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NOTE

Gentiana lutea L. subsp. *lutea* seed germination: natural versus controlled conditions

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Abstract: *Gentiana lutea* L. subsp. *lutea* is a geophyte that occurs in the central-southern European mountains. We investigated its germination ecophysiology with the main aim of evaluating seed germination behaviour under controlled and natural conditions. Seeds from four natural sites were tested for germination requirements under a range of different temperatures, and by comparing the results with those obtained under both cold stratification at 5 °C and treatment with GA_3. We simultaneously carried out germination tests at each collecting site, and soil temperatures were recorded. Our results indicate that germination under controlled conditions is limited by high temperatures (25 °C), below which GA_3 has a remarkable effect on germination percentages. Cold stratification did not have an influence on the germination response, thus suggesting that a temperature of 5 °C is not sufficient to break seed dormancy; this result was supported by soil temperatures. *Gentiana lutea* created a short-term persistent seed bank, and seeds mainly germinated in their first year. The germination under controlled conditions was similar in all accessions, while different responses were found in the field, where a potential influence of the aspect was observed. These findings underscore the importance of understanding germination behaviour in the field to define the real thermal requirements of a taxon.

Key words: Gentianaceae, cold stratification, field germination, gibberellins, mountain species.

Résumé : *Gentiana lutea* L. subsp. *lutea* est un géophyte qui pousse dans les montagnes du Centre-Sud de l'Europe. Les auteurs ont examiné l'écophysiologie de sa germination dans le but principal d'évaluer le comportement de la germination des semences dans des conditions contrôlées et naturelles. Les semences de quatre sites naturels ont été testées relativement à leurs besoins germinatifs à différentes températures et en comparant les résultats à ceux obtenus avec une stratification à froid à 5 °C et un traitement au GA_3. En même temps, des tests de germination ont été réalisés sur chaque site de récolte et les températures du sol ont été enregistrées. Les résultats des auteurs indiquent que la germination en conditions contrôlées est limitée par des températures élevées (25 °C), en dessous desquelles le GA_3 exerce un effet important sur les pourcentages de germination. La stratification à froid n'avait pas d'influence sur la réponse germinative, suggérant alors qu'une température de 5 °C n'est pas suffisante pour briser la dormance de la semence ; ce résultat était corroboré par les températures du sol. *Gentiana lutea* s'est créé une banque de semences persistante à court terme et les semences ont principalement germé au cours de la première année. La germination dans des conditions contrôlées était similaire chez toutes les accessions, alors que différentes réponses ont été trouvées sur le terrain, où une influence potentielle de l'orientation a été observée. Ces observations soulignent l'importance de comprendre le comportement germinatif sur le terrain afin de définir les conditions thermiques nécessaires réelles d'un taxon. [Traduit par la Rédaction]

Mots-clés : Gentianaceae, stratification à froid, germination sur le terrain, gibbérellines, espèces montagneuses.

Introduction

The broad objective of a seed germination ecologist is to explain how the timing of germination is controlled in nature, as well as which are the ecological and evolutionary origins and consequences of this timing (Baskin and Baskin 2014). Determining what controls the timing of seed germination in the field requires information on the seed environmental conditions in their habitat and how they interact from the time of seed maturation to germination (Baskin and Baskin 2014). This knowledge is

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also important because it contributes to a better understanding of certain biological concepts such as plant reproductive strategies, life history traits, adaptation to habitats, and physiological processes (Baskin and Baskin 2014). In the study of seed germination ecology, other kinds of questions come to mind. For example, how do the climate conditions under which seeds develop influence the requirements for dormancy breaking and germination?

In seasonal climates (as in our study case) temperature is usually the main environmental factor controlling seed germination in moist soils (Fenner and Thompson 2005). Plants in environments that are exposed to cold field temperatures have been found to be more likely to possess some form of seed dormancy than species living in milder environments (Jurado and Flores 2005). Seed dormancy prevents precocious seedling emergence and damage during winter conditions (Körner 2003). Seed dormancy is naturally broken through exposure to low-temperature conditions characterised by a snow cover period and, experimentally, through cold stratification methods (Baskin and Baskin 2014).

