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El siguiente artículo fue publicado en *Botanical Sciences*, 92(1): 123-129 (2014); y lo puede consultar en <http://dx.doi.org/10.17129/botsci.18>

HEAT SHOCK EFFECT IN BREAKING PHYSICAL DORMANCY IN SEEDS OF *LUPINUS ELEGANS* AND *L. ROTUNDIFLORUS* FROM JALISCO, MEXICO

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Abstract: Most wild *Lupinus* spp. (Fabaceae) grow in pine and pine-oak forests with natural and induced fires. Their seeds have physical dormancy, which can be broken in response to appropriate environmental signals, such as high temperatures, humidity, and fire. We applied heat treatments to break seed dormancy of *L. elegans* and *L. rotundiflorus* from the State of Jalisco, Mexico, with different storage times (0, 1, and 2 yr for *L. elegans* and 0, 1, 2, 3, and 4 yr for *L. rotundiflorus*). One set of seeds were immersed in boiling water for 5, 10, and 15 s, another set were incubated in wet or dry sand at 100 °C, 120 °C, and 150 °C during 60 min and 90 min. Germination trials were set at 25 °C with 12 h of irradiance per day. For both species, no germination resulted after incubation at 120 and 150 °C. For *L. rotundiflorus*, germination was higher for seeds incubated in wet sand for 60 min than for those incubated for 90 min. Fresh seeds of *L. rotundiflorus* showed higher germination than stored seeds across treatments. After scarification with boiling water, fresh seeds of *L. elegans*, germinated more than those stored for 1 and 2 year, whereas incubation in wet and dry sand resulted in higher germination for 1 and 2 year old seeds than for fresh seeds. Our results may be useful for further studies to understand and predict ecological plant responses in Mexican forests.

Key words: boiling water, dry sand, seed germination, wet sand.

Resumen: La mayoría de las especies silvestres de *Lupinus* (Fabaceae) crecen en bosques de pino y pino-encino, en donde se presentan incendios naturales e inducidos. Sus semillas tienen latencia física, la cual puede ser rota en respuesta a señales ambientales como altas temperaturas, humedad y fuego. En esta investigación, se aplicaron tratamientos de calor para romper la latencia de semillas de *L. elegans* y *L. rotundiflorus* del estado de Jalisco, México, con diferentes tiempos de almacenamiento (0, 1 y 2 años para *L. elegans* y 0, 1, 2, 3 y 4 años para *L. rotundiflorus*). Un grupo de semillas fue sumergido en agua hirviendo por 5, 10 y 15 segundos, otro grupo fue incubado en arena húmeda o seca a 100, 120 y 150 °C durante 60 y 90 minutos. Las semillas se pusieron a germinar a 25 °C con fotoperíodo de 12 horas. Para ambas especies, no hubo germinación en semillas incubadas a 120 y 150 °C. Para *L. rotundiflorus*, la germinación fue más alta en semillas incubadas en arena húmeda por 60 minutos que por 90 minutos. Las semillas frescas de *L. rotundiflorus* mostraron mayor germinación que las almacenadas, en los distintos tratamientos. Después de la escarificación con agua hirviendo, las semillas frescas de *L. elegans* germinaron más que las almacenadas por 1 y 2 años, mientras que para las semillas almacenadas por 1 y 2 años la incubación en arena húmeda y seca resultó en mayor germinación que para las semillas frescas. Estos resultados podrían ser útiles para entender y predecir respuestas ecológicas de las plantas en bosques mexicanos.

Palabras clave: agua hirviendo, arena húmeda, arena seca, germinación.

