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Improving seed quality in large-scale production of native seed

By

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I, Maria Marin, declare that this doctoral dissertation, submitted in partial fulfilment of the requirements for the award Doctor of Philosophy, in the School of Earth and Environmental Sciences, University of Pavia, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

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Improving seed quality in large-scale production of native seed

Abstract

The use of native species has been increasingly advocated to achieve the sustainable recovery of degraded areas and in Europe considerable effort has also been invested into grassland restoration using such species. However, little attention has been paid to the evaluation and significance of aspects of the seed quality of native species, which may have implications for the success of these projects. The current research examined three aspects of seed quality in the context of commercial seed production and the use of seed in the restoration of vegetation.

Firstly, the effect of plant and seed responses to a range of maternal environments during seed production was examined using the perennial herb *Primula vulgaris* as a model. Different shading conditions, imposed in the field over two seasons during seed maturation, produced a range of red : far-red light ratios (R:FR) received by the parent plants. The competitiveness of *P. vulgaris* plants grown in the different environments was evaluated though measurements of plant gas exchange (leaf conductivity to water vapour), membrane integrity (relative electrolyte leakage), leaf chlorophyll concentration, photosynthetic efficiency, specific leaf area and above ground plant biomass; the weight and number of seeds produced was also assessed. This revealed the phenotypic plasticity of *P. vulgaris* and that growth, competitiveness and seed production were favoured in shaded conditions. Subsequent germination of the harvested seeds at five temperatures (5 – 25^{o}C) and high to low R:FR revealed that there was no effect of shading of the maternal plant on seed quality, either the rate of germination or final germination. In addition, the maternal environment did not affect the subsequent light and temperature requirements for germination. An increased germination capacity in response to a higher R:FR for all maternal environments suggested the potential for seedling establishment under vegetative shade only in the presence of canopy gaps.

Secondly, the germination potential and dormancy-breaking requirements of 113 commercially available seed lots of eight European native species was examined. This revealed a wide range in the germination of seed lots within a species and surprisingly low levels of dormancy. Alternative and more rapid methods to the routine germination methods were subsequently developed. A tetrazolium testing protocol was developed that predicted germination of each of the eight native species in only two days. In addition, the electrical conductivity (EC) of seed soak water provided a quick and inexpensive measure of final germination in a day and even within a day. However, its application may be limited to large and exalbuminous seeds, such as *Cyanus segetum*, which are dominated by the embryo and lack an endosperm. In *C. segetum* EC was highly predictive of both the percentage germination (radicle emergence) and the mean germination time.

The third aspect of seed quality investigated was its impact on establishment and growth of *Rhinanthus minor*, a hemi-parasite. Ten seed lots from commercial sources were sown in the field and their germination characteristics were investigated in the laboratory. Field emergence was influenced by both the radicle emergence (%) of each lot and the length of the lag period from the beginning of imbibition to germination (mean germination time), which is indicative of seed vigour. Seeds from four lots with >90% radicle emergence were then germinated to radicle emergence and sown in pots alongside plants of two host species, *Lotus corniculatus* and *Holcus lanatus*. Plant establishment, height and flowering density were evaluated for the hemi-parasite, while plant biomass was measured for both *R. minor* and its host. A longer lag period (lower vigour) was associated with higher levels of seedling mortality and lower plant vigour, in terms of plant height and biomass accumulation, and was also reflected in the parasitic impact of the seed lots, with the least vigorous seed lot having no impact on the biomass of its host, *Lotus corniculatus*. These observations highlight that the quality of the seed is significant to the potential use of *R. minor* as a hemi-parasite.

In conclusion, this thesis has provided new information regarding the seed quality of wild herbaceous species in the context of commercial production, with important practical implications. It has shown that the conditions experienced by

native plants in cultivation can be optimised in terms of plant productivity, without implications for seed quality or germination requirements. The evaluation of the germination capacity of individual seed lots from different suppliers provided evidence of quality problems in the European native seed market. The development of the radicle emergence and conductivity tests for application to native species will help avoid future restoration failure due to poor seed quality. Finally, this study has shown that seed quality can have a fundamental role in the establishment and growth of one native species, the hemi-parasite *Rhinanthus minor*.

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Chapter 1

Introduction

1.1. Introduction

There has been a marked increase in degraded and altered areas of natural vegetation throughout the world over the last fifty years (Dobson *et al*., 1997; Van Andel and Aronson, 2012). Nearly two-thirds of the world's ecosystems have been degraded (Merritt and Dixon, 2011) with clear consequences for biodiversity. Climate change now places additional pressures as it is predicted to become a major threat to biodiversity in the 21st century (Dawson *et al*., 2011). Ecological restoration is the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed (SER, 2004) and is increasingly recognized as a priority action for habitat conservation (Menz *et al*., 2013). Fundamental to the achievement of global terrestrial restoration targets is the need for high-quality seed (Broadhurst *et al*., 2008; 2016; Nevill *et al*., 2016). However, the shortage of seeds of wild species for contemporary restoration is well recognized (Merritt and Dixon, 2011). Furthermore, the current and future demand for seeds exceeds the volume that can be sourced from the wild, where populations of native species risk depletion (Tischew *et al*., 2011; Broadhurst *et al*., 2015). Thus, commercial seed production will be central to overcome shortfalls in native seed availability (Nevill *et al*., 2016). Therefore, attention needs to be addressed to critical aspects of ecological restoration such as plant conservation strategies, seed-based research and seed supply (Merritt and Dixon, 2011).

Species-rich grasslands are an example of a habitat that has been in constant decline throughout the twentieth century in many parts of Europe (Poschlod and Wallis De Vries, 2002; Duprè *et al*., 2010; Wesche *et al*., 2012; Wilson *et al*., 2012; Haslgrübler *et al*., 2015). As a result, the restoration of species-rich grasslands is now one of the main targets of habitat re-creation projects (Fry *et al*., 2017). However, these projects are often limited due to the depletion of seed banks (Bossuyt and Honnay, 2008), limited seed dispersal in fragmented landscapes (Bischoff, 2002) and a lack of native seed supply in many European countries. Furthermore, Merritt and Dixon (2011) reported that typical seed establishment rates in biodiversity restoration projects are less than 10% due to substandard storage before seed use, lack of seed pre-treatments for

dormancy release and lack of precision in delivering seeds to sites at the appropriate time.

The Native Seed Science Technology and Conservation (NASSTEC) initial training network is a European consortium of academic institutions and commercial seed companies brought together to maximise the success of grassland restoration projects in Europe. The specific objectives of NASSTEC are to fill the existing gaps in native seed science, conservation and use, promote the European native seed industry and facilitate the transfer of knowledge between academia, industry and environmental management authorities. NASSTEC comprises three sub-programmes: (1) *in situ* seed sampling for the selection of species aimed at restoring Alpine, Atlantic, Continental and Mediterranean grassland habitats; (2) seed biology characterisation to generate information on seed germination, storage and stress tolerance; and (3) production and deployment of seed for the implementation of large-scale seed production, the establishment of achievable quality standards and the development of a sustainable certification system. The present work falls within the third NASSTEC sub-programme and was developed at Scotia Seeds, a private native seed company. The research focussed on native seed produced in the commercial environment, from field production to seed quality evaluation and the implications for seed quality in the field.

The implementation of large-scale seed production of wild plant species is fundamental to overcome shortfalls in seed availability and to help restore the great variety of ecological systems (Nevill *et al*., 2016). In addition to the availability of seed, successful restoration will depend upon the access to high quality and appropriately sourced germplasm (Broadhurst *et al*., 2008). However, there is a lack of studies investigating seed quality of native species in commercial seed production and further research is required to optimise plant establishment in restoration (Miller *et al*., 2016). Agricultural species have been widely investigated in relation to seed yield and seed quality, with many countries having either minimal germination standards for commercial seed lots or requiring truth in labelling in which the germination percentage has to be stated on the seed packet. Therefore, these species might be considered a good model for setting

the basis for quality control in native species, which is lacking in many European countries.

The universal quality standard for agricultural species is the germination test. Other aspects include genetic identity and purity, freedom from pathogens and pests and seed vigour (Matthews *et al*., 2012). Seed quality in crop seeds is mainly influenced by the conditions during seed maturation and subsequently by seed ageing, which can occur while the seed is still on the plant, during processing and, most commonly, in storage (Matthews *et al.*, 2012). Likewise, seed quality in native species will be affected by the conditions during seed development, collection and post-harvest handling and seed storage, while also being influenced by the source population characteristics and pollinator behaviour (Hay and Probert, 2013). Furthermore, critical issues associated with the use of native seed for restoration, such as viability, dormancy state and ability to germinate (Miller *et al*., 2016), may vary between and within populations in relation to the conditions under which the seed matured (Gutterman, 2000). Therefore, restoration measures starting from bare ground and depending on seedling recruitment will also depend upon environmental maternal effects (Bischoff and Müller-Schärer, 2010; De Vitis *et al*., 2014). The influence of the maternal environment on native seed quality needs to be further investigated as it could play a fundamental role in the development of multiplication strategies for agriculturally propagated native material.

Seed quality assessment is the next key step towards achieving high-quality native seed, which will in turn maximise the seed potential for field emergence, seedling survival and the ability of plants to become lasting components of restored vegetation. The lack of methods and routine protocols for assessing seed quality in native species (Ryan *et al.*, 2008; Nevill *et al.*, 2016) could be overcome by adopting some of the strategies widely investigated and used in agricultural species. Methods for the evaluation of seed quality in these species are well developed (ISTA, 2017). Laboratory germination testing assesses the ability of seeds to germinate under optimum conditions for germination (ISTA, 2017). There are guidelines for a wide range of agricultural species, including detailed information on how to break dormancy when present (ISTA, 2017). For germination of native species this entails identifying and

providing the appropriate environment that stimulates germination and/or overcomes dormancy in the wild. The tetrazolium (TZ) test is also used in screening agricultural and horticultural seed lots as it provides a quick estimate of the percentage of viable seeds in a given sample, even in seeds with dormancy (ISTA, 2011).

A second physiological aspect of seed quality, in addition to germination, is seed vigour, which is a characteristic of seeds having a high level of germination (ISTA, 2017). Evidence from agricultural crop seed shows that seed vigour influences the rate and uniformity of germination and growth, emergence under unfavourable environmental conditions, and germination after storage (Powell and Matthews, 2012). Vigour tests are based on an ageing / repair hypothesis, whereby ageing is the main cause of vigour differences, which can be repaired during early germination, before radicle emergence (Powell and Matthews, 2012). These test methods include the assessment of the mean germination time (MGT), a single early count of radicle emergence (RE) and the electrical conductivity (EC) test (ISTA, 2017). The mean germination time describes the mean lag period between the beginning of seed imbibition and the appearance of the radicle through the seed coat (Matthews and Khajeh-Hosseini, 2006), while the EC test measures the leakage of electrolytes from bulks of seeds into soak water (Mavi *et al*., 2016).

As a last step to promote seed quality evaluation and therefore improve seed quality in the large-scale production of native seed, attention needs to be paid to the implications that this has in the field. Merritt and Dixon (2011) reported that the failure of viable seeds to germinate and establish as seedlings represents the largest bottleneck in the restoration of plants using broadcast seed. They argue that this may be the result of inappropriate germination conditions and complex dormancy mechanisms. However, little is known about how the success and effectiveness of restoration projects can be influenced by characteristics of the seed that are revealed in seed quality, such as germination and vigour. Therefore, there is the need to investigate the outcome of seed quality differences on the plants subsequently produced. Previous studies have mainly addressed the effect of seed origin on plant performance, based on the premise that locally

adapted seed will guarantee successful restoration outcomes (McKay *et al*., 2005; Bischoff *et al*., 2010; Vander Mijnsbrugge *et al*., 2010).

The present research project focused on seed production, establishment and growth with implications for the large-scale deployment of good quality native seed. Firstly, seed quality in production was evaluated in relation to environmental maternal effects. Specifically, the plant and seed responses to a range of maternal light environments were investigated in *Primula vulgaris*, a species commonly used in restoration projects. Secondly, the quality of the seed already available on the European native seed market was assessed, in terms of germination potential and purity, in seed lots of eight European native species and a baseline of seed quality was drawn. In order to facilitate future seed quality evaluation in these species the project focused on potential testing methods. A rapid assessment of seed performance was investigated using a modification of the tetrazolium test for application to native species. The potential of the electrical conductivity test and of early counts of radicle emergence to quickly predict seed germination in some native species was also investigated. Finally, this project examined the practical outcome of seed quality differences in one native species, *Rhinanthus minor*, which is used in many seed mixtures.

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Chapter 2

Responses of *Primula vulgaris* to light quality in the maternal and germination environments

T*he work presented in this chapter will be submitted for publication as:*

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Abstract

Dormancy release in response to light has been widely documented in the small-seeded model species *Arabidopsis thaliana*. Research on *Arabidopsis* also showed that phytochromes mediate dormancy and germination responses to seasonal cues experienced during seed maturation on the maternal plants. However, there is a lack of studies investigating the specific effect of the maternal light environment on seed germination in native wild species. This is particularly important given its practical application in the context of environmental restoration. The present study investigated the plant and seed responses to a range of maternal environments in *Primula vulgaris*. Plants were grown in the field under four shading treatments (from low to high ratio of red to far-red light, R:FR) for two vegetative seasons. Leaf and seed traits were assessed in response to the light treatments. Seed germination behaviour was investigated from these four maternal environments (pre-dispersal) at seven light and five temperature treatments (post-dispersal). Thinner leaves, larger leaf area and greater chlorophyll content were found in plants growing in reduced light quality (R:FR). A canopy in the maternal environment led to increased seed size and yield, although the shading conditions experienced by the maternal plants had no effect on seed germination. Seeds responded strongly to the cues experienced in their immediate germination environment. The rate of, and final, germination were always enhanced under less filtered light conditions, highlighting negative germination responses to a vegetative canopy during germination. The observed phenotypic trait variation plays a major role in the ability of *P. vulgaris* to grow in a wide range of light conditions. However, the increased germination capacity in response to a higher R:FR for all maternal environments suggests potential for seedling establishment under vegetative shade only in the presence of canopy gaps.

2.1. Introduction

Light provides a key energy resource for photosynthesis, while also signalling plants with important spatial and temporal information about their surrounding environment (Franklin, 2008). Plants possess several different photoreceptors to perceive light of specific wavelengths, from UV-B to the near infrared (Kami *et al*., 2010; Sheerin and Hiltbrunner, 2017). Among these, phytochromes respond to red (R) and far-red (FR) light, which enables plants to detect vegetation using the R:FR ratio (Casal and Sánchez, 1998). Unfiltered daylight contains an approximately equal proportion of R and FR light (R:FR ≈ 1.2), but below the canopy that ratio is lowered up to 0.2 – 0.3 as a result of red absorption by photosynthetic pigments (Smith, 2000; Tiansawat and Dalling, 2013), and can be further reduced to 0.1 beneath leaf litter layers (Vásquez-Yanes *et al.*, 1990). The low R:FR signal of reflected light can reveal the presence and proximity of neighbouring vegetation, enabling plants to initiate adaptive developmental strategies to either tolerate or avoid shade (Franklin, 2008).

Contrasting light environments can affect both the plant and the subsequent behaviour of the seed it produces. There is a considerable intra- and interspecies variation in the response of plants to light, with some species expressing different phenotypes by altering plant traits in response to changes in the environmental conditions. This is known as phenotypic plasticity (Schlichting, 1986; Sultan, 2000). Species growing in shade have adapted their photosynthetic structures to function optimally even under low-light conditions (Ruberti *et al*., 2012). In *Rubia peregrina* and *Phalaris arundinacea* this led to thinner leaves and larger leaf area (Navas and Garnier, 2002; Her-Turoff and Zedler, 2007), while in the fern *Athyrium pachyphlebium* photosynthetic efficiency at low light intensities was optimised by an increase in the chlorophyll content (Huang *et al.*, 2011). On the other hand, woody and herbaceous species exposed to high light generally exhibit a decreased fraction of biomass allocated to leaves and an increased allocation to roots, as well as thicker leaves with a low specific leaf area (Poorter and Nagel, 2000). Additionally, as shown for ten species, acclimation to high light results in the re-allocation of nitrogen between the various pools involved in photosynthesis (Evans and Poorter, 2001).

Light is also an important signal for seed germination as it provides information on the seed proximity to the soil surface or about the presence of competing plants (Pons, 2000). Finch-Savage and Leubner-Metzger (2006) commented that it is a matter of debate whether light, as well as temperature, regulates dormancy. However, they proposed that since seeds can germinate in the dark following exposure to light, light must stimulate a response in the seed, which is the last step in the dormancy-breaking process, rather than the first step in germination. The control of seed germination by red and far-red light is one of the earliest documented phytochrome-mediated processes (Casal and Sánchez, 1998) as it provides important information about the optimal time and place for seedling establishment (Carta *et al.*, 2017). In a study on temperate herbaceous species, Milberg *et al*. (2000) found that a light requirement for germination is more likely in small- than in large-seeded species, suggesting that light response and seed mass could have co-evolved. Subsequently, in a study examining 27 temperate forest herbs, small-seeded species were only found to germinate in micro-sites with high R:FR (Jankowska-Blaszczuk and Daws, 2007).

Dormancy release in response to light has been widely documented in the small-seeded model species *Arabidopsis thaliana* (Finch-Savage *et al*., 2007; Footitt *et al*., 2013; Huang *et al*., 2015). Research on *Arabidopsis* also showed that phytochromes mediate dormancy and germination responses to seasonal cues experienced during seed maturation on the maternal plants (Donohue *et al*., 2007). The effects of both maturation- and imbibition-light treatments on germination of *A. thaliana* were evaluated by Leverett *et al*. (2016). Assessments of germination following maturation of seeds in white light and under a simulated canopy (green filter) revealed strong, positive germination responses to the simulated vegetative canopy in the maternal environment. These responses persisted as seeds were induced into secondary dormancy (Leverett *et al*., 2016). Recently, the photoperiod experienced by the maternal plants of *Arabidopsis* was shown to influence germination more strongly than the photoperiod experienced during seed imbibition (Imaizumi *et al*., 2017). These findings are however limited to genotypes of this model species (*Arabidopsis thaliana*).

Previous work has shown that maternal plants can alter the dispersal or germination requirements of their progeny (Roach and Wulff, 1987), acting as a

mechanism of adaptive phenotypic response to environmental heterogeneity (Mousseau and Fox, 1998). In a study on four herbaceous species (*Silene noctiflora, Sinapis arvensis, Spergula arvensis, Thlaspi arvense*) from different populations, Andersson and Milberg (1998) found large variations in seed dormancy between individuals, populations and seeds harvested in different years, as a result of the maternal environment. However, there is a lack of studies investigating the specific effect of the maternal light environment on seed germination in native wild species. This is particularly important given its practical application in the context of environmental restoration, a global business worth at least $1.6 trillion annually (Merritt and Dixon, 2011). Germination is the first crucial growth stage in the regeneration of wild species (Dürr *et al*., 2015) and seeds vary in their degree of germinability between and within populations, mainly in relation to the local conditions under which they matured (Gutterman, 2000). As a result, restoration measures depending on seedling recruitment are sensitive to environmental maternal effects (Bischoff and Müller-Schärer, 2010; De Vitis *et al*., 2014).

Primula vulgaris (Primulaceae) is a perennial herb with a North Atlantic and Mediterranean European distribution, reaching its northern margin in Norway and as far south as North Africa (Hultén and Fries, 1986). The species occurs in both open and shaded habitats, and can be found in woodlands, hedgerows, grasslands and other communities such as heaths (Jacquemyn *et al*., 2009). *P. vulgaris* seed is characterised by a non-deep physiological dormancy (Baskin and Baskin, 2014). However, Valverde and Silvertown (1995) found that a cold pre-treatment only affected germination rate, while it had no effect on final germination in plants from seven different populations.

Given its extensive geographical range and habitat flexibility (Whale, 1984), *P. vulgaris* is a good model for the study of intraspecific phenotypic plasticity in herbaceous species. Furthermore, a detailed understanding of its germination behaviour, considering also the effects exerted by the maternal environment, could provide practical information fundamental for the rehabilitation of natural populations of *P. vulgaris*. The habitats in which the species can be found have undergone severe degradation in north-western Europe, forcing it to survive in semi-natural habitat remnants and increasing the need for ecological

management practices and complementary restoration measures (Endels *et al*., 2002; Jacquemyn *et al*., 2010; Van Geert *et al*., 2015).

In this study, we have used *Primula vulgaris* as a model to evaluate plant and seed responses to four shading treatments, applied to adult plants. First, we assessed morphological and physiological leaf trait variations of *P. vulgaris* plants in response to the different light environments, as a measure of phenotypic plasticity. Next, we measured the variation in seed weight and number of seeds produced per plant in relation to the shading treatment. Finally, seed germination behaviour was investigated on seeds from four maternal environments (pre-dispersal) at seven light and five temperature imbibition treatments (post-dispersal) with the aim to assess the effect of the maternal environment on temperature and light requirements for germination.

2.2. Materials and Methods

2.2.1. Experimental plots and plant material

Field plots were set up at Scotia Seeds, Brechin (East Scotland, 56°69´99´´ N, 2°65´56´´ W), in early December 2014, on a site characterized by sandy loam soils with pH = 5.8. Previously, seeds of *Primula vulgaris* had been collected from a natural grassland population (56°26´72´´N, 3°04´94´´W) and grown in the field at Scotia Seeds. Adult plants were subsequently used for the study. The experimental site was divided into 20 plots (4 × 2 m each) and 54 individuals of *P. vulgaris* were transplanted into each experimental plot. Spacing between plants was 25 cm within a row and 40 cm between rows in each plot. From May to October 2015 and 2016 the plants were covered with a range of shading nets (45%, 73% and 85% reduction in available light; Premier netting; LBS Horticulture Ltd, Colne, UK) spread over a wooden framework, 1.5 m high, and placed on all sides. Five plots were left uncovered (C-0), while 15 were shaded (five for each condition: S-45, S-73 and S-85). Hence, four different shading treatments were assembled, each replicated five times and randomly distributed.

Climatic data (air temperature and relative humidity) and total irradiance (0.35 – 2.5 μm) were recorded hourly by EL-USB-2 data loggers (Laskar Electronics Inc., Salisbury, UK) and tube solarimeters (Delta-T Devices Ltd, Cambridge, UK), respectively, placed at a height of 1 m in each experimental treatment over July and August 2015. Soil moisture sensors (ML3, Delta-T Devices Ltd, Cambridge, UK) and data loggers (DL6, Delta-T Devices Ltd, Cambridge, UK) were used to monitor soil water content in the four treatments over the same study period. The photosynthetically active radiation (PAR) and the red and far-red ratio (R:FR) were recorded between 12.00 and 14.00 pm (solar time) on two selected sunny days in summer (13/08/2015 and 6/08/2016) with portable sensors (SKP 215 and SKR 110, respectively, Skye Instruments, Powys, UK).

2.2.2. Maternal plant responses to light

Leaf conductance to water vapour (g_L, mmol m^{-2} s^{-1}) was measured on three leaves per experimental plot (for a total of 15 measurements per shading treatment) using a portable porometer (AP4, Delta-T Devices Ltd, Cambridge, UK) calibrated at the beginning of each measurement session. Measurements were performed between 12.00 and 14.00 pm (solar time) on two selected sunny days in summer (13/08/2015 and 6/08/2016).

Cell membrane integrity was assessed using electrolyte leakage tests on the same dates as g_L. Twenty leaf disks (0.8 cm diameter) were punched from at least three leaves per plot and inserted in a test tube containing 20 ml of deionized water. Samples were left for 3 h at room temperature and the initial electrical conductivity (C_i) of the solution was assessed using a bench conductivity meter (4310, Jenway, Stone, UK). Samples were then subjected to three freezing (1h at -20 °C) and thawing cycles (1h at lab temperature) in order to cause complete membrane disruption and electrolyte leakage. The final electrical conductivity of the solution (C_f) was determined and the relative electrolyte leakage (REL) was calculated as $(C_i/C_f) \times 100$.

Chlorophyll concentration was also measured in August 2015 and 2016, on the same day and time as when g_L was assessed. An optical meter (CCM-200,

Opti-Sciences, Inc., Hudson, USA) was used to estimate chlorophyll concentration *in situ* of at least three leaves per plot. A single universal optical/absolute chlorophyll relationship derived by Parry *et al*. (2014) was used to relate the output from the CCM-200 to absolute chlorophyll concentration in µmol m^{-2}. The photosynthetic efficiency was estimated on the same day-time in 2016 by chlorophyll a fluorescence emission measurements, performed on two leaves per individual (ten leaves per treatment). Measurements were done with a portable fluorimeter (Handy Pea, Hansatech, Norfolk, UK) on leaves previously darkened for 20 min and F_v/F_m was calculated as a measure of the quantum yield of PSII.

Specific leaf area (SLA) was measured on five fully expanded young leaves per plot (for a total of 25 leaves per treatment), collected in July 2015 and 2016. Leaves were scanned and the area was measured using image analysis software (ImageJ 1.46r, NIH, Maryland, USA). Each leaf sample was placed in the oven at 60 °C until a constant weight was measured by a four place electronic balance. Specific leaf area was calculated by dividing leaf area by the corresponding leaf dry weight (cm^2 g^{-1}). Single plants of *P. vulgaris* and the other species present in an area of 0.25 m^2 were sampled in each experimental plot by cutting the plants at the root–stem transition zone. The above-ground portions were oven-dried at 60 °C until a constant weight was measured and their total dry mass recorded.

2.2.3. Seed details

Seeds of *P. vulgaris* were collected from all experimental plots on July 30th-31st 2016 at the time of natural dispersal. Following collection seeds were air-dried at ambient temperature and low relative humidity for two weeks, cleaned from any plant or inert matter, weighed and then stored at 15 °C and 15% relative humidity prior to the commencement of experimental work in November 2016. Seed weight (SW) was determined on 70 individual seeds per plot (350 seeds per treatment), while seed yield (SY) was expressed as number of seeds collected per plant.

2.2.4. Seed responses to a canopy in the maternal environment

Seeds from five field plots (biological replicates) of each shading treatment (maternal environment) were used for germination assays which were conducted at the Millennium Seed Bank, Wakehurst Place, UK. Coated polyester filters (089, 322, 122, 088, 322+121; Lee Filters, London, UK, Table 2.1) were used to germinate seeds in low to high R:FR (Daws *et al*., 2002; Pearson *et al*., 2003; Jankowska-Blaszczuk and Daws, 2007; Tiansawat and Dalling, 2013; Leverett *et al*., 2016). Comparisons of the rate of, and final, germination were made in the dark, light (12 h photoperiod) and a range of canopy (12 h photoperiod) treatments at five temperatures (5, 10, 15, 20 and 25 °C). Five replicates were set up for each combination of maternal environment, imbibition light and temperature treatments. In total, four maternal environments × seven light × five temperature treatments were tested and each combination was replicated five times.

Twenty five seeds per replicate were sown in Petri dishes on 1% (w/v) agar and four dishes at a time, each from a different maternal environment, were placed in open-topped aluminium foil containers (153 × 153 × 34 mm). Twenty five containers were left uncovered as controls, twenty five were placed and sealed in aluminium bags for the dark treatments and the remaining 125 were covered with a layer of coated polyester filter (R:FR of light transmitted through the filters is listed in Table 2.1). The aluminium containers were then placed in plastic bags to prevent water loss during the test and transferred to their treatments in walk-in (5, 15, 20 and 25 °C; 15-20 µmol m^{-2} s^{-1} PAR) and LMS 250A (LMS Ltd, UK; 10 °C; 40-50 µmol m^{-2} s^{-1} PAR) incubators. The filters created different R:FR ratios in the incubators (Table 2.1) because of differences in the R:FR of the light source. However, it was possible to identify filters that achieved similar values of R:FR (Table 2.2). These values were averaged and hereafter used in the analysis (R:FR 0.0; 0.4; 0.9; 2.1; 5.2; Table 2.2). During incubation germination was scored in a dark room under a green light (0.34 µmol m^{-2} s^{-1} PAR) every 2-7 days until the plateau was reached for all treatments except in the dark, as this was only scored once, at the end of the experiments (33 weeks after the commencement of the test). The criterion for germination was the emergence of the radicle from the seed coat. Germination percentage was

scored as the number of germinated seeds per total number of viable seeds in each dish. Remaining seeds were considered viable if they were firm after germination had plateaued, while soft, mouldy seeds were considered to be non-viable.

TABLE 2.1. Red and far-red ratio (R:FR) of light transmitted by different filters in the germination experiments at five constant temperatures. For the same filter, R:FR differs between incubators because of differences in the R:FR of the light source.

Filter Serial No.	R:FR				
	5 °C	10 °C	15 °C	20 °C	25 °C
088	5.36	2.25	5.57	5.64	5.54
089	0.97	0.39	1.01	1.01	1.14
122	1.95	0.89	2.03	2.07	2.15
322	0.85	0.41	0.94	0.93	1.02
322+121	0.40	0.22	0.44	0.43	0.43
No filter	9.67	4.00	9.75	10.04	9.52

TABLE 2.2. Final light treatments considered for the analysis and filters used to achieve them at each temperature. The R:FR values (0.4; 0.9; 2.1 and 5.2) are the mean among incubators.

Temperature	DARK	Filter Serial No.			
		R:FR 0.4	R:FR 0.9	R:FR 2.1	R:FR 5.2
5 °C	Aluminium bag	322+121	322	122	088
10 °C	Aluminium bag	322	122	088	No filter
15 °C	Aluminium bag	322+121	322	122	088
20 °C	Aluminium bag	322+121	322	122	088
25 °C	Aluminium bag	322+121	322	122	088

2.2.5. Statistical analysis

All statistical analyses were performed using GenStat 17th edition (VSN International, Hemel Hempstead, UK) and R v. 3.3.3 (The R Foundation for Statistical Computing, Vienna, Austria). Significant differences between field treatments, including environmental parameters, leaf traits, plant biomass, seed weight and yield were assessed with One-way-ANOVA. Pairwise differences were tested using a *post hoc* Tukey's test. Final germination was analysed using

Generalised Linear Models (GLM) with binomial distribution and logit link function. First, maternal environment, imbibition light and their interaction were included as fixed factors and their effect was assessed at each germination temperature. Subsequently, imbibition light, temperature, and their interaction were included as fixed factors to evaluate their effect on seed germination. After model fitting, to assess the significance of main effects and interactions, the Wald's χ^2 was calculated.

Germination progress curves were obtained for each sowing replicate (combination of maternal environment, imbibition light and temperature) by fitting a sigmoidal curve with Boltzmann function to the data, using OriginPro 9th edition (OriginLab, Northampton, USA). Estimates of time (t_g, days) taken for cumulative germination to reach different percentiles (g) for successive increments of 10% germination were interpolated from the germination progress curves. Germination rate was calculated as $1/t_g$. Germination rates were analysed using Generalised Linear Models (GLM) with normal distribution and identity link function after data were log transformed. First, maternal environment, imbibition light and their interaction were included as fixed factors and their effect was assessed at each germination temperature. Subsequently, imbibition light, temperature, and their interaction were included as fixed factors to evaluate their effect on the rate of germination. After model fitting, to assess the significance of main effects and interactions, the Wald's χ^2 was calculated.

Subsequently, a Principal Component Analysis (PCA) was conducted to examine the relationships between light parameters (PAR and R:FR in the field), plant traits (above ground biomass, F_v/F_m and SLA), seed traits (seed yield and seed weight) and seed germination at different imbibition R:FR treatments. Data on final germination at 5 °C were used for the analysis as this was the only treatment that allowed germination in all light conditions.

