Seed germination capability of four endemic species in the Central Apennines (Italy): relationships with seed size

Anna Rita Frattaroli (*), Luciano Di Martino (**), Valter Di Cecco (*), Rosangela Catoni (**), Laura Varone (**), Marco Di Santo (**) & Loretta Gratani (***)


Seed germination capability of *Adonis distorta*, *Androsace mathildae*, *Aquilegia magellensis* and *Campanula fragilis* subsp. *cavolinii* endemic species of the Central Apennines (Italy) were analyzed. Seed traits varied significantly among the considered species. In particular, seed volume was the largest in *Adonis* (91.642 ± 16.851 mm³) and the lowest in *Campanula* (0.029 ± 0.008 mm³). The seed coat thickness ranged from 31 ± 10 µm in *Adonis* to 9 ± 1 µm in *Campanula*. Pretreatments were carried out to improve seed germination. Seed germination did not happen in *Adonis* and *Androsace* in response to the applied treatments (i.e., 0, 250, 500 ppm gibberellic acid, GA3) and the cold-wet stratification. A 65% increase of germination was observed after the pre-treatment with 500 ppm GA3 in *Aquilegia* which could be justified by an endogenous non-deep physiological dormancy. The final germination percentage increased by 26% in *Aquilegia* and decreased by 89% in *Campanula* after the cold-wet stratification treatment. The obtained results were used to define germination protocols which could be used in reinforcement projects for the wild populations of the considered endemic species as a means of reducing their extinction risk.

Keywords: endemics, seed germination, seed size, seed dormancy.


En este trabajo se ha analizado la capacidad germínativa de cuatro especies endémicas de los Apeninos centrales: *Adonis distorta*, *Androsace mathildae*, *Aquilegia magellensis* y *Campanula fragilis* subsp. *cavolinii*. Los rangos de las semillas variaron significativamente entre las especies, particularmente se encontró que el volumen de semillas en *Adonis* (91.642 ± 16.851 mm³) era el más alto y en *Campanula* el más bajo (0.029 ± 0.008 mm³). El grosor de la cubierta seminal se encontraba desde 31 ± 10 µm en *Adonis* hasta 9 ± 1 µm en *Campanula*. Se realizaron algunos pretratamientos para mejorar la germinación como fue la adición de distintas concentraciones de ácido giberélico (i.e., 0, 250, 500 ppm giberellic acid, GA3) o la estratificación frío-caliente. Sin embargo no detectamos germinación ni en *Adonis* ni en *Androsace*. En el caso de *Aquilegia* se observó un aumento del 65 % de la germinación después de pretratarla con 500 ppm GA3, lo cual queda justificado por una dormancía fisiológica endógena no muy profunda. El porcentaje de germinación final se incrementó en *Aquilegia* en un 26%, mientras que decreció un 89% en *Campanula* después del tratamiento de estratificación frío-caliente. Los resultados obtenidos fueron utilizados para definir los protocolos que deben ser utilizados para mejorar la conservación de especies endémicas en riesgo de extinción.

Palabras clave: especies endémicas, germinación, tamaño de semilla, dormancia
INTRODUCTION

