Seed germination of *Schinus molle* L. (Anacardiaceae) as related to its anatomy and dormancy alleviation

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Abstract

The seeds of *Schinus molle* are referred to as displaying physical dormancy because of their water-impermeable endocarp. Therefore, this work aimed to evaluate the germination of *S. molle* seeds as related to environmental conditions, scarification, storage time and seed anatomy. Various experiments were conducted to test the alleviation of dormancy in newly collected and stored seeds. Acid-scarified seeds incubated under continuous light at 25°C showed greatest vigour and germination. The separation of seeds by specific gravity revealed a higher germination percentage for those seeds that sank. In addition, dry storage alleviated dormancy with a remarkable increase in the various germination parameters. Overall, germination traits decreased after prolonged storage, but even after 12 months the means for germination parameters for stored seeds were still higher than those of newly collected ones. *S. molle* seeds remain attached to parts of the fruit mesocarp and endocarp. The mesocarp contains several layers of parenchyma showing secretory cavities. The endocarp consists of three layers of sclereids surrounding the embryo. Acid scarification strongly changed the structure of the external layers in the mesocarp, digesting parenchyma cells and removing the contents from both parenchyma cells and the secretory cavities; this improved water uptake during imbibition, which occurred only at the carpellary hilar slit. In conclusion, *S. molle* seeds are positively photoblastic and show physiological dormancy which can be alleviated by acid scarification and dry storage. Seeds can be stored for over 12 months without significant losses in germination parameters compared to newly collected seeds.

Introduction

Schinus molle L. (Anacardiaceae), known as the pepper tree, occurs in the subtropical regions of South America, including areas of Argentina, Bolivia, Chile and Brazil, but is cultivated in many countries (Lim, 2012; Silva-Luz and Pirani, 2013). The fruit of Anacardiaceae is a drupe and the mesocarp is usually resinous (Simpson, 2006; Pell *et al.*, 2011). In the genus *Schinus* the endocarp of the drupe is comprised of three layers of sclereids, classified as the Anacardium-type endocarp, that remains attached to seeds (Wannan, 2006; Pell *et al.*, 2011; Oliveira and Mariath, 2015). This Anacardium-type structure of endocarps is referred to as the cause of seed physical dormancy (Li *et al.*, 1999a). However, the dormancy of *S. molle* seeds was suggested by preliminary evidence as physiological (Morfin, 1985; Jøker *et al.*, 2002). Therefore, the causes of dormancy of *S. molle* seeds are still unclear and must be investigated to improve plant production.

Seed dormancy has been defined as the incapacity of viable seeds to germinate under favourable conditions (Bewley, 1997; Bentsink and Koornneef, 2008). The dormancy response is controlled by environmental factors such as light, temperature and seed storage conditions (Bentsink and Koornneef, 2008). Dormant seeds cause problems in crop plant production and must be treated to improve the germination parameters.

Schinus molle is cultivated worldwide as trees for urban areas, and is also important as a medical plant and for the restoration of areas polluted by heavy metals (Iponga *et al.*, 2008; Lim, 2012; Pereira *et al.*, 2016). Despite its widespread use, seed germination of this species is usually very low (Demelash *et al.*,

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2003). However, the reasons for seed dormancy in this species are still unclear, and the effects of seed scarification and storage on germination have never been tested. Since *S. molle* has high potential for use for crop and medical proposes, enhancing seed germination would greatly benefit plant production. Thus, the objective of our work was to evaluate the germination of *S. molle* seeds as related to environmental conditions, scarification, storage time and seed anatomy.

Materials and methods

Seed sampling

Fruits from a population of 25 plants of *S. molle* were collected in the southern region of Minas Gerais state, Brazil (21°25′45″S and 45°56′50″W) in October 2014. Samples were taken to the laboratory and dried at room temperature. The exocarps were removed manually from dried fruits. Seeds (here we call 'seeds' the seed plus parts of mesocarp and endocarp) were surface sterilized with 50% sodium hypochlorite for 10 min, washed twice with distilled water and then dried at 35°C for 72 h.