2.3. Results

2.3.1. Climatic data

The maximum and minimum daily temperatures, recorded in July and August 2015, did not differ among experimental treatments (data not shown) and averaged 21 °C (range 16 – 28 °C) and 9 °C (range 3 – 14 °C), respectively. Relative air humidity ranged between 51 and 92%, while the soil moisture content averaged 0.31 $m^3\ m^{-3}$ (range 0.23 – 0.42 $m^3\ m^{-3}$), during the same study period and across all experimental treatments, indicating that there was no effect of the shading screens on air temperature, humidity and soil moisture (data not shown). Total irradiance (0.35 – 2.5 µm) varied between experimental treatments (Fig. 2.1) and averaged 0.25 kW m^{-2} for C-0 (range 0.06 – 0.41 kW m^{-2}), 0.18 kW m^{-2} for S-45 (range 0.05 – 0.31 kW m^{-2}), 0.09 kW m^{-2} for S-73 (range 0.02 – 0.15 kW m^{-2}) and 0.08 kW m^{-2} for S-85 (range 0.02 – 0.13 kW m^{-2}) during July and August 2015. Light quantity and quality differed significantly among treatments, with mean PAR values ranging from 413 µmol $m^{-2}\ s^{-1}$ (S-85) to 1451 µmol $m^{-2}\ s^{-1}$ (C-0) and R:FR between 0.35 (S-85) and 1.41 (C-0; Table 2.3).

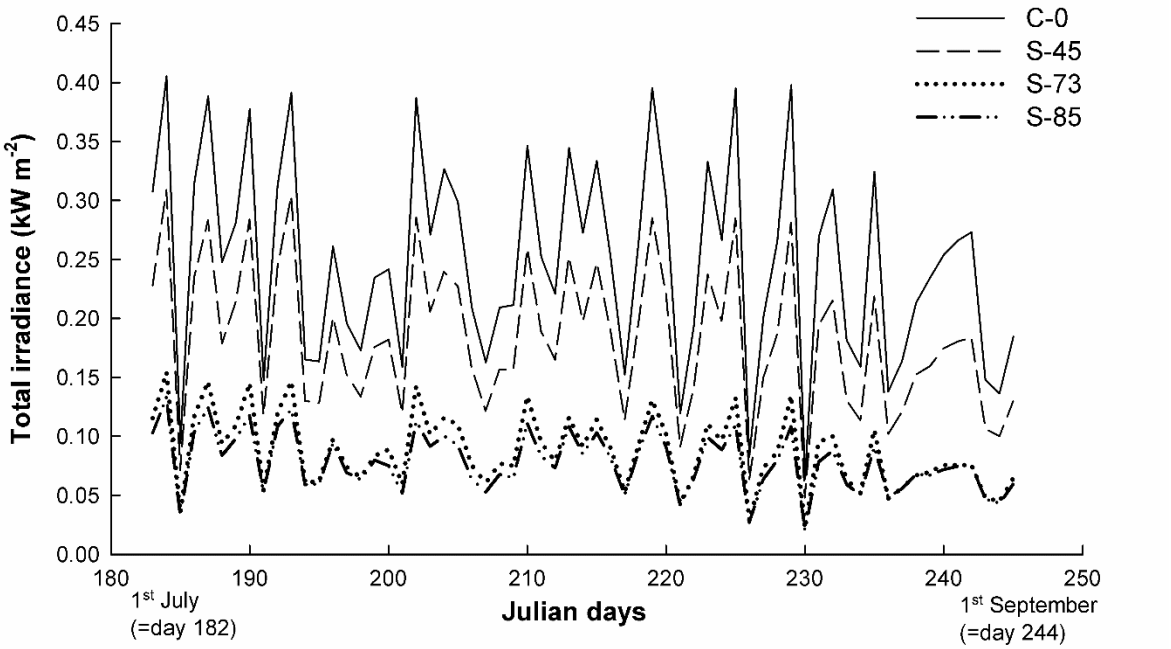

FIGURE 2.1. Total irradiance recorded in the four field treatments in low to high shading (0 – 85%; C-0, S-45, S-73, S-85) between July 1st and September 1st 2015.

2.3.2. Maternal plant responses to light

Leaf conductance to water vapour was assessed in 2015 and 2016 on two sunny days in mid-August and averaged 269 ± 12 mmol m^{-2} s^{-1} in 2015 and 289 ± 10 mmol m^{-2} s^{-1} in the following year across treatments (Fig. 2.2A). The average relative electrolyte leakage measured on the same dates was 12.7 ± 1.7% and 19.5 ± 1.7% in 2015 and 2016, respectively (Fig. 2.2B). For these physiological parameters no significant differences were found among the four treatments. In summer 2015 the specific leaf area averaged 146 cm^2 g^{-1} in the C-0 plots, while significantly higher values were recorded in the S-73 and S-85 plots, with a mean SLA of 209 cm^2 g^{-1} (Fig. 2.2C). During the following summer the differences in SLA among treatments were even greater, averaging 186 cm^2 g^{-1} (C-0), 226 cm^2 g^{-1} (S-45) and 270 cm^2 g^{-1} (S-73 and S-85; Fig. 2.2C). The leaf chlorophyll concentration revealed significantly higher values ($P < 0.001$) for plants growing in plots S-73 and S-85, both in 2015 (382 and 380 µmol m^{-2}, respectively) and 2016 (375 and 431 µmol m^{-2}, respectively) compared with the ones from plots C-0 and S-45 (Fig. 2.2D). Significant differences ($P < 0.001$) among treatments were also revealed by F_v/F_m measurements (Table 2.3). Plots S-73 and S-85 displayed the highest F_v/F_m (0.838) and progressively lower values were recorded for S-45 (0.812) and C-0 (0.785).

TABLE 2.3. Photosynthetically active radiation (PAR) and red and far-red ratio (R:FR) as recorded in the four field treatments with low to high shading (0 – 85%). Above-ground biomass of a plant of *Primula vulgaris* (BP) and of the species competing with it (BC), as measured within a sampling area of 0.25 m^2, in low to high shading (0 – 85%). Chlorophyll fluorescence (F_v/F_m), single seed weight (SW) and seed yield (SY) for *P. vulgaris* plants growing in low to high shading (0 – 85%). Means are reported ± s.e. Identical letters indicate no significant differences as tested using one-way ANOVA followed by a post hoc Tukey's test.

	No shading (C-0)	45% shade (S-45)	73% shade (S-73)	85% shade (S-85)
PAR (µmol m^{-2} s^{-1})	1450 ± 44 a	860 ± 56 b	453 ± 47 c	413 ± 35 c
R:FR	1.41 ± 0.05 a	0.86 ± 0.03 b	0.55 ± 0.06 c	0.35 ± 0.03 d
BP (g m^{-2})	21 ± 9 a	19 ± 5 a	28 ± 10 a	32 ± 9 a
BC (g m^{-2})	172 ± 17 a	93 ± 22 b	36 ± 16 c	28 ± 7 c
F_v/F_m	0.785 ± 0.027 a	0.812 ± 0.018 b	0.839 ± 0.010 c	0.836 ± 0.008 c
SW (mg)	0.954 ± 0.001 a	1.100 ± 0.001 b	1.192 ± 0.001 c	1.301 ± 0.001 d
SY (seeds $plant^{-1}$)	75 ± 16 a	165 ± 34 ab	260 ± 28 bc	284 ± 24 c

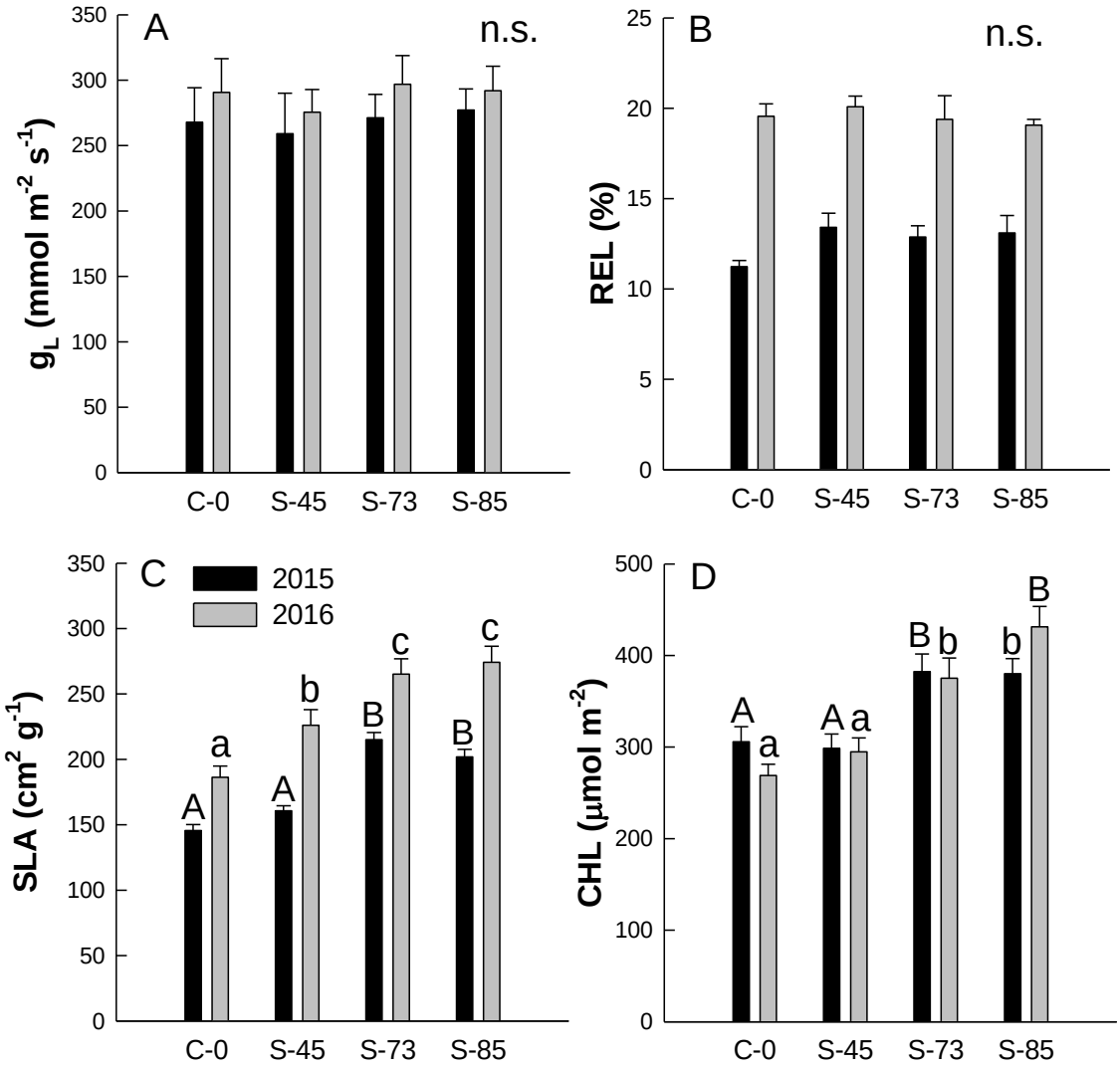

FIGURE 2.2. Values of (A) leaf conductance to water vapour (g_L), (B) relative electrolyte leakage (REL), (C) specific leaf area (SLA) and (D) leaf chlorophyll concentration (CHL), recorded for *Primula vulgaris* grown in low to high shading (0 – 85%; C-0, S-45, S-73, S-85) on August 13th 2015 (black columns) and August 6th 2016 (grey columns). Means are reported ± s.e. Identical letters and n.s. indicate no significant differences among treatments as tested using one-way ANOVA followed by a post hoc Tukey's test.

At the end of summer 2015 the biomass accumulation of *P. vulgaris* and the species competing with it was measured within an area of 0.25 m^2 in each experimental plot (Table 2.3). When grown in open conditions the biomass of a plant of *P. vulgaris* averaged 21 ± 9 g, while slightly higher but not statistically different values were recorded in shaded conditions (*e.g.* S-85: 32 ± 9 g; Table 2.3). The competitiveness of other species, mainly grasses, was light dependent as in open conditions their biomass averaged 172 ± 17 g m^{-2}, while significantly

lower values were recorded for shaded conditions, ranging from 93 ± 22 g m^{-2} (S-45) to 28 ± 7 g m^{-2} (S-85; Table 2.3). Seed weight of *P. vulgaris* varied significantly ($P < 0.001$) among treatments and was negatively correlated with the R:FR experienced during seed maturation on the maternal plants (Table 2.3). A similar trend was found for seed yield as this was significantly higher in S-85 plots (284 ± 24 seeds $plant^{-1}$) compared to the C-0 plots (75 ± 16 seeds $plant^{-1}$; Table 2.3).

2.3.3. Seed responses to a canopy in the maternal environment

Final germination percentage was positively correlated with R:FR experienced during seed imbibition for all maternal environments and incubation temperatures (Fig. 2.3). Indeed, a significant effect of the imbibition R:FR was found at all temperatures (Table 2.4). Maternal canopy did not affect final germination at any temperature and no significant interaction was found with incubation light (Table 2.4). Maximal final germination percentages for all maternal environments were recorded at 10 °C and R:FR ≥ 2.1 and averaged 95 ± 2% (C-0), 98 ± 1% (S-45), 95 ± 1% (S-73) and 96 ± 1% (S-85; Fig. 2.3).

Since the maternal environment did not affect subsequent germination it was not considered further and the analysis focused on assessing the effect of light and temperature on germination. Final germination percentages were positively correlated with R:FR and negatively with temperature, reaching the optimum at 10 °C and then decreasing again at 5 °C (Fig. 2.4). However, imbibition temperatures equal to or greater than 10 °C did not allow seeds to germinate in the dark, whereas 5 °C did allow this (Fig. 2.4). Statistical analysis confirmed these observations as final germination was significantly affected by both light ($P < 0.001$), temperature ($P < 0.001$) and their interaction ($P < 0.001$; Table 2.5).

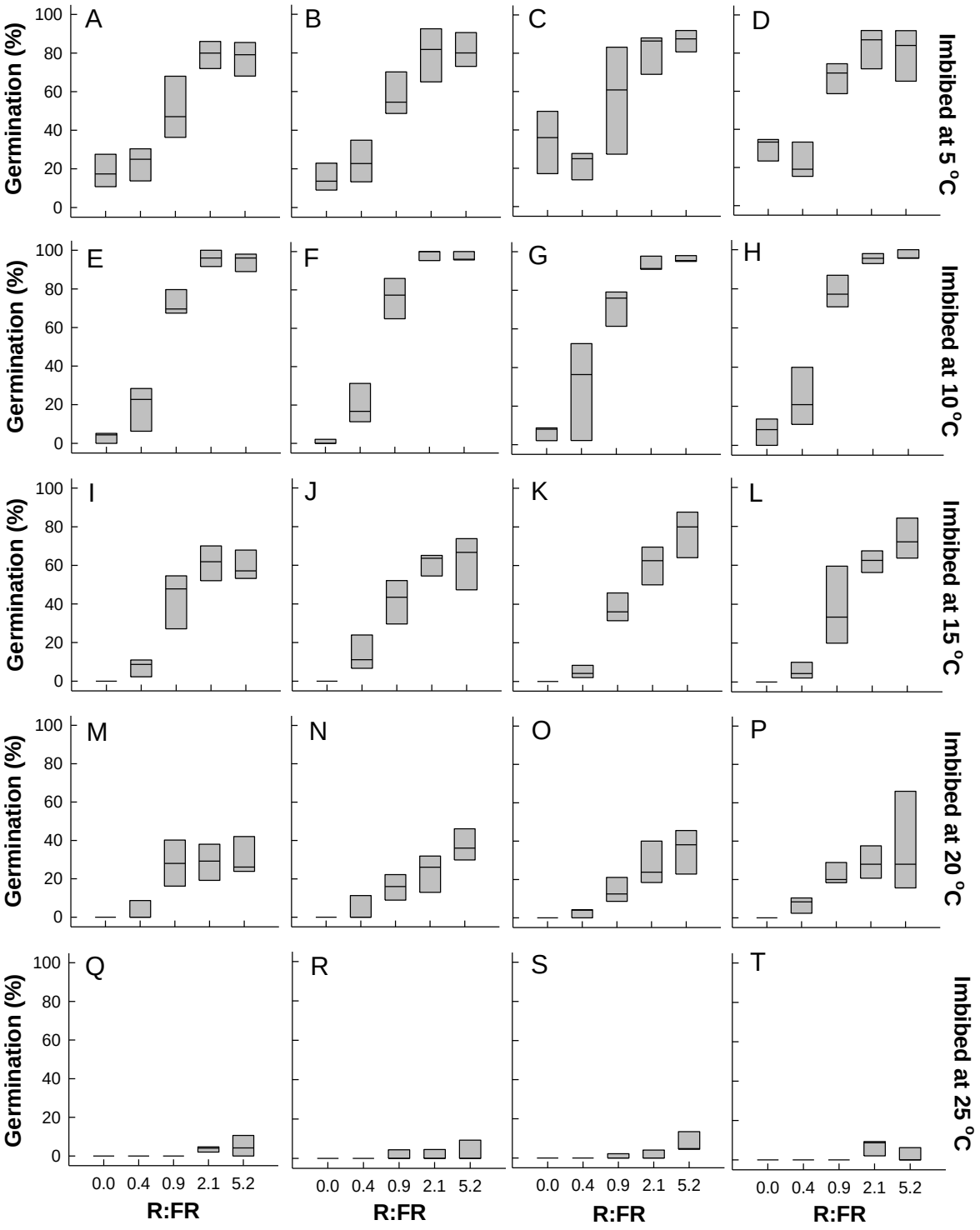

FIGURE 2.3. Final germination of seeds of *Primula vulgaris* matured under no shade (A, E, I, M, Q), 45% canopy (B, F, J, N, R), 73% canopy (C, G, K, O, S), 85% canopy (D, H, L, P, T) and then imbibed in low to high red and far-red ratio (R:FR; 0.0 – 5.2) at either 5 °C (A, B, C, D), 10 °C (E, F, G, H), 15 °C (I, J, K, L), 20 °C (M, N, O, P) or 25 °C (Q, R, S, T). Black horizontal lines within boxes represent median germination percentage. Box hinges indicate 75th and 25th percentiles.

TABLE 2.4. Effects of seed maternal environment ('Maternal'), light quality during seed imbibition ('Imbibition') and their interaction on final germination at five temperatures, based on logit-linked generalized linear models with binomial distribution. Wald coefficients and *P*-values are reported. Significant parameters ($P < 0.05$) are in bold.

Source	d.f.	Imbibition at 5 °C		Imbibition at 10 °C		Imbibition at 15 °C	
		χ^2	*P*	χ^2	*P*	χ^2	*P*
Maternal	3	4.900	0.185	2.400	0.490	0.700	0.873
Imbibition	4	271.100	**< 0.001**	409.200	**< 0.001**	224.100	**< 0.001**
Maternal × Imbibition	12	7.744	0.797	6.435	0.885	15.280	0.250

Source	d.f.	Imbibition at 20 °C		Imbibition at 25 °C	
		χ^2	*P*	χ^2	*P*
Maternal	3	2.830	0.423	1.360	0.715
Imbibition	4	78.420	**< 0.001**	16.540	**0.004**
Maternal × Imbibition	12	7.185	0.837	11.930	0.462

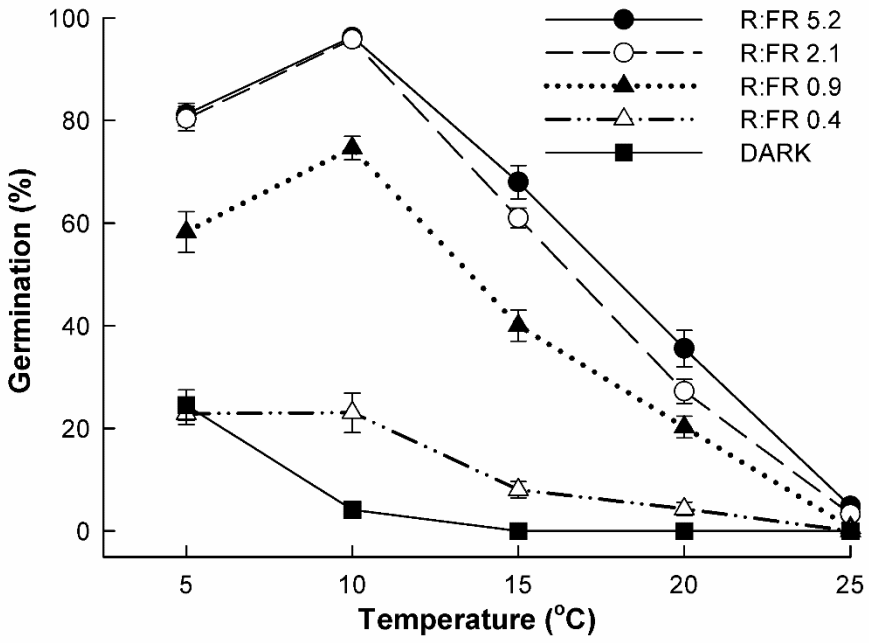

FIGURE 2.4. Final germination of seeds of *Primula vulgaris* (data were merged for all maternal environments) at five constant temperatures (5 – 25 °C), imbibed in light with low to high red and far-red ratio (R:FR; 0.0 – 5.2). Means ± s.e. are reported.

TABLE 2.5. Effects of the light and temperature during seed imbibition and their interaction on final germination, based on logit-linked generalized linear models with binomial distribution. Wald coefficients and *P*-values are reported. Significant parameters ($P < 0.05$) are in bold.

Source	d.f.	χ^2	*P*
Light	4	1079.300	**< 0.001**
Temperature	4	906.400	**< 0.001**
Light × Temperature	16	122.300	**< 0.001**

In general, a negative correlation was found between the time needed to reach 20% germination (t_{20}) and the R:FR during seed imbibition, with higher R:FR resulting in significantly faster germination (Fig. 2.5; Table 2.6). However, as seen for final germination, the maternal environment did not affect the rate of germination or show an interaction with imbibition light (Table 2.6). Further analysis focused on assessing the effect of light and temperature on the rate of germination, merging all maternal environments (Fig. 2.6). It was possible to measure germination rate of one percentile only (10) within a range of imbibition light (R:FR 0.4 – 5.2) and temperature (5 – 20 °C). The rate of germination was positively correlated with R:FR and temperature, increasing from 0.01 d^{-1} (5 °C and 0.4 R:FR) to 0.05 d^{-1} (20 °C and 5.2 R:FR; Fig. 2.6). However, the rate of germination remained unaffected when temperature increased from 5 to 10 °C at lower R:FR (0.4 and 0.9), while it increased by 28% and 35% at a R:FR of 2.1 and 5.2, respectively (Fig. 2.6). Statistical analysis revealed a significant effect of both light ($P < 0.001$), temperature ($P < 0.001$) and their interaction ($P < 0.001$) on the rate of germination (Table 2.7).

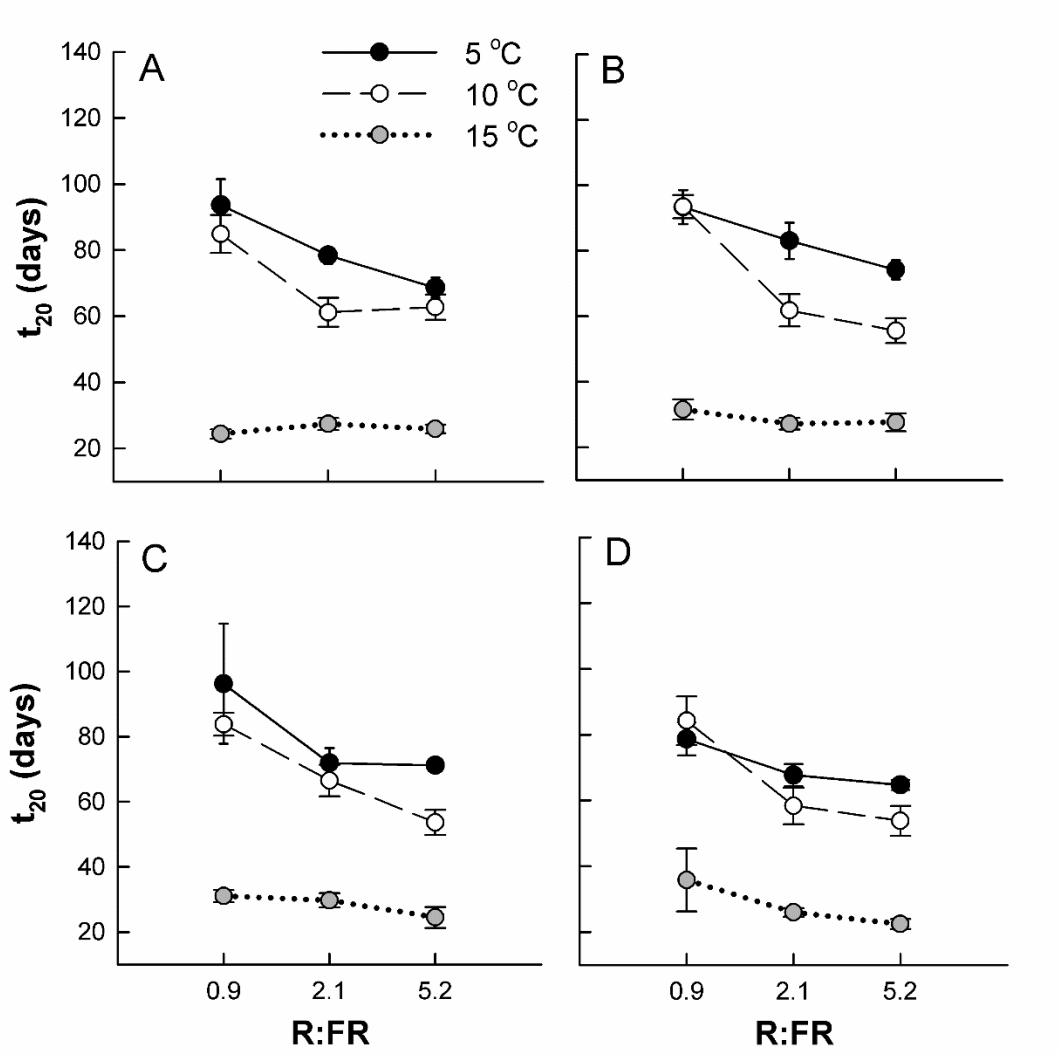

FIGURE 2.5. Time (days) to 20% germination (t_{20}) of seeds of *Primula vulgaris* matured under no shade (A), 45% canopy (B), 73% canopy (C), 85% canopy (D) and then imbibed in light with low to high red and far-red ratio (R:FR; 0.9 – 5.2) at either 5 °C, 10 °C or 15 °C. Means ± s.e. are reported.

TABLE 2.6. Effects of seed maternal environment ('Maternal'), light quality during seed imbibition ('Imbibition') and their interaction on the rate of germination ($1/t_{20}$) based on identity-linked generalized linear models with normal distribution (data were log transformed). Wald coefficients and *P*-values are reported. Significant parameters ($P < 0.05$) are in bold.

Source	d.f.	Imbibition at 5 °C		Imbibition at 10 °C		Imbibition at 15 °C	
		χ^2	*P*	χ^2	*P*	χ^2	*P*
Maternal	3	6.220	0.115	2.250	0.528	1.622	0.657
Imbibition	2	21.740	**< 0.001**	80.630	**< 0.001**	7.264	**0.033**
Maturation × Imbibition	6	1.908	0.924	4.691	0.588	6.996	0.341

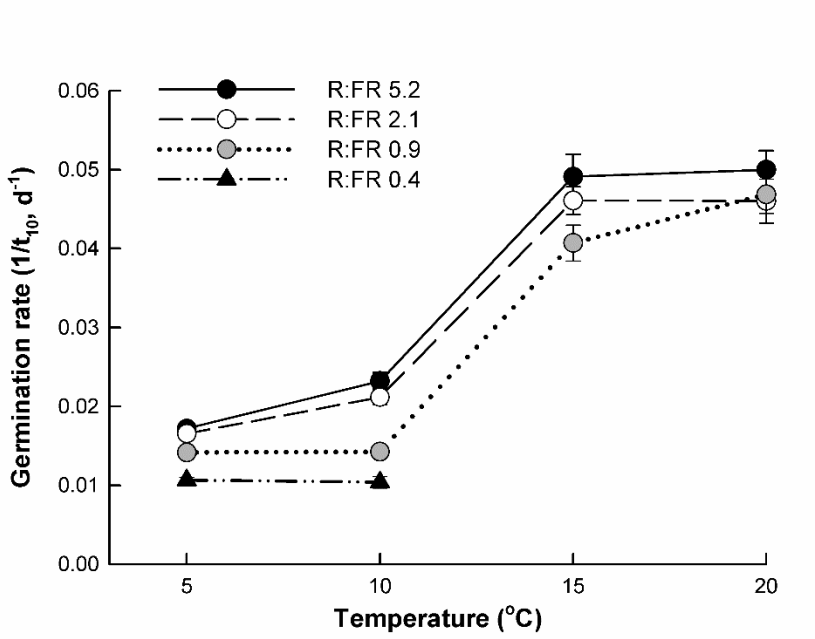

FIGURE 2.6. Germination rates ($1/t_{10}$) of seeds of *Primula vulgaris* (data were merged for all maternal treatments) at four constant temperatures (5 – 20 °C), imbibed in light with low to high red and far-red ratio (R:FR; 0.4 – 5.2). Means ± s.e. are reported. At the lowest R:FR (0.4) it was not possible to calculate t_{10} at 15 and 20 °C due to the low germination percentages reached.

TABLE 2.7. Effects of the light and temperature during seed imbibition and their interaction on germination rates ($1/t_{10}$) based on identity-linked generalized linear models with normal distribution (data were log transformed). Wald coefficients and *P*-values are reported. Significant parameters ($P < 0.05$) are in bold.

Source	d.f.	χ^2	*P*
Light	3	155.100	**< 0.001**
Temperature	3	933.800	**< 0.001**
Light × Temperature	7	30.010	**< 0.001**

A principal-component Analysis (PCA) biplot (Fig. 2.7) shows that from the projection of light parameters, plant and seed traits on the plane composed by the two first explanatory axes (74% variance), three distinct groups of variables can be defined. The first axis (54% variance) was negatively correlated with PAR (Pearson's r = -0.96), R:FR in the field (Pearson's r = -0.94) and the biomass of species competing with *P. vulgaris* (Pearson's r = -0.94), which represented the first identified group of variables. The same axis was positively correlated with a second group of variables, formed by seed yield (Pearson's r = 0.88), seed weight (Pearson's r = 0.84) and leaf physiological traits (*i.e.* SLA, F_v/F_m; Pearson's $r \geq$ 0.83). On the other hand, the second PCA axis (20% variance) was positively

correlated with seed germination at a range of R:FR (Pearson's *r* ranged from 0.51 to 0.73), *i.e.* the third group of variables. The variables included in the first and second group identified by the PCA analysis, namely light environment and vegetative (leaf) and seed yield traits, were mutually related. Indeed, light quality and quantity were positively correlated with the biomass of species competing with *P. vulgaris* (Pearson's $r \geq 0.94$) and negatively related to plant traits associated with the response to light (SLA, F_v/F_m), seed weight and yield (Pearson's *r* ranged from -0.81 to -0.88). Similarly, the biomass of species competing with *P. vulgaris* was negatively correlated with seed weight (Pearson's $r = -0.73$) and seed yield (Pearson's $r = -0.81$). In contrast, seed germination traits were not correlated with other variables. Furthermore, the field treatments were ordinated along the first PCA axis, from C-0 to S-85, confirming the absence of environmental maternal effects on seed germination.

