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Research

Chemical plasticity in the fine root construct of *Quercus* spp. varies with root order and drought

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Summary

- Fine roots of trees exhibit varying degree of plasticity to adapt to environmental stress. Although the morphological and physiological plasticity of roots has been well studied, less known are the accompanying changes in the chemical composite (chemical plasticity) of fine roots, which regulates both root function and soil carbon sequestration.
- We investigated the changes in quantity, composition and localization of phenolic compounds in fine root orders of *Quercus alba* and *Quercus rubra* subjected to drought stress.
- In both species the total quantity of lignins varied only by root orders, where the distal (first and second) root orders had lower lignin compared to higher orders. Despite a lower lignin content, the distal root orders had higher content of guaiacyl lignin and bound phenolics that would provide a greater meshing of lignocellulosic matrix, and thus a higher tissue integrity. Unlike lignins, drought altered the quantity and composition of tannins. In *Q. alba*, the ellagitannins decreased in the distal root orders exposed to drought, while the fiber-bound condensed tannnins increased. The lower content of ellagitannins with antimicrobial properties under drought reveals an adaptive response by fine roots to promote symbiotic association, as evidenced by the higher colonization of ectomycorrhizal fungi.
- Our study revealed that, when exposed to drought, the composition of heteropolymers are strategically varied across fine root orders, so as to provide a greater root function without compromising the tissue protection.

Introduction

Fine roots (diameter ≤ 2 mm) are highly structured organs of plants that perform essential absorptive and transport functions (McCormack *et al.* 2015). Fine roots represent 14–27% of net primary production in terrestrial ecosystems (Jackson *et al.*, 1997) and account for 33% of annual litter inputs, thus contribute to a significant proportion of carbon (C) sequestered in the soil (Richter *et al.*, 1999; Freschet *et al.*, 2013; Xia *et al.*, 2015). Under future climates that are predicted to have more frequent and intense drought, the adaptations of fine roots to efficiently forage for resources would be critical to sustain plant productivity (Brunner *et al.*, 2015; Schlesinger *et al.*, 2016). Morphological and physiological adaptations are key traits that allow roots to sustain plant growth demands under varied environmental conditions (Hodge, 2005; Smithwick *et al.*, 2013; Valverde-Barrantes *et al.*, 2013, 2015). These environment-driven plastic responses of fine roots often encompass changes in the compound-specific chemistry (chemical plasticity) of root tissues, which help these organs to capture the soil resources efficiently. Apart from the changes in the total content (quantity) of compounds within the tissue matrix, the root chemical plasticity also encompasses changes in the composition and localization of molecules within the three-dimensional root matrix that could regulate the root functions. However, unlike the well-documented physiological and morphological plasticity, the chemical plasticity in plant roots is strikingly less well understood. Intimate knowledge of the chemical construct of fine roots is vital to gain a finer-level understanding of the root functions and to forecast the potential role of these organs in facilitating soil C sequestration.

Fine roots exhibit tremendous structural and functional diversity within the broader diameter class (Wang *et al.*, 2015). Within the 2-mm-diameter class, based on their branching pattern, fine roots can be classified into different orders, and these branch orders differ in their functions. The distal first- and second-order roots predominantly perform an absorptive function while the higher-order roots that have larger diameter and vasculature have transport and structural functions (McCormack *et al.*, 2015). The absorptive roots have a lower diameter, higher specific root length (SRL), and higher nitrogen (N), whereas the transport roots are characterized by the large diameter, low SRL and low N. Moreover, these root orders differ in their mycorrhizal association, where the mycorrhizal colonization is higher in absorptive than in transport roots. The above morphological and anatomical traits differ between different root orders (McCormack *et al.*, 2015) and in response to environmental stress such

as drought (Brunner *et al.*, 2015). Along with the branch order- and environment-specific changes in morphology, the chemical construct of fine roots may also vary across the different branching orders, especially when exposed to nonoptimal growing conditions. However, the varying degree of chemical plasticity across the different root orders is less well known.

Fine roots are constantly exposed to environmental stressors such as resource heterogeneity, mechanical impedance, and pest and pathogens in their immediate soil environments. Thus, unlike leaves, roots always face an optimization challenge in which they need to construct the tissues to defend against biotic and abiotic stressors while concurrently maximizing their resource uptake functions (Weemstra *et al.*, 2016). These contrasting functions are, in part, achieved through the alteration in the content, composition and localization of small molecules (< 1500 Da) and heteropolymers, including lignins, tannins and suberins in root tissues. Although it is well known that environmental stress such as drought often alters the tissue chemistry of plants (Moura *et al.*, 2010), a majority of these studies focused on leaf tissues (Brunner *et al.*, 2015; Suseela *et al.*, 2015) and are mostly confined to tracking elemental composition and operationally defined compound classes (Preston *et al.*, 2009) that are less reflective of the overall biological functions.

Chemical protection of plant tissues is a function of both quantity and composition of defense compounds. Along with the proportional abundance of different compound classes within the tissue, for heteropolymeric compounds such as tannins and lignins, the chemical diversity within the class is a key regulator of the biological function. The chemical diversity includes the identity of the monomers, the inter-unit linkages connecting these monomers within the polymer, and the degree of polymerization (Kraus *et al.*, 2003; Suseela & Tharayil, 2018). Along with the compositional changes, the spatial arrangement/localization of these polymers within the three-dimensional root matrix, and the degree of integration between the heteropolymers and the cellulosic matrix (Gibson, 2012; Vanholme *et al.*, 2012; Ding *et al.*, 2014) would also influence the overall root functions. The plasticity in their chemical construct significantly helps fine roots to adapt to environmental stress, such as drought. However, as they vary in their biological functions, when exposed to biotic/abiotic stress, not every heteropolymer in roots would undergo similar changes in composition. For example, the deposition of lignins that contribute rigidity and hydrophobicity enhances the protection of tissues from biotic and abiotic stressors. But, the increased deposition of lignin would also reduce the mechanical flexibility of roots, water uptake, and mycorrhizal colonization, thus compromising the absorptive functions of fine roots. Thus, the differential response of chemical construct of the roots to soil resource heterogeneities has important implications for root functions and root-derived soil C sequestration. Although plants have a remarkable ability to alter the chemical composition and localization of heteropolymers within the tissue matrix in response to abiotic stress (Dixon & Paiva 1995), such adaptations in roots are remarkably less understood (Suseela & Tharayil, 2018).

Here we hypothesized: that in accordance with the different root orders that perform unique functions, the chemical construct of the fine roots would vary across the root branching orders; that within the same branch order, the chemical construct of the fine roots would be influenced by the soil resource availability such as drought, and this chemical plasticity will be more pronounced in lower-order fine roots; that lower-order roots with higher SRL and higher tissue N would be better protected through an efficient integration of lignocellulosic matrix than through a higher deposition of structural defense such as lignin; and that, based on their function, the compositional diversity within a heteropolymer class would be influenced by both root order and soil resource environment.

To test these hypotheses, we chose two oak species *Quercus alba* L. (white oak) and *Quercus rubra* L. (red oak), dominant tree species in the eastern US that differ in their tolerance to drought – white oak being more tolerant to drought than red oak (Abrams, 2003). We focused on the drought-induced variation in quantity, composition and localization of two phenolic heteropolymers – lignins and tannins – that contribute up to 30% of the plant biomass (Boerjan *et al.*, 2003). Lignin, the second most abundant biopolymer after cellulose, is deposited in the secondary cell wall and provides structural integrity and hydrophobicity to the cell wall (Boerjan *et al.*, 2003; Vanholme *et al.*, 2012). Lignin is composed of three monomeric units, namely syringyl (S), vanillyl (V) (guaiacyl (G)), and p-hydroxyphenyl (H) lignin. The lignin matrix in plants is a function of the proportional abundance of monomers, with lignins abundant in G-units resulting in branched lignins that are more recalcitrant compared with linear S-rich lignins. Similar to lignins, tannins are also ubiquitous in plants and are the second most abundant polyphenols after lignin. Tannins (proanthocyanidins) are broadly classified as condensed tannins (CTs; polymers of catechin or gallocatechin units linked by C-C interflavan bonds) and hydrolyzable tannins (HT; polymeric esters of gallic acid with glucose). Further the hydrolyzable tannins are classified as ellagitannins (ETs) and gallotannins based on the presence and absence of intramolecular C-C coupling between gallayol groups, respectively (Suseela & Tharayil, 2018). The protective functions of tannins are commonly attributed to its antioxidant and antifeedant capacity, which in turn vary with their composition. For example, tannins that are structurally more flexible readily complexes with macromolecules such as cellulose and proteins than tannins with interflavan or intergalloyl covalent linkages (Le Bourvellec & Renard, 2012), and tannins with higher degree of hydroxylated monomers (galocatechins/prodelphinidins) have a greater protein complexation capacity (Kraus *et al.*, 2003). Along with lignins and tannins, we also assessed the quantity of monophenolics that crosslink lignins with polysaccharides (bound-phenols), thus providing a measure of the integration of phenolic matrix with structural carbohydrates within the root tissues. In addition to the chemical plasticity, we also characterized the root morphology and anatomy of different root orders to examine whether the chemical plasticity of different fine root orders varies consistently with the morphological and anatomical traits.

