THE UNIVERSITY
of EDINBURGH

Regeneration of Scots pine (*Pinus sylvestris* L.) under drought

Sarah L. MacAllister

PhD - University of Edinburgh - 2016

Declaration

I, Sarah MacAllister, hereby certify that this thesis has been written by me and that it is an accurate record of work I have carried out in the pursuit of research for my PhD. This work has not been previously submitted for the application of a higher degree.

Sarah MacAllister

Summary

The risk of trees dying from drought is predicted to increase in the future for many forests. Regeneration is the process of renewing mature forest with subsequent generations. Different species may be expected to have their own regeneration 'niche': environmental conditions necessary for regeneration to occur. Mortality of the seedling bank is often said to be a population 'bottleneck'. Seedlings are more vulnerable to drought than mature trees; therefore, understanding how drought affects a species' regeneration niche can help us to determine the impact on a population. Scots Pine (*Pinus sylvestris* L.*)* is the most widely distributed pine tree, inhabiting a variety of ecological conditions throughout Europe and Siberia. The thesis aims to deepen understanding of drought-induced mortality, while also analysing variation in the drought response of *Pinus sylvestris* seedlings, *i.e*, the potential for regeneration under drought. Three experiments were carried out using seeds from different populations of origin (provenances) across the north-south axis of the European range of *Pinus sylvestris*. Seeds were collected from different provenances. Along with other differences in climate and soil, these provenances span a gradient of water availability: from wet (Scotland) to intermediate (Austria, Poland) to dry (Spain). The first experiment used a proxy for severe and rapid water deficit to test germination, seedling responses and survival; the second experiment involved a dry down treatment and seedling physiological responses were analysed at the level of metabolite changes (*i.e* small molecules of metabolism); the third experiment investigated the effects of seed weight and maternal parentage on germination and seedling drought responses. In general, seedlings from Spain showed higher seed weight and faster germination, as well as higher investment in roots and stems and lower specific leaf area (*i.e* higher

biomass per unit area) than seedlings from other provenances and were least affected by drought. Seedlings from Austria showed the most reduction in root length and higher photochemical stress was indicated under drought. The improved ability of seedlings from Spain compared with other provenances to quickly germinate and establish indicates local adaptation to drought in the regeneration niche. At the molecular level, drought stress increased expression of two antioxidant enzymes. Metabolomics is an emerging molecular approach that aims to capture a picture of all metabolite changes in a tissue sample. This is the first time a study into drought effects on the foliar metabolome of *Pinus sylvestris* seedlings – or indeed of any pine tree species at the seedling stage - has been carried out. The metabolic snapshot of leaves was taken at two time points during drought and showed higher concentrations of metabolites involved in osmoprotective and antioxidant capabilities.

Abstract

Drought-induced tree mortality is a phenomenon affecting many forest ecosystems and is predicted to increase under ongoing climate change. Forest stability partly depends on regeneration: the process of renewing mature forest with subsequent generations. As seedlings are more susceptible to drought effects than mature trees, mortality of the seedling bank can represent a major bottleneck controlling forest structure and species composition. Scots Pine (*Pinus sylvestris* L.*)* is the most widely distributed of the *Pinus* species, covering a broad latitudinal gradient of ecological conditions. The thesis aims to deepen understanding of drought-induced mortality, while analysing intra-specific variation in the phenotypic and metabolic profile of *Pinus sylvestris* seedlings subjected to drought stress. I also consider the relevance of the results to the broader conceptual framework of drought-induced mortality. The experiments utilise seeds from different populations of origin (provenances) across the north-south axis of the European range of *Pinus sylvestris*, in order to determine the extent of regeneration capacity in this species under drought. Seeds were collected from different populations (provenances) that, along with other climatic and edaphic differences, span a gradient of water availability: from wet (Scotland) to intermediate (Austria, Poland) to dry (Spain).

In Chapter 2, the effects of osmotic stress on the initial seedling establishment stage were studied by comparing phenotypic responses across provenances. Seedling germination, early growth, osmotic stress tolerance and survival were investigated using a polyethylene glycol irrigation treatment as a proxy for rapid and severe drought. Treatment, provenance and interaction effects were found for rate of

germination, final proportion of seeds germinated, seedling size, and superoxide dismutase activity (an antioxidant enzyme). Root investment was affected by both provenance and time to germination. Although there was no significant effect of provenance on survival, a trend towards increased probability of survival under osmotic stress was indicated for the southernmost (driest) as compared with the northernmost (wettest) provenance.

Chapter 3 investigates the responses of older seedlings (at 10 months) to a drying down of soil moisture for 40 days. Morphological and physiological data were collected to assess intra-specific and intra-population variation in the seedling stress response under drought. A metabolomics analysis using Ultra performance Liquid chromatography followed by mass spectrometry (UPLC/MS) was carried out to investigate whether metabolic markers could be identified that are suggestive of heightened oxidative stress and whether populations in different climatic and edaphic environments show variation in metabolic activity under drought. Preliminary results suggest large intra-population variability yet clear differentiation in metabolic responses to drought over the time course of the experiment. Univariate and multivariate analyses indicated that among the most significant increases in response to drought were those involved in osmoprotective and antioxidant capabilities, including the free amino acid proline and a quercetin derivative (a flavonoid). Interestingly, provenances, either under experimental drought or not, did not show significantly different metabolite profiles, even though provenance and its interaction with drought treatment did significantly affect seedling biomass and photochemical efficiency.

In Chapter 4 the effects of provenance, maternal parentage and seed weight on germination rate, final germination percentage, as well as seedling drought responses in biomass allocation and the expression of selected antioxidant genes were analysed. Seed weights were measured individually and seed weight was found to have a strong positive effect on: germination rate, seedling dry weights, and number of needles. Expression of two antioxidant enzymes increased under drought. Seed weight was strongly determined by provenance and maternal parentage as well as their interaction. However, root to shoot biomass allocation depended on provenance and maternal effects that were not mediated by seed weight effects. Principal component analysis indicated that the Spanish provenances could be characterised by a higher root to shoot ratio and stem weight. Specific leaf area was also found to be lowest for the Spanish provenances.

Acknowledgements

Most of all I thank my supervisor, Kyle Dexter, without whose guidance, tireless optimism and support my project would not have been successfully completed. Also, my sincere thanks to Andrew Hudson, my biological sciences supervisor, for sharing his in-depth molecular laboratory know how and for always responding patiently and kindly. I am grateful for the opportunity to do this PhD and early supervision given by Maurizio Mencuccini and Yann Salmon, as well as their continued interest in the project.

Thanks to Stephen Cavers and Annika Telford, as well as Alistair Jump and Luis Matias, for the seeds used in these experiments and for general advice on working with *Pinus sylvestris*. I am also thankful to Sophie Haupt and Pat Watson for arranging growth room space, caring for the seedlings between experiments and arranging for control group seedlings to be kept alive and passed on for landscaping and conservation projects.

I am indebted to many people for their patient assistance while I learned laboratory techniques. Thanks to: Andrew Hudson for helping with getting set up with RNA extraction and cDNA synthesis; Justin Goodrich for showing me how to run an electrophoresis agarose gel; Aitor De Las Heras for his help with the plate reader during the enzyme assay; Alistair McCormich for his demonstration of using the fluorometer; and Pumi Perera and Dave Kelly for advising me on using the Lightcycler for RT-PCR.

Special thanks to Ulf Sommer and Jasper Engel for their metabolomics expertise in carrying out the complicated metabolomics work flow, as well as their guidance throughout metabolite extraction and statistical interpretation.

I am very grateful to the Natural Environment Research Council for funding my research under grant NE/I011749/1.

Finally, for your wisdom and trust throughout my life and this endeavour, thank you Grandma Ann, Grandma Marge, Mum, David, Rachael and Dad. This thesis is dedicated in loving memory of my Grandads, Harry and Bryan.

For being by my side always, thank you James. Also, thanks for finding the following quote:

> *I consider a tree.*
>
> *I can look on it as a picture: stiff column in a shock of light, or splash of green shot with the delicate blue and silver of the background.*
>
> *I can perceive it as movement: flowing veins on clinging, pressing pith, suck of the roots, breathing of the leaves, ceaseless commerce with earth and air – and the obscure growth itself.*
>
> *I can classify it in a species and study it as a type in its structure and mode of life.*
>
> *I can subdue its actual presence and form so sternly that I recognise it only as an expression of law – of the laws in accordance with which a constant opposition of forces is continually adjusted, or of those in accordance with which the component substances mingle and separate.*
>
> *I can dissipate it and perpetuate it in number, in pure numerical relation.*
>
> *In all this the tree remains my object, occupies space and time, and has its nature and constitution.*
>
> *It can, however, also come about, if I have both will and grace, that in considering the tree I become bound up in relation to it. The tree is now no longer It. I have been seized by the power of exclusiveness.*
>
> *To effect this it is not necessary for me to give up any of the ways in which I consider the tree. There is nothing from which I would have to turn my eyes away in order to see, and no knowledge that I would have to forget. Rather is everything, picture and movement, species and type, law and number, indivisibly united in this event.*

Martin Buber (1923), Ich und Du, p6

Contents

List of Figures

List of Tables

1 General Introduction

1.1 Local adaptation and phenotypic plasticity

> *"Just as the genotype is the sum of all its genes, the phenotype is the sum of the physical, metabolic and physiological traits that define an individual, each of which can contribute to the reproductive fitness in different, albeit interrelated, ways: i.e the entire phenotype is the vehicle of evolution."*
>
> *Karl Niklas (1997)*

Micro-evolutionary processes of selection, gene flow, genetic drift and inbreeding can have pronounced effects on genetic variation of a population and potentially lead to genetic differentiation – and local adaptation. Local adaptation occurs when strongly divergent natural selection has eliminated phenotypes with deleterious or less well-adapted traits and thereby changed the frequency of alleles for adaptive genes in the standing genetic variation of a population, resulting in higher fitness of the population under the prevailing environmental conditions (Kawecki & Ebert 2004). Multiple traits can underlie local adaptation to an environmental condition, deriving from pleiotropic genes and co-adapted gene networks. Thus, changes in allele frequency of genes involved in epigenetic processes can also be a locally adaptive response. Since 'the entire phenotype is the vehicle of evolution', there are many aspects of biological complexity to be considered when assessing the implications of natural selection on an organism. Beyond the level of genetics and epigenetics are the metabolites and

proteins that constitute a cellular and tissue level response and then the conglomeration of organs and whole plant transport and resource allocation trade-offs, which include investment in the next generation.

Phenotypic plasticity describes the genetically determined capacity of one genotype to respond to environmental cues and give rise to multiple distinct phenotypes, which may involve alternate forms and combinations of physical, metabolic, physiological and phenological traits (Johannsen 1911, Bradshaw 1965). In Theophrastus's Enquiry into Plants (c. 350 - 287 BC), as well as describing altitudinal effects on species composition, he highlights the importance of environment in bud phenology at the intraspecific level: 'Again there are differences of time between individual trees of the same kind, according to the locality; those in the marshes bud earliest…second to them those in the plains, and latest those in the mountains' (book I, volume III, pp. 179). Thus, phenotypic plasticity enables organisms to tolerate fluctuating environmental conditions or even grow over a wider ecological range (Woltereck 1909; Via & Lande 1985). As phenotypic plasticity itself is genetically determined, it can also be subject to micro-evolutionary processes resulting in local adaptation. Moreover, as phenotypic plasticity increases phenotypic variance, it has been suggested that natural selection can shift adaptive phenotypes to fixation without a speciation event (West-Eberhard 1989). Not only local adaptation but also adaptive radiation and speciation have been proposed to be connected with phenotypic plasticity, whether this is continuous in nature and exhibits a norm of reaction or discontinuous and results in discrete phenotypes (polyphenism) (for review see Pfennig *et al.* 2010).

Intraspecific variation in drought response may be attributable to local adaptation, where alleles vary in frequency and have corresponding phenotypic variance that is heritable. Populations that occur under different environmental conditions are more likely to diverge under selection for traits that maximise fitness to the conditions. Of course, intraspecific variation can also occur at the within population level. Adaptive genetic differentiation can be detected using a population genetics approach by separating the genetic variation in quantitative traits (Qst) from that of neutral genetic variation (Fst) and/or by separating among population variance from within population variance using family structured provenance transplantation – or common garden - trials (McKay & Latta 2002). Detection of local adaptation is complicated because multiple traits may underpin a coherent response. Adaptive phenotypes may depend on traits with underlying co-adapted gene networks and the extent of phenotypic plasticity in a species; thus, pleiotropic genetic correlations may constrain population adaptive differentiation while phenotypic plasticity may be under selection to increase.

Trees have long generation times and therefore evolve slowly by *de novo* mutation and recombination. Despite this limitation, adaptive potential is considered high because of large effective population sizes, allogamous mating systems, great fecundity and differential selection acting on large juvenile populations: all of these factors contribute to the maintenance of high standing genetic diversity (Petit & Hampe 2006). Furthermore, there is a high capacity for phenotypic plasticity in traits such as wood formation, stomatal development and bud and reproductive phenology. This mediates the diversification of phenotypes upon which natural selection can act (West-Eberhard 1986).

Provenances are areas within which similar ecological and climatic characteristics are found and thus, local adaptation or acclimation to site conditions may be expected. The potential for local adaptation in forest tree populations has long been indicated by the results of provenance trials (transplantation experiments) that show latitudinal clines in phenology, growth and abiotic stress tolerance (for review see Alberto *et al.* 2013).

1.1.1 Gene flow

Gene flow can be an important process counteracting genetic differentiation of populations. Mayr (1947) argued that gene flow impedes speciation, so that divergence is largely restricted to allopatric mechanisms. There is often extensive gene flow between tree populations, mediated by dispersal of pollen and seed. Paternal genes are inherited in chloroplasts via pollen, while maternal genes are transmitted via seed in mitochondria and, in the case of conifers, the megagametophyte tissue enveloping the embryo. Zygotic genes are also transmitted via seed and are activated in the fertilized embryo (the zygote). Although the ratio of pollen to seed flow varies according to species and the mode of inheritance, both enable spread of adaptive or maladaptive alleles from source populations (Hu & Ennos 1997; Hu & He 2006). In wind pollinated (*anemophilous)* trees, gene flow via pollen can be more extensive (20 to nearly 200 times higher) than via seeds (Ennos 1994). Of gymnosperm species, 98% are anemophilous (Faegri & van der Pijl 1979). Evolution has led to the ability of pollen to rapidly dehydrate upon shedding, through microapertures (Tekleva *et al.* 2007).

Thus not only is the pollen weight reduced to enable effective dispersal by wind, but pollen is also invulnerable to damage by low humidity during aerial transit (Bohrerova *et al.* 2009). In *Pinus sylvestris*, long distance pollination was found to contribute to genetic variation in isolated Spanish stands, since ~4% of matings occurred with immigrated pollen from distances of >30km and even 100 km (Robledo-Arnuncio & Gil 2005; Robledo-Arnucio 2011). The relative contribution of paternal gene flow from exotic plantations to relict Iberian stands of *P. sylvestris* was found to be higher than maternal or zygotic gene flow, indicating significantly higher pollen dispersal than seed dispersal (Unger *et al.* 2014). Across the north-south axis of Finland, pollen transported over long distances (>100 km) can be important in fertilising female strobili that become receptive before local male pollen shedding begins, and even afterwards in ongoing pollen competition (Varis *et al.* 2009, Ertl *et al.* 2012).

Exotic gene flow, as well as gene flow from an ecologically distinct population, may hinder local adaptation by dismantling co-adapted gene networks or weakening the effect of divergent selection. Experimental crosses in an annual plant suggest that gene flow is most beneficial between populations within the same environmental conditions, rather than gene flow from central to edge populations, which undermined developmental timing adapted to the warmer edge environment (Sexton *et al.* 2011). Similarly, experimental crosses between *Pinus sylvestris* at the northern range edge and central range showed reduced frost tolerance compared with crosses between individuals from northern edge populations (Aho 1994).

1.1.2 The functional genome

1.1.2.1 Genome size, karyotype and chromosomal organisation

Plants can attain very large genome sizes by the insertion of repetitive sequences, especially through retrotransposon expansion (Kumar & Bennetzen 1999). Compared with most vascular plants, conifer genomes are particularly large and characterised by repetitive sequences (Ahuja & Neale 2005, Kovach *et al.* 2010). Interspecific variation in genome size within the genus *Pinus* ranges from 2C = 38 to 72 pg (Zonneveld 2012). Seed size has been found to be positively correlated with genome size at the interspecific level within the genus *Pinus* (Grotkopp *et al.* 2004, Ahuja & Neale 2005). However, there are mixed results at the intraspecific level with regard to genome size variation. Some studies of conifer species have found intraspecific variation in nuclear DNA content (Miksche 1968; Dhir & Miksche; Wyman *et al.* 1997), while others have not (Wakamiya *et al.* 1993;Bogunic *et al.* 2007; Bogunic *et al.* 2011).

Within the coniferous family Pinaceae, the diploid karyotype is found in the majority of species, meaning that there are two copies of every gene. This chromosomal organisation has been highly conserved through molecular divergence and speciation events. Nkongolo & Meyes-Smith (2012) carried out karyotype analyses comparing genera within Pinaceae and found that the genus *Pinus* was the least derived, or ancestral, in terms of karyotype parameters.

Transposable elements (TE) are DNA sequences that either relocate by excision and insertion by transposases (transposons) or via intermediating RNA (retrotransposons). These may help explain the large genome size of conifers. The silencing of

transposable elements by chromatin modifications and post-transcriptional silencing is thought to maintain genome stability and avoid deleterious mutations. McClintock (1984), upon discovering transposable elements, posited that the activation of TE occurred in response to stress. Subsequently, a number of stresses have been found to activate TE, including: UV exposure, temperature, radiation, wounding, and pathogen infection (Capy *et al.* 2000; Ito *et al.* 2011; Cavrak *et al.* 2014; Voronova *et al.* 2014). Federoff (2012) contends that it is the flexibility inherent in the epigenetic machinery that has allowed proliferation of transposition and expansions in genome size and complexity. Indeed, Morse *et al.* (2009) used massively parallel DNA sequencing of Cot fractionated genomic DNA in *Pinus taeda*, which revealed that the majority of the sequence complexity (all novel sequences contained by a genome) could be explained by the contribution of retrotransposon derivatives.

1.1.2.2 Gene expression, epigenetics and stress

Plant responses to stress that do not involve changes in allele frequency may still involve changes to the way in which genes are expressed. Epistasis describes the modifying interaction that one or multiple genes have on the expression of another gene. Furthermore, epistatic effects on metabolic networks and signalling pathways can arise because genes interact indirectly through downstream changes in products that might be necessary precursors to the formation of intermediary metabolites or signalling molecules. In out-crossing populations, including forest tree species, multi-allelic epistatic interactions may occur and affect the resulting phenotypic variation in a population (Tong *et al.* 2011). In *Populus tremula*, Ma *et al.* (2010) demonstrated stronger covariance of allelic effects for photoperiodic genes than for control genes,

which accounted for the majority of observed phenotypic variation rather than individual single nucleotide polymorphisms.

Whole genome shotgun sequencing is an approach that has enabled the identification of 28,354 genes in the *Picea abies* genome (Nystedt *et al.* 2013), while the number of genes within the *Pinus taeda* genome is estimated to be ~ 50,000 (Neale *et al.* 2014). However, the capacity of a genotype to give rise to appropriate phenotypic responses (physically, physiologically and metabolically) can depend on the expression of relatively few functional genes. In *Populus euphratica*, a cascade of drought-induced physiological responses (stomatal closure, shoot growth cessation, root growth reduction and oxidative stress induced compounds) were related to transcriptional changes that involved only 1.5% of genes on a micro-array (Bogeat-Triboulot *et al.* 2007).

A transcription initiation complex comprises promoter sequences that precede the gene coding sequence and DNA binding proteins, respectively the cis-acting elements and trans-acting factors. Expression quantitative trait loci (QTL) identification can reveal the relative importance of cis- or trans-acting regulation of differential gene expression in organisms with a full genome sequence; for example, trans-acting QTL are more important in tissue-dependent gene sub-functionalisation than cis-acting QTL (Kloosterman *et al.* 2012). Gene expression can be tightly regulated by trans-acting factors and changes in transcription factor regulatory networks are one way in which epistatic effects can alter gene expression and hence phenotype. Firstly however, epigenetic marks signal whether the chromatin is more open and accessible for

transcription (euchromatin) or densely packed and effectively silenced (heterochromatin). Thus, the role of epigenetic regulation in enabling developmental transitions, acclimation to environmental cues, and even transmission of traits to the subsequent generation might also explain how plants endure stress without changes in allele frequency.

The concept of an epigenetic stress memory in plants is gathering evidential support in research using *Arabidopsis thaliana*. Transgenerational systemic acquired resistance in inoculated *A. thaliana* progeny was transmitted via pathogen induction of DNA hypomethylation in genes involved in priming salicylic acid inducible promoters with a chromatin mark facilitating gene expression (Luna *et al.* 2012). Transgenerational methylation and a parental low humidity-induced stomatal phenotype are heritable in *A. thaliana* progeny because of siRNA-mediated silencing at *SPEECHLESS* (Tricker *et al.* 2013a). Furthermore, priming the epigenetic response to high vapour pressure deficit increased drought tolerance of *A. thaliana* (Tricker *et al.* 2013b).

Post transcriptional regulation offers added complexity to the regulation of patterns of gene expression. Small non-coding RNAs of only ~20-25 nucleotides in length, such as single-stranded microRNAs (miRNAs) and double-stranded small interfering RNAs (siRNAs) down-regulate gene expression. These have a key role in plant stress responses, including drought and oxidative stress (Shukla *et al.* 2008), as well as in co-ordinating seed germination and sensing stress to ensure germination is carried out under conditions that will allow survival (Martin *et al.* 2010).

Trees also have epigenetic patterns that are responsive to environmental conditions. Within a natural population of a mangrove shrub, ecotypes exposed to salinity stress exhibited a more divergent DNA methylation profile than genetic profile (Lira-Medeiros *et al.* 2010). Clone history accounted for divergent transcriptome remodelling in *Populus* subjected to drought and was further associated with DNA methylation profiles (Raj *et al.* 2011). In *Picea abies*, miRNAs expressed during embryogenesis have been implicated in orchestrating a temperature-dependent epigenetic memory by targeting genes that influence the timing of bud phenology (Yakovlev *et al.* 2010). However, the use of epigenetic recombinant inbred lines is not considered feasible given the long generation times of tree species, and so the association mapping of specific epigenomic regions with variation in phenotypic traits is currently limited to short-lived model organisms, such as *Arabidopsis* (Brautigam *et al.* 2013). Nevertheless, the discovery of abundant miRNAs in xylem tissue of *Populus* with target genes primarily involved in cell wall biosynthesis are highly suggestive of the role of epigenetic regulation in facilitating the plasticity of wood formation (Paiva *et al.* 2008; Puzey *et al.* 2012). This adds to the complicated picture of the role of epigenetic mechanisms in both enhancing phenotypic plasticity and creating novel genetic variation for natural selection.

1.2 Physiological responses to drought

1.2.1 Hydraulic and carbohydrate dynamics of trees under drought

Drought effects can disrupt plant fluid dynamics, internal resource distribution networks and metabolic pathways; hence, prolonged or severe episodes are associated with mortality events. The theoretical framework of physiological mechanisms of tree

mortality currently focuses on hydraulic failure, where fluid transport breaks down, and carbon starvation, where metabolic demands are unmet owing to depletion of non-structural carbohydrates (McDowell *et al.* 2008). Further to these hypotheses, phloem transport failure under drought owing to the coupling of xylem and phloem fluid transport has been suggested to contribute to failure to supply the non-structural carbohydrates (NSC) essential to plant metabolism (McDowell & Sevanto 2010).

Tree physiology is co-ordinated at a whole-plant level through vascular streams of transpiration and mass flow, which are conducted via xylem and phloem tissue respectively. Xylem cells are cast inwards from a lateral meristem (cambium) that actively divides and generates new tissue over the course of a growing season, thereby linking new leaves to water and nutrients, and maximising hydraulic efficiency by enlarging the total sapwood area. Xylem fluid transport is uni-directional towards the canopy. Phloem tissue is located on the outer rim of the lateral meristem and non-structural carbohydrates are translocated bi-directionally by an osmotically driven hydrostatic pressure gradient (Munch 1930). The intermediary meristem between xylem and phloem is hardly an osmotic barrier, being only a few cells thick, so there is a coupling of vascular carbon-water transport. This coupling has been represented in process-based models of fluid transport within trees (Hollta *et al.* 2009).

1.2.2 Hydraulic pathways and water-use efficiency

Water use efficiency refers to the ratio of the rate of carbon assimilation to the transpiration rate. Increased stomatal conductance raises the internal CO_2 concentration of the leaf, corresponding to an increased carbon assimilation rate. However, water is simultaneously lost via transpiration and this leads to a critical

trade-off between carbon and water under drought. Plasticity in stomatal conductance occurs at both the developmental level, manifesting in changes to stomatal number and size, and also at the physiological level, whereby drought effects are mediated by osmotic and chemical signalling of falling leaf water potential, resulting in loss of guard cell turgor and stomatal closure.

Flow against gravity along the soil-plant-atmosphere continuum is ensured by negative water potential gradients generated via evaporation (E, m^3 H_2O m^2 s^{-1}) from gas-exchange surfaces, or:

$$E = K_h(\psi_{soil} - \psi_{leaf} - \rho g h) \quad (1)$$

Where K_h is hydraulic conductivity and the water potential gradient is represented by the difference between leaf and soil water potential ($\psi_{soil} - \psi_{leaf}$) minus water potential due to gravity ($\rho g h$); ρ is water density (10^3 kg m^{-3}), g is the assumed gravitational constant (9.8 m s^{-2}) and h is tree height. Leaf water potential falls by an extra 0.01MPa per vertical metre (Tyree & Zimmerman 2002). Thus, trees are more at risk of discontinuity than other vascular plants owing to their greater height and the ensuing hydraulic path length (Niklas *et al.* 1994, Meinzer *et al.* 2010).

Regulation of hydraulic path continuity is mediated at the leaf level by osmotic and chemical signalling of falling leaf water potential, resulting in loss of guard cell turgor and stomatal closure. The leaf water potential threshold that elicits stomatal closure has been used to place species along a spectrum from isohydry (lower threshold) to

anisohydry (higher threshold). Isohydric stomatal behaviour is deployed to safeguard leaf water potential, but halts gas-exchange and incurs a carbon penalty under protracted droughts (McDowell *et al.* 2008). The isohydric strategy is also linked to higher xylem vulnerability to embolism, as plants rely on stomatal regulation of xylem tensions rather than alternative strategies of cavitation resistance (Taneda & Sperry 2008; Meinzer *et al.* 2010).

Alterations in maximal stomatal conductance (gs_{max}), to both water vapour and CO_2, can be achieved through adapted size (length by width) and density (number per unit area) of stomatal pores in the developing epidermis. There is a strong negative correlation between stomatal size and density across the Phanerozoic stomatal fossil record, along with a positive correlation between water-use efficiency and atmospheric CO_2 (Franks & Beerling 2009). Mutant *Arabidopsis* plants with lower stomatal density and greater stomatal size exhibit reduced transpiration rates and improved tolerance to water deficit (Doheny-Adams *et al.* 2012). The mechanism by which stomatal development is modified has been elucidated through characterisation of *Arabidopsis* mutants in wax biosynthesis, which suggest that epidermal wax composition influences the stomatal index (Holroyd *et al.* 2002).

Drought signals typically reduce stomatal number per leaf via signal networks involving sugar, ABA and transpiration (Quarrie & Jones 1977; Franks & Farquhar 2001). Low humidity of mature leaves triggers decreased stomatal density of new, non-exposed leaves in *Populus trichocarpa* × *P. deltoides* and *Arabidopsis thaliana* (Lake *et al.* 2001; Miyazawa *et al.* 2006). Meanwhile, high light experienced by

mature leaves in *Arabidopsis* and *Nicotiana* triggers a high stomatal index in young leaf primordia (Thomas *et al.* 2003; Coupe *et al.* 2006). Intra-canopy variation in 11 leaf traits of 6 deciduous trees indicated that stomatal density was plastic throughout the canopy, according to differences in leaf exposure (Sack *et al.* 2006). Accounting for canopy heterogeneity improved model simulation of canopy transpiration in a forest site dominated by *Populus tremuloides*, especially for shaded trees (Loranty *et al.* 2010). Exposure to elevated CO_2 (500 umol mol^{-1}) increased stomatal density and the number of stomatal rows in *Pinus sylvestriformis* and *P. koraiensis* needles; however, this did not impact stomatal conductance and therefore suggests the relationship between stomatal density and number does not necessarily correlate with functional variation in stomatal conductance (Zhou *et al.* 2013).

Aside from developmental plasticity in maximal stomatal conductance, physiological plasticity in stomatal conductance is facilitated by hormonal and metabolic signal transduction. Under drought conditions, abscisic acid (ABA) induces plasma membrane NADPH oxidases to produce hydrogen peroxide (H_2O_2) in guard cells (Guan *et al.* 2000). Accumulation of H_2O_2 activates calcium-permeable channels and increases the cytosolic Ca^{2+} concentration of guard cells, thereby triggering stomatal closure by obstructing K^+ channels (Schroeder & Hagiwara 1989). In addition to ABA effects on guard cell turgor, leaf hydraulic conductance is decreased by ABA in both wild-type and ABA-insensitive mutants of *Arabidopsis thaliana* (Pantin *et al.* 2013). Guard cell ABA-mediated control of stomatal closure is induced by sucrose and additionally mediated by a hexose phosphorylating enzyme involved in sugar-sensing,

indicating a potential feedback involving the product of photosynthetic activity and stomatal conductance (Kelly *et al.* 2013).

Although gymnosperms exhibit lower guard cell sensitivity to ABA than angiosperms (Brodribb & Cochard 2009; McAdam *et al.* 2011), isohydric regulation of stomatal aperture in *Pinus radiata* (Pinaceae) is driven by ABA, and there is no anisohydric switch to water potential-mediated closure (Brodribb & McAdam 2011). Isohydric stomatal behaviour is deployed to safeguard leaf water potential, concurrently halting gas-exchange and incurring oxidative damage and a carbon penalty under protracted droughts (McDowell *et al.* 2008). Thus, carbon starvation is a risk following early stomatal closure halting photosynthetic carbon fixation, along with immobilised non-structural carbohydrate (NSC) reserves (McDowell 2011). For example, regional drought-induced mortality rates of relatively isohydric *Pinus edulis* trees are significantly greater than for the sympatric, relatively anisohydric species, *Juniperus monosperma* (Breshears *et al.* 2005).

Defoliation is a common drought avoidance response in mature trees of deciduous species, where accelerated leaf senescence and abscission reduce canopy size, thereby alleviating evaporative demand. Collectively, cavitation in xylem of distal segments (leaves, roots and branches) can lead to hydraulic conductance failure more rapidly than in stem tissue and can elicit drought-deciduous behaviour (Tyree *et al.* 1993; Sperry *et al.* 2002; Bouche *et al.* 2015). Such leaf shedding can be a hydraulically mediated response to drought. Defoliated crowns also reduce nutrient losses (Marchin *et al.* 2010), but they entail reduced carbon assimilation and, where drought is

prolonged, can cause depletion of non-structural carbohydrates (Poyatos *et al.* 2013). In evergreen conifer tree species, there are long-lived needles that represent a high resource investment of nutrients and secondary compounds and the wax cuticle protecting the needles reflects this. Once large-scale crown defoliation has occurred it will take years for conifers to recover the same crown area through successive needle cohorts and the loss of photosynthetic tissue has a corresponding larger impact on evergreen than deciduous tree species. For example, defoliation in *Pinus sylvestris* presages reduction in non-structural carbohydrates and leads to mortality (Galiano et al. 2011).

Root growth is compromised in severe drought episodes and water uptake is regulated by aquaporins governing the permeability of the root cortex. Since the Casparian strip diverts water from the apoplastic to the symplastic pathway, changes in membrane hydraulic properties are significant (Cochard 2006). *Populus nigra* genotypes adapted to xeric or mesic growing conditions differ in both the degree of stomatal control and the expression of aquaporins under restricted irrigation. While drought-sensitive trees up-regulated aquaporin genes in vascular root tissue and induced stomatal closure, drought-adapted individuals down-regulated expression of root aquaporins and maintained stomatal conductance (Cocozza *et al.* 2010).

1.2.3 Carbohydrate translocation and sink-source interactions

Hydraulic failure by xylem embolism may occur if water cavitation in transporting conduits reaches a critical extent (Cochard 2006). Physiological impacts of hydraulic failure are manifold and include: loss of conductivity, runaway embolism, impeded

phloem translocation, tissue dehydration, blocked photosynthetic function, senescent distal organs, and carbon costs associated with refilling xylem and repairing damaged tissue (Brodribb 2010; McDowell 2011). A mechanistic link between hydraulic failure and tissue senescence is indicated by fine root death after coarse root cavitation in pot trials of *Populus tremuloides* (Anderegg *et al.* 2012). Process-based models have been validated showing that phloem functioning breaks down concomitantly with xylem dysfunction (Hollta *et al.* 2009). The root system can become isolated from mass flow carbohydrate delivery and source-sink dynamics are disconnected.

Carbon starvation can result from stomatal closure halting photosynthetic carbon fixation, depending on the duration of stomatal closure. Furthermore, non-structural carbohydrate (NSC) supply can become immobilised as a result of phloem function breaking down concomitantly with xylem dysfunction (Hollta *et al.* 2009; McDowell 2011). Phloem turgor loss can be higher in anisohydric species such as *Juniperus monosperma*, where flexible stomatal regulation leads to xylem water potentials plummeting to -8 MPa during drought, compared with isohydric species such as *Pinus edulis,* where xylem water potentials remain > -3 MPa (McDowell *et al.* 2013). Distal organs can thereby become isolated from mass flow carbohydrate delivery, disconnecting source-sink dynamics. Respiratory carbon isotope signatures from drought-stressed roots in 7 year old *Picea abies* indicate carbon metabolism switching to starch reserves and NSC levels falling in the root system, but not the canopy. Indeed, only root system carbon pools were strongly reduced upon tree death (Hartmann *et al.* 2013).

In the few studied plant species, including *Arabidopsis* and several cereals, soluble sugars (sucrose and hexoses) have been demonstrated to have a dynamic role in signalling and regulating expression of genes involved in photosynthesis, osmolyte production and sucrose metabolism and phloem loading, in order to mediate stress responses that included drought (for review see Rosa *et al.* 2009). Under conditions of drought-induced stomatal closure, soluble sugars have been found to increase concomitant with reduced starch in the leaf (Rodriguez-Calcerrada *et al.* 2011). In mature *P. sylvestris*, increased soluble sugar content in the sapwood is a predictor of drought-induced mortality (Camerero *et al.* 2015). In drought stressed *Pinus edulis* seedlings, foliar sugars (sucrose, fructose, glucose) decreased by 30% while starch content increased immediately prior to drought-induced mortality (Adams *et al.* 2013). Alexou *et al.* (2013) found that the developmental stage of seedlings affected physiological and metabolic drought responses in *Pinus halepensis*. While 3 month old seedlings increased starch and Rubisco degradation under drought, reflected in higher sugars and amino acids in phloem exudate, the 1 year old seedlings had higher gas exchange and did not degrade starch and Rubisco, but instead increased the antioxidant ascorbic acid under drought.

Drought effects on phloem transport and carbon availability are mediated through changes in xylem water potential, stomatal conductance and photosynthesis. However, oscillatory circadian clock effects on solute transport may also be mediated at the level of gene expression and hormonal signalling. For example, the gene *TOC1* is implicated in drought responses by regulating stomatal behaviour and interacting with solute

transport, but also shows circadian rhythms and is upregulated by ABA (Legnaioli *et al.* 2009; Pokhilko 2013).

Thus, current drought-induced tree mortality theory is centred on the importance of water and solute transport and carbon metabolism, with limited consideration of additional facets of plant metabolism, such as osmoregulatory and antioxidant defences. However, these might be especially relevant physiological processes for younger trees with shorter hydraulic path lengths. From a decadal through-fall exclusion experiment in the Amazon, it appears that hydraulic failure is the leading cause of death for taller trees as compared with smaller trees, from a combination of higher evaporative demand and the increased hydraulic path length (Rowland *et al.* 2015).

1.2.4 Antioxidant and osmoregulatory defences

Oxidative stress is a prominent side-effect of physiological responses to severe, mortality-inducing drought that can drive premature senescence of distal organs, such as leaves, thus carrying implications for the whole-plant carbon budget and hydraulic dynamics. The extent of oxidative damage and hence tissue turnover is governed by the activity of particular metabolic pathways and the capacity of antioxidant defences to avert an imbalance of reactive oxygen species (ROS). Reactive oxygen species (ROS) affect a variety of intra-cellular components, being highly diffusible and significantly more abundant under stress.

Both enzymatic and non-enzymatic ROS scavengers maintain ROS equilibrium and combat drought-induced oxidative stress (Cruz De Carvalho 2008). At the frontline of enzymatic defence is superoxide dismutase (SOD), a metalloenzyme that dismutates superoxide (O_2^-) to the less cytotoxic reactive oxygen species hydrogen peroxide (H_2O_2) and oxygen (O_2). Three groups are differentiated on the basis of their metal cofactors and subcellular locations: FeSODs in chloroplasts, MnSODs in mitochondria and peroxisomes, and structurally divergent Cu/ZnSODs in chloroplasts, cytosol and the extracellular space (Alscher *et al.* 2002). Metabolic modelling of the SOD-Ascorbate-Glutathione cycle with simulated loss of SOD resulted in O_2^- being processed via alternative non-enzymatic redox reactions that significantly elevated H_2O_2, though providing a compensatory route during low SOD activity (Polle 2001).

Following stomatal closure and dwindling intracellular CO_2, photorespiration enables increased consumption of photosynthetic electrons, providing an alternative route to carbon fixation that dissipates energy and averts photo-damage (Guan *et al.* 2004). Photorespiratory products are elevated in chloroplasts and shuttled to peroxisomes for oxidation, generating H_2O_2. Catalase, another antioxidant enzyme important in redox regulation, has a high Km value and therefore a lower efficiency for scavenging H_2O_2 at low concentrations than the other main scavenger of H_2O_2, ascorbate peroxidase (mM rather than μM range). Thus, catalase is able to function as a bulk scavenger of H_2O_2 under stress (Huang 1983; Mittler 2002). Indeed, peroxisome localised CATs detoxify the resultant H_2O_2 that is estimated to contribute >70% of drought-induced H_2O_2 (Noctor *et al.* 1998). Therefore, peroxisome proliferation ameliorates cellular redox equilibrium under various abiotic and biotic stressors (Lopez-Huertas *et al.*

2000; Castillo *et al.* 2008). Cytokinin can trigger increased rates of photorespiration and higher peroxisomal CAT activity, thereby protecting photosynthetic apparatus in transgenic *Nicotiana tabacum* overexpressing cytokinin during water stress (Rivero *et al.* 2009).

Sugars have a central position in relation to major ROS-producing processes. Application of the herbicide atrazine to *Arabidopsis* seedlings initiates the production of singlet oxygen, yet expression of chloroplastic *SOD* is enhanced by combination with sugar treatment rather than atrazine treatment alone (Sulmon *et al.* 2006). Conversely, sucrose starvation triggers activation of oxidative stress genes, including catalase (Contento *et al.* 2004).

Electrons channelled through the respiratory mitochondrial electron transport chain can be diverted from the cytochrome c pathway that generates a proton-gradient for ATP synthesis, instead flowing through the alternative oxidase (AOX). Treatment with an inhibitor of the AOX pathway in drought-stressed *Triticum aestivum* significantly decreased PSII quantum yield; however, only 10% of the reduction was attributable to AOX inhibition (Bartoli *et al.* 2005). In *Arabidopsis* mutants with a defunct AOX pathway, the resulting imbalance in ROS was offset by elevated photorespiration (Strodtkotter *et al.* 2009).

Glutathione (GSH) is an important reducing substrate for ROS detoxification, functioning as an antioxidant by scavenging hydrogen peroxide, singlet oxygen, superoxide and even hydroxyl radicals (Miller *et al.* 2010). GSH accumulation

compensates in catalase deficient mutants and in plants where catalase activity has been reduced by antisense technology (Chamnongpol *et al.* 1996; Willekens *et al.* 1997). Transgenic poplar (*Populus tremula x Populus alba*) generating higher levels of reduced GSH are less susceptible to photoinhibition stress than the wild-type species (Foyer *et al.* 1995). Photorespiration modulates the antioxidant glutathione pool by generating glycine, the substrate for glutathione biosynthesis (Foyer & Noctor 2000). Furthermore, the redox state of glutathione regulates CuZn-Sod gene expression *in vivo* in plants (Wingsle & Karpinski 1996).

Photo-oxidative stress results from over-reduction of the photosynthetic electron transport chain, generating excited triplet-state chlorophyll at PSII and singlet oxygen ($^1O_2$). Excessive lipid peroxidation by $^1O_2$ entails cellular death in *Arabidopsis* leaves (Triantaphylides *et al.* 2008). In the Mediterranean plant *Salvia officinalis*, the mechanism of leaf senescence under photorespiratory conditions is linked to degradation of chloroplast antioxidant defences by $^1O_2$; the protection afforded by low molecular weight lipophilic antioxidants is lost and the thylakoid membrane is damaged, leading to chloroplast degradation and senescence (Munne-Bosch *et al.* 2001). In particular, α-tocopherol is valuable in termination of lipid peroxidation chain reactions (Fryer 2002). Additionally, the role of a ligand-binding protein in protecting against lipid peroxidation was validated by autoluminescence visualisation of oxidative stress in *Arabidopsis* mutants with knock-out or overexpression of the chloroplastic lipocalin AtCHL (Levesque-Tremblay *et al.* 2009). De-epoxidisation of xanthophylls is vital in non-photochemical quenching (NPQ) of chlorophyll a fluorescence, facilitating the dissipation of excitation energy; this is particularly

required in chloroplasts of emerging leaves, where the photosynthetic apparatus is being progressively assembled (Latowski *et al.* 2011).

Flavonoids are a large class of compounds that are involved in photo-protection. Anthocyanins are water-soluble pigments generated via the flavonoid pathway that accumulate in vacuoles and epidermal tissue and are implicated in buffering abiotic stress, such as drought and UV-B, by filtering excess quanta and precluding chlorophyll excitation, saturation of the photosynthetic electron transport chain and overproduction of superoxide radicals (Gould 2004; Guo *et al.* 2005). Whereas *Arabidopsis* mutants lacking a key enzyme of the xanthophyll cycle readjusted during photo-acclimation and recovered PSII efficiency after 10-12 days, flavonoid-deficient mutants lacking chalcone isomerase were unable to compensate (Harvaux & Kloppstech 2001). Additionally, anthocyanins directly scavenge free radical species with a higher efficiency than ascorbate or α-tocopherol (Rice-Evans *et al.* 1997).

In a meta-analysis of 50 studies into foliar antioxidant defences across tree species in response to drought, Wujeska *et al.* (2013) found that while evergreen tree species increased membrane- bound protection by higher zeaxanthin and tocopherol, deciduous tree species preferentially accumulated water-soluble antioxidants (ascorbic acid and glutathione).

1.2.4.1 Osmotic adjustment

Osmotic adjustment involves accumulation of compatible solutes that constrain turgor loss at low water potentials, as well as protecting cellular membranes and proteins, and

compatible solute accumulation is non-toxic even at high concentrations (Zhang *et al.* 2002). Typical solutes involved in osmotic regulation are sugars, sugar alcohols, amino acids, organic acids, or inorganic ions (Munns 2005). Accumulation of sugars has been reported for a wide range of plant species under osmotic stress, induced both by drought and salinity (Chen & Jiang 2010). However, osmoregulation often involves a co-ordinated solute response; for instance, high accumulation of proline in *Arabidopsis thaliana* is an integrated response to osmoregulatory signals that include ABA and sucrose (Verslues & Bray 2006). Nguyen-Queyrens and Bouchet-Lannat (2003) observed intraspecific variation in osmotic adjustment of three year old *Pinus pinaster* saplings under drought stress. A slower imposition of PEG-induced osmotic stress favoured osmotic adjustment across 5 provenances in *Pinus canariensis* and was positively correlated with drought duration at the provenance site (Lopez *et al.* 2009).

Proline is an amino acid that acts as a molecular chaperone and compatible solute, protecting the antioxidant enzyme hydration shell under water stress. Exogenous application of proline thus increased antioxidant enzyme activities, mitigating salinity stress to a greater extent than betaine, another amino acid with an osmoprotective role (Hoque *et al.* 2007). Metabolite analysis indicated that proline was a drought stress marker in *Miscanthus x giganteus*, as the accumulation of metabolites in the proline synthesis pathway coincided with stomatal closure and growth cessation (Ings *et al.* 2013). Both proline and sugar accumulation have been found in *Pinus taeda* seedlings under drought stress (Meier *et al.* 1992). Additionally, proline acts as an antioxidant itself, by scavenging hydroxyl radicals (Smirnoff & Cumbes 1989). There is also

evidence for interaction between compatible solutes. For example, proline metabolism is responsive to sugar sensing mechanisms in *Arabidopsis* (Hellman *et al.* 2000).

1.3 Drought effects in the regeneration niche

> *"Now the ways in which they come into being are fairly simple; they all grow either from seed or from a root. [...] Such we must suppose are the ways in which wild trees originate, apart from the spontaneous ways of which natural philosophers tell. Anaxagoras says that the air contains the seeds of all things, and that these, carried down by the rain, produce the plants; while Diogenes says that this happens when water decomposes and mixes in some sort with the earth."*
>
> *Theophrasus (c. 350 - 287 BC), Enquiry into Plants, I, III, pp. 163.*

Observations made by Aristotle's contemporary and student, Theophrastus, provide some of the earliest insights into reproductive phenology and plant regeneration. Here Theophrastus has identified seeds as being the means of reproduction in plants. However, he refers to and partly supports the views of renowned philosophers, both of whom emphasise a requirement for rain and water in the soil. Theophrastus also writes about the variation in reproductive phenology between cultivated trees and natural populations: 'Such then are the differences as to the time of shedding and ripening their fruit between the wild as compared with cultivated trees, and likewise as compared with one another.' (pp. 185). Furthermore, he comments on the effect that drought can have on fruiting: 'There are also diseases of the fruits themselves, which occur if the winds and rains do not come in due season' (volume IV, pp. 397).

Much later, Grubb (1977) outlined the concept of a species' regeneration niche. He defined the regeneration niche as the biotic and abiotic requirements for an individual plant to re-establish following mortality of the mature plant. Whether increasing drought episodes would shift plant community species and functional composition is partly a question of how regeneration niches would be affected, directly and indirectly, by drought. Following a severe drought episode there will typically be both high mortality of the current establishing seedlings (often 100 %), owing to their less developed root systems, and differential mortality of mature trees. Adult trees with a longer hydraulic pathlength are more likely to suffer hydraulic failure, while mature trees of intermediate size that have a sufficiently developed root system to tap water sources deeper in the soil profile but have shorter hydraulic pathlengths may survive. Thus, there remains potential for regeneration to be a key stabilising process offsetting the impact of drought events that differentially affect the mature demographic.

Variation in leaf traits of both seedling and adult Bolivian tree species is strongly related to the regeneration niche rather than adult niche (Poorter 2007). Similarly, a mismatch between the altitudinal distribution limit of seedlings and adult trees was found for 13 out of 17 tree species in the French mountains, reflecting a disparity between the effects of warming on the regeneration niche and adult niche (Lenoir *et al.* 2009).

Water deficit can negatively affect viable seed production at a number of stages in the reproductive cycle. The effect of drought on reproductive phenology at the ecosystem scale is understudied compared to foliar phenology, since budburst and leaf senescence

are more easily observable through satellite imagery and canopy-level cameras than flowering and pollen and seed dispersal (Shen *et al.* 2015). However, both observational and experimental approaches have demonstrated that the development of female and male flowers can be perturbed by drought. For example, in *Pinus monticola* the development of strobili is associated with water stress: water deficit occurring during early summer, when strobili maturation happens or late summer in the preceding year leads to aborted development (Rehfeldt *et al.* 1971). In an Italian population of *Picea abies* monitored over 30 years, trees inhabiting north facing slopes exhibited higher seed production than those on south facing slopes, which was suggested to be linked to the higher frequency of water stress on the southern aspect (Mencuccini *et al.* 1995).

The final stage in regeneration, seedling establishment, has been used as an indicator of the effects of climate change on species assembly in plant communities (Sternberg *et al.* 1999; Kullman 2002; Lloret *et al.* 2009). Drought limitation of new recruitment and survival of establishing seedlings shows interspecific variation; for example, the density of *Pinus edulis* seedlings and saplings decreased following multi-year droughts and mortality was >50%, whereas the density of *Juniperus monosperma* seedlings and saplings increased, over 42 sites in North Arizona (Redmond *et al.* 2015).

Morphological and biochemical differences in early seedling performance can be influenced by maternal seed provisioning. Zas *et al.* (2013) reported that seed weight in *Pinus pinaster* was largely determined by whether conditions of the maternal environment were stressful or favourable. Differences in minimum temperature faced

by *P.pinaster* populations at different altitudes also explained variation in seed weight and germination rate (Correia *et al.* 2014). Germination rate and percentage increase with higher seed weight (Cedan *et al.* 2011). In *Pinus nigra*, higher seed weight increased emergence and seedling growth rate (Tiscar and Lucas 2010). In *Pinus halepensis*, higher seed weight increased seedling height and diameter and seedling survival of the first summer (Blade &Vallejo 2008). In *Pinus halepensis,* the effect of seed weight was more prolonged but had also disappeared by the second growing season to be replaced in significance by the maternal parent identity (Blade &Vallejo 2008). Higher seed weight has also been found to be positively correlated with emergence and early seedling root allocation, potentially facilitating drought avoidance (Lloret *et al.* 1999; Wennstrom *et al.* 2002). Establishing seedlings suffer high mortality rates during drought, owing to an underdeveloped root system, which precludes water uptake from deeper soil layers, and so increasing maximum root length is an important trait in drought avoidance.

