University of Pavia

Department of Earth and Environmental Science

PhD in Earth and Environmental Sciences, CICLE XXX

Comparative seed biology of European temperate forest herbs

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Academic Year 2016-2017

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CICLE XXX -Curriculum NASSTEC (2014-2017)

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By

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To all the seeds that lost their

lives to produce these results

Where have all the flowers gone?
Long time passing
Where have all the flowers gone?
Long time ago

Pete Seeger

CERTIFICATION

I, Cristina Blandino, declare that this thesis, submitted in partial fulfilment of the requirements for the award Doctor of Philosophy, in the School of Earth and Environmental Sciences, University of Pavia, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution. This thesis contains work prepared for publication, some of which has been co-authored.

Cristina Blandino

August 20th, 2017

AKNOWLEDGEMENTS

I would like to thank my family, first, for encouraging me to apply for this PhD, and then for their moral support during all these years, and my partner, Giacomo, for being close to me. It has been a formative, and sometimes surrealistic, experience from both the professional and human point of view. A huge thank you goes to Prof. Hugh Pritchard and Dr. Eduardo Fernàndez Pascual, for their infinite patience and availability in supervising me and to Giles Laverack, for his initial guidance and the interesting exchanges of ideas on restoration and landscape conservation. Thank you also to Rosemary Newton for introducing me to this job, her initial supervision and the always fruitful conversations. The list of people to thank for the help, that in different form, gave me in these years is huge, and some of them are listed at the end of each experimental chapter. All the collegues at the Millennium Seed Bank and at Wakehurst Place have always been kind and made me feel welcome all the time. I've always received help when asking a question but invaluable, for the realization of the experiments, was the technical support of John Adams, Keith Manger and Pablo Gomez Barreiro. During my secondment at the Jardìn Botanico Atlantico, Alvaro Bueno Sanchez provided me not only professional supervision but also shelter and friendship, for which I will always be grateful. For the same reasons I thank Maria Marin and David Boldrin. Finally, thank you to all the people in the NASSTEC consortium and especially to the fellow ESR that have been to me, more than mere collegues, a group of friends and to Antonio Da Costa Teixeira, with whom I shared this adventure since the beginning.

ABSTRACT

Herbaceous species of European ancient woodland understories are affected by land use and climate change. Their distribution can be at risk because of their poor capacity to colonize isolated forest patches. The objective of this thesis was to compare the regeneration strategies of those species, with a focus on their germination traits.

A database of ancient woodland seed traits was created by reviewing published information for 208 indicator species. The database included seed germination traits, embryo:endosperm ratio and other traits related to the plant regeneration strategy. Field surveys were conducted to compare the understories of old and recent woodlands in Spain and England. Community-weighted means of several seed traits were calculated to assess the functional regeneration ecology of the species that naturally colonize mature and recent forest patches. *Conopodium majus* (Apiaceae) was chosen as a model for further experimental work, as a morphologically dormant species that represent the ancient woodland regeneration strategy described in previous chapters. The temperature and chemical cues for embryo growth were investigated using sectioning and image analysis of seeds at different stages of development. Embryo response to desiccation at different stages of development was explored. Nine populations of *Conopodium majus* were sampled across a latitudinal transect of the distribution range of the species. The temperature control of embryo growth was investigated in the laboratory and in the field and compared with local climate.

Two groups of understory species were described according to their regeneration traits. The first group included late flowering species possessing seeds with physiological dormancy that germinated in spring and had a requirement for light, cold stratification

and high germination temperatures. Those species produced many small seeds and were taller, suggesting a good colonizing capacity. Species of the second group had seeds with morphophysiological dormancy, and were able to germinate in absence of light following a warm stratification. They produced few big seeds on short stems, suggesting a poor colonizing capacity. Species of the first group were abundant in the recent plantation sites while species of the second one characterized the plots on mature forests. *Conopodium majus* seeds lost viability if stored at relative humidity higher than 60%. They showed a narrow germination windows with respect to temperature conditions and were still able to germinate after being dried to 15% and 60% Rh after 84 days of imbibitions at 5˚C. Optimal temperatures for embryo growth and germination varied, across all populations, between 2.5 and 5˚C, ceiling temperature between 11.5 and 15.5 ˚C and base temperature between – 10.5 and -1.7 ˚C. Germination in the field peaked in the months of January and February and the field observation agreed with the predicted germination timing modelled on local climate data.

Forest specialists can be differentiated from other forest species on the basis of their germination traits. The identification of regeneration strategies characteristic of poor colonizing understory species provided the basis for planning restoration interventions for European temperate forest understories according to species germination strategies. A protocol for handling and storage of *Conopodium majus* seeds was developed and the method used to characterize its germination across its latitudinal range could give insights on how different scenario of predicted climate change can affect this species, characteristic of the Atlantic European biogeographic region, in the face of climate change.

ABBREVIATIONS AND ACRONYMS

AWI – Ancient Woodland Indicators

CWM – Community weighted mean

EIV – Ellenberg indicator values

FMDA – Factorial analysis for mixed data

GLM – Generalized Linear Model

HCPC – Hierarchical clustering on the principal components

MD – Morphological dormancy

MPD – Morphophysiological dormancy

ND - Non dormant

NMDS – Non metric multidimensional scaling

PCA – Principal component analysis

PD – Physiological dormancy

PY – Physical dormancy

PYPD – Physical + Physiological dormancy

SLA – Specific leaf area

TV – Terminal velocity

TZ - Tetrazolium Chloride

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CHAPTER 1

INTRODUCTION

Forests have high species richness and the most diverse terrestrial ecosystems in the world are found in tropical forests. However, during the last 300 years, the areas of the world covered in forest have reduced by 50% (Millennium Ecosystem Assessment, 2005).

Temperate forests are found in both hemispheres between the 25 and 50° latitude (Encyclopedia Britannica, 1997). They host less species than their tropical counterpart but still provide a wide range of important ecosystem services, mainly in the form of climate control, soil formation, waste treatment, provision of food and raw materials, estimated as 302 $/ha/y in 1994 (Costanza et al., 1997).

Temperate forests are characterized by seasonal cycles, with an alternation of cold winters and warm summer, even though the severity of the seasonal climate can vary within different forest types depending on their latitude. The climatic limiting factor to plant growth can be constituted by cold temperatures during winter and by drought during summer, especially at lower latitudes. Another important factor that seasonally influences plant growth in deciduous temperate forests is light availability. In fact, light availability, especially at ground level, has a peak during spring, before the tree canopy closes. Temperatures start to rise but the tree canopy is still open so that light availability is not a problem. As a consequence, species adapted to temperate deciduous forest environment, have developed a series of mechanism to tune their life cycle to this predictable seasonal environment.

Plants adapted to this habitat have, therefore, developed life cycles that take in account the seasonal availability of resources and these adaptations are reflected by their reproductive phenology. In particular, early spring is the season when the condition are more favourable to develop the more energetically demanding phases of life cycles.

In Europe, 96% of the forests have been modified by human management (Bastrup-Birk al., 2016) and the legacy of past agricultural land use on their structure can be still

detected after centuries of abandonment. For example, Dambrine et al. (2007) found, observing the species composition and the soil chemistry of a France old forest, legacies of its past agricultural use dating back to Roman times.

However, after centuries of intense agricultural use, the industrial revolution and changes in agricultural practices, led to an abandonment of cultivated land in Europe and forest cover has gradually started to recovery, leading to an increment in forest cover the las few decades (Bastrup-Birk al., 2016). When former agricultural land is abandoned, the secondary succession is slow and the new forest that results often has a different species composition compared with its original state (Peterken and Game, 1984). When forests are actively restored, the management intervention often focuses only on the reintroduction of trees and shrubs, whilst the understory layer is often neglected (Francis and Morton, 2011).

The understory layer of temperate forests account for the greater number of plant species and has an important ecological role because it constitutes the environment in which regeneration takes place for all the species (Gillam, 2007). The herbaceous layer also plays a role in the recycling of nutrients and in setting the interaction with above ground flora. Some common life history traits characterize forest understory herbs of temperate forests. For example, numerous studies have noted the prevalence of early flowering geophytes with the ability to vegetatively reproduce (Hermy et al., 1999; Whigham, 2004).

According to Grime ‘s (1974) definition of ecological strategies, typical herbaceous understory plants are stress tolerators because they can survive in an environment in which the light resource is often limited. Species indicators of an history of continuous forest cover (“Ancient Woodland Indicators”, “AWI”), are known for being poor colonizers with a low seed dispersal capacity and for not forming a long lived soil seed

banks (Hermy et al., 1999; Verheyen et al., 2003). Consequently, the species composition of the soil seed bank of mature forests is very different from the above ground vegetation and is enriched with opportunistic, small seeded species that are able to quickly colonize an area of disturbance when more light is available (Bossuyt et al., 2002; Bossuyt and Honnay, 2008).

Cramer et al., (2008), describe two types of threshold that can be crossed when an old field evolves towards a secondary succession into forest: a biotic and an abiotic threshold. The first is crossed when the composition of the new habitat lacks species that could potentially grow there but whose dispersal is limited by distance from a propagule source or, as it is the case for many forest herbs, by a low colonizing capacity (Verheyen at al., 2003). In these cases, habitat restoration should focus on reintroducing these species. Also mature forests are different from recent ones in their physical environment. Therefore, a second threshold may exist relating to abiotic conditions, i.e., the physical environment is now not suitable for certain species that were present before habitat degradation. Apart from the obvious differences in light availability between old and very young plantations, that result in enhanced competition from light demanding species, a change in soil chemistry due to agricultural use can remain for years after canopy closure (Dambrine et al., 2007). When the abiotic threshold has been crossed, restoration intervetions should seek to recreate the physical conditions for plant establishment (Cramer et al., 2008).

Another, important, difference between mature and recent forests is that the first provide a more heterogeneous landscape, with gaps in the canopy, presence of dead wood and uneven aged trees. Such a landscape provides an higher diversity of ecological niches. These features are often missing from recent forest that present a much more homogeneous environment and a soil impoverished in C and N and enriched in P, if set on past agricultural soils (Flinn and Marks, 2007; Verheyen et al., 1999).

Forest understory restoration by seed: what are the challenges?

Habitat restoration by seed addition is an approach that has been widely used in a range of habitats, with variable degrees of success. For example, in his review, Turnbull et al. (2000) state that it is not seed limitation but establishment failure that often limits the success of a restoration intervention.

Few attempts have been made to restore the understory of recent plantation with woodland species and, when carried out, the taxa selected have often been characteristic of forest hedge habitats (Francis and Morton, 2001).

In 1977, Grubb defined the concept of "regeneration niche" and stated that the environmental conditions required in the early stages of life by a plant (e.g., germination, seedling establishment) can differ from the habitat requirements of the adult specimen. As a consequence, the knowledge of the ecology of seed germination and seedling development is pivotal in developing successful restoration intervention by seed. In fact, seed broadcasting in recent woodlands, in which the environmental conditions for plant establishment are not yet present, can be unsuccessful because the germination requirement (e.g. presence/absence of light, temperature fluctuations, temperature stratification), of temperate forest understory species are often complex (Baskin and Baskin, 2019; Vandelook, 2009) and are an indication of their adaptation to stable and predictable settings.

Seed dormancy is a mechanism that prevents seed germination in conditions that are not suitable for seedling development**.** Dormancy to spread germination in time is also common in ruderal species, usually as a function of seed polymorphism that ensures germination across many years and assures survival also through unfavourable years (Baskin and Baskin, 2014). In a review of seed dormancy and its correlation with plant

traits, Jurado and Flores (2005) found that seed dormancy was more common in plants from environments with a marked seasonality and, especially, if frost or drought stress can occur during the year. Seed dormancy is also more common on herbaceous species and does not depend on seed size. Dormancy could therefore be considered a mechanism that can tune seed germination to the seasonal cycles of the habitat.

To study seed dormancy in the context of temperate forests can therefore highlight the different mechanisms that have been developed by plants to ensure survival and reproduction in a predictable but stressful environment where the main limiting factor is light availability. The different mechanisms of germination do reflect the ability of species to colonize heterogeneous habitat patches inside a forest and the definition of these traits can help land managers and conservationists to select the species to reintroduce and the methods to handle them. In fact, as it is the case for some species with complex and long germination requirements (e.g. *Ruscus aculeatus*) and low seed production (e.g. *Mercurialis perennis*), seed broadcasting can be costly and ineffective and reintroduction by plug plants may have to be considered.

The objective of this thesis is therefore to collect evidence on the germination requirements of woodland understory species and to verify which germination traits differ more between mature and recent forests. An approach focused on the expression of functional traits more than plant identity should be followed in order to define the ecosystem roles that are missing in the habitat to restore. Compared with plant vegetative traits, seed germination traits are used less in ecology studies because it can take longer to generate such comparative data. Consequently, they are not often available in online databases (Jiménez-Alfaro et al., 2016); an exception being that of Durr et al (2015; https://doi.org/10.1594/PANGAEA.829536). Clearly the germination trait is an important descriptor of the regeneration ecological niche of a species and thus the

evolution of diversity in plant communities.

Objectives and outline of the thesis

This thesis begins from a general overview of the regeneration strategies of herbaceous species frequently present in forest understory of European temperate forests. A literature review of the published germination studies will allow the identification of the different regeneration niches of these taxa, that reflect their adaptation to a heterogeneous forest landscape (Chapter 2). The data collected will be summarized and, using statistical ordination methods, groups with similar germination strategies will be described.

As the physical environment can vary between forest patches of different age, consideration needs to be given to whether the strategies identified in the review also reflect the ecological differences amongst forests. To achieve this objective a field survey was conducted of the understory community of recent and ancient forests. Two sites were compared in the middle and in the south of the European Atlantic biogeographic region to verify if forests, within the same potential vegetation association, that were geographically distant: 1) show the same dynamics of understory recolonization; and 2) have plant communities with a comparable representation of germination strategies. In order to describe these dynamics a functional trait approach was used and community weighted means (CWM) of those traits were compared between sites. The effect of the physical environment on the functional structure of the herbaceous understory was investigated to define if abiotic thresholds to forest recolonization had been crossed between the recent and the reference forests.

Finally, to narrow the focus on the germination characterization of typical woodland species, *Conopodium majus* (Apiaceae) was selected to develop a more in depth study of germination ecology and desiccation tolerance. This species was selected for two reasons:

its importance in the ecological restoration of ancient woodlands in Europe; and because it is an understudied, yet potential model, species on which to study germination strategy and species regeneration via seed. *C. majus* seed has morphological dormancy and the species exhibits environmental plasticity across its range. It is characteristic of oligotrophic meadow communities, especially in the northern range of its distribution, and can tolerate relatively closed and more open sites often on soils that are poor in nutrients. The habitats colonized by *C. majus* are also relatively stable and the species has a predictable seasonal (life) cycle. Therefore, this thesis reports the fine adaptation of the species to the seasonal cycle and the temperature regulation of its dormancy giving an interpretation of its environmental plasticity in an ecological perspective in relation to various forest landscapes. Moreover, given the strict dependence on temperature for embryo growth and germination for this species, we aimed to develop a thermal model of embryo growth that could predict the emergence in the field of *C. majus* and define its thermal germination niche.

In conclusion, the questions this thesis aims to answer are:

1) Are there germination traits that are prevalent within herbaceous understory species characteristic of ancient, temperate woodlands (Chapter 2)?
2) Do understories communities of ancient and recent woodland diverge in the representation of these traits and is this divergence influenced by the physical environment (Chapter 3)?
3) What are the germination strategies that define environmental plasticity in a species (*Conopodium majus*) that is regarded as indicator of both forest and meadow communities (Chapter 4)?
4) Is it possible to define the thermal germination niche of the seeds and embryos of *Conopodium majus* adding significantly to understanding of morphological

dormancy in understory species (Chapter 5)?

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CHAPTER 2

REGENERATION FROM SEED IN HERBACEOUS UNDERSTORY OF ANCIENT WOODLANDS OF TEMPERATE EUROPE

ABSTRACT

Understory species of European ancient woodland are subject to land use change and their distribution can be at risk from their poor capacity to colonize isolated forest patches. Here we compiled a list of 208 species regarded as indicators of ancient woodland in Atlantic and Continental Europe and, since regeneration is crucial in the establishment of a new plant population, collated their associated traits indicative of regeneration strategies. Embryo morphological type and the embryo: endosperm ratio were measured for 106 species and literature on their germination strategy was reviewed. Trait data on dormancy type, stratification requirements, germination temperatures, response to light and to fluctuating temperatures was collated and their relationship with adult plant environmental preferences, vegetative traits and regeneration traits (seed yield and reproductive phenology) explored for 57 species using statistical ordination method. Three groups of species with different germination strategies were identified on the basis of habitat preference and reproductive phenology. Firstly, those with a preference, as adult plants, for shaded habitats, tend to have a morphological seed dormancy and can germinate in the dark and at low temperatures, and these can be separated in autumn and late winter germinators with shoots emerging in early spring. Secondly, species with a preference for gaps and forest hedges tend to have physiological dormancy, with seeds needing light and high temperatures for germination such that emergences is in spring after cold stratification. Our analysis underlines how critical it is to time the reintroduction of woodland understory species to match their germination strategy and to consider the spatial habitat preferences of the adult plant.

KEYWORDS: Germination traits, Regeneration niche, Seed dormancy, Seed germination, Temperate forest understory.

INTRODUCTION

Ancient woodlands are defined as forests that have not been cleared for a certain period, the duration of which differs throughout Europe depending on the landscape history of each region (Hermy et al., 1999). Ancient Woodland Indicators (AWIs) are trees, shrubs and herbaceous species often associated with ancient forests (Peterken, 1974).

AWIs are stress tolerating species with slow colonizing capacity due to their heavy seeds, low seed production, and limited long distance dispersal mechanisms (Hermy et al., 1999; Verheyen et al., 2003). They are also scarcely represented in the soil seed bank (Bossuyt et al., 2002; Bossuyt and Honnay, 2008).

Consequently, natural colonization of forests may take many years due to poor seed availability. The presence of AWIs can thus be taken as an indicator of a long history of forest cover at a site (Peterken, 1974). However, their traits also make AWIs vulnerable to human-induced environmental changes (e.g., deforestation, habitat fragmentation, climate change) (Verheyen et al., 2003).

The herbaceous understory accounts for more plant species richness than other forest strata and it is the one with the highest rates of extinction (Gilliam, 2007). It can influence the composition of the canopy layer by competitive interaction with the juvenile stages of overstory species that, on their part, influence the composition of the understory by regulating light availability and litter cover (Gilliam, 2007).

Current reforestation efforts usually restore only the tree layer (McClain et al., 2011; Francis and Morton, 2001). Yet it is known that the natural recolonization of the core forest understory species, may be very slow (Bossuyt and Hermy, 2000; Brunet et al., 2011; Peterken and Game, 1984) and should be the focus of good restoration practice

(Blakesley et al., 2013). While successful trials have restored the understory of amenity woodlands by sowing generalist species, some characteristic AWI species have been avoided due to their complex germination requirements and scarce commercial availability (Francis and Morton, 2001). A first step towards the better inclusion of AWIs in reforestation efforts is to define their identity.

Association of a species with a particular mature forest can vary across Europe due to climatic, geological and biogeographical reasons (Hermy et al., 1999) and within the same forest, several ecological niches can be identified so that, species able to colonize gaps and edges of a woodland may differ from those with an optimum in closed canopy conditions.

Community assemblages can be defined by sharing certain ecological requirements (Ellenberg and Leuschner, 2010) and plant functional traits (Diaz et al., 2016; Westoby, 1998) that describe the ecology of the adult plant. Nonetheless, differences in the regeneration niches provide an important contribution to the species richness of a vegetal community (Grubb, 1977). An approach based on adult (life history) and regeneration (seed dispersal) traits has been used to distinguish British AWIs from other woodland species (Kimberley at al., 2013) and to describe the persistence of understory species under different forest management methods in Denmark (Graae and Sunde, 2000).

Regeneration traits determine the colonisation capacity of understory herbs and the ecological niche or forest successional stage in which each species can successfully establish. In trait based studies, the more widely used regeneration trait is seed dry mass, followed by seed dispersal type (Jimenez-Alfaro et al., 2016). Although some correlations of seed dry mass with seed production or seedling growth rate have been proposed , seed mass is not always the most informative trait (Larson & Funk, 2016). Few studies measure

the difficult to measure internal morphology and germination traits (Jimenez-Alfaro et al., 2016), the latter showing high intraspecific variation (Larson and Funk, 2016).

Stratification requirements for dormancy breaking and germination temperatures function as season detection mechanisms (Probert, 2000), while the germination requirement for light or temperature fluctuations is a gap detection mechanism that allows micro-niches within a forest to be colonised (Pearson et al., 2002). Seed dormancy is a mechanism that prevents seeds germinating in a time or in a place that are not favourable for seedling establishment (Baskin and Baskin, 2004). Therefore, the type of dormancy and the processes required to break it can be good descriptors of the regeneration niche of a species. However, information on germination traits is rarely represented in plant trait databases (Kattge et al., 2011) and a systematic literature search, including data published in non peer-reviewed journals, is needed (Haddaway et al., 2015).

The aim of this work is therefore to synthesise the current knowledge on the diversity of herbaceous plant regeneration traits (including germination) of species described in the literature as AWIs for the temperate broadleaf forests of Atlantic and Continental Europe, and to identify groups of species with common regeneration strategies. Then the germination traits were compared with adult plant environmental preferences, such as Ellenberg Indicator Values (EIV) (Ellenberg and Leushner, 2010), and with functional traits related to the regeneration strategy of the plant. Based on this information, specific regeneration strategies were identified and their implications for forest restoration discussed.

MATERIALS AND METHODS

Developing the AWI species list

A list of species regarded as AWIs in temperate Europe was compiled by merging published data on species from eight North Western and Central European countries (Hermy et al., 1999; Verheyen et al., 2003) based on statistical testing in relation to land use history and species distribution (Kimberley et al., 2013; Perrin and Daly, 2010; Schmidt et al., 2014; Wulf, 2003). Lists based on author's knowledge of species association with AW, were available for England (Kirby, 2006) and Scotland (Crawford, 2009). Our focus was only on the herbaceous understory layer and all trees, ferns, shrubs and vines were not included. Listed species were checked for synonyms and the Latin names were standardized per the Plant List (http://www.theplantlist.org/, accessed 16th March 2016).

Compilation of traits from databases

As a proxy of the species ecological requirements, its EIVs for temperature, continentality, light, soil moisture, nitrogen and pH were obtained from Ellenberg and Leuschner (2010). Values of the Leaf-Height-Seed (LHS) traits (specific leaf area, plant height and seed dry mass; Westoby (1998)) were downloaded from the TRY database (Kattge et al. 2011).

The LHS traits describe the ecological strategy of a plant according to its position on a volume formed by three axes, each corresponding to a trait. Conversely to Grime's CSR (Competitor-Stress tolerant-Ruderal) strategy (Grime, 1974), in which each axis of the scheme describes a life strategy resulting from the interaction of different functional traits, the LHS scheme is easier to measure for more species, allowing comparisons across

different habitats and life forms. The axes of the LHS strategy have been compared to those of the CSR (Westoby, 1998); but, since each one represents a single functional trait, they do not depict the complexity of a plant response to the environment.

For this reason and to highlight the importance of the first stages of plant life, some other traits related to plant regeneration from seed were also obtained from TRY: flowering month, number of seeds produced per plant, seed width and length and seed terminal velocity (Bond-Lamberty et al., 2002; Campetella et al., 2011; Cerabolini et al., 2010; Ciocarlan, 2009; Dainese and Bragazza, 2012; Everwand et al., 2014; Fitter and Peat, 1994; Freschet et al., 2010; Fry et al., 2014; Gachet et al., 2005; Garnier et al., 2007; Green, 2009; Hickler, 1999; Hill et al., 2004; Kattge et al., 2009; Kleyer et al., 2008; Kühn et al., 2004; Moretti and Legg, 2009; Milla and Reich, 2011; Ordonez et al., 2010; Paula et al., 2009; Peco et al., 2005; Pierce et al., 2007a; Pierce at al., 2007b; Pierce et al., 2013; Prentice et al., 2011; Price and Enquist, 2007; Royal Botanic Gardens, Kew, 2011; Sandel et al., 2011; Shipley; 1995, Shipley, 2002; Spasojevic and Suding, 2012; Van Bodegom et al., 2008; Vergutz et al., 2012; Vile, 2005; Wirth and Lichstein, 2009; Wright et al., 2004). When more than one record of a species trait was available in TRY, the average was calculated according to the available data and the outliers (those records whose value differed more than three standard deviations from the average of the species) removed. Diaspore is defined as the part of a plant that is dispersed for reproduction and can be a seed or a fruit (Bewley and Black, 2006). For ease of communication diaspores will be referred to as seeds.

Embryo measurements

For 106 of the 127 endospermic AWI species listed, seeds were obtained from the collections of the Millennium Seed Bank of the Royal Botanic Gardens, Kew, or collected in the wild. Twenty seeds of each species were imbibed on 1% agar-water for 24 hours. Thereafter, the seeds were cut longitudinally and photographs taken of the internal seed structure, including the embryo, using a camera (Carl Zeiss Axiocam Colour) mounted on a Stemi SV 11 Microscope (Carl Zeiss, Welwin Garden City, Herts, UK) microscope. Only the 10 most representative photographs per species were measured, discarding immature, malformed or badly cut seeds. Embryo and internal seed areas were measured using the software Axiovision 3.1.2.1 (Carl Zeiss Vision GmbH) and the ratio between them calculated. This parameter was defined as "embryo:endosperm ratio" (E:E) because the internal area of the seeds measured not occupied by the embryo was always filled with endosperm. Embryo types were classified following Martin (1946). The categories of "micro" (seeds < 0.2 mm long) and "dwarf" (seed 0.3 to 2 mm long) were referred to seed size rather than embryo morphology. Following the revision of Martin's classification in Baskin and Baskin (2007), species with micro seeds were assigned to the "undifferentiated" category while species with "dwarf" seeds where classified according to their embryo morphology.

Literature search for germination traits

Because reliable germination traits were for the most part unavailable in databases, a systematic search strategy was devised to obtain these traits from published journal articles. A Boolean search string was built including: (1) all the species names connected between them with an "OR" operator; and (2) the following string: "AND (seed AND (germination OR dormancy))". To include also those papers on woodland understory plants where no species name appeared in the title or abstract, a second string was used

in which, instead of the species list, the following terms were included: “ancient woodland indicators”, “woodland understory species”, “woodland herbs”, and “forest understory”. The strings were used to run searches in the Web of Science, accessed in June 2016. Initially, 924 papers were found. These were filtered by title and abstract, at first keeping only the ones that clearly referred to genera and species in the list (197) and then filtered again keeping only the ones who included germination experiments (55). Any additional references noted in the bibliography of the papers examined were added to the review (25). Finally, a reference was included in the review if it described the germination of at least one of the species in the list. The studies in which the seeds were exposed to only one germination temperature were excluded unless other parameters, such as the effect of light, fluctuating temperature or a dormancy breaking treatment were also investigated. Wherever possible the following information was recorded for each of the 80 included paper: the number of temperatures tested, the number and type of dormancy breaking treatment applied, the use of fluctuating temperature, the exposure to light, the origin (cultivated or wild) of the seed lots used, their geographical provenance, if natural germination phenology was studied and if the seeds tested were fresh or had been dried (Annex I).

The following germination traits were collected: response to light and fluctuating temperatures, dormancy type, stratification requirements, minimum, maximum and effective germination temperatures. The effective germination temperature was the condition resulting in the highest seed germination. Maximum and minimum temperatures were defined as the temperature extremes permitting some seeds to germinate. When positive germination responses were recorded for more than one temperature condition across different studies, the mean was calculated. The majority of published data related to seed testing at constant temperatures. When alternating

temperatures were used, the mean constant temperature was included in the dataset, taking into account the time spent at the warmer and cooler phases.

When it was not possible to obtain the original paper, data on dormancy type for some species were obtained from the literature reviewed by Baskin and Baskin (2014). Germination data were produced by the authors for *Conopodium majus*, *Hypericum androsaemum* and *Stachys sylvatica*.

From the literature review data on the dispersal season, defined as the season when seeds were collected, and germination season, defined as the season in which seeds were observed to germinate in the field or in a garden experiment, were also recorded. When such data wasnot available, the ECOFLORA database, accessed on May, 2017, was consulted (Fitter and Peat, 1994).

The distribution of the germination traits was visualized with stacked column plots for the qualitative variables and boxplots for the quantitative. Finally, a data matrix including 28 ecological, vegetative and regeneration traits was built.

Data exploration

Quantitative data were checked for normality with the Shapiro-Wilk test. Specific leaf area, plant height, seed dry mass, number of seeds produced per plant, diaspore length and width, germination temperatures and embryo:endosperm ratio were not normally distributed. However, the natural logarithm of the traits had a normal distribution, except embryo:endosperm ratio, which had a bimodal distribution, and minimum germination temperature, for which it was necessary to apply a quadratic transformation in order to obtain a normal distribution of the data. Effective and maximum germination temperature were not normalized either with a logarithmic or with a quadratic transformation but their

distribution appeared to be closer to normal without any transformation (Shapiro-Wilk p value = 0.00012 and 0.0018 respectively).

The distribution of the data was visualized with boxplots. A correlation matrix using the Pearson coefficient was also calculated for all the numerical traits, using only pairwise complete observations, to check if there was any correlation (threshold set at 0.6) between variables.

To explore the variability in the EIV, a Principal Component Analysis (PCA), ("PCA.Ellenberg") was performed on 191 of the listed species for which these data were available. The statistical significance of the correlation of each variable with the principal components was tested with a t-test.

The relationship between seed yield and dispersal traits (seed length and width, seed dry weight, seed number per plant and seed terminal velocity) was assessed on 68 species with complete trait data, with a PCA ("PCA.morph") in order to select, for further analysis, only the variables that contributed most to the first two axes. A Factorial Analysis for Mixed Data (FAMD) (Pages, 2004), was performed on 106 species using the three phenology traits ("FAMD.phenology").

Another FAMD ("FAMD.regeneration"), encompassing 57 species form the original list, was calculated including the following germination and regeneration traits: dormancy type, response to light, stratification requirements, effective germination temperature, flowering month, germination season, and the natural logarithm of seed dry mass, embryo:endosperm ratio and plant height. The latter was considered a regeneration trait because it is an important proxy for seed long distance dispersal ability (Thomson et al., 2011). From the two FAMDs, species representing the rarest categories of the variables considered ("germination in autumn and spring" for germination season from

FAMD.phenology and “PYPD” and “PY” for dormancy type and “cold+warm” for stratification requirement from FAMD.regeneration) were excluded as outliers. The EIV for light, moisture, nutrients and pH were transformed in categorical variables according to their belonging to the four quartiles of their distribution for the species used in FAMD.regeneration. They were used as supplementary variables to evidence the relationship between regeneration strategies and environmental preferences, colouring the species according to their EIVs values in the FAMD.regeneration’s map of the individuals. The Ellenberg indicators for continentality and temperature were not used because their correlation to the first two components of PCA.Ellenberg was low. Also the type of embryo was used as a supplementary variable. The statistical significance of the correlation of each quantitative variable with the first two dimensions of the FAMDs was tested with a t-test while for the qualitative variables a one way ANOVA model was calculated for each dimension, using, as explanatory variables, the coordinates of the individuals for the variable examined. To verify if each qualitative variable had a significant influence on the dimension an F-test was performed. Student t- tests verified, for each dimension, if the average coordinates of the individual falling in each category were significantly different from the average coordinates of all the individuals. The tests were performed by the function “dimdesc” of the R package “FactoMiner” (Lê et al., 2008). Clusters of individuals, grouped for their reproductive phenology and for their regeneration strategies, were described using the Hierarchical Clustering on Principal Components (HCPC, Husson et al., 2010) on FAMD.phenology and FAMD.regeneration. Euclidean distance was calculated between all the species in the maps of individuals, taking in account only the first two dimensions of the FAMDs. Clusters were aggregated using the Ward method and 40 iterations were performed. The association of each variable with the clusters was described, for the quantitative variables, by the difference between

the average of the variable in the group and its average in the whole dataset and tested using a hypergeometric test (v test, Lebart et al., 1997). The association was significant for values of the v test greater than 1.96. The sign of the v test indicated if the value of the mean of the cluster is greater or smaller than the overall mean. The association of the qualitative variables with each cluster was tested with a chi-square test and the representation of each category of the qualitative variables in the cluster was described comparing its frequency in each cluster with its overall representation. The significance of this association was tested using a hypergeometric test (Husson et al., 2010). Also the supplementary variables, who did not contributed to the calculation of FAMD and HCPC, were included in the description of the clusters obtained. Each cluster was named after the species closer to its centre. All data analyses were performed with the statistical software R (R Core Team, 2015).

ECOLOGICAL REQUIREMENTS AND ADULT PLANT TRAITS OF EUROPEAN AWI

The final species list included 208 AWI species distributed in 45 families and 124 genera. The most represented families were Poaceae (19 species), Orchidaceae (16 species), Ranunculaceae (15 species) and Cyperaceae (14 species) while the most represented genera were *Carex* with 13 species and, *Hypericum* with six species. Following the Europa 2000 biogeographical classification (European Environment Agency, 2016) the species list appeared dominated by plants described as AWIs only for the European Atlantic region (103); 92 species were common to both Atlantic and Continental Europe; six were considered AWIs only in Continental Europe. This Atlantic biased distribution can be explained because the sources used to build the species list

referred mostly to countries or regions located in this area (Crawford, 2009; Kimberley et al., 2013; Kirby, 2006; Perrin and Daly, 2010).

Ecological requirements (Ellenberg Indicator Values)

EIVs are widely used to describe the ecology of a species (Diekmann, 2003). They are ordinal categories that express habitat preference. For the present study the EIVs for light, nitrogen, soil moisture, temperature, continentality and pH were considered. These values were available for 191 of the species in the list (92%). All the EIVs range between 1 and 9, excluding soil moisture, that varies between 1 and 12. However, values between 10 and 12 only apply to aquatic plants that were not the subject of this study.

The indicators for temperature, light, nitrogen and moisture had all a median value of 5. Continentality had a median of 3, that indicates a lower seasonal and daily thermal variation. The relatively low value for continentality may be due to a bias in the available list of species, as 103 species have an exclusive European Atlantic distribution according to the Natura 2000 classification (European Environment Agency, 2016), and are adapted to a more oceanic climate. But the average value of the continentality EIV was low (3.33) also if calculated only for the AWI exclusive to Continental Europe. An alternative interpretation of these low values can be that daily temperature fluctuation during the warmest months is buffered by the forest canopy, creating a microclimate more oceanic than in an adjacent open area.

The pH indicator had a median value of 6, slightly above the average. It ranges from 2 to 9, indicating a broad spectrum of pH preferences amongst woodland species. This pattern could be expected for a list of species collated from studies conducted in different plant communities and geographical regions across Europe.

The light EIV ranged between 1 and 9, probably describing species ranging from forest edges and gaps to plants adapted to deep canopy cover, although an average of 5 indicated species that prefer semi-shaded habitats. The high variance of the EIV for light might be expected as the AWIs have been compared at a regional scale and along a latitudinal gradient. In fact, species with broad ecological requirements, such as, *Conopodium majus*, can be both a meadow community indicator in part of their distribution range (Rodwell, 1998) and an indicator of undisturbed ancient forests in others.

When the Ellenberg Indicator Values were analysed with a PCA (Fig. 1, Annex II), the first two components explained 56% of the variance. The first component (35 % of variance) represented mainly variation in nitrogen and pH requirements. Both indicators were positively correlated with the axis positions (Pearson = 0.86 and 0.71, respectively) together with the EIV for moisture (Pearson = 0.16). In contrast the EIV for light had a negative correlation (Pearson = -0.48) with the first component, placing, on the right side of the PCA biplot, shade tolerant species with preference for richer and more basic soils. The second component had the strongest, positive, correlation with the EIVs for moisture, light, and nutrients (Pearson = 0.72, 0.64, and 0.33 respectively), while temperature and pH had a significant negative correlation with it (Pearson = -0.33 and -0.16). Therefore the second component separated species from open, colder and wet habitats in the upper quadrants of the biplot from species from drier and closed-canopy sites. Fewer species are associated with high values of the light EIVs than those with lower values (Fig. 1). Temperature and continentality were the EIVs with the lower contribution coefficient to the first two components, and were excluded from further analysis.

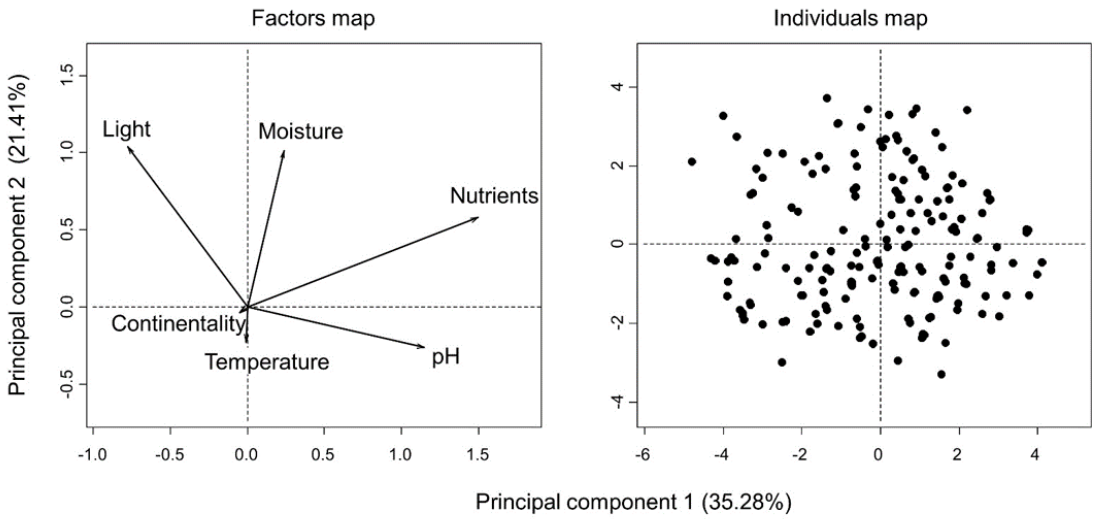

Fig. 1: Principal Component Analysis of Ellenberg Indicator Values for 191 European Ancient Woodland Indicators. The percentages express the proportion of variance explained by each component.

Adult plant traits

Of the three variables used in the Leaf-Height-Seed (LHS) scheme to describe a plant species ecological niche (Westoby, 1998), specific leaf area (SLA) and plant height refer to the adult plant. The mean value for SLA is 32 mm^2/mg, calculated on 184 AWI species. This trait describes the proportion of resources allocated to photosynthesis with respect to the dry mass produced and tends to be greater in species from shaded environments. It is the inverse of LMS (leaf area per mass) that is reported to range between 5 and 1507 g/m^2 in Diaz et al., (2016) for 10490 plant species worldwide. The equivalent average LMA value for the European AWI species is 301 g/m^2, placing them on the lower part of the global distribution of this trait. This lower than average investment in leaf construction may be explained by the need of having a broad leaf surface, to maximise light absorbance

in shaded understory environment. The ability to quickly produce leaves when the canopy starts to close in spring can be traded off with the leaf quality intended as robustness. AWI adult plant height had average and median values of 0.4 m and >75% of the species have an adult plant height < 0.6 m. This trait has been demonstrated to be more important than seed mass in influencing seed dispersal distance (Thomson et al., 2011) while SLA does not influence directly regeneration and therefore was not considered for further analysis.

REGENERATION TRAITS OF EUROPEAN ANCIENT WOODLAND INDICATORS

Seed yield and dispersal traits

Seed production per plant influences dispersal: the more seeds produced, the greater the probability of a long-distance dispersal event. Information was available for 184 species from the list. The trait distribution was skewed, with 75% of the species producing less than 1400 seeds per plant and only ten species, from the families Campanulaceae, Compositae, Hypericaceae, Ericaceae, Onagraceae and Orchidaceae, producing more than 30000 seeds. Species which produce more seeds tend to be from families or genera that lack endosperm while endospermic seeds are generally produced in smaller quantities and seeds possess greater dry mass. However, there are some exceptions to this trend, represented by some endospermic but small seeded genera of families defined by Martin (1946) to have “dwarf” seeds, like Campanulaceae, Plantaginaceae and Saxifragaceae. Seed production per plant and seed dry mass are negatively correlated (Pearson = -0.65), confirming the interspecific trade-off between these two variables widely demonstrated by other authors (Shipley and Dion, 1992).

Seed dry mass, available for 182 species, was not normally distributed but was skewed towards lighter seeds, with 75% of these species having a value < 4.28 mg. It reached a maximum value of 197 mg, represented by *Ruscus aculeatus* (Asparagaceae).

Seed length ranged between 0.37 and 14.5 mm and seed width between 0.1 and 10 mm. As for seed dry mass, data was not normally distributed skewed towards smaller values. These traits were available for 80% and 62% of the species in the list respectively. Both were positively correlated with seed dry mass (Pearson = 0.7 and 0.9, respectively) and negatively with seed number per plant (Pearson = -0.50 and -0.67, respectively).

Seed terminal velocity, measured in m/s, expresses the maximum rate of fall of a seed after dispersal and it is directly proportional to seed mass. Data were available for 48% of the species in the study and the variable was normally distributed, with a mean value of 3.03 m/s. Seed terminal velocity can also be influenced by seed shape since round seeds tend to accelerate more during their fall and reach a higher final velocity. A low terminal velocity allows wind dispersed seeds to travel greater distances and its importance, in relation to release height, increases with dispersal distance (Tackenberg et al., 2003).

A PCA including all the seed external morphology variables plus seed number and terminal velocity (Fig. 2) showed that the first component was enough to explain 75% of variance in these traits and another 15% was explained by the second component. Seed mass and number were the traits that contributed the most to both the first two components. Seed mass was positively correlated with length, width and terminal velocity (Pearson = 0.74, 0.89 and 0.73, respectively); indicating that heavier seeds tend to be bigger and fall faster. Seed number was negatively correlated with seed mass and width (Pearson = -0.64 and -0.67 respectively). The trade-off between seed number and seed mass has been described extensively in seed ecology literature (Muller-Landau, 2010,

Venable, 1992). These two variables had the strongest (-0.90 and +0.90 respectively) opposite, significant ($p < 0.01$, Annex II) correlation with the first PCA component, therefore, for further analyses only seed mass was kept as a proxy of the variation in all traits related to yield and dispersal.

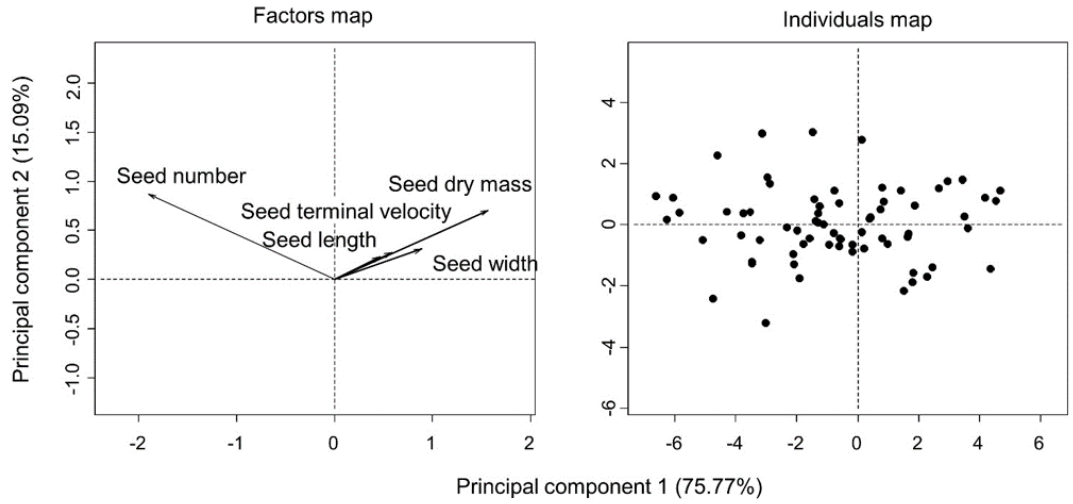

Fig. 2: Principal Component Analysis of seed yield and dispersal related traits for 68 European Ancient Woodland Indicators. The percentages express the proportion of variance explained by each component.

