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Discovering the type of seed dormancy and temperature requirements for seed germination of *Gentiana lutea* L. subsp. *lutea* (Gentianaceae)

Alba Cuena-Lombraña[1,2], *Marco Porceddu*[1,2,*], *Caterina Angela Dettori*[1] *and Gianluigi Bacchetta*[1,2]

[1] *Centro Conservazione Biodiversità, Dipartimento di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Viale S. Ignazio da Laconi, 11–13, Cagliari 09123, Italy*
[2] *Banca del Germoplasma della Sardegna (BG-SAR), Hortus Botanicus Karalitanus (HBK), Università degli Studi di Cagliari. Viale Sant'Ignazio da Laconi, 9–11, Cagliari 09123, Italy*
*Correspondence address. Centro Conservazione Biodiversità, Dipartimento di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Viale S. Ignazio da Laconi, 11–13, Cagliari 09123, Italy. Tel: +39-0706753806; Fax: +39-0706753509; E-mail: porceddu.marco@unica.it

Abstract

Aims
There are a number of mechanisms that regulate germination; among these, seed dormancy, one of the most important, is an adaptative mechanism in plants to promote survival by dispersing germination in space and time until environmental conditions are favourable for germination. The main goals of this study were to determine the temperature requirements for seed dormancy release and germination of *Gentiana lutea* subsp. *lutea*, to identify the class and level of seed dormancy and to suggest an optimal germination protocol.

Methods
Seeds belonging to two different localities were subjected to various pre-treatments, including cold stratification (0 and 5°C), warm stratification (25/10°C) and different combinations of these, and then incubated at a range of constant temperatures (5–25°C) and 25/10°C. Embryo growth during pre-treatments and incubation conditions were assessed at different times by measuring the embryo to seed length ratio (E:S ratio). The final germination percentage (FGP) and the germination rate (t_{50}) were calculated.

Important Findings
Fleshy mature seeds of *G. lutea* subsp. *lutea* have linear underdeveloped embryos. Cold stratification at 0°C was effective in overcoming the physiological dormancy (PD) and promoted embryo growth and subsequent germination. After cold stratification at 0°C, both the root and the shoot emerged readily under a wide range of temperatures. *G. lutea* subsp. *lutea* seeds showed an intermediate complex morphophysiological dormancy (MPD). As regards the optimal germination protocol for this taxon, we suggest a period of cold stratification at ca. 0°C followed by seed incubation at 10–20°C. The optimal germination temperatures found for seeds of this taxon, as well as its pre-chilling requirement at 0°C, suggest that it is well adapted to a temperate climate; this behavior highlights an increasing threat from global warming for *G. lutea*, which could reduce the level of natural emergence in the field, prejudicing also the long-term persistence of the natural populations in Sardinia.

Keywords: embryo growth, global warming, morphophysiological dormancy, pre-chilling requirement, seed germination, threatened species

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INTRODUCTION

Seed germination is a crucial process for seedling establishment and survival in nature (Fenner and Thompson 2005). There are several mechanisms that regulate seed germination; among these, one of the most important is seed dormancy, which promotes survival by dispersing germination in space and time until environmental conditions are favourable for germination (Baskin and Baskin 2014).

Seed dormancy and germination serve as an important focal point for understanding the direct and indirect, via seed maturation and mass, impacts of climate change, as well as soil seed bank formation when combined with other climate influenced factors (Walck *et al.* 2011). At the core of plant regeneration or distribution, temperature and water supply are critical drivers for seed dormancy (initiation, break) and germination. In the particular case of mountain species, changes in both the severity and duration of winters may impact some species, with at least some of their seeds remaining dormant. Thus, increased research in understudied ecosystems, on key issues related to seed ecology, and on evolution of seed traits is needed to more fully comprehend and plan for plant responses to global warming (Walck *et al.* 2011).

Our study focuses on *Gentiana lutea* L. *s.l.*, a perennial herb with wide latitudinal and distributional ranges throughout the Central-Southern European mountains, where it can be found at an altitude range of 800–2500 m a.s.l. (Anchisi *et al.* 2010). This species has a considerable economic importance in several European countries due to the bitter substances contained in its roots, which are used to prepare bitters and liqueurs, as well as pharmaceuticals such as anti-inflammatory agents and diuretics (Carnat *et al.* 2005; Nastasijević *et al.* 2012). It is included in Annex D of the European Habitats Directive, is reported to be threatened as a result of root harvesting practices and of global warming due to its distribution, which is restricted mainly to the upper sectors of the mountains (Gentili *et al.* 2013). From a conservationist point of view, this taxon, was catalogued as least concern at the European level (Bilz *et al.* 2011) and as near threatened in the Italian Red List (Rossi *et al.* 2016). More recently, Fois *et al.* (2016) assessed it as being endangered (*sensu* IUCN, 2012) in Sardinia, where the whole distributional area is limited to the Gennargentu Massif (Central-Eastern part of the island). These authors predicted a decrease of the environmental niche of *G. lutea* in Sardinia due to a reduction of its altitudinal range towards higher elevations, based on future climate scenarios.

