



# 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<sub>3</sub>). This study aimed to investigate modifying effects of elevated O<sub>3</sub> on poplar root response to nutrient addition.

**Methods** In pot experiment, young trees of an O<sub>3</sub>-sensitive Oxford poplar clone (*Populus maximoviczii* Henry × *berolinensis* Dippel) growing in soil with three

levels of P (0, 40 and 80 kg ha<sup>-1</sup>) and two levels of N (0 and 80 kg ha<sup>-1</sup>) were exposed to three levels of O<sub>3</sub> (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<sub>3</sub> 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<sub>3</sub>. 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<sub>3</sub> prevented acquisition of available N, rendering N fertilization of young poplar plantations in such conditions economically and environmentally questionable.

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## 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 anthropogenic 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<sub>x</sub> emissions from fossil fuel and biomass combustion and NH<sub>3</sub> escape from agriculture (Rennenberg et al. 2010). Net primary productivity of N limited ecosystems may respond positively to N addition (LeBauer and 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 (Utraiainen 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<sub>3</sub>) is a secondary air pollutant that is formed by the oxidation of carbon monoxide and volatile organic compounds in the presence of N oxides and sunlight. Since 1980, emissions of O<sub>3</sub> precursors have shifted from middle and high latitudes of northern hemisphere towards equator, where the potential for O<sub>3</sub> formation is much greater due to sunlight and intense heat. Since O<sub>3</sub> is transported globally, rising O<sub>3</sub> precursor emissions at low latitudes have a potential to affect O<sub>3</sub> concentrations on a global scale (Zhang et al. 2016). Poplars as fast-growing trees are more sensitive to O<sub>3</sub> 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<sub>3</sub> response (e.g. Pell et al. 1995; Schmutz et al. 1995; Maurer and Matyssek 1997; Utraiainen and Holopainen 2001b; Watanabe et al. 2012; Yuan et al. 2017). For *Populus tremuloides* it was shown that O<sub>3</sub> 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 suboptimal N levels, absence of biomass reductions due to O<sub>3</sub> was explained by compensatory response of accelerated leaf abscission which is providing sufficient recycling of nutrients for slow-growing plants (Pell et al. 1995). Under elevated O<sub>3</sub>, 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<sub>3</sub> 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<sub>3</sub> were studied on Norway spruce as well, and they did not have much effect on susceptibility to O<sub>3</sub> in this species, which is known to have relatively low sensitivity to O<sub>3</sub> (Weigt et al. 2012). Zhang et al. (2018a) have investigated interactive effect of N, P and O<sub>3</sub> on Oxford poplar clone and reported greater reduction in total biomass due to O<sub>3</sub> by higher N levels. P mitigated O<sub>3</sub> 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<sub>3</sub> (Zhang et al. 2018a). For the same experiment it was reported that both N and P addition decrease reactive oxygen species in leaves at ambient and 1.5 x ambient O<sub>3</sub>, but not at 2.0 x ambient O<sub>3</sub> (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<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> (Marzuoli et al. 2009; Hoshika et al. 2018). We hypothesized that O<sub>3</sub> 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<sub>3</sub>-induced effects. Greater effects of O<sub>3</sub> 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<sub>3</sub>-sensitive poplar clone *Populus maximoviczii* Henry × *berolinensis* Dippel (Oxford poplar clone; sensitivity to O<sub>3</sub> 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 (Table 1). P was added as 0, 0.5 and 1.0 mM KH<sub>2</sub>PO<sub>4</sub> solution according to Lewis and Strain (1996) and N was added as 0 and 5 mM solution NH<sub>4</sub>NO<sub>3</sub> according to Thomas et al. (1994). 200 mL solution of NH<sub>4</sub>NO<sub>3</sub> or KH<sub>2</sub>PO<sub>4</sub> with different concentrations as described above were added to soil twice a week during the whole treatment period. At the same time, KCl was supplied into the soil that did not receive KH<sub>2</sub>PO<sub>4</sub> to keep an equal amount of K among all treatments (Tissue and Lewis 2010; Mao et al. 2014). Levels of added P and N were in agreement with native N and P concentrations in soils, for detailed explanation see Zhang et al. 2018a, b. The plants were irrigated to field capacity every 2–3 days to prevent water deficit. For soil pH and concentrations of N and P at the end of experiment see Zhang et al. (2018a).

Potted cuttings were exposed to three levels of O<sub>3</sub>, ambient (referred as AA - control), 1.5 x AA (medium) and 2.0 x AA (high) at O<sub>3</sub> free air controlled exposure (FACE) facility (Paoletti et al. 2017) in Sesto Fiorentino, Italy, from May 1st 2016 to October 1st 2016, when destructive harvesting was performed. Each of 18 treatment combinations (Table 1) was replicated in three plots of the FACE facility, each replicate with three poplar plants.

