SEED LONGEVITY IN STORAGE OF ALPINE PLANT SPECIES

Thesis dissertation

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Seed longevity in storage of alpine plant species

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Certification

I, Malaka Madhuranga Wijayasinghe, declare that this thesis, submitted in partial fulfilment of the requirements for the award Doctor of Philosophy at Department of Earth and Environmental Sciences, University of Pavia, is wholly my own work unless otherwise referenced or acknowledge. This document has not been submitted for qualification at any other academic institution.

Malaka Madhuranga Wijayasinghe
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Statement of contribution

Three chapters (chapter 2, 3 and 4) presented in this thesis have been prepared as manuscripts. Andrea Mondoni, Louise Colville, Alma Balestrazzi, Hugh W. Pritchard and Maria Tudela-Isanta contributed to all three chapters, and Thomas Abeli contributed for chapter 3.

List of Publications

PAPERS IN INTERNATIONAL JOURNALS WITH PEER-REVIEW

As a first author

1. **Wijayasinghe M**, Balestrazzi A, Colville L, Pritchard HW, Tudela-Isanta M, Mondoni A. 2017. Effect of Seed and Ecological Traits on Seed Longevity: A Case Study of Alpine Asteraceae. *In preparation, Seed Science Research as a target journal.*
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3. **Wijayasinghe M**, Balestrazzi A, Colville L, Pritchard HW, Tudela-Isanta M, Mondoni A. 2017. Effect of seed priming on seed longevity of alpine plants: Implication for ex situ seed conservation and native seed industry. *In preparation, Seed Science Research as a target journal.*

As a co-author.

4. Paparella S., Araújo SS, Rossi G, **Wijayasinghe M**, Carbonera D, Balestrazzi A. 2015. Seed priming: state of the art and new perspectives. Plant cell reports, 34, 1281-1293. doi: 10.1007/s00299-015-1784-y.
5. Tudela-Isanta, M, Fernández-Pascual E, **Wijayasinghe M**, Orseningo S, Pritchard HW, Rossi G, Mondoni A. 2017. Habitat-related seed germination traits in alpine habitats. Ecology and Evolution. *Accepted.*
6. Tudela-Isanta, M, Ladouceur ER, **Wijayasinghe M**, Pritchard HW, Mondoni A. 2017. Seed germination niche contributes to limit some plant species distributions to calcareous or siliceous alpine bedrocks. Alpine Botany. *Accepted with major revision.*
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COMMUNICATIONS IN INTERNATIONAL CONGRESSES

ORAL PRESENTATIONS

As a speaker

1. **Wijayasinghe M,** Tudela-Isanta M, Mondoni A, Balestrazzi A, Colville L and Pritchard HW. 2017. Importance of seedling conversion on estimates of seed longevity in alpine plants. I International Conference (NASSTEC)- Seed Quality of Native Species- Ecology, Production & Policy, London, UK.
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As a co-author (not the speaker)

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POSTER PRESENTATIONS

As a presenter

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4. Ladouceur E, Frischie S, Dello Jacovo E, Hernandez-Gonzalez, Tudela-Isanta M, Teixeira A, Marin M, Blandino C, Lopez- Egido L, Abbandonato H, **Wijayasinghe M**. August 2015. Native Seeds for Plant Conservation and Grassland Restoration in Europe: A mission for science and policy. XXVII International Congress for Conservation Biology, Montpellier (France)
5. Tudela-Isanta, M, **Wijayasinghe M**, Rossi G, Mondoni A. September 2015. Patterns of seed germination within alpine grasslands of different provenience and habitats. III International Plant Science Conference (IPSC), Pavia (Italy).
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"In the sky, there is no distinction of east and west; people create distinctions out of their own minds and then believe them to be true." – Buddha

ABSTRACT

Species with short-lived seeds are known to abundant in alpine flora. Therefore, significant concern for successful *ex situ* conservation of alpine plants, which represent one of the groups most sensitive to the direct and indirect human impacts on plants diversity. Seed longevity has been investigated predominantly in relation to taxonomic and macroclimatic differences, while little is known about the variation within closely related taxa, growing under the same climate. Therefore, seed longevity of 18 alpine species Asteraceae was compared using artificial ageing (AA; 45 $^{\circ}$C and 60 % RH) to ascertain the influence of seed and species-specific ecological traits (i.e. seed mass, soil pH and moisture) on seed longevity. The estimates of p_{50} (estimate the time for viability to fall to 50 %) ranged between 1.63 and 40.03 d. Soil moisture significantly influenced seed longevity, with seeds of species growing on dry soil showing higher p_{50} than those from wetter soil. Conversely, seed mass and soil pH did not significantly contribute to longevity differences across species, though species from acid soils tend to be shorter lived than those from basic soils. Plant ecological traits, linked to condition at the plant growing site may play crucial roles in the prediction of seed lot longevity in air-dry storage including seed bank conditions.

Germination test by means of radicle protrusion is often used as an assessment of seed viability (i.e. seed longevity studies). Therefore, there is a possibility that radicle protrusion alone may over-estimate viability compared with normal germination (i.e. radicle plus cotyledon emergence), thereby seed longevity. However, the extent of such overestimation across species and the factors contributing to it are not yet well understood. Therefore, seed life span of 35 alpine species was studied by evaluating both radicle emergence and normal germination during artificial ageing (AA). Estimates of p_{50} based on radicle emergence ($p_{50\ (RE)}$) were significantly higher than estimates based on normal germination ($p_{50\ (NG)}$) in 18 (51.4 %) out of the 35 species tested, suggesting radicle emergence may not be a reliable indicator of the capacity of seeds to complete the germination process, thereby leading to an overestimation of seed longevity. Therefore, in accordance with these results, the actual seed longevity of several alpine species may be lower than previously reported, highlighting that *ex situ* storage of alpine seeds might be even more problematic than currently thought. The coefficient of OESL developed here and its correlates (i.e. seed type, soil pH and seed longevity) may be used to prioritize species' vulnerability to *ex situ* storage and to optimize viability testing, thereby reducing labour costs and enabling more effective conservation of seed collections.

Alpine species are short-lived and most of them are incapable of becoming a seedling. Therefore, long-term storage of these species could be a problematic even under conventional seed banking conditions. As a solution, seed priming can be used to increase both seed longevity and seedling recovery. Therefore, I investigated the potential for priming to increase the longevity of six alpine species using a range of water potentials (hydro and osmo-priming). According to this study, priming treatments had a significant positive effect on seed longevity ($p_{50\ (RE)}$ and $p_{50\ (NG)}$). In particular, hydro-priming was the most successful seed priming treatment to enhance both $p_{50\ (RE)}$ and $p_{50\ (NG)}$ and decreased the overestimation.

The information provided in this study on wild alpine plants may fill some knowledge gap about how to monitor and improve seed viability in storage, which may have important implications high quality seeds both long- and short-term *ex situ* storage, such as in seed banks and native seed industry, respectively. In particular, I highlighted that normal germination (i.e. radicle plus cotyledon emergence) should be used to monitor seed viability during storage, that species from more humid soil may have higher possibility to show short-lived seeds and that, alpine seeds are short-lived, their longevity can be significantly improved using easy and inexpensive techniques, such as hydro and osmo-priming.

ABBREVATION

RE - radicle emergence

NG - normal germination (radicle plus cotyledon emergence)

OESL - Over estimation of seed longevity

p_{50} - estimate the time for viability to fall to 50 %

$p_{50\,(RE)}$ - time taken for radicle emergence to fall to 50%

$p_{50\,(NG)}$ - time taken for NG to fall to 50%

eRH - relative humidity

v - viability (in normal equivalent deviates, NED) of the seed lot after p days in storage

K_i - initial viability (NED) of the seed lot

σ - time (d) for viability to fall by 1 NED (i.e. the standard deviation of the normal distribution of the seed deaths over time)

AA - artificial ageing test/experimental storage tests

LiCl - Lithium chloride

g – grams

d - days

MPD - Morpho-Physiological Dormancy

CHAPTER 1

General Introduction

Plant conservation

Plant genetic resources have been defined as the "genetic material of plants, which is of value as a resource for the present and future generations of people" (IPGRI, 1994). The ultimate goal of genetic resources conservation is to ensure that the maximum possible genetic diversity of a taxon is maintained and available for potential utilization (Maxted and Guariano, 2003). Since the availability of resources are limited, these resources should use in the most effective and efficient manner. In this regard, careful selection of which species to be prioritize for conservation is crucial. This can be valued through an integration of its conservation biology, the intended conservation purpose, the economic cost of conservation option and current conservation status (Maxted *et al*., 1997). *Ex situ* and *in situ* are the two approaches to conservation of plant genetic resources. Whilst *in situ* conservation measures should always be the priority of conservation actions, *ex situ* conservation can complement and assist these actions (Glowka *et al*., 1994). In *in situ* approach genetic resources conserve in the natural habitat. while, *ex situ* approach conserves genetic resources outside the native habitat/environment of the plant concerned, and this generally comprises of storing seed at low temperature, maintaining plants in the field gene banks or botanical gardens, *in vitro* storage (Maxted *et al*., 1997).

Ex situ seed conservation

Banking of seeds *ex situ* is an effective method of conservation because it is easy, allows for international exchange of genetic materials and it is cost effective (Pritchard, 2004). There are more than 1750 seed banks in the world established for the *ex situ* seed conservation of plant diversity, the majority of which conserve crop diversity, storing a combined total of about 4.6 million accessions of 64 food and forage crops (FAO, 2014). Given the concern about the loss of plant diversity globally, there is clearly an urgent need to advance programs on the *ex situ* conservation of wild plant diversity to complement *in situ* conservation efforts (Pritchard, 2004). In this regard, seed banks have been established at local, regional, national and global level to store many thousands of samples of seeds from wild species for long-term storage (i.e. Millennium seed bank project dedicated to conservation of wild plants).

Seed storage behaviour

Seeds are classified on the basis of their ability to withstand desiccation as orthodox (desiccation tolerant and longer-lived) or recalcitrant (desiccation intolerant and short-lived) (Roberts, 1973). Orthodox seeds are able to tolerate drying to a very low moisture content

percentage (≤ 3- 7 % fresh weight), while recalcitrant seeds do not tolerate drying. As a result, recalcitrant seeds die rapidly under the same gene-banking conditions used to store orthodox seeds (Walters *et al*., 2013). Therefore, recalcitrant seeds pose the ultimate *ex situ* seed conservation challenges (Bewley *et al*., 2013), alternatively, cryogenic storage (usually in liquid nitrogen, at -196 °C) is currently being used for long term seed storage (Walters *et al*., 2013). Fortunately, most of the angiosperm species (75 - 80 %) produce seeds with orthodox seeds that can survive prolong storage at -20 °C (Hey and Probert, 2013). In this study, I only focused on orthodox seeds and further descriptions are based on orthodox seeds.

Seed deterioration during storage

Loss of seed viability due to seed deterioration or seed ageing with time is an inevitable process that start soon after seed dispersal. Deterioration in seed quality is associated with the accumulation of cellular damage to macromolecules including lipids, proteins and DNA (Waterworth *et al*., 2015). However, under appropriate conditions, seeds are able to survive for long (Copeland and McDonald, 2001). For example, seeds of the lotus (*Nelumbo nucifera* Gaertn.) collected from a dry ancient lakebed remain viable for about 1300 years (Shen-Miller, 2002). To promote survival in the dry state, seeds have evolved effective mechanisms that protect cellular macromolecules and cellular structures against the adverse effects of desiccation (Waterworth *et al*., 2015). Synthesis of sugars (e.g. raffinose), heat shock proteins, and late embryogenesis abundant proteins reduce the damage upon rehydration (Bewley and Black, 1994). Oxidative stress due to production of reactive oxygen species (ROS) is a major cause of cellular deterioration in aged seeds (Kranner *et al*., 2010; Bailly, 2004). ROS damage to macromolecules may be caused by a cellular compartmentalization, while lower levels of metabolism in the quiescent state result in lower activity in repair pathways (Kranner *et al*., 2010). As a solution, seed priming can be used to facilitate cellular repair processes without the completion of germination in order to enhance seed quality (Paparella *et al*., 2015).

Seed viability equation

Time, storage temperature and seed moisture content are the main factors that determine quality during storage (Elis and Roberts, 1980). Reduction of seed moisture content is critical to maintain viable seeds and this can be achieved by decreasing the air relative humidity (eRH). The relationship between seed moisture content and eRH can be represented by a moisture soption isotherm (Walters, 1998).

Improved viability equation (Ellis and Roberts, 1980) are based on the notion that the longevity of a seed lot through time, at a given storage temperature and moisture content of the seeds, follows a normal distribution. Seed survival curves are plotted as probit percentage viability against *p*, line of negative slope 1/sigma (Finney, 1971). Therefore, the viability of a seed accession can be predicted by the equation (Ellis and Roberts, 1980).

$$v = K_i - p/\sigma$$

where v is the viability (in normal equivalent deviates, NED) of the seed lot after p days in storage, K_i is the initial viability (NED) of the seed lot, and σ is the time (d) for viability to fall by 1 NED (i.e. the standard deviation of the normal distribution of the seed deaths over time) (Ellis and Roberts, 1980). This equation can be used to infer the estimation the half viability period (p_{50}), that has been to use to describe seed longevity for seed lots (Hay *et al*., 2006; Probert *et al.*, 2009; Mondoni *et al*., 2011).

Seed storage conditions in seed banks

Fortunately, the conventional long-term storage techniques appear to be apply for all mature orthodox-seeded species (Linington, 2003), and orthodox seeds respond to drying and cooling in predictable ways (Ellis and Roberts, 1980). Seed banks store seeds according to the conventional gene standards (FAO, 2014). All seed samples should be dried to equilibrium in a controlled environment of 5-20 °C and 10-25 percent of relative humidity depending upon species to maximized the longevity during storage. After drying, as soon as the seeds have reached the desired moisture content, are stored at -18 °C in hermitically closed containers for long-term storage (FAO, 2014).

Viability testing

Proper seed storage conditions maintain germplasm viability, but even under excellent conditions viability declines. Therefore, it is necessary to assess viability periodically. According to the gene-bank standards on seed viability monitoring, the initial germination value should exceed 85 % for most cultivated crop species, but lower percentage could be accepted for some specific accessions and wild and forest species (FAO, 2014). Despite the occurrence of general seed conservation standards (see for example, ENSCONET guidelines; https://www.luomus.fi/sites/default/files/files/collecting_protocol_english.pdf), specific groups of native plants may present different requirements, and it is crucial to investigate them in order to successfully store these seeds. Seed banks storing wild species follow gene

bank standards (FAO, 2014), where most of the theories and protocols are derived from studies on crops.

The purpose of viability monitoring is to detect loss of viability during long-term storage before viability has fallen below the threshold for regeneration. Therefore, it is very important to measure seed viability. A range of tests are available for determining the viability of seeds, the most common being by seed x-ray, cut test, tetrazolium test, excised embryo test, electrical conductivity test and germination test (Gosling, 2003; ISTA, 2013), each giving a different value of seed viability. Therefore, it is very important to select reliable viability test to assess the performance of the seeds. Several authors have pointed out the importance of correct use of "viable" and "germinable" terms, because, viable seeds are not necessarily capable of germinating into normal seedlings, and therefore viable and germinable are not the same (Gosling, 2003; Copeland and McDonald, 1995). Viability tests aim to determine the proportion of a seed lot that is either alive or dead, a germination test determines the potential of a seed lot to produce normal healthy seedlings (Gosling, 2003). However, the practical definition of viability depends on the context in which it is used; here, and for ecologist in general, a viable seed is the one that can produce a normal seedling and a healthy plant (Bradbeer, 2013). Indeed, seed germination is the most reliable test to assess the performance/quality of seeds because it reflects their capacity to develop a heathy seedling. However, germination test is sometimes impractical due to various reasons, such as it is a time-consuming method and difficult to perform on deep dormant seeds. For these reasons, farmers, horticulturalists, conservationists and seed scientists tend to use other viability testing methods (see above) even though they could overestimate the seed viability. In this context, germination test may also provide misleading results when used to infer seed lot viability, depending on the method of scoring. For example, radicle protrusion as a proxy of viability may overestimate the longevity seeds (Ellis and Roberts, 1981), compared with normal germination (i.e. radicle plus cotyledon emergence), though the extent to which this overestimation could occur across species, as well as the factors that contributing to it are not yet well known. To better understand these issues, in chapter 3 of this study investigated the lifespan of seeds of 35 alpine species by either considering radicle emergence or full germination (i.e. radicle plus cotyledon emergence). We specifically focused on alpine species since they are expected to be short lived in air-dry storage (Mondoni *et al*., 2011) and, therefore, represent an important case study in the context of *ex situ* seed conservation.