Seed internal morphology

Eleven categories of embryos, following Martin, (1946) and Baskin and Baskin (2007) classification systems, were identified for all the species in this study. Species with bent, folded or investing embryo had negligible or any endosperm, while it could be present or not in species with linear and spatulate embryos. All other categories identified (broad, capitate, lateral, peripheral and rudimentary) included only endospermic seeds. All

Orchidaceae (16 species), Orobanchaceae (three species) and three parasitic Ericaceae (*Monotropa hypopitys, Orthilia secunda* and *Pyrola minor*) possess micro seeds with undifferentiated embryos (Martin, 1946) and were not dissected as well as species from genera reported to have no endosperm. Table 1 reports the number of species assigned to each embryo type category.

The ratio between embryo and endosperm area was measured for 106 endospermic species and a value of 1 was assigned to all species lacking endosperm, producing this data for 79% (165) of the species in this study (Table 1). The ratio varied between values of 0.01 and 1 and the data was not normally distributed , even after being transformed to natural logarithms. The average E:E value was 0.48 with 28% of the species having a ratio of 1.

Even being morphological traits, embryo type and the relative proportion of embryo and endosperm can be indicative of the type of dormancy (Forbis et al., 2002, Baskin & Baskin, 2007). In fact all species with rudimentary and 33 % of the species with linear embryos in the present study have been reported to have a morphological component to their dormancy. Low E:E ratios may signify that the embryo needs to grow and develop before germination can occur.

Reproductive phenology

Flowering month, expressed as month number, was available for 98% of the species. Flowering occurred from February to September. The trait was normally distributed and the mean of 6 (June) indicated that the average understory AWI tends to flower in late spring/early summer.

Table 1: Embryo type and embryo:endosperm ratios of European Ancient Woodland indicators. Embryo types are listed from smaller to greater values of embryo:endosperm ratio. Numbers in brackets indicate the number of species measured to calculate the mean ratio for each embryo type category.

Embryo type	N. species	N. endospermic species	Mean ratio	SD ratio
Rudimentary	19	19 (13)	0.03	0.01
Capitate	14	14 (13)	0.09	0.02
Broad	4	4 (4)	0.11	0.05
Lateral	19	19 (16)	0.12	0.03
Linear	52	45 (38)	0.28	0.33
Peripheral	6	6 (6)	0.59	0.08
Spatulate	52	20 (16)	0.82	0.27
Bent	12	0	1	0
Folded	3	0	1	0
Investing	5	0	1	0
Undifferentiated	22	NA	NA	NA

Dispersal season was available for 57% of the species (118 records), with the majority (63 species) possessing seeds which are dispersed in summer (June, July, August). Thirty five species dispersed seeds in the autumnal months (9-11)) and only 20 species in the spring (months 3-4) . No species disperse seeds in winter. Two-thirds of species that

disperse seeds in autumn have seeds requiring cold stratification (24 out of 35, 69%), thus avoiding germination and seedling emergence before the coldest months of the year.

The season during which seeds were most likely to germinate was obtained for 69% (144) of the species in the AWI list. This information was produced by observation of seedling emergence in the field or in garden experiments. In species with epicotyl dormancy a significant gap can exist between germination, usually in the autumn, and seedling emergence in spring (Barton and Schroeder, 1942; Eriksson, 1994; Mondoni et al., 2008; Mondoni et al., 2009; Mondoni et al. 2013; Kondo et al., 2004; Takagi, 2001). Delayed seedling emergence can be observed also when, after an autumnal radicle emergence, the growth of the shoot continues but becomes slower with the colder temperatures (Newton et al., 2015). The majority (94) of the species in the study for which the data were available, were reported to germinate in spring, 11 in summer, 29 in autumn and one, *Vicia sepium*, can germinate either in autumn or spring (Vandelook, unpublished data). The final nine species are known to germinate in late winter when the temperatures is still low and the forest canopy open.

The relationship between the phenology of different phases of the reproductive cycle were explored with a FAMD (Fig. 3, Annex II) for 106 species. The first dimension explained 28% of the variance in the data and was positively correlated with flowering month (Pearson = 0.87) and significantly explained by dispersal season ($R^2 = 0.74$, $p < 0.01$). It ordered species from early flowering and spring dispersal to late flowering and summer or autumn dispersal. The second dimension explained 21% of the variance and separated species that disperse and germinate in the later months of the year from species that disperse and germinate in the earlier part of the year ($R^2 = 0.63$ and $p < 0.01$ for dispersal season and $R^2 = 0.60$ and $p < 0.01$ for germination season). Three different

strategies were identified on the basis of species clustering on the first two components (Fig 3, Annex II):

1) *Adoxa moschatellina* group: includes 23 early flowering species mostly dispersed in spring (87% of the species in the cluster, representing all the spring dispersed species of the dataset) or in summer, that germinate in the autumn of the same year or during the vegetative season of the year following dispersal;

2) *Carex sylvatica* group: includes 45 species, characterized by spring flowering. All were dispersed in summer and the majority of them (87%) germinated in the spring of the next year or in late winter (11%);

3) *Lysimachia nemorum* group: includes 38 late flowering species that disperse seeds mostly in summer (87%) or in autumn (13% of the species in the group, representing all the autumn dispersed species of the dataset). Half of the species in the group germinate in the spring of the following year while the rest can germinate in summer or autumn in either the same year or in the year following dispersal.

The strategies 1) and 2) are favourable to species that grow in deciduous forest habitats because crucial steps (flowering in the *Adoxa moschatellina* group and germination in the *Carex sylvatica* group) of their reproductive cycle can progress in the early months of the year, when the tree canopy is still open.

Since the information carried by flowering month and dispersal season had a similar trend (Fig. 3), only flowering month was considered for further analysis, and because this trait was available for more species. The above described traits are summarized in Figure 4.

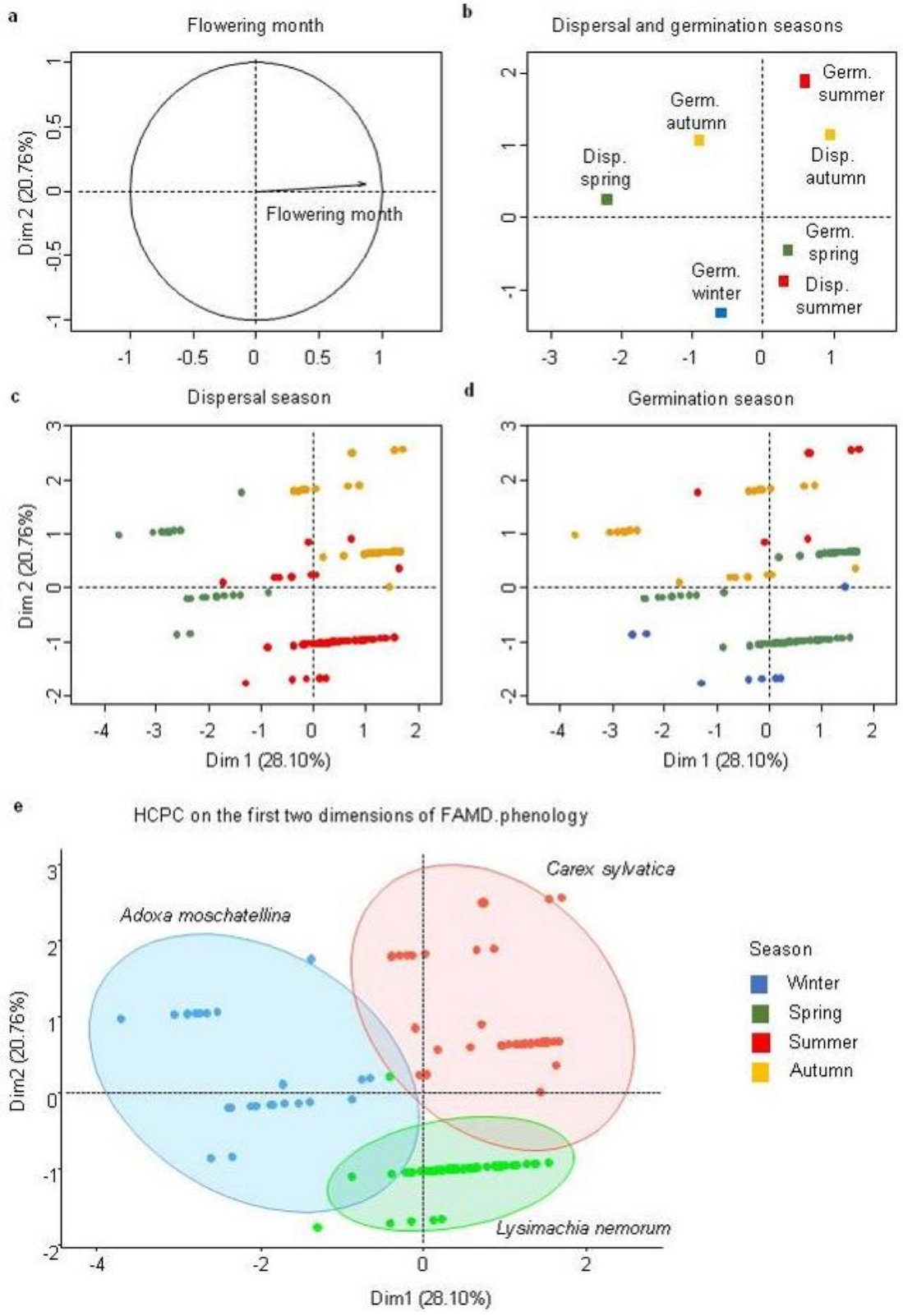

Fig 3: Factorial analysis of Mixed Data (FAMD) of phenological traits and hierarchical clustering on its principal components (HCPC). The centre of each cluster is marked by a black lined circle.

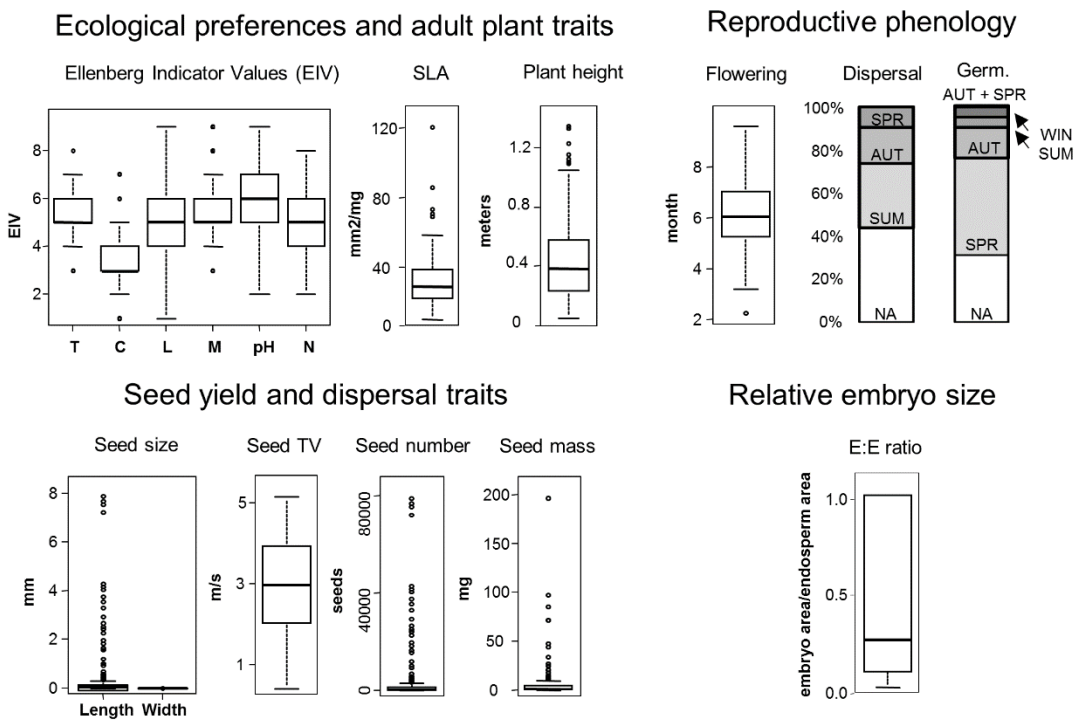

Fig. 4: Distribution of Ellenberg Indicator Values, reproductive phenology, regeneration and adult plant traits of European Ancient Woodland Indicators. Data are not transformed. AUT = autumn; SPR = spring; SUM = summer; WIN = winter; NA = data not available.

Seed germination

Germination traits were available for 132 out of 208 species in the original list. Data collated from the literature review are shown in Table 2.

Dormancy type

Seed dormancy is a mechanism that prevents seeds from germinating even if in the presence of adequate conditions of temperature and water availability (Baskin and Baskin, 2004). The ecological reasons for the presence of dormancy are to avoid seedlings developing in adverse seasons, or to spread seed germination over the years to ensure cohort survival in case a generation is lost during a particular year. In temperate forest

understories, the main factors that can limit seedling survival and development are the lack of light during the summer months, when the tree canopy is closed, and the cold temperatures during winter.

Data on dormancy type were available in the literature for 55% of the species, i.e., 115 out of 208 AWIs (Annex II). For some of these species, when not explicitly specified in the studies, dormancy type was deduced from the results of the experiments reported. Physiological dormancy (PD) is described for seeds that are water permeable but where a physiological inhibition prevent radicle emergence (Baskin and Baskin, 2014). It was the most prevalent dormancy class (71 species). Morphological dormancy (MD) is present in seeds with small embryos that need to elongate before germination can occur. If an additional physiological block to germination is present this dormancy type is defined "morphophysiological" (MPD) (Baskin and Baskin, 2004). From the 115 AWIs with data on dormancy type, 38 possessed MPD. Physical dormancy (PY) relates to a physical barrier to germination, e.g., seed coat impermeability to water. Only four were reported to have PY and one, *Geranium robertianum*, (Vandelook and Van Assche, 2010) to have a combination of physical and physiological dormancy (PYPD) (Fig. 5).

The finding of this review mirror the distribution of dormancy type described by Baskin and Baskin (2014) for nemoral understory species.

Stratification requirement

Species' seeds with physiological dormancy may require a defined period imbibed at a specific temperature before the block to germination is removed. These may be cold (C: 0-10°C) or warm (W: 10-25°C) to reflect the winter or summer average temperatures, respectively, of the distribution range of a species. Some species may require a

combination of W followed by C stratification (W+C) or the opposite (C+W) while some species are able to germinate without any stratification period.

From the list of AWIs, information about the stratification requirement in environmental chambers was available for 53% (110) of the species. Out of these, 69% just required C , 12% required W, 9% required W+C and one species, *Paris quadrifolia* (Vandelook, unpublished data), required C+W. Only 8% of the records stated no need for stratification (Fig. 5). Sometimes cold stratification was achieved by sowing the seeds in pots in the open during the winter months. In species with epicotyl dormancy an additional C stratification period was required after radicle emergence to break the dormancy of the shoot (Eriksson, 1994; Mondoni et al., 2008; Mondoni et al., 2009; Mondoni et al. 2013; Kondo et al., 2004; Takagi, 2001).

Germination temperature

Germination temperatures were the only numerical variables among the germination traits reviewed (Fig. 5). The effective temperature for germination ranged between 2 °C (*Hordelymus europaeus*, Ten Brink et al., 2013) and 33.5 °C (*Scyrpus sylvaticus*, Grime et al., 1981) and was positively correlated with reported maximum germination temperatures (Pearson = 0.66). among the temperatures tested. The term "optimal germination temperature" was avoided, since often not enough temperatures were compared (Annex I). This data was available for 52% of the species in the list (108 AWI). The mean temperature was 16.6°C (SD 5.5, SE 0.5) and the median 17.5°C, The data were not normally distributed ($p = 0.0007$ in Shapiro-Wilk's test). An average effective temperature for germination at 16.6 °C can be indicative of a prevalence of germination during the spring and summer months.

The data was in agreement with the average germination temperature of 15°C reported by Baskin and Baskin (2014) for temperate woodland understory herbs. Temperature extremes for germination varied considerable amongst species. The minimum germination temperature, defined as the lowest temperature at which germination occurred, between the temperatures tested in each paper, was available only for 38% of the species (79) and it ranged between 0°C and 20°C (SD 4.1, SE 0.46) while the maximum germination temperature was available for 78 species and ranged between 10°C and 38°C (SD 7.57, SE 0.85). The average minimum temperature for germination was 9.08 °C while the median of the distribution was 10 °C. The mean maximum germination temperature was 25.5 °C and the median of the distribution was at 27.5 °C. Minimum and maximum germination temperatures were not normally distributed ($p < 0.01$ in Shapiro-Wilk's test). Only the effective temperature was retained for further analysis. Minimum temperature of germination was not used because less data were available and maximum temperature was excluded because it correlated with the effective temperature.

Temperature fluctuations

Data on the effect of fluctuating temperatures were available for only 48 species, representing 23% of the AWIs. Half of them had a preference for alternating temperature, while the rest were divided between species indifferent to fluctuating temperatures (23%, 19 species) or requiring constant temperature to germinate (13 species). The latter group is mostly formed by species with MPD (10 out of 13). The need for temperature fluctuation is a requirement typical of species with small seeds, that are buried close to the soil surface (Probert, 2000). Germination of small seeds too deep in the soil may consign the seedling to die as there insufficient reserves to support growth up to the soil surface. . Species with small seeds are also better colonizers than larger seeded species

and tend to form a persistent soil seed bank (Thompson et al., 1993). Stronger temperature fluctuation can be a feature of gaps in the tree canopy and it is demonstrated that strong temperature fluctuation can significantly trigger germination in tropical, forest edge species (Wood et al., 2000). Species able to form a persistent soil seed bank tend to have seeds requiring light to trigger seedling development.

Light

The requirement of light for germination can be considered another gap detection mechanism (Pearson et al., 2002). As for temperature fluctuation, the response to light for germination in forest species is more common in small seeded species (Jankowska-Blaszczuk and Daws, 2007). Two parameters that describes the light available for germination have been used in the articles analysed. First, photosynthetic active radiation (PAR), that describes the quantity of light appropriate for photosynthesis (ranging from 400 and 700 nm of wavelenght), was by far the most used and indicated the quantity of light provided by the incubators used in the studies. Second, was the ratio between red (R) and far red (FR) ((660 nm and 730 nm wavelength respectively) which can strongly influence germination (Jankowska-Blaszczuk and Daws, 2007; Probert et al., 1985; Bewley and Black, 1994). The optimal R:FR ratio for germination was available only for 20 of the species in the list and was thus not used in the analysis.

The effect of light presence or absence on germination was available for 105 of the 208 species in the AWIs list (i.e., 50 %). Of these, 68 required light for germination, while 26 germinated both in light and complete darkness (Fig.5). Eleven germinated better in dark than in light, all of which are monocotyledons that can be grouped in three categories: orchids (*Dactylorhiza maculata*, *Epipactis helleborine*, *Neottia ovata*, *Platanthera clorantha),* large, endospermic seeded geophytes from the order Asparagales *(Allium ursinum, Colchicum autumnale, Convallaria majalis, Galanthus nivalis,*

Maianthemum bifolium, Narcissus pseudonarcissus) and a grass*, Bromus ramosus*. Orchids have been reported to germinate better in absence of light (Van Waes and Debergh, 1986) because they need to establish a symbiotic relationship with fungi in order to germinate (Baskin and Baskin, 2014) and the absence of light may be indicative of incorporation in the soil, where the symbiotic fungi can be found. Conversely, large endospermic seeds can have enough reserves for the embryo to grow and germinate even if not close to the soil surface and photoinhibition of germination has been reported to be more frequent in the order Asparagales (Carta et al., 2017).

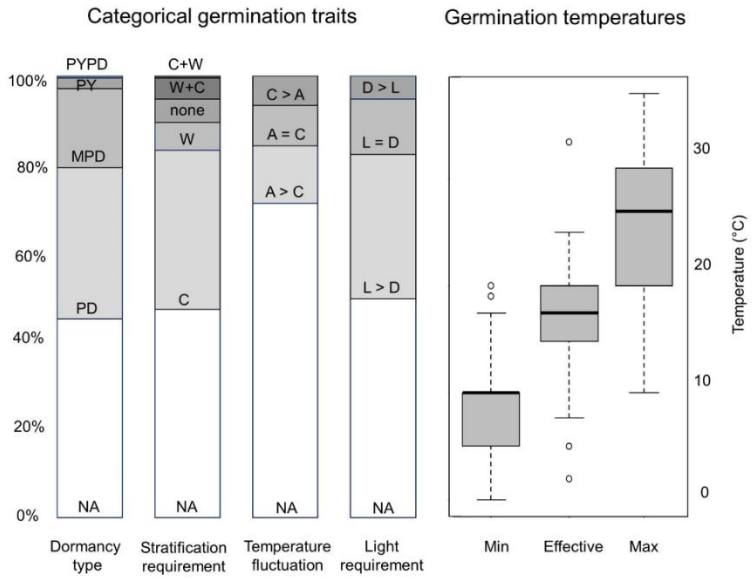

Fig. 5: Seed germination traits of European Ancient Woodland Indicators. PYPD = physiological + physical dormancy; PY = physical dormancy; PD = physiological dormancy; MPD = morphophysiological dormancy; C = cold stratification; W = warm stratification; W+C = combination of warm + cold stratification; NS = no needs for stratification; A>C = species requiring alternate temperatures for germination; C=A = species indifferent to temperature fluctuation; C>A = species requiring constant temperature for germination; L>D = species requiring light for germination; D>L = species requiring dark for germination; L=D = species indifferent to light for germination.

Table 2: Seed germination traits of European Ancient Woodland Indicators. PY = physical dormancy; PYPD = physiological + physical dormancy; ND = non dormant; PD = physiological dormancy; MPD = morphophysiological dormancy; L>D = species requiring light for germination; D>L = species requiring dark for germination; L=D = species indifferent to light for germination; A>C = species requiring alternate temperatures for germination; C=A = species indifferent to temperature fluctuation; C>A = species requiring constant temperature for germination; NA = data not available.

Species	Dormancy type	Light requirement	Temperature fluctuation requirement	Stratification	Effective germination temperature	References
Aconitum napellus L.	MPD	L > D	A > C	cold	13	Herranz et al., 2010
Actaea spicata L.	MPD	NA	NA	NA	NA	Baskin and Baskin, 2014
Adoxa moschatellina L.	MPD	NA	NA	warm + cold	20	Baskin and Baskin, 2014; Vandelook, unpublished data
Agrimonia eupatoria L.	PD	L = D	A > C	cold	17 .5	Grime et al., 1981; Vandelook, unpublished data
Ajuga reptans L.	NA	L > D	NA	NA	17 .5	Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007
Allium ursinum L.	PD	D > L	A = C	warm + cold	10	Ernst, 1979; Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Vandelook, unpublished data
Anagallis minima (L.) E.H.L.Krause	PD	L > D	NA	cold	NA	Salisbury, 1969
Anemone nemorosa L.	MPD	L = D	C > A	warm	15	Graae et al., 2009; Grime et al., 1981; Mondoni et al., 2008
Anemone ranunculoides L.	MPD	NA	C > A	warm	12 .5	Mondoni et al., 2009
Aquilegia vulgaris L.	MPD	L > D	NA	cold	NA	Grime et al., 1981
Arum maculatum L.	PD	L = D	C > A	cold	16	Grime et al., 1981; Pritchard et al., 1993
Brachypodium pinnatum (L.) P.Beauv.	PD	L = D	C > A	cold	20	Grime et al., 1981; Ten Brink et al., 2013

Species	Dormancy type	Light requirement	Temperature fluctuation requirement	Stratification	Effective germination temperature	References
Brachypodium sylvaticum (Huds.) P.Beauv.	PD	L > D	C > A	cold	15 .6	Graae et al., 2009; Grime et al., 1981; Ten Brink et al., 2013; Thompson, 1989
Bromus benekenii (Lange) Trimen	PD	L > D	A = C	cold	10	Ten Brink et al., 2013
Bromus ramosus Huds.	PD	D > L	A = C	cold	20	Grime et al., 1981; Ten Brink et al., 2013
Calamagrostis epigejos (L.) Roth	ND	NA	NA	no	22	Baskin and Baskin, 2014
Campanula latifolia L.	MPD	NA	NA	cold	17 .5	Grime et al., 1981
Campanula trachelium L.	PD	L > D	A = C	cold	20	Ten Brink et al., 2013
Carex acutiformis Ehrh.	PD	L > D	NA	cold	20 .5	Schütz and Rave, 1999
Carex brizoides L.	PD	L > D	NA	cold	23 .6	Schütz and Rave, 1999
Carex elongata L.	PD	L > D	A > C	cold	25	Schütz, 1997a; Schutz, 1997b
Carex laevigata Sm.	NA	NA	NA	cold	NA	Grime et al., 1981
Carex pallescens L.	PD	L > D	NA	cold	16	Schütz and Rave, 1999
Carex paniculata L.	PD	L > D	A = C	cold	23 .6	Grime et al., 1981; Schütz, 1997a; Schutz, 1997 b; Schütz and Rave, 1999
Carex pendula Huds.	PD	L > D	A > C	cold	23 .6	Brändel and Schütz, 2005; Schütz and Rave, 1999
Carex pilulifera L.	PD	L > D	NA	cold	16	Schütz and Rave, 1999
Carex remota L.	PD	L > D	A > C	cold	20 .5	Brändel and Schütz, 2005; Schütz, 1997a; Schutz, 1997b; Schütz and Rave, 1999; Ten Brink et al., 2013
Carex strigose Huds.	PD	L > D	NA	cold	16	Schütz and Rave, 1999
Carex sylvatica Huds.	PD	L > D	A > C	cold	18 .3	Graae et al., 2009; Grime et al., 1981; Schütz and Rave, 1999; Ten Brink et al., 2013
Ceratocapnos claviculata (L.) Lidén	MPD	L = D	NA	cold	15	Voss et al., 2012
Chrysosplenium alternifolium L.	PD	L > D	NA	cold	15	Jankowska-Blaszczuk and Daws, 2007
Chrysosplenium oppositifolium L.	NA	NA	NA	NA	17 .5	Grime et al., 1981
Circaea alpina L.	PD	NA	NA	cold	NA	Nichols, 1934; Baskin and Baskin, 2014

Species	Dormancy type	Light requirement	Temperature fluctuation requirement	Stratification	Effective germination temperature	References
Circaea lutetiana L.	PD	L = D	NA	cold	17 .5	Graae et al., 2009; Jankowska-Blaszczuk and Daws, 2007; Slade and Causton, 1979
Cirsium vulgare (Savi) Ten.	NA	L > D	A > C	no	19 .5	Doucet and Cavers, 1997; Grime et al., 1981; Lincoln, 1981; Michaux, 1989
Clinopodium vulgare L.	NA	L > D	NA	NA	NA	Grime et al., 1981
Colchicum autumnale L.	MPD	D > L	NA	cold	NA	Jung et al., 2011
Conopodium majus (Gouan) Loret	MPD	L = D	C > A	cold	5	Grime et al., 1981, Blandino, unpublished data
Convallaria majalis L.	PD	D > L	C > A	warm + cold	5	Barton and Schroeder, 1942 in Kondo et al., 2015
Corydalis cava (L.) Schweigg. & Körte	MPD	L = D	C > A	warm + cold	5	Mondoni et al., 2013
Corydalis solida (L.) Clairv.	MPD	L = D	NA	warm + cold	7 .7	Jankowska-Blaszczuk and Daws, 2007; Vandelook and Van Assche, 2009
Dactylis glomerata L.	PD	L > D	A > C	cold	18 .3	Grime et al., 1981; Probert et al., 1986; Qiu et al., 2008; Stanisavljević et al., 2011
Dactylorhiza maculata (L.) Soó	MPD	D > L	NA	NA	23	Van Waes and Debergh, 1986
Daphne mezereum L.	PD	NA	NA	cold	NA	Piotto and De Noi, 2003
Deschampsia cespitosa	NA	L > D	A > C	no	20	Davy, 1980; Grime et al., 1981
Deschampsia flexuosa (L.) P.Beauv.	PD	L > D	NA	NA	20	Grime et al., 1981; Scurfield, 1954
Digitalis purpurea L.	PD	L > D	A > C	cold	20 .5	Grime et al., 1981; Thompson, 1989; Vranckx and Vandelook, 2012
Epilobium angustifolium L.	PD	L > D	NA	cold	21	McLean, 1967; Myerscough, 1980; Nichols, 1934
Epilobium montanum L.	NA	L > D	NA	no	17 .5	Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007
Epipactis helleborine (L.) Crantz	MPD	D > L	C > A	no	23	Van Waes and Debergh, 1986
Festuca altissima All.	PD	L = D	A = C	cold	10	Ten Brink et al., 2013
Festuca gigantea (L.) Vill.	PD	L > D	A = C	cold	24	Grime et al., 1981; Ten Brink et al., 2013

Species	Dormancy type	Light requirement	Temperature fluctuation requirement	Stratification	Effective germination temperature	References
Ficaria verna Huds.	MPD	L = D	A = C	warm	10	Taylor and Markham, 1978; Vandelook, unpublished data
Fragaria vesca L.	PD	L > D	A = C	cold	23	Grime et al., 1981; Nichols, 1934; Thompson, 1968; Vandelook, unpublished data
Gagea lutea (L.) Ker Gawl.	MPD	NA	A = C	warm + cold	10	Kondo et al., 2004
Galanthus nivalis L.	MPD	D > L	C > A	warm	15	Newton et al., 2013, 2015
Geranium robertianum L.	PYPD	L = D	NA	cold	15 .5	Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Meisert, 2002; Slade and Causton, 1979; Van Assche and Vandelook, 2006; Vandelook and Van Assche, 2010
Geranium sanguineum L.	NA	L > D	NA	no	17 .5	Grime et al., 1981; Meisert, 2002
Geranium sylvaticum L.	NA	NA	NA	no	9 .5	Meisert, 2002
Geum rivale L.	PD	L > D	A > C	cold	19 .7	Graves and Taylor, 1988; Grime et al., 1981; Nichols, 1934; Ten Brink et al., 2013
Glechoma hederacea L.	PD	L > D	NA	NA	NA	Baskin and Baskin, 2014; Grime et al., 1981
Helleborus foetidus L.	MPD	NA	NA	cold	NA	Baskin and Baskin, 2014
Hepatica nobilis Mill.	MPD	NA	NA	warm	15	Nomizu et al., 2004
Heracleum sphondylium L.	MPD	NA	NA	cold	10	Jauzein and Mansour, 1992
Hordelymus europaeus (L.) Jess. Ex Harz	PD	L = D	A = C	cold	2	Ten Brink et al., 2013
Hyacinthoides non-scripta (L.) Chouard ex Rothm.	MPD	NA	C > A	warm	10	Grime et al., 1981; Slade and Causton, 1979; Vandelook and Van Assche, 2008a
Hypericum androsaemum L.	PD	L > D	A > C	cold	21 .5	Abdalla and McKelvie, 1980; Blandino, unpublished data
Hypericum hirsutum L.	PD	L > D	A > C	cold	20	Grime et al., 1981; Ten Brink et al., 2013
Hypericum montanum L.	NA	L = D	NA	NA	NA	Grime et al., 1981

Species	Dormancy type	Light requirement	Temperature fluctuation requirement	Stratification	Effective germination temperature	References
Hypericum perforatum L.	PD	L > D	A > C	cold	17 .5	Beckmann et al., 2011; Campbell, 1985; Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Pérez-García et al., 2006; Ten Brink et al., 2013
Hypericum pulchrum L.	PD	L > D	NA	NA	NA	Grime et al., 1981
Hypericum tetrapterum Fr.	NA	L > D	NA	NA	NA	Grime et al., 1981
Impatiens noli-tangere L.	PD	NA	NA	cold	10	Perglová et al., 2009
Impatiens parviflora DC.	PD	NA	NA	cold	15	Coombe, 1956; Grime et al., 1981; Perglová et al., 2009
Lamium galeobdolon (L.) L.	PD	L = D	NA	cold	10	Graae et al., 2009; Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Packham, 1983
Lathyrus linifolius (Reichard) Bassler	PY	L = D	A = C	warm	17 .5	Grime et al., 1981, Vandelook, unpublished data
Lilium martagon L.	MPD	NA	NA	NA	20	Parić et al., 2008
Luzula pilosa (L.) Willd.	NA	L > D	NA	no	16	Grime et al., 1981
Luzula sylvatica (Huds.) Gaudin	NA	L > D	NA	no	17 .5	Grime et al., 1981
Lysimachia europaea (L.) U.Manns & Anderb.	PD	L > D	NA	cold	20	Hiirsalmi, 1969
Lysimachia vulgaris L.	PD	L > D	A > C	cold	17 .8	Dillon and Reichard, 2014; Maas, 1989
Maianthemum bifolium (L.) F.W.Schmidt	MPD	D > L	NA	cold	20	Jankowska-Blaszczuk and Daws, 2007; Kosiński, 2008
Melampyrum pretense L.	PD	L = D	A = C	warm	8 .7	Masselink, 1980
Melica nutans L.	PD	L > D	NA	warm	17 .5	Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007
Melica uniflora Retz.	PD	NA	NA	cold	15	Graae et al., 2009
Mercurialis perennis L.	PD	L = D	A > C	warm + cold	10 .5	Graae et al., 2009; Grime et al., 1981; Gillot, 1925 in Jefferson, 2008; Vandelook unpublished data
Milium effusum L.	PD	L > D	C > A	cold	18 .5	Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Meisert, 2002; Slade and Causton, 1979; Thompson, 1989

Species	Dormancy type	Light requirement	Temperature fluctuation requirement	Stratification	Effective germination temperature	References
Moehringia trinervia (L.) Clairv.	PD	L > D	A > C	warm	15	Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Vandelook et al., 2008
Myosotis sylvatica Hoffm.	NA	L > D	NA	NA	NA	Grime et al., 1981
Narcissus pseudonarcissus L.	MPD	D > L	C > A	warm	10	Newton et al., 2013, 2015; Vandelook and Van Assche, 2008a
Neottia ovata (L.) Bluff & Fingerh.	MPD	D > L	NA	NA	23	Van Waes and Debergh, 1986
Orchis mascula (L.) L.	MPD	L > D	NA	cold	25	Valletta et al., 2008
Oxalis acetosella L.	PD	L > D	NA	cold	16 .2	Graae et al., 2009; Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Packham, 1978
Paris quadrifolia L.	MPD	L = D	A = C	cold + warm	10 .5	Jankowska-Blaszczuk and Daws, 2007
Phyteuma spicatum L.	MPD	L > D	NA	cold	NA	Jankowska-Blaszczuk and Daws, 2007; Wheeler and Hutchings, 2002
Pimpinella major (L.) Huds.	MPD	NA	NA	cold	5	Grime et al., 1981
Platanthera chlorantha (Custer) Rchb.	MPD	D > L	NA	NA	23	Van Waes and Debergh, 1986
Poa nemoralis L.	NA	L > D	A > C	no	10	Jankowska-Blaszczuk and Daws, 2007; Ten Brink et al., 2013
Polygonatum multiflorum (L.) All.	PD	NA	A = C	warm + cold	NA	Vandelook, unpublished data
Polygonatum odoratum (Mill.) Druce	MPD	L = D	NA	cold	23	Takagi, 2001
Potentilla sterilis (L.) Garcke	PD	L > D	A > C	NA	15	Vandelook, unpublished data
Primula elatior (L.) Hill	PD	L > D	A = C	cold	17 .5	Ahmad and Hitchmough, 2007; Taylor and Woodell, 2008; Ten Brink et al., 2013 Browne, 1995 in Taylor, 2008
Primula vulgaris Huds.	PD	L > D	NA	cold	17 .5	Ahmad and Hitchmough, 2007; Grime et al., 1981
Pulmonaria officinalis L.	PD	NA	A > C	cold	10 .5	Vandelook, unpublished data
Ranunculus auricomus L.	MPD	L = D	A = C	warm	5	Vandelook, unpublished data
Ranunculus lanuginosus L.	NA	L = D	NA	cold	NA	Jankowska-Blaszczuk and Daws, 2007
Ranunculus repens L.	MPD	L > D	NA	cold	20	Harris et al., 1998

Species	Dormancy type	Light requirement	Temperature fluctuation requirement	Stratification	Effective germination temperature	References
Rumex sanguineus L.	PD	L > D	A > C	cold	18 .3	Grime et al., 1981; Ten Brink et al., 2013; Van Assche et al., 2002
Ruscus aculeatus L.	MPD	NA	NA	NA	20	D'Antuono and Lovato, 2004
Sanicula europaea L.	MPD	L > D	C > A	cold	5	Vandelook and Van Assche, 2008b
Scirpus sylvaticus L.	NA	L > D	NA	NA	33 .5	Grime et al., 1981
Scrophularia nodosa L.	PD	L > D	A > C	cold	22 .5	Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Vranckx and Vandelook, 2012
Sedum telephium L.	NA	L > D	NA	NA	NA	Grime et al., 1981
Senecio sylvaticus L.	NA	L > D	NA	NA	NA	Grime et al., 1981
Serratula tinctoria L.	PD	L > D	NA	cold	22	Grime et al., 1981
Silene dioica (L.) Clairv.	PD	L > D	A > C	cold	21 .6	Grime et al., 1981; Slade and Causton, 1979; Ten Brink et al., 2013; Thompson, 1989
Solidago virgaurea L.	PD	L > D	NA	cold	15	Grime et al., 1981; Pietikäinen et al., 2005
Stachys sylvatica L.	PD	L = D	A > C	cold	18 .3	Grime et al., 1981; Jankowska-Blaszczuk and Daws, 2007; Slade and Causton, 1979; Blandino unpublished data
Stellaria holostea L.	PD	L = D	A > C	warm + cold	17 .5	Graae et al., 2009; Jankowska-Blaszczuk and Daws, 2007; Ten Brink et al., 2013; Vandelook et al., 2008
Stellaria nemorum L.	PD	L > D	A > C	cold	19 .3	Jankowska-Blaszczuk and Daws, 2007; Ten Brink et al., 2013; Vandelook et al., 2008
Succisa pratensis Moench	PD	L > D	A > C	cold	14 .7	Adams, 1955; Grime et al., 1981; Maas, 1989
Teucrium scorodonia L.	PD	L > D	NA	cold	NA	Grime et al., 1981; Hutchinson, 1968
Trifolium medium L.	PY	NA	NA	NA	NA	Grime et al., 1981
Trollius europaeus L.	MPD	L = D	A = C	warm + cold	14	Grime et al., 1981; Hitchmough et al., Corr, 2000; Maas, 1989; Milberg, 1994

Species	Dormancy type	Light requirement	Temperature fluctuation requirement	Stratification	Effective germination temperature	References
Vaccinium myrtillus L.	PD	L > D	NA	cold	20	Baskin et al., 2000; Grime et al., 1981
Valeriana officinalis L.	PD	L > D	NA	NA	23 .9	Grime et al., 1981; Hassell et al., 2004
Veronica montana L.	PD	NA	NA	cold	NA	Slade and Causton, 1979; Vandelook, unpublished data
Veronica officinalis L.	NA	L > D	NA	NA	NA	Grime et al., 1981
Vicia sepium L.	PY	L = D	A = C	warm	23	Vandelook, unpublished data
Viola mirabilis L.	PD	NA	NA	cold	NA	Berg and Redbo-Torstensson, 1999
Viola palustris L.	PD	L > D	NA	cold	18 .7	Grime et al., 1981; Jensen, 2004
Viola riviniana Rchb.	PD	NA	NA	cold	17 .5	Berg and Redbo-Torstensson, 1999; Grime et al., 1981

REGENERATION STRATEGIES IN ANCIENT WOODLAND UNDERSTORIES

In order to describe the germination strategies of the European AWIs, a FAMD (Fig. 6 a-b, Annex II) was performed for those traits that in the previous ordination analysis explained most of the variance (seed mass, flowering and germination phenology), plant height, E:E ratio and the following germination traits: dormancy type, stratification requirement, light requirement and effective temperature for germination. The latter were the most frequently described germination traits in the literature review (Table 2). Complete information on these traits were available for nearly one third of the species (i.e., 57 species or 29% of the total). Only these species were included in this ordination.

The first component explained 29% of the variance and all the continuous variables used contributed positively and significantly to it, excluding seed mass that had a negative correlation coefficient (Annex II). All the qualitative variables were highly significant ($p < 0.01$). Stratification and light requirements for germination were the qualitative variables that contributed most to explain the first axis (R^2 respectively = 0.59 and 0.55, $p < 0.01$). The distribution of the categories along the first component separate the species in two different regeneration strategies:

1) Species with seed PD that require cold stratification and light and germinate in spring. These species had higher germination temperatures and small seeds with an high E:E ratio. The adult plants tended to be tall and to flower later in the year.

2) Species with seed MD that required a W or a combination of W+C . Germination is photoinhibited or indifferent to light and happens in autumn. These seeds tended to be large with relatively small embryos and a preference for lower effective

germination temperatures.. The adult plants tended to be shorter than in the first group and to flower earlier.

It can be considered that from the right to the left of the graph there is a gradient of growing association with closed canopy situations expressed by lower numeric values of the coordinates on this component for species more associated with closed forests (Fig.6 a-b; Hermy et al., 1999). The position of each species in this axis then indicates its affinity with ancient woodland as expressed by its regeneration strategy. Of the EIVs included as categorical supplementary variables in the analysis only the indicators for light and moisture requirement were significantly explained by the first component of the FAMD (light $R^2 = 0.23$, $p < 0.01$, moisture $R^2 = 0.14$, $p = 0.04$) with higher values of the indicators associated with the species on the right side of the individuals' map (Fig. 6 c-d).

The second component of the FAMD explained 13% of the variance. The only quantitative variable significantly correlated to this dimension was E:E ratio (Pearson = 0.49, $p < 0.01$) while the contribution of all the qualitative variables was significant (Annex II). The second component mostly ordered the species from the second group (affinity with closed canopy situations), while species from group one were clustered together around the first component. Relative embryo size and effective germination temperature had a positive correlation with the second component while plant height, flowering month and seed dry mass were negatively correlated with it (although not significantly, Annex II). Germination season was the categorical traits with the strongest association with the second component ($R^2 = 0.61$, $p < 0.01$) that was also contributed to by light requirement ($R^2 = 0.23$, $p < 0.01$), dormancy type ($R^2 = 0.21$, $p < 0.01$) and stratification requirements ($R^2 = 0.18$, $p = 0.05$). No one of the EIVs contributed to

explain the second dimension (Annex II), and embryo type was the only significant supplementary variable ($p = 0.02$).

Hierarchical clustering on the two principal components of FAMD.regeneration defined three groups of species :

1) *Carex brizoides* group: the majority of the species analysed (41) were clustered together around the positive values of the first component of FAMD.regeneration (Fig. 6). All the species in the group possessed PD and the majority of them germinated in spring (85%) after a C stratification (95%) and in presence of light (82%). Small seeds with relatively big embryos, high germination temperatures and late flowering significantly ($p < 0.01$) characterized this cluster, that is formed by plants taller than the average of the dataset ($p = 0.03$). All species with summer germination and capitate, peripheral or investing embryos are part of this group.

2) *Narcissus pseudonarcissus* group: only six species were part of this group. All of them germinated in the autumn, after a W or W+C. This cluster included the majority (80%) of the plants with seeds that are photoinhibited for germination. The mean flowering time, plant height and germination temperature of these species was significantly smaller ($P < 0.01$) than the overall average and their seeds were significantly bigger ($p = 0.01$). Also average embryo size was relatively smaller than the overall ($p = 0.05$). Dormancy type contributed significantly ($p < 0.01$) to the definition of the cluster even though half of the species possessed MPD and half PD. Seeds in this group had endospermic seeds with linear or spatulate embryo. Epicotyl dormancy or a slow development of the shoot delay emergence until late winter or spring;

3) *Polygonatum odoratum* group: the ten species that formed this cluster were characterized by MPD (80% of the species in the group, representing 72% of the species with MPD) and half of them possessed rudimentary embryos. Low germination temperatures and small relative embryo size were significantly associated to this group ($p < 0.01$, Annex II) as well as having big seeds ($p = 0.02$, Annex II), while light was not required for germination in 70% of the species. The majority of the species (70%) germinated in spring after a cold stratification and the rest germinated in winter.