Dormancy alleviation tests

Newly collected samples were divided into the following groups: complete drupes (T1), seeds (T2), seeds mechanically scarified by using 25 g of sand in a mortar and pestle for 10 min (T3), seeds heat scarified in water at 80°C for 10 min (T4) and seeds acid scarified using 20 ml of sulphuric acid (H_2SO_4) for three different time intervals: 1 (T5), 3 (T6) and 5 min (T7) (Fig. 1). After acid scarification, the acid was neutralized with 40 ml of sodium bicarbonate and then seeds were washed twice with distilled water. All samples (treatments T1–T7) were placed under three
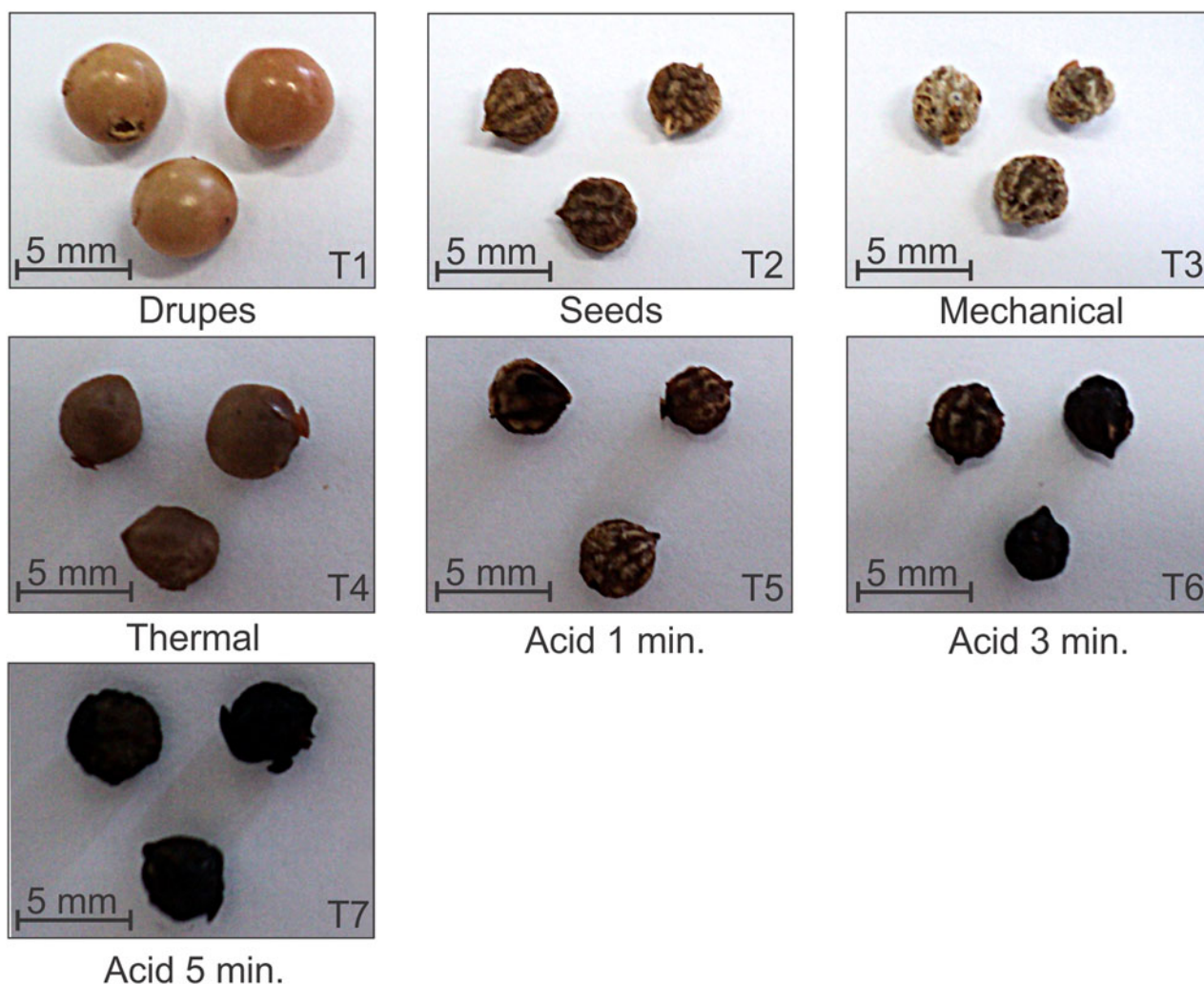

Figure 1. Dormancy alleviation methods using newly collected seeds. T1 = drupes, T2 = seeds, T3 = mechanically scarified, T4 = thermally scarified, T5 = acid scarified for 1 min, T6 = acid scarified for 3 min, T7 = acid scarified for 5 min.

photoperiods: 0 (dark), 12 or 24 h of light. The experiment was designed as a factorial 7 × 3 and two replicates of 25 seeds were used. For all scarification treatments, sets of 25 seeds were submitted separately to each method. Seeds were germinated in uncovered black plastic boxes (Gerbox; J. Prolab, São José dos Pinhais, Brazil) containing filter-paper sheets moistened with 3.5 ml of distilled water and placed at a controlled temperature of $25 \pm 2°C$ in a BOD-type chamber (Novatecnica, Piracicaba, Brazil). The experiment was carried out for 38 d, germination (%) was monitored daily and the speed index (GSI) was calculated. Germination percentage was calculated from the equation: $G\% = (n/N) \times 100$, where n = the number of germinated seeds and N = the total number of seeds (25). The GSI was calculated from the equation: $GSI = n/T_1 + n/T_2 + n/T_3 + \ldots + n/T_{38}$, where n = the number of germinated seeds and T = time in days (T_1 = day 1, T_2 = day 2 … T_{38} = day 38).

Electrical conductivity tests were performed to evaluate seed vigour, with four replicates of 25 seeds subjected to the same scarification treatments and further placed in 75 ml of distilled water at 25°C in the dark for 24 h, and then the electrical conductivity was measured with a conductivity meter (W12D; Bel Engineering, Monza, Italy).

Separation of seeds by specific gravity

After the previous experiment, the best scarification and light periods were selected for further testing. Floating seeds when separated in water may be non-viable. Thus, for the separation by specific gravity, sets of 3000 seeds were placed in a beaker containing 500 ml of distilled water and then separated into three groups after 15 min: non-treated (not placed in water), floating and sunken seeds. After separation, one seed set was subjected to scarification with H_2SO_4 for 1 min and one other was not scarified. The seeds were placed in uncovered black plastic boxes (Gerbox) containing filter-paper sheets moistened with 3.5 ml of distilled water and maintained at $25 \pm 2°C$ and 24-h photoperiod for 38 d. The experiment was conducted in a factorial 3 × 2 design with five replicates of 25 seeds. The germination percentage and the GSI were evaluated.

Dry-storage test

The dry-storage test was performed on seeds stored at two different temperatures [4°C (in a refrigerator) and 22°C (room temperature)] in paper bags. Seeds were sampled every 3 months for 1 year, starting with newly collected seeds (storage time zero). Stored seeds were sampled and then subjected to scarification with H_2SO_4 for 1 min. The seeds were germinated in uncovered black plastic boxes (Gerbox) containing filter-paper sheets moistened with 3.5 ml of distilled water, at a controlled temperature of $25 \pm 2°C$ in a BOD-type chamber with 24-h photoperiod. The experiment was conducted in a factorial 4 × 2 design with six replicates of 25 seeds. The germination percentage and the GSI were evaluated.

Anatomy of seeds

Some newly collected seeds were acid scarified for 1 min and others were non-scarified. Both sets of seeds were fixed in FAA solution (formaldehyde, acetic acid and 70% ethanol) for 72 h and stored in 70% ethanol. Seeds were dried in increasing ethanol concentrations (70%, 90% and 100%) at 2-h intervals. The samples were embedded in Historesin according to the manufacturer's instructions (Leica Microsystems, Wetzlar, Germany). Sections were obtained using a semi-automated rotary microtome (Yidi YD-335; Jinhua Yidi Medical Appliance Co., Ltd, Zhejiang, China). The sections were stained with 1% toluidine blue and mounted on slides with Canada balsam (O`Brien *et al.*, 1964). The slides were photographed using a microscope attached to an image capture system (CX31, Olympus, Tokyo, Japan).