Artificial ageing test (AA)

At its most complex, the mathematical characterisation of the sensitivity of seeds to moisture content and temperature has resulted in the generation of seed viability constants that can be used to predict seed longevity (Ellis and Roberts, 1980). Within parameters these constants are species-specific, but the use of thousands of seeds in their determination means that the generation of constants is not always feasible, especially for wild species (Probert et al. 2009; Hay and Probert, 2013). Similarly, storage experiments to determine seed longevity with standard seed banking conditions (e.g. 3-7 % moisture content and -18 °C) may require long-term monitoring before there is any evidence of viability falling (Kochanek *et al.*, 2009). There are many alternative and quicker means of estimating seed longevity, including artificial ageing test (AA). This test has been initially developed to estimate the relative storability of seeds (Delouche and Baskin, 1973), exposing seeds to two environmental variables, high temperature and high relative humidity, which cause rapid seed deterioration. As a result, viability loss of a seed lot occurs within days to months rather than years (Rajjou and Debeaujon, 2008). Therefore, AA has been used successfully to rank and predict relative seed longevity (low, medium and high) of hundreds of species (Probert *et al*., 2009; Mondoni *et al*., 2011). In this study, 45 °C and 60 % RH condition was used as AA condition or accelerate ageing condition.

Correlates of seed longevity of wild plants

The collection and storage of seed of wild plants is a major strategy for plant biodiversity conservation. Prediction and understanding of inter-specific differences in seed longevity is crucial for the effective management of seed banks because it underpins the selection of viability re-test intervals and hence regeneration or re-collection strategies (Probert *et al*., 2009). However, species-specific constants for the improved viability equations are only available for less than 100 species (Liu *et al*., 2008) in which majority of them are crop species. Generating species-specific constant is not very feasible for wild plants and, therefore, prediction of seed longevity in gene bank is problematic for these species (Probert *et al*., 2009). It follows that, the identification of reliable and robust correlates of longevity to develop models are important (Daws *et al*., 2006). Investigations on such correlates provides the potential for predicting the longevity of untested species and also contributes to understand ecological and plant/seed physiological traits associated with seed longevity. In a broader context, seed ageing data from AA can be used to ascertain the relationship between seed longevity and seed morphological, physical, and physiological traits, and also the plant

ecology (Merritt *et al*., 2014). Seed longevity parameters acquired from artificial ageing tests, have been shown to vary in relation to taxonomy, seed characteristics and macroclimate at the geographic origin of the species (Probert *et al*., 2009; Walters *et al*., 2005; Mondoni *et al*., 2011; Merritt *et al*., 2014). However, variation seed longevity is still wide both within closely related species (i.e. within plant family or genus) and macroclimate, suggesting that other factors might be involved in driving longevity differences across species. Therefore in 2nd chapter focused on closely related taxa (i.e. species within family Asteraceae) from a macroclimatic uniform area (i.e. alpine) to investigate the effects seed and species-specific ecological traits on seed longevity. I decided to focus on alpine species since they are known to have short-lived seeds in storage (Mondoni *et al*., 2011; Merritt *et al*., 2014). Alpine plants are considered as one of the groups of species most sensitive to the direct and indirect threats to ecosystem caused by land use and climate change (Korner, 2003; Parolo *et al*., 2008). Therefore, there are significant concerns for both successful *ex situ* and *in situ* conservation of alpine plants. Additionally, the large species and microhabitat diversity found in alpine habitats provide an ideal context to assess changes in longevity response related with plant ecology.

Seed priming

Priming provides controlled hydration of seeds to a level that allows pre-germination metabolic activity to proceed, but prevents the actual emergence of the radicle after priming, the seeds can be dried back to the initial moisture content (Bradford, 1990). A wide variety of priming treatments are available to enhance seed vigour, although hydropriming and osmopriming are commonly used methods to prime seeds (McDonald, 1999). However, the success of seed priming is strongly correlated to plant species/genotype and physiology, seed lot and vigour, as well as to the priming method applied (Perera and Cantliffe, 1994).

Priming has been shown to be both beneficial and detrimental to subsequent longevity (Tarquis and Bradford, 1992; Probert *et al*., 1991; Powell *et al*., 2000; Butler *et al*., 2009; Hill *et al*., 2007). Especially, differences in the effect of priming on the germination and longevity of low and high quality seed lots have been reported in many studies (Powell *et al*., 2000; Demir, 2003; Butler *et al*., 2009). Powell *et al*., (2000) suggested that low vigour seeds may benefit from priming, whereas high vigour seeds are not. Since seed longevity of alpine species are very low when compared to other species, seed vigour of those species seems

low. Therefore, positive effect of seed priming on longevity may possible even for freshly collected seeds.

The possibility of extending storage life of seeds has obvious practical implications, especially for germplasm conservation of inherently short-lived wild species (Probert *et al*., 1991) such as alpines (Mondoni *et al*., 2011) and native seed industry. Moreover, most of the alpine species poor seedling conversion, that can be improved by seed priming technique. Therefore, in 4th chapter, I investigated the potential for priming to increase the longevity of six alpine species using range of water potentials (hydro and osmo-priming). We specifically focused on these alpine species since they are short-lived and radicle emergence percentage was substantially higher than normal germination (see chapter 3) in artificial ageing (AA).

Seed priming studies on wild plants have revealed that priming may have a rejuvenating effect on seed as shown in *Digitalis pupurea* L. (Butler *et al*., 2009) and *Ranunculus sceleratus* L. (Probert *et al*., 1991). However, the effects of priming on seed longevity in wild alpine species has not been investigated yet but the possibility of its effectiveness will be highly species-dependent and might vary across plant populations (Paparella *et al*., 2015).

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CHAPTER 2

Effect of Seed and Ecological Traits on Seed Longevity: A Case Study of Alpine Asteraceae

ABSTRACT

Seed longevity has been investigated predominantly in relation to taxonomic and macroclimatic differences, while little is known about the variation within closely related taxa, growing under the same climate. In this study, seed longevity of 18 alpine species Asteraceae was compared.

Mature seeds were subjected to an artificial ageing (at 45 °C and 60 % RH) and sampled at regular intervals for germination testing. The time taken for viability to fall to 50% (p_{50}) was determined using probit analysis and used as a measure of relative seed longevity between seed lots. The influence of seed and species-specific ecological traits (i.e. seed mass, soil pH and moisture) on seed longevity was investigated.

The estimates of p_{50} ranged between 1.68 and 40.03 d. Soil moisture significantly influenced seed longevity, with seeds of species growing on dry soil showing higher p_{50} than those from wetter soil. Conversely, seed mass and soil pH did not significantly contribute to longevity differences across species, though species from acid soils tended to be shorter lived than those from basic soils.

Seed longevity of alpine Asteraceae was low, but showed large variations across species, which was mostly explained by soil moisture conditions of the species growing habitat. Plant ecological traits, linked to microhabitat conditions may play crucial roles in the prediction of seed lot longevity in air-dry storage including seed bank conditions.

INTRODUCTION

The longevity of seeds is an important plant trait that allows *ex situ* conservation of plant germplasm, for tens or even hundreds of years (Walters *et al*., 2005). For orthodox species, storage temperature and moisture content of the seed are the two main factors determining longevity in storage. Loss of seed viability is inevitable even though the longevity is extended under the low air temperature and low seed moisture content (Smith *et al*., 2003). Therefore, Uncertainty in how long seed lots of a species might survive in storage makes it desirable to estimate their longevity prior to storage (Walters, 2003) and to periodically monitor viability (FAO, 2014). Storage experiments to determine seed longevity under standard seed banking conditions (e.g. 3-7 % moisture content and -18 °C) may require long-term monitoring before there is any evidence of viability falling (Walters *et al*., 2005; Kochanek *et al*., 2009). As an alternative method, artificial ageing (AA) test has been used to estimate seed longevity and rank species or seed lots relative to each other. The relative longevity in the AA can be used as an indicator of seed longevity under seed bank conditions (Probert *et al*., 2009) and also persistence in the soil seed bank (Long *et al*., 2008).

The time taken in storage for viability to fall to 50 % as a measure of seed longevity, has been shown to vary predominantly in relation to macroclimate at the geographic origin of the species. For example, seeds from cool, wet climates are predicted to be shorter-lived in air dry storage than those from warm, dry climates (Probert *et al*., 2009; Walters *et al*., 2005). Consistently, seeds from high altitude alpine species were short-lived in storage than closely related species from warmer and drier lowland locations (Mondoni *et al*., 2011). However, seed longevity also varied considerably within closely related alpine species (i.e. within plant family). For example, seed longevity of alpine plants varied from 4.7 to 37.5 d and it ranged between 7.1 - 34.2 d and 11.5 - 37.5 d in Asteraceae and Caryophyllaceae, respectively (Mondoni *et al*., 2011). Since these species are growing in the same biogeographic zone (i.e. alpine) and macro area (i.e. Italian Alps), their seed longevity differences clearly indicates that other factors may influence for this particular plant trait, rather than taxonomy and macroclimate (see above).

Prediction of longevity based upon seed traits has been reported in several studies. For example, large endospermic seeds were shorter-lived than non-endospermic seeds (Probert *et*

al., 2009), seeds with folded embryo were longer-lived than other embryo types (Merritt *et al*., 2014) and seed mass was positively correlated with seed longevity (Merritt *et al*., 2014). However, closely related species, such as alpine Asteraceae, are expected to show small variation of seed traits (i.e. due to the convergence of taxonomic and environmental effect). Conversely, alpine Asteraceae show large ecological amplitude, inhabiting different soil types and soil moisture levels (Aeschimann *et al*., 2004), indicating that species microenvironment may play crucial role when assessing seed longevity. Kochanek *et al*. (2011) and subsequent observations (e.g. Mondoni *et al*., 2014; Bernareggi *et al*., 2015) reported significant transgenerational changes seed longevity, associated with changes of the parental growth environment, thereby highlighting important plastic responses.

The steep environmental gradients (e.g. temperature and water) found within a few meters in the alpine life-zone (Graham *et al*., 2012) provide an ideal context to assess changes in longevity in relation to the local micro environmental conditions. Consequently, aimed to investigate the influence of species-specific seed and ecological traits on seed longevity. To minimize potential taxonomic bias, we focused on 18 alpine species of Asteraceae previously found to show large variation in p_{50} (Mondoni *et al*., 2011) and that inhabit different microhabitats, in calcareous and siliceous alpine bedrocks. Difference between calcareous and siliceous substrate is mainly explained by chemical differences in the soil such as pH and soil moisture (Michalet *et al*., 2002). Such substrate differences are a crucial driver for the floristic composition of the alpine plant communities (Korner, 2003). Specific research aims were: 1) to investigate seed longevity under artificial ageing condition and 2) to whether seed traits (i.e. seed mass) and plant ecological traits (i.e. soil moisture and soil pH) are associated with seed longevity.

MATERIALS AND METHODS

Seed collection

Fresh mature seeds of 11 alpine Asteraceae (Table 1) were collected at the time of natural dispersal from both calcareous and siliceous grasslands at the Stelvio National Park (46.54288N; 10.42933E; 2565m asl) and in Val Dosdè (Sondrio Province), in August 2015. Species were chosen based on their presence and abundance at the study area. After collection, seeds were held under air-dry seed bank conditions (15 % RH, 15 °C) at the plant germplasm bank of the University of Pavia (Italy) until use. All the species had high viability in germination test.

Table 1. Seed longevity parameters, seed characteristics and plant ecological traits of alpine species from Asteraceae.

Species name	Longevity rank	p_{50} ± s.e. (days)	K_i ± s.e. NED	$1/\sigma$ ± s.e. (day^{-1})	σ (days)	Soil moisture [b]	Soil pH type [c]	Seed mass (g) [d]
Hypochaeris uniflora Vill.	1	1.68 ± 0.36	0.95 ± 0.21	0.56 ± 0.01	1.78	intermediate	acidic	3.31
Solidago virgaurea L.	2	3.12 ± 0.38	1.32 ± 0.25	0.42 ± 0.07	2.37	intermediate	neutral	0.55
Senecio doronicum (L.) L.	3	5.31 ± 0.85	1.37 ± 0.12	0.24 ± 0.04	4.21	intermediate	basic	1.80
Senecio incanus L. *subsp. incanus*	4	5.40 ± 0.79	1.08 ± 0.19	0.19 ± 0.02	5.10	dry	acidic	0.89
Hieracium intybaceum All.	5	5.95 ± 2.10	0.36 ± 0.08	0.06 ± 0.01	16.67	dry	acidic	0.65
Leucanthemopsis alpina (L.) Heywood	6	7.15 ± 0.70	1.53 ± 0.22	0.21 ± 0.03	4.68	intermediate	acidic	0.38
Artemisia umbelliformis Lam. [a]	7	10.90 ± 0.87	1.63 ± 0.25	0.15 ± 0.02	6.74	intermediate	neutral	0.29
Leontodon hispidus L.	8	12.30 ± 1.12	1.38 ± 0.20	0.10 ± 0.01	9.90	intermediate	neutral	1.20
Arnica montana L	9	13.38 ± 1.01	2.26 ± 0.27	0.17 ± 0.02	6.03	intermediate	acidic	1.20
Achillea clavenae L. [a]	10	14.59 ± 0.95	1.54 ± 0.17	0.11 ± 0.01	9.45	dry	basic	0.43
Cirsium spinosissimum (L.) Scop [a]	11	15.30 ± 1.45	1.09 ± 0.18	0.08 ± 0.01	14.09	wet	neutral	2.17
Achillea moschata Wulfen [a]	12	19.30 ± 1.97	1.99 ± 0.23	0.10 ± 0.01	9.70	intermediate	acidic	--*
Antennaria dioica (L.) Gaertn.	13	21.75 ± 1.19	2.13 ± 0.23	0.09 ± 0.01	10.54	dry	acidic	0.06
Artemisia glacialis L. [a]	14	26.56 ± 0.92	1.86 ± 0.20	0.08 ± 0.01	13.20	dry	neutral	--*
Centaurea scabiosa L. [a]	15	27.30 ± 2.54	1.28 ± 0.26	0.05 ± 0.01	21.43	dry	basic	6.60
Leontopodium alpinum Cass.	16	27.35 ± 2.95	2.38 ± 0.29	0.09 ± 0.02	11.58	dry	basic	0.12
Achillea millefolium L. [a]	17	27.40 ± 1.28	2.16 ± 0.19	0.08 ± 0.01	12.72	dry	neutral	0.20
Hieracium pilosella L.	18	40.03 ± 2.58	1.44 ± 0.18	0.03 ± 0.01	29.14	dry	neutral	0.20

. --*- data did not find; [a]- seed longevity data from Mondoni et al. (2011); [b,c]- soil moisture and soil pH data from Aeschimann et al. 2004 ; [d]- Seed mass (1000 seeds, g) data from Royal Botanic Gardens Kew's Seed Information Database (2017).

