Seed Pre-Treatments of Native Species for Optimal Germination and Establishment in *in-situ* Restoration Programmes

Thesis dissertation

From top to right: *Malva moschata*, *Fragaria vesca*, *Senecio vulgaris* and *Trifolium pratense*

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Seed pre-treatments of native species for optimal germination and establishment in *in-situ* restoration programmes

By

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Abstract

In this work I investigate seed quality of wild species and explore seed pre-treatments with the potential to enhance seed performance by assessing seed viability, seed germination and seedling establishment under optimum and stress conditions. Detailed germination experiments have been performed on a range of wild species at seed batch level. Seed were tested under a number of conditions with a set of temperatures, light conditions and additives (active ingredients). Characteristics that were evaluated were final germination, germination speed, uniformity of germination and seedling establishment. Following this, seed treatments with the potential to improve seed and seedling quality were studied to understand the relationship between responsiveness to treatments and the ecology of the species and the provenance and seed maturity of the seed batches. Different compounds with the potential to stimulate seed germination were tested using priming and coating. Primed and coated seeds were tested in various substrates (paper, low nutrient and high nutrient soil) to investigate the suitability of these techniques to improve seed quality of species to be used in restoration.

Little is known on tetrazolium testing of wildflower seeds. Therefore, in Chapter 2 a tetrazolium based method was developed in order to quickly assess the viability of seed batches. Moreover, the usefulness of this method for cultivated and wildflower seeds was determined. The number of non-viable seeds in a seed batch plays a large role in the final number of germinated seeds and will determine if seed improving treatments are sensible to be implemented, therefore, assessing seed viability is of remarkable importance. The method demonstrates that grinded seeds are able to reduce tetrazolium if seeds are viable. Hence the intensity of the colouring, generated by a homogenate of seeds is related to the proportion of viable seeds present in the sample.

Chapter 3 describes the responses of 23 wildflower species to a wide range of germination conditions. The hypothesis was that species from different habitats will have different germination requirements while species from a similar habitats have comparable ecological requirements. The conditions considered were: variation in light conditions and the absence or presence of nitrate, karrikins or gibberellic acid. The conclusion of this study was that the hypothesis had to be rejected. However, it was observed that species could be grouped by their light response and responsiveness to compounds, which suggested that the habitat required for seeds to germinate may differ from habitat required for plants to complete their life cycle.

Seed quality and characteristics are not only determined by genetics (in other words species) but also by time of harvest, production location and after ripening (storage). Hence in Chapter 4 the impact of production locations on seed characteristics is studied. Seed batches of *Arabis alpina* harvested at different locations are characterised and the effect of priming treatments on germination of the different batches is assessed. The main conclusion is that the germination characteristics of a single seed batch of a species are not representative for the other seed batches obtained for that species and that there is no single treatment that enhances all seed batches of a single species.

While in Chapter 4 variations in provenance were considered, in Chapter 5 the impact of harvest time and storage on the quality of primed, coated or untreated seeds is studied. Priming and coating were studied because of their proved usefulness in the seed industry and, thus, their potential in wildflowers. From the results obtained, priming and coating techniques are shown to be able to enhance germination of the wild species studied under optimal and stress conditions; however effectiveness of the treatments varies among seed batches.

To conclude, the main results from this study demonstrate that seed performance and responsiveness to treatments among species and more importantly different seed batches of a single species are highly variable. This heterogeneity among seeds can be due to the genetic make-up of the species, the seed maturity stage at collection and the impact of the environment during seed development, maturation and storage, among others. However, seed treatments can in some cases be applied in order to improve seed quality.

Contents

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Author's declaration

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

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Statement of contribution

This thesis is based on the following articles (published or in preparation). I have planned and performed the experimental work and written a significant portion of the manuscripts.

1. Lopez Del Egido, L., Navarro-Miró, D., Martinez-Heredia, V., Toorop, P. E., & Iannetta, P. P. (2017). A Spectrophotometric Assay for Robust Viability Testing of Seed Batches Using 2, 3, 5-Triphenyl Tetrazolium Chloride: Using *Hordeum vulgare* L. as a Model. *Frontiers in plant science*, 8.
2. Lopez del Egido, L., Toorop, P. E. and Lanfermeijer, F. C. Seed Enhancing Treatments: Comparative Analysis of Germination Response across 23 Wild Species (in preparation)
3. Lopez del Egido, L., Toorop, P. E. and Lanfermeijer, F. C. Seed Priming Improves Germination of *Arabis alpina* under Thermo-inhibiting Conditions (in preparation)

Statement of style

This thesis has been prepared in journal article compilation style format. Each chapter has been written with the aim of publication in a scientific journal. Due to this fact, some overlap among chapters is evident, particularly in concepts related to the physiology of seeds and the description of the treatments. Exceptionally, the following chapters have been written in a different format, because of their content: Chapter 1 (General Introduction), Chapter 5 (Comparison of responsiveness to treatments across genera, within genus and across seed batches) and Chapter 6 (General discussion).

Involved institutions

NASSTEC includes 7 full and 3 associated partners from 4 EU Member States, and it interconnects four different sectors: private companies, NGOs, public land governance bodies and academic institutions.

The 7 full partners are the following:

- University of Pavia (UNIPV)
- Syngenta B.V. (SYN)
- Semillas Silvestres SL (SESIL)
- Scotia Seeds Limited (SSE)
- Millennium Seed Bank, Royal Botanic Gardens (KEW)
- The James Hutton Institute (JHI)
- Museo delle Scienze (MUSE)

The 3 associated partners are the following:

- Jardín Botánico Atlántico S.A (JBA)
- The National Trust for Scotland (NTS)
- Botanic Gardens and Parks Authority (BGPA)

Chapter 1

General introduction

Background information

Destruction of natural habitats due to human activities continues to increase. In 2002, the United Nations estimated that over 70% of the natural habitats on Earth will be lost by 2030 (United Nations Environment Programme, 2002). Furthermore, recent assessments on the conservation state of habitats and species appeared "unfavourable" for 77% of the habitats and 60% of the species (European Environment Agency, 2015). Considering this high loss rate, conservation and restoration of wild vegetation in natural habitats seem critical. Restoration of vegetation can be achieved by planting young individuals or by sowing seeds. Sowing seeds in a heavily damaged ecosystem can be compared with sowing seeds on a barren agricultural field. Hence, an agricultural-like approach for restoration by seeds can be considered. Successful restoration via seeds requires knowledge on seed quality, germination characteristics, such as dormancy, stress tolerance and conditions for germination and seedling establishment for the species involved. Germination characteristics of cultivated species have been studied for years (Maguire, 1962; Tekrony et al., 2005; Willenborg et al., 2005; Magneschi and Perata, 2009), especially for the application in production for agriculture (ISTA, seed companies). However, not much is known about the biology of wild species (Vander Mijnsbrugge et al., 2010) and even less is known about the impact and usefulness of seed enhancement treatments for wild species.

The Marie Curie ITN programme NASSTEC was created to increase the knowledge on native plant species for restoration purposes. NASSTEC is a multidisciplinary programme that "plans to interconnect the public and private sector through the establishment of a European doctoral 'school' with the aim of integrating knowledge in plant ecology, genetics, molecular biology, taxonomy, ecology, conservation, seed biology, environmental science, agricultural botany, crop science, breeding and horticulture in order to develop the native seed industry in Europe". This scientific and training project includes twelve research projects, which are comprised under three sub-programmes: A) *In-situ* seed sampling, B) Seed biology characterisation and C) Production and deployment of seed. Looking at the large areas to restore (Menz et al., 2013) it is obvious that a large amount of high quality seeds will be needed to completely restore them. Therefore, my PhD project studies seed biology in order to: (1) produce standard procedures for seed quality assessment (expanded in Chapter 2); (2) develop seed pre-treatments to improve seed germination and improve stress tolerance of seeds; and (3) produce germination protocols for testing and delivering high numbers of germinated seeds of wild species for restoration (investigated in Chapter 3 – 5). My project falls under sub-programme "C" of the NASSTEC programme.

Seed physiology

Germination consists of a series of sequential complex processes. It commences with imbibition of water by the quiescent dry seed, followed by re-establishment of cellular structures and mobilisation of seed reserves (metabolism). Finally, it is completed when the embryo, usually the radicle extends to penetrate the structures that surround it (Bewley and Black, 1994; Nonogaki et al., 2007). Seeds have evolved receptors and a signal transduction network to integrate the various signals from the environment into a response. Thus, when seeds perceive favourable conditions they will initiate the process of germination (Steadman, 2004). The factors that are critical for seed germination are: water, light, temperature, oxygen and nutrients (Koller and Hadas, 1982).

An important aspect of seed quality is seed viability. A viable seed is defined as the seed that has the potential to germinate and develop into a seedling, or in other words, the seed is alive (Copeland and McDonald, 1999). Seed viability is affected by a series of factors: mechanical injuries, maternal environment, maturity at harvest, genetic factors and storage conditions (Sadhu, 1989). There are currently different methods to determine the viability of a seed batch; viability can be assessed by a tetrazolium chloride (TTC) test, a cut test, a germination test, X-rays and a buoyancy test. The TTC test is a biochemical assay that assesses the viability of the seeds at the individual seed level by determining the capability of the seeds to reduce TTC. This becomes visible as staining patterns in the individual seeds. On the other hand, the germination test is the most commonly used method, especially for flower seed testing (McDonald and Kwong, 2005). However, a germination test can be time-consuming depending on the germination speed of the species tested. The TTC method is a relatively quick test, which allows to obtain results in 48 hours; however, seeds need to be inspected by one, thus it is a labor intensive method. TTC methods also require special skills to conduct and to interpret the results, which do not always reflect seed metabolism. Moreover, the current protocols are not easily applied to wild species, as they have been developed to test seeds of agricultural species, such as grasses, cereals, small legumes, vegetables, and tree species, but not flower species by instance (International Seed Testing Association, 2013). Because it appears to be difficult to assess seed viability with the currently available TTC methods, especially for wild species, in Chapter 2 we present an improved TTC method that has the advantage of allowing testing of seed batches rather than individual seeds. Moreover, it is an objective method and results are quickly and without much effort obtained.

It is important to note that TTC methods allow us to evaluate the viability of the seeds; therefore, they do discriminate between potentially germinating (viable, including non-dormant and dormant) and dead seeds (non-viable); however, they do not separate viable seeds that will

germinate (non-dormant) from viable seeds that will not (dormant). Dormancy is difficult to define, and it seems to be poorly understood (Hilhorst, 1995; Bewley, 1997; Basbouss-Serhal et al., 2015). Nonetheless, a "dormant" seed could be defined as a seed which is viable, but fails to germinate given favourable environmental conditions (Baskin and Baskin, 2004). Dormancy is regulated by abscisic acid (ABA)/ gibberellins (GA) hormones balance in the seeds (Karssen and Lacka, 1986); under unfavourable conditions, seeds increase the ABA content, which promotes dormancy and, therefore, inhibits germination; while under favourable conditions, seeds synthesise GA, which promotes germination. Dormancy is an ecological strategy that allows seeds to synchronise germination with the optimal moment for the seedlings to survive (Baskin and Baskin, 1998). Therefore, species with different ecology and dormancy types are hypothesised to germinate after the integration of different signals, such as light, the clearings in the canopy (by a wildfire or other catastrophic event), the presence of nutrients, *etc*., which suggest favourable conditions for the seeds to germinate. Different signals exist to stimulate germination and to break dormancy (Grubb, 1977; Bewley and Black, 1982); these signals may only occur in the habitat the species has been adapted to. However, it is also important to distinguish the terms "primary dormancy" and "inhibition of germination". Both terms can be used to describe a viable seed that fails to germinate. In the former case, favourable germination conditions are present, but seeds do not germinate (Bewley, 1997); while in the latter, unfavourable germination conditions prevent seeds to germinate but germination will start when seeds are placed back under favourable environmental conditions (Hills et al., 2003; Baskin and Baskin, 2014).

In a dormant seed, germination will not take place even if the seed is placed under favourable conditions (Bewley, 1997). We have to distinguish, however, the various types of seed dormancy(Baskin and Baskin, 1998, 2001 and 2004). Primary dormancy is the state in which freshly mature seeds fail to germinate under a range of conditions, and five classes are distinguished: Class A) Physiological dormancy; Class B) Morphological dormancy; Class C) Morphophysiological dormancy; Class D) Physical dormancy; and Class E) Combinational dormancy. Non dormant seeds can enter secondary dormancy when they are subjected to unfavourable germinating conditions, that prevents them to germinate (Arc et al., 2013; Baskin and Baskin, 2014). Secondary dormancy is induced by unfavourable temperature (thermodormancy; Baskin and Baskin, 2014), light (photodormancy; Lambton, 1985) and a combination of darkness and temperature (skotodormancy; Lambton, 1985; Baskin and Baskin, 2014). In the germination process three phases (Fig. 1) are distinguished based on water uptake kinetics as described by Bewley (1997) and Nonogaki (2007): initial uptake of water or imbibition as a result of the matric and osmotic potential quiescent seed (Phase I), followed by a phase with

a slow water uptake during which seed cell structures and physiology are organised (Phase II), and, after the decision for germination has been taken, higher water uptake, which will lead to cell expansion and radicle protrusion (Phase III). Inhibition of germination occurs when environmental factors stop seeds entering to Phase III. Dormant seeds will imbibe and enter to Phase II (Fig. 1), but the germination process will be stopped at a certain point and the dormant seed will not enter Phase III. Non-viable seeds will take up some water due to the fact that even dead seeds are dry and will attract water, but less than a viable seeds, because metabolism will not beginand no additional osmolytes will be produced from the reserve compounds present in the seeds. Non-viable seeds will never proceed to Phase II (Fig. 1; Taylor et al., 1992).

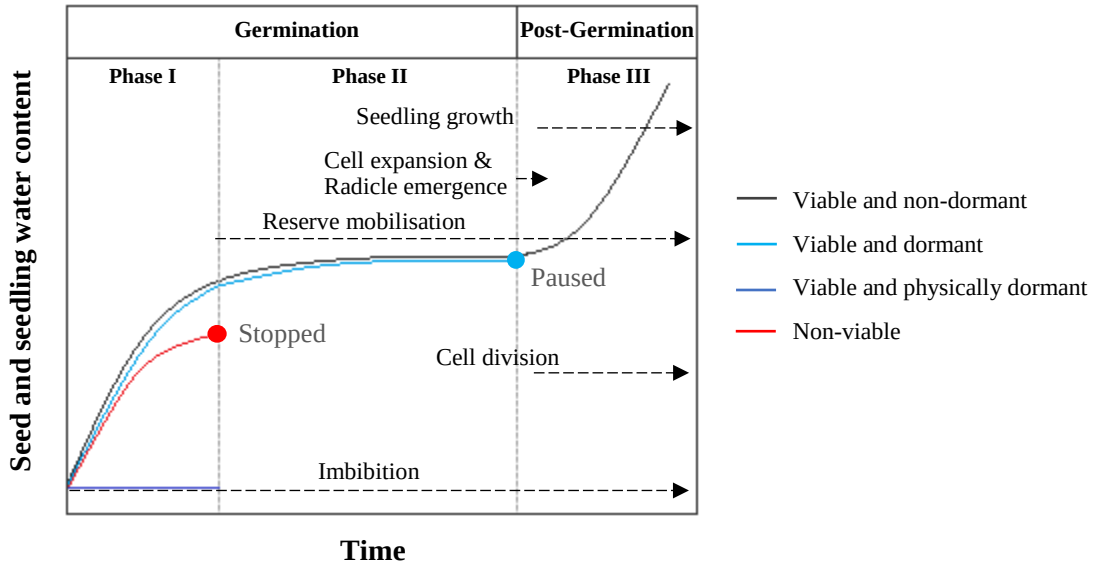

Figure 1. Time course of physical and metabolic events occurring during germination (Phases I and II) and early seedling growth (Phase III). The time taken for these events to occur varies between species and is influenced by germination conditions. The black curve shows the water uptake over time of a viable non-dormant seed. The blue line represents a viable dormant seed. The process of germination can be paused at every moment between the beginning of Phase II and III. The red line represents a non-viable seed. Modified from Nonogaki 2007, 2010 and Bewley 1997.

In nature, both primary dormancy and inhibition of germination are advantageous strategies for the survival of the seed as they regulate the timing of germination with the presence of favourable conditions during the germination season (Steadman, 2004). Therefore, dormancy and inhibition of germination will ensure seeds to develop into seedlings and seedlings to develop into mature plants only when favourable conditions are present. Depending on the type of signal seeds will become dormant or their germination will become inhibited. If conditions represent a short period of unfavourable conditions seeds will tend to become inhibited, in this way, they will be able to

germinate as soon as these conditions become favourable for germination. If unfavourable conditions last longer seeds will become secondary dormant. However, for restoration and cropping purposes dormancy can be an issue as it results in poor, delayed and non-uniform germination (Gubler et al., 2005; Arc et al., 2013), while for these purposes one wants 100% germination due to the investments made. On the contrary, for a restoration project to be successful, low and variable levels of dormancy in the used seed batches might be beneficial. Low levels of dormancy might lead to a wider germination window (less uniform germination in agricultural terminology), which might favour successful establishment of the wild species reducing the risk of the whole population being exposed to one single deleterious event. Moreover, low levels of germination caused by heat waves, for example, could compromise the soil seedbank, due to all the seeds from the population germinating in a narrow window of space (Ooi et al., 2012) and causing poor seedling recruitment (Graae et al., 2009) when environmental conditions are favourable for survival.

Application of seed treatments in the seed industry

To alleviate primary dormancy, avoid inhibition of germination and the induction of secondary dormancy, seed treatments can be applied (Hsiao and Quick, 1985; Carpenter and Boucher, 1991; Cantliffe et al., 2000; Anese et al., 2011). As defined by the International Seed Federation (2014) "Seed treatments are the biological, physical and chemical agents and techniques applied to seed to provide protection and improve the establishment of healthy crops". Seed treatments have been shown to also enhance the quality of seed batches in agriculture by improving uniformity, germination speed and final germination (Doran, 1983; Haynes et al., 1997). Seeds are important and valuable assets for farming (Bewley et al., 1986) and, hence, high germination numbers under a wide range of conditions are of outmost importance (Finch-Savage, 1995; Bettey et al., 2000). The seed industry has recognised this and, thus great importance has been given to the study and development of seed treatments. Seed treatments include priming, coating, scarification and stratification of crops (Willenborg et al., 2004; Ashraf and Foolad, 2005; Shanmugavalli et al., 2007; Gisbert et al., 2009; Paparella et al., 2015). Priming is generally described as a hydration process that initiates germination preventing radicle emergence (Heydecker and Coolbear, 1977; Taylor and Harman, 1990). Coating is defined as a process to add materials to the seed (Roos and Moore III, 1975; Taylor and Harman, 1990), we specifically refer to a film coat of material(s), which involves addition of a polymer to the seed which does not involve any change in seed size or shape. Scarification refers to any mechanism that leads to seed coat rupture (Vilela and Ravetta, 2001) and stratification is the process of subjecting seeds to cold or warm and moist conditions

(Tran and Cavanagh, 1984; Rees, 1997). Various seed priming techniques have been developed, these include soaking seeds in water, in inorganic salt solutions, in organic osmotic solutions and in biological compounds, specifically called hydropriming, halopriming, osmopriming and biopriming respectively (Paparella et al., 2015). In this thesis, hydropriming treatments are investigated as they have been proven to enhance germination not only in crop species (Parera and Cantliffe, 1994; McDonald, 1999), but also in some wild species (McDonald and Kwong, 2005; Anese et al., 2011). Therefore, we wanted to test the potential of such a treatment to enhance germination of other species where the potential of the treatment has not yet been described. Although it might seem marginal, the study of the seed biology of wild species is also important for the seed industry. Crop breeding makes use of related wild species as a source of new native traits, such as increased resistance to biotic and abiotic factors (Bessey, 1906; Hoisington et al., 1999; Zamir, 2001; Munns et al., 2002; Poehlman, 2013; Castañeda-Álvarez et al., 2016), and thus availability of plants and therefore germinating seeds for crossing from wild populations into the domesticated crops is needed. Moreover, crop plants have gone through numerous genetic bottle-necks (Doebley, 1992; Buckler and Thornsberry, 2002) which could have resulted in corrupted mechanisms of germination control at the expense of breeding for yield (Donald and Hamblin, 1983), amongst others. Hence the study of related wild species could shed light on the regulation of germination in crop species.

Species of study

Several species have been studied within this project and they were selected using seven criteria: I) the species are native to the alpine meadows, which are threatened communities (Beniston et al., 1996; Sundseth, 2009); II) species are suitable for use in restoration; III) species are closely related to a commercial or scientific reference species; IV) species are able to potentially support exchange knowledge, protocols and treatments between these species and their commercial counterparts and *vice versa*; V) species share germination issues with some of the commercial crops; VI) the species are indicative of means to optimise other groups of native species; and VII) the species are common and seed material is immediately available in high numbers allowing a prompt start of the experimental part of the project.

Seed morphology and seed characteristics of the chosen species are greatly diverse (Fig. 2) as these species belong to different ecology types. Therefore, the hypothesis we wanted to test was whether the ecology and seed requirements were responsible for the different germination and treatment requirements. Hence, the germination characteristics of each of these species was studied in regard to germination conditions, which included light, temperature and active ingredients.

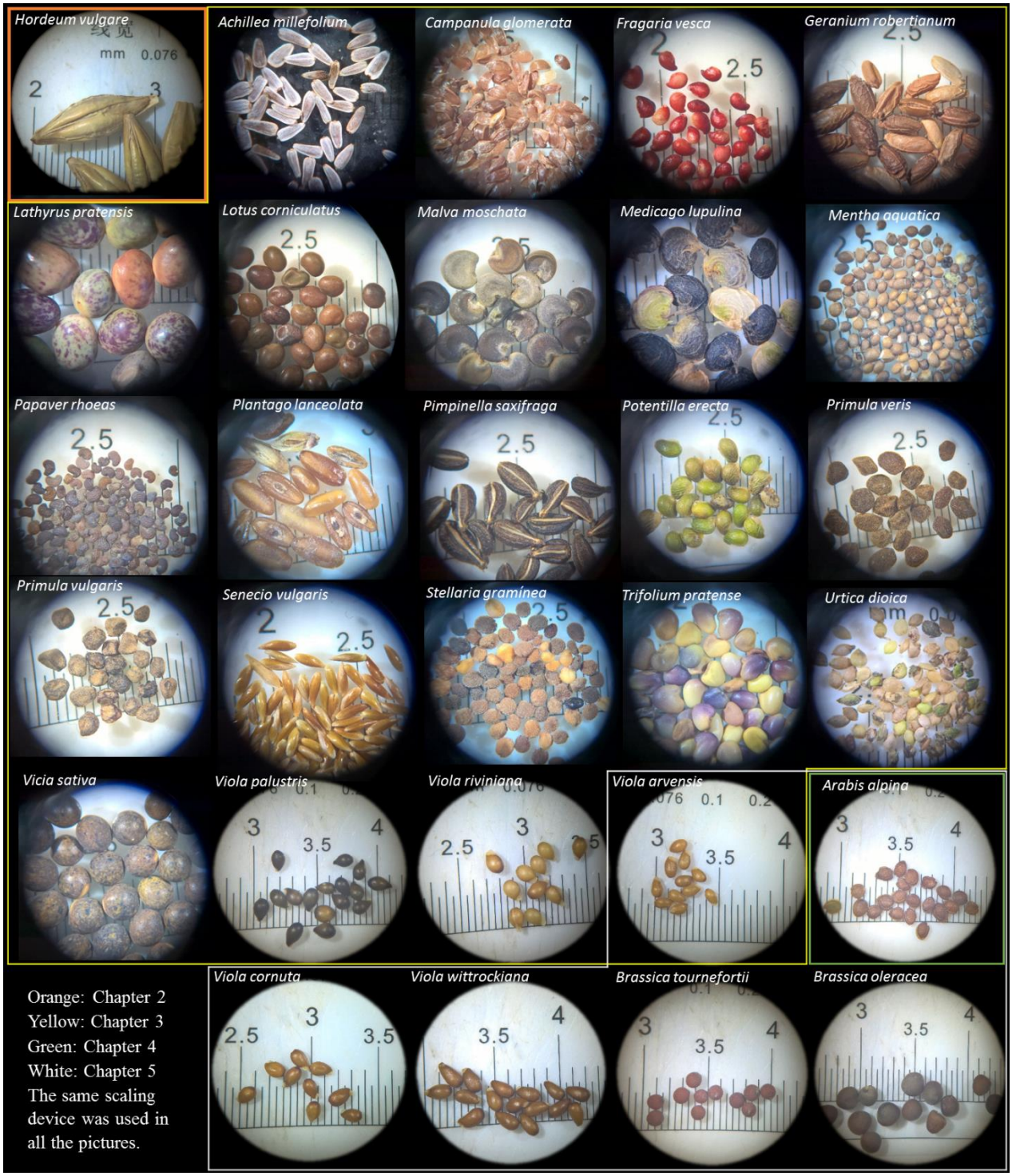

Figure 2. Seeds from the 29 species that have been studied in this thesis. Species are coloured according to the chapter they are studied in: orange (Chapter 2), yellow (Chapter 3), green (Chapter 4), and white (Chapter 5).

Aims and structure of the work

The aim of this project was to study seed treatments to improve seed performance and seedling quality for the use in restoration. Successful restoration of an ecosystem depends on the successful establishment of a large number of diverse species and thus, I studied the germination

characteristics of 29 indicative study species. I treated those species with active ingredients and studied the effect of delivery systems for those compounds.

Usually the first step to be taken in seed testing is to identify the quality of a seed batch. The quality of a seed batch depends on a range of characteristics, but viability of the seeds is one of the main ones. Viability is a crucial seed characteristic to recognise as it is the starting point for any further work; because of that, different pathways can be taken in order to assess seed viability. The tetrazolium (TTC) staining method is one of the options, however, current TTC protocols are performed at a single seed basis and they exist mainly for cropped species. Hence, we developed a method to assess viability more rapidly, using barley (*H. vulgare*) as a model species described in Chapter 2. We chose barley due to the large amount of seed available and its large seed size. However, the main objective to develop this method was to provide an improvement for assessing seed viability of small seeded species, thus preliminary results on *Viola cornuta* are shown in the supplementary material section although they were not included in the published article. The homogenate of seeds processed for TTC indicate a strong link to seed quality and provides a new and novel approach likely to have broad application across agrigultural and potentially to native species testing.

Once we have obtained the knowledge on the viability of a seed batch, we can proceed to apply seed treatments to improve seed quality if necessary; hence, next, my research has studied the potential of available commercial and experimental seed treatments for wild flower seeds and, to develop treatments for those seeds. The study of seed treatments and seed additives include on the one hand, the compounds that can improve seed vigour (performance), and, on the other hand, the delivery system for these compounds. In Chapter 3 to 5, I investigated the compounds that can be used to improve germination. Specifically, in Chapter 3, I explored the effect of various compounds (gibberellic acid, nitrate, sodium hypochlorite, etherel and smoke derived compounds) or in generally called, active ingredients (AI), on the germination of a range of species with different ecology types; however, only one batch of seeds per species was tested. Therefore, in the two latter Chapters, I focus on the sensitivity of different batches belonging to the same species to treatments, in this case of the species *Arabis alpina* (Chapter 4), and to changes in sensitivity during storage with seeds from *Brassica tournefortii* and *Viola arvensis* (Chapter 5). In Chapter 4, I test the compounds studied in Chapter 3 using priming. For this, I used *A. alpina*, which is a wild relative from the cropped Brassica. *Arabis alpina* has germination issues which are similar to some crops, such as leek, lettuce and spinach (Gelmond, 1965; Karssen, 1980; Atherton and Farooque, 1983; Valdes et al., 1985). These species all display thermo-dormancy and, therefore, findings in *Arabis* might be used in these crops and knowledge available from studies with those crops might

give leads for solving the *Arabis* problem. At this point, we drew the hypothesis that by applying seed treatments we could overcome the inhibitory conditions for germination and enhance performance of the seeds at stress conditions. In Chapter 5, I examine the sensitivity profile of species to treatments using: A) a set of *Viola* species, here we hypothesise that phylogenetically related species will have a similar responsiveness to treatments; B) two different seed batches of *Brassica tournefortii*, used due to the high variability of this wild weed species, with the hypothesis that both priming and coating techniques are useful technologies to stimulate germination; and C) four *Viola arvensis* seed batches, where we investigated how the effect of treatments change over-time with after-ripening.

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Chapter 2

Viability testing using a newly developed tetrazolium test

PROTOCOLS ARTICLE

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A Spectrophotometric Assay for Robust Viability Testing of Seed Batches Using 2,3,5-Triphenyl Tetrazolium Chloride: Using *Hordeum vulgare* L. as a Model

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Abstract

A comparative analysis was carried out of published methods to assess seed viability using 2,3,5-triphenyltetrazolium chloride (TTC) based assays of seed batches. The tests were carried out on seeds of barley (*Hordeum vulgare* cv. Optic) as a model. We established that 10% [w/v] trichloroacetic acid (TCA)/methanol is superior to the acetone and methanol-only based methods: allowing the highest recovery of formazan and the lowest background optical density (OD) readings, across seed lots comprising different ratios of viable and dead seeds. The method allowed a linear-model to accurately capture the statistically significant relationship between the quantity of formazan that could be extracted using the method we developed and the seed temperature-response, and seed viability as a function of artificially aged seed lots. Other quality control steps are defined to help ensure the assay is robust and these are reported in a Standard Operating Procedure.

Introduction

The tetrazolium (2,3,5-tryphenyl tetrazolium chloride, TTC) test to assess seed viability from cut single seeds was originally developed by Lakon (1949) as a rapid (1–2 days) method to replace germination based assessments which could take several weeks. The test relies on the reduction of the colourless and water soluble 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) to an insoluble red compound (formazan). This reduction occurs as a consequence of hydrogen ions donated to the TTC upon dehydrogenase activity in metabolically active tissues, such as the seed embryo (Junillon et al., 2014). Consequently, seed viability is usually determined using a topographical method (visual observation) to characterise the pattern and intensity of formazan staining pattern and the intensity of coloration for individual seed embryos (Copeland and McDonald, 2001). The TTC-test is commonly used to assess the viability of seeds that have failed to germinate (Parreño-de Guzman et al., 2011; Brar et al., 2013; Rami and Patel, 2014).

There are internationally recognised methods for TTC seed viability testing (International Seed Testing Association (ISTA), 2014), which can be as reliable as germination tests for seeds of some species and purposes (*Rubia fruticosa* Ait., Marrero et al., 2007); orchids (Custódio et al., 2016); grasses (Soares et al., 2016); and *Cucumis anguria* L. (Paiva et al., 2017). While the topographical method requires extensive experience to achieve an accurate interpretation, germination tests are still commonly used (Mastouri et al., 2010; Moreira et al., 2010; Van Treuren et al., 2013; Ntuli et al., 2015). In addition, there is confusion in the literature regarding seed pre-treatments and choice of extraction protocols and especially the solvent used to extract the formazan which is formed.

TTC testing is also used to test the viability and intensity of metabolic activity in other types of biologically active specimens including plant parts e.g., fine-roots of Norway spruce (*Picea abies* L. Karst, Ruf and Brunner, 2003), plant leaves and stems; fungi (*Aspergillus niger*, Ghaly and Mahmoud, 2007) and *Corylus avellana* pollen long term stored viability (Novara et al., 2017). In some cases, the formazan produced during the staining period is extracted in a liquid, the optical density of which is quantified using spectrophotometry (using light of wavelengths at or close to 484 ηm). However, formazan extraction is still not widely used to determine seed viability although there have been some attempts to standardise and develop the assay for this purpose (Harty et al., 1972; Norton, 1985; Zhao et al., 2010), and a comparison of the protocols (and those developed here) are provided in Table 1.

In 1997, Vankus described the time-consuming limitations of the current TTC protocols (Vankus, 1997). Also, Gaspar-Oliveira et al. (2011) and Zeng et al. (2014) also disclosed time-consuming pre-conditioning and preparations steps for TTC testing. Therefore, a robust high throughput technique to test seed viability in seed batches would be a great benefit as such a method would not require time-consuming seed pre-treatments such as dehulling or dissection. In a comparative analysis of glacial acetic acid with methanol, Harty et al. (1972) established the latter as a better solvent for formazan extraction from TTC-treated milled seeds. However, we hypothesised that the seed batch process could be improved. For example, the physiological state of the test material used by Harty et al. (1972) was "natural," and not standardised under controlled experimental conditions. Also, potentially important steps could be added such as "seed bleaching" (Peters, 2000), which can remove pigments that would otherwise have been extracted and caused high optical densities in the test extracts and control samples. The method used by Norton (1985) used whole bisected seed kernels (testa removed), which is time consuming and therefore cannot be used for high-throughput processing. Furthermore, the dissected seeds were incubated with 1% [w/v] TTC for periods of up to 4 h and formazan extraction was achieved by homogenisation in acetone (95% [v/v]). Also, it is important to note that later publications have highlighted that acetone is inferior to methanol for formazan extraction (Zhao et al., 2010). Additionally, Norton (1985) applied TTC to the dissected half-kernels: that is, the reduction potential of the whole tissue was not assessed, and imbibition of the dissected kernels on filter-paper moistened with TTC solution was advocated, which may not lead to standard treatment of the test material. Furthermore, important extraction conditions such as the homogenisation period and TTC incubation temperature were not specified. However, Norton (1985) did standardise the quantities of formazan recovered for the weight of kernels which were treated. Similarly, Zhao et al. (2010) established the recovery of formazan by homogenization and incubation of excised

embryos was greater for 10% TCA:acetone [v/v] than 80% acetone:water [v/v]. As this method also required dehulling it is therefore still time consuming. Also, seed viability was assessed indirectly using the quantity of malondialdehyde (MDA; a breakdown product of lipid peroxidation, and therefore cell damage/death). That is, seed viability was not tested by germination nor had the test samples been standardised: for example, to contain known proportions of viable and dead seeds, or treated to control seed vigour.

It therefore appears that the TTC based assays currently available in the literature do not yet describe a single robust or high-throughput spectrophotometric assay to assess seed viability of seed lots. This short-coming may be linked to the profusion of information regarding the TTC assays for other types of metabolically active tissue which may have led to conflicting and sub-optimal approaches. Crucially, it should be noted that formazan production is correlated with staining time (Harty et al., 1972; Mikuła et al., 2006), TTC concentration (Steponkus and Lanphear, 1967; Harty et al., 1972; Junillon et al., 2014) and incubation temperature for seed imbibition and TCC reduction where this is done on seed extracts. Equally, the quantity of formazan extracted and exclusion of secondary compounds that may interfere with the assay depends upon the type of solvent used and the extraction protocol: such as the extent to which moisture is removed from the test material after incubation in TTC. In addition, the quantity of formazan extracted should be standardised for the weight of seed tissue which is tested. We note that the vapour pressure (evaporation rate), for each formazan-carrying solvent is highly variable with: acetone > methanol > ethanol. These solvents may therefore differentially affect the stability of optical densities recorded using the spectrophotometry for TTC-based assays. The relative capacity of solvents to directly affect the OD which is recorded seems untested. Additionally, no recommendations seem to have been made to ensure that the solvent-formazan extract should be treated to minimise evaporation during storage and reading. This manuscript therefore uses seeds of barley (*Hordeum vulgare* L. cv. Optic) in a model approach based on significant modifications and developments of Harty et al. (1972), Norton (1985) and Zhao et al. (2010). The aim of the approach was to establish a more-robust and accurate assay to quantify seed viability.

