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Temperature affected the formation of arbuscular mycorrhizas and ectomycorrhizas in *Populus angustifolia* seedlings more than a mild drought

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ABSTRACT

Arbuscular mycorrhizal (AM) plants and fungi associate with lower soil organic matter, higher pH, lower phosphorus and higher nitrogen than ectomycorrhizal (EM) ones. However, soil conditions correlate with climatic factors, and we suggest that temperature and humidity have also direct roles in the success of mycorrhiza types. The hypothesis here is that EM perform better at low temperatures than AM, and AM resist drought better than EM.

Narrowleaf cottonwood (*Populus angustifolia* E. James) forms both AM and EM. We grew seedlings in soil at 14, 20 and 26 °C in factorial combinations with adequate watering and a cyclic mild drought for 4 and 7 weeks.

As hypothesized, the percent of EM root tips was largest at 14 °C, while the proportional root length with AM was largest at the two higher temperatures. However, unlike expectations, drought increased EM formation slightly, while the AM colonization was lower in the dry treatment. Plant growth was reduced more by low temperature than drought. Root branching was more prominent at low temperature and root length and mass growth at higher temperatures.

Soil nutrient availability did not provide a direct explanation to the results, as both soluble soil N and P were the same in 14 and 20 °C, while the change in mycorrhiza colonization took place between these temperatures. Differences in root morphology (root branching vs length) may affect the proportions of the mycorrhiza types at different temperature regimes. The most likely explanation to the differential colonization is that temperature affects AM and EM fungi in a different way. In nature, temperature and humidity regimes are tightly correlated, and temperature as such may be a stronger determinant for the success of mycorrhiza types than has been previously considered. The poorer performance of AM in low-temperature and drought conditions may reflect stress avoidance rather than stress tolerance by AM fungi.

1. Introduction

Mycorrhizas are symbioses between plants and fungi, where the plant provides photosynthates in exchange of mineral nutrients taken up by the fungus. Sometimes the relation is not mutually beneficial (Jones and Smith, 2004), but here we consider mutualistic symbioses. Arbuscular mycorrhiza (AM) is the most wide-spread type of mycorrhizas both across the plant kingdom and over different vegetation zones, while ectomycorrhizas (EM) are most common in woody plants in the cool regions of the world. There has been increasing interest in comparing arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) mutualisms in recent decades. The ability to utilize different forms of nitrogen (N) and phosphorus (P) has been considered to be a critical determinant for the success of these mycorrhiza types (Read, 1991; Read and Pérez-Moreno, 2003). The transformations of N and P are complex, affected by plant and litter properties, soil biota and climatic factors, and these cannot all be disentangled in the field. We have suggested that temperature and humidity affect the success of mycorrhiza types with their hosts more directly than has been previously assumed. The overall hypothesis is that AM are favoured by higher temperatures than EM; and that EM are favoured by ample (but not excessive) water, while AM are more drought resistant (Lehto and Zwiazek, 2011).

There is evidence for low AM colonization in cold environments from many types of studies, starting from the occurrence of host plants in

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different regions (Read, 1991). Gradient studies show almost lacking AM colonization in arctic regions as opposed to boreal (Olsson et al., 2004; Kytöviita, 2005). Similarly, higher elevation montane sites have been shown to harbour relatively more EM than AM both because of the composition of the vegetation (Read and Haselwandter, 1981) and larger proportion of EM in plants hosting both AM and EM types (Gehring et al., 2006). A large-scale study using eddy covariance showed that CO_2 fluxes from EM-dominated sites were more tightly controlled by temperature and AM-dominated sites by precipitation; this indicates that the corresponding mycorrhiza type is still more active/competitive in the corresponding conditions (Vargas et al., 2010). In a global data analysis, the distribution of AM was positively correlated with warm-season temperature, although also positively correlated with the occurrence of frost; the correlation with frost was linked to the predominance of AM plants in grasslands in continental regions in the world, having substantial seasonal variation in temperature (Soudzilovskaia et al., 2015). In contrast to AM, gradient studies in northern Europe show increasing amounts of conifer EM towards higher latitudes in relation to needle biomass (Helmisaari et al., 2009) or stand basal area (Ostonen et al., 2011). Also, changes in the EM fungal community composition, rather than changes in proportional colonization have been shown with decreasing temperature in the field (Bueé et al., 2005; Ruotsalainen et al., 2009; Fernandez et al., 2017).

In natural field conditions, high latitude as well as high altitude sites have usually more humid climates (precipitation minus evapotranspiration), and separating temperature effects from humidity effects requires experimental studies. There is more research available on the direct effects of temperature on AM than EM, and AM formation has been frequently observed to be reduced by low temperature (Hayman, 1974, Liu et al., 2004; Gavito and Azcón-Aguilar, 2012; Carvalho et al., 2015). Parke et al. (1983) used the EM species *Pseudotsuga menziesii* and *Pinus ponderosa* plus the AM species *Trifolium subterraneum* as bait plants. The temperature response of mycorrhiza formation followed a similar bell-shaped pattern between 7.5 and 35 °C in the three species, with the optimum at 18–25 °C. However, the most common AM fungus in this study was the fine endophyte, *Glomus tenue*, which tends to prevail in extreme environments and is not classified as mycorrhizal anymore (Olsson et al., 2004). In contrast, Domisch et al. (2002) observed that the EM colonization rate in one-year-old Scots pine (*Pinus sylvestris*) was about 100% in new short roots grown even at 5 °C soil temperature, while the total number of mycorrhizas per seedling was higher at the higher temperatures, up to 17 °C. The function of the two mycorrhiza types at different temperatures has not been compared previously. However, if the nutrient uptake is consistently low compared to the carbon drain by the fungus, plants may develop mechanisms for becoming less susceptible to AM formation in cold environments (Kytöviita, 2005).

Winter survival of AM hyphae has been shown in frost temperatures down to −12 °C (Addy et al., 1994) and EM fungi in pure culture down to −30 and −48 °C (Lehto et al., 2008). To compare AM and EM fungi (AMF and EMF), we tested whether soil-borne propagules were viable after severe frost treatments. The results lent support to the expected better frost tolerance of EMF, as EM formation was not affected by soil exposure to even −130 °C, but AM formation was reduced after frost (Kilpeläinen et al., 2016). The poorer performance of AM may have been because of loss of viable vegetative mycelium and slower mycorrhiza formation from spores.

We interpret these earlier results as cold-stress avoidance in AM fungi rather than stress tolerance. This classification uses Levitt's (1980) terminology, where plant resistance is divided into avoidance or tolerance. Avoidance strategies enable plants or fungi to circumvent severe physiological stress, for example by death of aboveground parts and vegetative mycelium during the most unfavourable times of the year; while tolerance means physiological endurance of for example intracellular dehydration to tolerate intercellular freezing. In contrast to the reliance of AMF on persistent spores in the soil (Brundrett and Abbott, 1994), the survival of EMF depends on their mycelium, as the dispersal of spores is short-termed and unpredictable. The survival of mycelium compared to spores has the advantage that the mycelium is ready to absorb nutrients early in the spring; but this is dependent on the ability of the fungus to function at low temperatures.

Drought is known to reduce EM colonization (Cudlín et al., 2007; Lehto and Zwiazek, 2011) while the effect of drought on AMF has varied in different studies, showing decrease, not detectable change or even increased colonization with drought (Augé, 2001). In the analysis of Soudzilovskaia et al. (2015), EM occurrence was positively correlated with low seasonal variability in precipitation, in addition to the soil factors pH and C/N ratio. However, direct comparisons of the degree of reduction by drought in the two types are still rare. In the field, relatively more AM than EM have been shown in dry conditions both in gradients (Lodge, 1989), seasonality studies (Querejeta et al., 2009) and common-garden watering experiments (Gehring et al., 2006). By contrast, a previous soil drought did not affect EM formation in the EM bait plant *Betula pendula*, although in *Alnus incana*, forming both types, EM colonization was negatively affected by previous soil drought (Kilpeläinen et al., 2017). In the AM bait plant *Trifolium repens* there was also a soil-drought legacy effect in the most severe treatment, especially a decrease in spore formation (Kilpeläinen et al., 2017). Therefore, the hypothesis of better performance of AM after drought did not gain support in the study of Kilpeläinen et al. (2017), but similarly as in the frost-legacy study (Kilpeläinen et al., 2016), there appeared to be a delay in AM formation after the adverse soil treatments.