Artificial ageing

Seeds were removed from the drying room and equilibrated to the laboratory environment (ca. 20 °C; 50% RH). Equilibrium RH (eRH) was monitored using a data logger (TinyTag DataLogger), and it took 5 days for seeds to reach. Once seeds had equilibrated, they were sealed in a 300 × 300 × 130 mm electrical enclosure box (Ensto UK Ltd, Southampton, UK), over a non-saturated solution of LiCl (anhydrous, Laboratory Reagent Grade, Fisher Scientific UK Ltd, Leicester, UK) at 60 % RH and placed in an oven (ASAL, Srl, Apparecchi Scientifici Attrezzature Laboratori) at 45 °C.

One sample of 60 seeds of each species was retrieved from the AA environment after 0, 1, 3, 5, 7, 10, 15, 20, 30, 40 and 50 days for germination testing. For each species, three replicates of 20 seeds each were sown on 1 % agar in distilled water containing 250 ppm GA_3 (Sigma-Aldrich Company Ltd, Dorset, UK) in 55 mm Petri dishes and incubated at 25 °C, light (12 hours) and 15 °C dark (12 hours) in a LMS 250A cooled incubator (LMS Ltd, Sevenoaks, UK). Radicle emergence of 1-2 mm in length was recorded separately every 5 days for 40 or 70 days after sowing. Excess water accumulating in the Petri dish lids during the incubation period was removed in order to minimize the risk of damping off.

Determination of seed and ecological traits

Information on species' ecological traits such as preferred soil pH (acidic, basic or neutral pH) and moisture condition (dry, wet and intermediate condition) was obtained from Flora Alpina (Aeschimann *et al*., 2004). Importantly, species preferring to inhabit dry and intermediate soil moisture sites were equally represented (n = 9 and 8, respectively), while only one was found in wet soil and therefore excluded from statistical analysis. Seed mass of each species was obtained from Royal Botanic Gardens Kew's Seed Information Database (2017). Moreover, in addition to the empirical results of ageing experiments carried out here (see above), longevity data of seven additional Asteraceae (obtained using the same experimental approach as here) were derived from Mondoni *et al*., (2011). Consequently, seed longevity data of 18 alpine Asteraceae were eventually considered in this study. Certain characters such as presence of endosperm, embryo type, life form was not considered for data analysis because those characters were same across species.

Data analysis

Probit analysis was performed using GenStat Release 11.1 (VSN International Ltd, Oxford, UK) to estimate the time for viability (assessed as radicle emergence) to fall to 50 % (p_{50}) through fitting of the basic viability equation:

$$v = K_i - (p/\sigma)$$

where v is the viability (in normal equivalent deviates, NED) of the seed lot after p days in storage, K_i is the initial viability (NED) of the seed lot, and σ is the time (d) for viability to fall by 1 NED (i.e. the standard deviation of the normal distribution of the seed deaths over time) (Ellis and Roberts, 1980). Survival curves were plotted using Origin software (OriginLab, Northampton, MA), fitting K_i and $1/\sigma$ values which were obtained from the probit analysis.

GLM (ANOVAs) was performed to test for the effect of soil pH and soil moisture (and their interactions) on seed-lot characteristics (p_{50}, K_i and σ). Seed mass values were transformed to square root values to meet normality. Linear regression was performed between p_{50} and square root of seed mass. All data (i.e. seed longevity parameters and square root of seed mass) were tested for normality using the Shapiro-Wilk test prior to analysis. Analysis of the data was performed with SPSS statistical software version 21.

RESULTS

Variation of longevity parameters across species

In all 18 species, seed viability declined with increasing duration of the ageing treatment, with a wide variation in the time taken for viability to fall to 50 % (p_{50}; Table 1). p_{50} ranged between 1.68 d for *Hypocheris uniflora* to 40.03 d for *Hieracium pilosella,* with an overall mean of 15.82 ± 2.52 days. Initial seed quality (K_i) was lowest for *Hieracium intybaceum* (0.36 NED, 64.1 %) and highest for *Leontopodium alpinum* (2.38 NED; 99.1 %) with an overall mean of 1.59 ± 0.10 NED (94.4 %). σ ranged between 1.78 to 29.14 d for *Hypocheris uniflora* and *Hieracium pilosella* respectively, with an overall mean of 10.52 ± 1.63.

Correlates of longevity parameters

Soil moisture condition significantly influenced seed longevity (Table 2). In particular, seeds collected from dry soil had significantly higher p_{50} than seeds from intermediate soil (Table 2,

Figure 1A). Normal distribution of the seed deaths over time (σ) was significantly differed between two soil moisture categories (one way ANOVA, df=1, F = 10.25, P = 0.006; Figure 1B), while initial seed quality (K_i) was similar (one way ANOVA, df = 1, F = 0.11, P = 0.918). Therefore, variation in p_{50} values between dry and intermediate soil categories was mainly explained by variation in σ. Soil pH and interaction between soil pH and moisture condition did not significantly affect for p_{50} (Table 2). However, species from acidic soil tended to have short-lived seeds than other soil pH types (Figure 2). Finally, seed mass varied across species, ranging between 0.06 to 6.6 g (1000 seeds), though longevity did not correlate with it (Linear regression; R^2 = 0.277, B = - 4.441, t = -1.126, P = 0.277).

Table 2. Results of GLM (ANOVAs) performed to investigate the effect of soil moisture, soil types and their interactions on p_{50}.

Variance	SS	df	MS	*F*-value	*P*-value
Soil moisture	649.2	1	649.2	11.53	0.006
Soil pH	281.44	2	140.72	2.49	0.127
Soil pH*soil moisture	412.36	2	206.18	3.661	0.06

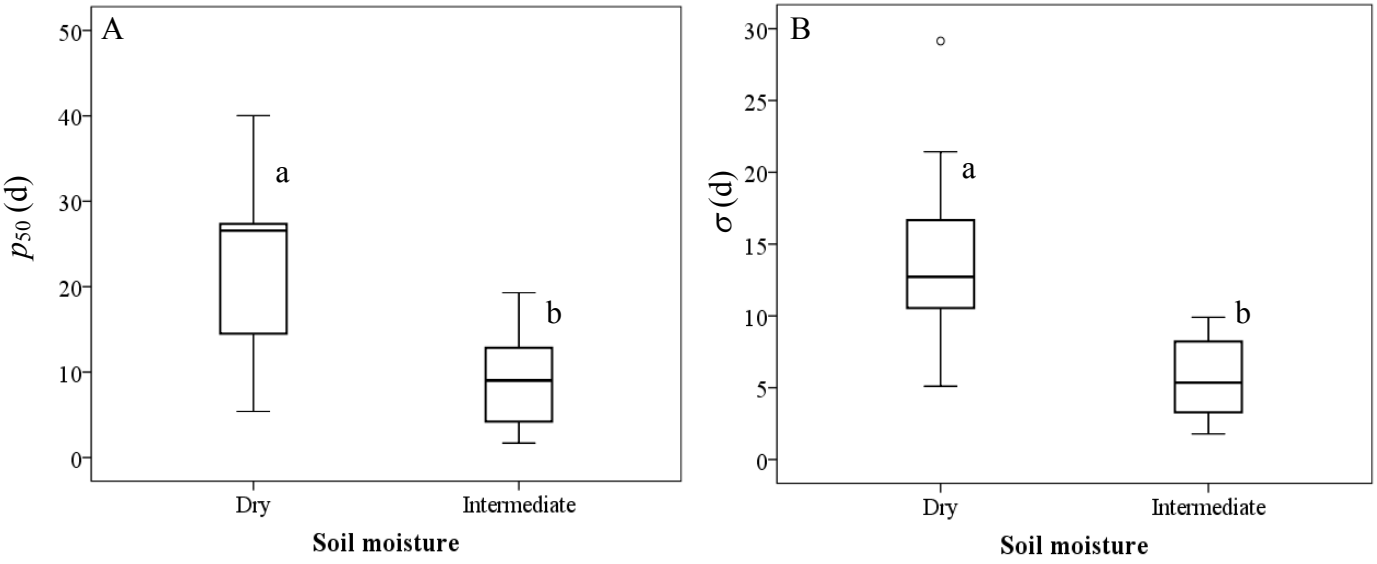

Figure 1. A) Box plots comparing p_{50} for dry and intermediate soil moisture levels B) Box plots comparing σ for dry and intermediate soil moisture levels. Mid-line of the boxplot shows the mean value and whiskers span the 5th to 95th percentile. Open circle represents an outlier. Different lowercase letters indicate significant differences (P < 0.05; one way ANOVA).

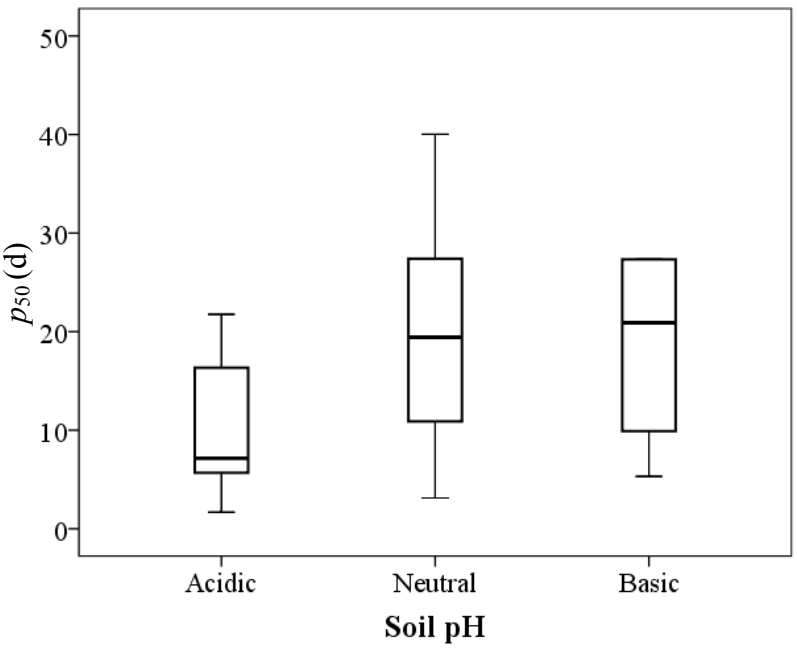

Figure 2. Boxplots comparing the p_{50} for acidic, neutral and basic soil pH levels ($P > 0.05$; one way ANOVA). Mid-line of the boxplot shows the mean value and whiskers span the 5th to 95th percentile.

DISCUSSION

Seed longevity has been shown to vary predominantly in relation to taxonomy and macroclimate at the geographic origin of the species both at global (Probert *et al*., 2009; Walters *et al*., 2005) and local scales (Mondoni *et al*., 2011). However, seed longevity varied significantly within closely related taxa and macroclimates, highlighting that further studies are needed to better understand drivers of longevity across species. To this end, in the present study, seed longevity of eighteen alpine Asteraceae based on AA was related with species-specific ecological traits (i.e. soil moisture). The estimates of p_{50} ranged between 1.68 and 40.03 d, showing a wide range of longevity across these species. Both initial seed quality (K_i) and distribution of seed death in time (σ) contributed to such variation in p_{50}.

Results also showed that seed longevity was significantly affected by soil moisture condition of the maternal environment, with species inhabiting dry soil producing longer-lived seeds than species inhabiting wetter soils (i.e. intermediate). Such a variation of p_{50} linked with soil moisture levels was explained only by differences in distribution of seed death in time (σ), while initial seed quality (K_i) was similar among soil moisture conditions. Consequently, despite showing high initial seed quality, seed collections of alpine species growing in

intermediate soil may need to be re-tested more frequently during storage to monitor their potential decline in viability than species from dry soil. Consistently, Kockanek *et al*., (2011) reported that seeds with narrower distribution of seed deaths in time (σ) were produced under wet soil condition compared to dry soil. Therefore, soil moisture condition may be a good indicator of seed longevity in storage and may have implications for *ex situ* seed conservation. At a global scale, Probert *et al*., (2009) hypothesized that prolonged longevity in the dry state evolved as an adaptation to either climatic drying, or the invasion of hot, dry environments. Interestingly, our results may further confirm this hypothesis showing that the same pattern may also occur at a local environmental scale (i.e. within microhabitat).

Soil pH has been related to soil seed bank persistence, with species from acid soils being associated with longer seed persistence than species from basic soil (Basto *et al*., 2015). Here, soil pH did not have a significant effect on seed longevity under AA, though species from acidic soil tended to have shorter-lived seeds than those from other soil types, suggesting that further studies are needed to clarify the role of pH as driver of longevity across species.

In accordance with previous studies (Probert *et al*., 2009; Walters, 2005), seed mass was not significantly correlated with seed longevity, but did show a positive trend. In contrast, Merritt *et al*. (2014) found a significant but weak positive correlation between seed mass and longevity, and speculated that the wide range of seed mass across species might strengthened the relationship i.e. more number of larger-seeded and small-seeded species into the analysis. Supporting this view, seed mass of our species was similar with less number of smaller and larger-seeded species (0.06 - 6.6 g/1000 seeds), confirming that seed mass may be a good longevity predictor only if there is a greater variation of this trait across species.

Finally, despite soil moisture contributing to explain most of the p_{50} variation across the species tested here, the large differences of p_{50} found within each soil moisture type (from 1.68 to 19.3 d and 5.4 to 40.3 d for intermediate and dry soil, respectively) suggest that other factors could be involved in determining the longevity of our seeds. In this regard, little is known about the effect of autogamy on seed longevity. However, autogamy (or apomixis), in which asexual formation of seeds directly from the maternal tissues of the ovule, may produce highly viable uniform seeds when compared to seeds from sexual reproduction (Ramulu, 1999), suggesting it may have an effect on their longevity too (i.e. in particular through σ). Interestingly, autogamy is common in alpine plants (Horandi, 2011, i.e. mostly in families Asteraceae,

Poaceae and Rosaceae), being found, for example, in the genus *Hieracium* and *Leontopodium*. *H. pilosella* and *L. alpinum* investigated in this study showed the highest seed longevity (but in *H. intybaceum* longevity was low), suggesting that further studies are needed to investigate the role that this particular plant trait may have on seed longevity.

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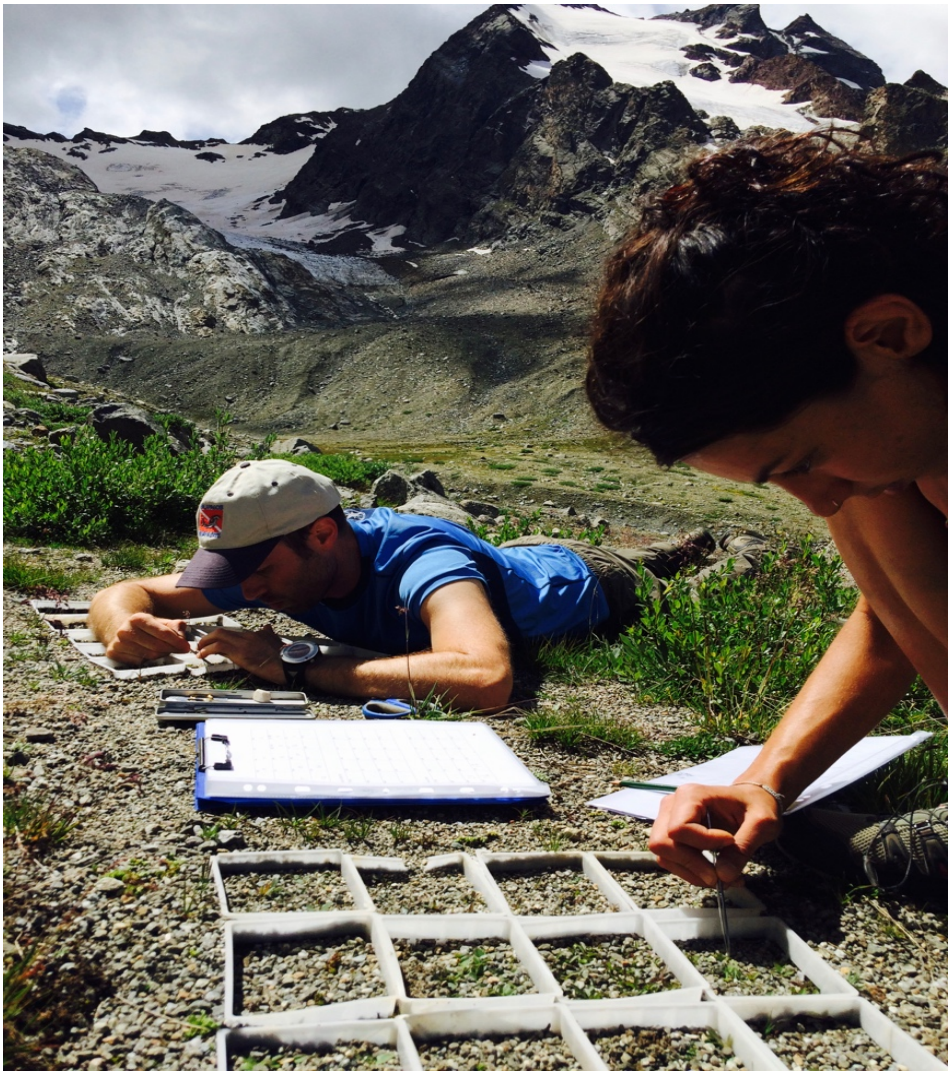
CHAPTER 3

Importance of Seedling Conversion on Estimates of Seed Longevity in Alpine Plants



ABSTRACT

Germination is considered to be the most reliable test to assess seed performance in storage, though radicle protrusion alone may over-estimate it, compared with normal germination (i.e. radicle plus cotyledon emergence). However, the extent of such overestimation across species and the factors contributing to it are not understood. Here, the seed life span of 35 alpine species was studied by evaluating both radicle emergence and normal germination.

