Elevated ozone prevents acquisition of available nitrogen due to smaller root surface area in poplar

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Abstract

Aims

Poplars are ecologically and economically important tree genus, sensitive to ozone (O_3). This study aimed to investigate modifying effects of elevated O_3 on poplar root response to nutrient addition.

Methods

In pot experiment, young trees of an O_3-sensitive Oxford poplar clone (*Populus maximoviczii* Henry × *berolinensis* Dippel) growing in soil with three levels of P (0, 40 and 80 kg ha^{-1}) and two levels of N (0 and 80 kg ha^{-1}) were exposed to three levels of O_3 (ambient – AA, 1.5 x AA, 2.0 x AA) at a free air exposure facility. After one growing season, root biomass, fine root (<2 mm) nutrient concentrations and ratios, and fine root morphology were assessed.

Results

Nitrogen addition resulted in an up to +100.5% increase in coarse and fine root biomass under AA, and only up to +46.3% increase under 2.0 x AA. Elevated O_3 and P addition had a positive effect, while N had a negative effect on P concentrations in fine roots. Nitrogen limitation for root growth expressed as a N:P ratio was more pronounced at elevated O_3. Nitrogen addition increased root surface area per soil volume by +78.3% at AA and only by +9.9% at 2.0 x AA.

Conclusions

Smaller root surface area per soil volume at elevated O_3 prevented acquisition of available N, rendering N fertilization of young poplar plantations in such conditions economically and environmentally questionable.

Keywords

Fine roots, nitrogen, phosphorus, O_3-FACE, *Populus maximoviczii* × *berolinensis*

Introduction

Poplars are an ecologically and economically important tree genus. Natural poplar stands and poplar plantations cover globally more than 75 million ha and 8.6 million ha, respectively (FAO, 2012). The extent of poplar plantations is expected to increase due to afforestation of abandoned agricultural land and increasing interest in bioenergy plantations (FAO 2012).

Plant growth in the majority of terrestrial ecosystems is limited by the availability of nitrogen (N) and/or phosphorus (P). Optimal N:P ratios for growth vary with species, growth rate, plant age and plant parts (Güsewell 2004). Establishment of poplar plantations and early growth is often supported by application of fertilizers as hybrid poplars have high nutrient demands. To achieve maximum productivity, nutrients should be supplied in optimal balance (Guillemette and DesRochers 2008). Meanwhile, natural stands are dependent on the local soil conditions, but in the recent decades antrophogenic inputs of nutrients by run-off from agriculture into the water table and deposition of N from the atmosphere are gaining importance (Güsewell 2004, Rennenberg et al. 2010). Human induced atmospheric N deposition is the consequence of NO_x emissions from fossil fuel and biomass combustion and NH_3 escape from agriculture (Rennenberg et al. 2010). Neto primary productivity of nitrogen limited ecosystems may respond positively to nitrogen addition (LeBauer & Treseder 2008). On the other hand, increased atmospheric N deposition may result in disturbance of normal plant metabolism, induction of mineral imbalances, reduction of frost hardiness, increase in sensitivity to air pollutants and other environmental stressors, and indirect effects on P nutrition by negative effects on mycorrhizal colonization of roots (Utriainen and Holopainen 2001a, Lang et al. 2016). Response of poplar species which originate from floodplain forests with open N cycle (inflow of N from external sources) to excessive load of N might be different from tree species that grow in N limited natural environment (Rennenberg et al. 2010).

Tropospheric ozone (O_3) is a secondary air pollutant that is formed by the oxidation of carbon monoxide and volatile organic compounds in the presence of nitrogen oxides and sunlight. Since 1980, emissions of O_3 precursors have shifted from middle and high lattitudes of northern

hemisphere towards equator, where the potential for O_3 fomation is much greater due to sunlight and intense heat. Since O_3 is transported globally, rising O_3 precursor emissions at low latitudes have a potential to affect ozone concentrations on a global scale (Zhang et al. 2016). Poplars as fast-growing trees are more sensitive to tropospheric ozone (O_3) in comparison to slowly growing species (i.e., evergreen trees) due to their high stomatal conductance (Novak et al. 2005, Marzuoli et al. 2009). Several studies performed on different tree species have shown a modifying effect of N on O_3 response (e.g. Pell et al. 1995, Schmutz et al. 1995, Maurer and Matyssek 1997, Utriainen and Holopainen 2001b, Watanabe et al. 2012, Yuan et al. 2017). For *Populus tremuloides* it was shown that O_3 significantly reduced biomass at optimal rates of N fertilization, while there was no effect at suboptimal and excessive N fertilization (Pell et al. 1995). At sub-optimal N levels, absence of biomass reductions due to O_3 was explained by compensatory response of accelerated leaf abscision which is providing sufficient recycling of nutrients for slow-growing plants (Pell et al. 1995). Under elevated O_3, N acquisition is impaired, probably due to reduced stomatal conductance and consequently lower water transport into the plant (Luedemann et al. 2005, Weigt et al. 2012). Up to our knowledge, P interaction with O_3 was considered in only one study performed on Norway spruce, but no consistent interactive effects were reported (Wallin et al. 2002). N and P interactions with O_3 were studied on Norway spruce as well, and they did not have much effect on susceptibility to O_3 in this species, which is known to have relatively low sensitivity to O_3 (Weigt et al. 2012). Zhang et al. (2018a) have investigated interactive effect of N, P and O_3 on Oxford poplar clone and reported greater reduction in total biomass due to O_3 by higher N levels. P mitigated O_3 induced reduction in biomass when no N was added to substrate, but no effect of P was observed at higher N levels. Positive correlation between soil P and leaf P was suppressed under elevated O_3 (Zhang et al. 2018a). For the same experiment it was reported that both N an P addition decrease reactive oxygen species in leaves at ambient and 1.5 x ambient O_3, but not at 2.0 x ambient O_3 (Podda et al. 2019).

Tree roots are poorly studied although they are essential for tree survival and growth. They are responsible for water and nutrient uptake from soil, storage of carbon compounds and physical

support of the tree. With process of fine root turnover, they play an important role in belowground cycling of carbon and nutrients, such as N, P, Mg and Ca (Brunner and Godbold 2007). Moreover, they influence soil biophysical and mechanical properties, and soil microbial communities. They protect soil from erosion and shallow landslides on slopes and riverbanks (Stokes et al. 2014). Through the support of mycorrhizal fungi, whole belowground part of the forest ecosystem acts as a complex functioning unit via common mycelial networks (Kraigher et al. 2013).