Gentiana lutea L. grows in the mountains of Central and Southern Europe, at altitudes ranging from 800 to 2500 m a.s.l., which suggests that its seeds may have a cold stratification requirement for germination (Favarger 1953). Controversial information on the type of dormancy is reported for *G. lutea* seeds; for example, Nikolaeva et al. (1985) described a nondeep complex morphophysiological dormancy (MPD), whereas Pérez-García et al. (2012) showed that seeds of this species exhibit nondeep PD. For this reason, understanding the factors controlling seed germination and comparing seed responses in nature versus under laboratory conditions is particularly important for resolving the information incoherence.

Gibberellins (GAs) play an important role in a number of physiological processes related to plant development (Finch-Savage and Leubner-Metzger 2006). In particular, gibberellic acid (GA_3) can enhance dormancy release, embryo growth, increase germination rate, and promote seed germination under a wide range of temperatures (Mattana et al. 2012). In some cases, GA_3 can replace the pretreatment and temperature requirements for germination and embryo growth (Baskin and Baskin 2014; Rhie et al. 2015), and therefore could indirectly allow us to test the viability of seeds in an alternative way.

As regards germination in natural sites, seeds of some temperate Gentianaceae may retain their viability for at least three years in soil seed banks and may germinate in spring or early summer (Thompson et al. 1997). However, seeds of several herbaceous *Gentiana* species from the Alps were classified as having transient soil seed banks (Cerabolini et al. 2003). The experimental examination of the time when germination occurs in natural sites, the understanding of the behaviour of seeds in the soil, combined with the investigation of their germination under laboratory conditions are all crucial for an effective management of plant species (Hesse et al. 2007).

In this work, we examined the germination of *G. lutea* L. subsp. *lutea* by studying the effects of prechilling and GA_3 on dormancy breaking and seed germination under laboratory conditions, as well as by evaluating seed germination behaviour in natural growing sites. We were specifically interested in two questions: (*i*) are there any differences in the seed germination behaviour of *G. lutea* subsp. *lutea* seeds under controlled and natural conditions, and (*ii*) if differences are present, could the germination behaviour in the field be useful to identify the real thermal requirements (of a taxon) in controlled conditions?

Materials and methods

Study species

Gentiana lutea subsp. *lutea* (Gentianaceae; hereinafter *G. lutea*) is a perennial herbaceous plant over 1 m tall, with rhizomatous and branched roots. In summer the plant produces inflorescences up to 120 cm, with yellow flowers (3–4 cm) grouped in pseudowhorls with corolla divided into five open lobes (Gentili et al. 2013). This taxon has been included in the European Habitats Directive (92/43/EEC) Annex V, in the EU Wildlife Trade Regulation No.338/97 and in the EU Policy species (Allen et al. 2014).

Seed lot details

Mature fruits (capsules) of *G. lutea* containing well-developed ripe seeds were sampled in August 2013 from four representative natural growing sites (Table 1), two from the Gennargentu Massif, located in central-eastern Sardinia (Is Terre Molentes "IS" and Trainu Murcunieddu "TM") and two from the Natural Park Fuentes Carrionas in Northern Spain (Peña Santa Lucia "SL" and Peña Carazo "PC"). All of them are found at an altitudinal range of 1300–1500 m a.s.l., are exposed either to the northwest or to northeast and are characterized by siliceous metamorphic substrate (Table 1). Seeds were taken from at least 30 randomly selected plants in each growing site. The collected seeds showed a similar degree of ripeness, as observed from their colour and hardness. Seeds were manually cleaned, discarding any visually malformed seeds (empty, parasitized, or not completely formed), and then stored at room temperature (ca. 20 °C and 40% relative humidity) for ca. one week until they were used in the germination tests.

