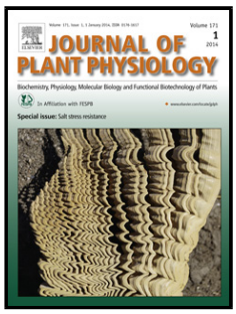

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Paths of water entry and structures involved in the breaking of seed dormancy of *Lupinus*

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Summary

Physical dormancy is the water impermeability of the seed coat caused by one or more palisade cell layer(s) called macrosclereids. The specialised structure for water entry sites is the water gap, which serves as a detector of environmental cues for germination. In Fabaceae, the water gap is the lens, although another seed structure for water entry could exist. In this study, we identified the initial site of water entry, observed the hydration of a cushion-like structure near the radicle, described the anatomy of the water gap, and analysed the association of anatomical seed traits with the initial site of water entry and the imbibition velocity of six species of *Lupinus* from the State of Jalisco, Mexico. Dye tracking with a toluidine blue solution was used to identify the initial site of water entry. The anatomical description was performed using conventional microtechnique and a light microscope. The entry of the toluidine solution into seeds of *L. montanus* was observed after 6 h, followed by *L. exaltatus* and *L. mexicanus* after 18 h and *L. elegans*, *L. reflexus* and *L. rotundiflorus* after 48 h. The site of water entry was the lens in *L. elegans*, *L. exaltatus*, *L. reflexus* and *L. rotundiflorus* and the micropyle in *L. mexicanus* and *L. montanus*. The cushion-like structure was responsible for water accumulation in embryo imbibition. Significant differences among anatomical seed traits such as thickness in the hilar region, the counter-palisade layer, cushion-like structure, epidermis, hypodermis, and innermost parenchyma layer were found among the species.

Keywords: Seed anatomy; *Lupinus*; Physical dormancy; Water entry path; Water gap

Introduction

Seeds with physical dormancy have water-impermeable seed or fruit coats and are unable to imbibe water when placed in a moist environment (Baskin and Baskin 2000; Venier et al. 2012). Physical dormancy has been demonstrated or inferred to occur in some or all species of the angiosperm plant families Anacardiaceae, Bixaceae, Biebersteiniaceae, Cannaceae, Cistaceae, Convulvulaceae, Curcubitaceae, Dipterocarpaceae, Fabaceae, Geraniaceae, Lauraceae, Malvaceae, Nelumbonaceae, Rhamnaceae, Sapindaceae, Sarcolaenaceae, Sphaerosepalaceae, and Surianaceae; however, it has not yet been reported in gymnosperms (Gama-Arachchige et al., 2013). The seed or fruit coat is water-impermeable due to the presence of one or more layers of palisade cells called macrosclereids. Further, the seed/fruit becomes water-permeable via the opening of a small specialised anatomical structure within the coat, which is recognised as the water gapt (Baskin and Baskin 1998; Baskin et al., 2000; Baskin, 2003; Jayasuriya et al., 2007, 2008; Hu et al., 2008; Turner et al., 2009; Gama-Arachchige et al., 2013). The water gap closes during maturation drying and opens in response to appropriate environmental signals, such as high temperature, humidity or fire, and it is the initial point of water entry into the seed (Baskin and Baskin, 2000; Baskin et al., 2000; Baskin, 2003; Jayasuriya et al., 2007, 2008). When the water gap opens, physical dormancy is broken.

The water gap location, anatomy, morphology and origin of the water gap differs among and even within families (Baskin et al., 2000; Jayasuriya et al., 2009). Twelve different water gap regions in seven families previously have been characterised: carpellary blister gap, chalazal oculus, gap adjacent to the hilum, bulge gaps adjacent to the hilum, hilar slit, pseudolens gap, lens gap, lens slit, chalazal slit, chalazal blister gap, micropylar slit and circumlinear endocarp suture (Gama-Arachchige et al., 2013).