Knowledge of rare species life-cycle and reproductive traits is essential for identifying limits to population growth and persistence (Bevill & Louda, 1999) especially in threatened wild species. Seed germination is a critical stage for the establishment of the plant (Youssef & al., 2012) as its success is determinant for plant species propagation (Rajou & al., 2012). Each species has specific requirements for seed germination (Harper & al., 1970; Meyer & Monsen, 1991; Schütz & Milberg, 1997) which involves particular features of seed and environmental factors (Baskin & Baskin, 1998). Many authors (Jakobsson & Eriksson, 2000; Bonito & al., 2011; Lönnberg & Eriksson, 2013) underline the relationship between seed germination and seed size. Seed size represents the amount of maternal investment in the individual offspring (Leishman & al., 2000). Generally, large seeds have a greater germination success than small seeds (Pizo & al., 2006). Seed coat plays an important role in embryo nutrition during seed development and against detrimental agents from the environment (Mohamed-Yassee & al., 1994; Weber & al., 1996). Seed coat-imposed dormancy is part of the seed survival strategy of many species (Wekker, 1981; Kelly & al., 1992; Schütz, 2000). Moreover, seed coat exerts a germination-restrictive action most of the time by being impermeable to water and/or oxygen or by its mechanical resistance to radical protrusion. Seed dormancy is a trait that has been acquired by many species through selection for the ability to survive in unfavourable environments (Bevwy & al., 2013). Seed dormancy can be viewed as an adaptive mechanism for survival in a seasonally variable environment (Westoby, 1981). Under natural conditions, dormant seeds are exposed to changes in environmental factors (e.g. light, temperature, moisture) which lead to cyclical changes in the dormancy state (Finkelstein & al., 2008). Many high mountain plants produce seeds with different types of dormancy to avoid germination in the year of seed dispersal and favour rapid emergence after snowmelt (Billings & Mooney, 1968; Baskin & Baskin, 1998; Shimono & Kudo, 2005). Several germination traits have been claimed to be specific to high-altitude species (i.e. rapid onset of germination after snowmelt and high seed viability; Giménez-Benavides & Milla, 2013).

Endemic species are a significant feature of the Mediterranean mountains (Gómez-Campo, 1985; Väre & al., 2003; Sainz & Moreno, 2002; Galván & al., 2002; Escribá & al., 2007; Fuente & al., 2011) because of the high number of speciation events that have occurred (Martín-Bravo & al., 2010). Mediterranean mountains are characterized by a high genetic diversity with many populations being genetically unique (Ruiz-Labourdette & al., 2012). Predictions of climate change indicate that this genetic diversity could be disturbed significantly in the future (Thuller & al., 2005). Moreover, Mediterranean mountains are considered one of the most threatened ecosystems in the European Union (Gómez-Campo, 1987; European Community, 1992). Thus, many efforts should be addressed to improve the conservation strategies for Mediterranean mountain species considering that the survival of endemic and threatened species is based on different and complementary conservation approaches and techniques (IUCN, 2002). The definition of germination protocols, in particular for species characterized by small populations and for which data are missing, could be an important step in this direction.

The aim of this work focused on seed germination capability of four endemic species growing in the Central Apennines, in Italy. In particular, we analysed the influence of different pre-treatments on seed germination for Adonis distorta Ten., Androsace mathildae Levier, Aquilegia magellensis F. Conti & Soldano and Campanula frigilis Cirillo subsp. cavolinii (Ten.) Damboldt. The results may be used for conservation projects of the wild populations of these endemic species.

MATERIALS AND METHODS

STUDY SITE AND SPECIES

Experiments were carried out in the Majella Seed Bank within the Botanical Garden Michele
Tenore (42° 2' 59'' N; 14° 11' 34''E; 650 m a.s.l., Italy) on seeds collected from the wild populations of *Adonis distorta*, *Aquilegia magellensis* and *Campanula fragilis* subsp. *cavolinii* growing on Mount Majella. Hereafter, to facilitate reading, each species will be referred to the name of Genus. All the species are endemic of the Central Apennines and are included in the Regional Red List (CONTI & al., 1997). In particular, *Adonis* (Ranunculaceae) grows in the Marche, Umbria, Lazio and Abruzzo regions (CONTI & al., 2005) on high-altitude screes (2000-2500 m a.s.l.) characterized by small clasts (PIGNATTI, 1982). In Abruzzo, populations of few individuals grow in the Gran Sasso Massif, Velino Mount, Sirente Mount and Majella Mount. *Adonis* is included in the II and IV Annex of the Habitat Directive (Habitat Natura 2000) and categorized as DD (Data Deficient) in the IUCN Red List. *Aquilegia* (Ranunculaceae) is endemic of the Abruzzo region (CONTI & al., 2005) where it grows at Gran Sasso Massif and Majella Mount (2500-2900 m a.s.l.) (PIGNATTI, 1982) on cracks of limestone cliffs, mainly in the northern exposure. It is included in the II and IV Annex of the Habitat Directive (Habitat Natura 2000) and categorized as DD (Data Deficient) in the IUCN Red List. *Campanula* (Campanulaceae) grows in Lazio, Abruzzo, Molise and Campania regions (CONTI & al., 2005) on limestone cliffs from 200 to 1800 m a.s.l. (PIGNATTI, 1982). To date, there are no studies regarding the conservation strategies and seed germination capability of the selected species.