### Sampling of roots and investigation of fine root morphology

For root morphological analyses, only roots from AA and 2.0 x AA O<sub>3</sub> treatments were selected to get insight into root responses to both extremes as root analyses are very time-consuming. One pot per replicate treatment was randomly selected, resulting in three pots from AA and three pots from 2.0 x AA treatment for each nutrient combination. From each selected pot, a subsample of a soil core with 274 mL soil corer was taken. Exact volume of a soil core was measured volumetrically, by submerging the soil sample into measuring cylinder. Roots from the soil core were cleaned off the soil. Cleaned roots were divided into fine roots (<2 mm) and coarse roots (>2 mm).

Afterwards, roots were scanned on Epson Perfection V700 Photo scanner in trays covered by water. 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 the next section. 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

**Table 1** Treatment combinations and amounts of nutrients added to the pots with young poplar trees obtained from cuttings

O <sub>3</sub> level	P treatment label	Amount of P per ha (kg ha <sup>-1</sup> )	Amount of P per poplar cutting (mg seedling <sup>-1</sup> )	N treatment label	Amount of N per ha (kg ha <sup>-1</sup> )	Amount of N per poplar cutting (mg seedling <sup>-1</sup> )
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

O<sub>3</sub> levels: AA – ambient (control), 1.5 x AA and 2.0 x AA. Loadings of nutrients are expressed for the time period from May 1st 2016 to October 1st 2016

tissue paper and air-dried. The remaining roots (that were not 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 min 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, respectively. The organic carbon present in the samples of dry roots was oxidized into carbon dioxide (CO<sub>2</sub>), and nitrogen compounds to nitrogen oxides (NO<sub>x</sub>) 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<sub>2</sub>. NO<sub>x</sub> were reduced to elemental N. Then 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<sub>3</sub>, 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 or 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<sub>3</sub>, N and the O<sub>3</sub> x N interaction, but not by P either alone nor in interaction with other factors (Fig. 1, Table 2). Relative to ambient O<sub>3</sub> treatment (AA), coarse root biomass decreased by 26.7 and 31.4% in medium and high O<sub>3</sub> 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<sub>3</sub> was –35.3 and –21.5%

relative to control in medium and high O<sub>3</sub> treatments, respectively, while in treatments with N addition, biomass decrease was -22.0 and -36.8% in medium and high O<sub>3</sub> treatments, respectively. Coarse root biomass at the highest O<sub>3</sub> exposure was increased by +46.3% due to N addition (but no significant difference), while at medium and control O<sub>3</sub>, N addition increased coarse root biomass by +119% and +81.6%, respectively (Fig. 1a).

Fine root biomass decreased due to O<sub>3</sub> by -34.2% and -34.5% in medium and high O<sub>3</sub> treatments, respectively, while N stimulated fine root biomass by +72.1%. In treatment with no added N, fine root biomass decrease due to O<sub>3</sub> was -28.5 and -16.1% relative to control for medium and high O<sub>3</sub> treatments, respectively, while in treatments with N addition, biomass decrease was -37.1 and -43.7% for medium and high O<sub>3</sub> treatments, respectively. Fine root biomass at the highest O<sub>3</sub> exposure was increased by +34.6% due to N addition (but no significant difference), while at control O<sub>3</sub>, 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<sub>3</sub> 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<sub>3</sub> (Fig. 2a). Concentration of N in fine roots was not responsive to any of the main effects, and only weakly to O<sub>3</sub> x P interaction without any clear pattern (Fig. 2b).

Concentration of P in fine roots was affected by O<sub>3</sub>, N and P as single factors and by the interaction of O<sub>3</sub> with both nutrients (Fig. 2c, d). 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<sub>3</sub> 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<sub>3</sub> x P (Fig. 2c) and O<sub>3</sub> x N (Fig. 2d) were shown as larger increases in P concentration in fine roots under elevated O<sub>3</sub> at lower P and N loads. The effect of P fertilization on P concentration in fine roots was therefore higher at ambient O<sub>3</sub> level (+60.9%) compared to the high O<sub>3</sub>

level (+19.3%), but plants grown with elevated O<sub>3</sub> levels had higher basal P concentrations per se (Fig. 2c).

