

HOW DIFFERENT REPRODUCTION PROTOCOLS CAN AFFECT THE GERMINATION OF SEEDS: THE CASE OF THREE STENOENDEMIC SPECIES ON MT. OLYMPUS (NC GREECE)

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ABSTRACT

Mt. Olympus, the highest mountain in Greece, is a biosphere reserve and a magnet for countless visitors. In the wider area of Olympus, at least 1,700 species and subspecies of plants are recorded, 26 of which are endemic. Most of the endemics only occur in the high or subalpine zone, which is expected to be strongly affected by climate change or in specific microsites that might be affected by several other factors. Thus, the unique flora of the mountain will probably become extinct. *Ex situ* conservation can be utilized to prevent and even reverse this trend and preserve plant diversity for future generations. The aim of the present study was to develop reproduction protocols for the endemic species on Mt. Olympus, *Centaurea incompleta*, *Centaurea litchorea* and *Viola striis-notata*, to facilitate their mass production, either for *ex situ* conservation or reintroduction into their natural habitats, if necessary. Seeds of the target species were collected in summer 2021. In a sample of the collected seeds, the embryo viability was checked using sequentially 1% w/v tetrazolium solution and Evans blue solution concentration of 0.25% w/v. As for the germination tests, two treatments were used to terminate seed dormancy: (a) cold stratification at ± 2 °C, and (b) imbibition in gibberellic acid (250 ppm) for 48 hours. The results showed that more than 75% of the embryos in the fertile seeds were viable. In the seed germination tests, treatment with gibberellic acid resulted in germination percentages for *Centaurea incompleta* and *Viola striis-notata* are equal to or very close to the seed viability percentage. In contrast, no treatment was successful for *Centaurea litchorea*, as the control germination percentage was higher.

Keywords: endemics; gibberellic acid; plants; seed germination; stratification; viability test

Introduction

The conservation of biodiversity is one of the most significant global issues that scientists face. The intensity and range of human effects on habitat loss and degradation has resulted in a reduction in biodiversity at an unprecedented rate (Rogan and Lacher 2018). It is estimated that, at least, 25% of the world's plant species are threatened with extinction due to habitat loss (Holtz et al. 2022). Endemic species have the highest rates of global extinctions, as they usually have a limited geographical distribution, small population sizes and low adaptive capacity (Kraus et al. 2022). Moreover, these species are also being gradually more affected by climate change, at both species and community levels, which will eventually result in a modified plant distribution (Román-Palacios and Wiens 2020). Southern European Mountain systems are among the ecosystems most affected by climate change (Engler et al. 2011) and the endemic mountain plants there are expected to be severely stressed (Dagnino et al. 2020; Manes et al. 2021).

In the southern part of Europe, the Balkan Peninsula acted as a glacial refugium for many vascular plants and is one of the main biodiversity centres in Europe (Thompson 2005; Hewitt 2011; Nieto Feliner 2014;

Rešetnik and Španiel 2022). Mount Olympus is one of the important mountains in the Balkans and the highest in Greece (2,918 m a. s. l.). It is situated in the southern-east part of the north-central (NC) floristic region in Greece and currently at least 1,700 plant species and subspecies are recorded in its wider area (c. 25% of the Greek flora). Of these, 60 are Greek endemics and 28 local endemics (Strid 1980; Strid and Tan 1986; Strid and Tan 1991; Tsiftsis and Antonopoulos 2017). To protect its unique wildlife (flora and fauna), Mount Olympus was established by the Greek Government as Greece's first national park.

Protecting plants in their natural environment (*in situ* conservation) is the main method used in their conservation. However, *in situ* conservation is not always efficient, despite the efforts and resources invested in it (Johnson et al. 2017). In such cases, conserving plants away from their natural habitat (*ex situ* conservation) could be more efficient way of protecting endangered species from external threats. Moreover, material for reintroduction, translocation, reinforcement, and habitat restoration can be produced by *ex situ* conservation. This material can be utilized to halt and even reverse the extinction trend and preserve plant diversity for future generations (Mounce et al. 2017).