Seed dormancy is a very common adaptive plant strategy in unpredictable and harsh environments (Jurado and Moles, 2003; Jurado and Flores, 2005). Seeds with physical dormancy (PY) have water-impermeable seed or fruit coat, and are unable to imbibe water when placed in a moist envi-

ronment (Baskin and Baskin, 2000; Venier *et al.*, 2012). Impermeability of seed or fruit coat is caused by one or more water impermeable palisade cell layers that form a physical barrier for water entry (Baskin and Baskin, 1998; Baskin *et al.*, 2000). PY as a seed trait has been documented for 17 fa-

milies of angiosperms, like Cistaceae, Convolvulaceae, Fabaceae, Malvaceae, and Rhamnaceae (Baskin and Baskin, 1998; Baskin *et al.*, 2000; Baskin, 2003; Gama-Arachchige *et al.*, 2010; Karaki *et al.*, 2012).

High temperatures have been suggested to break PY (Baskin and Baskin, 2000; Pérez-Sánchez *et al.*, 2011). Temperature mediates natural breakdown of water impermeable seed layers in two main ways: (1) climatic, through the heating effect of solar radiation on the surface layers of dry soils, coupled with night-time cooling, resulting in a combination of exposure to high temperatures and wide temperature fluctuations, and (2) fire-related through the brief but intense heating on the surface layers of soil caused by fires (Probert, 2000). Several genera of the Fabaceae, like *Lupinus*, grow in pine and pine-oak forests with natural and induced fires (Espinoza-Martínez *et al.*, 2008; Martínez *et al.*, 2008; Martínez-Hernández and Rodríguez-Trejo, 2008; Zuloaga-Aguilar *et al.*, 2010, 2011). *Lupinus* seeds have been found to have PY (Burns, 1959; Quinlivan, 1968; Dehgan *et al.*, 2003; Acosta-Percástegui and Rodríguez-Trejo, 2005; Hernández *et al.*, 2008; Martínez *et al.*, 2008; Bolin, 2009; Aldrete-Chávez *et al.*, 2010; Zuloaga-Aguilar *et al.*, 2010; 2011; Elliot *et al.*, 2011), and high temperatures have been suggested to break seed dormancy in several species as *L. varius* (Quinlivan, 1968) and *L. exaltatus* (Zuloaga-Aguilar *et al.*, 2010; 2011), but not in *L. texensis* (Davis *et al.*, 1991), or *L. leptophyllus* (Aldrete-Chávez *et al.*, 2010). Although, high temperatures can break seed dormancy, they can also affect seed viability either through accelerated “ageing” (Daws *et al.*, 2007), or by the impact of high temperatures on cellular processes (Probert, 2000), but this remains unexplored for *Lupinus* spp.

Seeds of *Lupinus* spp. are orthodox (desiccation-tolerant) (Ellis *et al.*, 1987; Garnczarska *et al.*, 2009); thus, *ex situ* conservation of *Lupinus* may be achieved through seed storage. The effect that aging through storage and high temperatures might have on breaking seed dormancy of *Lupinus* spp. deserves exploring.

Lupinus includes 300 to 500 species occurring in the Mediterranean region and America. Usually they are annual or perennial herbs or shrubs. They have palmately compound or rarely simple leaves, and produce spiraled or whorled arrangement of flowers in a raceme (Sousa and Delgado, 1993). In Mexico nearly 100 wild species occur, 15 are native to the state of Jalisco (Ruiz and Sotelo, 2001).

The objective of this study was to evaluate the effect of combined pretreatments of elevated temperature and humidity, in breaking physical dormancy and promote germination of fresh and stored seeds of two *Lupinus* species (*L. elegans* H.B.K and *L. rotundiflorus* M.E.Jones) common to Jalisco, Mexico. We expected that heat may promote germination as a strategy to establish when competition is low, different temperatures represent fire intensities and also accelerate aging processes.

Materials and methods

Study species and seed collection. *Lupinus elegans* is a shrubby legume that grows in disturbed areas in pine and pine-oak forests in Central Mexico, from 1,700 to 3,000 m in elevation (Ruiz-Moreno *et al.*, 2000). It is a common ruderal species; it incorporates nitrogen into the soil and has potential for restoration practices (Alvarado-Sosa *et al.*, 2007). In Jalisco it is found in Autlán, Bolaños, Cuautitlán, Gómez Farías, Mezquitic, and Tecolotlán (Ruiz-Moreno *et al.*, 2000; Ruiz-Moreno and Zamora-Natera, 2006).

