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Author(s):
Bachofen, Christoph

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Pine regeneration under future climate conditions: effects of local adaptation and acclimation

A dissertation submitted to attain the degree of
DOCTOR OF SCIENCES OF ETH ZÜRICH

presented by
CHRISTOPH BACHOFEN

MSc ETH in Biology
born September 1, 1983
citizen of Uster (ZH)

accepted on the recommendation of

Prof. Dr. Jaboury Ghazoul, examiner
Prof. Dr. Maurizio Mencuccini, co-examiner
Dr. Thomas Wohlgemuth, co-examiner
Dr. Barbara Moser, co-examiner

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Table of contents

Summary

Forest regeneration relies on the successful initial establishment of seedlings that tolerate the environmental conditions to which they are exposed. Pines are stand-forming at growth-limited sites at the cold and dry forest edge. Frost damage and drought stress are thus important ecological factors limiting pine seedling establishment. At their southern distribution limit, pines have been repeatedly observed to suffer from drought-mortality, and the predicted increase of temperatures and duration of drought events is expected to lead to even higher mortality rates. Regular exposure to drought might have led to the evolution of particular morphological and physiological traits that resulted in local adaptation of pine populations at the southern distribution limit. In Europe, bioclimatic envelope models therefore predict a range shift of more drought-tolerant Mediterranean pine species or populations to higher latitudes following climate change. The main objective of this thesis was to assess whether the regeneration potentials of *Pinus sylvestris* L., *Pinus nigra* Arnold and *Pinus halepensis* Mill. populations from different Mediterranean locations are higher than that of *P. sylvestris* from Central Alpine locations under future climatic conditions.

The aim of the *first chapter* was to study the relationship between local adaptation to drought and biomass partitioning in pine seedlings subjected to different water regimes. Shoot and root biomass of a total of 1890 *P. sylvestris* and *P. nigra* seedlings of nine origins was measured and the interactive effects of the water regimes, age, size and seed origin on biomass partitioning determined. Oxygen isotope fractionation was measured as an estimate of stomatal conductance and related to the seed origin. Less drought adapted seedlings initially responded to a constant dry climate by increasing root biomass fraction by 6%, and to severe drought by reducing root biomass fraction by 15%. Seedlings from dry locations had higher stomatal conductance and maintained similar biomass partitioning as under well-watered conditions, indicating an effect of high root biomass partitioning on assimilation and growth. Acclimation of the seedlings resulted in similar biomass partitioning in all water regimes in the subsequent year. Local adaptation to drought was thus most important for first-time drought-responses, whereas acclimation resulted in similar growth responses thereafter.

In the *second chapter* we focused on the effect of lethal drought-stress and future elevated CO_2 concentrations to the carbon metabolism of pine seedlings. We repeatedly measured biomass, starch and soluble sugars in needles, stem and roots in a total of 1890 *P. sylvestris* and *P. nigra* seedlings of nine origins. The drought resulted in 9.4% mortality in the aftermath. In the first season, drought led to 44% lower biomass and 48% higher starch concentrations. Elevated CO_2 did not reduce drought mortality, and resulted in an additional increase of needle starch concentrations from 2.98% to 4.01%, which indicates impaired phloem transport. The elevated carbon storage of drought-stressed seedlings is thus expected to be a result of the severe growth limitations and continued assimilation during the drought. In the second season, starch concentrations were not elevated in response to drought (except *P. sylvestris* roots) and damage from drought was minor, indicating acclimation.

The *third chapter* focuses frost limitations of drought-adapted Mediterranean pine seedlings. We compared late winter and spring cold hardiness of nine *P. sylvestris*, *P. nigra,* and *P. halepensis* populations of Mediterranean origin with that of Central Alpine *P. sylvestris*. We did not observe local adaptation to frost of the pine populations and cold hardiness did only differ between species. Both *P. sylvestris* and *P. nigra* seedlings maintained extremely wide safety margins between 34.2 K and 14.9 K (March 22 and April 19) throughout spring, whereas *P. halepensis* might suffer from regularly occurring low temperatures in late winter in Central Alpine valleys. A wide range of drought-tolerant populations of *P. sylvestris* and *P. nigra* exhibited thus no local adaptation to frost. Range shift from the Mediterranean to

Central Europe is thus not limited by intermittent cold temperature extremes in spring.

Overall, the findings of this thesis revealed a very high drought tolerance of all pine seedlings in the second and third growing season. Local adaptation to drought was observed for biomass partitioning, but not for carbon storage or cold hardiness. Rapid acclimation led to markedly lower drought responses of the seedlings in subsequent years, which shows that short-term drought responses might lead to underestimating the resistance of pine seedlings to future climatic conditions.

Zusammenfassung

Zur Verjüngung von Wäldern braucht es eine erfolgreiche Ansiedlung von Jungpflanzen, welche den Umweltbedingungen standhalten können, denen sie ausgesetzt sind. Föhren sind bestandesbildend an Standorten, wo Kälte und Trockenheit das Baumwachstum limitieren. Frost- und Trockenstress sind deshalb wichtige ökologische Faktoren, welche die Ansiedlung von Föhren-Sämlingen begrenzen. In ihrem südlichen Verbreitungsgebiet leiden Föhren oft an hoher Mortalität, die durch Trockenheit verursacht wird. Diese wird durch den prognostizierten Temperaturanstieg und länger anhaltende Trockenperioden noch verstärkt werden. Regelmässiger Trockenstress könnte jedoch auch zur Ausbildung von morphologischen und physiologischen Merkmalen geführt haben, welche sich in lokaler Anpassung von Populationen am südlichen Verbreitungsgebiet zeigen. Verbreitungsmodelle prognostizieren deshalb eine Ausbreitung trockenheitstoleranter Mediterraner Föhrenarten und -populationen in nördliche Europäische Breitengrade unter zukünftigen Klimabedingungen. Das Ziel dieser Doktorarbeit war es, zu beurteilen, ob die Verjüngung von Mediterranen Populationen von *Pinus sylvestris* L., *Pinus nigra* Arnold und *Pinus halepensis* Mill. sich von derjenigen der Zentralalpinen *P. sylvestris* Populationen unter zukünftigen Klimabedingungen unterscheidet.

Im *ersten Kapitel* wurde untersucht, ob bei unterschiedlicher Wasserverfügbarkeit ein Zusammenhang zwischen lokaler Anpassung an Trockenheit und der Verteilung der Pflanzenbiomasse in Wurzel und Spross besteht. Spross- und Wurzelbiomasse wurde an insgesamt 1890 *P. sylvestris* und *P. nigra* Sämlingen aus neun Herkunftsgebieten gemessen, und die wechselwirkenden Effekte von Wasserverfügbarkeit, Alter, Grösse und Herkunft auf die Biomassenverteilung

bestimmt. Sauerstoff-Isotopen wurden gemessen, um die stomatäre Leitfähigkeit der verschiedenen Populationen abzuschätzen. Sämlinge, die nicht trockenangepasst waren, reagierten zunächst auf konstant trockenes Klima mit einer Erhöhung der Wurzelbiomasse um 6%, und auf starken Trockenstress mit einer Reduktion um 15%. Trockenangepasste Sämlinge zeigten eine höhere stomatäre Leitfähigkeit und behielten gleiche Biomassenverteilung wie unter ausreichender Bewässerung bei, was auf einen positiven Effekt von hoher Wurzelbiomasse auf Assimilation schliessen lässt. Die Akklimatisierung führte zu einer ähnlichen Biomassenverteilung der Sämlinge unter allen Bewässerungsbedingungen im Folgejahr. Anpassung an lokale Trockenheit war daher vor allem während der erstmaligen Trockenreaktion ausschlaggebend, wohingegen danach Akklimatisierung zu ähnlichen Wachstumsreaktionen unter allen Bewässerungsbedingungen führte.

Im *zweiten Kapitel* wurde der Effekt von letalem Trockenstress und erhöhter CO_2-Konzentration auf den Kohlenstoffmetabolismus der Föhrensämlinge untersucht. Dazu haben wir wiederholt Biomasse, Stärke- und Zuckerkonzentration in Nadeln, Stämmen und Wurzeln von insgesamt 1890 *P. sylvestris* und *P. nigra* Sämlingen aus neun Herkunftsgebieten gemessen. Die Trockenheit führte zu 9.4% Mortalität im Anschluss an die Trockenperiode. In der ersten Saison führte die Trockenheit zu einer um 44% verringerten Biomasse und einer um 48% erhöhten Stärkekonzentration. Erhöhte CO_2-Konzentrationen reduzierten die Mortalität nicht und führten zur einer weiter erhöhten Stärkekonzentration in den Nadeln (von 2.98% auf 4.01%), was auf einen reduzierten Phloemtransport schliessen lässt. Die erhöhte Kohlenstoffspeicherung bei trockengestressten Sämlingen ist daher das Resultat der starken Wachstumslimitierung und fortgesetzten Assimilation bei Trockenheit. In der zweiten Saison waren die Stärkekonzentrationen bei trockengestressten Sämlingen nicht mehr erhöht (mit Ausnahme von *P. sylvestris*-Wurzeln), und Schäden durch die Trockenheit gering, was auf Akklimatisierung schliessen lässt.

Das Ziel des *dritten Kapitels* war es, Frostlimitierung von trockenangepassten Mediterranen Föhrensämlingen zu untersuchen. Dazu haben wir die Frosttoleranz von neun Mediterranen *P. sylvestris*, *P. nigra* und *P. halepensis*-Populationen im Spätwinter und Frühling mit derjenigen Zentralalpiner *P. sylvestris*-Populationen verglichen. Wir haben keine lokale Anpassung an Frost beobachtet, aber die Frosttoleranz unterschied sich zwischen den Arten. Sowohl *P. sylvestris*, als auch *P. nigra nigra*-Sämlinge hatten sehr breite Sicherheitsmargen zwischen 34.2 K und 14.9 K (22. März und 19. April) im ganzen Frühling, wohingegen *P. halepensis*-Sämlinge voraussichtlich unter den wiederkehrenden Frosttemperaturen in Zentralalpinen Tälern schaden nehmen. Unterschiedlich trockentolerante Mediterrane *P. sylvestris* und *P. nigra* Populationen zeigten somit keine lokale Anpassung an Frost. Die prognostizierte Ausbreitung vom Mittelmeerraum nach Mitteleuropa ist für diese Arten deshalb nicht limitiert durch periodische Frühlingsfröste.

Zusammenfassend zeigen die Resultate dieser Dissertation, dass Föhrensämlinge ab dem zweiten Standjahr extrem trockenangepasst sind. Anpassung an lokale Bedingungen war anhand Biomasseverteilung zu beobachten, aber nicht anhand von Kohlenstoffspeicherung oder Frosttoleranz. Die schnelle Akklimatisierung führte zu deutlich geringeren Trockenreaktionen der Sämlinge im Folgejahr, was aufzeigt, dass Schlussfolgerungen aus kurzzeitigen Trockenreaktionen zur Unterschätzung der Widerstandsfähigkeit von Föhrensämlingen gegenüber Klimaeffekten führen können.

General Introduction

Background

Forest regeneration

Seedling establishment in forest gaps following disturbances is essential for ensuring the persistence of forests. The success of forest regeneration depends on a multitude of factors that act during the various regeneration phases (Grubb, 1977; Pardos *et al.*, 2005). Regeneration from soil or aerial seed banks relies on factors such as the interval of seed production (Pardos *et al.*, 2005), the number and size of seeds (Greene *et al.*, 1999) and the distance from a seed tree (Moser *et al.*, 2010). During emergence and initial establishment of a seedling, survival is determined by the environment to which it is exposed (Collins & Good, 1987). The regeneration niche usually differs from the habitat niche of adult individuals (Grubb, 1977). In particular, seedlings are considered to be more susceptible to environmental stress such as drought or frost than adult trees. Important ecological factors limiting seedling establishment include light and water availability, frost damage, litter cover, competition with grasses or shrubs and herbivory (Grubb, 1977; Zackrisson *et al.*, 1995; Keeley & Zedler, 2000; González-Martínez & Bravo, 2001; Castro *et al.*, 2004; Pardos *et al.*, 2005). The relative importance of these factors depends on life history traits of tree species in the particular forest community.

The regeneration niche of Pinus sylvestris

Conifers are generally adapted to disturbed sites or sites with low nutrients and predominate in locations where they face little competition from other plants (Keeley & Zedler, 2000). Pines, in particular, can be considered "aggressive post-disturbance invaders" (Keeley & Zedler, 2000) with a high light and moderate soil fertility requirement. They are thus stand-forming at disturbed or growth limited sites at the cold and dry forest edges (Keeley & Zedler, 2000). Regeneration of conifers may thus be primarily limited by low water availability and cold temperatures. One of the most frost-tolerant species among *Pinus* is *P. sylvestris* L. with a wide distribution area, ranging from Siberia to the Mediterranean (Fig. 1) and with populations that are adapted to local climatic conditions (Keeley & Zedler, 2000). Local adaptation potentially affects the relative importance of limiting environmental factors for regeneration. In Mediterranean climates, for instance, frost plays a lesser role than drought (Castro *et al.*, 2004), in contrast to the boreal climate (Zackrisson *et al.*, 1995). At the northern distribution limit, seedling growth is primarily suppressed by light and nutrient competition with adult trees (Kuuluvainen, 1994; Valkonen, 2000). In contrast, *P. sylvestris* prefers moderate light conditions that lower evapotranspiration at the southern distribution limit in the Mediterranean Basin (Pardos *et al.*, 2007), where summer drought is one of the most limiting factors for seedlings establishment (Castro *et al.*, 2005; Pardos *et al.*, 2005; Moreno-Fernández *et al.*, 2015). In valleys of the Central Alps, drought limits seedling establishment (Weber *et al.*, 2007; Moser *et al.*, 2010; Rigling *et al.*, 2013) and winter temperatures regularly fall below -10 °C. Forest regeneration in the Central Alps therefore relies on seedlings that are adapted to drought, but also to the recurring low winter temperatures.

Adaptations of pines to drought stress

Most pines are adapted to drought, either by tolerating or avoiding desiccation (Rundel & Yoder, 1998). General responses of trees to drought stress include reduced growth (i.e. reduced cell division and expansion; Hsiao, 1973), reduced stomatal conductance (Buckley, 2005) associated with increased water-use efficiency (Klein *et al.*, 2013; Lévesque *et al.*, 2014), and other processes inherent to plants, such as reduced protein synthesis and suppressed respiration (Hsiao, 1973). The magnitude and type of response depends on the severity of drought (Hsiao, 1973). For instance, growth is reduced at a lower drought intensity than photosynthesis (Boyer, 1970; Muller *et al.*, 2011; Fatichi *et al.*, 2014). Furthermore, the responses can be modified by environmental conditions, such as the stomatal response to drought, which is amplified under high irradiance (Rundel & Yoder, 1998). In many locations, pines are at their dry distribution limit and suffer mortality from severe drought events (Hartmann *et al.*, 2015), such as *P. sylvestris* in northern Spain (Martínez-Vilalta & Piñol, 2002; Castro *et al.*, 2004; Galiano *et al.*, 2010) and Central Alpine valleys (Dobbertin *et al.*, 2007; Rigling *et al.*, 2013; Schuster & Oberhuber, 2013) and *Pinus edulis* in south-western North America (Breshears *et al.*, 2005; Gaylord *et al.*, 2013). With increasing temperatures, drought stress is expected to increase and lead to elevated mortality, particularly at the dry distribution limits of the species (McDowell *et al.*, 2011; Martínez-Vilalta *et al.*, 2012; Allen *et al.*, 2015). *P. sylvestris*, the "model species" for studies on drought-mortality (Martínez-Vilalta *et al.*, 2012), is assumed to be severely growth limited and suffer from elevated drought mortality in large part of Europe with changing climatic conditions (Reich & Oleksyn, 2008).

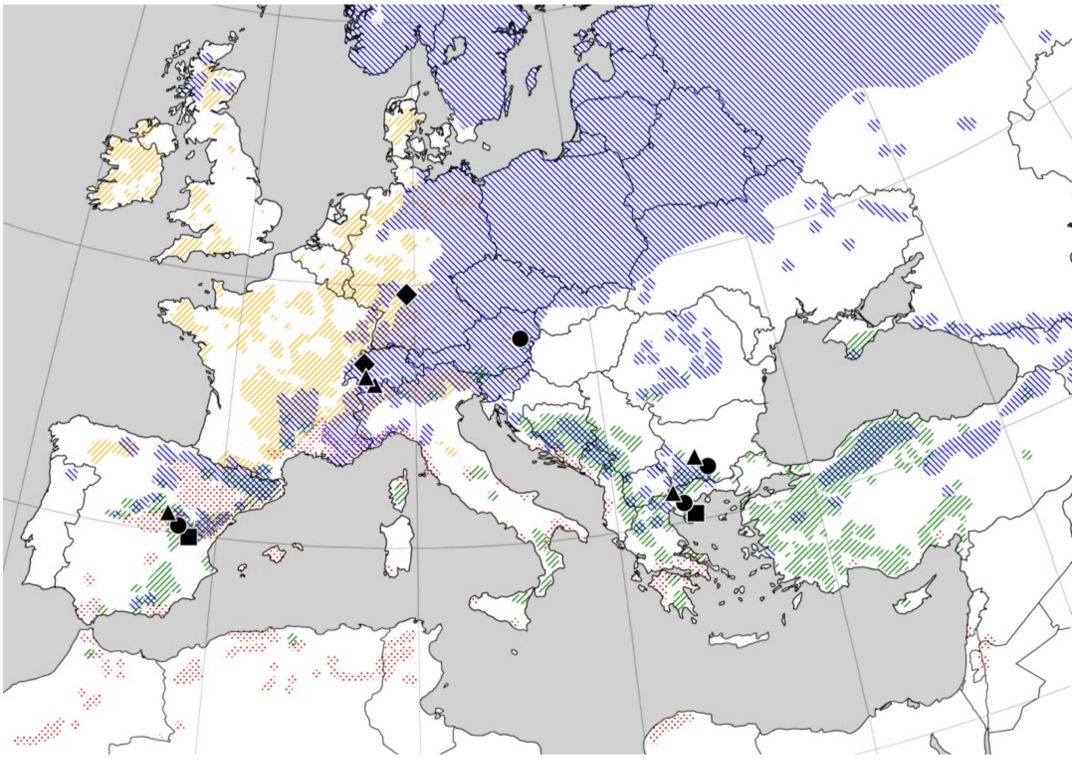


Fig 1. Distribution of *Pinus sylvestris* (blue dash), *Pinus nigra* (green dash), *Pinus halepensis* (red dots) and *Pseudotsuga menziesii* (yellow dash) in Europe and the Mediterranean, edited after EUFORGEN (www.euforgen.org) and (Schmid *et al.*, 2014). The locations of the seed sources are indicated with triangles (*P. sylvestris*), circles (*P. nigra*), squares (*P. halepensis*) and diamonds (*P. menziesii*; additional information s. Table 1).

Frost tolerance of pines

Cold temperatures are one of the most decisive abiotic factors that shape the distribution of tree species worldwide (Sakai & Larcher, 1987). Resistance to frost is therefore crucial for forest regeneration (Colombo *et al.*, 2001). It is strongly related to the temperatures in the distribution range of a species (Kreyling *et al.*, 2015) and can be as low as -90 °C in *P. sylvestris* during winter (Bannister & Neuner, 2001). Species differ largely their resistance to frost (Strimbeck *et al.*, 2007). Furthermore, populations from the same species have been repeatedly shown to differ in their frost resistance, potentially in relation to low temperatures at their origin (Beuker *et al.*, 1998; Aldrete *et al.*, 2008; Kreyling *et al.*, 2012). Resistance to cold temperatures changes dramatically during the season, particularly in spring (Repo, 1992) when cold hardiness is at its lowest (Weiser, 1970). Dehardening in spring is mainly driven by temperature (Repo, 1992; Leinonen *et al.*, 1997) and is tightly coupled with tree phenology (Lenz *et al.*, 2013; Vitasse *et al.*, 2014). It has been considered the most critical time during the year, limiting the distribution of temperate tree species (Parker, 1963). Spring cold hardiness is of particular interest, because bud burst is expected to advance in spring due to increasing temperatures (Parmesan & Yohe, 2003), but frost intensities and durations are expected to remain constant (Vavrus *et al.*, 2006; Kodra *et al.*, 2011), which could lead to an increase in frost damage (Beuker *et al.*, 1998; Gu *et al.*, 2008).

Interrelations of drought and frost tolerance

Frost and drought may select for contrasting growth strategies: high frost tolerance is linked to later bud brake, resulting in a shorter growing season length (Lenz *et al.*, 2013). In drought-tolerant *P. sylvestris* from Mediterranean regions, growth is, however, restricted to spring due to summer drought (Castro *et al.*, 2004). Selection for drought-tolerance might thus counteract selection for frost tolerance. Is has indeed been suggested that the genetic differentiation of drought-tolerant *P. sylvestris* populations from Iberia (Soranzo *et al.*, 2000) is related to reduced frost tolerance (Cheddadi *et al.*, 2006). There are, on the other hand, physiological adaptation that are related for frost and drought tolerance, such as an increased activity of enzymes that protect from oxidative stress (Blödner *et al.*, 2005) and xylem that is resistant to cavitation from both drought and winter desiccation (Lens *et al.*, 2013). Such interrelations might have an important effect on the drought- and frost-tolerances of populations from different locations of the wide distribution range of *P. sylvestris*.

Effects of increased CO_2 on tree growth

Atmospheric CO_2 concentrations have been rising since more than 150 years as a result of human activities. The pre-industrial concentration of 278 ppm is expected to more than double and reach 550–850 ppm by 2100 (*IPCC*, 2007). An immediate effect for plants is the enhanced carboxylation rate by Rubisco, thus an increased CO_2 assimilation (Franks *et al.*, 2013). Consequently, tree growth could be stimulated by what is referred to as 'CO_2 fertilization' (Zhu *et al.*, 2016). There are, however, several interacting processes in plants that may restrict CO_2 fertilization effects (Leuzinger & Hättenschwiler, 2013). For forests, the effect of increased CO_2 appears, indeed, to be limited (Bader *et al.*, 2013; Lévesque *et al.*, 2014; Camarero *et al.*, 2015) and to depend heavily on other growth conditions, such as nutrient availability (Körner, 2006; Norby *et al.*, 2010). Furthermore, plants have been shown to react to increased CO_2 with changes in stomatal conductance in order to reduce water loss (Picon-Cochard & Guehl, 1999; Franks *et al.*, 2013). Reduced stomatal conductance decreases the water loss by leaf transpiration, but also lowers the CO_2 uptake. Prolonged drought might thus lead to decreased CO_2 assimilation and consequently to reduced non-structural carbohydrates (NSC), potentially leading to higher tree mortality (McDowell *et al.*, 2011). With higher concentrations of atmospheric CO_2, diffusion into the leaves is enhanced and more CO_2 is taken up at a given stomatal conductance, resulting in a higher water-use efficiency (WUE; Battipaglia *et al.*, 2013). Stomatal conductance could therefore be reduced during drought while CO_2 uptake is largely maintained. An elevated concentration of atmospheric CO_2 might thus mitigate negative effects of drought stress on plants (Wullschleger *et al.*, 2002; Leuzinger & Körner, 2007). Effects on stomatal conductance and growth are, however, species-specific and depend on the actual drought conditions and other environmental effects (Hättenschwiler & Körner, 2000; Keel *et al.*, 2006; Linares *et al.*, 2009) and higher WUE does not necessarily translate to higher growth rates (Bernal *et al*., 2011; Peñuelas *et al*., 2011; Lévesque *et al*., 2014). Consequently, interactive effects of increased CO_2 and drought on trees cannot be precisely anticipated and need to be studied for the particular species and environment.

Research objectives and study design

Research objectives

The main objective of this doctoral thesis was to compare the regeneration potentials of *Pinus sylvestris* L. populations from different locations of the southern distribution limit to more drought-adapted *P. nigra* Arnold and *P. halepensis* Mill. populations under future climatic conditions. Mediterranean pine populations have been shown to exhibit a larger investment in roots than northern populations (Matías *et al*., 2014; Taeger *et al*., 2015), which is considered an essential trait for performance under future climatic conditions. In chapter 1 we examined whether the drought-tolerance of pine seedlings is related to a higher investment in root biomass under experimental drought. In particular, we tested whether (1) the root biomass increases under experimental drought, and if (2) investment in root biomass and stomatal conductance is related to the drought-tolerance of the populations and thus allows for higher growth rates under drought. An elevated concentration of atmospheric CO_2 (eCO_2) under future climate conditions might enhance growth and mitigate drought stress (Körner, 2006; Leuzinger & Körner, 2007). It is also expected to affect concentrations of non-structural carbohydrates (NSC) in plants (Runion *et al*., 1999; Körner, 2003), which might be depleted under prolonged drought (McDowell, 2011). In chapter 2 we therefore tested how eCO_2 affects the regeneration of the different pine populations under drought by potentially alleviating NSC depletion. We hypothesized that (1) drought does not lead to carbon depletion, but higher NSC concentrations in all plant organs, that (2) elevated CO_2 enhances growth of well-watered, but not drought-stressed seedlings, and will only lead to accumulation of NSC in the latter, and that (3) growth reduction and NSC accumulation is lower in drought-tolerant seedlings. Mediterranean pine species and populations that are more drought-tolerant are expected to shift their distribution range to Western and Central Europe under future climatic conditions (Hanewinkel *et al*., 2013). A higher drought tolerance of Mediterranean pine populations might, however, come at the cost of lower cold tolerance. In order to assess whether frost is a limiting factor for the predicted northward shift of Mediterranean tree species in Europe, we compared the cold hardiness of the Mediterranean populations of the three pine species with that of Central Alpine *P. sylvestris*. In chapter 3 we examined (1) to what degree seedlings of Mediterranean origin tolerate low temperature extremes, and whether drought-adapted Mediterranean populations are more prone to frost damage than Central Alpine populations and (2) whether pre-exposure to summer drought affects cold hardiness of the seedlings. In addition, the regeneration potential of the non-native *Pseudotsuga menziesii* under dry climatic conditions was assessed, as *P. menziesii* is considered a drought-tolerant alternative to *Picea abies* under future climatic conditions in Central Europe (additional work; Eilmann & Rigling, 2012).

Species, study site and experimental design

Pinus sylvestris L. has been frequently used in studies focusing on drought limitations, because it is an important and widely distributed tree species in European forests. It ranges almost continuously from Siberia to the Iberian Peninsula, and shows local adaptation to drought in southern populations (Fig. 1; Matías & Jump, 2012; Richter *et al*., 2012). While it is the most drought tolerant native conifer species in Central Alpine valleys, dominating forests at elevations below 1000 m a.s.l., it is stand-forming above 1300 m a.s.l. at its southern distribution limit (Richter *et al*., 2012) where temperatures are too low for oaks. *Pinus nigra* Arnold is discontinuously distributed in the northern Mediterranean and the Pannonian basin and is considered more drought tolerant than *P. sylvestris* (Rouget & Richardson, 2001; Eilmann & Rigling, 2012; Richter *et al*., 2012) but still frost resistant (Climent *et al*., 2009). It has therefore been considered a substitute for the less drought-tolerant *P. sylvestris* under future climatic conditions in Central Europe (Thiel *et al*., 2012). *Pinus halepensis* Mill. is the most drought tolerant but least frost resistant Mediterranean pine species in this study. It is found at lowest elevations scattered across the Mediterranean, at desert or steppe margins (Fady *et al.*, 2003). In Mediterranean mountains, the three species form distinct belts along an elevational gradient with *P. sylvestris* at highest elevations, succeeded by *P. nigra* at lower elevations and *P. halepensis* at the bottom. *Pseudotsuga menziesii* (Mirb.) Franco is a widespread tree species in western North America ranging from Canada to Mexico (Little, 1971). *P. menziesii* var. *menziesii* was introduced and is being cultivated in Europe since the late 19th century (Kowarik & Rabitsch, 2010) and is today the most important exotic tree species in Switzerland with a share of 0.2% (Brändli, 2010).

Table 1. Seed origins, respective mean precipitation sums of the driest month ('Precip'), and mean monthly minimum temperatures of the coldest month ('Min Temp') (1950–2000; www.worldclim.org) of the species and populations sowed in the common garden at Leuk (Switzerland). Regions include the Mediterranean ('Med'), Central Alpine ('C. Alp.'), Pannonian basin ('Pann.'), Central European ('C. Eur.') and North-western America ('NW A.').

Species	Region	Country	Locality	Elevation (m a.s.l.)	Lat (°)	Long (°)	Precip (mm)	Min temp (°C)
P. sylvestris	Med	Bulgaria	Jundola	1405	42.05	23.83	35	-7.2
	Med	Greece	Serres	1333	41.24	23.58	29	-5.2
	Med	Spain	Ademuz	1542	40.08	-1.08	34	-3.2
	C. Alp.	Switzerland	Leuk	570	46.29	7.61	49	-3.6
	C. Alp.	Switzerland	Visperterminen	1363	46.27	7.91	90	-6.2
P. nigra	Pann	Austria	Bad Fischau	344	47.83	16.13	36	-5.7
	Med	Bulgaria	Dobrostan	1167	41.90	24.93	40	-6.3
	Med	Greece	Parthenonas	644	40.13	23.86	18	0.5
	Med	Spain	Ademuz	1195	40.09	-1.38	26	-1.9
P. halepensis	Med	Greece	Parthenonas	353	40.12	23.81	16	1.6
	Med	Spain	Aras del Olmo	1056	39.92	-1.15	24	-0.6
P. menziesii	C. Eur.	Switzerland	Biel	700	47.21	7.37	74	-4.0
	C. Eur.	Germany	Taubersbischofsheim	370	49.63	9.65	45	-2.7
	NW A.	USA	Snoqualmie	500	47.53	-121.83	41	0.2

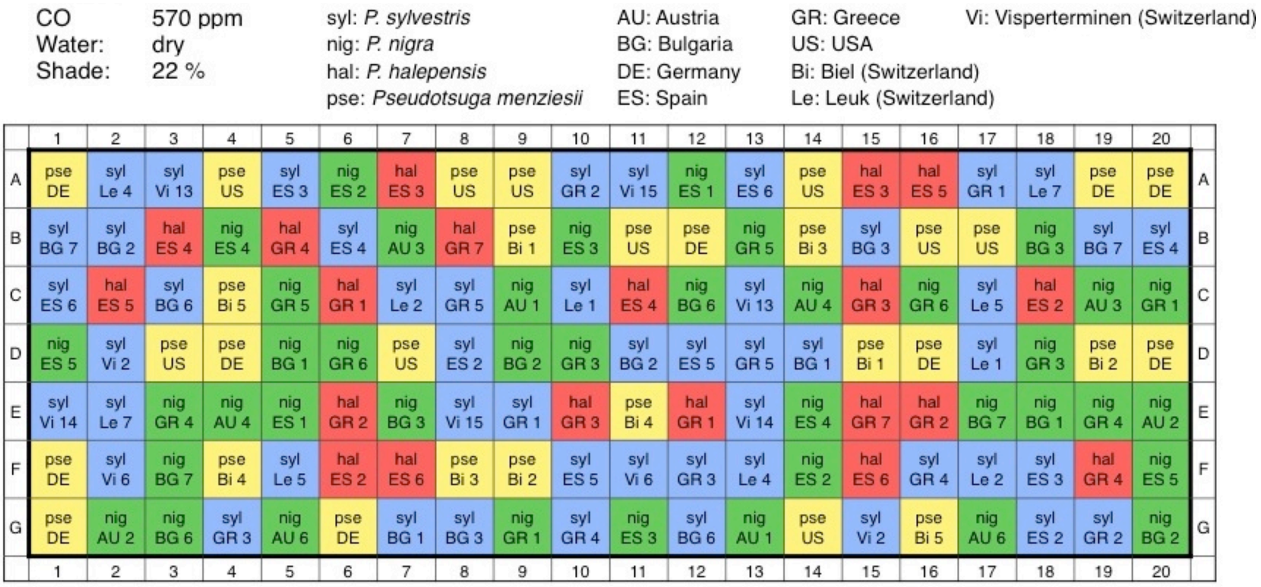
In winter 2011/2012, seeds of a total of 11 pine populations were collected, each from five maternal lineages, amounting to 55 seed sources. The seeds of five *P. sylvestris* populations were collected in Spain, Greece, Bulgaria and at two sites in Switzerland (Table 1). The seeds of three *P. nigra* populations were collected at locations close to the *P. sylvestris* populations (Spain, Greece, Bulgaria) and a fourth at the northernmost site of the species' distribution (Austria). The seeds of two *P. halepensis* populations were collected at locations close to the *P. sylvestris* and *P. nigra* populations (Spain, Greece). In addition, *P. menziesii* var. *menziesii* seeds were provided by commercial harvests from a population from the western USA (WA) and two naturalized populations in Europe (Germany and Switzerland).

Fig 2. Experimental site near Leuk (VS, Switzerland) with mesocosms covered by white shade cloths and opened mobile rainshelters.

