MODULATION OF GROWTH AND DEVELOPMENT OF TREE ROOTS IN FOREST ECOSYSTEMS

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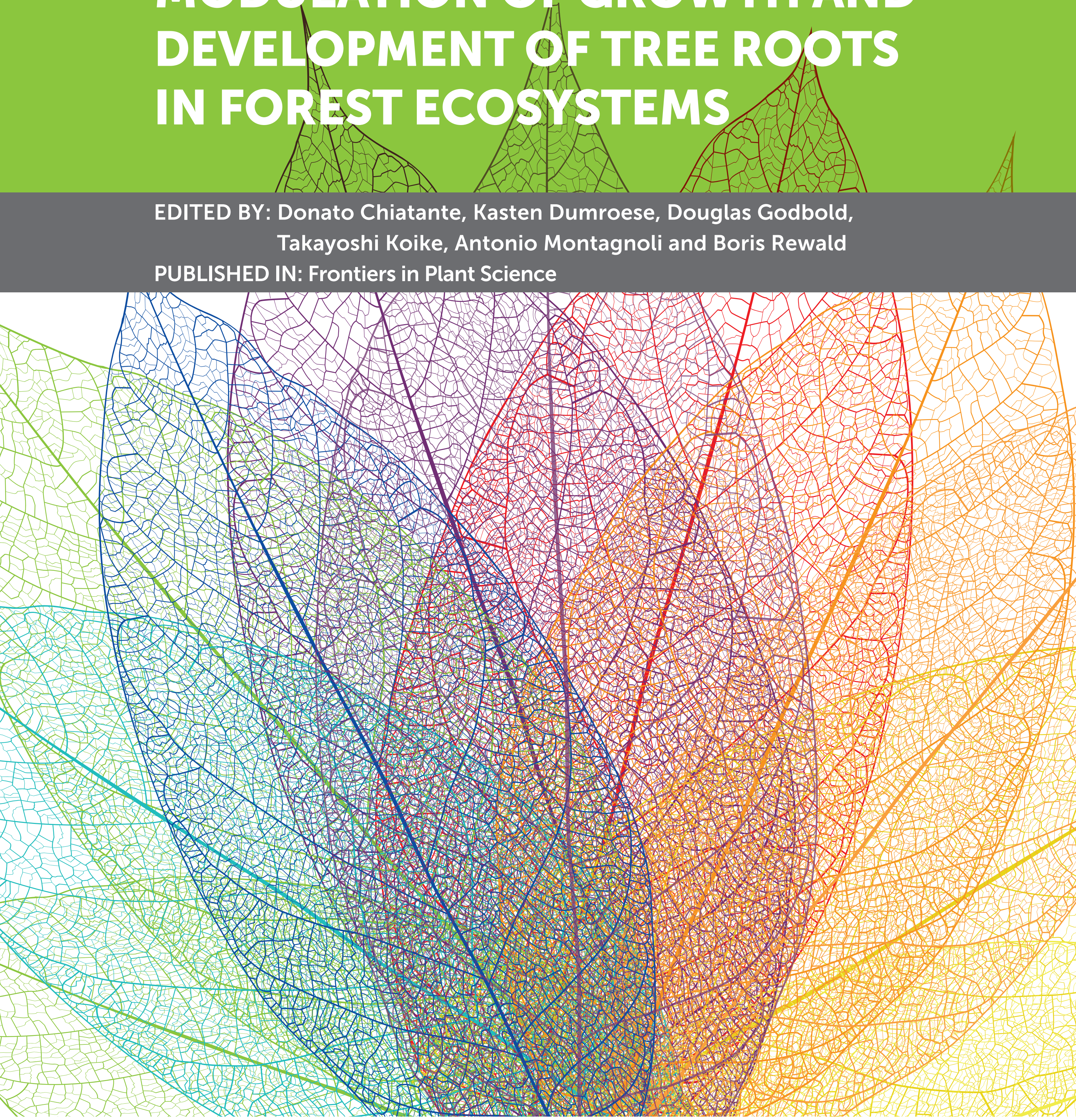

frontiers Research Topics

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MODULATION OF GROWTH AND DEVELOPMENT OF TREE ROOTS IN FOREST ECOSYSTEMS

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Editorial: Modulation of Growth and Development of Tree Roots in Forest Ecosystems

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Editorial on the Research Topic

Modulation of Growth and Development of Tree Roots in Forest Ecosystems

More research on the above-ground compartment of plants has been completed compared to the belowground plant compartment. Much of this disparity is likely ascribable to the methodological difficulties of studying plant roots, the associated rhizosphere, and related traits (Freschet et al., 2021a). During the past two decades, a focus on methodological advancements has rendered root investigations more feasible and more standardized in terms of quantitative approaches (Bao et al., 2018; Montagnoli et al., 2018; Atkinson et al., 2019; Chiatante et al., 2019; Cabal et al., 2021; Freschet et al., 2021b). This has allowed and encouraged a greater number of scientists to examine the hidden half of plants, thereby helping to reduce this long-standing knowledge discrepancy (Ryan et al., 2016; Freschet et al., 2021b).

These recent studies covered a wide range of root aspects, spanning from sub-cellular levels (Jung and McCouch, 2013; Wachsman et al., 2015; Slovak et al., 2016; Chiatante et al., 2021) to the community and ecosystem scales (Hopkins et al., 2013; Li et al., 2018; Freschet et al., 2021b). In association with the overall increase of root studies, scientific journals have published several thematic special issues covering diverse viewpoints, from a fundamental biological approach to identifying root phenotypes for a better understanding of responses to environmental cues. Finally, the accumulation of data regarding root system traits led to the establishment and development of databases representing local to global scales (Kattge et al., 2020; Guerrero-Ramirez et al., 2021; Iversen et al., 2021; Montagnoli et al., 2021), allowing scientists to perform reviews and meta-analyses and create new developmental models that enhance our understanding of root complexity in terms of dynamics, form, and function (Wang et al., 2018, 2019, 2021; Carmona et al., 2021).

The evidence of the effects of global climate change on vegetation in general, and on root systems in particular, continues to challenge our understanding (Norby and Jackson, 2000; Brunner et al., 2015; Zhang et al., 2019; Masyagina et al., 2021). Although forests represent an important ecosystem for reducing the impacts of climate change, climate change itself and the environmental stressors exacerbated by it (e.g., fire, drought, wind, and flooding) strongly affect forests and the services they provide (Wagner et al., 2014; Locatelli, 2016; Nordström et al., 2019). Analyzing the effects of these factors on the growth and development of single trees, and their complex organization into forest, is necessary for understanding and increasing the resilience of this ecosystem. Responses of forest ecosystem functions to climate change are strongly linked to changes in compositional and structural complexity (Ehbrecht et al., 2021). Indeed, a more complex forest ecosystem may respond differently to new and variable conditions, enhancing both ecosystem functional stability and services sustainability (Brockerhoff et al., 2017; Messier et al., 2019). This functional tree network is dependent on below-ground complexity (i.e., root plasticity and symbiotic relationship of roots with microbes such as fungi) and its integration with the above-ground structure (Freschet et al., 2021b). Unfortunately, despite more work being reported on roots, knowledge of the below-ground forest remains scarce, requiring an in-depth understanding of root function, complexity, and relationship with microbes. Thus, the 12 papers within this Frontiers in Plant Science Research Topic represent a contribution toward amplifying the knowledge regarding tree root development at the single–tree and forest levels. The aim is to reveal the impact of environmental stressors on root growth and how the sum of these impacts at the individual tree level may endanger the performance and resilience of entire forest ecosystems.

Seven manuscripts focus on the relationship between roots and water, revealing how plants plastically respond to the changing of water availability through the modulation of different root traits. In particular, Lihui et al., in regions with different precipitation regimes and slope aspects, highlighted how variations in the belowground rooting depth may be a key functional trait determining plant survival and growth in drought-prone regions. Similarly, Fujita et al., showed how potted seedlings subjected to different depths of waterlogging adapt to hypoxic conditions by changing the depth of their fine root distribution. Seedlings subjected to different drought and soil substrate conditions in pots maintained a low cost-benefit ratio in root system development under drought conditions by varying the morphological traits of different fine root branching orders and non-structural carbohydrate content (Ji et al.). In line with these findings, a forest-scale study with trees exposed to extreme drought conditions revealed that the responses of fine roots of different species are not linked to a sole ecological strategy (Nikolova, Bauerle et al.). Using a split-pot design to apply spatially heterogeneous soil moisture, Hart et al. discerned a differential mass or reserve allocation between above- and belowground organs as well as within the root system, suggesting that different portions of plant organs might respond autonomously to local conditions. A shovelomics approach within afforestation stands of arid lands showed that root biomass was correlated positively with water availability and negatively with fertilizer application (Nyam-Osor et al.). Finally, Withington et al., in a common garden experiment with different temperate tree species, suggested that annual absorptive fine-root growth response to extreme precipitation or drought events can be exacerbated across years through a "legacy effect."

The response of roots and their microorganisms to forest management activities is another important Research Topic. Koczorski et al. in a short rotation coppice system found that the main factors controlling fungal communities were soil properties and the level of fungal community association with the trees. In a mixed stand, Veselá et al. showed how management after disturbance (i.e., windstorm) significantly affected the composition of ectomycorrhiza on a species-specific basis but did not affect the potential connections of trees via their ectomycorrhizal symbionts. Nikolova, Geyer, et al. found that subdominant trees on the edges of strip cuttings presented a strong increase of root growth relative to shoot growth that persisted 7–8 years and probably supported the need to enhance wind firmness through improving root anchorage. Differences and/or similarities between stems and roots in response to mechanical constraints were examined by De Zio et al. They found an antagonistic interaction of auxin and cytokinin signaling that was highly organ-dependent and resulted in the production of reaction wood on the stretched side of stems and on the compressed side of roots. Finally, using the ingrowth core method, Noguchi et al. found that fine root growth of understory plants was increased by Sphagnum moss. This finding is key to better understanding belowground carbon dynamics, especially in forests sites where the shallow permafrost layer varies in thickness.

Together, these 12 studies provide new evidence on how roots, rhizospheres, and microorganisms are coordinated with the aboveground tree organs into an integrated whole organism that responds and adapts to changing environmental conditions. This research offers important insights into the ecosystem function of current and future forests. Yet, however, a main challenge in tree root research is to gain a complete understanding framework of integrated responses to the constant change of environmental conditions from the molecular level upscaled to tissue, single organism, and forest community scales, the later requiring a comprehension of biological communication mediated by microorganisms. This would lead to a more detailed understanding of carbon dynamics and carbon use efficiency of the entire tree and forest.

AUTHOR CONTRIBUTIONS

AM conceived and produced the first draft. BR provided important insights to the first draft. DC, DG, and TK commented on the final draft. AM and RKD equally contributed to all revisions leading to the final manuscript.

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Fine-Root Traits Reveal Contrasting Ecological Strategies in European Beech and Norway Spruce During Extreme Drought

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Trees adjust multiple structural and functional organ-specific characteristics, “traits”, to cope with diverse soil conditions. Studies on traits are widely used to uncover ecological species adaptability to varying environments. However, fine-root traits are rarely studied for methodological reasons. We analyzed the adaptability of the fine-root systems of European beech and Norway spruce to extreme drought within species-specific tree groups at Kranzberger Forst (Germany), focusing on the seasonality of morphological, physiological, and biochemical key traits in view of carbon (C) and nitrogen dynamics. We hypothesized that fine roots of both species adjust to seasonal drought: with beech representing a “fast” (i.e. with fast C turnover), and spruce a “slow” (i.e. with long-term C retention) ecological strategy. We identified three functional fine-root categories, based on root function (absorptive or transport fine roots), and mycorrhizal status of the absorptive fine-roots (mycorrhizal or non-mycorrhizal). Solely the non-mycorrhizal absorptive roots adjusted in a species-specific manner supporting fine-root ecological strategy hypothesis. During drought, beech produced thin ephemeral (absorptive non-mycorrhizal) fine roots with high specific fine-root area and high respiratory activity, representing fast C turnover and enabling effective resource exploitation. These adjustments reflect a “fast” ecological strategy. Conversely, spruce absorptive fine roots did not respond to the soil moisture deficit by growth but instead increased root suberization. Drastically lowered respiratory activity of this functional category facilitated C retention and structural persistence during drought, indicating a “slow” ecological strategy in spruce. Absorptive mycorrhizal fine roots maintained respiration throughout the drought event in both tree species, but in spruce this was the only fine-root category with high respiration. This suggests, that spruce relies heavily on mycorrhizal associations as a method of drought resistance. Accumulation of non-structural carbohydrates and high C concentrations were observed in the transport fine roots of both species, indicating drought-induced osmotic protection of these roots. Thus, functional classification enabled us to determine that fine-root branches of each species are not tied to one sole ecological strategy. The suggested approach helps to better understand the complex interplay between structure and function belowground.

Keywords: ecological strategy, extreme drought, *Fagus sylvatica*, fine-root classification, fine-root traits, morphology, non-structural carbohydrates, *Picea abies*

INTRODUCTION

Plants and especially long-lived trees have evolved a variety of structural and functional characteristics ("traits") both above- and belowground to optimize the use of heterogeneous spatiotemporal resources (Lavorel and Garnier, 2002; Rennenberg et al., 2006; Freschet et al., 2018). Analyses of plant traits make it possible to decipher species-specific trade-offs in adapting to resource limited site conditions (Iversen et al., 2017; McCormack et al., 2017; Brunner et al., 2019). Belowground traits include root morphology and physiology along with mycorrhizal associations in relation to seasonal and soil variation (Laliberté, 2017). Although more than 300 root traits have been identified across individual studies (Iversen et al., 2017), they remain underrepresented in global trait databases (Ma et al., 2018). Frequently, the lack of root trait data is a direct result of methodological sampling difficulty (Joslin et al., 2000; Pregitzer, 2002; Brunner et al., 2015), challenges in integrating outcomes from varying environments, diverse measurement techniques and complex species-specific stress responses (Iversen et al., 2017).

Fine roots, commonly defined as <2 mm in diameter (Böhm, 1979), are the most physiologically active plant components of a root system. Shifts in fine-root diameter can serve as a proxy for root water/nutrient uptake capacity (Zobel et al., 2006; Tobner et al., 2013). However, studies often fail to determine diameter thresholds that indicate changes in root function but, instead, tend to rely on arbitrary thresholds. The thinnest fine-root fraction (e.g., < 1 mm) can better reflect belowground adjustments to resource availability (Leuschner et al., 2001; Zobel et al., 2007). Such thin fine-root laterals, typical of many deciduous tree species, sometimes referred to as fibrous or feeder roots (Sutton and Tinus, 1983), do not undergo secondary growth, are short-lived, and display high N concentration and respiration rate (RR). These laterals are ephemeral, turning over at rates similar to deciduous leaves (Eissenstat et al., 2013). Thin highly ephemeral roots typify a "fast-strategy" and represent one extreme of the whole-plant economic spectrum (Reich, 2014, but see also Withington et al., 2006). While, coarse, slow-growing fine-roots exemplifies a "slow-strategy" (Wang et al., 2016; Leuschner and Meier, 2018). Perennial plants with fine-roots of similar diameter may differ in form and function, which makes diameter-based root trait and biomass data difficult to interpret (Iversen et al., 2017). Therefore, an alternative classification based on defined functional groups provides an improved alternative when comparing across species and sites (Freschet and Roumet, 2017).

The "fast-slow" plant economics spectrum defined by Reich (2014) depicts a range of adaptive organ-specific strategies as basic ecological features of plant life forms. According to Reich (2014), plant organs, i.e. roots and shoots of an individual species, should conform to a resource use strategy with implications for whole-plant performance and community assembly (but see Tobner et al., 2013). Species with rapid resource turnover, so-called "fast" species (Reich, 2014), produce short-lived organs for rapid resource capture and translocation. "Slow" species, however, have long-lived organs but prolonged retention of resources. In the context of roots, "slow" species should possess long-lived fine-roots, low specific fine-root length (SRL, among other related traits) and strongly rely on ectomycorrhizal associations (ECM) (Agerer, 2001; Brundrett, 2002; Bergmann et al., 2020).

We aimed to determine if fine-root strategies of adult beech and spruce trees follow similar ("fast" vs. "slow") patterns as those found in the leaf economics spectrum. This study utilized the prolonged, extraordinarily hot and dry, summer conditions that prevailed over wide regions of Western and Central Europe in 2003 (Rebetez et al., 2006) including the study site, Kranzberger Forst (Freising, Germany) (Raspe et al., 2004). Fine-root production and fine-root recovery rate were unaffected in beech during drought in 2003 (Nikolova et al., 2009). In contrast, fine root production and biomass recovery rate decreased by almost a factor of six in spruce during the drought year. We therefore hypothesized that beech and spruce represent belowground "fast" and "slow" plant strategies with corresponding fine-root traits. To this end, we examined the fine-root carbon (C) and nitrogen (N) status, morphological parameters, [fine root diameter (D), specific fine-root area (SRA)], and physiological parameters, [fine-root RR and concentration of non-structural carbohydrates (NSC)] in response to seasonal drought. Fine-root samples were classified into three categories, based on fine-root function and mycorrhizal abundance. This classification enabled (1) the quantification of seasonal progressive drought responses within functionally defined fine-root classes, and (2) an *in situ* comparison of fine-root traits between beech and spruce to distinguish underlying mechanisms of belowground drought adaptation.

MATERIAL AND METHODS

Site Conditions and Climate

The study was conducted at a mixed European beech-Norway spruce (*Fagus sylvatica* L./*Picea abies* [L.] H. Karst) stand at

Kranzberger Forst near Freising, Germany in 2003 (Matyssek et al., 2010). The site is composed of two groups of approximately 70-year-old beech trees, each surrounded by spruce trees, which were taller although younger by about 20 years (Pretzsch et al., 2010; Häberle et al., 2012). Rooting depth was about 1 m in a Luvisol (FAO classification) which had developed from Loess over Tertiary sediments and limited to approximately 1 mby a compacted hardpan layer. Litter layer depth was about 5 cm under the spruce canopy and 3 cm under the beech. The C:N ratio within the upper 10 cm of soil ranged between 14 and 17, with the highest values occurring under beech (Schuhbäck, 2004). Soil nutrients and water were non-limiting during average growth years.

The study site is classified as temperate based on a 30-year record (1971–2000) of mean daily air temperature (T) and annual precipitation (P) (7.8°C and 786 mm, respectively), with periods of snow cover between December and February (Nikolova et al., 2009). During the 2003 growing season, extreme weather conditions were recorded at Kranzberger Forst (Nikolova et al., 2009): mean T was 3.2 °C higher, and P was 30% lower relative to the long-term seasonal averages. In the same year, a drought period occurred from August through September (**Figure 1**), imposing tree water limitations during the late summer. Soil moisture differed between the two tree species starting in the spring of 2003 (Nikolova et al., 2009), a direct result of the ability of spruce to take up and transpire water before beech flushed its leaves (Beier, 1998). Available soil water was completely depleted in 2003 under spruce by mid-July, under beech by mid-August, respectively. This resulted in a longer period of exhausted soil water availability for spruce (i.e., 75 d in spruce vs. 45 d in beech). Additionally, soil temperature ($T_{(0)}$) was monitored at 0 cm soil depth, i.e. at the border of the humus layer and mineral soil.

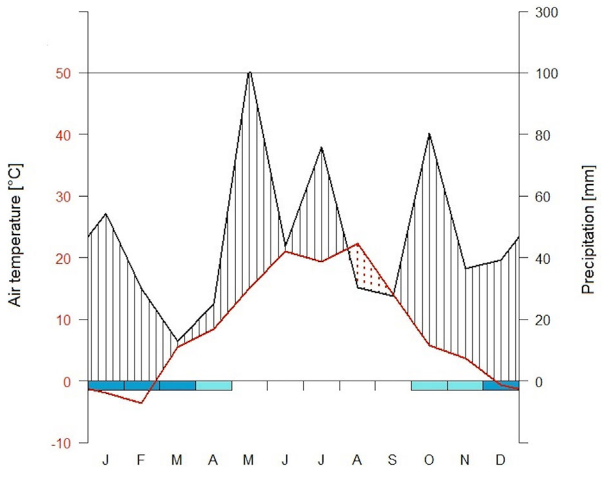

FIGURE 1 | Climate diagram at Kranzberger Forst during the study year of 2003. Scales of air temperature T (red line) and precipitation P (black line) according to Walter and Lieth (1960), i.e. 10°C correspond to 20 mm, respectively. Arid period (when P < 2×T) is filled in dotted red vertical lines, wet periods are filled in black lines. Cold months (when absolute daily minimums ≤ 0°C) are shown in dark blue, probable frost months (when absolute monthly minimums ≤ 0°C) are shown in light blue [using the R package climatol (Guijarro, 2020)].

Fine-Root Categories and Sampling

Measurements of fine-root parameters were organized in four sampling campaigns: spring (April, May), early summer (June, July) with exhausted soil water only under spruce, late summer (August) with exhausted soil water under beech and spruce, and autumn (October, November) when available soil water partially recovered under both tree species (Nikolova et al., 2009).

To characterize fine-root trait differences between beech and spruce, three functional fine-root categories were distinguished among sampled fine-root branches that reflect the commonly used < 2 mm in diameter classification, further called "rootlets" based on (1) root function (absorptive or transport fine roots), and (2) mycorrhizal status of the absorptive fine roots (mycorrhizal or non-mycorrhizal) (**Figure S1**):

- Absorptive foraging fine roots (FR): fast-growing, non-mycorrhizal fine roots with primary xylem, primarily serving for soil exploration (Guo et al., 2008; Zadworny and Eissenstat, 2011);
- Transport fine roots (TR): non-mycorrhizal fine roots with secondary xylem which fulfill the role of water transport and starch and nutrient storage (McCormack et al., 2015);
- Absorptive mycorrhizal fine roots (MR): intensely branched fibrous fine roots, enlarging the plant absorptive surface by related symbionts (Agerer, 2001; McCormack et al., 2015).

In the experimental forest site, 7–10 sampling positions were randomly selected and marked within both beech and spruce groups. At each sampling position, one rootlet was entirely extracted from the topsoil, i.e. from the humus layer and the upper 10 cm of the mineral soil. Rootlets were subdivided into the three fine-root categories and dried to a constant weight, DW (g) (i.e., DW_{FR}, DW_{TR}, and DW_{MR}) during the four sampling campaigns in 2003.

Dry masses were used to calculate the proportion of functional fine-root categories to individual measured parameters (e.g. RR, C, N, NSC) for an individual rootlet for each sampling campaign and tree species.

$$X^i = \frac{X_{FR} \times DW^i_{FR} + X_{TR} \times DW^i_{TR} + X_{MR} \times DW^i_{MR}}{DW^i} \qquad (1)$$

where X^i is the parameter X calculated for the rootlet *i*; X_{FR}, X_{TR} and X_{MR} are parameter levels each as derived from sub-samples of the fine-root categories FR, TR, and MR; $DW^i{}_{FR}$, $DW^i{}_{TR}$, and $DW^i{}_{MR}$ are dry masses (g) each of fine-root categories within a rootlet *i*, with DW^i as total rootlet dry mass.

In some cases the transition from absorptive to transport fine-roots occurs gradually and may vary across species (McCormack et al., 2015). In a small pre-experiment, we determined fine root anatomy for our species to identify functional breakpoints. To this end, serial transverse sections (50 µm, from distal to proximal) were obtained in May and August on 7–10 fine-root segments of each tree species using a cryomicrotome (Frigocut, Reichert-Jung, Heidelberg, Germany). After staining with safranin and astrablue, the fine-root cross sections were mounted on glass slides and examined with a Trinocular Phase Contrast microscope (Leitz ARISTOPLAN, Leitz Meßtechnik GmbH, Germany) equipped with a digital color camera system (KAPPA model CF 20/4 DX; Kappa GmbH, Gleichen, Germany) and Kappa ImageBase 2.2 software. Anatomical study was, however, not in focus of the present investigation.

Measurement of Fine-Root RR

The fine-root RR (nmol CO_2 g^{-1} s^{-1}) was measured by differential infrared gas analysis, IRGA (CIRAS-2, PP-Systems, UK) in combination with an open-chamber system (PLC Conifer, PP-Systems, UK). The window size of the conifer cuvette (70 x 50 mm) allowed RR measurement of fine-root sub-samples with a fresh weight ≤ 0.6 g. Corresponding sub-samples were taken from each fine-root category, cleaned by brushing off soil particles and dead root ramifications, and transferred into mesh bags each (60 x 40 mm, mesh size of 50 µm) to protect the analyzer from contamination. Empty bags were also run in preceding tests to ensure the absence of air contamination. Each mesh bag with its root sub-sample was immediately placed in the IRGA cuvette, operated with air humidity fixed to 90%, flow rate of 0.2 l min^{-1}, and incoming CO_2 concentration of 400 µl l^{-1}. No CO_2 contamination was detected in empty cuvettes with concentrations between 400 and 1,000 µl l^{-1} (also see Burton and Pregitzer, 2002). Cuvette temperature (T_c) was set according to the measurement protocol (see below, ca. 7, 15, and 22°C). Root respiration was recorded upon stabilization, within 3–5 min after closing the cuvette. Since fine roots were not rinsed before measurements, microbial respiration included in the measurement RR, but was likely negligible (i.e. < 5% of RR, according to Burton and Pregitzer, 2003). Rates of microbial respiration per mass unit of soil debris are orders of magnitude lower than those of respiring, mass-related root tissue (Zak et al., 1999).

The relationship between fine-root development (see methodological details in Nikolova et al., 2006) and weather conditions (data not shown) permitted data pooling in four sampling campaigns in 2003, i.e., of April 15, April 24 and May 9 (spring), June 27 and July 4 (early summer), August 22 and 24 (late summer), and October 28, 30, and November 3 (autumn). Each time, RR was assessed for each fine-root category and tree species. Three T_c levels (7, 15, and 22°C) were applied to determine the temperature response of RR. To this end, extracted rootlets were covered with wet paper, transported to the lab in plastic bags and stored at approximately 12°C until measurement, within 3 h after sampling. Preliminary tests found RR remained stable within that time period. On each sampling date, three to six RR replicates were measured per root category, tree species and T_c level. A fresh root sub-sample was inserted at each temperature change. The RR response to T_c was examined for each fine-root category and sampling date by exponential regressions using *van't Hoff* equation:

$$RR = \rho \times e^{\theta \times Tc} \tag{2}$$

where ρ and θ are model coefficients, and RR is the RR of the respective fine-root category in beech or spruce. By means of Eq. 2, RR of each fine-root category was normalized to T_c = 10°C (i.e., RR^{10}). RR^{10} was then calculated for each excavated rootlet and sampling date (Eq. 1). In addition, respiratory Q_{10} were determined for each fine-root category:

$$Q_{10} = e^{10 \times \theta} \tag{3}$$

with Q_{10} as the RR response to 10°C temperature change, and θ as model coefficient. Short-term Q_{10} per each sampling date was distinguished from seasonal long-term Q_{10} (Burton and Pregitzer, 2003). A Q_{10} of about 1.0 reflects low temperature dependence of RR and thus low metabolic activity of roots (e.g., dormancy), whereas high Q_{10} indicates highly active metabolism. RR upon determination for each fine-root category (nmol CO_2 g^{-1} s^{-1}) was scaled to the entire rootlet (Eq. 1). In September 2004, a RR reference measurement was taken at a temperature of 10°C in the three beech and spruce fine-root categories.

Fine-Root Morphology

Four to six root samples per fine-root category of both beech and spruce were optically scanned after RR analysis (Scanner STD4800, Regent Instruments Inc., Canada). The scanner had an optical resolution of 300 dpi and pixel size of 0.085 mm which allowed measurements of root diameters > 0.18 mm (Biernacki and Lovett-Doust, 2002). Stored digital images where processed in batch mode using WinRHIZO™ Pro analysis software (Regent Instruments Inc., Canada) to assess the SRA (cm^2 g^{-1}) and D (mm) of each fine-root category from each of the four sampling campaigns in 2003.

Carbon and N Analysis

Scanned samples were then analyzed for C and N on 4–6 root sub-samples per fine-root category. Samples were dried at 65°C, milled and analyzed by combustion in an elemental analyzer (Leco, CHN1000, USA). Individual beech and spruce rootlet C and N concentrations were calculated according to Eq. 1. In September 2004, the fine-root samples used as RR reference were additionally analyzed for C and N content.

Non-Structural Carbohydrate Analysis

In each sampling campaign, four to six rootlets per tree species were harvested and prepared for sugar and starch concentration analyses. Cut rootlets were covered with wet paper and transported in a plastic bag to the lab to avoid root damage and desiccation. Sub-samples from the three fine-root categories were carefully cleaned of soil particles and dead root ramifications by light brushing, frozen in liquid N, and stored at −80°C until analysis. The root samples were then ground manually in liquid N. Lyophilized, sugars (glucose, fructose, sucrose), and starch (hydrolyzed to glucose) were extracted according to Fleischmann et al. (2009) before measuring concentrations by HPLC. In spruce samples, pinitol was additionally identified (using expertise by M. Popp, University of Vienna, Austria). Total sugar concentration (TSC, mg g^{-1}) was additionally calculated as the sum of all analyzed sugars, inclusive pinitol in case of spruce. Individual beech and spruce rootlet non-structural carbohydrates concentrations were calculated according to Eq. 1.

Data Analysis

Seasonal effects (i.e., between the sampling campaigns) on fine-root parameters (i.e. SRA, D, non-structural carbohydrates, N and C concentrations, and C:N) were tested for each tree species by a factorial two-way ANOVA including fixed factors "category" and "sampling campaign". The interaction term "category" × "sampling campaign" was also included to test whether the responses of different fine-root categories depend on the sampling timing. Model residuals were tested for normality (Shapiro-Wilk test) and variance homogeneity (Levene test). For multiple comparisons, subsequent Tukey-HSD post hoc tests (HSD.test function of the *agricolae* package in R; Mendiburu, 2020) were performed. The temperature effect on RR was assessed in each fine-root category by an exponential fit (Eq. 2), based on datasets measured at three temperature levels over four sampling campaigns in 2003. Effect of N on RR was analyzed with individual beech and spruce rootlets by standardized major axis regression models (*smatr* R package; Warton et al., 2012), using RR^{10} for standardization. Non-parametric *U*-test (Mann-Whitney *U* test) was used to test for significant differences between two sample units. Statistical evaluation was performed with SPSS (version 13.0, SPSS INC., Chicago, IL, USA) and R (version 3.4.4; R Development Core Team, 2018). In all analyses, differences at $p < 0.05$ were considered significant.

RESULTS

Fine-Root Categories

Foraging roots (FR) constituted between 17–35% of the total beech rootlet biomass with a maximum of 35% in late summer and a minimum of 17% in autumn (**Figure 2**; **Table S1**). Transport fine roots (TR) contributed between 20-42% of the total rootlet biomass, with a minimum of 20% in late summer and a maximum of 42% in autumn, while mycorrhizal absorptive fine roots (MR) were consistently approximately 40% of the total rootlet biomass throughout the entire study period.

For spruce, FR contributed substantially less to the total rootlet biomass compared to beech and ranged between 10% in late summer and 3% in autumn (**Figure 2**; **Table S2**). Transport fine roots accounted for 37–53% of the total spruce fine-root biomass with a maximum of 53% in spring and a minimum of 37% in autumn. Absorptive mycorrhizal fine-roots contributed approximately 40% in spring through late summer and reached a maximum of 60% in autumn.

In spring, FR in both beech and spruce had whitish surface, but later, in early summer, spruce FR started to change color to yellow and brown (**Figure S1**). At the same time, beech FR remained whitish but appeared thinner and started to branch (**Figure S2**). In late summer of 2003, anatomical differences were apparent between beech and spruce FR: in beech, the living primary cortex was white, while in spruce, the primary cortex turned brown and shriveled (i.e., likely died), and the root surface suberized during drought (**Figure S1**).

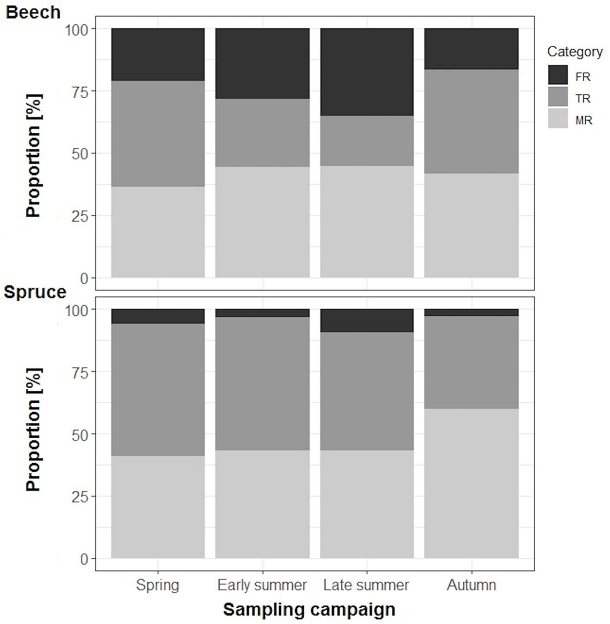

FIGURE 2 | Average proportions (%) of three fine-root categories in the biomass of individual beech (n=7–8) and spruce (n=8–10) rootlets: FR = absorptive foraging roots with primary xylem, TR = transport roots with secondary xylem, MR = absorptive mycorrhizal fine roots.

Fine-Root Morphology

In beech, SRA and D differed between fine-root categories depending on the season of the sampling campaign (**Table 2**). Beech FR SRA increased significantly from 167 $cm^2\ g^{-1}$ in spring to a maximum of 533 $cm^2\ g^{-1}$ in autumn. Beech SRA of the TR and MR categories were highest in early summer (78 and 520 $cm^2\ g^{-1}$, respectively), but reached lowest levels in late summer (58 and 388 $cm^2\ g^{-1}$); however this change was significant only for MR (**Table 1**). In beech, MR had generally the thinnest diameters (0.61–0.67 mm) and TR, had, in contrast, the largest diameters (1.40–1.62 mm). Interestingly, FR had diameters that were similar to TR at the beginning of the growing season (1.72 mm), but became thinner in early summer when their diameters were similar to those of MR (0.56 mm; **Table 1**).

In spruce, SRA differed between fine-root categories and sampling campaigns with both factors independent from each other (**Table 2**). Spruce FR, in contrast to beech, had low seasonal variation of SRA (**Table 1**), reaching maximal levels in early summer (168 $cm^2\ g^{-1}$), and minimal in late summer (140 $cm^2\ g^{-1}$). TR had highest SRA in spring (109 $cm^2\ g^{-1}$) and lowest in autumn (78 $cm^2\ g^{-1}$). However, the changes in SRA of FR and TR were not significant relative to the other samplings. Spruce MR had the highest SRA among spruce roots with a maximum in

TABLE 1 | Specific fine-root area (SRA, cm^2 g^{-1}) and diameter (D, mm) in (A) beech and (B) spruce fine-root categories, assessed at four sampling campaigns during 2003.

Sampling campaign	Fine-root categories*					
	FR		TR		MR	
	SRA	D	SRA	D	SRA	D
A) Beech						
Spring	167 ± 15^{d}	1.72 ± 0.22^{a}	68 ± 6^{e}	1.47 ± 0.08^{bc}	440 ± 35^{abc}	0.67 ± 0.06^{d}
Early summer	482 ± 84^{abc}	0.67 ± 0.05^{d}	78 ± 4^{e}	1.40 ± 0.08^{c}	520 ± 64^{ab}	0.61 ± 0.05^{d}
Late summer	430 ± 172^{bc}	0.69 ± 0.12^{d}	58 ± 4^{e}	1.62 ± 0.14^{ab}	388 ± 103^{c}	0.65 ± 0.08^{d}
Autumn	553 ± 83^{a}	0.56 ± 0.05^{d}	65 ± 8^{e}	1.51 ± 0.10^{bc}	465 ± 73^{abc}	0.65 ± 0.07^{d}
B) Spruce						
Spring	145 ± 39^{cd}	1.37 ± 0.28^{cd}	109 ± 30^{de}	1.54 ± 0.25^{bc}	310 ± 27^{ab}	0.84 ± 0.04^{e}
Early summer	168 ± 36^{c}	1.20 ± 0.22^{d}	88 ± 17^{e}	1.65 ± 0.13^{b}	327 ± 30^{a}	0.80 ± 0.04^{e}
Late summer	140 ± 28^{cd}	1.26 ± 0.40^{d}	83 ± 11^{e}	1.75 ± 0.18^{ab}	299 ± 60^{ab}	0.78 ± 0.07^{e}
Autumn	143 ± 29^{cd}	1.27 ± 0.40^{d}	78 ± 10^{e}	1.91 ± 0.20^{a}	286 ± 21^{b}	0.75 ± 0.04^{e}

Means ± 1 standard deviation (n = 4–6). Differences between means sharing a letter (a = highest value) are not statistically significant (ANOVAs provided separately for beech (A) and spruce (B) with Tukey-HSD post hoc tests, $p < 0.05$).

** FR, absorptive foraging fine roots; TR, transport fine roots; MR, absorptive mycorrhizal fine roots.*

SRA in early summer (327 cm^2 g^{-1}), and a minimum in autumn (286 cm^2 g^{-1}). Spruce D varied significantly between fine-root categories depending on the season of the sampling campaign (**Table 2**). Remarkably, no general seasonal adjustment of D was observed in spruce. In spruce, the diameter of FR roots was generally larger compared to beech FR, but in contrast to beech did not change with season (1.20–1.37 mm; **Table 1**). In spruce, TR had the largest diameter gradually increasing from 1.54 mm in spring to maximum of 1.91 mm in autumn. The MR category had the thinnest diameter (0.75–0.84 mm); however, the MR were coarser compared to the same category in beech.

Fine-Root Respiration

In beech, FR had the highest RR^{10} and temperature sensitivity (**Figure 3**; **Table 3**): RR^{10} ranged between 17.06 nmol CO$_2$ g^{-1} s^{-1} in spring and 9.46 in autumn, and Q_{10} was highest in early summer (i.e., 2.20) but lowest in spring and late summer (i.e., around 1.4). In contrast, TR had the lowest RR^{10} (4.14–2.40 nmol CO$_2$ g^{-1} s^{-1}) and temperature sensitivity: Q_{10} even dropped to 1.09 in the extremely dry month of August (i.e., late summer). On average, MR had RR^{10} levels of 4.5 CO$_2$ g^{-1} s^{-1} (late summer) to 8.9 nmol CO$_2$ g^{-1} s^{-1} (spring), with the lowest Q_{10} of 1.16 in late summer.

Compared to beech, all fine-root categories of spruce had lower respiration levels and less variation in RR^{10} throughout the entire study period. Spruce FR had 2–3 times lower RR^{10} but comparable Q_{10} except early summer when Q_{10} in spruce FR was extremely low (i.e., 1.11; **Figure 3**; **Table 3**). Spruce TR had the lowest RR^{10} and Q_{10}, ranging between 2.6 and 3.5 nmol CO$_2$ g^{-1} s^{-1}, and 1.09 and 1.37, respectively. The highest RR^{10} and temperature sensitivity were found in spruce MR (**Figure 3**; **Table 3**): RR^{10} maximum in spring (8.15 nmol CO$_2$ g^{-1} s^{-1}), and Q_{10} maximum of 1.80 after precipitation returned in autumn (**Figure 1**).

Rootlet RR at mean daily soil temperature (RR$^{T(0)}$) and at a standard temperature of 10°C (RR^{10}) were higher in beech than spruce over the entire study period (**Figure S3**). In late summer, rootlet RR$^{T(0)}$ decreased significantly in both tree species despite higher soil temperature (T$_s$). Beech rootlets had the highest RR^{10} in spring (9.90 nmol CO$_2$ g^{-1} s^{-1}). For the rest of the season, rootlet RR were similar in both species with the seasonal RR^{10} minimum occurring in late summer. In autumn 2004, rootlet RR^{10} was, in both tree species, 30–50% higher compared to autumn 2003, with higher increases in spruce.

Carbon and N Status

Carbon and N concentrations varied among transport versus absorptive fine-root categories and sampling periods (**Table 2**). In beech, FR and MR had the lowest C concentration in spring (400 mg g^{-1} to 420 mg g^{-1}) that increased over the course of the season up to nearly 430–450 mg g^{-1} (**Table 4**). Transport root C concentration was generally higher than in the other two categories, and similarly increased across the entire sampling period (i.e., from 467 mg g^{-1} to 476 mg g^{-1}). During late summer, FR reached maximal N concentrations among all categories (27.2 mg g^{-1}); however, these patterns shifted by autumn where FR and MR had similar N concentrations (nearly 19 mg g^{-1}). This similar N level was the result in a drop in N in the FR roots at the end of vegetation period. Across the entire study period, TR had the lowest N concentration among all categories, ranging from 6.8 mg g^{-1} in early summer to 10.1 mg g^{-1} in autumn. The C:N ratio was lowest in FR (a minimum of 16.6 mg g^{-1} in late summer and a maximum of 29.5 mg g^{-1} in spring), and highest in TR (a minimum of 47.2 mg g^{-1} in autumn a maximum of 69.5 mg g^{-1} in early summer), with lowest seasonal variation in MR category (23–32 mg g^{-1}). When calculated per rootlet, C:N of beech rootlets were the lowest in late summer, a result of the high proportion of high N containing FR roots that were present at this time (**Table 4**, **Figure S4**).

In spruce, C was more stable across all fine-root categories and months (**Table 4**) with lowest levels in FR during spring (448 mg g^{-1}) and highest in MR during autumn (482 mg g^{-1}). In contrast to beech, TR was not the C-richest category in spruce, but was similar to MR (C differences between both categories were not significant). Absorptive mycorrhizal roots had generally the highest N levels, with the exception of early summer when N

TABLE 2 | ANOVAs outcome for key fine-root parameters, assessed at four sampling campaigns during 2003 in fine-root categories of beech and spruce. Main factors are "category" and "sampling campaign" as well as their interaction term. Total number of samples for beech was 135, and for spruce 167.

Tree species		**Beech**		**Spruce**	
Response variable	***df***	**MS**	***F* value**	**MS**	***F* value**
SRA					
Category	2	2183794	315.9***	747037	662.9***
Sampling campaign	3	97257	14.1***	6423	5.7***
Category × sampling campaign	6	63994	9.3***	1305	0.3 ns
D					
Category	2	9.7	1031.0***	13.1	501.7***
Sampling campaign	3	0.6	67.5***	0.1	1.6 ns
Category × sampling campaign	6	0.6	66.4***	0.2	6.3 ***
C concentration					
Category	2	94.8	56.1***	9.1	54.3***
Sampling campaign	3	32.3	19.1***	7.1	42.5***
Category × sampling campaign	6	5.4	3.2*	0.8	4.35**
N concentration					
Category	2	5.9	273.3***	2.6	176.3***
Sampling campaign	3	0.7	33.4***	0.3	17.58***
Category × sampling campaign	6	0.6	26.5***	0.3	18.11***
C/N					
Category	2	5243	469.8***	936.3	118.5***
Sampling campaign	3	276.3	24.8***	94.7	12.0***
Category × sampling campaign	6	200.0	17.9***	91.7	11.6***
Saccharose					
Category	2	554.2	30.6***	228.1	7.8**
Sampling campaign	3	310.4	17.1***	1208	41.5***
Category × sampling campaign	6	154.1	8.5***	150.4	5.2***
Glucose					
Category	2	180.0	40.8***	62.9	12.1***
Sampling campaign	3	86.3	19.5***	417.8	80.2***
Category × sampling campaign	6	48.2	11.0***	187.6	36.0***
Fructose					
Category	2	284.1	44.9***	188.1	34.8***
Sampling campaign	3	104.9	16.6***	162.5	30.1***
Category × sampling campaign	6	23.7	3.8**	52.2	9.7***
TSC					
Category	2	1889	42.5***	3096	28.6***
Sampling campaign	3	1168	26.3***	5676	52.5***
Category × sampling campaign	6	73.4	1.7 ns	864.2	7.9***
Starch					
Category	2	7.3	34.2***	7.8	9.3***
Sampling campaign	3	1.4	6.4**	127.5	151.9***
Category × sampling campaign	6	3.7	17.1***	7.1	8.4***
Pinitol					
Category	2	nd	nd	884.9	105.2***
Sampling campaign	3	nd	nd	182.1	21.6***
Category × sampling campaign	6	nd	nd	51.0	6.1***

For multiple comparisons, Tukey-HSD post hoc tests ($p < 0.05$) were performed.

*df, degrees of freedom; MS, mean square. F value with significance levels ***$p < 0.001$; **$p < 0.01$; *$p \leq 0.05$; ns $p > 0.05$; nd = not defined.*

decreased substantially (14.7 mg g^{-1}) to the N-levels of FR (i.e., nearly 16 mg g^{-1}). Similar to beech, TR had the lowest N concentration among the fine-root categories, especially in early and late summer (10–12 mg g^{-1}). In contrast to beech, spruce rootlets had the highest C:N in early summer, which was due to the reduced N at the beginning of the drought period (**Table 3**; **Figure S4**). In autumn 2004, N content in spruce rootlets was nearly 30% higher compared to autumn 2003, indicating N limitation in spruce at the end of the growing season in 2003 (**Figure S4**).

Relationship Between RR and N Concentration

In both tree species, RR^{10} was positively correlated to the corresponding N concentration of the rootlets (**Figure 4**). In beech, RR^{10} peaked in response to N in spring ($\beta = 7.27$, $p < 0.001$), compared to early spring in spruce ($\beta = 12.18$, $p < 0.001$; **Table 5**). Beech RR^{10} responded positively to N across all sampling dates (coefficient β between 4.97 and 7.27), while spruce responded weakly (late summer: $\beta = 0.61$, $p = 0.001$), or not at all (autumn: $\beta = 3.65$, $p = 0.478$; **Table 5**).

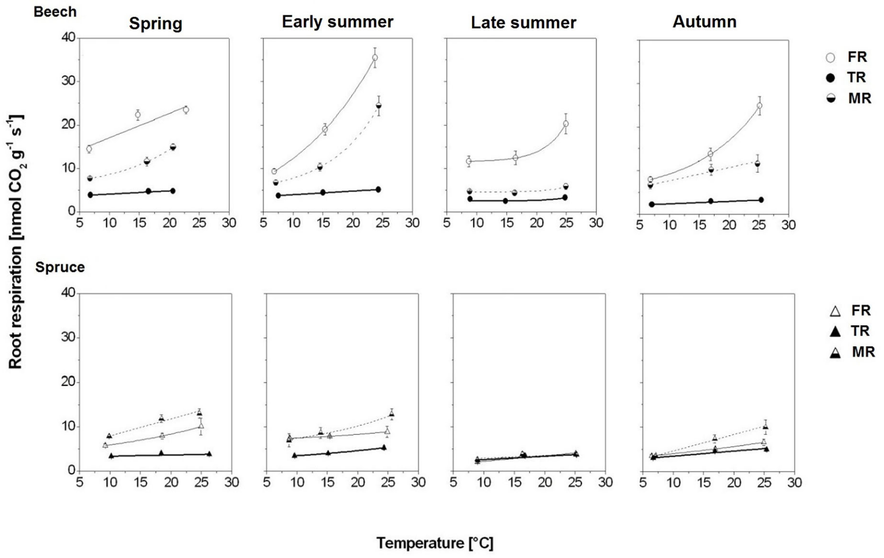

FIGURE 3 | Seasonal response of root respiration rate (RR) to temperature in beech and spruce per fine-root category, given as exponential response functions of absorptive foraging fine roots FR (solid thin line), transport fine roots TR (solid bold line), and absorptive mycorrhizal fine roots MR (dashed line); means ± 1 standard error (n = 4–6). Model statistics are provided in **Table 3**.

Non-Structural Carbohydrates

All studied non-structural carbohydrates varied among fine-root categories and sampling periods (**Table 2**). Interestingly, the seasonal responses of NSC in beech were similar among the studied fine-root categories, i.e. were independent on the sampling timing. During spring and early summer 2003, beech FR had the lowest TSC concentration 38 mg g^{-1} (**Table 6**; **Figure S5**), which increased by 40–60% during late summer the second half of the growing season as a result of an increase in sucrose and fructose (in late summer), and glucose concentrations (in autumn). Transport fine-roots of beech had lowest TSC also in spring (35.2 mg g^{-1}), but peaked in the second half of the growing season also due to enhanced fructose and glucose concentration. Absorptive mycorrhizal fine-roots of beech had low TSC during spring and early summer (approximately 20 mg g^{-1}), but levels doubled during late summer and autumn similar to FR. The three fine-root categories of beech showed however different patterns of starch allocation during the study period (**Table 6**): highest concentration was detected in FR in spring (3.26 mg g^{-1}) and steadily decreased through the rest of the growing season (**Figure S5**). In TR starch was extremely low in spring and then completely exhausted in early summer. Interestingly, the starch levels in TR recovered in late summer and in autumn; this seasonal change was however not significant. Absorptive mycorrhizal roots had oscillating starch levels, with minimal levels in late summer, but accumulated starch in autumn (1.86 mg g^{-1}).

Spruce fine-root categories had more variable seasonal patterns in NSC fractions compared to beech (**Table 2** and **Table 6**). Foraging non-mycorrhizal roots had highest TSC in early summer (123.9 mg g^{-1}), and lowest in autumn (38.4 mg g^{-1}), with glucose, fructose and pinitol influencing the seasonal variation (**Figure S5**). Total sugar concentration in TR of spruce also had the highest levels in early summer (84.1 mg g^{-1}), but minimum in autumn (44.4 mg g^{-1}), although the seasonal variation, driven mainly by sucrose, was less pronounced than in FR. In MR, TSC had highest levels in spring (61.8 mg g^{-1}), and lowest in autumn (32.8 mg g^{-1}) due to a simultaneous decrease in the concentration of all analyzed sugars. Starch concentrations in fine-root categories of spruce were highly variable at the beginning of the study (6–11 mg g^{-1} in spring), but then decreased five-fold and leveled out at similar concentrations for the remainder of the study (1–2 mg g^{-1}; **Table 6**). Interestingly, TR had in spring starch levels of 10.85 mg g^{-1} that were approximately two-fold greater than FR or MR roots but, then decreased five-to-ten-fold to levels typical for the other two categories.

At the beginning of the growing season, beech rootlets had two times lower TSC relative to spruce (**Figure 5**). During late summer and autumn, however, TSC of beech rootlets increased by 50%. In spruce, TSC was only slightly enhanced in early summer, remained stable into late summer, but decreased in autumn. In both species, the seasonal dynamics of TSC was mainly driven by sucrose which contributed 55–65% to TSC. In general, the higher sucrose levels during spring and summer period as well as the permanent presence of pinitol resulted in the higher TSC in spruce relative to beech fine-roots. Rootlet starch concentration in beech did not show pronounced seasonal dynamics (**Table 6**, **Figure 5**). Despite higher levels of starch in spruce during spring, by the end of the growing season starch

TABLE 3 | Root respiration rate (RR) in response to temperature per fine-root category of beech (A) and spruce (B) at four sampling campaigns during 2003.

Samplingcampaign	Fine-root category*)	Model parameters		Q_{10}	R^2	RR^{10}(nmol $CO_2g^{-1}s^{-1}$)
		ρ (nmol $CO_2g^{-1}s^{-1}$)	θ (°C)			
A) Beech						
Spring	FR	12.66	0.029	1.35	0.83	17.06
	TR	3.51	0.047	1.18	0.96	4.14
	MR	5.58	0.017	1.60	0.99	8.90
Early summer	FR	5.53	0.079	2.20	0.99	12.16
	TR	3.33	0.018	1.20	0.97	4.04
	MR	3.79	0.075	2.12	0.99	8.02
Late summer	FR	8.06	0.035	1.41	0.85	11.38
	TR	2.54	0.009	1.09	0.25	2.77
	MR	3.90	0.015	1.16	0.60	4.50
Autumn	FR	5.11	0.062	1.85	0.99	9.46
	TR	1.94	0.022	1.24	0.93	2.40
	MR	5.55	0.031	1.37	0.95	7.59
B) Spruce						
Spring	FR	4.18	0.035	1.42	0.99	5.93
	TR	3.28	0.007	1.10	0.43	3.52
	MR	5.75	0.035	1.42	0.94	8.15
Early summer	FR	6.79	0.010	1.11	0.68	7.53
	TR	2.61	0.029	1.37	0.99	3.49
	MR	4.90	0.039	1.47	0.99	7.19
Late summer	FR	1.52	0.041	1.50	0.88	2.28
	TR	2.03	0.026	1.30	0.82	2.64
	MR	2.43	0.019	1.20	0.61	2.95
Autumn	FR	2.85	0.04	1.40	0.99	4.00
	TR	2.62	0.027	1.30	0.89	3.44
	MR	2.47	0.058	1.80	0.96	4.41

Exponential fit ($RR = \rho e^{\theta T_c}$) describes the relationship between RR and T_c (Eq. 2), with ρ and θ being model parameters; Q_{10} calculated as $Q_{10} = e^{10\theta}$ (Eq. 3); R^2 as coefficient of determination (n = 4–6); RR^{10} at standard T of 10°C.

**FR, absorptive foraging fine roots; TR, transport fine roots; MR, absorptive mycorrhizal fine roots.*

levels were similar in both beech and spruce (i.e., about 1.0–1.5 mg g^{-1}) (**Table 6**; **Figure 5**).

DISCUSSION

Our study presents a novel look at beech and spruce fine-root ecological strategies (cf. Weemstra et al., 2017) by directly comparing key morphological and physiological traits of three functionally defined fine-root categories. We hypothesized that the fine-roots of both species would adjust to seasonal drought differently with beech following a “fast”, and spruce a “slow” ecological strategy. However, we determined that each tree species employs diverse ecological strategies dependent on the functional fine-root categories investigated (**Figure 6**).

Morphological Traits

The non-mycorrhizal absorptive roots adjusted in a species-specific manner supporting fine-root ecological strategy hypothesis. In beech, SRA of FR had the highest seasonal variation among all categories that more than doubled from spring through autumn. Enhanced SRA of FR was accompanied by a distinct reduction in mean diameter to the levels typical of mycorrhizal MR. Such a diameter decline may be associated with optimized uptake of scarce resources (Zobel et al., 2007; Ma et al., 2018). During drought, thin ephemeral roots are “shed” by species which are capable of root regrowth upon soil rewetting (Meier and Leuschner, 2008). The ability to “shed” roots and to initiate new root growth when available resources are present, represents an effective adaptation to drought, as long as the trees can afford the C demand (Brunner et al., 2015). This “shed-regrowth” mechanism as detected here in FR allows beech to regain fine-root biomass after drought (Leuschner et al., 2001). While substantial fine-root growth after drought comes at a C cost, the ability to grow new absorptive fine-roots with high SRA is essential for continued water uptake, demonstrating a “fast” ecological strategy for FR (**Figure 6**).

In contrast, spruce FR showed no seasonal variation of SRA. Instead, spruce FR became pigmented from white to brown during the dry midsummer (Blaschke et al., 2006; Nikolova et al., 2006). Also during this time, the roots temporarily stopped growing which was not the case in previous years (i.e. 1999, 2000, and 2002) when a sufficient water supply was present (Nikolova et al., 2010). The high degree of suberization of the FR can reduce radial hydraulic conductivity and may act as a physical barrier to the movement of water/nutrients into the plant (Steudle, 2000). Development of more suberized and lignified roots in drying soils has been described in plants with limited access to water, e.g. desert plants (Nobel and Huang, 1992) as well as in *Quercus ilex* and *Vitis vinifera* (Brunner et al., 2015 and the references therein) and *Pinus halepensis* (Leshem, 1970) and may serve as a mechanism to decrease water loss from roots. Orlov (1957) similarly observed color progression in absorbing roots of spruce under natural conditions and related

TABLE 4 | Concentration of carbon (C, mg g^{-1}) and nitrogen (N, mg g^{-1}), and the C:N ratio in the fine-root categories of (A) beech and (B) spruce at four sampling campaigns during 2003.

Sampling campaign	Fine-root categories*									Rootlet		
	FR			TR			MR					
	C	N	C:N	C	N	C:N	C	N	C:N	C	N	C:N
A) Beech												
Spring	400 ± 9.3^{e}	13.8 ± 2.0^{d}	29.5 ± 3.7^{cd}	467 ± 3.3^{ab}	9.6 ± 0.7^{e}	49.0 ± 3.3^{b}	420 ± 1.2^{de}	16.4 ± 1.0^{cd}	25.6 ± 1.3^{cde}	*442 ± 2.9*^{a}	*15.8 ± 1.5*^{a}	*28.3 ± 2.8*^{a}
Early summer	444 ± 9.4^{bcd}	22.0 ± 2.0^{b}	20.3 ± 1.9^{ef}	473 ± 2.5^{ab}	6.8 ± 0.6^{e}	69.5 ± 6.4^{a}	458 ± 2.2^{abc}	14.2 ± 1.1^{d}	32.5 ± 2.9^{c}	*459 ± 2.2*^{b}	*14.5 ± 1.2*^{a}	*31.8 ± 2.6*^{a}
Late summer	449 ± 6.0^{abc}	27.2 ± 1.4^{a}	16.6 ± 0.9^{f}	473 ± 1.3^{ab}	9.4 ± 1.1^{e}	50.9 ± 5.9^{b}	458 ± 4.3^{abc}	18.4 ± 1.2^{bc}	24.8 ± 1.6^{cde}	*458 ± 2.7*^{b}	*19.4 ± 1.8*^{b}	*23.8 ± 2.3*^{b}
Autumn	431 ± 3.1^{cd}	19.2 ± 0.9^{bc}	22.5± 2.2^{def}	476 ± 8.4^{a}	10.1 ± 0.5^{e}	47.2 ± 3.0^{b}	447 ± 2.2^{bcd}	19.7 ± 2.8^{b}	23.0 ± 2.7^{def}	*446 ± 2.4*^{a}	*15.7 ± 0.6*^{a}	*29.2 ± 1.1*^{a}
B) Spruce												
Spring	448 ± 5.7^{d}	12.7 ± 0.7^{efg}	35.2 ± 1.6^{bc}	463 ± 2.1^{bc}	12.0 ± 1.0^{fg}	38.9 ± 3.2^{ab}	463 ± 3.2^{bc}	22.2 ± 0.7^{a}	20.9 ± 1.6^{f}	*462 ± 0.5*^{a}	*17.6 ± 0.6*^{a}	*26.3 ± 0.9*^{a}
Early summer	450 ± 3.1b^{d}	16.5 ± 0.9^{cd}	27.4 ± 1.9^{de}	462 ± 6.1^{bc}	10.4 ± 0.8^{g}	44.9 ± 4.4^{a}	466 ± 3.3^{bc}	14.7 ± 1.0^{de}	31.9 ± 2.2^{cd}	*464 ± 0.8*^{b}	*13.0 ± 0.4*^{b}	*35.8 ± 1.0*^{b}
Late summer	460 ± 4.0^{c}	14.0 ± 0.7^{def}	32.9 ± 1.6^{bcd}	462 ± 1.9^{bc}	11.8 ± 1.4^{fg}	39.6 ± 5.2^{ab}	467 ± 0.5^{bc}	19.2 ± 0.5^{bc}	24.3 ± 0.7^{ef}	*464 ± 0.5*^{b}	*15.5 ± 0.7*^{c}	*30.1 ± 1.3*^{c}
Autumn	467 ± 2.7^{bc}	16.6 ± 2.1^{cd}	28.7 ± 4.3^{de}	470 ± 6.6^{a}	14.5 ± 0.9^{def}	32.7 ± 2.1^{cd}	482 ± 4.0^{a}	20.5 ± 1.2^{a}	23.6 ± 1.4^{ef}	*474 ± 0.8*^{c}	*16.8 ± 0.4*^{a}	*28.2 ± 0.7*^{ca}

Means ± 1 standard deviation (n = 4–6). Differences between means sharing a letter (a = highest value) are not statistically significant (ANOVAs provided separately for beech (A) and spruce (B) with Tukey-HSD post hoc tests, p < 0.05). Values representative for entire rootlets were calculated according to Eq.1 (see also ***Figure S4****).*

**FR, absorptive foraging fine roots; TR, transport fine roots; MR, absorptive mycorrhizal fine roots.*

this to the senescence of the cortex. The present study is, to our knowledge, the first study to report a shift in color/suberization in spruce fine-roots in response to drought. Such adjustment of FR may reflect an earlier senescence of the spruce fine-roots in order to enhance C retention during harsh drought, thus supporting the "slow" ecological strategy (**Figure 6**).

Physiological Traits

Beech had higher RR than spruce across all root types. In beech, this high RR was driven by FR, despite this category containing roots of a greater diameter compared to MR (e.g., in spring). This finding conflicts with other studies that report a decrease in RR with root diameter (e.g., Pregitzer et al., 1998; Di Iorio et al., 2016). Absorptive roots, like FR, represent primary roots with active cell divisions within the apical meristem, and a determinate growth pattern (Dubrovsky, 1997; Heimsch and Seago, 2008). Thus, the stimulation of root respiration and growth during the summer dry periods could be a result of phytohormonal control (Chapman et al., 2003; Perrot-Rechenmann, 2014) likely leading to temporary dysfunction of the primary-root apical meristem but stimulation of lateral-root production. Dubrovsky (1997) suggested the purpose of such dysfunction is to allow for the formation of lateral roots which quickly elongate (i.e. respire) and then slow down and finally cease elongation only a few days after emergence. In cactus, Dubrovsky (1997) related this adjustment to temporary water availability in arid environments (see also Chapman et al., 2003). In the present study, similar mechanisms may have induced a formation of lateral-like ephemeral FR roots with high SRA in beech. These young FR maintained the high RR of the beech rootlets during the rest of the growing season, thus representing a "fast" ecological strategy (**Figure 6**). Consequently, the formation of ephemeral root parts may represent a competitive advantage of beech, enabling rapid proliferation when resources are available (Nikolova, 2007; Bauerle, in prep.) or may play a role in hydraulic redistribution when present (Caldwell et al., 1998; Bauerle et al., 2008; Zapater et al., 2011).

During spring, spruce had the highest RR and Q_{10} in FR. During late summer, when soils were extremely dry, spruce FR possessed the lowest RR but had the highest metabolic activity (as reflected by high Q_{10}) among all three fine-root categories. The suberized exodermis protects the FR from desiccation (Cruz et al., 1992; Steudle, 2000), however at the price of C expenditure for respiration. Since the proportion of FR in the rootlet biomass was low (<10%) in spruce, this fine-root category did not drive the RR of rootlets for this species.

Overall, in both tree species, MR had medial RR, with the exception of spruce in spring and late summer, when MR had the highest respiration among all root categories. This could be explained by the relatively high percentage of non-mycorrhizal root tips (ca. 45%; Nikolova, 2007), which are fast-growing (Brundrett, 2002) and were produced in new root flushes during spring. In contrast, in late summer, the respiration activity of MR was likely needed to support the high fungal colonization rate (over 90%; Nikolova, 2007). In spruce rootlets,

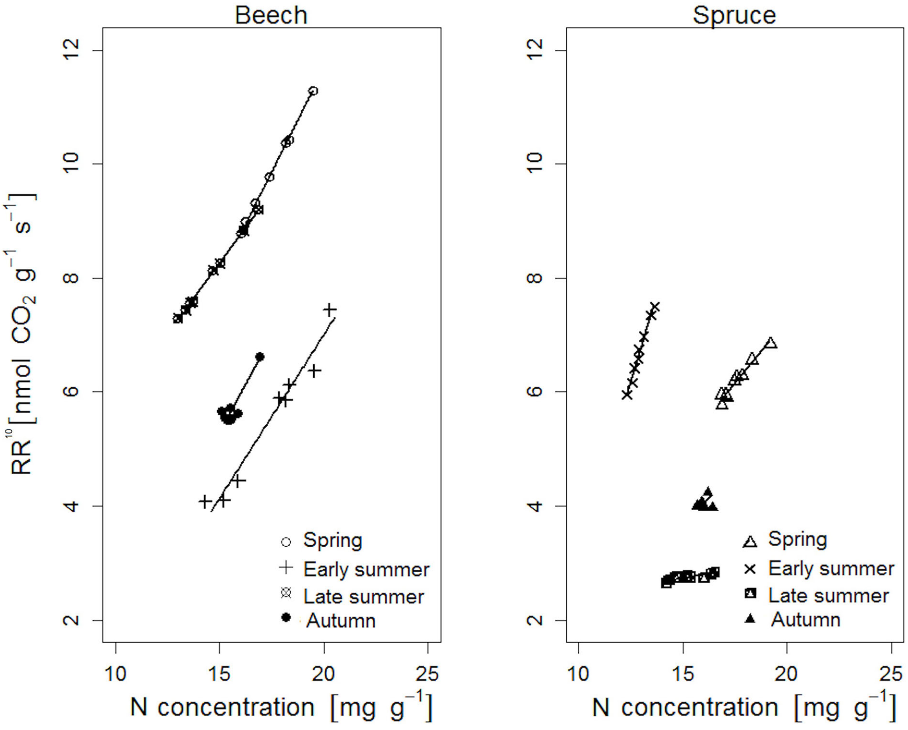

FIGURE 4 | Relationship between root respiration rate at a standard temperature of 10°C (RR^{10}) and nitrogen concentration of individual beech and spruce rootlets. Lines represent a linear fit (regression statistics are provided in **Table 5**).

MR is likely driving the respiration activity during drought, thereby following a "fast" ecological strategy (**Figure 6**).

Not surprisingly, respiration was low in TR in both tree species, as such aged root sections (cf. Solly et al., 2018) with secondary growth serve water/nutrient transport rather than resource uptake (Lobet et al., 2014) indicating a "slow" ecological strategy. The lowest Q_{10} was also measured in TR (around 1.10), as RR approached the level of maintenance respiration (RR^{10} around 2.5 nmol CO$_2$ g^{-1}s^{-1}, in both tree species), in the absence of high temperature sensitivity (Burton et al., 1998).

Carbon and N Concentrations

In beech rootlets, C concentration was the highest during early and late summer. This corresponds to the seasonal C trend reported for beech fine-roots from adult coppice forests in Italy's Prealps (Terzaghi et al., 2013). In contrast, C concentration of spruce rootlets consistently increased through the growing season, reaching higher levels than beech rootlets by the end of the growing season. In both tree species, C dynamics of the rootlets seemed to depend on the proportion of TR, which was the root category with the highest C concentration.

TABLE 5 | Linear fit (RR^{10} = $\alpha + \beta$N) between root respiration rate (RR) at a standard temperature of 10°C (RR^{10}; nmol CO$_2$ g^{-1} s^{-1}) and nitrogen concentration (N, mg g^{-1}) in individual rootlets of (A) beech and (B) spruce at four sampling campaigns during 2003.

Sampling campaign 2003	Intercept, α (upper/lower limit)	Slope, β (upper/lower limit)	n	R^2	p
A) Beech					
Spring	−2.86 (– 3.30/–2.43)	7.27 (7.03/7.52)	7	0.99	<0.0001
Early summer	0.81 (0.56/1.03)	4.97 (4.82/5.13)	8	0.99	<0.0001
Late summer	−4.48 (−6.46/−2.50)	5.63 (4.73/6.98)	8	0.96	<0.0001
Autumn	−4.71 (−9.54/0.12)	6.67 (4.27/10.44)	8	0.78	0.003
B) Spruce					
Spring	−1.64 (−3.16/−0.12)	4.46 (3.68/5.40)	9	0.95	<0.0001
Early summer	−9.05 (−10.87/−7.23)	12.18 (10.85/13.67)	8	0.99	<0.0001
Late summer	1.81 (1.42/2.20)	0.61 (0.40/0.92)	10	0.74	0.001
Autumn	−1.79 (−6.81/3.21)	3.65 (1.67/7.95)	9	0.07	0.478

Here, α (intercept) and β (slope) are regression coefficients, n is the number of the analyzed individual rootlets, R^2 represents the measure of determination, p gives the level of significance of the regression equations (standardized major axis regression models). Upper and lower limits of α and β are shown in brackets.

TABLE 6 | Non-structural carbohydrate concentrations in fine-root categories of (A) beech and (B) spruce in 2003.

A) Beech

Fine-root category	**Sampling campaign**	**Sucrose (mg g^{-1})**	**Glucose (mg g^{-1})**	**Fructose (mg g^{-1})**	**TSC (mg g^{-1})**	**Starch (mg g^{-1})**
FR	Spring	24.4 ± 3.3^{bc}	7.3 ± 0.9^{cde}	8.2 ± 1.7^{cde}	38.2 ± 3.9^{bc}	3.26 ± 1.05^{a}
	Early summer	20.7 ± 5.1^{bcd}	9.5 ± 1.8^{bc}	8.5 ± 1.6^{cde}	38.8 ± 8.2^{bc}	1.56 ± 0.38^{bc}
	Late summer	38.6 ± 4.5^{a}	8.3 ± 2.7^{bcd}	12.1 ± 3.4^{bcd}	60.1 ± 6.0^{a}	1.42 ± 0.51^{bc}
	Autumn	29.1 ± 7.2^{b}	18.9 ± 3.3^{a}	8.4 ± 1.9^{cde}	52.6 ± 10.3^{ab}	0.56 ± 0.08^{cd}
TR	Spring	23.8 ± 1.6^{bc}	3.8 ± 0.5^{de}	7.4 ± 1.2^{de}	35.2 ± 2.7^{c}	0.55 ± 0.41^{cd}
	Early summer	21.9 ± 1.1^{bc}	8.7 ± 0.5^{bcd}	13.6 ± 0.7^{abc}	44.2 ± 1.8^{bc}	0^{d}
	Late summer	19.4 ± 6.2^{cd}	12.0 ± 3.2^{b}	17.9 ± 5.2^{a}	49.5 ± 9.9^{abc}	0.84 ± 0.29^{bcd}
	Autumn	20.5 ± 2.7^{bcd}	9.8 ± 3.8^{bc}	17.5 ± 6.3^{ab}	48.1 ± 8.4^{abc}	0.70 ± 0.35^{cd}
MR	Spring	12.1 ± 3.6^{de}	3.7 ± 1.4^{e}	4.4 ± 1.3^{e}	20.1 ± 5.9^{d}	0.20 ± 0.04^{d}
	Early summer	8.8 ± 1.5^{e}	4.4 ± 0.8^{de}	4.9 ± 1.0^{e}	18.1 ± 2.8^{d}	0.17 ± 0.02^{d}
	Late summer	24.9 ± 5.1^{bc}	5.3 ± 1.1^{cde}	8.2 ± 1.9^{cde}	38.7 ± 6.7^{bc}	0^{d}
	Autumn	24.1 ± 1.9^{bc}	4.5 ± 0.7^{de}	7.9 ± 4.1^{cde}	37.0 ± 3.1^{c}	1.86 ± 0.20^{b}
Rootlet	Spring	*19.6 ± 0.8*^{c}	*4.5 ± 0.3*^{c}	*6.4 ± 0.2*^{c}	*30.6 ± 1.3*^{c}	*1.02 ± 0.27*^{ac}
	Early summer	*15.8 ± 0.8*^{d}	*7.0 ± 0.4*^{b}	*8.3 ± 0.4*^{b}	*31.1 ± 1.4*^{c}	*0.94 ± 0.10*^{ac}
	Late summer	*28.3 ± 1.7*^{a}	*7.9 ± 0.7*^{b}	*10.6 ± 1.4*^{a}	*46.9 ± 1.5*^{a}	*0.82 ± 1.14*^{b}
	Autumn	*23.3 ± 0.5*^{b}	*9.1 ± 0.7*^{a}	*12.1 ± 0.7*^{a}	*44.5 ± 1.0*^{b}	*1.18 ± 0.09*^{a}

B) Spruce

		Sucrose (mg g^{-1})	**Glucose (mg g^{-1})**	**Fructose (mg g^{-1})**	**Pinitol (mg g^{-1})**	**TSC (mg g^{-1})**	**Starch (mg g^{-1})**
FR	Spring	51.9 ± 11.5^{a}	7.4 ± 4.5^{cde}	13.6 ± 3.8^{bc}	15.1 ± 4.0^{b}	88.1 ± 22.8^{b}	5.67 ± 1.75^{b}
	Early summer	41.0 ± 7.9^{abc}	32.8 ± 5.1^{a}	20.8 ± 2.8^{a}	29.2 ± 1.1^{a}	123.9 ± 14.0^{a}	1.40 ± 0.22^{c}
	Late summer	40.7 ± 2.8^{abc}	9.0 ± 3.8^{cd}	8.5 ± 1.9^{cde}	24.8 ± 7.4^{a}	83.0 ± 13.1^{bc}	0.58 ± 0.13^{c}
	Autumn	20.6 ± 2.6^{e}	1.2 ± 0.6^{f}	3.5 ± 0.9^{e}	13.0 ± 3.1^{bc}	38.4 ± 2.7^{fg}	1.27 ± 0.50^{c}
TR	Spring	30.1 ± 2.9^{cde}	5.9 ± 1.3^{def}	12.7 ± 1.0^{bc}	6.8 ± 1.3^{cd}	55.5 ± 5.0^{defg}	10.85 ± 1.50^{a}
	Early summer	43.5 ± 5.7^{ab}	14.7 ± 1.8^{b}	16.1 ± 3.5^{ab}	9.8 ± 1.9^{bcd}	84.1 ± 10.7^{bc}	1.88 ± 1.50^{c}
	Late summer	36.7 ± 1.4^{bc}	12.4 ± 1.4^{bc}	17.0 ± 2.3^{ab}	12.1 ± 2.6^{bc}	78.3 ± 3.0^{bcd}	0.90 ± 0.54^{c}
	Autumn	21.4 ± 7.3^{de}	5.4 ± 1.7^{def}	12.0 ± 4.1^{bc}	5.6 ± 1.7^{d}	44.4 ± 13.5^{efg}	2.10 ± 1.38^{c}
MR	Spring	37.1 ± 3.3^{bc}	9.6 ± 0.9^{bcd}	9.3 ± 0.9^{cd}	5.7 ± 0.4^{d}	61.8 ± 4.6^{cde}	6.20 ± 0.74^{b}
	Early summer	35.3 ± 2.6^{bc}	7.4 ± 0.6^{cde}	10.0 ± 0.5^{cd}	6.5 ± 0.9^{cd}	59.1 ± 3.5^{cdef}	1.40 ± 0.83 ^{c}
	Late summer	33.1 ± 3.8^{bcd}	7.4 ± 1.6^{cde}	9.0 ± 1.1^{cd}	7.3 ± 2.4^{cd}	56.9 ± 5.8^{def}	0.96 ± 0.06^{c}
	Autumn	21.1 ± 3.3^{e}	3.2 ± 0.6^{ef}	4.6 ± 0.5^{de}	4.0 ± 1.0^{d}	32.8 ± 4.7^{g}	0.76 ± 1.17^{c}
Rootlet	Spring	*35.3 ± 0.7*^{b}	*8.0 ± 0.2*^{c}	*10.9 ± 0.2*^{b}	*6.7 ± 0.3*^{b}	*60.9 ± 1.0*^{c}	*7.99 ± 0.31*^{a}
	Early summer	*38.9 ± 0.5*^{a}	*11.1 ± 1.2*^{a}	*10.3 ± 0.3*^{b}	*8.5 ± 0.8*^{a}	*68.8 ± 3.3*^{a}	*1.59 ± 0.04*^{b}
	Late summer	*35.3 ± 0.4*^{b}	*9.7 ± 0.5*^{b}	*12.5 ± 0.8*^{a}	*6.5 ± 1.2*^{bc}	*64.1 ± 1.3*^{b}	*0.90 ± 0.02*^{c}
	Autumn	*21.2 ± 0.1*^{c}	*4.4 ± 0.3*^{d}	*8.9 ± 0.7*^{c}	*5.4 ± 0.3*^{c}	*39.8 ± 0.9*^{d}	*1.56 ± 0.11*^{b}

Means ± 1 standard deviation (n = 4–6). Means with the same letter (a = highest value) are not statistically significant (ANOVAs provided separately for beech (A) and spruce (B) with Tukey-HSD post hoc tests, $p < 0.05$). Values representative for entire rootlets were calculated according to Eq.1 (see also ***Figure 5****).*

Fine-root category: FR, absorptive foraging fine roots; TR, transport fine roots; MR, absorptive mycorrhizal fine roots, TSC, total sugar concentration (sucrose, glucose, fructose, and pinitol in case of spruce).

Beech rootlets only had significantly higher N concentration compared to spruce in late summer, which possibly resulted from the enhanced proportion of ephemeral roots with highest N. In rootlets, our results did not support the traits described by Reich (2014) for the foliar economic spectrum, that species with "fast" strategies, i.e. beech, will have higher N concentration compared to the "slow"-species, i.e. spruce. In contrast, our findings are in line with Weemstra et al. (2017) who found that root traits do not necessarily correlate with leaf traits, in particular in species with more conservative root traits (i.e. with thick roots and long root lifespan) such as (evergreens) conifers. Concerning these tree species, the discrepancy in leaf and root traits may result from the confounding effect of mycorrhiza on the seasonality in water and soil resources uptake.

According to Gordon and Jackson (2000) the average C:N ratio of fine roots <2 mm is 43:1 across a broad range of ecosystems and biomes. In beech fine-roots, Terzaghi et al. (2013) reported a C:N ratio from 40:1 to 90:1. Both studies reported on values which are much higher than the C:N ratio from our study. This discrepancy occurred as a result of the higher N concentration we found in beech and spruce rootlets (13–19 mg g^{-1}). This higher N concentration of rootlets in the trees from Kranzberger Forst is, however, not surprising, as it reflects the high levels of N deposition measured at that time in similar forests in Bavaria (20–25 kg ha^{-1} a^{-1}; Raspe et al., 2018).

In our study, N concentration was a significant predictor for root RR of the rootlets (see also Burton et al., 1998; Ceccon et al., 2016), but the seasonal dynamics in these relationships was species-specific, in particular during the harsh drought in late summer. In beech, the N status varied largely within the rootlets, indicating, in this tree species, an adaptable fine-root system to a patchy soil environment. In contrast, spruce rootlets were more uniform and likely showed a temporal 'dormancy' during late summer, in response to increased soil moisture deficits. The differential N–RR relationship outlines contrasting coping strategies for beech and spruce in the presence of drought: Fast mobilization and use of internal C stores for new fine-root growth to ensure sustained resource uptake in beech, but reduced fine-root growth and uptake via suberization of FR in spruce to prevent resource loss.

Non-Structural Carbohydrates

Non-structural carbohydrates are crucial in mitigating drought stress in plants (O'Brien et al., 2014; Hartmann and Trumbore, 2016; Hartmann et al., 2018). During drought, there is a distinct trade-off between growth and reserve accumulation, eventually leading to a decline of NSC concentrations in above-ground organs (McDowell, 2011) with simultaneously enhanced reallocation to roots (Brunner et al., 2015). Stored carbohydrates are important particularly in deciduous species that need to rely on the stored reserves to initiate leaf and root growth (Chapin et al., 1990; Landhäusser et al., 2012). In beech rootlets from our study, TSC increased through the growing season. This dynamic was largely driven by the sucrose content of the FR, which had the highest TSC reserves during late summer, to meet the C demand for respiration and regrowth. Physiologically active FR allow an increased water uptake and transfer to aboveground organs (Badri and Vivanco, 2009; Karst et al., 2016). TSC also remained high in transport fine-roots. The observed TSC accumulation in TR may lower the osmotic potential (Smirnoff, 1995; Brunner et al., 2015; Hommel et al., 2016), allowing for prolonged functionality of the aging roots responsible for transport and storage under drought. Such enhanced accumulation of assimilates reveals a "slow" ecological strategy of transport fine-roots in beech (**Figure 6**). In MR, sucrose, glucose and fructose concentrations almost doubled during the second half of the growing season, probably to maintain the high ectomycorrhizal colonization under drought (Shi et al., 2002).

In spruce rootlets, lowest levels of TSC were detected late in the growing season, corresponding to the enhanced proportion of MR with depleted sugars and starch concentration in autumn. In spruce FR and TR, highest TSC concentrations were found during the dry late summer, which is in line with temporally increased soluble sugars in roots in response to drought (McDowell, 2011; Müller et al., 2016). Thus, in both fine-root categories (FR and TR) a "slow" ecological strategy to drought adaptation was detected (**Figure 6**). Such temporal sugar accumulation may result from photosynthesis exceeding water-limited growth demands (Körner, 2003). Drought-induced TSC accumulation may be a common mechanism for survival during periods of stress in spruce. The high root concentrations of glucose, fructose, and pinitol may not only decrease the root water potential, facilitating water absorption from dry soil (Lambers et al., 1998) but also aid in sustaining and extending the mycelial network (Ekblad et al., 2013). For example, sustaining roots with a mycorrhizal association during drought requires sucrose to be hydrolyzed to glucose and fructose, and then consumed by both the fungal partners and root cortical cells (Nehls and Hampp, 2000). Although such use of sucrose is relevant for maintaining the respiration of MR in both tree species, spruce in particular, appears to rely more heavily on its mycorrhizae to withstand drought (Paradiso et al., 2019 and references therein). Under recurring prolonged episodes of drought, the persistence of drought-adapted ECM fungi can aid in tree survival, where trees with functional associations tolerating soil water potentials as low as −3 to −5.5 MPa (Smith and Read, 2008).

In this study, fine-root starch concentrations in both tree species were 10–100-fold lower than previously reported values for adult trees under natural growing conditions (e.g., Brunner et al., 2002; Barbaroux et al., 2003; Terzaghi et al., 2016; Rosinger et al., 2020). Soil water availability differed between the two tree

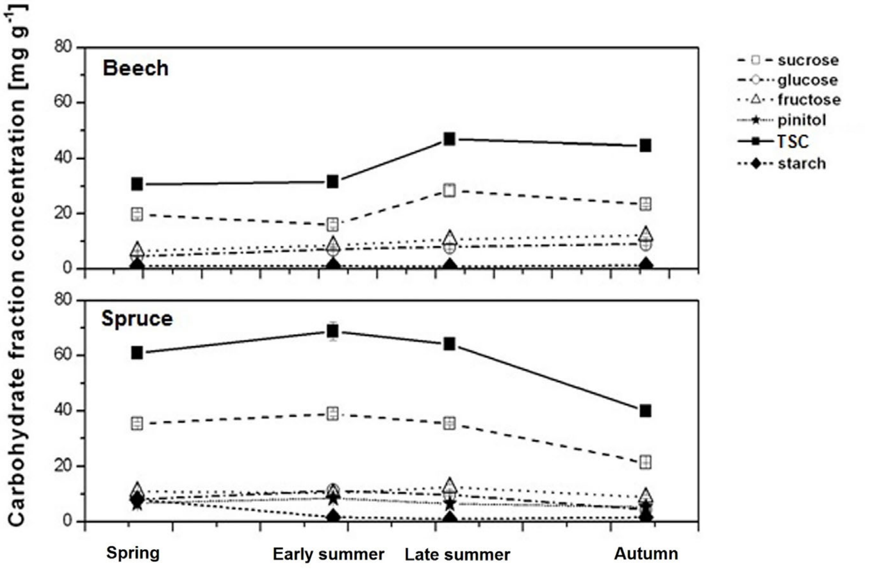

FIGURE 5 | Concentrations of the carbohydrate fractions (sucrose, glucose, fructose, pinitol) and the total sugar concentration TSC (i.e. integral of sucrose, glucose, fructose, and, in case of spruce, pinitol) together with starch concentration in individual rootlets of beech and spruce during 2003 (means ± 95% confidence interval, n = 7–10).

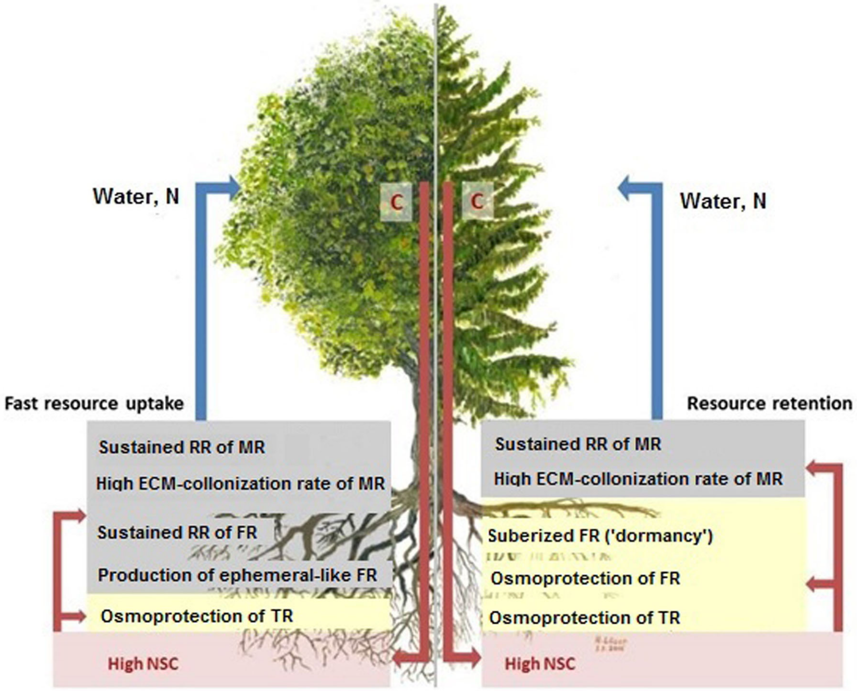

FIGURE 6 | Scheme of the two different ecological strategies of beech (left) and spruce (right) fine-roots under seasonal drought. Red arrows show the transport of C (assimilates, NSC) from leaves into root structures and functions, blue arrows show the returns of soil resources (e.g., water, N) from roots to leaves. The fine-root traits associated with "fast" ecological strategy are indicated in grey, those associated with "slow" ecological strategy are shown in yellow. The NSCs (red lettering) are crucial in linking and controlling above and belowground processes. ECM-colonization data according to Nikolova, 2007. Abbreviations see text.

species starting in the spring of 2003 (Nikolova et al., 2009), a direct result of the ability of spruce to take up and transpire water before beech flushed its leaves (Beier, 1998). This resulted in a longer period of exhausted soil water availability for spruce (i.e., 75 d in spruce vs. 45 d in beech). Experimental studies show starch concentration in roots to strongly decrease with the strength of stress (Thomas et al., 2002; Braun et al., 2004), which may result in higher mortality of the non-mycorrhizal fine roots, and/or in reduced fungal diversity of the mycorrhizal roots (Shi et al., 2002; Pena et al., 2010). In beech, the low starch concentration may have predetermined the short lifespan (Marshall and Waring, 1985; Nikolova, 2007) and, thus, the ephemeral character of beech fine roots during exceptional drought. In spruce, fine roots with reduced growth and low starch reserves may eventually represent a starch degradation into glucose, needed to support the mycorrhizal network. This would be reasonable as fungi use sugar alcohols such as arabitol and mannitol to enhance their osmotic strength during drought (Shi et al., 2002), and to enlarge their mycelium to proliferate into deeper and wetter mineral soil horizons (Ekblad et al., 2013). The heavy seed production that occurred for both tree species during 2003 (Dietrich et al., in prep.) could also have induced a major reallocation of the mobile C-pool into reproductive organs (Körner, 2003), additionally declining the stored starch in roots.

Specifics of the Study

The present study was a part of a larger project, where a comprehensive investigation on the belowground effects of an experimentally enhanced ozone regime was conducted from 2002 through 2004 (Matyssek et al., 2010). While this study was not explicitly set-up to study species specific drought effects we were able to capture this response through several of our samples. The systematic measurement of root RR and other related parameters was planned only for 2003 (cv. Nikolova, 2007), and only short methodological checks on these parameters were done during the non-limited years 2002 and 2004. Nevertheless, other parameters recorded systematically from 2002 to 2004 such as the "autotrophic" and "heterotrophic" soil RR, the fine-root production and the fine-root recovery rate (Nikolova et al., 2009), as well as the total soil RR, the standing fine-root biomass, the amount of annually produced fine-root biomass and its $\delta^{13}C$ signature (Nikolova et al., 2010) depicted contrasting responses of both species during 2003 with spruce being more effected by extreme drought compared to beech. This result is not surprising considering the different growth habits including leaf physiology, branching architecture (funnel-like in beech), rooting depth (shallow-rooted in spruce) of deciduous vs. evergreen species (Nikolova et al., 2009). But the underlying mechanisms of such species-specific adjustments to seasonal drought still remained unclear. Despite the limitation of not having reference data from "normal" conditions, the present study makes use 1/100 (1 in 100 years) drought event to analyze the belowground adaptability of these two most important Central European tree species (Ellenberg, 1996). Our investigation presents novel seasonal data on a broad range of fine-root traits and suggests possible mechanisms of adaptability, which should be tested in further experiments on adult forest trees.

CONCLUSIONS

We found for fine-roots in beech and spruce, that each species is not tied to one sole ecological strategy in coping with drought. In beech, the youngest absorptive FR followed the "fast" strategy, i.e. short-lived roots, enlarged specific fine-root area, and high root RR. High ECM colonization typical of mycorrhizal roots also represents a "fast" ecological strategy. These adjustments indicated enhanced C turnover, which facilitate effective acquisition of available belowground resources and rapid translocation of resources to aboveground organs. Transport fine-roots with developed secondary xylem, however, followed a "slow" strategy, as NSC increased during drought, possibly preventing resource efflux and root desiccation. Overall, during seasonal drought, beech fine-root traits largely reflected a "fast" strategy, particularly the youngest absorptive fine-roots.

In contrast, fine-root traits of spruce reflected largely a "slow" strategy. Suberized foraging fine-roots and the transport fine-roots had larger diameters and higher NSC levels facilitating long-term C retention during drought. Such adjustments can protect spruce fine-roots against desiccation and lower C required for respiration. However, absorptive mycorrhizal fine-roots were more indicative of the "fast" strategy. Therefore, the resource acquisition in spruce during drought seems to largely rely on mycorrhizal fungi.

