1 **Different belowground responses to elevated ozone and soil water deficit in**
2 **three European oak species (*Quercus ilex*, *Q. pubescens* and *Q. robur*)**

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Highlights

- combined effects of ozone and water deficit on roots and ectomycorrhizae not

known

- greater effects of ozone were observed in well-watered oak plants

- effects were complex, species-specific and root-trait specific

- belowground responses may change water, nutrient and carbon cycling in plants

Graphical abstract
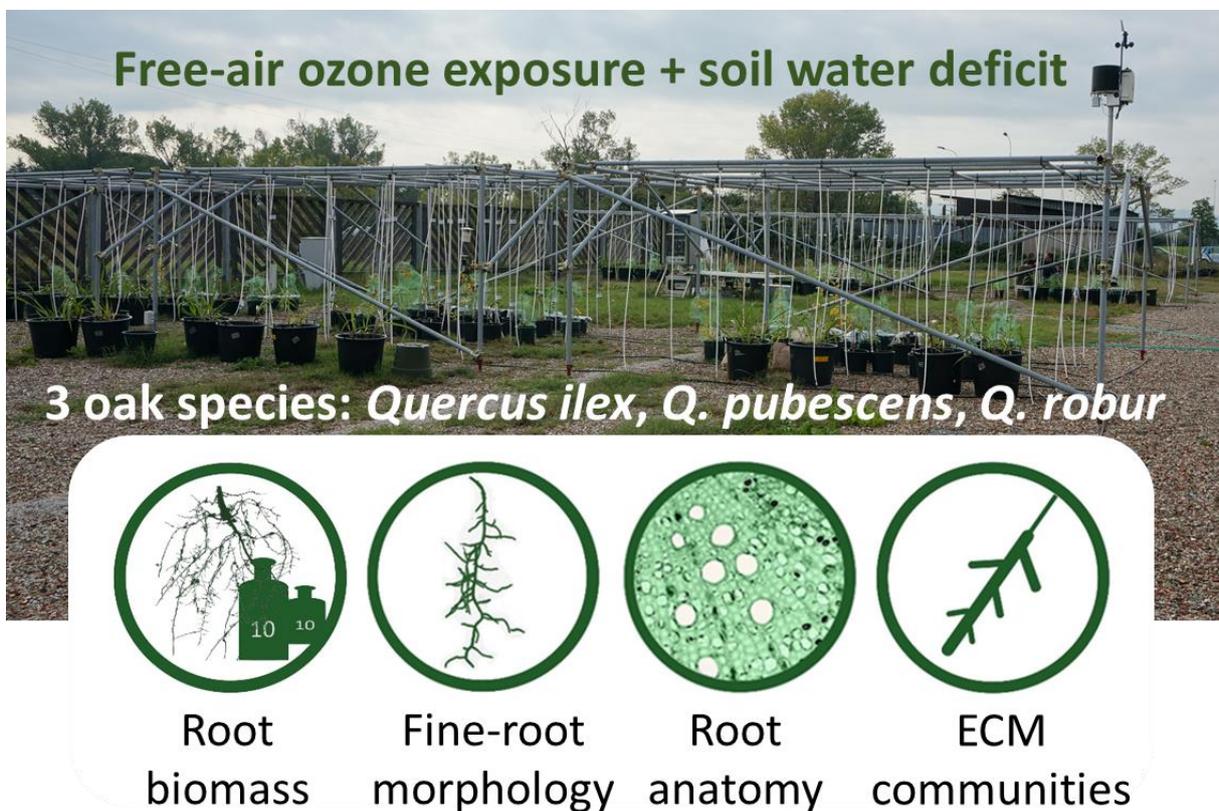
Abstract

Effects on roots due to ozone and/or soil water deficit often occur through diminished

belowground allocation of carbon. Responses of root biomass, morphology, anatomy and

ectomycorrhizal communities were investigated in seedlings of three oak species with

different water use strategies: *Quercus ilex* L., *Q. pubescens* Willd. and *Q. robur* L., exposed to combined effects of elevated ozone (ambient air and 1.4 x ambient air) and water deficit (100% and 10% irrigation relative to field capacity) for one growing season at a free-air ozone exposure facility. Effects on root biomass were observed only due to water deficit. A general reduction in coarse root biomass by -26.8% and in fine root biomass by -13.1% was observed. Effect on coarse root biomass was the most prominent in *Q. robur* (-36.3%). Root morphological changes manifested as changes in proportions of fine root (< 2 mm) diameter classes due to ozone and water deficit in *Q. pubescens* and due to water deficit in *Q. robur.* In addition, a reduction in mean fine root diameter (-8.49%) in *Q. robur* was observed due to water deficit. Changes in root anatomy were observed as increased vessel density (+18.5%) due to ozone in all three species, as reduced vessel tangential diameter (-46.7 %) in *Q. ilex* due to interaction of ozone and water, and as generally increased bark to secondary xylem ratio (+47.0%) due to interaction of ozone and water as well. Water deficit influenced occurrence of distinct growth ring boundaries in roots of *Q. ilex* and *Q. robur.* Of 14 ectomycorrhizal taxa altogether, 12 occurred in fully watered conditions and only 7 under water deficit. Twelve taxa were recorded at ambient air and 9 at elevated ozone. Water deficit shifted the ectomycorrhizal community towards dominance of stress-resistant species. It reduced relative abundance of *Tomentella* sp. 2 and increased relative abundance of *Sphaerosporella brunnea*. *Thelephora* sp. was present only under water deficit. Response in root traits in investigated species occurred both due to elevated ozone and water deficit, with stronger effects of water deficit. As expression of stress effects varies between root traits, the combined analysis of root traits is necessary to obtain a complete picture of belowground response.

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1. Introduction

Tropospheric ozone (O_3) is a phytotoxic air pollutant that is produced by photochemical oxidation of carbon monoxide and hydrocarbons in the presence of nitric oxides. Ozone is transported with air masses from polluted areas with high concentrations of its precursors, consequently affecting air quality on regional, intercontinental and hemispheric scales (Monks et al. 2015). Globally, tropospheric ozone concentrations have increased by 30% since the pre-industrial era (Young et al. 2013), while the predictions of future trends deal with several challenges and uncertainties (Monks et al. 2015, Ridley et al. 2017). In some parts of the world, emissions of ozone precursors are still rising, thereby affecting ozone concentrations on a global scale (Verstraeten et al. 2015). Ozone affects plant metabolism through the generation of free radicals and has the potential to modulate plant response to water deficit through interference with stomatal control mechanisms (Hayes et al. 2012, Hoshika et al. 2015, Monks et al. 2015). Plants often face a water deficit, even outside arid or semi-arid areas (Chaves et al. 2002). Global warming has already increased drying over many land areas in the last 30 years and is expected to further increase soil moisture deficit in space and time over the 21st century (Dai 2011).

Different studies on the interactive effects of ozone exposure and water deficit in trees have resulted in contradictory findings. Either a protective role of water deficit against ozone stress - by means of decreasing ozone uptake due to stomatal closure (Temple et al. 1992, Paoletti and Grulke 2005, Hoshika et al. 2015, Gao et al. 2017, Hoshika et al. 2018) - or no interactive effect of ozone and water deficiency (Le Thiec et al. 1994, Karlsson et al. 2002, Matyssek et al. 2006, Alonso et al. 2014) have been suggested. Although the primary site of ozone effects are leaves, responses in roots occur through diminished allocation of carbohydrates belowground

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77   (Matyssek et al. 2010). In some cases, root responses develop even before shoot effects are

78   observed (Andersen 2003). Ozone has an effect on cytokinin levels in leaves depending on the

79   age of the tree, resulting in contrasting responses of roots and associated mycorrhizal fungi in

80   adult trees and seedlings (Kraigher et al. 2008). Although water deficit has a similar negative

81   effect on belowground allocation of carbon (Hagedorn et al. 2016) as ozone, studies of the

82   interactive effects of ozone and water deficit on belowground parts of trees are limited to

83   investigations of total root biomass as a part of total biomass measurements or carbon

84   allocation patterns (reviewed by Agathokleous et al. 2015).

