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Seed germination of six Iberian endemic species – a contribution to enhance plant conservation

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ABSTRACT

Biodiversity has been degraded all around the world due to anthropogenic factors. To counteract this worldwide tendency, ecological restoration programs are expanding. In these programs, the use of native herbaceous species, particularly those endemic or threatened, can be promoted by seeding them but germination requirements for most of these species are unknown. Our research meant to fill this gap of information for six Iberian endemic species suitable to use in restoration actions - *Digitalis thapsi*, *Ferula communis* subsp. *catalaunica*, *Linaria amethystea* subsp. *amethystea*, *Pterocephalidium diandrum*, *Sanguisorba hybrida*, and *Silene scabriflora* subsp. *scabriflora*. Their seed germination behaviour was analysed to establish germination protocols for conservation actions. Three germination conditions were used to simulate different sown seasons. Seeds of all species were tested without pre-treatment and subjected to a cold stratification. Seeds of *S. hybrida* and *P. diandrum* were additionally tested with a scarification treatment. All species reached their better germination performance under autumn conditions (15/10 °C; 8/16 hours). In conclusion, for all the six Iberian endemic species tested, germination speed and percentage were suitable for their use in native plants reinforcement.

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Introduction

In the past century, biodiversity has declined all around the world due to anthropogenic factors, which are one of the major driving factors of biodiversity loss (Benítez-López et al. 2010). Ecological restoration can be used to counteract this degradation in a wide range of situations and environments and is becoming a key strategy to reduce biodiversity losses and guarantee the provision of ecosystem services (Lesica and Allendorf 1999; Bullock et al. 2011). An essential part of any restoration project is usually the reestablishment of native vegetation, since vegetation provides habitat for wildlife fauna (Santos et al. 2007) and is a requirement for ecosystem functioning (Hölzel et al. 2012).

Linear infrastructures, like roads, spread throughout a great variety of landscapes (Benítez-López et al. 2010) and should be considered when designing restoration programs, because their green marginal areas can be used to lessen their negative impacts on biodiversity (Damschen et al. 2006; Santos et al. 2007; Simões et al. 2013). Road corridors can be a crucial refuge for native plants (Zeng et al. 2010), namely in the Mediterranean region, where most of the traditional semi-natural grasslands were destroyed due to land-use changes (Blondel & Aronson 1999). The active promotion of native plants allows greater control of species composition and distribution than natural regeneration (Stanturf et al. 2014), guarantees that the restoration area is used also by native plants with conservation importance and, simultaneously, diminishes the possibility of exotic invasive species propagation.

Seeding patches with endemic or threatened species, capable of surviving and prospering in stressful environments, like road verges, promotes genetic diversity increase, and thus the population's ability to respond to future adaptive challenges, and is in accordance with the "International principles and standards for the practice of ecological restoration" (Gann et al. 2019). Furthermore, it is very important to use locally harvested seeds because it assures that the genetic diversity of local ecotypes is maximized (Lesica and Allendorf 1999; Dietrich et al. 2015; Toscano et al. 2018). However, in fragmented landscapes, it might be necessary to collect seeds from wider distances and multiple sources to obtain sufficient genetic diversity and, eventually, multiplying them in agricultural areas to obtain enough amount of propagules to achieve functional and resilient communities (Gann et al. 2019).

To accomplish ecological restoration, it is essential to select species suitable to the environmental conditions of the restoration area but also to know their germination behaviour, since germination capacity and seed viability are

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key factors in the success of restoration efforts, especially when stored seeds are used (Bhatt et al. 2019).

Nevertheless, this information is often unknown for native wild species, especially for rare and/or endemic species for which propagating material is more difficult to obtain (Navarro and Guitián 2003; Baskin and Baskin 2004; Cerabolini et al. 2004; Godefroid et al. 2010), and species of the highly diverse Mediterranean region are no exception (Toscano et al. 2018). This preliminary knowledge of seed germination requirements can be used to help to decide if a species could be used and how, and to establish a better formulation for commercial seed marketing (Toscano et al. 2018).