Lupinus rotundiflorus is found in open slopes growing alongside *Acacia* spp., shrubs of *Leucaena* spp., and arborescent *Ipomoea* spp. It occasionally grows in pine-oak forests from 1,500 to 2,500 m in elevation, and often occurs in disturbed areas (McVaugh, 1987). In Jalisco it occurs in Mascota, Talpa, and Tapalpa (Ruiz-Moreno and Zamora-Natera, 2006).

The seeds of *Lupinus elegans* were obtained from “Sierra de Quila” at 1,780 m a.s.l. and those of *L. rotundiflorus* from “Tapalpa”, Jalisco at 2,350 m a.s.l. The seeds were collected from at least 30 plants per species, and we obtained ca. 50 g of clean seed per species. We analyzed fresh, and 1 and 2 yr dry stored seeds of *L. elegans*; and fresh, and 1, 2, 3, and 4 yr dry stored seeds of *L. rotundiflorus*. We considered fresh seeds those collected two months (*L. rotundiflorus*) and four months (*L. elegans*) before the beginning of the lab experiment. All aged seeds were stored in paper-bags at room temperature (22 °C).

Anatomical study of physical dormancy (PY). Samples of three seeds were fixed in formalin-ethanol-glacial acetic acid (Ruzin, 1999), and longitudinally sectioned. Samples were mounted on a carbon double-sided adhesive tape on metal pins and coated with sputter gold to analyze the structure of the testa with a scanning electron microscope (Quanta™ 200, FEI, OR, 20.00 Kb).

Breaking dormancy in intact seeds. The following experiments were applied to intact (not mechanically scarified) seeds in an attempt to break PY: (1) seeds dipped in boiling water for 5, 10, and 15 s; (2) seeds heated for 100, 120, and 150 °C for 60 and 90 min on wet and dry sand. Treated and control seeds from both experiments were tested for germination at 25 °C in a 12/12 hr light/dark germination chamber (model Lumistell ICP-19d-c/iv) for 30 days. We used a complete random design, with five replicates of 20 seeds for each treatment. Seeds were placed in Petri dishes with water-agar as substrate sealed with parafilm (Parafilm M, Pechiney Plastic Packaging, Chicago, Ill.). Germination percentages in each treatment were compared with those of control (non-scarified seeds incubated) to determine the effectiveness of the dormancy-breaking treatment. Germination was determined with emergence of the radicle. We

analyzed fresh and 1 and 2 yr dry stored seeds of *L. elegans*, and fresh and 1, 2, 3, and 4 yr dry stored seeds of *L. rotundiflorus*.

Statistical analyses. We conducted a two-way ANOVA for the germination percentage data of each species for each experiment. Data were arcsine transformed to achieve normal distribution.

Storage time (five for *Lupinus rotundiflorus* and three for *L. elegans*) and pre-treatment were the factors analyzed in the ANOVA for each experiment. Tukey tests were performed to determine the differences in means among treatments. All analyses were performed using JMP version 8.0.

Results

Anatomical study of physical dormancy (PY). We provide direct evidence that fresh and stored seeds of the studied species possess PY by describing the basic structure of seed testa that results in a hard seed coat of *Lupinus* spp. We found in the seed testa longitudinal sections, four layers inwards from the surface: the cuticle, the epidermis, the hypodermis, and the inner parenchyma. The first barrier to imbibition is the waxy cuticle. The next layer is the epidermis, composed of thick-walled macrosclereids with the long axis oriented perpendicularly to the surface, arranged in a compact palisade layer. 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. The fourth innermost layer is parenchymatous, with six rows of tangentially collapsed endosperm remnant cells. At the hilum there is one external layer of macrosclereids forming a counter-palisade layer placed above the palisade layer (Figure 1).