The practice of plant conservation using *ex situ* propagation has been around for several years. The reproduction of wild plants from seed is a generally effective conservation action. However, there are many uncertainties, concerning appropriate species-specific propagation techniques, for most plant species of conservation concern, particularly rare and/or endemic species (Cerabolini et al. 2004). Nevertheless, studying seed germination is crucial because it is an essential step in the successful reproduction of a given plant species (Baskin and Baskin 2014).

Germination tests are the most effective method for providing a protocol for *ex situ* conservation (Katsalirou et al. 2019; Margreiter et al. 2020). Knowing the viability level is crucial for developing a reproduction protocol, since it reveals the potential reproduction dynamics, because germination is usually a lengthy process. Such knowledge will allow for the calculation of the number of seeds needed to reproduce a certain number of individuals for restoration purposes.

The seeds of many species of plants often undergo periods of dormancy with innate mechanisms securing the appropriate timing of seed germination in the wild (Baskin and Baskin 2014). To break the seed dormancy of a species, it is necessary to use different pre-treatments depending on the type of dormancy (e.g., morphological, physiological) and its intensity (deep, light, or intermediate) (Baskin and Baskin 2001). Usually, the type of dormancy in a population of a plant species is strongly affected by the climate, without this being a panacea (Cotado et al. 2020). For example, it is quite common for species that grow in colder environments or on northern slopes to produce seeds with physiological dormancy. Cold stratification is suggested as a trigger to initiate germination, because this pre-treatment imitates the low winter temperatures that prevail in their natural environment. Although this pre-treatment is simple, it is quite effective, especially for species that originate from high altitudes.

The pre-treatment of seed with various hormones has replaced the time-consuming stratification that is often required to initiate the germination of deeply dormant seeds. The hormones are naturally present in the seeds and appear to be the keys to breaking dormancy and initiating germination. It is commonly thought that dormancy release is due to an increase in the levels of cytokinin (KIN) or gibberellic acid (GA3) or both, even if it is not yet completely clear how exactly they work (Shu et al. 2016). However, placing seeds in a hormone solution seems to have a positive effect on terminating dormancy in several species and at the same time, germination is more uniform (Bewley and Black 1985).

Although optimal germination protocols are available for numerous species of plants, the specific requirements of narrowly distributed species (e.g., endemics, species with narrow niche) are usually unknown. Therefore, the aim of the present study was to determine the

germination requirements of three stenoendemic species on Mount Olympus (*Centaurea incompleta* Halácsy, *Centaurea litochorea* T. Georgiadis and Phitos and *Viola striis-notata* [J. Wagner] Merxm. and W. Lippert) and to evaluate the effects of cold stratification and the use of gibberellic acid (GA3) for improving germination.

Materials and methods

Species studied and seed collection

In total, the following three stenoendemic species, characterized by different ecological preferences, were studied:

- Centaurea incompleta* Halácsy is one of the rarest endemic species on Mt. Olympus, known only from three microsites on limestone rocks, occurring at 400–800 m (Strid 1980).
- Centaurea litochorea* T. Georgiadis and Phitos is an endemic and known only from a few microsites on the eastern and southern slopes. It prefers rocky slopes at 950–1,800 m on limestone (Strid and Tan 1991; Constantinidis 2009).
- Viola striis-notata* (J. Wagner) Merxm. and W. Lippert is a rare subalpine species, which occurs at 2,400–2,900 m, where it is exclusively found growing in mobile screes (Strid 1980; Strid and Tan 1986).

Mature seeds of *Centaurea incompleta*, *C. litochorea* and *Viola striis-notata* were collected from Mt. Olympus during the summer of 2021, extracted from the infructescence of plants and kept at room temperature until February 2022, when viability and germination tests began.

