PLANT SOCIOLOGY

formerly FITOSOCIOLOGIA

Volume 54 (1) - Suppl. 1 - June 2017

EDITO DALLA SOCIETA' ITALIANA DI SCIENZA DELLA VEGETAZIONE ONLUS - PAVIA - DIRETTORE RESPONSABILE PROF. E. BIONDI - SUPPLEMENTO 1 - VOLUME 1 - I° SEMESTRE 2017

USJ 1875
Université Saint-Joseph de Beyrouth
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Project funded by the EUROPEAN UNION

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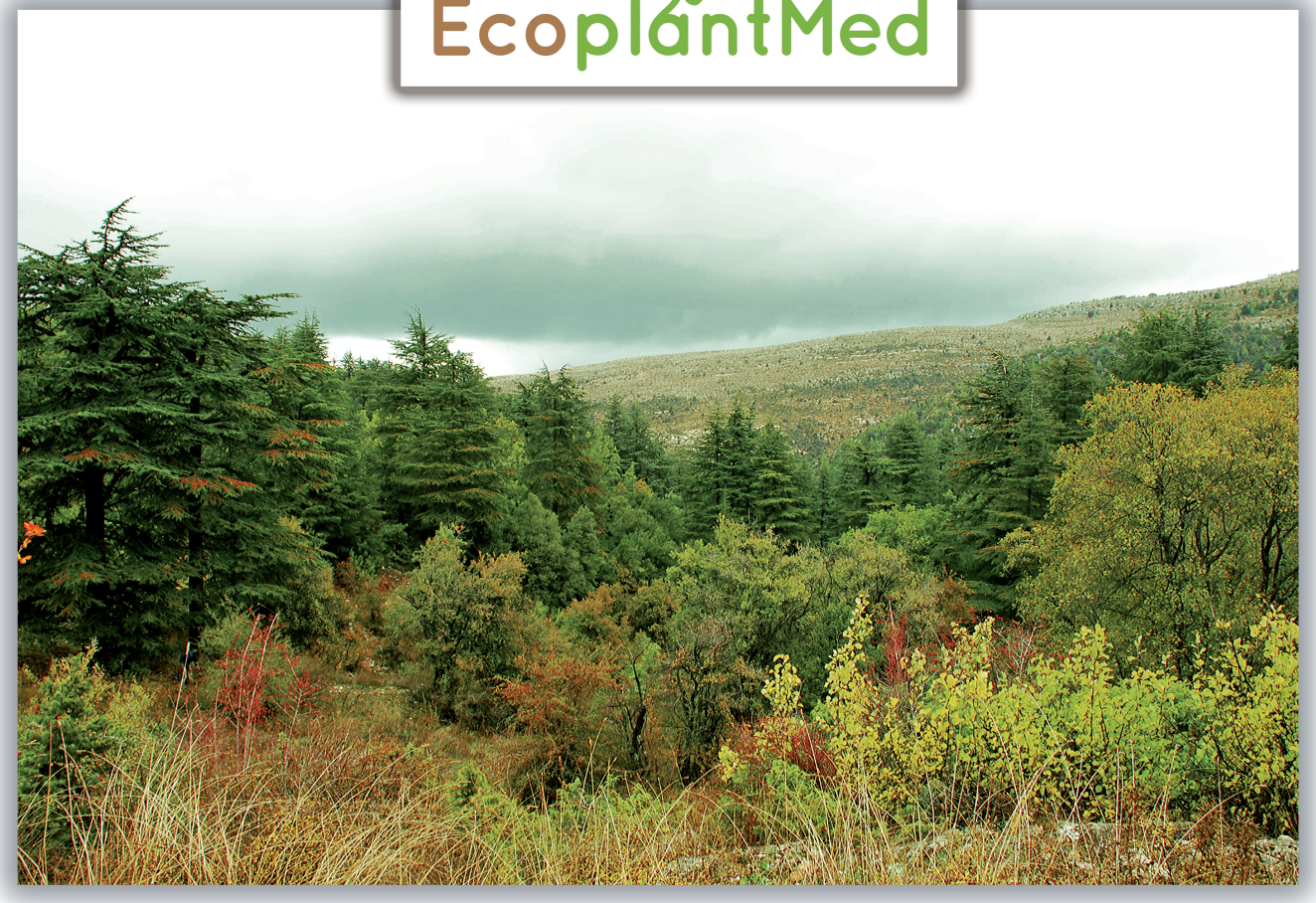