The present study took advantage of the naturally occurring severe drought during the summer of 2003. However, the question remains as to what extent do beech and spruce trees recover from successive years of drought. Such scenarios are realistic considering the recent droughts of 2018 and 2019 in Central Europe. Our findings indicate that beech trees could likely suffer from C starvation during successive drought events since root regeneration can deplete the C reserves under drought-impaired photosynthesis. Spruce's survival lies heavily in the ability of mycorrhizal communities to survive recurring drought and continue to contribute to water/nutrient uptake and plant vitality. If instead the mycorrhizal association becomes a competitor during drought (Kariman et al., 2018) and therefore a dwindling C supply in weakened trees then the fate of spruce is also likely dire.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PN, IB, RM, K-HH, and HB conceptualized the main research questions. PN collected data and performed the data analyses. PN wrote the manuscript, RM, TB, K-HH, HB, and IB revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01211/full#supplementary-material

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Reaction Wood Anatomical Traits and Hormonal Profiles in Poplar Bent Stem and Root

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Reaction wood (RW) formation is an innate physiological response of woody plants to counteract mechanical constraints in nature, reinforce structure and redirect growth toward the vertical direction. Differences and/or similarities between stem and root response to mechanical constraints remain almost unknown especially in relation to phytohormones distribution and RW characteristics. Thus, *Populus nigra* stem and root subjected to static non-destructive mid-term bending treatment were analyzed. The distribution of tension and compression forces was firstly modeled along the main bent stem and root axis; then, anatomical features, chemical composition, and a complete auxin and cytokinin metabolite profiles of the stretched convex and compressed concave side of three different bent stem and root sectors were analyzed. The results showed that in bent stems RW was produced on the upper stretched convex side whereas in bent roots it was produced on the lower compressed concave side. Anatomical features and chemical analysis showed that bent stem RW was characterized by a low number of vessel, poor lignification, and high carbohydrate, and thus gelatinous layer in fiber cell wall. Conversely, in bent root, RW was characterized by high vessel number and area, without any significant variation in carbohydrate and lignin content. An antagonistic interaction of auxins and different cytokinin forms/conjugates seems to regulate critical aspects of RW formation/development in stem and root to facilitate upward/downward organ bending. The observed differences between the response stem and root to bending highlight how hormonal signaling is highly organ-dependent.

Keywords: bending stress, auxins, cytokinins, metabolite profiling, UHPLC-MS/MS

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INTRODUCTION

Mechanical stimuli (e.g., rain, wind, gravity, soil impedance, wounding, and bending) can considerably influence plant growth and development. Plants have developed sensory mechanisms to detect mechanical perturbations and to induce a suite of responses (anatomical, physiological, biochemical, biophysical, and molecular) collectively termed "thigmomorphogenesis" (Jaffe and Forbes, 1993; Braam, 2005; Dumroese et al., 2019). Thigmomorphogenesis can be considered as an adaptive response allowing individual plants to mitigate mechanical stress that occurs in their natural environment.

Over the past decades, different typologies of mechanical stress (in term of duration, targeted plant organ, or plant developmental stage) have been used to investigate thigmomorphogenic response in a wide range of plant species. Meanwhile, this phenomenon was documented in all types of plants, indicating its wide conservation (Jaffe and Forbes, 1993). Several short-time scale studies indicate a transient cessation of plant growth upon a mechanical stimulus. Conversely, continued long-term stem flexing led to an increase in root/shoot dry weight ratios, without affecting root length or total biomass, compared to unflexed plants (Garner and Björkman, 1999).

Beyond the macroscopically visible morphogenic changes, mechanical stress also affects wood mechanical properties (Chiatante et al., 2003; Montagnoli et al., 2020). In general, vascular cambium of trees growing in a windy environment produces a specific wood called "flexure wood" (FW) by increasing secondary xylem production and decreasing the elastic modulus in comparison to normal wood (NW) formed in absence of stimulus. In windy natural settings, bending occurs in a non-symmetric way, with the leeward portion of the stem experiencing more compression stress and the windward part more tension stress. Hence, what is really FW? The anatomy and specific functions of FW are poorly understood, but it needs to function in both compression and tension, due to the alternating sway, to reduce the risk of mechanical failure of the stem (Telewski, 1989; Kern et al., 2005; Koehler and Telewski, 2006).

Gymnosperms and angiosperms have evolved another strategy to counteract the bending constraint with secondary growth, which implies the asymmetrical formation of the so-called reaction wood (RW). This secondary xylem tissue is anatomically different from the NW and from the opposite wood (OW), commonly located on the opposite side of RW and characterized by properties between NW and RW (Timell, 1986). It, contrary to FW, does not form in response to swaying but due to displacement of the stem or root for times much longer than the presentation time (minimal time to produce a response) of gravitropism (Gardiner et al., 2016).

In gymnosperms, the RW is referred to as compression wood (CW) and develops on the lower side of leaning stems or branches, whereas, in dicotyledonous angiosperms, it forms on the upper side and is called tension wood (TW; Du and Yamamoto, 2007).

TW (more contractile) is often characterized by the formation of a gelatinous layer within the fiber cell wall (G-fibers) thought to be poorly lignified, and mainly composed of cellulose with the potential additions of arabinogalactan and xyloglucan differing from the normal fiber cell wall (Nishikubo et al., 2007; Bowling and Vaughn, 2008). Conversely, the typical CW contains more lignin, and has a flatter microfibril angle and lower cellulose content than NW and TW, being thus characterized by a high rigidity (Pilate et al., 2004). Stems and roots subjected to similar mechanical forces may develop extremely different RW. Indeed, in poplar, bending induces TW formation on the "upper" convex (tension) side of the stem or branch (Du and Yamamoto, 2007). Conversely, in poplar root, De Zio et al. (2016) for the first time observed that a CW similar to gymnosperm stems was formed in the "lower" concave (compression) side after bending.

Authors observed many similarities between FW and RW (CW or TW) formed in both gymnosperms and angiosperms (Butterfield and Li, 2000; Kern et al., 2005). However, the effect of elementary stresses (swaying, compression, and tension loadings) on wood anatomy, structure, and function is not completely known.

In the last 2 decades, computer modeling has helped to understand how mechanical forces are loaded on plant organs showing patterns coherent with direct measurement and able to explain the induced alterations (Danjon et al., 2005; Yang et al., 2014, 2016; Montagnoli et al., 2020). It is evident that tension and compression loadings induce complex signal transduction pathways that involve other factors such as phytohormones, which are still poorly understood, especially at the root level (Du and Yamamoto, 2007).

Functional role of auxin in plant response to mechanical stress has been an active area of research on woody stems. However, findings on the relationship between endogenous auxin levels and the formation of CW or TW are still scarce and sometimes contradictory, thus remaining to be elucidated (Du and Yamamoto, 2007). Hellgren et al. (2004) found that the formation of TW and CW in poplar and pine bent stems, respectively, are not mediated by changes in the indole-3-acetic acid (IAA) level in the cambial tissues. On the contrary, Funada et al. (1990) and Du et al. (2004) detected higher amount of endogenous IAA at the side of the cambial region forming CW. In line with these findings, we also found that compression forces induce wood formation with the intermediation of high IAA levels (De Zio et al., 2016), which could act as a spatial regulator of cambial activity, enhancing cell division rate and conferring key positional information to the cells of the cambial zone surrounding tissues for differentiation/RW initiation. Unlike IAA, the role of cytokinins (CKs) in RW formation has seldom been investigated (Little and Pharis, 1995; Mellerowicz et al., 2001). In *planta*, CKs occur in four principal forms: (i) the nucleotides (iPRMP, *t*ZR5MP, *c*ZRMP, and DHZMP) which are produced by *de novo* biosynthesis and then converted to other derivatives; (ii) the free bases (iP, *t*Z, *c*Z, and DHZ) which are considered to have the highest activity together with (iii) the ribosides (iPR, *t*ZR, *c*ZR, and DHZR) which are also preferably transport; and

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Abbreviations: BS, Bending sector; CKs, Cytokinins; CZ, Cambial zone; ***c***Z, *cis*-Zeatin; ***c***Z7G, *cis*-Zeatin-7-glucoside; ***c***Z9G, *cis*-Zeatin-9-glucoside; ***c***ZOG, *cis*-Zeatin O-glucoside; ***c***ZR, *cis*-Zeatin riboside; ***c***ZR5'MP, *cis*-Zeatin riboside-5'-monophosphate; ***c***ZROG, *cis*-Zeatin riboside O-glucoside; DHZ, Dihydrozeatin; DHZ7G, Dihydrozeatin-7-glucoside; DHZ9G, Dihydrozeatin-9-glucoside; DHZOG, Dihydrozeatin O-glucoside; DHZR, Dihydrozeatin riboside; DHZRMP, Dihydrozeatin riboside-5'-monophosphate; DHZROG, Dihydrozeatin riboside O-glucoside; IAA, Indole-3-acetic acid; IAAsp, IAA-Aspartate; IAGlu, IAA-Glutamate; iP, N-Isopentenyladenine; iP7G, N^6-Isopentenyladenine-7-glucoside; iP9G, N^6-Isopentenyladenine-9-glucoside; iPR, N^6-Isopentenyladenosine; iPRMP, N^6-Isopentenyladenosine-5'-monophosphate; FW, Flexure wood; oxIAA, 2-Oxindole-3-acetic acid; OW, Opposite wood; PCA, Principal component analysis; RW, Reaction wood; SPE, Solid-phase extraction; ***t***Z, *trans*-Zeatin; ***t***Z7G, *trans*-Zeatin-7-glucoside; ***t***Z9G, *trans*-Zeatin-9-glucoside; ***t***ZOG, *trans*-Zeatin O-glucoside; ***t***ZR, *trans*-Zeatin riboside; ***t***ZR5'MP, *trans*-Zeatin riboside-5'-monophosphate; ***t***ZROG, *trans*-Zeatin riboside O-glucoside.

(iv) the glucosides which are temporary (*O*-glycosylation) or permanent (*N*-glycosylation) storage of inactivated forms (Spíchal, 2012). The *O*-glucoside, together with the ribosides, represents the form less susceptible to degradation by cytokinin oxidase and readily converted to the free base forms (Kieber and Schaller, 2014). Among the free bases, *c*Z is believed to have a lower activity compared to *t*Z or iP, which are generally considered the most biologically active CKs positively controlling overall plant growth (Schafer et al., 2015).

Despite the well-established functions of CKs in cell division, tracheid differentiation, and lignin biosynthesis (Aloni et al., 2006), no direct relation between CKs and RW formation was found in bent stem. Conversely, in the bent root, we found that specific and distinct CK types/forms in the vascular tissue control RW formation toward the compressed side (De Zio et al., 2016, 2019). Although evidence on the involvement of plant hormones has been provided (Trupiano et al., 2012b; Miyashima et al., 2013; Ursache et al., 2013; De Zio et al., 2016), differences and/or similarities existing between poplar bent roots and stems, especially in relation to the different intensity of tension and compression forces perception, remain unknown.

In the present study, firstly we assume that static bending in woody root and stem would induce an asymmetrical distribution of mechanical forces along the different stretched and compressed sides and sectors (bent sector and above/below the bent sector). Secondly, the different forces perceived by the two bent organs would produce also an asymmetrical phytohormones accumulation, able to trigger the formation of RW with differentiated characteristics in anatomical traits and chemical composition. To test our hypotheses, the woody *Populus nigra* plant responses to static, non-destructive, mid-term bending treatment was investigated along the stem and root axes, in stretched and compressed sides, by (i) developing a theoretical model to assess the type and magnitude of mechanical forces distribution, and (ii) analyzing anatomical features, chemical composition, and IAA and CKs metabolites profiling.

MATERIALS AND METHODS

Plant Material and Bending Conditions

Static non-destructive mid-term (5 months) bending constraint was applied to 2 year-old *P. nigra* plants ($n = 5$). The root bending simulation was performed by tying taproots around right angle curved steel nets, as previously described in De Zio et al. (2016). The same bending angle (~90°) and similar supporters were used to impose the stress at stem level of other five poplar plants (Supporting information 1). All plants were grown for 5 months in a growth chamber at 22°C and 60–70% humidity with a 16 h photoperiod simulated by LED lights (λ_{420}–λ_{740}) and a photosynthetically active radiation of 350 μmol m^{-2} s^{-1} (Light Meter HD2302.0, Delta Ohm, Caselle di Selvazzano, Italy), ensuring controlled conditions.

At the end of the 5-months of bending treatment, a detailed spatial sampling and analysis was performed. Firstly, from both root and stem were taken three longitudinal sectors, each 5 cm long: (1) above bending sector (ABS), corresponding to the region just above the bending zone; (2) bending sector (BS), representing the point of maximum bending; and (3) below bending sector (BBS), corresponding to the region just below the bending zone. In the case of bent roots, ABS was localized at 12–17 cm distant from the root collar, BS at 17–22 cm, and BBS at 22–27 cm. Distances were inverted in the case of stem (ABS at 22–27 cm, BS at 17–22 cm, and BBS at 12–17 cm). Secondly, each region (ABS, BS, and BBS) was further divided lengthwise into two parts to collect both the convex (CX) and concave side (CE; **Supplementary Figure S1**). Immediately upon collection, the samples were frozen in liquid nitrogen and stored at −80°C for chemical analysis and hormone profiling or fixed in formalin-acetic acid-alcohol (FAA, 5:5:90) for anatomical investigations.

Models of Bending Forces Distribution

Mechanical forces distribution along the bent stem and root models was performed by Mecway finite element analysis package (version 9.0; Mecway, 2014), considering the diameter and wood mechanical property of the two organs. At the beginning (t_i) and at the end (t_f) of the bending treatment, stem and root diameters of 15 plants were measured by using ImageJ 1.41o software (Wayne Rasbanb, National Institute of Health, United States). As already described in Fourcaud et al. (2008) and in Montagnoli et al. (2020), plant material was considered isotropic, uniform, and elastoplastic with a density of 1,000 kg/m^3, with Young's modulus of 5 GPa and a Poisson's ratio of 0.3. Bending stress was applied to a total axis length of 15 cm through a forced displacement at the narrow end of the stem or root. Tension and compression forces were calculated considering the mesh average characterized by a total of 15 sections (1 cm each). Plant diameters from five adjacent sections were used to compose a specific organ sector (ABS, BS, and BBS) and, comprehensively, were used to construct a 2D section of the organ. A 3D model was derived by revolving the 2D section on this longitudinal axis.

Anatomical Investigations

Each bent root and stem sector (ABS, BS, and BBS) fixed in FAA was dehydrated using a graded ethanol series (10, 30, 50, 70, 95, and 100%) and embedded using the Technovit 7,100 resin system (Heraeus Kulzer, Wehrheim, Germany) based on 2-hydroxyethyl-methacrylate.

Samples were sectioned into cross-sections (12 μm thick) using a sliding microtome. Finally, sections were stained in Toluidine Blue O (Parker et al., 1982) for 1 min. Sections were photographed using an Olympus BX63 light microscope equipped with an Olympus DP72 camera. Images were analyzed by ImageJ 1.41o software (Wayne Rasbanb, National Institute of Health, United States). In order to define the convex and the concave sides precisely, a 45°C rotated graphic crosswise object was applied, having the center of the primary xylem stele as the anchor point. In the convex and concave sides of three bent stem and root sectors, the following parameters were measured: cambial cell number (CCN), cambial zone thickness (CZT; µm), vessel wall thickness (VWT; µm), fiber wall thickness (FWT; µm), relative xylem thickness (RXT%), relative phloem thickness (RPT%), relative vessel area (RVA), relative vessel number (RVN), specific vessel area (SVA), and specific vessel number (SVN). Measurements were carried out in the areas formed after the application of bending (**Supplementary Figure S1**) following calculation reported in De Zio et al. (2016).

Lignin and Carbohydrate Determination

Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) was used to analyze lignin and carbohydrate content of bent woody root and stem samples. For the analysis, 50 µg (±10 µg) of ball-milled (MM400, Retsch, Germany) wood powder was applied to a pyrolyzer equipped with an auto sampler (PY-2020iD and AS-1020E, Frontier Lab, Japan) connected to a GC/MS (Agilent 7890A/5975C; Agilent Technologies AB, Sweden). The pyrolysate was separated and analyzed according to Gerber et al. (2012). After chromatograms processing, peaks were automatically classified and integrated. Classification defined peaks into syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H)-type lignin, carbohydrates (C), according to the highest abundant *m/z* channel.

Phytohormones Measurement

Indole-3-acetic acid (IAA) metabolites and CKs were extracted from 20 mg of fresh weight stem/root material (each pooled from five poplar plants), as described in De Zio et al. (2019). Vacuum-dried auxin and cytokinin fractions were dissolved in 10% methanol and stored at −20°C until ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC-MS/MS) analysis.

Separation and determination of compounds was performed using a 1,290 Infinity LC system and 6,490 Triple Quadrupole LC/MS system (Agilent Technologies). Auxins mass analysis was done according to Novák et al. (2012), while CKs mass analysis was carried out in accordance with Novák et al. (2008). IAA metabolites were expressed as pg. mg^{-1} of dry weight, while CKs as pmol g^{-1} of dry weight. MassHunter software (version B.05.02; Agilent Technologies) was used to determine the concentrations of all examined compounds, using stable isotope dilution method.

Statistical Analysis

When needed, variables were square root or log transformed, to ensure normal distributions and equal variances for the use of parametric statistics. As anatomical data did not follow the normal distribution, nonparametric statistics were applied. The Kruskal-Wallis multiple-comparison test was used to compare anatomical measurements for the two plant organs (stem and root), sectors (ABS, BS, and BBS), and sides (CX and CE). The Mann-Whitney U-test was used for pairwise comparison of anatomical measurements among root sectors for each of the two sides and to compare convex and concave sides within each sector. A 95% significance level was applied to analysis with nonparametric methods. For phytohormones analysis, a one-way ANOVA was used to compare different plant organs (stem and root), sectors (ABS, BS, and BBS), and sides (CX and CE). *Post-hoc* LSD-tests were conducted to detect overall differences between convex and concave sides for each sector of each plant organ. Analyses were applied on a 95% significance level. All statistical analysis was carried out using statistical software package SPSS 17.0 (SPSS Inc., Chicago IL, United States).

Finally, in order to investigate variance among different analytical dataset obtained from each sector of both the organs, we performed a principal components analysis (PCA). The main anatomical parameters (CCN, RXT, and RPT) and phytohormones (IAA and CK free forms – *c*Z, *c*ZR, *t*Z, *t*ZR, iP, iPR, and DHZ), together with total lignin amount, were analyzed by using FactoMineR package in R (Husson et al., 2014; R Core Team, 2020).

RESULTS

Mechanical Force Distribution Model

The modeling of mechanical force distribution along bent stem and root axis showed maximum values of compression and tension forces in BS compared to ABS and BBS sectors of both organs (**Figure 1**). In general, the magnitude was higher for compression forces, rather than tension; both of them were greatest in the BS and were dissipated away from it, showing higher values in BBS than ABS.

All forces increased from the initial (t_i) to the final (t_f) phase of the bending application with different intensities depending on the sector analyzed (**Figure 1**). In stem, from t_i to t_f, tension forces increased in the ABS and BS, remaining almost unchanged in the BBS, while compression forces greatly increased in the ABS and BBS and were similar in the BS.

In root, tension forces strongly increased in the BS, whereas remained almost unchanged in the ABS and BBS; compression forces increased slightly in the BS, 3-fold in the ABS, and unchanged in the BBS (**Figure 1**).

Anatomical Traits

Cross-sectional anatomical analysis of the bent stems showed that CCN did not differ among sectors, and for the ABS was lower in the convex side than in the respective concave side. No differences were detected among sectors and sides for the thickness of CZT (**Table 1**). The RXT did not differ among the three sectors while was larger in the convex side of both ABS and BS than the respective concave sides (**Figure 2** and **Table 1**). The specific vessel number (SVN) and vessel wall thickness (VWT) did not differ among sectors, but it was higher in the concave side of ABS than in the respective convex side. The relative phloem thickness (RPT) did not differ between the two sides within each specific sector but showed the highest and the lowest values in the concave BBS and ABS, respectively, and intermediate value in the BS (**Table 1**). The fibers wall thickness (FWT) did not differ between the two sides within each specific sector

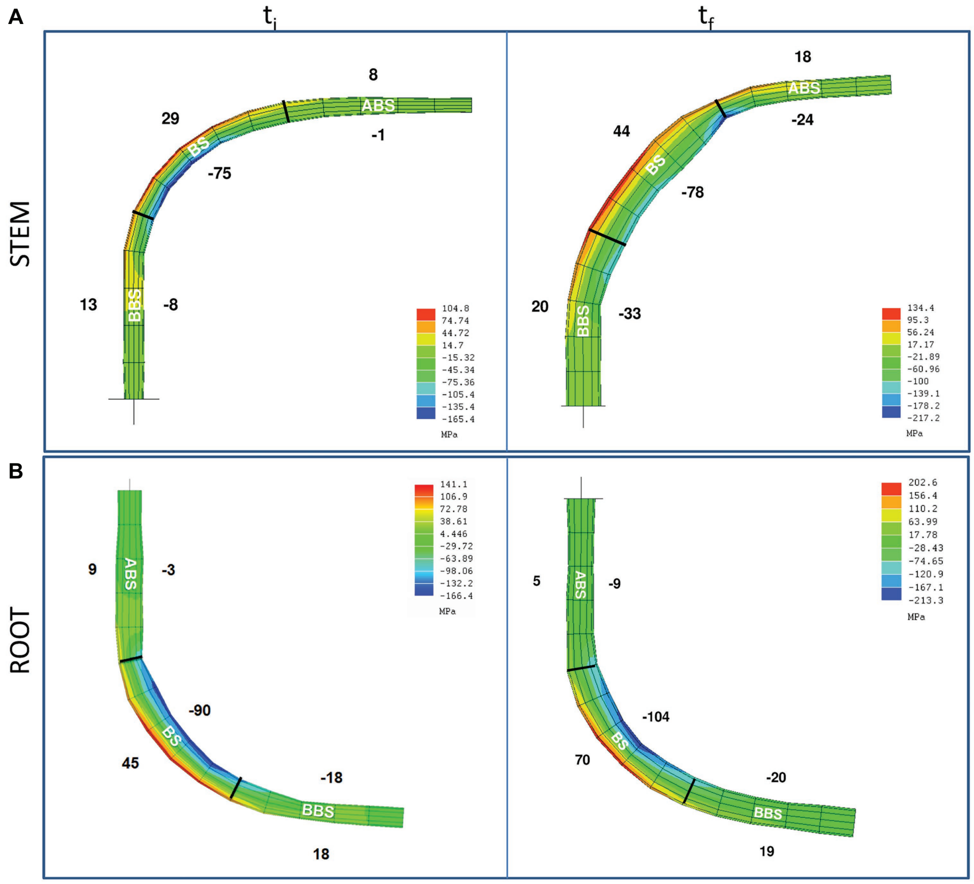


FIGURE 1 | Model of the mechanical forces distribution along *P. nigra* bent stem and root. Mechanical forces distribution along the stem **(A)** and root **(B)** main axis, at the beginning (t_i) and the end (t_f) of bending treatment. Average value (MPa) of the mechanical force magnitudes are indicated on the corresponded concave (negative values – compression) and convex (positive values – tension) sides of three bent sectors (ABS, BS, and BBS). ABS, above bending sector; BS, bending sector; BBS, below bending sector.

and showed a lower value in convex BS only compared to BBS. In addition, fiber cell walls of convex ABS and BS were characterized by the formation of gelatinous layer (G-layer; magnification in **Figure 2**). Specific vessel area (SVA) did not differ among sides within each specific sector but showed the highest and the lowest value in the both sides of BBS and ABS, respectively (**Table 1**).

Cross-sectional anatomical analysis of the root showed that the CCN and RXT did not show any significant difference among sectors, whereas the CZT and RPT, in the concave side, were higher in both BS and BBS than in the ABS (**Figure 2** and **Table 1**). However, all previous parameters (CCN, CZT, RXT, and RPT) measured in the concave side of both BS and BBS were higher than in the respective convex side (**Figure 2** and **Table 1**). SVN and VWT did not differ within sectors and showed the lowest and highest values only on the convex side of the ABS and BS, respectively, with intermediate value in the BBS (**Table 1**). FWT did not show any significant difference among sides and sectors, whereas the SVA differed only between different sectors on the concave sides showing low, middle, and high value in ABS, BS, and BBS, respectively (**Table 1**).

Carbohydrate and Lignin Content

The comparison of Py-GC/MS data among different bent stem sectors showed that carbohydrates were higher in the convex side of BS than BBS and unchanged in ABS, whereas in the concave sides, they were higher in ABS than BBS and unchanged in BS (**Figure 3A**). Total lignin in the convex sides was lower in BS than BBS, due to a lowest S-type lignin, and unchanged in ABS (**Figure 3B**). No variations were observed along different sectors of concave sides. Furthermore, within sectors, convex ABS and BS were characterized by higher carbohydrates amount and lower total lignin content compared to the opposite concave sides (**Figures 3A,B**); variations resulted mainly due to a lower amount in G- and S-type lignin, whereas H-types remained unchanged (**Figure 3B**).

In case of bent root, results revealed that in the concave side, carbohydrates were lower in ABS than BBS and unchanged compared to BS (**Figure 3C**) whereas in the convex side, no

TABLE 1 | Stem and root anatomical traits.

		STEM			ROOT		
		Sector			Sector		
Anatomical parameter	**Side**	**ABS**	**BS**	**BBS**	**ABS**	**BS**	**BBS**
CCN	CX	**3.42 ± 0.37***	4.72 ± 0.52	4.29 ± 0.53	4.31 ± 0.29	**4.46 ± 0.29***	**4.42 ± 0.23***
	CE	**4.65 ± 0.32***	4.56 ± 0.67	4.08 ± 0.35	4.62 ± 0.16	**5.62 ± 0.40***	**5.65 ± 0.47***
CZT	CX	14.04 ± 1.69	18.65 ± 3.00	17.27 ± 1.93	24.28 ± 1.78	**25.18 ± 2.13***	**22.37 ± 1.63***
	CE	18.17 ± 2.81	21.82 ± 3.20	18.01 ± 1.48	**26.87 ± 1.52**^{b}	**32.72 ± 1.88**^{a,*}	**35.45 ± 2.70**^{a,*}
RXT	CX	**10.44 ± 1.60***	**12.26 ± 1.28***	10.13 ± 2.81	4.94 ± 1.09	**5.72 ± 0.92***	**6.31 ± 1.06***
	CE	**6.47 ± 2.09***	**5.70 ± 1.21***	7.69 ± 1.00	8.06 ± 0.70	**9.67 ± 0.60***	**10.23 ± 1.14***
SVN	CX	**275 ± 51***	303 ± 98	458 ± 54	**348 ± 11**^{b}	**420 ± 28**^{a}	**396 ± 74**^{a,b}
	CE	**434 ± 13***	258 ± 100	552 ± 48	291 ± 51	332 ± 31	377 ± 41
VWT	CX	**1.15 ± 0.13***	1.29 ± 0.06	1.36 ± 0.07	1.65 ± 0.12	1.73 ± 0.13	1.76 ± 0.12
	CE	**1.47 ± 0.15***	1.28 ± 0.11	1.45 ± 0.12	**1.74 ± 0.11**^{b}	**2.03 ± 0.05**^{a}	**2.04 ± 0.11**^{a,b}
RPT	CX	8.24 ± 1.19	9.43 ± 0.36	9.31 ± 0.67	16.14 ± 1.36	**17.07 ± 1.34***	**18.88 ± 0.80***
	CE	**6.90 ± 1.69**^{b}	**7.79 ± 0.25**^{a,b}	**9.10 ± 0.86**^{a}	**16.75 ± 0.88**^{b}	**21.66 ± 1.06**^{a,*}	**21.26 ± 0.77**^{a,*}
FWT	CX	**0.71 ± 0.07**^{a,b}	**0.63 ± 0.04**^{b}	**0.79 ± 0.04**^{a}	0.88 ± 0.04	0.80 ± 0.04	0.84 ± 0.04
	CE	0.77 ± 0.05	0.66 ± 0.06	0.76 ± 0.03	0.97 ± 0.05	0.88 ± 0.05	0.86 ± 0.06
SVA	CX	**5.59 ± 0.91**^{b}	**7.82 ± 3.00**^{a,b}	**14.30 ± 2.39**^{a}	30.8 ± 2.9	34.8 ± 1.7	33.2 ± 5.1
	CE	**9.17 ± 1.21**^{b}	**8.10 ± 3.07**^{b}	**22.01 ± 2.43**^{a}	**25.9 ± 3.6**^{b}	**31.5 ± 2.3**^{a,b}	**38.0 ± 2.7**^{a}

Anatomical parameters were analyzed for stem and root in the convex (CX) and concave (CE) sides of three bent stem and root sectors (ABS, BS, and BBS). Reported values are the mean of five replicates (±SE). Bold value indicates significant differences ($p < 0.05$) between the three bent sectors of the same side (different letters) and between convex and concave sides of the same sector (asterisk). ABS, above bending sector; BS, bending sector; BBS, below bending sector; CCN, cambial cell number; CZT, cambial zone thickness; RXT, relative xylem thickness; SVN, specific vessel number; VWT, vessel wall thickness; RPT, relative phloem thickness; FWT, fiber wall thickness; and SVA, specific vessel area.

significant change was found. Total lignin showed an opposite trend in the concave sides, being higher in ABS than BBS and unchanged compared to BS, while it was completely unvaried in the convex side (**Figure 3D**). The change in lignin content was mainly due to the alternation in S-lignin type amount. Convex and concave sides of all three sectors showed similar carbohydrate content (**Figure 3C**). Lignin content increased only in the concave ABS compared to the opposite convex side and unchanged in the two sides of BS and BBS. The increase in total lignin amount in the concave ABS was due to an increase of G- and S-type lignin (**Figure 3D**).

Phytohormones Measurement

Hormone profile showed specific distribution patterns of IAA and CKs and related metabolites in the three sectors of two bent organs (stem and root), underlining differences among them and between the convex and concave side of each sector (**Figures 4, 5**).

In bent stems sectors, the comparison of IAA content showed that in both convex and concave sides, IAA was higher in BS and BBS than ABS and remained unchanged between the two sides of all three bent sectors (**Figure 4A**, light gray).

IAGlu, in the convex sides, was below the limit of detection (<LOD) in ABS, while increased in BS and BBS (**Figure 4B**, light gray). In the concave sides, it followed the same IAA trend, but reached the maximum in BBS. Between stem sides, IAGlu was lower in the convex ABS and higher in convex BS compared to the concave opposite side, while no variation was observed between the two sides of BBS.

The IAAsp resulted <LOD in both sides of BS and BBS and high in those of ABS, reaching the maximum in the concave side (**Figure 4C**, light gray).

The oxIAA did not show any variation among sectors but it was low in all convex side in respect to the concave side (**Figure 4D**, light gray).

In bent root, IAA amount was lower in the convex BS and BBS sides than opposite concave sides and convex ABS. Furthermore, no significant variations were observed among concave side sectors and within the convex and concave ABS (**Figure 4A**, dark gray).

The comparison of IAGlu, among sectors, revealed that in convex side was lower in BS and BBS than ABS while in concave side was higher in BS than ABS and unchanged compared to BBS. Furthermore, within sectors, it was lower in the concave ABS and higher in the concave BS compared to the opposite side, while values were comparable in the two sides of BBS (**Figure 4B**, dark gray).

Analyzing IAAsp amount, among sectors of the convex and concave sides, an opposite gradient was observed. In detail, in the convex side, it was lower BS and BBS than ABS, while in the concave side, it was higher in BBS than ABS and BS. Furthermore, between sides, it was lower on the concave ABS and higher in the concave BBS compared to the opposite sides, while similar values were found between BS sides (**Figure 4C**, dark gray).

As for the catabolic product oxIAA, in the convex side, it was higher in ABS and BS than BBS, while in the concave side, oxIAA showed the highest value in BS. Between sides, it decreased only in the concave ABS compared to the opposite side (**Figure 4D**, dark gray).

Distribution of different CKs between sectors and sides of the bent stem and root were analyzed in detail (**Figure 5**). CKs were specifically categorized according to their side-chain structure into *t*Z-, *c*Z-, DHZ-, and iP-types (**Figures 5A,C**),

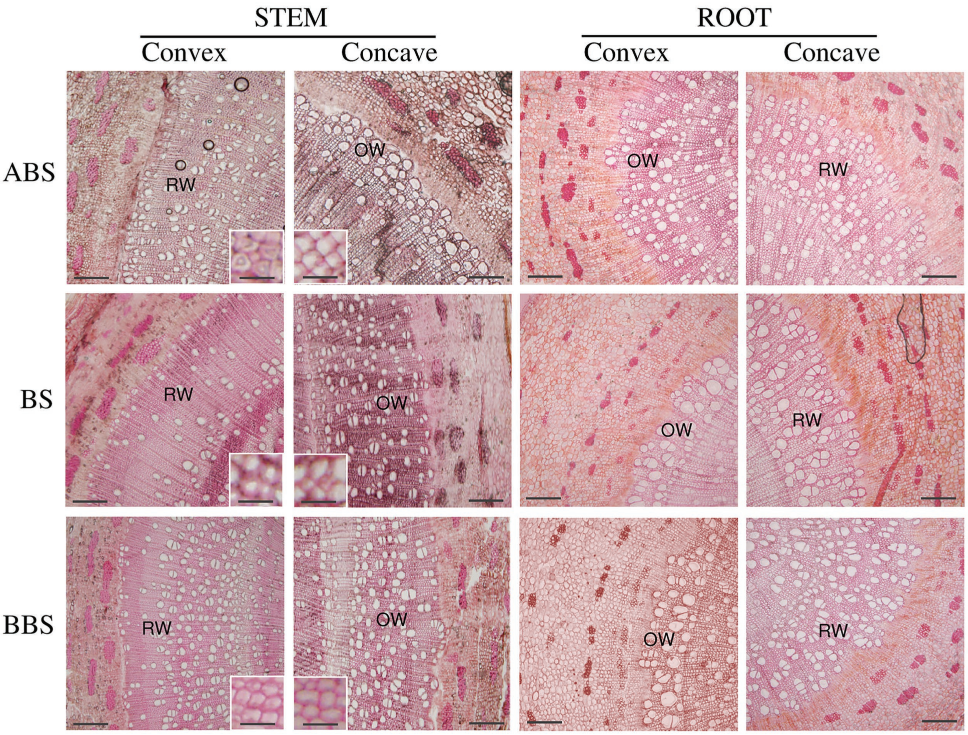


FIGURE 2 | Anatomical bent stem and root cross-sections. Cross-sections of the convex and concave sides of three bent stem and root sectors (ABS, BS, and BBS) stained with Toluidine Blue O. Scale bar = 20 μm. Magnification shows secondary wood fiber cell wall characteristics. Scale bar = 2 μm.

specifying the amount of each form/conjugate in the heat maps (**Figures 5B,D**).

In bent stem, total CKs amount was the highest in ABS and BS of the convex side (**Figure 5A**) due to the main accumulation of the *t*ZROG, DHZ, and iPR in ABS and the *t*Z, *t*ZR, *c*Z, *c*ZR, *c*ZOG, and *c*ZROG forms/conjugates in ABS and BS (**Figure 5B**). In the concave side, total CKs was the lowest in ABS, due to a low amount in *t*Z- and *c*Z-type, without any significant differences in the accumulation of specific forms/conjugates (**Figures 5A,B**).

Between stem sides, *t*Z and *t*ZR were mainly accumulated in the convex ABS and BS with respect to the opposite concave site and of DHZ, DHZOG, *t*ZROG, *c*ZR, and iPR only in convex ABS (**Figure 5B**). Thus, all CK-types were high in the convex ABS and *t*Z-type in the convex BS (**Figure 5A**).

In bent root, although total CKs were unchanged between and within sectors, the DHZ-type was slightly accumulated in the concave BBS (**Figure 5C**). Furthermore, some specific forms/conjugates were differentially accumulated between and within sectors (**Figure 5D**). In detail, among sectors, in the convex side, high iP amount was found in ABS and BS, and <LOD in BBS. In the concave side, *c*Z and iP were higher in BS and BBS than ABS (<LOD), together the *t*Z and *t*ZR5’MP in BS and the *t*ZROG and DHZ9G in BBS (**Figure 5D**). Comparing the two sides, the *t*ZR5’MP and the iP resulted <LOD in the convex ABS and BBS, respectively. The iP was <LOD also in the concave ABS together the *c*Z; finally, the *t*ZR5’MP was high in the concave BS and the *t*ZROG and DHZ9G in the concave BBS (**Figure 5D**).

The ratios of total and active CKs to IAA were also calculated (**Figure 6**) as total, sum of all CK metabolites detected, and active CKs, representing the sum of CK bases and ribosides. In the convex sides of three bent stem sectors, total and active CKs ratios showed the highest value in ABS while no variation was observed in the concave sides. In bent root, BBS showed the maximum value among sectors of the convex sides while no variation was observed between those of the concave sides. Similar trends were shown by both total and active ratios when analyzing differences between sides. In the stem, the content of the CKs and IAA resulted almost the same in both sides of BS and BBS, whereas in the ABS convex, the CKs levels were significantly higher (about 2-fold) compared to IAA (**Figure 6A**). In root, ABS and BS showed a similar content of two hormones in both root sides, whereas in the BBS concave, the IAA level was significantly higher than to that of CKs, especially considering the CKs active form (**Figure 6B**).

Principal Component Analysis of Main Stem and Root Traits According to Bending Sectors

The PCA scatter plots of principle component 1 (PC1) and 2 (PC2) obtained for the three sectors (ABS, BS, and BBS)

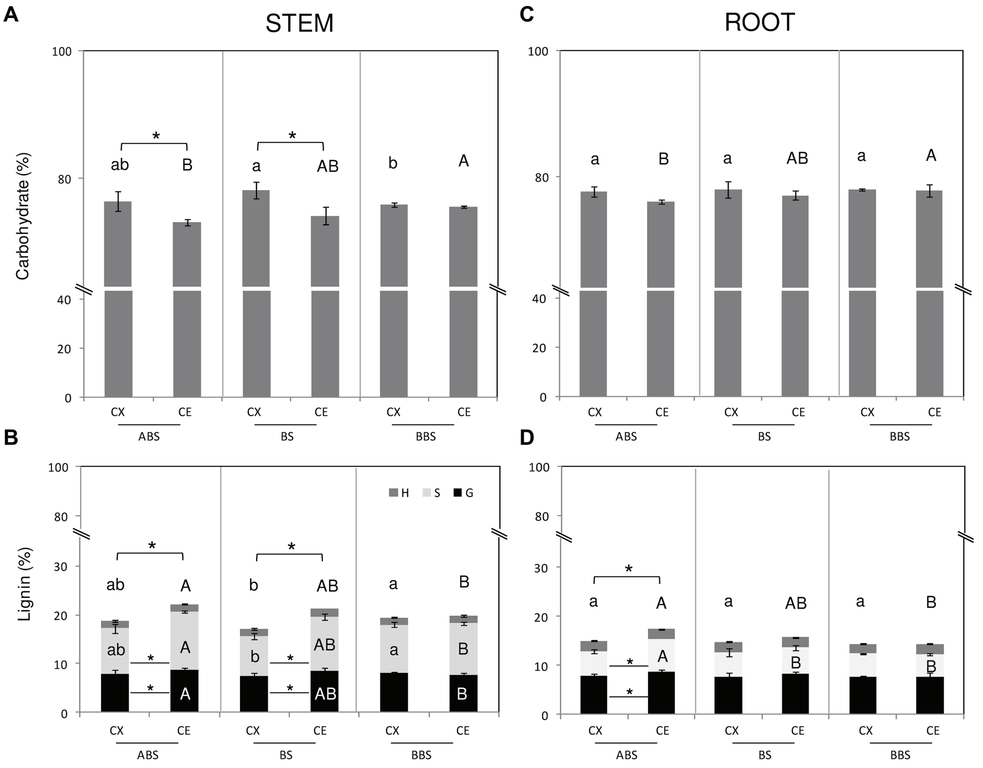


FIGURE 3 | Carbohydrate and lignin content of *P. nigra* stem and root. Total carbohydrate and lignin content from CX and CE sides of ABS, BS, and BBS are indicated in the **(A)** and **(B)** for the bent stem and in the **(C)** and **(D)** for the bent root. Percentage value represents the mean of five independent samples ±SD analyzed by Py-GC/MS. Significant differences (*post-hoc* LSD-tests, $p < 0.05$) among the CX sectors are indicated by lower letters whereas those among CE sectors by capital letter. Significant differences (*post-hoc* LSD-tests, $p < 0.05$) between sides of the same sector are indicated by asterisk. Differences in total lignin amount are indicated above the histograms while differences in Syringyl- (S-), Guaiacyl- (G-), and p-Hydroxyphenyl- (H-) types lignin are indicated inside the histograms. ABS, above bending sector; BS, bending sector; BBS, below bending sector; CX, convex side; CE, concave side.

of bent stem and root are illustrated in **Figure 7**. From the analysis, in stem, we found a cumulative percentage of PC1 and PC2 accounted for 79.7, 82.5, and 65.2% in ABS, BS, and BBS, respectively. In particular, it was clear that all CKs, IAA, RXT, and RPT were grouped in ABS plot, suggesting that these parameters had a positive correlation among themselves. Also in BS, we observed a good closeness of all the vectors representing CKs and anatomical variables (RXT, RPT, and CCN), meaning their reciprocal high correlation. However, this trend is not emerging for BBS in which a correlation among some CKs (*t*Z, *t*ZR, *c*ZR, iP, and iPR), IAA, RPT, and RXT is clear.

In root, the PC1 and PC2 cumulative percentage accounted for 73.5, 75.2, and 76.9% in ABS, BS, and BBS, respectively. In detail, considering data related to ABS, the scenario seems similar to what is already described for BBS of the stem: anatomical vectors seem to be correlated with IAA and some CKs (*t*Z, *c*Z, and iP) vectors. In the case of BS, we observed that CKs and IAA vectors are next to, and thus correlated to, all anatomical vectors. Finally, in the case of BBS, some CKs (*t*Z, *c*Z, and iP) vectors are spread and not closely related to anatomical vectors that conversely were grouped together with IAA vector.

DISCUSSION

The modeling of mechanical forces distribution along *P. nigra* L. stem and root axis showed a different intensity of compression and tension forces in the concave and convex sides, respectively, in all the three sectors ABS, BS, and BBS analyzed. However, in bent stem, compression forces increased during time (from t_i to t_f) on concave ABS and BBS, whereas tension forces increased on the convex ABS and BS. Confirming our previous findings (Trupiano et al., 2012b), bent root showed the highest increment of tension forces in the convex side of BS and compression forces in the concave side of ABS. Thus, based on model, in BS, where the maximum of forces intensity was recorded, only the tension forces increased during time in both organs.

Anatomical features and chemical composition variations observed in the convex and concave sides of the three bent woody stem and root sectors were strongly related to both the type of mechanical forces (compression or tension) and the intensity of mechanical force-displacement. In particular in bent stem, compression forces induced the development of secondary phloem on the concave side of BS and BBS, whereas tension forces promoted the formation of RW on

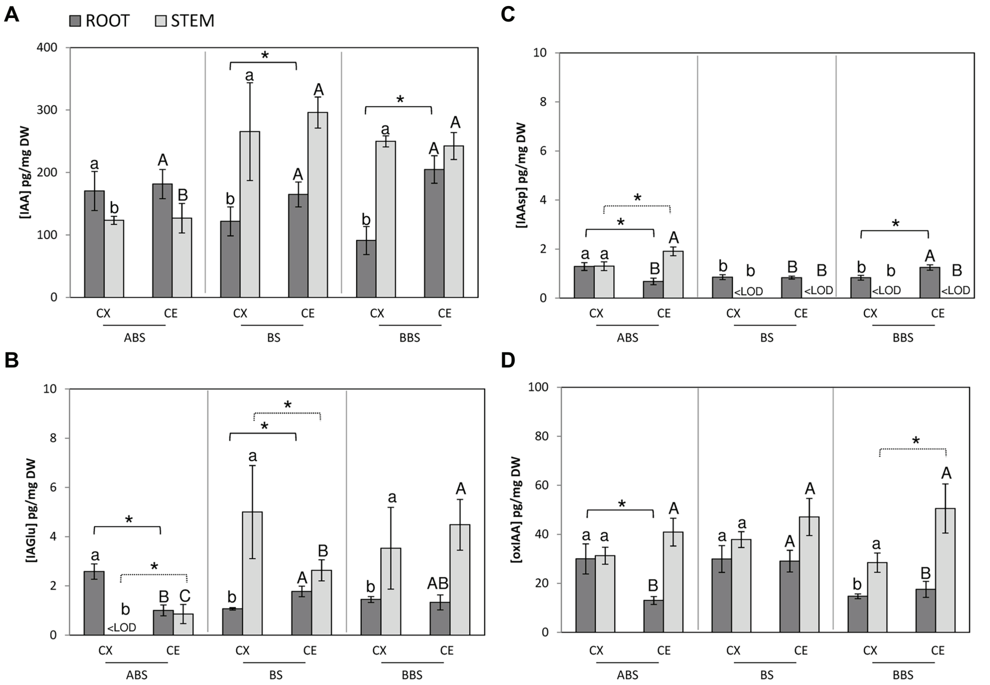
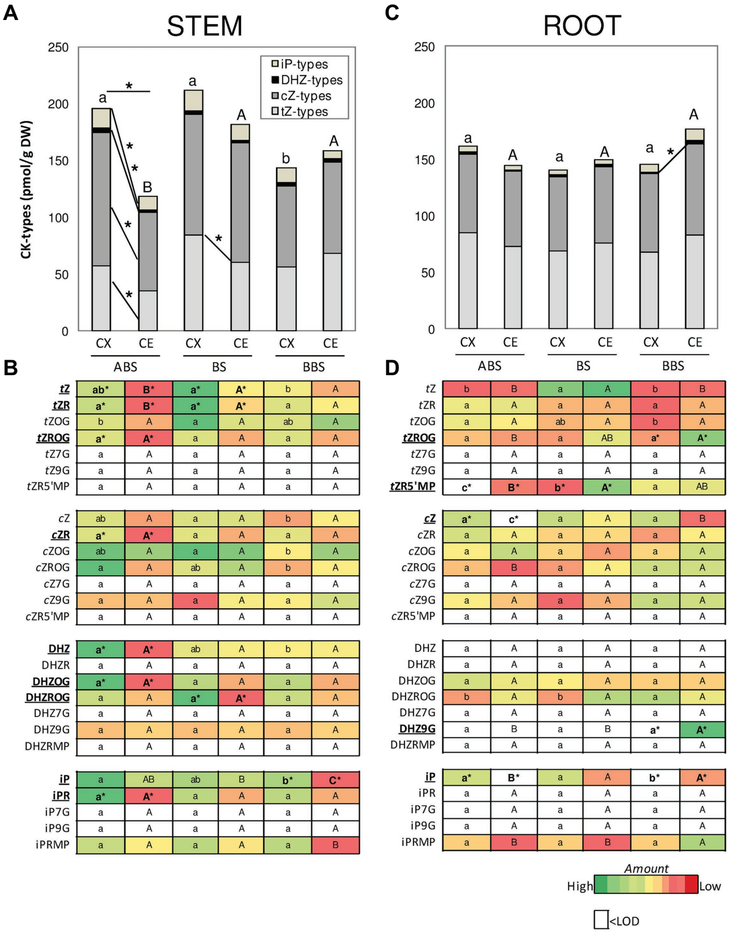


FIGURE 4 | Auxin metabolites profiling in different bent sides and sectors of *P. nigra* stem and root. Concentrations of IAA **(A)**, IAGlu **(B)**, IAAsp **(C)**, and oxIAA **(D)** were analyzed by UHPLC-MS/MS. The values are expressed in pg. mg^{-1} of dry weight (DW). Data represent the mean of three independent extractions ±SD. All significant differences (*post-hoc* LSD-tests, $p < 0.05$) between the three bent sectors in convex and concave sides are indicated by minuscule and capital letter, respectively. Significant differences (*post-hoc* LSD-tests, $p < 0.05$) between sides of the same sector are indicated by asterisk and continuous line for root or dashed line for stem. ABS, above bending sector; BS, bending sector; BBS, below bending sector; CX, convex side; CE, concave side.

the convex ABS and BS, characterized by low vessel number, poor lignifications, high carbohydrate, and G-layer in fiber cell wall, as widely reported in the literature (reviewed in Plomion et al., 2001).

This designed asymmetrical structural organization could represent the best engineering solution to counteract mechanical deformation, and the observed anatomical changes could be important for postural control and to guarantee transport in deforming condition (Mellerowicz and Gorshkova, 2012; Gril et al., 2017).

In bent root, the thicknesses of the cambium and phloem, as well as the area and number of vessel and their wall thickness, were higher in the concave BS and BBS, characterized by the highest magnitude of compression forces compared to ABS. Indeed, compression forces also triggered a significant increment of cambium cell number, xylem, and phloem traits in the concave BS and BBS with respect to the convex side. Thus, according to our previous result (De Zio et al., 2016), the increase of compression forces triggered RW formation, characterized by high relative vessel number and area in the concave BS and BBS of bent root. Moreover, the Py-GC/MS analysis showed high total lignin amount in convex ABS due to high accumulation of S- and G-lignin types, but it did not reveal any significant differences between the two sides in BS and BBS, even though a slight tendency toward higher lignin content was present in the concave sides. All our previous investigation revealed an increase of lignin content in the concave BS and BBS after 6, 12, 13, and 14 months of bending treatment by using Doster and Bostock (1988) method; this data, led us to hypothesize that secondary wood of the concave BS and BBS has characteristic more similar to gymnosperm CW than angiosperm TW (Scippa et al., 2008; Trupiano et al., 2012a, 2014; De Zio et al., 2016, 2019). The disagreement with our previous data could be related to the detection limit of Py-GC/MS technique or duration of the bending treatment, set to 5 months in the preset work. Indeed, evidences showed that Py-GC/MS technique is very accurate in distinguishing lignin-types (H-, G-, and S-type) sub-structures, while it seems inaccurate for the absolute lignin quantification (van Erven et al., 2017). However, it is reasonable to hypothesize that observed differences could be mainly related to the complexity of xylogenesis process influenced by seasonality of annual rhythm (Plomion et al., 2001). Indeed, our previous investigation showed that after 6 months of bending (Scippa et al., 2008; De Zio et al., 2016), lignin slightly increased in the concave BS and BBS, whereas it was strongly accumulated after 12, 13, and 14 months of bending (Trupiano et al., 2012b). Thus, environmental conditions, specially temperature and photoperiod, affecting rate and timing of wood formation (cambium division, cell expansion, followed by the ordered deposition of a thick multilayered secondary cell wall, lignification, and cell death) could regulate the intensity of lignin deposition to determine



B

	ABS		BS		BBS	
	CX	CE	CX	CE	CX	CE
_t_Z	**ab***	**B***	**a***	**A***	b	A
_t_ZR	**a***	**B***	**a***	**A***	a	A
*t*ZOG	b	A	a	A	ab	A
_t_ZROG	**a***	**A***	a	A	a	A
*t*Z7G	a	A	a	A	a	A
*t*Z9G	a	A	a	A	a	A
*t*ZR5'MP	a	A	a	A	a	A
*c*Z	ab	A	a	A	b	A
_c_ZR	**a***	**A***	a	A	a	A
*c*ZOG	ab	A	a	A	b	A
*c*ZROG	a	A	ab	A	b	A
*c*Z7G	a	A	a	A	a	A
*c*Z9G	a	A	a	A	a	A
*c*ZR5'MP	a	A	a	A	a	A
DHZ	**a***	**A***	ab	A	b	A
DHZR	a	A	a	A	a	A
DHZOG	**a***	**A***	a	A	a	A
DHZROG	a	A	**a***	**A***	a	A
DHZ7G	a	A	a	A	a	A
DHZ9G	a	A	a	A	a	A
DHZRMP	a	A	a	A	a	A
iP	a	AB	ab	B	**b***	**C***
iPR	**a***	**A***	a	A	a	A
iP7G	a	A	a	A	a	A
iP9G	a	A	a	A	a	A
iPRMP	a	A	a	A	a	B

D

	ABS		BS		BBS	
	CX	CE	CX	CE	CX	CE
*t*Z	b	B	a	A	b	B
*t*ZR	a	A	a	A	a	A
*t*ZOG	a	A	ab	A	b	A
_t_ZROG	a	B	a	AB	**a***	**A***
*t*Z7G	a	A	a	A	a	A
*t*Z9G	a	A	a	A	a	A
_t_ZR5'MP	**c***	**B***	**b***	**A***	a	AB
_c_Z	**a***	**c***	a	A	a	B
*c*ZR	a	A	a	A	a	A
*c*ZOG	a	A	a	A	a	A
*c*ZROG	a	B	a	A	a	A
*c*Z7G	a	A	a	A	a	A
*c*Z9G	a	A	a	A	a	A
*c*ZR5'MP	a	A	a	A	a	A
DHZ	a	A	a	A	a	A
DHZR	a	A	a	A	a	A
DHZOG	a	A	a	A	a	A
DHZROG	b	A	b	A	a	A
DHZ7G	a	A	a	A	a	A
DHZ9G	a	B	a	B	**a***	**A***
DHZRMP	a	A	a	A	a	A
iP	**a***	**B***	a	A	**b***	**A***
iPR	a	A	a	A	a	A
iP7G	a	A	a	A	a	A
iP9G	a	A	a	A	a	A
iPRMP	a	B	a	B	a	A

FIGURE 5 | Amounts of different CK-types/forms and conjugates in *P. nigra* bent stem and root. The total amount of *t*Z-, *c*Z-, DHZ-, and iP-types is illustrated in the graph for stem **(A)** and root **(B)** and the corresponding level of each CK-form/conjugate in the heat maps **(C,D)**. Different colors in the heat map indicate the abundance of each CK-form/conjugate in the different samples ($n = 3$): green and red colors indicate, respectively, relative high and low abundance while white color indicates value below the limit of detection (<LOD). Significant differences (*post-hoc* LSD-tests, $p < 0.05$) between the three bent sectors (ABS, BS, and BBS) of the same sides (CX or CE) are indicated, respectively, by lower and capital letter, while those between sides of the same sector are indicated by asterisk. ABS, above bending sector; BS, bending sector; BBS, below bending sector; CX, convex side; CE, concave side.

specific anatomical characteristics of the secondary xylem (Begum et al., 2008; Antonova et al., 2014).

Cambial activity in stem and root resulted differently affected by bending. In detail, in root, the RW formation in the concave BS and BBS was accompanied by an increase of cambial cell number, whereas, in stem, CCN decreased or was unchanged in the convex ABS and BS, respectively. In general, more cambial cells indicate more cambial activity and higher growth rate. The increased growth rate was accompanied by an increase in cambial cell division and, thus, the number of xylem mother cells, able to redesign an anatomical structure optimally tapered for hydraulic purpose (Sorce et al., 2013). Under deforming conditions, as those induced by bending, the control of cambial cells number and their successive differentiation pattern represent important traits to regulate vessel number/size to ensure xylem hydraulic efficiency (Sorce et al., 2013). Therefore, we can hypothesize that the decrease in cambial cell number, observed in the convex ABS stem, could be mainly associated to an increase of cambial cell differentiation rates rather than an absolute decrease in cell division. Furthermore, tension forces, here highly perceived, could control cambial cell division/differentiation rate. Conversely, in root, as reported by Montagnoli et al. (2020), both mechanical force types seem to be equally responsible for the unidirectional RW production toward the concave BS and BBS and in particular, through the compression-related stimulation and tension-related inhibition of cambium activity on their concave and convex sides, respectively.

However, it is well-known that mechanical constraints are the stimuli and that other factors, such as phytohormones, are responsible for controlling the characteristics of either stem or root RW.

The role of auxins in the differentiation of vascular tissue, during both normal development and mechanical constraint is well-documented, although most of the information come

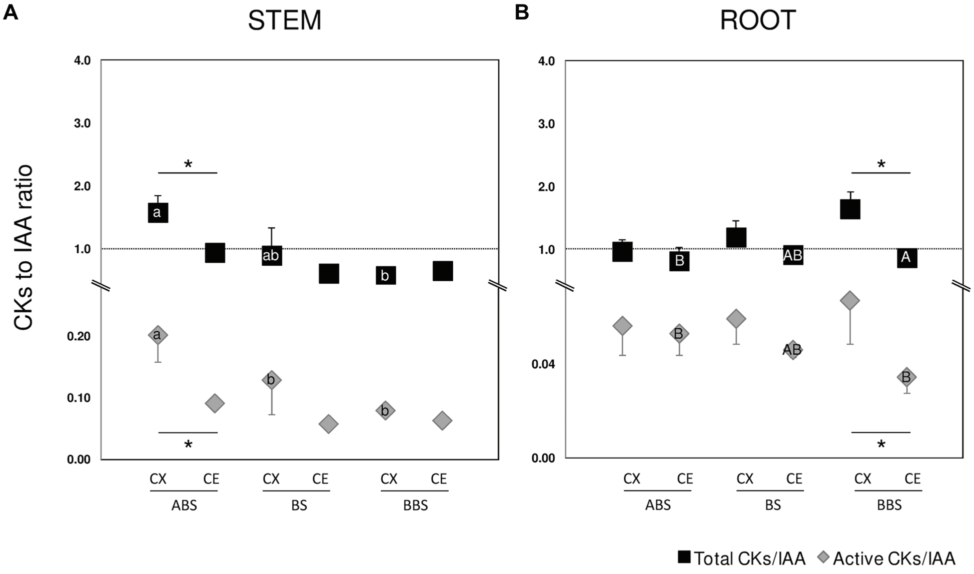


FIGURE 6 | Ratio of total/active CKs to IAA. Stem **(A)** and root **(B)** ratio between the total content of CKs and IAA are indicated by black squared (total CKs/IAA) while ratio between the content of CKs active forms (sum of CK bases and ribosides) and IAA are indicated by gray diamonds (active CKs/IAA). Error bars indicate SD (n = 3). Significant differences (Student's t test, $p < 0.05$) between the three bent sectors (ABS, BS, and BBS) along CX and CE sides are indicated by lower and capital letter, respectively, while those between sides of the same sector are indicated by asterisk. ABS, above bending sector; BS, bending sector; BBS, below bending sector; CX, convex side; CE, concave side.

from experiments at stem level involving applications of exogenous IAA or IAA-transport inhibitors (Du and Yamamoto, 2007; Tocquard et al., 2014). Despite CKs having a well-established function in cell division, increasing cambium sensitivity toward auxin, and acting as major regulators of wood quality and quantity (Aloni et al., 2006), they have seldom been investigated in relation to RW formation.

A widely accepted model suggests that in the bent stem, the TW forms in the region deficient in IAA, whereas CW is induced by an increase of auxin concentration (for review, see Timell, 1986; Little and Savidge, 1987; Srivastava, 2002). However, Hellgren et al. (2004) demonstrated that TW formation was not linked to any alteration in the balance of endogenous auxin. Moyle et al. (2002) proposed that in poplar stem, initiation of RW after bending stress is caused by an altered auxin sensitivity of specific cells rather than a redistribution of auxin in wood-forming tissues. Moreover, it is widely documented that low IAA concentrations result in slow differentiation, which permits more cell expansion before secondary wall deposition, resulting in wide vessels and a lower vessel density (Bhalerao and Bennett, 2003; Sorce et al., 2013).

In the present study, we found that in poplar bent stems the concentration of the bioactive auxin (IAA) was not linked to any redistribution between the two sides of the three bent sectors, although the lowest amount was found in ABS. It is possible that light stimulus change synergizes with those produced by bending to induce a redistribution of the auxin gradients only in cambial cells. The gradient here hypothesized could be important to enhance firstly cambial cell division and successively their differentiation rate to redirect stem growth orientation upward, against the gravitational pull and towards the light source (Vandenbrink et al., 2014).

In root, as hypothesized in our previous works (De Zio et al., 2016, 2019), the increased IAA level in the concave side could trigger the stress-related anatomical changes in the concave BS and BBS, expressed through the RW formation, due to an increase of cambial activity (Sundberg et al., 2000; Du et al., 2004).

The IAA endogenous levels are tightly controlled through biosynthesis, degradation, transport, and conjugate formation (Casanova-Sáez and Voß, 2019). Interestingly, in this study, IAA metabolites (IAAsp, IAGlu, and oxIAA) closely followed the IAA profile in all sides and sectors of the two bent organs. Despite the functions of IAA, conjugates are still under investigation, it has been proposed that they may serve as storage and protection against IAA oxidative degradation, where an IAA optimum must be guaranteed (Ljung, 2013; Tran and Pal, 2014). Normally, IAA conjugates are present in much lower quantities compared to oxIAA (Pencík et al., 2013; Vayssières et al., 2015); in fact, we noticed, oxIAA > IAGlu > IAAsp concentrations in all three bent stem and root analyzed sectors.

Brunoni et al. (2020) in a feeding experiment using labeled IAA, showed that in Norway spruce, IAAsp was the primary IAA catabolite originating from *de novo synthesis*, highlighting the production of IAAsp as the favorite route for IAA degradation. The group II of GRETCHEN HAGEN 3 (GH3) family of acyl-acid-amido synthetases is demonstrated to be active on IAA amount, playing an important role in catalysis of conjugation reaction in several species and

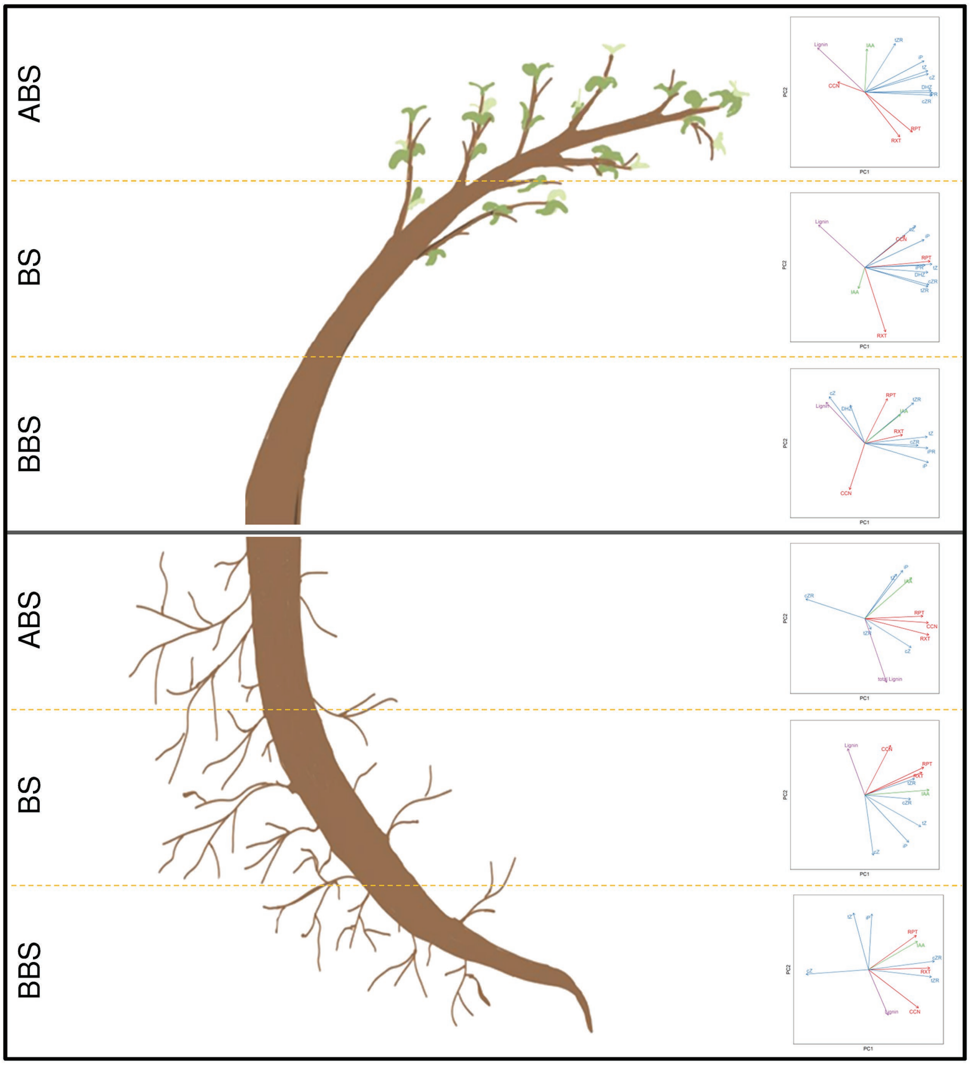


FIGURE 7 | Model summarizing correlation among anatomical, phytohormonal and lignin dataset in bent stem and root. The correlation among main anatomical parameters (CCN, RXT, and RPT – red vectors), phytohormones (IAA – green vectors; CKs free forms – *c*Z, *c*ZR, *t*Z, *t*ZR, iP, iPR, and DHZ – blue vectors) and total lignin content (Lignin – purple vectors) were analyzed by using Principal Component Analysis (PCA). Scatter plots show data variability within each sector (ABS, BS and BBS) of bent stem and root. Data were computed by using FactoMineR package in R and plotted by the two first principal components (PC1 and PC2). Vectors indicate direction and strength of each variable to the overall distribution. CCN, cambial cell number; CKs, cytokinins; *c*Z, *cis*-zeatin; *c*ZR, *cis*-zeatin riboside; DHZ, dihydrozeatin; IAA, indole-3-acetic acid; iP, N-isopentenyladenine; iPR, N^6-isopentenyladenosine; RXT, relative xylem thickness; RPT, relative phloem thickness; *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside.

different growth conditions (Ludwig-Müller, 2011). In *Arabidopsis thaliana, iaasp is* a major conjugate, formed by *AtGH3.1–6*, while IAAGlu formation is mediated by *AtGH3.17* (Staswick et al., 2005). In addition, *AtGH3.6*, *AtGH3.5*, and *AtGH3.17* are possible targets of the ARF8 auxin response factor (Tian et al., 2004).

Teichmann et al. (2008) demonstrated that GH3::GUS was strongly induced in response to poplar stem bending, concluding that auxin conjugation is involved in adjusting wood development in response to stress.

According to these evidences, an asymmetrical modulation of specific *GH3* genes in the three bent sectors and sides, that in turn control asymmetrical IAA-amino acid conjugates accumulation, could contribute to the modulation of specific signaling pathways and anatomical alteration we observed in the different sectors of two bent organs.

Depending on how the hormonal response pathways are integrated and on how their biosynthesis and metabolism are related, signals triggered by IAA may be enhanced or dampened, thus yielding additive/synergistic or competitive effects.

Another important hormone group, the CKs, could be a key signal to maintain appropriate levels of auxin biosynthesis (Jones and Ljung, 2011) but they have seldom been investigated in relation to RW formation (De Zio et al., 2019).

Our investigation showed that in poplar bent stems, all CK-types were high in the convex ABS and the *t*Z-type in the convex BS; only in the convex ABS, total and active CKs/IAA ratio was significantly more elevated (about 2-fold) relatively to the opposite concave side. In bent roots, although no variations were observed between different sides and sectors, the total DHZ-type slightly increased in the concave BBS where the CKs/IAA ratio significantly decreased compared to the opposite convex side.

Considering that distinct CKs types/forms are abundant in different sides and sectors of the two organs, each CK type could have specific roles in some important processes, such as cell division/proliferation, cell elongation/differentiation, and xylogenesis (Svacinová et al., 2012). In particular, in poplar bent stem, we may hypothesize that the increase of CKs free bases observed in the convex ABS and BS, together with other forms readily converted to the free base, could have a key role in the control of TW formation, especially in ABS, in which the CKs/IAA ratio was particularly elevated. Indeed, in an *Arabidopsis thaliana* quadruple isopentenyltransferases (*ipts*) mutant, defective for genes encoding free *t*Z and iP biosynthetic enzymes, a loss of vascular cambium activity and a lack of secondary xylem was observed (Matsumoto-Kitano et al., 2008). Also Immanen et al. (2016), exploring the effect of enhanced CKs signaling on vascular architecture, observed a dramatically increased of secondary development in poplar stem.

Conversely, in root, the lack of detection of *t*ZR5'MP in the convex ABS could be consistent with the reduced levels of all *t*Z-type forms. The *t*ZR5MP and *t*ZROG in the concave BS and BBS, respectively, could help to maintain the optimal *t*Z-type levels providing the source for free base conversion (Antoniadi et al., 2015). In *Arabidospis* root, *t*Z regulates the amount of PIN auxin efflux proteins (PIN1, 3 and 7) to create an auxin signaling maximum in protoxylem cells (Bishopp et al., 2011a,b). Auxin here accumulated is in turn able to promote the transcription of AHP6, a negative regulator of cytokinin signaling. The AHP6-mediated inhibition of cytokinin signaling, confines the cytokinin response to the procambial cells, defining vasculature patterning (Mahonen et al., 2006; Bishopp et al., 2011a). Thus, we may suggest that IAA and CKs spatial changes here observed could be important to control specification of vascular pattern (protoxylem identity) also in poplar woody roots.

Finally, the accumulation of DHZ9G form in the concave BBS of bent root, an irreversible *N*-glucosylation inactivation of DHZ (Kieber and Schaller, 2014; Schafer et al., 2015), could be an indication that DHZ-types, compared to the *t*Z-type, might have here a secondary role in RW formation and in gravitropic response (Kollmer et al., 2014). Indeed, here, where also the lowest CK/IAA ratio was found, especially considering the CKs active form, an antagonistic interaction of these two major hormone groups could be proposed to regulate critical aspects of root organogenesis/development and to facilitate downward organ bending and, thus, gravitropic response (Aloni et al., 2006; Waidmann et al., 2019). According to Waidmann and Kleine-Vehn (2020), cytokinins seem to be confirmed a central antigravitropic determinant.

CONCLUSION

Static non-destructive mid-term bending triggered the formation of a RW in both *P. nigra* stem and root, each with specific characteristics due to the intensity and type of mechanical forces perceived and related signaling activated in the convex and concave sides of three bent stem and root sectors (ABS, BS, and BBS).

Summarizing all information, in bent stem, the high tension forces in the convex ABS and BS tended to form RW characterized by low vessel number, poor lignifications, high carbohydrate, and G-layer in fiber cell wall. In the bent root, confirming our previous results, the increase of compression forces in the concave BS and BBS triggered RW formation, characterized by high relative vessel number and area. However, here, we did not notice any significant variation in total lignin amount, probably for the duration of bending treatment or the detection technique limit, thus, in this respect, more in-depth analysis is necessary to better assess the observed discrepancy.

These structural organizations may represent the best engineering solution to guarantee postural plant control and water transport in deforming condition and to prepare the bent stem and root to move towards and away, respectively, axial negative-gravitropic growth.

The observed differences between stem and root response to bending highlight how hormonal signaling is highly organ-dependent. Reasonably, the light, gravity and bending signals could synergize in modulating phytohormone gradients in the stem, which is different from that produced by the alone alteration of gravity bending-induced in root. In detail, an antagonistic interaction of CKs and IAA, with opposite trends in bent stem and root, seems to regulate organ-specific response to mechanical constraints. In stem, the CKs free bases could have a key role in the control of unidirectional RW formation, whereas the IAA could be specifically and asymmetrically accumulated only in the cambium zone to induce an earlier and more rapid RW production than in the bent root. Conversely, in root, a key role of IAA in the promotion of cambial cell division and RW initiation was confirmed. Here, a proper active reserve of *t*Z-type could provide the source for the free base conversion important to control vascular cell type identity and development. Thus, CKs are confirmed as central antigravitropic determinant to facilitate upward/downward organ bending in the altered condition of growth orientation.

Further research should be conducted to investigate differences between swayed (dynamic bending) and fixed (static bending) compression and tension forces loading on trees. This information could be critical for understanding how plants maintain/improve their structural integrity in natural mechanical stress conditions (wind, snow, and rain loadings).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

GSc and DC conceived the project and provided important insights during the research activities. AM and MT contributed to the experimental system set up and the anatomical analysis. KL supervised and supported the phytohormones experiments. EZ performed hormonal analysis and lignin/carbohydrate determination. MK and IA provided technical and analytical assistance during hormone measurements. GSf modeled the mechanical forces distribution. EZ, DT, MT, and GSf performed the data analysis. EZ, AM, and DT performed data interpretation and manuscript preparation. DT finalized the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articless/10.3389/fpls.2020.590985/full#supplementary-material

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Remarkable Similarity in Timing of Absorptive Fine-Root Production Across 11 Diverse Temperate Tree Species in a Common Garden

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Long-term minirhizotron observations of absorptive fine roots provide insights into seasonal patterns of belowground root production and carbon dynamics. Our objective was to compare root dynamics over time across mature individuals of 11 temperate trees species: five evergreen and six deciduous. We analyzed the timing and growth on 1st-and 2nd-order roots in minirhizotron images down to a vertical depth of 35 cm, as well as monthly and total annual length production. Production patterns were related to total annual precipitation of the actual and previous year of root production over 6 years. The main or largest peak of annual fine-root production occurred between June and September for almost all species and years. In most years, when peaks occurred, the timing of peak root production was synchronized across all species. A linear mixed model revealed significant differences in monthly fine-root length production across species in certain years (species x year, $P < 0.0001$), which was strongly influenced by three tree species. Total annual root production was much higher in 2000–2002, when there was above-average rainfall in the previous year, compared with production in 2005–2007, which followed years of lower-than-average rainfall (2003–2006). Compared to the wetter period all species experienced a decline of at least 75% in annual production in the drier years. Total annual root length production was more strongly associated with previous year's ($P < 0.001$) compared with the actual year's precipitation ($P = 0.003$). Remarkably similar timing of monthly absorptive fine-root growth can occur across multiple species of diverse phylogeny and leaf habit in a given year, suggesting a strong influence of extrinsic factors on absorptive fine-root growth. The influence of previous year precipitation on annual absorptive fine-root growth underscores the importance of legacy effects in biological responses and suggests that a growth response of temperate trees to extreme precipitation or drought events can be exacerbated across years.

Keywords: absorptive fine roots, deciduous, evergreen, precipitation, root phenology, root production, seasonal belowground dynamics, temperate tree species

INTRODUCTION

Seasonal patterns of fine-root growth often reflect a plant's ability to capture water and nutrients, to balance competing demands for carbon from different organ systems and to adjust to changing climatic conditions. Compared to aboveground production, seasonal patterns of fine-root production are much less understood, especially with regards to inter- and intra-annual variations of environmental conditions. Thus, improved understanding of the controls over seasonal fine-root growth is important for estimating plant resource partitioning as well as belowground net primary production.

Absorptive roots of trees are non-woody, short-lived, most distal roots to the proximal root attached to the plant stem, exuding primary and secondary metabolites, with very small diameters (e.g., < 1 mm) (McCormack et al., 2015a). They are ephemeral structures that are important for resource acquisition and microbial interactions. Growth of absorptive roots may be influenced by both intrinsic (i.e., endogenous) and extrinsic (i.e., exogenous) factors (Tierney et al., 2003; Comas et al., 2005). For example, intrinsic factors, such as internal competition for carbohydrates amongst plant organs (Farrar and Jones, 2000), can decrease fine-root production during times of production of leaves (Dougherty et al., 1979; Konopka et al., 2005), wood (Côté et al., 2003), or reproductive organs (Comas et al., 2005). Examples of extrinsic factors that can decrease fine-root growth include: low soil moisture (Newman, 1966; Comas et al., 2005), high soil moisture (Leuschner et al., 2001); low soil temperature (Tryon and Chapin, 1983), low solar radiation, (Edwards et al., 2004), and low nutrient availability (King et al., 2002). Interactions of moisture, temperature, and other environmental variables as well as fluctuations of these factors can result in seasonal patterns of high periods (peaks) and low periods (troughs) of fine-root growth.

Surprisingly, the number and scope of studies of fine-root growth periodicity amongst different species is quite limited. Because of challenges to assessing fine-root growth patterns including the large amount of work involved and the time investment to collect, as well as, analyze data, most journal articles on fine-root phenology report only 1 year of data (Lyr and Hoffman, 1967; Hendrick and Pregitzer, 1992; Ruess et al., 1998). In an early study, Sen (1962) reviewed 20 publications on tree root periodicity, with some papers reporting continuous summer growth (one peak) and others a bimodal pattern of annual growth (two peaks). Sen (1962) also reported that root growth periodicity was linked to both intrinsic factors (e.g., the age of the trees, and the species) and extrinsic factors (e.g., environmental conditions like temperature and precipitation) based on the studies he reviewed. However, except for the 3 years of data from McCormack et al. (2014); McCormack et al. (2015b), we are unaware of any studies addressing patterns in annual production across multiple growing seasons and for multiple, mature, even-age temperate tree species with differing leaf phenology and leaf lifespans. Both total annual fine-root production and fine-root growth periodicity are important metrics to understanding the ecology of fine roots.

Over the past 100 years, a variety of root phenology papers have reported on the number of major peaks of root growth observed. There is currently a discrepancy as to the number of peaks and their timing, with many authors reporting one (Brundrett and Kendrick, 1988; Ruess et al., 2003; McCormack et al., 2014), but others reporting two major peaks of root growth (Engler, 1903; Leibundgut et al., 1963; Norby et al., 2004; Noguchi et al., 2005). Various explanations have been proposed to account for a bimodal pattern of root production. For example, during bud break, fine-root growth is often depressed because the shoots may outcompete the roots for carbohydrates (Webb, 1976; Bloom et al., 1985; Joslin et al., 2001), which must all be from stored starch (Keel et al., 2006; Helmisaari et al., 2015; Solly et al., 2018). Later in spring, root growth is often observed to be extremely low during leaf production when again there is a large demand aboveground for carbohydrates (reviewed in Anderson, 2003). Fine-root growth may also be limited by low soil moisture availability in late spring or summer (Newman, 1966; Joslin et al., 2001; Comas et al., 2005; Bauerle et al., 2008), leading to a second root production peak in late summer or autumn generally associated with increased rain and soil moisture (Merritt, 1968) and lower competition for carbohydrates by aboveground organs. Even with seemingly sufficient summer precipitation, high air temperatures and low soil moisture holding capacity can lead to decreased soil water availability in the summer (Lowry, 1962; Weber and Nkemdirim, 1998), thereby, decreasing root production in the middle of the growing season (McDougall, 1916). Reasons for a single peak of fine-root growth during summer include the high demand for water and nutrients and the availability of soil water coupled with warm temperatures. During fall, fewer fine roots are produced because long fine-root lifespans (Withington et al., 2006) result in minimal turnover of roots produced earlier that year, and due to potential competition by mycorrhizal fungi for shared carbohydrates. However, there appears to be no consistent pattern for type of tree (e.g., evergreen vs. deciduous; arbuscular mycorrhizal vs. ectomycorrhizal), location (e.g., N. America vs. Europe), or method of root data collection (e.g., sequential cores vs. minirhizotrons) influencing the root growth patterns observed and reported.

Plant communities typically consist of multiple, interspersed species where identification of fine roots by species has traditionally been difficult, if not impossible. In particular, species identification of roots from minirhizotron images can be problematic because roots are typically not as distinctive as leaves are for identification. For many community-focused or forest ecosystem-focused publications, this is not a problem as the authors research objectives are on stand-level trends. However, it is a challenge for understanding differences in fine-root growth amongst species as well as factors that influence seasonal growth. While many publications over the past 100 years, have focused on one species (Krueger and Trappe, 1967; Ruess et al., 1998; Norby et al., 2004) or were done in mixed-species communities (Brundrett and Kendrick, 1988; Hendrick and Pregitzer, 1992; Joslin et al., 2001), single-species plots are a useful way to overcome issues with root identification while examining species growing in close proximity. Monoculture plots improve our ability to identify species-specific (intrinsic) and or environmental (extrinsic) factors. A common garden setting gives the benefit of plots being spatially in close proximity with similar environmental conditions, while permitting data collection on roots of known species that can be linked to the aboveground production. A shortcoming of this well-established approach is that root growth in single-species plots may not represent that of mixed-species communities, because inter- and intraspecific can result in different patterns of fine-root productivity (González de Andrés et al., 2018; Salahuddin et al., 2018; Zwetsloot et al., 2019).

Previous studies on fine-root growth of temperate tree species report that both intrinsic and extrinsic factors influence fine-root phenology. Abramoff and Finzi (2015) emphasized this idea with a research review of 63 articles covering 25 years on rhizosphere processes across plant species and types (grasses, forbs, and trees). Due to the similarity in extrinsic factors for individuals growing in a common garden, we felt that such a system is necessary for distinguishing between intrinsic and extrinsic factors influencing root growth. For example, if species-specific differences in leaf habit (evergreen or deciduous) are linked to differences in timing of root growth, this would suggest that intrinsic factors like carbohydrate demand dominate control of fine-root growth periodicity. Temperate, evergreen tree species have the capacity to photosynthesize and produce new carbohydrates for a longer period of time during a growing season compared to deciduous tree species (Schulze et al., 1977; Richardson et al., 2010), although their total annual production may be similar (Aerts, 1995), In the spring, evergreen root growth can begin before new leaf growth, combining the usage of stored starch (Endrulat et al., 2010; Epron et al., 2012; Wang et al., 2018) with new photosynthates from existing leaves. The same is true for an extended growing season in the fall, when favorable environmental conditions provide continued carbohydrates for possible belowground root production in evergreens after the deciduous species have dropped their leaves (Wang et al., 2018). In contrast, if timing of root growth is largely independent of leaf habit and better predicted by annual variation in abiotic factors like precipitation, this would suggest that extrinsic factors strongly influence fine-root growth periodicity.

Water availability is an important extrinsic factor influencing fine-root growth. Evergreen species are in general less susceptible to short periods of reduced water availability than temperate deciduous species (Eamus, 1999). The evergreen habit has been repeatedly linked to species that are able to tolerate drier conditions (Aerts, 1995). Because of their lower susceptibility to low water availability, it is reasonable to predict that evergreen species will be able to continue root production during years of lower than average rainfall at a greater level than deciduous species growing in the same environment.

To help address these knowledge gaps of the influence of intrinsic vs. extrinsic factors on root growth, we examined a 6 year data set of fine-root production of mature individuals of 11 temperate tree species. We had three main objectives. First, we examined the influence of leaf habit on timing of root growth. We hypothesized that fine-root growth of evergreen species would consistently occur earlier or later in the growing season than deciduous species, consistent with their longer leaf duration. Our second objective was to determine peak fine-root production in each year for each species and whether one or two peaks of growth occurred. We expected species to vary in root production peaks, based on differences in aboveground leaf habit and different tolerances to and strategies to cope with soil moisture deficits. Third, we determined to what extent total annual fine-root production would be influenced by yearly precipitation, as a major extrinsic factor influencing belowground growth. We hypothesized that tree investment in root growth would be more strongly reduced in years with limited precipitation in the deciduous species compared to the evergreen species.

MATERIALS AND METHODS

Field Site

Our field site was a common garden planting in the Siemianice Experimental Research Forest in west-central Poland, near the village of Biadaszki (51°14.87′N, 18°06.35′E, altitude: 150 m). The field site consisted of two adjacent plantings with 14 species total, nine species per planting, and with four species duplicated between plantings (Szymański, 1982). Species were planted in species-specific, 20 m × 20 m plots in each of three blocks, with a total of 27 plots per planting. Trees were planted at 1 m × 1 m spacing in 1970 (with 1 year-old seedlings) and in 1971 (with 2-year-old seedlings) resulting in mature trees of the same age in the adjacent plantings at the time of our study. Each planting had a fairly uniform topography (quite flat) with very few understory plants due to the high tree density (Withington et al., 2003). Soils were generally nutrient-poor loamy sands (average 80% sand and 15% silt) and classified as fine-loamy, mixed, Mesic Kanhaplic Haplustalfs, and sandy, mixed, Mesic Typic Ustipsamments (Mueller et al., 2012; **Supplementary Material S1** for soil type descriptions). For this experiment, we observed root phenology for 11 of the 14 temperate tree species at the site: 5 deciduous species *Acer pseudoplatanus* L., *Acer platanoides* L., *Fagus sylvatica* L., *Quercus robur* L., and *Tilia cordata* Mill.; 1 deciduous gymnosperm *Larix decidua* Mill.; and 5 evergreens *Abies alba* Mill., *Picea abies* (L.) Karst., *Pinus nigra* Arnold, *Pinus sylvestris* L., and *Pseudotsuga menziesii* (Mirbel) Franco. Detailed information regarding descriptions of this site were presented in Reich et al. (2005) and Hobbie et al. (2006).

The regional climate is considered transitional between maritime and continental. From 1968 to 1997, the long-term average annual precipitation was 591 mm, with about half falling in 5 months, from May to August (Data from Forestry Experimental Station, Siemianice, Poland). The long-term temperature average was 8.24°C with a mean growing season of about 213 days, calculated as the number of days with an average temperature $\geq$ 5°C (Szymański and Ceitel, 1989; Ceitel and Wawro, 1999a,b). Over the study period (2000–2007, data for 2003 were unavailable, but see Ciais et al., 2005 about Europe-wide drought in 2003), the average annual precipitation (rainfall, not including snowfall) and temperature were 581 mm and 8.81°C, respectively, very similar to the long-term average. However, the total annual precipitation during the study period varied more than twofold, with a high of 866 mm in 2,000 and lows of 412 mm and 382 mm in 2005 and 2006 (**Figure 1**), respectively. In 2005 and 2006, the months of low average precipitation coincided with monthly average temperatures of 18°C, resulting most likely in uncommon periods of temporary water limitations in the growing season (**Figure 1**). Each year in March (1999–2002, 2004), monthly average air temperatures were above 3°C (**Figure 1**) when bud break occurred (personal observations, J. Withington). Leaf expansion continued with increasing temperatures until about the end of April to mid-May depending on year (personal observations, J. Withington and M. Goebel). Leaf fall in the deciduous species occurred about October each year, when average (24 h/day) air temperatures were about 10°C (**Figure 1**, gray bars in **Figures 2**, **3**). Needle fall in the evergreens was continuous during the year with peaks in autumn (unpublished data of leaf litter traps, J. Oleksyn).

Fine Root Production

We examined fine root images collected from 2000 to 2007 which were previously used to establish fine-root lifespan estimates and cumulative root production in a common garden setting (Withington et al., 2003, 2006). Minirhizotron images were collected using a minirhizotron camera and associated image capture software (Bartz Technology Corp., Carpinteria, CA) starting in May 1999, 6 months after tube installation. Images were collected in 1999 at 2–4 week intervals, but sampling intervals were lengthened in 2000–2007 to a 1 month sampling interval due to the extended longevity of the roots of all study species (Withington et al., 2006). Six years of data from 2000 to 2007 are presented in this paper. Data from 1999 are not included: production in the first year was very high for all species, most likely as a result of disturbance from tube installation (Joslin and Wolfe, 1999). Data from 2003 to 2004 are also excluded due to equipment problems leading to minimal and sporadic sampling. In this paper we define seasons based on the days of the Equinoxes and the Solstices.

While the raw data we used (root images) for this current study overlap with two previous papers, our 2003 paper focused on cumulative root production in relation to the minirhizotron tube materials and the 2006 paper focused on median root lifespan and cumulative production. None of these topics are included in this paper. Since the current study examines questions beyond the scope of our original research, certain data that would be useful to the present study (such as more detailed information on shoot phenology and soil temperature measurements) are not available. In addition, while we recognize that mycorrhizae may influence root growth rate (Resendes et al., 2008) and root longevity (McCormack and Guo, 2014), any analysis of mycorrhizal type [e.g., arbuscular (AM) vs. ectomycorrhizae (EM)] on our root production patterns would be compromised because only two species (both *Acer*) were AM.

We focused solely on the first- and second-order absorptive roots, which for our species were < 1 mm diameter. This upper limit is smaller than that used by other researchers, but this limit allowed us to have data which consisted mainly of 1st and 2nd order roots across all 11 of our temperate tree species, but we acknowledge this limit may include some woody roots (Pregitzer et al., 2002; McCormack et al., 2015a). Compared to sequential soil cores, non-destructive technology, such as minirhizotron observation systems and their associated fixed tubes, provide more accurate data on timing of root growth as the measurements are made in the same location over time, removing the issue of confounding temporal variability with spatial variability as well as the confounding effects of multiple soil and root disturbances. Due to the small size of the minirhizotron windows and the majority of roots being first order and, to a limited extent, second order [defining a root in a given order from root tip to its base, not as a segment (*sensu* Withington et al., 2006)], we observed little production of older and higher-order roots in our images.

In a previous study at this field site, we found that tubes made from acrylic plastic provided root standing biomass estimates more consistent with standing crop estimated from soil cores than did tubes made from cellulose acetate butyrate plastic (see Withington et al., 2003); therefore, only observations from acrylic tubes were used in this study. Three tubes were installed per plot, three plots per species in Nov. 1998, keeping the tubes at least 3 m from the plot borders. We used only two *A. alba* plots (six tubes total) because the third plot had become overgrown with a different tree species. *Picea abies* is the one species represented in both plantings with a total of 6 plots. One tube of each of the following species was damaged and lost over the course of the experiment: *A. pseudoplatanus*, *Q. robur*, *T. cordata*, and *L. decidua*, so these species had data from eight tubes total. The minirhizotron tubes were 60 cm long and had an inside diameter of 5.2 cm and a wall thickness of 6.4 mm. The tubes were installed at an angle of 30° from vertical, providing a vertical viewing depth of maximal 35 cm. The tubes were scribed with a strip of 1 × 1.25 cm windows down the upper side. Tops of tubes were wrapped in black electrical tape and sealed with a rubber stopper to keep light and rain out of the tubes.

Root production, numbers of roots per unit viewing area (no. m^{-2}), was determined by counting the roots born on each sampling date and summing within plots. If we only used tip counts, this would not be a good comparison of growth across all of our species. Some of the species make very short terminal roots (e.g., *Pinus* spp.) while other make much longer terminal roots (e.g., *Acer* spp.). Therefore, we converted root count to an estimate of root length for each species. The numbers of roots were converted to root length (cm m^{-2}) using regression equations of the relationship between number of roots and root length determined for each species (Withington et al., 2006). Briefly, we used a subset of images that included all fine roots present in all tubes for five dates distributed across the years 1999–2003 and representing each season to obtain these regression equations. Root length in each subsample was determined using WinRHIZO Tron (Regent Instruments, Quebec, Canada) and then correlated with the total number of roots in each image ($r^2 > 0.88$ for all 11 species; Withington et al., 2006).

