

Factors controlling seed germination of the Iberian critically endangered *Pseudomisopates* (Antirrhinaceae)

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Abstract Many seeds are dormant when shed from the mother plant. This unique characteristic of plants poses challenges in conservation and many different treatments have traditionally been used to break dormancy. When germination only occurs under certain circumstances, recruitment may be insufficient and the viability of plant populations may be threatened. A marked dormancy was previously identified in the Iberian critically endangered species *Pseudomisopates rivas-martinezii*. The present study aimed to determine the magnitude of dormancy by estimating seed viability under different germination treatments: heat, ash, stratification, gibberellin addition, and darkness. Our results indicate that there were significant differences in viability across plant populations and treatments. Maximum seed germination was obtained under ash addition, although cold stratification alone gave a considerable enhancement. However, gibberellic acid did not improve germination and darkness had a diminishing effect. In conclusion, a cold period is required for the species to germinate, coupled with the effects of fire: (1) ash, (2) vegetation clearings, and (3) resprouting, which are major factors triggering seed germination. Although these conditions are widely found in the field, we hypothesize that a more dramatic situation may have tackled this

species before the increase of human-mediated fires in the past millennia.

Keywords Ash · Darkness · Dormancy · Fire · Mediterranean · *Pseudomisopates rivas-martinezii* · Seed germination

Introduction

Seed germination is a critical stage in the life cycle of sexually reproducing plants (Caswell 1989). Dormancy can prevent germination when conditions are suitable for germination, but the probability of survival and growth of the seedling is low. There are three main different types of seed dormancy (Baskin and Baskin 1989): (1) morphological dormancy, due to an undeveloped embryo; (2) physical dormancy, caused by seed or fruit coat impermeability to water, and (3) physiological dormancy, where inhibitors limit germination enzymes and hormones, so that a chemical change needs to take place in the seed. Physiological dormancy is the most common and allows a more flexible response to the environment than the other two types of dormancy.

When germination only occurs under certain circumstances, recruitment may be insufficient to compensate mortality or maintain genetic diversity, threatening the viability of plant populations (Keith 1996). Therefore, this unique characteristic of plants poses challenges in conservation and many different treatments have traditionally been used to break dormancy, including temperature, scarification of the seed coat or the application of hormones, mainly gibberellins, or other chemicals that have been proven to be effective (Bewley and Black 1994). Environmental factors such as chilling and light promote

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a decrease in the concentration of the germination inhibitor, abscisic acid (ABA), which in turn induces the synthesis of gibberellins (GA). These changes in ABA:GA balance eventually result in wall expansion to permit radicle emergence (Finkelstein et al. 2008). Fire is frequent in most Mediterranean ecosystems, and has shaped their composition, ecology, and evolution in time. For instance, fire-prone communities in the Californian chaparral and South African fynbos are dominated by species which exhibit remarkable similarities in their germination response to fire elements, such as charred wood and smoke. This germination behaviour has arisen independently in distantly related families and is interpreted as convergent evolution (Keeley and Bond 1997). Moreover, fire has also long been part of man's activity since it has been widely used as a management tool in natural ecosystems. As such, it can affect soil, water, vegetation, wetland, air, and cultural aspects; therefore, a correct evaluation and cost-benefit analysis among these components has to be achieved to make a proper use of fire in managing ecosystems (DeBano et al. 1998). In Mediterranean-type ecosystems, many plant species are adapted to germinating soon after the occurrence of fire (Lloret et al. 1999). Fire plays a key role in breaking seed dormancy in some of the most species-rich families of the Mediterranean: *Fabaceae*, *Poaceae*, *Asteraceae* and *Cistaceae* (Clemente et al. 1996; Pérez-Fernández and Rodríguez-Echevarría 2003; Quintana et al. 2004). These plants can behave as resprouters, seeders or both when exposed to fire (Keeley and Fotheringham 2000; Konstantinidis et al. 2005; Ojeda Copete et al. 2005). Under highly seasonal conditions resprouters seem to be the dominant form, whereas seeders are more dominant in milder Mediterranean climates (Ojeda Copete et al. 2005).