Materials and Methods

Study species

Oaks play an essential role in ecosystem functioning in the eastern US and are economically valued for its timber. In general, oak species are tolerant to chronic water stress and are considered as xerophytic species as compared with several other mesophytic tree species (Abrams & Nowacki, 1992; Fei *et al.*, 2011). The tolerance to drought varies between the different oak species. Several studies have reported that *Q. alba* occurs in drier sites whereas *Q. rubra* is dominant in mesic sites (Abrams, 1990, 2003; Poulos, 2009; Renninger *et al.*, 2014) . The difference in drought tolerance between white oak and red oak can be attributed to the difference in a range of above-ground morphological and physiological traits (Abrams, 1990). For example, *Q. alba* exhibits higher leaf conductance, stomatal closure for fewer days, and lower leaf water potential than does *Q. rubra* (Abrams, 1990; Keyser & Brown, 2016). *Quercus alba* also developed less elastic tissue than *Q. rubra* during drought, where low tissue elasticity can help to maintain a favorable gradient for the uptake of water from drying soils (Abrams, 1990).

Experimental setup

One-year-old bare-root seedlings of *Quercus alba* L. and *Quercus rubra* L. were planted in 60 l pots filled with 55 l of 2 : 1 sand : soil mixture. The branch roots were trimmed off before planting. To provide the microbial inoculum, including ectomycorrhizal (EcM) fungi associated with oak trees, the soil used in this study was collected (up to 10 cm deep) from the understories of monoculture oak stands in the Clemson Experimental Forest, Clemson University. The water-holding capacity and field capacity of the above sand–soil mixture was determined gravimetrically before planting the seedlings, and water content was continously monitored throughout the experiment using time domain reflectometry (TDR) waveguides installed vertically in the pots. The field capacity of our mesocosm was 19% volumetric water content. The moisture treatments included an ambient treatment (75% field capacity, *c.* 14% volumetric water content) and a drought treatment (25% field capacity, *c.* 5.0% volumetric water content). Based on the innate adaptation of species to drought stress and the high percent of sand in the growing medium, the low moisture treatment (25% field capacity) in our study may represent moderate drought stress and not an extreme drought condition. Previous studies under similar drought treatment resulted in the modified growth of these species rather than a completely arrested growth under field conditions (Top *et al.*, 2017). The soil moisture was continuously monitored using TDR waveguides, and the pots were watered with distilled water at regular intervals to maintain the treatments at ± 5% of the respective moisture content (Supporting Information Fig. S1). Moisture treatments in each species were replicated five times and were blocked within the glasshouse to account for environmental variation. The plants were fertilized with 200 ml of full-strength Hoagland's solution at monthly intervals. The plants were harvested at 15 months after the start of the treatment. At harvest, the pots were cut open, and roots were carefully washed in running water to remove the soil. Each plant was divided into shoots, leaves and roots. We selected 10 intact branches of roots that had four root orders per plant. We separated the roots into the first, second, third and fourth orders based on their branching pattern as per Pregitzer *et al.* (2002). These different root orders were subjected to morphological, anatomical and detailed chemical analysis, as described in the following sections. For the root morphology and chemical analysis, the first- and second-order roots were taken together as these represent the absorptive roots (McCormack *et al.*, 2015), and our previous analyses have demonstrated comparable chemistries across this broader group (Wang *et al.*, 2015).

Ectomycorrhizal fungal association To assess the mycorrhizal colonization, from each plant we selected 10 intact branches with one to four root orders and counted the number of EcM root tips under a dissecting microscope (×10–40 magnification) based on the difference in color, texture and branching patterns (Brundrett *et al.*, 1996). The percentage colonization of EcM in roots was calculated as: (no. of mycorrhizal root tips)/(no. of vital root tips) × 100.

Root morphology and anatomy The roots from each order were scanned using a desktop scanner, and the images were processed with WinRhizo (Regent Instruments Inc., Québec, Canada). After imaging, the scanned root orders were dried and weighed. The root tissue density (mg cm^{-3}), SRL (m g^{-1}) and specific root area (SRA; g cm^{-2}) were calculated as per Zadworny & Eissenstat (2011); and Chen *et al.*, (2013). The different root orders from first to fourth were collected in 70% ethanol and then fixed overnight in 4% formaldehyde solution. The roots were then paraffin-embedded, sectioned using a microtome to 20 µm thickness, and mounted on glass slides (five to six sections per root order per treatment) for confocal microscopy imaging. An upright confocal fluorescence microscope (Zeiss LSM 710) was used for the microscopy study. The microscope objective was Zeiss EC Plan-Neofluar 10X NA 0.3. A laser excitation source with 490 nm wavelength (Zeiss Intune) produces both transmitted bright-field image and fluorescence image simultaneously. The lignin fluorescence within the wavelength range 543–628 nm was selected.

Root chemical analyses For the root chemical analysis, the first + second, third and fourth root orders were dried at 40°C and powdered using a Geno/Grinder® (SPEX SamplePrep 2010, Metuchen, NJ, USA). The percentage C and N in the different orders of roots were analyzed using an elemental analyzer (Carlo Erba NA 1500 Elemental Analyzer; Thermo Scientific, Lakewood, NJ, USA). The different orders of roots from both ambient and drought treatments were subjected to the following analysis to capture the compound-specific chemistry.

Condensed tannins The amount of CT was quantified using the acid-butanol assay modified from Porter *et al.* (1986).

Approximately 30 mg of the finely-ground root tissue was extracted three times with 3 ml of 100% methanol by overnight shaking. After centrifugation, the supernatant was pooled, and 2 ml of the pooled supernatant were dried down under N gas at 40°C. Three milliliters of the butanol : HCl (95 : 5 v/v) reagent with Fe as catalyst was added, and the tubes were incubated at 90°C for 60 min. For the analysis of the nonextractable or fiber-bound tannins, the residue remaining after the third methanol extraction was dried, weighed into glass tubes, combined with 6 ml of the butanol : HCl reagent and incubated as described earlier. The amount of depolymerized anthocyanidins in the samples was quantified using a spectrophotometer (Jasco V-550 UV/VIS; Jasco Analytical Instruments, Easton, MD, USA) by measuring the absorbance at 550 nm, and the tannins were quantified using cyanidin as the standard.

Hydrolyzable tannins The hydrolyzable tannins (HTs) were quantified after methanolysis of the pooled extract (extractable HTs) or the methanol-extracted residue (bound HTs), in the presence of H_2SO_4 at 85°C. Ellagic acid (from ETs), and methyl gallate (from gallotannins) that was generated during the hydrolysis were quantified in samples using high-pressure liquid chromatography (HPLC) coupled to a UV-detector. Samples were analyzed with a Shimadzu quaternary pump UFLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an autosampler, inline degasser, and UV-visible diode array detector. Separations were performed on a C18 column (150 × 3 mm, 3 µm; Phenomenex, Torrance, CA, USA). The separation of the compounds was achieved by a gradient elution of 0.1% formic acid in water and acetonitrile. The limit of detection was defined as having a signal-to-noise ratio of 10, and all values reported are based on the peak area at 272 nm.

Composition of ellagitannins All analyses were performed using an Ultimate 3000 HPLC (Thermo Scientific, Waltham, MA, USA) coupled to an Orbitrap Fusion (Thermo Scientific) Tribrid mass spectrometer (MS) equipped with an electrospray ion source (Bowers, *et al.*, 2018). The pooled methanol extract of the root sample was analyzed after seperation on an Acquity UPLC HSS T3 column (150 × 2.1 mm, 1.8 µm; Waters Corp., Milford, MA, USA) maintained at 30°C. A gradient program utilizing water containing 0.1% formic acid as mobile phase A and acetonitrile as mobile phase B was employed. Solvent B was increased from 10% to 90% in 15 min and a subsequent re-equilibration for 5 min at 10% B at a flow rate of 0.20 ml min^{-1}. The MS was operated in a negative ionization mode with a data-dependent fragmentation (MS^2 HCD-CID) method (Bowers *et al.*, 2018). The interface conditions were as follows: emitter voltage, −2600 V; vaporizer temperature, 325°C; ion transfer tube, 325°C; sheath gas, 55 (arb); aux gas, 10 (arb); and sweep gas, 1 (arb). Detailed mass spectrometric parameters are provided in Methods S1. Metabolites were classified as ellagitannins following the unique fragmentation pattern generated by the reporter-ion triggered MS^2 (Bowers *et al.*, 2018).

Bound phenolics and lignins Bound phenols and lignin fraction were estimated on the residue remaining after the methanol extraction. The residue was extracted with 6 ml of freshly prepared 1 M NaOH (pre-sparged with Ar for 30 min) incubated at 90°C for 3 h. The tubes were centrifuged, and 4 ml of the supernatant was transferred to a new glass tube, and the pellet was washed twice with 5 ml deionized water, dried and kept for the extraction of lignin fraction. The base hydrolysate was extracted using 2 ml ethyl acetate, and 1 ml of the ethyl acetate was transferred into a GC vial and stored at −20°C. Lignin was estimated by quantifying the monolignols derived from the CuO-oxidation of the residual pellet obtained after the extraction of the bound phenols (see Methods S2 for detailed methodology on lignin depolymerization).

Quantification of bound phenol and lignin fraction Twelve phenolic monomers, including *p*-hydroxybenzoic acid (PAD), *p*-hydroxyacetophenone (PON), *p*-hydroxybenzaldehyde (PAL), vanillic acid (VAD), acetovanillone (VON), vanillin (VAL), ethyl vanillin (EVAL), syringic acid (SAD), acetosyringone (SON), syringaldehyde (SAL), cinnamic acid (CiAD), *p*-coumaric acid (CAD), ferulic acid (FAD) and 3,5-dihydroxy-benzoic acid (DiOHBA), were measured in bound-phenol and lignin fractions. Three classes of phenols, namely vanillyl (V = VAD + VON + VAL), syringyl (S = SAD + SON + SAL) and cinnamyl phenols (C = CAD + FAD), and the total lignin-derived phenols ($V + S + C$) were calculated (Wang *et al.*, 2015). The percentage of guaiacyl lignin (% V) was calculated as the percentage of guaiacyl monomers in total lignin. The phenolic compounds in the two fractions were derivatized using *N*-methyl-*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS) before gas chromatography- mass spectrometry (GC-MS) analysis. Details of derivatization and GC-MS parameters are provided in Methods S3.