Dates of root initiation were recorded as the date halfway between the actual sampling date and previous sampling date, with one exception. Beginning in the second year (2000), the time interval from the first sampling date of the year was adjusted to

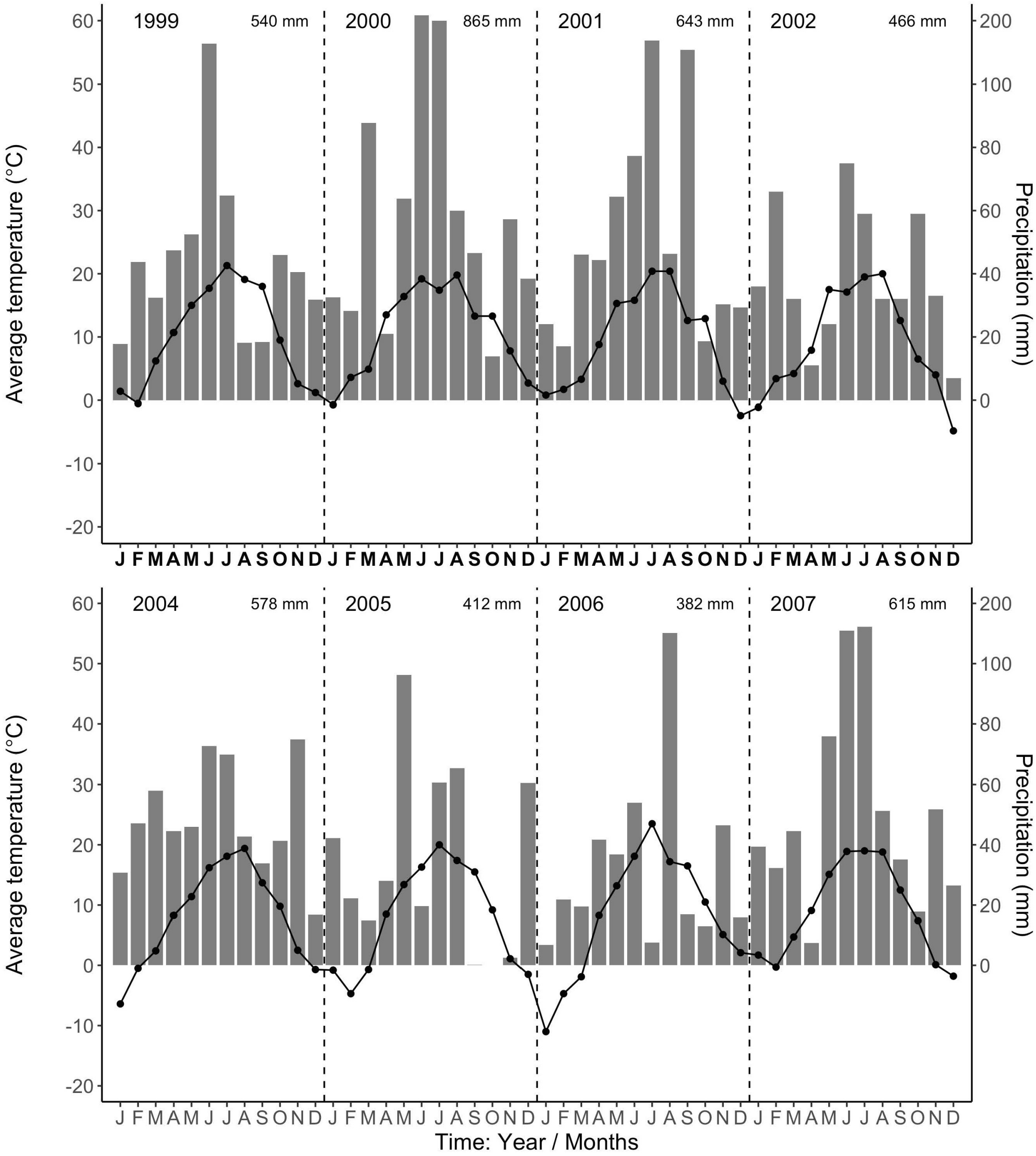

FIGURE 1 | Average monthly air temperatures (°C, point-line) and total monthly precipitation (mm, gray bars) from the Forestry Experimental Station, Siemianice, Poland (1999–2007). The temperature and precipitation data are in fixed proportions, 10°C corresponding to a precipitation of 20 mm, allowing the characterization of periods with indications of water limitation (Gaussen and de Phillips, 1958; Walter and Lieth, 1960). Months where the temperature point exceeds the precipitation bar indicate possible deficit in water availability. The total annual precipitation is stated in the upper right corner for each year. Data for the year 2003 are not available.

assume all new roots were initiated on 1 February or later. While it has been shown that root growth can occur at air temperatures close to freezing (0°C) (Gaul et al., 2008), we chose to use a more conservative estimate for root growth at air temperatures above 2–3°C (Krueger and Trappe, 1967; Schenker et al., 2014; McCormack et al., 2015b) to be sure we had confidence that root growth was possible in all 11 of our species. Average temperatures of about 3°C occurred in February from 2000–2004 (**Figure 1**), thus, our choice of 1 Feb. From 2005 to 2007, the average temperatures were lower in February and did not reach this level until March or April, constituting a longer soil freeze; however, we continued to use a February start date to simplify sampling protocols across years.

We calculated absorptive fine-root length production (RLP) (m m^{-2} viewing area) by taking the average across the three tubes per plot, based on total amount of fine roots produced in each plot, allowing for similar weighting of each plot to calculate seasonal patterns (plots are our replicates, not the individual

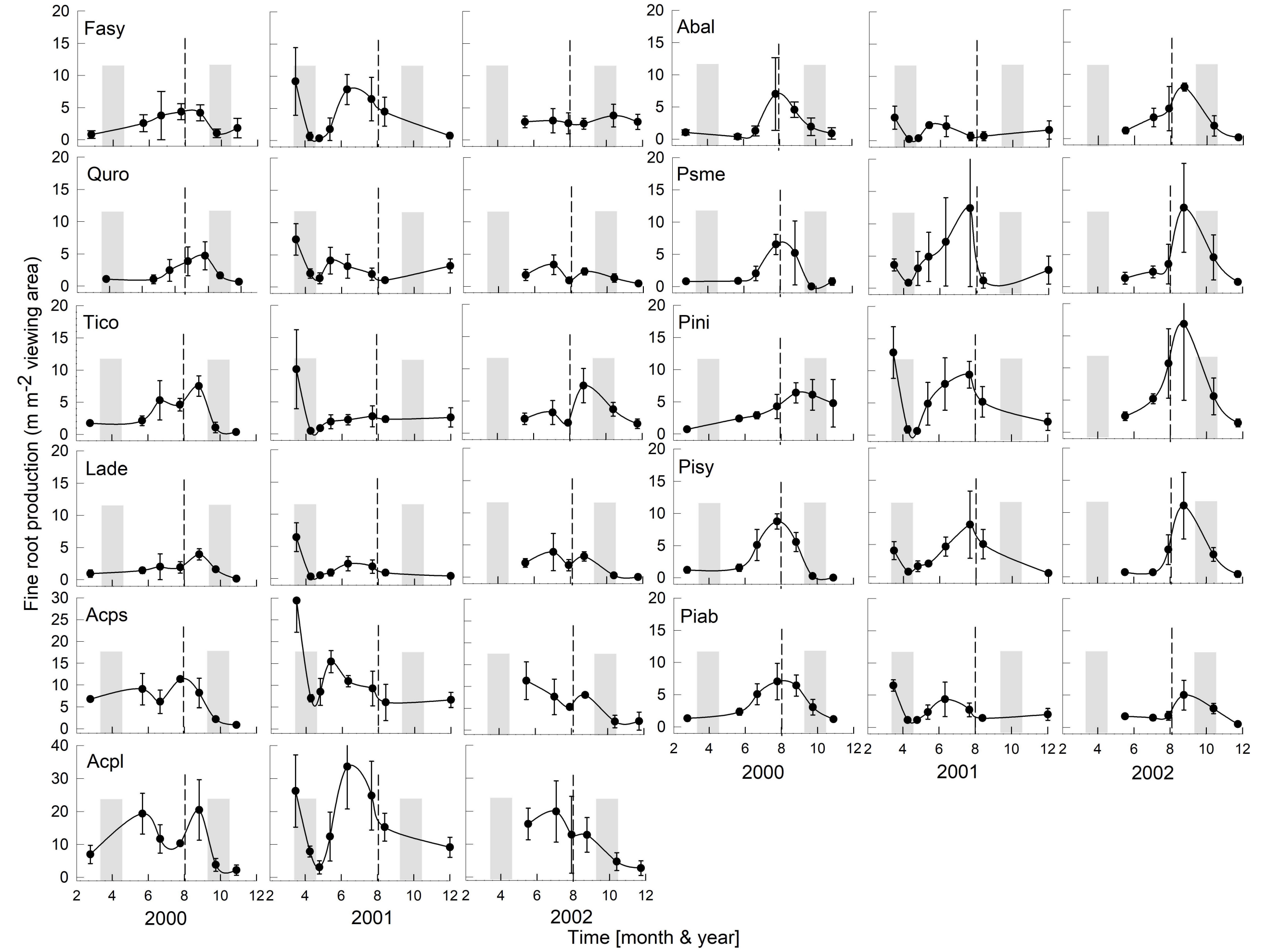

FIGURE 2 | Seasonality of monthly averaged fine-root length production (± SE) of 11 temperate tree species growing in a common garden for the years 2000–2002. Species are abbreviated with the first two letters of their genus and species name. Gray bars indicate observed bud break and leaf flush (mid-March to mid-May) as well as leaf fall (mid-September to end of October) of each year. The dashed line is the center of the observed growing season (June–October). Each plot was weighted equally in calculating the seasonal patterns (3–6 plots per species; see section "Materials and Methods" for details). Root production data in this figure were adjusted to 28 day intervals to account for uneven image recording dates within a year. The first data point in each year assumed that root growth did not occur in December or January (see section "Materials and Methods"). Note, the *y*-axis range for all species is 0–20, except for Acps it is 0–30 and Acpl it is 0–40.

tubes). In 2000, one *F. sylvatica* plot was excluded from the analysis; it contained fewer than five roots total produced over the year and production was too low to accurately assess seasonality when the other plots for the same species had dozens of roots in the same time period (see comment on large error bars in Discussion). These calculations were followed by multiple comparisons, testing differences within a year and differences between years within species.

Absorptive fine-root length production was the response variable of interest and was recorded for each plot at each time point. To analyze the RLP we thus used a linear mixed model, because each plot was repeatedly measured over years and calendar months. Plot was a random effect and species, month, species * year, species * month were fixed effects in the model. We did not test the interaction (year * month) because RLP was only calculated for certain months of actual image collections and therefore it was not appropriate. This analysis was followed by multiple comparison tests, using Bonferroni or Tukey correction when needed.

We also ran linear mixed-effect models to associate total annual RLP to species and annual precipitation (both, current- or previous-year), as well as their interactions (species * current- or previous-year precipitation) as fixed effects. Finally, we calculated the correlation coefficient between species annual root production and the total annual precipitation of the previous or current year. For more in-depth resolution, we separated the analysis into monthly periods of the year, in order to identify influential periods of precipitation within a year: (a) the general growing season based on our observed root production 7 months (Apr-Oct), and (b) the last 9 months of the year (Apr-Dec) (**Supplementary Table S2**). For all statistical analyses we used JMP (version 12, SAS, Cary, North Carolina, United States)

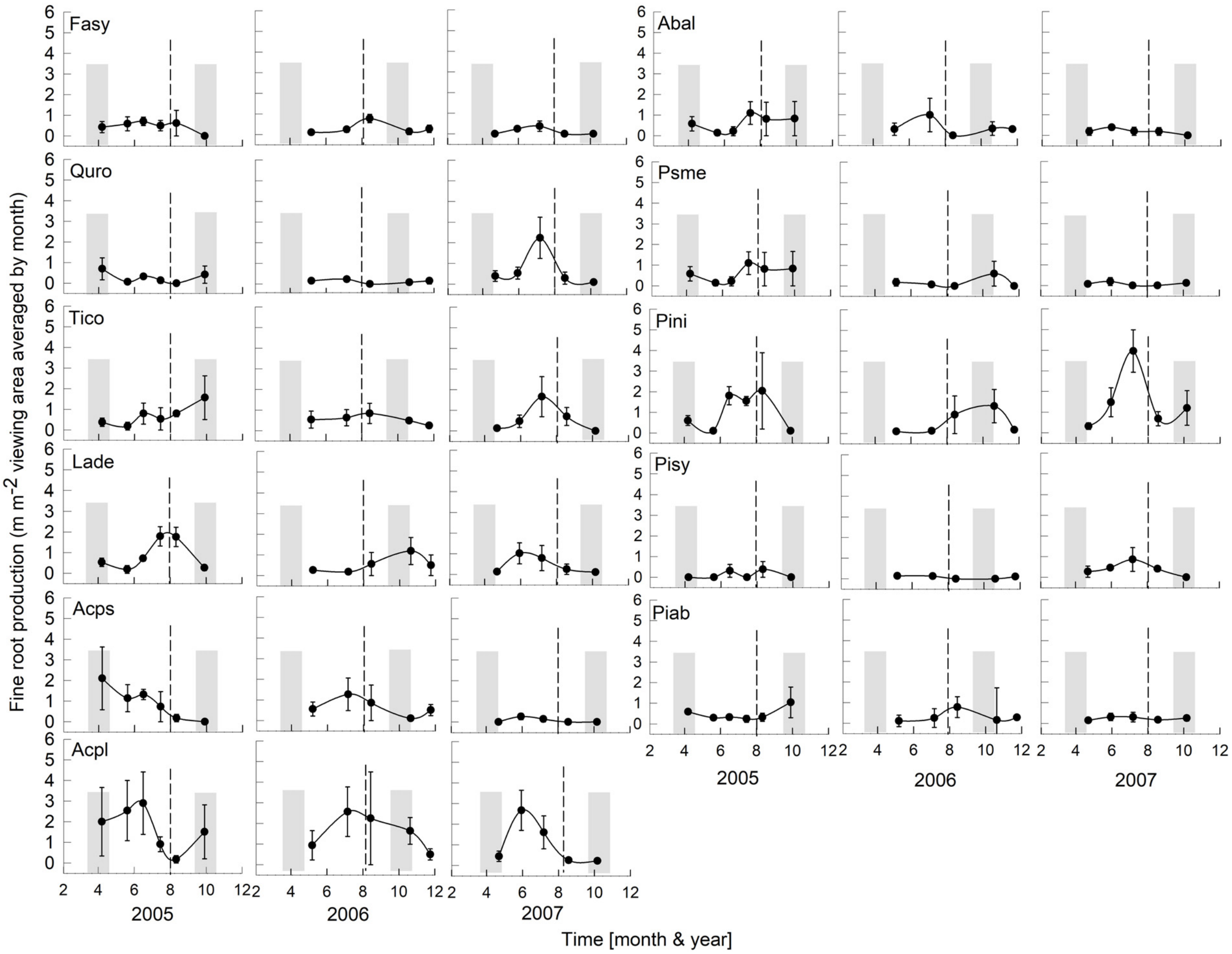

FIGURE 3 | Seasonality of monthly averaged fine-root length production (± SE) of 11 temperate tree species growing in a common garden for the years 2005–2007. Species are abbreviated with the first two letters of their genus and species name. Gray bars indicate observed bud break and leaf flush (mid-March to mid-May) as well as leaf fall (mid-September to end of October) of each year. The dashed line is the center of the observed growing season (June–October). Each plot was weighted equally in calculating the seasonal patterns (3–6 plots per species; see section "Materials and Methods" for details). Root production data in this figure were adjusted to 28 day intervals to account for uneven image recording dates within a year. The first data point in each year assumed that root growth did not occur in December or January (see section "Materials and Methods").

and SAS statistical software (Version 9.4; SAS, Cary, North Carolina, United States).

RESULTS

Seasonal Pattern of Fine Root Production

Overall, tree species had remarkably similar timing of root growth within a given year. During 6 years of observations, we found the main or largest peak of root production occurred, in general, between June and September (**Figures 2**, **3**), with distinct peaks also occurring sometimes during the spring season (April). In 2000, a main peak occurred in late summer across all 11 species, with evergreen's peak occurring 1 month earlier (**Figure 2**, August) than the deciduous species (**Figure 2**, September). In this year, *A. platanoides* was the only species with two peaks during the growing season. In 2001, an early peak occurred in March for all 11 species, with a second peak occurring in summer (Jun.-Aug.) for six species (*A. platanoides, A. pseudoplatanus, F. sylvatica, P. abies, P. nigra, P. sylvatica*). In 2002, a main peak occurred in September for all five evergreen species and one deciduous species; the two Acer species had early summer peaks, while *F. sylvatica*, *Q. robur* and *L. decidua* did not exhibit a peak in fine-root growth that year (**Figure 2**). In the last 3 years of observations (2005–2007), a period of extremely low root production, fewer species exhibited a growth peak (five: 2005, eight: 2006, six: 2007, **Figure 3**). The peaks during the last 3 years were also more variable in magnitude (height of peak) and duration (width of peak), and timing was not as synchronous with peaks spread out from April to October.

We were only able to record spring observations (Feb–Apr.) in 3 years (2000, 2001, 2005) due to winter conditions, such as heavy snow accumulations (**Figures 2**, **3**). We observed a spring peak of root growth, in two of those years, 2001 and 2005. In 2001, which followed an overall very wet year (2000: 865 mm, **Figure 1**), all 11 species showed an early growth peak in March. In 2005, which followed a year of average precipitation (2004: 578 mm), there was an early peak of root growth only in *A. pseudoplatanus* and *Q. robur,* even though the latter was small, while *A. platanoides* had a broad peak of absorptive fine-root growth from April until the end of June. We noted no evidence that evergreen species had more root growth either earlier in the year or later in the year than deciduous species, even though they are considered capable of photosynthesis during these periods due to their evergreen habit.

After the observational analyses, we quantified the differences in the timing of root length production. Total monthly root length production peaks varied significantly amongst species ($P < 0.0029$), by month ($P < 0.0001$) and by year ($P < 0.0001$) of occurrence (**Table 1** and **Figures 2**, **3**). The interaction term species * month was not significant, and therefore this term was removed from the model. The effect of year on peak root length production depended on species (interaction species * year, $P < 0.0001$). When comparing the interactive effects of species and year, three species contributed to the significant differences in monthly root length production (**Table 1**) as noted in our qualitative observations above, namely *A. platanoides* and *A. pseudoplatanus,* in the years 2000, 2001, and 2002, and *P. nigra* in addition in the year 2002 (**Table 2**). These few significant differences are a reflection of the moderate to high variability of fine-root length production (indicated by large standard errors) for individual species. The large standard errors at certain observation dates indicate that timing of peak production in that species varied greatly from plot to plot in that particular year (**Figures 2**, **3**). Conversely, some periods of small standard error, such as typically observed in the months of April and October, indicated very consistent seasonal timing among the plots of that species.

Total Annual Fine Root Production

During the two 3 year periods of observations, the average annual root length production (RLP; m m^{-2} viewing area) was higher in the deciduous species plots than in the evergreen species plots (**Table 2**); however, this relationship reverses if the values for the *Acer* species are removed. The largest annual average production of total root length per plot for 10 out of 11 species occurred in 2001 and the lowest annual average production was in 2006 or 2007, depending on individual species (**Table 2**).

There was a notable decrease in total annual RLP between the years 2002 and 2005, with the drought year of 2003 in the interval. The annual production for the first 3 year period was similar within species and much higher than the production for the latter 3 years, which all had lower production. The annual precipitation for 1999–2002 was much higher than for 2003–2007 (**Figure 1**). Absorptive fine-root growth decreased along with precipitation. The difference in RLP between the wetter years (2000–2002) and the drier years (2005–2007) was striking with a decrease of 73–90% in the deciduous species and 81–96% in the evergreens (**Table 2**).

TABLE 1 | Linear mixed model shows the effect of species, month, and year on root length production [RLP (m m^{-2} viewing area)] from 2000 to 2002 and 2005 to 2007.

Parameter	d.f., denDF*	*F*-value	*P*-value
Species	10, 25	3.9	<0.0029
Month	10, 1,163	18	<0.0001
Year	5, 1,165	36.9	<0.0001
Species * Year	50, 1,165	3.7	<0.0001

**d.f, degrees of freedom; denDF, denominator degrees of freedom.*

When relating RLP with previous year precipitation, our model predicted that in years with reduced precipitation (mean − 1 SD), annual root length production did not differ significantly amongst species. In contrast, in wet years (mean + 1 SD), there were species differences with the strongest influence on RLP by the two maple species (*Acer* spp.) (**Table 3**).

From our linear mixed model, total annual RLP was associated with the actual year's ($P = 0.003$) and previous year's precipitation ($P < 0.001$), but only the effect of previous year precipitation on RLP depended on species (interaction species * previous year annual precipitation, $P < 0.014$) (**Supplementary Table S1**). The residual variance component was reduced by 15% when controlling for the precipitation of the current year of root production in the model that included only the main effect of species. However, when controlling for precipitation of the previous year of root production, the model was a much better fit with the residual variance dropping by 59%. Furthermore, the interactions of species and previous year's precipitation was highly significant and the model including the interaction between previous year precipitation and species reduced the residual variance by 42% compared to the model with species alone (**Supplementary Table S1**).

To more closely examine the relationship between precipitation and RLP, we categorized species into groups based on leaf habit. Total annual precipitation of the previous year was significantly correlated ($P < 0.005$) with root length production over the 6 years of observations for both groups, the six deciduous and five evergreen species. We found similar significant correlations when grouping the five Angiosperms, as well as smaller groups: the two *Acer* spp., and *Q. robur* + *F. sylvatica* + *T. cordata* together (**Table 4**). The correlation coefficients range from 28 to 32% for the deciduous and evergreen groups (leaf habit), whereas these coefficients increase to 58–59% in narrower groupings, i.e., the two subgroups of Angiosperms, the *Acer* spp. and *Q. robur* + *F. sylvatica* + *T. cordata*. Total annual precipitation of the actual year was only significantly correlated with RLP for two groups, the five evergreens and *Q. robur* + *F. sylvatica* + *T. cordata*; however, the correlation coefficients were lower than for the previous year precipitation for these groups (**Table 4**).

To determine if there was a particular seasonal time period of the influence of precipitation on annual RLP, we also considered precipitation in time periods less than the full year. For the previous year, both the precipitation of the growing season (7 months, Apr-Oct) and of the last 9 months were significantly

TABLE 2 | Average annual absorptive fine-root length production for each species from 2000 to 2002, and 2005 to 2007.

Species	2000	2001	2002	2005	2006	2007	Total yrs avg.	2000-2002* yrs avg.	2005-2007† yrs avg.
				(m m^{-2} viewing area)					
Acer platanoides	122 (32)	158 (55)	153 (60)	16 (10)	18 (8.7)	9.8 (0.6)	80 (20)	144 (26)	15 (4.0)
Acer pseudoplatanus	77 (14)	112 (18)	83 (33)	9.7 (5.9)	8.6 (5.1)	0.7 (0.5)	48 (12)	91 (13)	6.4 (2.7)
Fagus sylvatica	26 (4.0)	38 (6.3)	34 (5.2)	4.2 (0.2)	2.6 (0.1)	1.0 (0.2)	18 (5.1)	33 (7.0)	2.6 (0.5)
Quercus robur	21 (7.4)	31 (10)	21 (8.3)	2.9 (1.0)	1.9 (0.8)	5.8 (1.3)	14 (3.5)	25 (4.7)	3.5 (0.8)
Tilia cordata	32 (8.2)	32 (12)	33 (8.6)	5.7 (0.9)	6.7 (2.7)	4.6 (1.5)	19 (4.1)	32 (4.9)	5.7 (1.0)
Larix decidua	17 (2.1)	17 (4.2)	24 (10)	6.5 (0.5)	5.2 (0.6)	4.1 (1.1)	13 (2.5)	20 (3.7)	5.3 (0.5)
Deciduous Avg.	49 (11)	65 (15)	58 (16)	7.5 (2.0)	7.2 (2.0)	4.3 (0.8)			
Abies alba	23 (5.2)	15 (0.2)	30 (5.5)	4.9 (0.7)	5.8 (0.1)	1.9 (0.4)	13 (2.8)	22 (5.7)	4.2 (0.9)
Picea abies	36 (5.8)	27 (6.2)	22 (4.4)	4.3 (1.7)	6.5 (2.9)	2.2 (1.1)	17 (2.8)	28 (3.3)	4.2 (1.0)
Pinus nigra	36 (6.7)	53 (11)	63 (25)	7.6 (0.6)	4.1 (1.9)	12 (2.7)	29 (6.8)	51 (9.1)	8.0 (1.5)
Pinus sylvestris	30 (5.3)	31 (12)	25 (7.0)	0.8 (0.0)	1.4 (0.2)	3.5 (0.4)	15 (4.3)	29 (4.3)	1.3 (0.6)
Pseudotsuga menziesii	22 (10)	41 (31)	34 (21)	14 (6.9)	2.2 (1.9)	0.8 (0.8)	19 (6.6)	33 (12)	5.6 (2.9)
Evergreen Avg.	31 (3.4)	33 (6.1)	33 (6.6)	6.5 (1.7)	4.2 (1.0)	4.0 (1.3)			

** 2000–2002. These years had average to above average amounts precipitation.*

† 2005–2007. These years had below average precipitation.

There were 2, 3, or 6 plots per species with 2 or 3 tubes per plot (see section "Materials and Methods" for details). Data represent numbers of new roots for each sampling day converted to root length (m m^{-2} viewing area). Standard errors of the mean are given in parentheses.

correlated with RLP (all categories, $P < 0.01$) over the 6 years of observations. The correlation coefficients for the partial years were similar to the 12 month correlation for all of the groups (**Supplementary Table S2**). For the actual-year, when looking at the same two time periods, the evergreen group was significant for both, the 7 month and the 9 month precipitation interval (both $P < 0.05$) while the *Q. robur* + *F. sylvatica* + *T. cordata* group was significant for the 7 month period ($P = 0.03$). The correlation coefficients for these three relationships were much lower than the those for the previous year or parts of the previous year but were similar to those for the actual year (**Supplementary Table S2**).

DISCUSSION

Seasonal Patterns of Fine Root Growth

Despite widely different leaf habits and phylogeny, the seasonality of fine RLP was generally synchronized across both evergreen and deciduous tree species within a given year (**Figures 2**, **3**); therefore, our data do not support our hypothesis that evergreens consistently have earlier root growth than deciduous species. After leaf expansion, root production often increased and peaked in June-August or September-October and dropped off later in the year (**Figures 2**, **3**). This alternation of shoot and root growth (or asynchronicity of growth) has been reported repeatedly in studies with seedlings (Reich et al., 1980; Thaler and Pages, 1996; Makoto et al., 2020), with mature trees (e.g., *Q. alba*: Reich et al., 1980), and with mixed biome data sets (Abramoff and Finzi, 2015). However, this pattern of alternation of root and shoot growth in spring is not always the case. Leibundgut et al. (1963) reported almost parallel growth in above- and belowground organs for 1 year-old seedlings of three evergreen and four deciduous species. McCormack et al. (2015b) also reported synchronous growth of leaves and roots in liriodendron tulipifera.

Our 3 years of early spring observations limit general statements about early RLP; however, we observed that root growth can occur before bud break and leaf expansion. We do note that 2000 was the wettest year, which in combination with mild winter temperatures (**Figure 1**), presumably contributed to the increased peak growth for all our 11 study species in spring 2001. However, the 2005 spring root production (that occurred only in the two *Acer* species) followed an average precipitation year in 2004, along with lower winter temperatures (**Figure 1**). Together these observations support the view that previous year rainfall can also impact new root growth early in the next year.

While the RLP was often synchronized across species in a particular year, the timing of peak root growth was not consistent from year to year within species, suggesting a strong influence of extrinsic factors. It is difficult to determine which extrinsic factors might have the greatest influence on absorptive RLP, considering all of the species were exposed to similar environmental factors, including precipitation, soil water content and air temperature. In general, our results are consistent with those of McCormack et al. (2012); McCormack et al. (2015b) who reported five of their six study species had large variability in timing of peak root production among years, suggesting that various environmental conditions were influencing the root production peaks.

One or Two Peaks of Root Production? It Depends

Our data support the commonly held notion of one peak of absorptive fine-root growth during a growing season. Many authors have reported a single root growth peak in the late spring to summer months (Hendrick and Pregitzer, 1992;

TABLE 3 | Summary of parameter estimates predicting individual species' annual fine-root production (m m^{-2} viewing area) based on the annual average precipitation of the previous years for all 11 temperate tree species for 6 years of observation (2000–2002 and 2005–2007).

Species	Fine-root production estimate (m m^{-2})		
	Dry year (−1 SD)	Center (mean)	Wet year (+ 1 SD)
Acer platanoides	28 a	79.6 a	131.2 a
Acer pseudoplatanus	11.6 a	48.4 ab	85.3 b
Fagus sylvaticus	4.7 a	17.7 c	30.6 c
Quercus rober	4.9 a	14.1 c	23.3 c
Tilia cordata	9.7 a	19 c	28.3 c
Larix decidua	14.3 a	16.3 c	18.3 c
Abies alba	8.4 a	13.3 c	18.2 c
Picea abies	10 a	17.1 c	24.1 c
Pinus nigra	12.2 a	29.3 bc	46.4 c
Pinus sylvatica	9 a	17.6 c	26.1 c
Pseudotsuga menziesii	4.8 a	19 c	33.2 c

Different letters signify significant differences amongst species ($P < 0.05$). Dry years are indicated by −1 SD and wet years by +1 SD. Production estimates for dry years (−1 SD) showed no significant differences.

TABLE 4 | Correlation coefficient (r^2) and *p*-values of tree species categories' annual total fine-root length production (m m^{-2}) and annual precipitation data.

Species' categories	Actual 12 months	Previous 12 months
Angiosperms	n.s.	0.28 *0.002*
Quro, Fasy, Tico	0.23 0.04	0.59 *0.0002*
Acpl, Acps	n.s.	0.58 *0.004*
Deciduous Angio + Lade	n.s.	0.25 *0.002*
Evergreens	0.15 0.019	0.32 *0.0003*

Italics indicate $P < 0.05$; n.s, not significant. Species are abbreviated with the first two letters of their genus and species name. Angio: Angiosperms axis range for all species is 0–10, for Acpl it 0–20.

Ruess et al., 2003; McCormack et al., 2014; Montagnoli et al., 2019), a pattern often linked to warm air and soil temperatures and consistently moist soil conditions. Reich et al. (1980) reported synchrony of root growth flushes across *Quercus* spp. seedlings; however, our study is the first to report that mature tree species individuals with widely different phylogeny and leaf habit have similar root growth patterns in a given year, despite annual variability.

We observed late summer absorptive fine-root growth peaks in 2 years; specifically, in 2000 (four deciduous species) and 2002 (nine deciduous and evergreen species). Moreover, if a late peak occurred, it was the only peak for the year. These data differ from those reported in the literature (e.g., Engler, 1903) and could be due to the limitation of our observations to a depth of 30 cm. Peaks of root growth may occur at different depths during a year; shallow observation depths may not observe second root flushes occurring deeper in the soil. Working with 1–4 year old seedlings in a walk-in rhizotron, Lyr and Hoffman (1967) reported two peaks of growth in eight species, with the second peak in the late summer at depths of about 1 m and deeper. Münzenberger et al. (2003) also recorded a second growth peak in autumn across several species at depths of more than 1 m. Burke and Raynal (1994) found a single fine RLP peak at 0–10 cm, between June and September, and two root production peaks at deeper depths (10–20, 20–30, 30–40, and 40–50 cm).

Tierney et al. (2003) proposed that the number of root growth peaks is related to the geographical location of the study site and the presence or absence of summer drought conditions. They observed in a temperate forest that fine-root production tracked temperatures with usually one peak in mid-summer. Our root production data do not support their conclusion; rather, our data suggest that linking root peak production and summer droughts may not always be true. We observed one peak of production for 5 years in Poland (**Figures 2**, **3**); however, in 2001 (wettest year), six species had two peaks of growth with the second peak in summer (Jun–Aug.). There were no second peaks observed in the drier observation years (2005–2007). Other studies in geographically similar locations like Germany, Switzerland and Denmark (Resa, 1877; Büsgen, 1901; Ladefoged, 1939) reported two peaks, with the second in autumn.

Total Annual Fine Root Production

Our additional objectives were to quantify total annual absorptive fine-root length production (RLP) among species, the phenology of fine-root growth, and to evaluate the linkage between total annual RLP and precipitation. We observed that the six deciduous tree species had greater annual RLP than the five evergreen species (due to the large production in the *Acer* spp.) and that total annual production for both groups varied more than ninefold between the highest production year (2001) and the lowest production year (2007) (**Table 2**). We also found total annual RLP was low in 2005–2007 following the extreme drought year of 2003, when European air temperatures in July reached on average 6°C above the long-term means, and annual precipitation deficits were as high as 300 mm, 50% below long-term precipitation averages (Raspe et al., 2004; Ciais et al., 2005). We suggest that the multiple-year drought conditions caused by below-average precipitation (2003, 2005, 2006; **Figure 1**) might have contributed to the long-term decline in RLP (**Table 2**; e.g., Olesinski et al., 2011). It is likely that RLP in 2005–2007 was low due to some combination of the limited vertical depth of observations in our minirhizotron tubes (max. 35 cm) and because the investment in root growth could not be sustained by the trees in the common garden due to chronically low precipitation across multiple years. However, drought does not consistently decrease absorptive fine-root production (Büttner and Leuschner, 1994) but can instead stimulate root production (Teskey and Hinckley, 1981; Leuschner et al., 2001), and this may have occurred at depths below our observation depth. Other extrinsic factors may also influence total annual root production, although we do not have data to evaluate these.

It is unlikely that the low root production in 2007 at the end of the 6 years of observation was due to effects of the acrylic tubes (Withington et al., 2003). Other long term minirhizotron studies did not observe a decrease in root growth after 6 years (Norby et al., 2004; Pritchard et al., 2014).

In addition, our observations from mature trees are similar to those of Atkinson (1983) who reported differences of 6–20-fold in yearly maximum and minimum RLP of apple (*Malus domestica*) over 10 years. Atkinson ascribed the differences he observed to planting response and to changes in seedling physiology. However, this reasoning seems to be insufficient for explaining our observations in relatively mature temperate trees.

Our final objective was to calculate average total annual absorptive fine RLP for each species and assess the correlation of RLP with cumulative precipitation over various time periods. We found significant correlations between total annual RLP with current-year precipitation as well as with previous-year's precipitation for our 11 species. However, when we considering the residual variance, as well as correlation coefficients, previous-year precipitation had a greater effect on fine-root growth compared to current year precipitation (**Tables 3**, **4**). These results underscore the influence of water availability (precipitation, an extrinsic factor) on plant growth, both in the current as well as the previous year. In a study of *Abies balsamea* root growth with respect to drought, Olesinski et al. (2011) also reported the importance of the previous-year's precipitation at influencing fine-root growth of trees under drought in the current year. Hadegorn et al. (2016) linked the buffering of root growth in beech under short-term drought conditions to the accumulation of carbohydrates in the root system, permitting renewed or sustained root growth after a period of drought stress. Similarly, Mazza et al. (2014) and Rita et al. (2014) reported that in years of decreased precipitation, the influence of a previously wet year had a significant impact on maintaining radial growth. In addition, there is some evidence that photosynthates of spring and summer may remain in the aboveground parts of the trees, whereas photosynthates produced in fall tend to go to the roots, both for direct root growth and for starch storage (Endrulat et al., 2010; Epron et al., 2012; Adams and Eissenstat, 2014). Collectively, these findings suggest that a legacy effect of annual precipitation may often carry over into the next year resulting in continued root growth when the year before had sufficient precipitation, but the actual year may not.

Environmental Conditions Matter

For temperate trees, species, or leaf habit, exert less influence on root growth dynamics than often assumed, with a greater response from environmental influences. Our results concur with numerous other studies: root growth is plastic to environmental conditions. However, we suggest that the "environmental conditions" linked to absorptive fine-root growth are rarely as simple as some researchers may suggest. This is notable when trying to determine the absorptive fine-root growth patterns of a single species in a specific region (i.e., *Picea abies*). Engler (1903); Ladefoged (1939), and Leibundgut et al. (1963) all report two peaks for *P. abies*, an early spring and autumn peak. Our study found *P. abies* to have only one peak in 4 years (late summer: 2000, 2002, and 2006; autumn: 2005), while only 2001 had two peaks (spring and mid-summer). To reconcile this variability across studies, we consider Engler's (1903) study conditions. Neither Engler's nor our site experienced low summer precipitation followed by a wet autumn (conditions generally associated with two annual peaks), yet we observed different patterns in root production for the same species at similar latitudes (Switzerland and Poland). It is possible that the differences in root growth peaks were not due to precipitation differences but to differences in site soil characteristics. Soil characteristics and temperature interact with precipitation to influence soil moisture availability (Lowry, 1962; Weber and Nkemdirim, 1998). Engler's research site (Adlisberg, 670 m), had a soil that was nutrient-rich and loamy, while our site, at 150 m, has a sandy, relatively nutrient-poor soil. Differences in (a) soil water retention at the two sites, (b) temperature regimes in lower vs. higher elevation, and (c) individual age could all contribute to the discrepancies in fine-root phenology between Engler's study and our own. The year-to-year variability of seasonal patterns of root growth, as illustrated here, suggests that a more comprehensive understanding with more detailed plant and environmental measurements are needed to adequately predict patterns of root growth in temperate trees.

CONCLUSION

The relatively consistent growth patterns across 11 tree species for a particular year underscore the benefit of a multi-species, long-term study for looking at absorptive fine-root production. Our demonstration of relatively synchronous fluctuation in the peak periods of root growth across multiple years for 11 species suggests a large influence of environmental conditions on root growth. The linkage of current year total RLP with previous-year rainfall indicates the importance of buffering resulting in lags in biological responses (i.e., legacy effects), and thus of long-term (>3 years) studies for root phenology. Long-term, species-specific studies with comprehensive environmental and physiological measurements will continue to advance our understanding of how tree species allocate carbon belowground and the relative role of environmental conditions in shaping these dynamics.

DATA AVAILABILITY STATEMENT

Root data will be submitted to the Fine-Root Ecology Database (FRED) jointly housed through the TRY Plant Trait Database and ORNL DAAC. Iversen CM, Powell AS, McCormack ML, Blackwood CB, Freschet GT, Kattge J, Roumet C, Stover DB, Soudzilovskaia NA, Valverde-Barrantes OJ, van Bodegom PM, Violle C. 2018. Fine-Root Ecology Database (FRED): A Global Collection of Root Trait Data with Coincident Site, Vegetation, Edaphic, and Climatic Data, Version 2. Oak Ridge National Laboratory, TES SFA, U.S. Department of Energy, Oak Ridge, Tennessee, U.S.A. Access on-line at: https://doi.org/10.25581/ornlsfa.012/1417481.

AUTHOR CONTRIBUTIONS

JW and DE planned and designed the specific research described herein. PR and JO organized the broader project on-site of which this was a component and facilitated use of the research site. JW, BB, and MG collected and analyzed the data. JO contributed data. JW and MG wrote the manuscript, with assistance from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.623722/full#supplementary-material

Supplementary Table 1 | Linear mixed model root length production and annual precipitation.

Supplementary Table 2 | Correlation coefficients by species groupings for RLP and each actual and previous year precipitation (9, 7 months).

Supplementary Description 1 | USDA Soil type descriptions.

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Different Waterlogging Depths Affect Spatial Distribution of Fine Root Growth for *Pinus thunbergii* Seedlings

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The increase of waterlogged environments at forests and urban greenery is of recent concern with the progress of climate change. Under waterlogging, plant roots are exposed to hypoxic conditions, which strongly affect root growth and function. However, its impact is dependent on various factors, such as waterlogging depth. Therefore, our objective is to elucidate effects of different waterlogging depths on *Pinus thunbergii* Parl., which is widely used for afforestation, especially at coastal forests. We conducted an experiment to examine growth and morphology of fine roots and transpiration using 2-year-old seedlings under three treatments, (1) control (no waterlogging), (2) partial waterlogging (partial-WL, waterlogging depth = 15 cm from the bottom), and (3) full waterlogging (full-WL, waterlogging depth = from the bottom to the soil surface, 26 cm). As a result, fine root growth and transpiration were both significantly decreased at full-WL. However, for partial-WL, fine root growth was significantly increased compared to control and full-WL at the top soil, where it was not waterlogged. Additionally, transpiration which had decreased after 4 weeks of waterlogging showed no significant difference compared to control after 8 weeks of waterlogging. This recovery is to be attributed to the increase in fine root growth at non-waterlogged top soil, which compensated for the damaged roots at the waterlogged bottom soil. In conclusion, this study suggests that *P. thunbergii* is sensitive to waterlogging; however, it can adapt to waterlogging by plastically changing the distribution of fine root growth.

Keywords: fine root growth distribution, fine root morphology, fine root color, in-growth core, coastal forest restoration

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INTRODUCTION

With the progress of climate change, it is predicted that precipitation regimes will change and extreme rainfall events will occur more frequently, consequently increasing waterlogged environments (Kundzewicz et al., 2014). As the diffusion of oxygen in water is 10,000 times slower than it is in air (Greenwood, 1961), oxygen availability for root respiration becomes limited under waterlogging (Blom and Voesenek, 1996), strongly affecting root growth and function. The risk of waterlogging stress is expected to increase at not only forests but also urban afforestation sites, as waterlogging occurs due to not only climate but also topography and soil properties, such as flat areas, clay soils, and poor drainage (Valipour, 2014).

Recently, coastal *Pinus thunbergii* Parl. forests have been gaining attention after the disastrous tsunami which occurred at pacific coast line of northeastern Japan in March 2011 (Sakamoto, 2012; Tanaka et al., 2013). Restoration of these sites is currently undergoing; however, in some cases, soil layers of the plantation growth base are of poor permeability and drainage due to the use of machinery (Ono et al., 2016), which results in waterlogged conditions after rainfall (Shinomiya et al., 2016). Fujita et al. (2020) reported that under waterlogging, fine root growth of *P. thunbergii* is severely inhibited. Furthermore, root damage such as partial root death and decay was observed from the decrease in root tissue density and darkening in root color. Therefore, there is a concern that *P. thunbergii* is sensitive to waterlogging and information on the effect of waterlogging is of urgent need.

Previous research works have found that species with high tolerance show morphological and physiological responses to waterlogging, such as the production of lenticels (Kozlowski and Pallardy, 2002; Shimamura et al., 2010) and adventitious roots (Yamamoto et al., 1995; Li et al., 2006; Steffens and Rasmussen, 2016) which are responses that support the entrance and transportation of oxygen. On the other hand, for species that are sensitive to waterlogging, avoidance responses are not observed and under long-term waterlogging, inhibition of fine root growth (Schmull and Thomas, 2000), induced root decay (Colin-Belgrand et al., 1991), and decrease in root biomass have been reported (Kozlowski, 1997). However, these results are obtained from relatively strong waterlogging stress conditions where the waterlogging depth reaches the soil surface and the whole root system is exposed to waterlogging.

The impact of waterlogging stress on plants is highly dependent on factors such as duration (Pryor et al., 2006), timing (Wang et al., 2015; Roitto et al., 2019), and depth (Jiang and Wang, 2006) of waterlogging. Under natural conditions, waterlogging depths varies. On the other hand, fine roots show high plasticity to environmental factors (Ostonen et al., 2007). Therefore, when the waterlogging is "partial (localized)," it is suggested that fine root growth responses will differ to when the whole root system is exposed to waterlogging. It is hypothesized that under partial waterlogging, localized growth is triggered, where growth is inhibited under waterlogging, and it is continued under non-waterlogged soils. Furthermore, as partial waterlogging would be less stress, responses and adaptions that could not be observed under strong waterlogging conditions would be elucidated, enabling a better understanding of waterlogging response for sensitive species.

Fine roots (diameter < 2 mm) play a vital role in plant growth and survival by absorbing water and nutrients from the soil. Hence, the impact and the damage of waterlogging on fine roots strongly influence these functions, which are essential for above-ground activity. From previous studies, above-ground activity such as transpiration is generally decreased under waterlogging due to the decrease in root water absorption and/or stomatal conductance caused by the decrease in root hydraulic conductance (Aroca et al., 2012). Therefore, to understand the effect of waterlogging on fine roots, not only growth (mass) but also the function (quality) in relation with above-ground activity must be evaluated.

Our research objective is to elucidate effects of different waterlogging depths on fine root growth distribution, fine root morphology, and transpiration of *P. thunbergii* seedlings. Our hypotheses are as follows:

(1) Under partial-waterlogging, fine root growth of *P. thunbergii* seedlings is inhibited at waterlogged bottom soil, whereas fine root can continue to grow at non-waterlogged top soil, likewise to control.
(2) Transpiration of *P. thunbergii* seedlings is decreased under full waterlogging, and the decrease in transpiration is more significant compared to partial waterlogging as the whole root system is exposed to waterlogging.

MATERIALS AND METHODS

Plant Material and Waterlogging Treatment

Two-year-old *P. thunbergii* seedlings were purchased from a commercial tree nursery. At the end of March, 45 seedlings were planted in 1/2,000 a Wagner pots (500 cm^2, depth 30 cm with a drainage hole) with Akadama soil deriving from the loamy B horizon of an Andisol (Nagakura et al., 2004). After transplantation, seedlings were grown under natural conditions at the experimental nursery of Tohoku Research Center, Forestry and Forest Products Research Institute. After 2 weeks, a 2,000-fold diluted liquid fertilizer (N:P: K = 6, 10, and 5%, Hyponex Japan, Osaka, Japan) was applied with 1 L of water. The amount of nutrients given per pot was approximately 30, 50, and 25 mg of N, P, and K, respectively. Pots were placed on wooded boards and were watered regularly (once a week, 2–4 L) until the waterlogging treatment began. The experimental period was approximately 6 months, from the end of March 2019 until the beginning of October 2019. Since there is a possibility that waterlogging indirectly affects fine root growth through suppressed aboveground growth, waterlogging treatments were started at the beginning of August when shoot and needle elongation had stopped in order to minimize such indirect effects (**Table 1**). Furthermore, it was aimed at a time when temperatures are high and seedlings are more likely to experience water stress.

In our experiment, two different waterlogging depths were set. For full waterlogging (full-WL), the drainage hole was blocked and water was added until the water table reached the soil surface (waterlogging depth = 26 cm from the bottom). For partial waterlogging (partial-WL), the waterlogging depth was set at 15 cm from the bottom of the pot. This was done by setting the end of the drainage tube at 15 cm height of the pot so that the water would overflow from the set depth (**Supplementary Figure 1**). The waterlogging depth was maintained by adding water at 1–2 day interval. For control, the drainage hole was kept open and was watered regularly throughout the experiment period according to the weather (at least once a week, 2–4 L). The waterlogging treatment was done from the beginning of August till the beginning of October (2 months). Each treatment was consisted of 15 seedlings.

Soil Oxidation–Reduction Potential

The soil oxidation–reduction potential (Eh, mV) was measured by a platinum electrode, a reference electrode, and a logger (FV-702, Fujiwara Scientific Co., Ltd., Tokyo, Japan) (Hara, 2013a,b). Probes were set in pots for the control and full-WL treatment, respectively ($n = 2$). The probes were placed at approximately 10 cm depth from the soil surface. Eh vales were recorded at 2–5 day intervals.

Root Measurements

In total, three in-growth cores (diameter, 32 mm; height, 30 cm; 2 mm mesh) were used for each pot. All in-growth cores were placed in the pot at the time of transplanting the seedlings. In each pot, two in-growth cores were placed on each side of the seedling, approximately 10 cm apart from the seedling and were used to measure fine root growth during the whole experimental period (IG_{total}). The third in-growth core was also placed approximately 10 cm from the seedling, perpendicular to the other two IG_{total}. This in-growth core (IG_{wl}) was covered with a thin plastic sheet to prevent root penetration before the waterlogging treatment. The plastic sheet was carefully taken away at the time of starting the waterlogging treatment, enabling fine roots to penetrate the in-growth core during the waterlogging period.

All in-growth cores were harvested at the beginning of October and stored at 4°C until further analysis. In-growth cores were separated into two sections, the "top part (11 cm from the soil surface)" and "bottom part (15 cm from the bottom of the pot)" (**Supplementary Figure 1**). For control, both the top and the bottom part were free from waterlogging. For full-WL, both the top and the bottom part were waterlogged. On the other hand, at partial-WL, the top part was free from waterlogging and the bottom part was waterlogged. Fine root growth, morphology, and brightness were analyzed separately for the top and the bottom part.

TABLE 1 | Seedlings size after transplantation, before, and after waterlogging treatment.

Measurement	Treatment	After transplantation	Before WL treatment	At end of treatment
Stem base diameter (mm)	Control	7.9 ± 0.3*a*	10.1 ± 0.2*a*	11.4 ± 0.2*a*
	Partial-WL	8.0 ± 0.2*a*	10.2 ± 0.1*a*	11.9 ± 0.1*ab*
	Full-WL	8.0 ± 0.2*a*	10.1 ± 0.2*a*	12.4 ± 0.2*b*
Height (cm)	Control	23.2 ± 1.0*a*	36.7 ± 1.1*a*	38.3 ± 1.2*a*
	Partial-WL	21.3 ± 0.5*a*	37.0 ± 0.7*a*	38.1 ± 0.7*a*
	Full-WL	22.2 ± 0.5*a*	36.0 ± 1.4*a*	37.2 ± 1.2*a*

Mean (±SE) of stem base diameter and height of seedlings after transplantation (April 18th), before (August 2nd) and right before the waterlogging treatment ended (September 25th, $n = 15$) for control, partial waterlogging (partial-WL) and full waterlogging (full-WL). Results of the Tukey–Kramer's multiple comparison test are shown with different italic letters indicating statistical differences between treatments ($p < 0.05$).

The soil from the in-growth cores was thoroughly washed out with water on a very fine sieve (sieve aperture, 250 μm). Fine roots were carefully picked from the sieve and carefully washed with a brush. After washing, 10 seedlings from each treatment were randomly chosen and fine roots with a flatbed scanner (GT-X980, EPSON). Scans were made without any image correction and under the same light conditions at 800 dpi. After scanning, fine roots were dried at 70°C for 72 h, and then measured for dry weight (W_{dr}). For the left five seedlings from each treatment, only W_{dr} was measured. Fine root growth (mg cm^{-3}) per pot was calculated from W_{dr} in IG_{total} and IG_{wl}. For IG_{total}, fine root growth of each seedling was calculated by adding W_{dr} of the two in-growth cores and dividing it by the total soil volume of the in-growth core.

The scanned images were analyzed with WinRHIZO Pro (2012b) (Regent Instruments, Inc., Quebec, Canada) for root length (L_r, cm), root projected area (A_{pr}, mm^2), and root volume (V_r, cm^3). Mean root diameter (D_r), specific root length (SRL), and RTD were obtained for evaluating fine root morphology and were calculated from the following equations:

$$D_r\ (mm) = \frac{A_{pr}}{L_r}$$

$$SRL\ \left(m\,g^{-1}\right) = \frac{L_r}{W_{dr}}$$

$$RTD\ \left(g\ cm^{-3}\right) = \frac{W_{dr}}{V_r}$$

For fine root morphology obtained from IG_{total}, D_r was calculated by dividing total A_{pr} by total L_r from the two in-growth cores. For SRL, it was calculated by dividing total L_r by total W_{dr} of the two in-growth cores. For RTD, it was calculated by dividing total W_{dr} by total V_r of the two in-growth cores.

For root color, root brightness was evaluated from the scanned images by image analysis carried out by Image J. A brightness histogram (0–255) of the scanned images was calculated as area ratio of the total number of pixels showing root region. Regions showing roots were determined by binarization of images, and brightness was set as the average of colors (R, G, B).

Transpiration and Needle Traits

Transpiration was estimated by measuring evapotranspiration and evaporation from pots, where the former and latter were estimated by weighing water loss from pots with seedlings ($n = 15$ per treatment) and without seedlings ($n = 3$), respectively. Days of measurement were chosen so that measurement days were all fully sunny and rainless. Before the transpiration measurement, waterlogging was temporally released. Then, all pots were watered until the water ran out from the drainage hole at the bottom to ensure that they were well-watered. Measurements were done during nighttime (20:00–21:30) to minimize transpiration and evaporation during the measurement period. Pots were measured for the first weight and were re-weighed the next night for the second weight. Transpiration per seedling (T, kg pot^{-1}) was estimated from the following equation by using the first weight and second weight of pots with seedlings (M_{s1}, M_{s2}) and without seedlings (M_{ws1}, M_{ws2});

$$T\left(kg\ pot^{-1}\right) = \{M_{s1}(kg\ pot^{-1}) - M_{s2}(kg\ pot^{-1})\} - \{M_{ws1}(kg\ pot^{-1}) - M_{ws2}(kg\ pot^{-1})\}$$

Here, "M_{S1}–M_{S2}" and "M_{WS1}–M_{WS2}" correspond to evapotranspiration and evaporation, respectively. For evaporation, the averaged value obtained from the three pots without seedlings was used. For some pots of full-WL, transpiration after 4 and 8 weeks of waterlogging showed negative values, where the averaged evaporation exceeded evapotranspiration (−0.03 to −0.005 kg). For these pots, transpiration was calculated as zero (six pots). After measurements, pots were filled with water once again for partial-WL and full-WL. In total, pots were released from waterlogging for 1.5 days during the transpiration measurement.

Dry weight of total needles, and fresh and dry needle weight were weighed for current year needles randomly sampled (15–20 needles) from the top part of current year shoot right after seedling harvest in October (n = 10–11). Sampled needles were scanned (800 dpi) and then were dried in an oven at 80°C for 72 h before weighing for dry weight. Needle water content (NWC) and leaf mass per area (LMA) were measured to evaluate needle traits. NWC was calculated as follows using W_{fn} (fresh needle weight, g) and W_{dn} (dry needle weight, g),

$$NWC\ (\%) = \frac{W_{fn} - W_{dn}}{W_{fn}} \times 100$$

Needle area was calculated as needle projected area (A_{pn}, m^2), which was obtained from Image J. LMA was calculated as follows,

$$LMA(g\ m^{-2}) = \frac{W_{dn}}{A_{pn}}$$

Statistical Analysis

The Levene's test was used to check homogeneity of variance. When homogeneity was ensured, statistical difference between treatment groups was tested by Tukey–Kramer's multiple comparison test, if not Steel–Dwass multiple comparison test was used. All analysis was done using R (version 3.6.1, The R foundation for Statistical Computing Platform). For fine root growth and morphology, the difference between treatment groups was tested separately at the top and bottom part. One-way ANOVA was used to test the difference between control and partial waterlogging for IG_{wl}.

RESULTS

Soil Oxidation–Reduction Potential

Soil oxidation–reduction potential (Eh, mV) for full-WL was decreased after approximately 5–7 days after the waterlogging treatment began and was approximately below 300 mV throughout the experiment, except when the Eh value increased due to the temporal release of waterlogging for transpiration measurement. The Eh value decreased again within 1 week, after refilling the pot with water. For control, Eh value was approximately 500–550 mV throughout the experiment (**Figure 1**).

Fine Root Growth

Comparing results of IG_{total} between treatment groups, total fine root growth (top + bottom) did not differ between treatment groups. However, at the top part (11 cm from the soil surface), fine root growth was significantly increased at partial-WL (**Figure 2A**) compared to control. At the bottom part (15 cm from the bottom), fine root growth did not differ among treatments.

From IG_{wl}, it was found that fine root growth during waterlogging was decreased for full-WL (**Figure 2B**) at both the top and the bottom part. For partial-WL, total fine root growth did not differ from control. However, compared to control, fine root growth was significantly increased at the top part.

Fine Root Brightness and Morphology

Fine roots of *P. thunbergii* were generally brown or relatively dark brown at the top and bottom parts of control. Using image analysis, root color was quantitatively analyzed as root brightness (**Figures 3A–D**). Concerning IG_{total}, control and partial-WL showed a similar histogram peak at the top part where it was not waterlogged for these two treatments. On the other hand, at the bottom part of partial-WL and full-WL, root brightness showed a similar peak, where the histogram peak was at a darker brightness compared to control. For IG_{wl}, similar results to IG_{total} were observed. At the top part for control and partial-WL, a similar histogram peak was observed. At the bottom, the histogram peak for partial-WL and full-WL was darker compared to control.

Concerning fine root morphology, mean D_r, SRL, and RTD were evaluated from image analysis (**Table 2**). For IG_{total}, D_r was increased compared to control at the bottom part of partial-WL and full-WL, where it was waterlogged. SRL did not differ among treatments at both the top and the bottom part. RTD was decreased compared to control at the bottom part for full-WL.

For IG_{wl}, D_r was increased compared to control at the top part of partial-WL (**Table 3**). SRL was decreased at the top part for partial-WL. At the bottom part, difference between control and partial-WL was not observed. RTD did not differ between control and partial-WL at both the top and bottom part. For full-WL, fine root morphology could not be evaluated as hardly any fine roots could be obtained from the in-growth cores.

Transpiration and Needle Traits

Transpiration was measured before waterlogging, after 4 and 8 weeks of waterlogging (**Figure 4**). Before the waterlogging treatment, transpiration did not differ among treatments. After 4 weeks of waterlogging, transpiration was decreased at both partial-WL and full-WL, and full-WL showed a smaller value compared to partial-WL. After 8 weeks of waterlogging, only full-WL was significantly decreased compared to control and partial-WL and there was no significant difference between control and partial-WL.

Results of total needle dry weight and current year needle traits are shown in **Table 4**. The total dry weight of needles did not differ

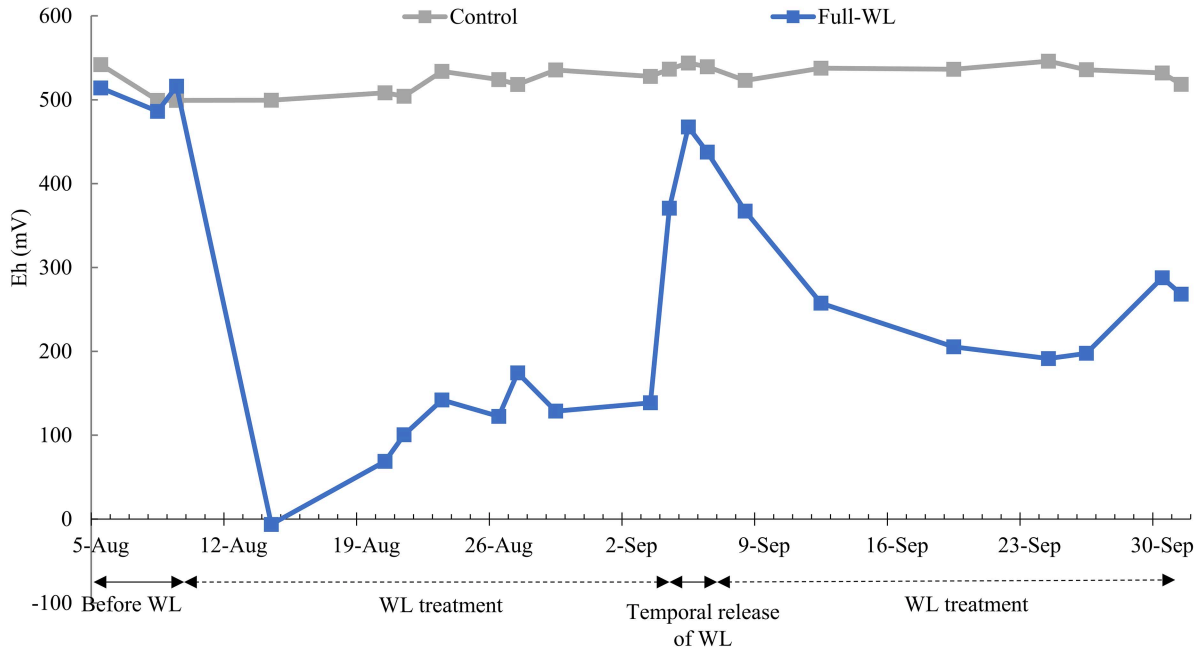
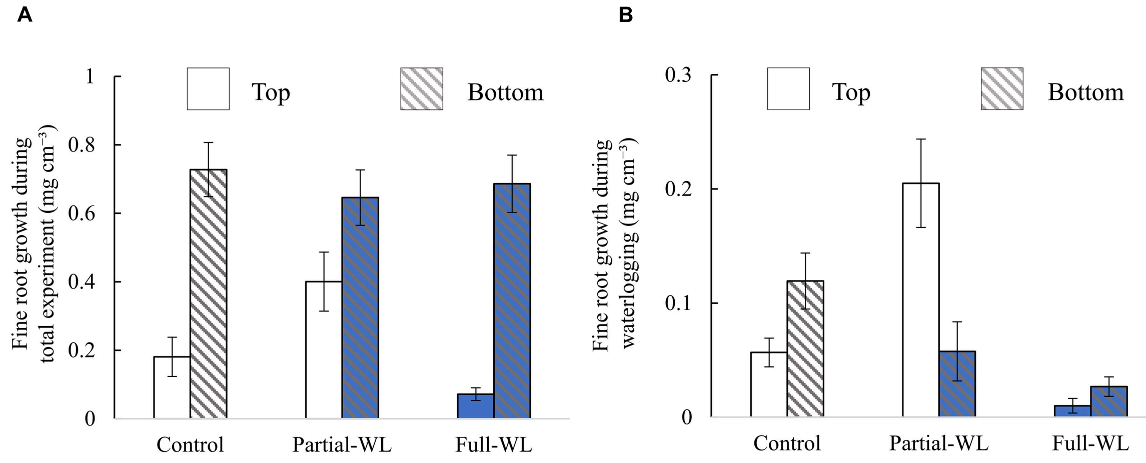


FIGURE 1 | Mean soil oxidation–reduction potential of control and full waterlogging (WL) ($n = 2$).



Treatment	Total	Top part	Bottom part
Control	*a*	*a*	*a*
Partial-WL	*a*	*b*	*a*
Full-WL	*a*	*a*	*a*

Treatment	Total	Top part	Bottom part
Control	*a*	*a*	*a*
Partial-WL	*a*	*b*	*ab*
Full-WL	*b*	*c*	*b*

FIGURE 2 | Mean fine root growth (±SE) during **(A)** the whole experiment period (IG_{total}) and **(B)** waterlogging treatment period (IG_{wl}) ($n = 15$ for each treatment and position). Solid bars are results from the top part (11 cm from soil surface), and hatched bars are results from the bottom part (15 cm from the bottom). Bars which are colored with blue are results obtained from waterlogged soils. The result of Tukey–Kramer's multiple comparison test or Steel–Dwass multiple comparison test (IG_{total}: top, IG_{wl}: total and top part) is shown in the table below the figures. Statistical difference was tested among treatments at the top part, bottom part, and total (top + bottom), respectively. Different italic letters show statistical difference ($p < 0.05$).

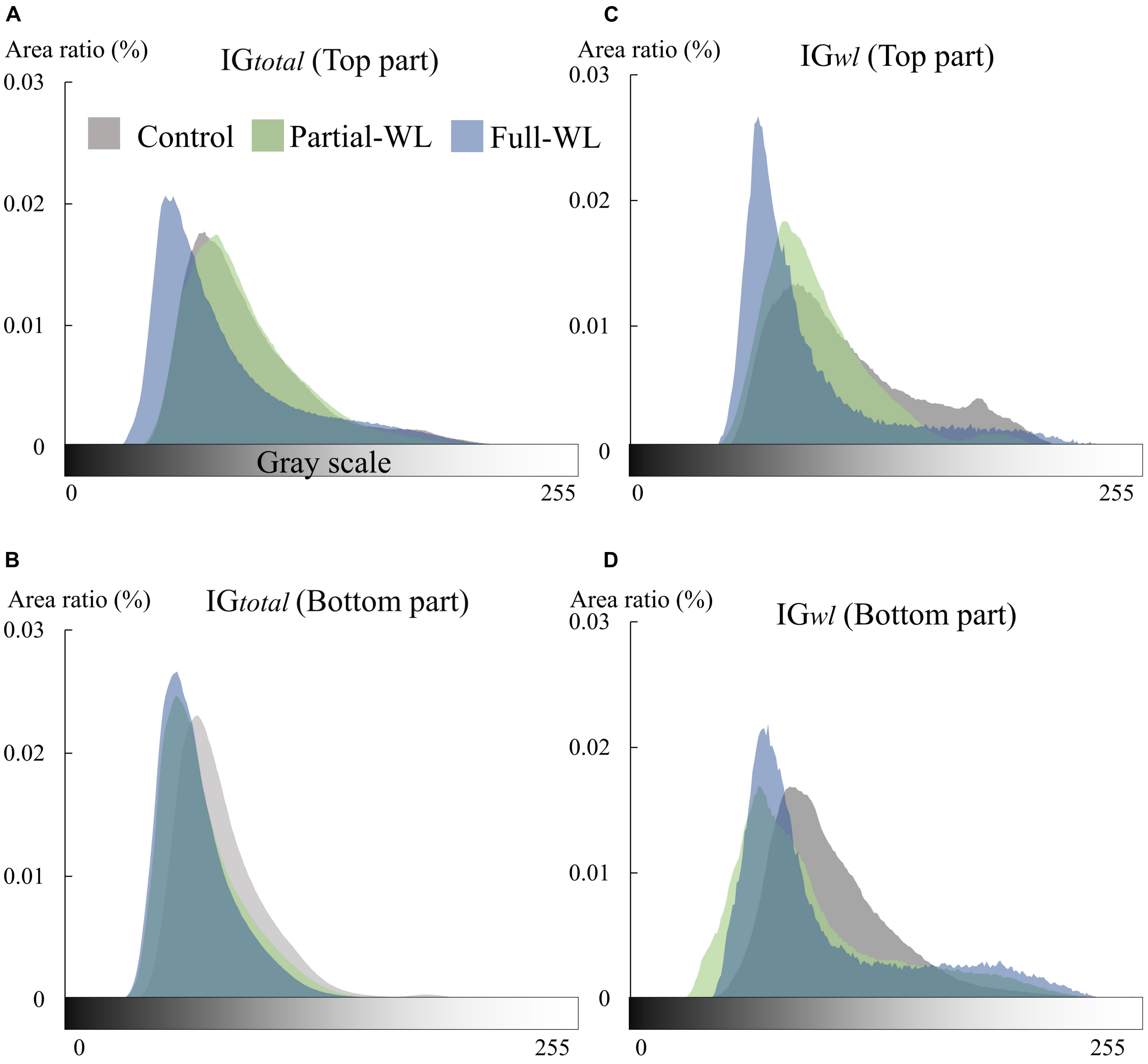


FIGURE 3 | Histogram of root brightness (0–255) of IG_{total} (n = 7–10) and IG_{wl} (n = 8–10) at the top (11 cm from the soil surface) and bottom (15 cm from the bottom), respectively **(A–D)**. Gray, green, and blue show results of control, partial waterlogging (partial-WL), and full waterlogging (full-WL), respectively.

among treatments. On the other hand, concerning current year needle traits, NWC was significantly decreased at full-WL, and values did not differ between control and partial-WL. LMA was significantly increased at partial-WL and full-WL, and the highest value was observed at full-WL.

DISCUSSION

In this study, soil oxidation–reduction potential (Eh) for full-WL was decreased to <300 mV throughout the period of the waterlogging treatment (**Figure 1**), with the exception when Eh increased during the temporal release of waterlogging for transpiration measurement. This suggests that full-WL created an oxygen-limited, anaerobic/hypoxic condition compared to control (Pearsall and Mortimer, 1939; Pezeshki and DeLaune, 2012). Increment of stem base diameter at full-WL also indicated that *P. thunbergii* seedlings were affected by waterlogging (**Table 1**). Previous studies on other pine species such as *Pinus densiflora* reported that stem diameter increment under waterlogging was due to increased bark thickening (stem hypertrophy) (Yamamoto et al., 1987). Therefore, it is suggested that the stem base diameter increase observed at full-WL was a similar response to *P. densiflora*.

Under full-WL, fine root growth was severely inhibited, suggesting that fine roots of *P. thunbergii* are relatively sensitive to waterlogged conditions (**Figure 2B**). Under partial-WL, fine root growth was suppressed only at the bottom part where it was waterlogged. In contrast, at the top part, fine root

TABLE 2 | Fine root morphology obtained from IG_{total}.

Position	Treatment	Root diameter (mm)	SRL (m g^{-1})	RTD (g cm^{-3})
Top part	Control (*n* = 9)	0.45 ± 0.03*a*	27.0 ± 2.0*a*	0.25 ± 0.02*a*
	Partial-WL (*n* = 10)	0.52 ± 0.01*a*	23.7 ± 4.0*a*	0.24 ± 0.03*a*
	Full-WL (*n* = 7)	0.52 ± 0.02*a*	22.5 ± 1.7*a*	0.22 ± 0.01*a*
Bottom part	Control (*n* = 10)	0.42 ± 0.01*a*	26.0 ± 2.0*a*	0.29 ± 0.02*a*
	Partial-WL (*n* = 10)	0.49 ± 0.02*b*	23.3 ± 1.1*a*	0.24 ± 0.01*ab*
	Full-WL (*n* = 9)	0.50 ± 0.02*b*	22.3 ± 2.0*a*	0.24 ± 0.01*b*

Mean (±SE) root diameter, specific root length (SRL), and root tissue density (RTD) obtained from IG_{total} (n = 7–10) of control, partial waterlogging (partial-WL), and full waterlogging (full-WL). Values in black show results obtained from non-waterlogged soils, and values in blue show results obtained from waterlogged soils. Statistical difference among treatments was tested at the top part and the bottom part, respectively. Results of the Tukey–Kramer's multiple comparison test or Steel–Dwass multiple comparison test (top and bottom of RTD) are shown with different italic letters indicating statistical differences between treatments ($p < 0.05$).

TABLE 3 | Fine root morphology obtained from IG_{wl}.

Position	Treatment	Root diameter (mm)	SRL (m g^{-1})	RTD (g cm^{-3})
Top part	Control (*n* = 8)	0.40 ± 0.02	37.3 ± 3.8	0.23 ± 0.01
	Partial-WL (*n* = 9)	0.51 ± 0.02***	28.1 ± 2.1*	0.23 ± 0.05
Bottom part	Control (*n* = 10)	0.44 ± 0.03	36.4 ± 3.9	0.22 ± 0.01
	Partial-WL (*n* = 9)	0.53 ± 0.05	30.8 ± 4.3	0.19 ± 0.02

*Mean (±SE) root diameter, specific root length (SRL), and root tissue density (RTD) obtained from IG_{wl} (n = 8–10) of control and partial waterlogging (partial-WL). Values in black show results obtained from non-waterlogged soils, and values in blue show results obtained from waterlogged soils. Statistical difference was tested between treatments at the top part and bottom part, respectively. Results of one-way ANOVA are denoted as: *$p < 0.05$; ***$p < 0.001$. Results from full-WL are not shown as hardly any roots could be obtained from in-growth cores.*

growth was significantly increased compared to values of both control and full-WL. These results support hypothesis (1), although the enhancement of fine root growth compared to control at top part was not expected before the experiment. This result indicates that fine root growth of *P. thunbergii* shows high plasticity in their vertical distribution under partial waterlogging.

From field survey, Hirano et al. (2018) reported that *P. thunbergii* growing at shallow groundwater level (sea side) allocates more biomass to the root system, especially to horizontal roots (age 32–58 years). This results in a "plate root system" with shallow vertical roots and long horizontal tap roots. In contrast, at deep groundwater level (land side), deeper vertical roots presented a "tap root system." For partial-WL, although fine root growth was inhibited under waterlogging at the bottom part, it was enhanced at the top part, resulting in a similar shape to the "plate root system." Although our results were obtained from a short-term experiment, it reflects the high plasticity of root systems observed in mature trees of *P. thunbergii* at different groundwater depths under natural conditions.

Although high plasticity was observed concerning the spatial distribution of fine root growth under partial waterlogging, response in root morphology mainly indicated root damage. New roots are generally white and turn brown or dark brown with development (Comas et al., 2000; Wells and Eissenstat, 2001). On the other hand, fragile black roots generally indicate dead roots (McClaugherty et al., 1982). Wang et al. (2015) reported that roots turn black under waterlogging for *Betula pendula* and *Betula pubescens* seedlings, and the proportion of black-colored roots was increased under waterlogging possibly due to the deposition of iron. As the deposition of iron/manganese oxides on roots is suggested to interfere with nutrient uptake (Levan and Riha, 1986), the darkening of root color may be indicating damage of fine roots such as the decrease in water absorption function, as transpiration was also decreased under waterlogging. Furthermore, as the darkening in root color and the decrease in RTD was observed at the bottom part of full-WL

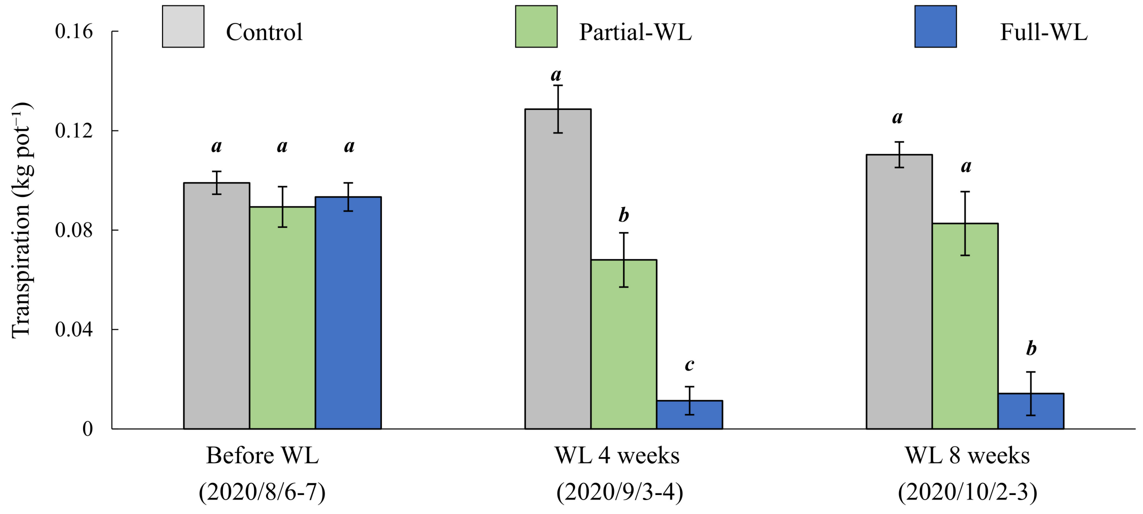


FIGURE 4 | Mean transpiration (±SE) before waterlogging treatment (August 8–6), 4 weeks after waterlogging (September 3–4), and 8 weeks after waterlogging (October 2–3). Gray bars, green bars, and blue bars are the result of control, partial waterlogging (partial-WL), and full waterlogging (full-WL), respectively (*n* = 15). Results of the Tukey–Kramer's multiple comparison test are shown with different italic letters indicating statistical differences ($p < 0.05$) between treatments at each measurement period.

TABLE 4 | Traits of current year needles.

Treatment	Total needle dry weight (g)	NWC (%)	LMA (g m^{-2})
Control	30.7 ± 1.3*a*	60.9 ± 0.4*a*	268.2 ± 8.7*a*
Partial-WL	27.5 ± 3.4*a*	59.2 ± 0.5*a*	296.1 ± 6.0*b*
Full-WL	28.5 ± 2.5*a*	53.1 ± 0.5*b*	346.0 ± 8.8*c*

Mean (±SE) values of total needle dry weight (g), needle water content (NWC), and leaf mass per area (LMA) of the control, partial waterlogging (partial-WL), and full waterlogging (full-WL), respectively (n = 10–11). Total needle dry weight was obtained from whole seedlings. Needle water content and leaf mass per area were obtained from randomly sampled current year needles. Results of the Tukey–Kramer's multiple comparison test are shown with different italic letters indicating statistical differences ($p < 0.05$).

(**Figure 3B** and **Table 2**), not only malfunction but root death was also suggested.

Concerning mean D_r, it was increased at the bottom part for full-WL (**Table 2**). This increase in D_r was possibly due to the decrease in branching and number of root tips due to the inhibition of growth under waterlogging, or the shedding of root tips due to root death, leaving only the relatively thicker fine roots (Repo et al., 2017).

The increase in D_r was also observed at the top part of partial-WL for IG_{wl} (**Table 3**). Here, it is suggested that this increase in D_r is due to a different mechanism than the increase under waterlogging, as the top part of partial-WL is not waterlogged and change in root color was not observed. Under non-waterlogged soils, the increase in D_r may have been due to the production of pioneer roots. Pioneer roots are roots with a larger diameter, suggested to be more readily to defend against abiotic challenges and long exploration in soils (Zadworny and Eissenstat, 2011). Therefore, as the root system was partially under waterlogging stress, pioneer roots may have been preferentially produced to explore soil at the top part and make a new root system to compensate for the damaged roots at the bottom part.

Although total needle dry weight did not differ among treatments, transpiration was significantly decreased at both partial-WL and full-WL after 4 weeks of waterlogging, in which transpiration for full-WL was more decreased than partial-WL (**Figure 4**). This result followed our hypothesis (2) and other waterlogging research measuring transpiration (Rodríguez-Gamir et al., 2011; Repo et al., 2016). It is suggested that transpiration was decreased due to root damage such as the decrease in water absorption function and/or root death, which was indicated by the decrease in root brightness and the decrease in RTD.

At partial-WL, transpiration recovered to a value which did not differ from control after 8 weeks of waterlogging (**Figure 4**). It is assumed that this recovery was enabled by the enhanced growth of new fine roots at the top part (**Figure 2B**), and this worked to recover the balance between water absorption by fine roots and transpiration. Under partial waterlogging, it is suggested that *P. thunbergii* took the strategy of compensation growth of fine roots against waterlogging stress. This result supports research results where it was reported that investments are made to roots where roots can achieve most nutrient and water acquisition (Hodge, 2004; Dresbøll et al., 2013).

Needle traits of current year needles were also affected by waterlogging. NWC of current year needles was significantly decreased at full-WL, possibly due to water stress caused by the decrease in water absorption by fine roots, indicating that needles were experiencing conditions similar to drought stress. LMA was significantly increased at both partial-WL and full-WL (**Table 4**). Under waterlogging, the reason for the observed increase has been reported to be the accumulation of starch and/or soluble carbohydrates, as carbon fixation exceeds carbon demand for growth (Poot and Lambers, 2003) and root demand is significantly decreased (Gravatt and Kirby, 1998). Although the decrease in fine root growth was significant for full-WL, it was not at partial-WL. Therefore, the increase in LMA may be due to another factor for partial-WL, and further investigations must be made on this point.

In this study, we examined effects using fresh water. We believe that our results give valuable results on effects of different waterlogging depths on *P. thunbergii*. However, at coastal forests, there is not only the concern of waterlogging due to rainfall, but also the additional concern of the rise in sea level due to climate change, resulting in higher groundwater with salinity stress (Plane et al., 2019). Therefore, in future studies, other factors such as waterlogging stress combined with salt stress should be investigated for a better understanding on growth of *P. thunbergii* trees planted at coastal forests.

CONCLUSION

This study showed that different depths of waterlogging significantly affect spatial distribution of fine root growth during the waterlogging treatment. Under partial waterlogging, fine root growth was significantly increased at the top part, which enabled the recovery of transpiration. Although *P. thunbergii* is sensitive to waterlogging, according to the depth of waterlogging, it can plastically change fine root growth distribution and compensate for the damaged fine roots by enhancing new fine root growth where it is not waterlogged. This potential acclimation strategy may also explain root system architecture in mature coastal *P. thunbergii* forests under high groundwater level (plate root system), although results of this present study were limited to short-term responses of seedlings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SF, KN, and TT conceived and designed the experiments and wrote the manuscript. SF and KN performed the experiments and analyzed the data. TT supervised the research. All authors read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Root Biomass Distribution of *Populus sibirica* and *Ulmus pumila* Afforestation Stands Is Affected by Watering Regimes and Fertilization in the Mongolian Semi-arid Steppe

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Desertification of the semi-arid steppe of Mongolia is advancing very rapidly, motivating afforestation efforts. The "Green Belt" joint project (Government of Mongolia and Republic of Korea), which aims to mitigate soil degradation and develop agroforestry activities through the planting of a forest shelterbelt, is one such response. In these plantations, tree growth has been supported by different watering regimes (no watering, 2, 4, and 8 L h^{-1}) and by two types of soil fertilization (NPK and Compost). The present paper analyses the effect of these techniques on soil chemistry and root biomass partitioning of *Populus sibirica* (Horth ex Tausch) and *Ulmus pumila* (L.) tree species. In July 2019, at the plantation site in Lun Soum, Tuv province (Mongolia), six trees were excavated by hand in each treatment, the root system was divided into taproot and five diameter classes (0–2; 2–5; 5–10; 10–20; > 20 mm), and the biomass was measured. Soil organic matter, macronutrients, and pH were also measured. The addition of fertilizers in the long-term did not enhance the soil chemical properties. The build-up of root biomass in both species correlated positively with increasing levels of the watering, while the application of fertilizers led to root growth suppression. For most of the root classes and both species, an irrigation level of 4 L h^{-1} was sufficient to yield the highest biomass and could be recommended for afforesting the semi-arid steppe of Mongolia. The root biomass of *P. sibirica* was more dependent on the watering regimes and of *U. pumila* was more negatively influenced by the application of fertilizers, indicating that *U. pumila*, due to the its lower water need, could be suitable for afforesting semi-arid environments. Our experiments suggest that afforestation practices in the semi-arid steppe of Mongolia should be supported by a prior analysis of plants' needs, soil type, dose, and type of fertilizers to be applied. Knowledge of the root response to the supporting techniques is necessary for choosing the best one for the plantation and, thus, to develop a sustainable and successful strategy to restore these degraded lands.

Keywords: siberian poplar, siberian elm, land degradation and desertification, forest shelterbelt, restoration, tree roots

INTRODUCTION

A considerable percentage (78%) of Mongolian arid and semi-arid lands are highly degraded and prone to desertification (Tsogtbaatar, 2004, 2009). To the aim of counteracting soil degradation and promoting agroforestry activities, in 2008 the Mongolian and South Korean governments jointly promoted a forested shelterbelt in the Mongolian arid and semi-arid lands named the "Green Belt" Project (Lee and Ahn, 2016; Byambadorj et al., 2020). The establishment of a new tree plantation in these lands represents a difficult task especially when extreme periods of cold and heat waves overlie the constant condition of water shortage. The interplay of these adverse environmental factors may explain why afforestation and reforestation efforts often are unsuccessful (Choi, 2004; Cao, 2008; Wang et al., 2010). Therefore, a careful selection of both plant material and management techniques is necessary to support adequate survival and rapid development of the planted trees, ensuring the success of the restoration interventions (FAO, 2004; Mansourian et al., 2005; Thomas et al., 2014). In particular, the tree species selection must take into consideration their current suitability and adaptability as well as the resistance to future environmental conditions (Ffolliott et al., 1995; Meli et al., 2014; Lu et al., 2017). However, the selection of tree species is also crucial for providing ecosystem services (Gamfeldt et al., 2013; Reisman-Berman et al., 2019). Indeed, forests provide a range of functions that are fundamental to sustaining terrestrial systems through the regeneration of soil and biodiversity, water conservation, groundwater recharge, dust and flood prevention (MEA., 2005; Chazdon et al., 2009; Abson et al., 2014). Also, forests directly provide vital provisioning services such as biomass for materials and energy (Vitousek et al., 1986; Rojstaczer et al., 2001; Felton et al., 2020), food, recreation, and sheltering areas (Mander et al., 2007; Nassauer and Opdam, 2008; Scherr and McNeely, 2008; Lovell and Johnston, 2009; Chirwa and Mahamane, 2017). Thus, a selection of tree species that can be used in afforestation of dryland areas should also aim to provide at the best the above-mentioned ecosystem services, taking into account other biological characteristics (Reisman-Berman et al., 2019).

The implementation of management techniques is also of fundamental importance for afforestation (Siyag, 2014; Zhang et al., 2016). In nutrient-deficit soils when plant productivity is one of the main objectives, fertilization is considered essential (FAO Soil Portal, Management of some problem soils; Marschner, 1995; Siyag, 2014). Fertilization with inorganic materials is the main practice, but organic manures also can supply nutrients in slowly available forms and improve soil physicochemical properties. The best approach is to use a mixture that includes macro- and micro-nutrients (Gregory, 2008; Giehl et al., 2013). However, the most commonly used fertilization mixtures include macronutrients such as nitrogen (N), phosphorous (P), and potassium (K). Furthermore, in semi-arid environments, the success of afforestation measures will strongly depend on the adoption of an adequate watering regime. It is well-known that watering regimes affect water-use characteristics (Li et al., 2013), water consumption (Gao et al., 2011), and protein content in plants due to the variations of water availability in the soil. Yao et al. (2016) have reported that afforestation of semi-arid or arid lands could lead to variations of the soil moisture content in the upper 30 cm of soil. The regime adopted depends on the water available on site and the chemical, and physical nature of the soil. Indeed, soil texture, structure, drainage and solid material composition (mineral and organic) affects its water-storing capabilities, drainage and evaporation (Bonsu, 1997; Scherer et al., 2013; BIO Intelligence Service., 2014; Amooh and Bonsu, 2015). In particular, in semi-arid environments, soil evaporation accounts for about 30% loss of the total precipitation contributing significantly to the depletion of water in the soil profile (Wallace, 1991; Bonsu, 1997). Water absorbed moves quickly through sandy soils, but they retain very little in comparison with clay soil. Therefore, for an efficient water use, irrigation in sandy sites should be frequent and for short periods, to obtain a broader wetting area, providing more soil volume for roots to exploit, avoiding water moving beyond the root zone and contributing to soil leaching (Goldy, 2012).

Recently, an experimental site located in Lun soum (Tov province), has been used to evaluate the suitability of two tree species, *Populus sibirica* hort. ex Tausch and *Ulmus pumila* L. (Cho et al., 2019; Byambadorj et al., 2020). The *Populus* genus was selected for its high productivity, rapid re-sprouting capability, and easy and inexpensive propagation through cuttings (Mao et al., 2008; Stanturf and van Oosten, 2014; Jo and Park, 2017). The *Populus* genus has been used for almost 20 years in afforestation of Chinese steppes with appreciable results (Hu et al., 2008). The *Ulmus* genus has been chosen for its great adaptability to grow in soils with limited water and nutrient availability (Moore, 2003; Engelbrecht et al., 2005). For example, after 10 years of growth, the morpho-physiological analysis of both species highlighted that *P. sibirica* is characterized by higher productivity with respect to *U. pumila* as a consequence of its higher photosynthetic efficiency (Cho et al., 2019; Byambadorj et al., 2020). The lower productivity found in *U. pumila* trees has been related to a slower growth habit despite the higher water use efficiency than *P. sibirica* (Cho et al., 2019).

Management techniques such as watering regime and fertilization, are under investigation to test their efficiency to foster survival rate, growth, and long-term development of both tree species. Byambadorj et al. (2020) demonstrated that both survival rate and growth performance of *P. sibirica* trees depend strictly upon the applied management techniques whereas *U. pumila* trees are characterized by higher survival rates even without the application of irrigation. Moreover, increments observed in stem height and diameter could be positively correlated to watering regimes and negatively to fertilizers addition in the soil (Byambadorj et al., 2020).

Since soil characteristics may vary between types of soil and as results of watering and fertilization as well as the addition of amendments (Amendola et al., 2017; Montagnoli et al., 2021), the root developmental plasticity has been a major determinant for the success of land plants (Wilkinson, 2000; Hodge, 2004; Morris et al., 2017). The root system can be subdivided according to differences in diameter or tissue quality (Vogt et al., 1991; John et al., 2002; Zobel and Waisel, 2010; Montagnoli et al., 2018) of the various components, which correspond to different functions and carbon (C) input to the soil. In particular, the taproot represents the principal component of the root system (Esau, 1965) from which axis all lateral roots originate by the reiteration of a molecular and physiological branching mechanism (Chiatante et al., 2018). The taproot functions largely as tree anchorage, water transport, and storage of nutritional reserves (Noquet et al., 2001; Meuriot et al., 2003; Yang et al., 2014, 2016; Dumroese et al., 2019). Among all the lateral roots, the coarse fraction supports the development and function of the fine roots network (Di Iorio et al., 2011), transports water, and provides mechanical anchorage of the plant to its rooting environment. The finest fraction (diameter < 2 mm) of the root system represents the first plant-soil interface. It plays a crucial role in plant survival potential through associated mycorrhizae (Finér et al., 2011) that function in water and nutrient uptake and transport and exude carbohydrates that stimulate microbial activity (Coutts, 1983; Resh et al., 2003; Guo et al., 2013; Sun et al., 2017; Montagnoli et al., 2019a).

Roots regulate its architecture in response to signals in their local soil environment, such as water and nutrient availability, in addition to genetically determined developmental programmes (Chiatante et al., 2005; Montagnoli et al., 2018). Indeed, the continuous fluctuation in space and time of soil water and nutrients may induce the root system to respond through plastic morphological adaptation (Hodge, 2003; Hwang et al., 2007; Giehl et al., 2013) determining the volume of soil explored by a root system, and significantly impacting efficiency in acquiring resources (Morris et al., 2017). This might be particularly true for trees living in afforestation sites of the Mongolian steppe, which may use different strategies for their roots to adapt to variations in seasonal climates as well as different management techniques such as fertilization and watering (Ma et al., 2018).

We hypothesized that biomass partitioning of *P. sibirica* and *U. pumila* root systems would be affected by different management techniques and directly related to the enhancement of soil chemical characteristics. In particular, we would expect a higher fraction of fine roots associated with lower watering regimes and a higher fraction of coarse roots associated with higher watering regimes. To test our hypotheses, according to the watering regimes and fertilization type, soil chemical analysis was performed and root biomass was analyzed as a function of diameter classes. Our objective was to use a "shovelomics" approach, where a complete root system is excavated by hand, to understand how *P. sibirica* and *U. pumila* tree species modify their root system biomass partitioning and architecture through the complete hand-excavation of the roots system and its dissection into diameter classes.

MATERIALS AND METHODS

Site Characteristics

The experimental site is located in Lun soum (Tuv province, Mongolia; 47°52'15.43″N, 105°10′46.4″E) on the right bank of the Tuul River, 135 km west of Ulaanbaatar at an elevation of 1,130 m a.s.l. The site extends for 2 ha within the forest nursery of the South Korea-Mongolia Joint *Green Belt* Plantation project in the Middle Khalkha dry steppe region (Ulziykhutag, 1989) that has been greatly degraded by intense livestock grazing.

The annual average temperature is 0.6 ± 0.45°C, and a summer average temperature is 16.29 ± 0.41°C. The mean air temperature of the warmest month (July) is 16°C, while that of the coldest month (January) is −22°C. The length of the growing season varies between 110 and 130 days. The average annual precipitation during the experiment (2000–2019) was 196 mm, according to the Lun soum weather station (NAMEM (The National Agency for Meteorology Environmental Monitoring of Mongolia), 2019). Precipitation usually occurs between June and August and accounts for 80–90% of the total annual rainfall. The mean annual potential evapotranspiration is 752 mm.

Vegetation is typical of the genuine dry bunchgrass steppe dominated by xerophytic and meso-xerophytic graminoids [e.g., *Stipa krylovii* Roshev. , *Cleistogenes squarrosa* (Trin.), *Agropyron cristatum* (L.) Gaertn, *Artemisia frigida* (Willd.), and, in degraded lands *Artemisia adamsii* (Besser), *Carex duriuscula* C.A. Mey. , *Leymus chinensis* (Trin.)] (Ulziykhutag, 1989; Lavrenko et al., 1991). Soil type is classified as Kastanozems (Loamic) type, more than 1 m and deep, immature, lacking horizontal development. The hardness of the topsoil is 4.5 kg cm^{-2}, while that of the subsoil is 1.7 kg cm^{-2}, as the topsoil is drier than the subsoil (IUSS Working Group WRB., 2015; Batkhishig, 2016). See **Table 1** for chemical-physical details of the soil characteristics.