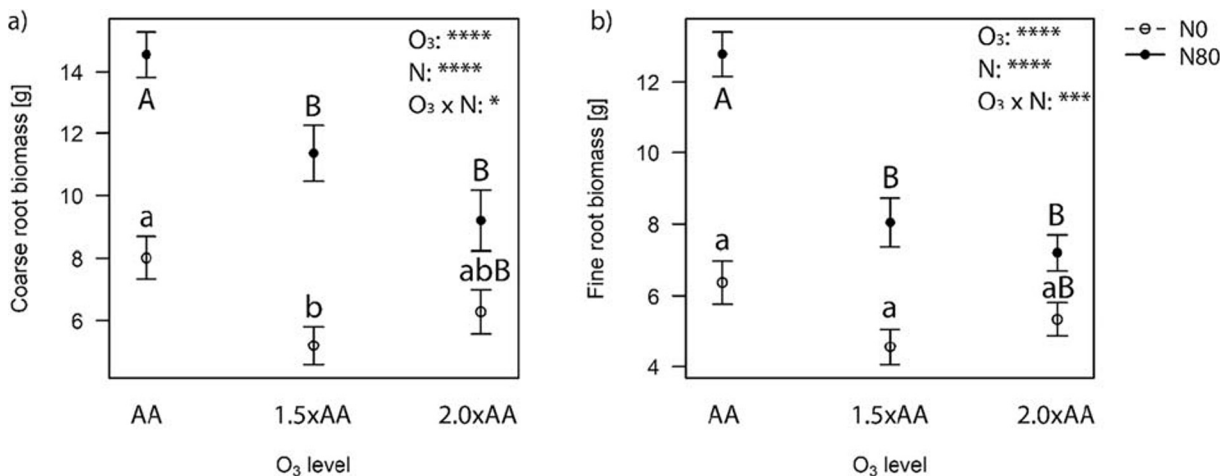
C:N ratio was weakly affected by O<sub>3</sub> x P interaction, but no clear response pattern was evident (Fig. 2e). Mean overall N:P ratio in fine roots was 3.34. It was strongly affected by main factors O<sub>3</sub>, N and P. In addition, weak interaction effects of O<sub>3</sub> x P (Fig. 2f) and N x P were observed. Main effect of O<sub>3</sub> was observed as decreased N:P ratio under elevated O<sub>3</sub> levels. As expected, higher N and lower P loads as single factors resulted in higher N:P ratio. Interaction effect between O<sub>3</sub> and P on N:P ratio was observed as significantly higher N:P ratio at ambient O<sub>3</sub> relative to both elevated O<sub>3</sub> levels at P0 and P40, while at P80, the N:P ratio was similar for all O<sub>3</sub> concentrations (Fig. 2f).

#### Fine root morphology

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

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

The highest proportion of fine root system in O<sub>3</sub>-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<sub>3</sub> had a significant positive effect on proportion of roots in 0.0–0.1 mm diameter class (+5.8%). On the other hand, O<sub>3</sub> significantly decreased the percentage of roots in 0.1–0.2 mm diameter class (-4.66%), Fig. 4b. Addition



**Fig. 1** Interactive effects of nitrogen (N) addition and ozone (O<sub>3</sub>) exposure on coarse (a) and fine root (b) biomass (mean values ± SE,  $n = 9$ ) in an O<sub>3</sub>-sensitive poplar clone subjected to three different levels of O<sub>3</sub> (AA - ambient, 1.5 x AA and 2.0 x AA), two levels of N (N0 – no added N, N80 – 80 kg N ha<sup>-1</sup> per exposure period) and three levels of P. As P effects and its

interactions with O<sub>3</sub> and N were not statistically significant, they are not shown here. ANOVA *P* values for O<sub>3</sub>, N and their interaction are indicated: ns, not significant, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001. Different uppercase and lowercase letters indicate statistically significant differences at *P* < 0.05 for separate comparison of each N treatment and each O<sub>3</sub> level

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).

any change in fine root morphology due to O<sub>3</sub> may have a profound effect on nutrient use efficiency and biomass gain of the whole tree.

## Discussion

In this study we have shown for the first time that effects of elevated atmospheric O<sub>3</sub> 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,

## Fine and coarse root biomass

Negative effects of O<sub>3</sub> 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)

**Table 2** Results of three-way ANOVA for coarse and fine root biomass of an ozone-sensitive poplar clone subjected to three levels of ozone (O<sub>3</sub>), two levels of nitrogen (N) and three levels of phosphorus (P)

	Degr. of freedom	Coarse roots		Fine roots	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
O <sub>3</sub>	2	<b>12.6</b>	<b>&lt;0.0001</b>	<b>24.8</b>	<b>&lt;0.0001</b>
N	1	<b>70.4</b>	<b>&lt;0.0001</b>	<b>78.7</b>	<b>&lt;0.0001</b>
P	2	0.11	0.8938	0.07	0.9310
O <sub>3</sub> x N	2	<b>3.46</b>	<b>0.0423</b>	<b>9.12</b>	<b>0.0006</b>
O <sub>3</sub> x P	4	0.77	0.5511	2.42	0.0664
N x P	2	1.76	0.1873	0.89	0.4210
O <sub>3</sub> x N x P	4	1.77	0.1564	1.33	0.2772

Values with *P* < 0.05 are presented in bold

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<sub>3</sub> 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<sub>3</sub> level biomass was stimulated by N addition by +81.6% and +100.5% for coarse and fine roots, respectively, while at high O<sub>3</sub> exposure, the N stimulation was only +46.3 and +34.6% for coarse and fine roots, respectively. At elevated O<sub>3</sub>, 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<sub>3</sub> concentration (Zhang et al. 2018a), transfer of N to photosynthetic apparatus might be impaired under elevated O<sub>3</sub> (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<sub>3</sub>, N can be complexed with phenolics (Andersen 2003). Zhang et al. (2018b) reported that N aggravate O<sub>3</sub> induced respiratory carbon loss. High N concentrations under O<sub>3</sub> 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<sub>3</sub> 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).