Journal of the Italian Society for Vegetation Science

Plant Sociology, Vol. 54, Suppl. 1, June 2017, pp. 53-59
DOI 10.7338/pls2017541S1/08

PLANT SOCIOLOGY
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Seed germination and conservation of two endemic species from Central Apennines (Italy)

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Abstract
The Mediterranean mountains are one of the most threatened ecosystems in Europe, and endemic species are a significant feature of this environment. The definition of germination protocols for endemic, rare or threatened species is an important step for their conservation. The aim of this work was to analyze seed germination of *Phyllolepidum rupestre* Ten. Trinajstić and *Crepis magellensis* F. Conti & Uzunov, two endemic species growing in small populations in the Majella Nation Park (Central Apennines, Italy). The effects of temperature (5, 10, 15, 20, 25, 25/10 and 20/10°C), irradiance and gibberellic acid (250 and 500 ppm) on seeds germination were considered. A protocol for the *in situ* reintroduction was also developed. The results highlight a significant effect of temperature on seed germination. In particular, seed germination for *P. rupestre* and *C. magellensis* was 70.58 ± 3.75 % and 97.30 ± 3.13% at 20°C, respectively. These protocols can be used in reinforcement projects for wild populations.

Key words: conservation, endemic species, germination ecology, Mediterranean mountains, protected areas, restocking.

Introduction

Forecasted models on climatic changes suggest variations in the distribution area of plant species (Diaz *et al*., 2003; Medail & Quezel, 2003; Nogués-Bravo *et al*., 2007; Dirnböck *et al*., 2011; Engler *et al*., 2011). In particular, the forecasted increase of air temperature could determine the loss of many European high-mountain plant species (Neuner *et al*. 1999; Neuner & Pramsohler 2006, Engler *et al*., 2011). Furthermore, the large presence of endemic species in the Mediterranean mountains of the South Europe (Gomez-Campo, 1985; Martín-Bravo *et al*., 2010) makes these ecosystems much more vulnerable. Mediterranean mountains are characterized by a high genetic diversity with many populations considered genetically unique (Ruiz-Labourdette *et al*., 2012). The anthropic disturbance, the habitat fragmentation and the presence of small populations are among the most important factors that expose species to risk (Drury, 1974; Rabinowitz, 1981; Snogerup, 1985; Lavergne *et al*., 2004). Thus, it is important to improve conservation strategies, particularly for endemic and threatened species growing in the Central Apennines (IUCN, 2001). The definition of the germination protocols for species characterized by small populations could be an important step in this direction, considering that seed germination is a critical stage for plant establishment (Cerabolini *et al*., 2004; Youssef *et al*., 2012).

In this context, the main objective of this research was to define the germination protocols of two endemic species growing in the Central Apennines: *Crepis magellensis* F. Conti & Uzunov and *Phyllolepidum rupestre* (Ten.) Trinajstić. To date, there are no studies regarding seed germination of these considered species. Seed germination studies are key tools in conservation programs, because they can be used for management programs and species reintroduction (Ortega-Baesa & Rojas-Aréchiga 2007).

Crepis magellensis (*Asteraceae*) is an endemic threatened species exclusive of the Majella National Park (Conti & Uzunov, 2011). It is a rosulate hemicryptophyte species growing in few areas on calcareous stones (Conti & Bartolucci, 2012) from 2550 to 2773 m a.s.l. It has 6 mm long achenes with a pappus of 4.6-5.8 mm and a fully differentiated and developed embryo which occupies the whole seed.