The climate of the Mount Majella, is characterized by a mean minimum air temperature \((T_{\text{min}})\) of -3.9 ± 2.2 °C (February), a mean maximum air temperature \((T_{\text{max}})\) of 22.3 ± 0.1 °C (July-August) and a mean annual air temperature \((T_{\text{mean}})\) of 7.6 ± 6.5 °C. Total annual rainfall is 1343 mm. Snow fallen from December to April (Meteorological Station of Passo Lanciano, Ch, 42° 18' 62''; 14° 09' 87'', data for the period 2000-2012, cetemps.aquila.infn.it).

**SEED COLLECTION**

Freshly-matured seeds of *Adonis, Androsace, Aquilegia* and *Campanula* were collected from the small wild populations growing on Mount Majella, from August to September 2010, in the fruiting period and immediately before dissemination, according to HAY & SMITH (2003). The mother plants were randomly selected, according to MARSHALL & BROWN (1983) (Table 1). In particular, seeds of *Adonis* were collected at 2675 m a.s.l, on the north-northeast facing of Mount Focalone (42° 6' 18'' N; 14° 7' 10'' E); seeds of *Androsace* at 2760 m a.s.l in the north facing of Mount Amaro (42° 5' 12'' N; 14° 7' 14'' E); seeds of *Aquilegia* at 1225 m a.s.l on the south-west facing of the Eremo di San Giovanni (42° 9' 14'' N; 14° 4' 50'' E), and seeds of *Campanula* at 750 m a.s.l in the north-east facing of Lama dei Peligni (42° 0' 09'' N; 14° 8' 44'' E). Seeds \((n = 300\) for *Adonis, Androsace* and *Aquilegia; n = 2000* for *Campanula*) were immediately transported to the Botanical Garden.

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Life Form</th>
<th>Chorotype</th>
<th>Altitude</th>
<th>Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Androsacea mathildae</em></td>
<td>Primulaceae</td>
<td>ChPulv</td>
<td>End.App.Abr.</td>
<td>2760.</td>
<td>NNE</td>
</tr>
<tr>
<td><em>Adonis distorta</em></td>
<td>Ranunculaceae</td>
<td>HScap</td>
<td>End.App.C.</td>
<td>2675</td>
<td>NNE</td>
</tr>
<tr>
<td><em>Aquilegia magellensis</em></td>
<td>Ranunculaceae</td>
<td>HScap</td>
<td>End.App.C.</td>
<td>1225</td>
<td>SW</td>
</tr>
<tr>
<td><em>Campanula fragilis</em></td>
<td>Primulaceae</td>
<td>ChSuffr</td>
<td>End.App.C.</td>
<td>750</td>
<td>NE</td>
</tr>
</tbody>
</table>
SEED TRAITS MEASUREMENTS

At the Majella Seed Bank seeds were surface-sterilized in a 1% sodium hypochlorite solution for 1-10 minutes, according to the seed coat type (Bacchetta & al., 2006), then rinsed in distilled water. The seed coat after the imbibition in distilled water was dried and then the experiment was carried out. Seeds were placed in paper bags at 5°C until the germination experiment started. Three groups of 100 seeds each for Adonis, Androsace and Aquilegia, and two groups of 1000 seeds each for Campanula (seeds < 0.010 mg) were weighed to measure seed fresh mass (SM), according to Cerda & Garcia-Fayos (2002). The reduced number of seeds used was justified by the small number of the wild populations of the considered species in Majella National Park. In particular, there were few Adonis populations for a total number of ca. 1500 plants, and few Androsace populations for a total number of ca. 400 plants (non-published data from the Park). Despite Aquilegia being largely distributed in respect to the other two species (van Gils & al., 2012), there are no data about the number of populations and the number of plants per population. No data about number and size of populations are available for Campanula.

