EFFECTS OF STRATIFICATION AND TEMPERATURE ON GERMINATION OF HIGH-ALTITUDE SPECIES FROM KOSCIUSZKO NATIONAL PARK

Euphrasia sp. Photo: K. Downs

Aciphylla glacialis Photo: A. Orme

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Effects of stratification and temperature on germination of high-altitude species from Kosciuszko National Park

Abstract

While the distribution and general ecology of Australian high-altitude species is increasingly well-studied, little is known about the seed biology of individual herbs. It is not known, for example, which species require low temperatures and/or cold stratification for germination and dormancy break, and whether these requirements would be fulfilled in a warmer climate. In this study, we investigated optimum temperatures for germination, and response to dormancy alleviating treatments of cold stratification and gibberellic acid (GA_3), for 20 species collected from high altitudes (860-1960m elevation) in Kosciuszko National Park, south-eastern Australia. While many of these species occur generally only at relatively high altitude (e.g. *Coronidiium waddelliae* 1170-1500m), a number have wider distributions (e.g. *C. scorpiodes* 1-1300m).

Germination response to temperature was highly variable among species. Temperature ranges and optima with and without periods of stratification (4, 8, 12 weeks) were determined for each species. For example, without stratification, low temperatures (5-19°C) were optimal for germination of *Aciphylla glacialis, Oreomyrrhis eriopoda* and *Arthropodium* sp. B. Moderate temperatures were favoured by *Brachyscome* sp. 1 sensu P.S.Short (1999) and *Epilobium gunnianum*, while Wahlenbergia had a higher temperature optimum (25°C). The endemic, high altitude species *Euphrasia collina* ssp. *diversicolor* exhibited poor germination at all temperatures. Two species (*Epilobium gunnianum* and *Oreomyrrhis eriopoda*) germinated to some extent at all temperatures (10, 15, 20, 25°C, 20/5°C and 20/10°C). Cold moist stratification response was positive for seven species, while GA_3 substituted for or improved upon cold stratification for *Derwentia* and *Wahlenbergia*. In general, the seven Asteraceae species germinated well at a wide range of temperature responses and showed little or no response to stratification or GA_3, with the exception of *C. waddelliae* which had faster germination after stratification.

This information was synthesized to determine the dormancy status of the species collections. Ten species appeared to be non-dormant, although there were indications of a degree of physiological dormancy in several. One species had deep physiological dormancy (*Viola bentonicifolia*) and another had physical dormancy (*Acacia pravissima*). The type and depth of morphophysiological dormancy varied for the other seven species (*Aciphylla glacialis, A. simplicifolia, Oreomyrrhis erriopoda, Wahlenbergia ceracea, W. gloriosa, Derwentia perfoliata*) including one which was deeply dormant (*Euphrasia collina* ssp. *diversicolor*).

Understanding the germination response to temperature has the potential to improve models of species response to pressures such as climate change and improve seed utilisation for rehabilitation, as well as clarify seed germination requirements for conservation seed banks and restoration.

Introduction

Seed germination is a critical stage in the life cycle of plants, particularly when considering the effects of global warming on high-altitude species. This is due to the dependence of these species on specific temperature regimes to stimulate germination and ensure seedling development coincides with favourable growing conditions (Mondoni *et al*. 2008 and 2009; Milbau *et al*. 2009). Understanding germination response to temperature has the potential to improve models of species response to climate change (Mondoni *et al*. 2008 and 2009; Milbau *et al*. 2009; Ooi *et al*. 2009), particularly as a large proportion of species regenerate from seed (Walsh and McDougall 2008; Venn and Morgan 2009). Information about germination can also improve the success rate of using seed for rehabilitation (Kaye 1997; Giménez-Benavides *et al*. 2005), which is critical to ongoing restoration of the former Snowy Hydro Scheme sites within Kosciuszko National Park (L. MacPhee, personal communication).

More than 119 seed collections from Kosciuszko National Park have been banked at the NSW Seedbank of the Botanic Gardens Trust, located at The Australian Botanic Garden, Mount Annan in south-western Sydney. We utilised 20 of these collections made at high altitudes (Table 1) to investigate the germination requirements for herb species collected from high altitudes. All but one of the species were collected from alpine or sub-alpine areas at altitudes between 1100 and 2000m. Fifteen of the species utilised are diagnostic taxa for one or more treeless vegetation communities in the Australian Alps (McDougall and Walsh, 2007), and 11 of these have ranges that extend into the area defined as alpine for Kosciusko National Park (≥ 1830m, Costin *et al*, 2000).