Seed viability tests

Two random samples (i.e., repetitions) of 25 seeds each were used for the estimation of seed viability. Initially, the seeds were soaked in water for 12 hours after the testa of each seed was abraded using a dissecting needle in order to make it easier to remove the embryos. Seeds were grouped into two categories during seed dissection: filled (contained an embryo) and empty (did not contain any gametophytic tissue). Empty seeds or seeds with atrophic embryos were considered non-viable (Figs 1a,b). The viability of the extracted embryos was determined by staining them sequentially with two dyes. The method used for staining was:

- Staining with a 1% w/v tetrazolium chloride solution (abbr. TTZ) (ISTA 1999), and
- Staining with 0.25% w/v Evans blue solution (Busso et al. 2005; Busso et al. 2015)

The embryos were treated with tetrazolium chloride solution, and the embryos that were not stained and therefore initially considered to be non-viable were then immersed in a solution of Evans blue dye. Thus, the percentage of non-viable embryos is the percentage of stained embryos after the Evans blue test. Consequently, the percentage of non-germinable seeds is the

sum of the percentages of empty seeds and those with a non-viable embryo. Both solutions were used due to the different properties of their active substances. Tetrazolium chloride solution only stains tissues red that are metabolically active (so-called viable tissues), whereas the Evans blue solution only stains dead tissues, which results in an accurate evaluation of embryo viability (Busso et al. 2015).

In the Evans blue staining of embryos they were left in the dye for 30 min, then examined every 30 min for 6 hours to determine the time of imbibition. There was no difference in the staining patterns of embryos after 30 minutes (Figs 1c,d) in both species of *Cen-*

taurea, whereas even the viable embryos of *Viola striis-notata* turned blue after two hours in Evans blue dye (Figs 2a,b). Therefore, the staining pattern in *Viola striis-notata* was estimated after staining with Evans blue dye for 90 min.

An embryo with more than 50% of its surface tissue stained red in TTZ was considered viable. Unstained TTZ embryos that did not turn blue in Evans blue dye were also considered viable (Figs 2c,d). Finally, the embryos were grouped into four categories, stained and unstained in TTZ, as well as stained and unstained in Evans blue dye. Average percentages in each category were calculated based on 25 seeds in each repetition.