Materials and Methods

Seed Imbibition and Germination

Initial tests assessed batches containing mixtures of viable (99.8% germination), and dead (by dry autoclaving at 120°C, 20 min) barley (cv. Optic) caryopses, hereafter referred to as seeds. A series

of standard test samples were prepared (in triplicate) by combining viable:dead seeds to a total of 100 seeds (*ca*. 7.5 g). The inclusion of dead seeds [w/w] was either: 0, 20, 40, 60, 80, or 100%. Seeds were left to imbibe overnight between water-saturated tissue paper inside a sealed container and incubated in the dark at 20°C. Rarely, any seed with a protruding shoot-born root was removed prior to processing for treatment with TTC.

Quantification of seed germination across a temperature series (10, 15, 20, 25, and 30°C), was also performed in the dark and scored when protrusion of the first shoot-born root was evident (*ca*. 1 mm; Tillich, 2007): these conditions were also used to confirm the efficacy of dry autoclaving to kill the seeds. It should also be noted that seeds for treatment and the sterile distilled water (SDW) and SDW-pre-soaked paper-tissues for imbibition were pre-incubated at their respective treatment temperatures for 2 h.

Germination tests were performed using 6 replicates of 50 seeds *per* Petri dish (300 *per* temperature), sown on to 3 MM Whatmann paper and incubated for 12–14 h in a sealed container inside a controlled environment cabinet (in darkness) before watering with 8 mL of SDW. Germination was scored at the same hour every day for 10 days and seeds with protruded shoot-born roots were removed.

Controlled Ageing Treatment

Barley seeds cv. optic were placed in glass vials inside an electrical enclosure box (catalogue number OABP303010B, Ensto UK Ltd., Southampton) sealed with a clear lid, above a solution with *ca*. 250 g LiCl added to 1 L of distilled water, producing 70% relative humidity. The seeds were placed for 2 weeks at 20°C to allow equilibration to the high humidity, and subsequently at 45°C to allow ageing. Seeds were sampled after 0, 8, 18, 25, and 31 days ageing. After each ageing period, 6 samples of 50 seeds were removed for germination testing, and 4 samples of 50 seeds for formazan extraction. The samples for formazan assay were dried on silica gel and stored at 15% RH at 15°C until extraction. Germination tests were carried out immediately by sowing the 6 replicates of 50 seeds on 1% [w/v] agar dishes. Dishes were incubated at 15°C under a 8 h photoperiod and germination was scored frequently up to 3 weeks. Germination was considered complete when emission of the first shoot-born root was detectable >1 mm. For the TTC extraction, three replicates were used in the initial tests, whereas for the controlled-aged samples, four replicates were used.

Tetrazolium Assay

The assay was performed on three replicates of 100 seeds in the case of the viable:dead seeds (1,800 seeds in total) and, on 4 replicates of 50 seeds in the case of the controlled aged seeds (1,200 seeds in total). Therefore, the volumes of solutions described below were used in the first case, for the second, the volumes used were halved. Imbibed seeds were bleached using 7 mL of 3% [v/v] hydrogen peroxide (Sigma-Aldrich, #H1009) for 10 min. before rinsing twice with SDW. Seeds were briefly blotted dry between tissue paper before grinding. The treated seeds were then homogenised by grinding for 1 min (in coffee grinder; James Martin by Wahl ZX595 Mini Grinder, 150 W), and transferred to a fresh 50 mL tube with 15 mL of 1% [w/v] TTC stock solution Sigma-Aldrich, #T8877, in 5 mM potassium phosphate buffer, pH 7.2, prepared as described in Peters (2000). The TTC stock solution was kept in the dark at 4°C as it is light-sensitive (Ghaly and Mahmoud, 2007). The samples were then incubated at room temperature for 4 h in the dark without shaking before centrifugation (5,100 rpm for 5 min, Sigma 4K-15) and the supernatant was removed. The stained tissues were rinsed twice with SDW and the residual TTC solution was eliminated by vacuum drying using a Buchner funnel.

The recovered paste was treated for formazan extraction by freezing (with liquid N_2), and grinding in a mortar and pestle, to which 7 mL of solvent was added. The incubation conditions varied according to the methods trialled here (see Table 1), which were either: I, Harty et al. (1972), methanol (100%) for 15 h at 30°C; III, Zhao et al. (2010) 10% TCA/acetone [v/v] 5 min. at room temperature; IV, 10% TCA/ acetone; V, 10% TCA:methanol, and; VI, acetone only: the final three solvents applied using incubation conditions of 15 h at 30°C. After incubation, the samples were centrifuged (15 min, 5,100 rpm), and *ca*. 2 mL of the supernatant was transferred to a 2 mL microfuge tube and re-centrifuged (14,680 rpm for 30 min at room temperature, Sigma 1-15K).

Immediately before reading the optical density (OD) of the recovered solvent, the sample (1.5 mL) was transferred to a new microfuge tube and re-centrifuged as before. Absorbance's at 484 ηm (using at ELx800™ Absorbance Reader, BioTek® Inc.), were acquired for the technical replicates (300 µl each) dispensed into spate wells of a 96-well flat bottom "ELISA plate" (Nunc MaxiSorp®, manufacturers code 439454). The values obtained were corrected for background using the average reading for the solvent only replicates. The percentage variation between the three sub-samples was calculated with the following formula (1):

$$\text{Variation (\%)} = \frac{\text{(Highest OD)} - \text{(Lowest OD)}}{\text{(Highest OD)}} * 100 \qquad (1)$$

TABLE 1 | A summary of methods examined to assess seed viability in seed lots using the TTC-assay.

Method	Sources	Test species	Test material	Conditions for incubation of test material with TTC			Conditions for formazan extraction			
				Pre-treatment	Buffer	Time/Temp.	Homogenisation	Drying	Solvent for homogenate	Incubation
I	c.f. (Harty et al., 1972)	Barley	Whole seeds	Homogenised fresh using mortar and pestle (<0.5 mm)	1% TTC in citrate-phosphate buffer (pH 7.4)	4 h/21°C*	None	Buchner funnel only	Methanol	15 h/30°C
II	Norton, 1985	Peas	Kernels	None	1% TTC in water	4 h (room temp.)	Homogenised (fresh in blender)	None	Acetone	None
III	c.f. (Zhao et al., 2010)	Maize	Kernels	None	0.1% TTC 50 mM Tris-HCl buffer (pH 7.6)	4 h/21°C*	Homogenised under liquid-N_2 using a mortar and pestle	None	10% TCA/acetone	5 min/room temp. (21°C)
IV	This study	Barley	Whole seeds	Bleached/sterilised seeds homogenised in blender, 1 min.	1% TTC in 5 mM Potassium phosphate buffer (pH 7.2)			Buchner funnel and overnight (21°C)	10% TCA/acetone	15 h/30°C
V									10% TCA/methanol	
VI									Acetone	

The table describes the assays as comprising two main stages which are the conditions for: (1) incubation of the test material with TTC, and; (2) formazan extraction. Methods I–III describe protocols for three methods reported in the peer-reviewed literature and these were assessed in parallel with methods IV–VI. Methods IV–VI were developed in the course of the research reported in this manuscript, and they combine aspects of methods I and III with other new steps (defined in ***Table 3*** *and proposed as a recommended "standard operating procedure"). *Denotes a modification to the TTC conditions for comparative purposes with methods V–VI. The original TTC incubation conditions for Harty et al. (1972) were 2 h, 30°C, and for Zhao et al. (2010) were 24 h, 30°C.*

Formazan Standard Curve

The relationship between the optical density and formazan concentration was determined using series of standard solutions of 0, 10, 20, 40, 60, 80, and 100 mg mL^{-1} red formazan; (1,3,5-triphenyltetrazolium formazan, 90% pure, Sigma Aldrich #93145). The optical densities of the standards were also corrected against background (solvent only), before plotting the standard curve and fitting the linear-model.

Statistical Analysis

Linear models were fitted using Microsoft Excel 2010 for Windows 7. Regression analysis was performed with GenStat v14.2. Results were considered significant at $P < 0.05$.

Results

The formazan extraction conditions of Zhao et al. (2010; 5 min. at room temperature; Table 1), recovered the least formazan in both viable and dead seed types (Table 2), probably as a function of the limited extraction time (Table 1). High and similar recoveries were achieved with acetone, 10% TCA:acetone or 10% TCA:methanol (at 15 h, 30°C; Table 2). The different solvent types gave variable OD readings as shown from the three technical replicates: variation being calculated according to formula [1]. Variability in the OD readings is also apparent from the SEs (Table 2). The extraction solution (methanol) and approach originally proposed by Harty et al. (1972) gave more stable OD readings, while Method V (10%TCA:methanol, and longer TTC incubation time), also gave stable OD readings and allowed greater formazan yields (27%), than the Harty-method. The variation in OD readings which were recorded when recovering formazan using the different solvent types (Table 2) is most likely caused as a function of their relative vapour pressures. These vapour pressures may also have influenced the consistency of extraction. This data also indicated that unstable OD readings were more likely when acetone was used for either viable or dead seed extractions. On this basis, we can exclude the use of acetone.

TABLE 2 | Formazan recovered (±SE, μg mL^{-1}) from milled 100%-viable and -dead batches of *Hordeum vulgare* (cv. Optic) seed that had been incubated with TTC.

Method number	Sources	Extraction solvent	Seed type	Formazan recovered	
				μg mL^{-1}	% Variation
I	Harty et al., 1972	Methanol*	Viable	39.3 ± 3.3	8.31
			Dead	13.7 ± 0.9	6.23
III	Zhao et al., 2010	10% TCA/acetone	Viable	16.2 ± 7.0	43.32
			Dead	6.6 ± 1.5	22.70
IV	This study	10% TCA/acetone	Viable	47.0 ± 7.8	16.65
			Dead	16.6 ± 7.4	44.84
V		10% TCA/methanol*	Viable	45.6 ± 7.4	16.30
			Dead	13.2 ± 1.1	8.31
VI		Acetone	Viable	40.3 ± 7.9	19.61
			Dead	17.0 ± 5.8	33.88

*Formazan extraction used Methods defined in order (as **Table 1**), with: I, c.f. Harty et al. (1972), methanol; III, c.f. Zhao et al. (2010), 10% TCA/acetone with 5 min. extraction time at room temperature (21°C); IV, modification of Zhao et al. (2010), (hatched bar); V, 10% TCA:methanol; and, VI, acetone. Also shown is the % Variation in the quantity of formazan extracted from replicates (n = 3), for each Method with either 100%-viable or -dead seeds. The data distinguish methods which provide data of low variability at the point of optical density measurement, and these are denoted "*." All extractions were carried out by incubation at 30°C for 15 h with the exception of Method III which used 5 min. at 21°C. Data was not acquired for Method II (Norton, 1985), though how Method II may perform under out test conditions is illustrated using Method VI.*

It is also noted that optical densities recorded in the 100% dead-seeds samples is related to pigments removed from seeds during extraction, and the resultant extract appears yellow, not red. These values present a background OD that is unrelated to metabolism but should be taken into account when calculating formazan production. Furthermore, we advocate that seeds are bleached and sterilised with 3% hydrogen peroxide (H_2O_2), prior to the TTC application. This sterilising treatment excluded the possibility of TTC reduction due to microbial contaminants (Peters, 2000) and also reduced the colour of the barley seeds testa, thus suppressing the otherwise higher background-OD level (data not shown).

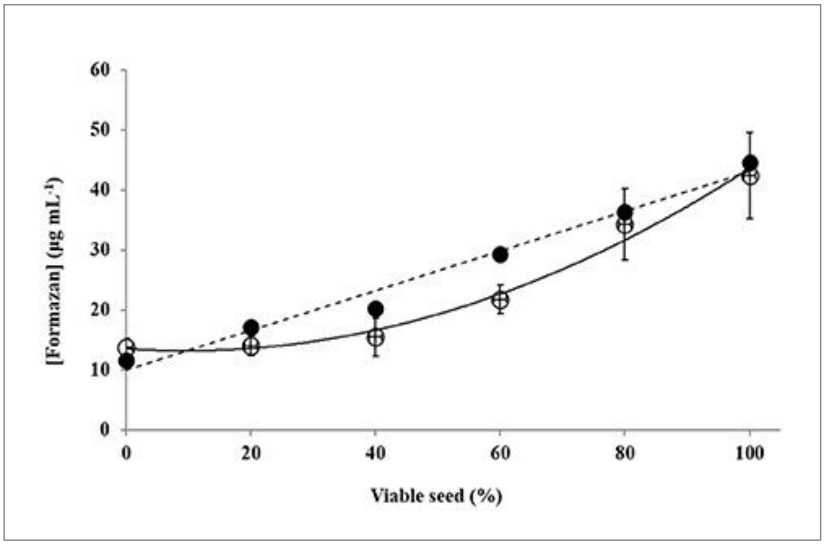


FIGURE 1 | A comparison between concentration of formazan that was extracted (±SE, µg mL^{-1}) from the TTC-incubated seed homogenate using either Method I of Harty et al. (1972; ◦, methanol) and Method V (•, 10% TCA:methanol; see Table 1). Each method allowed the fitting of polynomial- (—, solid-line) and linear- (- - -, dashed line) models, respectively.

The two methanol based methods were therefore used to assess the relationship between the concentration of formazan recovered and seed lots standardised to contain set portions of viable and dead seeds (Figure 1). The Harty-Method showed a polynomial relationship (Figure 1; $y = 0.0037x^2 - 0.0711x + 13.526$; $R^2 = 0.9851$; $P < 0.001$), while Method V (10% TCA:methanol), identified a linear relationship (Figure 1; $y = 0.3316x + 9.9714$; $R^2 = 0.9714$; $P < 0.001$). The variation in the OD for these two methods were also compared (Table 2), and showed no significant difference. This highlighted that the Harty-method had no power to discriminate between seed batches containing 0–40% live seeds, as the curve (Figure 1), was effectively flat in that range. Using Method V the concentration of formazan (µg mL^{-1}) extracted from the barley seed standards (Figure 2) was quantified using standards which fitted a linear model ($y = 0.0272x - 0.0166$; $R^2 = 0.9987$; $P < 0.001$, data not shown). Regression analysis of data for samples "proportion of viable barley seeds in the seed-lot tested" and "concentration of formazan" showed a statistically significant linear relationship (Figure 2; $y = 0.0318x + 1.1615$; $R^2 = 0.9804$; $P < 0.001$). These linear relationships between seed viability and higher recovery of formazan (Figure 1) using Method V (10% TCA:methanol), and across all viable: dead-seed proportions (Figure 2), means that we recommend 10% TCA:methanol for formazan extraction.

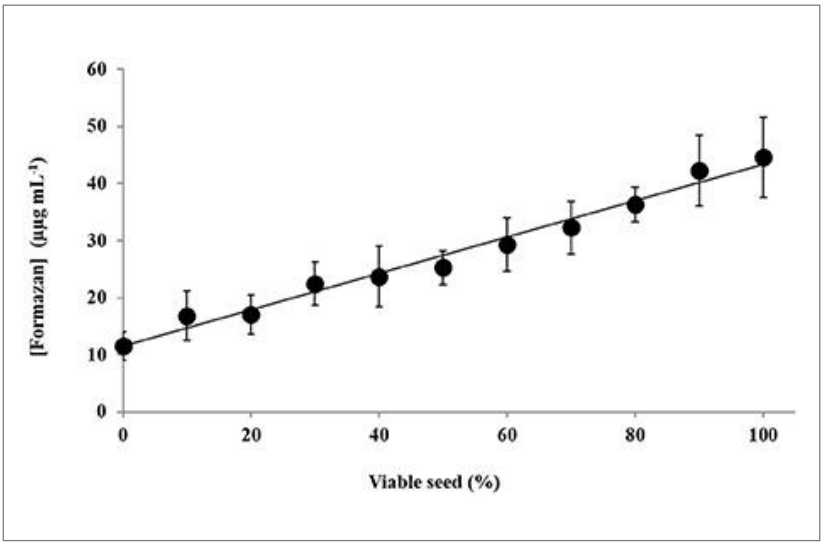
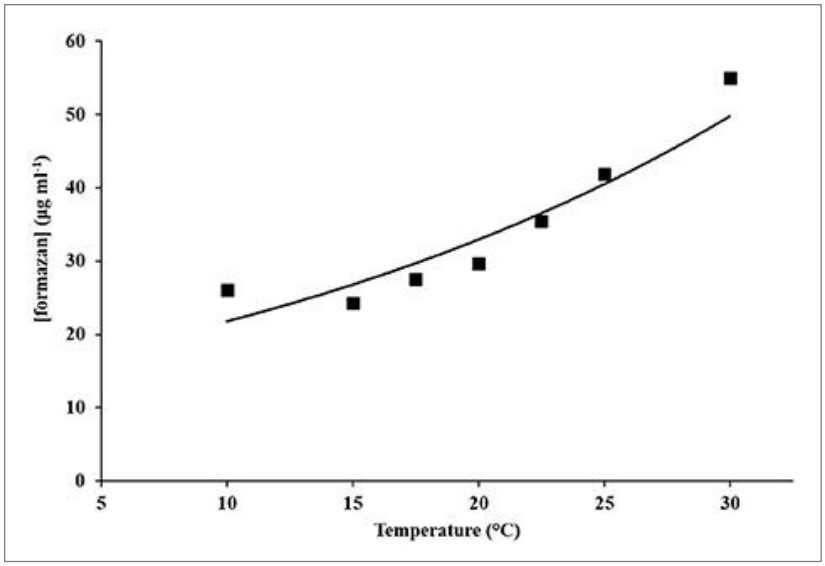


FIGURE 2 | Data acquired using Method V (10% TCA:methanol; see Table 1) showing the quantity of formazan extracted (•, µg mL^{-1}) plotted against the proportion of viable seeds in the test material. The solid line shows the linear-model which was fitted.

Method V was also applied to examine the relationship between the concentrations of formazan recovered from 100% viable seed lots and formazan concentration extracted from seeds whose response was controlled using a temperature series (Figure 3). This analysis found a significant linear relationship between the concentration of formazan which could be recovered and log final germination at the different temperatures (Figure 3; $y = 14.414e^{0.0413x}$, $R^2 = 0.8646$; $P < 0.001$). These results demonstrate that a linear model can also be used to predict seed temperature response (final germination) from formazan concentration over that temperature range for which their relationship is monotonic: which for the barley seeds used here was 10–30°C. At a high temperature, reactivation of metabolism in more rapid and this is reflected in a high formazan extraction. Nevertheless, at high temperatures percentage of germination is low, because of thermo-inhibition. Therefore, germination and metabolic reactivation are uncoupled events. This counter-intuitive result demonstrates the importance of temperature control.

FIGURE 3 | The relationship between temperature of germination (°C) vs. average concentration of formazan extracted (▪, µg ml^{-1} ± SE).

Standard errors were smaller than the symbols.



Method V was then applied to seeds after controlled aging, which showed a significant linear relationship between the concentration of formazan recovered and log final germination percentage of the seed batches with varying viability (Figure 4; $y = 10.185x + 41.711$, $R^2 = 0.9335$; $P < 0.001$).

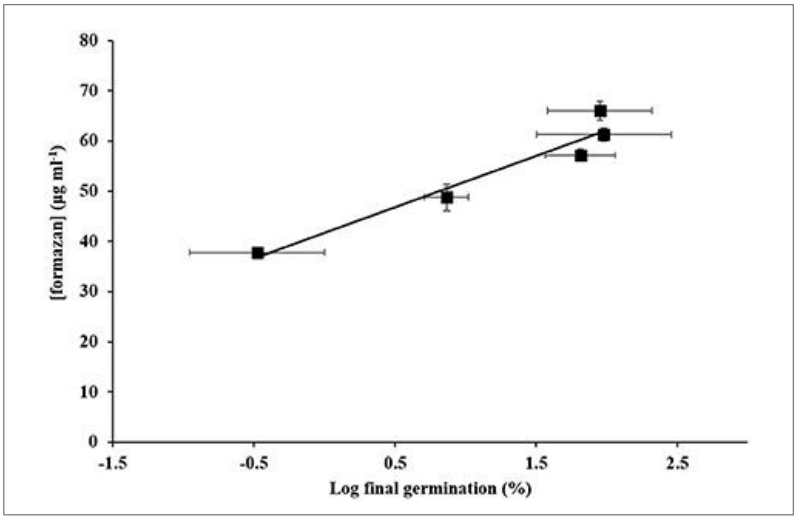


FIGURE 4 | The relationship between final germination after controlled ageing and formazan extracted: log final germination (%) vs. concentration of formazan extracted (▪, µg ml^{-1} ±SE).

The data reported here highlights the risks of using acetone to test viability of bulk seed lots. A standard operating procedure is suggested and this was described in the Table 3.

This provided a detailed standard operating procedure for the 10% TCA:methanol based technique, and it represents a significant development of Harty et al. (1972). A justification of the steps involved is given in the Discussion which follows.

TABLE 3 | A standard operating procedure for the spectrophotometric assay to test the viability of seed batches: using minimum of three technical replicates of barley (*Hordeum vulgare* cv. optic) seed as a model.

1. Place the seeds between water saturated tissue paper to imbibe over-night (ca. 16 h) in a sealed container.*
2. Remove any seeds with protruded shoot-born roots.
3. Dry the seeds and transfer a fixed weight (7.5 g, *ca.* 100 seeds), to a 50 mL container.
4. Bleach/sterilise the seeds by soaking in 7 mL 3% hydrogen peroxide (Sigma-Aldrich, #H1009) for 10 min.
5. Wash 2 times with 20 mL of sterile distilled water.
6. Dry the seeds with tissue paper before grinding in blender for 1 min (James Martin by Wahl ZX595 Mini Grinder, 150 W)
7. Transfer all the seed flour to a fresh 50 mL tube.*
8. Add 15 mL of TTC (Sigma-Aldrich, #T8877) in 1% [w/v] with 5mM potassium phosphate buffer, ph 7.2 (Peters, 2000).
9. Homogenise by vortexing for 15 s.
10. Incubate for 4 h in darkness at room temperature (21°C).*
11. Centrifuge at 5,100 rpm for 5 min. (Sigma 4K-15) and remove the supernatant[a].
12. Suspend residue in 20 mL sterile distilled water and re-centrifuge (as 11); repeat this step.
13. Transfer the residue to filter-paper (Whatmann No.3) on a Buchner-funnel[b].
14. Remove excess moisture from the residue under vacuum.
15. Place the entire residue into a sterile container to dry over-night (21°C).
16. Grind the whole dried residue to a powder using a mortar and pestle.
17. Re-grind the residue after the addition of liquid N_2[c].
18. Dry at 30°C 10 min. and decant the fine powder to new 50 mL centrifuge tubes.
19. Add 7 mL of 10% TCA (Sigma-Aldrich, #T6399) [w/v] /methanol and vortex 3 × 30 s.*
20. Incubate the samples overnight (15 h) at 30°C.
21. Centrifuge 15 min. at 5,100 rpm at room temperature (Sigma 4K-15).
22. Remove 2 mL of the extract and transfer to microfuge tube.
23. Centrifuge the microfuge tube for 30 min. at 14,680 rpm at room temperature (Sigma 1-15K).
24. Transfer 1.5 mL of the supernatant to fresh 1.5 mL microfuge tube[d].

OPTICAL DENSITY (OD) DETERMINATION

25. Re-centrifuge 1.5 mL extract for 20 min. at 14,680 rpm at room temperature (Sigma 1-15K).
26. Dispense 300 µl (extract, controls, blanks), to separate wells of flat-bottomed ELISA plate (Nunc MaxiSorp®, manufacturers code 439454).
27. Read the optical density of each at 484 nm (using at ELx800™ Absorbance Reader, BioTek® Inc.).
28. Correct the OD s by subtracting the background (solvent-only/ TTC-untreated controls).
29. Use the average reading of the three technical replicates.

DATA ANALYSIS

30. Correct test data: subtract average background of the exaction solvent-only control.
31. Convert the OD to µg mL^{-1} formazan (Sigma Aldrich #93145) using the linear-model of standard samples[e].

**This highlights steps of the protocol which may need modified and/or standardised for seeds for other species according to their parameters such as thousand seed weight and/or the (metabolically active) embryo weight relative to that of the endosperm.*
[a]Ensure all of the supernatant is removed as this may interfere with OD measurements.
[b]Ensure all the residue is transferred to the filter-paper.
[c]Ensure that there is no loss of material during grinding in the mortar.
[d]Ensure that residue is not re-suspended during pipetting.
[e]The standard samples are prepare by dissolving formazan in extraction solvent.

Discussion

TTC based assays have proven reliable to test viability in plant tissues such as the inner bark tissues of *Quercus serrata* Murray (Shimomura and Hasebe, 2004), roots from Norway spruce (*P. abies* (L) Karst (Ruf and Brunner, 2003), grape roots (*Vitis vinifera*, Comas et al., 2000) among others. However, the use of a spectrophotometric assay for testing of seed viability and germination response to temperature is not commonly used. It would appear that the profusion of information from the use of TTC to test viability and temperature response in other fields of biology is married to a lack of unanimity among seed-based tests. It is therefore important that a standard protocol that provides greater clarity is established. Among the variables, we acknowledge that TTC incubation time may vary from 2 to 3 h (for barley, Grzybowski et al., 2012), to 24 h or more (e.g., maize, Zhao et al., 2010). In addition, TTC concentration may range from 0.1% (e.g., Zhao et al., 2010), to 1% (Harty et al., 1972; Norton, 1985), and both these variables may correlate with formazan production (Steponkus and Lanphear, 1967; Harty et al., 1972; Mikuła et al., 2006). Thus, in order to obtain an optimal formazan production and facilitate the high-throughput nature of the assay: incubation of pre-soaked ground seeds in 1% [w/v] TTC in potassium phosphate buffer for 4 h provided the optimal balance of rapidity with high levels of formazan production. The pH of the TTC solution and the temperature at which it was administered was also standardised to improve the reliability, reproducibility and accuracy of the method. The Tetrazolium Testing Handbook (Peters, 2000), establishes a range of acceptable pH's which range from 6.5 to 7.5, and temperatures ranging from 20 to 40°C, and the standard protocol described here falls within the limits recommended.

The formazan produced in the staining reaction is water-insoluble and so the moisture content of the samples may affect the extraction efficiency. Harty et al. (1972) used the Buchner funnel to reduce the moisture after washing the samples. However, the moisture remains within the residue and this may influence the extraction efficiency. For this reason, the method we describe here also involved drying the residue after filtration. Additionally, the efficiency of formazan extraction is also dependent on the extent to which the stained tissue is disrupted and so grinding tissues after solvent application is recommended (Ruf and Brunner, 2003). Therefore, we explain that the stained tissues were re-ground to a very fine powder using a mortar and pestle with liquid-N_2 prior to the addition of solvent.

The choice of extraction-solvent used is also very important and the most commonly reported solvent is ethanol (Ruf and Brunner, 2003; Shimomura and Hasebe, 2004; Ghaly and Mahmoud, 2007), followed by methanol (Harty et al., 1972), acetone (Norton, 1985) and 10% TCA/acetone

(Zhao et al., 2010). However, while ethanol has the lowest vapour pressure supporting low variability during spectrophotometrics, it is also least efficient at extracting formazan (Harty et al., 1972; Zhao et al., 2010). While this inefficiency may be compensated using heat (above 80°C; Steponkus and Lanphear, 1967; Stattin and Lindström, 1999; Verleysen et al., 2004; Mikuła et al., 2006), the method also releases compounds which lead to high background and/or high ODs at 484 ηm which are not related to formazan production (Ruf and Brunner, 2003). As a consequence ethanol was discarded as a suitable solvent.

In the previous studies (Harty et al., 1972; Zhao et al., 2010), TTC reduction was also assessed in seed-batches of mainly viable seeds. We therefore considered it important that this study assess the accuracy and reliability of the methods by examining variation between samples containing high proportions of dead seeds too. Towards that end, it was also necessary that the presence of pigments which may confound the accuracy of the test be limited and the use of seed-bleaching also helped in this respect too. The presence of TCA in the extraction solvent appears to help considerably in this respect, most likely as a function of its capacity to help precipitate proteins and degrade other polar particulates that would otherwise have reduced the optical densities recorded (Figure 1). Most significantly, we found that the method proposed by Harty et al. (1972; methanol only), cannot distinguish between seed-batches containing <40% viable seeds (Figure 1). Efficient TTC reduction was demonstrated to represent seed viability of aged seeds on a logarithmic scale, allowing the use of this assay in predicting viability of unknown samples due to ageing.

The findings described here also demonstrate that reduction of TTC is dependent upon seed incubation temperature, and that reduced formazan production may be the result of either an ineffective temperature control or/and reduced viability. However, the choice of barley seeds and the cultivar Optic in particular is not insignificant in this regard. Optic is a popular variety of choice by the growers and whisky distillers for its capacity for complete and uniform germination (99.9% viability for the batch used here), which are essential attributes for cropping and malting (respectively), and is used as a standard control variety to improve this crop for the brewing and distilling industries (c.f. Booer and HGCA, 2001; Koliatsou and Palmer, 2003). The consistent performance of cv. Optic in seed tests reported here, is also reflected in response curves to the temperature treatments that were imposed (Figure 3). This illustrates that the reduced formazan staining is a function of incubation temperature due to: (1) delayed development of metabolic activity including dehydrogenase enzyme activity at lower temperatures, and; (2) slower imbibition at lower temperatures, resulting in delayed development of metabolic activity (e.g., Patanè et al., 2006).

We conclude therefore that 10% TCA:methanol appears superior to any acetone and methanol-only based methods. The 10% TCA:methanol (Method V), based method we describe (Table 3) allowed: (1) the highest recovery of formazan; (2) the lowest levels of background stain which was detected at 484 ηm, and especially in samples containing high levels of dead seeds; (3) minimum variation between technical replicates that could occur on spectrophotometric recording; (4) the fit of a linear-model to accurately capture the relationship between the levels of formazan extracted and seed viability, and; (5) allowed a log-linear model to accurately capture the relationship between the level of formazan extracted and germination in response to temperature.

The seed-batch method proposed here requires as little as 5 of manual work for 20 samples, independent of the number of seeds in each sample. In a standard TTC assay where seeds are cut in half to assess viability of the embryo, up to 1 h is required *per* sample of 100 seeds (M. Marin, personal communication, November 30, 2016). However, this time may vary due to seed size, quality of the seed lot, and the individual performing the assessment. Furthermore, the method presented here it is objective and does not need of specialised training, as OD of extracted formazan indicates the viability of the seed batch. Additionally, dissected seeds do not need to be assessed under a microscope. Thus, the method we present has the potential to be less laborious, and provides an objective assessment based on a large number of seeds.

Future work should develop the improved method described here to test the utility of the method to predict the viability of seed batches for a greater variety of species and seed types. In the case of small seeds, sufficient weight (numbers) may be required to obtain a sufficient quantity of formazan, even in the minimum volume necessary (60 µl), for OD measurements in an ELISA-plate reader. On this basis, we highlight that the seed weight (number) volumes used may need to be standardised relative to important seed parameters such as thousand seed weight, or metabolically active embryo to endosperm ratio.

Author Contributions

LL performed the final assessment of the method we present and led the final drafting of the manuscript. Her efforts have proven the utility of our method to assess seed viability, temperature response and seed vigour in aged seeds. DN carried out the laboratory work and methodological development on live and dead seeds, and drafted the initial version of the manuscript. His work highlighted the confounding effects of extraction protocol, and in particular solvent choice. VM is a research assistant who had a working knowledge of barley seed testing and supported DN in the

execution of the laboratory based tasks. Victor also helped develop the optimised standard operating procedure (SOP). PT is a molecular seed biologist who helped conceive the original idea in academic discussions with PPMI, and was pivotal in developing and conceptualisation the data for peer-review. PI conceived the original idea for this project in discussions (with PT). He was the Principal Investigator who led and oversaw the laboratory work, and helped finalise the manuscript and SOP. All authors contributed to the writing of the manuscript, approved the final version, and agree to be accountable to all aspects of the work.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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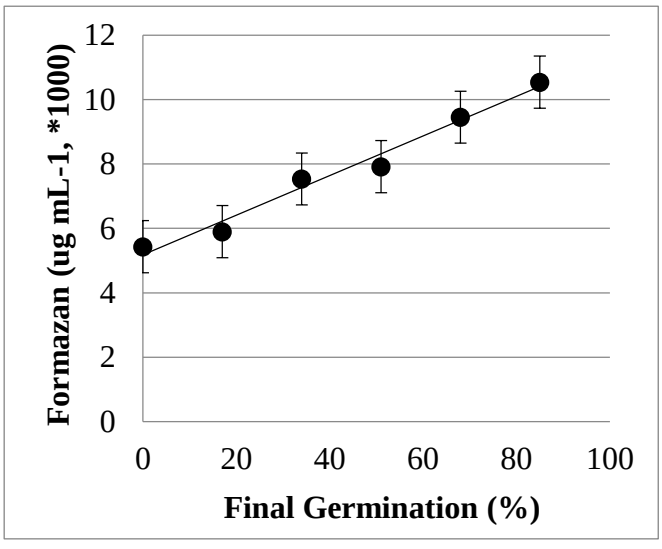
Supplementary material of unpublished data

Materials and Methods

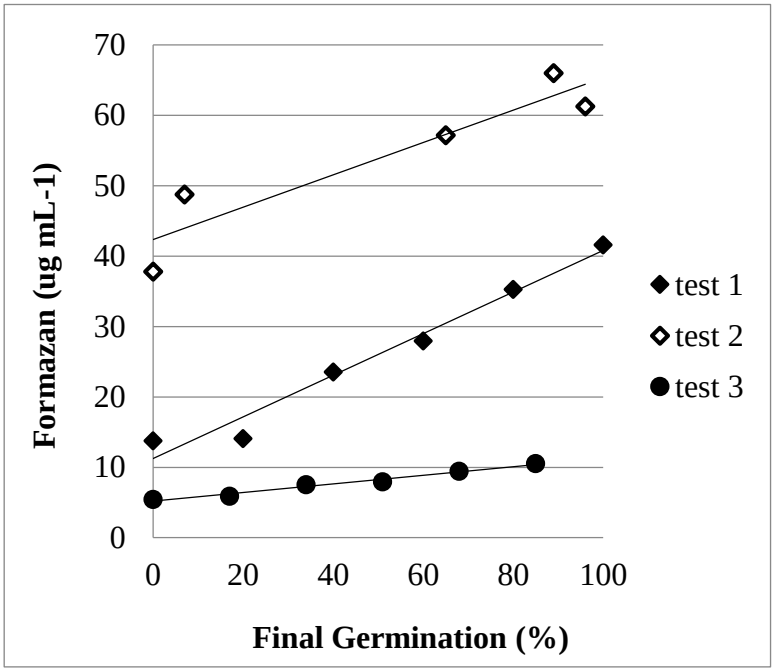
Initial tests assessed batches containing mixtures of viable (85% germination), and dead (by dry autoclaving at 120°C, 20 min) *Viola cornuta* seeds. A series of standard test samples were prepared (in triplicate) by combining viable:dead seeds to a total of 200 seeds (*ca*. 0.16 g). The inclusion of dead seeds [w/w] was either: 0, 17, 34, 51, 68, or 85%.