Phyllolepidum rupestre (*Brassicaceae*) is an endemic, rare and threatened species of Abruzzo (Scoppola & Spampinato, 2005). It is a suffruticose chamaephyte species growing on calcareous gravels and cliffs from 2000 to 2700 m a.s.l. (Pignatti, 1982). *P. rupestre* has a sub-round silicula (5-7 mm), a seed length of about 3.2 mm and a fully differentiated and developed embryo which occupies the whole seed.

Materials and Methods

The Seed collection

Seed collection was performed according to stan-

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dard protocols (ISTA, 2006) in the fruiting period of each *taxon,* immediately before dispersal, according to Hay & Smith (2003) and Baskin & Baskin (2014). For each *taxon*, a number of plants ranging from 50 to 200 (depending on the population size) were randomly selected and sampled (Marshall & Brown, 1983). A threshold of 20% of the total mature seeds collected from the mother plants was never exceeded (Way, 2003; ENSCONET, 2009). In particular, seeds of *C. magellensis* (n. = 1200) were collected on September 2014 in the Taranta Valley (42° 3' 00" N; 14° 6' 00" E, 2457 m a.s.l.) and seeds of *P. rupestre* (n. =1200) were collected on August 2014 near Mount Murelle (42° 7' 00" N; 14° 7' 00"E, 2080 m. a.s.l.).

The collected seeds were manually cleaned and stored at the Majella Seed Bank (Botanical Garden Michele Tenore, 42° 2' 59" N; 14° 11' 34"E; 650 m a.s.l., Italy) in controlled air temperature (20°C) and humidity (40%) conditions for a period of 30 days to ensure seed maturation and assess the health status of the harvested material (Schmidt & Jøker, 2001). After 30 days, seeds were stored at 5°C in paper bags until the beginning of the germination experiments. In addition, a part of the collected seeds was preserved in a freezer at -20°C after the dehydration step (about 50 days at 15°C and 15% RH).

Germination experiments

Specific conditions for the species germination were analyzed by testing the effects of different factors applied in sterile conditions. The germination experiments were preceded by a soaking phase placing seeds in demineralized water at room temperature in the darkness for 24 hours. Subsequently, for *C. magellensis* the sterilization phase was carried out placing seeds for 2 min in 10% H_2O_2, 1 min in 70% ethanol and 2 min in hypochlorite solution containing 3% active chlorine + Tween 20. *P. rupestre* seeds were placed for 2 min in 10% H_2O_2 and 2 min in hypochlorite solution containing 3% active chlorine + Tween 20.

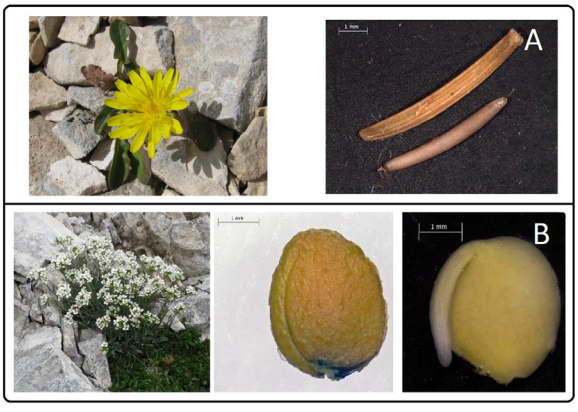

Fig. 1 - A) *Crepis magellensis* flowers in the natural environment, fruit (achene) and embryo; B) *Phyllolepidum rupestre* flowers in its natural environment, seed and embryo.

Germination tests were performed in Petri dishes. Water (25 ml per each dish) was provided from the culture medium consisting of agar at 1% (Gonçalves *et al.*, 2008). In the control test, media pH was adjusted to 5.75 (pH meter Crison pH 25) before autoclaving at 121°C for 20 min (Murashige & Skoog, 1962; Gonçalves *et al.*, 2008) by adding an HCl and NaOH 0.1 M solution.

Germination tests were performed in a growth chamber (Panasonic MLR-351) equipped with cool-white fluorescent tubes (400-700nm) providing a photon flux density (PFD) of 70 μmol (photon) m^{-2} s^{-1}. Tests were performed in a range of constant temperatures (i.e. 5°C, 10°C, 15°C, 20°C and 25°C) alternating temperature regimes (i.e. 25/10°C and 20/10°C). Seeds were exposed to light radiation for 12 hours per day in all the considered tests. In alternating temperature regimes, the period of 12 hours of light corresponded to the higher temperature period regime.

