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ORIGINAL PAPER

Drought resistance of *Pinus sylvestris* seedlings conferred by plastic root architecture rather than ectomycorrhizal colonisation

Barbara Moser · Tabea Kipfer · Sarah Richter · Simon Egli · Thomas Wohlgemuth

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Abstract

- ***Context*** Increased summer drought is considered as a threat to the regeneration of *Pinus sylvestris* in the Central Alps. To a certain degree, seedlings are able to mitigate negative effects of drought by altering root/shoot ratios. But, seedlings may also enhance access to water and nutrients by cooperation with ectomycorrhizal fungi.
- ***Aims*** We tested the importance of both mechanisms for drought resistance of *P. sylvestris* seedlings during early establishment and assessed whether differences occur between topsoil and deeper soil layers.
- ***Methods*** Biomass allocation and colonisation of fine roots by ectomycorrhizal fungi were assessed in seedlings grown for 6 months in a common garden under different precipitation scenarios: constant drought (March–September), summer drought (June–September), and wet conditions.
- ***Results*** Root/shoot ratios increased from 0.6 under wet conditions to 0.8 under drought conditions, irrespective of the onset of the drought (March vs. June). In both drought scenarios, seedlings had shorter roots in the topsoil, increased the number of root tips per root length in both soil layers, but did not alter the colonisation rate of root tips by ectomycorrhizal fungi.
- ***Conclusion*** We conclude that plasticity in root architecture is an important mechanism for drought resistance of *P. sylvestris* during early seedling establishment.

Keywords Central Alps · Common garden experiment · Ectomycorrhizal diversity · Phenotypic plasticity · Root/shoot ratio · Summer drought

1 Introduction

At the southern range limit of *Pinus sylvestris* in the Mediterranean and the Central Alps, increased frequency and duration of summer drought, as projected for the next century (IPCC 2013), are expected to severely limit the species' capacity for growth (Bigler et al. 2006; Büntgen et al. 2013) and regeneration (Castro et al. 2004; Moser et al. 2010). Successful seedling establishment is a prerequisite for the longer-term persistence of a species (Lloret et al. 2009), but early life stages are particularly susceptible to changes in environmental conditions (Lenoir et al. 2009). A limited root system with no or restricted access to ground water makes seedlings more prone to drought stress, especially during the first year of establishment. As a means of adaptation, *P. sylvestris* seedlings of both Mediterranean and Central Alpine origin are known to increase biomass allocation to roots when water resources are limited (Matías and Jump 2014; Richter et al. 2012). Alternatively, seedlings may enhance access to resources, particularly to water and nutrients, by association with ectomycorrhizal fungi (EcM). EcM root

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Contribution of the co-authors The co-authors jointly developed the idea and designed the experiment. The field work was carried out by Sarah Richter and Tabea Kipfer, supported by Thomas Wohlgemuth and Barbara Moser. Tabea Kipfer carried out the laboratory work with support by S. Egli. Barbara Moser and Tabea Kipfer analysed the data and wrote the manuscript with editorial advice by the co-authors.

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B. Moser (✉) · T. Kipfer · S. Richter · S. Egli · T. Wohlgemuth
Forest Dynamics, Swiss Federal Research Institute WSL,
Zuercherstrasse 111, 8903 Birmensdorf, Switzerland
e-mail: barbara.moser@wsl.ch

tips and associated mycelial strands and hyphae extend the root system, which allows plants to explore a larger soil volume at the expense of delivering carbon to the EcM fungi but avoiding the costs of root production (Lehto and Zwiazek 2011). EcM fungi and fine roots are often concentrated in the upper 0–20 cm of the soil (Hacke et al. 2000; Helmisaari et al. 2009), which increases the risk of desiccation and concomitant nutrient shortage during long drought events (Gaul et al. 2008). Many species, though, can colonise deeper soil layers under dry conditions. Mycorrhizal colonisation of pine species has been found to decrease in response to drought (Kennedy and Peay 2007; Valdés et al. 2006). It is not clear, however, if EcM colonisation decreased because of uneconomically high costs for seedlings to deliver photosynthate to associated fungi or due to altered root architecture, which may involve root proliferation in deeper soil layers, where EcM fungi are less abundant. Drought can also affect EcM species composition (Shi et al. 2002; Swaty et al. 2004), which, in turn, may modulate drought resistance of the host, since beneficial effects vary between fungal species (Kipfer et al. 2012; Lehto 1992). Thus, the question arises whether drought resistance of *P. sylvestris* seedlings is mainly determined by mycorrhizal colonisation, by biomass allocation to roots in general, or by differential root growth in different soil layers.

