Variability of physical dormancy in relation to seed mechanical properties of three legume species

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Abstract

Many Fabaceae species have a hard and impermeable seed coat that requires scarification for germination. The variability within a batch and between batches of the same species can be very high, resulting in inefficient dormancy breaking. The main objectives of this study were to investigate the variability in hardseededness among four populations of the legume species *Medicago polymorpha*, *Trifolium glomeratum* and *T. subterraneum*, and to evaluate the effect of the best scarification treatments on the mechanical properties of the seed coat. These species have very small seeds that hinder scarification procedures. Different treatments, including sanding and temperature changes, were studied. Results varied among species, and the most efficient treatment for breaking dormancy was determined for each one. For each species, wide variability was observed in the best scarification treatment within and among populations. The effect of the best scarification treatment on the seed coat of each species was examined by means of scanning electronic microscopy (SEM). Evident differences were found in *T. glomeratum*, as the treatment used was sanding. In *T. subterraneum*, scarified seeds (by thermal treatment) showed wider hilum opening. No differences were observed in *M. polymorpha* seeds.

Introduction

Legumes play an important role in agriculture, not only as a source of carbohydrates and proteins for human and animal consumption but also as providers of ecosystem services by improving nitrogen levels in the soil (Herridge *et al.*, 2008; Crews *et al.*, 2016). In addition, legumes play a potentially significant part in enhancing soil carbon sequestration (Abberton, 2010). Annual pasture legumes have been shown to be an important factor in sustainable agriculture by improving soil quality (Walsh *et al.*, 2013) when used in an integrated crop-livestock production system based on rotation of cereal crops with annual legume pasture (Walsh *et al.*, 2001). Conservation of the genetic diversity of annual pasture legumes, therefore, plays an important part in adaptation to climate change, particularly in the Mediterranean area.

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The management of legume seeds is difficult due to the small seed size of some species and their physical dormancy due to a hard seed coat. Physical dormancy in legumes is the result of an impermeable seed coat caused by the presence of one or more palisade layers of lignified cells (macrosclereids) (Baskin *et al.*, 2000; Baskin and Baskin, 2014). Impermeability has been attributed to properties including seed coat thickness, composition of the cuticle and testa pigments (Smykal *et al.*, 2014). Under natural conditions, the seed coat becomes permeable by the action of environmental factors. The specific site of water entry after seed scarification (either naturally occurring or under experimental conditions) has been studied in several legume species and is still a matter of debate (Smykal *et al.*, 2014; Hudson *et al.*, 2015). In some legume species, an anatomical structure in the impermeable layer, called the lens, functions as a “water gap” (Baskin *et al.*, 2000; Baskin, 2003).

Phenotypic variation of seed coat permeability can be the result of maternal genetic and environmental influences (as reviewed by Hudson *et al.*, 2015) and therefore, populations from different origins could show different responses to dormancy breaking treatments. The mechanical properties of legume seed coats have been studied in relation to their variability among species and populations (Rybinski *et al.*, 2014, 2015) and with regard to possible damage imposed by processing operations (harvesting, cleaning, sorting, transport and storage). Mechanical properties of seeds and fruits have also been related to their water content and size (Saiedirad *et al.*, 2008), and to their adaptation for ant dispersal (Rodgers, 1998).

Medicago polymorpha L. (toothed medick or burclover), *Trifolium subterraneum* L. (burrowing clover or subterranean clover) and *T. glomeratum* L. (clustered clover) are annual legumes of particular interest with regard to their use in mixed pastures for intensive pasturage and also as ground cover in some agricultural settings such as olive groves (Repullo-Ruibérriz *et al.*, 2014). A pre-chilling procedure is recommended for improving germination of genera with physical dormancy such as *Medicago* and *Trifolium* (ISTA, 2011). However, this treatment is not widely applicable due to inter- and intra-specific variability in germination response found among species of the same genus and even among populations (Hudson *et al.*, 2015; Renzi *et al.*, 2016).

