Salicaceae endophyte inoculation alters root biomass allocation and architectural traits in *Populus* across nitrogen environments

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Abstract

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Background and Aims

Salicaceae endophytes have demonstrated capacity to promote growth in *Populus*, and several strains are diazotrophic, yet their effects on root functional and architectural traits remain poorly characterized. Understanding how these endophyte inoculation and nitrogen regimes shape below-ground functional traits is critical for evaluating their potential as biological inoculants in nitrogen-limited plant production and bioenergy systems. We hypothesized that endophyte inoculation would reduce root-to-shoot ratio and shift biomass allocation toward coarse roots, consistent with relaxed nitrogen foraging if the dominant mechanism were alleviation of nitrogen limitation. Because many of these endophytes also produce auxin, we further considered an alternative, nitrogen-independent prediction in which inoculation directly stimulates root proliferation and deeper rooting irrespective of nitrogen supply. We therefore evaluated root architectural responses across contrasting nitrogen environments to distinguish these competing expectations.

Methods

Three complementary experiments were conducted using tissue cultured *Populus* plants. Two pot-based experiments examined biomass allocation and root functional class responses to endophyte inoculation. A hydroponic experiment using a rhizobox imaging system and image-based phenotyping with RhizoVision Explorer was conducted across two independent experimental rounds to characterize root system architectural responses. Twelve architectural traits were

extracted from final harvest images and analyzed using two-way ANOVA with experimental round and block as fixed covariates.

Key Results

Endophyte inoculation significantly increased net photosynthetic rate, total plant biomass, and coarse root biomass under low nitrogen, while reducing root-to-shoot ratio across contrasting nitrogen environments, indicating effects that were not solely dependent on nitrogen availability. The same nitrogen-independence was evident in the rhizobox experiment, where inoculated plants developed deeper, more extensive, and more vertically oriented root systems than mock-inoculated controls regardless of nitrogen level. Across both growth systems, therefore, inoculation shifted plants toward greater root growth and altered biomass allocation independently of nitrogen supply. Principal component analysis of shoot and root functional traits showed that endophyte inoculation drove a coordinated whole-plant transition from nitrogen acquiring toward biomass-accumulating phenotypes, explaining 60.9% of total trait variance along a single multivariate axis.

Conclusions

Our results indicate that inoculation with Salicaceae endophyte consortia can alter root architectural traits and shift above- and below-ground carbon partitioning in *Populus*. The inoculation-driven reduction in root-to-shoot ratio was statistically consistent across high and low nitrogen, with no significant interaction. This pattern aligns more closely with the alternative, nitrogen-independent prediction we proposed. Because nitrogen fixation was not directly measured, this distinction remains an inference. These findings position the endophyte consortia as candidates for improving performance in *Populus* production systems where nitrogen inputs are constrained. Future studies are recommended to examine endophyte colonization persistence under field soil conditions, quantify nitrogen fixation and phytohormone contributions in planta to determine whether the responses documented here are reliably expressed at ecologically relevant scales.

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1 Introduction

The global transition toward renewable energy has increased interest in lignocellulosic bioenergy crops such as *Populus*, which are valued for their rapid growth, ease of propagation, and adaptability across diverse environments (Taylor, 2019; Stanton *et al.*, 2002; Tuskan, 1998). However, sustaining high productivity in these systems is constrained by nitrogen availability, which is frequently the most limiting nutrient in both natural and managed poplar systems *(Gan et al.*, 2016; Taylor, 2025). Conventional reliance on synthetic nitrogen fertilizers improves yield but incurs substantial ecological and economic costs (Li, 2024; Namuhan *et al.*, 2024; Taylor & Menge, 2021), including greenhouse gas emissions, nutrient leaching, and soil degradation (Galloway *et al.*, 2003; Bowen *et al.*, 2020; Wang *et al.*, 2024). These challenges highlight the need for sustainable alternatives to support nitrogen acquisition in bioenergy crops (Knoth *et al.*, 2014; Doty, 2016; Taylor & Menge, 2021; Holland *et al.*, 2023; Sher *et al*., 2024a).

Plant-associated microorganisms offer promising pathways to reduce dependence on external nitrogen inputs (Koch & Sessitsch, 2024; Zhao *et al*., 2024). In particular, Salicaceae endophytes, microbes capable of fixing atmospheric nitrogen within plant tissues, have been identified in *Populus* and shown to enhance growth, biomass accumulation, and nitrogen use efficiency under nutrient-limited conditions (Doty *et al*., 2009; Xin *et al*., 2009a; Doty, 2011; Khan *et al*., 2012; Knoth *et al*., 2013; Knoth *et al*., 2014; Moyes *et al*., 2016; Doty, 2016). In addition to nitrogen fixation by some of the members of these microbe communities, Salicaceae endophytes can promote host growth through other mechanisms, including phytohormone production, making them relevant for improving productivity in low-input systems. Despite growing evidence of endophyte-mediated benefits, most research has focused on aboveground responses or nitrogen

dynamics (Philippot *et al*., 2013), with limited attention to how plant root systems structurally and functionally respond to endophyte colonization (Lynch *et al*., 2023). Because roots are the primary organs for resource acquisition, understanding their response is critical for linking microbial interactions to whole-plant performance.

Root system architecture (RSA) is a highly plastic trait that integrates intrinsic developmental programs with environmental signals, allowing plants to adjust root growth, branching, and spatial distribution in response to resource availability (Ingram & Malamy, 2010). Under nitrogen limitation, plants often modify RSA by increasing root allocation (root to shoot ratio), enhancing branching, and altering root spatial deployment to improve nutrient foraging efficiency (Gonin *et al*., 2023; King *et al*., 2021; Lynch *et al*., 2023). These allocational & architectural adjustments are closely linked to functional differentiation within the root system. Functional differentiation within root systems is well established, with fine roots specializing in nutrient and water uptake and exhibiting high plasticity, while coarse roots primarily provide transport, storage, and structural support (Butler *et al*., 2010; Van Do *et al*., 2016; Freschet *et al*., 2021). This plasticity is particularly evident in *Populus*. Under nitrogen starvation, *Populus* roots increase primary and lateral root elongation and lateral root proliferation (Wei *et al*., 2013). Fine root length and surface area increase substantially relative to plants under normal nitrogen supply (Luo *et al*., 2015). Conversely, fine root proliferation in poplar plantations is promoted by nitrogen addition only when soil nitrogen is low, and declines once soil nitrogen becomes abundant (Yan and Dai, 2019), illustrating that fine roots adjust their growth bidirectionally in response to nitrogen availability. Shifts in biomass allocation among these functional classes represent a key mechanism by which plants balance the carbon cost of root construction against the benefit of acquiring the most

limiting resource, allowing them to sustain nutrient and water uptake under environmental stress (Butler *et al*., 2010; Adamek *et al*., 2011; C. Wang *et al*., 2012; Gao *et al*., 2024; Van Do *et al*., 2016; Freschet *et al*., 2021). However, whether Salicaceae endophyte inoculation influences these allocation patterns and associated architectural traits remains largely unexplored. This knowledge gap is particularly pronounced in *Populus*, where empirical evidence linking Salicaceae endophyte inoculation to shifts in root functional and architectural traits is still limited. This gap constrains our understanding of how endophytes may influence belowground architecture, resource acquisition strategies, and plant performance under varying nitrogen availability.

