UNIVERSIDAD POLITÉCNICA DE MADRID
Escuela Técnica Superior de Ingeniería de Montes, Forestal y del Medio Natural

"Harnessing Plant Biotechnology for Enhanced Disease Resistance: Insights into Biopriming, Biopesticides, and Epigenetic Memory Mechanisms"

DOCTORAL THESIS

Submitted for the degree of Doctor by:

Serine Soudani
Master's in Plant Biotechnology

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Abstract

This thesis delves into innovative biological approaches aimed at improving plant resistance against high impact phytopathogens in both forest and non-forest settings. Specifically, it involves the use of *Artemisia absinthium* essential oil (AEO) as a natural treatment to enhance tomato resistance against the fungal phytopathogen *Fusarium oxysporum f. sp. radicis-lycopersici* (*Fol*), achieved through biopriming and the induction of epigenetic-related transcriptional changes. Additionally, a methodology was developed for the "*in vitro*" study of the transcriptional response of *Quercus suber* (*Qs*) clones to the oomycete pathogen *Phytophthora cinnamomi* Rands (*Pc*). Furthermore, the thesis explores the resistance of transgenic *Quercus suber* explants overexpressing antimicrobial compounds.

Overall Thesis Objective:

This thesis endeavors to advance the field of plant biotechnology by showcasing the effectiveness of biological methods in strengthening plants against phytopathogens. Natural products are used as treatments to induce an epigenetic effect on the plant. The goal is to provide a thorough understanding of the molecular mechanisms involved in enhancing plant resistance. This research sets the stage for the development of sustainable and eco-friendly strategies in agriculture. Additionally, a methodology "*in vitro*" is presented to enable the comparative study of resistance to *Phytophthora cinnamomi* in *Quercus* species.

Chapter 1: Utilizing *Artemisia absinthium* Essential Oil to improve resistance by producing Epigenetic changes in Tomato Plants

The first chapter investigates the application of AEO to modulate the epigenetic landscape of tomato plants, specifically targeting *Fol* resistance. Through detailed analyses, including DNA and RNA modification and gene expression studies, we aim to elucidate the molecular mechanisms underlying the enhanced resistance of tomato plants to *Fol* *Fol*lowing treatment with AEO.

Chapter 2: Epigenetic Insights into *Quercus suber* Response to *Pc* Infection

The second chapter delves into the epigenetic dynamics of *Qs* during infection with *Pc*. In this chapter a protocol was established to assess *Qs* resistance to this oomycete through “*in vitro*” comparative analysis among different clones. This method facilitated the characterization of plant’s molecular response to *Pc*. This investigation aims to uncover the molecular intricacies of the plant's response, providing valuable insights into potential epigenetic mechanisms that contribute to enhanced resistance against *Pc*. Additionally, the same method was employed to determine the plant´s resistance to genetically modified *Qs* explants overexpressing antimicrobials.

Resumen

Esta tesis explora enfoques biológicos innovadores destinados a mejorar la resistencia de las plantas contra fitopatógenos de alto impacto tanto en entornos forestales como no forestales. Específicamente, implica el uso del aceite esencial de *Artemisia absinthium* (AEO) como tratamiento natural para mejorar la resistencia del tomate frente al fitopatógeno fúngico *Fusarium oxysporum f. sp. radicis-lycopersici* (*Fol*), logrado mediante la biopriming y la inducción de cambios transcripcionales relacionados con la epigenética. Además, se desarrolló una metodología para el estudio "*in vitro*" de la respuesta transcripcional de clones de *Quercus suber* (*Qs*) al patógeno oomiceto *Phytophthora cinnamomi* Rands (*Pc*). Además, la tesis explora la resistencia de explantes transgénicos de *Quercus suber* que sobreexpresan compuestos antimicrobianos.

Objetivo general de la tesis:

Esta tesis se propone avanzar en el campo de la biotecnología vegetal al mostrar la efectividad de los métodos biológicos en el fortalecimiento de las plantas contra fitopatógenos. Se utilizan productos naturales como tratamientos para inducir un efecto epigenético en la planta. El objetivo es proporcionar una comprensión detallada de los mecanismos moleculares involucrados en la mejora de la resistencia de las plantas. Esta investigación sienta las bases para el desarrollo de estrategias sostenibles y respetuosas con el medio ambiente en la agricultura. Además, se presenta una metodología "*in vitro*" para facilitar el estudio comparativo de la resistencia a *Phytophthora cinnamomi* en especies de *Quercus*.

Capítulo 1: Utilización del aceite esencial de *Artemisia absinthium* para mejorar la resistencia mediante la producción de cambios epigenéticos en las plantas de tomate.

El primer capítulo investiga la aplicación de AEO para modular el paisaje epigenético de las plantas de tomate, centrándose específicamente en la resistencia a *Fol*. A través de análisis detallados, incluidos estudios de modificación de ADN y ARN y expresión génica, nuestro objetivo es elucidar los mecanismos moleculares que subyacen a la resistencia mejorada de las plantas de tomate a *Fol* después del tratamiento con AEO.

Capítulo 2: Ideas epigenéticas sobre la respuesta de *Quercus suber* a la infección por *Pc*.

El segundo capítulo profundiza en la dinámica epigenética de *Qs* durante la infección con *Pc*. En este capítulo se estableció un protocolo para evaluar la resistencia de *Qs* a este oomiceto mediante un análisis comparativo "*in vitro*" entre diferentes clones. Este método facilitó la caracterización de la respuesta molecular de la planta a *Pc*. Esta investigación tiene como objetivo descubrir las complejidades moleculares de la respuesta de la planta, proporcionando información valiosa sobre los posibles mecanismos epigenéticos que contribuyen a una resistencia mejorada contra *Pc*. Además, el mismo método se empleó para determinar la resistencia de la planta a explantes de *Qs* genéticamente modificados que sobreexpresan antimicrobianos.

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Abbreviations and acronyms

AEO	*Artemisia absinthium* essential oil
Fol	*Fusarium oxysporum sp. Oxysporum radicis lycopersici*
Fox	*Fusarium oxysporum*
Dpi	Days post-inoculation
MTT	Thiazolyl blue tetrazolium bromide
DAB	3,3’ Diaminobenzidine tetrahydro-chloride hydrate
MeJA	Methyl Jasmonate Acid
ACC	1-aminocyclopropane 1-carboxylic acid
SA	Salicylic Acid
PDB	Potato Dextrose Broth Medium
RPMI	Roswell Park Memorial Institute medium
IBA	Indol-3-Butyric Acid
Pc	*Phytophthora cinnamomi* Rands
SAMt	*S-adenosylmethionine transferases*
NRPD2	*DNA- dependent RNA polymerase IV and V subunit 2*
EB	Endophytic Bacteria
Qs	*Quercus suber*
QsSE	*Quercus suber* somatic embryo
FW	Fresh weight
FWL	Fresh Weight Loss
m6A	N6- methyladenosine
5 mC	5-Methylcytosine
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone lysine methyltransferase
RdDM	RNA-directed DNA methylation
CYP	Cytochrome P450
LCMS	Liquid Chromatography Mass Spectrometry
GCMS	Gas Chromatography coupled with Mass Spectrometry
ROS	Reactive Oxygen Species
EDTA	Ethylenediaminetetraacetic acid

I Introduction

Since our species arrival on the evolutionary scene, food was acquired by gathering it from the wild. In the Neolithic revolution period, over 10,000 to 5,000 years ago, agriculture emerged independently in several regions of the world by domesticating various plants and animals. This period was a significant turning point in human history as the agriculture was considered then, as a reliable source of food production (Smith, 2001). Over time, advancements in agricultural techniques led to the utilization of chemical products and molecules to protect plants against biological attack, because agriculture and domestication of plants contributed to incidence of phytopathogens specifically associated to crops and harvesting methods (Aguilar-Marcelino et al., 2020; Ul Haq & Ijaz, 2020). However, studies have unequivocally demonstrated the severe toxicity of these substances to humans health: leading to severe cancers (Sabarwal et al., 2018), neurological disorders, hormonal disturbances, and reproductive issues (Nicolopoulou-Stamati et al., 2016); it affects also animals health: by causing the decline of populations such as raptorial birds (Mitra Anindita *et al* 2011), intoxication (Berny & Berny, 2007), affect the microbiome of animals (Syromyatnikov et al., 2020); the environment is the most affected about chemical pollution since it causes an imbalances withing the ecosystem, loss of soil fertility, and deterioration of marine life (Laxmishree & Nandita, 2017).

Furthermore, it was revealed by preliminary surveys in foreign countries, that multiple types and varieties of chemical pesticides to protect crops from diseases and pests are improperly used by many farmers, as their knowledge is based on information provided by dealers and sellers of farming supplies, What may cause serious damages to them and to the environment (Al-Zaidi et al., 2011).

As mentioned previously, agriculture confronts numerous challenges, encompassing both abiotic and biotic stresses. Among these challenges, phytopathogens stand out prominently. This thesis will delve into the detailed study of two specific phytopathogens.

I.1 Fusarium oxysporum f.sp. radicis-lycopersici

The fungi *Fusarium oxysporum* (*Fox*) is a widely distributed fungal species complex, reproduces spores both asexually predominantly, and sexually by less than 20% only. It can be found in various habitats such as plants, soil and water, where they exist as parasites endophytes, or saprophytes (Rana et al., 2017). Cumulatively, *Fox* strains have the potential to induce wilt or root rot in an extensive array of host plants, including numerous economically significant crops (Lievens et al., 2009; McGovern, 2015). This fungal species complex was first identified in USA in 1929, after its spread in all of Africa, East Asia, Europe, Latin America, United Sates, and China (Buruchara & Camacho, 2000; Harter, 1929).

Its life cycle involves the production of three types of asexual infectious spores: macroconidia, microconidia, and chlamydospores. Despite extensive research, no sexual stage has been identified for this species (Booth, 1971; McGovern, 2015), despite other *Fusarium* species where the sexual stage has been reported to exhibit less than 20% sexual spore reproduction (Rana et al., 2017). The microconidia are uninucleate and exhibit poor germination efficiency, ranging from 1 to 20% (Ebbole & Sachs, 1990). On the other hand, macroconidia are multinucleate and demonstrate rapid germination. Chlamydospores result from structural modifications of vegetative hyphae or thick-walled conidial cells and accessory spores, showing diversity in macroconidia shape, micro-conidiophore structure, and chlamydospore formation for *Fox* (Beckman 1987). Most research indicates that the invasion process starts with the development of a hyphal network around root hairs, progressing to penetration and colonization of the epidermis, and eventually reaching the vascular tissues of the root. The colonization within these vascular tissues extends to the stem or the entire plant, resulting in phloem blockage, internal stem discoloration, and overall plant wilt. Infected plants exhibit symptoms such as stunted growth, complete wilting, widespread chlorosis, and necrosis on the leaves (Chen et al., 2019; R. Joshi, 2018; Rana et al., 2017; Takken & Rep, 2010).

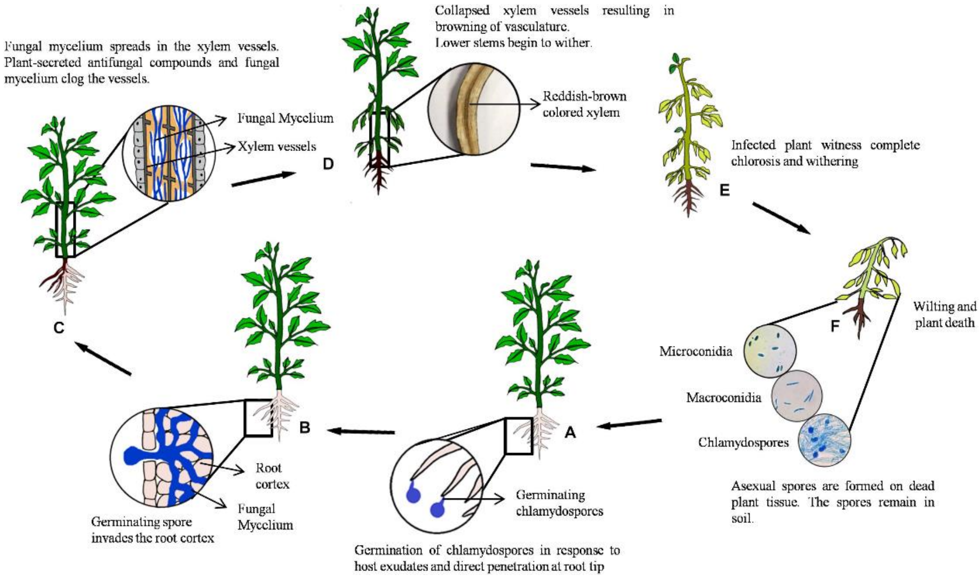


Figure 1: *Life cycle of Fusarium oxysporum f.sp. radicis-lycopersici (Jangir et al., 2021).*

Host plants at risk from pathogenic strains of *Fox* include numerous ornamental plants (Lecomte et al., 2016) as well as various agricultural crops, such as tomatoes (Soudani et al., 2022), potato tubers (Lastochkina et al., 2020), beans (Chen et al., 2019), rice, and watermelon (Hao et al., 2010), as well as grapevines (Reveglia et al., 2018). Additional host plants of conifers such as *Acacia koa* (Shiraishi et al., 2012)*,* teak or *Tectona grandis* (Raman & Muthukathan, 2015)*,* tree bean (Singh et al., 2022), olive trees (Trabelsi et al., 2017); More hosts are listed in the review of (Edel-Hermann & Lecomte, 2019). This study was focused on *Fol,* which notably affects both forest and non-forest species.

I.2 Tomato as An Important Crop:

One of the first target species of *Fusarium oxysporum* is *Solanum lycopersicum*, many studies have been realised on the response of tomato to this pathogen (Abdesselem et al., 2016; Lievens et al., 2009; McGovern, 2015; Nirmaladevi et al., 2016; Takken & Rep, 2010), including a very high number of researches about how to stop the invasion of the pathogen on these varieties (Kriaa et al., 2015), or how to make it tolerant to *Fol* infection (Król et al., 2015; Soudani et al., 2022)**.** Tomato (*Solanum lycopersicum L.*) has a major worldwide economic importance, as it ranks as the world's largest vegetable crop *Fol*lowing potatoes and sweet potatoes (Marchant, 2006). It is a key source of national agricultural income to attract foreign exchange to so many countries like Algeria (Abdesselem et al., 2016), , Egypt (Hassan et al., 2018), Thailand (Rosset et al., 1999), Turkey (Esengun et al., 2007), Spain (Larramendi et al., 2008) including more EU countries, China and USA (FAO, 2005).

In terms of health, studies indicate that the intake of tomatoes is linked to a lower risk of all-cause mortality and a reduced likelihood of developing certain types of cancer, including lung, prostate, stomach, cervical, breast, oral, colorectal, oesophageal, and pancreatic cancers (Bhowmik et al., 2012; Delian et al., 2017). Tomatoes also show effectiveness in preventing other diseases such as coronary heart disease and cerebrovascular disease (Li et al., 2021), as well as conditions like gastroesophageal reflux disease or heartburn, allergies, kidney and cardiovascular disorders (Bhowmik et al., 2012), body aches, arthritis, and urinary problems (Salehi et al., 2019). This health efficiency is attributed to the nutritional properties of tomatoes, as they are a rich source of essential minerals and vitamins, including vitamin C, potassium, *Fol*ic acid, and carotenoids such as lycopene (Delian et al., 2017). When tomatoes and tomato-based products are consumed, they contribute to the absorption of carotenoids and lycopenes in the human serum (Salehi et al., 2019). Lycopene, a key component, undergoes in the blood for better absorption and possesses the ability to inhibit adenosine deaminase, what makes tumor growth slow down. Additionally, tomatoes contain other beneficial compounds like neoxanthin, lutein, α-cryptoxanthin, α-carotene, β-carotene, cyclolycopene, and β-carotene 5, 6-epoxide. These components work together synergistically to provide health benefits, however further research is needed in this area (Agarwal & Rao, 2000; Perveen et al., 2015a; Przybylska & Tokarczyk, 2022). In plants, carotenoids are the pigments synthesized during fruit ripening and responsible for the final red color of the tomato (Perveen et al., 2015), they are also involved in photosynthesis and photoprotection (Tao et al., 2007), while lycopene has an antimicrobial effect against bacteria and fungi such as Candida albicans by inducing apoptosis via ROS production and mitochondrial dysfunction (Choi & Lee, 2015)

In science, Tomato serves as a commonly employed model crop for studying not only fruit development but also a range of physiological, cellular, biochemical, molecular, and genetic processes and its adaptability during cultivation in growth chambers or greenhouses (Schwarz et al., 2014). Due to their crucial significance, a complete sequence and assembly of the tomato genome was released on Sol Genomics Network (SGN): http://solgenomics.net/, also the https://www.tomatogenome.wur.nl/ database is employed to uncover and explore genetic variation in tomatoes, or to use in commercial breeding lines since tomatoes exhibit limited genetic diversity and the breeding focused on a narrow range of desirable agricultural traits, also considers old breeding material, which may serve as a source of intriguing alleles lost during domestication. A study has reported that the tomato genome size is approximately 950 Mb and this diploid plant has 12 chromosomes (Emmanuel & Levy , 2002; Okabe & Ariizumi, 2016; Salava et al., 2021).

Recent decoding efforts have elevated the tomato genome's status as one of the foremost vegetable crops boasting a high-quality reference genome (Seymour & Rose, 2022). This milestone has shed light on its genome's properties and unlocked novel avenues for targeted breeding of specific traits, fostering sustainable crop production practices (Seymour & Rose, 2022). Moreover, the abundance of genetic resources associated with tomato, including numerous accessions and phenotype databases, facilitates comprehensive research endeavors (Petit et al., 2021). Furthermore, research into genomic structural variation in tomato, particularly its impact on plant immunity, has been a focal point. Such structural variations encompass changes ranging from greater than 30 base pairs to several megabases (Ranjan et al., 2012).

Tomato serves as an exemplary model species for exploring fruit development due to its rich genetic resources, short life cycle, and ease of genetic transformation (Su et al., 2021). Tomato mutants have been valuable tools for genetic studies and breeding. However, only a few mutants have been characterized at the molecular level. Leveraging existing mutant resources and combining them with novel mapping approaches can accelerate tomato quality enhancement and yield improvement (Salava et al., 2021)

In conclusion, the tomato genome provides invaluable insights into diverse aspects of plant biology, breeding, and evolutionary processes, serving as a pivotal resource for advancing the realm of crop science. It has facilitated the analysis and identification of numerous gene families, encompassing both well-established and novel genes, including both housekeeping such as *β TUBULINE* and *β ACTIN*, and regulatory genes including *WRKY* (Huang et al., 2012), therefore numerous studies have been conducted on various aspects, such as its responses to biotic stress: the study of cellular function and gene induced in response of tomato to chlorosis virus (Kriaa et al., 2015; Pandey et al., 2023; Şahin-çevik et al., 2019), abiotic stress: and in tomato it was detected that it requires abscisic acid for histone 1 to be induced during water deficit (Bray et al., 1999), development (Chen & Bradford, 2000; Sitrit et al., 1999), and fruit ripening (Lang et al., 2017).

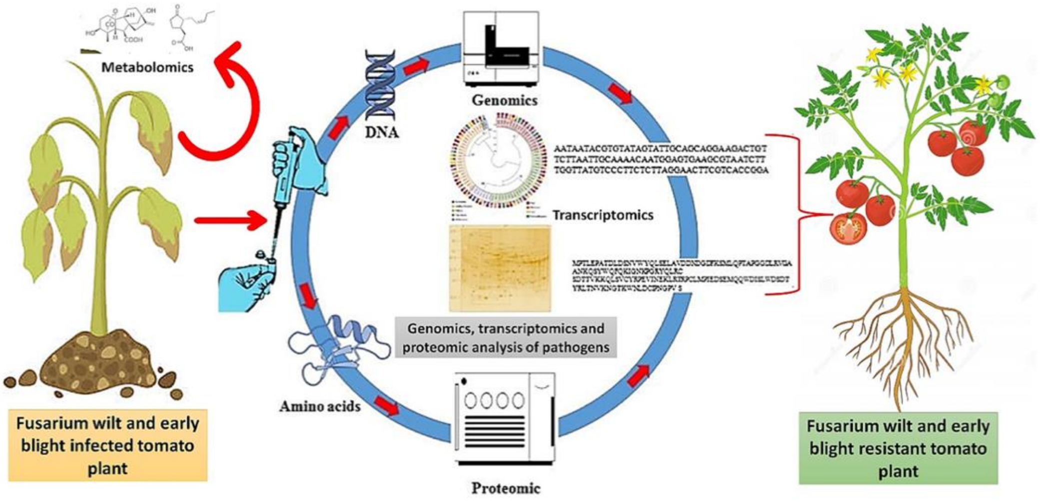


Figure 2: *Representative illustration of two molecular methods employed to enhance tomato's defense against phytopathogens (Pandey et al., 2023).*

I.3 The Use of Bioproducts as Pesticides:

Worldwide policies aiming to reduce reliance on toxic pesticides may encompass various strategies, it involves: the implementation of integrated pest management strategies (Bueno et al., 2021), taxation, and rigorous enforcement of regulations (Sapbamrer et al., 2023). The tightening of environmental regulations and policy frameworks within a Regulatory Framework is being applied such as the EU framework Regulation (EC) No 1107/2009 aiming to achieve sustainable use of plant protection products by promoting integrated pest management (Sustainable use of plant protection products—Publications Office of the EU, 2020) (Hu, 2020) and to reduce by 50% the use and risk of chemical pesticides by 2030 (Sustainable use of pesticides- Publication of the European commission, 2022). And European Food Safety Authority (https://www.efsa.europa.eu), whom their purpose is to maintain stringent standards and safeguard the safety and quality of food consumed by the public, and funding complex forms of social organization (Wells, 2016).

According to European Commission policy, the development of new strategies and natural sources of biopesticides is one of the challenges that must be addressed to diminish levels of pesticide residue in food and feed and the corresponding risks for human health. Consequently, there is a growing need to develop new environmentally friendly biopesticides, to achieve more sustainable production methods that meet consumer and societal expectations (Werrie et al. 2020) and are considered as excellent alternative to synthetic chemicals/molecule that are toxic and have a poor biodegradability (Prabha et al., 2016). The biopesticide potential of the millions of compounds acting directly to protect crops is growing exponentially in tandem with agricultural requirements around the globe (Montanarella & Panagos, 2021). These natural compounds will also help protect the aquatic and agroforestry environment, soil health and biodiversity.

Aromatic plants, studied for their fungistatic, insecticidal, larvicidal and bactericidal compounds, are currently one of the main sources of natural compounds in the biosphere (Andrés et al., 2012; Arraiza et al., 2018; Gonzalez-Coloma et al., 2012). In addition to these direct effects on plant pests and diseases, EOs can induce plant defenses (priming effects) resulting in better protection (Kesraoui et al., 2022). Properties of EO against a wide range of organisms have been well covered in *in vitro* assays, however, their mechanisms of action have scarcely been investigated (Raveau et al., 2020). The primed defence is mostly related to endogenous and exogenous signalling molecules that increases plant immune responses (Mustafa et al., 2019). The direct and indirect effects of essential oils for sustainable crop protection was more detailed in a review in collaboration with ICA-CSIC group (Kesraoui et al., 2022)

I.3.1 Direct Effects of *Artemisia absinthium* Essential Oil As Fungicides, Nematicides, Insecticides:

Previous studies have provided insights into the mechanisms of action of essential oils (EOs) in fungal cells (Duduk et al. 2015, Hong et al. 2015, Karimi et al. 2016, Parra Amin et al. 2021, Kalhoro et al. 2021, Li and Liu et al. 2021), as evidenced by experiments assessing cell wall alterations (Roques 2011, Scorzoni et al. 2017) or gene expression changes (Reuveni et al. 2020, Li and Liu et al. 2021, Mani-López et al. 2021). These actions include inhibiting fungal cell wall formation (Shahina al. 2018) and ergosterol biosynthesis, affecting mitochondrial function by inhibiting electron transport (Jensen-Pergakes et al. 1998, OuYang et al. 2016), and causing depolarization of mitochondrial membranes (Wu et al. 2006, Bakkali et al. 2008). This disruption in energy metabolism may slow down transcription and translation processes (Li and Liu et al. 2021). Furthermore, EO has been shown to reduce the minimum inhibitory concentration (MIC) of fluconazole and the expression of drug efflux pump genes (Keereedach et al. 2020).

Recent studies have investigated the potential of essential oils (EOs) and their constituents as commercial products for managing nematodes (Isman, 2020). EOs from various plant families have demonstrated promising activity against root-knot nematodes, including Meloidogyne spp. Understanding the mode of action of EOs is essential for effective nematode control (Isman, 2020). Reported neurotoxic effects on nematodes include inhibition of acetylcholine esterase (AChE) enzyme activity by low molecular weight terpenoids, although this bioactivity does not always correlate with in vivo toxicity in target insect species (Isman & Tak, 2017). Additionally, GABA and acetylcholinesterase inhibition have been observed (Andrés et al., 2012; Priestley et al., 2003). EO treatment has been shown to strongly suppress nematode egg hatching and juvenile infectivity (Andrés et al., 2017).

I.3.2 Indirect Effect of *Artemisia absinthium* Essential Oil (Epigenetic Effect of Bioproducts on The Plant):

The plant defense response is accompanied by an extensive transcriptional reprogramming of defense-related genes. The well-known processes which enable systemic acquired resistance (SAR), pathogen triggered immunity response (PTI) and enhanced triggered immunity (ETI) form part of the priming process in plants (Durrant & Dong, 2004; Macho & Zipfel, 2014; Nishimura & Dangl, 2014; Zipfel & Oldroyd, 2017).

French biologist Jean-Baptiste Lamarck proposed the theory of 'soft inheritance,' suggesting organisms could pass on acquired traits (Loison, 2018). However, Charles Darwin's theory of evolution by natural selection, published in 1859, rejected Lamarck's ideas (Galera, 2017). Gregor Johann Mendel later introduced the "Laws of Inheritance," supplanting the concept of acquired traits (Smith & Gericke, 2015).

Despite initial interest in Lamarckism, biologists of the time had limited understanding of molecular mechanisms underlying trait inheritance (Hackenberg & Mandrioli, 2023). Integration of Darwin's theory with advancements in genetics led to the development of neo-Darwinian evolution theory (Danchin et al., 2019).

Today, evolution is understood through genetics and mutations, which create genetic variation affecting traits. Most evolutionary biology models propose changes in nucleotide sequences as the primary molecular mechanism behind heritable phenotypic changes (Kumar et al., 2017).

The term epigenesis was first used by Conrad Waddington explaining that it’s the process by which genotypes lead to phenotypes in the course of development (Waddington, 1957). The term epigenetic was defined later by Arthur Riggs and his colleagues explain that it’s the study of mitotically and/or meiotically heritable changes in gene expression that occur without changes in the DNA sequence (Russo., Martienssen., & Riggs 1996; Shaikh et al., 2022).

Epigenetic marks allow to show the start and the end of genes: including **histone modification**, **DNA,** and **RNA modification**, they do not alter the DNA sequence, as mentioned in Riggs definition previously, but instead allow or silence gene activity and the subsequent production of proteins that guide the growth and development of an organism, direct and maintain cell identity (Fessele & Wright, 2018; Gibney & Nolan, 2010; Haider et al., 2021; Korochkin, 2006; Liang et al., 2020).

In the case of **DNA modification**, the identification and thorough examination of 6mA DNA modification as a novel epigenetic marker have been recent focal points in research (Liang et al., 2020; Ratel et al., 2006). . DNA methylation occurs when a methyl group is introduced to cytosine or adenosine within the CpG dinucleotide—a sequence where cytosine is *Fol*lowed by guanine and connected by a phosphate present in genomic DNA (gDNA) across prokaryotes, archaea, and eukaryotes (He et al., 2020). While this modification is infrequent in CpG islands and intergenic regions, it is more commonly observed in repetitive elements elements (Boyko

& Kovalchuk, 2013) The enzymatic establishment of DNA methylation, orchestrated by entities often referred to as "writers," predominantly involves DNA methyltransferases (DNMTs) (Shaikh et al., 2022). This process creates a resilient epigenetic mark, as during each cell division, DNMT1 faithfully replicates the methylation pattern onto the newly formed daughter DNA (Huh & Rim, 2013; Law & Jacobsen, 2010). DNA methylation in CpG islands initiates gene silencing through dual mechanisms: first, it is associated with the development of a repressive chromatin structure; second, it hinders the binding of transcription factors, effectively curtailing gene expression (Huh & Rim, 2013).

RNA modification happens in the non-coding RNAs that are composed of three different classes: endogenous small interfering RNAs (endo-siRNAs), PIWI-associated RNAs (piRNAs), and long non-coding RNAs (Yang et al., 2016).

The best-known function for miRNAs is the repression of gene expression by either mRNA destabilization and/or inhibition of translation. The miRNA sequence confers specificity to recognize particular mRNAs. In most cases, the 3'UTR of the target mRNAs is bound by the miRNA through imperfect complementary base pairing (Yang et al., 2016). While miRNAs are processed from long, single- stranded transcripts to form a short hairpin structure, small interfering RNAs (siRNAs) are generated from long, perfectly complementary, double-stranded RNAs and function as an additional level of gene regulation and as a cellular defence mechanism against foreign and deleterious nucleic acids such as virus RNA and transposable elements (Yang et al., 2016).

Long ncRNAs (lncRNAs) are a group of diverse heterogeneous ncRNAs that are longer than 200 nucleotides. LncRNAs have a wide range of functions, including the regulation of gene expression in cis and in trans, the regulation of epigenetic chromatin modification, post-transcriptional processes as well as structural functions (Yang et al., 2016).

PIWI-piRNA complexes silence Transposable elements both at the transcriptional level – by attracting repressive chromatin modifications to genomic targets – and at the post transcriptional level – by cleaving TE transcripts in the cytoplasm. Impairment of the piRNA pathway leads to overexpression of TEs, significantly compromised genome structure (Tóth et al., 2016).

Over 160 varieties **of RNA modifications** have been uncovered, with a majority occurring in transfer RNA (tRNA) and ribosomal RNA (rRNA). The initial discovery of mRNA modification dates back to m6A, which was first observed in mammals and subsequently identified in various eukaryotic organisms, including plants (Liang et al., 2020). This modification assumes crucial roles in regulating gene expression, encompassing both transcriptional and post-transcriptional processes. m6A, characterized as a reversible modification, undergoes installation, removal, and recognition by methyltransferases (writers), demethylases (erasers), and m6A-binding proteins (readers), respectively (Tang et al., 2023).

While the literature elucidating the impact of **histone modifications** on transcription is intricate and continually evolving, three overarching principles are believed to be implicated: (i) Post-translational modifications (PTMs) exert a direct influence on chromatin structure, modulating its higher-order conformation and thereby operating in cis to regulate transcription; (ii) PTMs disrupt the binding of proteins that interact with chromatin (trans effect); (iii) PTMs draw specific effector proteins to the chromatin (trans effect) (Gibney & Nolan, 2010).

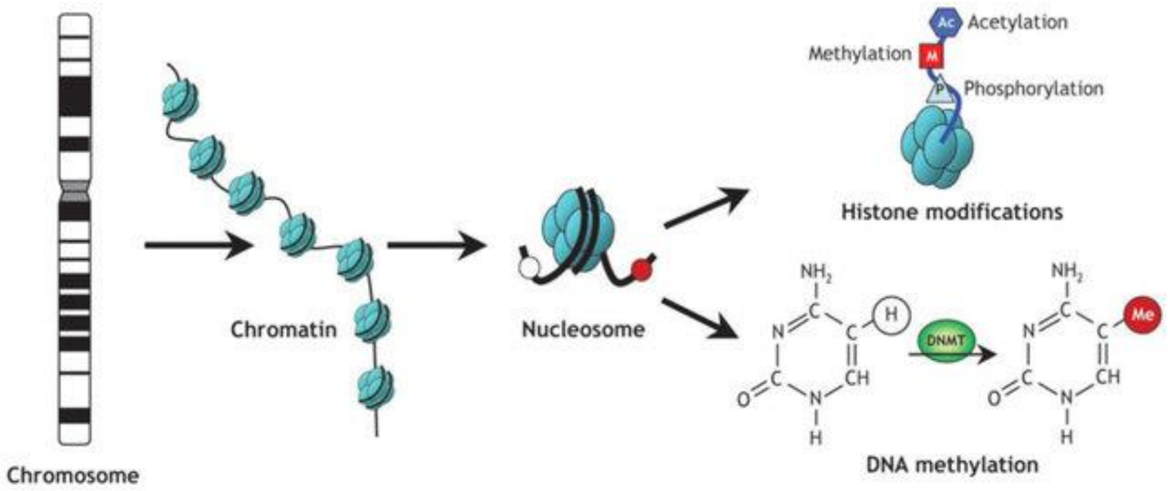


Figure 3: *Schematic of epigenetic modifications on histone and DNA (Fessele & Wright, 2018).*

The regulation of **stress memory in plants** may involve not only DNA methylation but also the post-translational modification of histones (HPTMs), along with the positioning and spacing of nucleosomes. These factors collectively influence the overall packaging and accessibility of individual regulatory elements (Gallusci et al., 2023; Mladenov et al., 2021). Numerous efforts have been undertaken to enhance stress tolerance by inducing stress memory over time(Rani et al., 2021). One promising approach involves activating priming responses and targeted epigenetic modifications of the plant's genome. The priming stimulus serves as the initiator, triggering defense priming and establishing a persistently enhanced state of plant defense readiness (Espinas et al., 2016; Martinez-Medina et al., 2016; Mladenov et al., 2021). Early to date, few studies have been conducted on the transcriptomic and metabolomic responses of tomato seeds coated with essential oil to combat phytopathogens or on the assumed "*de novo*" molecular synthesis of immunity-related compounds for priming effect of such treatment (Ben-Jabeur et al., 2015; Kaplan et al., 2006; Rocha et al., 2019).

In this thesis, the understanding of how coating tomato seeds with essential oils enhances their response to combat phytopathogens was expanded. Additionally, epigenetic effects of essential oils on plants were investigated.

I.4 Seed Priming, Technic of Treatment

Priming, traditionally utilized to facilitate synchronized seedling growth, seed germination, enhance seedling vigor, and optimize physiological performance to establish a stable crop stand, has recently emerged as a potent tool for promoting sustainable agriculture. This method has proven effective in mitigating various abiotic stresses, including salinity (Kahveci et al., 2021), drought (Klein et al., 2017), cold (Dahal & Bradford, 1990), and heavy metal stresses (Lutts et al., 2016). Additionally, it enhances plants' resistance to biotic stresses while simultaneously promoting the overall growth of crop plants (Dahal & Bradford, 1990; Delian et al., 2017; Paul et al., 2022) .

- Effect of Priming on The Mechanisms of The Plant:

The cellular changes induced by priming remain unclear, but studies have explored alterations at biochemical, physiological, and molecular levels. Modern omics tools have contributed to understanding some of the mechanisms behind priming (Paul et al., 2022): **Aquaporins (AQPs)** are widespread water-channel proteins facilitating rapid and passive transcellular and intracellular water transport in plants. Gene expression in spinach during seed osmopriming and post-priming indicate an up-regulation within 2 - 4 days of priming, researchers hypothesize that this increase aims to facilitate pressure potential-driven cell expansion and enhance the germination potential of primed seeds (Chen et al., 2013). **The accumulation of non-toxic compatible solutes** such as proline, and sugars which align with plant metabolism serves as a crucial mechanism for salt tolerance in plants. This process facilitates a reduction in cell osmotic potential, enabling the plant to undergo osmotic adjustment in response to stress conditions (de Oliveira et al., 2011; Ibrahim, 2016).

Priming can stimulate the activities of cell wall hydrolases, as detailed in the review by (Swaminathan et al., 2022). Specific immune signalling pathways are activated to counteract biotic or abiotic forces, including cascades dedicated to strengthening the cell wall structure.