Results and Discussion

Using Method V the concentration of formazan (μg mL^{-1}) extracted from the *Viola* seed standards were quantified (Supplementary Fig. 1). Regression analysis of data for samples "proportion of viable *Viola* seeds in the seed-lot tested" and "concentration of formazan" showed a statistically significant linear relationship (Supplementary Fig. 1; $y = 0.0615x + 5.1832$; $R^2 = 0.9782$; $P < 0.05$). This means that we recommend 10% TCA:methanol for formazan extraction. The results of the *Viola* tests are compared to the barley tests (Supplementary Fig. 2). Note that the scale of the values for formazan extracted is much lower in the *Viola* tests, this is due to the fact that the amount of *Viola* seed per sample was much smaller (0.15g for *Viola versus* 7g for Barley).



Supplementary Figure 1. Data acquired using Method V (10% TCA:methanol; see Table 1) showing the quantity of formazan extracted (•, μg mL−1) plotted against the proportion of viable seeds in the test material. The solid line shows the linear-model which was fitted. Note that formazan values have been multiplied by 1000. Comparable to Figure 2 of the published paper.



Supplementary Figure 2. Comparison among the three tests performed: Test 1: Barley test (dead seeds obtained by autoclaving; $y = 0.296x + 11.231$; $R^2 = 0.9715$); Test 2: Barley test (dead seeds obtained by controlled ageing; $y = 0.2297x + 42.371$; $R^2 = 0.8711$); and Test 3: Viola test (dead seeds obtained by autoclaving; $y = 0.0615x + 5.1832$; $R^2 = 0.9782$).

Chapter 3

Responsiveness of 23 species to a range of seed enhancing compounds

Seed Enhancing Treatments: Comparative Analysis of Germination Characteristics of 23 Key Herbaceous Species in European Restoration Programs

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Abstract

The response of seeds from 23 wild plant species to a range of seed enhancing treatments was studied in order to assess the potential of treatments to improve germination in restoration projects. These treatments were exposure to the smoke-derived compound karrikinolide (KAR_1), potassium nitrate (nitrate), and to plant growth regulator gibberellic acid; all described as cues of germination in native species. We tested the hypothesis that sensitivity of the 23 species with different dormancy classes to these compounds is related to their ecological niche. The three ecological niches we considered were open land, open-pioneer and woodland, each with its own characteristics. Hence, the germination of a species will be adapted to different light conditions and other environmental signals related to the niche. As representatives of environmental signals, the effects of KAR_1, nitrate and GA_3 on germination were studied. Two of these signals are associated with a reduced competition (KAR_1 and nitrate). We also investigated the effect of different light regimes (constant light, absence of light and 12h photoperiod) and compounds on germination parameters, which included final germination, germination rate and uniformity of germination. The results showed a wide variation of responsiveness of the different species to the three compounds, which was also affected by the light conditions. No interaction was found between responsiveness to compounds and ecology group. Additionally, no single treatment increased the germination of all the tested species, indicating that different species require unique treatments to improve germination. However, final germination and germination rate were affected by light conditions which relates to the ecology of the species.

***Keywords*:** enhancing treatments, germination, ecological niche, karrikinolide, potassium nitrate, gibberellic acid, seeds, restoration

Introduction

Plants perceive biotic and abiotic signals from their surroundings (Segarra et al., 2006; Wasternack, 2007). As a consequence, they have elaborated sophisticated signal transduction networks to respond to their changing environment (Forcat et al., 2008). Decisive moments in the plants' life cycle are germination and seedling establishment. Hence, controlling germination in relation to time, abiotic and biotic environment is critical for the success of the individual. The main factors that influence germination and plant development are abiotic and they include light, temperature, water status and nutrients (Koller and Hadas, 1982; Karssen and Lacka, 1986). However, plants also use biotic signals to "decide" whether to germinate (Topham et al., 2017). In this latter case, plants and seeds use abiotic signals such as nitrate (NO^-_3), karrikins (KAR), ethylene and red/far red ratio as indicators for their biotic environment (Vázquez-Yanes and Smith, 1982; Pons and van der Toorn, 1988; Pons, 1989; Alboresi et al., 2005; Fujita et al., 2006; Nelson et al., 2012; Waters et al., 2013).

Germination is a complex process. It commences with the uptake of water by the quiescent dry seed, followed by the mobilisation of reserves and it is completed when the embryo extends and breaks through the structures that surround it (Bewley and Black, 1994). However, seeds have elaborated mechanisms to spread germination, seed dormancy being one of them. The definition of seed dormancy is complex and dormancy appears to be a poorly understood phenomenon (Hilhorst, 1995; Bewley, 1997; Nonogaki et al., 2010); yet a "dormant" seed could be defined as a viable seed that does not germinate given a specific period of time although favourable environmental conditions are present (Bewley, 1997). Dormancy is an ecological strategy that allows seeds to synchronise germination with the optimal season for the seedlings to survive. Hence, this strategy is linked to the habitat constrains in which the species grow and is the key mechanism regulating establishment and environmental opportunities. However, in agriculture and restoration seeds are used under conditions that deviate from their usual natural habitat and establishment of a high percentage of seedlings is desired. Therefore, seed dormancy can constitute a problem, as dormancy in a seed batch leads to low germination and poor seedling establishment, and thus, to economic loss (Finch-Savage, 1995) and increased risk of failure of a restoration effort. Hence, like agriculture, restoration might require seed enhancing treatments in order to improve germination (Baskin and Baskin, 1998; Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006).

Nitrate has been suggested to be a germination cue in situations of high nutrient availability. Such a condition appears nearby agricultural fields due to the application in fertilisers (Swaine and

Whitmore, 1988; Scholefield et al., 1993; Sekhon, 1995) or in canopy gaps created by treefall, which provides litter (Dalling and Hubbell, 2002) or a fire event. This is because nitrate is taken-up by the plants, therefore high availability of nitrate in the environment may suggest minor competition. However, different soil types vary in their level of nutrients (Vitousek, 1984; Hooper et al., 2005; Tittonell et al., 2005) and, therefore, of nitrogen.

In regard to smoke-derived compounds, karrikins and specially the butenolide 3-methyl-2H-furo[2,3-c]pyran-2-one, termed karrikinolide (KAR_1) (Merritt et al., 2007; Dixon et al., 2009), have been successfully isolated from smoke (Smith et al., 2003; Nelson et al., 2012) and shown to be the major active compound to promote germination (Smith et al., 2003; Flematti et al., 2004; van Staden et al., 2006; Flematti et al., 2009). This compound has been described to stimulate germination of fire dependant and independant species (Flematti et al., 2004). Karrikinolide is active at concentrations as low as 10^{-9} M (Flematti et al., 2004; Stevens et al., 2007; Soós et al., 2012; Waters et al., 2014). KAR_1 is suggested to act via the gibberellins (GAs) synthesis pathway in *Arabidopsis*, by first binding to the KARRIKIN INSENSITIVE 2 receptor, which is a protein in the family of α/β -hydrolases; and subsequently, this complex binds to the F-box protein MAX2 (Waters et al., 2013; Waters et al., 2014). One of the hypotheses is that this complex interacts with proteins in the gibberellin signalling pathway (Janssen and Snowden, 2012; Meng et al., 2016). Others suggest that KAR_1 triggers GA biosynthesis by enhancing the expression of GA biosynthetic genes (Nelson et al., 2009). KAR_1 is naturally produced during wild fires and degradation of plant material; processes which can be related to open spaces (Grubb, 1977). Therefore, KAR_1 may also be present in habitats not associated with wild fires, such as alpine meadows and KAR_1 can be considered a signal for new open spaces, absence of competition and nutrient availability (Grubb, 1977; Chiwocha et al., 2009; Morffy et al., 2016; De Cuyper et al., 2017).

Gibberellins (GAs) are endogenous growth factors of plants and have been described to play a key role in germination (Achard et al., 2008; Harberd et al., 2009). As described by Karssen and Lacka (1986), germination is regulated by the interaction of promotive hormones, such as gibberellins, and the inhibitory hormone, abscisic acid (ABA); and changes in the balance of these promoters and inhibitors may result in germination or no germination. Environmental signals affect this balance (Bassel, 2016; Topham et al., 2017) and as such affect the decision. The application of exogenous GA_3 interferes with the endogenous control system and leads to increased levels of internal GA, which influences the GA/ABA balance towards germination (Karssen and Lacka, 1986; Finch-Savage and Leubner-Metzger, 2006; Nonogaki et al., 2010). Several factors trigger GA synthesis, including light and temperature (Derkx and Karssen, 1993; Olszewski et al., 2002;

Mutasa-Göttgens and Hedden, 2009). GA has been reported to substitute for light requirement, chilling and for after-ripening, and therefore, stimulate germination (Donoho and Walker, 1957; Fogle, 1958; Toole and Cathey, 1961; Curtis and Cantlon, 1963). Hence, GA_3 may be able to stimulate germination in the absence of light in species that require light to germinate.

Light quality plays a major role in the development of the plant (Fankhauser and Chory, 1997). In order to respond adequately to quality, quantity and the direction of light plants (and seeds) have evolved a large set of receptors. Phytochromes, chlorophyll, cryptochromes and phototropins are each specific for the perception of specific wavelengths (Briggs and Olney, 2001). Phytochromes (P) are able to sense light and have two different chemical conformations that are inter-convertible (Butler et al., 1959; Koning, 1994). These two forms are named by the colour of light they have maximal absorbance at: Pr absorbs red light (660 nm) and Pfr absorbs far-red light (730 nm) (Smith, 1975; Morgan and Smith, 1976). After the absorption of red light, which triggers a conformational change in Pr the phytochrome changes into Pfr (Sharrock, 2008). Far-red causes the phytochrome to be converted back to Pr configuration (Butler et al., 1959). Cryptochromes and phototropins are known to be the two types of blue light receptors in plants (Lin, 2002). The quality and amount of light that the plants perceive in the different ecosystems may vary. For instance, in woodlands, under canopy, the red/far-red ratio is lower than in open habitats. Hence, light requirements for plant growth and development and, thus, germination and seedling establishment per species might vary.

In open landscapes there is a high availability of light (Niinemets and Kull, 1994), while light is limited in the woodland environments (Harrington et al., 1989; Clark et al., 1996). Therefore, open-land or ruderal species are adapted to high levels of light (Grime, 1977). Open-pioneer or shade-intolerant species (Brokaw, 1987) could be described as a subclass within "open-field species", but they have additional characteristics (Coley, 1983; Dalling and Hubbell, 2002): they require light to germinate (Bazzaz, 1979; Whitmore, 1989; Kettenring et al., 2006), they can appear at the early succession stage, they have rapid germination, they are fast growing, they have high rates of dry-matter production (Baker, 1965) and they tend to have a bigger seed size (Dalling et al., 1998; Jensen and Schrautzer, 1999). On the contrary, woodland or shade-tolerant species can survive under the canopy and exhibit slow growth (Grime and Hunt, 1975).

Because of the diversity in types, quality, quantity and combination of signals in the various ecology types, requirements for germination must vary across species from different ecology types (Vázquez-Yanes and Smith, 1982; Brokaw, 1987; van der Meer et al., 1998). Note that here, ecology type and habitat are used indistinctly. Hence, interaction and integration of the different

signals can be expected and is needed to time germination. The response to these signals may also be related to the disturbance processes that occur in the ecosystem, such as response to smoke-derived compounds after a wild fire (Dixon et al., 1995), response to nitrates in areas of agricultural land abandonment (Grantz et al., 1998) or response to GA due to internal or external factors, such as after ripening or light incidence after the creation of a gap in the canopy (de Souza and Válio, 2001).

Germination characteristics of some cultivated species have been investigated for years (Maguire, 1962; Tekrony et al., 2005; Willenborg et al., 2005; Magneschi and Perata, 2009) specially for the interest in agriculture (ISTA, seed companies). However, little is known about the effect of seed enhancing treatments on germination of wild species. Therefore, in this work we study germination characteristics of 23 wild species in order to relate germination responsiveness to the application of GA_3, KNO_3 and KAR_1 with the habitat of the species. We investigate germination of species of different ecologies, which include open-field, open-pioneer and woodland species.

Materials and methods

Plant material

Viola arvensis Murray

Seeds of field pansy (Bachthaler et al., 1986) grown under glasshouse conditions were obtained in 2014 from Syngenta (Enkhuizen). In order to produce the seeds used in this study, plants were grown in the local glasshouses (The Netherlands) and fruits were collected in July 2015. The pods were placed for 2 weeks in a drying room at an average of 20-25°C with ca. 40% RH. Seeds were cleaned and stored at 15°C, 30% RH. Seeds were used for germination tests approximately 6 months after harvest.

Other species

Seeds from the other species were obtained from Scotia Seeds, a native Scottish seed producer (Brechin, UK). Seeds had been harvested from the wild and produced as crops for the number of generations as specified (Table 1).

Table 1. Species used in this study. Table contains species name, species abbreviation (ID), species family, dormancy type, year of harvest, source of seeds (producer) and number of generations for seed production.

Species name	ID	Family	Dormancy type	Year of harvest	Producer	Number of Generations[2]
Achillea millefolium	Am	Asteraceae	PD	*2015*	SSE[1]	NA
Campanula glomerata	Cg	Campanulaceae	PD	2015	SSE	3
Fragaria vesca	Fv	Rosaceae	PD	2014	SSE	2
Mentha aquatica	Ma	Lamiaceae	PD	2014	SSE	1
Papaver rhoeas	Pr	Papaveraceae	MPD	2013	SSE	14
Pimpinella saxifraga	Ps	Apiaceae	MPD	2015	SSE	4
Primula veris	Pve	Primulaceae	PD	2015	SSE	4
Primula vulgaris	Pvu	Primulaceae	PD	2014	SSE	3
Urtica dioica	Ud	Urticaceae	ND	2010	SSE	1
Geranium robertianum	Gr	Geraniaceae	PY + PD	2014	SSE	3
Lathyrus pratensis	Lp	Fabaceae	PY	*2015*	SSE[1]	NA
Lotus corniculatus	Lc	Fabaceae	PY	2015	SSE	3
Malva moschata	Mm	Malvaceae	PY	2015	SSE	4
Medicago lupulina	Ml	Fabaceae	PY	2009	SSE	5
Potentilla erecta	Pe	Rosaceae	PD	2014	SSE	1
Trifolium pratense	Tp	Fabaceae	PY	2014	SSE	3
Vicia sativa	Vs	Fabaceae	PY	2013	SSE	5
Senecio vulgaris	Sv	Asteraceae	PD	2004	SSE	0
Stellaria graminea	Sg	Caryophyllaceae	PD	2014	SSE	1
Plantago lanceolata	Pl	Plantaginaceae	PD	2013	SSE	4
Viola arvensis	Va	Violaceae	PD	2015	Syngenta	NA
Viola palustris	Vp	Violaceae	PD	2015	SSE	1
Viola riviniana	Vr	Violaceae	PD	2015	SSE	1

[1] Outsourced from Scotia Seeds (SSE); unable to retrace the harvest year.
[2] After collection from the wild.
NA stands for not available.
Note the difference between "2015" and "*2015*". The first is the actual year of harvest, while the second is the year the seed lot was obtained by SSE from another producer.

Dormancy type: Non-dormant (ND), Physiological (PD), Morphophysiological (MPD), Physical (PY) and Combinational (PY + PD). Baskin and Baskin 1998, 2004.

Germination experiments

Twenty five seeds were used *per* replicate and a total of 4 replicates per treatment and species were sown. Custom made transparent and black six-compartment trays (Voges Verpakking bv, Hillegom, The Netherlands) were used for the germination tests; the former trays were used for experiments with light and, the latter, for experiments in darkness. Trays contained six 75 cm^2 wells, 2.5 cm deep with lid. Each well contained an 64 cm^2 18-layered water absorbent paper (Zell-Pak) covered by an 64 cm^2 12 Steel-Blue Seed Germination paper (Anchor Paper Co., USA) and wetted with 28 mL of demineralised water or solutions as indicated. Trays were closed with a lid

to avoid evaporation and placed in a 20°C cabinet (IVL Van Leeuwen, The Netherlands). During incubation, germination was scored as radicle emergence twice a day for the first six days of germination and, subsequently, once a day until germination ceased. This was considered when no germination occured in the following 7 days. Germinated seeds were removed after they were scored. Germination was scored when the radicle protruded >1 mm. For the samples germinated under dark conditions germination was scored in a room under safe green light until the end of the test. *Potentilla erecta*, *Urtica dioica* and *Mentha aquatica* were tested at 25°C for the full test set as it was demonstrated that seeds of these species germinated poorly at 20°C (Brändel, 2006; Taylor, 2009). Light was applied by LEDs (660 nm) in the case of continuous light or by fluorescent tubes (Lumilux OSRAM L36W/840) in the case of a day and night rhythm. Seeds in constant darkness were transferred to constant red light when germination had ceased. This treatment is labelled as "DL".

Some species were dormant and required pre-treatments to break dormancy and allow germination. These were scarification using sand paper for *Lotus corniculatus, Trifolium pratense, Medicago lupulina, Malva moschata, Vicia sativa* and *Geranium robertianum*; incising with a scalpel for *Lathyrus pratensis*; and stratification for 7 days at 5°C for *Plantago lanceolata.* In the case of large scale restoration, species that require scarification would need to be prepared differently, as sand paper and use of scalpel are time consuming methods. Instead, seeds may be placed in a "coating pan" with sand paper attached.

Seed were germinated on water, 10 mM KNO_3, 1 mM GA_3 or 1 µM KAR_1. The 1 µM KAR_1 solution was prepared by diluting stock solution of 50 mM KAR_1 in DMSO 50,000-fold in water. The treatments were tested under three different light conditions: constant red light (RL), constant dark (D) and a photoperiod of 12h white light and 12h dark (WLD) and, additionally, constant red light after germination had ceased in constant darkness (DL, for FG analysis only).

At the end of the germination experiments, non-germinated seeds were cut open and only apparently viable seeds were used to calculate germination parameters and to perform statistical analysis. Seed batches do sometimes contain a large amount of empty or non-viable seeds, thus in order to assess the potential of the treatments to enhance germination, non-viable seeds were discarded from the analyses.

The following species were not included in the germination rate analysis due to: 1) very low final germination, or 2) very rapid germination, which resulted on scoring final germination on the first scoring data point; in these two cases, germination rate could not be calculated. Some species were not included under any of the light conditions (*Senecio vulgaris*, *Primula veris*, *Urtica dioica* and

Viola palustris), some others were only excluded for the dark treatment (*Campanula glomerata*, *Potentilla erecta* and *Viola riviniana*). Similarly to the analysis of germination rate, the following species were not included in the uniformity analysis. Some species were not included under any of the light conditions (*S. vulgaris*, *U. dioica* and *V. riviniana*), some others were only excluded in the dark treatment (*C. glomerata*, *Fragaria vesca*, *Primula vulgaris*, *Potentilla erecta* and *Mentha aquatica*).

Seedling quality

Seeds were placed on paper with 1 mM GA_3, 1 µ KAR_1, 10 mM KNO_3 solutions and water in the standard germination trays; with three replicates of 25 seeds per experimental treatments and species. Trays were placed in customised climate rooms (Nijssen Koeling BV, Leiden-Holland) at constant 20°C under white light (fluorescent tubes; Lumilux OSRAM L36W/840) with a 12h/12h photoperiod and 95% relative humidity. Germination was assessed as in the previous experiment, but seeds were not removed after germination. Seedling quality was assessed once, 4-6 days after final germination (FG), depending on the species at the stage of open cotyledons. All seedlings from all treatments belonging to one species were assessed at the same time. Seedlings were taken from the germination trays and arranged on a pre-wetted blue paper 15 x 25 cm, together with a colour palette and a ruler. High resolution photographs were taken and images were analysed using Image J (Schneider et al., 2012), with the smart root plug-in (Lobet et al., 2011; Fig. 1).

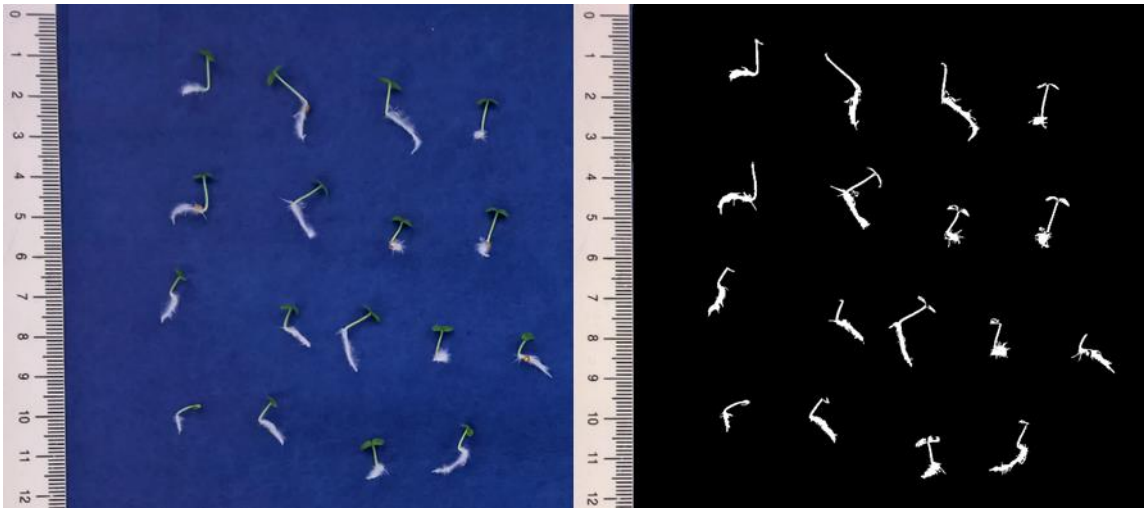

Figure 1. Seedlings of *Viola arvensis* eight days after sowing. White light (left) and processed (right) images; converted images were used for the analysis.

Statistical analysis

Final germination percentages (FG), time required for 50% of viable seeds to reach germination (t50) and time interval between 84% and 16% of viable seeds germinated (U_{8416}) were calculated using Microsoft Excel 2013 and Germinator software (Joosen et al., 2010) respectively. No t50 or U_{8416} were calculated in some cases due to very low final germination. Germination rate (GR) was later calculated by taking the reciprocal of t50. Relative uniformity (RU) was assessed rather than absolute uniformity (U). RU was calculated by dividing U_{8416} by t_{50}. The reasoning behind this is that RU is independent from germination rate and, therefore, it explains better how uniform the individual seeds from a seed batch are.

All statistical analysis were performed in R (R Core Team, 2016). First, final germination data of the various treatments was analysed separately for each species and light conditions using GLM with binomial error distribution and logit link function, in order to identify significant effects of the treatments. Germination rate and uniformity data were analysed with one-way analysis of variance, using the Kruskal-Wallis rank sum test (Hollander and Douglas, 1973). Further Mann-Whitney-Wilcoxon Test analyses were performed for germination rate data when Kruskal-Wallis test appeared significant (Hollander and Douglas, 1973). In order to perform analysis with quantitative (FG, GR and RU) and qualitative variables (ecology type), factor analysis of mixed data (FAMD) were performed using the FactoMineR package (Lê et al., 2008). Data on uniformity of germination (RU) was analysed, however, due to missing values because of final germination being lower than 10%, open-pioneer and woodland groups only contained one species. Therefore, results of this type of analysis were not conclusive.

For the treatments with lack of binomial variation, such as 0 or 100% for final germination, data was manually transformed to 1 or 99% in order to be able to include these results in the Generalised Linear Models (GLM).

Results

Relative uniformity (RU) was analysed, but we found no significant effect. In the FAMD with FG and GR of species clustered in relation with the species ecology (Fig. 2), dormancy type (Fig. 3A) and established strategies (Grime et al., 1988; Fig. 3B). Both FG and GR were further analysed for effects of light and compound treatments. All germination data is presented in the supplementary material (Table S1, Figure S1), while Table 2 and 3 show summarised data.

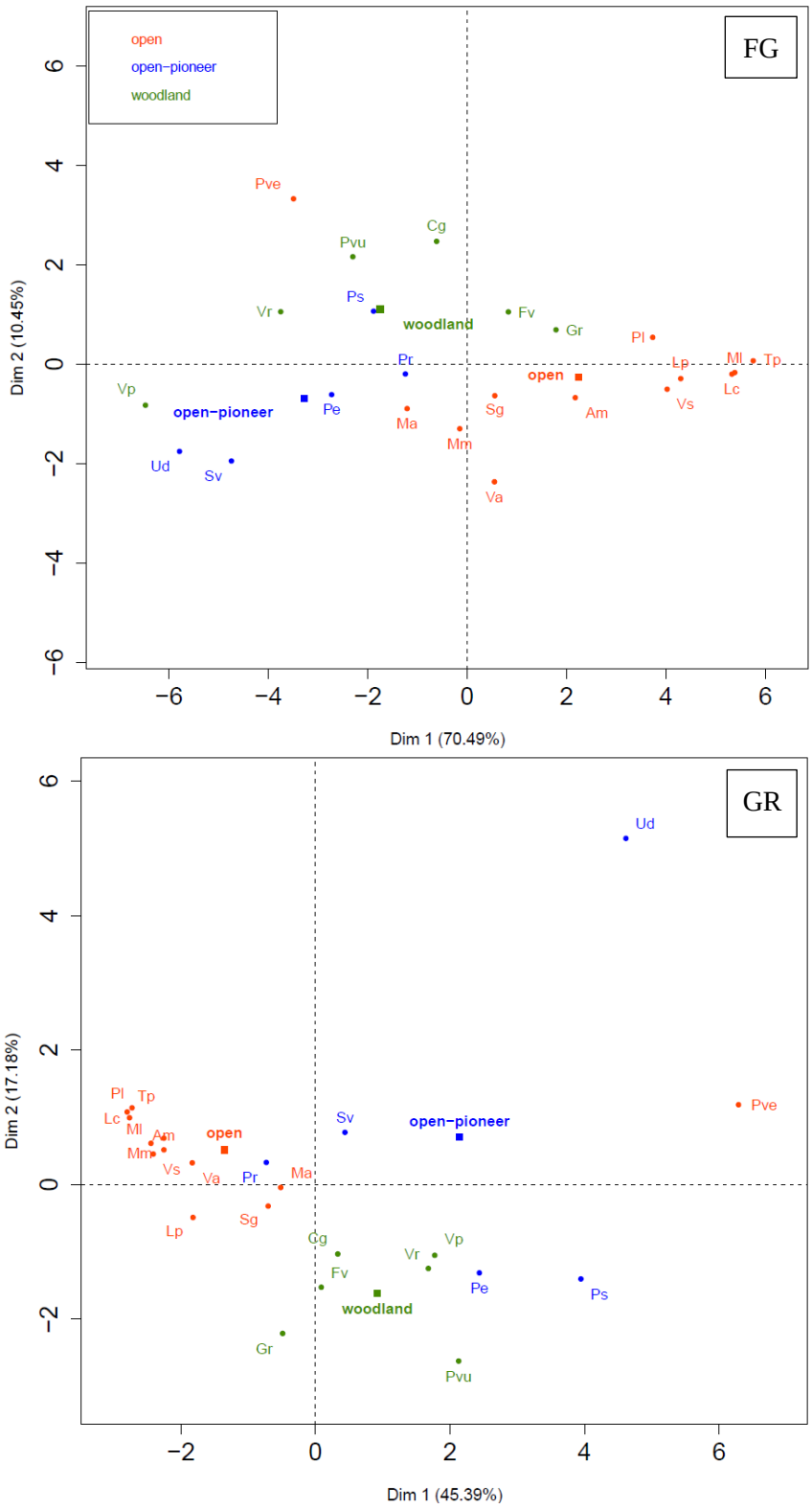

Figure 2. Individual factor map for final germination (FG) and germination rate (GR). Analysis included FG and GR of the 23 species mentioned in Table 1, under the three light conditions (constant red light, constant darkness and 12h white light photoperiod). For FG analysis, data from constant red light after germination ceased in constant darkness was also included. Analysis also included data on germination with the application of four compounds (GA_3, KAR_1, KNO_3 and water). Species are coloured by ecology type: open (red), open-pioneer (blue) and woodland (green).

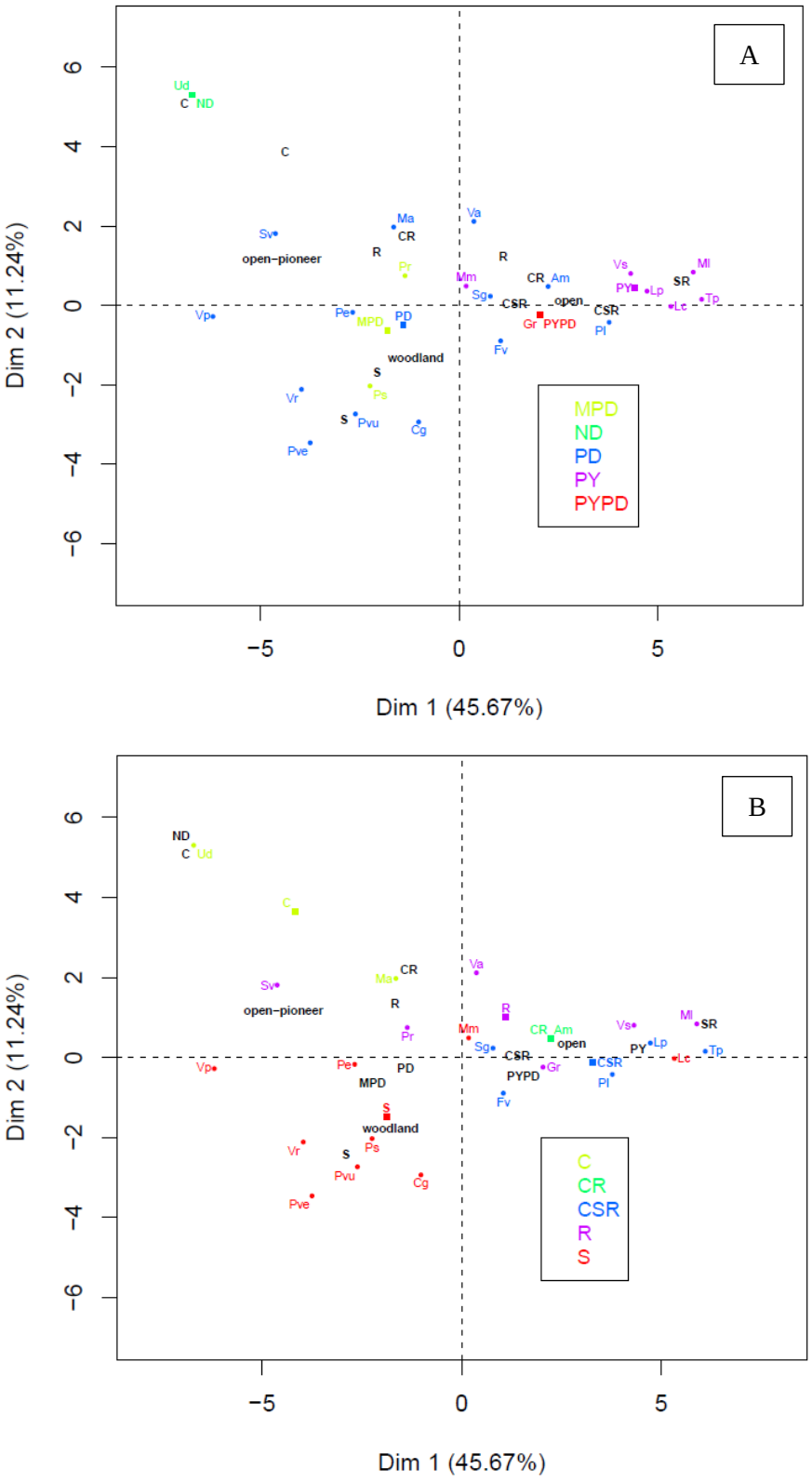

Figure 3. Individual factor map for final germination (FG). Species are coloured by dormancy type (A): Morphophysiological (MPD, yellow), Non-dormant (ND, green), Physiological (PD, blue), Physical (PY, magenta) and Combinational (PY + PD, red) and by established strategy (B): competitor (C, yellow), competitive-ruderal (CR, green), C-SR strategist (CSR, blue), ruderal (R, magenta), stresstolerator (S, red). Analysis included FG of the species in Table 1, under the three light conditions (constant red light, constant darkness and 12h white light photoperiod). Analysis included data on germination with the application of four compounds (GA_3, KAR_1, KNO_3 and water).

Effect on final germination (FG)

The results showed a wide variation of responsiveness to the three compounds and light conditions for the different species. We discriminated four groups when considering responsiveness to compounds on FG: 0) no effect of the treatments was observed at any of the light conditions; 1) compounds stimulation of germination occurred only in darkness; 2) compounds stimulated germination in all three light conditions; and finally 3) nitrate stimulated germination in constant and alternating light conditions (Table 2 and Supplementary Table 1).

Table 2. Final germination (FG, percent) data. FG in three light conditions: constant red light (RL), 12h photoperiod with white light (WLD) and constant darkness (D), and differences between light treatments (RL-D, WLD-D, RL-WLD). Ecology types: open (O), open-pioneer (OP) and woodland (W). No effect of the compound (o). Significant stimulation or inhibition (-) of FG by the different compounds is shown for: gibberellic acid (G), karrikinolide (K) and potassium nitrate (N). Cells from columns 6-8 are coloured from blue to red to represent increasing FG values. Cells from columns 9-11 are coloured in black if FG was higher under constant darkness, in grey if higher in the WLD condition and red if FG was higher in RL. Species full name (Table 1). FG values under the three stimulatory compounds is shown in Supplementary Table 1.

species	ecology	RL compound stimulation	WLD compound stimulation	D compound stimulation	FG in RL	FG in WLD	FG in D	RL-D	WLD-D	RL-WLD	group	FG summary
Ud	OP	N	N	o	4	21	2	3	20	-17	3	α
Sv	OP	o	o	o	5	9	5	0	3	-3	0	α
Vp	W	o	o	o	2	2	1	1	1	0	0	α
Mm	O	o	o	o	50	35	57	-7	-22	15	0	γ
Lc	O	o	o	o	90	96	95	-4	2	-6	0	β
Tp	O	o	o	o	94	98	95	-2	3	-4	0	β
Ml	O	o	o	o	96	96	97	-1	-1	0	0	β
Vs	O	o	o	o	97	83	97	0	-14	14	0	β
Lp	O	o	o	o	97	94	96	1	-2	4	0	β
Pl	O	o	o	G	93	90	90	3	0	3	1	β
Am	O	o	o	GNK	84	85	73	11	12	-1	1	β
Gr	W	o	o	GN	91	87	46	45	40	5	1	β
Sg	O	o	o	GN	80	75	29	52	47	5	1	β
Fv	W	o	o	GNK	80	84	23	57	62	-4	1	β
Ma	O	GN	NK	GNK	46	33	18	28	15	13	2	γ
Pvu	W	GN	GN	G	47	42	22	25	20	5	2	γ
Cg	W	G	GK	G	65	77	26	39	51	-11	2	γ
Vr	W	GK	GNK	G	19	42	9	11	34	-23	2	γ
Pe	OP	GK	G	G	24	34	6	18	29	-11	2	γ
Ps	OP	GN-	GN-K-	G	42	49	20	22	28	-7	2	γ
Pr	OP	GN	G	G	48	24	34	14	-10	23	2	γ
Va	O	K-N	GN	GNK	66	23	68	-2	-45	43	2	γ
Pve	O	G	G	G	25	25	25	0	-1	1	2	γ

Legend for column 12 and 13:

Group
- 0 no response
- 1 only response in the D
- 2 response to compounds in all three light conditions
- 3 response to KNO_3 in light conditions

FG summary
- α low FG in RL
- β high FG in RL
- γ intermediate FG in RL

Group 0

Within this group we observed two types of absence of response to compounds. Either final germination under control conditions was already close to 100%, thus no room was left for GA_3 stimulation, this happend for the physical dormant species tested (*L. corniculatus, T. pratense, M. lupulina, V. sativa* and *L. pratensis*) or germination was low under control conditions (*S. vulgaris, V. palustris* and *M. moschata*). Germination dependency to light varied between being not sensitive (*S. vulgaris, V. palustris, L. corniculatus, T. pratense, M. lupulina,* and *L. pratensis*) and light/dark photoperiod being inhibitory (*V. sativa* and *M. moschata*).