Species may benefit from direct or indirect factors of fire. Direct effects include (1) high temperatures generated by the fire, which stimulate the germination of serotinous (Cowling and Lamont 1987) and hard seeded species (Keeley 1991; González-Rabanal and Casal 1995; Pérez-Fernández and Rodríguez-Echevarría 2003); (2) smoke-derived gasses (Baxter et al. 1994; Dixon et al. 1995; Keeley and Fotheringham 1997); (3) chemicals contained in charred or ashed wood (Brown 1993; Enright et al. 1997), or (4) the release of nitrogenous compounds in the soil from plant biomass combustion (Humphreys and Craig 1981; Thanos and Rundel 1995; Pérez-Fernández and Rodríguez-Echevarría 2003). Indirect effects are related to the changes that occur in the environment after fire that enhances site quality for germination and recruitment. These include (1) formation of vegetation clearings, which benefits some species by decreasing competition and increasing levels of light, thus favouring growth and survival (Valverde and Silvertown 1995; Menges and Hawkes

1998); (2) acceleration of the mineralization of organic matter, making inorganic nutrients more readily available (Wells et al. 1979; Certini 2005); or (3) soil sterilization which alters microbial pathogens populations (Wicklow 1988; Ahlgren 1974).

The aim of this study was to determine the factors that influence seed germination in *Pseudomisopates rivasmartinezii* (Antirrhinaceae). This endangered species shows extensive clonal growth and although plants flower profusely, seeds at many sites seemed to be unviable, as shown by preliminary germinability tests, and no seedlings have been observed in the field (Martínez Rodríguez et al. 2004). To unravel the factors triggering seed germination (1) the level of seed viability in three populations of the species was determined, (2) the presence of dormancy was investigated by applying cold stratification and gibberellins, and (3) the direct and indirect effects of fire were studied by applying heat, ash, and darkness.

Materials and methods

Study species

Pseudomisopates rivasmartinezii (Scrophulariaceae) is an endangered species and monotypic genus endemic to the Gredos Mountain Range in the Spanish Central Mountain System. It has been classified as Critically Endangered following the IUCN criteria in the Red List of Spanish Vascular Flora (Martínez Rodríguez et al. 2004). The species occurs from 1,400 to 1,990 m.a.s.l. in *Cytisus oromediterraneus* shrublands, one of the most common habitats of Gredos. In this habitat, the species mostly occupies clearings among shrubs, where plants show higher vigour than plants growing in dense shrublands. There are two main distribution centres located 20 km apart, comprising five and three populations, respectively (Vargas and García 2008). The species is self-incompatible (Amat et al. 2010) and reproduces vegetatively by an underground stolon network, with connections between ramets that can span over one metre. Flowers bloom during July and August. Fruiting occurs during August and September. Fruits can contain between 1 and 24 seeds, which are dispersed by barochory.

Study areas

In Mediterranean mountains the natural vegetation above tree line is a shrubland dominated by several fabaceous species (*piornal*). The dominant species in our study areas are *Cytisus oromediterraneus*, *Genista cinerascens* or *Echinospartum barnadesii*. This area has a Mediterranean Continental climate type with a distinct dry summer period,

a mean annual rainfall of about 897 mm and an average annual temperature ranging from -1 to 15 °C.