Herbaceous species were mostly chosen because they are common in soil seedbank samples from Australian alpine sites and have the potential to contribute to vegetation changes in response to climatic changes (Venn and Morgan 2010). One woody species (*Acacia pravissima*) was included for contrast. Recent studies in Australia have shown that subalpine plant species, particularly forbs, respond differently to experimental warming, with changes in timing and proportion of seed maturation (Jarrad *et al*. 2008). In addition, the proportion of herbs and graminoids increases with altitude, while shrub cover decreases, although these proportions may be affected by climate changes (Pickering and Green 2009). The seed biology of herb species growing at high altitudes is poorly understood, while tree species have been included in many previous studies (Green 1969; Beardsell and Mullett 1984; Turnbull and Doran 1987; Ferrar *et al*. 1988; Battaglia 1993 and 1997; Battaglia and Read 1993; Close and Wilson 2002).

All species used in this study are Australian endemics (Botanic Gardens Trust 2010). Several species have been included in ecological monitoring studies to date: *Aciphylla glacialis* and *Euphrasia collina* ssp. *diversicolor* on summits in the Snowy Mountains (Pickering *et al*. 2008), *Oreomyrrhis eriopoda* in snow-persistence and vegetation studies (Edmonds *et al*. 2006) and *Wahlenbergia ceracea* as a potential species for phenological monitoring (Gallagher *et al*. 2009). *Aciphylla glacialis*, *Euphrasia collina* and *Oreomyrrhis eriopoda* were all found in the standing vegetation of alpine summits but not the soil seedbank (Venn and Morgan 2010).

The aims of this study were to determine: which of these high altitude seed collections display dormancy; what type of dormancy mechanisms are present; which species require a period of cold stratification (or gibberellic acid) for germination; and, which species have germination requirements that are likely to be fulfilled in a warmer climate. For each species, therefore, we determined: the temperature optima for germination; the response to cold stratification (simulating a period under snow cover in the natural environment); and the response to gibberellic acid (a possible substitute for cold stratification). Seed measurements and germination results were used to classify dormancy for each species.

Methods

Study species

Species were chosen from NSW Seedbank collections made in Kosciuszko National Park, at altitudes greater than 850m, in February 2004 or February 2007 (Table 1). Following collection, seeds were dried at 15°C and 15% relative humidity, cleaned of debris, vacuum sealed in foil packets and stored at -18°C in the NSW Seedbank prior to testing.

Seed characteristics and dormancy classification

Seeds were dissected and measured to: 1. calculate embryo:seed length, 2. determine the presence/absence of endosperm, 3. assign embryo type according to the classification system of Martin (1946). These data were used to assist in dormancy classification. Dispersal unit length was measured for 20 dry seeds, while seed and embryo length were measured for ten imbibed seeds of each species. Seeds were photographed using an Olympus Colorview Soft Imaging System camera (2005) mounted on a binocular microscope, with subsequent measurements made using analySIS LS Starter Version 2.6 Life Science software (2007) (Olympus Soft Imaging Solutions GmbH, Germany). Mean seed air dry weight was measured on three replicates of 50 seeds, with seeds equilibrated at 15% RH and 15°C, and then converted to individual seed weights. For very small seeds, the number of seeds per gram was estimated by counting the number of seeds in three subsamples of known weight. Seed characteristics and germination results were used to classify dormancy for each species using the system of Baskin and Baskin (2004).

Seed germination and viability

Prior to germination testing, foil packets containing seeds were held at room temperature for at least 24 hours, then opened to allow seeds to equilibrate to room humidity for at least 12 hours. Seeds of *Acacia pravissima* were scarified prior to use. Initial experiments on seven species commenced in August 2008. Five replicates of 20 or 25 seeds were placed on water agar (0.7%) in glass petri dishes and incubated at constant temperatures of 10, 15, 20, 25°C, and alternating temperatures of 20/5°C and 20/10°C (with and without GA_3), with a 12/12 h light/dark cycle. The same temperature treatments (± 20/5°C) were then applied to most species following stratification at temperatures ≤5°C for 4, 8 and 12 weeks. *Brachyscome* sp. 1 sensu P.S.Short (1999) was tested at a more limited range of temperatures (10, 15, 20/5°C and 20/10°C) as there were fewer seeds available for testing. Germination following stratification for this species and two others (*Epilobium gunnianum* and *Euphrasia collina* ssp. *diversicolor*) was tested at 20/10°C only.