In order to understand the reproductive mechanisms of *G. lutea*, the study of its germination ecophysiology is fundamental. Embryos in some Gentianaceae seeds are small at the time of dispersal and may increase by 57–182% before the radicle emerges from the seed (Baskin and Baskin 2005). Martin (1946) included seeds of Gentianaceae in the 'dwarf' seed category. Baskin and Baskin (2007) revised Martin's embryo type classification system and included this family within the 'linear underdeveloped' embryo. The presence of underdeveloped embryos would mean that seeds may have either morphological (MD) or morphophysiological (MPD) dormancy, depending on whether physiological dormancy (PD) occurs in the embryo or not (Baskin and Baskin 2004; Nikolaeva 1969). Seeds of many *Gentiana* species need stratification for germination (Baskin and Baskin 2014 and references therein). Nikolaeva *et al.* (1985) reported a non-deep complex MPD in seeds of *G. lutea*, while Pérez-García *et al.* (2012) showed that seeds of this species exhibit non-deep PD.

Seeds from populations encountering long periods with snow cover and adverse winter conditions would require longer periods of cold stratification for germination than those from populations exposed to milder winters (Jurado and Flores 2005). For many species, 5°C is optimal for seed dormancy breaking and, in general, it is the temperature which is most often used in this kind of research for pre-chilling treatments (Baskin and Baskin 2014), but in some cases temperatures below 5°C are more effective. For example, *G. purpurea* L. germinated around 60% after a treatment of 0°C for 7 months in the darkness (Orsenigo *et al.* 2015) and Cuena-Lombraña *et al.* (2016) reported that a chilling temperature of 5°C alone is not enough to break seed dormancy in Spanish and Sardinian populations of *G. lutea* L. subsp. *lutea*. In addition, after analyzing the soil temperature recorded in the natural sites, these authors supposed that a temperature of 0°C may be effective in overcoming PD in seeds of this taxon.

In this work, we investigate in depth the seed germination ecophysiology of *G. lutea* subsp. *lutea* in order to: (i) evaluate which temperature regime is most effective in promoting embryo growth, breaking seed dormancy and enhancing germination; (ii) identify the class and level of seed dormancy and (iii) suggest an optimal germination protocol for this taxon.

MATERIAL AND METHODS

Study species and area

Gentiana lutea subsp. *lutea* (hereafter *G. lutea*) is a perennial rhizomatous herb with a European distribution, although it is mainly present in the mountain ranges of Central-Southern Europe, i.e., in Sardinia, Corsica, the Iberian, Italian and Balcan Peninsulas and in the Alps, rarely in the Caucasus and in Anatolia (Renobales 2012). The distribution range in Sardinia consists of a vast population located in the Gennargentu Massif, where it is found in different nuclei or scattered individuals (Fois *et al.* 2015). The Gennargentu Massif (Fig. 1), situated in Central-Eastern Sardinia, is an independent biogeographical sector with a surface of ca. 721 km^2 and consists of a system of summits and windy ridges at 1400–1500 m a.s.l., with four peaks at more than 1800 m a.s.l. (Fenu *et al.* 2014). The average annual temperature of the study area varies from ca. 12°C (at an altitude of about 1000 m a.s.l.) to ca. 7°C in the higher elevations (around 1800