The aim of our study was to investigate EcM colonisation and concomitant root architecture in *P. sylvestris* seedlings in response to drought. In a common garden experiment with controlled precipitation regimes, we tested (1) to what degree *P. sylvestris* seedlings alter biomass allocation to roots in response to drought, (2) whether root growth depends on the onset of the drought (March vs. June), (3) how allocation patterns differ between upper and deeper soil layers and (4) whether mycorrhizal colonisation rate or EcM species richness change under dry conditions.

2 Materials and methods

2.1 Study site and simulated precipitation regimes

The study was part of a common garden experiment carried out in 2009 at the bottom of the Rhone valley in Leuk, Valais, Switzerland (46° 18′ 33″ N to 7° 41′ 10″ E, 610 m a.s.l.; Richter et al. 2012). The experiment included three precipitation regimes, one simulating wet conditions of the current Rhone valley climate, where seedlings should not experience water stress, and two regimes simulating climate projections for the Central Alps likely to be reached by the end of the twenty-first century (Beniston 2012; Schär et al. 2004). Under the *wet* precipitation regime, a total of 433 mm of water was added to experimental units from 18 March to 17 September 2009 (i.e. 72 mm month^{-1}), which corresponds to the average April–September precipitation of the ten wettest years of the past century (156 % of the 1900–2007 average) at the nearby MeteoSwiss climate station in Visp (46° 18′ 10″ N to 7° 50′ 34″ E, 639 m a.s.l. and 596 mm annual precipitation, −1.3 and 19.4 °C average January and July temperature, respectively). The *dry* regime simulated a 20 % reduction in precipitation with respect to the 1900–2007 average and consisted of 218 mm of water added during the 6 months of the experiment (36 mm month^{-1}). The *med* regime simulated a Mediterranean precipitation pattern with high rainfall during spring (72 mm month^{-1} from 18 March to 31 May; equal to the *wet* regime) followed by a dry summer season (36 mm month^{-1} from 1 June to 17 September; equal to the *dry* regime). The respective amount of water was added manually to the containers twice a week after sunset, on two consecutive days.

2.2 Experimental set-up and protocol

Experimental units consisted of wooden containers with a surface area of 50×60 cm and 50-cm depth. The containers were filled with 33 cm of sand and gravel from the local Rhone river bed (hereafter referred to as deeper soil layer) and covered by 12 cm of sieved topsoil (moder humus) from a nearby *P. sylvestris* stand. The two soil layers were designed to simulate *P. sylvestris* forest soils of the Rhone valley, which have shallow topsoil and low water retention capacity. The topsoil was inoculated with EcM fine roots according to Marx and Kenney (1982). EcM roots were excavated in a *P. sylvestris* stand with mature trees as well as natural regeneration, chopped into 0.5 cm pieces and mixed with the topsoil (1 l of chopped roots per 3 l topsoil). The effectiveness of this method is corroborated by the fact that the measured colonisation rates were comparable to those found in similar forest ecosystems (Kipfer et al. 2010, 2011).