Seeds were collected during the dispersal period of *P. rivas-martinezii*, at the end of the summer 2009 and stored in paper bags at room temperature. Seeds from a total of three populations (comprising both centres of distribution) were collected for study. The first one is located in the Serrota Mountain (Cepeda de la Mora, Ávila, Spain) which comprised an area of approximately $14,400$ m². The dominant shrub species was *C. oromediterraneus*. The second population is located in Conventos (Hoyos del Espino, Ávila, Spain) that comprised an area of approximately $12,100$ m². Vegetation consisted of *C. oromediterraneus* and *E. barnadesii* shrubland. *P. rivas-martinezii* plants were distributed in patches of different sizes throughout the area of both populations. The third population is located in Garganta Lóbrega (Candeleda, Ávila, Spain) that comprised an area measuring approximately $100,500$ m². The dominant shrub species was *C. oromediterraneus*. This last site was burnt in the year 2006 and *P. rivas-martinezii* has intensely occupied the recovering shrubland.

Germination experiment

Seed germination in mountain species often requires the application of dormancy-breaking treatments (Körner 2003). Thus, in this study most seeds were pretreated with a cold-wet stratification period of 2 months at 4 °C to simulate natural climatic conditions. Next, all seeds were surface sterilised with 2 % sodium hypochloride. Germination experiments were conducted in plastic Petri dishes (9 cm diameter) lined with glass beads and covered with filter paper disks and moistened with either distilled water or an ash solution, according to the test. Germination experiments were carried out in an incubator equipped with cool-white fluorescent tubes under a 16-h light/8-h dark photoperiod at a constant temperature of 20 ± 1 °C. These experimental conditions were chosen to emulate the environmental thermoperiods and photoperiods that a seed would experience on the soil surface in spring. This season is characterized by melting snow and rainfalls that provide enough soil moisture to activate seed germination. We considered the following treatments:

High-temperature treatment (HIGHTEMP)

Seeds were dry heated in an oven for 5 min at 110 °C prior to stratification. This treatment was selected to simulate the extreme conditions potentially encountered by seeds on the surface or near the surface of the soil during fire and test seed survival (Christensen and Muller 1975; DeBano et al. 1979).

Low-temperature treatment (LOWTEMP)

Seeds were dry heated in an oven for 5 min at 70 °C prior to stratification. This treatment simulates the conditions potentially encountered by seeds in the soil during fire (Christensen and Muller 1975; DeBano et al. 1979).

Ash treatment (ASH)

The effect of ash on germination was studied by watering wet-cold stratified seeds with a suspension of ash (Keeley and Fotheringham 2000). This solution was obtained from the combustion of leaves and branches from several representative species from the shrubland. Biomass was burned until there was no plant material left. The proportion of combusted species used was about 60 % shrubs and 40 % grasses, simulating the proportion found in its natural habitat. Ash was diluted with distilled water to a concentration of 10 g of ash per litre of water.

Gibberellic acid treatment (GA)

The breakdown of dormancy by gibberellins was studied by imbibing a set of untreated seeds in a solution of Gibberellic Acid (GA₃) at a concentration of 150 mM, pH 6.5.

Stratified gibberellic acid treatment (STR_GA)

Seeds that had been previously cold-wet stratified were imbibed in a solution of Gibberellic Acid (GA₃) at a concentration of 150 mM, pH 6.5.

Darkness treatment (DARK)

Petri dishes were wrapped in foil paper to avoid light from reaching previously wet-cold stratified seeds. This treatment was selected to simulate the effect of shrub closure on seeds, since it has been seen to affect flowering display (Amat et al. 2013).

Stratified control (STR_CONTROL)

Seeds were wet-cold stratified and incubated with distilled water at 4 °C for 2 months.

Control (CONTROL)

Untreated seeds were incubated with distilled water.

Each treatment was represented by 5 replicates containing 25 seeds each and the germination rate was expressed as a percentage. The germination criterion was defined as the emergence of the radicle and was recorded every 2 days over a period of 60 days. At the end of the

period, non-germinated seeds were individually checked for viability (embryo and endosperm intact). When treated seeds were in apparent good condition, viability was accounted for by a Tetrazolium test, so that final results could be adjusted considering non-viable seeds. In designing the experiment, seed availability of this endangered species was a limiting factor. Therefore, treatments were considered on the basis of relevance to a presumed fire-prone mountain species, and so only eight treatments were conducted to test the different effects of fire in the ecosystem, the presence of dormancy and finally the effect of darkness.