Plant species forming both types of mycorrhizas are useful models in experimental studies comparing AM and EM formation and function, because the responses of the fungi and the plant-fungus interactions to treatments are not as much confounded with those of host plants as is the case when using different hosts for AMF and EMF. *Eucalyptus* spp. have been used successfully (Jones et al., 1998; Chen et al., 2000; Holste et al., 2017), as well as *Salix repens* (van der Heijden and Kuyper, 2001), *Alnus incana* (Kilpeläinen et al., 2016, 2017, 2020), *Quercus agrifolia* (Egerton-Warburton and Allen, 2001) and *Quercus costaricensis* (Holste et al., 2017). These species can play an important role in understanding the effects of environmental factors on the formation and function of EM and AM. However, many of them have a preference for AM or EM (e.g. van der Heijden and Kuyper, 2001; Kilpeläinen et al., 2019), or form AM only when very young (Arveby and Granhall, 1998; Chen et al., 2000; Egerton-Warburton and Allen, 2001). By contrast, some cottonwood species, such as narrowleaf cottonwood (*Populus angustifolia* E. James) show substantial flexibility in the EM or AM formation and they are especially suitable test species for studying the effects of the environment on mycorrhiza formation (Khasa et al., 2002; Gehring et al., 2006; Meinhardt and Gehring, 2012).

Here we report an experiment with factorial combinations of temperatures 14, 20 or 26 °C and cyclic mild drought or adequate watering on the mycorrhiza formation in seed-grown narrowleaf cottonwood. The hypotheses tested were that plants grown at lower temperature would form more EM and plants at the higher temperature would form more AM, while drought would affect EM formation more negatively than AM formation. Growth, root morphology and foliar nutrient accumulation were measured to provide clues to the possible reasons for, or consequences of the altered plant mycorrhizal status.

2. Materials and methods

Seeds of narrowleaf cottonwood (*Populus angustifolia*) were collected from mother trees of five different genotypes from high-elevation sites in northern Arizona, courtesy of Prof. C.A. Gehring, in June 2016. The attached cotton was removed, and seeds were transported by courier to Joensuu, Finland.

Seeds were germinated in pots with an autoclaved mixture 1 part peat (Kekkilä peat with no fertilizer or lime, Luonnonturve, 2 mm sieve) and 5 parts perlite (Plante-perl, Nordisk Perlite, Denmark) in a walk-in

growth chamber (Conviron GR77, Controlled Environments, Winnipeg, MB, Canada) under fluorescent tubes (VHO 215 W, Sylvania Cool White, Sylvania, USA) in 90% relative humidity, 16-h day at 20 °C, 8-h night at 16 °C. Cooling/warming rate was 5 °C h^{-1}.

We collected soil from three sites in and near Joensuu, Finland, to be used as a substrate and simultaneously as a source of EMF and AMF inoculum after transplanting. In previous trials, native European aspen (*Populus tremula*) formed preferentially EM and no arbuscules (Kilpeläinen et al., 2019), but other poplar species form both types (Khasa et al., 2002). Therefore, we expected to obtain a range of compatible EM, and possibly AM, fungi by collecting soil under *Populus* trees and seedlings, and a range of AM fungi by additional sampling of meadow soil. The sites were (1) the Joensuu arboretum *Populus* plantation with European aspen, hybrid aspen (*P.* × *wettsteinii* = *tremula* × *tremuloides)*, white poplar (*P. alba*), laurel-leaf poplar (*P. laurifolia*), balsam poplar (*P. balsamifera*) and Berlin poplar (*P.* × *berolinensis* = *P. laurifolia* × *P. nigra*) with mainly grass and forb undergrowth (62°35.97′N, 29°43.3′E), (2) Joensuu city forest park, Linnunniemi, under native *Populus tremula* trees and seedlings (62°36.5′N 29°43.2′E) and (3) Havukanaho meadow (63°3.75′N, 29°52.3′E) in Koli national park with diverse grass and forb vegetation (Kilpeläinen et al., 2016). The soil was sampled to a depth 0–20 cm with a corer of 3 cm diameter in early July. The soils from the three sites were sieved (6 mm), removing larger roots and stones and mixed in the volume proportions 1: 1: 1. The homogenized soil was mixed with perlite in proportions 2 soil: 1 perlite.

The germinated seedlings were transplanted to plastic pots (soil compressed to 185 ml, pot height 80 mm, top diameter 61 mm and base diameter 48 mm) four weeks after sowing. The same batch of soil-perlite mixture was used in all pots. We allowed for a 10-day rooting period after transplanting before applying the treatments, in otherwise the same growing conditions as before, but adding light from incandescent lamps, (60 W, Oy Airam, Finland). Day/night PAR was ca. 350/0 μmol m^{-2} s^{-1}.

The experimental treatments were chosen to represent a wide range of temperatures occurring in boreal and temperate regions during the growing season, but not extremes (e.g. Kubin and Kemppainen, 1994; Repo et al., 2007, 2014), and a mild cyclic drought, which is a more usual condition than severe drought in these vegetation zones. The target soil humidity in the dry treatment (see below) was determined by measuring leaf temperature with a portable infrared thermometer with a laser sight and macro-optics (Optris LS, Optris GmbH, Berlin, Germany) daily during water withholding; an increase of ca. 2.5 °C in leaf temperature compared to well-watered controls indicated stomatal closure. At this stage, the dry treatment pots were watered to saturation. Based on pot weights, soil water content reached ca. 18% in each cycle. The treatments were factorial combinations of temperature and watering, temperature having three levels (14/10, 20/16 or 26/22 °C day/night, abbreviated as T14, T20 and T26) and the water regime having two levels (restricted and sufficient, W0 and W1).

Groups (blocks) of six seedlings were formed at random for each of the 5 origins (mother tree genotypes). The six combinations of temperature and watering treatments were allocated at random to the seedlings within each group. The numbers of plants per origin varied, being 30 for two origins, 12 for two origins and 6 for one at the first harvest, providing 15 plants per combination of temperature and watering regime at harvest 1. At harvest 2, the numbers of plants were the same, except with 6 additional plants for the origin that had 6 at H1, therefore 16 plants per treatment factor combination. Two harvests were done, at 4 (H1) and 7 weeks (H2) after start of treatments.

The temperature regimes were assigned at random to three identical growth rooms. The daytime target air humidity was set to the same vapour pressure deficit (VPD), 0.80 kPa in the temperature regimes, corresponding to relative humidities of 50, 66 and 76%. The same RH values were set for both night and day. However, controlling air humidity at 50% RH at 10 °C was not possible due to technical limitations of the growth chambers, and the VPD remained at about 0.63 kPa. Pots were weighed regularly and watered to saturation when they had reached the same target weight corresponding to the water regime at all temperature regimes. Four pots with no plants but filled with the same soil-perlite mix were placed in each growth room and watered at the same time as the well-watered seedlings. These pots were kept in the rooms until harvest 2 and used for soluble soil-nutrient determinations. During the first 3 weeks after the start of treatments plants received only water, but later all the plants received additionally 25 ml per week of a complete nutrient solution containing 40 mg N dm^{-3} and other nutrients in proportion (Riddoch et al., 1991).

At each harvest, plant height was measured to the nearest mm with a ruler. Plants were severed at the root collar and leaves and stems were dried at 40 °C to constant mass and weighed. Subsamples of the root systems from the depths of 0.5–3.0 cm and 4.0–6.5 cm were taken from each pot for mycorrhiza observation. The subsamples were cleared and thereafter stained with methyl blue (Grace and Stribley, 1991; Kilpeläinen et al., 2016). EM root tips and non-mycorrhizal root tips were counted under a stereo microscope, and the EM proportion is expressed as percent EM root tips of the total number of root tips. AM arbuscules, vesicles, hyphae and spores as percent root length were quantified with the gridline intersection method using a stereo microscope (Giovanetti and Mosse, 1980). When necessary, roots were additionally observed under a light microscope at higher magnification.

Estimates for the total number of EM root tips and root tips per plant were computed based on the dry masses of the subsamples and the remaining parts of the root systems. Total root length in the subsamples was calculated following Tennant (1975) and the specific root length (SRL, m g^{-1}) was calculated using the dry masses of the subsamples. Total root length was estimated also for the whole root system based on the dry masses of the subsamples and remaining parts, as well as the total length with AM hyphae per plant.