Incubator temperatures were accurate to within 1°C. Gibberellic acid, where used, was incorporated into agar at 250ppm. Germination, defined as radicle emergence, was monitored weekly and any germinated seeds were removed. Germination tests were terminated after 3-4 months, when germination had ceased or become sporadic, as recommended by Milbau *et al*. (2009).

At the end of the experiments, any remaining seeds were cut-tested and examined under the microscope to determine viability. Viability was expressed as the percentage of germinated + viable seeds out of the total number of seeds per replicate. Germination percentages were adjusted for viability, however the unadjusted germination percentages were used for analysis to anable the detection of any change in viability caused by the treatments applied. The mean time to germination (MTG) was calculated for cold stratification experiments using the following equation:

$$MTG = \Sigma (n \times d)/N$$

Where n is the number of seeds germinated between scoring intervals, d is the incubation period in days at that time point and N is the total number of seeds germinated (Cochrane and Probert 2006).

Statistical analysis

The effects of temperature/stratification treatments on total germination and MTG were tested on raw data in GenStat v.10 using the non-parametric Kruskal-Wallis test.

Results

Seed characteristics and viability

Seeds ranged in size from large (*Aciphylla* and *Oreomyrrhis*) to very small (*Wahlenbergia ceracea*) and exhibited a variety of embryo types including linear, investing, spathulate and dwarf (data not shown). All species had high viability (≥ 80%) in initial and subsequent tests.

Germination

All species, except *Aciphylla simplicifolia* and *Euphrasia collina* ssp. *diversicolor*, germinated to some degree, at one or more temperature regimes, without prior stratification (Table 2). Both *A. simplicifolia* and *E. collina* ssp. *diversicolor* required stratification to initiate germination; the latter germinated to only 4% ± 2.5 even after 12 weeks stratification.

The optimal temperatures for germination without stratification varied greatly among the remaining species (Table 2, selected species in Fig. 1). In terms of % germination, species fell into one of five categories:

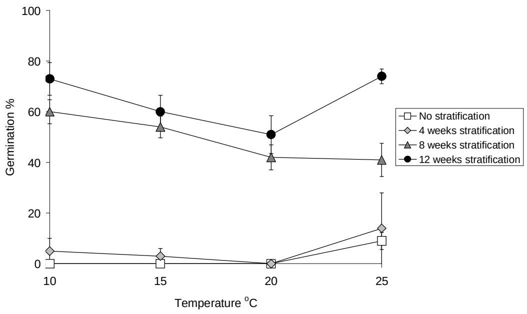
1. germinated equally well at all temperature regimes ≥10°C (four species, *Brachyscome tenuiscapa* var. *pubescens, Podolepis robusta, Xerochrysum subandulatum* and *Geum urbanum*),
2. germinated equally well at all temperature regimes ≥ 15°C (two species, *Poa hiemata* and *Coronidium waddelliae*),

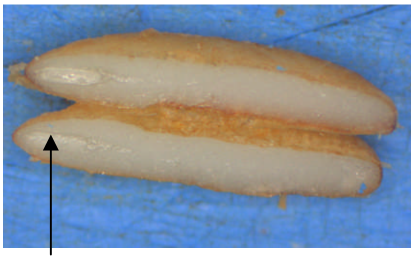
3. germinated optimally at low temperatures of 10°C or less (five species, *Aciphylla glacialis*, *A. simplicifolia*, *Arthropodium* sp. B, *Viola betonicifolia*, *Oreomyrrhis eriopoda*,
4. germinated optimally at moderate temperatures of 15-20°C and/or alternating temps of 20/5 and 20/10°C (three species, *Euchiton involucratus*, *Epilobium gunnianum*, *Brachyscome* sp. 1),
5. germinated optimally at 25°C (three species, *Acacia pravissima*, *Wahlenbergia ceracea* and *W. gloriosa*).

The response to stratification, in terms of % germination, fell into four main categories:

1. no significant effect, species germinated equally well with or without stratification (8 species, $P > 0.05$),
2. significantly improved germination across all temperature regimes (5 species, $P \leq 0.05$),
3. significantly improved germination for some temperature regimes (2 species, $P \leq 0.05$),
4. significantly reduced germination across some or all temperature regimes (4 species, $P \leq 0.05$) (data not shown).