This study is aimed to determine the more suitable germination protocol for six herbaceous species that are endemic to the Iberian Peninsula - *Digitalis thapsi* L., *Ferula communis* subsp. *catalaunica* (Pau) Sánchez-Cux. & M.Bernal, *Linaria amethystea* subsp. *amethystea* Hoffmanns. & Link, *Pterocephalidium diandrum* (Lag.) G.López, *Sanguisorba hybrida* (L.) Nordb., and *Silene scabriflora* subsp. *scabriflora* Brot. These species occur naturally in the landscapes cut by roads, but they are increasingly less frequent and abundant mainly due to land use changes. Their conservation interest, and the fact that they proved to be suitable to use along linear infrastructures, makes them good candidates to use in restoration programs aimed at the promotion of native vegetation in these green corridors.

Materials and methods

Seed collection

For this study, six herbaceous species endemic to the Iberian Peninsula were selected (Table 1). According to Castroviejo (1986-2012), these species respect the following criteria: they have the ability to survive in semi-arid and/or disturbed environments, an extended flowering period (approximately three months), and are therophytes or hemicryptophytes, which is in accordance with some of the species selection criteria to revegetation projects suggested by Hitchmough (2004), Karim and Mallik (2008), and Bretzel et al. (2012). The nomenclature and characterization of the species followed IPNI (2020) and Flora Iberica (Castroviejo 1986-2012), respectively.

Seeds from the six species under analysis were collected from plants growing in *montado* areas at the end of their growing season, during springs or summers of 2016-2018, according to the species. Less than 20% of the seeds available were collected at each site to avoid major damage to the donor populations and to assure natural regeneration (Royal Botanic Gardens Kew 2016). For each species, the same seed lot - i.e., seeds belonging to the same batch of seeds, with a single reference number, origin, and history as is defined by Tazi et al. (2018) - was used for all the experience, except for *P. diandrum* in which two seed lots were used due to the reduced number of seeds available.

All species were collected in central Alentejo (Portugal) and the geographical coordinates were recorded using GPS technology (Garmin Montana 680t). The seeds were then cleaned, dehumidified to 15% RH and stored in glass containers, and maintained in the dark at room temperature until germination tests were performed in August 2018. The eventual deleterious effect of storage was assessed by viability tests (Table 2). Visibly damaged seeds were excluded from the experiments. Seed weight was determined according to ISTA rules for seed testing (ISTA 2015) by extrapolation of the average weight of ten 100-seed replicates, chosen randomly. Seed length and width were also measured with DinoCapture 2.0, the associated software of the digital microscope ™Dino-Lite.

Germination tests

Seeds were germinated in plastic Petri dishes (9 cm diameter) with agar (1%; VWR Chemicals) in a germination chamber (FITOCLIMA S600PLH - Aralab 1680; Portugal). The counting of germinated seeds was carried out daily over a period of 21 days, and germinated seeds were removed from the Petri dishes. Germination is considered effective when the radicle (or other embryonic tissue) emerges from the tissues (Bewley et al. 2013); however, effective germination was only considered when a radicle with at least half of the seed size emerged. Non-germinated seeds were cut in half to examine them, with the aid of a magnifying glass when required, and those with a well-developed, white, and firm embryo were considered viable as recommended by Gosling et al. (2003). For each species, 100 seeds were assigned to each germination condition, distributed into four 25-seeds replicates. Petri dishes were re-randomized daily to ensure no systematic effects due to position within the chamber (Yang et al. 1999; Cerabolini et al. 2004).

All species were subjected to cold stratification (17 days at 4 °C), to investigate whether a period of cold was required to break dormancy, and two species (*S. hybrida* and *P. diandrum*) were additionally tested with a scarification treatment (a small cut of the seed coat with a scalpel, without damaging the embryo) due to their seed morphology (Castroviejo 1986-2012). Seeds without treatment, that is, seeds that were neither cold stratified nor scarified, were used as controls.

For most of the tested species, the germination requirements are unknown and the information for taxonomically related species was not consistent (Royal Botanic Gardens Kew 2016; ENSCONET 2017). Therefore, three germination conditions were tested to determine the influence of the main environmental factors (temperature and light period) for all species and for each treatment (without treatment, cold stratification, and scarification).