Only the Ellenberg Indicators for light and moisture had significant and positive association to the first component of the FAMD.regeneration ($p < 0.01$ and $p = 0.04$, respectively) while none were associated to the second component (Fig. 6, c-d, Annex II). Those indicators describe differences in habitat preference and, being the species of the *Carex brizoides* group clustered around the positive values of the first dimension. These can then be considered more associated with open and wet situations, as can be gaps in the tree canopy or in riparian forests. In fact, streams within a forest are found in relatively open areas, where more light is available and extreme temperatures are less buffered than under the canopy or leaf litter.

The requirement for C can be then an important adaptive mechanism for species in this group to avoid germination during the winter months. Germination phenology is strictly related with stratification requirements. Most of the species in *Polygonatum odoratum* and *Carex brizoides* groups required a C period before being able to germinate and can be defined as late winter/spring germinators. Those groups were already described by Grime et al., (1981) who found differences between two types of species requiring C: perennial species with big seeds that can germinate and do not form a soil seed bank and

arable or marsh plants with small seeds that require light for germination and participate to the soil seed bank

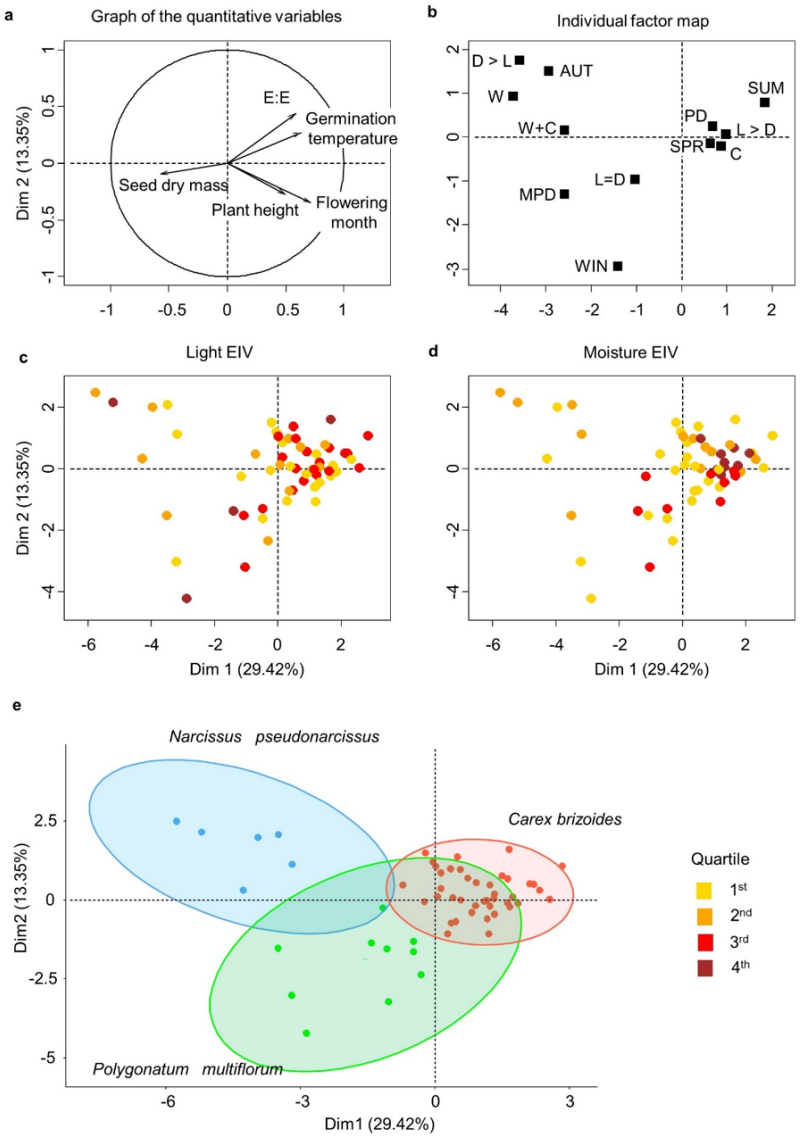

Fig 6: Factorial Analysis of Mixed Data (FAMD) of regeneration traits and hierarchical clustering on its principal components (HCPC). The centre of each cluster is marked by a black lined circle. Graphic **a)** describe the distribution of the numerical variables while **b)** indicate the distribution of the categorical traits. Graphics **c)** and **d)** indicate how the

(continued from p. 57) Ellenberg Indicator Values (EIV) for light and moisture vary across the species. The dark red dot represents the species with highest values for the variable (> than the 4th quartile of its distribution) while the yellow dots are the species with the lowest values (< than the 1st quartile). PD = physiological dormancy; MPD = morphophysiological dormancy; C = cold stratification; W = warm stratification; W+C = combination of warm + cold stratification; L>D = species requiring light for germination; D>L = species requiring dark for germination; L=D = species indifferent to light for germination; AUT = germination in autumn; SPR = germination in spring; SUM = germination in summer; WIN = germination in winter.

In fact, the more favourable season for germination in temperate, broadleaved woodland environments is spring, when the risk of frost has passed but the canopy is not yet closed, enabling a high availability of light for a developing seedling.

The production of many small seeds and the high stature of the plants make the species of the *Carex brizoides* group more adapted at long distance seed dispersal than the species in the other groups and the requirements of light for seed germination is indicative of gap detection mechanisms (Pearson et al., 2002). Overall, species from this group can be regarded as good colonizers (Verheyen et al., 2003) adapted to germinate in forest edge and canopy gaps as soon as the winter ends. Endospermic seeds with linear or rudimentary embryos are rare in this group, being associated with species having a seed MPD while all the species in *Carex brizoides* group have seed PD (Tables 3 and 6).

Species more associated with closed canopy forest (*Narcissus pseudonarcissus* and *Polygonatum odoratum* groups), had lower values of the Ellenberg Indicators for light and moisture, even though single species in the clusters can have higher requirements for

light as adult plants (Annex II). They present traits (early flowering, short plants that produce big seeds) of species regarded as bad colonizers (Verheyen et al., 2003).

Within these two groups different strategies can be identified but the indifference to light or the requirement of dark for germination can indicate preference for shady habitats or ability to germinate under leaf litter. The relationship between germination in the dark and seed size has been explored (Jankowska-Blaszczuk and Daws, 2007, Carta et al., 2017) and the ability of temperate forest species with high seed mass to germinate in the dark is confirmed in this analysis (Fig 6, a-b). Two of the species that can germinate in the dark, *Conopodium majus* and *Narcissus pseudonarcissus* (Annex II), have an EIV for light of 8, thus indicating a separation between the ecological niche of the seed/seedling stage and of the adult plant.

Species of the *Narcissus pseudonarcissus* cluster are the shortest plants and the earliest to flower. They usually disappear in late spring, after seed dispersal and are replaced by species of the *Polygonatum odoratum* and *Carex brizoides* groups (Newton et al., 2013, 2015).

Early flowering woodland plants take advantage of a season in which the tree canopy is still open and there is plenty of light available for flowers and fruits development. The habitats of early and late flowering species are then separated in time but not necessarily in space. Species of the *Narcissus pseudonarcissus* group germinate in autumn, after a W (or W+C) stratification and at lower temperatures than species from the *Carex brizoides* group. Autumn can also be a favourable season for germination, where temperatures are still relatively high and the canopy is starting to open, but is not as good for seedling emergence because the winter frost can damage them. So, forest species that germinate in the autumn can have epicotyl dormancy or a very slow growing shoot. While the radicle emerges and establishes itself in the autumn and during the winter, securing a place for

the seedling in the forest floor, the shoot will start to grow only after a C stratification (Mondoni et al., 2008 , Takagi, 2001), or will keep growing but at a very slow pace during the coldest months (Newton et al., 2013, 2015; Vandelook and Van Assche, 2008), assuring the emergence of the seedling after the risk of frosts has passed. This strategy is adopted by species that are either photoinhibited or indifferent to light for germination. Absolute dark can be a cue of deep burial in the soil or under leaf litter, a situation in which an endospermic seed can sustain radicle growth in an environment that is safer from predation, temperature extremes or desiccation than the soil surface. A good example is *Galanthus nivalis*, which also has desiccation sensitive seeds (Newton et al., 2013).

Even though the relative embryo size is small, only half of the species in the group possess MD . In fact, three of them have been reported to have linear embryos that grows very little (*Hyacinthoides non-scripta*; Vandelook and Van Assche, 2008) or do not grow (*Allium ursinum* and *Convallaria majalis*; Vandelook, 2009). In these cases it is likely that the seeds germinate and the embryo keeps absorbing nutrients from the endosperm after germination, acting like an haustorium. This type of germination has been described for the genus *Yucca* (Horner and Arnott, 1966) and can be a further adaptation to germination in the dark because it allows the shoot to start developing before emerging from soil or leaf litter.

Species of the *Polygonatum odoratum* group are comparatively taller and later flowering than species of the *Narcissus pseudonarcissus* cluster but are characterized by smaller embryos and the lower germination temperatures. Most possess big seeds with underdeveloped embryos (and thus seed MPD) that are dispersed in summer and the rudimentary embryo type is the most represented in the group. The embryos start to grow when the temperatures drops in autumn and during the winter and most of the species in

the group germinate after a C stratification in late winter or early spring of the year after dispersal. These species take advantage of the weeks in which the canopy is still open but the temperature is still too cold for species from the *Carex brizoides* group to germinate. Even though germination can happen in absence of light, no species from this group are photoinhibited. This group differentiated from the *Carex brizoides* cluster on the basis of dormancy type and embryo morphology, two characters that are highly associated and preserved in plant evolution (Finch-Savage and Leubner-Metzger, 2006; Forbis et al., 2002; Baskin and Baskin, 2004; Willis et al., 2014).

Seed MPD has been reported as the earlier type of dormancy by Willis et al., (2014) and there is agreement between different authors that a morphological component of dormancy is an ancestral character of angiosperms (Baskin and Baskin, 2004; Forbis et al., 2002; Finch-Savage and Leubner-Metzger, 2006; Willis et al., 2014), associated with a low relative embryo size (Forbis et al., 2002; Martin, 1946). Willis et al., 2014 stated that some dormancy classes occurs more in certain lineages but observed also a high degree of convergent evolution. In the *Polygonatum odoratum* cluster, 80% of the species possess seed MPD; these belong to Apiaceae, Asparagaceae, Papaveraceae and Ranunculaceae families which are reported to show this type of dormancy in, respectively, 63, 86, 94 and 96% of the studied species (Baskin and Baskin, 2014; Willis et al., 2014).

Species of the *Polygonatum odoratum* and *Narcissus pseudonarcissus* groups share a common timing of early spring emerging seedlings, although the two groups achieved it with different germination strategies. Early emergence represents a competitive advantage in seasonal temperate forests and is an indication of adaptation to this habitat. Of the clusters identified, *Carex brizoides* and the other two clusters present distinct seed dormancy types and germination strategies.

Only species from the latter two groups, that shares various traits associated with slow colonizer forest herbs (Verheyen et al., 2003), have seed MPD. This dormancy type has been reported as an important feature of herbaceous species of temperate forests in the Northern hemisphere (Baskin and Baskin, 2014) and its frequency in this type of habitat may be explained by the stable and moist condition that characterize it, allowing the seeds with underdeveloped embryo to remain imbibed for long enough to complete embryo growth. As an example, Vandelook et al., (2012) found, in Apiaceae, a negative correlation between relative embryo size and seed mass, plant longevity, shade requirement and precipitation: species with lower relative embryo size were expected to be more common in stable and moist environment like closed canopy temperate forests.

A convergent evolution in germination strategies between species derived from different evolutionary lineages can be observed in all the clusters described. For example, the *Narcissus pseudonarcissus* cluster is mostly composed by species from the Asparagales order but also two dicotyledons with a similar strategy, *Mercurialis perennis* and *Anemone nemorosa*, participate in it. The *Polygonatum odoratum* group is more heterogeneous and includes also species with PD (*Arum maculatum* and *Hordelymus europaeum*) that share with the others the indifference to light and low temperature germination requirements. A convergent evolution of similar germination strategies in temperate forest herbs from different lineages was already observed (Vandelook, 2009).

CONCLUSIONS

Implications for forest management

Within the 57 species analysed in FAMD.regeneration a clear distinction can be made between: (1) good colonizer species able to develop also in disturbed vegetation and that

benefit from gaps in and edges to the forest (*Carex brizoides* group); and (2) core forest species, well adapted to survive in a stable environment but limited in colonizing new habitats both by their low colonization capacity and by their more complicated dormancy breaking requirements (*Narcissus pseudonarcissus* and *Polygonatum odoratum* groups). These findings on a new data set of AWIs in Europe are in agreement with Verheyen et al., (2003). That study, which analysed life history and regeneration traits of understory species from temperate Europe and North America, found that dispersability is a limiting factor to colonization and that poor colonizers usually have complicated seed dormancy breaking requirements. For this reasons new forest areas should be established as close as possible to ancient forests that can act as source of propagules (Honnay et al., 2002). When this is not possible, reintroduction of the species with the lower colonizing capacity will be necessary. Core woodland species with low colonizing capacity should be prioritized for reintroduction because their natural colonization of a recent forest is more unlikely than for species of the *Carex brizoides* group.

When using seeds for ecological restoration of temperate woodland understory the type of dormancy and the needs for stratification are a major constraint that can influence the success of a reintroduction. It is then important, to assess for each species, the best strategy for reintroduction based on its regeneration traits and, if seeds are used, to take into account its germination strategy when planning the time and place of sowing. For example, good colonizer species can differ in their response to disturbance and gap size because, while some only germinate when a gap in the tree canopy is perceived, others need just a disturbance in the herb or root layer to trigger germination (Jankowska-Blaszczuk and Grubb, 1997). Further investigation of the response to different levels of R and FR radiation can give insight to species habitat preference inside the forest and provide indications for habitat management.

A limitation to recruitment for core forest species with big seeds is that they do not form a persistent soil seed bank (Bekker et al., 1998, Thomson et al., 1997) and, after deforestation, their seeds get soon depleted from the soil (Honnay et al., 2002). Enrichment by ruderal species that form a long lived soil seed bank can mean that recent forests on former arable land can take 100 years without any disturbance before the soil seed bank is depleted (Bossuyt and Hermy, 2002). Management to avoid such ruderal species from reproducing and competing with reintroduced core forest species, e.g. by minimising gaps in the canopy and disturbance in the herb layer, should be practiced (Honnay et al., 2002).

Species that produce few big seeds are difficult to produce in quantities sufficient for broadcast sowing and are best reintroduced as plug plants produced in a nursery. It has been demonstrated that big seeded species are often more limited by seed availability than small seeded ones (Clark et al., 2007) and that seedling emergence and development is a more critical stage of plant development than seed germination (Turnbull et al., 2000).

If seeds are used for reintroduction, the sowing time should be decided according to the stratification requirements and germination seasons of the species and it may be necessary to programme many sowings during the year according to the requirements of the species to be reintroduced.

Gaps in knowledge

Few attempts have been made to summarize the breadth knowledge on germination strategies and embryo morphology of temperate woodland understories (Grime et al., 1981, Baskin & Baskin 2014, Vandelook 2009) and there is need for further investigations

to fill the gaps in knowledge. No information was readily available on the germination of 76 of the AWIs, often species belonging to genera with a difficult taxonomy (e.g. *Hieracium*, *Pulmonaria*) or to groups for which germination is known to be difficult to achieve in laboratory (e.g., orchids or parasitic plants). The majority of the studies on the germination of 22 AWIs with micro seeds were not included because seeds were germinated in buried mesh nets and so no data on germination temperature or other germination traits were available. However, this gap may be filled using the seasonal averaged soil temperatures for the region in the year in which the experiments were performed. When orchid seeds are germinated in the laboratory, the main focus of the studies tends to be testing different growing media using a single germination temperature (Nadarajan et al., 2011) and this is also the case for other species with small seeds (Seglie et al., 2012).

AKNOWLEDGMENTS

The ideas behind this work was developed together with Rosemary Newton. This study would not have been possible without the collaboration of Eduardo Fernandèz Pascual and Hugh Pritchard, that provide valuable statistical and conceptual advice. Thank you also to Janet Terry for helping in obtaining from the Millennium Seed Bank the seeds used for the embryo measurements. The involvement of Eduardo Fernández Pascual, Hugh W. Pritchard and Rosemary Newton in revising the manuscript was precious and fundamental. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°607785. The study has been supported by the TRY initiative on plant traits (http://www.trydb.org). The TRY initiative and database is hosted, developed and maintained by J. Kattge and G. Boenisch (Max

Planck Institute for Biogeochemistry, Jena, Germany). TRY is currently supported by Future Earth/bioDISCOVERY and the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig. We are grateful to Filip Vandelook for sharing unpublished germination data. The Royal Botanic Gardens, Kew received grant-in-aid from Defra.

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ANNEX I

List of the papers that contributed to the literature review and characteristics of the seed lots used and of the experiments tested in them.

Seed source: Wi = wild, Cu = cultivated; Seed origin.: AM = North America, AS = Asia, EU = Europe, OC = Oceania; N. AWI: number of ancient woodland indicators in the reference; N. Temp: number of temperatures tested, Therm. = a thermogradient plate was used; Temp. fluct.: effect of temperature fluctuations tested, Light: effect of light on germination tested; Move along: move along experiment performed; Phen. : seed germination phenology observed; Gas : gibberellins used; C: cold stratification; W: warm stratification; W+C : warm + cold stratification; Scar: seed scarification applied; N : nitrates applied; Other: other dormancy breaking treatment applied.

Reference	Seed source	Seed origin	N. AWI	N. Temp	Temp. fluct.	Light	Move along	Phen.	GAs	C	W	W + C	Scar	N	Other
Abdalla and McKelvie, 1980	NA	EU	2	1	no	no	no	no	yes	yes	no	no	no	no	yes
Adams, 1995	Wi	EU	1	3	no	no	no	no	no	no	no	no	no	no	no
Ahmad and Hitchmough, 2007	Cu	EU	2	1	no	no	no	yes	no	yes	no	no	no	no	no

Reference	Seed source	Seed origin	N. AWI	N. Temp	Temp. fluct.	Light	Move along	Phen.	GAs	C	W	W + C	Scar	N	Other
Barton and Schroeder, 1942	NA	NA	1	NA	NA	NA	no	NA	no	yes	no	no	no	no	no
Baskin et al., 2000	Wi	EU	1	3	no	yes	no	yes	no	yes	no	no	no	no	no
Beckmann et al., 2011	Wi	OC	1	3	no	no	no	no	no	no	no	no	no	no	no
Berg and Redbo-Torstensson, 1999	Wi	EU	2	NA	no	no	no	yes	no	no	no	no	no	no	no
Brändel and Schütz, 2005	Wi	EU	2	16	yes	yes	no	yes	no	yes	no	no	no	no	no
Browne, 1995	NA	NA	1	NA	NA	yes	no	NA	no	yes	no	no	no	no	no
Campbell, 1985	Wi	OC	1	NA	NA	yes	no	NA	no	no	no	no	no	no	no
Coombe, 1956	Wi	EU	1	1	no	yes	no	NA	no	yes	no	no	no	no	no
D' Antuono and Lovato, 2004	Wi	EU	1	1	no	no	no	no	no	no	no	wc	yes	no	no
Davy, 1989	NA	EU	1	4	yes	yes	no	NA	no	no	no	no	no	no	no
Dillon and Reichard, 2014	Wi	AM	1	3	no	no	no	no	no	yes	no	no	no	no	no

Reference	Seed source	Seed origin	N. AWI	N. Temp	Temp. fluct.	Light	Move along	Phen.	GAs	C	W	W + C	Scar	N	Other
Doucet and Cavers, 1997	Wi	AM	1	1	yes	yes	no	no	no	yes	no	no	no	no	no
Ernst, 1979	Wi	EU	1	4	no	no	no	no	no	yes	yes	no	no	no	no
Gillot, 1925	NA	EU	1	3	NA	NA	na	yes	NA	NA	NA	NA	NA	NA	NA
Graee et al., 2009	Wi	EU	10	1	no	no	no	no	no	yes	yes	cw	no	no	no
Graves and Taylor, 1988	Wi	EU	1	20	no	no	no	no	no	no	no	no	no	no	no
Grime et al., 1981	Wi	EU	67	31	yes	yes	no	no	no	yes	no	no	yes	no	no
Harris et al., 1998	Wi	AM	1	4	no	no	no	no	no	no	no	no	no	no	no
Hassell et al., 2004	Cu	AM	1	10	no	no	no	no	no	no	no	no	no	no	no
Herranz et al., 2010	Wi	EU	1	6	yes	yes	no	yes	no	yes	no	no	no	no	no
Hiirsalmi, 1969	Wi	EU	1	3	no	yes	no	no	yes	yes	no	no	no	no	no
Hitchmough et al., 2000	both	EU	1	1	no	no	no	no	yes	yes	no	wc	no	no	yes
Hutchinson, 1968	Wi	EU	1	NA	NA	yes	no	NA	no	yes	no	no	no	no	no

Reference	Seed source	Seed origin	N. AWI	N. Temp	Temp. fluct.	Light	Move along	Phen.	GAs	C	W	W + C	Scar	N	Other
Jankowska-Błaszczuk and Daws, 2007	Wi	EU	22	1	no	yes	no	no	no	yes	no	no	no	no	no
Jauzein and Mansour, 1992	Wi	EU	1	5	no	NA	no	no	no	yes	no	no	no	no	no
Jensen, 2004	Wi	EU	1	2	no	yes	no	yes	no	yes	no	no	no	no	no
Jung et al., 2011	Wi	EU	1	1	no	yes	no	yes	no	no	no	wc	no	no	no
Kondo et al., 2004	Wi	AS	1	7	yes	no	no	yes	no	no	no	wc	no	no	no
Kosiński, 2008	Wi	EU	1	1	no	yes	no	yes	no	yes	no	no	no	no	no
Lincoln, 1981	NA	AM	1	4	yes	yes	no	no	no	no	no	no	no	no	no
Maas, 1989	Wi	EU	3	1	yes	yes	no	no	yes	yes	no	no	no	yes	yes
Masselink, 1980	Wi	EU	1	8	yes	yes	no	yes	yes	yes	yes	no	no	no	no
McLean, 1967	Wi	AM	1	5	no	no	no	no	no	yes	no	no	yes	no	no
Meisert, 2002	both	EU	3	1	no	no	no	no	no	no	no	no	yes	no	yes
Michaux, 1989	Wi	OC	1	Therm.	no	no	no	yes	no	no	no	no	no	no	no
Milberg, 1994	Wi	EU	1	Therm.	no	no	no	yes	no	yes	no	no	no	no	no
Mondoni et al., 2008	Wi	EU	1	4	no	no	yes	yes	yes	yes	yes	no	no	no	no

Reference	Seed source	Seed origin	N. AWI	N. Temp	Temp. fluct.	Light	Move along	Phen.	GAs	C	W	W + C	Scar	N	Other
Mondoni et al., 2009	Wi	EU	1	8	yes	no	yes	yes	yes	yes	yes	wc	no	no	no
Mondoni et al., 2013	Wi	EU	1	8	yes	yes	no	yes	yes	yes	yes	no	no	no	no
Myerscough, 1980	Wi	NA	1	NA	no	yes	no	no	no	no	no	no	no	yes	no
Newton et a;., 2015	Wi	EU	2	12	yes	yes	yes	yes	no	no	yes	no	no	no	no
Newton et al., 2013	Wi	EU	2	8	yes	yes	no	no	no	no	yes	no	no	no	no
Nichols, 1934	Wi	AM	3	NA	no	no	no	no	no	yes	no	no	no	no	no
Nomizu et al, 2004	Cu	AS	1	3	no	no	no	yes	no	no	no	no	no	no	no
Packham, 1978	Wi	EU	1	1	no	no	no	NA	no	yes	no	no	no	no	no
Packham, 1983	Wi	EU	1	1	no	no	no	NA	no	yes	no	no	no	no	no
Parić et al., 2008	Wi	EU	1	1	no	no	no	no	no	no	no	no	yes	no	yes
Pérez-García et al., 2006	Wi	EU	1	3	yes	yes	no	no	yes	no	no	no	no	no	yes

Reference	Seed source	Seed origin	N. AWI	N. Temp	Temp. fluct.	Light	Move along	Phen.	GAs	C	W	W + C	Scar	N	Other
Perglová et al., 2009	Wi	EU	2	3	no	no	no	yes	no	yes	no	no	no	no	no
Pietikäinen et al, 2005	Wi	EU	1	NA	NA	NA	no	yes	no	yes	no	no	no	no	no
Piotto and De Noi, 2003	NA	NA	1	NA	NA	no	no	no	yes*	yes*	no	no	no	no	no
Pritchard et al 1993	Wi	EU	1	Therm.	yes	yes	no	no	no	yes	yes	cw	no	no	no
Probert et al., 1986	both	EU	1	2	yes	yes	no	no	no	no	no	no	no	no	no
Qiu et al., 2008	Cu	NA	1	14	yes	no	no	no	no	no	no	no	no	no	no
Salisbury, 1969	Wi	EU	1	NA	NA	yes	no	yes	no	no	no	no	no	no	yes
Schutz and Rave, 1999	Wi	EU	9	6	yes	yes	no	no	no	yes	no	no	no	no	no
Schutz, 1997a	Wi	EU	3	6	yes	yes	no	yes	no	yes	no	no	no	no	no
Schutz, 1997b	Wi	EU	3	2	yes	yes	no	no	no	no	no	no	no	no	no
Scurfield, 1954	NA	NA	1	3	yes	yes	no	NA	no	no	no	no	no	no	no
Slade and Causton, 1979	Wi	EU	9	1	no	yes	no	no	no	yes	no	no	yes	yes	yes

Reference	Seed source	Seed origin	N. AWI	N. Temp	Temp. fluct.	Light	Move along	Phen.	GAs	C	W	W + C	Scar	N	Other
Stanisavljević et al., 2011	Cu	EU	1	1	no	no	no	no	no	yes	no	no	no	no	no
Takagi, 2001	Cu	AS	1	5	no	yes	yes	yes	yes	yes	no	cw	no	no	yes
Taylor and Markham, 1978	Wi	EU	1	3	no	yes	no	no	no	yes	no	no	yes	no	no
Ten Brink et al., 2013	both	EU	19	4	yes	yes	no	no	no	yes	no	no	no	no	no
Thompson, 1968	Cu	EU	1	NA	NA	yes	no	no	yes	no	no	no	no	no	yes
Thompson, 1989	Wi	EU	4	2	yes	yes	no	no	no	no	no	no	no	no	no
Valletta et al., 2008	Wi	EU	1	1	no	yes	no	no	no	yes	no	no	yes	no	no
Van Assche and Vandelook, 2006	Wi	EU	1	3	no	no	no	yes	no	no	no	no	yes	no	no
Van Assche et al., 2002	Wi	EU	1	24	yes	yes	no	yes	no	no	no	no	no	no	no
Van Waes and Debergh, 1986	Cu	EU	4	2	yes	yes	no	no	yes	yes	no	no	no	yes	yes

Reference	Seed source	Seed origin	N. AWI	N. Temp	Temp. fluct.	Light	Move along	Phen.	GAs	C	W	W + C	Scar	N	Other
Vandelook and Van Assche, 2008a	Wi	EU	2	7	yes	no	yes	yes	no	no	yes	no	no	no	no
Vandelook and Van Assche, 2008b	cu	EU	1	5	yes	yes	yes	yes	yes	yes	yes	no	no	no	no
Vandelook and Van Assche, 2009	Wi	EU	1	5	yes	no	yes	yes	yes	no	yes	no	no	no	no
Vandelook and Van Assche, 2010	Wi	EU	1	14	yes	yes	yes	yes	no	yes	yes	no	yes	no	no
Vandelook et al., 2008	Wi	EU	3	5	yes	yes	no	yes	no	yes	yes	no	no	yes	no
Voss et al., 2012	Cu	EU	1	4	yes	no	no	yes	no	yes	no	no	no	no	yes
Vranckx and Vandelook, 2012	Wi	EU	2	15	yes	yes	no	yes	no	yes	yes	no	no	yes	no
Wheeler and Hutchings, 2002	NA	EU	1	NA	NA	NA	no	yes	yes	yes	no	no	no	no	no

ANNEX II

Outputs from the ordination analysis

PCA Ellenberg: Pearson correlation coefficient of each Ellenberg Indicator Value with the first two dimensions of a PCA calculated for 191 European Ancient Woodland Indicators. The p-value of the t-test indicate if the correlation coefficient differs significantly from zero.

EIV	PC1	p-value	PC2	p-value
Continentality	-0.04	0.57	-0.03	0.67
Light	-0.48	**< 0.01**	0.64	**< 0.01**
Moisture	0.16	**0.01**	0.72	**< 0.01**
Nutrients	0.86	**< 0.01**	0.33	**< 0.01**
pH	0.71	**< 0.01**	-0.16	**0.02**
Temperature	-0.008	0.9	-0.33	**< 0.01**

PCA seed dispersal and yield: Pearson correlation coefficient of each external morphology and seed yield trait with the first two dimension of a PCA calculated for 68 European Ancient Woodland Indicators. The p-value of the t-test indicate if the correlation coefficient differs significantly from zero.

Trait name	PC1	p-value	PC2	p-value
Dispersule length	0.68	**< 0.01**	0.33	**< 0.01**
Dispersule width	0.81	**< 0.01**	0.38	**< 0.01**
Seed dry mass	0.90	**< 0.01**	0.40	**< 0.01**
Seed number per plant	-0.90	**< 0.01**	0.41	**< 0.01**
Seed terminal velocity	0.74	**< 0.01**	0.26	**0.03**

FAMD.phenology: Description of the first two dimensions and of the clusters of species identified on them by the HCPC (Hierarchical Clustering on Principal Components) function (Husson et al., 2010). The Pearson correlation coefficient describe the correlation of the quantitative variables with each of the first two dimensions of the FAMD and a t-test describe if the correlation is significant. The R^2 of one way ANOVA was calculated to test the association of each qualitative variable with each of the first two dimensions. The p-value is provided by a Fisher test. Student t- tests check, for each category, if the average of the coordinates along each dimension of the sub groups of individuals defined by that category are significantly different from the average of the coordinates of all the individuals. The association of each variable with the clusters is described by a v test for the quantitative variables and by a chi-square test for the qualitative variables. The representation of each category in the clusters is described by the % of species with that category in the cluster. The frequency of each category in each cluster is then compared with its overall representation and tested with a v test. All test are considered significant if $p < 0.05$ (in bold).

FAMD.phenology	**Dimension 1**		**Dimension 2**		***Narcissus pseudonarcissus***		***Carex sylvatica***		***Lysimachia nemorum***	
Quantitative variables	Pearson corr. coeff.	p-value	Pearson corr. coeff.	p-value	v test	p-value	v test	p-value	v test	p-value
Flowering month	0.87	< **0.01**	0.05	0.57	-6.2	< **0.01**	0.78	0.43	4.52	< **0.01**

FAMD.phenology

Qualitative variables	R^2	p-value	R^2	p-value		p-value	df		p-value	df		p-value	df
Dispersal season	0.74	< **0.01**	0.64	< **0.01**		< **0.01**	4		< **0.01**	4		< **0.01**	4
Germination season	0.18	< **0.01**	0.6	< **0.01**		< **0.01**	6		< **0.01**	6		< **0.01**	6
Categories	Estimate	p-value	Estimate	p-value	% species	v test	p-value	% species	v test	p-value	% species	v test	p-value
Dispersal spring	-1.89	< **0.01**	0.07	0.21	86.96	8.84	< **0.01**	0	-4.66	< **0.01**	0	-4.09	< **0.01**
Dispersal summer	0.62	**0.03**	-1.04	< **0.01**	13.04	-4.05	< **0.01**	100	9.65	< **0.01**	86.84	9.75	< **0.01**
Dispersal autumn	1.27	< **0.01**	0.96	< **0.01**	0	-4.05	< **0.01**	0	-6.53	< **0.01**	13.16	-5.78	< **0.01**
Germination winter	-0.46	0.16	-1.62	< **0.01**	8.69	0.27	0.79	11.11	1.11	0.27	31.58	1.77	0.07
Germination spring	0.49	< **0.01**	-0.76	< **0.01**	43.48	-2.24	**0.02**	86.67	4.2	< **0.01**	15.79	2.62	< **0.01**
Germination summer	0.73	0.23	1.61	< **0.01**	4.35	-0.39	0.07	0	-2.37	**0.02**	2.63	-1.37	0.17
Germination autumn	-0.77	< **0.01**	0.76	< **0.01**	43.48	2.64	< **0.01**	2.22	-4.43	< **0.01**	50	-2.21	**0.03**

FAMD.regeneration: Description of the first two dimensions and of the clusters of species identified on them by the HCPC (Hierarchical Clustering on Principal Components) function (Husson et al., 2010). The Pearson correlation coefficient describe the correlation of the quantitative variables with each of the first two dimensions of the FAMD and a t-test describe if the correlation is significant. The R^2 of one way ANOVA was calculated to test the association of each qualitative variable with each of the first two dimensions. The p-value is provided by a Fisher test. Student t- tests check, for each category, if the average of the coordinates along each dimension of the sub groups of individuals defined by that category are significantly different from the average of the coordinates of all the individuals. The association of each variable with the clusters is described by a v test for the quantitative variables and by a chi-square test for the qualitative variables. The representation of each category in the clusters is described by the % of species with that category in the cluster. The frequency of each category in each cluster is then compared with its overall representation and tested with a v test. All test are considered significant if $p < 0.05$ (in bold).

FAMD.regeneration	**Dimension 1**		**Dimension 2**		***Narcissus pseudonarcissus***		***Polygonatum odoratum***		***Carex brizoides***	
Quantitative variables	Pearson corr. coeff.	p-value	Pearson corr. coeff.	p-value	v test	p-value	v test	p-value	v test	p-value
lnplant.height	0.49	< **0.01**	-0.21	0.12	-2.58	**0.01**	-0.50	0.62	2.18	**0.03**
flowering.month	0.72	< **0.01**	-0.25	0.06	-4.40	**0.00**	0.20	0.84	2.84	**0.00**
lnseed.dry.mass	-0.57	< **0.01**	-0.04	0.74	2.43	**0.01**	2.34	**0.02**	-3.65	**0.00**
lnemb.end.ratio	0.57	< **0.01**	0.49	< **0.01**	-1.94	**0.05**	-3.94	**0.00**	4.66	**0.00**

effective.germ.t	0.61	< **0.01**	0.24	0.07	-2.80	**0.01**		-2.70	**0.01**		4.20	**0.00**

Qualitative variables	R2	p-value	R2	p-value	p-value	df		p-value	df		p-value	df
dormancy	0.41	< **0.01**	0.31	< **0.01**	< **0.01**	2		< **0.01**	2		< **0.01**	2
light.requirement	0.55	< **0.01**	0.23	< **0.01**	< **0.01**	4		< **0.01**	4		< **0.01**	4
stratification	0.59	< **0.01**	0.18	**0.05**	< **0.01**	4		< **0.01**	4		< **0.01**	4
germination.season	0.47	< **0.01**	0.61	< **0.01**	< **0.01**	6		< **0.01**	6		< **0.01**	6
embryo.type	*0.19*	*0.07*	*0.25*	**0.02**	< **0.01**	*12*		< **0.01**	*12*		< **0.01**	*12*
ell.l.cat	*0.23*	< **0.01**	*0.02*	*0.83*	*0.12*	*6*		*0.12*	*6*		*0.12*	*6*
ell.f.cat	*0.14*	**0.04**	*0.13*	*0.06*	**0.02**	*6*		**0.02**	*6*		**0.02**	*6*
ell.n.cat	*0.00*	*0.99*	*0.04*	*0.56*	*0.66*	*6*		*0.66*	*6*		*0.66*	*6*
ell.r.cat	*0.05*	*0.47*	*0.01*	*0.88*	*0.65*	*6*		*0.65*	*6*		*0.65*	*6*

Categories	Estimate	p-value	Estimate	p-value	% species	v test	p-value	% species	v test	p-value	% species	v test	p-value
PD	1.59	**0.00**	0.93	**0.00**	50.00	-1.70	0.09	20.00	-4.61	**0.00**	100.00	5.58	**0.00**
MPD	-1.59	**0.00**	-0.93	**0.00**	50.00	1.70	0.09	80.00	4.61	**0.00**	0.00	-5.58	**0.00**
L.>.D	2.23	**0.00**	-0.36	0.86	0.00	-3.27	0.00	30.00	-2.36	**0.02**	82.93	4.39	**0.00**
L.=.D	0.19	**0.01**	-1.04	**0.03**	33.33	0.41	0.68	70.00	3.09	**0.00**	14.63	-2.98	**0.00**
D.>.L	-2.42	**0.00**	1.40	**0.00**	66.67	3.74	**0.00**	0.00	-0.90	0.37	2.44	-2.33	**0.02**
cold	2.59	**0.00**	-0.83	**0.01**	0.00	-4.53	**0.00**	80.00	-0.25	0.80	95.12	3.65	**0.00**
warm	-1.93	**0.00**	-0.29	0.57	50.00	2.72	**0.01**	10.00	0.20	0.84	2.44	-2.33	**0.02**
warm.+.cold	-0.66	**0.00**	1.12	**0.00**	50.00	2.72	**0.01**	10.00	0.20	0.84	2.44	-2.33	**0.02**
germ.winter	-0.70	0.22	-2.91	**0.00**	0.00	-0.48	0.63	30.00	2.42	**0.02**	2.44	-1.83	0.07
germ.spring	1.04	**0.00**	0.17	0.64	0.00	-3.81	**0.00**	70.00	-0.30	0.76	85.37	2.98	**0.00**

germ.summer	2.24	0.11	0.96	0.33	0.00	-0.37	0.71	0.00	-0.59	0.55	7.32	0.91	0.36
germ.autumn	-2.58	**0.00**	1.78	**0.00**	100.00	4.94	**0.00**	0.00	-1.31	0.19	4.88	-2.84	**0.00**
rudimentary	-1.95	**0.02**	-1.58	**0.00**	16.67	0.50	0.62	50.00	3.58	**0.00**	0.00	-3.69	**0.00**
Categories	Estimate	p-value	Estimate	p-value	% species	v test	p-value	% species	v test	p-value	% species	v test	p-value
capitate	0.77	0.11	0.07	0.98	0.00	-1.05	0.30	0.00	-1.56	0.12	24.39	2.23	**0.03**
lateral	0.41	0.38	-0.21	0.48	0.00	-0.96	0.34	10.00	-0.45	0.65	19.51	1.16	0.25
linear	-0.77	0.11	0.27	0.49	66.67	1.84	0.07	40.00	0.74	0.46	21.95	-1.95	0.05
peripheral	0.58	0.40	0.51	0.45	0.00	-0.58	0.56	0.00	-0.90	0.37	12.20	1.34	0.18
spatulate	0.28	0.50	0.88	0.05	16.67	0.13	0.90	0.00	-1.43	0.15	19.51	1.16	0.25
investing	0.67	0.69	0.07	0.99	0.00	-0.13	0.89	0.00	-0.22	0.82	2.44	0.36	0.72
1st.l	0.71	0.56	0.08	0.97	33.33	-0.15	0.88	30.00	-0.45	0.65	39.02	0.51	0.61
2nd.l	-0.69	**0.02**	0.30	0.52	50.00	1.58	0.12	20.00	-0.04	0.97	17.07	-1.11	0.27
3rd.l	1.42	**0.01**	0.03	0.85	0.00	-1.85	0.06	30.00	-0.33	0.74	41.46	1.57	0.12
4th.l	-1.44	**0.04**	-0.41	0.45	16.67	0.81	0.42	20.00	1.43	0.15	2.44	-1.83	0.07
1st.f	-0.22	0.80	-0.13	0.47	33.33	-0.57	0.57	50.00	0.29	0.77	46.34	0.17	0.87
2nd.f	-1.16	**0.03**	0.70	**0.03**	66.67	2.29	**0.02**	10.00	-0.98	0.33	19.51	-0.90	0.37
3rd.f	0.17	0.61	-0.80	0.05	0.00	-0.96	0.34	40.00	1.99	0.05	12.20	-1.10	0.27
4th.f	1.21	**0.02**	0.23	0.59	0.00	-0.96	0.34	0.00	-1.43	0.15	21.95	2.06	**0.04**
1st.n	-0.09	0.77	-0.02	0.74	33.33	0.31	0.76	20.00	-0.56	0.58	29.27	0.29	0.77
2nd.n	0.15	0.70	-0.38	0.18	16.67	-1.31	0.19	50.00	0.41	0.68	46.34	0.57	0.57
3rd.n	0.01	0.97	0.27	0.32	33.33	0.95	0.34	10.00	-0.59	0.56	17.07	-0.17	0.87
4th.n	-0.08	0.88	0.12	0.66	16.67	0.50	0.62	20.00	0.95	0.34	7.32	-1.13	0.26
1st.r	-0.10	0.99	0.07	0.77	50.00	0.39	0.70	30.00	-0.80	0.42	43.90	0.41	0.68
2nd.r	0.71	0.16	0.23	0.57	0.00	-1.05	0.30	10.00	-0.59	0.56	21.95	1.34	0.18
3rd.r	-0.54	0.25	-0.16	0.53	33.33	0.12	0.90	50.00	1.28	0.20	26.83	-1.17	0.24
4th.r	-0.08	0.99	-0.13	0.81	16.67	0.64	0.52	10.00	0.20	0.84	7.32	-0.58	0.56

CHAPTER 3

COMPARATIVE FUNCTIONAL DIVERSITY OF REPRODUCTIVE TRAITS IN OLD AND RECENT TEMPERATE FOREST UNDERSTORIES

ABSTRACT

The poor colonization capacity of herbaceous forest species compromises their responses to land use and climatic changes. As a result, European recent forests on former agricultural land present an impoverished herbaceous understory. Plant functional traits can be used to compare the functional diversity of old vs recent forests, informing successful restoration actions. However, little attention has been paid to the reproductive traits of the herbaceous understory, and how they influence novel community assembly in recent forests. This study took place in two locations at the centre (UK) and southern limit (Spain) of the European Atlantic biogeographical region. In each location, a pair of old and recent temperate deciduous oak forests was sampled. For each of the four forests, understory species abundances were surveyed bimonthly in ten plots during one year. The physical environment of each plot was measured (soil pH, P, N and C content and light availability). Plant reproductive traits were recorded for all the understory herbaceous species present in the plots (two adult plant traits, two reproductive phenology traits, seven seed yield and dispersal traits and 14 seed dormancy and germination related traits); and community-weighted means (CWM) were calculated for each trait and plot. At both locations, old and recent understories presented significant differences in their reproductive traits. Old forest communities had shorter plants that flowered earlier and were more dependent on vegetative reproduction. They produced less seeds, which were heavier and wider, with faster terminal velocity. The germination of these seeds occurred at lower temperatures and was less dependent on light. In general, this variation in functional traits was associated with specific abiotic filters, and more so to soil chemistry than to light availability.

KEYWORDS: Community weighted means, Forest age, Functional traits, Seed germination strategy

INTRODUCTION

Forest cover in Europe is increasing following land use change but, at a local scale, forest fragmentation is intensifying, with more than 35% of forests placed in mosaic landscapes that are fragmented by agricultural and urban lands (Bastrup-Birk et al., 2016; Flinn and Vellend, 2005). Moreover, the herbaceous understory layer of forests that have been established on previously agricultural land (hereafter "recent forests") can be influenced by prior use; and differences in composition and species richness can be expected compared with the understory of woodlands that have not been clear felled in historically recorded times (hereafter "old forests"). In particular, "recent forests" are generally more impoverished in forest specialist species (Dzwonko and Loster, 1989; Peterken and Game, 1984) especially if the recent forest patches are isolated (Bossuyt and Hermy, 2000; Brunet et al., 2011). These differences can be the consequence of the old fields having crossed biotic or abiotic novelty thresholds (Cramer et al., 2008). A biotic threshold has been crossed when forest species are locally extinct and the source of propagules is far enough away to require active intervention, i.e., reintroduction. An abiotic threshold has been crossed when the new environmental conditions of the old field are not suitable for the completion of the life cycle of the species compared to conditions that were present before the conversion to agriculture. In the natural recolonization of old agricultural fields towards forest ecosystems, a restoration intervention may be needed if any of these two types of thresholds have been crossed.