85   The role of roots with associated mycorrhizal symbionts in water and nutrient acquisition from

86   soil and transport to aboveground parts is crucial for tree survival and growth. Moreover, they

87   play a key role in ecosystem processes (Bardgett et al. 2014, Ellison et al. 2017, van der Linde

88   et al. 2018). Given the enormous plasticity of roots in relation to their environment (Bardgett

89   et al. 2014), their responses to a combination of ozone and water deficit could be substantial.

90   In aboveground parts of three oak species (*Quercus ilex, Q. pubescens* and *Q. cerris*), biometric

91   plasticity accounted for 40-50% of phenotypic plasticity in response to ozone and water deficit

92   (Cotrozzi et al. 2016).

93   Mediterranean Europe is a hotspot of high tropospheric ozone in summer due to intense solar

94   radiation, high temperatures and very dry conditions (Millán et al. 1997). For this area, further

95   decrease in soil moisture in summer is expected to occur in 2021-2050 due to global changes

96   (Kovats et al. 2014). The main objective of our study was to investigate the effects of elevated

97   ozone and water deficit on tree root biomass, morphology, anatomy and ectomycorrhizal

98   communities. Seedlings of three common European oak species with different levels of

99   adaptation to water deficit were used. Holm oak (*Quercus ilex* L.) is an evergreen

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100 sclerophyllous drought-tolerant Mediterranean species, distributed in the central-western

101 part of the Mediterranean basin (de Rigo and Caudullo 2016); pubescent oak (*Q. pubescens*

102 Willd.) is a deciduous or semi-deciduous tree of south-eastern Europe with the ability to

103 withstand moderate summer drought (Pasta et al. 2016), while pedunculate oak (*Q. robur* L.)

104 is a deciduous species widespread in Europe with preference for moist soils (Eaton et al. 2016).

105 These species are all ectomycorrhizal. As a typical representative of sclerophyllous vegetation

106 with low gas exchange rates and high constitutive levels of antioxidants (Paoletti 2006, Alonso

107 et al. 2014), *Q. ilex* was hypothesized to suffer less ozone damage to belowground parts in full

108 water supply conditions compared to *Q. pubescens* and *Q. robur*. In water deficient conditions,

109 *Q. ilex* was expected to suffer from combined effects of water deficit and ozone due to its

110 keeping stomata open (i.e. anisohydric behaviour), which leads to ozone uptake into plants

111 even under water deficit, whereas in *Q. robur* only effects of water deficit were postulated

112 due to its isohydric strategy, which results in the limitation of ozone uptake due to stomatal

113 closure under water deficit. Finally, *Q. pubescens* was expected to show an intermediate

114 behaviour.

115 In detail, we hypothesized that ozone and water deficiency as single factors would: (a)

116 decrease root biomass and change root morphological properties, b) induce root anatomical

117 changes, such as vessel size, vessel density and tissue ratios and c) reduce mycorrhization and

118 shift ectomycorrhizal communities towards stress-resistant types. For the combined factors,

119 we hypothesized that: d) greater negative effects of ozone on roots and ectomycorrhizal

120 communities occur for well-watered plants, and e) species-specific effects occur due to the

121 different strategies of these plant species to cope with water deficit.

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2. Materials and methods

2.1 Origin of seedlings

Two-year-old *Q. ilex* seedlings of provenance LRBS 45 (Pineta di Classe, Ravenna, Italy) were

obtained from the tree nursery Vivaio di Pieve S. Stefano (Arezzo, Italy). Seedlings were raised

from seeds in pots (10 x 10 x 30 cm), using a substrate mixture composed of 1/3 local soil

(calcareous geological bedrock), 1/3 white Lithuanian peat and 1/3 calcareous local river sand.

To this mixture, pumice from Viterbo (Italy) was added. Two-year-old seedlings of *Q.*

pubescens of provenance SR. n. 024-EmR Monte Fuso (Parma, Italy) and *Q. robur* of

provenance LRBS n.301 Sile Park (Treviso, Italy) were obtained from a tree nursery in

Collecchio (Parma, Italy). Seedlings were raised from seeds in pots (10 x 10 x 30 cm) using a

VigorPlant (Vigorplant Italia srl, Fombio, Italy) substrate that contains peat, pumice,

compound mineral fertilizer (NPK) and limestone. Seedlings were chemically treated against

Curculionidae beetles. Substrates were not sterilized, and seedlings were not fertilized. Weed

removal was performed by hand.

2.2 Experimental treatments

In spring 2015, the seedlings were transported to Sesto Fiorentino near Florence in central

Italy (43° 48' 59" N, 11° 12' 01" E, 55 m a.s.l.). Five seedlings per species were destructively

harvested and their root systems checked for ectomycorrhizas according to the procedure

described in section 2.4. List of the ectomycorrhizal taxa initially present is provided in

supplementary material (SM, S1.1, Tab. S1). Further, 36 seedlings per species were

transplanted into 10 L pots using a substrate composed of peat (60%), pumice (25%) and

nursery soil (15%). From June 1 until October 15 2015, they were exposed to two levels of

146   ozone: ambient air concentration (AA), and 1.4 x ambient concentration (1.4 x AA) at the free-

147   air ozone exposure facility called FO₃X (Free air O₃ eXposure) in Sesto Fiorentino in hot-

148   summer Mediterranean climate. A detailed description of the facility, including performance

149   testing, is provided in Paoletti et al. (2017). Daily ozone averages, daytime (9:00-16:00) means,

150   maximal values of hourly means and cumulated ozone exposure above the threshold value of

151   40 ppb (AOT40) for the experimental period are provided in Table 1.

152   Plants were exposed to two watering regimes by means of drip irrigation. Fully watered plants

153   (control) received 1.2 L of water daily (referred to as 100% water and corresponding to field

154   capacity) and plants with water deficiency received 10% of the control level (Hoshika et al.

155   2017). Pots were protected from rain by plastic sheaths installed during rain 1 cm above the

156   topsoil in the pot. Precipitation was recorded by a Watchdog station, Mod. 2000 (Spectrum

157   Technology, Inc., Aurora, IL, USA). Soil water content was recorded by EC5 sensors connected

158   to an EM5b data logger (Decagon Devices, Pullman, WA, USA) in one plot per each treatment.

159   Soil water content remained close to soil field capacity (0.295 m^3 m^{-3}, Paoletti et al. 2017)

160   throughout the experiment in fully watered seedlings, while treatments with 10% water

161   supply remained well below the soil field capacity most of the time (Table 1).

162   Each watering regime was combined with two levels of ozone exposure. Three replicated plots

163   (5 m × 5 m × 2 m) with three seedlings per species were assigned to each treatment, resulting

164   in 9 seedlings per each combination of species, water deficit and ozone.

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166   **2.3 Sampling and preparation of roots**

167   Destructive harvesting of seedlings was performed in October 2015. Aboveground parts were

168   removed, and pots were kept refrigerated until analysis. Root biomass measurements were

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169 performed on 5-9 seedlings of each species per treatment (i.e. combination of ozone and

170 water deficit) while analyses of root morphology, anatomy, and ectomycorrhizal communities

171 were performed on three seedlings of each species per treatment, one seedling of each

172 species from each treatment replicate plot.

173 The root system was taken out of the pot and soil was gently shaken off. The root system with

174 the adhering soil was soaked in water and fine roots (< 2 mm in diameter; Pregitzer (2002))

175 were cut away from coarse roots (≥ 2 mm in diameter) by scissors. To avoid tearing off the

176 fine roots, no force was used to separate fine roots that were interwoven and glued together

177 with soil. The same quantity of fine roots (2.03 ± 0.18 g dry weight) from each seedling was

178 randomly selected regardless of the root system size (in the biggest root systems this amount

179 was approximately 25%) to obtain a subsample for analysis of ectomycorrhizal communities.

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181 **2.4 Characterization of ectomycorrhizal communities**

182 A subsample of fine roots was subjected to analysis of ectomycorrhizas under an Olympus SZH

183 (Olympus, Tokyo, Japan) dissecting microscope. Ectomycorrhizal root tips were separated

184 from non-ectomycorrhizal and individual ectomycorrhizal morphotypes were separated based

185 on their morphological and anatomical characteristics (Agerer 1991). Morphological and

186 anatomical descriptions of ectomycorrhizas were performed according to Agerer (1991) and

187 DEEMY (Agerer and Rambold 2004–2017). Generally, ectomycorrhizal mantles were thin.

188 Quantification of each ectomycorrhizal morphotype was done by scanning root tips and

189 analysis using WinRhizo (Regent Instruments Inc., Ville de Québec, Canada) software.