Effect of pretreatments on seed germination. For both species a higher germination percentage was observed for seeds pretreated in boiling water than for control seeds and those from the hot wet and dry sand experiment.

Boiling water. There was a significant effect of pretreatments ($F = 48.19$, $df = 3$, $P < 0.001$) on the germination of *Lupinus elegans* seeds. Boiling water for 5, 10, and 15 s resulted in higher germination than control seeds (Table 1). There was also a significant effect of seed age ($F = 19.26$, $df = 2$, $P < 0.001$) in that pretreated fresh seeds germinated less ($30 \pm 4.12\%$) than pretreated seeds after 1 yr ($57 \pm 6.31\%$) or 2 yr ($49.5 \pm 7.09\%$) of dry storage. Interaction between pre-treatment and seed age was not significant (Figure 2; $F = 1.55$, $df = 6$, $P = 0.18$).

For *Lupinus rotundiflorus* seeds, there was a significant effect of pretreatments on germination ($F = 82.00$, $df = 3$, $P < 0.0001$); boiling water for 5, 10, and 15 s resulted in higher germination than the control seeds (Table 2). There

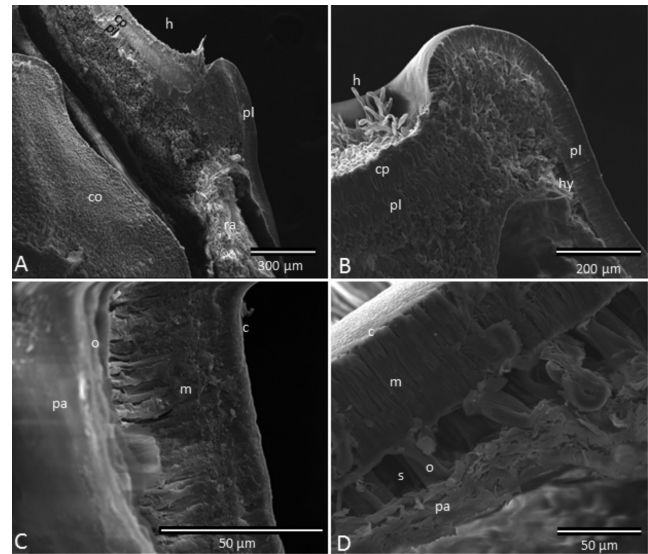


Figure 1. Seed testa longitudinal section SEM micrograph of *Lupinus*. A, C. *L. elegans*; B, D. *L. rotundiflorus*. A, B. Hilum region. C. Detail of the cuticle and the palisade layer. D. Detail of the epidermis structure. c, cuticle; co, cotyledon; cp, counter palisade; h, hilum; hy, hypodermis; m, macrosclereids; o, osteosclereids; pa, parenchyma; pl, palisade layer; ra, radicle; s, intercellular spaces.

was also a significant effect of seed age ($F = 12.54$, $df = 4$, $P < 0.0001$); on average pretreated fresh seeds showed higher germination ($71.5 \pm 7.65\%$) than seeds pretreated after 1 yr ($55 \pm 5.23\%$), 2 yr ($42.5 \pm 4.83\%$), 3 yr ($61.5 \pm 4.92\%$), or 4 yr ($53.5 \pm 5.91\%$) dry storage. Interaction between heat exposure in boiling water and storage time was significant ($F = 2.1350$, $df = 12$, $P = 0.0233$) in that 2 yr old seeds had similar low germination across pretreatments. Regardless of storage time, seeds germinated more after pre-treatment than control seeds. Fresh seeds showed higher germination than older seeds with pretreatments (Figure 3).