Fabaceae, which includes approximately 800 genera and 20,000 species that are widely distributed and adapted to different habitats (Sousa and Delgado, 1993; Black et al., 2006; Smýkal et al., 2014), has a high frequency of physical dormancy (Rodrigues-Junior et al., 2014). The lens is the water gap and detector of environmental signals for the break in dormancy and the germination of many, but not all, legumes (Hanna, 1984; Baskin et al., 2000; Baskin, 2003; Hu et al., 2008; Gama-Arachchige, 2013). Further, the lens regulates the rate of water uptake into the seed (Manning and Van Staden, 1987; Perissé and Planchuelo, 2004; Smýkal et al., 2014).

Lupinus Tourn. (Fabaceae) species are widely distributed in America, the Mediterranean and North Africa and cover a wide range of climatic conditions. There are both wild and crop species within the genus (Naganowska et al., 2003). In Mexico, there are approximately 100 wild species, of which 15 are native to the state of Jalisco (Ruiz and Sotelo, 2001). In the South American species *L. albus* and *L. angustifolius*, the water pathway at the beginning of imbibition is related to the lens and the pores within it, but the hypodermis, the intercellular spaces between the osteosclereids, the tracheid bar and a structure covering the radicle identified as a cushion-like structure are involved in the process of imbibition (Perissé and Planchuelo, 2004). The tracheid bar is composed of vertically oriented tracheoids surrounded by parenchyma cells, and is found immediately below the hilar fissure of seeds of papilionoid legumes, such as species of *Lupinus* (Pitot, 1936; Hyde, 1954; Lersten, 1982; Perissé and Planchuelo, 2004).

Previous studies have suggested that the epidermis and hypodermis of seeds of *Lupinus* species are the tissues that determine the PY, as an increase in the thickness of these two tissues also increases the water-impermeability of seeds (Petrova, 2002). However, there is no experimental evidence to support this hypothesis.

Seed dormancy of *Lupinus* can be broken with heat treatment (Quinlivan, 1968; Robles-Díaz et al., 2014; Zuloaga-Aguilar et al., 2011, 2014), but the initial site of water entry after this treatment is unknown. In our research, we studied six species of *Lupinus* to (1) identify the initial site of water entry, (2) observe the hydration of the cushion-like structure, (3) describe the anatomical structure of the water gap, and (4) analyse the relationship between anatomical seed traits, initial site of water entry and imbibition velocity.

Materials and methods

Study species and seed collection

We selected six species of *Lupinus* distributed in the state of Jalisco, Mexico: *Lupinus elegans* Kunth a shrub that grows in disturbed pine and pine-oak forests in Central Mexico, from 1,700 to 3,000 m above sea level (asl) (Ruiz-Moreno et al., 2000); *L. exaltatus* Zucc., which grows in open areas in the middle of a pine-oak forest, as a weed in disturbed or cultivated areas and roadside in the mountains of the Transverse Volcanic Sierra between 1,800 and 2,000 m asl (McVaugh, 1987); *L. mexicanus* Cerv. ex Lag., which grows in grasslands and desert scrub and is easily adapted to disturbed habitats, between 1,800 and 2,200 m asl on the Central Plateau and in the upper basin of the Rio Santiago (McVaugh, 1987); *L. montanus* Kunth which grows in pine-oak forest and alpine meadows from 2,500 to 4,100 m asl (Acosta-Percástegui and Rodríguez-Trejo, 2005); *L. reflexus* Rose which grows in coniferous forest, cespitose grassland and also volcanic soils above the timberline at altitudes from 2,700 to 3,600 m asl (McVaugh, 1987); and *L. rotundiflorus* M.E. Jones which grows in open slopes, dry scrub, and sometimes in pine-oak forest and is easily adapted to habitat disturbances, from 1,500 to 2,500 m asl (McVaugh, 1987).

The seeds were collected from at least 40 plants per species and were cleaned and stored in paper bags at room temperature until analysis.