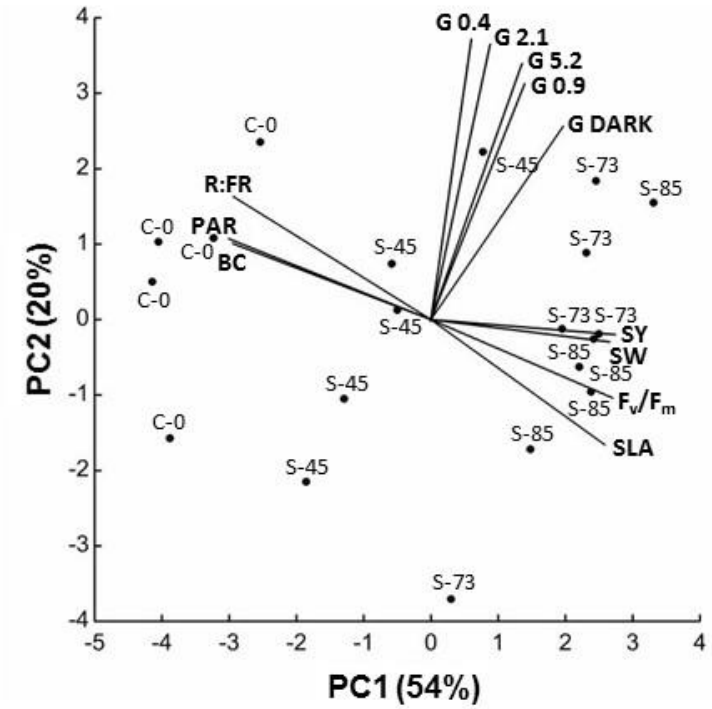

FIGURE 2.7. Biplot of light parameters, plant physiology, seed yield and germination traits of *Primula vulgaris* on the plane represented by the first two components of Principal Component Analysis (PCA; PC1: 54% of variation; PC2: 20% of variation). The individuals represent each field treatment. Acronyms of parameters and traits: R:FR (red and far-red ratio); PAR (photosynthetically active radiation); BC (above ground biomass of competing species); F_v/F_m (quantum yield of PSII); SLA (specific leaf area); SY (seed yield); SW (seed weight); G 5.2 to G DARK (germination at imbibition light ranging from 5.2 to 0.0 R:FR). Field treatment acronyms: C-0 (no shade); S-45 (45% shade); S-73 (73% shade); S-85 (85% shade). Five replicates for each field treatment.

2.4. Discussion

The present study has illustrated the effect of light intensity and quality on plants of *Primula vulgaris* as well as on the subsequent germination behaviour of the produced seeds. We have highlighted the successful ability of *P. vulgaris* to grow in a range of light intensities through several adaptations at morphological and physiological levels. The thinner leaves, larger leaf area and greater chlorophyll content found in plants growing in low light intensities enable *P. vulgaris* to maximise understory light capture and provide evidence of its shade tolerance. Differences in the light environment of the maternal plants and during seed maturation affected seed mass and yield, which increased in more shaded conditions. However, the maternal environment did not affect subsequent seed germination in any combination of imbibition light and temperature. On the other hand, seeds responded strongly to the cues experienced in their immediate environment, *i.e.* during germination. The rate of, and final, germination were always enhanced under brighter conditions, highlighting negative germination responses to a vegetative canopy during germination. Canopy dynamics have therefore strong implications for the recruitment of *P. vulgaris*, with plant establishment occurring mainly in less shaded areas or in canopy gaps.

The growth of natural populations of *P. vulgaris* in a wide range of light conditions is possibly due to the remarkable phenotypic plasticity of the species, as shown here. This plasticity was revealed in a number of physiological assessments. Plants in all light treatments retained a good water status, revealed by the measurements of leaf conductance to water (Fig. 2.2A), and assessments of electrolyte leakage suggested no differences in cell membrane integrity (Fig. 2.2B), the latter often being used to quantify the injury to plant tissues caused by environmental stresses (Prášil and Zámečník, 1998; Bajji *et al*., 2001). Acclimation to low light resulted in increased SLA (Fig. 2.2C), with shaded plants exhibiting a greater leaf area per unit of biomass invested. In work on ten species, including six herbaceous species, Evans and Poorter (2001) observed that increases in SLA were associated with increased nitrogen content per unit leaf area and the amount of light that could be intercepted per unit leaf dry mass, leading to a greater absorption of photons in comparison with the low SLA leaves.

A further response to changes in the light environment was the increase in chlorophyll content per unit leaf area in shaded conditions (Fig. 2.2D), which has been previously associated with an increase in chlorophyll *b* (Chl *b*; Huang *et al*., 2011). A relatively high Chl *b* content (*i.e.* lower Chl *a*/*b* ratio) in shaded leaves results in a higher proportion of light harvesting, while low Chl *b* in leaves in high-light conditions facilitates photoprotection of the reaction centre of PSII by reducing light absorption (Feng 2008; Huang *et al*., 2011; Catoni *et al*., 2015). Dark-adapted F_v/F_m is used as an indicator of plant photosynthetic performance, with optimal value of around 0.83 measured for most plant species (Maxwell and Johnson, 2000). The values measured in the present study (0.79 and 0.84 in plants grown in sun, *i.e.* unshaded, and shaded conditions, respectively; Table 2.3) reflect the potential quantum efficiency of the PSII in *P. vulgaris* in different conditions. The lower F_v/F_m that we observed in unshaded conditions supports the general assumption that F_v/F_m will decline when shade-adapted plants are exposed to high-light stress (Anderson and Aro, 1994; Huang *et al*., 2011).

The light environment of the maternal plants, and therefore during seed maturation, clearly influenced seed mass and seed production per plant, with an increase in both as light intensity was reduced by shading (Table 2.3). Wellstein *et al*. (2013) have previously recorded phenotypic adaptation of seed mass within four grassland species (*Sesleria nitida*, *Lotus corniculatus*, *Astragalus sempervirens*, *Thymus longicaulis*) growing in two habitats, differing in temperature and water availability. Many studies have shown that seed mass is positively correlated to the initial seedling size and the food reserves available during early seedling life across species as well as within species (Leishman *et al*., 2000; Westoby *et al*., 2002). Generally, in heterogeneous habitats, larger seedlings produced by larger seeds are more tolerant to stresses such as shade, drought, or defoliation, while smaller seeds are more likely to occupy and win less stressful sites (Muller-Landau, 2010). The effect of shading in the maternal environment on seed mass of *P. vulgaris* may therefore contribute to the ability of the seed produced to subsequently survive and compete. The larger seeds produced under shade would be better able to compete in the stress of a shaded environment due to their greater reserves and larger seedlings, while the smaller seeds from higher light environments would succeed in less stressful sites.

The increased seed yield, *i.e.* number of seeds produced per plant of *P. vulgaris*, in more shaded conditions (Table 2.3) contrasts with the observations from Valverde and Silvertown (1995), who found a general positive correlation between plant fecundity (flower, capsule and seed production) and light conditions in *P. vulgaris*. In open conditions, natural populations of *P. vulgaris* are most often found in areas where grass growth is less vigorous (Jacquemyn *et al*., 2009), while in our study there was a high abundance of neighbouring species, mainly tall grasses in the uncovered (C-0) plots. Indeed, we found that at higher light intensities the biomass accumulation of the herbaceous cover (species other than *P. vulgaris*) within the experimental plots (Table 2.3; Fig. 2.7) increased. This resulted in tall grasses overgrowing plants of *P. vulgaris* and may offer an explanation for the reduction in seed set in the uncovered and high light intensity plots, possibly due to competition from the grass species and reduced pollination (Piper *et al*., 1984; Boyd *et al*., 1990). The increased seed mass recorded in *P. vulgaris* in more shaded conditions may also result from reduced competition with other species. The effect of competition from neighbouring plants was illustrated by Platenkamp and Shaw (1993) who showed that seed size of the annual species *Nemophila menziesii* was significantly affected by the competition experienced by the mother plants, with the average seed mass being 23% smaller for mother plants competing with the introduced grass *Bromus diandrus*.

The present study also evaluated the effect of the environment during germination on the ability of the seed produced in different maternal environments to germinate. In these germination experiments we simulated the light conditions that maternal plants experienced in the field (Table 2.3), which were similar to those experienced by natural populations of *P. vulgaris* (R:FR 1.13 – 0.23; data collected from natural populations at Wakehurst Place, UK). The maternal environment did not influence seed germination, with similar responses to the germination environment from all shading treatments. However, final germination was higher in brighter conditions at all imbibition temperatures, while in the dark and under a range of simulated vegetative canopies germination was lower (Fig. 2.3; Table 2.4). Canopy dynamics have therefore strong implications for the recruitment of *P. vulgaris*, with germination and hence plant establishment more likely to occur in less shaded areas or in canopy gaps. Valverde and Silvertown

(1995) found that seedling emergence in the field was positively correlated with diffuse light, varying from 0.4% in a population under the canopy, to 17.2% in the population in a gap. Subsequently, they found positive population growth rates in canopy gaps and negative under closed canopy, mainly due to limited recruitment in more shaded areas and increased growth and reproduction (seed set and seedling establishment) in bright patches (Valverde and Silvertown, 1998). Indeed, the patchy distribution of *P. vulgaris,* with a generally higher abundance in lightly shaded areas, indicates that the species favours canopy gaps (Jacquemyn *et al*., 2009), as previously seen for other woodland herbs, such as *Cynoglossum virginianum* (Cipollini *et al*., 1993) and *Calathea ovandensis* (Horvitz and Schemske, 1986).

In all light conditions, final percentage germination was influenced by temperature (Fig. 2.4) with the optimum constant temperature for maximum final germination being generally 10 °C. However, an imbibition temperature equal or greater than 10 °C did not allow seeds to germinate in the dark as it did at 5 °C, highlighting an interaction between light and temperature treatments as previously found for a range of gap colonizing species (Bell *et al*., 1995; Teketay, 1998). Seed dispersal in *P. vulgaris* takes place in summer and most seeds germinate in the following spring (Jacquemyn *et al*., 2009), with mean monthly temperatures for the studied area in the period 1971-2000 (Meteorological Station of the James Hutton Institute, Dundee) ranging from 5.3 °C (March) to 10.0 °C (May). Our data also showed a positive association between temperature increase and germination rate (Figs. 2.5 and 2.6). This suggests that the species could positively respond to alternating temperatures, depending on rapid exploitation of temporarily favourable conditions as a gap colonizer (Grime *et al*., 1981). Furthermore, the amplitude of diurnal alternation in temperature becomes greater near the soil surface following removal of the insulating canopy or litter, helping seeds to detect the appearance of a gap and to sense their depth in the soil (Thompson and Grime, 1983; Teketay, 1998).

PCA analysis confirmed the strong response of *P. vulgaris* plants to changes in the light quality of their environment, with leaf parameters such as SLA and F_v/F_m being negatively correlated with R:FR and PAR (Fig. 2.7). Furthermore light availability in the plant environment was related to the size of

the seed produced, suggesting a maternal effect. However, there was no correlation between the maternal environment and subsequent seed germination capacity (Fig. 2.7), in terms of either final germination (Table 2.4) or germination rate (Table 2.6). Indeed, germination was inhibited at low R:FR and in the dark for all maternal environments (Fig. 2.3). This supports the view that cues experienced by the offspring are likely to be more reliable predictors of their immediate environments (DeWitt *et al*., 1998) than are parental environmental effects.

Summarising, this study has found evidence of phenotypic plasticity in *Primula vulgaris* in relation to habitat light availability as plants responded to their environment through morphological and physiological adaptations. A canopy in the maternal environment led to increased seed size and yield, although the conditions experienced by the maternal plants had no effect on seed germination and therefore seed quality. The environment during seed germination significantly affected the ability of the seed to germinate. Increased germination capacity occurred in brighter conditions, suggesting potential for seedling establishment under a vegetative shade only in the presence of canopy gaps. Therefore, the environmental cues experienced by the offspring in its immediate environment over-rode those that were experienced during its maturation in the parental environment.

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Chapter 3

Tetrazolium staining predicts germination of commercial seed lots of European native species differing in seed quality

The work presented in this chapter has been published:

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Abstract

The germination of 113 commercially available seed lots taken from eight European native species was evaluated and the requirement for dormancy-breaking treatment (250 mg l^{-1} gibberellic acid, GA_3; cold stratification) investigated. Laboratory germination, assessed as radicle emergence, of seed lots of single species from different suppliers was highly variable, ranging from 0 to 99%. This highlighted the problem of seed quality in the European native seed market. GA_3 gave small increases in germination, indicative of little dormancy, in six species (*Centaurea nigra*, *Cyanus segetum*, *Knautia arvensis*, *Prunella vulgaris*, *Silene vulgaris* and *Valeriana officinalis*) and the effectiveness of dormancy-breaking treatments did not differ between suppliers (*i.e.* seed origin). In *Papaver rhoeas*, GA_3 increased germination of some lots indicating intra-specific variation in dormancy. Only a cold stratification treatment significantly enhanced germination in *Rhinanthus minor*. A tetrazolium (TZ) testing protocol was developed for the eight species which predicted germination of each species in only two days. Furthermore there was a predictive relationship (r^2 = 0.95) between TZ staining and germination across all species. We therefore propose that TZ staining could be used as a rapid routine method for assessing the germination of many native species, even when dormancy is present.

3.1. Introduction

Degraded and altered areas of natural vegetation have become a problem in many countries leading to a growing awareness of an increasing need for habitat restoration (Dobson *et al*., 1997; Van Andel and Aronson, 2012). The soil seed bank is an important potential seed source for such restoration (Bakker and Berendse, 1999). However, seedling recruitment from the seed bank cannot be relied upon because of the absence of target species and the high dominance of early successional species (Bossuyt and Honnay, 2008). Recent reviews indicate that the lack of target species in the seed bank of restoration sites and dispersal limitation in fragmented landscapes (Bischoff, 2002) can be overcome successfully by species introduction (Hedberg and Kotowski, 2010; Hölzel *et al*., 2012; Kiehl *et al*., 2014), revealing the need for seeds of native wild species of local origin (Merritt and Dixon, 2011; Broadhurst *et al*., 2016). However, the increased demand for native seeds for grassland restoration purposes is not met by an adequate supply in many European countries. Furthermore, Merritt and Dixon (2011) reported that typical establishment rates in biodiversity restoration are less than 10% due to substandard storage before seed use, lack of seed pre-treatments for dormancy release and lack of precision in delivering seeds to sites at the appropriate time. Thus, both seed quantity and quality limit the extent to which restoration goals can be met by direct seeding (Gibson-Roy *et al*., 2007).

The evaluation of seed quality in agricultural crops is well established. Laboratory germination tests have been used for years to provide vital information on the percentage of seeds in a sample that have potential to germinate and produce normal seedlings under favourable conditions (AOSA, 2010; ISTA, 2015). Guidelines for breaking dormancy before or during the germination test are given for some species (AOSA, 2010; ISTA, 2015), but this is not usually a major problem in agricultural species. A quick estimate of the percentage of viable seeds in a given sample can also be provided by the tetrazolium (TZ) test (ISTA, 2011) which is often used in screening agricultural and horticultural seed lots. The test is based on the activity of dehydrogenase enzymes in respiring tissue that reduce the 2,3,5-triphenyl tetrazolium chloride to the stable and insoluble red formazan (Lakon, 1942). It is possible to analyse seed viability by identifying the

stained areas in the embryo, even in dormant seeds (ISTA, 2011). Thus, the TZ test may be used to reveal seed viability, potentially even in seeds with dormancy or in cases where a very quick estimate of germination potential is required (ISTA, 2011). Guidelines for applying the TZ test for viability are available for three genera (*Centaurea*, *Papaver* and *Silene*) used in this study (ISTA, 2011).

Little attention has been paid to the importance of seed quality of native species (Vogel, 2002), with great implications for the success of grassland restoration in Europe. Methods for quantifying seed quality are often limited for native species (Haslgrüber *et al*., 2014) and there are neither regulatory levels nor industry standards in most European countries (Ryan *et al*., 2008). A major factor inhibiting the development of germination testing amongst producers, traders and users of native seeds is the presence and range of types of dormancy found in native species (Baskin and Baskin, 2014), which has restricted the adoption of routine testing (Ryan *et al*., 2008). Moreover, a wide range of studies has indicated large intra-specific variation in germination requirements between geographically distinct plant populations (Probert *et al*., 1985; Ronnenberg *et al*., 2008; Hamasha and Hensen, 2009). Keller and Kollmann (1999) observed a number of origin-specific patterns in germination for five herbaceous species commonly used in ecological compensation studies, while Perez-Garcia *et al*. (1995) found great germination variability among populations of two herbaceous species widely distributed in the Mediterranean region. Inter-population variability in final germination was also found within species of the genus *Lavatera* (Santo *et al*., 2015). Karlsson and Milberg (2008) hypothesised that local adaptations occur through changes mainly in dormancy strength. Such intra-specific differences could limit the development of germination tests and dormancy-breaking treatments that apply to all lots of one species.

The objectives of the present study were to: (1) investigate the germination potential and purity of commercially available seed lots of eight European native species; (2) examine the requirement for dormancy-breaking treatment (GA_3, cold stratification) across the seed lots and species, to evaluate the importance of seed origin in determining germination requirements; (3) investigate a rapid assessment of potential seed performance using a modification of the tetrazolium test for application to native species.

3.2. Materials and Methods

3.2.1. Seed material

Samples from commercial seed lots of *Centaurea nigra*, *Cyanus segetum*, *Knautia arvensis*, *Papaver rhoeas*, *Prunella vulgaris*, *Rhinanthus minor*, *Silene vulgaris* and *Valeriana officinalis*, produced by 24 European seed suppliers from seven countries (Table 3.1), were obtained in the period November 2014 – February 2015. A total of 113 seed samples were obtained across the species and suppliers. The samples will be referred to as seed lots in this paper. For wild species seed companies do not frequently have more than one seed lot of a species available for sale. Thus, for each sample, the seed lot and seed supplier are the same. The listed species were selected as ones that are widespread and native to Europe and are commonly found in grasslands across the continent. These species are also in common use in restoration projects. The seed lots were stored at 15 °C and 15% relative humidity until the experiments were completed (within one year) in the laboratory at Scotia Seeds.

A questionnaire was sent to the 24 European suppliers from whom seeds were purchased for details about the location and year of production, field management, seed processing, and storage.

Table 3.1. Species, origin, number of seed lots tested and time to maximum germination.

Species	Origins	Number of lots	Time to maximum germination (weeks) *
Centaurea nigra	DE, GB	11	3-4
Cyanus segetum	CH, DE, ES, FR, GB, IT, SE	17	2-4
Knautia arvensis	CH, DE, FR, GB, SE	13	3-4
Papaver rhoeas	CH, DE, ES, FR, GB, IT, SE	16	2-4
Prunella vulgaris	CH, DE, ES, FR, GB, IT, SE	14	2-4
Rhinanthus minor	DE, GB	17	13-37
Silene vulgaris	CH, DE, ES, FR, GB, IT, SE	14	1-4
Valeriana officinalis	CH, DE, ES, FR, GB, SE	11	2-4

3.2.2. Purity test

The purity analysis was made on a working sample containing at least 2500 seeds for each lot. After weighing, the working sample was manually separated into three component parts: pure seed, seeds from contaminating species and inert matter. The percentage of each part was determined by weight.

3.2.3. Germination tests

Four replicates of 25 seeds per lot were placed in 90 mm-diameter Petri dishes on germination paper (Whatman, GE Healthcare Life Sciences) moistened with either 2.5 ml distilled water (control) or 2.5 ml gibberellic acid (GA_3; 250 mg l^{-1}), in order to release dormancy and enhance germination. The Petri dishes were then placed in plastic bags to prevent water loss during the test and held under controlled conditions at an alternating temperature of 25/10 °C, with a diurnal period of 12 hours-light and 12 hours-darkness.

Cold stratification was applied to three species as an additional dormancy-breaking treatment following information on previously applied germination protocols for these species (Vandvik and Vange, 2003; Ter Borg, 2005; Golmohammadzadeh *et al*., 2015). In the case of *Knautia arvensis* and Papaver rhoeas, seeds were placed onto germination paper moistened with 2.5 ml distilled water and put into an incubator at 5 °C in the dark for six weeks, then transferred to germinate at 25/10 °C, with a diurnal period of 12 hours-light and 12 hours-darkness. *Rhinanthus minor* seeds were kept on moist germination papers at 5 °C for the length of the test (37 weeks).

During incubation in all germination conditions (water, GA_3, cold stratification), germination was scored weekly as radicle emergence and seeds were removed when radicle emergence had occurred. The length of the germination period ranged from one week (*Silene vulgaris*) to 37 weeks (*R. minor*; Table 3.1). Normal seedlings were not assessed as there are no guidelines for native species and assessments are subject to individual interpretation.

3.2.4. Tetrazolium test

The TZ test was carried out on four replicates of 25 seeds drawn at random from a representative fraction of the submitted sample for each lot and the following procedures were developed for the different species:

1. Seed pre-conditioning: seeds were soaked in distilled water for 18 hours at 20 °C. Preconditioning allows activation of the enzyme system, makes the tissue less fragile, and softens the seed coat, ensuring proper development of the stain in the tissue and more reliable results (AOSA, 2010; ISTA 2011).

2. Preparation: seeds were prepared by exposing the tissue prior to staining to allow easier penetration of the tetrazolium solution by either:

    a) cutting laterally and removing the distal end of the cotyledons (*Centaurea nigra, Cyanus segetum, Knautia arvensis, Prunella vulgaris* and *Valeriana officinalis*); or

    b) cutting along the edge of the seed (*Rhinanthus minor*); or

    c) cutting longitudinally a thin slice from the edge of a seed (*Silene vulgaris*); or

    d) piercing the seed with a needle through the endosperm (*Papaver rhoeas*).

3. Staining: prepared seeds were immersed in 1% 2,3,5-triphenyl tetrazolium chloride solution at 30 °C for 22 hours.

After the staining, seeds were evaluated as viable or non-viable on the basis of the staining patterns and the soundness of seed tissue:

a) Viable seeds: light carmine, entire seed evenly stained (*Centaurea nigra*, *Cyanus segetum*, *Knautia arvensis* and *Rhinanthus minor*) or entire embryo evenly stained (*Papaver rhoeas*, *Prunella vulgaris*, *Silene vulgaris* and *Valeriana officinalis*).

b) Non-viable seeds: any part of the seed unstained, unevenly stained or necrotic; excessive damage to radicle or cotyledons (*Centaurea nigra*, *Cyanus segetum*, *Knautia arvensis* and *Rhinanthus minor*); any part of embryo unstained, any damage to radicle, hypocotyl or cotyledons (*Papaver rhoeas*, *Prunella vulgaris*, *Silene vulgaris* and *Valeriana officinalis*).

3.2.5. Statistical analysis

All statistical analyses were performed using GenStat 17th edition (VSN International, Hemel Hempstead, UK). Germination proportion data were analysed for each species using Generalised Linear Models (GLM) with binomial distribution and logit link function. Germination treatments (water control, gibberellic acid and cold stratification), seed supplier and their interaction were included as fixed factors. After model fitting, to assess the significance of main effects and interactions, the Wald's χ^2 was calculated. The mean and standard error (s.e.) of each treatment were estimated with GLM based on actual data.

The significance of correlations was tested using Spearman's rank correlation. All results were considered statistically significant at $P \leq 0.05$. Determination coefficient values and regression equations were determined between seed viability (quantified using the tetrazolium test) and total germination for the eight tested species to highlight the relationship between variables and assess the prediction potential of the tetrazolium test.

3.3. Results

Testing seed purity revealed differences in the percentage of pure seed found in some of the samples (data not shown). Purity was generally above 90%, although in the case of *Valeriana officinalis*, purity ranged from 69.9 to 99.6% and for *Rhinanthus minor*, from 37.7 to 99.9%. The percentage of contaminating species was less than 1% for the majority (62%) of the accessions, while it ranged from 1

to 10% for 36% of the samples and reached 62% in one lot of *R. minor* (data not shown).

The mean maximum total germination percentage, irrespective of germination treatment, for all lots and species was 58% and the frequency distribution of germination percentages showed that 18% of the tested lots had a mean germination higher than 90%, while for 12% of lots, the mean germination was lower than 10% (Fig. 3.1). Large differences in the germination percentage in water were found between the single lots of the same species that had been received from different seed suppliers (Figs. 3.2 and 3.3). A highly significant effect ($P < 0.001$) of seed lot/supplier was found for all the species considered (Table 3.2). *Silene vulgaris* and *Prunella vulgaris* showed marked variability in the germination of the lots with some consisting of mainly dead seeds, even though the majority had very high germination levels. This meant that the mean germinations in water for lots of these species were 75% (*S. vulgaris*, range 0 – 99%) and 67% (*P. vulgaris*, range 6 – 96%). Two species, *Cyanus segetum* and *Centaurea nigra*, showed less variability among the lots, with an average germination of 64% (range 26 – 87%) and 50% (range 29 – 81%), respectively. Two of the remaining species, *Knautia arvensis* and *Valeriana officinalis*, were characterised by a low average total germination, 19% (range 0 – 46%) and 29% (range 0 – 62%), respectively.

The species tested showed different responses to the dormancy-breaking treatments (Figs. 3.1 and 3.2). Gibberellic acid promoted dormancy release in all species tested except *Rhinanthus minor*. However, the overall level of this increase was quite small for most species. Germination therefore increased after GA_3 treatment by an average of only 7% and 8% for *Centaurea nigra* and *Valeriana officinalis*, respectively and the seed lot had no effect on the response to treatment. There was no significant effect of GA_3 treatment on seed germination for *Cyanus segetum* and *Silene vulgaris* (Table 3.2). The germination of *Prunella vulgaris* increased in 10 out of 14 seed lots after GA_3 treatment ($P < 0.001$; Fig. 3.2). In this case germination was also significantly affected by the lot ($P < 0.001$), and there was an interaction between lot and treatment ($P < 0.001$; Table 3.2). This interaction resulted from the marked 60% increase in germination

after GA_3 treatment compared with the water control (lot W) and lower germination after GA_3 for lots O, E, D and T (Fig. 3.2).

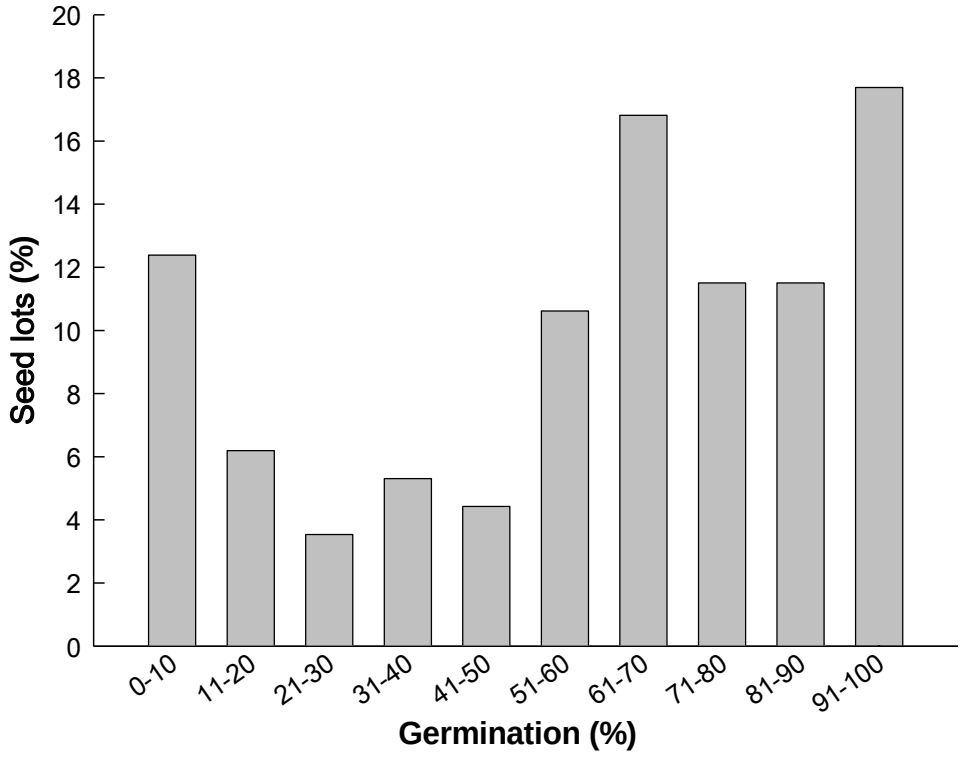

FIGURE 3.1. Frequency distribution of the germination percentage of the 113 tested seed lots of native seeds of eight species. The germination data is based on the maximum germination obtained in any of the germination treatments (*i.e.* water, GA_3 or cold stratification).

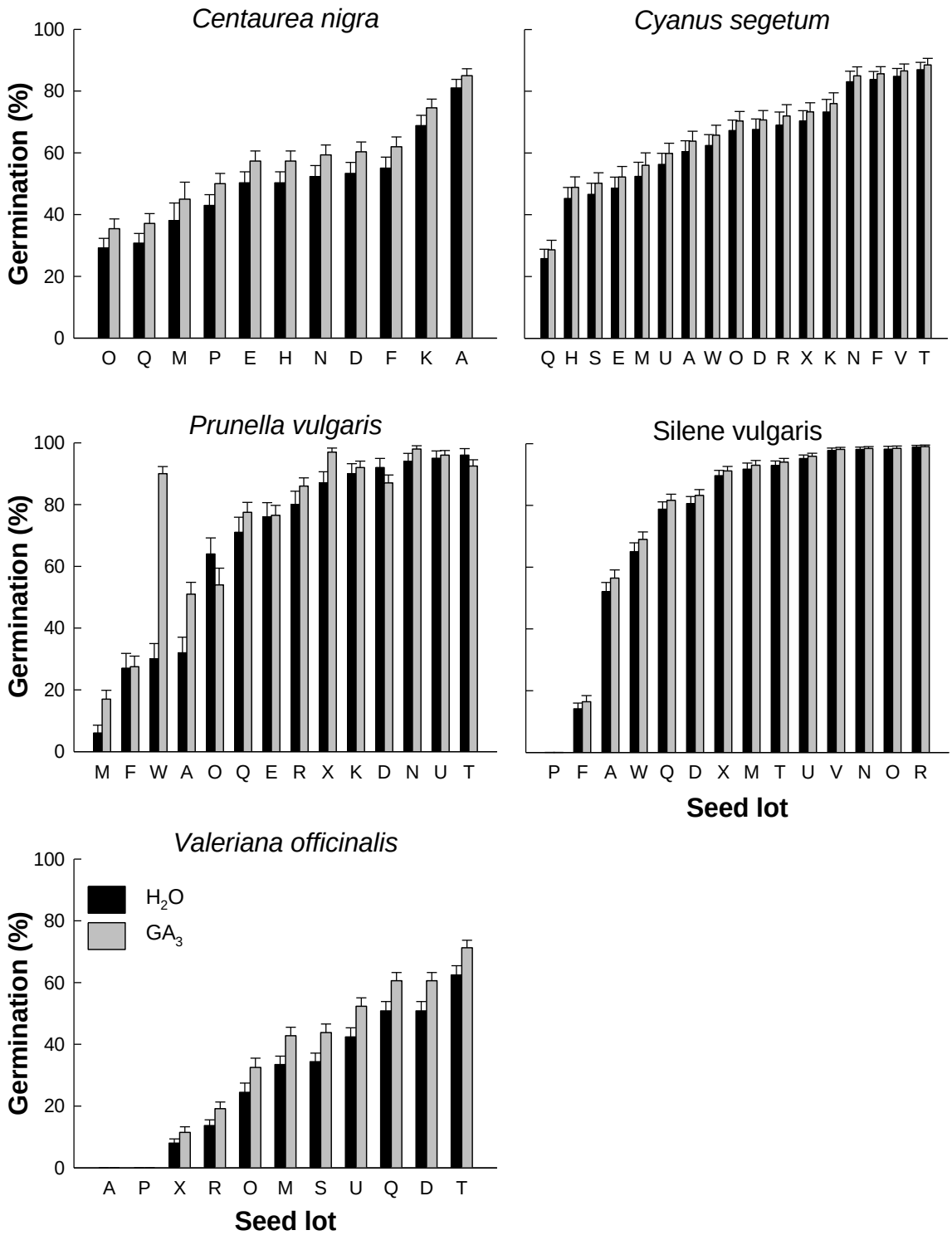

FIGURE 3.2. Final germination, with or without GA_3 as a dormancy breaking treatment, of seed lots from 18 European seed suppliers (indicated by the capital letters). Data were analysed by fitting GLMs (binomial distribution, logit link function) to the data; the fitted estimates and their s.e. are shown.