Germination tests under controlled conditions

For each accession, four replicates of 25 seeds were sown on the surface of 1% agar water in 90 mm diameter plastic Petri dishes and incubated in the light (12 h light : 12 h dark) under a range of constant temperatures (5, 10, 15, 20, and 25 °C) and under an alternating temperature regime (25–10 °C). In the alternating temperature re-

Table 1. Locations, collection data, and seed lot details for the Sardinian and Spanish sites of *Gentiana lutea*.

Locality (Region, State)	Locality code	Collection date	Altitudinal range (m a.s.l.)	Habitat	Coordinates (WGS84 datum)	Aspect	Number of individuals
Is Terre Molentes, (Sardinia, Italy)	IS	20/08/2013	1460–1505	Open grasslands[a]	40°02′43″N, 09°19′91″E	35° NE	>1000
Trainu Murcunieddu, (Sardinia, Italy)	TM	21/08/2013	1324–1372	Open grasslands[a]	40°03′29″N, 09°19′25″E	280° NW	>1000
Peña Santa Lucia, (Castilla y León, Spain)	SL	27/08/2013	1409–1429	Wet meadows, hay meadows and open woods[b]	42°54′38″N, 04°38′15″W	270° NW	100–500
Peña Carazo, Castilla y León, Spain)	PC	25/08/2013	1491–1526	Wet meadows, hay meadows and open woods[b]	42°57′34″N, 04°34′25″W	15° NE	100–500

Note: All substrates were Metamorphic–phyllite except for Peña Santa Lucia, which was Metamorphic–metaquartzite.
[a]Fois et al. 2015.
[b]Renobales 2012.

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gime, the 12 h light period coincided with the higher temperature period. The effect of cold stratification (CS) at 5 °C was tested for a period of three months, after which the seeds were incubated as detailed above. The effect of gibberellic acid (GA_3, 250 mg·L^{-1}) in the agar substrate was tested under the same range of germination temperatures. Seeds were scored three times a week and germination was defined as the emergence of a visible radicle (≥1 mm). All germination tests started in September 2013 (ca. one week after seed collection) and were conducted at the same time using the same environmental test chamber (Sanyo MLR-351) equipped with white fluorescent lamps (FL40SS.W/37 70-10 μmol·m^{-2}·s^{-1}). At the end of the germination tests (for a minimum of 90 days), when no additional germination had occurred for two weeks, a cut test was carried out to determine the firmness of remaining seeds and the firm seeds were considered viable (Porceddu et al. 2013).

Germination tests under natural conditions

Within two weeks from the date of collection, a total of 15 replicates of 35 seeds were placed in fine-mesh polyester envelopes and buried in the soil at a depth of ca. 3 cm in each of the four natural growing sites (Table 1). Three replicates were exhumed at intervals of about three months from September 2013 to June 2014 (with two intermediate spring exhumations in April and May 2014; for a total of five exhumations; Supplementary data, Table S1[1]). Retrieved envelopes were analysed in the laboratory, where they were washed under running water and opened. The number of germinated seeds was recorded, and a cut test was carried out to check the viability of any remaining nongerminated seeds. Seeds with fresh white endosperm and healthy embryos were considered viable. Soil temperatures at the level of the envelopes were recorded at 90 min intervals using data loggers (TidbiTw v2 Temp logger, Onset Computer Corporation, Cape Cod, Massachusetts, USA).

Data and statistical analyses

For each germination trial under controlled conditions, the final germination percentage (FGP) and the germination rate (T_{50}) were calculated. The FGPs were calculated as the mean of the four replicates ± SD on the basis of the total number of filled seeds. T_{50} was determined as the time in days required to reach 50% of germination (Bravo et al. 2014); when 50% germination was not reached the value was not calculated.