The obstacles to colonization of recent forests by forest specialist species include seed limitation, e.g., if the species lacks the seed dispersal capacity to reach the new habitats. In addition, there may be a recruitment limitation after the new habitat is reached, such that seeds are unable to germinate and to establish a viable population. Key traits that are associated with herbaceous species in temperate woodlands are the production of few, big

seeds that are dispersed prevalently by ants (Hermy et al., 1999), and that do not form long-lived soil seed banks (Bossuyt et al., 2002; Bossuyt and Honnay, 2008). Because of these features, seed limitation may be one of the main reasons for the absence of woodland specialist herbs from "recent forests" (Verheyen and Hermy, 2004). Additional recruitment limitations can relate to woodland specialists tending to have more complex seed dormancy breaking requirements than species from open habitats (Grime et al., 1981; Ten Brink et al., 2013). One other variable regarding forest history is that "recent forests" tend to present a more homogeneous physical environment with less differentiation of micro-niches (Flinn and Marks, 2007). Changes in the abiotic environment following forest clear cutting and agricultural use can also affect recruitment. Even after the return to forest conditions (natural secondary forest or reforestation), these differences remain and can be a problem for highly specialized species. For example, in "recent forests" that have established on former agricultural land, pH and nutrients (P and Ca) levels tend to be higher while carbon and total nitrogen content is lower (Beaten et al., 2011; Brunet et al, 2011; Verheyen et al., 1999). Light availability and quality can also be different between recent and old forests, but this is a difference that is more likely to lessen once the canopy closes (Flinn and Marks, 2007).

Aerts and Honnay (2011) highlight the importance to focus on tree functional diversity when planning forest restoration, rather than have an approach based only on the increase of species richness. Functional diversity measures the role of organisms in a community and is more informative than species presence-absence data (Mouillot et al., 2011). A functional approach can also be beneficial for the restoration of the herbaceous layer. To this end, the relationship between understory functional traits and their physical environment needs to be investigated. Species life-history traits have been used to compare the understory communities of forests of different age (Kelemen et al 2014;

Patrick et al., 2008; Verheyen et al., 2003) and to demonstrate the effect of forest age, area and isolation on the functional types (Patrick et al., 2008). Most of the traits that have been used are related to the adult plant ecology (life form, vegetative traits). The seed traits that are more often included relate to dispersal and colonizing capacity (seed size, seed dry mass, number of seeds produced per plant, seed longevity in the soil seed bank) rather than to the regeneration niche (Grubb 1977). Information on germination traits (dormancy breaking treatment, response to light, germination phenology, germination temperature) is clearly important when considering the assembly of plant communities. Quantification of the germination response provides a framework to compare species niche competitiveness, including under climate change scenarios (Seal et al., 2017). Importantly, it has also been demonstrated that the type of information carried by germination traits is independent from the information provided by vegetative traits (Hoyle et al., 2015). However, to our knowledge only Verheyen et al. (2003) included in his study dormancy breaking requirements; and Ten Brink et al. (2013) used experimental germination data to compare congeneric species from woodlands and open habitats.

Therefore, the aim of this study was to investigate how functional reproductive traits vary between forest understories of different age; and how the abiotic environment drives this variation. The study was set in the European Atlantic region. Two locations with a similar history of land use were chosen, in southern England and Northern Spain. In each location, a pair of forests sites of different age were investigated. In each forest site, ten plots were surveyed to measure plant reproductive traits as Community Weighted Means (CWMs). CWMs are a functional trait measure that permits to weight the value of a trait for its abundance in a given plot (Lavorel et al., 2008). Therefore it is not species identity but their ecological niches that are compared with the CWM approach. Communities that

are relatively different in their specie pools can be compared in this way. In this study system, two hypotheses were tested:

1) CWMs will differ between old and recent forest sites
2) CWMs can be predicted by the environmental filters of each forest type

MATERIALS AND METHODS

Study area

The European Atlantic region represents 18% of the territory of the European Union but is one of the most populated and intensely managed areas. Only 13% of the territory of this region is covered by forests and they are at risk from fragmentation and urbanization (Condé et al., 2002). The study was set in two locations representing the central and the southern portions on the European Atlantic biogeographic region: 1) the Loder Valley Nature Reserve, West Sussex, UK (51°03′N, 00°05′W); and 2) the Tragamón Oak Grove, Jardín Botánico Atlántico, Asturias, Spain (43º 30' N, 05º 31' W) (Fig. 1).

These two locations represent, respectively, the core and the southern edge of the Atlantic biogeographical region in Europe. The potential vegetation in both locations would be mixed deciduous forests dominated by *Quercus robur* and *Fraxinus excelsior* (EUNIS code G1.A1: *Quercus* - *Fraxinus* - *Carpinus betulus* woodland on eutrophic and mesotrophic soils). Nonetheless, at present two distinct forest types may be identified in both locations: an old forest, not clear-felled for more than 100 years; and a recent forest, younger than 25 years. Both old forests had had a history of coppicing that stopped in 1987 at the English location (UK.O) and at the beginning of the 21st century at the Spanish location (SP.O) (Rozas, 2005).

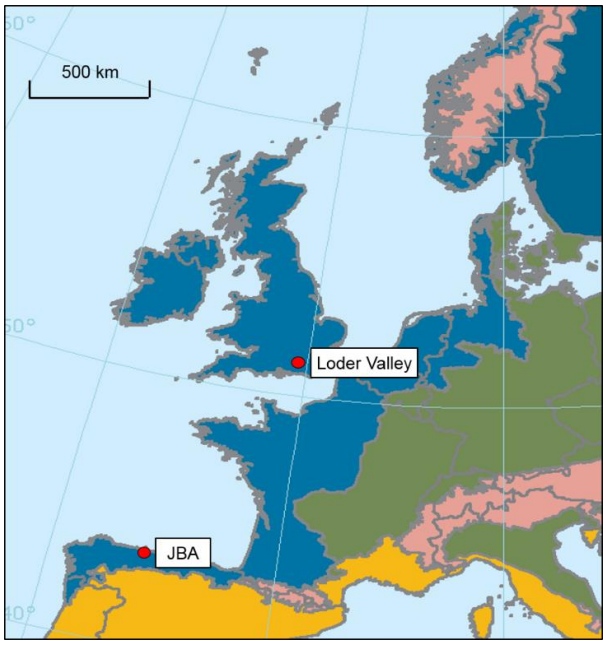

Fig.1: Atlantic European region (image modified from https://www.eea.europa.eu/data-andmaps/figures/biogeographical-regions-in-europe-2). The two study locations are indicated by the red dots.

The present management applied to both is of no intervention and natural dynamics are allowed to develop. Both in the UK and Spain, the recent forests have been planted with broadleaved native trees that occur also in the nearby old forest. The recent forest in the UK (UK.Y) was planted in a site where a former conifer plantation had been felled but with a history of agricultural use dating back to the beginning of the 19th century; whilst the one in Spain (SP.Y) had been planted on the site of former agricultural land. The ancient and restored woodlands are separated by less than 1 km in both locations.

Bimonthly vegetation sampling

Ten permanent quadrats of 25 m^2 were established within each forest type in September (UK) and October 2015 (Spain) (Fig. 2). Quadrats in the UK were regularly arranged on

a rectangular grid of 50 x 40 m. In Spain, it was not possible to maintain the regular grid because the study areas were irregularly shaped and crossed by footpaths. The grid design was therefore arranged to suit the topography of the place and this brought some plots to have a distance of less than 15 m from their centre. The vegetation of each quadrat was sampled every two months from October 2015 to August 2016. In each sampling date, flowering plants were identified and their abundance recorded using a percentage cover scale adapted from the GLORIA Field Manual (Pauli at al., 2015): 0.125%, 0.25%, 0.5%, 1%, 2%, 5%, 10 % and 10% increases above that. The flowering and fruiting phenology of the understory plants (excluding ferns, vines, shrubs and trees) was also recorded. Additionally, the total cover of the canopy, mosses, ferns, litter, dead wood, rocks and bare soil was recorded applying the same scale as above. Nomenclature was standardized against The Plant List (http://www.theplantlist.org, accessed on 30th June 2016).

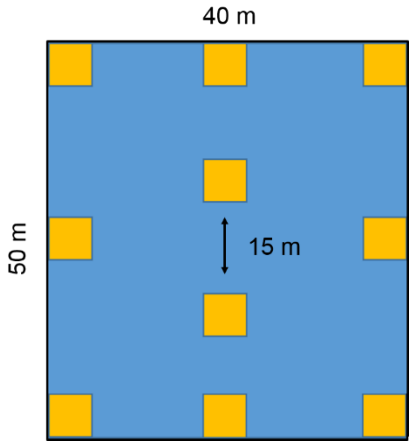

Fig. 2: Outline of sampling design with plot disposition in the study areas.

Abiotic parameters

Soil

Soil samples were collected from each plot in the winter of 2016 to measure soil water content, pH, available phosphorous, mineral nitrogen and carbon content. Five soil cores were taken, from the four corners and from the centre of each plot, using a 5-cm diameter

corer. Before taking the soil samples the leaf litter and the first cm of soil were removed. Each core was taken to a depth of 10 cm. The five soil cores of each plot were pooled in the same plastic bag and mixed, in order to have a sample representative of the whole plot. The bags were then sealed to avoid water loss and the samples were transported within a week to the laboratory of the Royal Botanic Gardens Kew, Wakehurst Place, UK for processing and measurement of pH and initial water content. Each sample was passed through a 3 mm sieve and weighed. In order to estimate the initial water content of the soil samples, a subsample was weighed at 0.0000 mg precision, placed in oven at 107 °C for 17 h and then weighed again. The percentage decrease in weight was equated to its initial water content. To measure the pH, from each sieved sample, three pseudo-replicates of 12.5 g were placed in 25 ml glass flasks and dissolved in 20 ml deionized water. The vials were shaken for 2 h and then left to rest for 10 min. The pH of the watery solution was measured using a pH meter (Jenway Model 3505, Fisher Scientific UK Ltd, Loughborough, UK). This protocol was adapted from Van Reeuwijk (2002).

The samples were then left to dry in a controlled humidity room at a temperature of 15 °C until reaching 40% RH before being transported to the James Hutton Institute (Scotland) for elemental analysis and available phosphorous measurements. There, the samples were dried in an oven at 60 °C for 48 h and their moisture content was measured. For each sample three pseudo-replicates were prepared and used for all the analyses. In order to account for changes in the pH of the distilled water during the processing of the samples, the pH measurements were repeated at the JHI using, instead of water, a buffer solution 0.01 M $CaCl_2$. Available P was measured with the Olsen method (Olsen et al., 1954). A 0.5 M solution of $NaHCO_3$ in distilled water was prepared and 2 ml of this solution was placed in plastic tubes together with 2 mg of oven-dried soil. The tubes were then shaken for 1 h using roller tubes to mix well the soil with the bicarbonate solution

and then centrifugated for 5 min at 4500 rpm in order to stabilize the compound. From the watery suspension 2 ml aliquots were taken and placed in Eppendorf tubes. For each pseudoreplicate, 15 µg were placed in the wells of a 96 well plate together with 185 µg of distilled water and 100 µg of Malachite Green solution. Malachite Green is acidic and reacts with the P in solution. A calibration curve was produced based on a line of wells was filled with solutions of known P content. In addition, two samples of a 'standard' soil (i.e., of known chemical composition) and three samples of $NaHCO_3$ aqueous solution were added to the plate to have a further reference. The plates were left to rest for 1 h before being placed in a scanner (Multiskan GO, Therno Scientific) and the amount of available P was measured colorometrically. The soil originating from the Spanish location was too dark to be processed with this method because of its high content of organic matter. The analysis was therefore repeated after flocculating the organic matter with the addition of 1.2 M H_2SO_4 and centrifugating the samples again. The proportion of the solution to use was then adjusted taking in account the amount of H_2SO_4 added. The concentration of P in the plate extracts was interpolated, for each value, using the equation of the linear regression of the P concentration over absorbance of the calibration curve. The concentration of P in the sample was then calculated from the concentration of the P in the plate extract taking in account the dilution of the extract and the initial moisture content of the sample. Elemental Analysis (EA) permits the determination of the content of C, N and H in organic or inorganic compounds. The sample is combusted in pure oxygen under static conditions and the electrical conductivity value of the elements is subsequently measured. The samples were ball milled for 4 min at a radial oscillation frequency of 23 s^{-1}, using a grinding mill (Retsch MM200, Retsch GmbH, Dusseldorf, Germany), and placed in tin containers. The EA was performed at using a CE440™ Elemental Analyzer according to the manufacturer's recommendations (Exeter

Analytical, Coventry, UK). From this analysis the percentage of C and N was obtained and the ratio between them calculated.

Light measurements

Light availability (photosynthetic active radiation, PAR) and the ratio between red and far red radiation (R/FR ratio) was measured twice, around the summer and winter solstices of 2016, using a photometer (PAR Quantum Sensor for PAR and 660/730 nm Sensor for R/FR, Skye Instruments, Powys, UK). Light measures for all the quadrats within each location were taken on a same day with a homogeneous sky cover, between 12:00 a.m. and 14:00 p.m. In each quadrat, ten measurements were taken and averaged. These were based on five measuring points, at the four corners and the centre of each plot, at both ground level and 1 m above the ground. For comparison, light was also measured at the same time in a nearby open area, at ground level and 1 m above ground. Finally, the maximum (winter) and minimum (summer) light availability of each quadrat was expressed as the percentage decrease in the quadrat as compared to the open area.

Temperature

Two dataloggers measuring temperature were placed at the centre of each study location (Tinytag View 2, Gemini Dataloggers Ltd., Chichester. UK). The first was fixed on a pole 10 cm above the soil. The second datalogger was sealed in an aluminium bag to avoid damage and buried at 5 cm of depth. The dataloggers were programmed to record every 30 minutes and left in the field for the duration of the experiment. Monthly average, minimum and maximum temperatures were calculated for each location.

Plant functional traits

For each understory species, a matrix of traits was collected, including traits that describe the ecology of the adult plant and traits that refer to its regeneration strategy, with a focus on dispersal, reproductive phenology and germination (Table 1).

Literature/database traits

The relative importance of vegetative vs reproduction by seed was obtained from Grime et al. (2007). The following functional traits were obtained from the TRY database (www.try-db.org, Kattge et al., 2011): specific leaf area (SLA), plant height, seed dry mass, seed number per plant, average seed production per plant, seed terminal velocity and seed persistence in the soil (Bond-Lamberty et al., 2002; Cornelissen et al., 2003; Dainese and Bragazza, 2012; Everwand et al., 2014; Fitter and Peat, 1994; Fry et al., 2014; Green, 2009; Hill et al., 2004; Kattge et al., 2009; Klimesova and De Bello, 2009; Kleyer et al., 2008; Kühn et al., 2004; Medlyn et al., 1999; Moretti and Legg, 2009; Milla and Reich, 2011; Ordonez et al., 2010; Paula et al., 2009; Prentice et al., 2011; Price and Enquist, 2007; Royal Botanic Gardens, Kew, 2011; Sandel et al., 2011; Scherer-Lorenzen et al., 2007; Schweingruber and Landolt, 2005; Spasojevic and Suding, 2012; Van Bodegom et al., 2008; Vergutz et al., 2012; Vile, 2005; Wirth and Lichstein, 2009, Wright and Sutton-Grier, 2012). Seed dormancy type, stratification and light requirements for germination and effective germination temperature were gathered from a review of the literature (see Chapter 2), including a compendium of germination information (Baskin and Baskin, 2014). Month of flowering and fruiting were assigned based on the vegetation sampling, or obtained from the ECOFLORA database (http://ecoflora.org.uk/, Fitter and Peat, 1994).

Table 1: Traits collected for the understory herbaceous species sampled in the experimental plots. G = germination, C = cotyledon emergence. Categorical traits were transformed in binary for the statistical analysis, and the presence/absence of each category level was used to calculate the CWM.

Trait	Measure unit	Life stage	Source
SLA	mm^2/mg	Adult	www.try-db.org
Plant height	m	Adult	www.try-db.org
Seed dry mass	mg	Regeneration	www.try-db.org
Seed terminal velocity	m/s	Regeneration	www.try-db.org
Seed persistence in the	years	Regeneration	www.try-db.org
Seed number per plant	seed number	Regeneration	www.try-db.org
Flowering month	month	Regeneration	Field survey
Fruiting month	month	Regeneration	Field survey
Seed length	mm	Regeneration	Laboratory
Seed width	mm	Regeneration	Laboratory
Vegetative reproduction	category	Regeneration	Grime et al, 2007
Embryo endosperm	mm^2/mm^2	Regeneration	Laboratory
Dormancy type	category	Regeneration	Literature review
Stratification	category	Regeneration	Literature review
Light requirement	category	Regeneration	Literature review
Germination	° C	Regeneration	Literature review
T_{50} G	days	Regeneration	Germination
T_{50} C	days	Regeneration	Germination
EHS T_{50} G	° C /day	Regeneration	Germination
EHS T_{50} C	° C /day	Regeneration	Germination
% not viable seeds	proportion	Regeneration	Germination
Final germination	proportion	Regeneration	Germination
Final cotyledon	proportion	Regeneration	Germination
Lag between G and C	days	Regeneration	Germination
Germination strategy	category	Regeneration	Germination

Seed sampling and morphological measures

Seeds were sampled within each forest location to measure additional traits related to regeneration strategies. Sampled individuals were selected from outside the sampling areas, but as close to these as possible. Seed length and width were measured on samples of 25 seeds from each collection. Seeds were photographed and the image was analysed with the software ImageJ 1.45s (Wayne Rasband, USA). For "length" the largest dimension of the seed was measured and for "width" the shortest, taking into account the fact that the seeds may have been flat in shape. The relative embryo size was measured as the ratio between embryo and endosperm area (E:E) in 10 randomly selected seeds from each collection. The seeds were imbibed on 1% agar-water for 24 h. Thereafter, they were cut longitudinally and photographs taken of the internal seed structure, using a camera (Carl Zeiss Axiocam Colour) mounted on a Stemi SV 11 Microscope (Carl Zeiss, Welwin Garden City, Herts, UK) microscope. Embryo and internal seed areas were measured using the software Axiovision 3.1.2.1 (Carl Zeiss Vision GmbH) and the ratio between them calculated.

Germination experiment

A move-along germination experiment was conducted to provide data on the germination timing under simulated natural conditions. Separate experiments were conducted for species collected in both locations (England and Spain), and, if a same species was collected in the two countries, they were considered as different populations. For each population, four 8 cm diameter Petri dishes containing 1% agar and 25 seeds each were prepared. Seeds were sown fresh, within two weeks from collection. Dishes were placed in one of two incubators (LMS Cooled incubators, LMS Ltd, Sevenoaks, UK), one for each geographical location. Incubators were set to replicate the seasonal average maximum and minimum temperatures and photoperiod in the two locations. The

temperature cycle of the incubators was based on the temperature means of 30 years provided by the Norwegian Meteorological Institute and Norwegian Broadcasting Corporation (Crawley, England, http://www.yr.no) and the Agencia Estatal de Meteorología (Gijon station, Spain, http://www.aemes.es) for the English and Spanish locations respectively. During the experiments, the incubator settings changed according to the seasonal and daily fluctuaction in temperature and photoperiods to replicate naturally occurring changes in temperature and light. Artificial "seasons" were settled on the basis of the meteorological data available (Table 2). Higher daily temperatures corresponded to the hours of light. Experiments in conditions of absolute dark were not performed as none of the taxa tested has been reported to be sensitive to photoinhibition (Carta et al., 2017). Species were entered in the experiment at different times, depending on the season of eed dispersal. The first germination tests started in June 2016 with temperatures corresponding to "late spring"/"early autumn" and finished in July 2017 with temperatures corresponding to "summer" (Table 2). Only one species, *Hypericum perforatum*, collected in October 2015, was entered in the experiment in autumn 2015, during a pilot experiment testing the move along regime for shrubs and vines (data not shown). The duration of each season is reported in Table 2. Seed germination, defined as 2 mm radicle emergence, and cotyledon emergence from the seed coat were scored every week, and the germinated seedlings were removed. After 48 weeks from the date of sowing (336 days), the experiments were stopped and the non-germinated seeds were cut open and classified as apparently normal, empty or infected. Only viable seeds were taken into account when calculating germination proportions. The following traits were finally collected from the germination experiments: 1) time to reach 50% germination ("$T_{50}G$", expressed in days) and cotyledon emergence ("$T_{50}S$", expressed in days); 2) environmental heat accumulated above 0C ("EHS", environmental heat sum, expressed

in degree days "d°C") to $T_{50}G$ and $T_{50}S$; 3) difference in time between $T_{50}S$ and $T_{50}G$; 4) percentage of not viable seeds; and 5) final percentages of germination and cotyledon

Table 2: Artificial "seasons" of the move along experiment. Temperatures for the two locations are reported in ˚C. The higher temperature phase corresponded to the hours of light.

Season	Year	Temperature UK	Temperature SP	Light	Weeks
Late spring	2016	16/6	18/11	12/12	4*
Early summer	2016	18/8	21/14	16/8	6
Summer	2016	22/11	22/16	14/10	4
Late summer	2016	18/8	21/14	16/8	4
Early autumn	2016	16/6	18/11	12/12	4
Late autumn	2016	10/2	15.5/7.5	10/14	6
Winter	2016/17	7/0	14/5	8/16	8
Early spring	2017	10/2	15.5/7.5	10/14	10
Late spring	2017	16/6	18/11	12/12	4
Late summer	2017	18/8	21/14	16/8	6
Summer	2017	22/11	22/16	14/10	4**

Notes: * species collected in June 2016 only received 2 weeks at "late spring" conditions. ** the last species added, only received 3 weeks at "summer" condition in 2017 because the 48 weeks of the experiment were completed by then.

emergence. The EHS was calculated as the daily sum of the average temperature experienced by each species from the date of sowing. For the species that did not reach 50% of germination or cotyledon emergence by the end of the 48 weeks a T_{50} of 336 d and its corresponding EHS was assumed. $T_{50}G$ and $T_{50}S$ were estimated visually. In order to use a single value for each species in the subsequent statistical analysis, the germination traits obtained experimentally were averaged between populations for 10 species that

were collected in both sites. Germination patterns were assessed visually, according to the shape of the germination curve and treated as binary variables.

Statistical analyses

The vegetation plots were ordered according to the species composition of different layers using non-metric multidimensional scaling (NMDS, Fig. 3). A correlation matrix (Annex I) was calculated with the environmental parameters using the Pearson Correlation Coefficient. Traits that were highly correlated (Pearson > 0.7) were not included in subsequent analysis. A PCA was then performed to describe the abiotic environment of the plots surveyed (Fig.4). A data matrix with 31 adult plant and regeneration traits was produced. For each of the traits described above, community weighted means (CWM) were calculated for each quadrat using the package function "functcompt" in the package "FD" for R 3.4 (Lalibertè et al., 2014). CWMs weight the trait value of each species by its abundance in a quadrat, and represent the dominant trait value in the community. Qualitative traits (as preferred regeneration strategy, dormancy type, and stratification and light requirement for germination) were transformed to binary values giving to each species a value of "1" or "0" for each category depending if they possessed it or not. CWM were initially calculated for each combination plot*visit and then averaged between all the visits. The CWMs of each trait were analysed with the Shapiro-Wilk and Levene tests, to check for normality and homoscedasticity, respectively. The traits that did not comply with either were log- or sqr- transformed. The traits that complied initially or after transformation were analysed with a 2-way factorial ANOVA, to investigate the effect of forest age, country and the interaction between these two factors. ANOVA models were simplified by stepwise backward selection. The effect of forest age and location was investigated through Generalised Linear Models (binomial, logit) followed by a stepwise backward selection for the traits that came from a binomial distribution.

When an interaction term resulted in significance the Tukey post hoc test was applied to test differences between the four treatment combinations (age * country) (Tukey, 1949). The Kruskal-Wallis test was used to investigate the effect of age and location factors on the following environmental parameters: µg C, N and P per gram of soil, soil pH, PAR and R/FR ratio in summer and in winter (average of the measurement taken at 0 and 1 m) because they did not met the assumptions of normality and homoscedasticity to be analysed with ANOVA. When an interaction term resulted in significance the post hoc Dunn test was applied (Dunn, 1964). To evaluate the effect of each environmental parameter, including forest age and location, on the CWM of each functional trait, linear models for the normally distributed traits and GLM for the ones with binomial distribution were calculated. A correlation matrix was calculated on the CWM using the Pearson coefficient with a threshold at 0.7 (Annex I). Finally, two separated PCAs, for England and Spain, were calculated using the abiotic parameters and the CWM that 1) were significantly different between the four study sites; 2) were not correlated (Fig.8).

RESULTS

Species survey

In total, 111 species were surveyed between the different forest layers. In UK 43 species of herbs, two species of vines, 14 trees and one shrub were recorded. In Spain there were 47 species of herbs, four vines, 24 trees and six shrubs.

Understory

Of the 75 herb species 28 were exclusive to the Loder Valley Nature Reserve, 32 were exclusive of the Tragamón Oak Grove and 15 were present in both sites (*Ajuga reptans, Brachypodium sylvaticum, Carex divulsa, Carex pendula, Carex sylvatica, Circaea*

lutetiana, Euphorbia amygdaloides, Geranium robertianum, Glechoma hederacea, Potentilla sterilis, Teucrium scorodonia, Urtica dioica, Veronica chamaedrys, Veronica montana and *Viola riviniana*). SP.O was the site in which the lower number of species was recorded (18) and with the lower average abundances (only four species had an average abundance between seasons ≥ 0.1). It shared ten of its species with SP.Y, which had the highest species richness (39) of all the studied sites. UK.O and UK.Y had respectively 27 and 26 species and shared ten species.

Four species could be identified only to the genera level: *Rumex sp*. and *Sonchus sp*. in England and *Taraxacum sp*. and *Crocus sp*. in Spain. These species, that only appeared in some of the surveys and whose abundance was very low, were not included in the calculation of the CWM because it was not possible to collect trait values for them. *Hyacinthoides non-scripta*, present only in the Loder Valley Nature Reserve, was extremely abundant in both the young and the old forest and its abundance masked any other difference in the CWM calculated between the two sites. To highlight these differences, *H. non-scripta* was removed from the analysis. Therefore, the CWM were calculated using 70 species.

Canopy, shrubs and vines

The canopy cover varied from 10% measured in December in UK.O to 95%, measured in October in the SP.O. The minimum canopy cover in Spain was 40% reached in December in plots of both woodlands and the maximum canopy cover in UK was 90%, recorded in summer (June and August) plots of both forests. In both locations, the principal canopy forming species was *Quercus robur,* followed, in Spain, by the evergreen *Laurus nobilis*. The average canopy cover in UK was 50 % (± 23.5 SD) and in Spain was 76% (±14 SD). The difference between minimum and maximum canopy cover is more prominent in England, because of the lack of dominant evergreen canopy forming species.

In England the shrub layer was constituted mainly by the fern *Pteridium aquilinum* and *Rubus sp.* while more species (*Daboecia cantabrica, Ligustrum vulgaris, Prunus spinosa*, *Rosa sempervirens, Ruscus aculeatus*) concurred to its composition in the Spanish forests where *P. aquilinum* and *Rubus sp*. were anyway the most abundant species. It was not possible to taxonomically identify the *Rubus ssp.* in the two study locations so they were referred as the same entity at genus level. The abundance of *Pteridium aquilinum* and *Rubus sp.* varied seasonally and was greater in areas with lower canopy cover. *Hedera helix* and *Lonicera peryclimenum* were the only vines present in the English sites while in Spain, in addition to these species, also *Rubia peregrina* and *Tamus communis* were recorded. *Hedera helix* constituted the main cover species at ground level of the SP.O plots that were poorer in species.

Other cover parameters

Leaf litter covered up to 90% of the plot surface in the winter season, in plots from both English forests and from SP.O. This site maintained a high amount of litter also in summer. The minimum leaf litter cover (1-2%) was recorded in the UK.O during spring (April) when the abundance cover of the herbaceous *Hyacinthoides non-scripta* was so intense as to mask the litter. The cover of mosses varied between 0 and 20% while dead wood was recorded between 0% in many young woodland plots of the Spanish location to 60% in the plot number three of the UK.O, were a fallen tree lay across the plot. Presence of surface rocks and bare soil patches was negligible and was considered no further in the assessments.

NMDS ordination of the vegetation survey

Non metric multidimensional scaling (NMDS) was used to represent the difference in cover type between the four forest communities investigated (Fig.3). The

multidimensional space described by the species abundances for the tree layer revealed a predominance of SP.Y that, being the species richest site, included most of the space described by SP.O. The two English locations did not overlap between forest type and appear distinct from the Spanish ones. The space that described the composition and species abundance for the shrub layer is neatly separated between the two countries but not between forest types. A NMDS that summarised the relative abundance of the herbaceous species of the understory showed separation between location and a consistent overlapping between the two Spanish sites. On the opposite, other cover parameters measured during the field surveys (canopy, *Rubus sp*., ferns, mosses, litter, dead wood, rocks and bare soil) still showed separation between countries and an overlap between forest types.

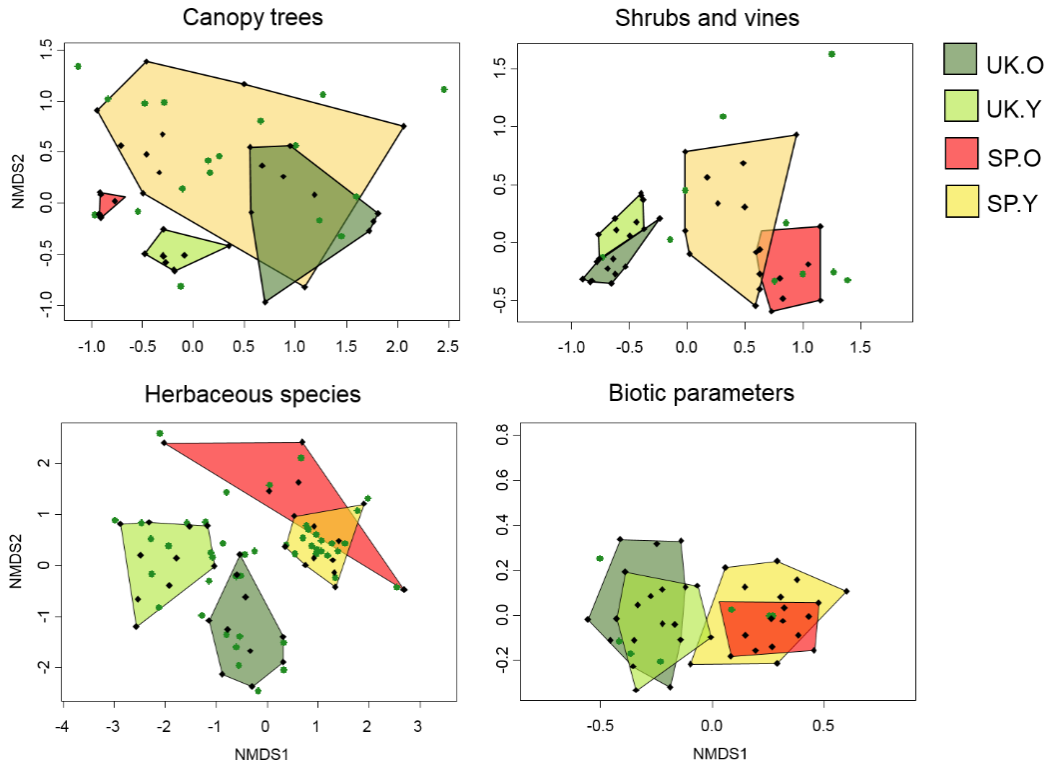

Fig.3: Non metric multidimensional scaling (NMDS) of the biotic components between all the forest types.

Abiotic factors

Soil properties

The average pH of the soil was 5.0 and it ranged from 4.1 to a maximum value of 7.4. The interaction between forest age and country was significant and a post hoc test demonstrated that the pH of SP.Y was significantly higher than all the other areas. Overall, the sites in Spain had an average pH higher than the English ones.

The quantity of available P varied between 0.31 to 42.88 μg/g with an average value of 13 μg/g and it was significantly different between the four forests. A post hoc test confirmed a significant difference in the P levels between the UK.O and UK.Y and between them and both Spanish forests. The English location had a significantly higher amount of P in the soil compared with the Spanish and UK.Y had more P than UK.O. In contrast, both SP.O and SP.Y forests were not significantly different in the amount of available P in their soils.

The total content of C ranged from 11501 to 134826 μg/g with only two plots exceeding a value of 100000 μg/g in the SP.O. The average value was 32053 μg/g. The interaction between age and country was significant and a post hoc test revealed that the average concentration of C in the soil of the SP.O was significantly higher than all the other sites.

The amount of N per gram of soil varied between 888 and 12500 μg/g with an average value of 2704 μg/g. This variable describes the total amount of atomic nitrogen rather than the amount of mineral nitrogen species available for plants. Forests differed for N content in the soil both with respect to their age (older forest had more N) and to the country of provenance (Spanish sites had more N than the English) and the interaction between these two factors was significant being the N content of SP.O significantly higher than the other sites.

Light

The PAR measured in summer varied between 1.2 and 5.8. PAR was, on average, significantly higher in England than in Spain. A post hoc test showed that the old forest in Spain had the significantly lowest values of PAR in summer but there were no differences between forests of different ages in the two countries. In winter, the PAR varied between 1.2 and 11, with an average value of 5.1. The interaction between age and country was significant and the post hoc test separated UK.O and SP.Y as these had higher levels of PAR than the other sites. Within the four forests, the average decrease in PAR in comparison to an open area was of 98% in summer and 75% in winter.

The R/FR ratio ranged in summer between 0.11 and 0.58 with an average value of 0.35. The interaction between age and location was significant and a post hoc test demonstrated significant differences in R/FR ratio between the two locations with forests in England having, on average, lower values than the Spanish. There was a significant similarity between the two young forests. The average decrease in R/FR ratio compared to the open was of 85% both in England than in Spain. Finally, the R/FR measured in winter ranged between 0.4 and 1.05 with an average value of 0.87. The interaction between country and age was significant and a post hoc test showed significant differences between the two Spanish forests and similarities between the two English ones. SP.O had the highest filtration of light during winter while the UK.O had the lowest. The two young forests were not significantly different in R/FR. The results described are summarized in Fig. 4 and Table 3.

A correlation matrix (Annex I) calculated between the above described traits showed a strong positive correlation between soil C and N (Pearson = +0.96) and a negative correlation between available P and pH (Pearson = -0.75). Also the R/FR ratio in winter was positively correlated with available P (Pearson = +0.69) and negatively correlated

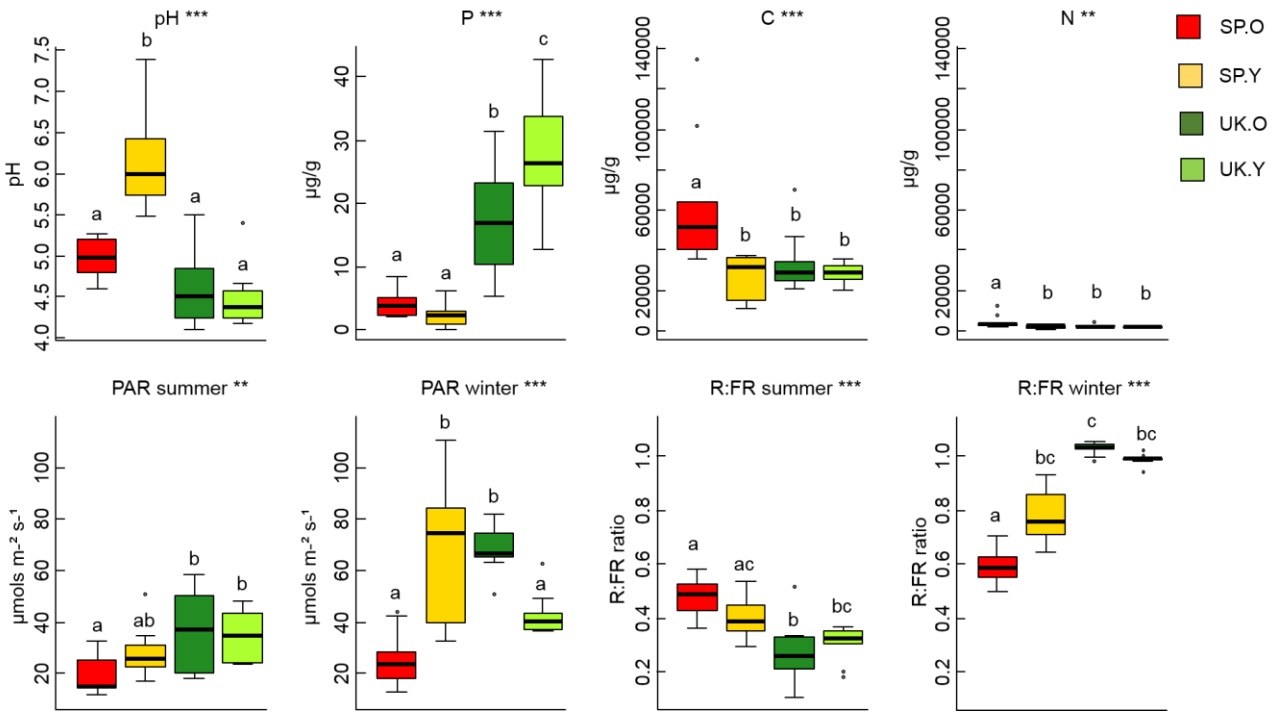

Fig. 4: Comparison of the four forests according to soil and light environment. Different letters indicate significant difference between sites. Asterisks indicate the significance of the interaction between forest age and location. . *** = $p < 0.0001$; ** = $p < 0.01$; * = $p < 0.5$.

with R/FR in the summer (Pearson = -0.65). Therefore the following variables were removed from further analysis to avoid autocorrelation: C in the soil, pH and R/FR in winter. A PCA was run with the remaining variables (Fig 5). The first axis explained 42% of the variability in the data and all the variables were significantly correlated with it (Annex I). It separated the English from the Spanish plots, ordering from left to right, plots with high N and low P content in the soil, low PAR and high R/FR. The second axis explained 23% of the variability and separated plots with more open canopy and lower P and N in the upper part of the biplot from pots with more closed canopy and higher P and N content.

Table 3: Environmental parameters (average ± SE) and effect of forest age, location and their interaction (Kruskal-Wallis test):

	UK.O	UK.Y	SP.O	SP.Y	Age		Location		Age	
					χ	p	χ^2	p	χ^2	p
Soil										
pH	4.6 ±	4.5 ±	4.9 ±	6.2 ±	1	0.2	19	<	26	<
C (µg/g)	33846	28667±	62753±	26834	8	**0.0**	5.	**0.01**	18	<
N (µg/g)	2293 ±	1885 ±	4486.5	2152 ±	5	**0.0**	6.	**0.00**	13	**0.00**
P (µg/g)	17.6 ±	27.9 ±	4.2 ±	2.4 ±	0	0.9	28	<	30	<
Light										
PAR sum	37.3 ±	34.8 ±	18.9 ±	28.3 ±	2	0.1	10	**0.00**	13	**0.00**
PAR win	68.3 ±	42.8 ±	25.9 ±	68.4 ±	0	0.3	2.	0.12	23	<
R:FR sum	0.3 ±	0.3 ±	0.5 ±	0.4 ±	0	0.6	19	<	22	<
R:FR win	1.0 ±	1.0 ±	0.6 ±	0.8 ±	0	0.8	29	<	35	<
Temperature										
Air ˚C	11.4	11.7	14.0	15.0						
Ground ˚C	10.8	10.9	13.8	13.8						

According to the physical environment illustrated by the parameters here described, the forests of the two countries appear to be different. If only the Spanish sites are considered, a clear difference between young and old forest can be observed while the two English forests appear to be quite similar. The situation with more open canopy corresponds to the plots of SP.Y, which is also the one with the highest number of herbaceous species. In comparison, the higher level of light filtration imposed by the canopy in summer can be found in UK.Y. The two recent forests therefore appear to have more extreme environmental differences between them than the two old ones.

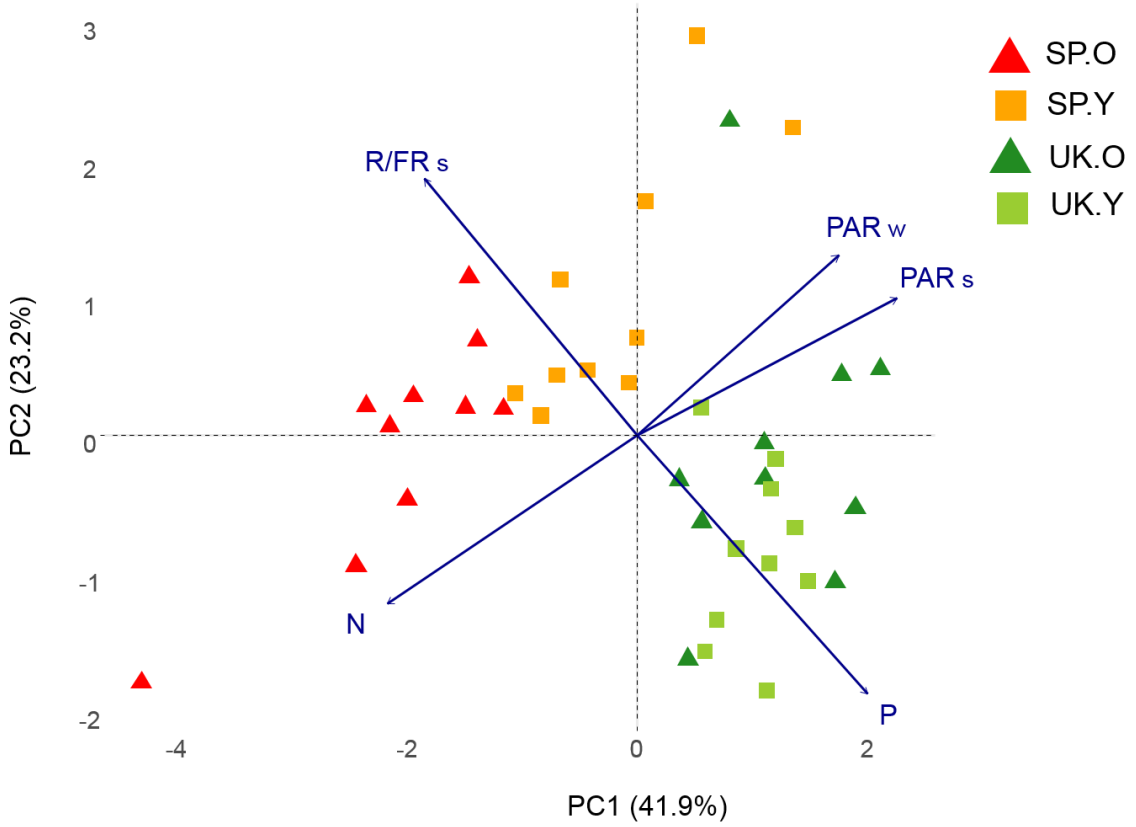

Fig. 5: Principal Component Analysis of the environmental parameters with lower autocorrelation. R/FR s = red/far red ratio in summer, PAR w = Photosynthetic Active Radiation in winter, PAR s = Photosynthetic Active Radiation in summer, P = available phosphorous, N = nitrogen content in soil, SP.O = old forest in Spain, SP.Y = recent forest in Spain, UK.O = old forest in England, UK.Y = recent forest in England.

Temperature

The average air temperature for the year was c.a. 4 °C higher in Spain than in England (Fig. 6).