Time-consuming procedures have to be used in order to carry out viability testing or multiplication cycles. The high number of hard seeds or damaged embryos that may result after some scarification treatments could not only under-represent the viable seeds but also impose selection on the germplasm (Martin and Guerrero, 2014).

The main objectives of this investigation were (1) to study the hardseededness of several populations of *M. polymorpha*, *T. subterraneum* and *T. glomeratum*, establishing a simple scarification procedure that could be used to avoid selection (non-germinated or damaged seeds), and (2) to study the effect of different scarification treatments on the mechanical properties and morphology of the seed coat, and the resulting effect on germination potential.

Materials and methods

Seed material

Seeds of four different populations of *M. polymorpha*, *T. subterraneum* and *T. glomeratum* were obtained from the seed bank of the Centro de Investigaciones Científicas y Tecnológicas de Extremadura (Spain; table 1). They were harvested in 2013 after multiplication at the centre; germination experiments took place between November 2013 and February 2014. Seeds were stored under laboratory conditions (at approximately 23°C, under darkness, 20-25% relative humidity) until the start of trials.

Table 1. Collection data of seeds from four populations of three legume species.

Species	Population code	Accession number in Spanish National Inventory	Collection site (Province, Country)
<i>Medicago polymorpha</i>	MP1	LO8983	Huelva, Spain
	MP2	LO8985	Sevilla, Spain
	MP3	LO8992	Cádiz, Spain
	MP4	LO8998	Cádiz, Spain
<i>Trifolium subterraneum</i>	TS1	LO0011	Badajoz, Spain
	TS2	LO3570	Sardinia, Italy
	TS3	LO3595	Badajoz, Spain
	TS4	LO9027	Málaga, Spain
<i>Trifolium glomeratum</i>	TG1	LO6669	Baixo Alentejo, Portugal
	TG2	LO8150	Badajoz, Spain
	TG3	LO9024	Málaga, Spain
	TG4	LO9039	Cádiz, Spain

Seed scarification and germination

Seeds from all populations were subjected to each of eight scarification treatments before sowing:

- *Sanding* (SD). Seeds were gently abraded between two sheets of fine sandpaper.
- *Wet heat* (WH). Seeds were immersed in boiling water, which was then immediately removed from the heat source; seeds remained in the water at room temperature for 24 hours.
- *Dry heat* (DH). Seeds were heated at 100°C in an oven for 30 (DH1) or 60 minutes (DH2) and then left to equilibrate to room temperature.
- *Liquid nitrogen 1* (LN1). Seeds were immersed in liquid nitrogen (-196°C) inside a polypropylene cryovial for 30 minutes and then left to warm at room temperature.
- *Liquid nitrogen 2* (LN2). Seeds were stored at -20°C for 24 hours inside a polypropylene cryovial, immediately immersed in liquid nitrogen for 30 minutes and then left to warm at room temperature.

- *Freezing 1* (FR1). Seeds were stored at -80°C inside a polypropylene cryovial for 24 hours and then left to warm at room temperature.
- *Freezing 2* (FR2). Seeds were stored at -20°C inside a polypropylene cryovial for 24 hours, followed by -80°C for 24 hours and then left to warm at room temperature.
- *Freezing + heat* (FH). Seeds were stored at -80°C inside a polypropylene cryovial for 24 hours, subsequently removed from the cryovial, immersed in water at 90°C for five seconds and then left to dry on a paper towel at room temperature.

After each scarification treatment, seeds were set to germinate. Non-scarified seeds were used as controls. Four replicates of 25 seeds each per population and treatment were sown on top of two sheets of filter paper (Filter-Lab®), previously moistened with 3.5 mL distilled water, in 70 mm-diameter glass Petri dishes. Seeds were incubated at 25°C for 16 hours in the light and 15°C for eight hours in the dark each day; light was provided by cool white fluorescent tubes with an irradiance of 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Filter papers were rewetted with distilled water as required. Dishes were checked three times a week over a 21-day test period for *M. polymorpha* and *T. glomeratum*, and over 30 days for *T. subterraneum*. Germination was defined as seedling production, both normal (with intact, fully-developed cotyledons and radicle) and abnormal (with broken radicle or cotyledons). Germination percentages of normal and abnormal seedlings were determined at the end of the experimental period. The final germination percentage was based on the sum of both values.