Taeger *et al.* (2015) found that seedlings of *P. sylvestris* showed morphological plasticity under drought by increasing taproot length and root to shoot ratios. There was higher root growth plasticity in southwestern provenances, which was suggestive of local adaptation, with a trade-off with growth rate under conditions of adequate moisture. Seedling maximum rooting depth was positively related to drought survival in five Mediterranean tree species. The only *Pinus* species in the study (*P.halepensis*) developed the shallowest rooting depth and thus had increased susceptibility to drought (Padilla & Pugnaire 2007). However, in another study the ratio between

rooting depth and total leaf area was more significant than rooting depth *per se* in predicting drought survival time (Lopez-Iglesias *et al.* 2014).

Screening 27 seedlings from open-pollinated families of *Pinus ponderosa* for drought tolerance and putatively corresponding functional traits revealed no differences among provenances in stomatal density, gas-exchange parameters (net photosynthesis and conductance to H_2O) and predawn water potential; instead, shorter needle lengths were found in drought-tolerant seedlings (Cregg 1994). However, Donnelly (2015) found a negative correlation between the number of stomatal rows and occurrence along a longitudinal gradient of water availability for 8 population of *P. sylvestris* in Scotland.

The rhizosphere is an essential part of a plant species' ecological niche and mycorrhizae are important associated organisms. Mycorrhiza form symbiotic connections with the majority of extant vascular land plants, exchanging nutrients for photosynthetically-derived carbon. For plants, this represents an efficient carbon allocation strategy, as mycorrhizal hyphae extend over a greater volume of soil and gain access to pore sizes that exclude fine root diameters (Smith & Read 1997; Raven & Edwards 2001, Allen 2009). The nature of the plant-fungal interface differs between arbuscular mycorrhiza (AM) and ectomycorrhiza (EM). AM trigger invagination of the root cell membrane and inhabit arbuscule structures, whereas EM generate a fungal mantle sheathing the root system (Taylor *et al.* 2009).

Drought effects on the rhizosphere that perturb mycorrhizae might have effects of reducing nutrient availability and amplifying water deficit, since mycorrhizae are

implicated in improving access to water as well as nutrients. Both AM and EM are capable of penetrating regolith substrate and bedrock, thereby making previously unavailable plant water sources available (Egerton-Warburton *et al.* 2003; Bornyasz *et al.* 2005). Hydraulic redistribution is the vertical or lateral transfer of water from moister towards drier zones in the soil profile via roots or hyphae; thereby water transfer is accelerated in comparison with bulk soil water flux. A role for mycorrhiza in hydraulic redistribution to neighbouring plants has begun to emerge; through upregulation of plant root aquaporin membrane protein genes when in association with EM (Marjanović *et al.* 2005) and AM fungi (Uehlein *et al.* 2007; Li *et al.* 2012), through leakage of water from hyphae into the rhizosphere, and directly by hyphal connections between plants (Egerton-Warburton 2007). Furthermore, there is evidence to suggest that AM hyphae conduct water (through both apoplastic and symplastic pathways) and thereby increase hydraulic conductivity of rhizosphere soil; for example, Auge *et al.* (2004) showed that AM colonisation of soil was the single most important factor determining drought tolerance of *Vigna unguiculata* seedlings, more significant than whether the plants were colonised.

Forming mycorrhizal associations at the seedling establishment stage depends on the availability of compatible mycorrhizal species in the rhizosphere. Mycorrhizal community composition is strongly dependent on forest age class and plant community composition (Johnson et al. 2005; Wu et al. 2013). If drought effects shift species composition, there can be a concomitant shift in mycorrhizal species abundance that restricts availability for establishing seedlings. For example, Haskins *et al*. (2005) found that the increasing dominance of *Juniperus communis* over *Pinus edulis* owing

to its higher drought tolerance had the effect of increasing its associated AM fungi. Thus, *P. edulis* seedlings were less likely to encounter compatible EM fungi.

1.4 Study organism: *Pinus sylvestris*

1.4.1 Distribution and genetic differentiation

Pinus sylvestris L. is the most widely distributed species in the *Pinus* genus, which comprises 110-120 species and is the largest genus in the coniferous family Pinaceae. The range of *P. sylvestris* extends from northern Finland to Turkey and from western Spain to eastern Siberia. Populations are found in both boreal and Mediterranean climatic regions in Europe (Figure 1.2.1). Thus, disparate ecological conditions are encountered within the range of *P. sylvestris*, a corollary of which is a capacity for local adaptation. For example, latitudinal and longitudinal clines in phenology of budburst and growth have been observed (Andersson & Fedorkov 2004; Notivol *et al.* 2007; Chmura *et al.* 2012).

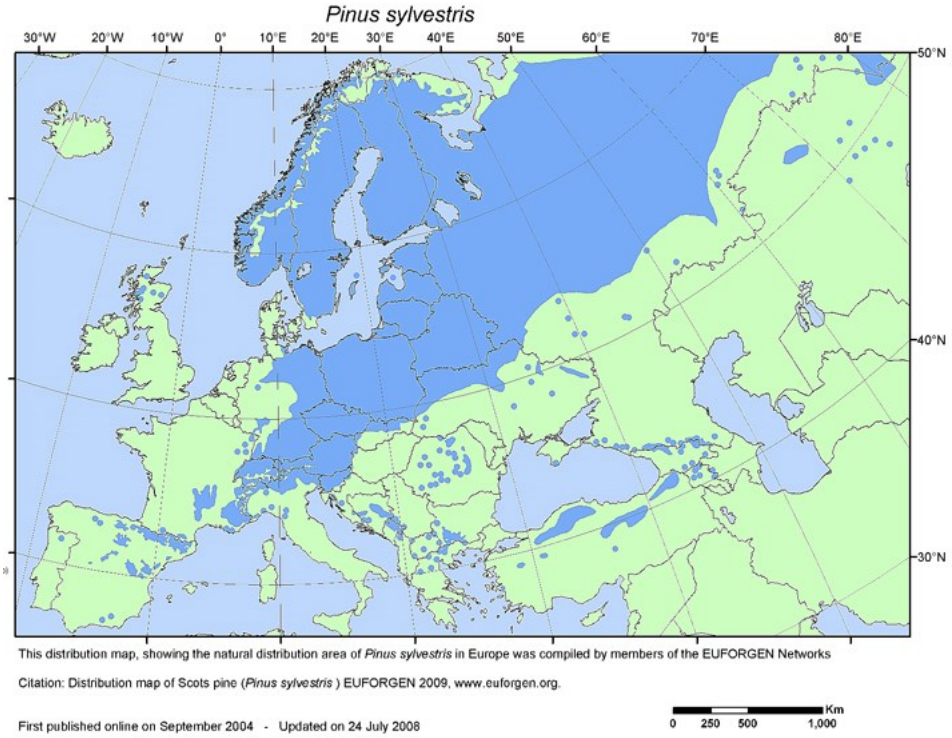

Figure 1.4.1 European distribution map of Pinus sylvestris (www.euforgen.org, accessed 02.10.14).

Genome size is typically very large in *Pinus* (20 to 40 Gb), though the karyotype is constant at 2N = 24 (Wakamiya *et al.* 1993). The most recent estimate of genome size for *P. sylvestris* is 1C (haploid) = 22474 Mbp or 22.98 pg (Fuchs *et al.* 2008). Estimates made by other authors are between 1C = 21.25 – 27.8 pg (Valkonen *et al.* 1994, Joyner *et al.* 2001, Bogunic *et al.* 2003). The genome is characterised by repetitive sequences, in common with other *Pinus* genomes, with 70 to 75% of the *P. sylvestris* genome consisting of repetitive sequences (Kole *et al.* 2007). Although the lack of a reference genome precludes estimates of the number of genes in the *P. sylvestris* genome, *de novo* transcriptome assembly revealed that less than 0.5% of unigenes identified (19 659 in total) were putative retrotransposon sequences and 48% matched known proteins (Wachowiak *et al.* 2015). Hizume *et al.* (2002) found that the pattern of

chromosomal location of eight 18S rDNA loci could distinguish *P. sylvestris* from three other *Pinus* species. Intraspecific variation in rDNA patterns are also apparent, with 13 rDNA phenotypes found in populations of *P. sylvestris* across Finland (Karvonen & Savolainen 1993). However, neutral molecular markers, including rDNA, were found to be poor predictors of population differentiation in bud phenology, an adaptive trait (Karhu *et al.* 1996). The large, out-crossing populations of *P. sylvestris* have resulted in a strongly homogenising effect of gene flow on genetic differentiation at nuclear gene loci, with shared patterns of nucleotide polymorphism across populations (Kujala & Savolainen 2012, Wachowiak *et al.* 2014). However, the effects of selection were detectable at several dehydrin loci involved in cold tolerance across a longitudinal cline (Wachowiak *et al.* 2009), as well as bud phenology (Hurme *et al.* 2000). Furthermore, Wachowiak *et al.* (2014) identified outlier nuclear loci of regulatory genes involved in epigenetic control of gene expression, signal transduction and cellular metabolism, possibly signifying that adaptive phenotypic plasticity might be occurring. Variation in telomeric repeat lengths has also been found in *P. sylvestris*, potentially carrying implications for oxidative stress tolerance by limiting DNA damage (Aronen *et al.* 2012).

1.4.2 Drought effects on the regeneration niche of Pinus sylvestris

According to Grubb, regeneration consists of five stages: 1) viable seed production; 2) seed dispersal; 3) germination; 4) initial establishment and 5) seedling establishment. The first stage in regeneration, viable seed production, involves not only successful fertilisation but also seed maturation. In a stand of *Pinus sylvestris* in Lithuania, a rainfall manipulation experiment using interception by roofs was carried out, which

resulted in a decrease in the germinability of the pollen and also a reduced proportion of fruiting trees (Ozolincius *et al.* 2009). At the seed maturation stage drought is known to be a major factor in reducing the seed size of *Pinus sylvestris* from Sierra Nevada (Southeast Spain), where trees consistently produce heavier seeds when cones ripen in rainy years (Castro 1999).

The second stage in regeneration is seed dispersal. Cone opening and seed release for *P. sylvestris* occurs in spring, with most seeds rapidly dispersing over 14-21 days in southern France (Debain *et al.* 2007) and 50% of seeds dispersing over 18-28 days in Sweden (Hannerz *et al.* 2002).Variation in seed dispersal velocity in *P. sylvestris* is low, with a strong positive correlation between seed size and wing area meaning that seed wing loading remains constant across a range of seed sizes (Debain *et al.* 2003a, 2003b). Regeneration frequently falls dramatically after strong fires due to the lack of serotinous cones in this species, and *P. sylvestris* almost disappears from burned areas, often being replaced by *Quercus* species or shrubs (Retana *et al.* , 2002; Vilà-Cabrera *et al.* , 2012).

The third stage in regeneration is seed germination. *P. sylvestris* has little or no seed dormancy and therefore does not form a persistent seed bank (Castro 1999, Castro *et al.* 2005). Although the regeneration niche of *P. sylvestris* is limited by low temperatures in northern range margins, summer drought constrains regeneration at the southern range edge, in relictual high altitude populations in Mediterranean-type ecosystems (Hurme *et al.* 1997; Martínez-Vilalta and Piñol 2002; Bigler *et al.* 2006). However, this is not owing to problems with seed production and dispersal (Debain *et*

al. 2003; Mendoza *et al.* 2009; Zamora *et al.* 2010). In Mediterranean regions, germination is restricted to spring and autumn because of the seasonality of precipitation. However, post-dispersal seed predation has a high impact during the autumn and therefore the majority of germination leading to seedling establishment occurs in the spring (Castro *et al.* 2005).

Following the initial three stages of the regeneration cycle is seedling establishment. This stage has a particularly high failure rate and is considered a bottleneck in *P. sylvestris* regeneration (Matias *et al.* 2011). Across its European range, higher seed weight classes are from southern populations (Reich *et al.* 1994). However, the effect of seed weight on improving seedling performance in Spanish populations of *P. sylvestris* has only been demonstrated to last one growing season, after which maternal parent identity accounts for variation in seedling performance (Castro 1999).

Although in Mediterranean regions spring and summer precipitation is predicted to decline under climate change, in the Sierra Nevada Mountains rainy summers of low frequency (7-40 years) are also predicted (Rodrigo 2002) and may provide an opportunity for seedling establishment (Matias 2010). In a Mediterranean mountain population in central Spain, microsites with conditions of partial cover and high soil water content during the summer were positively related to *P. sylvestris* seedling establishment (Barbeito *et al.* 2009). Mendoza *et al.* (2009) compared the contribution of seed limitation to establishment limitation at an inter-annual level for woody species across different habitats within a Mediterranean mountain forest (including *P. sylvestris*), finding that seed limitation was inversely proportional to adult tree density,

whereas establishment limitation was high across all sites and linked to inter-annual variability in precipitation reducing the abundance of suitable microsites. Lopez-Iglesias *et al.* (2014) used functional trait data to explain variation in time to drought-induced mortality of seedlings for 10 Mediterranean tree species. They found that a set of three evergreen species, including another *Pinus* species, *P. pinea*, had low RGR and deeper roots in relation to transpiring leaf area and increased drought survival time.

Nutrient availability might not be as essential during the seedling establishment phase as it is later on in seedling-to-sapling development (Matias 2011). However, the effect of drought in shifting community composition of plants and their associated mycorrhizae could have a subsequent effect on regeneration success of *P. sylvestris*. In Mediterranean regions, the higher drought tolerance of *Quercus ilex* is anticipated to result in its increasing recruitment and dominance over *P. sylvestris* under future drought scenarios, with regeneration of *P. sylvestris* limited to narrow windows of opportunity for recruitment provided by rainy summers (Matias 2011; Galiano *et al.* 2012; Carnicer *et al.* 2014). This would not however have the effect of eliminating compatible mycorrhizae, since *Pinus sylvestris* forms EM associations, including with the most common EM species associated with *Q. ilex*, *Cenococcum geophilum* (Johnsson *et al.* 1999; Claveria and De Miguel, 2005; Aucina *et al.* 2007; Scattolin et al., 2014). Yet there might be an effect on seedling establishment related to EM species identity. Kipfer *et al.* (2010) found that of four EM species, only *Suillus granulatus* improved *P. sylvestris* seedling performance in terms of shoot growth. *S. granulatus* is an EM commonly associated with conifers. In a mycological survey of *Q. ilex* forest, this species was recorded only in plots containing *Pinus pinaster* (Zotti *et al.* 2013).

Hence, drought effects on mycorrhizal composition could also potentially affect *P. sylvestris* regeneration.

1.5 Forecasting *Pinus sylvestris* range dynamics

In bioclimatic models, climatic variables are correlated with current species distributions to quantify the climatic niche, or the 'climate-envelope', which can then be used to predict range shifts under future climate scenarios (Heikkinen *et al.* 2006). Although these models assume equilibrium between distribution and current climate, additional factors governing niche space are evident from species survivorship outside their simulated climate-envelope, *e.g.* via transplant studies (Lewis 2006). The value of bioclimatic modeling lies in providing a species-level assessment and pinpointing areas of concern. There can be a strong regional disparity in species extinction risk, and species-rich regions with a high proportion of endemic species (species existing exclusively in the region) have been identified as particularly vulnerable to climatic change induced extinction (Enquist 2002; Thomas *et al*. 2004; Malcolm *et al*. 2006). Species distribution models (SDM) use a correlative approach to relate environmental variables to the occurrence of species. Thuiller *et al.* (2005) projected late 21st century species distributions for 1350 European plant species under seven climate change scenarios. Regional variation was found to exist, with mountain region species particularly threatened and boreal regions more resistant. Palaeoecological analyses may also identify the relative importance of different drivers, for example by hind-casting species range shifts during the late Quaternary (Froyd & Willis 2008).

However, correlative projections of extinction risk that rely on the static climate-envelope concept may be unjustified because many additional factors determine species distributions. Climatic variables are incongruent in scale with other factors regulating species ranges; SDMs do not represent demography, biotic interactions or reproductive phenology (Guisan & Thuiller 2005, Thuiller *et al.* 2013). Furthermore, climate model grid cell sizes are not typically of a sufficiently fine-scale resolution to capture climatic heterogeneity due to topographical complexity. Population demographics are difficult to model effectively, and there may be a mismatch between the juvenile and adult niche of species (Lenoir *et al.* 2009). For example, in the Andalusia forest region of southern Spain, there was a facilitative effect of *Pinus* species on the *Quercus ilex* regeneration niche, enabling seedling establishment (Urbieta *et al.* 2011). Climate envelope boundaries also differed between seedlings and adults in six coniferous tree species across the dry domain of the western US; the seedling climate envelope boundary suggested range contraction rather than expansion for the montane species compared with subalpine species (Bell *et al.* 2014). Biotic interactions are also influential in determining species' ranges. For example, Bond (1989) hypothesised that conifer regeneration would be restricted by competition intensity with angiosperms in their regeneration niche. Indeed, relative growth rate in seedlings of 7 early successional angiosperm tree species were higher than the maximum attained by conifer seedlings (Becker 2000).

More recently, the importance of focusing on demography in modelling species range dynamics has been noted (e.g., special issue Ecography 37, 2014). The demographic perspective encompasses the influence of environmental and evolutionary drivers on

plant functional traits as well as biotic interactions that combine to affect demographic changes. Snell *et al.* (2014) propose that dynamic vegetation modelling (DGVM) approaches incorporate multiple interacting processes, including: the onset of reproduction being responsive to increased atmospheric CO_2, a temperature dependent masting frequency, wind dispersal linked to local turbulence and air flow and a refined submodel for seedling establishment. With regard to the need for improvements in fine-scale modelling of seedling establishment, Snell *et al.* (2014) advocate increased experimental studies and parameterisation techniques. Both mortality and intraspecific variability in traits owing to local adaptation are also highlighted as processes omitted from DGVMs and that require further investigation.

Drought-induced tree mortality is a phenomenon affecting dry forest ecosystems and is predicted to increase under ongoing climate change (Allen *et al.* 2010; Steinkamp & Hickler 2015). As migration of forest trees is not expected to proceed at a sufficient pace to keep up with changing climate, populations are dependent on their resilience to extreme climatic events, such as heat-waves or severe drought (Petit *et al.* 2008; Aitken *et al.* 2008). However, population resilience does not only amount to the capacity of mature individuals to withstand lethal drought episodes. Both the reproductive capacity of adults and the subsequently regenerating seedling bank can be affected by drought, with implications for future generations. Investigating the mechanism of drought-induced mortality at the seedling stage is also warranted, in order to obtain a comprehensive picture of drought effects on population dynamics and community assembly.

Pinus sylvestris forest mortality is documented as occurring at its xeric range edge in relict populations owing to summer drought stress. The capacity for local adaptation will partly depend on the existing variation in traits related to drought tolerance available for selection. The stage of seedling establishment is considered a population bottleneck in the sense that high seedling mortality rates act as a filter, removing less well-adapted individuals.

1.6 Scope of thesis

The aim of this thesis is to test if drought impacts on seedling establishment in the species *Pinus sylvestris* vary depending on the origin of seed, *i.e* the combined effect of any adaptive genes found in parental genotypes, epigenetic mechanisms involving changes to the way in which genes are expressed and transgenerational seed provisioning. Since the study organism has a large genome size that is not fully assembled and mapped with quantitative trait loci (QTLs), disentangling transient epigenetic changes from adaptive allelic changes are beyond the scope of the thesis. In addition, clonal genotypes are not being used; rather seeds were collected from open pollinated trees in unmanaged, natural stands. Thus, the focus will generally be on characterising phenotypic variation at the level of genetic and environmental (G x E) interactions resulting from monitoring the effect of a drought treatment on seedlings from different population genetic backgrounds. In order to address this aim, a transplant experimental approach has value in investigating the extent of differentiation in phenotypic traits among populations from different conditions. Seedling performance under drought evaluated at the critical establishment bottleneck

stage and compared across different populations of origin will facilitate understanding of regeneration potential for this species.

The primary research questions are:

(1) To what extent does drought stress reduce *Pinus sylvestris* seed germinability, germination rate and early seedling growth and survival?

(2) What are the mechanisms by which drought impacts seedling growth and mortality?

(3) Is there differentiation of seedling drought responses at the morphological, physiological and metabolic level among populations from environments with and without severe summer drought?

Three experiments were used to assess seedling drought response and the extent of intraspecific variation. In Chapter 2 the effects of osmotic stress on the initial seedling establishment stage of the regeneration niche are studied by comparing phenotypic responses across provenances at morphological, physiological and molecular levels. Chapter 3 studies the responses of older seedling (10 months) to a drying down of soil moisture for 40 days. Along with measurements of biomass and photochemical efficiency, a non-targeted metabolomics analysis was carried out to investigate metabolic changes. Chapter 4 investigates the effects of seed size across provenances on seed germination rate, final germination percentage, as well as seedling drought responses in biomass allocation and the expression of selected antioxidant genes.

2 Provenance effects on germination and seedling performance under osmotic stress in *Pinus sylvestris*

2.1 Abstract

The frequency of extreme weather, including drought, is expected to increase under future climate change. This poses a major challenge for forest communities, particularly for populations at the xeric edge of tree species ranges, where species may be near their physiological drought tolerance limits. Whether or not these populations at the xeric edge show local adaptation for drier conditions will be critical for their survival. Here, we assess whether *Pinus sylvestris* seeds from four provenances across the species' range show differential ability to germinate, grow and survive, under a controlled experimental treatment that simulates drought stress. We inflicted this treatment by adding polyethylene glycol to the irrigation water to induce osmotic stress, both at the seed stage, to simulate effects of episodic low soil water potentials in the post-dispersal environment, and at the seedling stage, a particularly critical life stage for tree survival. We found limited evidence for an influence of the post-dispersal seed environment on subsequent germination, growth or survival of seedlings. Meanwhile, simulated drought conditions had large effects on seedling growth and performance, and this was modulated by provenance. Our results suggest that there is differentiation amongst populations for drought adaptation, with populations of *Pinus sylvestris* at its xeric southern range edge showing better seedling performance and survival than those from regions with low water deficit.

2.2 Introduction

Anthropogenic climate change is projected to lead to increased frequency and severity of extreme temperatures and precipitation (IPCC 2014). Despite knowledge of widespread dieback triggered by drought and heat in all major forest biomes (Allen *et al.* 2010) and documentation of variation in species mortality rates (Mueller *et al.* 2005; Breshears *et al.* 2009; Martinez-Vilalta *et al.* 2010; Rigling *et al.* 2013), the underlying mechanisms of mortality, species-specific vulnerability and population level resilience are poorly known for most tree species. Regeneration can be a key stabilising process offsetting the impact of events that decimate the mature demographic. Seedling establishment is a vital stage in a species' regeneration niche, defined by the biotic and abiotic requirements for an individual plant to re-establish following mortality of the mature plant (Grubb 1977). Owing to high mortality levels during seedling establishment of the regeneration niche, this stage represents a major bottleneck to recruitment into a population. However, the impact of drought at the stage of seedling establishment is understudied, despite the heightened mortality rates and increased sensitivity to climate change of this demographic (Lloret *et al.* 2004, 2009). It is therefore of interest to uncover to what extent severe drought stress brought on by a globally changing climate could impact the natural regeneration capacity of forest tree species and potentially initiate range retraction at the seedling establishment bottleneck.

Local adaptation occurs when divergent natural selection has eliminated phenotypes with deleterious traits and thereby changed the frequency of alleles in a population, resulting in higher mean fitness of the population under the prevailing environmental

conditions (Kawecki and Ebert 2004). Evidence from common garden experiments indicates that a number of forest tree species exhibit local adaptation resulting from divergent selection acting on populations with high standing genetic variation facilitated by long distance gene flow (Kremer 2010). Provenances are areas within which similar ecological and climatic characteristics are found and so local adaptation to site conditions may be expected.

Pinus sylvestris L. is the most widely distributed species in the genus *Pinus*, which comprises 110-120 species and is the largest genus in the coniferous family Pinaceae. The range of *P. sylvestris* extends from northern Finland to Turkey and from western Spain to eastern Siberia. Thus, populations are found in both boreal and Mediterranean climatic regions in Europe. Because of spanning such an extreme climatic gradient, *Pinus sylvestris* is an ideal species to study the effects of local adaptation. As a corollary to encountering disparate ecological conditions in its range, latitudinal and longitudinal clines in phenology of budburst and growth have been observed that testify to a capacity for local adaptation (Andersson & Fedorkov 2004; Notivol *et al.* 2007; Chmura *et al.* 2012).

The drought stress response not only depends on avoidance strategies, for example by adjusting biomass allocation and investing in deeper roots, but also traits that enable tolerance of unavoidable water deficit. These include changes that enable protection of photosynthetic machinery and cellular integrity by osmoregulatory and antioxidant defences, in order to facilitate recovery after a drought episode. Because of stomatal closure to limit water loss, a prominent physiological side effect of drought is oxidative

stress, whereby reactive oxygen species (ROS) accumulate from disrupted photosynthetic pathways. Therefore, the drought stress response not only depends on morphological and physiological traits, but also antioxidant defences. At the frontline of enzymatic defence is superoxide dismutase (*SOD*), a metalloenzyme that dismutates superoxide (O_2^-) to the less cytotoxic reactive oxygen species hydrogen peroxide (H_2O_2) and oxygen (O_2) (Alscher *et al.* 2002).

Osmoregulatory and antioxidant networks in seedlings can be affected by drought conditions and or seed wetting at the post-dispersal phase. This has been shown through seed priming experiments, which involve pre-treatment with solutions that enable germination to initiate metabolically followed by re-drying. In studies on seed priming in both annual and perennial plants, researchers observed increased antioxidant enzyme activities and upregulation of stress related genes that enhance stress tolerance (see review by Chen and Arora 2013).

The aim of this study was to test for differentiation among *Pinus sylvestris* provenances in their adaptation to drought stress, by examining variation in germination and early seedling growth and survival under an experimental drought treatment. Osmotic stress was induced using an osmotically adjusted solution, which serves as a proxy for low soil water potentials encountered in the regeneration niche during summer droughts (Perdiguero *et al.* 2012). High molecular weight polyethylene glycol (PEG $\geq M_n$ 6000) is a solute that has been used extensively to control water potential in drought stress experiments, as it is not taken up by plant cells and has no

toxic effects apart from inducing osmotic stress (Murillo-Amadaor *et al.* 2002; Turkan *et al.* 2004; Verslues *et al.* 2006).

Open pollinated seeds were collected from four provenances spanning a climatic gradient of water availability. These seeds were split into 3 treatment groups, with no, low and high concentrations of PEG solution applied. This seed priming treatment was thereafter crossed with a treatment on the subsequent seedlings where the irrigation water either did or did not contain PEG. We hypothesised that: 1) the experimental drought treatment would have a more negative impact on seedlings from provenances with higher soil water availability, owing to local adaptation in the provenances experiencing drought stress; and 2) seed priming would enhance SOD activity and seedling performance under the experimental drought treatment.

2.3 Materials and Methods

2.3.1 Seed provenances

Climatic characteristics of the four populations from which the open pollinated seeds were collected are presented in Table 2.3.1. Mean annual cumulative precipitation (ppn) was obtained from 1901 to 2015 from the CRU TS3.10 Dataset (Harris et al. 2014). Mean soil pF value (pF expresses the force with which different quantities of water are retained by the soil (Woodruff 1940)) in the summer month with the maximum mean pF value (July/August) from 1990 to 2015 were obtained from the European Drought Observatory (edo.jrc.ec.europa.eu/ on 20.02.15). Climatic water

deficit values for these sites were obtained from the Chave et al. 2014 data layer. The seeds were provided and prepared as follows: cones were oven dried at 40°C for 48 hours and then the seeds were extracted and the seed wings were removed from the seeds (A.Perry and L.Matias pers comm). Five mature trees were sampled per population from Rothiemurchus, Pernitz and Jarocin, whereas a minimum of 15 mature trees were sampled from Sierra Nevada. Seeds were collected and stored at <4˚C in 2010 and stored at 4 ˚C for 6-9 weeks prior to this experiment. These conditions are in accordance with Forestry Commission seed storage and pre-treatment guidelines for *Pinus sylvestris*. *Pinus sylvestris* seed viability can deteriorate with storage owing to the reduction of antioxidant protection from tocopherols (Vitamin E) leading to lipid oxidation and electrolyte leakage, therefore seed ageing and reduced germinability. However, comparison of germination percentage in seed lots stored for 3-21 years showed that even though 21 year old seeds contained 52% less tocopherol, germination capacity remained relatively high (75%) (Tammela *et al.* 2005).

Table 2.3.1 Populations included in the study with coordinates and mean altitude of the sampled sites within populations. Latitude: Lat.; Longitude: Lon. Decimal degrees: DD . Altitude: Alt. Metres above sea level: m a.s.l. Annual cumulative rainfall, climatic water deficit, and summer month soil moisture retention (pF) at provenance sites during the period 1901 to 2015. Mean seed weight was determined by weighing 100 seeds.

Provenance site	Lat. (DD)	Lon. (DD)	Alt. (m a.s.l)	Mean annual cumulative ppn (±SD)	Climatic water deficit (mm/yr)	Mean soil water retention (pF) for driest summer month (1990-2015)	Mean seed weight (g), n=100
Rothiemurchus, Scotland	57.15	-3.77	318	1040 ± 128	-5.92	2.32	0.0098
Pernitz, Austria	47.91	16	500	1060 ± 136	-76.92	3.38	0.0106
Jarocin, Poland	51.97	17.48	120	540 ± 79	-212.87	2.7	0.0076
Sierra Nevada, Spain	37.05	-3.27	1825	497 ± 119	-463.92	5.65	0.0110

2.3.2 Germination conditions and monitoring

The 12 week experiment (22.09.14 until 15.12.14) was conducted using controlled environment chambers at the University of Edinburgh (UK) under constant conditions with diurnal cycles of 16 h light at 100 µmol m^{-2} s^{-1} and 8 h darkness. Relative humidity was 65% (day) and 50% (night), with a constant day/night temperature of 21˚C. This is the current mean temperature at the southern range limit of this species during July and August (Matias and Jump 2014).

For the seed priming pre-treatment, 100 seeds were transferred to petri dishes (20 seeds/dish) with two filter papers moistened with 2.5 ml of a solution containing the osmotic agent polyethylene glycol (PEG 8000) (Sigma–Aldrich, Dorset, U.K) at one of two concentrations: 10% (100 gl^{-1}), with a water potential of −0.66 MPa, and 30% (300 gl^{-1}), with a water potential of −1.53 MPa, prepared according to Michel (1983). Seed priming was carried out over 7 days and petri dishes kept in the growth room. Seeds were dried to their original moisture content following the priming treatment. Dry, unprimed seeds were transferred to petri dishes with two dry filter papers and were considered the control treatment.

A randomized block design was used and blocks (32 cell plug-trays) rotated once a week to minimise variation attributable to block position within the growth chamber. One seed was sown per cell in 2:1 peat and sand mixture with perlite to aid drainage with a propagator lid to maintain constant humidity. Mycorrhizal inoculation was applied with 50 g TNC Mycorr^{Multi} per 15 L soil medium, which contained the

following ectomychorrizal species: *Rhizopogon amylpogon; R. fulvigleba; R. rubescans; R. villosuli; Laccarria laccata; Pisolithus tinctorius and Scleroderma sp*. Germination was monitored over 28 days, which is considered the upper time limit for germination in *Pinus sylvestris* (Nygren 1987; Castro 1999; Zhu *et al.* 2006). Germination was defined as radicle emergence 1–2 mm in length from the tegument. Germinated seeds were counted every 24 h until day 28, at which point ungerminated seeds were immersed in water for 48 h, and then examined using forceps for viability.

2.3.3 Osmotic stress irrigation treatment

Eight weeks after the sowing date, emerged seedlings were randomly assigned either to osmotic stress or control irrigation regimes for two weeks. For the osmotic stress treatment, the distilled water used for irrigation contained 30% (300 gl^{-1}) polyethylene glycol (PEG 8000), producing a water potential of −1.53 MPa. To compare with values obtained in the field, a study of water potential changes during a summer drought in a boreal *Pinus sylvestris* stand showed that soil water potential declined to -2 MPa in the upper soil profile and to -1 MPa in the lower soil profile (Duursma *et al*. 2008).

2.3.4 Seedling biomass and relative water content

Seedlings were harvested following the end of the 14 day irrigation treatment. Seedlings were cut at the root collar to determine biomass (g) of root and shoot separately. Roots were carefully washed, and shoot and root fresh weight obtained before being wrapped individually in lab roll and hydrated in a tray of distilled water for 2-4 hours, in order to obtain the turgid weight and subsequently determine the relative water content (%) of stems and roots. Dry weight was obtained after oven drying for 48 hours at 60 ˚C. Root to shoot dry matter ratios were calculated.

2.3.5 Maximal photochemical efficiency of photosystem II

Chlorophyll fluorescence emission was measured with a MiniPAM fluorometer (Walz, Effeltrich, Germany) using the fibre optic tip maintained with a leaf clip on the middle portion of a single needle. Successive durations of dark adaptation were tested until the maximal fluorescence level (F_m) was constant and 10 minutes was the minimum time required. Following dark adaptation for 10 mins, the initial level (F_o) of chlorophyll fluorescence was established by a 2 s pulse of far-red light. The maximal fluorescence level (F_m) was determined with a 0.8 s pulse of high intensity (4620 μmol m^2 s^{-1}) saturating actinic light. Hence, the maximal quantum yield at PSII was calculated as $F_v/F_m = (F_m - F_o)/F_m$. Measurements were taken for 5 seedlings/provenance/treatment at 12 pm after 7 days of the PEG irrigation treatment.

2.3.6 Superoxide dismutase enzyme assay

For the assay, 3 seedlings per provenance x treatment combination were harvested at 12 pm after 14 days of the PEG irrigation treatment. Crude enzyme extracts for the determination of *SOD* activities were prepared by homogenizing a whole seedling (100-200 mg) with a pestle and mortar under ice-cold conditions (4°C) in 3 ml of extraction buffer, containing: 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100. Following centrifugation at 10 000 × *g* for 20 min, the supernatant fraction was used in the assays.

Superoxide dismutase (*SOD*) activity was assayed by the enzymatic inhibition of the reduction of WST-1, a water soluble tetrazolium salt that is decolourised by superoxide ($[O_2^-]$), producing water soluble blue formazin, using the Sigma *SOD* assay kit (Sigma-aldrich, Dorset, U.K) and following the manufacturer's instructions. Therefore *SOD*

activity, which removes superoxide, can be quantified by measuring the decrease in the colour development at 440 nm. *SOD* activity was determined in a 96-well plate in a reaction mixture containing: 20 µl of enzymatic extract, 20 µl ddH_2O, 200 µl WST-1 working solution, 20 µl dilution buffer, and 20 µl enzyme working solution. Blank wells did not include enzymatic extract and served as a negative control. The plate was incubated at 37˚C for 20 mins. *SOD* activity was then assayed by inhibition activity quantified by measuring the decrease in colour development at 440 nm using a 96 well plate reader.

2.3.7 Survival

Survival was determined at the end of the 14 day PEG irrigation treatment by assessing whether the seedlings had wilted or showed signs of chlorophyll degradation (*i.e* shoot browning). Both seedlings that were classified as dead and as alive were included in the biomass measurements, however, only living seedlings were included in the measurements of *SOD* activity, relative water content and maximal efficiency of photosystem II. The proportion of seedlings surviving per provenance was calculated by dividing the number of surviving seedlings with the number of initially germinated seedlings.

2.3.8 Statistical analyses

The sample of seeds for germination analysis (per provenance and osmopriming treatment) was n = 100. Germination for each seed priming treatment was as follows: 265 (control), 281 (10% PEG), and 271 (30% PEG). Survival data represented a minimum (per provenance and treatment) of n = 60 seedlings from a total of 718 seedlings for control irrigation treatment and n = 30 seedlings from a total of 114 for PEG irrigation. The sample size used for measurements of biomass, relative water

content and chlorophyll fluorescence was a minimum of n=5 (Table 2.4.1, 2.4.2), while the sample size for the superoxide dismutase assay was a minimum of n = 3 (per provenance and treatment) (Table 2.4.2). Where the assumption of homoscedasticity for ANOVA was unmet, data were log transformed to improve normality of residuals before analysis.

Germination rate was analysed using Kaplan–Meier estimates of survivor functions for binomial data and the non-parametric Cox proportional hazard model, which is a time to event analysis based on the distribution of germination times of individual seeds rather than cumulative germination (Crawley 2007; McNair *et al.* 2012). Variation in germination and survival was analysed using generalized linear models with a binomial response and a logit link function. Binomial proportion confidence intervals were calculated using the *binom* package in R (Dorai-Raj 2014).

Analyses of variance (ANOVA) were used to test for effects of PEG seed priming and irrigation treatments, provenance and their interactions. Total shoot and root biomass, biomass allocation (root:shoot ratio), relative shoot and root water content, Fv/Fm and *SOD* activity were the response variables. Post-hoc pairwise comparisons were carried out using Tukey HSD (honest significant difference) tests. Pearson's correlations were calculated to assess relationships amongst the response variables. Where traits were not measured on the same individuals, mean values were used across treatments and provenances. In these cases, confidence intervals for the correlation coefficient were obtained by bootstrapping using the *boot* package in R (Canty & Ripley 2011). A total of 9999 bootstrap samples were generated and the correlation coefficient was

considered significant if the bias-corrected and accelerated percentile interval (BCa) did not include zero (More *et al.* 2012). We also used this approach to assess the correlation between mean trait values for seedlings from a given site (*i.e.* provenance) and treatment with environmental parameters of the sites. The R statistical programming environment was used for data analyses (R Core Team 2015).

2.4 Results

2.4.1 Germination

Of the 1200 seeds initially sown, 832 successfully germinated (69%). The rate of germination was affected by provenance, with Sierra Nevada (SN) showing faster germination rates than Rothiemurchus (RM), though not significantly faster than Jarocin (JAR) or Pernitz (PER) (Figure 2.4.1; Table S2.6.1). Provenance significantly affected the final proportion of seeds that germinated as well, with Sierra Nevada showing a significantly higher final germination proportion than Rothiemurchus (SN: $z= 3.09$, $p= 0.0019$, RM: $z= -3.36$, $p <0.001$), though not significantly higher than Jarocin or Pernitz. There was no significant difference in germination proportion between the 10% and 30% PEG seed priming treatment and there was no effect on final germination percentage. However, both 10% and 30% PEG seed priming treatment increased germination rate relative to control, unprimed seeds.

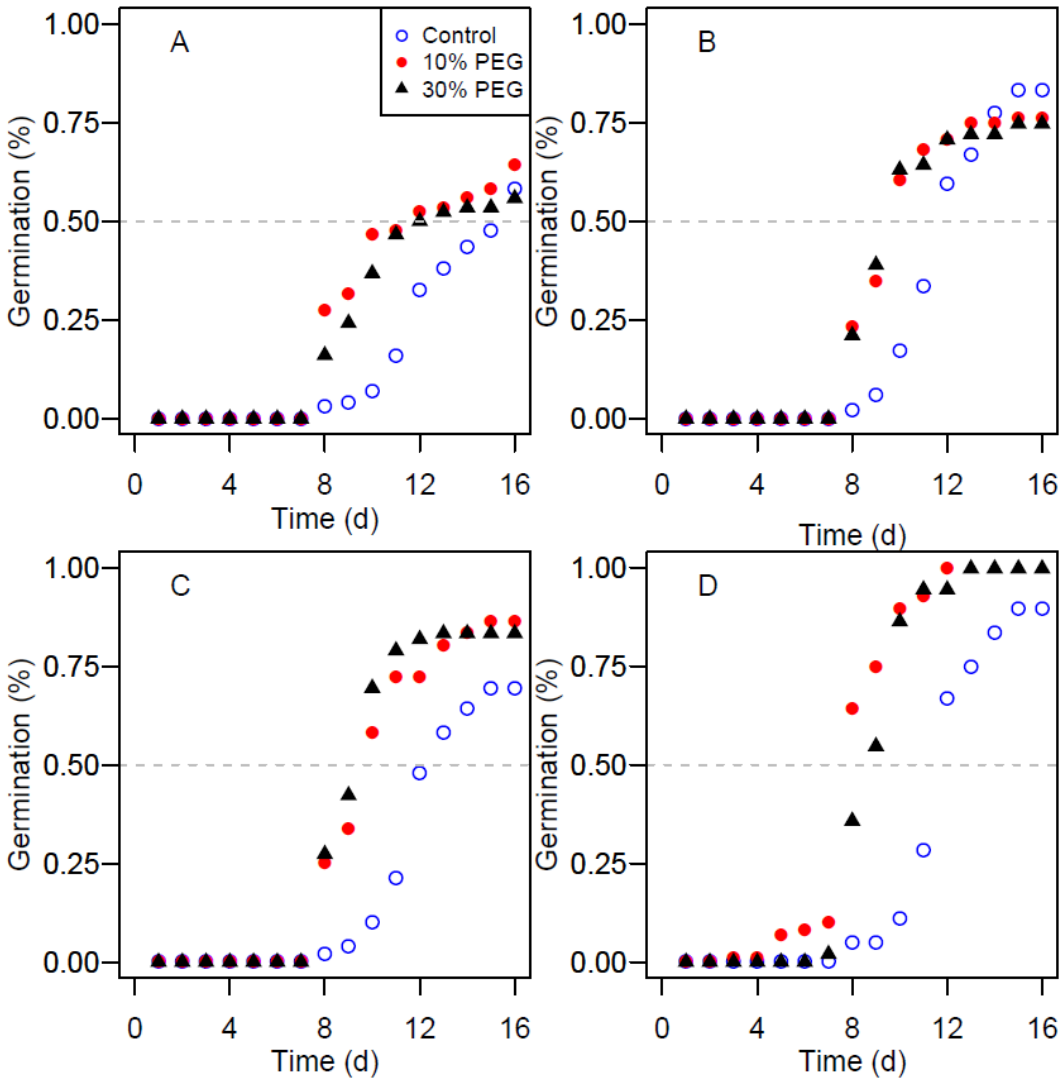

Figure 2.4.1 Cumulative germination of *Pinus sylvestris* seeds from 4 provenances: Rothiemurchus, Scotland (A), Pernitz, Austria (B), Jarocin, Poland (C), and Sierra Nevada, Spain (D). Treated seeds were primed for 7 days at two levels: 10% and 30% PEG solution. Control seeds were dry and unprimed.

2.4.2 Seedling biomass and relative water content

The ANOVA analyses showed that all seedling biomass parameters were unaffected by seed priming but were affected by the PEG irrigation compared with the control irrigation treatment (Table 2.4.1). In general, seedlings under the control irrigation

treatment were significantly larger than seedlings irrigated with water containing PEG. These seedlings had higher total, shoot and root biomass (Table 2.4.1). Time to germination included as a covariate in subsequent ANOVA had a significant effect on total biomass and root dry weight, though other response variables were unaffected (Table 2.4.1). There are also significant differences amongst provenances in biomass. Seedlings from Sierra Nevada and Pernitz showed higher shoot, root and total biomass than Jarocin and Rothiemurchus under control conditions (Figure 2.4.2). Notably, there were significant interactions between provenance and the PEG irrigation treatment, indicating that the simulated drought significantly decreased biomass in all provenances except Rothiemurchus. A significant effect was also detected of the PEG irrigation treatment and provenance on biomass allocation, but there was no significant interaction here (Table 2.4.1). Seedlings from Jarocin had the lowest root:shoot ratio, while those from the Sierra Nevada had the highest root:shoot ratio. Seedlings which were irrigated with water containing PEG had lower root:shoot ratios than control seedlings (Figure 2.4.2B). Shoot relative water content (RWC) was significantly lower in Jarocin than the other provenances and root relative water content was lower in seedlings from Jarocin than Sierra Nevada, and to a lesser extent from Pernitz (Figure S2.6.3). There was an interaction between provenance and PEG treatment for both shoot and root RWC.

2.4.3 Maximal photochemical efficiency of photosystem II

Maximal photochemical efficiency of photosystem II (F_v/F_m) was significantly lowered by the PEG irrigation treatment (Table 2.4.2; Figure 2.4.2C). However, there was no statistically detectable difference among provenances or effects of seed priming.

2.4.4 Superoxide dismutase enzyme assay

Relative *SOD* activity significantly decreased under the PEG irrigation treatment (Figure 2.4.2D). The strongest inhibition effect could be observed for Rothiemurchus (range 21–87%). Indeed, Rothiemurchus was the only provenance that showed a significant decrease in *SOD* activity in response to PEG on its own (TukeyHSD: $p<0.001$). There was a seed priming effect, with priming at 30% PEG producing a significant increase in *SOD* activity compared with seed priming at 10% or no priming. There were also significant interaction effects, between provenance and both seed priming and PEG irrigation.

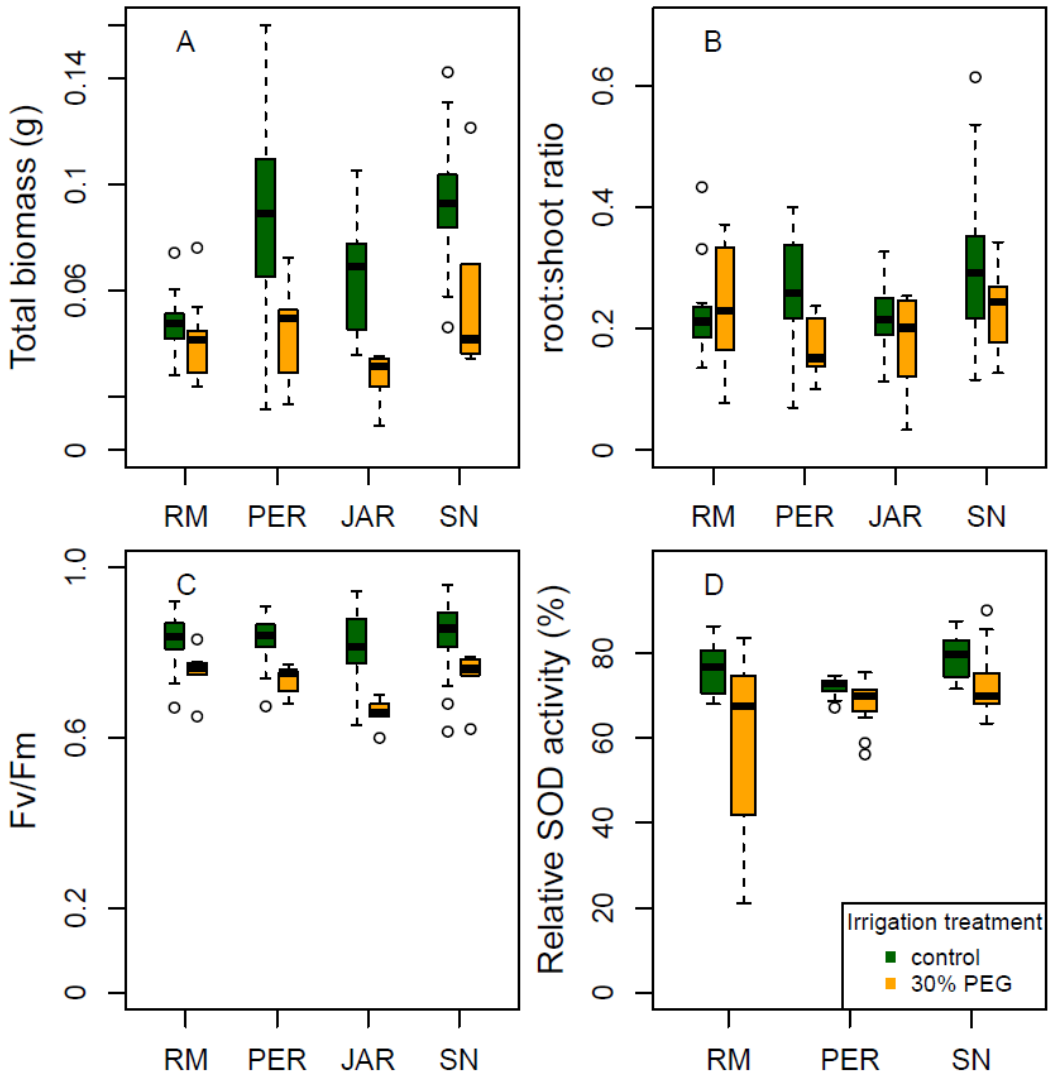

Figure 2.4.2 Total biomass (g) (A), Root to shoot dry weight ratio (B), maximal photochemical efficiency of photosystem II (F_v/F_m) (C), and superoxide dismutase (*SOD*) enzyme activity (D) of *Pinus sylvestris* seedlings from different provenances following: (a) 14 days of irrigation with 30% PEG (Polyethylene glycol 8000) or distilled water (control). Provenances are: Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin, Poland (JAR), and Sierra Nevada, Spain (SN). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

Table 2.4.1 Summary of ANOVA significance levels for the studied fixed factors over response variables using data collected after 14 days of osmotic stress by polyethylene glycol treatment. Sample size (per provenance and treatment), n = 5. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

		Root to shoot ratio		**Total biomass**		**Shoot dry weight**		**Root dry weight**	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment (T)	1	5.7	**0.01 ***	49.9	**<0.001 *****	52.3	**<0.001 *****	32.1	**<0.001 *****
Provenance (P)	3	2.8	**0.04 ***	9.1	**<0.001 *****	9.2	**<0.001 *****	6.0	**<0.001 *****
T x P	3	1.14	0.33	2.9	**0.038 ***	2.9	**0.038 ***	2.44	**0.06 .**
Seed priming (SP)	2	1.17	0.31	2.21	0.9	0.9	0.4	0.01	0.98

Table 2.4.2 Summary of ANOVA significance levels for the studied fixed factors over response variables using data collected after 7 days (Fv/Fm) or 14 days of osmotic stress by polyethylene glycol treatment. Sample size (per provenance and treatment), n = 5 for Fv/Fm, Shoot RWC and Root RWC, and n=3 for SOD activity. Fv/Fm: maximum potential PSII efficiency; *SOD*: superoxide dismutase. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

		Fv/Fm		***SOD* activity**		**Shoot RWC**		**Root RWC**	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment (T)	1	38.3	**<0.001 *****	24.9	**<0.001 *****	69.5	**<0.001 *****	150.6	**<0.001 *****
Provenance (P)	3	1.58	0.19	7.6	**<0.001 *****	6.2	**<0.001 *****	3.6	**0.016 ***
T x P	3	1.07	0.36	4.48	**0.014 ***	17.6	**<0.001 *****	6.7	**<0.001 *****
Seed priming (SP)	2	0.59	0.55	4.4	**0.014 ***	1.40	0.25	2.0	0.13
SP x P	4	1.30	0.26	4.5	**0.002 ****	--	--	--	--

2.4.5 Survival

Of the 832 successfully germinated seedlings, 599 survived to the end of the experiment. Survival was significantly reduced by application of PEG irrigation treatment, with only 41% survival compared with 77% for the control treatment (Table S2.6.3; Figure 2.4.3). There were no significant provenance effects or interactions between provenance and treatment on survival. However, it is notable that in the drought treatment mortality was highest relative to controls for seedlings from Rothiemurchus, the location with highest water availability, and lowest for seedlings from the Sierra Nevada, which has the lowest water availability.