Data analysis

Differences in seed viability among populations were first evaluated by a Generalized Linear Model comparing the categorical variable population and the binary response seed viability. Seed viability for each population and treatment was estimated as the proportion of germinated and non-germinated but viable seeds, as shown by the Tetrazolium test. Differences in seed germination of viable seeds were analysed with a Generalized Linear Mixed Model, fit by the Laplace approximation, studying the relation between the categorical variable treatment and the binary response number of germinated seeds, with population as random factor. Pairwise differences were assessed using the non-parametric Chi-squared test. Analyses were performed using the package ‘lme4’ written for R (R Development Core Team 2008) and JMP version 7.

Time to germination was strongly right-censored since 87 % of the viable seeds did not germinate irrespective of the treatment applied (i.e. 13 % of the observations were

censored). Therefore, data were analysed by Survival Analysis. Differences in time to germination were assessed by a regression failure-time analysis studying the relation between treatment nested within population, and day of germination, using the log-normal distribution. The germination index number of days for 50 % of the total number of seeds germinated (T_{50}) was also calculated. Analyses were performed with JMP version 7.

Results

Seed viability varied between populations ($\chi^2 = 75.51$; $p < 0.0001$) and was significantly higher in Lóbrega (0.45) than in Serrota (0.29; $\chi^2 = 64.77$; $p < 0.0001$) and Conventos (0.31; $\chi^2 = 43.47$; $p < 0.0001$); where as Serrota and Conventos were not significantly different ($\chi^2 = 0.14$; $p = 0.14$).

There were significant between-treatment differences in germination rates (Fig. 1; Table 1; Supplementary Appendix 1). ASH treatment had the highest germination rate ($\beta_{ASH} = 1.479 \pm 0.381$; $p = 0.0001$), but there were also significant differences between treatments, largely due to low results of GA ($\beta_{GA} = -1.486 \pm 0.615$; $p = 0.0001$) and CONTROL ($\beta_{CONTROL} = -2.207 \pm 0.791$; $p = 0.005$). The population effect had a standard deviation of 0.627, indicating that there is a certain level of variation between populations. This variation is due to the significantly higher germination rates observed in Serrota population (GLM, $p < 0.0001$). However, since interaction between treatment and population was not significant (Supplementary Appendix 1), and results are equivalent in all populations, data for treatments across populations have been pooled together for further analysis and figures.

Fig. 1 Accumulated germination of *Pseudomisopates rivasmartinezii* in response to germination treatments, expressed as per cent of viable seeds germinated. ASH: ash addition; STR_CONTROL: stratification; STR_GA: stratification with GA₃; HIGHTEMP: preheating at 110 °C with stratification; LOWTEMP: preheating at 70 °C with stratification; DARKNESS: no light; GA: GA₃ addition; CONTROL: untreated seeds. Bars on the right show treatments of seeds that were stratified (S) and seeds that were not stratified (NS)