At harvest 2, the soil in each of the 12 pots with no plants was dried at 40 °C. Ten grams of the soil was mixed with 100 ml 1 M KCl, shaken for 1 h and filtered (filter paper Schleicher & Schuell 589/1). NO_3–N and NH_4–N concentrations were determined from the samples by flow injection analyzer (FIAstar 5012, Tecator, Sweden). Other nutrients were analyzed with ICP-OES (Iris Intrepid II XSP, Thermo Elemental, Franklin, MA, USA) after ammonium acetate extraction in pH 4.65 (Halonen et al., 1983). At this harvest, all leaves from pairs of plants of the same origin within each treatment were pooled to have large enough samples for nutrient analyses (n = 8 per temperature and watering treatment combination). Dried leaves were ground to powder with a mortar. Nitrogen was determined with an element analyser (Varian). For the other nutrients, subsamples were digested in HNO_3 and H_2O_2 in Teflon containers (method based on Epa 3051 in microwave oven (MARS5). The nutrient concentrations were determined with the ICP-OES. Technical replicates were used to check the consistency of the analysis results. Foliar nutrient contents (total amount of a nutrient in the foliage of a seedling) were computed by multiplying the concentration by the mean dry mass of the leaves of the two seedlings in each pooled sample.

A randomized complete block design was used. Origin (O) was treated in the analysis as a block factor and harvest (H), temperature (T) and watering (W) as experimental factors. Origin had five levels, harvest two levels (H1 and H2), temperature three levels (T14, T20 and T26) and watering two levels (W0 and W1). Factorial ANOVA was used, and results discussed using $P < 0.05$ as threshold. In the case of significant interactions with harvest, the effects of temperature and watering were tested separately within each harvest. When a significant interaction was detected between temperature and watering, each watering regime was analyzed separately. Tukey's test was used to test effects of temperature levels when ANOVA indicated significance of this main effect.

3. Results

3.1. Mycorrhizal colonization

The colonization of EM (proportion of root tips, $P_H < 0.001$) increased between harvests. As there was an interaction ($P_{H\times T} = 0.010$), the data from the two harvests were analyzed separately. Within H1 (Fig. 1a), the proportion of EM was largest at T14 ($P_T = 0.019$), and it differed significantly from T26 (P = 0.018). Within H2 (Fig. 1b), T14 also had the highest EM proportion ($P_T < 0.001$), differing from T20 and T26 (Tukey's test P < 0.001), while T20 and T26 did not differ from each other. As also the interaction $P_{T\times W} = 0.011$ at H2, the watering effect was analyzed separately within each temperature. Within T14 and T26, there were more EM in the dry treatment ($P_W = 0.023$), within T20 there was no difference ($P_W = 0.173$) and within T26, the $P_W = 0.065$.

Between the harvests, the colonization of AM increased (root length with hyphae and arbuscules, both $P_H < 0.001$), as well as the occurrence of AM spores ($P_H = 0.001$) (Fig. 2). The main effect of temperature was significant also for the proportions of AM hyphae (Fig. 2a and b) and arbuscules (Fig. 2c and d) ($P_T < 0.001$) and AM spores ($P_T = 0.032$) (Fig. 2e and f) but in contrast to EM, the proportion of root with AM hyphae and arbuscules were both lower at T14 than at T20 and T26 (Tukey's test $P < 0.001$) which did not differ from each other. There were more spores in T26 than T14 (Tukey's test P = 0.022). There were more AM hyphae ($P_W = 0.020$) and arbuscules ($P_W = 0.004$) in the well-watered plants, and there were no interactions.

Very few vesicles were observed, and the data did not fulfil the assumptions of ANOVA. There were no vesicles at 14 °C and the dry treatment at 20 °C. In the well-watered treatment at 20 °C and both watering treatments at 26 °C there were 1–4 plants with a few vesicles at both harvests.

3.2. Root morphology

Most of the root length and branching indices were affected by harvest time and temperature. The main effect of watering was significant only for the length of root with arbuscules. Most of the interactions were not significant.

Between H1 and H2, the number of root tips per root length showed little change, but the number of EM tips per root length increased ($P_H = 0.013$) (Fig. 3a and b). In T14, there were more root tips and EM tips per root length ($P_T < 0.001$ for both), and Tukey's test showed difference between T14 and the higher temperatures ($P < 0.001$) which did not differ from each other. In contrast, the numbers of root tips per root mass ($P_H < 0.001$) – but not EM tips per root mass – decreased between the harvests (Fig. 3c and d). The temperature main effect was $P_T < 0.001$, and T14 had more root tips per mass than T20 and T26 which did not differ from each other (Tukey's test $P \leq 0.012$). Specific root length (root length per unit mass) decreased from H1 to H2 ($P_H < 0.001$) (Fig. 4a and b). Although SRL was consistently lower at the higher temperatures suggesting more mass production relative to length, this effect did not reach significance ($P_T = 0.111$). These results indicate that the root mass increased more than root tip formation and extension growth between the harvests. In other words, there were thicker and more sparsely branched roots in older seedlings.

Calculated per plant, the total root length ($P_H = 0.008$) and number of tips ($P_H = 0.003$) increased between harvests (Fig. 5a–d). Also, the total amount of root colonized by mycorrhizal fungi per plant increased between harvests: AM hyphal length and length with arbuscules per plant and total number of EM tips per plant, all $P_H = 0.001$. The total root length and length of root with AM hyphae and arbuscules were highest at the highest temperature ($P_T < 0.001$), T14 differing from T20 and T26 (Tukey's test, $P \leq 0.014$) which did not show difference (Fig. 5a). Watering main effect was significant only for the length of root with arbuscules ($P_W = 0.026$). The overall mean ± S.E. in the dry treatment was 0.29 ± 0.041m and in the watered treatment 0.42 ± 0.053m (not shown separately in Fig. 5a and b). The total number of tips per plant was highest at the highest temperature ($P_T = 0.005$) and the temperature main effect on EM total number was not significant (Fig. 5c and d).

To sum up the temperature effects on root morphology, root tip formation increased relatively least with increasing temperature (number of root tips per root length decreased), then the length (SRL decreased, although not significantly), and most the mass. Also, from H1 to H2, root mass increased more than root length and tips (number of root tips per mass decreased, SRL decreased). However, the relation between root tips and root length did not change with time.

3.3. Plant size

The main effects of H, T and W for plant height were significant ($P \leq 0.001$; Fig. 6a and b). As there were significant interactions ($P_{H\times T} = 0.009$, $P_{H\times W} = 0.025$, $P_{H\times T\times W} = 0.005$), the two harvests were subjected to ANOVA separately. At H1, temperature was far from significance, but the drought treatment already reduced plant height marginally ($P_W = 0.080$). Additionally, at H2, both the T and W main effects ($P_W < 0.001$) and the interaction were significant ($P_{T\times W} < 0.001$). Therefore, W was analyzed separately within each T treatment. In T14, W was not

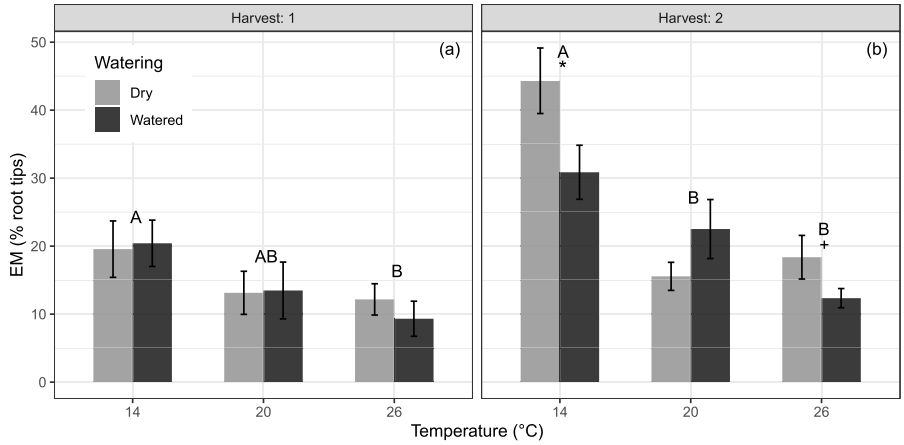

Fig. 1. Mean percentage (±SE) of ectomycorrhizal root tips of all root tips in *Populus angustifolia* at two harvests (a), (b) in three temperature and two water regimes (n = 15–16). The harvest effect was significant. Means for a temperature treatment with the same letter do not differ (Tukey's test, $P < 0.05$). A significant difference ($P < 0.05$) between water regimes is shown by asterisk, and $0.05 < P < 0.10$ by + sign.