Dry and wet sand. There were differences in germination of *Lupinus elegans* seeds between pretreatments ($F = 10.69$, $df = 4$, $P < 0.0001$). Germination was higher for seeds after wet sand incubation for 60 and 90 min at $100\text{ }^{\circ}\text{C}$ ($29 \pm 6.05\%$ and $32.33 \pm 6.91\%$, respectively) than for those incubated in

Table 1. Mean final germination (\pm SE) of *Lupinus elegans* seeds dipped in boiling water for 5, 10 and 15 s. Different letters indicate differences ($P < 0.05$) between means.

	Pretreatment	Germination percentage
	Control	7.33 ± 3.66^b
Dipped in boiling water for	5 sec	59.00 ± 3.66^a
	10 sec	58.33 ± 3.66^a
	15 sec	57.33 ± 3.66^a

Table 2. Mean final germination percentages (\pm SE) of *Lupinus rotundiflorus* seeds dipped in boiling water for 5, 10 and 15 s. Different letters indicate differences ($P < 0.05$) between means.

	Pretreatment	Germination percentage
Dipped in boiling water for	Control	20.2 \pm 2.46 ^b
	5 sec	70.2 \pm 2.69 ^a
	10 sec	69.8 \pm 2.69 ^a
	15 sec	67.0 \pm 2.69 ^a

dry sand ($13.33 \pm 4.13\%$ and $22.66 \pm 5.68\%$, respectively) at the same temperature and control seeds ($7.33 \pm 1.68\%$). Seeds exposed to temperatures of 120 and 150 °C did not germinate. Storage time influenced germination ($F = 83.83$, $df = 2$, $P < 0.0001$), in that the highest germination was obtained for seeds stored for 1 yr ($42 \pm 3.76\%$), followed by seeds stored for 2 yr ($17.2 \pm 3.48\%$) and fresh seeds ($3.6 \pm 0.98\%$). The interaction between pretreatment and storage time was significant ($F = 5.38$, $df = 8$, $P < 0.0001$), in that 1 yr old seeds had higher germination than fresh seeds, and 2 yr old seeds after wet and dry sand incubation (Figure 4).

For *Lupinus rotundiflorus*, seeds exposed to temperatures of 120, and 150 °C did not germinate. Wet sand incubation

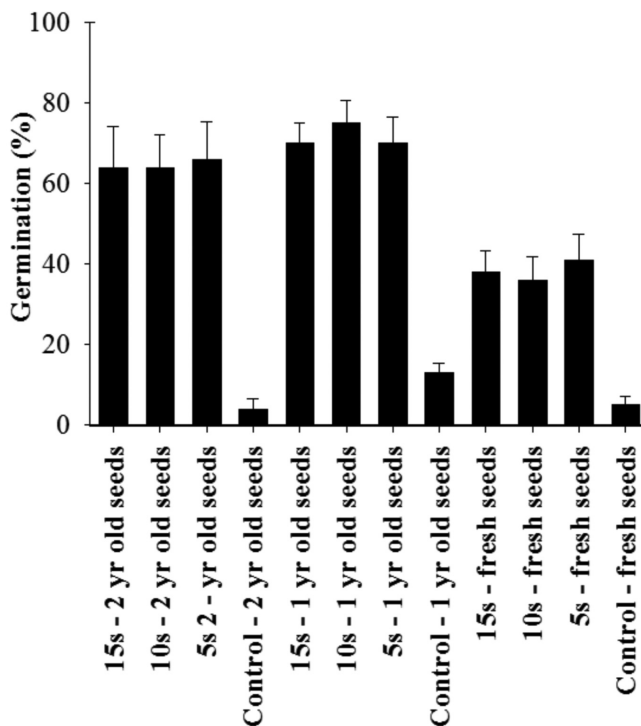


Figure 2. Mean final germination (\pm SE) of *Lupinus elegans* seeds of different aging (fresh, 1 and 2 yr) after dipped in boiling water for 5, 10 and 15 s. Interaction between boiling and seed age was not significant ($P = 0.18$).