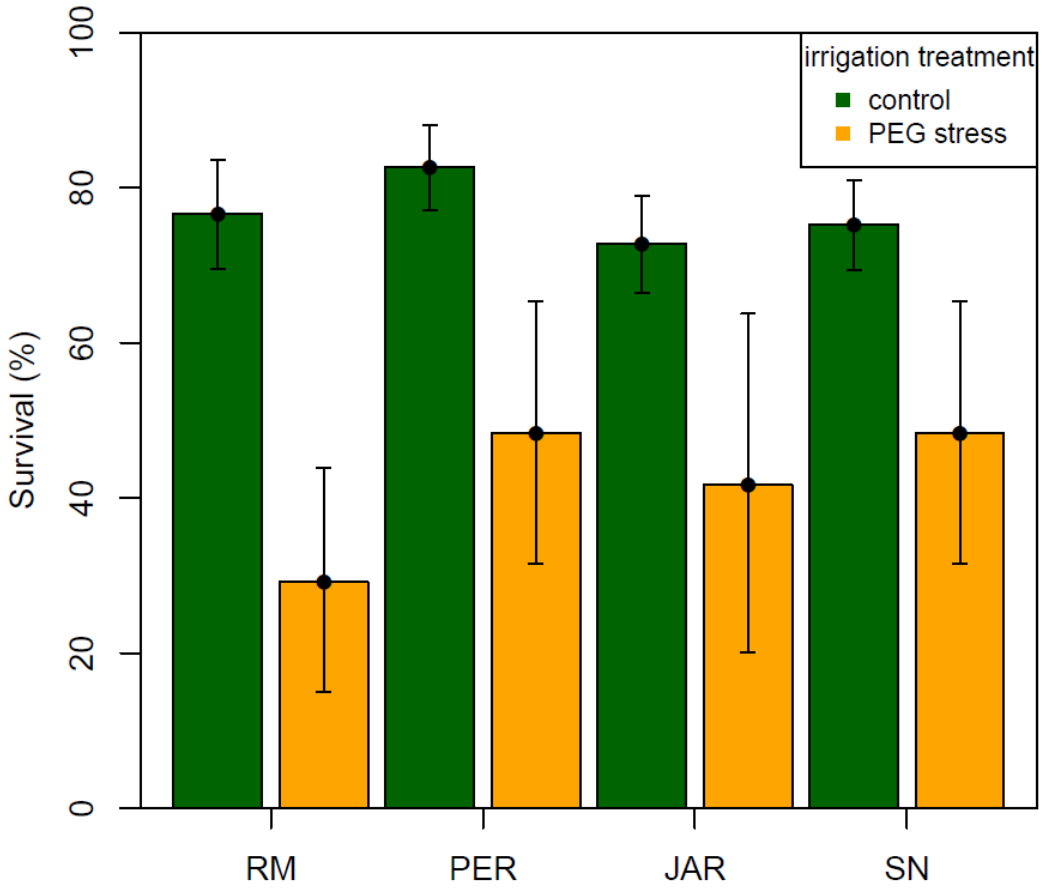

Figure 2.4.3 Survival (%) of *Pinus sylvestris* seedlings from 4 provenances following: (a) 14 days of irrigation with 30% PEG (Polyethylene glycol 8000) or distilled water (control). Provenances are: Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin, Poland (JAR), and Sierra Nevada, Spain (SN). Bars represent 95% binomial proportion confidence intervals.

2.4.6 Relationships among climatic and trait variables

A strong positive correlation was found between soil pF and root:shoot ratio under control irrigation treatment. Other site climatic variables did not correlate with trait

variables (Table S2.6.2). Under control irrigation, there were significant correlations between dry weights and root and shoot relative water content.

Under PEG irrigation, Fv/Fm was significantly correlated with shoot and root relative water content, as well as root dry weight. The shoot dry weight of seedlings was significantly positively correlated with seedling survival of PEG irrigation. Mean shoot dry weight and root relative water content were positively correlated with mean seed weight, which was positively correlated with survival (Table S2.6.2).

2.5 Discussion

At this early life stage of *Pinus sylvestris*, provenance was found to have pronounced effects on germination, seedling growth and survival. Germination rate and final germination proportion as well as seedling size was higher for seeds from the southernmost provenance at the xeric range edge of the species distribution in Spain compared with the wettest provenance, from Scotland. The most marked reduction in antioxidant enzyme (*SOD*) activity and survival was for seedlings from the wettest provenance. The provenance from the xeric range edge also showed higher root to shoot ratio, which was strongly positively correlated across provenance sites with soil moisture availability.

2.5.1 Germination, seed weight and seed priming

The germination rate and proportion of Sierra Nevada seeds was higher than for Rothiemurchus; these provenances also differed in terms of the maternal environment, particularly for climatic water deficit (Table 2.3.1). One trait in which Sierra Nevada

plants are locally adapted might be through the production of larger seeds relative to Rothiemurchus and Jarocin. However, when comparing similar seed weight classes in *Pinus sylvestris*, southern and central provenances were found to have a higher germination percentage than provenances from northern latitudes (Reich *et al.* 1994). In any case, in our study, any genetic differentiation is not distinguishable from the effects due to different environments experienced by maternal trees.

A positive correlation was found between mean seed weight and shoot dry weight. This result is in agreement with the positive relationship between mean provenance seed weight and the number of cotyledons, though not root length, previously identified across ten provenances of *P. sylvestris* from the southwestern to the central part of the range (Taeger *et al.* 2013). However, as seed weight was only analysed as a mean value for each provenance in this study and in Taeger *et al.* (2013), it is difficult to compare these findings to studies analysing seed weight effects within populations and families (Castro 1999; Castro *et al.* 2008). In a further study where seeds were weighed individually (Chapter 4), provenance was found to be significant in increasing germination rate independently of seed weight, as well as having a positive effect on seedling biomass.

Seeds that were primed with PEG solutions germinated more quickly relative to un-primed control seeds (Figure S2.6.1), in agreement with other studies of seed osmopriming (Hallgren 1989; Naglreiter *et al.* 2005). In *Pinus* species, rates of germination in seeds primed with PEG solutions of negative water potential between -0.8 MPa and 1.2 MPa were enhanced compared to un-primed seeds (Hallgren 1989;

Naglreiter *et al.* 2005). As control seeds were un-primed and remained dry, the comparison between seed priming in a solution without PEG and solutions with PEG could not be made. However, it is interesting to note that there was no significant difference between seed priming in solutions of different osmotic potential; this suggests that unless germination is inhibited by a threshold level of low water potential, seed priming effects are similar.

2.5.2 Seedling growth and performance

Under control conditions Sierra Nevada seedlings invested significantly more biomass in the root system than northern and central provenances, Rothiemurchus and Jarocin. A more developed root system is a key adaptation to summer drought, enabling enhanced water uptake (Lloret *et al.* 1999; Markesteijn and Poorter, 2009). Thus, it was expected that seedlings from Sierra Nevada, the provenances with the lowest rainfall would invest more in the root system. This might be attributable to an effect of the maternal seed environment or drought adapted alleles, since Sierra Nevada received the lowest mean annual rainfall and also had the lowest soil water availability, as indicated by the higher soil pF value of 3.6 compared to the values between 2 and 2.8 for the other provenances. Both Jarocin and Sierra Nevada had low mean annual precipitation, respectively 540 mm and 497 mm (Table 2.3.1). However, Jarocin had the lowest root to shoot ratio and was significantly different from Sierra Nevada (Figure 2.4.1B). Soil water retention depends on factors additional to total precipitation amount, which might have contributed to the disparity between the soil pF of Sierra Nevada and Jarocin despite their similar annual precipitation.

Both Spanish and Austrian provenances were observed to have higher mean seed weight and higher shoot and root dry weight under control conditions than Scottish and Polish provenances. Furthermore, the positive correlation between shoot dry weight and larger seed size and seedling survival suggests intraspecific variation in traits that are potentially related to adaptive differentiation among provenances. These differences may be due to maternal trees producing differently sized seeds in the different populations, owing to differences in the maternal environment or genetically determined differences in seed investment. Zas *et al.* (2013) found strong maternal parentage effects on seed weight variability in a study of seed orchard genotypes of *Pinus pinaster*; however, they caution that analysis of the fitness consequences of seed weight variability for the maternal trees would be needed in order to conclude that this had an adaptive value.

Irrigation treatment with PEG impacted all growth and physiological parameters considered in the study, as well as seedling survivorship. Decreases in shoot and root dry weights under PEG irrigation were observed that potentially indicate rapid growth cessation. Furthermore, seedling responses suggest provenance differentiation in the extent to which osmotic stress reduced biomass. Gibberellic acid (GA) governs the rate of cell division and elongation, through downstream elimination of growth-repressing DELLA proteins. Growth restraint is therefore achieved via reduced GA leading to the accumulation of DELLAs (Richards *et al.* 2001). The plant hormone abscisic acid (ABA) and reactive oxygen species (ROS) are hypothesised to interact with DELLA proteins and thereby modulate growth restraint under stress (Golldack *et*

al. 2013). Thus, local adaptation to drought involving rapid and appropriate growth cessation might involve differential expression of genes related to this pathway.

However, the fast rate of the imposed water deficit (at a level of ~1.5 MPa day^{-1}) is likely to have exceeded the threshold for acclimation in biomass allocation. This is consistent with a comparison of fast and slow imposed osmotic stress using PEG by Lopez *et al.* (2009), where the slow treatment (0.1 MPa day^{-1}) down to -1 MPa effected an increase in root dry weight but the fast treatment (0.15 MPa, day^{-1}) down to -1.5 MPa and control treatments did not.

Although the maximum photochemical efficiency of PSII (Fv/Fm) (a commonly used stress indicator) was strongly impacted by the PEG treatment and correlated with tissue relative water content, there was not any detectable provenance or provenance by treatment interaction effects. In Mediterranean populations of *Pinus pinaster*, intraspecific variation was detectable in photochemical parameters at the population level in response to drought in the field (Corcuera and Notivol 2015). Local adaptive genetic differentiation in photochemical capacity was also found in *Pinus sylvestris* saplings of Scottish families and populations under a glasshouse drought experiment (Salmela *et al.* 2011). However, intraspecific variation was not found in *Pinus canariensis* under PEG (6000 MW) induced osmotic stress (Lopez *et al.* 2009). Possibly there is a different osmotic stress effect on photochemical parameters when induced by PEG irrigation rather than water withdrawal that obscures signs of adaptive genetic differentiation, or possibly these are species or ontogenetic-specific responses.

Pinus canariensis has a restricted geographic range compared with *P. sylvestris*, being limited to the Canary island archipelago (Climent *et al.* 2004).

Although osmopriming had no effect on biomass allocation patterns or the maximal efficiency of photosystem II, total superoxide dismutase (*SOD*) activity was enhanced in seedlings that had been primed with 30% PEG. In terms of provenance differences, *SOD* activity decreased most markedly under the PEG irrigation treatment for seedlings from Rothiemurchus. Chai *et al.* (2005) found that in banana plantlets the more drought sensitive cultivar showed lower *SOD* activity both before and after PEG treatment. Antioxidant systems involve complex regulation by both enzymatic and non-enzymatic metabolic networks, within which the activity of the enzyme superoxide dismutase is embedded (Cruz De Carvalho 2008). Experiments using rice report that seed priming with osmotic solutions can enhance antioxidant defences. For example, primed rice seeds had less lipid peroxidation and higher *SOD* and catalase (*CAT*) activities than non-primed seeds as well as improved growth rate (Ella *et al.* 2011; Goswami *et al.* 2013). *SOD* activity decreasing under PEG simulated drought stress has also been found in other studies (Panda & Khan 2004, Abbasi *et al.* 2015). In this study, the low sample size for the *SOD* assay (n=3) means that these results must be interpreted with caution. Low sample size has been shown to lead to higher error rates in statistical analyses, for example in detecting genetic diversity of fragmented populations (Nazareno & Jump 2012). However, other studies have also reported using a similarly low number of replicates (2-3) for biochemical analyses, presumably owing to time constraints (Dhindsa *et al*. 1981, Kimmerer *et al*. 1987, Schenk *et al*. 2000, Chakraborty *et al*. 2001, Zhu *et al*. 2004).

2.5.3 Relevance of traits for survival

Survival was reduced by osmotic stress treatment in all provenances. Although no provenance showed a significantly higher survival rate than other provenances under the PEG treatment, Rothiemurchus did show the highest increase in mortality under the drought treatment relative to control, while the seedlings from the Sierra Nevada showed the least impact of drought on survival. Furthermore, this Spanish provenance showed faster germination and a higher root to shoot ratio than the Scottish and Jarocin provenances. Although in this study, time to germination had a significant effect only on root dry weight and survival was apparently not influenced, earlier seedling emergence has been linked to slightly increased seedling survival in *P. sylvestris* (Richter *et al.* 2012). Matias and Jump (2014) found a significant disparity in seedling survival between provenances from Kevo, Finland and Spanish provenances, including Sierra Nevada, with higher survival of drought in the southern provenances that emerged earlier and invested a higher proportion of biomass in the root system.

The correlation between mean seed weight and survivorship is suggestive of a link between maternal investment in seed weight and adaptation to osmotic stress during initial seedling establishment. The higher seed weight of southern populations is thought to enable seedlings to emerge earlier than from northern and central European populations, potentially enabling a root system to quickly establish, with the higher biomass investment in roots being critical for surviving Mediterranean summer droughts (Castro 2006; Padilla and Pugnaire 2007; Castro *et al.* 2008; Matias & Jump 2014). Indeed, in Chapter 4, where seed weights were measured individually, seed weight was also found to have a strong positive effect on seedling dry weights and

number of needles. Seed weight was strongly determined by provenance and maternal parentage as well as their interaction. Thus, seed provisioning dependent on the maternal environment has an effect on early seedling growth in *Pinus sylvestris*. Although provenance effects independent of seed investment seem to be responsible for differences in seedling root investment, the positive effect of seed weight on seedling size seems to translate to increased survival to osmotic stress.

The lower SLA in seedlings from Sierra Nevada as compared with Rothiemurchus might indicate local adaptation in this leaf trait (Figure S2.6.3B). Physiological differences between seedlings may have arisen because of this shift in biomass allocation. A high specific leaf area (SLA) implies a high surface to volume ratio of leaves; hence improved water use efficiency is related to lower values of SLA. However, Delzon (2015) point out that the lack of correlation between SLA and forest water availability suggests that it is premature to identify SLA as a key functional trait for drought tolerance.

Meanwhile, as well as the highest root:shoot ratio found for the Spanish provenance, the strong positive correlation between soil moisture availability and root:shoot ratio across provenances indicates that this is an adaptive trait in drought tolerance for *Pinus sylvestris*.

2.5.4 Conclusions

The effects of osmotic stress on the initial seedling establishment stage were studied by comparing phenotypic responses across provenances. The findings of this study are that seed germination and seedling responses to osmotic stress differentiate by

provenance. Treatment, provenance and interaction effects were found for rate of germination, final proportion of seeds germinated, seedling size, and superoxide dismutase activity (an antioxidant enzyme). Root investment was affected by both provenance and time to germination. Local adaptation to the drier conditions in the Spanish provenance is likely to account for the faster germination and higher root investment. Although there was no significant effect of provenance on survival, a trend towards increased probability of survival under osmotic stress was indicated for the southernmost (driest) as compared with the northernmost (wettest) provenance.

2.6 Supplementary material

Table S2.6.1 Final germination proportion and time to 50% germination for 4 provenances under seed priming treatments of 0%, 10% and 30% PEG.

Provenance	**Osmopriming**	**Final germination proportion (%)**	**Time to 50% germination, (days)**
Rothiemurchus	control	55	15-16
	10 % PEG	60	12
	30 % PEG	53	13
Pernitz	control	74	11-12
	10 % PEG	69	9-10
	30 % PEG	68	9-10
Jarocin	control	64	12-13
	10 % PEG	76	9-10
	30 % PEG	74	9-10
Sierra Nevada	control	78	11-12
	10 % PEG	87	7-8
	30 % PEG	84	8-9

2.6.2 Significance of Pearson's correlations between mean annual cumulative precipitation (PPN) and mean values for seed weight (SW), root to shoot dry weight ratio (R:S), shoot dry weight (SDW), root dry weight (RDW), shoot relative water content (SRWC), root relative water content (RRWC), maximum potential PSII efficiency (Fv/Fm), and survival proportion (S) of the four provenances, for the control regime (below grey diagonal) and PEG irrigation regime (above grey diagonal). The correlation coefficient ± the standard error of the simulated values is reported. Significant p-values are highlighted in bold and italicised if p-values are only marginally significant.

	CWD	pF	PPN	SW	R:S	Total DW	SDW	RDW	SRWC	RRWC	Fv/Fm	S
CWD					r: -0.19 ±0.74, p= 0.8	r: -0.49 ±0.77, p= 0.5	r: -0.46 ±0.74, p=0.53	r: -0.53 ±0.83, p=0.46	r: 0.14 ±0.64, p= 0.85	r: 0.06 ±0.68, p= 0.93	r: 0.11 ±0.65, p=0.88	r: -0.15 ±0.6, p=0.84
pF					r: 0.29 ±0.76, p= 0.7	r: 0.81± 0.47, p= 0.18	r: 0.81±0.46, p=0.18	r: 0.75±0.67, p=0.24	r: 0.26 ±0.68, p=0.73	r: 0.28±0.6, p=0.7	r: 0.28 ±0.66, p=0.71	r: 0.59 ±0.45, p=0.4
PPN					r: -0.02 ±0.19, p=0.97	r: -0.02 ±0.22, p=0.97	r: 0.01 ±0.22, p=0.98	r: -0.16 ±0.21, p=0.83	r: 0.55 ±0.15, p=0.44	r: 0.42 ±0.22, p=0.57	r: 0.52 ±0.18, p=0.47	r: 0.34 ±0.66, p=0.65

SW	r: -0.20 ±0.71, p= 0.79	r: 0.62 ±0.4, p= 0.37	r: 0.29 **±** 0.08, p=0.7		r: 0.45 ±0.18, p=0.54	**r:0.93 ±0.11, p=0.06**	**r: 0.95 ±0.11, p=0.04 ***	r: 0.78 ±0.12, p=0.21	r: 0.87 ±0.19, p=0.12	r: 0.81 ±0.17, p=0.18	r: 0.87 ±0.18, p=0.12	**r: 0.99 ±0.019, p=0.007 ***
R:S	r: -0.72 ±0.66, p= 0.27	**r: 0.95 ± 0.43. p=0.04**	r: -0.29 **±** 0.11, p=0.7	r: 0.80 **±** 0.08, p= 0.19		r: 0.58 ±0.12, p=0.41	r: 0.48 ±0.15, p=0.51	r: 0.82 ±0.05, p=0.17	r: 0.66 ±0.16, p=0.33	r: 0.81 ±0.14, p=0.18	r: 0.69 ±0.20, p=0.3	r: 0.34 ±0.7, p=0.65
Total DW	r: -0.62 ±0.49, p= 0.37	r: 0.8 ±0.11, p=0.19	r: -0.29 **±** 0.125, p=0.7	r: 0.55± 0.07, p= 0.44	r: 0.85 ±0.086, p=0.14		**r: 0.99 ±0.006, p=0.007**	**r: *0.92 ±0.05,* p=*0.07***	r: 0.77 ±0.11, p=0.22	r: 0.76 ±0.16, p=0.23	r: 0.77 ±0.17, p=0.22	*r: 0.90 ±0.13, p=0.09*

SDW	r: -0.62 ±0.49, p= 0.37	r: 0.78 ±0.11, p=0.21	r: -0.31 **±**0.12, p=0.68	r: 0.50 **±**0.09, p=0.49	r: 0.82 ± 0.10, p=0.17	**r: 0.99 ±0.006, p=0.002**		r: 0.87 ±0.09, p=0.12	r: 0.75 ±0.13, p=0.24	r: 0.72 ±0.17, p=0.27	r: 0.76 ±0.17, p=0.23	r: ***0.93 ±0.07,*** p=***0.06***
RDW	r: -0.6 ±0.52, p=0.39	r: 0.84 **±**0.09, p=0.15	r: -0.22 **±**0.12, p= 0.7	r: 0.68 **±**0.06, p=0.31	r: 0.91 ±0.03, p=0.085	**r: 0.98 ±0.02,** p=**0.012**	**r: 0.97 ±0.05, p=0.02 ***		r: 0.73 ±0.08, p=0.26	r: 0.8 ±0.13, p=0.19	**r: 0.98 ±0.17, p=0.015**	r: 0.71 ±0.4, p=0.28
SRWC	r: -0.66 ±, p=0.33	r: 0.76 ±0.1, p=0.2	r: -0.4 **±**0.11, p=0.59	r: 0.39 **±**0.10, p=0.6	r: 0.77 ± 0.11, p=0.22	**r: 0.98, ± 0.047, p= 0.017**	**r: 0.9 ±0.048, p = 0.008 ** **	**r: 0.94 ± 0.06, p=0.05 .**		**r: 0.97 ±0.09, p=0.02 ***	**r: 0.99 ±0.16, p=0.001 ****	r: 0.85 ±0.8, 0.14
RRWC	r: -0.56 ±0.6, p=0.43	r: 0.74 **±**0.46, p= 0.25	r: -0.25**±** 0.12, p= 0.74	0.51 0.09, 0.48	r: 0.85 ± 0.08, p=0.14	**r: 0.99 ±0.07 , p=0.004**	**r: 0.99 ±0.08, p=0.002**	**r: 0.97 ±0.06, p=0.02 ***	**r: 0.97 ±0.07, p=0.02 ***		**r: 0.98 ±0.17, p=0.01 ***	r: 0.76 ±0.78, 0.23

Fv/Fm	r: -0.5 ±0.77, p= 0.43	r: 0.85 ±0.49, p=0.14	r: -0.11 ±0.11, p= 0.88	r: 0.9 ±0.1, p=0.09	r: 0.91 ±0.10, p=0.08	r: 0.59 ±0.12, p=0.40	r: 0.54 ±0.12, p=0.45	r: 0.7 ±0.10, p=0.29	r: 0.46 ±0.11, p=0.53	r: 0.52 ±0.10, p=0.47		r: 0.84 ±0.8, 0.15
S	r: 0.17 ±0.63, p=0.82	r: 0.26 ±0.6, p=0.7	r: 0.58 ±0.5, p=0.41	r: 0.76 ±0.12, p=0.23	r: 0.51 ±0.4, p=0.48	r: 0.60 ±0.11, p=0.39	r: 0.57 ±0.5, p=0.42	r: 0.66 ±0.58, p=0.33	r: 0.48 ±0.57, p=0.51	r: 0.62 ±0.46, p=0.37	r: 0.46 ±0.55, p=0.53	

Table S2.6.3 Akaike's information criterion (AIC) values for Cox Proportional-Hazards Regression model of germination rate and generalized linear models of germination proportion and seedling survival

	Germination rate	**Germination proportion**	**Survival**
Fixed effects	*AIC*	*AIC*	*AIC*
treatment*provenance + osmopriming	na	na	938.17
treatment*provenance	na	na	937.44
osmopriming*provenance	119805.6	1449.82	992.66
treatment+provenance	na	na	934.34
osmopriming+provenance	119818.3	1446.86	989.74
treatment	na	na	934.98
osmopriming	120128.1	1483.30	989.89
provenance	120069.8	1444.93	988.57
treatment+germination day	na	na	936.79
provenance+germination day	na	na	938.68
treatment * provenance + germination day	na	na	939.22
1	120379.3	1481.37	988.74

Table S2.6.4 Summary of ANOVA significances. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

		Total Leaf Area		**Crown density**	
	d.f	*F*	*P*	*F*	*P*
Treatment (T)	1	Na	Na	Na	Na
Provenance (P)	3	3.26	**0.02 ***	4.8	**0.003 ****
T x P	3	Na	Na	Na	Na
Seed priming (SP)	2	2.03	0.13	1.35	0.26

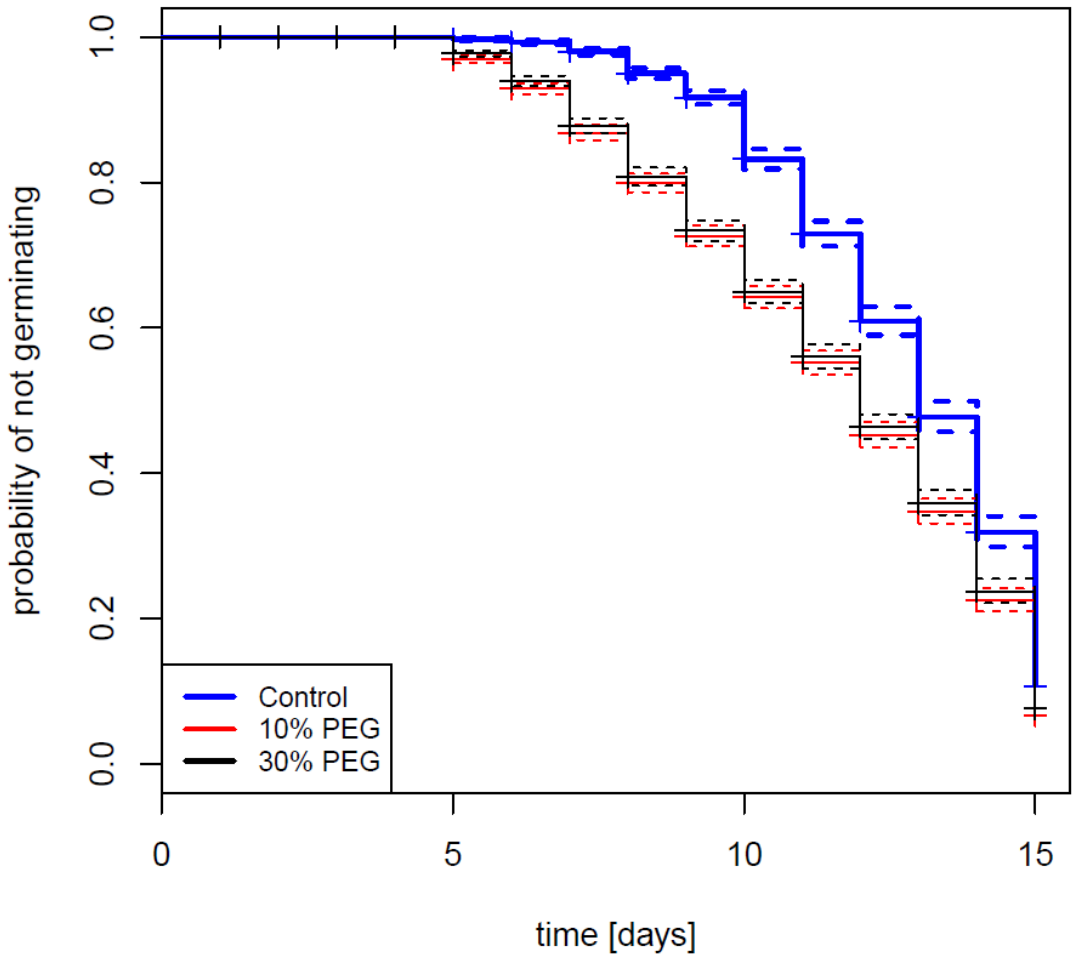

Figure S2.6.1 Kaplan-Meier estimates of survivor functions applied to germination data. Treated seeds were primed for 7 days at two levels: 10% and 30% PEG solution. Control seeds were dry and unprimed. 95% confidence intervals are represented by the broken lines.

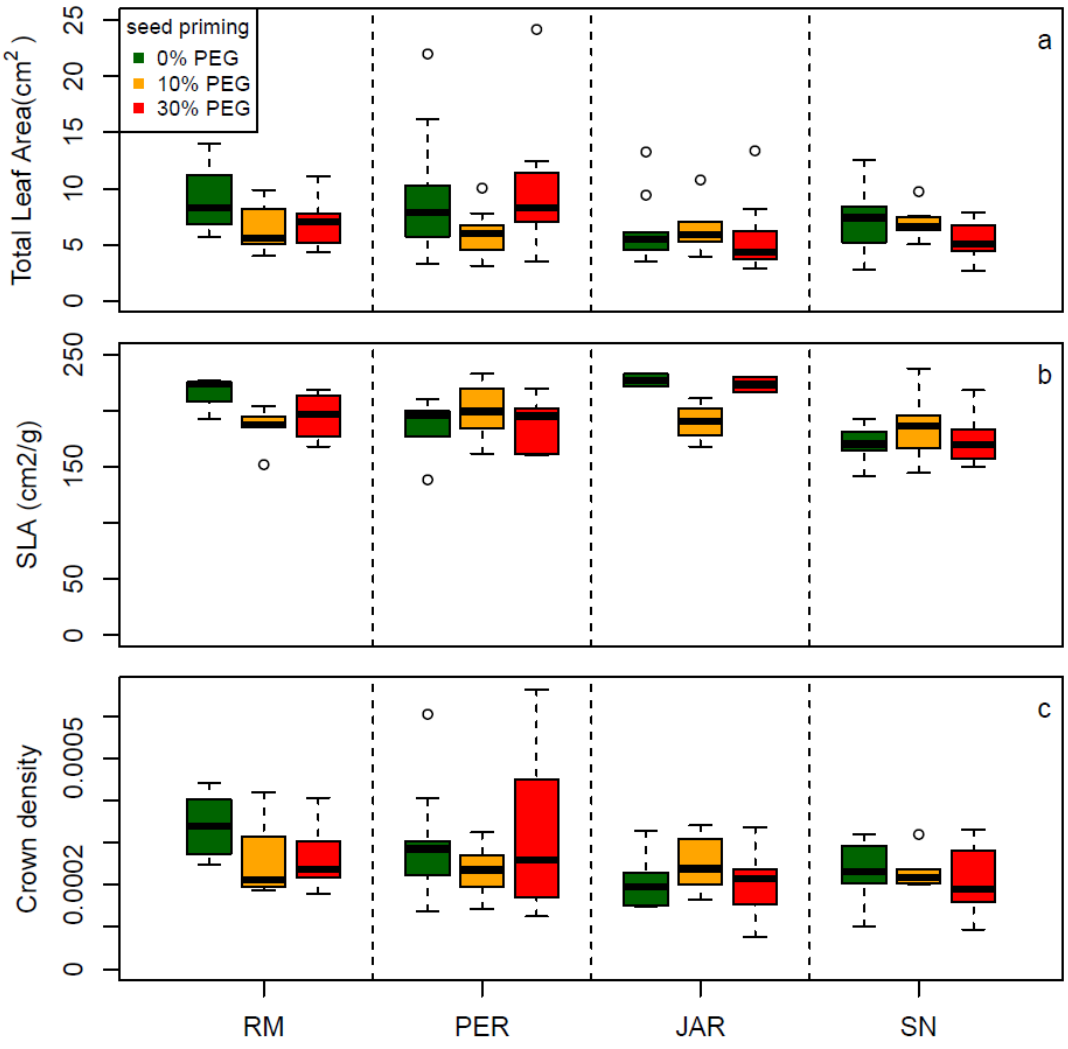

Figure S2.6.2 Total Leaf area (TLA, cm^2), Specific leaf area (SLA, cm^2/g^{-1}) and Crown density, or the ratio of leaf area to crown hull area, of *Pinus sylvestris* seedlings across the 4 provenances and in response to seed priming at: 0%, 10% or 30% PEG solution for 7 days. Provenances are: Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin, Poland (JAR), and Sierra Nevada, Spain (SN). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

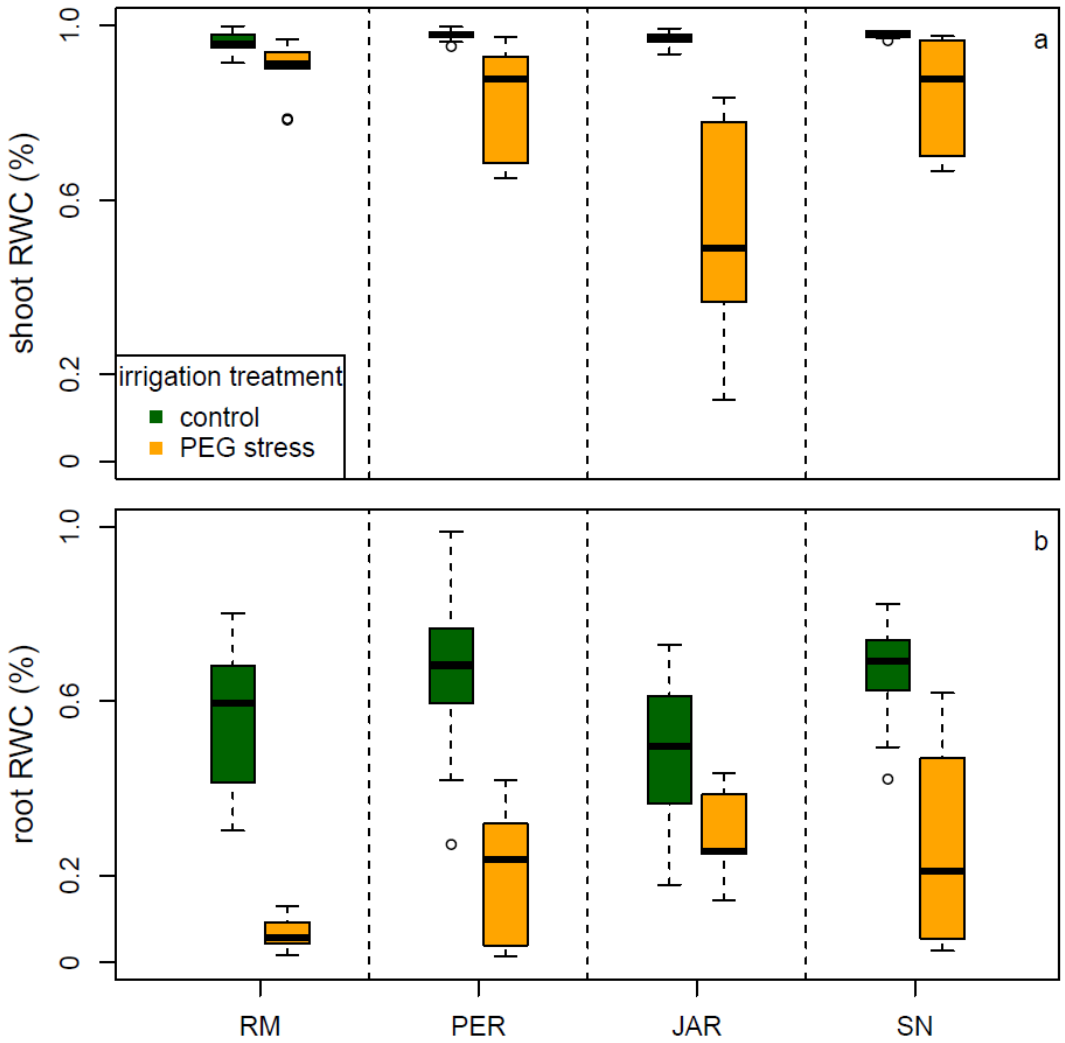

Figure S2.6.3 Shoot and root relative water content (RWC) (%) of *Pinus sylvestris* seedlings from 4 provenances following: (a) 14 days of irrigation with 30% PEG (Polyethylene glycol 8000) or distilled water (control), and (b) seed priming for 7 days in solutions of different concentrations of PEG. Provenances are: Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin, Poland (JAR), and Sierra Nevada, Spain (SN). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

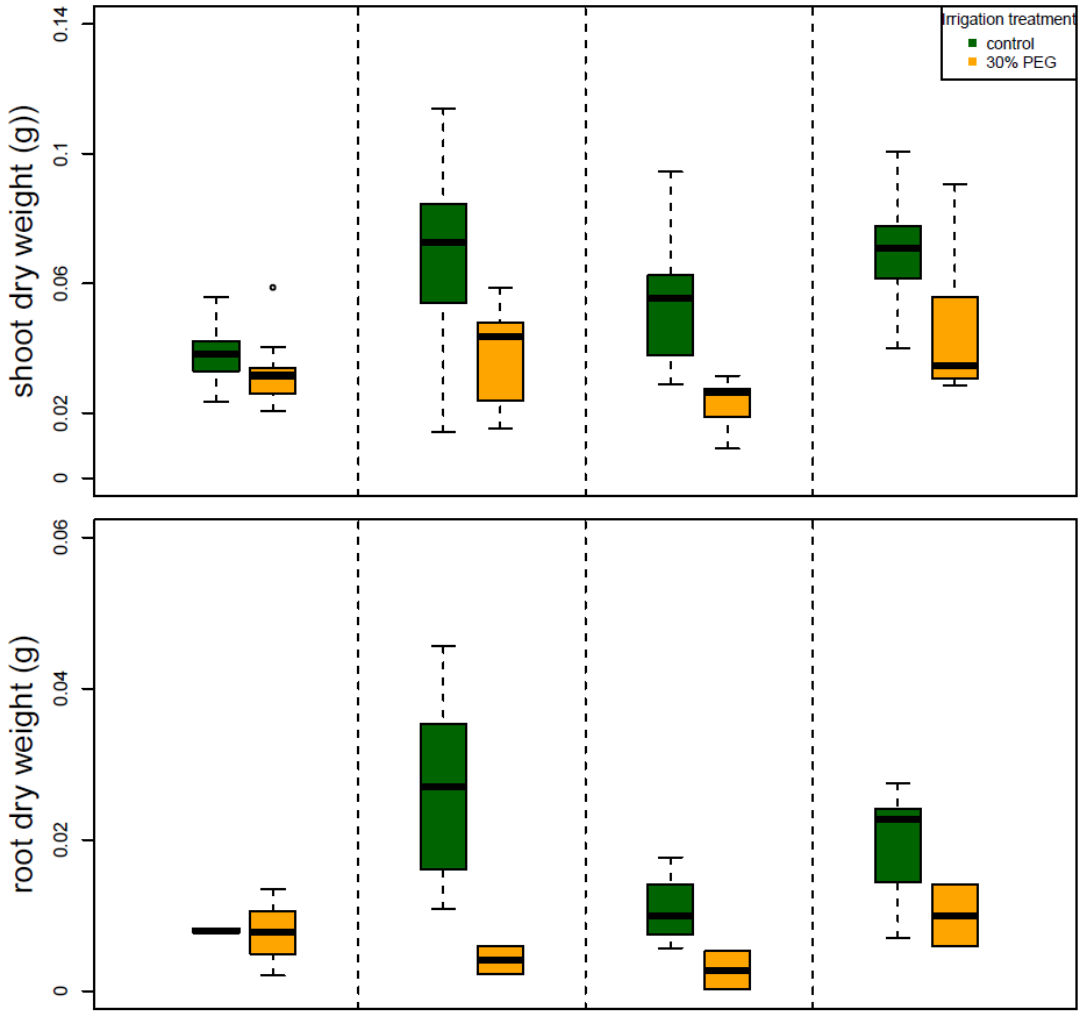

Figure S2.6.4 Shoot dry weight (g) and root dry weight of *Pinus sylvestris* seedlings from 4 provenances in response to: (a) 14 days of an irrigation treatment with 30% PEG (Polyethylene glycol 8000) or distilled water only (control), and (b) seed priming for 7 days in solutions of different concentrations of PEG. Provenances are: Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin, Poland (JAR), and Sierra Nevada, Spain (SN). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

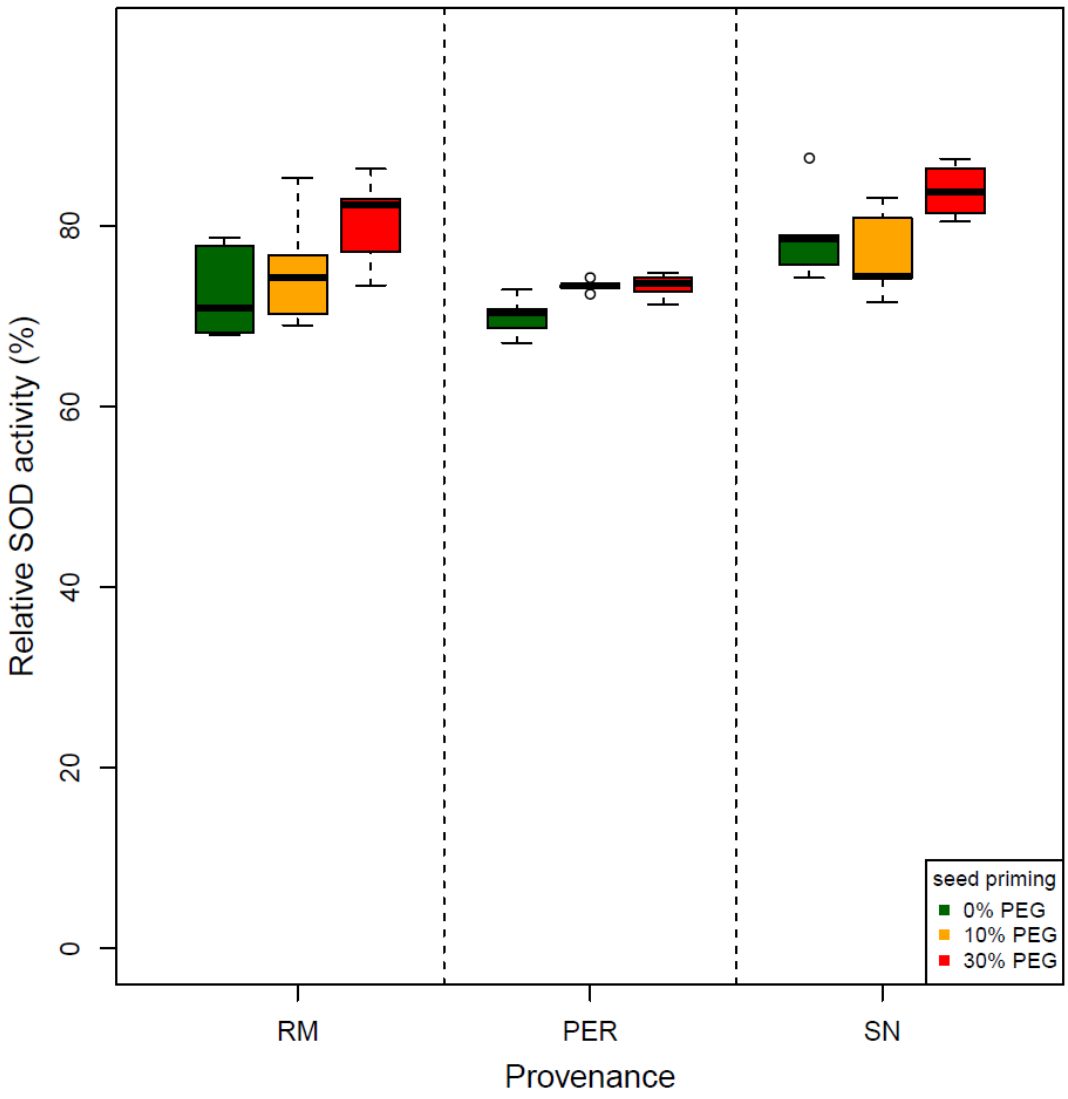

Figure S2.6.5 Relative superoxide dismutase (*SOD*) enzyme activity in 10 week old seedlings exposed to seed priming with different concentrations of PEG. WST-1 is a water soluble tetrazolium salt that is decolourised by superoxide, therefore *SOD* activity, which removes superoxide, can be quantified by measuring the decrease in the colour development at 440 nm. Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

3 Drought effects on growth and metabolism of *Pinus sylvestris* seedlings

3.1 Abstract

The primary objective of this study was to assess biological variation in the metabolic drought stress response of seedlings among four populations of *Pinus sylvestris*. Metabolite composition of needles was examined through metabolite profiles generated by liquid chromatography coupled with mass spectrometry and a series of statistical analyses to test for the effects of provenance, treatment and time over the course of the drought experiment. The drought treatment consisted of a dry down by water withdrawal. Additional measurements were taken of seedling biomass allocation traits and photochemical efficiency. We found that drought reduced seedling biomass and photochemical efficiency and that the magnitude of the effect varied by provenance. Meanwhile, metabolic profiles of drought stressed seedlings were significantly different from control seedlings, but the metabolic effects of drought did not differ by provenance. Drought and the duration of drought interacted significantly in affecting metabolite accumulation, enabling identification and putative annotation of metabolites involved in a drought stress response. Among the principal metabolites that showed differential abundance in response to drought were those involved in osmoprotective and antioxidant capabilities, including the free amino acid proline and a quercetin derivative (a flavonoid). However, of the top metabolites important in the drought time interaction, most were unknown and potentially represent novel findings for metabolic responses to drought.

3.2 Introduction

Fluctuations in environmental conditions necessitate appropriate plant responses. Summer drought is currently a major constraint on seedling establishment in southern populations of *Pinus sylvestris* in Mediterranean climates (Castro *et al.* 2005). Climate change models project increasing irregularity in precipitation, leading to a higher likelihood of drought episodes that might hamper seedling recruitment of *P. sylvestris*. Phenotypic plasticity is the genetically determined capacity of an individual to be responsive to stimuli and co-ordinate phenotypic changes at the level of anatomy, growth and metabolism (Bradshaw 2006). The capacity for phenotypic plasticity in *P. sylvestris* is suggested by its extensive geographic range, which encompasses heterogeneous ecological conditions. Both phenotypic plasticity and local adaptation could have a role in enabling resilience to drought and the seedling response is of particular importance, since it represents a bottleneck in terms of higher mortality rates (Castro *et al.* 2005; Matias *et al.* 2011).

Relative to structural and morphological plasticity, metabolism occurs over shorter time-scales and is therefore a more immediate reflection of the plant response to environmental stressors, including drought. Metabolites are low molecular weight chemicals that are the intermediary and end products of metabolism, including both primary metabolites such as: sugars, fatty acids and amino acids, and secondary metabolites such as: hormones, flavonoids, alkaloids and terpenoids. Some metabolites have bioactive properties, with an in vivo function pertaining to their biochemical structure; for example, alkaloids are toxic to a range of pests and pathogens (Facchini 2001), flavonoids have a photoprotective role (Agati et al. 2012),

and the amino acid proline can function in an osmoprotective role, and as an antioxidant by scavenging free radicals (Smirnoff & Cumbes 1989; Szabados & Savoure 2009).

Drought stress is discernible at the level of plant metabolic phenotypes. Oxidative stress is a potential side-effect of physiological responses to severe drought caused by the accumulation of cytotoxic reactive oxygen species (ROS). The extent of oxidative damage is governed by the activity of particular metabolic pathways and the capacity of antioxidant defences to avert an imbalance of ROS (Cruz de Carvalho *et al.* 2008). Zhao *et al.* (2015) compared foliar metabolome responses of *Nicotiana* from different growing districts; plants growing in the province with higher temperature, sunshine hours and water deficit showed increased metabolites of the shikimate-phenylpropanoid pathway, associated with a requirement for antioxidants to protect against oxidative stress. Bowne *et al.* (2012) found that subjecting wheat cultivars to drought-induced increases in amino acids such as proline, tryptophan and branched amino acids (leucine, isoleucine, and valine) for all cultivars, while tolerant cultivars also showed a slight decrease in organic acids. Metabolite analysis indicated that proline was a drought stress marker in *Miscanthus x giganteus* (Poaceae), as the accumulation of metabolites in the proline synthesis pathway coincided with stomatal closure and growth cessation (Ings *et al.* 2013).

Metabolomics is the study of the collection of all metabolites – the metabolome - in a cell, tissue, organ or even a whole organism (e.g. in studies of *Daphnia* spp.). Thus, the metabolome represents an integrated molecular phenotype of gene expression and

protein activity interacting with environmental cues and provides dynamic insights into the response of a biological system to perturbation (Fiehn 2002). Furthermore, in a non-targeted metabolomics study that aims to capture a picture of global metabolism rather than specific known metabolic pathways, discovery of novel pathways or interactions is made possible (Hall 2006). Liquid chromatography followed by mass spectrometry (LC/MS) is suitable for both targeted and non-targeted metabolomics and is a preferred technique for maximising detection of metabolites (Buscher *et al.* 2009, Jonsson *et al.* 2005, Nordstrom *et al.* 2008, Viant & Sommer 2012).