Histochemical studies

All histochemical studies were performed on seeds imbibed for 48 h in distilled water. Free-hand sections were performed with the aid of a steel blade, and further semi-permanent slides were prepared according to specific methodologies proposed by Johansen (1940). Detection of tannins was performed with a solution containing 2% ferrous sulphate, 10% formalin and 90% distilled water. After 24 h of immersion in ferrous sulphate solution, the sections were washed in distilled water and mounted on the slides using 50% glycerol (v/v). For the detection of lipids, the 0.5% Sudan III (w/v) solution was used, placing sections in this solution for 10 min and then washing in distilled water. Sections were mounted using 50% glycerol (v/v). The slides were photographed using a light microscope attached to an image capture system (CX31, Olympus, Tokyo, Japan).

Lignin was detected using fluorescence microscopy analysis. Cross-sections of seeds were placed in a solution containing distilled water and 0.1% berberine hemi-sulphate (w/v) for 1 h and then washed in distilled water. Further, sections were kept in 0.5% aniline blue (w/v) solution for 30 min and then washed twice with distilled water. Sections were mounted in a solution of 0.1% $FeCl_3$ (w/v) in 50% glycerol (v/v) (Brundrett *et al.*, 1988). The observations were made with a fluorescence microscope (BX60, Olympus) equipped with a cooled

monochrome camera (Olympus). Images were captured with ultraviolet excitation/emission wavelengths of 358–461 nm (Brundrett *et al.*, 1988).

Dye-tracking test

A dye-tracking experiment was performed to determine the water-uptake pathway throughout the mesocarp and endocarp and how water arrived at the inner parts of the seed. Seeds were incubated in 0.5% toluidine blue solution (w/v) at room temperature for 48 h. Seeds were sampled and subjected to the same plant microtechniques described above in the 'Anatomy of seeds' section. The slides were photographed using a microscope attached to an image capture system (CX31, Olympus).

Seed imbibition curve

Imbibition curves were generated for non-scarified seeds and for those scarified with H_2SO_4 for 1 min. Sets of 25 seeds were placed in uncovered black plastic boxes (Gerbox) containing filter paper moistened with 3.5 ml of distilled water and incubated at $25 \pm 2°C$. The seed fresh mass was measured at 30-min intervals until constant fresh mass was obtained.

Statistical analysis

Statistical analyses were performed using the SISVAR 5.0 software (Ferreira, 2011). Before parametric analysis, data were submitted for a normal distribution test (Shapiro–Wilk). In addition, data were subjected to analysis of variance, and means were compared by the Scott–Knott test at 5% probability or subjected to regression analysis.

Results

Dormancy alleviation

There was no significant interaction between the scarification and photoperiod ($P > 0.05$). However, all acid scarification treatments showed higher germination percentages compared to other scarification methods (Fig. 2A). Regarding the photoperiod, the highest germination percentage was found at constant light (Fig. 2B). No differences in the GSI were promoted by the scarification treatments ($P = 0.24$); however, the 24-h photoperiod increased this parameter (Fig. 2C). In addition, all the scarification treatments reduced the electrical conductivity of the seeds (Fig. 3).
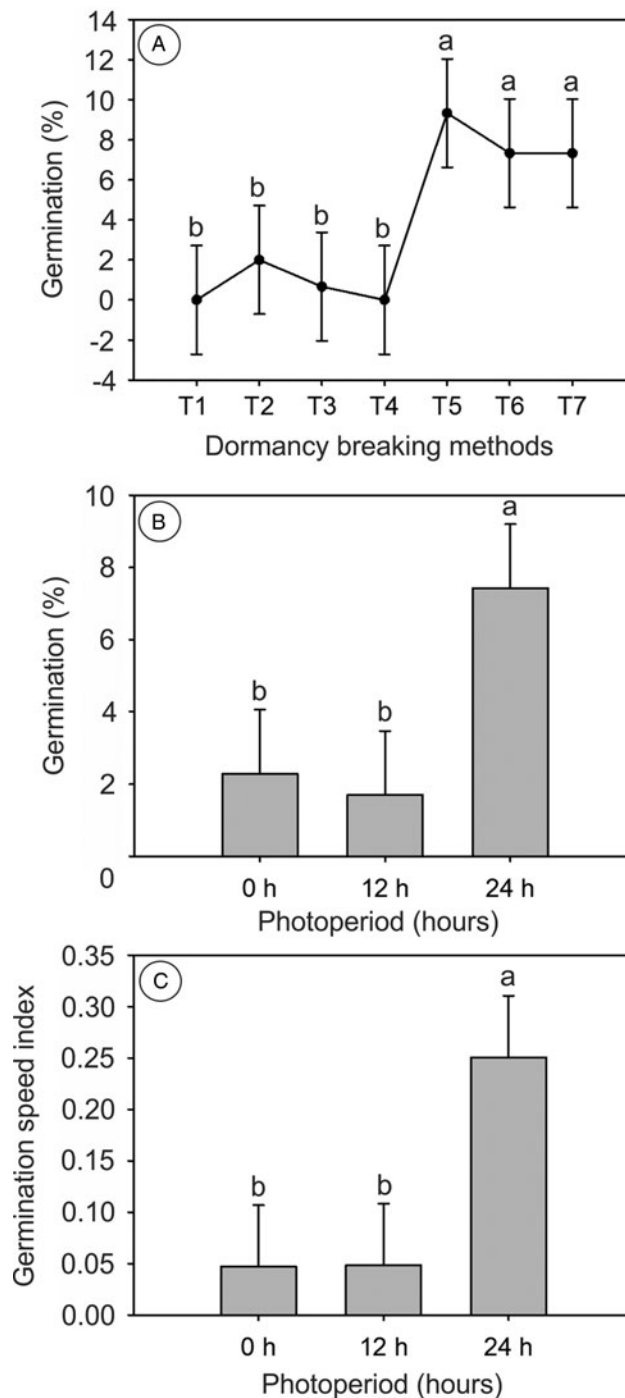

Figure 2. Germination and germination speed index (GSI) of newly collected seeds of *S. molle*. Data shown in (B) and (C) are combined from all treatments. Means followed by the same letter do not differ by the Scott–Knott test at the 5% significance level. T1, Drupes; T2, seeds; T3, mechanically scarified; T4, thermally scarified; T5, acid scarified for 1 min; T6, acid scarified for 3 min; T7, acid scarified for 5 min.