Priming tightly regulates chromatin modification, activate DNA repair DNA methylation and histone modifications that are closely linked to chromatin reconfiguration, in plant adaptation to different biotic stresses (Espinas et al., 2016), **enhance ROS during priming, Priming memory phenomena,** Plants have developed adaptive mechanisms to protect themselves against the harmful effects of repeated stress events, one of these mechanisms involves the ability to remember previous stress exposures, which is referred to as "priming" (Haider et al., 2021). Plants activate signalling pathways upon recognizing pathogens, triggering defense reactions. Increasing evidence suggests that epigenetic mechanisms play a direct role in the establishment of plant immune memory (Hilker & Schmülling, 2019; Ramirez-Prado et al., 2018). Priming is “the physiological state that enables cells to respond to very low levels of a stimulus in a more rapid and robust manner than non-primed cells”. A transgenerational and non-transgenerational or short term-inheritance of defense-related priming, improving the stability of this priming effect, has recently been shown in plants (Gully et al., 2019; Molinier et al., 2006; Pastor et al., 2013)

- Technics Used in Priming:

Seed priming involves a pre-sowing treatment wherein seeds undergo treatments based on imbibition, enabling them to enter the initial, reversible stage of germination without allowing radical protrusion through the seed coat (Lutts et al., 2016). Thus, there is several methods of priming that has been developed during its use:

Hydro-priming involves soaking the seeds in water before sowing and may or may not be *Fol*lowed by air-drying of the seeds (Nawaz et al., 2013). This approach proves effective against harmful fungi, enhances seed quality (Mustafa et al., 2019), ensures quick and synchronized germination, and promotes better seedling growth (Paul et al., 2022). The absence of additional chemical agents makes this method cost-effective and environmentally friendly (Lutts et al., 2016).

Halo priming involves using salt solutions such as NaCl, KCl, KNO_3, K_3PO_4, KH_2PO_4, $MgSO_4$, and $CaCl_2$ (Lutts et al., 2016). Studies have demonstrated that seedlings from primed seeds exhibit higher dry weight and total chlorophyll content, lower lipid peroxidase levels, increased activity of glucocorticoid receptors regulating development, metabolism, and immune response under osmotic stress, as well as reduced total phenolics and increased accumulation of proline, glycine betaine, and total soluble sugars (Srivastava et al., 2010). Further mechanisms are elucidated in detail in (Wojtyla et al., 2016).

Osmo priming is a pre-sowing treatment where seeds are soaked in osmotic solutions like polyethylene glycol (PEG), mannitol, sorbitol, and glycerol. This initiates the first stage of germination without radicle protrusion. The osmopriming process involves exposing seeds to low-water-potential solutions (Lei et al., 2021; Lutts et al., 2016). It induces the upregulation of aquaporins (Paul et al., 2022) and enhances seed germination under aging and salinity conditions by promoting a more robust antioxidant system (Delian et al., 2017; Lei et al., 2021).

Solid matrix priming, designed to regulate seed water uptake, emerges as a viable alternative to osmopriming due to the expensive nature of osmotic agents and challenges associated with aeration (Lutts et al., 2016; Mustafa et al., 2019). This approach not only improves field performance but also enhances germination, seed vigor, seedling emergence, and growth in both optimal and low-temperature environments (Lutts et al., 2016). The decline in the activity of superoxide dismutase (SOD) and catalase (CAT), recognized as key enzymes in preserving seed longevity during storage, has been identified (Wojtyla et al., 2016).

Different seed priming methods can be used for enhancing disease resistance in plants. Biopriming and hormonal priming are the most appropriate and effective one (Mustafa et al., 2019).

Hormonal priming involves the pre-treatment of seeds with various hormones, such as salicylic acid, ascorbate, kinetin, and others, aiming to enhance the growth and development of seedlings (Nawaz et al., 2013). Gibberellic acid, when used in hormonal priming, induces favorable metabolic reactions in plant seeds, improving seed germination performance and seedling establishment in saline conditions (Ibrahim, 2016). Methyl jasmonate, employed in priming, mitigates the adverse effects of PEG stress on seed germination and seedling growth, also enhancing resistance to biotic stress (Król et al., 2015; Sheteiwy et al., 2018). Additionally, salicylic acid seed priming has been shown to enhance seedling emergence, root and shoot length, seedling fresh and dry weights, as well as leaf and root scores under optimal and chilling temperatures. This improvement is attributed to the activation of antioxidants, maintenance of high tissue water contents, and reduction in membrane permeability of the seeds (Farooq et al., 2008).

Biopriming is the technique that this thesis is focused on, where natural products are used to improve seed resistance to stress. This method involves generally seed imbibition in antagonistic microbes (Singh et al., 2021) or by enhancing microorganisms by priming the seed with beneficial microorganisms (Mustafa et al., 2019). Essential oils (EOs) can also act as priming molecules both in biotic and abiotic plant stress response (Arraiza et al., 2018; Bertrand et al., 2021; Kesraoui et al., 2022). They can be an effective and sustainable tool to control seedborne diseases (Klein et al., 2017; Spadaro et al., 2017).

An alternative strategy for enhancing plant resistance against pathogens involves identifying species that possess genetic material resistant to specific phytopathogens. This brings us to the second objective of this PhD study, which centers on investigating the resistance of *Quercus suber* (*Qs*) to *Phytophthora cinnamomi* Rands (*Pc*) infection. In 2019, the National Program for the Conservation and Improvement of Genetic Resources of Holm Oak and Cork Oak was initiated by the "Genetic Improvement and Physiology" subgroup of the Drought Working Group, under the Ministry for Ecological Transition and Demographic Challenge. This program aims to identify naturally tolerant individuals to *Pc* for the preservation of genetic materials. These selections were made based on careful considerations from previous research projects(Cuenca et al., 2022). UPM's involvement in this program began with a contract established with TRAGSA, in which our group participated. Our objective was to optimize a methodology for studying the *in vitro* resistance of *Qs*. to *Pc*.

I.5 *Phytophthora cinnamomi* Rands

Phytophthora cinnamomi, also known as "cinnamon fungus" (Wilson et al., 2003), is a soil-borne water pathogen that has the potential to infect over 3000 plant species, including economically and ecologically significant evergreen oaks such as *Qs*. It was discovered in Sumatra on cinnamon trees in 1922 by Rands, it has spread to or been found in over 70 countries all over the world (Zentmyer, 1985).

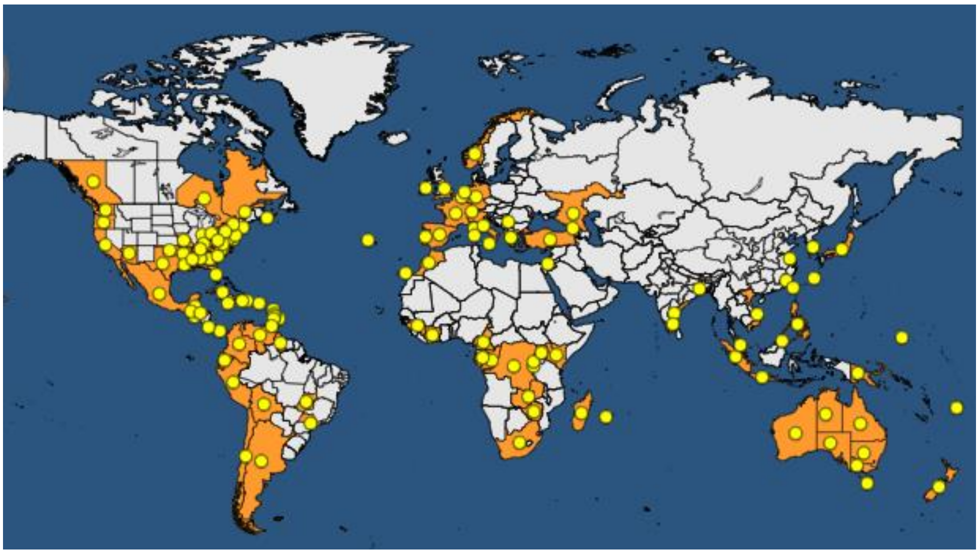


Figure 4: *Map showing distribution of Phytophthora cinnamomi world-wide, corresponding to EPPO (Updated: 29.06.2023).*

This pathogen is implicated in the observed decline of these oak species in the Iberian Peninsula over recent decades because once this organism infiltrates the soil or water, it spreads like wildfire throughout its environment. The decline is facilitated by *Pc's* secretion of effector molecules like elicitins, which enhance disease effectiveness during host colonization (Coelho et al., 2021). This pathogen can proliferate in the soil, generating asexual, biflagellate motile zoospores. These zoospores are attracted to fine roots of susceptible plants, where they attach and develop ramifying hyphae (Horta et al., 2008). *Pc* infects a diverse range of hosts, ranging from acacia through great numbers of primarily woody plants, including chestnut, pineapple, eucalyptus (over 100 species), several species of pine, many ornamental plants including azalea, camellia, rhododendron, and various conifers (Zentmyer, 1985). Symptoms include wilting, decreased fruit size, collar rot, gum exudation, necrosis, leaf chlorosis, leaf curl, and stem cankers, it can even mess with the transpiration process from roots to shoots (Coelho et al., 2006; Engelbrecht et al., 2021; Ruiz-Gómez et al., 2019; Vivas et al., 2021). Upon the initial identification of *Pc*, it was initially classified within the fungi kingdom due to its resemblance to "true fungi" in terms of hyphal growth polarization, the presence of vegetative spores adapted for dispersion by wind or water, and its utilization of specific infection strategies. In contrast to authentic fungi, this soil-borne pathogen predominantly undergoes its life cycle in a diploid state, features cell walls composed of cellulose and β-glucans instead of chitin and produces biflagellated zoospores. Another deviation from typical fungal characteristics is its inability to synthesize sterols, making it resistant to polygenic antibiotics like pimaricin. However, *Pc* does require sterols for sporulation (Boughanmi et al., 2022; Chen & Zentmyer, 1970; Ruiz-Gómez et al., 2019).

In studies conducted by (Engelbrecht et al., 2021), a high-quality reference genome sequence and annotation for *Pc* were generated using transcript evidence from RNA-Seq data. The assembly revealed a larger genome size for *Pc* than previously estimated from draft versions what facilitated the identification and characterization of various pathogenicity-related genes. In planta RNA expression analysis identified several up-regulated pathogenicity genes during infection, crucial for future functional characterization studies (Reitmann et al., 2017).

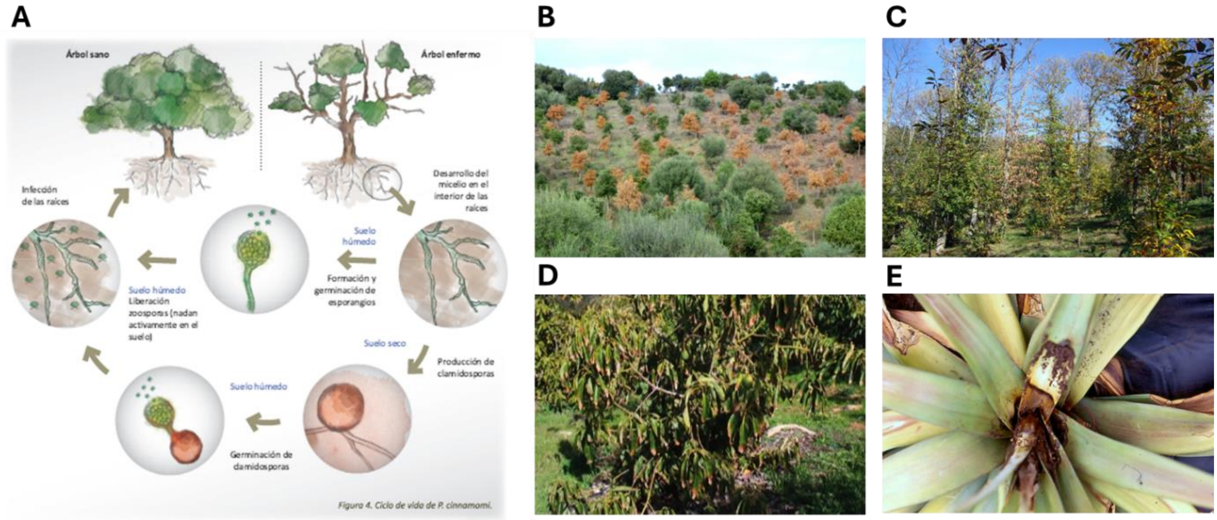


Figure 5: *Phytophthora cinnamomi life cycle (Source: Instituto de Investigación y Formación Agraria y Pesquera), and Symptoms of Host Plant Infection: (B) Quercus suber, (C) Castanea sativa, (D) Avocado, (E) Ananas comosus. Photo sources: B.Scanu and A.Francesch.*

Our study is focused on optimizing a protocol for inoculating *Qs* with *Pc*, focusing on both practical aspects and molecular insights. By refining inoculation techniques and experimental conditions, reproducibility and accuracy in disease induction experiments have been improved. Concurrently, molecular analyses have provided valuable insights into the underlying mechanisms of host-pathogen interactions, elucidating key pathways and molecular markers associated with the response of *Q. suber* to *Pc* infection. The optimization of inoculation protocols coupled with molecular insights offers promising avenues for improving disease management strategies and enhancing the resilience of cork oak forests against *Pc*.

I.6 *Quercus suber*:

The cork oak is not only economically significant but also plays a vital role in maintaining ecological balance and supporting diverse ecosystems. It is an enduring evergreen tree with a remarkable lifespan of over 200 years belonging to the family Fagaceae. Indigenous to Mediterranean countries, cork oak covers about 2.35 million hectares, primarily in Portugal (676,000 ha), Spain (500,000 ha), Algeria (480,000 ha), and Morocco (400,000 ha) (Burgarella et al., 2009; Latifa Belhoucine, 2011; Petroselli et al., 2013; Touhami et al., 2020; Vágner et al., 2005; Vieitez et al., 2012).

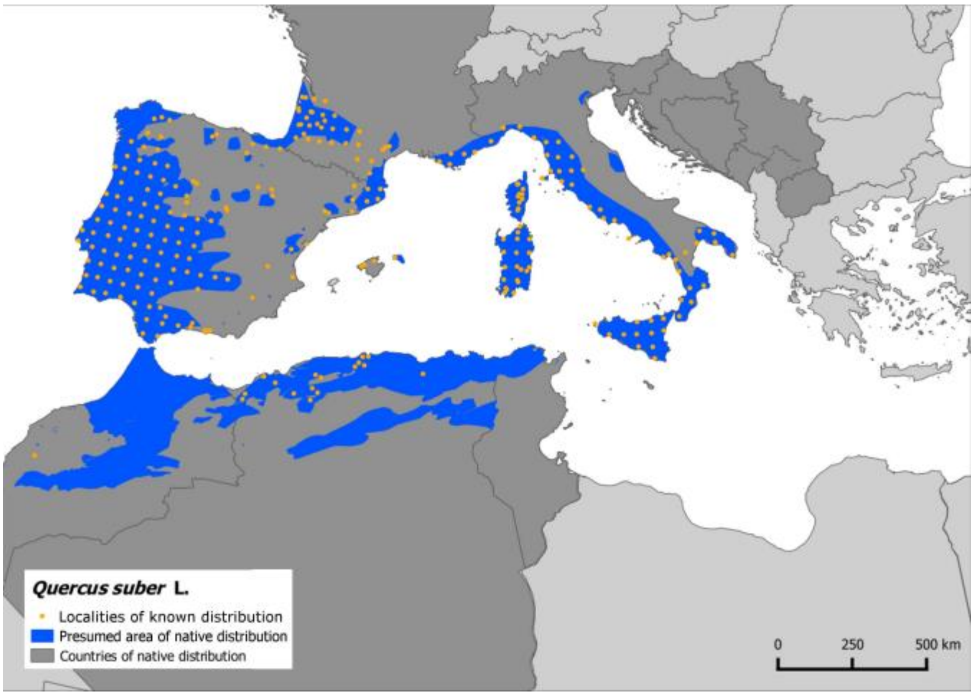


Figure 6*: Distribution map of Quercus suber (source: FAO 2015)*

The cork trees find application in numerous diverse fields. It is a deciduous tree providing raw material to produce lumber, cork, silicone, and charcoal, moreover, used for soil and water conservation, especially for the afforestation of difficult site conditions, thus playing an important role in developing the local economy and protecting ecological balance (Pang et al., 2021; Saiz-Fernández et al., 2022; Wei et al., 2020). Cosmetic enterprises started being interested in this species since consumers are more caring about environmental issues in response to this demand, the cosmetic industry is actively adopting practices that align with environmental consciousness (Mota et al., 2022). Cork's remarkable impermeability to liquids and its efficacy as an insulator for heat, sound, and vibration are well-documented (Gil, 2015). Additionally, cork oaks are prized for their drought tolerance and their ability to thrive in low soil quality conditions, making them crucial in the fight against desertification (Karam et al., 2011). In Spain, the oak woodlands of la Dehesa serve as the ideal environment for fattening Iberian pigs on acorns, a tradition that yields high-quality jamón, or air-dried ham (Huntsinger et al., 2013).

Significant progress has been made for obtaining specific oak species tolerant to diseases, particularly in the area of clonal propagation via organogenesis and somatic embryogenesis (SE) that is also considered the best regeneration method for producing "*in vitro*" propagation of tolerant individuals (Cuenca et al., 2022) as well as transgenic plants in hardwood species as the regeneration capacity is higher (Romano & Martins-Loucao, 1992; Serrazina et al., 2022; Vieitez et al., 2012).

In this PhD, two distinct approaches were employed to select *Quercus*-tolerant explants, obtained through different methods. Both types of plant material were acquired in collaboration with other research groups. One approach involved obtaining pre-selected clones of *Qs* through "*in vitro*" micropropagation and subsequent selection based on their characteristics in greenhouses. These clones were obtained from axillary buds (containing endophytes).

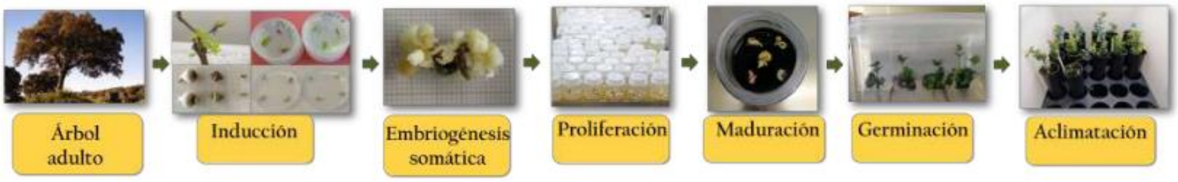


***Figure 7**: Induction process of somatic embryogenesis and production of somatic seedlings in adult cork oaks (Cuenca Valera et al., 2022).*

The other approach involved the micropropagation of somatic embryos (Martínez et al., 2019), where the resistance to *Pc* of transgenic explants obtained through biotechnological transformation approaches was analysed, similar to previous methods used by this group of researchers (Serrazina et al., 2022). Specifically, those lines were selected because they expressed higher levels of chestnut antimicrobials than the wild types, which were previously identified to be involved in plant resistance in chestnut trees (Serrazina et al., Under review).

I.7 Epigenetic Effect of *Phytophthora cinnamomi* on *Quercus suber*:

In *Q. suber*, there's insufficient data on the genes encoding chromatin regulators. To address this gap, (Silva et al., 2020) and (Alves et al., 2022) conducted sequence-based searches and phylogenetic analyses to identify enzymes potentially involved in histone and DNA modifications, thus contributing to the deposition of epigenetic marks. (Silva et al., 2020) identified eight DNA methyltransferases (DNMTs) and three DNA demethylases (DDMEs). Additionally, histone modifiers responsible for methylation (35), demethylation (26), acetylation (8), and deacetylation (22) were also identified. In the study of (Alves et al., 2022), focusing on Fagaceae proteins, orthologs of DNMTs, DDMEs, HATs, HDACs, HMTs, and HDMTs were sought across *Fagus*, *Quercus*, and *Castanea genera*. Utilizing blast searches in available genomes and RNA-seq data, homologs of seven DNMTs, three DDMEs, six HATs, 11 HDACs, 32 HMTs, and 21 HDMTs proteins were identified. The insights gained from this research lay the foundation for future investigations into the epigenetic regulation within the economically significant Fagaceae family.

Studies conducted by (Rodríguez-Sanz et al., 2014) detected early epigenetic markers in both embryogenesis pathways from microspores and immature zygotic embryos in cork oak. Other group characterized the epigenetic and hormonal profile during maturation of somatic embryos in *Qs* by analysing global DNA methylation and abscisic acid, revealing a decrease in 5-methyl-deoxycytidine as embryos matured, while ABA peaked during the maturation phase. Cold stratification induced germination ability, coinciding with reduced 5-mdC and ABA content. Immunohistochemical analyses revealed spatial-temporal regulation during embryogenesis, highlighting the role of ABA and epigenetic control in somatic embryo maturation and germination (Pérez et al., 2015). Additional studies turned interest to the differential DNA methylation patterns that are related to phellogen origin and quality of *Qs* cork, they discovered the potential role of cytosine methylation in the modulation of differential phellogen activity either involved in localized cell death or in pore production, resulting in different cork qualities (Inácio et al., 2017). The distribution of epigenetic marks for histone modifications at lysine residues H3 and H4, along with DNA methylation, was analysed in the nuclei of mature pollen cells of the Angiosperm tree *Qs*. The analysis revealed a high level of silent epigenetic marks (5-mC and H3K9me2) on the chromatin of the generative nucleus, with a prevalence of active marks (H3K9me3 and H4Kac) in the vegetative nucleus. These findings provide insight into the species' pollination/fertilization timing strategy (Ribeiro et al., 2009).

In examining the epigenetic impact of *Pc* infection in *Qs*, limited research has been conducted on this specific subject to date. While a few studies have explored the response of *Qs* when infected with *Pc*, there remains a notable scarcity of investigations addressing the epigenetic aspects of this interaction. Therefore, the assays and results obtained in this thesis contribute to the characterization of marker genes associated with *Qs* resistance to *Pc* and investigate the transcriptional modifications of epigenetic-related genes occurring in *Qs* in response to it.

II Material and Methodology

II.1 Biological Material:

Fusarium oxysporum was obtained from CECT (# 2715), Valencia, Spain. The fungus was cultivated on potato dextrose broth medium (PDB) at 28°C for 8 days in darkness (Berrocal-Lobo et al. 2002). The spores were collected in sterile water, filtered through three slides of a sterile gauze pad, quantified using a Neubauer chamber (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), and stored in 20% (v/v) glycerol at -80°C until use.

Phytophthora cinnamomi Rands (*Pc*) was kindly supplied by Beatríz Cuenca (TRAGSA), obtained from Solla's team from the Faculty of Forestry, Institute for Dehesa Research (INDEHESA), Universidad de Extremadura, Plasencia, Spain and obtained from the Center for Research and technology of Extremadura (CICYTEX), located in Mérida, Spain. The protocol was inspired from (Byrt & Grant, 1979) and developed in the lab as explained in the paper of (Del Castillo-gonzález et al., 2024) where 6 steps where *Fol*lowed, here explained briefly:

1. Growth of inoculum in a potato dextrose agar medium (PDA), using a sterile Miracloth disk watered with 1 mL of water, the plate is incubated at 24ºC in dark, allowing observation of mycelium development on the Miracloth over several days;
2. Growth of hyphae in liquid culture, the Miracloth is transferred in a medium consisting of a fresh V8 juice 100 mL/L, 2 g/L of calcium carbonate, .02 g of β -sitosterol dissolved in 5 mL of ethanol, 15 g/L of Difco agar. The pH was adjusted to 6.5. HEPES at 0.1 M was added to the medium and adjusted to the desired volume with distilled water, and autoclaved. The plates were incubated in darkness at 24 °C for 14 days;
3. Sporangium induction, the hyphae is transferred *Fol*lowing the protocol of (A. R. Hardham et al., 1991) to an Erlenmeyer flask containing a 10% clarified V8 liquid medium as described previously but without agar, the solution was filtered and autoclaved. The Erlenmeyer was incubated in an orbital shaker (100 rpm) at 24 °C, under fluorescence light, for 48 h to induce sporangial production;
4. Step 4: sporangium induction in mineral salt solution, consisting of 0.01 M of calcium nitrate (CaN_2O_6), 0.005 M of potassium nitrate (KNO_3) and 0.004 M of magnesium sulphate heptahydrate ($MgSO_4$ $7H_2O$), it was autoclaved then enriched with 1 mL/L of a chelated iron solution (13.05 g/L of Ethylene-dinitrile-acetic acid mixed with 7.5 g/L of potassium hydroxide (KOH), and 24.9 g/L of iron sulphate (II), and heptahydrate ($FeSO_4$ $7H_2O$, 99%), it was filtered with a 0.22-μm diameter Millipore filter). The culture was incubated for 24h at 24 °C with fluorescence light in an orbital shaker at 100 rpm.
5. Induction of zoospores, the disc containing sporangia was transferred to a 50-mL Falcon tube containing 20 mL of sterile 4 °C free nuclease water for molecular biology. The tube was then placed in an orbital shaker and kept at 100 rpm at 4 °C for 1.5 h and in absence of light.
6. The resulting solution was filtered through three slides of a sterilized gauze pad, and quantification of the zoospores was performed in a Neubauer chamber.

II.2 Plant Material:

In the chapter 1, at the outset of the study, two essential oils were evaluated for their toxic effects on three different pathogens. The primary objective was to identify the most effective essential oil in terms of toxicity towards these pathogens to facilitate the selection of the most promising essential oil to be further investigated in subsequent research involving plants.

The flowering plants of *Artemisia absinthium* var. Candial were harvested in 2019 and their essential oil (AEO) was extracted by vapor pressure in a semi-industrial stainless steel plant equipped with two 3,000 L vessels, as described by (Julio et al., 2015). AEO was provided by el Instituto de Ciencias Agrarias (ICA)- Consejo Superior de Investigaciones Científicas (CSIC) group.

Tomato seeds (*Solanum lycopersicum* L., var. Marmande) were provided by Ramiro Arnedo S.A. (La Rioja, Spain). The dried seeds were stored and maintained at a temperature of 4°C until they were ready for use.

In the chapter 2- part 1, the clones were obtained from axillary buds *in vitro* coming from different origins including University of Valencia, IMIDRA (Dra. Mar Ruíz Galea laboratory), IIAG-CSIC y TRAGSA (Beatríz Cuenca Laboratory, Palencia), from the project “Development of an *in vitro* test for the evaluation of resistance to *Pc* in holm oak and cork oak genotypes through Micropropagation” (Ref. TSA0069316. Entity: Ministry of Agriculture, Fisheries, and Food, Subdirectorate General of Forest Policy. National Program for Conservation and Improvement of Genetic Resources of holm oak and cork oak against the Oak Decline Syndrome. FEADES. Manager: TRAGSA).

Figure 8: *Origin of Quercus suber plant material.*

The chapter 2- part 2 was realised in collaboration with BioISI-Biosystems & Integrative Sciences Institute, Universidade de Lisboa and Sede Santiago de Compostela, Consejo Superior de Investigaciones Científicas (MBG-CSIC) with Dra. Elena Corredoira laboratory. For the genetic transformation, three embryogenic lines induced from leaves derived from centenary trees were used (Hernández et al. 2003). These embryogenic lines have been maintained by secondary embryogenesis, with subcultures every 6 weeks, in proliferation medium (Serrazina, Corredoira et al, Under review).

II.3 Plant Growth Conditions :

In chapter 1, tomato seeds were subjected to water stratification at 4ºC one day prior their use. The *Fol*lowing day, ten seeds per well were placed on sterile filter paper in a 12-well plate, and

500 µl of sterile distilled water was added. The plate was then placed in an Aralab chamber (Lisbon, Portugal) under controlled environmental conditions, including a 50% humidity level, a temperature of 24°C during the day and 18°C at night, a 16-hour light/8-hour dark photoperiod, and a constant light intensity of 150 mE/m^2 per second for all experiments. After 5 days of growth, an additional 500 µl of water was added to the seedlings to prevent dry stress.

In the chapter 1, Part 1, tomato was subjected to different types of kinetic, sharing a common objective: Comprehending the changes associated with epigenetic occurring in tomatoes during its development. Time points selected during the short term kinetic are: “after 12 hours in cold-hydropriming; time 0 in water exposed to light, then after 30 minutes, 1, 3, 6, 12 and 24 hours”. And Time points selected during the long term kinetic are: “time 0 exposed to light and in water, then at 1, 2, 3, 7 and 12 days". Additionally, a kinetic was realised during cold hydro-priming, where dry tomato seeds were subjected to hydropriming at 4ºC in the dark. The weight of seeds used per Falcon tube was 0.7g (equivalent to 240 seeds), with the addition of 50 mL of Type I water for molecular biology obtained using a filter machine (Aristerra 2011 S.L., Pamplona, Spain). Samples were collected at: “dry state, time 0 in water and then 2, 4, 8, 10 and 12 hours”.

In chapter 2, the explants of *Qs* are initially received in agar medium inside a culture tube, they are incubated in an Aralab chamber in the same conditions of tomato’s growth (explained previously). They are acclimated to their new environment for a week, and then transferred to new sterile culture tubes (DeWit tubes) containing 1 mL of sterile water and maintained for an additional two days for further acclimation before proceeding to the inoculation step with *Pc* zoospores.

II.4 Coating of tomato Seeds with *Artemisia absinthium* Essential Oil:

The seeds are pre-germinated in water at 4°C overnight. A treatment of AEO at 10 mg/mL of ethanol is prepared, *Fol*lowed by a dilution to 0.5 mg/mL. Another ependorph is prepared with 5% ethanol for the controls, which is the percent of ethanol that AEO treatment has. Each seed is immersed in 0.5 mg/mL of AEO for 1 second and then placed on the aluminum foil (15x10 cm) to dry in the laminar flow cabinet. Same process for the control seeds, using the 5% ethanol treatment. Plates with filter paper in the bottom of each pot is prepared. When the seeds are dry, 10 seeds per pot are placed for each treatment (water or pre-treated with AEO), and the plates inside the hydroponic system, previously sterilized and moistened, are placed in the growth chambers.

II.5 Coating of Tomato Seeds with Hormones to Improve Germination:

Tomato seeds underwent the same coating technique employed for essential oils. This process entailed immersing the seeds in hormone solutions and subsequently drying them on aluminum foil. The hormones and their respective concentrations used were as *Fol*lows: Salicylic acid (0.025 and 0.1 mM), Methyl Jasmonate Acid (0.01, 0.1, 0.25, and 2.5 mM), and 1-aminocyclopropane-1-carboxylic acid (0.25, 10 uM). *Fol*lowing drying, the seeds were placed in plates inside the hydroponic system, previously sterilized and moistened and transferred to a growth chamber with controlled environmental conditions (outlined in Section II.3).

II.6 Coating Tomato Seeds with Pathogen Extracts:

Mycelium of two pathogens were grown in plates with Potato dextrose agar previously for 14 days. The mycelium is then extracted, filtered carefully and freshly used. This protocol cannot be detailed due to its use for future industrial purposes.

Figure 9: *Steps prior initiating the experiment: dry after coating, placing coated seeds in the plates, put to grow in the chambers.*

II.7 Inoculation of Coated Tomato Seeds with *Artemisia absinthium* Essential Oil on Soil:

The seeds are coated with AEO as described previously. The soil mixture contains vermiculite and soil in a 1:3 ratio, respectively. Four seeds per pot are placed on the soil surface and watered with 150 mL, evenly distributed among all the pots, while 1 litter is added to the bottom of the plate containing the pots. Each seed is individually inoculated with 10^6 spores/mL. During the first days of germination, the plate is covered with film before being sprayed with distilled water to maintain stable watered conditions and humidity, as tomatoes require a significant amount of moisture during germination. On day 5 of growth, 12 holes are made in the film along the plate to allow oxygen penetration to the plantlets. On day 7, the film is removed to allow the elongation of the plantlets' stems without inducing stress.

Figure 10: Assay of tomato infected with Fusarium oxysporum *on* soil.

II.8 Antifungal Bioassays of *Artemisia absinthium* Essential Oil on *Fusarium oxysporum* :

The experiment of this section was performed in ICA-CSIC, supervised by Azucena González-Coloma and Maria Fe Andrés. The antifungal activity of AEO was tested on three *Fol*.

A conidial suspension was prepared from a potato dextrose agar (PDA) plate, and its concentration was measured using a Neubauer chamber. The final concentration of the suspension was 10^5 spores/mL. In a 96-well plate, the susceptibility of the spores was tested using 100 µL of Roswell Park Memorial Institute medium (RPMI) to support cell viability, 80 µL of DMSO (1%) as a negative control (solvent and drug-free), and 80 µL of Amphotericin B (5 µg/mL) as a positive control. AEO and SEO were tested separately at different concentrations: (0, 5, 50, 500, and 1000 µg/mL), with 4 replicates used for each treatment. After 24 hours of incubation, the antifungal activity was determined using an MTT staining (5 mg/mL) as an indicator of spore viability, proliferation, and cytotoxicity (Berrocal-Lobo et al., 2009; González-Coloma et al., 2002). Menadione (1 mM) and 25 µL of RPMI medium per well were added and incubated for 3 hours at 37°C. After incubation, the solution was removed from each well, and 200 µL of isopropanol acid (95% isopropanol with 1M HCl) was added and incubated for 30 minutes. The resulting-colored solution was quantified by measuring absorbance at 630 nanometers using a multi-well spectrophotometer and the Gen2.01 program (González-Coloma et al., 2012).

The inhibition test of the mycelial growth was conducted in 12-wells plate as described by (Bailen et al., 2013) with slight modifications. PDA medium was prepared, and 5 mg/mL of MTT was diluted in water. Ethanol was used as a negative control (2% final concentration). In a sterile falcon, 1950 µL PDA medium, 10 µL of MTT, and 40 µL ethanol were mixed and agitated in a vortex, then poured into each well. AEO stock was prepared at 50 mg/mL and diluted to different concentrations to be tested: 0.1, 0.25, 0.5, and 1 mg/mL, and 4 replicates were used for each treatment. The plates were covered with aluminum foil and incubated at 27°C for 5 days (Morales-Sánchez et al., 2021). The fungal colonies were quantified using the ImageJ program by measuring two perpendicular diameters of the colony, and the area (cm^2) was calculated using the formula:

$$\boldsymbol{Area} = Diameter \times \pi$$

II.9 Micropropagation and Growth of *Quercus suber*:

The protocol of micropropagation of *Qs* embryos is based on (Manzanera & Pardos, 1990), where culture techniques for the micropropagation of cork oak tissue was investigated. The study achieved optimal growth and proliferation with mineral media containing low ion concentrations, specifically Sommer's formulations. Root induction was facilitated using low IBA concentrations in the rooting medium and high sucrose levels.

II.9.1 Micropropagation Step:

To micropropagate the somatic embryos (SE), the medium was containing in every litter: 10 mL of macronutrients solution,1 mL of micronutrients, 10 mL of ferric solution, and 10 mL of vitamins, 30 g/l of saccharose, 6 g/l of agar, and 0.1 mg/l of BAP to promote cell division and shoot proliferation.

The macronutrients solution was prepared as described in Sommer et al 1975, it is an aqueous solution that contains: 15 g/l of Calcium chloride dihydrate, 100 g/l of Potassium nitrate, 83 mM of Sodium dihydrogen phosphate dihydrate, 25 g/l of Magnesium sulfate heptahydrate, 20 g/l of Ammonium Sulfate and 30 g/l of Potassium chloride.

Micronutrients was prepared *Fol*lowing Murashige-Skoog 1962, it is an aqueous solution that contains: 0.62 g/l Boric acid, 1.69 g/l of Manganese (II) sulfate, 0.86 g/l Zinc sulfate, 0.083 g/l of Potassium iodide, 0.025 g/l Sodium molybdate dihydrate, 0.0025 g/l of Copper (II) sulfate and 0.0025 g/l of Cobalt (II) chloride. The Ferric-EDTA solution, contains 2.78 g/l of Ferrous sulfate heptahydrate and 3.72 g/l.

The cofactors solution was prepared also *Fol*lowing Murashige-Skoog 1962, it is an aqueous solution that contains: 0.2 g/ of Glycine, 10 g/l of Mio-inositol, 0.5 g/l of Nicotinic acid, 0.5 g/l pyridoxine- HCl, 0.1 g/l of Thiamine- HCl, 0.2 g/l of Ascorbic acid. pH was adjusted to 5.5 to 5.7 and the solution is autoclaved for 20 minutes at 120ºC.

Culture tubes are also cleaned and autoclaved. The medium is poured by 10 mL approximately in each tube and cooled before use. Sterile forceps of 14.5 cm is used to transfer the SE from petri dishes to culture tubes, closed with the led and a micropore tape to allow oxygen to penetrate to the tissue. After transfer, it is left two weeks to one month in the same medium before transfer to a fresh one. The growth of new organs was *Fol*lowed weekly.

In the second to the third month, new organs start to appear, and the embryos that have new shoots are selected to be cut and micropropagated into a new culture tube.

II.9.2 Rooting Step:

When embryo has developed enough shoots, it is transferred to a rooting medium consisting of the same macro and micronutrients, vitamins, ferric solution described previously, adding 1% of saccharose and 25 mg/l of Indol-3-Butyric Acid (IBA) to promote root growth and development.