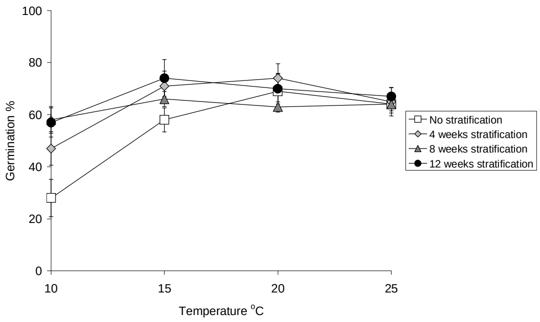
Though % germination was improved by stratification for only seven of the species tested, stratification significantly reduced mean time to germination at a greater range of temperatures for 14 species (Table 2).





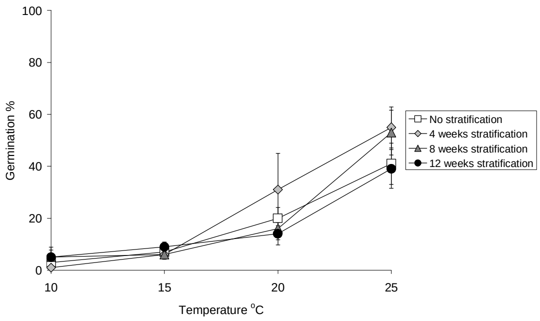
a. *Aciphylla glacialis.*

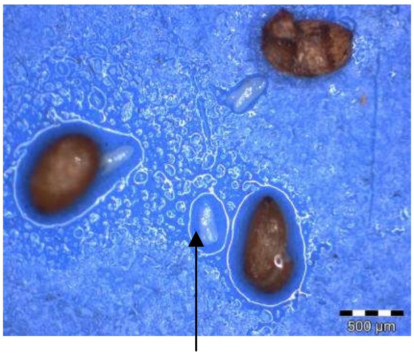
Embryo type: linear (small)



b. *Coronidium waddelliae.*

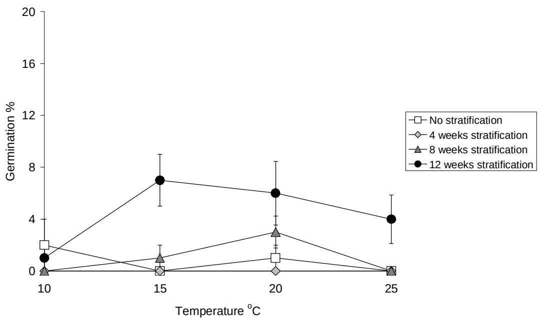
Embryo type: spathulate

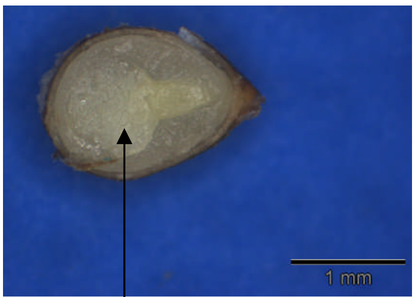




c. *Wahlenbergia gloriosa*.

Embryo type: dwarf





d. *Viola bentonicifolia*.

Embryo type: spathulate

Fig. 1 Final germination % (average ± standard error) for selected species a. *Aciphylla glacialis*, b. *Coronidium waddelliae,* c. *Wahlenbergia gloriosa*. D.*Viola bentonicifolia*. Seeds were incubated at 10, 15, 20 and 25°C following 0, 4, 8 or 12 weeks moist stratification at 2.5°C. Seed and embryo morphology of species is also presented.

Dormancy classification

The seeds of all but one species readily imbibed water, thus ruling out physical dormancy. *Acacia pravissima* was classified as physically dormant as its seeds did not imbibe water without scarification. Germination occurred without stratification and within 30 days for 12 species (*Arthropodium*, *Brachyscome* spp., *Coronidium* spp., *Epilobium, Euchiton, Geum, Poa, Podolepis*, *Wahlenbergia ceracea* and *Xerochrysum*). Three of these four species had fully-developed embryos and were therefore classified as non-dormant according to Baskin and Baskin (2004) (data not shown but see for example, the embryo types in Fig. 1). For several of these species, stratification improved the germination percentage and the MTG which may indicate that the seed had some level of dormancy. Both *Wahlenbergia* species have dwarf seed and a relatively small embryo in relation to seed size (data not shown), indicating morphological dormancy. *Aciphylla* and *Oreomyrrhis* display morphophysiological dormancy (MPD) with a low embryo:seed length ratio and germination taking >30 days unless cold stratification is applied (Table 2). MPD for *Oreomyrrhis* is non-deep Type 1, as gibberellic acid and cold stratification promoted germination, and low temperatures were optimal for germination. MPD for both *Aciphylla* species is intermediate in depth, as gibberellic acid did not promote germination and cold stratification of 2-3 months was optimal. *Euphrasia* has a moderate embryo:seed ratio (0.79), and though optimal germination conditions were not identified, there was no response to gibberellic acid and minimal response to cold stratification, so deep MPD is likely for this species.