Plant Material and Management Techniques

In May 2011, 2-years-old seedlings of *Ulmus pumila* (grown from seeds) and *Populus sibirica* Tausch (obtained from 20 cm cuttings) were acclimated in the open Greenbelt project nursery and transplanted into holes 60 cm deep with a diameter of 50 cm. At transplanting time, *U. pumila* seedlings were 51 ± 1.14 cm in height with a diameter at root collar of 0.33 ± 0.01 cm, whereas *P. sibirica* seedlings were 68 ± 2.94 cm in height with a diameter at root collar of 0.51 ± 0.02 cm. Immediately after transplanting, a sufficient level of watering was supplied to individual trees by compensating non-leakage (CNL) button drippers.

For each tree species, twelve plots were established in total: 4 plots characterized by the four watering regimes only with 32 seedlings per watering regime; 4 plots with different watering regimes and NPK addition with 16 seedlings per watering

TABLE 1 | Profile characteristics of the experimental site Kastanozems (Loamic) soil type.

									Particle size distribution (%)		
Depth (cm)	pH	Carbon (%)	Organic matter (%)	Nitrate-Nitrogen (mg kg^{-1})	Electrical conductivity (dS m^{-1})	P_2O_5 (mg/100 g)	K_2O	Rock content > 2 mm (%)	Sand (2–0.05 mm)	Silt (0.05–0.002 mm)	Clay (< 0.002 mm)
0–10	7.77	0	0.892	6.03	0.060	1.02	10.2	0.42	68.6	21.9	9.5
10–20	7.33	0	0.806	7.89	0.024	0.81	7.8	1.21	71.8	19.0	9.2
20–30	7.20	0	0.813	7.40	0.026	1.06	6.7	0.76	71.5	19.2	9.3
30–40	7.17	0	0.880	7.82	0.025	0.68	5.5	1.55	72.1	18.7	9.2
40–50	7.26	0	0.842	6.41	0.025	1.77	5.5	1.36	72.5	17.4	10.1
50–60	7.18	0	0.801	6.69	0.028	0.77	5.5	1.02	71.9	17.6	10.5
60–70	7.23	0	0.824	8.17	0.033	1.60	5.5	1.08	71.8	18.3	9.9
70–80	7.17	0	0.734	6.69	0.035	1.48	5.5	1.19	71.8	18.4	9.8
80–90	7.30	0	0.512	7.50	0.074	1.27	5.5	1.26	76.2	13.2	10.6
90–100	8.04	0.95	0.248	7.47	0.073	1.10	5.5	0.18	77.7	12.1	10.2

Data refer to two undisturbed plots outer the forest canopy for a total depth of 1 meter at 10 cm interval.

regime; 4 plots with different watering regimes and compost addition with 16 seedlings per watering regime. Seedlings were planted following rows distance of 2.5 m, and with a north-south orientation to ensure maximum light availability during the whole day (Johnson and Brandle, 2009) (**Figure 1**).

After seedling stabilization, water emitters were placed at a distance of 10 cm from the seedling, and four different watering regimes were applied: no watering (control), $2\ L\ h^{-1} = 0.25\ mm\ m^{-2}$, $4\ L\ h^{-1} = 0.5\ mm\ m^{-2}$, $8\ L\ h^{-1} = 1\ mm\ m^{-2}$. Seedlings were watered twice a week for the entire vegetative season (from the beginning of May to the end of August). The duration of each irrigation event was 5 h, provided through dripper buttons differing by the capacity of deliverable water. The irrigation hose system was connected to a water tank with a capacity of 50 m^3. In each watering regime, two plots received a fertilizer treatment comprised of 500 g of solid granules of NPK or composted sheep manure (hereafter named compost). Fertilizers were mixed with natural soil before seedling transplantation.

Soil Sampling Strategy and Chemical Analyses

To assess the effects of management techniques on soil chemical properties, at the beginning of July 2019 three soil samples were collected for each of the two tree species (*U. pumila* and *P. sibirica*), management techniques (four watering regimes alone and associated with NPK, and compost addition) and soil depth (0–20, 20–40, and 40–60 cm hereafter reported as mean 60 cm depth) at 20 cm distance from the stem (within the edges of the original planting hole), for a total of 216 samples. Soil samples were air-dried and passed through a 2 mm sieve. Soil organic matter (SOM) was measured by the $K_2Cr\neg O_7 - H_2SO_4$ oxidation method of Walkley and Black (Nelson and Sommers, 1996). Calcium carbon concentration was determined by the volumetric method (ASTM D4373-96, 1996). The pH was determined for a 1:2.5 air-dried soil/distilled water mixture using a glass electrode pH meter (Mongolian National Standard ISO 10390:2001, Mongolian National Standard ISO 10390:2001). The electrical conductivity (EC) was determined for a 1:5 air-dried soil/distilled water mixture using a platinum electrode. Available phosphorus (P_2O_5) was measured by molybdenum blue colorimetry, after $(NH_4)_2CO_3$ digestion (Mongolian National Standard 3310:1991, Mongolian National Standard 3310:1991). Nitrate-nitrogen (NO_2-N) was determined by using CH_3COONa digestion and spectrocolorimetry. Potassium (K_2O) was analyzed by flame spectrometry methods [SSIR (Soil Survey Investigations Report No.42), 2004].

Biomass Measurement

In mid-July 2019, 10 years after seedling transplantation, 6 trees of each species and management technique were cut at the root collar and measured for height and DBH (see Byambadorj et al., 2020). For these 6 replicates for each thesis, we hand-excavated the root system to ∼0.8–1 m in depth and to a distance of 1 m from the trunk. After cutting roots that were still attached to the soil, the root systems were carefully lifted and carried to an in-site field laboratory set up at the Lun soum nursery facility.

Roots were measured by a digital caliper at the branching point, detached and segregated by diameter classes according to the classification firstly suggested by Böhm (1979) and latterly revised by Zobel and Waisel (2010). In detail, all roots with diameters below 2 mm were selected as fine roots (FR). Roots with diameter above 2 mm were divided into 4 classes as follow: small (SR; $2 < d < 5$ mm), medium (MR; $5 < d < 10$ mm), large (LR; $10 < d < 20$ mm), and very large (VLR; $d > 20$ mm). Moreover, the main root (hereafter termed taproot, TR), originating from seeds in the case of *U. pumila* and stem cutting in the case of *P. sibirica*, was also selected. To obtain biomass data, each root sample belonging to a specific diameter class was oven-dried at 105° C until constant weight and then weighed. In the case of root samples with a large volume, a 100 g fresh weight subsample was oven-dried and used to estimate the specific wood gravity to be applied to the whole sample.

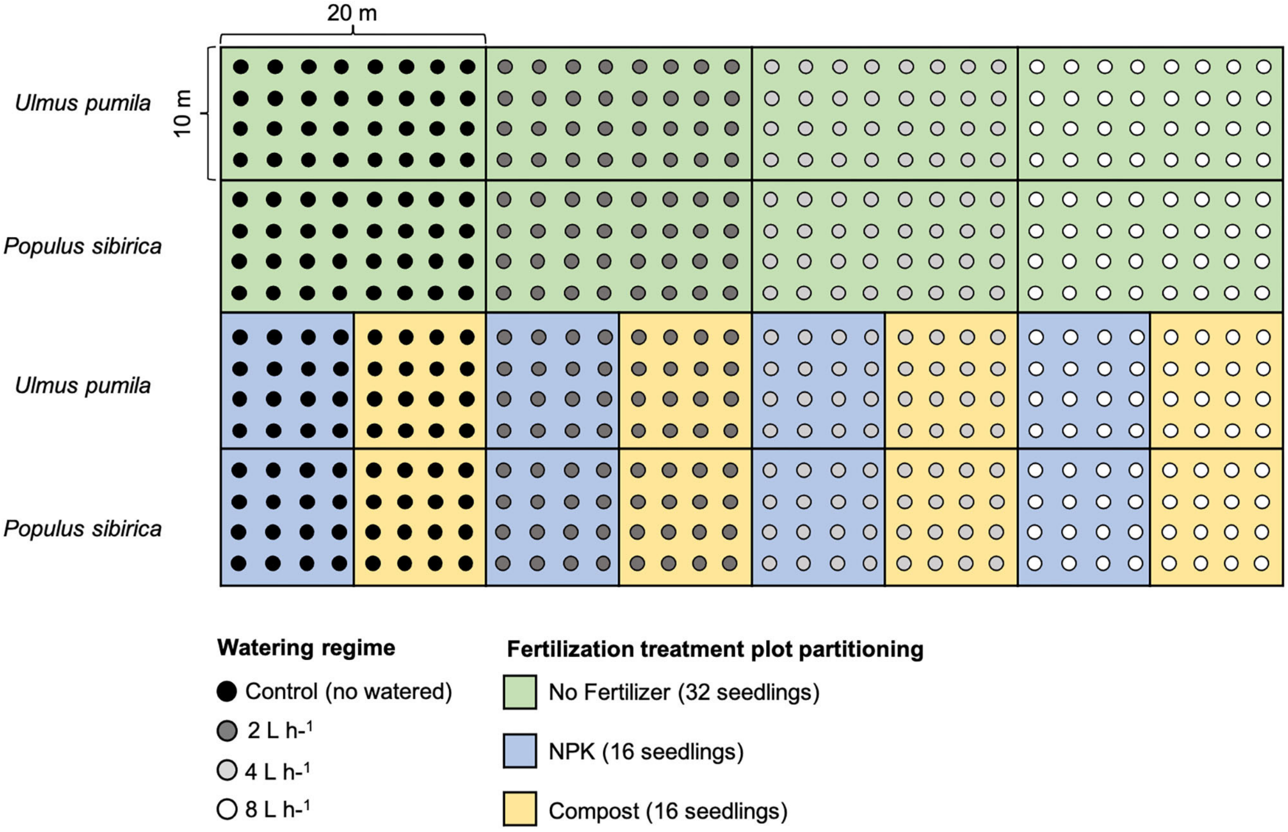

FIGURE 1 | Planting scheme of the afforestation site. Green rectangular area (20 × 10 m) indicates the watering-only treatment (no fertilizers) with 32 seedlings for each of the watering regime. Light blue square areas (10 × 10 m) indicate the NPK fertilization with 16 seedlings for each of the watering regime. The yellow square areas (10 × 10 m) indicate Compost fertilization with 16 seedlings for each of the watering regime. Areas of the upper rows refer to *Ulmus pumila* tree plantation while areas of the lower rows refer to the *Populus sibirica* tree plantation. Black dots are relative to control trees (no watered), dark gray dots indicate 2 L h^{-1} watered trees, light gray dots indicate 4 L h^{-1} watered trees, and white dots indicate 8 L h^{-1} watered trees.

Statistical Analysis

Soil statistical analysis was computed using the SAS software package, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). One-way analysis of variance (ANOVA) with Duncan's multiple range test (DMRT) was used for multiple comparisons of soil chemical properties among treatments and species. Differences were considered significant at $p < 0.05$.

For root biomass, a two-way ANOVA for each root class was carried out to test the effect of nutrient and water regime treatments. When needed, the dependent variables were square-root or log-transformed to ensure normal distributions and equal variances. Omega squared values (ω^2) for each predictor were calculated in order to show the variation in the dependent variable attributable to the independent variable (predictors). *Post-hoc* LSD-tests were conducted to detect overall differences between watering regimes and type of nutrient treatments. For the ratio between each root class and total root biomass, a categorical principal component analysis (CATPCA) was performed to represent the variability of our dataset to a nominal (nutrient treatment) and a numerical (water regime) independent variable. Moreover, to quantify how the root system growth was influenced by both independent variables, a linear regression model was calculated by relating total root biomass with water regime for each nutrient treatment and species. Differences were considered significant at $p < 0.05$. Statistical analysis was carried out with SPSS 17.0 (SPSS Inc., Chicago IL, USA).

RESULTS

Soil Chemical Properties

Soil pH under the *Ulmus pumila* was not influenced by the different watering regimes (**Table 2**). Values of pH for the soil amended with NPK showed a significant decrease of 12.5% only in non-watered soil (control). The same pattern was found in the Compost addition (**Table 2**). A slight but insignificant decrease was found in non-watered soil while no differences were detected when different watering regimes were added (**Table 2**). Soil organic matter (%) did not significantly change in watering-only soil when different watering regimes were considered. No watered soil treated with NPK addition showed a slight significant increase of soil organic matter (**Table 2**).

When watering regimes were added to the NPK, organic matter values decreased to levels measured for watering-only soil. The highest values of organic matter were measured in Compost treated soil with no watering (control) (**Table 2**). When watering regimes were added to the Compost treated soil, organic matter significantly decreased to the same values measured for watering-only soil. Nitrate-Nitrogen concentration in the soil was not influenced by the watering-only regimes. When both NPK and Compost were added, soil Nitrate-Nitrogen concentration significantly increased with respect to the watering-only soil (around 177% for both treatments; **Table 2**). Also, in this case the addition of watering regimes did not significantly change the Nitrate-Nitrogen soil concentration. Both phosphorus (P_2O_5) and potassium (K_2O) did not significantly change if watering regimes and fertilizers were considered (**Table 2**).

Soil pH under *P. sibirica* watering-only treatment progressively decreased with increasing level of watering regimes (**Table 2**). When NPK was added soil pH for both control and 2L h^{-1} was similar to the watering-only soil and was significantly higher when 4 and 8 L h^{-1} watering regimes were applied (6.7 and 6.2%, respectively; **Table 2**). When Compost was added to the soil, pH values were significantly higher when 8 L h^{-1} watering regime was applied (106% in respect to the control value). Soil organic matter for watering-only soils did not differ among the different watering regimes (**Table 2**). Similarly, in the case of soil treated with NPK, organic matter did not differ from the watering-only soils and did not show significant variation among different watering regimes with the only exception of the of 4 L h^{-1} watering regime. In the case of soil treated with Compost, organic matter was significantly higher in the 4 and 8 L h^{-1} among watering regimes (around 25% for both treatments; **Table 2**). Nitrate-Nitrogen did not significantly change with watering regimes in the watering-only soil with the exception of the of 4 L h^{-1} watering regime. When both NPK and Compost treated soil were analyzed, Nitrate-Nitrogen concentration was higher than watering-only soils (42.6 and 61.6%, respectively; **Table 2**). Higher values among watering regimes were found in NPK treated-soils when 4 and 8 L h^{-1} of water were added. When Compost was added to the soil, no differences were found among watering regimes. Both phosphorus (P_2O_5) and potassium (K_2O) did not significantly change if watering regimes and fertilizers were considered (**Table 2**).

Plant Survival Rate

Six months after transplanting in the field (September 2011), the survival rate of seedlings was 100% for both *U. pumila* and *P. sibirica*. Overall survival rate of all treatments 8 years later (2019) was 92% for *U. pumila* and 82% for *P. sibirica*. In particular, the number of surviving *P. sibirica* trees was reduced to 41, 13, and 37%, respectively for control (no watering), control plus NPK, and control plus compost management techniques. In the case of *U. pumila* trees survival rate was reduced to 62% only for control plus NPK management technique.

Root Biomass Partitioning

Fine Roots (FR)

In *P. sibirica* trees the different watering regimes significantly influenced the FR biomass ($p < 0.001$) accounting for 28.3% of the data variation (**Table 3**). The FR biomass measured in trees supported by different watering regimes (2, 4, and 8 L h^{-1}) was significantly higher than in control trees (5, 4, and almost 9-fold, respectively; **Figure 2A**). FR biomass did not differ between the three watering regimes despite that trees grown with the 8 L h^{-1} watering regime presented the highest values. On the contrary, ANOVA analysis did not reveal a significant influence of the fertilization treatment ($p = 0.733$) (**Table 3**). *P. sibirica* trees treated with the addition of compost showed the same pattern with the highest and lowest values in the case of 8 L h^{-1} and no watering regimes, respectively. No differences in FR were detected among control and 2 and 4 L h^{-1} treated trees. When soil was fertilized with NPK, the highest values of FR biomass were found with trees treated to 8 L h^{-1} watering regime, despite that the differences with values found in trees treated with 4 and 2 L h^{-1} watering regimes were not significant. Data on control trees are missing due to mortality of all trees. Although not significantly different, slightly higher values of FR were detected in 8 L h^{-1} trees subject to watering only in comparison with trees subject to watering and NPK addition (**Figure 2A**).

In *U. pumila* trees the different watering regimes significantly influenced the FR biomass ($p = 0.001$) accounting for 15.8% of the data variation (**Table 3**). The FR biomass measured in trees supported with different watering regimes only, were the highest and the lowest in 8 L h^{-1} and control trees, respectively, while intermediate values were measured for trees subject to the addition of 2 and 4 L h^{-1}, which were similar each other (**Figure 2B**). Fertilization treatment significantly influenced the FR biomass ($p = 0.041$) accounting for 5% of the data variation (**Table 3**). When the soil was fertilized with compost, FR values were similar across all watering regimes and of lowest magnitude in comparison with trees subject to watering only. When NPK was added to the soil the lowest value of FR was measured across all watering regimes and management treatments considered. FR values measured for trees with 4, and 8 L h^{-1} were similar each other and compared to the other management techniques while FR values for the 2 L h^{-1} where the highest (**Figure 2B**).

Small Roots (SR)

In *P. sibirica* trees the different watering regimes significantly influenced the SR biomass ($p < 0.001$) accounting for 25.9% of the data variation (**Table 3**). The biomass of SR measured in trees supported by the 8 L h^{-1} watering regime was the highest measured across all regimes (**Figure 2C**). Trees supported by the 4 L h^{-1} regime had intermediate values of SR. The SR biomass for trees supported by the addition of 2 L h^{-1} watering regime did not differ from the control trees, which showed the lowest biomass values (**Figure 2C**). Fertilization treatment significantly influenced the SR biomass ($p = 0.010$) accounting for 8.7% of the data variation (**Table 3**). Trees supported by the addition of compost showed biomass values 2.2-fold significantly higher than unwatered trees only in the case of 8 L h^{-1}. Moreover, SR biomass of the trees treated with 4 and 8 L h^{-1} plus compost

TABLE 2 | Soil chemical properties for different management techniques and watering regimes for *Ulmus pumila* and *Populus sibirica*.

Plant species	Management technique	Watering regimes	pH	Organic matter (%)	Nitrate-Nitrogen (mg kg^{-1})	P_2O_5 (mg/100 g)	K_2O
U. pumila	Watering only	Control	7.83 ± 0.10^{a}	0.82 ± 0.05^{def}	3.1 ± 0.4^{d}	0.84 ± 0.12^{b}	4.7 ± 1.6^{b}
		2 L h^{-1}	7.68 ± 0.05^{a}	0.94 ± 0.03^{bcde}	3.5 ± 0.3^{d}	0.60 ± 0.84^{b}	5.5 ± 1.8^{ab}
		4 L h^{-1}	7.55 ± 0.07^{a}	0.90 ± 0.02^{cde}	2.0 ± 0.4^{d}	2.80 ± 1.19^{a}	6.7 ± 1.8^{ab}
		8 L h^{-1}	7.43 ± 0.02^{a}	0.96 ± 0.06^{bcd}	2.7 ± 0.4^{d}	0.78 ± 0.43^{b}	6.3 ± 2.1^{ab}
	Watering and NPK	Control	6.85 ± 0.03^{b}	1.12 ± 0.12^{b}	7.8 ± 0.4^{abc}	0.78 ± 0.18^{b}	9.0 ± 1.2^{a}
		2 L h^{-1}	7.87 ± 0.19^{a}	0.75 ± 0.09^{f}	7.1 ± 0.4^{bc}	0.63 ± 0.41^{b}	8.2 ± 2.7^{ab}
		4 L h^{-1}	7.84 ± 0.14^{a}	0.90 ± 0.03^{cde}	6.8 ± 0.7^{bc}	1.00 ± 0.14^{ab}	6.6 ± 0.7^{ab}
		8 L h^{-1}	7.76 ± 0.14^{a}	0.89 ± 0.06^{cdef}	7.1 ± 0.2^{bc}	0.65 ± 0.30^{b}	5.5 ± 1.2^{ab}
	Watering and compost	Control	7.35 ± 0.42^{ab}	1.50 ± 0.10^{a}	7.7 ± 0.4^{abc}	1.31 ± 0.65^{ab}	9.0 ± 1.2^{a}
		2 L h^{-1}	7.78 ± 0.12^{a}	1.04 ± 0.03^{bc}	8.1 ± 0.3^{ab}	1.24 ± 0.41^{ab}	7.1 ± 1.0^{ab}
		4 L h^{-1}	7.85 ± 0.29^{a}	0.78 ± 0.04^{ef}	6.2 ± 0.7^{c}	1.08 ± 0.35^{ab}	6.3 ± 1.4^{ab}
		8 L h^{-1}	7.88 ± 0.13^{a}	0.82 ± 0.04^{def}	9.4 ± 1.8^{a}	0.63 ± 0.20^{b}	5.5 ± 1.2^{ab}
P. sibirica	Watering only	Control	7.75 ± 0.02^{a}	0.90 ± 0.02^{ab}	5.2 ± 0.6^{e}	0.43 ± 0.17^{c}	7.5 ± 1.9^{a}
		2 L h^{-1}	7.30 ± 0.13^{bc}	1.00 ± 0.11^{a}	5.5 ± 0.1^{e}	0.77 ± 0.09^{abc}	6.7 ± 1.2^{a}
		4 L h^{-1}	7.15 ± 0.04^{cd}	0.84 ± 0.08^{abc}	9.6 ± 0.1^{bcd}	0.82 ± 0.04^{abc}	7.8 ± 2.3^{a}
		8 L h^{-1}	6.79 ± 0.06^{d}	0.89 ± 0.00^{ab}	6.0 ± 0.3^{e}	0.85 ± 0.27^{abc}	5.9 ± 1.0^{a}
	Watering NPK	Control	7.65 ± 0.25^{ab}	0.77 ± 0.07^{bc}	7.9 ± 0.4^{d}	0.58 ± 0.21^{bc}	7.5 ± 1.9^{a}
		2 L h^{-1}	7.47 ± 0.10^{abc}	0.88 ± 0.08^{ab}	7.9 ± 0.5^{d}	0.40 ± 0.11^{c}	6.3 ± 0.8^{a}
		4 L h^{-1}	7.63 ± 0.18^{ab}	0.69 ± 0.02^{c}	10.3 ± 0.4^{abc}	0.50 ± 0.09^{c}	7.5 ± 2.6^{a}
		8 L h^{-1}	7.21 ± 0.16^{c}	0.86 ± 0.07^{ab}	11.4 ± 0.1^{a}	0.60 ± 0.07^{abc}	9.8 ± 2.6^{a}
	Watering compost	Control	7.29 ± 0.18^{bc}	0.77 ± 0.08^{bc}	10.4 ± 1.3^{abc}	0.70 ± 0.12^{abc}	6.3 ± 0.8^{a}
		2 L h^{-1}	7.45 ± 0.02^{abc}	0.77 ± 0.01^{bc}	9.6 ± 0.8^{cd}	1.09 ± 0.22^{ab}	7.1 ± 1.6^{a}
		4 L h^{-1}	7.32 ± 0.01^{bc}	0.95 ± 0.04^{a}	11.2 ± 0.4^{abc}	0.47 ± 0.16^{c}	7.1 ± 1.6^{a}
		8 L h^{-1}	7.75 ± 0.19^{a}	0.98 ± 0.04^{a}	11.3 ± 0.9^{abc}	1.14 ± 0.33^{a}	9.0 ± 3.5^{a}

Values represent the mean of 3 soil depth (0–20, 20–40, and 40–60 cm) and 3 samples ($n = 3$) ± SE. Letters indicate significant differences among factors for each species.

was significantly lower (−36.3 and −50.9%, respectively) than that of trees treated alone with the same watering regime; the same pattern was observed for trees supported by the addition of NPK (−28.3 and −59.2%, respectively). For this treatment condition, unwatered trees had a 100% mortality and thus were not detectable, while trees watered at the three levels showed the same values of lower magnitude when compared with watered trees alone (**Figure 2C**).

In *U. pumila* trees the different watering regimes significantly influenced the SR biomass ($p = 0.002$) accounting for 14.2% of the data variation (**Table 3**). Values of SR biomass measured in trees under different watering regimes were the highest and the lowest in 8 L h^{-1} and control trees, respectively, while trees subject to the addition of 2 and 4 L h^{-1} had intermediate values (**Figure 2D**). Fertilization treatment significantly influenced the SR biomass ($p = 0.001$) accounting for 12.7% of the data variation (**Table 3**). When compost was applied, SR biomass was not significantly different from irrigation alone (**Figure 2D**). SR biomass in the compost plus watering of 8 and 2 L h^{-1} treatments was significantly lower than values measured for trees with the same watering regimes only, while no differences were detected for control and 4 L h^{-1}. When NPK was applied, SR biomass was higher in trees watered at 2 and 4 L h^{-1} in comparison with unwatered trees (**Figure 2D**) while the 8 L h^{-1} trees did not differ from unwatered trees. Finally, values of SR biomass in trees treated with NPK were similar to those measured for compost trees across all watering regimes, and trees supported with 8 L h^{-1} had less SR biomass than trees with watering regimes only (**Figure 2D**).

Medium Roots (MR)

In *P. sibirica* trees the different watering regimes significantly influenced the MR biomass ($p = 0.027$) accounting for 9.6% of the data variation (**Table 3**). MR biomass was similar across the three levels of watering (2, 4, and 8 L h^{-1}), and a significant difference was detected only between no watered plants (control) and the 8 L h^{-1} watering regime, which was 2.5-fold higher (**Figure 3A**). ANOVA analysis did not reveal a significant influence of the fertilization treatment ($p = 0.561$) on the MR biomass (**Table 3**). Trees fertilized with compost had similar MR biomass values across all watering regimes. Furthermore, these values did not differ from MR biomass of irrigated trees. Similarly, trees fertilized with NPK showed the highest and the lowest values of MR biomass at 2 and 8 L h^{-1} respectively, with intermediate values for the 4 L h^{-1} trees (**Figure 3A**). Also, MR biomass of trees fertilized and watered did not differ from for trees irrigated the same watering regime alone. In the case of *U. pumila* trees ANOVA analysis did not reveal a

TABLE 3 | Two-way ANOVA results (predictors: WAT, water regime; FER, fertilization treatments) for biomass of each root diameter class.

Plant species	Root class (biomass)	Predictor	*F*	*p*	ω^2
P. sibirica	Fine root	WAT	10.26	**< 0.001**	0.283
		FER	0.31	0.733	0
	Small root	WAT	8.80	**< 0.001**	0.259
		FER	4.95	**0.010**	0.087
	Medium root	WAT	3.32	**0.027**	0.096
		FER	0.58	0.561	0
	Large root	WAT	0.46	0.715	0
		FER	0.67	0.515	0
	Very large	WAT	7.23	**0.001**	0.264
		FER	0.78	0.467	0
	Tap root	WAT	14.97	**< 0.001**	0.398
		FER	2.54	0.089	0.029
	Total root	WAT	14.75	**< 0.001**	0.395
		FER	0.65	0.525	0
U. pumila	Fine root	WAT	6.00	**0.001**	0.158
		FER	3.39	**0.041**	0.050
	Small root	WAT	5.81	**0.002**	0.142
		FER	7.48	**0.001**	0.127
	Medium root	WAT	0.06	0.980	0
		FER	1.58	0.214	0.017
	Large root	WAT	6.67	**0.001**	0.165
		FER	6.36	**0.003**	0.104
	Very large	WAT	2.57	0.062	0.058
		FER	0.70	0.502	0
	Tap root	WAT	6.26	**0.001**	0.171
		FER	1.30	0.281	0.006
	Total root	WAT	2.99	**0.001**	0.155
		FER	1.90	0.058	0.041

F-value (F), p-value (p), and omega square value are shown. Bold values indicate statistical significance ($p < 0.05$).

significant influence of either watering ($p = 0.980$) or fertilization ($p = 0.214$) treatments (**Table 3**). Indeed, trees did not show any difference in MR biomass among all watering regimes and fertilizers used (**Figure 3B**).

Large Roots (LR)

In *P. sibirica* trees the ANOVA analysis did not reveal a significant influence on the LR biomass of both watering ($p = 0.715$) and fertilization ($p = 0.515$) treatments (**Table 3**). LR biomass in *P. sibirica* trees differed only for the 4 L h^{-1} trees (**Figure 3C**). When trees were additionally treated with compost, the highest and the lowest values of LR biomass were measured, respectively for the unwatered, 2 and 4 L h^{-1} trees. The trees watered at 8 L h^{-1} had intermediate values (**Figure 3C**). Trees fertilized with NPK did not show any difference across the three levels of watering regimes, and these values did not differ from trees irrigated alone (**Figure 3C**).

In *U. pumila* trees the different watering regimes significantly influenced the LR biomass ($p = 0.001$) accounting for 16.5% of the data variation (**Table 3**). LR biomass of *U. pumila* trees supported by 8 L h^{-1} was 2.5-fold significantly higher than biomass of unwatered trees (**Figure 3D**). LR biomass for 2 L h^{-1} trees was intermediate, being similar to the 8 and 4 L h^{-1} and to the no watered trees. Fertilization treatment significantly influenced the LR biomass ($p = 0.003$) accounting for 10.4% of the data variation (**Table 3**). When compost was added, no differences were detected across all irrigation treatments. LR biomass for trees treated with compost plus irrigation was similar to trees with irrigation alone; the only exception was the 8 L h^{-1} trees, which were 47.8% significantly lower (**Figure 3D**). When NPK was added, trees supported by 2 L h^{-1} had the highest LR biomass, which was similar to the trees supported by the 8 L h^{-1} watering-only regime.

Very Large Roots (VLR)

In *P. sibirica* trees the different watering regimes significantly influenced the VLR biomass ($p = 0.001$) accounting for 26.4% of the data variation (**Table 3**). Biomass of VLR of trees supported by the 2 and 8 L h^{-1} watering regime was significantly higher, 3.6 and 9.3-fold, respectively, than unwatered trees, which showed the lowest values (**Figure 4A**). ANOVA analysis did not reveal a significant influence of the fertilization treatment ($p = 0.467$) (**Table 3**). Compost additions had no effect on VLR biomass

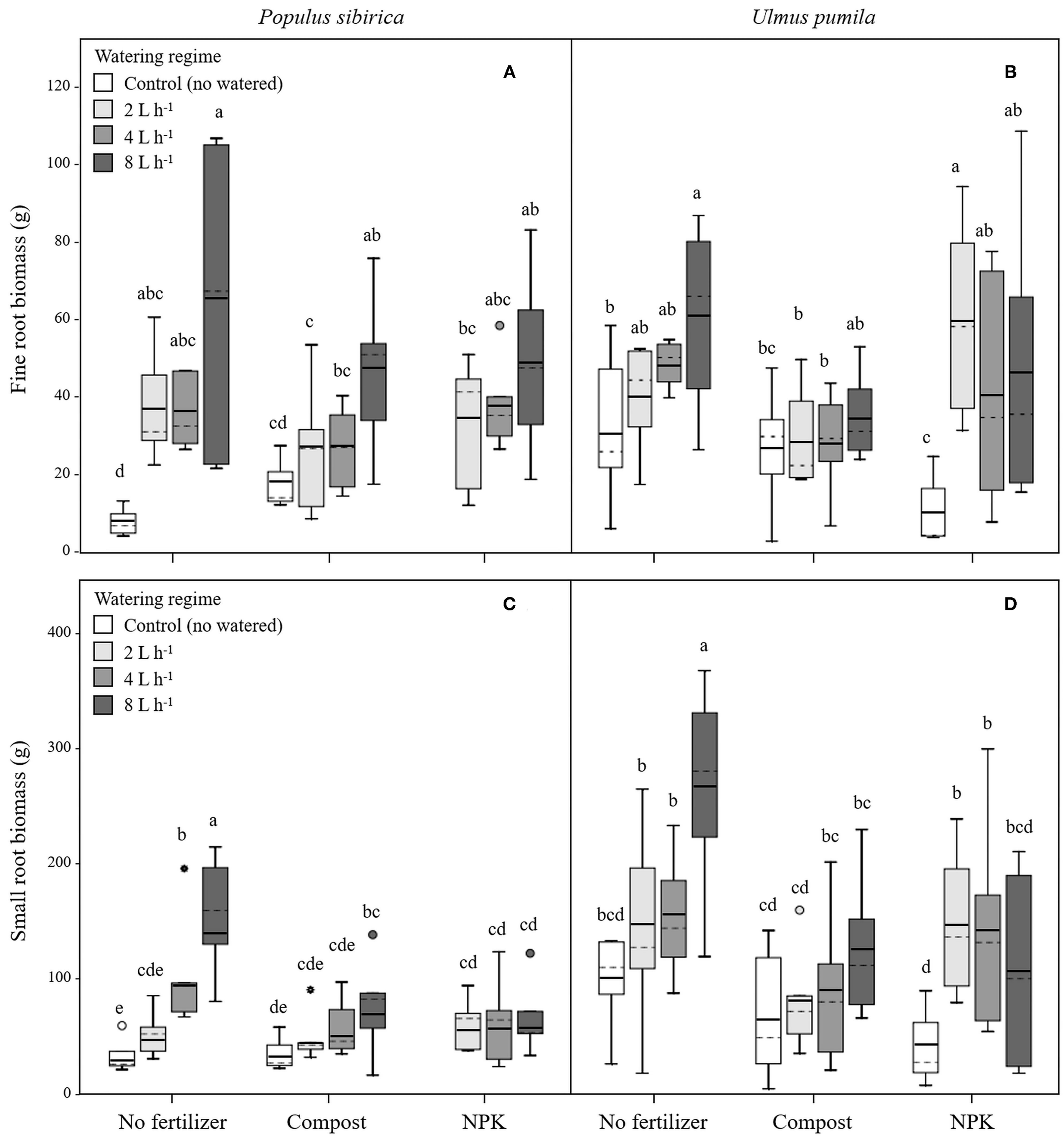

FIGURE 2 | Fine and small root biomass for *P. sibirica* **(A,C)** and *U. pumila* **(B,D)** for different watering regimes and by different fertilization treatments. Different letters indicate significant differences ($p < 0.05$) among watering regimes within each fertilization treatment. Vertical boxes represent ~50% of the observations and lines extending from each box are the upper and lower 25% of the distribution. Within each box, the solid horizontal line is the mean value and the dotted line is the median.

across the three levels of watering. Both 2 and 8 L h^{-1} plus compost were the same as the highest value of biomass measured for the trees watered at 8 L h^{-1} alone (**Figure 4A**). Addition of NPK showed the highest and the lowest values of VLR biomass for 8 and 2 L h^{-1}, respectively; 4 L h^{-1} had intermediate values (**Figure 4A**).

In the case of *U. pumila* trees ANOVA analysis did not reveal a significant influence of either watering ($p = 0.062$)

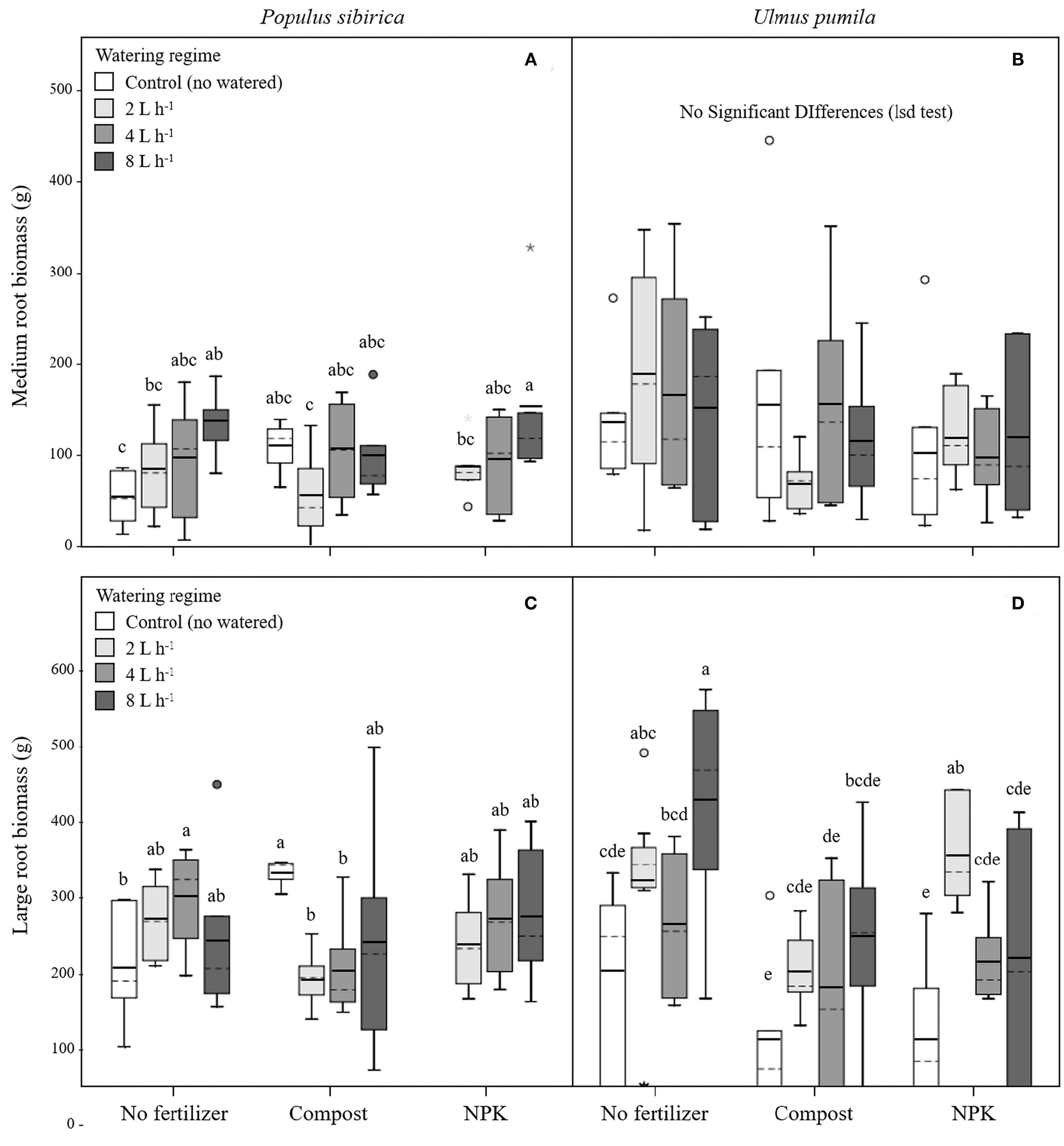

FIGURE 3 | Medium and large root biomass for *P. sibirica* **(A,C)** and *U. pumila* **(B,D)** for different watering regimes and by different fertilization treatments. Different letters indicate significant differences ($p < 0.05$) among watering regimes within each fertilization treatment; absence of letters reflects that no significant difference was detected. Vertical boxes represent ~50% of the observations and lines extending from each box are the upper and lower 25% of the distribution. Within each box, the solid horizontal line is the mean value and the dotted line is the median.

or fertilization ($p = 0.502$) treatments (**Table 3**). VLR biomass values were significantly higher in trees treated by 8 L h^{-1} than unwatered and 2 L h^{-1} trees; trees in the 4 L h^{-1} treatment had intermediate values (**Figure 4B**). When compost was added VLR biomass values did not differ from those measured for trees supported by watering alone. The NPK treatment produced the highest and the lowest VLR biomass values for the 2 L h^{-1} and unwatered trees. Trees treated with 4 and

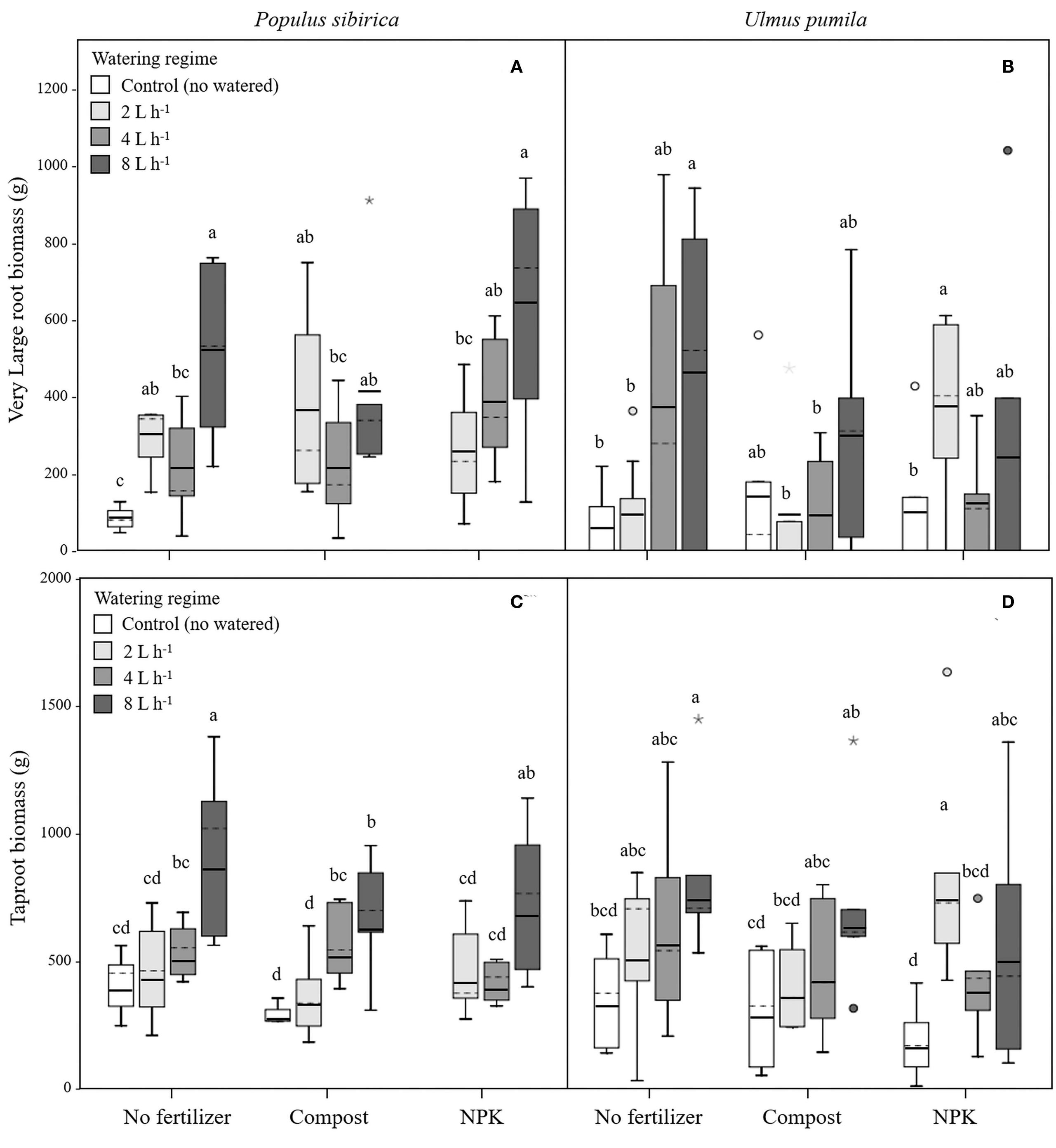

FIGURE 4 | Very large and tap- root biomass for *P. sibirica* **(A,C)** and *U. pumila* **(B,D)** for different watering regimes and by different fertilization treatments. Different letters indicate significant differences ($p < 0.05$) among watering regimes within each fertilization treatment. Vertical boxes represent ~50% of the observations and lines extending from each box are the upper and lower 25% of the distribution. Within each box, the solid horizontal line is the mean value and the dotted line is the median.

8 L h^{-1} had intermediate values. VLR values were similar to those measured for trees supported with watering regime only (**Figure 4B**).

Tap Root (TR)

In *P. sibirica* trees the different watering regimes significantly influenced the TR biomass ($p < 0.001$) accounting for 39.8% of the data variation (**Table 3**). TR biomass measured in *P. sibirica* trees treated with 8 L h^{-1} was significantly higher (1.7 and 2.2-fold, compared to 4 and 2 L h^{-1}) than in trees in the other watering levels including the unwatered (**Figure 4C**). ANOVA analysis did not reveal a significant influence of the fertilization treatment ($p = 0.089$) on the TR biomass (**Table 3**). Compost addition had the highest values of TR biomass in both the 4 and 8 L h^{-1} treatments and the lowest for 2 L h^{-1} and unwatered trees (**Figure 4C**). TR biomass of 8 L h^{-1} trees with compost addition was 27.4% significantly lower than trees at the same level of watering alone. Similarly, *P. sibirica* trees treated with added NPK had the highest TR biomass values for trees supported with 8 L h^{-1} and the lowest for the 2 and 4 L h^{-1} treatments (**Figure 4C**). The highest TR values detected for 8 L h^{-1} trees were similar to the values measured for trees supported with same watering level only.

In *U. pumila* trees the different watering regimes significantly influenced the TR biomass ($p = 0.001$) accounting for 17.1% of the data variation (**Table 3**). *U. pumila* trees had the highest values of TR biomass when trees were watered with 8 L h^{-1} (**Figure 4D**). TR biomass gradually declined with lower levels of watering, with the lowest values for unwatered trees. Fertilization treatment did not influence the TR biomass ($p = 0.281$) (**Table 3**). A similar pattern was observed when compost was added although TR biomass for the three watering levels did not differ from each other (**Figure 4D**). TR biomass did not differ across the watering regimes with NPK addition with the only exception the trees supported with 2 L h^{-1}, which had the highest mean biomass value.

Total Root Biomass (TRB)

In *P. sibirica* trees the different watering regimes significantly influenced the TRB ($p < 0.001$) accounting for 39.5% of the data variation (**Table 3**). *P. sibirica* had the highest TRB values for trees supported with 8 L h^{-1} and the lowest in the unwatered trees (**Figure 5A**). Trees in the 2 and 4 L h^{-1} watering regimes showed intermediate values. Similar patterns were observed when compost and NPK was added, although 8 and 4 L h^{-1} did not differ from each other. Indeed, ANOVA analysis did not reveal a significant influence of the fertilization treatment ($p = 0.525$) (**Table 3**).

TRB biomass of *U. pumila* trees followed the same trend as was observed for *P. sibirica* with the highest and the lowest values measured for trees at the highest watering level and the unwatered trees (**Figure 5B**). For this tree species watering treatment significantly influenced the TRB ($p = 0.001$) while fertilization treatment had no influence on the data variation ($p = 0.058$).

Categorical Principal Component Analysis (CATPCA) of Biomass Data

The first two principal components (PC1 and PC2) accounted for 68% (*P. sibirica:* PC1 54.2%, PC2 13.8%) and 74.7% (*U. pumila:* PC1 57.3%, PC2 17.4%) of the data variance. The loading plots of each root diameter class (**Figure 6**) was distributed along the PC1 axis with positive correlation to the water regime (higher the irrigation, higher the root mass) for both tree species, although *U. pumila* (**Figure 6B**) was influenced with lower magnitude than *P. sibirica* (**Figure 6A**). The only exception was represented by LR for *P. sibirica* and MR for *U. pumila* that were better explained by the PC2 axis showing no correlation with water regime. Moreover, the analysis of PC1 coordinate showed that fertilization treatments, being a categorical variable, had a significant influence on root biomass, although of less intensity than water regime (**Figure 6**). Root biomass of *U. pumila* was influenced by the fertilizers with higher magnitude than *P. sibirica*.

Regression Biomass Growth Model

Regression growth models were obtained from the relationship of the total root system biomass (TRB) with different watering regimes for each of the fertilizer types used and the two tree species analyzed (**Figure 7**). In the case of *P. sibirica*, TRB significantly increased ($p < 0.001$; $F = 35.322$) with increasing level of watering but the addition of Compost and NPK reduced the magnitude of this increase (**Figure 7A**). The model developed for *U. pumila* data showed a significant increase ($p < 0.001$; $F = 15.109$) of TRB with the increasing of the level of watering (**Figure 7B**). When fertilizers were added the magnitude of this increase was lowered in comparison with unfertilized trees (**Figure 7B**). In particular, TRB for trees fertilized with NPK did not show a significant relationship ($p = 0.47$) with the increasing level of watering (**Figure 7B**).

Root Class to Total Root Biomass Ratio

The highest and the lowest percentage of *P. sibirica* biomass was found for the TR and the FR, which represented, respectively, almost 50 and 3% of the total root biomass (**Table 4**). Taproot biomass showed the highest increase without both water and fertilizers (i.e., watering only treatment and control watering regime trees), which was almost 25% higher among all the other treatments (**Table 4**). When NPK was added, TR/TRB did not change with watering regime (**Table 4**). For Compost addition, TR/TRB was the highest only with the 4 L h^{-1} watering regime. FR/TRB was the lowest for controls for watering only trees, and did not change across watering regimes and fertilizers (**Table 4**). The same pattern was observed for SR and MR to TRB ratio with unremarkable changes across different watering regimes and fertilization types (**Table 3**). LR/TRB for watered only trees did not differ between control, 2, and 4 L h^{-1}; although it was 55.9% significantly lower for trees watered with 8 L h^{-1} (**Table 4**). LR/TRB did not differ among watering regimes in the case of NPK addition, while it was the highest in the control compared to watered trees when Compost was added (**Table 4**). VLR/TRB for watered only trees was the lowest for controls while no differences were detected between watering regimes (**Table 4**). When NPK was added, the highest VLR/TRB value was found for 4 L h^{-1} watering regime, although not significantly different from the 8 L h^{-1} treatment. The same pattern was found when Compost was added with the highest VLR/TRB value for trees watered with 2 and 8 L h^{-1} (**Table 4**).

In *U. pumila* the highest and the lowest percentage of biomass was found for the TR and the FR, which represented, respectively, almost 40 and 3% of the total root biomass (**Table 4**). TR, FR, and

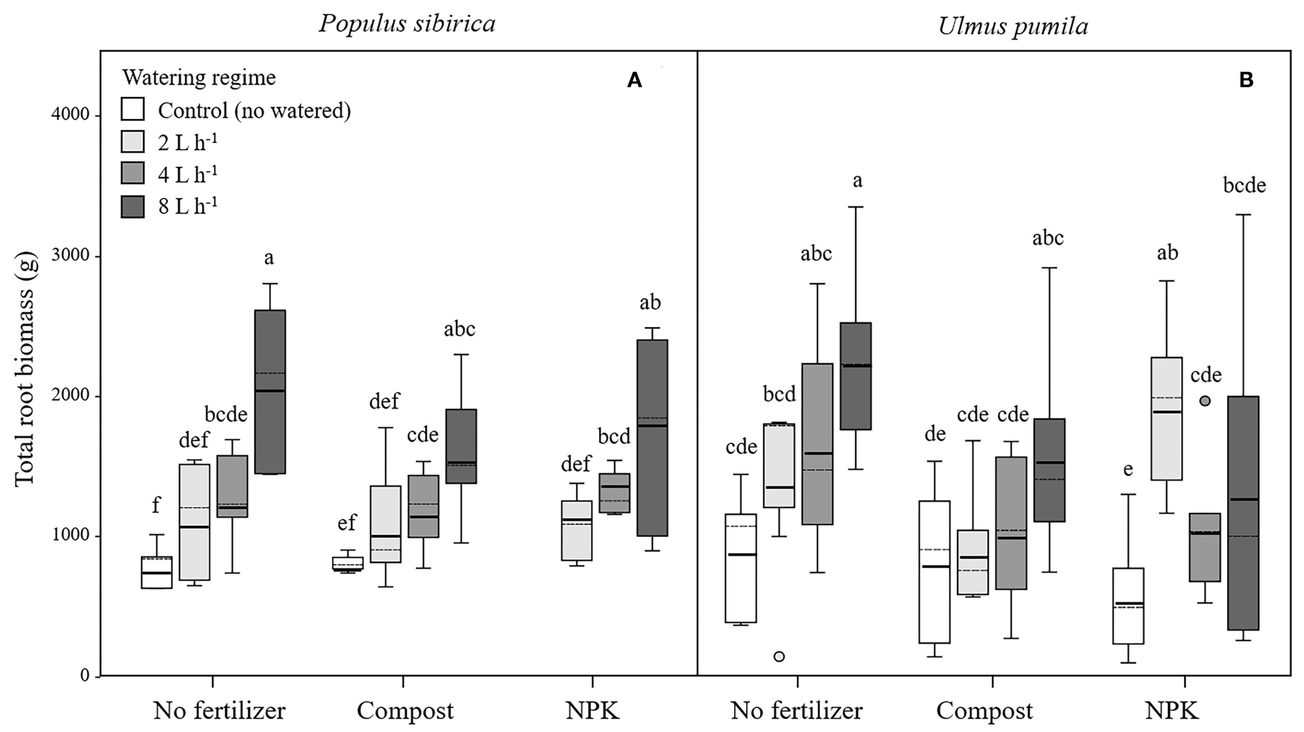

FIGURE 5 | Total root biomass for *P. sibirica* **(A)** and *U. pumila* **(B)** for different watering regimes and by different fertilization treatments. Different letters indicate significant differences ($p < 0.05$) among watering regimes within each fertilization treatment. Vertical boxes represent ~50% of the observations and lines extending from each box are the upper and lower 25% of the distribution. Within each box, the solid horizontal line is the mean value and the dotted line is the median.

SR to TRB ratio did not show any significant differences between watering regimes and fertilization types (**Table 4**). MR/TRB did not differ among watering regimes when watering only trees were analyzed. In the case of NPK addition the highest and the lowest MR/TRB value was found, respectively, for control and 8 L h^{-1} watering regimes. Trees treated with Compost did not show significant variation of MR/TRB between watering regimes (**Table 4**). Finally, LR and VLR to TRB ratio did not show significant differences among fertilization types and watering regimes (**Table 4**).

Categorical Principal Component Analysis (CATPCA) of Root Class to Total Root Biomass Ratio

The first two principal components (PC1 and PC2), accounted for 53.2% (*P. sibirica*; PC1 29.9%, PC2 23.3%) and 49.9% (*U. pumila*; PC1 30.0%, PC2 19.9%) of the data variance.

In the case of *P. sibirica* both fertilization and watering regimes influenced the ratio of root class with total root biomass. In particular, VLR/TRB increased with the increase of watering level (PC-1 axis) and FR/TRB increased with fertilization (PC-2 axis), although the relationship is weaker (**Figure 8**; **Table 4**). Moreover, TR/TRB showed an inverse relationship with fertilization while LR/TRB had an inverse relationship with watering regime.

The ratio of biomass of each root class and the total root biomass in *U. pumila* was scarcely influenced by the watering regimes (**Figure 8**). On the contrary, fertilization affected the biomass ratio (PC-2 axis) and in particular TR/TRB showed a strong increasing relationship with the addition of fertilizers, while SR and LR decreased with fertilization, although less significantly (**Figure 8**; **Table 4**). FR, MR, and VLR to TRB ratio were slightly lying on PC-1 axis and they were independent of the treatments.

DISCUSSION

In the present study, *P. sibirica* and *U. pumila* species were differentially influenced by both watering regime and fertilization. *P. sibirica* root biomass was more influenced by the watering than *U. pumila*, which on the contrary was influenced to a greater extent by fertilization. These findings are in line with previous work dealing with aboveground morpho-physiological traits (Cho et al., 2019), which highlighted that *P. sibirica* in water deficit conditions has a higher water requirement than *U. pumila*.

The explanation for this difference may be related to the biological characteristics of the two species. Indeed, although *Populus* species are fast-growing requiring a relatively higher water supply (Rhodenbaugh and Pallardy, 1993; Isebrands and Richardson, 2014; González et al., 2016), *P. sibirica* is a drought-resistant species adapted to drier and warmer conditions (Tsogtbaatar, 2004; Pearce et al., 2006). On the other hand, *U. pumila* was found to adopt morpho-physiological plasticity

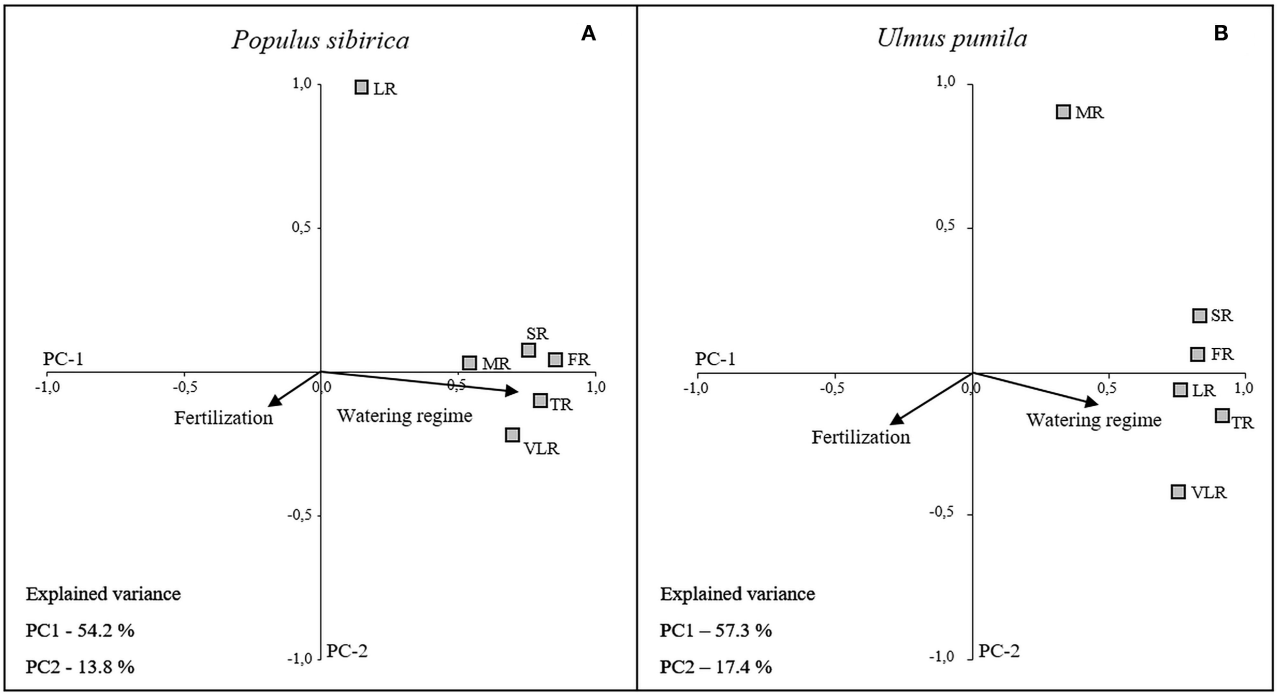

FIGURE 6 | Categorical Principal Component Analysis (CATPCA) biplot of *P. sibirica* **(A)** and *U. pumila* **(B)** indicating the different root classes biomass projection in relation with watering regime and fertilization. Tightly clustered variables are positively related, while variables at different ends of the figure are negatively related. Arrows represents the mean influence magnitude for each factor.

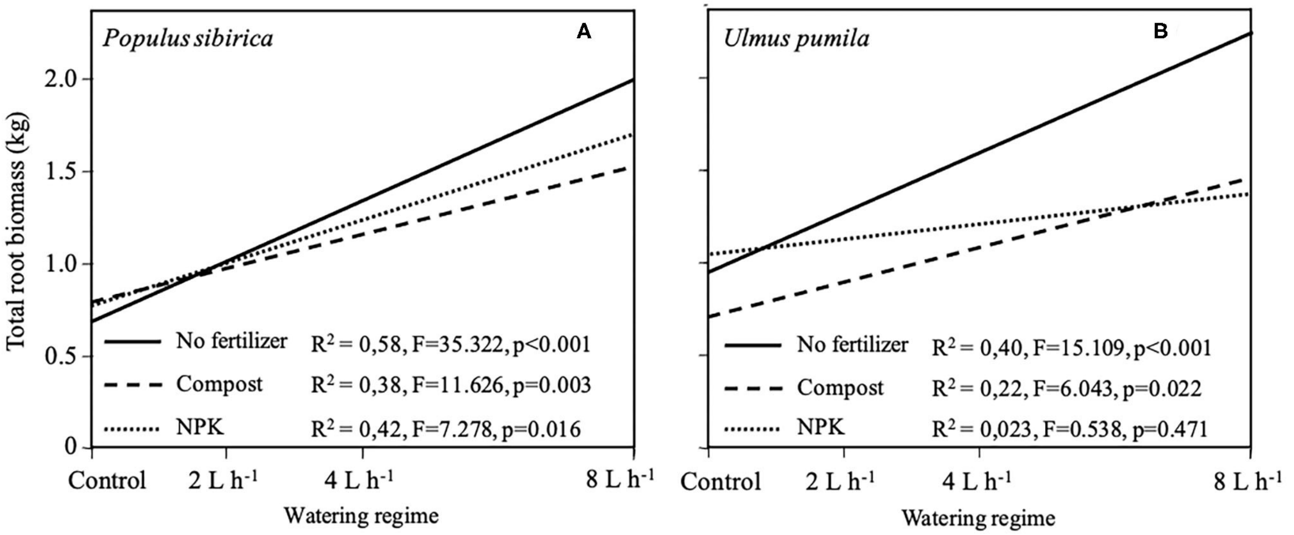

FIGURE 7 | Regression models of total root biomass for *P. sibirica* **(A)** and *U. pumila* **(B)** calculated by destructive sampling in relation with different watering regimes and fertilization treatment (No fertilizers, solid line; Compost, dashed line; NPK, dotted line).

mechanisms (e.g., reduced leaf size and increased leaf thickness) to counteract arid environmental conditions (Park et al., 2016; Qin et al., 2016).

For both species analyzed, the root biomass increased together with the increase of watering levels, independently of the diameter class considered. This has been related to the lowered photosynthetic CO_2 assimilation as the water deficit increased (Tezara et al., 2002; Li et al., 2011; Xu et al., 2015). In particular, for most of the root classes, biomass was remarkably lower in unwatered trees compared with watered trees, while no

TABLE 4 | Ratio between biomass of a specific root class (TR, FR, SR, MR, LR, and VLR) and the biomass of the total root system (TRB) according to two studied plant species (*U. pumila* and *P. sibirica*), management techniques (watering only, watering + NPK, and watering + Compost), and different watering regimes.

Plant species	Management technique	Watering regimes	% TR/TRB	% FR/TRB	% SR/TRB	% MR/TRB	% LR/TRB	% VLR/TRB
U. pumila	Watering only	Control	43.5 ± 6^{abc}	3.7 ± 1.9^{a}	12.7 ± 6.4^{a}	19.4 ± 12.1^{ab}	16 ± 13.2^{ab}	4.7 ± 7.3^{a}
		2 L h^{-1}	41.1 ± 5.7^{abc}	5.2 ± 5.8^{a}	12.1 ± 3.9^{a}	15.6 ± 7.7^{ab}	20.7 ± 10.4^{a}	5.3 ± 8.5^{a}
		4 L h^{-1}	38 ± 6.4^{c}	3.3 ± 1.5^{a}	9.4 ± 5.4^{a}	12.8 ± 9.7^{b}	17.3 ± 8.1^{a}	17.7 ± 16^{a}
		8 L h^{-1}	37.2 ± 5.7^{c}	2.9 ± 1.3^{a}	12.4 ± 4.2^{a}	7 ± 5.4^{b}	22 ± 13.3^{a}	18.4 ± 16.8^{a}
	Watering and NPK	Control	45.2 ± 10.1^{abc}	4.5 ± 3^{a}	8.6 ± 4.4^{a}	26.5 ± 11.5^{a}	6.4 ± 9.5^{ab}	12.1 ± 16.4^{a}
		2 L h^{-1}	48.5 ± 6.1^{ab}	3.5 ± 0.7^{a}	9.7 ± 3.9^{a}	9.1 ± 4.1^{b}	21.7 ± 5.5^{a}	7.4 ± 12.3^{a}
		4 L h^{-1}	50 ± 8.9^{a}	3.3 ± 1.5^{a}	9.4 ± 5.4^{a}	19.3 ± 14.4^{ab}	12.2 ± 15.3^{ab}	5.8 ± 9^{a}
		8 L h^{-1}	47.3 ± 7.2^{abc}	2.6 ± 1^{a}	8.9 ± 3.7^{a}	9.5 ± 7.7^{b}	14.9 ± 7.9^{ab}	16.9 ± 11.6^{a}
	Watering and compost	Control	39.8 ± 8.2^{bc}	3 ± 2.9^{a}	9.8 ± 3.9^{a}	26.5 ± 17^{a}	11.8 ± 13.2^{ab}	9 ± 14.8^{a}
		2 L h^{-1}	42.6 ± 8.8^{abc}	3.1 ± 0.7^{a}	7.9 ± 2.9^{a}	6.8 ± 2.6^{b}	20.7 ± 10.1^{a}	19 ± 12^{a}
		4 L h^{-1}	41.7 ± 9^{abc}	4 ± 2.6^{a}	13.8 ± 5.1^{a}	10.2 ± 5.3^{ab}	21.2 ± 9.1^{a}	9.1 ± 7.6^{a}
		8 L h^{-1}	48.6 ± 6.5^{ab}	5.3 ± 2.7^{a}	9 ± 1.8^{a}	16.3 ± 11.4^{ab}	12 ± 10.6^{ab}	8.8 ± 14.1^{a}
P. sibirica	Watering only	Control	58.3 ± 9^{a}	1.1 ± 0.5^{b}	4.2 ± 1.3^{a}	7.6 ± 4.7^{ab}	22 ± 12.2^{b}	6.7 ± 5.7^{de}
		2 L h^{-1}	44.6 ± 5.8^{bcde}	3.7 ± 1.2^{a}	5.7 ± 3.2^{a}	7.5 ± 2.8^{ab}	23.3 ± 10.7^{b}	15.1 ± 12.3^{cde}
		4 L h^{-1}	48.2 ± 8.5^{bc}	2.3 ± 1.2^{ab}	7 ± 5.9^{a}	7.6 ± 5.6^{ab}	21.7 ± 6.5^{b}	13.1 ± 9.4^{cde}
		8 L h^{-1}	46.8 ± 4.6^{bcd}	3.4 ± 2.5^{a}	7.9 ± 2.2^{a}	7 ± 2.4^{b}	9.7 ± 4.7^{c}	25.2 ± 7.1^{abc}
	Watering and NPK	Control	–	–	–	–	–	–
		2 L h^{-1}	46.1 ± 12.3^{bcd}	3.5 ± 1.6^{a}	6.2 ± 2^{a}	9.2 ± 5^{ab}	20.2 ± 9.9^{bc}	14.8 ± 15.9^{cde}
		4 L h^{-1}	35.3 ± 5.5^{d}	3 ± 0.7^{a}	5 ± 2.4^{a}	7.6 ± 4.5^{ab}	18.1 ± 7.9^{bc}	30.9 ± 12.3^{ab}
		8 L h^{-1}	46.9 ± 5.8^{bcd}	2.9 ± 0.7^{a}	4.1 ± 1.7^{a}	10.6 ± 7.6^{ab}	16.3 ± 9.4^{bc}	20.1 ± 17.8^{abcd}
	Watering and compost	Control	40.9 ± 3.1^{cde}	2.4 ± 1.2^{ab}	4.7 ± 2.7^{a}	14.2 ± 3.8^{a}	37.7 ± 5.3^{a}	0^{e}
		2 L h^{-1}	37.2 ± 3.9^{de}	2.5 ± 0.8^{ab}	4.4 ± 2.3^{a}	5.6 ± 4.6^{b}	16.3 ± 7.9^{bc}	34 ± 10.8^{a}
		4 L h^{-1}	51.8 ± 8.9^{ab}	2.6 ± 1.3^{ab}	5.4 ± 3.1^{a}	9.1 ± 4.6^{ab}	13.6 ± 5^{bc}	17.4 ± 9.9^{bcd}
		8 L h^{-1}	45.5 ± 6^{bcd}	3.3 ± 1.5^{a}	5.3 ± 3^{a}	7.3 ± 4.9^{b}	12.1 ± 8.4^{bc}	26.5 ± 8.8^{abc}

Letters indicate significant differences ($p < 0.05$) among factors for each species.
TR, taproot; FR, fine root; SR, small root; MR, medium root; LR, large root; VLR, very large root; TRB, total root biomass.

significant difference was detected between trees grown with 4 and 8 L h^{-1} watering regimes.

Our model highlighted that although root biomass increased with the increasing level of watering, this increment seemed to be lowered when root biomass was analyzed for trees grown with fertilizers. Interestingly, for *P. sibirica*, root biomass did not differ between fertilized and unfertilized trees when no watering and a 2 L h^{-1} regime were considered. At higher levels of watering, the addition of fertilizers seemed to induce a reduction of root growth. In the case of *U. pumila,* this reduction in root growth when fertilizers were applied was already detectable with the 2 L h^{-1} watering regime and was even of greater magnitude with higher levels of watering.

This clear reduction of root growth may be related to both the rate and time of application as well as to the type of fertilizer used in our study, which are important factors for meeting plant needs particularly in dry/arid lands (Abubaker et al., 2020).

Maturity and stability are important parameters for quality assessment of organic fertilizers (Gómez et al., 2006), which refers to the degree of decomposition of phytotoxic organic substances produced during the active composting phase and to the absence of pathogens (Wu et al., 2000). The sheep manure compost that was used in the present work might be compared to unmatured compost characterized by a high concentration of ammonium, organic acids, and other compounds that can be phytotoxic and inhibit root growth (Zucconi et al., 1981b; Chanyasak et al., 1983a,b; Wong, 1985). Although these chemicals do not induce lasting toxic effects in the environment (Zucconi et al., 1981a), long-term effects can occur from unstabilized organic material due to ammonium immobilization by soil microorganism, which makes it unavailable for plant utilization resulting in deficiency problems (Bengston and Cornette, 1973; Terman et al., 1973; Busby et al., 2007; Alburquerque et al., 2012; Abubaker et al., 2015). Furthermore, the low performance of digested manure might be due to the increased concentration of NH_4^+ (Risberg et al., 2017), which can be lost to the atmosphere by volatilization especially from sandy soil (Ni et al., 2012) and has been generally related to a decrease of root growth (Zhang et al., 2017), or more specifically to the inhibition of the primary root elongation, root biomass (Tian et al., 2008; Giehl and von Wiren, 2014; Morris et al., 2017; Abubaker et al., 2020), and deep roots development (Comfort et al., 1988).

Similarly, the reduction of root growth measured in plants grown with NPK addition might be related to the high

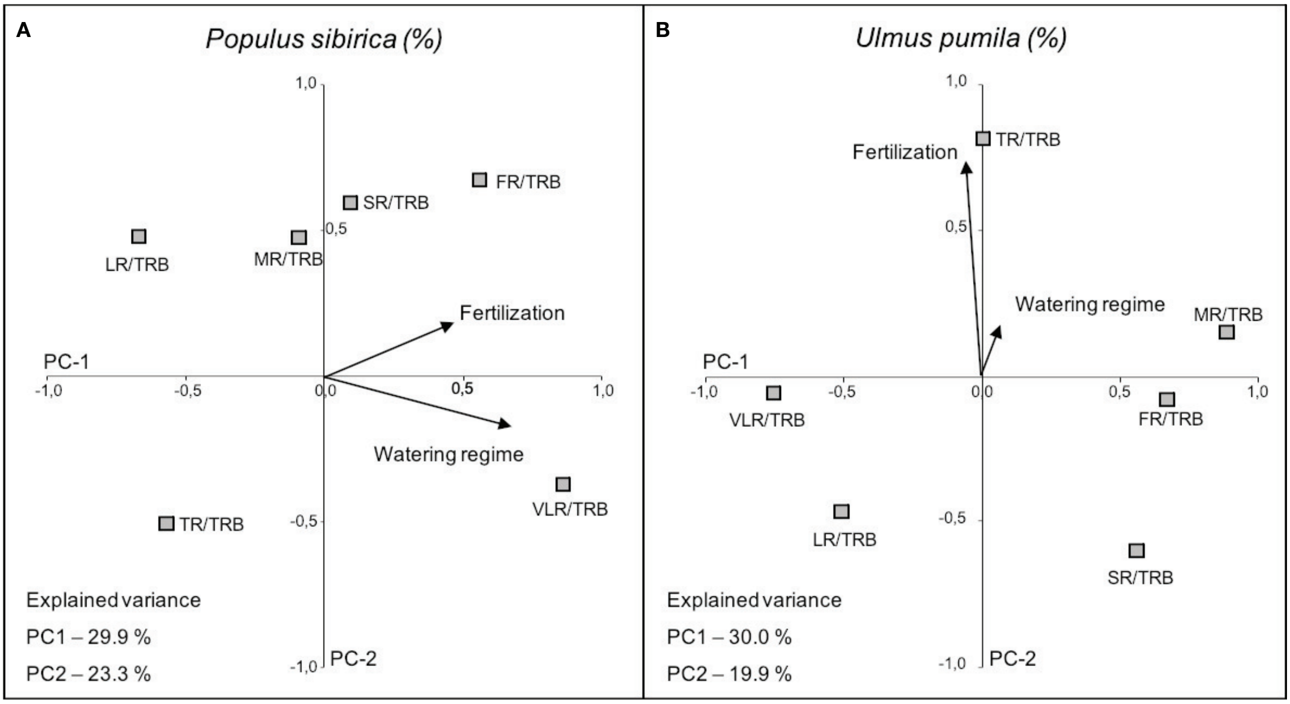

FIGURE 8 | Categorical Principal Component Analysis (CATPCA) biplot of *P. sibirica* **(A)** and *U. pumila* **(B)** indicating the percentage values of the ratio between different root classes and total root biomass projection in relation with watering regime and fertilization. Tightly clustered variables are positively related, while variables at different ends of the figure are negatively related. Arrows represents the mean influence magnitude for each factor.

application rate, which led to a large quantity of urea-based fertilizer considered phytotoxic (Chen et al., 2020). Also, high N fertilizer application rates to such an early growth stage of seedlings, may induce a growth priority to the aboveground structure, thereby repressing root growth without affecting shoot biomass (Dong et al., 2008; Rogato et al., 2010; Zhang et al., 2017).

These hypotheses to account for the lower root growth are further supported by the findings of soil analysis showing that after 10 years the nitrogen concentration of fertilized soils was almost double that of unfertilized soil, independently of the watering regime considered. Also, the level of nitrogen for the fertilized soil measured within the afforested site was similar to values measured outside. These results suggest a depletion of nitrogen attributable to the tree cover compared to grass and herbaceous cover, which was probably off-set by the fertilization. It is likely that fertilization has balanced the loss of nitrogen that would have occurred due to the tree growth. In our work, however, this demand seems to be different for the two species analyzed. *U. pumila* soil had slightly lower values of nitrate than *P. sibirica* independent of the application of fertilizers and the level of watering. This is in line with previous works (Van der Salm et al., 2006; Wei et al., 2010) that found differences in soil nitrogen across tree species used to afforest former grassland or agricultural lands. Together with the consumption of nitrogen due to the change in land cover, nitrogen leaching might be jointly responsible for low values found under canopy cover in our study for unfertilized soil. Indeed, Callesen et al. (1999) showed that the N leached from newly established forests on previously agricultural land may be higher than those from old-growth forests. Leaching of nitrate is a risk when the availability of inorganic N exceeds the demand from plants and microorganisms (Aber et al., 1989; Gundersen, 1991), and, as in our study, may be induced by the increased input of N due to fertilization (Rosenqvist et al., 2007). This mechanisms has been found to be most severe in sandy soils, similar to the soil type of our study site, that usually have a low retention of nutrients and 20–80% of applied nutrients or chemicals leach or runoff to ground and surface waters (Campbell et al., 1985; Sims et al., 1998; Manevski et al., 2015; Matichenkova et al., 2020).

Both *P. sibirica* and *U. pumila* trees showed the highest biomass investment in the taproot that corresponded, respectively, to almost 50 and 40% of the total root biomass. It is important to underline that since the *P. sibirica* trees were produced from cuttings the taproot we are referring to does not correspond to the seminal root but to the main root axis that may have replaced the pivotal role of the taproot. Indeed, Yang et al. (2014, 2016) found that the taproot contributed the most to anchorage strength, thus it is reasonable that our studied species have allocated the greatest root biomass to the taproot to ensure their stability. Furthermore, our biomass values are in line with values reported in literature that found from 50 to over 75% of belowground biomass allocated to the taproot (Kinerson et al., 1977; Van Lear and Kapeluck, 1995; Laclau, 2003; Miller et al., 2006). In addition, both species showed the lowest biomass values for the fine root category (d < 2 mm), accounting for the 3% of the total root biomass. This finding is in line with general agreement that fine roots rarely represent more than 5% of total tree biomass, although their annual production amounts to 33–67% of the total annual net primary production in most ecosystems (Matamala et al., 2003; McCormack et al., 2015; Montagnoli et al., 2018). Furthermore, *U. pumila* trees allocated more biomass in roots with small and medium diameters (SR and MR) whereas *P. sibirica* allocated more biomass in roots with large and very large diameter (LR and VLR).

These patterns in biomass allocation partitioning across the different root diameter classes changed when management practices were considered. Indeed, since the soil is spatially and temporally highly heterogeneous as a result of the soil type but also of different management practices (Mangalassery et al., 2014), plant roots may regulate their architecture (i.e., growth and branching) in response to soil environmental factors such as water, temperature, mechanical forces, and nutrient availability in addition to genetically determined developmental programs (Montagnoli et al., 2012, 2014, 2019a, 2021; Morris et al., 2017; Dumroese et al., 2019). This highly adaptable behavior, termed developmental plasticity, has been a major determinant for the success of land plants (Wilkinson, 2000; Hodge, 2004). Therefore, the different allocation of biomass among all components of a root system, further modified in relation to management practices, could be considered as a homeostatic response to factors that most limit plant growth (Bloom et al., 1985).

Our data suggest that the two species used different biomass partitioning strategies to face changes in soil characteristics due to the watering regimes and fertilization. In particular, *P. sibirica* increased the VLR components when levels of watering increased. This might be related to the need of *P. sibirica* to enhance the function of water transport from the roots to the stem as water availability increased. Moreover, enlarging the VLR fraction would probably be related to the enhancement of the root mechanical component (i.e., main first order lateral coarse roots) that has to sustain an outgrown tree structure (Dumroese et al., 2019; Montagnoli et al., 2019b, 2020). On the other hand, fertilization was found to increase the FR component suggesting a probable increase of the fine root web to face the higher availability of nutrients that should have occurred once fertilizers were applied (Ahlström et al., 1988; Wang et al., 2019). *U. pumila* did not show noticeable variations in biomass partitioning among different root classes in relation with the increase of levels of watering. This is in accordance with the lower sensitivity of *U. pumila* to the watering levels then *P. sibirica*. However, when fertilizers were applied *U. pumila* trees increased the TR component, reducing the SR and LR components. This might be related to the taproot accumulation function of N-reserves and vegetative storage protein (Noquet et al., 2001; Meuriot et al., 2003) that could be enhanced due to the higher nutrient availability.

Finally, in the present study there was a lack of influence of fertilization on soil organic matter. This could be due to the texture of the soil (70% sand and about 10% clay) and to the relatively low organic enrichment caused by a slow humification. Various authors have observed that organic carbon content and stabilization in the soil increase with increasing clay content (Ali et al., 1966; Ladd et al., 1977; Merckx et al., 1985; Feller and Beare, 1997; Six et al., 2002; Manna et al., 2005; Banger et al., 2009) since it provides protection to soil organic matter and makes it relatively inaccessible through aggregation against microbial and enzyme attack (Mtambanengwe et al., 2004; Strong et al., 2004; Banger et al., 2009). Moreover, in sand textured soils, decomposition of animal manure used in our study is faster than in fine textured soils (van Veen and Kuikman, 1990; Strong et al., 1999; Six et al., 2002), resulting in lower organic carbon (Bossuyt et al., 2001; Six et al., 2001), and short-time soil organic matter accumulation (Chivenge et al., 2011).

CONCLUSIONS

Although soil chemical characteristics were not enhanced over the long-term by the addition of fertilizers, our findings highlighted that increasing the levels of watering directly increased the root biomass development for both *U. pumila* and *P. sibirica*. However, this increment did not significantly differ when 4 and 8 L h^{-1} levels of watering were compared suggesting that an irrigation level of 4 L h^{-1} would yield high root biomass development with low levels of water consumption, and could be recommended for economical, sustainable, and effective reforestation in the semi-arid steppe of Mongolia. Our findings highlighted a possible reduction of root growth occurring when fertilizers were applied. This is unfortunate and might be due to the interplay of type and dose of fertilizers together with the sandy soil that characterized the study area. We therefore strongly suggest to implement afforestation intervention with an *a priori* analysis of the combination of type, dose, and chemistry of fertilizers, soil type, and plant needs for future application. Also, the two species showed a different root biomass developmental pattern with *P. sibirica* being more dependent on the water supply. These findings might be explained by the eco-physiological characteristics of the two species, suggesting that *U. pumila* might be selected for afforesting semi-arid environments with the lowest water consumption. Our data showed the higher and lower root biomass investment, respectively, in the taproot and fine root classes. Finally, the application of different management practices supporting tree growth modified the biomass partitioning across different root diameter classes. Such modification indicated a change in the overall root architecture that has been developed to ensure better tree performance in response to changes in the soil environment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

BN-O, DC, and AM conceived the research project. BN-O provided primary funding. AM, BN-O, S-OB, and DC developed the study plan. AM and DC dealt with the methodological approach and equally contributed to data interpretation. S-OB and BN-O were responsible for field excavation and data collection. S-OB and MT performed the data analysis. GSS, BP, and DC provided important insights into the research process. DC prepared the original draft. AM wrote the manuscript and dealt with revision process and finalization. JS and BN-O edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Splitting the Difference: Heterogeneous Soil Moisture Availability Affects Aboveground and Belowground Reserve and Mass Allocation in Trembling Aspen

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When exploring the impact of resource availability on perennial plants, artificial treatments often apply conditions homogeneously across space and time, even though this rarely reflects conditions in natural systems. To investigate the effects of spatially heterogeneous soil moisture on morphological and physiological responses, trembling aspen (*Populus tremuloides*) saplings were used in a split-pot experiment. Following the division of the root systems, saplings were established for a full year and then subjected to either heterogeneous (portion of the root system exposed to non-lethal drought) or homogeneous (whole root system exposed to non-lethal drought or well-watered) treatments. Above- and belowground growth and non-structural carbohydrate (NSC) reserves (soluble sugars and starch) were measured to determine how allocation of reserves and mass between and within organs changed in response to variation in soil moisture availability. In contrast to saplings in the homogeneous drought treatment, which experienced reduced shoot growth, leaf abscission and fine root loss, saplings exposed to the heterogeneous conditions maintained similar aboveground growth and increased root system allocation compared to well-watered saplings. Interestingly under heterogeneous soil moisture conditions, the portion of the root system that was resource limited had no root dieback and increased carbon reserve concentrations, while the portion of the root system that was not resource limited added new roots (30% increase). Overall, saplings subjected to the heterogeneous soil moisture regime over-compensated belowground, both in mass and NSC reserves. These results indicate that the differential allocation of mass or reserves between above- and belowground organs, but also within the root system can occur. While the mechanisms and processes involved in these patterns are not clear, these responses could be interpreted as adaptations and acclimations to preserve the integrity of the entire sapling and suggests that different portions of plant organs might respond autonomously to local conditions.

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This study provides further appreciation of the complexity of the mechanisms by which plants manage heterogeneous conditions and offers evidence that spatial and temporal variability of resource availability, particularly belowground, needs to be accounted for when extrapolating and modeling stress responses at larger temporal and spatial scales.

Keywords: split-pot experiment, drought, structural mass, non-structural carbohydrates, *Populus tremuloides*

INTRODUCTION

As the climate changes, the stochasticity of precipitation events is predicted to increase, and droughts are expected to become more intense and more frequent (IPCC, 2013). These changes have the potential to produce novel soil moisture conditions for many species (Harte and Shaw, 1995; Fridley et al., 2011; Metz and Tielbörger, 2016). Root systems of long-lived plants, such as trees will likely need to acclimate both morphologically and physiologically to these changing soil moisture conditions to ensure long-term survival. Controlled drought studies of potted plants have provided valuable insights into how species may respond to a drier future; however, these studies have several drawbacks. One issue is that the soil medium of pot-grown plants tends to be unrealistically homogenous, yet spatial and temporal heterogeneity in moisture is an inherent characteristic of soil ecosystems (Loranty et al., 2008; Guswa, 2012; Vereecken et al., 2014). Water availability varies both horizontally and vertically throughout a soil profile and is driven by topographical variability (e.g., hillslopes), soil pedogenesis and associated differences in soil properties (Chamran et al., 2002; Tromp-van Meerveld and McDonnell, 2006), vegetation cover and climate dynamics (Berry et al., 2006; Legates et al., 2010; Seneviratne et al., 2010). Therefore, as emphasized by Hutchings and John (2004), controlled studies investigating plant responses to heterogeneous distributions of environmental resources, whether that be moisture, space, light, or nutrients, are necessary to strengthen our understanding of plant growth and behavior, especially for the prediction of species responses and forest dynamics under future climate scenarios.

There has been considerable exploration of how trees and seedlings respond morphologically and physiologically to drought conditions (Breda et al., 2006; Brunner et al., 2015). However, knowledge of plant responses to hetero- vs. homogeneous soil moisture conditions is lacking, in particular how plants may alter the allocation of resources to maintain plant functionality and potentially survival in response to spatial variation. Recognizing how perennial plants balance the allocation of remobilized and newly acquired carbon between and within above- and belowground organs (Bloom et al., 1985; Chapin et al., 1990; Eissenstat, 1997) to structural components (Poorter and Nagel, 2000; Poorter et al., 2012), such as cellulose, hemicellulose and lignin, and/or to non-structural components (Magel et al., 2000; Dietze et al., 2014) such as soluble sugars, starch and secondary compounds, is critical to our understanding of plant stress responses. Based on studies simulating drought conditions that are spatially homogeneous, plants are known to respond to increasing water stress by reducing shoot growth, shedding leaves, reducing stomatal conductance and accumulating solutes in aboveground tissues to maintain turgor and limit xylem cavitation and desiccation (Rood et al., 2000; Arango-Velez et al., 2011; Galvez et al., 2011; Claeys and Inzé, 2013; Buckley et al., 2017). Belowground, as soil water potential decreases and the rhizosphere progressively dries, common responses include structural root growth and/or accumulation of solutes in root tissues to maintain a more negative water potential than the surrounding soil (Meier and Leuschner, 2008; Markesteijn and Poorter, 2009; Galvez et al., 2013). Yet when assessing belowground responses, we must consider that under natural conditions, soil moisture availability is often heterogeneous, and since root systems are capable of exploring and proliferating into favorable patches of soil resources (Drew, 1975; Hutchings and de Kroon, 1994), there is also the potential for distinct morphological and physiological adaptations within a root system depending on the conditions experienced by different parts of a root system (Gersani and Sachs, 1992). Thus, characterizing how carbon is allocated within perennial plants that are subjected to more natural heterogeneous soil moisture conditions could provide more accurate insights into drought avoidance and tolerance mechanisms, as well as how those impact our understanding of hydraulic failure and/or carbon starvation responses (McDowell et al., 2008; Sala et al., 2010; Pinheiro and Chaves, 2011; Adams et al., 2017).

To investigate how newly assimilated and remobilized carbon may be allocated within both aboveground and belowground organs in response to spatially variable soil moisture conditions, we selected a widely distributed tree species, trembling aspen (*Populus tremuloides* Michx.). Trembling aspen is well-known for its clonal root system, which is essential for its regeneration (root suckering) after disturbance (Peterson and Peterson, 1992; DesRochers and Lieffers, 2001; Frey et al., 2004; Wiley et al., 2019). The clonal root system of aspen is large and consists of interconnected lateral roots which can span across large gradients of soil moisture availability (Day, 1944; Snedden, 2013). While responses to both severe and moderate drought have been previously studied in aspen seedlings and large trees (Braatne et al., 1992; Frey et al., 2004; Hogg et al., 2008; Galvez et al., 2011; Anderegg, 2012), no studies have determined how aspen's drought response—particularly allocation patterns within different portions of a root system—is modulated by the heterogeneity of soil water availability.

The objective of this study was to characterize the morphological and physiological response of aspen saplings that had all or portions of their root systems exposed to progressive, non-lethal drought conditions. Specifically, we assessed the influence of hetero- and homogeneous soil moisture conditions on the aboveground and belowground allocation of non-structural carbohydrate (NSC) components

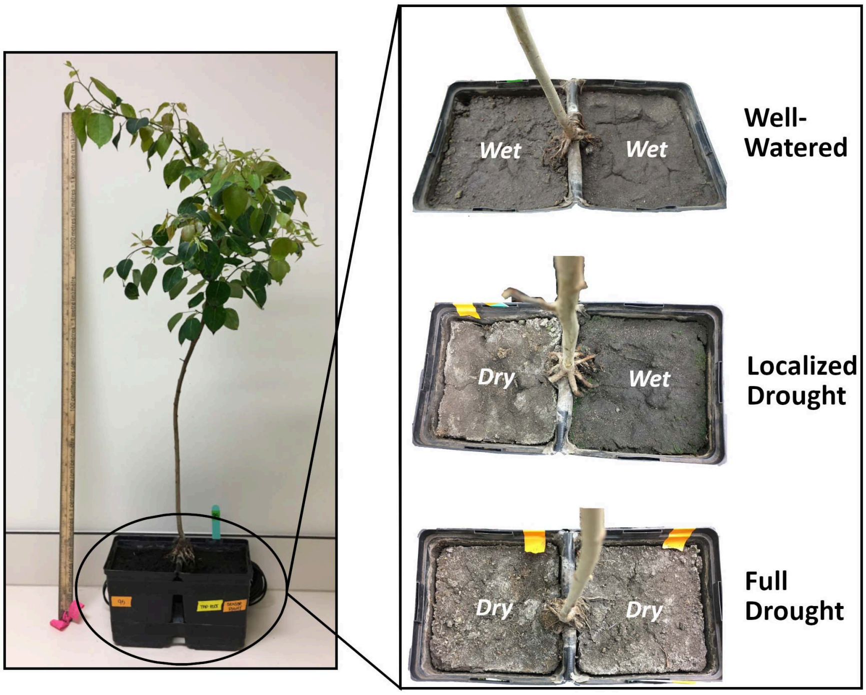

FIGURE 1 | Aspen sapling grown with spatially separated root system using a split-pot design. Aspen saplings were subjected to one of three watering treatments [well-watered (WW), localized drought (LD) or full drought (FD)] characterized by differences in soil moisture availability.