Statistical analysis

We analyzed root morphology and root chemistry data using a two-way ANOVA with root order and moisture treatments as the main factors. The data were transformed wherever necessary to satisfy the assumptions of normality. The differences among individual treatments were assessed using Tukey's honestly significant difference multicomparison test (SigmaPlot v.14; Systat Software Inc., Chicago, IL) The high-resolution MS data were analyzed with partial least-squares discriminant analyses (PLS-DA; MetaboAnalyst 3.0; Xia *et al.*, 2012; Suseela *et al.*, 2015). A heat map was generated to visualize the responses of different metabolites across different moisture treatments and root orders. Hierarchical clustering analysis was performed based on both treatments and metabolites.

Results

Across the species and the treatments, the EcM colonization was limited to the first- and second-order roots. The *Q. alba* plants

Table 1 Diameter of different orders of fine roots measured using WinRhizo.

Average root diameter (mm) by order				
		Root orders		
Species	Treatment	1 + 2	3rd	4th
Quercus alba	Drought	0.551	0.486	1.065
Quercus alba	Ambient	0.374	0.563	0.935
Quercus rubra	Drought	0.506	0.460	0.953
Quercus rubra	Ambient	0.358	0.473	0.755

subjected to drought had 60 ± 12% EcM association in the first + second-order roots compared with the 7 ± 5% EcM association in the first + second-order roots subjected to ambient conditions (Fig. S2). Compared with *Q. alba*, the EcM colonization of *Q. rubra* roots were lower; the first + second root orders of *Q. rubra* had 32 ± 7% and 10 ± 4% EcM colonization in drought and ambient treatments, respectively. The average diameter measurements showed that the first + second root orders from the ambient treatment had a lower diameter than first + second root orders from the drought treatment (Table 1). The difference in root diameter in the third-order roots was more accurately captured by the confocal microscopy. The third-order roots of both *Q. alba* and *Q. rubra* exhibited a visible difference in the anatomy when subjected to the drought and ambient treatments. The third-order roots of both *Q. alba* and *Q. rubra* from the ambient treatment had a large cortical parenchymatous tissue surrounding the endodermis (Fig. 1a,c). However, in the drought treatment, the third-order roots were devoid of the cortex in both *Q. alba* (Fig. 1b) and *Q. rubra* (Fig. 1d).

In both species, SRL and SRA decreased with increasing root order in the ambient treatment. In *Q. alba*, the first + second root orders exposed to drought had lower SRL (Fig. 2a,b) and specific root area (SRA; Fig. S3) compared with the first + second orders of roots of trees exposed to the ambient treatment. However, this pattern was not observed in the third- and fourth-order roots of *Q. alba*, where neither SRL and SRA varied between drought and ambient treatments. In *Q. rubra*, the SRL and SRA followed a similar trend as *Q. alba* except that the third-order roots exposed to drought treatment had higher SRL and SRA compared with the ambient treatment. The effect of moisture treatments on root tissue density varied with root order in both species (Fig. 2c,d). In *Q. alba*, drought increased root tissue density in the first + second root orders (Fig. 2c). In the fourth-order roots, higher tissue density was observed in roots from the ambient treatment than from the drought treatment in both species (Fig. 2c,d). The root : shoot ratio and specific leaf area of both species decreased in the drought treatment relative to the ambient treatment (Table S1).

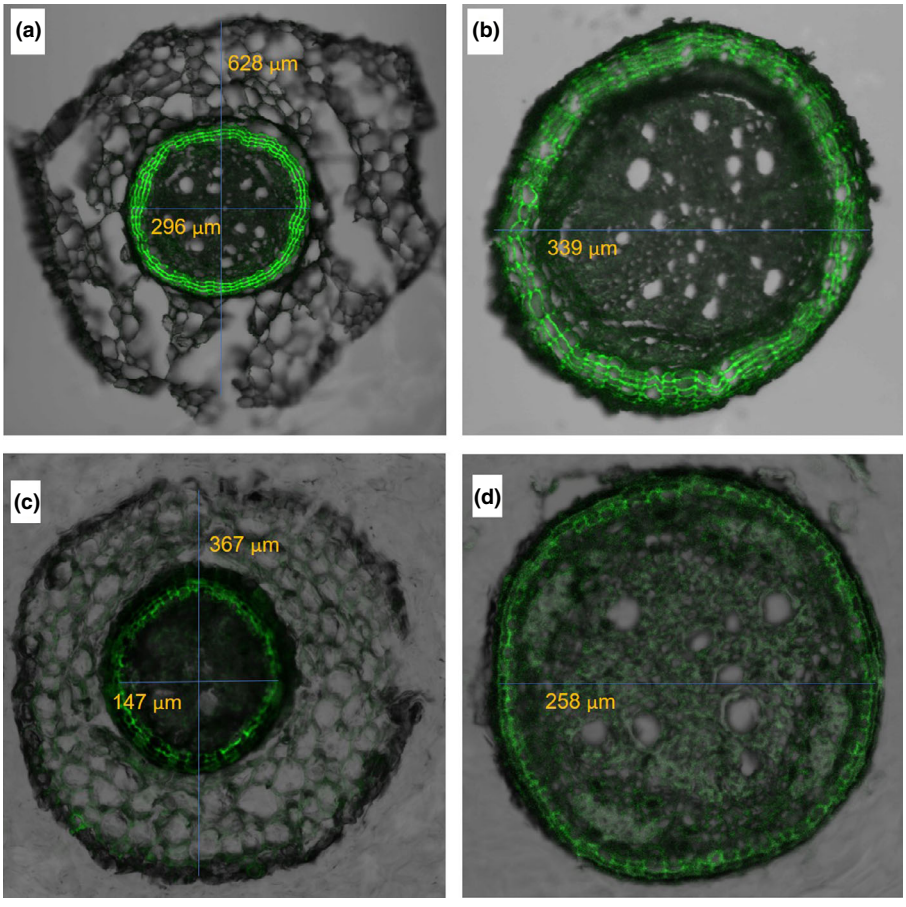

Fig. 1 Confocal microscopy analysis of the cross-section of the third-order roots of *Quercus alba* subjected to ambient (a) and drought treatments (b) and *Quercus rubra* subjected to ambient (c) and drought treatments (d). Representative samples are shown in the figures.

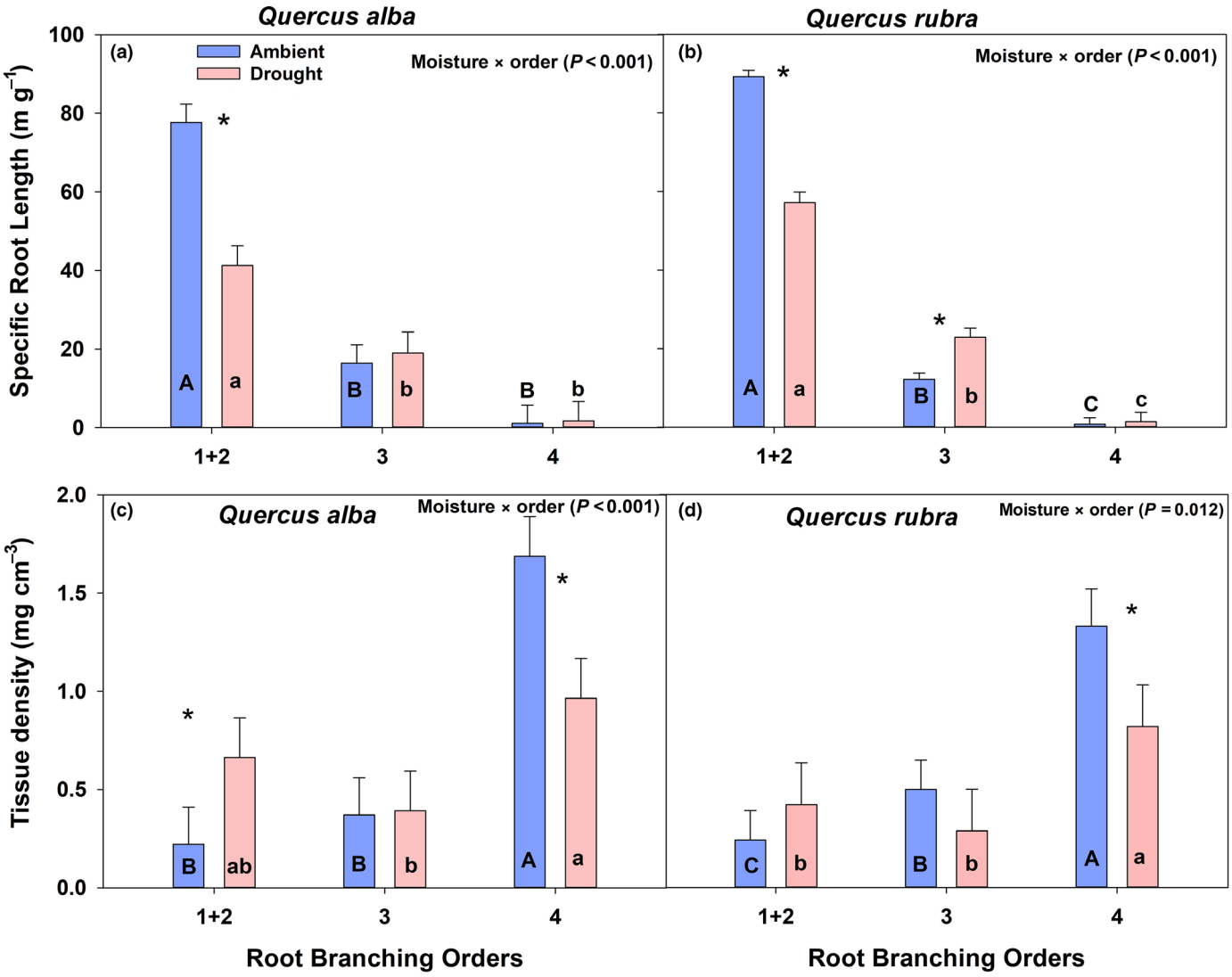

Fig. 2 Change in the morphology (specific root length and root tissue density) of the fine roots of different orders of *Quercus alba* (a, c) and *Quercus rubra* (b, d) across ambient and drought treatments. Values represent means ± SE ($n = 5$). Bars with different uppercase letters indicate a difference (Tukey's honestly significant difference (HSD)) between ambient treatments along root orders, and bars with different lowercase letters indicate a difference between (Tukey's HSD) drought treatments along root orders. Asterisks indicate a difference between the ambient and drought treatments within a root order. Root branching orders: 1+2, first + second orders; 3, third order; 4, fourth order.