In our study, effects of O_3 stress in conjunction with different levels of nutrient (N, P) addition were investigated in poplar roots. Up to our knowledge, this is the first study that investigates combination of O_3 effects with N and P in tree roots. Study was performed on rooted cuttings of Oxford poplar clone. Aboveground parts of this clone are highly sensitive to O_3 (Marzuoli et al. 2009, Hoshika et al. 2018). We hypothesized that O_3 will negatively affect root growth and modulate root response to N and P addition. On the other hand, imbalanced nutrient addition was postulated to affect root susceptibility to O_3-induced effects. Greater effects of O_3 on roots were expected under high nutrient addition. Root response was measured as fine and coarse root biomass, C, N and P concentrations and ratios in fine roots, and fine root morphology.

Methods

Propagation, planting and ozone/nutrient treatments

Cuttings of O_3-sensitive poplar clone *Populus maximoviczii* Henry × *berolinensis* Dippel (Oxford poplar clone; sensitivity to O3 demonstrated by Marzuoli et al. (2009) and references therein) were propagated in December 2015 and kept refrigerated until February, when they were potted into small pots. Cuttings were transferred outside in March 2016 and replanted in April 2016 into 10 L pots using a peat : sand : local soil mixture in 1:1:1 ratio. Local soil had a sandy-loam texture and a slightly acidic pH. To potting substrate three levels of P in combination with two levels of N were added (Tab. 1). P was added as 0, 0.5 and 1.0 mM KH_2PO_4 solution according to Lewis and Strain

120 (1996) and N was added as 0 and 5 mM solution NH_4NO_3 according to Thomas et al. (1994). 200 mL
121 solution of NH_4NO_3 or KH_2PO_4 with different concentrations as described above were added to soil
122 twice a week during the whole treatment period. At the same time, KCl was supplied into the soil
123 that did not receive KH_2PO_4 to keep an equal amount of K among all treatments (Tissue and Lewis
124 2010, Mao et al. 2014). Levels of added P and N were in agreement with native N and P
125 concentrations in soils, for detailed explanation see Zhang et al. 2018a and Zhang et al. 2018b. The
126 plants were irrigated to field capacity every 2–3 days to prevent water deficit. For soil pH and
127 concentrations of N and P at the end of experiment see Zhang et al. (2018a).
128
129 Potted cuttings were exposed to three levels of O_3, ambient (referred as AA - control), 1.5 x AA
130 (medium) and 2.0 x AA (high) at O_3 free air controlled exposure (FACE) facility (Paoletti et al., 2017) in
131 Sesto Fiorentino, Italy, from May 1st 2016 to October 1st 2016, when destructive harvesting was
132 performed. Each of 18 treatment combinations (Tab. 1) was replicated in three plots of the FACE
133 facility, each replicate with three poplar plants.
134
135 ***Sampling of roots and investigation of fine root morphology***
136 For root morphological analyses, only roots from AA and 2.0 x AA O_3 treatments were selected to get
137 insight into root responses to both extremes as root analyses are very time-consuming. One pot per
138 replicate treatment was randomly selected, resulting in three pots from AA and three pots from 2.0 x
139 AA treatment for each nutrient combination. From each selected pot, a subsample of a soil core with
140 274 mL soil corer was taken. Exact volume of a soil core was measured volumetrically, by submerging
141 the soil sample into measuring cylinder. Roots from the soil core were cleaned off the soil. Cleaned
142 roots were divided into fine roots (<2 mm) and coarse roots (>2 mm).
143 Afterwards, roots were scanned on Epson Perfection V700 Photo scanner in trays covered by water.
144 Scans were analysed with WinRhizo (Regent Instruments Inc., Ville de Québec, Canada) software to

obtain mean root diameter, length of roots per each fine root diameter class (i.e. length of all roots whose diameter fit into selected diameter span) and number of root tips. Percentage of root length in each fine root diameter class was calculated by dividing root length of selected diameter class by total root length in the soil core. Finally, root biomass was assessed as explained in chapter 2.3. Morphological and biomass data were combined to obtain specific root length and specific root tip density. Data were calculated per volume of soil where relevant.

Determination of root biomass

In addition to roots from AA and 2.0 x AA, also roots from 1.5 x AA treatments (which were not subsampled for root morphological analyses) were used for biomass determination. After scanning, subsamples of roots from AA and 2.0 x AA treatments were transferred onto tissue paper and air-dried. The remaining roots (that were removed by soil corer) from AA and 2.0 x AA pots were also cleaned and separated into fine and coarse roots. The subsample and the remaining roots from the pots were then dried in dryer at 70°C. Afterwards, paper bags with dried roots were transferred into desiccator, left to cool down and weighted on a SCALTEC SBC-31 analytical scale. Weights of the subsample and the remaining roots were summed. Roots from 1.5 x AA treatments were processed according to the procedure described for the remaining roots from the AA and 2.0 x AA pots.

Determination of total carbon, nitrogen and phosphorus concentrations in fine roots

Dried fine root samples were grinded for 2 minutes at 3000 rpm (Grindomix GM 200, Retsch, Germany) to obtain a fine homogeneous powder. Total C and total N content in fine roots were determined by dry combustion method using LECO TruSpec C/N analyzer (ISO 1998:13878, Cools and De Vos 2010, Hoshika et al. 2013). 150 and 200 mg of powdered fine roots were used for determination of total C and total N, respecitively. The organic carbon present in the samples of dry

roots was oxidized into carbon dioxide (CO_2), and nitrogen compounds to nitrogen oxides (NO_x) and elemental N by heating of the sample to a temperature of at least 900°C in the presence of oxygen and in the absence of CO_2. NO_x were reduced to elemental N. Than, the quantity of gasses produced was measured using infrared and thermal conductivity detectors.

Content of total P was determined by inductively coupled plasma–optical emission spectroscopy (ICP–OES) in 300 mg samples of powdered roots. Digestion (mineralization) was performed in a microwave oven and for extraction of dried fine roots a mixture of nitric acid and oxygenated water was used (Rautio et al. 2016).

Statistics

Data (root biomass, nutrient content, root morphological parameters) were tested with three-way ANOVA for factors O_3, N and P, and their interactions. Assumption on equality of variances was tested with Levene test. Planned contrasts were used to test for significant differences between specific factors of factor combinations if they were found significant in ANOVA. All tests were performed at $p<0.05$ significance level. Data was analyzed in R environment (R Core Team, 2017).