Generalized linear models (GLMs) were used to evaluate the effect of treatments and incubation temperature on the FGP and the T_{50}. Significant differences highlighted by GLM were then analysed by a post hoc pairwise comparison *t* test (with Bonferroni adjustment). A log link function and Poisson error structure was used for analysing the T_{50}, while a logit link function and quasibinomial error structure was used for analysing the FGP. GLM with a logit link function and quasibinomial error structure and *F* tests with an empirical scale parameter instead of chi-squared on the subsequent ANOVA were used to overcome residual over dispersion (Crawley 2007). All statistical analyses were carried out using R version 3.1.3 (R Core Team 2015).

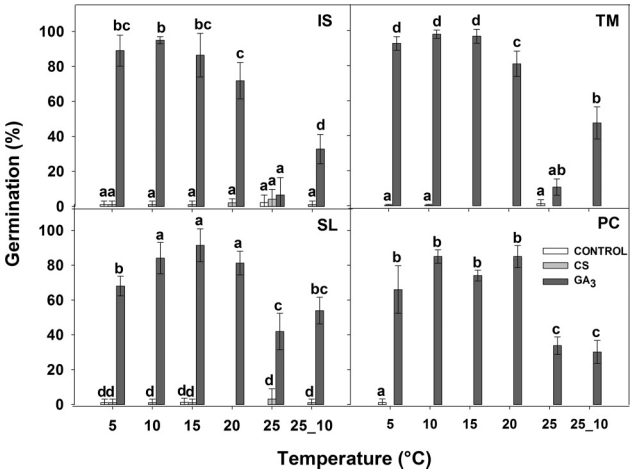
Results

Seed germination under controlled conditions

GLMs highlighted a statistically significant ($P < 0.001$) effect on FGP (dependent variable) for all three tested factors (Treatment; Temperature; Locality), as well as for their two-way and three-way interactions. The FGP of the control test was < 3% for all temperatures and accessions. Cold stratification did not significantly increase the FGP relative to control seeds ($P > 0.05$). Conversely, GA_3 treatment had a significant effect ($P < 0.001$) on the FGP (Fig. 1), with more than 50% seed germination occurring at a temperature range from 5 to 20 °C. The highest FGPs

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2016-0030.

Fig. 1. Final germination percentage achieved at the end of germination tests after each pre-treatment (Control; CS, 5 °C for three months; GA_3, 250 mg·L^{-1} of GA_3 in the germination substrate). Data are the mean of four replicates (±SD). Temperatures, treatments, and their interaction are statistically significant ($P < 0.001$) as determined using generalized linear models. Post hoc pairwise *t* test comparisons in each locality were carried out for each germination temperature, and bars with different letters indicate significant ($P < 0.05$) differences.



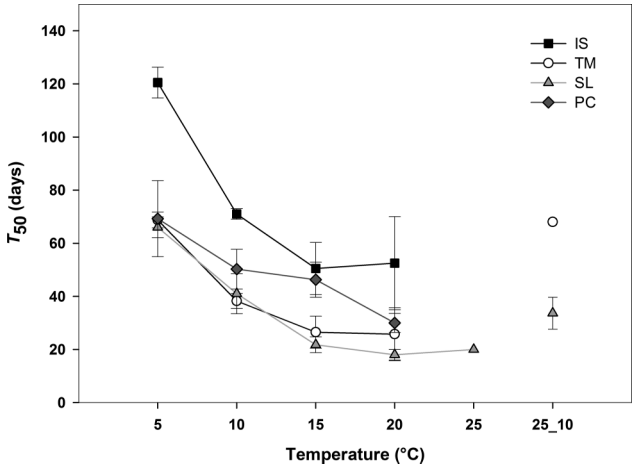
under this treatment were generally observed at 10 °C and 15 °C. A considerable reduction in the germination ability was found in all accessions at 25 °C and at 25–10 °C; almost all of these values were statistically different compared with those obtained at 5–20 °C ($P < 0.05$).