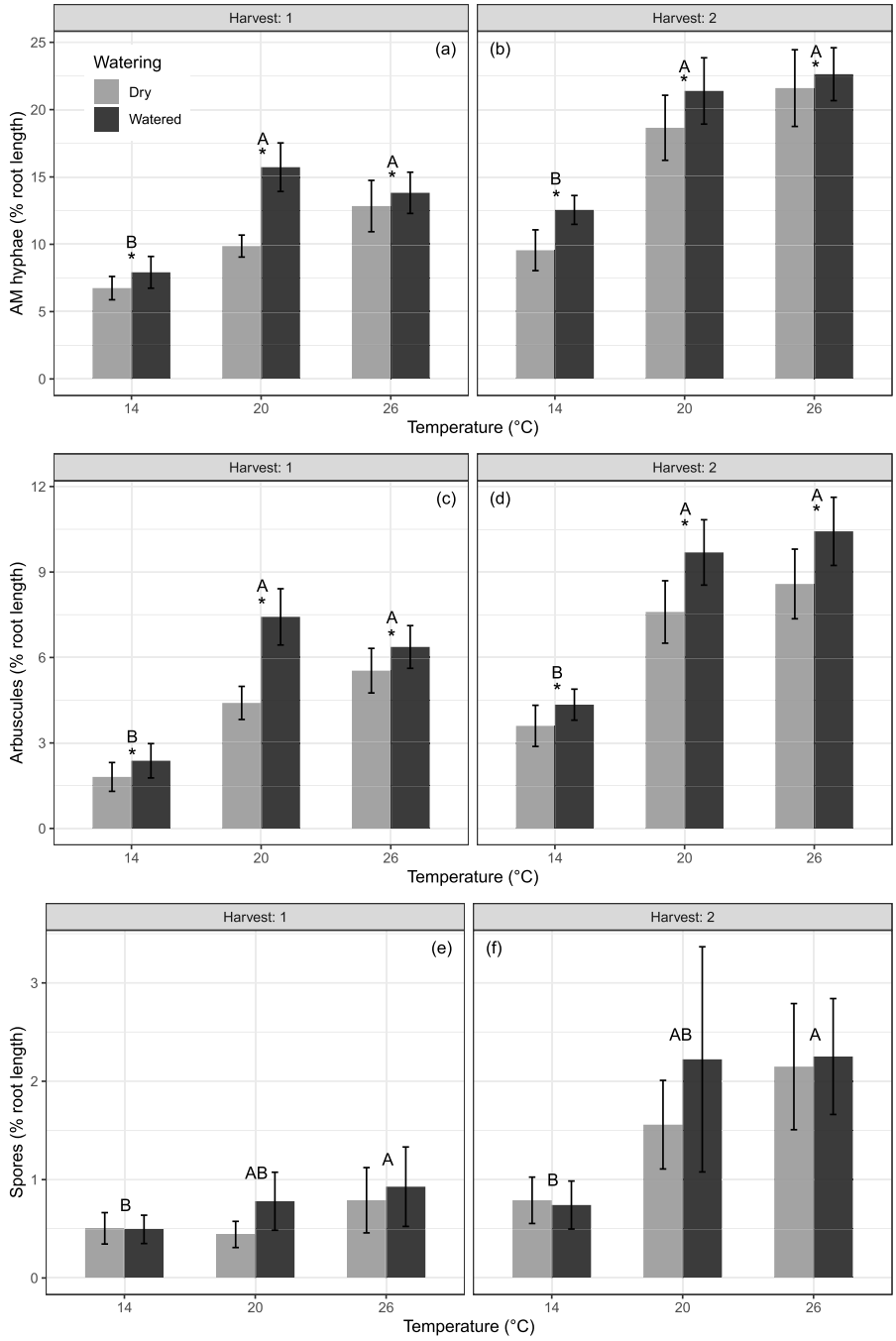

Fig. 2. Percent of root length with AM hyphae (a), (b), arbuscules (c), (d) and AM spores (e), (f) in *Populus angustifolia* at two harvests in three temperature and two water regimes (n = 15–16, means ± SE). The harvest effect was significant in each case. Means for a temperature treatment with the same letter do not differ (Tukey's test, $P < 0.05$). A significant difference ($P < 0.05$) between water regimes is shown by asterisk.

significant, but within T20 and T26 effect $P_W < 0.001$.

The plants more than doubled their dry mass between the harvests ($P_H < 0.001$; Fig. 6c and d). Plant dry mass was generally more reduced by low temperature ($P_T < 0.001$) than by the dry treatment ($P_W = 0.003$); the difference between T14 and T26 at H2 was about three-fold, while the difference between W and D at its largest, at H2 in T26 was 20% (Fig. 6). As the interaction H × T was significant ($P_{H\times T} < 0.001$), the dry mass was tested at each harvest. At both harvests, the main effects of temperature were significant (both $P_T < 0.001$), and the result of Tukey's test was the same, indicating that all temperature treatments differed; hence the interaction was caused by the larger treatment differences at H2. When the ANOVA was repeated using a ln transformation, the interaction was not significant, which confirms the conclusion.

The relative masses of leaves, stems and roots did not differ among harvests and watering regimes (main effects), but the temperature affected them ($P_T \leq 0.006$ for leaf, root and stem) (Fig. 7a and b). The stem mass ratio (SMR) was largest at T14 while T20 and T26 did not differ from each other. The interaction H × T was significant for leaf and root ($P \leq 0.001$), and the harvests were tested separately. At H1, the highest temperature treatment had more leaves and less roots than others, and T20 was intermediate ($P_T \leq 0.001$).

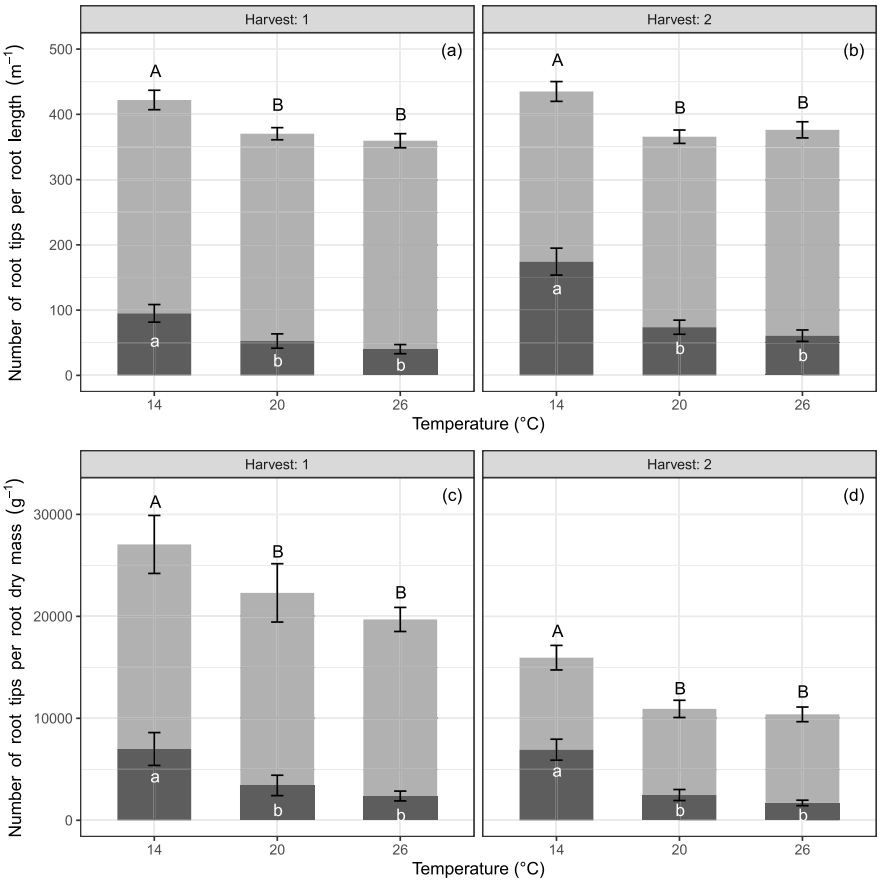

Fig. 3. Total numbers of root tips (whole bar) and numbers of ectomycorrhizal root tips (black) per unit root length (a), (b) and unit root mass (c), (d) in *Populus angustifolia* at two harvests in three temperature regimes (n = 15–16, means ± SE). Water regimes combined because of no significant difference. The harvest effect was significant for EM root tips per root length and for total number of root tips per root mass. Means for a temperature treatment with the same letter do not differ (Tukey's test, $P < 0.05$), capital letter for total number and lower case letter for ectomycorrhizal number.