Separation of seeds by specific gravity

The seeds that sank showed both higher germination percentage and GSI as compared to those that floated (Table 1). In addition, acid scarification significantly
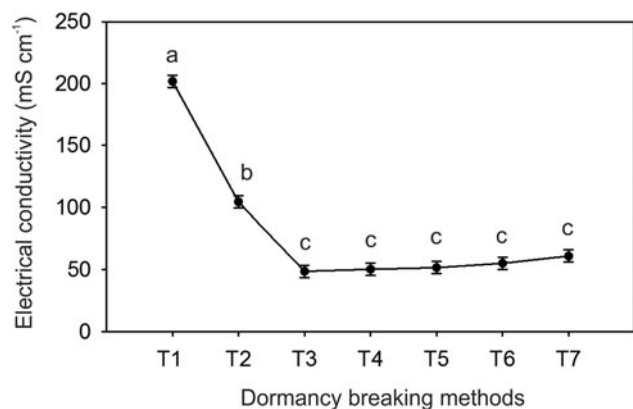
Figure 3. Electrical conductivity (mS cm^{-1}) of newly collected seeds subjected to different dormancy breaking methods. Means followed by the same letter do not differ by the Scott–Knott test at the 5% significance level. T1, drupes; T2, seeds; T3, mechanically scarified; T4, thermally scarified; T5, acid scarified for 1 min; T6, acid scarified for 3 min; T7, acid scarified for 5 min.

enhanced the germination percentage and GSI of the seeds that sank but not of the floating seeds (Table 1). The seeds that sank were viable, showing the mesocarp, endocarp and the fully developed embryo closely attached to endocarp (Fig. 4A). However, floating seeds showed large empty spaces and non-viable embryos (Fig. 4B).

Dry storage

Storage time and temperature promoted differences in germination parameters; however, no significant interaction between these factors was found ($P > 0.05$). Germination percentage and GSI showed higher means for all storage times compared to newly collected seeds; however, both variables decreased after longer storage times (Fig. 5A and C). In addition, seeds stored at 22°C showed higher germination percentage and GSI (Fig. 5B and D).

Seed anatomy

S. molle seeds displayed an external part containing several layers of parenchyma cells (pc) and secretory cavities (sc) surrounded by sclerenchyma. These tissues were comprised of attached parts from the mesocarp (Fig. 6). Acid scarification killed all cells in the mesocarp, and cytoplasm and cell content were lost (Fig. 6). The acid scarification also changed the seed surface, darkening and revealing open secretory cavities (Fig. 6A and B). Untreated cells were turgid and round, while acid-scarified cells showed irregular cell walls and had lost content (Fig. 6). Likewise, the secretory cavities in acid-scarified seeds were empty and surrounding cells appeared dead (Fig. 6). The endocarp consisted of three layers of sclereids surrounding the embryo (Fig. 6) plus the one-layered vestigial seed coat, as reported by Carmello–Guerreiro and Paoli (2005) for *Schinus* species.

Histochemical studies

Tannin histochemical tests showed negative results, as positive tannin-containing vacuoles were expected to be of blue-green colour (Fig. 7A and B). However, lipids were detected within secretory cavities (as red-stained content) and although some remained present, most lipid content was removed by acid scarification (Fig. 7C and D). Lignin detection by fluorescence showed an intense response from endocarp and external cell layers of the mesocarp (Fig. 7E). The lignin content of the endocarp was not affected by scarification; however, the external layers of mesocarp showed lignin content in untreated seeds but not in the scarified ones (Fig. 7F).

Dye-tracking test

The surface of *S. molle* seeds showed a region related to radicle protusion during germination (Fig. 8). This region was closed in untreated seeds but acid scarification promoted its opening and, hence, exposed the embryo (Fig. 8B). Figure 8C shows that only mesocarp parenchymal cells were stained. Toluidine blue cannot reach the inner parts of the seed unless it goes through the carpellary slit, were no secretory cavities were found and a suture line was visible (Fig. 8D). Therefore, seed imbibition occurs mainly in the region of the carpellary slit were mesocarp parenchyma is found.

Table 1. Germination and GSI of *S. molle* seeds separated by specific gravity, then scarified with H_2SO_4 for 1 min or not scarified, and maintained at a 24-h photoperiod

	Germination (%)			GSI		
	Untreated	Floated	Sank	Untreated	Floated	Sank
Not scarified	5bA	0aA	6bA	0.13bA	0.00aA	0.16bA
Scarified	25aA	2aB	33aA	0.94aA	0.05aB	1.16aA