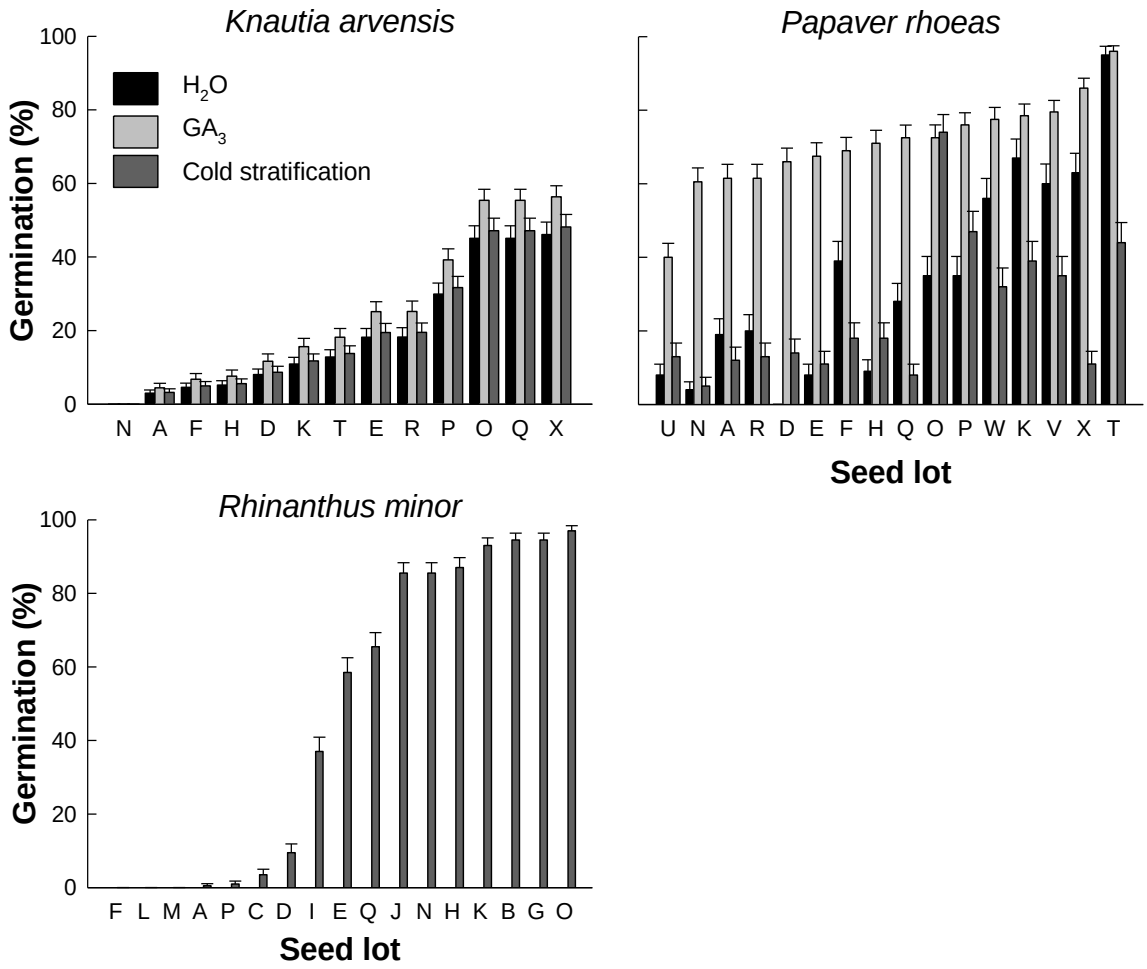

FIGURE 3.3. Final germination, with or without cold stratification or GA_3 as dormancy breaking treatments, of seed lots from 23 European seed suppliers (indicated by the capital letters). Data were analysed by fitting GLMs (binomial distribution, logit link function) to the data; the fitted estimates and their s.e. are shown.

Two dormancy-breaking treatments (GA_3 and cold stratification) were applied to three species (Fig. 3.3) and again, the main effect was from the GA_3 treatment. This treatment resulted in small but significant increases in the germination of *Knautia arvensis* ($P < 0.001$) and the application of a cold stratification treatment did not affect germination capacity in comparison with the water control ($P = 0.180$; Fig. 3.3); there was no interaction between seed lot and treatment ($P = 0.296$; Table 3.2). The GA_3 treatment clearly increased the germination of *Papaver rhoeas* and in this case the response varied according to lot (Table 3.2). In four lots with low germination in water (N, D, E, H), there was a large (mean 61%) increase in germination in the presence of GA_3. The cold stratification treatment also enhanced germination compared with the water

control in the lot from supplier O (Fig. 3.3). Five lots with higher germination in water and hence possibly lower dormancy (W, K, V, X and T), showed a smaller mean increase of percentage germination when treated with GA_3, in comparison with the water control. These lots were also characterised by a significant reduction in germination following the cold stratification treatment (Fig. 3.3). In contrast to all other species tested, GA_3 did not enhance the germination of *R. minor*, where germination only occurred following a cold stratification treatment for 13 – 37 weeks (Fig. 3.3).

TABLE 3.2. Generalised Linear Models (GLM) fitted to the results of the seed lot/supplier × germination treatment experiments for seeds of eight native species. GLM had binomial error distribution and logit link. Wald coefficients and *P*-values are reported. Significant parameters ($P < 0.05$) are in bold.

Species	Supplier		Treatment		Interaction	
	χ^2	*P*	χ^2	*P*	χ^2	*P*
Centaurea nigra	191.700	**<0.001**	9.900	**0.002**	14.080	0.136
Cyanus segetum	354.900	**<0.001**	2.800	0.094	20.620	0.067
Knautia arvensis	514.900	**<0.001**	19.100	**<0.001**	27.600	0.296
Papaver rhoeas	243.600	**<0.001**	433.500	**<0.001**	210.300	**<0.001**
Prunella vulgaris	501.500	**<0.001**	21.400	**<0.001**	83.700	**<0.001**
Rhinanthus minor	1463.000	**<0.001**	551.000	**<0.001**	3.121	1.000
Silene vulgaris	918.800	**<0.001**	3.200	0.073	6.520	0.832
Valeriana officinalis	369.400	**<0.001**	20.600	**<0.001**	10.200	0.345

The tetrazolium staining (viability) was highly predictive of the maximum total germination, irrespective of germination treatment, for all eight native species (Fig. 3.4), providing rapid identification of low quality lots in just two days. In six species a coefficient of determination (r^2) of greater than 0.94 indicated that over 90% of the variation in germination was accounted for by the TZ staining assessment (Fig. 3.4A, C, E, F, G, H). In two species, *C. segetum* and *P. rhoeas*, the r^2 values were lower, 0.83 (Fig. 3.4B) and 0.81 (Fig. 3.4D), respectively, but were still very highly significant ($P < 0.001$). When the data for all eight species was combined, there was a clear single universal relationship across all species between viability (TZ staining) and total germination (Fig. 3.5).

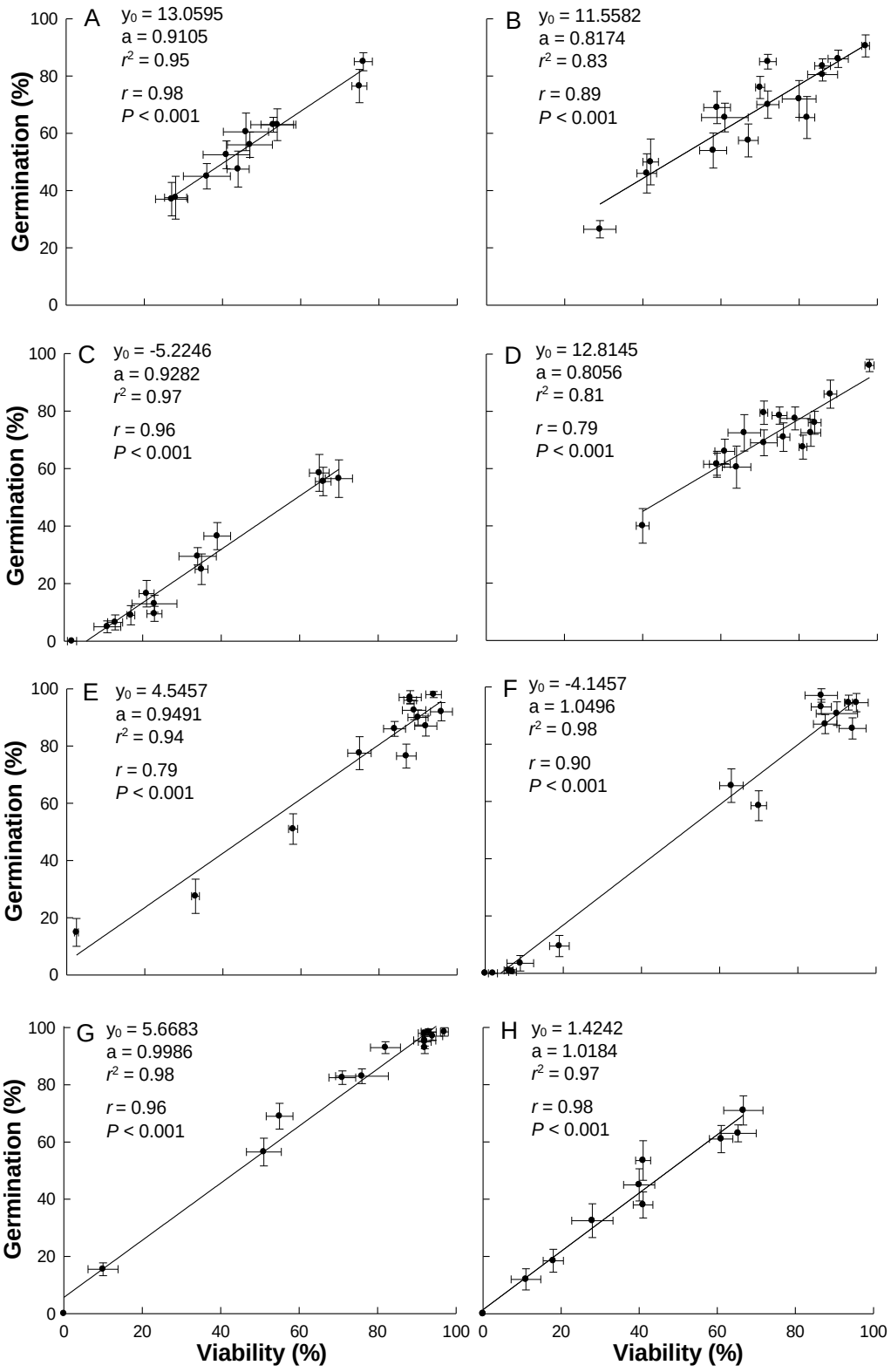

FIGURE 3.4. Relationship between seed viability (quantified using the tetrazolium test) and total germination for *Centaurea nigra* (A), *Cyanus segetum* (B), *Knautia arvensis* (C), *Papaver rhoeas* (D), *Prunella vulgaris* (E), *Rhinanthus minor* (F), *Silene vulgaris* (G) and *Valeriana officinalis* (H). In addition to correlation coefficient *r* and corresponding *P*-value from Spearman's rank correlation analysis, the constant y_0, coefficient a and r^2 are reported in each panel upon fitting with equation $y = y_0 + a*x$. Means are reported ± s.e.

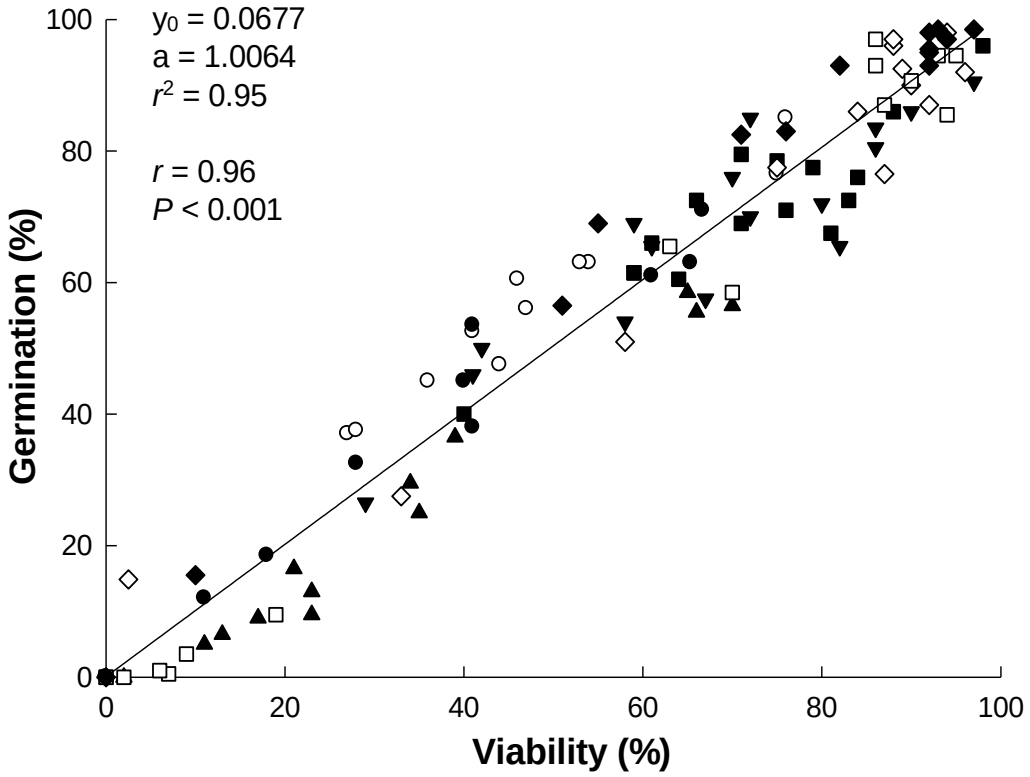

FIGURE 3.5. Relationship between seed viability (quantified using the tetrazolium test) and total germination for all the eight species (*Centaurea nigra* (○), *Cyanus segetum* (▼), *Knautia arvensis* (▲), *Papaver rhoeas* (■), *Prunella vulgaris* (◊), *Rhinanthus minor* (□), *Silene vulgaris* (♦) and *Valeriana officinalis* (●)) combined. In addition to correlation coefficient *r* and corresponding *P*-value from Spearman's rank correlation analysis, the constant y_0, coefficient a and r^2 are reported in each panel upon fitting with equation $y = y_0 + a*x$.

The results of the questionnaire revealed that harvesting method may be an important determinant of seed quality (Fig. 3.6). Germinations following multiple hand harvests, in which seed producers aim to select only mature seeds on each harvest occasion were greater than after single mechanical harvests when seeds typically include a range of maturity levels. Mean germination was markedly higher after multiple hand harvests of *Knautia arvensis* (44% germination, range 18 – 55%; single mechanical harvests, 8% germination, range 4 – 16%) and *Silene vulgaris* (multiple harvests, 91%, range 69 – 99%; single harvests, 24%, range 0 – 56%), although there was also a significantly higher germination after multiple harvests of *Prunella vulgaris* (multiple harvests, 78%,

range 51 – 96%; single harvests, 63%, range 28 – 78%) and *Cyanus segetum* (multiple harvests, 84%, range 66 – 89%; single harvests, 68%, range 29 – 87%).

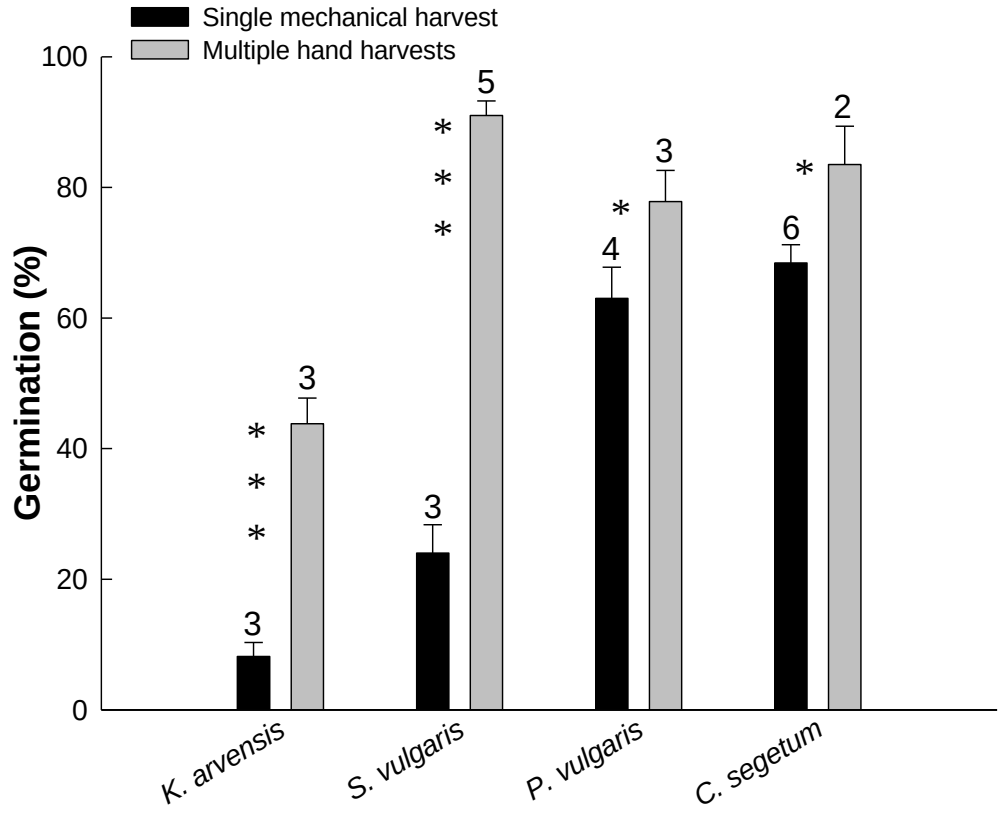

FIGURE 3.6. Final germination depending on weather there was single or multiple harvesting of seed lots of *Knautia arvensis, Silene vulgaris, Prunella vulgaris* and *Cyanus segetum*. Data were analysed by fitting GLMs (binomial distribution, logit link function) to the data; the fitted estimates and their s.e. are shown. * and *** indicate statistically significant differences between experimental categories ($P < 0.05$ and $P < 0.001$, respectively). The number above each bar indicates the number of seed lots/suppliers for each mean.

3.4. Discussion

This study highlighted a great variability in the germination capacity, indicated by radicle emergence, of individual seed lots from different suppliers, both with and without dormancy-breaking treatments. This provides experimental evidence of quality problems in the European native seed market. For the species used in this study, gibberellic acid was effective in breaking dormancy for all species tested except *R. minor*. There was no effect of seed lot on the effective dormancy-

breaking treatment for six of the eight tested species. Since each lot originated from a different supplier, this infers that the origin of the seed lots did not influence the effective dormancy-breaking treatment. A most useful practical finding was that the tetrazolium test can be used as a highly repeatable method that is predictive of germination in native seeds, providing rapid identification of low quality lots in just two days.

Seed availability among producers was highly variable, despite the abundance and common use of these species in restoration projects. Some species were widely available, *e.g. Cyanus segetum* and *Papaver rhoeas* were traded by more than 70% of the suppliers, while others like *Centaurea nigra* and *Valeriana officinalis* were purchasable from only 45% of the suppliers. The range in seed quality that we observed agrees with the observations of Ryan *et al*. (2008), who surveyed the quality of native seeds available on the UK market and found a great variability in the quality of the lots, with most of the suppliers providing seeds with poor germination in at least one species. For instance, germination ranged from 0 to 90% in the case of *Primula veris* and from 7 to 87% for *Leucanthemum vulgare* (Ryan *et al*., 2008). In the eight species we tested we also recorded highly variable values of final germination between samples of one species, ranging from 0 to 99% (Figs. 3.2 and 3.3). Seed lots containing only dead seeds were found in four of the eight tested species. In *R. minor*, 20% of the lots available on the market consisted of dead seeds. These observations highlight the need for quality control on the European native seed market, which would avoid significant restoration failure over the coming years. The harvest method may influence the final quality of the seeds produced, as seen here in four species (Fig. 3.6). Multiple hand harvests resulted in consistently higher levels of germination than a single mechanical harvest. This suggests that multiple hand harvests may, where possible, be the preferred harvest method to produce high quality seed. However, it may also be that rather than there being differences in the seed maturity, the process of mechanical harvest may have damaged the seed leading to the reduced germination.

In most of the tested species, germination levels above 90% were recorded among the samples suggesting the possibility of establishing high but achievable quality standards for these species. The generally low germination levels

recorded in the clonal herb *K. arvensis* might relate to the limited importance of the seed and seedling stages in clonal plants (Harper, 1977). Indeed, seed regeneration has generally been considered a rare event in clonal species, primarily linked to initial establishment at new sites (Vandvik *et al*., 2003). However, the limitations highlighted in the germination of *K. arvensis* need to be addressed by future research in order to assure successful field establishment of this species.

The application of GA_3 significantly enhanced germination in *Centaurea nigra*, *Knautia arvensis*, *Papaver rhoeas*, *Prunella vulgaris* and *Valeriana officinalis* (Figs. 3.2 and 3.3). However the increase in germination was small in most species suggesting that the poor germination recorded in some lots was not related to dormancy but to the presence of a large proportion of dead seeds. This is confirmed by the significant relationship found between the TZ test results and maximum final germination (Figs. 3.4 and 3.5), highlighting that dormancy was absent or had been broken in all the seed lots leading to matching values of germination and viability. The only instances in which there was a large increase in germination following GA_3 application were in *Papaver rhoeas* and one seed lot of *Prunella vulgaris*. The role of GA_3 in the promotion of germination is well known in a range of wild species (Rogis *et al*., 2004; Zhang *et al*., 2006; Foley and Chao, 2008; Golmohammadzadeh *et al*., 2015), including a wide range of conservation taxa (Cochrane *et al*., 2002).

In contrast to all other species tested, GA_3 did not enhance the germination of *Rhinanthus minor*, where germination only occurred when a cold stratification treatment was applied (Fig. 3.3). *R. minor* seed is characterised by an intermediate physiological dormancy (Baskin and Baskin, 2014) and many attempts have been made to reduce the requirement for cold stratification, including the scarification of seeds, the removal of the testa, extremes of temperature, leaching to remove any coat inhibitors, and chemical treatment with gibberellins, cytokinins and host root extracts (Gibson and Watkins, 1991). However none of the treatments was shown to increase germination (Gibson and Watkins, 1991; Westbury, 2004).

Germination characteristics of seeds of the same species collected in different locations commonly differ in the degree of dormancy (Baskin and Baskin, 2014). In this study, only *Papaver rhoeas* showed different levels of dormancy between seed lots from different suppliers and hence, origin. Germination was enhanced by GA_3 only in some lots, the same ones where the length (six weeks) of the cold stratification treatment was too short to release dormancy. Golmohammadzadeh *et al*. (2015) recorded the highest germination of *P. rhoeas* by combining cold stratification (45 days) and gibberellic acid treatments. In the present work, some lots that showed little evidence of dormancy had reduced germination following cold stratification. This may indicate that the cold treatment induced secondary dormancy as suggested by Cadman *et al*. (2006). The patterns observed in this study are in agreement with Karlsson and Milberg (2007) and enable the *Papaver* taxa to perform as winter annuals in warmer climates, but also as summer annuals in colder climates. Surprisingly, there was only one dormant lot of *Prunella vulgaris* (Fig. 3.2).

The present study highlighted the very low levels of dormancy across different lots *i.e*. origin in five tested species. Two species, *Cyanus segetum* and *Silene vulgaris*, germinated readily without a dormancy-breaking treatment. Keller and Kollmann (1999) also recorded high germination levels of *Cyanus segetum* under basic conditions of light, temperature and moisture. Moreover, they found a minimal variation among different origins of the same species, as observed in the responses to diurnal temperature fluctuation and day length. In *Centaurea nigra*, *Knautia arvensis* and *Valeriana officinalis*, where GA_3 released the low levels of dormancy, it did so regardless of seed origin. Similarly a common dormancy-breaking treatment, cold stratification, could be applied to seed lots of *Rhinanthus minor* with different origins. Ter Borg (2005) have also found the same pattern of dormancy and germination for seed samples of *R. minor* from a wide range of climates. The evolution of a requirement for a potentially very long stratification period to prevent untimely germination, in combination with the capacity to accelerate the last phases of the germination process when conditions become favourable, ensured its success during migration through a wide range of habitats (Ter Borg, 2005). In contrast, the variation in dormancy that we have seen in *Papaver rhoeas* and *Prunella vulgaris* could be related to environmental

factors, genetic variation or a combination of both (Santo *et al*., 2015). Moreover, it could result from the selection pressure associated with harvesting seeds across successive generations, which can lead to artificial selection for specific traits and performance of native populations (Chivers *et al*., 2016).

Even though there was little dormancy in many seed lots, GA_3 was the most useful dormancy-breaking treatment. This suggests that the same dormancy-breaking treatment and germination method may work for many species and that requirements vary between different origins of the same species only in a limited number of cases. This would limit the need to develop testing protocols for different species of commercially available native seeds.

Our work has revealed a TZ testing protocol that enables rapid evaluation of seed quality in native species, with a clear prediction of germination achieved in only two days, regardless of the dormancy status of the seeds (Figs. 3.4 and 3.5). This method contrasted with the long period of time necessary to achieve maximum germination (Table 3.1), saving from 1 to 37 weeks testing time and delivering faster results to seed producers. The strong correspondence between seed viability and maximum total germination percentage data, irrespective of germination treatment, also lead to the conclusion that there was a large proportion of dead and non-dormant seeds in a number of lots available on the market. TZ test protocols are available for four of the species used in this study (ISTA, 2011). However, to the best of our knowledge, this is the first data that clearly shows that the staining assessments relate to the actual germination of seed lots of these species and a further four species.

In summary, the quality of native seeds varied widely between European suppliers, which could be explained in part by the harvesting method for four species. Dormancy was present only in a limited number of seed lots. The significant correlation found between the TZ test results and final germination across all species revealed a common pattern across species and families (Fig. 3.5) which suggests that the TZ test could be applied to native species in general for routine evaluation of their germination. This is particularly important in today's global seed market, where the industry needs reliable information regarding the

germination potential of seed lots within a short time to make fast decisions in marketing and planting seeds (Soares *et al*., 2016).

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Chapter 4

Potential of the electrical conductivity of seed soak water and early counts of radicle emergence to assess seed quality in some native species

The work presented in this chapter has been submitted for publication:

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Abstract

The potential of the electrical conductivity (EC) test to predict final germination was evaluated in seed lots from seven native species. In four of the seven species tested (*Cyanus segetum*, *Prunella vulgaris*, *Valeriana officinalis* and *Centaurea nigra*) EC was indicative of the final germination (radicle emergence; RE), with high levels of leakage seen for lots with low germination. Single seed measurements of solute leakage from two species confirmed the link between high leakage and the failure to germinate, while in *Cyanus segetum* high EC was also associated with slow germination (after 42 hours). Reduced EC and earlier RE following a pre-hydration treatment in *C. segetum* supported the hypothesis that metabolic repair may occur during early imbibition. A single early count of RE (at 42 hours) also predicted germination ($r^2 \geq 0.858$) for 12 seed lots of *C. segetum*. Therefore, both measurements of solute leakage from seeds using EC and early counts of RE have potential to predict the germination of seed lots from native species. The use of the EC test may be dependent on the structure of the seed, but the RE test could be applied to a wider range of species and predict both germination and vigour differences.

4.1. Introduction

Seed quality of native, wild, species is key to habitat restoration projects as it determines the potential of a seed to germinate and establish a plant. The awareness of its importance is rising globally (Gibson-Roy *et al.*, 2007; Merritt and Dixon, 2011; Broadhurst *et al.*, 2016; Nevill *et al.*, 2016), including amongst contractors and other practitioners involved in the large-scale reintroduction of native species (Haslgrübler *et al.*, 2014). However, a recent study by Marin *et al.* (2017) provided experimental evidence of quality problems in the European native seed market, with highly variable values of germination and viability between samples of the same species. Indeed, seed lots containing only non-germinating seeds were found in four of the eight tested species (Marin *et al.*, 2017). Nevertheless, there is still a lack of methods and routine protocols for assessing seed quality in native species (Ryan *et al.*, 2008; Nevill *et al.*, 2016). Laboratory germination testing is the most common approach for seed quality evaluation in native species as it provides information on a range of cues (*e.g.* temperature and light conditions) that, singly or in combination, may be needed, particularly if dormancy is present, for successful germination and emergence of seedlings under field conditions (Gibson-Roy *et al.*, 2007). However, the broad range of germination responses exhibited across native species (Baskin and Baskin, 2014) and the long periods of time necessary to achieve germination (Marin *et al.*, 2017) suggest the need for the development of effective and quicker alternatives.

The tetrazolium test is a valid alternative to germination testing as it enables rapid evaluation of seed quality in native species, with a clear prediction of germination achieved in only two days, regardless of the dormancy status of the seeds (Marin *et al.*, 2017). Recent evidence from agricultural crop seeds points to another potentially applicable test to native species, the electrical conductivity (EC) test. This test measures the leakage of electrolytes from bulks of seeds into soak water and has been developed as a seed vigour test, indicative of the field emergence of lots of high and acceptable levels of normal germination. The EC test has been validated by ISTA for four grain legumes: garden peas, soybeans, *Phaseolus* beans and chickpea, and radish (ISTA, 2017). Legumes with large,

normally living, cotyledons are good candidates for the EC test to indicate field emergence, because they still germinate in the laboratory even with considerable areas of dead tissue on their cotyledons, provided that critical areas of the embryo remain living (Matthews and Powell, 2006).

Assessments of the leakage of electrolytes using bulk seed samples have also been associated with assessments of normal standard germination. In artificially aged seeds of *Brassica* spp., higher EC was seen from non-germinating seeds and seeds that gave rise to abnormal seedlings (Mirdad *et al.*, 2006). Similarly, commercial seed lots with higher proportions of seeds that failed to produce a radicle, or that produced abnormal seedlings, gave higher EC in bulk tests in cabbage (Demir *et al.*, 2008a) and oilseed rape (Wagner *et al.,* 2012) and in artificially aged radish seed (Demir *et al.*, 2012). More recently, Mavi *et al.* (2016) showed that in commercial seed lots of radish, the EC of bulks of seed after 1, 3, 5 and 24 hours of soaking was closely related to the proportion of non-germinating seeds and abnormal seedlings as well as to seeds that had slower RE. This was confirmed by the significant differences in leakage from single non-germinating and germinating seeds after 3 and 5 hours soaking (Mavi *et al.*, 2016). Similar relationships may also be a feature of some native species.

Other test methods have been developed to assess seed quality in agricultural species. The mean germination time (MGT), which is calculated from frequent counts of germination and is the average delay between imbibition and radicle emergence, was found to be predictive of emergence performance in pepper (Demir *et al.*, 2008b), maize (Matthews and Khajeh-Hosseini, 2006; Khajeh-Hosseini *et al.*, 2009), watermelon, melon and cucumber (Mavi *et al.*, 2010). A single early count of radicle emergence (RE) was predictive of MGT in oilseed rape (Matthews *et al.*, 2012a) and radish (Mavi *et al.*, 2014) and subsequently of emergence. Therefore, MGT and early counts of RE may be potentially useful tests for seed quality evaluation in native species.