In the first case, seeds were incubated with a photoperiod of 8 hours of light and 16 hours of darkness with an alternating temperature of 15/10 °C, to simulate autumn conditions. In the second case, winter conditions were simulated, and seeds were incubated at alternating temperatures during day and night, set respectively at 15/5 °C, with a photoperiod of 8 hours of light and 16 hours of darkness. Lastly, in the third case, spring environmental conditions were simulated, and seeds were incubated at 20/10 °C, with a photoperiod of 12 hours of light and 12 hours of darkness. All light conditions were obtained using cool white fluorescent lamps

Table 1. Life form, coordinates of the sites of germplasm collection, and seed dimensions of the species collected for the study. The acronyms used mean, respectively: Apia – Apiaceae, Capri - Caprifoliaceae, Cary – Caryophyllaceae, Hemi – Hemicryptophyte, Plan – Plantaginaceae, Rosa – Rosaceae, and Ther – Therophyte. Dimensions do not include floral appendices.

Species	Botanic Family	Life Form	1,000 Seed Weigh (g)	Seed length and width (mm ± SE)	Geographical Coordinates	Year of collection
Digitalis thapsi	Plan	Hemi	0.07	0.74 ± 0.03; 0.48 ± 0.02	38°32′3.94"N 7°59′59.24"W	2016
Ferula communis subsp. *catalaunica*	Apia	Hemi	12.00	11.07 ± 0.37; 5.45 ± 0.27	38°33′29.11"N 8° 9′43.20"W	2017
Linaria amethystea subsp. *amethystea*	Plan	Ther	0.07	1.42 ± 0.02; 1.24 ± 0.02	38°42′6.36"N 7°29′37.44"W	2017
Pterocephalidium diandrum	Capri	Ther	1.31	3.53 ± 0.09; 0.64 ± 0.03	38°31′37.04"N 8° 1′5.51"W	2017
Pterocephalidium diandrum	Capri	Ther	1.72	3.69 ± 0.12; 0.62 ± 0.02	38°31′37.04"N 8° 1′5.51"W	2018
Sanguisorba hybrida	Rosa	Hemi	1.26	2.24 ± 0.06; 0.96 ± 0.04	38°31′37.62"N 8° 2′7.54"W	2016
Silene scabriflora subsp. *scabriflora*	Cary	Hemi/ Ther	0.16	0.84 ± 0.03; 0.66 ± 0.02	38°32′48.52"N 7°56′6.34"W	2016

Table 2. Viability percentage (mean ± 95% confidence interval), viability percentage range and indication of dormancy for each lot of seeds used.

Species (lot of seeds)	Viability percentage (mean ± 95% CI)	Viability percentage range	Indication of dormancy
Digitalis thapsi	98.00 ± 0.95	84-100	0.39
Ferula communis subsp. *catalaunica*	40.50 ± 4.41	16-60	0.25
Linaria amethystea subsp. *amethystea*	94.33 ± 2.36	88-100	0.69
Sanguisorba hybrida	87.83 ± 4.39	76-100	0.81
Silene scabriflora subsp. *scabriflora*	98.50 ± 1.04	92-100	0.18
Pterocephalidium diandrum (2017)	95.00 ± 2.87	88-100	0.59
Pterocephalidium diandrum (2018)	64.00 ± 5.01	48-84	0.59

(80 µmol m^{-2} s^{-1}; OSRAM 18 W/21-840, Portugal). For *P. diandrum*, the 2017 lot was used for winter conditions, whilst the 2018 lot was used for the other two conditions.

At the end of the experiments, the following parameters were calculated:

1. Viability percentage (%) = (V x 100)/NT, where V is the number of viable seeds (germinated seeds plus viable seeds that did not germinate) and NT is the total number of seeds. The viability percentage was calculated for each lot except for the seeds used in the scarification pre-treatment, because when they were scarified most of the empty seeds were identified and eliminated of the trial.
2. Germination percentage (%) = (N × 100)/(NT-E), where N is the number of germinated seeds, NT is the number of total seeds and E is the number of empty seeds.
3. Mean germination time (MGT) = $\sum(n_i \times t_i)/\sum n_i$, where n_i is the number of seeds germinated on day i, and t_i is the number of days from the beginning of the germination test until the day i. MGT is a measure of the rate and time-spread of germination (Bewley et al. 2013).
4. Indication of dormancy = 1 - (seed germinated %/viability%). The calculation was made for each lot only with the results of untreated seeds and the seed germinated % was calculated including the empty seeds. The higher the fraction obtained, the greater the probability of the seed is dormant at that time, and an index ≥0.4 was used as the threshold value to indicate dormancy (Offord et al. 2004).