Many Salicaceae endophytes produce the phytohormone auxin (indole-3-acetic acid, IAA), a primary regulator of root initiation, elongation, and lateral root development. Several strains isolated from wild *Populus* and *Salix* produce substantial quantities of IAA in vitro (Xin *et al*., 2009a, Xin *et al*., 2009b; Khan *et al*., 2012). Auxin secretion has been described as one of the most common mechanisms by which these endophytes stimulate host root growth (Khan *et al*., 2012). Importantly, nitrogen fixation and auxin production may affect root growth in different ways. While alleviating nitrogen limitation would be expected to reduce the root foraging response typically observed under nutrient stress, auxin production could directly stimulate root growth regardless of nitrogen availability. The net effect of endophyte inoculation on the root system may therefore reflect the combined and potentially counteracting influence of improved nitrogen nutrition and auxin-mediated root stimulation.

In this study, we investigated how endophyte inoculation and nitrogen availability shape (i) whole-plant biomass allocation, including root-to-shoot partitioning, (ii) the distribution of biomass

among root functional classes, and (iii) root system architectural plasticity in *Populus*. To achieve these, we conducted 3-complementary experiments: 2-pot experiments examining biomass allocation responses- one isolating the effects of endophytes alone, and one incorporating contrasting nitrogen treatments alongside endophyte inoculation, and a hydroponic experiment designed to characterize root system architectural traits through image-based phenotyping under controlled nutrient conditions. We hypothesized that endophyte inoculation would reduce the root-to-shoot ratio in *Populus* by alleviating nitrogen limitation, thereby reducing the plant's reliance on extensive root foraging. Because several Salicaceae endophyte strains are diazotrophic and may supply fixed atmospheric nitrogen to the host, we expect if nitrogen supplementation is the operative mechanism, this reallocation of carbon toward shoot tissue to manifest across contrasting nitrogen environments. We also hypothesized that this shift in biomass partitioning would be reflected in relatively reduced fine root biomass and greater investment in coarse roots, as relief from nitrogen stress typically downregulates the proliferation of fine roots, the primary organs of nitrogen acquisition, freeing carbon for structural and transport functions. Further, we hypothesized that endophyte inoculation would alter root architectural traits, including root length, tip number, branching intensity, and spatial distribution, given that nitrogen availability is a well-established driver of root architectural plasticity (Hodge, 2004; Gruber *et al*., 2013). Under nitrogen limitation, plants typically intensify localized root proliferation as a foraging response; endophyte-mediated improvements in nitrogen status are expected to relax this behavior, promoting a more evenly distributed root system. However, many of these endophytes are also known to produce auxin, a plant hormone that directly promotes root growth (Xin *et al*., 2009b). If auxin production is the primary mechanism, inoculated plants would be expected to develop larger and more extensive root systems regardless of nitrogen availability. This prediction

contrasts with the expectation that alleviating nitrogen limitation would reduce the need for root foraging under nutrient stress. Comparing plant responses across different nitrogen levels therefore provides some insight into whether the observed changes are more consistent with improved nitrogen availability or direct growth stimulation by endophytes. Because nitrogen fixation was not directly quantified in this study, these hypotheses test the predicted consequences of endophyte-mediated nitrogen supplementation rather than fixation itself, and the observed responses are interpreted in light of both nitrogen-mediated and auxin-mediated mechanisms.

2 Methods

Plant material and study overview

Three experiments were conducted at two University of Washington (Seattle) facilities. Pot experiment I was carried out in an indoor plant grow room in the School of Environmental and Forest Sciences, whereas pot experiment II and rhizobox experiment III were conducted in a controlled greenhouse at the Center for Urban Horticulture (CUH). Experiments I and II used tissue culture-propagated hybrid *Populus* 'H11-11' (n = 18 across both experiments; 8 in experiment I and 10 in experiment II), while experiment III used tissue culture-propagated *Populus trichocarpa* (Nisqually-1) (n = 17). The three experiments differed in their experimental factors, growth system, and measured traits, and are described separately below; Table 1 summarizes the key differences. Throughout this study, mock-inoculated plants are designated NI, plants inoculated with the Mix I consortium Mix I, plants inoculated with the Mix II consortium Mix II, and in experiment III, which used ten-strain consortium Mix III inoculated plants are designated EI and mock-inoculated plants NI.

Endophyte consortia and inoculum preparation

Two endophyte consortia were used in the pot experiments. The Mix I consortium comprised nine strains (eight bacteria and one fungus: 11R-A, 11R-BB, 11R-BC, PTD1, R10, SD1, WP1, WP5, and WW5), originally isolated from wild *Populus* and *Salix* species (**Table 2**). The Mix II consortium comprised ten strains: the nine Mix I strains plus HT1-2. Experiment III used a single ten-strain Mix III consortium (PTD1, WP5, WW5, R10, SD1, HT1-2, HT1-9, 11R-A, 11R-BB, and 11R-BC). Strains were selected on the basis of demonstrated nitrogen-fixing ability, capacity to enhance host productivity, and tolerance to abiotic stress. Annotated draft genomes have been deposited in NCBI GenBank under BioProjects PRJNA1058978, PRJNA247595, PRJNA247570, and PRJNA32711.

Table 1: Overview of designs and treatments used in each experiments

Experiments	Treatments	Replicates	Individual per replicate
Experiment I: Indoor-grown experiment	Mock-inoculated, Mix I-inoculated, Mix II -inoculated.	8	1
Experiment II: Greenhouse-grown experiment	Mock-inoculated- low N, Mock-inoculated- high N, Mix I- low N, Mix I- high N, Mix II- low N, Mix II- high N.	10	1
Experiment III: Rhizobox experiment	Mock-inoculated – low N, Mock-inoculated – high N, Mix III inoculated – low N, Mix III inoculated – high N.	18	1

Each strain was cultured individually in 10 mL MG/L medium (Cangelosi *et al*., 1991) without biotin and incubated overnight at 30°C with shaking. Cells were harvested by centrifugation (6,000 × g, 10 min, 4°C), washed, and resuspended in nitrogen-free medium (NFM; Doty *et al*., 2009). Each strain suspension was adjusted to OD_{600} = 0.1 with NFM, and equal volumes of all strains were combined to produce a final inoculum at OD_{600} = 0.1, ensuring equal representation of all strains. Prior to inoculation, tissue culture-propagated plants were established in Woody Plant Medium (Phytotech) supplemented with 1 μg mL^{-1} indole-3-butyric acid to promote rooting. Rooted apical cuttings of uniform developmental stage were rinsed in sterile deionized water, submerged in 100 mL of the consortium suspension, and co-cultivated for 4–5 days under sterile conditions before transfer to individual vessels containing NFM until transplanting. Control (NI) plants underwent an identical procedure using NFM in place of the consortium suspension.

2.1 Experiment I: Indoor-grown experiment

2.1.1 Experimental design

Experiment I was established in the plant grow room with 48 tissue culture-propagated hybrid *Populus* 'H11-11' plants assigned to one of three inoculation treatments (NI, Mix I, Mix II). Plants were transplanted into one-gallon HDPE nursery pots filled with a 6:4 (v/v) vermiculite:sand mixture pre-wetted with tap water. Following transplanting, pots were placed under a mist tent for one week to promote root establishment and then moved to the indoor bench, where plants acclimated for 14 days prior to the initiation of nitrogen treatments. Pots were arranged in a randomized complete block design with eight blocks, each containing one replicate per treatment, and each pot was placed on a plastic saucer to prevent cross-contamination of inoculation

treatments through fertigation run-off. A 14/10 h light/dark photoperiod was provided by supplemental lamps.