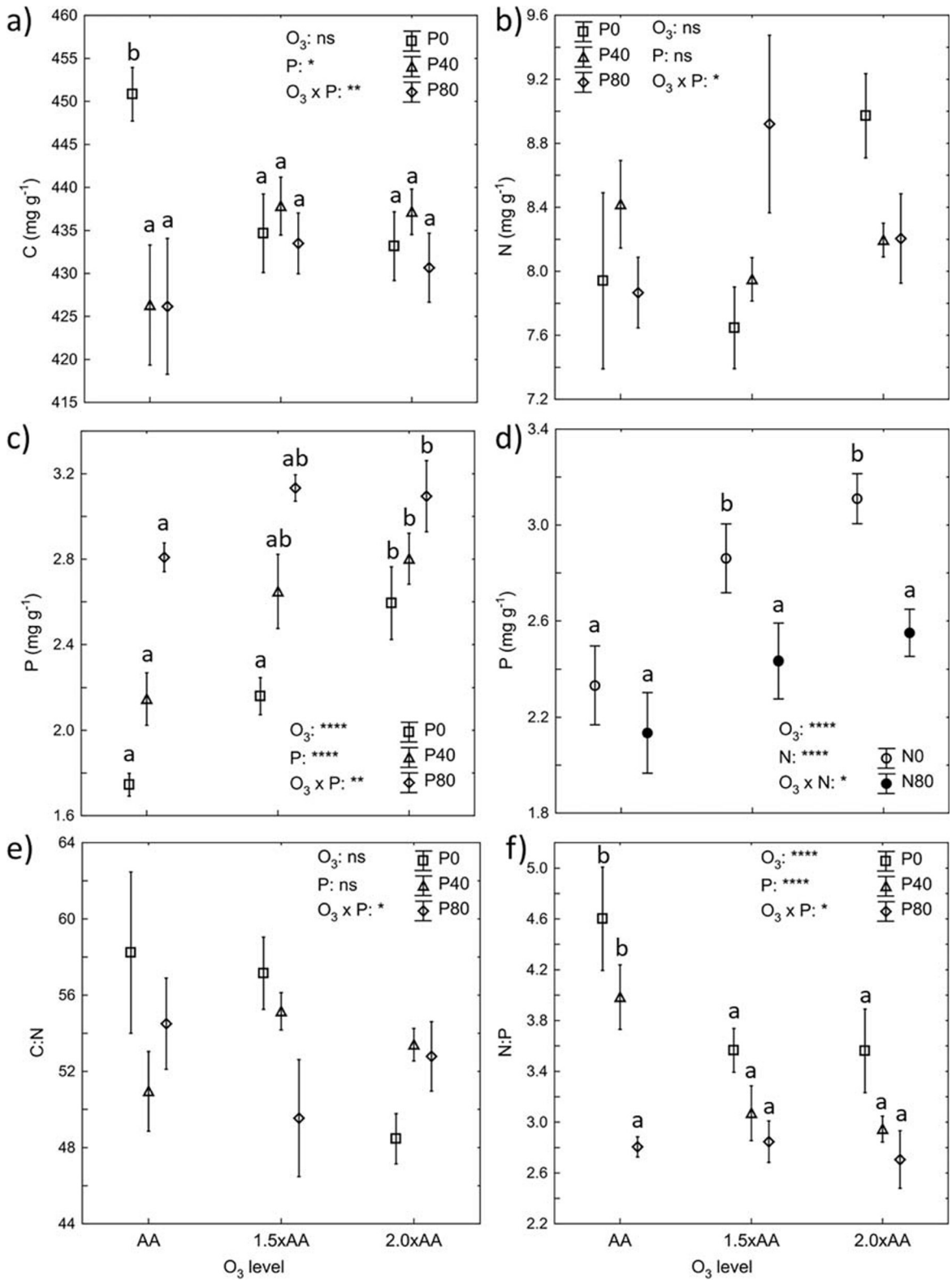
#### Carbon, nitrogen and phosphorus concentrations in fine roots

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<sup>-1</sup> for C and 8.24 vs. 11.1 mg g<sup>-1</sup> for N), while average P concentrations

were higher (2.57 vs. 0.92 mg g<sup>-1</sup>) (Gordon and Jackson 2000).