Group 1

This group was characterised by species which responded to compounds only in the absence of light. All the species in this group presented physiological dormancy. In this group germination in the presence of any light in the water treatment was greater than in darkness (*P. lanceolata, A. millefolium, G. robertianum, S. graminea* and *F. vesca*). For all these species except for the two later, germination in the presence of any light was also higher in the presence of chemical treatments.

Group 2

This group was characterised by species being responsive to several compounds in the three light conditions. This was in line with the fact that FG values were low and intermediate under control conditions. Therefore, there was room for germination improvement. In this group, the germination of the species varied among light conditions; in some cases germination was reduced by darkness (*P. vulgaris, C. glomerata, P. saxifraga, M. aquatica* and *P. erecta*), germination was optimal in light/dark photoperiod (*C. glomerata*, *V. riviniana, P. erecta* and *P. saxifraga*), germination was inhibited in light/dark photoperiod (*V. arvensis* and *P. rhoeas*) or insensitive to light (*P. veris*).

Group 3

Urtica dioica was considered an independent case due to the fact that its germination could only be stimulated by nitrate when in the presence of any light (continuous and alternating). Light photoperiod played an important role in germination of this species as final germination was > 5 times higher in this condition compared to the constant light and darkness conditions, where FG did not reach 5% (Supplementary Table 1).

FG was significantly different between germination of species in the three ecology groups (Fig. 4 and 5; $P < 0.001$). FG of open land species was in average the highest, followed by woodland species and lowest for the open-pioneer species. Moreover, FG of species from open habitats was not affected by the light condition ($P > 0.001$), while FG of open-pioneer and woodland species was light dependent ($P < 0.001$). In both open-pioneer and woodland species, germination was lower in the dark ($P < 0.001$). However, no differences were observed between RL and DL in the open-pioneer species. For woodland species this was not the case ($P < 0.05$). Moreover, no differences existed on the effect of compounds to final germination among the three ecology types, that is, germination of species in the three groups were stimulated by GA_3 only (Fig. 5).

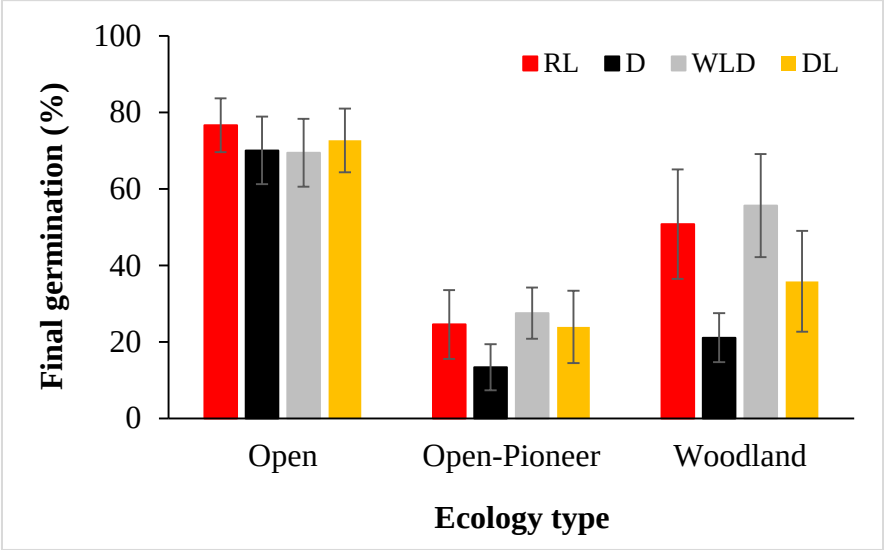

Figure 4. Final germination (percentage ± SE) of tested species under various light conditions grouped by ecology type. The three ecology types are: open, open-pioneer and woodland; for each of the four light conditions (constant red light, RL; constant darkness, D; 12h white light photoperiod, WLD; constant red light after germination ceased in constant darkness, DL). DL includes FG in D.

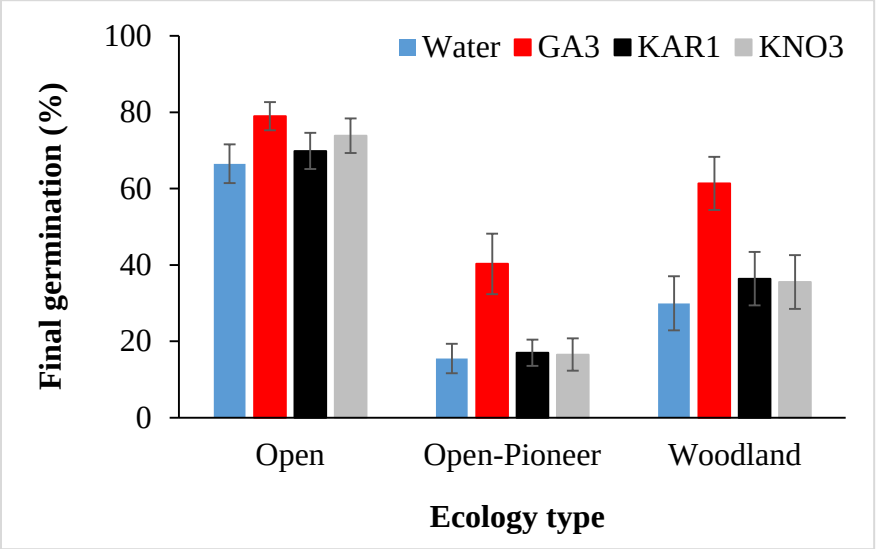

Figure 5. Final germination (percentage ± SE) of tested species with various compounds grouped by ecology type. The three ecology types are: open, open-pioneer and woodland; for each of the four compounds: water, gibberellic acid (GA_3), karrikinolide (KAR_1) and potassium nitrate (KNO_3).

Effect on germination rate (GR)

Compounds affected germination rate of 7 out of the 23 species, mainly by GA_3. Species could be classified in two groups accordingly to their responsiveness: 0) no effect or 1) effect of compounds increased germination rate.

Table 3. Germination rate (GR, /day) data. GR under three light conditions: constant red light (RL), 12h photoperiod with white light (WLD) and constant darkness (D), and differences between light treatments (RL-D, WLD-D, RL-WLD). Ecology types: open (O), open-pioneer (OP) and woodland (W). No effect of the compound (o). Significant stimulation or inhibition (-) of GR by the different compounds is shown for: gibberellic acid (G), karrikinolide (K) and potassium nitrate (N). Cells from columns 6-8 are coloured from blue to red to represent increasing GR values. Cells from columns 9-11 are coloured in black if GR was higher under constant darkness, in grey if higher in the WLD condition and red if FG was higher in RL. Species full name (Table 1). GR values under the three stimulatory compounds is shown in Supplementary table 1.

species	ecology	RL compound stimulation	WLD compound stimulation	D compound stimulation	GR in RL	GR in WLD	GR in D	RL-D	WLD-D	RL-WLD	group
Ud	OP	o	o	o	<10*	<10*	<10*	<10*	<10*	<10*	0
Sv	OP	o	o	o	0.11	0.33	0.13	-0.03	0.20	-0.22	0
Vp	W	o	o	o	0.06	<10*	<10*	<10*	<10*	<10*	0
Mm	O	o	o	o	0.58	0.46	0.41	0.17	0.05	0.12	0
Tp	O	o	o	o	1.09	0.77	0.83	0.25	-0.07	0.32	0
Ml	O	o	o	o	0.61	0.56	0.88	-0.27	-0.32	0.05	0
Vs	O	o	o	o	0.84	0.43	0.6	0.24	-0.17	0.41	0
Lp	O	o	o	o	0.29	0.19	0.27	0.02	-0.08	0.10	0
Sg	O	o	o	o	0.16	0.2	0.14	0.03	0.06	-0.03	0

Fv	W	o	o	o	0.11	0.16	0.11	0.01	0.06	-0.05	0
Ma	O	o	o	o	0.21	0.27	0.14	0.07	0.13	-0.06	0
Cg	W	o	o	o	0.19	0.21	0.14	0.05	0.07	-0.02	0
Vr	W	o	o	o	0.19	0.23	<10*	<10*	<10*	-0.04	0
Pe	OP	o	o	o	0.05	0.06	<10*	<10*	<10*	-0.01	0
Pr	OP	o	o	o	0.38	0.32	0.26	0.12	0.06	0.06	0
Va	O	o	o	o	0.33	0.28	0.34	-0.01	-0.06	0.05	0
Ps	OP	G	o	o	0.08	0.06	<10*	<10*	<10*	0.02	1
Pve	O	G	o	o	<10*	<10*	<10*	<10*	<10*	<10*	1
Pvu	W	G	o	G-	0.05	0.07	0.51	-0.46	-0.44	-0.03	1
Gr	W	o	GK	o	0.14	0.07	0.15	-0.01	-0.08	0.07	1
Am	O	o	o	GK	0.64	0.47	0.26	0.38	0.22	0.17	1
Lc	O	o	N-K-	GK	0.73	0.77	0.54	0.19	0.23	-0.04	1
Pl	O	o	GK	GNK	0.82	0.73	0.39	0.43	0.34	0.08	1

* "< 10" indicates final germination being lower than 10%, therefore data could not be calculated.

Group 0

The species in group 0 did not respond to compounds. Within this group, species could be further classified into slow germinating (low germination rate; *Lathyrus pratensis, Stellaria graminea, Fragaria vesca, Senecio vulgaris, Viola palustris, Mentha aquatica, Campanula glomerata, Viola riviniana, Potentilla erecta, Papaver rhoeas* and *Viola arvensis*) and fast germinating (high germination rate; *Urtica dioica, Malva moschata, Trifolium pratense, Medicago lupulina* and *Vicia sativa*). This group included the three species in the Violaceae family.

Group 1

The species in group 1 responded to at least one compound in any light condition. From the species that responded to treatments, three were affected only in the dark (*A. millefolium, L. corniculatus* and *P. lanceolata*) and had fast germination in all light conditions (GR > 0.64/day). Three others were only affected in constant light (*P. saxifraga*, *P. veris* and *P. vulgaris*) and had slow germination (GR < 0.15/day). The GR of *G. robertianum* was stimulated by GA_3 and KAR_1 only under variable light conditions.

Germination rate was significantly different between species in the following ecology groups (Fig. 1): open *vs* open-pioneer species and open *vs* woodland species ($P < 0.001$). No differences were observed between open-pioneer *vs* woodland species ($P > 0.05$). Moreover, germination rate within each of the three ecology groups was not affected by the compounds ($P > 0.05$). All the species within each ecology group showed a similar response in germination to light conditions ($P > 0.05$; Fig. 6), except for woodland species. However, germination rate is significantly higher in

the species from open habitat compared to the low germination rate for the open-pioneer and woodland species ($P < 0.001$; Fig. 6). Furthermore, no differences in germination speed existed on the effect of compounds among the three ecology types (Fig. 7).

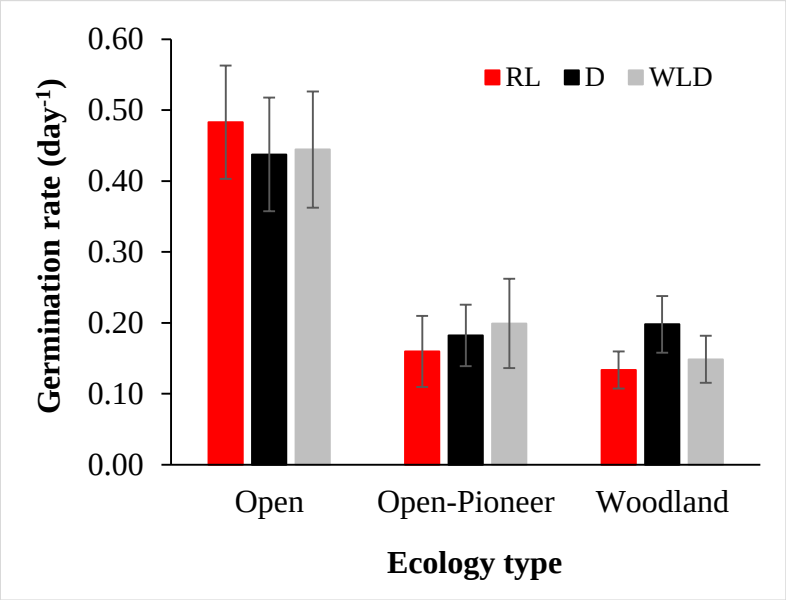

Figure 6. Germination rate (day^{-1} ± SE) of tested species under various light conditions grouped by ecology type. The three ecology types are: open, open-pioneer and woodland; for each of the four light conditions (constant red light, RL; constant darkness, D; and 12h white light photoperiod, WLD).

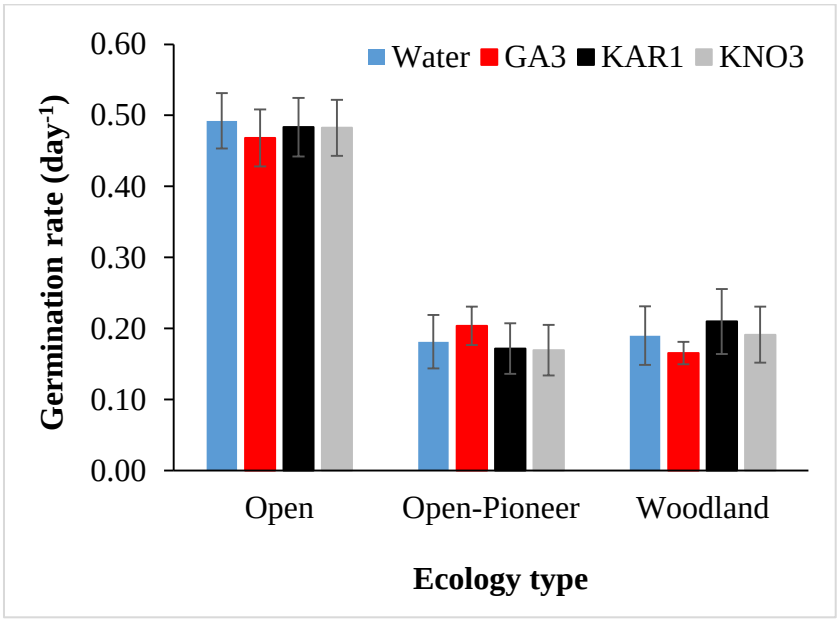

Figure 7. Germination rate (day^{-1} ± SE) of tested species with various compounds grouped by ecology type. The three ecology types are: open, open-pioneer and woodland; for each of the four compounds: water, gibberellic acid (GA_3), karrikinolide (KAR_1) and potassium nitrate (KNO_3).

Effect on seedling quality

Positive response to treatments on final germination tended to also have a positive effect on seedling establishment. However, results on the quality of seedlings showed a different response to the treatments in some species. In the case of *Viola arvensis*, for example, seedlings are significantly shorter with the application of nitrate ($P < 0.05$; Fig. 8); however seeds germinated to 72 ± 18% mean final germination compared to the control, where germination was of 4 ± 4% mean. Seedlings from other species, such as *Trifolium pratense* and *Medicago lupulina* also displayed elongated seedlings with GA_3 application (results not shown), but no effect was found on seedling quality with nitrate application.

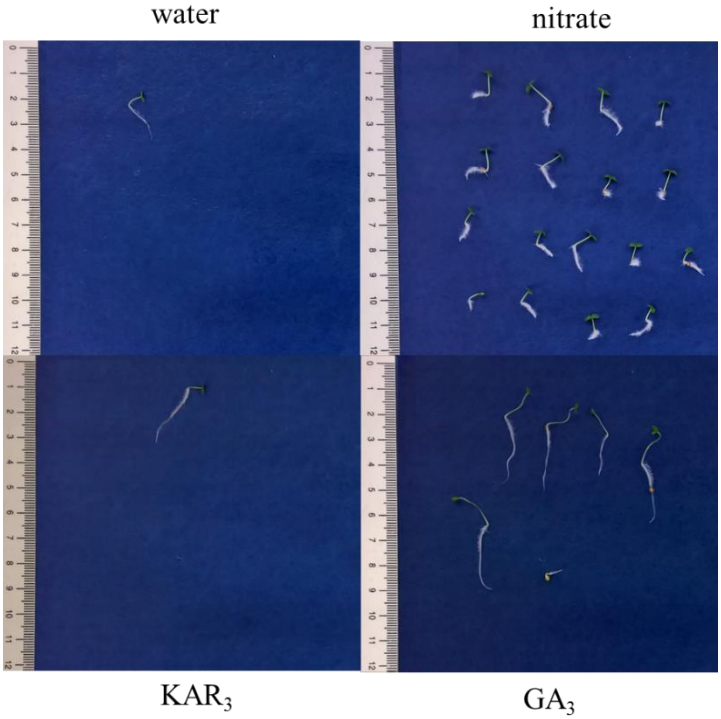

Figure 8. White light images of seedlings of *Viola arvensis* eight days after sowing. Seeds were treated with: water, 10 mM nitrate, 1 mM gibberellic acid (GA_3) or 1 µM karrikinolide (KAR_1).

Discussion

We propose that at least three signals are indicative for the environment in relation with plant establishment and, consequently, with germination requirements: GA_3, nitrate and KAR_1. Therefore, the species would follow different strategies in response to these treatments. GA_3 has been described to stimulate germination by changing the hormone balance of germination promoters and inhibitors (Karssen and Lacka, 1986). This change in GA_3 content happens because proteins in the GA_3 pathway act as a hub to integrate signals from the environment (Golldack et al., 2013; Pozo et al., 2015; Topham et al., 2017). Therefore, when seeds perceive environmental condition that would allow the seedling to survive, signals are integrated and GA is synthesised. Moreover, Baskin & Baskin (2004) has described the use of GA_3 to break physiological dormancy.

Nitrate indicates highly nutritious soils; and in woodlands, where there is a strong competition for nutrients, nitrate levels are low (Pons, 1989). Therefore, high levels of nitrate in the soil can be indicative of a gap in the vegetation (Pons, 1989; Ritter and Vesterdal, 2006). That can be explained by the fact that when a lot of vegetation is present, demands for nitrate are high and the soil gets depleted in this nutrient, amongst others. KAR_1 is one of the active ingredients in smoke, which is a signaling compound that has been isolated from wildfire smoke based on their ability to stimulate seed germination (Flematti et al., 2013). Not only are they produced by combustion of organic material but also from decomposition of dead vegetation (Dixon, personal communication, March 2017). Therefore, KAR_1 indicates recently opened spaces and organic soil with no competition by other vegetation.

In summary, 15 out of 23 species responded positively to any of the treatments, which had been described to have physiological or morphological dormancy (Table 1, Supplementary Table 1); from the 8 that did not respond to any of the treatments, 5 are species in the Fabaceae family, which physical dormancy had been removed prior seeds were sown and showed already a final germination larger than 90% on water. Germination of most of the species was significantly increased by the application of treatments under dark conditions, especially in the case of GA_3 application. GA_3 increased the final germination of seeds from 14 species (Supplementary Table 2), but it also appeared to inhibit the germination of 1 species. The latter may be due to scoring final germination before seeds reached the plateau, therefore, values of final germination in the GA_3 treatment may appear to be lower than they actually were. GA_3 stimulation is in line with the fact that GA_3 has been described to be able to substitute for requirement of light (Leubner-Metzger, 2003), chill (Baskin and Baskin, 1970) and after-ripening (Leubner-Metzger, 2002), which appears to hold true for some of the tested species. Nitrate stimulated germination of seeds from 10 species (Supplementary Table 2). This is in line with the fact that these species are known to be nitrophiles or to have high Ellenberg N Index values (>6; Pyatt, 1997; Hill et al., 1999; Pitcairn et al., 2006). Surprisingly, nitrate and KAR_1, however, inhibited germination of some of the tested species compared to the water control. This only happened under light conditions and not under constant darkness.

KAR_1 has been described so far as a germination stimulant, usually improving final germination and germination speed of a range of species, these included species from fire-prone and non-fire-prone habitats (Flematti et al., 2004; Waters et al., 2013). In this study, KAR_1 increased the germination of 7 species (Supplementary Table 2). However, we describe the case of *V. arvensis* and *P. saxifraga* species in which karrikin inhibits germination, which has hitherto only been observed in grasses (Long et al., 2011). Additionally, *L. corniculatus* also showed a reduction in

the germination rate with KAR_1. To explain this response we considered the fact that KAR_1 is modified under light should this have an UV component, resulting in KAR-dimers (Stasinopoulos and Hangarter, 1990). However, LED light with no UV component was used for part of the tests, so it is doubtful if the dimers will form. The same KAR_1 solution was tested in *Brassica napus* under the same light conditions and final germination and germination rate were increased (data not shown). Furthermore, Stasinopoulos & Hangarter (1990) tested the effect of the dimers and they found that the dimers are less efficient in activating germination than KAR_1 but do not inhibit. Soós et al., (2012) described that some other smoke-compounds, such as TMB, can inhibit germination, but stimulation of germination still occurred when TMB was applied in combination with KAR_1. In the manuscript from Lopez del Egido et al., (in prep.) on the reduction in final germination in several seed batches of *Viola arvensis* by KAR_1, we discuss that this reduction in final germination might be due to the ecology of the species. Seeds from the three species, which germination is reduced, appear to be agricultural weeds (Smith, 1952; Marshall et al., 2003). Therefore, inhibition of germination by KAR_1 under light condition may signal an undesirable situation for seedling establishment. However, the interaction of KAR_1 and light is complex and would require more careful analysis of the niche in nature, such as investigating the effect of light quality (red/far-red) and quantity from the ecological side; nonetheless, the interaction at a physiological level is of equal importance and would also need to be further investigated.

Stimulation of germination by KAR_1 is explained by KAR_1 stimulating the gibberellins synthesis (Nelson et al., 2009; Waters et al., 2013). The observation that seven species showed an increased final germination both after GA_3 and KAR_1 application (Supplementary Table 2) is in line with this mechanism. Species in which germination was only stimulated by GA_3 must then have lost the sensitivity to KAR_1 or KAR_1 is unable to reach the side of its action. However, species in which germination is only stimulated by KAR_1 implies that KAR_1 affects endogenous GA_3 levels, while exogenous GA_3 is not able to change internal GA_3 levels. In the cases where both GA_3 and KAR_1 application increased final germination or germination rate, the use of nitrate may be recommended, as nitrate would increase final germination and also improve seedlings quality. This is because in some species, such as *Viola arvensis*, seeds treated with GA_3 produced thin elongated seedlings (Marth et al., 1956). Elongated seedlings are associated with cell wall loosening (Kamisaka et al., 1972), which may have negative effects, such as making seedlings weak, more exposed and less resistant (Kamisaka et al., 1972). Other negative effect caused by the application of GA_3 is a less root growth and extension of the vegetative state, which causes plants to flower at a later time (Marth et al., 1956).

In four species both final germination and germination rate increased together compared to the control in water with the application of the compounds, while for most of the species the effect of the compounds was either on final germination or on germination rate alone.

The responsiveness pattern of species to compounds was related to the dormancy type, however, it was not related to the ecological niche. Additionally, no single treatment increased the germination of all the tested species, indicating that different species require unique treatments to improve germination. However, the requirement for light conditions is an important factor, which relates to the ecology type of the species. The species clustered by their ecology type both for final germination and for germination rate (Fig. 2). Moreover, species within a similar ecology type responded to light conditions in a comparable manner (Fig. 4 and 6). Species from open habitats germinate to a high proportion and in a fast way, which is in line with Shipley and Parent (1991). This may be explained by the fact that conditions that allow germination last for a short period of time in an open habitat and, therefore, habitat is more competitive compared to woodland, thus high and fast germination are required to compete with the surrounding vegetation (Kos and Poschlod, 2010). Studies in arid zones (Boeken and Gutterman, 1990) have demonstrated that species from more extreme habitats germinated earlier and faster than those in less extreme habitats. In a woodland habitat, soil conditions, such as temperature fluctuation are dampened by the vegetation (Morecroft et al., 1998; Aanderud et al., 2011). Moreover, soil moisture content and nutrient availability are higher under a forest canopy than in an area with no trees (Klopatek et al., 1998; Niklaus et al., 1998; Conant et al., 2004). This means that moisture in the soil would last longer in woodland habitats, which might may allow the seeds to germinate for a longer period of time, thus no fast germination is required. Contrary to what it is usually described (Botkin, 1981), our results show that pioneer species have a slow germination. However, slow and low germination in this group may be due to the fact that the seeds from 4 out of 5 species in this group either displayed some dormancy (*P. rhoeas*; Milberg and Andersson, 1997; Cirujeda et al., 2006), were inhibited by constant temperatures (*P. erecta*; Grime, 1981; and *U. dioica*; Thompson et al., 1977) or required a chilling period (*P. saxifraga*; Hovstad and Ohlson, 2008). Contrastingly to species in Chilean temperate rain forest (Figueroa and Lusk, 2001), the species we have studied exhibit a relationship between seed and seedling light requirements, understood as requirement for germination and light present in the plant habitat; and, therefore, ecology type can be used to delimit light response in open, open-pioneer and woodland species. Our results are in line with Swaine and Whitmore (1988) in tropical rain forests.

The fact that final germination in darkness and red light differed in the open-pioneer species, but no differences were observed between RL and DL suggests that darkness inhibited germination.

However, germination could be rescued by transferring seeds to red light to the same level of germination as seeds which were germinated in red light from the start of the test. Woodland species also had an increased germination when transferred from darkness to red light (DL), but final germination did not reach the same level as seeds germinated in red light from the start of the test. This suggests that darkness induces secondary dormancy in about 30% of the seeds from woodland species. This difference may be due to the fact that in the two habitats, there is a different proportion of perennial or annual life forms, and, therefore, germination differs. Some studies have shown that in the situation of a fire event, establishment of seedlings of perennial species was higher than those of annual species in woodlands (Hobbs and Atkins, 1991). The same authors described that in open habitats, regeneration of perennials was inhibited by the presence of an abundant annual cover. Moreover, perennial plant species produce seeds more than once in their life (Mooney et al., 1986), therefore they will depend less on a seed set. These have also been described to be short-lived (Stöcklin and Fischer, 1999; Clarke et al., 2000). Annual species, however, will usually reproduce only once; therefore, annual species are more dependent on their seeds to persist in a given environment. In order to persist, annual plants tend to produce higher numbers of seeds in a season, which are longer-lived than perennial species (Primack, 1979; Jurado and Flores, 2005). Thus, in order to maintain a soil seed bank annual species may produce seeds with more selective germination requirements than perennials species (Jurado and Westoby, 1992). To allow this persistence in soil and to spread establishment risks over time, annual species may have developed dormancy to regulate germination in a higher proportion of species than perennials have (Rees, 1996; Baskin and Baskin, 1998; Jurado and Flores, 2005). These results consider a European situation of young and highly disturbed landscapes, therefore, the simplistic model presented here may not apply to highly biodiverse landscapes outside Europe. If the studied species were to be used in ecological restoration, species specific pre-treatments would need to be applied *prior* sowing. This would be GA_3 for species like *P. saxifraga* or *P. veris*, which germination was improved with the application of this treatment; KNO_3 for *V. arvensis* or *S. graminea*; and KAR_1 for *M. aquatica* or *C. glomerata*.

Conclusion

From the results obtained, we conclude that seeds from some of the tested species were stimulated by the application of compounds, while others were insensitive to them. This was related to the dormancy type of the species, however, this response to compounds was not common among the species that share the same habitat. Therefore, species present in a similar habitat do not necessarily germinate in the same way given the germination cues present in the habitat (nutrient availability

and low competition). Instead, germination of species from a similar habitat showed a similar response to light. Other ecological signals may drive germination of the species that did not show a response to treatments (*U. dioica*, *S. vulgare* and *V. palustris*), which would need to be further investigated.

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Supplementary material

Supplementary Table 1. Germination data of seeds germinated under constant red light (RL), under constant darkness (D) and 12h white light photoperiod (WLD). Treatments: water, karrikinolide (KAR_1), nitrate (KNO_3), gibberellic acid (GA_3). Parameters calculated: Final germination percentage of viable seeds (FG); germination rate (GR); and relative uniformity (RU), calculated from dividing t50 by the time interval between 16% and 84% of viable seeds to germinate.

		RL			D			WLD		
sp	**compound**	**FG (%) ± SD**	**GR (1/day) ± SD**	**RU ± SD**	**FG (%) ± SD**	**GR (1/day) ± SD**	**RU ± SD**	**FG (%) ± SD**	**GR (1/day) ± SD**	**RU ± SD**
Achillea millefolium	water	19.9 ± 0.90	0.64 ± 0.29	1.16 ± 0.76	12.0 ± 4.04	0.26 ± 0.05	0.38 ± 0.34	23.0 ± 1.73	0.47 ± 0.02	0.37 ± 0.11
	GA3	22.3 ± 1.70	0.49 ± 0.05	0.73 ± 0.36	21.0 ± 1.73	0.32 ± 0.01	0.38 ± 0.08	18.0 ± 3.00	0.50 ± 0.01	0.40 ± 0.12
	KAR1	20.9 ± 1.95	0.61 ± 0.25	1.17 ± 1.14	21.6 ± 1.99	0.36 ± 0.04	0.67 ± 0.31	21.0 ± 2.65	0.44 ± 0.03	0.50 ± 0.16
	KNO3	20.1 ± 2.54	1.07 ± 1.30	0.96 ± 0.64	17.4 ± 1.13	0.27 ± 0.04	0.36 ± 0.1	23.0 ± 1.00	0.43 ± 0.03	0.46 ± 0.08
Campanula glomerata	water	12.8 ± 4.66	0.19 ± 0.01	0.35 ± 0.12	0.4 ± 0.89	0.14 ± NA	NA	17.7 ± 2.89	0.21 ± 0.01	0.38 ± 0.01
	GA3	20.0 ± 4.24	0.24 ± 0.01	0.29 ± 0.09	22.8 ± 2.39	0.22 ± 0.01	NA	20.7 ± 2.08	0.25 ± 0.01	0.26 ± 0.06
	KAR1	15.4 ± 4.04	0.19 ± 0.01	0.36 ± 0.11	1.6 ± 1.14	0.10 ± 0.00	NA	21.3 ± 1.53	0.23 ± 0.01	0.31 ± 0.02
	KNO3	14.0 ± 2.74	0.17 ± 0.01	0.41 ± 0.11	0.8 ± 0.45	0.13 ± 0.03	NA	17.0 ± 2.65	0.20 ± 0.01	0.27 ± 0.02
Fragaria vesca	water	14.2 ± 3.70	0.11 ± 0.02	1.00 ± 0.58	0.6 ± 0.55	0.11 ± 0.05	NA	17.7 ± 3.79	0.16 ± 0.03	0.61 ± 0.22
	GA3	16.6 ± 1.14	0.13 ± 0.02	1.04 ± 0.35	5.6 ± 2.70	0.08 ± 0.02	NA	16.3 ± 0.58	0.18 ± 0.01	0.67 ± 0.1
	KAR1	14.6 ± 2.19	0.14 ± 0.02	1.12 ± 0.36	9.8 ± 2.77	0.10 ± 0.01	NA	13.0 ± 2.65	0.17 ± 0.01	0.71 ± 0.3
	KNO3	17.8 ± 2.77	0.13 ± 0.02	0.96 ± 0.42	2.4 ± 0.55	0.12 ± 0.02	NA	13.7 ± 2.08	0.13 ± 0.03	1.13 ± 0.17
Geranium robertianum	water	20.6 ± 2.07	0.14 ± 0.05	1.65 ± 0.37	5.6 ± 3.05	0.15 ± 0.04	1.10 ± 0.42	19.7 ± 2.31	0.07 ± 0.01	1.28 ± 0.31
	GA3	20.8 ± 2.68	0.26 ± 0.07	0.93 ± 0.26	18.6 ± 2.70	0.19 ± 0.02	1.01 ± 0.59	20.3 ± 3.51	0.13 ± 0.03	1.17 ± 0.49
	KAR1	20.6 ± 1.52	0.17 ± 0.06	1.48 ± 0.29	7.6 ± 2.19	0.18 ± 0.02	1.12 ± 0.36	18.7 ± 2.52	0.10 ± 0.02	1.49 ± 0.36
	KNO3	21.0 ± 1.22	0.15 ± 0.05	1.23 ± 0.29	11.6 ± 3.21	0.18 ± 0.03	1.15 ± 0.2	19.7 ± 3.21	0.08 ± 0.02	1.42 ± 0.32
Lotus corniculatus	water	19.6 ± 3.05	0.73 ± 0.32	0.50 ± 0.25	21.0 ± 2.12	0.54 ± 0.08	0.52 ± 0.31	20.3 ± 4.04	0.77 ± 0.07	0.46 ± 0.13
	GA3	19.6 ± 2.07	0.92 ± 0.35	0.45 ± 0.28	21.8 ± 1.30	0.78 ± 0.29	0.41 ± 0.28	21.3 ± 1.15	0.78 ± 0.02	0.55 ± 0.34
	KAR1	22.0 ± 1.58	0.65 ± 0.17	0.57 ± 0.47	20.2 ± 3.42	0.72 ± 0.34	0.25 ± 0.13	21.0 ± 1.73	0.66 ± 0.02	0.63 ± 0.18
	KNO3	20.8 ± 2.28	0.74 ± 0.34	0.43 ± 0.28	20.6 ± 0.89	0.91 ± 0.36	0.26 ± 0.08	20.7 ± 3.21	0.66 ± 0.03	0.51 ± 0.06
Lathyrus pratensis	water	23.0 ± 1.22	0.29 ± 0.01	0.58 ± 0.12	22.2 ± 2.39	0.27 ± 0.05	0.62 ± 0.27	23.3 ± 1.53	0.19 ± 0.01	0.68 ± 0.12
	GA3	21.6 ± 1.34	0.29 ± 0.02	0.53 ± 0.07	22.4 ± 1.67	0.33 ± 0.04	0.59 ± 0.29	22.3 ± 1.53	0.21 ± 0.03	0.71 ± 0.11
	KAR1	22.2 ± 0.84	0.27 ± 0.02	0.61 ± 0.11	22.8 ± 1.30	0.25 ± 0.05	0.55 ± 0.24	21.7 ± 2.52	0.22 ± 0.03	0.84 ± 0.07
	KNO3	23.0 ± 2.00	0.25 ± 0.02	0.57 ± 0.15	22.4 ± 1.14	0.28 ± 0.03	0.55 ± 0.07	22.3 ± 1.15	0.19 ± 0.01	0.69 ± 0.13
Mentha aquatica	water	6.8 ± 2.39	0.21 ± 0.05	0.85 ± 0.41	1.4 ± 1.14	0.14 ± 0.03	NA	6.3 ± 0.58	0.27 ± 0.08	0.83 ± 0.45
	GA3	7.4 ± 3.13	0.24 ± 0.08	0.91 ± 0.35	6.8 ± 3.96	0.17 ± 0.06	NA	5.7 ± 0.58	0.26 ± 0.06	1.98 ± 0.77
	KAR1	8.0 ± 3.08	0.23 ± 0.05	1.12 ± 0.75	5.0 ± 2.35	0.16 ± 0.03	NA	11.7 ± 4.73	0.20 ± 0.03	1.05 ± 0.19
	KNO3	7.0 ± 3.00	0.18 ± 0.02	0.61 ± 0.62	4.6 ± 2.97	0.12 ± 0.03	NA	9.3 ± 4.73	0.24 ± 0.07	1.06 ± 0.22
Medicago lupulina	water	22.2 ± 1.79	0.61 ± 0.12	1.22 ± 0.72	22.6 ± 2.30	0.88 ± 0.14	1.64 ± 0.77	20.3 ± 2.89	0.56 ± 0.10	0.74 ± 0.39
	GA3	22.0 ± 1.58	0.51 ± 0.10	1.20 ± 0.65	20.6 ± 2.07	0.70 ± 0.28	1.36 ± 0.81	19.0 ± 3.61	0.51 ± 0.02	0.63 ± 0.09
	KAR1	21.6 ± 1.67	0.56 ± 0.05	1.33 ± 0.39	23.0 ± 1.00	0.85 ± 0.53	0.94 ± 0.69	21.3 ± 2.08	0.57 ± 0.06	0.87 ± 0.11
	KNO3	20.2 ± 1.30	0.44 ± 0.06	0.90 ± 0.23	21.2 ± 0.84	0.68 ± 0.22	1.42 ± 0.89	18.3 ± 2.31	0.42 ± 0.01	0.76 ± 0.17
Malva moschata	water	11.6 ± 3.13	0.58 ± 0.26	2.02 ± 0.88	12.8 ± 2.05	0.41 ± 0.19	2.00 ± 0.54	10.0 ± 1.73	0.46 ± 0.01	0.37 ± 0.24
	GA3	9.6 ± 2.70	0.57 ± 0.18	1.79 ± 0.7	11.0 ± 2.00	0.41 ± 0.14	2.70 ± 2.53	7.7 ± 2.52	0.66 ± 0.09	0.66 ± 0.39
	KAR1	11.2 ± 3.03	0.35 ± 0.12	1.59 ± 1.83	10.4 ± 3.51	0.25 ± 0.14	4.09 ± 2.64	8.3 ± 1.53	0.51 ± 0.17	1.10 ± 0.49
	KNO3	8.2 ± 4.27	0.36 ± 0.11	1.23 ± 1.2	10.0 ± 1.22	0.31 ± 0.07	2.37 ± 1.38	8.7 ± 1.53	0.57 ± 0.14	0.46 ± 0.17