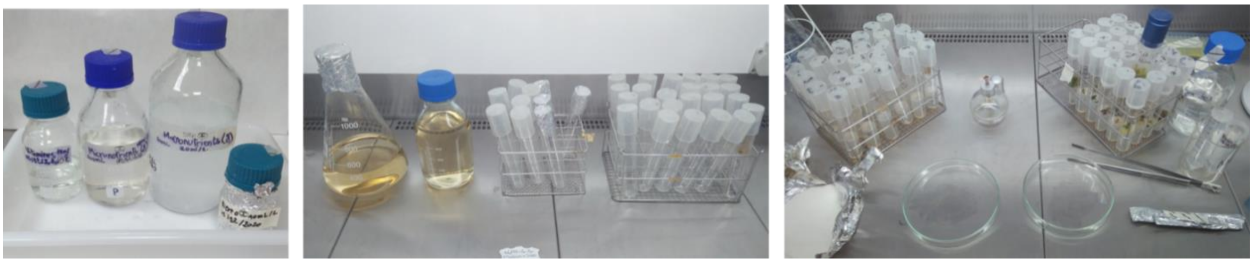


Figure 11: *Stock solutions prior the preparation of the culture medium, preparation of culture tubes and culture medium, the transfer of the embryos into the new medium.*

II.10 *Quercus suber* Endophytic Bacteria and Treatments:

Endophytic bacteria (EB) appeared after the step of rooting; different methods have been adopted to clean the explants from it before transferring to a new clean and fresh medium: Explants were first cleaned with 1 mL of Sodium hypochlorite 0.2% in aseptic conditions before being transferred to a new clean medium. While treatment with the antibiotic cetoxine, two different concentrations were used within the medium to inhibit the growth of endophytic bacteria. A stock solution of 100 mg/L was prepared, and this was added to the medium to achieve final concentrations of 0.5 mg/L and 1 mg/L.

The quantification of endophyte levels to evaluate the antibiotic efficiency was conducted after two weeks according to the *Fol*lowing scale: 0. No contamination, 1. Level 1, 2. Level 2, 3. Level 3, 4. Full of bacteria. (See Figure 12 for a visual representation of the contamination levels by endophytic bacteria as described previously).

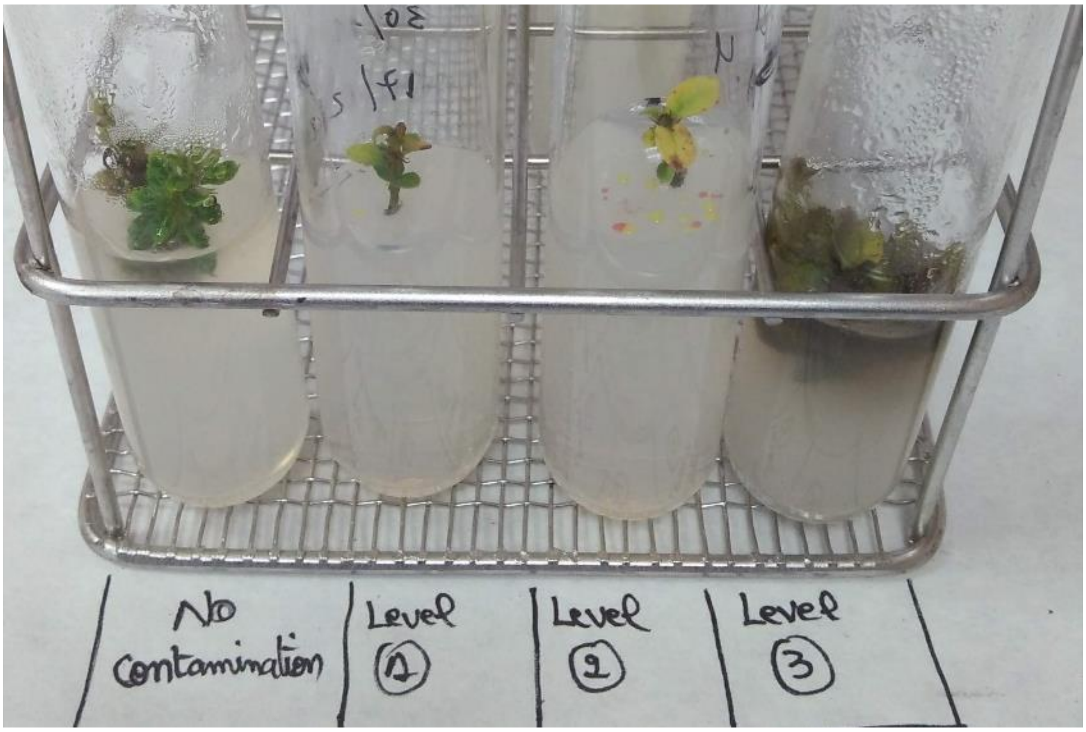


Figure 12: *The levels of endophytes present in Quercus suber after 2 weeks of in vitro growth.*

Initially, we suspected contamination in the samples, but upon further investigation, we confirmed that they were actually endophytes. Endophytes are microorganisms that reside within the plant tissues and are not considered contaminants. These endophytes likely originated from the natural soil environment and subsequently colonized the plants (Afzal et al., 2014).

II.11 Inoculation of The Plants:

The coated tomato seeds were subjected to continuous exposure to the fungus in a hydroponic setup that comprised 500 µl of *Fol* (10^6 spores/mL) in water. As for the control samples, they received a treatment of 500 µl of sterilized distilled water with a corresponding glycerol dilution. The plants that underwent inoculation were transferred to a growth chamber (Aralab S.L, Lisbon, Portugal) and were cultivated in the growth conditions previously indicated (see section II.3). To track the progression of the disease, the parameters specific to each assay were measured.

In the case of *Qs*, after complications arose during micropropagation with the appearance of endophytic bacteria, we stopped the process and, in collaboration with other groups, obtained explants ready for inoculation. The endophytes were isolated for genome sequencing to use for further studies.

In the quest to optimize the inoculation technique for *Qs* infection, various protocols were trialed. Among them was the aeroponic system, which involved immersing the explant roots in 5 mL of water infused with *Pc* mycelium (Figure 13-A). However, this method proved ineffective, causing notable stress not only in the infected plants but also in the control group (Figure 13-B).

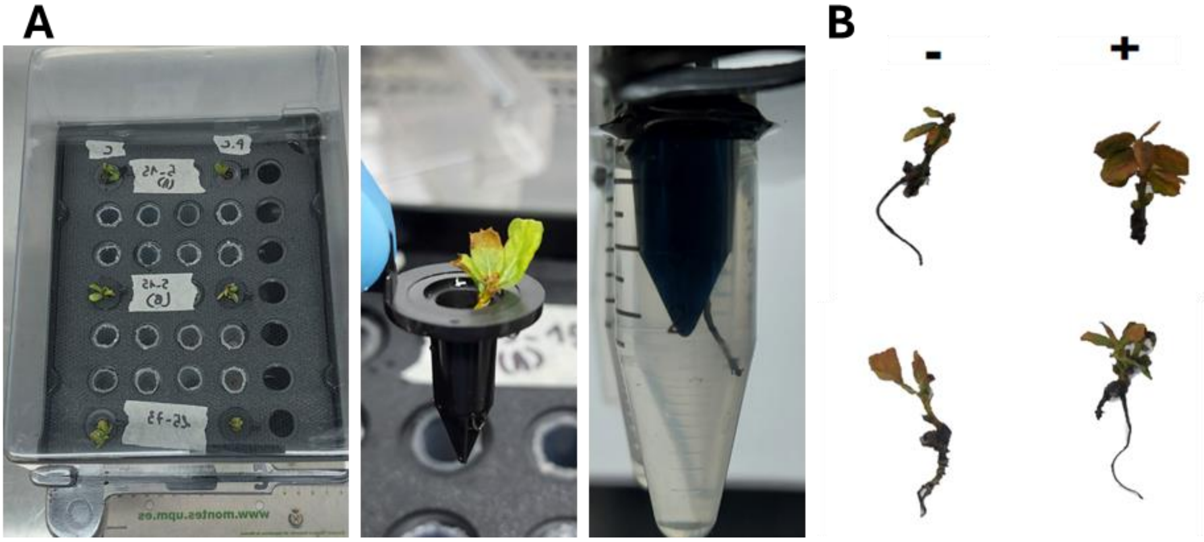


Figure 13: *Inoculation of Quercus suber with Phytophthora cinnamomi using araponic system.*

Finally, the protocol that demonstrated efficacy involved dividing the explants into two groups for each clone: a control group and an infected group. The plants in the infected group were inoculated with *Pc* zoospores at a final concentration of 10^7 zoospores/mL. These zoospores were obtained as explained previously (see section II.1). Both groups' culture tubes were covered with aluminum foil at the root level of the explant to keep this part in the dark and prevent exposing the zoospores to light, which could affect their ability to infect the roots.

Figure 14*: Quercus suber explants inoculated with Phytophthora cinnamomi covering the root part.*

II.12 Disease Symptoms:

To quantify disease symptoms, various parameters are considered and analysed. These parameters encompass a range of factors such as: disease ratio, length of the plant, fresh weight loss and water content, necrotic cells.

II.12.1 Disease Ratio :

In chapter 1, the *Fol*lowing criterium were considered to quantify disease ratio of tomato seedlings at 7 dpi, where: 0. no symptoms and normal germination (shoots and roots longer than 2 cm); 1. Delayed germination, showing radicle with no shoot; 2. Shoot and root measuring less than 2 cm; 3. Necrotic germinated seeds; 4. necrotic ungerminated seeds. And at 12 dpi the criterium considered were: 0. no symptoms; 1. growth inhibition of shoots and roots and leaf chlorosis; 2. leaf necrosis and chlorosis; 3. leaf chlorosis and root necrosis; 4. decayed seedlings. Bars: (1 cm).

In chapter 2, disease symptoms of infected *Qs* were *Fol*lowed for 4-5 days maximum, using a modified infection criteria described by Soudani et al. (2022) as *Fol*lowed: 0. No symptoms, 1. Leaf chlorosis and light necrosis on roots, 2. Apparent necrosis on leaves and light necrosis on roots, 3. High necrosis on leaves and roots, 4. Decayed explant. We considered leave necrosis and cell death on leaves as a secondary effect of the root necrosis produced by *Pc* on roots.

II.12.2 Fresh Weight Loss and Water Content:

FWL was calculated in tomato infected with *Fol*, were the fresh weight of controls and infected explants were measured at 12 dpi; the *Fol*lowing formula was calculated:

$$\boldsymbol{FWL} = \left[\frac{FW\ control}{FW\ infected}\right] x\ 100$$

To measure the Fresh weight loss (FWL) of *Qs* infected with *Pc*, the FW was measured at 0 and 4 dpi, and the FWL was calculated *Fol*lowing the formula:

$$\boldsymbol{FWL} = \left[\frac{FW\ (4\ dpi)}{FW(\ T0)}\right] x\ 100$$

$$\textbf{FWL (\%)} = 100\ \% - FW\ (\%)$$

To assess the water content, tomato seedlings tissue's fresh weight and dry weight were measured. The fresh plants were subjected to oven-drying at a temperature of 85ºC for 2 days, after which they were weighed as dry weight. The moisture content of each individual sample was then determined using the *Fol*lowing calculation:

$$\boldsymbol{Water\ content}\ (\%) = \left(\frac{Fresh\ weight - Dry\ weight}{Fresh\ weight}\right) \times 100$$

II.12.3 Measurement of Cell Necrosis

To detect cell death and necrosis both tomato and *Qs* tissue were stained using the same protocol, of (Fernández-Bautista et al., 2016). The trypan blue solution was prepared by combining 10 mL of lactic acid (85% w:w), 10 mL of phenol (TE balanced buffer, pH 7.5-8.0), 10 mL of glycerol (99%), 10 mL of distilled water, and 40 mg of Trypan blue, resulting in a final concentration of 10 mg/mL. The seedlings of tomato were subjected to staining for 20 minutes, *Fol*lowed by rinsing with 100% ethanol overnight, while *Qs* explants were rinsed for two days until all the chlorophyll was extracted, resulting in a transformation where the healthy tissue of the seedlings appeared white while the necrosed areas turned black. Subsequently, the stained plants were preserved in 60% glycerol until microscopic observation.

II.12.4 Reactive Oxygen Species (ROS) Production in Tomato Seeds and Seedlings:

To assess the (ROS) production in coated and inoculated tomato seeds, 3,3' Diaminobenzidine tetrahydro-chloride hydrate (≥96%) (DAB) was employed, *Fol*lowing the established methodology described in the literature (Berrocal-Lobo et al., 2010).

The procedure involved treating the seeds and seedlings with DAB solution (1 mg/mL) treatment under vacuum for a period of 2 minutes, then covered with aluminium foil and left at room temperature for 2 hours. *Fol*lowing this incubation, the DAB solution was carefully removed, and the tissue was immersed in 100% ethanol for 2 hours to extract the chlorophyll of the tissue. Finally, the tissue samples were placed in a solution of 60% glycerol for preservation. The DAB staining process was conducted at specific time intervals, including 30 minutes, 1 hour, then 1, 4, 7, and 12 days post-inoculation (dpi).

II.12.5 Callose Deposition in Tomato and *Quercus suber*:

To quantify callose deposition in tomato seeds and seedlings, the aniline blue staining method was used (Masachis et al., 2016). Initially, seeds or leaves were treated with 100% ethanol for 24 hours. Subsequently, in dark, the treated samples were stained using a 0.1 mg/mL water solution of aniline blue (Sigma-Aldrich, St. Louis, MO, United States) for 30 minutes period. *Fol*lowing the staining process, the tissue was carefully rinsed with distilled water and then placed in 60% glycerol at a temperature of 4°C. Finally, the samples were mounted on microscopy slides for further analysis.

Callose deposition in *Qs* was detected by Aniline Blue staining applying an adapted procedure of the method described by Chen and Fang (2016). Briefly, 0.1% aniline blue was prepared in 0.1 M of Monosodium phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$; pH=9) and 2% glycerol (v/v). The explants were immersed in the solution overnight in darkness. The stained samples were stored at 4^oC until use the root is cut for microscopic observation.

Microscopy:

To visualize DAB and trypan blue staining, bright light was utilized, while aniline blue staining was detected using a DAPI/UV filter for fluorescence microscopy. The imaging was performed using a stereomicroscope (model A292/21 Microscopy iScope IS.3153-PLFi/6 with Fluorescence—IS.3153-PLi/6, nEWF 10x/22) equipped with various lenses (Plan Fluarex PLFi, 4, 10, 20, 40, and 100 oil lenses) including fluorescence filters (Blue, Green, UV-DAPI, and Red). The microscopy setup was supported by Microsercon SLU, based in Madrid, Spain. For image capture, a charge-coupled device (CCD) digital cooled camera (A292/21 Euromex 20

MP USB 3.0) with a 1-inch CMOS sensor was employed to obtain high-resolution digital photographs. ImageJ Software and dedicated plug-in tools were utilized for the analysis and measurement of aniline blue (callose) and DAB signals in image processing and quantification.

II.12.6 Chlorohphyll Content

To determine the total chlorophyll A, B, and carotenoid content, the method described by (Lichtenthaler and Wellburn 1983) was employed with slight modifications. Initially, 500 mg of fresh leaves were carefully collected and placed in a sterilized Eppendorf tube containing borosilicate glass beads that had been pre-chilled in liquid nitrogen. Subsequently, the samples were ground in 5 mL of 90% acetone and subjected to centrifugation at 3,000 g for a duration of 10 minutes. The resulting supernatant was carefully collected, and the absorbance of the samples was measured at three specific wavelengths (662 nm, 644 nm, and 470 nm) using a spectrophotometer (Hach DR 2000, Hach Co., Loveland, CO, United States). The pigment content was then calculated according to the specifications outlined by the authors and expressed as milligrams per 100 grams of fresh weight (mg/100 g fw).

$$\boldsymbol{Ca}\ (\boldsymbol{nm}) = (13.96 \times A_{665}) - (6.88 \times A_{650})$$

$$\boldsymbol{Cb}\ (\boldsymbol{nm}) = (24.96 \times A_{650}) - (7.32 \times A_{665})$$

$$\boldsymbol{Ct}\ (\boldsymbol{nm}) = \left(\frac{(1000 \times A_{470}) - (2.05 \times C_a) - (114.8 \times C_{b)}}{245}\right) \times 100$$

Where Ca is Chlorophyll A, Cb is Chlorophyll B, and Ct is Carotenoids.

II.13 Extraction and Purification of RNA:

For each plant, a protocol was devised based on the tissue composition to extract high-quality RNA suitable for further analysis.

II.13.1 RNA Extraction of Tomato Seedlings:

For every sample, 3 plantlets were collected in sterile Eppendorf tubes containing zirconox zirconio-cerio beads (1.4-1.7 mm, Lumaquin, S.A.) that has been proven efficiency in breaking the solid structure of the seeds. The Eppendorf tubes are then placed in nitrogen and disrupted using a Sylamate for 6 seconds (x2). Total RNA was extracted from frozen tomato tissue, with separate analyses performed on the roots and aerial parts (shoots). The TRIzol Reagent (Invitrogen, Carlsbad, CA, United States) was utilized, *Fol*lowing the manufacturer's protocol, and chloroform was used in the extraction process. To eliminate any residual genomic DNA, the RNA samples were subsequently treated with the High Pure RNA Isolation Kit (Roche, Manheim, Germany. Cat. No: 11828665001). The RNA samples underwent various analyses to ensure both quantity and quality. Quantity assessment was performed using a Nanodrop spectrophotometer (UV-Vis ACTG Gene UVS—99, range 200 to 850 nm), while quality was assessed using the Qubit 4.0 fluorometer (Thermo Fisher Scientific, Madrid, Spain). Before proceeding to the next step, the RNA samples were visualized on a 1% agarose gel and stained with GelRed (Nippon, Japan).

II.13.2 RNA Extraction of Tomato Seeds:

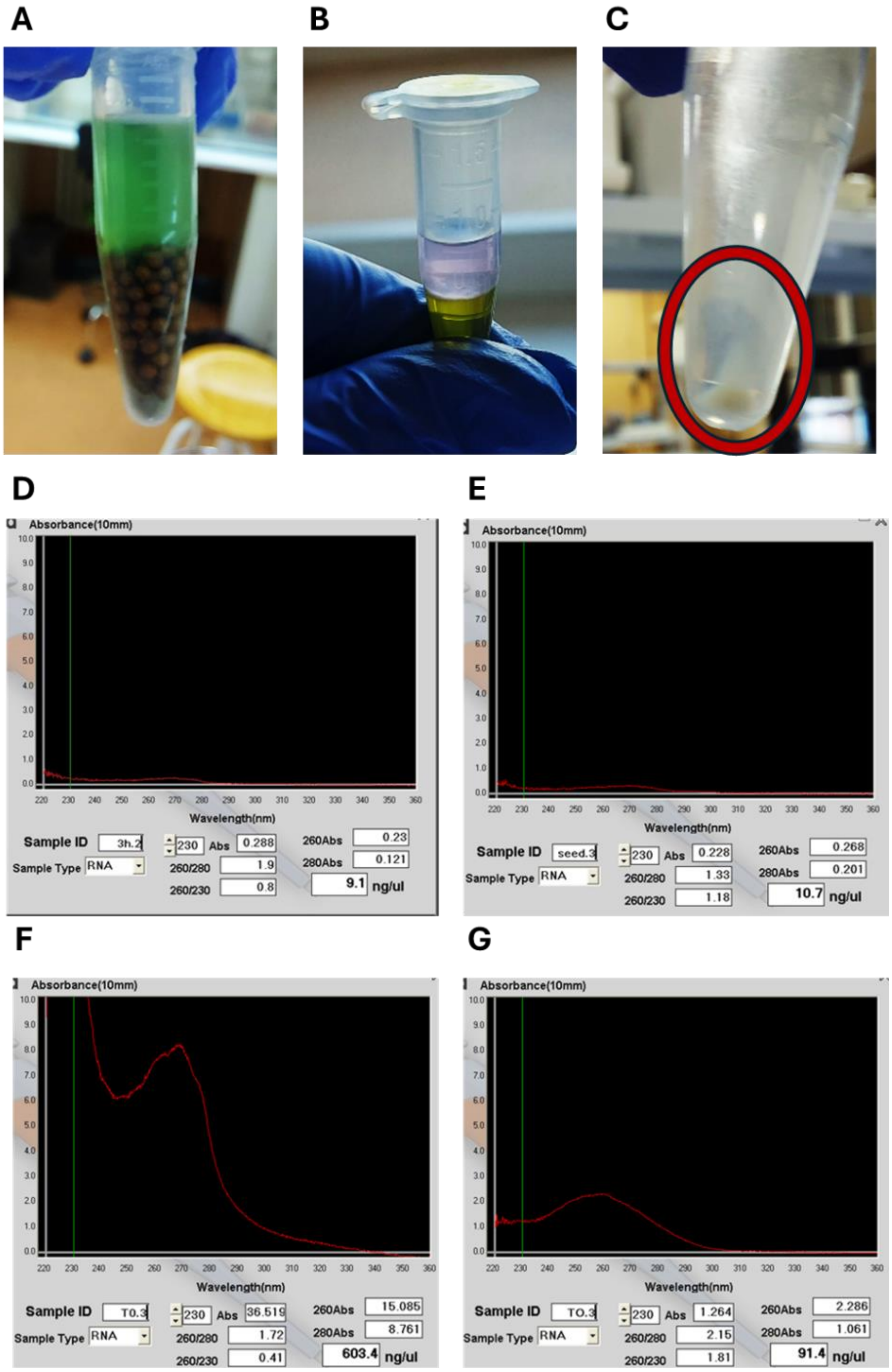
Initially, several methodologies were tested before getting the right protocol to extract tomato seeds RNA: the use of High Pure RNA Isolation Kit Roche Kit (Manheimm, Germany. Cat. No:

11828665001) *Fol*lowing manufacturer's protocol, and NZY Total RNA Isolation kit (MB13402). Both protocols failed since the seeds have complex compounds that interferes with the RNA during the extractions (Figure 15 D-E). For seed RNA extraction, finally, two steps were settled in the protocol. The first step allows the extraction of nucleic acid, while the second is to purify the RNA as *Fol*lows: To maintain RNA integrity and dissolve cell components, 800 µL of TRIzol (NZYTech; MB185) was added to preserve RNA integrity and dissolve cellular components, the mixture was homogenized for an additional 10 seconds. The homogenate was then centrifuged at 12,000 rpm/4ºC for 5 minutes, and the resulting supernatant (Figure 15-A) was transferred to a clean tube. To remove proteins and precipitate the purified RNA, 300 µL of chloroform was added, and the mixture was incubated at room temperature for 2 minutes and centrifuged again at 12,000 rpm/4ºC for 5 minutes. The resulting supernatant (Figure 15-B) was transferred to a new tube containing 1 mL of isopropanol, which was manually mixed to precipitate the RNA. The mixture was then incubated at -20ºC for 30 minutes and centrifuged at 12,000 rpm/4ºC for 30 minutes. The resulting pellet (Figure 15-C) was washed three times with 70% ethanol and air-dried at room temperature for approximately 20 minutes. The dried pellet was resuspended in 15 µL of nuclease-free water and RNA obtained was quantified by measuring 1.5 µL of the sample using a NanoDrop spectrophotometer (Figure 15-F), where it appears that the absorbance is high from the presence of additional components. The RNA was further purified using the NZY Total RNA Isolation kit (MB13402), *Fol*lowing the manufacturer's instructions. The RNA obtained was quantified by measuring 1.5 µL of the sample using a NanoDrop spectrophotometer (Figure 15-G), and the purity was established according to the criteria of Wilfinger, Mackey, and Chomczynski 1997.

II.13.3 RNA Extraction from *Quercus suber* Roots:

The *Qs* explant samples were collected in aluminum foil and immediately submerged in liquid nitrogen, then stored at -80ºC until further use. The roots were ground into a powder using a mortar and pestle, transferred into a sterile 1.5 mL Eppendorf tube. Two methods of RNA extraction were tested with industrial kits. Figure 16 shows nanodrop profiles. It appears that both methods allow the extraction of RNA from the root of *Quercus suber*, but the RNA extracted with CTAB buffer *Fol*lowed by RNeasy Plus Mini Kit of Qiagen (Figure 16-B) is of high quality and can be used for useful analyses such as QRT-PCRs and RNA-seq.

The new protocol used for extraction was adopted from the method described by (Chang et al., 1993) with some modifications. The buffer of extraction was prepared combining the *Fol*lowing components: 2% hexadecyltrimethylammonium bromide (CTAB), 2% Polyvinylpyrrolidone K30 (PVP), 100 mM Tris-HCl (pH 8.0),25 mM Ethylenediaminetetraacetic acid (EDTA), 2M Sodium Chloride (NaCl), 0.5g/L spermidine. It was mixed and the final pH of the solution was adjusted to 8.0 and autoclaved 20 minutes at 120ºC. Before each use, the buffer was heated at 65ºC. To prepare the sample, a solution of the buffer with 2% β-mercaptoethanol was created, and 800 µl of this solution was added to the ground tissue. The mixture was vortexed until the tissue was thoroughly mixed with the solution. The mixture was incubated at 65ºC for 2 minutes. Next, a mixture of Chloroform: isoamyl alcohol (24:1) was added to each sample and mixed with the solution. The samples were then centrifuged at 5000 rpm, 25ºC for 15 minutes. The supernatant was carefully transferred to a new microtube, and 0.25 volume of 10M Lithium Chloride (LiCl) was added. The resulting mixture was stored at 4ºC overnight to allow for the precipitation of RNA. Afterward, the samples were centrifuged at 7500 rpm, 4ºC for 30 minutes. A pellet formed at the bottom of the tubes, and the supernatant was carefully removed. The pellet was then air-dried. The purification of RNA was carried out *Fol*lowing the instructions provided in the RNeasy Plus Mini Kit (Qiagen). RNA samples were analysed to check quantity using a NanoDrop (UV-Vis ACTG Gene UVS—99. 200 to 850 nm). The methodology employed in this protocol relied on a CTAB extraction buffer supplemented with PVP and β-mercaptoethanol was to effectively eliminate polysaccharides and inhibit the oxidation of phenolic compounds (Sánchez et al., 2016).



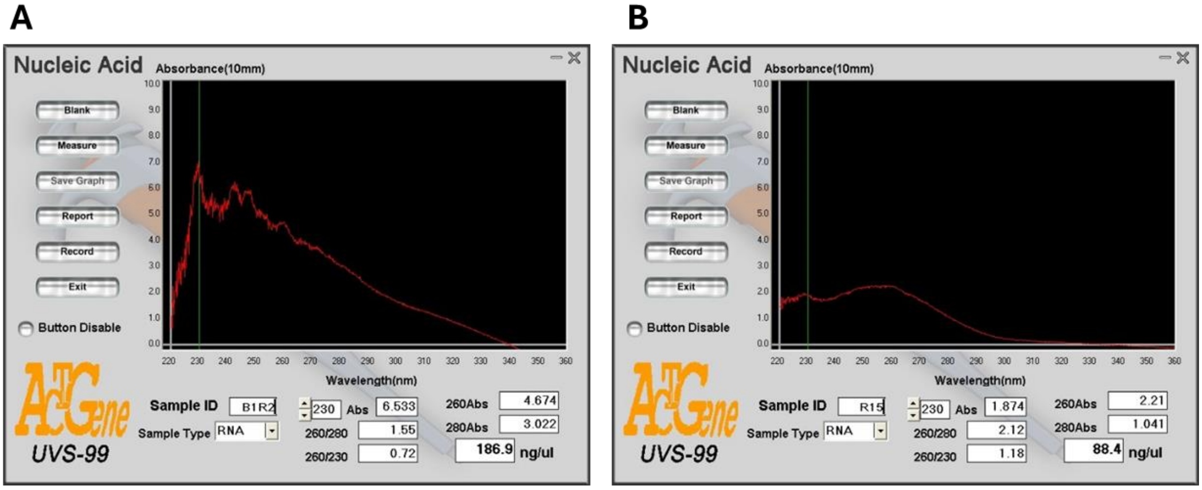
D
Sample ID 3h.2
Sample Type RNA
230 Abs 0.288
260/280 1.9
260/230 0.8
260Abs 0.23
280Abs 0.121
9.1 ng/ul

E
Sample ID seed.3
Sample Type RNA
230 Abs 0.228
260/280 1.33
260/230 1.18
260Abs 0.268
280Abs 0.201
10.7 ng/ul

F
Sample ID T0.3
Sample Type RNA
230 Abs 36.519
260/280 1.72
260/230 0.41
260Abs 15.085
280Abs 8.761
603.4 ng/ul

G
Sample ID T0.3
Sample Type RNA
230 Abs 1.264
260/280 2.15
260/230 1.81
260Abs 2.286
280Abs 1.061
91.4 ng/ul

Figure 15: *Key Steps in RNA Extraction (A, B, C) from Seeds and RNA profiles, (D) profiles obtained from the Roche kit (E) and Nzykit, (F) dried pellet after resuspension in 15 µL of nuclease-free water, (G) Purified RNA.*



A

Sample ID: B1R2
Sample Type: RNA
230 Abs: 6.533
260/280: 1.55
260/230: 0.72
260Abs: 4.674
280Abs: 3.022
186.9 ng/ul

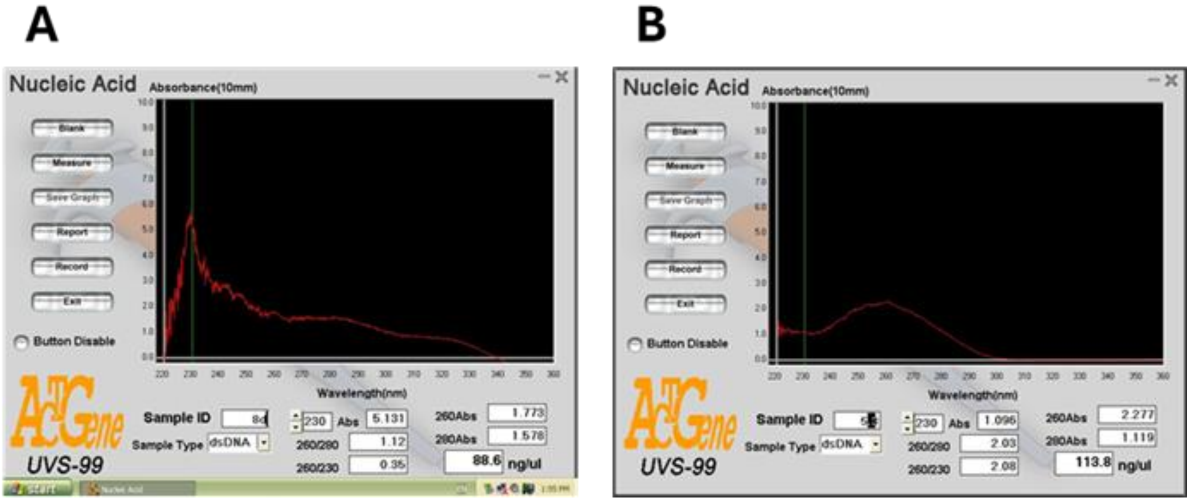
B

Sample ID: R15
Sample Type: RNA
230 Abs: 1.874
260/280: 2.12
260/230: 1.18
260Abs: 2.21
280Abs: 1.041
88.4 ng/ul

Figure 16: *Nanodrop profiles of two different methods of RNA extracted from Quercus suber roots. (A) NZY Total RNA Isolation kit. (B) CTAB Followed by RNeasy Plus Mini Kit of Qiagen.*

II.14 DNA Extraction from Seeds.

The EpiMag kit (Epigentek, NY; Reference: # P-1022) was initially used to extract DNA from tomato seeds, with 10 seeds per sample. However, upon measuring the final DNA sample, the nanodrop profile revealed a high absorbance at 230, indicating poor purification of the DNA solution (17-A). While the DNA extraction from seeds conducted with the DNeasy Plant Mini Kit (Hilden, Germany: Cat. No: 69204), appears to be of high quality as shown by the nanodrop profile in Figure (17-B), where the absorbance ratio A260/280 for DNA is 2.03 and A230 is low. These samples are earmarked for subsequent methylomic analysis.



A

Sample ID: 8
Sample Type: dsDNA
230 Abs: 5.131
260/280: 1.12
260/230: 0.35
260Abs: 1.773
280Abs: 1.578
88.6 ng/ul

B

Sample ID: 5
Sample Type: dsDNA
230 Abs: 1.096
260/280: 2.03
260/230: 2.08
260Abs: 2.277
280Abs: 1.119
113.8 ng/ul

Figure 17: *Nanodrop profiles of DNA extracted from tomato seeds. (A) using EpiMag High Throughput DNA Isolation Universal Kit, (B) Neasy isolation kit.*

Characterization of Nucleic Acid Purity:

During the initial phase of RNA extraction process which involves the use of TRIzol that is effective in removing substantial DNA molecules, it may not efficiently eliminate plasmid DNA or DNA fragments. This inefficiency could pose challenges when performing subsequent PCR applications (Rio et al., 2010). The Nanodrop quantification measuring the absorbance at 260 nm to 280 nm absorbance ratio serves as an indicator of RNA purity (Okamoto & Okabe, 2000). In molecular biology, references have stated that an A260/280 ratio between 1.5 to 2.0 for RNA signifies a "pure" nucleic acid preparation without proteins, a lower value suggests potential protein contamination. In the case of DNA, the absorbance varies between 1.5 to 2.0. Remarkably, nucleic acids distinctly absorb light at 260 nm in the ultraviolet range (Wilfinger et al., 1997). Therefore, the DNA derived from the DNeasy isolation Kit demonstrates notable quality (Lee et al., 2020).

II.15 Quantitative Real-Time PCR (qRT-PCR) Experiments:

I.1.1 cDNA Synthesis:

All cDNA of all the experiments were synthesized using NZY First-Strand cDNA Synthesis Kit according to the manufacturer's protocol. The cDNA solution was incubated at 25 °C for 10 min, then at 50 °C for 30 min, Inactivate the reaction by heating at 85 °C for 5 min, and then chill at 4ºC until use. The quantitative real-time (qRT-PCR) experiments was performed using a FG, Power Syber Green PCR Master mix (Thermofisher) with reactions at a final volume of 20 µl per well. The *Fol*lowing standard thermal cycling profile was used for the QRT-PCR: 95°C for 10 min; 40 cycles of 15 s at 95°C and 1 min at 60ºC; in the melting curve stage the temperature used was 95°C for 15 s, 60ºC for 1 min and 95ºC for 15 s.

I.1.2 Quantification of Gene Expression by qRT-PCR:

The qPCR experiments were performed using a fluorescence green Power Sybr Green PCR Master mix (Thermofisher, USA) with reactions at a final volume of 20 µl per well, using three biological replicates for each treatment or time points. The qRT-PCR reactions were conducted in a DNA Engine One-Step QRT-PCR machine (Thermo Fisher Scientific, USA).

In chapter 1, gene-specific primers were designed using the Primer Express 2.0 program (Applied Biosystems, Foster City, CA, USA), or designed manually, *Fol*lowing a melting temperature between 52-58ºC, GC content between 45-58 %, hairpins formed by intramolecular interaction within the primer is tolerated when ΔG is -3 kcal/mol or less, selfdimers formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself is tolerated at ΔG of -3 kcal/mol or less, heterodimers formed by intermolecular interaction between sense and antisense primers, were tolerated at ΔG of -3 kcal/mol or less. A repeat of a di-nucleotide occurring many times consecutively were avoided. The Amplicon Length were no less than 100 bp and no longer than 500 bp (Apte & Daniel, 2009). Find primers efficiency in table 4. Primer self-hybridization and dimer formation were evaluated using the Oligo 6.0 program (Molecular Biology Insights, West Cascade, CO, United States) or by Integrated DNA Technologies (IDT) website: https://eu.idtdna.com/. Primers with annealing Specificity was confirmed using the Basic Local Alignment Search Tool (BLAST) from National Center of Biotechnology Information (NCBI). The amplification efficiency of each primer pair was calculated according to the manufacturer's instructions (Bio-Rad, Hercules, CA, United States), with only primers exhibiting efficiencies above 90% being used for assays. The specific gene primers employed for quantitative real-time PCR are provided in the table 4.

Data acquisition was performed using the One-Step PCR Applied Biosystem Analysis software (Version 2.01), and changes in transcript levels related to a housekeeping gene (in chapter 1, part 1 and chapter 2, part 2) were determined using the $2^{(-\Delta\Delta CT)}$ method (Livak & Schmittgen, 2001). While the absolute quantification method (chapter 1, part 2 and chapter 2, part 1) involves creating a standard curve using serial dilutions of a known quantity of the target gene and the resulting data is used to plot a standard curve, which relates the threshold cycle (Ct) values obtained from the qPCR to the initial concentration of the target gene (Figure 18-A). Once the standard curve is established, the Ct values obtained from the experimental samples are plotted on the curve to determine their corresponding target gene concentrations. This allows for the calculation of the absolute quantity of the target gene in the sample based on its Ct value and the standard curve (Figure 18-B) (Dhanasekaran et al., 2010).