Seed length (L, longest axis), width (W, intermediate axis) and thickness (T, shortest axis) (20 seeds per species) were measured, according to Cerda & Garcia-Fayos (2002); from these data, seed surface (S = L x W), volume (V = L x W x T), density (D = SM/V) and the ratio S/SM (surface/mass) were calculated. To characterize seed shape, the Eccentricity Index (E.I. = L/W) was used (Balkaya & Odabas, 2002). Seed coat thickness was measured at 3 or 4 points of each seed by a stereo-microscope (Leica Wild M10), according to Tunjaï & Eilıott (2012).

GERMINATION EXPERIMENT

The seed germination experiment was carried out on seeds of the considered species which were transferred to an agar medium (7 g l⁻¹) (Morgan & al., 1997). Media was integrated with 4.4 g l⁻¹ of Murashige Skoog salts (MS; Murashige & Skoog, 1962) because it provided nutrients to allow the seedlings growth (Pence, 1999). We followed this procedure because we wanted to cultivate the seedlings in the Botanical Garden and use them in reinforcement programs of wild populations in Majella National Park.

The pH was stabilized at 5.5 and autoclaved at 120 °C and 2 atm for 20 min (Cerabolini & al., 2004). Seeds were transferred to Petri dishes (90 x 10 mm each) and sown under a laminar flow hood. Petri dishes were transferred to the growth chamber for 30-d incubation (Bacchetta & al., 2006). Germination tests were performed in a light and temperature controlled growth chamber (Angelantoni Ekoch 700, Italy) at 20° C constant temperature and photoperiod of 12 h in the light and 12 h in the dark (Bacchetta & al., 2006). The chamber was equipped with cool-white fluorescent tubes providing a photon flux density (PFD) of 22 µmol (photon) m⁻² s⁻¹. Seeds showing radicle emergence were recorded as ‘germinated’ (Côme, 1970).

The following treatments were carried out for the considered seed types: control treatment (0 ppm GA3 treatment); 250 ppm GA3 treatment; 500 ppm GA3 treatment; cold-wet stratification treatment. Each of the considered treatments consisted of two replicate of 25 seeds each (Cerabolini & al. 2004) for Adonis, Androsace and Aquilegia, and 50 seeds for Campanula. The number of germinated seeds was counted every day for 30 days to evaluate the dynamics of germination, according to Bacchetta & al. (2006). The low number of seeds per replicate and the low number of replicates in each experiment was due to the limited seeds availability because of the species were rare and there were few populations (Mattaña & al., 2012).

PRE-GERMINATION SEED TREATMENT

Gibberellic acid (GA3)

Seeds were imbibed for 24 h in either 250 ppm and 500 ppm GA3 and distilled water (0 ppm, control) (Rodrguezs Pérez, 1993).

COLD-WET STRATIFICATION TREATMENT

The seeds were subjected to a cold–wet stratification treatment to simulate chilling conditions under snow-pack typical of high elevation moun-
The pots were filled with sand and soil and wetted with distilled water to ensure humid (Bacchetta & al., 2006). The pots were wrapped in aluminium foil and stored in a refrigerator at 5 °C for 3 months before germination tests. The refrigerator (Angelantoni EKOFRIGOLAB 1500) was equipped with a display that show continuously the temperature inside, and a microprocessor control system with audible and visual alarm systems.

The cold-wet treatment was extended up to 9 months for Adonis and Androsace seeds.

DATA ANALYSIS

The dynamics of germination was determined by the Weibull function (Weibull, 1951; Johnson & Kotz, 1970) from the following formula:

\[ y = M \{ 1- e^{[k(t-z)]c} \} \]

where y was the germination percentage at time t (days); M the final germination at 30 d; z the germination delay; c the curve shape parameter (ranging from 0 to 3) obtained by optimising the sum of the squared differences.