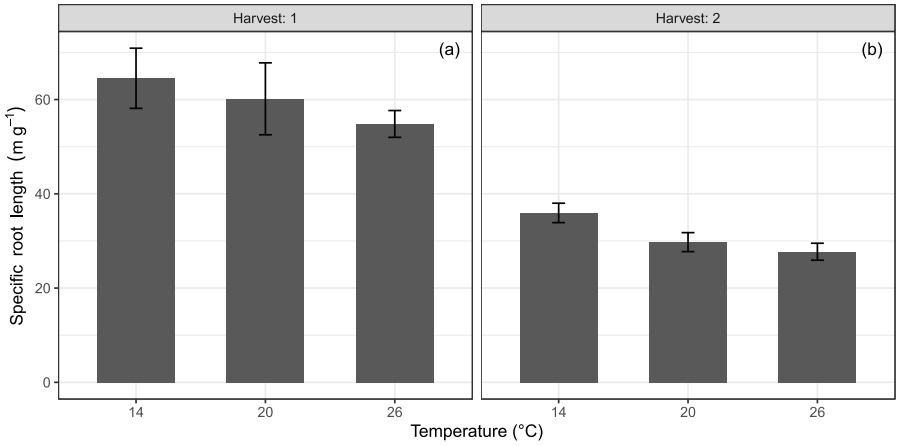

Fig. 4. Specific root length (root length per unit root mass) in *Populus angustifolia* at two harvests (a), (b) in three temperature and two water regimes (n = 15–16, means ± SE). Water regimes combined because of no significant difference. Harvest effect significant.

3.4. Soil elements

At H2 there was substantially more extractable NO_3–N than NH_4–N in the soils of the pots with no plants. The concentrations of both soluble N forms and their sum were similar in 14 and 20 °C and about twice as high at 26 °C ($P_T < 0.001$) (Fig. 8a). The proportion of nitrate and ammonium of total-N was the same in all temperature treatments.

The ammonium acetate soluble P was not clearly affected by temperature as it was only slightly lower in the intermediate T20 than extremes ($P_T = 0.075$) (Fig. 8b). Soluble S showed the same pattern as N, being highest in T26 and not significantly different between T14 and T20 (Table 1). The Ca and Mg concentrations were lowest at T14 and similar between T20 and T26. The Mn concentration was highest at T14 and similar between T20 and T26.

3.5. Foliar nutrients

The temperature treatments affected the Ca, Cu, Fe, Mn and N concentrations significantly, but W did not, although the P_W for N was 0.073; only W affected K and Zn; and both T and W affected Mg and P

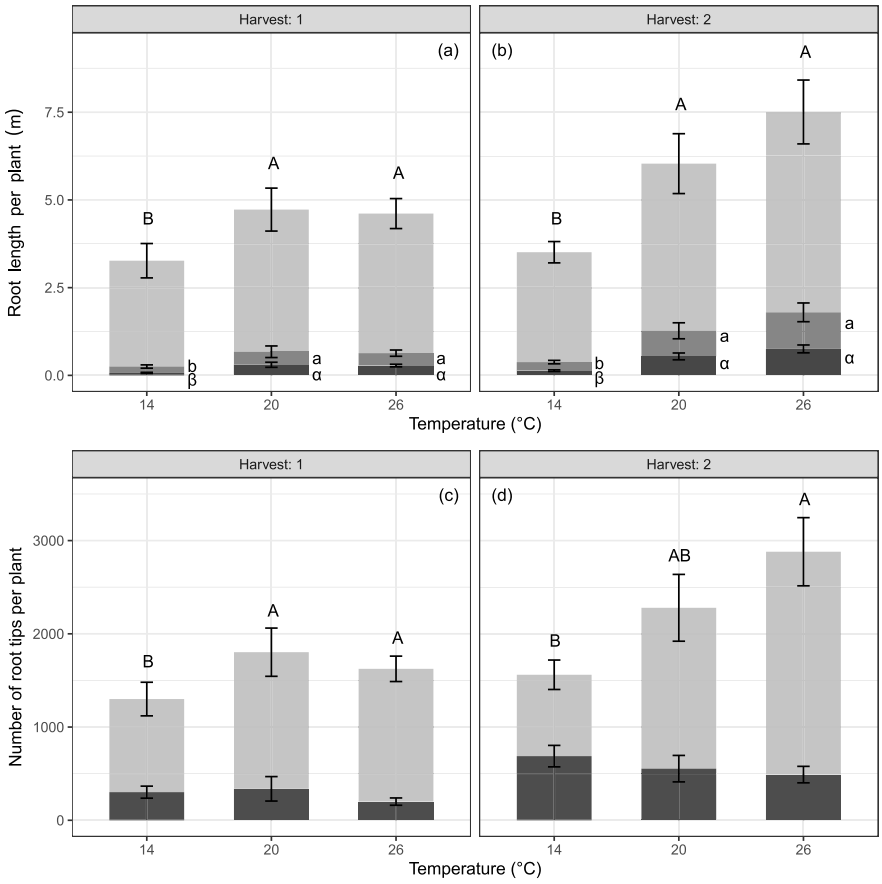

Fig. 5. Total root length per seedling (whole bar) and root length with AM hyphae (dark grey) and arbuscules per plant (black) (a), (b). Total number of root tips (whole bar) and number of EM tips per plant (black) (c), (d) in *Populus angustifolia* at two harvests in three temperature and two water regimes (n = 15–16, means ± SE). Water regimes combined because of no significant difference. Harvest effect significant in each case. Means for a temperature treatment with the same letter do not differ (Tukey's test, $P < 0.05$), capital letter for total length or number; lower case letter for root length with hyphae or ectomycorrhizal number; Greek letter for arbuscules.

(Table 2). Sulfur did not show significant effects, although S concentrations were highest in T14 ($P_T = 0.070$). There were no T × W interactions. The patterns among the temperature treatments varied from one element to another (Table 2). The Ca, Cu, Mg, Mn, and S concentrations were highest at T14. Nitrogen and Fe concentrations were highest in T20 and lowest in T26. Phosphorus concentrations were highest in T14 and lowest in T20. The watered treatment had significantly higher K, Mg, P and Zn concentrations than dry. However, the N concentration was slightly lower in the larger plants in the watered treatment.

All nutrient contents (foliar concentration × leaf dry mass) were increased by increasing temperature, following from the larger biomass increment (Table 3). The W effect was significant for Ca, K, Mg, P and Zn, as was also the interaction T × W. For these nutrients, the contents were always largest in T26, but in the watered treatment, the temperature regimes separated more clearly from each other, the contents being similar between the watering regimes in T14.

4. Discussion

We tested the hypotheses that ectomycorrhizal fungi (EMF) would be more competitive at lower temperatures and arbuscular mycorrhizal fungi (AMF) at higher temperatures in the same root systems, and that the EM formation would be more sensitive to drought. We found clear evidence for the hypothesis regarding temperature, as the EM were more successful at 14 °C and AM at 20 and 26 °C. However, the result on the role of water availability was the opposite to that hypothesized: EM colonization was slightly higher in the dry treatment while the AM showed consistent preference to the well-watered condition.

It should be noted that the temperature treatments were very different from each other, while the cyclic drought treatment was rather mild. This is shown in the growth of the plants, as the aboveground parts were 2.5–3 times as large at 26 °C than 14 °C, but the reduction with the drought treatment was about 20%. The drought treatment did not affect the root mass ratio, which also shows that the treatment was mild, as severe drought tends to reduce root growth but less than shoot growth, which leads to increased root mass ratio (Cudlín et al., 2007).