The lower-case letters compare the scarified seeds (columns) and the uppercase the seeds which were separated by specific gravity (lines). Means followed by the same letter in columns or lines do not differ by the Scott–Knott test at the 5% significance level.
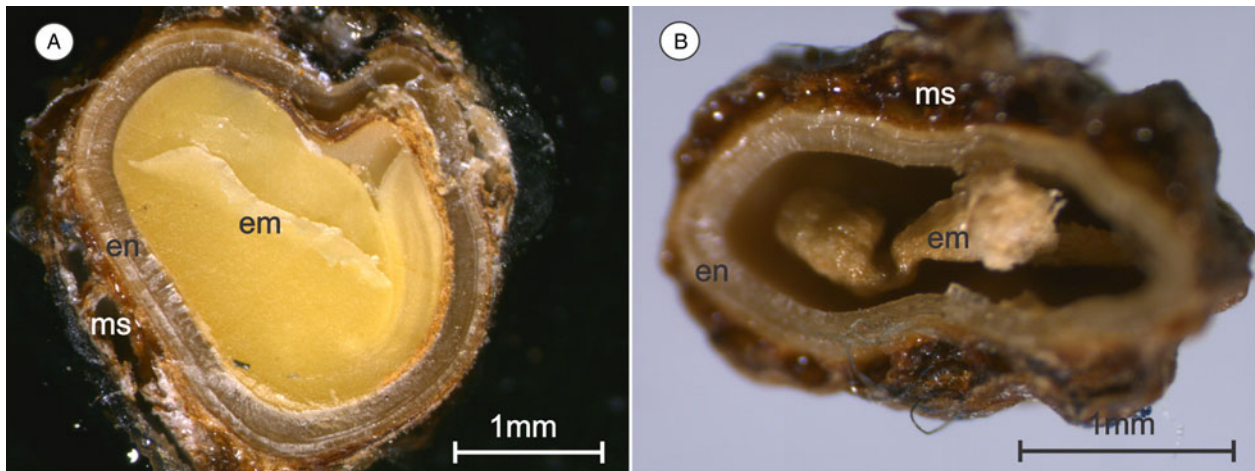
Figure 4. Morphology of sunken (A) and floating (B) *Schinus molle* seeds. In (B) the floating seed shows empty spaces and a non-viable embryo. ms, mesocarp; en, endocarp; em, embryo.
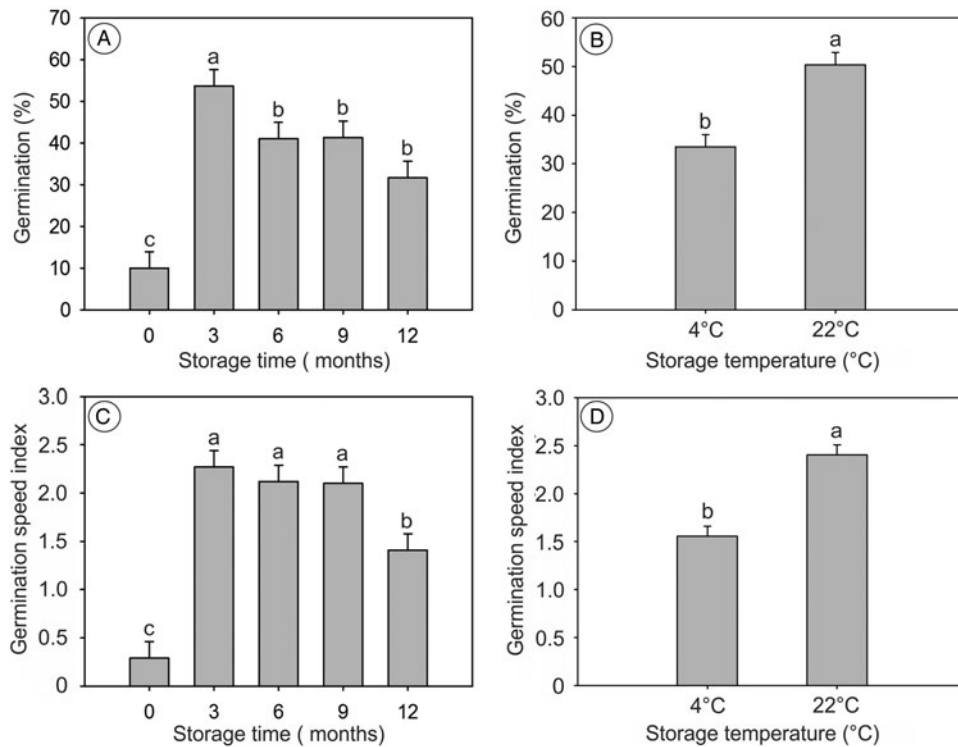

Figure 5. Germination and GSI of *S. molle* seeds stored at 4 and 22°C, for 12 months, with a 24-h photoperiod and scarified with H_2SO_4 for 1 min. In (A) and (C) the storage time zero refers to newly collected seeds. (B) and (D) show data combined for all storage times. Means followed by the same letter do not differ by the Scott–Knott test at the 5% significance level.

Seed imbibition curve

Imbibition curves showed constant increasing mass for both scarified and non-scarified seeds (Fig. 9). However, scarified seeds completed imbibition after 18 h while non-scarified ones achieved this point later, after 45 h. Soaked *S. molle* seeds showed an average of 40 mg fresh mass in both treatments, corresponding to twice the initial mass (Fig. 9).