Results

Fine and coarse root biomass

Biomass of coarse and fine roots was significantly affected by O_3, N and the O_3 x N interaction, but not by P either alone nor in interaction with other factors (Fig. 1, Table 2). Relative to ambient O_3 treatment (AA), coarse root biomass decreased by 26.7 and 31.4 % in medium and high O_3 treatments, respectively (Fig. 1). General stimulation of coarse root biomass due to N addition was +80.2%. In treatment with no added N, coarse root biomass decrease due to O_3 was -35.3 and -21.5% relative to control in medium and high O_3 treatments, respectively, while in treatments with N

addition, biomass decrease was -22.0 and -36.8% in medium and high O_3 treatments, respectively. Coarse root biomass at the highest O_3 exposure was increased by +46.3% due to N addition (but no significant difference), while at medium and control O_3, N addition increased coarse root biomass by +119% and +81.6%, respectively (Fig. 1a).

Fine root biomass decreased due to O_3 by -34.2% and -34.5% in medium and high O_3 treatments, respectively, while N stimulated fine root biomass by +72.1%. In treatment with no added N, fine root biomass decrease due to O_3 was -28.5 and -16.1% relative to control for medium and high O_3 treatments, respectively, while in treatments with N addition, biomass decrease was -37.1 and -43.7% for medium and high O_3 treatments, respectively. Fine root biomass at the highest O_3 exposure was increased by +34.6% due to N addition (but no significant difference), while at control O_3, N addition increased fine root biomass by +100.5% (Fig. 1b).

Carbon, nitrogen and phosphorus concentrations in fine roots

Concentration of C in fine roots was affected by P supply, and by O_3 x P and N x P interactions. With increasing P, the C concentration in fine roots decreased, but this effect was observed just at control O_3 (Fig. 2a). Concentration of N in fine roots was not responsive to any of the main effects, and only weakly to O_3 x P interaction without any clear pattern (Fig. 2b).

Concentration of P in fine roots was affected by O_3, N and P as single factors and by the interaction of O_3 with both nutrients (Fig. 2c, 2d). Ozone as a single factor had a stimulatory effect on P concentrations in fine roots, with up to +26.7% increase in fine root P at the highest O_3 level applied. Effect of N treatment as single factor on P concentration in fine roots was negative, P in fine roots was reduced by -14.3% in N80 relative to N0. Effect of P as single factor on concentration of P in fine roots was observed as up to +39.1% increase in fine root P with increasing P supply. Interactive effects of O_3 x P (Fig. 2c) and O_3 x N (Fig. 2d) were shown as larger increases in P concentration in fine roots under elevated O_3 at lower P and N loads. The effect of P fertilization on P concentration in fine

218 roots was therefore higher at ambient O_3 level (+60.9%) compared to the high O_3 level (+19.3%), but
219 plants grown with elevated O_3 levels had higher basal P concentrations per se. (Fig. 2c).

220 C:N ratio was weakly affected by O_3 x P interaction, but no clear response pattern was evident (Fig.
221 2e). Mean overall N:P ratio in fine roots was 3.34. It was strongly affected by main factors O_3, N and
222 P. In addition, weak interaction effects of O_3 x P (Fig. 2f) and N x P were observed. Main effect of O_3
223 was observed as decreased N:P ratio under elevated O_3 levels. As expected, higher N and lower P
224 loads as single factors resulted in higher N:P ratio. Interaction effect between O_3 and P on N:P ratio
225 was observed as significantly higher N:P ratio at ambient O_3 relative to both elevated O_3 levels at P0
226 and P40, while at P80, the N:P ratio was similar for all O_3 concentrations (Fig. 2f).

227

228 ***Fine root morphology***

229 Effect of N and N x O_3 interaction was observed on several fine root morphological parameters: root
230 surface area per soil volume, root length density, root tip density and branching density (Fig. 3),
231 although many parameters did not show any response to any factor (Table 3, Table S1). The
232 magnitude of effects was different depending on the morphological parameter. Overall, root surface
233 area per soil volume significantly increased (by +39.5%) in treatments with added N. Increase in root
234 surface area per soil volume due to N addition at ambient O_3 level was +78.3%, while at 2.0 x
235 ambient O_3, this increase was only +9.9%, and was not statistically significant (Fig. 3).

236

237 Similar response to N as for root surface area was found also for root length density (+70.3 at
238 ambient O_3 vs. +1.01% increase at 2.0 x ambient O_3), root tip density (+67.7% increase vs. -2.02%
239 decrease) and branching density (+85.4 vs. +11.4% increase), which were all positively correlated
240 with root surface area (Pearson r ranging from 0.82 to 0.95). Interestingly, for all these morphological
241 parameters there was a significant positive effect of high O_3 at N0, while at N80, this effect was
242 reversed (Fig. 3).

The highest proportion of fine root system in O_3-sensitive poplar clone was found in 0.1-0.2 mm diameter class (more than 50%), followed by 0.0-0.1 mm diameter class (more than 20%) (Fig. 4a). O_3 had a significant positive effect on proportion of roots in 0.0-0.1 mm diameter class (+5.8%). On the other hand, O_3 significantly decreased the percentage of roots in 0.1-0.2 mm diameter class (-4.66%), Fig. 4b. Addition of nitrogen had a significant negative effect (-3.39%) on proportion of roots in 0.0-0.1 mm diameter class (Fig. 4c). Other diameter classes were not affected by any of the parameters (Table 4).

Discussion

In this study we have shown for the first time that effects of elevated atmospheric O_3 on certain parameters of root growth and root chemical composition of poplar plants were modulated by N or P availability. As fine roots are crucial for the uptake of water and nutrients from soil, any change in fine root morphology due to O_3 may have a profound effect on nutrient use efficiency and biomass gain of the whole tree.

Fine and coarse root biomass

Negative effects of O_3 on coarse and fine root biomass in this study were stronger (up to -31.4% for coarse roots and up to -34.5% for fine roots) than for total biomass (up to -26%) of the plants from the same experiment reported by Zhang et al. (2018a), while stimulation of biomass by N addition was lower (80% for coarse roots and +72% for fine roots) compared to total biomass (+97%, Zhang et al. 2018a). Preference for biomass allocation to aboveground parts is typical for trees of productive habitats, such as poplars. Decrease in root biomass due to O_3 is reported in approx. 40% of studies (Agathokleous et al. 2015). Ozone had a modifying effect on response of root biomass to N, as at

ambient O_3 level biomass was stimulated by N addition by +81.6% and +100.5% for coarse and fine roots, respectively, while at high O_3 exposure, the N stimulation was only +46.3 and +34.6% for coarse and fine roots, respectively. At elevated O_3, photosynthetic N use efficiency is generally decreased (Watanabe et al. 2013, Shang et al. 2019). Although in the same plants the N concentrations in leaves were increased with N addition regardless of O_3 concentration (Zhang et al. 2018a), transfer of N to photosynthetic apparatus might be impaired under elevated O_3 (Watanabe et al. 2013, Shang et al. 2019). Instead to photosynthetic apparatus, N is allocated to cell walls and other undefined components that might be important in oxidative stress defence (Shang et al. 2019). In leaves senesced due to O_3, N can be complexed with phenolics (Andersen 2003). Zhang et al. (2018b) reported that N aggravate O_3 induced respiratory carbon loss. High N concentrations under O_3 exposure may increase lipid peroxidation and have detrimental effects on chlorophyll fluorescence, resulting in decreased biomass yield (Calatayud et al. 2006). Pell et al. (1995) suggested that at low growth rates due to N limitation compensatory responses to O_3 stress are sufficient to prevent detectable losses in total biomass.