In order to verify the light effect on seed germination, a preliminary test was carried out for each of the considered species (four replicates of 20 seeds for each species at 20°C in the darkness), by wrapping dishes in two layers of aluminum foil. To avoid any exposure to irradiance, seeds in this experiment were only scored once, at the end of the test (Mattana *et al.*, 2009).

To test the effects of gibberellic acid on the germination dynamic, sterilized seeds were soaked for 24 hours at room temperature and in the darkness, in solutions containing 250 ppm and 500 ppm GA_3 (95% purity, Sigma, USA), respectively, and sterile demineralized water (control at 0 ppm GA_3) (Rodriguez Perez, 1993; Chien *et al.*, 2011; Rhie *et al.*, 2015). Afterwards the pretreated seeds (with GA_3) were sown in Petri dishes with 1% agar and tested for germination at a constant temperature of 20°C and 12h of light. Seeds were recorded as "germinated" when the radicle appeared (Côme, 1970). The germination control was carried out daily for the first 10 days from the beginning of the test, and then 3 times a week (i.e. on Monday, Wednesday and Friday) for 50 days which are required to assess the germination dynamic. After 60 days from the end of the germination tests, the viability of any remaining seeds was checked by a cut test (ISTA, 2006). The final germination percentage was calculated on the basis of the total number of filled seeds (Mattana *et al.*, 2009).

Seed conservation

To evaluate the germination rate after storage, experiments were carried out on seeds subjected to a long-term conservation process (Roberts, 1973; Ellis & Roberts, 1980; Bacchetta *et al.*, 2006; ISTA, 2012). In particular, seeds were tested after a conservation pe-

riod of more than 365 days at -20°C replicating the test conditions that had given the best results on fresh seeds in terms of the final germination percentage without pretreatments (i.e. 20°C for *C. magellensis* and 20/10°C for *P. rupestre*).

Data analysis

With regards to the experimental design a risk (α=0.05 and β=0.10) with a 25% threshold detectability was selected. Considering that previous tests showed a standard deviation (S.D.) of 10% and a normally distribution of germination data, 4 Petri dishes were considered.

To understand the dynamics of germination, Z, the onset of germination (necessary time, in days, to observe the first seed germinated) and T_{50} (the half-germination time, i.e. number of days for reaching 50% of the final germination), were calculated. T_{50} was calculated by the equation of Coolbear *et al*. (1980), modified by Thanos & Doussi, (1995):

$$T_{50} = \frac{[(\frac{N}{2}) - N_1 \times (\frac{T_2}{T_1})]}{N_2 - N_1} + T_1$$

where N was the final germination percentage, N_1 the percentage of seeds germinated slightly lower than N/2, N_2 the percentage of seeds germinated slightly higher than N/2, and T_1 the number of days that corresponded to N_1, and T_2 the number of days that corresponded to N_2.

In order to analyze differences in the germination percentage, in T_{50} and Z among the considered treatments, a T-test and one-way ANOVA (followed by Tukey's test for multiple comparisons) were carried out. Differences among treatments were considered significant at p<0.05. All statistical analysis were performed with the R a language and environment for statistical computing.

Results

The light effect (i.e. darkness *vs* light) on *C. magellensis* final seed germination did not produce significant differences (T-Test, p>0.0.5) (97.2 ± 3.3% and 97.3 ± 3.1% in the darkness and in the light, respectively).

The germination percentage showed significant (p<0.05) differences at 20°C, 25°C, 20/10°C and 25/10°C (Fig. 2).

As regards to the dynamics of germination (Fig. 4), the analysis of T_{50} and Z were in line with the germination percentage at the different temperatures. In particular, at the highest temperatures (i.e. 25°C and 25/10°C), there was a reduction of the germination rate (p_{T50} =0.0012; p_Z <0.001, excluding data at 5°C and 10°C).