In a separate experiment, *M. polymorpha* seeds that had been subjected to sanding and had failed to germinate after 15 days were sanded, resown and germination observed after a further seven days. This procedure was repeated two days later.

Seed mechanical properties

Seed resistance to loading was measured under a quasi-static compression test performed with a universal testing machine (Texture Analyzer TA XT PLUS), equipped with a 30 kg load cell. The measurement resolution was ± 0.001 N in force and ± 0.001 mm in deformation. Compression was performed on individual seeds under a configuration of two flat parallel plates (between flat platform of Texture Analyser and flat probe), with the following settings: pre-test and post-test speeds of 0.50 mm second⁻¹, test speed of 0.10 mm second⁻¹ and trigger force of 0.049 N. The target mode selected to end each test was the compression distance, which was adjusted taking into account the seed size of each species: 0.70 mm (*T. subterraneum*), 0.50 mm (*M. polymorpha*) and 0.20 mm (*T. glomeratum*). Each seed was positioned horizontally with its major axis being normal to the direction of loading (except for *T. glomeratum* due to near spherical shape). To compare untreated seeds (controls) and scarified seeds by the best treatment (those resulting in highest final germination for each species), the force-deformation curves were recorded and processed to determine the following parameters: the maximum force (N) until rupture, when the seed hull undergoes failure during compression, and the rupture energy (mJ) or toughness (Saiedirad *et al.*, 2008). This last parameter is defined as the energy required to crush the hull, determined as the area under the curve up to the point of rupture (Baumler *et al.*, 2006). This study could not be carried out in the TS2 population of *T. subterraneum* due to insufficient number of seeds or in scarified

seeds of *T. glomeratum* due to their small size. Due to the high variability in seed sizes, both inter-species and inter-population, the surface area and weight of individual seeds were determined and their mechanical properties related to them. Seed surface area was determined by image by placing seeds horizontally (except for *T. glomeratum* due to their near spherical shape) under a standard camera, and measuring the area using Image J program. In the case of *T. glomeratum*, individual weights could not be measured due to small seed size, and two replicates of approximately 400 control seeds each were used. Additional variables calculated for each seed were breaking strength under compression stress, estimated as maximum force per unit area (N mm^{-2} , MPa), and specific energy or energy per unit mass (J g^{-1}). Twenty seeds were used per population for control and scarification treatments.

Seed coat observation by SEM

The seed coat was examined by SEM (JEOL 6335 F, at 15 kV, after gold metallisation for four minutes) to detect possible alterations caused by scarification treatment. Seeds from one population of each species were studied, both control seeds and seeds scarified by the method that resulted in the highest germination rate: MP1 for *M. polymorpha*, TS3 for *T. subterraneum* and TG2 for *T. glomeratum*. Seeds were placed either on their side or showing the hilum area. Ten seeds were observed per species and treatment.

Statistical analysis

The number of normal seedlings (normal germination) was analysed, among treatments for each population and among populations for each species and treatment, by logistic regression for events data and pairwise comparisons were carried out using, for the adjustment for multiple comparisons, the least significant difference (LSD) method with a 5% significance level (Agresti, 2002). Analyses were carried out using the Generalised Linear Models Procedure of the program IBM SPSS Statistics v20. The mechanical properties of seeds data were subjected to ANOVA (IBM SPSS Statistics v20).

Results

Scarification treatments

For non-scarified seeds (controls), the proportion of hard seeds ranged from 91 to 96% (*M. polymorpha*), 87 to 94% (*T. subterraneum*) and 98 to 100% (*T. glomeratum*), with no significant differences (at $P = 0.05$) among populations (figures 1, 2 and 3).