Potentilla erecta	water	3.2 ± 2.59	0.05 ± 0.03	0.72 ± 0.46	0.2 ± 0.45	NA	NA	5.7 ± 2.31	0.06 ± 0.01	0.54 ± 0.14
	GA3	7.8 ± 3.56	0.08 ± 0.02	1.31 ± 0.4	3.4 ± 2.30	0.10 ± 0.01	NA	9.3 ± 0.58	0.08 ± 0.01	1.33 ± 0.23
	KAR1	7.0 ± 2.12	0.06 ± 0.01	1.22 ± 0.53	0.2 ± 0.45	0.08 ± NA	NA	8.0 ± 2.65	0.07 ± 0.01	0.76 ± 0.34
	KNO3	2.2 ± 1.30	0.04 ± NA	0.61 ± NA	0.6 ± 0.55	0.04 ± NA	NA	2.7 ± 1.53	0.05 ± NA	0.38 ± NA
Plantago lanceolata	water	22.6 ± 1.52	0.82 ± 0.68	1.19 ± 1.64	19.2 ± 3.96	0.39 ± 0.03	0.62 ± 0.41	19.3 ± 3.06	0.73 ± 0.01	0.89 ± 0.12
	GA3	23.0 ± 2.00	0.57 ± 0.15	0.42 ± 0.24	24.4 ± 1.14	0.62 ± 0.18	0.81 ± 0.49	21.7 ± 0.58	0.83 ± 0.04	0.27 ± 0.18
	KAR1	23.4 ± 1.14	0.57 ± 0.10	0.60 ± 0.32	22.6 ± 0.55	1.01 ± 0.91	1.35 ± 1.09	20.0 ± 3.00	0.85 ± 0.02	0.44 ± 0.46
	KNO3	22.6 ± 1.14	0.67 ± 0.36	0.71 ± 0.52	22.4 ± 2.41	0.58 ± 0.13	1.07 ± 0.75	21.7 ± 2.08	0.71 ± 0.04	1.05 ± 0.38
Papaver rhoeas	water	5.6 ± 3.51	0.38 ± 0.04	0.79 ± 0.74	3.0 ± 1.73	0.26 ± 0.07	0.63 ± 0.3	2.7 ± 1.15	0.32 ± NA	0.65 ± NA
	GA3	19.8 ± 3.27	0.34 ± 0.06	1.31 ± 1.02	17.8 ± 1.30	0.30 ± 0.02	1.32 ± 0.46	13.0 ± 2.65	0.34 ± 0.04	0.64 ± 0.15
	KAR1	7.6 ± 3.58	0.30 ± 0.10	1.03 ± 1.15	5.4 ± 1.82	0.33 ± 0.10	0.90 ± 0.26	3.3 ± 0.58	0.36 ± NA	1.12 ± NA
	KNO3	13.2 ± 3.56	0.37 ± 0.09	0.88 ± 0.26	4.8 ± 2.39	0.33 ± 0.04	1.10 ± 0.01	5.3 ± 3.06	0.34 ± 0.07	0.60 ± 0.05
Pimpinella saxifraga	water	6.6 ± 4.56	0.08 ± 0.02	0.79 ± 0.53	0.2 ± 0.45	NA	NA	14.7 ± 1.15	0.06 ± 0.00	0.98 ± 0.12
	GA3	19.4 ± 1.95	0.13 ± 0.02	0.66 ± 0.1	19.6 ± 3.78	0.11 ± 0.01	0.49 ± 0.21	20.7 ± 0.58	0.12 ± 0.02	0.82 ± 0.46
	KAR1	6.8 ± 2.77	0.06 ± 0.02	0.74 ± 0.4	0.2 ± 0.45	NA	NA	10.3 ± 3.51	0.06 ± 0.01	0.45 ± 0.07
	KNO3	3.2 ± 1.92	0.06 ± 0.01	0.76 ± 0.06	0.2 ± 0.45	NA	NA	3.0 ± 1.73	0.06 ± 0.01	0.99 ± 0.37
Primula veris	water	0.2 ± 0.45	NA	NA	0.2 ± 0.45	NA	NA	0.3 ± 0.58	NA	NA
	GA3	24.4 ± 0.89	0.07 ± 0.00	0.36 ± 0.05	23.0 ± 1.41	0.06 ± 0.00	0.33 ± 0.14	23.7 ± 1.15	0.05 ± 0.01	0.59 ± 0.07
	KAR1	0.2 ± 0.45	NA	NA	0.2 ± 0.45	NA	NA	0.3 ± 0.58	NA	NA
	KNO3	0.2 ± 0.45	NA	NA	0.4 ± 0.89	NA	NA	0.3 ± 0.58	NA	NA
Primula vulgaris	water	6.0 ± 3.08	0.05 ± 0.01	0.88 ± 0.51	0.6 ± 0.55	0.51 ± 0.29	NA	2.7 ± 1.15	0.07 ± NA	2.51 ± NA
	GA3	19.0 ± 3.08	0.07 ± 0.01	0.75 ± 0.18	16.0 ± 3.32	0.06 ± 0.00	NA	15.0 ± 1.00	0.07 ± 0.01	0.87 ± 0.3
	KAR1	5.4 ± 2.30	0.05 ± 0.00	1.12 ± 0.13	0.2 ± 0.45	0.72 ± NA	NA	4.3 ± 1.53	0.04 ± 0.00	0.58 ± 0.01
	KNO3	11.4 ± 2.41	0.04 ± 0.00	0.85 ± 0.28	1.2 ± 0.84	0.58 ± 0.24	NA	9.0 ± 3.46	0.04 ± 0.01	0.64 ± 0.01
Saxifraga graminea	water	14.0 ± 2.74	0.16 ± 0.02	0.98 ± 0.26	3.4 ± 2.51	0.14 ± 0.06	1.57 ± 1.13	13.3 ± 4.51	0.20 ± 0.01	0.72 ± 0.16
	GA3	16.6 ± 2.07	0.15 ± 0.03	1.30 ± 0.8	6.6 ± 2.70	0.16 ± 0.03	0.77 ± 0.41	18.0 ± 2.65	0.15 ± 0.02	1.24 ± 0.34
	KAR1	16.2 ± 2.68	0.17 ± 0.02	0.86 ± 0.41	6.8 ± 2.95	0.17 ± 0.04	0.64 ± 0.38	16.0 ± 1.73	0.21 ± 0.02	0.63 ± 0.16
	KNO3	18.6 ± 1.14	0.18 ± 0.01	0.79 ± 0.2	8.0 ± 1.87	0.17 ± 0.01	0.59 ± 0.16	16.3 ± 3.06	0.19 ± 0.03	1.07 ± 0.53
Senecio vulgaris	water	1.7 ± 1.53	0.11 ± 0.04	NA	1.0 ± 1.00	0.13 ± 0.08	NA	2.3 ± 1.15	0.33 ± 0.17	NA
	GA3	0.3 ± 0.58	0.18 ± NA	NA	2.0 ± 1.00	0.18 ± 0.17	NA	1.7 ± 1.15	0.50 ± 0.28	NA
	KAR1	1.3 ± 1.15	0.18 ± NA	NA	1.7 ± 0.58	0.15 ± 0.02	NA	2.0 ± 2.00	0.25 ± 0.00	NA
	KNO3	2.0 ± 1.73	0.10 ± 0.02	NA	0.7 ± 0.58	0.13 ± 0.08	NA	2.7 ± 3.06	0.35 ± 0.17	NA
Ttrifolium pratense	water	21.6 ± 2.51	1.09 ± 0.20	0.56 ± 0.42	20.6 ± 2.79	0.83 ± 0.23	1.23 ± 0.74	22.7 ± 0.58	0.77 ± 0.09	0.78 ± 0.03
	GA3	21.8 ± 1.79	1.06 ± 0.22	0.61 ± 0.47	21.6 ± 2.70	1.03 ± 0.25	0.49 ± 0.3	22.7 ± 1.53	0.77 ± 0.03	1.17 ± 0.23
	KAR1	21.4 ± 0.89	0.77 ± 0.24	0.68 ± 0.42	22.6 ± 0.89	0.96 ± 0.20	0.76 ± 0.4	22.3 ± 1.53	0.80 ± 0.04	0.86 ± 0.02
	KNO3	22.4 ± 2.70	0.97 ± 0.30	0.57 ± 0.39	21.0 ± 2.65	0.81 ± 0.29	0.69 ± 0.3	21.7 ± 1.53	0.72 ± 0.06	0.83 ± 0.08
Urtica dioica	water	0.2 ± 0.45	NA	NA	0.4 ± 0.55	NA	NA	0.3 ± 0.58	NA	NA
	GA3	1.2 ± 0.84	0.23 ± 0.07	NA	0.8 ± 0.84	0.24 ± 0.09	NA	1.3 ± 0.58	0.30 ± 0.33	NA
	KAR1	1.0 ± 1.73	NA	NA	0.2 ± 0.45	NA	NA	0.3 ± 0.58	NA	NA
	KNO3	1.8 ± 2.17	0.08 ± NA	NA	0.2 ± 0.45	0.34 ± NA	NA	18.0 ± 2.00	0.06 ± 0.01	NA
Viola arvensis	water	15.8 ± 5.36	0.33 ± 0.01	0.23 ± 0.1	11.8 ± 3.56	0.34 ± 0.01	0.16 ± 0.05	1.0 ± 1.00	0.28 ± 0.00	NA
	GA3	15.6 ± 4.98	0.32 ± 0.05	0.52 ± 0.36	15.0 ± 4.53	0.30 ± 0.07	0.69 ± 0.8	3.7 ± 2.08	0.31 ± 0.00	NA
	KAR1	10.2 ± 4.15	0.31 ± 0.01	0.31 ± 0.06	17.4 ± 1.34	0.34 ± 0.01	0.24 ± 0.1	0.7 ± 0.58	0.28 ± 0.00	NA
	KNO3	24.8 ± 1.10	0.35 ± 0.01	0.20 ± 0.05	23.6 ± 2.07	0.34 ± 0.01	0.20 ± 0.09	18.0 ± 4.58	0.30 ± 0.02	NA

Viola palustris	water	0.2 ± 0.45	0.06 ± NA	NA		0.2 ± 0.45	NA	NA		0.3 ± 0.58	NA	NA
	GA3	1.0 ± 1.00	NA	NA		0.2 ± 0.45	NA	NA		0.7 ± 1.15	NA	NA
	KAR1	0.2 ± 0.45	NA	NA		0.2 ± 0.45	NA	NA		0.7 ± 0.58	NA	NA
	KNO3	0.2 ± 0.45	NA	NA		0.2 ± 0.45	NA	NA		0.3 ± 0.58	NA	NA
Viola riviniana	water	0.7 ± 0.58	0.19 ± 0.00	NA		0.3 ± 0.58	NA	NA		4.7 ± 0.58	0.23 ± 0.04	0.57 ± 0.41
	GA3	9.0 ± 7.21	0.18 ± 0.01	NA		6.7 ± 2.08	0.21 ± 0.05	NA		11.3 ± 2.52	0.21 ± 0.04	0.60 ± 0.24
	KAR1	9.0 ± 9.00	0.17 ± 0.03	NA		0.7 ± 1.15	0.19 ± NA	NA		16.3 ± 3.21	0.21 ± 0.02	0.49 ± 0.09
	KNO3	0.3 ± 0.58	0.19 ± NA	NA		0.3 ± 0.58	NA	NA		8.3 ± 2.08	0.19 ± 0.02	0.39 ± 0.11
Vicia sativa	water	23.6 ± 2.07	0.84 ± 0.46	1.09 ± 1.01		22.2 ± 1.92	0.60 ± 0.25	0.93 ± 1.15		19.3 ± 1.15	0.43 ± 0.02	0.60 ± 0.08
	GA3	23.0 ± 2.35	0.69 ± 0.45	0.75 ± 0.92		21.2 ± 2.59	0.55 ± 0.11	0.94 ± 0.9		22.0 ± 1.00	0.43 ± 0.03	0.65 ± 0.09
	KAR1	20.6 ± 1.67	0.48 ± 0.05	0.60 ± 0.39		23.0 ± 1.73	0.48 ± 0.11	0.83 ± 0.84		20.3 ± 0.58	0.48 ± 0.04	0.55 ± 0.12
	KNO3	22.2 ± 2.28	0.58 ± 0.15	1.08 ± 0.99		23.0 ± 0.71	0.55 ± 0.07	0.50 ± 0.42		21.3 ± 1.15	0.48 ± 0.05	0.90 ± 0.16

Species (sp), Final germination (FG), Germination rate (GR), relative uniformity (RU), Standard deviation (SD).

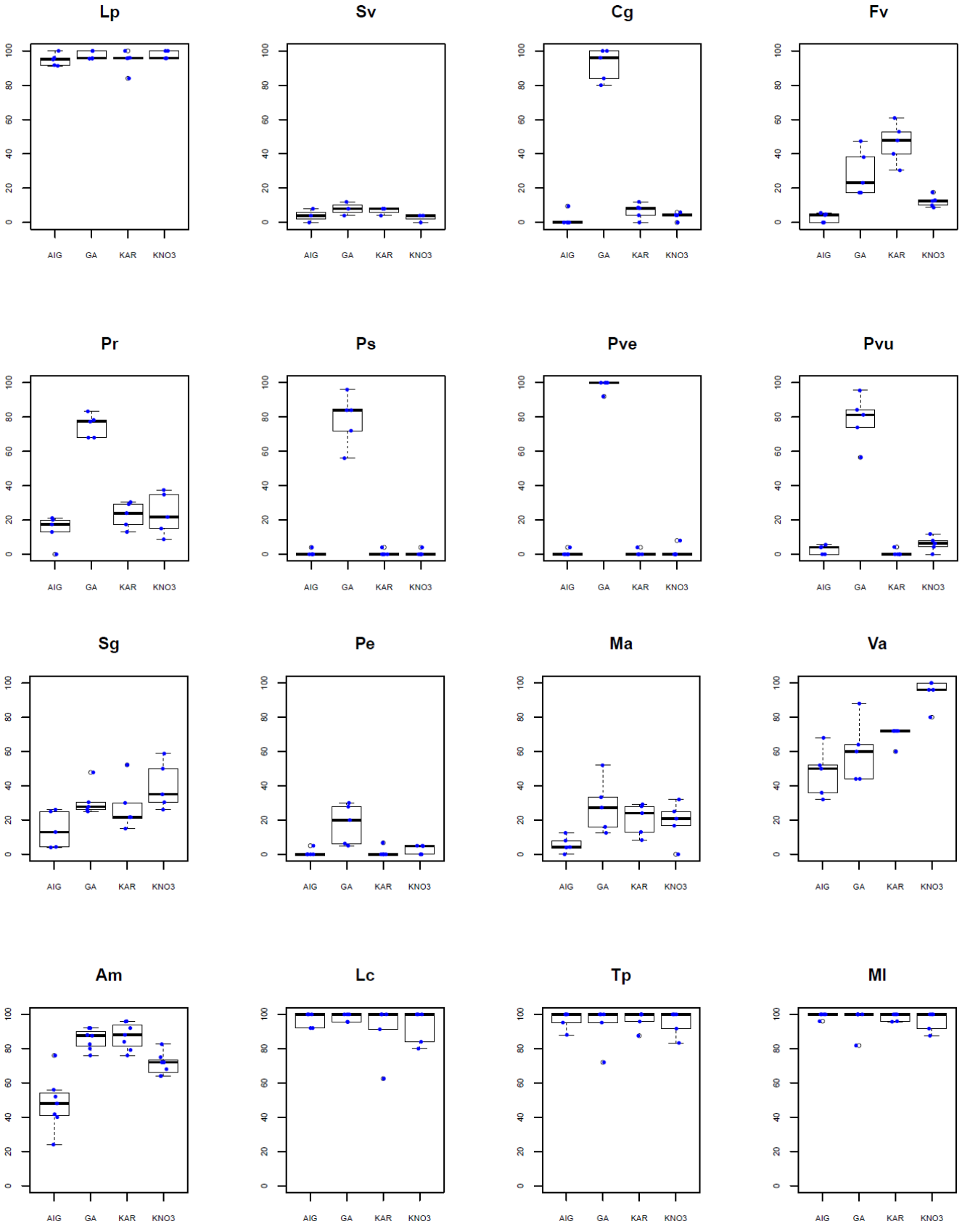
NA stands for Not Available; GR or RU could not be calculated due to FG ⩽ 10%

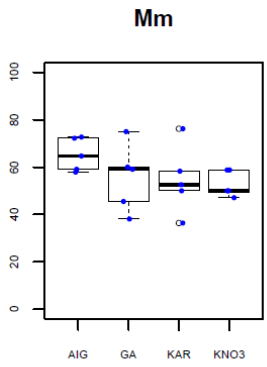
Supplementary Table 2. Species with light, chilling or after-ripening requirement to germinate which final germination was stimulated by any of the compounds.

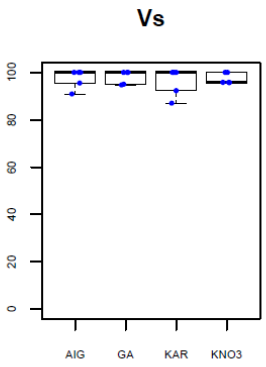
Species name	Stimulation of final germination by	Requirement to germinate	Reference
Achillea millefolium	GA_3	light	McDonald and Kwong, 2005
	nitrate		
	KAR_1		
Fragaria vesca	GA_3		
	nitrate		
	KAR_1		
Campanula glomerata	GA_3	light	McDonald and Kwong, 2005
	KAR_1		
Papaver rhoeas	GA_3	light	McDonald and Kwong, 2005
Potentilla erecta	GA_3	light	McDonald and Kwong, 2005
	KAR_1		
Primula veris	GA_3	light	McDonald and Kwong, 2005
Primula vulgaris	GA_3	light	McDonald and Kwong, 2005
Campanula glomerata	GA_3	chilling	Grime et al., 1981
Papaver rhoeas	GA_3	chilling	Grime et al., 1981
	nitrate		
Primula veris	GA_3	chilling	Grime et al., 1981
Primula vulgaris	GA_3	chilling	Grime et al., 1981
	nitrate		
Pimpinella saxifraga	GA_3	chilling	Grime et al., 1981
Mentha aquatica	GA_3	chilling	Grime et al., 1981
	nitrate		
	KAR_1		
Viola palustris	GA_3	chilling	Grime et al., 1981
Viola riviniana	GA_3	chilling	Grime et al., 1981
	nitrate		
	KAR_1		
Viola arvensis	GA_3	after-ripening	Lopez del Egido et al., in prep.
	nitrate		
	KAR_1		
Urtica dioica	nitrate		
Geranium robertianum	nitrate		Vandelook and Van Assche 2010
Stellaria graminea	nitrate		
Gibberellic acid (GA_3) and karrikinolide (KAR_1) GA_3 could, in the case of requiring chilling, overcome the need of a stratification period			

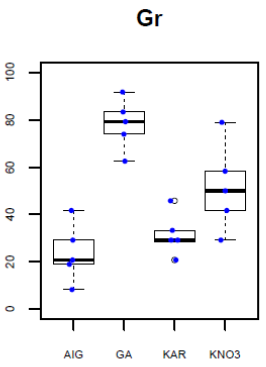
Supplementary Figure 1. Final germination percentage of the 23 studied species under the three light conditions and with the application of compounds. Light conditions are: constant darkness, constant red light and 12h photoperiod of white light. Compounds are: water (AIG), gibberellic acid (GA), karrikinolike (KAR) and potassium nitrate (KNO3). Species full names can be viewed in Table 1.

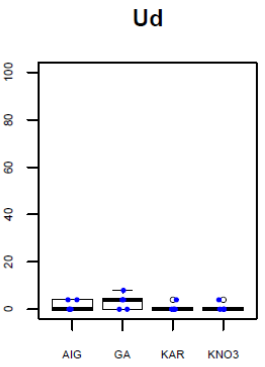
constant darkness

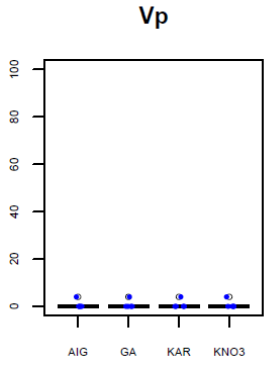


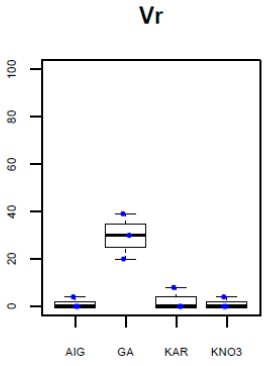


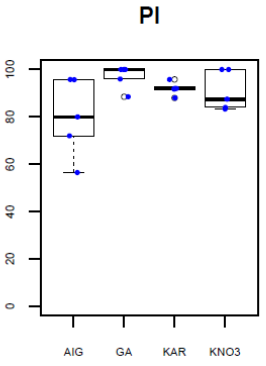




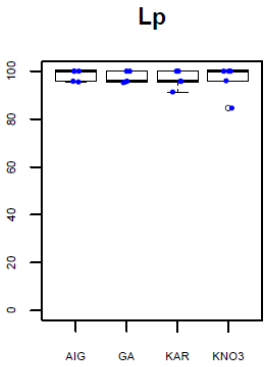


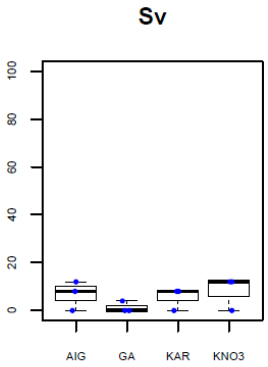


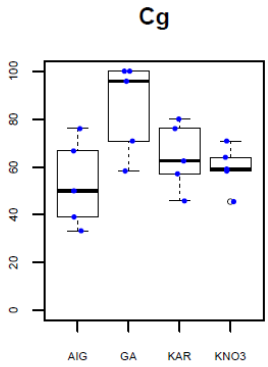


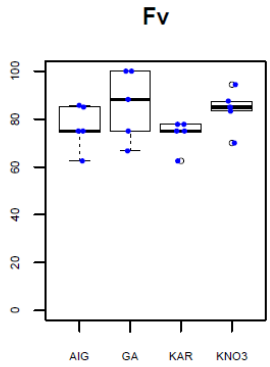


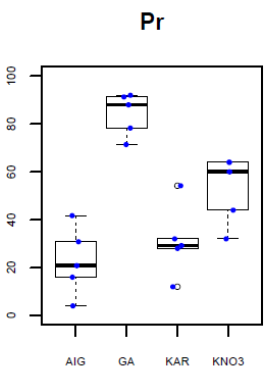
constant red light

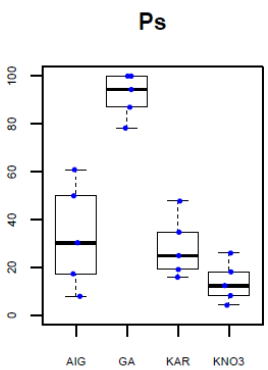


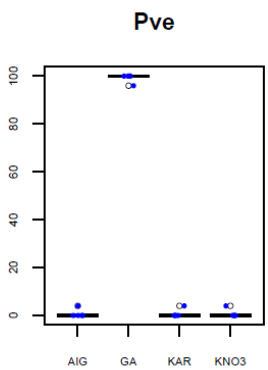


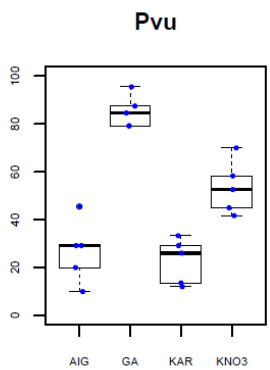


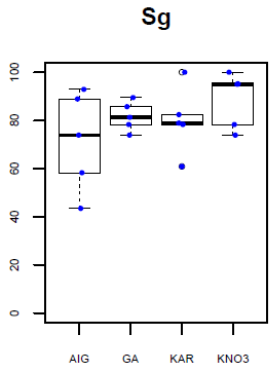








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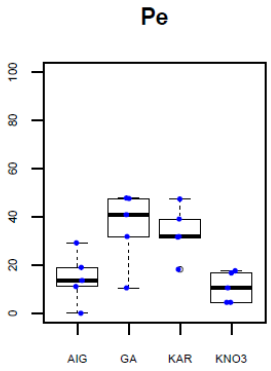
GA
KAR
KNO3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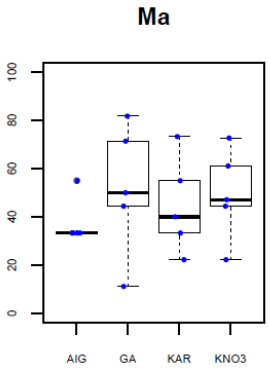
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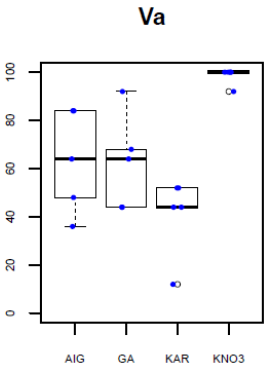
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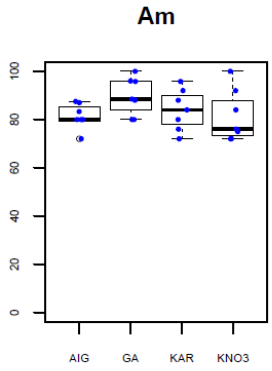
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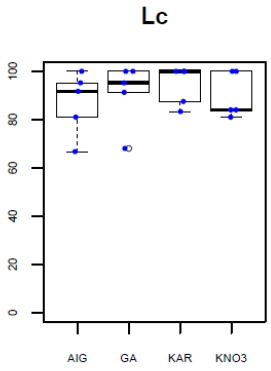
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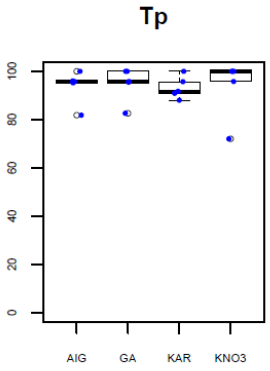
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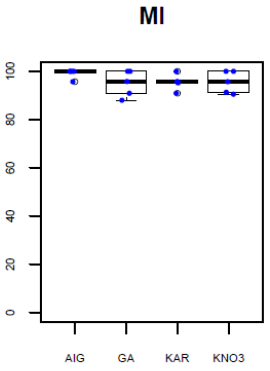
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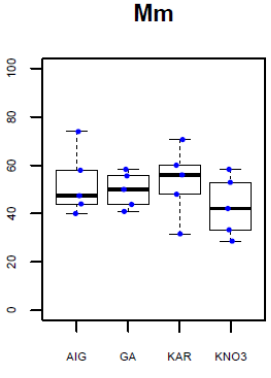
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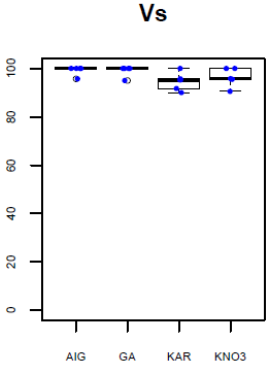
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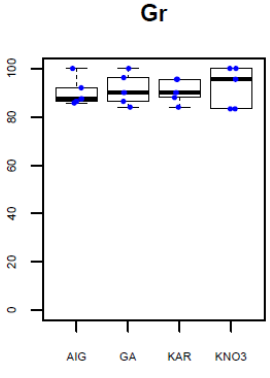
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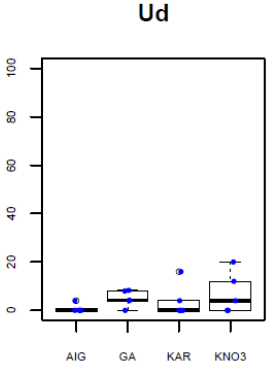
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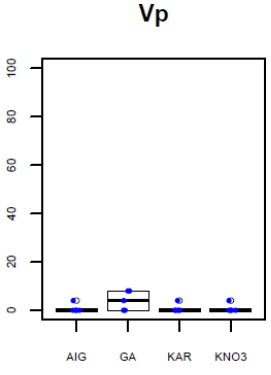
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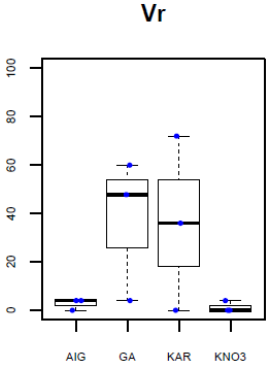
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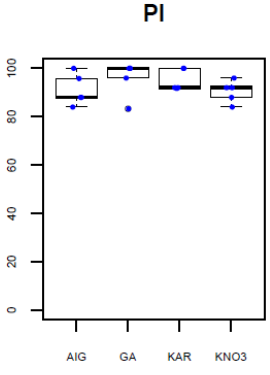
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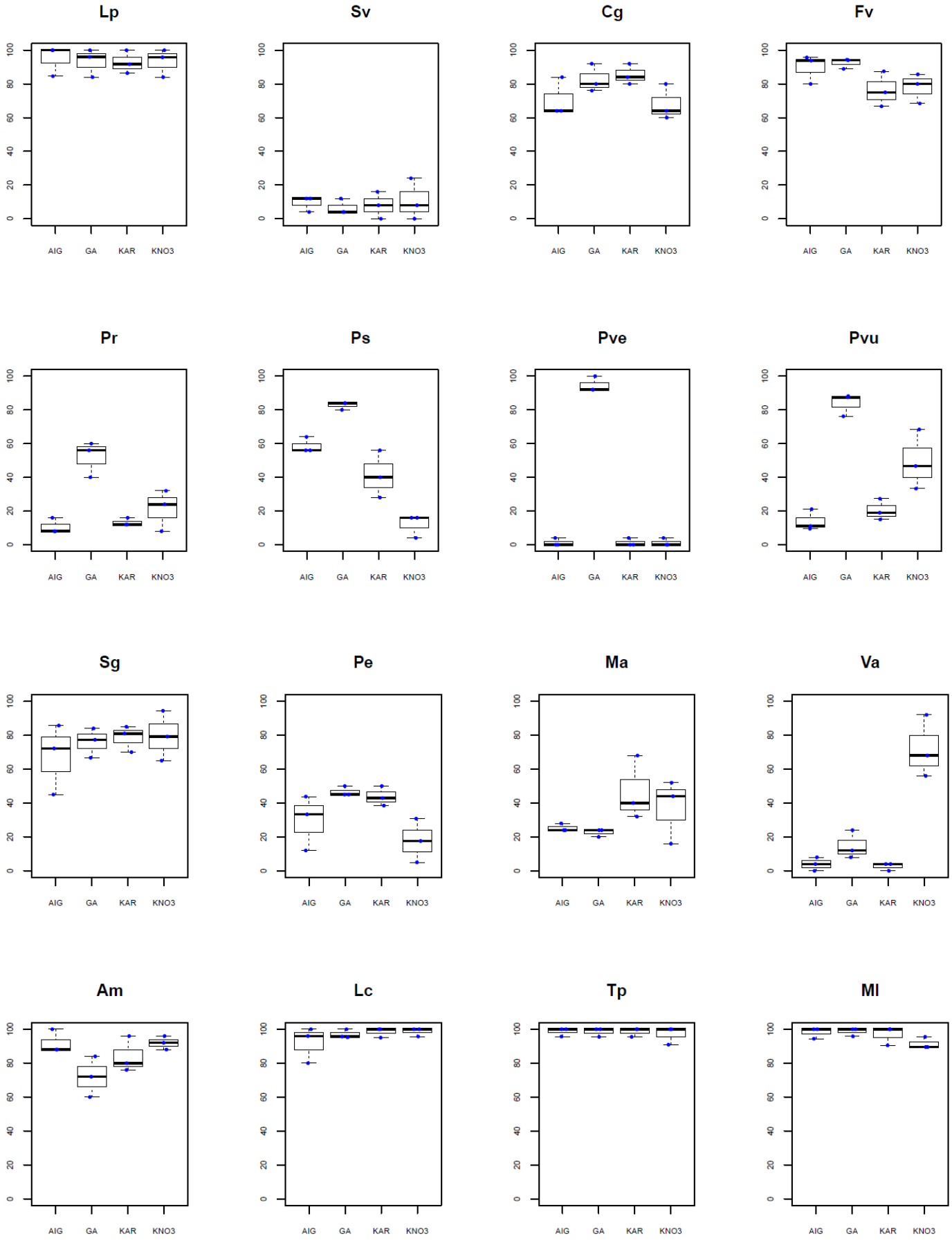

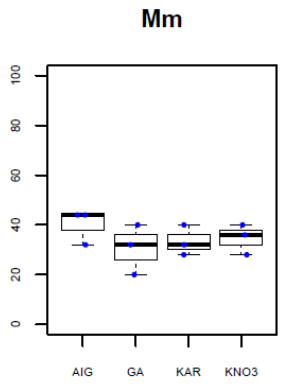
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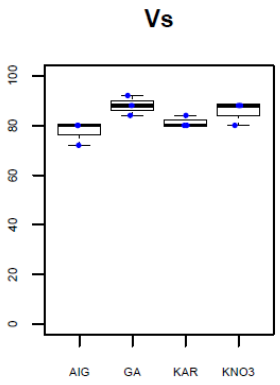
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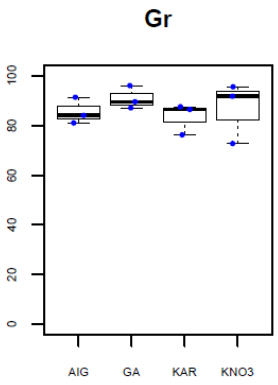
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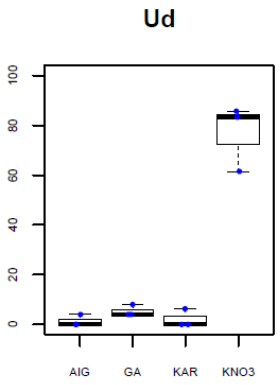
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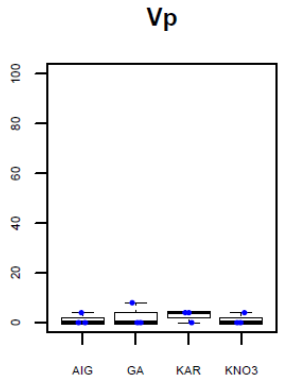
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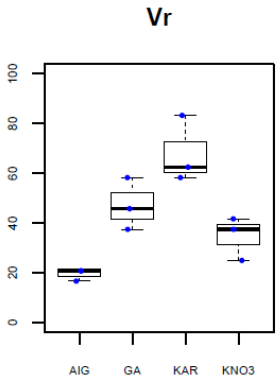
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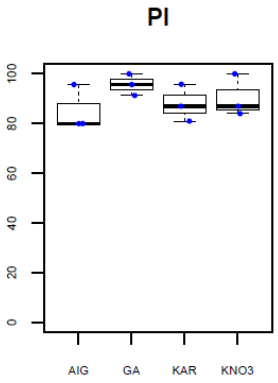
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Chapter 4

Effect of priming on four batches of *Arabis alpina*

Seed Priming Improves Germination of *Arabis alpina* under Thermo-inhibiting Conditions

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Abstract

Seed enhancement techniques are widely used to improve germination of crop species, but the usefulness of these techniques have been poorly studied for wild species. In some occasions, seed germination and seed and seedling stress resistance of crops have been proven to be improved, , by seed treatments. Hence such treatments might also be useful in large scale restoration projects. Here we investigate priming as a technique to enhance the germination of four seed batches of *Arabis alpina* under germination inhibitory conditions, evoked by high temperature and darkness. Responsiveness to the applied priming treatments (soaking in water or in solutions of gibberellic acid, karrikinolide, nitrate and sodium hypochlorite) varied among the seed batches. Final germination and germination rate of two of the batches were stimulated under inhibitory conditions with the priming in gibberellic acid and karrikinolide solutions, while no effects were observed in the other two seed batches. The conclusion is that priming can enhance germination of *Arabis alpina* seeds, however, seed batches from different sources can display a large variation in the responsiveness to the treatments.