The final germination percentage did not show significant differences (p>0.05) in response to different GA_3 concentrations (97.3 ± 3.1% in the control treatment, 95.8 ± 2.8 % in the pre-treatment with 250 ppm GA_3 and 93.4 ± 5.0% at 500ppm GA_3). On the contrary, the use of GA_3 produced significant differences in the germination dynamic, reducing both T_{50} and the onset of germination (p_{T50}<0.001, p_Z<0.001) (Fig. 2).

The final germination percentage did not show significant differences (T-test, p>0.05) between control treatment seeds and seeds stored at -20°C, resulting 97.3 ± 3.1% and 97.2 ± 5.6 %, respectively (Fig. 6).

The light effects on *P. rupestre* (i.e. darkness *vs* light) did not produce significant differences (T-Test, p>0.0.5) in the final germination percentage (70.6 ± 3.8 % and 59.2 ± 13.4 % in the darkness and in the light, respectively).

The tests carried out on *P. rupestre* at different temperatures (Fig. 3) showed two peaks at 20°C and 20/10°C. The results of one-way ANOVA showed significant differences (p<0.05) in the final germination percentage varying from 26.3 ± 6.3 % (at 25/10°C) to

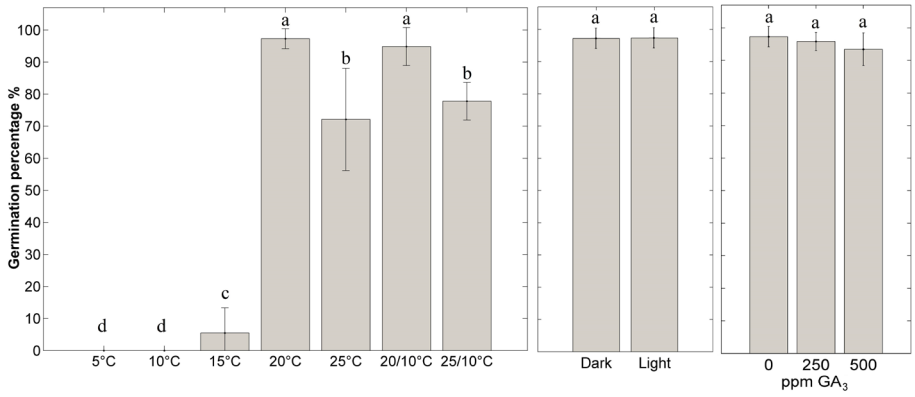

Fig. 2 - Values of the final germination percentage of *Crepis magellensis* in response to the considered treatments. Values with different letters are significantly different (one-way ANOVA, Tukey tests, p <0.05).

67.5 ± 6.3 % (at 20/10°C).

The analysis of T_{50} and Z highlighted significant differences among treatments, showing the best values at 20°C and 20/10°C (Fig. 5).

The use of different GA_3 on *P. rupestre* seeds did not determine significant differences in the germination dynamic. In particular, the final germination percentage was 59.2 ± 13.3% in the control treatment (0 ppm GA_3), 74.1 ± 12.7% at 250 ppm GA_3 and 69.5 ± 14.0 % at 500ppm GA_3. *P. rupestre* seeds remained viable after storage at -20°C, since no significant differences (T-test, p>0.05) in the final germination percentage between seeds stored in control condition (59.2 ± 13.3%) and in a freezer at -20°C (67.5 ± 13.2%) was observed (Fig. 6).

These results show that the gibberellins does not significantly improve seed germination (Figs. 2 and 3).