Carbon concentrations in fine roots were significantly higher in treatment with no P addition under ambient O<sub>3</sub> but decreased under elevated O<sub>3</sub> and different nutrient combinations. Under low P conditions, as it was the case in treatment with no P addition, trees allocate more resources to fine roots (McCormack and Guo 2014), while decreased C allocation to belowground is well-known response to O<sub>3</sub> stress occurring due to retention of carbohydrates in leaves and premature loss of foliage in the fall (Andersen 2003). For example, decreased C concentration in fine roots under elevated O<sub>3</sub> was reported for sun grown beech seedlings (Železnik et al. 2007). Interestingly, increased C concentration in fine roots was associated with P and not with N, although P was not limiting for growth of our poplar plants. Possible explanation could be that plants need to invest more resources in acquisition of P from natural soils (such as used in treatment with no added P), as P in natural soils is adsorbed, precipitated, or present in organic form and as such not easily available for uptake (Schachtman et al. 1998). Carbon is also needed to support ectomycorrhizal fungi in exchange for P (Smith and Read 2008). Indeed, the colonization rate with ectomycorrhizal fungi was significantly higher in treatments with P0 compared to P80 (unpublished data). As only around 10% of root tips were colonized and mycorrhizal communities were not affected (unpublished data), we assume that ectomycorrhizal fungi are not the reason for increased C concentration in fine roots.

Concentrations of N in fine roots in our study were neither affected by N or O<sub>3</sub>, while in leaves of the same plants they were affected just by N (Zhang et al. 2018a). This indicates that ability of fine roots for acquisition of N was unaltered despite O<sub>3</sub> stress. Similarly, Weigt et al. (2012) reported that labelled N and total N concentrations in beech and spruce roots were not significantly affected by O<sub>3</sub> treatment. Absence of O<sub>3</sub> effect on root N concentration in poplars was reported also by Schmutz et al. (1995) and Zak et al. (2007), while Haberer et al. (2007) observed decrease in N concentration of beech fine roots under elevated O<sub>3</sub>. Studies of Schmutz et al. (1995) and Zak et al. (2007) emphasized that total N content (i.e. total N acquired from soil) was lower due to negative effects of O<sub>3</sub> on biomass. As negative effects of O<sub>3</sub> on biomass were also observed in our study, total acquired N under elevated O<sub>3</sub> was also lower in our case. In our experiment, N was