Kew words: *Arabis alpina*, germination, light, provenance, restoration, seed priming treatments, temperature.

Introduction

Plants need to respond to signals from their biotic and abiotic surroundings in the various stages of their life-cycle (seed, seedling, mature plant and flowering plant) in order to maximise their fitness (Bewley and Black, 1994). As a consequence, they have developed sophisticated signal receptors and signal transduction networks to respond to signals from the environment (Segarra et al., 2006). Seeds perceive these signals in order to decide whether or not to germinate (Topham et al., 2017). Because seeds form the dispersal mechanism to migrate within or between ecosystems seeds are a crucial stage of the plant life cycle (Bewley and Black, 1994; Bentsink and Koornneef, 2008). The main factors that influence seed germination are light, temperature, water availability and soil nutrients (Bassel, 2016). The signals derived from these factors are perceived and integrated through hormone (abscisic acid (ABA)/gibberellins (GA)) and gene expression thresholds and trigger the stimulation or inhibition of germination (Karssen and Lacka, 1986; Finch-Savage et al., 2007; Bassel, 2016).

Light plays a major role in the development of the plant life (Fankhauser and Chory, 1997). Plants (and seeds) have evolved receptors in order to respond to the quality, quantity and direction of light. Plants use a combination of light receptors (phytochromes, chlorophyll, cryptochromes and phototropins) to perceive light of various wavelengths (Briggs and Olney, 2001). Temperature also plays an important role in germination (Freeman, 1973; Mott, 1974; Adams, 1999). The effect of different light and temperature conditions (Thompson et al., 1977; Lambton, 1985) as well as the interaction of these two factors at constant and fluctuating conditions have widely been investigated in germination studies (King, 1975; Murdoch et al., 1989; Kebreab and Murdoch, 1999; Válio and Scarpa, 2001; Fernández-Pascual et al., 2015; Galíndez et al., 2017).

Germination includes a series of complex processes. It commences with the uptake of water by the quiescent dry seed, and it is completed when the embryo extends to penetrate the structures that surround it (Bewley and Black, 1994). A seed will usually germinate when the conditions accommodate survival of the individual (Steadman, 2004). However, seeds can experience inhibition of germination under suboptimal ambient conditions. This is to time seed germination with favourable germination conditions in order to allow seedlings survival (Steadman, 2004). Inhibition of germination is of great economic importance (Bettey et al., 2000), as it can constitute a problem for industrial production or ecological restoration when seeds are sown in the field under the wrong environmental conditions. Therefore, treatments may be required to improve the ability of seeds to germinate. Priming is usually described as a hydration process that allows germination

to start, without radicle emergence to occur (Heydecker and Coolbear, 1977). Priming has been proven to be a useful seed treatment to improve germination and seedling establishment (Parera and Cantliffe, 1994; McDonald, 1999; Chiu et al., 2002), both under favourable and unfavourable conditions, such as salt, drought stress and high temperatures (Carpenter and Boucher, 1991; Kaya et al., 2006). However, the extended exposure of hydrated seeds to adverse germination conditions may induce secondary dormancy or inhibition of germination (Baskin and Baskin, 1998; Probert, 2000; Finch-Savage and Leubner-Metzger, 2006). Because of that, priming is a widely applied commercial practice (Valdes and Bradford, 1987; Cantliffe et al., 2000; Schwember and Bradford, 2005). In the seed industry, seed pre-treatments that include addition of active ingredients, such as gibberellic acid (GA_3) have also been demonstrated to be a useful compound to improve germination (Kahn et al., 1957; Hsiao and Quick, 1985; Watkins et al., 1985; Groot and Karssen, 1987). However, the usefulness of these treatments has not yet been proven to hold true for a wide range of wild species. Therefore, we hypothesise that priming treatments may improve germination (final germination and germination rate) under optimal and non-optimal germination conditions.

Arabis alpina belongs to the Brassicaceae family, which is currently estimated to comprise about 372 genera and 4,060 species (Koch et al., 2010; The Plant List, 2013). This species is closely related to other members of the family, including *Arabidopsis thaliana* and *Brassica* species, and several family members are important crops (Koch et al., 2000; Bailey et al., 2006; Clauss and Koch, 2006). The germination of *Arabis alpina* (Alpine rock cress) is largely influenced by light and temperature (Lopez del Egido et al., in preparation). The effect of germination of alpine species to low temperatures has received considerable attention, but little is known about the effect high temperatures have on it (Ting et al., 2014; Orsenigo et al., 2015). Therefore, here, we investigate the potential of seed treatments to improve the germination of *Arabis alpina* at high temperatures, including the control by light. Although the species may not currently be subject to 25-30°C often in its natural situation, this may change due to global warming. We investigated priming as method to overcome any inhibition by high temperatures. The effectiveness of the treatments in relation with the exposure time of the seeds to the compounds and the moisture content of the seeds at sowing time was also studied.

Materials and Methods

Germination assays

Germination assays were performed with four batches of *A. alpina* (Table 1) at a range of temperatures from 5 to 35°C, at intervals of 5°C under constant white light (WL), red light (RL) or darkness (D). Constant white light was applied by fluorescent tubes (Lumilux OSRAM L36W/840) and red light was applied by LEDs (Illumitex: Dual 48" Eclipse with (24) 660 nm LEDs – Dimmable). Custom made transparent and black six-compartment trays (Voges Verpakking bv, Hillegom, The Netherlands) were used for the germination tests. Trays were 30 cm x 21 cm, containing six 81 cm^2 wells, 2.5 cm deep with lid. Each well contained an 64 cm^2 18-layered water absorbent paper (Zell-Pak) covered by a 64 cm^2 12 Steel-Blue Seed Germination paper (Anchor Paper Co., USA) and wetted with 28 mL of demineralised water or the solutions indicated. Trays were closed with a lid to avoid evaporation and pre-incubated in a cabinet (IVL Van Leeuwen, The Netherlands) for 1 h at each temperature prior the seed sowing. Soil tests were carried out using 1 cm of soil in the same trays. Two type of soils were used: a high-nutrient soil (HNS, peat-based compost, Jiffy Substrates) and a low-nutrient soil (LNS, sand-based soil, Jiffy) wetted with water (10-15ml/g soil). Twenty five seeds *per* replicate and at least 4 replicates *per* treatment and batch were used. Germination was scored twice a day for the first three days of germination and, subsequently, once a day until germination ceased. Germination was scored as completed when the radicle protruded >1 mm. Germinated seeds were removed after scoring. Germination in soil was scored as completed upon breaking of the seedling through the soil surface. For the samples germinated under dark conditions germination was scored once a day in a room under safe green light until germination ceased. At the end of all the experiments, non-germinated seeds were cut open and only apparently viable seeds were used to calculate germination parameters and to perform statistical analysis.

The amount of seed available to us was limited in the case of batch 3 and batch 4; therefore, we had to prioritise the treatments to be tested and not all treatments and temperatures could be included in the tests.

Priming treatments

Priming experiments were performed on the four batches of *A. alpina* (Table 1), which were stored at 15°C, 30% RH. Compounds, priming time and light condition were considered explanatory variables for final germination. The compounds considered were: gibberellic acid (10 mM GA_3; Duchefa Biochemie; Haarlem), karrikinolide (1 µM KAR_1; Syngenta Crop Protection), nitrate (10

mM KNO_3; Sigma-Aldrich; Madrid), sodium hypochlorite (1% (w/v) NaOCl; Brenntag Nederland B.V.; Loosdrecht) and etherel-A (0.005%; Bayer; Mijdrecht), which were compared to dry seed and seeds primed in distilled water (water); the priming times considered were: 0.5, 2, 4 and 8h. Seeds were placed in 50 mL beakers containing 20 mL of one of the pre-treatment solutions (Table 2) and stirred for the duration of the treatment. After pre-treatment, seeds were rinsed with water twice and placed in a 4.5 x 7 cm net bag in a drier with forced air at ambient temperature and relative humidity (21°C and 40-45% RH) to dry over 3 days. Seeds that were pre-treated with any of these treatments are denominated "primed seeds". When testing the effect of the treatments on germination two controls were considered: the first one included non-primed seeds sown on water, and the second one primed seeds in water in order to test the effect of the solvent alone. However, not all the treatments were tested on all the seed batches (Table 2).

Table 1. **Details of the studied seed batches of *Arabis alpina.*** Table contains batch number, year of harvest, source of seeds, production type and production altitude.

Batch number	Year of harvest	Seed Provider	Provenance	Production Type	Production Altitude (m above sea level)
1	2015	B & T World Seed[1]	The Netherlands	Cropped	0
2	2015	Everwilde Farms	USA	Cropped	NA
3	2015	Jardin Botanico Atlantico	Spain	Wild collected	2,500
4	2016	Jardin Botanico Atlantico	Spain	Wild collected	2,500

[1] Based in Paguignan, France

NA not available.

Table 2. Compounds tested per each of the 4 *Arabis alpina* seeds batches: etherel, gibberellic acid (GA_3), karrikinolide (KAR_1), potassium nitrate (KNO_3), sodium hypochlorite (NaOCl) and water.

	Treatment		Batch number			
	compound	**priming time (h)**	**1**	**2**	**3**	**4**
Treatments	etherel	2	no	yes	no	yes
	etherel	4	yes	yes	no	yes
	GA_3	0	yes	yes	no	yes
	GA_3	2	yes	yes	yes	yes
	GA_3	4	yes	yes	no	yes
	KAR	2	yes	yes	yes	yes
	KAR	2	yes	yes	no	yes
	KNO_3	0.5	no	yes	no	no
	KNO_3	2	yes	yes	yes	yes
	KNO_3	4	yes	no	no	yes
	NaOCl	0.5	yes	yes	yes	yes
	water	0	yes	yes	yes	yes
	water	0.5	yes	yes	yes	yes
	water	2	yes	yes	yes	yes
	water	4	yes	yes	no	yes
	water	8	yes	yes	yes	no

Statistical Analyses

Final germination percentages (FG) and time required for 50% of viable seeds to reach germination (t_{50}) were calculated using Microsoft Excel 2013 and the Germinator software (Joosen et al., 2010) respectively. In some cases no t_{50} values were calculated due to very low final germination. Germination rate (GR) was calculated as the reciprocal of t_{50}. For the treatments with lack of binomial variation, such as 0 or 100% for final germination, data was manually transformed to 1 or 99% in order to be able to include those results in the Generalised Linear Models (GLM). Comparison of germination parameters (FG, GR and uniformity) and batches across temperatures and pre-treatments to establish optimal and sub-optimal germination conditions and effectiveness of the treatments were performed in R-Project (R Core Team, 2016). Binomial data was analysed using generalized linear models (GLMs) with a logit link-function as used to analyse similar data (Gorecki et al., 2012; Mojzes and Kalapos, 2016). Boxplots were constructed with the lattice (Sarkar, 2008) and the ggplot2 (Wickham, 2010) packages. Differences were considered significant when $P < 0.05$.

Results

Comparison of germination under light and darkness at a range of temperatures

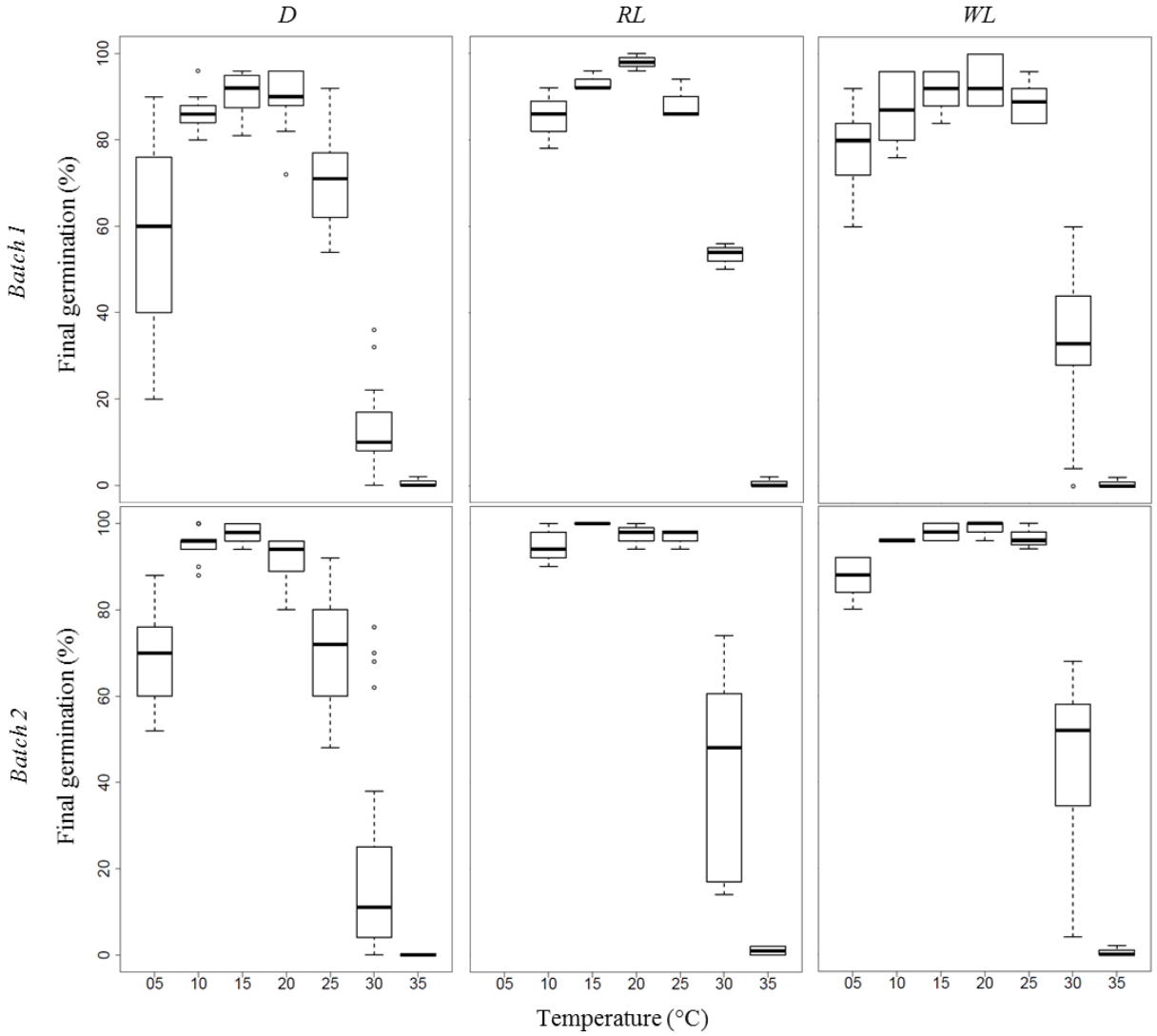


Figure 1. Boxplots show final germination percentage (FG) of two seed batches under light conditions and a range of temperatures. Batch 1 (three upper panels) and 2 (three bottom panels) final germination under three constant light conditions (darkness, D; red light, RL; and white light, WL) across seven temperatures (5, 10, 15, 20, 25, 30 and 35°C). No data is available for germination at 5°C under RL. The central rectangle spans the first to the third quartile (the interquartile range or IQR) and the mean FG (thick central line). The whiskers above and below the box indicate the variability outside the upper and lower quartiles, the minimum and maximum values. Outliers are represented with an empty circle.

For both cultivated seed batches 1 and 2 (B1 and B2 respectively), germination under white and red light showed no difference for both FG and GR at similar temperatures (Fig. 1 and 2, $P > 0.05$). For batch 1 and 2, germination in the dark at 5, 25 and 30°C is significantly lower from germination at 10, 15 and 20°C ($P < 0.001$). Under white and red light, only germination at 3 °C is significantly different from 20°C in batch 2 ($P < 0.001$), while in batch 1, also 5°C is significantly lower ($P <$

0.001). Therefore, 20°C was considered the optimum temperature and RL the optimal light condition for the other experiments.

Final germination at 30°C was significantly lower under dark conditions for both seed batches. In darkness, above the optimal temperature GR was negatively correlated with temperature. Under light conditions GR was positively correlated with temperature and germination speed increased until 25°C (Fig. 2). At 5°C, germination was slower than at 20°C ($P < 0.001$; Fig. 2), but seeds were still able to germinate to 80 to 90% in the light, for B1 and B2 respectively (Fig. 1), while germination in the dark varied from 55 to 70%. Overall, batch 2 germinated the fastest across most of the conditions (5-20°C in WL; 5-15°C in RL; and 5, 10, 20 and 30°C in D; $P < 0.05$; Fig. 2).

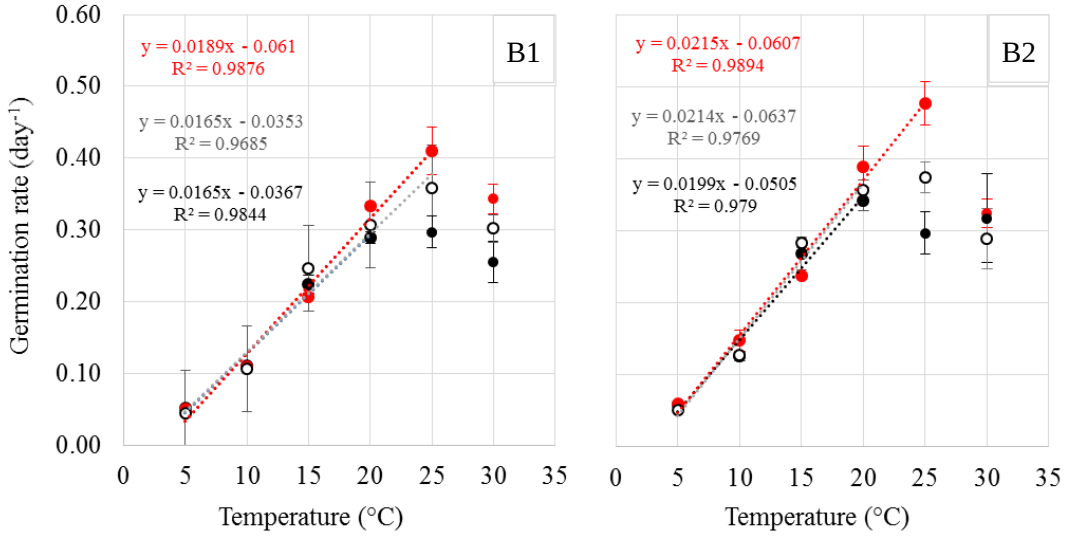


Figure 2. Germination rate (mean ± SE) across temperatures for *Arabis alpina*. Germination under red light (red symbols), white light (open symbols) and darkness (filled symbols) of batches 1 and 2. Germination rate (mean ± SE). Equations (red: RL; grey: WL; black: D) in the graphs describe the fitted lines through the data points belonging to the temperatures from 5 to 25°C (RL and WL) or to the temperatures from 5 to 20°C (Darkness).

Batch 3 and 4 did not germinate at 30°C under darkness, but germination was inhibited already at 25°C (Fig. 5), therefore, the impact of treatments on germination of batches 3 and 4 were tested at this temperature instead of at 30°C. When germination of batch 3 and 4 was tested at 20°C under light conditions final germination was 65±12%, which was lower than batch 1 and 2, but transfer to plates containing a 10 mM GA_3 solution increased germination to 95%. However, seeds from batch 3 and 4 germinated slower and with less uniformity than batch 1 and 2 ($P < 0.05$). This was due to completion of germination close to 30 days after sowing compared to 10 days for batch 1 and 2, even if germination started 2-4 days after sowing.

Effect of the treatments

A condition of 20°C in red light was considered optimal, since FG peaked near 100% for both seed batches 1 and 2 and the GR was high. Based on the fact that both FG and GR declined considerably at 30°C in the dark this condition was considered supra-optimal and inhibiting. Therefore, treatments were tested at this condition to test their potential to overcome these inhibitory conditions.

Effect of the treatments on final germination (FG)

At 20°C under constant light and constant darkness, final germination was close to 100%, and none of the treatments increased FG ($P > 0.05$). As an example, figure 3 shows the effect of hydropriming treatments on FG and GR in batch 2, both at the optimum 20°C and supra-optimum 30°C under darkness. At high temperatures and dark conditions, when comparing treatment to the not primed control seeds, priming with etherel, GA_3, KAR_1, KNO_3, NaOCl and water affected FG significantly ($P < 0.05$) for batch 2 (Table 3). However, the length of the priming treatment affected the impact of the various treatments (Table 3). Nevertheless, if we considered the ½ and 2 h water primed samples as the control for the ½ h and the 2 h priming treatments respectively, only ½ h

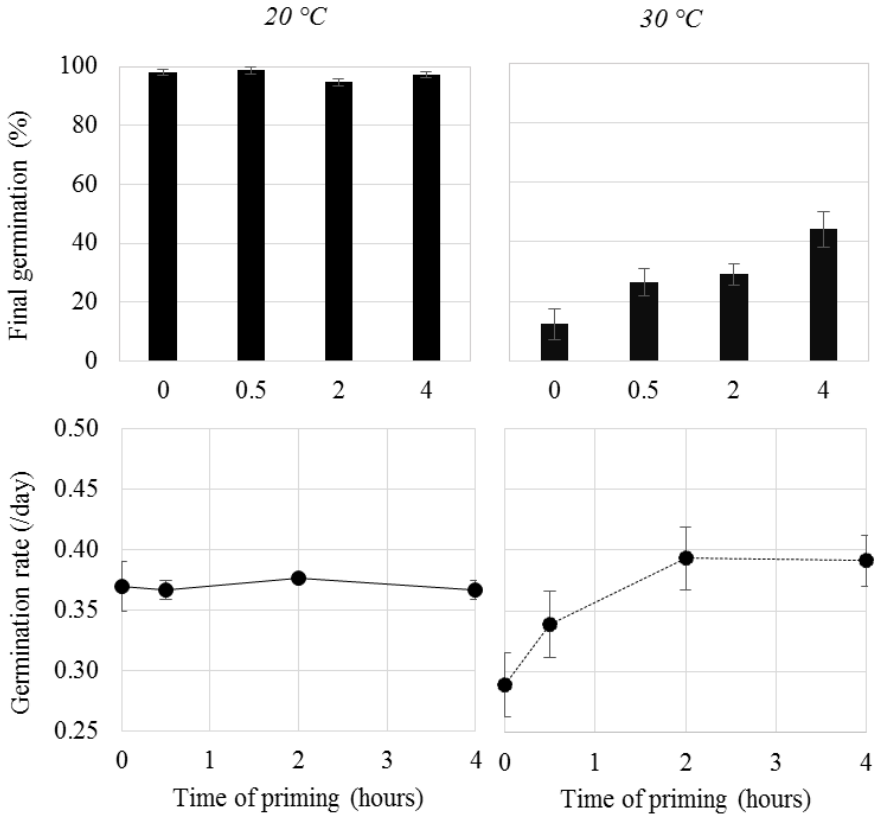


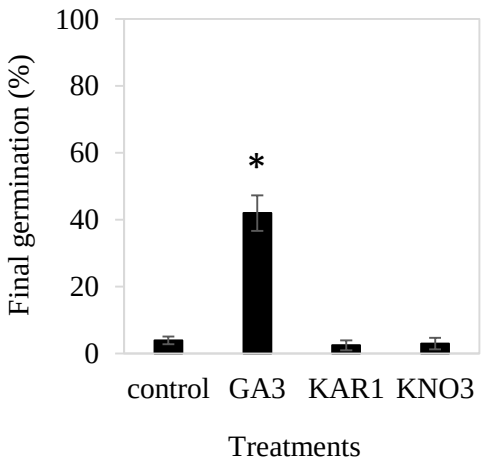
Figure 3. Germination parameters (mean ± SE) of *Arabis alpina* after priming. A) Final germination percentage (upper panels) and B) germination rate (bottom panels) of unprimed control (0) and hydroprimed seeds (0.5, 2 and 4 h) of batch 2 under constant darkness at 20 °C (left panels) and 30°C (right panels).

NaOCl and 2 h GA_3 had an additional effect on germination. This is in line with the fact that GA_3 is the only compound that stimulated final germination when it is added to the germination medium (Fig. 4).

Table 3. Final germination percentage (mean ± SD) at 30°C darkness of batch 1 and 2 of *Arabis alpina* after treatments. This were: etherel, gibberellic acid (GA_3), karrikinolide (KAR_1), potassium nitrate (KNO_3) sodium hypochlorite (NaOCl) and water treatments compared to the unprimed control.

Treatment		Batch 1		Batch 2	
Compound	**Priming time (h)**	**FG (%) ± SD**	**Significance**	**FG (%) ± SD**	**Significance**
Control	0	11.50 ± 5.62		18.6 ± 14.65	
Etherel	2	6.50 ± 1.73		32.0 ± 2.83	*
Etherel	4	12.25 ± 8.38		25.0 ± 1.41	
GA_3	0	70.25 ± 4.99	**	96.0 ± 5.66	**
GA_3	2	31.14 ± 9.63	**	40.1 ± 12.49	**
GA_3	4	36.00 ± 13.04	**	52.0 ± 5.66	**
KAR_1	2	19.25 ± 5.74	*	34.0 ± 2.83	*
KAR_1	4	19.75 ± 0.96	*	47.0 ± 1.41	**
KNO_3	1/2	-		24.0 ± 5.66	
KNO_3	2	23.50 ± 7.68	**	34.4 ± 6.07	**
KNO_3	4	18.00 ± 11.69		-	
NaOCl	1/2	33.33 ± 4.62	**	46.8 ± 11.21	**
Water	1/2	22.80 ± 7.16	**	26.3 ± 9.53	*
Water	2	21.89 ± 13.93	**	29.0 ± 7.21	*
Water	4	15.00 ± 5.20		44.2 ± 12.63	**
Water	8	26.80 ± 7.82	**	36.0 ± 6.48	**
$P < 0.05$ (*), $P < 0.001$ (**)					

Figure 4. **The impact of active ingredients on paper germination of batch 2 of *Arabis alpina*.** Germination was performed on 1 mM gibberellic acid (GA_3), 1 µM karrikinolide (KAR_1) and 1mM potassium nitrate (KNO_3) at 30 °C and darkness. Bars represent mean of final germination with standard error. Four replicates of 50 viable seeds were used per treatment. "*" represents statistical significance ($P < 0.05$).



For batch 1, all the compounds tested, except etherel had a positive effect on final germination at 3 °C and darkness when these compounds were added to the germination medium or to the priming solution. These were: GA_3, KAR_1, KNO_3, NaOCl and water. NaOCl and GA_3 treatments increased final germination, not only as a result of the priming in a water-based solution, but also due to the application of the compound (Table 3; $P < 0.05$). No effect was observed between the control and treated samples when seeds were transferred to 20°C after germination had ceased at 30°C.

For batch 3 and 4, none of the treatments improved FG at the inhibitory conditions (Fig. 5 and 6; $P > 0.05$), except for GA_3 applied on the germination plate in batch 4 (Fig. 6; $P > 0.05$). However, when seeds were transferred to 20°C after germination had ceased at 25°C, samples treated with 2h GA_3, ½ h NaOCl and ½ h hydropriming showed an increased FG compared to the unprimed control for batch 3 ($P < 0.05$; Fig. 5). FG of batch 4 did not show any response to the change of germination conditions ($P > 0.05$; Fig. 6).

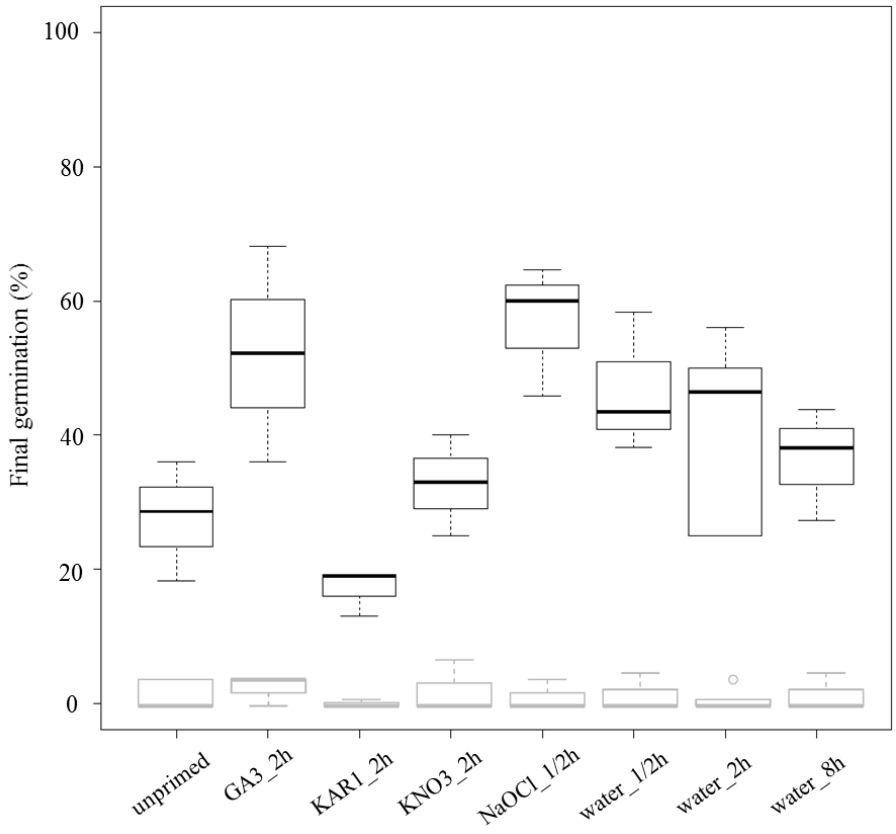


Figure 5. Boxplot shows final germination percentage of batch 3 at 25°C under constant darkness (light coloured boxplots) and at 20°C under constant red light (dark coloured boxplots), after reaching final germination at 25°C in constant darkness. Priming treatments included gibberellic acid for 2 hours (GA_2h), karrikinolide for 2 hours (KAR_2h), potassium nitrate for 2 hours (KNO3_2h), sodium hypochlorite for ½ hour (NaOCl_1/2h), and water for ½, 2 and 8 hours (water_1/2h, water_2h and water_8h). The central rectangle spans the first to the third quartile (IQR) and the mean FG (thick central line). The whiskers above and below the box indicate the variability outside the upper and lower quartiles, the minimum and maximum values.