Table 2: List of endophyte strains used in the experiments and their membership within a consortium.

Strain	Endophyte	Host	Consortia	N fixer	Auxin producer	References
PTD1	*Rhizobium* sp.	*P. trichocarpa x deltoides*	Mix I, II, III	-	Yes	Doty *et al*. 2005
WP5	*Rahnella aceris*	*P. trichocarpa*	Mix I, II, III	Yes	Yes	Doty *et al*. 2009
WW5	*Sphingobium* sp.	*Salix sitchensis*	Mix I, II, III	-	Yes	Knoth *et al*. (2014)
R10	*Rahnella aceris*	*P. trichocarpa*	Mix I, II, III	Yes	Yes	Hendrickson *et al*., 2025
SD1	*Azotobacter* sp.	*P. trichocarpa*	Mix I, II, III	Yes	Yes	Hendrickson *et al*., 2025
HT1-2	*Sphingomonas* sp.	*Pluchea carolinensi*	Mix I, II, III	-	-	Doty, unpublished
WP1	*Rhodotorula*	*P. trichocarpa*	Mix I, II	-	Yes	Xin *et al*. (2009b)
11R-A	*Azospirllum* sp.	*P. trichocarpa*	Mix I, II, III	Yes	Yes	Sher *et al*. (2024b)
11R-BB	*Sphingobium* sp.	*P. trichocarpa*	Mix I, II, III	-	-	Sher *et al*. (2024b)
11R-BC	*Herbiconiux* sp.	*P. trichocarpa*	Mix I, II, III	-	-	Sher *et al*. (2024b)
HT1-9	*Azorhizobium sp. (novel)*	*Pluchea carolinensi*	Mix III	Yes	-	Sher *et al*. (2024b)

Although experiment I was initially designed as a two-factor (inoculation × nitrogen) study, the plants were inadvertently exposed to drought stress midway through the trial, resulting in leaf abscission and compromised performance in some of the high nitrogen plants. Therefore, all high-nitrogen plants were excluded from analysis, and experiment I was analyzed as a single-factor design testing the effect of inoculation under low nitrogen. The retained low-nitrogen plants comprised the three inoculation treatments arranged across the original randomized complete blocks, so that the reduced dataset remained a balanced, randomized, and replicated single-factor design suitable for one-way ANOVA. Randomization and blocking were established at the outset of the experiment and were unaffected by the exclusion of high-nitrogen plants, and the low-nitrogen plants received their intended nitrogen and watering regime throughout. However, the low- and high-nitrogen plants shared a common above-ground bench environment, and that the drought stress and subsequent leaf abscission experienced by the some of the high-nitrogen plants may have altered the local micro-environment in ways that could have influenced the retained low-nitrogen plants. Because experiment I is consequently interpreted as an inoculation experiment under low nitrogen rather than as a test of the inoculation × nitrogen interaction, and because its principal findings are corroborated by the independently conducted experiment II (which retained both nitrogen levels) and experiment III, we consider the single-factor results valid while recognizing this limitation.

2.1.2 Nitrogen treatment

Fertigation was initiated two weeks after transplanting using a modified Hoagland nutrient solution (**Table 3**). Low-nitrogen plants received a one-eighth-strength solution applied at 60 mL per pot twice weekly.

2.1.3 Growth and leaf physiology measurements

Plant height was recorded weekly throughout the experiment. At harvest, chlorophyll content was measured on the youngest fully expanded, light-exposed intact leaf using a SPAD meter (SPAD 502 Plus, Spectrum Technologies Inc., Plainfield, IL, USA), and leaf area was quantified with a leaf area meter (LI-COR 3100, LI-COR Biosciences, Lincoln, NE, USA). Specific leaf area (SLA; $cm^2\ g^{-1}$) was calculated as total leaf area divided by leaf dry mass. Net CO_2 assimilation rate (A_{net}) was measured using a portable photosynthesis system (LI-COR 6800; LI-COR, Lincoln, NE, USA) with a 6 cm^2 chamber under saturating light (1,500 µmol $m^{-2}\ s^{-1}$ PPFD), 400 ppm CO_2, 40–60% relative humidity, 20°C leaf temperature, and 500 µmol s^{-1} flow rate. For each plant, the chamber was clamped to the youngest fully expanded, light-exposed leaf (three to four leaves distal to the apical meristem), at the center of the lamina away from the midvein.

2.1.4 Harvest, biomass, and root fractionation

At harvest, plants were destructively sampled and separated into leaves, stems, and roots (Fig. 1). Root systems were further subdivided into fine and coarse fractions: roots ≤ 2 mm diameter lacking lignified tissue were classified as fine roots, and roots > 2 mm with woody, lignified structure as coarse roots (Freschet et al., 2021). All components were oven-dried at 70°C to constant mass and weighed to the nearest 0.001 g. Total plant dry mass was the sum of leaf, stem, and root mass; root-to-shoot ratio was calculated as total root dry mass divided by total above-ground (leaf + stem) dry mass; and fine-to-coarse root ratio was calculated from the fine and coarse root masses.