The relative germination rate (k) was determined from the following equation:

\[ k = 1 / (T_{50} – z) \]

where T_{50} was the half-germination time (i.e. number of days in reaching 50% of final germination) calculated from the formula of Coolbear & al. (1980) modified by Thanos & Doussi (1995):

\[ T_{50} = [(N/2) – N_1) x (T_2-T_1)] / N_2-N_1 \]

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

Pearson’s correlation analysis was performed to evaluate the correlation among the considered seed traits (L, W, T, S, V, D, Ratio S/SM, E.I.).

One way ANOVA was performed to analyze differences in seed traits among the considered species followed by a post-hoc Tukey’s test to compare differences among means (Statistica, Stasoft, USA). Moreover, in order to test the interactive effect of the treatments and species on M, T_{50} and z, a 2×4 factorial design was performed by a generalized linear model (GLM) in R (R Development Core Team, 2011).

RESULTS

SIZE SEED

Seed traits of the considered species are shown in table 2. L ranged from 7.1 ± 0.4 mm in Adonis to 0.5 ± 0.1 mm in Campanula. W ranged from 3.92 ± 0.55 (Adonis) to 0.24 ± 0.02 mm (Campanula) and T from 3.31 ± 0.30 mm (Adonis) to 0.24 ± 0.02 mm (Campanula). S varied from 0.12 ± 0.02 mm² in Campanula to 27.6 ± 4.68 mm² in Adonis and V from 0.029 ± 0.008 mm³ in Campanula and to 91.6 ± 16.85 mm³ in Adonis. The mean value of D was 0.45 ± 0.16 mg mm⁻³ with Androsace having the highest value (0.62 ± 0.10 mg mm⁻³) and Adonis the lowest one (0.30 ± 0.05 mg mm⁻³).

<table>
<thead>
<tr>
<th>Species</th>
<th>L (mm)</th>
<th>W (mm)</th>
<th>T (mm)</th>
<th>S (mm²)</th>
<th>V (mm³)</th>
<th>D (mg mm⁻³)</th>
<th>Ratio S/SM</th>
<th>E.I.</th>
<th>Seed coat T (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adonis distorta</td>
<td>7.1±0.4a</td>
<td>3.92±0.55a</td>
<td>3.31±0.30a</td>
<td>27.6±4.68a</td>
<td>91.6±16.85a</td>
<td>0.30±0.05a</td>
<td>1.0±0.2a</td>
<td>1.82±0.24a</td>
<td>31±10a</td>
</tr>
<tr>
<td>Androsacea mathildae</td>
<td>2.3±0.2b</td>
<td>1.52±0.14b</td>
<td>0.70±0.06b</td>
<td>0.79±0.07b</td>
<td>2.48±0.49b</td>
<td>0.62±0.10b</td>
<td>2.4±0.4b</td>
<td>1.52±0.11b</td>
<td>27±5a</td>
</tr>
<tr>
<td>Aquilegia magellensis</td>
<td>1.9±0.1b</td>
<td>0.88±0.07c</td>
<td>0.79±0.07b</td>
<td>1.64±0.16c</td>
<td>1.29±0.16c</td>
<td>0.56±0.07b</td>
<td>2.3±0.2b</td>
<td>2.12±0.23c</td>
<td>26±2a</td>
</tr>
<tr>
<td>Campanula fragilis subsp. cavolini</td>
<td>0.5±0.1c</td>
<td>0.24±0.02d</td>
<td>0.24±0.01c</td>
<td>0.12±0.02d</td>
<td>0.029±0.008d</td>
<td>0.33±0.08a</td>
<td>13.2±2.5c</td>
<td>2.04±0.18ac</td>
<td>9±1b</td>
</tr>
</tbody>
</table>
The significantly highest (p<0.05) S/S_M ratio was found in *Campanula* (13.2 ± 2.5) and the lowest ratio in *Adonis* (1.0 ± 0.2). As regards to seed shape, E.I. ranged from 1.52 ± 0.11 in *Androsace* to 2.12 ± 0.23 in *Aquilegia*. The seed coat thickness was 31 ± 10 µm in *Adonis*, 26 ± 2 µm in *Aquilegia*, 27 ± 5 µm in *Androsace* and 9 ± 1 µm in *Campanula*.