T_{50} values within the range of temperatures from 5 to 20 °C decreased with increasing incubation temperatures, showing the same pattern in both the Spanish and the Sardinian accessions (Fig. 2). More specifically, from 5 to 20 °C, they ranged from 120 to 52 days for IS, from 68 to 25 days for TM, from 66 to 18 days for SL, and from 69 to 30 days for PC (Fig. 2). T_{50} values at 25 and 25–10 °C could not be calculated because the 50% rate of germination was not reached; the only exceptions to this pattern were found in SL (which showed T_{50} values of 20 days at 25 °C and 34 days at 25–10 °C, respectively), and TM (68 days at 25/10 °C; Fig. 2).

Seed germination under natural conditions

Seeds of *G. lutea* buried in each experimental site (Table 1) were exposed to a warm autumnal period (lasting ca. 44 days for Sardinian and ca. 40 days for Spanish accessions), with a mean recorded soil temperature of ca. 7–9 °C (Fig. 3). In the Sardinian sites the maximum soil temperature recorded in this period was ca. 15 °C (4 October 2013), whereas the minimum was ca. 0 °C (15 December, 2013). At the Spanish sites, the mean soil temperature was ca. 7–8 °C with a maximum of ca. 13 °C in SL (23 September 2013), and 11 °C in PC (16 October, 2013) and a minimum near 0 °C in both SL (since 14 December, 2013) and PC (18 December 2013; Fig. 3). After the warm autumnal period, seeds experienced a cold

Fig. 2. T_{50} (days ± SD) for *Gentiana lutea* seeds treated with GA_3 (250 mg·L^{-1} in the germination substrate) collected in each locality [for site abbreviations, see the text].



period (i.e., mean daily soil temperatures ≤5 °C) with a duration varying from 128 (PC) to 152 days (SL) (Fig. 3). At the time of the first exhumation (December 2013) seeds had remained dormant in all experimental sites (Supplementary data, Table S1). At the time of the second exhumation (March 2014) the majority of seeds were still dormant but viable in three out of four experimental sites. Germination occurred from April to May 2014 in IS, SL, and PC (third exhumation), when the mean daily soil temperatures were ca. 9 °C in IS and SL, and ca. 7 °C in PC (Fig. 3; supplementary Table S1). At the time of the fourth exhumation (May 2014), the germination was ca. 88% for IS, 75% for TM, 61% for SL, and 25% for PC (Fig. 3; Supplementary data, Table S1). At the time of last exhumation (June 2014) the percentages of empty seeds were high in all localities (approx. 48%–90%), probably due to the death of the seedlings inside the polyester envelopes (Supplementary data, Table S1).

The maximum germination percentages obtained in the field were found in TM and SL. GLMs highlighted a statistically significant ($P < 0.001$) effect on field germination (dependent variable) for both Date and Locality (Loc) factors, as well as for their two-way interaction (Loc × Date; $P < 0.001$).

Discussion

Untreated seeds of *G. lutea* did not germinate owing to seed dormancy. Also, cold stratification at 5 °C did not affect seed germination in all the accessions. This is in accordance with Pérez-García et al. (2012), who found that moist chilling the seeds of this species did not enhance germination. Seed germination of all accessions was promoted by GA_3; in fact, high germination percentages were generally obtained at each tested temperature for all localities. However, a decrease in the germination ability was detected at high (i.e., 25 °C) and at alternate (25–10 °C) temperatures. According to the temperatures recorded by the data loggers in the growing sites, our