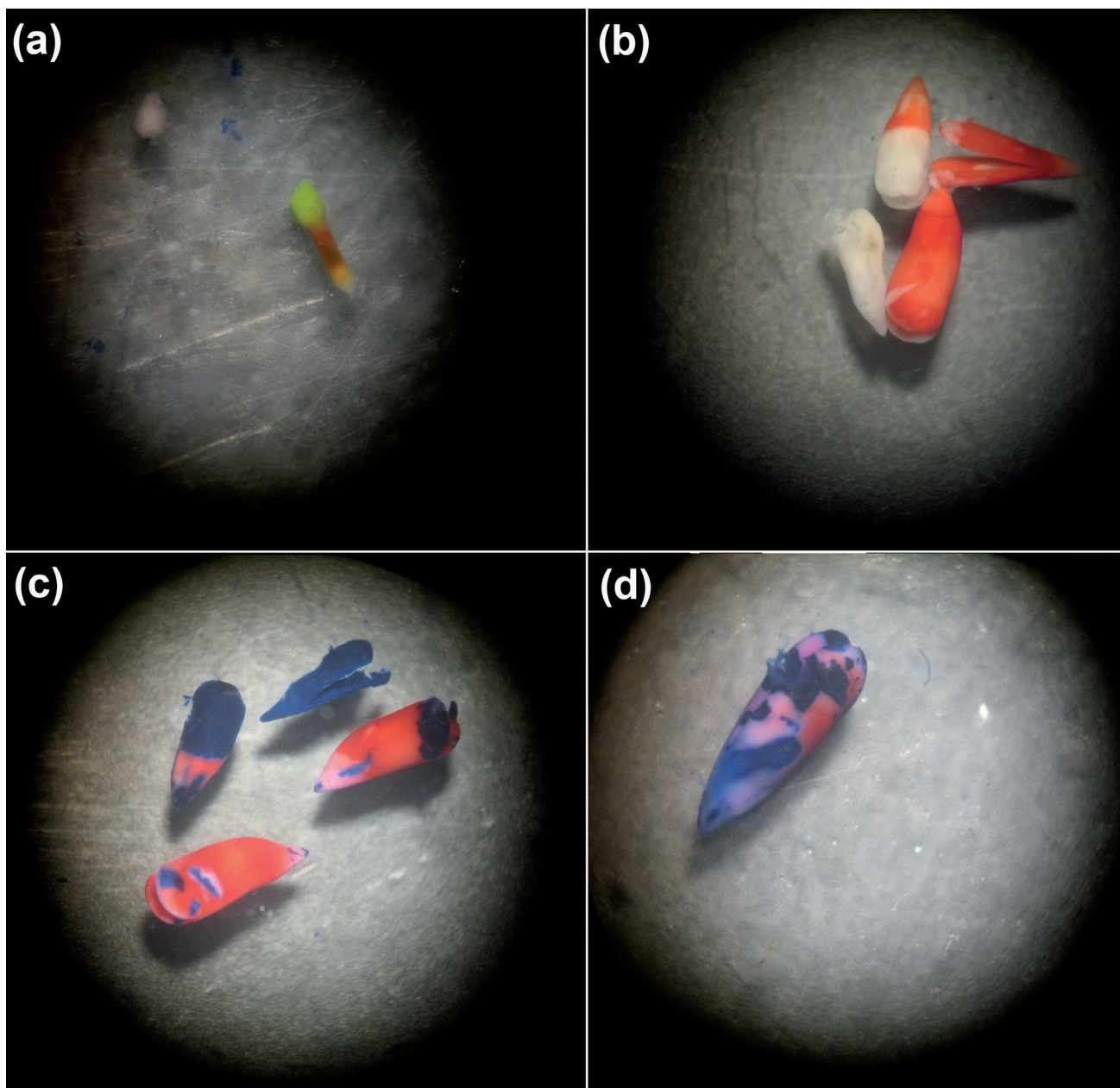


Fig. 1 (a) Atrophic (non-viable) embryo of *Viola striis-notata*; (b) Stained (viable), partially unstained (non-viable) and unstained using Tetrazolium (atrophic *Centaurea incompleta* embryos were considered to be non-viable); (c) Stained (non-viable) and unstained (viable) embryos of *Centaurea incompleta* using Evans blue; (d) Unstained (viable) embryos of *Centaurea litchorea* using Evans blue (local blue colouring in some areas is superficial due to slight damage caused by dissecting needle during extraction of the embryo).