In March 2012, the seeds were sown in a common garden in the Valais valley (Switzerland; 46°18'33''N, 07°41'10''E; 610 m a.s.l.; 19.1 ºC average temperature in July; -0.8 ºC average temperature in January; 600 mm annual precipitation; Fig. 2), a dry Central Alpine valley, where *P. sylvestris* is stand forming at low elevations and has been repeatedly observed to be drought limited (Bigler *et al.*, 2006; Rigling *et al.*, 2013). 60 mesocosms (200 cm x 80 cm x 50 cm) were filled with 30 cm of sand and gravel from the local Rhone riverbed, covered by 15 cm of humus (Oekohum GmbH, Herrenhof, Switzerland, Fig. 3). The two layers were designed to simulate the natural forest soils of the Rhone valley (Moser *et al.*, 2014). Each mesocosm was divided into 140 10 x 10 cm squares and 15 seeds of each pine seed source were sown in two randomly selected squares in March 2012 (two replicates per seed source, i.e. 10 replicates per population; Fig. 4). Similarly, 20 seeds of each *P. menziesii* population were sown in 10 randomly selected squares (10 replicates per population), as the maternal lineages of *P. menziesii* populations had not been determined. This resulted in a total of 8400 sowing quadrats with 15 or 20 seeds each. The 60 mesocosms were arranged in a split-plot design with five blocks (whole plots), each composed of two subblocks (split-plots) with six mesocosms (split-split-plots; Fig. 5). All mesocosms were watered regularly until May 31, 2012, to maximize germination.

Fig 3. Soil in the mesocosms, consisting of 15 cm topsoil (humus) and 30 cm subsoil (sand and gravel).

From June – September 2012, March – October 2013 and 2014 a CO_2 treatment was applied to the subblocks, which consisted of the two levels 'ambient CO_2' (aCO_2: 400 ppm) and 'elevated CO_2' (eCO_2: target concentration of 570 ppm, Fig. 4). The eCO_2 simulated the predicted concentration in 2100 assuming an increment of 20 ppm per decade (www.esrl.noaa.gov/gmd/ccgg/trends) and represents roughly twice the preindustrial concentration. Instead of the hexagonal arrangement used in free-air CO_2 enrichment (FACE) experiments, we chose a rectangular arrangement of the CO_2 injection tubes that fitted the shape of the mesocosms. Otherwise the same system as in Hättenschwiler *et al*. (2002) was used. To reduce costs, CO_2 release was interrupted when conditions were unfavourable for photosynthesis (temperature < 5 ºC, photon flux density < 30 $mmolm^{-2}s^{-1}$, air temperature > 35 °C) or wind speed exceeded 2 ms^{-1}.



CO 570 ppm
Water: dry
Shade: 22 %

syl: *P. sylvestris*
nig: *P. nigra*
hal: *P. halepensis*
pse: *Pseudotsuga menziesii*

AU: Austria
BG: Bulgaria
DE: Germany
ES: Spain

GR: Greece
US: USA
Bi: Biel (Switzerland)
Le: Leuk (Switzerland)

Vi: Visperterminen (Switzerland)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
A	pse DE	syl Le 4	syl Vi 13	pse US	syl ES 3	nig ES 2	hal ES 3	pse US	pse US	syl GR 2	syl Vi 15	nig ES 1	syl ES 6	pse US	hal ES 3	hal ES 5	syl GR 1	syl Le 7	pse DE	pse DE	A
B	syl BG 7	syl BG 2	hal ES 4	nig ES 4	hal GR 4	syl ES 4	nig AU 3	hal GR 7	pse Bi 1	nig ES 3	pse US	pse DE	nig GR 5	pse Bi 3	syl BG 3	pse US	pse US	nig BG 3	syl BG 7	syl ES 4	B
C	syl ES 6	hal ES 5	syl BG 6	pse Bi 5	nig GR 5	hal GR 1	syl Le 2	syl GR 5	nig AU 1	syl Le 1	hal ES 4	nig BG 6	syl Vi 13	nig AU 4	hal GR 3	nig GR 6	syl Le 5	hal ES 2	nig AU 3	nig GR 1	C
D	nig ES 5	syl Vi 2	pse US	pse DE	nig BG 1	nig GR 6	pse US	syl ES 2	nig BG 2	nig GR 3	syl BG 2	syl ES 5	syl GR 5	syl BG 1	pse Bi 1	pse DE	syl Le 1	nig GR 3	pse Bi 2	pse DE	D
E	syl Vi 14	syl Le 7	nig GR 4	nig AU 4	nig ES 1	hal GR 2	nig BG 3	syl Vi 15	syl GR 1	hal GR 3	pse Bi 4	hal GR 1	syl Vi 14	nig ES 4	hal GR 7	hal GR 2	nig BG 7	nig BG 1	nig GR 4	nig AU 2	E
F	pse DE	syl Vi 6	nig BG 7	pse Bi 4	syl Le 5	hal ES 2	hal ES 6	pse Bi 3	pse Bi 2	syl ES 5	syl Vi 6	syl GR 3	syl Le 4	nig ES 2	hal ES 6	syl GR 4	syl Le 2	syl ES 3	hal GR 4	nig ES 5	F
G	pse DE	nig AU 2	nig BG 6	syl GR 3	nig AU 6	pse DE	syl BG 1	syl BG 3	nig GR 1	syl GR 4	nig ES 3	syl BG 6	nig AU 1	pse US	syl Vi 2	pse Bi 5	nig AU 6	syl ES 2	syl GR 2	nig BG 2	G
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	

Fig 4. Example of sowing squares in one mesocosm (elevated CO_2, 'dry' climate, moderate shading). Numbers indicate the identities of the maternal lineages of the pines. Seeds of the five maternal lineages of each pine population were sown randomly in two quadrats: one in the left half (columns 1–10) and one in the right half (columns 11–20) of the mesocosm. No information of the maternal lineage of *Pseudotsuga menziesii* seeds was available.

Concurrently with the CO_2 treatment, automatic mobile rainshelters intercepted the natural rainfall and all mesocosms were irrigated weekly on two subsequent nights (Moser *et al*., 2014). The irrigation regime simulated two climates: a 'moist' and a 'dry' climate, corresponding to the 90th and the 10th percentile of the April–September precipitation sums from 1864 to 2011 in the Rhone valley (151 % and 67 % of the average precipitation). The two climates were each applied to three randomly assigned mesocosms in each subblock (Fig. 4). Additionally, a summer drought treatment ('drought') was implemented starting in the second growing season. The irrigation was interrupted in one of the three mesocosms of each irrigation regime in each subblock from June – October 2013 and 2014, resulting in a 'moist-drought' and 'dry-drought' treatment level (Fig. 4). The two remaining mesocosms of each irrigation treatment in each subblock continued receiving the same irrigation as before. Finally, a shading treatment was applied consisting of white knitted polypropylene shade cloths that blocked 22% (light shading) and 40% (deep shading) of the sunlight, respectively. The deep shading was applied to one each of the remaining mesocosms of each irrigation treatment in each subblock (Fig. 4). Hence, the shading treatment was fully crossed with the irrigation regime, but not with the drought treatment.

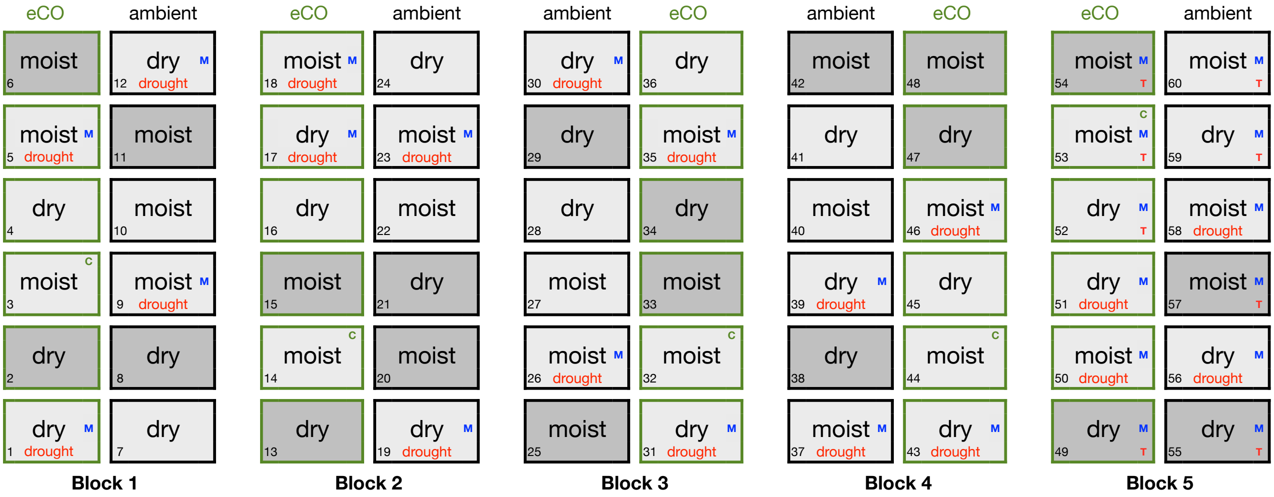


Fig 5. Arrangement of the 60 mesocosms: five blocks (whole plots) were each divided into two subblocks (split-plot). At this level, the CO_2 treatment (ambient or elevated CO_2, black and green frame) was applied. The subblocks each contained six mesocosms (split-split-plot) onto which a fully crossed combination of an irrigation regime and drought treatment was applied: a 'moist' and 'dry' irrigation regime and a summer drought treatment ('drought') or no drought. Two shading treatments were applied to the mesocosms: a light shading (22%, light grey) and a deep shading (40%, dark grey). The shading treatment was fully crossed with the irrigation regime, but not with the drought treatment. Soil moisture ('M'), temperature ('T') and CO_2 concentration ('C') was measured in the depicted mesocosms.

Structure of the thesis

The doctoral thesis is structured in three chapters. In the first part, I focus on the seedlings growth reactions to repeated water limitation, in particular biomass partitioning to shoot and roots. The second part deals with the carbon metabolism of the pine seedlings during repeated severe drought under eCO_2. The third part focuses on the frost tolerance of seedlings with different drought tolerances and aims at detecting restrictions of drought-tolerant populations to regenerate in regions with intermittent spring frosts. Due to the high number of seedlings in the whole experiment (105,131 seedlings in June 2012, thinned to 33,600 in April 2013), experiments and measurements were conducted on different subsets, depending on the particular research question (Table 2). *P. halepensis* seedlings showed a high degree of damage after the first winter (2012/2013) and almost all died after the second winter (2013/2014), most probably due to winter desiccation (see chapter 3). Therefore, this species was not included in the analyses in chapters 1 and 2. Regeneration of *P. menziesii* in dry climate was compared to *P. sylvestris* in additional work and is briefly presented after chapter 3. The effect of the shading treatment was not part of the thesis and will be analysed subsequently.

Table 2. Treatments and species included in chapters 1–3 and additional work.

Treatments	Chapter 1	Chapter 2	Chapter 3	Additional work
Irrigation	Yes	No	Yes	No
Drought	Yes	Yes	No	Yes
CO_2	No	Yes	No	No
Shading	No	No	No	No
Species				
P. sylvestris	Yes	Yes	Yes	Yes
P. nigra	Yes	Yes	Yes	No
P. halepensis	No	No	Yes	No
P. menziesii	No	No	No	Yes

Chapter 1: Biomass partitioning under water limitation is related to drought-tolerance of pine seedlings

The investment of limited resources (water, carbohydrates, nutrients) in root or shoot growth of seedlings is highly coordinated and has to ensure survival and optimal growth of the plant. Pine seedlings from locations with recurrent drought are expected to exhibit higher drought-tolerances, which should be reflected in their biomass partitioning under limited water availability. While there are several indications of a higher investment in root growth of *P. sylvestris* under drought (Moser *et al.*, 2014; Taeger *et al.*, 2015), there is no clear relationship with site conditions at the population's origin (Matías *et al.*, 2014; Taeger *et al.*, 2015). In this chapter, we try to identify a relationship between the drought-tolerance of a population and its biomass partitioning under water limitation. Seedlings of five *P.*

sylvestris and four *P. nigra* populations from sites with different aridity were grown under four different water treatments and analysed during the first three years of establishment.

The following research questions were addressed:

1) Is biomass partitioning related to the drought-tolerance of pine seedlings under moist and dry conditions?
2) How is biomass partitioning modified by soil water availability, plant size and ontogeny?

Chapter 2: No carbon limitation in pine seedlings under lethal drought

In order to reduce water loss, trees react to drought stress with decreased stomatal conductance. As a consequence, CO_2 uptake can be severely inhibited (Bréda *et al.*, 2006), which might lead to a life-threatening decrease of carbohydrates during sustained drought (McDowell *et al.*, 2008). Elevated CO_2 (eCO_2) could, on the other hand, mitigate the effects of drought-induced carbohydrate deficiency (Wullschleger *et al.*, 2002). In chapter 2, we tested the effect of drought and eCO_2 on the carbohydrate metabolism, growth and survival of pine seedlings from five *P. sylvestris* and four *P. nigra* populations. By comparing populations with different drought-tolerances, we were able to examine the importance of the carbohydrate metabolism for the drought adaptation of pine seedlings.

The following research questions were addressed:

1) What are the changes in starch and sugar concentrations in seedlings subjected to repeated lethal drought?
2) Does eCO_2 increase seedling growth, survival or starch and sugar concentrations under drought?
3) Are changes in starch and sugar concentrations related to the drought-tolerance of different populations?

Chapter 3: Cold temperature extremes during spring do not limit the range shift of Mediterranean pines into regions with intermittent frost

The adaptation of seedlings to drought might come at the cost of low frost tolerance. There are, on the other hand, interrelations between certain drought and frost tolerance traits of plants, such as an increased activity of protective enzymes (Blödner *et al.*, 2005) and xylem resistance (Lens *et al.*, 2013). In the third chapter, we investigated the limitations of drought-tolerant pines to regenerate in frost-prone regions. Cold hardiness was tested by artificially freezing the needles of the pine seedlings repeatedly during late winter and spring. A relationship between cold hardiness and drought tolerance was experimentally assessed by comparing seedlings subjected to a dry and wet climate during the previous growing season (Bachofen *et al.*, 2015).

The following research questions were addressed:

1) Is the cold hardiness of pine seedlings in a common garden related to the temperature at the seed origin?
2) Does exposure to a dry climate affect the cold hardiness in the following winter and spring?

Additional work: Root architecture might account for contrasting establishment success of *Pseudotsuga menziesii* var. *menziesii* and *Pinus sylvestris* in Central Europe under dry conditions

P. menziesii var. *menziesii* is the most important exotic tree species in Switzerland with a share of 0.2% (Brändli, 2010) and is considered to be relatively drought tolerant (Tschopp *et al.*, 2015). In this work, we assessed the early establishment of *P. menziesii* under drought conditions and its competitive ability in comparison to *P. sylvestris* seedlings.

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Chapter 1

Biomass partitioning under water limitation is related to drought-tolerance of pine seedlings

To be submitted as:

Christoph Bachofen[1,2], Barbara Moser[1], Frank Hagedorn[1], Thomas Wohlgemuth[1]

[1] Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zürcherstrasse 111, 8903 Birmensdorf, Switzerland

[2] Department of Environmental Sciences, Swiss Federal Institute of Technology, Universitätsstrasse 16, 8092 Zürich, Switzerland

Summary

- Biomass partitioning is assumed to be an important trait determining the drought-tolerance of trees, ensuring seedling survival and thus forest regeneration under future climatic conditions. It is however unclear, whether the drought-tolerance of different populations is related to a larger investment in root growth under drought.
- In order to detect a relationship between seed origin and biomass partitioning under drought, we grew *Pinus sylvestris* and *Pinus nigra* seedlings of nine different origins in mesocosms in a common garden and subjected them to a controlled irrigation regime and a summer drought treatment during three growing seasons. We measured the entire shoots and roots after the second and third growing season, and determined treatment effects, as well as effects of age, size and seed origin on biomass partitioning. Oxygen isotope fractionation was measured as an estimate of stomatal conductance and related to the seed origin.
- Seedling's changes in biomass partitioning in response to the drought were significantly related to the seed origin: less drought adapted seedlings responded strongly to the irrigation and summer drought, while seedlings from dry locations did not respond with changes in biomass partitioning. The responses differed largely between the two treatments: under dry irrigation, seedlings increased the root biomass fractions by 6%, whereas summer drought resulted in reduced root growth by 15%. In the third growing season, the drought did not result in biomass changes, which indicates acclimation. Stomatal conductance was positively related to the drought-tolerance of populations, indicating a positive effect of large root biomass partitioning on leaf transpiration.
- *Synthesis*: Local adaptation to drought enabled to maintain a larger root biomass fraction under severe drought. This allowed for higher stomatal conductance and higher overall growth of populations from dry locations. Acclimation to drought evened the differences between populations and resulted in similar biomass partitioning in all treatments. Local adaptation to drought was thus most important for first-time drought-responses, whereas acclimation became a key trait thereafter.

Introduction

Plant biomass partitioning into leaves, stem and roots is highly coordinated in order to achieve optimal growth and reproduction (Poorter *et al.*, 2015). Biomass partitioning has long been considered to follow general allometric rules, which uniformly apply to all plant species (Pearsall, 1927; Enquist & Niklas, 2002). Accordingly, constant scaling ratios between plant compartments, such as to the 3/4 power for leaf to root biomass, have been proposed for all plants, irrespective of species, size or environment (Enquist & Niklas, 2002). More recently, considerable differences related to phylogeny and functional groups have been disclosed (Poorter *et al.*, 2012, 2015). For instance, evergreen plants exhibit higher leaf mass fractions than deciduous species, and graminoids have higher root mass fractions than eudicotyledonous herbs (Poorter *et al.*, 2015). Biomass partitioning is subjected to trade-offs (Bloom *et al.*, 1985) and should result in optimal resource acquisition (Chapin, 1980; Tilman, 1988). Accordingly, plants will have to invest in the growth of the organ which accesses the most limiting resource (McConnaughay & Coleman, 1999; McCarthy & Enquist, 2007). Low water availability should, for instance, result in the development of larger root systems for a better access to soil water. It has indeed been repeatedly documented that tree species growing at drier sites allocate more biomass to roots and have deeper root systems than those growing at moist sites (e.g. Zimmer & Grose, 1958; Bongarten & Teskey, 1987; Hertel *et al.*, 2013). Similarly, tree seedlings increased investments in roots when growing under dry conditions in common garden experiments (e.g. Moser *et al.*, 2014; Taeger *et al.*, 2015). It is, however, unclear whether root growth in a common garden is related to the drought tolerance of different species or populations (Aranda *et al.*, 2010; Arend *et al.*, 2011; Matías *et al.*, 2014; Taeger *et al.*, 2015). Populations with different drought-tolerances are expected to differ heavily in their reaction to environmental changes (Valladares *et al.*, 2007), but how different populations respond to experimental drought is unclear (Arend *et al.*, 2011; Taeger *et al.*, 2015). Furthermore, a large number of experimental and field studies report an inverse or no relationship between tree root biomass and soil water status (e.g. Steele *et al.*, 1997; Leuschner *et al.*, 2004; Meier & Leuschner, 2008; Matías *et al.*, 2014; and literature within), particularly in long-term drought experiments (Joslin *et al.*, 2000).

Biomass partitioning in trees is difficult to measure under field conditions and studies often use ingrowth cores, which focus on fine root biomass at a particular rooting depth. Seedling studies, on the other hand, are often conducted on potted seedlings with a disturbed root architecture (Preisig *et al.*, 1979) and in the absence of intra- and interspecific competition, which is known to affect root development (Kroon, 2007; Messier *et al.*, 2009). Furthermore, biomass partitioning is related to plant size (Poorter *et al.*, 2015) and thus changes during tree growth (see literature in: Wilson, 1988; McCarthy & Enquist, 2007; Poorter *et al.*, 2012). Reduced resource availability (such as water) affects tree growth as well, and thus indirectly affects biomass partitioning. It therefore becomes difficult to detect the direct effect of the resource availability on biomass partitioning (McCarthy & Enquist, 2007). In other words, changes in biomass partitioning related to plant size that result from limited growth can easily be misinterpreted as an adaptive response to the treatment (McConnaughay & Coleman, 1999; McCarthy & Enquist, 2007). Thus, the effect of drought on biomass partitioning cannot be assessed without accounting for changes in plant size (Poorter & Sack, 2012).

In Europe, *P. sylvestris* is at its dry distribution limit in Central Alpine locations (e.g. Matías & Jump, 2014, Rigling *et al.*, 2013) and might therefore be particularly vulnerable to changes in temperature and precipitation. Mediterranean populations of *P. sylvestris* have been shown to exhibit a larger root to shoot ratio than northern populations if they are grown in a common garden (e.g. Matías *et al.*, 2014; Taeger *et al.*, 2015). A higher investment in root biomass might thus be an essential trait for performance under future climatic conditions. In this study, we tested whether the drought-tolerance of pine seedlings is related to a higher investment in root biomass under experimental drought. In a common garden, located in the Central Alpine Valais valley, we studied biomass partitioning of a total of nine populations of *P. sylvestris* (n=6) and *P. nigra* (n=5) seedlings subjected to two different irrigation regimes during three years. In addition to the irrigation regime, we applied a summer drought treatment during four months in the second and third year. By sowing seeds in large mesocosms (720 litre), we allowed for an unrestricted development of the root system, while enabling the excavation of the complete roots after the second and the third year. We studied biomass partitioning under the two irrigation regimes and the summer drought treatment of a spectrum of different drought-tolerant pine populations and hypothesized that (1) the root biomass fraction increases under drought, but decreases with plant size and ontogeny and (2) investment in root biomass is related to the drought-tolerance of the populations and we tested whether (3) stomatal conductance is positively related to the drought-tolerance of a population and thus allows for higher growth under drought.

Methods

Species, populations and study site

Pinus sylvestris L. ranges from boreal Siberia and Scandinavia to mountainous regions in the European Mediterranean. Local adaptation of *P. sylvestris* to drought has been studied repeatedly with seedlings in common garden experiments (e.g. Richter *et al.*, 2012; Matías *et al.*, 2014; Moser *et al.*, 2014; Taeger *et al.*, 2015). *Pinus nigra* Arnold thrives around the northern Mediterranean and resists drought better than *P. sylvestris* (Herrero *et al.*, 2013). We collected seeds from five *P. sylvestris* and four *P. nigra* populations in winter 2011/2012, using five maternal lineages per population (5 x 5 *P. sylvestris* + 5 x 4 *P. nigra* = 45 seed origins). Locations were selected along a precipitation gradient during the growing season. Accordingly, *P. sylvestris* populations come from two Central Alpine locations nearby the study site, one low and one high elevation, and three Mediterranean locations with low precipitation sums during summer and thus potentially drought-tolerant seedlings in Spain, Greece and Bulgaria (Fig. S1, Table 1). From the four *P. nigra* populations, we chose three that were geographically close to the Mediterranean *P. sylvestris* populations but at lower elevations, and one population from the northernmost location in the Eastern Alps, thus potentially more susceptible to drought. The seeds were sown in a common garden in the Valais valley (Switzerland; 46°18'33"N, 07°41'10"E; 610 m a.s.l.; 19.1 ºC average temperature in July; 600 mm annual precipitation). The Valais is a dry Central Alpine valley, where stand-forming *P. sylvestris* has been repeatedly observed to be drought limited at the valley bottom (Bigler *et al*., 2006; Rigling *et al*., 2013).

Table 1. Localities and respective mean precipitation sums of the driest month (1950–2000; www.worldclim.org) of the species and populations used in the common garden experiment at Leuk (Switzerland).

Species Region	Country	Locality	Elevation (m a.s.l.)	Lat (°)	Long (°)	Precip. driest month (mm)
Pinus sylvestris						
Mediterranean	Bulgaria	Jundola	1405	42.05	23.83	35
Mediterranean	Greece	Serres	1333	41.24	23.58	29
Mediterranean	Spain	Ademuz	1542	40.08	-1.08	34
Central Alpine, low	Switzerland	Leuk	570	46.29	7.61	49
Central Alpine, high	Switzerland	Visperterminen	1363	46.27	7.91	90
Pinus nigra						
East Alpine	Austria	Bad Fischau	344	47.83	16.13	36
Mediterranean	Bulgaria	Dobrostan	1167	41.90	24.93	40
Mediterranean	Greece	Parthenonas	644	40.13	23.86	18
Mediterranean	Spain	Ademuz	1195	40.09	-1.38	26

Experimental design and drought treatments

At the study site, 20 mesocosms (200 cm x 80 cm x 50 cm) were filled with 30 cm of sand and gravel from the local Rhone riverbed and covered by 15 cm of humus (Oekohum GmbH, Herrenhof, Switzerland). We designed the two layers to simulate the natural forest soils of the Rhone valley (Moser *et al*., 2014). We divided each mesocosm into 140 10 x 10 cm squares and sowed 15 seeds of each seed origin in two randomly selected squares in March 2012 (two replicates per seed origin, i.e. 10 replicates per population; Bachofen *et al*., 2015). The large mesocosms allowed natural root development and competition of the growing seedlings (Kroon, 2007; Messier *et al*., 2009). The 20 mesocosms were arranged in a split-plot design with five blocks (whole plots), each composed of four mesocosms (split-plots). We watered the soil daily until May 2012 to facilitate germination. From June to October 2012 and from March to October in 2013 and 2014, we applied a controlled irrigation regime to the mesocosms using automatic mobile rain shelters to intercept rainfall and a weekly automatic irrigation on two consecutive nights (Bachofen *et al.*, 2015). The irrigation regime simulated two climates: a 'moist' (P_{90}) and a 'dry' (P_{10}) climate, corresponding to the 90th and the 10th percentile of the summer season precipitation sums from 1864 to 2011 in the Rhone valley (151 % and 67 % of the average precipitation from April to September). We applied P_{90} and P_{10} to two randomly assigned mesocosms in each block. Additionally, we exposed mesocosms to a summer drought treatment ('drought' and 'no-drought') starting with the second growing season. From June to October in 2013 (D1) and 2014 (D2), we interrupted the irrigation in one of each P_{90} and P_{10} mesocosms in each block, resulting in a 'moist-drought' ($P_{90}D$) and 'dry-drought' ($P_{10}D$) treatment level. In the remaining P_{90} and P_{10} mesocosms in each block, we continued the irrigation until October ('no-drought').

Growth measurements

In October 2012, 2013 and 2014, we randomly chose one of the two squares of each seed origin per mesocosm, and cut one randomly selected seedling at ground level (Fig. 1). In 2012, only the P_{90} and the P_{10} mesocosms in every block were harvested, because the 'drought' treatment had not been effective until then. While we harvested shoots from all five blocks in 2012, we took samples in only four blocks in 2013 and 2014, because the mesocosms the fifth block served for root extraction. This resulted in a sample size of 450 seedlings in 2012 and 720 seedlings in 2013 and 2014. We measured seedling height from the base to the bud and dried the shoots at 60 °C to constant weight. In October 2013 and 2014, we excavated the entire root system of the seedlings of one randomly selected block by opening the mesocosms and carefully removing the humus and sand by hand from the heavily intertwined roots. We then measured the lengths of the taproot and the longest lateral root before drying the roots at 60 °C to constant weight.

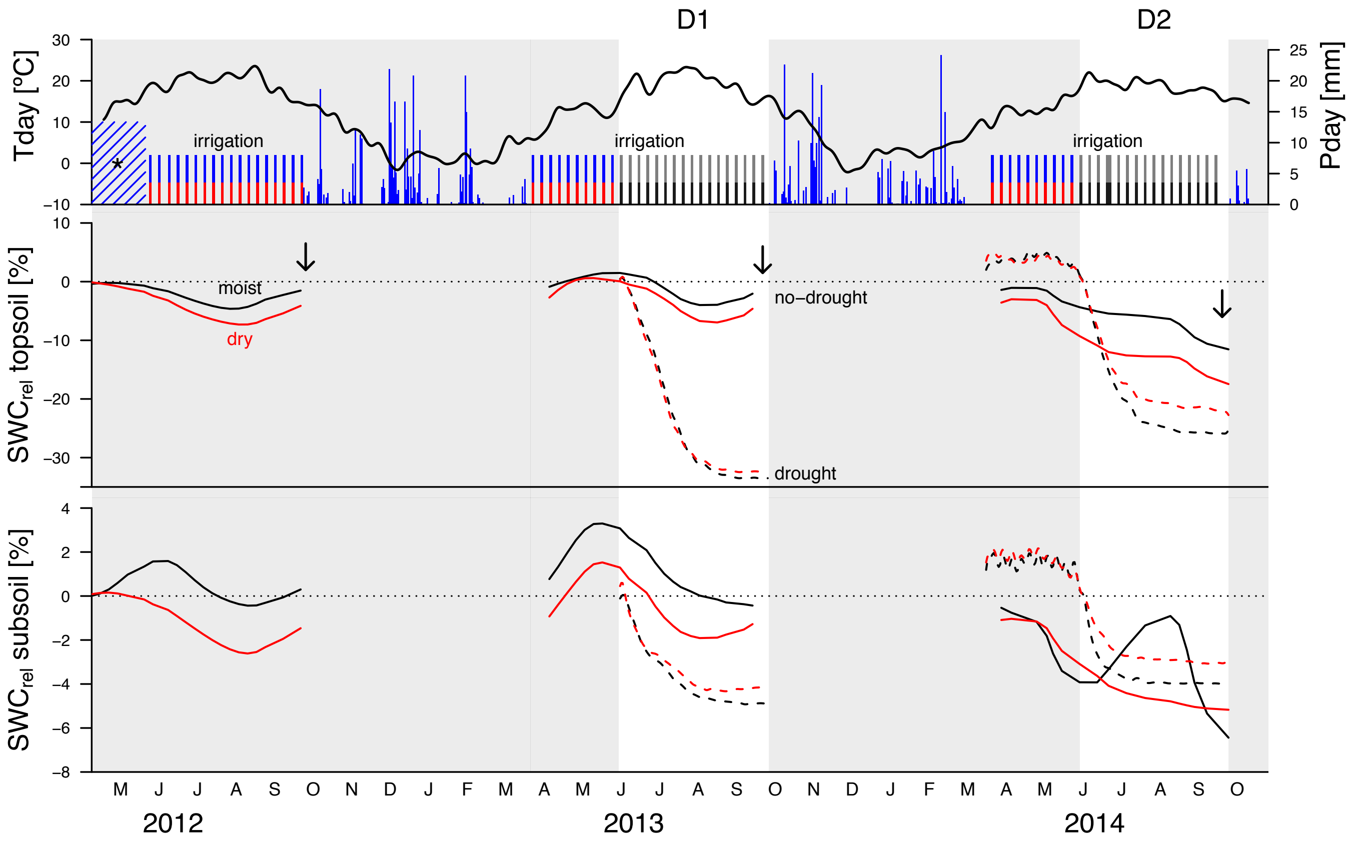

Fig 1. Daily air temperatures (Tday) and natural precipitation/irrigation (Pday) at the experimental site (top), and the relative volumetric soil water content (SWC_{rel}) in the topsoil and subsoil of the mesocosms during the experiment (bottom). SWC_{rel} is calculated as the difference to the first measurement of each year and shown for 'moist' (black) and 'dry' (red), and 'drought' (solid lines) and 'no-dryout' (dashed lines) mesocosms. D1 and D2 indicate the period of summer drought treatments; arrows indicate the time of seedling harvest for biomass measurements. The amount of water added to the mesocosms is depicted for the time when rainshelters were in operation. The asterisk denominates the time of daily watering until seedling emergence.

Statistical analyses of growth and biomass partitioning

We applied a linear mixed effects model (*lmer*, package *lme4*) to test the effects of the irrigation regime and the summer drought treatment on shoot biomass, root biomass, height and taproot length using the statistics program R (R Core Team, 2016). The two treatments (irrigation regime and summer drought), the species and the populations were fixed effects, the block identity and the maternal lineage random effects. The population means of shoot biomass, shoot height, root biomass and taproot length in 2013 and 2014 were related to the drought-tolerances of each population using a linear model (*lm*). The climatic water balance of the driest month at the seed origin of a population was used as an indicator for the population's drought-tolerance. The water balance was calculated as the difference between precipitation and potential evapotranspiration according to Thornthwaite (1948) using interpolated precipitation and temperature data from the WorldClim database (Hijmans *et al.*, 2005). A widely used value for describing biomass partitioning is the root to shoot biomass ratio. Since this parameter has several disadvantages, we used root biomass fraction, i.e. the root biomass divided by the total biomass, as suggested by (Poorter & Sack, 2012). Biomass partitioning of plants has been repeatedly observed to be related to plant size (i.e. 'allometric biomass partitioning'; Wilson, 1988; McCarthy & Enquist, 2007; Poorter *et al.*, 2012). It can be expected that the irrigation regime and the drought treatment affect plant biomass and thus the allometric biomass partitioning. However, the 'apparent' change in biomass partitioning needs to be distinguished from the 'true' change related to the treatment. To separate the direct effect of the treatment on biomass partitioning from an allometric partitioning effect, Poorter & Sack (2012) proposed to perform a regression of the root biomass fraction on the total plant size, including treatments as covariables. Here, we applied this approach and tested the effects of irrigation regime, species and summer drought on biomass partitioning (hypothesis 1) using a linear mixed effects model (*lmer*, package *lme4*). The maternal lineage of the seedlings was considered a random effect. The effect of the climatic water balance of the driest month at the seed origin of the populations on biomass partitioning (hypothesis 2) was tested using a linear model with population means of the root biomass fractions with the irrigation regime, the drought and the species identity as fixed effects.