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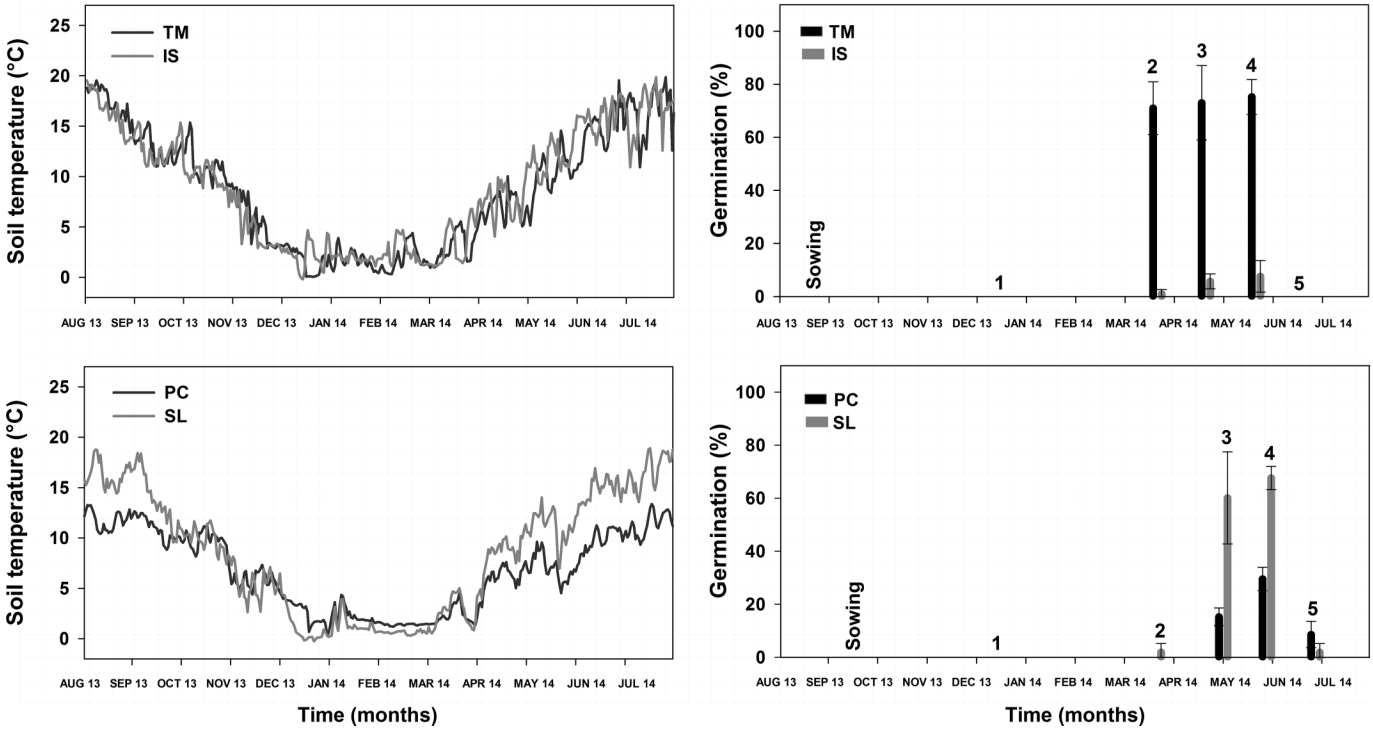
Fig. 3. Soil mean daily temperatures during the year (left) and germination in the field (right) for the Sardinian (TM; IS) and the Spanish (PC; SL) populations. Field germination data are the mean of three replicates of 35 seeds. 1, 2, 3, 4, and 5 correspond to the time that the seeds were exhumed.



experiments showed that the seeds did not experience a high temperature period, therefore suggesting that high temperatures represent a limit for the seed germination of this species, even in the presence of GA_3. In addition, *G. lutea* seeds from different sites were exposed to intermediate temperatures (~7 °C) post-seed-dispersal and to a cold period (~0–2 °C) before germination; we therefore suppose that 5 °C, the temperature that is most often used in this kind of research for prechilling treatments (Baskin and Baskin 2014), was probably not enough to break seed dormancy in this species, and that a temperature of about 0 °C might be more effective.