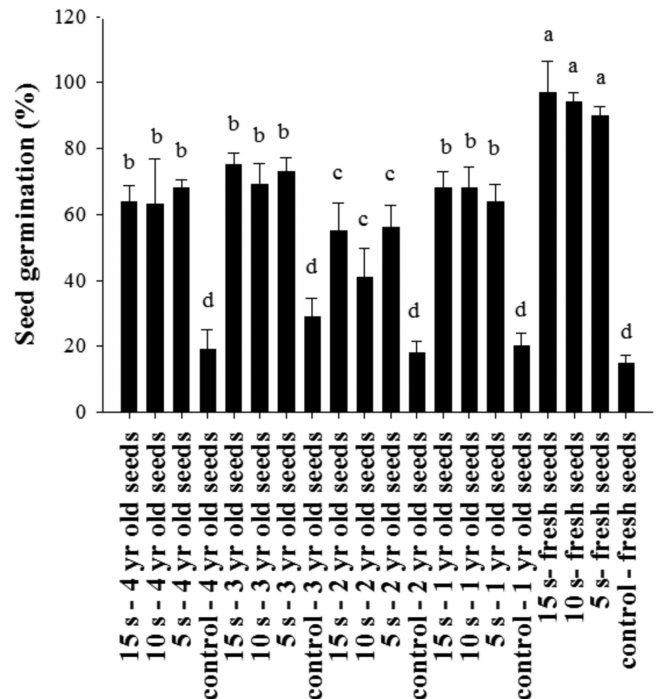


Figure 3. Mean final germination (\pm SE) of *Lupinus rotundiflorus* seeds of different seed aging (fresh, 1, 2, 3, and 4 yr) after dipped in boiling water for 5, 10 and 15 s. Different letters indicate differences ($P < 0.05$) between means for the interaction of boiling \times seed age.

for 60 min at 100 °C resulted in high germination ($55.8 \pm 4.48\%$) as well as dry sand incubation for 60 min ($60.6 \pm 4.47\%$), and 90 min ($54 \pm 4.58\%$) compared to seeds incubated in moist sand for 90 min ($32.4 \pm 5.43\%$) and control seeds ($20.2 \pm 2.07\%$, $F = 50.248$, $df = 4$, $P < 0.0001$). Storage time also affected germination, pretreated fresh seeds had higher germination ($73.4 \pm 6.28\%$) than pretreated seeds after 1 yr ($38.6 \pm 4.05\%$), 2 yr ($27.6 \pm 3.04\%$), 3 yr ($45.8 \pm 4.16\%$), or 4 yr ($37.6 \pm 3.58\%$) of dry storage ($F = 49.870$, $df = 4$, $P < 0.0001$). The interaction between storage time and pretreatment was significant ($F = 4.86$, $df = 16$, $P < 0.0001$), in that pretreated fresh seeds germinated more (at least 81%) than fresh untreated seeds ($15 \pm 2\%$), and control and pretreated stored seeds (Table 3).

Discussion

Seeds of the studied species were found to have structures that provide PY as for other species of Fabaceae with PY (de Souza and Marcos-Filho, 2001). Heat shock caused by high temperatures influenced the breaking of seed dormancy of studied *Lupinus* species. In general, the pretreatment that resulted in higher germination was boiling water. This result is similar to that obtained for *L. varius* by Quinlivan (1968) and Karaguzel *et al.* (2004), as well as for *L. lepidus* by Elliott