Precipitation regimes were applied to individual containers arranged in a randomised block design replicated five times (three treatments×five replicates=15 containers). In March 2009, 120 seeds per container were sown on a 5×5 cm grid, 40 of them originating from the local Rhone valley (for details see Richter et al. 2012). The others were 40 *P. sylvestris* and 40 *Pinus nigra* seeds from Mediterranean origin, but the seedlings from these seeds were not analysed for mycorrhizal colonisation. In the local Rhone valley provenance, seedling growth was measured in terms of needle length at the end of May (i.e. before the onset of the drought in the *med* treatment) and before harvest in September 2009. Needle length was defined as the distance from the insertion of the cotyledons to the tip of the longest needle. On average, 26, 9 and 29 out of the 40 Rhone valley seeds per container emerged in the *wet*, *dry* and *med* treatments, respectively. Seedling mortality was higher in the drought treatments so that after 6 months, an average of 24, 8 and 16 seedlings per container survived in the three treatments, respectively (Richter et al. 2012). Since it was not feasible to determine EcM colonisation on all seedlings, a random subset of five seedlings from the local Rhone valley provenance was excavated in each container, resulting in a total of 75 seedlings (three treatments × five containers × five replicates). If less than five seedlings had survived, additional seedlings were randomly selected in the remaining containers until an overall sample size of 25 seedlings was reached in each treatment. Treatment-wise comparison of needle length and biomass revealed no difference between the subset and the whole sample. The results reported here include only the subset of 75 seedlings. After excavation, seedlings were separated into shoot and root biomass. Roots were further divided into the parts grown in the topsoil (0- to 12-cm soil depth) and the deeper soil layer (13- to 45-cm depth), respectively. Root parts were stored in tap water at 2 °C and then scanned on a HP DeskScan at a resolution of 300 dpi. Cumulative root length was measured using the WinRhizo Software (Regent Instruments Inc., Canada). The total number of root tips was counted under a dissecting microscope, and each tip examined for the presence of EcM fungi. Ectomycorrhizal tips were classified into morphotypes based on morphological characteristics such as colour, mantle texture, type of ramification and presence of rhizomorphs following the procedure described by Agerer (1987–1997). From each seedling and morphotype, 3–5 root tips were frozen at −20 °C for later molecular identification. EcM formed by *Cenococcum geophilum* was considered sufficiently characteristic to be identified with certainty without molecular analysis. Dry weight of roots and shoots was measured after drying the plant material for 72 h at 60 °C. Molecular identification of EcM species is described in detail in Online Resource 1. Since not all morphotypes of a particular seedling could be identified at species level, the number of EcM species per seedling is given as the number of "operational taxonomic units" (OTUs). An OTU was defined as a morphotype whose DNA sequence differed from others by more than 98 %, regardless of whether it had been identified at species, genus or family level. A list with the names of successfully identified species and accession numbers of the corresponding database entry is given in Online Resource 2.

2.3 Statistical analyses

The layout of the experiment was a one-factor randomised block design replicated five times. Effects of precipitation regime on needle and root growth, and mycorrhizal colonisation were analysed with ANOVA followed by post-hoc pairwise Tukey HSD tests using SPSS Statistics (Release 19.0.0, SPSS Inc., 2010). Since randomisation is not complete in a randomised block design, the factor precipitation regime was tested against the block × treatment interaction and the factor soil layer against the residuals (Montgomery 2001). In cases where data did not meet assumptions of normality or homogeneity of variances, square root transformation improved data structure satisfactorily.

3 Results

3.1 Biomass allocation

P. sylvestris seedlings altered biomass allocation to needles and roots in response to both the constant drought (March–September) and the summer drought (June–September). A reduction in precipitation from 433 mm (*wet*) to 218 mm (*dry*) resulted in a 1.3 fold increase of the root/shoot ratio (Fig. 1a, b; Table 1). At the same time, absolute root biomass was 153.4±12.6 mg (mean±SE) under wet conditions compared to 82.3±11.2 mg under dry conditions (ANOVA of 75 seedlings with square root transformed response variable: $F_{2,8}=10.25$, $P=0.005$; Tukey's HSD, $P<0.001$). Similar to root biomass, cumulative root length was shorter under dry conditions (Fig. 1c). Reduced root growth was restricted to the topsoil layer, where seedlings produced less than half the amount of roots compared to the *wet* regime. Instead, seedlings increased the density of root tips under dry conditions (Fig. 2a). This adaptation occurred both in the topsoil and deeper soil layer, as well as in the *dry* and *med* treatments.

Measurements of needle length in June showed that seedling development was comparable in the *wet* and *med* treatments before the onset of the drought (ANOVA of 75 seedlings $F_{2,8}=4.88$, $P=0.033$ and Tukey's HSD, $P=0.989$), whereas needle length was smaller in the *dry* treatment (Tukey's HSD, $P<0.05$). During the summer months, however, seedlings drastically reduced needle growth in the *med* treatment (Fig. 1a; Table 1), and seedlings presumably invested more resources in root proliferation in order to cope with drought stress. This is indicated by the fact that at the end of the growing season seedlings in the *med* treatment had a similar root/shoot ratio and cumulative root length per soil layer as the ones in the *dry* treatment (Fig. 1b, c).