Seeds were subjected to artificial ageing (at 45 °C and 60 % RH) and regularly sampled for germination testing, considering both radicle and normal germination. A coefficient of overestimation of seed longevity (OESL) was estimated and its' correlates with seed (i.e. mass, type) and ecological traits (i.e. soil pH and moisture) were explored.

Seed longevity was significantly higher when radicle instead of normal germination was considered as a proxy of seed viability in half of the species tested, with most of this variation being explained by differences in the initial seed quality (K_i). OESL was highest for short-lived dwarf seeded species growing in basic soil.

Radicle emergence may not always be a reliable indicator of the capacity of seeds to complete the germination process, leading to an overestimation of seed longevity in storage. Therefore, seed (gene) banks should assess normal germination when conducting viability monitoring test, to avoid the risk of over estimating viability. The coefficient of OESL and its correlates (i.e. seed type, soil pH and seed longevity) may be used to prioritize species' vulnerability to *ex situ* storage and to optimize viability testing.

INTRODUCTION

During development, orthodox seeds acquire a set of physiological traits of significance to survival and regeneration (Bewley *et al.* 2013; Waterworth *et al*., 2015). First the seed becomes capable of germinating when removed from the parent plant. Then the seed becomes tolerant of rapid artificial drying, often coinciding with the attainment of maximum dry weight. Thereafter, during maturation drying, develops an increased ability to live longer under various humidity (RH or moisture content) and temperature conditions. However, seed deterioration begins soon after seed dispersal and even under international seed banking conditions viability loss during the storage is inevitable (Bewley *et al.*, 2013), likely as a result of cumulative oxidative stress (Kranner *et al*., 2006). Uncertainty in how long a seed lot of a species might survive in storage makes it desirable to estimate the potential longevity of a seed lot prior to storage (Walters, 2003) and critical to periodically monitor viability during storage (FAO, 2014).

Understanding inter-specific differences in seed longevity is crucial for the effective management of seed banks, particularly to take decisions on when to regenerate fresh seed, or recollect on the basis of the rate of viability loss based on re-test intervals (Probert *et al.*, 2009). At its most complex, the mathematical characterisation of the sensitivity of seed longevity to moisture content and temperature has resulted in the generation of seed viability constants that can be used to predict seed longevity (Ellis and Roberts, 1980). Within parameters these constants are species-specific, but the use of thousands of seeds in their determination means that the generation of constants is not always feasible, especially for wild species (Probert *et al*., 2009; Hay and Probert, 2013). Similarly, storage experiments to determine seed longevity with standard seed banking conditions (e.g. 3-7 % moisture content and -18°C) may require long-term monitoring before there is any evidence of viability falling (Kochanek *et al.,* 2009). There are many alternative and quicker means of estimating seed longevity, including artificial ageing (AA) in which seeds are exposed to elevated temperature and relative humidity to accelerate ageing so that viability loss occurs within days to months rather than years (Rajjou and Debeaujon, 2008). This has been used successfully to rank and predict relative seed longevity (low, medium and high) of hundreds of species under conditions similar to that in the tropics (Probert *et al*., 2009; Mondoni *et al*., 2011). Therefore, seed banks can use AA predict the longevity of seeds, which can be affected by the seed processing regime (Rajjou and Debeaujon, 2008; Delouche and Baskin,

1973).

In a series of AA studies, ecological correlates have been used to identify that seeds of understory herbs and alpine species often have rather short longevities (Mondoni *et al*., 2011; Ali *et al*., 2007). It is also a case that many of these species have complex dormancies that combines morphological and physiological barriers to germination. There are many means of assessing survival of a longevity test, including physically (cut test) and biochemically (tetrazolium staining). However, radicle emergence is usually used in the vast majority of studies (Butler *et al.,* 2009; Kochanek *et al*., 2009; Probert *et al*., 2009; Mondoni *et al*., 2011; Bernareggi *et al*., 2015; Long *et al*., 2008; Merritt *et al*., 2014). Normal germination is considered to be the most reliable test of seed quality (ISTA, 2013), since it measures the ability of a seed to develop into a viable plant. Differences in the temperature control for radicle emergence and normal germination are known, e.g. *Oenocarpus bataua* Mart. (Bastos *et al*., 2017). Moreover, after normal seedling (i.e. radicle plus cotyledon emergence) was found to be a better indicator of *Delphinium* and *Salvia* seed viability after storage (Kwong *et al*., 2001) and in Salix seeds after various storage treatments including cryopreservation (Popova *et al*., 2013). The possibility exists therefore, that using radicle protrusion alone as an estimate of survival of storage may over-estimate the ability of the seeds to produce a plant (Ellis and Roberts, 1981). The extent of overestimation may depend on the species and seed age (Ellis and Roberts, 1981; Tarquis and Bradford, 1992). In addition, it may be more a feature of seeds that have MPD (Morpho-Physiological Dormancy) where the conversion of the small embryo into a germinating seed is a more exacting process (Porceddu *et al*., 2016). Therefore, seed longevity may have been overestimated, although the extent of overestimation across species, and the factors contributing to it are not yet well known. For example, the imbalance between radicle and full seedling emergence may be due to poor seed quality and/or low seed longevity in general, which in turn, have been linked with plant and ecological traits including taxonomy, seed characteristics (i.e. embryo type and seed mass; Merritt et al. 2014), macroclimatic conditions (Probert *et al*., 2009; Mondoni *et al*., 2011) and maternal environments (i.e. soil moisture content and temperature; see chapter 2; Kochanek *et al*., 2011; Bernareggi *et al*., 2015; Mondoni *et al*., 2014).

In this study, seeds of 35 alpine species were subjected to AA and their longevity was determined based on both radicle emergence and radicle plus cotyledon emergence (i.e. normal germination). I specifically focused on alpine species since they are expected to be

short-lived in air-dry storage and have a preponderance of endospermic seeds (Mondoni *et al*., 2011). Moreover, alpine species show large variation of seed traits (e.g. embryo type, dormancy) and ecological needs (e.g. inhabiting diverse microhabitats), which may be good predictors of seed longevity (see above). Therefore, I hypothesized that: 1) longevity estimates based on radicle emergence and normal germination may be different and 2) the extent of such differences across species is driven by seed (i.e. mass, embryo type) and ecological traits (i.e. soil pH and moisture).

MATERIALS AND METHODS

Target species and seed collection

The studied species inhabit two of the Natura 2000 habitat types: '6230 - *Nardus*-rich species grasslands', formed on various types of siliceous rocks with acidic pH; and '6170 - Alpine and subalpine calcareous grasslands', formed on calcareous bedrocks with alkaline pH (92/43/CEE "Habitat" Directive classification) (European Commission, 2007). For simplicity, each habitat is referred to hereafter by its soil type (siliceous and calcareous).

Fresh mature seeds of alpine species were collected based on their occurrence and abundance in these two habitats at the time of natural seed dispersal from grasslands at the Stelvio National Park (46.54288N; 10.42933E; 2565m asl) and in Val Dosdè (46.41022N; 10.20268E; 2225m asl) (Sondrio Province, Italy), during August 2015. After collection, seeds were held under air-dry conditions (15% RH, 15 °C) at the plant germplasm bank of the University of Pavia (Italy) until use. Seed viability of all species was determined through germination testing and 35 species (14 families) having high viable seeds were selected for this study (Table 2).

Artificial ageing

Seeds were removed from the drying room and equilibrated to the laboratory environment (ca. 20 °C; 50 % RH). Equilibrium RH (eRH) was monitored using a data logger (TinyTag DataLogger), and it took 5 days for seeds to reach eRH. Once seeds had equilibrated they were sealed in a 300 × 300 × 130 mm electrical enclosure box (Ensto UK Ltd, Southampton, UK), over a non-saturated solution of LiCl (anhydrous, Laboratory Reagent Grade, Fisher

Scientific UK Ltd, Leicester, UK) at 60 % RH and placed in an oven (ASAL, Srl, Apparecchi Scientifici Attrezzature Laboratori) at 45 °C.

One sample of 60 seeds of each species was retrieved from the AA environment (at 45 °C and 60 % RH) after 0, 1, 3, 5, 7, 10, 15, 20, 30, 40 and 50 days for germination testing. For each species, three replicates of 20 seeds each were sown on 1 % agar in distilled water containing 250 ppm GA_3 (Sigma-Aldrich Company Ltd, Dorset, UK) in 55 mm Petri dishes and incubated at 25 °C, light (12 hours) and 15 °C dark (12 hours) alternating condition in a LMS 250A cooled incubator (LMS Ltd, Sevenoaks, UK). Radicle emergence (RE) of 1-2 mm in length and subsequent full emergence of cotyledons (normal germination, NG) were recorded separately every 5 days for 40 or 70 days after sowing. Excess water accumulating in the Petri dish lids during the incubation period was removed in order to minimize the risk of damping off.

Seed and ecological traits

Information on species' ecological traits such as preferred soil pH (acidic, basic or neutral) and moisture condition (dry, wet and intermediate condition) was obtained from Flora Alpina (Aeschimann *et al*., 2004). Seed mass (50 seeds) of each species were measured and seed type of each species (at least to the genus level) derived from Martin (1946) (Appendix table 1).

Data analysis

Probit analysis was performed using GenStat Release 11.1 (VSN International Ltd, Oxford, UK) to estimate the time for viability (assessed as radicle emergence or normal germination) to fall to 50 % (p_{50}) through fitting of the basic viability equation:

$$v = K_i - (p/\sigma)$$

where v is the viability (in normal equivalent deviates, NED) of the seed lot after p days in storage, K_i is the initial viability (NED) of the seed lot, and σ is the time (d) for viability to fall by 1 NED (i.e. the standard deviation of the normal distribution of the seed deaths over time) (Ellis and Roberts, 1980). Survival curves were plotted using Origin software (OriginLab, Northampton, MA), fitting K_i and $1/\sigma$ values which were obtained from the probit analysis.

General linear models (GLM; ANOVAs) were performed to test for differences in p_{50}, K_i and σ calculated using radicle emergence vs NG (RE/NG), and also between species. RE/NG emergence, traits and their interaction were treated as fixed factors, while replicates were treated as random factors. All data were tested for normality using the Shapiro-Wilk test prior to analysis.

According to results of the GLM, species were categorized into two groups based on whether or not there were significant differences in the estimation of p_{50}, K_i and σ using radicle emergence or NG. Binary logistic regression tests were performed to investigate the influence of ecological and seed traits on p_{50} and K_i categories (significant and non-significant).

The overestimation of seed longevity (OESL) was calculated for each species as a percentage, using the following equation:

$$\text{OESL} = \frac{p_{50\ (\text{RE})} - p_{50\ (\text{NG})}}{p_{50\ (\text{NG})}}\ 100\ \%$$

Where, $OESL$ is the coefficient of overestimation of longevity of a seed lot; $p_{50\ (\text{RE})}$ is the time taken for radicle emergence to fall to 50%; and $p_{50\ (\text{NG})}$ is the time taken for NG to fall to 50%. The use of such coefficient allows a better measure of overestimation of seed longevity as it equally accounts for either species with high or low seed longevity.

GLM was performed to test for the effect of seed mass, soil pH, seed type and soil moisture (and their interactions) on OESL. OESL values were $\log_{10}$ transformed to ensure normality. OESL values were not calculated for *Gentianella anisidonta* and *Phyteuma orbiculare,* having initial NG lower than 50 % (hence showing a negative $p_{50\ (\text{NG})}$). Analysis of the data was performed with SPSS statistical software version 21.

RESULTS

Seed longevity parameters: radicle emergence vs. normal germination

Seed viability declined with increasing duration of the ageing treatment in all species, with a wide variation in the time taken for radicle and NG to fall to 50 % across species [$p_{50\ (\text{RE})}$ and $p_{50\ (\text{NG})}$, respectively]. $p_{50\ (\text{RE})}$ ranged between 1.3 d for *Gentianella anisidonta* to 41.9 d for *Silene vulgaris* subsp. *glareosa*, while $p_{50\ (\text{NG})}$ ranged between -1.33 d for *Phyteuma*

orbiculare to 37.49 d for *Leontodon helveticus* (Table 2). $K_{i\ (RE)}$ was lowest (0.57 NED; equivalent to 71.6% germination) for *Bartsia alpina* and highest (2.98 NED ≈ 99.9%) for *Dryas octopetala* and $K_{i\ (NG)}$ ranged from -0.18 (42.9 %) to 2.34 (99 %) NED for *Gentianella anisidonta* and *Leontopodium alpinum*, respectively. $\sigma_{\ (RE)}$ ranged between 1.27 d to 29.14 d and $\sigma_{\ (NG)}$ ranged from 1.32 to 27.48 d for *Oxyria dygyna* and *Hieracium pilosella,* respectively (Table 2).

p_{50}, K_i and σ differed between species and p_{50}, and K_i also differed when calculated based on radicle emergence compared to NG (Table 1). Because p_{50} and K_i showed a significant species*RE/NG interaction (Table 1), further analyses were performed at the species level. σ did not show any significant difference between radicle emergence and full germination (table 1).

Table 1. Results of the General Linear Model (GLM) performed on seed longevity parameters (p_{50}, K_i and σ) data to investigate differences between species, radicle emergence and normal germination.