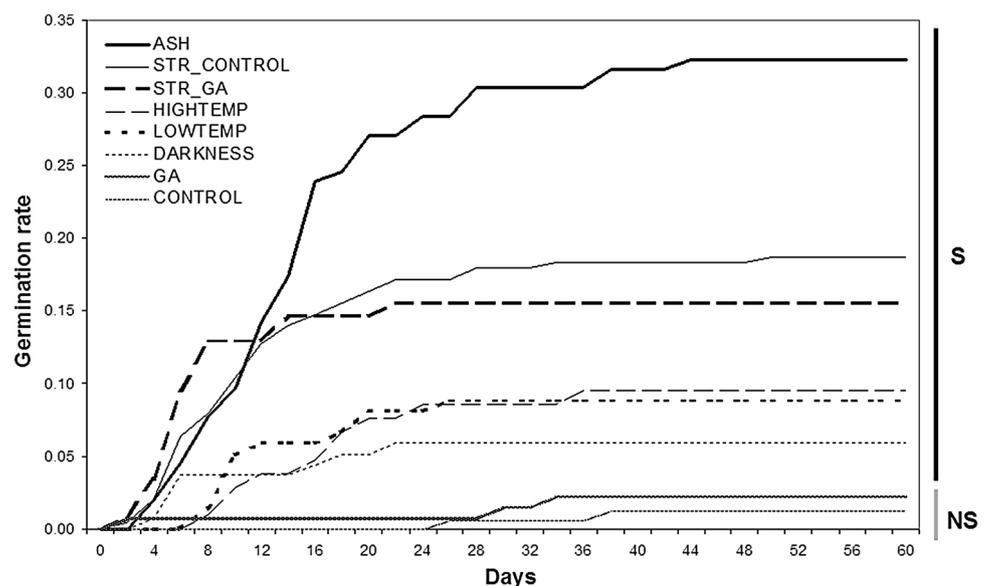


Table 1 Among-treatment differences in germination level (proportion of germinated seeds) for *Pseudomisopates rivas-martinezii* populations, and in germination timing as indicated by the T_{50} and the parameters of the failure-time analysis regression

Treatment	Germination level \pm SE				Germination timing	
	Serrota	Conventos	Lóbrega	Total	T_{50}	Parameter \pm SE
Intercept						2.56 \pm 0.08
ASH	0.70 \pm 0.07	0.20 \pm 0.06	0.19 \pm 0.05	0.34 \pm 0.04	14	0.19 \pm 0.18
STR_CONTROL	0.30 \pm 0.05	0.10 \pm 0.04	0.15 \pm 0.04	0.19 \pm 0.02	12	-0.04 \pm 0.17
STR_GA	0.23 \pm 0.06	0.09 \pm 0.06	0.11 \pm 0.05	0.16 \pm 0.03	6	0.02 \pm 0.11
HIGHTEMP	0.24 \pm 0.11	0.03 \pm 0.03	0.10 \pm 0.04	0.10 \pm 0.03	18	0.89 \pm 0.37
LOWTEMP	0.13 \pm 0.06	0.07 \pm 0.05	0.08 \pm 0.03	0.09 \pm 0.02	10	-0.18 \pm 0.11
DARKNESS	0.08 \pm 0.05	0.02 \pm 0.02	0.07 \pm 0.04	0.05 \pm 0.02	6	0.32 \pm 0.27
GA	0.05 \pm 0.03	0.05 \pm 0.04	0.00 \pm 0.00	0.03 \pm 0.01	34	-0.53 \pm 0.21
CONTROL	0.00 \pm 0.00	0.00 \pm 0.00	0.03 \pm 0.02	0.01 \pm 0.01	38	0.59 \pm 0.03

ASH Ash addition, STR_CONTROL stratification, STR_GA stratification with GA₃, HIGHTEMP preheating at 110 °C with stratification, LOWTEMP preheating at 70 °C with stratification, DARKNESS no light, GA GA₃ addition, CONTROL untreated seeds

Maximum seed germination was obtained with ASH, although STR_CONTROL alone gave a considerable enhancement in germination rate. However, differences were significant between these treatments ($\chi^2 = 11.64$; $p = 0.0006$). STR_CONTROL had significantly a higher germination rate than the CONTROL ($\chi^2 = 29.14$; $p < 0.0001$).

No significant differences were observed between the two heat treatments ($\chi^2 = 0.04$; $p = 0.837$). LOWTEMP had significantly lower germination than the STR_CONTROL ($\chi^2 = 4.774$; $p = 0.028$), as did HIGHTEMP ($\chi^2 = 3.099$; $p = 0.078$).