3.2 Mycorrhizal colonisation

The proportion of root tips colonised by EcM fungi did not differ between precipitation regimes and was on average 45.7 ±2.5 % (mean±SE) in the topsoil and 6.6±1.5 % in the deeper soil layer, respectively (Fig. 2b; Table 1). In the topsoil, higher EcM diversity in terms of number of operational taxonomic units (OTUs) was found in the *wet* compared to the *dry* and *med* treatments (Fig. 3). But the number of OTUs per unit root length did not differ between precipitation regimes (ANOVA of 75 seedlings $F_{2,8}=0.62$, $P=0.556$) indicating that the total number of EcM species that colonise a seedling is related to total root length. The most abundant EcM species were

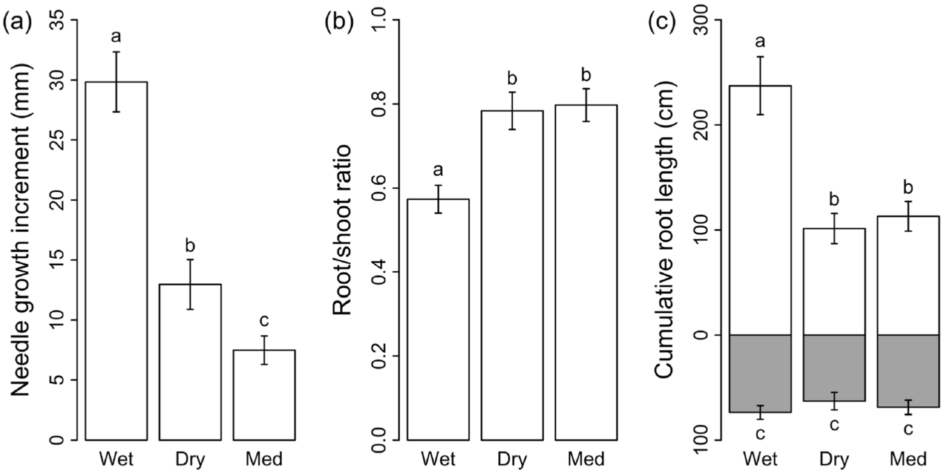

Fig. 1 Biomass allocation of *P. sylvestris* seedlings in relation to the precipitation regime (*wet*, *dry* and *med*): **a** Needle growth increment (May–September); **b** root/shoot ratio; **c** cumulative root length in the topsoil (0–12 cm depth; *white bars* above zero) and deeper soil layer (13–45 cm depth; *shaded bars* below zero), respectively. *Bars* represent means (±SE) of 25 seedlings (N=75). *Identical letters* above/below two bars indicate no statistical difference (ANOVA followed by Tukey's HSD tests)

C. geophilum (found on 57 % of all seedlings; topsoil layer only), an unidentified ascomycete (52 %), and *Rhizopogon roseolus* (32 %). A list with abundances of successfully identified species is given in Online Resource 3.

4 Discussion

Increased summer drought, as predicted for Central Europe under climate change, may considerably reduce resource availability for plants during the growing season. Depending on the phenotypic plasticity of individual species, altered resource availability may affect their competitive ability and thus ultimately species composition. Seedlings are expected to be particularly vulnerable to such changes due to their limited root system, thus differences in the occurrence of adults and juveniles along climatic gradients are often used to project the future distribution of tree species (Lenoir et al. 2010; Rabasa et al. 2013). It is well established that increased belowground biomass allocation enhances access to water and nutrients (Markesteijn and Poorter 2009). In *P. sylvestris* seedlings, adjustment of the root/shoot ratio has been observed both between provenances growing in different climates as well as within provenances (Cregg and Zhang 2001; Richter et al. 2012). Our results suggest that during early establishment, seedlings are even able to increase biomass allocation to roots within a few months, depending on seasonal changes in precipitation (Fig. 1b). The ability of local populations to react plastically to environmental conditions may be fundamental for the species' potential to withstand more frequent summer drought as predicted under climate change.