190 Scanning of non-ectomycorrhizal root tips was performed separately. Ectomycorrhizal

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191 colonization level was obtained as a ratio between the number of ectomycorrhizal root tips

192 and the total number of all root tips.

193 After scanning, one ectomycorrhizal system per morphotype was removed for molecular

194 characterization, while the remaining subsample was dried for biomass assessment.

195 Molecular characterization of ectomycorrhizal morphotypes was performed by PCR

196 amplification and sequencing of the complete internal transcribed spacer (ITS) regions in

197 nuclear ribosomal DNA (Gardes and Bruns 1993). DNA extraction was performed with a

198 DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and the ITS region was amplified with the

199 ITS 1f (CTTGGTCATTTAGAGGAAGTAA) and ITS 4 (TCCTCCGCTTATTGATATGC) primer pair

200 (White et al. 1990, Gardes and Bruns 1993) following the modified procedure described in

201 Grebenc and Kraigher (2007). After separation and excision of the amplified DNA from the

202 agarose gel and purification of the amplified fragments with Wizard SV Gel and PCR CleanUp

203 System (Promega Corp., Madison, WI, USA), sequencing was performed at a commercial

204 sequencing laboratory (Macrogen Inc., Seoul, South Korea). All sequences were preliminarily

205 checked for placement at the genus level. Subsequently, all available sequences for each

206 genus were retrieved from the GenBank and UNITE (Kõljalg et al. 2013) databases (retrieved

207 on March 1st, 2017) and identified using parsimony phylogenetic inference as in Sulzbacher et

208 al. (2016) after we removed short and low-quality sequences and performed a multiple

209 sequence alignment using a default multiple alignment strategy in MAFFT version 7 (Katoh

210 and Standley 2013). Identifications were based on the position of unknown sequences in

211 terminal clades of parsimony phylogenetic trees and names were used as given to closely

212 related sequences. For sequences manipulations and visualisation of phylogenetic outputs a

213 MEGA7 software (Kumar et al. 2016) was used. Reference sequences for ectomycorrhizal

morphotypes were deposited in GenBank database under accession numbers MH794930 - MH795054.

Species richness was calculated as the number of ectomycorrhizal taxa per seedling (Atlas and Bartha 1981), while estimation of diversity was performed by calculation of the Shannon diversity index (Shannon 1948). The relative abundances of individual ectomycorrhizal morphotypes were calculated as the number of root tips of an individual ectomycorrhizal morphotype divided by the total number of all ectomycorrhizal root tips.

2.5 Root morphology

All fine roots of each seedling were scanned by an Epson Perfection V700 Photo scanner (Seiko Epson Corp., Suwa, Nagano, Japan) in trays filled with water. Scans were analysed using WinRhizo software and results pooled per individual seedling to obtain mean root diameter, length of roots per each diameter class, number of branches and number of root tips. Diameter classes were set at 0.1 mm (i.e. 20 root diameter classes).

Coarse roots, fine roots and ectomycorrhizal morphotypes that were separated previously by morpho-anatomical investigations were air-dried, then oven-dried at 70°C for three days and kept desiccated over silica gel. Each root category per seedling was weighed separately by a SCALTEC SBC-31 (Denver Instrument, Bohemia, NY, USA) analytical scale. Morphological parameters were combined with fine root biomass to calculate specific root length (SRL), a ratio between root length and root biomass, and specific root tip density, a ratio between number of root tips and root biomass.

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2.6 Root anatomy

One 5-mm-long piece of 2-mm-thick root was randomly taken from each seedling and fixed in ethanol-formalin-acetic acid fixative. Two-mm-thick roots were chosen as they are representative for all roots with secondary growth, i.e. transport roots (Mrak and Gričar 2016). Fixed pieces of roots were dehydrated through ethanol series and embedded in paraffin. Cross sections (10-μm-thick) were prepared with a Leica EM2245 (Leica Biosystems Nussloch GmbH, Nussloch, Germany) rotary microtome and transferred to object slides. Paraffin removal was performed with UltraClear (J. T. Baker, Avantor Performance Materials B.V., Deventer, the Netherlands). Sections were stained with safranine (0.04%) and astra blue (0.15%) water mixture for better contrast of different tissues and cells, and finally mounted in Euparal (Waldeck, Münster, Germany). For more details see Mrak and Gričar (2016).

Cross sections were photographed in bright field under a Zeiss AxioImager Z2 (Carl Zeiss Microscopy, Jena, Germany) microscope at 200× magnification. To reveal the whole cross section in one photo, the panorama function of ZEN 2012 software (Carl Zeiss Microscopy GmbH, Jena, Germany) was used. On cross sections, the following parameters were measured with ZEN software: vessel tangential diameter (vessel lumen measured in tangential direction), vessel lumen area, distance of each vessel from the root centre, vessel density referred to the surface area of secondary xylem and to the conductive area in secondary xylem (Fig. S1 in SM), secondary xylem thickness, bark thickness and periderm thickness. Tissue thickness was measured at four measurement points per cross section. For each cross section, the presence of lenticels in bark (0 - absent, 1 – present) and distinctiveness of growth ring boundaries (0 - indistinct, 1 - inconspicuous, 2 – distinct) were visually observed and recorded. Only vessels in the outer 25% of secondary xylem were considered for comparison between

259   treatments. By using this approach, we aimed to exclude vessels formed before our

260   treatments as growth rings were not distinct in each sample. Indistinctiveness of growth rings

261   in roots is not uncommon and might not necessarily mean that the whole diameter was

262   formed within one year or that in the current year there was no growth at all (Mrak and Gričar

263   2016).

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265   **2.7 Root biomass**

266   Root biomass measurements were performed on 5-9 seedlings of each species per treatment

267   (i.e. combination of ozone and water deficit). For all the seedlings where ectomycorrhiza, root

268   morphology and root anatomy were not studied (i.e. 2-6 seedlings), the whole root system

269   was cleaned with a thicker brush and tweezers, to remove the adhering substrate, and divided

270   into coarse and fine roots. Then, coarse and fine roots were dried and weighed as described

271   above.

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273   **2.8 Statistics**

274   Statistical analyses were performed using the Dell Statistica version 13 data analysis software

275   system (Dell Inc., Tulsa, OK, USA). The statistical unit for all parameters was a seedling. For

276   statistical evaluation of differences between treatments, three-way analysis of variance

277   (ANOVA) was applied to assess effects of tree species, water deficit and ozone level as

278   independent factors and their interactions. Data were log or square-root transformed where

279   necessary to achieve the criterion of equality of variances, as tested with the Levene test

280   ($p < 0.05$). As a post-hoc test, the Tukey HSD test was used. Inherent interspecific differences

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281 were not further discussed unless necessary as they were not the objective of our study.

282 Differences in relative abundances of ectomycorrhizal fungi were studied with the Student t-

283 test for the effects of water and ozone and with the Kruskal-Wallis test for tree species, except

284 for the two most common taxa, where regular ANOVA was applicable. The Mann-Whitney U

285 test was applied for categorical variables.

286

287 **3. Results**

288 Figures and tables in the main text refer to statistically significant results, while the SM shows

289 all the data.

290

291 **3.1 Root biomass**

292 Biomass of coarse roots (Table S2) varied with species, water deficit and their interaction

293 (Table 2). *Q. ilex* had significantly lower biomass of coarse roots than the other two species

294 (Table S2). General reduction of biomass of coarse roots due to water deficit was -26.8%, but

295 in *Q. robur* (Fig. 1a) it added up to -36.3%. Biomass of fine roots (Table S2) varied with species

296 and water deficit (Table 2). *Q. robur* had significantly higher fine root biomass than the other

297 two species (Fig. 1b), while the effect of water deficit was evident as a general reduction in

298 fine root biomass (by -13.1%) (Fig. 1c).