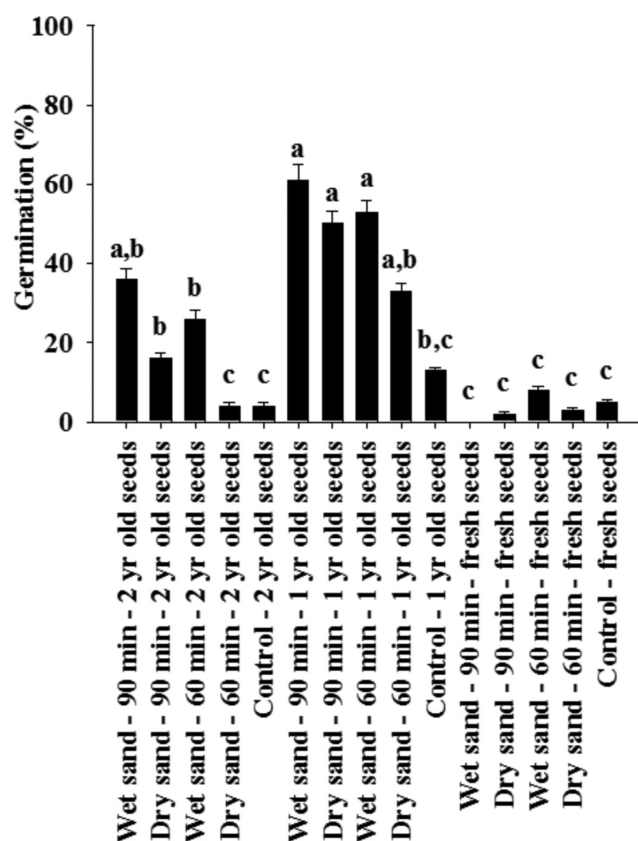


Figure 4. Mean final germination (\pm SE) of *Lupinus elegans* seeds of different aging (fresh, 1 and 2 yr) after heated for 100 °C for 60 and 90 minutes on wet and dry sand. Different letters indicate differences ($P < 0.05$) between means for the interaction of moist/dry heat \times seed age.

et al. (2011). However, it differs from results obtained by Bolin (2009) for *L. perennis*. For *L. polyphyllus* almost no germination was obtained after hot water exposure followed by 20 °C incubation, but 60% germination was obtained after chilling at 5 °C for six weeks to simulate winter followed by alternating diurnal temperatures of 15 °C/7 °C approximating spring conditions (Elliot *et al.*, 2011). This variation implies specific requirements among the species of *Lupinus* to break dormancy. Because *L. elegans* and *L. rotundiflorus* grow in high altitudes, the effect of chilling in breaking physical dormancy needs to be tested.

Pretreatment of wet or dry sand produced low germination percentages for *Lupinus elegans* (< 40% in wet sand and < 20% dry sand), and high germination for *L. rotundiflorus* (> 70% in wet sand and > 50% dry sand). This implies that species germination responses were not necessarily related to their phylogeny, but perhaps to their environment as suggested by Elliot *et al.* (2011) for three species of *Lupinus* of Washington, USA. These authors found that *L. lepidus* and *L. albicaulis*, species that are restricted to frequently burned prairies, had stronger responses to high pregermination tem-

Table 3. Mean final germination percentages (\pm SE) of *Lupinus rotundiflorus* seeds heated for 100 °C for 60 and 90 minutes on wet and dry sand. Different letters indicate differences ($P < 0.05$) between means for the interaction of moist/dry heat \times seed age.