Data analysis

Germination percentages and mean germination time of each species were modelled by fitting Generalized Linear Mixed Models (GLMM) with a logit link function and binomial error structure. Pre-treatment (without treatment, cold stratification, scarification) and germination conditions (15/10 °C – 8/16 h light, 15/5 °C – 8/16 h light, 20/10 °C – 12/12 h light) were considered as fixed factors, in order to analyse their effects and interaction on seed germination. Experimental blocks were included as a random factor, therefore accounting for pseudo-replication and seed lots variability. Models were performed taking into account the possible overdispersion of data and the significance level was set at 0.05. All statistical analyses were performed using IBM SPSS Statistics 25.

Results

Preliminary seed size and weight measurements (Table 1) guaranteed the homogeneity of the lots - seed size binomial confidence interval were low and weight coefficients of variation were in accordance with ISTA (2015) recommendations.

Overall, seeds viability (Table 2) was high with a mean percentage above 85%, except in the case of *F. communis* lot, which had viability mean percentage of 41%, and the *P. diandrum* lot of 2018, which had a mean viability percentage of 64%.

Two of the six species tested showed a low indication of dormancy, between 0.18 and 0.25 (*S. scabriflora* and *F. communis*, respectively), and the other two species, *L.*

amethystea, and *S. hybrida*, had an indication of dormancy considerably high, between 0.69 and 0.81, respectively (Table 2).

Three species (*D. thapsi*, *S. scabriflora* and *P. diandrum*) had, at least in one of the tested conditions, considerably higher final germination percentages (over 85%), and two species (*F. communis* and *L. amethystea*) reached final germination percentages between 50% and 60%. The remaining species, *S. hybrida*, had a germination percentage of less than 40% in all analysed conditions. Considering the whole trial, all species reached their maximum germination percentage under a photoperiod of 8 hours of light and 16 hours of darkness with an alternating temperature of 15/10 °C (corresponding to autumn conditions), with a corresponding MGT of less than 15 days (except *F. communis*, which had a MGT of 16 at its maximum germination percentage).

For most species, both germination percentage and MGT were mostly affected by the factor germination condition ($p \leq 0.05$). However, the germination percentage of *P. diandrum* and MGT of *S. hybrida* were affected by both factors (germination condition and pre-treatment; $p \leq 0.05$). *Silene scabriflora* was not affected by any of the tested factors. The effect of the experimental blocks was not significant in any of the GLMM, i.e., did not influence the effects of the treatment and condition on seed germination (percentage and MGT).

When comparing seeds without pre-treatment between the three different germination conditions, it could be observed that most species (*D. thapsi*, *L. amethystea*, *S. hybrida* and *P. diandrum*) had higher germination percentages and lower MGT with autumn conditions ($p \leq 0.05$) (Figure 1). Regarding germination percentage, the remaining species (*F. communis* and *S. scabriflora*) did not show significant differences between germination conditions, however *F. communis* germinated faster with either autumn or spring conditions. Seeds without pre-treatment of *L. amethystea* and *P. diandrum* had the lower germination percentage ($6 - 16\%$) in the spring condition, while *D. thapsi* and *S. hybrida* had the lower germination percentage ($8 - 23\%$) in the winter condition.

For seeds subjected to cold stratification we observed that the species *D. thapsi*, *F. communis*, *L. amethystea*, and *P. diandrum* had a better performance (higher germination percentages and lower MGT) with autumn conditions ($p \leq 0.05$). Germination percentages of *S. hybrida* in autumn and spring conditions were not statistically different, but germination was slightly faster in spring conditions ($p < 0.05$). *Silene scabriflora* did not show a significant preference for any of the conditions tested.

The species *S. hybrida* and *P. diandrum* were also tested with scarified seeds. In the case of *S. hybrida*, those seeds showed better germination percentage under autumn or spring conditions ($p \leq 0.05$) while the MGT was similar between all the conditions tested. The scarified seeds of *P. diandrum* germinated more with autumn conditions ($p \leq 0.05$), but the MGT was faster with either autumn or spring conditions. When considering autumn and spring conditions, scarified seeds of *P. diandrum* had a significantly higher germination percentage than seeds without pre-treatment or subjected to cold stratification ($p \leq 0.05$).