A significant correlation was verified among L, W, T, S and V (Table 3).

### SEED GERMINATION DYNAMIC

The seed germination dynamic of *Aquilegia* and *Campanula* in the control treatment and after 250 ppm GA3, 500 ppm GA3 and cold-wet treatments are shown in figure 1. Values of M, T_{50} and z for *Aquilegia* and *Campanula* are show in figure 2.

### CONTROL TREATMENT

In the control treatment M was 46 ± 8 % and 93 ± 1% in *Aquilegia* and *Campanula*, respectively. T_{50} was 12 ± 0 and 5.5 ± 0.7 days in *Aquilegia* and *Campanula*, respectively. The z was 9 ± 1 days in *Aquilegia* and the final germination took place 20 days after sowing, while in *Campanula* z was 3 ± 0 days and the final germination took place 20 days after sowing. In *Adonis* and *Androsace* M was 0%.

### 250 PPM GA3 TREATMENTS

Compared with the control M increased by 35% and 1% in *Aquilegia* and *Campanula* when the 250 ppm GA3 treatment was applied. The final germination took place 30 and 26 days after sowing, respectively. With respect to the control, T_{50} decreased by 4% in *Aquilegia* while in *Campanula* it did not vary significantly. In *Aquilegia*...
and **Campanula**, $z$ was $8 \pm 0$ and $3 \pm 0$ days, respectively. In **Adonis** and **Androsace** $M$ was $0\%$ after the treatment.

### 500 ppm GA3 treatments

After 500 ppm GA3 treatment, $M$ increased by $65\%$ in **Aquilegia** compared to the control, while it decreased by $10\%$ in **Campanula**. The final germination took place 28 and 16 days after sowing, respectively. $T_{50}$ was $11 \pm 0$ and $4.5 \pm 0.7$ days in **Aquilegia** and **Campanula**, respectively. In **Aquilegia** and **Campanula**, $z$ was $7.5 \pm 0.7$ and $2 \pm 1$ days, respectively. In **Adonis** and in **Androsace** $M$ was $0\%$ after the treatment.

### COLD- WET STRATIFICATION

In the cold-wet stratification treatment $M$ increased by $26\%$ in **Aquilegia** while it decreased by $89\%$ in **Campanula** compared to the control. The final germination happened 25 and 26 days after sowing, respectively. $T_{50}$ decreased by $46\%$ in **Aquilegia** with respect to the control while it increased more than $100\%$ in **Campanula**. Results also showed that $z$ was $2.5 \pm 0.7$ and $10 \pm 7$ days, respectively. In **Adonis** and **Androsace** $M$ was $0\%$ after the treatment. A low $M (< 10\%$) was observed 9 months after the cold-wet treatment.

### DISCUSSION

On the whole, the results of this study show significant differences in seed traits and germination capability in response to the considered treatments among the species.

The Pearson’s correlation analysis underlines a significant correlation among $L$, $W$, $T$, $S$, $V$ and $D$. In particular, $L$, $W$, $T$, $S$ and $V$ are the largest in **Adonis** and the lowest in **Campanula**, while in **Androsace** and **Aquilegia** have intermediate values. KIKUZAWA & KOYAMA (1999) underline that small seeds have a faster water absorption capacity than large seeds, since they have a larger surface area to mass ratio. Our results underline that $S/S_M$ ratio is the highest in **Campanula** ($13.25 \pm 2.5$) and the lowest in **Adonis** ($1.03 \pm 0.17$).