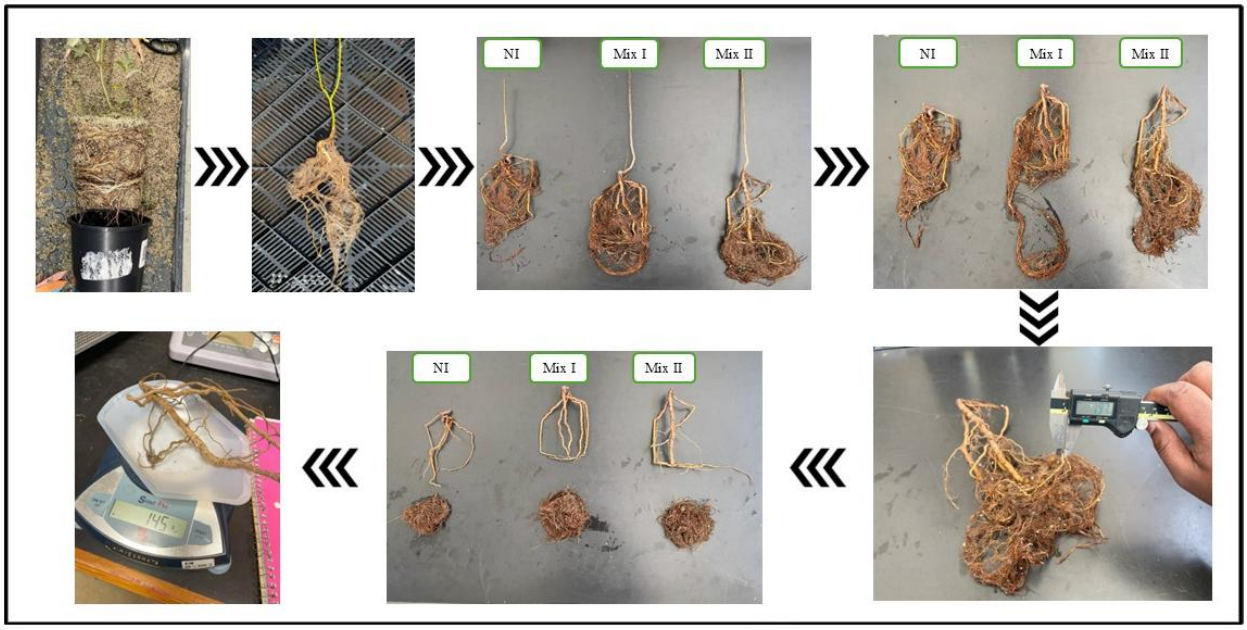

Fig. 1. Destructive harvest and root fractionation workflow in the indoor-grown experiment (Experiment I). At harvest, plants were removed from their pots and the root systems were separated from the substrate (top left and top centre). Whole root systems of representative mock-inoculated (NI) and endophyte-inoculated (Mix I, Mix II) plants are shown for comparison (top right). Root systems were then separated into fine and coarse fractions: roots ≤ 2 mm in diameter lacking lignified tissue were classified as fine roots, and roots > 2 mm with woody, lignified structure as coarse roots (Freschet *et al*., 2021); the separated coarse and fine fractions are shown for each treatment (bottom centre). Root diameter was measured with digital callipers to guide fraction assignment (bottom right). The separated root fractions were then oven-dried at 70 °C to constant mass and weighed to the nearest 0.001 g (bottom left).

2.1.5 Data analysis

Differences among inoculation treatments were assessed using one-way ANOVA, with pairwise comparisons among treatment means by Tukey's HSD test where ANOVA indicated significant or marginally significant effects. To examine multivariate relationships among shoot and root functional traits and the degree of phenotypic separation among treatments, principal component analysis (PCA) was performed on 17 morphological, physiological, and biomass traits measured

at final harvest (plant height, leaf number, leaf area, chlorophyll content, nodes per stem, phytomers per plant, SLA, net photosynthesis, stem weight, leaf weight, shoot weight, total root weight, coarse root weight, fine root weight, fine-to-coarse root ratio, root-to-shoot ratio, and coarse root-to-shoot ratio). Rows containing missing or non-finite values were excluded, and PCA was conducted on the correlation matrix using **princomp()** so that all traits contributed equally to the ordination. Biplot loading arrows were scaled by the product of each loading and the corresponding component standard deviation and adjusted to 85% of the maximum sample score radius; convex hulls were drawn per treatment using **stat_chull()** from the ggpubr package.

2.2 Experiment II: Greenhouse-grown experiment

2.2.1 Experimental design

Experiment II, carried out in the greenhouse at CUH, used 60 tissue culture-propagated hybrid *Populus* 'H11-11' plants assigned to the same three inoculation treatments (NI, Mix II, Mix II), crossed factorially with two nitrogen levels (high and low). Plants were transplanted into two-gallon HDPE nursery pots filled with the same 6:4 (v/v) vermiculite:sand mixture, established under a mist tent for one week, and acclimated on the greenhouse bench for 14 days before nitrogen treatments began. Pots were arranged in a randomized complete block design with ten blocks, each containing one replicate per inoculation × nitrogen combination. Saucers were placed under each pot to prevent fertigation cross-contamination. Plants were placed on the greenhouse bench with supplemental LED lighting (ZELION HL300 Grow White, OSRAM Sylvania Inc., Wilmington, MA, USA) and maintained the same photoperiod as experiment I.

2.2.2 Nitrogen treatment

Fertigation began two weeks after transplanting using a modified Hoagland solution (Table 3). High-nitrogen plants received a half-strength solution and low-nitrogen plants a one-eighth-strength solution, each applied at 60 mL per pot twice weekly.

2.2.3 Growth and harvest measurements

At harvest, plants were separated into leaves, stems, and roots, oven-dried at 70°C to constant mass, and weighed; total plant dry mass and root-to-shoot ratio were calculated as in experiment I. Roots were not subdivided into fine and coarse fractions in experiment II.

2.2.4 Data analysis

The main effects of inoculation, nitrogen, and their interaction were evaluated using two-way ANOVA with block included in the model, and pairwise comparisons among treatment means were conducted using Tukey's HSD test where effects were significant or marginally significant.

2.3 Experiment III: Rhizobox experiment

2.3.1 Experimental design and hydroponic rhizobox system

Experiment III, also carried out in the green house at CUH comprised two sequential rounds using independent batches of tissue culture-propagated *Populus trichocarpa* (Nisqually-1). Round 1 used 28-plants and round 2 used 40-plants. Within each round, half the plants were inoculated with the ten-strain Mix III consortium (EI) and half received mock inoculum (NI), crossed with two

nitrogen levels (high and low). A modified two-dimensional rhizobox system was adapted from Parasurama et al. (2023), consisting of clear acrylic plates paired with blue germination paper to enhance root-background contrast and enable non-destructive visualization of root development along a planar surface (Fig. S2). A black back panel restricted ambient light and promoted root growth along the imaging surface. Plants were established on pre-wetted germination paper with roots maintained in continuous contact with the substrate, secured with black HDPE sheets and binder clips, and placed in crates supported at a fixed angle (~60°) on a PVC frame to maintain consistent orientation. Each crate contained 4 L of modified Hoagland solution at the treatment-specific nitrogen concentration (Table 1), replaced every seven days and buffered to pH 6.3–6.8 with 0.1 M KOH. The lower 60-70 mm of germination paper was submerged to ensure uniform water and nutrient distribution.

2.3.2 Nitrogen treatment

The modified Hoagland nutrient solution used in the pot experiments with the same high- and low-nitrogen concentrations matched was applied as the hydroponic nutrient solution in Experiment III.

2.3.3 Root imaging and trait extraction

High-resolution root images were captured at biweekly intervals from an initial establishment timepoint through final harvest using a Canon EOS Rebel SL3 camera (Canon USA, Melville, NY, USA), with the black HDPE covering removed and two studio lights positioned perpendicular to the imaging surface to maximize root-background contrast (Fig. 2). Images were processed in

ImageJ (Schindelin *et al*., 2012) by enhancing contrast and applying color thresholding in YUV color space to generate segmented root images, which were refined in RhizoVision Explorer (Seethepalli et al., 2021) by removing background artefacts smaller than 2 pixels. Twelve root architectural traits were extracted from final harvest images: median number of roots, number of root tips, total root length, rooting depth, width-to-depth ratio, network area, solidity, perimeter, surface area, average root orientation, shallow angle frequency, and steep angle frequency. These traits capture complementary aspects of root system size, shape, and spatial deployment, and follow the definitions of Seethepalli *et al*. (2021). The median number of roots is the median count of roots intersected by horizontal line scans across all rows of the segmented image, and reflects the typical number of roots present along the width of the system. The number of root tips is the count of tip points in the skeletonized image and indexes the number of root apices, the primary sites of uptake and meristematic activity. Total root length is the summed length of the root skeleton and represents the overall extent of the system, while perimeter is the length of the root boundary and surface area is computed from the medial axis and root diameters, both indexing the contact area between the root system and its surrounding medium. Network area, the total number of root pixels in the segmented image, provides an integrated measure of overall root system size in the imaging plane. Rooting depth and width-to-depth ratio describe the vertical versus lateral deployment of the system: depth is the vertical extent of the root system in the image, and the width-to-depth ratio is the maximum width divided by depth, such that lower values indicate a deeper, narrower system and higher values a shallower, more laterally spreading one. Average root orientation is the mean orientation of medial axis pixels computed over local 40 × 40 pixel neighborhoods, and the shallow, medium, and steep angle frequencies are the proportions of these local orientations falling below 30°, below 60°, and below 90°, respectively, thereby describing

the angular distribution of roots. Solidity is the ratio of network area to convex hull area, where the convex hull is the smallest convex polygon enclosing the root system; higher solidity indicates a more compact, space-filling architecture and lower solidity a more open, exploratory one. Together, these traits provide a quantitative description of how endophyte inoculation and nitrogen availability shape both the magnitude and the spatial organization of the root system.