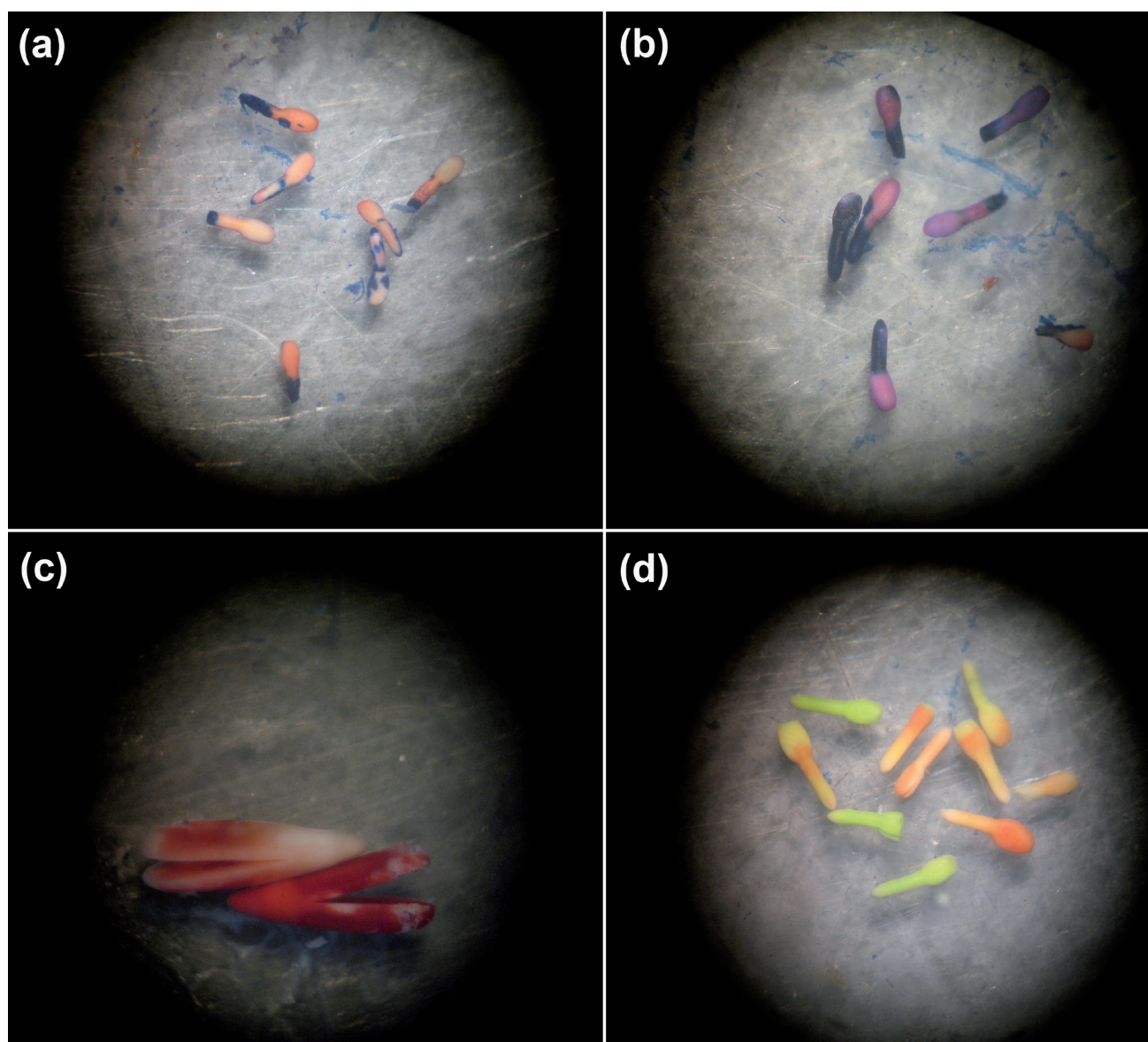


Fig. 2 (a) Stained (non-viable) and unstained (viable) embryos of *Viola striis-notata* using Evans blue after 90 min; (b) *Viola striis-notata* embryos using Evans blue after 2 hours; (c) Stained (viable) and partially unstained (non-viable) embryos of *Centaurea litchorea* using Tetrazolium; (d) Stained (viable) and unstained embryos of *Viola striis-notata* using Tetrazolium.

Germination test

Germination test was based on 4 repetitions of 50 seeds for *C. incompleta* and *C. litchorea* and 4 repetitions of 25 seeds for *Viola striis-notata*. Seeds in each repetition were visually inspected under a ZEISS STEMI 2000-C stereoscope and empty seeds were discarded and replaced. It should be noted here that, especially in *Centaurea* species, it is easy to distinguish empty from filled seeds when magnified.

