard 2010).

On the other hand, AMFs symbiosis generally results in increased plant transpiration and stomatal conductance rates (Auge, Augé 2001). Among other factors, both AMFs and ECMFs may increase water uptake and transport thanks to the action of extraradical hyphae. Correspondingly, mycorrhizal plants have been reported to withstand drought better than non-mycorrhizal ones (Ruiz-Sanchez et al. 2010). Water use efficiency (WUE) was found to increase as the result of increasing assimilation rates in higher proportion than transpiration ones in mycorrhizal *B. papyrifera* seedlings under irregular watering (Birhane et al. 2012). Although similar results have been reported in other species (Shamshiri et al. 2006, Kumar et al. 2016), the impact of mycorrhizae on WUE may depend on the plant species (Querejeta et al. 2003). Mycorrhizae may enhance shoot growth in host plants by means of improved nutrition and soil water acquisition, but below-ground biomass would also change, as far as the combined growth of plant roots and fungi mycelia are considered. As a result, alterations in plant

allometry as a result of the mycorrhizal infection may have an impact on long-term WUE. Moreover, any modification of plant WUE at the global scale may affect the hydrological cycle. In fact, it should be taken into consideration that plant transpiration does not only play a role in the hydrological cycle, but it is also a meaningful way to cool the atmosphere. A better understanding of the effects of the proposed liming and rhizocultural practices on the physiology and use of soil water by mycorrhizal plants may, therefore, contribute to achieve better solutions for current agriculture, forestry and environmental concerns.

4.4 Conclusions

In summary, agricultural and forestry systems must support the global increasing demands of food and fibre, facing at the same time delicate and outstanding issues as critical as fertilizers availability, C sequestration, sustainable development, and climate change. Therefore, new nature-based solutions are required to perform the transition towards a circular economic system and a green society. Rhizoculture would lead to decrease fertilization and increase soil C storage, resilience of plants, and even agricultural and forest production, through a set of techniques focused on intensifying the abundance, biomass, depth, and activity of roots and the symbiotic microorganisms and fauna associated to the rhizosphere. The hypotheses supporting this rhizocultural approach are based on imitating the promising possibilities of some fungal behaviour that alter their environment to favour symbiotic relationships, such as the hypotheses of the “*calcium(pH)-induced chlorosis*” and the “*Ca²⁺ salts paradox*”. Therefore, a deeper understanding of calcium salts management impacts, along with fungi and soil fauna management, and roots biology, will help (if discussed simultaneously) developing new rhizocultural technologies. They would also be based on the management of livestock and biomass (to increase ratios of symbiotic/saprophytic communities in the soil), agricultural and aquaculture residues (applying new recycling technologies), and new socioeconomic systems, that may significantly reduce the need for fertilizers. We believe that this approach will open the field for discussions on a new management of terrestrial ecosystems for the benefit of safe and sustainable food and forest production in a more environmentally friendly way.

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Luis Gonzaga GARCÍA MONTERO

Associate Professor

Dept. of Forest and Environmental Engineering and Management

Polytechnic University of Madrid UPM (www.upm.es)

E-mail UPM: luisgonzaga.garcia@upm.es

Skype: [luisgonzaga.garcia](https://www.skype.com/people/luisgonzaga.garcia)

Mailing address: U.D. Análisis Ambiental, E.T.S. Ingenieros de Montes

Ciudad Universitaria s/n, Madrid 28040, Spain

Tel.: (+34) 913365565; fax: (+34) 913336386

Mobile phone: (+34) 676323388