Pretreatment	Germination (%)
Wet sand - 90 min - 4 yr old seeds	19 \pm 1.5 ^d
Dry sand - 90 min - 4 yr old seeds	48 \pm 2.3 ^b
Wet sand - 60 min - 4 yr old seeds	49 \pm 2.1 ^b
Dry sand - 60 min - 4 yr old seeds	53 \pm 2.6 ^b
Control - 4 yr old seeds	19 \pm 1.6 ^d
Wet sand - 90 min - 3 yr old seeds	28 \pm 2.0 ^{c,d}
Dry sand - 90 min - 3 yr old seeds	58 \pm 3.0 ^b
Wet sand - 60 min - 3 yr old seeds	53 \pm 2.7 ^b
Dry sand - 60 min - 3 yr old seeds	61 \pm 3.1 ^b
Control - 3 yr old seeds	29 \pm 1.6 ^c
Wet sand - 90 min - 2 yr old seeds	14 \pm 1.0 ^d
Dry sand - 90 min - 2 yr old seeds	30 \pm 2.6 ^c
Wet sand - 60 min - 2 yr old seeds	41 \pm 2.2 ^c
Dry sand - 60 min - 2 yr old seeds	35 \pm 3.0 ^c
Control - 2 yr old seeds	18 \pm 1.9 ^d
Wet sand - 90 min - 1 yr old seeds	20 \pm 1.5 ^d
Dry sand - 90 min - 1 yr old seeds	43 \pm 2.5 ^c
Wet sand - 60 min - 1 yr old seeds	47 \pm 2.2 ^{b,c}
Dry sand - 60 min - 1 yr old seeds	63 \pm 3.5 ^{a,b}
Control - 1 yr old seeds	20 \pm 2.0 ^d
Wet sand - 90 min - fresh seeds	81 \pm 4.7 ^a
Dry sand - 90 min - fresh seeds	90 \pm 5.0 ^a
Wet sand - 60 min - fresh seeds	90 \pm 4.0 ^a
Dry sand - 60 min - fresh seeds	91 \pm 5.5 ^a
Control - fresh seeds	15 \pm 2.0 ^d

perature regimes. In contrast, *L. polyphyllus*, with a broader distribution showed less response to high pregermination temperatures, and minor response to fluctuating spring temperature regimes.

Temperatures above 100 °C adversely affected germination of our species, suggesting that they are not adapted to germinate after very intense fires. Loss of viability after heat was probably due to embryo damage, as reported by Keeley *et al.* (1985) for *Marah macrocarpus*, *Paeonia californica*, *Stipa coronata*, and *Zigadenus fremontii*, as well as by Cruz *et al.* (2003) for *Erica australis* at 150 °C. For some species in the Fabaceae, damage of seeds inhibiting germination has been found even at relatively low temperatures (> 90 °C). Martin *et al.* (1975) found that temperatures above 90 °C were lethal for seeds of *Cassia*, *Desmodium*, *Galactia*, and *Lespedeza*. Stored seeds of *Lupinus elegans* showed higher germination than fresh seeds, suggesting that physical dormancy is lost with age. Therefore, this species probably has

the ability to form seed banks in the soil. For *L. rotundiflorus* however, fresh seeds germinated more after being exposed to wet and dry hot sand, implying that physical dormancy can be easily broken by heat shock in the same year seeds were produced. This also implies that species germination responses are not necessarily related to their phylogeny.

Storage of seeds did not break their dormancy, but high temperatures did. High temperatures helped break seed dormancy, although high temperatures affected seed viability because 90 min treatments with dry or wet sand showed decreased germination compared with 60 min treatments for *Lupinus rotundiflorus*. Rapid 'accelerated' ageing by the impact of high temperatures might damage cellular structures (Daws *et al.*, 2007).

As it has been suggested for other species growing in harsh environments (Jurado and Flores, 2005), the hard and impermeable seed coat found for *Lupinus elegans* and *L. rotundiflorus* allows the seeds to endure the stressful high temperatures of open sites where these species occur. Our results on effect of heat on breaking seed dormancy is a first approach to investigate the regeneration biology of species in pine and pine-oak forests in Central Mexico and may be useful for further studies to understand and predict ecological plant responses in Mexican forests.

Acknowledgements

This study was supported by Consejo Nacional de Ciencia y Tecnología-Fondos Sectoriales-Secretaría de Medio Ambiente y Recursos Naturales (2006-1-23818). Robles-Díaz, E. was supported by a grant (090388) from Consejo Nacional de Ciencia y Tecnología during her master degree studies at Instituto Potosino de Investigación Científica y Tecnológica. Araceli Patrón (División de Biología Molecular-IPICYT) provided assistance with SEM. We thank two anonymous reviewers for contributing to improve the manuscript.

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Received: January 4th, 2013

Accepted: May 9th, 2013