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300 **3.2 Root morphology**

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301   Fine-root morphology (i.e. root length, mean root diameter, number of root tips, SRL, root tip

302   density, specific root tip density and branching density) varied significantly with oak species

303   (Tables S3, S4). The same was true for proportion of root length in each root diameter class

304   up to 1.5 mm root diameter (Tabs. S6, S7). Only the proportion of root length in the 0.0-0.1

305   mm root diameter class showed a significant interaction between species and water (Table

306   S7). In water deficient *Q. robur*, the percentage of the root system in the 0.0-0.1 mm diameter

307   class increased from 7.64% to 11.27% (Fig. 2a). Due to a strong species effect, fine root

308   morphological parameters were additionally tested separately for each species (Tables S5, S8).

309   No significant effect of ozone or water deficit on any morphological parameter was found in

310   *Q. ilex*, while there were some effects in *Q. pubescens* (Table S8) and *Q. robur* (Tables S5, S8).

311   For *Q. pubescens*, a slight but significant reduction of percentage (from 8.68% to 6.26%) of

312   fine root length in the 0.0-0.1 mm root diameter class was recorded due to elevated ozone

313   (Fig. 2b). In the same species, water deficit decreased the percentage of fine root length in the

314   1.3-1.4 mm and 1.5-1.6 mm root diameter classes (Table S6). In *Q. robur*, water deficit reduced

315   the mean root diameter by 8.49% (Table S3) and decreased the percentages of the root system

316   in the root classes 0.2-0.3 mm, 0.3-0.4 mm, 1.2-1.3 mm, 1.3-1.4 mm, 1.4-1.5 mm, 1.5-1.6 mm

317   and 1.6-1.7 mm (Fig. 2b, Table S6).

318

319   **3.3 Root anatomy**

320   Root anatomy showed significant effects of species just for vessel tangential diameter and

321   number of vessels, significant effects of ozone for vessel tangential diameter, vessel density

322   in secondary xylem and vessel density in the conductive part of secondary xylem, and no

323   significant effects of water (Table 3). Statistically significant interaction between species,

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324   water and ozone on mean vessel tangential diameter in roots was observed (Table 3). At

325   ambient ozone levels, the mean vessel diameter in *Q. ilex* was significantly larger than at 1.4

326   x AA (Fig. 3, Table S9). In this species, decrease in mean vessel diameter due to ozone was

327   more profound in fully watered plants (by 46.7% compared to 16.1% in water deficient plants),

328   leading to an effect where at ambient ozone, vessel tangential diameter was higher in fully

329   watered plants, whereas at 1.4 x AA vessel tangential diameter was higher in water deficient

330   conditions (Fig. 3). In *Q. pubescens and Q. robur* no significant effect on vessel tangential

331   diameter was detected (Fig. 3). Vessel density in whole secondary xylem was significantly

332   higher at elevated ozone, by 21.6% (Table S9). When vessel density was observed only in the

333   conductive part of secondary xylem, treatment with 1.4 x AA ozone resulted in a general

334   increase in vessel density by 18.5% (Fig. 4a). Mean vessel area was influenced by species and

335   ozone interaction. The most responsive was *Q. ilex*, where at 1.4 x AA, vessel area was reduced

336   by 53.4% compared to ambient ozone (Fig. 4b). Interaction between water deficit and ozone

337   significantly affected bark to secondary xylem ratio, which was significantly higher at 1.4 x AA

338   compared to AA in fully watered plants (Fig. 4c). Results for anatomical traits with no statistical

339   significance of any investigated effect are presented in SM (Tables S9, S10).

340   Regardless of oak species, water deficit affected the presence of lenticels in thin transport

341   roots (Table 4). In water deficient treatments, only 5.6% of samples had lenticels, while the

342   percentage of samples with lenticels increased to 33.3% in fully watered treatments (Table

343   S11). Besides this, investigated roots of fully watered *Q. ilex* had distinct growth boundaries in

344   all cases, while in water deficient conditions 50% of roots had indistinct growth boundaries.

345   On the other hand, 66.7% of investigated roots of fully-watered *Q. robur* had indistinct growth

346   boundaries, while in water deficient conditions, 83.3% of roots had distinct growth boundaries

347   (Table S11).

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3.4 Ectomycorrhizal communities

Ectomycorrhizal colonization of individual seedlings subjected to the experimental conditions ranged from 0.45% to 31.6%. None of the experimental conditions significantly affected ectomycorrhizal colonization (Table 5).

Altogether 14 ectomycorrhizal taxa were identified with molecular methods, 6 of them to the species level (3 of them with status cf.). The most common taxon was *Tomentella* sp. 2, which occurred on 30 seedlings out of 36 checked (83.3%), followed by *Sphaerosporella brunnea* (on 24 seedlings, 66.7%). Overall, 12 taxa occurred in fully watered conditions, and only 7 under water deficit. At ambient ozone, 12 taxa were recorded, while at 1.4 x AA, 9 taxa were found. *Q. ilex* hosted 13 taxa, *Q. pubescens* 7 taxa, and *Q. robur* 5 taxa. The most common morphotypes are presented in Table S12 in the Appendix.

None of parameters significantly affected ectomycorrhizal species richness and the Shannon diversity index (Table 5, Table S13). However, ectomycorrhizal species richness at ambient ozone level was higher in fully watered plants compared to water deficient plants (Table S13).

Significant effect of water (F=10.3, p=0.0037) was observed for relative abundance of *Tomentella* sp. 2. Relative abundance of this ectomycorrhizal fungus decreased under water deficit (Fig. 5). On the contrary, relative abundance of *Sphaerosporella brunnea* increased under water deficit (Student t-test, t=-2.30, p=0.0276). *Thelephora* sp. was present only under water deficit (Student t-test, t=-2.25, p=0.0310), while *Geopora cervina* occurred only on *Q. ilex* (Kruskal-Wallis ANOVA, H=8.71, p = 0.0128).

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4 Discussion

Root traits are increasingly recognized as drivers of many ecosystem processes, such as carbon, water and nutrient cycling, soil formation and structural stability (Bardgett et al. 2014, Ellison et al. 2017, Fort et al. 2017). To be able to predict responses of terrestrial ecosystems to expected future abiotic changes with higher accuracy, the response of root traits needs to be better investigated (Bardgett et al. 2014). In the experiment described here, the combined effects of elevated ozone and water deficit were studied on roots of seedlings of three oak species. AOT40 values revealed that the critical level of ozone for European forest trees, 5 ppm h (CRLTAP 2017), was exceeded even at the ambient (AA) level of exposure in the experimental site. AOT40 values recorded at the highest exposure level (1.4 x AA) were similar to those recorded in rural parts of Italy (Paoletti 2006). The level of water deficit was also realistic as a reduction of around 50% in the average SWC of the root soil layer is consistent with field conditions of water stress (Büker et al. 2012).

4.1 Root biomass

Water deficit and ozone stress have contrasting effects on root to shoot biomass ratio, with generally increasing root to shoot ratio due to water deficit (Brunner et al. 2015) and decreasing or zero effect due to ozone (Agathokleous et al. 2015). However, the effects on root biomass can be similar in both cases: fine (Cudlin et al. 2007, Brunner et al. 2015) and coarse (Fotelli et al. 2000, Kuster et al. 2013) root biomass is often reduced due to water deficit, while ozone resulted in fine, coarse or total root biomass reduction in 40% of studies and no effect in 60% of studies (Agathokleous et al. 2015). Responses cannot be generalized as in both cases stress severity and duration, genetic variation at the species and population

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393   levels and differences in physiology between young and adult trees all play a role (Cudlin et

394   al. 2007, Kraigher et al. 2008, Agathokleous et al. 2015, Brunner et al. 2015). In our study, only

395   effects of water deficit were observed. General reduction in coarse root biomass by 26.8%

396   compared to 13.1% in fine roots is consistent with interpretation of Kuster et al. (2013) who

397   suggest that fine roots are less affected due to water deficit because of their importance for

398   water and nutrient uptake. The effect of water deficit on coarse root biomass was the most

399   prominent in *Q. robur*, followed by *Q. pubescens* and *Q. ilex* which is consistent with the ability

400   of these three species to cope with water deficit. In *Q. robur*, water deficiency also reduced

401   the percentage of roots in certain diameter classes above 1 mm, which already have secondary

402   tissues (Mrak & Gričar 2016).

403

404   **4.2 Root morphology**

405   Effects of elevated ozone on root morphology were evident in *Q. pubescens* where the

406   percentage of the root system of the thinnest root diameter class (0.0-0.1 mm) was reduced

407   by 27.9%, indicating decreased carbon availability for fine root formation or maintenance due

408   to ozone stress. This finding is consistent with several negative effects of ozone fumigation on

409   fine root morphological parameters of *Fagus sylvatica* seedlings reported by Železnik et al.