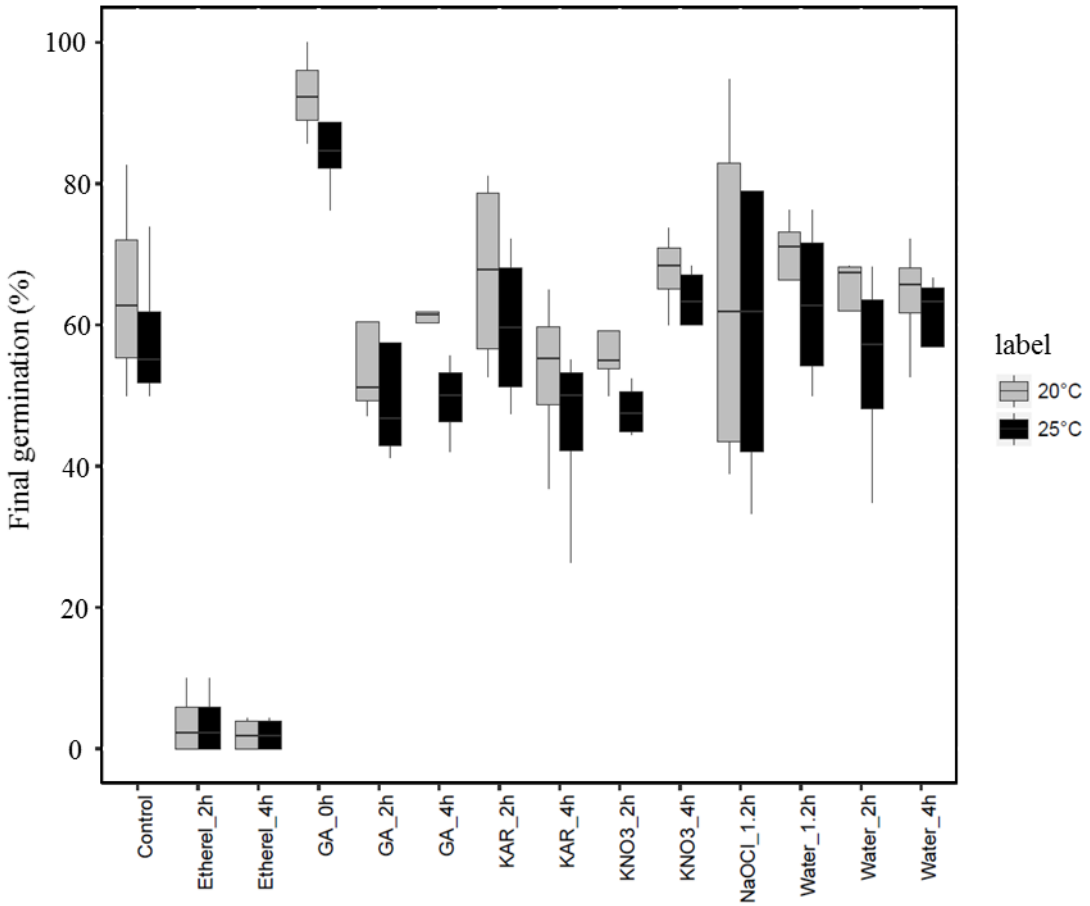


Figure 6. Boxplot shows final germination percentage of batch 4 at 25°C under constant darkness (light coloured boxplots) and at 20°C under constant red light (light coloured boxplots) after reaching final germination at 25°C in constant darkness. Priming treatments included priming with etherel for 2 and 4 hours (etherel_2h and etherel_4h), gibberellic acid on the germination plate (GA_0h) or during priming for 2 and 4 hours (GA_2h and GA_4h), karrikinolide for 2 and 4 hours (KAR_2h and KAR_4h), potassium nitrate for 2 and 4 hours (KNO3_2h and KNO3_4h), sodium hypochlorite for ½ hour (NaOCl_1/2h), and water for ½, 2 and 4 hours (water_1/2h, water_2h and water_4h). The central rectangle spans the first to the third quartile (the interquartile range or IQR) and the mean FG (thick central line). The whiskers above and below the box indicate the variability outside the upper and lower quartiles, the minimum and maximum values.

Effect of the treatments on germination speed (GR)

All priming compounds improved germination speed in batch 1 ($P < 0.05$). In all the treatments, except for 2 h priming in KAR_1, the enhancement in germination speed was due to the seeds being soaked in water, rather than the role of the compound. Interestingly, application of GA_3 on the germination plate, rather than during priming, reduced GR ($P < 0.001$), contrary to the positive effect of the compound on improving FG. Batch 1 germination speed was also improved by GA, KAR and hydropriming treatments, but not by etherel. FG values were very low for batch 3, therefore GR could not be calculated. For batch 4, none of the treatments had an effect on the GR.

Effect of the substrates on germination

Under red light at 20°C, there were no significant differences in the germination of batch 2 on the three substrates: paper, low-nutrient (LNS) and high-nutrient soil (HNS). However, at 30°C, germination of untreated seeds on paper and in the LNS were significantly lower from germination in the HNS (Table 4; $P < 0.001$). Primed seeds were tested for germination on paper and LNS and showed the same amount of germination on both substrates ($P > 0.05$). 2h GA_3, ½ h NaOCl and 4 h water treated seeds significantly increased FG compared to dry seeds (Fig. 7, $P < 0.001$) on both substrates, while no effect was observed with ½ h and 2 h priming with water ($P > 0.05$).

Table 4. Final germination percentage (mean ± SD) of untreated *Arabis alpina* seeds at 30°C red light. Germination tests were performed in three substrates: high-nutrient soil, paper and low-nutrient soil.

Substrate type	Final germination (%) ± SD
Paper	38.3 ± 28.6
Low-nutrient soil	40.0 ± 0.0
High-nutrient soil	73.0 ± 15.6

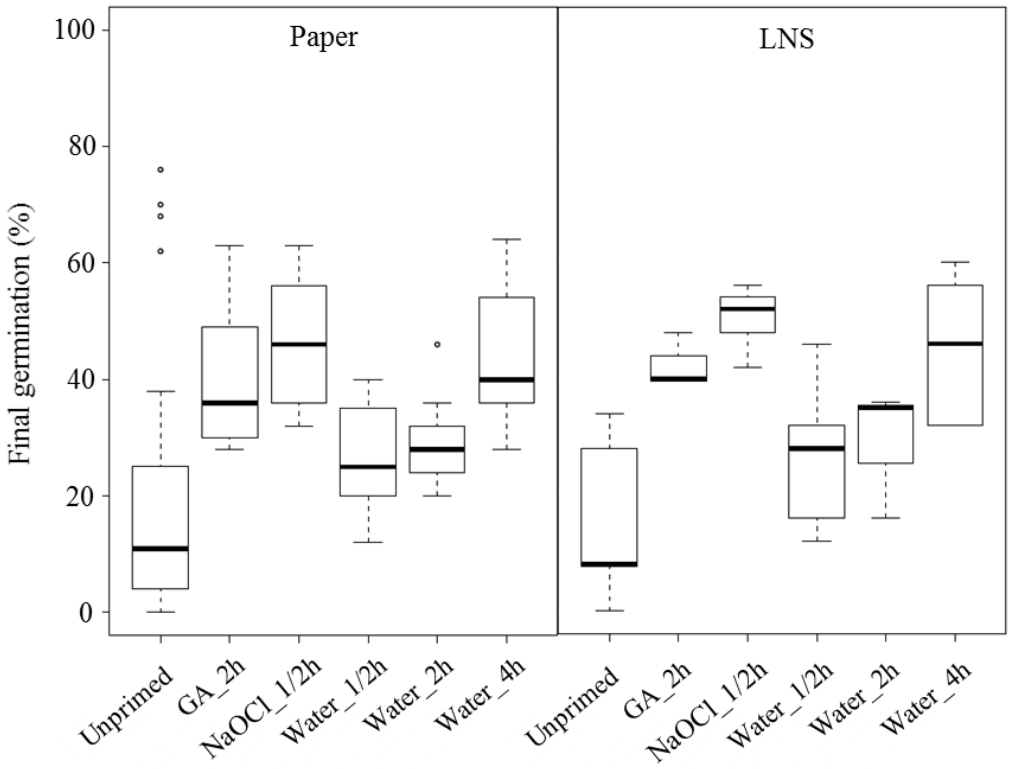


Figure 7. **Boxplots show final germination percentage of batch 2 under constant darkness at 30°C.** Seeds were germinated on paper or on low-nutrient soil (LNS) after priming in the various solutions: gibberellic acid for 2 hours (GA_2h), sodium hypochlorite for ½ hour (NaOCl_1/2h), and water for ½, 2 and 4 hours (water_1/2h, water_2h and water_4h). The central rectangle spans the first to the third quartile (the interquartile range or IQR) and the mean final germination (thick central line). The whiskers above and below the box indicate the variability outside the upper and lower quartiles, the minimum and maximum values. Outliers are represented with an empty circle. Outliers represent germination data points of experiments where temperature was set at 30°C, but actual temperature deviated from the settings.

Discussion

The purpose of this study was to investigate the effect of priming to improve the germination of *Arabis alpina* under conditions, which inhibit germination. For *A. alpina* these conditions are exposure to high temperatures when light is restricted. Inhibition of germination means that seeds do not germinate due to unfavourable conditions but upon removal of the inhibiting factor germination is completed (Bewley and Black, 1994). However, if seeds remain ungerminated even if transferred to favourable conditions seeds have become secondary dormant (Bewley, 1997). In this study, germination of seeds from some seed batches was rescued when placed back at optimal germination conditions (20° C light) after the incubation at 30°C in the dark (Fig 5 and 6). Hence, we conclude that they were (thermo)inhibited. Thermoinhibition has also been described for cropped species, such as leek (Gelmond, 1965); spinach (Atherton and Farooque, 1983); the cultivated lettuce (*Lactuca sativa*; Karssen, 1980; Valdes et al., 1985), and for wild relatives such as *Lactuca serriola* (Marks and Prince, 1982; Small and Gutterman, 1992). Moreover, light has also already been proven to be one of the factors that reduce thermoinhibition (Fielding et al., 1992; Toyomasu et al., 1998; Roth-Bejerano et al., 1999; Cantliffe et al., 2000). However, seeds from another seed batch did not recover after they were transferred to optimal conditions (data not shown), which leads us to suggest that this particular batch became secondary dormant under darkness.

Our results indicate that the applied priming treatments can have different effects on final germination and germination rate. Therefore, the treatments to be applied in order to enhance germination of a species will vary in relation to the parameter we want to affect. Moreover, variability of the response of seed batches to treatments needs to be considered. Our results showed differences in the germination responsiveness to the temperature and to the priming treatments of the four tested batches of *A. alpina.* This indicated an intra-specific variation in the germination of this species. The results showing that seed batches have different germination characteristics is consistent with Gray (1975) and Thompson *et al.* (1979), who described a variation in the response of different seed batches of a single species to inhibitory temperatures. The reason behind this difference in response might be due to the adaptations of the seed batch to the maternal environment and to the genetic make-up, as it was also proposed by Probert et al. (1985) and Hamasha and Hensen (2009). This leads us to suggest that provenance is a factor that needs to be taken into account in order to apply enhancing treatments to improve the germination of this species under high temperatures and darkness; thus, batch-specific priming treatments are required. To illustrate this, our results show that final germination and germination rate were

increased under inhibitory conditions in batch 1 and 2 when seeds were primed in GA_3, KAR_1, NaOCl and water solutions; while no effect was observed with the application of treatments to batch 3 and 4. These latter two seed batches were harvested in the same area in the wild but in different years. This may support the hypothesis that seeds collected in the same location may have a similar responsiveness to treatments, either due to genetics or comparable conditions during growth. Moreover, the high variability across seed batches of this native species may be indicative of taxa from young landscapes, such as the post-glacial environments in Europe, while less variability may occur in species from older landscape floras (Hopper, 2009). As described in Lopez del Egido et al. in prep., the ecophysiology of certain species may be tightly related to the space in time they appear in the succession process.

Seeds from batch 1, 2 and 3, resumed germination when they were transferred to optimal germination conditions, therefore, they were thermo-inhibited. However, seeds from batch 4 remained quiescent (Fig. 6), thus they became secondary dormant. Therefore seeds from the first three batches may have acquired stress resistant through priming. In this context we define stress resistance as being able to postpone the transition of being inhibited to becoming dormant. Conditions of high temperature and limitation of light can currently occur in forested and warm areas of the planet; and in the near future, temperatures are likely to increase during summer due to global warming, which occurs more rapidly in the alpine areas (Hoyle et al., 2013), affecting germination and seedling establishment (Graae et al., 2009; Milbau et al., 2009; Shevtsova et al., 2009). These conditions can also occur in non-natural environments when seeds are produced in chambers, in order to be used as seedlings in restoration projects.

Our results also suggest that pre-treatments do not only have an effect on improving germination under non-optimal conditions, but in some seed batches priming also increased the number of germinating seeds after transfer to more suitable conditions.Therefore, pre-treatments also icreased the resistance to the initiation of secondary dormancy (Fig. 5). In this later case, application of seed treatments would be useful for seeds germinated in places where they are subjected to fluctuating optimum – non-optimum temperatures.

Although variation across seed batches needs to be considered, recommendations can be made to improve the germination of *Arabis alpina*. We suggest using the following seed treatments for each of the three purposes: 1) If the purpose is to obtain higher final germination values, we propose 4 h water or GA_3 or ½ h NaOCl priming, as these three treatments gave the highest values

of final germination. When *A. alpina* is germinated on paper, addition of GA_3 in the germination plates could also be considered as a treatment to improve the percentage of final germination. 2) If the purpose is to improve germination rate, we propose 2 or 4 h hydropriming and KAR priming. 3) If the purpose is to enhance both final germination percentage and germination rate, the treatments that would, overall, give the best enhancement combinations are 2 or 4 h hydropriming.

The effect of the treatments on germination at the inhibitory conditions was consistent across various substrates when nutrient levels were low (paper and low nutrient soil). We did not assess the effect of the treatments in a high-nutrient soil (HNS), but we did test the germination of non-treated *A. alpina* seeds at high temperatures in the absence of light, and final germination percentages of HNS differed from paper and LNS results (Table 4).

Although some of the treatments, such as 2h GA_3 priming and 4h water priming in batch 2, gave similar final germination values (Table 3 and Fig. 7), several outcomes and factors need to be considered in order to decide for the best treatment. GA_3 priming is beneficial for seed germination; however, GA_3 has also been described to cause negative effects on seedling quality, such as seedling elongation and cell weakening (Kamisaka et al., 1972). In this case, GA_3 treated seeds may produce more vulnerable seedlings, which may not be able to resist certain environmental conditions. Resources or pollution into the environment could also be a factor to influence the choice of treatment to be applied. In this case, priming seeds in water may be a preferred treatment over GA_3 and NaOCl due to the costs and the secondary effects these later treatments would imply.

Based on the knowledge gained from this study on the germination of *A. alpina* general recommendations can be made on the process of choosing a treatment to enhance germination. First, seed viability needs to be assessed, as this determines the base quality of the seed batch. If the viability of the seed batch is close to zero, treatments will not be required. However, if the viability of the seed batch is high enough but germination is low, treatments could be applied in order to enhance germination. Secondly, seed characteristics need to be assessed. This could be done by testing the germination under different conditions to detect which of those germination could be improved. Examples of conditions to test germination in include a range of temperatures, water availability and light conditions. Thirdly, variability across seed batches needs to be determined, as some seed batches might be highly sensitive or insensitive to the environment, soaking time, and/or differences in genetics. Next, the outcome and use of the treatment needs to

be defined; this is in the sense of deciding whether to strive for a treatment that improves final germination, germination rate or both. Finally, various seed treatments could be explored.

Further experiments should be performed using more seed batches and batches with larger amount of seed available to see if there is a pattern in the treatment response due to factors such as provenance of the seed, production conditions and genetics. Additional research is needed to test other application methods of the compounds; this would also help improve protocols for germination of this species. Our results suggest a correlation between paper and soil tests in low-nutrient soil, but we could not perform field experiments; future work in which seeds are subjected to treatments and later sown in the field would give valuable data to corroborate our preliminary tests in soil. We believe this to be useful information to produce higher germinating seed numbers. This would be useful in seed production and restoration areas with thermal stress, where temperatures are increasing and being more severe due to climate change. This study on priming of *Arabis alpina* shows the benefits of seed technology and their effective use on native seed, as well as the suitability for usage in restoration. Furthermore, this work examplifies how to improve seed performance in large-scale sowing in restoration projects and could be a starting point to investigate other enhancing techniques and their effect on the germination and seedling establishment of wild species. Moreover, this study brings us a step forward on the understanding of the biology of native species in Europe and, therefore, the increased capacity to have more effective restoration in a European and global scale.

Conclusion

We conclude that *Arabis alpina* seeds germinate in high numbers and fast under optimal conditions, therefore, no priming was required to improve germination. Under non-optimal germination conditions, priming enhances the germination of *Arabis alpina* seeds, however, seed batches from different sources can display a large variation in the responsiveness to the treatments, which may also occur for seeds of other species. Thus, there is not a single treatment that improves germination in all seed batches. However, recommendations can be given on treatments that may improve the germination of *A. alpina*. The decision about which treatment to apply in order to enhance seed performance of any species should, therefore, take into consideration the aim of the work (final germination or germination speed) and the seed batch.

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Chapter 5

Application of treatments across genera, within genus and species

Treatment responsiveness across genera, within genus and across seed batches

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Abstract

Germination protocols that include seed treatments are often developed for single species or for a single genus; however, variation of species responsiveness to treatments is not often considered among these groups nor among seed batches within a species. A range of treatments, which include addition of smoke-derived-compounds, gibberellic acid (GA_3) and nitrate (KNO_3) and hydropriming were used to test the germination of five species (*Arabis alpina, Viola arvensis, V. cornuta, V. x wittrockiana* and *Brassica tournefortii*) and different seed batches of those species. Compounds were applied during germination or as pre-treatments via priming or coating. The effect on final germination was assessed. Results show a wide variation of responses to the treatments across species and seed batches. These differences seem to be mainly attributed to provenance and maturity state of the seed.

Kew words: *germination, priming, coating, smoke-derived-compounds, gibberellic acid, nitrate.*

Introduction

Viola arvensis, V. cornuta and *V. x wittrockiana* Gams. (*V. tricolor* var. *hortensis*) are highly related phylogenetically and belong to the Tricolores group in the Melanium section of the *Viola* genus (Yockteng et al., 2003); however, they differ in their habitat type (Clausen, 1926; Grime et al., 1988). The former is considered an arable species that occur in lowlands , while *V. cornuta* is considered an alpine woodland species; both found in the wild and, also cultivated in the case of *V. cornuta* (Clausen, 1926). *Viola x wittrockiana* is an ornamental species that has been cropped for over 50 years, it is derived by hybridization from several *V. tricolor* species (Yoshioka et al., 2006) and it has great importance in the flower market (McDonald and Kwong, 2005; Kelly et al., 2006). Performance of *Viola* species have been demonstrated to be affected by light and temperature (Niu et al., 2000), factors that may affect germination characteristics. To test the hypothesis that different species require different seed treatments in order to enhance seed germination, I investigate the germination of four species. From those, three are wild and semi-wild species (*Viola arvensis, Viola cornuta* and *Arabis alpina*) and one cultivated species (*Viola wittrockiana*). The germination response of these four species to four seed treatments (GA_3, KAR_1, KNO_3 and 2 hour-water priming) are studied under stress conditions.

Brassica tournefortii is a Mediterranean species, considered an invasive weed in many parts of the world (Bossard et al., 2000; Bangle et al., 2008; Warwick et al., 2009; Berry et al., 2014). This species belongs to the Brassicaceae family, which also includes *Arabis alpina* and the two main model plants (Beilstein et al., 2006; Liu et al., 2012): *Arabidopsis thaliana,* used as a model in experimental genetics; and *Brassica oleracea,* used because of its large phenotypic variation (Kennard et al., 1994; Camargo and Osborn, 1996). These are well studied species and have provided insight into several topics, such as the genetics of flowering time (Schranz et al., 2002), hybridization and gene silencing (Pires et al., 2004). As many other species, the germination of these three species in the Brassicaceae family has been shown to respond to smoke-related compounds (Chiwocha et al., 2009; Long et al., 2010; Gorecki et al., 2012), including smoke-responsive and non-smoke-responsive species (Flematti et al., 2004; Daws et al., 2007; Commander et al., 2009), and including crops and weeds (Daws et al., 2007; Light et al., 2009). Several factors affect the KAR-response behaviour of species (Merritt et al., 2006; Gorecki et al., 2012); these include exposure to light (Nelson et al., 2009), temperature (Merritt et al., 2006), hydration state of the seed (Long et al., 2010) and dormancy state, which is related to after-ripening time (Long et al., 2011).

Five thousand smoke-related compounds have been identified so far (Smith et al., 2003; Nelson et al., 2012); but the smoke-derived butenolide 3-methyl-2H-furo[2,3-c]pyran-2-one (karrikinolide, KAR_1) has been successfully isolated and shown to be the major active compound to promote germination (Flematti et al., 2004; van Staden et al., 2006; Flematti et al., 2009). Nevertheless, another compound in smoke, trimethylbutenolide (TMB), has been proven to be antagonistic to KAR_1 and inhibit germination in the presence of KAR_1 (Soós et al., 2012; Papenfus et al., 2015). Other compounds identified in smoke have been the two KAR_1-photodimers that appear from the degradation of KAR_1 by the effect of light (Scaffidi et al., 2012). These photodimers have been proven to be able to promote seed germination, but at concentrations of 10µM(Scaffidi et al., 2012); while KAR_1 can work at concentrations as low as one part per billion (10^{-9} M, Flematti et al., 2004; Stevens et al., 2007; Chiwocha et al., 2009). A number of cyanohydrin analogues, such as mandelonitrile, have also been identified in smoke and have been proven to hydrolyse in aqueous solutions to liberate cyanide, which has been found to be the active stimulant for seed germination when mandelonitrile was applied (Flematti et al., 2011; Nelson et al., 2012) in a number of plant species (Hendricks and Taylorson, 1972; Dziewanowska et al., 1979; Bethke et al., 2006; Baldos et al., 2015). Although the mode of action of cyanide in stimulating seed germination remains unclear it is thought to involve ethylene production (Oracz et al., 2008) and reactive oxygen species (Oracz et al., 2009). On the other hand, KAR are suggested to act in the gibberellins pathway in *Arabidopsis*, by first binding to the KARRIKIN INSENSITIVE 2 (KAI2) receptor, which is a protein in the family of α/β–hydrolases that has been described to be analogous to the gibberellin receptor GID1 (Ueguchi-Tanaka et al., 2005); and binding later to the F-box protein MAX2 (Waters et al., 2013; Waters et al., 2014), which interacts with DELLA proteins in gibberellin signalling. Therefore KAR_1 stimulates germination by enhancing expression of the GA biosynthetic genes GA3ox1 and GA3ox2 (Nelson et al., 2009). Strigolactones are another type of butenolide that, in rice, after binding a DWARF14 protein from the α/β-hydrolases family also bind MAX2 (Waters et al., 2013) and can control many aspects of plant physiology, such as plant branching and nutrient uptake (Zhao et al., 2013). Figure 1 shows some of the compounds carried in smoke (Nelson et al., 2012) and some have been proven to provide an effect when delivered in different seed technologies, such as priming (Long et al., 2010).

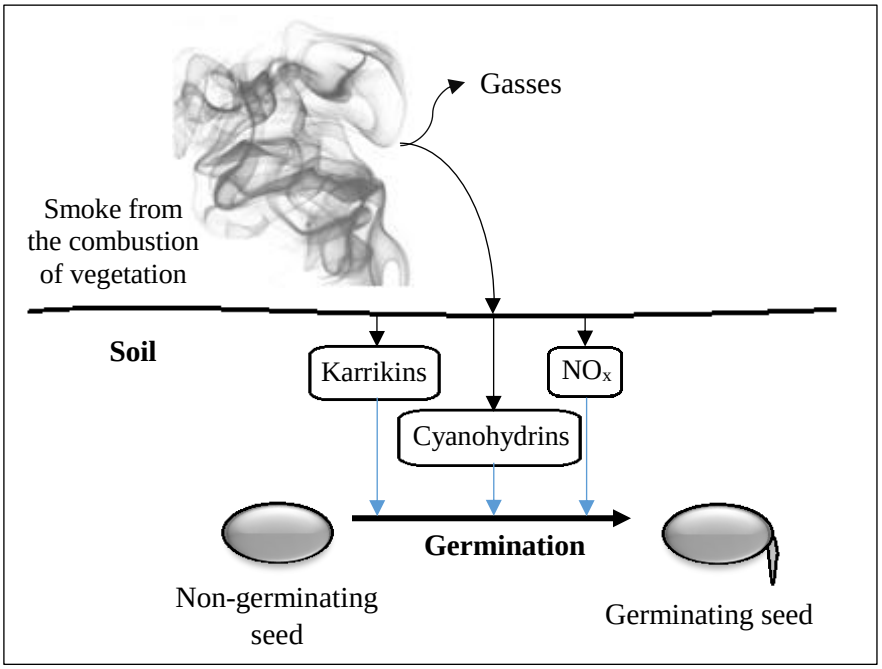


Figure 1. Figure shows germination stimulants derived from smoke. Adapted from Nelson et al., (2012).

Priming and coating are two systems to deliver compounds to the seed and enhance seed performance (Taylor et al., 1998). Both techniques are used in agriculture to improve postharvest seed quality, by improving stress tolerance and allowing release of seed dormancy, that leads to increased seed vigour, which includes final germination as well as germination rate and uniformity, and seedling development (Afzal et al., 2004; Ghassemi-Golezani et al., 2008; Anese et al., 2011). Seed hydropriming is a pre-sowing technique that involves the initiation of germination metabolism by controlling the hydration of the seeds and activating various metabolic processes, without allowing the seed to germinate, therefore it is a physiological treatment (Taylor et al., 1998; McDonald, 1999). The basic procedure consists in soaking seeds for a specific period, followed by a drying step to bring moisture content to a higher or to the same level as the originally dry seed. However, more sophisticated hydropriming treatments use different solutions to soak seeds, these can include the addition of active ingredients. Seed coating refers to “any process for the addition of materials to the seed” (Roos and Moore III, 1975; Taylor and Harman, 1990). In this study, we use the term "seed coating" to denote the application of a film coat of material(s), which involves addition of a polymer and a colourant to the seed that does not involve any change in seed size or shape. Application of seed treatments have been proven to provide advantages over application of compounds *in*-field, due to the fact that seed treatments target individual seeds, thus the amount of material used is relatively small compared to the amount needed to do field applications (Powell and Matthews, 1988).

There is a large variability in the response to treatments across species (Lopez del Egido et al. in prep.) and even within one single species and across seed batches (Lopez del Egido et al. in prep.). For this reason, this part of the project covers a more detailed study on this topic. The aim of this work was to investigate the effect of compounds that stimulate the germination of the tested species, the delivery systems that appeared to more adequately enhance seed quality and the effect of treatments at different levels: across genera and across species, and across seed batches within one species.

The choice of the tested species relied on how to best test the hypothesis of the study. First, we hypothesise that germination of a range of species will be improved under stress conditions by the application of seed treatments. The germination of *Arabis alpina, Viola arvensis, V. cornuta* and *V. wittrockiana* had been proven, in preliminary tests, to be inhibited at high temperatures. All the *Viola* species appear to belong to different habitats and to have different degrees of cropping, and, therefore, were selected to allow comparison in responsiveness to the treatments among species within the same genus. Degree of cropping refers to the possible adaptations that the species may have suffered due to breeding in cropping systems, and we consider wild, semi-wild and cropped for each species respectively. Secondly, we investigate if within one species seed batches with the same provenance would respond similarly to treatments and if the effect of those would change over time. For this we chose a species easy to crop in the glasshouse and with high yields, *V. arvensis*; therefore, all seed batches will be subjected to the same maternal environment. Thirdly, we compare a range of technologies to deliver seed enhancing compounds, thus we tested the germination of two seed batches of *Brassica tournefortii*, as this is a model species in the laboratory were priming and coating technologies were performed.

Materials and Methods

Seed material

Seeds of *Viola arvensis* Murray, common name field pansy (Bachthaler et al., 1986) were obtained from Syngenta (Syn, Enkhuizen) and Scotia Seeds (SSE, Scotland). In order to produce enough seeds for this study, plants from the Syngenta seed batch were grown in the local glasshouses (The Netherlands) and fruits were collected in July 2015. The pods were placed for 2 weeks in a drying room at an average of 20-25°C with ca. 40% RH. Seeds were cleaned and stored at 15°C, 30% RH. Seeds were used for germination tests approximately 6 months after harvest for the experiments across species. For the experiments over time, germination of four batches of *V.*

arvensis was tested, one from 2015 and three from 2016. The multiplied *V. arvensis* batch was used for all the experiments unless specified. Seeds from *V*iola *cornuta* L., common name Horned or Bedding Pansy (Kelly et al., 2006; Janicka and Dobrowolska, 2013) and *Viola wittrockiana*, were obtained from the *Viola* production sites from Syngenta. They were harvested in 2012 in Chili and in 2015 in Turkey respectively. Seeds of *Arabis alpina* were purchased in 2015 (Everwilde Farms). After drying and upon arrival, seeds from all batches were stored at 15°C, 30% RH.

Seeds of *Brassica tournefortii* were collected at maturity from a site in City Beach (6015, Western Australia; Long et al., 2010) in November 2015 and October 2016 from a wild population, and stored at 15°C, 30% RH. The two seed batches from *B. tournefortii* were tested in Australia for their response to a range of treatments (karrikinolide, KAR_1; smoke water, SW; mandelonitrile, MD; strigolactone, GR24; gibberellic acid, GA_3; potassium nitrate, KNO_3; trimethylbutenolide, TMB; KAR1-photodimer 1, D1; and KAR1-photodimer 2, D2). Smoke water was prepared as described in the International Network for Seed-based Restoration (2016) and diluted in water, to reach each of the tested concentrations.

Solution preparation

Solutions were prepared by serial dilution of stock solutions. GA_3 and KNO_3 stocks were freshly prepared and dissolved in water. Smoke water (SW) was produced by heating plant material at 175°C for 10 - 30 min and bubbling the smoke through water (International Network for Seed-based Restoration, 2016). Germination was tested for each of the compounds at a range of concentrations (Table 1); however, optimal concentration and priming time were used to perform priming and coating experiments (Fig. 3). Priming of samples was performed in an aerated solution of 1) 1 µM KAR_1, 2) 1:10 SW, 3) 1 mM GA_3, 4) 10mM KNO_3, and 5) water in a beaker for 1h and later rinsed in water. Coating of samples was performed in a RRC 150 Lab Coater (Centor Thai, Thailand) with 5g of seeds per treatment; the same priming solutions were used with the addition of 5% hydroxyethyl cellulose and 1 mL of dye. Dye was added to allow for visual evaluation of even distribution of the polymer (hydroxyethyl cellulose) onto the coated seeds.

Germination and seedling establishment testing

For the laboratory tests, 25 seeds were used per replicate and a total of 4 replicates per treatment were sown. Custom made transparent and black six-compartment trays (Voges Verpakking bv, Hillegom, The Netherlands) were used for the germination tests performed in the Netherlands.

Trays contained six 75 cm^2 wells, 2.5 cm deep with lid. Each well contained an 64 cm^2 18-layered water absorbent paper (Zell-Pak) covered by an 64 cm^2 12 Steel-Blue Seed Germination paper (Anchor Paper Co., USA) and wetted with 28 mL of demineralised water or solutions as indicated. Trays were closed with a lid to avoid evaporation and placed in a germination cabinet (IVL Van Leeuwen, The Netherlands). Germination tests were conducted under inhibitory conditions for the germination of the comparison study of *Viola* and *Arabis* species; seeds were placed at 30°C under continuous red light for the *Viola* species or continuous darkness for the *Arabis* species. Experiments testing the effect of treatments over time in four batches of *Viola arvensis* were performed at 20°C under continuous red light or continuous darkness; germination was tested at regular intervals from 0 month after harvest (MAH) and final germination percentage (FG %) was recorded.

In the case of the tests performed in the laboratory in Australia, the standard materials were used; this included germination tests in 90-mm Petri dishes containing two sheets of filter paper moistened with 15 mL of solution. Petri dishes were sealed with Parafilm M® to avoid evaporation and placed in a Biosyn 6000 OP cabinet at 20°C (Contherm, Korokoro, New Zealand) with 12h white light (TLD 36w/840 cool white, Philips) photoperiod. Primed and coated seeds were dried back with blowing air at 35°C after treatment for the first 24h and at room temperature for an extra 48h. Priming and coating experiments were only performed with the 2015 batch due to the stronger responsiveness to treatments. For the soil tests, primed and coated seeds were sown in randomised 14 x 8 x 4.6 cm trays containing a soil mix of 50% river sand - 50% native potting mix (Baileys , Kwinana, Western Australia), covered by a thin layer of sand and placed in the glasshouse at an average of 30/20°C day/night in March 2017 with no extra lightening. During incubation, germination was scored as radicle emergence at the end of the tests and germination in soil was scored as completed upon breaking of the seedling through the soil surface.

Statistical analysis

For the treatments with lack of variation in final germination, such as 0 or 100%, data was manually adjusted to 1 or 99% in order to be able to include these results in the Generalised Linear Models (GLM). All statistical analysis were performed in R (R Core Team, 2016). Final germination data were analysed separately for each species, seed batch and light condition using GLM with binomial error distribution and logit link function, in order to identify significant effects of the treatments. All the treatments were compared to the water control. Cut tests were performed at the end of the experiments and germination data is shown on 100% viable seeds.

Results

Comparison of germination across species

The results showed that under inhibitory conditions the three tested *Viola* species exhibit different responses to the applied treatments (Fig. 2). The germination of *Viola cornuta* was stimulated by nitrate ($P < 0.05$), *V. wittrockiana* was not stimulated by any of the tested treatments and *V. arvensis* and *A. alpina* were significantly stimulated by GA_3 ($P < 0.05$). Under optimum conditions all *Viola* species displayed germination numbers close to 85% and *Arabis* close to 100% (data not shown).

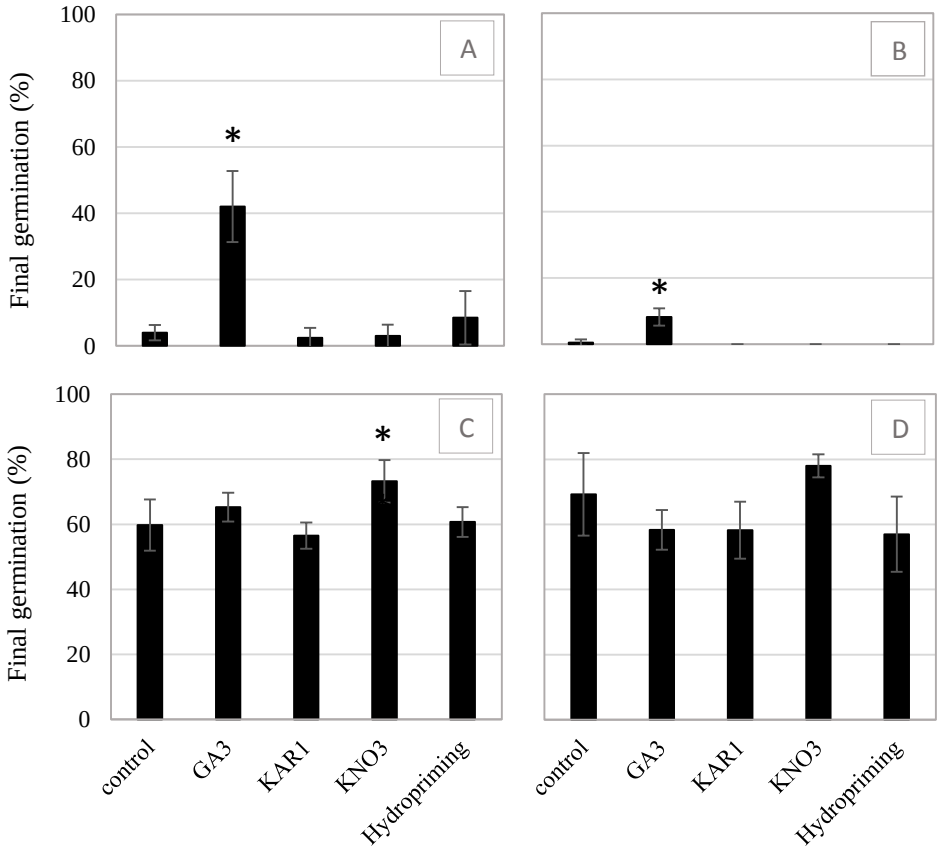


Figure 2. Final germination percentage of A) *Arabis alpina,* B) *Viola arvensis,* C) *V. cornuta* and D) *V. wittrockiana* under the application of four treatments. The following treatments were applied on the germination plate: gibberellic acid (GA_3), karrikin (KAR_1), potassium nitrate (KNO_3) and water (control and hydropriming). The hydropriming consisted in a 2h-water soaking pre-treatment followed by a drying step. Seeds were germinated under inhibitory conditions (30°C constant darkness for *A. alpina* and constant red light for the *Viola* spp). Significant results with $P < 0.05$ (*). Germination under optimal conditions is 80-100%.