The objectives of the current study were to: (1) investigate the potential of the electrical conductivity test to predict final germination of seed lots from seven native species in relation to seed structure; (2) examine the relationship between final germination and the length of the delay to radicle emergence (RE), as

indicated by the mean germination time (MGT) and early counts of RE; (3) determine if EC and single RE counts can be used as quick estimates of seed germination in some native species.

4.2. Materials and Methods

4.2.1. Seed material

Samples from commercial seed lots of *Centaurea nigra*, *Cyanus segetum*, *Knautia arvensis*, *Papaver rhoeas*, *Prunella vulgaris*, *Silene vulgaris* and *Valeriana officinalis* were obtained from various seed companies in Europe. A total of 83 seed samples were obtained across the species and suppliers. The seed samples will be referred to as seed lots in this paper.

4.2.2. Germination tests

For all seven species, eight replicates of 25 seeds per lot were placed in 90 mm-diameter Petri dishes on germination paper (Whatman, GE Healthcare Life Sciences) moistened with 2.5 ml gibberellic acid (GA_3; 250 mg l^{-1}) in order to release any dormancy and enhance germination (Marin *et al.*, 2017). The Petri dishes were then placed in plastic bags to prevent water loss during the test and held under controlled conditions at an alternating temperature of 25/10 °C, with a diurnal period of 12 hours-light and 12 hours-darkness. During incubation (four weeks), germination was scored weekly as radicle emergence (RE) and seeds were removed when RE had occurred.

The mean germination time was measured for *Cyanus segetum* during a subsequent germination test. Eight replicates of 25 seeds per lot were placed to germinate as described above and counts of radicle emergence were made twice a day for four weeks. The mean germination time (MGT) was calculated using the formula:

$$MGT = \Sigma(n_i \times t_i)/N$$

where n_i is the number of seeds that germinated (2 mm, radicle emergence) within consecutive intervals of time, t_i the time (hours) between the beginning of the test and the end of a particular interval of measurements, and N the total number of seeds that germinated. As the above formula illustrates, MGT is the average delay between the start of imbibition and radicle emergence (RE).

4.2.3. Bulk electrical conductivity (EC) measurements

Four replicates of either:

a) 25 seeds (*Centaurea nigra*, *Cyanus segetum* and *Knautia arvensis*); or

b) 50 seeds (*Prunella vulgaris* and *Silene vulgaris*); or

c) 100 seeds (*Valeriana officinalis*); or

d) 1000 seeds (*Papaver rhoeas*) per lot were weighed using an electronic 4-place balance and soaked in 10 ml distilled water for 24 hours at 20 °C. The conductivity of seed soak water was measured using a conductivity meter (4310, Jenway) after 24 hours and expressed per gram of seeds (µS cm^{-1} g^{-1}). In order to take into account the effect of possible electrolytes on the seed surface on the EC measurements a reading was taken two minutes after adding the water and subtracted from the 24-hour readings. The EC taken after two minutes never exceeded 24.10 µS cm^{-1} (recorded for one lot of *Knautia arvensis*).

Conductivity readings were taken at 3 and 5 hours, as well as 24 hours for 12 seed lots of *Cyanus segetum*.

4.2.4. Comparison of single seed conductivity measurements and germination

Twenty seeds were drawn randomly from each of the seed lots of *Centaurea nigra* (10 lots) and *Cyanus segetum* (13 lots). Single seeds were washed briefly in 8 ml distilled water for a few seconds, the water poured off and a further 8 ml water added to the seed. Electrical conductivity readings were taken for each seed after three and five hours at 20 °C and expressed per seed (µS cm^{-1} $seed^{-1}$). The seeds were then placed in 90 mm-diameter Petri dishes on germination paper moistened with 2.5 ml gibberellic acid (GA_3; 250 mg l^{-1}) so that germination

performance of each seed could be related to the EC readings. The plastic bags containing the Petri dishes were held under controlled conditions at an alternating temperature of 25/10 °C, with a diurnal period of 12 hours-light and 12 hours-darkness. Counts of radicle emergence were made twice a day for four weeks for both species.

4.2.5. Effect of hydration treatment and drying back

Three lots of *Cyanus segetum*, that were characterized as including a proportion of both earlier and later germinating seeds (before or after 42 hours) were subjected to a hydration treatment in two runs. During each run 25 seeds, drawn randomly from each lot, were weighed and washed briefly in distilled water for a few seconds. The 25 seeds were then placed in a 90 mm-diameter Petri dish on a moist germination paper to hydrate in air for five hours at 25 °C in the dark and subsequently allowed to dry back to their original weight on dry germination paper on the bench at 15 °C and 15% RH. Twenty five seeds from the same lots that had not been hydrated were used as a control.

The electrical conductivity of single seeds was measured after five hours, followed by germination for both the hydrated seed and the control, as described above. The moist germination papers on which 25 seeds had been hydrated were washed in 40 ml distilled water and the EC of the solution was measured. In order to take into account the EC due to the germination paper only, three replicates of moist germination papers with no seeds were each held in a Petri dish for five hours at 25 °C in the dark, subsequently washed and the EC of the solution was measured.

4.2.6. Internal morphology of seeds

Twenty seeds of each species and lot were dissected and observations were made with a stereomicroscope on a median longitudinal section. This was to determine if seeds of the seven tested species included an endosperm cell layer in the mature seed or if this is fully consumed during seed development as described by Yan *et al.* (2014). In addition, the seed internal morphology

observed for the target species was compared to that reported by Martin (1946), who described the embryo type of a range of species.

4.2.7. Statistical analysis

All statistical analyses were performed using GenStat 17th edition (VSN International, Hemel Hempstead, UK). Significant differences between experimental groups were assessed with unpaired Student's *t*-test and One-way-ANOVA. The significance of correlations was tested using Spearman's rank correlation, a Two-sample nonparametric test, to assess the strength and direction of any correlation between variables that do not follow a normal distribution. Determination coefficient (r^2) values and regression equations were determined to assess the prediction potential. Significance was evaluated in all cases at $P \leq 0.05$.

4.3. Results

The bulk electrical conductivity (EC) test of seed soak water was significantly related to total seed germination (radicle emergence achieved) of seed lots from four of the seven native species tested (Table 4.1). The Spearman's rank correlations were as follows: *Cyanus segetum,* $r = -0.906$ ($P < 0.001$); *Prunella vulgaris,* $r = -0.657$ ($P = 0.005$); *Centaurea nigra,* $r = -0.567$ ($P = 0.022$) and *Valeriana officinalis* $r = -0.479$ ($P = 0.044$) (Table 4.1). The negative correlation revealed that a greater leakage of electrolytes was associated with lower germination (Fig. 4.1), a relationship that was particularly strong for 17 commercially available seed lots of *Cyanus segetum* ($r^2 = 0.867$; Fig. 4.1A).

Almost all of the lots with germination above 50, 60 and 70%, could be identified by an EC reading of less than 900 µS cm^{-1} g^{-1} for *Centaurea nigra*, *Cyanus segetum* and *Prunella vulgaris*, respectively. In the case of *Valeriana officinalis*, germinations ranged from 0 to 71% and lots with germination above 40% could be mostly identified by an EC reading of less than 1600 µS cm^{-1} g^{-1}.

TABLE 4.1. Coefficient of correlation, *r*, and *P*-value from Spearman's rank correlation analysis between bulk electrical conductivity (EC) measurements of seed soak water and germination (percentage radicle emergence) for seeds of seven native species from six families. Species, families, number of seed lots, internal morphology of seeds and 1000 seed weight are reported.

Species	Family	Number of lots	*r*	*P*	Internal morphology of seeds	1000 seed weight, g
Cyanus segetum	Asteraceae	17	-0.906	**< 0.001**	Non-endospermic, large embryo	4.13
Prunella vulgaris	Lamiaceae	12	-0.657	**0.005**	Non-endospermic, large embryo	0.79
Centaurea nigra	Asteraceae	10	-0.567	**0.022**	Non-endospermic, large embryo	2.46
Valeriana officinalis	Valerianaceae	9	-0.479	**0.044**	Non-endospermic, large embryo	0.66
Knautia arvensis	Caprifoliaceae	11	-0.273	0.101	Endospermic, small embryo	6.44
Papaver rhoeas	Papaveraceae	15	0.070	0.201	Endospermic, small embryo	0.12
Silene vulgaris	Caryophyllaceae	9	-0.283	0.109	Endospermic, small embryo	0.87

Early bulk conductivity readings, taken at three and five hours, for 12 seed lots of *Cyanus segetum* were highly predictive of the EC at 24 hours, with r^2 values of 0.913 and 0.943, respectively (Fig. 4.2). The remaining three tested species (*Knautia arvensis*, *Papaver rhoeas* and *Silene vulgaris*) did not show a significant correlation between EC and germination (Table 4.1).

Levels of electrolyte leakage from single seeds were measured for two Asteraceae species (*Cyanus segetum* and *Centaurea nigra*) and compared to the seedlings subsequently produced by each seed (Tables 4.2 and 4.3). The overall mean EC was significantly higher for seeds that did not produce a radicle for both species ($P < 0.001$) and was consistently greater for non-germinating seeds for each seed lot in both species (as seen for *Cyanus segetum* in Table 4.2). Slower radicle emergence was also related to higher levels of leakage for *Cyanus segetum* (Table 4.2). This is clearly revealed by a comparison of leakage from the earlier germinating seeds (germination at and before 42 hours) with that from later germinating seeds (germination after 42 hours). Indeed, for the mean of all lots, EC was significantly greater for later germinating seeds ($P < 0.001$; Table

4.2). No differences in EC were seen in relation to the timing of RE in *Centaurea nigra* (Table 4.3).

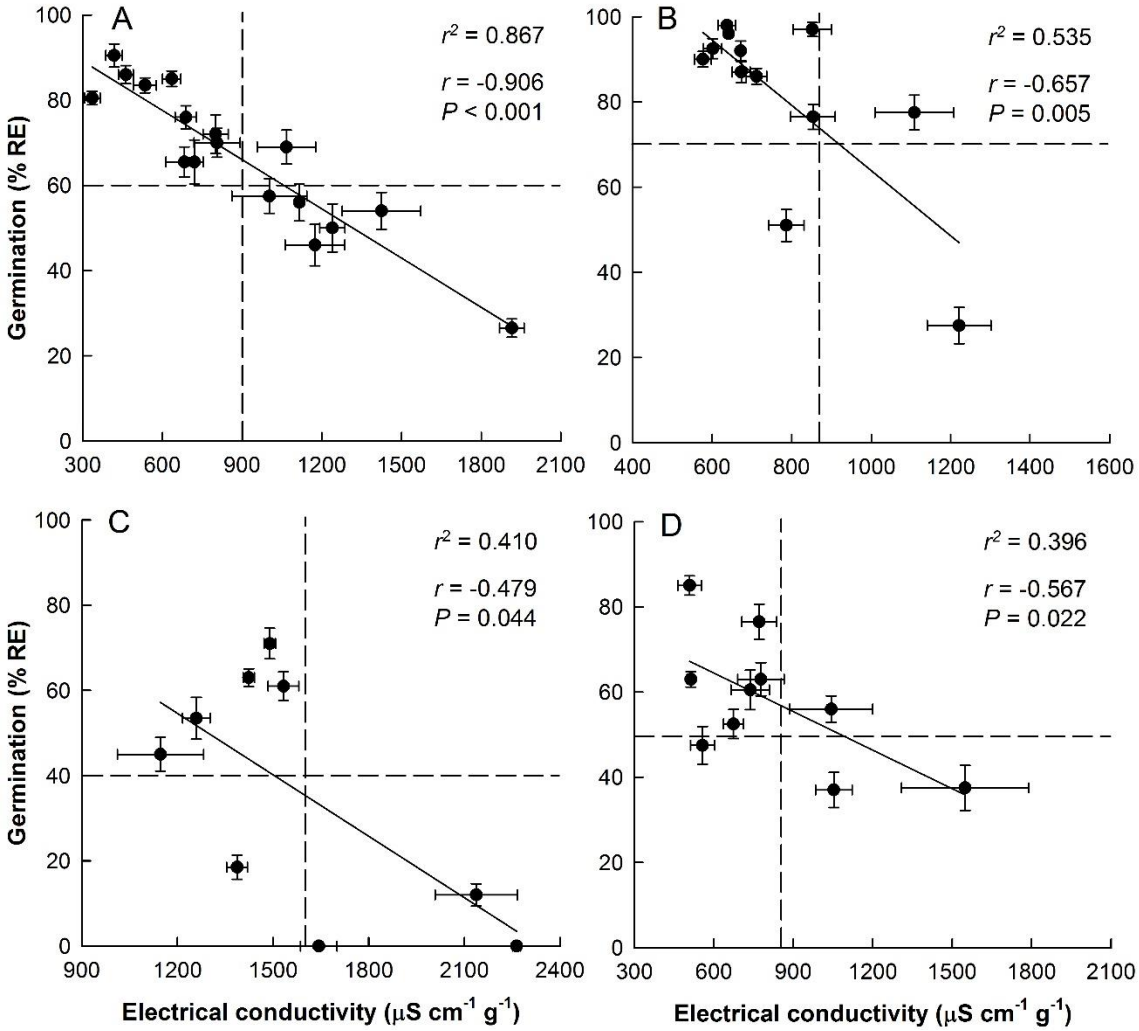

FIGURE 4.1. Relationship between bulk electrical conductivity (EC) measurements of (A) 17 seed lots of *Cyanus segetum*, (B) 12 seed lots of *Prunella vulgaris*, (C) 9 seed lots of *Valeriana officinalis*, (D) 10 seed lots of *Centaurea nigra* and their germination (percentage radicle emergence). Means are reported ± s.e. In addition to correlation coefficient, *r*, and corresponding *P*-value from Spearman's rank correlation analysis, the determination coefficient r^2 is reported in each panel upon fitting with equation $y = y_0 + a*x$.

The internal morphology observed (Table 4.1) was in agreement with that reported by Martin (1946). Seeds from the genera *Centaurea*, *Cyanus*, *Prunella* and *Valeriana* are characterized by a large embryo and lack an endosperm cell layer. *Knautia* spp. exhibit a peripheral embryo and a soft and usually watery-

fleshy endosperm. The seeds of *Papaver* spp. are similar. In the case of *Silene* spp., the embryo is peripheral and the endosperm is described as usually firm or hard and semi-translucent (Martin, 1946).

Both the length of the average delay to RE (mean germination time, MGT) and an early count of RE predicted final germination of *Cyanus segetum* (Fig. 4.3). The MGT for the 12 lots of *C. segetum* ranged from 37 to 98 hours (Fig. 4.3A) and was significantly correlated with final germination (r = -0.727), giving an r^2 of 0.638. The early count of RE at 42 hours was, in turn, significantly correlated with MGT (r = -0.818; P < 0.001; Fig. 4.3B). Consequently RE at 42 hours was highly predictive of final germination with a highly significant correlation coefficient giving an r^2 of 0.858 (Fig. 4.3C).

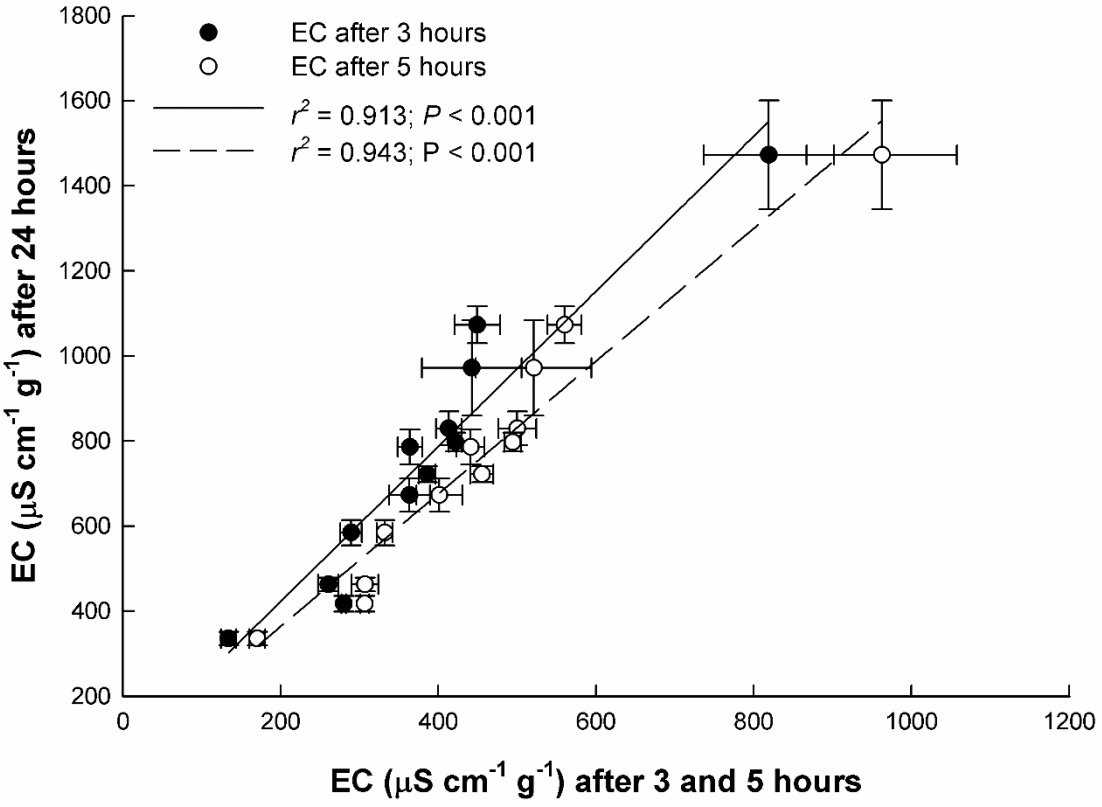

FIGURE 4.2. Linear regression between electrical conductivity (EC) readings of bulks of seeds from 12 seed lots of *Cyanus segetum*, after three (●, solid line) and five (○, dashed line) hours, and EC after 24 hours. Means are reported ± s.e. The determination coefficient, r^2, and P-value are reported.

TABLE 4.2. Mean electrical conductivity (EC, µS cm^{-1} $seed^{-1}$) of seed soak water (8 ml) of all single seeds from 13 seed lots of *Cyanus segetum* after 5 hours at 20 °C and for seeds that either germinate (produce a radicle) or fail to do so in their subsequent germination test. Also shown is the EC (µS cm^{-1} $seed^{-1}$) for seeds that produce a radicle before or after 42 hours. Values in parentheses are the number of seeds contributing to each value. *** indicates statistically significant differences between categories as tested using unpaired Student's *t*-test ($P < 0.001$).

Lot no	EC after 3 hours						EC after 5 hours					
	All seeds	No radicle produced		Germinated seeds	When RE ≤ 42h	When RE > 42h	All seeds	No radicle produced		Germinated seeds	When RE ≤ 42h	When RE > 42 h
1	2.85 (20)	2.96 (5)		2.82 (15)	2.77 (5)	2.84 (10)	3.23 (20)	3.57 (5)		3.11 (15)	3.01 (5)	3.17 (10)
2	3.40 (20)	3.69 (13)		2.86 (7)	2.56 (1)	2.91 (6)	3.97 (20)	4.41 (13)		3.16 (7)	2.81 (1)	3.22 (6)
3	6.02 (20)	6.43 (17)		3.73 (3)	- (0)	3.73 (3)	7.20 (20)	7.69 (17)		4.41 (3)	- (0)	4.41 (3)
4	2.59 (20)	2.67 (4)		2.57 (16)	2.60 (15)	2.14 (1)	2.99 (20)	3.10 (4)		2.96 (16)	2.98 (15)	2.64 (1)
5	3.40 (20)	3.49 (15)		3.12 (5)	- (0)	3.12 (5)	3.95 (20)	4.09 (15)		3.51 (5)	- (0)	3.51 (5)
7	3.10 (20)	3.22 (14)		2.84 (6)	- (0)	2.84 (6)	3.56 (20)	3.72 (14)		3.20 (6)	- (0)	3.20 (6)
8	3.01 (20)	3.10 (8)		2.95 (12)	3.15 (8)	2.55 (4)	3.71 (20)	4.10 (8)		3.44 (12)	3.69 (8)	2.96 (4)
9	2.99 (20)	3.01 (16)		2.92 (4)	2.72 (3)	3.50 (1)	3.49 (20)	3.53 (16)		3.33 (4)	3.08 (3)	4.09 (1)
10	3.02 (20)	3.31 (6)		2.90 (14)	2.83 (10)	3.07 (4)	3.44 (20)	3.85 (6)		3.26 (14)	3.16 (10)	3.52 (4)
11	3.13 (20)	4.00 (7)		2.66 (13)	2.40 (10)	3.52 (3)	3.56 (20)	4.67 (7)		2.96 (13)	2.66 (10)	3.96 (3)
14	4.05 (20)	4.43 (12)		3.47 (8)	3.38 (4)	3.56 (4)	5.05 (20)	5.48 (12)		4.41 (8)	4.38 (4)	4.44 (4)
15	1.89 (20)	2.04 (5)		1.84 (15)	1.85 (14)	1.82 (1)	2.17 (20)	2.35 (5)		2.11 (15)	2.11 (14)	2.07 (1)
17	3.59 (20)	3.85 (12)		3.19 (8)	2.99 (4)	3.40 (4)	4.33 (20)	4.70 (12)		3.76 (8)	3.39 (4)	4.13 (4)
Mean	3.31 (260)	3.81 (134)	***	2.78 (126)	2.60 (74)	*** 3.04 (52)	3.89 (260)	4.56 (134)	***	3.19 (126)	2.97 (74)	*** 3.50 (52)

TABLE 4.3. Mean electrical conductivity (EC, µS cm^{-1} $seed^{-1}$) of the seed soak water (8 ml) of all single seeds from 10 seed lots of *Centaurea nigra* and for seeds that either geminate (produce a radicle) or fail to do so in their subsequent germination test. Also shown is the EC (µS cm^{-1} $seed^{-1}$) for seeds that produce a radicle before or after 72 hours. Values in parentheses are the number of seeds contributing to each value. *** indicates statistically significant differences between categories as tested using unpaired Student's *t*-test ($P < 0.001$), while n.s. indicates the lack of significant differences.

Lot no	EC after 3 hours						EC after 5 hours					
	All seeds	No radicle produced		Germinated seeds	When RE ≤ 72h	When RE > 72 h	All seeds	No radicle produced		Germinated seeds	When RE ≤ 72h	When RE > 72 h
1	2.52 (20)	2.60 (8)		2.46 (12)	- (0)	2.46 (12)	2.77 (20)	2.87 (8)		2.70 (12)	- (0)	2.70 (12)
2	2.48 (20)	2.75 (4)		2.41 (16)	2.30 (6)	2.48 (10)	2.93 (20)	3.37 (4)		2.82 (16)	2.76 (6)	2.85 (10)
3	2.73 (20)	3.47 (5)		2.49 (15)	2.48 (11)	2.51 (4)	3.23 (20)	4.38 (5)		2.85 (15)	2.84 (11)	2.87 (4)
4	2.56 (20)	2.76 (7)		2.45 (13)	2.69 (3)	2.37 (10)	2.79 (20)	2.93 (7)		2.72 (13)	2.94 (3)	2.66 (10)
5	2.58 (20)	2.65 (16)		2.31 (4)	2.21 (1)	2.34 (3)	2.84 (20)	2.94 (16)		2.48 (4)	2.44 (1)	2.49 (3)
6	2.81 (20)	3.37 (8)		2.43 (12)	2.62 (3)	2.37 (9)	3.22 (20)	3.88 (8)		2.78 (12)	2.93 (3)	2.73 (9)
7	3.09 (20)	3.12 (18)		2.78 (2)	2.92 (1)	2.64 (1)	3.43 (20)	3.48 (18)		2.94 (2)	2.99 (1)	2.88 (1)
8	2.15 (20)	2.28 (6)		2.10 (14)	2.16 (3)	2.09 (11)	2.45 (20)	2.72 (6)		2.33 (14)	2.33 (3)	2.33 (11)
9	2.22 (20)	2.44 (4)		2.17 (16)	2.16 (11)	2.20 (5)	2.53 (20)	2.84 (4)		2.46 (16)	2.43 (11)	2.52 (5)
10	2.61 (20)	2.83 (14)		2.09 (6)	2.02 (1)	2.10 (5)	3.18 (20)	3.55 (14)		2.32 (6)	2.24 (1)	2.33 (5)
Mean	2.57 (200)	2.85 (90)	***	2.34 (110)	2.36 (40)	n.s. 2.34 (70)	2.94 (200)	3.30 (90)	***	2.64 (110)	2.67 (40)	n.s. 2.63 (70)

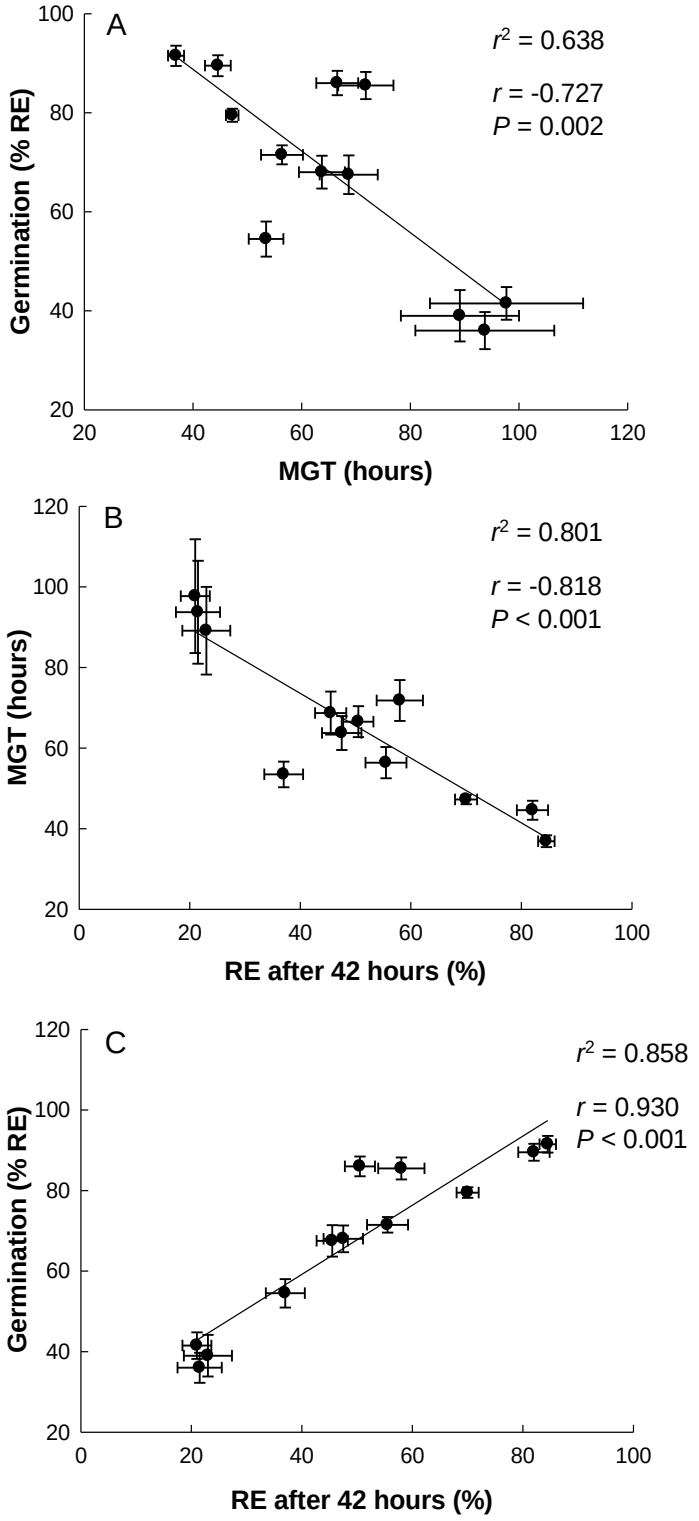

FIGURE 4.3. Relationship between (A) mean germination time (MGT) and final germination (percentage radicle emergence); (B) radicle emergence (RE) at 42 hours and MGT and (C) RE at 42 hours and final germination for 12 seed lots of *Cyanus segetum*. Means are reported ± s.e. In addition to correlation coefficient, *r*, and corresponding *P*-value from Spearman's rank correlation analysis, the determination coefficient, r^2, is reported in each panel upon fitting with equation $y = y_0 + a*x$.

TABLE 4.4. Mean electrical conductivity (EC, µS cm^{-1} $seed^{-1}$) of the soak water of single seeds from three lots (1, 14 and 17) of *Cyanus segetum* after five hours soaking at 20 °C. Seeds were either given a 5-hour hydration / drying treatment or were non-hydrated control seeds. The hydration treatment was performed twice and data were compared to the control. Seeds were identified as those that did not, or did, produce a radicle (number of seeds in parentheses) in a subsequent germination test. The percentage of germinated seeds that produced a radicle at, or before, 42 hours is also reported. Significant parameters ($P < 0.05$) are in bold.

Lot no.	Treatment	EC reading (µS cm^{-1} $seed^{-1}$)			RE ≤ 42 hours (%)
		All seeds	No radicle produced	Germinated	
1	Control	3.70 (25)	3.72 (4)	3.70 (21)	43%
	5-hour hydration 1st	2.09 (25)	2.27 (6)	2.04 (19)	47%
	P-value	**< 0.001**	**0.037**	**<0.001**	
	5-hour hydration 2nd	2.35 (25)	2.56 (4)	2.30 (21)	57%
	P-value	**< 0.001**	0.193	**<0.001**	
14	Control	3.81 (25)	4.20 (13)	3.38 (12)	58%
	5-hour hydration 1st	2.76 (25)	2.75 (14)	2.77 (11)	45%
	P-value	**< 0.001**	**0.002**	**0.011**	
	5-hour hydration 2nd	2.91 (25)	3.25 (12)	2.60 (13)	77%
	P-value	**< 0.001**	**0.047**	**0.002**	
17	Control	3.96 (25)	4.57 (13)	3.30 (12)	42%
	5-hour hydration 1st	2.47 (25)	2.66 (14)	2.23 (11)	91%
	P-value	**< 0.001**	**< 0.001**	**< 0.001**	
	5-hour hydration 2nd	2.50 (25)	2.62 (16)	2.71 (9)	67%
	P-value	**< 0.001**	**< 0.001**	**0.037**	
Mean	Control	3.82 (75)	4.30 (30)	3.51 (45)	47%
	5-hour hydration	2.51 (150)	2.74 (66)	2.38 (84)	62%
	P-value	**< 0.001**	**< 0.001**	**< 0.001**	

A hydration treatment, followed by drying back, of three lots of *Cyanus segetum* resulted in reduced leakage per seed in each of two repeat runs of the treatment, compared with a non-hydrated control (Table 4.4). The electrical conductivity of single seeds that produced a radicle was significantly lower in the hydrated seed (mean 2.38 µS cm^{-1} $seed^{-1}$) compared to the control seeds (3.51 µS cm^{-1} $seed^{-1}$). Lower leakage from hydrated seeds was accompanied by an increase in early RE (at or before 42 hours) with the overall mean for the three lots / runs increasing from 47% in the control to 62% following the hydration

treatment. This was particularly evident following the first hydration treatment in lot no 17, when the percentage of early germinating seeds increased from 42 to 91%. The reduction in leakage was also seen for the seeds that did not produce a radicle, from a mean for all lots / runs of 4.30 μS cm^{-1} $seed^{-1}$ for the control to 2.74 μS cm^{-1} $seed^{-1}$ for hydrated seed. The EC of the washings from the germination papers on which 25 seeds had been hydrated averaged 17.76, 19.66 and 20.02 μS cm^{-1}, for lots no 1, 14 and 17, respectively. The EC of the washings from the germination papers with no seeds, used as controls, averaged 14.03 μS cm^{-1}. Thus an estimate of leakage from individual seeds during hydration was low per seed and would have had little effect on the differences between the hydrated and control seed.