Seed anatomy

Five water-impermeable seeds of each species were fixed in 3% glutaraldehyde solution, dehydrated in an ethanolic series (10 to 100%) and embedded in glycol-methacrylate (Technovit® 7100, Heraeus Kulzer, DE). Transverse sections with a thickness of 3 µm were made with a rotatory microtome (RM2125RT, Leica Microsystems, DE), stained with brilliant cresyl blue 0.6% (Ruzin, 1999) and observed under a light microscope (DM 2000, Leica Microsystems, DE). Measurements were taken on two seeds of each species (n=12). Seeds sectioned longitudinally were mounted on a double-sided adhesive carbon tape on metal pins to analyse the structure of hilar region observed under a scanning electron microscope (QuantaTM 200, FEI, OR).

Initial site of water entry into seeds

Fifty seeds of each species were subjected to boiling water for 5s to break physical dormancy (Robles-Díaz et al., 2014) and non-treated seeds were immersed in saturated toluidine blue solution 0.5% at room temperature. Subsequently, two seeds were taken out of solution and sectioned longitudinally with a disposable microtome blade every 2 h for 48 h and observed under a stereomicroscope (Leica EZ4D, Leica Microsystems, DE) to determine the initial site of water entry into the seeds.

Cushion-like structure hydration

Longitudinal sections of five seeds of each species were obtained using a disposable microtome blade, and 0.1 µL of distilled water was immediately added on the tracheid bar and cushion-like tissue region at room temperature. This process was recorded with a digital camera (EC3, Leica Microsystems, DE) adapted to a light microscope (DM 2000, Leica Microsystems, DE) and an external light source (NI-150, Nikon Instruments, USA).

Statistical analyses

We conducted an analysis of variance (ANOVA) for each of the anatomical characters measured for the seed coat of the hilar region, counter-palisade layer, cushion-like structure, seed coat, osteosclereids and parenchyma layer. Tukey's test was performed as a multiple comparison test to find means that were significantly different among species. A correlation analysis (Pearson product-moment correlation coefficient) was applied between anatomical characters and time of imbibition to measure the degree of linear dependence between the variables. All analyses were performed using XLSTAT (2014.2.02 v, Addinsoft, NY, USA).

Results

Seed anatomy

In all species, the seed coat (outside to inside) has an epidermis, hypodermis, and inner parenchyma. The epidermis is composed of thick-walled macrosclereids with the long axis oriented perpendicularly to the surface, arranged in a compact palisade layer. There were significant differences among species for epidermis thickness ($n=12$, $df=5$, $F=63.886$, $P<0.0001$) (Fig. 1D), with *Lupinus exaltatus* (Fig. 2B) and *L. mexicanus* (Fig. 2D) having a

thicker epidermis than the other species. Under the epidermis, a single layer of osteosclereids separated by wide intercellular spaces forms the hypodermis, except under the hilum cleft, where it is absent. Additionally, there were differences among species for the thickness of the hypodermis (n=12, df=5, F=32.560, P< 0.0001) (Fig. 1E), with *L. montanus* (Fig. 2F) having the thickest layer. The innermost layer is the parenchyma, with six rows of tangentially collapsed endosperm remnant cells, which differed among the species (n=12, df=5, F=26.186, P< 0.0001) (Fig. 1F); *L. mexicanus* (Fig. 2D) had the thickest layer. The hilum has one external layer of macrosclereids forming a counter-palisade layer placed above the internal palisade layer which is the continuation of palisade tissue of the seed coat. *Lupinus reflexus* (Fig. 2G) was the only species without a counter-palisade layer; *L. mexicanus* (Fig. 2C) had the thickest layer (n=12, df=5, F=47.471, P<0.0001) (Fig. 1B). The thickness of the internal palisade layer varied with the species (n=12, df=5, F=18.777, P<0.0001) (Fig. 1A), with *L. reflexus* (Fig. 2G) having the highest value. Under the internal palisade layer, a hypodermic layer that consists of short irregularly shaped thick-walled cells (tracheoids) with large intercellular spaces forms the tracheid bar. Below the porous tissue is an extended layer of obliterated parenchyma cells, separating the seed coat from the embryo. In addition, parenchyma cells arranged in a cushion-like structure were in the distal portion that covers the radicle tip. This tissue varied among the species (n=12, df=5, F=4.844, P<0.002) (Fig. 1C), with *L. reflexus* (Fig. 2G) having the largest layer.