Air and soil humidities are tightly coupled with temperature, and it is challenging to separate the effects of temperature, humidity and nutrient availability even in a controlled environment, while in the field it is often impossible If the relative humidity is the same at different temperatures, there is larger atmospheric demand for water at higher temperature as the vapour pressure deficit (VPD) is higher. Stomatal conductance per unit leaf area may also be directly affected by temperature, although in constant VPD the effect is not large (Aphalo and Jarvis, 1991). Because of these factors, we aimed to have the same atmospheric demand for water in each temperature treatment by setting the target VPD the same. Although the target VPD was not always reached at 14/10 °C because of technical limitations of the chambers, the effect was small. Moreover, the plants grown at low temperature were much smaller, and therefore very likely with smaller leaf areas, lower whole-plant transpiration rates and less drying of the pots. Therefore we used an additional method for reducing the differences in water availability, watering the pots to saturation when the stomata closed at all temperatures, which should have compensated most of the difference in the humidity between the temperature treatments. The larger root mass ratio at the lowest temperature at harvest 1 suggests that low nutrient availability at low temperature had an overriding effect on the regulation of root vs. shoot growth allocation compared to putative drought stress in high temperature. The lower nutrient availability at 14 °C is shown by the lowest substrate concentrations of soluble N, Ca, Mg and S in this treatment. The difference in the root mass

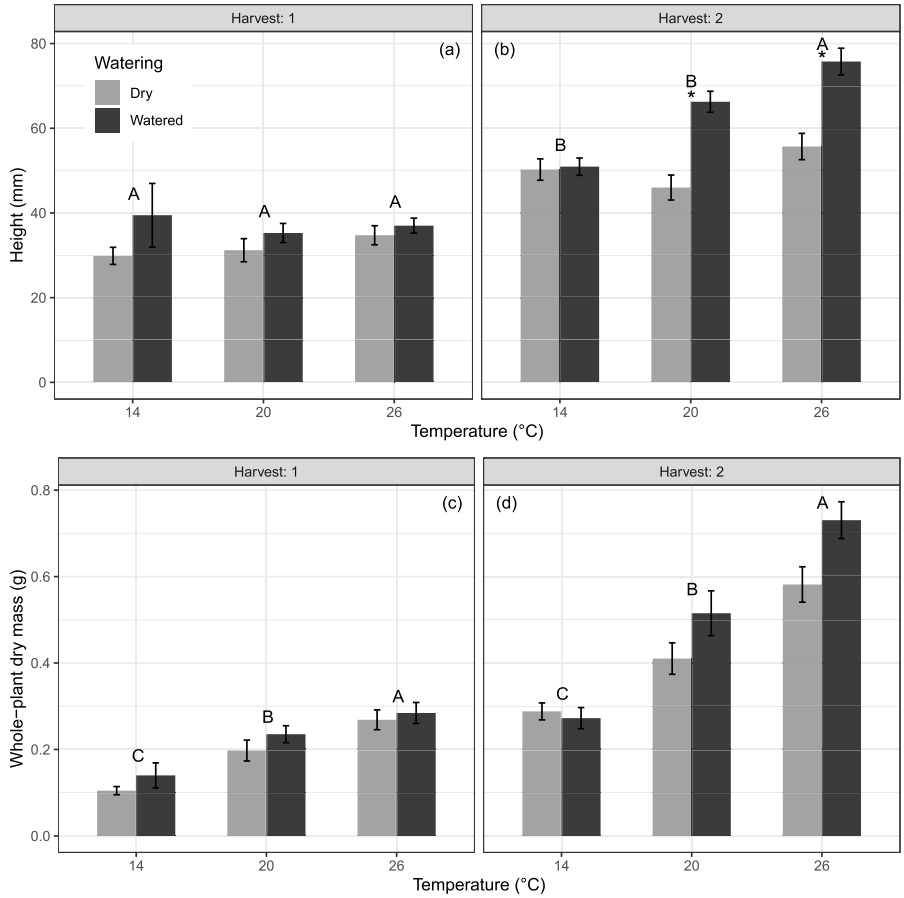

Fig. 6. Seedling height (a), (b) in *Populus angustifolia* at two harvests in three temperature and two water regimes (n = 15–16, means ± SE). A significant difference ($P < 0.05$) between water regimes is shown by asterisk. Total dry mass (c), (d). Harvest and watering main effect significant, for interactions see text. Means for a temperature treatment with the same letter do not differ (Tukey's test, $P < 0.05$) for both height and mass.

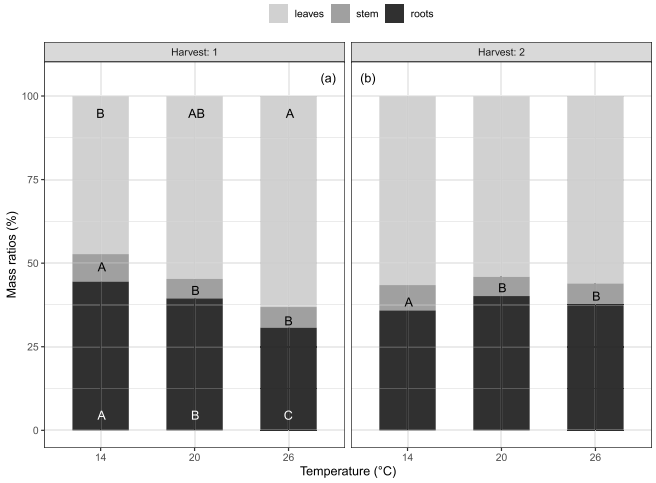

Fig. 7. Proportions (%) of leaves (light grey), stems (dark grey) and root systems (black) of total dry mass of *Populus angustifolia* at two harvests (a), (b) in three temperature regimes (n = 15–16, means ± SE). Water regimes combined because of no significant difference. Means for a temperature treatment with the same letter do not differ (Tukey's test, $P < 0.05$).

ratio lost significance by harvest 2, which is consistent with slightly increasing water stress with the growth and transpiration of the plants.

Possible mechanisms for the temperature responses include:

1) N versus P availability.
2) Covariation of root morphology with the mycorrhizal status.
3) Direct better performance of EMF than AMF at low temperature.

First, D. J. Read's theory emphasizes the role of soil formation and resulting differential N and P availability in the distribution of different mycorrhiza types (Read, 1991; Read and Pérez-Moreno, 2003). EM can access a broader range of N compounds including proteins, while AM are especially efficient at mineral P uptake. We have argued that because N is typically a limiting factor in cold climates, and P in dry climates, their direct influence cannot be easily disentangled from the direct effects of temperature and humidity (Kilpeläinen et al., 2016). In our present study, the foliar N and P levels were low, and therefore the conditions were suitable for differences between the mycorrhiza types to manifest, if N or P deficiency is a driving factor in the prevalence of mycorrhizal types. Both NO_3 and NH_4 extracted from soil in (well-watered) pots with no plants were ca. doubled at 26 °C compared to 20 and 14 °C, with no difference between the two lower temperatures. However, the largest differences in the colonization rates of the mycorrhiza types were between 14 and 20 °C, which indicates that N availability was not the only reason for the colonization difference. In contrast to N, the extractable P concentration in the substrate was not clearly affected by temperature. Phosphorus availability is generally restricted in dry soils (Marschner and Rengel, 2012), which is consistent with the present results on the higher foliar P concentrations in the watered treatment. However, the lower colonization by AM fungi in the dry treatment does not lend support to a role of P deficiency promoting AM formation in dry conditions, but rather, the increased EM in the dry treatment could point to

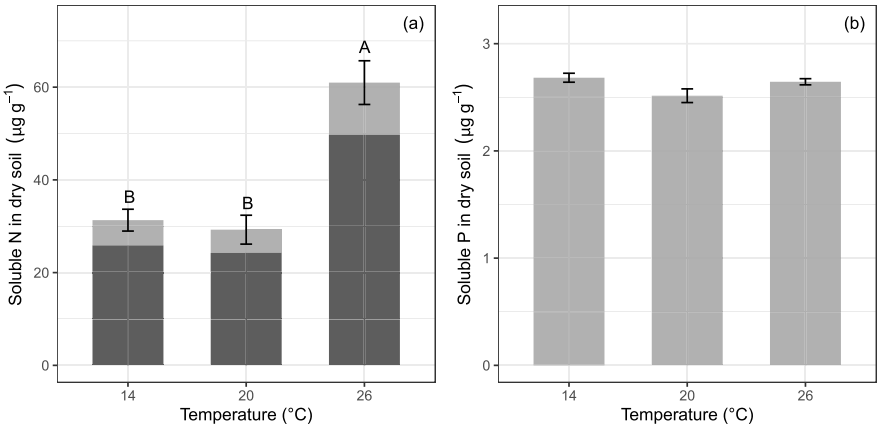

Fig. 8. Concentrations of NO_3–N and NH_4–N (a) and soluble P (b) in soil of plantless pots in three temperature regimes in the watered treatment at the end of the experiment. Means of total soluble N (NO_3–N plus NH_4–N) and P (n = 4, means ± SE) for a temperature treatment with the same letter do not differ (Tukey's test, $P < 0.05$).