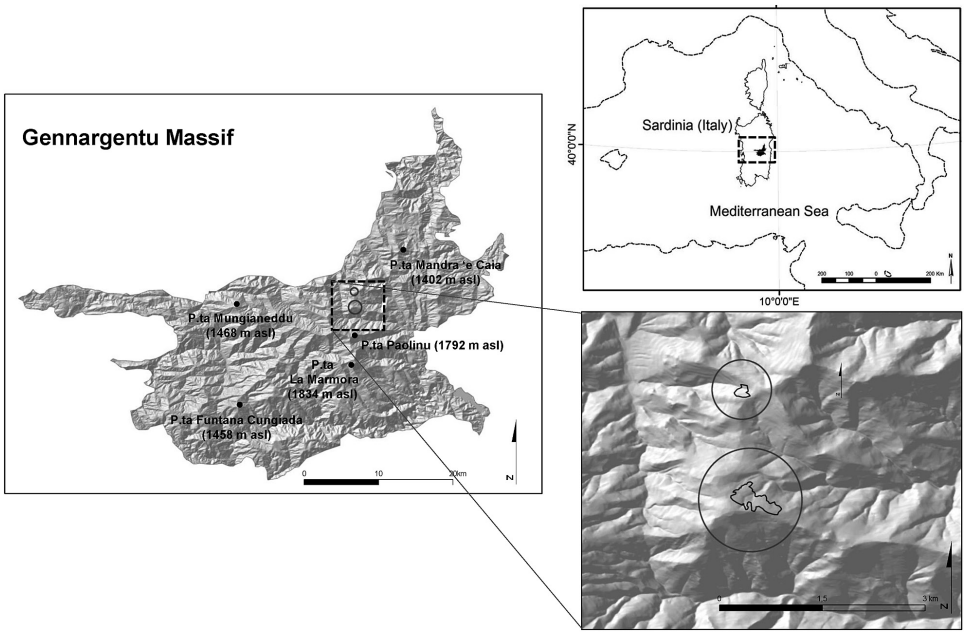

Figure 1: geographical location of Sardinia in the Mediterranean context, toponyms of the main peaks included in the Gennargentu Massif and sampling sites of *G. lutea*: Is Terre Molentes (IS) and Trainu Murcunieddu (TM).

m), with a snowfall period from 3 to 4 months (Secci *et al.* 2010).

During August 2014, mature fruits (capsules) of *G. lutea*, containing well-developed ripe seeds, were sampled in two representative Sardinian localities: Is Terre Molentes (IS), situated at 1460–1505 m a.s.l. and Trainu Murcunieddu (TM) at 1324–1372 m a.s.l. and with a linear distance between them of <10 km. These localities are characterized by similar habitat characteristics (open grassland) and soil substrate (metamorphic). Seeds were collected from at least 50 randomly selected plants in each 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, and stored at room temperature (ca. 20°C and 40% of relative humidity) for ca. two weeks until the start of the germination tests.

Seed germination test

In an attempt to break seed dormancy, several pre-treatments were applied to fresh seeds of *G. lutea*; the experimental design took into account also the soil temperatures recorded after seeds dispersal in the Sardinian natural sites (Cuena-Lombraña *et al.* 2016). The following treatments and pre-treatments were applied: (i) control, without any pre-treatment, incubated directly at germination conditions (see below for further details); (ii) cold stratification at 0°C for three months (C0); (iii) cold stratification at 5°C for three months (C5); (iv) warm stratification at 25/10°C for three months (W); (v) warm stratification (25/10°C for three months) followed by five months of cold stratification at 5°C (W + C5) and (vi) warm stratification followed by two different cold stratification periods, the first at 5°C for one month and the second at 0°C for three months (W + C5 + C0). The pre-treatment C0 was performed in dark conditions (0 h light/24 h dark) in order to simulate the snow cover period, while the other pre-treatments were carried out at an alternate photoperiod of 12 h light/12 h dark.

All the pre-treatments were conducted at the same time and started within two weeks after collection. As regards the germination tests, four replicates of 25 seeds for each experimental condition were sown on the surface of 1% agar water in 90 mm diameter plastic Petri dishes and incubated simulating a day/night cycle (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 regime, the 12 h light period coincided with the higher temperature period. Light was provided by white fluorescent lamps (FL40SS.W/37 70–10 µmol m^{-2} s^{-1}). Germinated seeds were scored three times a week; germination was defined as visible radicle emergence (≥1 mm) and germinated seeds were removed from the Petri dishes to guarantee no double counting. 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 the remaining seeds and the number of empty seeds. Firm seeds were considered viable (ISTA 2006).

For each germination trial, 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 (empty seeds were excluded). Germination rate (t_{50}) was determined as the

time (expressed in days) required to reach 50% of the germination percentage; this value was only calculated when the 50% of germination was reached.

Embryo measurements

Embryo and seed lengths were determined at different times by cutting the seeds longitudinally using laboratory tweezers and a scalpel, both during and after the pre-treatments (see Table 1 for details). Ten seeds for each sample interval (Table 1) were sectioned in half under a dissecting microscope and images of embryos acquired using a Zeiss SteREO Discovery.V8, with an objective Achromat S 0.63x, FWD 107 mm (Carl Zeiss MicroImaging GmbH) at 1.0× magnification, coupled to a Canon (Power shot G11) digital camera. Embryo and seed lengths were measured using the image analysis software ImageJ 1.41 (National Institutes of Health, Bethesda, MA, USA). Seed length was measured ignoring the seed coat (Mattana *et al.* 2012). The embryo to seed length ratio (i.e., E:S ratio) was calculated. The initial E:S ratio was calculated by measuring 20 randomly selected seeds before the start of the experiments; in order to take these measurements the seeds were sowed for 24 hours at room temperature on the surface of 1% agar water in 90 mm diameter plastic Petri dishes. The critical E:S ratio (i.e., the E:S ratio at the moment immediately prior to germination, when the seeds had a split seed coat but no radicle protrusion) was determined as the average E:S ratio of 20 seeds. The critical E:S ratio was also considered for those seeds that had germinated before measurements were taken (Porceddu *et al.* 2016; Vandelook *et al.* 2007).