Discussion

Seeds of Anacardiaceae species, such as *Rhus* L., usually show physical dormancy, which is promoted by an impermeable endocarp (Li *et al*., 1999b). The three-layered endocarp containing sclereids has been reported as the main cause of seed dormancy for *Schinus* species (Carmello-Guerreiro and Paoli, 2005). However, we found that parts of the mesocarp still
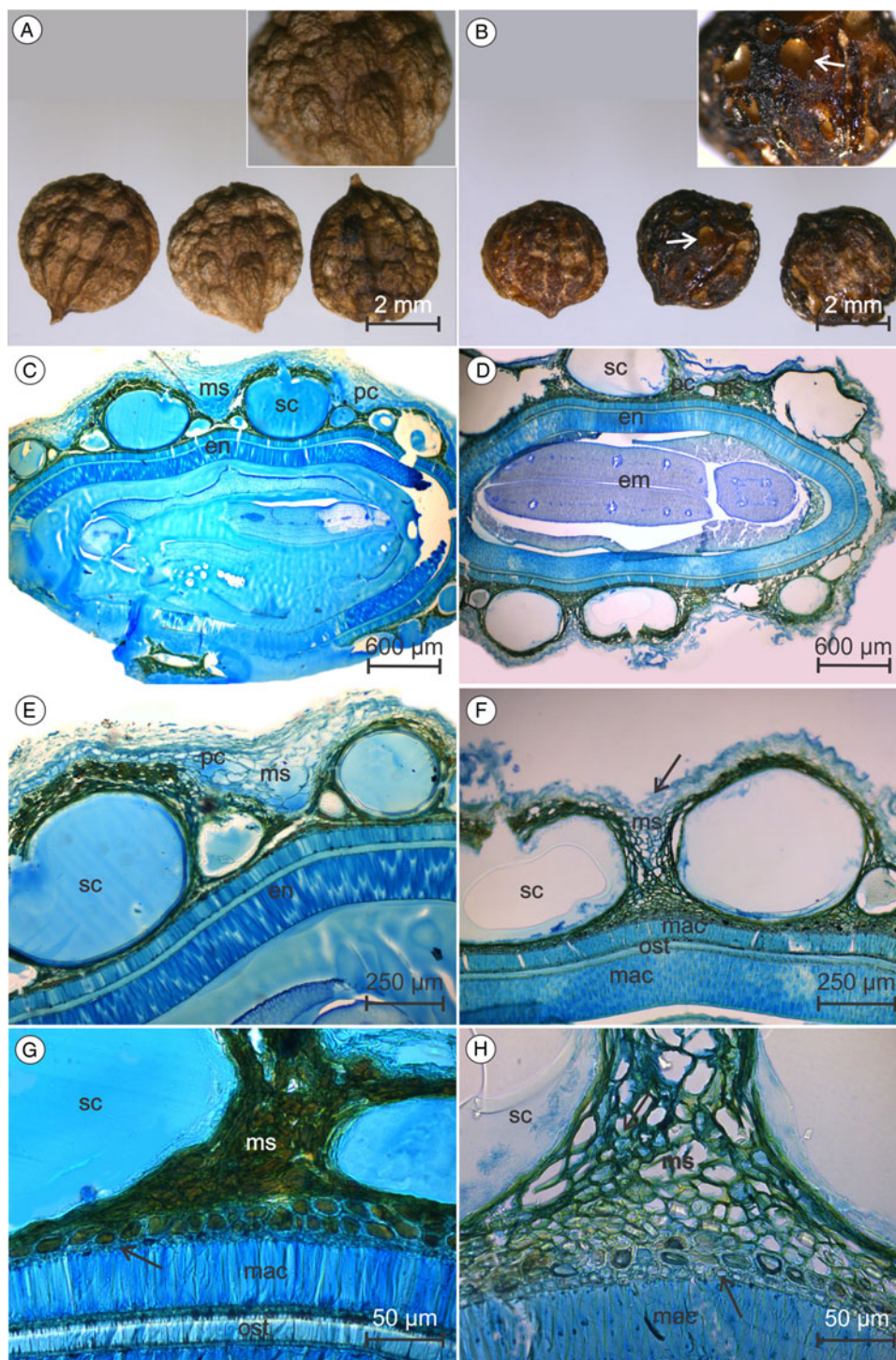
Figure 6. Morphological and anatomical structure of *S. molle* seeds. (A, C, E and G) Non-scarified seeds, and in (B, D, F and H) seeds were scarified with H_2SO_4 for 1 min. In (B) the endocarp and the embryo remained intact after scarification. The acid scarification removed part of the parenchyma layers in the mesocarp (arrow) compared to intact seeds (C and D). The scarification method killed all cells that still remained in the mesocarp. In these cells the cytoplasm and cell content were lost and only cell walls remained. The endocarp features three layers of palisade cells, composed of osteosclereids (ost) and macrosclereids (mac). In (F) the arrow indicates the outermost layer of the mesocarp. pc, parenchyma cells; ms, mesocarp; en, endocarp; em, embryo; sc, secretory cavity.
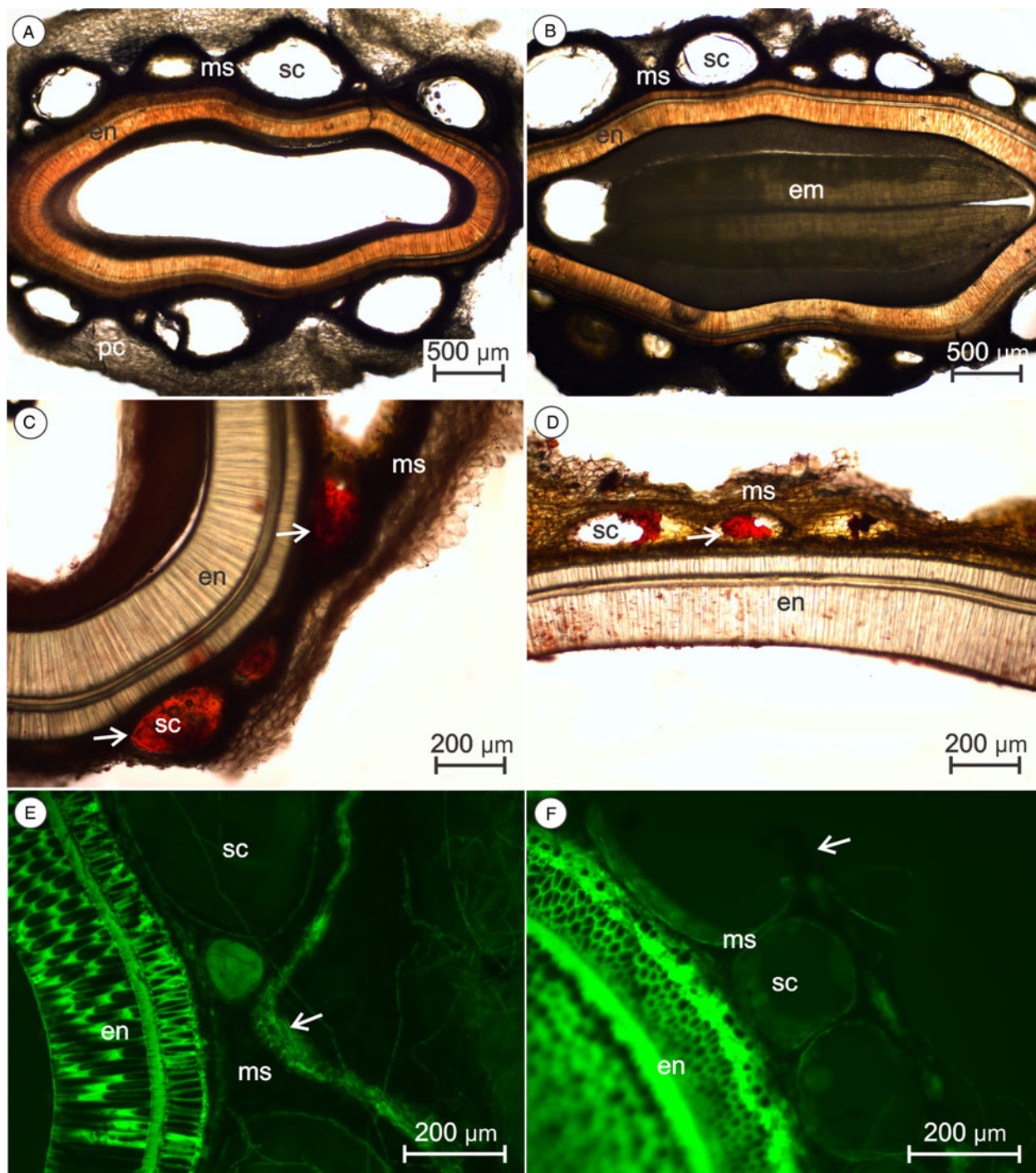
Figure 7. Histochemical tests of non-scarified (A, C and E) and acid-scarified (B, D and F) *Schinus molle* seeds. (A) and (B) show the ferrous sulphate test for tannin (not detected). (C) and (D) show the Sudan III test for lipids; arrows indicate lipids within secretory cavities. (E) and (F) show fluorescence detection of lignin; arrows indicate the outermost layer of the mesocarp, which was removed by acid scarification (F). ms, mesocarp; en, endocarp; em, embryo; sc, secretory cavity; pc, parenchymal cells.