In all populations of *M. polymorpha*, the highest percentages of normal seedlings (normal germination) were obtained after seeds had been immersed in liquid nitrogen following pre-treatment at -20°C for 24 hours (LN2). Direct immersion in LN (LN1), despite reducing hardseededness to a similar extent, resulted in a much higher percentage of abnormal seedlings (43 vs. 3%). Several other treatments resulted in percentages of normal seedlings between those resulting from the best treatment and control values: SD, FR1 and FH treatments. WH and DH treatments resulted in normal germination percentages not significantly different from those of control seeds in the four populations, except in

Figure 1. Final germination percentages (abnormal and normal seedlings) of seeds belonging to four populations of *Medicago polymorpha* after different scarification treatments: C, control seeds; SD, sanding; WH, wet heat; DH1 and DH2, dry heat; LN1 and LN2, liquid nitrogen; FR1 and FR2, freezing treatment; FH, freezing and heat. Full bar corresponds to normal seedlings and shaded bar to abnormal seedlings. For percentages of normal germination (normal seedlings) within a population, columns with the same letter are not significantly different by pair-wise comparison by LSD ($\alpha = 0.05$).

MP2 where the DH1 treatment resulted in higher normal germination percentages. The seeds from the SD treatment that had not germinated after 15 days (hard seeds or non-imbibed seeds) were subjected to further sanding, and this operation was repeated with non-germinated seeds after seven days. In all four populations, final germination rates reached 100% (table 2) and all seedlings were normal.

Seeds of *T. subterraneum* and *M. polymorpha* showed different responses to the same treatments. Immersion in liquid nitrogen did not result in the highest removal of hardseededness in all populations, and in those in which the proportion of hard seeds was low there was a higher number of abnormal seedlings than with other treatments. In all

four populations, the highest percentages of normal germination were obtained with WH and FH treatments. In two populations (TS3 and TS4), FR1 and SD, respectively, resulted in normal germination not significantly different from the best treatments. The response to other treatments varied between populations.

For all four populations of *T. glomeratum*, the best results were obtained after SD treatment. This species showed the highest proportion of hardseededness. The response to other treatments varied between populations. For TG1 and TG4, other treatments resulted in less than 20% normal germination; dry heat improved germination to > 40% in the other two populations.

Figure 2. Final germination percentages (abnormal and normal seedlings) of seeds belonging to four populations of *Trifolium subterraneum* after different scarification treatments: C, control seeds; SD, sanding; WH, wet heat; DH1 and DH2, dry heat; LN1 and LN2, liquid nitrogen; FR1 and FR2, freezing treatment; FH, freezing and heat. Full bar corresponds to normal seedlings and shaded bar to abnormal seedlings. For percentages of normal germination (normal seedlings) within a population, columns with the same letter are not significantly different by pair-wise comparison by LSD ($\alpha = 0.05$). NA, data not available (treatment not carried out due to shortage of seeds).

Regarding intra-specific variability, there were no significant differences among populations in normal germination of non-scarified seeds for the three studied species (figures 1, 2 and 3). *M. polymorpha* showed no significant differences among populations with the treatment that showed the best results (LN2) or with LN1, WH, DH2 and FH treatments. In contrast, *T. subterraneum* seeds showed significant differences between populations in all the treatments studied, with TS4 and TS3 being the ones with the best and worst performance, respectively. In *T. glomeratum*, only the treatment SD achieved a normal germination percentage above 50%. With this treatment, the differences between populations were significantly different, even though high germination percentages were observed (85-100%).

Figure 3. Final germination percentages (abnormal and normal seedlings) of seeds belonging to four populations of *Trifolium glomeratum* after different scarification treatments: C, control seeds; SD, sanding; WH, wet heat; DH1 and DH2, dry heat; LN1 and LN2, liquid nitrogen; FR1 and FR2, freezing treatment; FH, freezing and heat. Full bar corresponds to normal seedlings and shaded bar to abnormal seedlings. For percentages of normal germination (normal seedlings) within a population, columns with the same letter are not significantly different by pair-wise comparison by LSD ($\alpha = 0.05$).