The maximum temperature recorded varied by about the same amount, ranging from 29.8°C in UK.Y to 34.5 °C in SP.Y. The minimum air temperature also varied by about

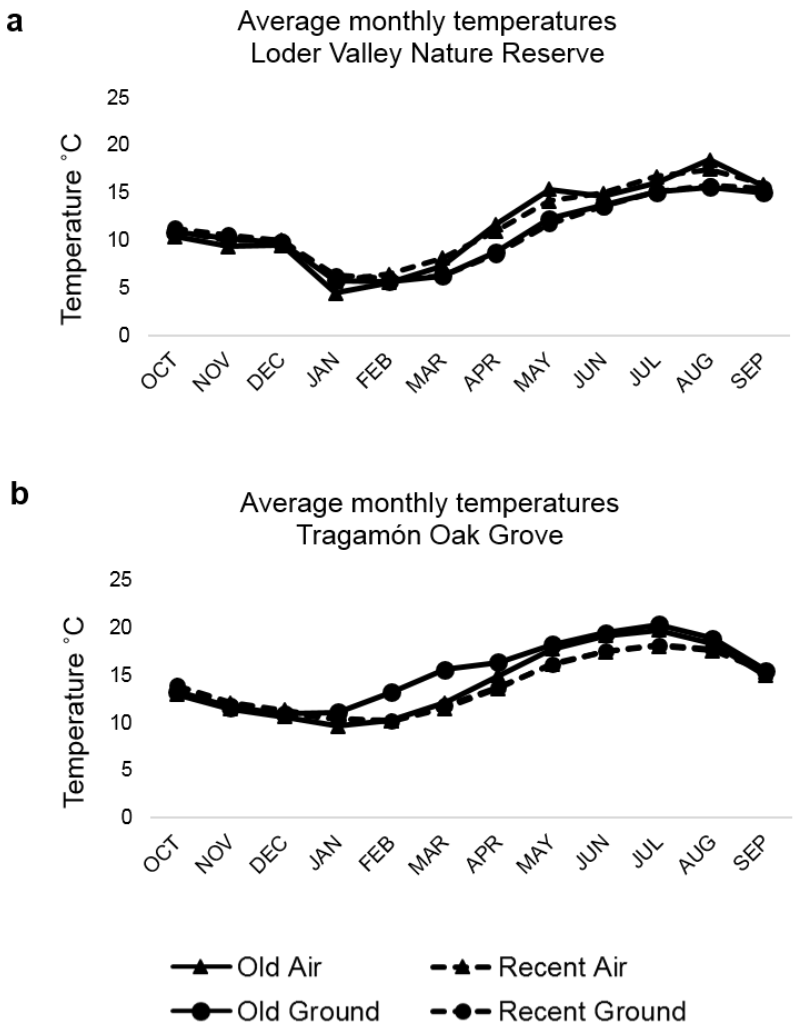

Fig. 6: Average monthly temperatures recorded from October 2015 to September 2016 in the two location. "Air" temperature were recorder 10 cm above the ground and "Ground" temperatures were recorded 5 cm below the ground.

4 to 5°C, from -0.2 °C recorded in the SP.O to -5.5 °C in UK.O. There was no great difference between forests of different ages in the same location regarding the average, minimum and maximum air temperature. However, the average daily temperature fluctuation differed such that it was more pronounced in the young forests than in the old ones; especially in SP.Y. The daily fluctuation in the ground / soil was smaller, being on average of 1.4 °C with little difference between the sites. The average yearly temperatures

recorded in the soil were very close to those recorded for the air, but there were no pronounced peaks of maximum of minimum.

Species traits

Community weighted means were calculated for 35 functional traits. Excluding the variables with a binomial distribution, the following traits were not normally distributed even after transformation: seed number per plant, SLA, $T_{50}G$ and $T_{50}S$. Therefore, these parameters were analysed with non-parametric tests whilst for the other non-binomial CWM, a factorial ANOVA was performed to investigate the differences between forest types and countries (Table 4). The significant effects of environmental traits on CWM are summarized in Table 5.

Adult plant traits

SLA was not significantly different between the four forests investigated, and none of the environmental parameters influenced this trait significantly. The recent forests had significantly taller plants. Plant height had a positive association with available P in the soil and a negative correlation with N content (Fig. 7).

Reproductive phenology

Flowering month spanned from a minimum of four (April) to a maximum of seven (July). Flowering was significantly earlier in older forests ($p < 0.001$), with a median value corresponding with the month of May while the median was closer to June in the young forests. There was a significant positive association between flowering month and available P in the soil and a significant effect of country, being the UK.O forest the earlier to flower. Fruiting month varied from mid-May in the SP.O to August in the UK.Y, but it was not significantly different between either countries or forest types. The only

environmental parameter that significantly affected fruiting month was the level of available P in the soil, being higher where the fruiting month was later.

Table 4: Effect of Age and Location on the CWM. Variables with a binomial distribution, in italics, were tested with Generalized Linear Model. Continuous variables were tested with a Factorial ANOVA. *** = $p < 0.0001$; ** = $p < 0.01$; * = $p < 0.5$.

Age	Location	Age x Location	No effect
Flowering month ***	*Immediate germination* *	*Delayed germination, one peak* *	SLA
Germination temperature **	*Delayed germination, one peak* **	Lag between germination and cotyledon emergence**	*Cold stratification*
Indifference to light ***	Lag between germination and cotyledon emergence ***	Persistence in soil seed bank **	Delayed germination, two peaks
Need for light **	Proportion not viable seeds **	Proportion not viable seeds ***	EHS T50G
Plant height ***	Relative embryo size *	Relative embryo size *	EHS T50S
Relative embryo size *	Seed dry mass ***	Seed length ***	Fruiting month
Seed dry mass ***	Seed terminal velocity *	Time to 50% cotyledon emergence **	*Max final cotyledon emergence*
Seed number ***	Seed width ***	Time to 50% germination ***	*Max final germination*
Seed terminal velocity ***			*More 50% seed dormant*
Seed width ***			*Morphological dormancy*
Vegetative reproduction *			*Physiologycal dormancy*
			Warm stratification

Seed dispersal and yield traits

The natural logarithm of seed dry mass was significantly higher in Spain compared to England and in the old forests compared to the young ones. The trait varied between 0.08 and 31 mg and the calculated CWMs were higher in the SP.O than in the other sites (median > 10 mg). Available P in the soil had a negative association with it while it was positively associated with N levels. The same pattern was found for seed terminal velocity and the natural logarithm of seed width. In seed length, a significant interaction between the two factors was demonstrated with higher values for the Spanish forests compared to the English. A post hoc test showed that, while there were no significant differences between the two Spanish forests on one side and the two old forests on the other, the two young forests were significantly different, and UK.Y had the lowest values of seed length. This trait had a negative association with available P in the soil and with the PAR in the summer while it had a positive association with the PAR in the winter. Finally, seed production per plant was higher in the younger forests. The trait varied between 41 and 13286 seeds per plant.

The square root of seed persistence in the soil had a significant interaction between age and country with higher values recorded for UK.Y and lower values for UK.O. The two Spanish forests were found to be not significantly different but they present an inverse trend, with SP.O having higher values of the CWM for seed persistence in the soil. The trait was measured in years and spanned from 0.01 to 1.2 years (Fig. 7). Seeds were significantly more persistent in areas with lower PAR in the winter.

Germination traits

Relative embryo size varied little. Although a significant interaction between forest age and country was demonstrated, a post hoc test did not show any difference between the

four woodlands. The trait ranged between 0.06 and 0.99 with a higher CWM recorded in the SP.O. Relative embryo size was significantly higher in areas with low PAR in the winter.

Only age was significant for germination temperature. Young forests had significantly higher values of this CWM. Available P in the soil was positively associated with higher germination temperatures, while plots with lower PAR in the winter tended to have lower germination temperatures. The following binary germination traits, that were collated in the literature review, were not significantly influenced by any factor and environmental parameter: presence of PD (recorded for 41 out of 70 species), presence of morphological dormancy (8 species) and need for cold stratification (35species).

The requirement for warm stratification was significantly influenced by the country factor, with higher CWM in the UK.O where two species showing this trait, *Anemone nemorosa* and *Mercurialis perennis*, were seasonally abundant. The requirements for light for germination were significantly influenced by forest age, with species that were indifferent to light being more abundant in the old forest and species that needed light more abundant in the young one. Finally, another important binary regeneration trait that was examined was the reliance on vegetative reproduction as the main method of reproduction. It was more common and significantly correlated with the age of the forest, i.e., found more often in the old forests, but was not significantly influenced by any of the environmental parameters examined.

Germination experiment

Fifty seed collection were tested in a move along experiment, 33 of them collected in UK and 18 in Spain (Annex II). From these collections, 10 species were in common but were tested at different temperatures, according to the site of collection. In order to provide a

single trait for these analysis, however, the values recorded for the same species across the two countries were averaged. The time to reach 50 % of germination and cotyledon emergence were not normally distributed. Both metrics were significantly affected by the interaction between age and country. A post hoc test demonstrated that the UK.Y had higher abundance of species that were quicker to reach 50% germination and cotyledon emergence, while species in the UK.O were significantly slower. The two Spanish forests did not differ significantly for these germination traits. Interestingly, a longer time required to reach 50% germination and cotyledon emergence was significantly associated with a lower amount of P in the soil. The natural logarithm of the interval, in days, between germination and cotyledon emergence was significantly influenced by the interaction between country and age of the forest. A post hoc test demonstrated that UK.O was the only site to be significantly different from all the other, with a higher lag between the two phenomena. This parameter too was significantly associated with low abundances of P in the soil.

The quantity of heat accumulated to reach 50% germination was not significantly influenced by age or by country, i.e., the four forests were rather similar. The only environmental parameter that had a significant effect on both traits was the availability of P in the soil, which was negatively associated with both the EHS for germination and for cotyledon emergence. The percentage of non-viable seeds was significantly higher in the UK.O, with a median of 50% of the seeds in a collection being non viable. The interaction between country and age was significant for the logarithm of this trait. Both maximum final germination and cotyledon emergence failed to show any significant relationship with any other parameters investigated.

Finally, four different types of germination strategy were identified based on the germination curves (Annex II):

1) Seed that germinated within three weeks from sowing (16 collections)
2) Seed that germinated partly within three weeks from sowing, stop for the winter and had a second germination peak after a cold stratification (14 collections)
3) Species that germinated only after cold stratification (13 collections)
4) Species that germinated sporadically but constantly and did not reached 50% germination (8 collections)

Only two species amongst those collected in the two countries had a different germination strategy between countries: *Carex sylvatica* and *Carex pendula* that shown a strategy of type 1) in the Spanish population and a strategy of type 3) in the English population.

Of the four strategies described, only two differed significantly in representation between the two forests. Species with strategy (1) were significantly less frequent in the UK, and the species with strategy (2) were significantly more in the UK.O but poorly represented in UK.Y.

DISCUSSION

Species composition and the environment

The species composition of the herbaceous layers between the recent and old woodland sites differed in both locations, even though the two pair of forests were separated by less than one km. These findings are in agreement with other studies that compared the herbaceous layer of recent and ancient woodland (Bossuyt and Hermy, 2000; Brunet et al., 2011; Peterken and Game, 1984). A contributory factor is likely to be that the two locations investigated presented different physical environments that influenced the development of their woodland communities. In particular, the soils have a higher content

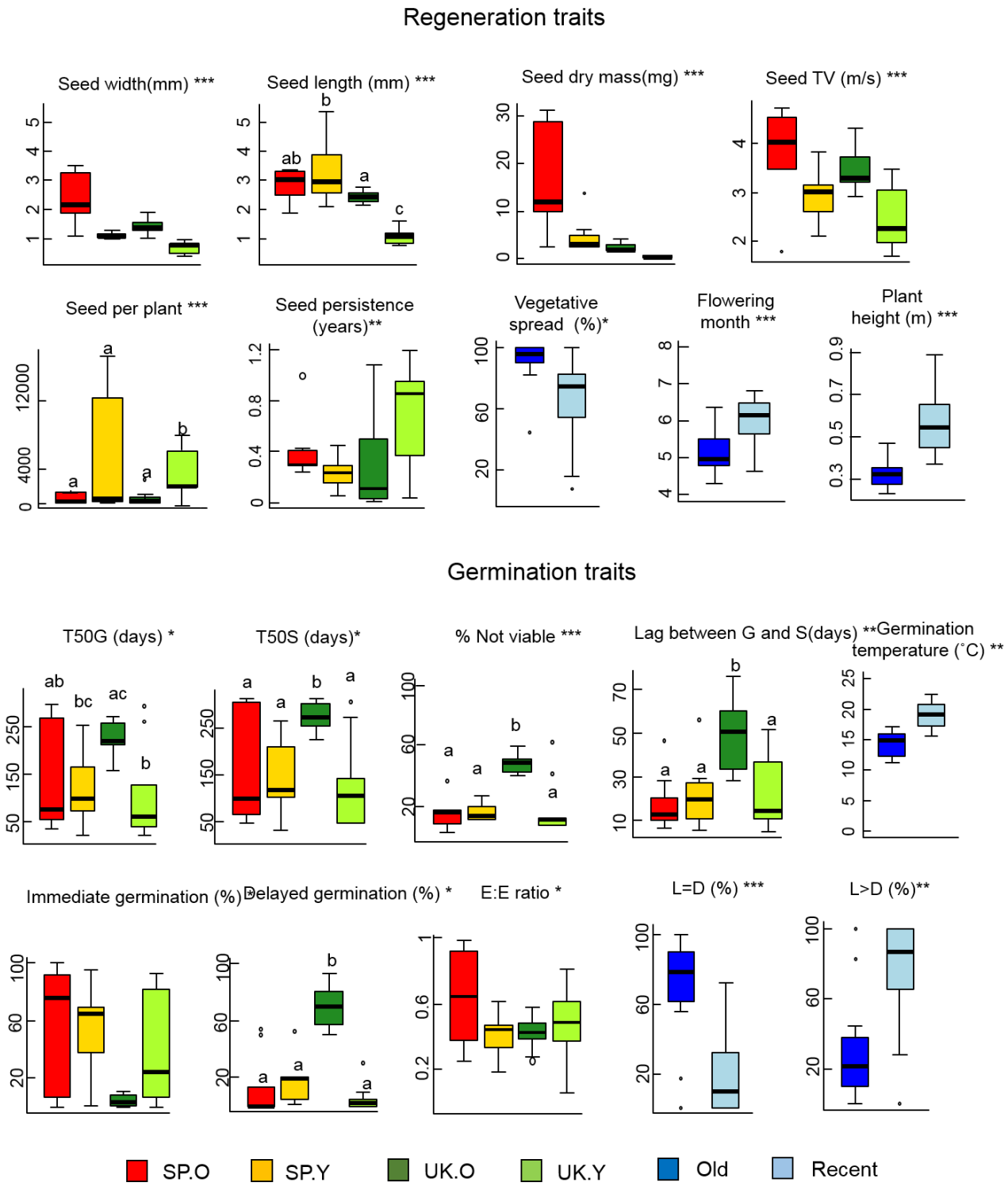

Fig. 7: Boxplots of the traits that were significantly different with respect to forest age and location (Table 4). When the interaction between the two factors was significant a post hoc test was performed and, in these cases, different letters indicate significant difference between sites. *** = $p < 0.0001$; ** = $p < 0.01$; * = $p < 0.5$.

Table 5: Effect of forest age, location and abiotic parameters on the CWM. Variables that were normally distributed were analysed with Linear Models. CWM calculated from traits with binomial distribution were analyzed with Generalized Linear Models. The – and + indicate a positive or negative effect of the parameter on the dependent variable. For the "Location" factor the sign indicates correlation with higher latitude (UK). Only significant interaction were reported. *** = $p < 0.0001$; ** = $p < 0.01$; * = $p < 0.5$.

Trait	Age	Location	P	N	PAR	PAR	R:FR
Reproductive phenology							
Flowering month	***	* -	** +				
Fruiting month			* +				
Seed yield and dispersal							
Plant height	***	* -	* +	* -			* -
Seed dry mass	***	** -	*** -	** +			
Seed number	** -						
Seed width	***		*** -	*** +			
Seed length	* +	* -				*** +	
Seed terminal velocity	***		*** -	* +			*-
Persistence in soil seed						* -	
Germination							
Germination temperature	***	*-	*+			*-	
Warm stratification		* +					
L = D	** +						
L > D	* -						
Germination traits							
E:E ratio						**-	
T50G		* +					
T50S		** +	*-				
EHS T50G			*-				
EHS T50 S		* +	**-				
Lag between T50G and		***	** -	*+			* -
Proportion of not viable	** +	** +				*+	
Immediate germination		* -					
Delayed germination		* +					

in available P in England and a higher pH in Spain. The two English forests have similar soil characteristics, with the main difference being the enrichment in P in the young woodland (UK.Y). The soil environment is more markedly different between the two Spanish forests that, even though they have a similarly low content of P, differ markedly in pH, and total C and N. SP.O, in agreement with patterns already observed (Beaten et al, 2011; Brunet et al, 2011; Verheyen et al., 1999), has higher content of C and N and lower pH than SP.Y.

Not only do the soil conditions vary, but also the availability of light differs between the two locations (Spain vs England). The English forests have higher PAR compared with the Spanish forest studies (Fig 5) and a marked seasonality in the degree of light filtration (e.g. R:FR ratio). Higher PAR level were found in those plots that were placed under a gap in the tree canopy, a situation more frequent in UK.O, that had higher spatial heterogeneity than the other forests. The ratio between red (R) and far red (FR) light (660 nm and 730 nm wavelength respectively) describe the density of canopy cover because the light that passes through a canopy is filtered in its spectral composition and has a lower proportion of red light (Holmes and Harry, 1977). Higher seasonal difference in the degree of light filtration in England can be explained by the scarcity of evergreen species in the tree layer. R:FR reach its highest values in winter and the lowest in summer, when the deciduous canopy trees have leaves. In Spain a distinctive element of the forest community is *Laurus nobilis*, which is absent from the English site. *L. nobilis* is an evergreen small tree with Mediterranean affinity, that characterizes the Atlantic forests of the Iberian peninsula (Bueno Sánchez and Fernández Prieto, 1991; Rodríguez Guitián et al., 2007) but is not native in the British Isles (Stace, 1991). A lower PAR in the winter can be explained for the SP.O by the important presence of the evergreen *Laurus nobilis* and *Hedera helix*, which prevents light reaching the forest floor even in winter, when the

deciduous components of the canopy lose their foliage. In this condition, herbaceous understory plants that rely on higher light availability in the early months of the year cannot develop. In UK.Y the low PAR in winter can be interpreted as a consequence of the absence gaps in the young canopy, the even age of the trees and their lower average distance.

An important difference between the two locations is also represented by their size and isolation from similar habitats. The Loder Valley Nature Reserve (UK) is set in a rural landscape, with many small woodlands connected by old wooded hedges. The Tragamòn Oak Grove Natural Monument (Spain) is set in a urban landscape, isolated from other forests and broken in two fragments by a local road, that run through it. Therefore, the low number of herbaceous species (18) recorded in SP.O, can be explained also by its isolation that could have led to a higher extinction rate of the species originally present (MacArthur and Wilson. 1963; Simberloff, 1976) and by an increased detrimental hedge effect (Laurance et al., 2007). Dzwonko and Loster (1992) found that site area is one of the more important factors that influences the number of species in a secondary forest but this is in contrast to the findings in this study, as more species are were found to be present in the SP.Y compared with SP.O and the two English sites have the same number of species.

When investigating species composition in vegetation studies it is also critical to appreciate that the management regime in relation to prior use can influence the outcome. SP.Y was planted for educational purposes, to illustrate the potential species that can grow in a temperate forest in Northern Spain, and was found to be richer in tree species compared to the other sites. Also, the abandonment of cultivation and the closing of the growing canopy clearly affected the SP.Y in which both ruderal species from open habitats and species indicator of woodland continuity, migrated from the nearby SP.O, are

present. SP.Y is consistently richer in species than SP.O (39 species vs. 18) and include also 10 of the species of the old forest. For this reason and due to the low number and low abundance of species in SP.O, the CWM of many of the traits examined are not significantly different between the two Spanish sites (Fig.7),

The two English forests, instead, have similar species richness (26 species in UK.O and 27 in UK.Y) and share 10 species. The difference in the CWM calculated for these sites is therefore more pronounced (Fig.7). Less tree species have been reintroduced in UK.Y than SP.Y but they are densely planted. The area is still homogeneous, with even aged trees and no gaps in the canopy. If compared with UK.O, UK.Y presents a more homogeneous environment with less ecological niches for specialist species, a feature that is common in post agricultural forests (Flinn and Marks, 2007).

Sabatini et al. (2014) found that the abiotic factors that influence species composition of the herbaceous layer in temperate forests can vary depending on the forest type. On the basis of this finding and of the differences (Fig. 3) in species composition and structure between the two reference forests (UK.O and SP.O) of this study, a separate description of the effect of abiotic environment and forest age on the functional structure of the study sites is necessary. With regard to the distribution of the functional traits of species in the herbaceous layers, their land use history and present physical conditions should be taken in account. (Fig.8)

Plant height and phenology

Plant height changed significantly between forests of different age being higher in the young ones. This result is in agreement with the literature reviewed in Chapter 2 and is correlated to the higher dispersal capacity of the species that have been able to colonize the young forests (Thomson et al., 2011).

Early flowering is confirmed to be a trait characteristic of old woodland specialists, as already described by Kelemen et al., (2014) and Verheyen et al. (2003). Winter or early spring flowering herbs are adapted to the seasonal changes in canopy cover of temperate deciduous forest and develop their reproductive cycle before the complete closure of the canopy and the emergence of later flowering species, avoiding the competition for light. It is not the case that the CWM for flowering phenology is significantly lower in the English location, where the forest canopy is formed only by deciduous trees.

Seed morphology and yield

Traits related to seed dispersal and yield (seed dry mass, seed length, seed width, seed terminal velocity, plant height and seed number per plant) are strongly correlated between them (Pearson > 0.7 , Annex I). In fact, they are influenced by the same environmental filters, in particular forest age but also P availability and N content in the soil (Table 5) that reflect the differences in soil chemistry reported by Beaten et al. (2011) for forests of different ages. Plants that produce many small seeds suffer less from seed limitation and are more likely to colonize recent forests that are not adjacent to propagule source (Verheyen et al., 2003). Alternatively, seed size in forest herbs has been found to be positively correlated with seedling establishment (Erlhén and Eriksson, 2000) but big seeds tend to suffer more from predation (Reader, 1993). The latter finding can explain why old forests have a higher abundance of species that rely on vegetative, other than sexual, reproduction and species that produce a higher proportion of non viable, empty seeds. Fuentes and Shupp (1998) demonstrated that specimens of *Juniperus osteosperma* that were more heavily attacked by seed predators (birds) had the highest proportion of full seeds: the production of empty seeds, therefore, reduces the pressure of predation on fertile seeds by confounding seed predators. A similar strategy is not to be excluded for old forest herbs such as *Euphorbia amygdaloides, Lamium galeobdolon* and *Mercurialis*

perennis, that were found to produce, respectively, 52, 94 and 54% of partially or totally empty seeds in UK.O.

Seed longevity in the soil

The ability to persist in the soil seed bank is also related to the colonization capacity and it has been demonstrated to be lower for bigger seeds (Bekker et al, 1998). The results from this study are in agreement with previous findings. Thus, the difference in the CWM for seed persistence in the soil are particularly evident between the two English forests, with persistent seeds significantly more abundant in UK.Y (Fig. 7). In fact, the soil seed bank gets quickly depleted by the short lived seeds when the land is cultivated (Bossuyt and Hermy, 2002; Honnay et al., 2002). Therefore, if dispersal limitation exists (e.g. distance from propagule source), the recent forest will lack the species that were both absent from the soil seed bank and that had not migrated after reforestation.

In contrast, the two Spanish sites are not different regarding the expression of this trait. In particular, a low value of the CWM for seed persistence in the soil was not expected for SP.Y; but this can be explained by higher abundance in this site of the two Poaceae species (*Brachypodium pinnatum* and *B. sylvaticum*) that do not form a persistent soil seed bank (Thompson et al.1997). *B. sylvaticum* is a indicator species for ancient woodland and the congeneric *B. pinnatum* is a species more adapted to open habitat (Ten Brink et al., 2013). Nonetheless, the two species can and do coexist. Their presence in SP.Y is indicative of an early stage of forest succession with large gaps and light availability. Clearly, the interpretation of a functional trait value with the CWM approach needs to take into account the local context and the ecology of the species that dominate the community. Notwithstanding this, the trait is negatively influenced by availability of PAR in winter: less persistent seeds are found in sites where light is more available in

winter, like deciduous mature forests or open young forest dominated by grasses that do not form a persistent soil seed bank.

Embryo morphology

Even if the interaction between age and location is significant, there are no significant differences between each pair of forests in their embryo:endosperm ratios (Fig.7). The median value is anyway higher in SP.O and the data appear to span a higher range in this site. This trend could be explained because in the SP.O only 18 herbaceous species were recorded and, from those, only four have an average across season abundance higher than 0.1%; three being non-endospermic species (*Geranium robertianum, Pulmonaria longifolia* and *Stachys sylvatica*) and one having a small E:E ratio (*Arum maculatum*). In contrast with the findings of Hoyle et al. (2013), that found the presence of endosperm being correlated with the germination strategy, the CWM of relative embryo size in this study is not correlated with any of the CWM of the germination traits considered (Annex I).

Seed germination

There was a strong correlation between the time to reach 50% germination and time to 50% cotyledon emergence and between these two traits and the amount of heat necessary to reach 50% of the event (Pearson > 0.7, Annex I). As in Hoyle et al., (2013), the germination strategies described did not correlate with any of the vegetative traits considered and neither with seed yield and dispersal traits.

The pattern that emerges from the results of the germination tests is of a slower germination in the old forests and a quicker germination in the young one. This concept is supported by the shape of the germination curves produced: species from the old forests in fact tend to germinate in a single peak after a period of cold stratification. In this way germination can be delayed to the most favourable period of the year that, in deciduous

forests, is usually spring. The interval between germination and cotyledon emergence, is significantly higher in the old woodland. This is also indicative of a strategy in which the plants first establish a deep root, germinating when the temperatures are still too cold for seedling survival and delay the emergence of the cotyledon to a warmer season. The delay can happen by means of epicotyl dormancy (e.g. *Anemone nemorosa*, Mondoni et al. 2008) or by a slow development of the shoot at cold temperatures (e.g. *Conopodium majus, Mercurialis perennis*) and gives the seedlings a competitive advantage of being well rooted when other species are starting to germinate.

Immediate germination, more abundant in the recent forest sites, is a characteristic of opportunistic species with a ruderal strategy that produce many seeds. Therefore, the opposite strategies recorded in the two habitats can be a consequence of seed limitation: better colonists tend to have a quicker germination and, if abiotic filters are removed, they are able to quickly establish a new populations. However, the majority of the temperate forest herbs possess dormancy, with PD and MPD being the more common types (Baskin and Baskin, 2014) and the seeds require a period of stratification before germination can happen. The more common stratification requirement in forest environment is cold stratification (Baskin and Baskin, 2014; Vandelook, 2009) and this trait was found to be equally represented between all the study sites. Species that require a combination of warm and cold stratification or that possess a morphological component in their dormancy are more characteristic of old forests, where a more stable environment permits the seeds to remain imbibed long enough to complete embryo growth. In the forests sampled, these species were proportionally rarer than the species that only possess PD and just require cold stratification and especially abundant in UK.O. In fact, the environment of the forest location was the most significant factor associated with the germination traits examined. P availability in the soil had an inverse correlation with all the traits related to seed

germination rate (Table 5), confirming that, on average, species from recent forests have a rapid germination.

Eleven of the species tested showed two germination peaks demonstrating heterogeneity in dormancy levels within the same population. The CWM for the "two peaks" germination strategy was not significantly different between sites and was not influenced by any of the environmental parameters. However this pattern of germination was more abundant in the two recent forests and was a feature of plants with relatively small seeds. Its ecological meaning can be to spread the germination between seasons so that there is: 1) quicker establishment of a population; and 2) incorporation of part of the seeds in the soil seed bank, thus enabling germination later in the year or in later years. Overall, the results of this study are in agreement with Ten Brink et al. (2013). These authors compared the germination strategies of a congeneric pair of species, one characteristic of woodland and the other characteristic of open habitat, and found that forest species have delayed germination more often and can germinate in the dark and at lower temperatures.

CONCLUSIONS

Two opposite scenarios have been described on the basis of the comparisons of the functional diversity between the four study sites:

1) In the Loder Valley Nature Reserve, the two forests compared have a similar abiotic environments (Fig. 5) but their herbaceous plant communities are different regarding their regeneration and seed germination traits (Fig.8).

2) In the Tragamón Oak Grove, the abiotic environment is more different between the study sites but some of the functional traits examined are not significantly different (Fig. 7). However, the expression of the traits associated with old

woodland or recent plantation is comparable with the findings relative to England (Fig. 8) and with the literature on the topic (Kelemen et al., 2014; Ten Brink et al., 2013; Verheyen et al., 2003).

Despite of this, the old and recent forest communities differed for several traits independently of the country. Both in England and Spain, old forest understories had shorter plants that flowered earlier and were more dependent on vegetative reproduction. They produced less seeds, which were heavier and wider, with faster terminal velocity. The germination of old forest understories occurred at lower temperatures and was less dependent on light.

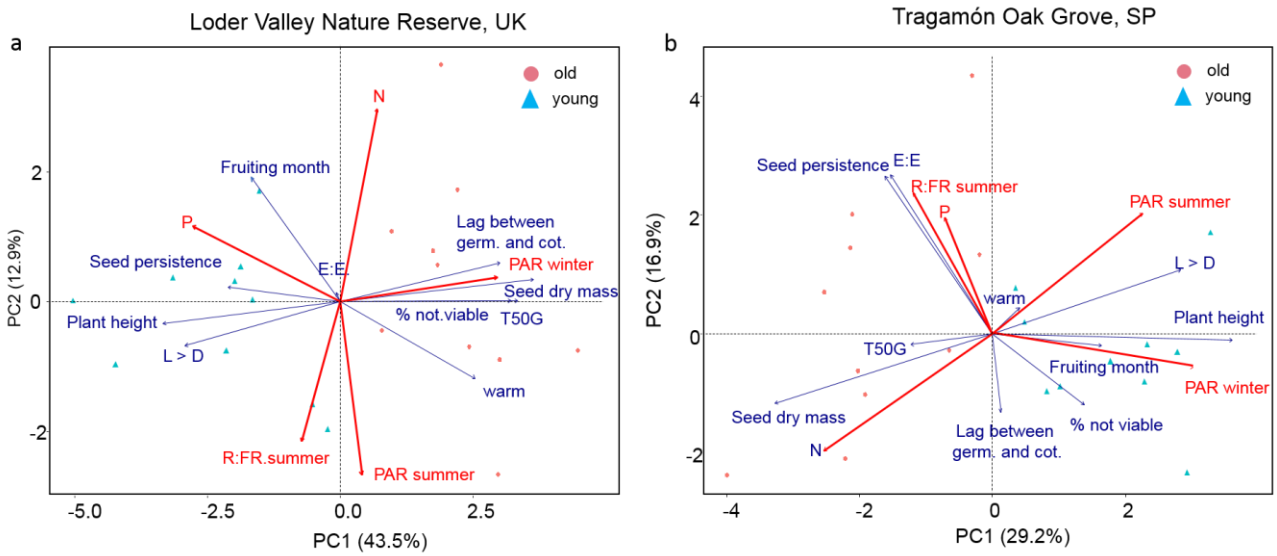

Fig.8: PCA of the environmental parameters and the CWM that were: 1) not correlated between them (Annex I); 2) significantly influenced by the environment (Table 5).

Two types of threshold can be crossed when a habitat is degraded (Cramer et al., 2008). The biotic threshold is crossed when the species disappear and, due to dispersal limitation, cannot recolonize the habitat. The composition of the new habitat is therefore different and seed addition can be useful if the abiotic environment has not been modified over a

threshold that would not allow species establishment. In UK.Y, only the biotic threshold has been crossed and seed limitation excludes from UK.Y some of the woodland specialist that are restricted to UK.O, in accordance with the findings of Verheyen and Hermy, (2004) for the woodland specialists *Anemone nemorosa* and *Primula elatior*. In this scenario, restoration of the old forest understories can be aided by introducing seed mixes that prioritize species with the traits that we have identified as characterizing the old forest.

In SP.Y not only the biotic threshold but also the abiotic one has been crossed, because the two forests differ significantly in their physical environment. However, due to the small size and impoverished understory of SP.O it would be advisable to compare the two sites with a third, larger area of the same potential vegetation to verify if a significant difference can be detected in the CWM between forests of different ages.

Natural recolonization of understory in restored forest can take more than one century for some species and reintroductions may be necessary (Bossuyt and Hermy, 2000) even though high species richness in a recent woodland can be achieved in 70-80 years if the forest is adjacent to a source of propagules (Brunet, 2007). However this study demonstrates that an increase in species richness does not correspond to a similar functional diversity of the new plant community and species reintroduction aimed to restore also ecosystem functioning may be necessary.

AKNOWLEDGEMENTS

The ideas behind this work was developed together with Eduardo Fernàndez Pascual. This study would not have been possible without the collaboration of Alvaro Bueno Sànchez, Ignacio Alonso Felpete and Elena Gaudìn at the Atlantic Botanic Garden and

without the support of John Adams at RBG Kew, Wakehurst Place. The involvement of Eduardo Fernandèz Pascual and Hugh W. Pritchard in revising the manuscript was precious and fundamental. Thank you also to Pete Iannetta, Geoff Squire and Tracy Valentine for their advice on soil analysis and for hosting me at the JHI and to Gillian Banks, David Boldrin, Lawrie Brown and Timothy George for their help with soil analysis and data interpretation. Finally, we are grateful to Filip Vandelook for sharing unpublished germination data. Special thanks go to Aya Matsushita, Eva Correia Alvarez, Filippo Guzzòn and Giacomo Biasi who helped with the fieldwork, and to Luìs Carlòn and Nicola Ardenghi who helped with plant identification. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°607785. The study has been supported by the TRY initiative on plant traits (http://www.trydb.org). The TRY initiative and database is hosted, developed and maintained by J. Kattge and G. Boenisch (Max Planck Institute for Biogeochemistry, Jena, Germany). TRY is currently supported by Future Earth/bioDISCOVERY and the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig.

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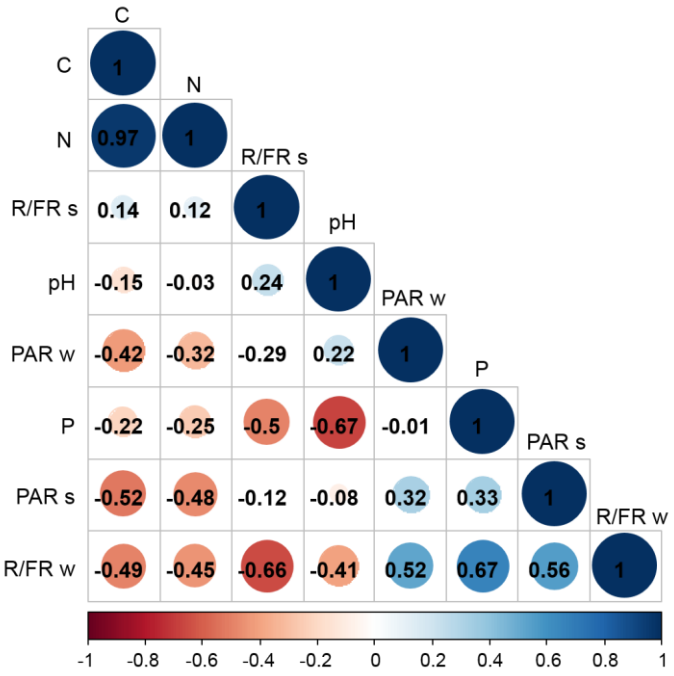
ANNEX I

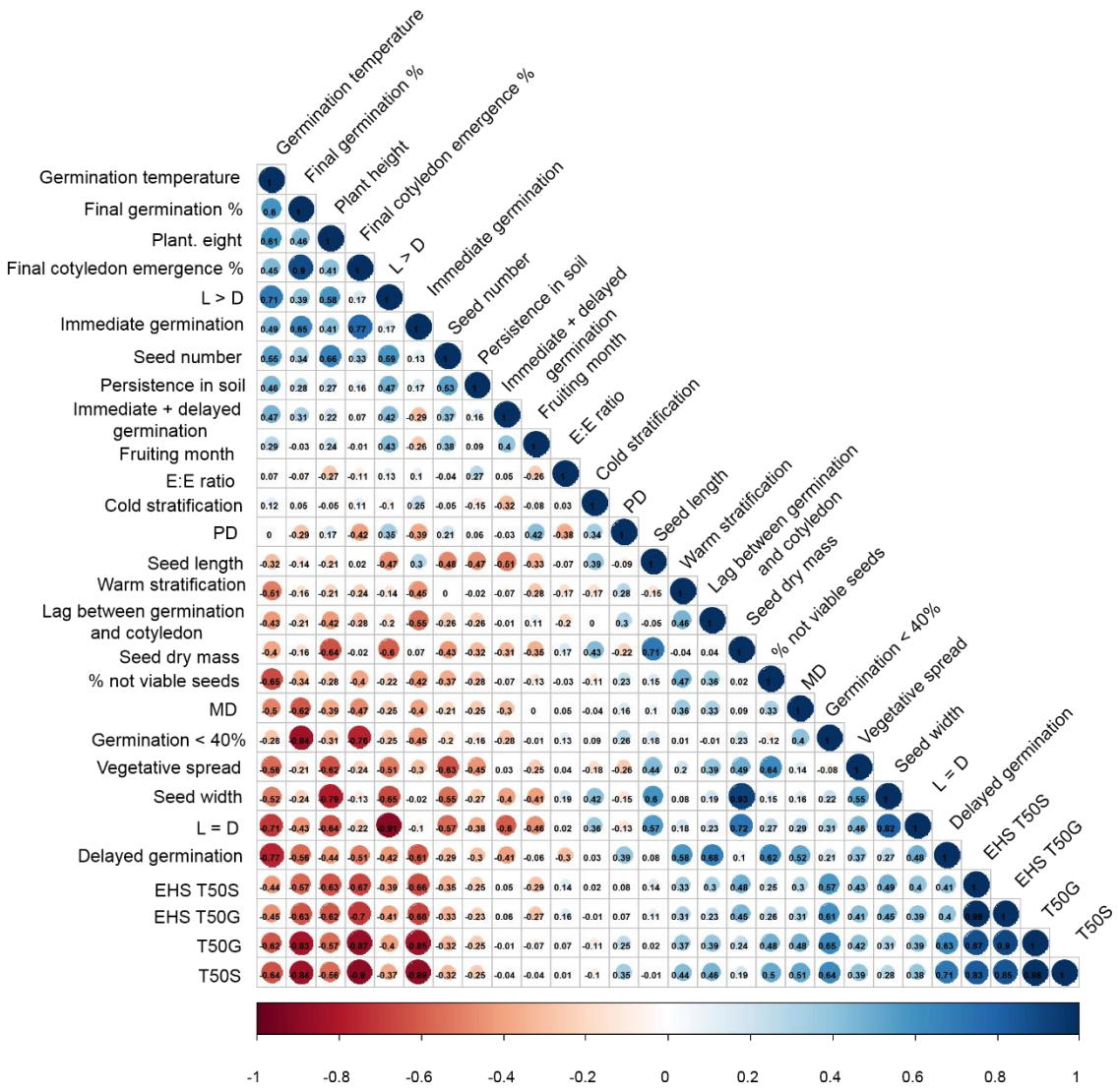
Correlation matrices of environmental variables and CWM.



Correlation matrix between all the environmental parameters: Pearson correlation matrix of soil and light parameters measured. When the correlation was significant, the values of the correlation coefficient are inscribed inside the circles. The quadrants were no circles are shown indicate a not significant correlation between the variables. R/FR s = red/far red ratio in summer, PAR w = Photosynthetic Active Radiation in winter, PAR s = Photosynthetic Active Radiation in summer, P = available phosphorous, N = nitrogen content in soil

Correlation of the environmental variables with the first two component of PCA in Fig. 5

Environmental parameter	PC1		PC1	
	Pearson	p.value	Pearson	p.value
P	0.64	0.00	-0.60	0.00
N	-0.70	0.00	-0.39	0.01
PAR s	0.73	0.00	0.32	0.04
PAR w	0.56	0.00	0.42	0.01
R/FR s	-0.59	0.00	0.60	0.00



Correlation matrix between all the CWM: Pearson correlation matrix of the CWM for all the traits considered in this study. When the correlation was significant, the values of the correlation coefficient are inscribed inside the circles. The quadrants were no circles are shown indicate a not significant correlation between the variables.

Correlation of the CWM with the first two component of PCA in Fig. 8a (England)

Only the variables that had a significant correlation with the first two axis are shown.

NS = not significant.

CWM	PC1		PC1	
	Pearson	p.value	Pearson	p.value
				NS
Seed dry mass	0.95	0.0		NS
Not viable seeds	0.87	0.0		NS
T50G	0.85	0.0		NS
Lag between G and S	0.79	0.0		NS
Seed persistence	-0.55	0.01		NS
L > D	-0.74	0		NS
Plant height	-0.89	0		NS
E:E ratio		NS	0.68	0.0
Fruiting month		NS	0.62	0.0
Warm stratification	0.66	0	-0.53	0.01

Correlation of the CWM with the first two component of PCA in Fig. 8b (Spain)

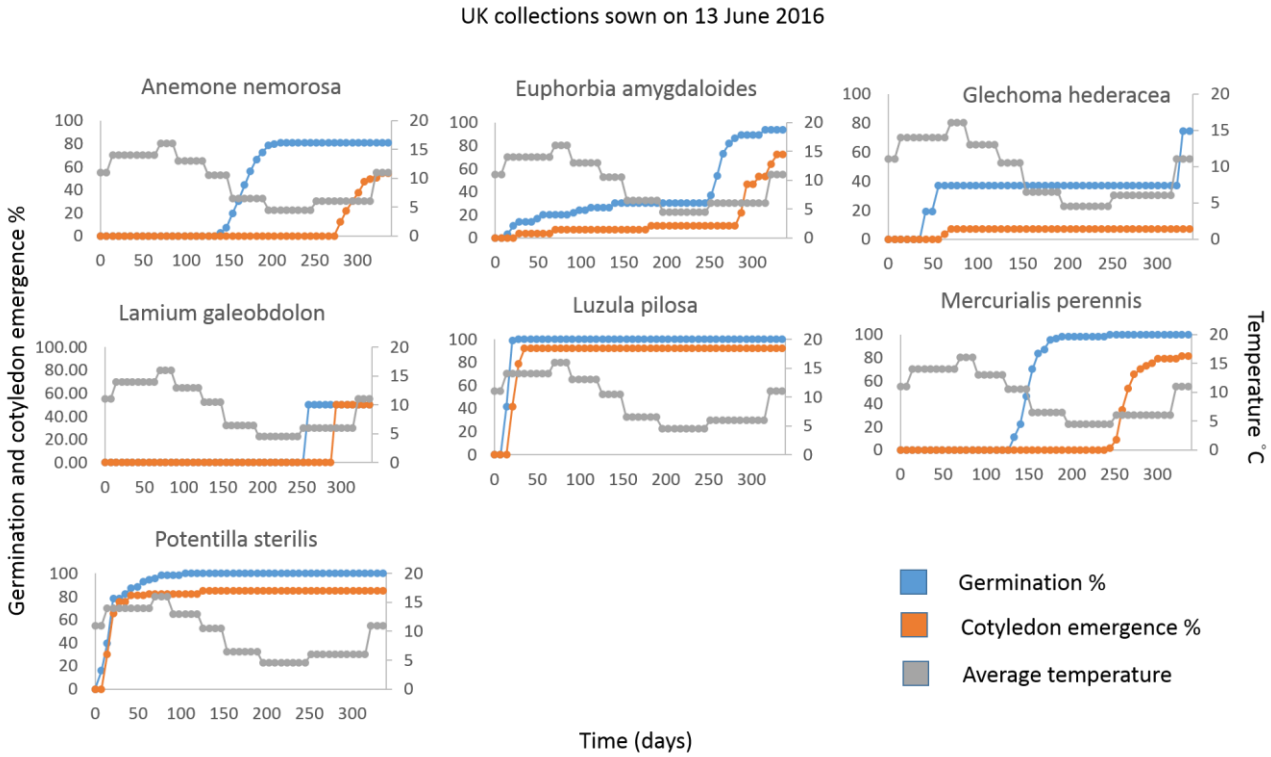
Only the variables that had a significant correlation with the first two axis are shown.