Disentangling the relative importance of genetic and environmental effects on plant metabolomes is a research focus of environmental metabolomics (Brunetti *et al.* 2013). Studies of several conifer and crop species have indicated that intraspecific genetic differences among plants may have less of an effect than the environmental conditions faced (Robinson *et al.* 2007; Matsuda *et al.* 2012). However, Barchet *et al.* (2013) found that of four hybrid poplar clones, one showed a different metabolic profile under drought stress related to reduced osmolyte accumulation. Natural variation in metabolic profiles has also been detected over latitudinal and environmental clines. For example, reconfiguration of metabolic networks during cold acclimation was found to result in a clear disparity in metabolic profiles of northern and central compared with southern provenances of adult *Picea sitchensis* in a common garden trial (Dauwe *et al.* 2012). Metabolic profiles were also found to be population specific in *Arabidopsis lyrata* ssp. *petraea* (Davey *et al.* 2008). In *Pinus pinaster* at 5 years old, metabolic differences were apparent across Mediterranean provenances in a common garden trial, and were related to aridity of the provenance site (Meijon *et al.* 2016).

This study aimed to investigate the drought stress response of *Pinus sylvestris* seedlings in their foliar metabolomes and to find out whether different *P. sylvestris* provenances can differ in their metabolic profiles. Additional measurements of biomass and photochemical efficiency were taken to determine if changes at the metabolic level accompany differences in seedling growth and physiological capabilities. In order to determine the relative importance of provenance on seedling growth and metabolism, seeds from four populations of *P. sylvestris* were grown under controlled environmental conditions that minimised the influence of environmental variability on the metabolome. The drought experiment was carried out by water withdrawal over 4 weeks to investigate differential responses by provenances. Traits relating to growth and physiology were measured in order to determine patterns of population variation in the traits and to see if any correlations with metabolic shifts could be ascertained. Metabolic profiles were statistically analysed in relation to the population of origin of the plant, as well as multiple time points over the dry-down, for both experimental and control treatments, in order to define the key compounds relating to metabolic variation in populations and drought treatment.

3.3 Materials and Methods

3.3.1 Experimental conditions and sampling

The experiment was carried out over 5 weeks from 03.07.15 to 07.08.15 in a controlled growth room with 10 month old seedlings from 4 provenances spanning a gradient of water availability from wet (Scotland), intermediate (Austria, Poland) to dry (Spain). Climatic characteristics of the four populations from which the open pollinated seeds

were collected are presented in Table 3.3.1. Mean annual cumulative precipitation (ppn) was obtained from 1901 to 2015 from the CRU TS3.10 Dataset (Harris et al. 2014). Mean soil pF value (pF expresses the force with which different quantities of water are retained by the soil (Woodruff 1940)) in the summer month with the maximum mean pF value (July/August) from 1990 to 2015 were obtained from the European Drought Observatory (edo.jrc.ec.europa.eu/ on 20.02.15). Climatic water deficit values for these sites were obtained from the Chave et al. 2014 data layer. The seeds were from open pollinated trees, with at least 5 maternal parent trees sampled per provenance site.

The growth conditions preceding the start of the experiment were as follows. One seed was sown per cell in a 2:1 peat and sand mixture with perlite to aid drainage and grown in a controlled environment chamber at the University of Edinburgh (UK) under constant conditions with diurnal cycles of 16 h light at 100 μmol m^{-2} s^{-1} and 8 h darkness. Relative humidity was 65% (day) and 50% (night), with a constant day/night temperature of 21˚C. This is the current mean temperature at the southern range limit of this species during July and August (Matias and Jump 2014). A randomized block design was used and blocks (32 cell plug-trays) rotated once a week to minimise variation attributable to block position within the growth chamber.

Table 3.3.1 Populations included in the study with coordinates and mean altitude of the sampled sites within populations.Latitude: Lat.; Longitude: Lon. Decimal degrees: DD . Altitude: Alt. Metres above sea level: m a.s.l. Annual cumulative rainfall, climatic water deficit, and summer month soil moisture retention (pF) at provenance sites during the period 1901 to 2015. Mean seed weight was determined by weighing 100 seeds.

Provenance site	**Lat. (DD)**	**Lon. (DD)**	**Alt. (m a.s.l)**	**Mean annual cumulative ppn (±SD)**	**Climatic water deficit (mm/yr)**	**Mean soil water retention (pF) for driest summer month (1990-2015)**	**Mean seed weight (g), n=100**
Rothiemurchus, Scotland	57.15	-3.77	318	1040 ± 128	-5.92	2.32	0.0098
Pernitz, Austria	47.91	16	500	1060 ± 136	-76.92	3.38	0.0106
Jarocin, Poland	51.97	17.48	120	540 ± 79	-212.87	2.7	0.0076
Sierra Nevada, Spain	37.05	-3.27	1825	497 ± 119	-463.92	5.65	0.0110

Two months before the experiment, the seedlings were re-potted into 7 x 7 x 8 cm pots with Levingtons M3 pot and bedding high nutrient mix (Everris, Ipswich, UK). The drought treatment consisted of complete withdrawal of irrigation that resulted in a steep decline in soil water content (Figure 3.3.1). During the experiment, 40 pots were weighed at 9 am on days 0, 14, 29 and 36. At the end of the experiment following plant harvesting, the pots oven dried at 70 °C for 48 hours to obtain the dry weight. The pot weight was subtracted from the weight measurements. The gravimetric soil water content (θ_d) (grams of water per gram of oven-dried soil) was calculated (Equation 3.3.1).

$$\theta_d = \frac{(\text{weight of wet soil}) - (\text{weight of dry soil})}{\text{weight of dry soil}}$$

Equation 3.3.1

Then volumetric water content (θ_{vd}) was calculated as follows, where θ_d = gravimetric soil water content, d_b = bulk density and dw = density of water:

$$\theta_{vd} = \theta_d \text{ x } \frac{d_b}{d_w}$$

Equation 3.3.2

Three harvests were made, at days t_1=0, t_2=11 and t_3=29 after the beginning of the water stress treatment. For each treatment at each time point, 4-7 seedlings per provenance were sampled for metabolomics and trait measurements. At t_1, only the control treatment was sampled to provide baseline data.

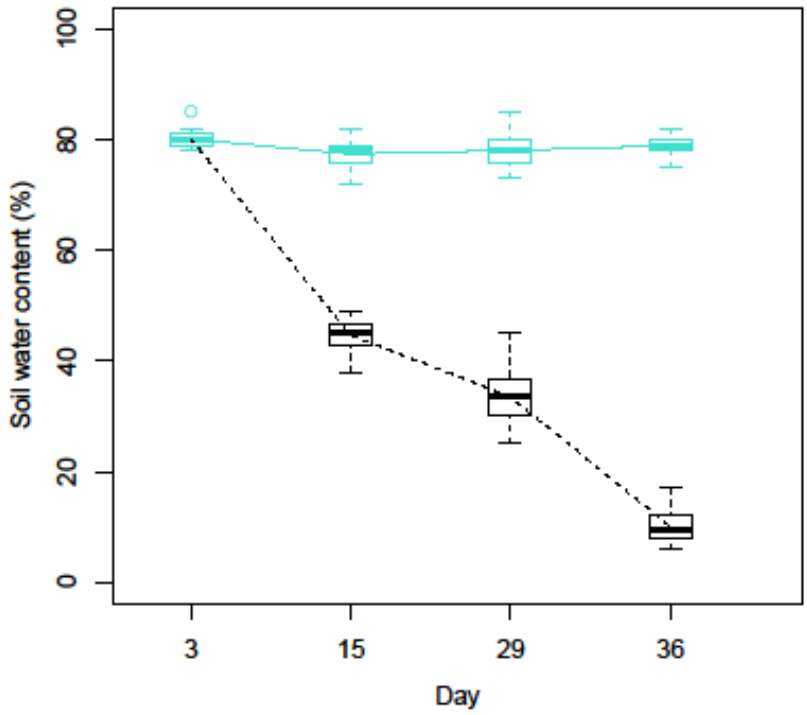

Figure 3.3.1 Soil water content (%) under control watering compared with drought treatment at day 3, 15, 29 and 36. Control treatment: pale blue; drought treatment: black.

3.3.2 Biomass measurements

Height and crown depth were measured for 15 seedlings per provenance at the start of the experiment (Figure S3.6.1). At the beginning of the experiment and at each sample point, 5 individuals were sampled per provenance per treatment. Fresh leaf weight was measured by separating all needles from the shoot and weighing using a balance accurate to ±0.0001 g (Sartorius, B120 S). After this, total leaf area (cm^2) was obtained by spreading the needles on a white sheet of A4 paper with a scale and then using a

flatbed scanner to scan the image. Image analysis of total area was conducted in ImageJ software (Image-J 136b; NIH, Bethesda, Maryland, USA).

Total needles were weighed for fresh weight (FW, g) and then saturated in vials of water for 24 hours in order to obtain the turgid weight (TW, g). Dry weight (DW, g) of total needles and stem tissue was obtained after oven-drying for 48 hours at 70 °C. The percent relative water content was then calculated as: RWC = (FW-DW)/ (TW-DW) x 100. Water deficit was calculated as WD = (TW-FW)/ (TW-DW). Specific leaf area was also calculated as follows:

$$\text{SLA} = \frac{\text{Fresh leaf area (cm2)}}{\text{Leaf dry weight (g)}}$$

Equation 3.3.3

Entire root systems were washed and arranged so as to avoid overlapping lateral roots on a clear plastic tray with a white background and a marker of known size. Images were taken with a digital camera and images were converted to binary and then analysed to obtain total root length using RootReader2D plugin (Clark *et al.* 2013) on ImageJ. Roots were oven dried at 70°C for 48 hours and weighed on a 4 decimal point balance to measure root biomass (g). Specific root length (SRL, m g^{-1}) was also calculated.

3.3.3 Maximal photochemical efficiency of photosystem II

Chlorophyll fluorescence emission from needles was measured by means of a Plant Efficiency Analyzer (Hansatech Instruments Ltd., Norfolk, England). The ratio of variable (F_v) to maximum fluorescence (F_m) was taken, since this value has been

widely used for assessing plant physiological status and the state of Photosystem II (PSII) (Krause and Weis, 1991). The ratio of variable (F_v) to maximum fluorescence (F_m), F_v/F_m, was measured for 5 seedlings per provenance. A comparison of values obtained for attached and detached needles was carried out and no significant difference found. The middle portion of one needle per seedling was placed in the centre of the leaf clip measuring area. Needles were detached and then dark adapted in leaf clips supplied with the analyser for 30 min at room temperature. Then the minimum fluorescence (F_o), maximum fluorescence (F_m), variable fluorescence ($F_v = F_m - F_o$), and the ratio F_v/F_m were recorded using a saturating intensity pulse for 0.7 s at 80% intensity level of photon flux density (4620 μmol m^{-2} s^{-1}). The maximal quantum yield at PSII was calculated as $F_v/F_m = (F_m - F_o)/F_m$.

3.3.4 Sampling for metabolomics

For downstream metabolite analyses, at each sample point, 5 individuals were sampled for foliar tissue per provenance per treatment at midday. These were different individuals than those sampled for trait measurements. Metabolite concentrations vary diurnally for both primary and secondary metabolites and so sampling at the same time of day is important to minimise variation among samples (Gibon *et al.* 2006, Urbanczyck-Wochniak *et al.* 2005). Furthermore, changes in metabolite concentration can occur very rapidly and so quenching of metabolism is essential; freezing immediately in liquid N_2 effectively halts enzyme activity and metabolism (Fiehn 2002). Entire shoots were immediately frozen in liquid N_2 to ensure no wounding response was elicited by removing tissue from the plant, and then stored temporarily

in frozen aluminium foil on dry ice. Samples were stored in a -80°C freezer until metabolite extraction.

3.3.5 Metabolite extraction

A monophasic extraction method was used to extract metabolites. The solvent comprised acetonitrile, methanol, and HPLC grade water (2:2:1) and was kept on wet ice to avoid evaporation. Frozen tissue was weighed into MK-28 Precellys homogenisation tubes (Stretton Scientific, UK) containing steel beads. The volume of solvent to be added was adjusted according to the fresh tissue weight and 5% extra acetonitrile and methanol added to compensate for estimated mean differences in relative water content. Following the addition of solvent, samples were vortexed and homogenised in a Precellys-24 bead-based homogenizer (Stretton Scientific Ltd., UK) at room temperature with two 3 minute pulses of 6800 rpm. The homogenate was transferred to a 1.75 ml eppendorf and half the volume of solvent added to ensure that any residual homogenate was transferred. Samples were placed on ice and vortexed for 10 s before the mixture was centrifuged at 19°C, 14000 rpm (19064 g-force), 10 min). Finally, 400 µl of supernatant was transferred to a new eppendorf and was stored at -80°C prior to speed vacuum drying (Wu *et al.* 2008). After centrifugation, equal volumes of the supernatant were transferred into a new glass vial and dried in a SpeedVac before storage at -80 °C. Taking up the samples in 100 µl water / methanol 1:1 with 0.1 % formic acid could not be achieved by vortexing alone and required 20 min of sonication. A QC sample was pooled from 10 µl each.

3.3.6 UHPLC-MS analysis (positive ion mode)

After centrifugation at 15000 rpm for 10 min at 4 °C (Biofuge), 20 µl per sample were pipetted into a 96-well plate in a controlled randomised order, with QC samples placed

equidistantly between them. The samples were analysed by UHPLC-MS on a Thermo Scientific Q Exactive mass spectrometer attached to a Thermo Dionex Ultimate 3000 RS system, equipped with a Thermo Hypersil Gold column (100 x 2.1 mm, 1.9 µm particles). Ultra-Performance LC (UPLC) uses smaller and more evenly shaped particles within the separation columns than traditional high performance LC (HPLC), thereby decreasing separation times and enhancing sensitivity and resolution (Wilson *et al.* 2005). Solvent A was 0.1 % formic acid in water and Solvent B was 0.1 % formic acid in methanol. Liquid chromatography was performed over 14 min at a flow rate from A to B of 400 µl/min, with 18 µl injections per sample, and MS start at 0.5 min, with the prior flow directed towards waste. Following analysis of preliminary test samples of aqueous and organic phases, data were collected in positive ion and profile mode, *m/z* 100-1000 Da.

Mass spectra were collected using the selected ion monitoring (SIM) stitching method with seven windows of overlapping 100 Da mass ranges (Southam *et al.* 2007). Prior to multivariate analysis, raw spectral data were processed as described in Kirwan *et al.* (2014). Peak signal filtering was applied using an 80 % sample filter (*i.e.* signals have to be found in at least 80 % of samples), applied per group to generate a Sample Filtered Matrix. The dataset was normalised using the probabilistic quotient normalisation (PQN) algorithm to correct for peak intensity differences, since the total sum of peak intensity should be 1.0. Missing values were interpolated using a K Nearest Neighbour algorithm (k=5) and then transformed using a generalised logarithm (g-log) to minimise heteroscedasticity.

3.3.7 Statistical and bioinformatics analyses

The normality of frequency distributions was tested on trait values by Shapiro-Wilk's W test. Where necessary, variables were arc sine transformed to improve the normality of model residuals before analysis. This applied to: crown percentage of shoot, maximum root length percentage of total root length, leaf RWC and F_v/F_m data. For time points 2 and 3, responses of traits were analysed by means of analysis of variance (ANOVA) with provenance and treatment as factors. Pearson's correlations were calculated for mean values of various parameters to check for relevant relationships. The software R version 3.2.2 was used for data analyses (R Core Team, 2015).

Metabolomics data were analysed using a combination of univariate and multivariate statistics, as warranted by the complexity and high dimensionality of the dataset. Principal component analysis (PCA) was used for an initial exploratory analysis of the data. First, the spread between the quality control samples was compared to that of the biological samples for quality assurance of the data. The QC samples were then removed from the data in further analyses. PCA scores plots were constructed and indicated that divergence between drought and controls occurred between time point 2 and 3. Therefore further analyses were carried out for time points 2 and 3 only for both drought and control treatment.

ANOVA, in combination with Benjamini–Hochberg false discovery correction ($\alpha = 0.05$), was used for univariate analysis of the data. First a full model with main effects drought, time point and provenance as well as their two-way and three-way interactions was fitted to the data. As provenance did not show an influence on results

(Table 3.4.4, 3.4.5) a reduced model with factors drought and time and their interaction was used for final univariate analyses of the data (Table 3.4.6). Next, significant peaks (in the reduced model) were filtered on the basis of their fold change, with a threshold of peaks with absolute log2 fold change values larger than 1.

Three further methods were used for multivariate analysis: multivariate analysis of variance (MANOVA), ANOVA simultaneous component analysis (ASCA), and regularized MANOVA (rMANOVA). Multivariate analysis of variance (MANOVA) is not well suited to datasets where the number of samples is considerably lower than variables, which is the case for metabolomics datasets. Therefore, analysis of variance combined with principle component analysis (ASCA) has been used in metabolomics studies (Smilde *et al.* 2005), though it is limited in that it does not include covariance among variables (metabolites). The development of a data-driven weighted average between ASCA and MANOVA, termed regularised MANOVA, has been proposed to solve these issues (Engel *et al.* 2015). When a factor or interaction was marked as significant, Multi-Group Sparse Discriminant Analysis (MGSDA) was used to visualize the differences between samples and to identify the peaks most related to this effect. MGSDA is an extension of sparse discriminant analysis used to select relevant variables from highly dimensional datasets (*i.e* far more variables than samples); for details see Gaynanova *et al.* (2015). Thus, the combined implementation of multivariate and univariate data analysis has been strongly recommended when analysing metabolomics datasets owing to the different limitations inherent to each approach (Karp *et al.* 2005; Goodacre *et al.* 2007; Vinaixa *et al.* 2012).

Putative metabolite identification was carried out by matching compound structures derived from peak m/z patterns to the online database Kyoto Encyclopedia of Genes and Genomes (KEGG), which covers a wide range of metabolic pathways (Kanehisa *et al*. 2016). Identification was carried out using the software MI-PACK by comparing the peaklist to compounds in the KEGG database using a 1 ppm error margin, thereby finding a range of reasonable molecular formulae for putative identification (Weber & Viant 2010). Since isomers and adducts are included and not distinguishable, the number of possible compounds increases over the m/z range. Thus, not all peaks could be assigned a putative metabolite identification even within the 1 ppm error margin.

3.4 Results

3.4.1 Seedling biomass and photochemical capacity

ANOVA analyses of biomass traits at the start of the experiment showed that there were provenance differences in height and SLA, but not in any other traits (Table 3.4.1). Seedlings from Pernitz had the most aboveground shoot growth. Meanwhile, SLA was marginally significantly lower for seedlings from Sierra Nevada as compared with Rothiemurchus (Table 3.4.1).

Table 3.4.1 Summary of ANOVA significance levels for the factor provenance over the main response variables, prior to the application of drought treatment. DW: dry weight; TLA: total leaf area; SLA: specific leaf area; F_v/F_m: maximum potential PSII efficiency of dark adapted seedlings; SRL: specific root length; Max RL: maximum root length. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

Day 0		**Height**		**Crown**		**Leaf DW**		**Root DW**		**TLA**		**SLA**		**F_v/F_m**		**SRL**		**Max RL**	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Provenance	3	**3.09**	**0.03 ***	1.0	0.39	0.12	0.9	1.8	0.16	1.2	0.3	**2.81**	**0.072 .**	1.8	0.17	0.23	0.87	1.7	0.19

Table 3.4.2 Summary of ANOVA significance levels for the studied factors (drought treatment and provenance) over response variables using data collected at day 11 of the drought stress experiment. DW: dry weight; TLA: total leaf area; SLA: specific leaf area; F_v/F_m: maximum potential PSII efficiency; SRL: specific root length; Max RL: maximum root length; R:S: root to shoot ratio. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

Day 11		**Shoot DW**		**Root DW**		**Total DW**		**TLA**		**F_v/F_m (day 12)**		**SRL**		**Max RL**		**R:S**	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment (T)	1	0.006	0.9	3.5	0.07	0.80	0.3	0.82	0.37	1.94	0.17	0.9	0.3	0.03	0.85	**11.08**	**0.002 ** **
Provenance (P)	3	0.59	0.62	0.78	0.5	0.5	0.6	0.16	0.92	**2.9**	**0.04 * **	1.6	0.2	2.24	0.12	0.64	0.59
T x P	3	1.56	0.2	0.17	0.9	0.8	0.4	0.49	0.69	0.8	0.48	0.9	0.4	0.42	0.73	1.4	0.24

Table 3.4.3 Summary of ANOVA significance levels for the studied factors (drought treatment and provenance) over the response variables using data collected at day 29 of the drought stress experiment. DW: dry weight; TLA: total leaf area; SLA: specific leaf area; F_v/F_m: maximum potential PSII efficiency; SRL: specific root length; Max RL: maximum root length; R:S: root to shoot ratio. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

Day 29		**Shoot DW**		**Root DW**		**Total DW**		**TLA**		**F_v/F_m (day 26)**		**SRL**		**Max RL**		**R:S**	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment	1	1.5	0.2	**20.5**	**<0.001 *****	**7.9**	**0.005 ****	**8.26**	**0.006 ****	**67.2**	**<0.001 *****	21.3	**<0.001 *****	**11.1**	**0.002 ****	**13.6**	**<0.001 *****
Provenance	3	1.2	0.29	0.49	0.68	0.7	0.5	0.45	0.71	**3.9**	**0.017 ***	1.15	0.35	0.5	0.67	0.59	0.6
T x P	3	**3.9**	**0.01 ***	**3.4**	**0.01 ***	**4.2**	**0.006 ****	1.3	0.2	**3.69**	**0.02**	0.5	0.66	0.8	0.4	1.59	0.19

ANOVA analyses indicate a significant decrease in total, shoot and root dry weight of seedlings under drought by day 29, along with significant provenance interactions with the drought treatment (Table 3.4.3; Fig 3.4.1A-C). Seedlings from Rothiemurchus were most affected under drought and showed reduced total, shoot and root dry weight, while the least reduction in biomass was found for the Spanish provenance. Root to shoot ratios significantly decreased under drought, though no provenance interaction effect was found (Fig 3.4.1D).

The provenance difference in SLA was not apparent on day 11 or 29 and the drought treatment had no significant effect on SLA. Drought treatment significantly decreased the specific root length and maximum root length by day 29 of the experiment; however, no provenance effects were detected (Table 3.4.3; Figure 3.4.2). However, drought significantly increased the maximum root length as a fraction of the total root length by day 29 of the experiment (ANOVA, F: 11.1, p=0.002) (Figure S3.6.4).

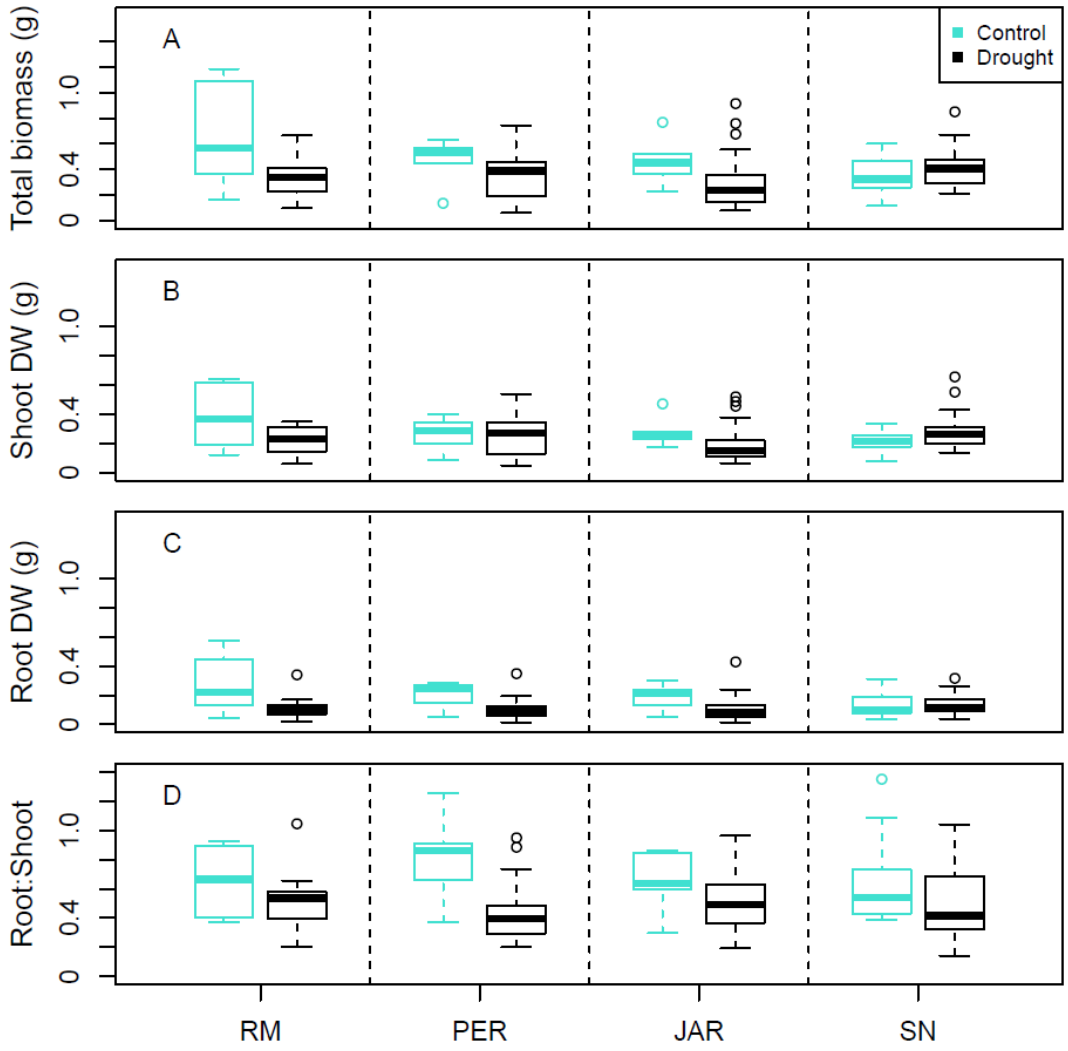

Figure 3.4.1 Total biomass (A), shoot dry weight (B), root dry weight (C) (n=10 (*i.e* root tissue from seedlings where foliar tissue was sampled for metabolomics as well), and root to shoot dry weight ratio (D) on day 29 of the experiment. Provenances are: Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin, Poland (JAR), and Sierra Nevada, Spain (SN). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

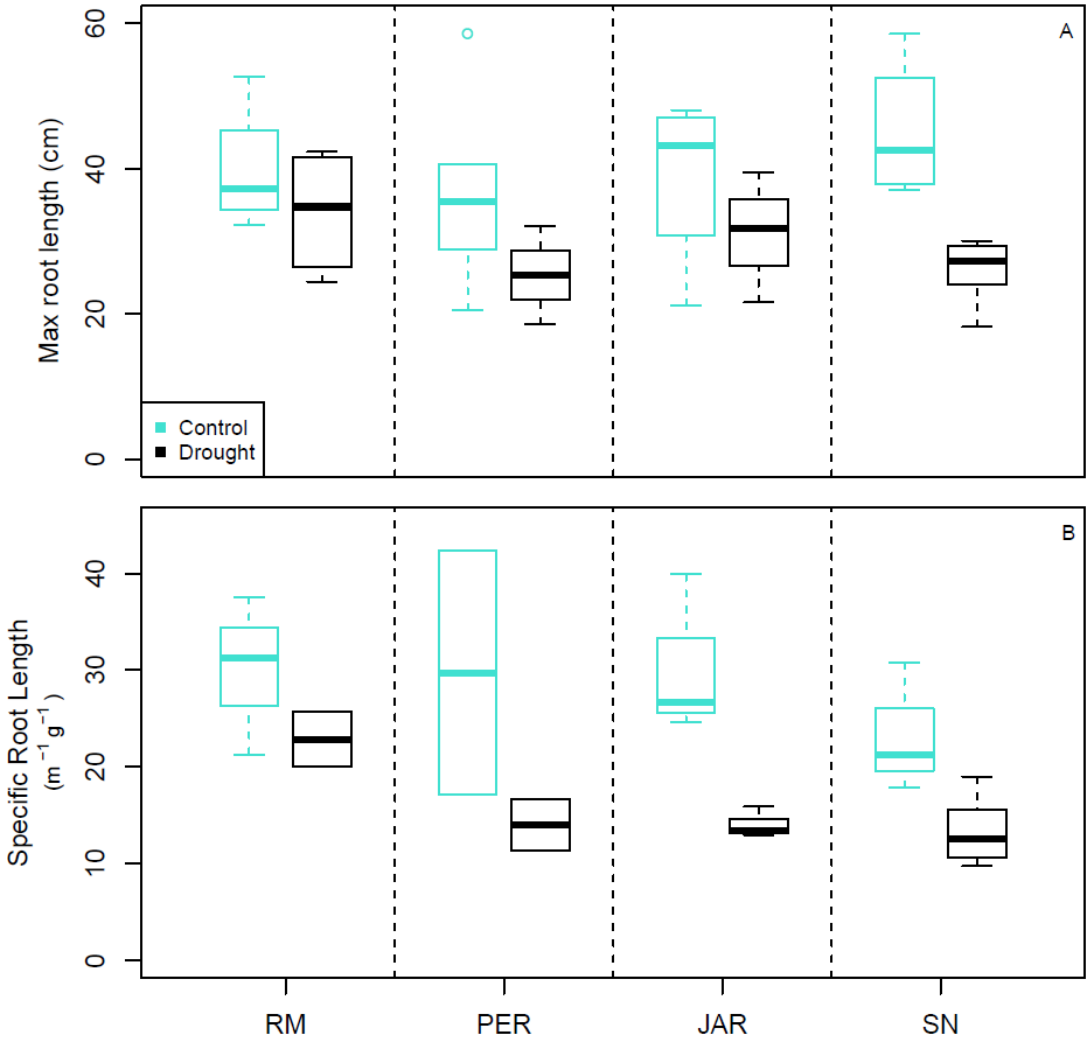

Figure 3.4.2 Maximum root length (A) and specific root length (m-1 g-1) (B) on day 29 of the experiment. Provenances are: Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin, Poland (JAR), and Sierra Nevada, Spain (SN). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

A provenance effect on F_v/F_m was detected on day 12 of the experiment, where Pernitz showed marginally lower F_v/F_m than Sierra Nevada and Rothiemurchus, though it was still within the healthy range of F_v/F_m, *i.e.*, not below 0.7 (Ritchie 2006). A significant effect of drought treatment on F_v/F_m was found after day 26 (Figure 3.4.3), corresponding non-linearly to the reduction in soil water content (Figure 3.4.4A). Only at day 33 was there a linear response of F_v/F_m as soil relative water content fell below 0.2 soil relative water content (Fig 3.4.4B). There was a provenance interaction with drought on day 26, as F_v/F_m decreased most in seedlings from Rothiemurchus and Pernitz, to a lesser extent in seedlings from Jarocin and was least reduced in seedlings from Sierra Nevada (Fig 3.4.3).

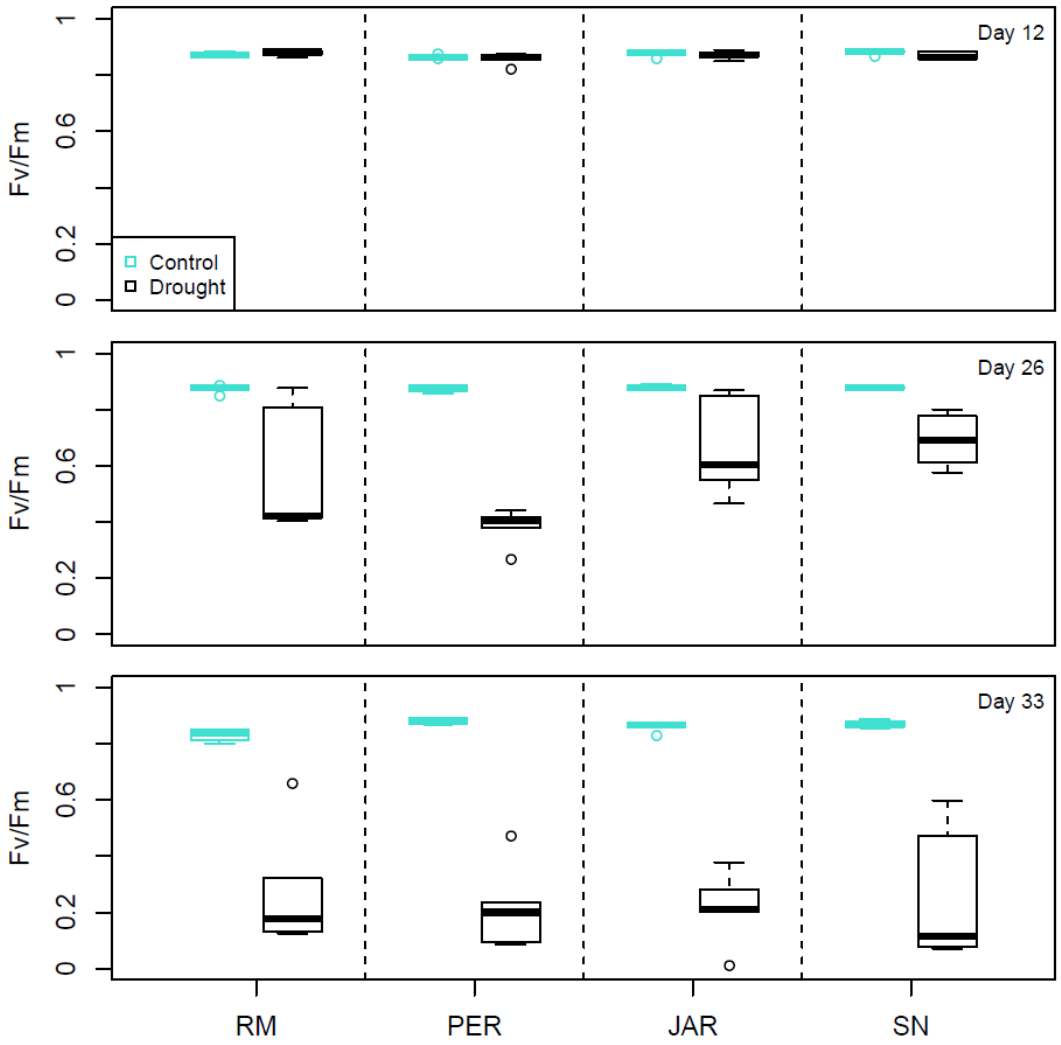

Figure 3.4.3 Maximal photochemical efficiency of photosystem II (F_v/F_m) of seedlings across provenances under drought or control watering at: day 12, 26 and 33 of the experiment. Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

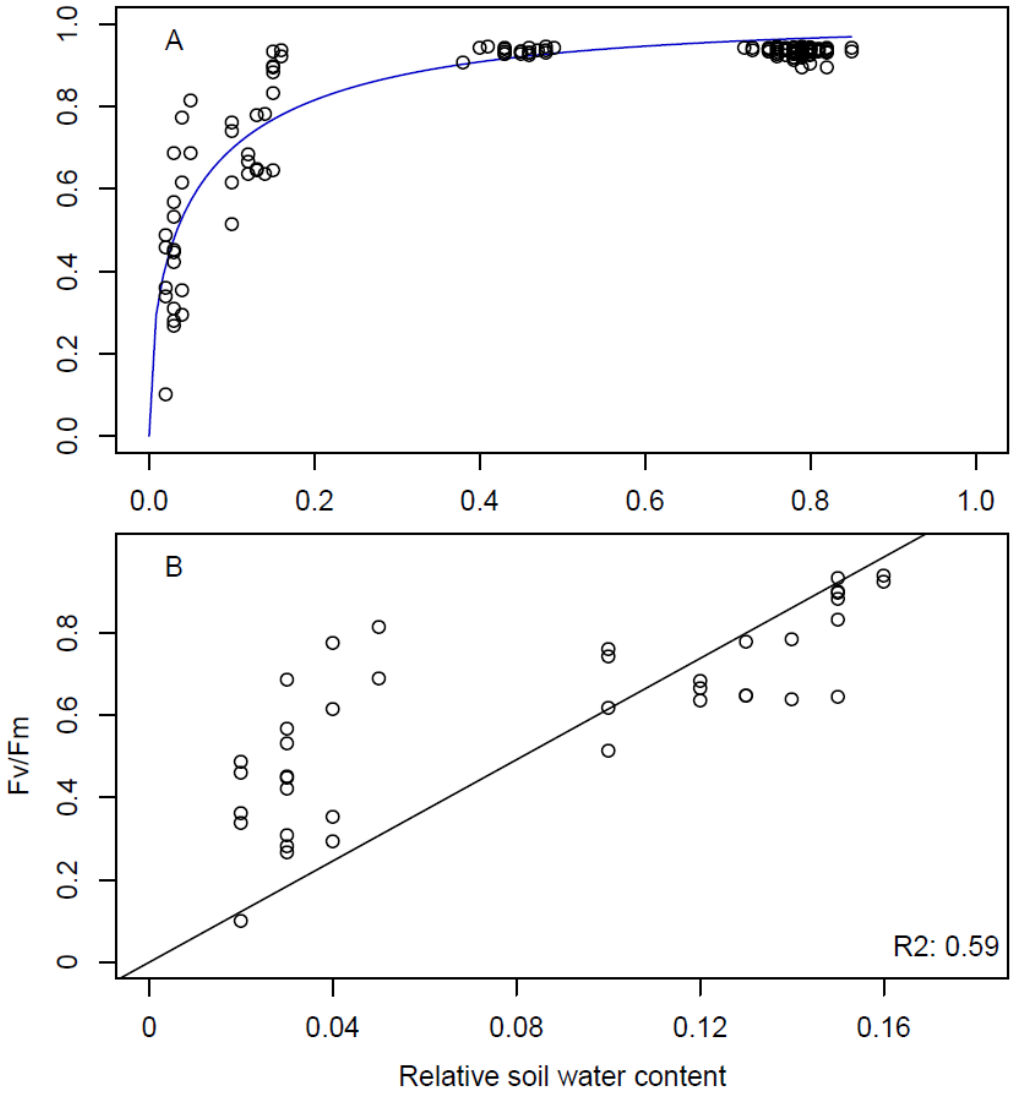

Figure 3.4.4 Non-linear relationship between maximal photochemical efficiency of photosystem II (F_v/F_m) and soil water content (Weibull 4 parameter fitted line) (A) and relationship between maximal photochemical efficiency of photosystem II (F_v/F_m) and soil water content below 0.2 (B).

3.4.2 Metabolomics

As indicated by the tight clustering of the QC samples visualised using PCA, the data was of acceptable quality for further analysis. In the PCA scores plot analysis, there was a clear separation of the drought samples at time point 3 for principle components 1 and 2 (Fig. 3.4.5). It was observed that provenance and all interactions involving provenance were not significant both from univariate analysis (Table 3.4.4) and from the multivariate analyses (Table 3.4.5). However, a significant interaction between drought and time in the experiment was found, indicating a divergence in metabolite composition between control and drought over time. ANOVA analysis indicated a large number of peaks that were affected by the interaction of drought and time (595), of which 89 showed a significant fold change, indicating that the metabolism of seedlings under drought changed differently through time compared to the control group (Table 3.4.6). All multivariate methods also showed significant differences for factors drought and time, and their interaction (Table 3.4.5).

Table 3.4.4 Summary of the univariate analysis of the data using an ANOVA model with all fixed effects: drought, time, provenance and their interaction. Significant peaks out of a total 4640 following the 80 % peak signalling filter.

Factor	# Significant peaks
Treatment	1302
Time	562
Provenance	0
Treatment x Time	561
Treatment x Provenance	0
Time x Provenance	0
Treatment x Time x Provenance	0

Table 3.4.5 Summary of the multivariate analyses of the data. Note that only factors and interactions involving treatment (drought vs control) and time were marked as significant (bold).

Model	PCA + MANOVA	ASCA	rMANOVA
Treatment	**8.867e-10**	**9.99e-04**	**9.99e-04**
Time	**2.101e-10**	**9.99e-04**	**9.99e-04**
Provenance	0.870	0.743	0.9810
Treatment x Time	**3.960e-12**	**9.99e-04**	**9.99e-04**
Treatment x Provenance	0.543	0.786	0.9870
Time x Provenance	0.052	0.084	0.7872
Treatment x Time x Provenance	0.877	0.578	0.9740

Table 3.4.6 Summary of the univariate analysis of the data using an ANOVA model with main effects drought and time and their interaction.

Factor	**Number of significant peaks**	**Number of significant peaks with significant fold change**	**Number of peaks up regulated**	**Number of peaks down regulated**
Drought	1309	68	51	17
Time	608	54	48	6
Drought x Time	595	89	44	45

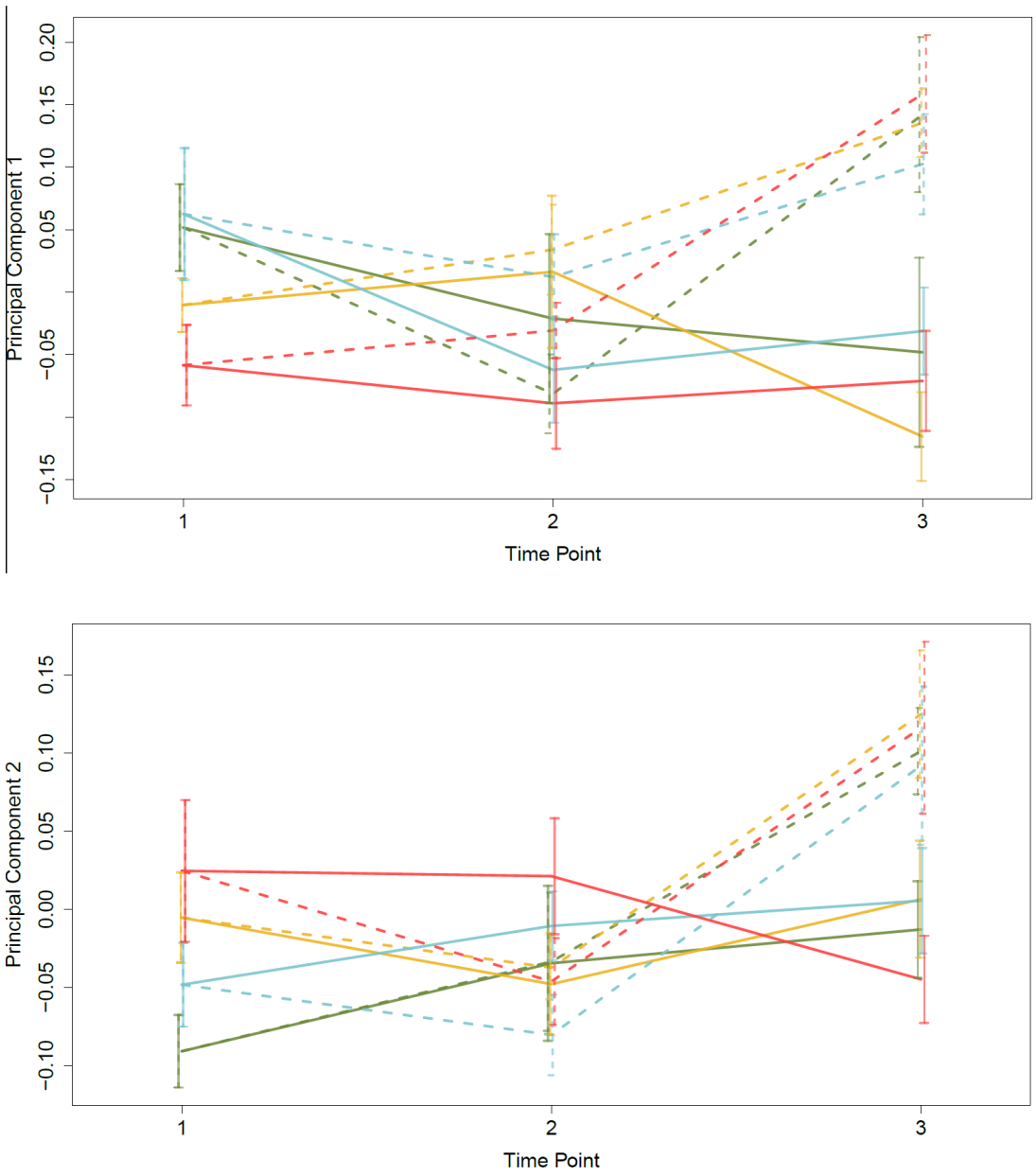

Figure 3.4.5 Mean lines ± standard errors for principle component 1 and 2. These two components together explain 20% of the variation in the metabolomics data. Drought treatment is indicated by broken line. Provenances are colour coded as follows: blue – Rothiemurchus; orange – Pernitz; green – Jarocin; red – Sierra Nevada.

Both univariate and multivariate analyses were used to select important peaks for metabolite identification. The most significant peaks with highest fold changes were selected from the ANOVA analysis for the drought by time interaction. The highest fold changes were used to discriminate peaks that increased in abundance to the greatest extent and therefore were more likely to represent biologically meaningful metabolite reduction or accumulation. Of the top ten significant peaks identified by ANOVA, 6 were classified as unknown. The top eight peaks that could be annotated (*i.e.*, excluding peaks with unknown identification) are presented in Table 3.4.7. Of these, the top 3 annotated metabolites that were significantly higher under drought were free amino acids: proline, tyrosine and tryptophan. For the significant factors of drought and time and their interaction, Multi-Group Sparse Discriminant Analysis (MSGDA) was used to visualize the differences between the samples and to determine which peaks in the data were most related to this effect (S Fig 3.6.5). Of the top ten significant peaks identified by MSGDA, 9 were classified as unknown. The top ten peaks that could be annotated (*i.e.*, excluding peaks with unknown identification) are presented in Table 3.4.8. From the MSGDA analysis the top 3 metabolites with putative identification were: an amino acid (tyrosine), a flavonoid (5,7-Dimethoxyflavone) and an isoindole (N-(p-Nitrobenzyl)phthalimide).

Table 3.4.7 Top 8 significant peaks with putative annotation from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for the interaction between drought and time from ANOVA analysis. The significant peaks with the highest absolute fold change are reported. The q-value is defined as Pr(null|Z≥z). It corresponds to the probability that peaks with a test-statistic with a value of z or larger are not significant (null). By only marking peaks with a q-value ≤ 0.05 as significant, the False Discovery Rate is maintained at 5%. The fold change between groups A (control) and B (drought) is reported as log2(b/a), where a and b correspond to the average peak intensity for samples in group A and B, respectively.

q-value	Fold change	KEGG ID hits	Putative annotation	Biological role of compound/Pathway(s) involved
8.46E-06	2.63	C00148, C00763	L-Proline	Proline; amino acid-related compounds (primary metabolism)
2.38E-05	2.42	C04368, C00082	L-Tyrosine	Tyrosine metabolism; amino acid-related compounds (primary metabolism)
3.63E-11	2.33	C00078, C00525	L-Tryptophan	Tryptophan metabolism; amino acid-related compounds (primary metabolism)
4.41E-11	2.25	C17245	5-Methylthio-pentanaldoxime	Glucosinolate biosynthesis (secondary metabolism)
7.56E-06	2.24	C09198	Terpenoid EA-I	Terpenoid biosynthesis (secondary metabolism)
0.001417	2.2	C12160	Myxalamid C	Fatty amide (secondary metabolism)
1.15E-10	2.09	C08313, C02596	Skatole	Indole (secondary metabolism)

Table 3.4.8 Top 10 significant peaks with putative annotation from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for the interaction between drought and time from Multi-Group Sparse Discriminant Analysis (MSGDA) analysis.

Rank (based on CV weights from MGSDA model)	KEGG ID hits	Putative annotation	Class of compound/Pathway(s) involved
8	C04368, C00082	Tyrosine	Tyrosine metabolism; amino acid-related compounds (primary metabolism)
13	C01265, C15052	5,7-Dimethoxyflavone	Flavone and flavonol biosynthesis (secondary metabolism)
16	C14265	N-(p-Nitrobenzyl)phthalimide	Isoindole (secondary metabolism)
19	C10283	4-Prenyloxyresveratrol	Stilbenoid biosynthesis (secondary metabolism)
30	C17615	Kukoamine A	Amine (primary metabolism)
48	C09233	Psychotridine	Alkaloids derived from tryptophan and anthranilic acid (secondary metabolism)
49	C11608, C08779	Nafenopin-glucuronide/ Rutaevin	Glucosiduronic acid/Steroid lactone (secondary metabolism) Carbohydrate acid derivative/Terpenoid biosynthesis (secondary metabolism)
52	C1078, C06317	2,6-Dimethoxyphenol/ Vanillyl alcohol	Phenol (secondary metabolism)

3.5 Discussion

This study aimed to investigate the foliar metabolic responses of *P. sylvestris* seedlings to drought, with a view to increasing the depth of our understanding of the mechanisms of drought-induced mortality, and to ascertain whether local adaptation in the metabolic response to drought would be discernible amongst provenances. Findings of metabolites important in the drought response included a shift towards the production of the compatible solute proline, as well as secondary metabolites and their precursors. Proline has a key role in osmoregulation and antioxidant defence, while the identification of compounds from aromatic amino acids and flavonoids shows that these metabolically costly pathways are upregulated under drought stress, even at the seedling stage. Interestingly, even though different provenances seemed to differ in their response to drought as evaluated via biomass and photochemical assays, there were no detectable metabolic differences amongst provenances either in controls or in interaction with the drought treatment.