Source of variation	p_{50}		K_i		σ	
	F-value	*P*-value	*F* -value	*P* -value	*F* -value	*P* -value
Intercept	1105.93	0.001	1444.94	0.001	365.56	0.003
Species	106.74	<0.001	18.61	<0.001	26.19	<0.001
Significant/non-significant	58.12	<0.001	122.13	<0.001	0.26	0.613
Replicates	2.97	0.054	2.42	0.092	3.23	0.043
Species*RE/NG	2.13	0.001	3.51	<0.001	0.96	0.532

All species showed higher $p_{50\ (RE)}$ than $p_{50\ (NG),}$ and the differences were statistically significant in 18 out of 35 species (GLM, $P < 0.05$, Table 2). Overall mean $p_{50\ (RE)}$ and $p_{50\ (NG)}$ values were 12.2 ± 3.8 d and 9.8 ± 3.6 d, respectively. The difference of 2.4 d represents a mean 24.49 % overestimation of $p_{50,}$ when calculated using radicle emergence, but was much larger than this for some species. Differences in $p_{50\ (RE)}$ and $p_{50\ (NG)}$ were assigned to different estimates of initial seed quality (K_i) for 11 species (Table 2), while for the other 7 species differences were not explained by K_i nor σ when analysed independently, indicating that their effects were cumulative. Two species had lower $K_{i\ (NG)}$ than $K_{i\ (RE)}$, but this did not contribute to differences in $p_{50\ (RE)}$ - $p_{50\ (NG)}$. The difference of overall mean K_i value between radicle (1.81 ± 0.2 NED) and NG (1.27 ± 0.2 NED) was 0.54 NED (42.52 % mean overestimation; and significant ($F = 11.764$; df = 1; $P < 0.05$). Conversely, differences in σ between the two growth quality criteria were not significant for any species (GLM, $P > 0.05$, Table 2).

Table 2. Seed longevity parameters for each species including initial seed quality (K_i), standard deviation of the distribution of seed life spans (σ) and time taken to fall viability to 50% (p_{50}) for both radicle emergence (RE) and normal germination (NG). Significant differences (Sig.) of p_{50} and Ki between RE and NG, are represented at $P \leq 0.05$ (**) (N.S. not significant). Also shown the overestimation of seed longevity (OESL).

Species	**p_{50} (days)**			**K_i (NED)**			**σ (days)**		**OESL**
	RE	NG	Sig.	RE	NG	Sig.	RE	NG	
Asteraceae									
Antennaria dioica (L.) Gaertn.	21.75	20.33	N.S.	2.13	2.04	N.S.	10.54	10.27	7.00
Arnica montana L.	13.38	12.03	N.S.	2.26	2.01	N.S.	6.03	6.16	11.25
Hieracium pilosella L.	40.03	35.97	N.S.	1.44	1.39	N.S.	29.14	27.48	11.28
Leontopodium alpinum Cass.	27.35	25.99	N.S.	2.38	2.34	N.S.	11.58	11.17	5.23
Leucanthemopsis alpina (L.) Heywood	7.15	6.64	N.S.	1.53	1.36	**	4.68	4.88	7.64
Leontodon helveticus Mérat	38.38	37.49	N.S.	1.69	1.61	N.S.	24.14	23.91	2.28
Senecio doronicum (L.) L.	5.31	4.38	N.S.	1.37	1.00	N.S.	4.21	4.60	21.05
Solidago virgaurea L.	3.12	2.34	N.S.	1.32	1.10	N.S.	2.37	2.16	32.87
Campanulaceae									
Campanula scheuchzeri Vill.	3.76	1.43	**	1.50	0.34	**	2.50	4.35	162.78
Campanula barbata L.	10.32	8.27	**	1.64	1.12	**	6.53	7.40	24.76
Campanula cochleariifolia Lam.	9.53	3.32	**	2.14	0.54	**	4.69	6.11	187.12
Phyteuma hemisphaericum L.	6.19	4.93	N.S.	2.02	1.70	**	3.08	2.90	25.57
Phyteuma orbiculare L.	4.81	-1.33	**	2.04	-0.14	**	2.71	6.94	-
Caryophyllaceae									
Silene acaulis (L.) Jacq.	27.00	25.46	N.S.	2.52	2.33	N.S.	10.87	11.09	6.02
Silene rupestris L.	33.23	25.13	**	2.13	1.99	N.S.	15.83	12.75	32.24
Silene vulgaris subsp. glareosa (Jord.) Marsden-Jones & Turrill	41.91	34.30	**	2.44	2.02	N.S.	17.35	17.13	22.21
Silene vulgaris (Moench) Garcke subsp. *Vulgaris*	24.39	20.77	**	2.11	1.65	N.S.	11.90	12.91	17.43
Gentianaceae									
Gentiana acaulis L.	10.01	5.27	**	1.98	1.04	**	5.16	5.22	90.10
Gentiana verna L.	2.35	0.08	**	1.16	0.05	**	2.19	2.34	2795.81
Gentiana anisodonta Borbás.	1.32	-0.25	**	0.93	-0.18	**	1.44	1.49	-
Juncaceae									
Luzula alpino-pilosa (Chaix) Breistr.	8.83	7.50	N.S.	2.11	1.66	N.S.	4.24	4.53	17.68
Onagraceae									
Epilobium fleischeri Hochst.	3.45	2.24	**	1.75	1.09	**	1.99	2.05	54.55
Orobanchaceae									
Bartsia alpina L.	1.55	0.95	N.S.	0.57	0.40	N.S.	2.74	2.60	62.24
Plantaginaceae									
Veronica bellidioides L.	12.64	8.58	**	2.22	2.11	N.S.	5.84	3.95	47.29

Table 2 continued.

Species	p_{50} (days)			K_i (NED)			σ (days)		OESL
	RE	NG	Sig.	RE	NG	Sig.	RE	NG	
Poaceae									
Festuca halleri All. subsp. Halleri	4.01	3.79	N.S.	2.08	2.07	N.S.	1.94	1.90	5.78
Festuca luedii (Markgr.-Dann.) Foggi, Gr. Rossi, Parolo & Wallossek	7.39	6.56	N.S.	2.02	1.90	N.S.	3.93	3.57	12.65
Festuca quadriflora Honck.	6.46	5.95	N.S.	1.82	1.77	N.S.	3.66	3.49	8.70
Helictotrichon versicolor (Vill.) Pilg.	4.98	3.79	N.S.	0.83	0.83	N.S.	6.17	4.78	31.48
Poa alpina L.	8.83	8.42	N.S.	0.77	0.71	N.S.	11.95	12.13	4.85
Poligonaceae									
Oxyria digyna (L.) Hill	3.27	2.87	**	2.65	2.30	N.S.	1.28	1.32	14.05
Primulaceae									
Primula farinosa L.	13.15	4.81	**	2.25	0.71	**	5.96	7.00	173.61
Rasaceae									
Dryas octopetala L.	6.54	4.53	**	2.99	1.62	N.S.	2.39	2.83	44.32
Geum montanum L.	4.28	3.38	**	0.82	0.58	N.S.	5.40	5.82	26.51
Saxifragaceae									
Saxifraga stellaris L.	9.52	7.02	**	2.22	1.03	**	4.40	7.02	35.52
Scrophulariaceae									
Pedicularis rostratocapitata Crantz	2.54	0.63	**	1.61	0.35	**	1.59	1.79	304.04

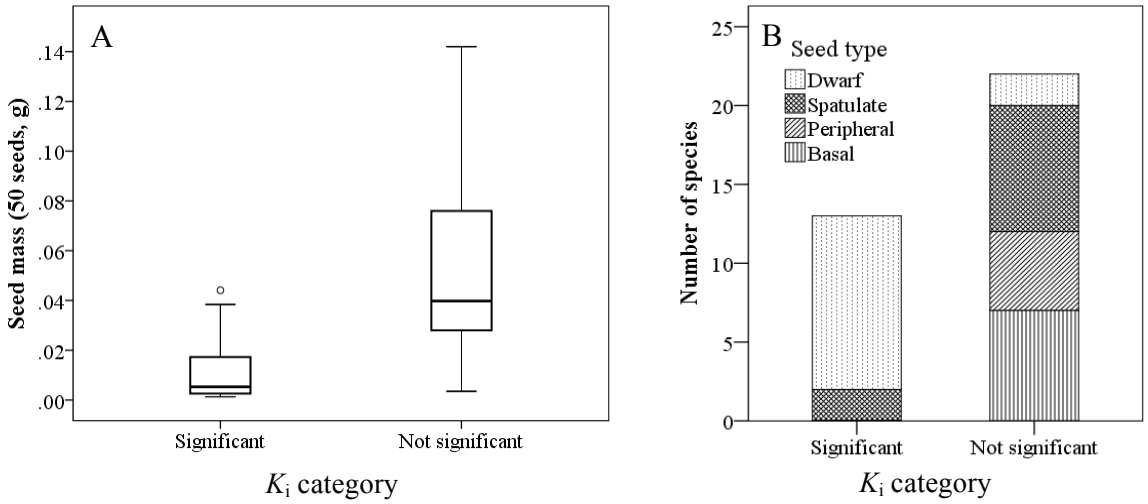


Figure 2. (A) Boxplots showing the seed mass distribution across species between K_i categories (significant and not significant). (B) Bar columns are showing the number of species with different seed type distribution between K_i categories (significant and not significant).

Table 3. Results of the Binary logistic regression performed on the significant and non-significant categories of both K_i and p_{50} data using different variables.

Source of variation	K_i			p_{50}		
	Wald (χ^2)	df	*P*-value	Wald (χ^2)	df	*P*-value
Seed mass	5.092	1	0.024	2.638	1	0.104
Seed type	7.858	3	0.050	6.895	3	0.075
Soil pH	1.319	2	0.517	2.477	2	0.290
Soil moisture	2.729	2	0.256	0.170	2	0.918

According to the logistic regression, seed traits (mass, type) and soil traits (moisture, pH) did not statistically contribute to the explanation of differences between $p_{50\,(RE)}$ and $p_{50\,(NG)}$ (Table 3). However, seed mass was statistically lower in species that had a significantly lower K_i than that of the other species (Table 3; Figure 2A). Additionally, seed type also contributed to differences in K_i with all 13 species that had signifcantly lower $K_{i\,(NG)}$ than $K_{i\,(RE)}$ having a dwarf embryo (Figure 2B).

Correlates of overestimation of seed longevity

The extent of overestimation of seed longevity (OESL) ranged widely from 2.28 % for *Leontodon helveticus* to 2795.81 % for *Gentiana verna* (Table 2). OESL was significantly associated with seed type, seed mass and p_{50} (Table 4). In particular, species having dwarf seeds had significantly higher OESL when compared to other seed types (Figure 3A). Seed mass (linear regression, R^2= 0.152, B = -7.176, t = -2.318, P = 0.027; Figure 3C) and longevity (linear regression, R^2= 0.275, B = -0.030, t = -3.432, P = 0.002; Figure 3D) were negatively correlated with OESL. No significant relations were found with soil type and soil moisture (Table 4), though species from basic soil tended to show higher OESL than those from acid and neutral soils (P = 0.155; Table 4, Figure 3B).

Moreover, an interaction between soil pH and seed type was significantly associated with OESL. Further analyses revealed that OESL in dwarf seeded species significantly differed between soil types (one-way ANOVA; F = 6.112; df = 2; P = 0.024). In particular, dwarf-seeded species growing in basic soil had higher OESL when compared with neutral and acidic soils (Figure 3B).

Table 4. Results of GLM (ANOVAs) performed to investigate the effect of seed types, soil pH, soil moisture and their interactions on OESL.

Source of variation	df	SS	MS	*F*-ratio	*P*-value
Seed type	3	6.168	2..056	9.721	0.000
Soil pH	2	1.437	0.719	1.984	0.155
Soil moisture	2	0.947	0.474	1.252	0.301
Seed type * Soil pH	11	9.182	0.835	5.618	0.000
Seed type * Soil moisture	8	0.160	1.160	1.291	0.289
Soil pH * Soil moisture	8	2.826	0.353	0.895	0.536

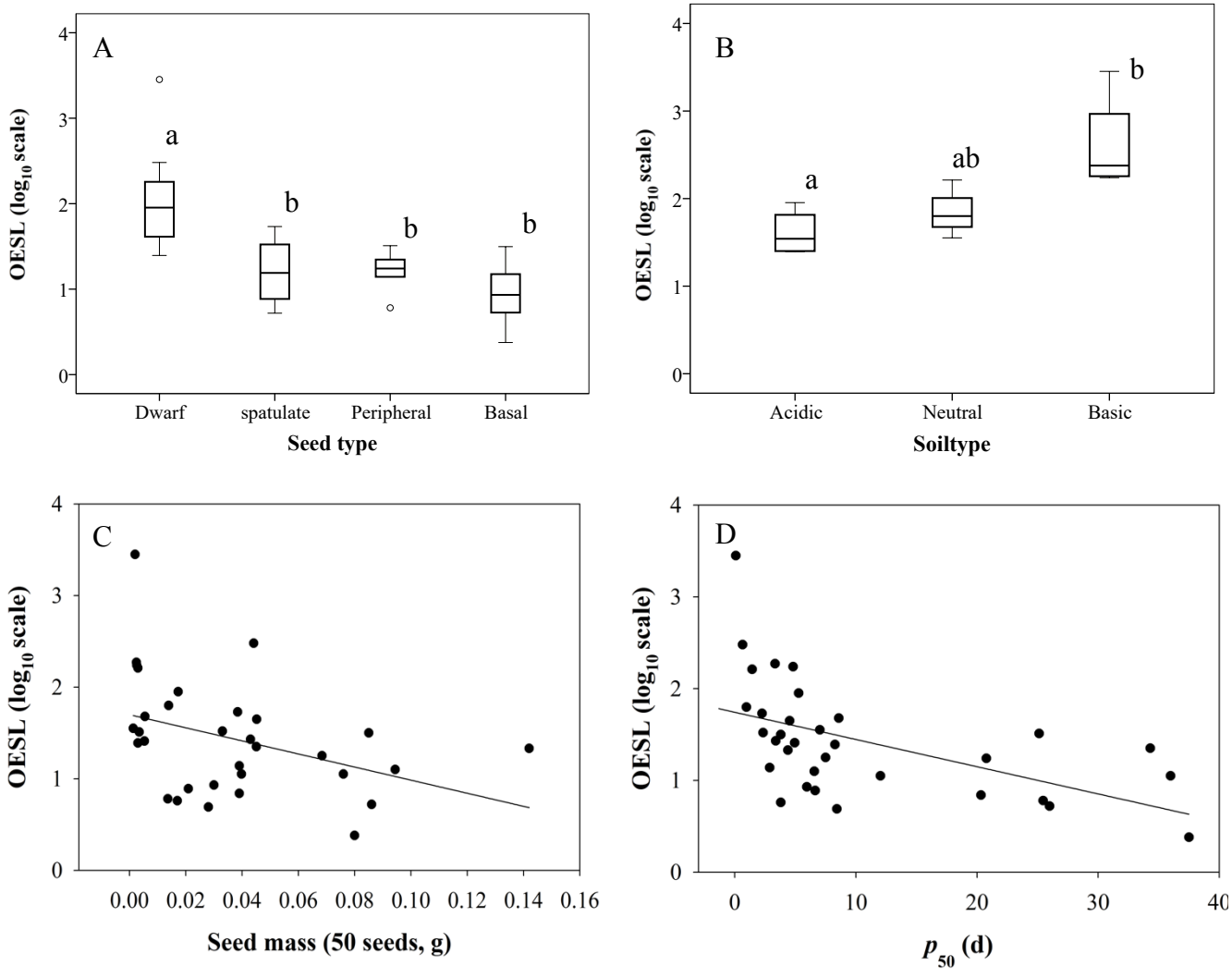


Figure 3. Correlates of OESL. (A) Box plots comparing OESL (log_{10} scale) between different seed types (B) Box plots comparing OESL (log_{10} scale) of dwarf seed types between different soil types. (C) Correlation of OESL and seed mass (50 seeds, g). (D) Correlation with OESL and seed longevity (filled circle, $p_{50\,(NG)}$). Different lowercase letters indicate significant differences ($P < 0.05$).