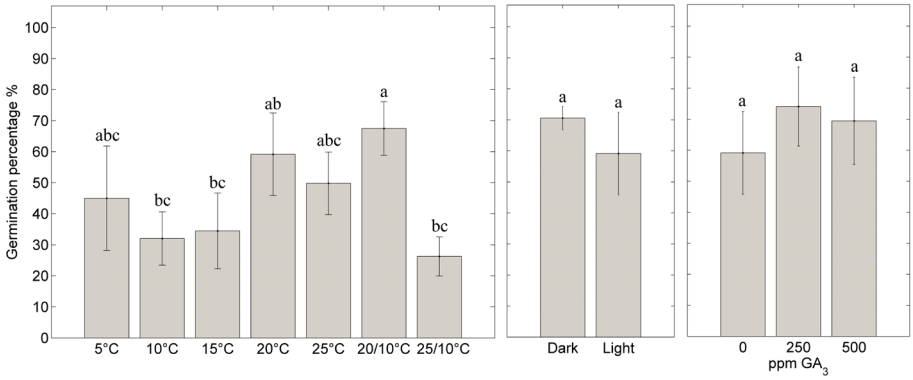

Fig. 3 - Values of the final germination percentage of *Phyllolepidum rupestre* in response to the different treatments. Values with different letters are significantly different (one-way ANOVA, Tukey tests, p<0.05).

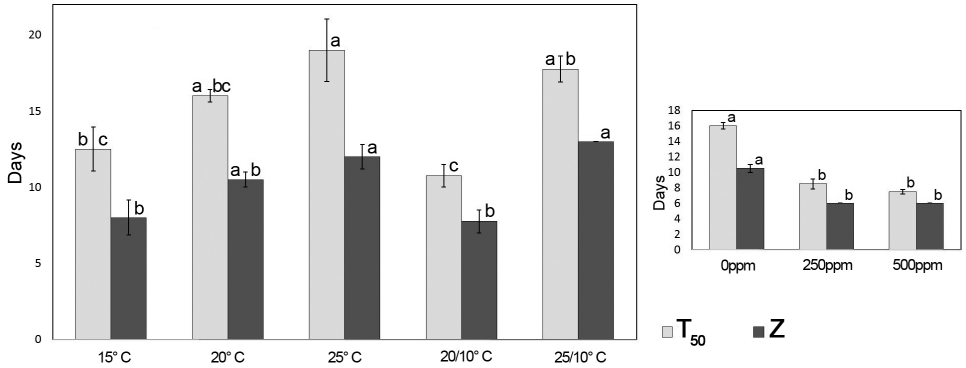

Fig. 4 - Values of the half-germination time (T50) and the onset of germination (Z) for *Crepis magellensis* in response to the different treatments. Values with different letters are significantly different (one-way ANOVA, Tukey tests, p<0.05).

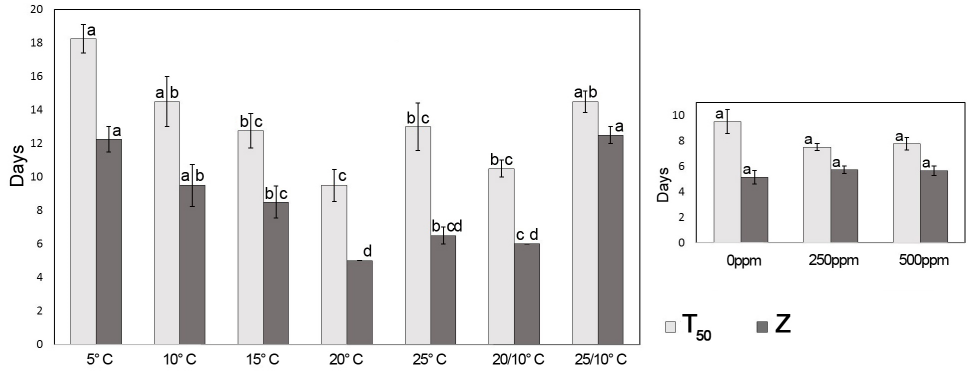

Fig. 5 - Values of the half-germination time (T50) and the onset of germination (Z) for *Phyllolepidum rupestre* in response to the different treatments. Values with different letters are significantly different (one-way ANOVA, Tukey tests, p<0.05).