Table 2. Accumulated germination percentages of four populations of *Medicago polymorpha* after three successive sanding treatments.

Population	Germination (%)		
	1 st sanding	2 nd sanding	3 rd sanding
MP1	36	71	100
MP2	53	69	100
MP3	65	75	100
MP4	43	85	100

Seed mechanical properties

In terms of maximum force (N) and energy (mJ), the highest mean value was obtained for *M. polymorpha* (62.1 ± 12.9 N; 6.9 ± 2.6 mJ), intermediate for *T. subterraneum* (36.4 ± 10.2 N; 4.0 ± 1.8 mJ), while *T. glomeratum* seeds broke with lower loads and energy (19.5 ± 3.4 N; 1.1 ± 0.3 mJ) (table 3). In terms of breaking strength (MPa) and specific energy (J g^{-1}), *T. glomeratum* seeds showed the highest average values (27.55 ± 0.48 MPa; 3.39 ± 0.51 J g^{-1}) as corresponds to the smaller size (in weight and area) of these seeds. The average weight of 1,000 *T. glomeratum* seeds (WTS) was 0.39 g, 18- and 10.5-times lighter than *T. subterraneum* and *M. polymorpha*, respectively. In addition, *T. glomeratum* stores up to 7-times and twice the energy per mass unit before fracture than *T. subterraneum* and *M. polymorpha*, respectively.

In contrast, there were significant differences among populations in *M. polymorpha* and *T. subterraneum* (table 3). In *M. polymorpha*, there were significant differences among populations both in their maximum load and their size: MP2 seeds had the highest surface area and WTS. This population also had the seeds with the highest maximum load (68.31 N) and energy to breakage (8.28 mJ), although these values were not significantly different from those obtained for MP1. However, as these seeds had the highest surface area, the breaking strength to seed failure was the lowest (11.86 MPa). With regard to specific energy, there were no significant differences ($P = 0.296$) among populations. Scarified seeds of *M. polymorpha* used for the mechanical properties' study had been subjected to the treatment that resulted in the highest percentage of normal seedlings (LN2). There was an overall effect (considering the four populations) of scarification treatment on specific energy values ($P = 0.036$), although not in the remaining mechanical properties studied ($P > 0.05$). On the other hand, when statistical analysis was conducted separately for each population, no significant difference ($P = 0.352$) was found between control and scarified seeds. In the four populations studied, energy, specific energy, maximum force and breaking strength were higher in control seeds than in scarified ones (table 3).

Mechanical properties of *T. subterraneum* seeds were studied only in three populations due to insufficient TS2 seeds. Again, there were significant differences between populations (control seeds) for all mechanical properties studied. Maximum force and energy were higher for TS1 and TS3 than for TS4. However, as TS1 had the largest seeds (surface area and mass), the energy per mass unit was similar to that of TS4. There was a significant effect ($P = 0.012$) of the scarification treatment (FH) in the maximum force

Table 3. Static load parameters (mean ± standard error) of seeds from different populations of three legume species.