Table 1
Ammonium acetate extractable nutrient concentrations in the soil-perlite mix in plantless pots held in the same conditions as experimental plants at temperatures 14, 20 and 26 °C (T14, T20 and T26). Probabilities <0.05 for the temperature effect from one-way ANOVA are bold. The same letter (or none) indicates that there was no difference between means in Tukey's test ($P < 0.05$). Means ± SE of four replicate pots.

Nutrient (µg g^{-1})	T14	T20	T26	P_T
Ca	219 ± 6.6b	240 ± 5.1a	240 ± 2.3a	**0.025**
K	17.6 ± 1.06	14.8 ± 0.68	16.9 ± 0.54	0.086
Mg	23.3 ± 0.98b	26.6 ± 0.63a	26.2 ± 0.53a	**0.022**
S	13.2 ± 1.04b	14.4 ± 0.53b	17.4 ± 0.53a	**0.008**
Cu	0.100 ± 0.004	0.092 ± 0.003	0.089 ± 0.001	0.096
Mn	19.9 ± 0.477a	17.3 ± 0.42b	17.0 ± 0.11b	**0.001**
Zn	2.36 ± 0.055	2.08 ± 0.21	2.40 ± 0.002	0.307

merits use of a combination of approaches, aiming at separating the temperature and watering treatment effects on nutrient uptake from their effects on soil nutrient availability. As the availability of other nutrients also depends on soil temperature and water conditions, they could play a role in the success of different mycorrhiza types in addition to N and P, and the relative availability of different nutrients depends on the particular soil.

Second, an interplay exists between root morphology and fungal colonization as affected by the temperature treatments. The root mass increased relatively more than root length and root tip numbers with increasing temperature. This change in root morphology, leading to fewer root tips per root length and per root mass at the higher temperatures might affect the relative success of the mycorrhiza types, because EM formation is dependent on the availability of suitable new short roots or root branchlets, while AM (*Arum* type) spreads along the root length

Table 2
Foliar nutrient concentrations of *Populus angustifolia* seedlings grown at three temperatures (T14, T20 and T26 denote 14, 20 and 26 °C) and two water regimes (restricted W0 and sufficient W1). Probabilities for the main effects of temperature and watering and their interaction. When T was significant ($P < 0.05$), the means followed by the same letter indicate no difference between the temperature treatments (Tukey's test, $P < 0.05$). Means ± SE of four replicates per treatment, each comprising pooled leaves of two plants in Harvest 2. P values < 0.05 are bold.

Nutrient	W	T14	T20	T26	P_T	P_W	$P_{T\times W}$
Ca (mg g^{-1})	0	3.99 ± 0.28a	2.98 ± 0.15b	3.49 ± 0.16a	**0.010**	0.247	0.141
	1	3.70 ± 0.03a	3.42 ± 0.25b	3.92 ± 0.22a			
K (mg g^{-1})	0	9.03 ± 0.87	7.49 ± 0.19	7.49 ± 0.42	0.466	**0.002**	0.345
	1	9.82 ± 0.82	10.11 ± 0.53	9.81 ± 0.78			
Mg (mg g^{-1})	0	1.79 ± 0.10a	1.29 ± 0.05b	1.55 ± 0.07 ab	**<0.001**	**0.038**	0.810
	1	1.93 ± 0.07a	1.41 ± 0.08b	1.77 ± 0.14 ab			
N (mg g^{-1})	0	7.77 ± 0.35 ab	8.44 ± 0.58a	7.05 ± 0.38b	**0.038**	0.073	0.920
	1	6.75 ± 0.77 ab	7.79 ± 0.35a	6.37 ± 0.44b			
P (mg g^{-1})	0	0.98 ± 0.025a	0.83 ± 0.017b	0.90 ± 0.021 ab	**0.021**	**0.003**	0.684
	1	1.06 ± 0.055a	0.96 ± 0.067b	1.04 ± 0.032 ab			
S (mg g^{-1})	0	0.97 ± 0.051	0.80 ± 0.058	0.83 ± 0.025	0.074	0.463	0.878
	1	0.98 ± 0.098	0.86 ± 0.044	0.89 ± 0.073			
Cu (µg g^{-1})	0	5.51 ± 0.24a	3.43 ± 0.28b	2.97 ± 0.057b	**<0.001**	0.619	0.938
	1	5.52 ± 0.48a	3.61 ± 0.24b	3.14 ± 0.29b			
Fe (µg g^{-1})	0	17.2 ± 1.20 ab	28.9 ± 4.42a	13.9 ± 1.00b	**0.014**	0.550	0.223
	1	19.3 ± 4.89 ab	20.8 ± 3.56b	15.2 ± 1.23a			
Mn (µg g^{-1})	0	109.3 ± 12.3a	63.3 ± 8.0b	105.8 ± 9.59a	**0.001**	0.569	0.807
	1	116.4 ± 9.71a	76.9 ± 6.67b	103.2 ± 8.19a			
Zn (µg g^{-1})	0	151.6 ± 12.1	126.6 ± 3.69	129.8 ± 4.85	0.464	**0.011**	0.457
	1	162.7 ± 17.1	157.3 ± 9.47	171.2 ± 17.1			

this effect. Generally, limited P availability promotes mycorrhiza formation by both types, and limited N at least that of EM (Smith and Read, 2008). Nutrient uptake by different mycorrhizas under stress conditions (Smith and Read, 2008). On the other hand, as the plants were largest at 26 °C, both the total root length per plant and number of root tips per plant were also largest at this temperature. The total root length

Table 3
Foliar nutrient contents (mass × concentration) of *Populus angustifolia* seedlings grown at three temperatures (T14, T20 and T26 denote 14, 20 and 26 °C) and two water regimes (restricted W0 and sufficient W1). When T was significant ($P < 0.05$), the means followed by the same letter indicate no difference between the temperature treatments (Tukey's test, $P < 0.05$). When T × W was significant, the T effect was tested separately within each watering treatment (Tukey's test, $P < 0.05$). Means ± SE of four replicate plants per treatment, each comprising pooled leaves of four plants in Harvest 2. *P* values < 0.05 are bold.

Nutrient	W	T14	T20	T26	P_T	P_W	$P_{T\times W}$
Ca (mg plant^{-1})	0	0.68 ± 0.089b	0.64 ± 0.055b	1.14 ± 0.085a	**<0.001**	**0.022**	**0.014**
	1	0.54 ± 0.045c	0.90 ± 0.074b	1.56 ± 0.138a			
K (mg plant^{-1})	0	1.51 ± 0.130b	1.62 ± 0.081b	2.46 ± 0.281a	**<0.001**	**<0.001**	**0.003**
	1	1.42 ± 0.146c	2.67 ± 0.24b	3.86 ± 0.217a			
Mg (mg plant^{-1})	0	0.30 ± 0.032b	0.28 ± 0.024b	0.50 ± 0.040a	**<0.001**	**0.014**	**0.046**
	1	0.28 ± 0.031b	0.37 ± 0.021b	0.71 ± 0.073a			
N (mg plant^{-1})	0	1.31 ± 0.098b	1.84 ± 0.205b	2.30 ± 0.191a	**<0.001**	0.782	0.244
	1	0.99 ± 0.129b	2.04 ± 0.161b	2.54 ± 0.256a			
P (mg plant^{-1})	0	0.17 ± 0.011b	0.18 ± 0.010b	0.29 ± 0.023a	**<0.001**	**0.002**	**0.015**
	1	0.15 ± 0.012c	0.25 ± 0.015b	0.41 ± 0.036a			
S (mg plant^{-1})	0	0.16 ± 0.014b	0.17 ± 0.020b	0.27 ± 0.017a	**<0.001**	0.088	0.125
	1	0.14 ± 0.015b	0.22 ± 0.013b	0.36 ± 0.051a			
Cu (μg plant^{-1})	0	0.94 ± 0.096 ab	0.75 ± 0.093b	0.97 ± 0.071a	**0.038**	0.179	0.141
	1	0.807 ± 0.102 ab	0.95 ± 0.072b	1.26 ± 0.163a			
Fe (μg plant^{-1})	0	2.90 ± 0.274b	6.15 ± 0.762a	4.57 ± 0.591a	**0.001**	0.646	0.369
	1	2.89 ± 0.683b	5.61 ± 1.20a	6.06 ± 0.606a			
Mn (μg plant^{-1})	0	18.8 ± 3.32b	15.0 ± 2.32b	35.0 ± 5.33a	**<0.001**	0.281	0.480
	1	17.02 ± 2.16b	20.0 ± 1.53b	41.1 ± 4.38a			
Zn (μg plant^{-1})	0	25.8 ± 3.53b	27.5 ± 2.05b	42.0 ± 1.43a	**<0.001**	**0.004**	**0.022**
	1	23.7 ± 3.25b	40.9 ± 0.91a	68.6 ± 9.97a			