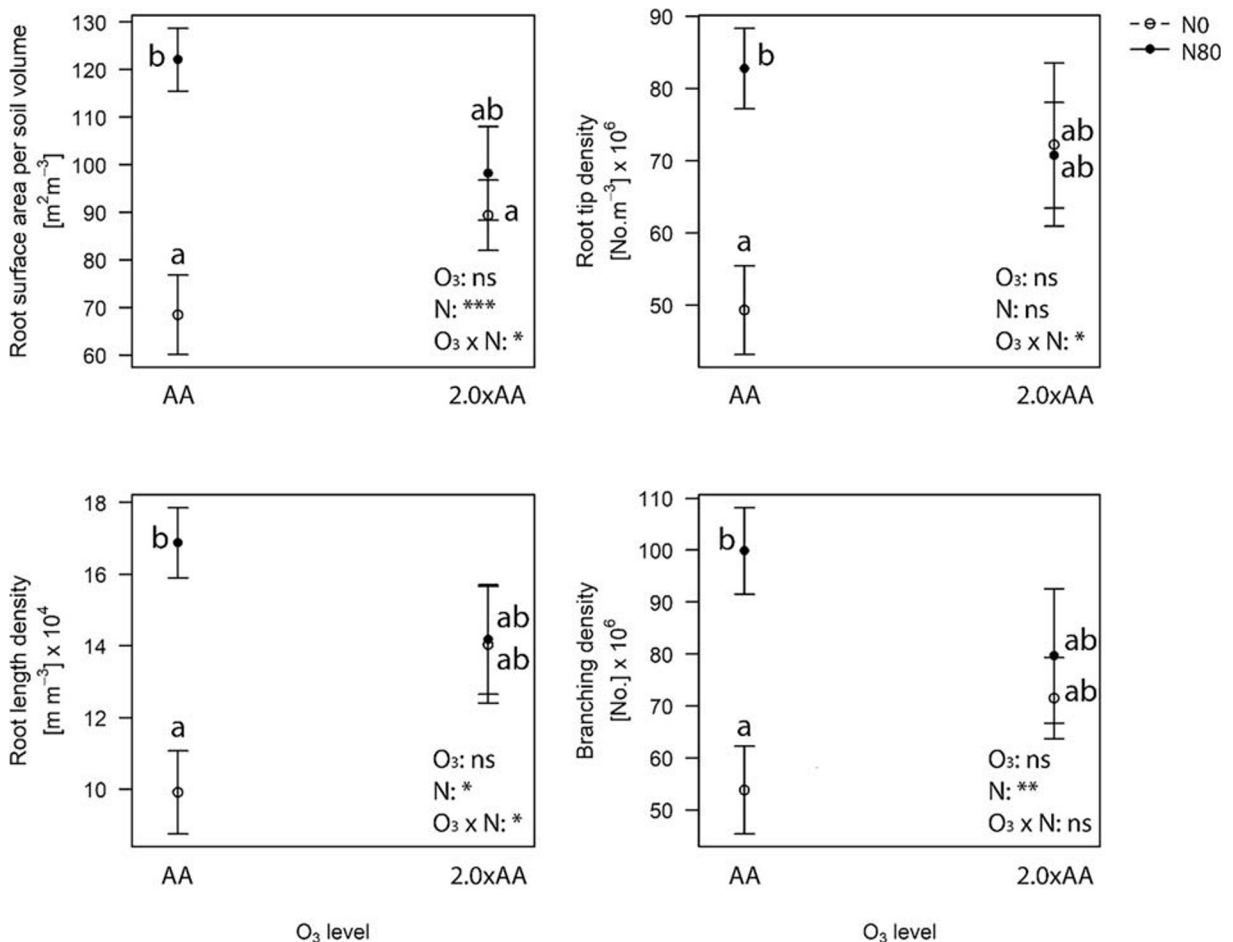




**Fig. 2** Mean values  $\pm$  SE ( $n = 3$ ) for C, N and P concentrations (panels a–d) and C:N and N:P ratios (panels e–f) in fine roots of an  $O_3$ -sensitive poplar clone subjected to three different levels of  $O_3$  (AA - ambient, 1.5 x AA, 2.0 x AA), two levels of N (0 and 80 kg ha<sup>-1</sup>)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

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 photosynthesizing 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



**Fig. 3** Interactive effects of N and  $O_3$  on selected fine root morphological parameters (mean values  $\pm$  SE,  $n = 9$ ) of  $O_3$ -sensitive poplar clone subjected to two different levels of  $O_3$  (AA - ambient, 2.0 x AA), two levels of N (N0 and N80) and three levels of P. ANOVA  $P$  values for  $O_3$ , N and their interaction are

indicated: ns, not significant, \* $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ . Effects of P and its interactions with  $O_3$  and N are not shown as they were not statistically significant. Different letters indicate statistically significant differences ( $P < 0.05$ )

**Table 3** Results of three-way ANOVA for fine root morphological parameters of O<sub>3</sub>-sensitive poplar clone

Df	Mean diameter		Tips per length		Branches per length		SRL		Specific root tip density		Root surface area per soil volume		Root tissue density		SRA		Root tip density		Branching density		Root length density		
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	
O <sub>3</sub>	1	2.08	0.1622	0.26	0.6176	0.85	0.3672	3.12	0.0903	2.85	0.1044	0.03	0.8565	2.09	0.1610	3.10	0.0909	0.44	0.5135	0.02	0.8956	0.29	0.5977
N	1	2.94	0.0993	0.02	0.8927	1.84	0.1877	0.50	0.4867	0.67	0.4213	<b>14.6</b>	<b>0.0008</b>	0.34	0.5646	0.04	0.8403	3.80	0.0629	<b>7.87</b>	<b>0.0098</b>	<b>7.20</b>	<b>0.0130</b>
P	2	0.06	0.9397	0.18	0.8392	0.90	0.4210	0.33	0.7251	0.50	0.6155	0.91	0.4142	2.25	0.1267	0.54	0.5904	0.59	0.5632	1.40	0.2656	0.88	0.4280
O <sub>3</sub> × N	1	0.01	0.9368	0.09	0.7650	0.16	0.6887	0.02	0.8955	<0.01	0.9622	<b>7.53</b>	<b>0.0113</b>	0.20	0.6568	0.15	0.7010	<b>4.53</b>	<b>0.0438</b>	3.84	0.0616	<b>6.63</b>	<b>0.0166</b>
O <sub>3</sub> × P	2	0.99	0.3879	0.27	0.7625	1.02	0.3751	0.31	0.7360	0.07	0.9310	0.11	0.8955	0.74	0.4857	0.46	0.6353	0.28	0.7601	0.05	0.9561	0.34	0.7129
N × P	2	0.78	0.4691	0.99	0.3868	0.06	0.9451	1.23	0.3110	1.29	0.2945	1.87	0.1762	0.50	0.6116	1.22	0.3147	1.86	0.1773	1.43	0.2586	2.28	0.1243
O <sub>3</sub> × N × P	2	0.65	0.5293	2.43	0.1091	1.22	0.3125	0.25	0.7804	0.16	0.8567	0.99	0.3880	0.28	0.7602	0.11	0.8966	0.15	0.8622	0.91	0.4176	1.04	0.3699