Absence of P effect on root biomass indicated that P was not a limiting nutrient for root biomass gain in the investigated soil. This was supported by low N:P ratios in roots (mean 3.34+/-0.11), which were well below N:P ratios of fine roots (<2 mm) from natural ecosystems, that is 12:1 (Gordon and Jackson 2000) and indicated that actually N and not P was limiting for root growth in our plants. The same was reported for total biomass from the same experiment (Zhang et al. 2018a).

Carbon, nitrogen and phosphorus concentrations in fine roots

Overall, average C and N concentrations in poplar fine roots from our experiment were lower than reported for fine roots of the same diameter range of adult trees from natural ecosystems (434 vs. 480 mg g^{-1} for C and 8.24 vs. 11.1 mg g^{-1} for N), while average P concentrations were higher (2.57 vs. 0.92 mg g^{-1}) (Gordon and Jackson 2000).

292 Carbon concentrations in fine roots were significantly higher in treatment with no P addition under
293 ambient O_3 but decreased under elevated O_3 and different nutrient combinations. Under low P
294 conditions, as it was the case in treatment with no P addition, trees allocate more resources to fine
295 roots (McCormack and Guo 2014), while decreased C allocation to belowground is well-known
296 response to O_3 stress occuring due to retention of carbohydrates in leaves and premature loss of
297 foliage in the fall (Andersen 2003). For example, decreased C concentration in fine roots under
298 elevated O_3 was reported for sun grown beech seedlings (Železnik et al. 2007). Interestingly,
299 increased C concentration in fine roots was associated with P and not with N, although P was not
300 limiting for growth of our poplar plants. Possible explanation could be that plants need to invest
301 more resources in aquisition of P from natural soils (such as used in treatment with no added P), as P
302 in natural soils is adsorbed, precipitated, or present in organic form and as such not easily available
303 for uptake (Schachtman et al. 1998). Carbon is also needed to support ectomycorrhizal fungi in
304 exchange for P (Smith & Read 2008). Indeed, the colonization rate with ectomycorrhizal fungi was
305 significantly higher in treatments with P0 compared to P80 (unpublished data). As only around 10%
306 of root tips were colonized and mycorrhizal communities were not affected (unpublished data), we
307 assume that ectomycorrhizal fungi are not the reason for increased C concentration in fine roots.

308 Concentrations of N in fine roots in our study were neither affected by N or O_3, while in leaves of the
309 same plants they were affected just by N (Zhang et al. 2018a). This indicates that ability of fine roots
310 for acquisition of N was unaltered despite O_3 stress. Similarly, Weigt et al. (2012) reported that
311 labelled N and total N concentrations in beech and spruce roots were not significantly affected by O_3
312 treatment. Absence of O_3 effect on root N concentration in poplars was reported also by Schmutz et
313 al. (1995) and Zak et al. (2007), while Haberer et al. (2007) observed decrease in N concentration of
314 beech fine roots under elevated O_3. Studies of Schmutz et al. (1995) and Zak et al. (2007) emphasized
315 that total N content (i.e. total N acquired from soil) was lower due to negative effects of O_3 on
316 biomass. As negative effects of O_3 on biomass were also observed in our study, total acquired N
317 under elevated O_3 was also lower in our case. In our experiment, N was apparently transported

predominantly aboveground to leaves as leaf N - and not root N - was increasing with N addition (see Zhang et al. 2018a). Similar results were reported by Schmutz et al. (1995) for poplar and by Maurer and Matyssek (1997) for birch. This is consistent with the statement of Newman and Hart (2006) that more nutrients are translocated to photosynthetisizing parts at increased nutrient availability to maximize carbohydrate synthesis.

On the other hand, P concentrations in roots increased with increasing O_3 level, while in leaves of plants from the same experiment they decreased under the highest O_3 level (Zhang et al. 2018a). This indicated that the uptake of P by the roots might remain at the same level, but the sink strength in leaves could decrease due to decrease in photosynthesis (Watanabe et al. 2013; Zhang et al., 2018b). Ozone caused a reduction of stomatal conductance in this poplar clone (Zhang et al., 2018b). The increased P concentration in roots might be related to the less efficient delivery of P by transpirational water stream (Cernusak et al., 2011). Alternatively, P could be translocated from senescing leaves or shoots back to the roots (Schachtman et al. 1998). Senescence-like symptoms in leaves are often associated with O_3 stress (see Watanabe et al. (2013) and references therein). Retranslocation rate of P from senescing to live leaves in birch, which is a tree species with indeterminate growth (as poplars), was markedly increased by elevated O_3 (Shi et al. 2016). Interestingly, P addition at ambient O_3 resulted in higher increase of P concentration in fine roots compared to elevated O_3 levels, but under elevated O_3, higher basal P concentrations were recorded under P0 treatment.

Mean C:N ratio in fine roots in our study was 53.4:1, which is higher than mean value reported for fine roots of adult trees from different biomes 43:1 (Gordon and Jackson 2000) and in the range of values 17.0 to 63.6:1 reported for fine roots of 32 temperate broadleaved tree species (Ferlian et al. 2017). This value may indicate N limitation relative to C. Although a weak interaction effect of P and O_3 on C:N ratio was detected, no clear pattern was recognisable. Kasurinen et al. (2005) reported no effect of O_3 on C:N ratio in birch roots.

343 In fine roots of our plants, mean N:P ratio was 3.34:1, while average N:P ratios for terrestrial plants in
344 natural conditions are 12-13 (Güsewell 2004). According to Gordon and Jackson (2000), N:P ratio in
345 fine roots is comparable to N:P ratios of leaves and shoots. Low N:P ratios indicate N limitation
346 (Güsewell 2004). As expected, N:P ratio in our study increased due to addition of N and decreased
347 due to addition of P, but despite this N limitation occurred all nutrient treatments. O_3 significantly
348 affected N:P ratio. In both, medium and high O_3, N:P ratio was significantly lower compared to
349 control. O_3 modulated the response of N:P ratio to P status. At P0 and P40, medium and high O_3
350 exposure resulted in higher N limitation in roots than at ambient O_3 levels.