Initial site of water entry

In the non-treated seeds, no staining was observed (lens closed) after 48 h, but in the treated seeds the time from immersion until staining visibly varied for each species. The shortest

time of immersion for staining was 6 h observed in *Lupinus montanus*; 18 h in *L. exaltatus* and *L. mexicanus* and the longest in *L. elegans*, *L. reflexus* and *L. rotundiflorus* with 48 h. The observed path of water entry into the seed of *L. elegans* (Fig. 3A), *L. exaltatus* (Fig. 3B), *L. reflexus* (Fig. 3C) and *L. rotundiflorus* was by the lens and in *L. mexicanus* and *L. montanus* (Fig. 3D) by the micropyle.

The correlation analysis showed that the time of imbibition was negatively associated with the thickness of the epidermis (macrosclereids) layer (n= 12, r = -0.857, P<0.0004), the thickness of the innermost parenchyma layer thickness (n= 12, r = -0.746, P<0.005), and the hypodermis (osteosclereids) layer (n= 12, r = -0.616, P<0.03), but was positively associated with the length of the cushion-like structure (n= 12, r = 0.625, P<0.03).

Cushion-like structure

The cushion-like structure is composed of parenchyma cells (Fig. 4). When we added water to this structure over the radicle tip the bright white tissue in the hilar end of the seed it progressively spread to the tracheid bar cell network, and the cushion-like structure increased in volume because it had a high capacity for water absorbance, as shown in *L. elegans* (Online Resource 1)., displacing the air and completely filling the intercellular spaces, as observed in *L. exaltatus* (Online Resource 2).

Discussion

The studied species *Lupinus elegans, L. exaltatus, L. mexicanus, L. montanus, L. reflexus* and *L. rotundiflorus* had the typical structures involved in physical dormancy (Valenti et al., 1989; Perissé and Planchuelo, 2004; Robles-Díaz et al., 2014). The seed coat layers, including the epidermis formed by macrosclereids and the hypodermis formed by

osteosclereids are the more representative tissues involved in the physical dormancy and are present in most legumes (Reeve, 1946; Petrova, 2002; Ma et al., 2004; de Souza et al., 2012; Venier et al., 2012; Perissé and Planchuelo, 2004; Robles-Díaz et al., 2014). Although the six species showed the typical anatomical structure of *Lupinus* seeds, we observed some differences in anatomical variables. For example, seeds of *Lupinus reflexus* did not have the counter-palisade layer, but they had a thicker palisade layer of the hilum region and a larger cushion-like structure than those of the other species (Fig. 1). Additionally, the hypodermis layer of osteosclereids was variable among species.

The imbibition time of *Lupinus* species varies between 6h and 48h, which differs from the 1 – 3 h observed for seeds of *Ipomoea lacunosa* (Jayasuriya et al., 2007), *Cuscuta australis* (Jayasuriya et al., 2008), *Cassia leptophylla* and *Senna macranthera* (de Paula et al., 2012) and three species of *Derris* (Jayasuriya et al., 2012). These differences may be due to the anatomical characteristics and location of the water gap. In the Fabaceae family, the lens is specialised for water uptake, in addition to the micropyle. These structures are relevant for imbibition in the seed after the breaking of dormancy (Hanna, 1984; Manning and Van Standen, 1987; Kelly et al., 1992; Valenti et al., 1995; Baskin et al., 2000, Baskin, 2003; Perissé and Planchuelo, 2004; Hu et al., 2008; de Paula et al., 2012; Venier et al., 2012; de Souza et al., 2012; Gama-Arachchige et al., 2013; Rodrigues-Junior et al., 2014). The hilum is a hygroscopic valve that allows for loss of water during seed drying, but it does not open unless additional drying occurs (Hyde, 1954). That is, the hilum does not open during seed incubation on a wet substrate.