Oxygen isotope fractionation ($^{18}O/^{16}O$)

We estimated stomatal conductance of the seedlings during the whole vegetation period by analysing stable oxygen isotope fractionation (^{18}O/^{16}O; Farquhar *et al.*, 2007). For this purpose, we analysed samples from 'moist' mesocosms (P_{90} and P_{90}D) from three blocks in order to reduce sample size. The needles of seedlings from five maternal lineages per population per species were pooled and ground with a ball mill (Retsch M200, Haan, Germany). For the ∂^{18}O analysis, 0.1 mg of needle powder was weighed into tin capsules and pyrolyzed with an elemental analyser (Euro EA, HEKAtech GmbH, Wegberg, Germany). The isotopic ratio of the oxygen (∂^{18}O [‰]) was subsequently determined with an isotope ratio mass spectrometer (IRMS Delta V Advantage, Thermo Fisher Scientific, Waltham, MA, USA). The ∂^{18}O values are expressed as the isotopic ratio in the sample (R_{sample}) relative to the international standard for oxygen isotope ratio ($R_{standard}$, VSMOW): $\partial_{sample} = (R_{sample} / R_{standard} - 1) \times 1000 + 23.87$. We tested the effect of the summer drought on oxygen isotope discrimination and its relation to the drought-tolerance of the populations (hypothesis 3) with a linear model, separately for 2013 and 2014. As fixed effects, we used the water balance at the seed origin, as well as summer drought treatment and species identity.

Results

Irrigation and soil water status

Seedlings in P_{10} mesocosms obtained less than half as much water as seedlings in P_{90} mesocosms during the controlled irrigation (44 % in all three years). In P_{90}D mesocosms, the drought from June to October in 2013 and 2014 resulted in a similar reduction of the precipitation sum over the whole growing season (41% March–October 2013 and 40 % March–October 2014). 'Dry' irrigation and 'drought' thus resulted in similar amount, but differently timed water availability. Hence, the total amount of irrigation received by P_{90}D mesocosms did not differ much from P_{10} mesocosms (95% in 2013 and 91% in 2014).

Treatment effects on seedling growth

Shoot growth of the seedlings was affected by both the irrigation regime and the summer drought, but to a very different degree (Fig. 2). In *P. sylvestris*, shoot biomass of P_{10} seedlings was 10% less than that of P_{90} seedlings after each vegetation period ($p < 0.05$), while in *P. nigra* P_{10} seedlings had only 2-4% less biomass than P_{90} seedlings ($p > 0.1$). No effect of the irrigation regime was observed for the root biomass of both species (Fig. S2). The summer drought treatment had, in contrast, a considerable effect on biomass: in $P_{90}D$ seedlings, shoot biomass was reduced in both species by 32-42% after D1 and 50-58% after D2, compared to P_{90} seedlings ($p < 0.001$). Root biomass was severely reduced as well by 29-67% after D1 and 43-56% after D2 ($p < 0.001$). Taproot length was reduced in $P_{90}D$ seedlings as well ($p < 0.001$), but to a much lower degree than root biomass, which led to a 2.2 (*P. sylvestris*) and 1.4 (*P. nigra*) times higher specific taproot length (taproot length per root weight unit) compared to P_{90} seedlings in 2013 (Fig. 3; $p < 0.001$ and $p < 0.01$). In 2014, respective differences decreased to 1.8 (*P. sylvestris*) and 1.3 (*P. nigra*) ($p < 0.001$ and $p < 0.01$).

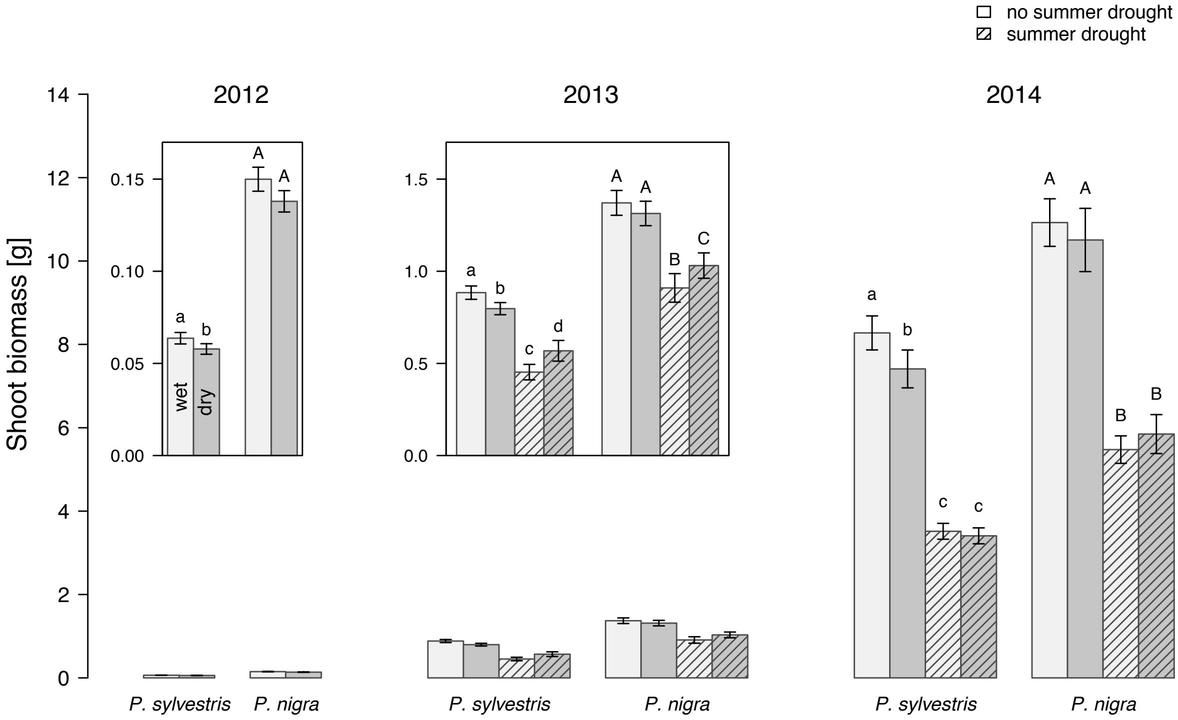

Fig 2. Shoot biomass (means ± se) of *P. sylvestris* and *P. nigra* seedlings at the end of the growing seasons of 2012–2014 under irrigation ('moist': light grey, 'dry': dark grey) and summer drought ('drought': shaded). Different letters denote significant differences between the treatments in each year; the two species were tested separately.

Shoot growth of the seedlings subjected to drought was strongly influenced by the previous irrigation regime (i.e. 'moist' or 'dry'; Fig. 2). As a result, the seedlings of both species from the $P_{10}D$ irrigation regime had significantly more shoot biomass than that of the $P_{90}D$ seedlings after D1 in October 2013 ($p < 0.05$ for both species). The effect was, however, no longer observed after D2 in October 2014 ($p > 0.1$). Similarly, taproots of seedlings from the $P_{10}D$ treatment were on average 21 % (*P.*

sylvestris) and 13 % (*P. nigra*) longer than those from the $P_{90}D$ treatment in October 2013 ($p < 0.001$ for both species), but not anymore after D2 in October 2014. In contrast to shoot biomass and taproot length, root biomass of 'drought' seedlings was not affected by the previous irrigation regime ($p > 0.1$).

Effect of the seed origin on growth

Seedling growth varied greatly among the nine *P. sylvestris* and *P. nigra* populations, with the most vigorous Spanish *P. nigra* (20.7 g) having almost 3 times more total biomass than the least vigorous *P. sylvestris* from higher elevation Switzerland (7.3 g) after three growing seasons. Shoot and root biomass was related to the climatic water balance at the seed origin of the populations in 2013 and 2014 ($p < 0.001$, Fig. 4). Likewise, shoot height and taproot length showed the same relation (2013: $p < 0.001$ and $p < 0.01$; 2014: $p < 0.001$ for both traits).

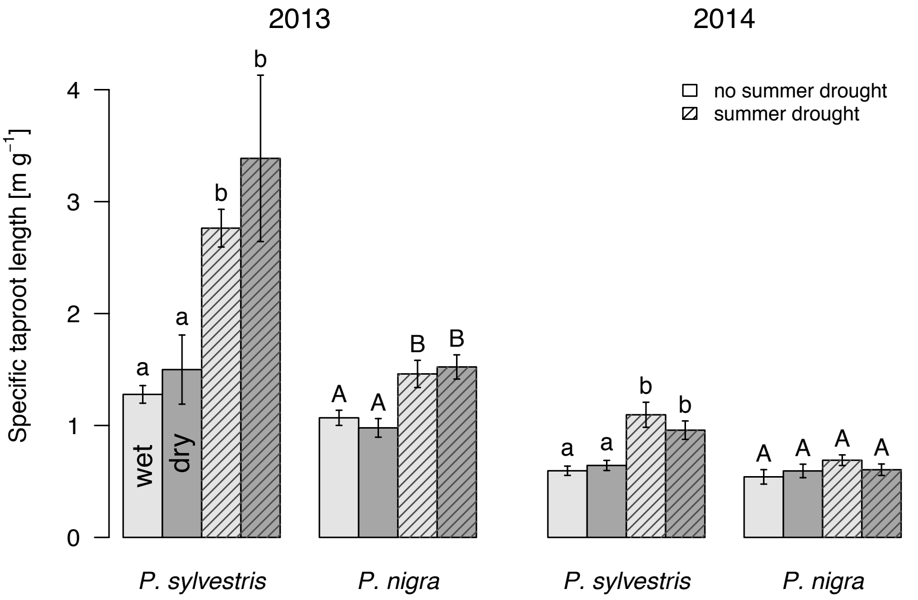

Fig 3. Specific taproot length (means ± se) of *P. sylvestris* and *P. nigra* seedlings after the second (2013) and third (2014) growing seasons under the irrigation regime ('moist': light grey, 'dry': dark grey) and summer drought ('drought': shaded). Different letters denote significant differences between the treatments.

Biomass partitioning in relation to ontogeny, plant size, treatments and seed origin

Root biomass fraction was significantly higher in 2013, compared to 2014 ($p < 0.001$, Fig. 5). In both P_{90} and P_{10} seedlings, it dropped from above 42 % in 2013 to below 30 % in 2014. In $P_{90}D$ and $P_{10}D$ seedlings, the effect was lower and dropped from around 35 % in 2013 to 30 % in 2014. The drought led to a markedly reduced root biomass fraction in 2013 ($p < 0.001$) and, to a lower extent, in 2014 ($p < 0.05$). The effect of the drought was related to plant size in both years ($p < 0.001$). Only in small seedlings, drought affected biomass partitioning, whereas in larger seedlings, it was similar to seedlings with continued irrigation during summer (Fig. 5). In contrast to the summer drought treatment, the irrigation regime affected root biomass fraction only little: P_{10} seedlings had a higher root biomass fraction than P_{90}, whereas $P_{10}D$ seedlings had a lower root biomass fraction than $P_{90}D$ ($p < 0.01$, Fig. 5). After D2 in 2014, the effect of the irrigation regime had disappeared ($p > 0.1$). In seedlings subjected to drought ($P_{10}D$ and $P_{90}D$), root biomass fraction correlated negatively with the water balance of driest month at the seed origin, i.e. seedlings from dry sites had higher root biomass fractions in 2013 ($p < 0.05$, Fig. 6). Non-droughted seedlings (P_{10} and P_{90}) did not exhibit an equivalent correlation ($p > 0.1$). After D2, the biomass partitioning was only marginally related to the water balance at the seed origin ($p = 0.053$, Fig. 6), and the differences of root biomass fraction related to the summer drought treatment had disappeared ($p > 0.1$).

Oxygen isotope fractionation in relation to drought

The fractionation of oxygen isotopes in the needles of the plants indicates a tight stomatal control of seedlings subjected to drought. After both D1 and D2, $\partial^{18}O$ was markedly higher in $P_{90}D$ than P_{90} seedlings ($p < 0.001$, Fig. 7), but differences were less distinct in 2014 than in 2013 ($p < 0.001$). The $\partial^{18}O$ was positively related to the climatic water balance at the seed origin in P_{90} and $P_{90}D$ seedlings in October 2013 ($p < 0.001$) and marginally in October 2014 ($p = 0.054$). This indicates that stomatal conductance over the whole growing season was higher in populations from dry locations, even under drought conditions during summer.

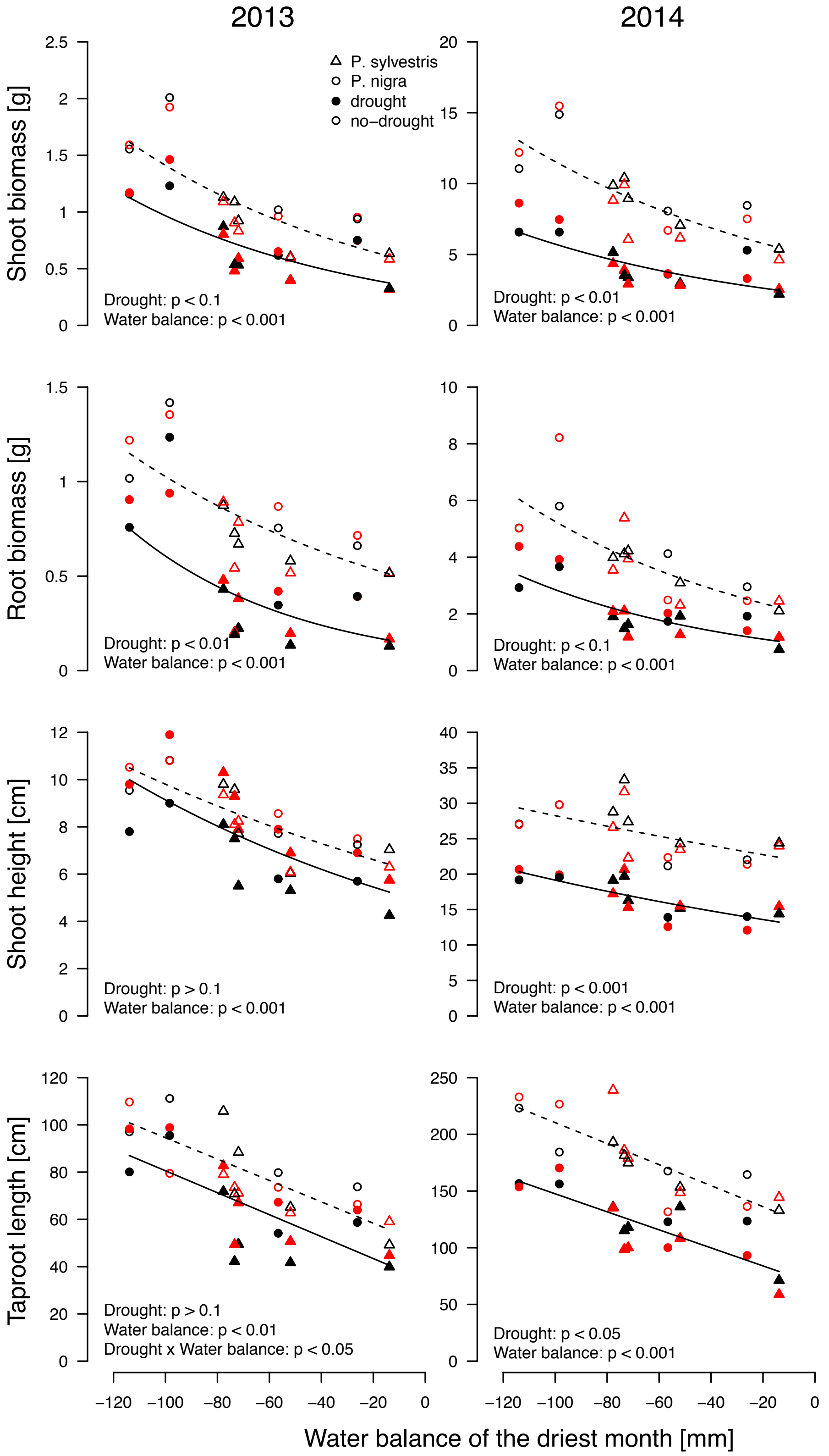

Fig 4. Shoot and root biomass, shoot height and taproot length (population means) of *P. sylvestris* (triangles) and *P. nigra* (circles) seedlings, in relation to the climatic water balance of the driest month at the seed origin in October 2013 and 2014. Seedlings are from the 'moist' (black), 'dry' (red), 'drought' (filled) and 'no-drought' (open) treatment levels.

Discussion

Both *P. sylvestris* and *P. nigra* seedlings reacted with contrasting biomass partitioning to the irrigation regime and the summer drought treatment. Root biomass allocation was slightly increased in seedlings subjected to the 'dry' irrigation regime, whereas it was heavily decreased in seedlings subjected to drought during four summer months (Fig. 4). The drought response differed largely between populations and was related to the climatic water balance at the seed origin (Fig. 5). Accordingly, populations from less dry locations showed a 15% reduction of the root biomass fraction in response to the drought and a 6% increase to 'dry' irrigation, whereas populations from dry locations showed no response to either irrigation regime or summer drought treatment (Fig. 6).

Biomass partitioning in response to the irrigation and drought

The higher root biomass fraction of populations from less dry origins under the 'dry' irrigation regime corresponds well to the 'optimal partitioning' theory (McCarthy & Enquist, 2007). It is also in accordance with common garden experiments using similar drought treatments (Moser *et al.*, 2014). 'Optimal partitioning' fails, however, to describe the large reduction of root biomass fraction as a response to the drought. The 'dry' irrigation regime and the drought did differ very little in the total amount, but very much in the timing of the provided water. It has previously been observed that the timing of drought may affect biomass partitioning (Leuschner *et al.*, 2004), which might be explained by different growth phenologies of shoots and roots. In the studied seedlings, shoot elongation was virtually terminated at the onset of the summer drought treatment and needle elongation one month thereafter. If root growth peaked later, the drought could have affected it to a much larger extent than shoot growth, thus resulting in the lower root biomass fraction. In contrast to shoot growth phenology, only few studies have been conducted on tree root phenology to date (Iivonen *et al.*, 2001; Abramoff & Finzi, 2015). But the available data strongly indicate a lag of 28±12 days of the maximum root growth compared to shoot growth in trees of temperate biomes (Abramoff & Finzi, 2015). In particular, root growth of *P. sylvestris* seedlings has been shown to coincide with the completion of shoot growth (Iivonen *et al.*, 2001). We therefore suspect that a later peak in root growth, compared to the shoot, must have resulted in the different responses of the two plant parts to the drought. In addition to the timing, the severity of the drought may also have caused the reduced, instead of an increased root biomass fraction. Phenotypic responses of plants are limited by large investment cost (Valladares *et al.*, 2007). Particularly under severe drought, the investment costs seem to be too large with respect to the benefit of root growth (Mainiero & Kazda, 2006). While the irrigation sums from March to October were similar in P_{10} and $P_{90}D$ mesocosms, the minimum soil water content differed largely between the two treatments (Fig. 1). In $P_{90}D$ seedlings, the low soil water resulted in severe growth limitations (see also chapter 2) and consequently, it may have been impossible for seedlings to respond to the stress with enhanced root growth.

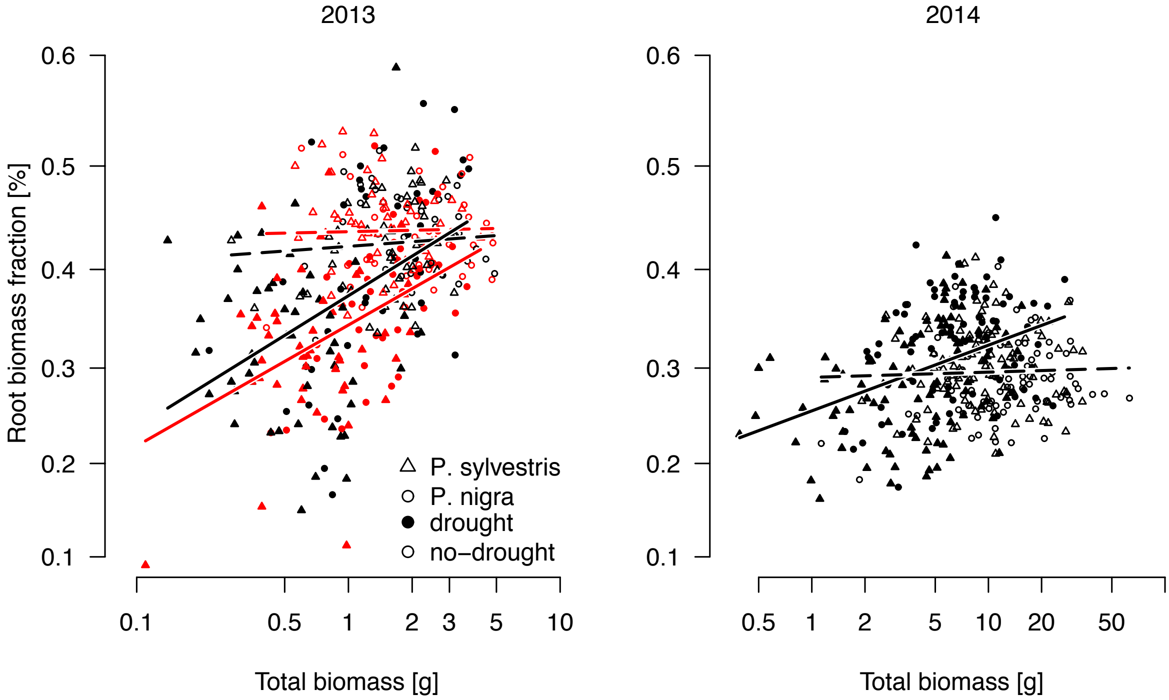

Fig 5. Root biomass fraction in relation to the total biomass of seedlings of both species in 2013 and 2014. Seedlings of the 'moist' and 'dry' (black and red) and 'drought' and 'no-drought' (closed and open symbols, continuous and dashed line) treatments are depicted when treatment effects were significant ($p < 0.05$).

Biomass partitioning in relation to the seed origin

The effects of the irrigation regime and the summer drought treatment on biomass partitioning were strongly related to the climatic water balance at the seed origin. We thus assume that the water balance is closely related to the drought-tolerance of the populations. Seedlings from the most drought-tolerant populations retained a similar root biomass partitioning under drought as under continued irrigation, whereas less drought-tolerant populations showed a strong responses to both treatments (Fig. 5 and 6). Thus, contrary to our expectations (hypothesis 1 and 2), root biomass partitioning of drought-tolerant populations did not increase as a response to experimental drought. An important trait of drought-tolerant pine populations therefore seems to be the continued, and not higher, investment in root growth during drought. The primary focus on leaf structures and leaf physiology in drought ecological studies (Leuschner *et al.*, 2004) might thus miss out on an important plant trait for drought-tolerance. The smaller responses of populations from driest origins to water limitations correspond well to earlier observations on populations of the same species (Richter *et al.*, 2012). In contrast to the study by Richter *et al.* (2012), the more drought-tolerant populations had, however, a higher competitive ability under well-watered conditions as well (Fig. 3 and 4).

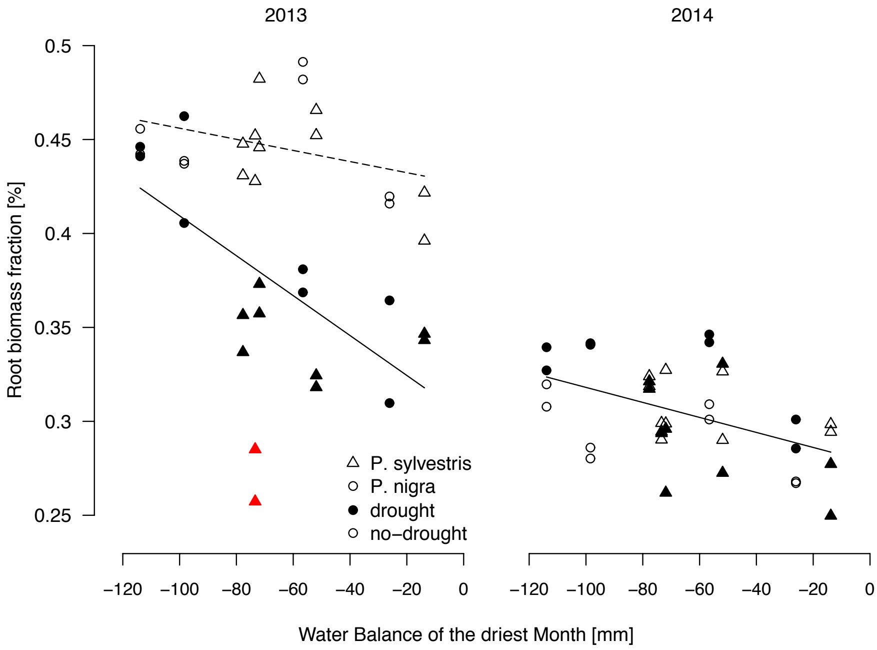

Fig 6. Root biomass fraction (population means) in relation to the climatic water balance of the driest month at the seed origin of seedling of both species in 2013 and 2014. Seedlings of the 'drought' and 'no-drought' (closed and open symbols, continuous and dashed line) treatment differed, whereas the irrigation regime had no significant effect. Red symbols indicate the autochthonous *P. sylvestris* population (Central Alpine, low).

Oxygen isotope fractionation in relation to drought and the seed origin

Stomatal conductance was, as expected, severely reduced in seedlings subjected to drought. As a consequence, $\partial^{18}O$ fractionation was higher in $P_{90}D$, compared to P_{90} seedlings in summer 2013 and 2014 (Fig. 7). As expected, oxygen isotope fractionation was negatively related to the drought-tolerance of the populations (hypothesis 3), thus drought-tolerant populations had a higher stomatal conductance than drought-susceptible populations during the whole vegetation period, particularly if grown under water limitations. Reduced stomatal conductance is related to reduced assimilation and thus in lower biomass production (Atkinson *et al.*, 2000). The strategy of the less drought-tolerant populations, to reduce stomatal conductance during drought, can thus be characterised as drought-avoidance. Conversely, the higher stomatal conductance of drought-tolerant populations resulted in higher growth, as was observed in this study (Fig. 3). These findings are in contrast to observations of a more conservative water use strategy of apparently more drought-tolerant pines in response to drought (Herrero *et al.*, 2013). The less conservative water strategy of the drought-tolerant populations might be explained by their larger root length (Fig. 4) that might allow for better water uptake. While large root biomass does not necessarily result in more effective water uptake, plants may change root architecture in order to reach water pools that are less rapidly depleted during dry spells, such as deeper soil layers (Ryel *et al.*, 2008). The survival of several Mediterranean shrubs under drought conditions was, for instance, related to rooting depth rather than species-specific root-shoot ratio (Padilla & Pugnaire, 2007). The most drought-tolerant populations had longest taproots (Fig. 4) and hence were able to maintain higher stomatal conductance during the drought.

Effect of seedling age and size on root biomass partitioning and specific root length

The measurements of root biomass fractions of 2-years and 3-years old seedlings provide a good means to test allometric partitioning theories. The observed changes in root biomass fractions (0.4 in 2-years old and 0.3 in 3-years old seedlings) are in accordance to recent findings of (Poorter *et al.*, 2012) and thus in strong contrast to predictions of a constant scaling ratio (Enquist & Niklas, 2002), and estimations of increasing root biomasses in woody plants (Wilson, 1988). We found, however, no relation between plant size and root biomass fraction in well-watered seedlings of the same age. This indicates that under well-watered conditions not the seedling size, but the ontogenetic stage determined the root biomass fraction of the seedlings. Similarly as with biomass partitioning, specific taproot length decreased with seedling age (Fig. 3). *Pinus* is known to develop long taproots during early development (Wilcox, 1968; Richter *et al.*, 2012). After the initial formation of a thin taproot in 2-years old seedlings, roots grew less in length and more in width in 3-years old seedlings. Root length is thus most critical in the first two years and thereafter, resources are invested in thicker and more outstretched roots.

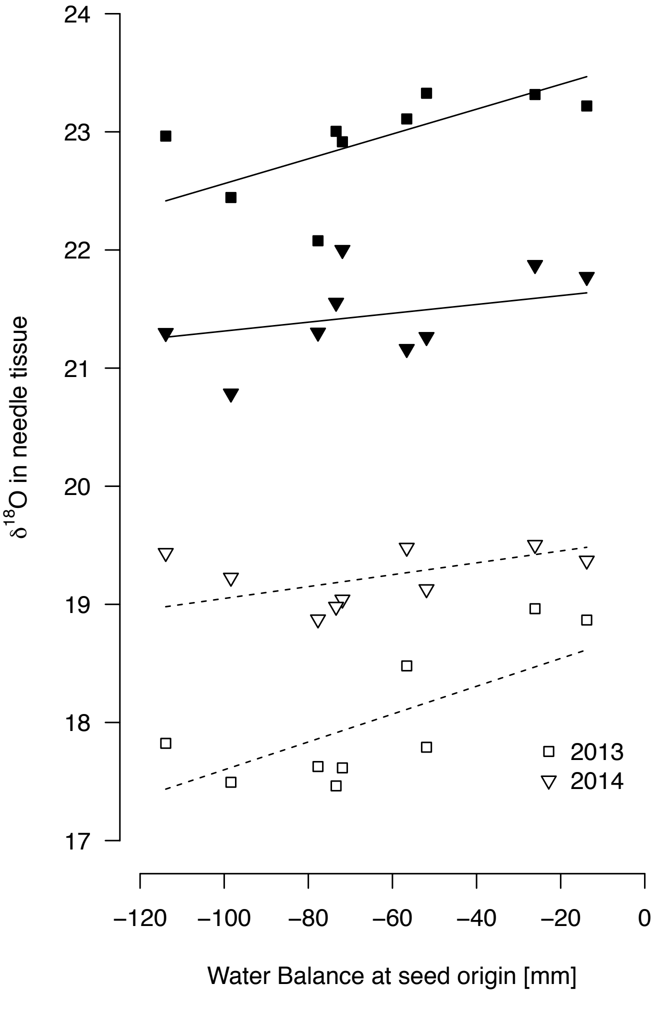

Fig 7. Fractionation of oxygen isotopes (population means) in the needle tissue of $P_{90}D$ and P_{90} (closed and open symbols) seedlings (both species) after the first and the second summer drought (squares and triangles) in relation to the climatic water balance at the seed origin of the populations.

Acclimation to drought

The response of seedlings to drought was affected by the previous irrigation regime. Accordingly, seedlings previously grown under the 'dry' irrigation regime reacted to drought in 2013 with less reduction of shoot biomass and taproot length (Fig. 2 and 4), which indicates drought acclimation. Similarly, severe changes in biomass partitioning of seedlings after the drought in 2013 were no longer observed in 2014 (Fig. 5 and 6). Both observations indicate acclimation resulting from previous water limitations. Physiological acclimation of plants to drought has been studied for long (Kozlowski & Pallardy, 2002; Bruce *et al.*, 2007; Walter *et al.*, 2013), but has been largely neglected in ecological experiments. In particular, the effects of physiological acclimation for tree growth under natural conditions and during multiple vegetation periods are largely unknown (Walter *et al.*, 2011, 2013). Drought experiments extending multiple vegetation periods (e.g. Joslin *et al.*, 2000) show changing responses between years. Potential causes for these changes are, for instance, the bud formation during summer, which influences leaf number and surface of the shoot in the following spring (Bréda *et al.*, 2006). In this study, the acclimation of $P_{10}D$ seedlings did not result in increased root biomass fraction, but led to a smaller root biomass fraction in 2013, compared to $P_{90}D$ seedlings (Fig. 5). This is a result of the enhanced shoot growth of $P_{10}D$ seedlings (Fig. 2). Acclimation to drought thus not only has the potential to reduce mortality under more severe drought (Martínez-Vilalta *et al.*, 2012), but also to enhance above-ground growth.

Conclusions

In this study, we observed a higher investment in roots under moderate continued water limitation, but reduced root biomass in response to a short and severe drought episode. Extreme drought episodes might thus affect growth and survival of seedlings to a much larger extent than prolonged moderate drought (see also: Lindner *et al.*, 2014). Resistance to severe drought episodes is thus an important trait for seedling survival. During D1, seedling responses were mainly determined by the drought-tolerance of the seed source, whereas in D2, acclimation resulted in similar responses of all populations. Short-term acclimation thus critically increases seedlings resistance to repeated drought. We also showed that higher root biomass partitioning of drought-tolerant pine populations is associated with higher stomatal conductance under drought. Longer taproots might be particularly important for continued water uptake from deep-water resources and thus for less severe growth limitations during drought stress.