351

352 **Fine root morphology**

353 Uptake of water and nutrients in plants is dictated more by root morphological properties such as
354 root length density and root surface area, than by root mass (Tachibana and Ohta 1983, Eissenstat
355 1992). Nitrogen addition in our experiment significantly increased root surface area, root length
356 density, root tip density and branching density, thereby improving capacity for N absorption. Our
357 results are supported by the finding of King et al. (1997) who reported that N addition increased root
358 surface area for +21% in two pine species. Response to N in our experiment was strongly modulated
359 by O_3. Under high O_3 levels, N addition resulted only in slight increase or even decrease in above
360 stated root morphological parameters. Therefore, supplementary N under high O_3 level cannot be
361 fully exploited as a resource for growth, as it was supported by biomass measurements. Slight
362 stimulatory effect of high O_3 at N0 on these parameters could be explained as investment into roots
363 to provide additional nutrients for repair of O_3-induced damage, while at increased N this mechanism
364 fails (Pell et al. 1995).

365 In addition, O_3 had a significant stimulatory effect on percentage of fine roots in 0.0-0.1 mm
366 diameter class, but on the other hand decreased the percentage of fine roots in 0.1-0.2 mm diameter
367 class. These are roots that are most active in absorption of nutrients and water (McCormack et al.

2015). In study on three oak species, Mrak et al. (2018) reported an O_3-induced decrease in percentage of fine roots in 0.0-0.1 mm diameter class for *Q. pubescens*. As investment of carbon for construction of thinner roots is lower (Eissenstat 1992), investment into 0.0-0.1 mm roots instead 0.1-0.2 mm roots in poplars might be a strategy to sustain the absorption of nutrients under stress. A trend for increase in specific root length (SRL, Table S1) (a parameter which describes length of roots constructed for certain amount of biomass) due to O_3 is consistent with the finding that the percentage of 0.0-0.1 mm roots increased with elevated O_3. Thinner roots (roots with higher SRL) have greater specific rates of water and nutrient uptake, which might be the reason why the concentration of P in roots is increased under elevated O_3. As C concentration in fine roots is positively correlated with branching order (Zadworny et al. 2015), with the thinnest absorptive roots belonging to the lowest branching orders, the increase in 0.0-0.1 mm diameter class would support the measured decrease in C concentration in fine roots of our plants due to elevated O_3.

Addition of N had a negative effect (-3.4%) on proportion of roots in 0.0-0.1 mm diameter class. There was also a non-significant trend for mean root diameter to increase with N addition. Eissenstat et al. (2015) reported that six arbuscular mycorrhizal species responded to fertilization on average with 11% increase in root diameter.

Conclusions

At highest O_3 exposure level, N fertilization did not significantly increase coarse and fine root biomass compared to treatment with no N fertilization. At elevated O_3, root surface area did not respond positively to N addition. Although N uptake capacity remained unchanged, smaller root surface area did not allow for the uptake of N quantity that could support biomass gain. N:P ratio indicated that elevated O_3 increased N starvation in young poplar plants. Although concentration of P in roots was increased under elevated O_3, this was not associated with biomass response. We could not unequivocally find out the reason for increased P in roots under elevated O_3, therefore further

studies would be needed. From the viewpoint of root growth, it could be suggested that in areas with high O_3 concentrations N fertilization of poplar plantations would not be economical neither environmentally friendly. Due to increasing importance of poplars in growing bioeconomy, the findings of this study are highly relevant for natural forest and plantation managers and and contribute to the knowledge on growth and nutrient use efficiency of aboveground parts under O_3 stress.

Declarations of interest

None.

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584

585 Table 1: Treatment combinations and amounts of nutrients added to the pots with young poplar
586 trees obtained from cuttings. O_3 levels: AA – ambient (control), 1.5 x AA and 2.0 x AA. Loadings of
587 nutrients are expressed for the time period from May 1st 2016 to October 1st 2016.

O_3 level	P treatment label	Amount of P per ha (kg ha^{-1})	Amount of P per poplar cutting (mg $seedling^{-1}$)	N treatment label	Amount of N per ha (kg ha^{-1})	Amount of N per poplar cutting (mg $seedling^{-1}$)
AA/1.5xAA/2.0xAA	P0	0	0	N0	0	0
				N80	80	392.5
	P40	40	196.3	N0	0	0
				N80	80	392.5
	P80	80	392.5	N0	0	0
				N80	80	392.5

588

589

590 Table 2: Results of three-way ANOVA for coarse and fine root biomass of an ozone-sensitive poplar
591 clone subjected to three levels of ozone, two levels of nitrogen and three levels of phosphorus.
592 Values with *P*<0.05 are presented in bold.

	Degr. of freedom	Coarse roots		Fine roots	
		F	*p*	*F*	*p*
O_3	2	**12.6**	**<0.0001**	**24.8**	**<0.0001**
N	1	**70.4**	**<0.0001**	**78.7**	**<0.0001**
P	2	0.11	0.8938	0.07	0.9310
O_3 x N	2	**3.46**	**0.0423**	**9.12**	**0.0006**
O_3 x P	4	0.77	0.5511	2.42	0.0664
N x P	2	1.76	0.1873	0.89	0.4210
O_3 x N x P	4	1.77	0.1564	1.33	0.2772

593

Table 3: Results of three-way ANOVA for fine root morphological parameters of O_3-sensitive poplar clone. SRL: specific root length, SRA: specific root area. Factors ozone (O_3), nitrogen (N), phosphorus (P) and their interactions were tested. Statistically significant effects at $P<0.05$ are marked in bold.

	Df	Mean diameter		Tips per length		Branches per length		SRL	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
O_3	1	2.08	0.1622	0.26	0.6176	0.85	0.3672	3.12	0.0903
N	1	2.94	0.0993	0.02	0.8927	1.84	0.1877	0.50	0.4867
P	2	0.06	0.9397	0.18	0.8392	0.90	0.4210	0.33	0.7251
O_3 x N	1	0.01	0.9368	0.09	0.7650	0.16	0.6887	0.02	0.8955
O_3 x P	2	0.99	0.3879	0.27	0.7625	1.02	0.3751	0.31	0.7360
N x P	2	0.78	0.4691	0.99	0.3868	0.06	0.9451	1.23	0.3110
O_3 x N x P	2	0.65	0.5293	2.43	0.1091	1.22	0.3125	0.25	0.7804