Gibberellic Acid (GA₃) did not produce any stimulatory effect on germination compared to controls. Germination of seeds from the GA treatment was not significantly different to that of CONTROL seeds ($\chi^2 = 1.15$; $p = 0.284$). Nor was the germination of STR_GA seeds significantly different to STR_CONTROL ($\chi^2 = 0.7$; $p = 0.403$). DARKNESS also had a diminishing effect in germination compared to STR_CONTROL seeds, even if it had been stratified itself ($\chi^2 = 14.13$; $p = 0.0002$).

Finally, only 13 % of the viable seeds ($N = 1,047$) had germinated at the end of the experiment. Survival analysis revealed significant between-treatment differences in germination timing (Log-Rank, $\chi^2 = 29.59$; $p < 0.0001$; Wilcoxon, $\chi^2 = 26.41$; $p < 0.0004$), with CONTROL seeds taking the longest time to germinate and STR_GA and DARKNESS seeds taking the shortest (Table 1). Treatment within population significantly affected germination timing ($\chi^2 = 56.844$; $p < 0.001$), whereas population did not ($\chi^2 = 3.031$; $p = 0.2196$).

Discussion

The different levels of viability observed in the three populations may be a consequence of different levels of

mate availability among populations. A previous study reported that lowered mate availability decreased levels of seed viability due to high clonality in this self-incompatible species (Amat et al. 2013). The higher abundance, density and vitality of plants in Lóbrega population promoted by the fire occurred in 2006, may have increased genetic diversity, mate availability and, therefore, seed viability. However, this higher viability did not result in higher germination rates.

The present study gives evidence of the high dormancy found in seeds of *P. rivas-martinezii* as previously reported (Martínez Rodríguez et al. 2004), with a germination of just 1 % on completely untreated seeds (CONTROL, Table 1). A stratification treatment is required for seed germination, as reported for many other mountain species (Körner 2003). In high mountain Mediterranean species cold-wet stratification increased germination, although a relatively high number of these species seemed to be ready to germinate without any treatment (Giménez-Benavides et al. 2005).

Furthermore, the effect obtained by applying gibberellic acid (GA₃) was not significantly different to controls. Gibberellins applied to seeds have been proved very effective in breaking the dormancy of many species that otherwise would require other stimuli that increase gibberellin biosynthesis, such as cold stratification or exposure to light (Derkx et al. 1994). In particular, GA₃ has been found to be active in breaking dormancy in other closely related Antirrhinaceae species, such as *Chaenorhynchus minus* (Arnold et al. 1996) or *Linaria vulgaris* (Bielefeld 1987). However, since sensitivity to different gibberellins seems to be highly species-specific (Arnold et al. 1996), a positive response to a different gibberellin in *P. rivas-martinezii* could be expected.

Darkness lowered germination ability, even if seeds had been wet-cold stratified. This response is congruent with

the spatial pattern of the species in its natural habitat, where it preferably occupies clearings and shows diminished flowering by shrub closure (Amat et al. 2013). Although usually light filtrates through canopy (Saatkamp et al. 2011), the behaviour observed may increase the chance of seedling establishment in clearings or more open sites. It also indicates that seeds will not probably need to be buried for germination. Reduced or lack of germination in darkness is a common result observed in another Antirrhinaceae species, *Linaria tursica* (Valdés et al. 1996), amongst other Iberian endemics (Cabello et al. 1998). However, Valdés et al. (1996) reported an increase in germination of seeds on soil in contrast to filter paper under darkness conditions.

Interestingly, although preheating did not lower seed viability, seeds showed a significant decrease in germination following heat treatments. This result indicates that although the heat doses applied (70° and 110° for 5 min) were not lethal for *P. rivas-martinezii* seeds, germination was not stimulated either. Hence, seeds that survive fire may subsequently germinate under different stimuli. Species in fire-prone environments can show different germinating behaviours. Some may germinate readily without treatment; some may be stimulated by heat, others by charred wood or by other environmental factors, such as light or sowing medium (Keeley 1987).