Chemical construct of fine roots

Tannins The total content of tannins varied across root orders and treatments in both species (Fig. 3). In *Q. alba*, the total tannins decreased with increasing root orders in the ambient treatment where the tannin content of first + second orders of roots was 47% higher than that of the third-order roots and twice as high as the fourth-order roots (Fig. 3a). In *Q. alba*, the total tannin content of the first + second orders of roots was not influenced by drought. However, compared with the respective orders in the ambient treatment, the total tannin content in roots of trees exposed to drought was increased by 72% in the third-order roots and was almost doubled in the fourth-order roots (Fig. 3a). In *Q. rubra*, the third-order roots in the ambient treatment had the highest tannin content, 30% and 45% higher than the first + second and fourth orders, respectively (Fig. 3b). Unlike *Q. alba*, compared with the ambient treatment, the drought treatment decreased the total tannin content in the first + second- and third-order roots of *Q. rubra* by *c.* 15% (Fig. 3b).

The tannin composition of *Q. alba* was predominated by CTs (45%) and HTs (55%). Among the HTs, ETs accounted for 50% of the total tannin content, whereas gallotannins contributed only 5% of the total tannins. Along with the quantitative changes in the total tannins, the composition of tannins in *Q. alba* also differed across root orders and treatments (Fig. 3a). Except for the first + second root orders in the drought treatment, HTs contributed by ETs were the most predominant tannin in *Q. alba* across all root orders and treatments (Fig. 3a). Compared with the respective ambient treatment, the CT doubled in the first + second-order roots exposed to drought, while ETs in the

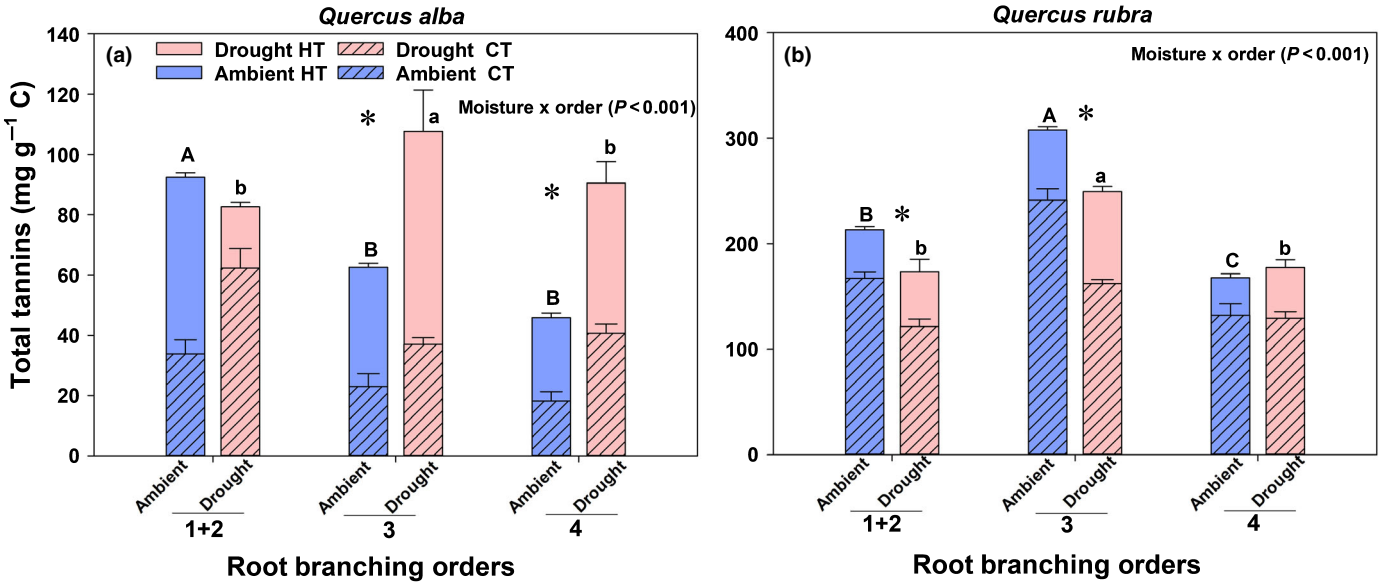

Fig. 3 Total tannins in the fine roots of different orders of *Quercus alba* (a) and *Quercus rubra* (b) across ambient and drought treatments. Values are means ± SE ($n = 5$). Bars with different uppercase letters indicate a difference (Tukey's honestly significant difference (HSD)) between ambient treatments along root orders and bars with the different lowercase letters indicate a difference between (Tukey's HSD) drought treatments along root orders. Asterisks indicate a difference between the ambient and drought treatments within a root order. HT, hydrolyzable tannins; CT, condensed tannins; root branching orders: 1+2, first + second orders; 3, third order; 4, fourth order.

same root orders decreased around four-fold in *Q. alba* (Figs 3a, 4a). However, the trend of ETs reversed in the third- and fourth-order roots of *Q. alba*, where drought doubled ETs in these two orders as compared with the respective root orders in the ambient treatment (Fig. 4a). Unlike the pattern of ETs, the total CT increased with drought across all root orders in *Q. alba* (Fig. 4c). The first + second-order roots had higher CT compared with the third- and fourth-order roots of *Q. alba* (Fig. 4c). Condensed tannins dominated the tannin profile of *Q. rubra* (Fig. 3b). Compared with the ambient treatment, plants exposed to the drought treatment had higher ETs across all order roots (Fig. 4b), whereas the CT decreased in first + second- and third-order roots of *Q. rubra* (Fig. 4d).

The association of tannins with tissue fiber (extractable vs fiber-bound) varied with respect to tannin types and treatments. In *Q. alba*, *c.* 80% of the CTs were bound to the cell wall (fiber-bound tannins) across treatments and different root orders (Fig. 4c) , whereas only *c.* 50% of the ETs were bound to the tissue fibers (Fig. 4a). The observed increase in total CTs within the same root orders in response to drought was almost exclusively contributed by the increase in bound CTs (Fig. 4c). The greatest increase in the amount of bound CTs was observed in first + second root orders, where the content of fiber-bound CTs doubled in response to drought (Fig. 4c). Despite significant variation in ET content across the treatments in *Q. alba*, the proportion of fiber-bound ETs was similar between the ambient and drought treatments (Fig. 4a). In *Q. rubra,* the fiber-bound proportion of ETs was similar between treatments within a root order, except for the third-order roots where the fiber-bound fraction of ETs doubled under the drought treatment (Fig. 4b). In *Q. rubra,* the fiber-bound proportion contributed to < 50% of the total CTs in the ambient treatment (Fig. 4d).

Detailed profiling of ETs using ultra-high-resolution MS identified differences in the composition of ETs in *Q. alba* root extracts across treatments and root orders (Fig. 5). Castalagin, a predominant ET in *Q. alba* and other major ETs decreased in the first + second order roots of drought compared to the respective root orders in the ambient treatment (Fig. 5). The chemical compositions of ETs in ambient first + second- and third-order roots were similar (Fig. 5). The hierarchical clustering and heat map showed a higher abundance of more hydrophobic ETs, as estimated from the retention time on a C18 column, in the first + second- and third-order roots exposed to ambient treatments. In *Q. alba*, the PLS-DA axis that accounted for 54% variation in the dataset separated the first + second and third root orders of the ambient from the rest of the treatments (Fig. S4a). Various classes of ETs that contributed to the described PLS projection, as identified from the variable importance in projection (VIP) scores, showed higher abundance in the first + second and third root orders of the ambient treatment (Fig. S4b). Even though less in overall quantity, the *Q. rubra* root extract exhibited a higher chemodiversity of ETs across the treatments (Fig. 6). Unlike in *Q. alba*, irrespective of the treatments, the same order roots of *Q. rubra* from ambient and drought treatments had similar chemical composition of ETs (Figs 6, S5). The first + second-order roots were abundant in less polymeric ETs compared with the third-order roots, which were proportionally abundant in higher polymeric ETs. Based on ETs with significant VIP scores, the third-order roots from the drought and ambient treatments had a higher abundance of ETs than did the other root orders (Fig. S5b).