Statistical analyses

Generalized linear models (GLMs) were used to evaluate the effect of pre-treatments and incubation temperature on the E:S ratio, 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. A log link function and quasipoisson error structure was used for analysing the E:S ratio. Quasibinomial and quasipoisson error structures and *F* tests with an empirical scale parameter instead of chi-squared on the subsequent analysis of variance were used in order to overcome residual overdispersion (Crawley 2007). All statistical analyses were carried out using R v. 3.1.3 (R Development Core Team 2015).

RESULTS

Effect of pre-treatments on embryo growth

GLMs indicated that both the pre-treatment and the locality factors had a significant effect on embryo growth ($P < 0.001$), while the interactions between pre-treatments and localities were not statistically significant ($P > 0.05$). The mean length of embryos from freshly mature seeds were 0.12 ± 0.01 cm in IS and 0.09 ± 0.01 cm in TM locality, while the seed lengths were 0.27 ± 0.02 and 0.24 ± 0.03 cm, in IS and TM, respectively. Hence, the initial E:S ratio in mature seeds was 0.44 ± 0.04 in IS and 0.39 ± 0.05 in TM (Fig. 2).

The final mean embryo length of cold stratified seeds at 0°C was 0.17 ± 0.02 cm (E:S 0.64 ± 0.07) in IS and 0.16 ± 0.02 cm (E:S 0.60 ± 0.06) in TM, while at 5°C it was 0.14 ± 0.02 cm (E:S 0.51 ± 0.07) and 0.12 ± 0.02 cm (E:S 0.46 ± 0.05) in IS and TM, respectively; no embryos achieved the embryo critical length for germination (Fig. 2) during these pre-treatments. Embryos in W stratified seeds reached mean lengths of ca. 0.14 cm (E:S of ca. 0.52) in both localities. Embryos that suffered two cycles of pre-treatments (i.e., W + C5 and W + C5 + C0) did not reach a critical E:S ratio for seed germination, but W + C5 + C0 reached the highest value for the E:S ratio (0.72 ± 0.05 in IS and E:S 0.71 ± 0.07 in TM) probably due to the long duration of the pre-treatment.

Table 1: description of the pre-treatments applied and experimental design for embryo growth measurements

Pre-treatment		Embryo growth measurements	
Code	Description	Total measurements	Measurement timing
0	Control	4	After 15 days, and 1, 2 and 3 months.
C0	0°C for 3 months (0/24 hours of light)	7	After 1, 2 and 3 months during cold stratification and after 15 days, and 1, 2 and 3 months after sowing for germination.
C5	5°C for 3 months (12/12 hours of light)	7	After 1, 2 and 3 months during cold stratification and after 15 days, and 1, 2 and 3 months after sowing for germination.
W	25/10°C for 3 months (12/12 hours of light)	7	After 1, 2 and 3 months during warm stratification and after 15 days, and 1, 2 and 3 months after sowing for germination.
W + C5	25/10°C for 3 months + 5°C for 5 months (12/12 hours of light)	10	After 1, 2 and 3 months during warm stratification, after 1, 2 and 3 months during cold stratification and after 15 days, and 1, 2 and 3 months after sowing for germination.
W + C5 + C0	25/10°C for 3 months + 5°C for 1 month (12/12 hours of light) + 0°C for 3 months (0/24 hours of light)	11	After 1, 2 and 3 months during warm stratification, after 1 month during cold stratification at 5°C, after 1, 2 and 3 months during cold stratification at 0°C and after 15 days, and 1, 2 and 3 months after sowing for germination.