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Supporting Information

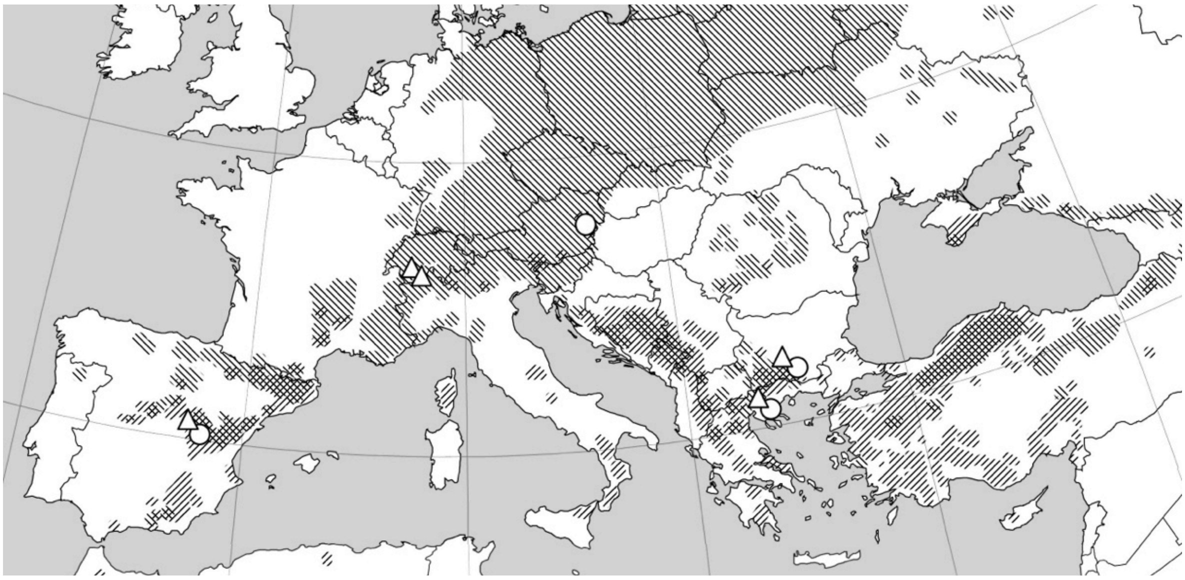

Fig S1. Distribution of *Pinus sylvestris* (right dash) and *Pinus nigra* (left dash) in Central and Southern Europe, edited after EUFORGEN (www.euforgen.org). The locations of the seed origins are indicated with triangles (*P. sylvestris*) and circles (*P. nigra*). Additional information s. Table 1.

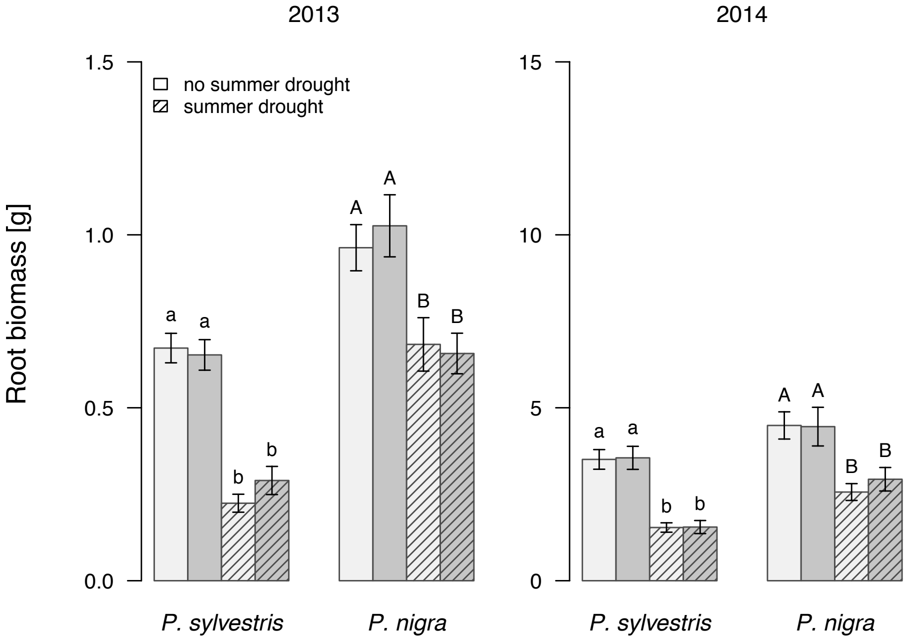

Fig S2. Root biomass (means ± se) of *P. sylvestris* and *P. nigra* seedlings after D1 (2013) and D2 (2014) under the combined moderate drought ('moist': light grey, 'dry': dark grey) and summer drought ('no-drought': blank, 'drought': shaded) treatments. Different letters denote significant differences between the combined watering treatments; the two species were tested separately.

Chapter 2

No carbon limitation in pine seedlings under lethal drought

To be submitted as:

Christoph Bachofen[1,2], Barbara Moser[1], Günter Hoch[3], Jaboury Ghazoul[2], Thomas Wohlgemuth[1]

[1] Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zürcherstrasse 111, 8903 Birmensdorf, Switzerland

[2] Department of Environmental Sciences, Swiss Federal Institute of Technology, Universitätsstrasse 16, 8092 Zürich, Switzerland

[3] Department of Environmental Sciences - Botany, University of Basel, Schönbeinstrasse 6, 4056 Basel, Switzerland

Summary

- More frequent drought episodes are expected to increase tree mortality and hamper regeneration in the future. The mechanisms linking drought-stress to tree carbon metabolism and its relation to mortality are highly disputed. Effects of future elevated CO_2 on tree carbon relations are largely unknown.
- We repeatedly measured the allocation of biomass, starch and soluble sugars to needles, stem and roots in a total of 9 populations of *P. sylvestris* and *P. nigra* seedlings growing under factorial combinations of a four-months summer drought and CO_2 treatment for two years.
- Drought stress led to 44% lower biomass and 48% higher starch concentrations in the seedlings after the first summer. Elevated CO_2 (eCO_2) resulted in additional increase of starch concentrations from 2.98% to 4.01% (+ 1.03%) in the needles of drought-stressed seedlings, whereas non-droughted seedlings allocated starch surpluses to stem and roots. Only 1.1% of the seedlings died during long-term drought, but post-drought mortality was considerable (9.4%) and was not reduced by eCO_2.
- We suggest that elevated starch concentrations in drought-stressed seedlings result from non-adaptive, sink-driven processes that are not related to survival or growth. Additionally assimilated carbon from eCO_2 was not allocated to storage of sink organs under drought, which indicates decreased phloem transport or larger turnover of starch to soluble sugars. The seedlings acclimated rapidly to repeated drought, which resulted in a remarkable drought-tolerance and emphasizes the role of acclimation in drought responses.

Introduction

Increased frequency and duration of drought have been identified as principle factors for tree growth declines (Lindner *et al.*, 2014), tree mortality (Rigling *et al.*, 2013; Anderegg *et al.*, 2013) and hampered tree regeneration (Cochrane *et al.*, 2014). Traits related to drought resistance include stomatal control, resistance to xylem embolism, and the storage and allocation of non-structural carbohydrates. Carbon starvation and hydraulic failure have been postulated as main mechanisms leading directly or indirectly to tree death (McDowell *et al.*, 2008). Hydraulic failure due to xylem embolism occurs as the xylem water pressure drops below a critical value and conduits become air-filled (cavitation) and thus incapable of transporting water (Lens *et al.*, 2013). Carbon starvation, on the other hand, is expected to be the result of prolonged, drought-induced stomatal closure, the consequential reduction of CO_2 assimilation and insufficient supply with non-structural carbohydrates (NSC) for metabolism (McDowell *et al.*, 2008). It might also result from reduced NSC transport from source to sink under drought stress due to impaired phloem function (Ruehr *et al.*, 2009; Sala *et al.*, 2010; Klein *et al.*, 2014). Phloem integrity, in return, depends on soluble sugars for the maintenance of a sufficient turgor pressure during changing environmental conditions (Sala *et al.*, 2012; Sevanto *et al.*, 2014), which directly links plant water and carbon relations.

The hypothesis of carbon-related tree mortality has raised a debate about the physiological mechanisms that link NSC storage with growth and mortality of trees (Sala *et al.*, 2010, 2012; Wiley & Helliker, 2012). Several experiments seem to confirm a decline of NSC in isohydric trees subjected to prolonged drought, but most these findings are based on experiments with potted trees (Piper, 2011; Mitchell *et al.*, 2013, 2014; Hartmann *et al.*, 2013a; Sevanto *et al.*, 2014, but see Dickman *et al.*, 2015). What levels of decline are damaging to trees and whether trees are carbon limited under natural conditions remains largely unclear (Sala *et al.*, 2010, 2012). There are indeed many examples where drought stressed trees exhibit elevated, rather than reduced concentration of NSC (see reviews in Muller *et al.*, 2011 and Rosas *et al.*, 2013, and e.g. Duan *et al.*, 2015). These observations suggest carbon sink limitation, i.e. the continuation of photosynthesis and CO_2 fixation under conditions where the allocation of carbon to sinks is limited by nutrient or water deficiency (Körner, 2003; Sala *et al.*, 2010; Palacio *et al.*, 2014). This is corroborated by evidence that growth is more sensitive to stress than photosynthesis (Boyer, 1970; Muller *et al.*, 2011; Fatichi *et al.*, 2014; Palacio *et al.*, 2014), which is known to lead to NSC accumulation in trees at their altitudinal range limit (Hoch & Körner, 2012). The notion that the accumulation of carbon during drought is mainly driven by reduced sink demands has been challenged repeatedly (Sala *et al.*, 2010, 2012; Wiley & Helliker, 2012; Dietze *et al.*, 2014). For instance, increased starch and soluble sugar concentrations in source organs could be the result of reduced phloem transport (Sala *et al.*, 2010), resulting in the starvation of sink organs (Hartmann *et al.*, 2013b). Many experiments that report an increase of NSC concentrations did not involve lethal drought events (but see e.g.: Duan *et al.*, 2015). It has thus been suggested that drought induced accumulation of NSC concentrations happens in an initial, non-lethal state of drought stress and should be regarded as an adaptive strategy (McDowell, 2011; Sala *et al.*, 2012; Dietze *et al.*, 2014 and literature therein) reducing the risk for carbon starvation under continuing drought conditions (Wiley & Helliker, 2012). To date, it remains unresolved whether the accumulation of carbon increases indeed the tree's likelihood for survival under prolonged drought (Dietze *et al.*, 2014), although work of O’Brien *et al.* (2014) with manipulated NSC concentrations of tropical tree seedlings indicates such a relationship.

A possibility to disentangle mechanisms of carbon storage and growth is to study trees subjected to a combination of elevated atmospheric CO_2 (eCO_2) and drought (McDowell, 2011; Fatichi *et al.*, 2014). In unlimited, expanding systems, eCO_2 has been shown to enhance growth during the first few years of CO_2 enrichment (Körner, 2006). In resource-limited systems, eCO_2 is not expected to directly enhance tree growth (Körner, 2003; Sala *et al.*, 2012), but might still lead to an increased concentration of NSC in the tree (Runion *et al.*, 1999; Körner, 2003; Duan *et al.*, 2013). It is not known, however, how the additional carbon will be used by the plant (Fatichi *et al.*, 2014). If a tree favours storage over growth, excess carbon should be transported to storage organs, but in the case of impeded phloem transport the additional carbon might remain in source organs. There, end-product inhibition might lead to reduced photosynthesis and stomatal conductance (Layne & Flore, 1995). Patterns of NSC allocation during drought and eCO_2 might therefore reveal the plant's strategies and abilities for carbohydrate transport and utilization. NSC measurements in trees subjected simultaneously to drought and eCO_2 are, to our knowledge, scarce, particularly for isohydric species. Allocation under these conditions has, for instance, been studied with respect to single organs (Tschaplinski *et al.*, 1993; Picon-Cochard & Guehl, 1999). Two studies considering different parts of the same plant report either inconsistent effects of eCO_2 and drought on NSC (Duan *et al.*, 2015) or carbon limitation at low (200 ppm) concentrations of CO_2 (Quirk *et al.*, 2013).

We studied carbon allocation of 2 years-old seedlings of two isohydric pine species, *Pinus sylvestris* and *Pinus nigra,* growing under factorial combinations of CO_2 and summer drought during two consecutive growing seasons in a common garden in the Central Alps (Valais, Switzerland). Changes of growth and NSC allocation patterns in relation to the summer drought and CO_2 treatment were tracked with measurements of biomass, starch and soluble sugar concentration in needles, stems and roots in early summer and early autumn (before and at the end of the drought) in both years. We tested whether additionally assimilated carbon under elevated CO_2 is used for increased storage or growth and if elevated CO_2 enhances survival. By growing seedlings from seed in mesocosms, we allowed for natural root development in the presence of inter- and intraspecific competition. Seeds of a total of nine populations from locations at the southern distribution limit with varying levels of summer precipitation were used. This should allow revealing a potential relationship between drought-adaptation and storage or growth during drought. We hypothesize that the allocation of NSC during drought is a sink-driven process and consequently expect that (1) the extreme drought during summer will not lead to carbon depletion, but higher NSC concentrations in all plant organs, (2) an elevated level of CO_2 will enhance growth of well-watered, but not drought-stressed seedlings, and will lead to accumulation of NSC in the latter, and (3) growth under drought will be positively related to a high drought-tolerance of different populations, whereas NSC concentrations might be higher in less drought-tolerant seedlings that exhibit higher sink limitations under drought.

Methods

Species, populations and study site

Pine species have been widely used in studies focusing on tree carbon limitations (s. literature in Palacio *et al*., 2014), because they exhibit a strong isohydric behaviour during drought (Zweifel *et al*., 2009). In Europe, *Pinus sylvestris* L. is a common species in forests, ranging from Siberia to the Mediterranean and showing local adaptation to drought in southern populations (Richter *et al*., 2012; Matías *et al*., 2014). *Pinus nigra* Arnold has a discontinuous distribution in the northern Mediterranean and is regarded as more drought tolerant than *P. sylvestris* (Richter *et al*., 2012). Seeds from five maternal lineages of five populations of *P. sylvestris* and four populations of *P. nigra* were collected in winter 2011/2012. The *P. sylvestris* populations come from two Central Alpine locations, one at low and one at high elevation, as well as three locations in the Mediterranean that were selected based on low precipitation sums during the driest month of the year, an indicator for drought-adaptation of the seed sources (Fig. S1, Table 1). Three Mediterranean *P. nigra* populations were from locations close to the Mediterranean *P. sylvestris* populations and a fourth from an Eastern Alpine location that corresponds to the northernmost distribution of the species. In March 2012, the seeds were sown in a common garden in the Valais valley (Switzerland; 46°18'33"N, 07°41'10"E; 610 m a.s.l.; 19.1 ºC average temperature in July; 600 mm annual precipitation), a dry Central Alpine valley, where *P. sylvestris* is stand forming at low elevations and has been repeatedly observed to be drought limited (Bigler *et al*., 2006; Rigling *et al*., 2013).

Table 1. Seed origin and respective mean precipitation sums of the driest month (1950–2000; www.worldclim.org) of the species and populations sowed in the common garden at Leuk (Switzerland).

Species	Region	Country	Locality	Elevation (m a.s.l.)	Lat (°)	Long (°)	Precipitation (mm)
P. sylvestris	Mediterranean	Bulgaria	Jundola	1405	42.05	23.83	35
	Mediterranean	Greece	Serres	1333	41.24	23.58	29
	Mediterranean	Spain	Ademuz	1542	40.08	-1.08	34
	Central Alpine	Switzerland	Leuk	570	46.29	7.61	49
	Central Alpine	Switzerland	Vispertermi nen	1363	46.27	7.91	90
P. nigra	East Alpine	Austria	Bad Fischau	344	47.83	16.13	36
	Mediterranean	Bulgaria	Dobrostan	1167	41.90	24.93	40
	Mediterranean	Greece	Parthenonas	644	40.13	23.86	18
	Mediterranean	Spain	Ademuz	1195	40.09	-1.38	26

Experimental design and treatments

In February 2012, 12 mesocosms (200 cm x 80 cm x 50 cm) were filled with 30 cm of sand and gravel from the local Rhone riverbed, topped by 15 cm of humus (Oekohum GmbH, Herrenhof, Switzerland), simulating natural forest soils of the Rhone valley (Moser *et al.*, 2014). The mesocosms were subdivided into 140 squares (10 cm x 10 cm), and 15 seeds of one species, population and maternal lineage were sown in two randomly selected squares in March 2012 (Bachofen *et al.*, 2015). This resulted in two replicates per maternal lineage per mesocosm and hence 10 replicates per population per mesocosm. Until May 2012, the soil was watered daily to facilitate germination (Fig. 1). The 12 mesocosms were arranged in a split-plot design with three blocks (whole plots) each of which consisted of two subblocks (split-plots) with two mesocosms (split-split-plots). From June – September 2012, March – October 2013 and 2014 a CO_2 treatment was applied to the subblocks, which consisted of the two levels 'ambient CO_2' (aCO_2: 400 ppm) and 'elevated CO_2' (eCO_2: target concentration of 570 ppm). The eCO_2 simulated the predicted concentration in 2100 assuming an increment of 20 ppm per decade (www.esrl.noaa.gov/gmd/ccgg/trends) and represents roughly twice the preindustrial concentration. Instead of the hexagonal arrangement used in free-air CO_2 enrichment (FACE) experiments, we chose a rectangular arrangement of the CO_2 injection tubes that fitted the shape of the mesocosms. Otherwise the same system as in Hättenschwiler *et al.* (2002) was used. To reduce costs, CO_2 release was interrupted when conditions were unfavourable for photosynthesis (temperature < 5 ºC, photon flux density < 30 mmolm^{-2}s^{-1}, air temperature > 35 °C) or wind speed exceeded 2 ms^{-1}. Concurrently with the CO_2 treatment, automatic mobile rainshelters intercepted the natural rainfall and all mesocosms were irrigated weekly on two subsequent nights (Fig. 1; Moser *et al.*, 2014) to a level of 16 mm, i.e. 416 mm from Mar–Sep. This corresponds to the top decile of natural rainfall at low elevations in the Rhone valley simulating conditions of no water stress (151% of the average March – September precipitation, measured from 1864–2011 at the MeteoSwiss station in Sion located 28 km to the East of the study site). Starting in the second growing season, a summer drought treatment was applied to the mesocosms. In each subblock, irrigation was completely stopped from June – October 2013 (D1) and June – October 2014 (D2) in one randomly selected mesocosm ('dry-out') and continued in the remaining one ('moist').

Seedling mortality

Emergence of the seeds in the mesocosms was recorded in June 2012 and seedling mortality in the whole experiment was determined in April 2013. Afterwards, we randomly chose four seedlings per square and cut the surplus to reduce seedling density. In May and September 2013, we recorded the mortality of 'dry-out' seedlings and in May 2014 and September 2014, of both the 'moist' and 'dry-out' seedlings selected for harvest. Seedlings were considered 'dead' when all the needles turned brown and brittle.

Biomass and NSC

In May and September 2013 and 2014 (Fig. 1), we randomly chose one of the two squares of each species, population and maternal lineage per mesocosm, and uprooted one randomly selected seedling. Only living seedlings were harvested for biomass and NSC measurements. Due to the destructive sampling for NSC measurements, mortality could not be assessed for the same individuals thereafter. We washed the roots and, in every mesocosm, pooled the five maternal lineages from the same species and population, resulting in 108 pooled samples per date. Within one hour of uprooting, the seedlings were shock heated with microwaves to stop enzymatic activity (two pulses of 20 s, 700 W; Popp *et al.*, 1996) and dried at 60 °C to constant weight. We separated the seedlings into four parts: roots, green needles, brown needles and stems (including twigs and buds) and measured the dry weights of each part. Biomass of brown and green needles was added together, whereas NSC analyses were only conducted on green needles. After grinding with a ball mill (Retsch M200, Haan, Germany), total non-structural carbohydrate (NSC) and soluble sugar (sucrose, fructose and glucose; SS) concentration of the roots, green needles and stem were measured according to Hoch *et al*. (2002), except that amyloglucosidase was used instead of clarase for starch digestion. Starch (St) concentration was calculated as the difference between NSC and SS.

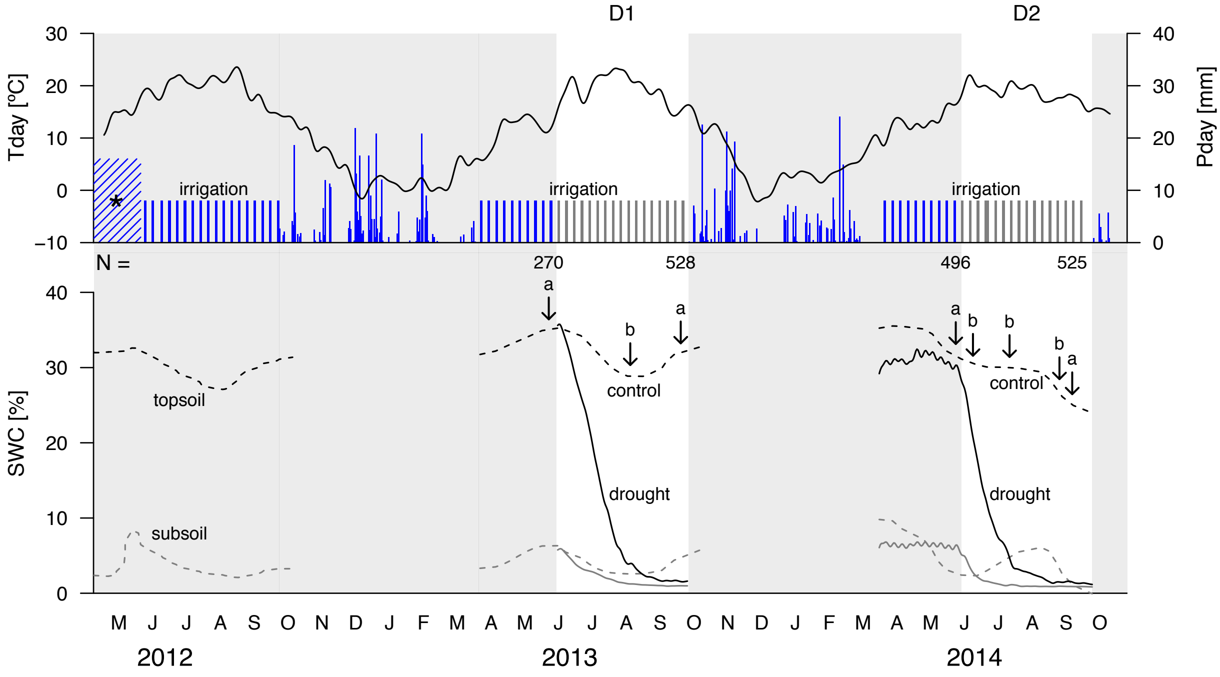

Fig 1. Daily air temperatures (Tday) and natural precipitation/irrigation (Pday) at the experimental site (top), and volumetric soil water content (SWC) in the mesocosms during the experiment (bottom). D1 and D2 indicate the period when summer drought treatments were applied (four months in 2013 and 2014, respectively). SWC is shown for 'moist' (dashed lines) and 'dry-out' mesocosms (solid lines) in the topsoil (5 cm depth) and subsoil (40 cm depth). The amount of irrigation is depicted instead of natural precipitation when rainshelters were in operation. The asterisk denominates the time of daily watering until seedling emergence. Arrows indicate the sampling dates for biomass and NSC measurements (a) and measurement dates for stomatal conductance (b).

Data analysis for Biomass and NSC

To test hypotheses (1) and (2), differences in biomass and St and SS concentrations between treatments, populations, date and plant parts were calculated by using a linear mixed-effects model (*lmer;* package *lme4*) with the statistics program R (R Core Team, 2016). Treatments, populations, dates, and plant parts were fixed effects, block and sub-block identity random effects. To identify the nature of significant interactions of fixed effects, we tested treatment effects on biomass St and SS concentration separately for the two species, at single dates, and with single plant parts with a linear mixed effects models (*lme*; package *nlme*). Multiple testing was corrected for with the 'Hommel' method. The effect of the drought-tolerances of the different populations on whole-plant biomass, St and SS concentrations after D1 and D2 (September 2013 and 2014, hypothesis 3) was tested with a linear mixed effects models with the summer drought treatment and species as fixed effects and the block identity as random effect. We used the climatic water balance of the driest month at the seed origin of the nine populations as indicator for drought-tolerance. The water balance was calculated as the difference between precipitation and potential evapotranspiration according to Thornthwaite (1948) using interpolated precipitation and temperature data from the WorldClim database (Hijmans *et al.*, 2005).

Stomatal conductance

We measured stomatal conductance on a small number of seedlings (12 in 2013, 24 in 2014) using a SC-1 leaf porometer (Decagon Devices, Pullman, WA, USA) on August 20/21 2013, June 25/26, July 24/25 and September 11/12 2014 (Fig. 1). In 2013, we measured seedlings from the Spanish populations of the two species, in 2014 from the Greek populations and the most northern populations of the two species (*P. sylvestris* from Leuk, Switzerland and *P. nigra* from Austria). Before the first measurements in 2013 and again in 2014, we randomly marked one seedling of the selected populations in each mesocosm. Stomatal conductance was measured over 24 h at 2h-intervals from 1 p.m. to sunset (approx. 9 p.m.) and from sunrise (approx. 7 a.m.) to 1 p.m. on the following day. A linear mixed effects model was used to test the effect of the summer drought and the CO_2 treatment on stomatal conductance at different times of the day (within three hours after sunrise and at noon). Additionally, the total daily stomatal conductance was estimated for each date by adding the area under the curve (AUC), i.e. the integrals of the stomatal conductance, of the afternoon and subsequent morning measurements. An ANOVA was used to test for the effect of the summer drought and CO_2 treatment and the populations on the AUC of stomatal conductance at each date. In 2014, pre-dawn water potential was measured using a portable Scholander pressure chamber (M-600, PMS Instruments Inc., Corvallis, OR, USA; Scholand *et al.*, 1965) at three dates during D2 (details s. Appendix S4).

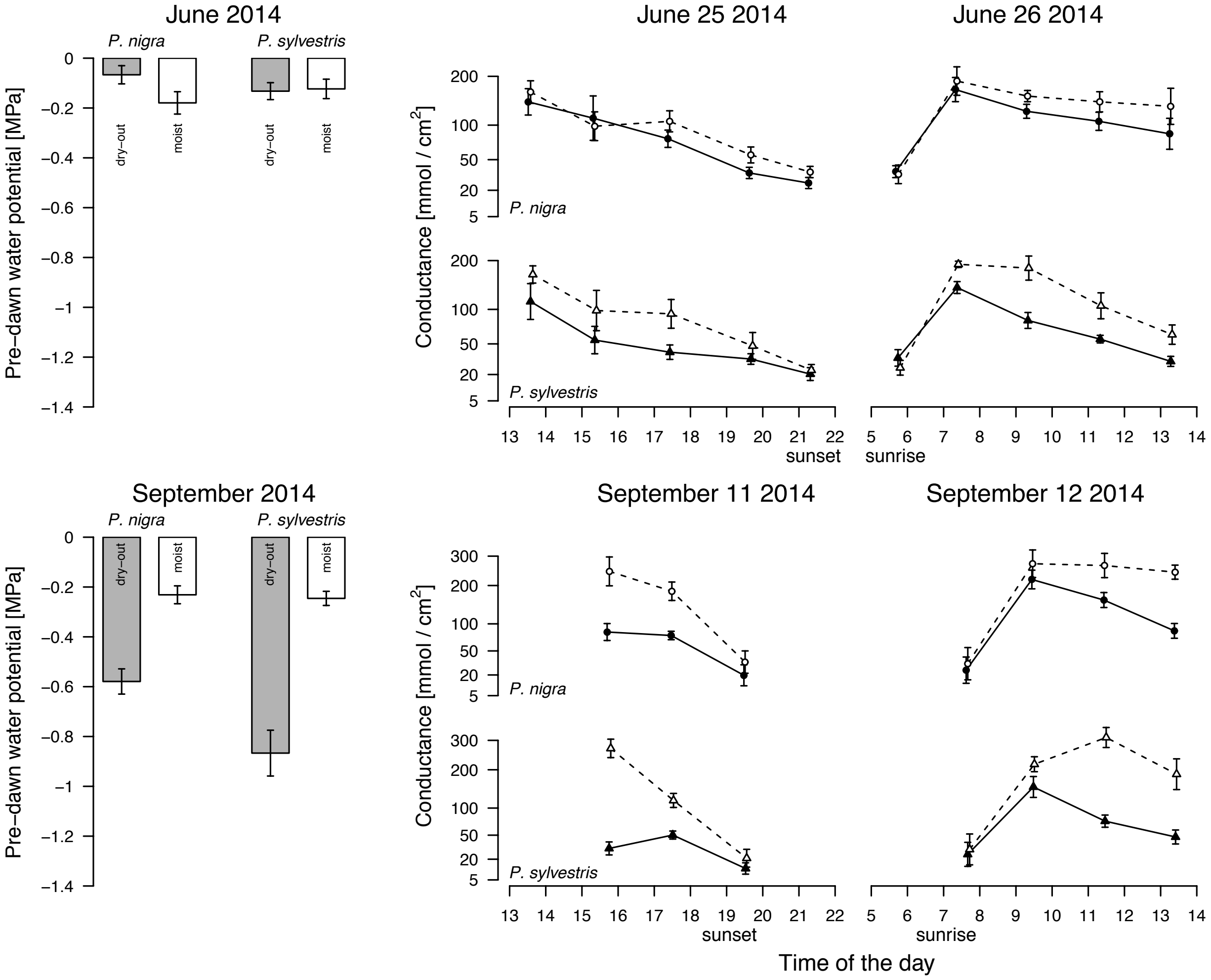

Fig 2. Stem pre-dawn water potential of 'dry-out' (black bars) and 'moist' (open bars) *P. sylvestris* and *P. nigra* seedlings and their respective leaf conductance during a 24 h measurement on June 25 and 26 and on September 11 and 12 2014 ('dry-out': black symbols; 'moist': open symbols).

Results

Soil water status and plant stomatal conductance

Soil water content (v/v) decreased gradually with a rate of 0.53 % (D1) and 0.69 % (D2) per day in the humus layer and 0.13 % per day in the sand layer (D1 and D2) after the irrigation was stopped in the 'dry-out' mesocosms in June 2013 and 2014 (Fig. 1). It fell below 5% in the humus layer and below 1% in the sand layer in late July 2013 and at the beginning of July 2014, respectively. Accordingly, the pre-dawn water potential of *P. sylvestris* and *P. nigra* seedlings was significantly lower in 'dry-out' than in 'moist' mesocosms from July to September ($p < 0.001$, Fig. 2). Stomatal conductance followed a distinct diurnal pattern in both 'moist' and 'dry-out' seedlings. At the end of D1 and D2, stomatal conductance increased quickly in the morning hours and reached the maximum level within three hours after sunrise (Fig. 2). 'Moist' seedlings retained high stomatal conductance until late afternoon, whereas 'dry-out' seedlings reduced conductance sharply at early noon (11:00–12:00; $p < 0.001$, except on June 25/26 2014: $p = 0.027$) and remained at a constantly lower level during the afternoon. The CO_2 treatment had no effect on stomatal conductance at the same time intervals ($p > 0.1$). The AUC of the conductance during 24 h was clearly reduced in 'dry-out' seedlings on all dates ($p < 0.001$), except on June 25/26 2014, when the AUC was only marginally lower ($p = 0.075$). Again, the CO_2 treatment had no effect on the AUC of stomatal conductance ($p > 0.1$).

Drought effects on biomass, NSC and mortality

Total biomass of all 'dry-out' seedlings was substantially lower compared to 'moist' seedlings after the experimental summer drought in the first year (D1; $p < 0.001$, Table 2.1). Roots and stems were most severely affected, whereas needles exhibited no significant growth reduction in either species (Fig. 3). Drought-stressed *P. nigra* seedlings compensated the reduced summer growth with higher root growth over winter and spring, and reached the same total biomass as 'moist' seedlings by May 2014, whereas drought-stressed *P. sylvestris* continued to lag behind 'moist' plants ($p < 0.001$). The experimental drought in the second year (D2) further limited the growth of both species, resulting in a severely reduced total biomass of 'dry-out' compared to 'moist' seedlings until the end of the second season (*P. nigra*, needles: 61.3 %, stem: 44.2 %, roots: 71.3 %; *P. sylvestris*, needles: 52.3 %, stem: 30.9 %, roots: 50.2 % of the 'moist' biomass). Starch and soluble sugar concentrations fluctuated heavily between late spring and late summer in 2013 and 2014 (Fig. 4). In 'moist' seedlings, mean whole-plant starch decreased from 10.2% to 1.7% between May and September 2013, increased to 6.1% in May 2014 and decreased again to 1.4% in September 2014. Starch concentration was elevated in the needles of 'dry-out' compared to 'moist' seedlings after D1 (*P. sylvestris* and *P. nigra*: $p < 0.001$, Table 2.2). In *P. nigra*, starch concentrations were also elevated in stem ($p < 0.001$) and roots ($p < 0.01$), whereas in *P. sylvestris* stem and root starch concentrations did not significantly differ from 'moist' seedlings (Fig. 4). In May 2014, starch concentration in the needles of 'dry-out' seedlings was still higher than in 'moist' seedlings in both species (*P. sylvestris*: $p < 0.001$, *P. nigra*: $p = 0.023$), but had returned to same concentrations as in 'moist' seedlings in *P. nigra* stems and roots. D2 did not affect starch concentrations in neither species, aside from an elevated root starch concentration in *P. sylvestris* ($p < 0.001$). In all other parts, starch concentrations of 'dry-out' seedlings stayed at or returned to levels of 'moist' seedlings during D2. Soluble sugar concentrations were higher in all parts of 'dry-out' compared to 'moist' seedlings at the end of D1 ($p < 0.001$, Table 2.3), particularly in the roots (*P. sylvestris*: +4.91%; *P. nigra* +4.86%, Fig. 4). In May 2014, sugar concentrations had returned to levels of 'moist' seedlings in most parts of both species. Only *P. sylvestris* needles retained higher sugar concentrations ($p = 0.044$). D2 resulted in an increase of sugar concentrations in all parts of *P. sylvestris* seedlings (needles: $p < 0.01$, stem and roots: $p < 0.001$), but only in the roots of *P. nigra* ($p = 0.035$). By the end of D1 in September 2013, only 6 out of 540 'dry-out' seedlings were dead. In contrast, by late spring of the following season (May 2014) we observed 9.4% mortality among the 'dry-out' seedlings (51 indiv.) and 0.6 % among 'moist' seedlings (3 indiv.). The species differed largely, with *P. sylvestris* exhibiting higher mortality under drought (44 indiv.) than *P. nigra* (7 indiv.; $p < 0.001$).