Species	Population	T	N	Seed morphological parameters ¹		Static load parameters ²			
				Seed area (mm ²)	Weight of 1,000 seeds (g)	Maximum force (N)	Breaking strength (MPa)	Rupture energy (mJ)	Specific energy (J g ⁻¹)
<i>M. polymorpha</i>	MP1	C	20	4.3 ± 0.7b	3.8 ± 0.7b	63.41 ± 15.35ab	15.04 ± 3.98a	7.01 ± 2.90ab	1.89 ± 0.80a
		S	20						
	MP2	C	20	4.0 ± 0.6c	3.4 ± 0.7c	58.62 ± 9.55b	13.93 ± 2.29a	6.04 ± 1.84b	1.73 ± 0.53a
		S	19						
	MP3	C	19	5.9 ± 1.0a	5.6 ± 1.3a	68.31 ± 13.48a	11.86 ± 2.93b	8.28 ± 2.98a	1.52 ± 0.63a
		S	19						
<i>T. subterraneum</i>	MP4	C	20	4.3 ± 0.7b	3.8 ± 0.8b	58.30 ± 10.74b	14.12 ± 3.06a	6.22 ± 2.10b	1.71 ± 0.61a
		S	20						
	TS1	C	19	6.6 ± 1.3a	8.7 ± 2.5a	40.48 ± 9.31a	6.11 ± 1.06ab	4.22 ± 1.69a	0.49 ± 0.12b
		S	20						
	TS3	C	16	5.8 ± 1.4b	7.3 ± 2.6b	37.28 ± 9.35a	7.07 ± 1.69a	4.54 ± 1.85a	0.62 ± 0.18a
		S	20						
<i>T. glomeratum</i>	TS4	C	9	4.9 ± 1.2b	5.3 ± 1.9c	26.33 ± 6.85b	5.56 ± 1.70b	2.35 ± 0.87b	0.46 ± 0.18b
		S	10						
	TG1	C	20	0.69 ± 0.1b	0.347 ± 0.007	18.40 ± 2.52a	27.12 ± 5.52a	0.97 ± 0.20a	2.80
		C	19						
	TG3	C	20	0.73 ± 0.1ab	0.436 ± 0.019	18.96 ± 2.36a	26.19 ± 3.90a	1.00 ± 0.21a	3.12
		C	20						

T, seed treatment; C, control; S, scarified by LN2 for *Medicago polymorpha*, FH for *Trifolium subterraneum*, or SD for *Trifolium glomeratum* (see text for details); N, number of tested seeds. For each species, mean values for control seeds within a column followed by the same letters are not significantly different ($P = 0.05$). ¹Seed morphological parameters for control (C) and scarified (S) seeds were not significantly different ($P = 0.05$) and mean value for both seed lots is showed. ²For each population, mean values for control (C) and scarified (S) seeds were not significantly different (NS) or significantly different at 0.05 level (*).

that seeds withstood before rupture; these differences ($P = 0.007$) were also observed in the breaking strength that seeds could tolerate, but not in terms of energy (toughness) ($P = 0.061$). However, when the variable studied was the specific energy, the differences were significant ($P = 0.01$). When control and scarified seeds were compared within each population, only in TS3 were all of these variables significantly different (table 3).

There were no significant differences ($P > 0.05$) among the mechanical properties of control seeds of *T. glomeratum* in the four populations studied. Due to the small size of these seeds, the mechanical properties of the scarified seeds could not be studied as these were beyond the limits of the device used.

Seed coat observation by SEM

Seed coats of control and scarified seeds were examined. The scarification procedure used was the one that resulted in the highest percentage of normal seedlings (LN2 for *M. polymorpha*, FH for *T. subterraneum* and SD for *T. glomeratum*). There were no external differences on the seed coat of control and scarified seeds of *M. polymorpha* (figure 4). Thin fissures and marks could be observed in both types of seeds on the lateral sides (figure 4A-D). On the area near the lens and the radicular lobe, wider fissures were observed both in control and scarified seeds (figure 4E-F, arrows). These cracks were also observed near the hilum (figure 4G-H). The size of the cracks was similar in both seed types; therefore, they were considered to be unrelated to the breakage of hardseededness.

In *T. subterraneum*, the seed surface had stellar patterns in patches (figure 5A-B), probably related to differential deposition of waxes. In this species, small fissures could be observed on the lateral sides of both control and scarified seeds (figure 5C-F). No fissures were observed around the hilum; however, one distinctive difference between both control and scarified seeds was that, in the latter, the hilum groove appeared more open (figure 5G-H). In *T. glomeratum*, the scars and fissures produced by sanding were clearly evident when observed using the SEM (figure 6).