3.5.1 Seedling foliar metabolome response to drought

There were significant differences in the metabolite composition of the control and drought treatment groups, with a clear divergence in metabolic profiles following four weeks of drought stress (Figure 3.4.5). A number of important significant peaks determined by univariate and multivariate statistics were not identifiable through interrogation of metabolic databases. These compounds might offer insights into novel pathways that are drought stress responsive, though further analyses would be necessary to uncover their functional properties. Compounds putatively identified as important in the drought by time interaction that were annotated in metabolite databases included primary and secondary metabolites. Only peaks that were

significant in the interaction between drought and time were selected, since drought and time effects were not additive, *i.e* we focused on metabolites that changed differently through time under drought compared to the control (non-drought) group. Free amino acids (proline, tryptophan and tyrosine; Table 3.4.7) produced by primary metabolic pathways were identified. Proline was the most significant annotated peak identified by ANOVA. Proline is an amino acid that functions as a compatible solute, carrying no net charge at physiological pH, by raising osmotic pressure in the cytoplasm and stabilising proteins and cellular membranes. Additionally, proline has been indicated to exhibit antioxidant capacity in free radical scavenging (Smirnoff & Cumbes 1989). Owing to its osmoprotective and antioxidant capacity, proline accumulates in a number of plant species under drought (Hayat *et al.* 2012). Furthermore, it is suggested that proline biosynthesis under stress may have a role in signalling as well as maintenance of NAD(P)/NAD(P)H ratios that enable metabolic pathways to function in generating secondary metabolites (Hare & Cress 1997). Accumulation of proline under water deficit occurs in an ABA-dependent manner and is also influenced by sugar availability. *Arabidopsis* mutants that are ABA-deficient (aba1, aba2, and aba3) or ABA-insensitive (abi4 and abi5) display insensitivity to sugar (Rook *et al.* 2001). Under water deficit exogenous ABA-induced proline accumulation was partially blocked in abi4 and this response was modified by additional sucrose treatment, indicative of a common regulatory mechanism to restrict proline accumulation when carbohydrate status is low (Verslues & Bray 2006).

Plants synthesise aromatic amino acids, such as tyrosine (Tyr) and tryptophan (Trp), via the metabolically costly seven step shikimate pathway, to which over 30 % of

photosynthetically derived carbon is directed (Maeda & Dudareva 2012). Aromatic amino acids (Trp, Phe, and Tyr) serve as precursors for indole glucosinolates, phytoalexins, alkaloids, lignins, flavonoids, isoflavonoids, and hydroxycinnamic acids (Dixon, 2001).

Tyrosine (Tyr) was identified as the most significant annotated peak by Multi-Group Sparse Discriminant Analysis (MSGDA) and tryptophan (Trp) was the second most significant annotated peak identified by ANOVA. Tyrosine concentration is responsive to light availability; for example, shade leaves of *Pinus ponderosa* seedlings showed higher Tyr levels both in well-watered and drought treatment groups (Vance & Zaerr 1990). Tyrosine hyperaccumulation in young shade leaves of *Inga umbellifera* has been linked to decreased insect larval performance, thus presenting as a rare example of an amino acid functioning as a defensive compound (Lokvam *et al.* 2006). Tryptophan biosynthetic enzymes have been shown to be up-regulated in response to oxidative stress treatment in *Arabidopsis* (Zhao *et al.* 1998).

Secondary compounds involved in plant defence and abiotic stress responses can be derived from the aromatic amino acids tyrosine and tryptophan. Glucosinolates are derived from aromatic amino acids, including Tyr and Trp, and have aldoximes as intermediates, such as 5-Methylthiopentanaldoxime that was identified as the fourth most significant peak by ANOVA (Table 3.4.7; Poulton & Moller 1993; Sanchez-Vallet *et al.* 2010). This class of compounds shows natural variation among populations of *Brassica oleracea* (Newton *et al.* 2009) and also in *Arabidopsis petraea* (Davey *et al.* 2008). Although the majority of glucosinolates are associated with biotic stress, there are also examples of accumulation of glucosinolates under drought (Rask

et al. 2000). Thus, the aromatic amino acid accumulation of tyrosine and tryptophan, as key precursors of glucosinolates, might be supportive of increased production of secondary metabolites as part of a pre-emptive defence response against pathogens during drought stress.

As well as their precursor molecules, secondary metabolites were also identified as significantly increasing under drought. Flavonoids are a large class of secondary metabolites that are responsive to drought stress and involved in photoprotection (Agati *et al.* 2010). Under drought stress, photo-inhibition is more of a risk. There is insufficient regeneration of $NADP^+$ by the Calvin cycle, owing to reduced CO_2 availability due to stomatal closure and yet ongoing exposure to continuous excessive light, which directs electron transfer to molecular oxygen, the electron acceptor in the so called Mehler reaction, resulting in superoxide radicals $O_2^{\cdot -}$ formed at PSI and endangering the photosynthetic apparatus (Cruz de Carvalho 2008). Furthermore, quercetin derivatives such as putatively identified in this study (5,7-Dimethoxyflavone; Table 3.4.8) are considered to be effective antioxidants (Agati *et al.* 2012). In a meta-analysis of 50 studies into foliar antioxidant defences across tree species in response to drought, Wujeska *et al.* (2013) found that evergreen tree species increased membrane-bound protection by higher zeaxanthin and tocopherol. However, in this study the metabolites identified with an antioxidant function were proline and a flavonoid. Needle age has been shown to affect the concentration of flavonoids in *Pinus pinaster*, with many flavonoids (including quercetin) having significantly higher concentration in juvenile needles compared with mature needles (de Miguel *et al.* 2016).

For conifer species, carbon-based secondary metabolites, terpenoids and phenolic compounds are expressed constitutively and are inducible to high concentrations that provide effective defence against many pests and pathogens. (Keeling & Bohlmann 2006). The generally held view is that drought stress precludes investment in defence and increases vulnerability to biotic attack, owing to the effect of drought on reducing the availability of non-structural carbohydrates. Anderegg *et al.* (2015) outline integration of biotic attack within the drought-induced mortality framework, using two insect guilds as case examples: bark beetles and defoliators. In both cases, the NSC pool is critical to the formation of carbon based secondary metabolites that form the tree's defence. However, it is possible that at the seedling stage, the metabolic drought stress has a different effect on the regulation of secondary metabolite production. The findings of this study indicate that secondary metabolites are increasing under drought, potentially suggesting that drought has a priming effect on plant defences.

3.5.2 Provenance variation in the seedling drought response

Total seedling biomass was reduced under drought treatment and there were significant interactions with provenance. Seedlings from the wettest provenance were most affected under drought in terms of biomass reduction, while seedlings from the driest provenance were least affected. Similarly, the photochemical efficiency of seedlings was least reduced under drought for the Spanish seedlings. However, metabolic profiles were not found to be significantly different among populations of *P. sylvestris* at this developmental stage (10 months old). Peak signal filtering was applied using an 80 % sample filter (*i.e.,* signals have to be found in at least 80 % of samples), though the lack of provenance effect was consistent at lower filter thresholds (50% and 10% filtered data).

Drought treatment decreased the maximum root length and no provenance effect was detected. However, biomass allocation patterns under drought differed between this controlled environment study and a field rainfall interception experiment across 10 provenances of *P. sylvestris*, where seedlings of southwestern provenances from France and Spain responded to drought with an increase in root length in the field (Taeger *et al.* 2015). This may be explained by the differing levels of drought stress experienced; whereas the rainfall exclusion experiment approximately halved precipitation, water withdrawal and complete soil dry down that occurred in this study might have inhibited root growth. Furthermore, the high nutrient medium is likely to have affected seedling root to shoot ratio, as increasing root investment in order to maximise nutrient uptake would be less important. However, since all provenances were similarly affected, provenance differentiation could still be investigated. Specific root length decreased in response to drought for all provenances. Olmo *et al.* (2014) conducted an analysis of specific root length responses to drought across seedlings of 10 tree species and did not find a consistent drought response in this trait. As well as depending on species identity, there can be an effect of ontogeny; for example, fine root production increases with age in *P. sylvestris* (Makkonen & Helmisaari 2002).

Provenance variation is detectable for several aboveground physiological traits in *P. sylvestris*. Oleksyn *et al.* (1998) compared 24 populations across the European range and found that the duration of the shoot elongation period of one-year-old seedlings was affected by temperature and photoperiod, as well as being shortest in seedlings from the northern populations compared with other populations. Furthermore, in a

common garden experiment with six populations spanning a latitudinal cline in Europe, higher foliar concentrations of nitrogen, phosphorous and magnesium were found for northern as compared with central provenances, potentially reflecting metabolic adaptation to nutrient limitation in colder northern environments (Olekysn *et al.* 2002). Furthermore, seasonal dynamics of nutrients and non-structural carbohydrates vary according to provenance, and this may relate to the phenology of shoot and root growth (Oleksyn *et al.* 2000).

Under drought, total leaf area was significantly reduced, but there was no needle abscission (personal observation). Rather, the drought is thought to have inhibited growth. Needles also showed a reduction in the maximal efficiency of photosystem II (F_v/F_m) by day 26 of the drought treatment, indicating that photosynthesis was compromised. F_v/F_m is relatively insensitive to drought and only declines when water stress becomes severe (Epron & Dryer 1992; Iijima *et al.* 2006; Ditmarova *et al.* 2010; Way *et al.* 2013). However, there was a provenance effect in that seedlings from Rothiemurchus were more affected by drought than seedlings from Sierra Nevada. Seedlings from Rothiemurchus were also most affected in terms of reduction in total, shoot and root biomass, indicating that this wetter provenance was susceptible to drought to a greater extent.

However, the metabolomics analysis did not detect differentiation in the metabolic profiles of seedlings according to provenance. Previous studies on intraspecific variation in conifers have found evidence for a stronger environmental than genetic signal on metabolomes of developing xylem in *Pseudotsuga menziesii* (Robinson *et*

al. 2007). However, differences in foliar metabolomes of *Pinus pinaster* were found to be strongly related to the aridity of the provenance site of origin, suggesting local adaptation (Meijon *et al.* 2016). Also, Du *et al.* (2015) found that *Pseudotsuga menziesii* seedlings showed a provenance specific drought response, with the interior drier provenance increasing aromatic amino acids. In this study, the univariate and multivariate analyses used indicated that metabolic phenotypes of *P. sylvestris* do not appear to be population specific and show local adaptation; or at least such differences did not have a sufficiently large effect size to be statistically detectable. This could reflect a limitation to local adaptation, such as insufficient standing genetic variation related to drought responsive metabolic pathways, resulting in conserved metabolic responses between populations of the range of *P.sylvestris*.

3.5.3 Conclusions

There was a strong metabolic response to drought in *P. sylvestris* needles at this seedling stage. Metabolites identified as important in this response included proline, a compatible solute and antioxidant, as well as secondary metabolites and their precursors involved in plant defence and photoprotection. While seedling biomass and photochemical efficiency was found to be most strongly reduced by drought for Rothiemurchus, the wettest provenance, there was a lack of a provenance effect on metabolic traits. Whether this result accurately reflects local adaptation of *P. sylvestris* populations, or was only a result of limited sample size being unrepresentative of populations is not clear. However, these findings do show the identification of important metabolite changes under drought as well as unknown compounds potentially of interest to further research.

3.6 Supplementary material

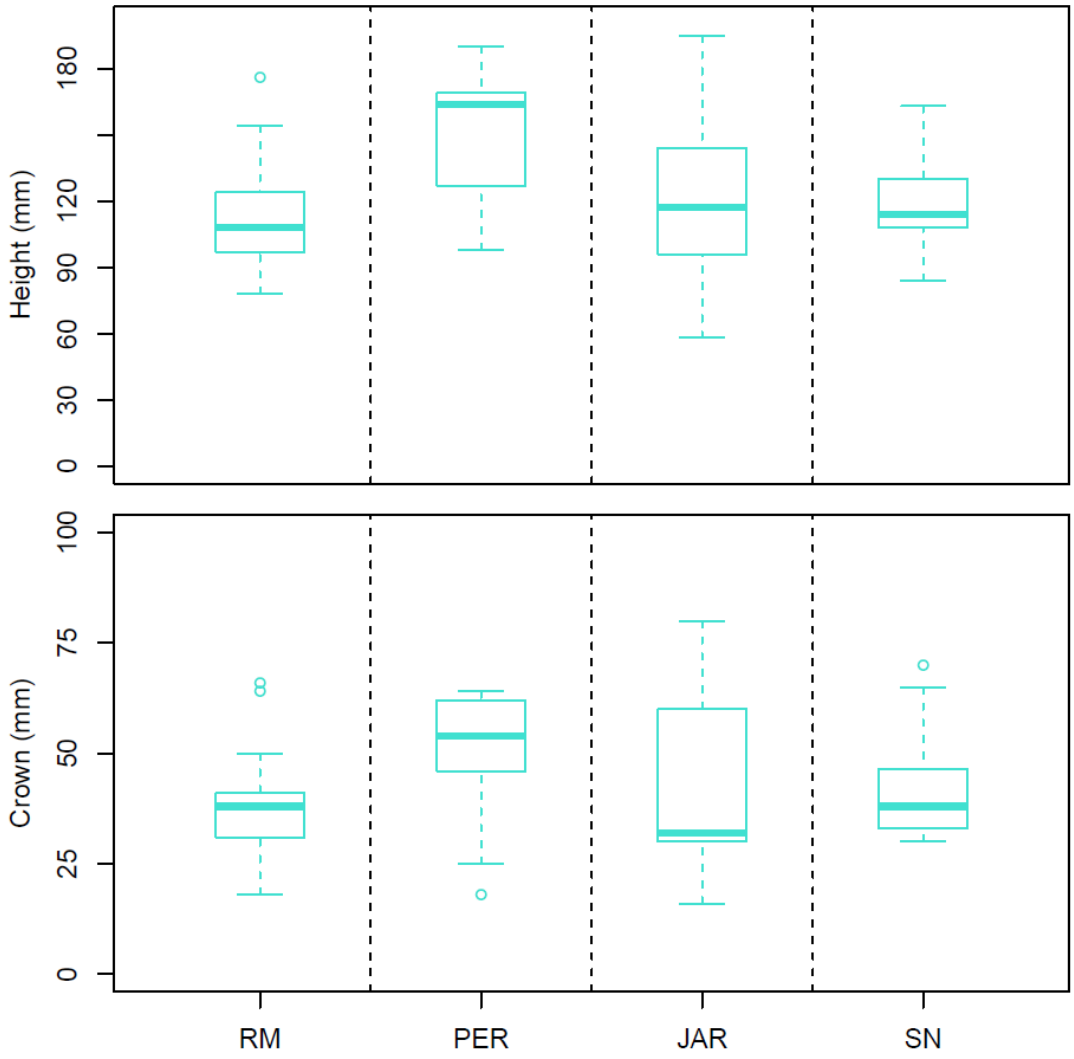

Figure S3.6.1 Initial aboveground height (mm) and crown depth (mm) for seedlings across provenances before the start of the treatment. Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

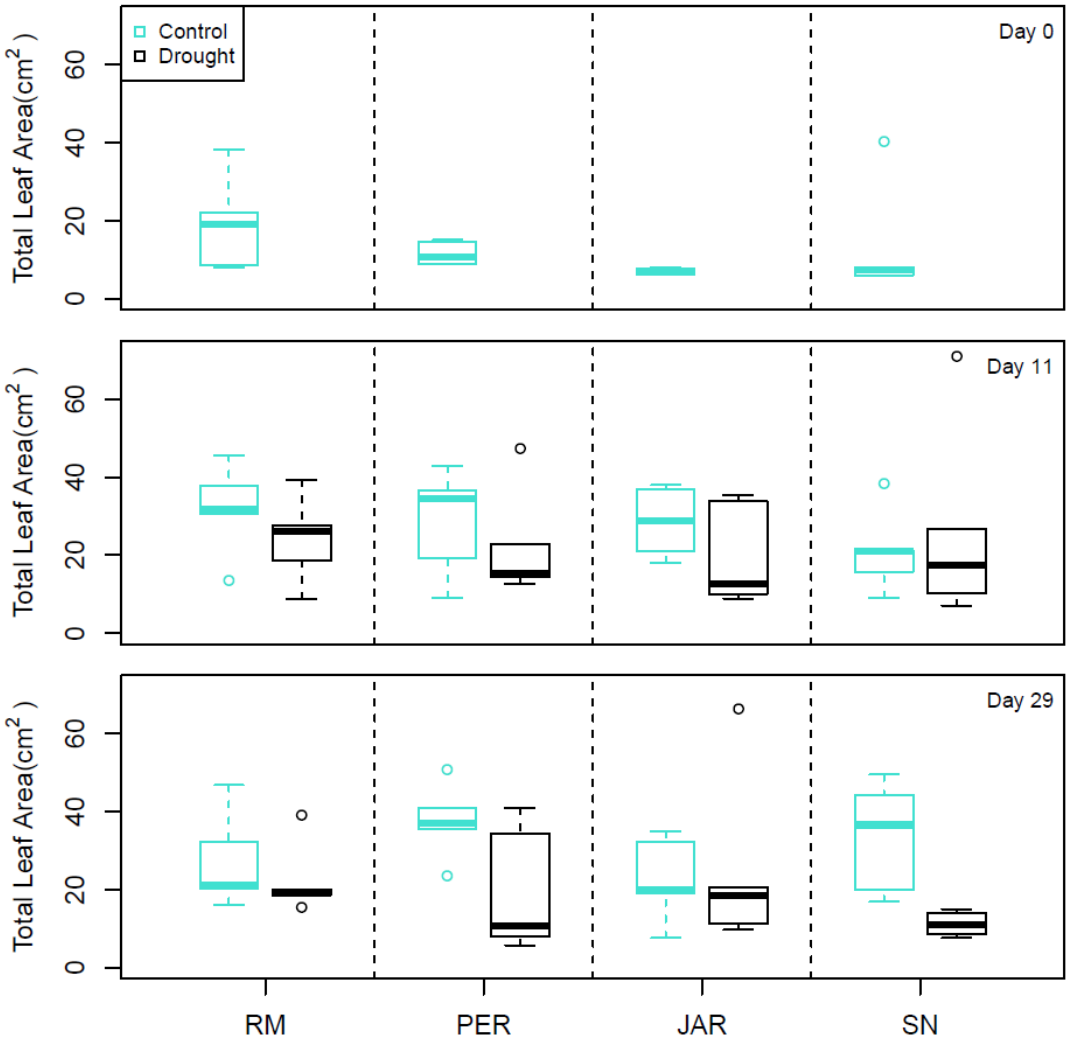

Figure S3.6.2 Total leaf area for seedlings across provenances at: day 0, 11 and 29 of the experiment. Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

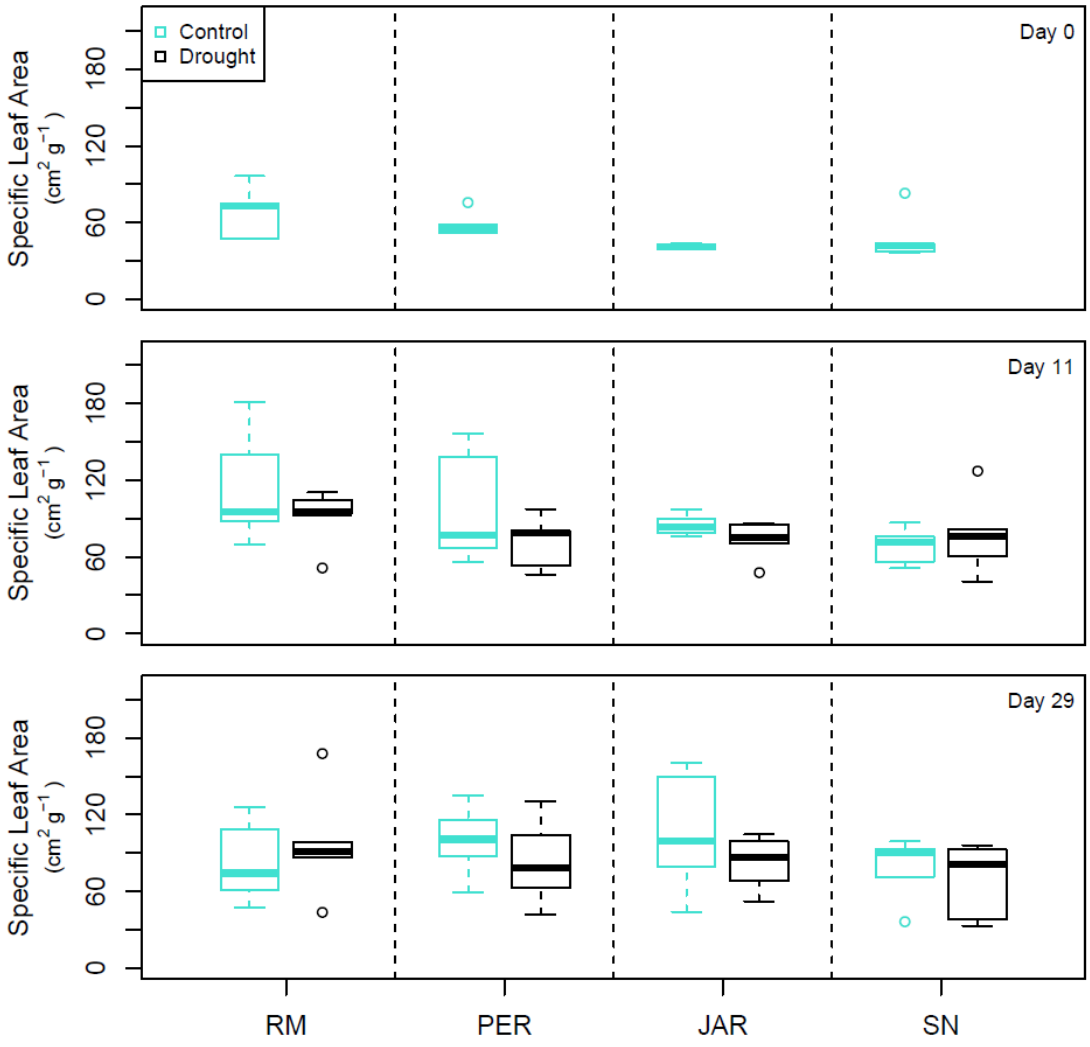

Figure S3.6.3 Specific leaf area (SLA, $cm^2\ g^{-1}$) of seedlings across provenances under drought or control watering at: day 0, 11 and 29 of the experiment. Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

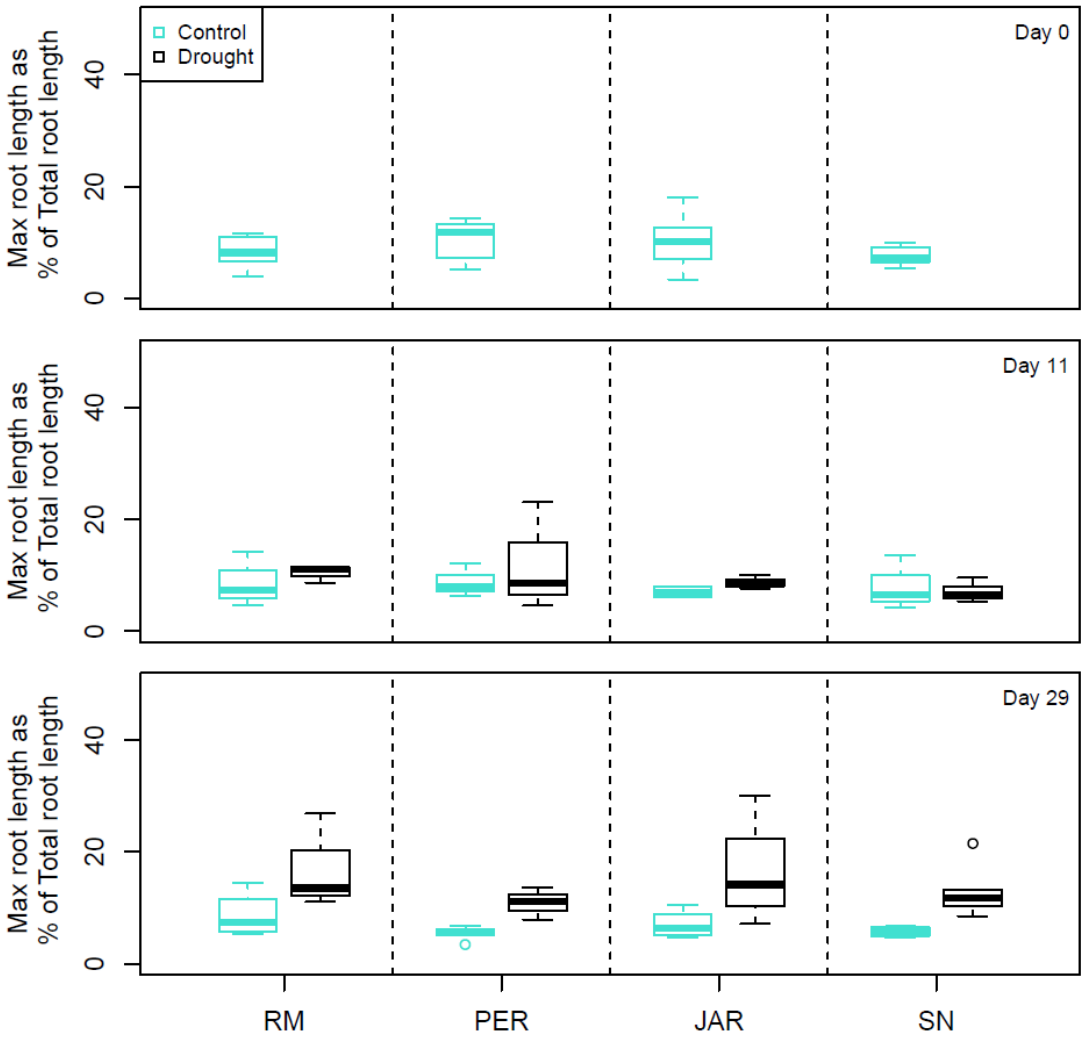

Figure S3.6.4 Seedling maximum root length as a percentage of total root length across provenances under drought or control watering at: day 3, 14 and 32 of the experiment. Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

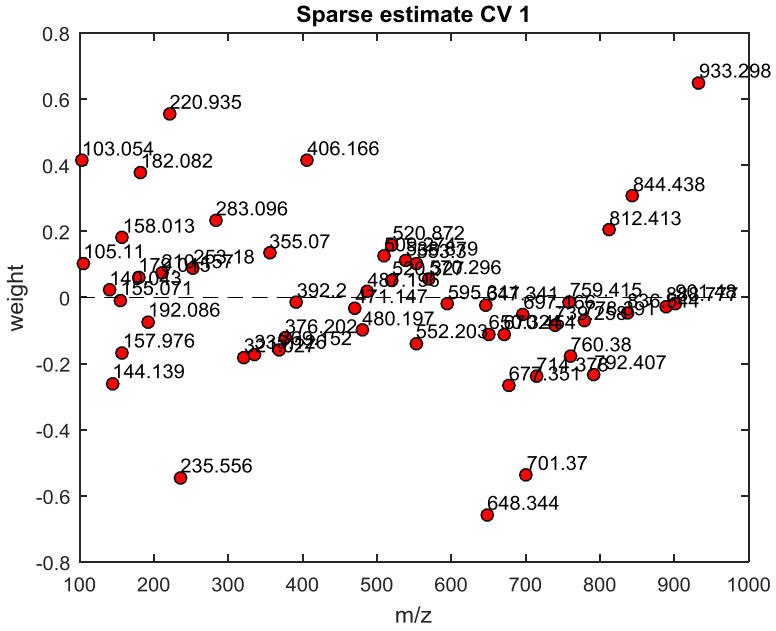

Figure S3.6.5 Variable importance for interaction treatment x time estimated by Multi-Group Sparse Discriminant Analysis (MGSDA). The standardised canonical coefficients of canonical variate (CV) 1 for the interaction effect were visualised and outlier peaks detected. Outlier peaks were then ranked based on their absolute CV weight.

4 Provenance, maternal parentage and seed weight effects on germination and early seedling drought stress responses

4.1 Abstract

This study was undertaken in order to better understand the risks to *Pinus sylvestris* populations (and thus much of the forests in Europe and Asia) under drought, which is predicted to increase under global climate change. The effects of provenance, maternal parentage, and maternal investment in seed weight may have implications for seedling establishment, in terms of germination timing, growth and survival under drought stress. A controlled environment chamber experiment was performed to analyse the effect of provenance, maternal parentage and individual seed weight on early seedling biomass traits and antioxidant gene expression under drought. Higher seed weight and earlier timing of germination increased needle number, needle and total biomass. Provenances differed in seed weight, which was also affected by the maternal parent. Of the three provenances with a half-sib family structure, the high altitude Austrian provenance showed effects of maternal parentage on seedling biomass traits. However, there were provenance differences in seedling drought responses independently of maternal parentage and seed weight, with seedlings from Austria being most affected by drought in terms of reduced total biomass, as well as maximum root length. Meanwhile, a group constituted by the Spanish provenances was visualised using principle component analysis and defined by higher seed weight,

high allocation to stem, and higher root to shoot ratio. Gene expression of the antioxidant enzyme superoxide dismutase (*SOD*) increased under drought treatment, which might be explained by its role in preventing damage to photosynthetic apparatus under drought stress.

4.2 Introduction

Drought will be a major issue facing future forest tree populations, particularly at the xeric range edge of species distributions. Provenances are areas within which similar ecological and climatic characteristics are found and thus, local adaptation or acclimation of populations to site conditions may be expected. Countering the process of adaptive genetic differentiation is that of gene flow (Mayr 1947). Despite having largely outcrossing populations with extensive gene flow, the potential for local adaptation in forest tree populations has long been indicated by the results of provenance trials (transplantation experiments) that show latitudinal clines in phenology, growth and abiotic stress tolerance (for review see Alberto *et al.* 2013). Seed germination and seedling establishment are a bottleneck stage in regeneration with high mortality rates (Lloret *et al.* 1999; Castro *et al.* 2005). Thus, traits that maximise offspring fitness to the conditions of the regeneration niche might be expected in locally adapted populations.

Although maternal parent trees share environmental conditions, intraspecific variation can still occur at the within population level. Maternal effects have been implicated in determining germination speed in *Pseudotsuga menziesii* (El-Kassaby *et al.* 1990). Similarly, both the maternal environment and maternal genetic effects contributed to

variation in time to germination for *Pinus pinaster* (Cendan *et al.* 2011) and larger seeds were found to germinate faster in *Pinus taeda* (Dunlap & Barnett 1983).

Seed weight is one measure of the extent of maternal investment in reproductive fitness that has been shown to vary at scales ranging from the individual (Zas *et al.* 2015) to across environmental gradients (Reich *et al.* 1994). Species with large seeds have higher survivorship during seedling establishment than species producing smaller seeds (Westoby *et al.* 1997). Under stressful environmental conditions, such as high altitude and drought stress, it has been proposed that selection pressure for maternal investment in larger seeds is higher than selection pressure for a higher number of smaller seeds (Westoby *et al.* 1992). Reich *et al.* (1994) compared the relationship between seed weight classes and seedling height for 24 populations in a controlled growth room experiment. There was an initial positive effect of seed weight on height that declined or disappeared by the age of 5–7 years among central and southern populations, but remained stable for northern populations. This finding is corroborated by other studies; for example, the effect of seed weight on seedling performance has been demonstrated to last one growing season for Spanish populations of *P. sylvestris*, after which maternal parent identity better explained variation in seedling performance (Castro 1999). Conversely, in Sweden the higher seed weight of orchard seed (~6 mg) relative to stand seed (~4 mg) was found to affect seedling growth traits for 5 years, improving growth performance under field conditions as compared with nursery conditions (Wennstrom *et al.* 2002). The higher seed weight of southern populations of *Pinus sylvestris* is thought to enable seedlings to emerge earlier than from northern and central European populations (Reich *et al.* 1994). This potentially enables a root

system to quickly establish, with the higher biomass investment in roots being critical for surviving Mediterranean summer droughts (Castro 2006; Padilla and Pugnaire 2007; Castro *et al.* 2008; Matias and Jump 2014).

Therefore, seed weight variation within populations is expected to be an important factor in enhancing seedling performance under drought stress. However, there may be other facets to local adaptation of southern provenances under drought. Antioxidant defences are one physiological response that protects plants from cellular damage under drought by regulating the cellular redox homeostasis. Three antioxidant enzyme genes were selected for gene expression analyses. Two directly encode the antioxidant enzymes superoxide dismutase and catalase, while glutathione synthetase is an important enzyme in the synthesis of an antioxidant, glutathione.

At the frontline of enzymatic defence is superoxide dismutase (*SOD*), a metalloenzyme that dismutates superoxide (O_2^-) to the less cytotoxic reactive oxygen species hydrogen peroxide (H_2O_2) and oxygen (O_2), and *SOD* isoforms are present in all subcellular compartments (Alscher *et al.* 2002). Cytosolic Cu-Zn *SOD* overexpression has been found to increase drought stress tolerance in *Nicotiana tabacum* (Faize *et al.* 2011). Metabolic modelling of the *SOD*-Ascorbate-Glutathione cycle with simulated loss of *SOD* resulted in O_2^- being processed via alternative non-enzymatic redox reactions that significantly elevated H_2O_2, though providing a compensatory route during low *SOD* activity (Polle 2001).

Glutathione sythetase (*GS*) is a key enzyme in the biosynthesis of glutathione (GSH). GSH is an important reducing substrate for ROS detoxification, functioning as an antioxidant by scavenging hydrogen peroxide, singlet oxygen, superoxide and even hydroxyl radicals (Miller *et al.* 2010). GSH accumulation compensates in catalase deficient mutants and in plants where catalase activity has been reduced by antisense technology (Smith *et al.* 1984; Smith 1985; Chamnongpol *et al.* 1996; Willekens *et al.* 1997). Transgenic poplar individuals (*Populus tremula x Populus alba*) generating higher levels of reduced GSH are less susceptible to photoinhibition stress than the wild-type species (Foyer *et al.* 1995). Photorespiration modulates the antioxidant glutathione pool by generating glycine, the substrate for GSH biosynthesis (Foyer & Noctor 2000). Furthermore, the redox state of glutathione regulates *SOD* gene expression *in vivo* in *Pinus sylvestris* (Wingsle *et al.* 1996).

Following stomatal closure and dwindling intracellular CO_2, photorespiratory production of glycolate is elevated in chloroplasts and shuttled to peroxisomes for oxidation. Photorespiration enables increased consumption of photosynthetic electrons, providing an alternative route that dissipates energy and averts photo-damage (Guan *et al.* 2004). Peroxisome localised catalase (*CAT*) detoxifies the resultant H_2O_2 that is estimated to contribute >70% of drought-induced H_2O_2 (Noctor *et al.* 1998). *CAT* has a high Km value and therefore a lower efficiency for scavenging H_2O_2 at low concentrations than ascorbate peroxidase (mM rather than μM range). Thus, *CAT* is able to function as a bulk scavenger of H_2O_2 under stress (Huang 1983; Mittler 2002).

This study aims to investigate the effects of provenance, maternal parent and seed weight on seed germination rate, final percentage and early seedling biomass allocation and responses to drought, in terms of expression levels, for genes that code for antioxidant enzymes (superoxide dismutase, catalase, glutathione synthetase).

4.3 Materials and Methods

4.3.1 Seed provenances

Details of provenance site information, seed collection and storage conditions for provenances included in this study are presented in Table 4.3.1. Mean annual cumulative precipitation (ppn) was obtained from 1901 to 2015 from the CRU TS3.10 Dataset (Harris et al. 2014). Mean soil pF value (pF expresses the force with which different quantities of water are retained by the soil (Woodruff 1940)) in the summer month with the maximum mean pF value (July/August) from 1990 to 2015 were obtained from the European Drought Observatory (edo.jrc.ec.europa.eu/ on 20.02.15). Climatic water deficit values for these sites were obtained from the Chave et al. 2014 data layer. A family structure was recorded for the following provenances: Shieldaig, Pernitz, Jarocin, whereas the Spanish provenances (Sierra Nevada and Sierra de Baza) comprise more maternal individuals per population, though without a family structure.

Table 4.3.1 Populations included in the study with coordinates and mean altitude of the sampled sites within populations.Latitude: Lat.; Longitude: Lon. Decimal degrees: DD . Altitude: Alt. Metres above sea level: m a.s.l. Annual cumulative rainfall, climatic water deficit, and summer month soil moisture retention (pF) at provenance sites during the period 1901 to 2015. Mean seed weight was determined by weighing 100 seeds.

Provenance site	Lat. (DD)	Lon. (DD)	Alt. (m a.s.l)	Mean annual cumulative ppn (±SD)	Climatic water deficit (mm/yr)	Max mean soil water retention (pF) for summer month	Mean seed weight (g) (n=100, ± SE)
Shieldaig, Scotland	57.51	-5.64	81	1980 ± 244	0.00	2.43	0.0067 ±1.4e-04
Pernitz, Austria	47.91	16	500	1060 ± 136	-76.92	3.38	0.0071 ± 1.7e-04
Jarocin, Poland	51.97	17.48	120	540 ± 79	-212.87	2.7	0.0052 ±7.8e-05
Sierra Nevada, Spain	37.05	-3.27	1825	497 ± 119	-463.92	5.65	0.0111 ±2.2e-04
Sierra de Baza, Spain	37.25	-2.75	2010	497 ± 138	-604	5.55	0.01044 ±2.2e-04

4.3.2 Germination conditions and monitoring

The 7 week experiment (16.01.16 until 05.03.16) was conducted within a controlled environment glasshouse at the University of Edinburgh (UK) under supplementary lighting for diurnal cycles of 16 h light and 8 h darkness. Supplementary lighting automatically switched off when the external sensor measured light levels above 800 PAR. The light level at bench height was a minimum of 140 μmol $m^{-2}s^{-1}$. Relative humidity was 65% (day) and 50% (night), with a constant day/night temperature of 21˚C. One seed was sown per cell in 32 cell plug-trays with a propagator lid in Levingtons M3 pot and bedding high nutrient mix (Everris, Ipswich, UK). A randomized block design was used and blocks (10) rotated once a week to minimise variation attributable to block position within the glasshouse. Germination was monitored over 28 days, which is considered the upper limit for germination in *Pinus sylvestris* (Castro 1999; Zhu *et al.* 2006). Germination was defined as radicle emergence 1–2 mm in length from the tegument.

4.3.3 Experimental conditions and sampling

Two weeks before the experiment, the seedlings were re-potted two in a pot, into 7 x 7 x 8 pots with Levingtons M3 pot and bedding high nutrient mix (Everris, Ipswich, UK). This was to prevent restriction of root growth. The occurrence of damping off fungus approximately two weeks post emergence led to mortality of some seedlings and so a copper-based fungicide (Subdue, Fargro Ltd, UK) was applied as a protective measure on 12.02.16. At 5 weeks after the sowing date, emerged seedlings from a given provenance and/or half-sib family were randomly assigned either to drought stress or control irrigation regimes for 2 weeks.

The drought treatment consisted of a withdrawal of irrigation that resulted in a steep decline in soil water content (S Figure 1). During the experiment, 32 pots were weighed at 9 am on days 3, 7 and 14. At the end of the experiment, the pots were oven dried at 70 °C for 48 hours to obtain the dry weight. The pot weight was subtracted from the weight measurements. The gravimetric soil water content (θ_d) content (grams of water per gram of oven-dried soil) was calculated (Equation 3.3.1).

$$\theta_d = \frac{(\text{weight of wet soil}) - (\text{weight of dry soil})}{\text{weight of dry soil}}$$

Equation 4.3.1

Then volumetric water content (θ_{vd}) was calculated as follows, where θ_d = gravimetric soil water content, d_b = bulk density and dw = density of water:

$$\theta_{vd} = \theta_d \text{ x } \frac{d_b}{d_w}$$

Equation 4.3.2

4.3.4 Seedling biomass measurements

Total needle area was obtained by separating all needles from the shoot, placing on a white background and then using a flatbed scanner to scan the image. Image analysis of total needle area was conducted in ImageJ (Image-J 136b; NIH, Bethesda, Maryland, USA). Dry weight (DW) of needles, stem and roots were obtained after oven drying for 48 hours at 70 ˚C. Specific leaf area (SLA) was calculated as follows:

$$\text{SLA} = \frac{\text{Leaf area (cm}^2\text{)}}{\text{Leaf dry weight (g)}}$$

Entire root systems were washed and arranged so as to avoid overlapping lateral roots on a clear plastic tray with a white background and a marker of known size. Images

were taken with a digital camera and images were converted to binary and then analysed to obtain total root length and maximum root length using RootReader2D plugin (Clark *et al.* 2013) on ImageJ. Roots were oven dried at 70°C for 48 hours and weighed on a 4 decimal point balance to measure root dry weight and calculate specific root length (SRL, m g^{-1}).

At the end of the study, seedlings in the drought treatment were re-watered to confirm mortality.

4.3.5 Real-time PCR analysis of Superoxide Dismutase, Catalase and Glutathione synthetase transcript levels

Antioxidant enzyme genes selected for monitoring of gene expression were: superoxide dismutase (*SOD*; enzyme important for converting superoxide (O_2^-) to the less cytotoxic reactive oxygen species hydrogen peroxide (H_2O_2) and oxygen (O_2); catalase (*CAT*; an important enzyme for bulk scavenging of H_2O_2); and glutathione synthetase (*GS*; enzyme of the cellular glutathione biosynthetic pathway). Target gene expression was relative to the expression of endogenous reference genes β-actin (*ACT*) and glyseraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primers were synthesized by Sigma-Aldrich (Dorset, U.K).

Whole seedlings were sampled after 14 days of drought treatment by immediately freezing in liquid N_2 and were stored in aluminium foil packets in a -80˚C freezer. Total RNA was extracted from leaf tissue from a total of 68 seedlings. For each treatment (control and drought), 3 seedling were sampled per family for provenances: Shieldaig (2 families), Jarocin and Pernitz (3 families each), while 5 seedlings were sampled per provenance for: Sierra Nevada and Sierra de Baza. Tissue was frozen in

liquid N_2 and then 100 mg weighed into 1.75 ml Eppendorf tubes, into which two 4 mm steel beads were added. A TissueLyser II (Qiagen) grinder mill was used to homogenise tissue and the blocks were cooled in a -80 ˚C freezer for 30 min so that tissue would not thaw. A grinding pulse of 3 min at 30 Hz was applied, the blocks were returned to the -80 ˚C freezer for 10 min to ensure the tissue would not thaw, and then a second pulse of 3 min at 30 Hz was applied. Total RNA was extracted using Spectrum™ Plant Total RNA Kit (Sigma) following the manufacturer's protocol. RNA quality was assessed by checking for RNA degradation on ethidium bromide 1.5% agarose gel by electrophoresis (Appendix). RNA was stored at -80 °C prior to downstream PCR. Samples with degraded RNA were discarded.

First-strand cDNA was synthesized from <1 µg of total RNA. Reverse transcription was carried out in a 10µl reaction volume containing: 10 µM of dNTPs mixture, 0.5 µl of 100 µM oligo d(T), 5× RT buffer, 40 U/µl of RNase Inhibitor, and 50 U/µl of MMLV Reverse Transcriptase. Reactions were incubated at 85 °C for 5 min, held at 4 °C, then 25 °C for 5 min, 42 °C for 60 min, 70 °C for 10 min and held at 4 °C. The cDNA obtained was diluted by adding 50 µl of nuclease free water and then stored at −80°C.

Primers for the endogenous reference and target genes were pre-published: β-actin (*ACT*) and glyseraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers were developed by Vuosku *et al.* (2009); catalase (*CAT*) and glutathione synthetase (*GS*) primers were developed by Muilu-Mäkelä *et al.* (2015); and superoxide dismutase

(*SOD*) primers were developed by Karpinska *et al.* (2001). Oligonucleotides were synthesized by Sigma-Aldrich (Dorset, U.K).

The reaction volume for PCR was 10 µl and reagents are listed in S Table 1. Primer efficiency was verified using a 10 fold cDNA dilution series (with 1, 0.1, 0.01 and 0.001 dilutions) and specificity verified by melt curve analysis. A single peak was observed in the melting curve for all the primers. Relative expression levels were calculated using the $2^{-\Delta Ct}$ method, whereby Ct values of catalase were normalized to the geometric mean C_t of two stable reference genes (β-actin and *GAPDH*) (Vandesompele *et al.* 2002). Each gene per provenance was analysed in at least 3 biological replicates and two technical replicates (*i.e* each sample was run twice for the three target genes).

4.3.6 Statistical analysis

Germination data were analysed following the approach derived from survival analysis proposed by Onofri *et al.* (2010). Survival analysis enables analysis of the timing of events such as germination which occur over time, and to quantify the effects of contributing factors. Germination functions were first constructed for each provenance, by using the Kaplan–Meyer (KM) estimators (Eqn 1), as implemented in the function *survfit*. Then, accelerated failure time (AFT) regression modelling was implemented using the R function *survreg* to compare germination functions. Factors accelerating germination shift the germination rate curves to the left. The most appropriate distributional form for germination times was selected by using the Akaike Information Criterion (AIC; Akaike 1974) and it was always log-normal.

Analyses of variance (ANOVA) were used to test for effects of drought stress treatment, provenance, and maternal parentage. Spanish provenances were excluded from the ANOVA analysis that included maternal parentage as a fixed effect. Seed weight effects were analysed using general linear models. Block was included as a random effect covariate. Total, needle, stem and root biomass, biomass allocation (root:shoot ratio), SLA, needle number, total root length, maximum root length, SRL, and relative gene expression of *SOD*, *CAT* and *GS* were the response variables. Post-hoc pairwise comparisons were carried out using Tukey's HSD. Bivariate relationships between traits were analysed using Pearson correlation analysis. We used principal component analysis (PCA) on correlation matrix amongst variables followed by factor analysis to determine interrelationships among traits explaining variation among provenances and treatment groups. The traits included in the PCA were total seedling dry weight, seed weight, needle dry weight, needle number, root dry weight, SRL, max RL and TRL. R software version 3.2.2 was used for data analyses (R Core Team 2015).

4.4 Results

4.4.1 Germination

Of the 600 seeds initially sown, 465 successfully germinated (78%). The final germination proportion was fairly consistent across provenances, ranging from 71% (Jarocin) to 86% (Shieldaig). The rate of germination was affected by provenance, with the largest contrast being between Sierra de Baza (SN) (fast) and Jarocin (slow) (Figure 4.4.1). Seed weight included in the individual provenance regression models for time to germination increased model accuracy for Pernitz, Sierra Nevada and

Shieldaig (Table S4.6.2). Seed weight included in the individual maternal half-sib family regression models for time to germination increased model accuracy for Pernitz and Shieldaig (Table S4.6.3).

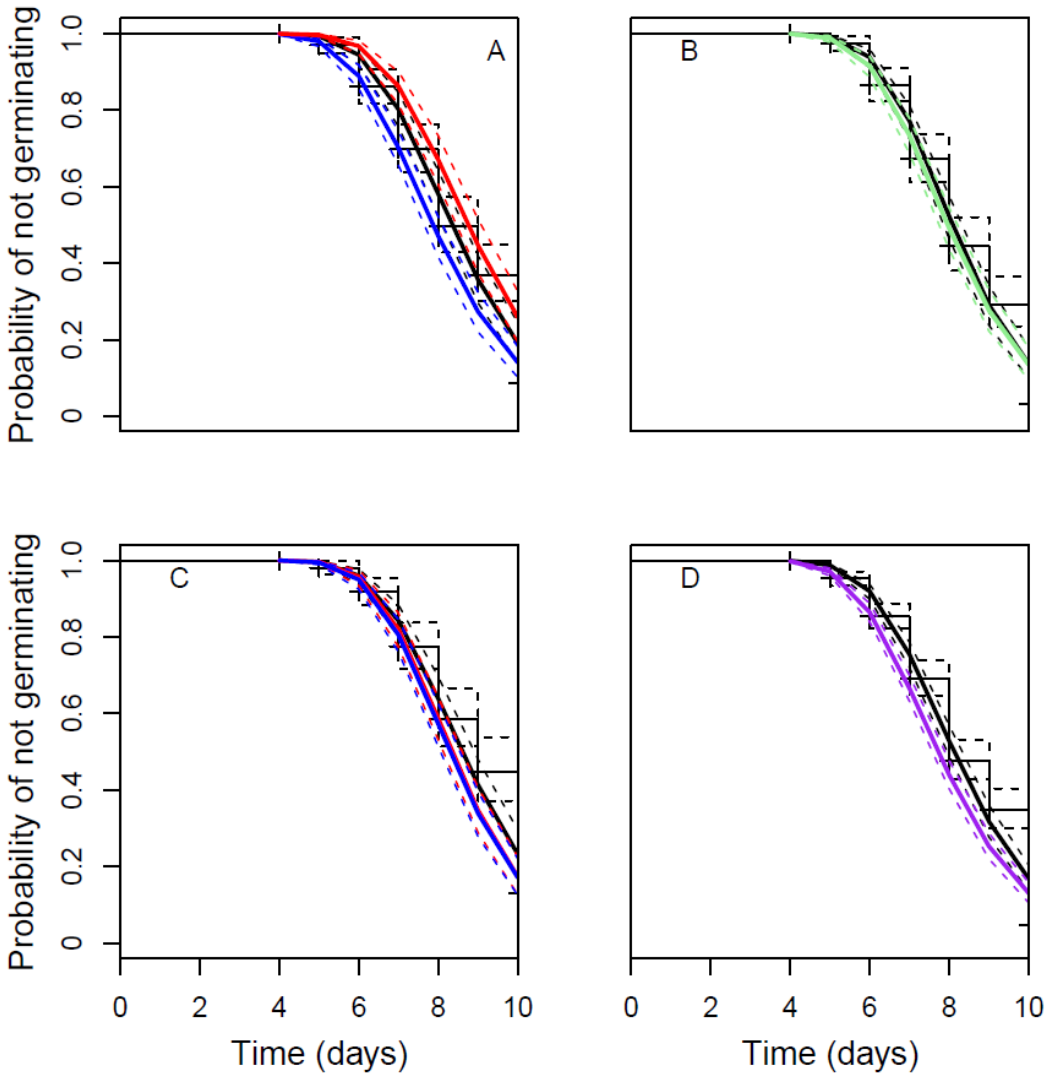

Figure 4.4.1 Regression models of time to germination for maternal parent families of provenances Pernitz (A), Shieldaig (B), and Jarocin (C). Sierra Nevada and Sierra de Baza are represented in (D) and do not have maternal parent structure.