(soluble sugars and starch) and of other mass components (non-NSC, mostly structural) using a split-pot experiment. We hypothesized that saplings subjected to heterogeneous soil moisture conditions would compensate for the partial stress by preferentially increasing carbon (i.e., structural mass and NSCs) allocation toward the root system, accompanied by a decrease in aboveground growth. We also hypothesized that under heterogeneous soil moisture conditions, a sapling would allocate relatively more carbon to the drought exposed portion of the root system compared to a root system that was exposed to a homogeneous drought, as under these soil moisture conditions carbon acquisition and investment into growth would be greatly reduced. Furthermore, we hypothesized that the portion of the root system exposed to non-limiting soil moisture conditions in the heterogeneous treatment would respond similarly to a root system that was exposed to homogeneous non-limiting conditions.

MATERIALS AND METHODS

Split-Pot Design

A split-pot apparatus was used to spatially split the root systems of each tree sapling to allow for portions of a common root system to be independently exposed to different soil moisture conditions (Gowing et al., 1990; Fort et al., 1997; Sakuratani and Higuchi, 1999; Hirota et al., 2004; **Figure 1**). Split-pots were constructed using two square Kordlock pots (10 × 10 × 14 cm tall) stapled together. A square section of rubber liner (Pond Building Series, reinforced PVC pond liner, plant compatible) was glued and sealed with waterproof caulking over the joining portion of the two pots to prevent any water transfer along the edges of the pot. Reinforced tape was wrapped around the two joined pots and a 2.5 cm foam block was glued between the two pots to increase rigidity of the split-pot apparatus. To monitor soil moisture conditions, one half of the split-pot had a matric potential sensor (dielectric water potential sensors MPS2, Decagon Devices, Inc., Pullman, WA, United States) installed through a hole in the pot wall and was sealed into position with waterproof caulking. A piece of very fine mesh was placed at the bottom of each pot to prevent soil loss during watering. A sifted mineral agricultural topsoil with a sandy-loam texture was used as a growing medium. Each pot was then filled with the same weight of sifted soil and compacted to the same soil volume in the pot. A soil water potential response curve was created for the soil at the same bulk density as found in the split-pots, using the pressure extractor method to assess hydraulic properties to ensure a better control of drought conditions (Reynolds and Clarke Topp, 2008; **Supplementary Figure 1**).

Fifty one-year old nursery grown containerized trembling aspen saplings (6 cm diameter and 15 cm deep) grown from open-pollinated seed sources of Central Alberta (Smoky Lake Forest Nursery, AB, Canada) were used. During planting, the existing root system of each sapling was carefully split by first removing some of the growing medium. Care was then taken to equally divide the root system to accommodate the split-pot design. Separation of the root system was accomplished by dividing the total number of major lateral roots in half and planting them in each pot with the root collar of the sapling sitting on the pot joint (**Figure 1**). The presence and position of a large root within the split-pot was recorded for each sapling in all treatments to assure similar root distribution across treatments. A small piece of burlap was wrapped around the root collar to cover the exposed section of the root system sitting on the joint between the pots, preventing desiccation and root death during early establishment of the saplings. Saplings were watered regularly and fertilized once using 10-52-10 N-P-K fertilizer (Agrium, Inc., Calgary, AB, Canada). The saplings were kept outside at the University of Alberta (Edmonton, AB, Canada) for 20 weeks (July 4th, 2016–November 21st, 2016) to fully establish and allow the root system to occupy both pots and produce a healthy crown.

To prevent root damage from soil temperatures that were well below freezing (<-5°C), the saplings were moved to a dark growth chamber in November 2016. The chamber was set at a constant temperature of -1°C for a period of 6 weeks to allow saplings to accumulate additional chilling hours. Saplings were watered regularly with a small volume of water during that time, approximately 20 mL weekly, to prevent soil desiccation. After the 6-week dormant period, saplings were exposed to progressively higher air temperatures and increased light conditions over a period of 7 weeks (**Supplementary Table 1**). In that period, temperature increased to a maximum of 18°C during the day and 16°C at night with 12 h of light [500 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR)] which simulated spring conditions (**Supplementary Table 1**). Relative humidity in the chamber was maintained at 60% throughout the period. During the 7-week spring period, saplings were watered daily, fertilized weekly with 50 mL of 1 g L^{-1} 15-30-15 N-P-K fertilizer (Agrium, Inc., Calgary, AB, Canada) and pots were rotated weekly to minimize spatial variability. Of the 50 saplings grown initially, only 29 saplings were considered healthy (i.e., successfully flushed and produced new large leaves and elongated new shoots) and were used for the remainder of the experiment.

Experimental Period and Application of Watering Treatments

The experimental period (4 weeks) started at the beginning of March 2017, during which the growth chamber conditions were set to 20°C both day and night with a 17-h light/7-h dark cycle, a relative humidity at 60% and PAR of 500 μmol m^{-2} s^{-1}. Initial measurements of height and root collar diameter (RCD) were taken on all saplings generating four groups with similar sapling size distributions (total $n = 29$). Six saplings were harvested at the beginning of the experimental period (Initial). The remaining saplings were separated into treatment groups based on three soil moisture regimes: eight saplings were assigned to have both pots watered to field capacity (homogeneous well-watered treatment: WW); another set of eight saplings had one pot watered to field capacity (wet pot) while the other pot underwent a progressive dry-down (dry pot) (heterogeneous soil moisture: localized drought treatment: LD); and the remaining seven saplings had both pots undergoing a progressive drought (homogeneous full drought treatment: FD) (**Figure 1**). For all saplings, soil water potential was recorded every 15 min in one of the wet pots in the WW treatment, one of the dry pots in the FD treatment, and in the dry pot of the LD treatment, using the installed soil water potential sensors connected to EM50 dataloggers (Decagon Devices Inc. Pullman, WA, United States). For the first week of the experimental period, saplings received water (only), but then for the remainder of the experiment, water that included a 2 g L^{-1} solution of 15-30-15 N-P-K fertilizer (Agrium, Inc., Calgary, AB, Canada). Saplings were moved weekly to different positions on the growth chamber benches to reduce any effects of spatial variability in the ambient conditions.

For the WW treatment and the wet pot of the LD treatment, at the start of each daytime period, each pot was watered to saturation and then allowed to drain freely reaching field capacity. For the dry pot of the LD treatment and the FD treatment, a progressive drought was applied. To apply the drought in the LD treatment, the initial starting weight at field capacity for the entire split-pot was determined at the start of the experimental period. During the experimental period, the wet portion of the split-pot was always re-watered to field capacity (watered to saturation and allowed to completely drain) at the beginning of the day, then the entire split-pot was weighed, and the difference to the initial (previous) weight was attributed to the water loss from the dry pot only. The dry pot was then watered with half of the water loss amount, based on the weight lost, thus contributing to a gradual decrease in soil water potential over a period of 4 weeks (**Supplementary Figure 2**). For the last 2 weeks of the experimental period, the soil water potential in the dry pots was maintained between -700 to -1200 kPa, with the pots receiving only small water additions (<5 g) during the last 4 days (**Supplementary Figure 2**). Soil water potentials were maintained within this range to avoid catastrophic drought-induced cavitation, as previous research has demonstrated that an average xylem pressure below -1200 kPa can produce a loss of hydraulic conductivity greater than 50% in aspen (Plavcova and Hacke, 2012; Fichot et al., 2015; Schreiber et al., 2016). A similar watering strategy was applied to the FD treatment; however, here both sides of the split-pot were subjected to the same progressive drought, with both pots receiving water in the amount replacing only half the water lost from the previous day and then maintained at the same range of water potential to achieve a similar drought intensity at the pot-level compared to the LD dry pot (**Supplementary Figure 2**).

Measurements

To monitor physiological responses, net assimilation and stomatal conductance were measured with a LI-6400XT portable photosynthesis system (LiCor, Inc., Lincoln, NE, United States) once a week throughout the experimental period on three saplings (two leaves each) randomly selected within each treatment. Leaf chamber light was set at 800 μmol m^{-2} s^{-1}, CO$_2$ was set at 400 ppm, incoming relative humidity at 60% and leaf temperature at 20°C to mimic the conditions in the growth chamber. Net assimilation and stomatal conductance were measured 2 h after the beginning of the daytime period.

At the end of the experimental period, the final height and RCD were measured on all saplings at harvest. Saplings were separated into leaves, stem (old and new primary growth) and the two portions of the split root system. Projected leaf area was measured for each sapling in the LD and the WW treatments using a LI-3100 area meter (LiCor, Inc., Lincoln, NE, United States). Leaf area could not be measured for the FD saplings, as dead leaves in the FD treatment were too brittle to measure. The portions of the root system were extracted separately from each side of the split-plot and kept separated. After careful removal of all soil, fresh root volume, which more closely represents the root surface area (estimate of water uptake potential), was measured for each portion of the split root system via the water displacement method (Harrington et al., 1994). All collected tissues from each sapling were dried for 1 h at 100°C to denature enzymes, followed by 48 h at 70°C to constant weight. Dried root samples were separated into coarse roots (diameter > 1 mm) and fine roots (diameter < 1 mm). All dried material was weighed, and samples were ground to a 40-mesh (0.4 mm) using a Thomas mini Wiley mill (Thomas Scientific, Inc., Swedesboro, NJ, United States) for subsequent NSC analysis.

Non-structural carbohydrates (NSC) were analyzed following the protocol described in Landhäusser et al. (2018). In brief, total soluble sugars were extracted in 80% hot ethanol followed by a phenol-sulfuric assay to determine their concentration colorimetrically by measuring the absorbance at 490 nm with a spectrophotometer. To determine starch concentration, the remaining pellet was digested with α-amylase (Sigma cat. no. A4551) and amyloglucosidase (Sigma cat. no. ROAMYGL). A peroxide-glucose oxidase/o-dianisidine reagent was then added to the resulting glucose hydrolysate. After incubation, concentrated sulfuric acid was added before measuring absorbance at 525 nm. Absorbance values were used to calculate sugar and starch concentrations by comparison with standard curves and expressed as percent of sample dry weight.

Calculations and Statistical Analyses

The following calculations were used to compare the effects of heterogeneous soil moisture vs. uniform soil moisture on the allocation of structural and non-structural (soluble sugars and starch) components between aboveground and belowground tissues and within the root system (i.e., between the split-pots). Treatment effects on growth were assessed using sapling height, RCD, and biomass. Height and RCD growth during the experimental period were calculated by subtracting the initial height and RCD measured on each treated sapling at the beginning of the experiment from the final height and RCD of that same sapling. To evaluate changes in leaf and stem mass that occurred during the 4-week experimental period (i.e., new leaf and new stem growth), the average of initial measurements, taken from the six destructively sampled saplings at the beginning of the experimental period, were subtracted from the individual final treatment measurements of leaf mass (which included dead leaves in the FD treatment) and the mass of primary stem growth. Specific leaf area was calculated by dividing total leaf area by total leaf dry mass. To determine mass allocation in saplings, the ratio of leaf, stem or root mass to total sapling mass was calculated for each sapling. Leaf, stem and root NSC mass (pools) were estimated by multiplying the total sugar and starch concentrations by the total dry mass of each sampled organ. Further, the remaining mass (hereafter called structural mass) that was not related to reserve mass of each organ was estimated by subtracting the respective NSC mass from the total dry mass of each organ. These measures were estimated to evaluate any differences in leaf structural mass, stem structural mass and root structural mass in response to our treatments. In addition, root structural density was calculated as a ratio of the structural mass of the entire root system (fine and coarse roots combined) over the measured root volume (fine and coarse roots combined), to explore potential changes in the morphological composition of the root system.

All data were analyzed using R statistical software v3.5.1 (R Development Core Team, 2018). Assumptions of normality and homoscedasticity were tested using the Shapiro-Wilks test and Levene's test for parametric analyses. If these assumptions were not met, removal of outliers and transformations were applied. The soil water potential data was fit with a logistic non-linear model using the nlme package (Pinheiro et al., 2016) to show the gradual dry down of soil within the split-pots (**Supplementary Figure 2**). No differences in root measures between the two sides of the split-pots were found in either well-watered or full-drought treatments using *t*-tests (data not shown; $p > 0.1$). Thus, in subsequent analyses, the two sides of the split-pots in these treatments are considered equivalent. Two statistical analyses were applied to the data. First, to test for differences among the initial harvest and three treatments for aboveground and combined belowground measures, one-way ANOVA was used followed by pairwise *post-hoc* tests with a Benjamini-Hochberg adjustment using the emmeans package (Lenth, 2019). Second, to understand how the localized drought (LD) treatment impacted *within*-root system response, a linear mixed model was used with pot-type (LD-dry, LD-wet, FD, WW) as a fixed factor. Individual sapling was included as a random factor to account for the fact that the same individual was repeatedly measured (2 pots per sapling). The initial presence/absence of a large root within a pot was also included as a random factor to account for the fact that a large root could impact pot-level variables like final root mass, volume, etc. The post-hoc tests for these models were restricted to the following planned comparisons: (1) dry pots vs. the wet pots of the LD treatment, (2) dry pots of the LD treatment vs. the FD treatment pots, and (3) wet pots of the LD treatment compared to the WW treatment pots. The Benjamini-Hochberg adjustment was used with the emmeans package. Differences among treatments or between sides of the split-pot were considered statistically significant at $\alpha = 0.1$. Estimated marginal means and standard errors are reported in the Results and Discussion sections.

RESULTS

Growth and Mass Allocation

Overall, saplings exposed to the localized drought (LD) had various aboveground measures that were similar in comparison to the well-watered (WW) saplings but were greater than those measures in saplings of the full drought (FD) treatment.

Although average height growth did not differ among the three treatments ($p > 0.18$; **Table 1**), the LD and the WW saplings had overall larger RCDs, with over three times more RCD growth compared to the FD saplings ($p < 0.01$; **Table 1**). Total aboveground dry mass was approximately 30% greater in the LD and the WW treatments compared to the FD treatment, for which no significant increase in aboveground mass occurred ($p < 0.01$; **Table 1**). Saplings in the LD and the WW treatments produced 1.74 and 2.34 g, respectively, of new leaf mass during the 4-week experimental period, while saplings in the FD treatment did not produce any new leaves ($p = 0.03$; **Table 1**). Saplings in the FD treatment also experienced partial browning of pre-existing leaves and significant leaf abscission prior to harvest. Specific leaf area was similar between the LD and the WW saplings (**Table 1**). During the experimental period, stem growth of the WW saplings was 1.27 g, which was similar to the stem growth of the LD saplings (0.87 g) and greater than the stem growth of the FD saplings (0.36 g; $p < 0.01$) (**Table 1**). The allocation of mass to leaves (25%) and stems (30%) was statistically similar among the three soil moisture treatments (**Table 1**), although the allocation to leaves in the FD treatment is likely lower if only live leaf mass had been considered. During the period when the soil water potential was maintained between −700 to −1200 kPa, there were no differences in the net assimilation rate and stomatal conductance between the LD saplings (7.74 ± 1.23 $\mu\text{mol}_{\text{CO2}}$ m^{-2} s^{-1} and 0.196 ± 0.029 mmol_{H2O} m^{-2} s^{-1}, respectively) and the WW saplings (8.62 ± 1.23 $\mu\text{mol}_{\text{CO2}}$ m^{-2} s^{-1} and 0.243 ± 0.029 mmol_{H2O} m^{-2} s^{-1}, respectively), whereas the FD saplings had significantly lower net assimilation and stomatal conductance compared to the other two treatments (-1.50 ± 1.73 $\mu\text{mol}_{\text{CO2}}$ m^{-2} s^{-1} and 0.019 ± 0.041 mmol_{H2O} m^{-2} s^{-1}, respectively, both $p < 0.01$).

TABLE 1 | Estimated marginal means (±1 standard error) of aboveground and belowground variables: height growth (cm), root collar diameter (RCD) growth (mm), total sapling mass (g), total aboveground mass (g), new stem growth (g), leaf mass (g), new leaf growth (g), specific leaf area (cm^2 g^{-1}), total root mass (g), total coarse root mass (g), total fine root mass (g), total root volume (cm^3), structural root density (g cm^{-3}), leaf mass ratio (%), stem mass ratio (%) and root mass ratio (%) prior to (INITIAL) and after three watering treatments (FD, full drought; LD, localized drought; WW, well-watered).

	INITIAL	FD	LD	WW
Height growth (cm)	NA	3.3 (1.3)a	4.1 (1.5)a	8.8 (2.6)a
RCD growth (mm)	NA	0.72 (0.26)b	2.50 (0.24)a	2.62 (0.24)a
Total sapling mass (g)	21.8 (2.1)b	22.6 (2.0)b	33.5 (1.9)a	32.1 (1.9)a
Total aboveground mass (g)	12.2 (1.2)b	12.5 (1.1)b	18.4 (1.1)a	19.7 (1.1)a
New stem growth (g)	NA	0.36 (0.20)b	0.87 (0.18)ab	1.27 (0.18)a
Leaf mass (g)	6.29 (0.58)b	5.40 (0.56)b	8.03 (0.53)a	8.63 (0.53)a
New leaf growth (g)	NA	0.27 (0.46)b	1.74 (0.43)a	2.34 (0.43)a
Specific leaf area (cm^2 g^{-1})	162.9 (6.4)a	NA	125.5 (4.7)b	123.7 (4.7)b
Total root mass (g)	9.58 (1.25)b	10.13 (1.16)b	15.03 (1.08)a	12.37 (1.08)ab
Total coarse root mass (g)	3.94 (0.65)*c*	5.66 (0.61)*bc*	8.03 (0.57)a	7.04 (0.57)ab
Total fine root mass (g)	5.64 (0.67)ab	4.47 (0.62)b	7.00 (0.58)a	5.33 (0.58)ab
Total root volume (cm^3)	59.1 (6.1)a	37.2 (5.7)b	63.1 (5.3)a	58.3 (5.3)a
Structural root density (g cm^{-3})	0.141 (0.01)*c*	0.247 (0.01)a	0.203 (0.009)b	0.186 (0.009)b
Leaf mass ratio (%)	29 (2)a	24 (2)a	24 (2)a	28 (2)a
Stem mass ratio (%)	26 (2)b	31 (2)a	31 (2)a	34 (2)a
Root mass ratio (%)	44 (2)ab	45 (2)a	45 (2)a	38 (2)b

Letters represent statistical difference (n = 29; α = 0.1) among treatments using post-hoc comparisons with a Benjamini-Hochberg adjustment. NA, not applicable.

Localized drought saplings had a total root dry mass (both pots combined) of 15 g, similar to the 12.4 g of WW saplings, and nearly 50% more compared to the FD saplings (10.1 g, $p = 0.01$) (**Table 1**). Full drought saplings had a total root dry mass that was similar to the initial saplings (**Table 1**). However, when comparing total root volume which relates to root surface area and its potential for water uptake, FD saplings also had a total root volume of 37.2 cm^3 at harvest, which was significantly lower than the LD and the WW saplings (63.1 cm^3 and 58.3 cm^3, respectively, $p = 0.01$ and $p = 0.02$, respectively), but also significantly lower than the average initial root volume of 59.1 cm^3 ($p = 0.03$) (**Table 1**). Root structural density of the root system, a measure that indicates potential changes in root system morphology, was overall higher in all three experimental treatments after the 4-week experimental period compared to the start of the study (0.141 g cm^{-3}; $p < 0.01$). However, while the root systems of the LD and WW saplings had similar root structural density at the end of the experiment (0.203 and 0.186 g cm^{-3}, respectively), root structural density in FD saplings was higher than both treatments (0.247 g/cm^3; both $p < 0.01$) (**Table 1**). Overall, the LD saplings and the FD saplings allocated a greater amount of mass toward the root system (45%) compared to the WW saplings (38%) ($p = 0.07$; **Table 1**). This suggests that the WW saplings allocated more mass to the aboveground variables, such as height growth, stem and leaf mass which all tended to be greater in the WW saplings, but we could not detect significant statistical differences between the LD and the WW saplings ($p = 0.2$, $p = 0.1$, and $p = 0.1$, respectively).

A closer analysis of belowground measurements between the dry and the wet root system portions in the split-pots of the LD saplings revealed distinct patterns of allocation. Under the localized drought conditions, saplings allocated more mass to the roots within the wet pot (8.33 g) than to the roots within the dry pot (6.70 g; $p = 0.01$; **Figure 2A**). This greater root mass in the wet pot can be attributed to an increase in fine root production compared to the dry pot ($p < 0.01$; **Figure 2C**). The portion of the root system contained in the wet pot of the LD treatment also had a greater mass (8.33 g), comprised of significantly more fine

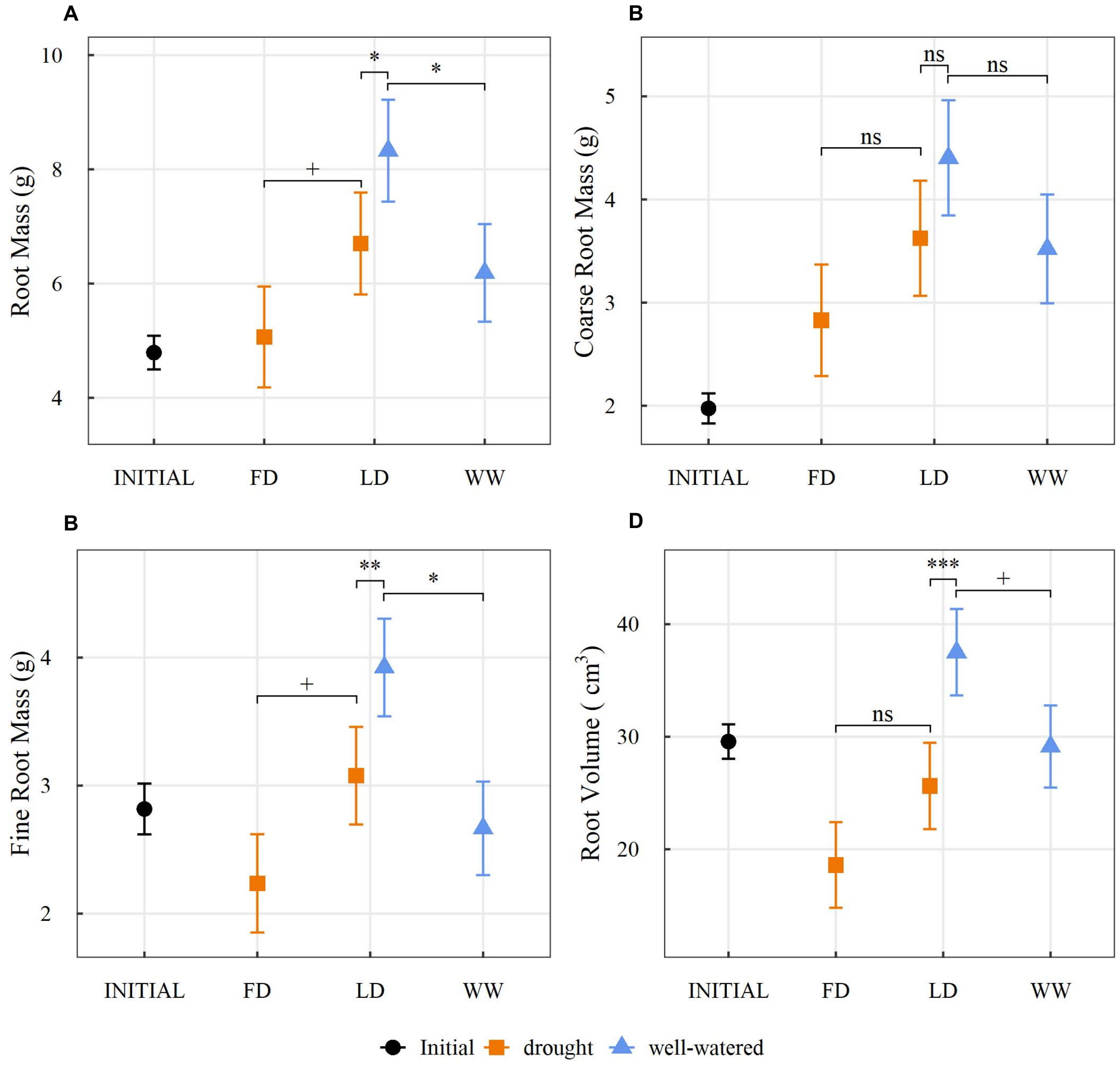

FIGURE 2 | Estimated marginal means (±1 standard error) of **(A)** root mass (g), **(B)** coarse root mass (g), **(C)** fine root mass (g) and **(D)** root volume (cm^3) within the split root system of saplings, prior to (INITIAL) and after the three watering treatments (FD, full drought**;** LD, localized drought**;** WW, well-watered) (orange: dry, blue: well-watered). Statistical differences between the three planned comparisons (FD vs. LD-dry, LD-dry vs. LD-wet, WW vs LD-wet) are indicated by + for $p < 0.1$, * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and ns for no significance ($n = 29$; $\alpha = 0.1$).

roots ($p = 0.02$; **Figure 2C**) than either section of the root systems in the WW treatment (6.19 g; $p = 0.03$) (**Figure 2A**). The root system portion contained in the dry pot of the LD treatment was greater in mass, with a significant increase in fine roots ($p = 0.09$; **Figure 2C**), compared to either root system portion in the FD treatment (5.06 g; $p = 0.08$; **Figure 2A**). When comparing root system volumes, which relate to root surface area and potential for water uptake, across the split-pots, the root system portion in the wet pot of the LD treatment had a greater volume (37.51 cm^3) than the root portion in the dry pot (25.63 cm^3; $p < 0.01$; **Figure 2D**). However, the root volume in the wet pot of the LD treatment was greater than the volume of the root portions measured in the WW treatment (29.14 cm^3; $p = 0.08$; **Figure 2D**). Although there was a trend for the dry portion of the root system in the LD treatment to have a greater volume (25.63 cm^3) than in the FD treatment (18.6 cm^3) the difference was not statistically significant ($p = 0.12$; **Figure 2D**).

Non-structural Carbohydrate Concentrations

At the end of the 4-week period, there were only a few differences in the starch concentrations in the aboveground tissues among the three watering treatments, while stark differences existed in the root NSC concentrations in response to the different soil moisture conditions. Aboveground, there were no differences in total NSC (sum of starch and soluble sugars) and sugar concentrations in leaf tissue among the three watering treatments, and these concentrations did not differ from the initial measurement (**Table 2**). However, the FD

TABLE 2 | Estimated marginal means (±1 standard error) of starch and soluble sugar concentration (% dry weight) for leaf and stem tissue, and of total non-structural carbohydrate (NSC) concentration (% dry weight) of leaf, stem and root (both pots combined) tissue, prior to (INITIAL) and after the three watering treatments (FD, full drought; LD, localized drought; WW, well-watered).

Organ		**INITIAL**	**FD**	**LD**	**WW**
Leaf	Starch	0.75 (0.15)a	0.20 (0.10)b	0.78 (0.13)a	0.66 (0.12)a
	Sugar	13.78 (0.58)a	13.53 (0.54)a	12.56 (0.50)a	12.69 (0.50)a
	NSC	14.62 (0.64)a	13.75 (0.63)a	13.37 (0.60)a	13.36 (0.60)a
Stem	Starch	0.38 (0.18)b	0.05 (0.12)b	1.31 (0.26)a	0.73 (0.19)ab
	Sugar	9.87 (0.55)a	8.29 (0.43)b	8.19 (0.39)b	7.34 (0.35)b
	NSC	10.28 (0.61)a	8.52 (0.62)a	9.66 (0.58)a	8.27 (0.58)a
Root	NSC	12.95 (1.64)a	12.85 (1.58)a	15.49 (1.48)a	12.90 (1.48)a

Letters represent statistical difference (n = 29; α = 0.1) among treatments using post-hoc comparisons with a Benjamini-Hochberg adjustment.

saplings had lower leaf starch concentrations (0.20%) compared to the LD and the WW saplings (0.78 and 0.66%, respectively, $p < 0.01$ and $p = 0.01$, respectively, **Table 2**). In comparison to the initial measurement, stem NSC concentrations did not change in LD saplings (10.28% vs. 9.66%, respectively), WW saplings (8.27%) or FD saplings (8.52%) (**Table 2**). Among treatments, the LD saplings had a similar stem starch concentration (1.31%) compared to WW saplings (0.73%; $p = 0.12$), yet a significantly greater starch concentration compared to the FD saplings (0.05%; $p < 0.01$) (**Table 2**). Only the LD saplings increased stem starch concentrations over the initial measurement of 0.38% ($p = 0.01$; **Table 2**). Stem sugar concentrations prior to the start of the experiment were 9.87% which decreased slightly during the experimental period ($p < 0.06$), but no differences were detected in soluble sugar concentrations in the stems among the three watering treatments (**Table 2**).

Belowground, the total NSC concentrations found across the entire root system (both pots combined) did not differ from the initial measurements or among the three watering treatments (**Table 2**). However, when the NSC concentrations of the root systems were compared between the split-pots, the roots in the dry pot of the LD treatment had higher NSC concentrations (18.35%) than the roots in the wet pot of the LD treatment (13.03%; $p < 0.01$; **Figure 3**). Furthermore, the roots in the dry pot of the LD treatment had higher NSC concentrations than the roots in the FD treatment (12.82%; $p = 0.03$) (**Figure 3**). In contrast, NSC concentrations of the roots in the wet pot of the LD treatment (13.03%) did not differ from those in the WW treatment (12.83%) (**Figure 3**). When broken down into soluble sugar and starch, roots in the dry pot of the LD treatment had higher soluble sugar (4.85%) and starch (13.44%) concentrations than the roots in the wet pot, with 3.51 and 9.49%, respectively ($p < 0.01$ and $p = 0.05$, respectively; **Figure 3**). The roots in the wet pot of the LD treatment had soluble sugar and starch concentrations similar to the roots in the WW treatment. The roots in the FD treatment had higher soluble sugar (9.54%) but lower starch (3.11%) concentrations compared to the roots in the dry pot of the LD treatment (both $p < 0.01$; **Figure 3**).

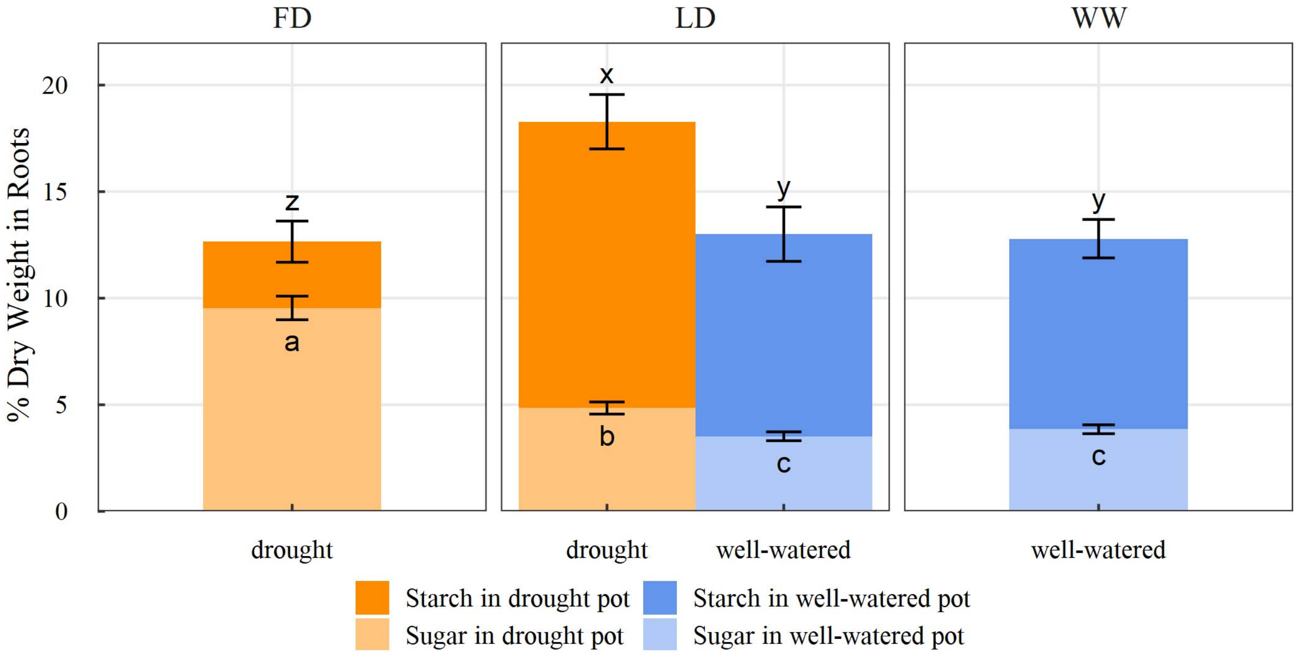

FIGURE 3 | Estimated marginal means (±1 standard error) of root starch and soluble sugar concentration (% of dry weight) within the split root systems of saplings subjected to three watering treatments (FD: full drought, LD: localized drought, WW: well-watered), (light orange: sugar concentration of roots in drought pot, dark orange: starch concentration of roots in drought pot, light blue: sugar concentration of roots in well-watered pot, dark blue: starch concentration of roots in well-watered pot). Letters indicate statistical differences among treatments and pot watering regimes (n = 29; α = 0.1) using pairwise comparisons with a Benjamini-Hochberg adjustment.

Allocation to Structural Mass and Non-structural Carbohydrate Pools

The NSC concentrations in the leaves, stem and roots of each individual sapling were used to estimate the structural mass and the NSC pool sizes and their relative allocation (% of total) in response to the soil moisture treatments. Of the total structural pool, the LD saplings allocated 24.2% to leaves, 32.1% to the stem, and 43.7% to roots (**Table 3**). There were no differences in the relative allocation of structural mass to leaves among the three soil moisture treatments (**Table 3**). Well-watered saplings allocated slightly more structural mass to the stem in comparison to the LD saplings ($p = 0.09$; **Table 3**). However, while the relative allocation of structural mass to the root system was similar for the LD and the FD saplings, the WW saplings allocated less structural mass to the root system (37.2%) compared to both treatments (both $p = 0.03$; **Table 3**). Of the total NSC pool (sum of soluble sugars and starch), the LD saplings allocated 25.3% to leaves, 22.3% to the stem, and 52.4% to roots (**Table 3**). The relative allocation of NSC to leaves, stems and the root systems were similar among the three soil moisture treatments (**Table 3**).

Differences in the structural mass within the two portions of the split root system in the LD saplings were driven by the localized soil moisture conditions. Roots in the dry pot had less structural mass than the roots contained within the wet pot ($p < 0.01$) but did not differ from the amount of structural mass of a root system portion found in the FD treatment (**Figure 4A**). In contrast, the roots in the wet pot of the LD treatment had 30% more structural mass compared to the roots in the WW treatment ($p = 0.01$; **Figure 4A**). LD saplings had similar NSC mass in their dry and wet pots, however, the dry portion of the root system had over double the NSC mass compared to either half of the root systems of the FD saplings ($p = 0.06$; **Figure 4B**). The NSC mass of roots in the wet pot of the LD treatment was not significantly different from either half of the root system in the WW treatment ($p = 0.4$; **Figure 4B**).

DISCUSSION

Our study demonstrates that the root systems of aspen saplings subjected to heterogeneous water availability (LD treatment) responded unlike saplings with root systems that were exposed to homogeneous soil moisture conditions [full drought (FD) or well-watered (WW)]. Based on the heterogeneous conditions in the LD treatment, saplings responded quickly by partitioning structural mass and NSC functionally across and within organs. As expected, saplings exposed to either the full or the localized water limitation increased overall allocation toward the root system (45%) compared to saplings growing in non-limiting conditions (38%). However, the saplings exposed to the full drought experienced reduced gas exchange, terminated aboveground growth, shed leaves and lost root volume. In contrast, the saplings exposed to the LD treatment maintained gas exchange and aboveground growth similar to the WW saplings, avoided root loss in the dry soil, while increasing root structural mass and volume in the wet soil. The different responses of the roots in the dry vs. wet soil under the localized drought treatment suggests the potential for some autonomy within root systems to adaptively adjust allocation depending on the soil conditions individual roots are exposed to.

TABLE 3 | Estimated marginal means (±1 standard error) of the relative allocation (% of total sapling pool type) of structural and non-structural carbohydrate (NSC) pools for leaves, stem and roots prior to (INITIAL) and after the three watering regimes (FD, full drought; LD, localized drought; WW, well-watered).

Organ	Pool type (%)	INITIAL	FD	LD	WW
Leaf	Structural	28.4 (1.7)a	23.1 (1.7)a	24.2 (1.6)a	26.9 (1.6)a
	NSC	33.8 (3.7)a	29.3 (3.7)a	25.3 (3.5)a	33.2 (3.5)a
Stem	Structural	27.5 (1.4)*c*	32.9 (1.2)ab	32.1 (1.1)b	35.7 (1.2)a
	NSC	21.2 (3.6)a	21.6 (3.3)a	22.3 (3.1)a	24.5 (3.1)a
Root	Structural	44.1 (2.0)a	44.0 (2.0)a	43.7 (1.8)a	37.2 (1.8)b
	NSC	44.3 (4.4)a	47.6 (4.5)a	52.4 (4.2)a	41.4 (4.2)a

Letters represent statistical difference ($n = 29$; $\alpha = 0.1$) among treatments using post-hoc comparisons with a Benjamini-Hochberg adjustment.

While the responses of aspen exposed to either homogeneous or heterogeneous drought appear to be consistent with the concept of functional equilibrium of biomass allocation or optimal partitioning theory (Brouwer, 1963; Thornley, 1972; Iwasa and Roughgarden, 1984; Bloom et al., 1985), where plants preferentially allocate biomass to the organ responsible for the uptake of the limiting resource (Poorter et al., 2012), the manner by which the saplings in both drought treatments arrived there is very distinct. In the LD saplings, the proportional increase in root mass was the result of a differential allocation toward the root system, while the increase seen in saplings in the FD treatment was mostly the result of a differential mortality of organs. Further, the saplings exposed to the heterogeneous water availability responded with significant increases in leaf, stem and root mass, but attained the higher root mass ratio (also root to shoot ratio) by allocating more carbon to root growth relative to shoot growth. In contrast, the increase in the root mass ratio of the FD saplings was the result of terminated primary growth and a greater net tissue loss in above- vs. belowground parts; this increase would have been even larger if we had discounted the abscised leaves in this treatment. Leaf and branch shedding have been hypothesized as key drought adaptations in *Populus* species, as it decreases transpiration loss through leaf area (Rood et al., 2000; Galvez et al., 2011). The loss in root mass in the FD saplings was more difficult to discern, as the root mass of the FD saplings was similar to the initial root mass, it would appear that the root system of these saplings was maintained during the drought conditions. However, this observation is not supported by the reduction in root volume of the FD saplings from the initial volume, indicating that significant root death occurred (**Table 1**). It appears most likely that fine root mass was shed in these root systems (**Figure 2C**), while coarse roots remained viable and alive (**Figure 2B**). This is further supported by an increase in structural root density we observed in the FD treatment (**Table 1**), which might also indicate that only roots with higher densities were maintained. In grasses it had been observed that roots with higher density tend to have longer life spans than roots with lower density which are more likely to

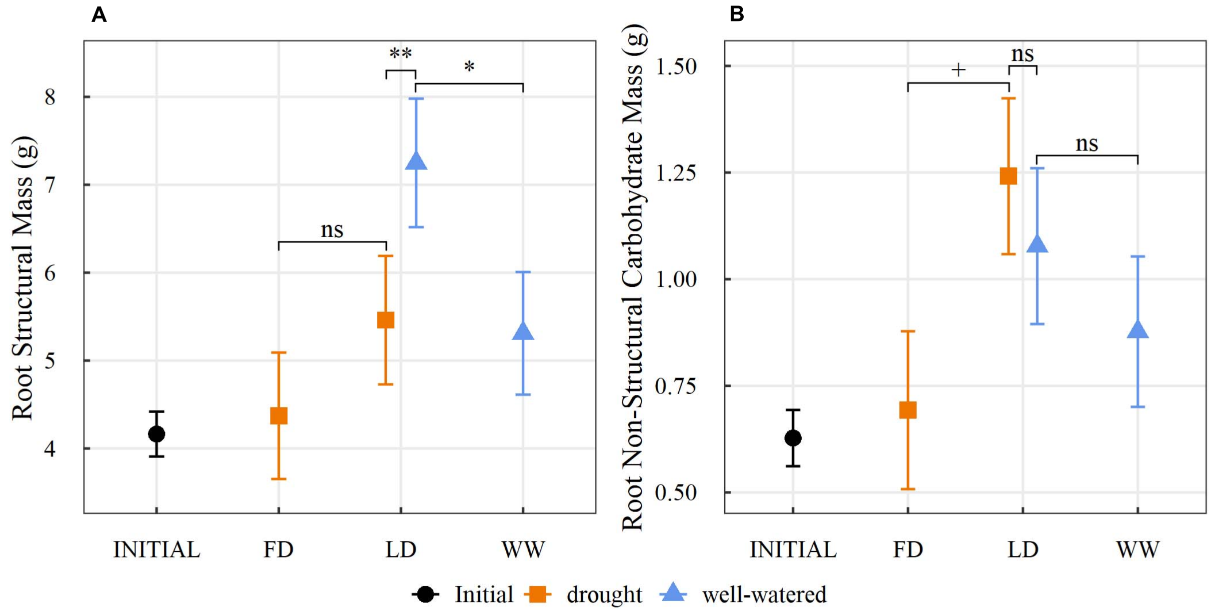

FIGURE 4 | Estimated marginal mean (±1 standard error) of **(A)** root structural mass (g) (the non-reserve portion of dry root mass) and **(B)** root non-structural carbohydrate mass (g) within the split root system for saplings prior to (INITIAL) and after the three watering treatments (FD: full drought, LD: localized drought, WW: well-watered) (orange: dry, blue: well-watered). Statistical differences between the three planned comparisons (FD vs. LD-dry, LD-dry vs. LD-wet, WW vs. LD-wet) are indicated by + for $p < 0.1$, * for $p < 0.05$, ** for $p < 0.01$ and ns for no significance ($n = 29$; $\alpha = 0.1$).

die (Ryser, 1996). Similar fine root deaths have been observed in other studies exploring drought and carbon limitation in aspen (Galvez et al., 2011, 2013; Wiley et al., 2017). Our observation of fine root and leaf abscission also supports the hypothesis that the more distal parts of plants whose role is primarily resource acquisition are potentially more expendable and/or more prone to damage under stress compared to other parts, such as larger diameter roots, stems and branches, which required significant investments over time and have additional crucial functions such as transport and storage within a plant (Kozlowski, 1973; Zimmerman, 1983; Tyree et al., 1993; Sperry and Ikeda, 1997; Landhäusser and Lieffers, 2012; Wiley et al., 2017). This may be especially important when considering a tree species (here aspen) with a root system that is adapted for long-distance resource acquisition (Snedden, 2013), as well as for the storage of NSC as reserves for post-disturbance regeneration (Wiley et al., 2019). Similar adaptations in root allocation have also been observed in other species growing in environments prone to water limitation and fire disturbance (Bell et al., 1996; Hoffman et al., 2004; Tomlinson et al., 2012).

The continued carbon acquisition in saplings under localized drought combined with an increased investment of carbon into the growth and maintenance of the root system allowed these plants to quickly adapt and generate new leaf mass and leaf area similar to the WW saplings, suggesting continued investment in leaf area development and its maintenance even under locally reduced water availability. Our results suggest that when a plant experiences drought but has access to areas in the soil that are less or non-limiting in moisture availability, the plant should be able to compensate. If the plant is able to use the portion of the root system that experiences the non-limiting conditions, it could potentially maintain the rest of the root system and continue to support aboveground growth and prevent leaf abscission. Interestingly, the LD saplings had overall similar total aboveground mass compared to the WW saplings, but they had less stem structural mass, suggesting that the WW saplings might have prioritized stem growth over other potential carbon allocation strategies. Instead, LD saplings increased stem starch concentrations from initial, which suggests a reserve storage strategy to potentially increase chances of rapid growth when conditions improve or for re-filling of xylem vessels when drought conditions worsen and cavitation occurs (Brodribb et al., 2010; Sala et al., 2012; Trifilò et al., 2017; Trugman et al., 2018). There were no differences in the overall NSC concentration of the combined root system among the three treatments. However, the FD saplings had two times higher soluble sugar concentration in the roots (9.54% dry weight) compared to the root system portion in the dry soil of the LD treatment (4.85% dry weight), which suggests a significant osmotic adjustment to improve the acquisition of water from the soil and avoid water loss back to the soil. This response is expected as it is well established that under drought stress, a higher solute concentration in roots will reduce their water potential and therefore increase the passive movement of water from the soil into the roots, in an attempt to relieve plant stress (Chaves, 1991; Gebre et al., 1998; Arndt et al., 2001; Kozlowski and Pallard, 2002; Galvez et al., 2011). Interestingly, while the localized drought conditions also led to an increase in soluble sugar concentration in the drought exposed portion of the root system, this increase was over 30% (4.85% dry weight) relative to the wet roots. This differential response of sugar concentration between the roots in the full and the localized drought might suggest a different strategy for how these plants cope with low soil water availability affecting only a portion of a root system (see below).

The fact that there was a striking difference between the degree of sugar accumulation between a full droughted root system (FD) and a partially droughted root system (LD) indicates that homo- or heterogeneous water limitations trigger different allocation responses within a root system. When faced with heterogeneous soil moisture availability, a root system showed very distinct patterns of structural and reserve mass allocation. Under the localized drought conditions, structural mass was allocated toward the portion of the root system that experienced the non-limiting condition and led to overall higher root mass and root volume. The increased allocation to root growth in the wetter soil likely allowed the saplings to compensate for the reduced water uptake in the part of the root system that experienced the low moisture availability, allowing the plant to maintain gas exchange and growth rates aboveground that were similar to those in saplings experiencing no water limitation. Additionally, the portion of the root system that experienced the limiting conditions likely preserved some of its functionality, as it maintained its mass and volume. We speculate that the preservation of the drying portion of the root system may have been favored in comparison to complete abscission, as the sapling had already invested in its establishment and the roots may be able to assist with future resource capture if the soil becomes rewetted. The benefits of maintaining a drying portion of a root system in comparison to complete abscission have been considered in other species subjected to heterogenous soil moisture conditions (Kosola and Eissenstat, 1994; Fort et al., 1997).

Since saplings in the LD treatment had access to water from the wet pot and the root system proliferated in these conditions, the demand for water supply from the dry portion of the root system was low, resulting in a reduced for need for osmotic adjustment via sugar accumulation (see above). However, the dry portion of the root system in a LD sapling accumulated significantly more starch than the wet portion of the root system, which might indicate a preferential allocation toward storage of reserves in the drier portion of the root system. These reserves would be available for future translocation or remobilization for growth, reproduction and/or other physiological processes such as osmotic adjustments in case drought conditions persist or worsen. This increase in NSC concentration in the dry portion of the root system could be driven by several possible mechanisms and processes within a plant. As mentioned previously, the lower soil water potential in the drought-exposed roots could have induced an active solute buildup for osmotic adjustment to allow for improved water uptake or the adjustment was more passive, where a lower turgor in the dry portion of the root system may have limited its growth, leading to an accumulation in NSCs due to reduced growth demand (Körner, 2003). Alternatively, the heterogeneous water availability could also have created a steeper gradient in water potentials across the entire root system, allowing for lateral water redistribution within the root system, increasing the hydration and with that the maintenance of functionality in the drought-exposed portion of the root system. Similar responses have been observed in other studies and species (Burgess and Bleby, 2006; Bleby et al., 2010; Prieto et al., 2012). By hydrating the roots experiencing water limitations from the portion of the root system that experienced less or non-limiting conditions, the risk of root cavitation and desiccation of the drought-exposed roots was likely reduced and these roots were more likely to maintain contact with the soil, enabling continued resource acquisition and other functional interactions such as mycorrhizae (Bleby et al., 2010).

This study highlights the importance of considering spatial heterogeneity of belowground resources when explaining above- and belowground responses of trees to stress. This is particularly important when studying mature trees that have extensive root systems. These trees most likely experience considerable vertical and lateral moisture gradients in the rooting space and within a root system. Since our application of the drought treatment was applied at the pot-level to assure that the drought at the root level was comparable, we recognize that the drought effects at the whole plant level were different among our treatments. Exploring these relationships and responses on these root systems in greater detail is further complicated by the generally poor accessibility of whole root systems (Hartmann et al., 2018). However, the results of our study demonstrate an adaptability and a multi-faceted response of root systems of a perennial species exposed to heterogeneous soil moisture environments. Depending on the soil moisture conditions, the root systems we studied exhibited plasticity in carbon allocation between structural mass and NSC, with differences in allocation between and within organs. The aspen saplings appeared to optimize functionality of the root system during water limiting conditions that affected only a portion of the root system by increasing root volume where water was locally available and preferentially accumulating additional NSC where root growth was limited. Our study highlights a need for exploring other potential measures of carbon allocation under stress, such as measures of total carbon and nitrogen. Short-term responses, like those noted here, will likely have impacts on how a plant will react to subsequent changes in stress conditions and might play a role in the adaptation and/or acclimation processes that have been observed in perennial plants exposed to stress over the short- and long-term (Rachmilevitch et al., 2008; Pomiès et al., 2017). In our short-term study, aspen saplings responded relatively rapidly to moisture stress by enhancing the functionality of the entire root system through adaptation [increase in root system size (LD) or leaf and root loss (FD)] and acclimation processes (accumulation of reserves), which can be considered beneficial even under prolonged drought conditions, as roots are a critical organ in aspen not only for resource uptake, but also for maintaining its resilience (i.e., vegetative regeneration) to disturbances.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AH, MM, and SL crafted the main research questions and experimental design. AH and MM collected the data and performed the data analyses. AH wrote the manuscript. MM, EW, and SL revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.654159/full#supplementary-material

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Management After Windstorm Affects the Composition of Ectomycorrhizal Symbionts of Regenerating Trees but Not Their Mycorrhizal Networks

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Due to ongoing climate change, forests are expected to face significant disturbances more frequently than in the past. Appropriate management is intended to facilitate forest regeneration. Because European temperate forests mostly consist of trees associated with ectomycorrhizal (ECM) fungi, understanding their role in these disturbances is important to develop strategies to minimize their consequences and effectively restore forests. Our aim was to determine how traditional (EXT) and nonintervention (NEX) management in originally Norway spruce (*Picea abies*) forests with an admixture of European larch (*Larix decidua*) affect ECM fungal communities and the potential to interconnect different tree species *via* ECM networks 15 years after a windstorm. Ten plots in NEX and 10 plots in EXT with the co-occurrences of Norway spruce, European larch, and silver birch (*Betula pendula*) were selected, and a total of 57 ECM taxa were identified using ITS sequencing from ECM root tips. In both treatments, five ECM species associated with all the studied tree species dominated, with a total abundance of approximately 50% in the examined root samples. Because there were no significant differences between treatments in the number of ECM species associated with different tree species combinations in individual plots, we concluded that the management type did not have a significant effect on networking. However, management significantly affected the compositions of ECM symbionts of Norway spruce and European larch but not those of silver birch. Although this result is explained by the occurrence of seedlings and ECM propagules that were present in the original forest, the consequences are difficult to assess without knowledge of the ecology of different ECM symbionts.

Keywords: disturbances, diversity, ectomycorrhizal fungi, exploration types, mycorrhizal networks

INTRODUCTION

Windstorms represent one of the major factors influencing European temperate forests (Fischer et al., 2013), and their intensity has been increasing during the last few decades (Gregow et al., 2017). Depending on wind severity, the influence of windstorms may extend from single trees to large areas of forests. Large windstorms, causing forest breakdown, affect the forest ecosystem in a complex way. Changes in forest structure lead to overall changes in microhabitat conditions, such as increased solar radiation connected to increased temperatures and changes in water availability (Gömöryová et al., 2008), nutrient cycling (Don et al., 2012), and vegetation cover (Dietz et al., 2020). Because European temperate forests mainly consist of trees living in symbiosis with ectomycorrhizal (ECM) fungi (Martin et al., 2016), these fungal symbionts also face these changes.

Severe windstorms generally lead to decrease in ECM fungal diversity and changes in their community composition (Egli et al., 2002; Ford et al., 2018; Vašutová et al., 2018). Subsequent management practices, such as clear-cut logging, usually enhance these changes as a possible consequence of soil mechanical damage (Ford et al., 2018; Vašutová et al., 2018). The main driver of fungal community shifts is the death of mature trees (Karst et al., 2014; Treu et al., 2014), because ECM fungi are obligate symbionts unable to live without their hosts (Baldrian, 2009; Lofgren et al., 2018). This results in an extinction of ECM fungal species associated with mature trees (= late-successional ECM fungi), while early-successional ECM fungi, with a low demand on photosynthates, survive on seedlings (Egli et al., 2002; Vašutová et al., 2018; Veselá et al., 2019b). Fungal inoculum may further persist in soil for some time as spores or hyphae emanating from dying or recently dead root tips (Simard, 2009; Guignabert et al., 2018). ECM species known to be able to survive or rapidly re-establish after disturbances are, e.g., *Cenococcum* sp., *Laccaria* sp., *Piloderma* sp., *Rhizopogon* sp., *Thelephora* sp., or *Wilcoxina* sp. (Visser, 1995; Jones et al., 2003; Kranabetter et al., 2004; Twieg et al., 2007).

The ability to succeed in young forest stages depends on various functional traits of the ECM fungal species, i.e., on their enzymatic activity, which enables them to decompose various substrates and mobilize nutrients (Velmala et al., 2014; Haas et al., 2018); spore traits which improve their resistance in soil (Jones et al., 2003); and morphology of mycorrhizas represented by exploration type which influences nutrient exploitation from soil and interconnection of trees through the mycorrhizal network (Agerer, 2001; Rosinger et al., 2018). For example, some *Piloderma* species secrete hemicellulases and hydrolytic enzymes involved in litter degradation, while *Wilcoxina* sp. is effective in degrading chitin (Velmala et al., 2014). Higher nitrate uptake and retranslocation to the plant host was also observed in *Laccaria laccata* compared with *Suillus bovinus* (Hobbie et al., 2008). The ascomycete species *Cenococcum geophilum* is supposed to be low carbon demanding due to having a thin mantel and short-distance exploration type (Guignabert et al., 2018; Defrenne et al., 2019), while long-distance explorers belonging to the genus *Rhizopogon* were found to dominate in dry, nutrient-poor areas, since they are able to transport resources from longer distances (Bakker et al., 2006).

Each individual tree can be associated with many ECM fungal species (Bahram et al., 2011), and each ECM fungal species differs in its specificity (Molina and Horton, 2015). ECM fungi with narrow host range (e.g., some *Leccinum*, *Rhizopogon*, and *Suillus* species) are usually restricted to a specific host genus, while ECM fungi with a broad host range (e.g., *Boletus*, *Paxillus*, and *Laccaria* species) are known to be associated with nearly every tree species that form ectomycorrhizae (Molina et al., 1992). ECM fungi with broad host ranges dominate in ECM fungal communities (Molina et al., 1992; Horton and Bruns, 1998; Taudiere et al., 2015; van der Linde et al., 2018; Defrenne et al., 2019; Rog et al., 2020). These fungi can interconnect different tree species and influence nutrient exchange among them (Simard et al., 1997; van der Heiden and Horton, 2009). On the other hand, almost all host tree species harbor in their symbiotic fungal assembly genus-restricted ECM fungi, which provide them exclusive access to resources (Molina et al., 1992). Nevertheless, ECM fungi with a broad host range may also show host preference depending on the ecological context (Lang et al., 2011), while genus-restricted ECM fungi may expand their host range in the absence of their primary host (Lofgren et al., 2018).

The ECM fungal community is a dynamic system interconnecting trees of the same species, but also different tree species through the mycorrhizal network (Simard et al., 1997; Klein et al., 2016; Rog et al., 2020). This phenomenon is not negligible when considering that up to approximately 40% of fine-root carbon can originate from mycorrhizal network exchange (Klein et al., 2016). Not only carbon can be transferred through the mycorrhizal network, but also water and other nutrients, or signaling molecules (Gorzelak et al., 2015). The mycorrhizal network is influenced by tree physiology, e.g., photosynthetic and growth rates, nutrient content, forest age, or infestation (Simard et al., 1997, 2012; van der Heiden and Horton, 2009; Song et al., 2015; Simard, 2018); the phylogenetic relationships among trees involved in the network (Rog et al., 2020); environmental conditions (Rosinger et al., 2018; Defrenne et al., 2019); and also by the ECM fungi themselves which protect their carbon donors (Song et al., 2015). This system is expected to be very dynamic and vulnerable to large disturbances, as demonstrated by Pec et al. (2020).

Severe windstorm damaged approximately 12,000 ha of mountain spruce forest in the Tatra Mts. (Slovakia) in 2004 (Fleischer, 2008). In most of the affected area, the fallen trees were extracted (EXT), while a small part was left for natural succession (NEX). To bring new perspectives to the discussion on the suitability of subsequent management, we were interested in how the windstorm and following timber harvest affected the ECM fungal community and its potential to form mycorrhizal networks among regenerating tree species, Norway spruce, European larch, and silver birch. We hypothesized that ECM fungi with a broad host range will prevail in plots with traditional management (e.g., fallen tree extraction) and that different tree species will therefore share a high percentage of ECM fungal species. On the other hand, plots left for natural succession will harbor more ECM fungi with narrow host range associated with target tree species, with fewer ECM fungal species shared among them. We also hypothesized that the number of ECM fungal species would decrease in managed plots due to soil compaction and mechanical damage to seedlings that survive and the ECM fungal extramatrical mycelium.

MATERIALS AND METHODS

Study Site

The research plots are situated in the Tatra Mts. (Slovakia) in the Tatra National Park (**Supplementary Figure 1**). This area was affected by severe windstorm in 2004 during which about 12,000 ha of forest was destroyed. The area was originally covered by a Norway spruce (*Picea abies*) forest with an admixture of European larch (*Larix decidua*), and Scotch pine (*Pinus sylvestris*). Currently, this area is dominated by young trees of Norway spruce, birches (*Betula* sp.), European larch, and European rowan (*Sorbus aucuparia*) in variable proportions depending on the microhabitat conditions. Locally, Scots pine, goat willow (*Salix caprea*), and silver fir (*Abies alba*) are present. The area is characterized by a cold climate and has a mean annual temperature of 5.3°C and a mean annual precipitation of 833 mm (Fleischer, 2008). The soils in the plots are dystric cambisols on glacial moraine deposits with a loamy sand texture, an acidic pH of approximately 3.0 (Don et al., 2012), and carbon and nitrogen percentages of approximately 11.9 and 0.6%, respectively (Vašutová et al., 2018).

Following this disturbance event, permanent research sites with different management regimes were established in 2005, and each research site is about 100 ha wide (Fleischer, 2008). Within the current research, we studied two treatments: (i) site with traditional management (EXT), where fallen trees were extracted and the locality was consequently reforested, and (ii) a site without management (NEX) left for natural succession, in which fallen trees were left. Ten circular plots (d = 12.6 m and P = 125 m^2) per treatment were selected so that there were at least two individuals of each tree species of interest, Norway spruce, European larch, and silver birch (*Betula pendula*), and the presence of other ECM tree species was avoided to minimize influencing the ECM fungal community.

Ectomycorrhizae on Tree Roots

ECM root samples (each consists of about 100 root tips) from 20 plots were collected during 2 years: three plots per treatment in September 2018 and seven plots per treatment in September 2019 (**Supplementary Table 1**). In each plot, two individuals for each tree species were selected, and three root samples from each individual tree were collected. The cover of trees, shrubs, herbs, mosses, and dead wood was visually estimated (**Supplementary Table 1**). Dead wood was classified into five categories (lying logs, piles of branches, root collars, stubs, and stumps).

The height and perimeter of each selected tree were measured (**Supplementary Table 1**). For each root sample, its depth, soil horizon, distance from the tree stem, and direction to other tree species were recorded (**Supplementary Table 1**). In total, 60 root samples from each tree species per treatment (2 trees × 3 root samples per plot × 10 plots) were sampled, with the exception of Norway spruce (due to the misidentification of one of the root samples, only 59 samples were analyzed).

The identity of the roots was guaranteed by direct tracing from a tree stem; in disputable cases, the identity was confirmed using chloroplast DNA sequence comparison against the GenBank database (Benson et al., 2007) using MegaBlast algorithm, with 97% similarity threshold as a molecular species criterion.

Processing of Root Samples

Each root sample was carefully washed under tap water, and all attached soil particles were removed using tweezers in a Petri dish with water. The root samples were examined under a stereomicroscope and documented (Canon 1,000D, Tokyo, Japan). The ECM root tips of each root sample were sorted into morphotypes according to their morphological features: color, presence of emanating hyphae and rhizomorphs, cystidia, and ramification (Agerer, 1991; Agerer and Rambold, 2004–2015). Each morphotype was excised and stored in a tube with silica gel. At least one morphotype per tree species and treatment was then used for molecular identification.

DNA Extraction and Molecular Identification

Genomic DNA was extracted from the selected ECM root tips using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was then used as a template for PCR amplification of the rDNA ITS region with the primer pairs ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993) for fungal species identification and of the noncoding region of chloroplast DNA with the primers *trn*L/*trn*F (Taberlet et al., 1991) for plant species identification. In case that we obtained multiple fungal amplicons from a root tip, we used primer pairs specific for basidiomycetous (ITS1F/ITS4B; Gardes and Bruns, 1993; Nikolcheva and Bärlocher, 2004) or ascomycetous fungi (ITS1/ITS4A; White et al., 1990; Nikolcheva and Bärlocher, 2004). When the amplicon separation was unsuccessful, we cut the bands directly from gel using QIAquick Gel Extraction Kit (Qiagen). PCR was performed in a 25-μl reaction mixture containing 1× reaction buffer (Bioline, London, United Kingdom), 500 nM of each primer, 0.75 U of MyTaq DNA polymerase (Bioline), and ∼20 ng of template DNA filled with dH$_2$O up to 25 μl. The amplification conditions were 94°C for 2.5 min, followed by 37 cycles at 94°C for 30 s, 55°C for 40 s, 72°C for 30 s, and a final extension at 72°C for 4.5 min for the fungal amplicons; 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1.2 min, and a final extension at 72°C for 10 min for the plant amplicons. Sequencing was performed by Macrogen Inc., Amsterdam, Netherlands, on an ABI 3730 XL automated sequencer (Applied Biosystems, Foster City, CA, United States).

Molecular Data Analysis

Sequence chromatograms were analyzed using Chromas Lite v.2.6.5 (Technelysium Pty Ltd, Brisbane, Australia), and the sequences were blasted against the UNITE (Abarenkov et al.,

2010) and GenBank (Benson et al., 2007) public databases. A 97% similarity threshold was used as a molecular species criterion and 90% similarity threshold as a molecular genus criterion (Nilsson et al., 2019; Vu et al., 2019). The sequences were deposited in GenBank (accession numbers: MT908274–MT908330). Based on the molecularly identified morphotypes, species names were given to all the documented morphotypes.

The assignment of ECM taxa into exploration types was made according to Agerer (2001), Agerer and Rambold (2004–2015), and our observations (**Supplementary Table 2**).

Statistical Analysis

The numbers of ECM fungal species shared by all tree species, a combination of two tree species, or unique for a single tree species were calculated for the entire dataset, EXT and NEX treatments and separately for each plot. The same approach was used in assessing the number of species with the exploration types with rhizomorphs (long-distance exploration type, medium-distance fringe exploration type, and medium-distance smooth exploration type), which could in particular provide networking.

The tree heights and perimeters per tree/tree species for the NEX and EXT treatments and the numbers of ECM fungal species per tree/tree species for the NEX and EXT treatments were tested by analysis of variance with Tukey's *post hoc* contrasts in R 3.6.2 (R Development Core Team, 2019).

Rarefaction curves, using R package mobr (McGlinn et al., 2018), were prepared for all tree species within the NEX and EXT treatments (**Supplementary Figure 2**).

Three datasets were prepared for relating the plot variables (n = 20), trees (n = 120), and root samples (n = 359). Presence/absence data (roots) and number of root samples with each morphotype (trees, plots) were used to avoid difficulties in the proper identification of not well-developed morphotypes. The datasets were separately analyzed by multivariate methods in Canoco 5 program (ter Braak and Šmilauer, 2012), using detrended correspondence analysis (DCA) and canonical correspondence analysis (CCA). DCA was used to compare the ECM fungal species compositions in plots, trees, and roots. The year of sampling was used as a covariate. CCA was used to identify (i) the effects of management, vegetation cover, and geographical position on ECM fungal species composition in plots; (ii) the effects of management, tree species, height, and perimeter on ECM fungal species composition in trees; and (iii) the effects of management, tree species, distance from tree stem, and soil horizon on the ECM fungal species composition in roots. The dataset trees was divided based on tree species identity, and the resulting datasets were further analyzed separately. Various environmental variables were tested as explanatory variables in CCA analyses. Year (for all datasets) and plot identifier in interaction with year (only for datasets roots and trees) were used as covariates. We used hierarchical split-plot design for permutations and permutated whole plots (i.e., keeping the data from one plot together) for testing the effect of management. These whole plots were permuted at random within the covariate year, and the two split plots (trees) were not permuted. The effect of tree species was tested by split-plot permutations (within the whole plot, i.e., the data from different tree species were permutated within the plot). The effects of explanatory variables were summarized in partial analyses after removing the effects of covariates. The Monte Carlo permutation test was used and only significant variables were selected.

The effects of tree species and management on the number of ECM fungal species for tree were analyzed by linear mixed-effects model fit by REML using the lme function in nlme package in R 3.6.2., with plot as a random effect. The effect of plot was tested by comparing the model without plot (made by gls function) and the model with plot (made by lme function) by function ANOVA.

The numbers of shared and specific ECM fungal species in NEX and EXT treatments on *P. abies*, *L. decidua*, and *B. pendula* were presented by Venn diagrams made in web application BioVenn (Hulsen et al., 2008).

Two root samples (19Pa_2b and 2Ld_1a) and one tree sample (9Pa) were excluded from analysis as outliers.

RESULTS

ECM Fungal Species and Potential ECM Networks

In total, 57 ECM taxa were detected (**Supplementary Table 2**): 51 taxa were assigned at the species level, two at the genus level, and four at the order level. There were no significant differences in the number of ECM fungal species per tree between the NEX and EXT treatments ($p > 0.05$, **Table 1**). However, differences among tree species were detected; silver birch had significantly higher number of ECM fungal species per tree than Norway spruce and European larch ($p < 0.005$), and Norway spruce and European larch did not differ significantly ($p > 0.05$, **Table 1**).

The tree heights and perimeters within a treatment were significantly greater for silver birch than for the other tree species ($p < 0.001$, **Table 1**). In EXT, tree heights ($p < 0.001$, **Table 1**) and perimeters ($p < 0.005$, **Table 1**) were significantly greater than in NEX.

Fourteen ECM taxa were found in all tree species, five species were detected in Norway spruce and European larch, seven in Norway spruce and silver birch, five in European larch and silver birch, and eight were unique to Norway spruce, 10 to European larch, and 14 to silver birch. When comparing the

TABLE 1 | Mean number of ectomycorrhizal (ECM) fungal species per tree, mean tree height, and mean tree perimeter in NEX (plots without management) and EXT (plots with traditional management) treatments and in Norway spruce, European larch, and silver birch.

	Number of ECM fungal species/tree	Mean tree height (m)	Mean tree perimeter (cm)
NEX	4.5 ± 0.3	3.7 ± 0.2	5.8 ± 0.5
EXT	4.4 ± 0.2	4.8 ± 0.3	7.9 ± 0.6
		NEX/EXT	NEX/EXT
Norway spruce	4.3 ± 1.4	3.0 ± 0.2/3.9 ± 0.4	4.3 ± 0.4/5.6 ± 0.7
European larch	3.2 ± 2	3.1 ± 0.3/4.7 ± 0.4	4.3 ± 0.6/7.1 ± 0.7
silver birch	5.5 ± 1.4	5.1 ± 0.5/5.9 ± 0.4	8.8 ± 1.1/11.1 ± 1.1

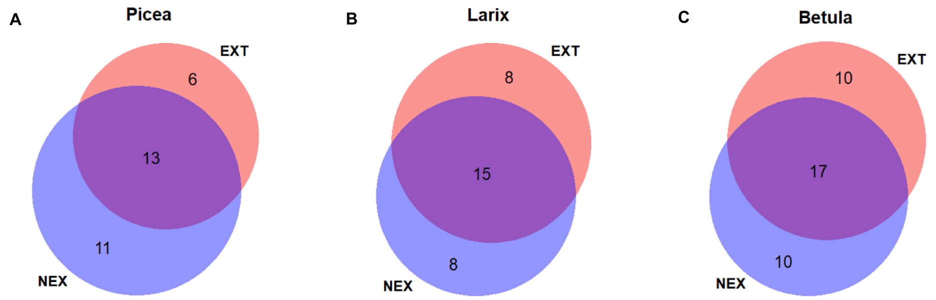

FIGURE 1 | Venn diagram displaying the numbers of shared and specific ectomycorrhizal (ECM) fungal species in NEX (plots without management) and EXT (plots with traditional management) treatments on **(A)** *Picea abies*, **(B)** *Larix decidua*, and **(C)** *Betula pendula*.

numbers of ECM fungal species in single tree species between the NEX and EXT treatments (**Figure 1**), only Norway spruce had a lower number of ECM fungal species in EXT (six species) compared with NEX (11 species). When focusing on the potential mycorrhizal networking among trees in individual plots, we found no significant differences between NEX and EXT in the numbers of ECM fungal species that were shared by all host trees, between individual pairs of host trees, or associated with a single host tree only.

Overall, 12 ECM species belonged to contact exploration type, 15 species to short-distance type, eight species to medium-distance smooth type, 12 species to medium-distance fringe type, and 11 species to long-distance type (**Supplementary Table 2**). When considering only the numbers of rhizomorph-forming ECM fungal species, i.e., medium-distance and long-distance exploration types, more ECM species shared by all tree species were found in NEX compared with EXT, but these differences were not significant (**Supplementary Table 3**). In case of NEX treatment, the potential networkers were *Cortinarius bataillei*, *C. croceus*, *Paxillus involutus*, *Piloderma olivaceum*, and *Thelephora terrestris*, and in case of EXT, the potential networkers were *P. olivaceum* and *T. terrestris*.

There were also no significant differences in the numbers of shared species among combinations of two tree species or in single tree species between NEX and EXT treatments.

Five ECM fungal species dominated in both treatments: *Cenococcum geophilum*, *Lactarius rufus*, *P. involutus*, *P. olivaceum*, and *T. terrestris* (**Table 2**). Most of these species were detected in all tree species and together accounted for 50.1% in the NEX plots and 51.7% in the EXT plots. The potential of these species to form mycorrhizal networks differed only for the case of *P. involutus*, which was absent in EXT on Norway spruce.

ECM fungal species that were unique to individual tree species were generally rare. For Norway spruce, *Piloderma bicolor* (0.5%, both NEX and EXT) was present in both treatments; in NEX, *Amphinema byssoides* (1.8%), Helotiales1 (0.8%), and *Imleria badia* (0.5%) were present; in EXT, *Tylopilus felleus* (0.8%) was present. For European larch, *Suillus gravillei* (in NEX 0.8% and in EXT 2.6%) was present in both treatments; in NEX, *Cortinarius cinnamomeus* (0.8%) was present; in EXT, *C. parvanulatus* (0.5%), *S. cavipes* (0.3%), and *S. viscidus* (1.8%) were present. For silver birch, *Leccinum holopus* (1.5% in both NEX and EXT), *L. variicolor* (in NEX 3.5% and in EXT 3.3%), and *Meliniomyces bicolor* (in NEX 0.5% and in EXT 0.8%) were present in both treatments; in NEX, *C. porphyropus* (0.5%), *Inocybe soluta* (0.8%), *Laccaria proxima* (0.5%), and *L. glyciosmus* (2.3%) were present; in EXT, *Amanita muscaria* (0.5%) and *Clavulina* sp. (0.5%) were present.

Management Effect on ECM Fungal Species Composition

Management significantly influenced the ECM fungal species composition in the studied plots and explained 11.7% of the detected variability (**Table 3**). The other studied environmental variables (vegetation and dead wood cover, geographic position, morphometric parameters of host trees—height, perimeter, and root placement in soil) had no significant effect.

Regarding the community composition of ECM fungal species based on the tree dataset, the only significant variables were tree species and management type (**Table 3** and **Figure 2**). The morphometric parameters of trees (height and perimeter) had no effect on the ECM fungal species composition. When the tree species were analyzed separately, only the ECM fungal species compositions of Norway spruce and European larch were affected by the management type (**Table 3** and **Figures 3A,B**). In Norway spruce trees, *C. croceus*, *Meliniomyces vraolstadiae*, *P. involutus*, *T. terrestris*, and *T. fibrillosa* were more abundant in NEX, whereas *A. byssoides*, *L. rufus*, and *T. asterophora* were more abundant in EXT. In European larch trees, *C. bataillei*, *L. laccata*, *P. olivaceum*, and *T. terrestris* were more abundant in NEX and

TABLE 2 | Dominant ECM fungal species present in NEX (plots without management) and EXT (plots with traditional management) treatments.

Dominant ECM fungi (% of the root samples)	Exploration type	NEX	EXT
Cenococcum geophilum	SD	11.8	13.6
Lactarius rufus	C	8.6	15.7
Paxillus involutus	LD	12.1	9.3
Piloderma olivaceum	MDf	8.3	6.7
Thelephora terrestris	MDs	9.3	6.4

C, contact type; LD, long-distance type; MDf, medium-distance fringe type; MDs, medium-distance smooth type; SD, short-distance type.

TABLE 3 | Results of canonical correspondence analyses (CCA) of ECM fungal community.

Dataset	Explanatory variables	Covariates	Explained variability	Pseudo-*F*	*p*
Roots	Tree species	Plot × year	4.7	7.9	0.001
	Management type	Year	1.0	3.4	0.001
	Soil horizon	Plot × year	2.1	2.2	0.001
Trees	Tree species	Plot × year	9.9	5.4	0.001
	Management type	Year	2.2	2.4	0.003
Picea abies	Management type	Year	5.3	2.1	0.003
Larix decidua	Management type	Year	4.6	1.8	0.019
Betula pendula	Management type	Year	*3.6*	*1.4*	*0.193*
Plots	Management type	Year	11.7	2.2	0.001

Datasets were based on the presence of ECM fungal species in plots, trees, and roots. Variations in ECM fungal community data explained by selected explanatory variables were summarized after removing the effects of covariates (year, interaction of plot and year).

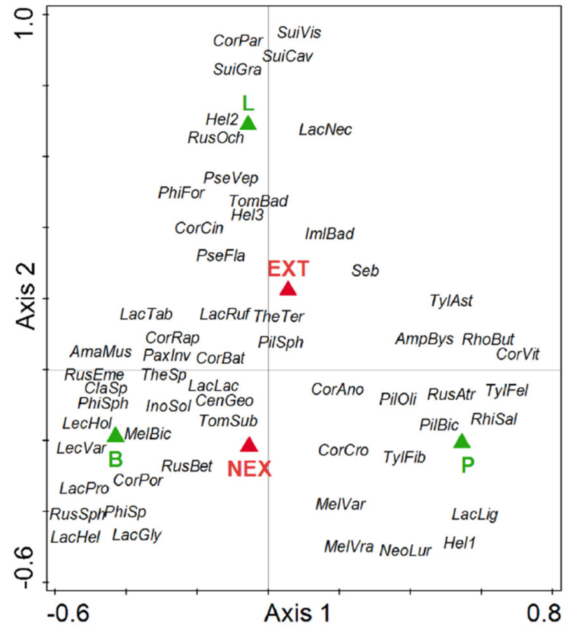

FIGURE 2 | Ordination diagram from canonical correspondence analysis (CCA) of ECM fungal species presence on tree species (P—*Picea abies*, L—*Larix decidua*, and B—*Betula pendula*) in NEX (plots without management) and EXT (plots with traditional management) treatments. Species: AmaMus—*Amanita muscaria*, AmpBys—*Amphinema byssoides*, CenGeo—*Cenococcum geophilum*, ClaSp—*Clavulina* sp., CorAno—*Cortinarius anomalu*, CorBat—*Cortinarius bataillei*, CorCin—*Cortinarius cinnamomeus*, CorCro—*Cortinarius croceus*, CorPar—*Cortinarius parvannulatus*, CorPor—*Cortinarius porphyropus*, CorRap—*Cortinarius raphanoides*, CorVit—*Cortinarius vitiosus*, Hel1—Helotiales1, Hel2—Helotiales2, Hel3—Helotiales3, ImlBad—*Imleria badia*, InoSol—*Inocybe soluta*, LacLac—*Laccaria laccata*, LacPro—*Laccaria proxima*, LacGly—*Lactarius glyciosmus*, LacHel—*Lactarius helvus*, LacLig—*Lactarius lignyotus*, LacNec—*Lactarius necator*, LacRuf—*Lactarius rufus*, LacTab—*Lactarius tabidus*, LecHol—*Leccinum holopus*, LecVar—*Leccinum variicolor*, MelBic—*Meliniomyces bicolor*, MelVar—*Meliniomyces variabilis*, MelVra—*Meliniomyces vraolstadiae*, NeoLur—*Neoboletus luridiformis*, PaxInv—*Paxillus involutus*, PhiFor—*Phialocephala fortinii*, PhiSph—*Phialocephala sphaeroides*, PilBic—*Piloderma bicolor*, PilOli—*Piloderma olivaceum*, PilSph—*Piloderma sphaerosporum*, PseFla—*Pseudotomentella flavovirens*, PseVep—*Pseudotomentella vepallidospora*, RhiSal—*Rhizopogon salebrosus*, RhoBut—*Rhodocollybia butyracea*, RusAtr—*Russula atrorubens*, RusBet—*Russula betularum*, RusEme—*Russula emetica*, RusOch—*Russula ochroleuca*, RusSph—*Russula sphagnophila*, Seb—Sebacinales, SuiCav—*Suillus cavipes*, SuiGra—*Suillus grevillei*, SuiVis—*Suillus viscidus*, TheSp—*Thelephora* sp., TheTer—*Thelephora terrestris*, TomBad—*Tomentella badia*, TomSub—*Tomentella sublilacina*, TylFel—*Tylopilus felleus*, TylAst—*Tylospora asterophora*, and TylFib—*Tylospora fibrillosa*.

Suillus spp. in EXT. The ECM fungal species composition of silver birch was not influenced by the studied variables (**Table 3** and **Figure 3C**). The ECM fungal species composition, based on the root sample dataset, was significantly affected by tree species, management type, and soil horizon (**Table 3** and **Figure 4**).

DISCUSSION

Potential Mycorrhizal Networks and ECM Fungal Diversity

Our study on the effect of different management practices on ECM fungal communities of regenerating trees in the post-windstorm area in the Tatra Mts. revealed no effect on dominant ECM fungi and their potential to form mycorrhizal networks. For both treatment types, five ECM fungal species with broad host range dominated and included both early-colonizers (*C. geophilum*, *P. involutus*, and *T. terrestris*) and late-successional ECM fungi (*L. rufus* and *P. olivaceum*; Visser, 1995; Twieg et al., 2007; Kim et al., 2021). The windstorm apparently affected forest ecosystem in such an extreme manner that the management type produced only insignificant adjustments and had only limited influence on the potential to form ECM networks.

In contrast to our hypothesis, we also found almost no difference in the total number of potentially shared ECM fungal species among different tree species between the traditionally managed plots (EXT) and plots without management (NEX). Even if we focus on ECM species with exploration types with rhizomorphs, we found no significant differences, although the numbers of species shared by all tree species slightly differ in favor of NEX. Exploration types with rhizomorphs are more carbon demanding compared with contact and short-distance types (Defrenne et al., 2019; Wasyliw and Karst, 2020); on the other hand, they are able to form common mycelial networks and exploit water and nutrients from a larger area (Simard et al., 2003; Bakker et al., 2006). So, a higher number of these species per plot in NEX could be explained by lower damage to the mycelium caused by management practices, since hyphae are able to emanate from dying or recently dead root tips for a short time (Simard, 2009).

Norway spruce was the only tree species, which showed a higher number of specific ECM fungal species in NEX than in EXT (**Figure 1**). This difference is also evident from rarefaction curves (**Supplementary Figure 2**). In contrast to other tree species in our study, Norway spruce is a shade-tolerant

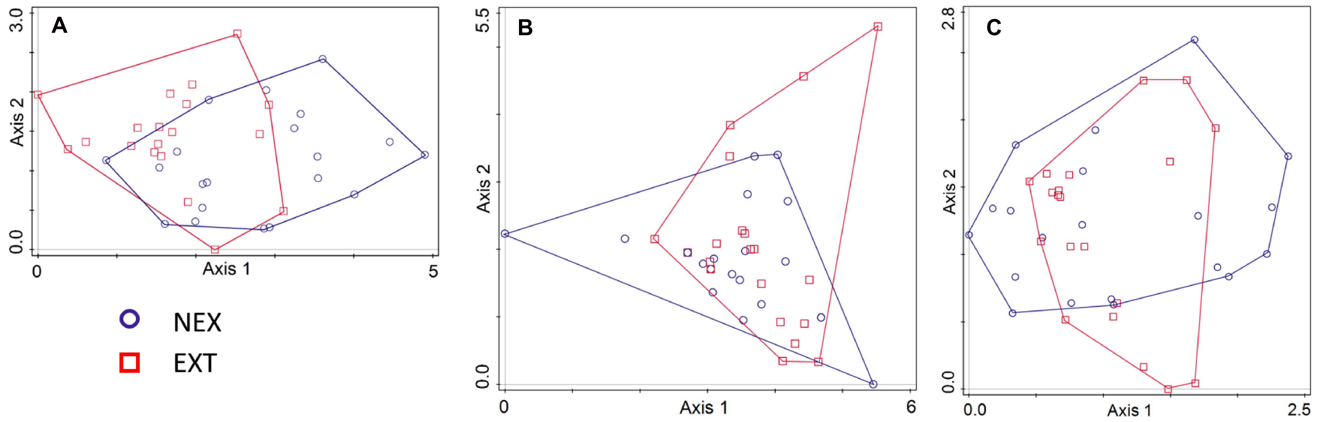

FIGURE 3 | Ordination diagram from detrended correspondence analysis (DCA) with the ECM fungal species composition in NEX (plots without management) and EXT (plots with traditional management) treatments for single tree species: **(A)** *Picea abies*, **(B)** *Larix decidua*, and **(C)** *Betula pendula*.

species, which is able to regenerate under mature forest canopies (Metslaid et al., 2007). Moreover, Norway spruce requires an adequate water supply. Gömöryová et al. (2008) detected lower soil moisture levels 2 years after the windstorm in EXT compared with NEX, which could, along with the increased insolation following tree removal, negatively affect spruce fitness. The better Norway spruce seedling fitness in NEX (although not supported by morphological characteristics) combined with the connectivity of the original forest could explain the differences observed. In contrast, both silver birch and European larch possessed the same numbers of specific ECM symbionts in both treatments. The highest ECM fungal species richness in silver birch can be the consequence of its larger dimensions related to its pioneer strategy and higher adaptability to clear-cut sites (Dubois et al., 2020). Similar findings were also reported by Twieg et al. (2007), who detected more ECM fungal species on paper birch than Douglas fir in young stands after clear-cut logging. However, in that case, the higher species richness of paper birch was also attributed to the presence of birch stumps.

No or small differences in quantitative parameters between treatments could be also related to a common problem in evaluating the responses of fungal communities to environmental factors or management. Generally, most fungal communities are composed of a small number of dominant species with a wide ecological valence that only respond to very pronounced gradients. These species are accompanied by few subdominant species and numerous rare species. Due to their rarity and the patchiness of ECM fungi dropping after 3–4 m (Lilleskov et al., 2004; Pickles et al., 2012), it is impossible to disentangle the random occurrence of these species and their response to environmental factors without including many more research plots and more intensive root sampling. Rosinger et al. (2018) found the majority of ECM taxa in one or two plots out of approximately 100 plots. While the overall fungal diversity in temperate forests is high, the species diversity at small scales is low and does not usually exceed 10–20 species (Tedersoo et al., 2003; Anderson et al., 2014). Thus, the theoretical absence of a few species for a total area of 10 times 125 m^2 could indicate larger losses at larger scales. However, this hypothesis must be verified by further research focusing on the ecology of rare ECM fungal species, in which all the above mentioned limitations would be taken into consideration (i.e., rarity and patchiness of ECM fungal species and sampling effort). The combination of fruit body monitoring together with environmental soil sequencing could be a promising nondestructive method, but unfortunately without data on the associations with host trees.

Effect of Management on ECM Fungal Species Composition 15 Years After a Windstorm

Interestingly, management is the only significant variable that explains ECM fungal species composition 15 years after a windstorm on the studied plots and for Norway spruce and European larch trees. The similarity of the ECM fungal communities did not reflect the phylogenetic relationships of the studied trees, which contradicts to the observations of Rog et al. (2020).

The observed differences in ECM fungi composition between NEX and EXT in Norway spruce are difficult to explain because there are no clear patterns in exploration types (Agerer, 2001) or spore characteristics (Bernicchia and Gorjón, 2010; Knudsen and Vesterholt, 2012). We also do not expect differences in ability to spread, because both treatments contain species with pileate and resupinate fruit bodies (Lilleskov and Bruns, 2005; Halbwachs et al., 2016). However, the affinity of *T. fibrillosa* and *T. terrestris* for NEX and of *A. byssoides* and *T. asterophora* for EXT has been previously observed in 4–9-year-old seedlings from a similar area in the Tatra Mts. (Vašutová et al., 2018), and these differences persist in Norway spruce between treatments. These differences are difficult to evaluate without detailed knowledge of the ECM fungal ecology and physiology. European larch trees are often occupied by *C. bataillei*, *L. laccata*, *P. olivaceum*, and *T. terrestris* in NEX and by *Suillus* spp. in EXT. Coleman et al. (1989) found several *Suillus* spp. to be drought tolerant, and in contrast,

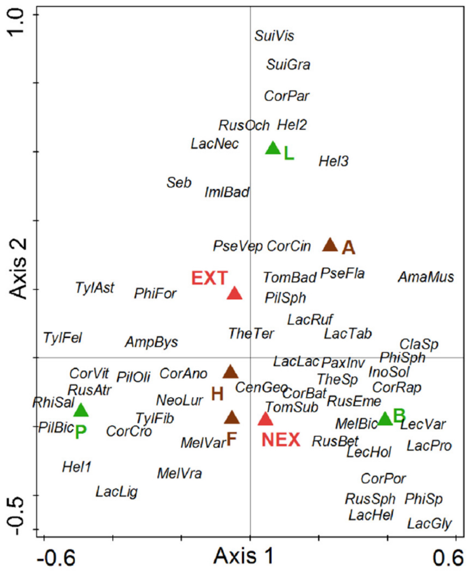

FIGURE 4 | Ordination diagram from canonical correspondence analysis (CCA) of ECM fungal species presence on tree roots in NEX (plots without management) and EXT (plots with traditional management) treatments for single tree species (P—*Picea abies*, L—*Larix decidua*, and B—*Betula pendula*) differentiated according to organic (F, H) and organo-mineral (A) soil horizons. Species: AmaMus—*Amanita muscaria*, AmpBys—*Amphinema byssoides*, CenGeo—*Cenococcum geophilum*, ClaSp—*Clavulina* sp., CorAno—*Cortinarius anomalu*, CorBat—*Cortinarius bataillei*, CorCin—*Cortinarius cinnamomeus*, CorCro—*Cortinarius croceus*, CorPar—*Cortinarius parvannulatus*, CorPor—*Cortinarius porphyropus*, CorRap—*Cortinarius raphanoides*, CorVit—*Cortinarius vitiosus*, Hel1—Helotiales1, Hel2—Helotiales2, Hel3—Helotiales3, ImlBad—*Imleria badia*, InoSol—*Inocybe soluta*, LacLac—*Laccaria laccata*, LacPro—*Laccaria proxima*, LacGly—*Lactarius glyciosmus*, LacHel—*Lactarius helvus*, LacLig—*Lactarius lignyotus*, LacNec—*Lactarius necator*, LacRuf—*Lactarius rufus*, LacTab—*Lactarius tabidus*, LecHol—*Leccinum holopus*, LecVar—*Leccinum variicolor*, MelBic—*Meliniomyces bicolor*, MelVar—*Meliniomyces variabilis*, MelVra—*Meliniomyces vraolstadiae*, NeoLur—*Neoboletus luridiformis*, PaxInv—*Paxillus involutus*, PhiFor—*Phialocephala fortinii*, PhiSph—*Phialocephala sphaeroides*, PilBic—*Piloderma bicolor*, PilOli—*Piloderma olivaceum*, PilSph—*Piloderma sphaerosporum*, PseFla—*Pseudotomentella flavovirens*, PseVep—*Pseudotomentella vepallidospora*, RhiSal—*Rhizopogon salebrosus*, RhoBut—*Rhodocollybia butyracea*, RusAtr—*Russula atrorubens*, RusBet—*Russula betularum*, RusEme—*Russula emetica*, RusOch—*Russula ochroleuca*, RusSph—*Russula sphagnophila*, Seb—Sebacinales, SuiCav—*Suillus cavipes*, SuiGra—*Suillus grevillei*, SuiVis—*Suillus viscidus*, TheSp—*Thelephora* sp., TheTer—*Thelephora terrestris*, TomBad—*Tomentella badia*, TomSub—*Tomentella sublilacina*, TylFel—*Tylopilus felleus*, TylAst—*Tylospora asterophora*, and TylFib—*Tylospora fibrillosa*.

L. laccata was not drought tolerant, which could partly explain the observed differences.

Although the studied sites were chosen to be the same as each other and the lack of differences in silver birch-associated ECM fungal community supported a good experimental design, we could not fully exclude the differences in ECM fungal community of original Norway spruce forest with European larch and Scotch pine admixtures (*Lariceto–Picetum*) that persisted in spores and as mycorrhizae on surviving seedlings. It would be interesting to determine whether these differences will persist 10–15 years later when ECM fungal diversity should be saturated after canopy closure (Visser, 1995; Twieg et al., 2007). More replications under different local conditions and with other tree species are necessary to generalize our results.

Regarding the effects of vertical distribution on the ECM fungal species composition, *Tylospora* spp., *Amphinema* spp., and *Meliniomyces* spp. showed an affinity for an organic layer, and *Tomentella* and *Pseudotomentella* spp. showed an affinity for an organo-mineral layer (**Figure 4**), as was previously observed by Tedersoo et al. (2003). They detected athelioid and thelephoroid species as indicators of organic layers, but *Tomentella* spp. were present in all horizons depending on the species. These findings support the importance of vertical niche partitioning, as was already discussed by Bahram et al. (2015).