Maximum seed germination was obtained by applying ash. Although the chemical compounds of ash were not identified, ash effects on *P. rivas-martinezii* germination are probably chemically mediated. Species that require the presence of charred wood to break dormancy are also likely to be dependent on the pH of the medium (Sanders et al. 1981; Keeley and Fotheringham 1998). However, *P. rivas-martinezii*, which grows in natural acidic soils, exhibited high germination rates on the alkaline medium of ash. Similarly, some *Cistus* species that grow in naturally acidic soils display high germination rates in both the acid medium smoke and the alkaline medium of charred wood. This behaviour suggests that chemicals other than pH seem to have an effect on the seed dormancy of these species (Pérez-Fernández and Rodríguez-Echevarría 2003). Charred wood stimulus is far more specific than heat at timing seedling establishment and some species are almost completely dependent on it; it determines the abundance of these species the first year after burning and in some cases even their disappearance until the next fire (Keeley and Keeley 1987).

Despite aboveground die-back, wildfires may have minimal impact on the survivorship of *P. rivas-martinezii* populations due to deeply buried stolons. Plants have been proven to resprout and flower abundantly after a fire occurred in 2006 in Lóbrega population, thus increasing seedling recruitment in postfire years (Bernardo García,

personal communication). This behaviour is widely extended in Mediterranean-climate regions, in which fire stimulates flowering of plants with deeply buried bulbs, corms or rhizomes (Horton and Kraebel 1995; Keeley and Bond 1997). Because soil seed banks may not build up between fires, on some sites resprouting is the predominant mode of reestablishment after fire. For instance, postfire recovery studies on Californian chaparral species have shown all species to be present the first growing season as vigorous resprouters, with seedlings being absent. However, the resprouts flowered and fruited abundantly, leaving abundant seedling recruitment in the second growing season (Keeley and Keeley 1984). This ability of some species to use both the sexual and vegetative methods to regenerate has been interpreted as an evolutionary response to fire (Trabaud 1984).

P. rivas-martinezii seeds seem to display intermediate physiological dormancy (Baskin and Baskin 2004), since seed coat is permeable, seeds required a period of cold stratification, and GA₃ did not increase germination. The response shown by this species suggests that around 20 % of the seeds will germinate readily upon wetting by winter rains if seeds remain near the soil surface, but another 15 % will remain dormant until exposed to ash. Most seeds will remain dormant, if buried below the level of light penetration or under dense canopy, since germination is inhibited in the dark. Similar results have been reported in other Californian chaparral and coastal sage species (Keeley 1987).

The increased viability and vigorous resprouting observed in Lóbrega population, together with the clear trigger effect of ash on germination of *P. rivas-martinezii*, indicates that we are dealing with a postfire species. Consequently, fire is not only a chemical stimulus for germination in this species, as results indicate that darkness inhibits germination. Thus, the reduction in competition provoked by fire would be another trigger to germination. These results suggest that fire has both direct and indirect effects on this species, since (1) it is a chemical stimulus for germination, (2) it creates clearings for seeds and (3) it enhances resprouting.

In conclusion, *P. rivas-martinezii* shows specificity to fire. The palynological record of the Gredos Range showed that before the twelfth century AD *Pinus sylvestris* forests were dominant, whereas the current landscape of the Gredos Range is clearly anthropogenic and includes a combination of forest patches, pastures and dense shrubby formations, mostly generated during the transition between the seventeenth and eighteenth centuries AD, when forest management activities, including fire and intense grazing, caused a progressive deforestation and the expansion of the current fire-prone shrubland (López-Merino et al. 2009). In this sense, despite being critically endangered the question

remains as to whether this plant is now favoured by human-mediated fire and underwent more critical stages in the past. Consequently, the conservation of this species may rely on the continuity of traditional land management practices.

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