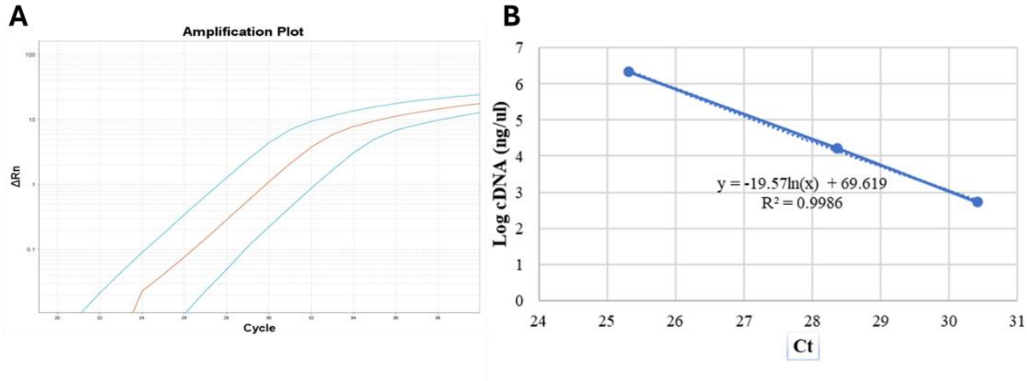


Figure 18: *Amplification plot and Standard curve for the absolute quantification of NRPD2 gene expression.*

In chapter 2, part 2, *β-Tubulin* was tested as housekeeping gene and *Cast-Gnk2-like* sequence oligonucleotides are based on previous study (Serrazina et al., 2022), these primers were designed for *Q.ilex*, but when tested on *Q. suber* they amplified effectively. The successful specificity of both oligonucleotides amplified in *Qs* was confirmed by getting a melting curve at the last step of corresponding qRT-PCR reactions (Figure 19). The *Gnk2-like* gene expression in the transgenic plants infected or non-infected was quantified using the $2-^{\lambda\lambda Ct}$ method respected to wild type explants and using three biological triplicates to each condition and assay. Three biological replicates were used from two different individuals for both control and inoculated explants, in both WT and *TRANSG 1,* respectively.

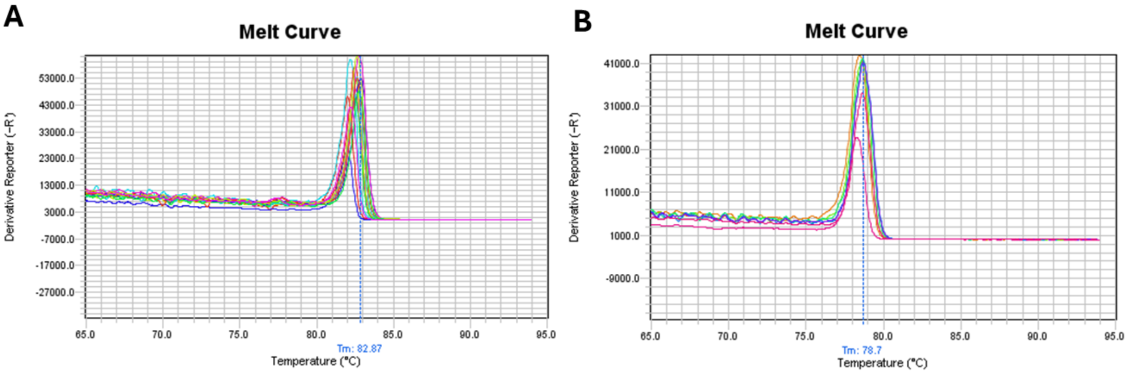


Figure 19: *Melting curve analysis of Quercus suber upon qPCR reactions with β Tubulin promoter primers (A) and Gnk2-like (B).*

II.16 Construction of RNA-Seq Libraries

RNA-seq data analysis, gene ontology enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes pathway analysis were conducted in collaboration with César Poza-Carrión.

Total RNA was extracted from three independent biological replicates *Fol*lowing the previously described method. To construct the Illumina sequencing libraries, 1 µg of total RNA was utilized for each sample, adhering to the manufacturer's instructions provided by the TruSeq Stranded mRNA LT Sample Prep Kit. The libraries were subsequently sequenced on the Illumina HiSeq 2500 platform (Biomarker Technologies), generating 150 bp paired end reads.

II.16.1 Analysis of RNA-Seq Data:

Each library yielded approximately 4 Gigabases (Gb) of high-quality paired end reads, with each read spanning 150 base pairs (bp). To assess the quality of the clean reads, a quality threshold of $Q < 20$ was employed. In small RNA sequencing, the reads obtained from current sequencing machines are often longer than the actual RNA molecules. Consequently, these reads may contain portions of the 3' adapter used during library preparation. To ensure accurate read mapping, it is necessary to identify and remove the 3' adapter from each read, taking into account potential sequencing errors (Martin, 2011), In our study, Cutadapt (https://pypi.org/project/cutadapt/) was employed to handle this task. HISAT2, a splice-aware aligner, was utilized to map the trimmed reads to the reference genome (Pertea et al., 2016). The tomato reference genome and gene model annotation files were obtained from the genome website browser (https://solgenomics.net, SGN release version SL2.50). To assemble known genes and transcripts, StringTie was employed in conjunction with the aligned reads (Kovaka et al., 2019). In chapter 2, part 1, Biojupies website (https://maayanlab.cloud/biojupies/) generated RNA-seq Data Analysis Notebooks, including: the clustergrammer, volcano plot and scatter plot.

II.16.2 Gene Ontology Enrichment Analysis and Kyoto Encyclopedia of Genes and Genomes Pathway Analysis:

Panther GO (http://www.pantherdb.org) was used for Gene Ontology (GO) enrichment. The GO enrichment analysis provided all the GO terms which were significantly enriched in the DEGs relative to the genomic background, and DEGs were filtered according to cellular components, molecular functions, and biological processes. KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/) is a main pathway-related database. Based on the comparison of the DEGs to the genomic background, pathway enrichment analysis pinpointed the enriched pathways.

II.16.3 Validation of RNA-Seq by quantitative Real-Time-PCR:

To validate RNA sequencing reading data, 1 µg total RNA was reverse transcribed into cDNA *Fol*lowing the previously described protocol for first strand synthesis using oligo (dT) primers. A qPCR was performed as previously described. The fluorescence signal was monitored automatically in each cycle. Relative expression levels of specific mRNAs were measured as previously described using the $2^{(-\Delta\Delta Ct)}$ analysis method, and expression values were normalized using the *β-Actin* gene. A regression line was calculated to analyse the correlation between Log2 RNA-seq readings and quantitative real-time PCR Ct results from twelve independent

RNA samples and five genes for each tissue. Three independent biological replicates were analysed for each sample.

II.17 Metabolomic Analysis:

Metabolomic analyses were performed in collaboration with ICA-CSIC, to tomato seedlings of 12 days and to the essential oil itself. These analyses are needed to understand how the plant's metabolic pathways are altered in response to the treatment. Analysing the essential oil itself allows the identification and quantification of its chemical constituents to identify bioactive compounds that may contribute to its antifungal properties. By correlating changes in plant metabolites with the composition of the essential oil, the mode of action of the essential oil can be understood.

II.17.1 Tomato Extract Preparation:

Treated and untreated tomato seedlings (12 days old, 12 seedlings per replica with three biological replicas) were frozen in liquid nitrogen and then extracted with methanol (MeOH). Extracts were filtered and kept at -20 ºC until High-Performance Liquid Chromatography and Mass Spectrometry analysis (HPL-MS). For Gas Chromatography coupled with Mass Spectrometry (GC-MS), the MeOH extracts were partitioned with dichloromethane (DCM), filtered and the solvent evaporated prior to GC-MS analysis.

II.17.2 Gas Chromatography Coupled with Mass Spectrometry Analysis of Essential Oil and Tomato Extracts:

The essential oil and DCM fractions of MeOH tomato extracts were analysed by GC-MS using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV). Sample injections (1 µl) were performed using an AOC-20i and equipped with a 30 m × 0.25 mm i.d. capillary column (0.25 µm film thickness) Teknokroma TRB-5 (95%) Dimetil- (5%) diphenylpolisiloxane. Working conditions were as *Fol*lows: split ratio (20:1), injector temperature 300 ºC, temperature of the transfer line connected to the mass spectrometer 250 °C, initial column temperature 70 °C, then heated to 290 °C, at 6 °C/min intervals. Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those found in the Wiley 229 and NIST Mass Spectral Database. All extracts (4 µg/µl) were dissolved in 100% DCM for injection. Pure compounds (salicylic acid, chlorogenic acid and methyl jasmonate from Sigma-Aldrich) were injected and analysed under the same conditions just described.

II.17.3 LCMS Analysis of Tomato Extracts:

Methanolic tomato extracts were analysed by liquid chromatography coupled with mass spectrometry (HPLC-MS) in a Shimadzu apparatus equipped with an LC- 20AD pump and a CTO-10AS VP column oven coupled to a mass spectrometer with a simple quadrupole analyser LCMS-2020 QP), with an electrospray ionization source (ESI). An ACE 3 C18 column (150 mm × 4.6 mm, 3 µm particle size) with an ACE3 C18 analytical pre-column was used for separation. The compounds were eluted with Methanol (LC-MS grade) (MeOH): MiliQ water with 1% acetic acid 5% MeOH for 5 minutes, *Fol*lowed by a gradient 5:100% MeOH for 30 minutes, 100% MeOH for 10 minutes and 100:5% MeOH for 8 minutes, with a flow rate of 0.5 mL/min. The nitrogen flow (drying gas for solvent evaporation) was 15L/min. Electrospray capillary potential was + 4.50 kV and a Full Scan was used in positive mode (m/z 100–700) with a potential of 1.40 kV and a capillary temperature of 250 ºC. Stock solutions of extracts were injected at 0.25 mg/mL with a 5 µl injection through an automatic injector (SIL-20A XR). All extracts (0.25 µg/µl) were dissolved in 100% MeOH for injection. Pure compounds (lycopene, carotene, salicylic acid, chlorogenic acid and methyl jasmonate from Sigma-Aldrich) were injected at 0.2 mg/mL and analysed under the same conditions as described above.

II.18 Global DNA and RNA Methylation Analysis:

Global DNA methylation levels of 5-methylcytosines (5mC) and Global RNA methylation levels of N6-methyladenosines (m6A) and 5-methylcytosine were determined by using the MethylFlash global DNA methylation 5 mC ELISA easy kit (# P-1030), EpiQuik m6A RNA methylation quantification kit (# P-9005) and MethylFlash 5mC RNA methylation ELISA easy kit (# P-9009) (Epigentek, Farmingdale, Nassau County, NY, USA), respectively, *Fol*lowing the manufacturer's instructions.

The m6A RNA methylation quantification kit is colorimetric. It includes a Developer Solution, which, after incubating at room temperature for 8 minutes away from light, causes the solution to turn blue in the presence of sufficient m6A (Figure 18-A.1). Adding the Stop Solution changes the color to yellow, and the absorbance is measured on a microplate reader at 450 nm within 2 to 15 minutes (Figure 18-A.2). The 5-mC RNA methylation ELISA easy kit is fluorescent. It contains Fluorescence Development Solution, which turns pink after incubating at room temperature for 4 minutes away from direct light in the presence of sufficient 5-mC products in RNA (Figure 18-B). The SpectraMax iD3 plate reader was utilized to quantify both colorimetric and fluorescence assays.

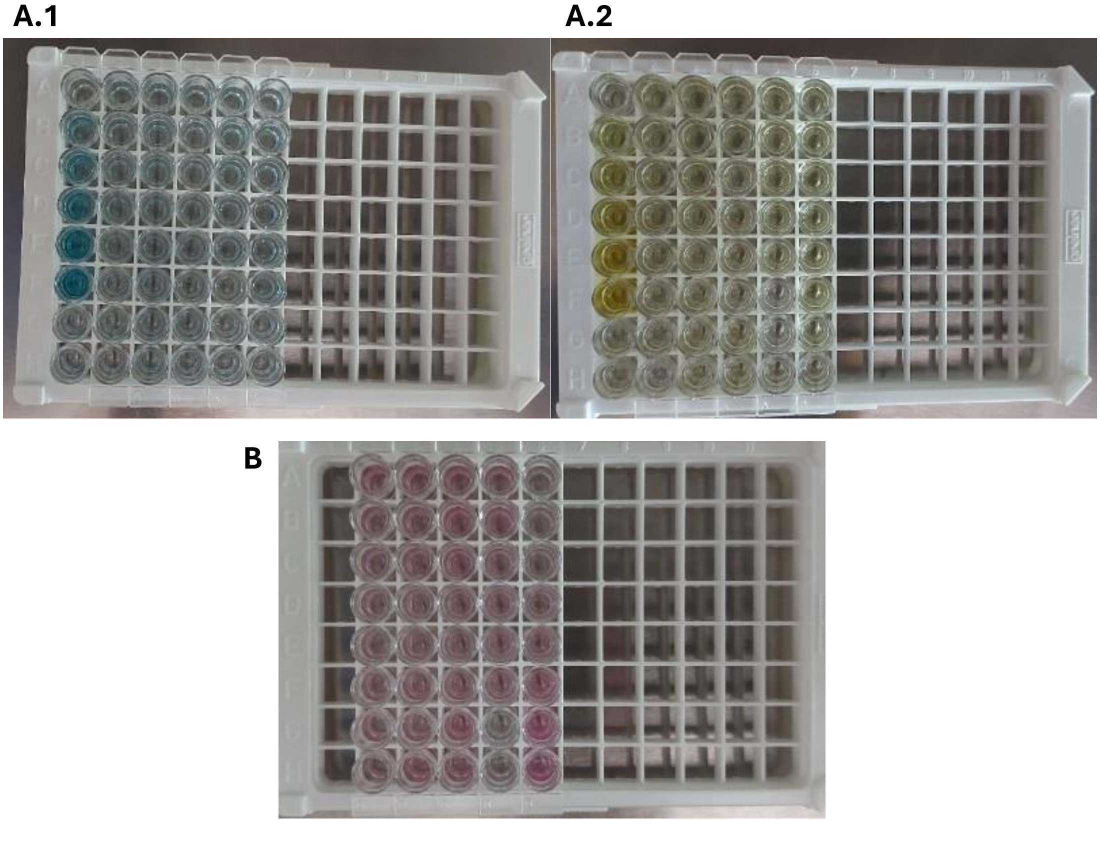


Figure 20: *Global DNA and RNA Methylation.*

To calculate the percentage of methylated DNA, a standard curve is generated, plotting the OD values against Positive Control (PC) at each concentration point. Next, the slope (OD/1%) of the standard curve is determined using linear regression, ideally focusing on the most linear part of the curve with at least four concentration points, including the 0 point. Finally, the percentage of methylated DNA (5-mC) in total DNA is calculated using the *Fol*lowing formula:

$$5 - mC\ \% = \frac{Sample\ OD - NC\ OD}{Slope\ \ \times S} \times 100$$

To calculate the percentage of 5-mC RNA, a standard curve is generated, plotting the RFU values against the PC at each percentage point. Next, slope (RFU/1%) of the standard curve is determined using linear regression, focusing on the most linear part with at least four concentration points, including the 0 point. Finally, the percentage of methylated RNA (5-mC) in total RNA is calculated using the *Fol*lowing formula:

$$5 - mC\ \% = \frac{Sample\ RFU - NC\ RFU}{Slope\ \ \times S} \times 100$$

Absolute quantification of m6A RNA methylation involves generating a standard curve by plotting the OD values (background-subtracted) against the amount of PC at each concentration point. The slope of the standard curve (OD/ng) is then determined using linear regression functions in Microsoft Excel. The most linear part of the standard curve, including at least four concentration points, is selected for optimal slope calculation. Finally, the amount and percentage of m6A in total RNA are calculated using the *Fol*lowing formulas:

$$m6A = \frac{Sample\ OD - NC\ OD}{Slope}$$

$$m6A\ \% = \frac{m6A\ (ng)}{S} \times 100$$

S is the amount of input sample DNA in ng, in this case 100 ng.

Statistical analysis:

The Stat Graphics Centurion XVI.II program (Stat Point Technologies, Inc., Warrenton, VA, United States) was used for all data analysis. A one-way analysis of variance (ANOVA) and Duncan’s mean comparison test were performed for all experiments and t-tests with a significance level of 0.05%. In the case of non-homogeneous variance, a non-parametric Kruskal– Wallis test was used.

Table 1: *DNA Primers used in this study.*

Genes	***Accession number***	***Primer sequence (5´-3´; forward/reverse)***	***Amplicon size (bp)***	R^2	***Reference***
HISTONE 1	Z11842.1	Forward: CTATCTGAGGCTGGAAAGAAG Reverse: GTA GAC TTC CTT GTC CTC TTG G	193	0.992	Soudani, 2023
LEH1	AJ224933	Forward: GTAGCAAAGCCGAAAGCAG Reverse: GGT CTT AGA TTT CAC ACT CTT CAC	189	0.994	
EXPANSIN 2	AF096776.1	Forward: GCA GCA CTA AGT ACA GCA C Reverse: GCA ATC CTC CTC TCC AAC	184	0.985	
NRPD2	Solyc08g075940.3	Forward: AAACTAAGACGTAAGCGCCG/ Reverse: AGAGGGCGCATAATCCTTCC	116	0.998	Poza-Carrión and Berrocal-Lobo, 2021
SAMt	Solyc04g040180.3	Forward: GATGTTGGTACTGGTAGTGGC Reverse: GAACATTTGGAACCTTTGCTGC	160	0.998	
WRKY33	Solyc09g014990.3	Forward: AGTAGGCCTGCTACTTCTTCC Reverse: ATGGTTGACAGTAATGCACCG	199	0.942	
β -Actin	Solyc11g005330.2	Forward: CAAGTTATTACCATTGGTGCTGAGA Reverse: TGCAGCTTCCATACCAATCATG	150	0.999	(Martín-Trillo et al., 2011)
Pc-β-TUBULIN	PHYCI_418545	Forward: CGCTGTCCGTGCACCAGCTTG Reverse: CATGTCCGGCATCACCACGTGC	161	1	(Del Castillo-gonzález et al., 2024)
Qs-β-TUBULIN	KJ563262	Forward: GCTCACTACCCCAAGCTT Reverse: GGAACCTCTGGAGGTTAAA	187	0.999	(Ebadzad & Cravador, 2014)
Qs-CHITINASE	KF704743	Forward: CCGTGGTGCCTTGCCTTTG Reverse: GCATCATGTGCTGAAGGCTG	177	0.994	
Qs β-TUBULIN	EE743717	Forward: CTGCGGTCGCTATGTTCCT Reverse: CCCTTGGCCCAGTTGTTTC	147	0.995	(Serrazina et al., 2022)
Cc_Gnk2		Forward: GGGGACCTAAAGCTTGACTCA Reverse: CATCGCAACAGTTGGGAAGTT	129	0.991	

III Results:

III.1 Chapter 1: Improvement of *Solanum lycopersicum* Resistance to *Fusarium oxysporum* Using Bioproducts.

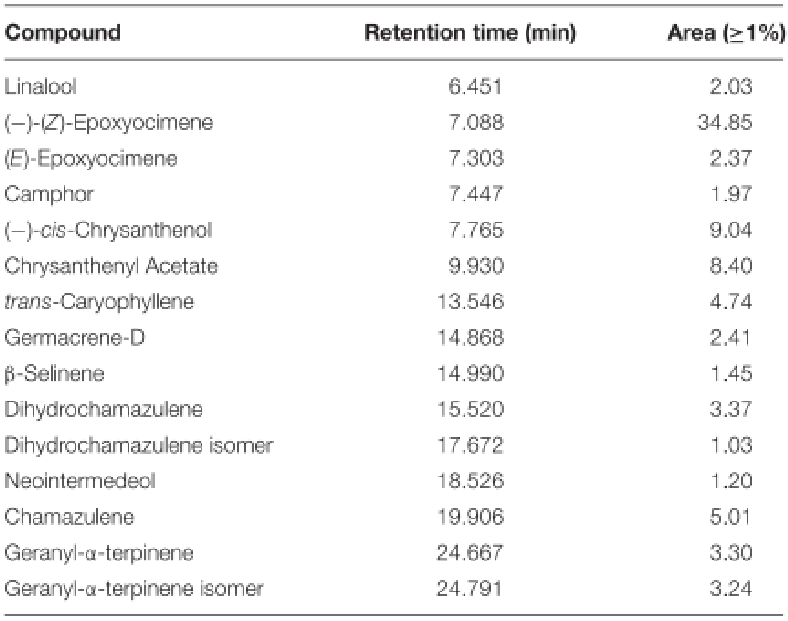
III.1.1 Part 1: Seed Coating Increase Tomato Seedlings Resistance Against *Fusarium oxysporum* Infection.

To comprehensively evaluate the protective effect of AEO on tomatoes against *Fol* infection, a series of analyses were conducted involving both seedlings and the essential oil itself. These analyses aimed to delve into various aspects of the interaction between AEO and the tomato plant's defense mechanisms, shedding light on its efficacy in combating *Fol* infection. Through rigorous examination of seedlings and thorough characterization of the essential oil, we sought to gain a deeper understanding of how AEO contributes to the plant's resilience against this pathogen.

III.1.1.1 *Artemisia absinthium* Essential Oil :

Before determining if AEO was active on tomato seeds first a chemical composition analysis was performed in collaboration with the investigation group BIOPLAG and ICA- CSIC. Table 2 shows the chemical composition of AEO, it was characterized as cis-epoxyocimene (35%), *Fol*lowed by cis-chrysanthenol (9,04%), chrysanthenyl acetate (8,40%), chamazulene (5,01%), and t-caryophyllene (4,74%). This composition was similar to that previously reported for other crops, highlighting the chemical stability of this plant variety (Julio et al., 2015).

Table 2: *Chemical composition of Artemisia absinthium essential oil tested.*

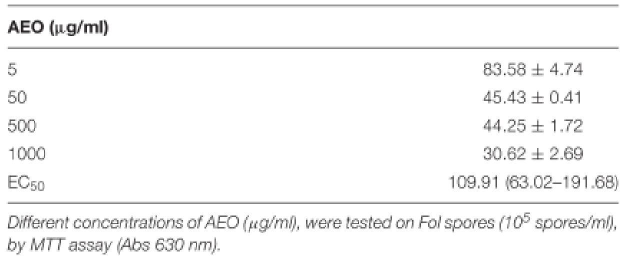


Compound	Retention time (min)	Area (≥1%)
Linalool	6.451	2.03
(−)-(*Z*)-Epoxyocimene	7.088	34.85
(*E*)-Epoxyocimene	7.303	2.37
Camphor	7.447	1.97
(−)-*cis*-Chrysanthenol	7.765	9.04
Chrysanthenyl Acetate	9.930	8.40
trans-Caryophyllene	13.546	4.74
Germacrene-D	14.868	2.41
β-Selinene	14.990	1.45
Dihydrochamazulene	15.520	3.37
Dihydrochamazulene isomer	17.672	1.03
Neointermedeol	18.526	1.20
Chamazulene	19.906	5.01
Geranyl-α-terpinene	24.667	3.30
Geranyl-α-terpinene isomer	24.791	3.24

III.1.1.2 Fungicidal Activity of *Artemisia absinthium* Essential Oil Against *Fusarium oxysporum* Phytopathogen:

With the objective of testing the potential direct activity of the oil on *Fol*, increasing concentrations of AEO were tested (0, 0.005, 0.05, 0.5, and 1 mg/mL), and the spore germination rate was analyzed. AEO exhibited strong fungicidal activity against *Fol* spores *in vitro* with a significant spore germination inhibition rate of 44.25% ± 1.72, compared to controls at 0.5 mg/mL, with an EC50 of 109.91 as shown in Table 3. Moderate or no fungicidal effects on spore germination were observed at concentrations below 0.005 mg/mL.

Table 3: *In vitro analysis of toxic activity of Artemisia absinthium essential oil on Fusarium oxysporum spores.*



AEO (μg/ml)	
5	83.58 ± 4.74
50	45.43 ± 0.41
500	44.25 ± 1.72
1000	30.62 ± 2.69
EC_{50}	109.91 (63.02–191.68)

Different concentrations of AEO (μg/ml), were tested on Fol spores (10^5 spores/ml), by MTT assay (Abs 630 nm).

III.1.1.3 *Artemisia absinthium* Essential Oil Effect on Seed Germination and Tomato Seedling Growth in Presence of *Fol*

To test for physiological effects of the AEO coating, seeds were treated with 0.5 mg/mL and 1 mg/mL of AEO and germinated in presence (+), or absence (-), of *Fol* (10^6 spores/mL). Germination rates were determined at 7 dpi, before seed germination was complete, and after 12 days once germination was finished.

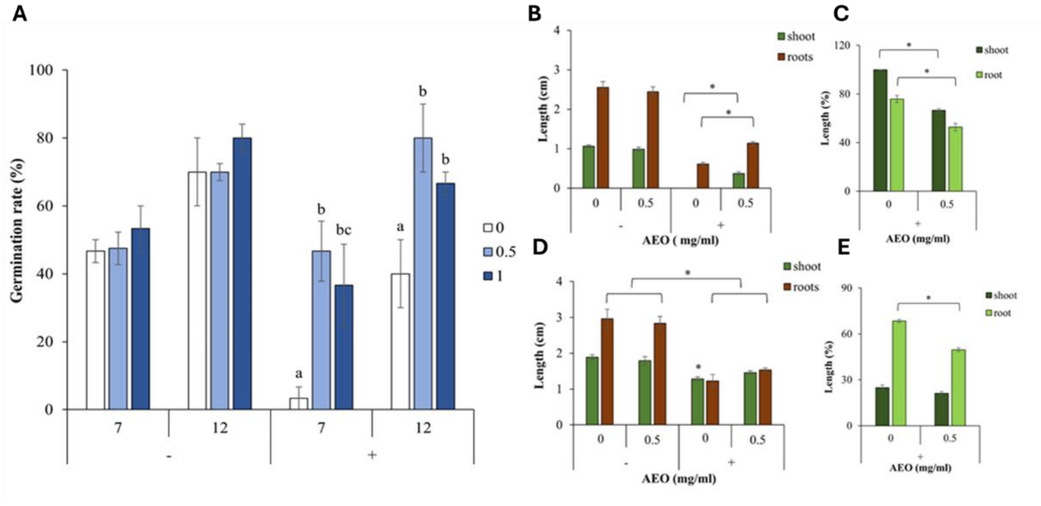


Figure 21: *Artemisia absinthium essential oil and Fusarium oxysporum effects on germination rate and growth of tomato seedlings.*

Figure 21A (-) shows that, in absence of the fungus, there were no significant differences in seed germination rate between AEO treated seeds with 0.5 mg/mL (light blue bars), 1 mg/mL (dark blue bars), or control watered seedlings (white bars). However, figure 21A (+), shows that in presence of *Fol* (+), the fungus severely inhibited germination in non-coated seeds (white bars), compared to the AEO treated ones with 0.5 mg/mL (light blue bars) and 1 mg/mL (dark blue bars). This shows that AEO contributed to seedling germination both at 7 and at 12 dpi in presence of *Fol*. Based on these results, the 0.5 mg/mL concentration of AEO, was chosen for further assays. Seedling root and shoot length was determined after 7 and 12 days in presence (+) and absence (-) of *Fol* (10^6 spores/mL) from pre-treated seeds with an AEO coating of 0.5 mg/mL (0.5), or 1 mg/mL (1). Figure 21B (-) shows that, at control plants, after 7 days, AEO did not affect shoot growth (dark green bars) or root growth (brown bars), in absence of the fungus (-). However, figure 21B (+) shows that, in presence of the fungus (C), AEO raises plant resistance to *Fol* as determined by increased root and shoot length, compared to controls. Figure 21C represents the percent of growth inhibition of shoots (dark green bars) and roots (light green bars) compared to controls in presence of the fungus (+). Growth inhibition of roots and shoots was higher for inoculated plants in absence of AEO. Figure 21D (-) shows that after 12 days, into control non inoculated plants (-), AEO did not affect shoot growth (dark green bars) or root growth (brown bars). Furthermore, as shown in Figure 21D, in presence of the fungus (+), an increase in root and shoot length happens, in AEO coated seedlings, showing that AEO contributes to plant tolerate to *Fol*. Figure 21E shows the percent of growth inhibition of shoots (dark green bars) and roots (light green bars) compared to controls in presence of the fungus (+). The inhibition observed on growth, was higher on inoculated plants in absence of AEO, in roots but was not observed in shoots. The * denotes a statistically significant difference using variance check (*P-value* ≤ 0.05) and Duncan test, between bracket samples at (B, C), or (*P-value* ≥ 0.05) and Kruskal-Wallis test at (D,E). The * on (D) denotes a statistically significant difference related to all the other treatments.

III.1.1.4 *Artemisia absinthium* Essential Oil Effect on Tomato Seedlings Disease Parameters

The disease parameters of the seedlings were studied under hydroponic conditions in the presence and absence of *Fol*. Disease ratios were measured considering the different stages of germination and development of tomato at 7 and 12 days. After 7 days, disease ratios were measured completion of seed germination, where: 0: no symptoms and normal germination (shoots and roots longer than 2 cm); 1: Delayed germination, showing radicle with no shoot; 2: Shoot and root measuring less than 2 cm; 3: Necrotic germinated seeds; 4: necrotic ungerminated seeds. And after 12 days during heterotrophic growth disease ratio was quantified, where: 0: no symptoms; 1: growth inhibition of shoots and roots and leaf chlorosis; 2: leaf necrosis and chlorosis; 3: leaf chlorosis and root necrosis; 4: decayed seedlings. The disease symptoms analysis indicated that the AEO treatment diminished the disease ratio both at 7 dpi (Figures 22A, C) and 12 dpi (Figures 22B, D).

AEO increased plant water content (15%, Figure 23A), and reduced fresh weight loss (FWL) (30%, Figure 23B). AEO also reduced other disease symptoms such as leaf necrosis (Figure 23C) and pigment content measured as total content of chlorophylls A and B (Figures 24A, B), and carotenoids (Figure 24C). Levels of all 3 pigments increased after 12 dpi compared to the corresponding controls into AEO treated seedlings (Figure 24D). The statistical differences using variance check (*P-value* ≤ 0.05) and Tukey test in (Figure 23-B) and (Figure 24-A and B), and Kruskal–Wallis test in (Figure 23-A) and (Figure 24-C).

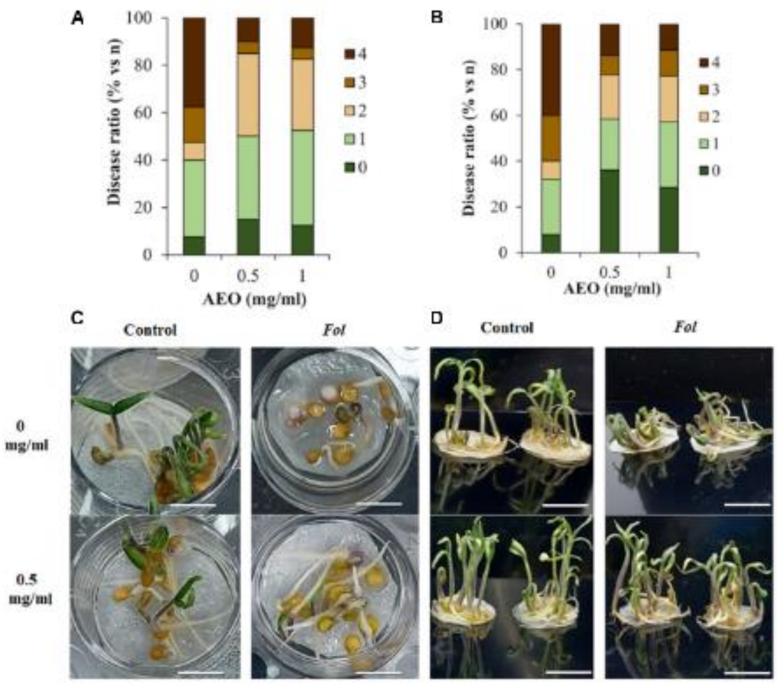


Figure 22: *Disease ratio under Artemisia absinthium essential oil treatment of 12 days seedling inoculated with Fusarium oxysporum. Bars: (1 cm).*

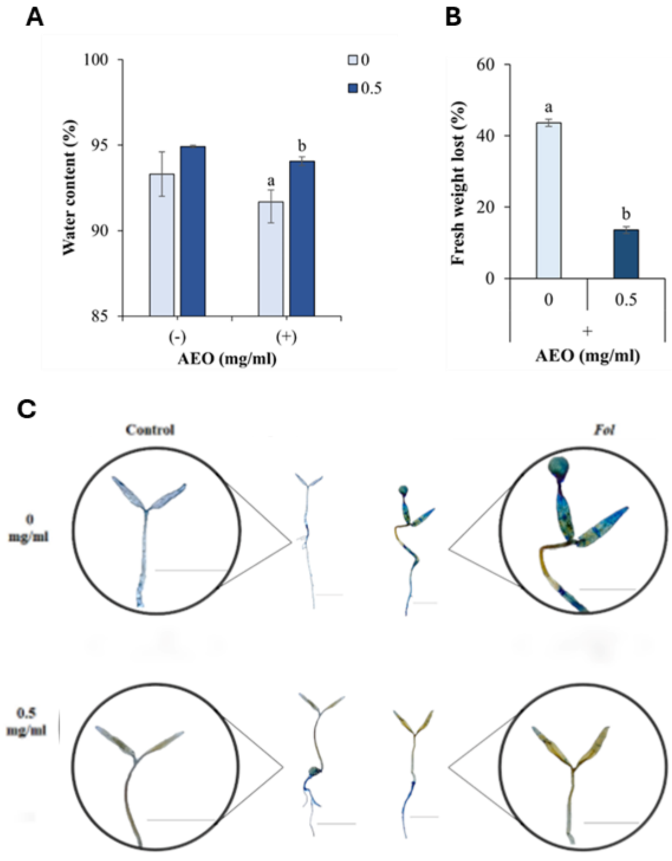


Figure 23: *Disease symptoms produced by Fusarium oxysporum in Artemisia absinthium essential oil treated tomato seedlings. (A) Water content, (B) Fresh weight loss, (C) Trypan blue staining showing necrosis induced by Fusarium oxysporum after 12 dpi. Amplification of TB staining of seedlings is shown in circles. treatment at concentrations of 0 (Controls at the top) and 0.5 mg/mL (at the bottom), and in absence (at the left) and in presence (at the right) of Fol. Bars (1 cm).*

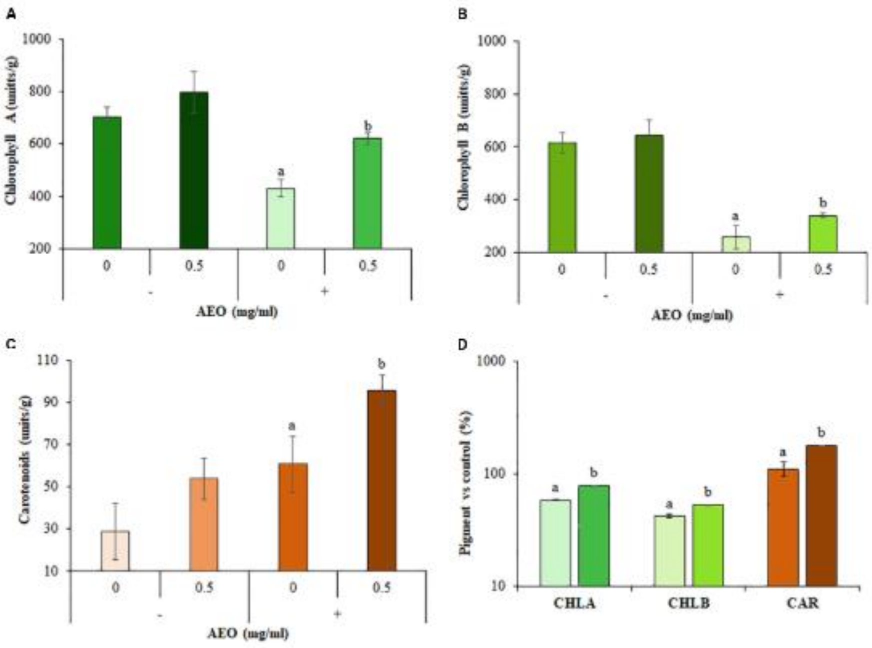


Figure 24: *Pigment content of tomato seedlings treated with Artemisia absinthium essential oil at 12 dpi. (A) Total content of Chlorophyll A (CHLA). (B) Total content of Chlorophyll B (CHLB). (C) Total content of carotenoids. (D) Pigment increase compared to respective controls*

The effect of AEO seed coating on reactive oxygen species production and callose deposition on seeds after germination was investigated to understand how AEO might protect seeds and seedlings against *Fol*. A kinetic study of seed response to AEO treatment (0.5 mg/mL) was conducted for this purpose. Coated seeds were stained with DAB and aniline blue to determine the reactive oxygen production (ROS) of seeds throughout the germination process and the effect of AEO on the callose deposition process, respectively. Staining intensity was measured after 30 min, 1 h and at 1, 4, 7, and 12 days after the coating treatment in the presence and absence of *Fol,* AEO or both treatments. As shown in Figure 25A, the AEO coating treatment increased callose deposition during the germination period, between days 4 and 7. Callose quantification (see section "Materials and Methods: II.12.4"), confirmed the observed increase in callose deposition. The increase in callose was maintained up to 12 days in the AEO treated, inoculated and non-inoculated seedlings, as represented in the histogram showing quantification of yellow pixels produced by callose measured with Image J program (Figure 25B). The * denotes a statistically significant difference using variance check (*P-value* ≥ 0.05) and Kruskal–Wallis test.