Effect of treatments over time across seed batches of *V. arvensis*

Three weeks after pods harvest, considered 0 month after harvest (MAH) due to seeds being dried and prepared for storage, germination of 4 batches of *V. arvensis* was tested on water (SDW),

gibberellic acid (GA_3), potassium nitrate (KNO_3) and karrikinolide (KAR_1) solutions. No germination was observed at this stage (Fig. 3). Final germination of the batches harvested in 2016 was highly similar, thus average of the three seed batches is shown with deviations. During storage germination under darkness increased in both seed batches. In parallel with this increase, seeds gained responsiveness to GA_3 and KNO_3, but sensitivity to both compounds was acquired later in the 2015 batch. In darkness, no effect of KAR_1 on seed germination was found. Under light conditions germination also increased; however when KAR_1 was present, either in the absence or presence of KNO_3, germination in the light was considerably lower than in the dark ($P < 0.05$). Two months after harvest nitrate increased final germination under darkness for batch of 2015 ($P < 0.05$), but no effect of the treatment was found under light conditions (Fig. 3). Three months after harvest there was no effect of light on final germination with the application of nitrate ($P > 0.05$). Interestingly, after storage, no difference was found between light and dark in the 2015 batch, while significant differences existed in 2016 batch ($P < 0.05$).

Experiments to test the germination responsiveness of the Scottish *V. arvensis* batch to the treatments were performed in order to test whether provenance affected responsiveness to treatments. This batch differed in its sensitivity to the various treatments: the Scottish batch was sensitive to GA_3 but not to KNO_3, while the two Syngenta batches (2015 and 2016) are reported to be highly sensitive to KNO_3 but not GA_3 (Fig. 4). However, the two seed batches showed a reduced final germination in the KAR_1 treatment under light conditions (Fig. 4).

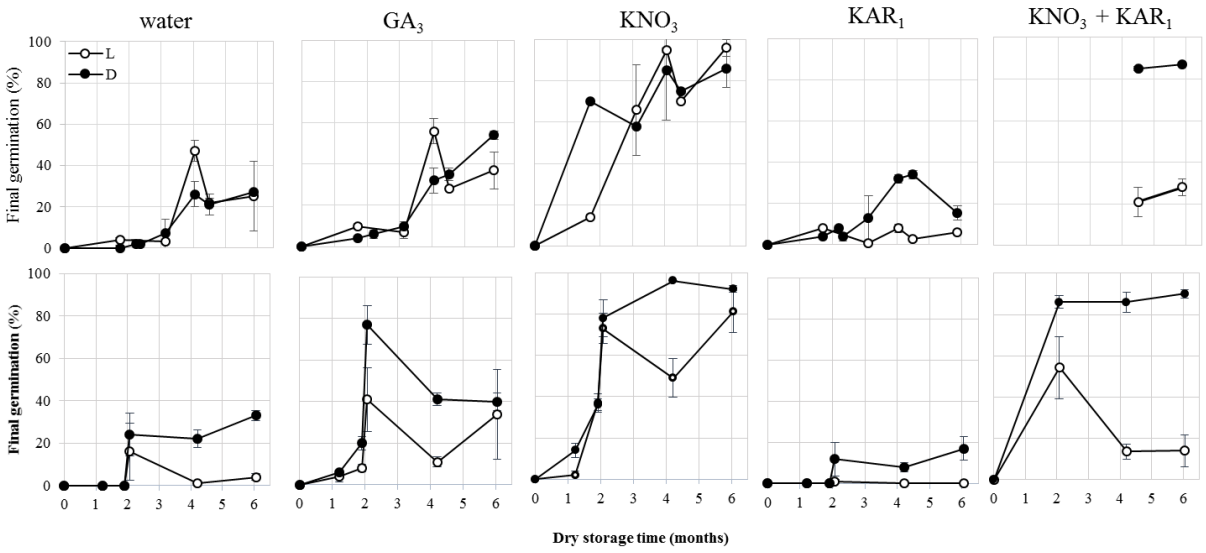


Figure 3. Final germination percentage (mean ± SE) of *Viola arvensis* batches harvested in 2015 (upper panels) and 2016 (bottom panels) during storage. Germination in the presence of: water (control), potassium nitrate (KNO_3), gibberellic acid (GA_3), karrikinolide (KAR_1) and a solution containing both potassium nitrate and karrikinolide (KNO_3 + KAR_1). Open circles (○) indicate germination under light and closed circles (●) indicate germination under darkness. Final germination of the batches harvested in 2016 was highly similar, thus average with deviations is shown.

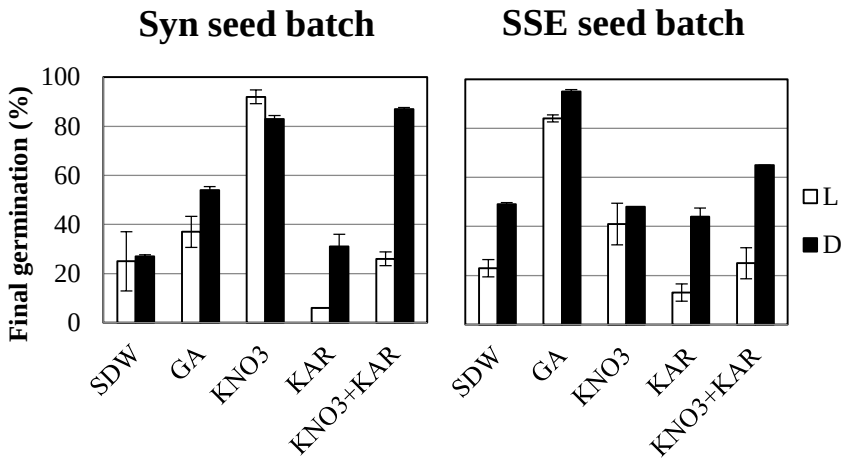
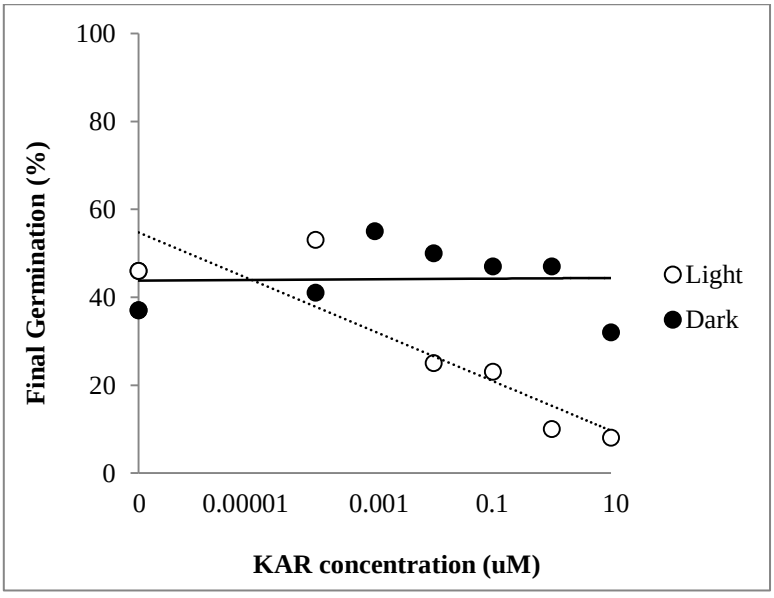


Figure 4. Final germination percentage (mean ± SE) of two batches of *Viola arvensis* produced at different locations. One of the batches was multiplied in the local glasshouses in the Netherlands (Syn) and the other was purchased from Scotia Seeds (SSE). White and black coloured bars show germination under constant light and darkness respectively. The treatments tested include: water (SDW), gibberellic acid (GA), potassium nitrate (KNO3), karrikinolide (KAR) and a solution containing KNO3 and KAR (KNO3 + KAR).

KAR_1 was tested at a range of concentrations on the germination of the 2015 *V. arvensis* (6 MAH). Parallel tests were performed with a seed batch from a cultivated *Brassica* species. Final germination of the *Brassica* species was increased compared to the control in water with the application of KAR_1 at any of the tested concentrations (data is not showed). However, there was no effect of KAR_1 in the final germination of *V. arvensis* in the dark (Fig. 5). Conversely, final germination of *V. arvensis* was inhibited with increased concentrations of KAR_1 under light conditions ($P < 0.05$).

Figure 5. Final germination of *Viola arvensis* germinated in constant light (○, open circles) and constant darkness (●, closed circles) at 20 °C. The seed batch tested was harvested in 2015. Germination tests were performed 6 months after harvest. Logaritmic curves were fitted for germination under dark data (solid line) and for light data (dashed line). X-axis is displayed in logaritmic scale.



Compounds and delivery systems using seed batches of *Brassica tournefortii*

Experiments were performed to test the sensitivity of two batches of *Brassica tournefortii* to a range of smoke-derived compounds (Fig. 6). Optimal concentration and priming time were used to perform priming and coating experiments (Fig. 7 and 8). Results showed that germination of batch 2015 was stimulated to almost 100% final germination with the application of GA_3, KAR_1 and SW, and to 45% in KNO_3 and 25% in MD ($P < 0.05$) when applied to the germination plate. Batch 2016 also responded with 100% final germination to GA_3, and 15-20% final germination on average when treated with GR24 and KAR_1 ($P < 0.05$). Batch 2016 was not tested for all the treatments.

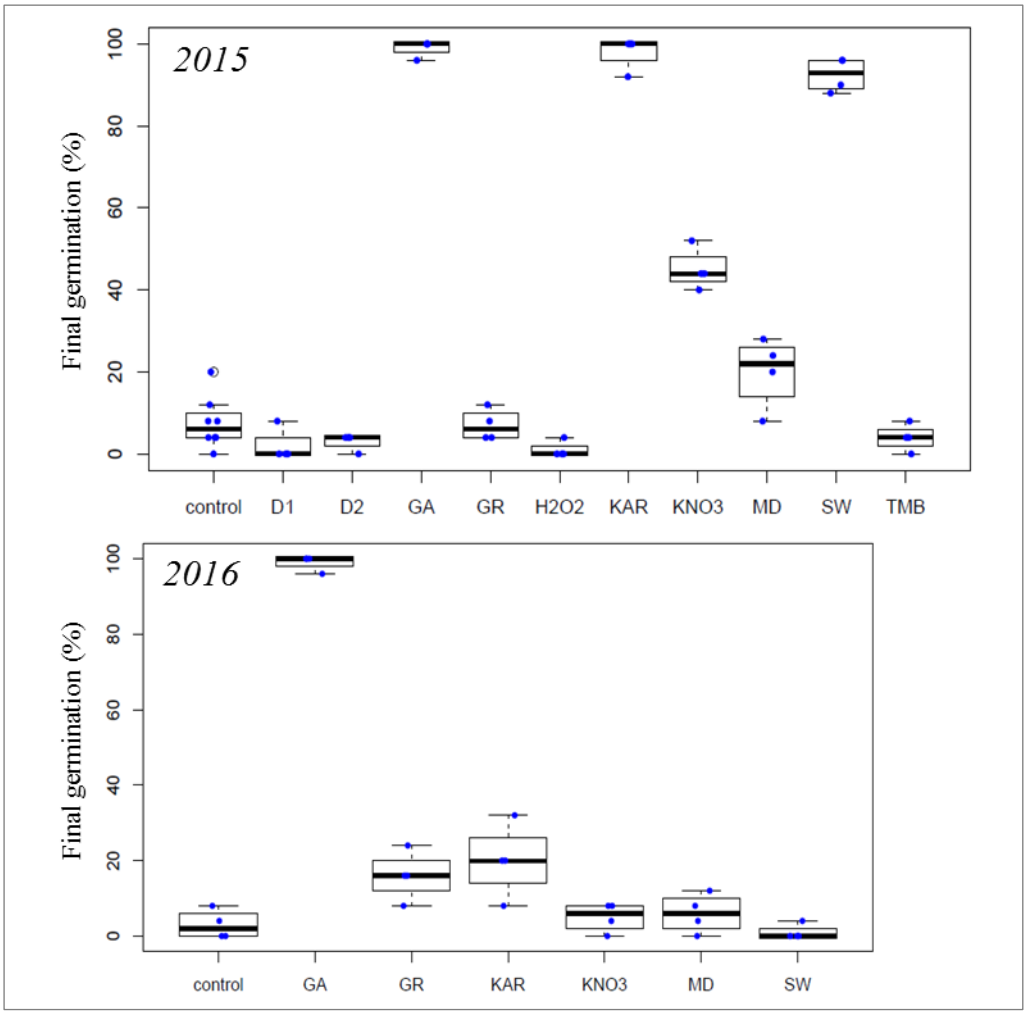


Figure 6. Boxplots show final germination percentage of *Brassica tournefortii* seed batches (2015 and 2016) under 20°C with a 12h white light/12h dark photoperiod when subjected to smoke-derived compounds compared to the control in water. These compounds were: KAR-photodimer 1 (D1), KAR-photodimer 2 (D2), gibberellic acid (GA_3), strigolactone 24 (GR), hydrogen peroxide (H_2O_2), karrikinolide (KAR_1), potassium nitrate (KNO_3), mandelonitrile (MD), smoke water (SW), and KAR antagonist (TMB). Batch 2016 was not tested for all the treatments as preliminary tests showed no effect. The central rectangle spans the first to the third quartile (the interquartile range or IQR) and the mean final germination (thick central line). The whiskers above and below the box indicate the variability outside the upper and lower quartiles, the minimum and maximum values. Outliers are represented with an empty circle.

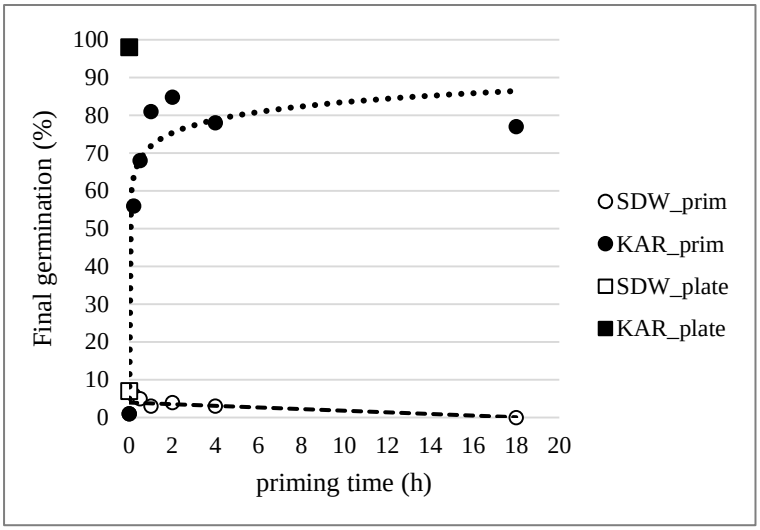


Figure 7. Priming time (hours, h) *versus* final germination percentage of *Brassica tournefortii* to obtain optimal priming time. Logarithmic fitted curves for priming in karrikin (dotted line for KAR priming; ●; y = 5.0735ln(x) + 71.771; R^2 = 0.9369) and linear fit for priming in water (dashed line for SDW priming; ○; y = -0.2155x + 3.934; R^2 = 0.434). Germination with the application of KAR (■) or water (□) in the germination plate is also shown.

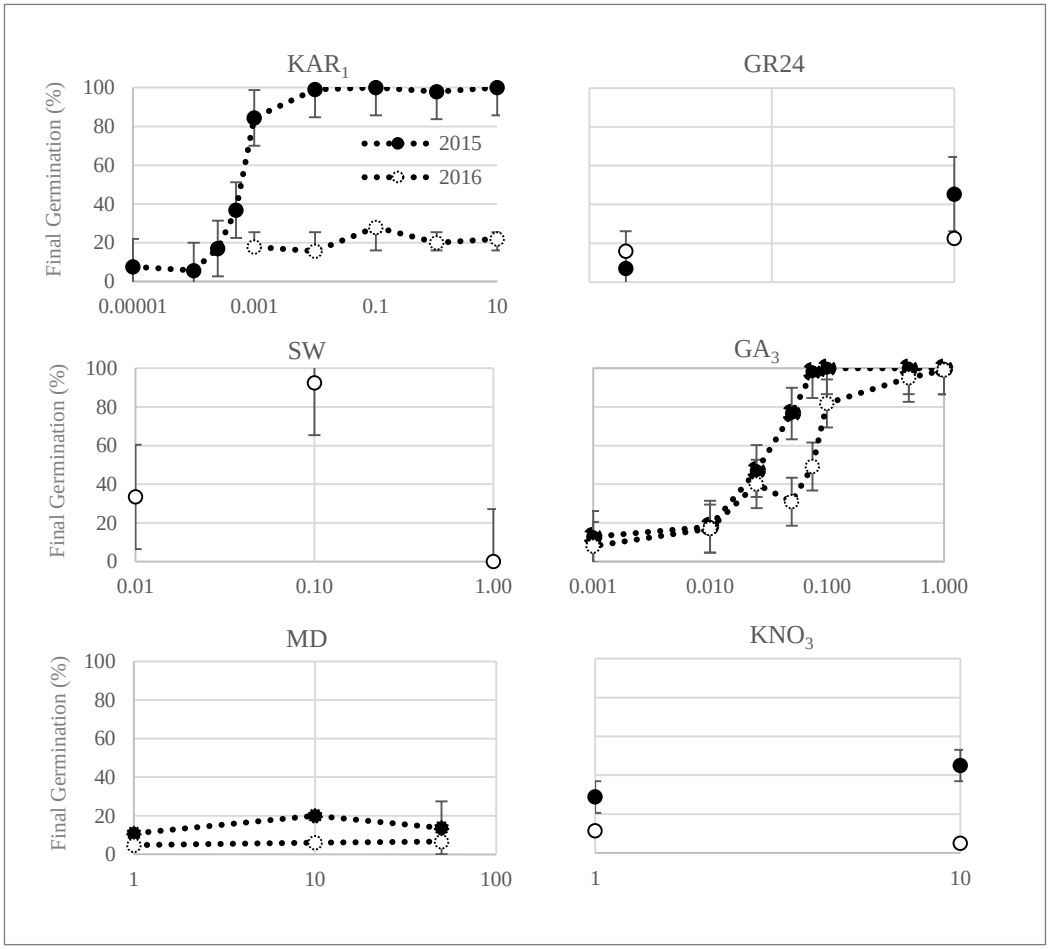


Figure 8. Final germination percentage (mean ± SE) of *Brassica tournefortii* seed batches harvested in 2015 (●, filled symbols) and 2016 (○, empty symbols) with the application six compounds. These were karrikin (KAR_1, µM), strigolactone (GR24, µM), smoke-water (SW), gibberellic acid (GA_3, mM), mandelonitrile (MD, µM) and potassium nitrate (KNO_3, mM). Germination under 20°C with 12h white light photoperiod.

In the case of *B. tournefortii*, KAR_1 increased final germination at 0.00025uM for both batches ($P < 0.05$). GA_3 also had a stimulatory effect on the germination or *B. tournefortii*, and it significantly affected germination at a concentration of 0.01 mM ($P < 0.05$).

Final germination on paper of primed seeds was increased by GA_3, KAR_1 and KNO_3; while germination in soil was increased by GA_3 and KAR_1, but not by KNO_3 (Fig. 9). Seeds coated with the same concentration as for the priming samples did not show an improved germination or improved establishment in soil after being treated with any of the compounds; however, the results differed when concentration of the coating solution was increased 5 and 10 times (data not shown). In this case, KAR_1 coated samples showed an improved final germination ($P < 0.05$), in contrast to no effect for the GA_3 treated samples. In all cases, germination on paper was comparable to seedling establishment in soil ($P < 0.05$).

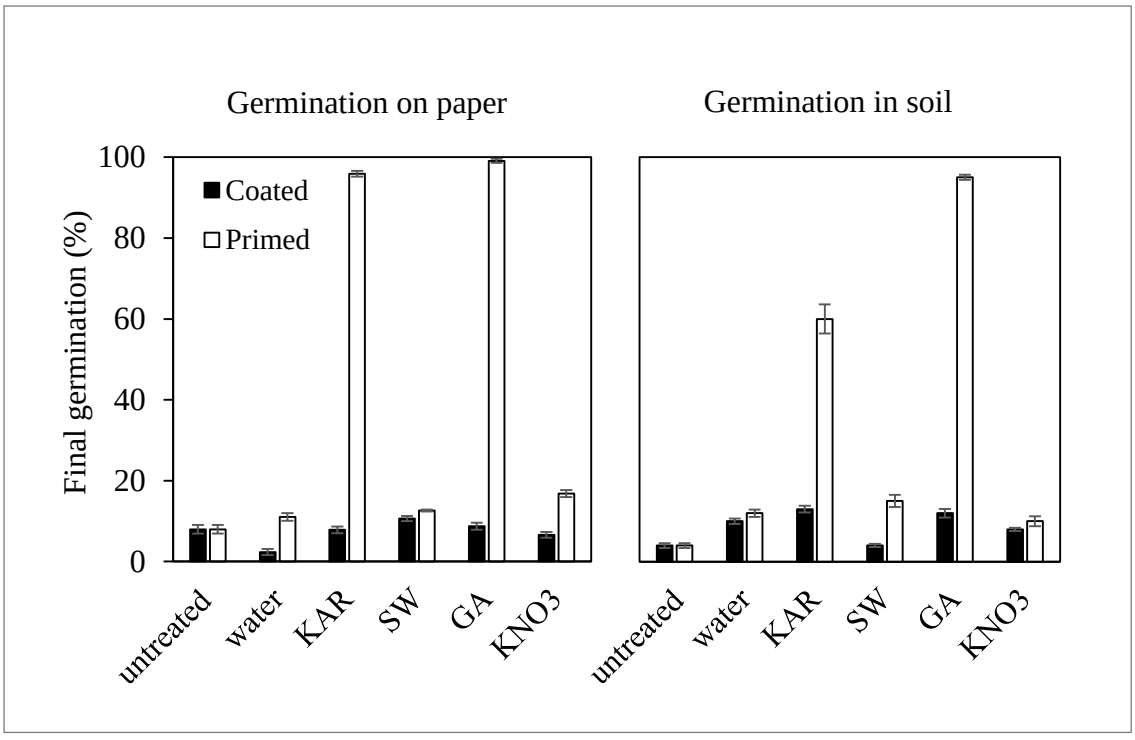


Figure 9. Final germination percentage (FG, mean ± SE) of coating (filled bars) or primed (empty bars) seeds of *Brassica tournefortii* harvested in 2015. FG of untreated seeds was compared to FG of seeds treated with the following compounds: water, karrikinolide (KAR), smoke water (SW), gibberellic acid (GA_3) and potassium nitrate (KNO_3). Germination tests were performed under 20°C with a 12h white light/12h dark photoperiod.

Discussion and conclusion

Comparison of germination across species

We hypothesised that the application of seed treatments would overcome inhibition of germination of cultivated and wild species under stress conditions. The fact that species have been domesticated has affected species phenotype (McCouch, 2004) and, domestication may also determine how species respond to the environment (Zohary, 2004; Nicotra et al., 2010). Therefore, domestication might explain the differences in the effectiveness of the treatments, due to loss of adaptation to environmental signals by the cropped species. We speculate that two reasons may be the cause for increased germination of the cultivated *Viola* species (*V. wittrockiana* and *V. cornuta*) under high temperature conditions: 1) selection for a specific trait, such as flower morphology, may have driven unintended selection on the ability to germinate at high temperatures, or 2) the cultivated species have adapted to the growth conditions, which include areas with a wide temperature window, in other words, with low and high temperatures, e. g., Guatemala and Chile (McDonald and Kwong, 2005). Moreover, none of the treatments stimulated the germination of *V. wittrockiana*, this might suggest that this species has been cropped extensively and selected to have a stable performance. On the other hand, *V. cornuta* (semi-cropped) and *V. arvensis* (wild) do respond to treatments, but not to the same ones.

Effect of treatments over time across seed batches of *V. arvensis*

It is not new that germination of seeds in the natural populations change over time due to external factors, such as temperature and soil moisture fluctuations (Bouwmeester, 1990; Bouwmeester and Karssen, 1992; Benech-Arnold et al., 2000), which affect internal seed characteristics, such as dormancy cycles (Moreno-Casasola et al., 1994; Batlla et al., 2003). However, this has also been shown to occur when temperature and relative humidity are constant during storage of seeds (Budelsky and Galatowitsch, 1999; Basbouss-Serhal et al., 2015). Here, the sensitivity of germination to seed additives during storage was investigated. Decrease in germination during dry storage may be linked to loss of seed viability (Gurusinghe and Bradford, 2001; Demir et al., 2011); while increase in germination may be related to reduction of seed dormancy and after-ripening (Li and Foley, 1997; Batlla et al., 2003; Leubner-Metzger, 2003; Basbouss-Serhal et al., 2015). The latter is in line with the results obtained for the four *V. arvensis* batches, in which germination of the control samples germinating on water increased with storage; and also sensitivity to the compounds increased with after-ripening. It is important to note that KAR_1 has been reported to stimulate germination in a wide range of species (Chiwocha et al., 2009), while the four *V. arvensis* batches show a consistent inhibition of germination with the application of

KAR_1 under light conditions. This inhibition of germination by KAR_1 in the light might be due to the fact that *V. arvensis* is not a pioneer species, but that it appears in a later stage of succession. The reason behind this might be that this species requires fertile, disturbed environments (Storkey, 2004) and protection from other vegetation in order to establish. *V. arvensis* has been described as a weed in agricultural fields (Froud-Williams et al., 1984; Baskin and Baskin, 1995; Marshall et al., 2003), which may be a situation comparable to a secondary stage in the succession where agricultural practices provide fertilisation and disturbance, and the crop provides this needed protection.

Despite difference in the production environment of the four Syngenta batches of *V. arvensis*, similar responsiveness to the treatments was found at 6 MAH. However, *V. arvensis* seeds produced in Scotland appeared to have a different sensitivity to treatments. This suggests that the production environment (provenance) plays an important role in the way different seed batches respond to the environment (Keller and Kollmann, 1999; Wingler et al., 2014). Differences may also exist in the genetics of these batches, which needs to be considered.

Compounds and Delivery systems using seed batches of *Brassica tournefortii*

Technologies to deliver compounds to enhance germination (priming and coating) were investigated. Four compounds stimulated germination when added during germination (KAR_1, SW, GA_3 and KNO_3); from these, all except SW also stimulated germination in when used to prime seeds; and only KAR_1 enhanced germination in coated seeds. KAR_1 significantly increased final germination at low concentrations, which is in line with previous studies (Flematti et al., 2004; Stevens et al., 2007; Soós et al., 2012; Waters et al., 2014). The difference between the effectiveness of the GA_3 and KAR_1 treatments to enhance germination of the two batches may be due to the after-ripening state of the seed batch. Batch from 2015 showed a positive effect both to GA_3 and KAR, while batch from 2016 showed a positive responsiveness only to GA_3. The results lead us to suggest that: 1) GA_3 is able to break strong primary dormancy, such as is the case for the 2016 batch; and 2) KAR_1 is only able to break weak dormancy, like in the case of the 2015 batch, which has lost strong dormancy during after-ripening. Apparently, the positive effect of the treatments is maintained across all delivery types; however, from the application types tested, priming seems a more reliable method to increase seed and seedling quality, as germination and seedling establishment could be improved in all cases through priming, but could not be improved in the case of GA_3 when applied during coating.

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Chapter 6

General discussion and conclusions

Application of seed treatments

Germination characteristics of cultivated species have been investigated for years (Maguire, 1962; Tekrony et al., 2005; Willenborg et al., 2005; Magneschi and Perata, 2009) ultimately to improve germination of these species. Many studies have been published on seed treatment techniques to improve germination of cropped species. These seed enhancement techniques include scarification (Haynes et al., 1997; Shanmugavalli et al., 2007); stratification (Haynes et al., 1997; Gisbert et al., 2009); immersion of seeds in hot water (Farajollahi et al., 2014); coating (Scott, 1989; Willenborg et al., 2004); and priming, defined as pre-soaking seeds in various solutions (Ashraf and Foolad, 2005; Shanmugavalli et al., 2007; Galahitigama and Wathugala, 2016). Various seed priming techniques have been developed: hydropriming, which consists of soaking in water (Fujikura et al., 1993); halopriming, which consists of soaking in inorganic salt solutions; osmopriming, which consists of soaking in solutions of different organic osmotica (Fujikura et al., 1993; Basra et al., 2005); and biopriming, which consists of hydration using biological compounds. These treatments are intended to improve germination (Mahmood et al., 2016; Singh et al., 2016), control pathogens (Jensen et al., 2004; El-Mougy and Abdel-Kader, 2008) and regulate plant growth (Meena et al., 2016). Priming has been proven to enhance the germination of cropped species under stress conditions (Cantliffe et al., 1984; McDonald and Kwong, 2005). However, much less is known about the biology of wild species and the potential of treatments to improve their germination. However, priming had not yet been investigated in a wide range of wild species, and neither had the application of other treatments. The purpose of this study was, therefore, to investigate the effect of treatments to improve the germination of a set of wild species, both under stress and optimum conditions.

Germination patterns across and within species

In Chapter 3 – 5, germination pattern across species and seed batches has been demonstrated to be highly variable, which had been also described in previous studies (Wagner et al., 2011; Wingler et al., 2014). Likewise, the effect of germination enhancing treatments on a range of species and seed batches also differed; this not only included the type of the treatment, but also the length of the treatment. In the case of priming, in order to obtain optimum seed quality of three different accessions or varieties of one single species different soaking times should be considered, as these varieties may have different requirements on soaking time (McDonald and Kwong, 2005). A series of factors may interfere with the way various species and seed batches respond to seed treatments, some of them are the ecology of the species, and provenance and seed maturity of the seeds.

Overall, some treatments have been shown to stimulate germination of a range of species, while others have proven to be species-specific and even batch-specific, therefore information can be given to provide advice on how to improve seed quality of a species when no information exists in the literature; however, because of this specificity the effect of the treatment on a specific seed batch cannot be guaranteed.

Agriculture *versus* nature: Which type of seeds shall we select for? Shall we select for homogeneity or heterogeneity?

Good selection of seed is the basis for the success of the future generations. However, the definition of what a "good" seed is may depend on who answers. In agriculture, seeds are produced to obtain genetically identical plants, with little or no variation in seed, seedling, plant and fruit characteristics. The mother plants will have been selected for high yields; resistances; fruit qualities, such as colour and shelf life (storability). Regarding germination, each seed should develop into a mature plant in order to have a high profitability, thus seed companies aim for rapid, uniform, 100% germinating seeds in order to reach this target. Also, the window for germination should be narrow, leading to all the seeds germinating at the same time, soon after they are sown, independently to the environmental conditions. Therefore there is a strong selection for specific traits, such as yield and reproductive characters (Nguyen and Sleper, 1983).

On the other hand, nature strives for other characteristics: yield and resistances are not the main characteristics to select for, unlike for seed companies; because of the changing environment selection will go towards variability, as in the diversity of responses relies the success and survival of the species (Herrera, 1998; Herrera et al., 1998; Van der Heijden et al., 1998). There is therefore, a selection for fitness and not traits. This involves high adaptability of the species to the environment and its conditions (Nathan and Muller-Landau, 2000; Koenig et al., 2003), and high plasticity in the response to these conditions. For that, individuals within a population will be selected for a high genetic diversity in order to respond differently to the biotic and abiotic surroundings. As an example, seeds will usually germinate after different intervals of time to ensure survival of some of the individuals from the offspring. The species will continuously try to adapt to the neighborhooding environmental conditions and evolve.

Typically, the characteristics that drive selection and definition of a "good" seed seem to conflict and refer to opposite things when comparing both situations. But in the two cases they describe a "good" seed for the purpose they target. Therefore, there appears not to be one definition of "good seed" or "good selection", as this will depend on the use of the seed and the aim of the selection.

A similar comparison could be made with the use of seed treatments. Application of treatments that lead to improvement of final germination, uniformity and germination speed would be preferred in the seed industry, while they may not be recommended for their use in natural conditions due to a preference for non-uniform seed germination in this situation. However, restoration of natural habitats may require seeds with high stress tolerance and seedlings of native species ready to germinate at a specific time, which could both be obtained by the use of pre-treated seeds. In this sense, restoration on a bare soil area could be seen as a cropping set up of wild species; therefore, seed and seedling requirements would not be that different from what is demanded in agricultural systems.

Conservation and restoration remarks

Although destruction of natural habitats continues to increase, awareness of this destruction is also increasing together with the acknowledgement of the importance for conservation (Banda et al., 2016) and ecological restoration (van Andel and Aronson, 2012). This has stimulated restoration and ecological landscaping designs focussing on the use of native plant materials (Milstein, 2005; Sacande and Berrahmouni, 2016), and since the last few years more people make use of wild species in restoration, private gardening, parks, residential and commercial developments, golf courses and amusement parks (McDonald and Kwong, 2005). Several studies describe the benefits obtained from ecosystems, denominated "ecosystem services" and conservation of wild nature (Costanza et al., 1997; Balmford et al., 2002; Primack, 2006; Swinton et al., 2007; Power, 2010; Alexander et al., 2016), and some surveys have shown that people value the increase in ecosystem services (Loomis et al., 2000), which could be gained by conservation and restoration of ecosystems. There is currently also an increasing concern about biodiversity loss, and as a result, there has been an increase in the use of wild flowers and the attemps to restore natural habitats (Menz et al., 2013) with the intention to improve the environment. Germplasm preservation has also gained attention, and for the last 60 years management of plant genetic resource has developed into long-term storage of seed in cold storage facilities, tissue culture, meristem preservation, *etc*. (Kew Royal Botanic Gardens; The New York Times Magazine, 2017). Also, restoration projects are currently considering native species and the importance of genetic diversity (Jones, 2005; Lindenmayer et al., 2008; Bischoff et al., 2010; Vander Mijnsbrugge et al., 2010; Thomas et al., 2014).

Closing statements

I believe this study provides novel information on a variety of topics. First, we have developed a new tetrazolium (TTC) based method to assess seed viability; further research could trial the method in a range of wild species to develop protocols for rapid assessment. Second, we have provided experimental data on the germination characteristics of 29 species and several seed batches under a range of light and temperature conditions. Furthermore, we have produced information on the responsiveness of several wild species to seed enhancing compounds and to the application of seed technologies. Moreover, we have not only compared the effect of treatments across different species, but also across seed batches. These include comparisons such as species from different habitats, cultivated *vs* wild species and seed batches at different maturity stages. The described methods and technologies could be useful not only in the seed industry, but also in seed restoration to deliver enhanced seeds with the potential to perform better under stress conditions. In summary, we believe this work provides useful information not previously described in the literature on the effect of maternal environment, ecology and seed maturity on the efficiency of seed treatments and seed quality. Restoration of vegetation by using seeds is a challenge due to the diversity in requirements of species and seed batches. Is, therefore, restoration with seeds of wild species as easy as some people may think?

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