4.4.2 Seedling biomass and allocation

The ANOVA analyses showed that all seedling biomass parameters were affected by the drought stress treatment compared to the control irrigation treatment (Table 4.4.1). In general, seedlings under the control irrigation treatment were significantly larger than seedlings under drought, with higher total, needle, stem and root biomass (Table 4.4.1; Fig 4.4.2A, C; S Figure 4.6.1A, B). Root to shoot ratio increased under drought treatment because needle biomass decreased more under drought than root biomass (Table 4.4.1; S Figure 4.6.1C). Seed weight and maternal parentage had an effect on total biomass independently of provenance (Table 4.4.1). There were also significant differences amongst provenances in biomass independently of seed weight. Seedlings from Sierra Nevada, Sierra de Baza and Pernitz showed higher needle, stem, root and total biomass than Jarocin and Shieldaig (Fig 4.4.2A; S Figure 4.6.1). Root to shoot ratio was generally lower in seedlings from Sierra de Baza than Sierra Nevada (S Figure 4.6.1C). Provenance had a significant effect on SLA and Tukey's HSD test revealed that the main difference was the lower SLA of the two Spanish provenances compared with Jarocin (Table 4.4.1). Additionally, there was a provenance seed weight interaction for root dry weight.

Table 4.4.1 Summary of ANOVA significance levels for the studied factors over response variables using data collected at day 14 of the drought stress experiment. Dry weight: DW; Specific leaf area: SLA; Specific root length: SRL; Maximum root length: Max RL. . Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

		Total DW		**Needles DW**		**Root DW**		**Stem DW**		**Root:shoot**		**SLA**		**Needle nb**		**SRL**		**Max RL**	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment (T)	1	**73.8**	**<0.001 *** **	70.1	**<0.001 *** **	21.7	**<0.001 *** **	**16.6**	**<0.001 *** **	**7.9**	**0.006 ** **	Na	Na	**195.4**	**<0.001 *** **	**5.4**	**0.02 * **	**18.23**	**0.0001 *** **
Provenance (P)	4	**11.0**	**<0.001 *** **	**7.8**	**<0.001 *** **	**7.6**	**<0.001 *** **	**4.9**	**0.0015 ** **	**2.4**	**0.057 . **	**5.79**	**0.02 * **	**4.7**	**0.003 ** **	2.0	0.11	**2.95**	**0.033 * **
Seed weight (SW)	1	**42.5**	**<0.001 *** **	**33.6**	**<0.001 *** **	**15.5**	**<0.001 *** **	**18.6**	**<0.001 *** **	0.005	0.93	**116.5**	**<0.001 *** **	**7.84**	**0.007 ** **	**4.3**	**0.049 * **	0.42	0.51
T x P	4	**3.6**	**0.01 * **	1.1	0.35	1.5	0.21	**3.4**	**0.013 * **	1.6	0.16	Na	Na	**5.43**	**0.002 ** **	1.4	0.23	**2.1**	**0.09 . **

Table 4.4.2 Summary of ANOVA significance levels for the provenances with maternal parent recorded (Shieldaig, Pernitz and Jarocin) over response variables using data collected at day 14 of the drought stress experiment. Dry weight: DW; Specific leaf area: SLA; Specific root length: SRL; Maximum root length: Max RL. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

		Total DW		**Needle DW**		**Root DW**		**Stem DW**		**R:S**		**SLA**		**Needle nb**		**SRL**		**Max RL**	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Maternal parent (M)	4	**6.14**	**<0.001 *** **	**5.4**	**0.002 ** **	**3.5**	**0.018 *** **	1.4	0.23	0.7	0.56	**13.38**	**0.002 ** **	**9.7**	**<0.001 *** **	0.98	0.46	**5.3**	**0.0019 ** **
Treatment (T) x M	3	1.02	0.39	0.9	0.44	**2.7**	**0.06 .**	0.84	0.47	1.4	0.24	Na	Na	2.3	0.119	0.14	0.86	0.3	0.81

There were significant interactions between provenance and drought on total biomass, with a higher drought effect found for seedlings from Pernitz and Sierra de Baza as compared with the other provenances. Needle number decreased under drought stress and there was provenance interaction, with all provenances showing a significant decrease except for Jarocin (Figure 4.4.2B). A drought effect on stem dry weight and maximum root length was only found for seedlings from Pernitz (Figure 4.4.2C, D).

Maternal parentage had a significant effect on total biomass for Pernitz, with higher needle number from maternal parents 6 and 10 as compared with maternal parent 5, which has a lower mean seed weight (Figure S4.6.3). Needle biomass and total biomass of seedlings from maternal parent 10 was also higher compared with maternal parent 5, whereas SRL was higher in seedlings from maternal parent 6 compared with maternal parent 5. There was a significant maternal parentage interaction with drought for root dry weight (Table 4.4.2), indicating that maternal parent 10 of half-sib seedlings from Pernitz was most affected by the drought treatment.

A significant effect of seed weight independent of provenance and maternal parentage was found for all biomass traits except for root to shoot ratio and maximum root length. Seed weight was strongly determined by provenance (ANOVA, F: 1425, $p<0.001$) and maternal parentage (ANOVA, F: 72, $p<0.001$), as well as their interaction (ANOVA, F: 7.3, $p=0.006$). Time to germination had an effect on the number of needles, needle and total biomass, as well as a treatment interaction effect on root dry weight (Table S 4.6.4). However, other variables were not influenced by time to germination.

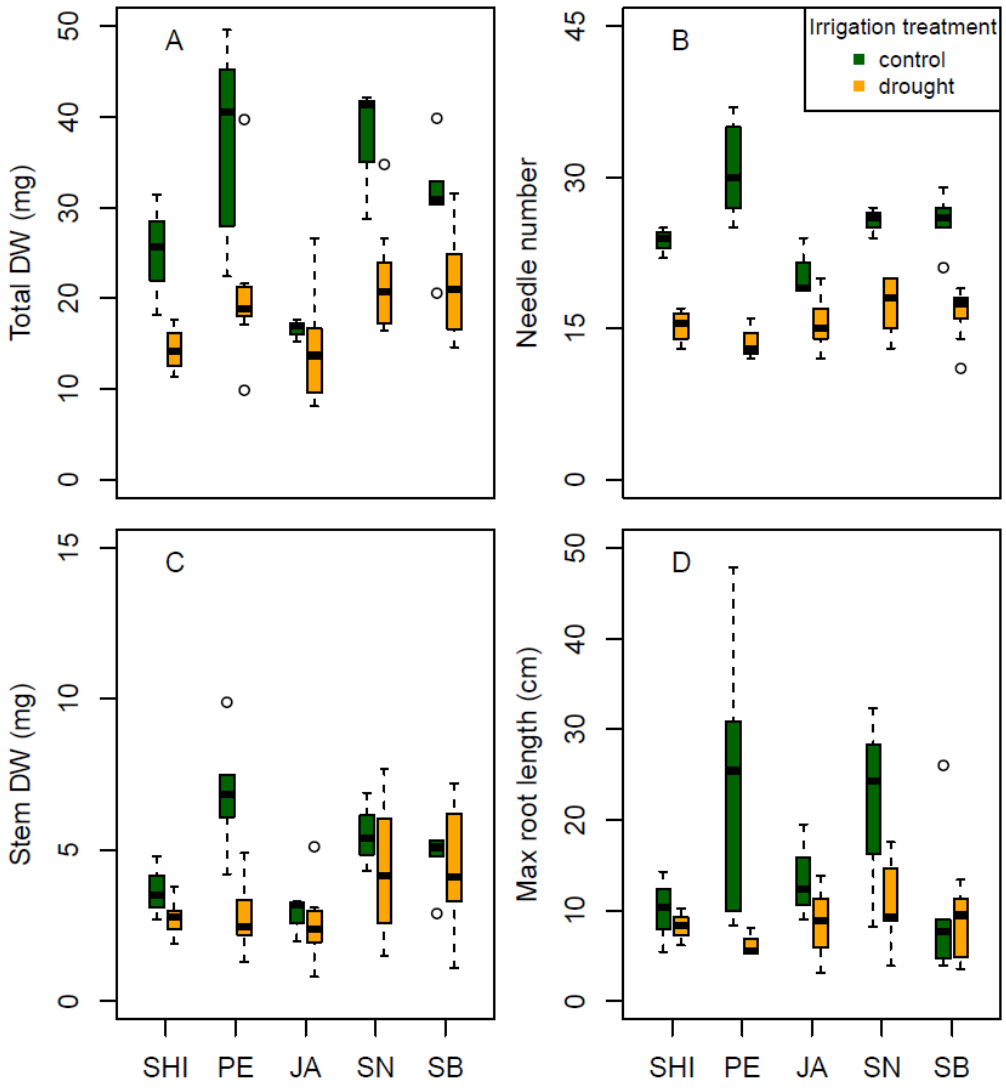

Figure 4.4.2 Total dry weight (mg) (A), Needle number (B), Stem dry weight (C), and maximum root length (cm) of *Pinus sylvestris* seedlings from different provenances following: (a) 14 days of drought stress or normal irrigation (control). Provenances are: Shieldaig, Scotland (SHI), Pernitz, Austria (PER), Jarocin, Poland (JAR), Sierra Nevada, Spain (SN) and Sierra de Baza (SB). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

4.4.3 Relative gene expression of SOD, CAT and GS

Relative to reference genes, expression levels of target genes was in the order *CAT>SOD>GS* across both control and drought treatment. Under drought stress, *SOD* were upregulated relative to reference genes (Figure 4.4.3, 4.4.4), but *CAT* expression remained stable (Figure S4.6.2). While *GS* remained at a lower expression level than the reference genes, expression was upregulated under drought. However, all of the antioxidant enzyme genes showed low expression profiles relative to reference genes. There were no significant effects of provenance and maternal parentage on relative gene expression, though seed weight had a marginally significant effect on *SOD* expression (Table 4.4.3).

Table 4.4.3 Summary of ANOVA significances for the fixed factors (drought treatment, provenance, maternal parent) over the response variables (gene expression) using data collected on data 14 of the drought stress experiment. Target gene expression relative to endogenous reference genes (β-actin and *GAPDH)* . *SOD*: superoxide dismutase; CAT: catalase; GS: glutathione synthetase. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: **.**

		SOD		***CAT***		***GS***	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*
Treatment (T)	1	**3.99**	**0.05 .**	0.03	0.84	**10.3**	**0.006 ***
Provenance (P)	4	0.66	0.62	0.64	0.63	1.6	0.22
Maternal parent (M)	4	1.1	0.37	0.77	0.59	0.79	0.64
Seed weight (SW)	1	3.4	**0.07 .**	0.02	0.88	1.2	0.3
T x P	4	2.0	0.12	0.32	0.86	0.024	0.97
T x M	3	0.77	0.5	0.37	0.77	0.62	0.45

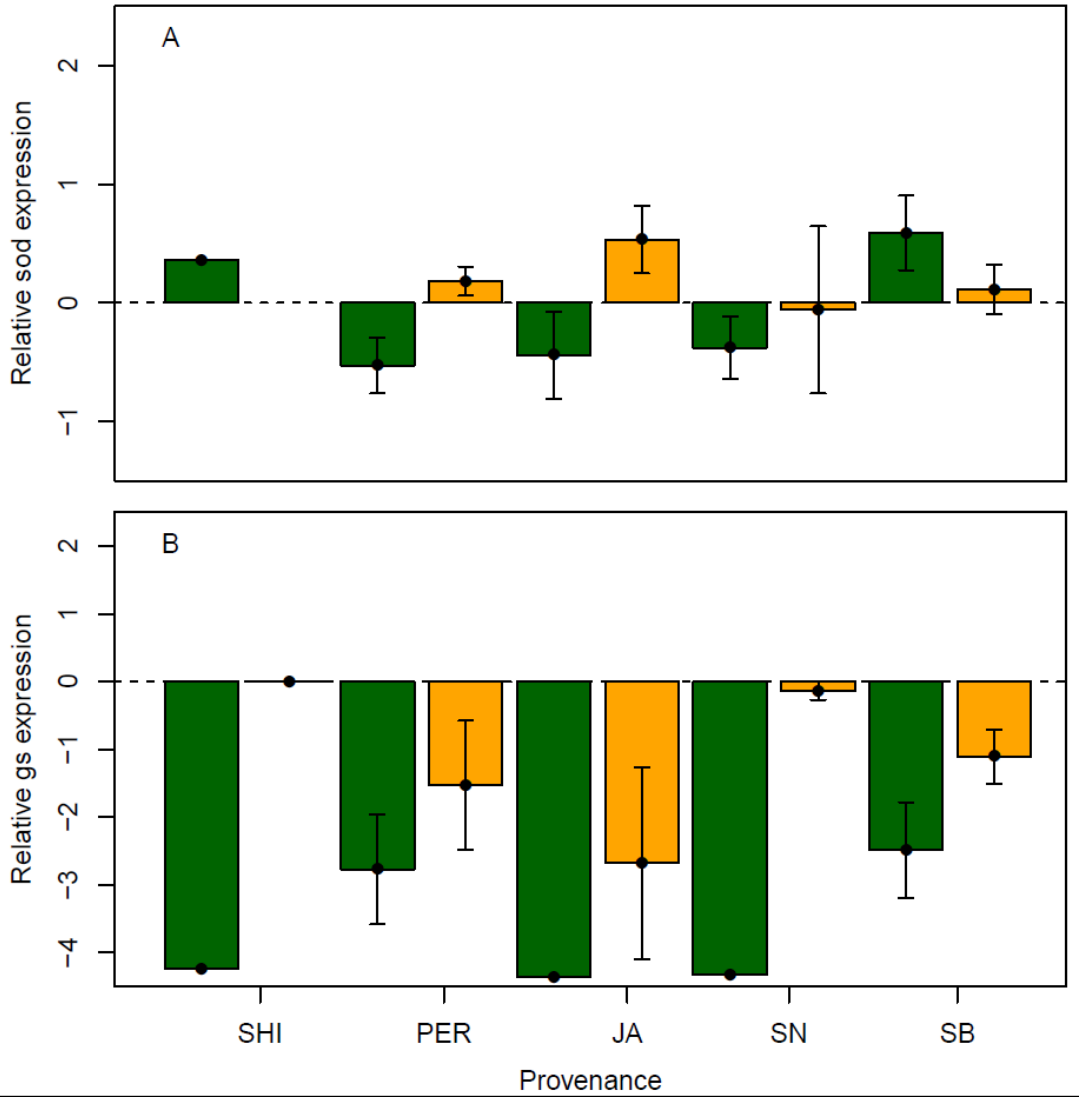

Figure 4.4.3 Relative expression of cytosolic superoxide dismutase (*SOD*) and glutathione synthetase (*GS*) to endogenous reference genes β-actin (*ACT*) and glyseraldehyde-3-phosphate dehydrogenase (*GAPDH*). Provenances are: Shieldaig, Scotland (SHI), Pernitz, Austria (PER), Jarocin, Poland (JAR), Sierra Nevada, Spain (SN) and Sierra de Baza (SB). Bars represent mean values ± standard error. Green bars: control treatment; orange bars: drought treatment.

4.4.4 Relationships among trait variables

The first and second axis of the PCA (eigenvalue > 1) together explained 74 % of the trait variation. The first component (PC1) explained 54 % of the total variance. There is a separation of groups on PC1 according to treatment, with the drought group positioned to the left and control to the right. Total dry weight was the variable that contributed the most to PC1. The second component (PC2) explained 15% of the total variance. Seed weight was the variable that contributed the most to PC2. According to PC1, a group constituted by the Spanish provenances was defined by higher seed weight, high allocation to stem, and higher root to shoot ratio.

Significant correlations between variables are presented in Table 4.4.4. Under drought conditions, root dry weight was positively correlated with seed weight, total dry weight and the maximum root length. Meanwhile, root to shoot ratio was positively correlated with stem dry weight and negatively correlated with needle dry weight.Under control conditions, seed weight was positively correlated with needle number and negatively correlated with specific leaf area, whileroot to shoot ratio was positively correlated with maximum root length, stem and root dry weight and SLA.

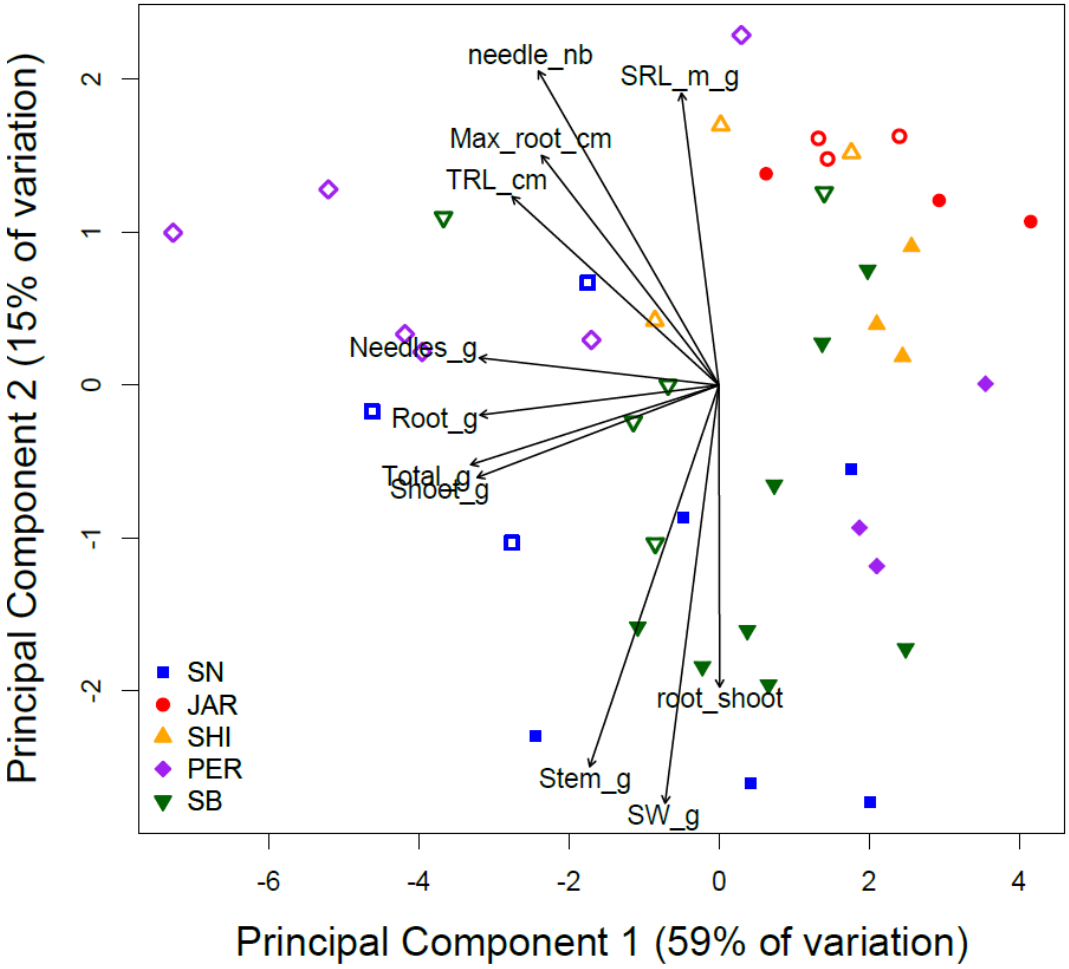

Figure 4.4.4 Principal components 1 and 2 for the seedling biomass traits. Open symbols represent control treatment and closed symbols represent drought treatment. Arrows show loadings of traits to the principal components. Provenances are: Shieldaig (SHI), Pernitz (PER), Jarocin (JAR), Sierra Nevada (SN), and Sierra de Baza (SB).

Table 4.4.4 Significance of Pearson's correlations between seed weight (SW), maximum root length (Max root), specific root length (SRL), total dry weight, needle dry weight, stem dry weight, root to shoot dry weight ratio (R:S), needle number and specific leaf area (SLA) of the five provenances, for the control treatment (below grey diagonal) and drought treatment (above grey diagonal). The correlation coefficient ± the standard error of the simulated values is reported. Significant p-values are highlighted in bold.

	SW	**Max root**	**SRL**	**Total DW**	**Needle DW**	**Stem DW**	**Root DW**	**R:S**	**#Needle**
SW		0.24, 0.26	-0.28, 0.35	**0.58, <0.001**	**0.45, <0.001**	**0.46, <0.001**	**0.42, <0.001**	0.07, 0.55	0.31, 0.14
Max root	r:0.005, p=0.98		0.31, 0.15	**0.572, 0.005**	**0.578, 0.004**	**0.409, 0.05**	**0.52, 0.012**	-0.01, 0.96	**0.61, <0.001**
SRL	-0.21, 0.35	0.02, 0.91		-0.04, 0.84	-0.03, 0.88	-0.08, 0.7	-0.02, 0.92	-0.03, 0.86	0.07, 0.72
Total DW	**0.62, 0.003**	**0.59, 0.005**	0.03, 0.89		**0.95, <0.001**	**0.515, <0.001**	**0.58, <0.001**	-0.2, 0.12	**0.57, 0.005**
Needle DW	**0.70, <0.001**	**0.47, 0.03**	-0.04, 0.85	**0.97, <0.001**		**0.31, 0.014**	**0.44, <0.001**	**-0.46, <0.001**	**0.65, <0.001**
Stem DW	**0.43, 0.05**	**0.45, 0.04**	0.32, 0.15	**0.83, <0.001**	**0.72, <0.001**		-0.009, 0.94	**0.29, 0.02**	0.33, 0.13
Root DW	0.36, 0.11	**0.82, <0.001**	0.002, 0.99	**0.87, <0.001**	**0.75, <0.001**	**0.71, <0.001**		0.20, 0.12	**0.409, 0.05**
R:S	-0.32, 0.16	**0.53, 0.01**	0.22, 0.33	0.14, 0.54	-0.09, 0.70	**0.44, 0.05**	**0.48, 0.03**		-0.2, 0.3
#Needle	**0.44, 0.04**	**0.61, 0.004**	0.16, 0.49	**0.86, <0.001**	**0.84, <0.001**	**0.75, <0.001**	**0.71, <0.001**	0.09, 0.68	
SLA	**-0.67, <0.001**	0.06, 0.79	0.105, 0.65	**-0.52, 0.017**	**-0.6, 0.005**	-0.33, 0.14	-0.3, 0.19	**0.48, 0.03**	**0.71, <0.001**

4.5 Discussion

Previous research has shown that provenances have different seed weight and differential ability to tolerate drought (Reich *et al.* 1994; Matias and Jump 2014). This seems to be related to increased total seedling biomass and preferential allocation to roots for southern populations. This study aimed to test whether provenances exert an effect on the seedling drought response independently of the effects of seed weight by using maternal half-sib families and weighing seeds individually. It was also hypothesised that expression of antioxidant enzymes that also help to combat drought stress might show provenance differences. Seedlings of different provenances were differentially impacted by drought and part, but not all, of this differential response was driven by provenance level differences in seed weight. Germination rate was higher for seeds of the Spanish provenances at the xeric range edge and the Austrian provenance compared with the wetter central and northern populations. However, these provenance differences were not evident in the expression of the antioxidant enzymes. Although superoxide dismutase and glutathione synthetase showed increases in expression under drought versus control, seedlings from different provenances or maternal parents did not differ in their expression level.

4.5.1 Germination and seed weight

The germination rate and proportion of Sierra de Baza seeds was higher than for Jarocin; these provenances also differed in terms of seed weight class, since Sierra de Baza mean seed weight (0.0104) was twice that of Jarocin (0.0052). Furthermore, seed weight added to the regression model fit indicated that intra-population variation in seed weight had a significant effect on germination rate. This suggests that increased seed weight accelerates time to germination. Indeed, seed weight has been found to

affect the timing of germination and early seedling growth in Spanish provenances (Castro 1999). This study extends this finding to provenances from central and northern parts of the *P. sylvestris* range.

Seed weight was found to significantly affect shoot dry weight and number of needles, independently of provenance and maternal parentage. Higher seed weight is correlated with improved early seedling growth performance in many *Pinus* species (Maltoni & Tani 1997; Blade *et al.* 2008; Correia *et al.* 2014) including *P. sylvestris* (Reich *et al.* 1994; Castro 1999; Wennstrom *et al.* 2002) and thus might represent an important aspect of local adaptation to a drier environment. Across the range of *P. sylvestris*, higher seed weight classes have been observed from southern populations (Reich *et al.* 1994). The higher impact of drought on root dry weight for seedlings of maternal parent 10 may be because of the higher potential for growth in these seedlings under control conditions. Similarly, a positive relationship between mean provenance seed weight and the number of cotyledons, though not root length, was identified across ten provenances of *P. sylvestris* from the southwestern to the central part of the range (Taeger *et al.* 2013). However, as seed weight was only analysed as a mean value for each provenance in Taeger *et al.* (2013), it is difficult to compare these findings to other studies analysing seed weight effects within populations and families (Castro 1999; Castro *et al.* 2008). In this study, seed weight and timing of germination was determined by provenance and maternal parentage as well as their interaction. However, root to shoot biomass allocation and maximum root length depended on provenance and maternal effects that were not mediated by seed weight effects, congruent with findings for *Pinus pinaster* (Zas *et al.* 2013).

4.5.2 Seedling biomass

Seedling total biomass decreased under drought, though root to shoot ratio increased because of a drought effect decreasing needle rather than stem biomass (Figure S4.6.1, Figure 4.4.2). This suggests prioritisation of stem and root tissue over needles under drought. Previously it has been shown that there may be evidence of local adaptation in root investment, with Spanish provenances showing significantly higher root system development under drought than northern Finnish provenances (Matias & Jump 2014). In this study, there was no interaction of treatment with provenance on root dry weight, but maximum root length was only significantly lower under drought for Pernitz.

Earlier germination time significantly increased seedling needle number and needle biomass and therefore total seedling biomass. The effect of increased seedling size with earlier germination time did not lead to an increase in root dry weight under control conditions; however, there was an interaction effect with drought indicating that earlier germination may enhance root biomass. However, this effect was not found for root to shoot ratio, maximum root length or specific root length. Date of emergence has been linked to improved seedling establishment by better shoot growth and survival of summer drought in Spanish populations of *P. sylvestris* (Castro *et al.* 2006). In this study, a maternal parentage effect detected for provenance Pernitz on total biomass and SRL seems related to seed weight, since maternal parent 5 produced a lower mean seed weight and seedling biomass. Thus, faster germination owing to higher seed weight might one of the reasons seedlings achieve larger seedling sizes.

4.5.3 Antioxidant gene expression

Two of the three genes related to the antioxidant stress response showed higher relative expression profiles under drought. Cytosolic *SOD* gene expression increased significantly under drought treatment. Similarly, upregulation of cytosolic *SOD* in *Nicotiana tabacum* and increased total *SOD* activity in *Phoenix dactylifera* has been reported under drought by water withdrawal (Faize *et al.* 2011; Benhiba *et al.* 2015). *SOD* levels increasing in response to drought might be explained by its role in preventing damage to photosynthetic apparatus under drought stress; specifically averting free radical activity at photosystem I by conversion to a less cytotoxic reactive oxygen species (Cruz de Carvalho 2008).

Although in this study there appeared to be an effect of drought on *GS* expression, the level of expression remained lower than that of reference genes indicating that the glutathione synthesis pathway was only marginally increased under drought and may not be functionally significant. Catalase is an important enzyme involved in detoxifying the reactive oxygen species generated during photorespiration, which increases under drought conditions of stomatal closure (Noctor *et al.* 1998; Guan & Scandalios 2000). However, in this study there was no detectable increase in *CAT* expression under drought. Similarly, in the grass species *Poa pratensis* and *Festuca arundinacea*, no difference in *CAT* transcript levels was found under drought treatment (Fu & Huang 2001; Bian & Jiang 2009). Muilu-Makela *et al.* (2015) also found that *CAT* and *GS* gene expression was unresponsive to drought in *Pinus sylvestris* needles. This is perhaps unexpected given previous research that suggests a key role of catalase in detoxifying approximately 70% of reactive oxygen species produced during

photorespiration under drought stress (Nocter *et al.* 2002). The co-ordinated response of components of antioxidant defence at different stages of drought stress might account for this. Thus, further research direction would benefit from time series experiments that monitor changing expression levels of antioxidant enzymes and enable better evaluation of the integrated plant response.

The role of non-structural (NSC) supply in mounting a drought stress response is supported by research indicating that there are active signalling networks that react to levels of NSC. Sugars have a central position in relation to major reactive oxygen species (ROS)-producing processes (for review see Couée *et al.* 2006). For example, application of the herbicide atrazine to *Arabidopsis* seedlings initiates the production of singlet oxygen, yet expression of the antioxidant enzyme superoxide dismutase (*SOD)* is enhanced in combination with sugar treatment rather than atrazine treatment alone (Sulmon *et al.* 2006). Conversely, sucrose starvation triggers activation of oxidative stress genes, including catalase (Contento *et al.* 2004).

4.5.4 Conclusions

Seedling drought responses involved biomass reduction and varied according to provenance, with seedlings from Austria being most affected by drought in terms of reduced total biomass, as well as maximum root length. Meanwhile, seedlings from the Spanish provenance showed higher seed weight, high allocation to stem, and higher root to shoot ratio. Disparity in the early seedling biomass allocation and drought stress response was also attributable to variation at the maternal parent level in terms of investment in seed weight. Although *SOD* and *GS* expression level increased under

drought, no provenance effect was found in gene expression of the antioxidant enzymes studied. An expression profile over time might better facilitate detection of provenance differentiation in antioxidant defence.

4.6 Supplementary material

Table S4.6.1 Reagents for real-time PCR reactions.

Reagents	**µl**
SYBER green mix (2X)	5
Forward primer	0.5
Reverse primer	0.5
Nuclease free water	3
Master mix total volume	9
cDNA (sample) or water (negative control)	1
Total (reaction volume)	**10**

Table S4.6.2 Akaike Information Criterion (AIC) scores for germination regression models with seed weight and maternal parentage across the five provenances. All regression models were fitted using a log normal distribution.

Provenance	**Regression model**	**AIC**
Pernitz	(Time, germ) ~ 1,	2052.3
Pernitz	(Time, germ) ~ seed weight (SW)	1984.6
Pernitz	(Time, germ) ~ maternal parent (M)	2039.89
Jarocin	(Time, germ) ~ 1	1747.98
Jarocin	(Time, germ) ~ SW	1765.75
Jarocin	(Time, germ) ~ M	1749.0
Shieldaig	(Time, germ) ~ 1	1488.36
Shieldaig	(Time, germ) ~ SW	1483.37
Shieldaig	(Time, germ) ~ M	1489.5
Sierra Nevada	(Time, germ) ~ 1	1349.4
Sierra Nevada	(Time, germ) ~ SW	1346.92
Sierra de Baza	(Time, germ) ~ 1	1650.614
Sierra de Baza	(Time, germ) ~ SW	1651.8

Table S4.6.3 Akaike Information Criterion (AIC) scores for germination regression models with seed weight across the maternal half-sib families for provenances Pernitz, Jarocin and Shieldaig. All regression models were fitted using a log normal distribution.

Regression model	**Provenance**	**Maternal parent**	**AIC**
(Time, germ) ~ 1	Pernitz	5	671.9
	Pernitz	6	781.2
	Pernitz	10	590.1
	Jarocin	1	538.4
	Jarocin	2	584.0
	Jarocin	4	629.9
	Shieldaig	7	726.1
	Shieldaig	8	764.6
(Time, germ) ~ seed	Pernitz	5	649.8
weight	Pernitz	6	770.9
	Pernitz	10	557.2
	Jarocin	1	540.1
	Jarocin	2	585.0
	Jarocin	4	624.6
	Shieldaig	7	724.2
	Shieldaig	8	762.7

Table S4.6.4 Summary of ANOVA significances for time to germination and interaction with treatment effects on seedling biomass traits. Significance codes for p-values: p<0.001: ***, p= 0.001: **, p = 0.01: *, p= 0.05: .

		Total DW		**Needles DW**		**Root DW**		**Stem DW**		**R:S**		**SLA**		**#Needle**		**SRL**		**Max RL**	
	d.f	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Time to germination (G)	1	**8.7**	**0.004** **	**8.8**	**0.004** **	1.6	0.2	2.2	0.13	1.0	0.31	0.05	0.8	**4.77**	**0.035** *	0.04	0.83	0.2	0.63
Treatment (T) x G	1	**9.1**	**0.003**	**9.6**	**0.002** **	**3.6**	**0.06** .	1.8	0.18	0.16	0.68	--	--	**6.95**	**0.01** *	0.71	0.4	0.009	0.92

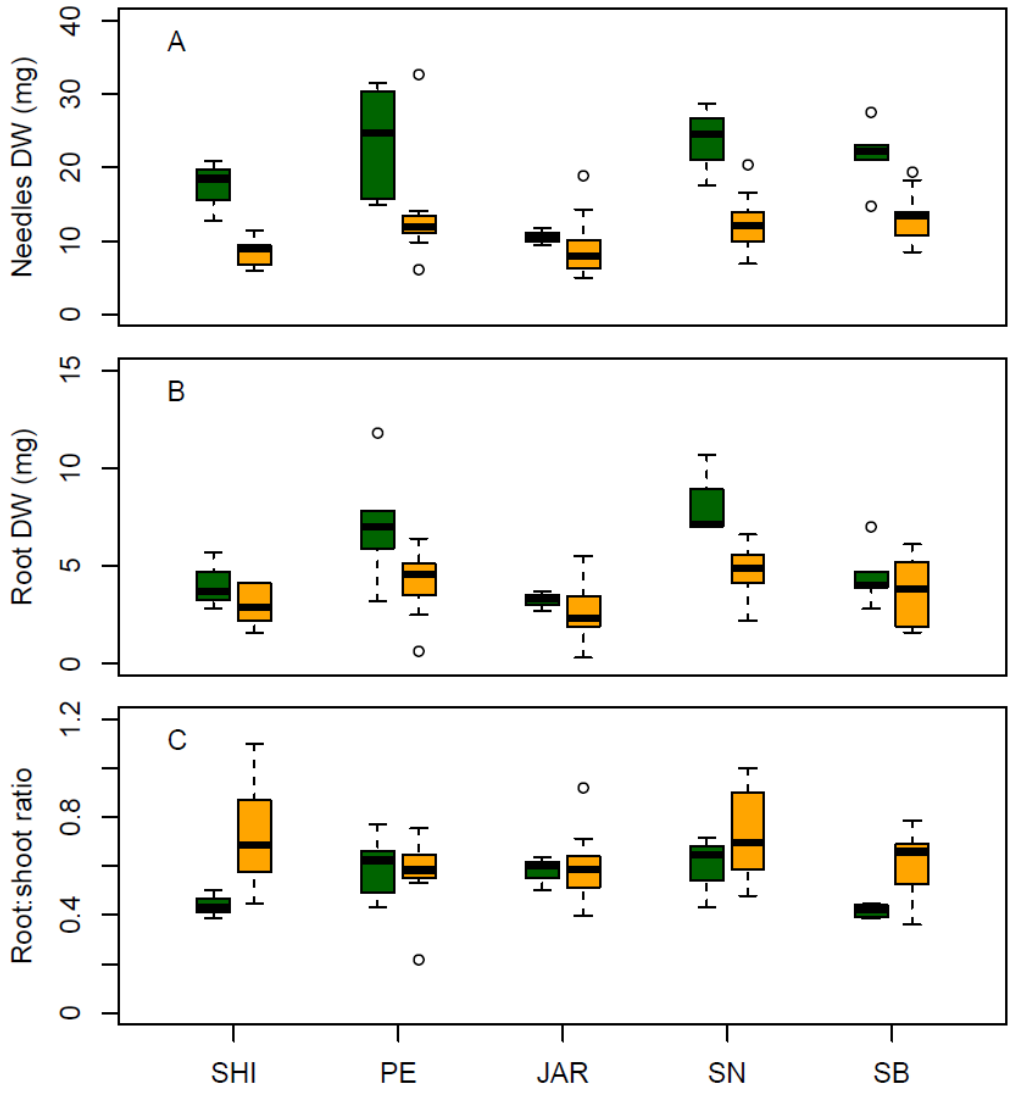

Figure S4.6.1 Needle dry weight (mg) (A), root dry weight (mg) (B), and root to shoot dry weight ratio (C) of *Pinus sylvestris* seedlings from different provenances following: (a) 14 days of drought stress or normal irrigation (control). Provenances are: Shieldaig, Scotland (SHI), Pernitz, Austria (PER), Jarocin, Poland (JAR), Sierra Nevada, Spain (SN) and Sierra de Baza (SB). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%) and the minimum and maximum values. Whiskers represent the most extreme data point that is no more than the range multiplied by the interquartile range from the box.

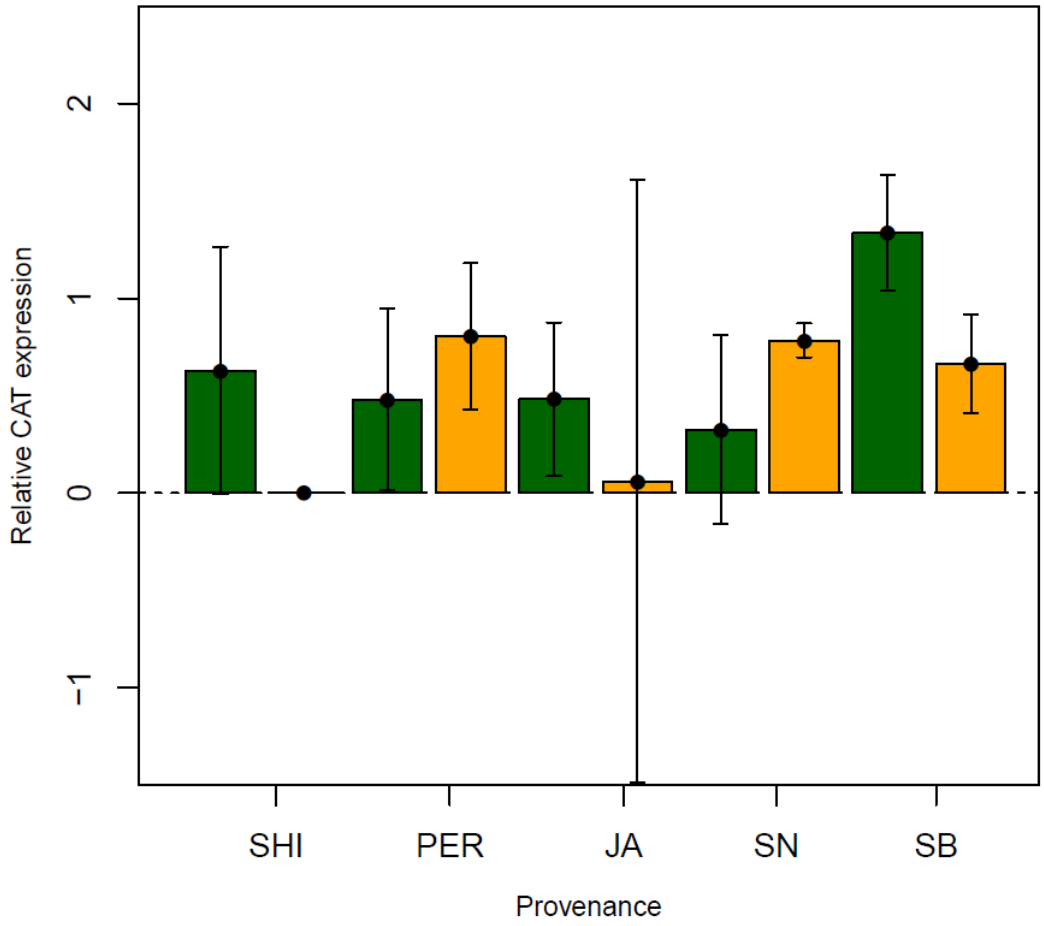

Figure S4.6.2 Relative expression of catalase (*CAT*) to endogenous reference genes β-actin (*ACT*) and glyseraldehyde-3-phosphate dehydrogenase (*GAPDH*). Provenances are: Shieldaig, Scotland (SHI), Pernitz, Austria (PER), Jarocin, Poland (JAR), Sierra Nevada, Spain (SN) and Sierra de Baza (SB). Bars represent mean values ± standard error.

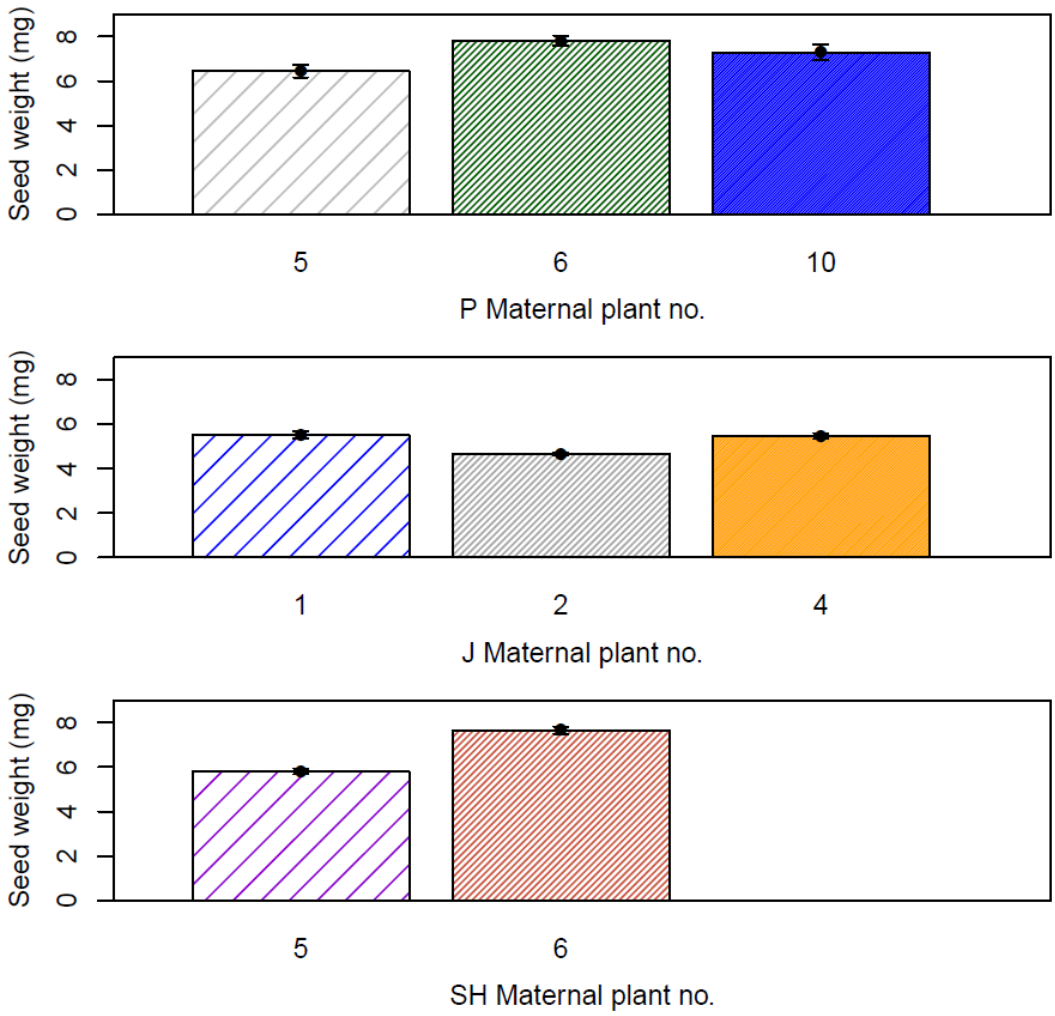

Figure S4.6.3 Seed weights for maternal half-sib families for provenances Pernitz (P), Jarocin (J) and Shieldaig (S). Bars represent mean values ± standard error.

5 General Discussion

Forest dieback accelerated by drought and heat at a regional scale has been documented in all major forest biomes (Allen *et al.* 2010) and is expected to worsen as anthropogenic climate change brings the risk of changed precipitation patterns and temperature extremes that are particularly expected to increase drought stress across forest ecosystems (IPCC 2007, 2014). Widespread tree mortality can have a strong positive feedback on local and regional climate, carbon dynamics and nutrient cycling (Anderson *et al.* 2011, Anderegg *et al.* 2013), as well as shifting plant community composition (Suarez *et al.* 2008, Kane *et al.* 2011). Dynamic global vegetation models (DGVMs) attempt to simulate, using ecophysiological principles, the effects of changing environmental conditions on plant functional types as well as the knock-on effects on ecosystem level biogeochemical and hydrological cycles and vegetation composition (Cramer *et al.* 2001, Prentice *et al.* 2007, Bonan *et al.* 2008). There is a recognised need to integrate process-based sub-models of tree mortality into DGVMs (McDowell *et al*. 2013), as well as other aspects of demography such as seedling recruitment (Snell *et al*. 2014).

The overarching aim of this thesis was to examine the effects that a change in drought timing and severity may have on seedling responses of *Pinus sylvestris*. The focus here is on the important recruitment bottleneck of early seedling establishment, characterised by high seedling mortality rates.

There is evidence supporting a capacity for local adaptation in *P. sylvestris* populations for various traits at the level of physiology and phenology (Oleksyn *et al.* 2002; Andersson & Fedorkov 2004; Notivol *et al.* 2007; Chmura *et al.* 2012). This is in common with many forest tree species that span extensive ranges encompassing heterogeneous environmental conditions (Aitken *et al.* 2008). At the seedling level in *P. sylvestris*, the role of local adaptation to drought is suggested from provenance experiments that have shown root growth is typically higher in seedlings from the xeric range edge of the species (Matias and Jump 2014; Taeger *et al.* 2015). However, the physiological susceptibility of seedlings to drought-induced mortality may not only involve changes in growth but also shifts in metabolic activity, for example, related to osmoregulation and antioxidant defences. Therefore, this study investigated provenance variation in seedling responses to drought in terms of changing biomass allocation, antioxidant gene expression and metabolite accumulation. By examining responses across provenances where summer drought is or is not a factor in the regeneration niche, these experiments aimed to test for adaptive variation in drought responses.

In this discussion chapter, the main conclusions drawn from the experiments are highlighted and framed in the wider context of drought effects on the *Pinus sylvestris* regeneration niche. First the seed investment, early seedling growth characteristics and drought stress responses for each of the four provenances will be summarised. It will be suggested that in the regeneration niche of *P. sylvestris* at its xeric range edge, local adaptation is responsible for the observed differentiation between seedlings from Spain and other provenances. The findings of the metabolomics study will then be

considered in light of the potential relevance to drought tolerance of *P. sylvestris* seedlings. Future directions for research will be suggested to include a more ecological focus, which takes into account biotic interactions, both stressful and beneficial. Finally, some general conclusions will be given for the thesis.

5.1 The regeneration niche under drought

5.1.1 Seed investment, timing of germination and seedling biomass allocation

The third experiment suggested an effect of provenance on maternal investment in seed weight as provenances differed in terms of seed weight class; typically the Spanish provenances (Sierra de Baza and Sierra Nevada) had higher seed weights. This is in agreement with findings of other studies of seed weight variation across the European range of *P. sylvestris* (Reich *et al.* 1994). Timing of seedling emergence has been shown to have an effect of enhancing seedling growth and performance under drought for a Spanish population (Castro *et al.* 2005). In this experiment, maternal parentage (if known) and seed weight improved the regression model fit of germination rate curves for all provenances except Jarocin. These findings suggest that intra-population variation in seed weight has an effect on timing of germination and potentially seedling emergence for Shieldaig and Pernitz as well as the two Spanish provenances. The effect of seed weight variation within individual maternal parent trees also had a strong effect on germination rate for the three provenances where maternal parent identity was recorded. However, Pernitz was the only provenance to

show effects of maternal parentage on seedling traits, probably owing to the higher mean seed weight produced by one maternal parent.

In the first experiment, a positive correlation was found between mean seed weight and survivorship of osmotic stress, reflecting the adaptive value of higher seed weight during initial seedling establishment. A trade-off in maternal fitness between producing fewer larger seeds and many smaller seeds is described in an optimal allocation model by Smith and Fretwell (1974). The model indicates that maternal fitness is balanced with offspring fitness by investing similarly across seeds. Zas *et al.* (2015) found that the maternal effect on seedling performance was strongly related to seed provisioning. The variation in seed weight for clonal genotype maternal parents was related to the environmental conditions of the seed orchard, with the greatest variation in seed weight under the more unfavourable site conditions of low winter temperatures, shallow soils and high winds. Hence, the conditions in which the maternal parent trees are growing in Pernitz as compared with Shieldaig and Jarocin might be more unfavourable, perhaps owing to altitudinal effects or the lower available soil water (as indicated by soil water retention values).

Under control conditions Sierra Nevada seedlings invested significantly more biomass in the root system than northern and central provenances, Rothiemurchus and Jarocin. There was also a positive correlation between soil moisture availability and root:shoot ratio across provenances. Higher root development of seedlings is a crucial early adaptation to drought (Markesteijn & Poorter 2009). However, the duration and intensity of drought stress is critical in determining seedling biomass allocation under

these conditions. Under moderate drought stress, it is possible for plants to sustain their aboveground growth and thus their competitiveness, though severe drought stress often manifests in an increase in the root biomass fraction at the expense of stem biomass (Poorter *et al.* 2012). In all experiments, compared with control seedlings drought stress reduced the total biomass of seedlings, as well as root biomass. However, the drought response of root to shoot ratio differed between the 5 week and 10 month old seedlings used in Chapters 3 and 4. Following two weeks of drought, 5 week old seedlings showed an increased root to shoot ratio because of reduced needle biomass rather than stem biomass, whereas root to shoot ratio decreased for seedlings at 10 months old. Conversely, two weeks of drought reduced specific root length (SRL, mg^{-1}) for the 5 week old seedlings, but not the 10 month old seedlings. This might be because of differences in fine root turnover, since the younger plants had not yet developed fine root biomass and fine root production increases with age in *P. sylvestris* (Makkonen & Helmisaari 2002). The SRL indicates how much root length is built per unit of root mass and higher SRL increases the root-soil interface (Kramer & Boyer 1995). However, the effects of drought on SRL depends on the root diameter (RD, mm) and root tissue mass density (TMDr, g cm), which have been found to concomitantly decrease and increase respectively under drought, thereby resulting in no change in SRL though fine root dynamics changed (Meier & Leuschner 2007; Olmo *et al.* 2014).