Most species had a similar germination percentage in all pre-treatments tested, within each germination condition (Figure 1). The exceptions were *P. diandrum* that had a better germination percentage when subjected to scarification under autumn and spring conditions ($p \leq 0.05$), and *S. hybrida*, that had a higher germination percentage under spring conditions either with cold stratification or with scarification ($p \leq 0.05$).

Regarding MGT, the species *D. thapsi*, *L. amethystea,* and *S. scabriflora* did not show significant differences between the pre-treatments tested within each germination conditions. Under spring conditions, *F. communis* (MGT of 19 days instead of 15 days) and *P. diandrum* (MGT of 14 days instead of 11 days) germinated slower with cold stratification when compared with the remaining pre-treatments. *Sanguisorba hybrida* seeds germinated faster with scarification or without treatment under autumn conditions and with scarification under spring conditions ($p \leq 0.05$).

Discussion

For most species, the viability of the tested lots was high (above 85%), which assures the seeds quality and their usability for future restoration actions (Farley et al. 2013; Bellairs and Caswell 2016). Although the viability of seeds can vary considerably among species and seed lots of the same species (Bellairs and Caswell 2016), the viability percentages obtained for the tested species (with exception of *F. communis* and 2018 *P. diandrum* lot) were similar to the seeds' viability of taxonomically related species (between 90-100%) recorded in other studies (Royal Botanic Gardens Kew 2016).

Determination of seeds' viability before their application in field actions can improve the results of restoration programs by allowing the rejection of a seed lot whose seeds have low viability. In case of lots with an average seeds' viability, like *F. communis* lot or 2018 *P. diandrum* lot, the seeds can still be used in field actions, but the sowing density should take that into account and thus be higher than usual. The viability was not the same between the two lots of *P. diandrum* (2017 lot − 95%; 2018 lot − 64%), probably due to environmental factors since the lots were harvested from the same population and the storage time could not be used to explain the difference between the viability of the seeds, as the oldest lot had the highest viability.

The majority of species tested (except *L. amethystea* and *S. hybrida*) did not show dormancy or had a low indication of dormancy (≥ 0.4, in accordance with Offord et al. 2004), and half of them had a germination percentage above 85% in at least one of the trial conditions. That makes those species good candidates to use in restoration actions, since no pre-treatment appears to be necessary to guarantee seeds' germination (Baskin and Baskin 2004; Grossnickle and Ivetić 2017). *Ferula communis* presented a low dormancy index, nevertheless, its final germination percentage was medium, even in germination conditions with a higher probability of