	Df	Specific root tip density		Root surface area per soil volume		Root tissue density	
		F	*P*	*F*	*P*	*F*	*P*
O_3	1	2.85	0.1044	0.03	0.8565	2.09	0.1610
N	1	0.67	0.4213	**14.6**	**0.0008**	0.34	0.5646
P	2	0.50	0.6155	0.91	0.4142	2.25	0.1267
O_3 x N	1	<0.01	0.9622	**7.53**	**0.0113**	0.20	0.6568
O_3 x P	2	0.07	0.9310	0.11	0.8955	0.74	0.4857
N x P	2	1.29	0.2945	1.87	0.1762	0.50	0.6116
O_3 x N x P	2	0.16	0.8567	0.99	0.3880	0.28	0.7602

	Df	SRA		Root tip density		Branching density		Root length density	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*

O_3	1	3.10	0.0909	0.44	0.5135	0.02	0.8956	0.29	0.5977
N	1	0.04	0.8403	3.80	0.0629	**7.87**	**0.0098**	**7.20**	**0.0130**
P	2	0.54	0.5904	0.59	0.5632	1.40	0.2656	0.88	0.4280
O_3 x N	1	0.15	0.7010	**4.53**	**0.0438**	3.84	0.0616	**6.63**	**0.0166**
O_3 x P	2	0.46	0.6353	0.28	0.7601	0.05	0.9561	0.34	0.7129
N x P	2	1.22	0.3147	1.86	0.1773	1.43	0.2586	2.28	0.1243
O_3 x N x P	2	0.11	0.8966	0.15	0.8622	0.91	0.4176	1.04	0.3699

599

600

601 Table 4: Results of three-way ANOVA for % of total fine root length in root diameter classes for O_3-
602 sensitive poplar clone. Factors O_3, N, P and their interactions were tested. Statistically significant
603 effects at *P*<0.05 are marked in bold.

	Df	% 0.0<.L.<=0.1		0.1<.L.<=0.2		% 0.2<.L.<=0.3		% 0.3<.L.<=0.4		% 0.4<.L.<=0.5		% sum 0.5-2 mm	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	F	p
O_3	1	**12.8**	**0.0015**	**23.5**	**<0.0001**	2.14	0.1561	0.26	0.6142	0.02	0.8972	<0.01	0.9712
N	1	**4.36**	**0.0477**	0.23	0.6385	2.84	0.1047	3.16	0.0881	2.86	0.1038	1.86	0.1854
P	2	0.02	0.9768	0.78	0.4696	0.51	0.6087	1.00	0.3828	0.63	0.5412	0.04	0.9577
O_3 x N	1	0.81	0.3774	2.69	0.1140	0.18	0.6737	0.03	0.8655	0.24	0.6301	0.16	0.6883
O_3 x P	2	0.93	0.4102	0.30	0.7402	0.77	0.4759	0.48	0.6248	1.08	0.3543	0.58	0.5652
N x P	2	0.46	0.6391	0.54	0.5889	0.23	0.7996	0.05	0.9505	0.10	0.9040	1.21	0.3160
O_3 x N x P	2	0.53	0.5968	2.35	0.1172	1.05	0.3652	1.58	0.2271	0.68	0.5164	0.49	0.6199

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605

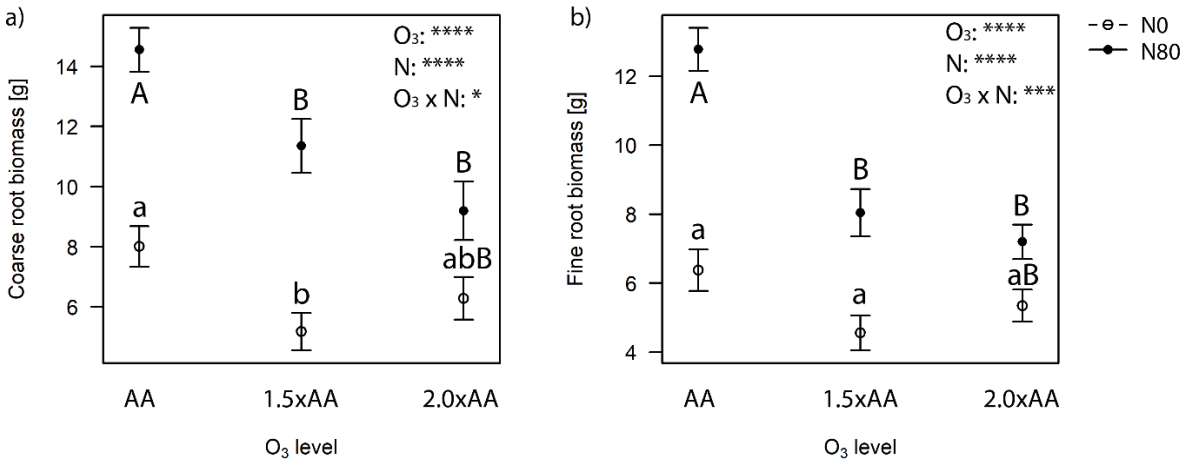
606 Fig. 1: Interactive effects of nitrogen (N) addition and ozone (O_3) exposure on coarse (a) and fine root
607 (b) biomass (mean values ± SE, n = 9) in an O_3-sensitive poplar clone subjected to three different
608 levels of O_3 (AA - ambient, 1.5 x AA and 2.0 x AA), two levels of N (0 N – no added N, 80 N – 80 kg N
609 ha^{-1} per exposure period) and three levels of P. As P effects and its interactions with O_3 and N were
610 not statistically significant, they are not shown here. ANOVA *P* values for O_3, N and their interaction
611 are indicated: ns, not significant, *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Different
612 uppercase and lowercase letters indicate statistically significant differences at P<0.05 for separate
613 comparison of each N treatment and each O_3 level.

614 Fig. 2: Mean values ± SE (n = 3) for C, N and P concentrations (panels a-d) and C:N and N:P ratios
615 (panels e-f) in fine roots of an O_3-sensitive poplar clone subjected to three different levels of O_3 (AA -
616 ambient, 1.5 x AA, 2.0 x AA), two levels of N (0 and 80 kg ha^{-1}) and three levels of P (0, 40 and 80 kg
617 ha^{-1}). In individual panels only statistically significant first-order interactive effects of O_3 with either N
618 or P are shown. Appropriate terms (main effecs and interactions) of three-way ANOVA and their
619 significance (ns, not significant, *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001) are added to
620 individual panels. More specifically, the differences in O_3 exposure levels within each N or P
621 fertilization level are shown; different letters indicate significant differences at P<0.05. Where letters
622 missing, no significant effects were found with planned contrasts.