4.4. Discussion

Electrolyte leakage from bulks of seed, assessed by the electrical conductivity (EC) of seed soak water, were indicative of the final germination (radicle emergence; RE) of four out of the seven species tested (*Cyanus segetum*, *Prunella vulgaris*, *Valeriana officinalis* and *Centaurea nigra*), with high levels of leakage (high EC) seen for lots with low germination. Single seed measurements of solute leakage from two species confirmed the link between high leakage and the failure to germinate. In *Cyanus segetum* the relationship between the length of the delay to radicle emergence (mean germination time; MGT) and an early (42 hour) count of RE suggested a single RE count also predicted final germination. The observation of reduced EC and earlier RE following a pre-hydration treatment in three seed lots of *Cyanus segetum* supported the hypothesis that metabolic repair may occur during early imbibition.

The EC of seed soak water clearly predicted differences in the final germination (percentage radicle emergence) in 17 commercially available seed lots of *Cyanus segetum* (Fig. 4.1A) and the seed lot of highest quality (RE: 91%; EC: 418 μS cm^{-1} g^{-1}) could be clearly distinguished from the one with the lowest final germination (RE: 27%; EC: 1915 μS cm^{-1} g^{-1}). Seed lots with a relatively high level of germination (above 60%) were identified as having EC readings of less

than 900 µS cm^{-1} g^{-1} (Fig. 4.1) for all but one lot of *Cyanus segetum*. An EC reading of below 900 µS cm^{-1} g^{-1} also identified seed lots of *Prunella vulgaris* and *Centaurea nigra* having germinations above 70% and 50%, respectively, while lots of *Valeriana officinalis* with above 40% germination had EC readings of less than 1600 µS cm^{-1} g^{-1} (Fig. 4.1). These levels of final germination would be considered low in crop species, but can occur frequently in commercial lots of native species (Ryan *et al.*, 2008; Marin *et al.*, 2017). The bulk EC after 24 hours was significantly correlated with earlier readings after 3 and 5 hours (Fig. 4.2), an observation also made in radish (Mavi *et al*., 2016). This suggests that even more rapid prediction of germination is possible (Fig. 4.2). The association between high levels of leakage and both slow and poor germination was supported by the higher EC readings from single seeds after 3 and 5 hours soaking for seeds of *Cyanus segetum* that subsequently germinated later (after 42 hours), and from non-germinating seeds of both *Cyanus segetum* (Table 4.2) and *Centaurea nigra* (Table 4.3).

The four species in which EC was related to final germination (Fig. 4.1) all had non-endospermous seeds with relatively large embryos (Table 4.1). *Cyanus segetum* had the largest embryo and showed the closest relationship (r^2 = 0.867, Fig. 4.1). It may be that a relatively large embryo is needed before differences in the extent of damaged and dead areas can be detected by seed leakage. In recent work on the same seed lots (Marin *et al.*, 2017), tetrazolium staining revealed a wide variation between lots of all species and that germination was correlated with the proportion of seeds showing complete staining of the embryos. This was suggested as a test indicative of germination in just two days. The EC test appears to be restricted only to seed with appropriate seed morphology, but even so can be completed within 3, 5 or 24 hours (Fig. 4.2) to give a prediction of germination. Further testing of other species showing similar morphology to *Cyanus segetum* would be a useful next step in developing this method of predicting germination further.

Calculation of the mean germination time for *Cyanus segetum* from daily counts of RE over four weeks revealed a significant relationship to both germination (Fig. 4.3A; r^2 = 0.638) and to a single early count of RE at 42 hours (Fig. 4.3B; r^2 = 0.801), resulting in a highly significant relationship between the 42

hour count and germination (Fig. 4.3C; r^2 = 0.858). This suggests that a 42 hour RE count can predict germination of *Cyanus segetum*, as seen for a 48 hour RE count in radish (Mavi *et al.*, 2016).

In previous work, early RE counts have been used to indicate potential differences in emergence, that is vigour, of maize (Matthews *et al.*, 2011), radish (Mavi *et al.*, 2014) and oilseed rape (Matthews *et al.*, 2012a). Seed lots at risk of poor emergence can be identified by a low early RE count and the RE test is included in the ISTA Rules as a validated vigour test for maize, radish and oilseed rape (ISTA, 2017). The use of early counts of RE to predict vigour in crop seeds suggests the possibility that similar counts in *Cyanus segetum* may not only indicate the ability to germinate, as shown in the present work, but also emergence in the field. Evidence for this would be a useful future objective, since the overall aim of the producers of native seed species is to provide seed that will emerge well and become lasting components of vegetation (Kiehl *et al.*, 2014).

The negative correlation found between EC and final germination resulted from greater leakage of non-germinating seeds, as seen by the single seed conductivity measurements for both *Centaurea nigra* and *Cyanus segetum* (Tables 4.2 and 4.3). The two categories of seeds were clearly identified by EC measurements after 3 and 5 hours, confirming that early readings are indicative of differences in final germination as seen for bulks of seeds. These observations are in agreement with previous work on radish (Demir *et al.*, 2012), oilseed rape (Wagner *et al.*, 2012) and cabbage (Demir *et al.*, 2008a). In the case of *Cyanus segetum* higher single seed conductivities were seen for seeds that were slower to radicle emergence, as observed for radish (Mavi *et al.*, 2014; Mavi *et al.*, 2016).

The significant reduction in electrolyte leakage and earlier radicle emergence following the pre-hydration treatment (Table 4.4) can be explained in terms of the ageing / repair hypothesis (Matthews and Khajeh-Hosseini, 2007; Matthews and Powell, 2011). Osborne (1983) suggested that all seeds undergo a period of DNA repair before germination. The ageing / repair hypothesis proposes that in aged seeds the slower germination, or prolonged lag period before radicle emergence, reflects the time needed to repair the deterioration that has accumulated during ageing (Matthews and Khajeh-Hosseini, 2007; Matthews

et al., 2012b; Powell and Matthews, 2012). The present work has shown that a reduced time to radicle emergence is also associated with reduced solute leakage from the seeds, as seen in radish (Mavi *et al.*, 2016). This raises the question whether metabolic repair before germination includes the repair of membranes, as well as DNA.

Our results showed a significant reduction in leakage following the hydration treatment for seeds that failed to produce a radicle, as well as for the germinating seeds (Table 4.4). This could be explained by the fact that a large proportion of viable tissue may still be found in non-germinating seeds. Indeed, damage may affect parts of the embryo that are essential for germination which therefore fails to occur, even though other areas of the seed may remain alive (ISTA, 2011). The living tissue in such seeds may therefore have undergone repair during hydration reducing the leakage from these parts of the seed.

In summary, we have shown that both measurements of solute leakage from seeds using EC and early counts of RE have potential to predict the germination (radicle emergence) of seed lots from native species. The use of the EC test may be dependent on the structure of the seed, but the RE test could well be applied to a wider range of species and predict both germination and vigour differences.

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Chapter 5

Germination characteristics of *Rhinanthus minor* influence field emergence, competitiveness and potential use in restoration projects

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Abstract

The facultative root hemi-parasite *Rhinanthus minor* is a widespread component of grasslands throughout Europe and North America. It is often used in grassland habitat restoration projects where it plays an important role in regulating ecosystem structure and function by suppressing dominant grasses and allowing other species to proliferate, potentially increasing plant diversity. This study presents the first evidence that seed quality is a factor in influencing the efficacy of *R. minor*, from establishment to its effect on the host plants. Ten seed lots from commercial sources were sown in the field and their germination characteristics were investigated in the laboratory. Seeds from four lots were germinated to radicle emergence and sown in pots alongside plants of two host species, *Lotus corniculatus* and *Holcus lanatus*. Plant establishment, height and flowering density were evaluated for the hemi-parasite, while plant biomass was measured for both *R. minor* and its host. Two aspects of seed quality influenced the field emergence of seed lots of *R. minor,* the radicle emergence (%) and the length of the lag period from the beginning of imbibition to germination (mean germination time), which is indicative of seed vigour. A longer lag period (lower vigour) was associated with higher levels of seedling mortality and lower plant vigour, in terms of plant height and biomass accumulation and was also reflected in the parasitic impact of the seed lots, with the least vigorous seed lot having no impact on the biomass of its host, *Lotus corniculatus*. Seed quality, specifically germination and vigour, can influence the establishment, survival, subsequent plant productivity and parasitic impact of *R. minor* in vegetation restoration projects. Seed quality is therefore a key factor to consider when predicting the impact of the hemi-parasite on community productivity and diversity.

5.1. Introduction

The use of native species has been increasingly advocated to achieve the sustainable recovery of degraded areas (Oliveira *et al.*, 2014), for example in post-mining rehabilitation (Barrett-Lennard *et al.*, 2016), land reclamation on mine spoil dumps (Juwarkar *et al.*, 2009) and road-slope revegetation (Bochet *et al.*, 2010). Wildflower plantings are also used to increase insect pollination in adjacent crop fields (Blaauw and Isaacs, 2014) and in Europe, considerable effort has been invested into grassland restoration due to the decline in the once widespread, traditionally managed, species-rich meadows (Fry *et al.*, 2017). The establishment of a diverse range of target species on many sites requires the reduction of either the soil fertility or the vigour of competitive plants (Bullock and Pywell, 2005). A direct approach to lowering soil fertility by removing the nutrient rich topsoil is however limited, being too time consuming and costly (Hölzel and Otte, 2003; Kiehl and Wagner, 2006). Successful recreation of target plant communities may therefore depend upon the introduction of key species that could be used to decrease the growth of competitive plants and enhance species diversity (Smith *et al.*, 2003).

The facultative root hemi-parasite *Rhinanthus minor* (Orobanchaceae) is a widespread component of grasslands throughout Europe and North America, known to infect and reduce the competitive dominance of fast growing grassland species (Fry *et al.*, 2017). *R. minor* exerts much of its influence by dramatically reducing host plant biomass (Rowntree *et al.*, 2014), with up to 73% of total plant community biomass reduction (Ameloot *et al.*, 2005). Gibson and Watkinson (1989) report at least 50 species from 18 plant families that can be parasitized by *R. minor*. However, the hemi-parasite was particularly effective in reducing the competitiveness of grasses (Poaceae) and legumes (Fabaceae; Cameron *et al.*, 2006; Rowntree *et al.*, 2014; Mudrák *et al.*, 2016), thereby allowing slower growing species to increase in abundance (Fry *et al.*, 2017). Like other parasitic plants, *R. minor* establishes cellular continuity with the xylem stream of its hosts via specialized organs known as haustoria, thereby extracting nutrients (Rowntree *et al*., 2014).

The hypothesis that the impact of hemi-parasites such as *R. minor* on community productivity and diversity is a function of resource supply (nutrient and light availability) has been widely studied (Bardgett *et al.*, 2006; Cameron *et al.*, 2009; Borowicz and Armstrong, 2012). *R. minor* colonization and persistence are also influenced by factors such as sward composition and management (Bullock and Pywell, 2005), and plant density (Ameloot *et al.*, 2005). However, there is a lack of information about the possible influence of seed quality on the efficacy of the hemi-parasite. Indeed, the seed used within previous studies was either obtained from a single commercial source (Smith *et al.*, 2003; Keith *et al.*, 2004; Westbury *et al.*, 2006; Westbury and Dunnett, 2007; Fry *et al.*, 2017) or gathered from the wild (Davies and Graves, 1998; Davies and Graves, 2000). Several seed sources have only been considered in the evaluation of the genetic diversity between *R. minor* populations (Houston and Wolff, 2012), but not in relation to the seed ability to germinate or to establish a plant. The establishment of *Rhinanthus* seed has only been referred to in terms of the number of plants produced for a given seed rate (*e.g.* Westbury and Dunnett, 2007).

The importance of seed quality in establishment is well recognised in crop species, with many countries having either minimum germination standards for commercial seed lots (the unit in which seeds are bought and sold) (*e.g*. above 80%), or requiring truth in labelling in which the germination percentage has to be stated on the seed packet. There are no such standards for wild species in Europe. High germination standards are possible for crop seeds as they are the product of many years of plant breeding and the result of production conditions using well-established harvest and processing techniques. The production of native species, such as *R. minor*, is less well established and the absence of breeding pressures means that crops tend to be more variable (Laverack *et al.*, 2006). Differences in the germination capacity of native species have been reported, with large variations between individual seed lots from different suppliers (Ryan *et al.*, 2008; Marin *et al.*, 2017). Dormancy is frequently a problem for the germination of wild species and *R. minor* is characterised by an intermediate physiological dormancy (Baskin and Baskin, 2014). Even when dormancy of *R. minor* was broken by a period of cold stratification ranging from 13 to 37 weeks, germination among commercial samples ranged from 0 to 97%

(Marin *et al.*, 2017). These differences in germination capacity were also identified in only two days using the tetrazolium test (Marin *et al.*, 2017).

A further aspect of seed quality seen in crop species, namely seed vigour, may play a role in the efficacy of *Rhinanthus* spp. Seed lots of crop species with similarly high levels of germination may show differences in field emergence due to differences in seed vigour (Matthews and Powell, 2011). Seed vigour influences the rate and uniformity of germination and growth, emergence under unfavourable environmental conditions, and germination after storage (Powell and Matthews, 2012). Vigour is largely determined by the physiological deterioration that occurs as seeds age at any stage of seed production. This may be while the seed is still on the plant, during processing and, most commonly, in storage (Matthews *et al.*, 2012). One aspect of germination that relates to vigour is the mean germination time (MGT), which describes the mean lag period between the beginning of seed imbibition and the appearance of the radicle through the seed coat (Matthews and Khajeh-Hosseini, 2006). The mean germination time has been found to be predictive of emergence performance in seed lots in maize (*Zea mays*; Khajeh-Hosseini *et al.*, 2009), watermelon (*Citrullus lanatus* var. *lanatus*), melon (*Cucumis melo*) and cucumber (*Cucumis sativus*; Mavi *et al.*, 2010), pepper (*Capsicum annuum*; Demir *et al.*, 2008) and radish (*Raphanus sativus*; Mavi *et al.*, 2014). The timing of radicle emergence also influences seedling size; early germination is associated with larger seedlings due to the longer time available for seedling growth after radicle emergence (Matthews *et al.*, 2012).

The overall aim of this study was to answer the question: could seed quality be a factor in influencing the efficacy of *Rhinanthus minor*, from establishment to its effect on the host plants? To our knowledge this is the first experimental study that has examined the role of differences in seed quality. Ten seed lots of *Rhinanthus minor*, obtained from commercial sources, were used with the specific objectives to: (1) evaluate the field performance of commercially available seed lots of *Rhinanthus minor*; (2) characterise different aspects of the seed quality of the lots and examine their relationship with field performance; (3) investigate the effect of several seed lots of the parasitic plant, each with high

laboratory germination (percentage radicle emergence), on two individual host species.

5.2. Materials and Methods

5.2.1. Seed material

Ten samples from commercial seed lots of *Rhinanthus minor* were purchased from seven UK seed suppliers (British Wild Flower Plants, Ecoseeds, Emorsgate Seeds, Goren Farm, Growing Wild, MAS Seeds and Wildflower Shop). The seed samples will be referred to as seed lots in this paper (Table 5.1). We obtained seeds of two host plants of *R. minor* from Emorsgate Seeds (*Holcus lanatus*) and Scotia Seeds (*Lotus corniculatus*).

5.2.2. Germination tests

Eight replicates of 25 seeds per lot of *R. minor* were placed in 90 mm-diameter Petri dishes on germination paper (Whatman, GE Healthcare Life Sciences, UK) moistened with 2.5 ml distilled water. The Petri dishes were then placed in plastic bags to prevent water loss during the test and held in an incubator (LMS 250A, LMS Ltd, UK) at 5 °C in darkness. These conditions have previously been shown to break dormancy of *R. minor* (Marin *et al*., 2017). During incubation, plates were checked every seven days for germination and seeds were scored as germinated once radicle emergence was > 2 mm and the mean germination time (MGT) was calculated using the formula:

$$MGT = \Sigma(n_i \times t_i)/N$$

where n_i is the number of seeds that germinated (2 mm, radicle emergence) within consecutive intervals of time, t_i the time (days) between the beginning of the test and the end of a particular interval of measurements, and N the total number of seeds that germinated. At the completion of each germination test (up to 40 weeks), non-germinated seeds were cut-tested and classified as apparently fresh, and therefore viable, or dead.

5.2.3. Seedling emergence in the field

Seedling emergence was carried out at Scotia Seeds, Brechin, Scotland. Five replicates of 200 seeds from each lot were sown in randomly distributed 1 m long rows in the field (December 2014). Emergence counts were taken every week for 10 weeks, with the appearance of cotyledons on the surface used as the emergence criterion. Field emergence was expressed as a percentage of the number of seeds sown. Emergence in the field was also expressed as a percentage of germination in the laboratory, that is, as relative field emergence.

5.2.4. Embryo growth

The embryo growth was evaluated for the four lots of *R. minor* with germination (percentage radicle emergence) greater than 90% (lots 1, 2, 3 and 4; Table 5.1). Fifteen replicates of 25 seeds per lot were placed to germinate following the same methodology as described above. The embryo length and areas of the embryo and endosperm within the imbibed seeds were determined the following day. One seed from each replicate Petri dish for a total of 15 seeds per lot was subjected to a tetrazolium test to determine the viability of embryos by cutting along the edge of each seed and soaking it in 1% 2,3,5-triphenyl tetrazolium chloride solution at 30 °C for 22 hours (Marin *et al.*, 2017). Next, ten viable seeds from each lot were dissected and observations were made with a stereomicroscope (Leica MZ FLIII, Leica Microsystems, Germany) and images were generated with a microscope camera (Axiocam 506 color, Zeiss, Germany). Embryo length, embryo and endosperm area were measured on a median longitudinal section using the software ImageJ (ImageJ 1.46r, NIH, USA). The tetrazolium viability test and observations on embryo growth were then made every week on 15 seeds per lot until the majority of seeds had germinated.

5.2.5. Rhinanthus minor performance when grown with host species

R. minor seeds from lots 1, 2, 3 and 4 were placed onto 90 mm-diameter Petri dishes containing damp filter paper in an incubator at 5 °C as described above. Seed lots were sown sequentially according to their MGT in order to produce, at the same time, seeds in which the radicle had emerged. The seeds of the host

plants (*Holcus lanatus* and *Lotus corniculatus*) were germinated to radicle emergence on damp filter paper at 20 °C and transplanted to 9 x 9 x 14 cm (0.8 l) pots (one seed per pot), containing six parts sand and one part John Innes No. 2 compost. After one month of host plant growth, a germinated seed of *R. minor* was sown in each pot containing a host plant. Thus there was one host plant and one parasitic plant in each replicate pot. Twenty replicates were set up for each combination of *R. minor* and host species for lots 1 and 2, while 24 and 25 replicates were used for lots 3 and 4, respectively. In addition, 25 individuals of each host species (*H. lanatus* and *L. corniculatus*) were grown without the hemi-parasite to act as controls. A total of 228 pots was set up and plants were grown in a glasshouse at the James Hutton Institute Dundee from March to September 2016. The average daily temperature ranged between 16 and 21 °C. Pots were supplied with tap water *ad libitum* and treatments arranged randomly on benches.

Seedling establishment of *R. minor* was assessed when the cotyledons appeared. Plant height was then assessed every seven days following establishment and the percentage of plants that were flowering was assessed weekly from the time that the first flower was observed (after ten weeks). The *R. minor* plants were allowed to set seed and the seeds produced were collected and their germination and MGT were assessed as described above. When the parasitic plants began to show signs of senescence all plant materials (*R. minor* and host plants) were dried at 60 °C until a constant weight was measured and their total dry mass recorded.

5.2.6. Statistical analysis

All statistical analyses were performed using GenStat 17th edition (VSN International, Hemel Hempstead, UK). Significant differences between experimental groups were assessed with unpaired Student's t-test and One-way-ANOVA. Pairwise differences were tested using a *post hoc* Tukey's test. The significance of correlations was tested using Spearman’s rank correlation. Determination coefficient values and regression equations were determined to assess the prediction potential. Significance was evaluated in all cases at $P < 0.05$. Mean ± standard error of the mean (s.e.) are reported.

5.3. Results

The seed lots of *Rhinanthus minor* showed a wide range in the percentage of germination (Table 5.1). Throughout this paper, the criterion for germination is radicle emergence (RE). Final germination (radicle emergence) was greater than 90% in four seed lots (1, 2, 3 and 4). Four of the remaining lots had germinations ranging from 59 to 87% (5, 7, 8 and 9), with two lots (6 and 10) having germinations as low as 38 and 37%, respectively. The average length of the lag period from the beginning of imbibition to radicle emergence, measured as mean germination time (MGT), was however significantly different between the high germinating lots 1 – 4, ranging from 58 to 109 days (Table 5.1; Fig. 5.7). The final emergence of these lots in the field, also differed and ranged from 9 to 45%. The other six lots also showed differences in their MGT values which ranged from 63 and 94 days, with field emergence percentages in the range 3 – 27% (Table 5.1).

Table 5.1. Details of seed quality of ten lots of *Rhinanthus minor*: production year, germination (percentage radicle emergence), percentage of dead seeds and mean germination time (MGT) of eight replicates of 25 seeds at 5 °C and field emergence of five replicates of 200 seeds.

Lot no.	Production year	Germination (% RE)	Dead seeds (%)	Field emergence (%)	MGT (days) at 5 °C
1	2014	93 ± 2	5 ± 1	44 ± 4	58 ± 0
2	2014	93 ± 2	6 ± 2	45 ± 4	59 ± 0
3	2014	95 ± 2	2 ± 1	26 ± 2	89 ± 2
4	2013	91 ± 3	5 ± 2	9 ± 1	109 ± 10
5	2014	71 ± 3	18 ± 3	17 ± 2	72 ± 3
6	2014	38 ± 3	51 ± 5	3 ± 1	94 ± 5
7	2014	82 ± 3	17 ± 3	25 ± 4	63 ± 2
8	2014	59 ± 4	33 ± 3	7 ± 1	84 ± 3
9	Unknown	87 ± 2	11 ± 2	27 ± 3	63 ± 1
10	Unknown	37 ± 4	51 ± 5	4 ± 1	88 ± 3

Two factors influenced the emergence of the seed lots in the field. Firstly, field emergence was, not surprisingly, related to the laboratory germination, lots with poor germination due to the presence of dead seeds having low emergence

(Fig. 5.1A; Table 5.1). Secondly, the significant correlation (r^2 = 0.724) for all seed lots between MGT and the relative field emergence (Fig. 5.1B), which indicates the percentage of germinable seeds that emerged in the field, suggests that the average lag period for the germinable seeds affected the ability to emerge. Thus the longer the lag period before radicle emergence (RE, *i.e.* higher MGT) the lower was the field emergence. However, the same significant relationship was seen for two groups of seed lots, the first consisting of seed lots with germinations (RE) greater than 90% (r^2 = 0.997, lots 1 – 4; Fig. 5.1B; Table 5.1) and the second with lower germinations (r^2 = 0.991, lots 5 – 10; Fig. 5.1B; Table 5.1).

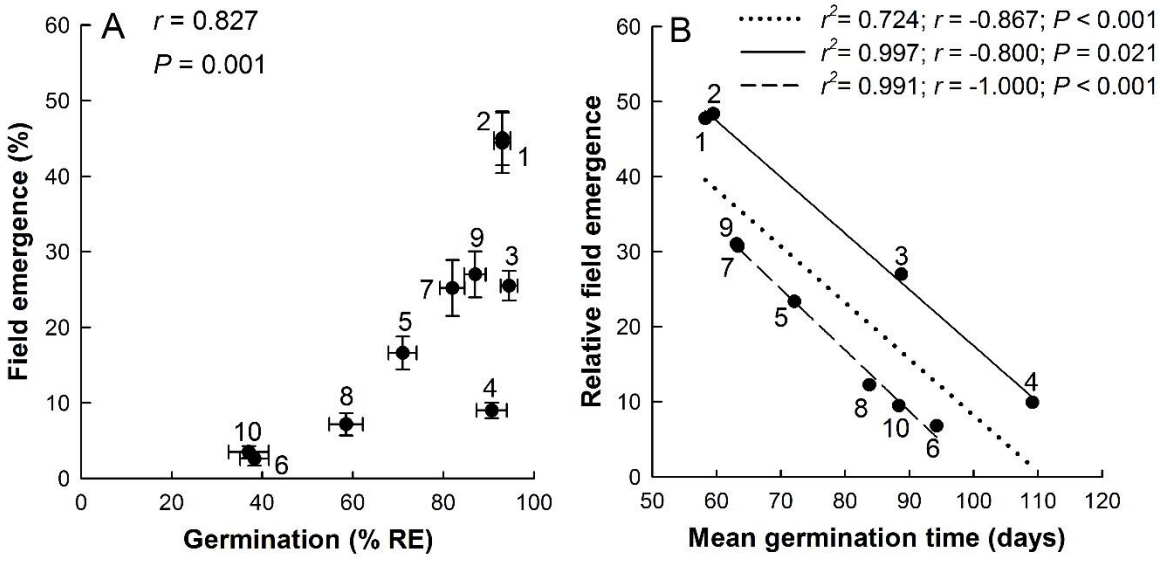

Figure 5.1. Relationship between germination (percentage radicle emergence) and field emergence of ten *Rhinanthus minor* seeds lots; means ± s.e. are reported (A). Relationship between mean germination time (MGT) and relative field emergence (dotted line: all lots, solid line: lots 1 – 4, dashed line: lots 5 – 10; B). Each point for relative emergence is the ratio between emergence (mean of five replicates of 200 seeds) and germination (mean of eight replicates of 25 seeds). Each point for MGT is a mean of eight replicates of 25 seeds. The number next to each point indicates the lot number. In addition to correlation coefficient, *r*, and corresponding *P*-value from Spearman's rank correlation analysis, the determination coefficient, r^2, is reported upon fitting with equation $y = y_0 + a*x$.

Comparisons of the length of the lag period (MGT) with final germination (% RE) revealed that the MGTs of eight seed lots were significantly correlated with their germination, thus indicating that lower seed quality lots (fewer

germinable seeds) are also characterized by a longer lag period (Fig. 5.2). However, lots 3 and 4 (germination > 90%) did not fit this correlation as their high MGT (indicative of a long lag period) was much greater than expected for seed lots with such a high germination (RE; Fig. 5.2). The MGT is based on counts of RE over time, therefore the frequency distributions of the percentage RE during germination might clarify why the two lots, 3 and 4, did not fit the general relationship shown by the other lots between MGT and final germination (Fig. 5.3). For the eight seed lots that fitted the correlation in Fig. 5.2, the frequency distribution clearly related to their final germination. Thus, uniform and rapid germination (RE) was found for lots 1 and 2 (high germination: 93%; Table 5.1; Fig. 5.3), followed by slightly later but uniform germination for lots 7 and 9 (germination: 82%, 87%; Table 5.1; Fig. 5.3). Lots 5 and 8 with lower germination (71%, 59%; Table 5.1; Fig. 5.3) showed an early peak in germination followed by some late germination. Germination was spread over a wide range in lots 6 and 10 which had even lower germinations (38%, 37%; Table 5.1; Fig. 5.3). In contrast lot 3, with high germination (95%) was characterized by slower germination than the other lots (1, 2) with high germination, beginning approximately five weeks later, even though germination was uniform. There were also a number of late germinating seeds. Lot 4, also with high germination (91%), had two peaks of radicle emergence, one at a similar time to lots 1 and 2, but also a second peak of germination 20 to 30 weeks later (Fig. 5.3).

Further work focused on the seed lots with high germination (lots 1, 2, 3 and 4) and examined the growth of the embryo and subsequent growth of both *R. minor* plants and two host plants. The growth of the embryo within the seed before germination occurred, under simulated winter conditions in the laboratory at 5 °C, revealed a correspondence between the timing of radicle emergence and embryo elongation (Fig. 5.4). Embryos grew from 1.89, 1.69, 1.69 and 1.39 mm to 2.81, 2.60, 2.43 and 2.44 mm, respectively for lots 1 – 4. Lots 1, 2 and 3 initially had significantly larger embryos, with embryo and endosperm ratio being 0.11 ± 0.01, 0.11 ± 0.01 and 0.12 ± 0.01, respectively. Lot 4 was characterized by a lower initial ratio of 0.08 ± 0.004 (Fig. 5.4). Embryos of the two rapidly germinating lots (lots 1 and 2; Table 5.1) occupied approximately 25% of the whole endosperm after 50 days on filter paper and there was no significant embryo

growth after this point prior to radicle emergence (Fig. 5.4). The majority of embryos from lots 3 and 4 reached similar values of embryo and endosperm ratio more slowly after 77 and 105 days, respectively.

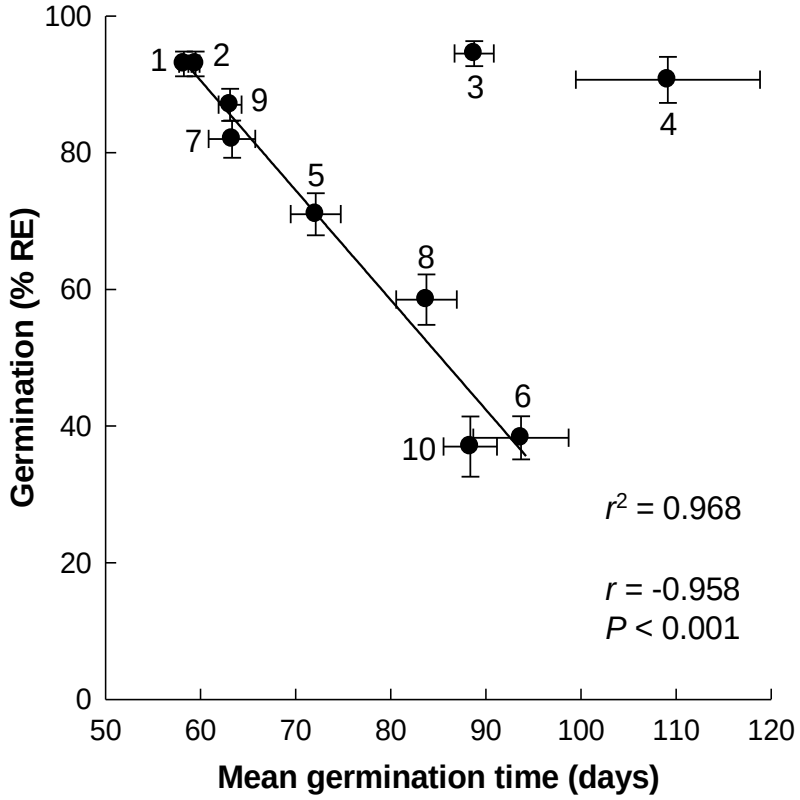

FIGURE 5.2. Relationship between mean germination time (MGT) and germination (percentage radicle emergence) of eight *Rhinanthus minor* seed lots; means ± s.e. are reported. Each point for radicle emergence and for MGT is a mean of eight replicates of 25 seeds. The number next to each point indicates the lot number. In addition to correlation coefficient, r, and corresponding P-value from Spearman's rank correlation analysis, the determination coefficient, r^2, is reported in the panel upon fitting with equation $y = y_0 + a*x$.

Differences were clearly identified in the development and growth of *R. minor* from the four seed lots when germinated seeds were transplanted to pots containing one host plant, either *Holcus lanatus* or *Lotus corniculatus*. Assessment of seedling mortality in the pots revealed losses of only 3 and 13% for lots 1, 2 and 3 while mortality reached 50% in lot 4 (Fig. 2.5A). Following seedling establishment, over 90% of the surviving plants from lots 1 and 2 flowered in the presence of *H. lanatus*, while slightly fewer plants flowered when

growing with *L. corniculatus*, 89 and 79%, respectively (Fig. 2.5B). However, lots 3 and 4 were characterized by similarly lower percentages of flowering plants, in the presence of *H. lanatus* (71%) and *L. corniculatus* (55%).