In terms of aboveground growth, seedling shoot biomass was positively correlated with seed weight. A lower specific leaf area (SLA) of Spanish provenances compared with other provenances was found. There is a co-ordinated morphological response to

shade and drought rather than a trade-off; root investment promotes drought tolerance and low SLA is considered adaptive under shade and potentially drought (Markesteijn & Poorter 2009). Furthermore, drought-tolerant species are also shade-tolerant in dry habitats (Markesteijn *et al.* 2011).

Hence, the low SLA might be a co-ordinated response to shade and drought in the regeneration niche of the Spanish provenances. Survival during the first summer is the main bottleneck for the seedling recruitment of woody species in Mediterranean regions (Castro *et al.* 2005; Mendoza *et al.* 2009; Matias 2011). The effect of summer drought on seedling establishment varies spatially and temporally. In Mediterranean regions, microhabitats that are sheltered by neighbouring vegetation benefit from increased soil moisture, lower soil temperatures and higher air relative humidity as compared with open microhabitats with high irradiation, where the higher seedling mortality rates are found. Owing to typical seedling mortality rates of 100 % in dry summers, recruitment in open microhabitats is restricted to unusually mild summers with sufficient precipitation (Castro *et al.* 2002; 2004). Regeneration is possible in sheltered microhabitats under dry summers, though the decreased light intensity also has a negative effect on seedling growth (Castro *et al.* 2004; 2005). Yet a process of facilitation by the presence of vegetation is deemed necessary for seedling establishment in drought afflicted Mediterranean climates (Matias 2011).

5.2 Seedling metabolic responses to drought

The main contribution of this thesis has been to identify important metabolites responsible for a shift in the foliar metabolome of *P. sylvestris* seedlings under drought stress (Chapter 3). This is the first time a study into drought effects on the foliar metabolome of *Pinus sylvestris* seedlings – or indeed of any *Pinus* spp. at the seedling stage - has been carried out. Hence, the identification of compounds from aromatic amino acids and flavonoids shows that these metabolically costly pathways are upregulated under drought stress even at the seedling stage.

Although metabolic profiles of drought stressed seedlings were significantly different from control seedlings, the metabolic effects of drought did not differentiate by provenance. The importance of ontogeny in needle metabolic responses is linked to morphology and physiology (de Miguel et al 2016; Canas *et al.* 2015). Therefore, the lack of a provenance effect was surprising given the morphological and physiological differentiation of seedlings from different provenances.

The key metabolite that showed differential abundance in response to drought involved in osmoprotective and antioxidant capabilities was the free amino acid proline. Osmotic adjustment involves solute accumulation that constrains turgor loss at low water potentials. Proline acts as a compatible solute and free radical scavenger, accumulating in a number of plant species under drought (Hayat *et al.* 2012). Furthermore, intra-specific variation in proline accumulation has been linked to improved drought tolerance. For example, drought tolerant genotypes of *Pinus*

radiata, as indicated by increased survival, showed increasing proline accumulation under drought stress (De Diego *et al.* 2015). Nguyen-Queyrens and Bouchet-Lannat (2003) observed intraspecific variation in osmotic adjustment of *Pinus pinaster* three year old saplings under drought stress, which was more pronounced in saplings from xeric sites. A slower imposition of PEG induced osmotic stress favoured osmotic adjustment across 5 provenances in *Pinus canariensis*, and was positively correlated with drought duration at the provenance site (Lopez *et al.* 2009). However, in *Arabidopsis* under combined drought and heat stress, proline accumulation is no longer favoured over sugar accumulation (Rizhsky *et al.* 2004). Thus, the likely combination of heat and drought stress under climate change might induce different metabolic responses in terms of osmoregulation.

Tyrosine and tryptophan are aromatic amino acids that were identified as accumulating in *P. sylvestris* seedlings under drought stress. Unlike animals, plants and micro-organisms synthesize aromatic amino acids through the metabolically costly seven stage shikimate pathway, to which ≥30% of photosynthetically fixed carbon is directed in vascular plants (Maeda & Dudareva 2012). Du *et al.* (2015) found that *Pseudotsuga menziesii* seedlings showed a provenance specific drought response, with the interior drier provenance increasing aromatic amino acids.

The identification of key metabolites in this study involved both univariate and multivariate statistical approaches, as is recommended for analysing metabolomics datasets (Goodacre et al. 2005; Vinaixa *et al.* 2012). Where information about correlated trends between metabolites is not required, univariate statistics can retain

more statistically significant peaks potentially of interest (Kenny *et al*. 2010). However, this is at the risk of increased false positives (Vinaixa *et al.* 2012). In this study, different metabolites were identified as being most significant by ANOVA (univariate) and multi-group sparse discriminant analysis (MGSDA). Another issue with this metabolomics study, in common with many metabolomics studies, regards the sample size. Analysis of variance (ANOVA) and multi-variate analysis of variance (MANOVA) assess the significance of the ratio of the variation within class to the variation between classes. However, a core underlying assumption is that of homogeneity of variances (homoscedasticity), which may very easily be violated for biological groups of small samples from a much larger population.

Of the top metabolites important in the drought time interaction, most were unknown and potentially represent novel findings of metabolic responses to drought. In many plant metabolomics studies, the aim of characterising all metabolites is limited by our inability to translate some statistically significant peaks into a functionally identifiable compound. The use of metabolomics data in conjunction with other –omics data has been strongly recommended in order to fully appreciate which metabolic pathways change under stress (Obata *et al.* 2010). For example, the combined use of transcriptomics and metabolomics data successfully uncovered novel regulators in redox control of photosynthesis (Kolbe *et al.* 2006).

Approaches such as '-omics' analyses of metabolites, transcripts and proteins attempt to study the functional genome. Gene annotation is the ultimate goal and '-omics' has facilitated the correlation of transcript and metabolite levels with genes for model plant

species such as *Medicago truncatula, Nicotiana tabacum* and *Arabidopsis* (Suzuki *et al.* 2005; Goossens *et al.* 2003; Hirai *et al.* 2004). However, compared with most vascular plants, conifer genomes are considerably larger and characterised by repetitive sequences (Ahuja & Neale 2005, Kovach *et al.* 2010) and *Pinus sylvestris* is no exception with a genome size of 1C (haploid) = 22474 Mbp (Fuchs *et al.* 2008). The number of genes within the *Pinus taeda* genome is estimated at ~ 50,000 (Neale *et al.* 2014) and a similar number might be expected for other *Pinus* species. Furthermore, in out-crossing populations such as those across the range of *Pinus sylvestris*, multi-allelic epistatic interactions may occur and affect the resulting phenotypic variation in a population (Tong *et al.* 2011). Thus, studying functional genomics is complicated in pines. In order to maintain or improve adaptive potential of *Pinus sylvestris* populations, recommendations for conservation practices centre on promoting natural regeneration or planting from seed stock of the same population (Salmela 2011). The justification for this restriction is that a high intra-population genetic variation exists and high out-crossing rates suggest that potential for adaptive genetic variation will remain high (Robledo-Arnucio 2011).

5.3 Future directions

5.3.1 Integrating drought and biotic stress responses

The role of multiple stresses in accelerating tree mortality is in need of further elucidation before it can be represented in process-based models (Anderegg *et al.* 2013). Biotic attack is an additional causal factor in tree mortality and trees weakened by drought stress are then predisposed to die from opportunistic pests and pathogens.

For conifer species, carbon-based secondary metabolites, terpenoids and phenolic compounds, are expressed constitutively, yet are inducible to high concentrations to provide defence against many pests and pathogens (Keeling & Bohlmann 2006). Anderegg *et al.* (2015) outline integration of biotic attack within the drought-induced mortality framework, using two insect guilds as case examples: bark beetles and defoliators. In both cases, the NSC pool is critical to the formation of carbon based secondary metabolites that form the tree's defence. These authors propose that the NSC pool serves as the fundamental link enabling the development of integrated model for drought and insect interaction in mortality, since the probability of infestation increases owing to compromised production of secondary metabolites to defend the tree. Metabolites that are not involved in the photosynthetic and central metabolic processes are known as secondary metabolites with no major involvement in normal plant growth and development. Secondary metabolites can function both in stress signalling and defence, though plants have to re-direct resources from primary metabolism. In this thesis, defence related secondary metabolites seemed to increase in abundance in drought-stressed seedlings. This shows that metabolically costly secondary metabolism can continue under drought stress. Thus, there may be implications for metabolic physiological changes resulting from co-ordinating a drought stress response with a defence response against biotic attack.

Defences to biotic attack can be constitutive or inducible. The constitutive defences may represent anatomical or biochemical barriers or mutualistic associations. For example, a role for endophytic fungi in limiting foliar pathogen damage has been shown for mature *Theobroma cacao* (Arnold *et al.* 2003) and *Pinus radiata* seedlings

(Martínez-Álvarez *et al.* 2016). Below ground, mycorrhiza seem to offer increased plant resistance against root pathogens; it is not always clear to what extent this is attributable to facilitated higher nutrient uptake improving host plant chemical defences or direct competition between the mycorrhiza and pathogen (Wehner *et al.* 2010). Only constitutive not inducible biochemical responses to pest induced defoliation were found for three pine species, suggesting a low capacity for defence (Hodar *et al.* 2015).

Hormonal and signalling cross-talk between metabolic stress responses elicited by different abiotic and biotic drivers has emerged in results of many plant science studies, carrying implications for the ultimate physiological response to multifactorial stress (Narusaka *et al.* 2004; Fujita *et al.* 2006; Miller *et al.* 2008; Atkinson & Urwin 2012). For example, hormonal signals affect equilibrium of reactive oxygen species under abiotic and pathogen stress. Systemic induced resistance (SIR) to pathogens involves changes in secondary metabolism co-ordinated by one or more hormones of salicylic acid, jasmonic acid, ethylene and ABA (Durrant & Dong 2004). In a natural population of *Picea abies* colonised by bark beetle, *Ips typographus*, trees with half stem sections treated with SA exhibited higher bark resistance to pest attack, as SA triggered SIR by delaying thiol degradation and thus promoting glutathione-mediated defence (Krajnc *et al.* 2011). SIR has also been demonstrated in the tree model pathosystem, *Pinus nigra – Diplodia pinea* (Blodgett *et al.* 2007). Volatile organic compounds (VOCs) involved in SIR can act as phytohormones, signalling to unaffected parts of the canopy (Frost *et al.* 2008), as well as 'pheromones', signalling to neighbouring plants that are primed to induce defences but do not actively produce

defensive compounds until attacked (Conrath *et al.* 2006), or even attracting predators of the infesting organism (Hilker *et al.* 2002). A capacity for SIR by emission of VOCs in *Pinus sylvestris* has been found (Mumm *et al.* 2003). There also appear to be interactions between multiple co-occurring stresses on VOC emission, including high light, temperature and pathogen (Holopainen & Gershenzon 2010). Integrative systems biology approaches, such as the complementary use of metabolomics and transcriptomics, might facilitate understanding of the genes and metabolic pathways affected by multiple stresses.

5.3.2 Mycorrhizal symbioses and plant facilitation

Under climate change, increased frequency of summer droughts might co-occur with changes in phenology and interspecific interactions, in particular the spread of pests and pathogens. Hence, the effect of drought on a species' regeneration niche is not only dependent on the adaptive capacity of that species to drought or indeed to multiple stressors. No organism exists in isolation and so any implications of research into the drought response of *Pinus sylvestris* regeneration might also consider its associated biota when formulating practical conservation advice. The process of plant facilitation has been shown to have a major influence on regeneration of woody seedlings during summer in Mediterranean, with higher seedling survival associated with shelter from shrubs (Matias 2011). The effect of drought in shifting community composition of plants and their associated mycorrhizae could have a subsequent effect in regeneration success of *P. sylvestris*. Kipfer *et al.* (2010) found that of four EM species only *Suillus granulatus*, improved *P.sylvestris* seedling performance in terms of shoot growth.

S.granulatus is an EM commonly associated with conifers; in a mycological survey of *Q.ilex* forest, this species was recorded only in plots containing *Pinus pinaster* (Zotti *et al.* 2013). Hence, drought effects on mycorrhizal composition could also potentially affect *P. sylvestris* regeneration.

5.4 Conclusions

Increasing drought risk under anthropogenic climate change threatens populations at the xeric range edge of *P. sylvestris*. Understanding the extent of divergence under conditions of severe drought is therefore important in order to assess the capacity of this species to adapt to future climate change. In most previous studies, the focus has been on the mature demographic when considering mechanisms of tree mortality in drought episodes. However, assessing population stability also requires an understanding of regeneration capacity.

This study provides evidence of divergence among four populations of *P. sylvestris* in germination and seedling establishment under drought stress. Provenances of *P. sylvestris* from central and southern parts of the European range differed in terms of seed weight and germination rate, with the Spanish provenance showing the highest mean seed weight and rate of germination. Furthermore, seedling biomass allocation of Spanish provenances shows higher investment in the root system and lower specific leaf area, indicating a co-ordinated phenotypic adaptation to drought stress. Meanwhile, at the intra-population level, maternal parentage has an effect on seed germination across provenances and a seed weight effect mediated by maternal parentage was also found for seedling biomass traits within the Austrian provenance.

As well as the reduction in biomass under drought, seedlings at nearly one year old showed a distinct metabolic response to drought. This involved accumulation of both primary and secondary metabolites related to osmoregulation and antioxidant defence. Furthermore, expression of two key antioxidant enzymes, superoxide dismutase and glutathione synthetase, increased under drought stress in seedlings during the initial establishment stage. Thus, a similar metabolic response to drought stress was found across provenances. This is suggestive of a similar capacity to respond to drought stress at the metabolic level in seedlings. Future research comparing the effect of single and multiple co-occurring stresses and beneficial associated organisms on *P. sylvestris* physiology could help provide additional insight into the variation in stress tolerance under climate change for this species.

Drought may become an important environmental driver of trait variation during seedling establishment and regeneration under climate change. In this work, findings of demonstrable intra-specific effects in growth and physiology at the seedling stage are suggestive of local adaptation in populations of this widespread conifer. Thus, consideration of intra-specific effects at the seedling stage will be necessary in developing models of the response of *P.sylvestris* to changing environmental conditions.

6 References

Adams, H.D., Germino, M.J., Breshears, D.D., Barron-Gafford, G.A, Guardiola-Claramonte, M., Zou, C.B., and Huxman, T.E., 2013. Nonstructural leaf carbohydrate dynamics of *Pinus edulis* during drought-induced tree mortality reveal role for carbon metabolism in mortality mechanism. *New Phytologist*, 197 (4), pp.1142–51.

Agati, G., Azzarello, E., Pollastri, S. and Tattini, M., 2012. Flavonoids as antioxidants in plants: location and functional significance. *Plant Science*, 196, pp.67-76.

Aho M.L., 1994. Autumn frost hardening of one-year-old *Pinus sylvestris* (L.) seedlings: effect of origin and parent trees. *Scandinavian Journal of Forest Research*. 9 (1-4), pp. 17–24.

Ahuja, M.R. & Neale, D.B., 2005. Evolution of genome size in conifers. *Silvae Genetica*, 54 (3), pp. 126–137.

Aitken, S. N., Yeaman, S., Holliday, J. a., Wang, T., & Curtis-McLane, S., 2008. Adaptation, migration or extirpation: climate change outcomes for tree populations. *Evolutionary Applications*, 1(1), pp. 95–111.

Akaike, H., 1974. A new look at the statistical model identification. *Automatic Control, IEEE Transactions on*, 19(6), pp.716-723.

Alberto, F.J., Aitken, S.N., Alía, R., González-Martínez, S.C., Hänninen, H., Kremer, A., Lefèvre, F., Lenormand, T., Yeaman, S., Whetten, R., Savolainen, O., 2013. Potential for evolutionary responses to climate change - evidence from tree populations. *Global Change Biology*, 19(6), pp.1645–1661.

Alexou, M., 2013. Development-specific responses to drought stress in Aleppo pine (*Pinus halepensis* Mill.) seedlings. *Tree Physiology*, 33(10), pp. 1030–42.

Allen, C.D., Macalady, A.K., Chenchouni, H., Bachelet, D., McDowell, N., Vennetier, M., Kitzberger, T., Rigling, A., Breshears, D.D., Hogg, E.T. and Gonzalez, P., 2010. A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *Forest ecology and management*, 259(4), pp.660-684.

Allen, J., Davey, H.M., Broadhurst, D., Rowland, J.J., Oliver, S.G. and Kell, D.B., 2004. Discrimination of modes of action of antifungal substances by use of metabolic footprinting. *Applied and environmental microbiology*, 70(10), pp.6157-6165.

Allen, J.W. and Shachar-Hill, Y., 2009. Sulfur transfer through an arbuscular mycorrhiza. *Plant Physiology*, *149*(1), pp.549-560.

Alscher, R. G., Erturk, N., & Heath, L. S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany*, 53(372), pp. 1331–41.

Anderegg, W.R., Hicke, J.A., Fisher, R.A., Allen, C.D., Aukema, J., Bentz, B., Hood, S., Lichstein, J.W., Macalady, A.K., McDowell, N. and Pan, Y., 2015. Tree mortality from drought, insects, and their interactions in a changing climate. *New Phytologist*, 208(3), pp.674-683.

Anderegg, W.R., Kane, J.M. and Anderegg, L.D., 2013. Consequences of widespread tree mortality triggered by drought and temperature stress. *Nature Climate Change*, 3(1), pp.30-36.

Anderegg, W.R., Plavcová, L., Anderegg, L.D., Hacke, U.G., Berry, J.A. and Field, C.B., 2013. Drought's legacy: multiyear hydraulic deterioration underlies widespread aspen forest die-off and portends increased future risk. *Global Change Biology*, 19(4), pp.1188-1196.

Anderson, R.G., Canadell, J.G., Randerson, J.T., Jackson, R.B., Hungate, B.A., Baldocchi, D.D., Ban-Weiss, G.A., Bonan, G.B., Caldeira, K., Cao, L. and Diffenbaugh, N.S., 2010. Biophysical considerations in forestry for climate protection. *Frontiers in Ecology and the Environment*, 9(3), pp.174-182.

Andersson, B. and Fedorkov, A., 2004. Longitudinal differences in Scots pine frost hardiness. *Silvae Genetica* 53(2), pp. 76-80.

Arnold, A.E. et al., 2003. Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences of the United States of America*, 100(26), pp.15649–15654.

Aronen, T. and Ryynänen, L., 2012. Variation in telomeric repeats of Scots pine (*Pinus sylvestris* L.). *Tree genetics & genomes*, 8(2), pp.267-275.

Atkinson, N.J. & Urwin, P.E., 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of experimental botany*, 63(10), pp.3523–43.

Augé, R.M., 2004. Arbuscular mycorrhizae and soil/plant water relations. *Canadian Journal of Soil Science*, *84*(4), pp.373-381.

Barbeito, I., Fortin, M. J., Montes, F., & Cañellas, I. (2009). Response of pine natural regeneration to small-scale spatial variation in a managed Mediterranean mountain forest. *Applied Vegetation Science*, 12(4), pp. 488–503.

Barchet, G.L., Dauwe, R., Guy, R.D., Schroeder, W.R., Soolanayakanahally, R.Y., Campbell, M.M. and Mansfield, S.D., 2014. Investigating the drought-stress response of hybrid poplar genotypes by metabolite profiling. *Tree physiology*, *34*(11), pp.1203-1219

Bartoli, C. G., Gomez, F., Gergoff, G., Guiamét, J. J., & Puntarulo, S., 2005. Up-regulation of the mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under drought conditions. *Journal of Experimental Botany*, 56(415), pp. 1269–76.

Bell, D. M., Bradford, J. B., & Lauenroth, W. K., 2014. Early indicators of change: divergent climate envelopes between tree life stages imply range shifts in the western United States. *Global Ecology and Biogeography*, 23(2), pp. 168–180.

Benhiba, L., Oussouf, M. & Abdellatif, F., 2015. Arbuscular mycorrhizal symbiosis enhanced growth and antioxidant metabolism in date palm subjected to long-term drought. *Trees*, 29(6), pp.1725–1733.

Benjamini, Y. and Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*. Series B (Methodological), pp.289-300.

Bian, S. and Jiang, Y., 2009. Reactive oxygen species, antioxidant enzyme activities and gene expression patterns in leaves and roots of Kentucky bluegrass in response to drought stress and recovery. *Scientia Horticulturae*, 120(2), pp.264-270.

Bigler, C., Bräker, O. U., Bugmann, H., Dobbertin, M., & Rigling, A., 2006. Drought as an Inciting Mortality Factor in Scots Pine Stands of the Valais, Switzerland. *Ecosystems*, 9(3), pp. 330–343.

Bladé, C. and Vallejo, V.R., 2008. Seed mass effects on performance of *Pinus halepensis* Mill. seedlings sown after fire. *Forest Ecology and Management*, 255(7), pp.2362-2372.

Blodgett, J.T., Eyles, A. and Bonello, P., 2007. Organ-dependent induction of systemic resistance and systemic susceptibility in Pinus nigra inoculated with Sphaeropsis sapinea and Diplodia scrobiculata. *Tree physiology*, *27*(4), pp.511-517.

Bogeat-Triboulot, M., Brosché, M., Renaut, J., Jouve, L., Le Thiec, D., Fayyaz, P., Vinocur, B., Witters, E., Laukens, K., Teichmann, T., Altman, A., Hausman, J., Polle, A.,K angasjärvi, J., Dreyer, E., 2007. Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in Populus euphratica, a poplar growing in arid regions. *Plant physiology*, 143(2), pp. 876–92.

Bogunic F, Muratovic E, Brown SC, Siljak-Yakovlev S., 2003. Genome size and base composition of five *Pinus* species from the Balkan region. *Plant Cell Reports*, 22(1), pp. 59-63.

Bogunić, F., Siljak-Yakovlev, S., Muratović, E., Pustahija, F. and Medjedović, S., 2011. Molecular cytogenetics and flow cytometry reveal conserved genome organization in *Pinus mugo* and *P. uncinata*. *Annals of Forest Science*, 68(1), pp.179-187.

Bohrerova, Z., Bohrer, G., Cho, K. D., Bolch, M. A. and Linden, K. G. 2009. Determining the viability response of pine pollen to atmospheric conditions during long-distance dispersal. *Ecological Applications*. 19(3), pp. 656-667.

Bonan, G.B. (2008) Forests and climate change: forcings, feedbacks, and the climate benefits of forests. *Science*, 320, 1444–1449.

Bond, W. J., 1989. The tortoise and the hare: ecology of angiosperm dominance and gymnosperm persistence. *Biological Journal of the Linnean Society*, 36(3), pp. 227-249.

Bornyasz, M.A., Graham, R.C. and Allen, M.F., 2005. Ectomycorrhizae in a soil-weathered granitic bedrock regolith: linking matrix resources to plants. *Geoderma*, *126*(1), pp.141-160.

Botkin D.B., Saxe H., Araújo M.B., Betts R., Bradshaw R.H.W., Cedhagen C., Chesson P., Dawson T.P., Etterson J.R., Faith D.P., Ferrier S, Guisan A., Skjoldborg Hansen A., Hilbert D.W., Loehle C., Margules C., New M., Sobel M.J., Stockwell D.R.B., 2007. Forecasting the effects of global warming on biodiversity. *Bioscience*, 57 (3), pp. 227-236.

Bouche, P.S., Delzon, S., Choat, B., Badel, E., Brodribb, T.J., Burlett, R., Cochard, H., Charra-Vaskou, K., Lavigne, B., Li, S. and Mayr, S., 2015. Are needles of *Pinus pinaster* more vulnerable to xylem embolism than branches? New insights from X-ray computed tomography. *Plant, cell & environment*.

Bowne, J.B. et al., 2012. Drought Responses of Leaf Tissues from Wheat Cultivars of Differing Drought Tolerance at the Metabolite Level. *Molecular Plant*, 5(2), pp.418–429.

Bradshaw, A. D., 1965. Evolutionary significance of phenotypic plasticity in plants. In E.W. Caspari, & J.M. Thoday (Eds.), *Advances in genetics* (Vol.13, pp.115-155).

Bradshaw, A.D., 2006. Unravelling phenotypic plasticity–why should we bother? *New Phytologist*, 170(4), pp.644-648.

Bräutigam, K., Vining, K.J., Lafon-Placette, C., Fossdal, C.G., Mirouze, M., Marcos, J.G., Fluch, S., Fraga, M.F., Guevara, M., Abarca, D. and Johnsen, Ø., 2013. Epigenetic regulation of adaptive responses of forest tree species to the environment. *Ecology and Evolution*, 3(2), pp.399-415.

Bray, J. & Maxwell, S., 1985. Multivariate Analysis of Variance: INTRODUCTION TO MULTIVARIATE ANALYSIS OF VARIANCE. In *Multivariate Analysis of Variance*. pp. 8–14.

Breiman, L.E.O., 2001. Random Forests. *Machine Learning*, 45, pp.5–32.

Breshears, D. D., Cobb, N. S., Rich, P. M., Price, K. P., Allen, C. D., Balice, R. G., … Meyer, C. W., 2005. Regional vegetation die-off in response to global-change-type drought. *Proceedings of the National Academy of Sciences of the United States of America*, 102(42), pp. 15144–8.

Breshears, D.D. et al., 2009. Tree die-off in response to global change-type drought: mortality insights from a decade of plant water potential measurements. *Frontiers in Ecology and the Environment*, 7(4), pp.185–189.

Broadhurst, D.I. & Kell, D.B., 2006. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics*, 2(4).

Brodribb, T. J., & Cochard, H., 2009. Hydraulic failure defines the recovery and point of death in water-stressed conifers. *Plant Physiology*, 149(1), pp. 575–84.

Brodribb, T. J., & McAdam, S. a M., 2011. Passive origins of stomatal control in vascular plants. *Science*, 331(6017), pp. 582–5.

Brodribb, T. J., Bowman, D. J. M. S., Nichols, S., Delzon, S., & Burlett, R., 2010. Xylem function and growth rate interact to determine recovery rates after exposure to extreme water deficit. *New Phytologist*, 188(2), pp. 533–42.

Brunetti, C., George, R.M., Tattini, M., Field, K. and Davey, M.P., 2013. Metabolomics in plant environmental physiology. *Journal of experimental botany*, p.ert244.

Büscher, J.M., Czernik, D., Ewald, J.C., Sauer, U. and Zamboni, N., 2009. Cross-platform comparison of methods for quantitative metabolomics of primary metabolism. *Analytical chemistry*, 81(6), pp.2135-2143.

Canas, R. a. et al., 2015. Understanding developmental and adaptive cues in pine through metabolite profiling and co-expression network analysis. *Journal of Experimental Botany*, 66(11), pp.3113–3127.

Capy, P., Gasperi, G., Biemont, C. & Bazin, C., 2000. Stress and transposable elements: co-evolution or useful parasites? *Heredity,* 85 (2), pp. 101–106.

Cartwright,N., 1983.How the laws of physics lie. Oxford UniversityPress, Oxford.

Castellanos MC, Medrano M, Herrera CM.,2008. Subindividual variation and genetic versus environmental effects on seed traits in a European Aquilegia. *Botany* 86: 1125–1132.

Castro J, Zamora R,Hodar JA, Gomez JM., 2005. Alleviation of summer drought boosts establishment success of *Pinus sylvestris* in a Mediterranean mountain: an experimental approach. *Plant Ecology*, 181, pp. 191–202.

Castro, J. et al., 2004. Seedling establishment of a boreal tree species. *Journal of Ecology*, pp.266– 277.

Castro, J. et al., 2008. Evidence that the negative relationship between seed mass and relative growth rate is not physiological but linked to species identity: a within-family analysis of Scots pine. *Tree physiology*, 28, pp.1077–1082.

Castro, J., 1999. Seed mass versus seedling performance in Scots pine: a maternally dependent trait. *New Phytologist*, 144(1), pp.153-161.

Castro, J., 2006. Short delay in timing of emergence determines establishment success in *Pinus sylvestris* across microhabitats. *Annals of botany*, 98(6), pp.1233–40.

Castro, J., Zamora, R. & Hódar, J., 2002. Mechanisms blocking *Pinus sylvestris* colonization of Mediterranean mountain meadows. *Journal of Vegetation Science*, 13, pp.725–731.

Cavrak, V.V., Lettner, N., Jamge, S., Kosarewicz, A., Bayer, L.M. and Scheid, O.M., 2014. How a retrotransposon exploits the plant's heat stress response for its activation. *PLoS Genetics*, 10(1), p.e1004115.

Cendán, C., Sampedro, L. & Zas, R., 2013. The maternal environment determines the timing of germination in *Pinus pinaster*. *Environmental and Experimental Botany*, 94, pp.66–72.

Chakraborty, U., Chakraborty, B. and Basnet, M., 2006. Plant growth promotion and induction of resistance in Camellia sinensis by Bacillus megaterium. Journal of basic microbiology, 46(3), pp.186-195.

Chai, T.-T.. C. et al., 2005. Water stress-induced oxidative damage and antioxidant responses in micropropagated banana plantlets. *Biologia Plantarum*, 49(1), pp.153–156.

Chatfield, C., 1995. Model Uncertainty, Data Mining and Statistical Inference. *Journal of the Royal Statistical Society*, 158(3), pp.419–466.

Chen J, Kallman T, Ma X *et al.*, 2012 Disentangling the roles of history and local selection in shaping clinal variation of allele frequencies and gene expression in Norway spruce (*Picea abies*). *Genetics*, 191(3), pp. 865–881.

Chen, H. and Jiang, J.G., 2010. Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. *Environmental Reviews*, 18, pp.309-319.

Chen, K. & Arora, R., 2013. Priming memory invokes seed stress-tolerance. *Environmental and Experimental Botany*, 94, pp.33–45.

Chmura, D.J., Rozkowski, R. & Chałupka, W., 2012. Growth and phenology variation in progeny of Scots pine seed orchards and commercial seed stands. *European Journal of Forest Research*, 131(4), pp.1229–1243.

Cochard, H., 2006. Cavitation in trees. *Comptes Rendus Physique*, 7(9-10), pp. 1018–1026.

Cocozza, C., 2010. Early effects of water deficit on two parental clones of *Populus nigra* grown under different environmental conditions. *Functional Plant Biology*, 37(3), pp. 244–254.

Conrath, U., et al., 2006. Priming: getting ready for battle. *Mol. Plant Microbe Interact*. 19, 1062–1071.

Contento, A.L., Kim, S.J. and Bassham, D.C., 2004. Transcriptome profiling of the response of *Arabidopsis* suspension culture cells to Suc starvation. *Plant physiology*, 135(4), pp.2330-2347.

Corcuera, L. & Notivol, E., 2015. Differences in photosynthetic activity might explain the large-scale shifts in pine recruitment in favour of oaks in continental Mediterranean climates. *Forestry*, 88(2), pp.248–256.

Correia, I., Santos, L., Faria, C., Nóbrega, C., Almeida, H. and David, T., 2014. Cone to Seedling—Variation between *Pinus pinaster* Provenances from Contrasting Altitudes. *Forest Science*, *60*(4), pp.724-732.

Couée, I. et al., 2006. Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of experimental botany*, 57(3), pp.449–59.

Cramer W, Bondeau A, Woodward FI, Prentice IC, Betts RA, Brovkin V, Cox PM, Fisher V, Foley JA, Friend AD et al. 2001. Global response of terrestrial ecosystem

structure and function to CO2 and climate change: results from six dynamic global vegetation models. *Global Change Biology* 7: 357–373.

Cruz de Carvalho, M.H., 2008. Drought stress and reactive oxygen species. *Plant signaling & behavior*, 3(3), pp.156–165.

Dauwe, R., Holliday, J.A., Aitken, S.N. and Mansfield, S.D., 2012. Metabolic dynamics during autumn cold acclimation within and among populations of Sitka spruce (*Picea sitchensis*). *New Phytologist*, 194(1), pp.192-205.

Davey, M.P., Burrell, M.M., Woodward, F.I. and Quick, W.P., 2008. Population-specific metabolic phenotypes of *Arabidopsis* lyrata ssp. *petraea*. *New Phytologist*, 177(2), pp.380-388.

de Miguel, M. De et al., 2016. Plant Physiology and Biochemistry Organ-specific metabolic responses to drought in *Pinus pinaster* Ait. *Plant Physiology and Biochemistry*, 102, pp.17–26.

Debain, S., Chadœuf, J., Curt, T., Kunstler, G., & Lepart, J., 2007. Comparing effective dispersal in expanding population of *Pinus sylvestris* and *Pinus nigra* in calcareous grassland. *Canadian journal of forest research*, *37*(4), pp. 705-718.

Debain, S., Curt, T., & Lepart, J., 2003a. Seed mass, seed dispersal capacity, and seedling performance in a *Pinus sylvestris* population. *Ecoscience*, pp. 168-1

Debain, S., Curt, T., Lepart, J., & Prevosto, B. (2003b). Reproductive variability in *Pinus sylvestris* in southern France: implications for invasion. *Journal of Vegetation Science*, 14(4), pp. 509-516.

Delzon, S., 2015. New insight into leaf drought tolerance. *Functional Ecology*, 29(10), pp.1247–1249.

Dhindsa, R.S. and Matowe, W., 1981. Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation. Journal of experimental botany, 32(1), pp.79-91.

Dhir, N. K. and Miksche, J. P., 1974. Intraspecific variation of nuclear DNA content in Pinus resinosa Ait. *Canadian Journal of Genetics and Cytology,* 16, pp. 77–83.

Diego, N. De et al., 2015. Metabolites and hormones are involved in the intraspecific variability of drought hardening in radiata pine. *Journal of Plant Physiology*, 188, pp.64–71.

Ditmarova L, Kurjak D, Palmroth S, KmetJ, Strelcova K., 2010. Physiological responses of Norway spruce (Picea abies (L.) Karst) seedlings to drought stress. *Tree Physiology*, 30, 205–213.

Dixon, R. & Strack, R., 2003. Phytochemistry meets genome analysis, and beyond ....*Phytochemistry*, 62(August 2001), pp.815–816.

Dixon, R.A., 2001. Natural products and plant disease resistance. *Nature*, 411(6839), pp.843-847.

Dobzhansky, T., 1937. Genetic nature of species differences. *American Naturalist*. 71, pp. 404-420.

Doheny-adams, T., Hunt, L., Franks, P. J., Beerling, D. J., Gray, J. E., 2012. Genetic manipulation of stomatal density influences stomatal size, plant growth and tolerance to restricted water supply across a growth carbon dioxide gradient. *Philosophical Transactions of the Royal Society B*, 367(1588), pp. 547–555.

Du, B., Jansen, K., Kleiber, A., Eiblmeier, M., Kammerer, B., Ensminger, I., Gessler, A., Rennenberg, H. and Kreuzwieser, J., 2015. A coastal and an interior Douglas fir provenance exhibit different metabolic strategies to deal with drought stress. *Tree Physiology*, 36, pp.148–163.

Duhem, P. 1906, tr 1962., The Aim and structure of physical theory, Athenum, New York.
Dunn OJ., 1961. Multiple comparison among means. *Journal of the American Statistical Association*, 56, pp. 52–64.
Durrant, W.E., Dong, X., 2004. Systemic acquired resistance. *Annu. Rev. Phytopathol*. 42, 185–209.
Duursma, R.A. et al., 2008. Predicting the decline in daily maximum transpiration rate of two pine stands during drought based on constant minimum leaf water potential and plant hydraulic conductance. *Tree physiology*, 28, pp.265–276.
Egerton-Warburton, L.M., Graham, R.C. and Hubbert, K.R., 2003. Spatial variability in mycorrhizal hyphae and nutrient and water availability in a soil-weathered bedrock profile. *Plant and Soil*, *249*(2), pp.331-342.
Egerton-Warburton, LM; Querejeta, JI; Allen, MF. 2007. Common mycorrhizal networks provide a potential pathway for the transfer of hydraulically lifted water between plants. *Journal of Experimental Botany*. 58(6):1473-1483.
El-Kassaby, Y. a. & Reynolds, S., 1990. Reproductive phenology, parental balance, and supplemental mass pollination in a sitka-spruce seed-orchard. *Forest Ecology and Management*, 31(1-2), pp.45–54.
Ella, E.S., Dionisio-Sese, M.L. & Ismail, A.M., 2011. Seed pre-treatment in rice reduces damage, enhances carbohydrate mobilization and improves emergence and seedling establishment under flooded conditions. *AoB plants*, 2011, p.plr007.
Engel, J. et al., 2015. Regularized MANOVA (rMANOVA) in untargeted metabolomics. *Analytica Chimica Acta*, 899, pp.1–12.
Ennos RA. 1994. Estimating relative rates of pollen and seed migration among plant populations. *Heredity,* 72(3), pp. 250–59.
Enquist, C. A. F. 2002. Predicted regional impacts of climate change on the geographical distribution and diversity of tropical forests in Costa Rica. *Journal of Biogeography.* 29, pp. 519–534.
Epron D, Dreyer E., 1992. Effects of severe dehydration on leaf photosynthesis in *Quercus petraea* (Matt) Liebl – photosystem-II efficiency, photochemical and non-photochemical fluorescence quenching and electrolyte leakage. *Tree Physiology*, 10, 273–284.
Eriksson, L., Johansson, E., Kettaneh-Wold, N. and Wold, S. (2001). Multi- and Megavariate Data Analysis: Principles and Applications, Umetrics Academy, Umea˚ .
Ertl, C. et al., 2012. Assessing the proportion of "extra-local" pollen by means of modern aerobiological and phenological records — An example from Scots pine (*Pinus sylvestris* L.) in northern Finland. *Review of Palaeobotany and Palynology*, 185, pp.1–12.
Facchini, P.J., 2001. Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. *Annual review of plant biology*, 52(1), pp.29-66.
Faegri, K. and van der Pijl, L. (eds.) 1979. The principles of pollination ecology. 3rd ed. Pergamon Press, Oxford, UK
Faize, M., Burgos, L., Faize, L., Piqueras, A., Nicolas, E., Barba-Espin, G., Clemente-Moreno, M.J., Alcobendas, R., Artlip, T. and Hernandez, J.A., 2011. Involvement of cytosolic ascorbate peroxidase and Cu/Zn-superoxide dismutase for improved tolerance against drought stress. *Journal of Experimental Botany*, *62*(8), pp.2599-2613.

Federoff, N. V., 2012. Transposable elements, epigenetics, and genome evolution. *Science* 338(6108), pp. 758–767.

Feise, R.J., 2002. Do multiple outcome measures require p-value adjustment?. *BMC Medical Research Methodology*, 2(1), p.1

Ferriere, R. & Fox, G., 1995. Chaos and Evolution. *Trends in ecology & evolution*, 10(12), pp.480–485.

Fiehn, O. University of California, Davis, CA, USA; 1st Metabolomics Conference, Japan 2005

Fiehn, O., 2002. Metabolomics–the link between genotypes and phenotypes. *Plant molecular biology*, *48*(1-2), pp.155-171.

Fisher, R. A. (1921). Studies in crop variation. I. An examination of the yield of dressed grain from Broadbalk. *Journal of Agricultural Science*, 11, 107–135.

Franklin, Laura R. 2005. Exploratory Experiments. *Philosophy of Science,* 72, 888–99.

Franks, P. J., & Beerling, D. J., 2009. CO_2-forced evolution of plant gas exchange capacity and water-use efficiency over the Phanerozoic. *Geobiology*, 7(2), pp. 227–36.

Frost, C.J., Mescher, M.C., Carlson, J.E. and De Moraes, C.M., 2008. Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiology*, 146(3), pp.818-824.

Froyd CA, Willis KJ., 2008. Emerging issues in biodiversity & conservation management: the need for a palaeoecological perspective. *Quaternary Science Reviews*, 27(17), pp. 1723–1732.

Fryer, M. J., Oxborough, K., Mullineaux, P. M., & Baker, N. R., 2002. Imaging of photo-oxidative stress responses in leaves. *Journal of Experimental Botany*, 53(372), pp. 1249–54.

Fu, J. and Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. *Environmental and Experimental Botany*, 45(2), pp.105-114.

Fuchs, J., Jovtchev, G. & Schubert, I., 2008. The chromosomal distribution of histone methylation marks in gymnosperms differs from that of angiosperms. *Chromosome research*, 16(6), pp.891–8.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K., 2006. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current opinion in plant biology*, *9*(4), pp.436-442.

Gibon Y, Usadel B, Blaesing OE, Kamlage B, Hoehne M, Trethewey R, Stitt M. (2006) Integration of metabolite with transcript and enzyme activity profiling during diurnal cycles in *Arabidopsis* rosettes. *Genome Biology*, 7, p. 76.

Golldack, D. et al., 2013. Gibberellins and abscisic acid signal crosstalk: living and developing under unfavorable conditions. *Plant cell reports*, 32(7), pp.1007–16.

Goodacre, R. and Kell, D.B., 2003. Evolutionary computation for the interpretation of metabolome data in Harrigan, G.G. and Goodacre, R. (Eds), Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis. Kluwer Academic Publishers, Boston, pp. 239–256.

Goodacre, R., 2005. Making sense of the metabolome using evolutionary computation: seeing the wood with the trees. *Journal of experimental botany*, *56*(410), pp.245-254

Goodman, N. 1983. Fact, fiction and forecast, 4th ed. Cambridge: Harvard University Press.

Goossens, A. et al. (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. *Proc. Natl. Acad. Sci. U. S. A*. 100, 8595–8600.

Goswami, A., Banerjee, R. & Raha, S., 2013. Drought resistance in rice seedlings conferred by seed priming : role of the anti-oxidant defense mechanisms. *Protoplasma*, 250(5), pp.1115–29.

Grotkopp, E. et al., 2004. Evolution of Genome Size in Pines (*Pinus*) and Its Life-History Correlates: Supertree Analyses. *Evolution*, 58(8), p.1705.

Grubb, P. J., 1977. The maintenance of species-richness in plant communities: the importance of the regeneration niche. *Biological Reviews* 52, pp. 107–145.

Guan L.M. & Scandalios J.G. (2000) Catalase transcript accumula- tion in response to dehydration and osmotic stress in leaves of maize viviparous mutants. *Redox Report*, 5, 377–383.

Guisan A, Thuiller W., 2005. Predicting species distribution: offering more than simple habitat models. *Ecology Letters*, 8(9), pp. 993–1009.

Hall, R.D., 2006. Plant metabolomics: from holistic hope, to hype, to hot topic. *New phytologist*, *169*(3), pp.453-468.

Hallgren, S.W., 1989. Effects of osmotic priming using aerated solutions of polyethylene glycol on germination of pine seeds. *Annals of Forest Science*, 46, pp.31–37.

Hamanishi, E.T., Barchet, G.L., Dauwe, R., Mansfield, S.D. and Campbell, M.M., 2015. Poplar trees reconfigure the transcriptome and metabolome in response to drought in a genotype-and time-of-day-dependent manner. *BMC genomics*, *16*(1), p.1.

Hampe A, Petit RJ, 2005. Conserving biodiversity under climate change: the rear edge matters. *Ecology Letters* 8(5), pp. 461–467

Hampe A., 2004. Bioclimate envelope models: what they detect and what they hide. *Global Ecology and Biogeography*, 13(5), pp. 469–476.

Hannerz, M., Almqvist, C., & Hornfeldt, R., 2002. Timing of seed dispersal in *Pinus sylvestris* stands in central Sweden. *Silva Fennica*, *36*(4), pp. 757-765.

Harborne, J.B., Baxter, H., 1999. The Handbook of Natural Flavonoids, Vol. 1. Chichester, Wiley.

Hare, P.D. and Cress, W.A., 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant growth regulation*, *21*(2), pp.79-102.

Hartmann, H., Ziegler, W., & Trumbore, S., 2013. Lethal drought leads to reduction in nonstructural carbohydrates in Norway spruce tree roots but not in the canopy. *Functional Ecology*, 27(2), pp. 413–427.

Hayat, S. et al., 2012. Role of proline under changing environments Role of proline under changing environments A review. *Plant signaling & behavior*, 7(11), pp.1456–1466.

Heikkinen R.K., Luoto M., Araújo M.B., Virkkala R., Thuiller W., Sykes M.T., 2006. Methods and uncertainties in bioclimatic envelope modelling under climate change. *Progress in Physical Geography*, 30(6), 751–777.

Herman, J.J. & Sultan, S.E., 2011. Adaptive transgenerational plasticity in plants : case studies , mechanisms , and implications for natural populations. *Frontiers in plant science*, 2(December), pp.1–10.

Hilker M, Kobs C, Varama M, Schrank K. 2002. Insect egg deposition induces *Pinus* to attract egg parasitoids. *Journal of Experimental Biology*, 205, 455–461.

Hirai, M.Y. et al. (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U. S. A*. 101, 10205–10210.

Hizume M., Shibata F., Matsuki Y., Garajova M., 2002. Chromosome identification and comparative analysis of four *Pinus* species. *Theoretical and Applied Genetics*, 105(4), pp. 491–497.

Hódar, J.A., Torres-Muros, L., Zamora, R., Pérez-Luque, A.J. and Senhadji, K., 2015. No evidence of induced defence after defoliation in three pine species against an expanding pest, the pine processionary moth. *Forest Ecology and Management*, 356, pp.166-172.

Holopainen, J.K. & Gershenzon, J., 2010. Multiple stress factors and the emission of plant VOCs. *Trends in Plant Science*, 15(3), pp.176–184.

Holroyd, G. H., Hetherington, A. M., & Gray, J. E., 2002. A role for the cuticular waxes in the environmental control of stomatal development. *New Phytologist*, 153(3), pp. 433–439.

Höltta T, Cochard H, Nikinmaa E, Mencuccini M (2009) Capacitive effect of cavitation in xylem conduits: results from a dynamic model. Plant Cell Environ 32:10–21.

Hoque, M. A., Okuma, E., Banu, M. N. A., Nakamura, Y., Shimoishi, Y., & Murata, Y., 2007. Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betaine by increasing antioxidant enzyme activities. *Journal of Plant Physiology*, 164(5), pp. 553–61.

Huang A.H.C., Moore T.S., Trelease R.N., 1983. Plant peroxisomes. Academic Press, New York, NY, USA

Hume D.,1748, tr 2007. An Enquiry concerning Human Understanding, 1st published 1748. Oxford, Oxford University Press.

Hurme P., Sillanpaa M.J., Arjas E., Repo T., Savolainen O., 2000. Genetic basis of climatic adaptation in Scots pine by Bayesian quantitative trait locus analysis. *Genetics* 156(3), pp. 1309–1322

Iijima H, Shibuya M, Saito H, Takahashi K., 2006. The water relation of seedlings of Picea jezoensis on fallen logs. *Can J For Res* 36: 664–670.

Ings, J., Mur, L. A.J., Robson, P. R. H., & Bosch, M., 2013. Physiological and growth responses to water deficit in the bioenergy crop *Miscanthus x giganteus*. *Frontiers in Plant Science*, 4(November), p. 468.

IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

IPCC. 2007. Climate Change 2007: synthesis report. In Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Eds. R.K. Pachauri and A. Reisinger. IPCC, Geneva, Switzerland, 104 p.

Ito, H., H. Gaubert, E. Bucher, M. Mirouze, I. Vaillant, and J. Paszkowski. 2011. An siRNA pathway prevents transgenerational retrotransposition in plants subjected to stress. *Nature* 472(7341), pp. 115–119.

Jasińska AK, Wachowiak W, Muchewicz E, Boratyńska K, Montserrat JM, Boratyński A. 2010. Cryptic hybrids between *Pinus uncinata* and *P.sylvestris*. *Botanical Journal of the Linnean Society*, 163(4), pp. 473– 85.

Johannsen, W., 1911. The genotype conception of heredity. *The American Naturalist,* 45(531), pp. 129-159.

Johnson, N. C., & Gehring, C. A., 2007. Mycorrhizas: symbiotic mediators of rhizosphere and ecosystem processes. *The Rhizosphere: An Ecological Perspective*, 73-100.

Jonsson, P., Johansson, A.I., Gullberg, J., Trygg, J., Grung, B., Marklund, S., Sjöström, M., Antti, H. and Moritz, T., 2005. High-throughput data analysis for detecting and identifying differences between samples in GC/MS-based metabolomic analyses. *Analytical chemistry*, 77(17), pp.5635-5642.

Joyner K.L., Wang, X-R., Johnston, J.S., Price H.J., Williams C.G., 2001. DNA content for Asian pines parallels New World relatives. *Canadian Journal of Botany,* 79: 192Y196.

Kane, J.M., Meinhardt, K.A., Chang, T., Cardall, B.L., Michalet, R. and Whitham, T.G., 2011. Drought-induced mortality of a foundation species (*Juniperus monosperma*) promotes positive afterlife effects in understory vegetation. *Plant Ecology*, 212(5), pp.733-741.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M., 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 44, D457-D462.

Kant, I., 1785, tr 2007. The Moral Law; Groundwork of the Metaphysics of Morals, 3rd Edition, 1st published in German in 1785. London, Routledge.

Karhu, A., Hurme, P., Karjalainen, M., Karvonen, P., Kärkkäinen, K., Neale, D., & Savolainen, O., 1996. Do molecular markers reflect patterns of differentiation in adaptive traits of conifers? TAG. Theoretical and Applied Genetics. *Theoretische Und Angewandte Genetik*, 93(1-2), 215–21.

Karpinska, B., Karlsson, M., Schinkel, H., Streller, S., Süss, K.H., Melzer, M. and Wingsle, G., 2001. A novel superoxide dismutase with a high isoelectric point in higher plants. Expression, regulation, and protein localization. *Plant Physiology*, *126*(4), pp.1668-1677.

Karvonen, P., & Savolainen, O., 1993. Variation and inheritance of ribosomal DNA in *Pinus sylvestris* L. (Scots pine). *Heredity*, 71(6), 614–622.

Kawecki, T.J. & Ebert, D., 2004. Conceptual issues in local adaptation. *Ecology Letters*, 7(12), pp.1225–1241.