410   (2007). Ozone effects were observed regardless of water deficit, which is reasonable in *Q.*

411   *pubescens* due to its intermediate sensitivity of gas exchange rates to soil water deficit

412   (Hoshika et al. 2017), i.e. ozone can enter into plants through stomata even under conditions

413   of soil water deficit. Reduced ratio of the thinnest root diameter class in *Q. pubescens*, i.e.

414   purely absorptive roots, has a potential effect on belowground and aboveground cycling of

415   water, carbon, and nutrients. The finest absorptive roots have the greatest absorptive

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capacity, respiration rate, nitrogen content and turnover rate (McCormack et al. 2015).

Individual trees with reduced quantity of absorptive roots could therefore suffer from

diminished uptake of water and nutrients, consequently producing less biomass, with possible

effects at ecosystem level.

On the other hand, effects of water deficit were observed as a 32.2% increase in the

percentage of roots in the thinnest root diameter class (0.0-0.1 mm class) in *Q. robur* and as

an 8.5% decrease in the mean root diameter of fine roots (< 2 mm). An increased proportion

of the thinnest roots could compensate at least partially for the reduced water availability, as

the thinnest roots have the greatest absorption capacity (McCormack et al. 2015). Similarly,

reduced mean root diameter would mean higher surface to volume ratio, which is a trait

associated with higher uptake and absorption capacity (McCormack et al. 2015). Specific root

length (SRL) apparently increased due to water deficit in both *Q. pubescens* and *Q. robur*, but

the difference was not statistically significant. Similar effects of drought on morphological

properties of fine roots were reviewed by Brunner et al. (2015). Absence of any changes in

fine root morphology in *Q. ilex* could be related to much lower SRL in this species compared

to *Q. pubescens* and *Q. robur*. Species with high SRL tend to have a more plastic root system

compared to those with lower SRL as investment costs of roots with high SRL are lower

(Eissenstat 1992). In addition, the three oak species might have a different phenology of fine

root growth (Radville et al. 2016), and therefore it is possible that the fine root growth in *Q.*

ilex had already ceased before the start of the experiment. There is very little known about

tree root growth phenology; in temperate trees, the peak in fine root growth occurs in spring

or early summer with an offset after the peak of shoot growth (Abramoff and Finzi 2015,

Delpierre et al. 2016), while in Mediterranean trees, fine root growth is presumably decoupled

from shoot growth (Abramoff and Finzi 2015).

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4.3 Root anatomy

Contrary to fine root morphology, *Q. ilex* showed a clear response to ozone in combination with water in the anatomical structure of thin transport roots. These roots are crucial for transport of water to the thicker roots that supply water to stem. At elevated ozone, mean vessel diameter in fully watered plants of *Q. ilex* decreased by 46.7%, whereas in water deficient plants it decreased only by 16.1%, indicating stomatal control of water loss and ozone uptake even in *Q. ilex*. Reduction in vessel size as a response to ozone treatment was also observed in stems of *Betula pendula* (Matyssek et al. 2002) and *Populus tremuloides* (Kostiainen et al. 2014) and is probably related to hormonal control. Vessel differentiation in roots is regulated by interplay of auxin and cytokinin (Aloni et al. 2006). Auxin is increasingly recognized as a mediator of stress adaptation responses in plants (Kazan 2013), and high regulation of cytokinin metabolism during the response to abiotic stress was shown as well (Zwack and Rashotte 2015). A reduction in mean vessel diameter by 46.7% decreases the theoretical conductivity by 91.9%, as calculated by applying the Hagen–Poiseuille law and assuming a perfectly circular cross-section of the vessels. A benefit of decrease in vessel diameter is increased hydraulic safety i.e. resistance to cavitation (Tyree and Zimmermann 2002). A decrease in vessel diameter in roots is also regarded as a response to drought stress (Brunner et al. 2015). Smaller vessel diameters in well water-supplied *Q. ilex* trees growing under elevated ozone would decrease the water conductivity of roots and consequently the water supply to the aboveground parts. On the other hand, in future ecosystems with frequent occurrences of soil water deficit, smaller vessel diameter would be beneficial as the vulnerability to cavitation would decrease. Smaller vessel diameter is usually correlated to

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463 higher vessel density (Savage et al. 2010). Indeed, higher vessel density in the conductive part

464 of the secondary xylem and in whole secondary xylem at elevated ozone was observed

465 regardless of the oak species, and this might be an early indicator of changes in vessel

466 diameter due to ozone exposure. In addition, in fully watered seedlings at elevated ozone

467 level, the ratio of bark to secondary xylem was increased in all oak species, suggesting higher

468 effects of ozone in full water supply conditions due to higher stomatal ozone uptake (Hoshika

469 et al. 2017) compared to water deficient conditions. The increased ratio of bark to secondary

470 xylem indicates reduced growth of secondary xylem compared to bark. The formation of

471 phloem which is part of the bark tissues is crucial for tree survival and therefore on the priority

472 list over secondary xylem for a tree under stress. The increased ratio of bark or phloem to

473 secondary xylem is one of the earliest anatomical indicators of decreased tree vitality (Gričar

474 et al. 2014). Inhibited xylem production due to ozone was reported also for stems of *Betula*

475 *pendula* (Matyssek et al. 2002).

476 Studies of growth rings in tree trunks have clearly shown the positive relationships between

477 quantity of precipitation and width of tree rings (Eilmann et al. 2009, Abrantes et al. 2013).

478 Since in roots sometimes growth rings are not present or are inconspicuous or wedging (Mrak

479 and Gričar 2016), the width of growth rings in our experiment could not be measured. Despite

480 this, we detected changes in occurrences of growth rings in roots of *Q. ilex* and *Q. robur* in

481 response to water deficit. However, changes in occurrence of growth rings were contradictory

482 in these two species, with *Q. ilex* having lower and *Q. robur* higher occurrence of growth rings

483 in water deficient seedlings. Dry growing season conditions are associated with both missing

484 tree rings (Leland et al. 2016) and false growth rings, i.e. intra-annual density fluctuations

485 (IADF), that are often observed in Mediterranean trees or shrubs as a response to water deficit

486 (Battipaglia et al. 2016). It is not excluded that growth rings in our samples were in fact IADF,

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especially because in root samples of *Q. robur* from temperate climate, growth rings were not observed (Mrak and Gričar 2016). In addition, well-watered seedlings showed higher occurrences of lenticels. Lenticels are normal structural components of periderm in stems and roots, increasing the permeability of periderm for water and air (Groh et al. 2002). It has been shown that in roots, secondary lenticels can be formed as a response to excessive soil moisture (Hahn et al. 1920).

4.4 Ectomycorrhizal communities

Colonization with mycorrhizal fungi can increase the ability of plants to resist environmental stress (Finlay 2008), but on the other hand, stressors that limit carbon allocation to roots, such as ozone, would be expected to decrease mycorrhizal colonization (Andersen 2003). In natural conditions, ectomycorrhizal colonization of roots is believed to follow the intermediate-host plant stress hypothesis (Swaty et al. 2004), with higher colonization at intermediate stress levels and lower colonization at severe stress levels. In our study, percentage of ectomycorrhizal colonization did not change in response to ozone or water deficit nor their interaction. In their meta-analysis, Cudlin et al. (2007) reported, that ozone has a small, but significant effect on decrease in ectomycorrhizal colonization, while Agathokleous et al. (2016) summarized several studies with contradictory effects. More than 50% of papers have shown no change in ectomycorrhizal colonization due to water deficit in review by Brunner et al. (2015).

In conditions of sufficient water availability, trees are able to support higher species richness of ectomycorrhizal fungi as they can supply more carbohydrates to belowground parts (Gehring et al. 2014). We observed similar yet not significant outcome in our study, as the

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highest species richness was observed in fully watered seedlings, but only at ambient ozone level. Reduced ectomycorrhizal species richness under elevated ozone was reported for hybrid larch in study of Wang et al. (2015). The strongest effect of water deficit was observed in the *Q. ilex* ectomycorrhizal community, where species richness decreased from 11 taxa in fully watered plants to only six under water deficit.