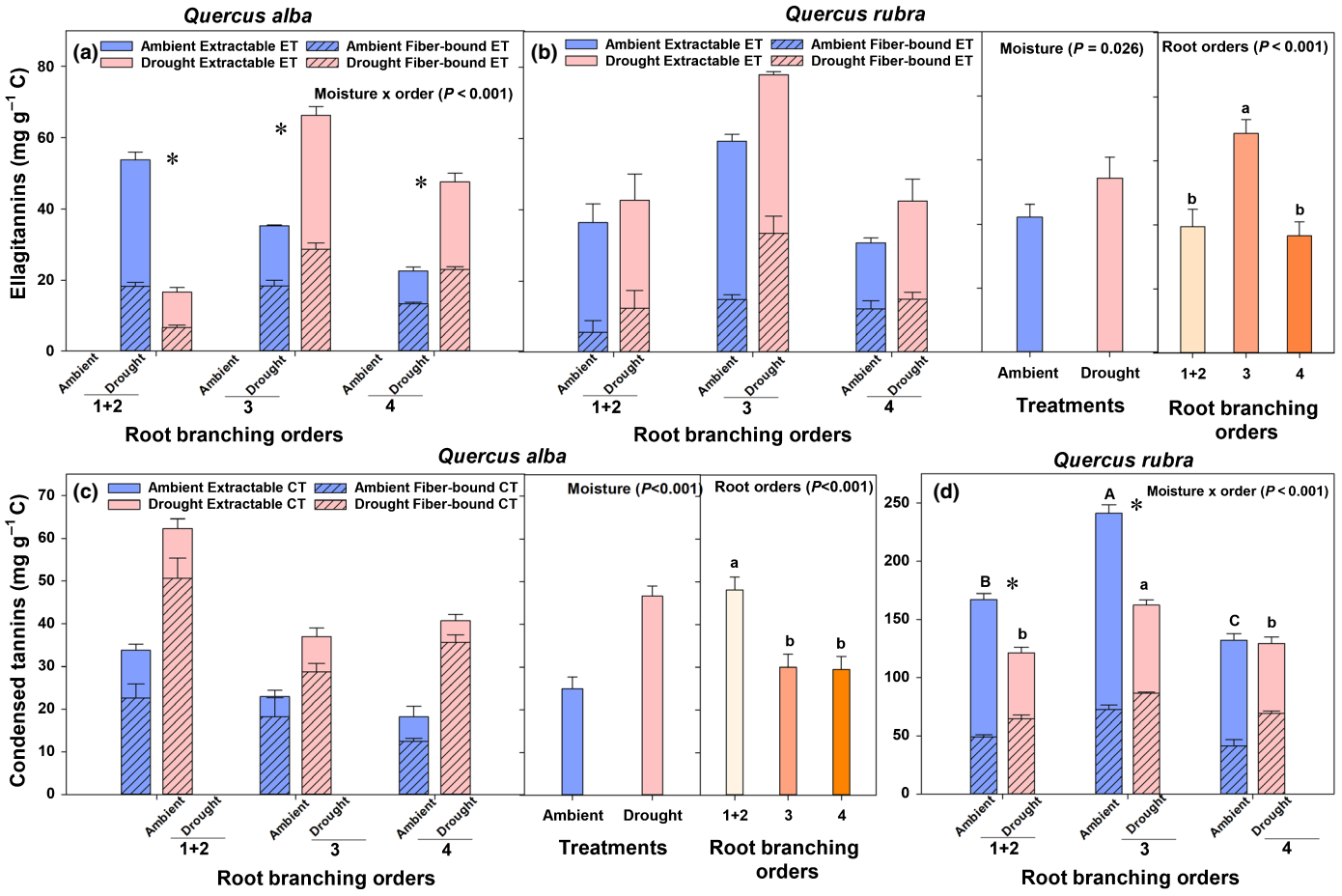

Fig. 4 Ellagitannins and condensed tannins in the fine roots of different orders of *Quercus alba* (a, c) and *Quercus rubra* (b, d) across ambient and drought treatments. Values are means ± SE ($n = 5$). Upper panel: bars with the same lowercase letters indicate no difference between root orders. Lower panel: (d) bars with different uppercase letters indicate a difference (Tukey's honestly significant difference (HSD)) between ambient treatments along root orders, and bars with the different lowercase letters indicate a difference between (Tukey's HSD) drought treatments along root orders. Asterisks indicate a difference between ambient and drought treatments within a root order. ET, ellagitannins; CT, condensed tannins; root branching orders: 1+2, first + second orders; 3, third order; 4, fourth order.

Lignin and bound phenolics The total syringyl + vanillyl + cinnamyl (SVC) lignin content increased with increasing root orders in both the species, with the fourth-order roots having double the SVC content than the first + second root orders (Fig. 7a,b). Unlike tannins, the SVC content of the roots was not influenced by drought treatments across the root orders. Despite an increase in total lignin content with increasing root orders, the proportion of guaiacyl lignin content decreased with increasing root order (Fig. 8a,b). The content of syringyl lignin increased with increasing root order, resulting in a higher proportional abundance of S units (Fig. S6). Unlike in *Q. alba*, the proportional abundance of G and S units in *Q. rubra* responded to treatments, where, compared with the third-order roots in the ambient treatment, the percentage of G units increased by 20% in similar order roots exposed to drought treatment (Fig. 8b) and drought decreased S units by 7% compared with the ambient treatment (Fig. S6b). In both species, across the root orders and treatments, the amount of bound-phenolics was higher in the first + second root orders. (Fig. 8). The content of bound phenolics showed a significant treatment response only in the first + second root orders in *Q. alba*, where the bound phenolics increased two-fold in the drought treatment compared with the ambient (Fig. 8c). In *Q. rubra*, across root orders, bound-phenolics increased with the drought treatment (Fig. 8d). In both species, bound phenolics: lignin ratio was twice higher in the first + second root orders compared with the third-and fourth-order roots (Fig. S7). Although, drought did not alter SVC lignin across species and root orders, in *Q.alba*, drought increased bound-phenolics: lignin ratio in the first + second root orders (Fig. S7).

Elemental N and C : N ratio In *Q. alba* and *Q. rubra*, the percentage N was higher in the first + second root orders compared with the third- and fourth root orders in both treatments (Fig. S8). In *Q. alba,* drought increased percentage N in first + second- and third-order roots (Fig. S8a) and the C : N ratio was lower in the first + second root orders compared with the ambient treatment (Fig. S8b). In *Q. rubra*, within the root order the N content doubled on exposure to drought (Fig. S8c), resulting in a two-fold decrease in the C : N ratio in the drought treatment as compared with the ambient treatment (Fig. S8d).

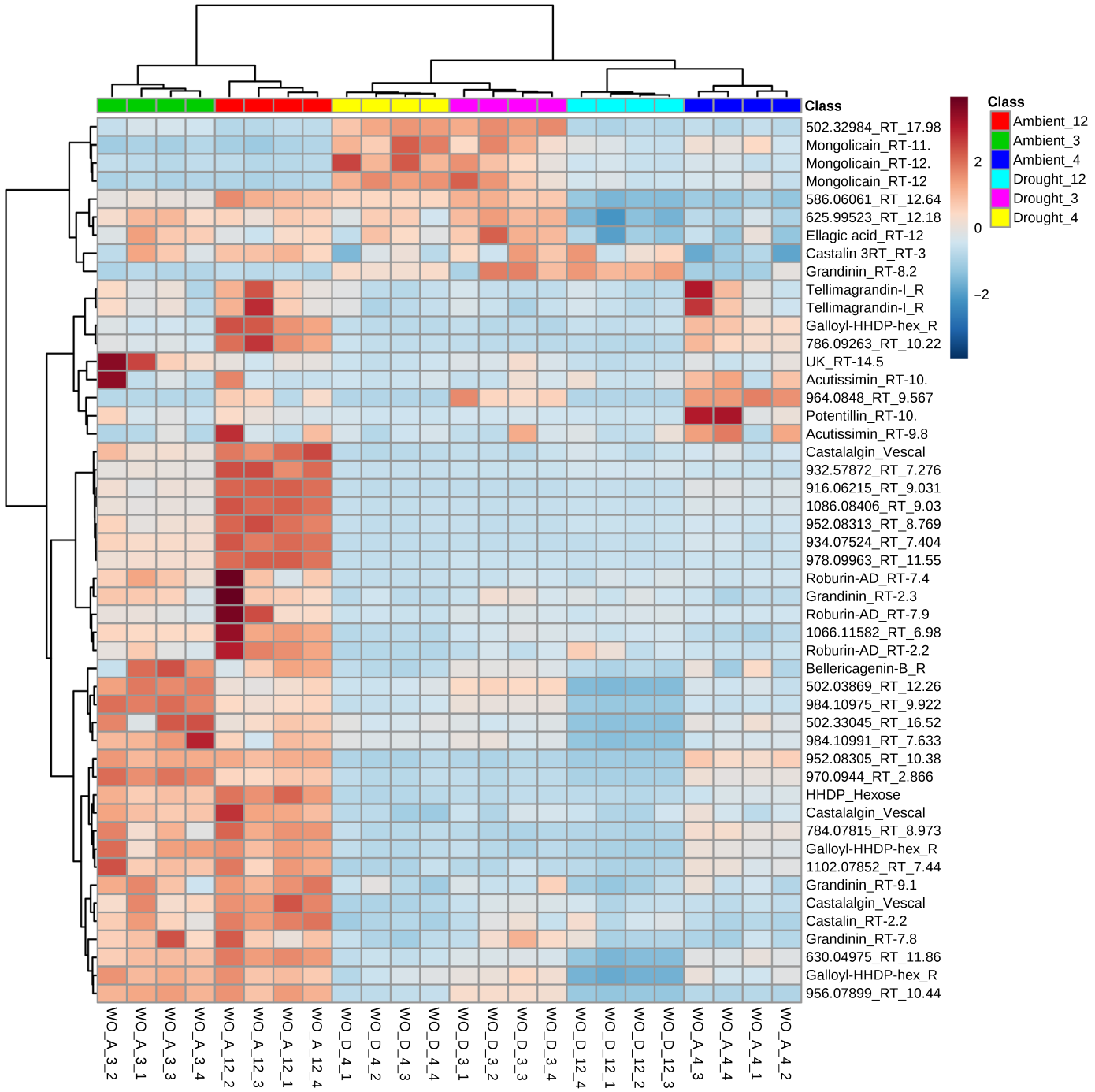

Fig. 5 Heat map and two way hierarchical clustering of the various ellagitannins in different orders of fine roots of *Quercus alba* exposed to ambient and drought treatments. Each column represents a replicate from a treatment, and each row represents a positively identified ellagitannin (metabolite/accurate mass_retention time). 12, first + second-order roots; 3, third-order roots; 4, fourth-order roots. Ellagitannins were identified following the targeted reporter-ion trigger method described in Bowers *et al*. (2018).