A high level of ROS production was also observed before germination in all seeds, as was a reduction in ROS production during seed germination (between 4 and 7 days) in control and AEO treated seeds in absence of the fungus (Figure 26A). The ROS level was high, on infected with *Fol*, even after the germination state. However, ROS levels in control seeds, seeds treated with AEO and AEO pre-treated and infected seeds, decreased once the germination process started (Figure 26A). The quantification of DAB signal in the histogram measured with Image J program confirmed those data (Figure 26B). A similar staining analysis was performed on seedling leaves but no ROS or callose depositions were observed in that tissue (data not shown). In day 4, the * denotes a statistically significant difference using variance check (*P-value* ≤ 0.05) and Duncan test. In day 12, the * denotes a statistically significant difference using variance check (*P-value* ≥ 0.05) and Kruskal–Wallis test. Tomato seeds were coated with 0.5 mg/mL of AEO.

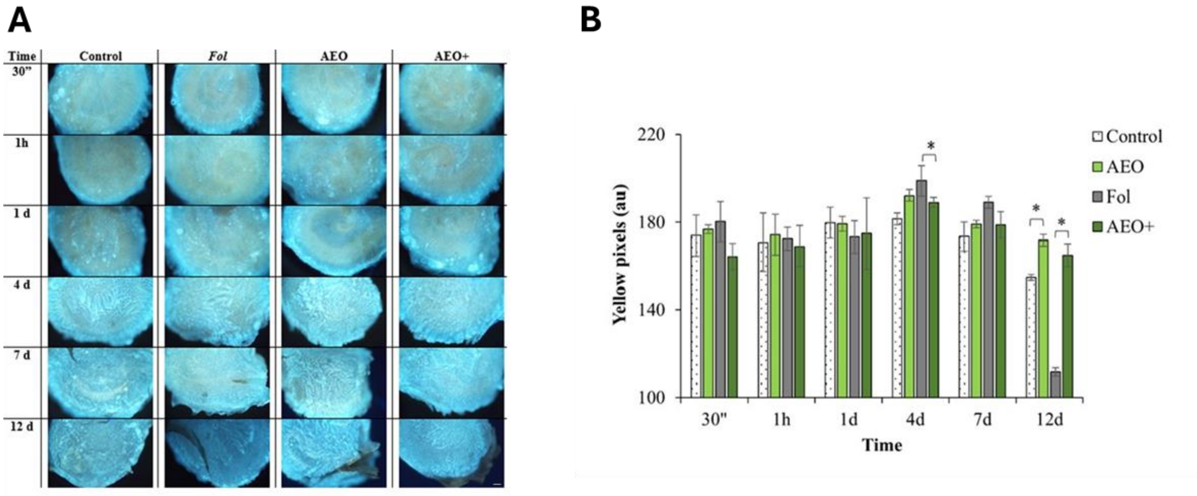


***Figure 25**: Kinetic of callose deposition on tomato seeds pre germination and post germination treated (AEO and FEO) or non-treated (Control and Fol) with Artemisia absinthium essential oil and infected with Fusarium oxysporum (Fol and FEO) or non-infected (Control and AEO). Bars (1 mm).*

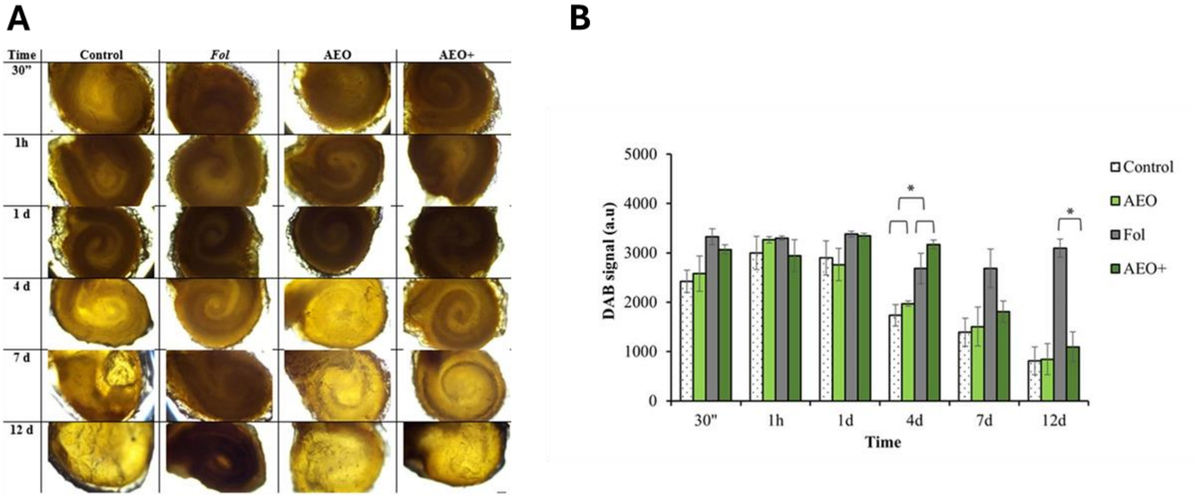


***Figure 26:** Kinetic of Reactive Oxygen Species Production (ROS) on tomato seeds. Bars (1 mm).*

III.1.1.5 Analysis of Seedling Response to *Artemisia absinthium* Essential Oil by RNA-Seq and Metabolomics:

While the short-term compounds involved in tomato's recognition of *Fol* have been described (Berrocal-Lobo & Molina, 2008; de Lamo & Takken, 2020; Kaplan et al., 2006) studies characterizing the long-term and "*de novo*" synthetized molecular compounds involved in plant tomato defense against *Fol* are still scarce. This work focuses on characterizing the effect that AEO has on tomato's long-term defense response to *Fol*. RNA-seq sequencing of plant genome after 12 dpi enabled us to determine the longer-term transcriptional changes produced by AEO in terms of the immunity response of tomato seedlings (see section Materials and Methods:

II.16). A volcano Plot performed based on DEG analysis (Figure 27A-C) allowed us to determine that the number of genes transcriptionally upregulated or downregulated was substantially higher in shoots than in roots, 1061 being induced in shoots compared to 526 in roots, 1174 genes repressed in shoots compared to 323 in roots (Figures 27B–D).

DEG analysis of shoot tissue using Venn diagram software (VENNY2.1: https://bioinfogp.cnb.csic.es/tools/venny/index.html); showed a significant number of genes that are transcriptionally induced or repressed (Figure 29A) by the AEO treatment, 925 genes upregulated and 1059 repressed, respectively. A small number of genes was sufficient to perform a DEG analysis of the roots. The KEGG analysis determining pathway enrichment (see section "Materials and Methods: II.16.2 ") enabled us to identify the metabolomic pathways involved in the biosynthesis of secondary metabolites, linolenic acid, phenylpropanoids, monoterpenoids (Mcgarvey & Croteau', 1995), amino acid degradation, and plant-pathogen interactions, i.e., the main pathways induced as part of the long-term defense response of tomato seedlings (Figure 29B, left panel). Significant inhibition of other metabolic pathways was repressed, including the biosynthesis of secondary metabolites, the primary metabolism of carbon and glyoxylate and dicarboxylate metabolism, showing a transcriptional plant response to AEO affecting some specific metabolic pathways but not others (Figure 29B, right panel). Raw data were submitted, GEO accession number GSE186754.

RNA-seq results, showed that *NRPD2* mediating in *de novo* cytosine methylation by RNA-directed DNA methylation pathway (RdDM) (López et al., 2011), is highly induced after 12 days of treatment with AEO in *Fol* infected seedlings (Figure 28-A). Therefore, qRT-PCR was performed to confirmed, in addition to *WRKY33* transcription factor, involved in the epigenetic control of plant defense against necrotrophs (Alvarez-Venegas et al., 2014; Ramirez-Prado et al., 2018) (Figure 28-C)and to *SAMt* that are DNA cytosine methylation markers for transcriptional RNA silencing (Pooggin, 2013) (Figure 28-B).

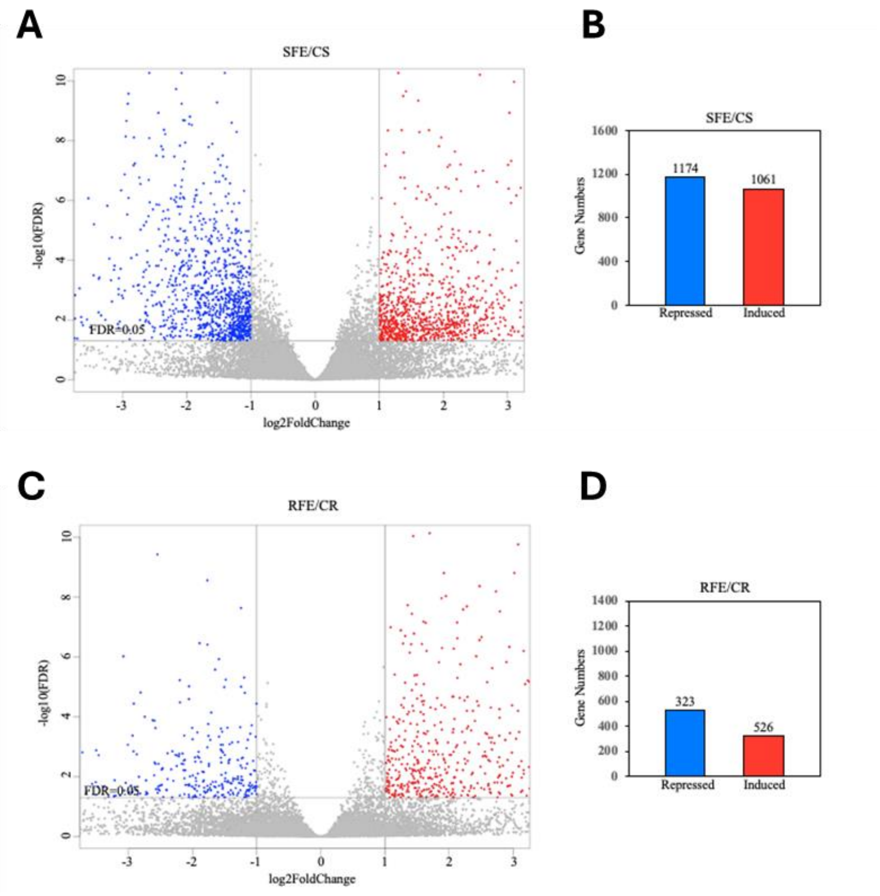


Figure 27: *Comparison of RNA-seq data between shoot and root assays. (A, C) Volcano plots made on shoots (upper) and roots (lower). Up-and downregulated DEGs are reported as red and blue dots, respectively. No DEG contribution represented as gray dots. (B,D) Number of induced or transcriptionally repressed genes corresponding to volcano analysis.*

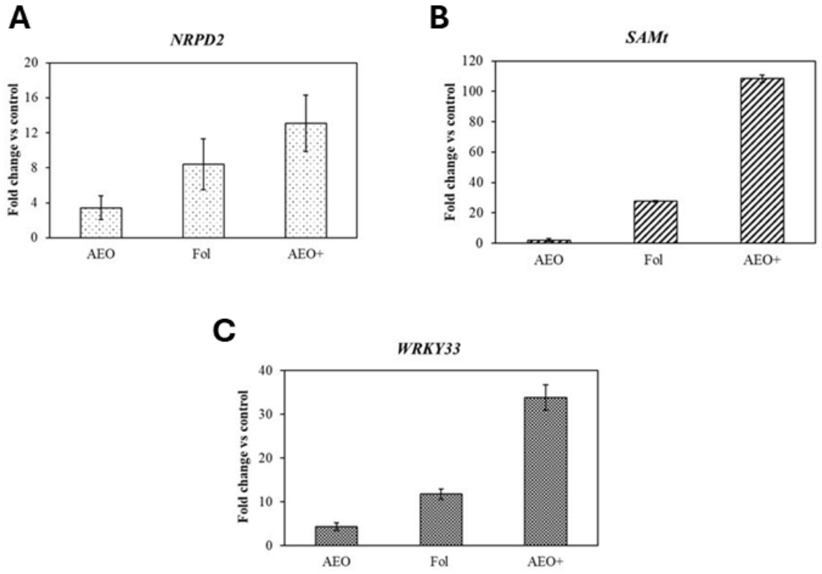


Figure 28: *Transcriptional levels of DNA-directed RNA polymerase IV and V subunit 2 NRPD2 (A), S-adenosyl-L-methionine-dependent methytransferase 2 SAMt (B), and the transcription factor WRKY33 (C), genes determined by qRT-PCR in tomato seedlings, after 12 days treated with Artemisia absinthium essential oil, infected with Fusarium oxysporum, or treated with Artemisia absinthium essential oil and infected with Fol.*

III.1.1.6 Metabolomic Effects of *Artemisia absinthium* Essential Oil on Tomato Seedlings:

Because tomato is an herbaceous plant very rich in metabolites and them give additional properties to tomato (Perveen et al., 2015), much of them involved into defense response against phytopathogens, such as lycopene, coumarin (López-Gresa et al., 2010; López-Gresa et al., 2011). It was deemed necessary and interesting to study the metabolomic changes induced in tomatoes by the effects of AEO. In collaboration with the ICA group (CSIC), specialists in this technique (Bertrand et al., 2021; Gonzalez-Coloma et al., 2012; Morales-Sánchez et al., 2021), mass ions of 12-day-old tomato seedlings were analyzed using liquid chromatography mass spectrometry (LCMS) which is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (LC) with the mass analysis capabilities of mass spectrometry (MS). This powerful combination allows for enhanced metabolites analysis (Pitt, 2009). The results revealed induced metabolites such as vanillic acid (179, MCH), coumarin (147, MCH), lycopene (537, MCH), and an unknown metabolite of m/z 529 potentially linked to lycopene (Table 4).

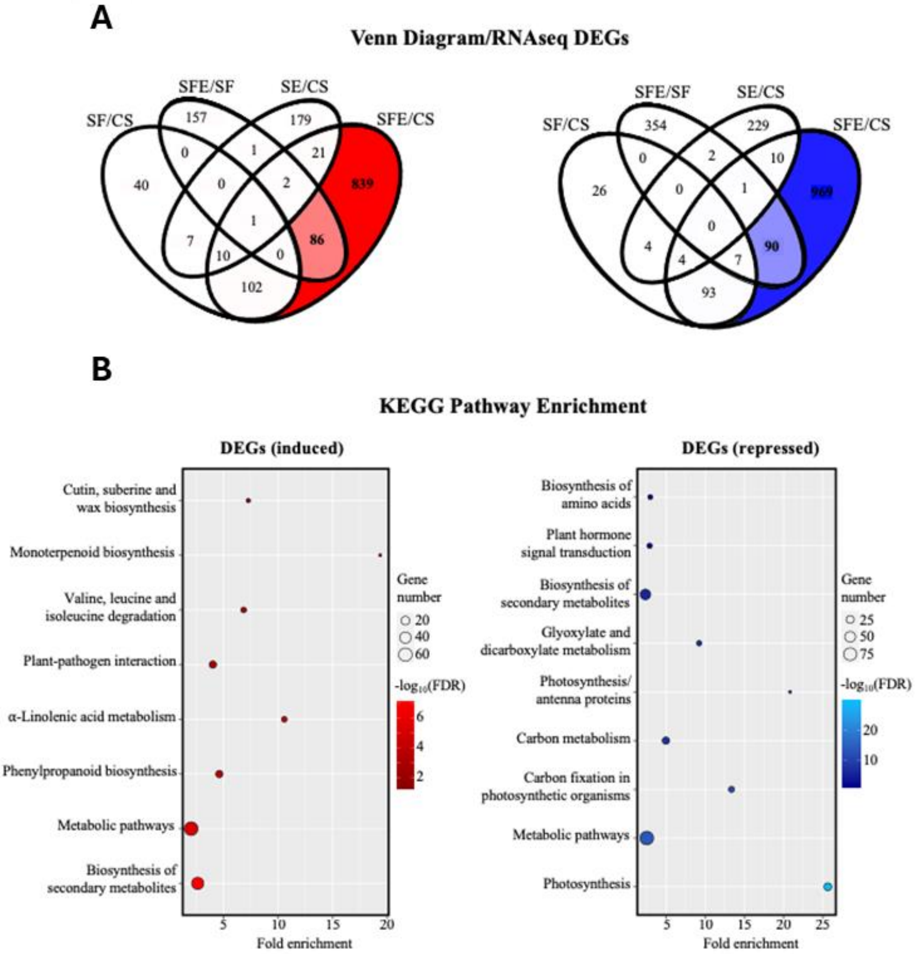


***Figure 29**: Analyses of RNA-seq data from seedlings (shoots). (A) Identification of differentially expressed genes (DEGs); Correspondences are (CS, control plants; SE, plants treated with Artemisia absinthium essential oil; SF, seeds inoculated with Fusarium oxysporum; and SFE, AEO coated seeds inoculated with Fol. (B) KEGG pathway enrichment analysis of DEGs obtained from Venn diagram in RNA-seq. Gene number corresponds to the number of target genes in each pathway. Fold enrichment is the ratio (observed/expected) of the number of DEGs in a certain pathway. FDR-value is corrected P-value. The color and size of the dots represents the range of the –log10(FDR)-value and the number of DEGs mapped to the indicated pathways, respectively. Top enriched pathways are shown in the figure.*

Table 4: *LC-MS m/z adducts of main metabolites detected in tomato seedlings extracts analysed by LC-MS.*

Retention time (min)	[M+H]+ m/z	Identification
2.93	179	Vanillic acid
3.49	147	Coumarin
32.10	537	Lycopene
34.53	529	Lycopene-related

All these compounds exhibited induction in the presence of AEO and *Fol*, with the compound of m/z (529) showing the strongest response (Figure 30). Gas Chromatography coupled with Mass Spectrometry (GCMS) analysis of the dichloromethane fraction of the MeOH extracts revealed several compounds, including palmitic acid methyl ester (MP), linoleic acid methyl ester (ML), oleic acid methyl ester (MO), stearic acid methyl ester (MS), and oleoamide (OA) (Figure 31). These results demonstrated a significant induction of the lipid oleoamide, even in the presence of AEO plus *Fol*.

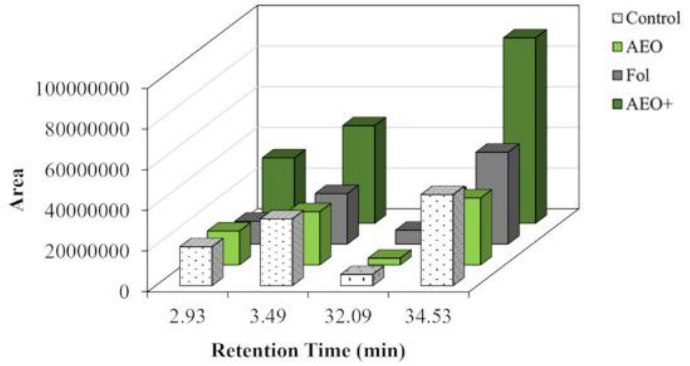


Figure 30: *Metabolomic analysis in Artemisia absinthium essential oil tomato seedlings. Metabolite induction is represented by arbitrary units. Correspondences are (CS, control plants; SE, plants treated with AEO; SF, seeds inoculated with Fusarium oxysporum; and SFE, AEO coated seeds and inoculated with Fol).*

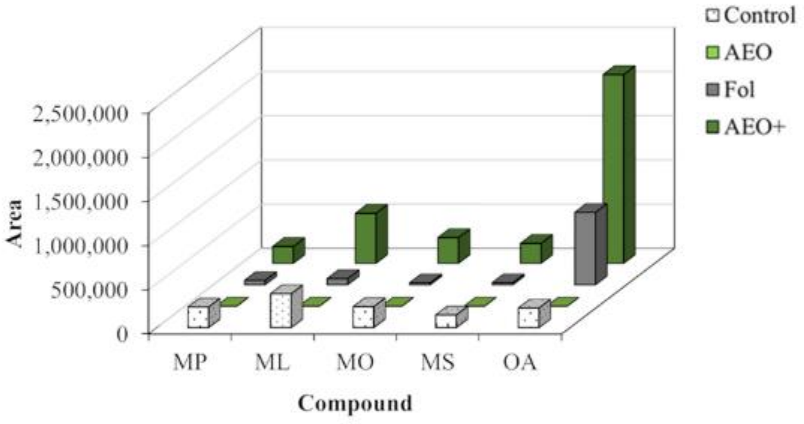


Figure 31*: GC-MS analysis of the DCM-soluble fraction of Artemisia absinthium essential oil tomato seedlings. The analysed compounds are palmitic acid methyl ester (MP), linoleic acid methyl ester (ML), oleic acid methyl ester (MO), stearic acid methyl ester (MS), and oleoamide (OA). CS, corresponds to control plants; SE, plants treated with AEO; SF, seeds inoculated with Fusarium oxysporum; and (SFE, AEO coated seeds, inoculated with Fol).*

III.1.1.7 Experiment in Soil: Effect of Coating Tomato Seeds with *Artemisia absinthium* Essential Oil Infected with *Fusarium oxysporum*:

To test the effects of tomato seed coating with AEO and test the oil's efficiency, three experiments were realised where the tomato seeds were coated with 0.5 mg/mL which is the same concentration that proved efficiency *in vitro*, and the disease symptoms were quantified at 7 days.

III.1.1.7.1 <u>**Germination Rate and Length of Shoots and Roots:**</u>

The figure (32-A) shows the results of seeds germination at 7 dpi, in absence of the fungus (-), there were no significant differences in seed germination rate between AEO treated seeds with 0.5 mg/mL (light blue bars) and control watered seedlings (white bars). However, in presence of the *Fol* (+) the fungus inhibited germination in non-coated seeds, compared to the AEO treated ones with 0.5 mg/mL showing a significative difference. This confirms that AEO contributed to seedling germination at 7 dpi, even in soil assays.

Seedling root and shoot length was also determined after 12 days in presence (+) and absence (−) of *Fol* (10^6 spores/mL) from pre-treated seeds with an AEO coating of 0.5 mg/mL (0.5). The figure (32-B) shows that in absence (-) of *Fol* the length of shoots are induced in seedlings treated withl AEO compared with controls with a significant difference, while it doesn't affect significantly the length of roots. In Presence of the fungus (+), AEO raises plant resistance to *Fol* as determined by increased root and significantly shoot length, compared to controls. In (A), statistical differences were assessed using variance check (*P-value* ≤ 0.05) and Duncan test. In (B), * indicates a statistically significant difference in leaves determined by variance check (*P-value* ≤ 0.05) and Duncan test, while no significant difference was observed in roots.

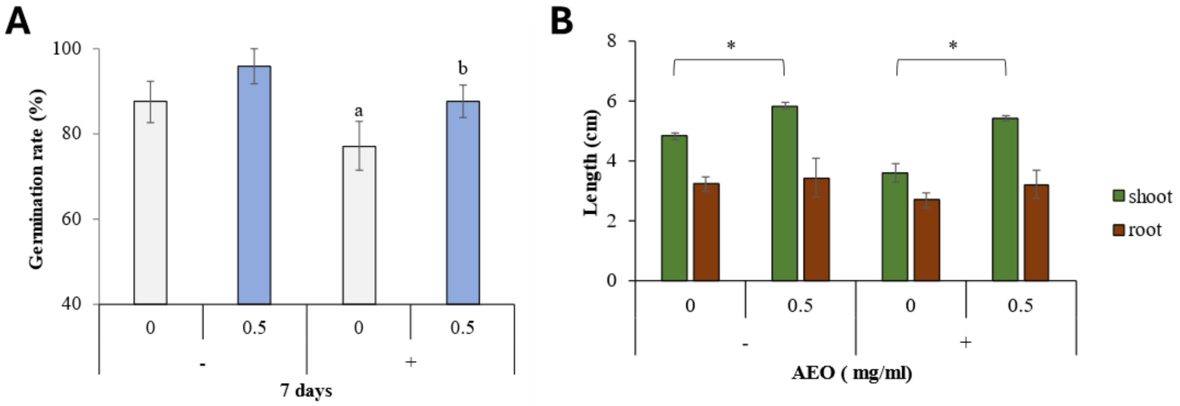


Figure 32: *Effects of* Artemisia absinthium essential oil *and* Fusarium oxysporum *on Germination Rate and Growth of* tomato *seedlings in* soil*.*

III.1.1.7.2 <u>**Effects on Fresh Weight and Water Content:**</u>

Figure (33) shows that coating with AEO significantly increases water content in seedlings compared to control when infected (+) or non- infected (-) with *Fol*. The treatment reduces also the fresh weight loss. Seedlings were inoculated with 10^6 spores/mL *Fol*. Results are shown after twelve days of inoculation. * Denotes a statistically significant difference using variance check (*P-value* ≤ 0.05) and Kruskal-Wallis test.

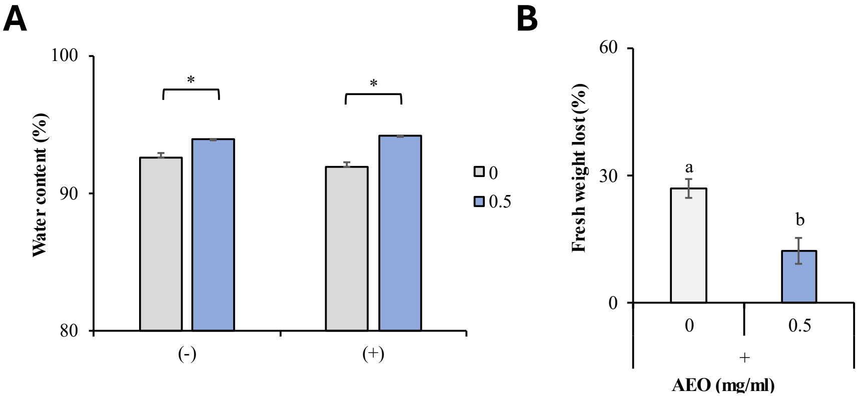


Figure 33: *Disease symptoms produced by Fusarium oxysporum in Artemisia absinthium essential oil -treated tomato seedlings in soil after 12 dpi. (A) Water content (%) in the absence (-) of Fol and in the presence (+), coated with AEO or without coating (0). (B) Fresh weight loss (%) of infected seedlings with Fusarium oxysporum.*

III.1.1.7.3 Leave Growth and Chlorophyll Content:

In Figure 34 (A and B), it does not appear that AEO affects pigments A and B compared to the control in the absence of *Fol* (-). However, it shows that the pigment content of chlorophyll A and B is increased when coated with AEO and infected with 10^6 spores/mL of *Fol* (+), while it is inhibited in the seedlings that are infected but not treated. The statistical analysis shows a significant difference using variance check (*P-value* ≤ 0.05) and Kruskal-Wallis test

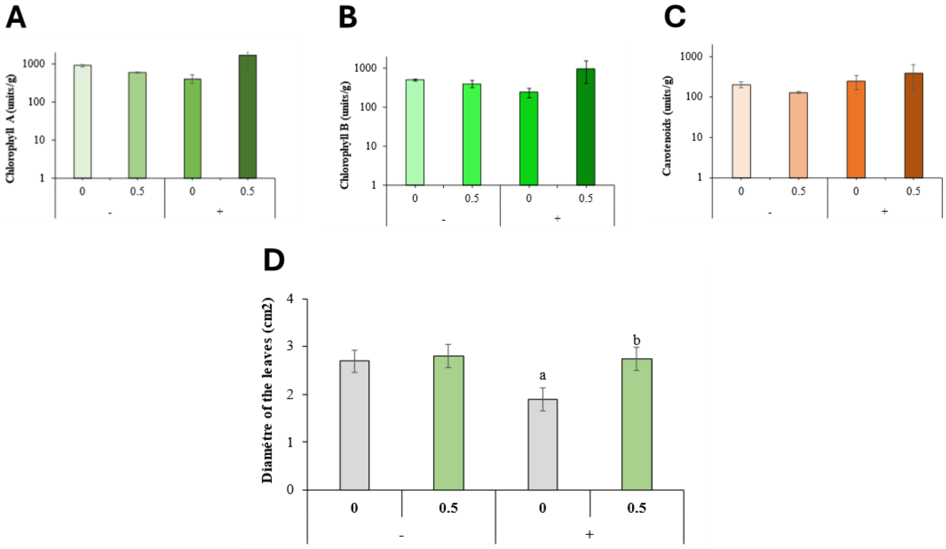


Figure 34: *Effect of Artemisia absinthium essential oil on seedling's leaves and their pigment content at 12 dpi of infection with Fusarium oxysporum in soil. (A) Chlorophyll A content (unit/g), (B) Chlorophyll B content (unit/g), (C) Carotenoids content (unit/g), (D) Diameter of the leaves (cm^2).*

III.1.1.8 Study on The Effect of Coating Tomato Seeds with Hormones on The Germination:

In addition to enhancing defense against pathogens, there was an interest in evaluating hormones as natural products through *in vitro* assays aimed at enhancing seed germination.

Germination was monitored daily up to day 7, a critical period for tomato seed germination, and appropriate concentrations for future investigations were identified.

III.1.1.8.1 **Salicylic Acid (SA) :**

Figure (35) shows that the hormone of SA increases germination rate at both concentrations 0.025 and 0.1 mM by 5% after 7 days of growth without a significant difference compared to the control that were coated with water. The statistical analysis using variance testing (*P-value* ≤ *0.05*) showed no significant changes.

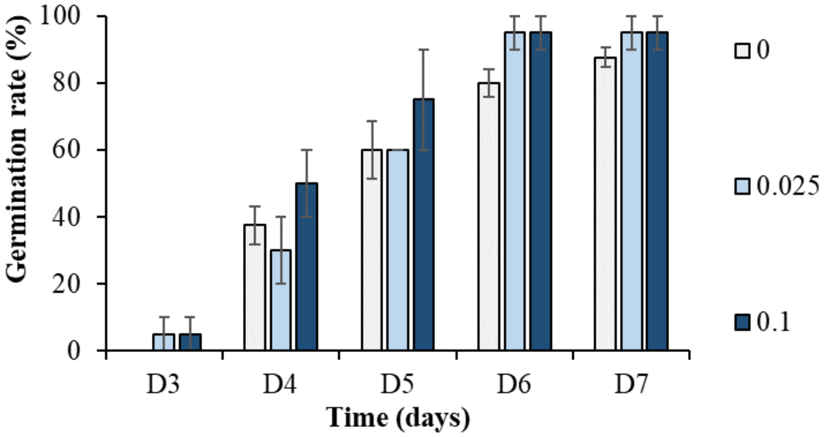


Figure 35: *Kinetic of germination rate from 3 to 7 days of tomato seeds coated with Salicylic acid at two different concentrations 0.025 mM and 0.1 mM, and 0 represents control watered seeds.*

III.1.1.8.2 **Methyl Jasmonate Acid (MeJA) :**

MeJA was tested on tomato seeds at different concentration: 0.01, 0.1, 0.25 and 2.5mM. The figure (36) shows that the hormone of MeJA increases germination rate at the concentrations of 0.25 and 2.5 mM by approximately 10% after 7 days of growth without a significant difference compared to the control that were coated with water. While the concentration at 0.1 mM seems to inhibit the germination. The statistical analysis in day 4, shows a significant difference using variance check (*P-value* ≤ 0.05) with Tukey test.

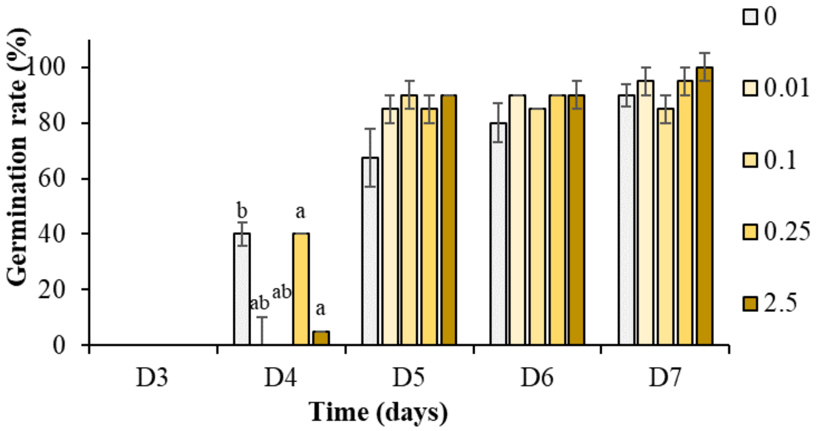


Figure 36: *Kinetic of germination rate from 3 to 7 days of tomato seeds coated with Methyl jasmonate acid at two different concentrations (mM) and 0 represents control watered seeds.*

III.1.1.8.3 1-Aminocyclopropane 1-Carboxylic Acid (ACC) :

Figure (37) shows that the hormone ACC increases the germination rate by 8% at a concentration of 10 uM after 7 days of growth, with no significant difference compared to the control coated with water (0), while it is only increased by 3% at 0.25 uM. The statistical analysis using variance testing (*P-value* ≤ 0.05) showed no significant changes.

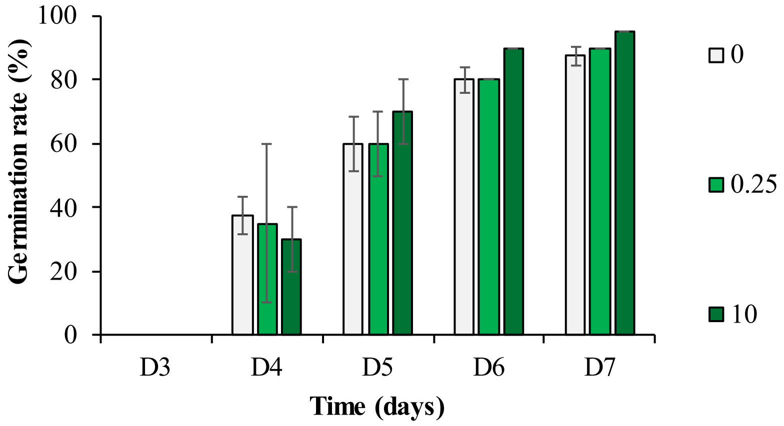


Figure 37: *Kinetic of germination rate from 3 to 7 days of tomato seeds coated with 1-aminocyclopropane 1-carboxylic acid at two different concentrations (uM) and 0 represents control watered seeds.*

III.1.1.9 Study on The Effect of Coating with Pathogen Extracts:

Other natural products were tested as putative plant growth promoters, namely extracts originally derived from pathogens. Two extracts of phytopathogens were used as coating of tomato seeds *in vitro* experiments. The effect of this treatment was monitored over 7 and 12 days. The parameters measured to asses this natural product effectiveness were: quantification of germination during the first days, measuring water content and length of shoots and roots to understand the physiological state and overall health of the seedlings at 12 days.

III.1.1.9.1 ***F. oxysporum* Extract :**

Because pathogens contain several antimicrobials as well as proteins activating plant defense responses, the effect of extracts obtained from *Fol* on tomato coating assays was studied. This pathogen extract was tested on tomato seed as a coating treatment at different concentrations (0, 0.005, 0.01, 0.1, 0.2, 0.5 %). The germination rate was *Fol*lowed up to 7 days and the figure (38-A) shows that this extract promotes seed germination at the lowest concentration (0.005, 0.01, 0.1%) until 100% of germination rate, while the high concentrations negatively affect germination compared to control seeds coated with water (0). This product also increases the water content of the plants after 12 days of growth, as can be seen in Figure (38-B), again at the lower concentrations (0.005, 0.01%). While the higher concentrations (0.2, 0.5%) appear to be toxic as the water content decreases compared to control seedlings (0). Figure (38-C) shows the length of shoots and roots measured at 12 days of growth, demonstrating that the concentration 0.01% of the treatment increases both shoot and root length in tomato seedlings while the higher concentrations (0.2, 0.5%) inhibits significantly their growth compared to control (0). The statistical analysis shows a significant difference using variance check (*P-value* ≤ 0.05) with Duncan test (A, B and C-shoots) and Kruskal-Wallis test (C-roots).