colonized by AM fungi increased with temperature (as did the proportional colonization), but the total number of EM tips per plant was about the same at all temperatures. Consequently, the relative decrease in EM with increasing temperature was because of the increasing number of root tips without EM. This contrasts with the results from a study on one-year-old *Pinus sylvestris* seedlings, where the roots did not outgrow the mycorrhizal fungi in the temperature range from 5 to 17 °C, but rather, the scarcity of short roots limited the EM formation (Domisch et al., 2002). This suggests the competition between fungi of the two types as a possible driver for the differences. However, the mechanism remains to be studied in more depth, and could involve active regulation of fungal infection by the plant as well as regulation of plant root metabolism by the fungi. Moreover, young seedlings of *Pinus ponderosa* and *Pseudotsuga menziesii* formed proportionally fewer EM at low temperatures (Parke et al., 1983).

The same pattern of relatively more increase in root mass than length and root tips as with temperature was observed with the growth between the harvests. Therefore, these responses to temperature may have been partly an indirect effect of the dependency of plant morphology and growth allocation on plant size. It has been shown that the morphology of young tree seedlings can depend more on their biomass than on chronological age (Aphalo and Lehto, 1997). In dual-mycorrhizal systems, during the early developmental phase colonization by AM often predominates but may be later replaced by EM (e.g. Arveby and Granhall, 1989; Chen et al., 2000). However, here the initial predominance of EM at low temperature and AM at high temperature became more distinct at harvest 2 compared to harvest 1. The time between harvest was 3 weeks, and in this time frame, the ambient temperature effect overpowered a possible temporal shift between the mycorrhiza types.

Third, EM and AM fungi may have direct physiological adaptations to different temperature ranges. EM fungi appear to have a better tolerance to freezing, as some EM strains survived the lowest test temperature −48 °C in pure culture even without acclimation (Lehto et al., 2008). Legacy effects of soil frost reduced AM colonization in subsequent favourable conditions, while EM colonization was not affected (Kilpeläinen et al., 2016). Lethal temperatures have not been determined for vegetative AM hyphae, partly because AM fungi cannot be grown in pure culture, and it is difficult to separate vegetative hyphae from spores. However, Addy et al. (1994, 1998) showed that AM hyphae retained their ability to colonize after exposure to a minimum −12 °C soil temperature, and methodology could be adjusted for testing lower temperatures.

In plants, exposure to low temperature leads to massive metabolic changes (Schultz et al., 2016), and some of these are known also in basidio- and ascomycetes (Robinson, 2001). Cell membranes are vulnerable to adverse low-temperature effects, and cold acclimation involves increased production of fatty acids and desaturation of membrane lipids also in fungi (Konova et al., 2009; Maggi et al., 2013). Different lipid metabolism could provide a clue to the relatively poor performance of AMF at low temperatures, as AMF take their lipids from their host plants (Jiang et al., 2017; Luginbuehl et al., 2017) while EMF synthesize them. Other possibilities include differences in carbohydrate and secondary metabolism, which contribute to cold acclimation (Schultz et al., 2016). Comparisons between AM and EM systems in these respects are missing, particularly in stress conditions. Recently, we found a much larger concentration of condensed tannins in EM than AM *Alnus* roots especially after a severe drought treatment (Kilpeläinen et al., 2020). Such differences are some of the possible mechanisms underlying the poor growth of mycelium and consequent low colonization rates of AM fungi at low temperatures (Liu et al., 2004), and/or the low nutrient uptake rates of AM at low temperatures (Gavito et al., 2003).

In humid temperate regions, carbohydrate availability is limited in winter because of low photosynthetic activity or dormancy in perennial plants, while the soil remains mostly unfrozen and fungi may not be able to down-regulate their maintenance respiration. By contrast, at very low temperatures the maintenance respiration is not a large carbon cost. Together with low-temperature survival, this could be a key to the different temperature responses of EM and AM fungi. A linear response of respiration to temperature between 11 and 23 °C was shown for 12 pure-cultured EM fungal species, and in nine of these there was no significant acclimation to prior growth temperature (Malcolm et al., 2008). This result suggests that the energy requirement of many EM fungi is strongly temperature dependent. By contrast, the respiration by AM external mycelium acclimated rapidly to an increase by 6 °C in soil temperature, showing no difference between the temperature treatments (Heinemeyer et al., 2006). If the cost of EM mycelium is higher than that of AM mycelia, as suggested by Gehring et al. (2006), the difference may increase with increasing temperature. The temperature response of the carbon and energy cost of the mycorrhizas of both types should receive more attention, particularly as the respiration of

mycorrhizal mycelium is among the largest ecosystem CO_2 sources alongside with that of plants and decomposer organisms.

The drought effect on mycorrhiza was opposite to that expected. However, it resembles our earlier results, where previous soil drought somewhat reduced AM formation and spore production in the AM bait plant *Trifolium repens* but did not affect EM formation in the EM bait plant *Betula pendula* (Kilpeläinen et al., 2017). In contrast, in the dual-mycorrhizal bait plant *Alnus incana* the EM were suppressed by drought, which was suggested to be due to competition by AM. In the present experiment, there was no evidence of a suppressive effect of drought on EM coexisting with AM; on the contrary, the percentage of EM root tips was higher in the dry treatment, concurrently with no effect of drought on root tip formation. Boreal AM fungi may not be as tolerant to drought as those in warmer and drier regions, which could be one explanation to the contrast between the present study and earlier results showing more AM formation in dual-mycorrhizal species in dry conditions (Gehring et al., 2006). Furthermore, it is possible that the dominance of EM in regions with relatively continuous precipitation (Soudzilovskaia et al., 2015) is driven more by the woody host plants' requirement for abundant water than that of the EM fungi.

To conclude, we found that low temperature increased the proportional EM colonization and inversely, higher temperature favoured AM colonization in competition in the same root systems. This is consistent with the low presence of AM in the boreal zone and even lower in tundra (Kytöviita, 2005). On the contrary, our result about increasing EM formation and decreasing AM in the drought treatment does not coincide with the distribution of AM in much drier habitats than EM. In the present state of knowledge, we suggest that many AMF may have a strategy of avoidance of low temperature and drought rather than tolerance. AM plants are most clearly dominant in grasslands (Soudzilovskaia et al., 2015; Davison et al., 2015), which are prevalent in continental climates with both low winter temperatures and summer drought. We suggest that in such conditions, avoidance may be a better strategy than tolerance for AM fungi. In dry grasslands, AMF show a clear drought avoidance behaviour, as they produce resilient spores in response to declining moisture, followed by rapid and opportunistic growth of new mycelium upon the return of favourable conditions (Jacobson, 1997). In contrast, the extensive EMF mycelium (e.g. Wallander et al., 2013) cannot be rapidly replaced by new mycelium after drought or after the winter, especially as the low soil temperatures in springtime limit fungal growth. Obviously, temperature cannot be a sole determinant of the distribution of different mycorrhiza types, but from our results it seems to be more important than has been previously thought.

Declaration of competing interest

We declare no conflict of interests.

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