attached to seeds may only reduce water uptake but not really cause seed dormancy. As shown in Fig. 9, seed imbibition still occurred in non-scarified seeds, albeit at a lower rate. However, histochemical studies showed that acid scarification removed part of the lipids and the lignin of mesocarp cells. These results corroborate the work of Morfin (1985) who reported

the inhibition of *S. molle* seed germination by the mesocarp. In addition, Jøker *et al.* (2002) suggested that the oil in the pericarp may inhibit germination. Likewise, according to Zahed *et al.* (2010) the essential oil of *S. molle* shows allelopathic effects by inhibiting the germination of wheat seeds. Thus, removing these compounds by scarification improved imbibition speed,
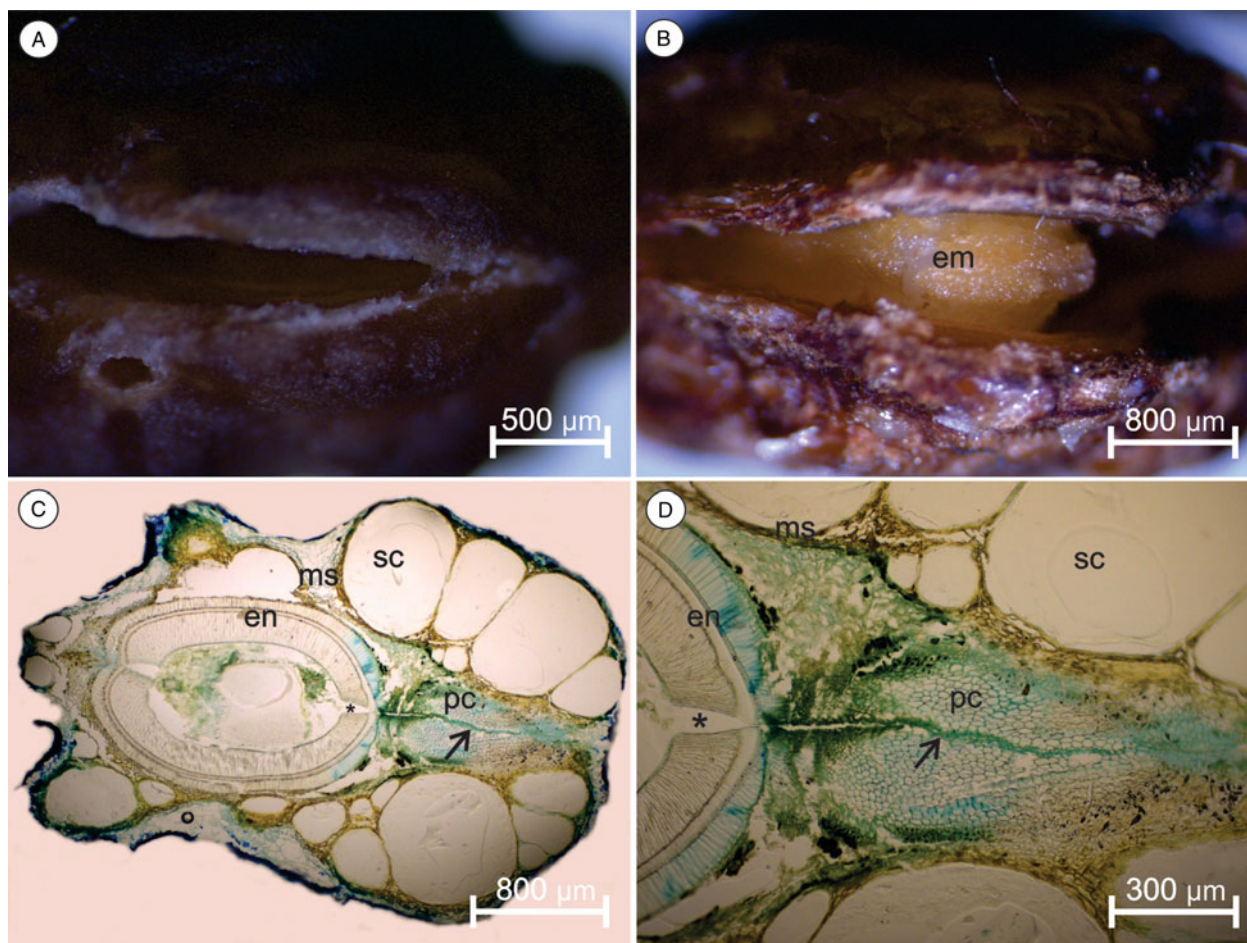
Figure 8. The water path during imbibition of *Schinus molle* seeds, tracked by toluidine blue infiltration. (A, B) The seed surface at the region of radicle protrusion in untreated (A) and scarified seeds (B) after 48 h of imbibition. In the acid-scarified seeds (B) the radicle-protrusion region is open, while it is closed in untreated seeds. (C, D) The path of the toluidine blue dye throughout the mesocarp. In (D) a carpellary hilar slit (arrow) is shown and the asterisk indicates a partial break. ms, mesocarp; en, endocarp; em, embryo; sc, secretory cavity; pc, parenchymal cells.