623 Fig. 3: Interactive effects of N and O_3 on selected fine root morphological parameters (mean values ±
624 SE, n = 9) of O_3-sensitive poplar clone subjected to two different levels of O_3 (AA - ambient, 2.0 x AA),
625 two levels of N (0 N and 80 N) and three levels of P. ANOVA *P* values for O_3, N and their interaction
626 are indicated: ns, not significant, *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Effects of P and its
627 interactions with O_3 and N are not shown as they are not statistically significant. Different letters
628 indicate statistically significant differences (P<0.05).

629 Fig. 4: Fine root system in O_3-sensitive poplar clone (mean value ± SE): a) Overall structure (n = 36).
630 b) Effect of O_3 (AA - ambient O_3 level, 2.0 x AA O_3 level) on fine root structure (n = 18), c) Effect of N

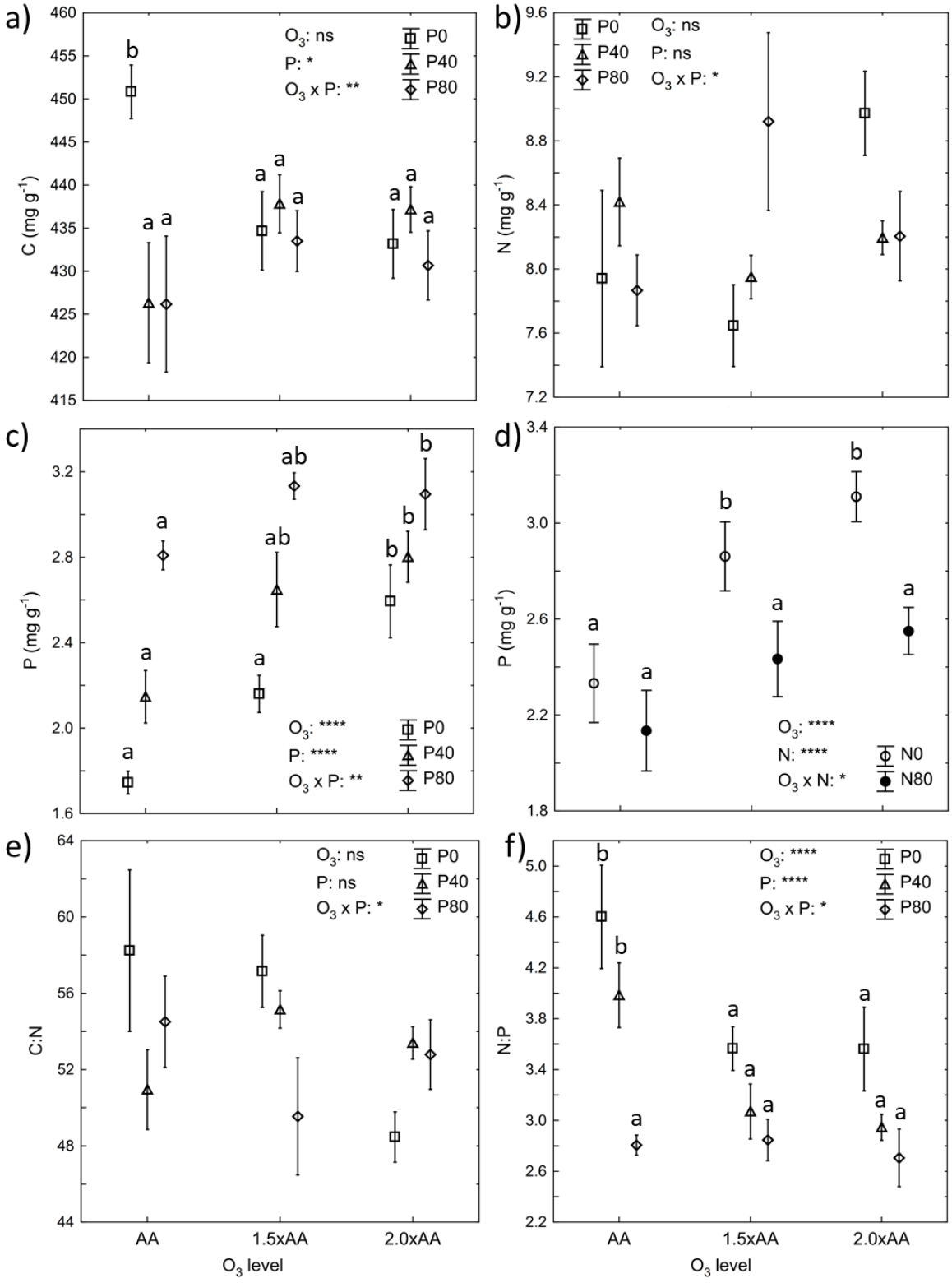
631 (N0 – no added N, N80 – 80 kg ha^{-1} N added) on fine root structure (n = 18). In b) and c) only two root
632 diameter classes are presented as in other classes no statistically significant effect of any studied
633 parameter was observed. Different letters in b) and c) designate statistically significant differences
634 (P<0.05).
635