Table 2.1 ANOVA results of differences in seedling biomass between populations, CO_2 treatments, drought treatments and plant parts in September 2013, May 2014 and September 2014.

	Biomass Sep 13				**Biomass May 14**				**Biomass Sep 14**			
Source of variation	Mean Sq	Df	F-value	p-value	Mean Sq	Df	F-value	p-value	Mean Sq	Df	F-value	p-value
Population	6.11	8, 224	129.09	**< 0.001**	4.68	8, 224	82.77	**< 0.001**	6.64	8, 224	124.40	**< 0.001**
CO_2	0.04	1, 8	0.87	0.377	0.01	1, 8	0.15	0.709	0.01	1, 8	0.18	0.682
Part	27.34	2, 224	577.92	**< 0.001**	12.63	2, 224	223.51	**< 0.001**	9.10	2, 224	170.52	**< 0.001**
Drought	5.47	1, 8	115.74	**< 0.001**	4.41	1, 8	78.10	**< 0.001**	9.86	1, 8	184.73	**< 0.001**
Population × CO_2	0.06	8, 224	1.35	0.220	0.10	8, 224	1.69	0.103	0.47	8, 224	8.86	**< 0.001**
Population × Part	0.03	16, 224	0.68	0.808	0.17	16, 224	2.93	**< 0.001**	0.05	16, 224	0.99	0.465
Population × Drought	0.22	8, 224	4.60	**< 0.001**	0.64	8, 224	11.29	**< 0.001**	0.60	8, 224	11.26	**< 0.001**
CO_2 × Part	0.03	2, 224	0.68	0.507	0.01	2, 224	0.15	0.863	0.02	2, 224	0.47	0.629
CO_2 × Drought	0.03	1, 8	0.71	0.425	0.00	1, 8	0.08	0.785	0.16	1, 8	3.05	0.119
Part × Drought	0.59	2, 224	12.48	**< 0.001**	1.13	2, 224	20.06	**< 0.001**	1.79	2, 224	33.59	**< 0.001**
Population × CO_2 × Part	0.01	16, 224	0.19	1.000	0.01	16, 224	0.12	1.000	0.01	16, 224	0.19	1.000
Population × CO_2 × Drought	0.09	8, 224	1.90	0.061	0.17	8, 224	3.02	**0.003**	0.13	8, 224	2.51	**0.013**
Population × Part × Drought	0.03	16, 224	0.53	0.929	0.01	16, 224	0.19	1.000	0.03	16, 224	0.58	0.897
CO_2 × Part × Drought	0.02	2, 224	0.46	0.629	0.01	2, 224	0.17	0.843	0.04	2, 224	0.71	0.495

Table 2.2 ANOVA results of differences in seedling starch concentrations between populations, CO_2 treatments, drought treatments and plant parts in September 2013, May 2014 and September 2014.

	Starch Sep 13				**Starch May 14**				**Starch Sep 14**			
Source of variation	Mean Sq	Df	F-value	p-value	Mean Sq	Df	F-value	p-value	Mean Sq	Df	F-value	p-value
Population	0.44	8, 224	9.05	**< 0.001**	9.35	8, 224	85.99	**< 0.001**	1.60	8, 224	18.46	**< 0.001**
CO_2	0.05	1, 6	1.04	0.347	0.51	1, 6	4.72	0.073	0.03	1, 8	0.30	0.602
Part	8.44	2, 224	173.34	**< 0.001**	21.04	2, 224	193.47	**< 0.001**	2.37	2, 224	27.21	**< 0.001**
Drought	0.92	1, 6	18.90	**0.005**	3.17	1, 6	29.15	**0.002**	0.00	1, 8	0.00	0.998
Population × CO_2	0.07	8, 224	1.49	0.162	0.11	8, 224	1.05	0.401	0.08	8, 224	0.94	0.487
Population × Part	0.21	16, 224	4.32	**< 0.001**	1.52	16, 224	13.99	**< 0.001**	0.19	16, 224	2.23	**0.005**
Population × Drought	0.40	8, 224	8.28	**< 0.001**	0.34	8, 224	3.14	**0.002**	0.13	8, 224	1.47	0.168
CO_2 × Part	0.03	2, 224	0.63	0.534	0.02	2, 224	0.19	0.830	0.15	2, 224	1.70	0.186
CO_2 × Drought	0.00	1, 6	0.00	0.965	0.35	1, 6	3.20	0.124	0.00	1, 8	0.05	0.825
Part × Drought	2.85	2, 224	58.62	**< 0.001**	2.86	2, 224	26.26	**< 0.001**	1.59	2, 224	18.27	**< 0.001**
Population × CO_2 × Part	0.03	16, 224	0.62	0.868	0.05	16, 224	0.48	0.954	0.03	16, 224	0.38	0.985
Population × CO_2 × Drought	0.16	8, 224	3.37	**0.001**	0.46	8, 224	4.24	**< 0.001**	0.26	8, 224	3.00	**0.003**
Population × Part × Drought	0.05	16, 224	0.94	0.522	0.08	16, 224	0.76	0.732	0.04	16, 224	0.44	0.970
CO_2 × Part × Drought	0.16	2, 224	3.23	**0.041**	0.03	2, 224	0.30	0.742	0.01	2, 224	0.07	0.931

Table 2.3 ANOVA results of differences in seedling soluble sugars (sucrose, fructose and glucose) concentrations between populations, CO_2 treatments, drought treatments and plant parts in September 2013, May 2014 and September 2014.

	Soluble sugars Sep 13				**Soluble sugars May 14**				**Soluble sugars Sep 14**			
Source of variation	Mean Sq	Df	F-value	p-value	Mean Sq	Df	F-value	p-value	Mean Sq	Df	F-value	p-value
Population	0.55	8, 224	16.26	**< 0.001**	0.14	8, 224	2.96	**0.004**	0.37	8, 224	6.59	**< 0.001**
CO_2	0.08	1, 6	2.48	0.167	0.01	1, 6	0.30	0.603	0.06	1, 6	1.09	0.336
Part	45.20	2, 224	1325.77	**< 0.001**	9.08	2, 224	185.56	**< 0.001**	30.19	2, 224	541.18	**< 0.001**
Drought	1.96	1, 6	57.47	**< 0.001**	0.27	1, 6	5.51	0.057	1.79	1, 6	32.05	**0.001**
Population × CO_2	0.06	8, 224	1.74	0.091	0.03	8, 224	0.54	0.824	0.03	8, 224	0.49	0.863
Population × Part	0.16	16, 224	4.83	**< 0.001**	0.26	16, 224	5.40	**< 0.001**	0.10	16, 224	1.73	**0.043**
Population × Drought	0.15	8, 224	4.52	**< 0.001**	0.05	8, 224	1.00	0.435	0.45	8, 224	8.15	**< 0.001**
CO_2 × Part	0.08	2, 224	2.20	0.113	0.07	2, 224	1.53	0.220	0.00	2, 224	0.07	0.931
CO_2 × Drought	0.01	1, 6	0.40	0.552	0.02	1, 6	0.36	0.569	0.00	1, 6	0.07	0.804
Part × Drought	0.66	2, 224	19.39	**< 0.001**	0.25	2, 224	5.07	**0.007**	1.53	2, 224	27.34	**< 0.001**
Population × CO_2 × Part	0.01	16, 224	0.27	0.998	0.02	16, 224	0.43	0.972	0.09	16, 224	1.55	0.084
Population × CO_2 × Drought	0.05	8, 224	1.52	0.152	0.05	8, 224	0.95	0.474	0.06	8, 224	1.11	0.354
Population × Part × Drought	0.03	16, 224	1.03	0.431	0.03	16, 224	0.52	0.937	0.03	16, 224	0.46	0.965
CO_2 × Part × Drought	0.03	2, 224	0.85	0.430	0.18	2, 224	3.66	**0.027**	0.23	2, 224	4.04	**0.019**

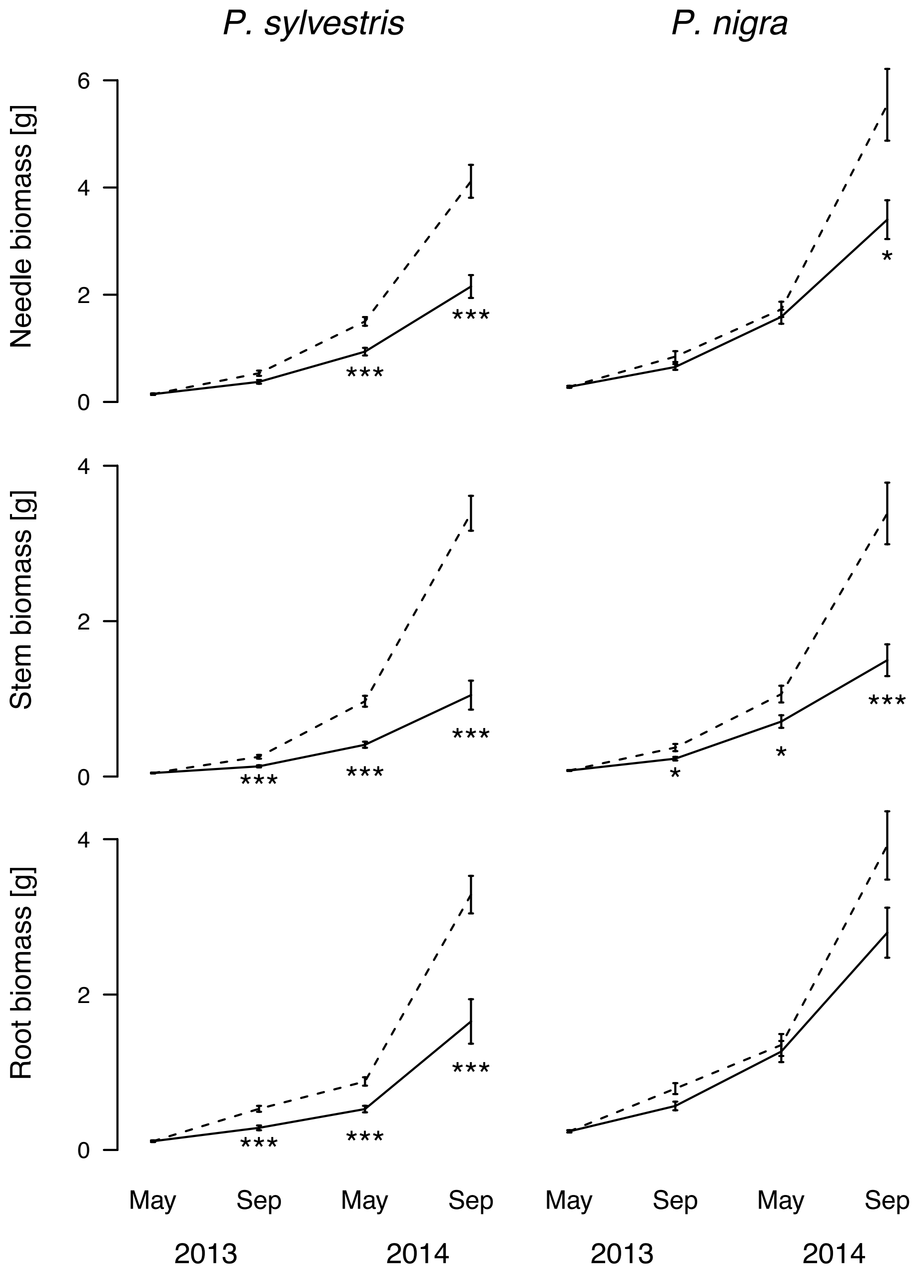

Fig 3. Biomass (means ± s.e.) of the needles, stem and roots of 'dry-out' (solid lines) and 'moist' (dashed lines) *P. sylvestris* and *P. nigra* seedlings in May and September 2013 and 2014. Stars indicate significant differences between the treatments at each date (* $p < 0.05$ and *** $p < 0.001$)

Effects of elevated CO_2 on biomass and NSC

Elevated CO_2 (eCO_2) did not affect biomass production in any part of neither species during the whole experiment (Fig. 5a and 5b, Table 2.1). Likewise, mortality did not differ between mesocosms with ambient and elevated CO_2 ($p > 0.1$). Starch concentrations were, on the other hand, elevated in specific parts of seedlings exposed to eCO_2 after D1 (Fig. 5c, Table 2.2). 'Moist' and 'dry-out' seedlings differed notably in the allocation of surplus starch: While the 'moist' seedlings mainly accumulated starch in roots (from 2.70% to 3.12%, i.e. +0.42%, $p = 0.032$) and stem (from 1.26% to 1.47%, i.e. +0.21%, $p = 0.011$) and not in the needles ($p > 0.1$), concentrations only increased significantly in needles of 'dry-out' seedlings (from 2.98% to 4.01%, i.e. +1.03%, $p = 0.019$) and not in stem or roots ($p > 0.1$). After D2, 'dry-out' and 'moist' seedlings exposed to eCO_2 had similar starch concentrations in all the parts of the plant (Fig. 5d). Soluble sugar concentrations were also elevated in eCO_2 seedlings (Fig. 5e and 5f), but increases were only small (approx. + 0.5%) compared to the effect of D1 (approx. + 5%). The surplus of sugar was differently allocated than the surplus of starch: in 2013, 'moist' seedlings showed no preference of any part of the plant, whereas 'dry-out' seedlings allocated additional sugars only in the needles and the roots ($p < 0.1$). After D2 in 2014, sugar concentrations decreased in the roots of 'moist' seedlings ($p < 0.01$) and in the stem ($p < 0.05$) of 'dry-out' seedlings.

Biomass, starch and soluble sugar concentrations in relation to the climatic water balance at the seed origin

Biomass of both 'moist' and 'dry-out' seedlings at the end of D1 and D2 (September 2013 and 2014) was strongly related to the climatic water balance of the driest month at the location of the seed sources (Fig. 6, $p < 0.001$). It accounted for a high proportion of biomass variability of drought-stressed (2013: $r^2 = 0.69$, 2014: $r^2 = 0.57$) and irrigated seedlings (2013: $r^2 = 0.67$, 2014: $r^2 = 0.61$). Starch concentrations of 'dry-out' seedlings after D1 and D2 were not related to the water balance (Fig. 6, $p > 0.1$). In 'moist' seedlings, starch concentrations were related to the water balance only in September 2014 ($p = 0.021$). Soluble sugar concentrations were only marginally related to the water balance in both 'moist' and 'dry-out' seedlings in September 2013 ($p = 0.066$ and $p = 0.056$), but not in September 2014 ($p > 0.1$).

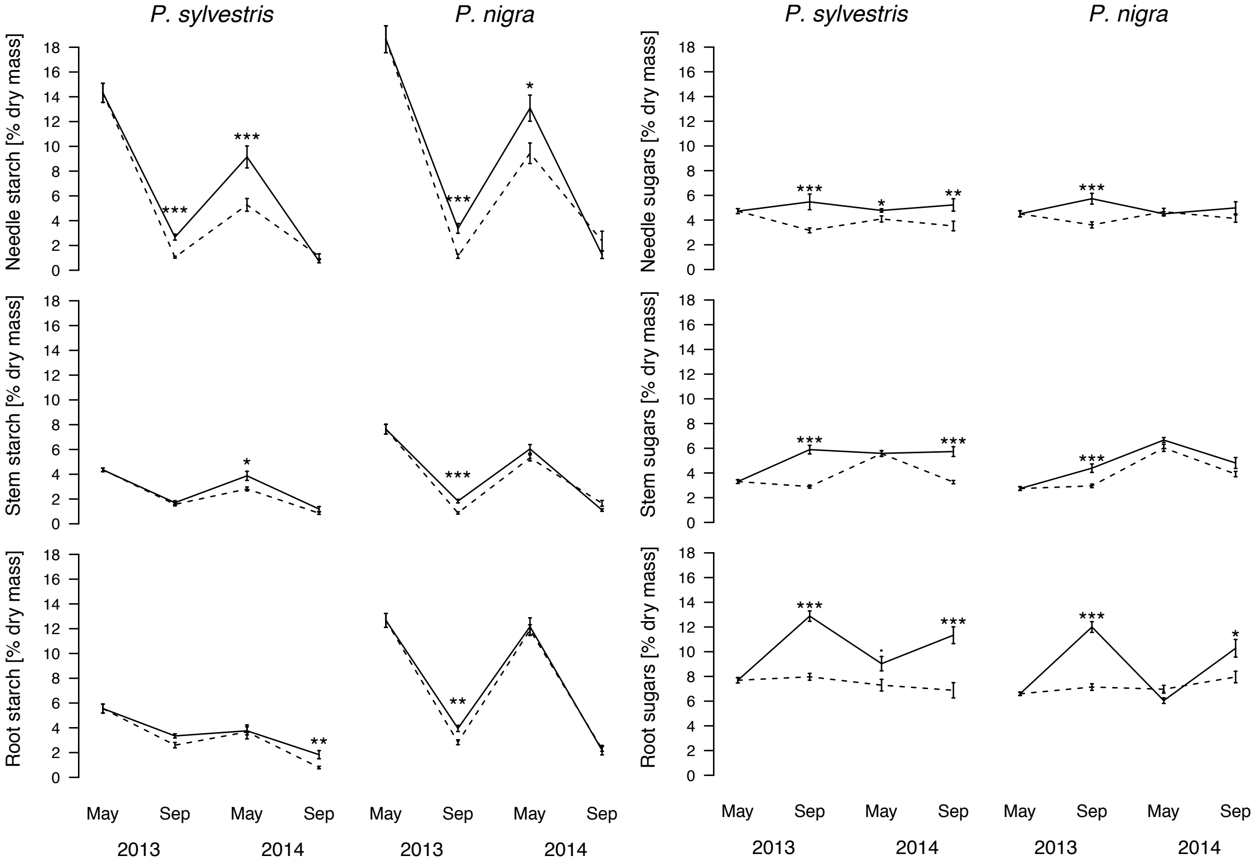

Fig 4. Starch (left panel) and soluble sugar (sucrose, fructose and glucose; right panel) concentrations (means ± s.e.) of the needles, stem and roots in 'dry-out' (solid lines) and 'moist' (dashed lines) *P. sylvestris* and *P. nigra* in May and September 2013 and 2014, as percentage of tissue dry mass. Starch concentrations were calculated as the difference between the total NSC and soluble sugar fraction. Stars indicate significant differences between the treatments at each date (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Discussion

Experimental drought during four summer months in two consecutive years resulted in severely impaired growth of 2-years old pine seedlings. While after the first drought (D1) in September 2013, only 6 out of 540 seedlings died, a new census in spring of the following year (May 2014) revealed significantly higher mortality in drought-stressed seedlings (9.4 %, 44 *P. sylvestris* and 7 *P. nigra*) in comparison to mortality in irrigated seedlings (0.6 %, 1 *P. sylvestris* and 2 *P. nigra*), which suggests a delayed mortality response. Four months of continuous drought during summer is only currently observed at the southernmost locations in Europe (Fig. S2), and are not representative of droughts elsewhere in Europe, although climate projections suggest more intense summer droughts in Central Alpine valleys such as the Valais (Appenzeller *et al*., 2011). While the severity of the experimental drought treatment is relatively extreme, the low mortality rate of the seedlings is remarkable. This might be related to the root architecture of pines, which form taproots that allow for water uptake from the subsoil in the first months after germination (Moser *et al*., in review).

Drought effects on starch concentrations and mortality

The responses of tree carbon storage to drought and its implications for growth and mortality are highly disputed (e.g. Hartmann *et al*., 2015; Körner, 2015; Sevanto & Dickman, 2015). Starch is the most prominent storage compound in trees (Hoch *et al*., 2003); we therefore focused on the effects of drought on starch concentrations together with its effect on mortality and growth. As hypothesized, we observed higher starch in drought-stressed compared to irrigated seedlings of both species after D1 (Fig. 4). In *P. nigra*, all three plant parts (needles, stem, roots) had elevated starch concentrations, whereas *P. sylvestris* seedlings exhibited higher starch concentrations only in the needles. We allowed for the development of a large undisturbed root system in the mesocosms (Moser *et al*., in review) and a slow, natural soil drying (<0.7 % per day, Fig. 1). Consequently, drought-stressed seedlings were able to assimilate carbon during the drought period, particularly in early morning hours (Fig. 2). Sustained carbon assimilation in combination with severely impaired growth (Fig. 3) likely resulted in elevated starch concentrations (Fig. 4). It has been suggested that NSC accumulate only in early stages of drought stress (McDowell, 2011; Sala *et al*., 2012; Dietze *et al*., 2014) and then decline as a result of prolonged, lethal drought (Hoch, 2015; Sevanto & Dickman, 2015). Here, we show that four months of severe and partially lethal drought led to elevated starch concentrations. While we can only speculate whether NSC concentrations might have declined with more sustained drought, our results demonstrate that a mortality of 9.4% resulted from water limitations that did not reduce carbon reserves, which is consistent with observations of Duan *et al*. (2015). We therefore infer that processes leading to seedling death originate independently of a possible starch decline, as suggested by (Anderegg & Anderegg, 2013). As previously suggested, hydraulic failure might thus induce mortality before decreased stomatal conductance might result in lethal reduction of carbon storage (Hartmann *et al*., 2013a).

Effects of elevated CO_2 on biomass, starch and soluble sugar concentrations

Contrary to our expectations, elevated CO_2 (eCO_2) did not enhance growth, in either irrigated or in drought-stressed seedlings in 2013 or 2014 (Fig. 5). Effects of eCO_2 on tree growth are highly dependent on test conditions (Körner, 2006; Handa *et al*., 2008), and our results indicate that seedling growth during the experiment was not fully decoupled from other limiting factors such as, for example, water (Camarero *et al*., 2015) and concomitantly nutrient availability (Norby *et al*., 2010). Nevertheless, elevated starch and soluble sugar concentrations in seedlings under eCO_2 and retained stomatal conductance indicate that additional carbon was assimilated. During D1, the eCO_2 treatment revealed a preference of irrigated seedlings for additional starch allocation to the roots (Fig. 5). Drought-stressed seedlings did, on the other hand, not allocate additional starch into stem or roots, indicating a limitation of carbon phloem loading (Körner *et al*., 1995) or phloem transport during drought (Sala *et al*., 2010; Reinhardt *et al*., 2015; Savage *et al*., 2016). Yet, concentration changes are only an indirect measurement of carbon fluxes. To confirm phloem impairment during drought (Sevanto & Dickman, 2015), direct measurements (as e.g. Ruehr *et al*., 2009; Blessing *et al*., 2015, both studies in beech) would be needed. In contrast to the starch, soluble sugar concentrations increased in all three parts of the irrigated seedlings with eCO_2, showing no preference of allocation to any part of the seedling during D1 (Fig. 5). In drought-stressed seedlings, sugar concentration only increased in needles and marginally in roots (Fig. 5). The elevated root sugar concentrations in drought-stressed seedlings are apparently in contrast to the hypothesized phloem impairment. We assume that carbon transport to the roots was not completely shut down during the first drought, and enough soluble sugars were transported or converted to increase root sugar concentrations for osmotic adjustment in order to maintain water uptake (Chen & Jiang, 2010; Brunner *et al*., 2015).

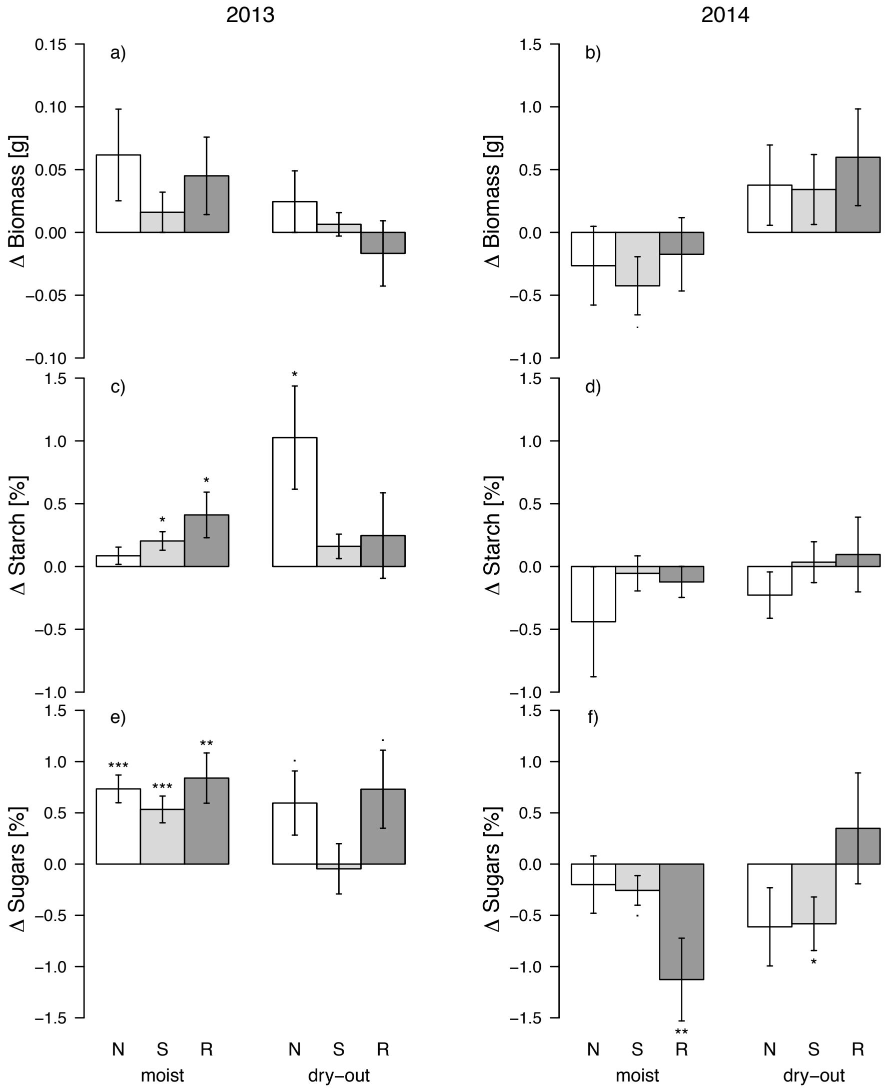

Fig 5. Effect of elevated CO_2 (eCO_2) on the biomass (a, b), starch (c, d) and soluble sugar concentrations (sucrose, fructose and glucose; e, f) of pooled *P. sylvestris* and *P. nigra* seedlings: mean difference between eCO_2 and aCO_2 (± s.e.) in needles ("N", white), stem ("S", light grey) and roots ("R", dark grey), in 'moist' and 'dry-out', and in September 2013 and 2014. Stars indicate a significant effect of eCO_2 (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) and points a marginal effect ($p < 0.1$).

Relationship between growth, storage and drought-tolerance of populations

It has been hypothesised that drought-induced increases of storage at the expense of growth is an adaptive property of the plant to prevent carbon starvation in the long term (McDowell, 2011; Dietze *et al.*, 2014). Indeed, experimentally carbon depleted (defoliated) tree seedlings prioritised carbon allocation to storage at the expense of growth (Wiley *et al.*, 2013). Accordingly, we should be able to observe a negative relation between growth and storage by comparing the differently drought-tolerant populations subjected to experimental drought. While biomass was clearly related to the climatic water balance at the seed origin of the populations, starch concentrations after D1 were not, indicating no relationship between storage and growth of 'dry-out' seedlings (Fig. 6). This in accordance to similar measurements of growth and NSC concentrations in drought-stressed *Pinus halepensis* (Klein *et al.*, 2014). It is therefore questionable whether plants that are not artificially carbon depleted react to drought-stress with preference for storage. In contrast, Wiley & Helliker (2012) assumed that if enhanced storage was an adaptive property of the plants during drought, populations that are regularly under risk of drought stress should have a higher storage than populations from humid locations. Again, our observations do not support this hypothesis. This indicates that under severe drought, growth is limited independently of storage.

Seasonal development of NSC concentrations

NSC concentrations of trees are subjected to strong seasonal changes related to growth phenology (Gough *et al.*, 2010). Starch concentrations of pine needles and sapwood usually peak in late spring and continuously decrease during summer and autumn until a minimum is reached in mid-winter (Fischer & Höll, 1991; Fischer & Höll, 1992; Oleksyn *et al.*, 2000; Hoch *et al.*, 2003). The observed decrease of starch concentrations between May and September (Fig. 4) can thus be associated to growth during that period (Oleksyn *et al.*, 2000). The seasonal starch fluctuations were much larger than the effects of the summer drought or CO_2 treatment (Fig. 4 and 5). It is therefore essential to include a control to the treatments, in order to distinguish treatment effects from seasonal changes, which are sometimes being neglected (e.g.: Adams *et al.*, 2009; Quirk *et al.*, 2013).

Acclimation to drought

While we measured significantly higher starch concentrations in drought-stressed seedlings than in irrigated plants by the end of D1, no differences were recorded after D2, except for *P. sylvestris* roots (Fig. 4). Likewise, there was no effect of eCO_2 on starch concentrations after D2 (Fig. 5). While we observed strong needle browning in many populations during D1, almost no needle browning occurred during D2. This indicates a large acclimation potential of seedlings exposed to repeated drought stress.

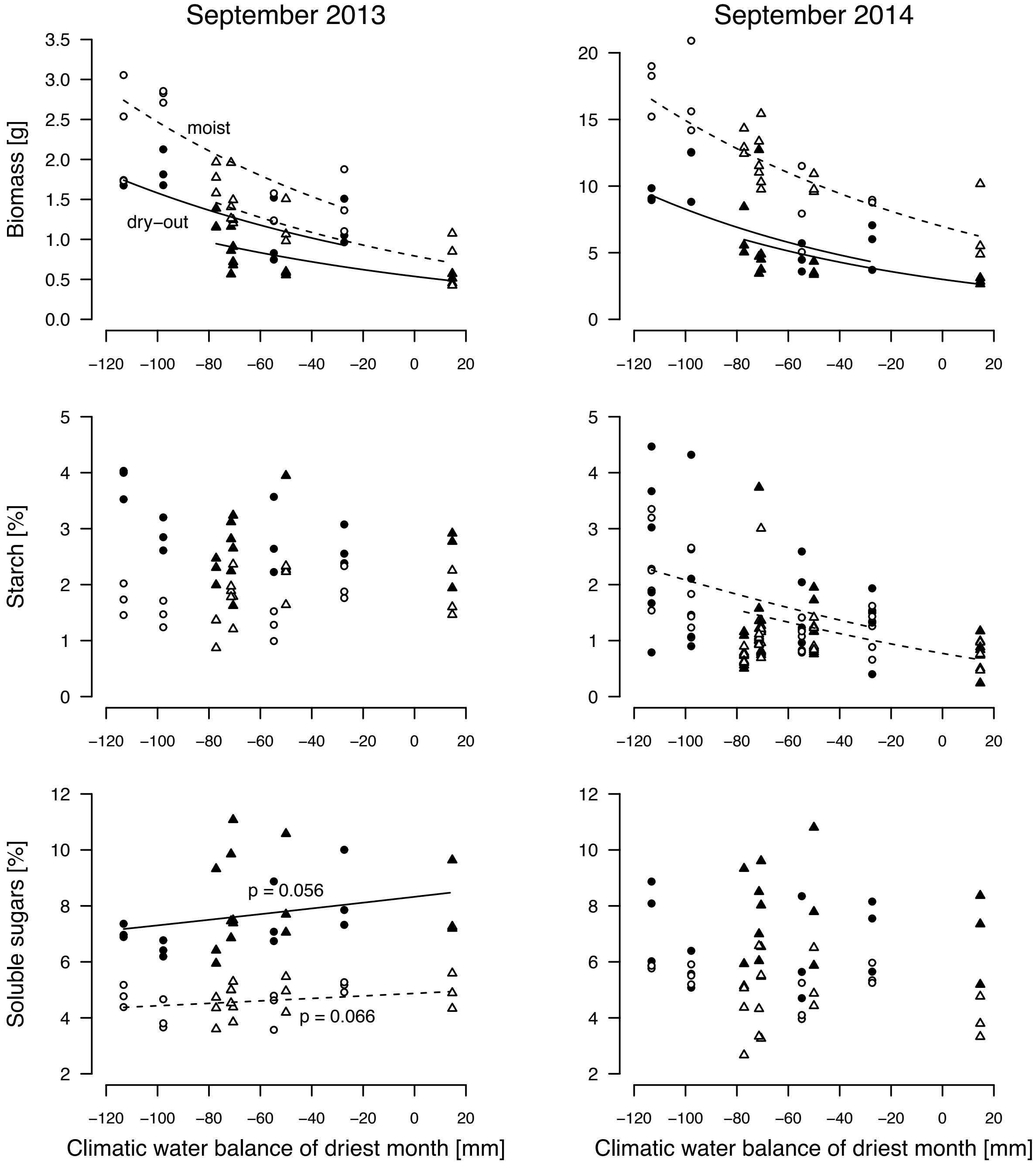

Fig 6. Whole-plant biomass, starch and soluble sugar concentrations of *P. sylvestris* (triangles) and *P. nigra* (circles) seedlings grown under 'dry-out' (filled symbols, solid line) and 'moist' conditions (open symbols, dashed line) in September 2013 and September 2014 in relation to the climatic water balance of the driest month at the seed origin.