We found physiological (seed germination) variability among *G. lutea* accessions in natural conditions. Our study confirmed that *G. lutea* created a short-term persistent seed bank, and seeds mainly germinated in their first year, as was also reported by Hesse et al. (2007). Germination in the natural sites occurred during spring and after a natural cold stratification period, when the average soil temperatures were ca. 5–12 °C. This is in accordance with the results obtained in the laboratory, where the optimal FGPs were generally recorded at temperatures between 5 and 15 °C. We found a one-month delay in germination in Spanish localities with respect to the Sardinian ones. It is well known that temperature is the main environmental factor regulating seed germination (Fenner and Thompson 2005), therefore the delay observed in Spanish sites is very likely the result of the lower soil temperature experienced by seeds, which could have influenced the timing of dormancy release and the beginning of seed germination.

Seed germination percentages obtained in the field differed among localities. Specifically, higher germinations were found in TM and SL, both of them characterized by a northwestern orientation, while the other two growing sites (IS and PC) are characterized by a northeastern orientation. The number of individuals in each site was also excluded as a potential factor promoting seed germination, because one Sardinian locality had more than 1000 individuals, whereas the Spanish localities had a lower number of individuals. Scherrer and Körner (2011) showed that mountain topography can cause temperature differences over very short horizontal distances, and this could also be the case for the sites in our study. The only dissimilarity observed in our experimental sites that might explain the difference in germination behaviour, and therefore the field germination response, is aspect. Interestingly, the potential of small-scale topographic complexity to drive microclimatic variation (Opedal et al. 2015) could cause the different intraspecific germination responses. In this framework, there is still relatively little information about the extent of intraspecific trait variation at small scales across sites differing in topographic complexity (Albert et al. 2010; Boucher et al. 2013). More studies are needed to gain further insights on the role that this environmental factor plays in the intra-specific variability of *G. lutea*.

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Our research goal was the evaluation of any differences in seed germination behaviour under controlled and natural conditions. Seed germination under controlled conditions was similar for all accessions, whereas we found different responses in the field. Intraspecific variation in seed germination may depend on local weather during the growth of the mother plant, soil quality, or other naturally occurring factors (Karlsson and Milberg, 2008). Seeds from nearby collecting sites (linear distance between them <10 km) and similar habitats characteristics (both in Sardinian and Spanish localities; see Table 1) did not show the same pattern as the natural sites. These results therefore suggest that the differences in FGP were probably influenced by the ecological conditions characterizing each specific site, such as microclimate, soil humidity, and (or) snow cover periods (Frattaroli et al. 2013). The differences in the germination response among natural sites could be interpreted as an efficient survival strategy for species growing under unpredictable environments (Giménez-Benavides et al. 2005). These results are relevant when planning territory management actions and when evaluating prediction models associated with germination responses as a results of global warming (e.g., Porceddu et al. 2013; Fernández Pascual et al. 2015). Although this approach is often very expensive and time-consuming, the study of seed germination behaviour in the field and its correlation with the annual trend of soil temperatures should be taken into account when planning experimental tests in controlled conditions; a multi-approach field/laboratory study could be a key element for identifying the real thermal requirements of a taxon.

Conclusions

These results are consistent with several previous studies investigating the relevance of the differences in the behaviour of seeds belonging to different accessions of the same taxon. The importance of such variability might be particularly high for those plant taxa that have a relatively wide distribution range but that at the same time have a high conservation value, such as *G. lutea*. Our experiments highlighted the existence of a considerable degree of physiological difference among seeds belonging to different growing sites. These differences must definitely be taken into account when planning ex situ conservation actions on this taxon and emphasize the importance of collecting and preserving seeds from multiple origins to maximize the genetic diversity of seed collections stored in germplasm repositories. Information on seed germination potentially has great monetary value. A knowledge of what controls the timing of germination enhances the planning for the effective control, propagation of threatened and (or) economically important plant taxa.

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