Although Wang et al. (2015) reported changes in relative abundance of ectomycorrhizal fungi due to elevated ozone, this was not found in our study. Relative abundance of ectomycorrhizal fungi in our study was affected only by water deficit. Water deficit significantly reduced relative abundance of *Tomentella* sp. 2, but increased relative abundance of *Sphaerosporella brunnea*. *Thelephora* sp. was present exclusively under water deficit in all three species. The most well-known species of *Thelephora* genus, *T. terrestris,* is a very common, fast-growing ectomycorrhizal symbiont in tree nurseries in many parts of the world. It occurs on a wide variety of soils in dry or wet conditions and is found in regenerating forests after severe disturbances (Colpaert 1999). It was shown to produce exocellular enzymes with saprotrophic activity (Burke and Cairney 2002). As such, it could potentially survive detrimental effects of water deficit on tree as it may supplement decreased supply of carbohydrates from the plant with other sources of carbon. Similarly, *S. brunnea*, one of the most abundant species in our experiment, is a very fast-growing fungus with both mycorrhizal and saprophytic characteristics, being able to degrade complex polymers such as cellulose and producing phenoloxidases (Danielson 1984). In stress conditions resulting in decreased carbohydrate supply to belowground parts, ectomycorrhizal fungal communities diverge towards communities dominated by a few generalist species (Gehring et al. 2014). Both elevated ozone (Wang et al. 2015) and water deficit (Shi et al. 2002, Richard et al. 2011, Nickel et al. 2017) were reported to affect community composition of ectomycorrhizal fungi. In our study, water

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deficit induced a shift of the ectomycorrhizal community towards dominance of stress-resistant species with the ability to produce exocellular enzymes with saprotrophic activity.

5 Conclusions

The effects of elevated ozone and water deficit on three oak species were species-specific and complex, suggesting that belowground responses to these stress factors should be studied holistically, as investigation of only one root trait or plant species can be misleading. The hypothesized decrease in root biomass for both stress factors was confirmed only for water deficit. Root morphological parameters were changed due to either ozone or water deficit in *Q. pubescens* and just due to water deficit in *Q. robur.* Root anatomical changes were observed due to different combinations of investigated factors or due to single factors. Ectomycorrhizal colonization levels did not change, but on the other hand, changes in relative abundances of ectomycorrhizal fungi were observed and were related to soil water deficit for three ectomycorrhizal taxa. Under stress conditions, a shift of the ectomycorrhizal community towards dominance of stress-resistant species was evident. The hypothesis on the lowest belowground effects of ozone in well-watered conditions and the highest in conditions of soil water deficit in *Q. ilex* compared to the other two oak species was not confirmed. The hypothesis of greater effects in well water-supplied plants was confirmed partially: In *Q. ilex*, reduction in mean vessel diameter due to elevated ozone was much greater in well-watered seedlings. In well-watered plants, ratio of bark to secondary xylem increased, indicating reduced secondary growth on the xylem side of the cambium. Although only a few responses were observed, these belowground effects of elevated ozone and soil water deficit have implications for ecosystem functioning in the sense of water, nutrients and carbon cycling.

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823   Tab. 1: Ozone and soil water content values over the experimental period (June 1 – October

824   15, 2015). AA – ambient ozone; H_2O – irrigation to field capacity. Part of the data are

825   summarized from Paoletti et al. (2017).

Treatment	24-h mean	Daytime mean (9:00-16:00)	Hourly mean MAX	AOT40
AA	35.0 ppb	45.9 ppb	93.4 ppb	17.8 ppm h
1.4 x AA	49.0 ppb	61.9 ppb	123.0 ppb	40.3 ppm h
100% H_2O	0.30 $m^3 m^{-3}$	0.29 $m^3 m^{-3}$	0.40 $m^3 m^{-3}$	
10% H_2O	0.14 $m^3 m^{-3}$	0.14 $m^3 m^{-3}$	0.40 $m^3 m^{-3}$	

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828   Tab. 2: Results of three-way ANOVA for coarse and fine root biomass for three species of oak

829   seedlings subjected to different levels of ozone and water deficit. Values with p<0.05 are

830   presented in bold.

		Biomass of coarse roots		Biomass of fine roots	
	Df	F	p	F	p
Species	2	**23.2**	**<0.0001**	**36.8**	**<0.0001**
Ozone	2	0.64	0.4247	0.44	0.5072
Water	2	**17.4**	**0.0001**	**4.66**	**0.0337**
Species x Ozone	4	0.43	0.6495	0.57	0.5673
Species x Water	4	**3.87**	**0.0248**	0.26	0.7752
Ozone x Water	4	<0.01	0.9450	0.55	0.4603
Species x Ozone x Water	8	0.17	0.8481	0.35	0.7051

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833 Tab. 3: Results of three-way ANOVA for the effects of oak species, ozone and water deficit on

834 root anatomical parameters. Values with p<0.05 are presented in bold.

	Mean vessel tangential diameter		Vessel density in sec. xylem		Vessel density in conductive		Mean vessel area		Bark to secondary xylem ratio		No. of vessels	
	F	p	F	p	F	p	F	p	F	p	F	p
Species	**6.43**	**0.0058**	1.89	0.1733	2.92	0.0734	3.40	0.0501	0.42	0.6602	**3.97**	**0.0325**
Ozone	**7.17**	**0.0132**	**6.26**	**0.0196**	**5.30**	**0.0303**	4.14	0.0531	3.41	0.0771	1.15	0.2943
Water	0.09	0.7651	0.15	0.7010	0.86	0.3624	0.04	0.8514	1.96	0.1746	0.12	0.7329
Species x Ozone	**13.0**	**0.0002**	0.25	0.7807	0.20	0.8212	**11.9**	**0.0003**	0.51	0.6093	0.14	0.8692
Species x Water	0.65	0.5323	1.80	0.1877	0.32	0.7269	0.25	0.7788	0.91	0.4151	2.26	0.1265
Ozone x Water	**6.09**	**0.0211**	<0.01	0.9731	0.61	0.4417	2.12	0.1582	**5.77**	**0.0244**	0.33	0.5703
Species x Ozone x Water	**4.19**	**0.0275**	**5.10**	**0.0142**	1.36	0.2746	3.30	0.0541	0.26	0.7726	0.37	0.6954

835

836 Tab. 4: Results of Mann-Whitney U test for the effects of ozone and water deficit on presence

837 of lenticels and distinctiveness of growth rings in 2 mm roots of three oak species subjected

838 to two levels of ozone and water deficit. Statistically significant effects (p<0.05) are presented

839 in bold.

		N	Presence of lenticels		Distinctiveness of growth rings	
			Z	p	Z	p
Ozone	Total	18	-1.22	0.2215	1.00	0.3180
	Q. ilex	6	-0.53	0.5948	0.96	0.3359
	Q. pubescens	6	-0.53	0.5948	-0.18	0.8586
	Q. robur	6	-0.83	0.4047	1.12	0.2608
Water	Total	18	**2.05**	**0.0401**	-0.37	0.7130
	Q. ilex	6	1.81	0.0705	**2.21**	**0.0269**

Q. pubescens	6	0.53	0.5948	-0.18	0.8586		
Q. robur	6	0.83	0.4047	**-2.34**	**0.0195**		

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841 Tab. 5: Results of three-way ANOVA for ectomycorrhizal (ECM) colonization, ECM species

842 richness and Shannon diversity index for three species of oak seedlings subjected to different

843 levels of ozone and water deficit. No statistically significant effects ($p < 0.05$) were found.

|		ECM colonization		ECM species richness		Shannon diversity index	
	Df	F	p	F	p	F	p
Species	2	2.07	0.1481	1.27	0.2995	0.31	0.7388
Ozone	1	1.18	0.2891	1.20	0.2852	0.01	0.9177
Water	1	1.04	0.3191	2.95	0.0987	1.26	0.2736
Species x Ozone	2	0.84	0.4434	0.68	0.5147	0.26	0.8002
Species x Water	2	0.34	0.7181	0.68	0.5147	0.44	0.6479
Ozone x Water	1	0.06	0.8158	4.12	0.0536	3.62	0.0692
Species x Ozone x Water	2	0.11	0.8944	1.56	0.2305	2.28	0.1240