Seeds were subjected to one of two treatments, cold stratification or GA3hormone. For the hormone treatment, the seeds were soaked in a solution of 250 ppm gibberellic acid for 48 hours at room temperature in the dark between two moistened filter papers. After 48 hours, the seeds were placed in Petri dishes on top of filter paper and wet sterile sand. As for the strati-

fication, the seeds were placed in Petri dishes on top of filter paper and wet sterile sand and were put in a refrigerator at 2 °C for two months. Upon completion of treatments, seeds were placed for germination in a growth chamber under alternating conditions of temperature (25/15 °C) and photoperiod (8/16 h, light/dark) for 12 weeks. To evaluate the treatment effectiveness, control Petri dishes were also put in the growth chamber, containing 4 × 50 untreated seeds of *C. incompleta* and *C. litchorea* and 4 × 25 untreated seeds of *Viola striis-notata*. Germination was recorded every seven days for 12 weeks. When the germination test ended, the non-germinated seeds were dissected, and any empty seed left removed. Germination percentages were corrected based on the total number of full seeds per repetition.

Data analysis

Germination data were checked for normality and homogeneity using Shapiro-Wilk's and Levene's tests, respectively, and found to meet both assumptions. Differences in mean values were checked using one-way ANOVA followed by Tukey's test at 5% level of significance.

Results

A significantly higher percentage of empty seeds (without embryo or any gametophytic tissue) was recorded during the viability test for both species of *Centaurea* (*C. incompleta*: 40%; *C. litochorea*: 54%; Table 1). However, the two-dye treatment revealed that the embryos in full seed were viable. In contrast, no empty seeds were recorded in the case of *Viola striis-notata* and the viability test also revealed a high percentage of potentially germinable seeds (80%). After the two-staining test, most of the full seeds was recorded as viable (*C. incompleta*: 93.3%; *C. litochorea*: 100%; *V. striis-notata*: 80%).

The highest percentage germination of *C. incompleta* (57.73%) was recorded after the seed was treated with GA3 and was significantly higher ($P < 0.001$) than the germination percentage after cold stratification (49.74%), which in turn was significantly higher ($P < 0.001$) than

the control (14.60%) (Fig. 3). It should also be noted that the maximum germination was recorded in the control and the cold stratification by the 4th week, whereas for the GA3 treatment it was by the 6th week.

Unlike in *C. incompleta*, the control rather than the treatment with GA3 or cold stratification of *C. litochorea* resulted in the highest germination (68.00%, 53.45% and 44.68% respectively) (Fig. 4). Statistically significant differences were only recorded in the comparison between the control and the cold stratification treatment ($P < 0.01$). The maximum germination in the cold stratification and control treatments was recorded by the 3rd and 4th week, respectively, whereas the percentage in the GA3 treatment increased rapidly until the 6th week, and then slightly up to the 10th week.

For *Viola striis-notata*, treatment of seed with GA3 hormone resulted in the highest germination (71.21%), whereas significantly lower germination percentages ($P < 0.001$) were recorded for seed either cold stratified or subjected to no treatment at all (control seeds) (Fig. 5). Although the percentage germination when cold stratification was used was higher than in the control, the differences were not statistically significant according to the one-way ANOVA and Tukey's test. The maximum percentage germination was recorded by the 4th (control) and 5th week (cold stratification and GA3).

Table 1 Results of the viability tests on the three species studied.

| Species | Seed category | | Tetrazolium staining | | Evans blue staining | | Total number of seeds with viable embryos | Total number of seeds that did not germinate | Total |
|-----------------------------|---------------|------------|--------------------------|--------------------------------|----------------------------|------------------------------|---|--|-------|
| | Empty seeds | Full seeds | Stained embryos (viable) | Unstained embryos (non-viable) | Unstained embryos (viable) | Stained embryos (non-viable) | | | |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) = (4) + (6) | (9) = (2) + (7) | (10) |
| <i>Centaurea incompleta</i> | 40% | 60% | 40% | 20% | 16% | 4% | 56% (93.33%*) | 44% | 100% |
| <i>Centaurea litochorea</i> | 54% | 46% | 36% | 10% | 10% | 0% | 46% (100%*) | 54% | 100% |
| <i>Viola striis-notata</i> | 0% | 100% | 60% | 40% | 20% | 20% | 80% (80%*) | 20% | 100% |

* Percentages are for full seeds.