Soluble sugar concentrations in roots and biomass, on the other hand, reacted similarly to D2 (Fig. 5), which highlights the importance of the osmotically active sugars for drought acclimation (Chen & Jiang, 2010; Brunner *et al.*, 2015). There are several mechanisms involved in ecological stress memory in plants, comprising the accumulation of osmoprotective proteins, the reduction of the photosynthetic apparatus and altered gene expression (Bruce *et al.*, 2007; Walter *et al.*, 2013). How these mechanisms are effective under natural conditions and whether they extend to multiple vegetation periods is largely unknown (Walter *et al.*, 2011, 2013). Changes in leaf morphology seem, however, to be more important for drought acclimation than changes in leaf physiological parameters (Limousin *et al.*, 2010). Shoot formation in trees is a process that unfolds over two years, and environmental conditions during the time of bud formation in the first year influence leaf number and surface area in the second year (Bréda *et al.*, 2006). Hence, the reduced shoot growth of the seedlings during D2, as a result of the experimental conditions during bud formation in D1, likely contributed to the drought acclimation of the seedlings. The acclimation and recovery during winter led to a markedly different drought-response in the subsequent year. Acclimation to drought thus reduced seedling mortality considerably.

Conclusions

Four months of drought during summer did not have immediate lethal effects on most *P. sylvestris* and *P. nigra* seedlings, but induced processes leading to mortality in almost 10% of the seedlings during the subsequent winter and spring. There was no evidence of carbon decrease in the 2-year old pine seedlings as a result of the drought. On the contrary, the severe drought resulted in elevated carbon storage, which is expected to be a result of the severe growth limitations. Stomata were never completely closed, and therefore seedlings assimilated enough carbon to surpass the low consumption during the drought. Supplying drought-stressed seedlings with elevated atmospheric CO_2 did not enhance post-drought survival, and we observed that additionally assimilated carbon was not utilized, but remained in the source organs of drought-stressed seedlings. Carbon storage may thus not directly affect seedlings survival in locations where drought periods are seasonally interrupted by recovery phases. After successful establishment in sporadic wet years (Moser *et al.*, 2015), pine seedlings are remarkably tolerant to severe droughts in their second and third year. Increased frequency of droughts may thus not result in widespread seedling die-off, but rather lead to constrained productivity of more drought-tolerant pine forests.

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Supporting Information

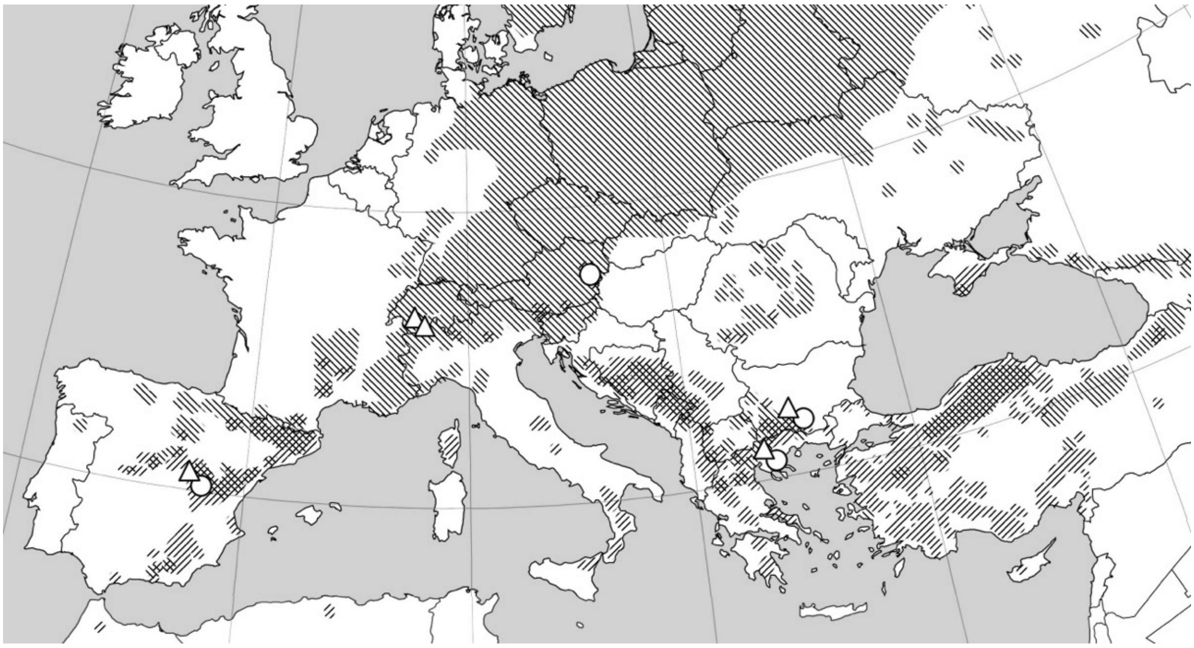

Fig S1. Distribution of *Pinus sylvestris* (right dash) and *Pinus nigra* (left dash) in Central and Southern Europe, edited after EUFORGEN (www.euforgen.org). The locations of the seed sources are indicated with triangles (*P. sylvestris*) and circles (*P. nigra*). Additional information s. Table 1.

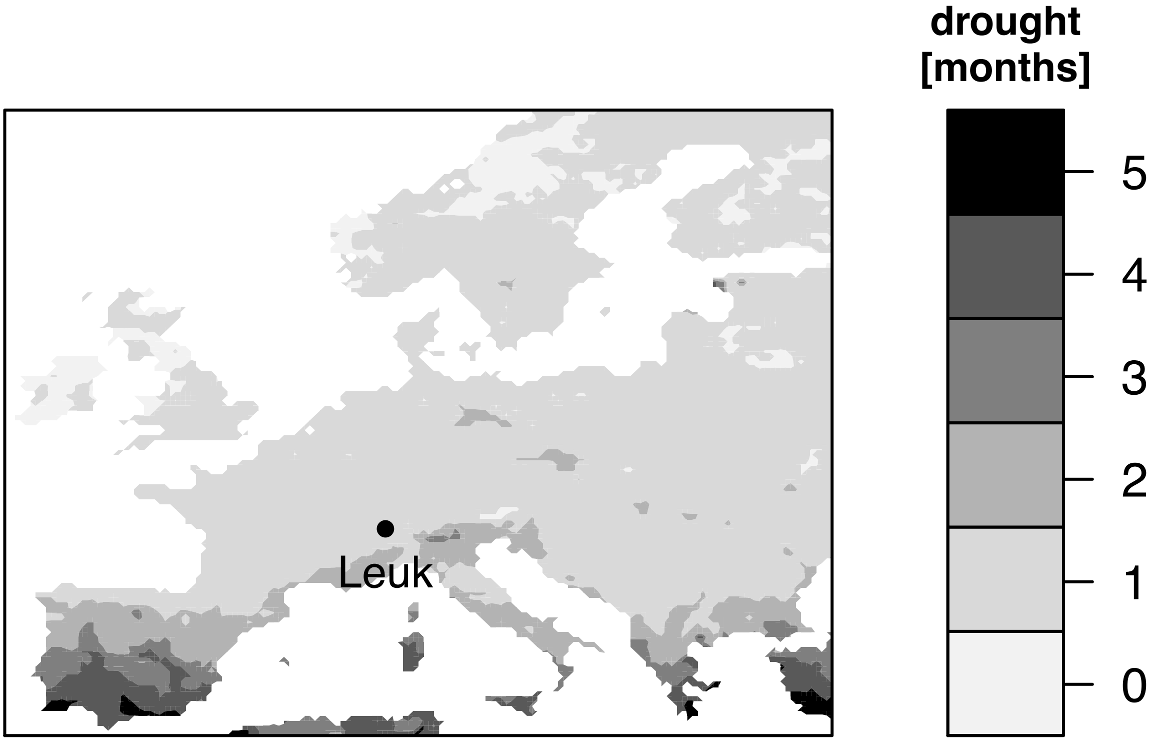

Fig S2. Duration of the longest drought period (< 0.1 mm daily precipitation) from 1960–2014 (ENSEMBLES gridded observational data, www.ecad.eu).

Methods S4. Pre-dawn water potential measurements

Pre-dawn water potential was measured using a portable Scholander pressure chamber (M-600, PMS Instruments Inc., Corvallis, OR, USA) on June 3, July 29 and September 10 2014 between 1 a.m. and 4.30 a.m.. At each date, we randomly selected one seedling from each combination of treatment, species and population per mesocosm. In every treatment-species-population combination, the replicates among maternal lineages were chosen in a Latin square design, i.e. a different maternal lineage was selected in each block. On each seedling, a randomly selected shoot from the current year was cut and measured within a minute. Because the data are heavily skewed, we tested the effects of the drought and CO_2 treatment on the water potential of the two species at each of the sampling dates with a non-parametric two-sample Wilcoxon test.

Chapter 3

Cold temperature extremes during spring do not limit the range shift of Mediterranean pines into regions with intermittent frost

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Christoph Bachofen[1,2], Thomas Wohlgemuth[1], Jaboury Ghazoul[2], Barbara Moser[1]
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[1] Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zürcherstrasse 111, 8903 Birmensdorf, Switzerland

[2] Department of Environmental Sciences, Swiss Federal Institute of Technology, Universitätsstrasse 16, 8092 Zürich, Switzerland

Summary

- Bioclimatic envelope models have predicted latitudinal range shifts of tree species in Europe following climate change. Accordingly, Mediterranean species will be able to migrate northwards if climatic conditions become warmer and dryer. Frost, on the other hand, is an important and recurring factor in temperate and boreal regions causing damage to buds and leaves, and potentially limiting the survival of Mediterranean tree species or populations at higher latitudes. Since species distribution models rely on average climatic parameters, they may underestimate the risk of frost damage from low temperature extremes.
- We measured the cold hardiness of *Pinus sylvestris*, *Pinus nigra* and *Pinus halepensis* seedlings from a total of 11 European populations growing in a common garden in a cold Central Alpine valley on seven dates between February and July 2013. On each date, needles were artificially frozen at several temperatures and the temperature estimated at which 50% of the needle tissue is damaged (LT_{50}; relative electrolyte leakage).
- Cold hardiness did not differ between populations of the same species and was not related to the minimum temperatures at the seed origin. In comparison to deciduous trees, Mediterranean *P. sylvestris* and *P. nigra* maintained extremely wide safety margins against frost throughout late winter and spring. By contrast, safety margins of *P. halepensis* were much narrower until March and winter cold hardiness was in the range of regularly recurring low temperature events in Central Europe.
- According to the measured LT_{50} values, the migration of a wide range of drought-tolerant populations of *P. sylvestris* and *P. nigra* from the Mediterranean to Central and Western Europe is not limited by intermittent cold temperature extremes in spring. They are notably as well adapted to frost as populations from Central Alpine origin.
- Differences in dehardening patterns between species demonstrate the importance of analysing cold hardiness repeatedly during potentially sensitive periods in order to predict species range shifts in the context of climatic change.

Introduction

In Central Europe, yearly drought periods are expected to extend by 3–9 days within a century (Lindner *et al.*, 2014). Populations or species of trees within the Mediterranean region are regularly exposed to long dry periods during summer. They are expected to be better adapted to the future climate of Central Europe than temperate species. Indeed, seedlings from Spanish *Pinus sylvestris* populations have been repeatedly shown to perform better under drought conditions than Central or Northern European populations (Richter *et al.,* 2012; Taeger *et al.*, 2013, Matías and Jump, 2014). Such results underpin expectations of a northwards shift of Mediterranean tree species to Western and Central Europe (Hanewinkel *et al.*, 2013). Nonetheless, drought tolerance is just one of several prerequisites for survival under future climatic conditions in Central Europe, and Mediterranean species might be vulnerable to other factors to which trees in more northerly climates are exposed. Sensitivity to frost, for instance, is a trait that limits the northern distribution of many tree species (e.g. Sakai and Larcher, 1987) and intensities and durations of cold extremes are expected to persist in Central Europe in the future (Kodra *et al.*, 2011). Frost tolerance often comes at the cost of later bud break resulting in a shorter growing season (Lenz *et al.,* 2013). This may be fatal in Mediterranean regions, where growth is restricted to spring due to sparse summer precipitation (Castro *et al.*, 2004). Genetic differentiation of drought tolerant Iberian populations of *P. sylvestris* (Soranzo *et al.*, 2000) have been suggested to be related to reduced frost tolerance (Cheddadi *et al.*, 2006). Frost might therefore constrain the northerly expansion of Mediterranean populations to Central Europe, where winter temperatures are considerably lower than in the Mediterranean.

Bioclimatic envelope models predict shifts of tree species to higher latitudes throughout Europe (e.g. Thuiller *et al.*, 2006, Hanewinkel *et al.,* 2013), but evidence for current northward migration of trees remains scarce (Zhu *et al.*, 2012, but see Boisvert-Marsh *et al.*, 2014, Fisichelli *et al.*, 2014). It is possible that predictions from bioclimatic envelope models are underestimating the limitations imposed by low temperature extremes. For instance, Svenning and Skov (2004) found marked discrepancies between the current distribution of tree species in Europe and their potential bioclimatic ranges based on monthly mean temperatures. These differences can be explained by management history and geographic limitation of post-glacial dispersal (Svenning and Skov, 2004), but also by the negligence of temperature extremes in bioclimatic models (Zimmermann *et al*., 2009). Kollas *et al*. (2013) suggested that the elevational and latitudinal range shift of deciduous tree species are limited by extremes of cold temperatures during bud break in spring. For conifers a similar frost-sensitive season seems to exist during dehardening in spring (Beuker *et al.,* 1998). Provenance trials with pine species suggest that the cold hardiness of trees is related to the temperature at the seed origin (Beuker *et al.,* 1998; Aldrete *et al.,* 2008; Kreyling *et al.,* 2012), which varies greatly given the wide distribution ranges. On the other hand, there is some evidence that frost resistance is physiologically related to drought tolerance in conifers (Blödner *et al*., 2005) such that prior exposure to drought stress increases cold hardiness (Kreyling *et al.,* 2012). This implies that drought tolerant Mediterranean species (or populations) might be pre-adapted to frost.

In order to assess whether frost is a limiting factor for the predicted northward shift of Mediterranean tree species in Europe, we compared the spring cold hardiness of drought-adapted populations of *P. sylvestris*, *Pinus nigra,* and *Pinus halepensis* of Mediterranean origin with that of Central Alpine *P. sylvestris*. Seedlings of all species and populations were grown in a common garden in the Valais valley (Central Alps, Switzerland), where winter temperatures regularly fall below -10 °C. In this region, *P. sylvestris* is growing at the dry limit of its wide distribution range and shows signs of increased mortality as a result of rising temperatures (Rigling *et al.,* 2013). We examined (1) to what degree seedlings of Mediterranean origin tolerate low temperature extremes between late winter and early summer, (2) whether drought-adapted populations from the Mediterranean are more prone to frost damage than Central Alpine populations, and (3) whether exposure to summer drought affects the cold hardiness of the seedlings in the following spring.

Materials and Methods

Species, study site, and experimental design

Pinus sylvestris L. ranges almost continuously from the Iberian Peninsula to Siberia. It is the most drought tolerant native tree species in the Central Alpine valleys, forming stands at elevations below 1000 m a.s.l. *Pinus sylvestris* forests are not found below 1300 m a.s.l. at their southern distribution limit in the Mediterranean (Richter *et al*., 2012). In Mediterranean mountains, *P. sylvestris* is succeeded towards lower elevations by *Pinus nigra* Arnold and *Pinus halepensis* Mill. at lowest elevations and driest locations. *Pinus nigra* is discontinuously distributed in the northern Mediterranean (Fig. 1) and is generally assumed to be more drought tolerant than *P. sylvestris* (e.g. Rouget and Richardson, 2001) but still frost resistant (e.g. Climent *et al.,* 2009). *Pinus halepensis* is the least frost resistant but most drought tolerant Mediterranean pine species in this study. It is found at desert or steppe margins in Spain and other areas scattered across the Mediterranean (Fady *et al.,* 2003; Fig. 1).

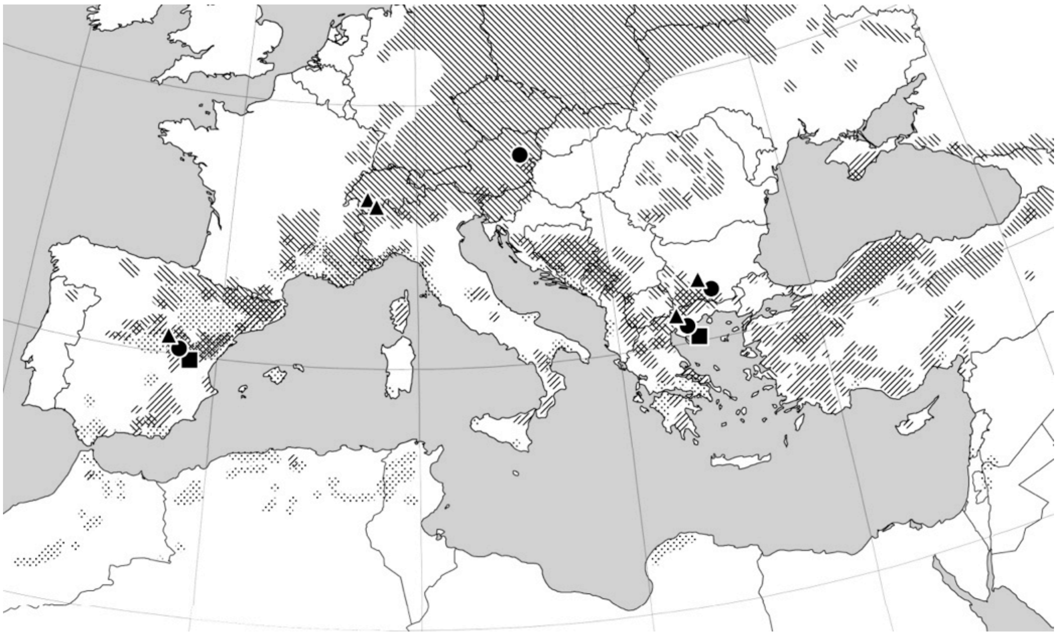

Fig. 1. Distribution of *Pinus sylvestris* (right dash), *Pinus nigra* (left dash) and *Pinus halepensis* (dots) in Central and Southern Europe, edited after EUFORGEN (www.euforgen.org). The locations of the seed sources are indicated with triangles (*P. sylvestris*), circles (*P. nigra*) and squares (*P. halepensis*; additional information s. Table 1).

Seeds from two to five populations per species and five maternal lineages per population were collected in winter 2011/2012 at two Central Alpine, one East Alpine and several Mediterranean locations (Table 1, Fig. 1). Precipitation during the driest month of the year was used to select driest regions in Bulgaria, Greece and Spain, where the three *Pinus* species occur along an elevational gradient (Fig. S1). An air-screen cleaner was used to remove empty seeds before sowing. Germination tests (200 seeds incubated on filter paper at 25/13 °C, day/night cycles and 90% humidity) showed germination rates >85% in all species and populations, except in *P. sylvestris* from Greece (72%) and *P. halepensis* from Spain (65%). In March 2012, the seeds were sown into six potting containers with a surface area of 200 cm × 80 cm each, located in a common garden near Leuk (Valais, Switzerland; 46°18'33''N, 07°41'10''E; 610 m a.s.l.; 590 mm annual precipitation; -0.8 ºC average temperature in January). The containers were filled with 30 cm of sand and gravel from the local Rhone riverbed, topped by 15 cm of humus (Oekohum GmbH, Herrenhof, Switzerland). The two layers were designed to simulate natural forest soils of the Rhone valley (Richter *et al.,* 2012). Individual containers were divided into squares of 10 cm x 10 cm, and 15 seeds from one species, population and maternal lineage were sown in two randomly selected squares (two replicates x five maternal lineages, i.e. 10 replicates per population). After germination, a controlled precipitation regime was applied container-wise during the vegetation period (May 24 – September 26 2012) by using an automatic mobile rain shelter to intercept rainfall and a weekly automatic irrigation on two consecutive nights (Richter *et al.,* 2012). The irrigation treatment simulated two climates: a 'dry' climate, corresponding to the lowest decile, and a 'wet' climate, corresponding to the top decile of the April–September precipitation sums from 1864 to 2011 in the Rhone valley (67% and 151% of the average, respectively, measured at the MeteoSwiss station in Sion located 28 km to the East of the study site). The six containers were arranged in a split-plot design with the irrigation treatment replicated three times. In all squares, 10–15 individuals had emerged until June 2012. Seedling mortality was negligible between June 2012 and February 2013.

Table 1. Seed origin and respective mean monthly minimum temperatures of the coldest month (1950–2000; www.worldclim.org) of the species and populations sowed in the common garden at Leuk (Switzerland).

Species	Region	Country	Locality	Elevation (m a.s.l.)	Lat (°)	Long (°)	Min temp (°C)
P. sylvestris	Mediterranean	Bulgaria	Jundola	1405	42.05	23.83	-7.2
	Mediterranean	Greece	Serres	1333	41.24	23.58	-5.2
	Mediterranean	Spain	Ademuz	1542	40.08	-1.08	-3.2
	Central Alpine	Switzerland	Leuk	570	46.29	7.61	-3.6
	Central Alpine	Switzerland	Visperterminen	1363	46.27	7.91	-6.2
P. nigra	East Alpine	Austria	Bad Fischau	344	47.83	16.13	-5.7
	Mediterranean	Bulgaria	Dobrostan	1167	41.90	24.93	-6.3
	Mediterranean	Greece	Parthenonas	644	40.13	23.86	0.5
	Mediterranean	Spain	Ademuz	1195	40.09	-1.38	-1.9
P. halepensis	Mediterranean	Greece	Parthenonas	353	40.12	23.81	1.6
	Mediterranean	Spain	Aras del Olmo	1056	39.92	-1.15	-0.6

Frost tolerance

The cold hardiness of the needles of the seedlings was determined bi-weekly from late February to May 2013 (Feb 15, Mar 8, Mar 22, Apr 5, Apr 19 and May 3), and after full dehardening in summer on July 22 2013. On each sampling date, we randomly selected one square of every maternal lineage and population per container. In the selected square, one random seedling was harvested, rinsed with de-ionized water, and 18 first-year needles were randomly selected, detached and distributed to nine plastic bags. The needles of the 5 maternal lineages were pooled container-wise (Aronsson, 1980), resulting in 10 needles per plastic bag and three replicates per treatment level ('wet' and 'dry'). In the 'dry' treatment, only four populations were sampled (*P. sylvestris* Leuk, Switzerland, *P. sylvestris* Spain, *P. nigra* Spain, *P. halepensis* Spain), due to the limited time available between the harvest of the needles and the freezing treatment. Second-year needles began to emerge in late April on all the seedlings and were collected in addition to first-year needles on May 3 and July 22. They were processed separately but in the same manner as the first-year needles to account for a possible difference in cold hardiness related to the ontogenetic state. Again, only four populations were sampled (*P. sylvestris* Leuk, *P. sylvestris* Spain, *P. nigra* Spain, *P. halepensis* Spain) due to time constraints.

The nine bags of each treatment combination and replicate were randomly allocated to 7 different freezing temperatures, a negative control (-80 ºC) and a positive control (4 ºC). The bags were wrapped in aluminium foil and kept at 4 ºC until they were placed in the freezers in the frost laboratory at the Botanical Institute of the University of Basel. The freezing of the samples started within 12 hours after sampling. The freezers allowed independent freeze-thaw cycles for each freezing treatment (details s. Lenz *et al.,* 2013): the samples were cooled at a rate of 3 K h^{-1}, kept at the target temperature (-5 ºC to -35 ºC in 5 K steps) for 4 h and thawed at 3 K h^{-1} until they reached a temperature of 4 ºC, at which they were maintained until further processing. On May 3 and July 22, the range of freezing temperatures was adjusted to the expected decrease in cold hardiness of the needles (-3 ºC to -21 ºC in 3 K steps). The adjustment was necessary to capture the tipping point between frost resistance and frost damage (s. below). Missing values resulted from single freezer failures on Feb 15 (-25 ºC), Mar 8 (-5 ºC), Mar 22 (-15 °C), Jul 22 (-12 ºC, -18 ºC).

The cold hardiness of the needles was determined with an adjusted version of the Relative Electrolyte Leakage (REL) method, which is an index of cell injury (Repo, 1992; Kreyling *et al.,* 2012). Strimbeck *et al.* (2007) demonstrated that REL values above 0.7 are associated with necrosis on more than 60% of the needle area in temperate *Pinus* species, whereas Sutinen *et al.* (1992) even found 100% browning of *P. nigra* needles at the same REL threshold under conditions of slow freeze-thaw cycles. After the freezing treatment, the needles were cut into pieces of 1 cm and incubated for 48 h at 4 ºC in 8 ml of de-ionized water containing 0.1% Triton-X (Sigma-Aldrich Chemie GmbH; Buchs, Switzerland). The electrolyte leakage was quantified as the conductivity of the solution at 25 ºC, measured with a Mettler Toledo SevenMulti S47. After the initial measurement, the solution was autoclaved at 121 ºC for 20 min, simulating total tissue damage. After incubation for another 48 h at 4 ºC, a second conductivity measurement was taken. The REL was then expressed as the ratio of the first measurement to the second measurement. Cold hardiness was estimated by a non-linear-regression of the REL in relation to the freezing temperature using the formula of Anderson *et al.* (1988). The temperature at which 50% of the tissue is damaged (LT_{50}) is estimated as the inflection point of the logistic regression (Repo *et al.,* 1996)

$$Y_T = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + e^{k(T_m - T)}}$$

where Y_T is the REL at temperature T, Y_{min} is the lower asymptote of the regression, Y_{max} is the upper asymptote of the regression, k is an index for the slope of the curve, and T_m is the inflection point of the curve. The curve was fitted using the *nls* function in the R package *stats* version 2.15.2 (R Core-Team, 2012). In *P. nigra* it has been shown that estimates of needle cold hardiness using LT_{50} values derived from electrolyte leakage measurements are highly correlated with estimates based on visual observations of more than 50% needle browning ($R^2 > 0.9$; Sutinen *et al.*, 1992). At the first two sampling dates, a large proportion of *P. halepensis* needles showed signs of desiccation combined with light coloration. REL analyses showed a high degree of needle damage at all freezing temperatures, including the positive control (4 °C). We suspect that the needle tissue had been damaged before the artificial freezing treatment, probably due to winter-embolism (Mayr *et al.,* 2002), which most likely occurred as the soil was frozen for several days in January. This data was therefore omitted from the analyses, and care was taken during subsequent sampling not to include needles with signs of winter-embolism. As a result, sample size in *P. halepensis* was reduced from February to mid-April.

Safety margins

The safety margins of individual populations against frost damage during the experimental period were calculated as the differences between the minimum temperatures measured at the study site in spring 2013 and the LT_{50} on a given sampling date. Temperature measurements were missing from March 30 to April 17 due to a malfunction of the data logger. For the calculation of the safety margins on April 5, interpolated temperature data from MeteoSwiss with a resolution of 2 km were used (comparison of interpolated data with data measured at the experimental site is shown in Fig. S2). Since safety margins against frost damage are related to the temperature development in a given season (Lenz *et al.*, 2013), the long-term risk of frost damage can only be approximately estimated. Return rates of frost events in

Sion were calculated similarly to Kollas *et al*. (2013), based on absolute daily minimum temperatures recorded at the MeteoSwiss station at Sion (482 m a.s.l.) between 1959 and 2012. Frost events were defined as a series of consecutive days on which the minimum temperature reached a given negative threshold, i.e. a frost event that lasted several days counts as a single event. Return rates of frost events were calculated for each month separately (October to April) as the length of the weather record (53 years) divided by the number of frost events in a given class (-2 °C to -20 °C in 2 °K intervals). Likewise, we calculated the return rates of a -20 °C frost event in January and a -10 °C event in April using daily gridded observational minimum temperatures for Europe between 1959 and 2012 provided by ENSEMBLES (EU FP6 project, http://ensembles-eu.metoffice.com and ECA&D project, http://www.ecad.eu, Haylock *et al*., 2008).

Seed origin

An analysis of variance (ANOVA) was applied to test for main and interaction effects of population and the sampling date on the LT_{50} of the seedlings grown under wet conditions. The potting container was treated as a random effect. The populations were treated as a fixed effect, because they were selected based on an indicator for their drought-adaptation (as previously described). Contrasts were used to test for pairwise differences between the three species. A pairwise comparison of the LT_{50} of the populations was conducted for each sampling date and each species separately, using a Tukey HSD post-hoc test with the 'Bonferroni' adjustment for p-values. Differences of the LT_{50} between the first-year needles and the second-year needles were analysed with a separate ANOVA with the two sampling dates, the needle age and the four populations (*P. sylvestris* Leuk, *P. sylvestris* Spain, *P. nigra* Spain, *P. halepensis* Spain) as fixed effects and the potting containers as a random effect.

In order to assess the degree of frost adaptation of individual species and populations, interpolated monthly minimum temperatures (averages of the years 1950–2000) were retrieved from the WorldClim database for each seed collection site (Hijmans *et al.,* 2005; www.worldclim.org). Linear regression was used to analyse the relationship between the LT_{50} and the minimum temperatures at the seed origin of *P. nigra* and *P. sylvestris* with the potting container as a random effect. As only two populations of *P. halepensis* were included in the experiment, this species was omitted from the analysis.

The effect of drought on cold hardiness

An ANOVA was used to test for the effect of the drought treatment, the populations and the date on the LT_{50} of the seedlings of the four populations (*P. sylvestris* Leuk, *P. sylvestris* Spain, *P. nigra* Spain, *P. halepensis* Spain) that were included in the sampling of the drought treatment.

Results

Temporal patterns of cold hardiness in spring

An increasing temperature at which 50% of the needle tissue was damaged (LT_{50}) was observed for all three pine species, until the maximum value was reached in late spring at the sixth sampling date (May 5; Fig. 2). Values from the last sampling in mid-summer (July 22) did not exceed the LT_{50} from the previous date and confirmed a constant cold hardiness since then. In late winter (February 15 and March 8), needle tissues of all *P. sylvestris* and *P. nigra* populations suffered only minor damage by freezing to as low as -35 °C. Consequently, the inflection point of the logistic regression was not captured by the selected freezing temperatures and the true LT_{50} could not be estimated (Appendix S3). Hence, the first two sampling dates were excluded from statistical analyses. From March to early April, the LT_{50} of *P. halepensis* was higher than the LT_{50} of both *P. sylvestris* and *P. nigra* (Fig. 2; ANOVA, $F_{1,16}$ = 244.6 and 248.1, $P < 0.001$ on March 22 and $F_{1,16}$ = 8.094 and 74.06, $P < 0.05$ and $P < 0.001$ on April 5). In late April, the situation changed, resulting in a lower LT_{50} of *P. halepensis* compared to *P. sylvestris* and *P. nigra* (Fig. 2; ANOVA, $F_{1,16}$ = 47.46 and 91.02, $P < 0.001$ on May 3 and $F_{1,16}$ = 16.15 and 56.45, $P < 0.001$ on July 22). The dehardening was thus less pronounced in *P. halepensis* than in *P. sylvestris* and *P. nigra* (significant species x date interaction;

Table 2). In *P. sylvestris* and *P. nigra*, the dehardening pattern was similar, with a strong frost tolerance in winter and a quick reduction of cold hardiness in spring (Fig. 2). Over all sampling dates, the difference in LT_{50} between these species was not significant (Table 2). At the level of individual sampling dates, the LT_{50} of *P. nigra* was lower than that of *P. sylvestris* in early April (Fig. 2; ANOVA, $F_{1,16} = 36.6$, $P < 0.001$ on April 5) but higher from May to July (Fig. 2; ANOVA, $F_{1,16} = 91.02$, $P < 0.001$ on May 3, and $F_{1,16} = 19.49$, $P < 0.001$ on July 22), which resulted in a significant species x date interaction over the whole experimental period (Table 2).

Table 2. ANOVA results testing differences in cold hardiness between populations and sampling dates. Contrasts were used to test for differences between the species.