As concerns germination it does not occur in **Adonis** and **Androsace** in response to treatments (control treatment, GA3 treatment, cold-wet treatment). Nevertheless, increasing the time of cold-wet stratification from three to nine months, the germination capability is below $10\%$. This result suggests that seeds from higher altitudes, such as **Androsace**, have a stronger dormancy than those from lower altitudes (i.e. **Aquilegia** and **Campanula**). We hypothesize that **Androsace** seeds have a deep physiological dormancy (sensu NIKOLAEVA, 1969) because dormancy is not completely broken by the stratification treatment. The same results are obtained for **Adonis**, in agreement with those of GODFROID & al. (2010) for other species of the same genus, who underline the lack of kno-
knowledge with regard to germination and dormancy for rare and threatened species. A morphological dormancy cannot be excluded for *Adonis* and *Androsace*; in particular, *Adonis* belonging to *Ranunculaceae* family is characterized by a rudimentary underdeveloped embryo of mature seeds (Martin, 1946; Finch-Savage & Leubner-Metzger, 2006), while *Androsace* is characterized by a linear underdeveloped embryo of mature seeds (Martin, 1946; Finch-Savage & Leubner-Metzger, 2006). The presence of an underdeveloped embryo of mature seeds suggests for these species both a morphological and physiological dormancy according to Baskin & Baskin (2004), Finch-Savage & Leubner-Metzger (2006). Nevertheless, in the present study we have not investigated this type of dormancy because a large amount of seeds would have been necessary, and considering that Crawford & *al.* (2007) suggest that only one germination treatment should be done when there are small seed amounts available for endemic species. Thus, in this case it is important to define an efficient protocol to enhance the germination success (Godefroid & *al.*, 2010).

Our results underline that treatment, species and their interaction significantly affect M, T50 and z as shown by GLM analysis. In particular, the interaction effect between treatment and species on M differs significantly for cold (t = -0.072, p<0.001) and 500 ppm GA3 (t = -3.314, p<0.01). With regards to T50 and z, the interaction effect between treatment and species differ significantly only for the cold-wet treatment (t = 7.260, p<0.01 and t = 4.095, p<0.05, respectively).

Debeaujon & Koornneef (2000) show the role of gibberellins in promoting seed germination. Exogenous application of GA3 overcomes seed dormancy in several species (Baskin & Baskin, 1998) promoting germination in some species that normally require cold stratification, light, or afterripening (Beulwy & *al.*, 1994). GA promotes the production of enzymes such as endo-b-mannanase, which loosen cell walls in the endosperm, thereby reducing resistance to radicle emergence (Beulwy, 1997; Groot & Karssen, 1987; Yamaguchi & Kamiya, 2002). Nevertheless, our results underline a different behaviour for *Aquilegia* and *Campanula* in response to GA3 treatments. In particular, *Aquilegia* is more responsive to GA3 treatments than *Campanula* which in turn shows a significant response only to the cold-wet treatment. In fact, the 500 ppm GA3 treatment increases the final germination by 65% in *Aquilegia* compared to the control and it can be justified by an endogenous non-deep physiological dormancy, according to the results of Nikolaeva (1969). On the contrary, the 250 and 500 ppm GA3 treatments do not significantly affect the final germination in *Campanula* compared to the control.

Meyer & Mosen (1991) suggest that populations normally encountering long periods with snow cover and adverse winter conditions require longer periods of cold stratification for germination than those exposed to milder winters. The cold-wet stratification improves germination in many high mountain species of eastern Europe and North America (Baskin & Baskin, 1998) and it is indicative of a physiological dormancy (Baskin & Baskin, 2005). Our results show a different response to the cold-wet treatment for the considered species. In particular, the 89% decrease in the final germination and the more 100% increase in the T50 more in *Campanula* compared to the control can be also related to the low seed size of as suggested by Schorhauffer (2006) who underlines that small seeds are more subjected to viability loss. This result can be interpreted as an adaptive consequence of the short period of exposure to snow, considering that this species grows at 750 m a.s.l. and snow covers the soil only for 15 days for one month during the year. On the contrary, *Adonis* and *Androsace* grow at a higher altitude (2675 m a.s.l. and 2760 m a.s.l., respectively) where snow persists for 7 to 9 months during the year. Moreover, the 93% germination percentage in the controls suggests a low level of physiological dormancy in *Campanula*, according to the results of Godefroid & *al.* (2010) for the species of the same genus. This result shows that cold-wet treatment does not necessarily promote germination.