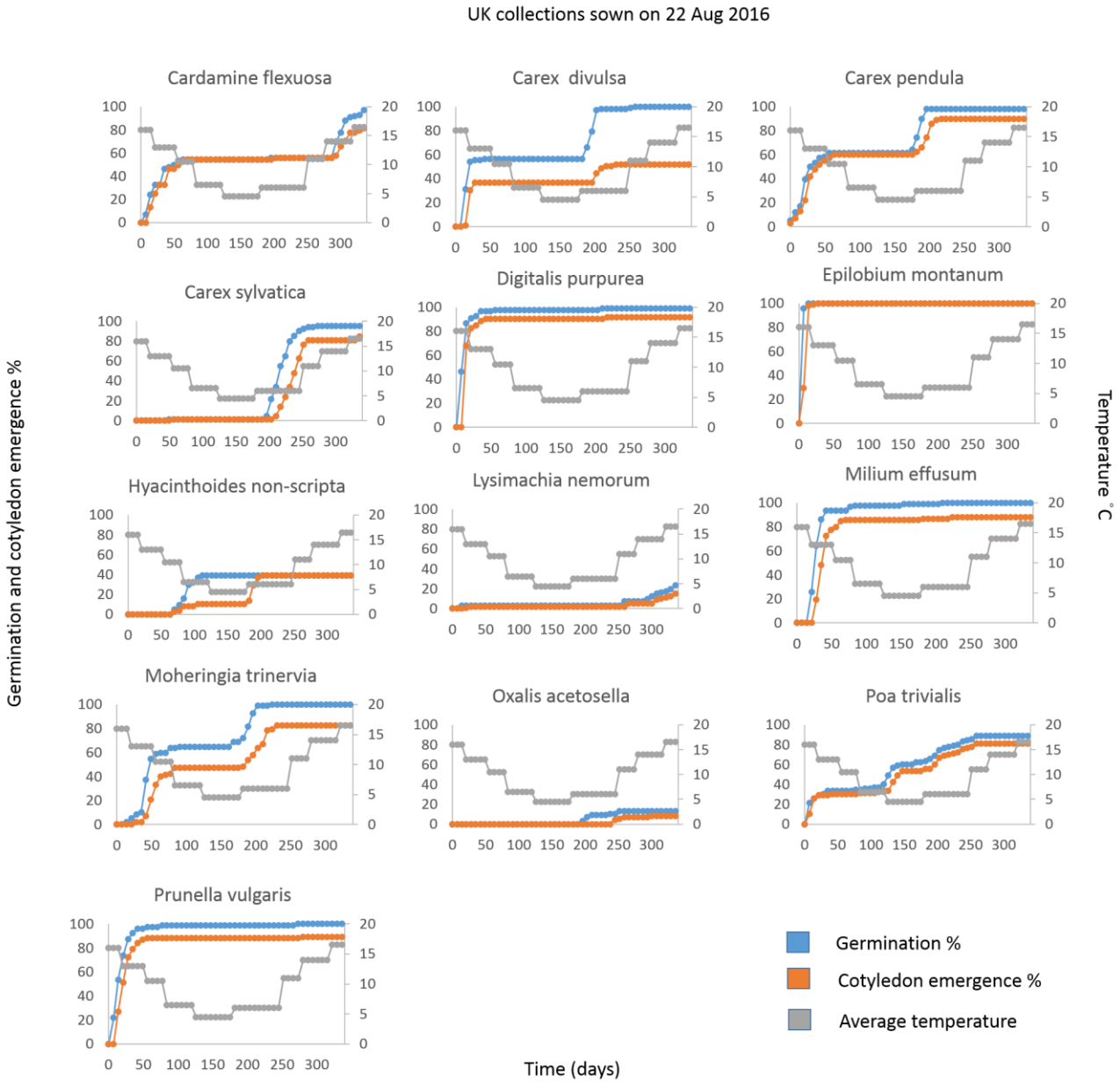
NS = not significant.

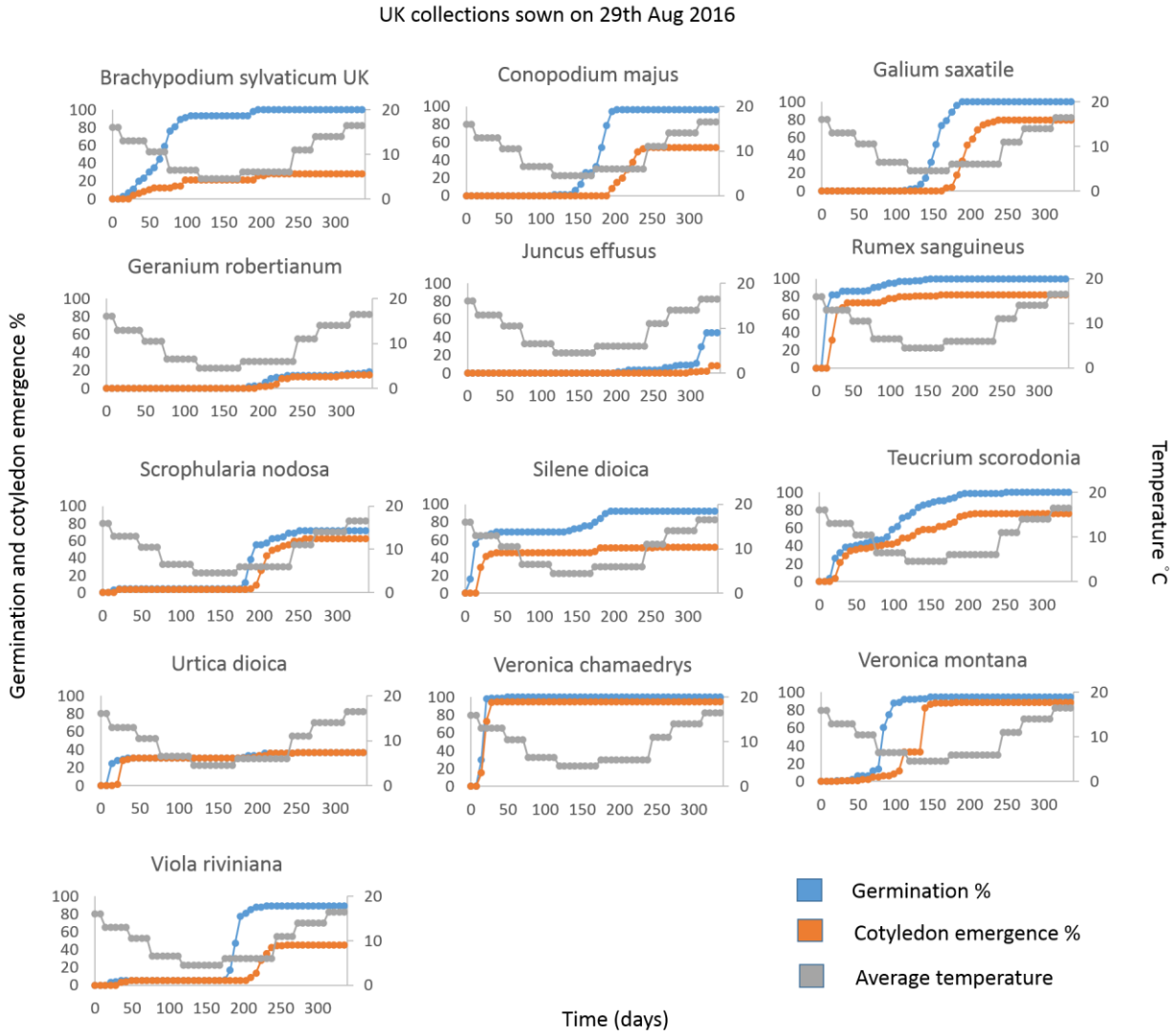
CWM	PC1		PC1	
	Pearson	p.value	Pearson	p.value
Seed dry mass	0.84	0.00		NS
Not viable seeds	-0.47	0.03		NS
L > D	-0.77	0.00		NS
Plant height	-0.90	0.00		NS
Fruiting month		NS	0.51	0.02
Lag between G and S		NS	0.49	0.02
Seed persistence		NS	-0.48	0.3
E:E ratio		NS	-0.83	0.0

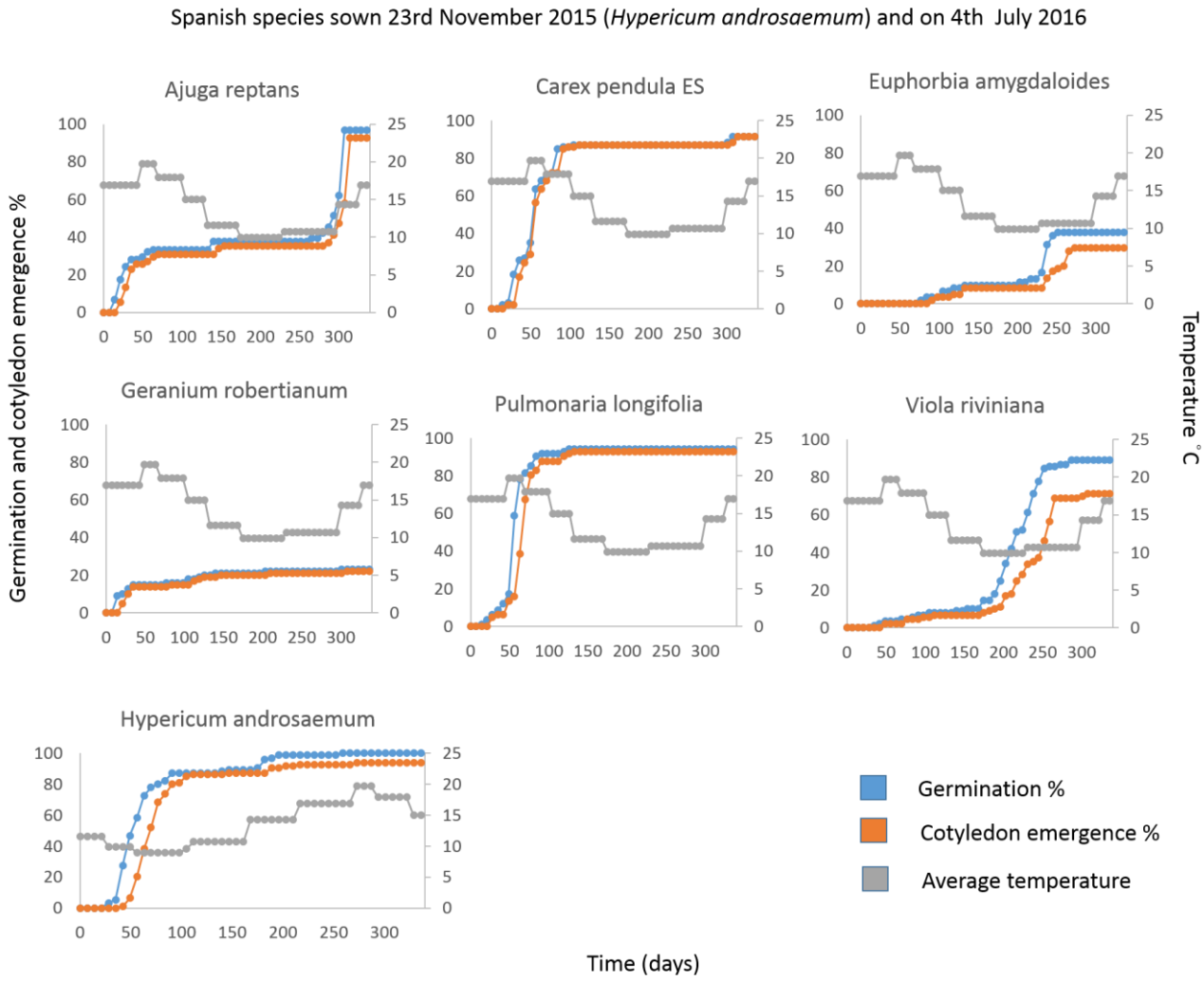
ANNEX II

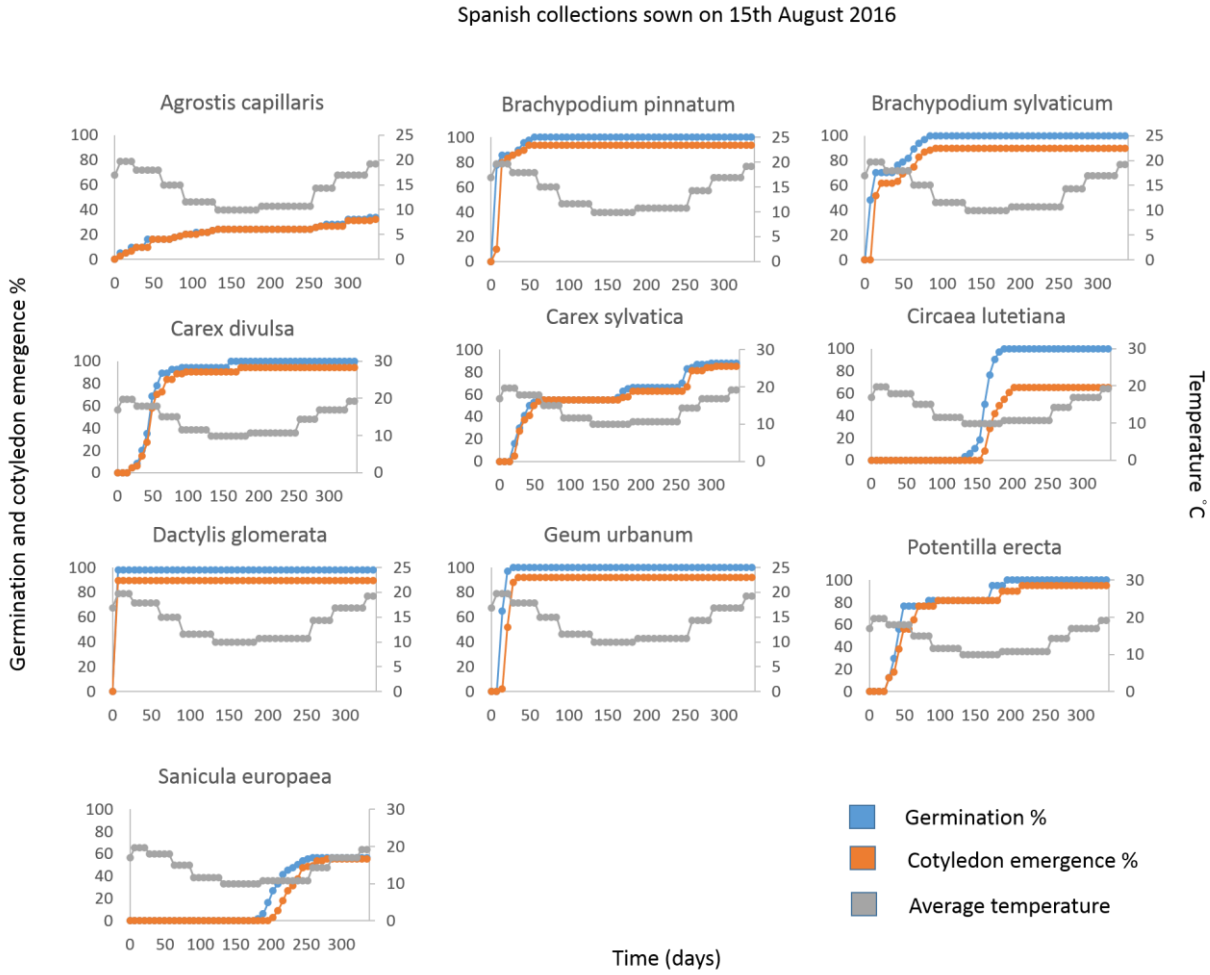
Germination charts of all the species tested, grouped per sowing date.




UK collections sown on 22 Aug 2016
Cardamine flexuosa
Carex divulsa
Carex pendula
Carex sylvatica
Digitalis purpurea
Epilobium montanum
Hyacinthoides non-scripta
Lysimachia nemorum
Milium effusum
Moheringia trinervia
Oxalis acetosella
Poa trivialis
Prunella vulgaris
Germination and cotyledon emergence %
Temperature °C
Time (days)
Germination %
Cotyledon emergence %
Average temperature


UK collections sown on 29th Aug 2016
Brachypodium sylvaticum UK
Conopodium majus
Galium saxatile
Geranium robertianum
Juncus effusus
Rumex sanguineus
Scrophularia nodosa
Silene dioica
Teucrium scorodonia
Urtica dioica
Veronica chamaedrys
Veronica montana
Viola riviniana
Germination and cotyledon emergence %
Temperature ˚C
Time (days)
Germination %
Cotyledon emergence %
Average temperature


Spanish species sown 23rd November 2015 (*Hypericum androsaemum*) and on 4th July 2016
Ajuga reptans
Carex pendula ES
Euphorbia amygdaloides
Geranium robertianum
Pulmonaria longifolia
Viola riviniana
Hypericum androsaemum
Germination and cotyledon emergence %
Temperature ˚C
Time (days)
Germination %
Cotyledon emergence %
Average temperature


Spanish collections sown on 15th August 2016
Agrostis capillaris
Brachypodium pinnatum
Brachypodium sylvaticum
Carex divulsa
Carex sylvatica
Circaea lutetiana
Dactylis glomerata
Geum urbanum
Potentilla erecta
Sanicula europaea
Germination and cotyledon emergence %
Temperature °C
Time (days)
Germination %
Cotyledon emergence %
Average temperature

CHAPTER 4

SEED MORPHOLOGY, EMBRYO GROWTH, GERMINATION AND DESICCATION TOLERANCE IN THE PSEUDO-MONOCOTYLEDONOUS GEOPHYTE *CONOPODIUM MAJUS* (APIACEAE), AN ANCIENT WOODLAND INDICATOR WITH MORPHOLOGICAL DORMANCY

ABSTRACT

Conopodium majus (Apiaceae) is considered an Ancient Woodland Indicator species in temperate Europe but it is also an importnat component of oligotrophic meadow communities whose distribution has been reported to decline in Europe. Attempts to reintroduce it by seeds have resulted in scarce seedling emergence possibly as a result of the seeds having a small embryo and thus morphological dormancy. The objective of this study was to characterize its germination ecology and its ability to survive in storage. The effects of temperature and chemical cues on embryo growth were characterised physiologically and morphologically, using sectioning and image analysis of seeds at different stages of embryo growth internally. *Conopodium majus* is a dicotyledonaous species yet the seeds only have a single cotyledon. Morphological analysis suggested that this structure is derived from a process of cotyledon fusion rather than abortion. The seeds had a narrow temperature range for germination and are relatively short-lived as considerable viability was lost when seeds were stored at 60% RH and 20°C for one year. Seeds imbibed at 5˚C for 84 days were still able to germinate after being dried back to 15% and 60% RH, although the germination rate was decreased. The results suggest that seeds of this species might only survive short-term in the soil seed bank and probably germinate slowly in the natural environment during which time a high level of desiccation tolerance is retained by the embryo whilst it is growing within the seed.

KEYWORDS

Conopodium majus, embryo morphology, embryo growth, desiccation tolerance, germination

INTRODUCTION

Temperate deciduous forests are habitats with a marked sesonality and it has been demonstrated that the majority of the species regarded as forest specialists in Europe possess seed dormancy Baskin and Baskin, 2014. One particular type of seed dormancy that is quite common in these habitats is morphological dormancy (MD). This occurs when the embryo is undifferentiated or underdevelopped at the time of dispersal and needs time to grow before germination can occurr (Baskin and Baskin, 2004). Embryo growth within the seed can delay germination timing until the end of a long and predictable unfavourable season. For this reason, embryo growth usually needs from several weeks to months of exposure to temperatures characteristic of that season. Good examples are summer temperatures indicating canopy closure in temperate woodland understories (Mondoni et al., 2008) or near zero temperatures indicating snow cover in alpine meadows (Forbis and Diggle, 2001). MD may also allow greater time for seeds to be dispersed by ants, a common feature of the temperate forest understory flora (Hermy et al., 1999). MD has been demonstrated to be highly conserved across some plant lineages (Martin, 1946; Finch-Savage and Leubner-Metzger, 2006) and had been considered to be an ancestral character in plant evolution from which other types of dormancy evolved (forbis et al., 2002). More recently, morphophysiological dormancy (MPD) has been suggested to be ancestral (Willis et al., 2014). Nonetheless, MD is quite common in geophytes of temperate meadows and forests (Baskin and Baskin, 2014).

In the Apiaceae family the presence of underdeveloped, linear, embryos has been described (Martin, 1946). The initial relative embryo size has been associated with habitat preferences, such that smaller embryos and colder temperatures for embryo growth in Apiaceae from forest habitats appears to be a consequence of adaptation to shady habitats (Vandelook et al., 2012). *Conopodium majus* is a vernal geophyte from the *Apiaceae*

family with a sub-Atlantic distribution. It is described as an Ancient Woodland Indicator species at both the British and European levels (Hermy et al., 1999; Kirby, 2006). This species grows both in open meadows and in woodlands, in soils poor on nutrients (Grime et al., 2007). Its establishment seems to be inhibited by competing vegetation iff introduced in productive meadows (Thompson and Baster, 1992). *Conopodium majus* seems to be a poor colonizer (Thompson and Baster, 1992) and its distribution in open meadows could be an indication of past woodland presence in the site (Grime et al., 2007). It regenerates mostly by seeds and there is little evidence that the species reproduces vegetatively (Lovett Doust and Lovett Doust, 1982). It does not form a long lived soil seed bank (Thompson et al., 1997). Seedlings are reported to emerge in late winter (January/February) (Roberts, 1979) and, as many other Apiaceae, the species may need a period of cold stratification to allow embryo growth.

These features make *Conopodium majus* an important target plant for ecological restoration in both woodland and oligotrophic meadow communities. However, its seed ecophysiology is poorly understood, possibly because the seed is difficult to study because of slow germination and reduced seedling survival. This study investigates the morphology, embryo growth, germination and desiccation tolerance of *Conopodium majus* seeds, in order to enable its use by the native seed industry. The ultrastructure of the seeds was analysed too because of the unusual feature of pseudomonocotyly in *Conopodium majus:* in fact this is one of the few dicotyledon that only develop a single cotyledon. The conditions for embryo growth and germination are investigated through manipulation of temperatures and addition of GA_3, in order to characterize MPD and MD break (Baskin and Baskin, 2014). Since *C. majus* is reported to grow in oligotrophic soils (Grime et al., 2007), different concentration of KNO3 were used to wether if its germination is inhibited by high concentrations of nitrates. Moreover, since the plant is

reported not to form a long-lived seed bank, and this feature is sometimes associated with low desiccation tolerance, the conditions of storage are investigated as well as changes in desiccation tolerance as embryo development progresses within the seed.

MATERIALS AND METHODS

Seed acquisition and initial measurements

Fresh seeds were collected on 28th August 2015, at Dalreoch Farm (56 44' 47" N, 3 32' 25" W; Perthshire, Scotland, UK). They were kept at ambient temperature and humidity for 3 days and sent to the Royal Botanic Gardens, Kew, Wakehurst Place where the experiments were started upon delivery. During the shipping the seeds were kept in sealed plastic bags to avoid desiccation.

Seed viability was tested using 1% aqueous solution of triphenyl tetrazolium chloride (TZ). Twenty seeds were allowed to rehydrate overnight at 20℃ and 100% RH and placed on 1% agar for 24 hours to become fully imbibed and reactivate their metabolism before being prepared for staining. A slice of seed coat was removed from the dorsal surface of each seed using a scalpel. The seeds were then covered with the TZ solution and incubated at 30℃ in the dark for 24 hours. To evaluate seed viability a longitudinal cut was made and the seed embryo located. The staining of embryo and endosperm was assessed. (Fig.1).

Fig.1: Dissected seed of *Conopodium majus* and extracted embryo. The uniform red staining of endosperm and embryo indicate that the seed is viable.

If part of the tissues were not red, the seeds were considered to be non viable.

The moisture content of fresh seeds was measured on arrival. Ten randomly selected seeds were weighted in a 0.0000 μg scale and placed for 17 hours to dry in a ventilated oven at 103 ˚C. The percentage water loss was then obtained from their dry weight. The fresh mass of 96 seeds was measured as well using the same balance.

Internal morphology

A sample seed, incubated at 5 ˚C for 56 days, was fixed in FAA soultion and sent to the University of Oxford Wellcome Trust Centre for Human Genetics to be scanned using a Micro-CT scanner (SkyScan 1172).

Effect of temperature on embryo growth and germination

Seeds were suspended in water overnight at 20 ˚C and sown the following day in 8 cm diameter Petri dishes containing 1% agar-water. For each treatment, four dishes with 25 seeds each were sown for the germination tests, plus 10 dishes of 20 seeds each for embryo growth measurements. The temperature tested were 0, 5 and 10 ˚C. Higher temperatures were avoided because a pre-screening showed that germination and embryo growth were inhibited at temperatures much greater than 10 ˚C (unpublished data). The effect of temperature fluctuation during day and night was tested using an alternating temperature regime of 10 ˚C during the day and 0 ˚C during the night. In all the treatments, the light alternated between 12 hours light and 12 hours dark that, in the fluctuating temperature regime, corresponded to the coldest phase (LMS Cooled incubators, LMS Ltd, Sevenoaks, UK).

Germination tests continued, with weekly scoring, until no seeds were observed germinating for four consecutive weeks.

Every 14 days a Petri dish containing 20 seeds was ramdomly selected from each treatment. In order to avoid mesuring non viable embryos, that could have stopped growing before the date of dissection, seeds were prepared for TZ staining: only seed that appeared viable were measured. Each seed was cut longitudinally and the embryo were extracted. Embryos and endosperms were photographed using a camera (Carl Zeiss Axiocam Colour) mounted on a Stemi SV 11 Microscope (Carl Zeiss, Welwin Garden City, Herts, UK) microscope, and their length measured using the software Axiovision 3.1.2.1 (Carl Zeiss Vision GmbH).

Effect of GA_3 and KNO_3 on embryo growth and germination

Using a temperature of 5 ˚C, that proved to be the more effective for embryo growth and germination in a pre-screening experiment (unpublished data), the effect of GA_3 and KNO_3 were tested. Seeds were sown in 8 cm diameter Petri dishes filled with 1% agar in which were added respectively a concentration of 250 mg/L of GA_3 (Kew Millennium Seed Bank standard testing concentrations) and 3, 10 or 30 mM of KNO_3. To obtain the desired concentration of KNO_3, 0.138, 0.461 and 1.38 g of compound were added to 5 mL of distilled water and mixed with a magnetic stirrer until completely dissolved. The solution was then added to 450 mL 1% agar-water solution, previously melted. The GA_3 solution was added to the melted agar only when it reached a temperature below 50 ˚C, to avoid denaturation. For each treatment four dishes of 25 seeds were sown for germination testing and 10 dishes of 10 seeds for embryo growth measurements.

Effect of cold stratification on germination

The effect of cold stratification on seeds imbibed at 5 °C for different amount of time was investigated to test if they do acquire the ability to germinate at warmer temperatures after

a cold stratification treatment. Eight replicates of 25 seeds were sown on 1% agar at 5 °C for 0, 6, 14 and 20 weeks and then moved to germination temperature of 10 °C and of 15/5 °C. A control, in which seeds were held at 5 °C and not moved to warmer temperatures was sown as well. Seeds were scored weekly after 20 weeks from sowing and all the treatments were terminated at the same time, after 33 weeks from sowing. Seedling emergence, defined as the stage in which the cotyledon was free from the seed coat, was scored as well as radicle emergence. A cut test was performed at the end of the experiment and all the seeds that were firm and possessed an embryo were considered viable.

Embryo growth in the soil

To monitor embryo growth in natural condition 20 bags of *Conopodium majus* were prepared and buried in the soil at a depth of 5 cm in Scotia Seeds farm (Angus, Scotland, 56°41'59''N, 2° 39' 21'' W). In each bag, 20 seeds were mixed with 20 g of the farm soil and placed on a mesh tissue that was then folded and stapled. Maximum and minimum air temperature were monitored every 3 days. Fortnightly a bag of seeds was recovered and sent to the laboratory for TZ testing and embryo measurement.

Storage behaviour

To test the effect of storage at different air RH%, fresh seeds of *Conopodium majus* were placed, upon arrival, in airtight jars suspended over a solutions of lithium chloride (LiCl) to create air RH of 15%, 60% and 80%. The quantities of LiCl used per 200 mL of de-ionised water were 147 g, 60 g and 34 g respectively (Gold and Hay, 2014). The temperature of the storage room was 20 ˚C. The seeds were stored for 0 months (control), 6 months or 1 year and 110 seeds were used for each treatment. After each storage period

four dishes of 25 seeds were tested for germination at 5 ˚C and scored weekly. The 10 remaining seeds were stained in TZ solution and assessed for viability.

Dessication tolerance during embryo development

The aim of this experiment was to test if and when *Conopodium majus* seeds lose viability when re-dried after the beginning of embryo growth. Two drying condition were used: equilibration with 60% and 15% RH, obtained through suspension over LiCl solution in an airtight container held at 20 ˚C (see method above). Four dishes of 25 seeds were sown on 8 cm diameter Petri dishes containing 1% agar-water and placed at 5 ˚C as a control treatment. Other seeds were randomly sampled using a seed counter and sown on 1% agar-water in four plastic boxes to be retrieved after 3, 28, 56, and 84 days since sowing. Each box contained 200 seeds that, after the sampling time, were removed from the agar, blotted with paper to remove excess water and split in two sub-samples. Each subsample of 100 seeds was then placed on a desiccation jar. Seed equilibrium RH (eRH) was monitored daily using a Rotronic hygrometer fitted with a HC2-AW sensor and USB interface, connected to laptop/PC running HW4-E software (Rotronic Instruments, UK) until the seeds reached the equilibrium with the jar RH. Then the seeds were rehydrated being suspended over water for 24 hours to avoid imbibition damage and sown on agar-water in four dishes of 25 seeds for germination testing. Germination was monitored weekly.

Statistical analysis

The effect of the temperature and chemical treatments on embryo growth was tested by fitting Linear Models to the embryo growth progression curves. In all the experiments, the effect of each treatment on the final germination percentages was tested be comparing

it against the control (5°C constant without any pretreatment) using Generalized Linear Models (binomial distribution, logit link). When all seeds of a treatment were non-viable, that treatment, obviously different, was not included in the analysis. All the statistical analysis were performed using the software R 3.4.0 (R Development Core Team, 2017).

RESULTS

Of the 20 seeds tested for viability, 18 had an embryo and endosperm that completely stained red with TZ solution (i.e., 90% viability). The initial moisture content of the seeds on receipt was 17.8% (± 5.5 SE) and their average fresh weight was 1.89 mg (± 0.06 SE). The initial relative embryo size was 0.12 (± 0.005 SE).

Ultrastructure of C. majus *embryo*

The *Conopodium majus* embryo appears to have a single, entire sheath, cotyledon (Fig. 2a). Using a light microscope and observing whole, excised, embryos, no trace of a second cotyledon was visible at any stage of embryo development and during germination. Soon after germination, a bulbil formed by enlargement of a portion of the hypocotyl, but no true leaves were produced until the second year after germination and these appeared to develop from the bulbil. No evidence of a second cotyledon was detected by Micro CT imaging of the transversal section of the seed (Fig. 2b).

Effects of temperature and chemicals on embryo growth and germination

A considerable difference in the rate of embryo growth between the temperatures tested was observed (Fig. 3). The optimal temperature for embryo growth was 5 ˚C and an

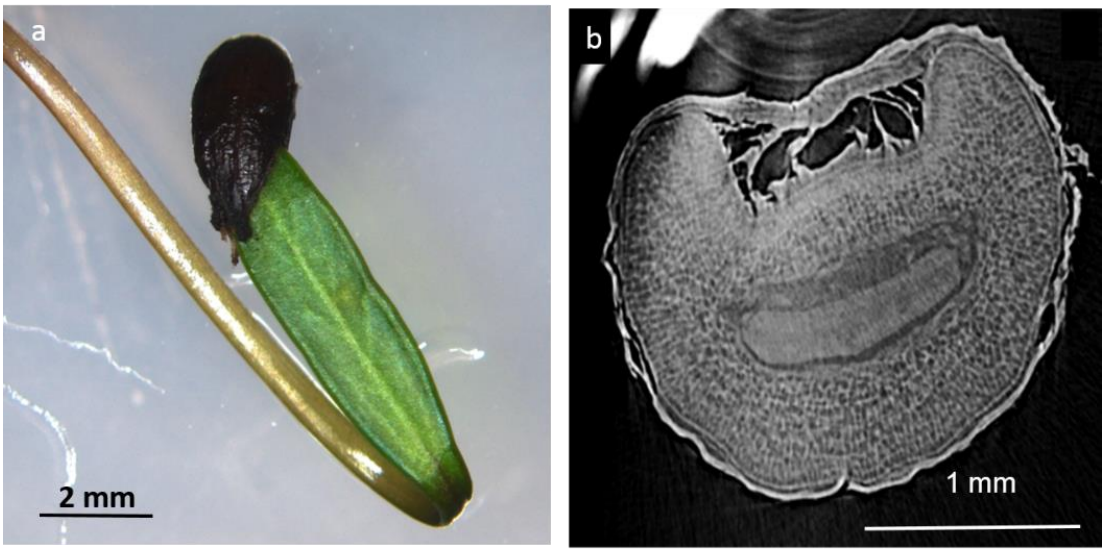


Fig 2: Embryo morphology in *Conopodium majus*. a) Cotyledon almost fully emerged from the seed coat. The photograph was taken using using a camera (Carl Zeiss Axiocam Colour) mounted on a Stemi SV 11 Microscope (Carl Zeiss, Welwin Garden City, Herts, UK) microscope. b) Transverse section of *C. majus* seed obtained with Micro CT scans (SkyScan 1172). The epicotyl portion of the embryo is already differentiated at this stage but the tissue does not show any discontinuity that can suggest the presence of two cotyledons.

increase in relative embryo size was already measured after 14 days from imbibition. In contrast, all the other treatments had a negative effect on the rate of embryo growth as compared with 5°C. In particular, the effect of alternating temperatures and of incubation at 10 ˚C had a significant negative effects on the rate of embryo growth (Table 1).

The slope of the linear model fitted was higher for the treatment in which GA_3 was added (0.007), that resulted in a significantly faster embryo growth compared to all the other treatments. A positive effect was detected also with the addition of a moderate amount of KNO_3 that, also, increased the rate of embryo growth compared to the control. The other

Table 1: Results from linear regressions of increasing relative embryo size over time in relation to the applied treatment. Slope and R^2 refer to the linear model fitted to the embryo growth curves (Fig. 1) and constrained to pass through a common origin defined by the value of the initial embryo:endosperm ratio.

Treatment	Estimate	SE	t value	p-value	Slope	R^2
0 ˚ C	-0.0534	0.0346	-1.5460	0.1269	0.0045	0.9637
5 ˚C / control	0.0006	0.0006	0.9620	0.3398	0.0055	0.9952
10 ˚C	-0.0035	0.0006	-5.8460	0.0000	0.0020	0.9447
10/0 ˚C	-0.0026	0.0006	-4.2860	0.0001	0.0027	0.9624
250 mg/L GA3	0.0019	0.0006	3.0840	0.0030	0.0073	0.9760
3 mM KNO_3	0.0016	0.0006	2.6780	0.0094	0.0065	0.9828
10 mM KNO_3	0.0008	0.0006	1.4000	0.1665	0.0060	0.9942
30 mM KNO_3	0.0008	0.0006	1.2540	0.2145	0.0057	0.9917

two concentration had a positive influence on the rate of embryo growth if compared to the control but they were not significantly different.

Germination started after 16 weeks from sowing in the GA_3 treatment. The final germination after 29 weeks of experimentation, are shown in Figure 4. A significant difference was detected when final germination was compared across treatments. In fact, final germination percentage was 0 in the seeds incubated at 10°C. Within the other treatments applied, 10/0°C was the only that significantly decreased final germination

compared to the control (25% vs 98%, $p < 0.001$).

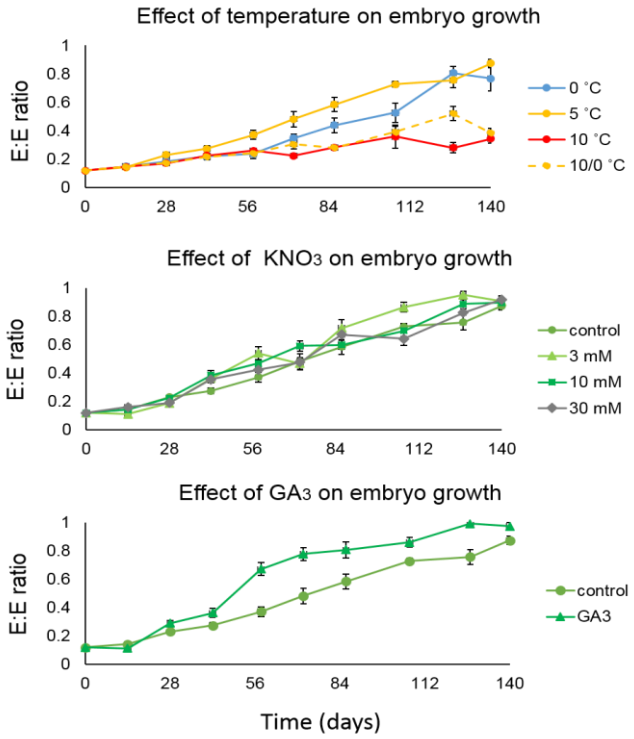


Fig.3: Increase on relative embryo size with time for seeds treated at different temperatures and with solutions of GA_3 or KNO_3 at 5°C.

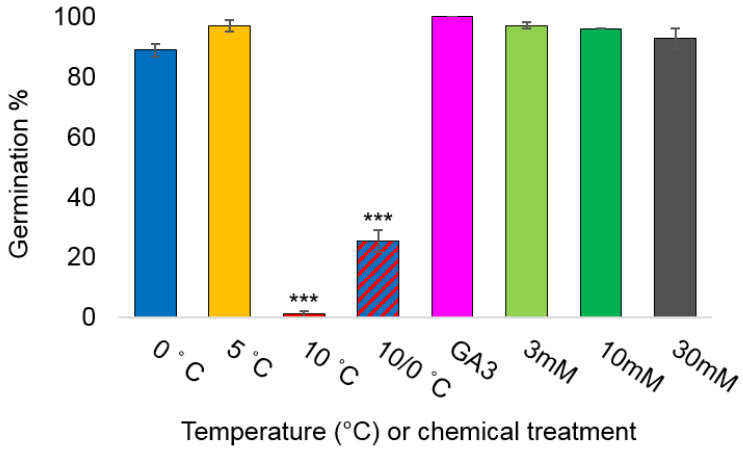


Fig 4: Final germination percentages between temperature and chemical addition treatments (250 mg / L GA_3 or 3 – 30 mM KNO_3). *** indicate significant ($p < 0.001$) difference with the control. Vertical bars indicate the binomial confidence intervals.

Effect of cold stratification on germination

Germination started during the cold stratification treatment. On the date of first scoring, after 20 weeks, there was already considerable? germination at the 5 °C control (31.6 ± 2.8 %). For the 20 week stratification treatments, 45 ± 4.4% germination was achieved for the seeds that were subsequently moved to 10°C and 35.6 ± 2.7 %germination for the seeds subsequently moved to 15/5 °C (Fig. 5). Only 13 ± 3 % and 3.1% ± 2.0 of the seeds cold stratified for 14 weeks and moved, respectively, to 10 and 15/5 °C, achieved germination after 20 weeks. No germination was observed in the seeds stratified for 6 weeks and in the 10 and 15/5 °C controls (i.e., constant treatment).

The final germination percentage, scored after 33 weeks from the beginning of the experiment, was higher in the 5°C control (97.0 ± 1.9%) and significantly ($p < 0.001$) lower in all the other treatments, except when 20 weeks of stratification were followed by a germination temperature of 10 °C (90 ± 1.2%). However, the increment in germination from the first scoring to the end of the experiment, was higher for the seeds in the 5°C control (+ 66%) than for the seeds that were moved to 10 (+ 45%) and 15/5 °C (+ 36 %).

The pattern was reversed for seedling emergence. On the date of first scoring (20 weeks after sowing), in a few seeds the cotyledon had emerged in the treatments that were stratified for 14 weeks (5% ± 1 for the treatments moved to 10 °C and 2.4 ± 1.2% for the ones moved to 15/5 °C) and for 20 weeks (2 ± 1% for the treatments moved to 10 °C and 2.3 ± SE 1.2 % for the ones moved to 15/5 °C). However, the rate of seedling emergence and their final percentage was higher in the seeds that were cold stratified for 20 weeks and then moved to 10 and 15/5 °C (66.0 ± 9.0% and 50.0 ± 4.7%, respectively) while it remained lower in the 5 °C control (40.6 ± 7.0%). Seeds that were stratified for 14 weeks

and then moved to 10 °C presented a shorter gap between germination and cotyledon emergence, compared to the seeds of the 5 °C control, but they had a lower final emergence percentage (21 ± 2.5% and 2.0 ± 1.2%, respectively) (Fig. 5).

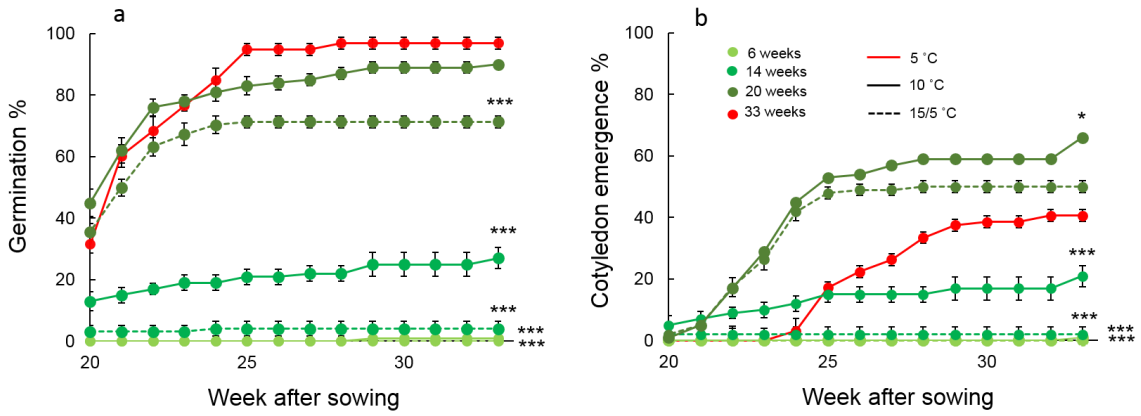


Fig. 5: Germination (a) and cotyledon emergence (b) after four different stratification treatments: 6, 14, 20, 33 (control) weeks at 5 ˚C. Differences in final germination between control, in which the seeds were held at 5 ˚C for all the 33 weeks of the experiment, and treatments were tested using generalized mixed models with logit-link function. *** = $p < 0.001$, treatment significantly different from control. The treatments that were not stratified and keept, for 33 weeks, at the germination temperatures of 10 and 15/5 °C are not shown because no germination occurred.

Embryo growth in the soil

Seeds were sown on 11th september 2016 and retrieved approximately every 14 days. The last sample was measured on 2nd March 2017, 173 days after the beginning of the experiment and it was the first one in which visible germination was recorded. In fact, half of the seeds in the last retrieved bag presented radicle emergence. The average E:E ratio of the whole sample, including germinated seeds, was 0.94 (±0.01 SE). As in the laboratory experiment, embryo growth in the field began within the first 14 days from

sowing (Fig.6).

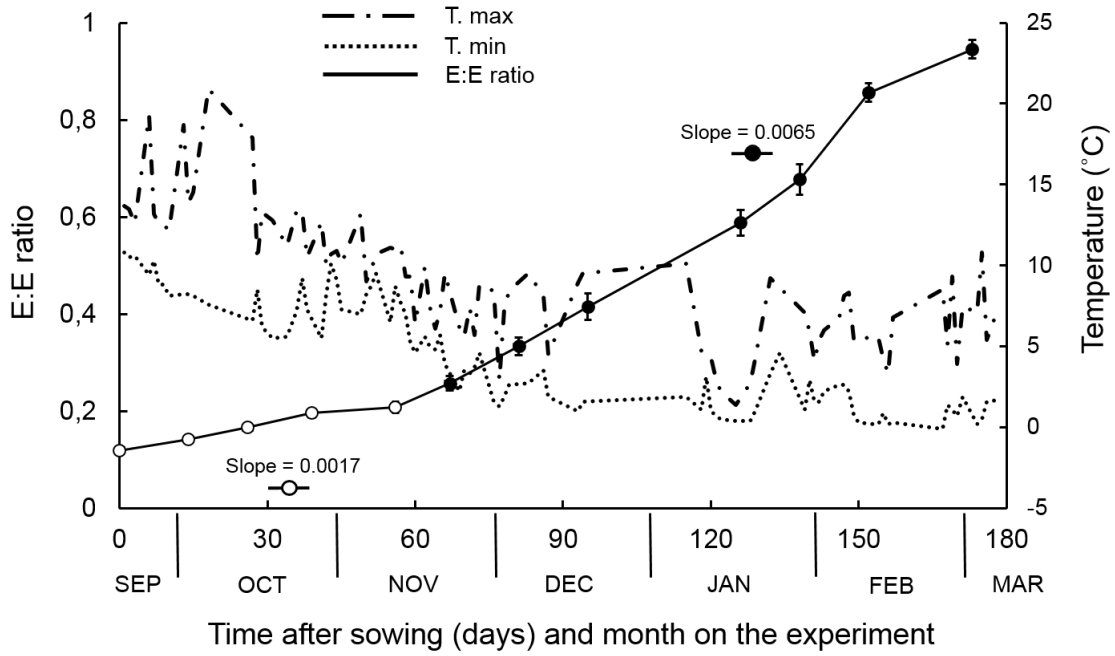


Fig. 6: Embryo development in seeds buried in the ground at the farm of Scotia Seeds Ltd, Angus, Scotland . The vertical bars indicate the standard error of the average E:E ratio of 20 seeds for each data point. The slopes (inset) were estimated by linear fits to the first five measurements (open circles) and to the remnant seven (closed circle) to highlight the differences in rate of embryo growth as minimum temperatures dropped regularly below 5 °C.