Previous studies have shown that the epidermis and hypodermis of *Lupinus* species are the tissues that determine physical dormancy, and with an increase in the thickness of these two tissues, the seed water-impermeability increases (Petrova, 2002). Our study showed that

species with a thicker seed coat epidermis, hypodermis, and inner parenchyma thickness had reduced times of imbibition after the treatment to break physical dormancy, but water entered through the micropyle.

Experimental studies have demonstrated that the lens is the point of initial water entry into the seed, while it controls the rate of water movement into the seed (Manning and van Staden, 1987; Lersten et al., 1992; Perissé and Planchuelo, 2004; Smýkal et al., 2014). Our study showed that the cushion-like structure may regulate water movements into the seed once the water has entered by the micropyle or the lens.

The hypodermis layer formed by porous tissue below the inner epidermis in the hilum region wraps around the radicle tip, and it is involved in water absorption. In *Lupinus,* this has been named the cushion-like structure (Perissé and Planchuelo, 2004) and is made of asterinoid tissue (Petrova, 2002), formed by parenchyma cells (Lersten et al., 1992; Perissé and Planchuelo, 2004). The cushion-like structure in the species of this study may be involved in water absorption because seeds with the largest cushion-like structure had the fastest time of imbibition. Once the tissue was hydrated, it joined the radicle, probably assisting in germination. Considering these findings, we are currently studying the chemical composition of this structure.

In conclusion, the six species of *Lupinus* had two paths of water entry: the lens (*L. elegans, L. exaltatus, L. reflexus* and *L. rotundiflorus*) and the micropyle (*L. mexicanus* and *L. montanus*). Seeds with a relatively thick seed coat (*L. mexicanus* and *L. montanus*) required less time for imbibition because the entry path was via the micropyle where the tracheid bar extends along the hilum, channelling the water directly to the radicle through the tracheoids (Lersten, 1982). The cushion-like structure, present in all species, is involved in the

imbibition of water after physical dormancy is broken because it is a hydrophilic tissue that is highly absorbent and flexible due to its significant water content.

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Figure Captions

Figure 1. Anatomical characters (mean ± S.E.) of the *Lupinus* seed coat (A) epidermis layer of hilum region thickness (B) counter-palisade layer of hilum region thickness (C) cushion-like structure length (D) epidermis thickness (E) hypodermis thickness, and (F) innermost parenchyma layer thickness. Different letters indicate significant differences ($P < 0.05$).

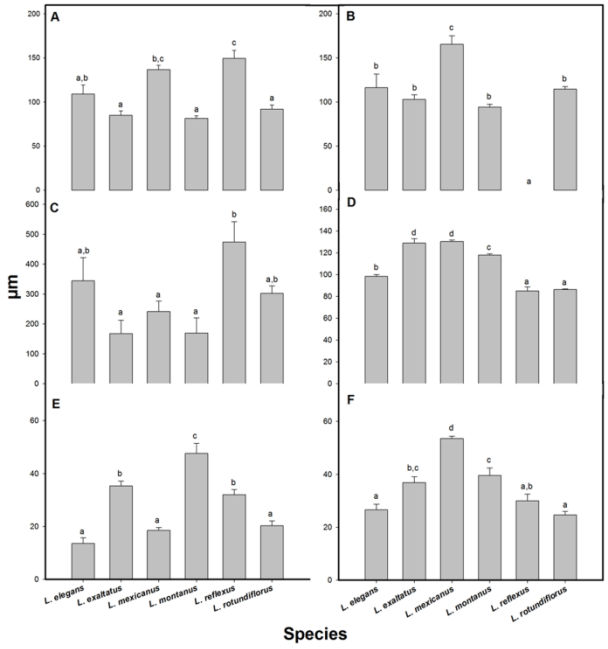

Figure 2. Longitudinal section of *Lupinus* seed coat (light microscope). From outside to inside the hilum has an external counter-palisade layer of macrosclereids except (G) *L. reflexus*, a single internal macrosclereids palisade layer (epidermis of the seed coat), the tracheid bar (layers of tracheoids with large intercellular spaces) and parenchyma cells arranged in a cushion-like structure :(A) *L. exaltatus*, (C) *L. mexicanus and* (E) *L. montanus*. The seed coat have an epidermis composed of thick-walled macrosclereids, a single layer of hypodermis composed of osteosclereids anda layer of inner parenchyma: (B) *L. exaltatus* (D) *L. mexicanus.* (F) *L. montanus* and (H) *L. reflexus*. cpl, counter-palisade layer; cls, cushion-like structure; e, embryo; os, osteosclereids; psl, palisade layer; pl, parenchyma layer; sc, seed coat; schr, seed coat of hilar region. Bar=50 µm.