Discussion

Germination response to temperature and cold stratification

The study species were highly variable in their germination response and timing at different temperatures and with different periods of cold stratification. The variety of observed responses to germination temperature is expected, as alpine environments exhibit significant spatio-temporal variability (Kaye 1997; Shimono and Kudo 2005). Even within a particular habitat, germination responses are unlikely to be consistent (Giménez-Benavides *et al*. 2005;

Shimono and Kudo 2005). For each species, germination is likely to vary between altitudes, populations and years (Giménez-Benavides *et al*. 2005; Venn 2007; Mondoni *et al*. 2008). Variability in germination is an important strategy to ensure species survival in unpredictable environments, reducing the risk of exposing the entire seedling cohort to poor growing conditions (Giménez-Benavides *et al*. 2005). For example, in the genus *Penstemon*, Meyer (1995) suggests that germination of most species combines predictive mechanisms (e.g. fulfilment of cold stratification requirements) with the potential for development of a persistent seed bank.

Cold stratification response was positive, in one or more respects, for all species except *Brachyscome tenuiscapa* var. *pubescens*, *Euchiton involucratus*, *Epilobium gunnianum, Euphrasia collina* ssp. *diversicolor* and *Poa hiemata* (Table 2) having similar effects to those observed in previous studies of alpine species: improving final germination, widening the range of temperatures for germination, decreasing germination time, and synchronising germination by reducing variability in time to germination (Shimono and Kudo 2005). Both cold stratification temperatures used in these experiments (5°C in initial studies and 2.5°C in subsequent stratification experiments) provide an adequate moist chilling treatment, defined by Baskin and Baskin (1998) as a period below 10°C. The temperatures are also within the range of soil temperatures likely to be encountered in the field in high altitude Australia, with average annual temperatures at 100mm depth of around 5°C and minimum temperatures under snow cover of -0.2°C (Pickering and Green 2009). A wide range of cold stratification temperatures have been reported as successful in breaking dormancy in studies of other alpine and high mountain species e.g. 0°C (Shimono and Kudo 2005); 1°C (Meyer *et al* 1995); 1-2°C (Wardlaw *et al*. 1989); 2.5°C (Forbis and Diggle 2001); 4°C (Cavieres and Arroyo 2000; Giménez-Benavides *et al*. 2005; Mondoni *et al*. 2008); 5°C (Kaye 1997).

Five species (*Aciphylla glacialis*, *A. simplifolia*, *Arthropodium* sp. B, *Epilobium gunnianum* and *Oreomyrrhis eriopoda*) were able to germinate at very low temperatures (≤ 5°C). Although not common, the ability to germinate at very low temperatures has been observed in other alpine and high mountain species including the Australian *Caltha introloba* (Wardlaw *et al*. 1989). The capacity to germinate at low temperatures may provide an advantage during a short growing season by allowing germination to begin under snow banks (Meyer *et al*. 1995; Forbis and Diggle 2001; Walck and Hidayati 2004). *Aciphylla glacialis* germinated optimally at low temperatures, similar to the Asian and North American *Osmorhiza* species (Walck *et al*. 2002, Baskin *et al*. 1995, Walck and Hidayati 2004) in the same family (Apiaceae). At high altitudes in Kosciuszko National Park (e.g. Charlotte Pass - 1755m, Perisher Valley -1735m and Thredbo - 1380m), mean winter temperatures (BoM 2010) are currently generally in the range of the laboratory stratification temperatures for *A. glacialis*, whilst at lower altitudes (e.g. Khancoban 337m, BoM 2010), temperatures may not be low enough for periods of time required for sufficient stratification. Verification of in-field germination of this and other species with low temperature requirements at different sites in Kosciuszko National Park would be useful.