Factors ozone (O<sub>3</sub>), nitrogen (N), phosphorus (P) and their interactions were tested. Statistically significant effects at  $P < 0.05$  are marked in bold  
SRL specific root length, SRA specific root area

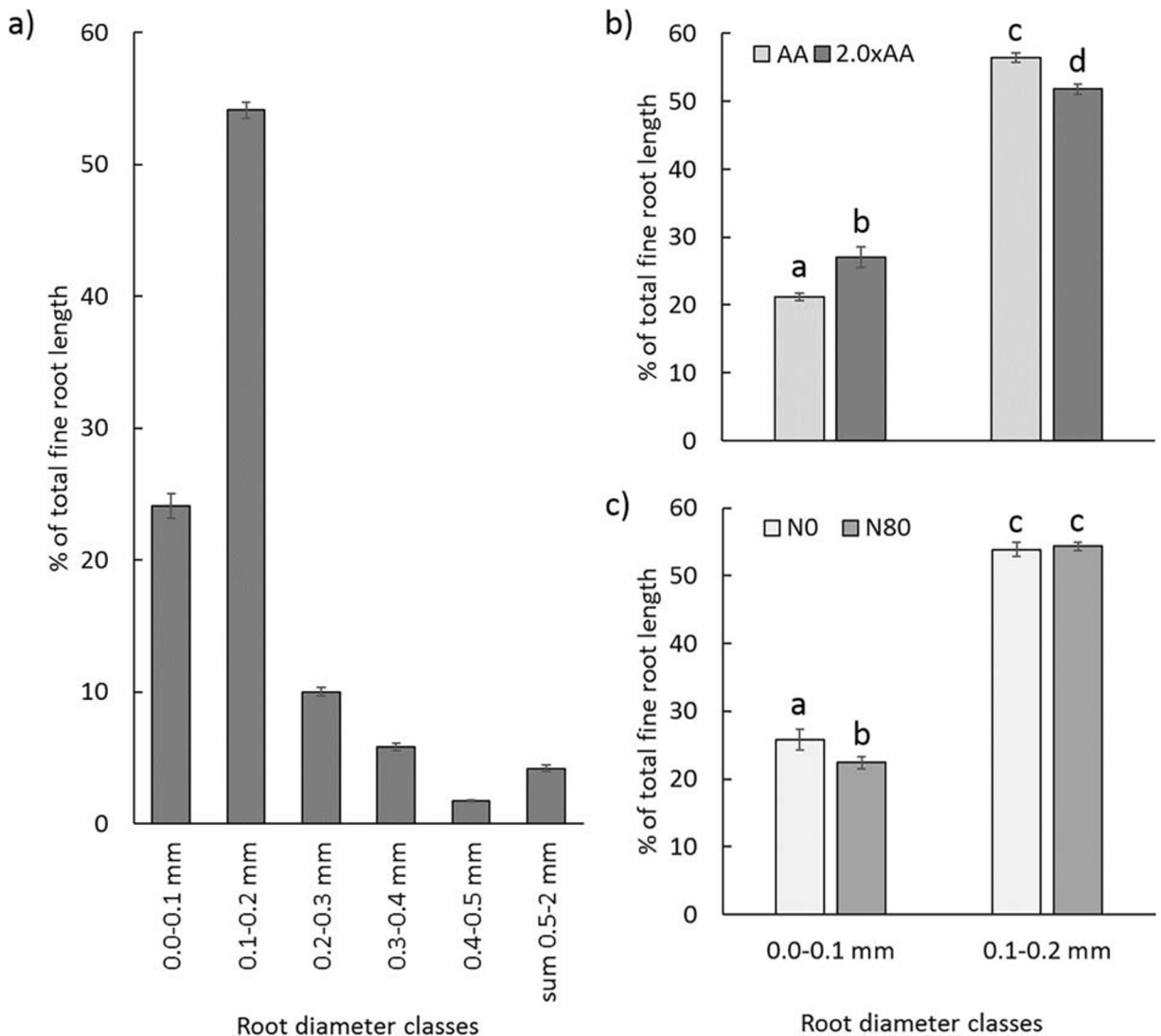
decrease due to decrease in photosynthesis (Watanabe et al. 2013; Zhang et al. 2018b). Ozone caused a reduction of stomatal conductance in this poplar clone (Zhang et al. 2018b). The increased P concentration in roots might be related to the less efficient delivery of P by transpirational water stream (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<sub>3</sub> 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<sub>3</sub> (Shi et al. 2016). Interestingly, P addition at ambient O<sub>3</sub> resulted in higher increase of P concentration in fine roots compared to elevated O<sub>3</sub> levels, but under elevated O<sub>3</sub>, 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<sub>3</sub> on C:N ratio was detected, no clear pattern was recognisable. Kasurinen et al. (2005) reported no effect of O<sub>3</sub> on C:N ratio in birch roots.