In conclusion, different post-windstorm management practices do not significantly influence the potential for mycorrhizal networking among Norway spruce, European larch, and silver birch, which is mostly provided by common ECM fungal species with broad ecological niches. Management type affects the ECM fungal species compositions on Norway spruce and European larch; however, the consequences are difficult to assess without knowledge of the ecology and physiology of the affected ECM fungi. Norway spruce in NEX harbored a higher number of unique species than in EXT, but extremely intensive sampling is required to evaluate the possible loss of rare species due to traditional management.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

PV, MV, FH, PC, and PF designed the research. PV, MV, and FH performed the research and collected the data. ME-J analyzed the data. PV, MV, and ME-J interpreted the data. PV and MV wrote the manuscript with the assistance from all co-authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.641232/full#supplementary-material

Supplementary Figure 1 | Map of the research area, Tatra Mountains National Park (TANAP), Slovakia (SK). Two management treatments, site without management (NEX) and site with traditional management (EXT), each with ten research plots.

Supplementary Figure 2 | Rarefaction curves showing the ECM species richness on tree species (P – *Picea abies*, L – *Larix decidua*, and B – *Betula pendula*) in NEX (plots without management) and EXT (plots with traditional management) treatments.

Supplementary Table 1 | Characteristics of the plots in NEX (plots without management) and EXT (plots with traditional management) treatments (geographic position, cover of trees, cover of dominant herbs and shrubs, and dead wood cover); characteristics of the selected trees (height and perimeter); and characteristics of selected roots (depth, soil horizon, distance from the tree stem, and direction to other tree species).

Supplementary Table 2 | List of ECM taxa, including exploration of each taxa, detected on root samples of the studied tree species (*Picea abies*, *Larix decidua*, and *Betula pendula*), including the numbers of root samples with taxon presence divided into NEX (plots without management) and EXT (plots with traditional management) treatments. For each taxon, the GenBank Accession Number, closest hit and percentage of similarity are shown.

Supplementary Table 3 | Presence/absence matrix showing rhizomorph-forming ECM fungal species in the plots in NEX (plots without management) and EXT (plots with traditional management) treatments.

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The Effects of Host Plant Genotype and Environmental Conditions on Fungal Community Composition and Phosphorus Solubilization in Willow Short Rotation Coppice

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Phosphorus (P) is an essential plant nutrient. Low availability of P in soil is mainly caused by high content of Fe_2O_3 in the clay fraction that binds to P making it unavailable. Beneficial microbes, such as P solubilizing microorganisms can increase the available P in soil and improve plant growth and productivity. In this study, we evaluated the effects of environmental conditions (climate, soil parameters), plant genotype, and level of plant association (rhizosphere or endophytic root organism) on the abundance and diversity of phosphorus solubilizing microorganisms in a *Salix* production system. We hypothesized that a lower number of endophytic fungi may possess the ability to solubilize P compared to the number of rhizosphere fungi with the same ability. We also expect that the plant genotype and the experimental site with its environmental conditions will influence fungal diversity. Two *Salix* genotypes grown in pure and mixed cultures were investigated for their fungal microbiome community and diversity in the rhizosphere and endosphere during two growing seasons. We found that the rhizosphere fungal community was more diverse. A general dominance of Ascomycota (*Dothideomycetes*) and Basidiomycota (*Tremellomycetes*) was observed. The classes *Agaricomycetes* and *Pezizomycetes* were more frequent in the endosphere, while *Tremellomycetes* and *Mortierellomycetes* were more abundant in the rhizosphere. Plot-specific soil properties (pH, total organic carbon, and nitrogen) significantly influenced the fungal community structure. Among the culturable fungal diversities, 10 strains of phosphate solubilizing fungi (PSFs) from roots and 12 strains from rhizosphere soil were identified using selective media supplemented with di-calcium and tri-calcium phosphates. The fungal density and the number of PSF were much higher in the rhizosphere than in the endosphere. *Penicillium* was the dominant genus of PSF isolated from both sites; other less frequent genera of PSFs were *Alternaria, Cladosporium,* and *Clonostachys*. Overall the main factors controlling the fungal communities (endophytic vs. rhizosphere fungi) were the soil properties and level of plant association, while no significant influence of growing season was observed. Differences between *Salix* genotypes were observed for culturable fungal diversity, while in metagenomic data analysis, only the class *Dothideomycetes* showed a significant effect from the plant genotype.

Keywords: diversity, fungal endophytes, phosphate solubilization, rhizosphere fungi, short rotation cropping, willow

INTRODUCTION

Phosphorus (P) is an essential plant nutrient provided by a non-renewable resource (Filippelli, 2008; Shen et al., 2011). Fluoroapatite is the main and non-renevable resource from which fertilizers are produced (Filippelli, 2008). Moreover, P compounds released during weathering are usually complex and are not immediately bio-available (Filippelli, 2008; Singh and Satyanarayana, 2011). Plants and microorganisms release extracellular phosphatases from roots and recover orthophosphate ions from mineralizing phosphor-organic compounds (Richardson and Simpson, 2011). Furthermore, P solubilization is a microbial-driven process of hydrolysis of organic and inorganic P compounds to simpler compounds that can be utilized by plants (Richardson and Simpson, 2011).

Phosphorus solubilizing microorganisms can increase the availability of P for plants from the soil P pool, reducing the need for P fertilization. More P solubilizing bacteria have been reported than P solubilizing fungi (PSFs), but the latter are more effective, as PSFs do not lose the ability to solubilize P after subculture (Zhang et al., 2011). There are reports of various PSF species, such as *Aspergillus* spp., *Penicillium* spp., *Trichoderma viride*, *Arthrobotrys oligospora*, *Cephalosporium* sp., or *Cladosporium* sp. (Khan et al., 2010; Patil et al., 2012; Sharma et al., 2012; Ram et al., 2015; Li et al., 2016; Alori et al., 2017). In certain cases, these fungi may form symbioses with plants, develop mycelial networks, produce plant growth-promoting metabolites, and increase plant P and nitrogen uptake from the soil, e.g., by increasing root surface area (Baum et al., 2009). Chatli et al. (2008) isolated PSFs belonging to the genera *Aspergillus* and *Penicillium* from the rhizosphere of willow (*Salix alba* L.) growing in the *trans*-Himalayan region. Few experiments have demonstrated that PSFs isolated from other host plants could be used to increase yields of tomato, maize, and wheat (Khan and Khan, 2002; Reyes et al., 2002; Sharma et al., 2012; Ram et al., 2015).

The European Union directive (April 2009) on the promotion of the use of energy from renewable sources (Directive 2009/28/EC) states that 20% of the total energy in Europe should be generated from renewable sources[1]. Generally modern agriculture is focused mostly on reaching high yields by the use of best-performing plant species grown in pure culture. Such pure cultures are often easier in maintenance than mixed cultures, but pure cultures have been shown to be more vulnerable to pests and diseases in many cases. Thus, the introduction of mixed genotypes plantations could limit the losses due to pests and diseases while significantly enhancing biodiversity (Hoeber et al., 2018; Schweier et al., 2019). This may improve the sustainability of biomass production on SRCs but our knowledge in this area is still limited. Additionally selection of tree species for SRC is critical and depends on the local climate and soil conditions. SRCs are generally confined to fast growing tree species, mainly from the genera *Populus*, *Salix*, *Eucalyptus*, and *Robinia* (Navarro et al., 2016). *Salix* species are fast growing trees that possess high economic value because of their high potential to contribute to renewable energy generation in Europe (Sevel et al., 2012; Weih et al., 2021). This woody crop can be planted on soils that are less suitable for farming of food crops and can be fertilized with sewage sludge, wastewater, or ashes which contain high amounts of nitrogen and phosphorus (Dimitriou and Aronsson, 2011). Many species of *Salix* can control P uptake and metabolism, although the corresponding mechanisms are still largely unknown (Rennenberg and Herschbach, 2013).

Rhizosphere and endophytic fungi play important roles in plant growth and development. Their ecology and function is different and depends on various parameters (Hrynkiewicz and Baum, 2012). The level of plant association may be affected by soil properties, climate, weather conditions, and host plant genotype. Likewise, understanding how the willow plantations in the form of monoculture and mixed genotypes could affect the overall microbial diversity is key information. This study bridges the gap by providing knowledge on the rhizosphere and endophytic fungal diversity in willow SRCs. Hence, the main aim of this research was to investigate the diversity of rhizosphere and endophytic fungi from two willow species, *S. dasyclados* (cultivar "Loden") and *S. schwerinii* × *S. viminalis* ("Tora"), as well as their mixture at two sites located in Germany and Sweden. The two species selected for the study are phenotypically different, and "Tora" is known for its high productivity compared to "Loden" (Hoeber et al., 2017). The mixture of two host species may result in increased diversity of fungi compared to growing the same species in pure culture. The two investigated experimental sites represent ECOLINK-Salix within a global tree diversity network (Verheyen et al., 2016). The two selected sites are similar in terms of planting time, design, and management (e.g., fertilization, timing of shoot harvests) but vary by local climate and soil conditions. This might provide more information about importance of climate and soil nutrient content on willow microbiome. We

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[1] https://ec.europa.eu/eurostat/web/energy/data/shares

evaluated the effect of climate, soil conditions, level of plant association, and planting design on the abundance and diversity of PSFs by applying culture-dependent and culture-independent (metagenomic) methods. Using culture-dependent techniques is critical as it allows application of this study for future research in using these strains as inoculants in plants. Moreover, culture-independent methods give more insight into the culturable and unculturable fungal diversity present in the two experimental sites. In addition, to assess the potential impact of PSFs on the P supply of *Salix* in fall, we tested the P solubilization efficiency of fungal isolates. We hypothesized that a lower number of endophytic fungi may possess the ability to solubilize P compared to the number of rhizosphere fungi with the same ability. Second, the site-specific climate and soil conditions and the *Salix* species genotypes may determine the abundance and diversity of total fungi and PSFs.

MATERIALS AND METHODS

Site Description and Sampling

The two experimental sites have been well-established SRCs since spring 2014 (Hoeber et al., 2018). The first experimental site is located in Uppsala, Sweden (59°49′13.4″N, 17°38′25.2″E), and the second is located in Rostock, Germany (54°03′41.0″N, 12°04′54.7″E). Both sites are former arable fields. The experimental site in Uppsala is located on fine-textured mineral soils, mainly Vertic Cambisols (according to IUSS Working Group WRB, 2015). The experimental site Rostock is dominated by Stagnic Cambisols developed from loamy sands.

The study sites vary by local climate and soil conditions. According to the Köppen–Geiger classification, the climate in Uppsala is boreal (Dfb: snow, fully humid with warm summers; Kottek et al., 2006). The average annual rainfall is 551 mm, and the average annual air temperature is 5.8°C (1970–2000; http://www.worldclim.org/current). Winters are usually not as cold as in other cities at similar latitudes due to the influence of the Gulf Stream. Rostock is situated on the Baltic coast in a warm temperate climate (Cfb: fully humid with warm summers). The average air temperature of Rostock is higher than that of Uppsala (8.4°C), but the rainfall sum is similar (600 mm).

The meteorological data obtained from https://www.worldweatheronline.com/showed similar trends in the 2018–2019 period for both studied localities. In general, these years were warmer than average (2009–2019). However, lower than average monthly temperatures were recorded in February (Rostock and Uppsala) and March (Rostock) 2018. The interannual variability in the monthly distribution of rainfall was high. The 2018 year was significantly drier and the 2019 year was much wetter than average (2009–2019; **Supplementary Table 1** and **Supplementary Figure 1**). Two *Salix* genotypes, "Loden" (*S. dasyclados*) [L] and "Tora" (*S. schwerinii* × *S. viminalis)* [T], were cultivated as pure cultures and mixtures [LT] at the two sites. The L genotype is characterized by shorter shoots and a larger leaf area than the T genotype (Hoeber et al., 2018).

Willow roots and soils were sampled from the two experimental sites during two seasons: Fall 2018 (Sweden – October 23rd, Germany – October 27th) and spring 2019 (Sweden – May 15th, Germany – May 18th). Both experimental sites were organized into three blocks, and the density of plants was 15,600 ha^{-1}. Blocks were divided into plots randomly planted with different *Salix* genotypes (**Figure 1**) and their mixtures in all possible combinations. Treatments L, T, and LT were selected for investigation since they were present at both experimental sites (**Figure 1**). There were three replicates per treatment (nine plots per experimental site, in total), and each plot was 9.6 m × 9.6 m.

Three samples per plot (81 from Sweden and 81 from Germany per season, 324 in total) were taken by digging root samples with adhering soil (15 cm × 15 cm × 15 cm) at a distance of 6 m from each other for microbiological analysis. The organic litter layer (up to 5 cm thick) was removed and topsoil (0–25 cm) was sampled. Samples were carefully transferred to collection bags and covered with a thin layer of soil to prevent drying. Collected samples were immediately transported to the laboratory and used for analyses.

Soil Physicochemical Analysis

Air-dried soil samples were passed through a 2 mm sieve. The total organic carbon (TOC) and total nitrogen (TN) contents were measured after dry combustion using a CHNS Vario Macro Cube elemental analyzer. Available phosphorus (P_{av}) in 1% citric acid (van Reeuwijk, 2002) was determined by a spectrophotometric method using spectrophotometer UV–Vis Rayleigh UV-1601 (van Reeuwijk, 2002), and the pH (in H_2O and 1M KCl) at 1:2.5 soil to solution ratio was determined by the potentiometric method using an Elmetron CP-105 pH-meter. **Table 1** shows differences in soil parameters on both sampling sites and two growing seasons.

Total Cultivable Fungal Density

Samples from fall 2018 were processed for cultivable fungal density and phosphate solubilizationability. The willow roots were carefully separated from the adhering rhizosphere soil. One and a half grams of roots were surface sterilized with 60% alcohol (3 min) by vigorous shaking and then washed three times in sterile 2% NaCl solution. The roots were washed in sterile 5% H_2O_2 solution (10 min) and rinsed three times in sterile 2% NaCl solution. The last wash of 2% NaCl was plated on R2A medium (BD Difco, United States; sterilization control). The surface sterilized roots (1 g) were homogenized using a sterile mortar and pestle under sterile conditions and then transferred to Falcon tubes containing 9 ml of sterile 0.5% NaCl solution. For the rhizosphere soil samples (from uppermost mineral part of soil affected by the plant roots), 1 g was transferred to Falcon tubes with 9 ml of sterile distilled water, and dilutions were performed.

For the total fungal density, potato dextrose agar (PDA) medium (BD Difco, United States) supplemented with tetracycline at 100 mg/l was used. Serial dilutions of 10^{-2} and 10^{-3} of the root samples and 10^{-3} and 10^{-4} of the rhizosphere soil were selected for spread plating. Plates were kept at 24°C, and the total number of fungal colonies on PDA medium was counted after day 7 and presented as colony forming units (c.f.u).

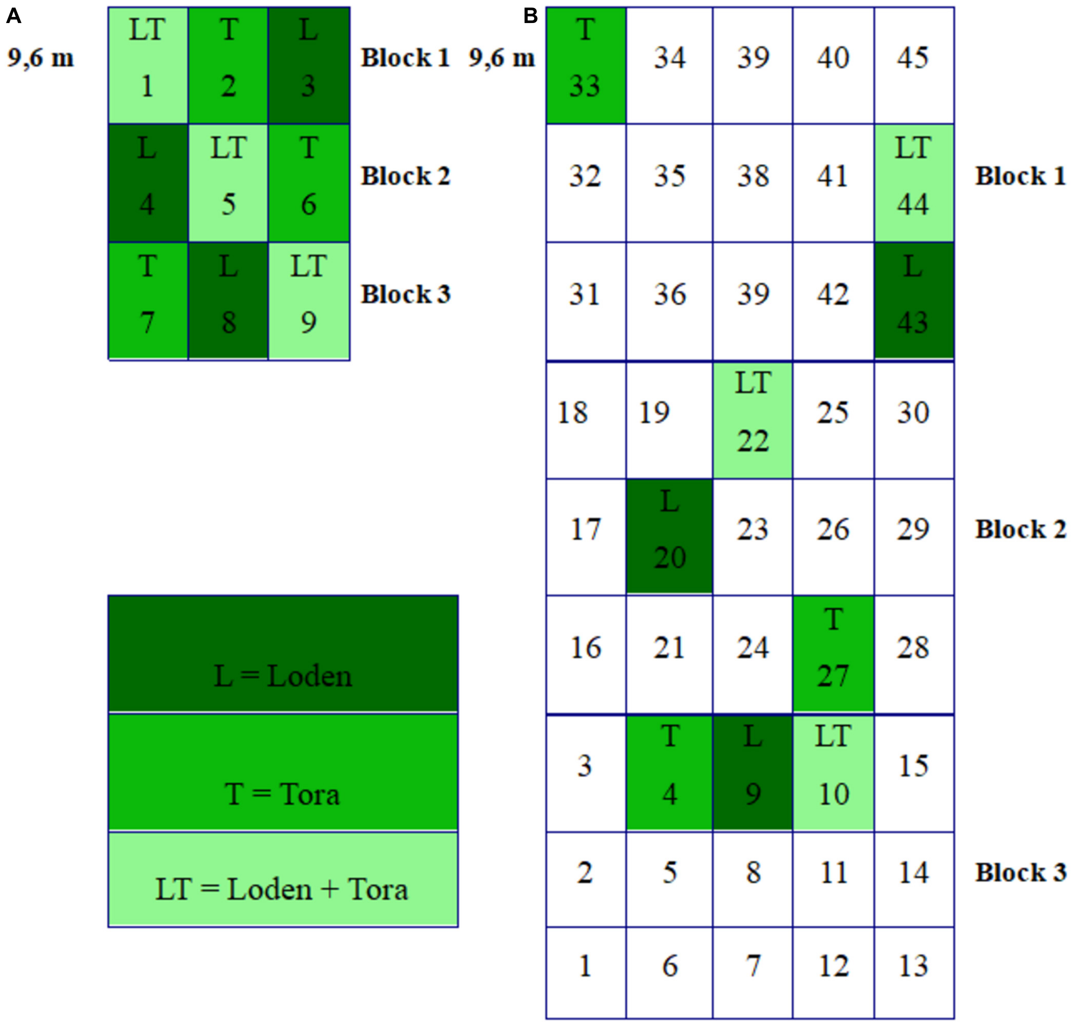

FIGURE 1 | (A) The randomized block design of the ECOLINK-Salix field trial in Germany (Rostock) consisting of 9 plots (each 9.6 m × 9.6 m) in three blocks with genotype monocultures and mixtures. **(B)** The randomized block design of the ECOLINK-Salix field trial in Sweden (Uppsala) consisting of 45 plots (each 9.6 m × 9.6 m) in three blocks with genotype monocultures and mixtures.

Screening PSFs From Roots and Rhizosphere Soil

The isolation and selection of PSFs were performed on three selective media (NBRIP, PVK; Nautiyal, 1999, and DCP; modified Pikovskaya, 1948; **Supplementary Table 2**) containing either tri- (NVRIP, PVK) or diphosphates (DCP). In total, 108 plates per medium were used to evaluate P solubilizing and non-PSF.

The serial dilutions from the roots and the rhizosphere soil (100 μl each) were spread plated on the three selective media. For NBRIP and PVK media, 10^{-5}–10^{-6} dilutions were used for both types of samples. For DCP medium, 10^{-3}–10^{-4} dilutions were used for the roots, and 10^{-4}–10^{-5} dilutions were used for the rhizosphere soil samples. Three technical replicates were prepared for each sample. Plates were kept at 24°C for 7 days and observed for fungal halos indicated phosphorous solubilization. Calculations were performed to determine the number of PSF and the total number of fungi growing on each of the three media. All PSF isolates unique to each plot were subjected to molecular identification. The fungal density was determined by the $\log_{10}$ [c.f.u. (g f.w. soil)$^{-1}$ or (g f.w. roots)$^{-1}$] values for both the total fungal count and those screened with selective media.

Molecular Identification of Fungal Strains

Twenty-two selected fungal isolates (12 from Germany and 10 from Sweden) were cultivated on fresh PDA medium (BD Difco, United States). Fungal DNA was isolated from fresh mycelium using the Plant and Fungi DNA Purification Kit (Eurx, Poland). The concentration of DNA was measured using a UV–Vis spectrophotometer (Thermo Scientific NanoDrop2000, United States). The ITS region was amplified using ITS1 (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) primers (Martin and Rygiewicz, 2005; Manter and Vivanco, 2007). PCR clean-up was carried out using a PCR/DNA Clean-Up Purification Kit

TABLE 1 | Characteristics of rhizosphere soils.

Site	Season	Genotype	TOC (%)	TN (%)	C:N	P_{av} (mg·kg^{-1})	pH(H_2O)	pH(KCl)
Sweden	Fall	L	1.69 ± 0.24*	0.16 ± 0.02*	10.3 ± 0.5b	101 ± 12.2b	5.7 ± 0.2b	5.3 ± 0.1
		T	1.59 ± 0.21*	0.16 ± 0.01*	10.2 ± 0.7	94.2 ± 13.6*	5.7 ± 0.1b	5.2 ± 0.2
		LT	1.65 ± 0.2*	0.16 ± 0.02*	10.4 ± 0.6	105 ± 28.1b*	5.7 ± 0.1	5.1 ± 0.1
	Spring	L	1.89 ± 0.29*	0.17 ± 0.02*	11.1 ± 0.5Aa	75.7 ± 20.1Ba*	6.0 ± 0.2Ba	5.2 ± 0.2
		T	1.72 ± 0.22*	0.17 ± 0.02*	10.2 ± 0.6B	93.6 ± 14.7A*	6.2 ± 0.2Ba	5.2 ± 0.1
		LT	1.63 ± 0.18	0.16 ± 0.02*	10.3 ± 0.5B	63.1 ± 19.8Ba	5.7 ± 0.2A	5.2 ± 0.1
Germany	Fall	L	1.21 ± 0.17	0.11 ± 0.02	11.3 ± 0.7*	101 ± 22.0Aa	6.0 ± 0.4*	5.8 ± 0.4*
		T	1.13 ± 0.13b	0.1 ± 0.01b	11.3 ± 0.7*	57.0 ± 7.3B	6.1 ± 0.3*	6.0 ± 0.3*
		LT	1.22 ± 0.21b	0.1 ± 0.01b	11.7 ± 1.0*	55.0 ± 12.3B	6.0 ± 0.3b*	5.7 ± 0.4*
	Spring	L	1.24 ± 0.2	0.11 ± 0.01	11.3 ± 0.9	50.5 ± 9.5b	6.2 ± 0.3*	6.1 ± 0.3*
		T	1.4 ± 0.24a	0.11 ± 0.01a	12.5 ± 1.3*	54.8 ± 11.3	6.3 ± 0.2*	6.2 ± 0.2*
		LT	1.49 ± 0.19a	0.12 ± 0.01a	12.4 ± 0.8*	57.3 ± 11.2	6.2 ± 0.2a*	6.1 ± 0.3*

*At each sampling site (Sweden and Germany), three plots per three blocks for each variant of the experiment (Loden, Tora, and mixture of both genotypes) were sampled. In total, 108 root and soil samples from both samplings were collected. Soil properties were compared by site, season, and genotype (plots: L-Loden, T-Tora, and LT-mixture). Values are means ± SDs (n = 9). The significant differences with $p < 0.05$ are marked by the following symbols: * – differences between sites in the same season, small letters – differences between seasons within one site, and capital letters – differences between genotypes on the same site and season.*

(EurX, Poland). The presence of ITS sequences was confirmed on a 1% agarose gel (1X TBE buffer) with the addition of Simply Safe dye (EurX, Poland). Samples were sent for sequencing to the Institute of Biochemistry and Biophysics PAS[2]. Contigs were assembled using Sequencher 5.4.6 software and compared with the NCBI database using BLASTn[3] to find sequences that showed the highest similarity to the assembled contigs. The DNA sequence generated for this study were deposited in the NCBI GenBank under the following accession numbers: MW342736–MW342757 (as shown in **Supplementary Table 3**). The phylogenetic tree was constructed using the NJ method in MEGA 7, and 1,000 bootstrap replicates were used to assess branching support (Tamura et al., 2013; Kumar et al., 2016). The *p*-distance method was calculated (Saitou and Nei, 1987). The phylogenetic tree was visualized using Interactive Tree of Life (iTOL) v3 (Letunic and Bork, 2016).

Metagenomic Analysis

Total DNA was extracted from 50 mg of lyophilized willow roots and rhizosphere soil samples using Plant and Fungi DNA Purification Kit (EURx, Poland) according to the manufacturer's protocol. Three biological replicates were prepared for each plot. The amount of isolated DNA was quantified fluorometrically (InvitrogeneQubit 2.0, United States) and the quality was assessed spectrophotometrically (Thermo Scientific NanoDrop 2000, United States) and the preparations were diluted to 1 ng/μl.

Fungal ITS amplicon libraries were generated in two-step PCR, as described by Thiem et al. (2018) using fungal primers (uITS1 and uITS2) then with M13 and M13R primers with P5/P7 adapters and barcodes (different MID sequences for each sample). Libraries were purified twice with Agencourt AMPure XP (Beckman Coulter) according to the manufacturer's protocol. The quality of the pooled libraries was assessed on a Bioanalyzer chip (Agilent) and they were quantified with KAPA Library Quantification Kit for Illumina Platform using LightCycler 480 (Roche) according to the manufacturers' protocols. The final pool was diluted to 4 nM, denaturated, mixed with 5% of PhiX control library and sequenced with the use of 2 × 300 cycles kit v.3 on a MiSeq machine (Illumina).

The resulting read pairs were denoised with dada2 (Callahan et al., 2016) and the fungal sequences were processed with ITSx (Bengtsson-Palme et al., 2013), and all fungal ITS1 sequences were used in the downstream analyzes. The reads were de-replicated and OTUs were constructed using vsearch (Rognes et al., 2016) at 0.03 dissimilarity level, then singletons as well as doubletons (OTUs consisting of one or two sequences only) were removed. The sequences were classified with naive Bayesian classifier (minimum 80% bootstrap support was required; Wang et al., 2003) using ITS1 extracted from UNITE v.7 (fungi), and the non-fungal sequences were removed. The final data were sub-sampled to 300 (fungi) sequences per sample 20 times, sequences names were mangled to reflect the iteration, the sets were pooled, de-replicated, and OTUs were constructed as described earlier. OTU tables were then averaged over the 20 subsamples and the entries were rounded to the nearest integer with a Perl script to yield the final tables.

Data Analysis

Statistical analysis for screening of PSFs was performed using the Statistica software package (version 13.0, StatSoft) based on three replicates for each sample variant. For total density of culturable strains (**Figure 2**) and PSF screening (**Figure 3**) nine replicates (three samples from each of three plots) for each genotype present on site were used. Normality was tested using Shapiro–Wilk test and homogeneity of variance using Levene's test. Samples that were outside of two standard deviations range from mean were removed. The one-way analysis of variance (ANOVA) was used to determine whether there are any statistically significant differences between the means of total fungi count for each genotype on PDA and selective media and PSF count for

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[2] http://oligo.ibb.waw.pl/

[3] www.ncbi.nlm.nih.gov/BLAST

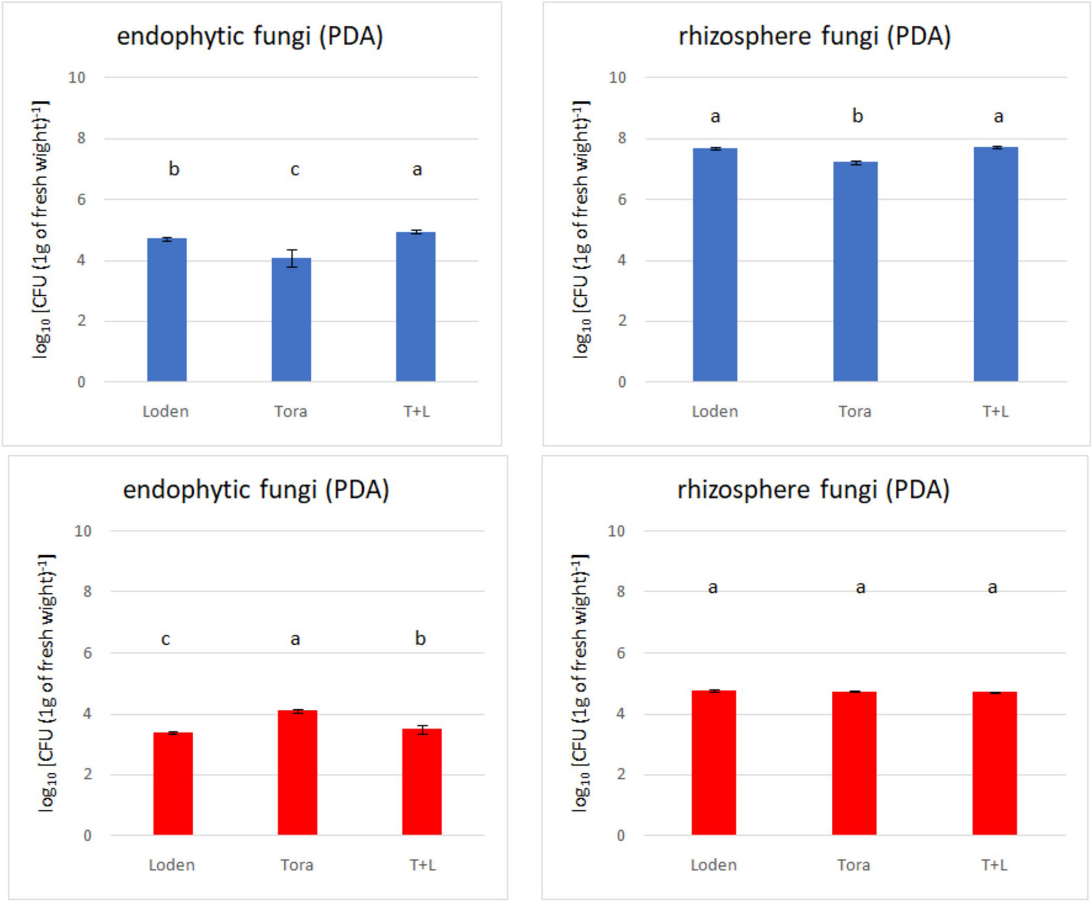

FIGURE 2 | Total fungal count (endophytes and rhizosphere) from two experimental sites (Sweden and Germany) in fall 2018. The upper panels (blue bars) indicate the total fungal counts for Swedish samples; the lower panels (red bars) indicate the total fungal counts for Germany samples.

genotypes for each experimental site. Upper case letters represent significant differences for PSF count for genotypes for each experimental site (**Figures 2**, **3**).

For metagenomic data analysis, Bray–Curtis distance matrices based on Wisconsin double-standardized OTU tables were calculated with vegdist in R. Non-metric multidimensional scaling (NMDS) and canonical correspondence analysis (CCA) analyzes were performed within R with vegan's metaMDS and cca functions. In case of CCA, forward selection procedure implemented in ordistep was used for stepwise model building. Significance of differences between sample clusters was assessed with ANOSIM and PERMANOVA in vegan's anosim and adonis functions, respectively. P-values < 0.05 was considered significant. Variance partitioning was performed with the varpart function. Significance of differences in means (number of observed OTUs, Shannon's H′, Shannon's E, taxa, and functional groups distribution) was assessed with ANOVA with *post hoc* Tukey's HSD analysis, unless assumptions of normality of data and/or homogeneity of variance were violated, in which case robust ANOVA implemented in raov of the Rfit package was used to check for general p-value. All figures were plotted with standard R graphic functions.

RESULTS

Soil Properties and Climatic Conditions at the Two Experimental Sites

As shown in **Table 1**, in Uppsala the rhizosphere soil parameters TOC, TN, C:N was higher while P_{av}, pH(H_2O), pH(KCl) was lower. ANOVA analysis revealed that the soil properties differed significantly between the experimental sites (**Table 1**). Soil samples from Sweden had higher contents of TOC, TN, and P_{av}, while soil samples from Germany were characterized by higher C:N ratios and pH values. Soil parameters at both experimental sites differed slightly in spring. Significant differences between genotypes were observed for the TOC (T and LT in Germany), pH (all plots in Sweden) and P_{av} content, which were higher in fall (L and LT plots in Sweden and L plots in Germany; **Table 1**).

Identification of Dominant PSFs and Total Fungal Density in Willow Genotypes and Mixtures

The overall density of cultivable fungi ranged from 3.38 to 4.94 log10 [measured as colony-forming units: c.f.u. (g d.w. roots)$^{-1}$]

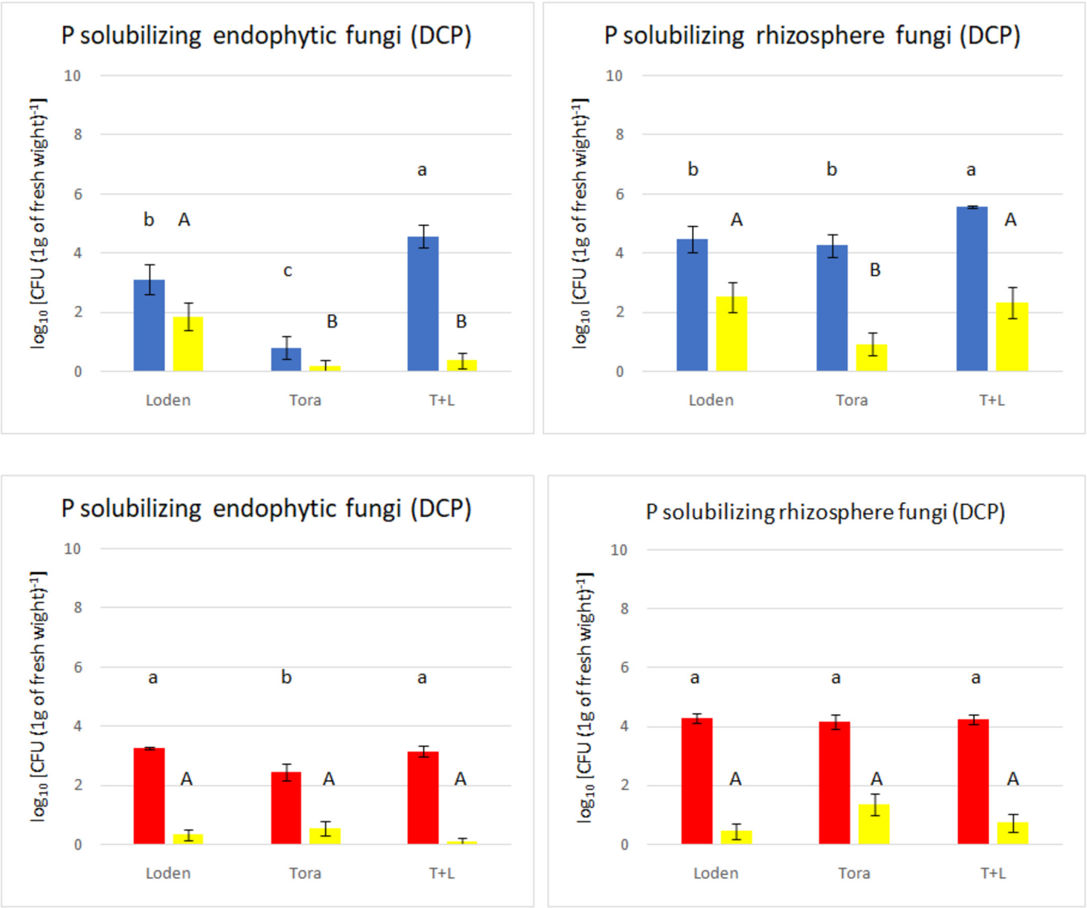

FIGURE 3 | Total non-P solubilizing and P solubilizing fungi count (endophytes and rhizosphere) from two experimental sites (Sweden and Germany) in fall 2018. The upper panels (blue bars) indicate the total fungal counts from selective media for Swedish samples; the lower panels (red bars) indicate the total fungal counts from selective media for Germany samples. The yellow bars indicate the number of phosphate solubilizing fungi found among the non-P solubilizing fungi.

for endophytes from both experimental sites. In the rhizosphere soil, the total fungal count ranged from 4.71 to 7.72 log10 [c.f.u. (g d.w. soil)$^{-1}$]. Significant differences among the analyzed willow genotypes were observed for endophytic fungal density (**Figure 2**), but this was not observed for rhizosphere fungi. The endophytic fungal density was lower than that of rhizosphere fungi. The difference in fungal density between the endophytes and rhizosphere soil was significantly greater in Swedish samples than German ones. Generally, the highest fungal density was recorded in LT samples from Sweden and T samples from Germany.

The medium supplemented with triphosphate (PVK) showed nofungal growth, and therefore, it was excluded from the analysis. The total number of culturable endophytic fungi ranged from 0.80 to 4.57 log10 c.f.u g^{-1}, among which PSF ranged from 0.12 to 1.86 log10 c.f.u. g^{-1} but showed no significant differences with the exception of Loden (only in Sweden). The endophytic fungal density was lower than that of rhizosphere fungi, ranging from 3.38 to 4.94 log10 c.f.u. g^{-1}, whereas rhizosphere fungi ranged from 4.75 to 7.72 log10 c.f.u. g^{-1}. The LT genotype showed the highest non-PSF density at both experimental sites (**Figure 3**). The endophytic fungal density was lowest for the Tora genotype at both experimental sites. The phosphate solubilizing ability of fungi from the Swedish site (found mostly in rhizosphere soil) was higher than that in fungi from the German site.

In total, 22 fungal strains were isolated from roots (10 strains) and rhizosphere soil (12 strains; **Figure 4**). *Clonostachys* and *Penicillium* were the dominant genera at the German site (30% for each), while at the Swedish site, *Penicillium* alone was the dominant genus (70%; **Figure 4**). Most of the strains found at the German site were isolated from the rhizosphere soil of T, in contrast to the Swedish site, where the majority was endophytic isolates from the L genotype. *Penicillium* was the only fungal genus occurring at both sites. The German site showed higher diversity in the rhizosphere, with five different fungal genera (*Penicillium*, *Clonostachys*, *Alternaria*, *Gibellulopsis,* and *Cladosporium*). For the Swedish site, the highest diversity was obtained for endophytes of the L genotype with three different species (*Penicillium*, *Talaromyces,* and *Juxtiphoma*). In total, 54% of the identified strains were isolated from T, 31% from L, and 13% from LT samples.

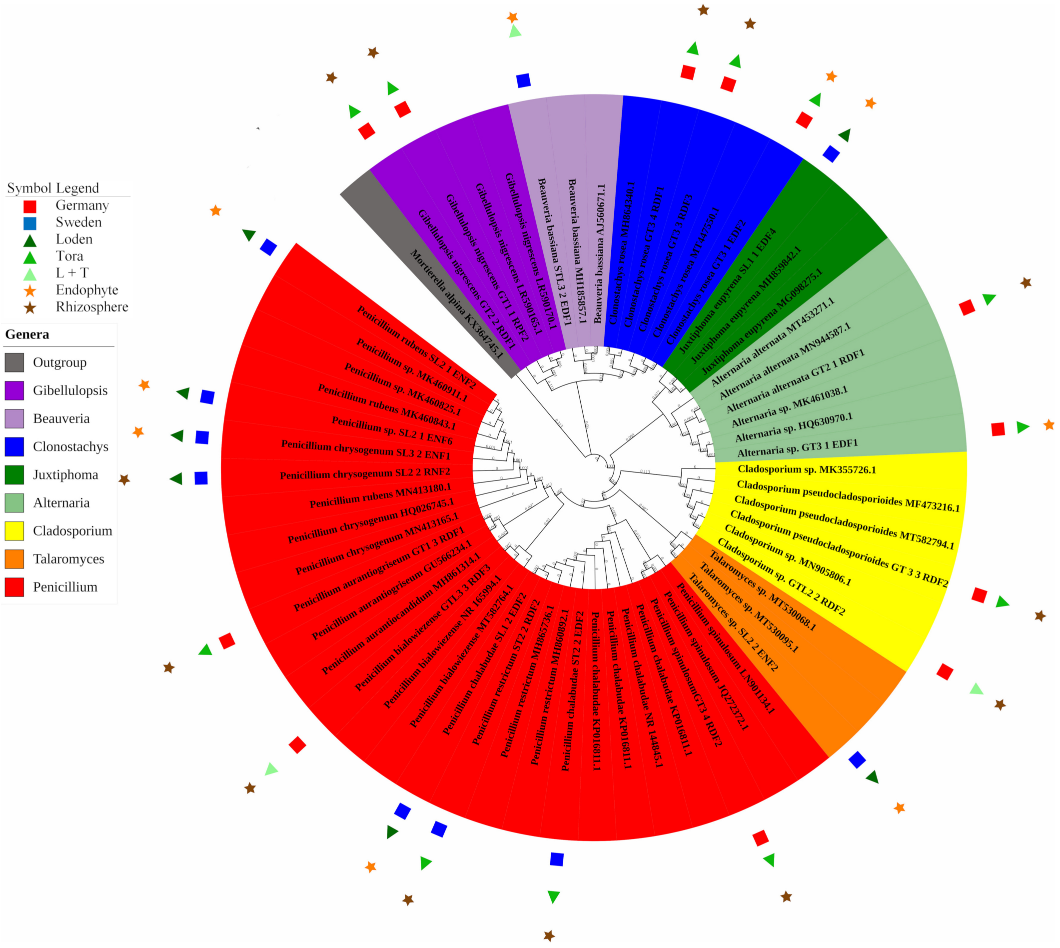

FIGURE 4 | Phylogenetic analysis of culturable fungi isolated from three willow genotypes classified at the genus level. For details of isolates with their GenBank accession numbers, see **Supplementary Table 3**. Reference sequences with closest BLAST match were used (strains without symbols) to construct the phylogenetic tree. The 22 fungal strains with PSF activity are marked with their respective symbols (see legend on the left).

The endophytes comprised 54% of the total number of identified fungi.

Experimental Site and Level of Plant Association Shaped the Community Structure in Willow SRCs

The alpha diversity of the fungal community was not influenced by genotype but by site or the level of fungi association with the plant (rhizosphere vs. endosphere; **Figure 5**). Diversity (H′), species richness (observed OTU number) and evenness (E) were higher in the rhizosphere than in the endosphere. Endophytic communities at the German site were more diverse and even harbored more OTUs than those at the Swedish site; however, there were no differences between seasons. At the Swedish site, alpha diversity was higher in spring than in fall. There were no differences between variants in rhizospheric communities.

The alpha diversity analysis did not show significant differences between genotypes. Overall, Shannon's diversity (H′), Shannon's evenness, and observed OTUs revealed significant differences between the endophytes and rhizosphere fungal diversity regardless of the experimental site and seasons. The endophytic diversity at the two experimental sites in Germany and Sweden was significantly different from that of the rhizosphere fungi. A significant effect of seasonality was observed only for endophytes from Sweden (**Figure 5**). The number of observed OTUs for rhizosphere fungal diversity showed a greater tendency than that of the endophytes, and this difference was prominent between the experimental sites in Germany and Sweden during the fall.

The NMDS analysis revealed that the fungal communities clustered according to the experimental sites, but this was not observed for seasons and genotypes (**Figure 6**). The grouping was significant for roots (PERMANOVA, $F = 0.5050$, df = 1, and $P = 0.0001$) and for soil (PERMANOVA, $F = 0.1830$, df = 1, and $P = 0.0001$). The differences in variance were not significant for roots (PERMDISP, $P = 0.579$) or soil (PERMDISP, $P = 0.2911$). The distance between the fungal communities in the two experimental sites was significantly larger for the rhizosphere soil than for roots.

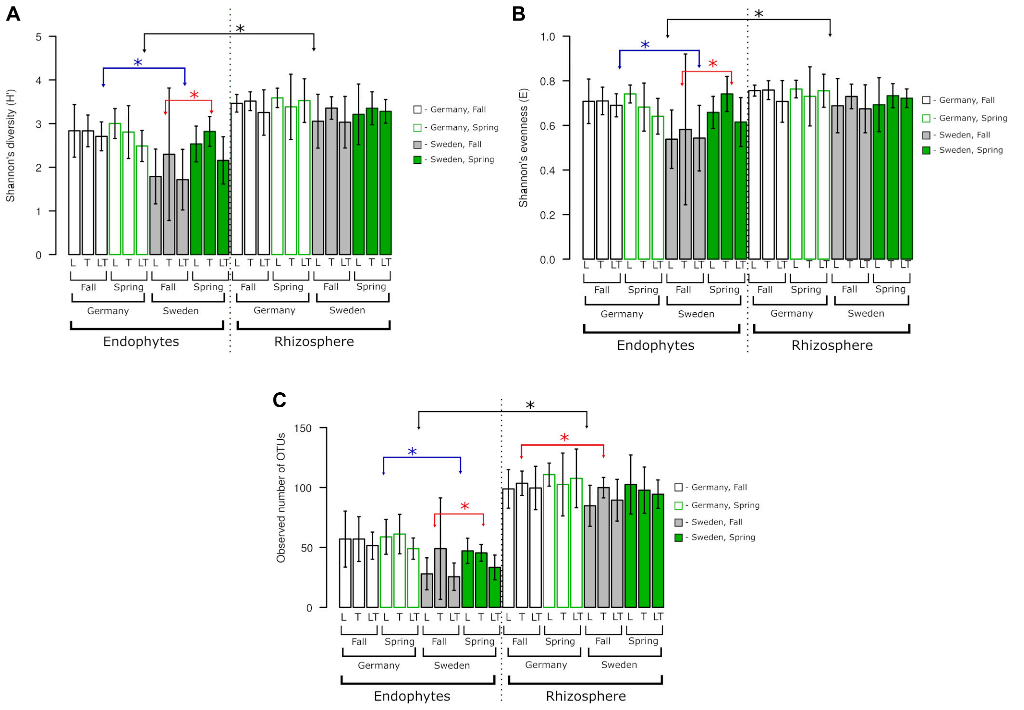

FIGURE 5 | Fungal species richness, diversity, and evenness across two experimental sites, rhizosphere soil and roots. OTUs constructed at 0.03 dissimilarity for fungal sequences. **(A)** Shannon's (H'), **(B)** Shannon's (E), **(C)** observed number of OTUs. Robust ANOVA with Tukey's *post hoc* analysis was used to assess the significance of differences between experimental sites, rhizosphere soil, and roots. Colors denote the following: black – significant difference between endophytes/rhizosphere. *Indicates significant difference between variants indicated by arrows.

The CCA showed that the fungal communities were significantly different between the two experimental sites only (**Figures 6B,D**). In the rhizosphere soil, total organic C and total N were the two environmental variables shaping fungal diversity at the Swedish experimental site (**Figure 6**). TN explained 7.5% of variance whereas TOC explained 2.4%. In contrast, the fungal communities in the roots of the German experimental site were mostly influenced by pH(H_2O), whereas at the Swedish experimental site, the most influential factor was TN (**Figure 6**). TN explained 2.5% of the variance, while pH(H_2O) explained 1.5%.

In conjunction with the culturable fungal diversity, the fungal libraries from the rhizosphere soil and endophytic community showed significant dominance of the phylum Ascomycota, followed by Basidiomycota and Mortierellomycota (**Supplementary Figure 2**).

At the class level, the fungal community was dominated by *Dothideomycetes* and *Leotiomycetes*, with significant differences seen for both endophytes and rhizosphere soil fungi. The above two classes were significantly different based on both the experimental sites and seasons as well (**Figure 7**). *Agaricomycetes* and *Pezizomycetes* showed high abundance and were exclusive to endophytes only. All classes except *Agaricomycetes* displayed significant differences between the two seasons. The rhizosphere soil fungal community was dominated by *Tremellomycetes* and *Mortierellomycetes*; the former showed significance among the two sites and seasons, while the latter showed only seasonal effects. *Dothideomycetes* was the only class exhibiting differences among genotypes.

At the family level, *Piskurozymaceae* showed greater fungal diversity among the rhizospheric fungi than the endophytes (**Figure 7**). *Helotiaceae* and *Phaeosphaeriaceae* were dominant and significantly represented in the Swedish site. *Tuberaceae* and *Herpotrichiellaceae* reads were found mostly in German samples, but the former did not show seasonal changes. Among the fungal endophytic communities, the effect of season was not observed for *Tuberaceae* and *Thelephoraceae.* In the rhizosphere fungal community, both sites were dominated by *Piskurozymaceae* and *Mortierellaceae,* mainly at the Swedish site. At the German site, the family *Plectosphaerellaceae* displayed a significant seasonal effect.

The endophytes *Paraphoma* and *Exophiala* were the most frequently occurring genera in both the experimental sites and seasons. The genus *Exophiala* was present in greater numbers at the German site than at the Swedish site; in contrast, the *Tetracladium* genus was present mostly at the Swedish site. On the other hand, the rhizosphere fungal libraries were dominated by reads of the genera *Solicoccozyma* and *Mortierella* mainly at the Swedish site. The rhizospheric fungi were significantly different in the two seasons and were more prominent at the Swedish site than at the German site.

At the species level, the endophytic fungal community consisted mostly of *Tetracladium* sp., *Paraphoma rhaphiolepidis* and *Exophiala salmonis,* whereas most of the identified fungi

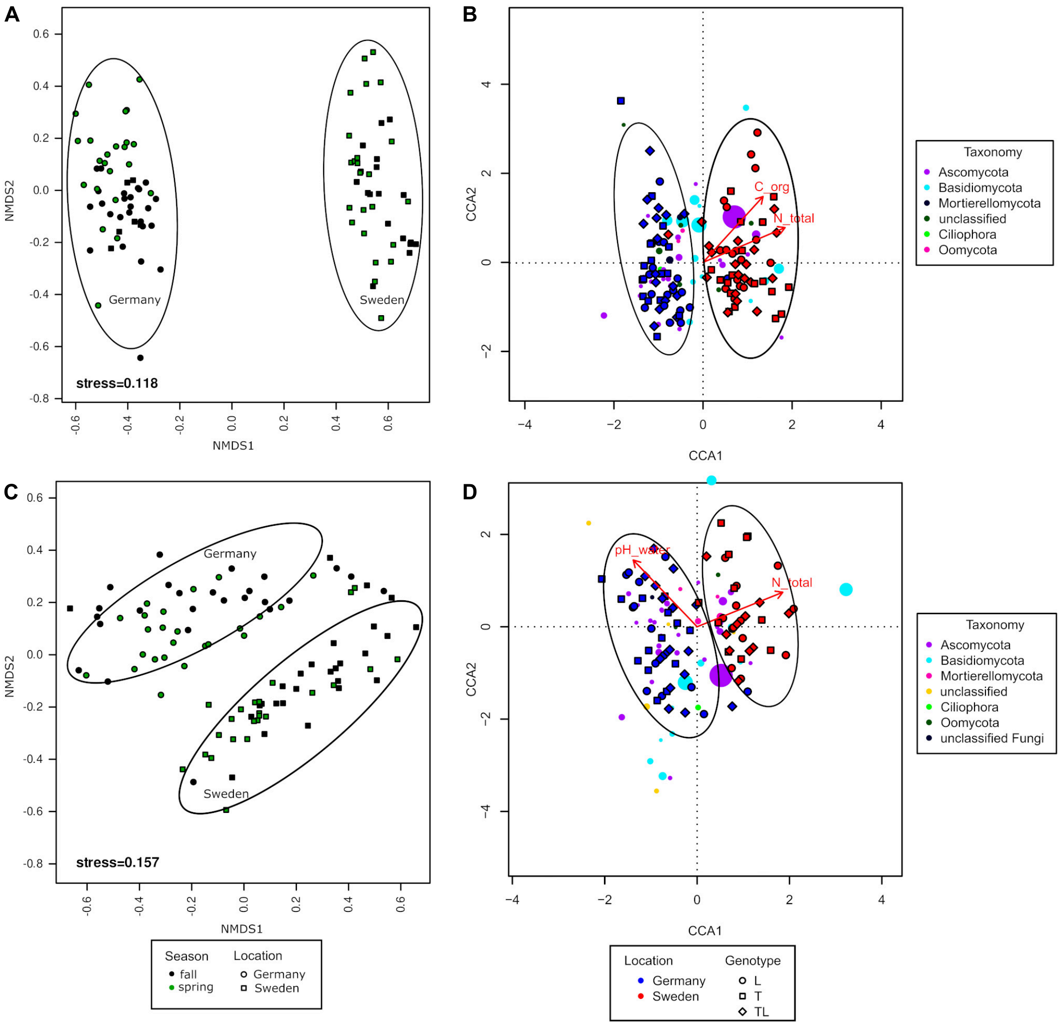

FIGURE 6 | Analysis of log-transformed and Wisconsin double-standardized Bray-Curtis dissimilarity matrix for rhizosphere soil and root fungal communities associated with *Salix* genotypes L (Loden), T (Tora), and LT (mixture) between two seasons (fall, spring) and two sampling sites (Germany and Sweden). **(A,C)** NMDS (non-metric multidimensional scaling analysis); **(B,D)** CCA (canonical correspondence analysis).

from the rhizosphere soil belonged to *Salicoccozyma terrea* (with the highest occurrence at the Swedish site). Based on the experimental sites, the rhizosphere fungi at the German site showed significantly more fungal reads belonging to *E. salmonis*. *E. salmonis* was the only species to show significant differences between the endophytic and rhizospheric communities at the German site.

DISCUSSION

This study is first in describing the PSF in woody SRC and comparing the fungal diversity and community structure between sites, seasons, and genotypes. The beta diversity revealed that experimental site drives the fungal community structure. Abundance as well as alpha and beta-diversity of fungal community were mainly driven by TOC, TN, and pH. We found *Penicillium* to be the dominant genus of PSF in the group of isolated fungal strains, while this genus was not detected in the metagenomic analysis. This may be due to the presence of other abundant fungal genera that may mask its presence. The level of plant association (endophytic or rhizosphere fungi) was the main factor driving fungal diversity and community structure (number of observed OTUs was greater for rhizosphere fungi than endophytic fungi). Differences for seasons and genotypes were present but were not particularly prominent.

Physico-Chemical Soil Properties and Climate Distinct at the Two Experimental Sites

To date, only a few studies have characterized the fungi associated with woody crops from different geographic locations, e.g., *S. alba* in India (Chatli et al., 2008), *Salix viminalis* in the United Kingdom (Barnes et al., 2018), *S. viminalis* and *S.* × *dasyclados* in Germany (Baum et al., 2006), and *S. viminalis*

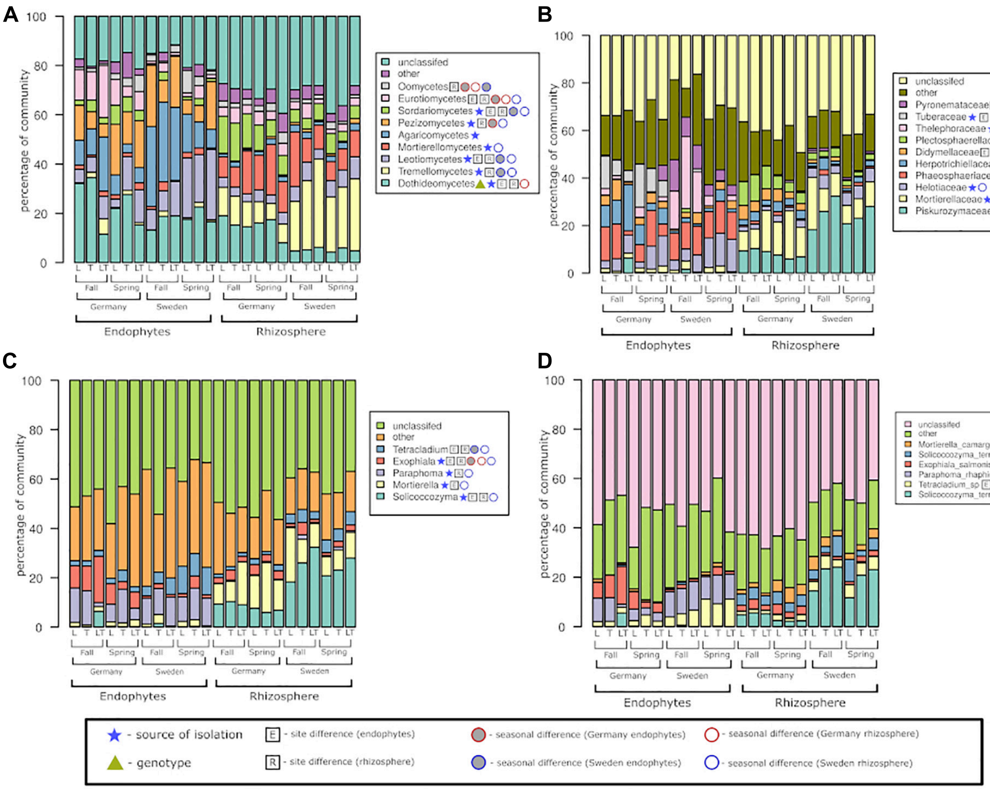

FIGURE 7 | Fungal community structure at the level of class **(A)**, family **(B)**, genus **(C)**, and species **(D)** among rhizosphere soil and roots, two experimental sites, and two seasons.

and *S. schwerinii* in Sweden (Hrynkiewicz and Baum, 2012). The soils in the investigated sites can be described as fertile and biologically active, with optimal properties for willow cultivation (Guidi et al., 2013). Soils at the Swedish site contained a higher content of TOC, TN, and P_{av} than soils at the German site, which likely resulted from primary soil properties related to their different pedogenesis (Vertic and Stagnic Cambisols) such as texture and nutrient abundance. However, the studied soils were moderately to slightly acidic, which undoubtedly favors the bioavailability of P (Yue et al., 2000; Richardson et al., 2009; Yadav et al., 2012). TN is frequently found to be a critical factor for fungal diversity (Allison et al., 2007; Lauber et al., 2008; Zhou et al., 2016). The available P in soil may influence fungal communities in the rhizosphere to some extent (He et al., 2016; Rosenstock et al., 2016; Williams et al., 2017). In our case, the Swedish site had significantly higher $P_{av,}$ but we observed no significant influence of this variable. This confirms N-limited rather than P-limited conditions, as is common at most arable sites (Williams et al., 2017).

A statistically significant short-term seasonal increase in TOC was recorded at the experimental site in Germany, but a similar trend was also observed at the experimental site in Sweden. This can be attributed to the leaf and root litter inputs in combination with no-till management. However, research by Hoeber et al. (2020) showed that climatic factors may significantly determine the rate of decomposition of leaf litter. These authors found that the decomposition rates in Germany were 43% faster than those in Sweden. The fine roots of willows can also be an important source of soil organic matter (Kahle et al., 2007). A slight decrease in P_{av} concentrations in spring suggested nutrient depletion. No effects of nutrient depletion on the yields were observed in Sweden. These results were supported by the findings of (Kahle et al., 2007).

Dominating PSF Identity and Total Fungal Density in Willows

Among the culturable diversities, the number of PSF was much higher in the rhizosphere fungi than in the endophytic fungi. This supports our hypothesis that lower number of endophytic fungi may possess ability to solubilize P than rhizosphere fungi. We found *Penicillium* to be the dominant genus of PSF isolates from the SRCs; other, less frequent isolates were of the genera *Alternaria, Cladosporium,* and *Clonostachys*. Similarly, Chatli et al. (2008) reported members of *Penicillium* to be among the dominant strains isolated from woody crop species. These strains were also abundant in the rhizosphere of *Salix* spp. in Lithuania (Repečkienė et al., 2009). The genus *Clonostachys* (isolated from the rhizosphere and endosphere) was specific to the German site, whereas *Juxtiphoma*, *Talaromyces,* and *Beauveria* were specific to the Swedish site. Apart from PSFs, we isolated non-PSF that were able to grow on selective media (NBRIP, PVK DCP). These fungi probably require low P concentrations to support growth. At the Swedish site, the fungal density in the three genotypes was found to range as follows: LT > Loden > Tora, in contrast to the German site, where it ranged as follows: Loden > LT > Tora. This effect might be based on the varying site-specific environmental conditions and their interactions with the genotypes.

Penicillium is commonly found as a PSF taxon (Chatli et al., 2008; Patil et al., 2012; Sharma et al., 2012). *Penicillium bilaiae* is even sold by NovoZymes as a bioinoculant enabling soil P mobilization. Levels of plant growth-promoting effects by *Penicillium* species were associated with increased uptake of P into shoots (Qiao et al., 2019). Although P solubilization ability is common in the genus *Penicillium*, various species and strains differ in their capacity to mobilize P due to differences in the secretion of organic acids, phosphatases, and phytase or in the operation of other P solubilizing mechanisms. The genus *Clonostachys* (isolated both from the endosphere and rhizosphere) was previously reported as an endophyte in *Salix* species growing in SRCs (Hosseini-Nasabnia et al., 2016). *Clonostachys rosea* is reported to be a mycoparasite in *Theobroma gileri* and as a biocontrol agent against *Phytophthora palmivora* and *Moniliophthora roreri* (ten Hoopen et al., 2003). To date, reports on the P solubilizing abilities of *Alternaria* and *Cladosporium* are scarce. There is not much information about PSF isolates from other woody plants. Schmidt et al. (2018) investigated endophytic community of poplar, species that is also commonly grown as SRCs but did not found any PSFs. Mishra et al. (2014) reported presence of several phosphorus solubilizing fungi in banana tree including *Fusarium* sp., *Trichoderma* sp. and present in this study *Penicillium* sp. In forest environment *Aspergillus* sp., *Cladosporium* sp., *Curvularia* sp. and several *Penicillium* species are frequently reported to possess high potential in P solubilization (Manoharachary and Nagaraju, 2017). There is several studies that use *Penicillium* species as bioinoculants that improve P availability for various plants (Omar, 1997; Ram et al., 2015; Yin et al., 2015; Li et al., 2016).

Multiple Factors Influencing Alpha Diversity and Fungal Community Structure in Willow SRCs

The rhizosphere fungal community was more diverse in culture independent (metabarcoding) analyses, suggesting strong selective pressure in the interior of willow roots. Similar observations are common both for bacterial (e.g., Kielak et al., 2008; Bulgarelli et al., 2012) and fungal (e.g., Hrynkiewicz et al., 2012; Yergeau et al., 2015; Thiem et al., 2018; Furtado et al., 2019) communities associated with various plants. The reason for this phenomenon could be that roots may act as a filter or selection barrier for fungal species present in the rhizosphere soil, which can result in a lower number of endophytes (Garbeva et al., 2004; Lauber et al., 2008). This might be especially valid for woody roots such as those of *Salix* spp.

The classes *Agaricomycetes* and *Pezizomycetes* were more frequent in the endosphere, while *Tremellomycetes* and *Mortierellomycetes* were more abundant in the rhizosphere. These facts could be explained by the former two classes comprising mostly ectomycorrhizal fungi (Li et al., 2018), whereas the latter contains mostly saprophytic organisms (Francioli et al., 2020). Yergeau et al. (2015) reported the dominance of the class *Dothideomycetes* in willows, and the same was observed in our study. Members of this class are mainly endophytes and epiphytes and can be lichenized or lichenicolous fungi (Schoch et al., 2009). Moreover, *Dothideomycetes* was the only fungal class whose abundance differed between genotypes. *Agaricomycetes* was the only class whose abundance did not differ between seasons, which was caused by large differences within variants (i.e., high standard deviation). Similarly, reports by Shakya et al. (2013) from poplar showed no seasonal effect for rhizosphere fungi at any of their investigated sites.

At species level fungal community was mostly build from six species. For endophytes *Tetracladium* sp., *P. rhaphiolepidis* and *E. salmonis* were the most frequent. All three species are known grass endophytes while *E. salmonis* was additionally reported as an animal pathogen (Maciá-Vicente et al., 2016; Ricks and Koide, 2019; Gomzhina et al., 2020). Besides being pathogenic in animals it was previously isolated from the roots of poplar, which is another commonly grown tree species in SRCs (Maciá-Vicente et al., 2016). Most of the fungal reads from the rhizosphere soil belonged to *S. terrea,* but no information is available on this fungal species. The other *Solicoccozyma* species found in this study, *S. terricola* is a well known psychrotolerant yeast used in lipid production (Filippucci et al., 2016; Stosiek et al., 2019; Tasselli et al., 2019). Lastly, *Mortierella camargensis* isolated from grassland soils showed ability to produce arachidonic acid and lipase activity (Botha et al., 1999; Miklós et al., 2012).

The absence of the PSFs cultured from our samples in amplicon libraries is probably due to technical reasons. First, universal primers used to generate libraries might be biased against particular sequence variants (SVs), which together with SV scarcity in samples might result in excluding them from libraries.

Unconstrained ordination revealed that the level of community association with plants (rhizosphere vs. endosphere) and experimental sites were the two most important factors grouping the samples. This is expected, as (i) the difference between rhizosphere and endophytic communities is frequently observed (Thiem et al., 2018) and (ii) the sites differed in both climatic and soil conditions. Indeed, soil environmental parameters (TOC, TN, and pH) significantly influenced the fungal community structure. A significant influence of pH was unexpected, as it is usually not a limiting factor for fungi. Out of four factors we hypothesized would influence fungal communities in willow, level of community association with the plant, experimental site location and season turned out to exert a significant impact on fungi, while the effect of tree genotype was not as prominent. This fact can be explained by (i) the spatial distribution of fungal mycelium in soil, i.e., mycelium is able to freely grow out of particular field boundaries, effectively canceling actual differences, and (ii) genetic differences between analyzed genotypes may cause little effect on the soil fungal microbiome.

CONCLUSION

The level of fungal community association with the plant (rhizosphere vs. root endophytes) is the most important factor shaping its diversity. The site, season, and planting design have a lower impact. The fungal diversity at the same level of plant association was mainly driven by soil properties such as TN, TOC, and pH. Among the culturable fungal diversities, *Penicillium* was dominant and commonly isolated genus as a P solubilizing taxon from both SRCs, while others less frequently isolated were the genera *Alternaria, Cladosporium,* and *Clonostachys*. Apart from PSFs, we isolated non-PSF that may require less P to support their growth. In general, a lower number of endophytic fungal strains possessed the ability to solubilize P compared to the number of rhizosphere fungal strains with this ability. The rhizosphere fungal community was generally more diverse than that in the endosphere at both willow SRC sites. This might suggest selective pressure on willow roots and emphasize the uniqueness of the fungal community. Fungal libraries of rhizosphere soil and endophytic communities showed significant dominance of the phyla Ascomycota followed by Basidiomycota and Mortierellomycota. The genus *Exophiala* was present in greater numbers at the German site, while the genus *Tetracladium* was present mostly at the Swedish site.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject, PRJNA716888; https://www.ncbi.nlm.nih.gov/genbank/, MW342736–MW342757.

AUTHOR CONTRIBUTIONS

PK participated in all analyses and wrote the first version of the manuscript. BF participated in preparation of manuscript. MG designed the bioinformatics pipeline, performed bioinformatics analyses, and participated in the preparation of the manuscript. PK and BF analyzed the results and the statistical output. PK, CB, and MW performed sampling at the locations, selected plant genotypes for the experiments, and provided input to the manuscript. PH did soil analyzes and participated in the preparation of the manuscript. KH designed and managed the field and lab experiments and participated in the preparation of the manuscript. All authors revised the manuscript and approve of its publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.647709/full#supplementary-material

Supplementary Figure 1 | Average temperatures and average rainfalls in Rostock (Germany) and Uppsala (Sweden) in 2018 and 2019.

Supplementary Figure 2 | Fungal community structure at the phylum level among rhizosphere soil and root samples, two experimental sites, and two seasons.

Supplementary Table 1 | Meteorological data from two experimental sites in Sweden (SE) and Germany (GER). The maximal monthly temperature (max temp), minimal monthly temperature (min temp), average monthly temperature (avg temp), precipitation (rain), and monthly sunshine hours (sun) in 2018–2019. Data source: https://www.worldweatheronline.com.

Supplementary Table 2 | Selective media (NBRIP, PVK, and DCP) used for the selection of phosphate solubilizing fungi.

Supplementary Table 3 | Identified fungi with accession numbers. S – Sweden, G – Germany, L – Loden, T – Tora, LT – mixture, E – endophyte, and R – rhizosphere fungi.

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Changes in Root–Shoot Allometric Relations in Alpine Norway Spruce Trees After Strip Cutting

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Silvicultural interventions such as strip cuttings can change the resource availability of the edge trees. This may alter tree allometry, as light regime, water, and nutrient availability can change at the forest edge. Increased root growth may optimize resource uptake and/or enhance tree anchorage to withstand the altered wind regime. However, little is known about the patterns of the root–shoot allometric responses to strip cuttings. In three alpine stands differing in climate, site productivity, and stand characteristics, we selected 71 Norway spruce trees and took increment cores from stems, root collars, and main roots. This enabled us to study changes in the long-term root-stem allometry for 46 years and short-term allometric responses to intervention. The effects of cutting were compared between edge trees and trees from the stand interior in 10 years before and after the intervention. The long-term allocation to roots increased with stem diameter, with the strongest effects on the regularly managed stand with the tallest and largest trees. These results support the allometric biomass partitioning theory, which postulates resource allocation patterns between different plant organs to depend on plant size. Strip cutting on north-facing slopes boosted edge-tree growth in all plant compartments and enhanced allocation to roots. This change in allometry started 2 years after cutting but disappeared 7–8 years later. In the post-cutting period, the highest root–shoot increase was observed in the small trees independent of the site. This indicates the change in growing conditions to have the strongest effects in formerly suppressed trees. Thus, the effect of such acclimation on the wind firmness of subdominant spruce trees is a question with high importance for optimizing cutting layouts in lowering post-cutting vulnerability to disturbance. The results from this case study contribute to a better understanding of the structural acclimation of spruce trees from high-elevation forests to new forest edges. However, for a more mechanistic understanding of environmental drivers, further analyses of tree-ring stable isotopes are recommended.

Keywords: strip cutting, mountain forests, Norway spruce, edge trees, biomass allocation, root-shoot allometric responses, tree size

INTRODUCTION

Investigations about growth allocation patterns in trees are fundamental to select the appropriate silvicultural treatments to improve the growth of economically important plant organs (Poorter and Sack, 2012; Poorter et al., 2012) and to understand the extent to which trees or tree stands are susceptible to disturbances (Wonn and O'Hara, 2001; Pretzsch et al., 2014). Changes in relative growth or size of different plant parts are the focus of allometric studies (Huxley and Tessier, 1936; Niklas, 2005) with root–shoot development being the best-analyzed allometric relationship (Shipley and Meziane, 2002; Mokany et al., 2006). The ability of a tree to shift biomass growth toward above- or belowground structures is a key trait for adapting to changing site conditions (Bolte et al., 2004; Dumroese et al., 2019) and depends on (1) environmental effects (e.g., duration and magnitude of changes in resource availability; Weinstein et al., 1991; Callaway et al., 1994), (2) phylogeny (e.g., differences between deciduous and evergreen species; Drexhage and Colin, 2001; Poorter et al., 2012), and (3) ontogeny (i.e., resource allocation patterns change with plant size; Shipley and Meziane, 2002; Niklas, 2005).

In forest stands, the allocation patterns of individual trees can be altered by management (Pretzsch et al., 2014). For example, the competition release induced by thinning may boost root expansion since more soil water becomes available to cover, among others, the increased transpiration demand for tree growth (Tyree, 2003). An overproportional belowground growth enhances the weight of the root-soil plate itself, which is an important structural component of tree anchorage and resistance to uprooting (Ennos, 1993). An altered wind regime due to cutting can also alter cambial growth as a direct response to tree movement, with accelerated growth close to the stem base (in the zone of the root collar), as compared to the upper stem parts (Nicoll and Ray, 1996; Nicoll and Dunn, 2000). However, quantitative information on these aspects is still scarce. Failure to understand how trees adapt structurally and functionally to competition-release may lead to erroneous conclusions about tree vigor and mechanical stability in response to disturbance and management.

For many conifers that tend to develop shallow and plate-formed root systems (Stokes and Mattheck, 1996; Polomski and Kuhn, 2001), the ability to redistribute resources to roots seems essential in adapting to imposed mechanical stress (Urban et al., 1994; Telewski, 1995; Stokes, 2002). For example, in a balsam fir-black spruce forest in Québec (Canada), retention cutting induced faster roots than stem growth (Pretzsch et al., 2014). The interpretation for the observed change in growth allocation was that the newly exposed trees needed to increase their mechanical stability against windthrow, by biomass allocation to the root system for stronger anchorage (see also Nielsen, 1995). Another study from a subalpine Norway spruce [*Picea abies* (L.) Karst.] forest in Switzerland showed growth releases in the edge trees after one-sided tree exposure to edge conditions, with higher radial growth at the stem base relative to breast height position (Bräker and Baumann, 2006). The authors supposed adaptive belowground growth of the edge trees to the new wind exposure. Hence, after a certain degree of competition-release, adapting tree growth would favor allocation belowground as this response can improve wind firmness of trees, i.e., would be beneficial for tree functionality and structural stability.

The effects of management on the relative tree-stem growth are related to tree size: small-sized trees react more strongly to thinning or strip cutting than larger trees (Epp et al., 2005; Vitali et al., 2016). The size of trees or their social position in closed stands results mainly from the lower availability of resources they can acquire (Pretzsch et al., 2018). A study in stands in the Solling Mountain region (Germany) showed dominant spruce trees [stem diameter at breast height (*dbh*) higher than 60 cm] to attend the highest root–stem ratios (Bolte et al., 2004). Tall and dominant spruce trees had not only larger root biomass but also showed higher anchorage than smaller and suppressed trees (Bolkenius, 2003). However, only view quantitative results exist about the time needed for spruce trees to acclimate to reduced canopy density (Urban et al., 1994; Pretzsch et al., 2014). Long adjustment periods would make edge trees vulnerable to ecological risks such as wind or snow break (Wonn and O'Hara, 2001) followed by bark beetle attacks (Jurc et al., 2006). So, the intended effects of silvicultural interventions may be outweighed by unintended side effects. A profound assessment of such effects is particularly important for designing future cutting strategies in the spruce forests of Central Europe, as the ongoing warming combined with the highly uncertain wind regime (Lindner et al., 2014) will challenge our ability to evaluate related ecological risks.

To promote natural regeneration, narrow slit-shaped gaps (20–30 m wide) have been cut since the 1980s in many of the Alpine forests of Switzerland dominated by spruce (Brang, 1998). However, 10–15 years after the intervention, in a significant number of cases, the trees surrounding these regeneration gaps were damaged by storm events or bark beetle outbreaks (Streit et al., 2009). As the intensity of natural disturbances in Alpine forests is expected to increase with climate change (Seidl et al., 2014; Bebi et al., 2017), the area covered by forest edges will also increase. This may particularly affect even-aged, spruce-dominated stands with a closed canopy (Stritih et al., 2021). Therefore, the quantification of timing and magnitude of the structural acclimation of remaining edge trees to gap cutting is of general interest.

Aboveground responses of spruce trees to large strip cuttings were recently quantified in high-elevation stands. After cutting, the radial growth temporally increased by 12–60% only in edge trees growing in north-facing sites, with stronger effects in small trees (20–30 cm *dbh*; Vitali et al., 2016). However, it remained unclear how edge conditions influence root growth. This is of particular interest as belowground allocation may optimize the form of the tree not only to enhance resource uptake but also to increase its ability to withstand the altered wind regime. Therefore, in summer 2017, we cored individual trees again on the same experimental sites (Vitali et al., 2016) but took samples from three compartments: stems, representative root collars, and main roots. Annual increment in roots and stems and their allometric relationships were applied as surrogate variables for tree-growth allocation to aboveground and belowground organs (Nikolova et al., 2011; Pretzsch et al., 2014). Although root–stem ratios are only a coarse indicator of acclimation processes affecting carbon (C) allocation (Poorter et al., 2012; Prescott et al., 2020), increment data provide good estimates of belowground and aboveground biomass growth (Vincent et al., 2009). To this end, the following analyses were done:

(1) Assessment of the long-term allometry for stand-interior trees to test if the relative allocation to roots is site- or tree-size-specific.

(2) Comparison of the short-term allometry change in stand interior vs. edge trees 10 years before and 10 years after the cutting year. The factors driving the change in post-cutting allometry were analyzed by statistical modeling.

MATERIALS AND METHODS

Study Area

The study was conducted in summer 2017 on 74 adult spruce trees, growing at two locations in the Upper Rhine Valley and a third one in the Lower Engadine in the Swiss Alps (Grisons, Switzerland). Our sampling used three permanent plots established in a previous study on radial growth changes in spruce after strip cutting (Vitali et al., 2016). Tree age and forest origin (naturally regenerated or planted forest) are known to influence tree allometry (Huxley and Tessier, 1936; Wang et al., 2008); therefore, we selected the stands Furna, Siat, and Sur En, which represent forests with trees originating from natural regeneration (**Table 1**). The three stands are composed of 100% spruce, and have been affected by neither management activities nor natural disturbances within the 20 years prior to strip cutting. The time since the year of strip cutting varies from 11 to 13 years. The three sites are located at elevations ranging from 1,500 to 1,680 m a.s.l. on slopes with an inclination between 50 and 70%. Siat has a south-facing aspect with winds predominantly from the north, whereas Furna and Sur En have a north-west aspect with winds prevailing from the south (**Table 1**).

In this study, Furna is the oldest (nearly 270 years old), and Sur En is the youngest (nearly 160 years old) stand. Furna and Siat were traditionally managed by repeated selection cutting, while Sur En was only sporadically managed. With an average width of 35 m, the strip in Sur En was two times smaller than the strips in Furna and Siat (**Table 1**). The edges of the three strips are oriented to East or North-East and receive direct radiation in the morning and around noon. Winds are prevailing from South-South-West or North-North-East, and Furna is the windiest site with a mean annual wind velocity of 5.6 m s^{-1} (**Table 1**).

The spruce stands differed in stand characteristics and climate. Furna has the lowest tree density (332 trees ha^{-1}, caliper cut-off size 8 cm) but the highest growing stock and basal area among all stands (1,029 and 71.2 m^2 ha^{-1}, respectively; **Table 1**). Siat has a similar stand density to that in Furna (i.e., 363 trees ha^{-1}), but a lower growing stock and basal area (668 and 47.6 m^2 ha^{-1}, respectively), and Sur En have the highest stand density (644 trees ha^{-1}) with the smallest growing stock (400 m^3 ha^{-1}) and lowest basal area (34.6 m^2 ha^{-1}). According to the site index of Keller (1978), showing the dominant tree height at an age of 50 years, Furna is the most productive site, and Sur En is the site with the lowest productivity (site index of 22 and 16, respectively; **Table 1**). Stand data were assessed according to the instructions from the Experimental Forest Management program (Forrester et al., 2019).

Daily means of air temperature (*Ta*) and precipitation sum (*Pr*) were obtained for each study site using the DAYMET software (Thornton et al., 1997). Furna and Siat from the Upper Rhine Valley have similar climatic characteristics with an annual mean *Ta* of nearly 3.5°C and an annual *Pr* amount of 1,300–1,500 mm. Sur En is located in the Lower Engadine, which is one of the driest regions of Switzerland and is characterized by a continental climate. To identify the water availability of the study sites, the annual de Martonne aridity index (DMI) was used (Maliva and Missimer, 2012). Furna and Siat are classified as "excessively humid" sites, whereas Sur En with DMI < 60 is a "very-humid" site (**Table 1**; for DMI classification refer to Pellicone et al., 2019). This results from the relatively low amount of *Pr* in the continental Lower Engadine (**Figure 1**).

Sampling Design

Characteristics of the Sample Trees

Eleven to sixteen vigorous dominant or codominant trees without visible damage by pathogens or rockfall were selected from the edge zone and the stand interior of each study site as sample trees. Trees with crown perimeters exposed to the cutting area were defined as edge trees and the remaining trees as stand-interior trees. We measured tree height *h*, *dbh*, stem diameter at a position of coring *ds*, live crown ratio (*Lc*) (calculated as the percentage of live crown length to the total tree height), and two perpendicular diameters of the sampled main roots at the coring positions dr_1 (in horizontal direction) and dr_2 (in vertical direction). As a measure of tree stability, we calculated the individual *h* to *dbh* ratio (*h/d* ratio). In addition, for documentation purposes, we sketched the main root architecture of each sampled spruce in relation to the cutting area.

Sampling Procedure

Core samples were taken from all trees using 400 mm Haglöf increment borers (5 mm in diameter). Three parts of the trees were sampled: stems, root collars, and main roots. In the stems, the same sampling procedure was applied to edge and stand-interior trees. Each sample tree was cored two times at 1.0 m stem height to avoid damage at the position of future *dbh* measurements on the future permanent plots. The stem increment cores were taken from two opposing directions perpendicular to the slope direction to minimize the potential bias caused by any reaction wood.

A sampling of root collars and roots differed between edge and stand-interior trees. From each edge tree, four horizontal main roots were sampled: two roots growing in the direction of the cutting area and two additional roots in the direction of the stand interior to account for effects of wind load and possible differences in soil water/nutrients availability on cambial activity. From the stand-interior trees, two main roots were sampled: one main root facing toward the cutting area and one growing in the opposite direction to avoid eventual bias from different wind

TABLE 1 | Site and stand description.

Site	Furna	Siat	Sur En
Latitude	46°54′07″N	46°47′58″N	46°48′43″N
Longitude	9°40′16″E	9°09′40″E	10°22′35″E
Elevation (m a.s.l.)	1,680	1,615	1,500
Aspect	North-west	South	North-west
Slope inclination (%)	59	51	72
Edge orientation	NE	E	NE
Main wind directions[a]	SSW/NNE	SSW/NNW	SSW/NNE
Mean annual wind velocity (m s^{-1})	5.6	3.7	3.0
Stand characteristics			
Stand origin	Natural regeneration	Natural regeneration	Natural regeneration
Management type	Selection cuttings	Selection cuttings	Sporadic
Year of last intervention before strip cutting	1990	1974	1960
Year of the strip cutting	2004	2004	2006
Average strip width (m)	70	70	35
Stand density (n ha^{-1})	332	363	644
Growing stock (m^3 ha^{-1})	1,029	668	400
Basal area (m^2 ha^{-1})	71.2	47.6	34.6
Dominant tree height (m)[b]	35.6	33.1	28.4
Stem *dbh* of dominant trees (cm)	61.5 ± 0.5	56.3 ± 0.4	41.2 ± 0.9
Site index[c]	22	20	16
Climate[d]			
Mean annual air temperature (*Ta*, °C)	3.5	3.4	4.2
Min/Max monthly annual air temperature (°C)	−0.2/7.8	0/7.6	−0.5/10.0
Mean air temperature during growing season (°C)	11.1	11.0	12.8
Annual precipitation sum (*Pr*, mm)	1,290	1,496	809
Precipitation sum during growing season (mm)	463	480	310
Annual De Martonne index (DMI)	96	111	57
Soil characteristics[e]			
Soil type	Albic Podzol	Albic Podzol	Skeletic Cambisol
Rooting depth (cm)	80	80	85
Available water capacity (mm/80 cm soil depth)	166	136	82
Humus type	Mor	Mor	Moder

[a] *Modeled wind data according to Windatlas (2017), maximal wind velocity is derived from the site-specific wind rose (Windatlas Schweiz, 2017).*

[b] *Average height of the 100 largest trees per hectare. The tree parameter data are given with the mean ± std. error. Plot size is 0.35 ha (Furna), 0.49 ha (Siat), and 0.45 ha (Sur En) ha; calipering threshold 8 cm.*

[c] *Site index according to Keller (1978), i.e., dominant tree height at age of 50 years. Low values indicate low site productivity.*

[d] *Climatic data were averaged over the period 1970–2016. The growing period is defined as June 1 to August 31.*

[e] *Soil description represents soil characteristics of the stand interior.*

loads due to the nearby edge. Roots that were nearly vertically oriented were not cored.

Each sample root was first cored at the root collar at a maximum distance of 10 cm from the trunk edge. The same root was then excavated, keeping damage to the soil as low as possible, and a second increment core was taken from the top at a distance of 30–100 cm from the trunk edge. In this range, missing rings are comparably rare, and cross-sections exhibit small eccentricity (Gärtner, 2007; Wrońska-Wałach et al., 2016). Horizontal dr_1 and vertical dr_2 root diameters were measured at the coring position by a slide caliper (precision 1 mm) to get an estimate of mean root diameter dr.

In the laboratory, cores that could not be measured due to rot were discarded from further analyses (**Supplementary Table 2**). This procedure provided an average of 3.8 main root cores per edge tree, and 1.9 main root cores for the trees from the stand interior. According to our sketches of the main root architecture, each tree had an average of 5.2 horizontal main roots available for coring. This means that in the present study we have analyzed 40% (for the stand-interior trees) to 75% (for the edge trees) of all horizontal main roots available for coring.

After a windthrow event in Sur En (August 8, 2017), several trees from the study plot and the surrounding forest were uprooted, exposing the root system. We used this chance to obtain an estimate of the main root eccentricity. To this end, 30 disks of eight roots and five trees were cut in distances of 30–160 cm from the trunk edge. The samples were then sanded and growth eccentricity from the pith was measured in four

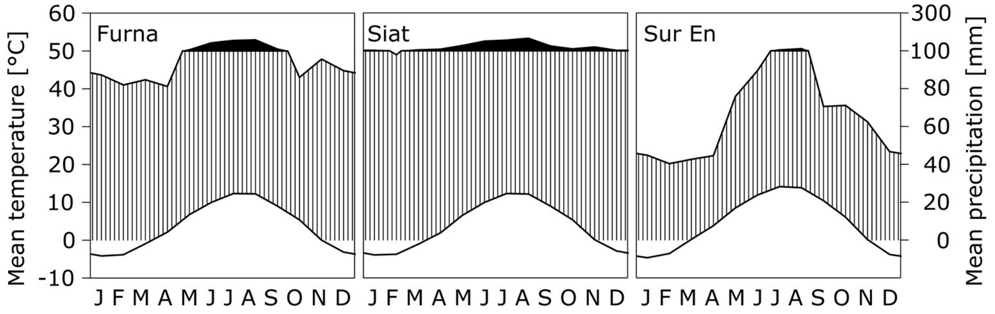

FIGURE 1 | Walter-Lieth climate diagrams representing the long-term averages (from 1970 to 2016) of monthly air temperature *Ta* (upper line) and monthly precipitation sum *Pr* (lower line) at the study sites Furna, Siat (both Upper Rhine Valley), and Sur En (Lower Engadine). The vertical axes for *Ta* and *Pr* are scaled with a ratio of 1:2, as after Walter and Lieth (1960–1967) a month is classified as arid if *Ta* in °C exceeds two times *Pr* in mm. Black areas indicate periods with $Pr > 100$ mm, and white areas show periods with $Ta < 0$°C. Note that the vertical axis is broken at $Pr = 100$ mm.

directions (for methodological details see Montagnoli et al., 2020) and related to the distance from the trunk edge. The eccentric growth of the main roots ended more than 100 cm from the trunk edge. At a closer distance, root radii at the upper part of the root were on an average 40% wider than the mean radius of the corresponding cross-sectional root disk (data not shown).

Tree-Ring Measurement and Dating Procedure

The increment cores were prepared with a core-microtome (Gärtner and Nievergelt, 2010) to maximize annual ring visibility. Ring widths were measured to the nearest of 1/100 mm using the Lintab 5 measuring system in connection with the software TSAP-Win (Rinn, 2012). As color differences between earlywood and latewood were occasionally low in the roots, chalk was applied to identify the annual ring border.

After visual cross-dating, COFECHA (Grissino-Mayer, 2001) was used to statistically check synchronization and dating of each stem series based on site-specific pointer years (**Supplementary Table 1**). The two stem cores of a tree were averaged to obtain a representative time series of the aboveground increment for each tree. The resulting mean series was used to cross date root collar and root cores of the same tree. For root and root collar cores, high *t*-values and a good optical fit with the stem chronology and the mean site chronologies (per position: edge, stand interior) were used as indicators for dating success. COFECHA routines were not used on the root samples as these often feature too few rings and the results proved to be not helpful for dating (Nikolova et al., 2011). Missing rings were found especially in the root samples, and represented on an average 1.5% of all rings in the specimens. No double rings were recorded across all studied plant compartments.

A total of 71 stems, 209 root collars, and 211 main root ring-width chronologies were built for edge and stand-interior trees on the three sites (**Table 2**). All cores that did not reach back at least 10 years before the respective year of cutting were excluded from the dataset. In preliminary analyses, no significant difference was found between the post-cutting annual increments related to the direction of root collar or main root growth (data not shown). Therefore, the root collar and main root increment series were averaged to produce one-time series representative for each of the root collars and the main root growth patterns of the respective tree. The common interval of all series was truncated to the period 1970–2016, as this interval enabled us to study the growth reactions of spruce trees before and after the year of strip cutting (i.e., 2004 in Furna nad Siat; 2006 in Sur En; **Table 1**), and was enough long for cross dating.

We used mean sensitivities (MSs; Fritts, 1976) of stems, root collars, and main root growths as indicators for high-frequency growth patterns of each of the studied tree parts to changes in environmental conditions. We calculated MS for each chronology as the difference between two successive rings divided by their mean (Biondi and Qeadan, 2008) using the function *sens1* of the R package "dplR" (Bunn, 2008). Then, the MS was averaged for the period of 46 years (1970–2016) within each studied tree compartment and study site. As a measure of the series intercorrelation, the cross date index (CDI) was calculated for the same period of 46 years using TSAP-WIN. In this study, we show MS and CDI analyses only for stand-interior trees, as the growth patterns of edge trees during the last 10–12 years were additionally influenced by strip cutting.

The ring-width series of stems, root collars, and roots were indexed to remove biological trends (e.g., the age trend) following standard procedures (Cook and Kairiukstis, 1990; Esper and Gärtner, 2001). The indexed chronologies were then averaged per tree compartment, tree position, and site.

Allometric Analysis

For the allometric analyses, we focused only on stems and main roots as indicators for above- and belowground tree growth (Cherubini et al., 2021). At the root collar, effects of tree swaying and reaction wood as a result of altered wind exposure can be expected (Wrońska-Wałach et al., 2016).

We applied the basic allometric equation (Huxley and Tessier, 1936), which describes how the studied plant organs main root and stem change with plant size:

$$\ln\left(dr_i\right) = \alpha_0 + \alpha \times \ln(ds_i) \tag{1}$$

TABLE 2 | Characteristics of the sampled trees (stems and roots) in summer 2017.