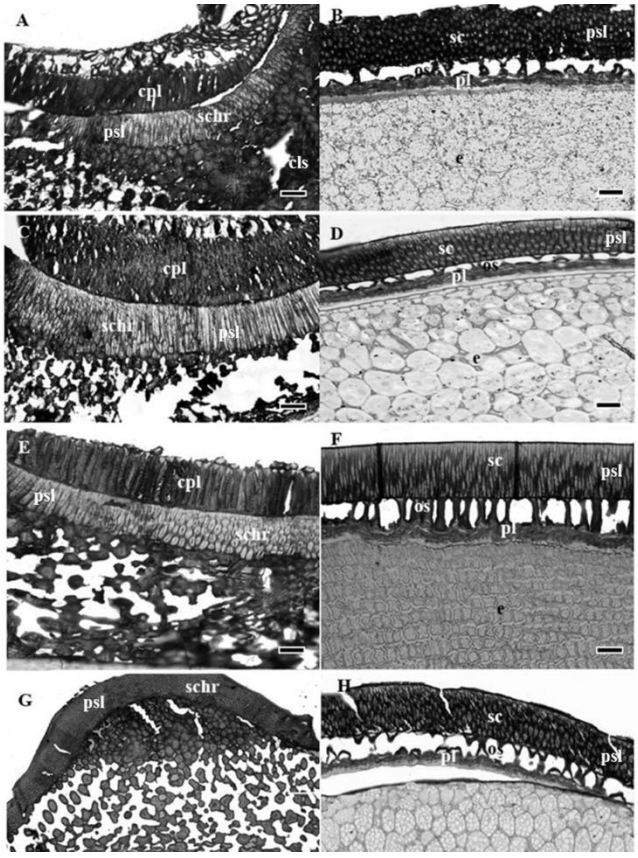

Figure 3. Entry of the dye (indicated by blue colour) after pre-treatment in *Lupinus* seeds by the lens: (A) *L. elegans* to 48 h (B) *L. exaltatus* to 18 h (C) *L. reflexus* to 18 h; and by the micropyle: (D) *L. montanus* to 6 h. cls, cushion-like structure; hr, hilar region; ls, lens; mi, micropyle; r, radicle.

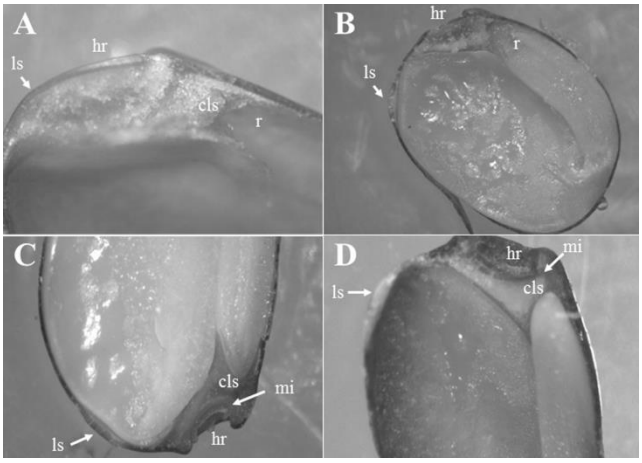

Figure 4. Longitudinal section of the hilar region of *Lupinus mexicanus* (SEM). The cushion-like structure is in the distal portion that covers the radicle tip aside the tracheid bar. cls, cushion-like structure;.cpl, counter-palisade layer;. hr, hilar region; m, macrosclereids; p, parenchyma; tb, tracheid bar. Bar=300 µm