In fine roots of our plants, mean N:P ratio was 3.34:1, while average N:P ratios for terrestrial plants in natural conditions are 12–13 (Güsewell 2004). According to Gordon and Jackson (2000), N:P ratio in fine roots is comparable to N:P ratios of leaves and shoots. Low N:P ratios indicate N limitation (Güsewell 2004). As expected, N:P ratio in our study increased due to addition of N and decreased due to addition of P, but despite this N limitation occurred all nutrient treatments. O<sub>3</sub> significantly affected N:P ratio. In both, medium and high O<sub>3</sub>, N:P ratio was significantly lower compared to control. O<sub>3</sub> modulated the response of N:P ratio to P status. At P0 and P40, medium and high O<sub>3</sub> exposure resulted in higher N limitation in roots than at ambient O<sub>3</sub> levels.

### Fine root morphology

Uptake of water and nutrients in plants is dictated more by root morphological properties such as root length density and root surface area, than by root



**Fig. 4** Fine root system in O<sub>3</sub>-sensitive poplar clone (mean value ± SE): **a** Overall structure (n = 36). **b** Effect of O<sub>3</sub> (AA - ambient O<sub>3</sub> level, 2.0 x AA O<sub>3</sub> level) on fine root structure (n = 18), **c** Effect of N (N0 – no added N, N80 – 80 kg ha<sup>-1</sup> N added) on fine

root structure (n = 18). In **b** and **c** only two root diameter classes are presented as in other classes no statistically significant effect of any studied parameter was observed. Different letters in **b** and **c** designate statistically significant differences (P < 0.05)

mass (Tachibana and Ohta 1983; Eissenstat 1992). Nitrogen addition in our experiment significantly increased root surface area, root length density, root tip density and branching density, thereby improving capacity for N absorption. Our results are supported by the finding of King et al. (1997) who reported that N addition increased root surface area for +21% in two pine species. Response to N in our experiment was strongly modulated by O<sub>3</sub>. Under high O<sub>3</sub> levels, N addition resulted only in slight increase or even decrease in above stated root morphological

parameters. Therefore, supplementary N under high O<sub>3</sub> level cannot be fully exploited as a resource for growth, as it was supported by biomass measurements. Slight stimulatory effect of high O<sub>3</sub> at N0 on these parameters could be explained as investment into roots to provide additional nutrients for repair of O<sub>3</sub>-induced damage, while at increased N this mechanism fails (Pell et al. 1995).

In addition, O<sub>3</sub> had a significant stimulatory effect on percentage of fine roots in 0.0–0.1 mm diameter class, but on the other hand decreased the

**Table 4** Results of three-way ANOVA for % of total fine root length in root diameter classes for O<sub>3</sub>-sensitive poplar clone

	Df	% 0.0 < .L. <=0.1 mm		0.1 < .L. <=0.2 mm		% 0.2 < .L. <=0.3 mm		% 0.3 < .L. <=0.4 mm		% 0.4 < .L. <=0.5 mm		% sum 0.5–2 mm	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
O <sub>3</sub>	1	<b>12.8</b>	<b>0.0015</b>	<b>23.5</b>	<b>&lt;0.0001</b>	2.14	0.1561	0.26	0.6142	0.02	0.8972	<0.01	0.9712
N	1	<b>4.36</b>	<b>0.0477</b>	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 <sub>3</sub> 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 <sub>3</sub> 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 <sub>3</sub> 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

Factors O<sub>3</sub>, N, P and their interactions were tested. Statistically significant effects at  $P < 0.05$  are marked in bold

percentage of fine roots in 0.1–0.2 mm diameter 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<sub>3</sub>-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<sub>3</sub> is consistent with the finding that the percentage of 0.0–0.1 mm roots increased with elevated O<sub>3</sub>. 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<sub>3</sub>. 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<sub>3</sub>.

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<sub>3</sub> exposure level, N fertilization did not significantly increase coarse and fine root biomass compared to treatment with no N fertilization. At elevated O<sub>3</sub>, 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<sub>3</sub> increased N starvation in young poplar plants. Although concentration of P in roots was increased under elevated O<sub>3</sub>, this was not associated with biomass response. We could not unequivocally find out the reason for increased P in roots under elevated O<sub>3</sub>, therefore further studies would be needed. From the viewpoint of root growth, it could be suggested that in areas with high O<sub>3</sub> 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 contribute to the knowledge on growth and nutrient use efficiency of aboveground parts under O<sub>3</sub> stress.

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#### Compliance with ethical standards

**Conflict of interest** None.

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