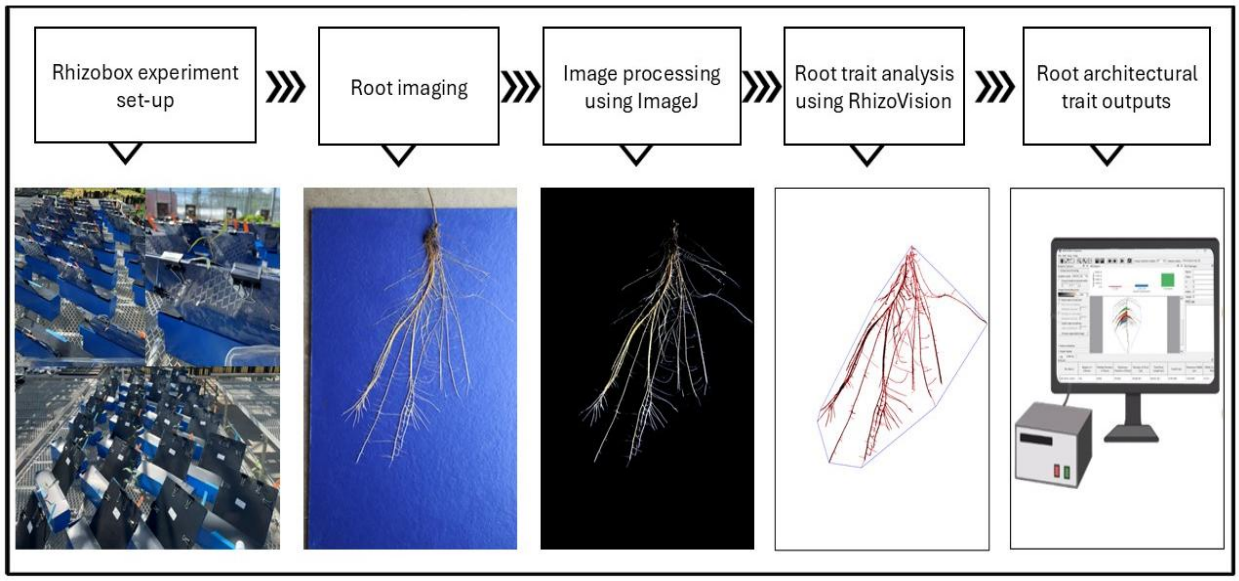

Fig. 2. Workflow for root system architecture assessment in *Populus trichocarpa* grown in the Rhizobox experiment III. Plants were cultivated in a two-dimensional rhizobox system with transparent acrylic plates and blue germination paper, which enabled non-destructive observation of root development against a high-contrast background. During imaging, the light-restricting cover was removed and root systems were photographed in place. Images were processed in ImageJ (Schindelin *et al*., 2012) by contrast enhancement and colour thresholding to generate segmented, high-contrast root images, which were then analyzed in RhizoVision Explorer (Seethepalli *et al*., 2021) to extract root architectural traits. These measurements were used to evaluate endophyte-mediated changes in root system architecture across contrasting nitrogen conditions.

2.3.4 Data analysis

Data from both rounds were analyzed jointly using two-way ANOVA, with experimental round and block included as fixed covariables to account for between-round variation in absolute trait values while preserving statistical power to detect treatment effects. The primary model was trait ~ Endophytes × Nitrogen + Round + Block. Pairwise comparisons between inoculation treatments were conducted using Tukey's HSD test, and effect sizes were calculated as η^2 from the combined model. To verify that final trait differences were not attributable to pre-existing variation at establishment, analysis of covariance (ANCOVA) was performed using initial trait values measured at the first imaging timepoint as covariables. Initial trait values did not differ significantly between inoculation treatments at establishment for 11 of 12 traits (all $p > 0.08$); solidity was excluded from ANCOVA because it showed a significant baseline treatment difference ($p = 0.036$). ANCOVA results were consistent with the primary ANOVA for all traits, confirming that observed treatment effects were not attributable to pre-existing size differences.

2.4 Statistical and graphing software

All analyses were performed in R v4.4.0 (R Core Team, 2024; https://www.R-project.org/). Effect sizes were calculated as partial eta-squared (η^2) using the effectsize package (Ben-Shachar *et al*., 2020), with values of 0.01, 0.06, and 0.14 interpreted as small, medium, and large effects, respectively (Cohen, 1988). Data processing and visualization used the tidyverse package (Wickham *et al*., 2019), and ordination plots were generated with ggplot2 and ggrepel. Results are presented as means ± standard error, with statistical significance determined at $\alpha = 0.05$.

3 Results

3.1 Experiment I: Indoor-grown experiment

3.1.1 Plant height

All three inoculation treatments showed steady height growth throughout the 280-day experiment, with time accounting for most of the variation in plant height ($\eta^2 = 0.916$, $p < 0.0001$; Fig. 3). Although treatments started at similar heights, their growth rates diverged progressively over time, reflected by a significant inoculation × time interaction ($\eta^2 = 0.083$, $p < 0.0001$). By day 280, Mix I plants reached the greatest heights (42.26 ± 1.83 cm, n = 8), followed by Mix II (38.33 ± 2.62 cm, n = 8), while NI controls remained considerably shorter (25.23 ± 0.69 cm, n = 6). At day 280, both consortia mix produced plants significantly taller than NI controls: Mix I by 17.87 cm ($p < 0.0001$) and Mix II by 13.42 cm ($p < 0.0001$). Mix I plants were also significantly taller than Mix II plants (4.45 cm difference, $p = 0.0036$), indicating that differences in strain composition between the two consortia influenced the extent of growth promotion under low nitrogen.