favouring seed germination in *S. molle*. Acid scarification is often reported to enhance germination in seeds with hard coats (Varela and Lizardo, 2010). Fruits showing hard parts attached to seeds had improved germination after acid scarification (Li *et al.*, 1999a). According to Puglia *et al.* (2015) the pericarp of *Glebionis coronaria* L. is a determinant of seed dormancy and its removal by scarification enhances germination. According to Hosni *et al.* (2011), 5.35% of the dry weight of mature *S. molle* fruits is composed of fatty acids and 1.15% of essential oils. All these compounds are hydrophobic and can reduce water uptake during seed imbibition. Apparently, removing the cytoplasm of parenchymal cells and contents of the secretory cavities improved imbibition speed and germination of *S. molle* seeds. In addition, the cell wall is comprised mainly of cellulose which is strongly hydrophilic and this may have favoured seed imbibition. As all acid-scarification treatments had the same effect, treatment for only 1 min can be used with satisfactory results for *S. molle* seeds. The seed imbibition curves

show that acid-scarified seeds achieve quicker imbibition than non-scarified ones, which take almost three times as long to complete the process. According to Rosental *et al.* (2014), the starting point of seed germination is the imbibition process that reactivates the metabolism of dry seeds, preparing for germination. Therefore, a quicker imbibition promoted by acid scarification in *S. molle* seeds may have enhanced seed metabolism, improving both overall germination and seed vigour. The water path during imbibition was located only at the carpellary hilar slit, which is a common trait in seeds containing palisade layers. As the carpellary hilar slit is comprised mainly of mesocarp parenchymal cells, the removal of its lipid and lignin content may have enhanced imbibition speed. Thus, despite previous reports of physical dormancy in Anacardiaceae seeds, our results show that in *S. molle* no real physical dormancy is present and acid scarification only increases imbibition speed.

Methods other than acid scarification had no effect on seed germination, although decreased conductivity
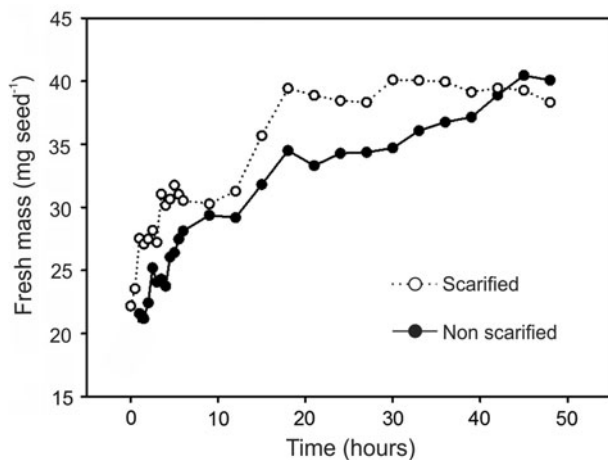
Figure 9. Imbibition curves of acid scarified and non-scarified *Schinus molle* seeds.

was found. Increased conductivity is related to the leakage of sugars, ions and other metabolites (Fessel *et al.*, 2006) and this method is proposed as a test for the physiological potential of seeds (Panobianco *et al.*, 2007). However, in this work, scarification methods probably reduced electrical conductivity by removing parts of the mesocarp attached to seeds. According to Carmello-Guerreiro and Paoli (2002) the mesocarp of *Schinus terebinthifolius* contains parenchyma, secretory cavities and calcium oxalate crystals. This structure is very similar to that of the *S. molle* seeds, and these tissues can retain sugars, ions and oils that may leak during seed imbibition, increasing electrical conductivity. Therefore, scarification removed these tissues and electrical conductivity cannot be related to seed vigour for this specific situation.

Photoperiod modulates the germination of photoblastic seeds (Oh *et al.*, 2006). Light is critical for the regulation of seed germination due to changes in the abscisic acid/gibberellin balance (Nambara *et al.*, 2010). According to Chen *et al.* (2013) seeds from tropical tree species only germinate in open areas, while light availability is commonly required particularly by small seeds. Therefore, *S. molle* shows positive photoblastic seeds, as constant light enhanced its germination.

According to Demelash *et al.* (2003), separating the seeds of *S. molle* using specific gravity led to a 22% improvement of germination in those that sank. We observed similar results, with 32% increased germination in seeds that sank. The separation of seeds by specific gravity is a common method to separate viable seeds (which sink) from dead or floating insect-damaged ones (Demelash *et al.*, 2003). Likewise, we found that floating seeds consisted of non-viable embryos and large empty spaces which made them float. Thus, the separation by specific gravity together with acid scarification can effectively improve seed germination in *S. molle* by removing the non-filled, non-viable seeds.

Several studies reported the increase in germination following dry storage, due to the reduction of levels of inhibitors in the embryo, mainly abscisic acid (Deno, 1993; Hidayati *et al.*, 2002; El-Keblawy and Al-Rawai, 2006; Bentsink and Koornneef, 2008). This can also be related to the increased receptiveness of signals given by gibberellins (Nambara *et al.*, 2010). The optimal period for breaking dormancy of *S. molle* seeds seems to be up to 3 months, since germination decreased at longer intervals. However, even after 12 months under dry storage, the means of the germination parameters were higher than for newly collected seeds. Therefore, the seeds of *S. molle* have physiological dormancy that can be alleviated during dry storage. Likewise, even seeds stored for 12 months were viable. This is an important trait for reforestation, since seeds can be stored and new plants can be produced for prolonged periods of time.

S. molle seeds stored at room temperature showed a higher germination percentage and vigour as compared to those stored at 4°C. This was also reported in *Arabidopsis*, where higher seed storage temperatures improved germination (Basbouss-Serhal *et al.*, 2016). However, this depends on species, since Escobar and Cardoso (2015) showed that the seeds of tropical trees show better performance when stored under low temperatures. Likewise, the physiological basis of improved germination under higher temperature is still unclear, since it depends on several traits which are specific for one particular species.

Conclusion

S. molle seeds are positively photoblastic and show physiological dormancy, which can be alleviated by acid scarification and dry storage. Seeds can be stored for over 1 year without significant decreases in germination parameters compared to newly collected seeds. Scarification enhances seed germination by the removal of lipid and lignin from mesocarp cells.

Conflicts of interest

None.

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