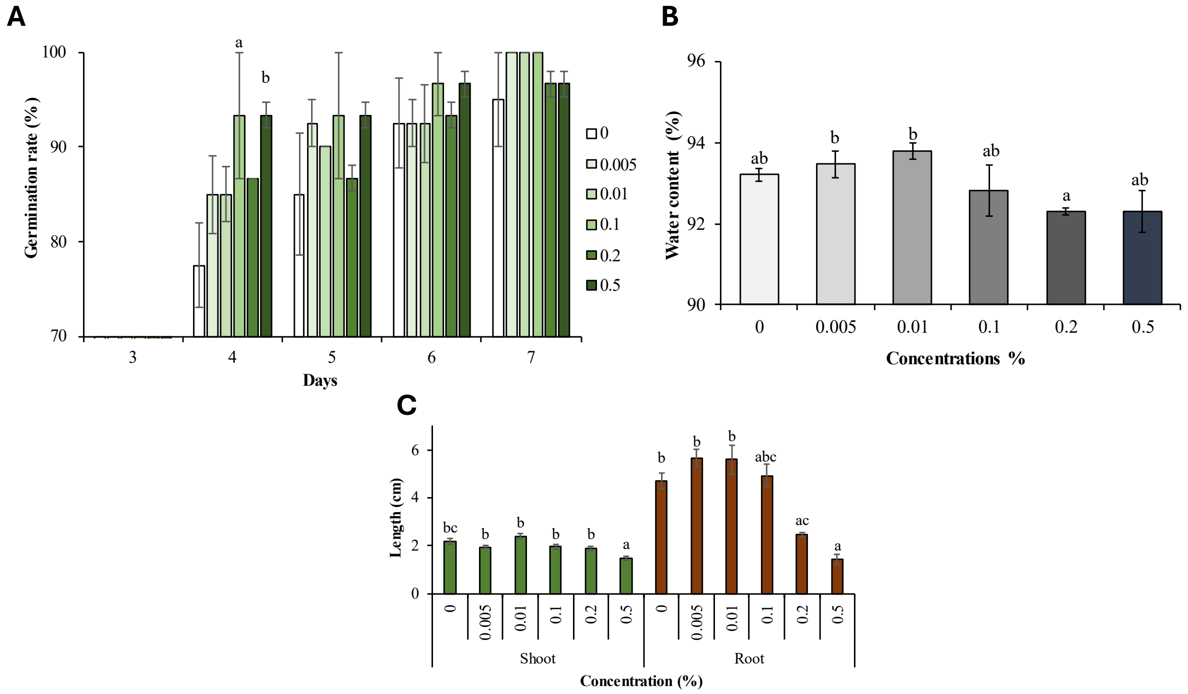


Figure 38: Effect of coating with Fusarium oxysporum extract at different concentrations (%) on tomato seedlings development compared to control (0). (A) Kinetic of germination rate (%) until 7 days. (B) Water content (%) after 12 days of growth. (C) Length of shoots and roots (cm) of tomato seedlings at 12 days of growth coated with Fusarium oxysporum extract.

III.1.1.9.2 ***Phytophthora cinnamomi* Extract:**

The *Pc* pathogen extract was tested on tomato seeds as a coating treatment at different concentrations (0, 0.01, 0.02, 0.2 and 0.5 %). The germination rate was *Fol*lowed for up to 7 days and the figure (39-A) shows that a 0.2% coating with this product promotes seed germination up to 100% of the germination rate, while the lower concentrations do not have much effect and the higher concentration of 0.5% inhibits germination compared to the control seeds coated with water (0).The *Pc* Product also enhances the water content of the plants at a concentration of 0.2% after 12 days of growth, as illustrated in Figure (39-B). Lower concentrations (0.01% and 0.02%) show minimal effect, while higher concentrations (0.5%) appear to be toxic, leading to a decrease in water content compared to the control seedlings (0). Figure (39-C) shows the length of shoots and roots measured at 12 days of growth, demonstrating that the concentration 0.2% of the treatment increases shoot length in tomato seedlings while concentrations 0.02% promote root growth significantly compared to control (0). The statistical analysis shows a significant difference using variance check (*P-value* ≤ 0.05) with Duncan test (A, B, C).

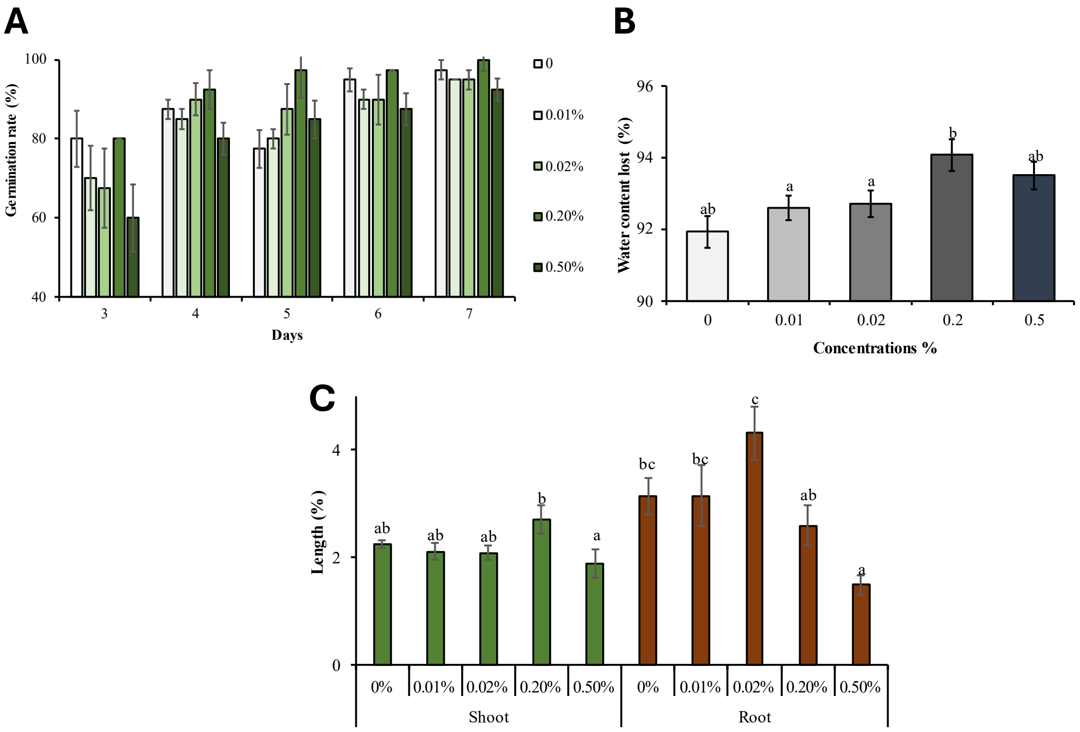


Figure 39: *Effect of coating with Phytophthora cinnamomi extract at different concentrations (%) on tomato seedlings development compared to control (0). (A) Kinetic of germination rate (%) until 7 days. (B) Water content (%) after 12 days of growth. (C) Length of shoots and roots (cm) of tomato seedlings at 12 days of growth coated with Phytophthora cinnamomi extract.*

III.1.2 Part 2: Seed Coating Prime Epigenetic Effect in Tomato Seeds at Early Stages of Development (Plant Memory).

Chapter 1, part 1, established a significant finding which is the induction of an epigenetic effect through the application of AEO *Fol*lowing a 12-day interval. The *Fol*lowing work was focused on comprehending tomato seed gene expressions throughout its developmental stages, both pre- and post-germination, since. The primary objective of this investigation was to ascertain whether the treatment involving this oil had a priming influence on the initial phases of seed growth. Thus, the confirmation of this hypothesis depended on first understanding the basic steps that come before this analysis.

Based on the physiology of tomato seeds, analysis was performed to determine if some marker genes on epigenetic changes described previously in tomato might be altered during the seed coating realised into our priming treatments (Bera et al., 2022; Spoelstra, 2002). Since the first step before seed coating with oils is the cold hydropriming of the seeds, this process allow the activation of the embryo and induce the germination process (Lutts et al., 2016).

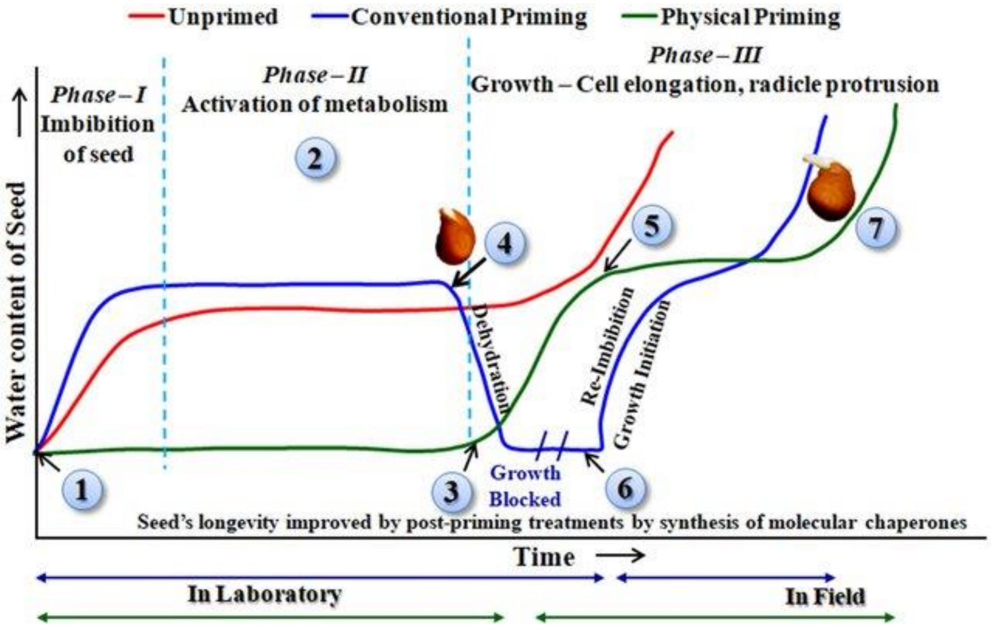


***Figure 40:** Seed hydration curves and different (physiological, biochemical and molecular) events in the seeds during the phases of germination in unprimed, Conventional primed and Physical primed seed* (Bera et al., 2022).

III.1.2.1 Understanding gene expression of tomato seeds during cold hydropriming:

The seeds were exposed to 12 hours of cold-hydropriming at 4°C in darkness (see methodology II.3). As it is shown in Figure (41), the expression of *HISTONE 1* (Figure 41-A) decreases in the first 2 hours and increases after 4 hours to 12 hours. The expression of *EXPANSIN 2* (Figure 41-B) is also reduced in the first 2 hours, then increases after 4 hours and stabilises until 12 hours. The expression of *NRPD2* and *SAMt* (Figure 41-C and D) is stable during cold hydropriming. The *LEH1* gene, when quantified, appears to exhibit no expression during the cold-hydropriming process. Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Duncan's test.

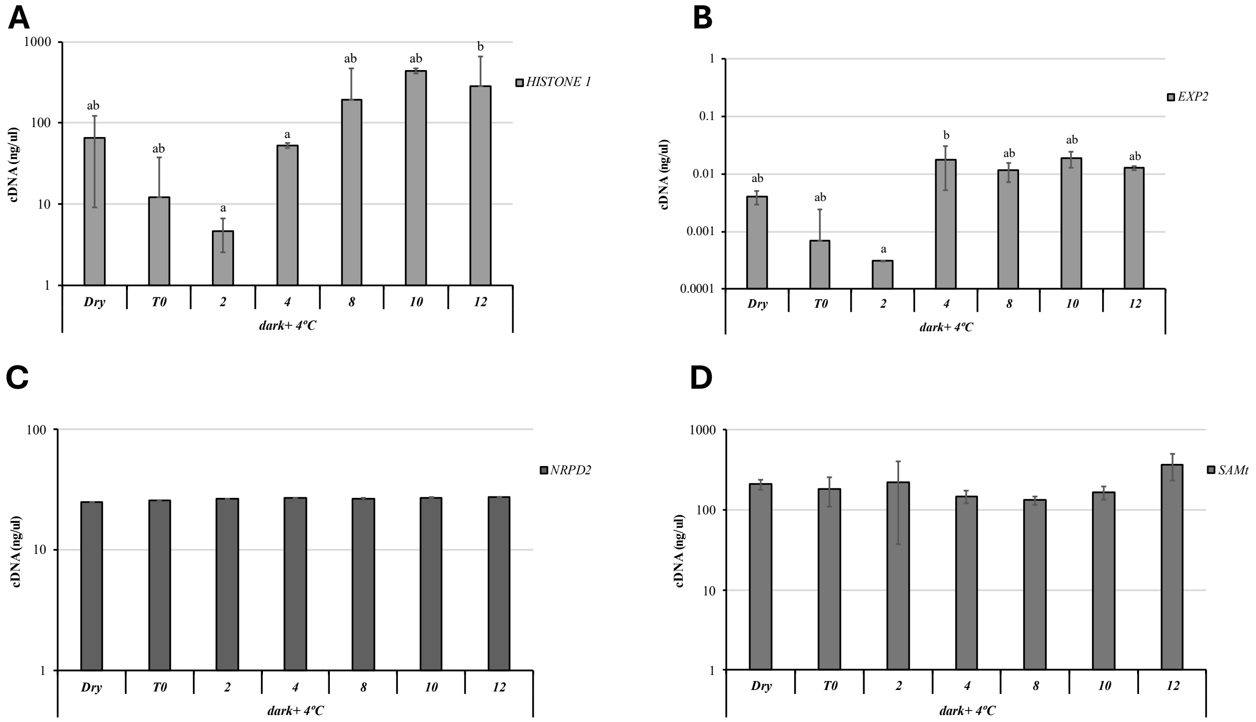


Figure 41: *Absolute quantification of genes expression during 12 hours kinetic of cold-hydropriming. (A) HISTONE 1, (B) EXPANSIN 2, (C) NRPD2, (D) SAMt.*

III.1.2.2 Understanding gene expression of tomato seeds prior germination:

After cold hydropriming, the seeds are prepared for transfer to growth conditions, and gene expression was quantified during the early stages prior germination under growth conditions (imbibed in water at 24°C and exposed to light see methodology II.3). The expression of *HISTONE 1* and *LEH1*, as depicted in Figure (42-A and C), significantly increases up to 12 hours and then decreases after 24 hours. Conversely, the expression of *NRPD2*, *EXPANSIN 2* and *SAMt* appears to continuously and significantly increase up to 24 hours (42-B, D and E). Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Duncan's test (A, B, D, E) and in (C) * denotes a statistical difference using the test of variance (*P-value* ≤ 0.05) with Kruskal-Wallis test.

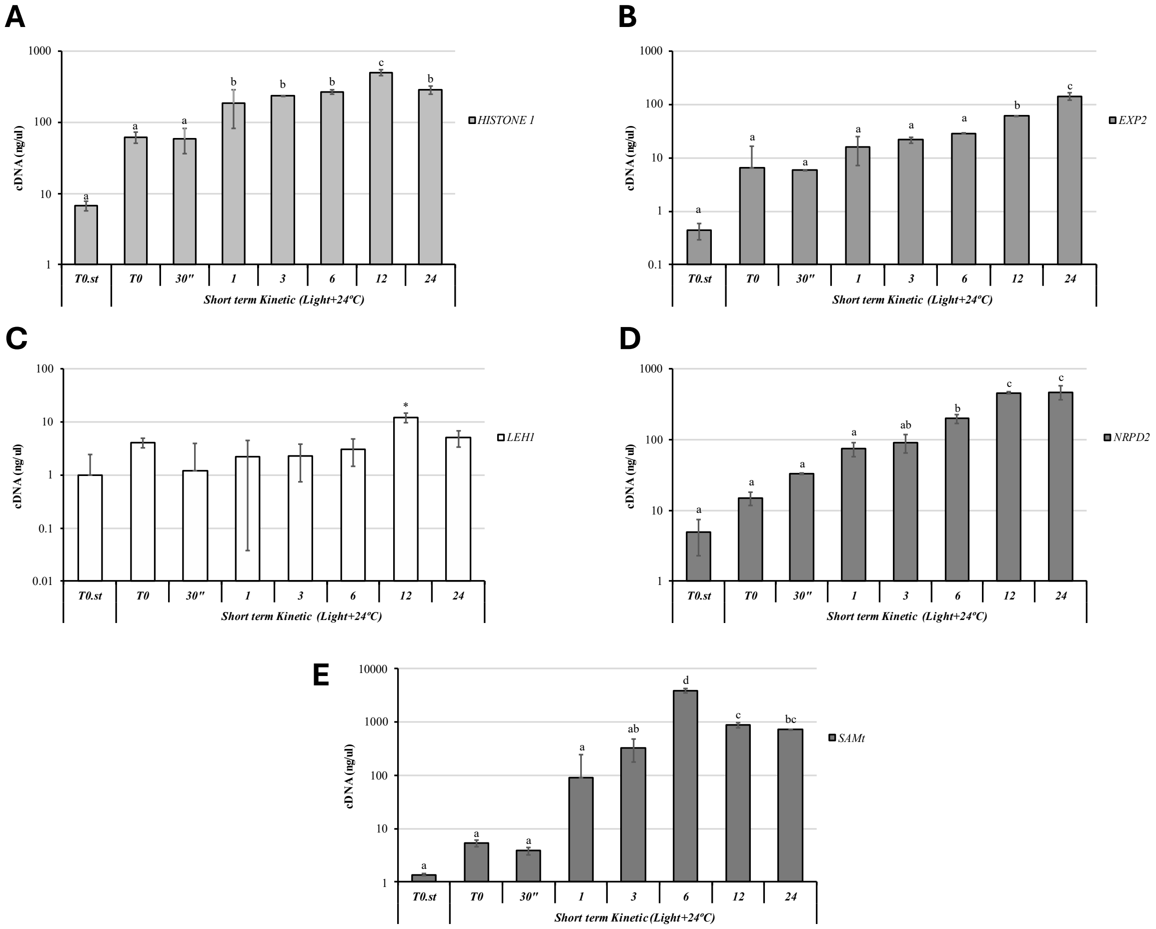


Figure 42: *Absolute quantification of genes expression during 24 hours kinetic in growth condition. (A) HISTONE 1(B) EXPANSIN 2 (C) LEH1 (D) NRPD2 (E) SAMt.*

III.1.2.3 Understanding gene expression of Tomato Seeds Pre- and Post-Germination:

After cold hydropriming, the seeds were transferred to growth conditions. The Gene expression was quantified before and after germination up to 12 days under these conditions (Figure 43). The expression of *HISTONE 1* increases significantly over the course of the days continuously until day 12 (Figure 43-A), while the expression of *EXPANSIN 2* increases after 2 days and then decreases significantly until day 12 (Figure 43-B). The expression of *LEH1* increases significantly until day 3 and then decreases significantly until day 12 (Figure 43-C). *NRPD2* expression is stable during the 12 days of growth (Figure 43-D). While *SAMt* increases significantly after 7 days up to 12 days (Figure 43-E). * Denotes a statistical difference using the test of variance (*P-value* ≤ 0.05) with Tukey test (A, B and E), and Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Duncan test (E, D).

The preceding qRT-PCR results validated the initial methylation process in tomato seeds before germination by monitoring the expression of genes responsible for methylation, such as S-adenosylmethionine transferases (*SAMt*) and DNA-directed RNA polymerase IV and V subunit 2 (*NRPD2*). These findings facilitated the identification of appropriate time points for subsequent investigations.

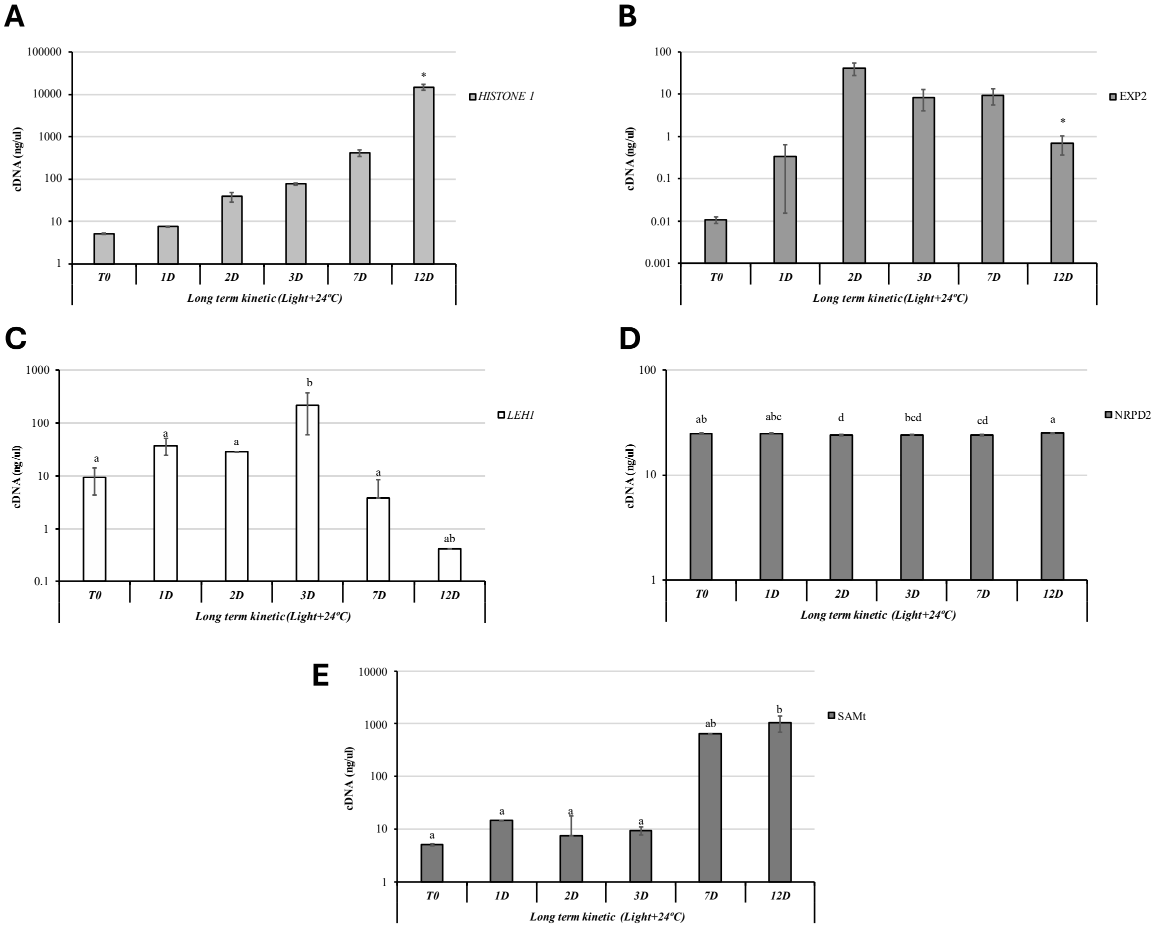


Figure 43: *Absolute quantification of genes expression during a kinetics in growth conditions from time 0 pre-germination until 12 days post germination. (A) HISTONE 1, (B) EXPANSIN 2, (C) LEH1, (D)NRPD2, (E) SAMt.*

III.1.2.4 Epigenetic- Related Transcriptional Changes on Coated Seeds with *Artemisia absinthium* Essential Oil after Inoculation with *Fol:*

Kinetics at short time intervals was realised onto coated seeds with AEO inoculated with 10^6 spores/mL of *Fol*, and in control water treated seeds, with and without inoculation. The transcriptional expression levels of three genes were included, those genes were previously involved into tomato methylation events and plant defense after 12 dpi (our previous Results in part 1: III.1.5). The levels of expression were quantified at short times up to 6 hours by qRT-PCR, the genes selected inlcuded *HISTONE 1*, *LEH1*, *NRPD2*, *SAMT* and *WRKY33*.

The figure (44-A and B) shows that the expression of *HISTONE 1*and *LEH1* increases slowly in the first moments under growth conditions, while it increases rapidly for *LEH1*, except after 6 hours, where infection appears to inhibit the expression of *LEH1*. Figure (44-C) shows that *NRPD2* increases after 3 hours in all seeds, with AEO-coated and *Fol*-infected seeds showing the highest expression. Up to 6 hours, expression continues to increase in seeds not infected with *Fol*, while it decreases in infected seeds. Nevertheless, AEO seems to induce *NRPD2* expression compared to the non-coated seeds. AEO coated and infected seeds appear to induce *SAMt* and *WRKY33* expression compared to control infected and non-infected seeds significantly especially after 30 minutes in growth conditions where it gets to its highest as showed by figure (44-D and E). Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Duncan test (C, D, E) and Kruskal-Wallis test (E- aft. Coating).

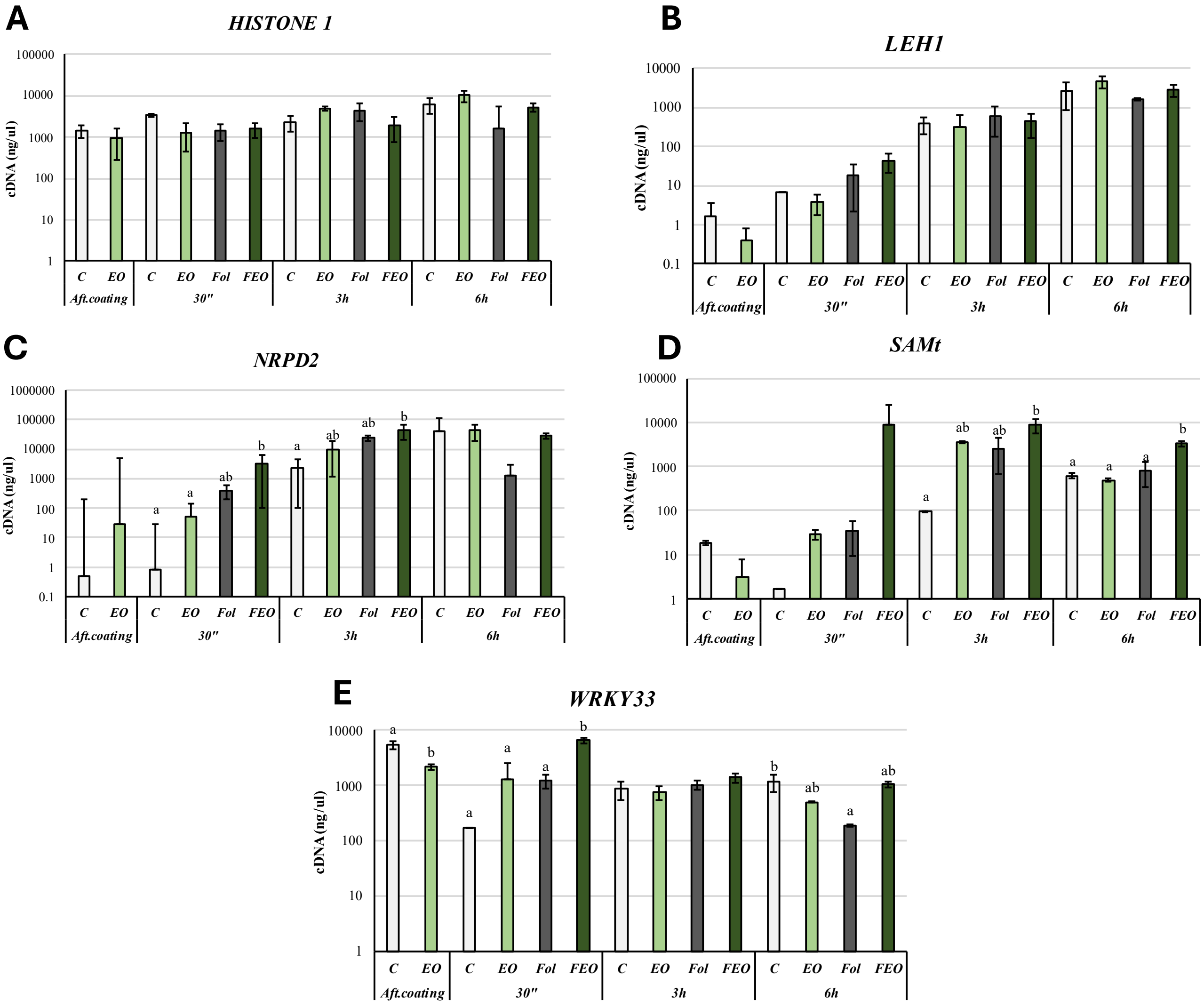


Figure 44: *Kinetic study of transcriptional changes on epigenetic-related genes. during growth conditions of tomato seeds from time "after coating" (Aft. Coating) until 6 hours, to quantify different gene expressions by absolute quantification qRT-PCR. (A) HISTONE 1 ,(B) LEH1, (C) NRPD2, (D) SAMt, (E) WRKY33.*

III.1.2.5 Quantification of Global Methylation Changes Occurring in Tomato Seeds by The Effect of AEO

To detect the methylation that happens on RNA, the levels of N6-methyladenosine was quantified, since it plays important roles in gene expression regulation, including transcriptional and post-transcriptional regulation (Tang et al., 2023); and the levels of 5-methylcytosine that is generally associated with repression of gene expression, using the EpiQuik m6A RNA Methylation Quantification Kit and MethylFlash 5mC RNA Methylation ELISA Easy Kit (explained in methodology: II.18). Interest was also directed towards detecting DNA methylation and quantifying 5-methylcytosine, which plays a significant role in regulating gene expression and TE silencing. For this purpose, the Global DNA Methylation 5 mC ELISA Easy Kit was employed.

III.1.2.6 Global m6A Methylation Changes on Total RNA:

The levels of 5-methylcytosine in RNA were quantified, to assess the extent of gene repression at early stages preceding seed germination, on seeds coated with AEO compared with control seeds shown in figure (45-A). And the levels of m6A in RNA, to know about gene expression regulation (49B-C). From the figure (45 A and B), It appears that AEO induces 5 mC levels up to 6 hours significantly compared to control, while m6A seems to be stable through time and the statistical analysis doesn't show a significant difference. the figure 45 (C) shows the levels of global m6A through short times, from thirty minutes, three hours and ten hours, during germination of seeds coated and non-coated with AEO and in presence or absence of *Fol*. It appears that both AEO and *Fol* induces m6A at 6 hours compared to control seeds. Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Duncan test (A, B) and Kruskal-Wallis test (C).

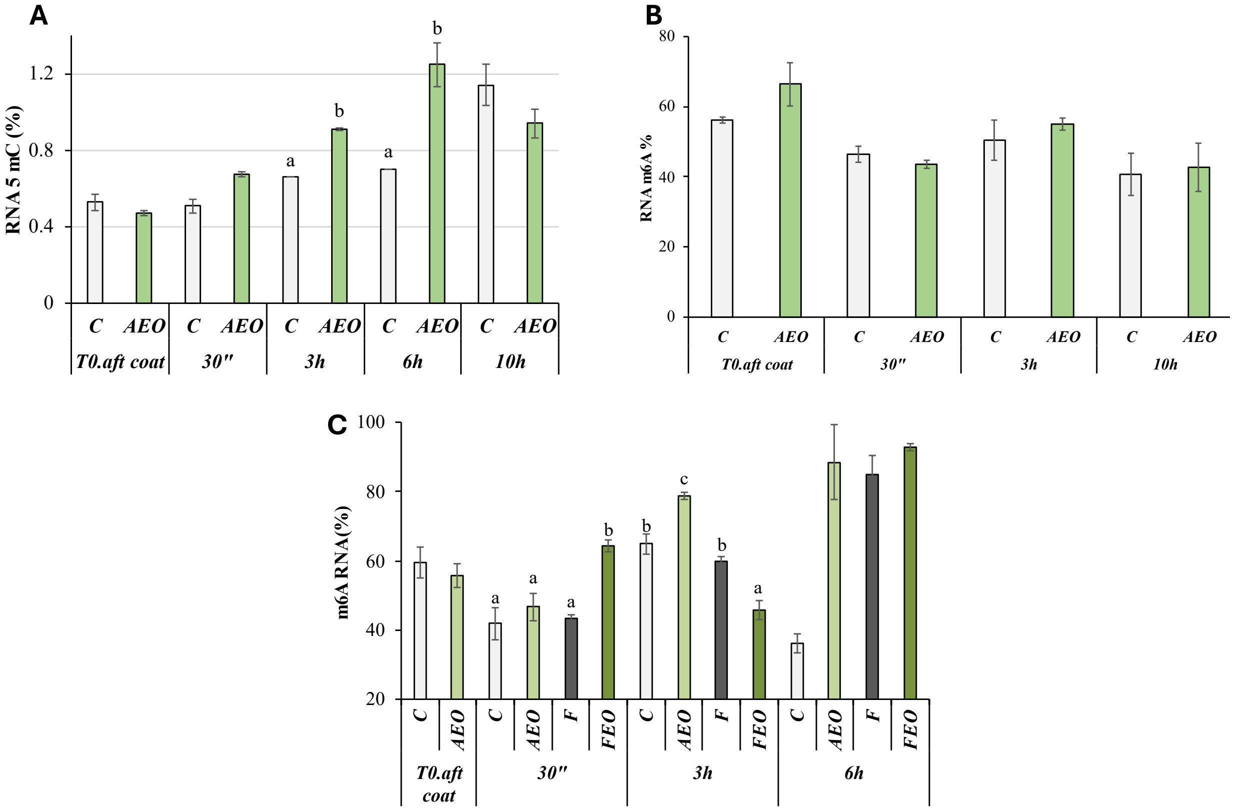


Figure 45: *Kinetic of Global methylation levels in RNA at early stages of treatments on tomato seeds coated with Artemisia absinthium essential oil or with water in control. (A) 5-methylcytosine, (C). (B) N6-methyladenosine, (C) N6-methyladenosine levels in tomato seeds coated with Artemisia absinthium essential oil or with water in control in absence or presence of Fusarium oxysporum (F and FEO).*

III.1.2.7 Global Methylation Level in DNA:

The figure 46 demonstrates levels of 5 mC in DNA through short times in growth conditions on seeds coated and non-coated with AEO and in presence or absence of infection with *Fol*. It appears that up to 3 hours AEO induced 5 mC levels compared to control seeds. Furthermore, the presence of *Fol* in AEO-coated seeds leads to a substantial increase in 5 mC levels. Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Tukey test. Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Tukey test. Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Tukey test.

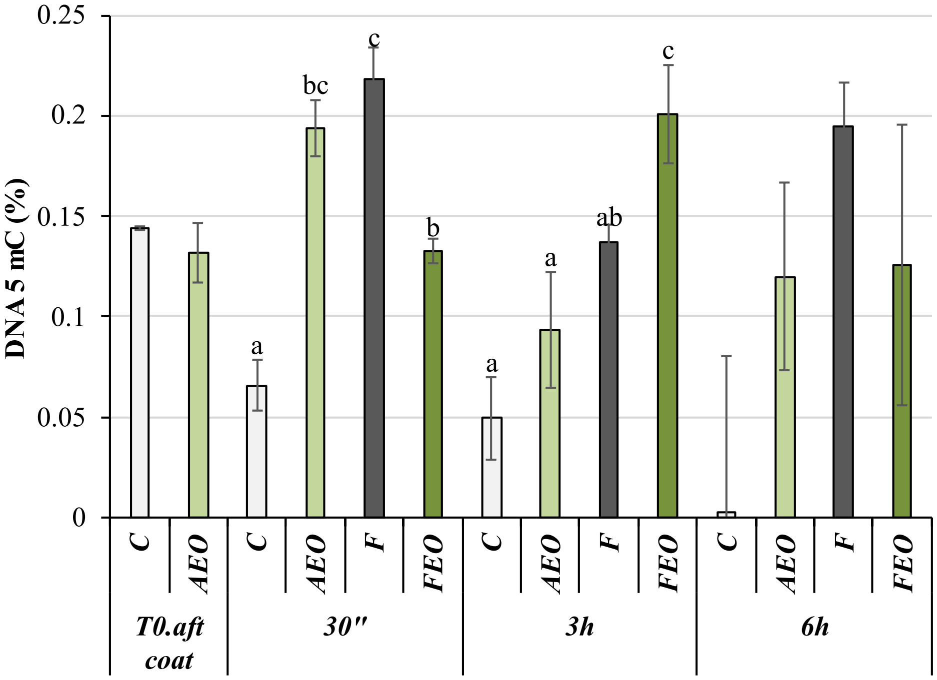


Figure 46: *Kinetic of Global methylation levels in DNA at early stages of treatments on tomato seeds coated with Artemisia absinthium essential oil or with water in control, and in presence or absence of Fusarium oxysporum infection.*

III.2 Chapter 2: Comparative *In vitro* Study on Resistance of *Quercus suber* Clones in Response to *Phytophthora cinnamomi* Rands

III.2.1 Part 1: Study of *Quercus suber* clones response to *Phytophthora cinnamomi* Infection

To assess the resistance of *Qs* clones to *Pc* infection, the somatic embryos needed to develop into juvenile explants with roots, as roots are essential for *Pc* penetration.