Discussion

Drought alters fine root mycorrhizal association, root morphology and anatomy

In both *Quercus* spp., the EcM colonization was limited to first + second root orders; however, the extent of this symbiotic association in response to drought was species-specific. The more drought-tolerant *Q. alba* (Abrams, 1990, 2003; Poulos, 2009; Renninger *et al*., 2014) had higher colonization of EcM than *Q. rubra,* which is less well adapted to drought. Previous studies have reported that the percentage of EcM colonization could vary with species (Mrak *et al*., 2019), and host specificity contributed by plant traits may influence the EcM-mediated drought

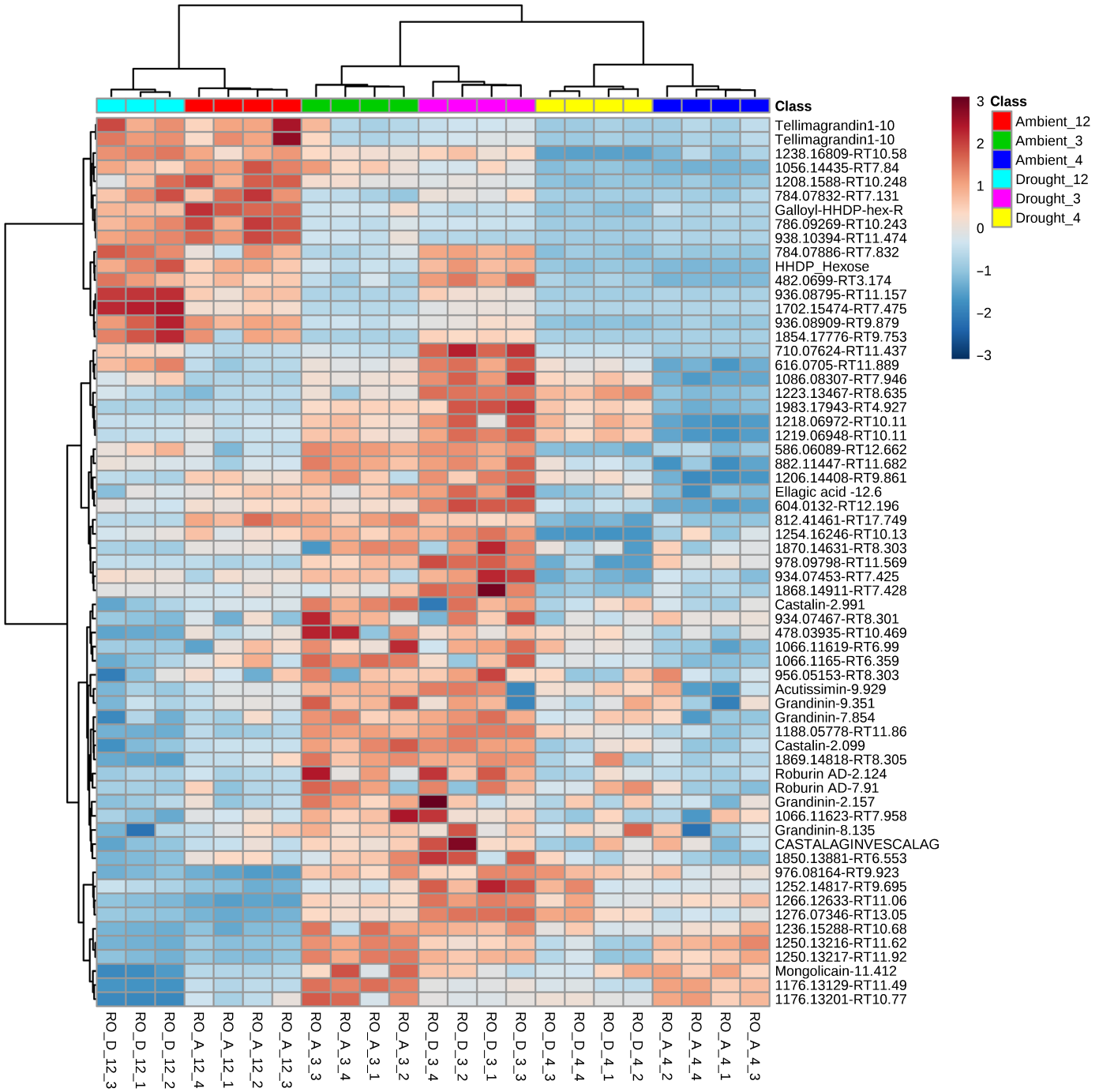

Fig. 6 Heat map of the composition of ellagitannins of different orders of fine roots of *Quercus rubra* exposed to ambient and drought treatments. Each column represents a replicate from a treatment, and each row represents a positively identified metabolite/accurate mass/retention time. 12, first + second-order roots; 3, third-order roots; 4, fourth-order roots. Ellagitannins were identified following the targeted reporter-ion trigger method described in Bowers *et al*. (2018).

tolerance in trees (Patterson *et al*., 2019). For example, compared with the high and low end of drought stress that decreased EcM colonization, pine trees subjected to moderate drought stress exhibited a two-fold increase in the EcM colonization (Swaty *et al*., 2004). The beneficial effects of EcM to increase plant tolerance to drought have been well characterized (e.g. Beniwal *et al*., 2010; Sebastiana *et al*., 2019), although the experimental findings on drought effects can be quite mixed (Lehto & Zwiazek, 2011). Water stress did increase oak EcM colonization in some studies (Dixon *et al*., 1980; Garcia de Jalon *et al*., 2020), although the opposite was also reported (poplar; Beniwal *et al*., 2010). As previous studies suggested, the actual response can be highly specific to the combination of fungi species, hosts and experimental conditions.

Along with EcM colonization, we also observed a decrease in SRL and SRA with drought in the first + second root orders in

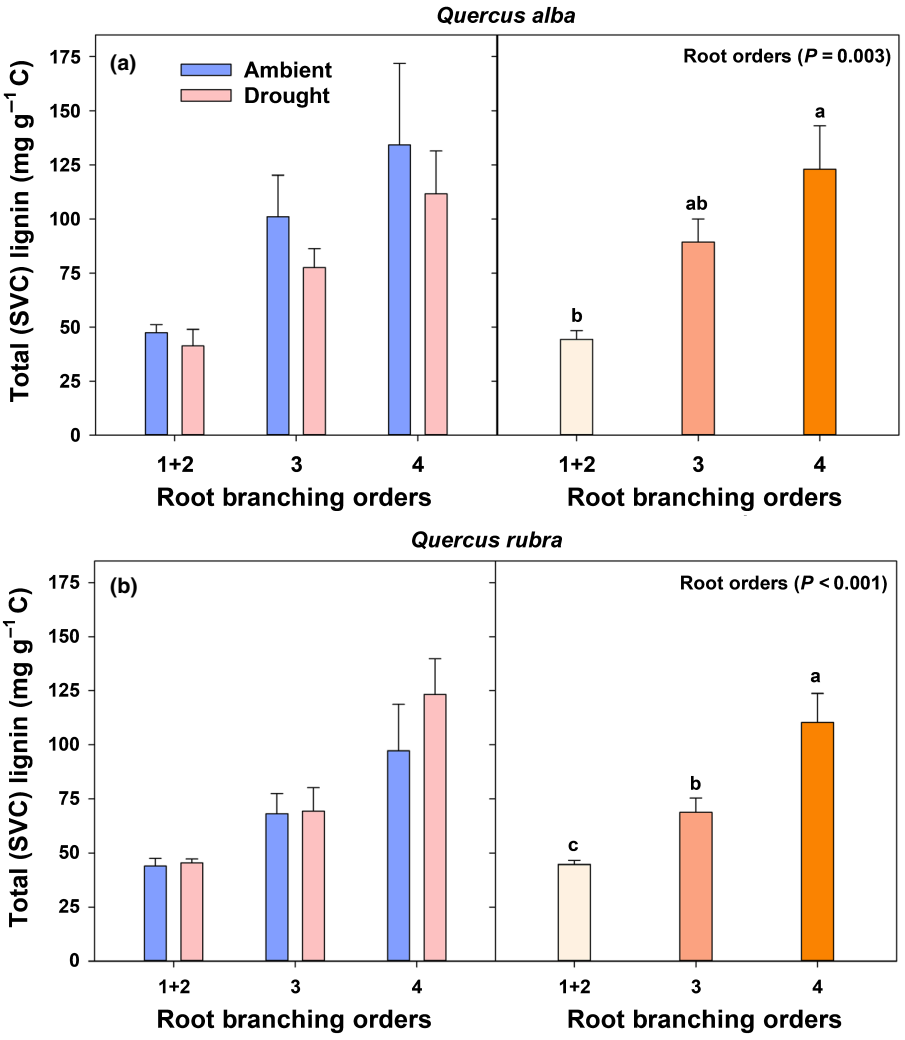

Fig. 7 Total syringyl + vanillyl + cinnamyl (SVC) lignins in the fine roots of different orders of *Quercus alba* (a) and *Quercus rubra* (b) in the ambient and drought treatments. Values are means ± SE ($n = 5$). Bars with different lowercase letters indicate a difference between root orders. Root branching orders: 1+2, first + second orders; 3, third order; 4, fourth order.