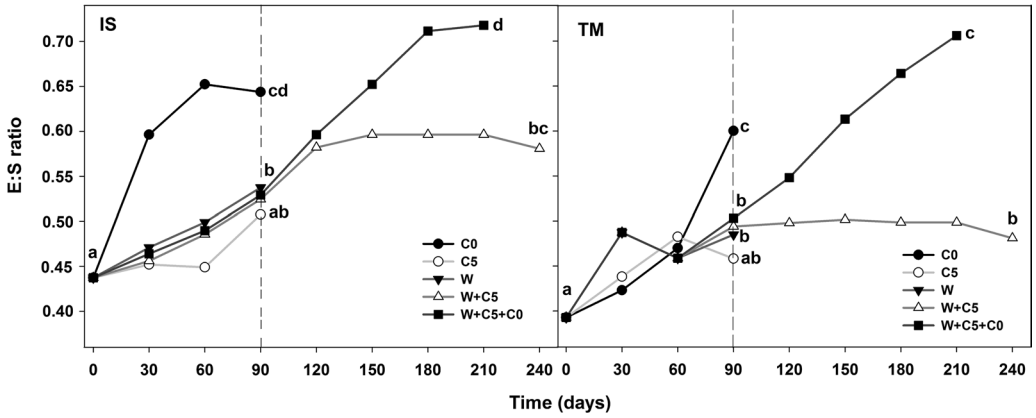

Figure 2: the effect of pre-treatment on embryo growth. Embryo:Seed (E:S) ratio at the beginning of the experiment and during pre-treatments. E:S ratio values are the mean of 10 seeds (±SD). GLMs were carried out, values with the same letter are not statistically different at $P > 0.05$ by *post hoc* pairwise *t*-test comparisons. TM = Trainu Murcunieddu and, IS = Is Terre Molentes. See Table 1 for further details about the pre-treatments.

In the first cycle no statistical differences ($P > 0.05$) were found between the initial E:S ratio and the final E:S in C5, while statistical differences were found in the other pre-treatments ($P < 0.05$) in both localities (Fig. 2). Considering the second cycle, the pre-treatment W + C5 + C0 was statistically different with respect to W + C5 ($P < 0.05$); the latter was statistically similar ($P > 0.05$) to C5 and W in both sites. The statistical results indicated that the effect of C0 and W + C5 + C0 on embryo growth was similar ($P > 0.05$) in both localities (Fig. 2).

Effect of incubation temperature on embryo growth during germination tests

The mean critical E:S ratio were 0.79 ± 0.08 for IS and 0.81 ± 0.06 for TM (Fig. 3). The embryo growth during the germination tests in the control treatment did not reach the mean critical E:S ratio in none of the localities and under none of the temperature conditions. The same occurred in the pre-treatments C5, W and W + C5. In the C0 pre-treatment the critical E:S ratio was achieved after 15 days at 5, 10, 15 and 20°C in both localities. At 25°C and alternate temperatures (25/10°C) more days were required to reach it, i.e., 90 and 60 days for IS and TM, respectively. In W + C5 + C0, the critical E:S ratio was achieved during the first two weeks of incubation in both localities and at all temperatures (Fig. 3). In general, the mean critical E:S ratio was achieved at all incubation temperatures only after the seeds had undergone a period at a temperature of 0°C (i.e., C0 and W + C5 + C0 pre-treatments), this was confirmed also by a lack of embryo growth after the W + C5 pre-treatment (Fig. 3).

Effect of pre-treatments on seed germination

Seeds treated for three months at 5°C (C5) did not germinate; W and W + C5 were also ineffective on seed germination. In general, all the seeds included in the experiment with 0°C in the pre-treatments (C0 and W + C5 + C0) achieved high germination percentages (Fig. 4). The differences in both pre-treatments, locality and temperatures were statistically significant ($P < 0.05$; Table 2) for FGP, as well as the two-way interactions between locality and temperatures ($P < 0.01$; Table 2). However, the two-way interactions between pre-treatments and locality, and between pre-treatments and temperature, as well as the three-way interaction (Treat × Loc × Temp), were not statistically significant ($P > 0.05$; Table 2). The differences highlighted by GLM for the t_{50} analysis were statistically significant ($P > 0.05$) for all analyzed factors.

In general, the percentage of empty seeds in *G. lutea* was less than 1–2%. In IS, after C0, more than 60% of FGPs were reached at 5, 10, 15 and 20°C (Fig. 4). At 25°C the FGP was ca. 20% and at 25/10°C it was >40%. The t_{50} values decreased (19.15, 8.43, 5.56, 4.80 days) from 5 to 20°C and at 25/10°C it was reached in ca. 4 days. In TM, high FGPs (>80%, Fig. 4) were achieved at 5, 10, 15 and 20°C after C0. At 25°C the FGPs were lower than 20% and increased up to about 60% in the alternate temperatures. As for the time to achieve 50% of the final germination, the values decreased (13.35, 6.45, 4.87 and 5.12 days; Fig. 4) with the increase of the incubation temperature from 5 to 20°C, while in the alternate temperatures regime (25/10°C) the t_{50} was reached after 5.90 days.

The effects of W + C5 + C0 highlighted a reduction in the time needed for germination in all localities and, in the majority of temperatures, it increased the FGP in IS and TM. As regards IS (Fig. 4), the FGP was ca. 80% at 5°C. At 10, 15 and 20°C the FGP reached values near 100%, whereas at 25°C it decreased to 22% and at 25/10°C to 48%. At 5°C the t_{50} was 16.07 days, while at 10, 15 and 20°C it was 5.45, 4.06 and 2.74 days, respectively, and at 25/10°C it was 3.58 days (Fig. 4). As regards TM, more than 70% of the seeds germinated at a temperature range comprised between 5 and 20°C and at alternate temperatures (25/10°C, Fig. 4); at 25°C the FGPs were less than 30%. As for the t_{50}, at 5°C it was 14.67 days, while at 10, 15, 20 and 25/10°C it decreased to 4.24, 2.86, 2.04 and 1.90 days, respectively (Fig. 4).