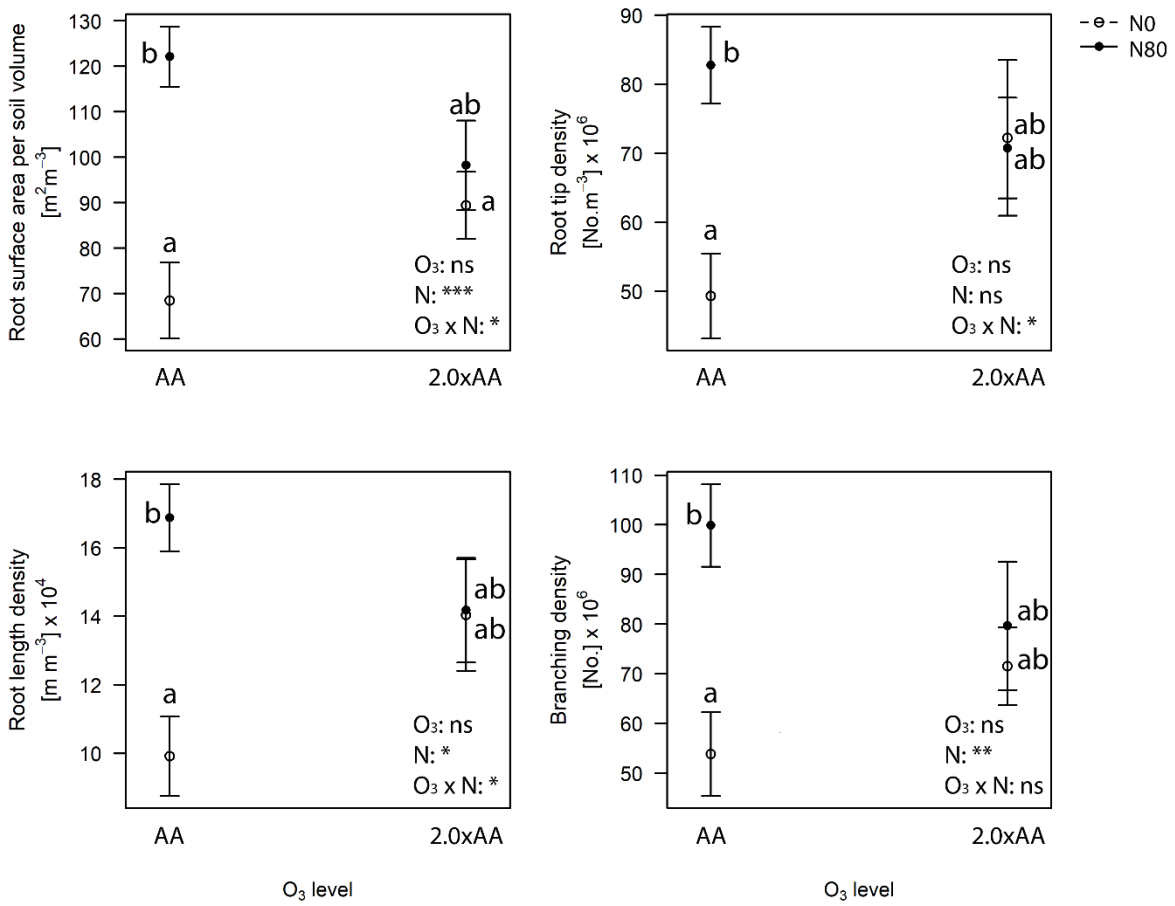
636

637 Fig. 1

638


a)
C (mg g-1)
O3: ns
P: *
O3 x P: **
P0
P40
P80
b)
N (mg g-1)
P0
P40
P80
O3: ns
P: ns
O3 x P: *
c)
P (mg g-1)
O3: ****
P: ****
O3 x P: **
P0
P40
P80
d)
P (mg g-1)
O3: ****
N: ****
O3 x N: *
N0
N80
e)
C:N
O3: ns
P: ns
O3 x P: *
P0
P40
P80
AA
1.5xAA
2.0xAA
O3 level
f)
N:P
O3: ****
P: ****
O3 x P: *
P0
P40
P80
AA
1.5xAA
2.0xAA
O3 level

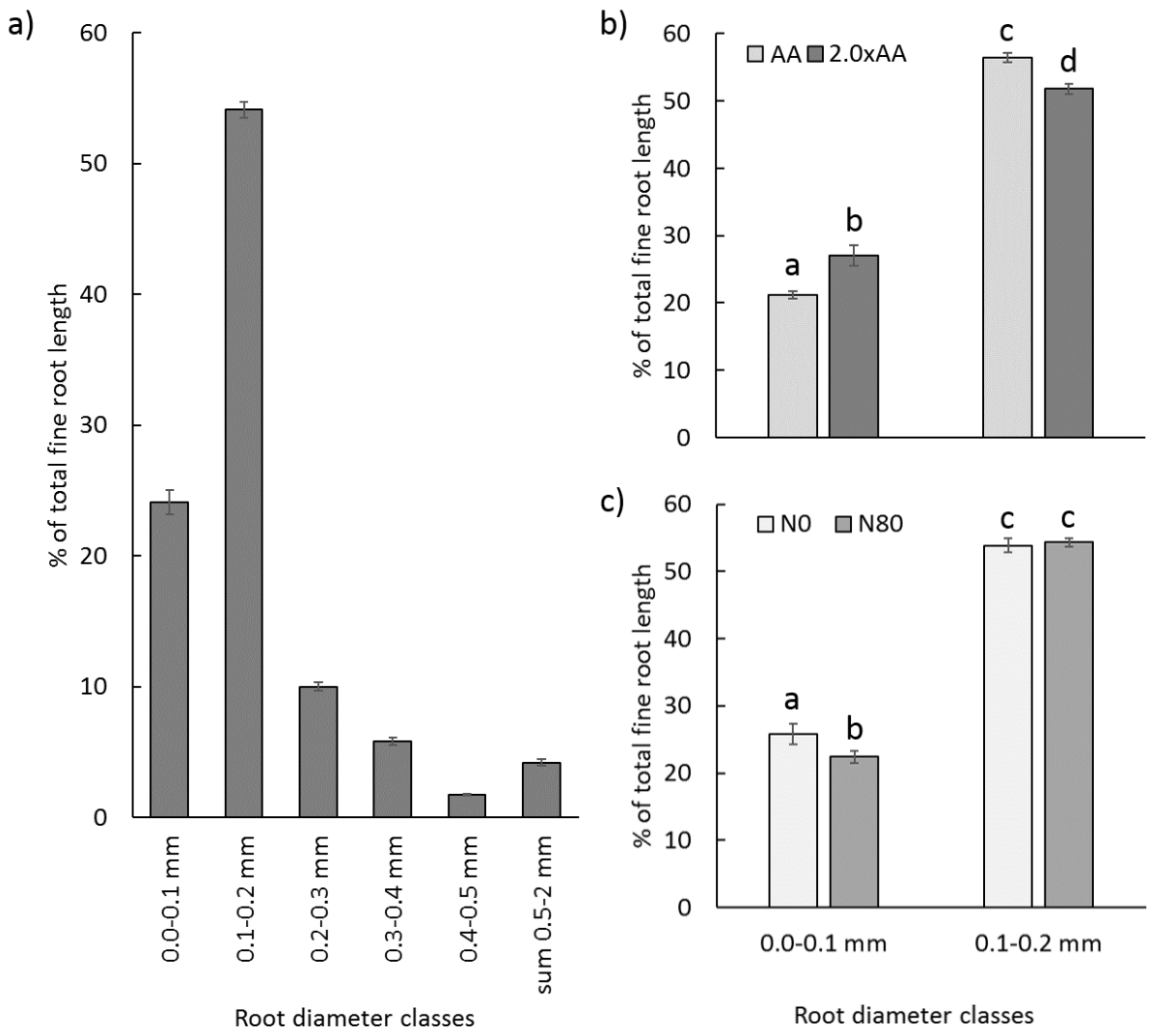
639
640 Fig. 2



641

642 Fig. 3

643


a)
% of total fine root length
0
10
20
30
40
50
60
0.0-0.1 mm
0.1-0.2 mm
0.2-0.3 mm
0.3-0.4 mm
0.4-0.5 mm
sum 0.5-2 mm
Root diameter classes
b)
AA
2.0xAA
a
b
c
d
c)
N0
N80
a
b
c
c
0.0-0.1 mm
0.1-0.2 mm
Root diameter classes

644

645 Fig. 4