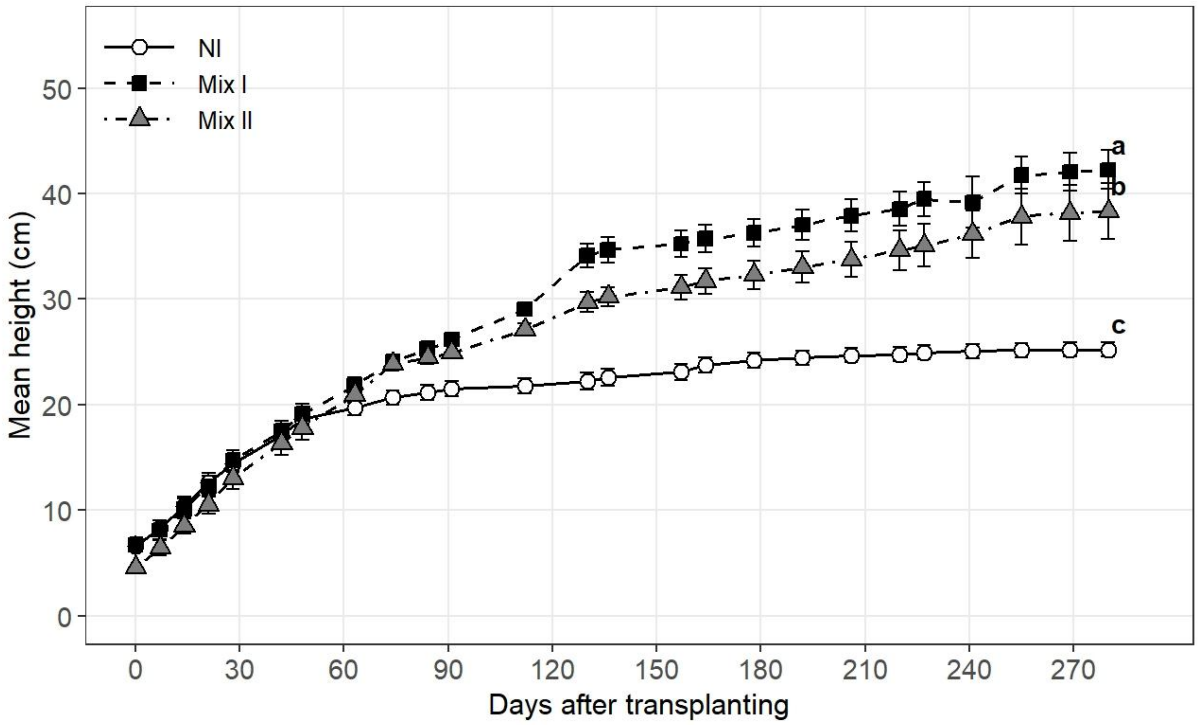

Fig. 3. Main effect of endophyte inoculation on plant height of *Populus* cv. H11-11 under low nitrogen conditions over 280 days after transplanting in experiment I (Indoor grown). Plants were mock-inoculated (NI, white circles), inoculated with a Salicaceae endophyte consortium (Mix I , black squares), or inoculated with a Mix II consortium (Mix II, gray triangles). Points represent treatment means ± standard error at each measurement timepoint (NI n = 6, Mix I n = 8, Mix II n = 8). Lines connect treatment means across timepoints. A significant inoculation × time interaction was detected ($p < 0.0001$, $\eta^2 = 0.083$). Letters denote significant pairwise differences between treatments at day 280 based on Tukey HSD post-hoc comparisons ($\alpha = 0.05$).

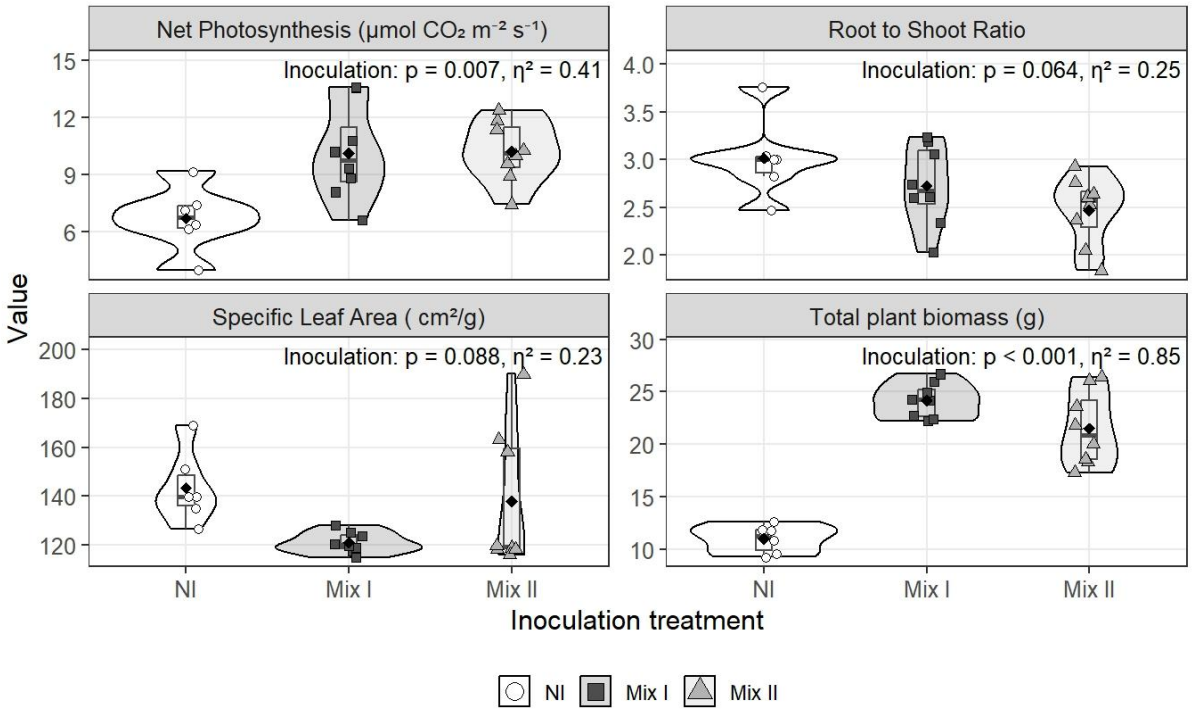

Fig. 4. Effect of endophyte inoculation on physiological and biomass traits of *Populus* cv. H11-11 under low nitrogen conditions at final harvest in experiment I (Indoor grown). Plants were mock-inoculated (NI, white circles), inoculated with a Salicaceae endophyte consortium (Mix I, dark gray squares), or inoculated with Mix II endophyte consortium (Mix II, light gray triangles). Violins show the data distribution, boxes indicate the interquartile range, horizontal lines indicate the median, and filled diamonds indicate the treatment mean. Individual data points are shown as jittered symbols (NI n = 6, Mix I n = 8, Mix II n = 8). P-values and η^2 shown in each panel are for the inoculation main effect from one-way ANOVA. Traits shown are net photosynthesis (µmol CO_2 m^{-2} s^{-1}), specific leaf area (SLA; cm^2 g^{-1}), total plant biomass (g), and root-to-shoot ratio.