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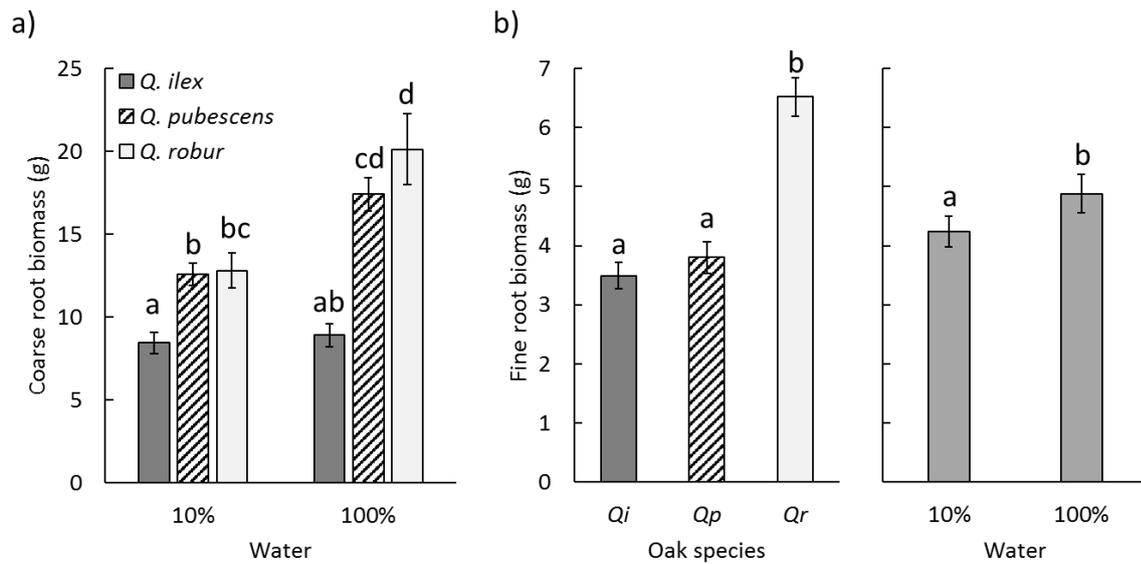
Fig. 1: Statistically significant effects on root biomass (mean ± std.err.) of (a) water and species interaction on coarse root biomass, (b) species and water deficit effects on fine root biomass of three oak species subjected to two levels of ozone (AA: ambient ozone and 1.4 x AA) and water deficit (irrigation at 10% and 100% of field capacity). Different letters mark significantly different results (Tukey HSD test, p<0.05, N=5-9). For all biomass values, see Tab. S2.

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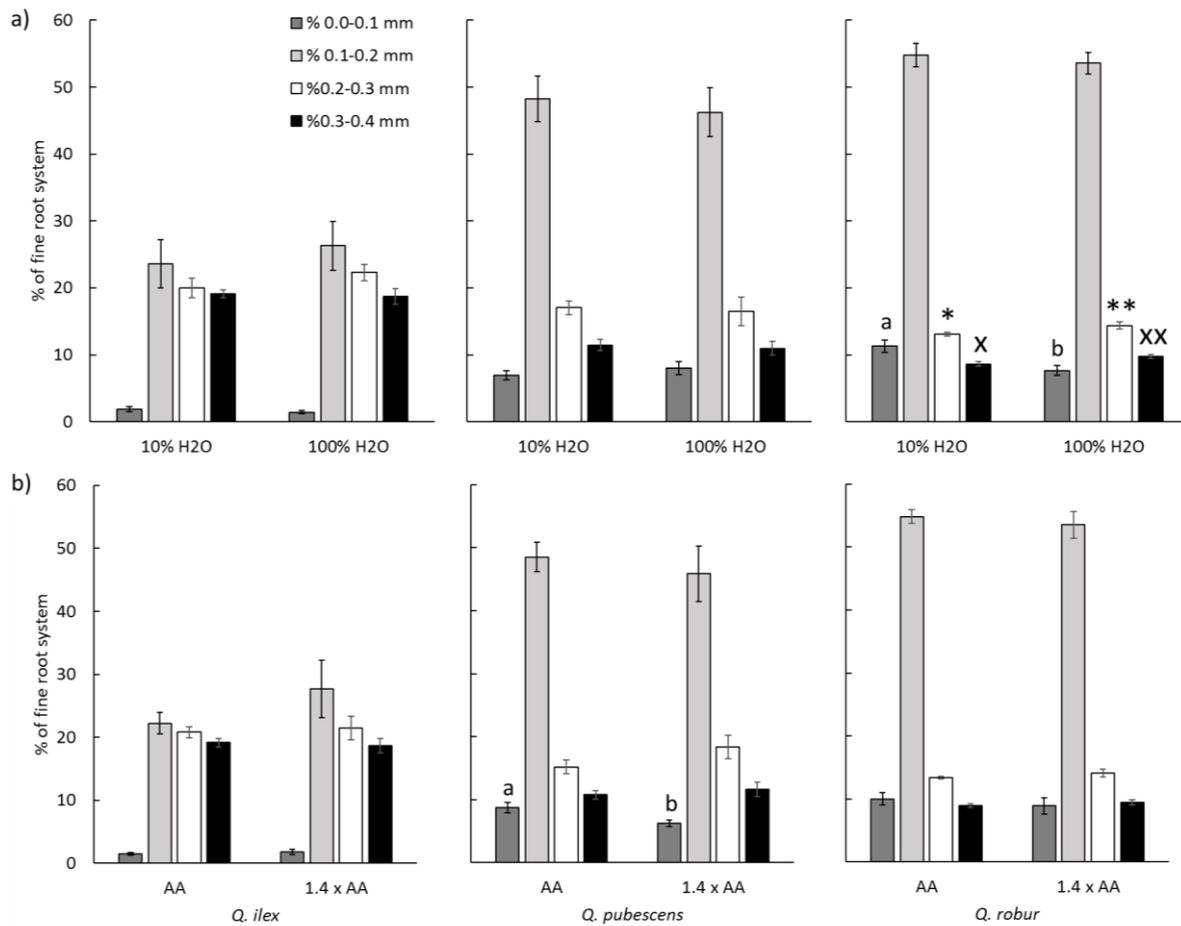
Fig. 2. Statistically significant effects on root morphology (mean ± std.err.) of (a) water and species interaction and (b) ozone and species interaction on percentage of fine root length in four diameter classes (0.0-0.1, 0.1-0.2, 0.2-0.3 and 0.3-0.4 mm) for three oak species subjected to different levels of ozone (AA: ambient ozone and 1.4 x AA) and water deficit (irrigation at 10% and 100% of field capacity). Only results for 0.0-0.4 mm are shown as they represent the majority (80-90%) of the fine roots. Different signs mark significantly different results (Tukey HSD test, $p < 0.05$, N=3). For all morphological results, see Tables S3-S8.
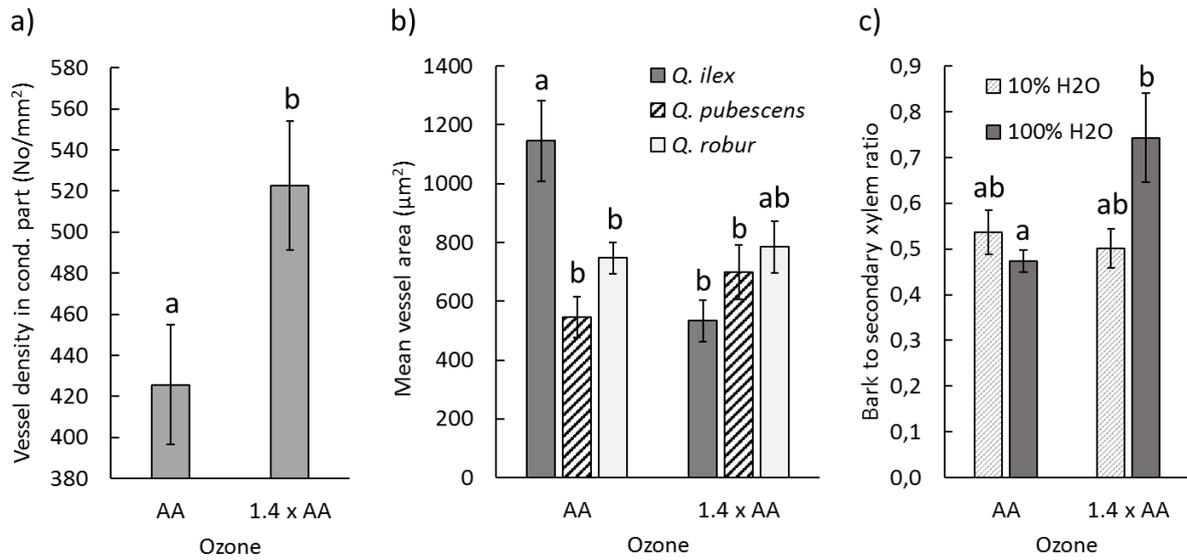
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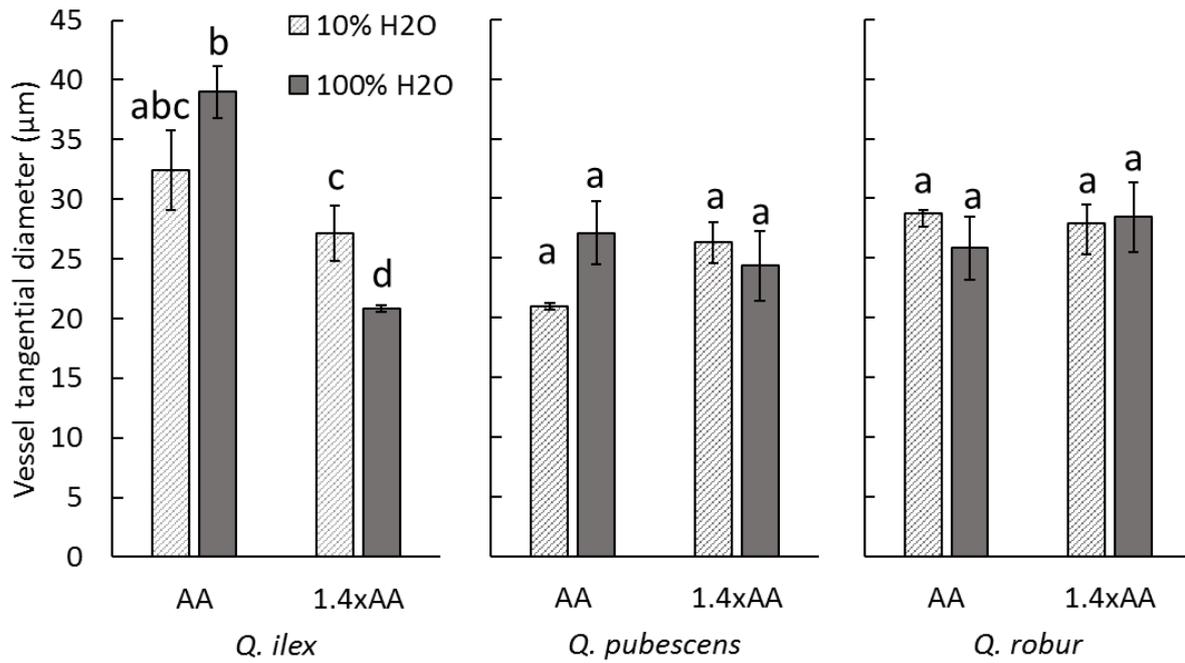
Fig. 3: Statistically significant effects on root anatomy (mean ± std.err.) of oak species, ozone and water on mean vessel tangential diameter in roots of oak seedlings exposed to two levels of ozone (AA, 1.4 x AA) and two levels of water deficit (10% and 100% irrigation). Different letters mark significantly different results (Tukey HSD test, $p<0.05$, N=3) for each graph separately.