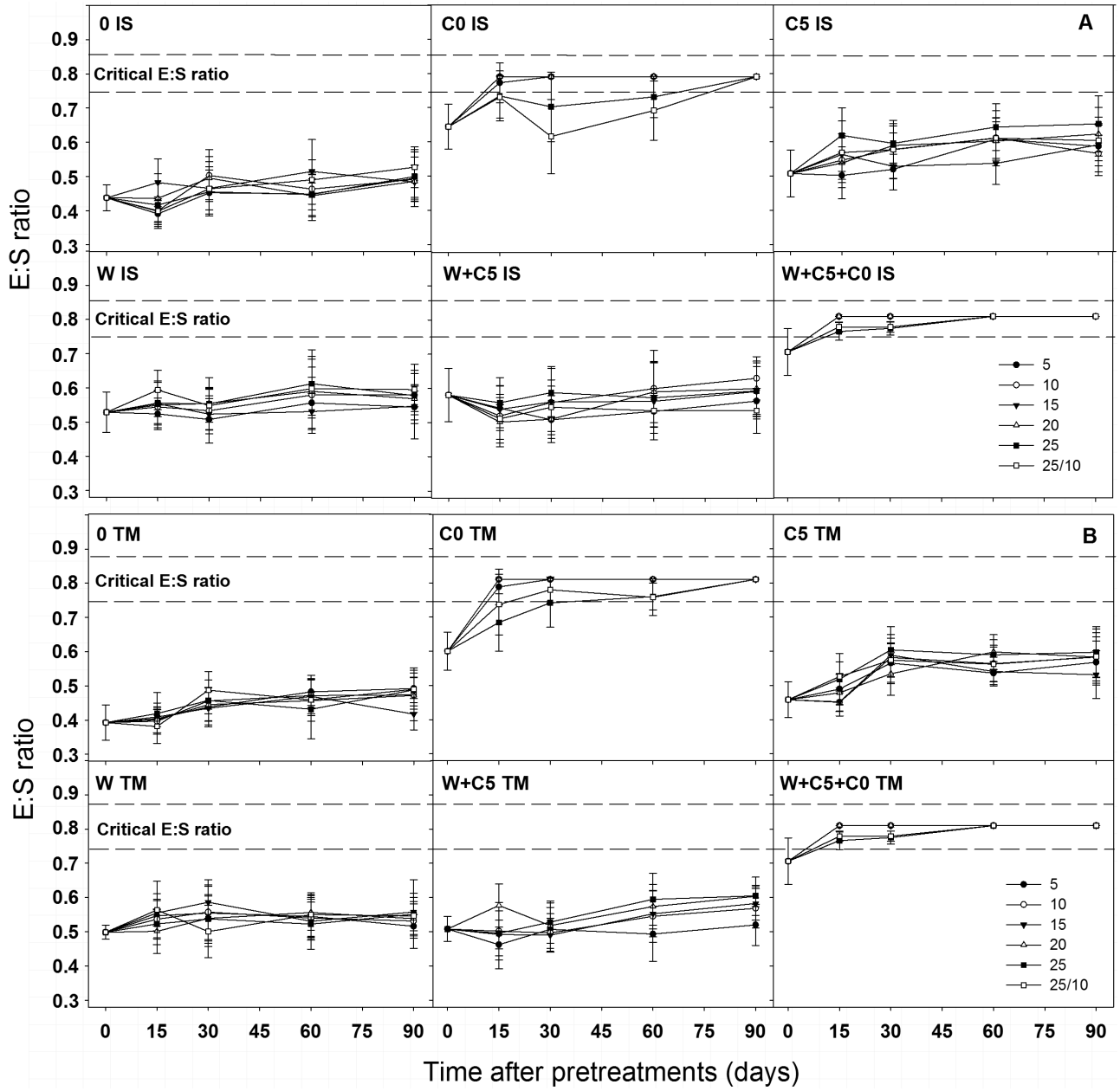

Figure 3: effect of germination temperature conditions on the E:S ratio in all pre-treatments. 5, 10, 15, 20, 25 and 25/10°C were the incubation temperatures during the germination tests. Upper graphics regard (A) IS, Is Terre Molentes locality and lower graphics (B) TM, Trainu Murcunieddu locality. The range of critical E:S ratio are indicated by dashed lines. See Table 1 for further details about the pre-treatments.