both the species. A change in these morphological parameters can occur through variations in root diameter and root tissue density (Ostonen *et al.*, 2007). The EcM short roots with a thick mantle increased the diameter and mass of the roots, resulting in a decrease in SRL and SRA in the first + second root orders formed under drought. However, the increase in tissue density in the first + second root orders was significant only in *Q. alba*. It should be noted that the relationship among SRL, RTD and diameter is not always straightforward owing to the adaptive responses of roots to soil resource availability (Kramer-Walter *et al.*, 2016). This uncertainty is particularly true for tree species where plants can increase the diameter while simultaneously decreasing SRL and RTD, thus creating a nonsignificant relationship between SRL and RTD (Comas & Eissenstat, 2009; McCormack *et al.*, 2012; Valverde-Barrantes & Blackwood, 2016). In addition to changes in root diameter, SRL and RTD, previous studies have shown that *Quercus* spp. with EcM associations had higher branching intensity and the highest proportion of first- and second-order roots than other species (Yahara *et al.*, 2019) which also reflect the adaptive response to soil resource limitation.

Our study also revealed considerable changes in the anatomy of the third-order roots when subjected to drought stress. The third-order roots formed under drought were thinner than the roots produced under ambient conditions. This difference in diameter was primarily attributed to the parenchymatous cortex in the roots from the ambient treatment. The loss of cortex in higher-order roots is typically associated with ontogeny and secondary development, which lowers the radial movement of water and ions across the roots (McCormack *et al.*, 2015) and reduces the chances of mycorrhizal colonization (Valenzuela-Estrada *et al.*, 2008). Under drought, this loss of cortex might be accelerated, resulting in an outer layer of endodermis that protects the pericycle and vascular tissues from drying out while concurrently maintaining a connection with the shoots and roots (Clarkson *et al.*, 1968; Jupp & Newman, 1987; Enstone *et al.*, 2002). These changes in root anatomy may have implications for root decomposition. For example, within the same root branching order, the roots produced in ambient treatment might be more prone to decomposition due to the presence of parenchymatous cells that are rich in labile substrates . Previous studies have shown that the root diameter and tissue density can also regulate decomposition

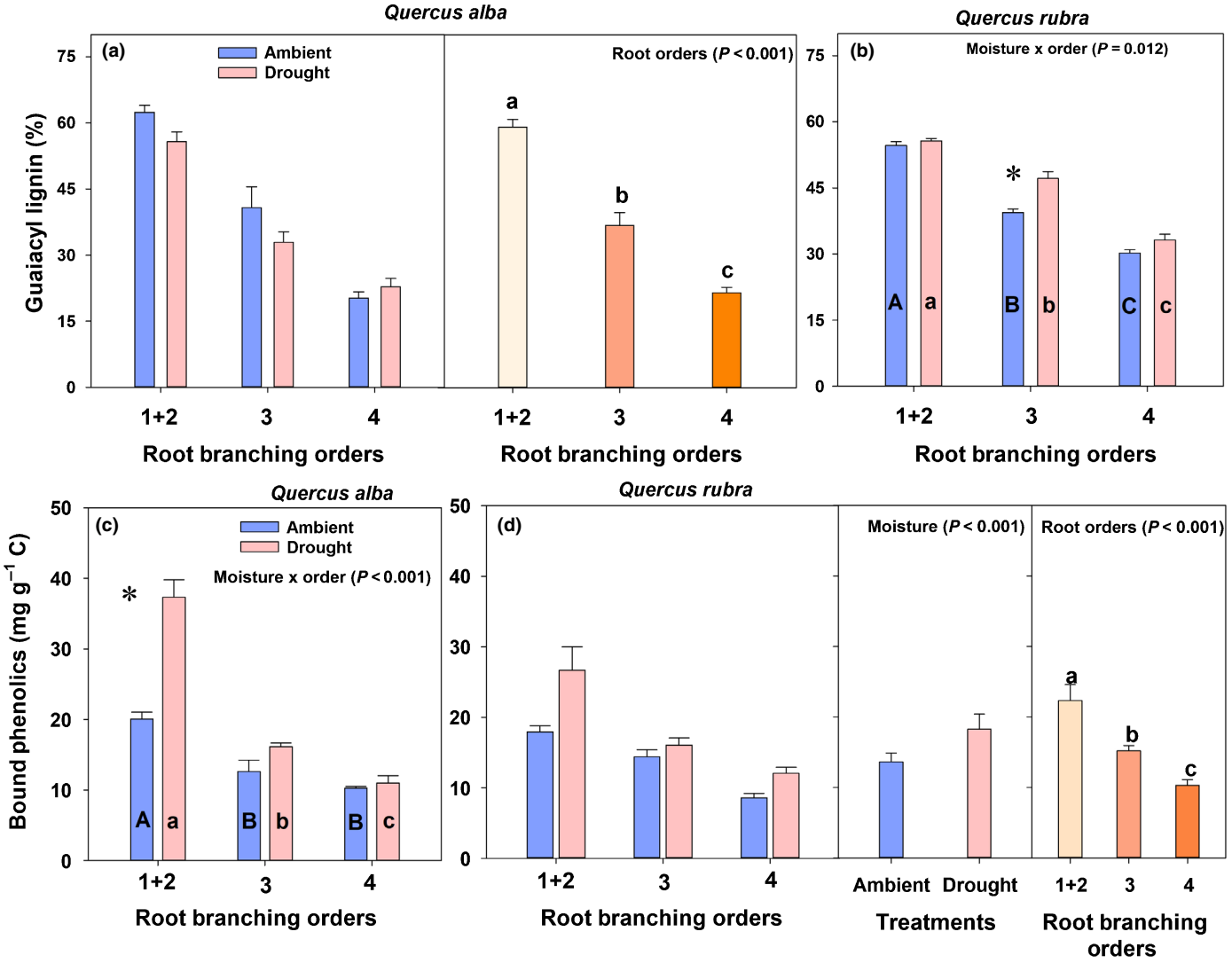

Fig. 8 Guaiacyl lignin and bound phenolics in the fine roots of different orders of *Quercus alba* (a, c) and *Quercus rubra* (b, d) in the ambient and drought treatments. Values are means ± SE ($n = 5$). Bars with different uppercase letters indicate a difference (Tukey's honestly significant difference (HSD)) between ambient treatments along root orders, and bars with different lowercase letters indicate a difference between (Tukey's HSD) drought treatments along root orders. Asterisks indicate a difference between the ambient and drought treatments within a root order. (d) Bars with different lowercase letters indicate a difference between root orders. Root branching orders: 1+2, first + second orders; 3, third order; 4, fourth order.

rates and the contribution of C to different soil organic matter pools (Mao *et al.*, 2011; Minerovic *et al.*, 2018).

Chemical construct of fine roots varies across the root branch orders

Different orders of fine roots vary substantially in their form and function (McCormack *et al.*, 2015). However, the underlying and/or ensuing changes in the chemical composition across these root orders might play a pivotal role in facilitating the resource uptake function of lower-order roots, and transport functions of the higher-order roots. We hypothesized that, similar to the functional and morphological differentiation across the fine-root order, the chemical construct of the fine roots will also be root order-specific. The compound-specific analysis of root orders supported the hypothesis that root chemical traits of the lower-order roots were reflective of the acquisition strategies. However, the magnitude of change varied between species and the compound classes.

The quantity of SVC lignin was lowest in the first + second-order roots and increased with increasing root order. This observation agrees with the related function of these root orders, where higher lignification along the xylem walls of the higher-order roots provides hydrophobicity that maintains the integrity of the water column in response to transpiration pull (Lourenco *et al.*, 2016; Kang *et al.*, 2019). A blanket deposition of this hydrophobic heteropolymer across all root orders in response to drought would significantly reduce the absorptive function of the lower-order roots, thus compromising the overall plant performance. The lower lignin quantity in lower-order roots could be partly regulated by ontogeny. As the metabolically active lower-order roots are also rich in N, and thus are more susceptible to pests

and pathogens, the overall protection of these organs that are quantitatively low in lignin could be enhanced by changing the composition of the lignin polymers (Lourenco *et al.*, 2016; Suseela & Tharayil, 2018). In our study, this was evident from the monomer composition of lignins across the root order. Even though the quantity of lignins in the distal first + second-order roots was lower, these lower-order roots were proportionately abundant in guaiacyl monomers (G) of lignin. As the 5C position of the phenolic ring is open for coupling reactions in G-units, the guaiacyl-rich lignins tend to be more branched, as compared with linear organization of syringyl-rich lignins. These condensed lignins that are relatively rich in C–C interunit linkages are more resistant to enzymatic depolymerization (Li *et al.*, 2016), and hence could provide greater protection to the root tissues against pathogens. Thus, despite a lower total lignin content, the lower-order roots could be better protected by the observed compositional changes in lignin. The lack of influence of drought on the lignin composition highlights the overriding effect the ontogeny may have on the monomer composition of lignin.