Seedlings invested proportionally more biomass in the root system under dry conditions, but absolute root biomass and cumulative root length after 6 months were both 46 % smaller than under wet conditions. In accordance with the study by Gaul et al. (2008) on *Picea abies*, we found considerably reduced root network in the topsoil under dry conditions (Fig. 1c). Root length reduction might primarily be a consequence of reduced fine root production during drought, as shown in a meta-analysis including a range of EcM-forming tree species (Cudlin et al. 2007). Contrary to our expectations, seedlings in the drought treatment did not increase root proliferation in the deeper soil layer, where the soil dries out slower than in the shallow topsoil. We suggest that either seedlings were unable to extend their root network due to drought-induced nutrient shortage in the topsoil, or root proliferation was not beneficial due to the scarcity of nutrients in the deeper sandy soil layer. Nevertheless, *P. sylvestris*

Table 1 ANOVA results for the effects of precipitation regime (*wet*, *dry* and *med*) and soil layer (topsoil and deeper soil layer) on needle growth increment (May–September), root/shoot ratio, cumulative root length, root tip density and EcM species richness (no of OTUs) of 6-month old *P. sylvestris* seedlings (N=75)

Source of variation	Precipitation			Soil layer		
	df	*F*	*P*	*df*	*F*	*P*
Needle growth increment (mm)*	2,8	17.53	*0.001*	–	–	–
Root/shoot ratio	2,8	7.85	*0.010*	–	–	–
Cumulative root length (cm)*	2,8	6.05	*0.022*	1,132	65.35	*<0.001*
Total root tip density (cm^{-1})*	2,8	12.08	*0.003*	1,130	12.32	*0.001*
EcM root tip density (cm^{-1})*	2,8	0.03	0.967	1,130	169.90	*<0.001*
EcM species richness	2,8	9.86	*0.005*	1,130	273.30	*<0.001*

* square-root transformed

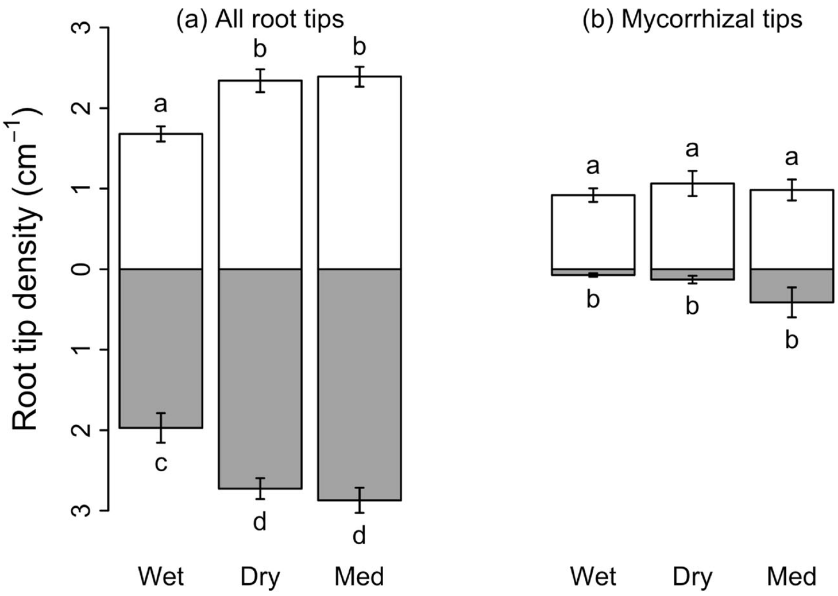

Fig. 2 **a** Total number of root tips and **b** number of mycorrhizal root tips per unit root length depending on precipitation regime (*wet*, *dry* and *med*) and soil layer (topsoil 0–12 cm; *white bars* above zero and deeper soil layer 13–45 cm; *shaded bars* below zero). *Bars* represent means (±SE) of 25 seedlings (N=75). *Identical letters* above/ below two bars indicate no statistical difference (ANOVA followed by Tukey's HSD tests)

seedlings potentially increased water uptake capacity under dry conditions by producing more root tips per unit root length in both soil layers (Fig. 2a).