DISCUSSION

Class of dormancy and dormancy-breaking temperature

Gentiana lutea has linear underdeveloped embryos. In general, if embryo growth and radicle emergence are completed in about 30 days under suitable conditions, seeds have only MD. On the other hand, if germination is delayed for more than 30 days and seeds require a dormancy-breaking treatment such as exposure to moist cold (0–10°C) and/or moist warm (≥15°C) stratification to germinate, they have MPD (Baskin and Baskin 2004; Nikolaeva 1977). In the simple kinds of MPD, embryos grow at relatively high temperature (≥10°C), while in complex kinds of MPD, embryos grow during cold stratification (Baskin and Baskin 2004; Baskin *et al.* 2008). In *G. lutea* fleshy mature seeds, both the root and the shoot emerged after cold stratification at 0°C; this temperature was effective in interrupting seed dormancy and promoting embryo growth and germination in this taxon. To help determine the class of dormancy it is necessary to obtain information also on the effectiveness responses to plant hormones on seed germination, in particular to gibberellic acid

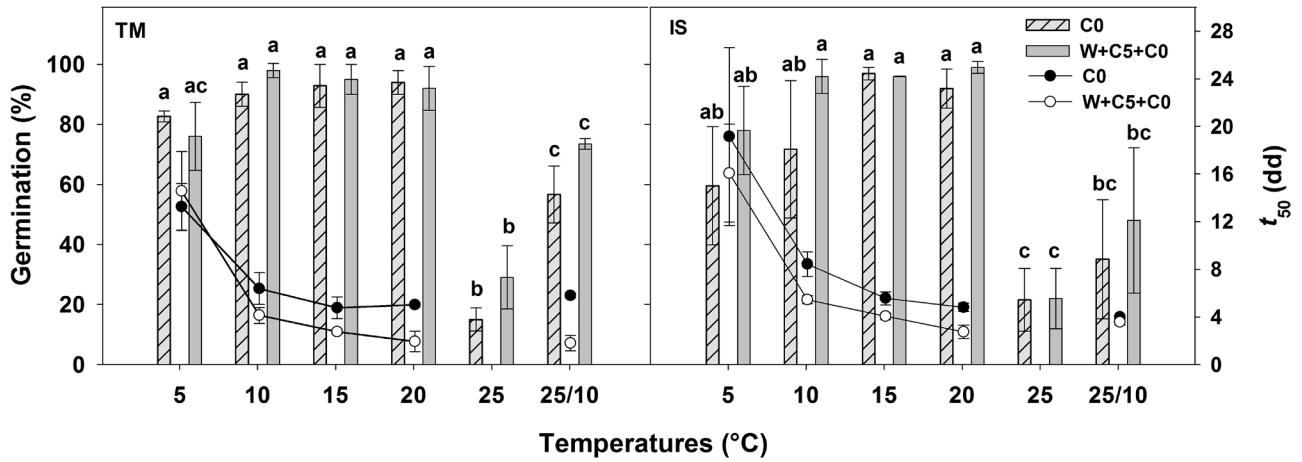

Figure 4: FGP (bars) and t_{50} values (points and lines) achieved at the end of the germination tests, after each pre-treatment (only the pre-treatment where germination occurred are included in this figure); *Post hoc* pairwise *t*-test comparisons were carried out for each germination temperature and bars with different letters indicate significant ($P < 0.05$) differences. TM = Trainu Murcunieddu; IS = Is Terre Molentes. Data are the mean of four replicates (±SD). See Table 1 for further details about the pre-treatments.

Table 2: GLM results of seed germination (FGP) of the following factors: pre-treatment (Treat: C0, W + C5 + C0), temperature (Temp: 5, 10, 15, 20, 25, 25/10°C) and locality (Loc: TM 'TrainuMurcunieddu'; IS 'Is Terre Molentes') and their interaction

	df	Deviance	Residual df	Residual deviance	*F*	*P* (>*F*)
Null			95	4343.2		
Treat	1	73.2	94	4269.9	10.1709	0.002113**
Loc	1	50.9	93	4129.0	7.0747	0.009630**
Temp	5	3421.2	88	797.8	95.0260	<2.2e−16***
Treat × Loc	1	7.7	87	790.1	1.0731	0.303720
Treat × Temp	5	64.6	82	725.5	1.7930	0.125118
Loc × Temp	5	91.0	77	634.5	2.5273	0.036471*
Treat × Loc× Temp	5	64.3	72	570.2	1.7861	0.126533

See Table 1 for further details about the pre-treatments. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$.