Source of variation	df	*F*	*P*
Populations	10	12.89	<0.001
P. halepensis vs. *P. sylvestris*	1	62.56	<0.001
P. halepensis vs. *P. nigra*	1	32.21	<0.001
P. sylvestris vs. *P. nigra*	1	3.33	0.071
Date	4	1880.30	<0.001
Populations x date	39	22.20	<0.001
Species x date: *P. halepensis* vs. *P. sylvestris*	4	341.87	<0.001
Species x date: *P. halepensis* vs. *P. nigra*	4	529.14	<0.001
Species x date: *P. sylvestris* vs. *P. nigra*	4	34.49	<0.001
Residuals	92		

Safety margins against spring frost

Safety margins for both *P. sylvestris* and *P. nigra* were broad during winter 2012/2013, with 33.6 K and 34.2 K, respectively, on March 22 2013 (Fig. 2). They then dropped to the lowest measure of 15.3 K and 14.9 K on April 19. Until mid-summer, safety margins increased again to 22.8 K and 21.0 K. This development was different for *P. halepensis*, which started with a much lower safety margin of 16.9 K on February 15, increasing to a mid-summer safety margin of 25.1 K with no depression during the dehardening period. During flushing (May 3), the LT_{50} of the second year needles were not different from the first year needles' LT_{50} (ANOVA, $F_{1,34} = 0.5$, $P = 0.472$), and the safety margins of the second year needles were equally wide.

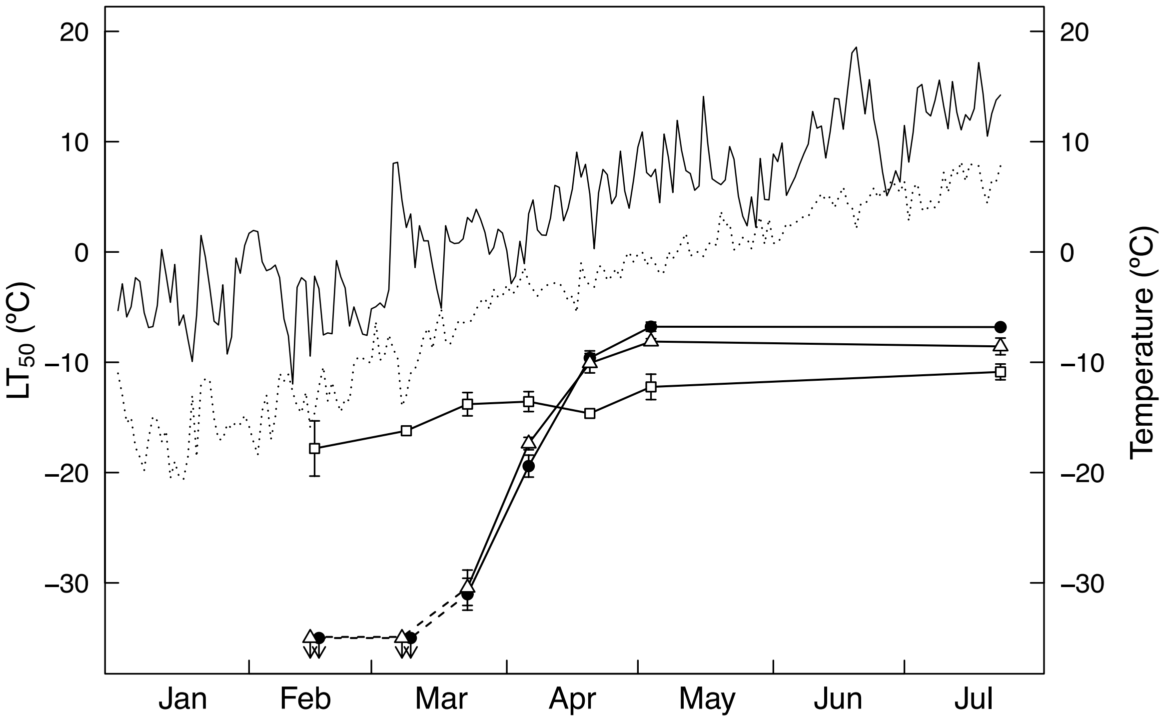

Fig. 2. Seasonal development of the cold hardiness, expressed as the temperature at which 50% of the first-year needle tissue is damaged (LT_{50}; mean ± s.e.) for *Pinus sylvestris* (open triangles), *Pinus nigra* (filled circles) and *Pinus halepensis* (open squares). Symbols with arrows indicate LT_{50} values below -35°C. The safety margins against frost damage are expressed as the difference of the LT_{50} to the daily minimum temperatures measured at the study site in Leuk during the experiment (solid line). The dotted line shows the absolute daily minimum temperature recorded since 1959 in Sion (data from MeteoSwiss).

During the period 1959–2012, temperatures dropped to -20 ºC only three times in Sion in January, an event never lasting more than a single day (Fig. 3a). In January, similarly low temperatures have only been recorded in continental regions of Central and Eastern Europe, and not in Western Europe (Fig. 3b). This implies that there is no regular risk of frost damage for *P. sylvestris* and *P. nigra* seedlings during winter, as LT_{50} values are far below -20 ºC until April (Fig. 2). In April, the coldest temperature measured between 1959 and 2012 was -5.5 ºC and only temperatures to -2 ºC occurred regularly (Fig. 3a). During the 53 reference years, frost events below -10 ºC in April, corresponding to the LT_{50} of *P. nigra* and *P. sylvestris* at that time of the year, were exclusively recorded in northernmost parts of Europe (Fig. 3c). Contrary, the risk of frost damage is much higher for the *P. halepensis* populations with LT_{50} values close to frost events of -16 °C in February and -10 °C in March that recur within 25 years (Fig. 2, Fig. 3a).

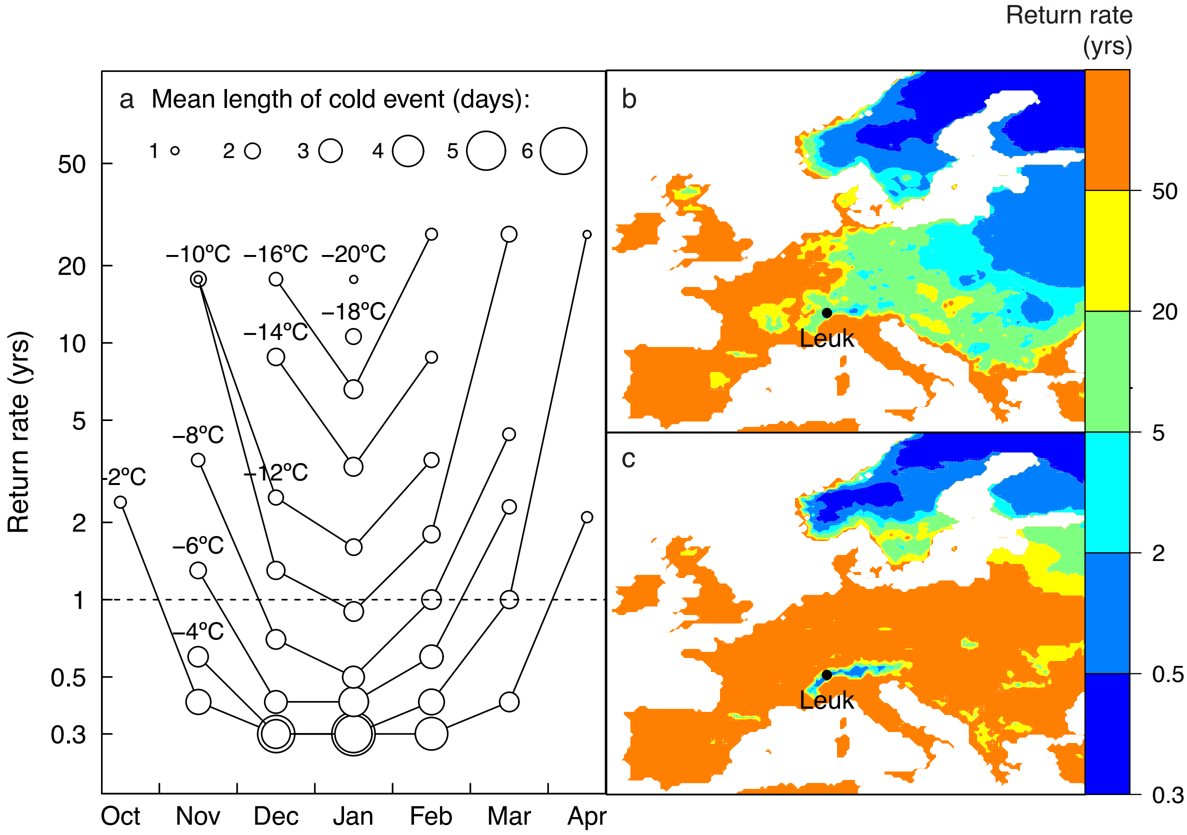

Fig. 3. Return rates of (a) frost events that occurred at the MeteoSwiss station in Sion (Switzerland; 482 m a.s.l.) from 1959–2012, (b) frost events below -20 °C in January and (c) frost events below -10 °C in April in Europe from 1959–2012 (ENSEMBLES gridded observational data, www.ecad.eu). The diameter of the circles in (a) indicates the average length of the corresponding cold event. Return rates of more than 50 years in (b) and (c) indicate no frost event within the covered period of time.

Seed origin

Within-species differences of cold hardiness were only analysed for *P. sylvestris* and *P. nigra* seedlings since sample size was too small in *P. halepensis* on the first four sampling dates. No differences between any of the *P. nigra* or *P. sylvestris* populations were found, except in the middle of dehardening at the beginning of April. At that time, *P. nigra* from Austria had a lower LT_{50} compared to *P. nigra* from Bulgaria and Greece (Tukey HSD, $P < 0.05$ and $P < 0.01$). No linear relationship was found between the cold hardiness and the minimum temperatures of the corresponding month (long-term average 1950–2000) at the seed origin of the populations (Fig. 4), except for *P. nigra* on March 22 ($P = 0.008$) and on July 22 ($P = 0.041$). Despite the statistically significant relationship, the range of the LT_{50} values of the *P. nigra* populations on July 22 was the smallest among all sampling dates and species, with the largest difference being less than 0.5 K. With a standard deviation of 0.34 K at that date, such a difference lies well within the uncertainty of the REL method. The significant relationship on March 22 is solely based on the values of the Greek population, which have a high leverage effect on the linear model (Cook's distance 0.31).

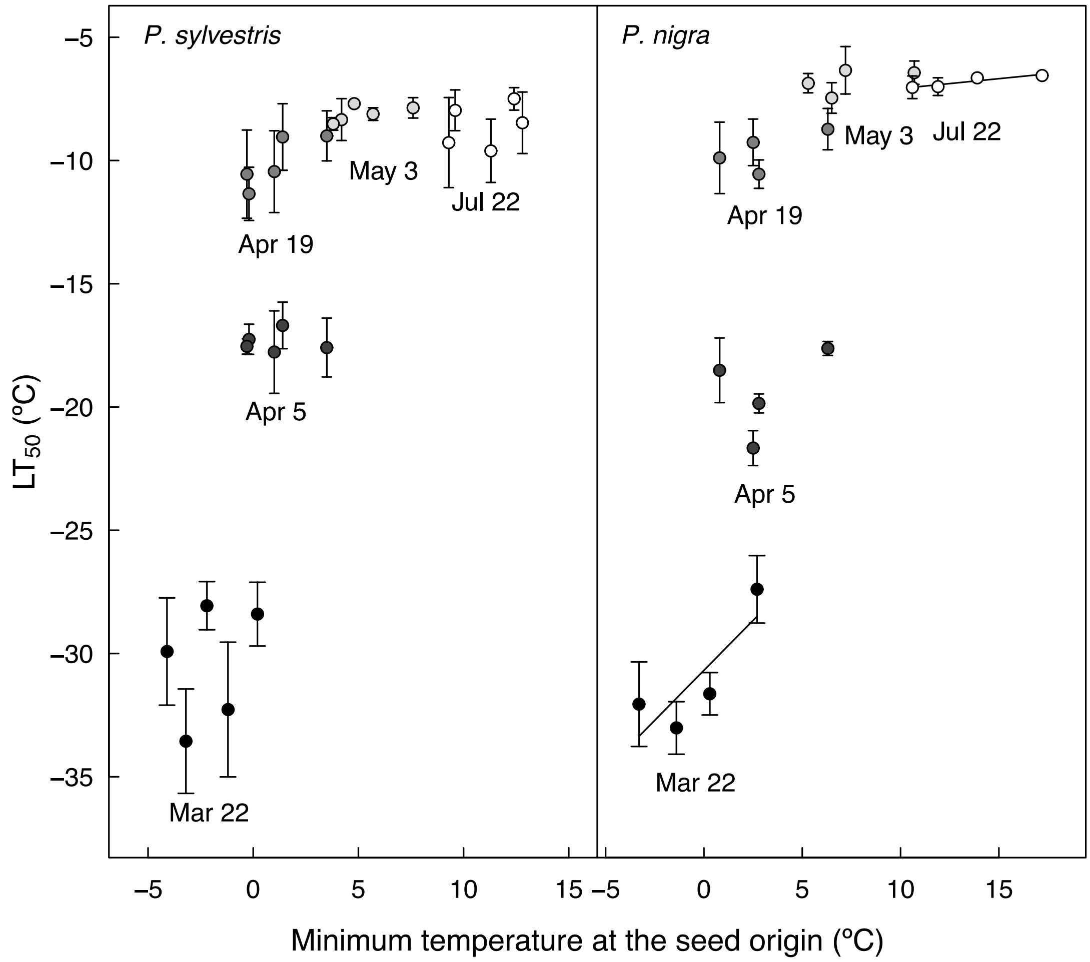

Fig. 4. Relationship between the monthly minimum temperature (average 1950–2000) at the seed origin and the cold hardiness (temperature at which 50% of the tissue is damaged, LT_{50}; mean ± SE) of (a) *Pinus sylvestris* and (b) *Pinus nigra* depending on sampling date. Linear relationships are significant for *P. nigra* on March 22 ($P = 0.008$) and July 22 ($P = 0.041$).

The effect of drought on cold hardiness

Plants in the 'dry' treatment received 44% (126 mm) of the amount of water irrigated in the 'wet' treatment (288 mm) between May 24 and September 23 2012. An effect of the drought treatment on the cold hardiness of the four tested populations could not be observed (Fig. S4, ANOVA, $F_{1,84} = 2.5$, $P = 0.119$). The seedlings in the 'dry' treatments showed the same dehardening patterns as in the 'wet' treatment.

Discussion

The studied populations of *Pinus sylvestris* and *Pinus nigra* were extremely frost tolerant during the whole study period from February to May and always exhibited a much higher cold hardiness than respective daily minimum temperatures measured at the experimental site during the study (Fig. 2). We observed a substantial decrease of the cold hardiness of the two species in the transition from late winter to spring (February–May). Drought-adapted Mediterranean populations of *P. sylvestris* and *P. nigra* were equally well adapted to spring frost as populations from Central Alpine origin during the whole study period. Even during the most sensitive phase in late spring, when buds and leaves were at the highest risk of frost damage, safety margins (differences between cold hardiness and the minimum temperatures) were still wide. The latter also holds for the exclusively Mediterranean *Pinus halepensis*. LT_{50} values based on relative electrolyte leakage (REL) are only an approximate measure of cold hardiness, as high electrolyte leakage from the needle tissue indicates needle damage but not necessarily seedling death (Sutinen *et al.*, 1992). We found strongly sigmoid response curves of the REL to freezing temperatures and hence clear tipping points of frost damage, except when cold hardiness was lower than the lowest freezing temperature (*P. sylvestris* and *P. nigra* in February and early March; Fig. S3). Strimbeck *et al.* (2007) observed needle necrosis whenever the REL exceeded 50%. We found REL of more than 50% in all samples of the negative control (-80 °C, Appendix S3), but only in a few samples at LT_{50} (7 out of 493). Overall, this implies that the LT_{50} in our study tends to overestimate frost damage and underestimate cold hardiness.

Local adaptation of pine trees to frost has been extensively studied (e.g. Beuker *et al.,* 1998; Aldrete *et al.*, 2008; Kreyling *et al.,* 2012). Some results indicate a clear relationship between a tree's phenology and the latitude of seed origin in both deciduous trees (Kreyling *et al.*, 2011) and conifers (Aldrete *et al.*, 2008; Kreyling *et al.,* 2012). In our study, the wide safety margins against frost damage (Fig. 2) contrast with the minor differences in cold hardiness among the populations of both *P. sylvestris* and *P. nigra* during the entire dehardening process from February to July (Fig. 4). The variation among populations never exceeded 20% of the safety margins at a respective date, not even during the most sensitive period of needle emergence in spring. On July 22, for instance, the relationship between the LT_{50} and the climate at the seed origin of *P. nigra* was significant, but the difference of LT_{50} between the most and the least cold hardy population was less than 0.5 K, which is ecologically irrelevant with respect to a safety margin against frost of more than 20 K. On March 22, the relationship between the LT_{50} and the monthly minimum temperature at the seed origin was significant in *P. nigra*, because the LT_{50} of the Greek population had a strong effect on the linear model. It is possible that the Greek *P. nigra* population is less adapted to frost, since this seed source is located at a considerably lower elevation compared to the other Mediterranean populations included in the study. If so, it remains unclear why this population only differed on March 22 and not on any other date during dehardening. Overall, our results indicate a low selective force and minor ecotypic differentiation of needle cold hardiness at the dry end of the *P. nigra* and *P. sylvestris* distribution ranges. We acknowledge, however, that there might be differentiation among populations from a wider temperature range or regarding traits, which we did not cover in our study (e.g. spring phenology, Aldrete *et al.*, 2008).

Compared to *P. sylvestris* and *P. nigra*, the cold hardiness of *P. halepensis* changed only little during the season (Fig. 2), which implies that the physiology of winter hardiness and the mechanism of dehardening differ fundamentally between these species. While the uniform cold hardiness of *P. halepensis* indicates a lack of a hardening/dehardening mechanism, it is surprising that the seedlings were frost resistant to more than 15 K below daily minimum temperatures in spring. A possible explanation is a correlation in physiological adaptation to both frost and drought tolerance, as e.g. increased enzyme activity to protect from oxidative stress (Blödner *et al.*, 2005), the formation of wood that is able to withstand xylem cavitation, or efficient repair mechanisms to rapidly restore xylem conductivity (Lens *et al.*, 2013). It is conceivable that such mechanisms are already activated by short-term exposure to severe drought, thus adaptation to frost might be triggered by seasonal drought prior to the frost season (Kreyling *et al.*, 2012, Walter *et al.*, 2013). Under experimental conditions, an extreme summer drought with a theoretical recurrence rate of 1000 years in Central Germany increased the frost tolerance of *P. nigra* seedlings in the following spring by roughly 4 K (Kreyling *et al.*, 2012). In our experiment, a reduction of summer rainfall by 33% compared to the long-term average did not affect frost tolerance in any of the four tested populations (Fig. S4). This suggests that, if at all, an interaction between drought acclimation and frost tolerance takes effect under extreme conditions only and may thus be inconsequential for the long-term recruitment success of the studied species.

Although cold hardiness of *P. sylvestris* has been shown to be lowest during needle emergence and shoot elongation (Repo, 1992), we found no difference in frost tolerance between first-year and second-year needles in any species, not even during earliest stages of emergence of the second-year needles. This contrasts with deciduous trees, where cold hardiness is tightly linked to the spring phenology (Lenz *et al.,* 2013). Here, a trade-off exists between early leaf out to maximize growing season length (*capacity adaptation*) and delayed shoot elongation to avoid frost damage (*survival adaptation*; Kramer *et al.,* 2000). The high safety margins of the studied pines (Fig. 2) exceeded those of Central European deciduous trees at bud break and during early leaf stages by 3–6 times (Lenz *et al.*, 2013). The ability of pines to photosynthesize while retaining a high cold hardiness (Repo and Leinonen, 2006) may allow for a delayed dehardening and shoot elongation compared to deciduous trees (Rötzer *et al.,* 2004; Michelot *et al.,* 2012), without limiting the length of the growing season.

A comparison of cold hardiness with seasonal return rates of cold temperature extremes in the Central Alpine Valais demonstrates that autochthonous *P. sylvestris* and Mediterranean *P. sylvestris* and *P. nigra* are all adapted to frost events that exceed the most severe frost conditions regarding the local records from 1959 to

2012, especially during winter (Fig. 2 and 3a). We assume that the cause for the wide safety margins can be explained by past climatic conditions during the Quaternary, when *P. sylvestris* was present at the frontier of the retreat and recolonization ensued by the last glaciation (Bennett *et al.,* 1991). Distribution models (Svenning *et al.,* 2008) and genetic analyses (Afzal-Rafii and Dodd, 2007) show that *P. sylvestris* and *P. nigra* survived the last glaciation not only in southern refugia, but also in patchy populations to the north of the Alps. An adaptive lag (Mátyás, 1990) to the climatic changes since the last glaciation likely preserved their adaptation to cold climates even in regions like the southern Mediterranean. The current return rates of cold temperature extremes at low elevations in the Valais valley are comparable to return rates in large parts of Central and Western Europe. This holds for both in winter and spring, although extreme winter frost is more common in continental regions of Central and Eastern Europe (Fig. 3b and c). We derive that the measured safety margins against needle frost damage hold for large parts of Central and Western Europe. It is likely that a northward shift of *P. halepensis*, which has the narrowest safety margin in mid-winter, is limited by regularly returning winter frosts of -20 ºC in Central and Eastern Europe. Although Western Europe seems to have a climate favourable to a future expansion of *P. halepensis*, the survival of this species might be hampered by other factors, as e.g. by winter-embolism due to frozen soil. Mediterranean populations of *P. sylvestris* and *P. nigra*, on the other hand, are most threatened by frost events of -10 ºC in April, temperatures that have only been reached in the Alps and in Northern Europe during the last 53 years (Fig. 3c). This suggests that a northward migration of these two species to Central and Western Europe is not limited by needle frost damage, neither in winter nor in spring.

In conclusion, our estimates of seedling cold hardiness confirm a discrepancy between the realized distribution range of these conifers and their potential range, which extends farther northwards under current climatic conditions (Svenning and Skov 2004). We therefore expect a low risk of frost damage under the predicted latitudinal range shift for both *P. sylvestris* and *P. nigra*. The predicted increase in temperature will most likely be accompanied by a loss of the coldest extremes in winter (Rebetez, 2001; Zubler *et al*., 2014), which will further reduce the risk of needle frost damage in *P. sylvestris* and *P. nigra*, and might even allow for the successful regeneration of the least cold hardy species, *P. halepensis*. By comparing several species and a wide range of populations over the whole dehardening period, we demonstrate that the species ranking of frost tolerance changes from winter to summer. Analysing frost tolerance at a single point in time may thus be misleading. Since the timing of the highest risk of frost damage differs between species, more detailed knowledge on species-specific dehardening patterns will be necessary in order to more reliably predict a tree species' future suitable range.

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Supporting Information

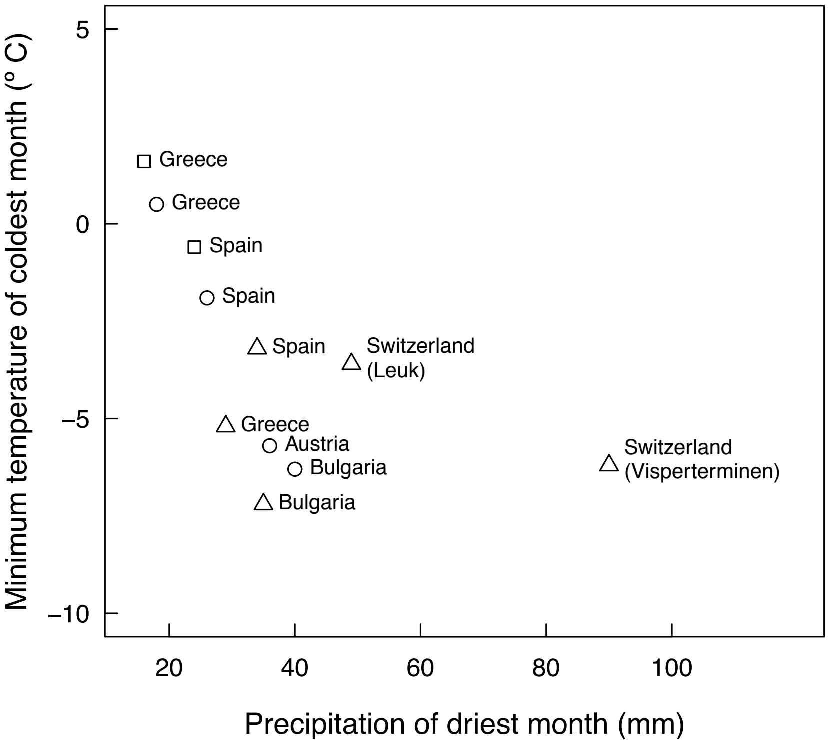

Fig. S1. Precipitation of the driest month and minimum temperature of the coldest month at the pine stands selected for seed collection: *Pinus sylvestris* (triangles), *Pinus nigra* (circles) and *Pinus halepensis* (squares). The interpolated average monthly minimum temperature and precipitation data were retrieved from the WorldClim database (1950–2000; www.worldclim.org).

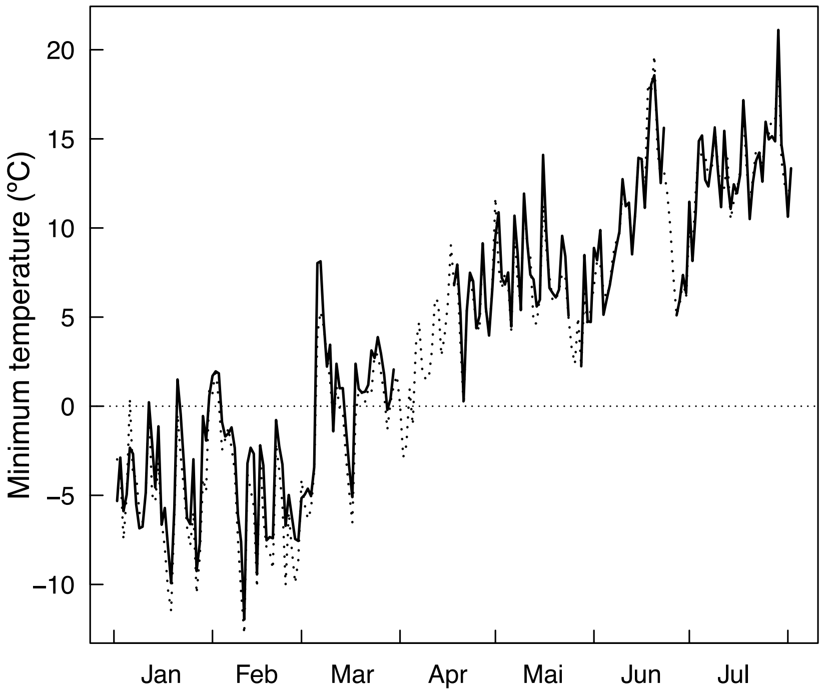

Fig. S2. Daily minimum temperature measured in spring 2013 at the study site near Leuk (solid line) with logger interruptions from March 30 to April 17, May 24–25 and June 23–26). The missing data was replaced by temperature data recorded in Sion and interpolated for the study site with a 2 km resolution (MeteoSwiss; dotted line).

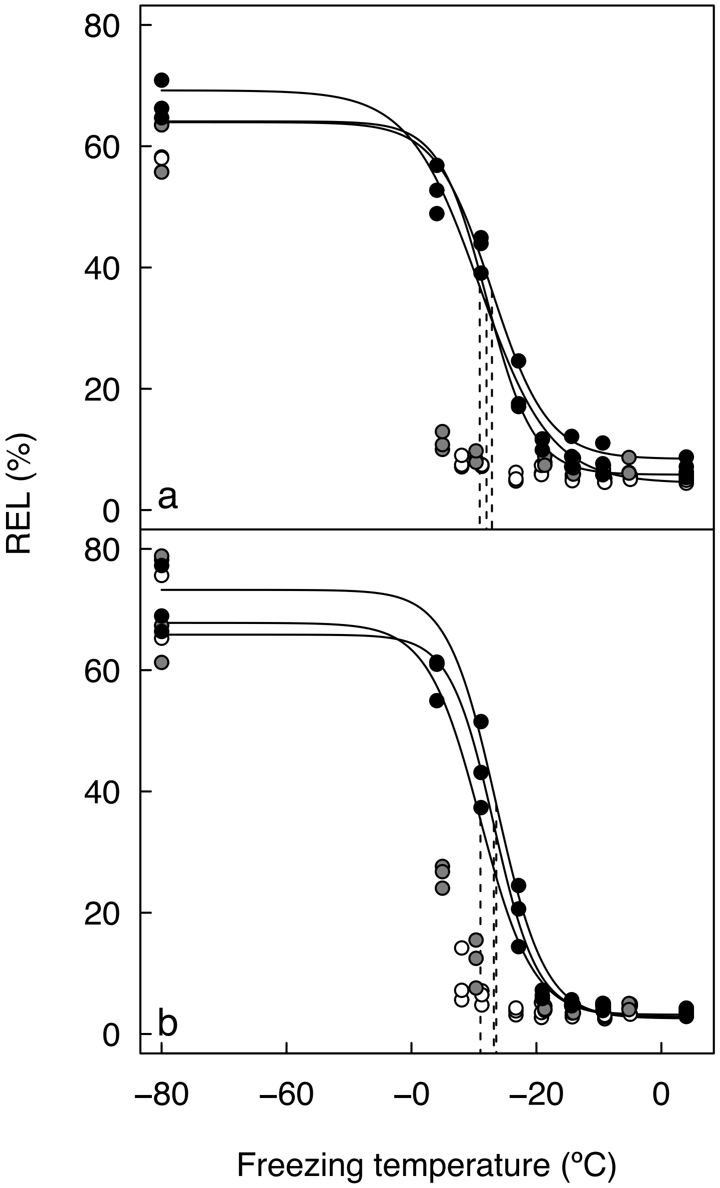

Fig. S3 Relative electrolyte leakage (REL) of first-year needles in relation to freezing temperatures on February 15 (open circles), March 8 (grey dots) and March 22 (black dots) of (a) *Pinus sylvestris* and (b) *Pinus nigra* from the Greek population. The dashed lines indicate the inflection points of the logistic regressions of the three replicates and represent the temperatures at which 50% of the needle tissue is damaged (LT_{50}). Logistic regression, and thus calculation of LT_{50}, was not performed before March 22 since the inflection point was not captured by the experimental freezing temperatures on these dates.

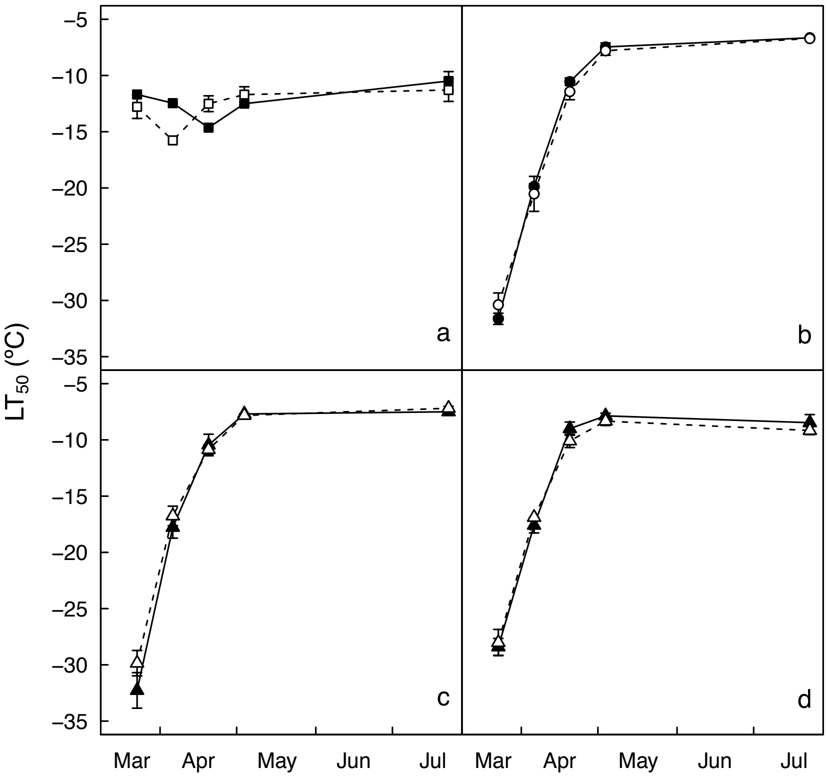

Fig. S4 Cold hardiness (temperature at which 50% of the tissue is damaged, LT$_{50}$, mean ± s.e.) of first-year needles of seedlings grown under 'wet' (solid line) or 'dry' (dashed line) conditions: (a) *Pinus halepensis,* Spain, (b) *Pinus nigra,* Spain, (c) *Pinus sylvestris,* Spain, and (d) *Pinus sylvestris,* Switzerland (Leuk).