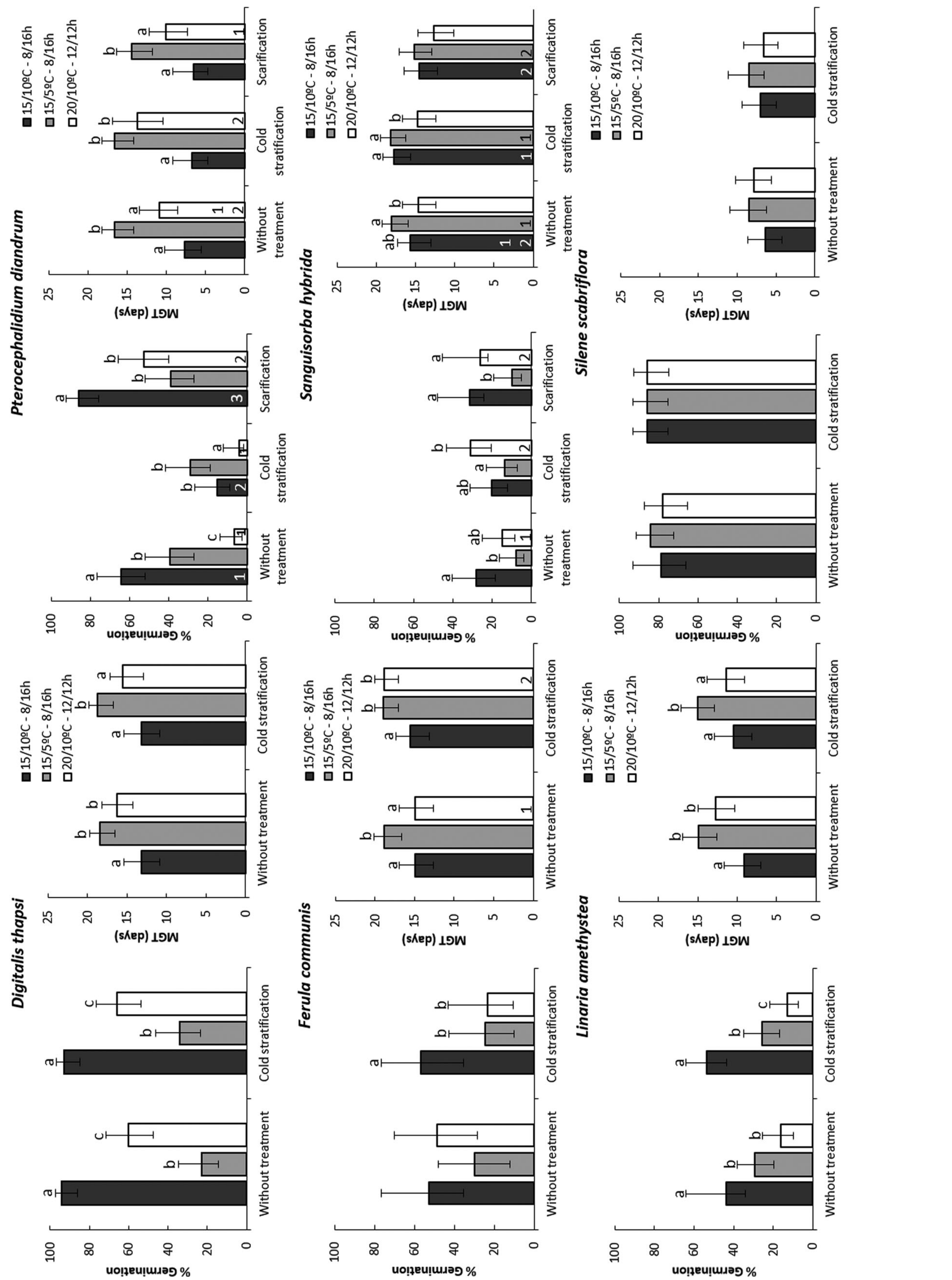

Figure 1. Germination percentage and mean germination time (MGT), and 95% binomial confidence intervals, for each germination condition (15/10 °C – 8/16 h: autumn conditions; 15/5 °C – 8/16 h: winter conditions; 20/10 °C – 12/12 h: spring conditions), distributed by pre-treatment tested: without treatment, with cold stratification or with scarification. Different letters mean significant differences between germination conditions within the same pre-treatment with a $p \leq 0.05$. Different numbers mean significant differences between pre-treatment within the same germination conditions with a $p \leq 0.05$. When no letters, regarding germination conditions, or no numbers, regarding pre-treatments, are added it means that there are no differences between the compared parameters.

success (autumn conditions). This is most probably due to the conditions of the lot used, which had a viability percentage of only 40%.

As expected, species reached their better germination performance (maximum germination percentage and lower MGT), regardless of the pre-treatment used, under autumn conditions (photoperiod of 8 hours of light and 16 hours of darkness with an alternating temperature of 15/10 °C), which is in accordance with the optimum germination season of most Mediterranean species, i.e., late autumn or beginning of winter season (Bell et al. 1993; Copete et al. 2009).

Regarding germination percentage, seeds without treatment of *F. communis* and *S. scabriflora* did not show a preference between germination conditions, and this may allow a wider time range for sowing, providing water is available.

The seeds without pre-treatment of *L. amethystea* and *P. diandrum* had low germination percentage (6 – 16%) in the spring condition, and this seems to indicate a mechanism to prevent germination in response to occasional summer rainfalls, when the probability of seedling survival in dry-summer Mediterranean ecosystems is low (Copete et al. 2009).