(Baskin and Baskin 2014). More recently, studies on seed germination ecology on seed of this species (Cuena-Lombraña *et al.* 2016), collected from the same localities of this study, demonstrated the positive effect of gibberellic acid (GA_3) on seed germination (FGP > 60%). Therefore, following the classification system *sensu* Baskin and Baskin (2014), we argue that *G. lutea* seeds have an intermediate complex MPD.

Ecological and conservation implications

Fruits of *G. lutea* are many-seeded capsules that ripen in summer, dispersal takes place in August and dissemination occurs through anemochory. Following dispersal time, once imbibed, embryos may start to grow inside the seeds. They are exposed to a slightly warm post-dispersal period (September, October and November with mean daily soil temperatures of ca. 10°C) before winter begins, and after the seed are exposed to one month of ca. 5°C before soil temperatures get lower, near 0°C (for a period of approximately of three months). Germination occurs in the following spring, when the embryo growth reaches the critical E:S ratio, and as we could show, after having experienced the low winter temperatures that break dormancy. Seeds must be adapted to germinate soon after winter, thus avoiding unfavourable conditions, and when temperature and soil moisture may be appropriate for germination (Jurado and Flores 2005; Meyer and Monsen 1991). The same pattern has been reported for some temperate species growing in high mountains of Sardinia, such as *Rhamnus persicifolia* Moris, which required cold stratification to break dormancy and relatively low temperatures for seed germination (Porceddu *et al.* 2013). Our results are in accordance with the concept of natural selection favouring germination patterns that reduce the probability of facing adverse environmental conditions for seedling establishment (Baskin and Baskin 2014).

Recently studies that modelled the distributions of plants in relation to climate reported that mountain species, specifically those located near the Mediterranean Basin, are disproportionately sensitive to climate change (up to 60% species loss by 2080; Nogués-Bravo *et al.* 2007; Thuiller *et al.* 2005). Firstly, accurate estimate of the effects of climate change in mountain

systems is difficult because of uncertainties associated with the climate scenarios and the existence of non-linear feedbacks between impacts (Nogués-Bravo *et al.* 2007). However, as far as *G. lutea* in Sardinia is concerned, we can hypothesize that a reduction of natural emergence in the field will happen as a consequence of the predicted reduction of the cold period in Mediterranean mountains. If this prediction turned out to be true, a reduction of the emergence in the field of this species could be possible, due to the inability to break seed dormancy, prejudicing also the long-term persistence of the populations. Furthermore, long-lived species and species with limited dispersal abilities (such as *G. lutea*) are thought to have higher conservatism of their realized niche (Pearson and Dawson 2003). The populations of *G. lutea* are therefore differently threatened throughout their distribution and thus require different measures of protection. The loss of potential habitat will be unequally distributed across the various mountain ranges in Europe; as a result, the species will probably be more sensitive to climate change in their southern distribution range. Actually, as predicted by Fois *et al.* (2016) under different scenarios and projections for global warming in Sardinian mountains, all extinctions would occur at the edge of the distribution and elevation gradient; according to it, *G. lutea* would tend to reduce its altitudinal range towards higher elevations. For these reasons, we believe that it is necessary to begin active conservation measures and invest resources and time to protect this species in Sardinia.

Optimal germination protocol

The optimal protocol of germination should definitely be taken into account when developing an *in-situ* conservation strategy involving the *ex-situ* cultivation of plants, an action that could effectively contribute to the reduction of the root harvest pressure on wild populations. In general, propagation from seed is actually relatively inexpensive and usually effective, but often the particularities of germination requirements are unknown or just partially known. On the basis of these results, we suggest that the optimal germination protocol for this species consists of a period (three months) of cold stratification at 0°C in dark conditions, followed by seed incubation at 10–20°C under photoperiod conditions of 12/12 hours. Considering the low differences in terms of FGPs between the two pre-treatments that promote germination (i.e., C0, with a three-months duration and W + C5 + C0, seven months), our study suggests that they both have the same effect on the seed germination response, therefore highlighting the importance of cold stratification at 0°C (or near) to break dormancy. In addition, we demonstrated that germination did not occur in the pre-treatment W + C5, which further emphasize the effectiveness of cold stratification at 0°C (C0).

Besides, determining the optimal germination protocol is also useful and necessary in view of the challenges related to climate change, as these are predicted to be particularly tough for mountain plant species, which are supposedly destined to a general upward migration under a warming climate (Gentili *et al.* 2015).

CONCLUSIONS

In conclusion, our study shows that seeds of *G. lutea* are characterized by intermediate complex MPD and that temperature is a critical environmental factor for germination to occur. These results are relevant to conservation of this species listed in the Habitats Directive and also considering the threat represented by global warming, as its distribution in Sardinia mainly regards the upper sectors of mountains. The present study is useful in programming management actions such as translocation programmes and population reinforcements in order to help preserve this plant of European interest and of high economic value.

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