Additional Work

Root architecture might account for contrasting establishment success of *Pseudotsuga menziesii* var. *menziesii* and *Pinus sylvestris* in Central Europe under dry conditions

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Barbara Moser[1], Christoph Bachofen[1,2], Jonathan D Müller[1], Marek Metslaid[3], Thomas Wohlgemuth[1]

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[1] Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zürcherstrasse 111, 8903 Birmensdorf, Switzerland

[2] Department of Environmental Sciences, Swiss Federal Institute of Technology, Universitätsstrasse 16, 8092 Zürich, Switzerland

[3] Estonian University of Life Sciences, Department of Forest Biology, Kreutzwaldi 64, 51014 Tartu, Estonia

Abstract

- Context: *Pseudotsuga menziesii* (Douglas-fir) is regarded as a promising species to maintain the productivity of Central European lowland forests given the projected increase of long dry spells.
- Aims: Will the species be able to regenerate from seed and spread outside plantations in a drier temperate Europe?
- Methods: We measured relative growth rate, biomass allocation, root architecture, and phenotypic plasticity of *P. menziesii* seedlings sown in a common garden and grown under current precipitation and prolonged drought, respectively. The species' competitive ability with respect to *Pinus sylvestris* L., the most drought tolerant native conifer in Central Europe, was assessed during three growing seasons.
- Results: *P. sylvestris* seedlings had higher relative growth rates than *P. menziesii* seedlings, first in terms of aboveground biomass and later in terms of shoot height. This resulted in heavier and taller seedlings after three growing seasons under both moist and dry conditions. Shorter vertical roots corresponded with lower survival of *P. menziesii* seedlings under dry conditions (Fig. 1).
- Conclusion: Fast root proliferation allows *P. sylvestris* seedlings to reach deeper water pools that are less rapidly depleted during transient drought. By contrast, the shallow root system might put *P. menziesii* seedlings at the risk of desiccation during prolonged dry spells.

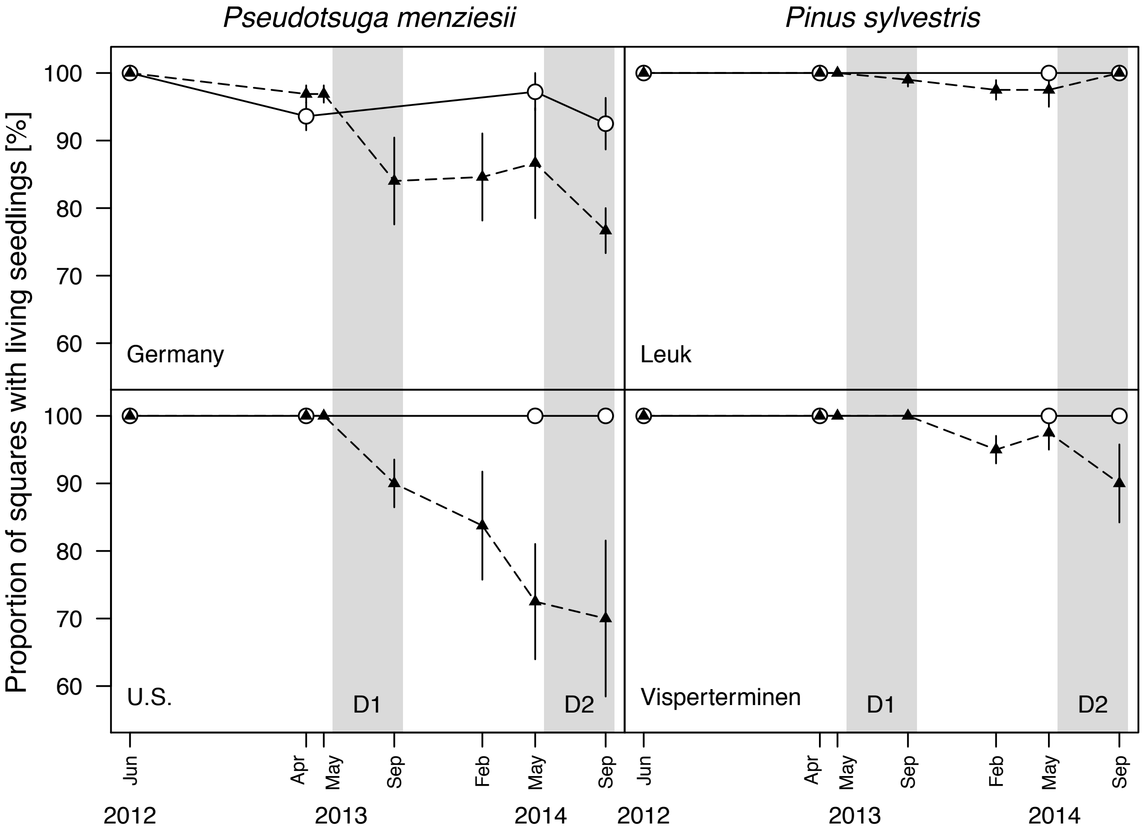


Fig 1. Survival of *Pseudotsuga menziesii* and *Pinus sylvestris* seedlings of different origin in the course of the experiment measured in terms of the proportion of squares containing at least one living seedling. Black line and open circles denote the control treatment, dashed line and triangles the drought treatment, vertical bars represent standard errors at the block level. Surveys in different treatments took place at different times and did not always include the same replicates, thus the proportion of squares with living seedlings can increase from one time step to the next. D1: summer drought June – September 2013; D2: summer drought June – September 2014

Synthesis

During early establishment, seedlings face numerous adverse factors limiting growth or survival, among which drought and frost are crucial. Seedlings have developed diverse mechanisms for stress-avoidance or stress-tolerance. The main objective of this doctoral thesis was to investigate strategies and limitations of European pine species and populations from the southern distribution limit with contrasting drought- and frost-tolerances to withstand future climatic conditions. Particularly, the aim was to (1) investigate whether the drought-tolerance of pine seedlings is related to biomass partitioning under drought (2) examine effects of future dry and high-CO_2 climate on the carbohydrate metabolism, and (3) assess frost limitations of pine populations from the dry distribution limit for the predicted northward shift in Europe. In a first part of the synthesis, the key findings of this doctoral thesis are summarized, conclusions drawn and the contribution of the thesis to the body of knowledge discussed. Second, methodological aspects of the experiment are reviewed, and third, the synthesis concluded with prospects for future research.

Key findings of the thesis

Local adaptation to drought and frost

The studied pine species and populations were in general very well adapted to drought, as indicated by the low mortality during the experiment (9.4% of drought-stressed seedlings, chapter 2). Populations from locations with dry summers were able to maintain higher growth rates in response to the first drought, which shows strong local adaptation to drought (chapter 1). We found a marked positive relationship between the drought-tolerance of the studied populations and root biomass fraction after the first drought episode (chapter 1). While such a relationship has been hypothesised repeatedly, direct evidence has been lacking (Arend *et al.*, 2011; Taeger *et al.*, 2015). Carbon storage of drought-stressed seedlings was, on the other hand, not related to the drought-tolerance of the populations, and was not the limiting factor for growth and survival during the two experimental droughts (chapter 2). Local adaptation to drought was thus important for morphological, but not physiological drought responses. Frost tolerance differed only between species, in particular between *P. halepensis* and the two other pine species. It was not related to the climate at the seed origin (chapter 3), thus local adaptation of populations of a particular species to frost was not determining seedling survival.

The amount of local adaptation thus differed largely between the measured traits. Low local adaptation of a trait may be due to genetic fixation within a species, resulting from the unconditional adaptive advantage of that trait (Kawecki & Ebert, 2004). A fixed trait will continue to be expressed even if the advantage is lost or it had become detrimental. The constantly high cold hardiness of *P. sylvestris* and *P. nigra* irrespective of the seed origin might, accordingly, be a result of the past distribution range of the two species. Distribution models (Svenning *et al.,* 2008) and genetic analyses (Afzal-Rafii & Dodd, 2007) showed that *P. sylvestris* and *P. nigra* survived the last glaciation not only in southern refugia, but also in patchy populations to the north of the Alps (see chapter 3). A high cold hardiness might thus be the result of genetic fixation of this trait in these populations. Local adaptation of carbon storage has been observed for *P. sylvestris* populations from Central- and Northern Europe (Oleksyn *et al.*, 2000). Here, we only observed a relationship between starch storage and seed origin in well-watered three-years old seedlings (chapter 2, Fig. 6), but not in drought-stressed or younger seedlings.

Acclimation to repeated drought and frost in the common garden

A key finding of this doctoral thesis is the observed very fast acclimation of pine seedlings to repeated extreme drought and thus their considerable ability to survive repeated dry spells. In June and July of the first year, the seedlings responded to the drought with only small changes in shoot height, because growth was nearly completed for that growing season (chapter 1). Root growth, on the other hand, which presumably peaks later in the growing season (Abramoff & Finzi, 2015), was severely inhibited by the first summer drought. Consequently, root biomass fraction was lower in drought-stressed seedlings and the first summer drought resulted in strong needle browning (Fig. 1) and, in a few cases, death of individual seedlings. Changes in starch storage were very large in response to the first summer drought (chapter 2): starch accumulated in the source organs, most presumably because the allocation of assimilates to sink organs was impaired by reduced phloem transport. In the following growing season, the same drought resulted in very different morphological and physiological responses. Seedlings reduced shoot growth in response to the drought much more and biomass partitioning was similar in drought-stressed and well-watered seedlings (chapter 1). Changes in carbohydrate storage in response to the drought were much less extreme: while sugar concentrations were still elevated in drought-stressed seedlings, similarly as after the first summer drought, starch concentrations were not higher than in well-watered seedlings (chapter 2). No needle browning occurred and mortality was not observed until autumn. Seedlings exhibited thus morphological acclimation to the drought (chapter 1), which most probably alleviated physiological stress reactions (chapter 2). Acclimation was also a key trait enabling seedlings to tolerate cold temperatures (chapter 3). This experiment did not include a winter-temperature treatment. Comparison with a common garden experiment in Spain (Climent *et al*., 2009) shows, however, that a much lower frost-tolerance of *P. nigra* and *P. sylvestris* is reached if they are grown in warmer regions, indicating a high acclimation potential of seedlings of these species to cold temperatures.

Morphological acclimation to drought might be much higher in seedlings than adult trees, because of the higher phenotypic inertia of adult trees (Valladares *et al*., 2007). In this experiment, the seedlings grew by a factor of 10 every year, hence the biomass partitioning could be adjusted dramatically from one growing season to the next. For instance, needles developed during a year with abundant water supply only account for a tenth of the needle biomass in the following year and thus contribute little to the total amount of water loss through transpiration. This allowed the seedlings to rapidly adjust to dry conditions. In adult trees, growth adjustments to dry conditions can take up to six or seven years after water availability is reduced (Feichtinger *et al.*, 2014). The fast acclimation to drought was thus most probably specific for the seedling stage of the pines.

Fig 1. Needle browning in a seedling of *P. sylvestris* from Spain subjected to summer drought in August 2013.

Contribution to the larger understanding of seedlings drought and frost relations

There is a vast body of literature on morphological and physiological responses of various pine seedlings to experimental drought. Many of them focus on initial drought responses of the seedlings (e.g. Galvez *et al.*, 2011; Matías *et al.*, 2014; Mitchell *et al.*, 2014; Duan *et al.*, 2015; Taeger *et al.*, 2015) or effects of a single long-term drought (e.g. Rosas *et al.*, 2013), but miss out on the responses to repeated drought with intermediary stress release (e.g. moist winters). Climate change is predicted to increase drought only during summer months in Switzerland, whereas in autumn, winter and spring similar precipitation is expected (Appenzeller *et al.*, 2011). The repeated severe summer drought with intermediary stress release thus corresponds closely to the predicted future climate.

In this doctoral thesis, I tried to add to the understanding of seedling's response to predicted climatic changes by assessing the morphology and physiology of both the local adaptation to drought, as well as the acclimation to repeated drought. The common garden experiment was designed to simulate natural regeneration, therefore seedlings were grown from seeds and a large rooting volume provided. Measurements of whole seedling biomass, including roots, and physiological parameters allowed to understand combined drought responses, for instance the effect of reduced stomatal conductance on whole seedling biomass.

Methodological aspects

CO_2 treatment

Two principal difficulties emerge with CO_2 enrichment experiments that are discussed in the following: (1) technical problems that arose during the three years of CO_2 enrichment and (2) difficulties with interpreting findings in CO_2 studies that are related to measurement and sampling methods and how they were addressed in this thesis.

Technical difficulties with FACE

In order to reduce costs, we interrupted the CO_2 enrichment when conditions were unfavourable for photosynthesis (temperature < 5 ºC, photon flux density < 30 mmolm^{-2}s^{-1}, air temperature > 35 °C). The nature of CO_2 enrichment experiments entails, however, that the application of CO_2 is repeatedly interrupted by wind gusts that carry away the enriched air and reduce CO_2 concentrations to ambient levels. Consequently, CO_2 release had to be interrupted when wind speed exceeded 2 ms^{-1}. Wind speeds of more than 2 ms^{-1} occurred at 56% of the favourable time for photosynthesis (MeteoSwiss station in Sion located 28 km to the East of the study site) during the three years of the experiment (Jun 2012 – Oct 2014). Starting in spring 2013, we closed the rainshelters when wind speeds exceeded 2 ms^{-1} and continued gassing in order to extend the operation time for the CO_2 enrichment. The rainshelters had, however, to be opened when temperatures rose above 25º C to avoid extreme heat below the rainshelter roofs. Consequently, the operation time for the CO_2 enrichment was reduced to 40% because of the wind. The distinctive wind system in the Valais valley (Fig. 2, Bigler *et al.*, 2006) thus interfered heavily with the CO_2 enrichment.

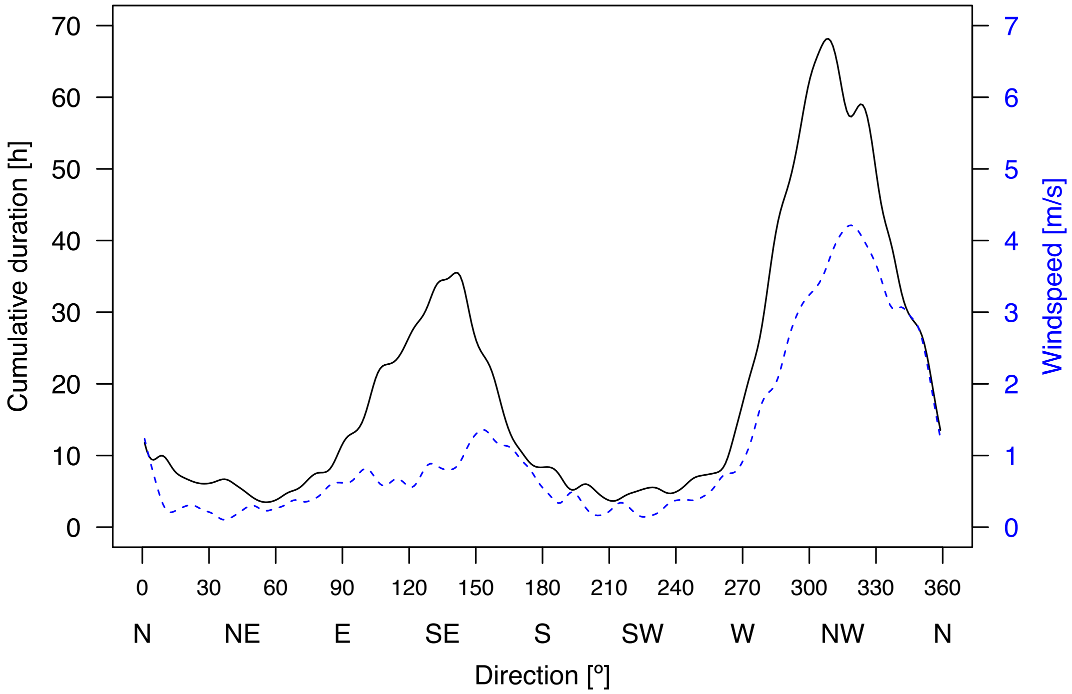


Fig 2. Direction and speed of wind flow at the study site in the Central Alpine Valais valley during the growing season in 2013 and 2014. Most and strongest wind flows came from Northwestern and Southeastern direction (katabatic winds).

In addition to the natural circumstances, repeated failures of the FACE-computer and freezing of the pressure-reducing valve further reduced the actual time for CO_2 enrichment to 42%. The CO_2 concentrations in mesocosms of the eCO_2 treatment amounted to 549 (2012), 529 (2013) and 494 ppm (2014) during the actual gassing time (target: 570 ppm, ambient: 400 ppm, Fig. 3). In conclusion, the FACE experiment successfully elevated atmospheric CO_2 when it was fully operative, but suffered from many issues due to the climate of the Valais and the technical implementation.

Interpreting results from CO_2 enrichment studies

Experiments with increased CO_2 have been conducted in growth chambers, greenhouses, or in the field for more than 25 years with diverse plant species and ecosystems, and free-air CO_2 enrichment (FACE) were first implemented in forests almost 20 years ago (Hendrey *et al.*, 1999). The interpretation of CO_2 effects on forests remains, however, still challenging (see for instance: Norby & Zak, 2011; Leuzinger & Hättenschwiler, 2013). The increased assimilation of CO_2 with higher atmospheric concentrations (Franks *et al.*, 2013) does, for instance, not necessarily translate to higher carbon pools in the plant, because of potential changes in respiration or carbon fluxes from the plant to the soil (Leuzinger & Hättenschwiler, 2013). Further, higher growth rates of plants grown under eCO_2 and sufficient nutrient and water supply are not representative of the reactions of seedlings in a natural environment, where water and nutrients limit the positive effects of eCO_2 (Körner, 2006; Norby *et al.*, 2010, and see review by Leuzinger & Hättenschwiler, 2013). Trees may also react to elevated CO_2 with a reduction of net CO_2 assimilation either physiologically, or by morphological changes (Leuzinger & Hättenschwiler, 2013). As a result, large initial reactions to eCO_2 gradually decline during the first years of observation (Luo & Reynolds, 1999; Körner *et al.*, 2005; Norby *et al.*, 2010; Leuzinger *et al.*, 2011). This reaction is even amplified by the usually performed step increase of CO_2 concentration in FACE experiments, instead of the actual occurring gradual increase in the atmosphere (Leuzinger & Hättenschwiler, 2013). Therefore, short-term effects of increased CO_2 cannot directly be extrapolated to ecosystem responses. Most of these issues have been addressed with the sampling methods used in this study. For instance, we measured the biomass of the entire seedlings, as well as the non-structural carbohydrate (NSC) concentrations of a composite sample from the entire seedlings. We are therefore able to quantify the effect of eCO_2 on whole-tree biomass and NSC concentrations in the entire plant. Further, we studied the effect of eCO_2 under seminatural growth conditions, where seedlings were limited by water and nutrient availability. We thus believe that responses of the seedlings to eCO_2 corresponded closely to that under future climatic conditions.

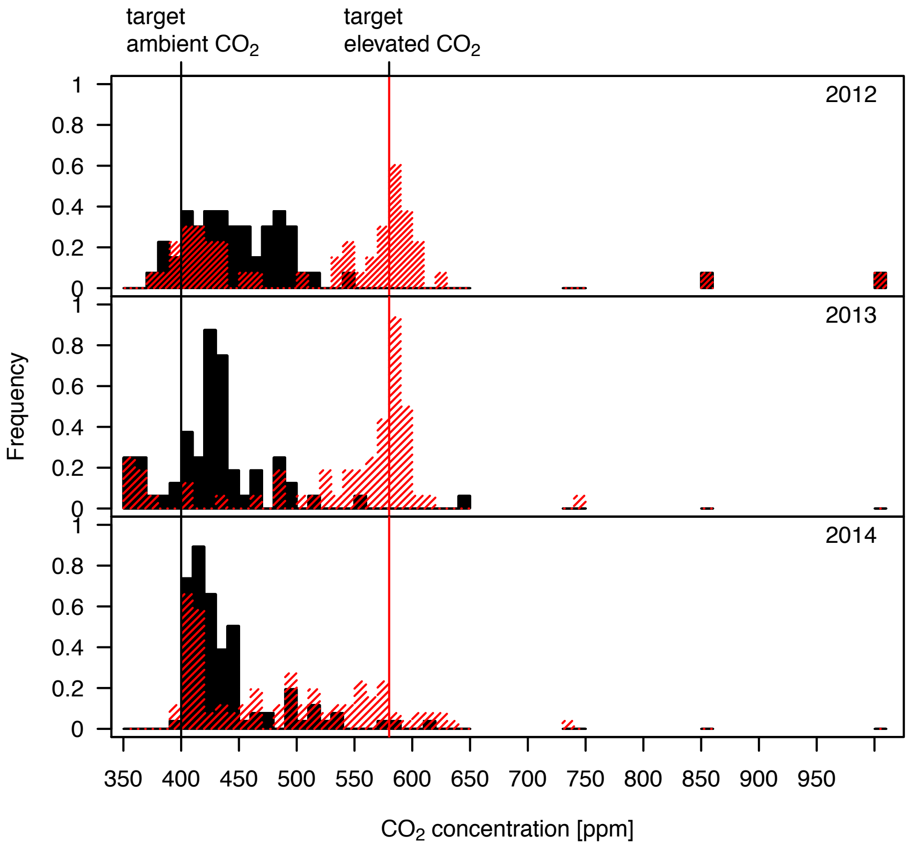


Fig 3. Distribution of the measured CO_2 concentrations in mesocosms of ambient (grey) and elevated CO_2 (red dash) in 2012, 2013 and 2014. Target concentrations (400 ppm, 570 ppm) are indicated with vertical lines.

The irrigation regime and drought treatment

The mesocosms were irrigated during two subsequent nights, which corresponds to the average natural rainfall pattern in the Valais valley during the growing season. In addition, a repeated irrigation during two subsequent nights proved to better humidify the soil and resulted in less leakage in a previous experiment (Richter *et al.*, 2012). Seedlings grown in mesocosms with the 'dry' irrigation regime received a little less than half the amount of water, but had 90–98% of the biomass of seedling in the 'moist' mesocosms (see chapter 1). In contrast, 'moist' seedlings subjected to the summer drought received a similar amount of water over the growing season as 'dry' seedlings, but only produced 42–50% of the biomass. In addition, needle browning was observed in seedlings after the severe summer drought (Fig. 3), which qualifies the summer drought as a disturbance event. Drought disturbance events hence affected seedling growth much more than the constantly low irrigation, confirming the impact of extreme events for ecosystems (Jentsch *et al.*, 2007) and the importance of occasional summer precipitation for regeneration (Matías *et al.*, 2012). In addition to the length, the timing of the drought event can have an important effect on seedling responses (Leuschner *et al.*, 2004). In grasslands, the timing of drought did, for instance, affect the various species of the examined community to a different degree (Zeiter *et al.*, 2015). While some species were more affected by spring drought, a large part suffered more from summer drought. In the Mediterranean, summer precipitation is very low, thus trees need to be adapted to dry summers. In the Valais valley, on the other hand, spring precipitation is on average much lower than in the Mediterranean (Moser *et al.*, 2015), thus *P. sylvestris* populations from the Valais may be better adapted to spring drought. The timing of an experimental drought thus critically affects the results. In this study, we applied a severe summer drought, in order to closely simulate the predicted climatic changes in Switzerland, which are estimated to comprise increasing drought only in summer months (Appenzeller *et al.*, 2011).

Prospects

Implications of the findings for the assisted migration of P. nigra

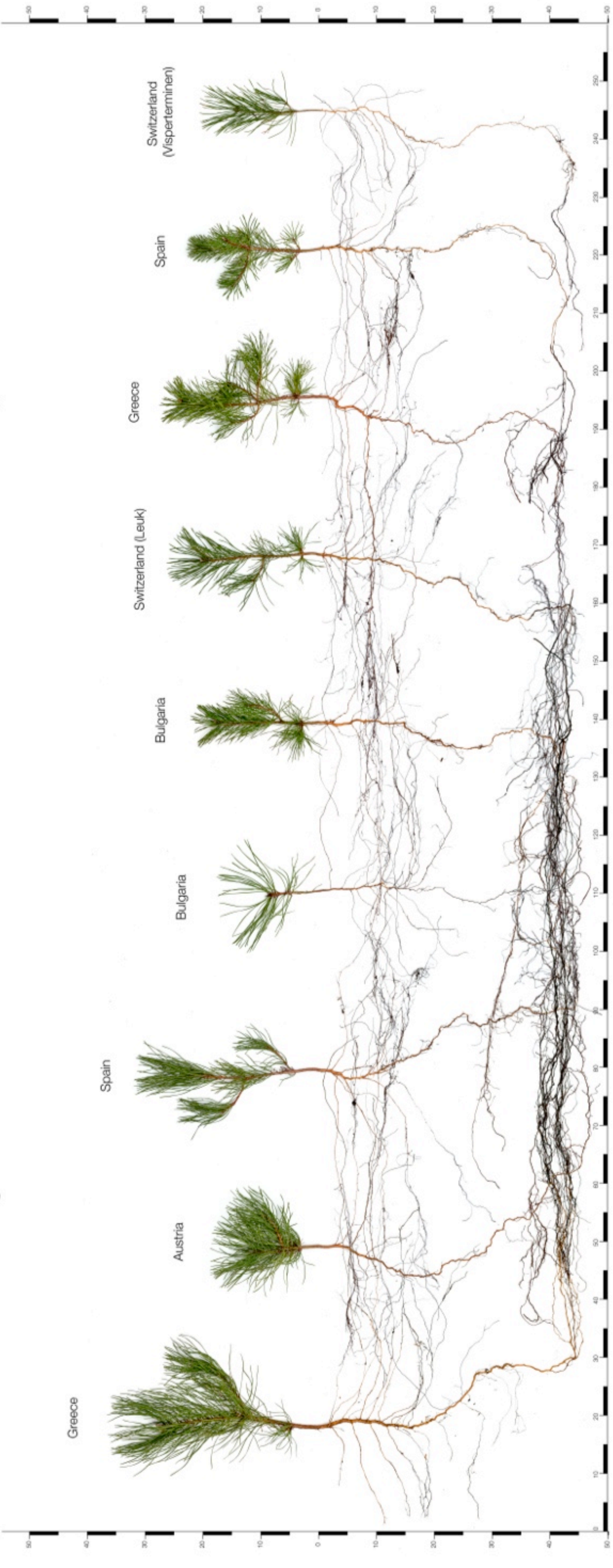
Rising temperatures are expected to lead to a poleward shift of tree species in Europe (Thuiller *et al.*, 2006; Hanewinkel *et al.*, 2013) so that in the long run, Mediterranean provenances and species might be better adapted to the future climate in Central Europe, where temperate forests currently prevail (Hanewinkel *et al.*, 2013). The natural migration rate of trees is, however, thought to be insufficient to keep up with projected climatic change (Aitken *et al.*, 2008; Vitt *et al.*, 2010), particularly in regions with dispersal barriers such as fragmented landscapes or high mountain ranges (Hoegh-Guldberg *et al.*, 2008). As a means to maintain forest productivity and ecosystem functioning under these circumstances, the assisted migration of appropriate tree species and provenances has been suggested (Gray *et al.*, 2011; Kreyling *et al.*, 2011). The focus of forestry related assisted migration lies in the movement of tree species within or just outside the species range (Ste-Marie *et al.*, 2011; Pedlar *et al.*, 2012; Williams & Dumroese, 2013), potentially minimizing the risk of adverse effects on the recipient ecosystems (Kreyling *et al.*, 2011). In undertaking assisted migration, it is important to carefully evaluate the adaptive traits of potential migrants in relation to the present and future climatic conditions at the target site (Chmura *et al.,* 2011; Gray *et al.,* 2011; Richter *et al.,* 2012). In this study, we found higher drought-tolerances of all *P. nigra* populations, compared to *P. sylvestris*. Mortality was lower (chapter 2), growth rates higher (chapter 1) and resistance to the summer drought superior. Previous observations of lower growth rates in moist conditions (Richter *et al.*, 2012) could not be confirmed. Contrary to our expectations, cold hardiness was not lower (chapter 3), rendering this species superior to the autochthonous *P. sylvestris* for all measured traits. Although the regeneration of the autochthonous populations is expected to persist under future climatic conditions, *P. nigra* might be considered for assisted migration into dry Central Alpine valleys because of its higher growth rates. There remain, however, particular open questions concerning the long-term survival under local conditions. For instance, several *P. nigra* trees growing at a dry site in the Swiss lowlands were severely infested with *Sphaeropsis sapinea*, a fungus infecting pine needles, in autumn 2015. These infections have been repeatedly observed after hailstorms in Swiss *P. nigra* afforestations (pers. comm. Valentin Queloz) and might render this species unsuitable at hailstorm-prone sites. Further, we have to consider the possibility that other important factors limiting the survival of *P. nigra*, such as prolonged winter desiccation, were not covered by this study.

Challenges and recommendations for future precipitation experiments

Precipitation experiments are a valuable tool for testing the effects of different climate scenarios while controlling for confounding effects (Beier *et al.*, 2012). Changes of natural precipitation resulting from climate change are more heterogeneous than changes in temperature or CO_2. It is thus necessary to conduct a wide range of precipitation experiments with different conditions. Most precipitation experiments use drought treatments that correspond to naturally occurring droughts (Beier *et al.*, 2012). Under future climatic conditions, drought episodes might exceed these scenarios (IPCC, 2012), therefore experiments applying severe drought might be of particular interest. Applying the severe summer drought a second time in the following growing season proved to produce one of the key findings of the doctoral thesis: the fast acclimation to repeated severe drought stress. Neglecting the acclimation potential of seedlings to drought would have resulted in the prediction of higher mortality rates due to drought. Many mechanisms of stress memory, such as the accumulation of osmoprotective proteins, the reduction of the photosynthetic apparatus and altered gene expression (Bruce *et al.*, 2007; Walter *et al.*, 2013) are well known. But the effects of these mechanisms on growth and survival of seedlings in natural conditions are largely unknown (Walter *et al.*, 2011, 2013). In addition to physiological acclimation, lagged morphological responses (Feichtinger *et al.*, 2015) are fundamental for the drought response. Experiments using repeated drought events are, however, scarce for trees (but see e.g. Liu & Dickmann, 1993; Stewart *et al.*, 1995). The acclimation effects and lagged responses of the seedlings to the drought demonstrate the importance for more experiments with repeated experimental droughts. Another key finding of the thesis was the different responses of drought-tolerant populations in root biomass partitioning to drought. However, seedlings or saplings are often transplanted from a nursery to the experiment, thereby disturbing the root system. The effects are severe in both bareroot and containerized seedlings and result, for instance, in a shorter taproot (Preisig *et al.*, 1979). Seedling responses to drought might thus be heavily affected by the disturbance of the root system and we thus recommend growing seedlings from seeds in place. This also allows analysing root architecture, which revealed in this experiment important species-specific strategies for drought-avoidance (Fig. 4, and see additional work).

Fig 4. Selected individuals of all *P. sylvestris* and *P. nigra* populations from a 'moist' (p. 138) and 'dry-out' (p. 139) mesocosm, depicted as growing in the mesocosms on September 2014.


Pinus nigra
Greece
Austria
Spain
Bulgaria
Pinus sylvestris
Bulgaria
Switzerland (Leuk)
Greece
Spain
Switzerland (Visperterminen)


Pinus sylvestris
Pinus nigra
Greece
Austria
Spain
Bulgaria
Bulgaria
Switzerland (Leuk)
Greece
Spain
Switzerland (Visperterminen)

Research gaps

In this doctoral thesis, a number of questions on the reaction of seedling to future environmental conditions have been answered. Several important questions remain open:

1. Soil composition

Seedlings were grown in substrate simulating the natural soil composition at the valley bottom in the Valais. The substrate consisted of sand and gravel from the local Rhone riverbed (pH 8.3) and humic topsoil (pH 5.9). Clay and silt fractions were minor, and therefore the field capacity of the substrate very low. We suspect that drought reactions might have differed in soils more capable of retaining a larger amount of water accumulated in the humid winter. Furthermore, species-specific preferences for particular soil-pH might affect overall growth and treatment responses. Drought reactions should therefore be tested under different soil compositions.

2. Soil nutrients

Soil nutrient availability may affect the reaction of plants to elevated CO_2 (Saurer *et al.*, 2003) and drought (Ghashghaie & Saugier, 1989). In this study, we did not include a nutrient treatment, but in future studies on tree seedlings, interactive effects of drought and nutrients might also be useful to investigate.

3. Competition with *Quercus pubescens*

Quercus pubescens has been shown to be more drought-tolerant than *P. sylvestris* (e.g.) (Weber *et al.*, 2007). In Central Alpine valleys, it could thus replace *P. sylvestris* at low elevations (Rigling *et al.*, 2013; Vacchiano & Motta, 2014). A major reason for the observed competitive replacement of *P. sylvestris* might, however, be changes in land-use (Vacchiano & Motta, 2014). Contrasting species-specific responses to drought (Zweifel *et al.*, 2009) might also favour *P. sylvestris* (e.g. short episodes of severe drought; Morán-López *et al.*, 2013). Seedling responses of the two species to different drought conditions should therefore be directly compared in future studies.

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Curriculum vitae

Christoph Bachofen

Born September 1, 1983 in Uster (ZH)