Keeling, C.I. & Bohlmann, J., 2006. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytologist*, 170(4), pp.657–675.

Kell, D.B., 2012. Scientific discovery as a combinatorial optimisation problem: How best to navigate the landscape of possible experiments? *BioEssays*, 34(3), pp.236–244.

Kelly, G., Moshelion, M., David-Schwartz, R., Halperin, O., Wallach, R., Attia, Z., … Granot, D., 2013. Hexokinase mediates stomatal closure. *The Plant Journal : For Cell and Molecular Biology*, 75(6), 977–88.

Kenny, L.C., Dunn, W.B., Ellis, D.I., Myers, J., Baker, P.N., Kell, D.B. and GOPEC Consortium, 2005. Novel biomarkers for pre-eclampsia detected using metabolomics and machine learning. *Metabolomics*, 1(3), pp.227-234.

Kimmerer, T.W. and MacDonald, R.C., 1987. Acetaldehyde and ethanol biosynthesis in leaves of plants. Plant Physiology, 84(4), pp.1204-1209.

Kipfer, T. et al., 2012. Growth response of drought-stressed *Pinus sylvestris* seedlings to single- and multi-species inoculation with ectomycorrhizal fungi. *PloS one*, 7(4), p.e35275.

Kloosterman, B., Anithakumari, A., Chibon, P.-Y., Oortwijn, M., van der Linden, G. C., Visser, R. G., & Bachem, C. W., 2012. Organ specificity and transcriptional control of metabolic routes revealed by expression QTL profiling of source--sink tissues in a segregating potato population. *BMC Plant Biology*, 12(1), 17.

Kolbe A, Oliver SN, Fernie AR, Stitt M, van Dongen JT, Geigenberger P., 2006. Combined transcript and metabolite profiling of *Arabidopsis* leaves reveals fundamental effects of the thiol-disulfide status on plant metabolism. *Plant Physiology*, 141: 412–422.

Kovach, A. et al., 2010. The *Pinus taeda* genome is characterized by diverse and highly diverged repetitive sequences. *BMC genomics*, 11, p.420.

Krajnc, A.U., Kristl, J. & Ivancic, A., 2011. Forest Ecology and Management Application of salicylic acid induces antioxidant defense responses in the phloem of Picea abies and inhibits colonization by Ips typographus. *Forest Ecology and Management*, 261(3), pp.416–426.

Kramer PJ, Boyer JS. 1995. Water relations of plants and soils. San Diego: Academic Press, Inc.

Kremer, A. et al., 2012. Long-distance gene flow and adaptation of forest trees to rapid climate change. *Ecology Letters*, 15(4), pp.378–392.

Kujala S., Savolainen O., 2012. Sequence variation patterns along a latitudinal cline in Scots pine (*Pinus sylvestris*): signs of clinal adaptation? *Tree Genetics & Genomes* 8(6):1451–1467.

Kullman, L., 2002. Rapid recent range-margin rise of tree and shrub species in the Swedish *Scandes. J. Ecol*. 90, 68–77.

Kumar, A. and Bennetzen, J.L. 1999. Plant retrotransposons. *Annual Review of Genetics*. 33: 479–532.

Lamy, J.-B., Delzon, S., Bouche, P. S., Alia, R., Vendramin, G. G., Cochard, H. and Plomion, C., 2014. Limited genetic variability and phenotypic plasticity detected for cavitation resistance in a Mediterranean pine. *New Phytologist,* 201(3), 874–886.

Lanz, C. et al., 2009. Radiation Metabolomics . 3 . Biomarker Discovery in the Urine of Gamma- Irradiated Rats Using a Simplified Metabolomics Protocol of Gas Chromatography-Mass Spectrometry Combined with Random Forests Machine Learning Algorithm Radiation Metabolomics. *Radiation research*, 172, pp.198–212.

Latowski, D., Kuczyńska, P. and Strzałka, K., 2011. Xanthophyll cycle – a mechanism protecting plants against oxidative stress. *Redox Report*, 16(2), pp.78-90.

Lenhard, J. & Winsberg, E., 2010. Studies in History and Philosophy of Modern Physics Holism , entrenchment , and the future of climate model pluralism. *Studies in History and Philosophy of Modern Physics*, 41(3), pp.253–262.

Lenoir, J., Gégout, J.C., Pierrat, J.C., Bontemps, J.D. and Dhôte, J.F., 2009. Differences between tree species seedling and adult altitudinal distribution in mountain forests during the recent warm period (1986–2006). *Ecography*, 32(5), pp.765-777.

Levesque-Tremblay, G., Havaux, M., & Ouellet, F., 2009. The chloroplastic lipocalin AtCHL prevents lipid peroxidation and protects *Arabidopsis* against oxidative stress. *The Plant Journal : For Cell and Molecular Biology*, 60(4), 691–702.

Lewis OT., 2006. Climate change, species–area curves and the extinction crisis. *Philisophical Transactions of the Royal Society B*, 361(1465), 163–171.

Li T, Hu YJ, Hao ZP, Li H, Wang YS, Chen BD., 2012. First cloning and characterization of two functional aquaporin genes from an arbuscular mycorrhizal fungus Glomus intraradices. *New Phytologist*, 197, 617–630.

Lira-Medeiros, C.F., Parisod, C., Fernandes, R.A., Mata, C.S., Cardoso, M.A. and Ferreira, P.C.G., 2010. Epigenetic variation in mangrove plants occurring in contrasting natural environment. *PLOS One*, 5(4), pp.1–8.

Lloret, F., Casanovas, C., & Peñuelas, J., 1999. Seedling survival of Mediterranean shrubland species in relation to root : shoot ratio, seed size and water and nitrogen use. *Functional Ecology*, 13(2), 210–216.

Lloret, F., Penuelas, J. & Estiarte, M., 2004. Experimental evidence of reduced diversity of seedlings due to climate modification in a Mediterranean-type community. *Global Change Biology*, 10, pp.248–258.

Lloret, F., Peñuelas, J., Prieto, P., Llorens, L., Estiarte, M., 2009. Plant community changes induced by experimental climate change: Seedling and adult species composition. *Perspectives in Plant Ecology, Evolution and Systematics*, 11(1), 53-63.

Lloyd, E.A., 1987. Confirmation of Ecological and Evolutionary Models. *Biology & philosophy*, 2, pp.277–293.

Lokvam, J., Brenes-Arguedas, T., Lee, J.S., Coley, P.D. and Kursar, T.A., 2006. Allelochemic function for a primary metabolite: the case of L-tyrosine hyper-production in *Inga umbellifera* (Fabaceae). *American journal of botany*, 93(8), pp.1109-1115.

López, R., Aranda, I., & Gil, L., 2009. Osmotic adjustment is a significant mechanism of drought resistance in *Pinus pinaster* and *Pinus canariensis*, 18(2), 159–166.

López, R., Rodríguez-Calcerrada, J. and Gil, L., 2009. Physiological and morphological response to water deficit in seedlings of five provenances of *Pinus canariensis*: potential to detect variation in drought-tolerance. *Trees*, *23*(3), pp.509-519.

Lopez-Huertas, E., Charlton, W.L., Johnson, B., Graham, I.A. and Baker, A., 2000. Stress induces peroxisome biogenesis genes. *The EMBO Journal*, *19*(24), pp.6770-6777.

Lopez-Iglesias, B., Villar, R., & Poorter, L., 2014. Functional traits predict drought performance and distribution of Mediterranean woody species. *Acta Oecologica*, 56, 10–18.

Loranty, M. M., Mackay, D. S., Ewers, B. E., Traver, E., & Kruger, E. L., 2010. Competition for light between individual trees lowers reference canopy stomatal conductance: Results from a model. *Journal of Geophysical Research*, 115(G4), G04019.

Lorenz, E.N.,1963. Deterministic non-periodic flow. *Journal of the Atmospheric Sciences*, 20, 130–141

Luna, E., Bruce, T.J., Roberts, M.R., Flors, V. and Ton, J., 2012. Next-generation systemic acquired resistance. *Plant Physiology*, 158(2), pp.844-853.

Ma, X. F., Hall, D., Onge, K. R. S., Jansson, S., & Ingvarsson, P. K., 2010. Genetic differentiation, clinal variation and phenotypic associations with growth cessation across the Populus tremula photoperiodic pathway. *Genetics*, *186*(3), 1033-1044.

Maeda, H. & Dudareva, N., 2012. The Shikimate Pathway and Aromatic Amino Acid Biosynthesis in Plants. *Annual review of plant biology*, 63, pp.73–105.

Mak, T.D., Laiakis, E.C., Goudarzi, M. and Fornace Jr, A.J., 2013. Metabolyzer: A novel statistical workflow for analyzing postprocessed lc–ms metabolomics data. *Analytical chemistry*, 86(1), pp.506-513.

Makkonen, K. & Helmisaari, H., 2002. Fine-root biomass and production in Scots pine stands in relation to stand age. *Tree Physiology*, 21, pp.193–198.

Malcolm, J. et al., 2006. Global Warming and Extinctions of Endemic Species from Biodiversity Hotspots. *Conservation biology*, 20(2), pp.538–548.

Marjanović, Ž., Uehlein, N., Kaldenhoff, R., Zwiazek, J.J., Weiß, M., Hampp, R. and Nehls, U., 2005. Aquaporins in poplar: what a difference a symbiont makes!. *Planta*, *222*(2), pp.258-268.

Markesteijn, L. & Poorter, L., 2009. Seedling root morphology and biomass allocation of 62 tropical tree species in relation to drought- and shade-tolerance. *Journal of Ecology*, 97, pp.311–325.

Markesteijn, L., Poorter, L., Bongers, F., Paz, H. and Sack, L., 2011. Hydraulics and life history of tropical dry forest tree species: coordination of species' drought and shade tolerance. *New Phytologist*, *191*(2), pp.480-495.

Martin, R. C., Liu, P.-P., Goloviznina, N. a., & Nonogaki, H., 2010. microRNA, seeds, and Darwin?: diverse function of miRNA in seed biology and plant responses to stress. *Journal of Experimental Botany*, 61: 2229–2234.

Martínez-Álvarez, P., Fernández-González, R.A., Sanz-Ros, A.V., Pando, V. and Diez, J.J., 2016. Two fungal endophytes reduce the severity of pitch canker disease in Pinus radiata seedlings. *Biological Control*, 94, pp.1-10.

Martinez-Vilalta, J. et al., 2010. Interspecific variation in functional traits, not climatic differences among species ranges, determines demographic rates across 44 temperate and Mediterranean tree species. *Journal of Ecology*, 98, pp.1462–1475.

Martinez-Vilalta, J., Pinol, J., & Beven, K., 2002. A hydraulic model to predict drought-induced mortality in woody plants : an application to climate change in the Mediterranean. *Ecological Modelling*, 155, 127–147.

Matías Resina, L., 2011. Efectos del cambio climático sobre la regeneración del bosque mediterráneo: una aproximación experimental. UNIVERSIDAD DE GRANADA. Available at: http://tdx.cesca.cat/handle/10803/17985.

Matías, L. & Jump, A.S., 2014. Impacts of predicted climate change on recruitment at the geographical limits of Scots pine. *Journal of Experimental Botany*, 65(1), pp.299–310.

Matías, L., Zamora, R., Castro, J., 2011. Repercussions of simulated climate change on the diversity of woody-recruit bank in a Mediterranean-type ecosystem. *Ecosystems* 14, 672–682.

Matsuda, F., Okazaki, Y., Oikawa, A., Kusano, M., Nakabayashi, R., Kikuchi, J., Yonemaru, J.I., Ebana, K., Yano, M. and Saito, K., 2012. Dissection of genotype–phenotype associations in rice grains using metabolome quantitative trait loci analysis. *The Plant Journal*, 70(4), pp.624-636.

Mayr E. 1947. Ecological factors in speciation. *Evolution* 1: 263–288.

McAdam, S. a M., Brodribb, T. J., Ross, J. J., & Jordan, G. J., 2011. Augmentation of abscisic acid (ABA) levels by drought does not induce short-term stomatal sensitivity to CO2 in two divergent conifer species. *Journal of Experimental Botany*, 62(1), 195–203.

McClintock B, 1984. The significance of responses of the genome to challenge. *Science* 226: 792–801.

McDowell, N. et al., 2013. Tansley review: Evaluating theories of drought-induced vegetation mortality using a multimodel – experiment framework. *New Phytologist*, 200, pp.304–321.

McDowell, N. G., 2011. Mechanisms linking drought, hydraulics, carbon metabolism, and vegetation mortality. *Plant Physiology*, 155(3), 1051–9.

McDowell, N., Pockman, W. T., Allen, C. D., Breshears, D. D., Cobb, N., Kolb, T., … Yepez, E. a., 2008. Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytologist*, 178(4), 719–39.

Mcdowell, N.G. & Sevanto, S., 2010. The mechanisms of carbon starvation : how , when , or does it even occur at all ? *New Phytologist*, 186(2), pp.264–266.

McDowell, N.G., Fisher, R.A., Xu, C., Domec, J.C., Hölttä, T., Mackay, D.S., Sperry, J.S., Boutz, A., Dickman, L., Gehres, N. and Limousin, J.M., 2013. Evaluating theories of drought-induced vegetation mortality using a multimodel–experiment framework. *New Phytologist*, 200(2), pp.304-321.

McKay JK, Latta RG, 2002. Adaptive population divergence: markers, QTL and traits. *Trends in Ecology and Evolution*, 17, 285–291.

McNair, J.N., Sunkara, A. & Frobish, D., 2012. How to analyse seed germination data using statistical time-to-event analysis: non-parametric and semi-parametric methods. *Seed Science Research*, 22(02), pp.77–95.

Meier IC, LeuschnerC(2007)Genotypic variation and phenotypic plasticity in the drought response of fine roots of European beech. *Tree Physiology* 28:297–309

Meijon, M. et al., 2016. Exploring natural variation of *Pinus pinaster* Aiton using metabolomics : Is it possible to identify the region of origin of a pine from its metabolites ? *Molecular Ecology*, 25, pp.959–976.

Meinzer, F. C., McCulloh, K. a, Lachenbruch, B., Woodruff, D. R., & Johnson, D. M., 2010. The blind men and the elephant: the impact of context and scale in evaluating conflicts between plant hydraulic safety and efficiency. *Oecologia*, 164(2), 287–96.

Mencuccini, M., Piussi, P. & Sulli, a Z., 1995. Thirty years of seed production in a subalpine Norway spruce forest : Patterns of temporal and spatial variation. , 76, pp.109–125.

Mendoza, I. et al., 2009. Recruitment limitation of forest communities in a degraded Mediterranean landscape. *Journal of Vegetation Science*, 20(2), pp.367–376.

Menni, C., Graham, D., Kastenmüller, G., Alharbi, N.H., Alsanosi, S.M., McBride, M., Mangino, M., Titcombe, P., Shin, S.Y., Psatha, M. and Geisendorfer, T., 2015. Metabolomic identification of a novel pathway of blood pressure regulation involving hexadecanedioate. *Hypertension*, *66*(2), pp.422-429.

Mikkelson, G.M., 2001. Complexity and verisimilitude: realism for ecology. *Biology and Philosophy*, *16*(4), pp.533-546.

Miksche, J. P., 1968. Quantitative study of intraspecific variation of DNA per cell in *Picea glauca* and *Pinus banksiana*. *Can. J. Genet. Cytol*. 10: 590–600.

Miller, G., Shulaev, V. & Mittler, R., 2008. Reactive oxygen signaling and abiotic stress. *Physiologia Plantarum*, 133, pp.481–489.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., & Mittler, R., 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell & Environment*, 33(4), 453–67.

Miyazawa, S.-I., Livingston, N. J., & Turpin, D. H., 2006. Stomatal development in new leaves is related to the stomatal conductance of mature leaves in poplar (Populus trichocarpaxP. deltoides). *Journal of Experimental Botany*, 57(2), 373–80.

Morgan, M. S., & Morrison, M. (Eds.).,1999. Models as mediators: Perspectives on natural and social science (Vol. 52). Cambridge University Press.
Morse, A.M. et al., 2009. Evolution of genome size and complexity in *Pinus*. *PLoS ONE*, 4(2), pp.1–11.
Mueller, R.C. et al., 2005. Differential tree mortality in response to severe drought: evidence for long-term vegetation shifts. *Journal of Ecology*, 93(6), pp.1085–1093.
Muilu-Mäkelä, R. et al., 2015. Water availability influences morphology, mycorrhizal associations, PSII efficiency and polyamine metabolism at early growth phase of Scots pine seedlings. *Plant Physiology and Biochemistry*, 88, pp.70–81.
Mumm, R. et al., 2003. Chemical analysis of volatiles emitted by *Pinus sylvestris* after induction by insect oviposition. Journal of Chemical Ecology, 29(5), pp.1235–1252.
Münch E., 1930. Die stoffbewegungen in der pflanze. Gustav Fischer, Jena, Germany.
Munne-Bosch, S., Jubany-Mari, T., & Alegre, L., 2001. Drought-induced senescence is characterized by a loss of antioxidant defences in chloroplasts. Plant, Cell and Environment, 24(12), 1319–1327.
Munns, R., 2005. Genes and salt tolerance : bringing them together.
Naglreiter, C. et al., 2005. Free radical generation in *Pinus sylvestris* and Larix decidua seeds primed with polyethylene glycol or potassium salt solutions. *Plant physiology and biochemistry : PPB*, 43(2), pp.117–23.
Narusaka, Y. et al., 2004. Crosstalk in the responses to abiotic and biotic stresses in *Arabidopsis* : Analysis of gene expression in cytochrome P450 gene superfamily by cDNA microarray. Plant *Molecular Biology*, 55, pp.327–342.
Narusaka, Y. et al., 2004. Crosstalk in the responses to abiotic and biotic stresses in *Arabidopsis* : Analysis of gene expression in cytochrome P450 gene superfamily by cDNA microarray. *Plant Molecular Biology*, 55, pp.327–342.
Nazareno, A.G. and Jump, A.S., 2012. Species–genetic diversity correlations in habitat fragmentation can be biased by small sample sizes. *Molecular ecology*, *21*(12), pp.2847-2849.
Neale D, Wegrzyn J, Stevens K, Zimin A, Puiu D, Crepeau M, *et al.* 2014. Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome Biology*, 15(3):R59
Neale, D.B. et al., 2014. Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome biology*, 15(3), p.R59.
Newton, E.L., Bullock, J.M. and Hodgson, D.J., 2009. Glucosinolate polymorphism in wild cabbage (Brassica oleracea) influences the structure of herbivore communities. *Oecologia*, 160(1), pp.63-76.
Nguyen-Queyrens, A. & Bouchet-Lannat, F., 2003. Osmotic adjustment in three-year-old seedlings of five provenances of maritime pine (*Pinus pinaster*) in response to drought. *Tree physiology*, 23(6), pp.397–404.
Nguyen-Queyrens, A., & Bouchet-Lannat, F., 2003. Osmotic adjustment in three-year-old seedlings of five provenances of maritime pine (*Pinus pinaster*) in response to drought. Tree Physiology, 23(6), 397–404.
Niklas, K.J., 1994. *Plant allometry: the scaling of form and process*. University of Chicago Press.
Nkongolo, M. Mehes-Smith, 2012. Karyotype evolution in the Pinaceae: implication with molecular phylogeny *Genome*, 55(10): 735-753

Noctor G, Foyer CH. Ascorbate and glutathione: Keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol,* 49,249-79.

Nordström, A., Want, E., Northen, T., Lehtiö, J. and Siuzdak, G., 2008. Multiple ionization mass spectrometry strategy used to reveal the complexity of metabolomics. *Analytical chemistry*, 80(2), pp.421-429.

Notivol E., Garcia-Gil M.R., Alia R., Savolainen O., 2007. Genetic variation of growth rhythm traits in the limits of a latitudinal cline in Scots pine. *Canadian Journal of Forest Research*, 37,540–551.

Nystedt B, Street NR, Wetterbom A, Zuccolo A, Lin Y-C, Scofield DG, *et al.*, 2013. The Norway spruce genome sequence and conifer genome evolution. *Nature*, 497(7451):579–84.

O'Malley, M. & Dupre, J., 2005. Fundamental issues in systems biology. *BioEssays*, 27(12), pp.1270–1276.

Obata, T. & Fernie, A.R., 2012. The use of metabolomics to dissect plant responses to abiotic stresses. *Cellular and molecular life sciences : CMLS*, 69, pp.3225–3243.

Oleksyn, J., Reich, P.B., Zytkowiak, R., Karolewski, P. and Tjoelker, M.G., 2002. Needle nutrients in geographically diverse *Pinus sylvestris* L. populations. *Annals of Forest Science*, *59*(1), pp.1-18

Oleksyn, J., Tjoelker, M.G. & Reich, P.B., 1998. Adaptation to Changing Environment in Scots Pine Populations across a Latitudinal Gradient. *Silva Fennica*, 32(2), pp.129–140.

Oleksyn, J., Zytkowiak, R., Karolewski, P., Reich, P.B. and Tjoelker, M.G., 2000. Genetic and environmental control of seasonal carbohydrate dynamics in trees of diverse *Pinus sylvestris* populations. *Tree Physiology*, *20*(12), pp.837-847.

Olmo, M., Lopez-Iglesias, B. and Villar, R., 2014. Drought changes the structure and elemental composition of very fine roots in seedlings of ten woody tree species. Implications for a drier climate. *Plant and soil*, *384*(1-2), pp.113-129.

Onofri, A., Gresta, F. & Tei, F., 2010. A new method for the analysis of germination and emergence data of weed species. *Weed Research*, 50(3), pp.187–198.

Ozolincius, R., Stakenas, V., Serafinaviciute˙ , B., Buozyte˙ , R., 2009. Effects of artificial soil drought on Scots pine fruiting, seed vitality, and pollen germination. *Ekologija* 55, 189–195.

Padilla, F.M. & Pugnaire, F.I., 2007. Rooting depth and soil moisture control Mediterranean woody seedling survival during drought. *Functional Ecology*, 21(3), pp.489–495.

Padilla, F.M., Miranda, J.D.D. & Pugnaire, F.I., 2007. Early root growth plasticity in seedlings of three Mediterranean woody species. *Plant and Soil*, 296(1-2), pp.103–113.

Paiva, J.A.P. et al., 2008. Molecular and phenotypic profiling from the base to the crown in maritime pine wood-forming tissue. *New Phytologist*, 178, pp.283–301.

Pantin, F., Monnet, F., Jannaud, D., Costa, J.M., Renaud, J., Muller, B., Simonneau, T. and Genty, B., 2013. The dual effect of abscisic acid on stomata. *New Phytologist*, *197*(1), pp.65-72.

Pearson RG, Dawson TP: Predicting the impacts of climate change on the distribution of species: are bioclimate envelope models useful? *Global Ecology & Biogeography* 2003, 12, 361–371.

Perdiguero, P. et al., 2012. Identification of water stress genes in *Pinus pinaster* Ait. by controlled progressive stress and suppression-subtractive hybridization. *Plant physiology and biochemistry : PPB*, 50(1), pp.44–53.

Perdiguero, P. et al., 2013. Molecular response to water stress in two contrasting Mediterranean pines (*Pinus pinaster* and *Pinus pinea*). *Plant physiology and biochemistry*, 67, pp.199–208.

Petit, R. J., Hampe, A., 2006. Some Evolutionary Consequences of Being a Tree. *Annual Review of Ecology, Evolution, and Systematics,* 37, 187–214.

Petit, R. J., Hu, F. S., & Dick, C. W., 2008. Forests of the past: a window to future changes. *Science*, 320, 1450–2.

Pfennig, D. W. Wund, M. A. Snell-Rood, E. C. Cruick- shank, T. Schlichting, C. D. and Moczek, A. P., 2010. Phenotypic plasticity's impacts on diversification and speciation, *Trends in Ecology and Evolution*, vol. 25, no. 8, pp. 459–467.

Pietsch, W., 2015. Aspects of Theory-Ladenness in Data-Intensive Science. In: [2014] Philosophy of Science Assoc. 24th Biennial Mtg (Chicago, IL). 82(December), pp.905–916.

Plato, c 380 BC, tr 1946. The Republic. Book VII, p249-268. c. 380 BC. The World Publishing Company, Cleveland.

Polle, a., McKee, I. & Blaschke, L., 2001. Altered physiological and growth responses to elevated [CO_2] in offspring from holm oak (*Quercus ilex* L.) mother trees with lifetime exposure to naturally elevated [CO_2]. *Plant, Cell and Environment*, 24(10), pp.1075–1083.

Polle, A., 2001. Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. *Plant Physiology*, 126(1), 445–62.

Poorter, H. et al., 2012. Biomass allocation to leaves, stems and roots: meta-analyses of interspecific variation and environmental control. *New phytologist*, 193(1), pp.30–50.

Poorter, L., 2007. Are species adapted to their regeneration niche, adult niche, or both? *The American naturalist*, 169(4), pp.433–442.

Popper, K (1958, tr 1968). The Logic of Scientific Discovery. 5th edition, 1st edition published in 1959. Hutchinson, London

Posada, D. & Buckley, T., 2004. Model Selection and Model Averaging in Phylogenetics : Advantages of Akaike. *Systematic Biology*, 53(5), pp.793–808.

Poskitt, A.D.S. & Tremayne, A.R., 1987. Determining a Portfolio of Linear Time Series Models. *Biometrika*, 74(1), pp.125–137.

Poulton JE, Møller BL: Glucosinolates. *Meth Plant Biochem* 9: 209–237 (1993).

Poyatos R, Aguade D, Galiano L, Mencuccini M & Martinez-Vilalta J., 2013. Drought-induced defoliation and long periods of near-zero gas exchange play a key role in accentuating metabolic decline of Scots pine. *New Phytologist*,

Prasad, A.M., Iverson, L.R. & Liaw, A., 2006. Newer Classification and Regression Tree Techniques : Bagging and Random Forests for Ecological Prediction. *Techniques for Ecological Prediction*, 9(December), pp.181–199.

Prasch, C.M. and Sonnewald, U., 2013. Simultaneous application of heat, drought, and virus to *Arabidopsis* plants reveals significant shifts in signaling networks. *Plant Physiology*, 162(4), pp.1849-1866.

Prentice, I., Bondeau, A., Cramer, W., Harrison, S., Hickler, T., Lucht, W., Sitch, S., Smith, B. & Sykes, M. (2007) Dynamic global vegetation model- ing: quantifying

terrestrial ecosystem responses to large-scale environmental change. Terrestrial Ecosystems in a Changing World Global Change — The IGBP Series (closed). (eds J.G. Canadell, D.E. Pataki & L.F. Pitelka), pp. 175–192. Springer, Berlin, Heidelberg.

Puzey, J.R. et al., 2012. Deep Annotation of Populus trichocarpa microRNAs from Diverse Tissue Sets. *PLoS ONE*, 7(3), p.e33034.

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raj, S. et al., 2011. Clone history shapes Populus drought responses. *PNAS*, pp.1–6.

Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B. and Meijer, J., 2000. Myrosinase: gene family evolution and herbivore defense in Brassicaceae. In *Plant Molecular Evolution* (pp. 93-113). Springer Netherlands.

Raven, J.A. and Edwards, D., 2001. Roots: evolutionary origins and biogeochemical significance. *Journal of Experimental Botany*, *52*(suppl 1), pp.381-401.

Redmond, M.D. et al., 2015. Woodland recovery following drought-induced tree mortality across an environmental stress gradient. *Global Change Biology*,

Rehfeldt, G. E.; Stage, A. R.; Bingham, R. T., 1971. Strobili Development in Western White Pine: Periodicity, Prediction, and Association with Weather. Forest Science, Volume 17, pp. 454-461(8)

Reich, P.B., Oleksyn, J. and Tjoelker, M.G., 1994. Seed mass effects on germination and growth of diverse European Scots pine populations. *Canadian Journal of Forest Research*, 24(2), pp.306-320.

Richards, D.E., King, K.E., Ait-ali, T. and Harberd, N.P., 2001. How gibberellin regulates plant growth and development: a molecular genetic analysis of gibberellin signaling. *Annual review of plant biology*, *52*(1), pp.67-88.

Richter, S. et al., 2012. Phenotypic plasticity facilitates resistance to climate change in a highly variable environment. *Oecologia*, 169(1), pp.269–79.

Rigling, A. et al., 2013. Driving factors of a vegetation shift from Scots pine to pubescent oak in dry Alpine forests. *Global Change Biology*, 19(1), pp.229–240.

Rivero, R. M., Shulaev, V., & Blumwald, E., 2009. Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. *Plant Physiology*, 150(3), 1530–40. doi:10.1104/pp.109.139378

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S. and Mittler, R., 2004. When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. *Plant physiology*, *134*(4), pp.1683-1696.

Robinson, A.R. et al., 2007. Metabolite profiling of Douglas-fir (*Pseudotsuga menziesii*) field trials reveals strong environmental and weak genetic variation. *New phytologist*, 174(4), pp.762–73.

Robledo-Arnuncio JJ, Gil L. 2005. Patterns of pollen dispersal in a small population of *Pinus sylvestris* L. revealed by total-exclusion paternity analysis. *Heredity* 94: 13–22.

Robledo-Arnuncio, J., 2011. Wind pollination over mesoscale distances: An investigation with Scots pine. *New Phytologist*, 190: 222-233.

Rodríguez-Calcerrada, J., Atkin, O. K., Robson, T. M., Zaragoza-Castells, J., Gil, L., & Aranda, I., 2010. Thermal acclimation of leaf dark respiration of beech seedlings experiencing summer drought in high and low light environments. *Tree Physiology*, 30(2), 214–24.

Rook, F., Corke, F., Card, R., Munz, G., Smith, C. and Bevan, M.W., 2001. Impaired sucrose-induction mutants reveal the modulation of sugar-induced starch biosynthetic gene expression by abscisic acid signalling. *The Plant Journal*, 26(4), pp.421-433.

Rosa, M., Prado, C., Podazza, G., Interdonato, R., González, J. a, Hilal, M., & Prado, F. E., 2009. Soluble sugars--metabolism, sensing and abiotic stress: a complex network in the life of plants. *Plant Signaling & Behavior*, 4(5), 388–393. doi:10.4161/psb.4.5.8294

Rowland, L. et al., 2015. Death from drought in tropical forests is triggered by hydraulics not carbon starvation. *Nature*, 528(7580), pp.119–122.

Salmela, M.J., 2011. Adaptive genetic variation in Scots pine (*Pinus sylvestris* L .) in Scotland. The University of Edinburgh.

Sanchez-Vallet, A. et al., 2010. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. *The Plant Journal*, 63, pp.115–127.

Savolainen O, Pyhajarvi T, 2007. Genomic diversity in forest trees. *Current Opinion in Plant Biology*, 10, 162–167.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C. and Manners, J.M., 2000. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proceedings of the National Academy of Sciences, 97(21), pp.11655-11660.

Schlichting, C. D. (2004) The role of phenotypic plasticity in diversification, in Phenotypic Plasticity: Functional and Conceptual Approaches, T. J. DeWitt and S. M. Scheiner, Eds., pp. 191–200, Oxford University Press.

Schwab, W., 2003. Metabolome diversity : too few genes , too many metabolites ? *Phytochemistry*, 62(April), pp.837–849.

Sexton, J.P., Strauss, S.Y. & Rice, K.J., 2011. Gene flow increases fitness at the warm edge of a species' range. *Proceedings of the National Academy of Sciences of the United States of America*, 108(28), pp.11704–9.

Shen, M, Piao, S,Dorji, T,Liu, Q, Cong, N,Chen, X,An, S,Wang, S,Wang, T,Zhang, G, 2015. Plant phenological responses to climate change on the Tibetan Plateau: research status and challenges. *National Science Review*.

Shukla, L. I., Chinnusamy, V., & Sunkar, R., 2008. The role of microRNAs and other endogenous small RNAs in plant stress responses. *Biochimica et Biophysica Acta*, 1779(11), pp. 743–748.

Slotkin RK,Martienssen R, 2007. Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet* 8, pp. 272–285

Smilde,A.K. et al., 2005. ANOVA-simultaneous component analysis (ASCA): a new tool for analyzing designed metabolomics data. *Bioinformatics*, 21, 3043–3048.

Smirnoff, N. & Cumbes, J., 1989. Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry*, 28(4), pp.1057–1060.

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London.

Smith, C.C. and Fretwell, S.D. (1974) The optimal balance between size and number of offspring. *The American Naturalist,* 108, 499–506.

Snell, R. S., Huth, a., Nabel, J. E. M. S., Bocedi, G., Travis, J. M. J., Gravel, D., … Lischke, H., 2014. Using dynamic vegetation models to simulate plant range shifts. *Ecography*, 1–14.

Sober, E., 2002. Instrumentalism, Parsimony and the Akaike Framework. *Philosophy of Science*, 69(September), pp.112–123.

Southern, J., Pitt-Francis, J., Whiteley, J., Stokeley, D., Kobashi, H., Nobes, R., Kadooka, Y. and Gavaghan, D., 2008. Multi-scale computational modelling in biology and physiology. *Progress in biophysics and molecular biology*, 96(1), pp.60-89.

Steinkamp, J, Hickler, T, 2015. Is drought-induced forest dieback globally increasing? *Journal of Ecology*, 103, pp. 31–43.

Sternberg, M., Brown, V.K., Masters, G.J., Clarke, I.P., 1999. Plant community dynamics in a calcareous grassland under climate change manipulations. *Plant Ecology*, 143, pp. 29–37.

Strange, K., 2005. The end of "naıve reductionism": rise of systems biology or renaissance of physiology ? *American Journal of Physiology - Cell Physiology*, 2520, pp.968–974.

Strevens, M. Notes on Bayesian Confirmation Theory. 2006. Available online: www.nyu.edu/gsas/dept/philo/user/strevens/Classes/Conf06/BCT.pdf (accessed on 1 March 2016). [Google Scholar]

Strevens, M., 2001. The Bayesian treatment of auxiliary hypotheses. *The British Journal for the Philosophy of Science*, *52*(3), pp.515-537.

Strodtkötter, I., Padmasree, K., Dinakar, C., Speth, B., Niazi, P. S., Wojtera, J., … Scheibe, R. (2009). Induction of the AOX1D isoform of alternative oxidase in A. thaliana T-DNA insertion lines lacking isoform AOX1A is insufficient to optimize photosynthesis when treated with antimycin A. *Molecular Plant*, 2(2), pp. 284–97.

Suarez, A.V. and Tsutsui, N.D., 2008. The evolutionary consequences of biological invasions. *Molecular Ecology*, *17*(1), pp.351-360.

Sulmon, C., Gouesbet, G., El Amrani, A. and Couée, I., 2006. Sugar-induced tolerance to the herbicide atrazine in *Arabidopsis* seedlings involves activation of oxidative and xenobiotic stress responses. *Plant cell reports*, 25(5), pp.489-498.

Suzuki, H. et al. (2005) Methyl jasmonate and yeast elicitor induce differential transcriptional and metabolic re-programming in cell suspension cultures of the model legume *Medicago truncatula*. *Planta* 220, 696–707

Svetnik, V., Liaw, A., Tong, C., Culberson, J.C., Sheridan, R.P. and Feuston, B.P., 2003. Random forest: a classification and regression tool for compound classification and QSAR modeling. *Journal of chemical information and computer sciences*, 43(6), pp.1947-1958.

Szabados, L. and Savoure, A., 2010. Proline: a multifunctional amino acid. *Trends in plant science*, *15*(2), pp.89-97.

Taeger, S., Fussi, B., Konnert, M. and Menzel, A., 2013. Large-scale genetic structure and drought-induced effects on European Scots pine (*Pinus sylvestris* L.) seedlings. *European Journal of Forest Research*, *132*(3), pp.481-496.

Taeger, S., Sparks, T.H. and Menzel, A., 2015. Effects of temperature and drought manipulations on seedlings of Scots pine provenances. *Plant Biology*, *17*(2), pp.361-372.

Taiz, L., Zeiger, E., 1998. Plant Physiology, second ed., Sineaur, Massachusetts.

Tammela, P., Salo-Väänänen, P., Laakso, I., Hopia, A., Vuorela, H. and Nygren, M., 2005. Tocopherols, tocotrienols and fatty acids as indicators of natural ageing in *Pinus sylvestris* seeds. *Scandinavian Journal of Forest Research*, *20*(5), pp.378-384.

Taneda, H., & Sperry, J. S., 2008. A case-study of water transport in co-occurring ring-versus diffuse-porous trees: contrasts in water-status, conducting capacity, cavitation and vessel refilling. *Tree Physiology*, 28(11), pp. 1641–51.

Tanevski, J., 2016. Learning stochastic process-based models of dynamical systems from knowledge and data. *BMC Systems Biology*, pp.1–17.

Taylor, L.L., Leake, J.R., Quirk, J., Hardy, K., Banwart, S.A. and Beerling, D.J., 2009. Biological weathering and the long-term carbon cycle: integrating mycorrhizal evolution and function into the current paradigm. *Geobiology*, *7*(2), pp.171-191.

Tekleva, M. V., Polevova, S. V. and Zavialova, N. E. 2007. On some peculiarities of sporoderm structure in members of the Cycadales and Ginkgoales. *Paleontol*. J. 41, pp. 1162-1178.

Theophrastus., c. 350 - 287 BC, tr 1916. Enquiry into plants, and minor works on odours and weather signs. Volume I and II. Heinemann, London.

Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, Collingham YC, Erasmus BFN, de Siqueira MF, Grainger A, Hannah L, Hughes L, Huntley B, van Jaarsveld AS, Midgley GF, Miles L, Ortega-Huerta MA, Townsend Peterson A, Phillips OL, Williams SE., 2004. Extinction risk from climate change. *Nature*, 427, pp. 145-148.

Thuiller, W. et al. 2013. A road map for integrating eco-evolutionary processes into biodiversity models. – *Ecol. Lett*. 16: 94–105.

Thuiller, W., Lavore, S., Araujo, M.B., Sykes, M.T., Prentice, I.C., 2005. Climate change threats to plant diversity in Europe. *Proceedings of the National Academy of Sciences*, 102, 8245–8250.

Tíscar, P., & Lucas, M., 2010. Seed mass variation, germination time and seedling performance in a population of Pinus nigra subsp. salzamannii. *Forest Systems*, 19(3), 344–353.

Tong C, Zhang B, Wang Z, Xu M, Pang XM, Si JN, Huang MR, Wu RL., 2011. Multiallelic epistatic model for an outbred cross and mapping algorithm of interactive quantitative trait loci. *BMC Plant Biol*, 11:148.

Tricker, P.J., López, C.M.R., Gibbings, G., et al., 2013a. Transgenerational, dynamic methylation of stomata genes in response to low relative humidity. *International journal of molecular sciences*, 14(4), pp.6674–89.

Tricker, P.J., López, C.M.R., Hadley, P., et al., 2013b. Pre-conditioning the epigenetic response to high vapor pressure deficit increases the drought tolerance of *Arabidopsis thaliana*. , pp.8–10.

Türkan, I. et al., 2005. Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant P. acutifolius Gray and drought-sensitive P. vulgaris L. subjected to polyethylene glycol mediated water stress. *Plant Science*, 168(1), pp.223–231.

Tyree M, Zimmerman MH. 2002. Xylem structure and the ascent of sap. Berlin: Springer.

Tyree, M.T., Cochard, H., Cruiziat, P., Sinclair, B. and Ameglio, T., 1993. Drought-induced leaf shedding in walnut: evidence for vulnerability segmentation. *Plant, Cell & Environment*, *16*(7), pp.879-882.

Uehlein, N., Fileschi, K., Eckert, M., Bienert, G.P., Bertl, A. and Kaldenhoff, R., 2007. Arbuscular mycorrhizal symbiosis and plant aquaporin expression. *Phytochemistry*, *68*(1), pp.122-129.

Unger, G.M., Vendramin, G.G. & Robledo-Arnuncio, J.J., 2014. Estimating exotic gene flow into native pine stands: zygotic vs. gametic components. *Molecular Ecology,* 23(22), pp.5435–5447.

Urbanczyk-Wochniak E., Baxter C., Kolbe A., Kopka J., Sweetlove L. J., Fernie A. R. (2005). Profiling of diurnal patterns of metabolite and transcript abundance in potato (*Solanum tuberosum*) leaves. *Planta*, 221, 891–90310.

Urbieta, I. R., García, L. V., Zavala, M. a., & Marañón, T., 2011. Mediterranean pine and oak distribution in southern Spain: Is there a mismatch between regeneration and adult distribution? *Journal of Vegetation Science*, 22(1), 18–31.

Valkonen, J.P.T., Nygren, M., Ylonen, A., Mannonen, L., 1994. Nuclear DNA content of *Pinus sylvestris* L. as determined by laser flow cytometry. *Genetica* 92, 203–207.

Vance, N.C. and Zaerr, J.B., 1990. Analysis by high-performance liquid chromatography of free amino acids extracted from needles of drought-stressed and shaded Pinus ponderosa seedlings. *Physiologia Plantarum*, *79*(1), pp.23-30.

Vandesompele, J. et al., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology*, 3(7), p. 34.

Varis, S. et al., 2009. The extent of south-north pollen transfer in Finnish Scots pine. *Silva Fennica*, 43(5), pp.717–726.

Vendramin, G.G. Fady, B González-Martínez, S., Hu, F.S. Scotti, I Sebastiani, F. Soto, A. Petit, R., 2008. Genetically depauperate but widespread: the case of emblematic Mediterranean pine, *Evolution* 62.

Verslues, P.E. & Bray, E., 2006. Role of abscisic acid (ABA) and *Arabidopsis thaliana* ABA-insensitive loci in low water potential-induced ABA and proline accumulation. *Journal of experimental botany*, 57(1), pp.201–12.

Via, S., & Lande, R., 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution*, 505e522.

Viant, M.R. & Sommer, U., 2012. Mass spectrometry based environmental metabolomics: a primer and review. *Metabolomics*, 9(S1), pp.144–158.

Vilà-Cabrera, A., Martínez-Vilalta, J., & Retana, J., 2014. Variation in reproduction and growth in declining Scots pine populations. *Perspectives in Plant Ecology, Evolution and Systematics*, 16(3), 111–120.

Vivas, M. et al., 2013. Environmental maternal effects mediate the resistance of maritime pine to biotic stress. *PloS one*, 8(7), p.e70148.

Voronova, A. et al., 2014. Stress-induced transcriptional activation of retrotransposon-like sequences in the Scots pine (*Pinus sylvestris* L.) genome. *Tree Genetics & Genomes*, 10(4), pp.937–951.

Vuosku, J. et al., 2009. One tissue , two fates : different roles of megagametophyte cells during Scots pine embryogenesis. *Journal of experimental botany*, 60(4), pp.1375–1386.

Wachowiak W, Balk P, Savolainen O., 2009. Search for nucleotide diversity patterns of local adaptation in dehydrins and other cold- related candidate genes in Scots pine (*Pinus sylvestris* L.). *Tree Genetics & Genomes* 5(1), pp. 117–132

Wachowiak W, Palme AE, Savolainen O., 2011. Speciation history of three closely related pines *Pinus mugo* (T.), *P. uliginosa* (N.) and *P. sylvestris* (L.). *Molecular Ecology*, 20(8), pp. 1729–43

Wachowiak, W., Trivedi, U., Perry, A., & Cavers, S., 2015. Comparative transcriptomics of a complex of four European pine species. *BMC Genomics*, 16(1).

Wachowiak, W., Wójkiewicz, B., Cavers, S., & Lewandowski, A., 2014. High genetic similarity between Polish and North European Scots pine (*Pinus sylvestris* L.) populations at nuclear gene loci. *Tree Genetics & Genomes*, pp. 1015–1025.

Wakamiya I, Newton RJ, Johnston JS, Price HJ., 1993. Genome Size and Environmental Factors in the Genus Pinus. *American Journal of Botany*, 80(11), pp. 1235-1241.

Way, D. a., Crawley, C. & Sage, R.F., 2013. A hot and dry future: Warming effects on boreal tree drought tolerance. *Tree Physiology*, 33(10), pp.1003–1005.

Weber R.J.M., & Viant, M.R. (2010). MI-Pack: increased confidence of metabolite identification in mass spectra by integrating accurate masses and metabolic pathways. Chemom Intell Lab Syst. 104,75–82. doi: 10.1016/j.chemolab.2010.04.010

Wehner, J. et al., 2010. Plant pathogen protection by arbuscular mycorrhizas: A role for fungal diversity? *Pedobiologia*, 53(3), pp.197–201.

Weigel, A.P., Knutti, R., Liniger, M.A. and Appenzeller, C., 2010. Risks of model weighting in multimodel climate projections. *Journal of Climate*, 23(15), pp.4175-4191.

Weigel, A.P., Liniger, M.A. & Appenzeller, C., 2008. Can multi-model combination really enhance the prediction skill of probabilistic ensemble forecasts ? *Quarterly Journal Of The Royal Meteorological Society*, 260, pp.241–260.

Wennström, U., Bergsten, U. & Nilsson, J.-E., 2002. Effects of Seed Weight and Seed Type on Early Seedling Growth of *Pinus sylvestris* under Harsh and Optimal Conditions. *Scandinavian Journal of Forest Research*, 17(2), pp.118–130.

West-Eberhard, M.J., 1986. Alternative adaptations, speciation, and phylogeny. Proceedings of the National Academy of Sciences of the United States of America,vol. 83 (5), pp. 1388–1392.

West-Eberhard, M.J., 1989. Phenotypic plasticity and the origins of diversity. *Annual review of Ecology and Systematics*, 20, pp. 249–278, 1989.

Wilson ID, Nicholson JK, Castro-Perez J, Granger JH, Johnson KA, Smith BW, Plumb RS. High resolution "Ultra performance" liquid chromatography coupled to oa-TOF mass spectrometry as a tool for differential metabolic pathway profiling in functional genomic studies. *J Proteom Res*. 2005;4:591–598.

Wingsle, G. & Karpinski, S., 1996. Differential redox regulation by glutathione of glutathione reductase and CuZn superoxide dismutase genes expression in *Pinus sylvestris* (L.) needles. *Planta*, 198, pp.151–157.

Woltereck, R., 1909. 'Weitere experimentelle Untersuchungen über Artveränderung, speziel über das Wesen quantitativer Artunterschiede bei Daphnien', Verhandlungen der deutschen zoologischen Gesellschaft 19, pp. 110–173.

Wu, Q.-S., Xia, R.-X. & Zou, Y.-N., 2008. Improved soil structure and citrus growth after inoculation with three arbuscular mycorrhizal fungi under drought stress. *European Journal of Soil Biology*, 44(1), pp.122–128.

Wujeska, A., Bossinger, G. & Tausz, M., 2013. Responses of foliar antioxidative and photoprotective defence systems of trees to drought: a meta-analysis. *Tree physiology*, pp.1–12.

Wyman, J., Laliberte, S. & Tremblay, M.F., 1997. Nuclear DNA content variation in seeds from 22 half-sib families of jack pine (Pinus banksiana, Pinaceae). *American Journal of Botany*, 84(10), pp.1351–1361.

Yakovlev, I.A., Fossdal, C.G. & Johnsen, Ø., 2010. MicroRNAs , the epigenetic memory and climatic adaptation in Norway spruce. *New Phytologist*, 187, pp.1154–1169.

Zamora, R., Hódar, J.A., Matías, L. and Mendoza, I., 2010. Positive adjacency effects mediated by seed disperser birds in pine plantations. *Ecological Applications*, 20(4), pp.1053-1060.

Zas, R., Cendán, C. & Sampedro, L., 2013. Mediation of seed provisioning in the transmission of environmental maternal effects in Maritime pine (*Pinus pinaster* Aiton). *Heredity*, 111(3), pp.248–55.

Zas, R., Cendán, C. & Sampedro, L., 2013. Mediation of seed provisioning in the transmission of environmental maternal effects in Maritime pine (*Pinus pinaster* Aiton). *Heredity*, 111(3), pp.248–55.

Zelinka, I., Chen, G. and Celikovsky, S., 2008. Chaos synthesis by means of evolutionary algorithms. *International Journal of Bifurcation and Chaos*, 18(04), pp.911-942.

Zhang, J.H., Nishimura, N., Okubo, A., and Yamazaki, S. 2002. Development of an analytical method for the determination of betaines in higher plants by capillary electrophoresis at low pH. *Phytochem. Anal*. 13(4), pp. 189–194.

Zhao, J., Last, R.L. & Williams, C.C., 1998. Induction of *Arabidopsis* tryptophan pathway enzymes and camalexin by amino acid starvation, oxidative stress, and an abiotic elicitor. *Plant Cell*, 10(March), pp.359–370.

Zhao, Y. et al., 2015. A metabolomics study delineating geographical location-associated primary metabolic changes in the leaves of growing tobacco plants by GC-MS and CE-MS. *Nature*, 9(April), pp.1–11.

Zhou, X. F., Jin, Y. H., Yoo, C. Y., Lin, X.-L., Kim, W.-Y., Yun, D.-J., Jin, J. B. (2013). CYCLIN H;1 regulates drought stress responses and blue light-induced stomatal opening by inhibiting reactive oxygen species accumulation in *Arabidopsis*. *Plant Physiology*, 162(2), 1030–41.

Zhu, Z., Wei, G., Li, J., Qian, Q. and Yu, J., 2004. Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (Cucumis sativus L.). Plant Science, 167(3), pp.527-533.

Zhu, J. et al., 2006. Effects of drought stresses induced by polyethylene glycol on germination of *Pinus sylvestris* var. mongolica seeds from natural and plantation forests on sandy land. *Journal of Forest Research*, 11(5), pp.319–328.

Zonneveld, B. J. M., 2012. Conifer genome sizes of 172 species, covering 64 of 67 genera, range from 8 to 72 picogram. *Nordic Journal of Botany*, 30, pp. 490–502.

Zotti, M. & Pautasso, M., 2013. Macrofungi in Mediterranean *Quercus ilex* woodlands : relations to vegetation structure, ecological gradients and higher-taxon approach. *Czech Mycology*, 65(2), pp.193–218.