III.2.1.1 Growth and Micropropagation of *Quercus suber* Obtained from Axillary Buds for Inoculation Assays:

The transfer of the somatic embryos to the culture medium led to the development of callus, new buds, and new shoots, as shown in Figure 47.

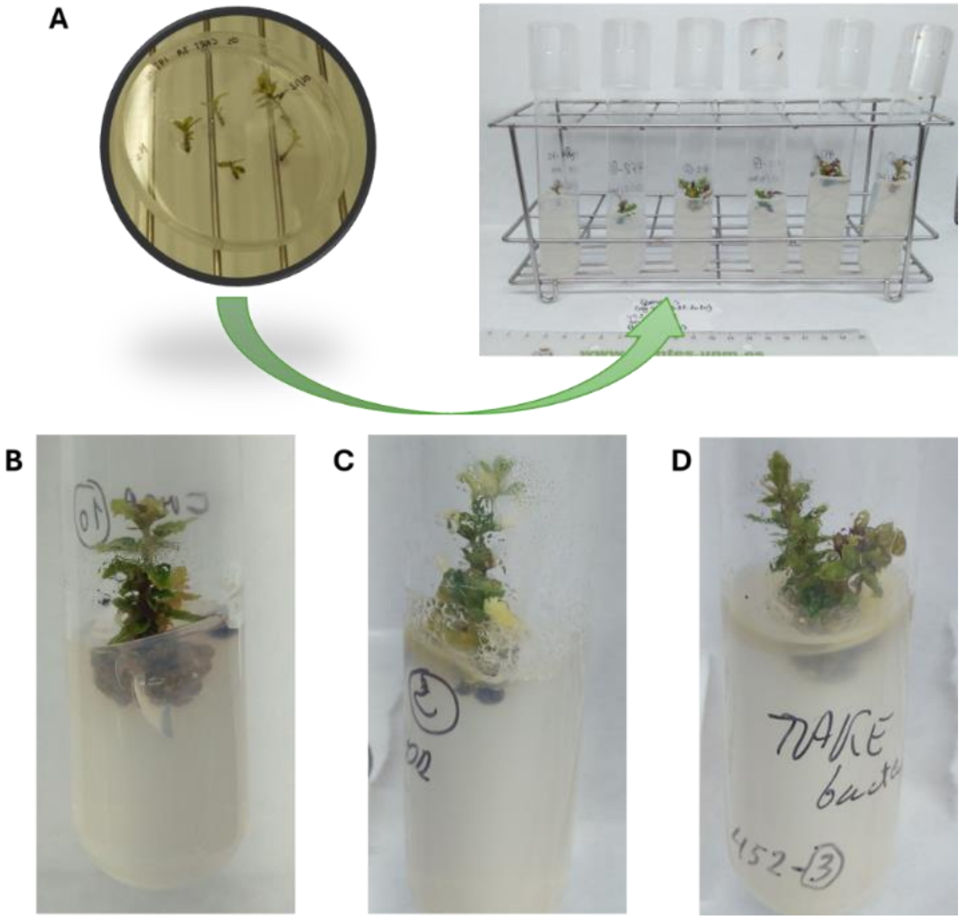


Figure 47: *Development of micropropagated Quercus suber somatic embryos after 47 days in medium growth. (A) Transfer of explants to growth medium. (B) Callus development. (C) buds development. (D) Growth of new shoots.*

III.2.1.2 Emergence of Endophytic Bacteria:

Severa non-identified bacteria appeared in the medium of the explants when transferred to the rooting medium (Figure 48-A), two different colonies were seen that can be differentiated by view by its colour where one is red and the other yellow. Figure 48 (B and C) shows a microscopic image where the morphological difference of the spores can be seen.

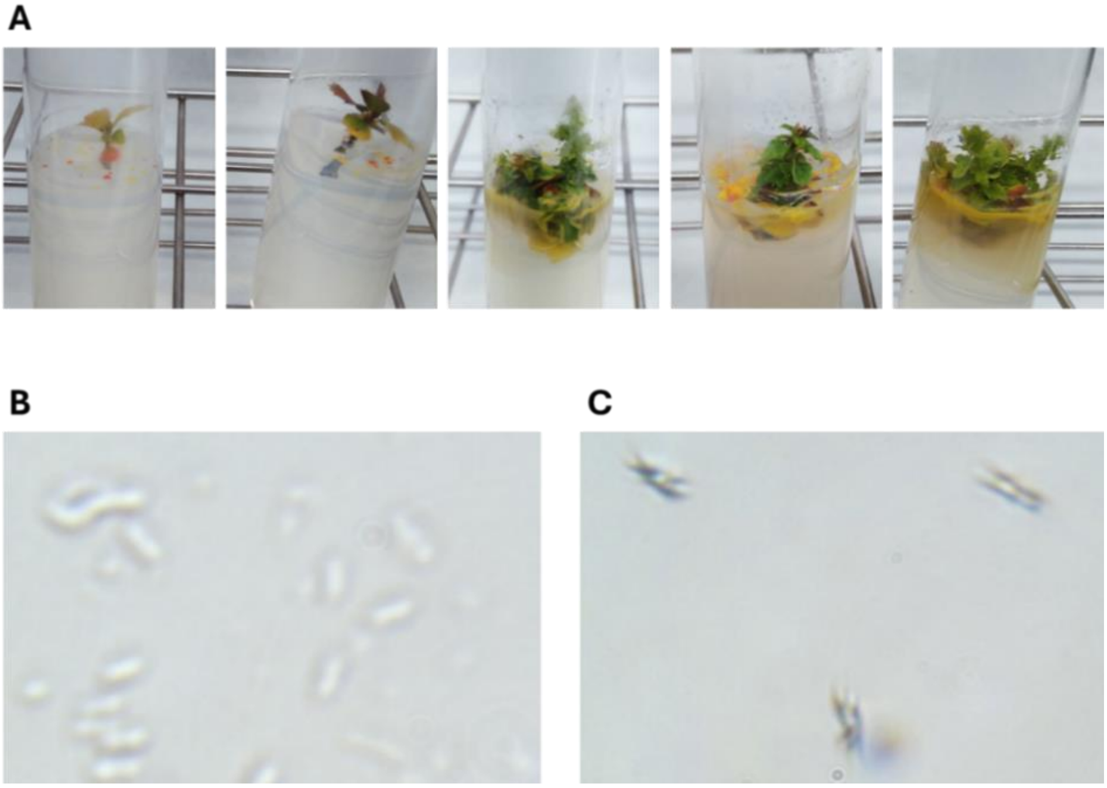


***Figure 48:** Emergence of the endophytic bacteria within Quercus suber somatic embryos. (A) Quercus suber somatic embryos contaminated with endophytic bacteria, (B) Red bacteria, (C) Yellow bacteria. Microscope amplification (x100).*

III.2.1.3 Treatment of Endophytic Bacteria with An Antibiotic:

Cleaning with 0.02% sodium hypochlorite does not seem to be efficient enough to disinfect *Qs* from EB after two weeks of cleaning as can be seen in Figure 49(A), where out of 5 clones, 4 tubes are at level 3 or at full of contamination level.

When treated with the antibiotic cetoxin at two concentrations (0.5 and 1 mg/mL), EB appears to increase at both concentrations, but at 0.5 mg/mL, Figure 49 (B) shows that more tissue was recovered at level 0 (no contamination).

After determining that the putative contamination remained after different treatments with antibiotics and several transfer to new tubes, we confirmed that they came from the plant, so we considered them as endophytes. Later sequenciation of them confirmed our hypothesis. Those strain are actually under study for using them as biocontrol tools to improve plant resistance against *Pc*.

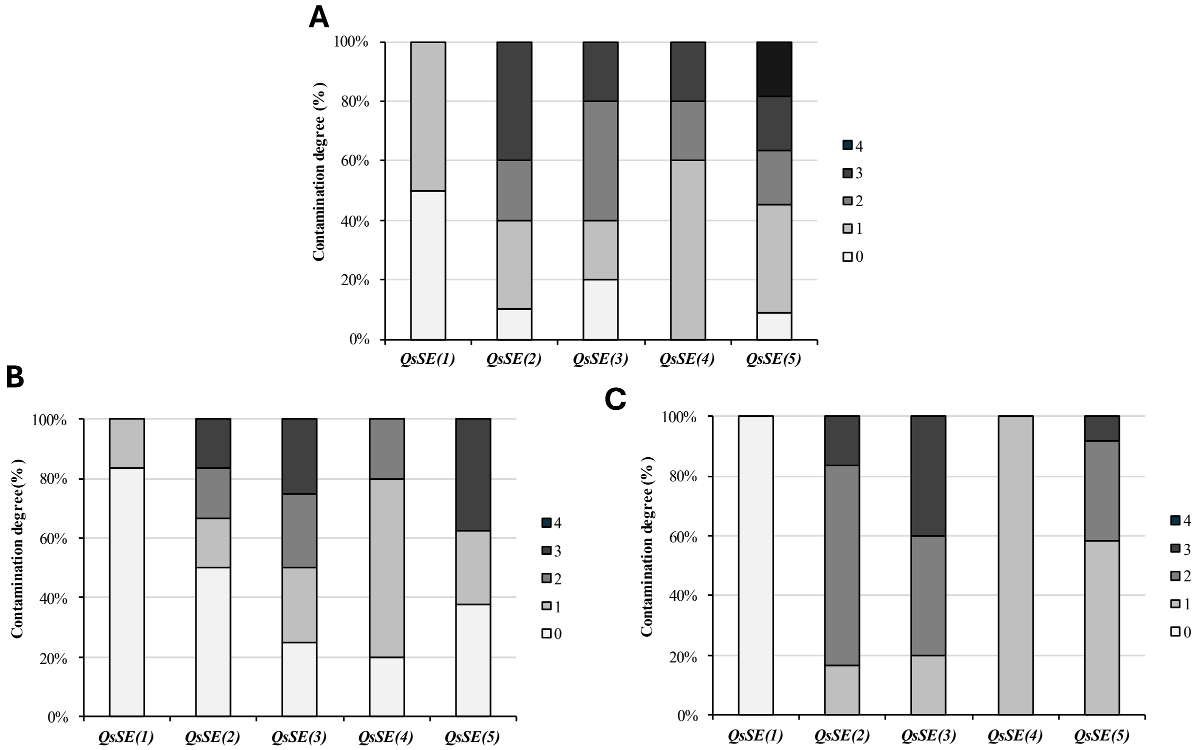


Figure 49: *Treatments used to clean Quercus suber somatic embryo (QsSE) from endophytic bacteria, where: 0. No contamination, 1. Level 1, 2. Level 2, 3. Level 3, 4. Full of bacteria. (A) EB levels (%) when disinfected with 0.02% of Sodium hypochlorite, (B) EB levels (%) after treatment with cetoxine antibiotic 0.5mg/mL, (C) EB levels (%) after treatment with cetoxine antibiotic 1 mg/l.*

III.2.1.4 Disease Parameters of *Quercus suber* infected with *Phytophthora cinnamomi* zoospores:

Because explants from axillary buds contained endophytic bacteria, the decision was made to conduct disease analysis using embryos obtained through somatic embryogenesis to avoid contaminations and other external biotic or abiotic stress factors. In collaboration with the group from la Universidad Complutense de Madrid, new explants with developed roots were received and prepared for inoculation with *Pc* zoospores. In vitro embryos obtained from somatic embryogenesis from IMIDRA and Tragsa were grown at Universidad Complutense de Madrid in the laboratory of Arantcha Gómez Garay, who was also involved in the Tragsa Contract (see acknowledgments). Explants at the early stages of developed roots and short shoots were acclimated at UPM and then inoculated with *Pc* zoospores (see methodology II.5).

Based on the disease symptoms (see methodology II.12.1), which characterize the infection with 10^7 zoospores/mL of *Pc* zoospores after 5 dpi, and where: 0. No symptoms, 1. Leaf chlorosis and light necrosis on roots, 2. Apparent necrosis on leaves and light necrosis on roots, 3. High necrosis on leaves and roots, 4. Decayed explant.

It is shown in Figure 50 (A), resistance levels determined comparatively: *QsClone(1)* to *QsClone(6)* exhibited the highest resistance, whereas *QsClone(7)* to *QsClone(14)* were identified as susceptible. Additionally, Figure 50 (B and C) validates the disease ratio quantification through measurements of root necrosis and fresh weight loss, particularly evident in the susceptible clones, where root necrosis exceeded 50% of the total root length.

As demonstrated in the figures, our established protocol facilitated the quantitative assessment of comparative resistance among *Qs* clones.

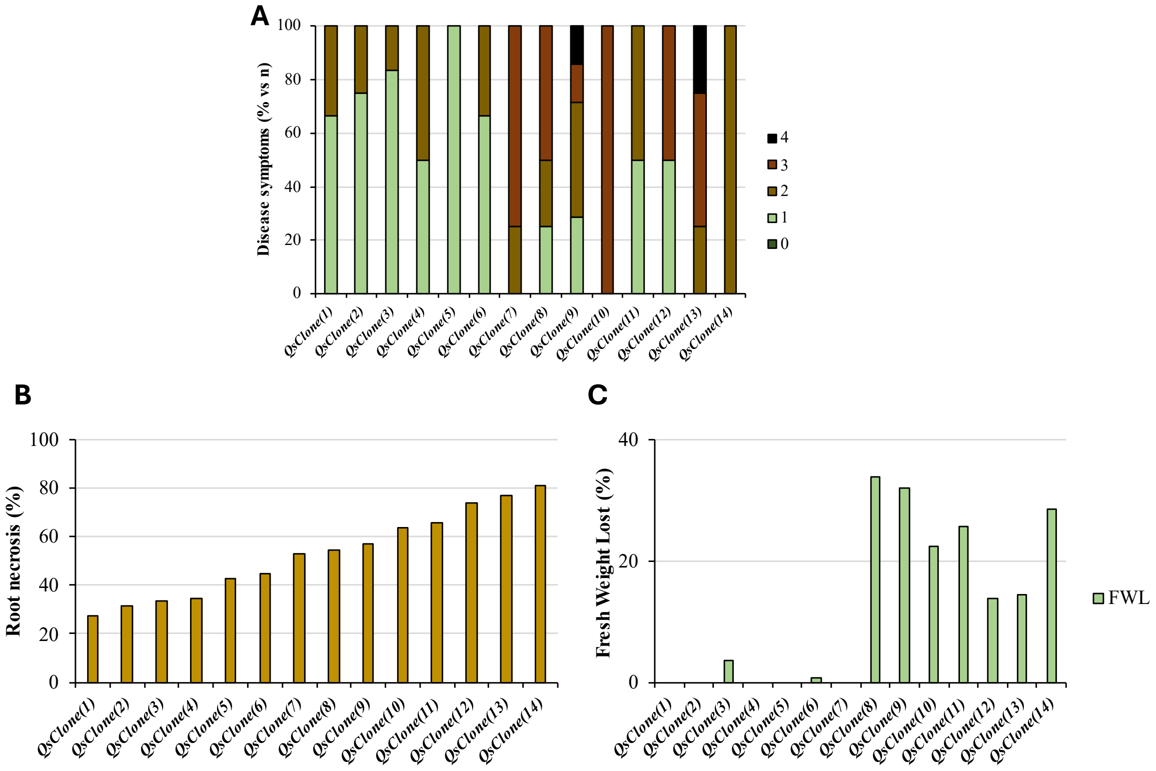


Figure 50: *Disease symptoms of Quercus suber clones infected with Phytophthora cinnamomi zoospores after 5 dpi. (A) Disease symptoms, (B) Root necrosis (%). (C) Fresh weight loss (%).* At least three explants were analysed per treatment, and three independent experiments.

III.2.1.5 Exploring Gene Expression Changes of *Quercus suber* roots Induced by Infection with *Phytophthora cinnamomi*:

Because the method we established enables us to determine resistance levels under axenic conditions, it also allows us to identify specific genes that might be inducible by *Pc*, while avoiding additional stressors present in forest or greenhouse conditions, such as drought, frost, or additional microorganisms. With this approach, we isolated RNA from *Qs* explants and analysed plant responses using RNA genome sequencing (see methodology II.16).

From the previous results, two clones were selected to study the response of *Qs* to *Pc* infection taking into consideration all the parameters measured previously. *QsClone(3)* was selected as the tolerant clone while *QsClone(9)* is considered a susceptible one according to the disease symptoms results. The susceptible clone exhibited sufficient "resistance" to provide adequate infected tissue for our purposes. However, the most susceptible clones were not chosen due to the extensive tissue necrosis, which hindered proper extraction of nucleic acids.

III.2.1.5.1 QRT.PCR to Detect *Phytophthora cinnamomi* in *Quercus suber* Roots and Genes Expressed in *Quercus suber*:

The primers specific to *Pc* were performed to identify and quantify the presence of *Pc* in the *Qs* indicated the presence of *Pc* in the susceptible clone, *QsClone(9)*, *Fol*lowing infection with zoospores. In contrast, the resistant clone, *QsClone(3)*, did not show any presence of the pathogen (Figure 51-A). The *β-TUBULIN* gene, which is required for the maintenance of basic cellular functions, was quantified to determine the damage that *Pc* can inflict on the plant. Figure 51 (B) shows that in *QsClone(3)*, *β-TUBULIN* is slightly inhibited in the infected plant compared to the control, while in *QsClone(9)* the gene is strongly inhibited in the infected plants compared to the control. The accumulation of *CHITINASE* in *Qs* has the capacity to boost plant defense reaction. Figure 51 (C) shows levels of expression of this gene where it seems that the plants infected with *Pc* have high expression then control trees in both lines. But in *QsClone(9)* infected seems to express higher quantity then the *QsClone(3)*. Statistical analysis shows a significant difference using the test of variance (*P-value* ≤ 0.05) with Tukey test (A and C) and Duncan test (B).

These findings substantiated the influence of *Pc* on *Qs* in both clones, confirming previous identifications of the susceptible and tolerant clone.

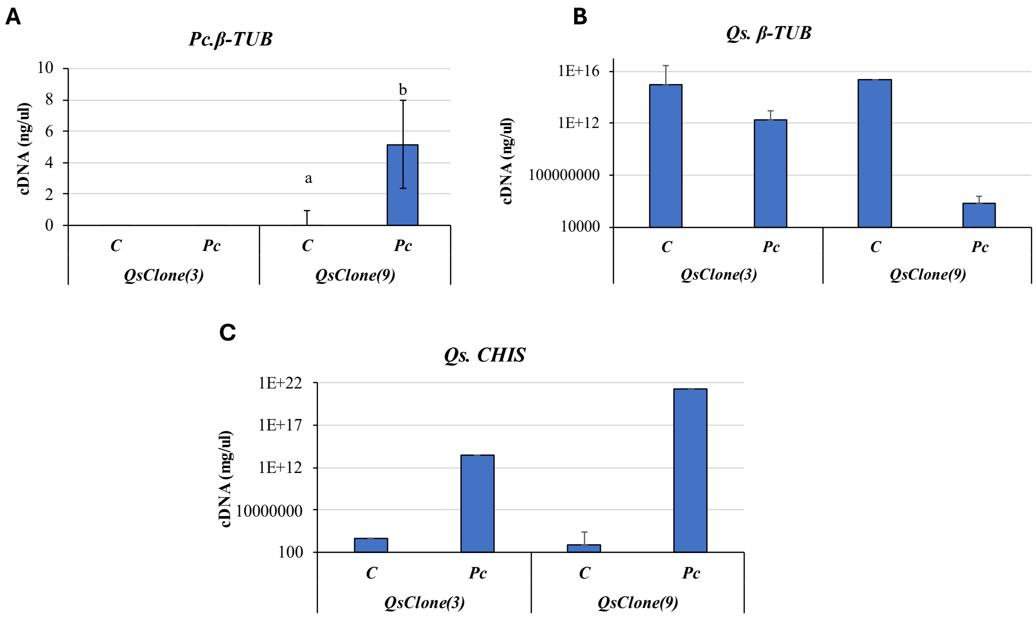


Figure 51: *Detection of Phytophthora cinnamomi infection and its effect on Quercus suber explant. (A) β-TUBULIN specific to Phytophthora cinnamomi (ng/ul), (B) β-TUBULIN specific to Quercus suber (ng/ul), (C) CHITINASE.*

III.2.1.5.2 RNA-seq to Explore Genes of Defense:

The analysis of RNA-seq data enabled us to identify 858 genes induced in *QsClone(3)* and 1276 genes repressed, while *QsClone(9)* represented 1134 genes induced and 916 repressed as represented in the Venny diagram (Figure 52-A). Principal Component Analysis of genes induces and repressed (*Fol*d change ≥ 1) in both clones were represented in a three-dimensional scatter plot, where each point represents a sample. Samples with similar gene expression profiles are closer in the three-dimensional space (Figure 52-B).

Figure (52-C) illustrates a Volcano Plot; this interactive scatter plot presents the log2-*Fol*d changes and statistical significance of individual genes derived from the gene expression analysis. Each point on the plot corresponds to a specific gene, with its position indicating both the magnitude of change in gene expression (log2-*Fol*d change) and the level of statistical significance associated with that change.

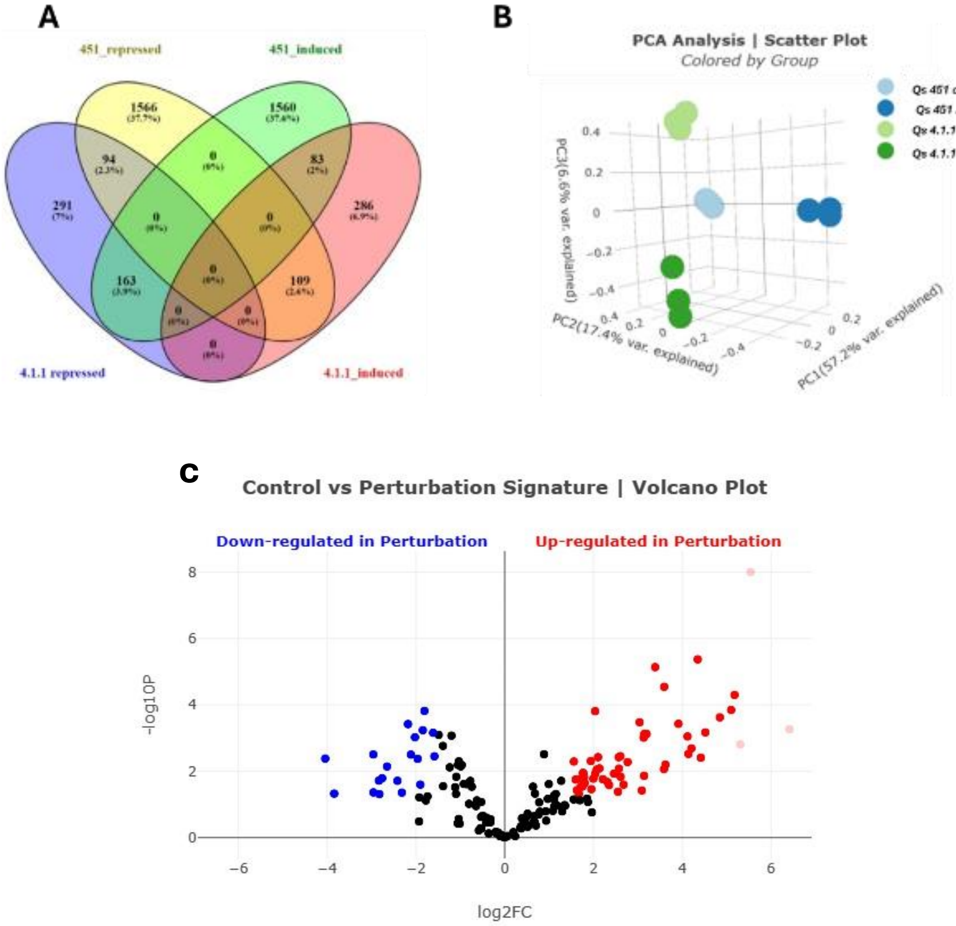


Figure 52: *Analysis of RNA-seq data to identify genes of resistance from Quercus suber roots clones infected with Phytophthora cinnamomi. (A) Venny diagram, (B) Three-dimensional scatter plot of Principal Component Analysis of genes induces in QsClone (3) and repressed in QsClone(9) (Fold change ≥ 1), (C) Volcano Plot presenting the log2-Fold changes and statistical significance of individual genes derived from the gene expression analysis.*

RNA-seq sequencing of explants genome after 5 dpi of *Pc* infection enabled us to determine genes of resistance induced within both clones, but where the expression is higher in the tolerant clone *QsClone(3)* than in the susceptible clone *QsClone(9)*, mainly: Hevein-like proteins that plays a role as antimicrobial or antipathogen (Berthelot, Peruch, and Lecomte 2016), Acidic endochitinase, WRKY transcription factor 75, Disease resistance-responsive (dirigent-like protein), Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein, phosphoenolpyruvate carboxykinase 1, pleiotropic drug resistance 12.

Moreover, the results of RNA sequencing allowed us to identify genes induced in *QsClone(3)* while it is repressed in *QsClone(9)*: Pectin esterase inhibitor related to the cell wall remodeling that occurs during diverse plant developmental processes (Pérez-Pérez., Carneros et al. 2019), Dehydration-responsive protein RD22, Lipoxygenase 2, Proline-rich protein 2, Thioesterase/thiol ester dehydrase-isomerase superfamily protein, Cytochrome P450, Glycosyl hydrolase proteins, Peroxidase 45, Serine carboxypeptidase 24. Cinnamyl alcohol dehydrogenase is present in cc but doesn't seem to be expressed in the *QsClone (9)*.

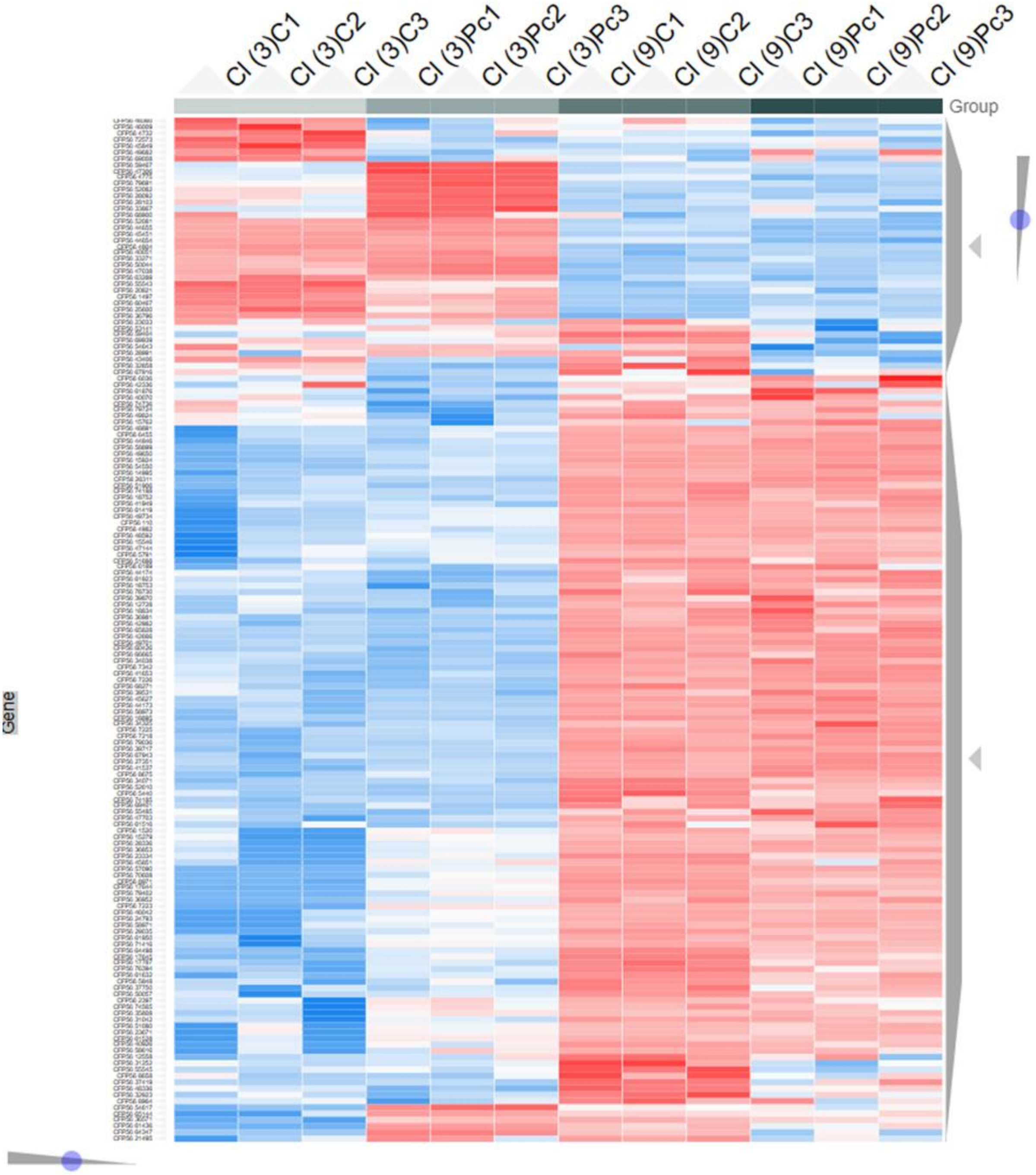


Figure 53: *Clustergrammer analysis that contains an interactive heatmap displaying gene expression for each sample in the RNA-seq dataset. Every row of the heatmap represents a gene, every column represents a sample of QsClone (3) or QsClone (9), infected (Pc) or non-infected (C), and every cell displays normalized gene expression values.*

III.2.1.5.3 **RNA-Seq to Explore Genes Related with Epigenetic:**

Based on the paper of (Silva et al., 2020), genes related to epigenetic were detected in our dataset of RNA-seq in order to understand the epigenetic changes affected by *Pc* infection in *Qs* roots. Genes related to histone modifications, DNA methylation and RNA directed DNA methylation were found as represented in table 5.

Table 5: *Marker genes related with epigenetic changes detected in Quercus suber roots when infected with Phytophthora cinnamomi.*

Type of epigenetic	Marker genes	ID
Histone lysine methyltransferase (HMT)	*SUVR4*	CFP56_36057
	SUVR5	CFP56_22604
	SUVR3	CFP56_29051
	CLF	CFP56_48440
	ATXR7	CFP56_14674
	ATXR2	CFP56_29061
	ATXR5	CFP56_53906
	SWN	CFP56_32660
	ASHH	CFP56_19033
	ATX2	CFP56_26820
	ATX3	CFP56_4889
	SUVH4	CFP56_1500
	ASHR3	CFP56_53193
	ASHH3	CFP56_26485
Histone acetyltransferase (HATs)	*HAC1*	CFP56_7357
	GCN5	CFP56_29024
	HAM1	CFP56_18129
	HAC1	CFP56_7357
Histone deacetylase (HDACs)	*HAD15*	CFP56_10834
	HAD9	CFP56_22884
DNA methyltransferase	*MET1*	CFP56_43329
	DNMT2	CFP56_40985
RNA-directed DNA methylation (RdDM)	*DMS4, RDM4*	CFP56_15104

III.2.2 Part 2: Response of *Quercus suber* Transgenic Clones to *Phytophthora cinnamomi* Infection

The *Fol*lowing studies were realised in collaboration with la Universidade de Lisboa and El Consejo Superior de Investigaciones Científicas (MBG-CSIC), Santiago de Compostela. The protocol of inoculation with *Pc* developed when working with cork oak clones (see chapter 2, part 1), was used to study the response of different transgenics of *Qs* to *Pc* infection. The results were published in Serrazina et al, Under review.

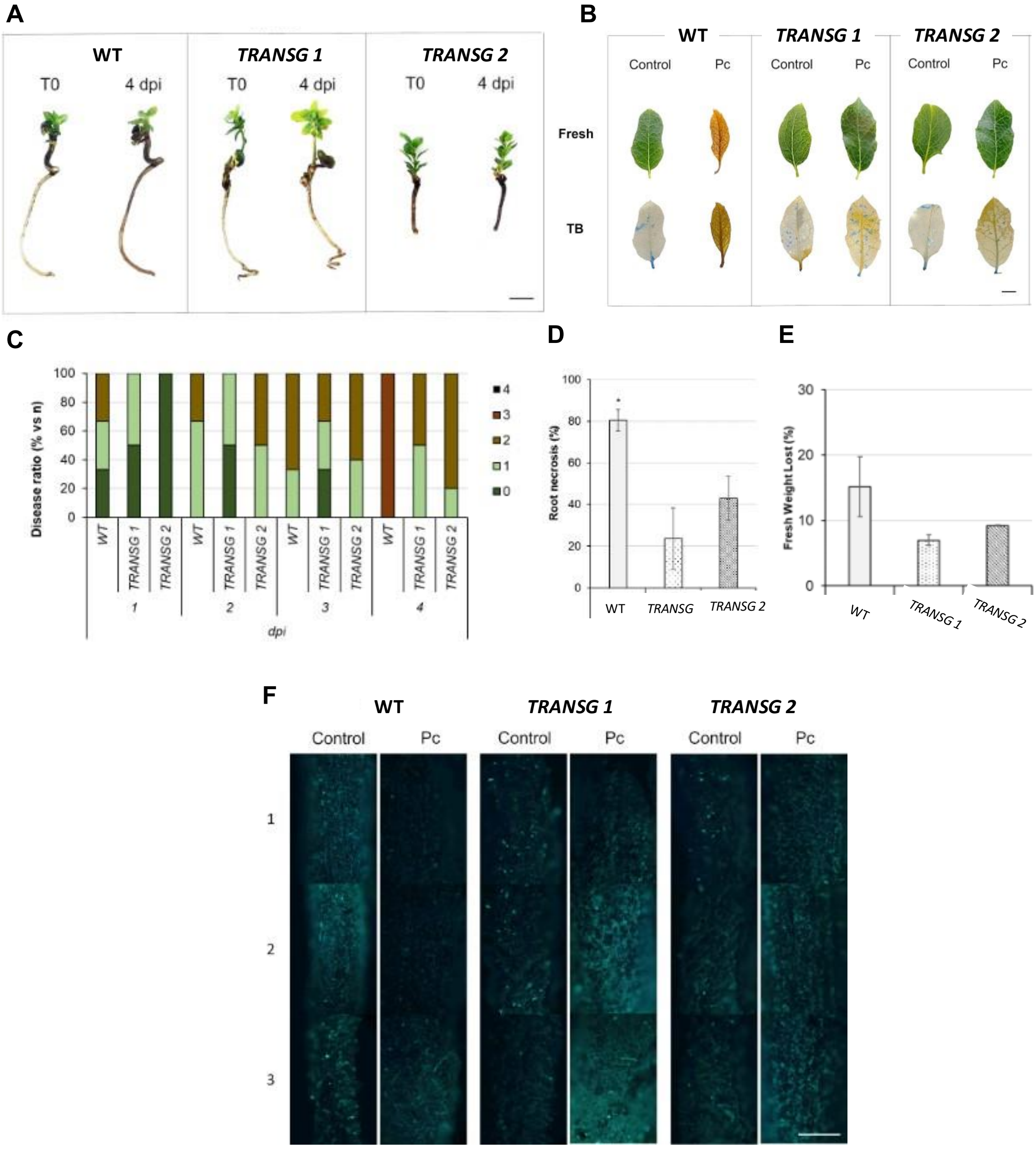


Figure 54: *Disease symptoms of Quercus suber transgenics infected with Phytophthora cinnamomi zoospores at 4 dpi. (A) Symptoms before T0 and after 4 dpi of Pc zoospores infection Bar: 1 cm. (B) Leaves symptoms on Fresh leaves and stained with Trypan Blue solution (TB) Bars: 1 mm. (C) Kinetic of disease symptoms ratio in percent at 1, 2,3 and 4 dpi (days post-inoculation), (D) Percentage of fresh weight lost in WT and the transgenics TRANSG 1 and TRANSG 2 infected with Pc. (E) Quantification of root necrosis percentage in WT and the transgenics TRANSG 1 and TRANSG 2 infected with Pc. (F) Staining with Aniline Blue to detect callose deposition on the root of WT and the transgenics TRANSG 1 and TRANSG 2. infected with Pc zoospores and non-infected explants on three different parts of the root (1. Differentiation, 2. elongation, and 3. meristematic part). The digital photos were acquired at 100x magnification. Bar: (1 mm).*

Figure 54 shows the disease symptoms of *Pc* infection on *Qs* where the infection is more apparent on the WT and the trangenic *TRANSG 2* at both levels root and leaves necrosis, while the transgenic *TRANSG 1* shows less symptoms (Figure 54 A and B), and where the disease symptoms (Figure 54-C) was quantified, where: 0. No symptoms, 1. Leaf chlorosis and light necrosis on roots, 2. Apparent necrosis on leaves and light necrosis on roots, 3. High necrosis on leaves and roots, 4. Decayed explant. The fresh weight loss (Figure 54-E) and percent of necrosis (Figure 54-D) quantified has confirmed the disease ratio results. The * in (D) denotes a statistically significant difference using variance check (*P-value* ≤ 0.05) and Tukey HSD test. The staining of roots with aniline blue has shown in Figure (54-F) that the intensity of callose deposition is higher in the transgenic *TRANSG 1* infected with *Pc*, while there is less signal in *TRANSG 2* and no signal at all in WT infected.