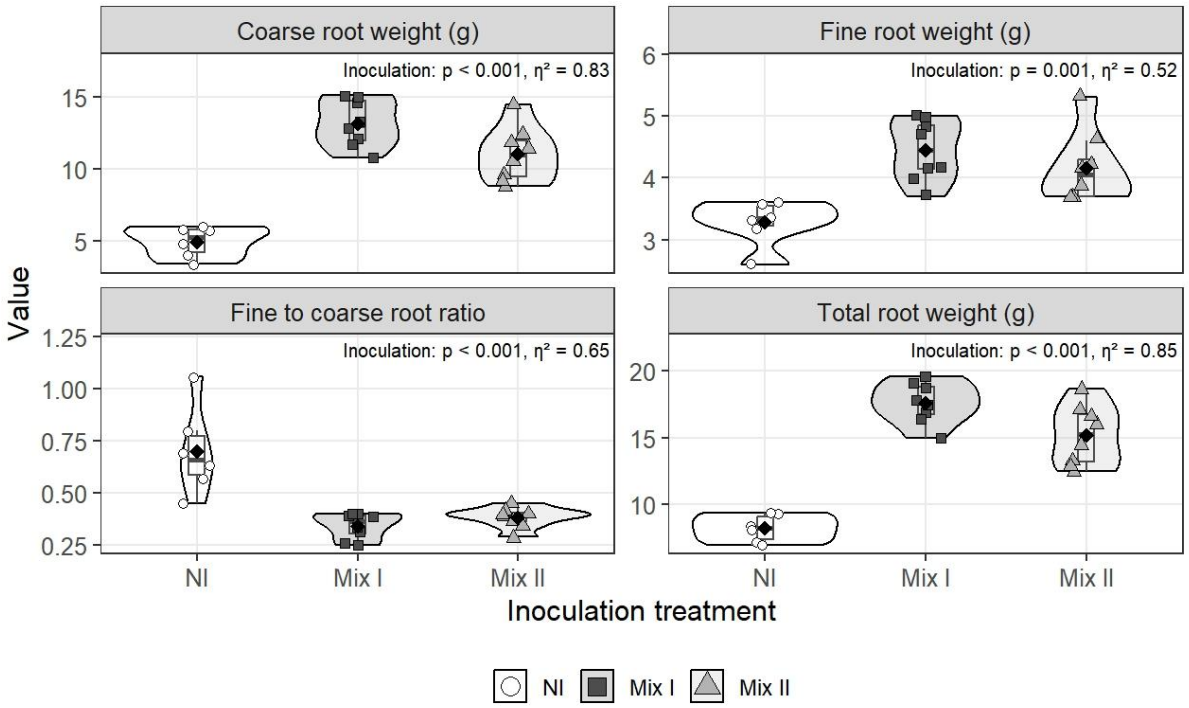

Fig. 5. Effect of endophyte inoculation on root biomass allocation traits of *Populus* cv. H11-11 under low nitrogen conditions at final harvest in Experiment I (Indoor grown). Plants were mock-inoculated (NI, white circles), inoculated with a Salicaceae endophyte consortium (Mix I, dark gray squares), or inoculated with Mix II endophyte consortium (Mix II, light gray triangles). Violins show the data distribution, boxes indicate the interquartile range, horizontal lines indicate the median, and filled diamonds indicate the treatment mean. Individual data points are shown as jittered symbols (NI n = 6, Mix I n = 8, Mix II n = 8). P-values and η^2 shown in each panel are for the inoculation main effect from one-way ANOVA. Traits shown are total root weight (g), coarse root weight (g), fine root weight (g), and fine-to-coarse root ratio.

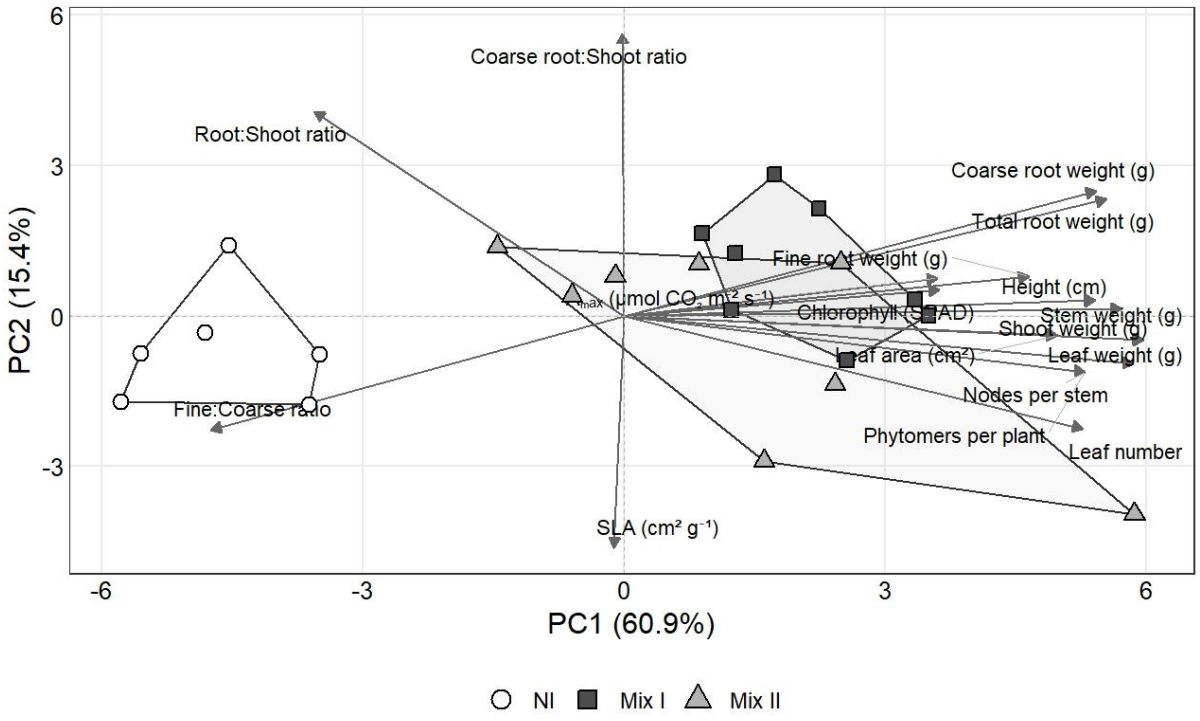

Fig. 6. Principal component analysis (PCA) of shoot and root functional traits of *Populus* cv. H11-11 under low nitrogen conditions across endophyte inoculation treatments in experiment I (Indoor grown). Plants were mock-inoculated (NI, white circles), inoculated with a Salicaceae endophyte consortium (Mix I, dark gray squares), or inoculated with Mix II endophyte consortium (Mix II, light gray triangles; NI n = 6, Mix I n = 8, Mix II n = 8). Shaded polygons represent convex hulls per treatment group. Arrows indicate the direction and relative contribution of each trait to the first two principal components, which together explained 76.3% of total variance (PC1 = 60.9%, PC2 = 15.4%). Trait abbreviations: Amax= net photosynthesis (μmol CO_2 m^{-2} s^{-1}); SLA = specific leaf area (cm^2 g^{-1}); SPAD = leaf chlorophyll content. PCA was conducted on correlation-scaled trait values using princomp() in R.

3.1.2 Net CO_2 assimilation

Net CO_2 assimilation (Anet) differed significantly among inoculation treatments ($p = 0.007$, $\eta^2 = 0.41$). Both Mix I(10.1 ± 0.88 μmol CO_2 m^{-2} s^{-1}, n = 8) and Mix II(10.2 ± 0.57 μmol CO_2 m^{-2} s^{-1}, n = 8) produced significantly higher assimilation rates than NI controls (6.7 ± 0.70 μmol CO_2 m^{-2} s^{-1}, n = 6; NI vs Mix I: $p = 0.014$; NI vs Mix II: $p = 0.011$). The two consortia did not differ from

each other ($p = 0.995$), indicating that the enhancement of assimilation was consistent regardless of consortium composition (Fig. 4).