Another factor that would enhance the lignin-derived recalcitrance of the tissue is the extensive bridging of the labile cellulosic matrix and recalcitrant lignin matrix by monophenols through the ester–ether linkages (Carpita, 1996). Thus, the observed higher abundance of bound phenols per unit of lignin in distal orders of roots in both species highlights another potential avenue by which organs that are lower in the overall lignin quantity are effectively protected- through the greater integration of lignocellulosic matrix. Additionally, tannins could also confer protection to the lignocellulosic matrix through their antifeedant and pro-oxidant activity (Top *et al.*, 2017). The species-specific higher abundance of total tannins in the lower-order roots of *Q. alba* could provide higher chemical protection to these roots without compromising their flexibility that is essential for navigating the soil matrix. This overall uniqueness of the chemical construct of fine-root orders unambiguously points to a sophisticated strategy in plants that maximize the resource uptake functions while concurrently adapting to unfavorable biotic and abiotic soil conditions. Our results indicate that the root order-specific tissue chemistry would protect the N-rich, lower-order roots from biotic stressors without compromising their resource uptake efficiency. The observed higher integration of lignocellulosic matrix along with the high amount of defense compounds, including tannins in the lower-order roots, could partly explain the lower decomposition rate of these root orders (Sun *et al.*, 2018) despite their lower lignin and higher N content.

Drought stress alters the chemical plasticity of fine roots across root orders and heteropolymers

The effect of drought on the chemical plasticity of fine roots was more evident from the quantity and composition of tannins than of lignins. The total lignin content in both species across the fine-root orders was unaffected by drought. The proportion of G units was also similar between drought and ambient treatments, except for the third-order roots of *Q. rubra*, indicating that, unlike in leaves, in roots, the total content and monomer composition of lignins are less affected by drought. However, the content of bound phenolics was influenced by drought, and the two-fold higher bound phenolics in the lower-order roots exposed to drought could reflect an extensive integration of cellulose-lignin matrix under environmental stress.

Unlike lignins, there were extensive changes in the quantity and composition of tannins in fine roots exposed to drought, and these changes were branch order- and species-specific. Although low in total tannin content compared with *Q. rubra,* the more drought-tolerant *Q. alba* exhibited drought-dependent compositional variation of tannins. The similar content of total tannin in lower-order roots of *Q. alba* across the treatments was facilitated by a doubling of HTs and a proportional decrease in CTs under drought. This pattern was reversed in third-order roots where the proportional abundance of HTs doubled in drought treatments, whereas CTs showed only a marginal increase. These changes in both quantity and composition of tannins highlight the ability of *Q. alba* to modulate the plant chemistry in the face of drought. Also, this reciprocal shift in abundance between HT and CT fits well with the biosynthesis of these two macromolecules. The biosynthesis of gallolyated HTs branches from 3-dehydroshikimic acid in the upstream shikimate pathway, whereas the CTs are biosynthesized from the phenylpropanoid pathway which is downstream of the shikimate pathway (Ossipov *et al.*, 2003; Suseela & Tharayil, 2018). Thus, preferential C allocation to one of the compounds would decrease the production of the other .

Within the ETs, the changes in chemical composition in response to treatments were evident at a much finer level. Irrespective of the root order, the composition of ETs in *Q. alba* showed a similar effect of the drought treatment where the first + second- and third-order roots of the drought treatment clustered separately from the first + second- and third-order roots of the ambient treatment. By contrast, the composition of ETs in *Q. rubra* changed with the root order irrespective of the drought treatment. Castalagin and vescalagin are the predominant ETs in oak species, and these ETs provide antimicrobial properties to oak wood (Zhang et al. 2015). The decrease in the content of castalagin, the most dominant ET in *Q. alba*, along with other major ETs in the first + second- and third-order roots developed under drought, indicate a modulation of chemical defenses in these lower-order roots. It is plausible that this observed down-regulation of ETs that have antimicrobial properties (Yoshida *et al.*, 2009) would be a strategic adaptive response in lower-order fine roots to facilitate symbiotic association with soil microbiota. This was supported by the higher colonization of EcM fungi in the first + second-order roots of *Q. alba* exposed to drought. The concomitant upregulation of CTs that have a lower pro-oxidant but a higher anti-oxidant activity (Barbehenn *et al.*, 2006) in these lower-order roots further supports this notion. The primary antimicrobial property of ETs is through the chelation of metals, especially iron, which is critical for microbial metabolism (Mila *et al.*, 1996). Compared with the ambient treatment, the flavano-ET mongolicain, which is a dehydrogenerative oxidation product of acutissimin (Pouysegu *et al.*, 2011), was the only ET abundant in lower-order roots developed under drought. The root order-

specific compositional similarity of ETs irrespective of the drought treatment in *Q. rubra*, along with the low EcM colonization of these roots, supports the key role that ETs might have in regulating the mycorrhizal colonization. Overall, the profile of ETs as influenced by the root order and drought depicts the modulation in chemical defense that potentially underlies EcM colonization of roots – the ET content in *Q. alba*, the more drought-adapted species, was downregulated with a compositional shift towards ETs that facilitate better EcM colonization. By contrast, the less drought-tolerant species, *Q. rubra*, did not exhibit a drought-induced compositional change of ETs.

Along with the compositional changes, the localization of tannins was influenced by the drought treatment. About 80% of the CTs across fine root orders and treatments in *Q. alba* were bound to the cell wall fiber, and the droughted treatment has proportionally higher CTs bound to the cell wall in first + second root orders. The greater integration of CTs to the cell wall would provide greater defense capacity and potentially greater drought resistance (Top *et al.*, 2017). The integration of CT to the cell wall matrix is similar in function to the higher proportion abundance of G lignins and bound phenolics, and the decrease in ETs in first + second root orders of *Q. alba* that was observed in this study. These changes in the composition and localization would provide greater integrity and protection to the lower-order roots without compromising absorption function and flexibility, while facilitating EcM colonization. Also, the higher percentage of fiber-bound CTs across fine-root orders in *Q. alba* can protect these roots long after their senescence, thus resulting in slower decomposition of these roots.

Chemical construct of fine roots: implications for root decomposition

The observed changes in the chemical construct of fine roots across root orders and when exposed to environmental stress such as drought may have potential implications for the decomposition of these roots. Despite the lower C : N, lignin : N content and nonwoody structure, which collectively should facilitate more rapid tissue decomposition, the distal first- and second-order roots (younger) decompose more slowly than third- and fourth-order roots (older) with a higher C : N (Hobbie *et al.*, 2010; Olajuyigbe *et al.*, 2012; Sun *et al.*, 2013, 2018). Recently, it was reported that the decomposition rate of the first-order roots was negatively correlated with the concentration of both bound phenolics and CT in their tissues (Sun *et al.*, 2018). From our results, it can be postulated that the lower decomposition of the first- and second-order roots could be partly a result of a more efficient integration of the lignocellulosic matrix that is made possible by the chemical plasticity across root branch orders and environmental variables.

Conclusion

The advantage of quantifying root traits under controlled conditions could also imply certain limitations when compared with natural field conditions as root traits can differ in the field as a result of environmental conditions and ontogeny (Mokany & Ash, 2008; Freschet *et al.*, 2017). Our study has some limitations as it is a greenhouse study conducted with seedlings compared with mature trees under natural field conditions; however, mutiple root traits of seedlings correlate well with that of mature trees (Kramer-Walter *et al.*, 2016). Our study presents several novel results related to the chemical plasticity of fine roots across root orders and drought. Most notably, our results reveal that, unlike leaves where lignin generally increases with drought, in fine root orders the drought did not alter the content of lignin . Although lignin content was lower in the lower-order roots, these roots were better protected as a result of higher integration of the lignocellulosic matrix, facilitated by the proportional abundance of G-lignin and bound phenols, and condensed tannins. The adaptation to drought was partly facilitated by changes in the content and composition of tannins, particularly in the acquisitive roots of the more drought-tolerant *Q. alba*, which potentially facilitated greater mycorrhizal association. Overall, our results revealed the uniqueness of the chemical construct of fine roots, which unambiguously points to a sophisticated strategy in trees that maximize the resource uptake functions while concurrently adapting to unfavorable biotic and abiotic soil conditions. The chemical plasticity of different fine-root orders has significant implications for the adaptation of trees to drought stress, and hence forest productivity and soil C sequestration.

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Author contributions

VS designed and conducted the experiment, analyzed the data, and wrote the draft of the manuscript. NT performed the high resolution mass spectrometry analysis and data interpretation. GO and DH performed the confocal microscopy imaging. All authors commented on the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Soil volumetric water content for the experimental period.

Fig. S2 Roots of *Quercus alba* seedlings subjected to ambient and drought treatments.

Fig. S3 Specific root area of the fine roots of different orders of *Q. alba* and *Q. rubra* across ambient and drought treatments.

Fig. S4 PLSDA and VIP scores of the composition of ellagitannins of different orders of fine roots of *Q. alba* exposed to ambient and drought treatments.

Fig. S5 PLSDA and VIP scores of the composition of ellagitannins of different orders of fine roots of *Q. rubra* exposed to ambient and drought treatments.

Fig. S6 Syringyl lignin in the fine roots of different orders of *Q. alba* and *Q. rubra* in the ambient and drought treatments.

Fig. S7 Percentage N and C : N ratio in the fine roots of different orders of *Q. alba* and *Q. rubra* across ambient and drought treatments.

Methods S1 Composition of ellagitannins.

Methods S2 Bound phenolics and lignin.

Methods S3 Phenol derivatization and GC-MS quantification.

Table S1 Root : shoot ratio and specific leaf area of *Quercus alba* and *Quercus rubra* exposed to ambient and drought treatments.

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