Along with altitude, soil temperatures are the main determinant of species composition on alpine summits which means that predicted changes in climate are likely to affect future plant composition (Pickering *et al.* 2004; Pickering and Green 2009). Increased temperatures (up to 2.6°C, Hennessy *et al*. 2003) are likely to be detrimental to germination of *Aciphylla* and other species with a preference for low temperatures. Germination of *Aciphylla*, *Arthropodium* and *Oreomyrrhis* may be lower or slower, given their low optimum germination temperatures, while *Wahlenbergia* may germinate in greater numbers, due to a higher optimum temperature. Germination of all species except *Epilobium* may be slower and restricted to a more narrow range of temperatures due to a lack of cold stratification, if field responses are similar to laboratory responses.

Dormancy, temperature requirements and climate change

The study species varied in their dormancy classification, from non-dormant to deeply dormant. Confirming dormancy classification by testing with fresh seeds is recommended (Baskin and Baskin 1998; Kaye 1997; Wang *et al*. 2010), as afterripening between collection and testing may have reduced the depth of physiological dormancy. Several collections that were deemed not dormant had, nevertheless, some indications of dormancy (e.g. *Brachyscome* sp. 1) which may indicate incomplete afterripening. Cold storage of seeds between collection and testing did not impact on seed viability but has the potential to change dormancy status and germination requirements (Wang *et al*. 2010).

The two widespread species collected from subalpine locations (*Arthropodium* and *Epilobium*) were both non-dormant, while the widespread high-altitude species *Oreomyrrhis* germinated at all temperatures and had non-deep physiological dormancy. The majority of the Asteraceae species studied were non-dormant (previous comments re. after ripening notwithstanding), as observed for *Brachyscome rigidula*, *B*. *spathulata* and four other species in the family Asteraceae (Venn 2007). A lack of dormancy and a wide temperature range indicates that these species may be more opportunistic in their germination timing, taking advantage of appropriate temperatures to begin germinating whenever soil moisture is available. Dormancy classification for *Wahlenbergia* was based on the presence of a small but developed embryo and germination taking >30 days. Embryo growth may be occurring as soon as temperatures are appropriate, with the lag time prior to germination simply required for embryo growth. If this was the case, which should be confirmed, the dormancy classification may not hold for this species.

Germination cues for *Aciphylla* and *Euphrasia* are more complex, with dormancy mechanisms present to restrict germination until cold stratification or other requirements are fulfilled. Further investigation is required to break dormancy in *Euphrasia collina* ssp. *diversicolor*, which germinated poorly under all test conditions in this study. Two European alpine *Euphrasia* species germinated at 5°C or varying low temperatures (3-10°C) but took up to three years (Liebst and Schneller 2008). Australian *Euphrasia collina* ssp. *tetragona* and ssp. 'NW Tasmania' germinated quickly (T_{50} <40 days) at 5°C and 15°C respectively, although these collections were made at lower altitudes (Wood 2009). Higher altitude collections of *Euphrasia* were more difficult to germinate, with *E*. *collina* ssp. *diemenica* (altitude 960m) germinating slowly at 5°C and *E*. *gibbsiae* (altitude 1255m) germinating very slowly with warm stratification followed by low temperatures (8 weeks at 20°C, then 5°C) (Wood 2009). *Euphrasia collina* ssp. *diversicolor* may require periods of stratification >12 weeks, a longer germination time and/or other treatments such as alternating cold and warm stratification to break dormancy.

Conclusions

While this study has useful recommendations about producing plants for restoration in a laboratory or nursery situation, the seed ecology of these species requires further study within natural habitats. The germination behaviour observed in the laboratory can be very different to that observed in the field, especially in highly variable alpine environments (see Shimono and Kudo 2005). Such variability has been observed in soil temperatures in the Australian Alps, with variation in soil temperatures leading to differences in the length of the growing season and date of thaw across sites and years (Pickering and Green 2009). This may impact on the germination response of each species across altitudes, populations and years (Giménez-Benavides *et al*. 2005; Mondoni *et al*. 2008). Variability in optimal germination temperature, dormancy class and timing of germination should be the expectation, rather than the exception, when testing seeds collected from high altitudes.

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Captions

Table 1: Study species, location, habitat, habit and distribution. KNP = Kosciuszko National Park. Habitat data from Costin *et al*. (2000) except for *Arthropodium* and *Brachyscome*, where data relates to field notes at time of collection. Habit and distribution data from PlantNet (Botanic Gardens Trust 2010).

Table 2:Optimum germination conditions for 20 high-altitude species. Seeds were germinated at 10, 15, 20, 25, 20/5 and 20/10 with and without stratification at 2.5°C for 4, 8 and 12 weeks.