The application of a period of cold (in this case 17 days at 4 °C) did not significantly improve the germination performance of any species at any germination condition. The exception was *S. hybrida* whose germination doubled under spring conditions with cold stratification when compared to seeds without treatment, suggesting that the seeds that germinate later in the year may benefit from the action of winter cold, simulated here by the subjection to a cold period (Baskin and Baskin 2004). Some of our results are in accordance with Navarro and Guitián (2003) who applied a similar pre-treatment (5 days at 4-5 °C) and did not have any effect on the final germination percentage of two Iberian endemic species of the Caryophyllaceae family (*Petrocoptis grandiflora* Rothm., and *Petrocoptis viscosa* Rothm.).

Scarified seeds of *S. hybrida* and *P. diandrum* were also tested and it was verified that the utilization of this pre-treatment did not alter the preference for germination under autumn conditions. Under autumn conditions, scarification of *S. hybrida* seeds did not improve germination. *Pterocephalidium diandrum* scarified seeds (86% of germination), however, germinated significantly better, but seeds without treatment (65% of germination) also had high final germination percentages. Therefore, scarifying seeds of *P. diandrum* improves the germination but is not essential and its use in the context of habitat conservation must take into account the practicality of this technique. As such, and since scarification can damage the embryo and make the seeds more susceptible to diseases or dehydration (Farley et al. 2013), we do not recommend it, except for very specific situations. For instance, seeds of *P. diandrum* and *S. hybrida,* germinated better under spring conditions, if they had been subjected to scarification. This means that *P. diandrum* and *S. hybrida* may be seeded until early spring providing water is available, and seeds are subjected to scarification.

In this study, experiments were conducted to test only the best conditions of photoperiod and temperature for seeds dispersed by natural processes. The reason for this is to find the best germination protocols mimicking natural conditions, for these would be less expensive to apply in restoration programs. Germination in dark conditions is strongly associated with open, disturbed and dry habitats (Carta et al. 2017), but was not considered because most of the seeds used are small and thus more likely to require light for germination (Pons 2000). Nevertheless, taking into account the germination results obtained, testing in the dark may contribute to increase final germination percentage of some species (namely *L. amethystea* and *S. hybrida*), and thus it should be considered in future studies.

Although the germination behaviour in the laboratory can be different from what actually happens in nature, laboratory germination results can give useful clues to promote species *in-situ*, if the species ecology and most common environmental conditions are considered (Toscano et al. 2018). Since herbaceous plants are commonly established by direct seeding, identifying the optimal sowing periods and the possible requirement for pre-treatments helps to ensure the successful establishment of wildflower communities (Hitchmough 2016). So, to define germination requirements for these endemic species is the first step for successful environmental restoration actions and can point out the best strategy for promoting them.

The six species tested seem suitable to be used in restoration programs, namely for seeding native herbaceous nuclei on marginal habitats like road verges, since they have high germination percentages and fast germination, without the application of any pre-treatment (Jinks et al. 2006; Toscano et al. 2018). Of all species tested, *S. hybrida* was the less suitable for use in restoration actions, since it had a poor germination, with 28% of germination at the more favourable conditions, and a high dormancy. Nevertheless, due to its conservation importance, more germination conditions should be tested, namely in the absence of light.

Another factor that favours the use of these species in restoration programs is that most of them reach high germination percentages in a relatively short time (MGT of 6-15 days) when autumn conditions were used, and this decreases the seeds' susceptibility to predation. Seeds' predation is one of the biggest problems of seeding, and thus ensuring that the time of inactivity of the sown seeds is minimum is an important step to achieve seeding success (Grossnickle and Ivetić 2017) and therefore this criterion should be included in the selection procedure of the species. Additionally, in Mediterranean areas, autumn is the adequate time of the year for sowing most of the species, since that is when the first rains occur, providing availability of water to trigger germination (Bell et al. 1993; Copete et al. 2009).

In conclusion, the six endemic species tested fulfilled the requirements to be used as ecological restoration in the south of the Iberian Peninsula. These species do not need pre-treatments to break dormancy, their germination percentages are medium to high and germination occurs in a short time if seeded in autumn.

Finally, more knowledge about the germination requirements of *S. hybrida* is still needed and studies to achieve baseline data on *in-situ* plant germination and development are henceforth required for all the species.

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