IV Discussion:

IV.1 Chapter 1: Improvement of *Solanum lycopersicum* Resistance to *Fusarium oxysporum* Using Bioproducts

In collaboration with ICA-CSIC research group specialised in Biopesticides, the study demonstrated the antifungal activity of AEO against *Fol* conidia and its lack of efficacy on *A. alternata* spores growth inhibition, similarly to the studies conducted by (Şesan et al., 2016). However, AEO appears to be efficient in inhibiting *Botrytis cinerea* spores germination in all concentrations tested. And the antifungal properties of AEO against *Fusarium* species mycelium and *Botrytis cinerea* was identified, where the analysis identified (-)-cis-chrysanthenol as the primary antifungal compound, *Fol*lowed by linalool (Julio et al., 2015).

In this study, it was determined that the extract not only directly affects the pathogen but also exhibits epigenetic effects at very low concentrations, altering the methylation pattern of RNA and DNA. These changes were found to enhance plant resistance to *Fol*.

The innovative technique employed to induce the resistance effect on tomatoes was seed coating. This method was selected based on previous studies indicating that alterations in seeds could induce epigenetic changes (Delian et al., 2017; Gonçalves et al., 2021; Jisha et al., 2012; Król et al., 2015; Rocha et al., 2019). Furthermore, seed coating enables the potential effects of priming treatments to be applied before embryo development, aiming to preserve these changes for future developmental stages (Delian et al., 2017; Paparella et al., 2015).

The results obtained in this thesis, allowed to determine that coating tomato seeds with a vapor pressure essential oil from *A. absinthium var. candia*l protected seed germination and seedling growth against *Fol*. Our results indicate that AEO protected seeds by directly affecting the fungus but also by the induction of a long-term response in terms of ROS production and callose deposition after germination. These effects were not detected in untreated seeds, where callose and ROS production increased during germination and decreased in the absence of the fungus. There are previous results describing effects of coated seed priming on tomato. Methyl jasmonate (MeJA) induces resistance against *B. cinerea* by priming defense mechanisms in tomato seeds (Król et al., 2015), mediating intra- and inter-plant communication and modulating antioxidant systems (González-Bosch, 2018). A pulsed magneto-primed technic was tested to improve germination, vigour and yield in cherry tomato seeds exhibited an upregulation in the activity of antioxidant enzymes, serving as a mechanism to counteract reactive oxygen species (Gupta et al., 2015). In this thesis, a long-term response from AEO seed treatment was detected, which maintained higher levels of ROS and callose up to 12 days.

Few studies have addressed callose deposition on tomato seeds in the presence of phytopathogens. (Pei et al., 2023) investigated the role of *β-1,3-GLUCANASE10* in regulating tomato development and disease resistance by modulating callose deposition. (Scalschi et al., 2015)silenced *OPR3* in tomato to explore the involvement of OPDA in facilitating callose deposition as part of the plant's defense mechanisms against *Botrytis cinerea*. Additionally, oxidative burst, antioxidant genes, and enzymes were found to be associated with callose formation in tomato's response to various taxonomic groups of Rhizoctonia spp. (Hosseini-

Zahani & Taheri, 2023). Notably, our study is the first to describe callose deposition on tomato seeds induced by *Fol* and an essential oil.

The staining employed to identify callose deposition suggest that AEO could penetrate seeds upon testa rupture, causing molecular alterations in seeds and undifferentiated embryonic cells. This likely contributes to the enduring resistance observed in seedlings after 12 days. Our RNA-seq findings imply that the AEO coating treatment might trigger specific "*de novo*" molecular changes detected at the transcriptional level after 12 days in contact with the fungus. However, the precise molecular mechanisms underlying these effects remain elusive.

This work measures several of the hormones involved in the activation of the short-term signaling pathways related to tomato plant defense (Hernández-Aparicio et al., 2021) such as salicylic acid, jasmonate, methyl-jasmonate, and ethylene. However, the signal levels were not changed significantly compared to controls (data not shown), indicating that they are not the principal molecules involved in the long-term resistance of tomato seedlings responding to *Fol*.

The RNA-seq analysis showed that some other genes related to plant defense responses might also contribute to this resistance, including genes involved in fatty acid metabolism, peroxidases, terpene synthases (Sun et al., 2016; Zhou & Pichersky, 2020), methyltransferases, and enzymes involved in gene silencing. Transcriptomic analysis showed enriched transcriptional induction on specific secondary metabolism pathways. The terpene synthases were transcriptionally induced. Lycopene and carotenoid synthesis was also transcriptionally induced by AEO in the DEG analysis as previously described for wheat (Colasuonno et al., 2017). The metabolomic analysis showed that the coating of tomato seeds with a vapor pressure essential oil from (AEO) induced several metabolites in tomato seedlings when infected with *Fol*, including the polar compounds vanillic acid and coumarin and the apolar ones lycopene, a metabolite of MC 528, and the fatty acid derivative oleamide, these latter two being the most highly induced compounds.

These results agree with the transcriptional induction of carotenoids and fatty acid metabolic pathways detected by RNA-seq analysis, and the increase in total chlorophyll A and B and carotenoids found in the pigment analysis. These results might suggest that AEO treatment induces *de novo* changes that remained in the presence of the fungus for long-term plant resistance. Vanillic acid is a phenolic allelochemical reportedly present in tomato plants (Méndez & Brown, 1971) which significantly improves salinity tolerance and plant growth performance when externally applied to tomato seedlings (Parvin et al., 2020). Coumarin is an antioxidant, antimicrobial, and growth promoter in plants (MU et al., 2019; Saleh et al., 2015; Santra & Banerjee, 2020) and also mitigates salt stress in tomato plants (Parvin et al., 2020). Lycopene is a red carotenoid pigment of MC (536) found in fruits and vegetables, including tomatoes (Stahl & Sies, 1996). Carotenoids are involved in photosynthesis and photoprotection in plants (Tao et al., 2007). Lycopene has an antimicrobial effect against bacteria and fungi such as Candida albicans by inducing apoptosis via ROS production and mitochondrial dysfunction (Choi & Lee, 2015). However, this is the first report on the induction of an apolar compound of MC (528) in tomato plants treated with AEO and *Fol*. Oleamide (an oleic acid derivative) content increased with UV-B2 treatment in an olive cultivar (Celeste Dias et al., 2018). The role played by oleamide in plants remains unclear, but could be involved in growth/development regulation, stress response, and pathogen interactions (Kim et al., 2013). In (Shao et al., 2016), the study investigates the growth inhibition and potential mechanism of oleamide against the toxin-producing cyanobacterium Microcystis aeruginosa NIES-843, where it resulted that there is a potential utilization of oleamide as an algicide in cyanobacterial bloom control. Other studies marked the inaugural mention of oleamide in olive, highlighting its role in enhancing the nutritional value of the cultivar 'Galega'. The observed increase in oleamide levels in response to UV-B exposure hints at a previously unrecognized protective function against UV-B irradiation (Celeste Dias et al., 2018).

Additional assays are needed to determine the specific genetic modifications that enable the transcriptional and metabolic changes responsible for the long-term resistance of tomato observed in this work. The possibility that epigenetic modifications are taking place in seeds resulting in "*de novo*" molecular modification for long-term resistance of aerial parts opens new perspectives for the use of priming tools. The RNA-seq analysis, also detected an inhibition of genes related to redox stress associated to ROS production during photosynthetic electron transport in the chloroplast. This response probably relates chloroplast stress with immunity response to *Fol*. Similarly, mitochondrial stress induced plant resistance, through chromatin changes, against phytopathogenic fungi and bacteria in Arabidopsis (López Sánchez et al., 2021). Therefore, the stress produced in organelles involved into primary metabolism, might contribute to plant immunity. Furthermore, the interactions between metabolomics, transcriptomics, redox regulation, and epigenetics as shown here, are under current study in other plant systems (Shen et al., 2016). Future experiments will be required to clarify our hypothesis, which revolves around the idea that seed epigenetic modifications enhance resistance. However, the specific targets of these modifications are still unknown, as this aspect is currently under investigation based on the global methylation analysis conducted in the latter part of this study.

Our RNA-seq results, confirmed by quantitative real time PCR, show that *NRPD2* (López et al., 2011) mediating in *de novo* cytosine methylation by RNA directed DNA methylation pathway (RdDM), is highly induced after 12 days of treatment with AEO in *Fol* infected seedlings. *NRPD2* was involved in efficient immunity response to *Botrytis cinerea* (López et al., 2011) and *Pseudomonas syringae pv tomato DC3000* in *Arabidopsis thaliana* (Zhang et al., 2021), and was necessary for reactive oxygen species (ROS) production, and activation of jasmonic acid and salicylic acid signaling pathways. Alterations in chromatin structure were necessary for efficient resistance to that fungus (Walley et al., 2008). To our knowledge, this is the first report relating *NRPD2* to tomato immunity response regulation to *Fol*. *NRPD2* was also highly induced at transcriptional level by cucumber mosaic virus (CMV) in *Arabidopsis* being essential for plant immunity and demonstrating the importance of the induction of that gene during plant defense response (Kanazawa et al., 2011). In line with our results, a previous work demonstrated in *Arabidopsis* that DNA methylation is involved in immunity against *Fol* (Le et al., 2014) since RdDM-related mutants showed enhanced susceptibility to this fungus, suggesting that *de novo* methylation process contributes to *Fol* immunity.

In addition, in this work the *WRKY33* transcription factor, involved in the epigenetic control of plant defense against necrotrophs (Alvarez-Venegas et al., 2014; Ramirez-Prado et al., 2018), was also induced specifically by AEO in presence of *Fol* in this work. This transcription factor showed increased levels on H3K4me3 on its promoter during *Botrytis cinerea* tomato infection (Crespo-Salvador et al., 2018). A significant increase was observed in two S-adenosyl-L-methionine-dependent methyltransferases, one of which exhibited over a hundred*Fol*d induction as determined by real-time PCR, in AEO-infected tomato seedlings after 12 days of treatment. S-adenosylmethionine transferases (*SAMts*) are responsible for maintaining the plant's methionine cycle (MTC) in the plant (Mäkinen and De, 2019). MTC connects ethylene and methylation pathways, where SAMs are DNA cytosine methylation markers for transcriptional RNA silencing (RGS), (Pooggin, 2013). The overexpression of SAMs in tomato increased tolerance to ROS stress (Gong et al., 2016) and prevented ROS accumulation in *Arabidopsis* (Jang et al., 2012). Our results detected very high levels of 1-aminocyclopropane-1-carboxylate oxidase 2 (ACCO), specifically involved in MTC cycle, for the synthesis of ethylene. Considering that our RNA sequencing results indicate that MTC cycle is altered by AEO primed tomato plants in presence of *Fol*, a molecular analysis including histone epigenetic marks and effects on the offspring will be necessary to characterize this *de novo* long-term resistance, produced by AEO.

In soil assays, AEO again proved its efficiency: it increased the germination rate and vegetative growth of *Fol*-infected plants compared to untreated plants. The main mechanisms by which AEO increases the resistance of plants to *Fol* infection are by reducing stress ethylene production, balancing the level of plant hormones, increasing antioxidant enzymes, and improving nutrient uptake due to their different chemical structure as described previously in (Soudani et al., 2022) and in the direct effect of EOs on crops in the review (Kesraoui et al., 2022).

Besides essential oils, hormones were evaluated in this thesis as a coating treatment for tomato seeds. ACC hormone did not affect the germination of tomato seeds at the tested concentrations, suggesting that higher concentrations may be more efficient, considering ACC's role as a growth regulator (Polko & Kieber, 2019). Previous research has shown that priming basil seeds with SA improved plant growth and mineral uptake (Kulak et al., 2021), while priming tomato seeds with SA increased fruit yield (Chakma et al., 2021), and SA priming improved germination in rice (Shatpathy et al., 2018). Therefore, our results suggest that SA priming can positively influence tomato seed germination. Studies by (Król et al., 2015) have demonstrated that MeJA improves resistance to *Fol* in tomatoes and inhibits spore germination and mycelial growth. Although its effect on germination has not been shown, MeJA was evaluated as a seed coating treatment to enhance tomato germination, as discussed in the results section. Additionally, its efficacy has been demonstrated in enhancing the quality and health attributes of various fresh fruits (Reyes-Díaz et al., 2016).

After testing essential oils and hormones, pathogenic extracts were evaluated as biological control agents to enhance plant characteristics. Our studies utilized two different extracts from distinct fungi (*Fol* and *Pc*), both of which demonstrated a positive impact on the germination and growth of tomatoes. Few studies have explored the use of fungus extracts as biocontrols. In a study by (Ibrahim et al., 2021), endophytic fungi isolated from medicinal plants were identified as a potential source of active compounds. These compounds exhibited antioxidant properties, as evidenced by assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric ion reduction. Additionally, phytochemical analysis revealed the presence of phenols, tocopherols, sterols, terpenoids, and fatty acids. While other studies focus on synthesizing nanoparticles through fungi or fungal extracts, fungi stand out as an ideal option for metallic nanoparticle production for their ability to secrete a large amount of proteins, which enhances productivity and makes them easy to use in laboratory settings (Madhavi et al., 2023). Agriculturally, nanoparticles derived from fungi have found applications in plant disease management, as it was seen in studies using nanoparticles of the fungus *Trichoderma harzianum*, that secretes a large number of hydrolytic enzymes and is used for its ability to inhibit the growth of plant pathogens (Ahluwalia et al., 2014).

In the latter segment of this chapter, attention turned to identifying epigenetic changes in the genes of tomato seeds treated with AEO, particularly in their initial response to *Fol* infection. The goal of this investigation is to discover potential biomarkers for the early detection and defense against this destructive pathogen, consequently, early short time points were selected to study. Similar studies were conducted previously by (Crespo-Salvador et al., 2018) determining histone epigenetic marks in *Arabidopsis* and tomato genes in the early response to *Botrytis cinerea* at 24 and 33 h after inoculation. To attain our objective, it is essential to comprehend the germination process of tomato seeds. Drawing on the thesis by (Spoelstra, 2002) and our prior RNA-seq findings, a set of genes was chosen to monitor their expression during the seeds' early stage.

HISTONE 1 is a linker histone, a protein in the cell nucleus that plays a role in the organisation of chromatin and influences gene expression. In tomatoes, four genes were discovered and studied that show that their expression depends on increased ABA levels during water stress. Among these genes is LE20, which codes for a protein with H1 histone domains, thus it was renamed *HISTONE 1*. The role of *HISTONE 1* in the structural protection of chromatin is excluded (Scippa et al., 2004; Spoelstra, 2002). Based on (Spoelstra, 2002), two functionally different linker histones were examined for their expression during and after germination: *HISTONE 1* and *LEH1*. According to (Bray et al., 1999), *HISTONE 1* is involved in the control of gene expression during plant stress. The overall gene expression of *HISTONE 1* have demonstrated in a previous study that it is low during tomato seed germination, but our long-term kinetic shows that the expression of this gene increases with time up to 12 days. While expression of the *LEH1* gene increased during germination and was absent during cold stratification of tomato seeds, our results are consistent with the work of (Spoelstra, 2002). However, neither the infection with *Fol* or the priming with AEO seem to significantly affect the linker histones expression in the short term kinetic.

During seed germination of dicotyledonous plants, certain cell wall hydrolases and non-enzymatic substances such as expansin are required for this process, as it increases the extensibility of the cell wall in the radicle (Chen et al., 2019; Spoelstra, 2002). This is evident in our results, as the expression of *EXPANSIN 2* gradually increased in the first hours of growth conditions until germination after 2 days and decreased after 12 days of growth because germination was complete.

Nuclear RNA Polymerase D2 (*NRPD2*) is the second largest subunit of the nuclear Pol IV and V, which function in RNA-directed DNA methylation and epigenetic regulation of gene expression driven by small interfering RNAs (siRNAs) (López et al., 2011; Yan Zhang et al., 2021). *NRPD2* is stable during cold priming in water, but when transferred to a growing condition, the expression of this gene increases in the first hours as the seeds adapt to the new conditions, which are considered stress conditions. Once adapted, it remains stable for up to 12 days. In Arabidopsis, it was demonstrated that *NRPD2* gene expressing is sensible to the change of temperature (Popova et al., 2013), this explains the increase of *NRPD2* during short term kinetic, since the seeds were transferred from 4ºC (during cold priming) to 24ºC (growing conditions). Moreover, infecting the seeds with *Fol* and the priming with AEO induces significantly RNA-directed DNA methylation especially after 3 hours in the seeds coated with AEO and infected with *Fol*.

S-adenosyl-methionine transferases (*SAMt*) play a variety of roles in the biosynthesis of important plant products related to disease resistance, plant growth and development (Joshi & Chiang, 1998) and in the transfer of methyl groups to various biomolecules, including DNA, proteins and low-molecular-weight secondary metabolites that cause epigenetic changes (Handy et al., 2011; Struck et al., 2012). In the case of our kinetics, SAMt is strongly expressed after 6 hours under growth conditions, which leads us to believe that natural methylation occurs in the first hours before germination and is induced again after 7 and 12 days due to the growth and developmental changes. However, when coated with AEO and infected with *Fol*, *SAMt* is significantly expressed after 3 hours compared to the control. Thus, AEO and *Fol* infection led to a high activity of methyl group transfer, which triggers an epigenetic effect. Moreover, within 30 minutes of being in a growth condition, the seeds already exhibited recognition of self and non-self-molecules, as evidenced by the induction of SAMt expression in all treated seed groups (EO, *Fol*, and FEO) compared to the control (C).

WRKY33 is a gene responsible for modulating the expression of SA and JA responsive genes that increase resistance to necrotrophic pathogens, and is considered a marker of H3K4me3, (Crespo-Salvador et al., 2018). Our results show that AEO increases the expression of *WRKY33* in the absence or presence of *Fol* spores. The hypothesis suggests that this results in an increase in methylation of H3K4me3, but further confirmation is required through additional experimentation.

These results led us to realise that the first significant epigenetic changes in tomato seeds coated with AEO and infected with *Fol* spores occur principally before 3 hours during short-term kinetics and at very short times after treatments. Therefore, further studies are focused on this time point.

It was imperative to quantify and compare the methylation patterns occurring during the early stages of tomato seed development, influenced by both AEO treatment and *Fol* infection. By quantifying deposition of m6A in RNA, the results show that AEO significantly induces the deposition of m6A in RNA, leading to regulation of gene expression, as previously demonstrated by QRT-PCR. However, upon infection with *Fol*, the deposition of m6A is strongly suppressed in seeds primed with AEO and infected at 3 hours. This is explained by previous research showing that specifically in tomato, m6A deposition is generally negatively correlated with transcript abundance, suggesting a role for m6A in accelerating RNA decay (Liang et al., 2020; Zhou et al., 2019). RNA N6-methyladenosine is the most common internal chemical modification in eukaryotic mRNA and plays an important role in the regulation of gene expression (transcriptional and post-transcriptional regulation), embryonic development, fruit maturation and stress responses (Tang et al., 2023). In addition to post-transcriptional regulation, regulation of transcription and chromatin state have also been identified as important roles of RNA m6A modification (Liang et al., 2020).

In the case of 5mC methylation, it has been demonstrated that it is induced significantly by AEO. The mechanism and cellular function of RNA 5-methylcytosine remain unclear, but’it's recognized as a crucial modification with diverse roles, including regulating stress responses, stem cell proliferation, and RNA metabolism (David et al., 2017). In Arabidopsis, 5mC in mRNA is identified as a new marker in the epitranscriptome, playing a vital role in gene networks driving plant development (Cui et al., 2017). Further studies in plants revealed nuclear-specific tRNA methylation and widespread rRNA methylation across genomes, with Arabidopsis RMTase likely methylating rRNA within organelles. Unlike vertebrates, mitochondrial and chloroplast tRNAs in plants aren't methylated (Burgess et al., 2015). 5mC stabilizes RNA structures by promoting base stacking and enhancing thermal stability of hydrogen bonding with guanine, suggesting its role in stabilizing rRNA *Fol*ding. Modifications within tRNA core affect structure and stability, while those near the anticodon influence tRNA function by affecting codon-anticodon interactions (Bohnsack et al., 2019; Brzezicha et al., 2006).

By quantifying 5-methylcytosine methylated in DNA, the results showed induction of methylation in coated seeds infected with *Fol* significantly, while it is repressed in non-coated seeds and infected, this is aligning with studies that shown that wilt-infected plant cells with a pathogen, DNA tends to be undermethylated (demethylated) (Guseinov & Vanyushin, 1975; Tirnaz & Batley, 2019). Cytosine methylation has generally been associated with repression of gene expression when present in prompter regions, but it might also enhance the gene expression by recruiting methylation-specific transcription factors (Kumar et al., 2018).

Thus, AEO is inducing an epigenetic change at early stages of seed development increasing resistance of tomato seeds resistance to *Fol*. From these kinetic results, 3 hours was selected from the time intervals for future analysis of methylomics on DNA and RNA at the hole tomato genome, in order to look for specific targets involved into seed protection.

IV.2 Chapter 2: Comparative *In vitro* Study on Resistance of *Quercus suber* in Response to *Phytophthora cinnamomi* Rands

Currently is well known that endophytic bacteria colonize the internal tissue of the plants with no apparent negative effects on their host (Tashi-Oshnoei et al., 2017). The yellow bacteria isolated (see results section: III.2.7) was identified initially as *Luteibacter sp.* (Jaiswal et al., 2023). Studies have described its potential as active producers of cellulolytic and hemicellulolytic degrading enzymes, it is one of the mechanisms to bind polysaccharides, such as carbohydrate binding modules included in cellulases and hemicellulases (Jaiswal et al., 2023; Lasa et al., 2019). The red bacteria is initially identified as *Paenibacillus sp*, studies conducted on poplar inoculated with this bacteria demonstrated significant positive increase in lateral root number and density, fresh and dry root biomass, as well as a significant enhancement in adventitious root growth parameters (Vaitiekūnaitė et al., 2021). This may explain the emergence of this bacteria during the rooting stage of our micropropagation process. Further analysis is required to confirm the identification of both bacteria. Moreover, ongoing research aims to ascertain whether the presence of the endophytes detected on explants obtained from axillary buds of *Qs* could enhance the clones' resistance to *Pc*.

Several studies have explored the impacts of *Pc* on *Quercus spp*., with root rot emerging as a primary driver of oak decline. Within host plants, invasion by *Pc* results in root lesions, necrosis, reduced fine roots, and symptoms suggestive of water deficiency (Coelho et al., 2006; Ruiz-Gómez et al., 2019; Ruiz Gómez et al., 2015). In this study, a protocol was developed to quantitatively compare the resistance against *Pc* among different clones of *Qs* in axenic conditions. The advantage of this technique in determining plant resistance, compared to natural conditions, lies in the absence of abiotic or biotic stresses, such as drought, light intensity, insects, soil compounds, and other natural stressors. Under the tested conditions, the explants are solely exposed to the pathogen under controlled conditions, allowing for the identification of potential molecular changes specifically activated during the immune response against *Pc*.

This *in vitro* methodology enabled us to identify the most tolerant explants, which could prove valuable for replacing severely affected *Quercus suber* trees in the future. This objective stands as one of the primary aims of this study.

From the previous results, two clones were selected to study the response of *Qs* to *Pc* infection analysing the roots, taking into consideration all the parameters measured previously. *QsClone(3)* was selected as the tolerant clone while *QsClone(9)* is considered a susceptible one according to the disease symptoms results (see results section III.2.8). The susceptible clone exhibited sufficient "resistance" to provide adequate infected tissue for our purposes. However, the most susceptible clones were not chosen due to the extensive tissue necrosis, which hindered proper extraction of nucleic acids.

The RNA-seq analysis on the roots, revealed the presence of genes highly induced in the tolerant clone and downregulated in the susceptible one. Because our protocol is conducted under axenic conditions, devoid of any additional stressors, we posit that these genes are specifically implicated in the defense response of *Qs* against *Pc* infection.

The molecular responses were analyzed at 5 days post-inoculation (dpi) under our conditions, revealing that the plant's response to *Pc* is notably swift, likely due to the small tree's attempt to shield the roots from zoospore penetration.

It has previously been described that Phytophthora sp. invasion of the host is accompanied by the production and secretion of a number of enzymes that digest and degrade the underlying plant cell wall (Hardham, 2005), and *Phythophtora capsici* has been described to produce inhibitors of cell wall reinforcement in plants, in particular pectin methylesterase, which degrades plant pectin and causes maceration of plant tissue (Jia et al., 2009). However, RNA-seq data showed strong expression of pectinesterase/pectinesterase inhibitor 17 (PMEI) in the tolerant clones compared to the susceptible ones. These PMEI proteins have been shown to counteract the activity of PME while exhibiting antifungal properties against a number of pathogens such as *Fusarium oxysporum* f. sp. matthiole, Alternaria brassicicola, Botrytis cinerea and Verticillium wilt (Hayat et al., 2020). These results indicate that *Pc* attacks the integrity of the cell wall and likely degrades cell wall junctions and contributes to the penetration of zoospores into the roots (Hardham, 2020). It is plausible that esterases and other cell wall-related proteins could establish interactions between the pathogen and the root. Further testing is needed to confirm this hypothesis, although some work has shown interaction between *Pc* proteins and the host. In addition to PME, other cell wall proteins were detected to be induced by *Pc* on *Qs*: the **proline-rich protein 2 (PRP)** is a cell wall protein involved in the oxidative burst (O'Brien et al., 2012). Its implication in biotic stress resistance are limited to only few reports, their contribution has been well documented in providing resistance against viral (He et al., 2002), bacterial (Cecchini et al., 2015; Li et al., 2014), and fungal (Mellacheruvu et al., 2016) pathogens. Contemporary investigations suggest that the promoter region of PRP genes has drought inducible elements, and these cis-acting elements serve as binding sites for WRKY transcription factors, basic helix-loop-helix proteins and MYBs (Gujjar et al., 2018).

Moreover, **Glycosyl hydrolases (GHs)**, that are enzymes crucial for metabolizing various carbohydrate-containing compounds found in plant tissues (Minic, 2008) and that is also induced highly in the tolerant clone compared to the susceptible. During the colonization of hosts, plant-associated microbes like fungi and oomycetes release a range of GHs onto their cell surfaces and surrounding extracellular environments. These secreted GHs from plant-associated fungi and oomycetes serve diverse functions, many of which act as virulence factors (effectors) facilitating microbial host colonization. Their specific roles include nutrient acquisition, detoxification of antimicrobial compounds, manipulation of plant microbiota, and suppression or prevention of plant immune responses. Additionally, they can activate the plant immune system by functioning as microbe-associated molecular patterns (MAMPs) or by releasing damage-associated molecular patterns (DAMPs) due to their enzymatic activity (Bradley et al., 2022; Rafiei et al., 2021). **Cinnamyl alcohol dehydrogenase** was detected in our data in the tolerant clone but doesn't seem to be expressed in the susceptible. Previous studies provided evidence of up-regulation of this gene in infected roots of cork oak seedlings, suggesting a detoxifying role that may be inactive in susceptible hosts but partially or fully active in hosts displaying chronic decline symptoms (Coelho et al., 2006; Ebadzad & Cravador, 2014). **The dehydration-responsive protein** exhibited upregulation. In *Arabidopsis thaliana*, this gene plays a dual role in combating both drought and biotic stress responses (Ben Rejeb et al., 2014; Sakuma et al., 2006). This can be attributed to the fact that *Pc* induces root rot, thereby inducing drought stress, as previously described (San-Eufrasio et al., 2021; Sghaier-Hammami et al., 2013). Regarded as a defense-related gene, **Peroxidase 45** was detected in the RNA-seq analysis. Peroxidases (POXs) are known to facilitate the production of reactive oxygen species (ROS) while oxidizing plant hormones like indole-3-acetic acid (IAA) and defense-related compounds such as salicylic acid (SA) (Kawano, 2003; O'Brien et al., 2012).They play pivotal roles in various physiological processes, including wound healing and defense mechanisms against pathogen infections (Hiraga et al., 2001). A recent study investigated the proteomic and metabolomic responses of both *Quercus suber* and Q. *variabilis* infected with *Pc*. The study observed the upregulation of stress-related proteins, including peroxidases, superoxide dismutases, and glutathione S-transferases, alongside proteins involved in jasmonic acid metabolism (Saiz-Fernández et al., 2022). Moreover, various facto may switch peroxidases between generating and neutralizing reactive oxygen species (ROS) produced in response to phytopathogens (Minibayeva et al., 2015).

Additionally, **Cytochrome P450 (CYP)** that serves a vital function in higher plants was found highly expressed in the tolerant clone. CYP contributes to the biosynthesis of secondary metabolites, antioxidants, and phytohormones. The expression of numerous CYP genes is modulated in reaction to environmental stresses, and they also facilitate significant interactions between abiotic and biotic stress responses.(Cunha et al., 2023; Pandian et al., 2020). Moreover, **Lipid transfer proteins (LTPs)** which the function as antimicrobial peptides (AMPs) contributes to plant defense against pathogens (Carvalho et al., 2006) was expressed. LTPs possess the capability to bind and transport various hydrophobic molecules such as phospholipids, acyl CoA, and fatty acids of different sizes (Capote et al., 2018; Carvalho et al., 2006). While the exact mechanism of fungal growth inhibition remains unclear, LTP1 has been observed to permeabilize the fungal plasma membrane to fluorescent dye, suggesting potential involvement in halting fungal growth (Regente et al., 2005). In addition to their inhibitory activity in vitro, the transgenic expression of LTP genes has been shown to confer enhanced resistance to bacterial and fungal infections (Carvalho et al., 2006; García-Olmedo et al., 1995; Li et al., 2021).

In addition to the detection of defense genes, studies conducted by (Silva et al., 2020) were used to identify marker genes related to epigenetic process. These genes exhibit expression in the tolerant clone of *Qs*, whereas they are suppressed in the susceptible clone.

Amidst plant developmental shifts and reactions to stress, there is substantial restructuring of histone methylation and gene expression, facilitated by specific enzymes known as histone methyltransferases (HMTs) and demethylases (HDMs). HMTs catalyze histone lysine methylation using S-adenosyl methionine (SAM) as a methyl group donor, forming N-methyl protein adducts. Recent findings suggest that certain HMTs play a crucial role in governing metabolic gene expression in plants (Li et al., 2015; Liu et al., 2010; Shen et al., 2016; Thorstensen et al., 2011).

Histone acetyltransferases (HATs) and deacetylases (HDACs) catalyzes histone acetylation and deacetylation at the N-terminus of histone tails which play crucial roles in the regulation of eukaryotic gene activity (Zhao et al., 2015), since histone acetylation is frequently related to increased gene expression while deacetylation is associated with transcriptional repression (Hebbes et al., 1988). It also participate in control of defense priming (Espinas et al., 2016). HATs are regrouped into 4 families, in our data we detected 3 of them: GNAT (Gcn5-related N-acetyltransferase), MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60), CBP (cAMP-responsive element-binding protein-binding protein). While HDACs from 3 families we detected one: Histone deacetylase.

DNA methyltransferases are responsible for cytosine methylation, a crucial DNA modification governing gene expression. 5mC DNA methylation at gene promoters typically leads to gene silencing. However, the role of 5mC in coding regions have demonstrated in studies suggesting it correlates with active gene transcription. (Bewick & Schmitz, 2017; Silva et al., 2020; Zhang et al., 2006). In the dataset obtained analysing *Qs* roots infected with *Pc*, two families of DNA methyltransferases are identified: Methyltransferase (MET) and DNA Methyltransferase homolog 2 (DNMT2). MET is responsible for maintaining CG methylation in heterochromatic regions rich in transposable elements (TEs) and repeats, as well as within intragenic regions. while the specific role of the DNMT2 family in plant DNA methylation remains unclear (Alves et al., 2022; Ramos et al., 2013; Silva et al., 2020). The RNA-seq data also revealed the presence of RNA-directed DNA methylation (RdDM), a *de novo* methylation mechanism that triggers cytosine base methylation in DNA through small interfering RNAs (siRNAs) (Espinas et al., 2016). In plants, RdDM can modulate chromatin states using double-stranded RNA and resulting 24-nucleotide (nt) short RNAs (Popova et al., 2013).

In the work realised in collaboration with la Universidade de Lisboa and El Consejo Superior de Investigaciones Científicas (MBG-CSIC), Santiago de Compostela. Testing resistance of different transgenics of *Qs* to *Pc* infection. In the present study, we investigated whether overexpression of the *Cast_Gnk2-like* gene from *C. crenata* in somatic embryos of cork oak increases the resistance of the trees to *Pc*, the main cause of oak decline. Research findings have unveiled that this gene shares similarity with Ginkbilobin-2, discovered in *Ginkgo biloba*, which encodes an antifungal protein. This protein features a plant-specific cystine-rich motif serving as a lectin, and its capability to bind carbohydrates is closely linked with its antifungal properties. The interaction between lectins and mannose residues present on the cell wall of *Phytophthora* species has been shown to potentially disrupt and alter the structure of the cell wall (Colavolpe et al., 2023). This concept has already been tested on holm oak (Quercus ilex) to enhance its resistance against *Pc* infection. In an in vitro resistance assay, it was observed that transgenic plants could endure longer than the wild type (Serrazina et al., 2022). This finding might elucidate the resistance of transgenic *Quercus suber* against *Pc* infection, although further results will be forthcoming from (Serrazina et al., Under review).

V Conclusion

1. In the study, it was determined that an extract derived from *Artemisia absinthium* var. candial exhibits activity "*in vitro*" against *Fusarium oxysporum f. sp. radicis-lycopersici*.

2. The priming treatment with *Artemisia absinthium* essential oil enhanced seed germination and improved protection against *Fusarium oxysporum* during the germination process and seedling growth producing long term effects on plant resistance.

3. This research illustrates that priming with the essential oil *Artemisia absinthium* induces transcriptional changes in the genome of tomato Marmande seedlings, thereby enhancing resistance to *Fol*. These changes involve genes associated with the reduction of oxidative stress, the ethylene-mediated signalling pathway through MTC cycle, and methylation events.

4. AEO seed priming resulted in an increase in the production of metabolites in tomato seedlings, contributing to the defense response. This increase aligns with the observed transcriptional changes, particularly with regard to vanillic acid, coumarin, lycopene, oleamide, and an unknown metabolite of m/z 52.

5. AEO seed priming induced "*de novo*" epigenetic changes and methylation events mediated by master regulator genes involved in RNA-mediated methylation, such as *SAM* and *NRPD2*, thereby enhancing resistance to *Fol*.

6. The methylation events occur within a short timeframe by the effect of both *Fusarium oxysporum* infection and the treatment with *Artemisia absinthium* essential oil, leading to an increase in both DNA and RNA global methylation patterns.

7. In the study, an optimized "*in vitro*" protocol was developed to comparatively assess the resistance levels of *Quercus suber* explants to the highly impactful phytopathogen *Phytophthora cinnamomi* Rands.

8. The comparative analysis of resistance to *Phytophthora cinnamomi* among fourteen different *Quercus suber* clones demonstrated a strong correlation with resistance assessments conducted by other research groups in both greenhouse and forest environments.

9. Through genomic analysis comparing tolerant and susceptible *Quercus suber* explants, it was established that *Quercus suber* responds to *Phytophthora cinnamomi*, activating specific gene families involved in hindering direct pathogen attacks and reinforcing cell wall protection to prevent zoospore penetration through the roots.

10. Using our *in vitro* protocol, it was determined that the overexpression of defense-related genes from *Castanea* into *Quercus suber* transformed explants improved resistance against *Phytophthora cinnamomi* oomycete.

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