DISCUSSION

Radicle emergence vs NG

In this study, we have evaluated both radicle and radicle plus cotyledon emergence (i.e. normal germination) during AA. Normal germination was lower than radicle emergence for all species. As a result, estimates of p_{50} based on radicle emergence ($p_{50\ (RE)}$) were significantly higher than estimates based on normal germination ($p_{50\ (NG)}$) in 18 (51.4 %) of the 35 species tested. This suggests that radicle emergence may not be a reliable indicator of the capacity of seeds to complete the germination process, thereby leading to an overestimation of seed longevity in storage. Radicle emergence is widely used as a measure of viability in seed longevity studies, and it is therefore possible that the recovery potential has been overestimated in these investigations (e.g. Butler *et al*., 2009; Kochanek *et al*., 2009; Probert *et al*., 2009; Mondoni *et al*., 2011; Bernareggi *et al*., 2015; Hay *et al*., 2006; Merritt *et al*., 2014). For example, Mondoni *et al*., (2011) reported that seeds of alpine plants are short lived in storage. According to our results, the actual seed longevity of several alpine species may be lower than previously reported, highlighting that *ex situ* storage of alpine seeds under a range of conditions might be even more problematic than currently thought (Mondoni *et al*., 2011).

Standard deviation of the normal distribution of the seed deaths over time (σ) was similar for both quality assessment criteria. In contrast, 13 (40.6 %) of the 35 species had significantly lower $K_{i\ (NG)}$ than $K_{i\ (RE)}$, indicating that the ease of conversion from root to seedling emergence is straightforward in some species. As σ for RE and NG was not significantly different, this differential in conversion persists throughout storage, i.e. is not a simple function of seed quality. This group of species produced seeds with significantly lower mass when compared to the other 22 species, indicating that small seeds may be more likely fail to complete germination. Interestingly, this may explain why seed weight is often positively correlated with seed germination and seedling survival both among species (Marshall, 1986; Hutchinson, 1967) and within species (Schaal, 1980; Kitchen and Monsen, 1994; Krannitz, 1991).

Correlates of overestimation of seed longevity

A coefficient of overestimation of seed longevity (OESL) was used, which equally accounted in either species with high or low seed longevity. A very wide range of OESL was observed

across species depicting the considerable variability in seed germination performance. OESL was influenced by seed traits and species' ecology, indicating that such overestimation is not random across species, but rather determined by specific plant and ecological traits. In particular, OESL was highest for species with shorter-lived (low p_{50}), dwarf seeds and from basic soil.

In this context, seed traits (e.g. mass and embryo type) and the maternal environment are known to correlate with seed longevity. For example, seeds with folded embryos were significantly longer-lived than other embryo types such as dwarf or rudimentary (Merritt *et al.*, 2014). Moreover, positive correlation between seed size and seedling vigour has been documented for numerous species (Daws *et al*., 2003). In this study, dwarf seeded species had significantly high OESL when compared to other embryo types. Since dwarf seeded species are tiny seeds (0.3-2.0 mm long) with little storage reserves, and often have underdeveloped or undifferentiated embryos (e.g. 8 of the 13 dwarf-seeded species in this study; see Baskin and Baskin, 2007) as described by Martin (1946), these seeds may be more problematic in dry storage than large-seeded species with fully developed embryos. In contrast, in terms of soil seed bank, numerous studies have shown that persistent seeds tend to be smaller than transient seeds (Bakker *et al*., 1996; Bekker *et al*., 1998; Thompson, 1993). However, the evidence from a burial soil seed bank experiment on alpines shows that seed shape and hard-seededness are good predictors (smaller rounded seeds become buried more easily) of seed persistence in soil seed bank of alpine species, while seed size/mass seem to be less important (Schwienbacher *et al*., 2010).

The physical, chemical and biological properties of soil can directly or indirectly influence the absorption of water and nutrients by seeds, and in turn affect seed quality (Fageria *et al*., 2010). Nutrient deficiency at the time of flowering and bud initiation has a negative effect on seed vigour, viability and seedling performance (see Rengel, 1999; Fenner, 1992; Welch 1999). Deficiency of phosphorus and iron, and sometimes also manganese, zinc and copper are more common in calcareous soil than other soil types, and this may be related to the difference in OESL for dwarf seeds between soil types.

Implication for ex situ seed conservation and native seed industry

The results reported here provide important insights to how to improve longevity estimates whether for shorter- (seed industry) or longer-term (conservation) purposes. In particular, 1)

highlight a large overestimation of seed longevity when using radicle emergence, instead of NG as an assessment of recovery growth after storage, 2) used a new coefficient that quantifies conversion challenges post storage (the OESL) and show that high OESL is related to particular seed, embryo and plant ecological traits.

Moreover, this study confirms that seeds of many alpine plants are not only short lived in storage, but that the way in which recovery growth is assessed affects the interpretation of longevity data. In particular, species with high OESL, (i.e. those inhabiting calcareous grasslands and with dwarf seeds) need to be retested more frequently during storage. Conversely, in species with other embryo types (i.e. spatulate, basal and peripheral) and inhabiting acidic or neutral soils, radicle emergence may suffice as an indicator when it is impractical to measure normal germination. It follows that seed type and soil type are important considerations when selecting the most appropriate seed viability test and interval for the effective management of *ex situ* seed accessions of alpine. Taxonomy may guide such decisions, since dwarf seeds have been reported for several genera within families including Campanulaceae, Gentianaceae, Saxifragaceae and Scrophulariaceae, whilst species within the Asteraceae and Poaceae, for example, do not produce dwarf seeds (Martin, 1946). However, the definition of dwarf seeds based on size alone does not differentiate between the range of embryo and endosperm characteristics observed in small seeds (Baskin and Baskin, 2007). These may be more important factors for seed storage, and further investigation is needed for a clearer picture of the relationships between seed type and OESL.

CONCLUSIONS

In this study, we have provided clear evidence that radicle emergence may not always be a reliable indicator of the capacity of seeds to complete the germination process, thereby overestimating seed longevity in storage. We have developed the coefficient of OESL and identified correlates (seed type, soil type and seed longevity) that may be used to prioritize species' vulnerability to *ex situ* storage and optimize viability testing, thereby reducing labour costs and enabling more effective conservation of seed collections.

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APPENDIX

Table 1. Details of the species used in the study.

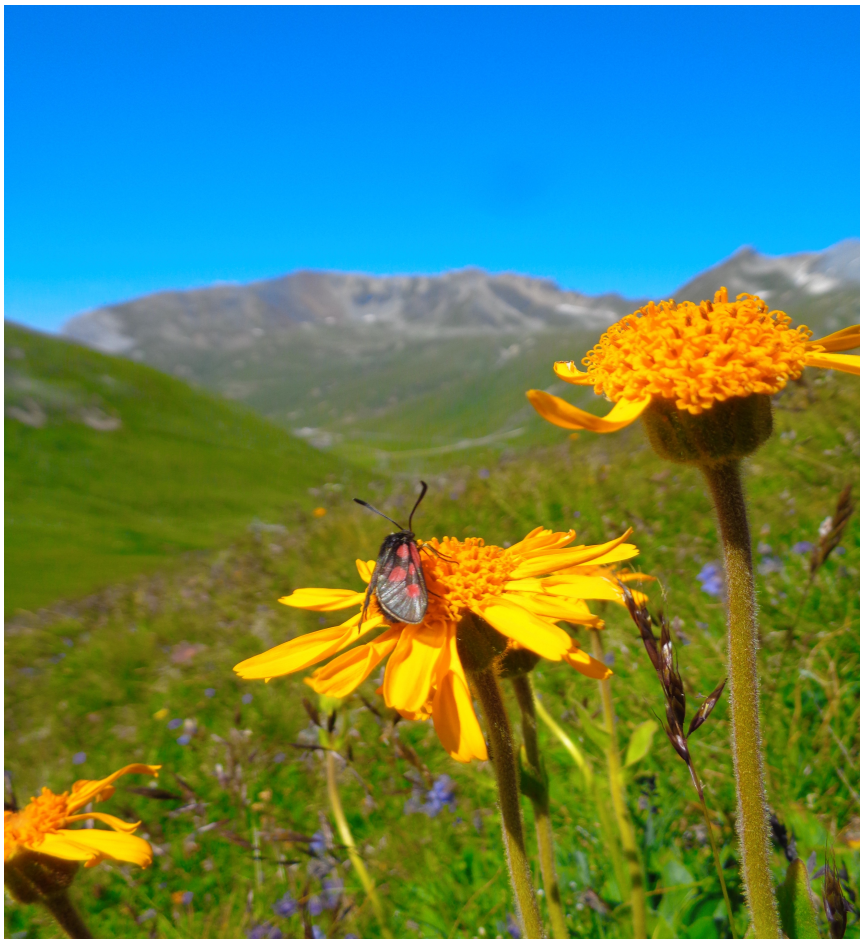
Plant species	Seed mass (50 seeds, g)	Seed type	Soil moisture	Soil pH
Asteraceae				
Leucanthemopsis alpina (L.) Heywood	0.0209	Spatulate	Intermediate	Acid
Solidago virgaurea L.	0.033	Spatulate	Intermediate	Neutral
Antennaria dioica (L.) Gaertn.	0.039	Spatulate	Dry	Acid
Hieracium pilosella L.	0.0398	Spatulate	Dry	Neutral
Arnica montana L.	0.076	Spatulate	Neutral	Acid
Leontodon helveticus Mérat	0.08	Spatulate	Neutral	Acid
Leontopodium alpinum Cass.	0.086	Spatulate	Dry	Basic
Senecio doronicum (L.) L.	0.142	Spatulate	Intermediate	Basic
Campanulaceae				
Campanula cochleariifolia Lam.	0.0025	Dwarf	Wet	Basic
Campanula scheuchzeri Vill.	0.003	Dwarf	Intermediate	Neutral
Campanula barbata L.	0.003	Dwarf	Intermediate	Acid
Phyteuma hemisphaericum L.	0.0053	Dwarf	Dry	Acid
Phyteuma orbiculare	0.0085	Dwarf	Intermediate	Basic
Caryophyllaceae				
Silene vulgaris (Moench) Garcke subsp. *Vulgaris*		Peripheral	Dry	Neutral
Silene rupestris L.	0.0035	Peripheral	Dry	Acid
Silene acaulis (L.) Jacq.	0.0136	Peripheral	Intermediate	Basic
Silene vulgaris subsp. glareosa (Jord.) Marsden-Jones & Turrill	0.0451	Peripheral	Dry	Basic
Gentianaceae				
Gentiana verna L.	0.002	Dwarf	Intermediate	Basic
Gentiana anisodonta Borbás.	0.0072	Dwarf	Dry	Basic
Gentiana acaulis L.	0.0173	Dwarf	Intermediate	Acid

Table 1 continued.

Plant species	Seed mass (50 seeds, g)	Seed type	Soil moisture	Soil pH
Juncaceae				
Luzula alpino-pilosa (Chaix) Breistr.	0.0684	Basal	Wet	Acid
Onagraceae				
Epilobium fleischeri Hochst.	0.0384	Spatulate	Intermediate	Neutral
Orobanchaceae				
Bartsia alpina L.	0.0139	Dwarf	Wet	Neutral
Plantaginaceae				
Veronica bellidioides L.	0.0055	Dwarf	Dry	Acid
Poaceae				
Festuca halleri All. subsp. Halleri	0.017	Basal	Dry	Acid
Poa alpina L.	0.028	Basal	Intermediate	Neutral
Festuca quadriflora Honck.	0.03	Basal	Dry	Basic
Helictotrichon versicolor (Vill.) Pilg.	0.085	Basal	Dry	Acid
Festuca luedii (Markgr.-Dann.) Foggi, Gr. Rossi, Parolo & Wallossek	0.0944	Basal	Intermediate	Acid
Poligonaceae				
Oxyria digyna (L.) Hill	0.039	Peripheral	Intermediate	Neutral
Primulaceae				
Primula farinosa L.	0.0026	Dwarf	Wet	Basic
Rasaceae				
Dryas octopetala L.	0.0452	Spatulate	Dry	Basic
Geum montanum L.	0.043	Spatulate	Intermediate	Acid
Saxifragaceae				
Saxifraga stellaris L.	0.0013	Dwarf	Wet	Neutral
Scrophulariaceae				
Pedicularis rostratocapitata Crantz.	0.0441	Dwarf	Dry	Basic

CHAPTER 4

Effect of Seed Priming on Seed Longevity of Alpine Plants: Implications for *Ex Situ* Seed Conservation and Native Seed Industry



INTRODUCTION

Prolong storage generally reduces seed viability and vigour, although the rate of deterioration varies among species and environmental conditions. Long term storage of desiccation tolerant seeds in the quiescent state is associated with cellular deterioration. This cellular damage is greatly exacerbated by adverse seed storage conditions and the consequent requirement for an extended repair period may underlie the delay to germination that is characteristic of low vigour seeds (Powell and Matthews, 2012). Seed vigour is closely related to seed longevity, as seeds of low vigour generally have shorter potential longevity than high-vigour seeds (Ellis and Roberts, 1981). However, enhancement of seed vigour in many species can be done by pre-germination priming treatments in which seeds are dried back following controlled hydration (Waterworth *et al*., 2015). However, priming treatments often have detrimental effects on storage life of the subsequent dried seeds (Perera and Cantliffe, 1994).

Priming provides controlled hydration of seeds to a level that allows pre-germination metabolic activity to proceed, but prevents the actual emergence of the radicle after priming, the seeds can be dried back to the initial moisture content (Bradford, 1990). A wide variety of priming treatments are available to enhance seed vigour, although hydro-priming and osmo-priming are commonly used methods (McDonald, 1999). During hydro-priming seeds are soaked in water (i.e. non-controlled water uptake) under optimal temperature with or without aeration (Taylor *et al*., 1998). Osmo-priming is a widespread pre-treatment procedure that involves treatment with osmotic solutions at low water potential facilitating the control water uptake (Paparella *et al*., 2015). The benefits (i.e. synchronous and fast emergence) of hydro-priming and osmo-priming have been widely reported (Paparella *et al*., 2015). However, the success of seed priming is strongly correlated to plant species/genotype and physiology, seed lot and vigour, as well as to the priming method applied (Perera and Cantliffe, 1994).

Following priming, dehydration and subsequent seed storage are crucial components of effectiveness of priming. For example, Gurusinghe and Bradford (2001) reported that slow-drying after priming likely to improve longevity. This slow drying may induce the synthesis of LEA (late embryogenesis abundant) proteins which may provide protective mechanisms that are beneficial to seed longevity (Gurusinghe *et al*., 2002). To facilitate handling and storage, the seeds should be carefully dried to an acceptable moisture level (i.e. 15 % eRH)

for long-term storage after the priming treatment. During dehydration after priming may involve removal of a large quantity of moisture from the seeds (Perera and Cantliffe, 1994).

Priming has been shown to be both beneficial and detrimental to subsequent longevity (Tarquis and Bradford, 1992; Probert *et al*., 1991; Powell *et al*., 2000; Butler *et al*., 2009; Hill *et al*., 2007). Especially, differences in the effect of priming on the germination and longevity of low and high quality seed lots have been reported in many studies (Powell *et al*., 2000; Demir, 2003; Butler *et al*., 2009). Powell *et al*. (2000) suggested that low vigour seeds may benefit from priming, whereas high vigour seeds are not. Certain alpine species have shown seedling conversion problem at initial period of storage and those species often had dwarf seeds (see chapter 3). In these cases, seed vigour is lower when compared to other alpines, indicating that benefits of seed priming on longevity may predictive even for fresh seeds. However, the effects of seed priming on longevity of wild alpine species has been not studied yet and the possibility of its effectiveness might be highly species-dependent Paparella *et al*., 2015).

The possibility of extending storage life of seeds has obvious practical implications, especially for germplasm conservation of inherently short-lived wild species (Probert *et al*., 1991) such as alpine plants, which are thought to be short lived in storage (See chapter 3, Mondoni *et al*., 2011) and have shown poor seedling conversion (see chapter 3). Therefore, here is an investigation of the potential for priming to increase the longevity and reduce seedling conversion problems of six alpine species using different water potentials (hydro and osmo-priming). Species were subjected to AA after different priming treatments and their longevity was determined considering both radicle emergence (RE) and radicle plus cotyledon emergence (i.e. normal germination, NG).