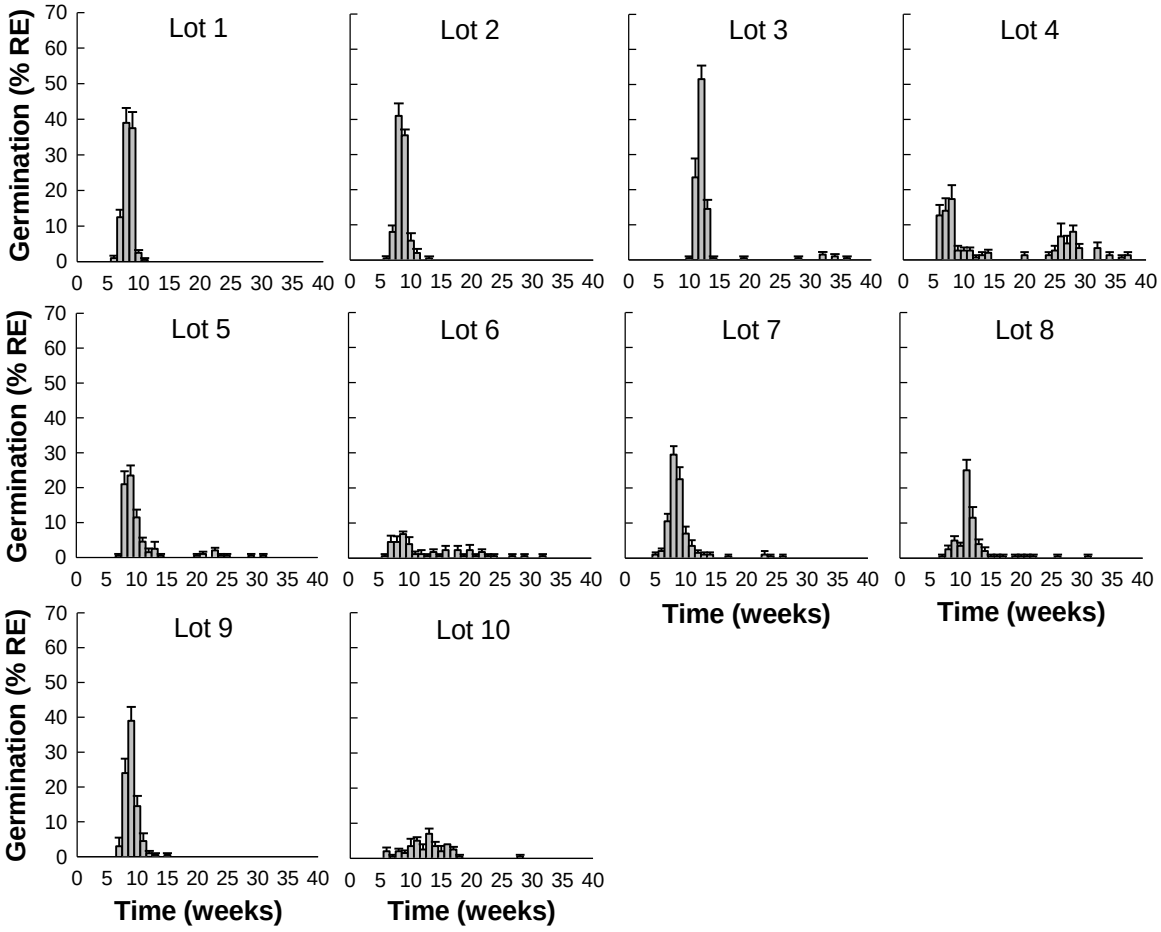

FIGURE 5.3. Frequency distributions of the germination (percentage radicle emergence) over time of the ten seed lots of *Rhinanthus minor*. Means are reported ± s.e.

There were significant differences in the plant height of the four lots of *R. minor*, measured at time of flowering (Fig. 5.5C), both in the presence of *H. lanatus* (P = 0.005) and *L. corniculatus* (P = 0.002), with the shortest plants recorded for the slowest germinating lot 4 (Fig. 5.5C). Significant differences in plant height (P = 0.002) between host species were observed only for lot 1, where plant height averaged 23 and 15 cm in the presence of *H. lanatus* and *L. corniculatus*, respectively (Fig. 5.5C). For the other three lots there was no significant difference (P > 0.05) in plant height on the basis of the host species. This trend was confirmed by the biomass measurements, although the large

within group variability reduced the significance of the differences between groups (Fig. 5.5D).

The two host species grown alongside *R. minor* were affected differently by the hemi-parasite. All four seed lots of *R. minor* resulted in a reduction in growth of the host species *H. lanatus* by an average 56% (Fig. 5.6). The growth of *L. corniculatus* was significantly ($P < 0.001$) reduced by lots 1, 2 and 3 leading to an average 51% reduction in its total biomass (Fig. 5.6). However, lot 4, with the least vigorous seed and seedling performance, did not result in a significant reduction of the host biomass (Fig. 5.6).

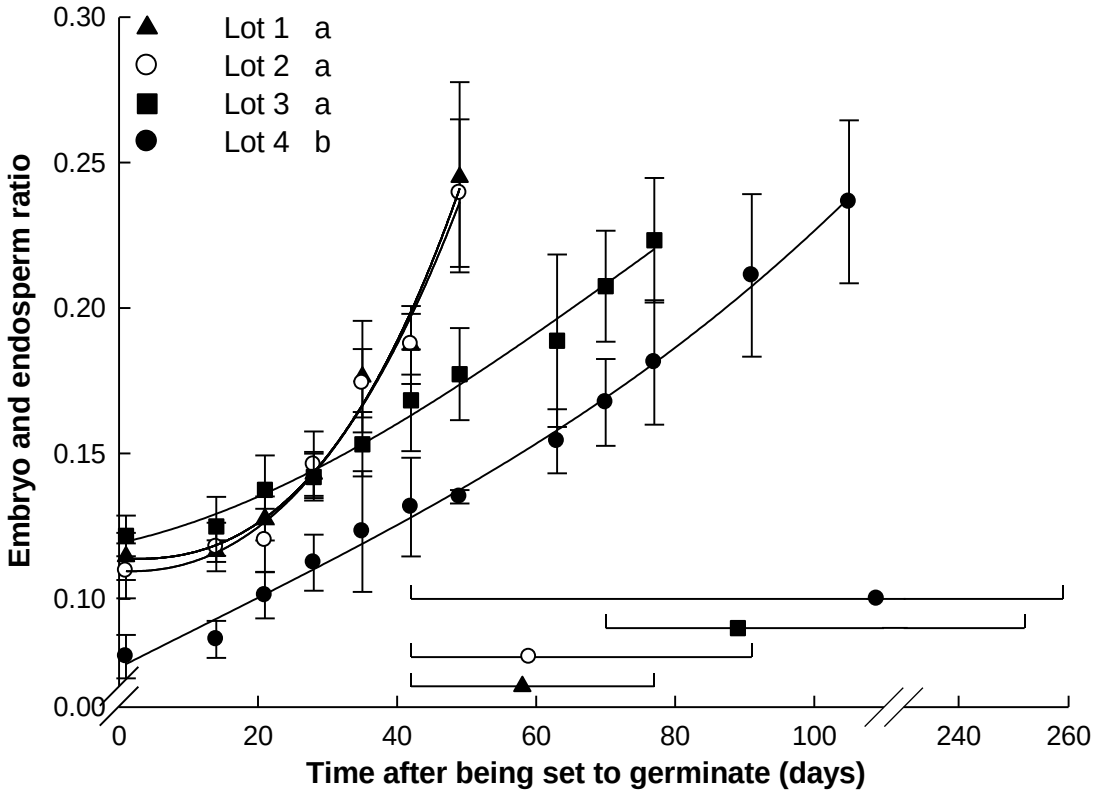

FIGURE 5.4. Embryo growth within the seed, expressed as embryo and endosperm ratio, in the laboratory at 5 °C, of the four seed lots of *Rhinanthus minor* with germination greater than 90%. Means are reported ± s.e. Identical letters indicate no significant differences between the initial embryo and endosperm ratio of the four seed lots as tested using one-way ANOVA followed by a post hoc Tukey's test. Regression curves are expressed by the following function: $y = y_0 + a*x + b*x^2 + c*x^3$. The horizontal bars indicate the start and end of the germination period for each lot, while the symbol in between indicates the mean germination time.

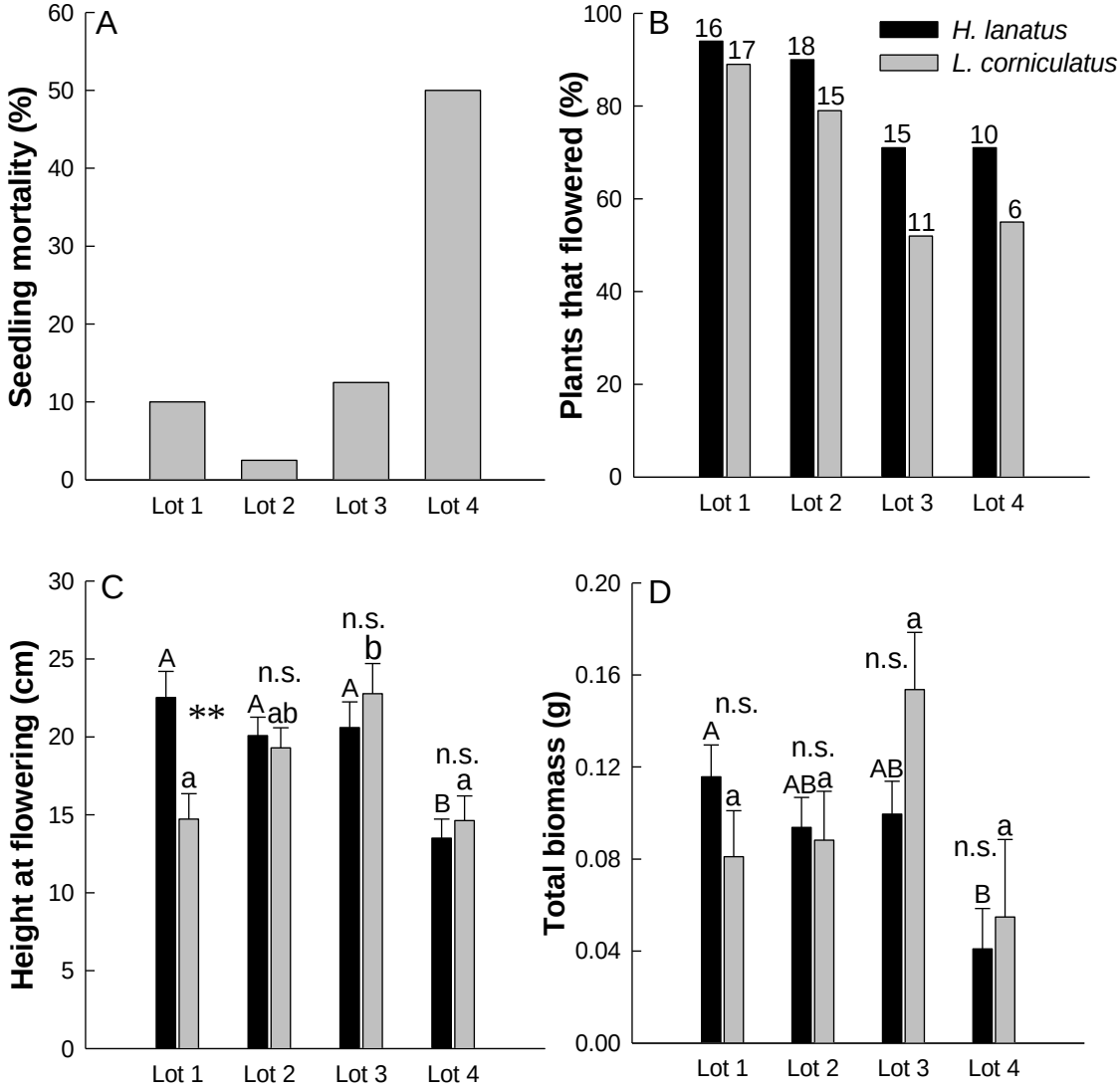

FIGURE 5.5. Percentage of seedling mortality after transplanting the germinated seeds of the four lots of *Rhinanthus minor* to pots containing the host species (A). Percentage of surviving *R. minor* plants that flowered (B) and height at time of flowering (C) for each combination of seed lot and host species. The number above each bar indicates the number of plants that flowered for each combination of seed lot and host species. Total biomass of *R. minor* plants that flowered, grown with either *Holcus lanatus* or *Lotus corniculatus*, for the four seed lots (D). Means are reported ± s.e. Identical letters indicate no significant differences as tested using one-way ANOVA followed by a post hoc Tukey's test. n.s. indicates the lack of significant differences in plant height within the same lot but between different host species. ** indicates a statistically significant difference at $P = 0.002$.

The mean germination time of a second generation of seeds was evaluated using seeds of *R. minor* collected from the plants grown in the glasshouse. A significant increase in MGT, indicative of an increase in the lag period to radicle emergence occurred in the second compared to the first generation for lots 1, 2 and 3 (Fig. 5.7). There was no significant difference in MGT between the generations of lot 4. As seen in the first generation, lots 3 and 4 germinated more slowly than lots 1 and 2 (Fig. 5.7). However, in contrast to the distribution of germination over time in the first generation (Fig. 5.3), the second generation of lots 3 and 4 showed more uniform and single peaks of germination (Fig. 5.8), although these were delayed compared to the more rapidly germinating lots 1 and 2.

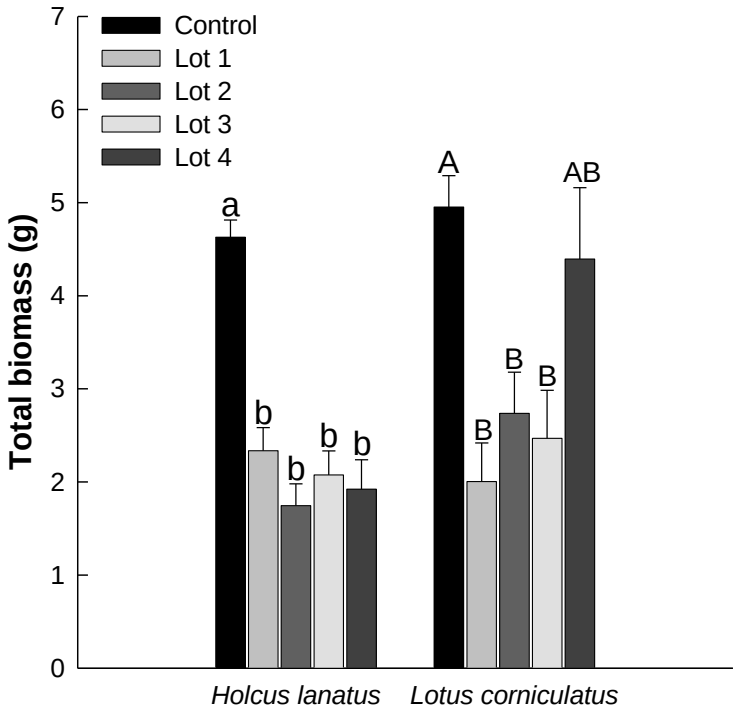

FIGURE 5.6. Total biomass (roots + shoots) of the host species *Holcus lanatus* and *Lotus corniculatus* grown without *Rhinanthus minor* (control) and in the presence of the parasitic plant from the four seed lots (only the pots containing a plant of *R. minor* that flowered were considered). Means are reported ± s.e. Identical letters indicate no significant differences as tested using one-way ANOVA followed by a post hoc Tukey's test.

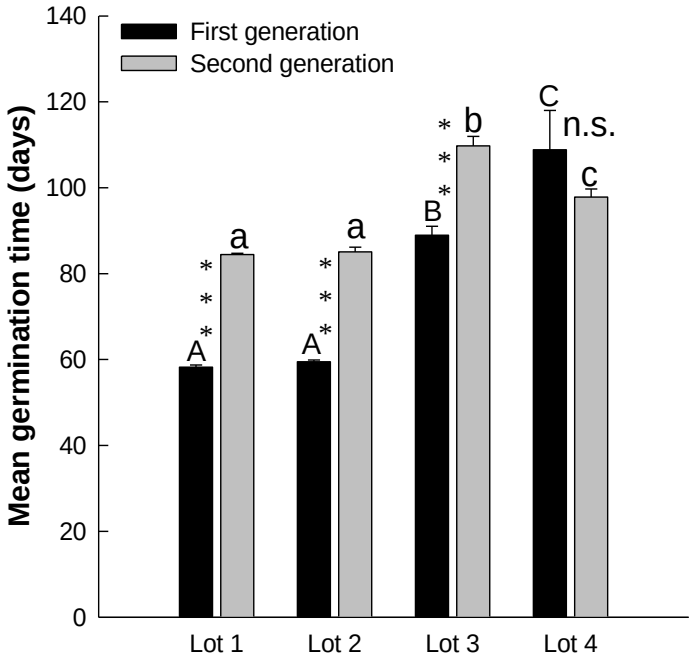

FIGURE 5.7. Mean germination time of the first and second generation for each lot of *Rhinanthus minor*. Means are reported ± s.e. Identical letters indicate no significant differences between lots as tested using one-way ANOVA followed by a post hoc Tukey's test. *** indicates a statistically significant difference at $P < 0.001$ between two generations of the same lot, while n.s. indicates the lack of significant differences.

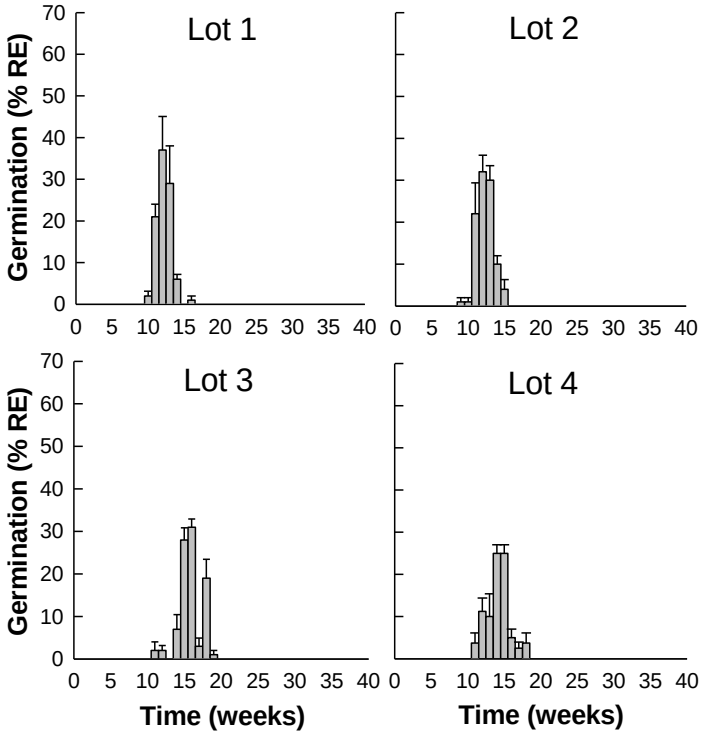

FIGURE 5.8. Frequency distributions of the germination (percentage radicle emergence) over time of the second generation for the four seed lots of *Rhinanthus minor*. Means are reported ± s.e.

5.4. Discussion

This study has illustrated that the quality of *Rhinanthus minor* seeds can influence their emergence, establishment and subsequent plant growth, as well as the effect of the hemi-parasite on the host species. Seed quality evaluation therefore appeared to be fundamental for predicting the impact of the hemi-parasite on community productivity and diversity. Two aspects of seed quality influenced the field emergence of seed lots of *R. minor,* the laboratory germination (% radicle emergence) and the mean germination time, which is indicative of seed vigour. The role of seed vigour was confirmed by the range in field emergence found for seed lots with uniformly high laboratory germination (> 90%), where a high MGT, that is, a long average lag period before radicle emergence, was associated with poor emergence. The differences in MGT were closely associated with the early embryo growth within the seed before radicle emergence. The longer lag period (lower vigour) was also associated with higher levels of seedling mortality and reduced plant vigour, in terms of plant height and biomass accumulation and was reflected in the parasitic ability of the seed lots, with the least vigorous seed lot having no impact on the biomass of its host, *Lotus corniculatus*.

The large variability in laboratory germination (radicle emergence; range 37 – 95%) among the ten seed lots of *R. minor*, was not due to dormancy as only a small percentage (1 – 12%) of non-germinated seeds were fresh (*i.e.* imbibed but not germinated) at the end of the germination test. Furthermore, previous work (Marin *et al*., 2017) has shown that germination at 5 °C breaks the dormancy of *R. minor* since the actual germination was similar to the viability assessed by tetrazolium staining which describes both germinable and dormant seeds. Not surprisingly, laboratory germination was indicative of the range in field emergence (3 – 45%) in our study (Fig. 5.1A), as suggested by Forcella *et al.* (2000). This supports the observations (Müller *et al.*, 2011; Mondoni *et al.*, 2015) that laboratory germination can overestimate field emergence in a range of native species, as it does in crop species (Matthews and Powell, 2011; Powell and Matthews, 2012). Indeed, environmental conditions such as moisture or thickness of the litter layer are known to influence the emergence of *R. minor* seedlings in the first year after sowing (Ameloot *et al.*, 2005; Mudrák *et al.*, 2016). However,

even when the differences in germination were accounted for by calculating the relative field emergence (the percentage of germinable seeds that emerged), the emergence ranged from 7 to 48% (Fig. 5.1B). This suggested that a further factor also influenced emergence.

The importance of seed vigour in determining the emergence of seed lots of *R. minor* was revealed by the linear correlation ($P < 0.001$) between relative field emergence and MGT of all seed lots (Fig. 5.1B). Thus seed lots with high MGT, that is, a long lag period before radicle emergence, also showed poor emergence compared to lots with a short lag period (low MGT). Interestingly, two different groups of seed lots showed the same significant relationship between MGT and relative field emergence (Fig. 5.1B). The first group included only lots with equally high laboratory germination (> 90%), while the second group included seed lots that germinated to a lower percentage (Fig. 5.1B). The differences in the emergence of the two groups may be attributable to the extent to which the seeds that germinated to the stage of radicle emergence were able to produce normal or abnormal seedlings. Abnormal seedlings, which in crop species are characteristic of seed lots with lower radicle emergence, are recognised as being less likely to emerge in the field and are hence not included in the germination percentage of commercial seed lots of crop species (ISTA, 2017). The four lots with high (above 90%) radicle emergence would be more likely to go on to produce mainly normal seedlings which would emerge successfully. In contrast the lots with lower radicle emergence would produce a greater proportion of abnormal seedlings that would fail to emerge, as suggested for maize by Khajeh-Hosseini *et al.* (2010).

Differences in the mean germination time have been related to the physiological age of seed, with older seed showing a longer lag period (high MGT) and producing lower levels of field emergence (Powell and Matthews, 2012), as seen here for lot no 4 (Table 5.1). This effect of natural ageing on MGT has been seen in cucurbit species (Mavi *et al.*, 2010), maize (Matthews and Khajeh-Hosseini, 2006), pepper (Demir *et al.*, 2008), oil seed rape (*Brassica napus*; Larsen *et al*., 1998) and radish (Mavi *et al.*, 2014). In her work on unaged and aged rye (*Secale cereale*) embryos, Osborne (1983) suggested that any damage to DNA is repaired during the lag period and that this is a necessary step

for the subsequent events leading to germination. There is also evidence of DNA repair as an early event during the lag period in other species, including maize (Vasquez-Ramos and Sanchez, 2003). It has therefore been suggested that aged, low vigour seeds require a longer lag period, and hence have a higher MGT, in order to repair the deterioration that has occurred during ageing (Matthews and Khajeh Hosseini, 2007; Matthews and Powell, 2011; Matthews *et al*., 2012; Powell and Matthews, 2012). This hypothesis is supported by the observation that a short period of imbibition followed by dehydration to the original moisture content reduced the length of the lag period (decreased MGT) needed before radicle emergence of maize (Matthews and Khajeh-Hosseini, 2007) and radish seeds (Mavi *et al*., 2016). In addition, seed priming, a well-established method of increasing the earliness of germination, in which seeds are partially hydrated in aerobic conditions, has been in part attributed to metabolic repair (Thornton and Powell, 1992; Ashraf and Bray, 1993; Thornton *et al*. 1993).

The differences in the lag period (MGT) of the four high germinating seed lots were related to observations of the elongation of the embryo before germination had occurred (Fig. 5.4). Slow and asynchronous germination was a feature of seeds with a small initial embryo and endosperm ratio as well as a slow average embryo elongation within the seed prior to germination. Cotyledons and radicle were clearly distinguishable within all seed lots at time of first measurement, as reported by Westbury (2004). However, embryos of *R. minor* continued to develop on damp filter paper and grew from an average 1.67 to 2.57 mm, occupying initially the 10% of the endosperm, which increased to 24% prior to germination (Fig. 5.4).

The differences in vigour revealed by the MGT continued to be evident after emergence, with lot no 4, which had the highest MGT showing 50% seedling mortality compared to the higher vigour lots (maximum 13% mortality, Fig. 5.5A). Furthermore, the vigour of plants from lot 4 was lower, in terms of plant height and biomass, even in the presence of *H. lanatus* or *L. corniculatus*, species that are considered to be suitable hosts (Rowntree *et al.*, 2014). Reduced seedling size has also been shown in low vigour seed lots of crop species such as in maize (TeKrony *et al*., 1989), leek (*Allium porrum*), cauliflower (*Brassica oleracea*) and onion (*Allium cepa*; Finch-Savage, 1986) and can be explained in terms of the

time of radicle emergence (Matthews and Khajeh Hosseini, 2006). Thus, at any one time, the low vigour lots that are slow to radicle emergence would have had a shorter period for seedling growth compared to the rapidly germinating high vigour seed lots. Furthermore, Larsen *et al*. (1998) found that MGT was highly correlated with time to emergence, plant growth and eventually yield in field trials for oil seed rape.

Differences in host biomass in the presence of *R. minor* revealed differences in the parasitic effects of the seed lots. Three lots significantly reduced the biomass of both the legume and the grass hosts, but the low vigour lot 4 did not reduce the growth of infected *L. corniculatus* hosts when compared with uninfected controls. This is an important and novel observation indicating that the seed vigour of ecosystems engineers, such as *R. minor*, can influence their effect on community structure. Rowntree *et al.* (2011) has shown, in a *Rhinanthus*–barley (*Hordeum vulgare*) system, that the outcome of infection for both host and parasite, in terms of host fitness, host tolerance, parasite virulence and transmission ability, depends on genetic variation within both partners. Here we add a further factor that can influence the efficacy of *R. minor*, highlighting that seed quality, in particular the mean germination time, is indicative of field performance of the hemi-parasite and its effect on the host plant. Moreover, this infers that seed source and quality is a factor that needs to be considered within future studies on the ecology and application of this species. This is in contrast to previous work in which either single seed lots of unknown quality, or seeds collected from the wild, have been used. Seed origin, germination capacity and timing of radicle emergence will all influence the efficacy of *R. minor*, from establishment to its effect on the host plants.

Assessment of the seed quality of seed produced by the four highly germinating seed lots revealed similar differences in the MGT for seeds of the second generation of seed to those seen in the first, suggesting that MGT of the four seed sources might have been genetically determined. Uniform and rapid germination was found for two lots, while this was delayed by approximately five weeks for the other two lots. The slower but uniform germination of the latter may be related to different degrees of dormancy, due to genetic variation between populations or ecotypes (Ter Borg, 2005; Rowntree *et al.*, 2011). Indeed, slight

variation of stratification temperatures may have strong effects on the germination rate of *R. minor* seeds (Ter Borg, 2005) thereby determining plant establishment and the outcome of infection by the hemi-parasite. However, Ter Borg (2005) pointed out that seed quality, including seed age, might explain part of the results.

In the present work it is possible, but unlikely, that differences in ecotype contributed to the differences observed. The seed lots originated from sources ranging from south-east and south-west England, to as far north as Cumbria and Scotland. If local adaptations contributed to emergence, the Scottish seed lot (lot 4) would be expected to be best adapted and hence respond best to the environmental conditions. In fact this had the poorest emergence of the four more highly germinable seed lots. Furthermore, all the observations of field emergence, radicle emergence, MGT and seedling growth are characteristics that reflect differences in seed ageing that have been seen in crop species (Matthews and Khajeh-Hosseini, 2007; Powell and Matthews, 2012).

Summarizing, this study suggests that seed quality can affect plant performance of *R. minor* as a result of germination capacity and seed vigour, playing an important role in community productivity and diversity. Substantial differences in germination (percentage radicle emergence) and MGT may be found amongst seed lots from different seed sources and influence plant productivity and its parasitic ability. The study also found that elongation and growth of the embryo of *R. minor* continued from dispersal to radicle emergence in damp conditions at 5 °C, filling the existing gap in the embryo anatomy of this species. A possible genetic variation was found in seed dormancy and/or optimal germination conditions. However, the impact that genetic variation of both host and parasite has on natural host–*Rhinanthus* communities remains to be investigated.

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Chapter 6

Conclusions and practical implications

Conclusions and practical implications

This research project has provided new information regarding the seed quality of wild herbaceous species in the context of commercial production, with important practical implications. It has shown that the conditions experienced by native plants in cultivation can be optimised in terms of plant productivity, without implications for seed quality or germination requirements. Subsequently, the evaluation of the germination capacity of individual seed lots from different suppliers provided evidence of quality problems in the European native seed market. Methods to assess seed quality were therefore developed to help avoid future restoration failure due to poor seed quality. The tetrazolium test provided a method for rapid identification of low quality lots in just two days in all tested species. Measurements of solute leakage from seeds highlighted the potential of using electrical conductivity to predict the germination of seed lots from native species with non-endospermic seed. Finally, this study has shown that seed quality can have a fundamental role in the establishment and growth of one native species, the hemi-parasite *Rhinanthus minor*. Work on *R. minor* illustrated that reduced seed germination and vigour result in the failure to emerge and establish plants that will contribute to the restoration or diversification of a plant community.

The role exerted by the maternal environment on the quality of the seed subsequently produced was investigated in *Primula vulgaris*. Differences in light availability during cultivation affected plant performance, with increased plant competitiveness and seed yield in more shaded conditions. However, seeds matured under different maternal environments did not differ in the ability to germinate, or show different requirements for germination. This has significance for the supply of native species, which will increasingly rely on commercially produced seed and less on seed sourcing directly from the wild. The development of cultivation protocols is therefore fundamental to facilitate seed harvesting, maximise seed yield and avoid highly competitive weeds that may over-compete low-growing crops as often seen in single species plantations. The present research has shown that for shade-tolerant species, such as *P. vulgaris*, successful plant cultivation can be achieved under artificial shade or as woodland crops. The lack of environmentally induced maternal effects would allow the use

of the seed subsequently produced to re-vegetate areas characterized by conditions different from those experienced by the mother plants in the field. On the other hand, the positive correlation found between final germination and light quality in *P. vulgaris* suggests potential for seedling establishment mainly in open areas or under a vegetative shade only in the presence of canopy gaps providing guidelines for the establishment of new understory species in woodland restoration.

Comparison of the seed quality of 113 seed lots that were being sold on the European native seed market revealed great variability in the germination capacity of individual seed lots from different suppliers, both with and without dormancy-breaking treatments. These observations illustrate the need for regular quality assessments as well as the need for native seed quality data to be made widely available and possibly the introduction of a voluntary certification scheme. This would require the development of quick and reliable methods for quality evaluation in native species. This study developed and tested a tetrazolium staining (TZ) testing protocol for native species. This enabled the rapid evaluation of seed quality in native species, with a clear prediction of germination achieved in only two days, regardless of the dormancy status of the seeds. Furthermore, a common pattern was found across species and families suggesting that the TZ test could be applied to native species in general for routine evaluation of their germination. Evidence from crop species pointed to another potentially applicable test to native species, the electrical conductivity (EC) test. The assessment of solute leakage from seeds using EC was related to final germination in four out of the seven tested species, all four being characterised by a non-endospermous seed. In particular, the test strongly predicted germination in one species (*Cyanus segetum*) that had the largest embryo. These observations suggest that embryo size and seed structure will determine the potential use of the EC test in other species in the future.