Fig. 4: Statistically significant effects on root anatomy (mean ± std.err.) of (a) ozone on vessel density in conductive part of secondary xylem, (b) ozone x species on mean vessel area and (c) ozone x water on bark to secondary xylem ratio in oak seedlings exposed to two levels of ozone (AA, 1.4 x AA) and two levels of water deficit (10% and 100% irrigation). Different letters mark significantly different results (Tukey HSD test, p<0.05, N=3). For all anatomical results, see Tables S9-S11.

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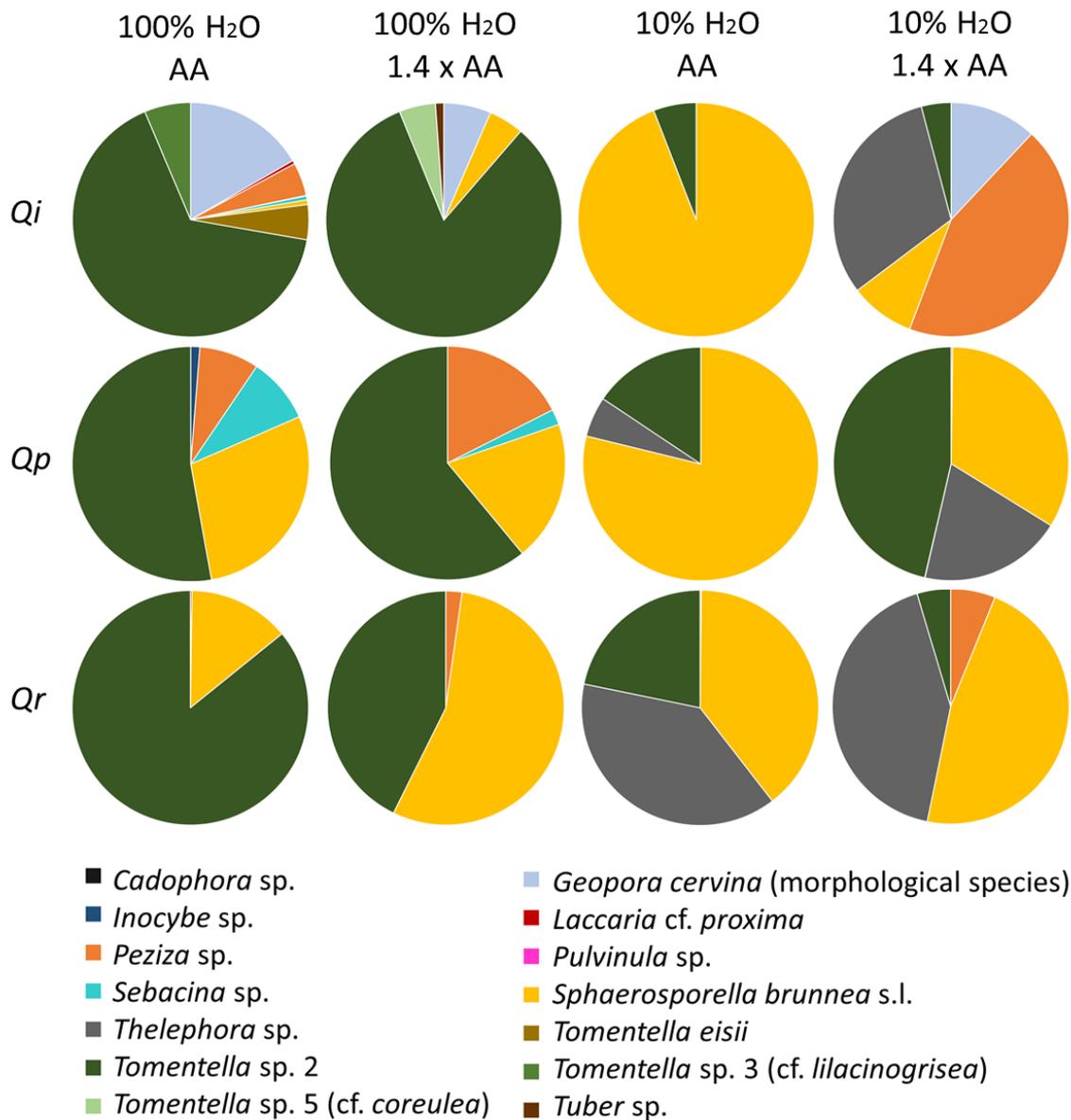
Fig. 5: Relative abundances (%) of ectomycorrhizal fungi from three oak species (Qi = *Q. ilex*,

Qp = *Q. pubescens*, Qr = *Q. robur*) subjected to different combinations of ozone and water

treatments. Ectomycorrhizal communities were analysed on three seedlings per species per

treatment. Abbreviation: AA=ambient ozone level.