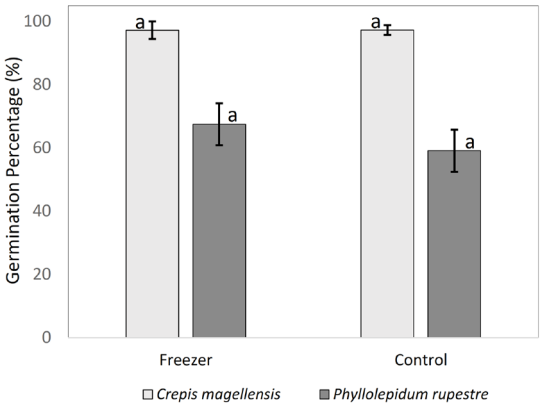

Fig. 6 - Values of the germination percentage in *Crepis magellensis* (at 20°C) and *Phyllolepidum rupestre* (at 20/10°C) for seeds stored at -20°C for 365 days and fresh seeds after a period of post-ripening (0 days at -20°C). Mean values with the same letters are not significantly different (T-test, p>0.05).

Discussion

Seed germination is a critical event in the plant life cycle and the timing of emergence is crucial for survival and reproductive success. Several abiotic stimuli such as light and temperature provide information about the environmental factors affecting seed germination (Nelson *et al*. 2009).

Our results highlight that seed propagation is a valid method for *ex situ* conservation programs both for *C. magellensis* and *P. rupestre*. Moreover, the results of the tests suggest the absence of the dormancy (ND) or a non-deep physiological dormancy (PD) which cannot be excluded, as the seeds may have undergone an after ripening process during dry storage at room temperatures (Geneve, 1998; Hamze & Jolls, 2000). The results are in accordance to those showed by Finch-Savage & Leubner-Metzger (2006) which identified the absence of the dormancy (ND) and of the physiological dormancy (PD) for both families (i.e. *Asteraceae* and *Brassicaceae*). Besides the basic requirement for water, oxygen and an appropriate temperature, seeds may also be sensitive to other factors such as light (Finch-Savage & Leubner-Metzge, 2006). Light controls the timing of seed germination, which may be crucial for seedling survival in the natural environment (Ortega-Baesa & Rojas-Aréchiga 2007). The analysis of the light effects (i.e. darkness *vs* light) on seed germination capacity shows the same final germination percentage in *C. magellensis* and in *P. rupestre*, suggesting that these seeds are not photoblastic (i.e. light insensitive seeds), according to the results of Takaki (2001).

With regard to the seeds response to different temperatures, both *C. magellensis* and *P. rupestre* giving the best results at 20°C. Nevertheless, a significant decrease in the final germination percentage and an increase in T_{50} and in the onset of germination were observed at 25°C between the two species. In particular, *C. magellensis* shows a limited temperature range of germination (about 10°C), while *P. rupestre* a wide range (about 20°C). The use of gibberellic acid (GA_3) has no statistical effects on the final seed germination percentage compared to untreated seeds, while a reduction of both T_{50} and the onset of germination is observed, even if significant only in *C. magellensis*. Observations carried out at the early cultivation stages for the two considered species prove that seedlings from seeds exposed to gibberellic acid are more vigorous than those without treatment. Moreover, important findings are also highlighted concerning the carrying out of *ex situ* conservation for the two species. In particular, seeds stored for a period of one year in a freezer at a temperature of -20°C maintain an excellent viability. The results of the germination tests show no significant differences in the germination dynamic between fresh and stored seeds.

On the whole, the results suggest that seed propagation is a valid tool for the *ex situ* conservation of *C. magellensis* and *P. rupestre*. Moreover, the observed different temperature range suggests that the considered species have different strategies of germination. According with Baskin & Baskin (1973, 1989), the species with seeds non-dormant or with non-deep physiological dormancy, have two different strategies of germination: after the dispersion or at the next vegetative season. The species that disperse seeds at the end of the vegetative season suggest that the seeds don't germinate immediately after their dispersion. *P. rupestre* seeds germinate at a wide temperature range, probably exploiting the immediate subsequent period to snow melting, with favorable growth conditions due to high soil moisture and reduced competitors. On the contrary, *C. magellensis* seeds that germinate at higher temperatures with small temperature range, shows a probably preference to germinate in the middle of the vegetative season when the weather conditions are more stable. The results supply information on *C. magellensis* and *P. rupestre* germination capability for which there are no data. Thus, these findings can be used in propagation projects to support conservation programs reducing the extinction risk of these endemic species.

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