The observed differences in seed dormancy of the considered species could also be related to the seed coat thickness, as suggested by Uubanska & *al.* (1979), Zuur-Islar (1982) and Schütz (2000). Seed coat thickness is larger in *Adonis*, *Aquilegia* and *Androsace* (28 ± 3, mean value) and the lo-
west in *Campanula* (9 ± 1). Large-seeded species invest proportionately greater resources into physical defences, such as a thick endocarp or seed coat, in response to high predation risks (Fenner, 1983; Blate & al., 1998; Moles & al., 2003). Nevertheless, the presence of a thick seed coat may delay germination by limiting oxygen exchange or by acting as a physical constraint to embryo growth (Norden & al., 2009).

Seed shape is an important determinant of seed dispersal, probable loss and moisture imbibitions (Cerdà & García-Fayos, 2002; Balkaya & Odabas, 2002). The results show that seed shape is elliptic for *Androsace*, egg-shaped for *Adonis* and long-sided for *Aquilegia* and *Campanula*. Differences in seed shape determine variations of the surface area that provides contact with the external environment (Grundy & al., 2003) and influence the response to burial depth in a different way. There is a significant correlation between the optimum emergence depth and seed shape (Thompson & al., 1993) where small and rounded seeds tend to persist in soil, while large and elongate or flattened seeds are transient in the soil (Thompson & al., 1994; Bekker & al., 1998). Thompson & al. (1993) suggest that ease of burial and rates of predation could be the mechanism underlying the relationship between seed size and shape and persistence in the soil.

On the whole our results give information on the relationship between seed traits and germination capability of the considered endemic species underlining the importance of the selected treatments to favour germination capability. Thus, based on the obtained results, germination protocols for the considered species can be suggested. In particular, the germination protocol for *Campanula* forecasts seed imbibition for 24 hours in distilled water, then moving seeds to Petri dishes with agar medium (7 g l⁻¹) integrated with 4.4 g l⁻¹ of Murashige Skoog salts and pH stabilized at 5.5. Petri dishes must be put into the growth-chamber at 20°C with 12/12 hour light and dark period.

With regards to *Aquilegia*, the protocol is the same as that for *Campanula* except carrying out the imbibition for 24 hours in 500 ppm GA rather than in distilled water.

We recommend a cold-wet treatment at least for 9 months to improve the germination capability for *Adonis* and *Androsacea*. Nevertheless, for these two species further research regarding their germination behaviour should be carried out. The recommended protocols may be used in reinforcement projects of the wild populations as a means of reducing the extinction risk of these endemic species.

**REFERENCES**


Bonito, A., Varoni, L. & Gratani, L. — 2011 — Relationship between acorn size and seedling morphological and physiological traits of Quercus ilex L. from different climates — Photosynthetica 49(1): 75-86.


Coolbear, P., Grierson, D. & Heydecker, W. — 1980— Os -

Debeaujon, I & Koornneef, M. — 2000— Gibberellin requirements for Arabidopsis seed germination is determined both by testa characteristics and embryonic Absciscic Acid — Plant Physiol. 122: 415-424.


IUCN — 2002— IUCN red list of threatened species— IUCN, Gland.


Martín-Bravo, S., Valcárcel, V., Vargas, P. & Luceño, M. — 2010— Geographical speciation related to Pleistocene range shifts in the western Mediterranean mountains — Taxon 59: 466-482.


Werker, E. — 1981 — Seed dormancy as explained by the anatomy of embryo envelopes — Israel J. Bot. 29: 22-44.


Received: 21 May 2013
Accepted: 30 July 2013