Tree position	Furna			Siat			Sur En		
	Stand interior	Edge		Stand interior	Edge		Stand interior	Edge	
N sampled trees	15	14		12	11		16	14	
Age of the five oldest trees (years)	269 ± 19	265 ± 7		213 ± 6	204 ± 11		173 ± 3	151 ± 10	
Tree height (*h*) (m)	34.0 ± 1.9	33.4 ± 3.2		30.9 ± 3.3	32.3 ± 4.6		30.0 ± 2.4	25.0 ± 4.2	
Stem diameter (*dbh*) (cm)	57.3 ± 5.5	57.9 ± 9.5		52.9 ± 8.1	57.0 ± 6.8		43.5 ± 6.7	34.8 ± 11.4	
h/d ratio	59.7 ± 5.2	58.7 ± 7.6		58.9 ± 5.4	56.6 ± 3.8		69.8 ± 7.2	74.9 ± 11.8	
Live crown ratio (*Lc*) (%)	74.7 ± 12.0	74.8 ± 12.5		61.8 ± 12.9	67.2 ± 9.4		62.6 ± 14.1	63.6 ± 10.2	
Root growing direction	Stand	Cut	Stand	Stand	Cut	Stand	Stand	Cut	Stand
Number of sampled roots	26	23	23	21	19	18	28	24	23
Root diameter *dr* (cm)	20.11 ± 9.7	19.2 ± 9.4	16.8 ± 7.6	21.1 ± 9.2	20.5 ± 9.6	19.4 ± 8.2	19.7 ± 8.0	18.0 ± 8.9	18.5 ± 9.9
Age of root samples (years)	75 ± 32	58 ± 28	73 ± 31	104 ± 48	121 ± 59	94 ± 42	96 ± 31	77 ± 29	72 ± 26

Data are shown as mean ± SE.

where α_0 is the allometric factor and α the allometric exponent, and dr_i and ds_i are the root and stem radii for the year i from the study period 1970–2016. Stem and root radii were calculated backward by subtracting the measured annual radial increment from the radius at survey time.

Logarithmic transformation of the observed radial increment data reduces the effect of outliers on the results, and the transformed data better meet the statistical assumptions of normal distribution and homoscedasticity (Poorter and Sack, 2012). The biologically relevant term in Equation (1) is the exponent α, which covers both radial increment and plant proportions in the long term, and equals 1.0 when plant growth is in a steady-state (Poorter et al., 2012). In this study, we only used the trees from the stand interior, representing undisturbed long-term growth. As this study is not based on biomass assessment and because of the eccentric form of the roots, α overestimates the relative increase of root biomass growth. Therefore, in the present work, α was used as a surrogate variable to analyze long-term changes in tree allometry between stems and roots, and not as a predictor for the absolute biomass allocation to these plant organs (Nikolova et al., 2011).

We applied the value pairs dr_i, dr_{i-1}, and ds_i, ds_{i-1} from consecutive years to calculate the period-wise slope α'_i, which represents the short-term allometry change between root and stem of individual trees within 1 year:

$$\alpha'_i = \frac{\ln\left(\frac{dr_i}{dr_{i-1}}\right)}{\ln\left(\frac{ds_i}{ds_{i-1}}\right)} \tag{2}$$

Both radii dr and ds for the year i were calculated from raw non-detrended ring widths.

Furthermore, we quantified the allometric changes induced by strip cutting in terms of percentage change in allometric slope α'_i (PCA, %), calculated as the relation between α'_i prior to (A_p) and after (A_a) the year of cutting. To this end, we used the formula:

$$PCA = \frac{A_a - A_p}{A_p} \times 100 \tag{3}$$

where A_p is the mean α'_i during the 10 years before the cutting, and A_a that during the 10 years after the cutting. Positive PCA-values indicate enhanced allocation to roots instead of stems following strip cutting. In this way, we have broadened the analysis of Vitali et al. (2016), who quantified the growth release of spruce trees from the same study sites as the percentage change in the mean annual radial increment between the two intervals, based on information from stems only.

Statistical Analyses

We took the nested structure of our data (on a tree level) into account and applied linear mixed models to compute the allometric exponent α for each study site. To this end, we tested for a difference in the root–stem allometries of spruce trees growing in the stand interior:

$$\ln(dr) = \alpha_0 + \alpha_1 \times \ln(ds) + \alpha_2 \times \text{site} + \alpha_3 \times \ln(\text{ds}_\text{j}) \times \text{site} + b \tag{4}$$

where dr and ds are the roots and stems radii, α_0 (allometric factor), α_{1-3} represent the fixed effects (i.e., the allometric coefficients; Equation 1), and b considers the random effects on the tree level. In this model, we included the interaction of "stem radius" × "site" to test if the long-term allometry of stand-interior trees is site-specific. Linear mixed models were fitted with the *lme4r* function from the "lme4" package in R. For multiple pairwise comparisons (contrasts), the *HSD.test* function of the "agricolae" package in R (Mendiburu, 2020) was applied.

To explain the change in PCA on a tree-level after strip cutting, we applied linear models with a backward step-wise selection. In the initial models, "stem radius prior to cutting" (*Rp*), "tree position," and "site" were included as fixed factors. We included *Rp* to test whether growth allocation following cutting depends on tree size prior to cutting.

$$PCA = c_0 + c_1 \times Rp + c_2 \times \text{tree position} + c_3 \times \text{site} + c_4 \times \text{tree position} \times \text{Rp} + c_5 \times \text{tree position} \times \text{site} \tag{5}$$

where Rp is the stem radius prior to cutting, c_0 is the intercept, c_{1-5} represents the fixed-effect coefficients. The interactions "tree position" × "Rp" and "tree position" × "site" were tested as we were interested to understand if the cutting effects on trees growing at different positions are size- or site-specific. Outliers with Cook's distance $> 8/N$ (N = dimension of the dataset) were excluded from the analysis and the model was then refitted (Crawley, 2012). Outlier exclusion (one edge tree in Siat and one stand-interior tree in Sur En) did not change the model outcome but improved the model fit. Model selection was done by the function *stepAIC* of the R package "MASS" (Ripley, 2021), and the model with the lowest Akaike Information Criterion (AIC) was retained as a final model. The interaction plot was visualized by the function *interact_plot* of the R package "interactions" (Long, 2021).

Parameter coefficients and fit (R^2) were used to evaluate model effects and suitability. Wilcoxon rank-sum test was applied to test differences in PCA of stand-interior and edge trees within each site after strip cutting. This and all other computations were performed by R version 3.4.4 (R Development Core Team, 2018).

RESULTS

Characteristics of the Sampled Trees

The oldest trees were found in Furna (up to 270 years), whereas trees in Sur En were the youngest, with a maximum age of about 160 years (**Table 2**). The largest sample trees were recorded in Furna (mean values of about 34 m in height and 58 cm in *dbh*), the trees in Siat were only slightly smaller, and the smallest trees were found in Sur En (height: 25–30 m, dbh: 35–44 cm), with somewhat larger dimensions in the stand interior than at the edge. The resulting mean *h/d* ratios were < 60 in Furna and Siat, but in Sur En, mean *h/d* ratios were 70 and 75 for stand-interior and edge trees, respectively (**Table 2**). The sampled spruces had a *Lc* of 62–75% with maximum values in Furna. On all sites, *Lc* was similar for edge and stand-interior trees (**Table 2**).

The average diameter of the main roots sampled varied largely within stands, tree positions, and growing directions and ranged between 16.8 and 21.1 cm while not showing any significant patterns related to their growing direction. Main roots were on an average 58–121 years old at the position of coring with the oldest roots detected in edge trees in Siat. Interestingly, the youngest roots were sampled in the oldest and coldest stand Furna, but had similar dimensions (diameters) as the roots sampled from the other two sites (**Table 2**).

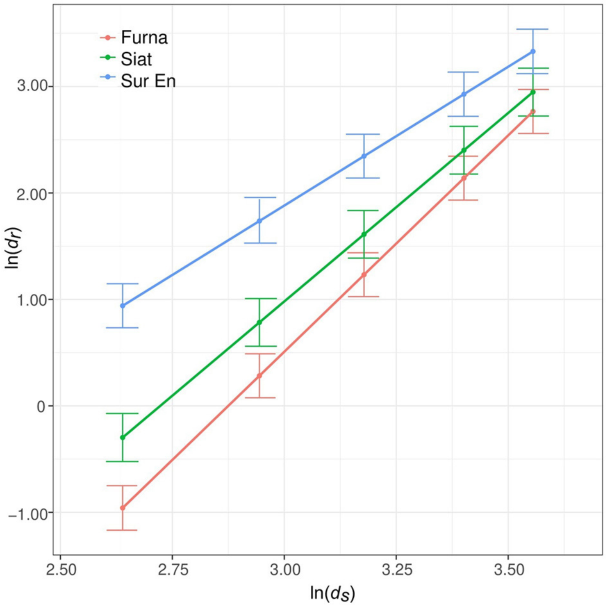

FIGURE 2 | Root–stem long-term allometry α (1970–2016) from the fitted model according to Equation (4). Its coefficients are shown in **Table 3**. Points indicate model estimates at five different stem radii covering a range of *ds* from 13 to 37 cm, and whiskers are the SE, with *dr* and *ds* being the root and stem radii (mm).

TABLE 3 | Estimated fixed effects for the long-term allometry (α) model (1970–2016) with the response variable ln(*dr*) of the stand interior trees (n = 37) in relation to ln(*ds*) and site [Furna, Siat, and Sur En; Equation (4)], with *dr* and *ds* being the root and stem radii (mm).

	Estimate	**Std. error**	***t*-Values**
(Intercept)	−11.68	0.29	−40.83***
ln(*ds*)	4.06[a]	0.06	66.31***
Siat	2.04	2.04	4.99***
Sur En	5.74	0.40	14.30***
ln(*ds*) × Siat	−0.52[b]	0.09	−6.10***
ln(*ds*) × Sur En	−1.46[c]	0.09	−16.37***

[a,b,c] *indicate significant differences between sites (compared to Furna).*
*Significance level ***$p < 0.001$.*

Long-Term Growth

Stem, Root Collar, and Root Growth

In trees from the stand interior, the mean annual tree-ring width of stems was higher in Furna (1.2 mm) and Siat (1.3 mm) than in Sur En (0.9 mm) (**Supplementary Table 3**; **Supplementary Figures 1–3**). Annual tree rings were widest in the root collars (from 1.8 cm in Sur En to 2.7 cm in Siat) and narrowest in the main roots (from 0.7 mm in Sur En to 1.0 mm in Siat; **Supplementary Figures 1–3**).

The MS of stand-interior trees was lowest in stems (0.14–0.23), followed by root collars (0.19–0.27), and roots (0.27–0.36; **Supplementary Table 3**). Overall, MS was highest in the driest site Sur En and lowest in Furna. Series intercorrelation captured by CDI was highest in stems (0.67–0.79) and lowest in roots (0.46–0.55) in all study sites. The highest MS and CDI values were found in all studied tree compartments of trees from Sur En.

Root–Shoot Allometry of Stand-Interior Trees

The long-term tree allometry described by the allometric exponent α differed between sites and was highest in Furna and lowest in Sur En (**Figure 2**, **Table 3**). Its values between 2.60 (in Sur En) and 4.06 (in Furna) illustrate a long-term site-dependent increase of growth allocation to roots with increasing stem diameter.

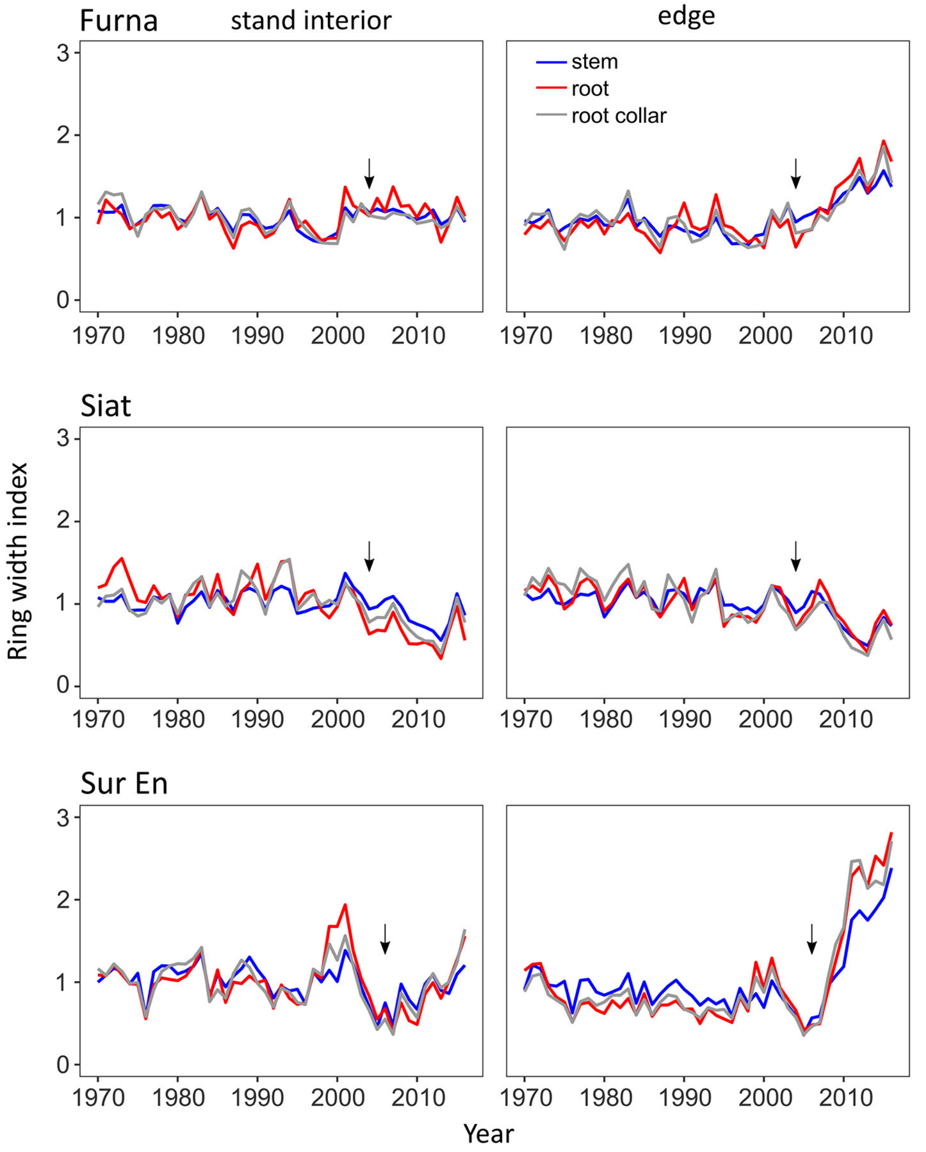

FIGURE 3 | Mean chronologies of the ring-width index for stand interior and edge trees in the three study sites Furna, Siat, and Sur En for the period 1970–2016. Arrows indicate the cutting year.

Effects of Strip Cutting on Tree Growth

Radial Growth of Edge and Stand-Interior Trees Before and After Strip Cutting

In Furna, the raw-data mean chronologies (**Supplementary Figure 1**) of edge trees show an overall increase of average annual ring width after strip cutting with the highest effects in root collars and roots. In Siat, no change in growth was detected in any of the studied tree compartments (**Supplementary Figure 2**), whereas in Sur En, all tree compartments reacted clearly with an increased average post-cutting radial increment (**Supplementary Figure 3**).

Before strip cutting, the year-to-year variation and the overall trend of indexed ring widths were similar in stand-interior and edge trees at all study stands and in all tree compartments (**Figure 3**). The distinct differences in the growth pattern of stand-interior and edge trees began to appear 2–3 years following the strip cutting (in Furna and Sur En) and lasted until the last measured year 2016. In Siat, trees from the stand interior and the edge showed similar growth patterns, which seemed independent of cutting.

Change in the Root–Shoot Allometry After Strip Cutting

Before cutting, the short-term allometry α' showed temporary differences for edge and stand-interior trees in Furna (in the period 1985–1995) and Siat (in the periods 1970–1980 and 1990–1997) with a high variance among both tree groups (**Figure 4**). A distinct increase in α' after strip cutting was detected in the edge

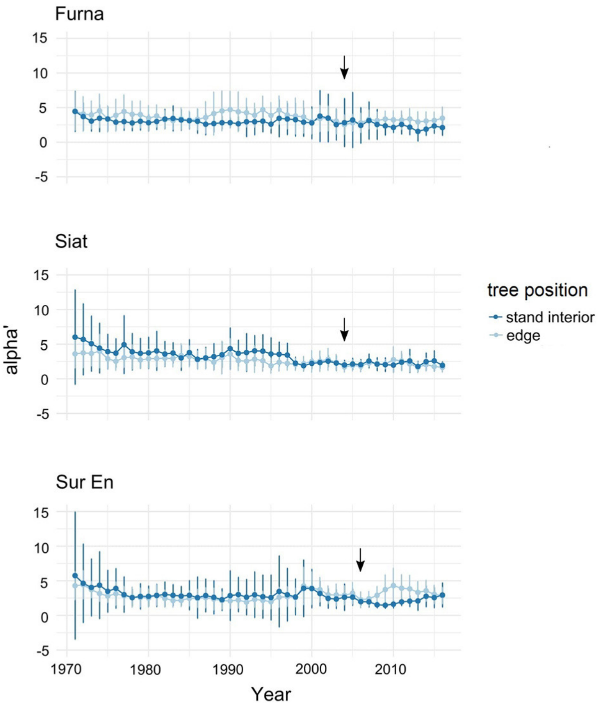

FIGURE 4 | Annual allometric slope α' (alpha' ± standard deviation) in edge and stand interior trees at the study sites Furna, Siat, and Sur En. Alpha' is calculated according to Equation (2) for the period 1971–2016. Arrows indicate the year of strip cutting.

trees of Sur En, and only a small change occurred in Furna, which indicates a higher allocation of growth to the roots than to stems on both sites. This change in growth started 2 years after cutting but disappeared after 7 years.

No change in PCA was detected in Furna, while significantly more belowground growth following cutting was found in edge trees from Siat and Sur En (**Figure 5**). Variation of PCA was strongest in Sur En in both stand-interior and edge trees. PCA was negative in the stand-interior trees from all sites, and positive in edge trees only from Siat and Sur En.

Factors Affecting the Changes in the Short-Term Allometry

In contrast to as could be expected from **Figure 5**, the factor "site" not significantly related to the change in PCA in spruce and was therefore omitted during model selection. Our final model was based on the variables "tree position," *Rp*, and included the interaction term "tree position" × *Rp*. The model explained 46% of the variation in PCA (**Table 4**). Nearly 25% of the variation was due to "tree position" and the other 20% were explained by the interaction of "tree position" with *Rp*. Allocation to roots generally decreased after the year of cutting in the stand-interior trees, but increased in the edge trees, with stronger allocation to main roots in small edge trees (**Figure 6**).

DISCUSSION

Strip cuttings are increasingly being practiced in the Swiss Alps to initiate natural regeneration in high-elevation spruce-dominated stands (Streit et al., 2009). The success of this approach is closely linked to the acclimation reaction of the remaining trees growing along the created forest edges, as post-cutting destabilization of the stand may occur (Nielsen, 1995; Girona et al., 2019). Therefore, the present study focuses on the post-cutting growth reaction of stems, root collars, and roots in edge spruce trees in relation to site and tree characteristics. Growth adjustments can optimize tree form for better resource uptake, but may simultaneously be inadequate to enable newly exposed trees to withstand stronger wind forces (Hanewinkel et al., 2015). In the following sections, we discuss the general growth patterns of spruce in the three studied tree compartments (stem, root collar, and roots), the long-term whole-tree (i.e., root–stem) allometry as depending on site and tree characteristics, as well as the factors controlling the allometric post-cutting (i.e., short-term) responses of edge trees. The possible site-and stand-specific effects of strip cutting on the tree and stand stability in high-elevation spruce forests are summarized as implications for practical applications.

Growth Patterns of Spruce Trees

In this study, we compare increments of stand-interior spruce trees from three Alpine stands differing in site characteristics and productivity. In cold climates such as those of the high-elevation forests, primarily temperature has been shown to control and stimulate tree radial growth (Hartl-Meier et al., 2014). However, the most narrow tree rings with the highest variability of annual growth were found at the comparatively warm but dry and least productive site Sur En. Thus, the high growth sensitivity to site fertility and water supply, which is typical for spruce from lowland forests (Nikolova et al., 2011; Van der Maaten-Theunissen et al., 2013) seems to be valid also in specific high-elevation locations.

The widest annual increments were detected in the root collars, which is the most important component in stabilizing the tree and conferring its resistance to uprooting (Nielsen, 1995; Stokes, 2002). A high increment in the root-collar zone resulting in higher biomass accumulation is most likely caused by increasing wind sway of the aboveground part when trees become dominant and more exposed to wind stresses (Ennos, 1993). The most uniform course in individual root-collar growth in the stand-interior trees at Sur En may reflect, alongside the less variable individual biomass productivity under continental climate conditions, the lowest wind firmness of the spruce trees within this stand. This low variation seems to reflect a failure to achieve individual adaptation to wind forces on this site and may relate to the higher stem density and structural homogeneity in the stand in Sur En as compared to the intensively thinned sites, Siat and Furna. In addition, openings in closed spruce stand relying on collective (i.e. tree-group) stability from the neighborhood may make trees after sudden exposure to edges more susceptible to subsequent disturbances (Nielsen, 1995). This destabilization due to management interventions might

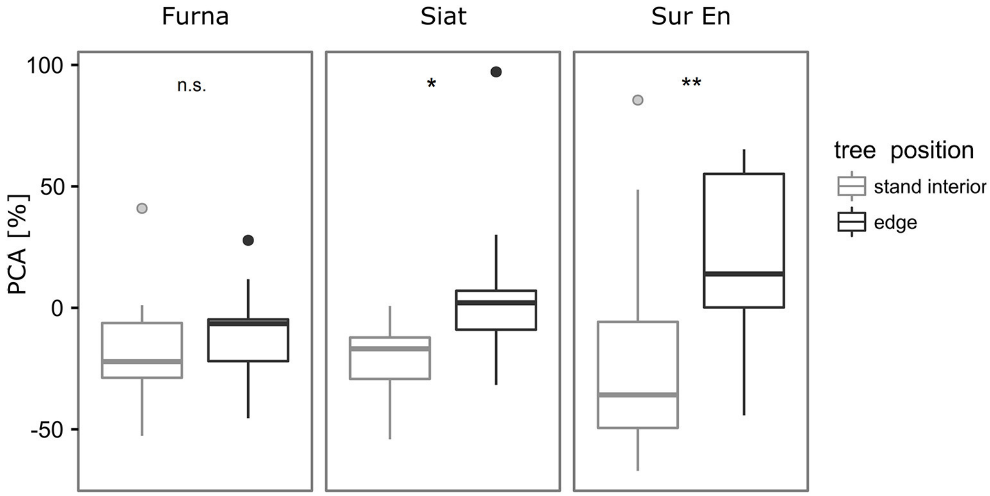

FIGURE 5 | Box plots of the percentage change in allometric slope α'_i (PCA, %) of stand interior and edge trees after strip cutting. Wilcoxon rank-sum test was applied to test differences in PCA of the stand interior and edge trees within each site, significance levels as follows: $^{*}p \leq 0.05$, $^{**}p < 0.01$, n.s. (not significant, $p > 0.10$). Individual points represent outliers.

have long-term effects on forest resistance if adjustments in edge trees need long time, and disturbance frequency, e.g., due to climate change, simultaneously increases as predicted for North European forests (Olofsson and Blennow, 2005; Blennow and Olofsson, 2008).

TABLE 4 | Estimated fixed effects of the model for predicting the percentage change in period-wise allometric slope α'_i (PCA).

	Estimate	Std. error	*t*-Value
(Intercept)	−60.15	20.31	-2.96^{**}
Tree position "edge"^{a}	127.38	24.98	5.10^{***}
Rp	1.38	0.78	1.77^{*}
Tree position "edge" × *Rp*	−4.00	0.96	-4.15^{***}

Only significant effects are shown $R^2 = 0.46$. *The variable selection and the variables are described in section Statistical Analyses.*

Significance levels: $^{*}p \leq 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$.

^{a}*Compared to tree position "stand interior".*

Long-Term Allometry in Different Site Conditions

In this study, the allometric exponent α, which represents the long-term root–stem allometry at the tree level, was site-specific, with the highest allocation to roots in Furna (4.06) and the lowest in Sur En (2.60). The long-term allometry of stand-interior trees likely follows the increase of site productivity, with biomass allocation to roots increasing with *dbh* (i.e., tree size). These results support the allometric biomass partitioning theory (Enquist and Niklas, 2001, 2002), which postulates resource allocation patterns between different organs to differ with plant size. Furthermore, Furna is the windiest among the sites studied. The outstanding large trees that are always exposed to the wind might have additionally increased the relative allocation to roots permanently adjusting to physiological, i.e., enhanced transpiration demand, and biomechanical (i.e. dynamic bending) stresses (Telewski, 2006).

In other studies, much lower α-values of 0.3–1.24 were found in woody and herbaceous plants (Müller et al., 2000; Enquist and Niklas, 2002; Mokany et al., 2006). The higher α-values from this study indicate much higher allocation to roots. Part of this may be attributable to an overestimation due to the eccentric growth of the sampled roots, their upper radii being on an average 40% wider than the mean radius of the corresponding cross-sectional root disc (cf. 48% for lowland spruce trees; Nikolova et al., 2011). Even after correction of α with the overestimation factor of 1.90, the long-term α estimates from this study (i.e., 2.14 for Furna, 1.86 for Siat and 1.37 for Sur En) are still higher than those reported for lowland spruce trees on relatively flat terrain (1.0–1.3; Nikolova et al., 2011). However, the values found here fit well to biomass estimation models parametrized for high-elevation spruce forest stands in Central Europe (2.31; Konôpka et al., 2011) or spruce plantations from the Northern Harz mountains (2.36; Drexhage and Gruber, 1999). The high α-values reported from mountain spruce forests might be related to the larger belowground investment due to genotypic adaptation to colder habitats (Oleksyn et al., 1998; Poorter et al., 2012) or for anchorage to stabilize trees on steep terrain (Chiatante et al., 2002; Dumroese et al., 2019) and suggest allometric trajectories to relate to elevation and/or relief. The latter should be proven by whole-biomass assessments *in situ* (cf. Bolte et al., 2004; Wang et al., 2008) and accounted for in-biomass estimation models.

Post-cutting Allometry Changes

Before strip cutting, short-term allometry α' values transiently differed between edge and stand- interior trees in Furna and Siat only, possibly reflecting post-cutting phases of allometric adjustment in the managed stands (Pretzsch et al., 2014). In

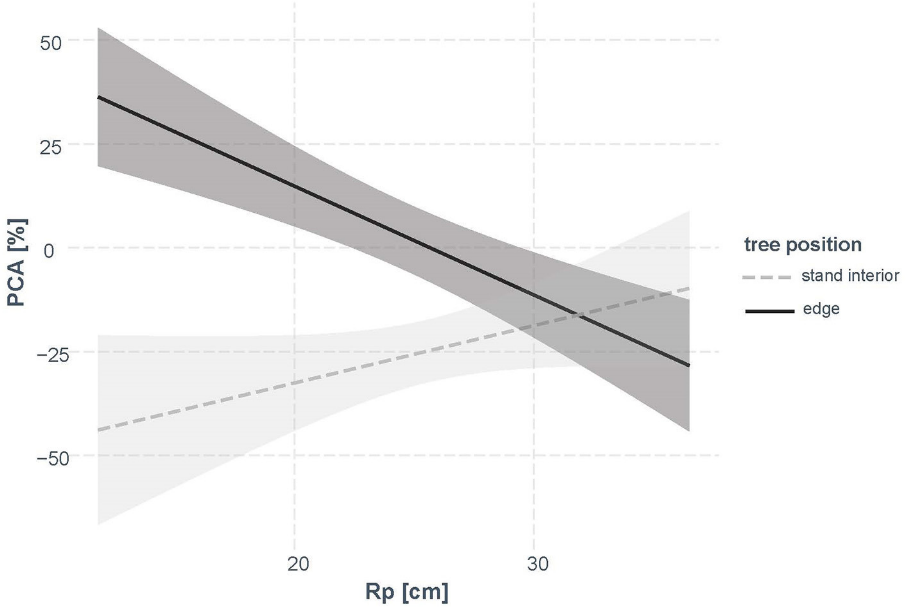

FIGURE 6 | Interaction plot of "tree position" and *Rp* (stem radius prior to cutting) according to the statistical model (**Table 4**). CI (95%) are shown in gray.

Furna, a periodic but minor increase of allocation to roots was detected in the group of edge trees, whereas in Siat, stand-interior trees have been experienced repeated released events in the past. Interestingly, since 1998, the allometry is flattening in Siat with only small individual variability, with the stem showing the highest increments among the studied tree compartments. This indicates that the sampled trees in the south-facing and selectively thinned site Siat had sufficient resources for equalized/balanced growth even before strip cutting. In Sur En, both tree groups had almost the same long-term allometry confirming the absence of management interventions and other significant disturbances after 1960 on this site.

Strip cutting causes abrupt ecological changes along the created forest edges. This kind of silviculture shapes structural contrasts of high magnitude between stocked and unstocked (i.e., cut) areas, thereby increasing disturbance severity by more overstory mortality (e.g., Goode et al., 2020). In previously thinned stands such as Furna and Siat, the effects on edge tree growth seem to be only moderate, as the edge-to-interior ecological gradient is relatively small (Matlack, 1993). The edge trees in Sur En seem to experience the most abrupt change in growing conditions as indicated by the change in post-cutting root–stem allometry (PCA). In this study, however, statistical analyses (**Table 4**) yielded no significant site effect on the change in PCA, indicating a general response of the edge trees. In addition, the strongest effects of enhanced post-cutting allocation to roots were detected in small trees. According to the resource limitation theory (Bloom et al., 1985), biomass shifts into roots would indicate primarily improved water/and nutrient availability for the small suppressed trees along edges. Small trees likely profited more than taller trees from the warmer and wetter soils along edges, a root-growth stimulating environment. During the first years after cutting, edge trees with their relatively well-developed crowns (*Lc* 63–75%) might have experienced a surplus of assimilates as a result of rapidly enhanced C uptake under still limited nutrients (e.g., P, N) availability, which excess of photosynthates might have first stimulated fine-root growth and supporting symbiotic associations (Nikolova et al., 2020; Prescott et al., 2020). Over time, such relocation to roots resulted in greater root–shoot ratios (Prescott et al., 2020). In this study, edge-tree adjustment to new light conditions, i.e., the increase of N and P uptake due to enhanced fine-root growth, seems to be completed 7 years after cutting, with stronger effects in formerly suppressed (and more nutrient-limited) small-sized trees.

Conclusion and Applications for the Praxis

The results suggest long-term allocation to roots to increase with tree size. This outcome supports previous works (e.g., Urban et al., 1994; Stokes, 2002) highlighting the increased allocation of resources to roots as a general mechanism of *Picea* to increase wind firmness with tree size. After one-sided removal of the canopy, small-sized spruces growing as subdominants showed a strongest change in allometry enhancing root growth. A time span of 7–8 years was necessary to allow tress to acclimate to the new growing conditions. Ecologically, the edge influence on tree growth is less important in forests that have been regularly managed and are therefore more structurally heterogeneous (Harper et al., 2005). In the case of homogenous stands with suppressed spruce trees, the post-cutting period is characterized by the high vulnerability of small-sized edge trees to windthrow and/or snow-/ice-break (Klädtke, 1999; Bachofen and Zingg, 2001). An increasing post-cutting vulnerability to a disturbance within the period of acclimation, e.g., by more overstory damage or mortality of subdominant edge trees, may represent a risk for stand integrity, as secondary risk factors such as bark beetle attacks might be provoked. In times of climate change with a predicted increase of extreme events like drought or biotic diseases, strip-cuttings in dense, unmanaged spruce forests should therefore be executed after a careful examination of associated risks.

The results from our case study contribute to a better understanding of the structural acclimation of spruce trees from high-elevation forests to new forest edges. However, for a more mechanistic understanding, an analysis of the interplay of environmental drivers such as light or water availability on the physiological responses of edge trees using tree-ring stable isotopes (Cherubini et al., 2021) would help.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

PN initiated and designed the study, provided project administration and supervision, and wrote the draft version of the manuscript. JG was the main responsible for lab and fieldwork. PN and JG analyzed the data. PN, PB, HG, SZ, and PC provided interpretations. PN, HG, PB, and SZ contributed substantially to the funding success. All authors contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.703674/full#supplementary-material

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Effects of Slope Aspect and Rainfall on Belowground Deep Fine Root Traits and Aboveground Tree Height

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The vertical root distribution and rooting depth are the main belowground plant functional traits used to indicate drought resistance in arid and semiarid regions. The effects of the slope aspect on the aboveground traits are visible but not the belowground deep root traits. We aimed to investigate the fine root traits of the locust tree (*Robinia pseudoacacia* L.) planted on southerly and northerly aspects, and the variations in the rooting depth in regions with different rainfall, as well as assessing how deep rooting, might affect the response to drought in a loess region. We selected three study sites with different rainfall amounts, with six sampling plots at each site (three each with southerly and northerly aspects). Soil core samples were collected down to the depth where no roots were present. The locust trees tended to develop deeper fine roots rather than greater heights. The tree height and diameter were greater for locust trees on northerly aspects, whereas trees on southerly aspects had significantly deeper rooting depths. Fine root traits (root length, root area, and root dry weight density) were higher in the southerly aspect for both Changwu and Ansai, but lower in Suide. The ratio of the root front depth tree height ranged from 1.04 to 3.17, which was higher on southerly than northerly aspects, and it increased as the rainfall decreased. Locust tree growth traits (belowground fine root and aboveground tree height) were positively correlated with the mean annual rainfall. The soil moisture content of the topsoil decreased as the rainfall decreased, but the pattern varied in the deep layer. Our results suggest that the variations in the belowground rooting depth under different slope aspects may be related to plant survival strategies. The vertical extension of the rooting depth and tree height may be key functional traits that determine plant growth in drought-prone regions.

Keywords: fine root, functional trait, root distribution, slope aspect, vertical tree extension

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INTRODUCTION

Determining plant functional traits might help us to understand the responses of plants to environmental variations (Reich et al., 2003). Solar radiation, temperature, and soil moisture are the main environmental factors that contribute to plant growth. The slope aspect is an important topographical factor because it can change the intensity and duration of solar radiation, thereby affecting the temperature and soil moisture in local areas (Cantón et al., 2004). The contrasting effects of northerly and southerly slopes can produce different climates, where the greater solar radiation on southerly slopes can increase the temperature and evapotranspiration to yield more arid conditions in the Northern Hemisphere (Finkel et al., 2001; Gutiérrez-Jurado et al., 2013). These climatic differences can cause plants to develop different functional traits, especially in terms of the tree height, canopy size, leaf structure, and branch density (Niinemets, 2006; Rozendaal et al., 2006). Finkel et al. (2001) and Nevo (2012) showed that the aboveground biomass was lower in a plantation on a southerly slope, whereas Kou et al. (2013) and Fan et al. (2012) obtained the opposite results. These differences of slope aspect and soil moisture in environmental factors could affect the aboveground morphological traits of plants (Vitória et al., 2019) and they might reflect the growth strategies employed by plants to resist drought (Liu and Ma, 2015). However, little information is available regarding the deep root traits of plants.

Fine roots (diameter < 2 mm) play important roles in the absorption of water and nutrients (Chahine, 1992), especially the deep fine roots, which can absorb deep water in the soil to maintain plant transpiration during the dry season (Nepstad et al., 1994; Liu and Diamond, 2005; Oliveira et al., 2005). The ability of plants to access water reflects its adaptation to drought (Vadez, 2014). The fine root distribution and the rooting depth are the main belowground functional traits that might determine the volume of soil water acquired, thereby characterizing the growth and survival strategies of plants in water-limited ecosystems (Collins and Bras, 2007). Akkermans et al. (2012) showed that the rooting depth was a major variable that controls evapotranspiration during the dry season in African forests according to soil–vegetation–atmosphere transfer models, and it might be a direct indicator of a plant's adaptability to drought. However, few studies have quantified the fine root distribution relative to rooting depth due to the difficulties of root sampling (Wang et al., 2009; Maeght et al., 2013; Freycon et al., 2014). In addition, studies have rarely investigated the deep fine root distributions under contrasting slope aspects (Kurze et al., 2017). Previous studies mainly concentrated on the differentiation of ecotypes on a large-scale aridity gradient (Wang and Gao, 2003; Volis, 2007; Kurze et al., 2017). According to the Hijioka et al. (2014), the aridity will intensify in many regions throughout the world in the present century and the annual precipitation will decrease. Thus, deep fine root traits in regions with different rainfall amounts are valuable data for assessing hydrobiological processes and the adaptability of plants to environmental variations.

The Loess Plateau region of China is an ideal area for studying the root distributions in a deep and homogeneous soil layer because the deep loess layer is not affected by any interference due to differences in the groundwater level. The locust tree (*Robinia pseudoacacia* L.) is an exotic species that has been planted widely in China to conserve soil and water since the 1950s due to its drought tolerance, rapid growth, and simple propagation (Wang, 2017). The area planted with locust trees since the 1950s exceeds 70,000 ha in the arid and semiarid Loess Plateau region (Guo et al., 1998). The locust tree is characterized by high water consumption and it can readily exhaust the available soil water. However, it is highly resistant to drought when the minimum survival requirement is maintained. Zhang et al. (2018) found that the fine root area density in locusts tree exhibited a decreasing trend from a wetter site to a drier site, and the fine roots exhibited similar rooting patterns along a precipitation gradient throughout the whole 3 m soil profile. Nicolescu et al. (2018) showed that the locust tree was fast growing with the maximum tree height being reached in the first 5 years and the peak diameter in the first 10 years. The locust tree has a high capacity for adaptability and it can be utilized in projects that aim to convert farmland into forestland, it can severely desiccate the deep soil layer (Jia et al., 2017), thereby resulting in environmental deterioration with low productivity and efficiency.

In the present study, we comprehensively analyzed the vertical extent comprising the tree height and the rooting depth for the locust tree. We hypothesized that the rooting depth would increase as the rainfall decreased and it was deeper in northerly than southerly aspect according to the theory of optimal resource partition which indicated that plant root could extend deeper layer to absorb deeper soil water to resist drought in drier areas. The main aims of this study were as: (1) to investigate the deep fine root distribution for locust trees under different slope aspects; (2) to analyze the trends in the fine roots in regions with different rainfall amounts; and (3) to assess the vertical extents of the rooting depth and tree height as key functional traits for determining plant growth. The results obtained in this study provide new insights into the vertical extent of the deep roots and the ratio of the belowground rooting depth relative to the aboveground tree height in areas with different rainfall amounts, thereby helping us to understand plant growth and survival strategies.

MATERIALS AND METHODS

Study Sites

This study was conducted during summer between July and August 2018 in the Loess Plateau region of China, where the deep loess layer and ground water depth are more than 50 m (Ma et al., 2013; Zhu et al., 2018). Thus, this region is an excellent area for studying deep roots. In our study area, we investigated three different sites in Changwu, Ansai, and Suide counties across a distance of 337 km. In this region, the climate is cold with four distinct seasons characterized by dry winters and warm summers in Changwu and Ansai (Dwb), and the arid conditions characterized by steppe and cold (Bsk) in Suide according to the Köppen-Geiger climate type (Peel et al., 2007). The highest sunlight, heat, and rainfall levels mostly occur in July, August, and September, and more than 50% of the total annual rainfall occur in these 3 months (**Table 1**). The locations of the sites are shown in **Figure 1**. We sampled the northerly and southerly aspects at all sites with three samples from each.

TABLE 1 | Main natural geographical characteristic and soil properties at sampling sites.

Site	Annual rainfall (mm)	Annual temperature (°C)	Altitude (m)	Annual sunshine hours (h)	Annual potential evapotranspiration (mm)	Cumulative temperature (≥ 10°C)	Soil bulk density (g cm⁻³)	Soil field capacity (%)	Soil wilting point (%)	Climate type	Geographical coordinates
Changwu	580	9.1	1,224	2226.5	1029.1	3,029	1.3	21.9	7.46	Cold with dry winter and warm summer (Dwb)	107°48'–107°58'E, 34°59'–35°18'N
Ansai	500	8.8	1,063	2395.6	1,127	3,121	1.28	18.4	4.5	Cold with dry winter and warm summer (Dwb)	108°05'–109°26'E, 36°30'–37°19'N
Suide	443	9.7	877	2615.1	1280.5	3,485	1.25	15.8	3.7	Arid with steppe and cold (Bsk)	110°04'–110°41'E 37°16'–37°45'N

Soil bulk density, soil field capacity, and soil wilting point were measured in 0–1 m layer. Climate was based on updated Köeppen-Geiger climate classification (Peel et al., 2007).

At each site, we selected six sampling plots on the upper slopes (10 × 10 m, with three on southerly aspects and three on northerly aspects) to quantitatively investigate the characteristics of the locust tree plantations. The sites were chosen carefully to minimize confounding environmental factors, where the altitude, inclination, and tree growth stage were similar on both aspects (three replicates). All sites were located in well-vegetated areas where the depth of the surface litter was about 1 cm in the locust tree stands. The sampling point was located on the same side of the tree along the contour line. The sampling point and two adjacent locust trees (2 m between the trees and 4 m between the tree rows) at a similar growth stage in the same row formed an isosceles triangle with a side length of 1.2 m from the tree trunk. We transformed the slope into flat ground with a shovel and fixed sampling shelves with two wooden baseboards (1.2 m in length and 0.3 m in width), where a plumb was used to determine the level. All weeds and the litter layer were removed every 10 days at 30, 20, and 10 days before sampling period within the sampling area to eliminate any interference from other plant roots.

The maximum rainfall infiltration depth was 2 m (Liu et al., 2010). We defined the deep soil as the soil layer below 2 m. Liu et al. (2014) showed that the deep soil moisture content (below 2 m) remained fairly stable with almost no seasonal variation based on long-term observations in the study area. Thus, we assumed that the deep soil moisture was not affected by the rainfall in the sampling period and it could represent the soil moisture regime consumed by plants over a long period. According to previous studies, the sampling period during the summer is characterized by the peak abundance of fine roots (Burke and Raynal, 1994), minimum soil water storage (Duan et al., 2017), and the most severe drought throughout the year. Therefore, the data collected represented the responses of the fine roots to the soil water regime and other environmental factors in the corresponding sampling period.

Stand Characteristics

The locust tree stands at the three sites had similar densities (5,000 trees ha^{-1}), with 2 m between the trees and 4 m between the tree rows, and the canopy was closed with coverage of more than 85%, except on the southerly aspect in Ansai where the coverage was 70%. The stands were all artificial forests, which were in middle age in Ansai (18 years) and mature age in Changwu and Suide (37 and 35 years) (**Table 2**); the trees were all at the growth period based on the regulations for the main tree species (LY/T 2908–2017, China), which define the age of locust trees as young at ≤15 years, middle at 16–25 years, and mature at 31–40 years. The tree height and mean diameter at breast height were determined. We measured the tree height with an optical height meter (CGQ-1, Harbin Optical Instrument Factory Co. Ltd., China) and the mean diameter with a measuring tape. First, we measured the horizontal distance between the trees and the optical height meter with a measuring tape. We then targeted the top of the tree through an observation hole and determined the sight point carefully

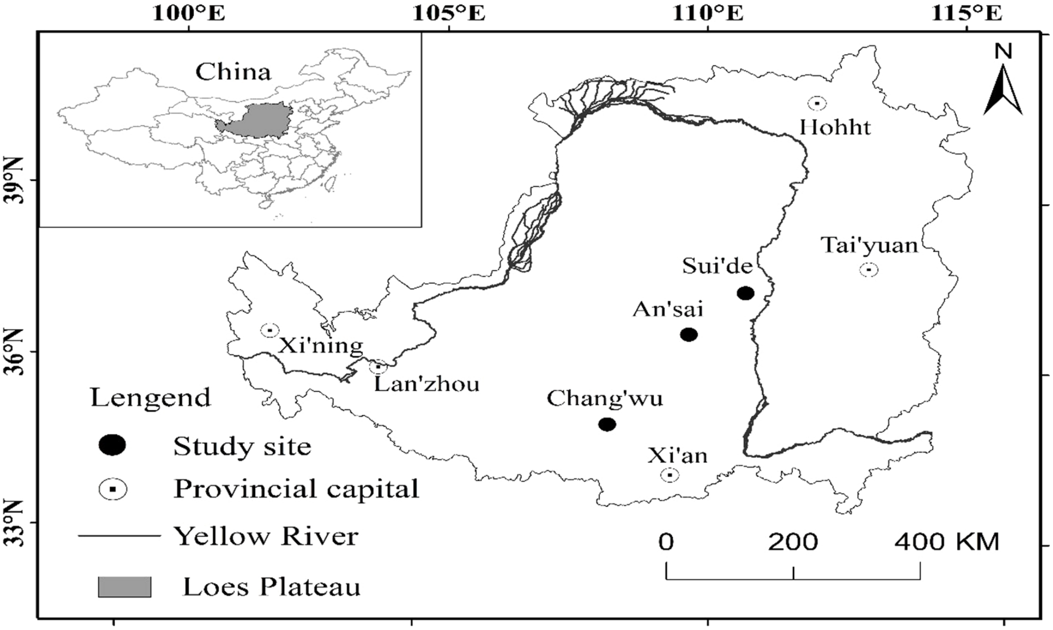

FIGURE 1 | Study sites for locust tree in loess region.

by aligning the pointer in the plumb position. We pulled the trigger and the pointer was locked. Hence, tree height was determined by the pointer scale values. The coverage represents the percentage of the vertical projection area of the plant aboveground relative to the sampling plot area, and it was calculated using the formula for the elliptical projected area of the canopy: $C = \pi XY / 4$, where X and Y denote the major and minor axis lengths, respectively (Phillips and Macmahon, 1981).

Fine Root Sampling and Analysis

Fine roots were sampled using the soil coring method (with a specialized soil auger made in China called a "Luoyang shovel" internal diameter = 16 cm), as described previously by Ma et al. (2013). In fact, a massive effort was done to achieve such work through very deep soil excavation. Luoyang shovel with larger internal diameter could greatly improve the sampling efficiency, it took about 2.5 h for two persons to drill down to 8 m depth, 4 h for two persons to 12 m depth, and 8 h for three persons to 18 m depth. Soil samples were collected at 20 cm intervals throughout the whole soil layer until no roots were found in two consecutive layers. We set the "two consecutive layers" criterion for root front depth which meant that root front extended downward to the depth where no root occurred below this depth in two consecutive layers. Deep roots were defined as roots found below 2 m. The vertical tree extension was defined as root front depth plus tree height.

Roots were separated from soil particles by washing through a 0.2 mm sieve under running tap water. The root samples were placed in plastic bags, tagged with identifiers, and stored at 4°C until they were processed. Tree roots were distinguished from grass roots based on their morphology and color (Sun et al., 2015). Dead roots were identified based on their color and flexibility, and then discarded (live roots were yellowish brown, whereas dead roots were dark). We found a few dead roots in the top 1.2 m soil depth and almost no decay roots occurred in deeper soil layer. Fine roots (diameter < 2 mm) were collected and placed on transparent rectangular plastic plates filled with water, which allowed the roots to spread and float on the surface, before scanning at a resolution of 300 dpi. The area, length, and diameter were measured for the fine roots with WinRHIZO (Regent Instruments Inc., Canada). The fine root length density (RLD, m m^{-3}) was defined as the root length per unit soil volume. D_{50} and D_{95} were used to denote the soil depths above which 50% or 95% of the total RLD were located, respectively. The fine root area density (RAD, m^2 m^{-3}) was defined as the root area per unit soil volume. The fine root dry weight density (RDWD, g m^{-3}) was defined as the dry weight of roots per unit soil volume, which was determined after washing and cleaning, and drying for 24 h at 65°C. Nothing was present in the deep layer except for soil and roots. We found only a few roots above 1 cm in diameter below 3 m. The eolian soil was homogeneous among the six sampling points at each site in the loess region. At present, the soil coring method is a feasible approach for identifying deep roots by actual field sampling.

Soil Moisture Analyses

Soil bulk density, soil field capacity, and soil wilting point were measured in 0–1 m layer in the laboratory. Soil bulk density and soil filed capacity were determined by the cutting ring method. The wilting point was measured following the method described by Wei et al. (2004), who defined the wilting point as the soil water content when the soil water suction was equal to 1.47 MPa.

After separating the roots, each soil core sample was dried to a constant weight at 105°C and the gravimetric soil moisture content was determined using the following formula.

$$\text{soil moisture}\left(\%\right)=\frac{\text{wet soil weight}-\text{dry soil weight}}{\text{wet soil weight}}\times 100\%$$

Soil organic carbon, soil total nitrogen, and soil total phosphorus concentration were measured on the southerly and northerly aspect in the whole sampling soil depth in Changwu (unpublished data); we found that soil nutrients were concentrated on surface layer (0–40 cm) and varied little with soil depth. Soil organic carbon content was larger on northerly than southerly aspect, while the total nitrogen and phosphorus content kept fairly consistent on different slope aspect. Soil organic carbon, total nitrogen, and phosphorus content were largest in Changwu, while they remained roughly similar values in Ansai and Suide (Zhang et al., 2018).

Statistical Analysis

A general linear model and multi-way analysis of variance were used to test the effects of the soil depth, slope aspect, site, and the interactions between the slope aspect × site, slope aspect × soil depth, site × soil depth, and slope aspect × soil depth × site on the fine root diameter, RLD, RAD, and RDWD. The slope aspect and site were treated as fixed effects, and the soil depth as a continuous variable and covariate. F-tests were conducted to assess the significance of the site, slope aspect, and their interaction. We log-transformed the root biomass values to satisfy the requirements for homoscedasticity and normality. All analyses were conducted with SPSS 11.0 (SPSS Inc., Chicago, Illinois, United States). Duncan's test was used to determine significant differences ($p < 0.05$).

RESULTS

Fine Root Traits

The fine roots of the locust trees exhibited similar trends, where RLD, RAD, and RDWD all decreased sharply in the top 2.6 m of the soil, varied slightly from 2.6–5 m, and then remained stable below 5 m (**Figure 2**). The maximum RLD and RAD occurred in the top 0.2 m on the northerly aspect in Ansai, and the maximum RDWD on the southerly aspect in Changwu. More than 50% of the total RLD was concentrated in the upper 2.2 m (except for 3.4 m on the northerly aspect in Changwu) and less than 5% below 8.6 m (**Figure 3**).

We found that trees on southerly aspects had higher RLD, RAD, and RDWD values, except for Suide (**Table 3**). The total RLD, RAD, and RDWD values decreased as the rainfall decreased on southerly aspects. However, the total RAD and RDWD increased on northerly aspects, while the total RLD values were similar in Ansai and Suide, and greater than those in

TABLE 2 | Main characteristics of locust tree stands.

Location	Slope aspect	Slope gradient	Age (years)	Annual cumulative PAR (mol m^{-2} s^{-1})	Maximum daily temperature	Stand height (m)	Diameter at breast height (cm)	The depth of the root front (m)	Ratio between the depth of root front and stand height	Whole vertical extension (m)	Coverage	Main understory vegetation
Changwu	North	37°	37	9,320	32.2°C	10.03 ± 1.73 a	10.53 ± 2.70 ab	10.4 ± 0.33DE	1.04	20.4	96%	*Rubus*
	South	36°	37	9,861	33.4°C	7.45 ± 1.57 bc	9.4 ± 2.56 abc	18 ± 0.78 A	2.42	25.5	98%	*Leymus*
Ansai	North	33°	18	9,440	34.5°C	7.75 ± 0.70 b	12.05 ± 2.78 a	11.8 ± 0.21 CD	1.52	19.6	95%	*Armoise*
	South	31°	18	9,988	37.1°C	6.59 ± 1.77 bc	6.89 ± 1.89 bc	14 ± 0.65 B	2.12	20.6	70%	*Agropyron*
Suide	North	29°	35	9,510	34.2°C	5.20 ± 0.79cd	7.53 ± 1.51 bc	9.6 ± 0.96 E	1.85	14.8	85%	Moss
	South	32°	35	10,062	36.8°C	4.04 ± 0.28 d	5.30 ± 1.41 c	12.8 ± 0.14BC	3.17	16.8	85%	*Armoise*

Different letters for stand height, diameter, and the depth of the root front indicate significant differences among sampling sites on different slope aspects ($n = 3$, $p < 0.05$).
PAR indicates photosynthetically active radiation. Whole vertical extension refers to the stand height plus the maximum rooting depth. Coverage represents the percentage of the vertical projection area of the plant above ground relative to the sampling plot area.

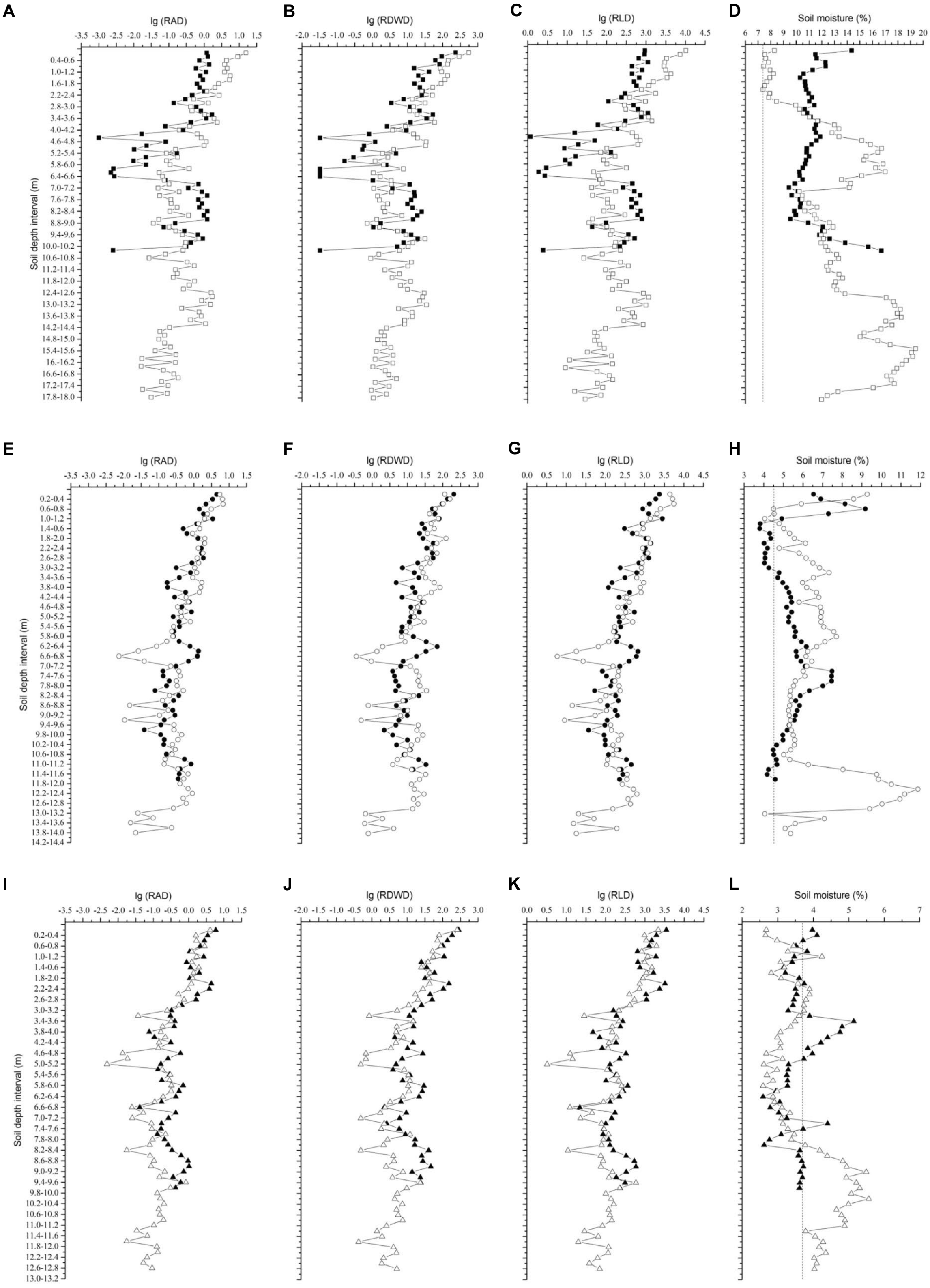

FIGURE 2 | Changes in RAD, RDWD, RLD, and soil moisture down to the root front with soil depth. RAD is the root area density (m^2 m^{-3}), RDWD is the root dry weight density (g m^{-3}), and RLD is the root length density (m m^{-3}). Dashed lines denote the wilting point at each site. Solid and empty rectangles, circles, and triangles represent northerly and southerly aspects at the sites Changwu **(A,B,C,D)**, Ansai **(E,F,G,H)**, and Suide **(I,J,K,L)**, respectively.

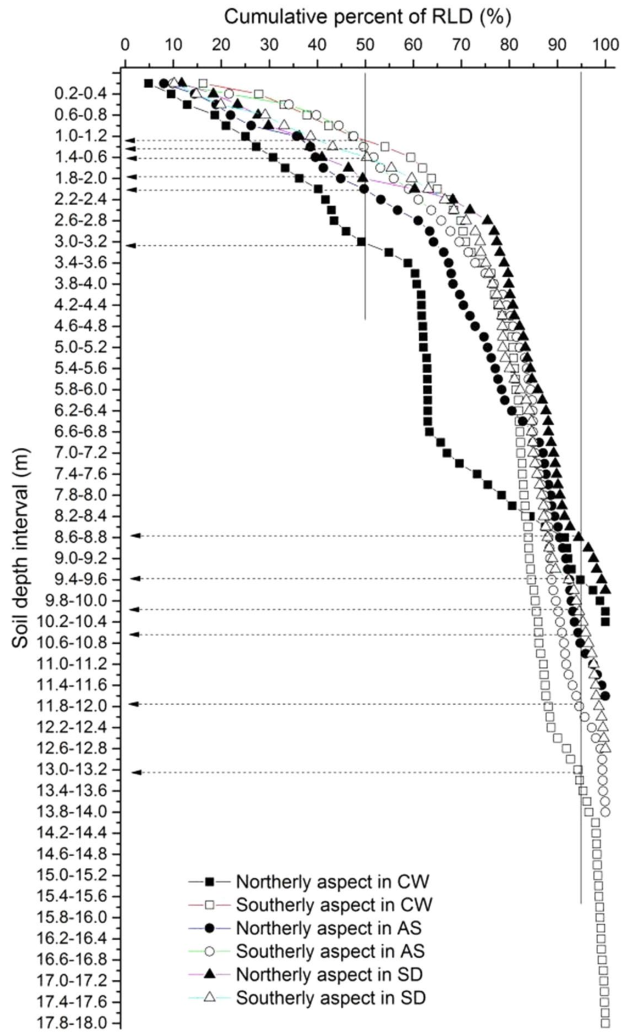

FIGURE 3 | Cumulative percentages of fine root length density down to the root front with soil depth. Solid lines denote the 50 and 95% total root length densities. Dashed lines are the corresponding soil depths above which 50 and 95% of the total root length densities are located. Solid and empty rectangles, circles, and triangles represent northerly and southerly aspects at the sites Changwu, Ansai, and Suide, respectively.

Changwu. Though fine root traits (RLD, RAD, and RDWD) decreased with the rainfall amount, there were no significant differences among the three sites (**Table 4**). The effects of the soil depth on RLD, RAD, and RDWD were highly significant ($p < 0.01$). The slope aspect had significantly different effects on RLD ($p < 0.01$) and RAD ($p < 0.05$) but not on RDWD. The mean root diameter varied little and it tended to be uniform throughout the whole soil depth (**Table 3**).

Vertical Tree Extension

Our results showed that the vertical extents of the trees varied among sites and slope aspect (**Figure 4**; **Table 2**). The tree height and diameter at breast height decreased as the rainfall amount decreased, and they were also higher on northerly than southerly aspects (**Table 2**). The mean root front depth also decreased as the rainfall amount decreased, but it was much deeper on southerly than northerly aspects in the same site, where the difference was significant. Root front depth ranged from 9.6 m on the northerly aspect in Suide to 18 m on the southerly aspect in Changwu. The heights of the locust trees tended to be shorter than the depths of the root front. The ratio between the belowground root front depth and aboveground tree height ranged from 1.04 to 3.17, which was higher on southerly than northerly aspects, and it increased as the rainfall amount decreased. The vertical tree extension values were also larger on southerly than northerly aspects but they decreased as the rainfall amount decreased (**Table 2**).

Soil Moisture

The mean soil moisture contents in the 0–2 m layer were higher on northerly than southerly aspects at each site, which were 11.55 and 7.8% in Changwu, 5.93 and 5.75% in Ansai, and 3.6 and 3.15% in Suide (lower than the wilting point of 3.7%), respectively. However, the tendency of mean soil moisture contents from 2 m to the root front depth was opposite, which were 11.32 and 14.1% in Changwu, 5.34 and 6.65% in Ansai, and 3.57 and 3.83% in Suide, respectively. We found that the mean soil moisture contents of the top soil layer were higher on northerly than southerly aspects at each site and they decreased as the rainfall decreased. The soil moisture contents varied below 2 m (**Figures 2D,H,L**).

TABLE 3 | Main characteristics of fine roots on different slope aspects.

Location	Slope aspect	Total root length density (m m^{-3})	Soil layer where the cumulative percentage of total root length density occurs (m) D_{50} D_{95}	Total root area density (m^2 m^{-3})	Total root dry weight density (g m^{-3})	Mean diameter (mm)
Changwu	North	19397.2 ± 190.2 e	3.4 9.8	28.3 ± 0.2 d	1032.6 ± 27.9 f	0.45 ± 0.08 a
	South	62967.3 ± 611.3 a	1.4 13.6	90.7 ± 7.8 a	2493.0 ± 69.6 a	0.43 ± 0.08 a
Ansai	North	29688.3 ± 277.5 c	2.2 10.8	45.5 ± 1.2 c	1541.9 ± 41.9 d	0.47 ± 0.08 a
	South	44395.9 ± 414.9 b	1.6 12.2	62.9 ± 8.9 b	2005.9 ± 54.4 b	0.48 ± 0.08 a
Suide	North	29651.1 ± 270.3 c	2.2 8.6	47.7 ± 0.2 c	1898.0 ± 50.7 c	0.54 ± 0.09 a
	South	21161.9 ± 201.5 d	1.6 10.2	30.3 ± 5.1 d	1121.4 ± 30.7 e	0.42 ± 0.07 a

Different lowercase letters for total root length density, total root area density, total root dry weight density, and mean diameter indicate significant differences among sampling sites on different slope aspects ($n = 3$, $p < 0.05$).

TABLE 4 | F-values for the effects of site, slope aspect, and depth, and the interactions between site × slope aspect, site × depth, slope aspect × depth, and site × slope aspect × depth based on fine root length density (RLD, m m^{-3}), fine root area density (RAD, m^2 m^{-3}), fine root dry weight density (RDWD. g m^{-3}), and fine root diameter (mm).

	RLD	RAD	RDWD	Diameter
Site	F2,372 = 0.901	F2,372 = 0.532	F2,372 = 0.351	F2,372 = 3.316
Slope aspect	F1,372 = 7.934**	F1,372 = 5.886*	F1,372 = 0.006	F1,372 = 0.759
Depth	F1,372 = 92.038**	F1,372 = 92.602**	F1,372 = 102.883**	F1,372 = 2.223
Site × Slope aspect	F2,372 = 12.060**	F2,372 = 13.987**	F2,372 = 6.441**	F2,372 = 0.649
Site × Depth	F2,372 = 1.134	F2,372 = 1.729	F2,372 = 1.291	F2,372 = 1.119
Slope aspect × Depth	F1,372 = 0.702	F1,372 = 0.460	F1,372 = 2.116	F1,372 = 18.109**
Site × Slope aspect × Depth	F2,372 = 4.912**	F2,372 = 5.758**	F2,372 = 2.013	F2,372 = 8.009**

**Significant difference at $p < 0.05$; **significant difference at $p < 0.0$.*

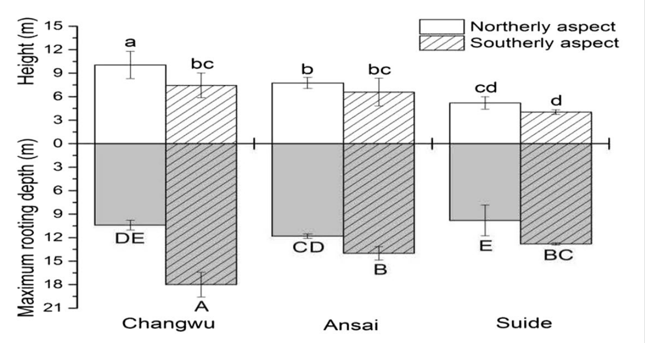

FIGURE 4 | Mean stand heights and the depth of the root front at Changwu, Ansai, and Suide. Vertical bars indicate standard deviations. Lowercase letters indicate significant differences in stand height and uppercase letters indicate significant differences in the depth of the root front ($n = 3$, $p < 0.05$).

DISCUSSION

Deep Root Traits

Our results indicated that the mean RLD, RAD, and RDWD decreased as the rainfall amount decreased. Locust trees on southerly aspects had higher root biomasses, but the opposite was found in Suide, possibly because the lower root diameter and weaker growth led to smaller biomasses (**Table 3**) due to severe soil moisture depletion lower than wilting point. We found that the total RLD on southerly aspects was approximately three times higher in Changwu compared with that in Suide (**Table 3**; 62967.3 m m^{-3} vs. 21161.9 m m^{-3}). A previous study also showed that the root biomass was four times higher in mesic populations (Kurze et al., 2017). Volis et al. (2002) and Mendola et al. (2015) determined a similar trend to our results for an annual grass. There may be intensified plant–plant competition for belowground water and nutrient resources in mesic environments (Metz and Tielbőrger, 2016). If the competition supersedes the effect of regional climate on the availability of belowground resource, i.e., less resources are available per individual in mesic sits, then the allocation of resources to the roots will be higher in a mesic environment compared with an arid environment (Kurze et al., 2017). However, these results were opposite to those predicted by optimal resource partitioning, where the investment in roots was expected to be higher under lower soil water conditions (Bloom et al., 1985). Aronson et al. (1992) also found that the allocation to roots was higher in an arid population during the early life stage. While this trend gradually reversed with age, thereby indicating that the maturing plant allocated more biomass to seed production rather than roots which might reflect a strategy for maximizing reproductive production at the expense of vegetative growth.

Pinheiro et al. (2016) found that the fine root densities decreased in the topsoil but remained roughly similar at depths of 0.25 and 6 m. We also found that RLD, RDWD, and RAD decreased sharply in the top soil, but they remained stable below 5 m (**Figure 2**). Schenk and Jackson (2002a) showed that D_{50} and D_{95} were located in the upper 0.3 m and 2 m, respectively. However, we found that D_{50} and D_{95} moved down to greater depths, especially D_{95}, according to the experimental field measurements. The cumulative percentage RLD did not differ greatly in the shallow layers between northerly and southerly aspects, where the difference was mainly due to roots extending deeper on southerly aspects (**Figure 3**). Christina et al. (2011) and Laclau et al. (2013) showed that less than 5% of the fine root biomass of *Eucalyptus grandis* seedlings was present below a depth of 8 m. We obtained similar results where less than 5% of the fine RLD was located below 8.6 m (**Figure 3**). Christina (2015) reported that very low densities of the fine roots of *E. grandis* below a depth of 10 m could meet most of the water requirements during dry periods after canopy closure. Indeed, an extremely low deep fine root density might contribute to improving tree survival in extremely arid regions. We speculated that variability in the rooting depth might be related to the survival of trees on different slope aspects in arid regions.

Soil Moisture

Plants can maintain a balance between the supply and demand of water by regulating the proportions of the aboveground and belowground plant parts (Tian et al., 2014). Tian et al. (2014) reported that the root: shoot ratio increased when *Haloxylon* seedlings suffered from severe water stress and a new water balance mechanism formed, which facilitated survival under soil moisture contents below 1.5%. We found that root front depth and root biomass (RLD, RAD, and RDWD) decreased as rainfall amount decreased and rainfall amount was positively related to soil moisture in the whole soil layer in three sampling sites. Severe soil moisture depletion up to the wilting point of 3.7% in Suide led to slow and stagnant growth, where the locust trees tended to be smaller and older, probably because of the rapid growth and high consumption of soil water in the early stage, and soil moisture could not meet the water requirements and environmental deterioration occurred accompanied by soil dry layer and degraded forest.

The infiltration of rainfall is quite complex and it is primarily influenced by factors, such as the soil texture, soil pore space, soil surface feature, slope position, and slope gradient. Ribolzi et al. (2011) found that the infiltration depth increased as the slope gradient increased. However, Jiang and Huang (1984) showed that the infiltration of rainfall decreased as the slope gradient increased due to the gradual decrease in the infiltration rate and increased runoff. Thus, the soil moisture content was higher on gentle slopes than steep slopes. In our study, we selected similar slope positions and soil surface feature to avoid the influence of rainfall infiltration. The soil moisture is an important factor for root growth (Zhou and Shangguan, 2007). We found that the mean soil moisture content in the 0–2 m depth was higher on northerly than southerly aspects at each site (**Figures 2D,H,L**), possibly due to the greater photosynthetically active radiation and higher evapotranspiration on southerly compared with northerly aspects. Our results are consistent with those reported by Deng and Zhang (2010), whereas Sun et al. (2007) obtained the opposite results in the 1 m surface layer. Wang (2017) found that the soil moisture content was higher in the topsoil and the tree height was lower on a shady slope compared with a sunny slope, where the main factor related to tree growth was soil aeration rather than the water content. We concluded that the soil moisture in the topsoil might have been affected by environmental factors, such as rainfall and soil transpiration, but the soil moisture content in the deep soil may have reflected differences of long-term rainfall in the soil and deep root characteristics, where an alternating pattern was detected at different sites (**Figures 2D,H,L**).

Effects of Slope Aspect on Tree Height and Rooting Depth

In contrast to our hypothesis, we found that the effect of the slope aspect was greater on the belowground plant parts than the aboveground parts (**Figure 4**). The locust trees had greater rooting depths and smaller tree heights on southerly aspects than northerly aspects, possibly because the southerly aspects could receive more solar radiation, thereby leading to greater transpiration and lower relative humidity, and the available soil moisture by locust trees in surface soil layer was less, thus exhibited smaller tree height. As we all know, the aboveground growth depended on the water taken up by the roots (Salmerón-Miranda et al., 2007), we found that root extended deeper in southerly than northerly aspect, which showed that root could absorb deeper water from deeper soil layer to sustain plant growth, while it did not indicate a lower water moisture content because the mean deep soil moisture content was calculated from 2 m to the root front depth., The reduced root in northerly aspect may reflect the strategy of belowground part to maximize reproductive allocation (Kurze et al., 2017). Thus, the locust trees appear to have adapted to the different water conditions caused by the different slope aspects, where they exhibited diverse survival strategies (vertical tree extension, i.e., tree height plus rooting depth) to cope with drought. Petrů et al. (2006) obtained similar results and found that the height of *Biscutella* was lower in arid populations. May et al. (2013) and Šímová et al. (2015) also found that the rooting depth differed greatly between northerly and southerly aspects, where the tree height varied along a regional rainfall gradient. However, Christina et al. (2011) showed that the vertical tree extents above and below ground were almost the same in *Eucalyptus* plantations in Brazil. Kurze et al. (2017) suggested that the relative investment in the root: shoot ratio was higher in mesic populations. However, we found that the ratio of the rooting depth relative to the tree height increased as the rainfall amount decreased, thereby indicating that the locust trees allocated more biomass to the belowground parts to cope with drought conditions.

Larson and Funk (2016) suggested that the rooting depth may be the key factor for differentiating the performance of plants under drought conditions. However, few previous studies have determined the correlation between the rooting depth and the mean annual rainfall. Bhattachan et al. (2012) reported that there was no obvious relationship between the rooting depth and mean annual precipitation in water-limited ecosystem. Zhou et al. (2020) found that the potential rooting depth was negatively correlated with the mean annual rainfall across Southern African savannas, and they suggested that the root strategies were highly diverse in more arid sites. They estimated that the rooting depth was 4.58 m for drought-resistant *Birrea* based on an exponential decay function. However, we determined deeper rooting depths by conducting full vertical excavations and based on actual measurements. Contrary to our hypothesis, we found that the rooting depth and vertical tree extension all decreased as the rainfall amount decreased (**Figure 4**). Yu et al. (2002) showed that annual rainfall of 550 mm was the boundary for the vegetation type distribution from forest steppe to forest vegetation. Our experimental areas had annual rainfall amounts ranging from 443 to 580 mm (**Table 1**). Thus, the difference in the annual rainfall combined with the evapotranspiration produced by the slope aspect could have affected plant growth, thereby reflecting the plant survival strategy. The locust trees were relatively vigorous in terms of both their aboveground and belowground parts in the areas with more rainfall, but not in arid areas because the low rainfall and poor water supply led to weaker growth. We considered that the rooting depth could be used as a single indicator to represent a plant's survival strategy, and the tree height combined with the rooting depth as two criteria to understand the strategy employed by a plant to adapt to drought stress.

The changes in the rooting pattern with stand age might vary according to the root adaptation strategies relative to the availability of water and nutrients. Zhang et al. (2018) obtained a curve with a single peak for the roots as the trees aged, and the coefficient of the rooting distribution to stand age (β) peaked in 18-year-old locust trees ($\beta = 0.9912$) with a greater rooting depth but then decreased sharply in 35-year-old stands ($\beta = 0.9847$) with a shallower rooting depth. Chang et al. (2012) found that the rooting patterns were similar for 8- and 30-year-old locust stands in the Loess Plateau region. However, we could only investigate 18-year-old locust trees in the area sampled in Ansai. In addition, we found that the 18-year-old locust trees in Ansai were in a vigorous growth stage, whereas the trees aged over 35 years were in a stage of decline in Suide and Changwu (**Table 2**). According to a study by Zhang et al. (2018), the rooting depth might have become shallower with age in Ansai, and it would be greater in Changwu than Ansai at the same stand age. The difference between Ansai and Suide means that it is also necessary to compare tree samples with similar ages.

The rooting depth and biomass allocation are important belowground functional traits (Liu and Ma, 2015). Our deep sampling procedure was laborious and time consuming, so we only sampled three trees on each slope aspect per site. The sample size was limited but given the valuable quantitative data obtained, we consider that sampling three trees may have been representative of the actual root distributions. We found only a few roots with lengths of about 1 cm below 2 m in the soil cores, and our previous study also showed that that deep root biomass varied little with the age of the trees (Ma et al. 2013). We concluded that a few deep root observed in our study might play an important role in improving locust tree resistance to drought and sustaining tree growth. Our sampling was an integration of annual growth that encompassed the water availability of the year given the limited infiltration of the periodic rainfall events. As described above, rainfall infiltration depth was no more than 2 m (Liu et al., 2010), deep soil moisture (below 2 m) would not affected by the periodic rainfall, i.e., the wettest part of the year versus both wet and dry period, it could reflect the effect of long-term meteorological factors on local area, rather than the short-term ones. Ma et al. (2012) also showed that the root density decreased with the horizontal distance from the tree trunk. Thus, the coring points selected in our study in the same position relative to the tree trunk might have avoided differences in the horizontal root distribution. The specific sampling positions might have influenced the shallow root distribution more than that of the deep roots. Therefore, comparing the deep root distribution at the same horizontal sampling position in different sites was a suitable approach.

The rooting depth is recognized as a key trait that underlies the resistance or resilience of trees under drought events (Nardini and Valentino, 2016). Our findings suggest that the vertical extension of the rooting depth and tree height might be key functional traits that determine plant growth in drought-affected areas. We found that the rooting depth of locust trees was positively correlated with the mean annual rainfall, but further studies of the shifts in the belowground root traits (e.g., root xylem size) and aboveground functional traits (e.g., leaf area) are needed to advance our understanding of the roles of the roots in the response to drought (Choat et al., 2018). Nevo (2012) suggested that differences in the slope aspect may be suitable for assessing the mechanisms that allow plants to adapt to different environments under climate change. Considering both the aboveground and belowground effects will be critical for predicting future changes in forest ecosystem function (Collins et al., 2020). Our results provide useful insights into how environmental factors might control the belowground and aboveground functional traits of plants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

ML and LX were responsible for manuscript drafting and editing. WY was contributed to experimental design and analysis. YJ was contributed to field sampling and root cleaning. CJ was contributed to interprate the data of work, and revise our manuscript critically. All authors contributed to the article and approved the submitted version.

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Fine Root Growth of Black Spruce Trees and Understory Plants in a Permafrost Forest Along a North-Facing Slope in Interior Alaska

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Permafrost forests play an important role in the global carbon budget due to the huge amounts of carbon stored below ground in these ecosystems. Although fine roots are considered to be a major pathway of belowground carbon flux, separate contributions of overstory trees and understory shrubs to fine root dynamics in these forests have not been specifically characterized in relation to permafrost conditions, such as active layer thickness. In this study, we investigated fine root growth and morphology of trees and understory shrubs using ingrowth cores with two types of moss substrates (feather- and *Sphagnum* mosses) in permafrost black spruce (*Picea mariana*) stands along a north-facing slope in Interior Alaska, where active layer thickness varied substantially. Aboveground biomass, litterfall production rate, and fine root mass were also examined. Results showed that aboveground biomass, fine root mass, and fine root growth of black spruce trees tended to decrease downslope, whereas those of understory Ericaceae shrubs increased. Belowground allocation (e.g., ratio of fine root growth/leaf litter production) increased downslope in both of black spruce and understory plants. These results suggested that, at a lower slope, belowground resource availability was lower than at upper slope, but higher light availability under open canopy seemed to benefit the growth of the understory shrubs. On the other hand, understory shrubs were more responsive to the moss substrates than black spruce, in which *Sphagnum* moss substrates increased fine root growth of the shrubs as compared with feather moss substrates, whereas the effect was unclear for black spruce. This is probably due to higher moisture contents in *Sphagnum* moss substrates, which benefited the growth of small diameter (high specific root length) fine roots of understory shrubs. Hence, the contribution of understory shrubs to fine root growth was greater at lower slope than at upper slope, or in *Sphagnum* than in feather-moss substrates in our study site. Taken together, our data show that fine roots of Ericaceae shrubs are a key component in belowground carbon flux at permafrost black spruce forests with shallow active layer and/or with *Sphagnum* dominated forest floor.

Keywords: active layer thickness, feather moss, fine root morphology, ingrowth core, *Sphagnum* moss

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INTRODUCTION

Permafrost ecosystems store huge amounts of carbon below ground (about 1,000 Pg), which accounts for 50% of global belowground organic carbon pool (Tamocai et al., 2009; Mishra et al., 2021). Although frozen soil can be a harsh environment for vascular plants, coniferous forests dominated with black spruce (*Picea mariana*) (Interior Alaska, Northwestern Canada) and larch (*Larix gmelinii* and *L. cajanderi*) (central and eastern Siberia) are widely distributed in permafrost regions (Viereck et al., 1983; Abaimov, 2010; Fujii et al., 2020), covering more than 20% of the boreal forest globally (Osawa and Zyryanova, 2010). Considering this large area of distribution, it is important to improve our knowledge of carbon dynamics in permafrost forests to better understand terrestrial carbon cycling. However, the information on plant growth in permafrost forests is still limited especially for belowground parts, although a few papers reported above and belowground productivity in those permafrost forests (Kajimoto et al., 1999; Ruess et al., 2003).

One of the dominant species of permafrost forests is black spruce (*Picea mariana*), which is well known to distribute widely across boreal zone of North America (Vogel et al., 2008). This wide distribution can be explained by high adaptability of this species to wide ranges of environmental conditions. One of the unique characteristics of black spruce stands in permafrost regions appears to be high carbon partitioning below ground. For example, it was reported that root-to-shoot biomass ratio (R/S ratio) in a permafrost black spruce stand was about 0.88 (Noguchi et al., 2012), which is three times as large as averaged R/S ratio of boreal forests (about 0.27) (Cairns et al., 1997). Furthermore, fine root production rates in other black spruce stands on permafrost were estimated to be 126–374 g m^{-2} $year^{-1}$, which were 14–17 times as large as litterfall production rates in those stands (Ruess et al., 2003). These data suggest that root growth dynamics are a key to understanding how the black spruce can survive in harsh environments such as cold and poorly drained permafrost (Viereck et al., 1983). However, it should be noted that most of the data on fine root production reported previously have been stand-level assessments that include both black spruce and understory plants (Ruess et al., 1996, 2003; O'Connell et al., 2003; Bond-Lamberty et al., 2004; Kalyn and Van Rees, 2006). Since understory plants were reported to make a significant contribution to aboveground productivity in permafrost or poorly drained black spruce stands (O'Connell et al., 2003; Ruess et al., 2003; Bond-Lamberty et al., 2004), it would be valuable to evaluate fine root growth of understory plants separately from black spruce trees to better understand how the belowground carbon flux is controlled in permafrost forests.

Permafrost conditions vary with latitudinal location or topography. Throughout Interior Alaska, permafrost is found on north-facing slopes and poorly drained lowlands (Viereck et al., 1983), where the thickness of the active layer (mineral soil layer with seasonal thaw) varies with slope position and aspect (Noguchi et al., 2016; Tanaka-Oda et al., 2016; Wolken et al., 2016). For example, it was reported that the active layer thickness and biomass of black spruce trees are greater in the upper slope than in lower slope, whereas biomass allocation to fine roots (i.e., fine root/aboveground biomass ratio) was greater in the lower slope (Noguchi et al., 2016). These results likely reflect acclimation of black spruce trees to colder and less fertile soils in the lower slope position with thinner active layer thickness (Tanaka-Oda et al., 2016).

Another factor influencing growth of trees on permafrost would be mosses and lichens which typically cover forest floor continuously and play an important role in regulating biophysical conditions of thick organic layer (O'Donnell et al., 2009; Terrier et al., 2014; Pacé et al., 2020), where more than 90% of black spruce fine roots were observed in the above-mentioned permafrost forest (Noguchi et al., 2016). Species composition of the forest floor moss community varies with environmental conditions such as drainage and canopy openness. Particularly, *Sphagnum* mosses dominate poorly drained wet sites whereas feather mosses, such as *Pleurozium schreberi* and *Hylocomium splendens,* dominate sites with better drainage (Bisbee et al., 2001). A recent paper reported that the mosses affected the growth of black spruce seedlings differently by species, in which 2-year-old seedlings grew better on feather mosses than on *Sphagnum* mosses, although such difference was not observed in 3-year-old seedlings planted in the field (Pacé et al., 2018).

Understory shrubs are known to often contribute significantly to aboveground productivity in black spruce forests (Ruess et al., 2003). However, little is known about the separate contributions of overstory trees and understory shrubs to fine root dynamics. Although a recent study showed greater contribution of understory plants to fine root biomass at a shallow than at a deep active layer stand (Noguchi et al., 2016), information is limited on how fine root growth of the trees and the shrubs vary in relation to gradients in abiotic and biotic conditions such as slope position, active layer thickness, and species composition of forest floor mosses. Thus, the first objective of this study was to quantify fine root growth rates of black spruce and understory plants in mature black spruce stands with varied active layer thickness across slope positions. Based on the previous reports on soil fertility (Tanaka-Oda et al., 2016) and fine root biomass (Noguchi et al., 2016) in our study site, we hypothesized that (1) fine root growth rate was lower but belowground allocation was greater on lower slopes with colder and less fertile soils and (2) contribution of understory plants to total fine root growth was greater on lower slopes. The second objective was to elucidate effects of different moss substrates on the fine root growth rates. Considering the data from seedling experiments (Pacé et al., 2018), we also hypothesized that (3) fine root growth was greater in feather than in *Sphagnum*-moss substrates. Finally, our third objective was to quantify variation in fine root morphology such as specific root length (SRL) across slope positions or in different moss substrates, which could explain how black spruce trees and understory shrubs acclimated to the varied abiotic and biotic conditions on permafrost.

MATERIALS AND METHODS

Study Site

This study was conducted in a ca. 100-year-old black spruce (*Picea mariana*) forest on a north facing slope in the Caribou Poker Creek Research Watershed (CPCRW) of University of Alaska Fairbanks (65°08′N, 147°30′W). The mean annual temperature and precipitation are −2.5°C and 400 mm, respectively (Petrone et al., 2006). This is the same black spruce forest studied by Noguchi et al. (2016) and Tanaka-Oda et al. (2016). We established one 14 m × 14 m plot at each of the upper, middle, and lower slope positions, where the elevation is 360, 330, and 260 m, respectively (**Supplementary Figures 1**, **2**). Hereafter, these plots will be designated as NE360, NE330 and NE260, respectively. The stand characteristics and photographs of the study plots are shown in **Table 1** and **Supplementary Figure 3**, respectively. Variations of environmental conditions across the three plots included 8–14 degrees in slope inclination, 34–42 cm in organic layer thickness, 63–113 cm in active layer thickness, and 3.1–4.6 g kg^{-1} in total soil nitrogen in A horizon of mineral soil. Stand densities and mean diameter at breast height (DBH) of black spruce trees taller than 1.3 m varied as 5,420–9,220 trees ha^{-1} and 2.7–5.1 cm, respectively. Major species of understory vascular plants included Ericaceae shrubs such as *Rhododendron* and *Vaccinium* spp. (Noguchi et al., 2016). Forest floor was continuously covered by feather mosses (*Pleurozium schreberi* and *Hylocomium splendens*), *Sphagnum* mosses and lichens.

Vegetation Survey

To estimate the aboveground biomass of black spruce trees, DBH was measured for all trees taller than 1.3 m in the three plots in August 2018. As for trees 0.5–1.3 m in height, diameter at stem base was measured in eight 2 m × 2 m subplots in each 14 m × 14 m plot. Aboveground biomass was then calculated using allometric equations established in Noguchi et al. (2012). To estimate the aboveground biomass of understory vascular plants, 50 cm × 50 cm plots were set at six locations around the side of each 14 m × 14 m plot and aboveground parts of the plants were harvested in July 2018. Samples were dried at 75°C for 48 h and weighed. Forest floor cover was recorded at nine 2 m × 2 m subplots in each plot by visual inspection. Mean area proportions covered by feathermoss (*P. schreberi* and *H. splendens*), *Sphagnum* moss, and others (including lichens) were then calculated.

Litterfall

Litterfall was collected using litter traps in the three plots. In September 2016, eight litter traps (20 cm × 27 cm basket) were set on the ground in each plot (**Supplementary Figure 2**). The litterfall samples were collected in September 2017, August 2018, and September 2019, transported to the laboratory, and divided into leaves, branches, and reproductive organs (e.g., cones) of black spruce trees, leaves of understory plants, and others. These samples were dried at 70°C for more than 48 h and weighed. Litterfall production rates of the three sampling periods were calculated and their averaged values were considered as the annual litterfall production rates. Data from one of the eight traps in NE360 were excluded from further analyses because it contained large amounts of debris of spruce shoots and cones dropped by squirrels, which was 13 times as large as the mean value from seven other traps (ca. 2002 vs. 157 g m^{-2} year^{-1}).

TABLE 1 | Stand characteristics of the black spruce stands in this study.

Plot		NE360	NE330	NE260
Slope aspect		NE	NE	NE
Slope position		Up slope	Mid slope	Low slope
Elevation	(m)	360	330	260
Slope inclination	(degree)	8	8	14
Organic layer thickness	(cm)	34 ± 3	42 ± 2	42 ± 2
Active layer thickness*	(cm)	113 ± 10	63 ± 5	74 ± 7
Soil N concentration*	(g kg^{-1})	4.6	3.6	3.1
Organic layer temperature**				
Under feather moss	(°C)	8.8	8.9	7.9
Under *Sphagnum* moss	(°C)	No data	9.4	7.0
Black spruce (H > 1.3 m)				
Stand density	(trees ha^{-1})	8,660	9,220	5,420
Mean DBH	(cm)	5.1 ± 0.2 x	2.9 ± 0.1 y	2.7 ± 0.2 y
Mean Height*	(m)	7.1 ± 0.6	4.5 ± 0.2	3.0 ± 0.2
Black spruce (H 0.5–1.3 m)				
Stand density	(trees ha^{-1})	2,210	9,150	8,700
Mean D_0	(cm)	1.2 ± 0.1	1.7 ± 0.1	1.9 ± 0.1
Aboveground biomass				
Black spruce	(kg m^{-2})	5.96	2.08	1.11
Understory plants	(g m^{-2})	43.6 ± 10.8 a	145 ± 31 b	142 ± 18 b
Forest floor cover***				
Feather moss	(%)	89	81	41
Sphagnum moss	(%)	2	16	38
Other	(%)	9	3	21

**Data from adjacent stands (Tanaka-Oda et al., 2016).*
***Mean growing season temperature (May–September 2018–2019) in the depth of 10 cm.*
****Data of NE360 and NE330 were from Noguchi et al. (2016).*
Different alphabetical letters indicate significant difference between the plots (a,b: Tukey-Kramer HSD test; x,y: Steel-Dwass test).

Fine Root Mass

To estimate fine root mass, core sampling was conducted in August 2018. In each plot, nine 2 m × 2 m subplots were selected, and one sample was taken from each subplot (**Supplementary Figure 2**). The core samples were collected by vertically inserting a metal tube (3.0 cm in inner diameter, 60 cm in length) to the depth of gravel or frozen soil that could not be penetrated. Maximum depth sampled by this procedure was 50 cm. The core samples were divided into organic and mineral soil layers and transported on ice to the laboratory and stored in a freezer until processed. From these core samples, organic layer thickness was calculated by subtracting the length of mineral soil core from the depth of tube insertion. In the laboratory, the core samples were spread in water and fine roots were manually picked up using tweezers. The samples were rinsed with water on a sieve with 0.5 mm openings to remove fine particles of soil and organic matter when necessary. The fine root samples were divided into those of black spruce trees and understory plants by their morphological characteristics (Noguchi et al., 2016). Those fine root samples were further divided into two groups by diameter classes of < 0.5 mm and 0.5–2.0 mm. Although we removed fragile roots as dead roots, a certain fraction of black or dark brown roots in the remaining samples might also have been dead. Therefore, we analyzed those fine root samples as "fine root mass" and not as "fine root biomass" in this study. Fine root samples were dried at 70°C for more than 48 h and weighed. Fine roots in mineral soil were not used for further analyses because more than 90 and 80% of fine roots of black spruce and understory plants, respectively, were present in the organic layer (data not shown).

Fine Root Growth Rate

Fine root growth rate was examined for 2 years (September 2017 to September 2019) with an ingrowth core method. Although data obtained by the ingrowth core method have been previously expressed as "fine root production rate" (Vogt et al., 1998), here, we described our data as "fine root growth rate" due to lack of information on fine root mortality and decomposition, which might contribute significantly to the amounts of fine root production (Hendricks et al., 2006; Osawa and Aizawa, 2012). Plastic mesh tubes with 2-mm openings, 3.2 cm in diameter, and 30 cm in length were used as the ingrowth cores. Two different substrates, dead feather mosses and dead *Sphagnum* mosses, were used. For the substrate preparation, moss shoots were collected near the study plots and packed gently in the mesh tubes after removing their top living (green) parts. Care was taken to remove any live or dead roots from the moss substrates prior to packing. Ingrowth cores were installed on 26–27 September 2017 at nine subplots in each of the NE360, NE330, and NE260 plots (**Supplementary Figure 2**). To prepare the installation holes, a metal tube smaller than the ingrowth cores (2.4 cm in diameter) was used to ensure good contact between the ingrowth core and surrounding soft organic layer. At NE330 and NE260 plots, a pair of ingrowth cores with feather and *Sphagnum*-moss substrates were set 20–55 cm apart in each subplot. At NE360, only feather moss was used to pack the ingrowth cores because the ground cover by *Sphagnum* mosses was little at the NE360 plot and surrounding area (**Table 1**).