Discussion

Ex situ conservation management of plant germplasm requires efficient methods to carry out germination procedures. These tests should consume as little time as possible, reflect actual viability of the seed population and avoid selection. In the case of species with hard seed coats (common in Fabaceae and Malvaceae families, for example), germination tests should produce a high percentage of germinating seeds without producing damage to the seedlings. This could be particularly difficult and time-consuming in the case of species with small seeds. Some seed banks use a two-step procedure: seeds are set to germinate after scarification, and non-germinated hard seeds at the first count are subjected to repeated treatment. This procedure could be time-consuming when many accessions have to be tested (Wang and Hanson, 2008). Scarification procedures for the species reported here have been studied before. Treatment with sulphuric acid for 30 minutes raised germination levels from 23.4 to 62.6% as a mean value for 53 accessions of *T. glomeratum*; however, this treatment was harmful for some of those accessions

Figure 4. Scanning electron microphotographs of *Medicago polymorpha* control seeds (A, C, E and G) or scarified seeds at -20°C for 24 hours and then LN for 30 minutes (B, D, F and H). General view of the lateral side showing marks (A and B), and detail of those marks (C and D); inset in (A) detail of seed coat. View of the ventral side showing cracks at the radicular lobe and lens side (arrows in E and F). View of hilum with cracks around them (G and H).

Figure 5. Scanning electron microphotographs of *Trifolium subterraneum* control seeds (A, B, C, E, G and I) or scarified seeds at -80°C for 24 hours and then five seconds in water at 90°C (D, F, H and J). General view of the lateral side (A), and detail of the star pattern (B); inset in (A) detail of seed coat. Fissures observed in lateral sides of both control and scarified seeds (C-F). View of hilum with a wider aperture in the scarified seed (G and I vs. H and J)".

Figure 6. Scanning electron microphotographs of lateral sides of *Trifolium glomeratum* control (A) and sanded seeds (B).

(Martin and Guerrero, 2014). The same treatment was also effective in *M. polymorpha* and *T. subterraneum* (Martin and De la Cuadra, 2004; Can *et al.*, 2009). Nevertheless, in other studies, germination of these species was not improved by scarification with sulphuric acid for 5 or 10 minutes (Siles *et al.*, 2016), probably due to too short a period of scarification or intra-specific variability. On the other hand, acid scarification could be risky for seeds and workers and should be avoided if possible, especially when many accessions have to be treated. Thermal treatments have also been used, and immersion in liquid nitrogen resulted in a high percentage of normal seedlings in *T. subterraneum* but not in *M. polymorpha* (Martin and De la Cuadra, 2004). Treatments based on extensive temperature gaps have been tested with several legume species (Martin and De la Cuadra, 2004; Patane and Gresta, 2006; Wang and Hanson, 2008; Can *et al.*, 2009; Tiryaki and Topu, 2014). In the present study, high percentages of normal seedlings (93-97%) were obtained in the four populations of *M. polymorpha* by storing seeds for 24 hours at -20°C before immersion in LN (figure 1). A similar treatment was used by Martin and De la Cuadra (2004) on one accession of *M. polymorpha*, but only 12% of normal seedlings were obtained, while 72% were abnormal. In contrast, in that same investigation, *T. subterraneum* responded positively to that same treatment with 96% of normal seedlings (figure 2); however, we obtained a variable response to this treatment depending on the accession (17-77% normal seedlings).

In nature, physical dormancy breaking in legume seeds implies a two-phase mechanism: firstly, a constant temperature is required (high or low depending on habitat), and secondly, as temperatures alternate, seeds become permeable by the opening of the lens (Smykal *et al.*, 2014). In *T. subterraneum* (a species from Mediterranean climates), temperature fluctuation between 30 and 60°C over some weeks or months allows the seed coat to become permeable, with a maximum daily temperature depending on the species: 30°C for *T. subterraneum* and 50°C for *M. polymorpha* (Smith *et al.*, 1996; Smykal *et al.*, 2014). In our experiments, for *M. polymorpha* and *T. subterraneum*, the best normal germination percentages were obtained with a thermal gap of approximately 170°C (treatments LN2 and FH, respectively; figures 1 and 2). Although these treatments had a similar thermal gap, the different maximum and minimum temperatures used (LN2 -20 to -196°C; FH -80 to 90°C) are likely to have influenced the final results.