Ultimately, the availability of high quality seed is fundamental to ensure planting success and population establishment. In this study, both the timing of germination and final germination of *Rhinanthus minor* seeds influenced their emergence, establishment and subsequent plant growth, as well as the effect of the hemi-parasite on the host species. These findings could be broadened to

species other than *R. minor* including those species that are regarded as playing a key role in the establishment of new plant communities. Seed quality will be a key factor to consider when predicting the impact of a species on community composition and diversity. Furthermore, this infers that seed source and quality need to be given greater attention within future studies on the ecology and application of native species. Seed origin, germination capacity and timing of radicle emergence will all influence the successful use of these species within restoration projects.

In the future, the success of ecological restoration will be enhanced by the availability of high-quality native seed, particularly in view of the increasing targets for restoration and the limited seed availability. Large-scale restoration will require more commercial seed production and further advancement in native seed research. Future studies should therefore focus on the potential risk that species may adapt to a seed-production environment resulting in artificial selection for specific traits during cultivation. Furthermore, all aspects related to seed based restoration and the technical knowledge associated with seed quality will need to become widely available to native seed producers, reinforcing the connection between industry and academia. The outcome will be a functioning network of academic and industry specialists able to produce and use European native seed effectively and efficiently.

Appendix I

Native seed supply and the restoration species pool

The work presented in this appendix has been published:

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LETTER

Native Seed Supply and the Restoration Species Pool

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Abstract

Globally, annual expenditure on ecological restoration of degraded areas for habitat improvement and biodiversity conservation is approximately $18bn. Seed farming of native plant species is crucial to meet restoration goals, but may be stymied by the disconnection of academic research in seed science and the lack of effective policies that regulate native seed production/supply. To illustrate this problem, we identified 1,122 plant species important for European grasslands of conservation concern and found that only 32% have both fundamental seed germination data available and can be purchased as seed. The *"restoration species pool,"* or set of species available in practice, acts as a significant biodiversity selection filter for species use in restoration projects. For improvement, we propose: (1) substantial expansion of research and development on native seed quality, viability, and production; (2) open-source knowledge transfer between sectors; and (3) creation of supportive policy intended to stimulate demand for biodiverse seed.

Introduction

One-tenth of global wilderness has been destroyed in the last two decades (Pennisi 2016), and two-thirds of terrestrial environments are officially classed as degraded (Merritt & Dixon 2011). Ecological restoration (ER) accelerates the recovery of a degraded ecosystem with respect to health, integrity, and sustainability (SER 2004), and is recognized as a key complementary action for habitat conservation. Current global *ER* targets aim to restore 150 million ha or 15% of degraded ecosystems by 2020 (Menz *et al.* 2013). The estimated $18bn/year restoration cost is far exceeded by the potential global ecosystem service benefits of $85bn annually (Menz *et al.* 2013). Critical to success is the urgent need for access to high-quality seed through the farming of native species, as part of a range of flexible strategies to improve *ER* (Broadhurst *et al.* 2016).

Several large-scale *ER* initiatives are underway globally, such as the Australian Gondwana Link (Merritt & Dixon 2011), the Bureau of Land Management U.S. initiatives (Oldfield & Olwell 2015), the African Great Green Wall (Sacande & Berrahmouni 2016), and the European Union (EU) Natura 2000 (European Commission 1992). Seed-based plant conservation and use strategies (Merritt & Dixon 2011; Royal Botanic Gardens Kew 2015), seed-based research (Jiménez-Alfaro *et al.* 2016), and seed supply all play critical roles in successful ER. However, native seed sourcing, collection, production, and storage is more challenging than for agricultural species (Bischoff *et al.*

2008; Broadhurst *et al.* 2008) for which cultivars have been bred to be stable, uniform, and distinct (European Commission 1966).

ER depends on selecting appropriate species to cope with abiotic and biotic characteristics of degraded habitats. In ecological communities, scientists describe the species pool as the set of species that potentially occur at a site (Zobel 1992). The conditions limiting or facilitating species assembly will determine successional and recovering legacies of a system, including responses following *ER* (Temperton *et al.* 2004). Hand-collecting seed in large quantities from a broad range of species is unrealistic for most *ER* projects and wild populations risk depletion. Often the material used is restricted to that available from commercial or institutional seed suppliers. The "*restoration species pool*" ("*RSP*"), or pool of species available from these seed suppliers, thus imposes a critical biodiversity filter in *ER* projects. Where native supply lacks, easily available agronomic or horticultural seeds are used as a substitute, which is ecologically unacceptable. A *RSP* of native species, which has been systematically sourced between and within populations and species distribution ranges, is necessary for the support of genetic diversity in seed supplies and restored ecosystems (Hoban & Schlarbaum 2014).

Seed yields and germination of wild species can be naturally low and variable (Fenner 2000), and while cropping of native species can facilitate controlled production, some seed ecological traits (Fenner & Thompson 2005) can determine obstacles to harvesting. Not all wild species are candidates for commercial production as variation in seed morphological traits necessitates the use of appropriate harvesting and conditioning equipment, the costs of which can be very high if a large number of species are being produced. Proper seed management from collection to postconditioning storage is essential to maintain seed viability, which is variable between suppliers and can be very low (Marin *et al.* 2017). These challenges require collaborative efforts between seed suppliers and researchers to fully realize the potential of providing native farmed seeds for ER. This encompasses research on seed germination, dormancy (a process that regulates germination so that plants emerge under environmental conditions favourable for seedling establishment; Table S1), seed traits relevant for *ER* (Jiménez-Alfaro *et al.* 2016), and other bottlenecks that can be encountered such as adaptations for cultivation or genetic diversity maintenance (Chivers *et al.* 2016). However, research findings are rarely accessible to public stakeholders involved in ER.

Here, we assess the potential of the *RSP* to meet conservation needs in European grasslands, which are priority habitats as detailed in European policies on nature conservation. Human-induced habitat loss has impacted grassland biomes to the greatest rate and extent, largely due to agricultural conversion and the lack of conservation protections (Hoekstra *et al.* 2005). This neglect is in stark contrast to the biodiversity value of temperate grassland habitats, which across continental Europe are global biodiversity hot spots (Wilson *et al.* 2012). Using European grasslands of conservation concern as a case study, we analyze how many species have both detailed seed quality data and commercial seed lots available across taxa and across three species groups of relevance to European policies on *ER*. Addressing the availability of seed and related scientific information is important for the design of effective policy, research agendas, the foci of commercial seed suppliers, and reducing the risk of falling short in reinstating functional ecosystems in *ER* (Menz *et al.* 2013).

Methods

Study systems and target species

The European initiative Natura 2000 aims to establish a network of diverse, representative high-quality protected habitats of conservation concern, much of which will require intensive *ER* (European Commission 1992). Our study is focused on six major temperate grassland habitat types of conservation concern in Europe: lowland meadows (Natura 2000 number: 6510); high altitude hay meadows (6520); dry grasslands (6210); species rich *Nardus* grasslands (6230); calcareous alpine grasslands (6170); and acidic alpine grasslands (6150).

We created a database of 1,122 target species with potential interest for *ER* within these habitats, regulated by EU legislation that affects strategies of seed quality and use (Table S2). This includes 116 *protected* species subjected to legal protection, in most cases endangered or narrow endemic species; 929 *indicator* species, which are indirectly protected when occurring in protected habitats but unregulated in seed production; and 77 *fodder* species controlled for quality as domestic stock feed (European Commission 1966; 2014), as well as for preservation of genetic diversity (European Commission 2010; Table 1).

To assess the availability of seed quality data, we collected trait information on germination temperature and dormancy type of the target species available from the *Seed Information Database* (Royal Botanic Gardens Kew 2008), and the most recent review of seed germination studies (Baskin & Baskin 2014). As these are the main traits related to the germinability of a seed lot, we assume that having this information implies a minimum contribution of the scientific community for a given

Table 1 Relevant legislation details related to each target species group

Species group	Description	Legislation	Impact
Protected species ($N = 116$)	Includes species of conservation concern, in most cases endangered or narrow endemics, listed by name in relevant policy, and occurring in focus habitats.	Specific species for which member states must protect and conserve when found to occur under Annex II & IV of the EU policy on Conservation of Natural Habitats Wild Fauna and Flora (European Commission 1992).	Species seed cannot be collected without a rigorous permit process.
Indicator species ($N = 929$)	Species that are diagnostic or dominant for any of the selected habitats at the continental scale according to Schaminée *et al.* (2016) and vegetation ecology literature (Georg Grabherr & Mucina 1993).	These species are indirectly conserved in Annex II as reflected in the designation of special protected areas for the habitats in that they occur under the EU policy on Conservation of Natural Habitats Wild Fauna and Flora (European Commission 1992).	Species are of interest for use in restoration and have no direct EU policy restrictions on their collection, reproduction, or use but may have local regulations.
Fodder species ($N = 77$)	Grass and legume species used for animal forage, also considered valuable for preservation of the natural environment and conservation of genetic resources in grasslands listed by name under relevant policies.	Specific species and genera important for domestic stock and grazing (European Commission 1966, 2010, 2014).	Controlled for quality including high purity standards and minimum germination thresholds in EU Commission Directive 1966. Expanded in Directive (2010) to include harvest method, seed weight, quantity, region of origin, source area (collection site and multiplication), habitat type, and year of collection. Native seed production cannot exceed 5% of the total commercial cultivar production market in their country.

N = number of species in each group.

species. A systematic online search was conducted from November 2014 to May 2016, and the lists of species available commercially as seed were downloaded, or requested to seed suppliers. As there are multiple seed sources in some countries, the supplier providing the highest number of target species was selected since the inclusion of smaller companies did not influence the total number of available species. This resulted in seed availability lists from 17 seed suppliers across 17 countries (Table S3). Species names were verified against *The Plant List* (Missouri Botanical Gardens, Royal Botanic Gardens Kew 2013). Possible limitations of these data are that species reported as available may be an overestimate as lists may be outdated, inaccurate, or in some cases represent cultivars rather than native species, particularly in the *fodder* group. Nonetheless, the list is an accurate representation of the current state of native seed acquisition in Europe. We use the term *supplier* instead of *producer* because in the majority of cases, seed is reproduced in a native seed farm or orchard, but in some cases seed may be hand-collected.

Analyses

Data were collected as binomial variables. To assess *Germination Data Availability (GDA)*, each species was assigned as data being available (1) or not (0). Similarly, species were either *Commercially Available (CA)* (1) or not (0).

The proportions (%) of species with *CA* and with *GDA* were calculated for each plant family represented in the target species list to elucidate taxonomic representation as a surrogate of phylogenetic variation. A Generalized Linear Model (*GLM*) was fitted to assess the variation of *CA* as a function of *GDA* and species groups. The *GLM* was computed with binomial error distribution and logit link function in order to assess the influence of policy groups and *GDA* (explanatory variables) on *CA* (response variable; $CA \sim GDA +$ policy group). All analyses were performed in *R Statistical Computing Language and Platform version 3.2.2* (R Core Development Team 2016), and Figures created in the package *ggplot2* (Wickham 2009) and *yarrr* (Phillips 2016). The package *Effects* (Fox 2003) was used to create probability estimates of *CA* based on each variable

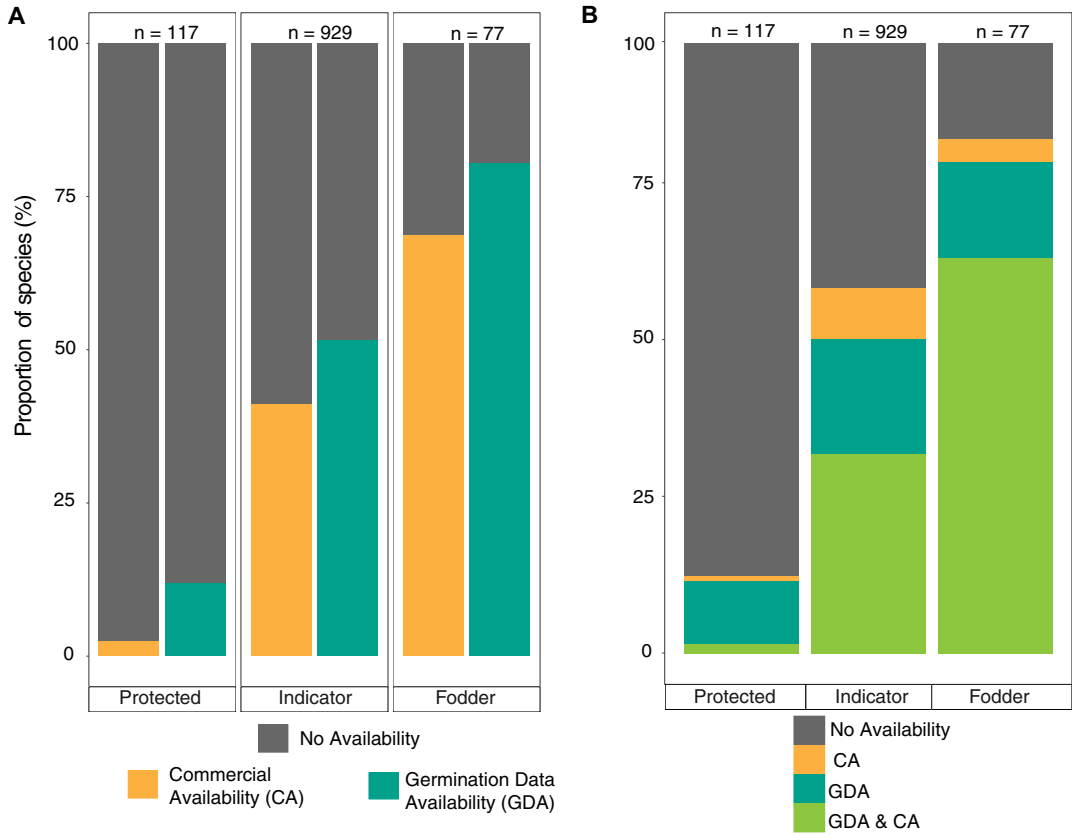

Figure 1 (A) Proportion (%) of species that are commercially available (*CA*) and with germination data availability (*GDA*) (B) proportion (%) of species that are commercially available (*CA*) with germination data availability (*GDA*), and with the combination of *CA* + *GDA*.
N: number of species represented within each group.

and package *PMCMR* for post hoc pairwise Kruskal-Wallis tests (Pohlert 2014).

Results

The 1,122 target species with potential interest for *ER* within European grassland habitats are spread across 59 plant families, with highest representation in Compositae (146 species) and with the top 5 and 10 families comprising 43% and 62% of the species list, respectively. Information on *GDA* and *CA* alone extended to 49% (i.e., 556) and 39% (i.e., 439) of target species, respectively (Figure 1A). Information for both seed *GDA* and *CA* details are available for only 32% (i.e., 358) of species on the target list (Figure 1B). Supplied seed is not available across all suppliers (Figure S1), although *indicator* and *fodder* species with *GDA* are available across a higher proportion of suppliers than those without *GDA* and with *protected* status (Kruskal-Wallis $s^2 = 338.81$, $P \leq 0.001$; Tables S2 and S4).

The majority of taxonomic families completely lacking *GDA* are also completely lacking *CA*, although the sample size is small in these cases (Figure 2, Table S5). The vast majority of families with large sample sizes have ~50% *GDA* and *CA*. Within this case study, there are seven families, spanning nine genera and 15 species, for which germination data are unknown. Twelve families (20% of total) lie within the lower quartile of *CA*, covering 158 species (14% of total).

Strong predictive patterns based on the *GLM* are exhibited for the estimate of *CA* of target species across all variables (Figure 3, Table 2). The model predicts that *protected* species have a 0.04 probability of being *CA*, *indicator* species 0.37 ($P < 0.001$), and *fodder* species 0.54 ($P < 0.001$; Figure 3A, Table 2). Species with no *GDA* have 0.13 probability of being *CA*, and species with *GDA* have a 0.58 probability of being *CA* overall ($P < 0.001$; Figure 3B). The combination of predictors (Figure 3B) provides a further level of outcomes. *Protected* species for which there is no *GDA* have 0.01 probability of *CA*; this probability increases to 0.11 when there is *GDA*. Comparable values for *indicator* species without and with *GDA* are 0.17 and 0.64, respectively; and 0.29 and 0.78 probability, respectively, for *fodder* species.

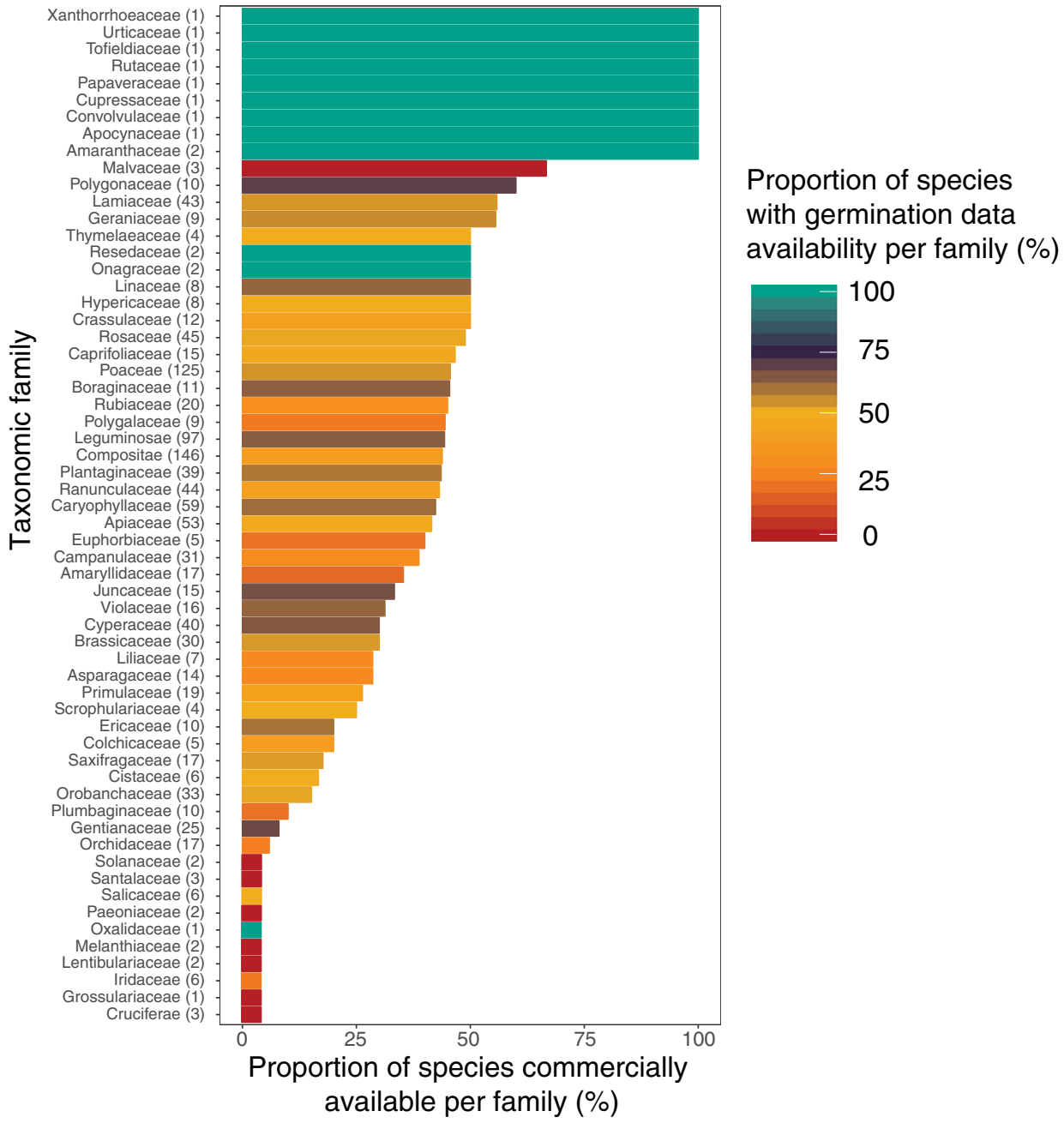

Figure 2 Bars show the proportion (%) of species per taxonomic family that have seed which has *commercial availability*. The degree and proportion of *germination data availability* is represented by the color scale according to the Seed Information Database (*Royal Botanic Gardens*, Kew 2008) and Baskin & Baskin (2014). The numbers in brackets next to each family name represents how many species are included in the data set from that given family.

Discussion

The *Restoration Species Pool in European grasslands*

To our knowledge, no studies have investigated the availability of commercial seed and related germination data for native seed in a large-scale case study. In Europe, the relatively high availability of native seeds for *fodder* species demonstrates that commercial availability of native seed is subject to economic demand and a long-standing regulatory framework. This framework follows an agricultural model meant for animal feed rather than Ecological Restoration (ER) (European Commission 1966), yet is recognized for *ER* use (European

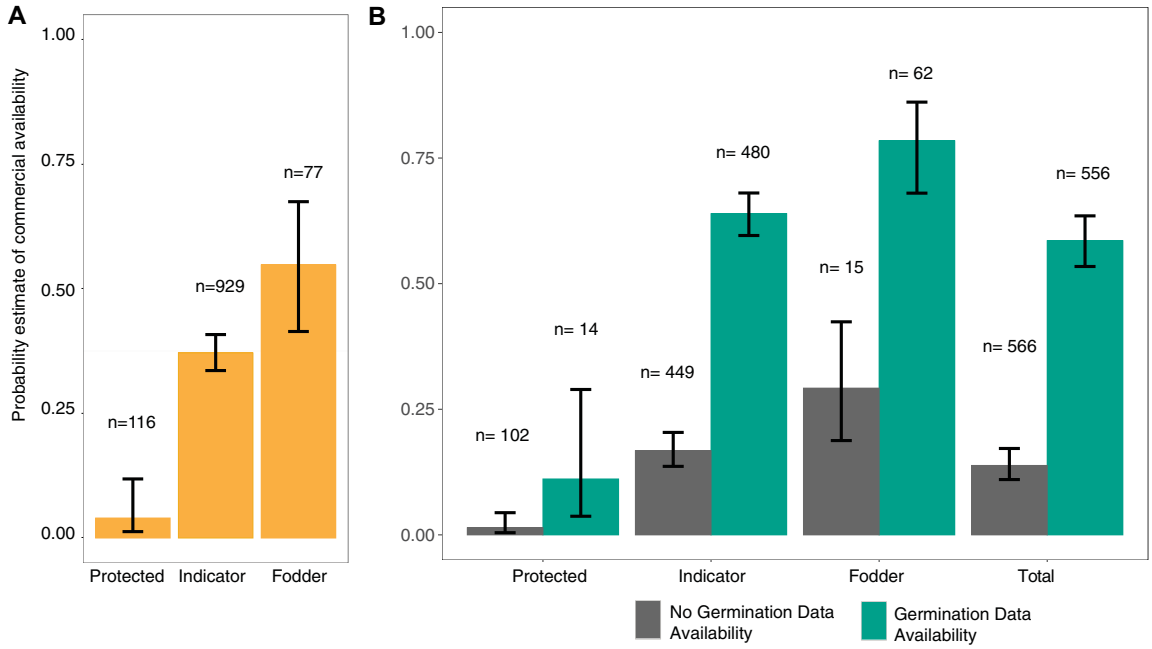

Figure 3 Predicted effect plots showing the *commercial availability* of species grouped per species category. Probability was estimated using *GLM* (binomial error, logit link) fitted to the commercial production data of each species (*commercial availability* ~ *germination data availability* + species group). The same model was used to fit each group, and results were grouped based on: (A) species groups (*protected*, *indicator*, *fodder*) (B) species group + *germination data availability*.
Bars represent the probability that a given group of species is commercially available. Brackets represent the upper and lower limits of that estimate.
N = number of species represented by each prediction.

Table 2 Generalized linear model (binomial error, logit link) analysis testing the effect of *germination data availability* and species group on *commercial availability*

Coefficient	Effect estimate	Standard error	Z	*P*
Intercept (protected)	−4.2541	0.6016	−7.071	< 0.001
Germination data availability	2.1759	0.1524	14.281	< 0.001
Indicator species	3.3685	0.6570	5.127	< 0.001
Fodder species	2.6502	0.6021	4.401	< 0.001

Results were estimated using *GLM* fitted to the commercial availability data for each species.

Commission 2010). The opposite trend is evident for *protected* species, as the availability of commercial and germination data is extremely low, despite their conservation concern in EU regulations. There are relatively high levels of *indicator* species represented in the Restoration Species Pool (RSP), but this does not necessarily indicate availability from many suppliers. Seed availability and use is compounded by the origin of the seed, as some supplies may not be appropriate for use in every region (Bischoff *et al.* 2008). Species for which there is a lack of *GDA* are also less likely to be *CA* and more likely to be omitted from the *RSP*. This indicates the urgent need for research and development on European grassland native seed biology, including knowledge transfer to support the commercial sector.

When there are little or no germination data for species within a family, congeneric species can offer predictions of potential dormancy (Table S1), that is, *implied dormancy*, and thus the type of environmental conditions to trigger germination (Baskin & Baskin 2014). Implied dormancy for the large majority of study species (~75%) indicates probable complex germination characteristics (Table S1). Currently, most revegetation projects in Europe have no requirement to improve biodiversity outputs, thus there is lack of consistent demand, and little capacity to improve the range of species with *CA*, particularly for species that may be complex to supply. Without change, *ER* of grassland habitats could continue to demonstrate species bias limiting biodiversity, facilitating the persistence of degraded systems in alternatively stable states (Suding *et al.* 2004). Improving the *RSP* will reduce risk in *ER* projects as a complementary conservation resource.

For the *RSP* to better support *ER*, industry also requires cooperative market sharing, improved provision

and storage strategies. In Australia, United Kingdom, and the United States, there are examples of government, community, or nonprofit groups working cooperatively with seed suppliers to enable the inclusion of species that have challenging seed traits in the commercial *RSP* supply chain. The U.S. Native Plant Program (Oldfield & Olwell 2015) contracts production of seed across all available suppliers, to partition demand and market share, then stored in government infrastructure for purchase. As a unique example in Europe, Germany has mandated that only native species may be used for all revegetation by 2020 (BNatSchG, Federal Ministry for the Environment, Nature Conservation, and Nuclear Safety 2010). Compared to German native seed demand in 2015, production of local native seeds must grow 10-fold to meet 2020 targets (Pers. Comm., Ann Kareen Mainz, Association of German Wild Seed Producers), an increase which will require expansion of their *RSP*. Demand creation, contracting, storage, and provision solutions must be developed in tandem to effectively expand *RSP* capacity.

Policy recommendations

Current legislation relating to *protected* (European Commission 1992) and *fodder* (European Commission 2010) species recognize the need to produce seed specifically for ER, but do not match the native seed market appropriately. Policy relating to the use of seed mixtures mandates that commercially produced seed must come from the same source area in which it is being used, and germination minimums are required (European Commission 2010), which are easily achievable in cultivars, but unrealistic for native species. These quality standards are too restrictive (Tishew *et al.* 2011), to which there is low adherence and enforcement, as they are contradictory to a much-needed industry with a small market niche. Supportive regulation is needed and future EU policy should require that all public revegetation projects use only native material. Creating demand through policy while aligning the contracting of supply offers immense potential to enable growth of the *RSP*. We strongly support initiation of policies to contract annual native seed production of baseline *indicator* and *fodder* species across available producers to store for large-scale projects. Policy should require vegetation biodiversity targets to be met in *ER* and revegetation. Sourcing and contracting of site-specific seed material beyond yearly *indicator* and *fodder* stores (including but not limited to *protected* species) should be required at project inception to allow time for realistic production. New policies should be designed to embrace consultation with the native seed industry and restoration professionals.

Conservation seed banks for native species can support these strategies in a small capacity and can provide access to relevant small-scale seed processing and quality assessment equipment (Nevill *et al.* 2016). The largest *ex situ* plant conservation programme globally, the *Millennium Seed Bank Partnership (MSBP*), managed by the Royal Botanic Gardens, Kew, UK, has successfully banked seeds of 13% of the world's wild species, aiming to bank 25% by the year 2020 (Royal Botanic Gardens Kew 2015). Seed from the *MSBP* has been used for small-scaled reestablishment, generally targeted for threatened species. An exemplar is FAO-RBG Kew "Africa's Great Green Wall" program within which collaborating country seed banks supply ~25,000 kg of seed per annum of about 200 species of trees, shrubs, and grasses (Sacande & Berrahmouni 2016). Nevertheless, a new form of *Restoration Seed Bank*s (Merritt & Dixon 2011) is needed if a sustainable seed supply chain of the right scale is to be supported for the *RSP*. To improve *ER* outcomes, wide expansion of current capacity and collaboration across sectors is needed to provide the requisite tons of native seed needed (Merritt & Dixon 2011). In addition, research in seed biology and vegetation science applied to seed sourcing, applications, and bottlenecks related to collection and use are required.

Current research in seed biology and regeneration processes remains specialized, in need of urgent expansion (Larson & Funk 2016). In addition, long-term interdisciplinary and collaborative open-source knowledge sharing platforms are needed to facilitate the exchange of research (Royal Botanic Gardens Kew 2015). We suggest future germination research focus on the development of efficient dormancy breaking treatments, the thermal control of germination (thresholds and rates), and improvements in native seed production practices for European grassland species not currently covered by the *RSP*. Integration of research and industry knowledge sharing where any research project connected to native seed germination delivers findings to the private sector could hold wide benefits. Research projects for *protected* or underrepresented taxa could ideally include commercial or cooperative seed production contracts for direct use in conservation and reintroduction as industry output components. Supplying *protected* species must be strictly designed, implemented, and controlled with the direct use of vanguard science through extremely collaborative approaches (Shirey *et al.* 2013).

Conclusions

Our analysis presents the first study investigating seed germination data availability and the commercial "*RSP.*" We present a continental case study, reflecting a global issue of global importance to habitat conservation. In

summary, we encourage further exploration and reconsideration of public policy, compilation of open-access knowledge sharing across sectors and multinational efforts to provide infrastructure and support, so as to expand and realise the full potential of the emerging native seed industry. Improving the breadth of seed biology research and knowledge sharing between sectors has potential to support the expansion of the commercial native seed market and the *RSP*. Improved commercial availability could reduce species bias and risk in ER.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1 Observed percentage (%) of suppliers (total 17) with *commercial availability* of seed with and without *germination data availability*.

RDI plots (raw data, descriptive and inference statistics) show jittered points of raw data, center bars indicate the mean of the data, beans outline the smoothed density of the data, whiskers mark the 10% and 90% quantiles of the data, and inference bands show the Bayesian 95% high-density interval inferential statistics for each group. Letters show statistical differences between groups (Table S4).

Table S1 Simplified seed dormancy types (adapted from Baskin & Baskin 2014)

Table S2 Full species list, associated category, and associated data

CA = commercial availability (yes [1], no [0]), *GDA* = germination data availability (yes [1], no [0]).

Table S3 Seventeen seed suppliers across 17 countries used for data collection

Table S4 Statistics representing differences between variables in the percentage of suppliers with seed of each species commercially available compared across species groups (Figure S1)

Kruskal-Wallis χ^2 test and post hoc pairwise Tukey and Kramer (Nemenyi) χ^2 test, *P*-value statistics, indicating significance between group variables. Germination data available = "+*GDA*," germination data not available = "–*GDA*."

Table S5 The complete data set summarized by taxonomic family in descending order of percentage of commercial availability (*CA*)

= number, % = percentage, Sp. = species, *CA* = commercially availability, *GDA* = germination data availability.

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