Storage experiment

Storage conditions had a significant effect on final seed germination (one way ANOVA p value > 0.05). A post hoc test revealed significant differences between the two storage treatments at 80% RH and all the others (Fig. 7), such that at 80% RH there was 100% seed mortality after six months.

A significant difference was detected when final germination was compared across

treatments. Final germination percentage was zero in all the seeds stored at 80% RH. Within the other treatments applied, only 12 months of storage at 60% RH significantly decreased final germination compared to the control (61% vs 98%, $p < 0.001$), (Fig.7). Seeds stored for six months at 60% RH maintained high viability but when seeds were kept in the same condition for one year their TZ viabiliy and final germination decreased to 54 and 61% respectively. (NB the two proportions are different because the tests were performed on different samples).

Finally, storage condition had an effect also on the onset of germination. Germination began 29, 10, 15 and 4 days earlier than the control in the 6 m 15% RH, 1y 15% RH, 6 m 60% RH and 1 y 60% RH treatments respectively. In contrast, the final germination of the first three was not significantly different from the control (Fig. 7 and 8).

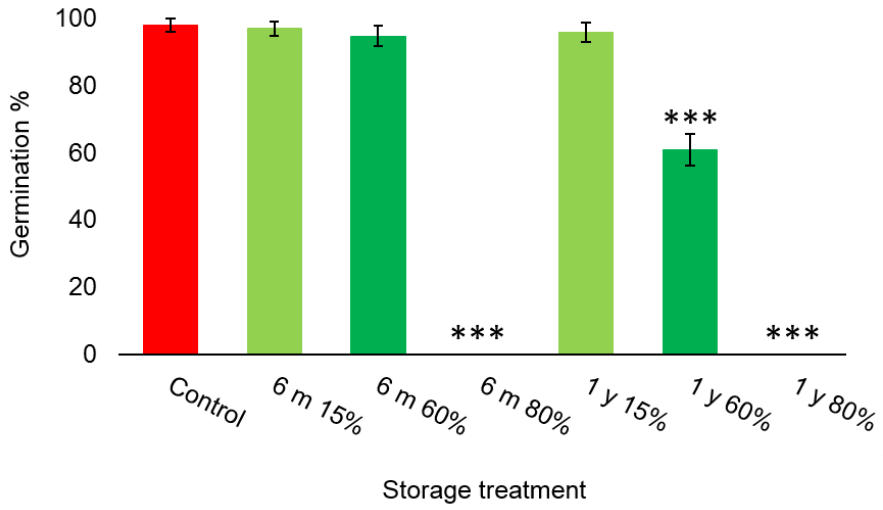


Fig 7.: Final germination at 5°C after storage at 20°C and 15-80% RH for 6 months (m) or one year (y). *** indicate significant ($p < 0.001$) difference with the control. Vertical bars indicate the binomial confidence intervals.

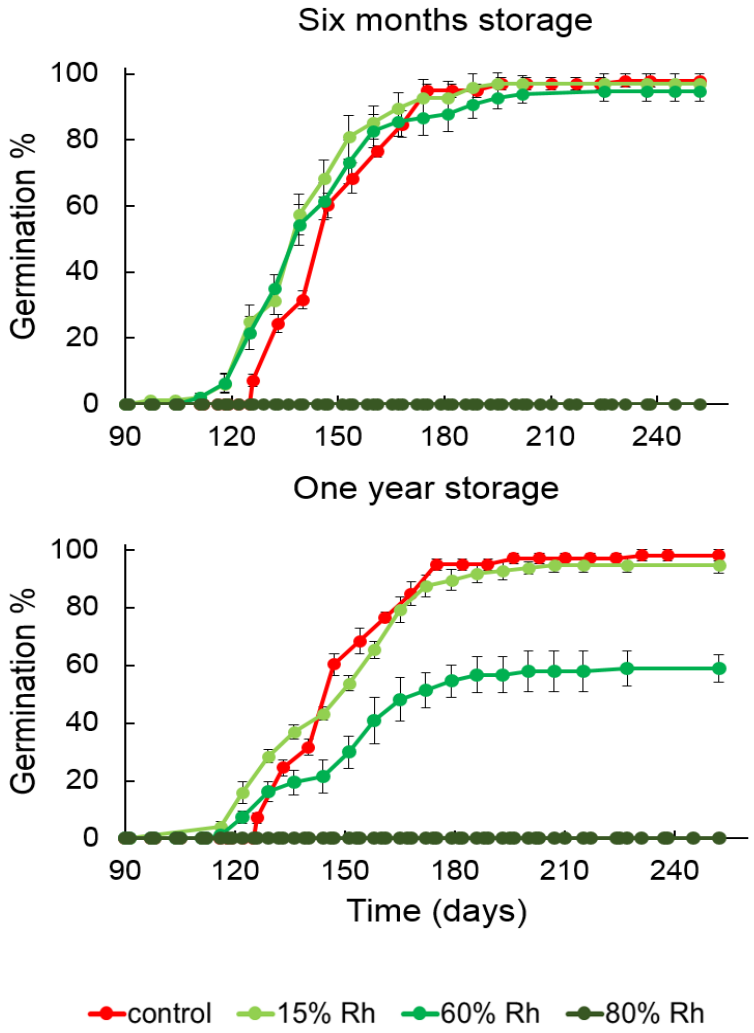


Fig 8: Germination progress curves at 5 °C after storage treatments at different RHs. Germination was not recorded until 97 days after sowing, explaing why the time series on the graph begins 90 days after sowing.

Dessication tolerance of imbibed seeds at different stages of embryo development

Visible germination was evident around 98 days after sowing in the control, and this was the time when the last dehydration treatment (84 days after sowing) was imposed (Fig. 9). In other words, an assessment was made as to whether seed desiccation tolerance was

lost when embryos were closer to the size for radicle emergence. The relative embryo size reached when sampled at the time of the last desiccation treatment was more than 50% the length of the endosperm.

The desiccation treatments had a significant effect on the final germination scored 462 d after. Final germination was significantly lower for seeds dehydrated to 15% RH after 84 days of embryo growth compared with the control (53% vs 98%, $p > 0.001$), (Fig. 10). The proportion of fresh but non germinated seeds was higher in the seeds desiccated after 84 days of embryo growth within the seed (Fig. 9) while the proportion of non viable seeds did not increase significantly (data not shown).

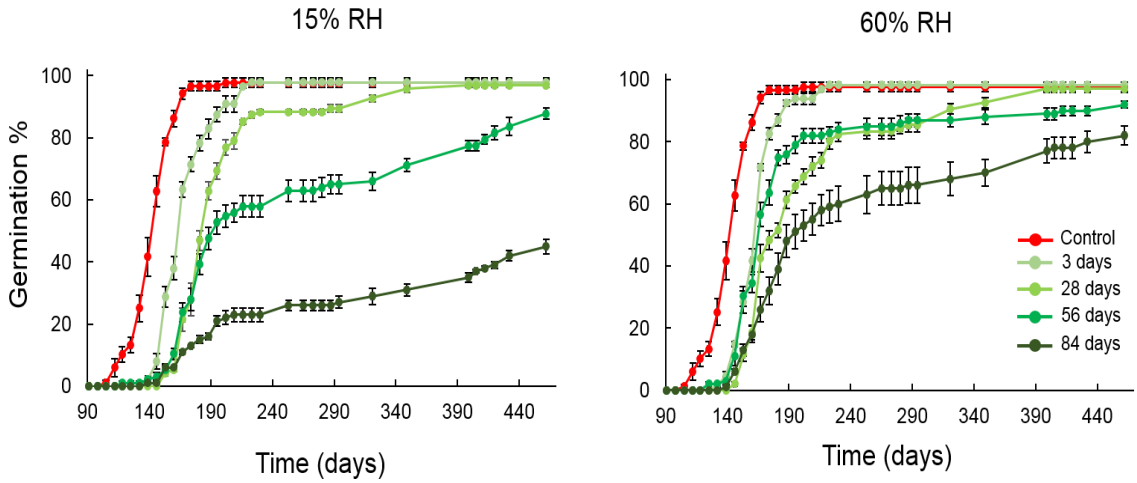


Fig. 9: Germination progress curves at 5 °C after fully imbibed seeds were dried back to 15 and 60% RH. The treatments were applied after 3, 28, 56 and 84 days from sowing. Germination is calculated only as the proportion of viable seeds.

The difference was not significant if the seeds were instead desiccated to 60% RH, altough a decrease in the final germination was recorded for all the treatments in which

desiccation was applied after 56 days since sowing.

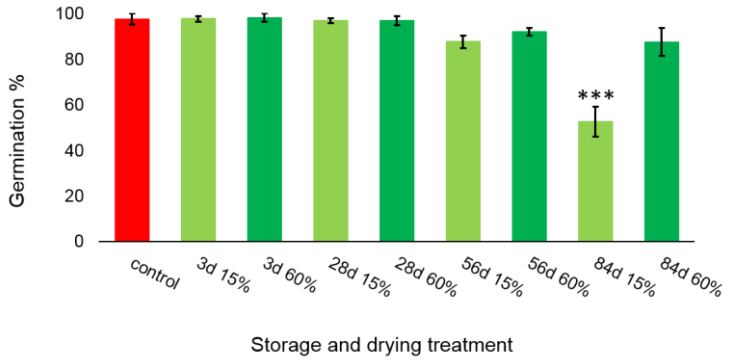


Fig. 10: Final germination at 5°C after desiccation to 15 or 60% RH at 20°C of seeds previously hydrated for 3-84 days at 5°C. *** indicate significant ($p < 0.001$) difference with the control. Vertical bars indicate the binomial confidence intervals.

DISCUSSION

Conopodium majus has a morphological component to its dormancy and embryo growth needs to be completed before the radicle can emerge. The initial relative embryo size on *Conopodium majus* was small compared with other members of the Apiaceae and this has been proven to be correlated with a slower germination rate and with the preference of the adult plant for shady habitats (Vandelook et al., 2012). A slower germination rate has an ecological meaning in stable and predictable habitats, such as a temperate deciduos forest, where the conditon for imbibition and embryo growth are available throughout the winter. Apiaceae from open habitats, where the environmental conditions can be more unpredictable, tend to have higher relative embryo sizes (Vandelook et al., 2012).

Conopodium majus showed a preference for low temperatures for embryo growth around 5°C, but was also able to germinate to a consistent proportion even if kept imbibed at 0 °C. In contrast, embryo growth was inhibited by temperatures > 10 °C. The optimal

temperature for embryo growth enables higher and faster germination and with no developmental arrest of embryo growth from the start of imbibition. The species can not therefore be classified as morphophysiologically dormant (MPD, sensu Baskin and Baskin, 2004) because, according to their classification of dormancy, there should be no embryo growth within 28 days and an increase in relative embryo size was recorded already after 14 days from sowing. The seeds therefore appear to have only morphological dormancy (MD). A similar behaviour has been reported by Mondoni et al. (2008) for the woodland indicator species *Anemone nemorosa* (Ranunculaceae), whose embryo started to grow immediately after dispersal. Differently from *Anemone nemorosa*, that responded positively to a warm stratification period (Mondoni et al., 2008), embryo development in *Conopodium majus* is inhibited by temperatures higher than 10 °C (data not shown) and growth starts only when temperatures become cooler in late autumn (Fig. 6).

In contrast with other woodland species, e.g., *Anemone nemorosa, Corydalis solida, Narcissus pseudonarcissus* and *Scilla bifolia* (Mondoni et al., 2008; Newton et al., 2013, 2015; Vandelook and Van Assche, 2008a, 2009), that possess MPD and whose embryos start to grow when the temperatures are still relatively high, *Conopodium majus* embryos only begins to growth when the temperatures drop below autumn levels.

Between other woodland Apiaceae that do not require warm stratification for embryo growth, *Sanicula europaea* shows a very similar behaviour regarding the coincidence between a low (5°C) optimal embryo growth and germination temperature and the lack of developmental arrest between the two phenomena (Vandelook and Van Assche, 2008b). *Aegopodium podagraria* and *Chaerophyllum temulum* (Vandelook et al., 2007, 2009), that also require cold temperatures fore embryo growth, can germinate over a wider range of temperature once the internal growth of the embryo within the seed is completed. Our results demonstrate in *Conopodium majus* thet the temperatures that

better promote embryo growth does not fully correspond with the temperature that promote cotyledon emergence. In fact, once the radicle is emerged the development of the seedling occurs at a faster rate if temperatures are higher than 5°C. Seedlings will eventually emerge also at a temperature of 5 °C but at a slower pace than at higher temperatures.

The majority of the Apiaceae are reported to have requirements for cold stratification and species from temperate zones germinate and emerge mainly in spring (Grime et al., 1981; Lovett Doust and Lovett Doust, 1982). In the field the time required to reach 50% germination can be up to six months, from dispersal in late summer/autumn to germination in early spring. The phenology of germination and seedling emergence in *Conopodium majus* is comparable to other woodland specialists (Chapter 2) whose germination is finely tuned with the seasonal environmental changes in temperate forest. That is, germination is regulated to happen in early spring when the canopy is still open and few herbaceous understory species have emerged. Thus, the competition for light and other resouces is still low. The species can be regarded as a spring ephemeral because it develops its reproductive cycle between January and May and, after this date, only the dry fruits bearing stems remain visible.

Embryo growth and gemination were significantly higher when seeds were exposed to a constant temperature regime of 0 or 5 °C rather than at temperatures fluctuating between 10 °C during the day and 0°C during at night. A possible explanation of this results is that 10 °C is supra-optimal for embryo growth in this species and, therefore, its developement was significantly slower for half the time of the experiment. The detection of fluctuating temperatures is a gap sensing mechanism and some species have a threshold of daily temperature fluctuation that can trigger their germination (Pearson et al., 2002). In contrast, species adapted to germinate in low light conditions and that possess big seeds

can also germinate in absence of fluctuating temperatures (Chapter 2). In the case of *Conopodium majus*, another factor to take in account is that embryo development takes place in coldest month of the year when the span of daily temperature fluctation is lower. Both embryo growth rate and germination are accelerated by the addition of GA_3 and by moderate amounts of nitrate but the differences with the control treatment were not significant. However, at the highest concentration of KNO_3 used, the final germination level appeared lower (Fig. 4) and it is not to be excluded that germination can be significantly depressed if even higher concentrations of nitrates are used. *Conopodium majus* in fact is a species adapted to oligotrophic soils (Grime et al., 2007) and a higher concentration of nitrogen can be a signal of potentially increasing competition. However, the decrement in final germination with the higher concentration of KNO3 may have been due also to a greater growth of bacteria and fungi that could have competed for oxygen in the environment of the Petri dish.

Seeds of *Conopodium majus* tolerate desiccation to 15% RH and do not loose viability for at least one year if stored dry at a temperature od 20°C. However, if the condition of storage are of higher humidity (60% RH), as it can be the case if measures to control the air RH are not implemented, the seeds loose half of their viability in one year. They completely lost viability and get infected by mold if kept at RH of 80%. To store seeds of this specie is therefore adviceable to control the conditions of seed storage or, in case this is not possible, to regenerate the collection by sowing them within six months from collection.

Seeds of *Conopodium majus* are not reported to form a persistent soil seed bank (Thompson et al., 1997) and the observations of this study point in the direction that is not possible to arrest the process of embryo development without reducing the viability of the seed once there has been extensive embryo growth. Therefore, all the viable seeds

produced in a year are likely to germinate within the first winter after dispersal and not become incorporated into the soil seed bank. Even if it has been demonstrated that species with morphological dormancy can persist in the soil for some years (Hawkins et al., 2007) a decrease in desiccation tolerance as the embryo develops is not uncommon. For example, *Panax ginseng* seeds exhibit greater desiccation sensitivitiy after drying back at different stages of embryo growth (0.10, 0.46 and 0.91 E:E ratio) (Han et al., 2016). As in the case of *C. majus*, seeds of ginseng exposed to higher level of desiccation showed a more marked decrease in germination. Similarly, in a study on the germination ecology of the Apiaceae *Lomatium dissectum* (Scholten et al., 2009), a species subject to seasonal habitat drought, seeds were tested for desiccation tolerance after a period of imbibed cold stratification. The treatment resulted in 30% decrease in viability and the surviving seeds shown a reduced germination rate that suggested entry into secondary dormancy. Their results are in agreement with the finding of this study where a decrease in final germination percentage and in germination rate was observed when drying back was imposed after the seed embryos grew to five times their initial size, although *Conopodiumn majus* seeds did not have a corresponding decrease in viability.

The effect of the degree of drying back was significantly different, with 15% RH being more stressful as there was a higher decline in the germination rate compared with drying to 60% RH. This deeper drying condition could be indicative of prolonged drought, as can be found in the seasonally dry weather to which *Lomatium dissectum* is exposed. Although such conditions are not likely to occur on most of the European Atlantic distribution of *Conopodium majus*, mountain populations at the southern boundary of the distribution range experience a continental Mediterranean climate where the capacity to survive desiccation and defer germination to the next year can be an advantage.

Dicotyledon species that develop seedlings with only one cotyledon is a rare phenomenon

known as "pseudomonocotyly." This morphological conditions has been described for a few families (Titova, 2000) and has been reported for many Apiaceae genera, where it appears to be a characteristic of tuberous geophytes (Degtjareva et al., 2009; Kljuykov et al., 2014; Haccius, 1952). It has also been reported earlier for *Conopodium majus* (Thomson, 1988). In his review on pseudomonocotyly, Heines (1979) affirmed that the origin of a single cotyledon in some Apiaceae species is a consequence to their adaptation as geophyte. The necessity to push the radicle and the hypocotyl deep in the soil may have led to the evolution of a cotyledonary tube originated by the fusion of the cotyledon petioles. He therefore advocated the theory of a fusion of the two cotyledons and not the suppression of one of them. A detailed anatomical description of embryo and seedling development in pseudomonocotyledonar Apiaceae has been only produced to date for the Asiatic genus *Acronema* (Kljuykov et al., 2014). However, no details have been provided so far on the development of the single cotyledon during its development inside the seeds. In a ripe seed of *Conopodium majus* the average initial embryo length is of 0.18 mm and at this stage the differentiation into a radicle and cotyledon is not yet clear. In seeds cold stratified for 56 days, however the distriction between radicle and cotyledon is evident but still there is no clear subdivision into two cotyledonary sheaths (Fig. 2, b). The fusion of the two cotyledons results even more evident in the young seedlings (Fig.2, b) so it can be concluded that *Conopodium majus,* in agreement with Heines (1979) statements; there is a single cotyledon that is derived by the fusion of two cotyledons and not by the abortion of one of them.

CONCLUSIONS

In conclusion, *Conopodium majus* seeds have morphological, physiological and germination characteristics that suit its natural environment in temperature woodlands.

The small embryo is capable of slow growth within the seed at low temperatures so that germination is timed for early spring. During embryo growth over winter the tissues become a little more sensitive to drying back under conditions indicative of very open sites and thus not optimal for seedling establishment. The evolution of fused cotyledons likely enables the radicle and the hypocotyl to be pushed deep into the soil and thus reduce the risk of such drying occurring.

AKNOWLEDGENTS

Many thanks to Giles Laverack and Fiona Hay for collecting and sending the seeds and for Maria Marin for realizing the germination phenology experiment and sending the seeds every two weeks. Thank you also to Keith Manger, Pablo Gomez Barreiro and John Adams for their precious technical help, to Aude Vernet for the Micro CT scan and to Eduardo Fernàndez Pascual and Hugh Pritchard for their advice and for revising the manuscript. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°607785.

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CHAPTER 5

FUNCTIONAL BIOGEOGRAPHY OF THE THERMAL THRESHOLDS FOR EMBRYO GROWTH IN *CONOPODIUM MAJUS*

ABSTRACT

Conopodium majus (Apiaceae) is a species with seeds that have morphological dormancy in which the development of the embryo is strictly controlled by temperature. There is no developmental arrest between embryo growth and germination. The species has an Atlantic oceanic distribution and its environmental plasticity defines it as an indicator of both woodlands and oligotrophic meadows. To date very few studies have approached the development of thermal models measuring the embryo development in a MD species and none have compared the regulation of embryo growth across the latitudinal distribution range of a species. Quantifying dormancy / germination in this way is therefore novel. Nine populations of *Conopodium majus* were sampled across a latitudinal transect from Spain to Norway. The temperature control of embryo growth was investigated in the laboratory and in the field and compared with the local climate. Optimal temperatures for embryo growth and germination varied, across all populations, between 2.5 and 5.2˚C, with ceiling temperatures between 12 and 20.5 ˚C and base temperatures between -6.6 and -2.7 ˚C. Germination in the field peaked in the months of January and February. The limiting factor to embryo growth related to higher temperatures and a significant correlation was described between the ceiling temperature and the bioclimatic environment of each population. In contrast, the optimal and base temperature were independent of local climate. The method used to characterize *C. majus* embryo development across its latitudinal range could give insights on how different scenario for predicted climate change can affect the regeneration of this species which is an important component of ancient woodlands in temperate Europe.

KEYWORDS: Cardinal temperatures for germination, Climate change, *Conopodium majus*, Embryo:endosperm ratio, Morphological dormancy

INTRODUCTION

The three aims of functional biogeography are to describe the distribution of functions along environmental gradients and across spatial scales; to use this information to explain the geographic distribution of organisms; and to predict their responses to environmental changes using trait-based predictive models (Violle et al 2014). A relevant aspect of plant function that has been underutilized by biogeographical studies is the physiological thermal control of plant reproduction (Bykova et al 2012), and especially seed germination. The temperature to which imbibed seeds are exposed affects their germination rate (Heydecker, 1977). This phenomenon can be described by the definition of the "cardinal" temperatures, i.e., the optimum temperature (T_o), at which the germination rate is maximal and the base (T_b) and ceiling (T_c) temperature that are, respectively, the coldest and the highest temperature at which the progress towards germination occurs. The measurement of these temperatures for a given species enables prediction of seed germination rate under different temperatures and, using a thermal time approach (Covell et al., 1986; Ellis et al., 1986; Hardegree, 2006; Pritchard and Manger, 1990), to predict the germination timing of a given proportion of the population based on heat accumulated and the time spent between T_b and T_c. Therefore, the cardinal temperatures are key parameters to develop models that explain the contribution of regeneration environmental envelopes on species distributions and responses to climatic changes.

In many species however, seed dormancy prevents seeds from germinating even in the presence of suitable conditions, with the objective of avoiding the exposure of seedlings to unfavourable environmental conditions for their development. The depth of dormancy and the timing of its release can be regulated by physical factors, as is the case of species with a hard seed coat that is not permeable to water, by chemical inhibitors, and by

temperature change (Probert, 2000). In particular, exposure of imbibed seeds to cold temperatures increases their response to gibberellic acids (Baskin and Baskin, 2014) and can reduce the requirement for other environmental signals (light, nitrate or temperature fluctuation) (Probert, 2000). In temperate environments, with a pronounced seasonality, the requirements for cold stratification can programme the seedlings to emerge after winter.

A particular case of seed dormancy occurs when the embryo is not completely developed and needs to grow to a critical size before germination can occur (morphological dormancy, MD) (Baskin and Baskin, 2014). Morphological dormancy is highly conserved in plant evolution (Forbis et al., 2002, Willis et al., 2014). Temperature is the main driver of morphological dormancy release, influencing the rate of development of the embryo, a mechanisms that allows the precise timing of germination (Stokes, 1952, Porceddu et al., 2017). The cardinal temperatures for the growth of the embryo can correspond or not with the ones for germination. In several species, such as *Aegopodium podagraria*, *Anthriscus sylvestris* and *Chaerophyllum temulum*, germination can occur over a wider range of temperatures after cold stratification has occurred (Baskin et al., 2000, Parthyal et al., 2009, Vandelook et al., 2007, Vandelook et al., 2009). Others like *Conopodium majus* and *Sanicula europaea* require a low temperature (5°C) both for embryo growth and germination (Chapter 4, this thesis; Vandelook and van Assche, 2008).

Biogeographical variation in several germination traits is well documented. For example, seeds of the tree *Aesculus hippocastanum* collected from across Europe had lower base temperatures for germination at the southern end of the distribution (Daws et al., 2004). The requirements for cold stratification can vary according to the local climate, as it has been demonstrated that populations from habitats with longer winters require a longer

period of cold stratification compared with populations from milder habitats (Allen and Meyer, 1998). However, to our knowledge much less research has been dedicated to intraspecific variation in morphological dormancy and embryo growth. Mondoni et al. (2008) compared morphological dormancy between mountain and lowland populations of the temperate woodland forb *Anemone nemorosa*. Embryo size at dispersal was similar in all the populations. Nonetheless, embryo growth at cool temperatures was faster in the mountain population. This suggest a capacity of morphological dormancy to adapt to local conditions, either by local adaptation or phenotypic plasticity, analogous to that of physiological dormancy. Further research is warranted, to measure the thermal thresholds for embryo growth across wider geographical scales, and investigate whether they vary in association with environmental gradients. To our knowledge, a study on temperature regulation of embryo growth across the whole latitudinal distribution of a species has not being performed yet.

Previously, we demonstrated that seeds of the geophyte *Conopodium majus* have morphological dormancy, and that embryo growth and germination occur continuously at a narrow range of temperatures around 5 °C (Chapter 4, this thesis). Such narrow and low temperature requirements for embryo growth can offer several benefits to conduct a functional biography study of embryo growth. First, such precision in thermal control in a relatively wide latitudinal distribution (from Spain to Norway) can expose the species to shifts in its temperature germination niche in a scenario of changing climate (Walck et al., 2011). Second, since embryo growth and germination occur seamlessly, it is possible to develop a relatively simple model based on embryo growth as a response to temperature. Third, since the temperature range is so narrow it will be possible to consider the role of both T_c and T_b in the regeneration niche.

In this study, data is presented on the functional biogeography of *Conopodium majus* in relation to the physiological thermal thresholds for embryo growth, a key reproductive process in many plant species. How these thresholds vary across the species distribution is assessed with the aim of testing the following two hypotheses:

1) Seed morphological traits and germination traits varies across populations sampled on a latitudinal transect;
2) Geographical and climatic factors can influence the requirements for embryo growth and germination in this species across its distribution.

MATERIALS AND METHODS

Study species

Conopodium majus (Apiaceae) is a geophyte with an European Atlantic distribution that ranges between Southern Spain to Central Norway (Tutin et al, 1968; http://www.gbif.org). As many *Apiaceae*, seeds of *C. majus* possess undeveloped, linear embryos (Martin, 1946) and germinate when they reach a length close to the full length of the endosperm (Chapter 4). For this reason, in this study the relative embryo size (embryo length/endosperm length, hereafter referred to as "E:E ratio") is used to describe embryo development and germination capability is defined as the point at which the E:E ratio is ≥ 1, i.e., the embryo is now guaranteed to germinate under suitable conditions. In *C. majus*, germination has been observed to occur both at 0 and 5 °C with a similar rate of embryo growth (Chapter 4, this thesis). Such low temperature requirements are indicative of germination in winter. For species adapted to develop in woodlands as well as oligotrophic meadows, the control of the germination process in this way can constitute

an adaptive advantage, allowing the seedlings to establish before the development of a tree canopy or of competing vegetation.

Seed collection

Mericarps of *Conopodium majus* were collected in the summer of 2016 from nine naturally occurring populations sampled across the western European latitudinal range of the species (Fig. 1).

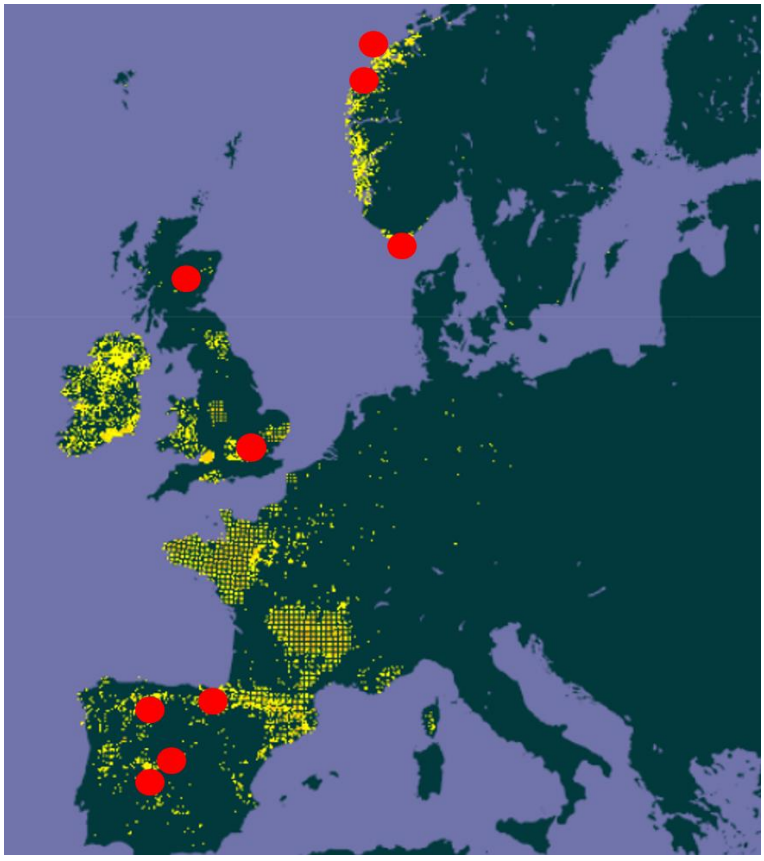

Fig. 1: Latitudinal transect across Europe of population samples used to study embryo growth in *Conopodium majus*.

Since the seed cannot be separated by the fruit in this species, the dispersal unit will be referred hereafter as a "seed". A population was sampled only if it consisted of at least 200 individual plants. Seeds were sampled from 50 plants within the population to ensure

a representative sample of the genetic variability of the population was secured. At least 4000 seeds were collected from each population. The two southernmost populations (coded "CHO" and "TRE") used in this study were sampled in the Gredos mountain range, in central Spain and belonged to *Conopodium majus* subsp. *marizianum* (Samp.) López Udias & Mateo. Another population from the same subspecies (coded "LEO") was sampled in the north of Spain on the Cantabrian Mountains. The nominal subspecies in fact only reaches the Pyrenean mountain range (http://www.gbif.org/) and was sampled in the Basque Country, Spain ("BAS"), south of England ("WAK"), Scotland ("SCO"), south of Norway ("FLE") and central Norway ("BER" and "HER") (Table 1). All seeds were collected between July and August 2016 and the experiments started within three weeks from seed collection. To avoid any change in morphological dormancy (by embryo growth at high humidity) seeds were kept at below full hydration under ambient condition on a laboratory bench until the beginning of the tests.

Table 1: Provenance of seeds used in the experiments

Population	Country	Location	Latitude	Longitude	Altitude (m.s.l.)
HER	Norway	Herdla	60 34'29.784" N	4 56' 53.627" E	37
BER	Norway	Bergen	60 20' 7.35 N	5 22' 17.79" E	97
FLE	Norway	Flekkeroya	58 4'5.34" N	7 59' 53.56" E	19
SCO	UK	Dalreoch Farm	56 44' 47.36" N	3 32' 25.03" W	252
WAK	UK	Wakehurst Place	51 04' 12.79" N	0 05' 28.28" W	114
BAS	Spain	Ondarre	43 01' 42.8" N	2 03' 55.7" W	809
LEO	Spain	El Tendero	42 54' 26,62" N	5 49' 25,87" W	1426
CHO	Spain	Central del Chorro	40 18' 26.17" N	5 40' 09.39" W	1398
TRE	Spain	Tremedal	40 22' 00.5" N	5 37' 57.20" W	1555

Initial measurements

Each collection was cleaned from debris and empty seeds were removed using a gravity seed separator machine. From each population, 10 seeds were selected randomly and

allowed to rehydrate overnight at 20℃ and 100% RH. The seeds were then placed on 1% agar-water for 24 hours to become fully imbibed and reactivate their metabolism. Thereafter, seeds were prepared for vital staining with 1% aqueous solution of triphenyl tetrazolium chloride (TZ). A slice of seed coat was removed from the dorsal surface of each seed using a scalpel and seeds were incubated in TZ solution at 30℃ in the dark for 24 hours. Each seed was then cut longitudinally and the embryo was extracted. Embryos and endosperms were photographed using a camera (Carl Zeiss Axiocam Colour) mounted on a Stemi SV 11 Microscope (Carl Zeiss, Welwin Garden City, Herts, UK) microscope and their lengths measured using the software Axiovision 3.1.2.1 (Carl Zeiss Vision GmbH). The initial relative embryo length was measured only for the seeds that stained red with the TZ, i.e., indicating viability; unstained seeds / embryos were discarded. Relative embryo size was used because it describes the growth of the embryo regardless the size of each seed.

From each population 100 seeds were placed in a controlled humidity room at 15% RH and left to dry. The dry seed weight of 100 replicates / individuals for each population was measured using a precision scale. The differences in seed dry mass and initial E:E across populations were represented with barplots (Figures 2 and 3).

Embryo growth in controlled temperature conditions

From each population and treatment, 16 subsamples of 15 seeds each were randomly taken and sown in separate, 8 cm diameter Petri dishes containing 1% agar-water substrate. Seeds were sown at -2.5 ℃, 0 ℃, 2.5 ℃, 5℃, 7.5 ℃ and 10℃ in incubators with a daily light regime of 12 hours of light and 12 hours of darkness. Every 14 days one subsample for each population and treatment was retrieved and the 15 seeds were placed for 24 hours in 1% TZ solution at 30℃ in the dark, after a slice of the seed

coat was removed. From this subsample, the embryo and endosperm length of 10 viable seeds was measured. In this species the radicle emerges when the embryo is fully grown and has reached the same length as the endosperm. Therefore, an E:E value of 1 was assigned to all germinated seeds. Seed measurement was stopped when the seeds ceased germinating. The experiment continued for 224 days, until all the 16 subsamples assessments were concluded.

Embryo growth in natural conditions

Embryo growth in the soil was recorded for three population representing the southern (CHO), middle (WAK) and northern (BER) distribution of the species. The experiment was replicated in two locations where *C. majus* naturally occurs: at Wakehurst Place, England (site of collection of the "WAK" population); and in a meadow on the periphery of Bergen, Norway (close to the site of collection of the "BER" population). Sixteen subsamples of 20 seeds for each population and experimental site, were mixed with 20 g of soil collected at the site and passed through a 3 mm sieve. Seeds and soil were placed in mesh net bags and buried at a depth of 5 cm. A datalogger that recorded soil temperature every 30 minutes was placed in each location (Tinytag View 2, Gemini Dataloggers Ltd., Chichester. UK and EasyLog USB-2, Lascar Electronics, in Norway). The seeds were buried in England on 1st September 2016 and in Norway on 14th September 2016. Every 14 days a bag for each population was retrieved and the soil washed. Seed bags buried in Norway were shipped to England for measurements. All the seeds retrieved were prepared for TZ staining and their embryo and endosperm lengths measured. It was easiest to measure the seeds when most of the seeds were not germinated. With an increasing number of germinated seeds and seedlings, the amount of empty seed coats left in the soil bags made it difficult to distinguish between mouldy

or germinated seeds. At this point, the experiment was terminated, representing nine measurements in Norway and thirteen in England.

Calculation of a thermal model for embryo growth

The average E:E ratio of 10 seeds for each population*temperature*time combination was calculated. All the temperatures for a same population had the same initial E:E ratio value a time of 0, while the maximum value was fixed at 1, after which the seed was considered to be able to germinate based on the evidence presented in Chapter 4. Since the data followed a sigmoidal growth distribution, except the treatments at -2.5 °C, a logistic model was fitted to each population * temperature combination using the software OriginLab 9.0. The models of each population were bounded between the initial value of E:E for that population and 1. A linear model was fitted to the -2.5°C treatments. From the equation of the logistic and linear models, it was possible to calculate the time expressed in days (tr) at which each temperature * population combination would have reached the following deciles of relative embryo size: 0.3, 0.4, 0.5, 0.6 and 0.7. Deciles < 0.3 could not be calculated because they were under initial E:E. Deciles > 0.7 were not calculated to keep the symmetry of the analyses regarding deciles of the population. For each treatment, the embryo growth rate was calculated as 1/tr.

For each population and decile, embryo growth rate was plotted against temperature. Each dataset was visually divided in a sub-optimal and supra-optimal range, using the point with the highest value of 1/tr as the dividing point. Liner regressions were fitted separately to the sub- and supra-optimal ranges. The intersection with the temperature axis of the sub-optimal and supra-optimal regression are, respectively, the base (T_b) and the ceiling (T_c) temperatures; these are the temperatures below and above which the embryo growth

rate was equal to 0. The optimal temperature (T_o), defined as the temperature at which the rate of embryo growth is estimated to be fastest, is the x-coordinate of the intersection point between sub-optimal and supra-optimal regressions. Then, for each population, the cardinal temperatures (T_b, T_c and T_o) were averaged across all the deciles calculated to define an average value of the population (Ellis et al., 1986). The regression lines of each decile were recalculated and forced to pass through a common origin defined by the average T_b (for the sub-optimal regressions) or the average T_c (for the supra-optimal regressions) (Hardegree, 2006). For the three populations of *Conopodium majus* subsp. *marizianum*, only the cardinal temperatures calculated for the relative embryo size of 0.4, 0.5 and 0.6 were used, because it was not possible to fit a supra-optimal regression to the 0.3 decile.

The slopes of these new linear regressions were then taken as a reciprocal to estimate the sub-optimal (θ_b) and supra-optimal (θ_c) thermal time for embryo growth. θ, expressed in degree days (°Cd), indicates the amount of thermal time units above T_b (θ_b) or below T_c (θ_c) that the seed has to accumulate for the embryo to reach successive E:E deciles. For each population, the deciles were plotted against θ_b and θ_c, expressed both as their value and as the natural logarithm of the value, and linear regressions were fitted to the data. The regressions fitted to θ and to log(θ) were compared in each case by their R^2 (Hardegree, 2006). The regression models with the highest R^2 were chosen to represent the rate of embryo growth as a function of thermal time for each population.

Validation of the model with field data

In order to compare the embryo growth predicted by the determined thermal time model with embryo growth in natural conditions, embryo growth in the field sites was plotted

against time. A logistic regression was fitted to these curves, and from the equations, the t_r to reach every decile of relative embryo growth was calculated. The units of thermal time required by each population to reach every t_r during the field experiment were calculated for both field locations using the data recorded by the loggers. In order to account for every temperature fluctuation during the day, the thermal time was expressed in "°C 30 min" and the heat accumulated by the seed was calculated for every 30 minutes data-logged. The difference (ΔT) between each temperature record and the population T_o (averaged between deciles) was summed. When the temperature was higher than the average T_c or lower than the average T_b the heat accumulated was considered = 0 and the difference (ΔT) between each temperature record and the T_o was summed.

The time necessary in the field to accumulate enough heat to reach the thermal time necessary for each t_r was compared with the t_r estimated from the embryo growth data. The time (in days) needed to sum enough heat to reach the θT_b and θT_c calculated in the model, for each t_r decile ($t_{r\ model}$) in each population was compared with the time needed by each population to reach the same decile of relative embryo growth in the field ($t_{r\ field}$). These estimates were then graphically compared expressing the different t_r in function of E:E (Fig.6).

Relationship between embryo growth and germination

Germination was scored for each independent sample before measuring the relative embryo size, and expressed as percentage of germinated seeds vs time. For each population, the germination data for the treatments at 2.5 and 5°C were fitted with the Boltzmann equation using the software OriginLab9. The other temperatures were not used because germination was too slow. For each population, from the fitted Boltzmann equation the day to reach 50% germination (t_{g50}) was calculated. The t_{g50} was then used

to calculate the corresponding E:E ratio at the same day using the logistic regression of the E:E data for the same treatment. For each population, the average E:E ratio corresponding to the t_{g50} for germination at the two temperatures used was displayed as the average E:E ratio for 50% germination in that population (Table 2). The average between all the populations represented the average for the species.

Relationship between environmental data and germination traits

The relationship between embryo development and seed germination traits and geographical and bioclimatic data was explored for each population. A data matrix was built including latitude, altitude, seed dry mass, initial E:E ratio, cardinal temperatures for each population and the following bioclimatic variables: average temperature of the months during which embryo growth occurs (Sep-Feb), precipitation of the driest month, average maximum temperature of the hottest month and minimum average temperature of the coldest month. The latter three variables were chosen because they can represent a limiting factor to plant life. Data was checked for autocorrelation using the Pearson correlation coefficient in order to exclude the variables with a strong autocorrelation. Finally a PCA was run on the dataset, scaling the axis (Fig.7).

RESULTS

Initial measurements

The initial relative embryo size ranged from an average value of 0.10 (±0.29 SD) for the population BER to an average value of 0.19 (±0.29 SD) for the population CHO (**Fig.2).**

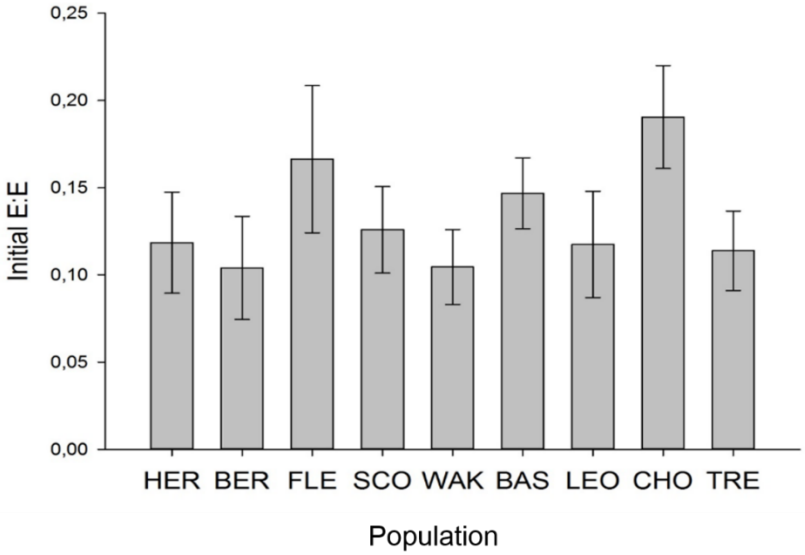

Fig. 2: Average initial E:E in seeds of all populations of *Conopodium majus* studied. Vertical bars indicate the standard deviations.

Average seed dry mass ranged just under two-fold from 1.20 mg (±0.38 SD) in TRE to 2.03 mg (±0.53 SD) in FLE (**Fig. 3**):

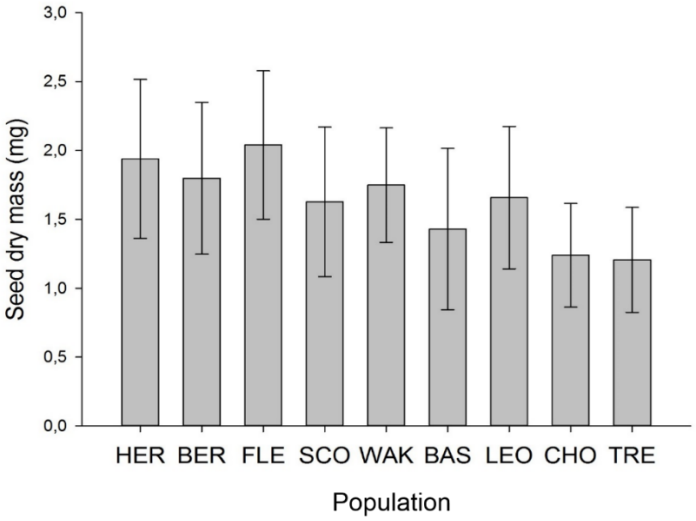

Fig. 3: Average seed dry mass for all populations of *Conopodium majus* studied. Vertical bars indicate the standard deviation.