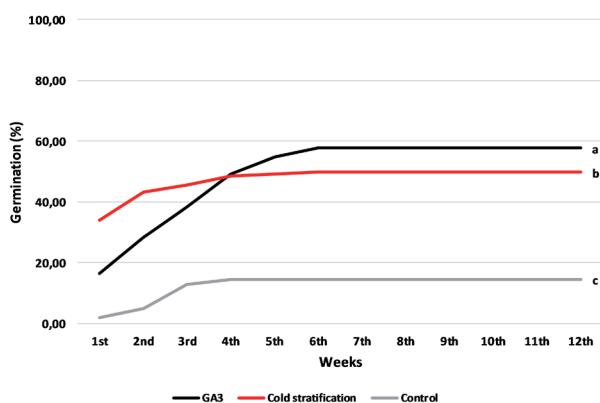


Fig. 3 Cumulative germination recorded for seed of *Centaurea incompleta* in the different treatments.

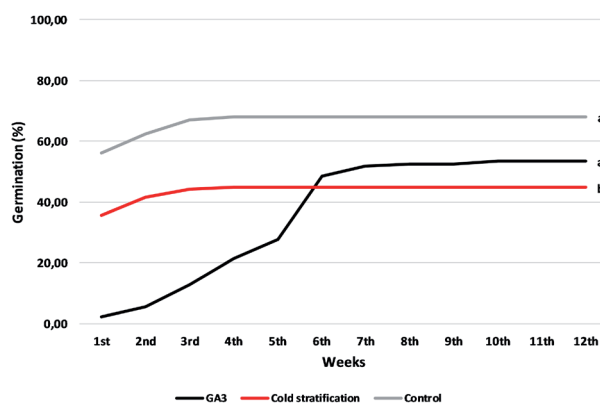


Fig. 4 Cumulative germination recorded for seed of *Centaurea litochorea* in the different treatments.

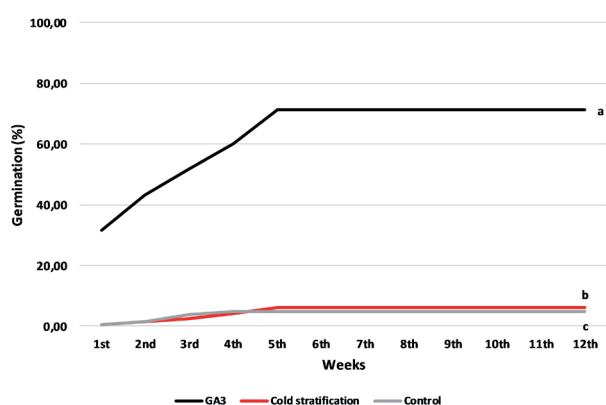


Fig. 5 Cumulative germination recorded for the seed of *Viola striis-notata* in the different treatments.

Discussion

Mount Olympus has a rich and diverse flora, which is characterized by a significant number of local endemic species (Strid 1980; Strid and Tan 1986; Strid and Tan 1991). Some of them occur widely above 2,000 m a. s. l. (e.g., *Potentilla deorum*, *Achillea ambrosiaca*), whereas others, despite their broad altitudinal range, occur at a limited number of microsites (e.g., *Centaurea litchorea*). Endemic species, and especially those that are range-restricted, are expected to become vulnerable and strongly affected by climate change or stochastic events (Trigas et al. 2012). The increasing threat to these species requires urgent action for their conservation. With this in mind, it is highly advisable to develop successful reproduction protocols with which species' genetic diversity will be preserved and their *ex situ* conservation, with a view to their reintroduction, will be guaranteed.