MATERIALS AND METHODS

Study species

Fresh mature seeds of six alpine species (*Primula farinosa* L.; *Gentiana acaulis* L.; *Gentiana verna* L.; *Pulsatilla alpina subsp. apiifolia* (Scop.) Nyman.; *Phyteuma orbiculare* L.; *Phyteuma hemisphaericum* L.) were collected at the time of natural dispersal from both calcareous and siliceous grasslands at the Stelvio National Park (46.54288N; 10.42933E; 2565m asl) and in Val Dosdè (46.41022N; 10.20268E; 2225m asl) (Sondrio Province), in

August 2015. After collection, seeds were held under air-dry seed bank conditions (15 % RH, 15 °C) at the plant germplasm bank of the University of Pavia (Italy) until use. Six species were selected in this study mainly considering previous results in which species having short-lived seeds with high percentage difference between radicle emergence (RE) and normal germination (radicle plus cotyledon emergence, NG) during AA.

Seed priming

Two priming methods were used for seed priming. For hydro-priming, seeds were soaked in water and for osmo-priming, seeds were exposed to osmotic solutions such as PEG (Poly ethylene glycol) and inorganic salts (i.e. KNO_3). Seed samples were removed from dry room (15 % RH, 15 °C) and equilibrated seeds at 20 °C over a non-saturated solution of LiCl giving 50 % eRH in a sealed plastic box. After equilibrations (approx. 5 days), seeds were placed in plastic containers (with filter papers) to soak seeds with distilled water (0 MPa), -2 MPa and -10 MPa and 2% KNO_3 for 3 days at 20 °C in the dark. Distilled water treatment (hydro-priming) was used as 0 MPa solution. -2 MPa was prepared by using PEG 8000 and non-saturated LiCl was used to create the environment of -10 MPa as described by Butler *et al*. (2009). Treated seeds were removed from the solutions and rinse with tap water until clean. Seeds were air dried over 24 hours and equilibrated again in 50 % eRH at 20 °C using non-saturated solution of LiCl over three-five days (the larger were the seeds the longer they needed to equilibrate). After equilibration, seeds were moved to AA.

Artificial ageing

Seeds were removed from the drying room and equilibrated to the laboratory environment (ca. 20 °C; 50% RH). Equilibrium RH (eRH) was monitored using a data logger (TinyTag DataLogger), and it took ca. 5 days for seeds to reach eRH. Once seeds had equilibrated they were sealed in a 300 × 300 × 130 mm electrical enclosure box (Ensto UK Ltd, Southampton, UK), over a non-saturated solution of LiCl (anhydrous, Laboratory Reagent Grade, Fisher Scientific UK Ltd, Leicester, UK) at 60 % RH and placed in an oven (ASAL, Srl, Apparecchi Scientifici Attrezzature Laboratori) at 45 °C.

One sample of 60 seeds of each species was retrieved from the AA environment after 0, 1, 3, 5, 7, 10 and 15 days for germination testing. For each species, three replicates of 20 seeds each were sown on 1 % agar in distilled water containing 250 ppm GA_3 (Sigma-Aldrich

Company Ltd, Dorset, UK) in 55 mm Petri dishes and incubated at 25 °C, light (12 hours) and 15 °C dark (12 hours) condition in a LMS 250A cooled incubator (LMS Ltd, Sevenoaks, UK). Radicle emergence of 1-2 mm in length (RE) and subsequent full emergence of cotyledons (Normal germination, NG) were recorded separately every 3 days for 30 days after sowing. Excess water accumulating in the Petri dish lids during the incubation period was removed in order to minimize the risk of damping off.

Data analysis

Probit analysis was performed using GenStat Release 11.1 (VSN International Ltd, Oxford, UK) to estimate the time for viability (assessed as radicle emergence or normal germination) to fall to 50 % (p_{50}) through fitting of the basic viability equation:

$$v = K_i - (p/\sigma)$$

where v is the viability (in normal equivalent deviates, NED) of the seed lot after p days in storage; K_i is the initial viability (NED) of the seed lot and σ is the time (d) for viability to fall by 1 NED (i.e. the standard deviation of the normal distribution of the seed deaths over time) (Ellis and Roberts, 1980). Survival curves were plotted using Origin software (OriginLab, Northampton, MA), fitting K_i and σ values which were obtained from the probit analysis.

The overestimation of seed longevity (OESL) was calculated for each species (each treatment) as a percentage, using the following equation:

$$\text{OESL} = \frac{p_{50\ (\text{RE})} - p_{50\ (\text{NG})}}{p_{50\ (\text{NG})}}\ 100\ \%$$

where, $OESL$ is the coefficient of overestimation of longevity of a seed lot. $p_{50\ (\text{RE})}$ is the time taken for radicle emergence to fall to 50% and $p_{50\ (\text{NG})}$ is the time taken for NG to fall to 50%. The use of OESL coefficient allows a better measure of overestimation of seed longevity as it equally accounts for either species with high or low seed longevity.

The effect of seed treatments on longevity (RE and NG) of each species was tested using one-way ANOVA, followed by Tukey's multiple comparison test. OESL values were $\log_{10}$ transformed before analysis to meet normality (OESL value was not calculated if the $p_{50\ (\text{NG})}$ value was a negative). All data were tested for normality using the Shapiro-Wilk test prior to analysis. Analysis of the data was performed with SPSS statistical software version 21.

RESULTS

Effect of seed priming on seed longevity

Primed seeds were longer lived than non-primed seeds ($p_{50\ (RE)}$) for all species except for *P. orbiculare*. In particular, hydro-priming significantly increased $p_{50\ (RE)}$ for *P. farinosa*, *G. verna* and *P. hemisphaericum* (see table 1, Figure 1). Interestingly, significant influence of seed priming on $p_{50\ (NG)}$ was prominent. Notably, hydro-primed seeds increased $p_{50\ (NG)}$ significantly than non-primed seeds in all the species (except *P. alpina*). However, *P. alpina* tended to improve normal germination (see Table 1, Figure 1, Appendix Figure 1).

$p_{50\ (RE)}$ and $p_{50\ (NG)}$ of non-primed seeds of *P. farinosa* was 13.2 and 3.42 d, respectively. In this species, although all priming treatments had positive effect on $p_{50\ (RE)}$, only 0 MPa and -10 MPa treatments significantly increased $p_{50\ (RE)}$ up to 22.17 and 23.52 d, respectively. Similarly, all priming treatments improved normal germination percentage ($p_{50\ (NG)}$) and only hydro-priming (0 MPa or distilled water treatment) significantly increased $p_{50\ (NG)}$ (16.37 d) when compared to other treatments (Table 1). Overall, hydro-priming significantly contributed to improve seed quality, thereby seed longevity.

$p_{50\ (RE)}$ and $p_{50\ (NG)}$ values for the non-primed seeds of *G. verna* were 2.97 and 1.16 d, respectively. $p_{50\ (RE)}$ increased significantly when seeds were treated with distilled water, -2 MPa and -10 MPa. $p_{50\ (NG)}$ was significantly improved only when seeds treated with distilled water. Even though, priming treatments improved $p_{50\ (RE)}$ of *Gentiana acaulis* when seeds were treated with distilled water and 2% KNO_3, increase was not statistically significant; on the other hand, $p_{50\ (NG)}$ was significantly increased when seeds were treated with distilled water.

Seed longevity of *Pulsatilla alpina* for untreated seeds was 4.78 and - 2.07 d for $p_{50\ (RE)}$ and $p_{50\ (NG)}$, respectively. Seed priming did not increase seed longevity significantly. However, seeds treated with distilled water increased $p_{50\ (RE)}$ to 5.02 d and $p_{50\ (NG)}$ to 2.98 d. Interestingly, for *Phyteuma orbiculare*, $p_{50\ (RE)}$ was reduced significantly for all primed seeds with respect to un-primed seeds. Conversely, $p_{50\ (NG)}$ increased significantly when seeds were treated with distilled water. $p_{50\ (RE)}$ and $p_{50\ (NG)}$ of un-primed seeds of *Phyteuma hemisphaericum* were 4.09 and 3.6 d, respectively. Both osmo-priming and hydro-priming treatments increased both $p_{50\ (RE)}$ and $p_{50\ (NG)}$ significantly. Conversely, 2 % KNO_3 treatment significantly reduced seed longevity.

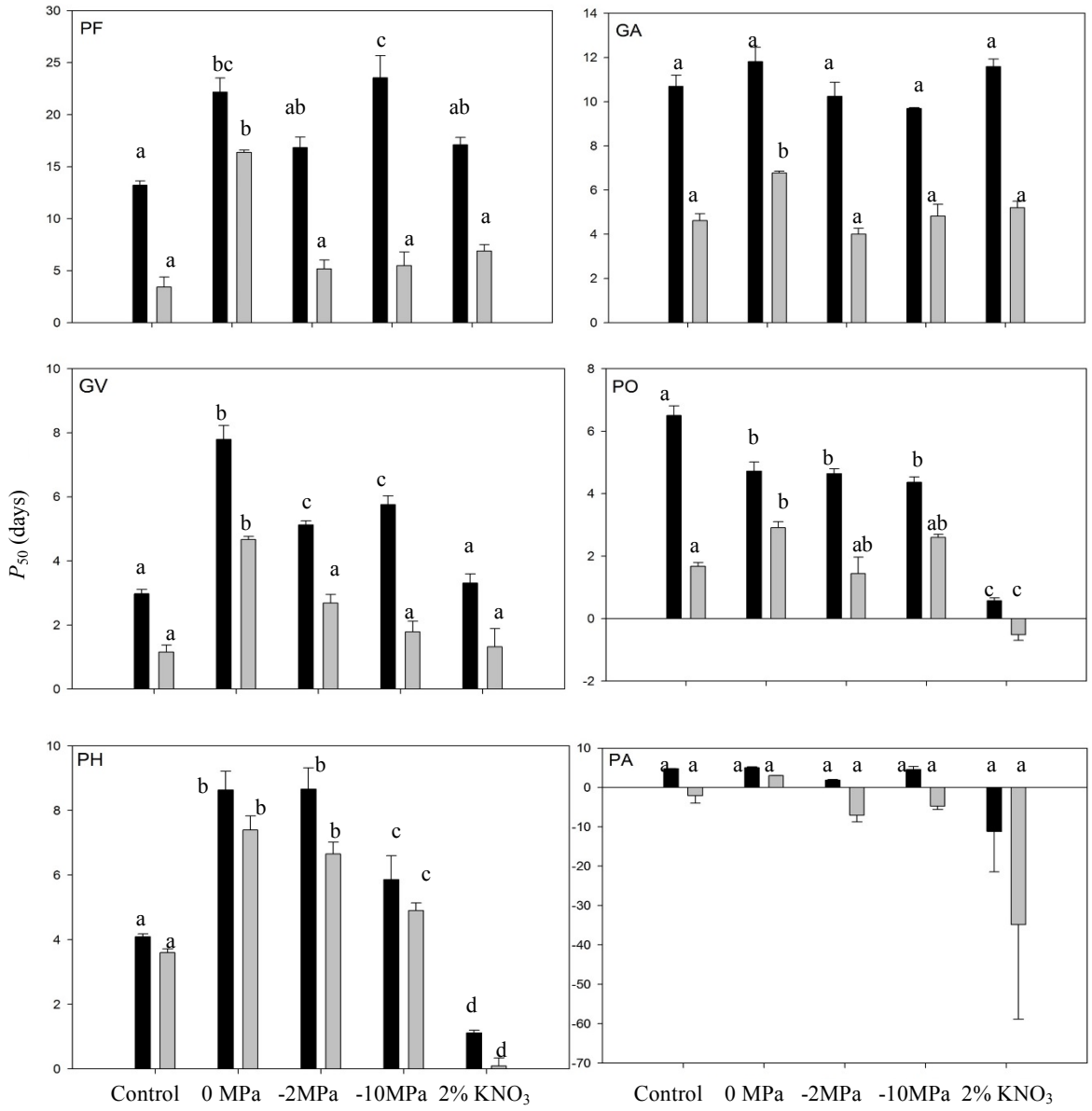


Figure 1. Bar charts showing the seed longevity ($p_{50\ (RE)}$ and $p_{50\ (NG)}$) after control and different priming treatments (black columns represent $p_{50\ (RE)}$ and grey columns represent $p_{50\ (NG)}$. Different lowercase letters indicate significant differences (one way ANOVA, $P < 0.05$) among treatments (RE or NG). PF, *Primula farinosa*; GV, *Gentiana verna*; GA, *Gentiana acaulis*; PA, *Pulsatilla alpina*; PO, *Phyteuma orbiculare*; PH, *Phyteuma hemisphaericum.*

Table 1. Results of one way ANOVA analysis comparing Seed longevity ($p_{50\ (RE)}$ and $p_{50\ (NG)}$) and OESL values among control (un-primed) and different priming treatments (0 MPa, -2 MPa, -10 MPa and 2% KNO_3).

Species	$p_{50\ (RE)}$			$p_{50\ (NG)}$			OESL		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Primula farinosa L.	4	11.29	0.001	4	34.57	0.000	4	7.76	0.004
Gentiana verna L.	4	57.44	0.000	4	18.43	0.000	4	1.63	0.242
Gentiana acaulis L.	4	3.33	0.56	4	9.53	0.002	4	3.07	0.068
Pulsatilla alpina subsp. apiifolia (Scop.) Nyman.	4	2.29	0.137	4	1.880	0.190	-	-	-
Phyteuma orbiculare L.	4	97.02	0.000	4	24.83	0.000	3	5.51	0.024
Phyteuma hemisphaericum L.	4	114.4	0.000	4	106.02	0.000	4	7.75	0.004

Effect of seed priming on OESL

Difference between radicle and normal germination was significant in un-primed seeds in all species ($p < 0.05$; data not shown) and, consequently, difference between $p_{50\ (RE)}$ and $p_{50\ (NG)}$ was also significant (Figure 1 and 2). Interestingly, such difference tended to decrease for primed seeds, although it was not significant for all species (Figure 2). Seed priming (i.e. Hydro-priming) decreased OESL significantly in two species (Table 1). In particular, distilled water or 0 MPa treatment (hydro-priming) decreased OESL for all species except *P. hemisphaericum* (Figure 2). In contrast, seeds treated with 2% KNO_3 increased OESL most of the time.