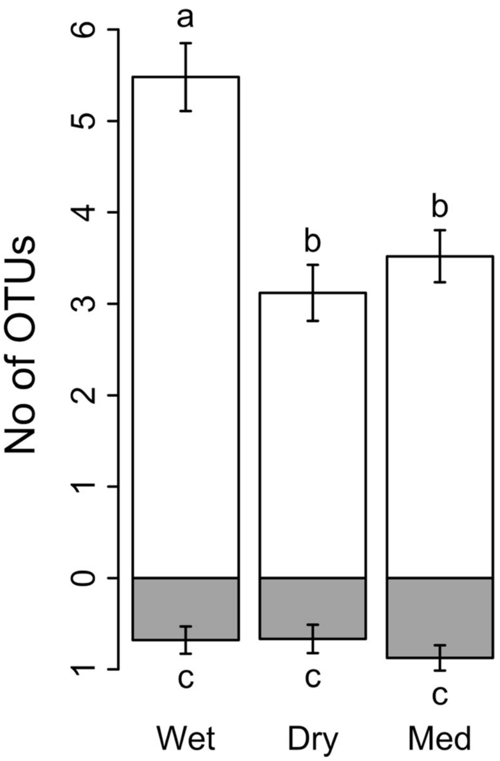

Fig. 3 Species richness of ectomycorrhizal fungi on *P. sylvestris* seedlings (in terms of operational taxonomic units, OTU) in relation to precipitation regimes (*wet*, *dry* and *med*) and soil layer (topsoil 0–12 cm; *white bars* above zero and deeper soil layer 13–45 cm; *shaded bars* below zero). *Bars* represent means (±SE) of 25 seedlings (N=75). *Identical letters* above/below two bars indicate no statistical difference (ANOVA followed by Tukey's HSD tests)

When soil dries, water is more and more retained in smaller pores, eventually becoming inaccessible to plant roots. Under such circumstances, water uptake by associated EcM fungal hyphae may become important for survival, especially for tree seedlings, whose root network does not yet reach deepest soil layers (Allen 2007; Brownlee et al. 1983). EcM fungi may also improve water relations indirectly, e.g. by enhancing nutrient status and thus water use efficiency of the seedlings (Lehto and Zwiazek 2011). While some studies found increased EcM colonisation rates of pine seedlings under drought (Davies et al. 1996), other experiments showed a decrease of seedling root colonisation (Kennedy and Peay 2007; Valdés et al. 2006). We did not find evidence for intensified cooperation between *P. sylvestris* seedlings and EcM fungi under dry conditions: the number of EcM root tips per root length was similar in all treatments (Fig. 2b) and EcM species diversity correlated with root length. This is not surprising since increased EcM colonisation would come at the cost of increased carbon allocation from seedlings to fungal symbionts at a time when CO_2 assimilation is likely to be reduced due to stomatal closure (Courty et al. 2010). Alternatively, EcM fungi may be directly affected by water limitation (Valdés et al. 2006). Individual EcM species differ in their ability to tolerate drought as well as their efficiency to capture nutrients under dry conditions (Lehto and Zwiazek 2011). Consequently, the results of experimental EcM studies may depend to a high degree on the substrate used to inoculate tree seedlings. Ideally, experiments would include a treatment where EcM are absent, but since EcM spores are persistent, wind dispersed propagules, control for such a treatment is unfeasible under common garden conditions. Despite these limitations, the most abundant EcM species found on the seedlings in the present study were equally predominant in bioassay studies in similar forest stands (Kipfer et al. 2010, 2011). Thus, we are confident that the inoculum used in our experiment is representative for Central Alpine *P. sylvestris* forests, which best regenerate naturally in gaps after disturbance. The majority of identified EcM species occurred only on a few seedlings, thus we are cautious to draw conclusions about species-specific differences in drought tolerance. While *R. roseolus* seemed to be more frequently associated with *P. sylvestris* seedlings under dry conditions, *Humaria hemisphaerica* was more often present in the *wet* treatment (Online Resource 3). EcM species can affect root morphology of their hosts (Smith and Read 1997), thus observed differences in root length between dry and moist conditions may also be a consequence of differential EcM species composition.

We acknowledge that the present study covers a very short, albeit decisive, phase during seedlings establishment. EcM species composition may differ between seedlings and mature trees, and changes during vegetation succession, e.g. after disturbance (Kipfer et al. 2011). Nevertheless, the fact that seedlings did not increase EcM root tip colonisation during drought but increased biomass allocation to root instead of shoot growth indicates that plasticity in root architecture is an important mechanism for *P. sylvestris* to cope with more frequent summer drought at its southern distribution limit in the Central Alps.

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