Here, the specific germination requirements of three stenoendemic species on Mount Olympus (*Centaurea incompleta*, *Centaurea litchorea* and *Viola striis-notata*) were determined to find the optimal treatment for maximizing their percentage germination. The two species of *Centaurea* studied (*C. incompleta* and *C. litchorea*) produced a high number of empty seeds (40% and 54%, respectively). This can be attributed to various reasons, such as pollination failure due to random environmental constraints (i.e., low spring temperature), genetic drift or correlated paternity if we consider that both species occur in isolated, small populations (Hardy et al. 2004; Bossuyt 2007). In addition, the reproductive (i.e., mating) system can affect successful pollination and later embryo formation (Zheljzakov et al. 2022). Even though there is no information in the literature on the mating system of the species of *Centaurea* studied, it is generally known that other species of *Centaurea* species are self-incompatible (e.g., Sun and Ritland 1998; Bellanger et al. 2015; Abrahamczyk et al. 2021). Thus, it is possible that self-incompatibility of these narrow endemic species in combination with their small effective population size

may lead to pollination failure, low embryo formation and consequently to an increased proportion of empty (non-viable) seeds.

Unlike the two species of *Centaurea*, the percentage of empty seeds recorded for *Viola striis-notata* was nil. This may be because unfertilized ovules are aborted (Miyajima 2006), which in turn might be related to abiotic stresses (Sun et al. 2004). Moreover, the genus *Viola* includes both cleistogamous and non-cleistogamous taxa (Marcussen et al. 2015). In the Northern Hemisphere, most species of *Viola* can produce cleistogamous flowers (Culley and Klooster 2007). Although cleistogamy is not reported for *V. striis-notata*, it is an adaptation ensuring seed production in harsh environments in which pollinators are rare or absent.

The tetrazolium chloride viability test is a standard procedure for estimating embryo viability and potential germination since it stains seed tissues that are metabolically active and has long been used in studies on different plant taxa (França-Neto and Krzyzanowski 2022). *Viola striis-notata* and *Centaurea incompleta* produced seeds with embryos that were not stained by tetrazolium dye. A percentage of them, also not stained by Evans blue, were considered viable. Thus, it is possible that some of the seeds are in deep dormancy, which would indicate intrapopulation variability in the expression of seed dormancy (Kildisheva et al. 2020). In contrast, all the seeds of *C. litchorea* with embryos that were not stained by the tetrazolium dye were unstained by Evans blue and were all considered to be viable. The three species studied differed in the percentages of full seeds and seeds with viable embryos. Although *C. litchorea* had the lowest percentage of full seed, they were all viable. In contrast to *C. litchorea*, all seeds of *V. striis-notata* were full, but only 80% of them were viable. This cannot be accounted for and should be the subject of future research.

The period of dormancy of the seeds of many species of plants varies depending on innate mechanisms that result in them germinating at the appropriate time in the wild. Species-specific research on the treatments required to overcome seed dormancy are required, but this can sometimes be extremely difficult and time consuming (Baskin and Baskin 2014). The existence of deep dormancy was verified in *C. incompleta* since GA3 and cold stratification resulted in higher percentages of germination than recorded in the control. Dormancy was also recorded in *Viola striis-notata*, in which seeds treated with GA3 germinated better than control seeds. However, this was not recorded in *C. litchorea* where both treatments resulted in lower percentages of germination than recorded in the control indicating a weaker dormancy, which was easily terminated, whereas the two treatments (cold stratification and GA3) delayed germination.

Species-specific reproduction protocols enabling propagation from seeds are very important for plant conservation and the ecological restoration of disturbed

habitats (Kildisheva et al. 2020). Although the treatments used did not increase the percentage germination above that of the control for one (*C. litochorea*) of the species studied, they were successful for two species (*C. incompleta* and *V. striis-notata*), as they terminated seed dormancy and initiated germination. However, in addition to the studies on germination and the conditions required for terminating dormancy, the successful transplantation and establishment of young seedlings in pots are also crucial for *ex situ* plant conservation.

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