DISCUSSION

In this study, both hydro-priming and osmo-priming had a significant positive effect on seed longevity ($p_{50\ (R)}$ and $p_{50\ (NG)}$) in most cases, while, significant negative effect was shown in several species when seeds were treated with 2 % KNO_3. High ion concentration in the embryo may affect germination adversely when inorganic salts (i.e. KNO_3) are used as osmotica in the priming process. For example, Brocklehurst and Dearman (1984) reported that lower germination percentage of leek seeds primed in KH_2PO_3 solution than PEG solution and they noted that ions from inorganic salt solutions are able to penetrate and accumulate inside seed during priming. Here, half of the species decreased seed longevity when seeds primed with 2 % KNO_3 suggesting inorganic priming solution may not effective

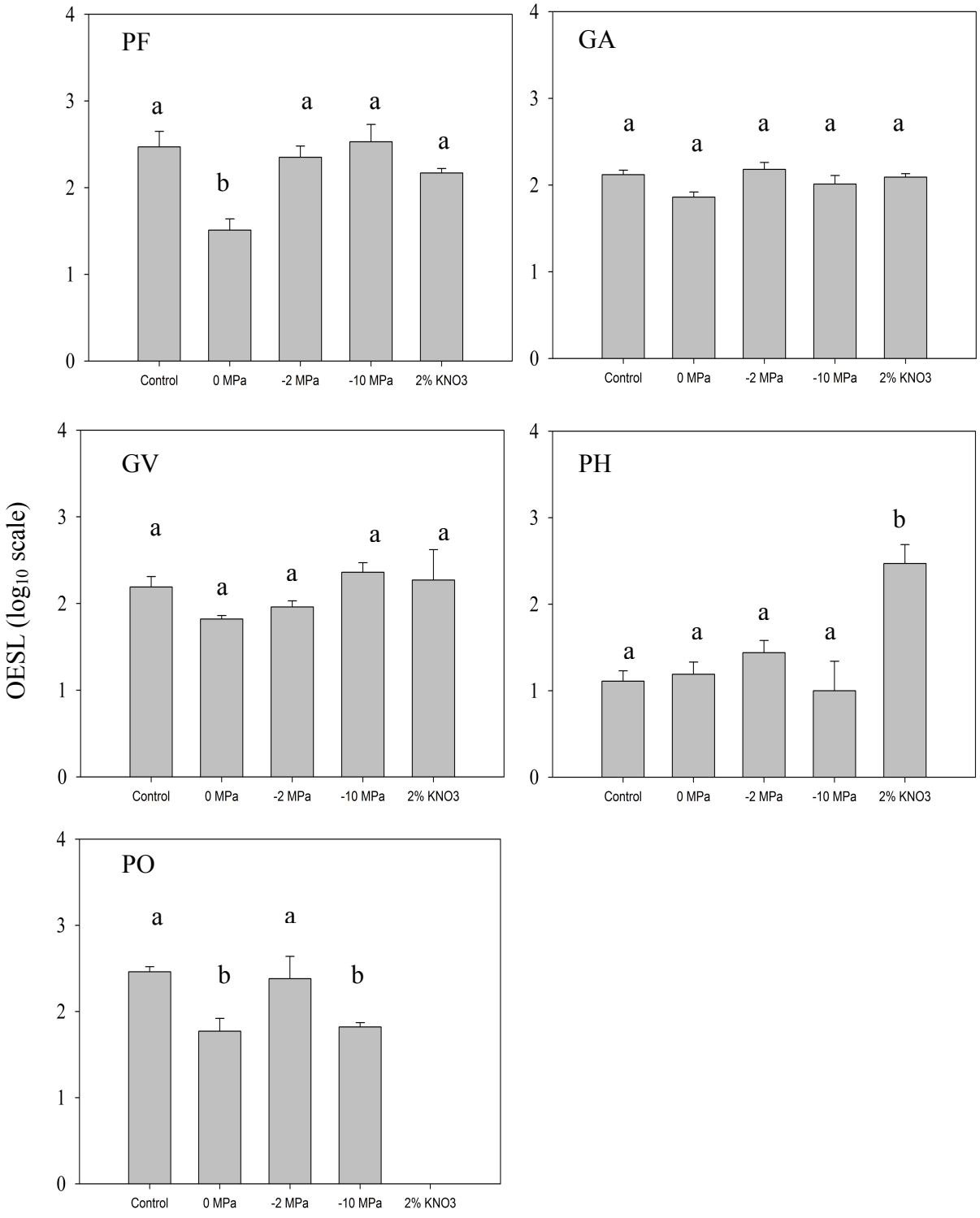


Figure 2. Column bars showing the OESL (log_{10} scale) values in different priming treatments of each species. PF, *Primula farinosa*; GA, *Gentiana acaulis*; GV, *Gentiana verna*; PH, *Phyteuma hemisphaericum*; PO, *Phyteuma orbiculare*. Different lowercase letters indicate significant differences ($P < 0.05$) among treatments.

on seed longevity in these alpine species. However, further studies should be done to get a clearer picture.

Hydro-priming was the most successful seed priming treatment to enhance both $p_{50\ (RE)}$ and $p_{50\ (NG)}$, being consistent in all species. Conversely, Butler *et al*. (2009) have reported that priming (- 1MPa) had a negative effect on newly collected mature *Digitalis purpurea* L. seeds, though priming could improve the subsequent longevity of mature seeds that had already experienced some experimental storage. Similarly, priming had negative effect on high viable seeds of *Brassica oleracea*, but had a positive effect on low vigorous seeds, suggesting that the effect of seed priming depend on the initial seed quality (Powell *et al*., 2000). Conversely, our results show that hydro and osmo-priming were effective treatments for fresh mature seeds of alpine plants (i.e. held in dry storage 15 $^{\circ}$C and 15 % RH for 2-3 months). Donà *et al*. (2013) reported that *Silene acaulis* L. from alpine locations are particularly vulnerable to oxidative damage during imbibition and concluded low level of seed viability of this species before transfer to experimental ageing condition. Moreover, fresh seeds of alpine plants often had poor seedling conversion success and they have shown low longevity (see chapter 3), may be an indication of low level of seed vigour (Ellis and Roberts, 1981). This low level of seed vigour could be one of the reasons why priming treatments had positive results in alpine species. Interestingly, while p_{50} of *Digitalis purpurea* was about 30 days (Butler *et al*., 2009), our highest p_{50} value for the alpine species was 13 days, indicating that the effectiveness of priming may be a feature of short-lived seeds. Moreover, most of the species tested in this study had underdeveloped or undifferentiated embryos (see chapter 3) at the time of seed dispersal, possibly with different maturity levels. During seed priming, these embryos can grow up to a certain level and may be more resistance for artificial ageing conditions. This could be another possible explanation why priming had positive effect. However, further studies are needed to be done to understand the effectiveness of priming on short-lived seeds in general.

Moreover, difference between $p_{50\ (RE)}$ and $p_{50\ (NG)}$ were significant in non-primed seeds, showing the incapability of seeds becoming a seedling after radicle has emerged. Interestingly, OESL decreased substantially when seeds were treated with distilled water (hydro-priming), showing that hydro-priming reduces the gap between radicle emergence and normal germination percentage. This is an interesting and novel finding highlighting that

despite alpine seeds may show low vigour (i.e. poor seedling conversion), it can be greatly enhanced by using feasible priming methods.

Merritt and Dixon (2011) have highlighted the increase demand for use of wild species for restoration projects and the role of *ex situ* seed banks to fulfil the demand by providing restoration-ready seeds. In this context, despite alpine seeds are expected to be short-lived in storage (Mondoni *et al*., 2011), our results show that the application of hydro and osmo-priming (easy and inexpensive techniques) may significantly improve their longevity in dry storage, allowing the use of good quality seeds in conservation and restoration practices. However, extensive repeated priming experiments should be done in different conditions in order to optimize a proper priming method for short-lived seeds.

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Appendix

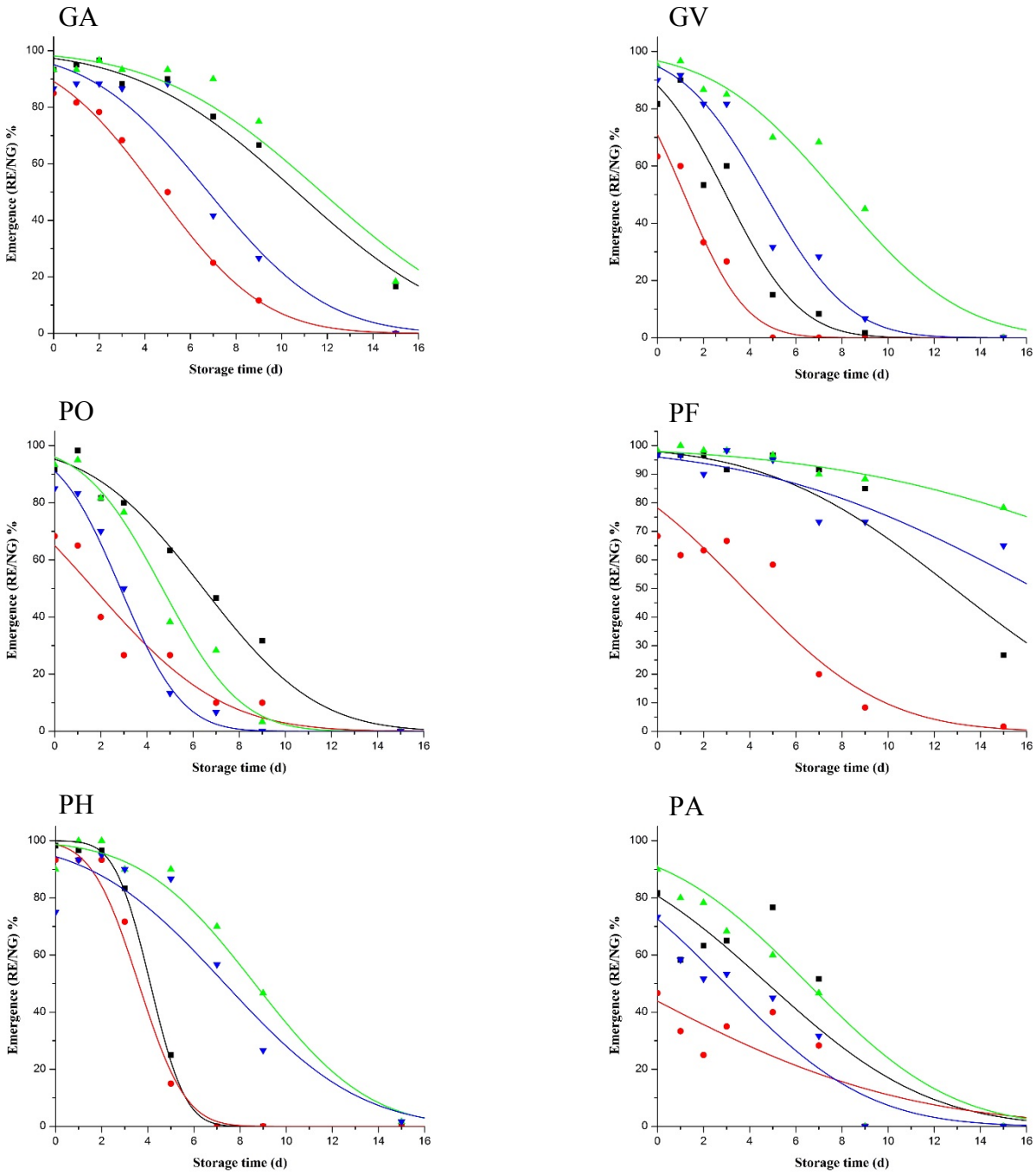

Figure 1. Survival curves fitted by probit analysis for control treatment (filled black square, black line (RE) and filled red circles, red line (NG)) and 0 MPa or hydro-priming treatment (filled green triangle, green line (RE) and filled inverted blue triangle, blue line (NG)). PF, *Primula farinosa*; GV, *Gentiana verna*; GA, *Gentiana acaulis*; PA, *Pulsatilla alpina*; PO, *Phyteuma orbiculare*; PH, *Phyteuma hemisphaericum*.

CHAPTER 5

Discussion and Conclusions

DISCUSSION AND CONCLUSIONS

Understanding species difference in seed longevity is crucial to the effective management of seed conservation collection because it underpins the selection of viability re-test intervals and hence regeneration or re-collection strategies (Probert *et al*., 2009). Our results clearly show that species with short-lived seeds are very abundant in alpine flora (Mondoni *et al*., 2011) when compared to other ecosystems, such as Australian flora which is abundant with long-lived seeds (see Merritt *et al*., 2014). Therefore, is significant concern for the successful *ex situ* conservation of alpine plants, which represent one of the groups most sensitive to direct and indirect human impacts.

Seed longevity has been investigated predominantly in relation to taxonomic and macroclimatic differences across species growing sites, while little has been given to explain variation shown within closely related taxa from the same climate. In this study, seed longevity of 18 alpine species from Asteraceae was compared. Seed longevity of alpine Asteraceae was low, but showed large variations across species, which was mostly explained by species-specific requirements for soil moisture, with species from wetter soils showing faster deterioration rate. Plant ecological traits, linked to microhabitats conditions may, therefore, play crucial role to predict seed lot longevity in air-dry storage, including seed bank conditions.

Germination is considered to be the most reliable test to assess seed quality in storage, radicle protrusion may over-estimate it, compared with normal germination (i.e. radicle plus cotyledon emergence). However, the extent of such overestimation across species and the factors contributing to it are not yet well understood. In the third chapter, I have evaluated both radicle and radicle plus cotyledon emergence (i.e. normal germination) of 35 alpine species during AA. Normal germination was lower than radicle emergence for all species. As a result, estimates of p_{50} based on radicle emergence ($p_{50\ (RE)}$) were significantly higher than estimates based on normal germination ($p_{50\ (NG)}$) in 18 (51.4 %) out of the 35 species tested. This suggests that radicle emergence may not be a reliable indicator of the capacity of seeds to complete the germination process, thereby leading to an overestimation of seed longevity in storage. Therefore, in accordance with these results, the actual seed longevity of several alpine species may be lower than previously reported, highlighting that *ex situ* storage of alpine seeds might be even more problematic than currently thought.

A coefficient of overestimation of seed longevity (OESL) was developed, which equally accounted for small difference of p_{50} in either species with high or low seed longevity. A very wide range of OESL was observed across species depicting the considerable variability in seed germination performance. OESL was influenced by seed traits and species' ecology, indicating that such overestimation is not random across species, but rather determined by specific plant and ecological traits. In particular, OESL was highest for species with shorter-lived (low p_{50}) dwarf seeds collected from basic soil. The results reported here provide important insights to improve longevity estimates either for short- (seed industry) or long-term conservation purposes. In particular,1) highlighted a large overestimation of seed longevity when using radicle emergence, instead of NG as an assessment of recovery growth after storage and 2) describe a new coefficient that quantifies conversion challenges post storage (the OESL) showing that high OESL is related to particular seed, embryo and plant ecological traits. Such correlates may be used to prioritize species' vulnerability to *ex situ* storage and optimize viability testing, thereby reducing labour costs and enabling more effective conservation of seed collections.

The possibility of extending storage life of seeds has obvious practical implications, especially for germplasm conservation of inherently short-lived wild species (Probert et al. 1991) such as alpines (Mondoni *et al*. 2011). Moreover, most of the alpine species are incapable of becoming a seedling and the possibility of recovery is possible by using seed priming technique. I investigated the potential for priming to increase the longevity of six alpine species using a range of water potentials (hydro and osmo-priming). According to this study, both hydro-priming and osmo-priming had a significant positive effect on seed longevity ($p_{50\,(R)}$ and $p_{50\,(NG)}$). In particular, hydro-priming was the most successful treatment to enhance both $p_{50\,(R)}$ and $p_{50\,(NG)}$. Moreover, hydro-priming decreased OESL value of tested species. Hydro-priming ultimately positively affected on two seed traits, that is seed longevity and seed quality (i.e. decreased gap between radicle emergence and fill emergences).

Merritt and Dixon (2011) have highlighted increase demand for wild species for restoration projects and the role of *ex situ* seed banks to fulfil the demand by providing restoration-ready seeds. In this scenario, information is available for the *ex situ* seeds conservation of wild plants, except for some species (Hay and Probert, 2013). Therefore, seed banks storing wild

species follow gene-bank standards (FAO, 2014), where most of the theory and protocols is derived from studies on crops. However, more studies are needed to identify proper seed dormancy breaking treatments, seed storage conditions, seed longevity patterns, seed priming techniques and seed viability tests on wild species (Hay and Probert, 2013). In this regard, a better understanding of the variability of seed longevity across species provides a foundation for future investigation on the mechanism of damage and protection during ageing stress (Walters *et al*., 2005). The information provided in this study on wild alpine plants may fill some knowledge gap about how to monitor and improve seed viability in storage, which may have important implications to conserve high quality seeds both for long- and short-term *ex situ* storage, such as in seed banks and native seed industry, respectively. In particular, I highlighted that normal germination (i.e. radicle and cotyledon emergence) should be used to monitor seed viability during storage, that species from more humid soil may have higher possibility to show short-lived seeds. Seeds of alpine species held in seed banks will need to be re-tested more frequently to monitor their potential decline in viability during storage, and alternative storage techniques such as cryopreservation should be considered for the shortest lived with high OESL cases. Despite alpine seeds are short-lived, it may possible that their longevity can be significantly improved using easy and inexpensive techniques, such as hydro and osmo-priming.

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