Embryo growth in controlled temperature conditions

The seeds survived cooling to -2.5 C but the embryo did not grow at this temperature; while it can grow and germinate at 0 °C. The increase in embryo size in some populations at the -2.5 °C treatment was therefore due to temperature fluctuations in the incubator temperature, presumably beyond its specification (i.e., ± 2°C). The peak that was seen in this treatment for some of the populations occurred because of a fault of the incubator, when ice formation close to the ventilation system prevented air flow circulation leading to an increase in temperature. When the temperature of -2.5 °C was re-instated the growth of the embryo stopped again.

The rate of embryo growth was strictly dependent on the temperature and the increase in embryo size can be appreciated already after 14 days of imbibition. For all the populations, the temperature treatments with the highest rate of embryo growth were 2.5 and 5 °C. Clearly 0 °C is sub-optimal for embryo growth rate, and 7.5 and 10 °C were supra-optimal (**Fig.4**).

Relationship between embryo growth and germination

The first germination was seen after 84 days of imbibition in the four Spanish populations at the temperatures of 0, 2.5 and 5 °C. The populations from WAK and BER first germinated after 112 days of imbibition. The last population to begin germinating was SCO, after 126 days of imbibition.

Germination occurred when the embryo reached the same length of the endosperm (E:E=1) and an average E:E = 1 corresponded to 100% germination in the sample.

The treatments that, after 32 weeks of imbibition had the highest average germination

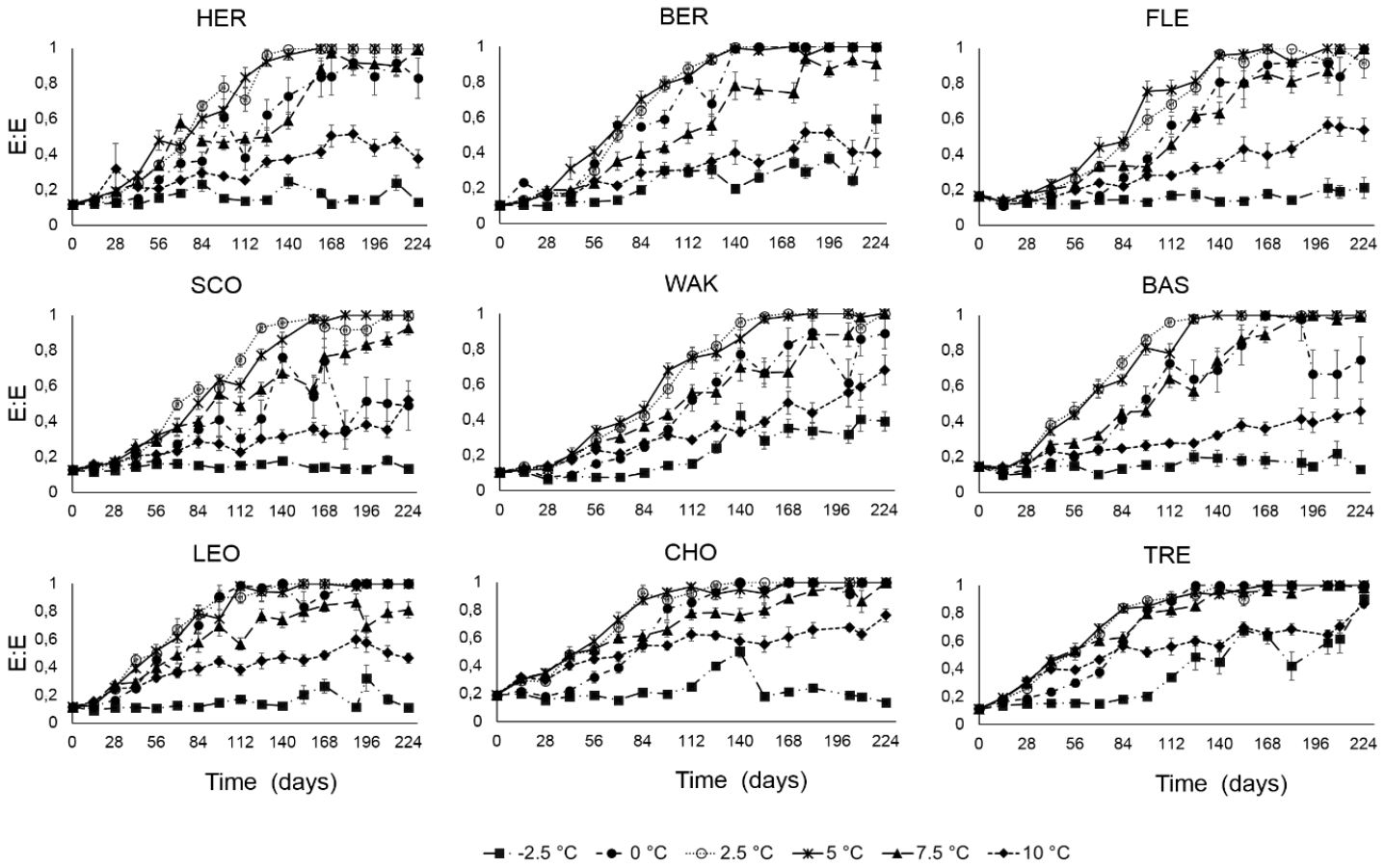

Fig. 4: Patterns of embryo growth (E:E ratio) for all the populations seeds of *Conopodium majus* and all temperatures tested. Each data point represent the average of ten replicate (± SE).

across all the populations were 2.5 °C, and 5 °C with, respectively, 97.7 and 98.4 % of seeds germinated in the last sampling. The lowest germination was observed at -2.5 and 10 °C. The population that reached, across all the treatments, the highest average germination at week 32 (the end of the experiment), was TRE (80% ± 32 SD) while the lowest was achieved by SCO (59%, ± 42 SD).

The time to reach 50 % germination (T50g), interpolated with the Boltzmann equation ranged between 111 (BAS) and 147 (FLE) at 2.5 °C and between 116 (LEO) and 150 (SCO) at 5 °C. The values of E:E corresponding to the estimated T50 (**Table 2**) in these two treatments were averaged between population and temperatures to describe a value of 0.89 (± 0.02 SD) for the species.

Table 2: Correspondence between the T50g and the estimated E:E ratio on this date for the two treatments (2.5 and 5 °C) that resulted in the highest germination.

		2.5 °C	5°C		2.5°C	5°C		2.5 °C	5°C
T50g		123	126		133	150		115	116
R2 Boltzmann	HER	0.99	0.97	SCO	0.99	0.99	LEO	0.99	0.95
E:E at T50g		0.89	0.87		0.87	0.88		0.91	0.89
T50g		123	125		131	140		117	124
R2 Boltzmann	BER	0.99	0.99	WAK	0.98	0.98	CHO	0.99	0.99
E:E at T50g		0.91	0.90		0.85	0.87		0.93	0.93
T50 germination		147	132		111	118		127	133
R2 Boltzmann	FLE	0.99	0.99	BAS	0.98	0.99	TRE	0.94	0.97
E:E at T50g		0.90	0.88		0.90	0.88		0.92	0.92

However, when interpreting this analysis it is important to consider that the original data of E:E ratio were based on averages of 10 individual seeds. This mean that at the

T50g, only 50% of the seeds would have reached an E:E =1, corresponding to radicle protrusion, while the others would have had an E:E ratio lower than the average (Fig.4). The SD of the original data at T50g should then be taken into account when interpreting its correspondence with E:E ratio.

Cardinal temperatures for embryo growth

In the three populations of *C. majus* subsp. *marizianum* it was not possible to calculate the supra-optimal regression for the E:E 0.3 decile because, in the case of CHO and TRE there was no decrease in the embryo growth rate with increasing temperature (i.e., there were insufficient data points against which to fit the line) and, in the case of LEO, the regression had a very low slope that led to unrealistically high temperatures. Therefore, for these populations the cardinal temperatures were calculated on the average of the 0.4, 0.5 and 0.6 deciles.

The sub-optimal and supra-optimal regression lines fitted estimated T_b and T_c to vary between deciles (c.f. the same regression in which the line was forced to pass through a value of T_b and T_c that was averaged between deciles). Since the R^2 of these last regressions was higher, the inverse of their slopes was used to define the thermal time at different deciles of embryo growth.

Between populations, T_b varied between -2.63 (SCO) and -6.65 °C (BER). In addition, To ranged from 2.54 (LEO) and 5.23 °C (CHO). Finally, Tc spanned 12.08 (BER) and 20.54 °C (TRE) (Table 3). Such low T_b means that the embryo growth of *C. majus* in its natural environment is limited by the higher temperatures because the low T_b for this species is seldom reached. Therefore, since the seeds are dispersed in late summer, there is no embryo growth until the temperatures drop in autumn. The relatively low Tc is, for

this species, more indicative of environmental limitation for the embryo growth than is T_b.

Table 3: Cardinal temperatures averaged between deciles (± SD). In order to have a symmetric results around the middle value, if the lower deciles were excluded because too close to the initial embryo size, the higher ones were excluded too.

Population	T_b	T_o	T_c	Deciles used
HER	-4.01 ± 0.57	4.26 ± 0.80	12.90 ± 1.86	0.3 - 0.7
BER	-6.65 ± 0.62	4.58 ± 0.02	12.08 ± 1.32	0.3 - 0.7
FLE	-3.90 ± 0.14	4.50 ± 0.07	13.70 ± 0.71	0.3 - 0.7
SCO	-2.63 ± 0.38	2.80 ± 0.25	14.42 ± 2.47	0.3 - 0.7
WAK	-6.20 ± 0.89	4.59 ± 0.11	14.44 ± 1.72	0.3 - 0.7
BAS	-2.75 ± 0.10	2.69 ± 0.10	13.07 ± 0.93	0.3 - 0.7
LEO	-3.17 ± 0.06	2.54 ± 0.03	14.64 ± 2.23	0.4 - 0.6
CHO	-4.09 ± 0.59	5.23 ± 1.05	20.48 ± 9.09	0.4 - 0.6
TRE	-6.47 ± 0.41	4.86 ± 0.04	20.54 ± 7.25	0.4 - 0.6

Model selection

The R^2 of the models obtained fitting embryo growth and log-normal (log °Cd) were slightly higher than the R^2 of the model obtained using normal distributed thermal times (°C) (Table 4). The only exception was constituted by the population CHO, for which the best model fit was obtained using the non-transformed thermal time values, thus describing a linear increase of relative embryo size with accumulated heat.

Table 4: R^2 of the linear models fitted to the relationship between E:E ratio and Θ or log Θ for the suboptimal and supraoptimal regressions across the deciles from 0.2 to 0.9 E:E.

Population	ΘT_b	log ΘT_b	ΘT_c	log ΘT_c
HER	0.92	0.94	0.95	0.99
BER	0.99	0.99	0.90	0.98
FLE	0.96	0.99	0.96	0.99
SCO	0.95	0.99	0.94	0.99
WAK	0.97	0.98	0.93	0.98
BAS	0.95	0.99	0.95	0.99
LEO	0.94	0.99	0.93	0.99
CHO	0.97	0.95	0.97	0.93
TRE	0.96	0.98	0.94	0.97

Embryo growth in natural conditions

The minimum temperature recorded in Norway in winter was -2 °C in mid-November while the highest was recorded at the beginning of the experiment, on 15th September 2016 (18.5 °C). In England the minimum temperature recorded was 1.6 °C at the end of January and the maximum 17.0 °C, recorded on the same day as the Norwegian site, during an autumn heat wave. Embryo growth in natural condition was faster, for all the population tested, in the northern most location of Bergen where daily average temperatures were lower than at Wakehurst, UK. However, in both sites the rate of embryo growth started to increase when the temperatures fell below 10 °C (**Fig. 5**).

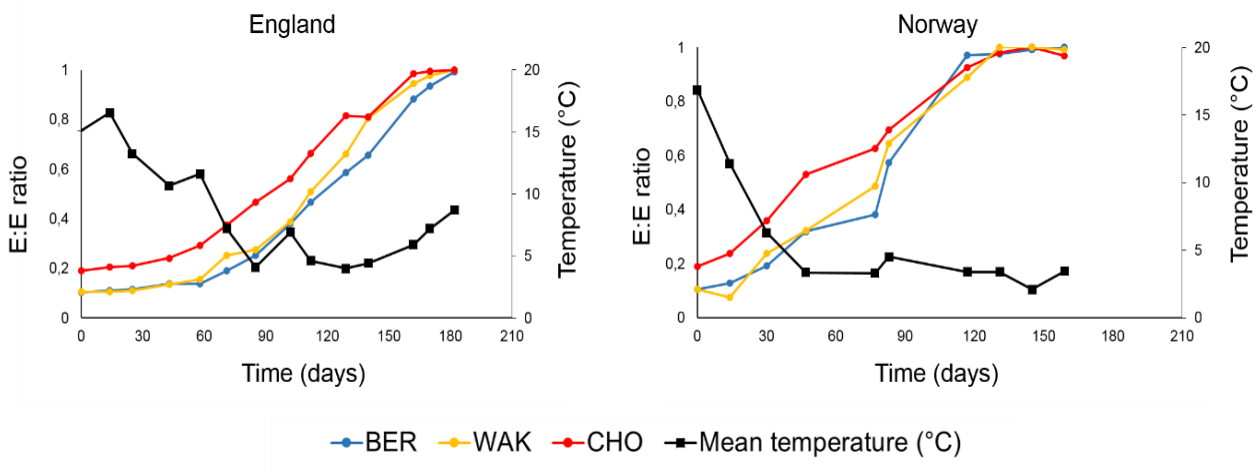

Fig. 5: Embryo growth in the field for buried seeds of *Conopodium majus*. Each data point represents the average E:E ratio of 20 seeds; soil temperature is also shown. The experiment started on 1st September 2016 in England and on 15th September 2016 in Norway. For each site, the experiment finished when all population reached 100% radicle emergence (corresponding to E:E =1).

Even if the southern population (CHO) had the greater initial E:E ratio, its growth rate was not different from the other populations tested. Eventually, the three growth curves tended to converge when an average E:E ratio approached 0.8 (Fig. 5). Germination in nature tended to peak in the months of January and February. Fitting a logistic regression to the curves permitted an estimation of the time, in days, to reach different deciles of relative embryo size (Table 5).

Comparison of the model with field data

The comparison of the thermal models against estimates of embryo growth in the field gave different results between the three populations, but was consistent between experimental sites (**Fig.7**).

Table 5: Time, in days, estimated to reach different deciles of embryo growth in the two field locations for buried seeds of *Conopodium majus*.

	Norway (start 15th September 2016)			England (start 1st September 2016)		
E:E ratio	BER	WAK	CHO	BER	WAK	CHO
0.2	63	43	10	82	79	37
0.3	70	53	28	95	92	66
0.4	76	61	38	106	101	79
0.5	81	69	48	115	109	90
0.6	85	78	59	125	118	100
0.7	91	88	72	137	128	112
0.8	98	101	91	151	141	128
0.9	109	126	128	176	162	155

Estimates of time to reach successive deciles of E:E ratio were similar if calculated using the ΘT_b and ΘT_c of the WAK population for both sites but higher than the Tr_g estimated from the logistic regression of embryo growth in the field. The BER population shown a rate of embryo growth that could be better predicted by the ΘT_c rather than by ΘT_b while both models diverged from the observed pattern of embryo growth in the southern population.

Environmental variability of germination traits

Excluding the minimum temperature of the coldest month, all the other bioclimatic and geographical variables (altitude, latitude, precipitation of the driest month and maximum temperature of the warmest month) were correlated (Pearson coefficient > 0.7 or < -0.7).

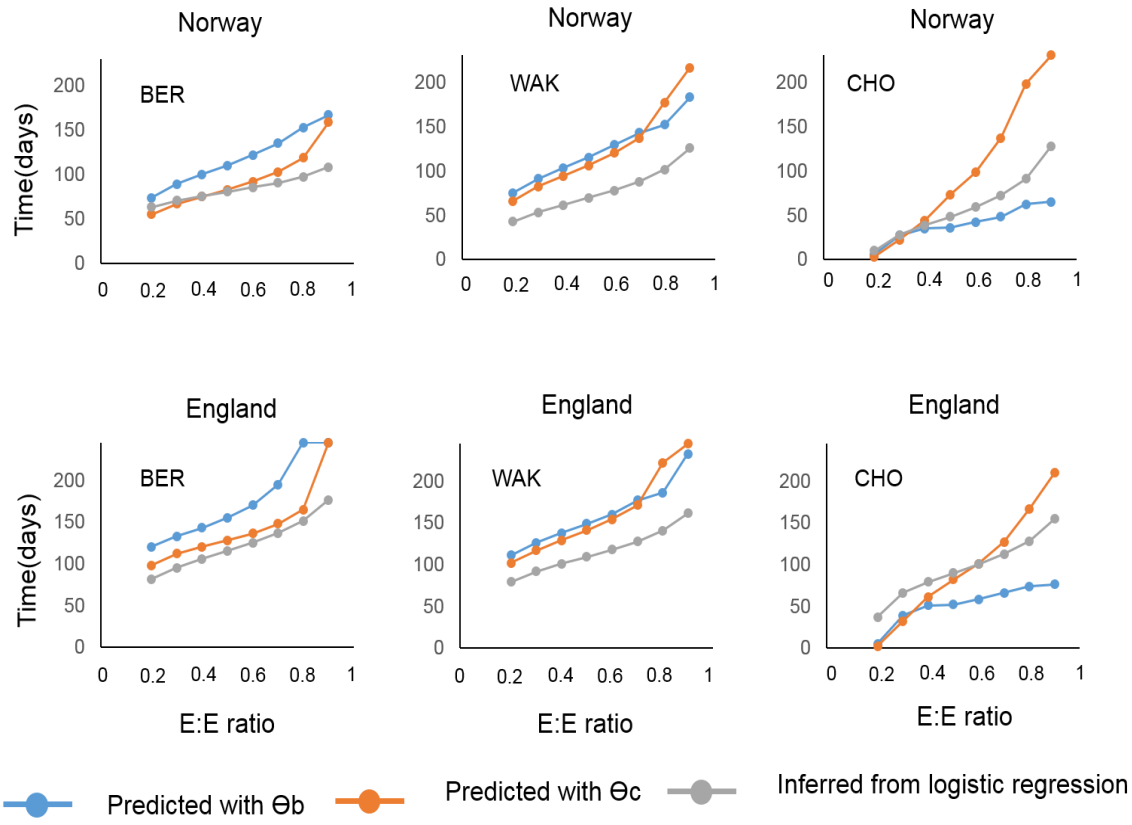

Fig. 6: Time (in days) required by each population of *Conopodium majus* seeds in each field location to reach different deciles of E:E ratio according to: 1) interpolation from the logistic regression of embryo growth in the field; 2) ΘT_b; and 3) ΘT_c obtained from the model.

The base temperature (T_b) was negatively correlated with T_o (Pearson = -0.72) while the T_c had a positive correlation with initial embryo size. Only the average temperature of the months during which embryo growth occurs (Sep-Feb) had a positive correlation (Pearson correlation coefficient > 0.6) with the minimum temperature of the coldest month.

A PCA (**Fig. 7**) ordered the populations according to their seed and germination traits and to the climate of the collection site. The first axis, that explained 51% of the variability in

the data, separated the mountain, southern populations from the northern, lowland ones. The axis was described mostly by geographic and bioclimatic variables and the only seed traits that had a significant correlation with it were seed dry mass and Tc. In particular, there was a strong negative correlation between precipitations of the driest month and Tc. Mountain populations of *C. majus* were located in the southern portion of the distribution range of the species and were characterized by higher maximum temperatures and more severe drought stress. The seeds from these populations had a lower dry mass but a greater initial relative embryo size than the northern, lowland populations.

The second axis explained 18.7 % of the variability in the data and had a significant correlation only with T_b and the minimum temperature of the coldest month. The two variables showed opposite trends, such that a higher minimum temperature corresponded to a lower T_b.

The T_o was negatively correlated only with the third axis (not shown in Fig. 7) of the PCA, that explained 15.8% of the variability in the data. Initial E:E ratio and the average temperature in the months during which embryo growth occurs were not significantly correlated with any of the first three axis in the PCA.

The two southern most populations, CHO and TRE, remained separated from the others: they came from the highest altitude and experience the strongest heat and drought stress. Of the remaining populations, SCO, ~~BAS~~ and LEO had the highest T_b, HER, FLE and BER the biggest seeds and WAK population came from a site with the lowest minimum temperature in the coldest month.

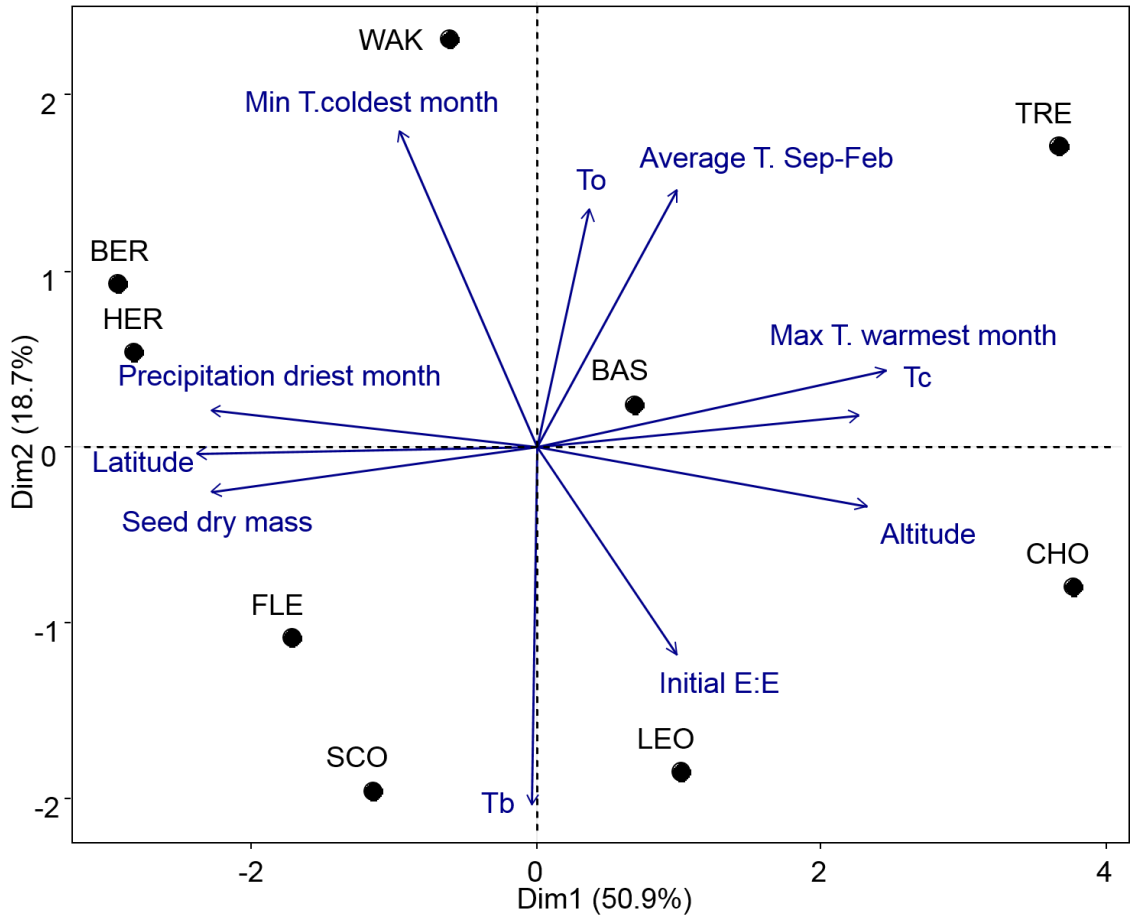

Fig. 7: Principal component analysis of seed traits in *Conopodium majus* and geographic and bioclimatic variables across Europe.

DISCUSSION

Conopodium majus shows considerable intraspecific variability in seed size across latitude. This has been observed for another species, *Sarracenia purpurea* (Ellison, 2001), with a similar seed morphology but with MPD rather than MD. Also, for *Sarracenia purpurea* the variation is seed size is not significantly affected by latitude. A positive effect of increased average annual precipitation on seed mass has instead been reported by Lemke et al., (2015) for the forest herb *Milium effusum* across a latitudinal

transect in Europe. Also for *Conopodium majus*, the populations with average higher seed dry mass were the ones growing in areas not subject to drought or high temperature stress. A possible explanation of this trend is that plants that live in less stressful environment can afford to allocate more resources on seed production.

However, having heavier seeds does not translate into the presence of more developed embryos. In fact, the initial relative embryo size was higher for two populations representing the two extremes of our latitudinal transect: CHO, in central Spain and FLE in southern Norway. Moreover, these two populations also had the lightest (CHO) and the heaviest (FLE) average seed dry mass. While seed dry mass had a strong correlation with local climate the same cannot be affirmed for the initial relative embryo size, whose pattern of variation across populations seemed more random. In our ordination analysis, initial E:E ratio was not significantly correlated ($p > 0.05$) with either the first or the second axis of the PCA but was the only variable to be represented and significantly correlated with the third. Its variation is therefore independent both from the climate and geographic parameters and from the cardinal temperatures that define PC2 (**Fig. 7**).

The narrow temperature optimum for embryo growth in *C. majus*, already reported in Chapter 4, was confirmed by testing temperatures at a finer resolution. In all the populations the higher rate of embryo growth is at 2.5 and 5 °C while almost no growth happens at -2.5 °C. The standard deviation of the average E:E in seed samples incubated at 0, 7.5 and 10 °C is much higher than in the other treatments, especially towards the end of the experiment, when the average E:E approximates unity. Since these temperatures are further from the optimum for embryo growth but not so 'stressful' as to inhibit it, the increased standard deviation in the sample can be explained by a more heterogeneous response of the individual seeds to these conditions. In fact, some seeds, even though remaining viable (red staining at the TZ test and no apparent malformations) did not

increase their embryo size at all at 0, 7.5 or 10 °C. But, while at the first two temperatures 100% of the seeds germinated eventually, embryo growth is too slow at 10 °C to culminate in germination during the 32 weeks of the experiment. Thus warmer winter temperatures in temperate forest environments as a result of climate change has the potential to inhibit the development of the embryo and thus negatively impact on germination and emergence of *Conopodium majus*.

All the populations considered are estimated to have a negative T_b, ranging from -6.7 °C in BER to -2.7 in BAS. Values of T_b lower than zero have been reported for some temperate trees, crops (mainly legumes) and wild plants but are not common (Durr et al., 2015). However, no values as low as -6.7 °C have been reported previously, the lowest being a T_b of -3.9°C for *Cryptantha minima* (Boraginaceae) and -4.5°C for *Krascheninnikovia lanata* (Amaranthaceae). The germination of *Cryptantha minima* at negative temperatures was explained by Wei et al., (2009) as an adaptation to take advantage of the water of the snowmelt in early spring and develop its annual cycle before the summer drought. In the case of *C. majus*, that is a perennial, this strategy could however offer some advantage at the southern range of its distribution, where summer drought can be a recurrent issue, as it has already been observed for Mediterranean subalpine species (Fernàndez Pascual et al., 2017). *Krascheninnikovia lanata* seeds show a positive effect of seed size on the ability to germinate at sub-zero temperatures (Wang et al., 2006). The authors demonstrate that bigger seeds had a higher concentration of sugars (glucose, raffinose and sucrose) that probably lower the freezing point of the seed tissues.

Our thermal models show the possibility for the *Conopodium majus* to germinate at sub-zero temperatures based on the rates of embryo growth at the range of temperatures tested and observing germination to eventually occur when the relative embryo size was equal

to one. In this sense we used embryo growth as a proxy for germination, thus comparing our results with other studies that observed germination at temperatures below 0°C. Such germination may seem counter-intuitive on the basis of expected conversion of water into ice at below zero temperatures. However, fully hydrated seeds of various species are known to have cell supercooling (i.e., ice nucleation) points around -5°C as a result of the effect of solutes depressing the onset temperature for ice formation (HW Pritchard, pers comm). Since the objective of the experiment was to monitor embryo development more than germination, the tests did not last long enough to produce a complete germination curve for the sub and supra optimal treatments. However incomplete germination curves have been added to the supplementary materials (Annex I).

There are no reports on sub-zero germination in Apiaceae but an optimal temperature for embryo growth of 2 °C has already been described for *Heracleum spondylium* (Stokes , 1953) and is not unlikely that this species, or others from the same family, could present equally low T_b for embryo growth. Field collected data (**Fig. 5**) and averaged climatic data from 2070-2000 (Fick and Hijmans, 2017) of the collection sites of the populations studied show that such low average temperatures are rare in the natural environment of *C. majus.* Therefore embryo growth is possible throughout the winter season and is limited by the higher temperatures in autumn. In fact, results from the ordination analysis (**Fig. 7**), showed that the T_b is independent from climatic and geographic factors and is not even correlated to seed size or initial E:E ratio. Therefore, as mentioned above, the limiting factor for this species is constituted by exposure to higher temperatures during the seed germination phase of the life cycle.

The optimum temperature for germination rate ranged between 2.5 and 5.2 °C (Table 3) and has a negative correlation with T_b, a phenomenon already reviewed by Durr et al., (2015). The populations with the higher T_b (BAS, LEO and SCO) also have the lower T_o

and therefore a narrower window of suboptimal conditions for embryo growth. Therefore, these populations are at greater risk of exposure to a reduced germination niche in the face of climate warming (Walck et al., 2011).

T_c varies between 12.1 and 20.5 °C and has a strong negative correlation with latitude and precipitation. Species from northern populations, that are less likely to experience long exposure to high autumnal temperatures, have lower values while the two southernmost populations, CHO and TRE, stand out for high T_c above 20 °C. Water stress is the main limiting factor for embryo development in these populations, that experience also a shorter winter and a more continental climate. The higher T_c can therefore be an adaptation to cope with higher daily fluctuations in temperatures that can prevent the embryo from growing during warmer, potentially desiccating parts of the day during late autumn or early spring. Moreover, embryo growth (and the potential to germinate) under cold (close to 0°C) will enable the start of growth during winter and emergence under the snow to avoid drought, as has been suggested to be the case for many sub-alpine species (Fernandez-Pascual et al., 2017).

CONCLUSION

In conclusion, *Conopodium majus* can be considered a model species for studying morphological dormancy due to its fine regulation of embryo growth by temperature and the coincidence between the temperature requirements for embryo growth and germination. To date only one study is known to have developed thermal models of embryo growth in a species of the Ranunculaceae family, *Aquilegia barbaricina* (Porceddu et al., 2017) and this work represents the first attempt to develop such a model on a species from Apiaceae family. The thermal models developed in this study can be used to predict shifts in its temperature germination niche caused by different climate

change scenarios. However, *C. majus* also shows an adaptation to the climatic environment along its latitudinal distribution that are expressed by the breadth of the temperature germination niche indicated by the cardinal temperatures of each populations. Because of this phenotypic plasticity in the species the development of population specific model is more appropriate to describe and predict germination timing in this species. Finally, since for this species the T_c is more associated with the local climate than T_o and T_b, the use of the supra-optimal thermal time analyses are more appropriate when modelling a shift in germination niche due to climate change.

AKNOWLEDGEMENTS

The ideas behind this work was developed together with Eduardo Fernández Pascual. This study would not have been possible without the collaboration of Alvaro Bueno Sánchez, Joseba Garmendia, Luis Carlòn, Sylvy Sandvick, Giles Laverack, Maria Marin, and Brith Natlandsmyr that all helped with seed collection. We are especially grateful to Brith Natlandsmyr also for performing part of the experiments in her own garden and for her commitment to the project.

The involvement of Eduardo Fernández Pascual and Hugh W. Pritchard in revising the manuscript was precious and fundamental. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°607785.

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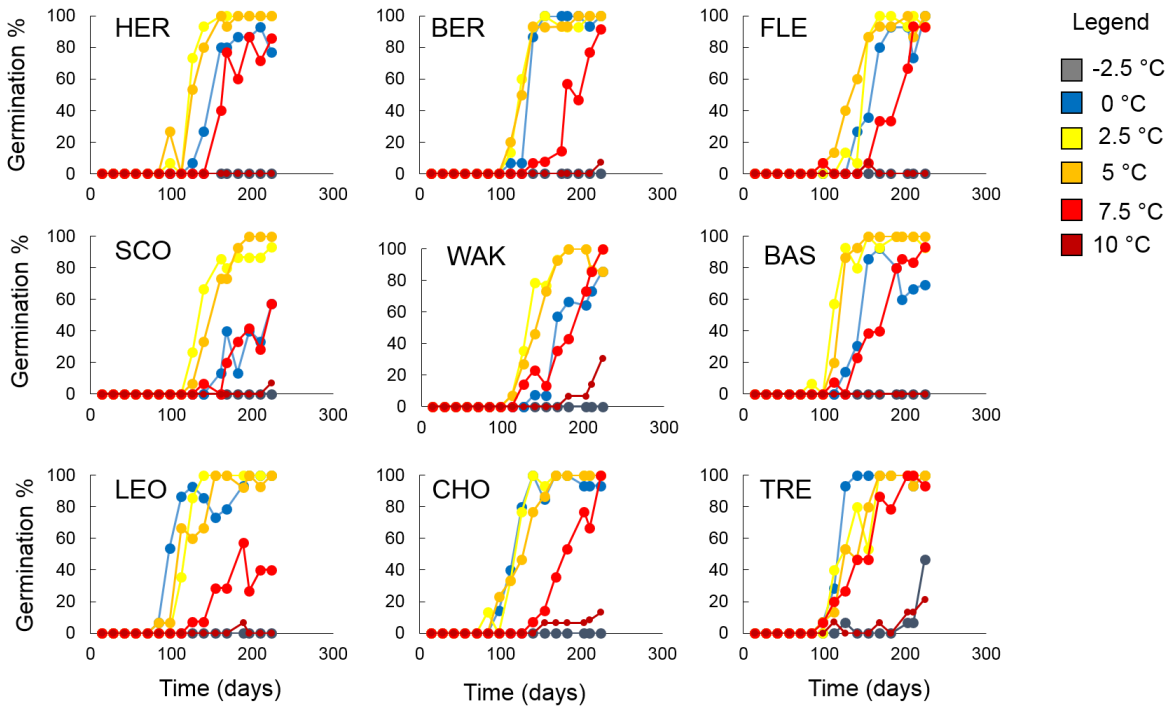
ANNEX I

Germination curves for all the populations of *Conopodium majus* tested. Each point represents the percentage germination based on the sample of 15 seeds used to test embryo growth. Empty and non-viable seeds were excluded from the calculation of the response.



CHAPTER 6

DISCUSSION AND CONCLUSIONS

DISCUSSION

The findings in this thesis identify three main germination strategies present in ancient woodland indicator species, and these reflect a different degree of specialization and adaptation to forest habitat. Species from forest hedges and gaps have the higher colonization capacity and are able to germinate in partially disturbed habitats (Pearson et al., 2002). In fact, alteration in light availability and temperature fluctuations signal to these species that an environmental change has happened that could favour their establishment. This group of species represent the majority of the woodland indicator species based on the literature review and analyses of the traits of such species presented in **Chapter 2**.

In contrast, forest herbs with a more restricted habitat specialization are adapted to regenerate in environments where light availability is limited and have evolved germination strategies that allow the seedlings to emerge in early spring, which is the more favourable season for their establishment. Morphological dormancy is more recurrent in this group of species and is strictly regulated by temperature. Germination can be programmed to happen in autumn, and in this case shoot development is delayed until the return of warmer temperatures. In this way the seedlings secure their place in the forest floor by developing their roots months earlier than shoot emergence (Mondoni et al., 2008). Species from this group do not require light for germination, which can take place also under the leaf litter, where the seedlings are more protected from extreme temperature fluctuations during the winter months.

Other forest specialists do germinate in winter when the temperatures are still low and are able to develop their seedlings in these conditions. In this way competition from later emerging species can be avoided. However, this strategy is not unique to temperate forest

species but can be found also within Mediterranean subalpine species subject to cold winters and dry summers (Fernàndez Pascual et al., 2017). These species do germinate under the snow during the period of snow melt and take advantage of the opportunity window constituted by the spring months before the onset of summer drought.

Species richness in recent woodlands could be equal to old woodland but the functional diversity expressed by the understory community is markedly different (**Chapter 3**). Species from forest hedges and ruderal plants with immediate germination are more represented in this habitat while ancient forest understories are characterized by species with low colonizing capacity, in agreement with the current literature (Hermy et al., 1999; Verheyen et al., 2003), and with delayed germination. Dispersal limitation is not the only explanation for this discrepancy: also abiotic differences in the physical environment, and especially in soil chemistry and spatial heterogeneity have to be taken in account when comparing understories of recent and ancient forests.

Further research on the evolution of winter germination at cold temperature can shed light on the environmental plasticity demonstrated by some woodland indicator species such as *Conopodium majus* (Apiaceae) (Fig. 1). This species is characteristic of both woodland and oligotrophic meadows communities (Grime et al., 2007) and is also found in montane habitats of central Spain, at the edge on its distribution. *C. majus* possess undeveloped embryos that need to increase their size about ten-fold before germination can occur (**Chapter 4**).

The process of embryo growth is strictly regulated by temperature and no interruption between embryo development and germination has been observed. The rate of embryo growth and its regulation by temperature are therefore essential in the timing radicle emergence to coincide with a season that is favourable for seedling establishment. By

monitoring germination phenology in its natural environment, early spring emergence was observed, and desiccation sensitivity of embryo at different stages of development was defined. *C. majus* seeds that have been dehydrated after the embryo has increased five times its original size can resume embryo growth but at a reduced rate. Further investigation on the physiological consequences of dehydration in this species can clarify if the decline in germination rate is caused by loss of viability or is an adaptative strategy that allows the species to persist in the soil until the "drought stress" has passed, as it has been hypothesized for *Lomatium dissectum* (Scholten et al., 2009).

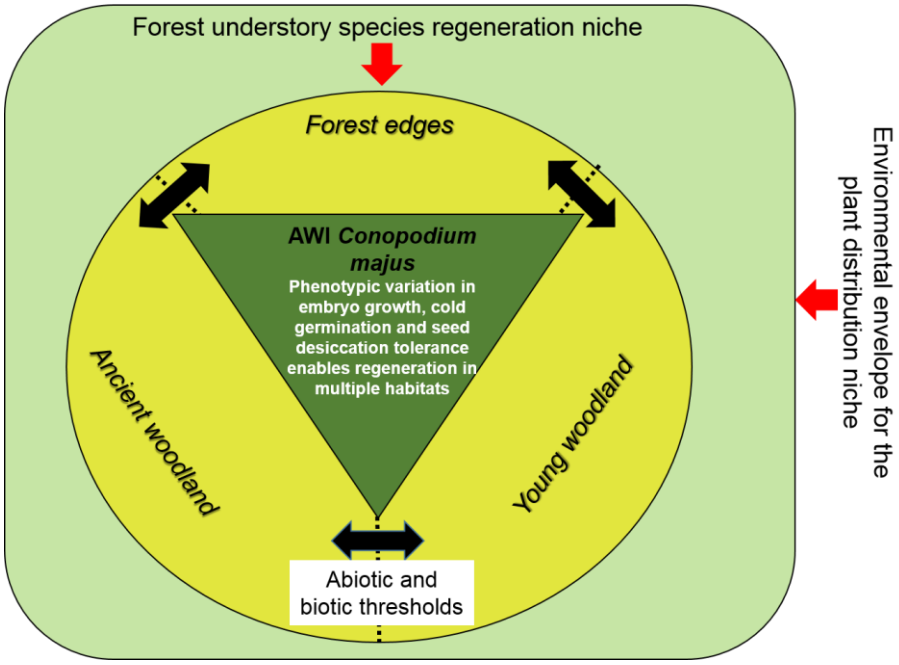


Fig. 1: Schematic representation of the the narrower confines of the species regeneration niche compared to the plant distribution niche. The Ancient Woodland Indicator species Conopodium majus exhibits phenotypic plasticity for the thermal control of embryo growth across its distributional range which permits it to occupy multiple habitats.

Embryo growth in *C. majus* happens at cold temperatures and the rate of growth is constant until radicle emergence. This feature made *C. majus* a good species to develop a thermal model of embryo growth (**Chapter 5**). Using a novel approach (Porceddu et al., 2017), cardinal temperatures for embryo growth were characterized for nine populations collected along the latitudinal range of the species and the variation in germination traits were compared with bioclimatic and geographical variables. Seeds of *C. majus* from northern population showed a narrower thermal amplitude for embryo growth than southern populations. The latter are found in Mediterranean-like mountain environment that experience high temperatures and drought stress during the summer. Germination temperatures of *C. majus* varied across its latitudinal distribution and higher ceiling temperatures were correlated to lower precipitation in the driest months. The description of the germination ecology of a species across its biogeographical distribution can provide an explanation for the variation in species distribution. Moreover, the development of a thermal model can be used to predict whether this variation can be expected to be affected by climate change. Developing this model further by applying it to other habitat restricted species with morphological dormancy will enable greater understanding of the role of germination niche in species responses to climate change (Walck et al., 2011).

CONCLUSIONS

In the past few years the number of published research in the field of restoration ecology has increased dramatically and growing importance is being given in restoring the functional structure of ecosystems (Young et al., 2005). Still, restoration of temperate forest herbaceous understory is a field that has been little developed because of the

complex germination requirement and limited desiccation tolerance of many specialist forest species. When planning forest understory restoration, it is critical that two matters are considered: 1) the regeneration strategies represented in the plant community being restored; 2) the characteristics of the physical environment where the intervention is going to take place. Moreover, a comparison of germination requirements across the distribution area of a species can provide useful information on its ecology and help to better define its regeneration niche and to predict the challenges that could be met for its conservation in a scenario of human driven environmental changes.

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OTHER ACHIEVEMENTS

Publications in peer-review journals

The following publication derived from a collaboration that I've initiated during my MSc:

Lemke, I. H., Kolb, A., Graae, B. J., De Frenne, P., Acharya, K. P., **Blandino, C**., Brunet, J., Chabrerie, O., Cousins, S.A.O., Decocq, G., Heinken, T., Hermy, M., Liira, J.,Schmucki, R., Shevtsova, A., Verheyen, K., Diekmann, M., 2015.Patterns of phenotypic trait variation in two temperate forest herbs along a broad climatic gradient. Plant Ecol. 216, 1523-1536.

Book chapters

Blandino, C., (2017) Restoration of the understory of European temperate deciduous forests. In: NASSTEC Handbook. Unpublished.

Blandino, C., (2017) How to study embryo development. In: NASSTEC Handbook. Unpublished.

Blandino, C., (2017) Germination and cultivation protocol for *Stachys sylvatica*. In: NASSTEC Handbook. Unpublished.

Conference presentations

Blandino, C., Frischie, S., Lopez Del Egido, L. Tusela Isanta, M., 2015. Dormancy and germination: their potential impact on seed production and plant establishment. Poster presentation at the 27th International Congress for Conservation Biology, Montpellier, France.

Public engagement:

Outreach article: Wrote the outreach article “Nuevo experimento de ecología forestal en el Jardín Botánico” for the Jardín Botánico Atlántico website about the forest ecology experiment started at this institution in October 2015.

Display: Participated to “Wild wood festival”, 27-25 May 2016, Wakehurst place, with a thematic display on NASSTEC and the germination of woodland understory herbs.

Talk: Participated to Lewes Seedy Saturday giving the talk: “Seed saving and its importance for research”. 4th February 2017, Lewes, UK

Mentoring:

Foreign consultant for Elza Makaradze, PhD student at Batumi Shota Rustaveli State University, Georgia for the following research project:

"Studying the population diversities of some the endangered species of herbaceous plants (Cyclamen adzharicum pobed, Galanthus woronowii Losinsk., G.rizehensis Stern.) of Adjara flora (South Colchis) ".