After 2 years of incubation, the ingrowth cores were collected on September 19–22, 2019. During sampling, the ingrowth cores were removed together with surrounding organic layer by cutting with hedge shears followed by removal of organic matter and roots outside the ingrowth cores using scissors. The ingrowth cores were divided into three 10 cm depth intervals and transported to the laboratory on ice and stored in a freezer until processed. Fine roots in the ingrowth cores were collected using the same procedure for above-mentioned fine root mass analyses. Roots that appeared to be fresh were separated as living roots based on their color and tensile strength for four ingrowth core samples from NE360 (feather moss substrate) and for four pairs of the samples from NE330 and NE260 (a pair included the feather and *Sphagnum*-moss substrates at a given sampling location). These living roots were subjected to analyses of fine root morphology as described below. After the root separation and morphological analyses, the samples were dried at 70°C for 48 h and weighed. Fine root growth rate was calculated as fine root mass divided by area of the ingrowth cores (8.0 cm^2) and by the incubation period (2 years). Since fine root growth was examined with feather and *Sphagnum* moss substrates, weighted mean of fine root growth rate was also calculated for the NE330 and NE260 plots considering proportion of ground cover by feather (F) and *Sphagnum* (Sp) mosses (except for "Other"). The F:Sp ratios used for the calculations were 0.84:0.16 and 0.52:0.48 (**Table 1**) in the NE330 and NE260, respectively.

Fine Root Morphology

Living roots collected from the ingrowth cores were spread in water on a glass petri dish. Then, samples were scanned using a flat-bed scanner (GT-980X, Epson, Suwa, Japan) at 800 dpi under constant brightness (80 in Epson Scan software) and back-lighting using a LED light panel (Treviewer A3-500, Trytec, Kyoto, Japan). The obtained root images were analyzed for length, volume, and diameter using the WinRHIZO Pro software (Regent Instruments, Quebec, Canada). From these data, specific root length (SRL, m g^{-1}) was calculated as the fine root length (m $sample^{-1}$) divided by fine root dry weight (g $sample^{-1}$), and root tissue density (RTD, g cm^{-3}) was calculated as the fine root dry weight (g $sample^{-1}$) divided by fine root volume (cm^3 $sample^{-1}$).

Soil Temperature and Moisture Monitoring

Temperature and water content in the organic layer were monitored at 10 cm depth using sensors and data loggers (5TE and Em50, METER Group Inc., Pullman, WA, United States). In the NE360 plot, one sensor was set under a feather moss patch, while in the NE330 and NE260 plots, one sensor was installed under each of a feather and a *Sphagnum* moss patches. The data were retrieved from data loggers in August 2018 and September 2019.

Statistics

One-way ANOVA was performed to examine variations of fine root parameters for each moss substrate, litterfall, and aboveground biomass of understory vascular plants across study plots (NE360, NE330 and NE260). When the variations were significant, Tukey–Kramer's HSD test was performed for multiple mean comparisons. To examine the effects of the moss substrates (feathermoss vs. *Sphagnum* moss) on the fine root parameters, split-plot ANOVA was conducted for each of the NE330 and NE260 plots, in which sampling locations within a plot were considered as random effect. Before the ANOVA, normality and equal variances of the dataset were checked by Shapiro–Wilk test and Bartlett test, respectively. When the normality or equal variance were not confirmed, the data were subjected to Box-Cox transformation. However, if the transformed data did not meet these conditions, non-parametric Steel–Dwass test was performed. All the statistical analyses were done using JMP 12.0 (SAS Institute, Cary, NC, United States).

RESULTS

Organic Layer Conditions

Organic layer thickness at NE360 plot (34 cm) was approximately 80% of that at the NE330 and NE260 plots (42 cm) (**Table 1**).

Although temperature and volumetric water contents (VWC) were monitored by only one sensor per each ground cover type (feather or *Sphagnum*-mosses) in each plot, mean organic layer temperature during the growing seasons (May–September of 2018–2019, 10 cm depth) appeared lower at NE260 (7.0–7.9°C) than at NE360 and NE330 (8.8–9.4°C) (**Table 1** and **Supplementary Figure 4**), while VWC were greater under *Sphagnum* mosses than under feather mosses (**Supplementary Figure 4**). The mean temperature (May–September of 2018) measured at bottom of the organic layer at adjacent stands of NE360, NE330, and NE260 were 7.4, 7.9, and 4.7°C, respectively (**Supplementary Figure 5**).

Aboveground Biomass and Litterfall

Diameter at breast height (DBH) of black spruce trees was significantly greater at NE360 than at NE330 and NE260, where abovegound biomass of black spruce trees was 5.96, 2.08, and 1.11 kg m^{-2}, respectively (**Table 1**). In contrast, aboveground biomass of understory vascular plants was significantly smaller at NE360 (ca. 44 g m^{-2}) than at NE330 and NE260 (ca. 140 g m^{-2}) (**Table 1**).

Total amounts of annual litterfall production ranged ca. 35–175 g m^{-2} $year^{-1}$, which was significantly smaller at NE260 than at NE330 and NE360 (**Figure 1**). Leaf litters of black spruce trees and understory plants accounted for 35–48 and 5–23%, respectively, of the litterfall. In general, litterfall from black spruce trees was greater in the order of NE360 > NE330 > NE260, whereas leaf litter production of understory plants was significantly greater at NE330 than in two other plots (**Figure 1**).

Fine Root Mass

At NE360, NE330 and NE260 plots, mean fine root mass (Diam. < 2.0 mm) of black spruce trees was 1,949, 1,489, and 804 g m^{-2}, respectively, while that of understory plants was 54, 352, and 212 g m^{-2}, respectively (**Figure 2**). The fine root mass at NE360 was significantly larger than in the NE330 and NE260 for black spruce trees, whereas that of understory plants was significantly smaller than at NE330 and NE260. Roots < 0.5 mm in diameter accounted for 81–90% of the fine root mass of black spruce trees, while that of understory plants accounted for 67–85%.

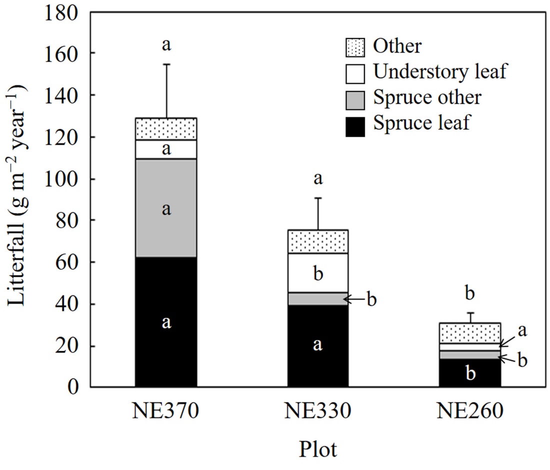

FIGURE 1 | Litterfall production rates in NE360, NE330, and NE260 plots. Black, gray, white, and dotted columns represent data on leaves of black spruce, other organs of black spruce, leaves of understory plants and others, respectively. Data shown are mean + SE (*N* = 7–8). Different alphabetical letters indicate significant difference between the plots within each organ (Tukey–Kramer HSD test).

Fine Root Growth Rate

In the feather moss substrate, fine root growth rate of black spruce trees tended to decrease downslope, which ranged from 20.3 to 37.0 g m^{-2} $year^{-1}$ (**Figure 3**). In contrast, the fine root growth rate of understory plants increased downslope significantly, which ranged from 9.3 to 33.7 g m^{-2} $year^{-1}$. As a result, variation of total fine root growth rate among the plots was little, ranging from 46.3 to 54.0 g m^{-2} $year^{-1}$. In the *Sphagnum* moss substrate (NE330 and NE260), fine root growth rates of black spruce, understory plants, and total were 14.7–17.2, 43.8–57.0, and 58.5–74.2 g m^{-2} $year^{-1}$, respectively, which were not significantly different between the plots (**Figure 3**). As for the fine root growth in feather moss substrate, the proportion of fine root growth in the depth of 0–10 cm to total fine root growth rate (0–30 cm) was significantly greater at NE260 than at NE360 for both black spruce trees (27 vs. 50%) and understory plants (23 vs. 47%) (**Figure 4**). In contrast, for the *Sphagnum* moss substrate, the proportions were similar between the two plots (NE330 and NE260), averaging 54%–56% and 43%–44% in black spruce and understory plants, respectively.

When fine root growth rate was compared between the different moss substrates, it was significantly greater in *Sphagnum* moss than in feather moss for understory plants at NE330 (**Table 2**). Although the difference was not significant, a similar trend was observed at NE260 (split plot ANOVA, $p = 0.094$). The fine root growth rate of black spruce and total fine root growth rate were not significantly different between the moss substrates.

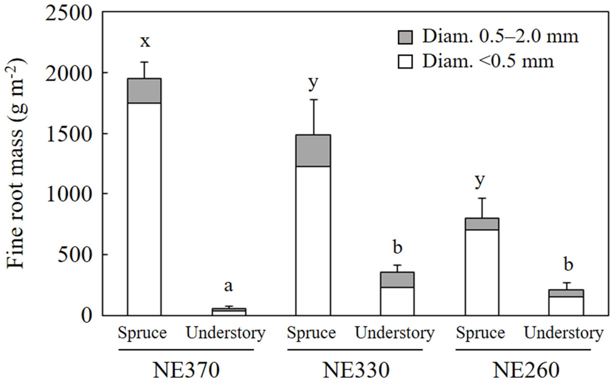

FIGURE 2 | Fine root mass of black spruce and understory plants in NE360, NE330, and NE260 plots. White and gray columns represent data on roots < 0.5 and 0.5–2.0 mm in diameter. Data shown are mean + SE (*N* = 9). Different alphabetical letters indicate significant difference between the plots for total fine root mass in < 2 mm in diameter (a–b, Tukey–Kramer HSD test; x–y, Steel–Dwass test).

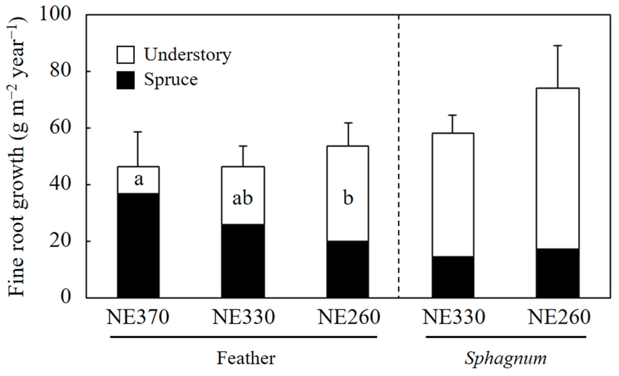

FIGURE 3 | Fine root growth rates of black spruce and understory plants in feather or *Sphagnum*-moss substrates at NE360, NE330, and NE260 plots. Black and white columns represent data on black spruce and understory plants, respectively. Data shown are mean + SE (*N* = 9). Different alphabetical letters represent significant difference between the plots within each species category in each moss substrate (Tukey–Kramer HSD test). No letter was given when significant variation was not detected among the plots by one-way ANOVA.

We examined belowground allocation by calculating ratios of fine root growth rates to leaf litter production rates. Weighted means of fine root growth rates based on feather and *Sphagnum* moss ground cover were used as plot means at NE330 and NE260. The obtained ratios of black spruce, understory plants and total vascular plants were 0.59–1.41, 1.08–12.38, and 0.65–3.77, respectively, which were greater in the order of NE260 > NE330 > NE360 (**Table 3**).

Fine Root Morphology

Mean specific root length (SRL), root tissue density (RTD), and diameter of black spruce fine roots in the ingrowth cores were ca. 42–50 m g^{-1}, 0.30–0.35 g cm^{-3}, and 0.28–0.29 mm, respectively, while those of understory plants were ca. 217–325 m g^{-1}, 0.29–0.37 g cm^{-3}, and.09–0.11 mm, respectively (**Figure 5**). In feather moss substrates, the SRL and diameter of understory fine roots were significantly different among the plots, whereas those of black spruce trees were not. RTD in the feather moss was not significantly different among the three plots for either black spruce or understory plants. As for roots in *Sphagnum* moss, fine root morphology did not vary significantly between the plots (NE330 and NE260). At NE260, on the other hand, RTD of black spruce was significantly lower in *Sphagnum* moss than in feather moss (**Table 2**). Although the difference was not significant, a similar trend of variation was found in the NE330 (split plot ANOVA, $p = 0.077$).

Contribution of Understory Plants

The contribution of understory plants to total stand aboveground biomass of vascular plants was relatively small across plots (0.7–11%) (**Figure 6**). However, the fine root mass of understory plants constituted between 2.1 and 18.1% of total stand fine root mass and was significantly lower at NE360 than at the other two plots. Stand-level contributions of understory plants to leaf litter and fine root growth rate averaged between 17–38% and 25–78%, respectively, in which the former was not significantly different across the plots, whereas the latter was significantly smaller at NE360 than at NE260. In addition, the contribution of understory plants to total fine root growth was significantly greater in *Sphagnum* substrate than in feather moss substrate at NE330 (**Table 2**). Although the difference was not significant, a similar trend was found at NE260 (Split plot ANOVA, $p = 0.061$).

DISCUSSION

Fine Root Growth in Relation to the Slope Positions

In this study, we investigated fine root growth rates of black spruce and understory plants separately, across different slope positions, while most of the previous studies have reported total (black spruce + understory plants) fine root production rates (O'Connell et al., 2003; Ruess et al., 2003; Kalyn and Van Rees, 2006). Since the effects of different permafrost conditions were not well known, we focused on the variation of fine root growth across slope positions, where active layer thickness varied. Our results on aboveground biomass, fine root mass, litterfall production rates, and fine root growth rates show that above and belowground growth of black spruce trees were smaller at lower slope than at upper slope (**Figures 1–3** and **Table 1**). However, the variation of fine root growth rates of black spruce was less evident (not significant) as compared with those of aboveground parameters. As a result, ratios of fine root growth rates to leaf litter production rates, for example, were greater at a lower slope, especially at NE260 plot (**Table 3**). These results suggest that belowground allocation of black spruce trees increased downslope, which supports our hypothesis 1. Similarly, our results show that the belowground allocation of understory plants was also greater at the lower slope than at upper slope (**Table 3**). However, in contrast to those of black spruce trees, above and belowground (bio) mass and growth of understory plants were significantly greater at the lower slope (NE330 and/or NE260 plots) than at NE360 (**Table 1** and **Figures 1–3**). For example, the fine root growth rate of understory plants in feather moss at NE260 (33.7 g m^{-2} year^{-1}) was 3.6 times as large as that at NE360 (9.3 g m^{-2} year^{-1}). Thus, as for understory plants, our results supported the hypothesis 1 for belowground partitioning, although the amount of fine root growth was greater at the lower slope, in contrast to our expectation.

Similar patterns of change in belowground allocation have often been observed in studies on other non-permafrost forests. For example, fine root biomass, growth, or allocation (e.g., fine root biomass per basal area) were negatively correlated with nitrogen mineralization rates (temperate deciduous broad-leaved forest) (Tateno et al., 2004) and annual precipitation (European beech forests) (Hertel et al., 2013) or positively correlated with soil C/N ratio (boreal-temperate Norway spruce, Scots pine and silver birch forests) (Ostonen et al., 2017). In our study site, Tanaka-Oda et al. (2016) showed that soil nitrogen concentration tended to be lower in the lower than in upper-slope. Furthermore,

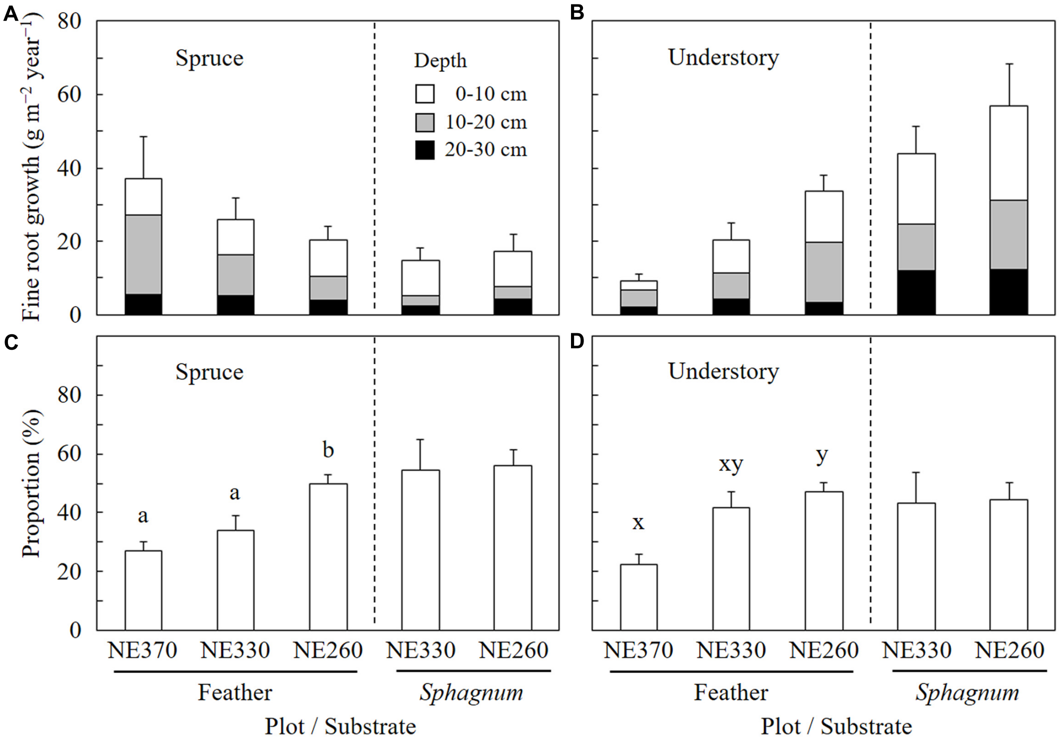

FIGURE 4 | Vertical distribution of fine root growth of black spruce and understory plants in feather or *Sphagnum*-moss substrates at NE360, NE330, and NE260 plots [**(A)** fine root growth rates of black spruce; **(B)** those of understory plants; **(C)** proportions of fine root growth at the depth of 0–10 cm in total fine root growth (0–30 cm) of black spruce; **(D)** those of understory plants]. White, gray, and black columns represent data in the depths of 0–10, 10–20, and 20–30 cm, respectively **(A,B)**. Data shown are mean + SE ($N = 9$). Different alphabetical letters indicate significant difference between the plots within each moss substrate (a–b, Tukey–Kramer HSD test; x–y, Steel–Dwass test). Data on fine root growth rates **(A,B)** were not subjected to the statistical analyses.

they demonstrated that foliar δ^{15}N of black spruce trees positively correlated with aboveground biomass and current shoot growth rates, suggesting that contribution of ectomycorrhizal fungi in nitrogen uptake increased when aboveground growth of black spruce trees was limited. Thus, greater belowground allocation at lower slope was likely driven in part by a response to lower nutrient availability (**Table 3**).

As mentioned above, fine root growth of understory plants was significantly greater at the lower slope than at the upper slope, although that of black spruce trees showed an opposite trend of variation along the slope. As a result, the contribution of

TABLE 2 | Results of split-plot ANOVA for effects of different moss substrates on fine root parameters.

		NE330		NE260	
		***F*-value**	***P*-value**	***F*-value**	***P*-value**
FRG	Spruce	5.22	0.052	0.24	0.64
	Understory (U)	**23.7**	**0.001**	3.61	0.094
	Total (T)	2.94	0.12	1.79	0.22
	U/T	**37.5**	**< 0.001**	4.74	0.061
SRL	Spruce	0.00	0.98	0.19	0.69
	Understory	0.08	0.79	0.34	0.60
RTD	Spruce	7.04	0.077	**35.2**	**0.010**
	Understory	0.08	0.80	2.38	0.22
Diameter	Spruce	0.08	0.79	0.09	0.79
	Understory	0.14	0.74	0.11	0.76

U/T, proportion of fine root growth rate of understory plants in total fine root growth rate.
Bold values indicate that the effect was significant ($p < 0.05$).

TABLE 3 | Weighted means of fine root growth rates (FRG), mean leaf litter production rates (LLP), and ratio of FRG/LLP in each plot.

	Plot	Spruce	Understory	Total
Fine root growth (FRG)	NE360	37.0	9.3	46.3
g m^{-2} year^{-1}	NE330*	24.2	24.2	48.4
	NE260*	18.8	44.9	63.7
Leaf litter production (LLP)	NE360	62.4	8.6	71.0
g m^{-2} year^{-1}	NE330	39.0	18.5	57.5
	NE260	13.3	3.6	16.9
FRG/LLP	NE360	0.59	1.08	0.65
	NE330	0.62	1.31	0.84
	NE260	1.41	12.38	3.77

**Fine root growth rate was calibrated by ground cover ratio between feather and Sphagnum-mosses for each of the NE330 and NE260 plot.*

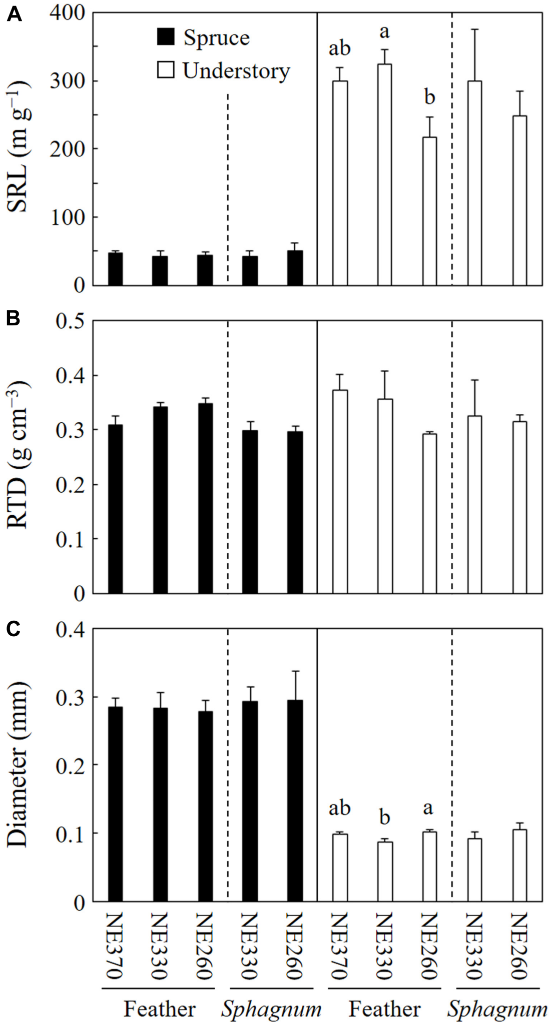

FIGURE 5 | Fine root morphology of black spruce and understory plants in feather or *Sphagnum*-moss substrates at NE360, NE330, and NE260 plots. Specific root length [SRL, **(A)**], root tissue density [RTD, **(B)**], and root diameter **(C)** are shown. Black and white columns represent data on black spruce and understory plants, respectively. Data shown are mean + SE (*N* = 4). Different alphabetical letters indicate significant difference between the plots within each species category with each moss substrate (Tukey–Kramer HSD test). No letter was given when significant variation was not detected among the plots by one-way ANOVA.

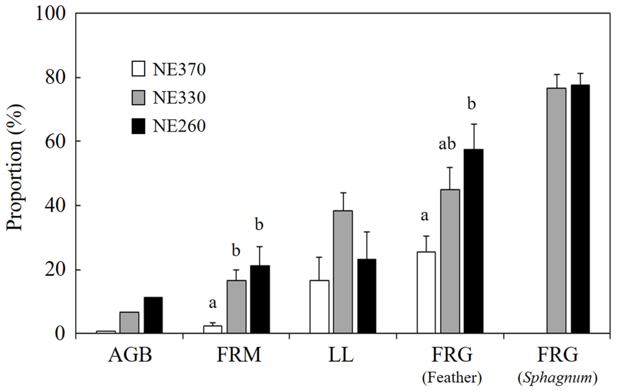

FIGURE 6 | Proportion of understory plants in total (black spruce + understory) aboveground biomass (AGB), fine root mass (FRM), leaf litter production (LLP), and fine root growth (FRG) in feather and *Sphagnum*-moss substrates. White, gray, and black columns represent data from NE360, NE330, and NE260 plots, respectively. Data shown are mean + SE (AGB, *N* = 1; LLP, *N* = 7–8; FRM and FRG, *N* = 9). Different alphabetical letters indicate significant difference between the plots within each of the above and belowground parameters (Tukey–Kramer HSD test).

understory plants to total fine root growth increased downslope, which supported our hypothesis 2. One of the reasons for the difference between the black spruce and understory plants in the patterns of variation in fine root growth was probably in a gradient of light availability for understory plants. Since lower stand density with smaller black spruce trees formed an open canopy stand (**Table 1** and **Supplementary Figure 3**), light availability for understory plants would be greater at the lower slope than at the upper slope, which could translate to increased plant growth. Another explanation could be differences in responses to belowground conditions. Although the proportion of fine root growth in surface organic layer (0–10 cm depth) was greater at the lower slope for both black spruce and understory plants, the former resulted from a decrease at deeper layer (10–30 cm depth), whereas the latter was due to increase in the fine root growth at surface layer (0–10 cm depth) (**Figure 4**). This suggests a possibility that the negative effects of belowground conditions (e.g., low temperature, **Supplementary Figures 4**, **5**) were little or negligible for understory plants as compared with those for black spruce trees. Thus, it seemed that variations of fine root growth across slope positions were controlled by mixed effects of above and belowground resource availability, although further study is needed to clarify the underlying mechanisms including competition between the plant species (Inderjit and Mallik, 1996).

Effects of Forest Floor Mosses on Fine Root Growth

As discussed above, smaller aboveground biomass of black spruce trees at lower slope could be linked to lower belowground temperature due to higher permafrost table in our study site (**Table 1** and **Supplementary Figures 4**, **5**). The results on the vertical distribution of fine root growth of black spruce trees showed that surface concentration of the fine root growth was more evident at lower slope than at upper slope, which might result from growth suppression of fine roots at the deeper layers with lower temperature (**Figure 4** and **Supplementary Figure 5**). However, our data on stand characteristics suggested that both active layer and organic layer thickness were similar between the low-slope (NE260) and mid-slope (NE330) plots, even though aboveground biomass of black spruce trees in the former plot was about 50% of that in the latter (**Table 1**). Therefore, other factors should also be considered to explain the growth variation of black spruce and understory plants across the slope positions.

In this study, we focused on differences in ground cover, which was highly heterogeneous along the toposequence, but in general, the proportion of *Sphagnum* moss relative to feather moss increased downslope (**Table 1**; Morishita et al., 2019; Toriyama et al., 2021). Our measurements investigating effects of different moss substrates showed that while fine root growth of black spruce was not strongly sensitive to the different moss substrates, *Sphagnum* moss positively affected fine root growth of understory plants as compared with feather moss (**Table 2**). Thus, our hypothesis 3 was not supported by the results of this study. A recent study using black spruce seedlings suggested that *Sphagnum* moss substrate could affect the growth of black spruce negatively due to lower nutrient availability, although the effects were likely to vary depending on growth conditions (greenhouse vs. field) and/or seedling characteristics (age) (Pacé et al., 2018). In our study, fine root growth of black spruce tended to be lower in *Sphagnum* than in feather-mosses at the NE330 (p = 0.052), but not at the NE260 (**Table 2**). There is a possibility that lower nutrient availability or lower pH within the *Sphagnum* moss substrates may decrease fine root growth of black spruce, but further study is needed to know whether the *Sphagnum* moss substrates have negative effects on the growth of black spruce fine roots. Another well-known characteristic of *Sphagnum* moss substrates is that they can retain higher amounts of water than feather moss substrates (O'Donnell et al., 2009; Terrier et al., 2014). This moisture condition could benefit fine root growth of understory plants more than that of black spruce, considering that fine roots of understory plants such as Ericaceae shrubs were much thinner and probably more vulnerable to occasional drought in feather moss substrate than those of black spruce (**Figure 4** and **Supplementary Figure 4**).

As for fine root morphology, root tissue density (RTD) of black spruce fine roots was lower in the *Sphagnum* than in feather-moss substrates, although we did not examine it at NE360 in this study (**Table 2**). This suggested that fine roots of black spruce in *Sphagnum* moss substrates might have higher physiological activity than in feather moss substrates (Makita et al., 2015). In contrast, such an effect was not observed in understory fine roots. Previous studies showed that RTD increased with decreasing soil resource availability (Makita et al., 2015; Kramer-Walter et al., 2016). Although nutrient availability in feather moss substrates is likely higher than in *Sphagnum* moss substrates (Pacé et al., 2018), occasional drought in feather moss might result in higher RTD in feather than in *Sphagnum* moss substrates in our study (**Table 2** and **Supplementary Figure 4**). However, a recent study reported that drought effect on RTD was not so simple, in which drought treatment increased RTD of distal (1st and 2nd order) roots of *Quercus alba*, while opposite responses were observed in higher (4th) order roots, and the effects varied with tree species (Suseela et al., 2020). Hence, further study is needed to understand why RTD of black spruce varied with the different moss substrates in our study site.

Taken together, our results suggested that black spruce responded to different moss substrates by changing fine root morphology (e.g., RTD) rather than the fine root growth rate in our study site, whereas the effects on understory plants were more evident in fine root growth rate than in fine root morphology (**Figure 5** and **Table 2**).

Implication to Carbon Dynamics

One of previous papers using the minirhizotron technique showed that fine root production rates in three permafrost black spruce stands in Interior Alaska averaged 126–374 g m^{-2} $year^{-1}$, which were 14–17 times as large as litterfall production rates (9–22 g m^{-2} $year^{-1}$) (Ruess et al., 2003). This suggests that the contribution of fine roots to net primary productivity (NPP) or belowground carbon input is much greater than leaves or aboveground litters, respectively. On the other hand, our results showed that fine root growth rates were 46–64 g m^{-2} $year^{-1}$ (**Table 3**), which were 0.26–1.8 times as large as aboveground litterfall production rates (35–176 g m^{-2} $year^{-1}$; **Figure 1**). These results suggested that the contribution of fine roots to NPP or belowground carbon input in permafrost black spruce stands varied with stand productivity, in which the contribution of fine roots was greater at lower productivity sites (**Table 3**; Ruess et al., 2003). Here, it should be noted that methods might also affect the estimates of fine root growth (production) rates. In general, fine root production rates estimated by the minirhizotron technique were greater than those by the ingrowth core method (Finér et al., 2011), which were also demonstrated in mature black spruce stands in Canada (Steele et al., 1997). This is probably because the ingrowth core method cannot detect mortality and decomposition of fine roots (turnover), which are, of course, occurring throughout the ingrowth period (Hendricks et al., 2006; Osawa and Aizawa, 2012).

We found that the contribution of understory plants to total stand-level fine root mass and fine root growth rates were greater at lower slope than at upper slope (**Figures 2**, **3**). Since SRL of understory plants was six times that of black spruce trees (**Figure 5**), an increased proportion of understory fine roots would likely enhance carbon flux through fine root turnover and respirations, because roots with smaller diameters generally have a shorter life span and greater physiological activity (Wells and Eissenstat, 2001; Pregitzer et al., 2002; Ruess et al., 2006; Makita et al., 2015). In our results, ratios of the fine root mass to the fine root growth rate of understory plants across the plots (4.7–15) were smaller than those of black spruce trees (43–53) (**Figure 2** and **Table 3**), suggesting that fine root turnover of understory plants was faster than that of black spruce trees. These findings suggest that belowground carbon flux in permafrost black spruce forests may increase by increasing the productivity of understory plants (under improved light conditions) with faster fine root turnover at shallower permafrost sites. Some previous studies on boreal forests estimated fine root (litter) production rates of trees and understory plants separately, in which, for example, understory plants accounted for 19–21% (black spruce forests; Steele et al., 1997), 6–29% (drained fen or bog forests with Scots pine, etc.; Bhuiyan et al., 2017), and 20–47% (Scots pine forests; Ding et al., 2021) of total fine root (litter) production, which were comparable or smaller than our data on understory contribution to fine root growth (25–78%; **Figure 6**). Therefore, to better understand belowground carbon flux in permafrost forests, it is recommended that future studies will focus more on the roles of understory plants such as Ericaceae shrubs.

Here, it should be noted that our data were obtained only from one experiment for 2 years. Although long-term data on fine root growth is not available, recent studies showed that stem radial growth of black spruce fluctuated with climatic factors such as summer temperature and precipitation, in which, interestingly, direction and/or strength of the climatic effects varied with site conditions such as slope position and active layer thickness (Wolken et al., 2016; Nicklen et al., 2021). In addition, another tree ring analysis showed that black spruce radial growth is also affected by soil hummock formation (mound rising) on permafrost, which causes tree leaning (Fujii et al., 2020). Considering these results, there is a possibility that the above and belowground growth of understory plants are also affected by these environmental factors directly, or indirectly through changes in overstory black spruce growth. Therefore, it is recommended that future studies will examine temporal (yearly) changes in fine root growth in relation to climatic factors and site conditions, in which it would be important to elucidate the contribution of each functional type (e.g., black spruce vs. understory shrub). Such studies will help us to understand belowground carbon flux in permafrost forests more comprehensively, and also help to predict effects of climate warming that may enhance permafrost thaw and cause shifts of plant communities (Finger et al., 2016).

CONCLUSION

This study quantified fine root growth of black spruce and understory plants during two growing seasons (2018–2019) in a permafrost black spruce forest in Interior Alaska. This black spruce forest is located along a north-facing slope, where active layer thickness and aboveground biomass of black spruce trees decreased downslope in general. Our major finding was that the fine root growth rate of understory plants increased downslope, whereas that of black spruce tended to decrease. As a result, understory root growth accounted for more than 50% of total fine root growth at the low-slope plot (NE260). Furthermore, we found that *Sphagnum* mosses, which often dominate forest floor at sites with the shallow active layer, were likely to benefit fine root growth of understory plants as compared with feather mosses. In conclusion, this study suggests that fine root growth of understory plants such as Ericaceae shrubs is a key to better understanding belowground carbon dynamics in permafrost forests especially at sites with the shallow active layer.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KN, YM, and TM conducted the fieldwork. KN performed the root analyses in the laboratory and prepared the manuscript. JT, YM, TM, and YK contributed to discussing and editing it. All authors contributed to the planning of this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.769710/full#supplementary-material

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Differential Variation in Non-structural Carbohydrates in Root Branch Orders of *Fraxinus mandshurica* Rupr. Seedlings Across Different Drought Intensities and Soil Substrates

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Non-structural carbohydrates (NSCs) facilitate plant adaptation to drought stress, characterize tree growth and survival ability, and buffer against external disturbances. Previous studies have focused on the distribution and dynamics of NSCs among different plant organs under drought conditions. However, discussion about the NSC levels of fine roots in different root branch orders is limited, especially the relationship between fine root trait variation and NSC content. The objective of the study was to shed light on the synergistic variation in fine root traits and NSC content in different root branch orders under different drought and soil substrate conditions. The 2-year-old *Fraxinus mandshurica* Rupr. potted seedlings were planted in three different soil substrates (humus, loam, and sandy–loam soil) and subjected to four drought intensities (CK, mild drought, moderate drought, and severe drought) for 2 months. With increasing drought intensity, the biomass of fine roots decreased significantly. Under the same drought intensity, seedlings in sandy–loam soil had higher root biomass, and the coefficient of variation of 5th-order roots (37.4, 44.5, and 53% in humus, loam, and sandy–loam soil, respectively) was higher than that of lower-order roots. All branch order roots of seedlings in humus soil had the largest specific root length (SRL) and specific root surface area (SRA), in addition to the lowest diameter. With increasing drought intensity, the SRL and average diameter (AD) of all root branch orders increased and decreased, respectively. The fine roots in humus soil had a higher soluble sugar (SS) content and lower starch (ST) content compared to the loam and sandy–loam soil. Additionally, the SS and ST contents of fine roots showed decreasing and increasing tendencies with increasing drought intensities, respectively. SS and ST explained the highest degree of the total variation in fine root traits, which were 32 and 32.1%, respectively. With increasing root order, the explanation of the variation in root traits by ST decreased (only 6.8% for 5th-order roots). The observed response in terms of morphological traits of different fine root branch orders of *F. mandshurica* seedlings to resource fluctuations ensures the maintenance of a low cost-benefit ratio in the root system development.

Keywords: non-structural carbohydrates, *Fraxinus mandshurica*, fine root, drought, soil substrate, root trait

INTRODUCTION

In recent decades, forest decline and death caused by high temperatures and extreme droughts have occurred on a large scale worldwide (Allen et al., 2010; Choat et al., 2012; Zhang et al., 2015; Martínez-Vilalta et al., 2016). Global climate change is predicted to cause tree death and is becoming increasingly serious, which inevitably affects carbon metabolism and balance in the plant and changes its physiological metabolic functions (Choat et al., 2012; Adams et al., 2013). Non-structural carbohydrates (NSCs), as important substances involved in the life process of plants, are primarily composed of soluble sugar (SS) and starch (ST); they largely reflect the carbon supply status of plants and affect the growth and development of plants (Richardson et al., 2013). When plants undergo drought stress, the stored NSCs can be used as a buffer to temporarily supply plants for their growth and metabolism (Dietze et al., 2014). In recent years, the different responses of NSCs among different plant tissues or organs under drought stress have been discussed deeply (Martínez-Vilalta et al., 2016; Furze et al., 2019; Deng et al., 2020; He et al., 2020; Zhang et al., 2020). Many studies have found that the NSC concentration in root tissue is highest except for the trunk, which has an important impact on the storage and distribution of NSCs in trees (Dietze et al., 2014; Mei et al., 2015; Ji et al., 2020). However, the performance of NSCs with fine roots (especially different functional root sequences) in response to drought is ignored. Therefore, understanding the variation in the composition and level of fine root NSCs under drought conditions is of great significance for better recognition of the carbon balance and dynamics of plant survival and growth (Hartmann and Trumbore, 2016).

Accumulating evidence from field and laboratory experiments showed that root biomass usually decreased when plants were exposed to drought conditions (Meier and Leuschner, 2008; Eldhuset et al., 2013; Hertel et al., 2013; Zhou et al., 2018; Wang et al., 2021). However, Olmo et al. (2014) found that the fine root with diameters less than 0.5 mm would have increasing biomass under water deficit. The root system plays a crucial role in plant growth and productivity, especially in resource-constrained environments. The morphological and physiological plasticity of root systems reflects important mechanisms by which plants obtain limited soil resources (Ristova and Busch, 2014; Rogers and Benfey, 2015). Many researchers have demonstrated that changes in root ST under drought conditions are related to plant survival, and root NSC reserves play an important role in repairing embolism and preventing self-death (Rodríguez-Calcerrada et al., 2017; Kannenberg et al., 2018). Oswald and Aubrey (2020) observed that the root ST concentration of *Pinus palustris* in xeric environments increased delayed in summer compared with mesic habitats. ST stored in the root system can promote root growth and maintain root osmotic potential, ensuring that plants can absorb more water (Ge et al., 2012; Camarero et al., 2016). However, previous studies have mostly focused on the overall NSC level of the root system (including coarse roots and fine roots) (Hoch and Körner, 2003; Landhäusser and Lieffers, 2012; Hartmann et al., 2013; Yang et al., 2021), and the response of fine root NSCs with functional root branch orders to drought is rarely considered (Aubrey and Teskey, 2018; Nikolova et al., 2020). Fine roots (≤ 2 mm) are the main organ for water and nutrient absorption, and the most active and sensitive part of the root system (McCormack et al., 2015; Ma et al., 2018). Additionally, Pregitzer et al. (2002) found that the lower-order roots were produced later; the younger the age, the larger the contribution; the lower-order roots have higher nitrogen content and the respiration metabolism ability; and they are more sensitive and fragile than the higher-order roots. In addition, previous studies have mostly compared the effects of a single drought or severe drought on the root system, but gradient studies are lacking (Hartmann et al., 2013; Kannenberg et al., 2018; Blackman et al., 2019; Zhang et al., 2020). McDowell et al. (2008) speculated that trees depleted NSC only under mild or moderate drought, while severe drought caused the xylem to form embolisms while NSCs were not depleted. Recently, a meta-analysis based on 52 tree species around the world indicated that variations in plant NSCs were related to drought intensity, and the net loss of carbohydrates from roots was the most obvious (He et al., 2020). Therefore, it is of great significance to understand the response of fine root NSCs with branch root orders to different drought intensities.

Root traits play a vital role in the acquisition and transportation of water and nutrients, and they can strongly affect plant growth, survival, and response to climate change (Bardgett et al., 2014; Kong et al., 2014; Iversen et al., 2017). Most previous studies have demonstrated that the drought shapes thinner and deeper root systems to improve the ability to capture water and nutrients (Chapman et al., 2012). Zhou et al. (2018) based on 128 drought experiments found that the drought increased the specific root length (SRL) of woody plants by 30%, and there was a significant positive correlation between plant–SRL and drought. Although numerous studies have reported the response of root traits (high SRL, low diameter) to drought or water deficit (Comas et al., 2013; Fort et al., 2017; Zhou et al., 2018, 2019; Lozano et al., 2020; Nikolova et al., 2020), few studies have focused on the synergistic changes and relationships between root NSC levels and root traits under stress conditions (Ji et al., 2020; Yang et al., 2021). Olmo et al. (2014) studied the drought resistance response of 10 woody tree species seedlings and found that SRL increased significantly under drought conditions. In addition, the drought usually accompanied by increasing temperature induced the accumulation of ST in very fine (<0.5 mm) and fine roots (0.5–1 mm) (Di Iorio et al., 2016). It is a strategy that when water is limited, plants can build longer roots with less carbon. Indeed, increasing the carbon input per unit produces a larger surface area, length of fine roots, and more fine roots, which could facilitate optimization of the cost-benefit ratio of fine roots (Eissenstat et al., 2000; Ostonen et al., 2007). Under drought conditions, thicker roots with transport and storage functions tend to preserve NSCs (Konôpka et al., 2007; Yang et al., 2021), whereas thinner roots with absorption functions are severely affected by the drought (Olmo et al., 2014). Yang et al. (2021) found that a significant correlation between the root NSC concentration, root architecture, and SRL occurred in *Phyllostachys edulis* seedlings under drought conditions, indicating that the sensitivity of NSC concentration to drought supported the plasticity of root architecture to a certain extent, and plants could build low-cost roots through more carbon investment. In addition, when plants adapt to drought stress, they balance the lack of tissue radial growth by increasing the concentration of NSCs in the growing parts (Dietze et al., 2014; Kannenberg et al., 2018). Therefore, exploring the coupled relationship between the variation in fine root NSCs and root traits under drought conditions will help us to further understand the response strategies of plants to water deficit.

Fraxinus mandshurica Rupr. is one of the main timber species in northeastern China. In Jilin Province, China, *F. mandshurica* plantations are distributed in the north-south latitudes, and the soil types they inhabit are roughly divided into three categories, namely, humus soil, loam soil, and sandy–loam soil. Weemstra et al. (2017) found that the SRL and root tissue density (RTD) of the fine roots of European beech and Norway spruce were not significantly different in clay and sandy soil, but the dry mass of fine roots in sandy soil (both species) was 10 times that in clay. Paudel et al. (2016) studied these interactions in the osmometer of an orchard and clay sandy–loam and found that soil type significantly affects the morphological traits of all root branch orders of *Citrus paradisi* Macf. The interaction of the root system with soil quality and water will cause changes in root growth, structure, and function (Paudel et al., 2016). To our knowledge, few studies have focused on the response of fine root NSCs to drought intensity gradients in different soil substrates, and even less is known regarding the coupled relationship between fine root NSCs and traits. In this study, 2-year-old *F. mandshurica* potted seedlings with different drought intensities and soil substrates were set up, and the variation and the coupled relationship of the fine root traits and NSC content of different root branch orders of *F. mandshurica* seedlings under different water and soil conditions were compared. It was hypothesized that (1) given different physicochemical properties, seedlings planted among different substrates will have variable root traits; drought will significantly change root traits to increase the ability to capture water, e.g., increase SRL and decrease diameter; (2) with the increase of drought intensity, the fine roots of seedlings subjected to drought stress will consume SS and accumulate ST content to survival under carbon-limited conditions; and (3) due to the trade-off between plant carbon investment and growth under drought conditions, the variation in fine root NSCs will be closely related to root traits.

MATERIALS AND METHODS

Experimental Site and Sapling Preparation

A controlled pot experiment was conducted at Xinli Town, Jingyue Development District, Changchun, which is located in Jilin Province, China (43°33′ N–44°41′ N, 125°19′ E–125°24′ E). The location has a temperate continental monsoon climate with a frost-free period of 140 days; and with a mean annual rainfall of 600–800 mm, which mainly falls from July to September and a mean annual temperature of 4.6°C.

The 2-year-old *F. mandshurica* Rupr. seedlings, of the Hongwei nursery of Lushuihe Forestry Bureau in Jilin Province, were used as experimental materials. They were transplanted into plastic pots (24 cm × 20 cm) in April 2017 and were placed in flat, open canopies before they started to bud. An equal number of pots were filled with humus, loam, and sandy-loam soil, respectively. The humus soil and loam soil were collected in a secondary forest (consisting of *F. mandshurica*, *Juglans mandshurica*, *Phellodendron amurense*, *Picea asperata*, and *Larix olgensis*) in the Lushuihe Forestry Bureau. The main soil type is a Eum-Orthic Anthrosol according to the Food and Agricultural Organization soil classification system. The sandy-loam soil consists of a mixture of equal volumes of loam (above mentioned) and sand. All potted seedlings were placed under a rain shelter (consisting of iron shelves and thick plastic). The soil surface was well ventilated throughout the experiment. For the experimental area, the pots were placed in rows 50 cm apart from the neighbors under full sunlight. They were kept well-watered prior to the application of drought treatments, and the gravimetric soil water content was initially maintained at field capacity. Fertilizer was not added during the experimental period.

Experimental Design and Sampling

Before the beginning of the drought experiment, three soil substrate samples were collected in July 2017 to determine the soil total nitrogen, total phosphorus, available phosphorus and soil physical structure, water content, and field water holding capacities. The field water holding capacity of humus, loam, and sandy-loam soil was 54.6, 36.4, and 20%, respectively. About 120 pots of cultured *F. mandshurica* seedlings (relatively uniform seedlings) were randomly selected for three different substrates, 40 pots per substrate, respectively. The basal diameter and height of each seedling were measured using a Vernier caliper with an accuracy of 0.01 mm and a tape measure with an accuracy of 0.1 cm prior to the application of drought treatments in July 2017, respectively (**Supplementary Table 1**).

The soil substrates and drought were conducted for a two-factor experiment. Three soil substrates were set with four water available gradients, the control (CK): approximately 80–85% of the maximum field water-holding capacity (FC); mild drought (T1): 60–65% of the FC; moderate drought (T2): 40–45% of the FC; and severe drought (T3): 20–25% of the FC.

About 10 seedlings were assigned to each treatment, totaling 120 seedlings (10 seedlings × four treatments × three soil substrates). Briefly, the soil moisture content of the pots was monitored by weight. The pots were weighed before the progressive drought experiment, and then, the theoretical weights for different drought intensities of the three substrates were calculated. These pots were thereafter controlled daily to maintain a constant weight until the experiment ended in September 2017. A detailed description of drought control was provided in a previously conducted study (Ji et al., 2020). All seedlings were not fertilized before and during the drought experiment.

In each experimental treatment, the roots of 10 seedlings were destructively sampled after 2 months of continuous drought stress. Roots were sorted carefully out of the soil, and root samples were washed free of soil particles with deionized water until the branching structure of the roots could be identified, and then put into the labeled pocket and stored in a portable refrigerator (2–3°C). Then, the root samples were divided into two parts: (1) root morphology analysis and biomass sample; (2) chemical properties analysis sample (for root chemical traits and NSCs). All samples were shipped back to the laboratory on the same day and stored in a freezer at −20°C. In this study, only live root samples were measured, and the dead roots were picked and discarded.

Soil Physicochemical Property

The soil physical and chemical properties of the three soil substrates were determined before drought stress (**Table 1**). The soil total nitrogen was determined by the Kjeldahl titration. The soil total phosphorus was determined by the sulfuric acid-perchloric acid-molybdenum anti-colorimetric method (Yang et al., 2018). Soil available phosphorus was extracted by double acid extraction, soil water content, and bulk density were determined by the ring knife method (Yang et al., 2018), and soil total porosity, aeration porosity, water absorption multiple, water seepage rate, and evaporation rate were measured by following the description of Wei et al. (2015).

Fine Root Morphological and Chemical Traits

In the laboratory, root samples for morphological analysis were carefully dissected with forceps on the basis of branch order, following the procedure described in Pregitzer et al. (2002) and Wang et al. (2006), with the distal non-woody roots regarded as first-order roots (1st-order root), and the next root segment was the 2nd-order roots. For the 4th- and 5th- order roots, root axes larger than 2 mm were separated from each other where necessary with scissors. Then, root samples were scanned with an Expression 10000XL 1.0 scanner at Northeast Forestry University (Epson Telford Ltd., Telford, United Kingdom). The average diameter, total length, and volume of root tips were determined with the root system analyzer software (WinRhizo 2004b, Regent Instruments, Inc., Québec, QC, Canada). These root samples were oven-dried at 65°C to determine the constant weight (nearest 0.0001 g) for fine root biomass and the SRL, specific root surface area (SRA), and RTD were calculated.

TABLE 1 | Physicochemical properties of three soil substrates.

Soil property	Humus soil	Loam soil	Sandy–loam soil
Bulk density (g cm^{-3})	1.18 ± 0.02b	1.35 ± 0.02a	1.32 ± 0.02a
Total porosity (%)	53.19 ± 1.12b	58.90 ± 0.91a	36.72 ± 0.95c
Aeration porosity (%)	25.61 ± 0.48a	23.52 ± 0.53a	13.01 ± 0.99b
Water absorption capacity	0.23 ± 0.01b	0.26 ± 0.01a	0.18 ± 0.01c
Penetrate rate (g min^{-1})	4.49 ± 0.04a	4.01 ± 0.12b	3.04 ± 0.18c
Evaporation rate (g h^{-1})	0.59 ± 0.01a	0.50 ± 0.01b	0.37 ± 0.01c
Total nitrogen (mg g^{-1})	7.09 ± 0.78a	3.11 ± 0.05b	1.20 ± 0.03c
Total phosphorus (mg g^{-1})	0.71 ± 0.04a	0.34 ± 0.09b	0.32 ± 0.01b
Available phosphorus (mg kg^{-1})	13.10 ± 0.82a	5.04 ± 0.21b	12.75 ± 0.69a

The different letters in the same line indicate significant differences among the different treatments ($p < 0.05$).

For root chemical analyses, a part of the root of the seedlings was classified quickly, and then, the fine root samples were placed at 65°C and dried for 48 h to constant weight. The dried fine root sample was ground and homogenized by using a ball mill instrument (RETSCH MM 400, Haan, Germany), and 1 g of the dry powder sample was weighed and pressed with an FYD-20 electric tableting machine (Nuoleixinda Technology Co., Ltd., Tianjin, China) to a boat with a thickness of 6 mm and a diameter of approximately 13 mm. For the pellet sample, the tableting conditions can be adjusted according to the actual conditions. The tableting conditions of the test were maintained at a pressure of 16 MPa for 3 min. The carbon, nitrogen, and phosphorus elements in the root samples after tableting were measured by a J200 Tandem laser spectroscopic element analyzer (Applied Spectra, Inc., Fremont, CA, United States).

Fine Root Non-structural Carbohydrate Concentration

The NSC concentration was defined as the sum of SS and ST concentrations that were measured using the anthrone method (Yemm and Willis, 1954). Root samples (0.1000 g) were placed into a 10 ml centrifuge tube, and 2 ml of 80% ethanol was then added. The mixture was incubated at 80°C in a shaking water bath for 30 min and then centrifuged at 4,000 rpm for 5 min. Two additional extractions from the pellets were carried out with 80% ethanol. The supernatant was retained, combined, and stored at −20°C for SS determination.

Starch was extracted from the ethanol-insoluble pellet after ethanol was first removed by evaporation. The ST in the residue was then released by boiling in 2 ml distilled water for 15 min. After cooling to room temperature, 2 ml of 9.2 M $HClO_4$ was added, and the mixture was shaken for 15 min. About 4 ml of distilled water was then added, and the mixture was centrifuged at 4,000 rpm for 5 min. A further extraction was carried out with

2 ml 4.6 M $HClO_4$. The supernatant was also retained, combined, and stored at −20°C for ST determination.

The soluble sugar and ST determination were performed based on the absorbance at 625 nm using the same anthrone reagent in a spectrophotometer (Yemm and Willis, 1954). Sugar concentration was calculated from the regression equations based on glucose standard solutions and ST concentration by multiplying glucose concentration with a conversion factor of 0.9 (Osaki et al., 1991).

Data Analysis

Normality and variance homogeneity requirements were met, and no data transformation was necessary. The effects of drought intensity, soil substrate, and root order as fixed factors on the fine root NSC concentration and morphological traits were tested using the three-way ANOVA by SPSS 19.0 (IBM Corp., Armonk, NY, United States). The differences in fine root traits of seedlings between different treatments were examined (Turkey's test, $\alpha = 0.05$); redundancy analysis (RDA) was performed on the fine root traits of seedlings under different treatments using the Canoco software (Version 4.56, Biometris Plant Research International, Wageningen, Netherlands). The Monte Carlo test was performed on the parameters in the RDA using R software (vegan package) (R Core Team, 2018). A multiple linear regression analysis was performed using the Sigmaplot 12.5 software (Systat Software Inc., San Jose, CA, United States) to analyze the influence of root traits on the fine root NSC (SS and ST) content in all treatments. All data are mean ± SE. All bar figures were drawn using Origin Pro 8.5 (OriginLab, Northampton, MA, United States).

RESULTS

Fine Root Biomass and Morphological Traits Among Root Branch Orders

Soil substrates, drought intensity, and root order had significant effects on the fine root biomass of *F. mandshurica* seedlings (**Supplementary Table 2**). With the increase in drought intensity, the fine roots biomass in the three substrates showed a progressive decrease (**Figures 1A–E**). The biomass of fifth-order roots had the highest coefficient of variation (among different drought intensities), which was 37.4, 44.5, and 53% in humus, loam, and sandy-loam soil, respectively (**Figure 1E**). Under the same drought treatment, the fine root biomass with all branch orders was the highest in sandy-loam soil and the lowest in humus soil. With the increase in drought intensity, the coefficient of variation of the fifth-order root biomass among different soil substrates was the highest, which was 61.8% (in CK), 36.6% (in T1), 57% (in T2), and 40.4% (in T3) (**Figure 1E**).

Soil substrate, drought intensity, and root order had significant effects on the SRL, SRA, and RTD of *F. mandshurica* seedlings (**Supplementary Table 2**). Under the same drought intensity, the SRL and SRA of all root branch orders in the humus soil were the lowest and showed a significant decrease with increasing root order (**Figures 2A–F**). The average diameter (AD) and RTD of all root branch orders were the highest and lowest in the humus and sandy-loam soil, respectively, and showed a significant increasing trend with ascending root order (**Figures 2G–I,J–L**). With the increase in drought intensity, the SRL and SRA of seedlings for all soil substrates increased significantly. Compared with CK, the SRL and SRA under T3 treatment increased significantly by 39% (variation range 19.1–88.6%) and 22.3% (variation range 9.9–37.4%), respectively. Compared with CK, the RTD of seedlings in all three soil substrates was the lowest under T3 treatment, especially in the 1st-root order, which decreased by 13.7% (humus soil), 10.7% (loam soil), and 28.6% (sandy-loam soil) (**Figure 2J**). The AD of all root branch orders was less affected by drought intensity (**Figures 2G–I** and **Supplementary Table 2**).

Fine Root Chemical Traits Among Root Branch Orders

The fine root carbon, nitrogen, and phosphorus contents of *F. mandshurica* seedlings were significantly different among different root branch orders. With increasing the root order, the fine root carbon and nitrogen contents showed a decreasing trend, whereas the fine root phosphorus content showed an increasing trend (**Supplementary Table 3** and **Figures 3A–F**). The soil substrate had a significant effect on the fine root carbon and nitrogen contents of *F. mandshurica* seedlings (**Supplementary Table 3**). The carbon and nitrogen contents of all root branch orders in sandy-loam soil were significantly higher than those of humus soil. The carbon and nitrogen contents of 1st-order roots in sandy-loam soil were the highest, which were 412.1 and 2.14 mg g^{-1}, respectively (**Figures 3A,B**). The drought had a significant effect on the fine root carbon and phosphorus content of *F. mandshurica* seedlings, and the phosphorus content of the first fourth root order under the T3 treatment was lower than that of the CK (**Figure 3F**).

Fine Root Non-structural Carbohydrate Content Among Root Branch Orders

Soil substrate, drought intensity, and root order had significant effects on the SS, ST, and total NSC content of the fine roots of *F. mandshurica* seedlings (**Supplementary Table 4**). Under the same drought treatment, the SS content of seedlings in humus soil was the highest for all root branch orders. With ascending root order, the SS content of fine roots of seedlings in humus soil was 107.9, 162.7, 125.7, 269.2, and 118.5% higher than those of sandy-loam soil, respectively (after the average intensity of the four droughts) (**Figures 4A–E**). In all soil substrates, the SS content of the fine roots decreased with the increasing drought intensity. With increasing root order, the SS content of fine roots under the T3 treatment was 51.3, 58.1, 62.1, 68.7, and 36.5% lower than that of CK, respectively (after the average of the three soil substrates) (**Figures 4A–E**). Under the same drought intensity, seedlings in sandy-loam soil had the highest ST and total NSC contents for all root orders. The ST and total NSC contents of lower-order roots in different soil substrates varied greatly. The ST and total NSC content of the 1st-order and 2nd-order roots in sandy-loam soil were 276.1, 231.1, 195.8, and 186.7% higher than those of humus soil, respectively (after averaging the intensity

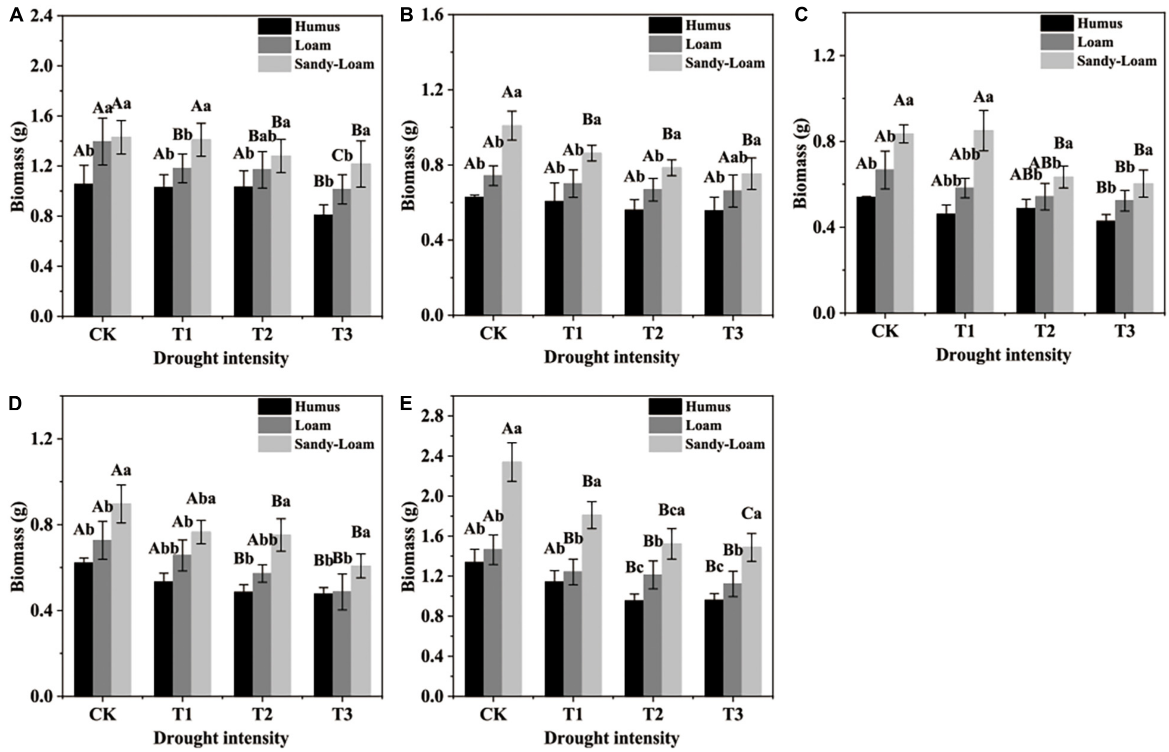

FIGURE 1 | The fine root biomass of *Fraxinus mandshurica* seedlings in different drought intensities and soil substrates. The different lowercase letters denote significant differences among soil substrates ($p < 0.05$). The different uppercase letters denote significant differences among drought intensities ($p < 0.05$). **(A)** 1st-order root; **(B)** 2nd-order root; **(C)** 3rd-order root; **(D)** 4th-order root; **(E)** 5th-order root. CK: control; T1: mild drought; T2: moderate drought; T3: severe drought.

of the four droughts) (**Figures 4A–E**). In all soil substrates, the fine root ST content generally increased with increasing drought intensity. The ST and total NSC contents of the 1st-order roots under the T3 treatment were 58.6 and 43.4% higher than those of the CK, respectively (**Figure 4A**).

Relationship Among the Fine Root Biomass, Traits, and Non-structural Carbohydrate Content

The root morphological and chemical traits of the first five orders of roots of *F. mandshurica* seedlings under different drought intensities and soil conditions were analyzed by RDA, and the results showed that the first two axes of the RDA explained approximately 65% of the total variation between all treatments (**Figures 5A–E**). The first and second ordination axes indicated the variations in fine root morphological traits, chemical traits, and biomass, respectively. Soil substrates and drought intensity in plots had a good degree of separation. The fine root morphological and chemical traits under all treatments were subjected to partial Monte Carlo tests. For the 1st-order roots, SS and ST exhibited the highest degree of the total variation in fine root traits, which were 32 and 32.1%, respectively (**Supplementary Table 5**). With increasing root order, the variation in root traits under ST decreased only 6.8% (for 5th-order roots) (**Supplementary Table 5**).

The Pearson correlation analysis showed that under different soil substrates and drought treatments, the first five orders of root morphological and chemical traits were significantly correlated with SS, SRL, and SRA were significantly negatively correlated with SS, whereas the AD and RTD were significantly positively correlated with SS (**Figures 6A–D**).

DISCUSSION

Our results highlighted several key findings related to NSC and fine root traits of *F. mandshurica* seedlings under different soil substrates and drought intensities. First, with the increase of drought intensity, the fine root biomass decreased significantly. Under the same drought intensity, there was higher biomass in relatively barren sandy-loam soils, and the coefficient of variation for fifth-order roots was higher than those of lower-order roots. Second, compared with chemical traits, the morphological traits of fine roots were more sensitive to soil substrates and drought intensities. With increasing drought intensity, the SRL and AD of all root orders increased and decreased, respectively. Finally, the fine roots in the humus soil had higher ST content and lower ST content. With the increase of drought intensity, the SS and ST content of the fine roots showed decreasing and increasing trends, and the NSC content of the fine roots significantly correlated with root morphological traits. The variation in fine

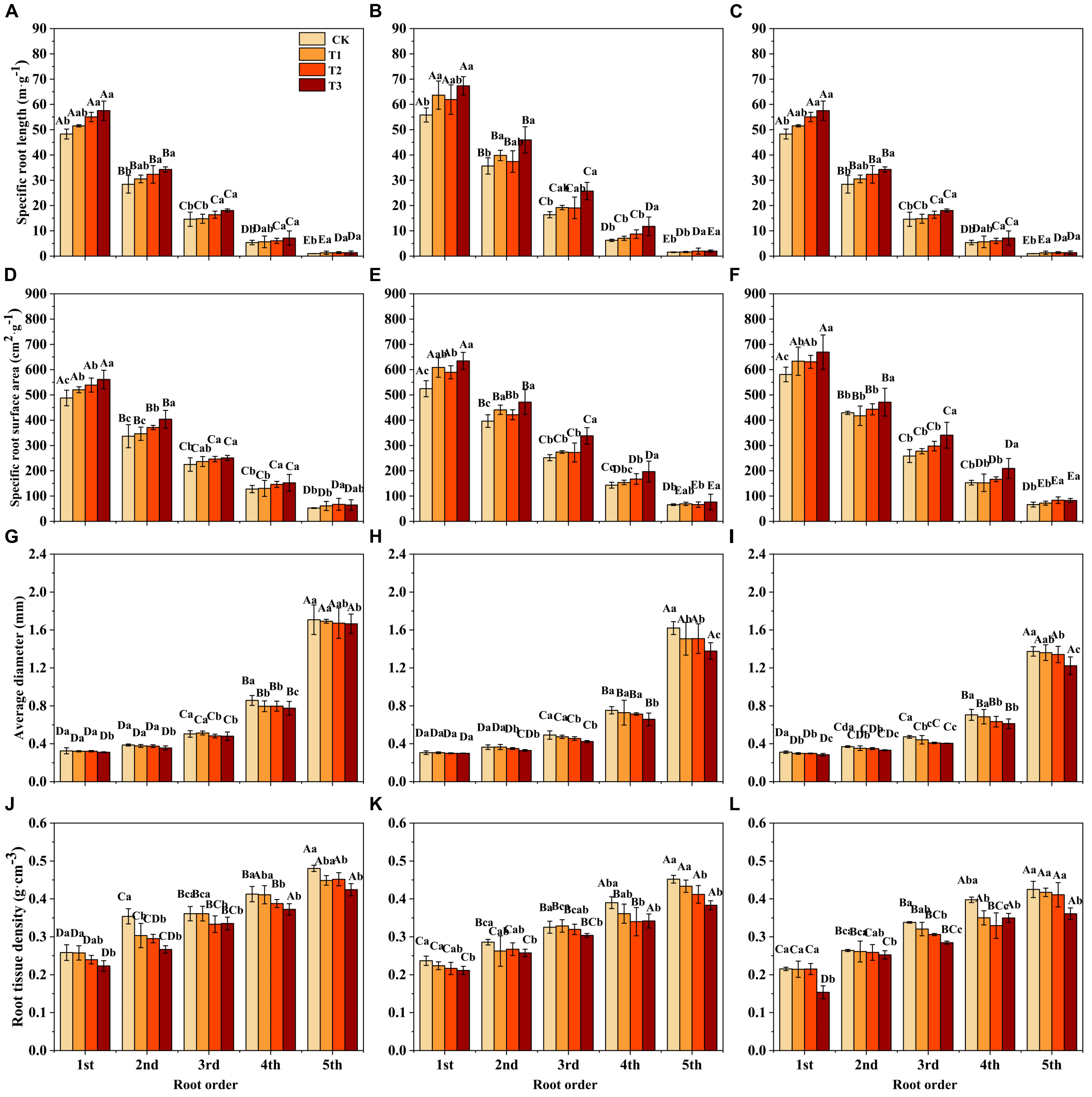

FIGURE 2 | The specific root length (SRL) **(A–C)**, specific root surface area (SRA) **(D–F)**, the average diameter (AD) **(G–I)**, and root tissue density (RTD) **(J–L)** of *Fraxinus mandshurica* seedlings in different drought intensities and soil substrates. The different lowercase letters denote significant differences among drought intensities ($p < 0.05$). The different uppercase letters denote significant differences among root branch order ($p < 0.05$). **(A,D,G,J)** Humus soil; **(B,E,H,K)** loam soil; **(C,F,I,L)** sandy-loam soil; CK: control; T1: mild drought; T2: moderate drought; T3: severe drought.

root NSC content in different soil conditions was supported to some extent by the plasticity of root morphology.

Response of Root Morphology and Biomass to Drought and Soil Substrate

In this study, the fine root biomass of seedlings in sandy-loam soil (soil nutrient relatively poor) was significantly higher than that in humus soil (soil nutrient relatively rich), which was consistent with those of previous studies (Hertel et al., 2013; Poorter and Ryser, 2015). Weemstra et al. (2017) demonstrated that the fine root mass and the growth rate of fine roots of *Fagus sylvatica* and *Picea abies* in sandy soil were three times and 10 times higher than those of clay soil, respectively. In response to increased nitrogen availability, Hendricks et al. (1993) proposed two hypotheses for fine roots: (1) a decrease in carbon allocation and an unchanged turnover rate, or (2) an unchanged carbon allocation and the increase in the turnover rate. For both cases, a decrease in fine root biomass was expected. Liu et al. (2020) found that the higher-order root biomass of *Pinus tabuliformis* seedlings under the 20% field water holding capacity treatment was 33.3% lower than that under the 80% field water holding capacity treatment. It was

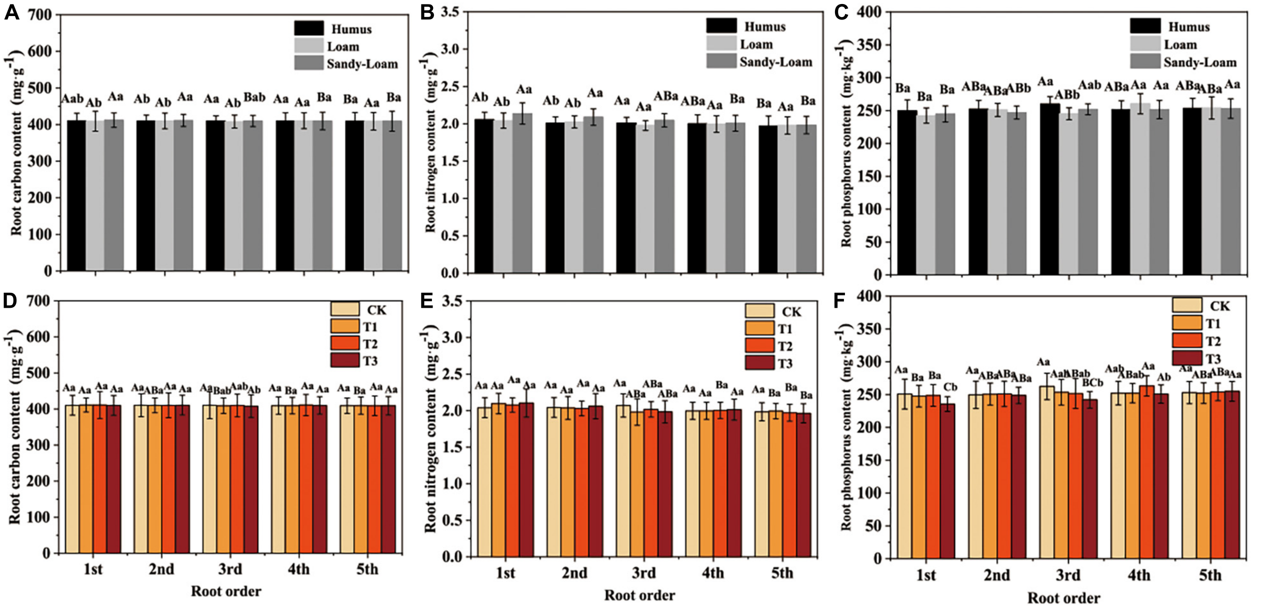

FIGURE 3 | The root carbon **(A,D)**, nitrogen **(B,E),** and phosphorus **(C,F)** of *Fraxinus mandshurica* seedlings in different soil substrates **(A–C)** and drought intensity **(D–F)**. The different lowercase letters denote significant differences among drought intensities ($p < 0.05$). The different uppercase letters denote significant differences among root branch order ($p < 0.05$).

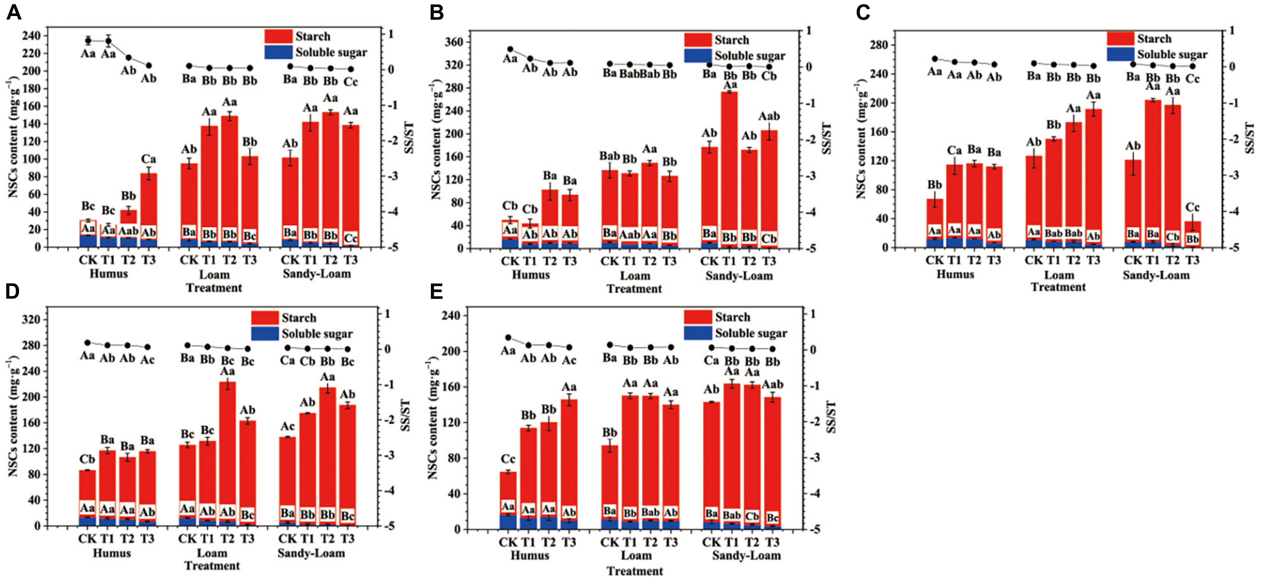

FIGURE 4 | The fine root non-structural carbohydrate (NSC) content of *Fraxinus mandshurica* seedlings in different drought intensities and soil substrates. The histogram represents the soluble sugar (SS) and starch (ST) content, and the line chart represents the SS-ST ratio (SS/ST). The different lowercase letters denote significant differences among soil substrates ($p < 0.05$). The different uppercase letters denote significant differences among drought intensities ($p < 0.05$). **(A)** 1st-order root; **(B)** 2nd-order root; **(C)** 3rd-order root; **(D)** 4th-order root; **(E)** 5th-order root. CK: control; T1: mild drought; T2: moderate drought; T3: severe drought.

found that the biomass of higher-order roots (e.g., fifth-order) had the highest degree of variation under different soil substrates and drought treatments, which was due to the different responses of fine roots of different root sequences to resource changes (Withington et al., 2006; Guo et al., 2008).

The morphological traits of fine roots, such as AD, SRL, and RTD, are important functional parameters that characterize or affect the water absorption efficiency and ability of roots. Generally, fine roots with smaller diameters and larger root lengths have higher water absorption efficiency

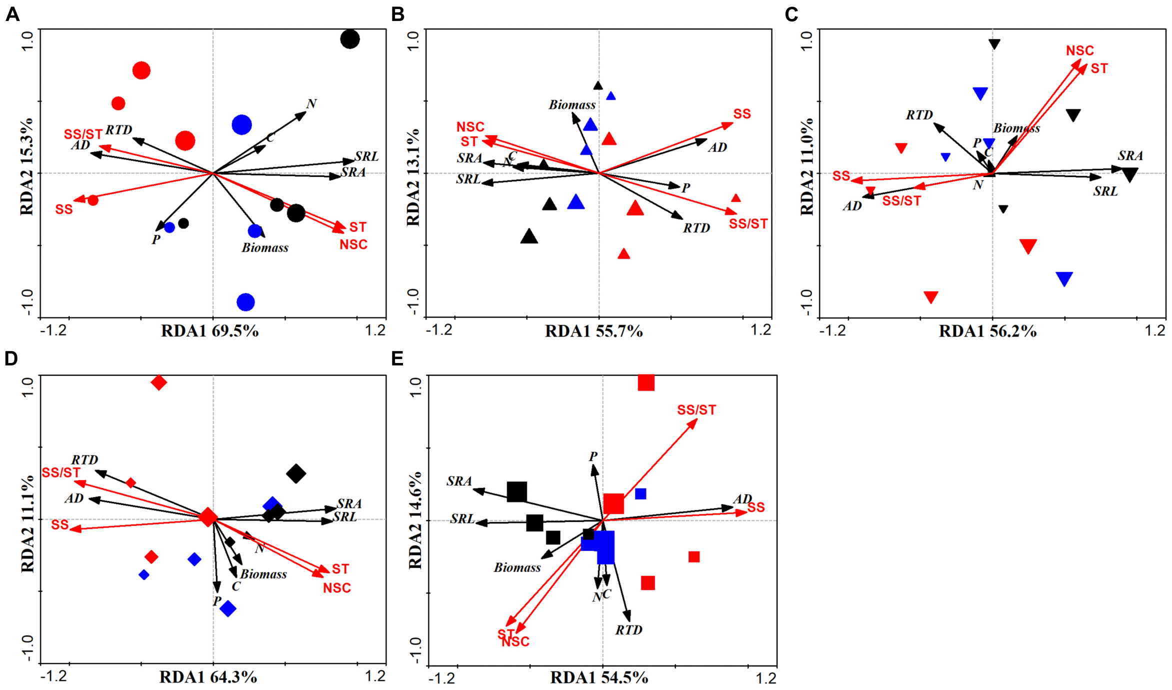

FIGURE 5 | Redundancy analysis of fine root morphological and chemical traits in different drought intensity and soil substrates. **(A)** 1st-order root; **(B)** 2nd-order root; **(C)** 3rd-order root; **(D)** 4th-order root; and **(E)** 5th-order root. Red symbols, humus soil; blue symbols, loam soil; black symbols, sandy-loam soil. SRL: specific root length; SRA: specific root surface area; AD: average diameter; RTD: root tissue density; SS: soluble sugar; ST: starch; C: root carbon; N: root nitrogen; P: root phosphorus. The greater symbols indicate the stronger drought intensity.

(Freschet and Roumet, 2017; Dhiman et al., 2018; Ma et al., 2018). Root morphological characteristics responded to drought here in accordance with our first hypothesis as well, that is the root system of seedlings in humus soil had lower SRL, lower SRA, higher AD, and RTD, which is consistent with the research of other scholars on the fine root traits of *Pinus tabulaeformis*, *L. olgensis,* and *F. mandshurica* with increased nutrient availability (Liu et al., 2009; Wang et al., 2013). Gruber et al. (2013) believed that soil nitrogen deficiency usually promoted the elongation of main roots and some lateral roots. Plants can increase the absorption capacity of the root system in two different ways to adapt to water or nutrient shortages, (1) increase root yield and maintain a larger absorption surface area (acquisition strategy), or (2) increase the efficiency per unit mass by changing the morphology and physiological condition of the root system (conservative strategy) (Lõhmus et al., 2006; Ostonen et al., 2007). In this study, with the increase of drought intensity, the SRL and SRA of the first five order roots increased significantly, and the RTD decreased significantly, this is a manifestation of *F. mandshurica* seedlings coping with water-deficit conditions. In addition, the results showed that the differences in various morphological indicators between root orders had reached a significant level, indicating that the fine roots of *F. mandshurica* had a high degree of morphological heterogeneity, which was consistent with the previous study of the plasticity of the morphological traits of the root system from the perspective of orders (Pregitzer et al., 1997, 2002; Wang et al., 2006). Cortina et al. (2008) found that *Pistacia lentiscus* seedlings could rapidly expand the length and surface area of fine roots when exposed to drought stress, and shaped fine roots to avoid damage to arid environments. Generally, the larger the SRL or SRA and the smaller the AD, the higher the water absorption efficiency of fine roots (Freschet and Roumet, 2017; Dhiman et al., 2018). The results of this study emphasized the adaptation strategies of the fine roots of *F. mandshurica* seedlings in three soil substrates to different drought intensities.

Effects of Drought and Soil Substrate on Root Non-structural Carbohydrate

Soluble sugar is an important osmotic adjustment substance for plants to tolerate arid environments. It can reflect the drought status of plants. Changes in its concentration can adjust the osmotic pressure of cells in plants to maintain normal physiological activities to adapt to drought stress (Quentin et al., 2015). In this study, compared with sandy-loam soil, the SS content of fine roots (especially lower-order roots) in humus soil was significantly increased, the ST was significantly decreased, the NSC was significantly reduced, and the SS-to-ST ratio was significantly increased. The development of fine roots accelerates the consumption of NSCs, and the roots decrease the ratio of SS to ST to cope with stress conditions. The increase in the ratio is beneficial for plants to adjust osmotic potential to maintain the transport channel between leaves and roots, and improve water

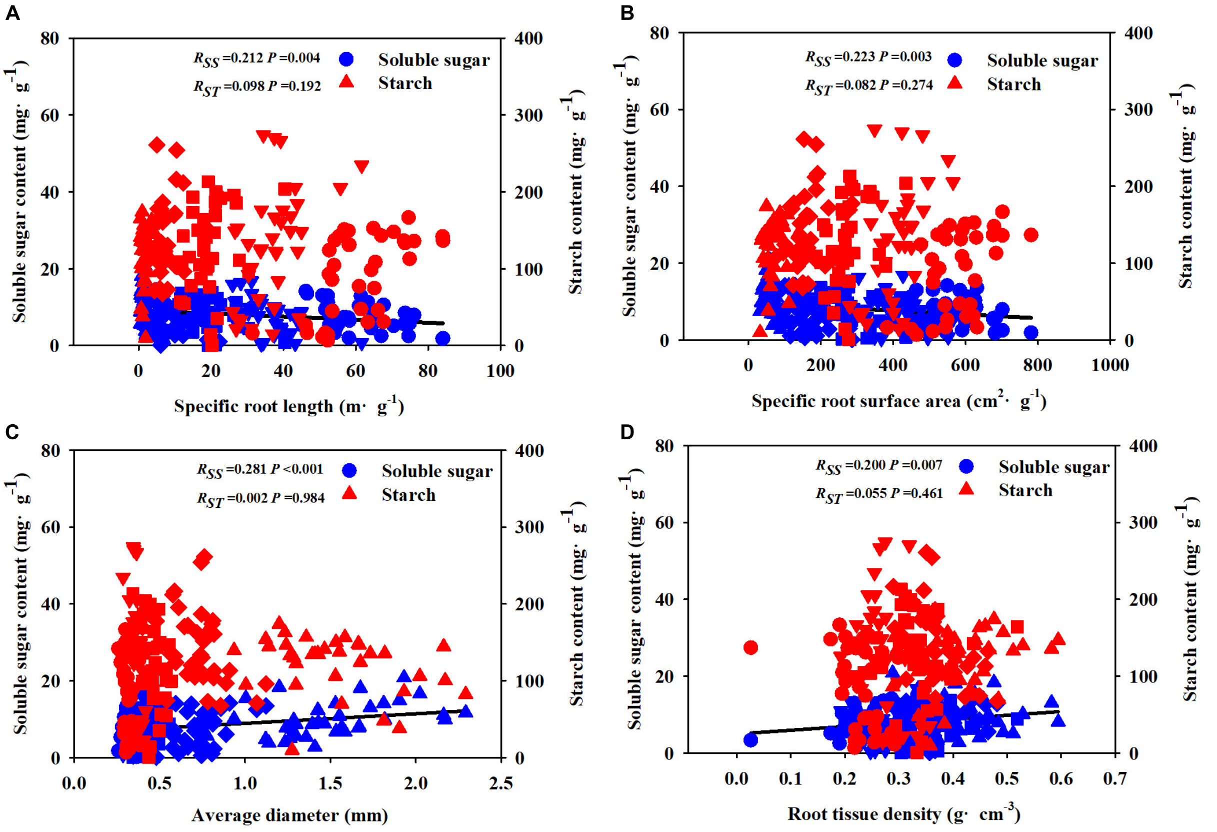

FIGURE 6 | Relationships between soluble sugar, starch concentration, and fine root morphological traits [SRL **(A)**, SRA **(B)**, AD **(C)**, and RTD **(D)**] during the whole experiment period. R: correlation coefficient; SS: soluble sugar; ST: starch; SRL: specific root length; SRA: specific root surface area; AD: average diameter; RTD: root tissue density; circle represents 1st-order root; down-triangle represents 2nd-order root; square represents 3rd-order root; diamond represents 4th-order root; up-triangle represents 5th-order root.

transport efficiency (Sala et al., 2012). Some studies have pointed out that lower-order roots may obtain greater carbon investment than higher-order roots because the main function of lower-order roots is to absorb water and nutrients (absorptive roots), whereas higher-order roots are responsible for transporting nutrients and supporting the entire root (Liu et al., 2020). The results showed that after 2 months of drought, the lower-order roots of *F. mandshurica* seedlings had more NSC content than the higher-order roots. This result implied that in the case of limited carbon, *F. mandshurica* preferentially allocated carbon to thin roots rather than thick roots, which was similar to the research results in hybrid poplar *Populus* × *canadensis* cv. *Eugeneii* (Kosola et al., 2001) and *P. tabuliformis* (Liu et al., 2020). Di Iorio et al. (2016) found that the ST content of very fine root (<0.5 mm) of *F. sylvatica* saplings in warming and drought treatment was significantly higher than that in warming conditions. Under carbon-limiting conditions, plants prioritize the distribution of fine roots. They could reduce carbon consumption (higher-order roots consume more carbon than lower-order roots). However, maintaining fine roots could absorb more water and nutrients (Finér et al., 2011). Liu et al. (2020) conducted short-term drought stress on *P. tabuliformis* seedlings and found that the amount of ^{13}C allocated to the first three roots 120 days after isotope labeling was significantly higher than that of the control in the moderate and mild drought, whereas the amount of ^{13}C allocated to fifth-order roots was significantly higher in the control. Some studies have suggested that carbon starvation caused by drought stress may only exist in belowground organs and has little to do with the aboveground parts. Specifically, thick roots are essential to alleviate the decline in the NSC content of the entire plant caused by drought (Hartmann et al., 2013; Kannenberg et al., 2018), and drought will cause the loss of phloem function, making the aboveground and underground parts uncoupled. When plants are subjected to drought stress, the NSC content of the aboveground organs will increase or remain stable for a short time, whereas the NSC content of the roots will decrease significantly (Sevanto et al., 2014).

Galvez et al. (2011) found that drought significantly increased the concentrations of SS and ST in the fine roots of *Populus tremuloides* seedlings. The results of this study supported the second hypothesis, with the increase in drought intensity, the variation in root NSCs was higher than that of root chemical traits, root SS content decreased, and ST and total NSC contents increased. A possible explanation for this might be that the path of NSCs to the fine roots of *F. mandshurica* seedlings was blocked after 2 months of drought stress, causing the carbohydrates produced by leaf photosynthesis unable to transport to the belowground organs. Fine roots could only rely on regulating their own NSC levels to cope with drought stress (Dietze et al., 2014). However, the inference presented in this study needs to be further verified by combining the dynamic changes in fine root NSC content under different periods of drought treatment. The results revealed the interaction of different soil substrates and drought intensities on the variations in the first five root order NSCs of *F. mandshurica* seedlings.

Linking the Fine Root Traits to the Non-structural Carbohydrate Level

The root system is the organ responsible for absorbing water and is the first responder to a variety of stresses. Because roots tend to grow in moist soil, the impact of water shortages can be minimized (Brunner et al., 2015; Weemstra et al., 2016). Under drought stress conditions, more carbohydrates are allocated to lower-order roots to promote root structure and growth (Liu et al., 2020). Although there is evidence that thick roots increase NSC accumulation under drought conditions (Yang et al., 2016), whether the variation in fine root traits is directly related to NSC accumulation remains to be explored. Previous research revealed that the root tip length and diameter of the seedlings (three broad-leaved tree species in the temperate zone) under drought conditions explained the higher variation in ST and SS contents (Ji et al., 2020). In this study, drought reduced the root biomass of *F. mandshurica* seedlings, changed the root traits of all root orders, and promoted the absorption of water and nutrients. This response may be related to the increased demand for osmotically active C compounds under drought conditions. NSCs can provide fuel for root respiration and are an important substrate for root growth and physiological regulation (George et al., 2003; Xu et al., 2008). In this study, SRL and SRA of all samples were significantly negatively correlated with the SS content of fine roots, and AD and RTD were significantly positively correlated with the ST content, which confirms the third hypothesis. When the root diameter is smaller, the level of NSC required to build and maintain a thin root per unit length of the plant would be lower than that of the thicker root (Guo et al., 2004). Ma et al. (2018) pointed out that the root diameter of herbaceous plants was thinner than that of woody plants, so more carbon could be easily distributed to the roots under drought conditions. Maguire and Kobe (2015) pointed out that drought stress may directly increase root mortality by depleting ST and SS reserves and indirectly inhibit the transport of photosynthetic products to roots (Hasibeder et al., 2014). Therefore, the results suggested that changes in NSCs caused by changes in environmental conditions were related to the variation in fine root morphological traits.

CONCLUSION

This project was the first comprehensive investigation of the interactive response of root branch order and fine root NSC levels of *F. mandshurica* seedlings to drought intensity and soil substrate. The results confirmed that the variation in higher-order fine root biomass was higher than that in lower-order roots under different soil substrates and drought intensities. Second, the fine roots of the seedlings in the humus soil (first 5th root order) had a higher SS content and lower ST content. With increasing drought intensity, the SS and ST contents of the fine roots showed decreasing and increasing trends, respectively. The variation in fine root NSC content was related to the variation in root morphological traits (SRL, SRA, AD, and RTD) induced by drought and soil substrate rather than root chemical traits. This study reveals the adaptation strategies of *F. mandshurica* seedlings to drought under different soil substrate conditions, thereby enhancing the understanding of the construction and maintenance of the root system of *F. mandshurica*, and contributing to optimizing soil water management in *F. mandshurica* plantations. In further research, it is necessary to combine ^{13}C-isotope-labeling technology to more deeply reveal the mechanism of carbohydrate distribution among different root branch orders under prolonged periods of drought.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LJ and YY conceptualized the main question. LJ, JW, and ZL conducted the fieldwork. LJ and YL collected data and performed the data analyses. LJ wrote the manuscript. LJ, YY, and LZ revised the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.692715/full#supplementary-material

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