Seed coat morphology and lipid content in several legume species were studied after exposure to field conditions (Zeng *et al.*, 2005). After two years, seeds of *M. polymorpha* and *T. subterraneum* showed more and larger fractures on the seed coat than control seeds, and water entered through those fractures or through the lens. In *T. subterraneum*, seed coat lipid content decreased, and this property was suggested to be related to seed softening. We found fissures (long and thin) and cracks in both control and scarified seeds of these two species (figures 4 and 5); therefore, an association between these coat alterations and germinability could not be established. The presence of cracks in the cuticle of the palisade layer of some soybean cultivars has been related to permeable coats, being more frequent in the dorsal side (Ma *et al.*, 2004). In our study, *T. glomeratum* seeds showed cracks on their surfaces after sanding (figure 6). *Vigna oblongifolia* A. Rich. seeds cracked at different sites depending on the scarification treatment used: in the hilum and extra-hilar region when treated with hot water and only in the hilum when treated

with sulphuric acid (Hu *et al.*, 2009). Conversely, in the same study, *Sesbania sesban* (L.) Merr. fractured in the lens with both treatments, and both control and treated seeds showed cracks in the hilum. We found no differences related to cracks or fissures between control and scarified seeds of *M. polymorpha* and *T. subterraneum* (figures 4 and 5). The only difference observed was in *T. subterraneum* where the hilar groove showed a wider opening in scarified seeds (figure 5). In several cultivars (with permeable and impermeable seed coats) of soya bean, the hilum groove was open, although it was not found to be the site of initial water entry (Ma *et al.*, 2004).

The resistance of seeds or fruits has been studied mainly in relation to processing or to compare different water contents or sizes (Baumler *et al.*, 2006; Saiedirad *et al.*, 2008). Seed mechanical properties of several vetch accessions were studied, revealing a wide variability in the resistance of seeds to static loading: from 20 to 70.5 MPa stress at maximum load or strength (Rybinski *et al.*, 2015). Of the three species investigated here, *T. glomeratum* had the strongest seeds, with a breaking strength of 27.6 MPa (mean value of four populations; table 3). This was expected due to their much smaller size, as was described for soya bean (Vincent, 1990). In addition, their specific energy also had the highest values, indicating an intrinsic high resistance to breakage in this species compared to the other two (table 3). This strength could be reflected in the more aggressive treatment required to remove seed dormancy in *T. glomeratum*, namely sanding. In *M. polymorpha* and *T. subterraneum*, there were differences among populations in breaking strength and toughness (table 3).

The scarification treatments did not have a significant effect on breaking strength in any of the populations of *M. polymorpha* (table 3). In *T. subterraneum*, a general effect of the treatment was observed (when all the populations were considered; table 3). However, within the population, there were only significant differences between control and scarified seeds in TS3. The energy required for breaking scarified seeds was lower, although not significantly different, than that of control seeds (table 3). This lack of statistical significance could be due to marked variation in the values, which could in turn be related to variability in the intrinsic hardseededness of each population. Variability in hardseededness is important as a ecophysiological strategy of species for spreading seed germination over time (Smykal *et al.*, 2014). The results obtained with *M. polymorpha* seeds with the best treatment (LN2) were not significantly different among populations (table 3). Similarly, differences in physical dormancy were found among wild populations and cultivars of *Vicia villosa* Roth (Renzi *et al.*, 2016).

The results of the present investigation show that species with seeds possessing a hard and impermeable coat respond differently to scarification treatments. In addition, we found inter-population variability in the mechanical properties of seeds, even when maternal plants have been cultured under similar environmental conditions. That variability is also demonstrated in the different responses of populations within a species to dormancy-breaking treatments. These results could suggest a strong genetic influence in determining variation in seed characters, and further studies are necessary to explore that influence and implications for the management of genebanks.

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