3.1.3 Specific leaf area (SLA)

SLA showed a marginally non-significant trend across inoculation treatments ($p = 0.088$, $\eta^2 = 0.23$), with inoculation accounting for a medium-to-large proportion of variation despite the absence of statistical significance, likely reflecting limited statistical power from small sample sizes. Mix I plants had the lowest mean SLA (121.0 ± 1.52 cm^2 g^{-1}, $n = 8$), followed by Mix II(138.0 ± 10.1 cm^2 g^{-1}, $n = 8$) and NI controls (143.0 ± 6.03 cm^2 g^{-1}, $n = 6$). Although the direction of the difference was consistent with a trend toward lower SLA in inoculated plants, no pairwise comparison reached significance after Tukey correction (NI vs Mix I: $p = 0.098$; NI vs Mix II: $p = 0.847$; Mix I vs Mix II: $p = 0.206$; Fig. 4).

3.1.4 Total plant biomass

Total plant biomass differed significantly among inoculation treatments ($p < 0.0001$, $\eta^2 = 0.85$). Both consortia produced greater biomass than NI controls: Mix I accumulated 24.2 ± 0.59 g ($n = 8$) and Mix II 21.5 ± 1.25 g ($n = 8$), compared with 11.0 ± 0.54 g ($n = 6$) in NI controls. Both consortia differed significantly from NI (Mix I vs NI: $p < 0.0001$; Mix II vs NI: $p < 0.0001$), while the two consortia did not differ from each other ($p = 0.105$), indicating that the biomass benefit of inoculation was consistent across consortium compositions under low nitrogen (Fig. 4).

3.1.5 Root-to-shoot ratio

Inoculation had a marginally non-significant effect on root-to-shoot ratio under low nitrogen ($p = 0.064$, $\eta^2 = 0.25$), with a large effect size suggesting a biologically meaningful trend likely limited by small sample size. Mix I plants had the lowest mean root-to-shoot ratio (2.73 ± 0.15, $n = 8$), followed by Mix II(2.47 ± 0.13, $n = 8$) and NI controls (3.02 ± 0.17, $n = 6$), although no pairwise comparison reached significance after Tukey correction (all $p > 0.05$; Fig. 4).

3.1.6 Distribution of biomass among root functional classes

All root biomass traits were significantly affected by inoculation, with effect sizes ranging from large to very large, indicating strong responses to inoculation under low nitrogen (Fig. 5). Both consortia produced substantially greater total root biomass than NI controls ($p < 0.0001$, $\eta^2 = 0.85$), with Mix I reaching 17.6 ± 0.54 g ($n = 8$) and Mix II 15.2 ± 0.79 g ($n = 8$), compared with 8.23 ± 0.41 g in NI controls ($n = 6$; both $p < 0.0001$). Mix I also accumulated significantly more total root biomass than Mix II($p = 0.029$), indicating that consortium composition influenced the extent of root growth promotion.

The increase in total root biomass was driven primarily by coarse root development. Coarse root weight followed the same pattern as total root weight ($p < 0.0001$, $\eta^2 = 0.83$), with MIX I(13.2 ± 0.57 g) and Mix II(11.0 ± 0.67 g) both exceeding NI controls (4.95 ± 0.44 g; both $p < 0.0001$), and Mix I exceeding Mix II($p = 0.039$). Fine root weight was also significantly greater in inoculated plants ($p = 0.001$, $\eta^2 = 0.52$), with Mix I(4.45 ± 0.17 g) and Mix II(4.16 ± 0.20 g) both exceeding NI controls (3.28 ± 0.15 g; Mix I vs NI: $p = 0.001$; Mix II vs NI: $p = 0.010$), while the two consortia did not differ from each other ($p = 0.483$).

Despite the greater absolute fine root biomass in inoculated plants, the fine-to-coarse root ratio was significantly lower in both inoculated treatments than in NI controls ($p < 0.0001$, $\eta^2 = 0.65$;Mix I: 0.34 ± 0.02;Mix II: 0.38 ± 0.02; NI: 0.70 ± 0.09; Mix I vs NI: $p < 0.0001$; Mix II vs NI: $p = 0.0003$). The two consortia did not differ from each other in this ratio ($p = 0.793$). This indicates that inoculation may stimulate coarse root development over fine root proliferation, shifting root allocation toward structural and transport functions regardless of consortium composition.

3.1.7 Principal component analysis

To assess the multivariate relationships among shoot and root functional traits across inoculation treatments, PCA was conducted on multiple morphological, physiological, and biomass traits measured at final harvest. The first two principal components collectively explained 76.3% of total variance (PC1 = 60.9%, PC2 = 15.4%), indicating a high degree of trait covariation captured by the ordination. PC1 broadly separated inoculated plants from NI controls, reflecting an overall endophyte-driven shift in functional strategy. Inoculated plants (Mix I andMix II) clustered in the positive PC1 region, associated with greater biomass allocation including coarse root weight, total root weight, shoot weight, stem weight, leaf weight, height, leaf area, net photosynthesis, and leaf chlorophyll content. In contrast, NI plants clustered in the negative PC1 region, where root-to-shoot ratio, fine-to-coarse root ratio, and specific leaf area loaded strongly, consistent with a nitrogen-foraging strategy under nitrogen limitation in which a greater proportion of biomass is allocated to roots relative to shoots (Fig. 6).

PC2 (15.4%) provided secondary separation primarily within the inoculated group. Coarse root-to-shoot ratio loaded positively along PC2, while fine-to-coarse root ratio and SLA loaded negatively, indicating that PC2 captures variation in root allocation strategy between and within treatments. Mix I plants tended toward higher coarse root-to-shoot ratios relative to Mix II, while several Mix II individuals scattered toward more negative PC2 values, consistent with greater variability in root allocation within that consortium. The clear spatial separation of convex hulls between NI and the two inoculated treatments along PC1, with minimal overlap, confirms that inoculation produced a consistent and coordinated shift across multiple functional traits rather than isolated effects on individual measurements. The partial overlap of Mix I and Mix II hulls indicates broadly similar functional responses to inoculation, with consortium-specific differences in root allocation captured along PC2.

3.2 Experiment II: Greenhouse-grown experiment

3.2.1 Total plant biomass

Nitrogen levels was the dominant driver of total plant biomass ($p < 0.0001$, $\eta^2 = 0.82$) in experiment II, with high-nitrogen plants accumulating substantially greater biomass than low-nitrogen plants across all treatments (HN mean range: 41.0–43.6 g; LN mean range: 12.7–15.6 g; LN vs HN difference: 27.97 g, $p < 0.0001$). In contrast, inoculation had no significant effect on total biomass ($p = 0.522$, $\eta^2 = 0.02$), and the inoculation × nitrogen interaction was also non-significant ($p = 0.846$, $\eta^2 = 0.006$), indicating that the endophyte effect on biomass did not vary with nitrogen availability. Block explained negligible variation ($p = 0.456$, $\eta^2 = 0.01$). Within each

nitrogen level, no pairwise differences in biomass were detected among inoculation treatments (all $p > 0.05$; Fig. 7).

3.2.2 Root-to-shoot ratio

Inoculation significantly reduced root-to-shoot ratio across both nitrogen levels ($p = 0.022$, $\eta^2 = 0.13$), as did nitrogen treatments ($p = 0.004$, $\eta^2 = 0.14$), while block accounted for a significant proportion of variation ($p = 0.029$, $\eta^2 = 0.09$), confirming that blocking was appropriate for controlling spatial heterogeneity within the greenhouse. The inoculation × nitrogen interaction was non-significant ($p = 0.916$, $\eta^2 = 0.003$), indicating that the endophyte-mediated reduction in root-to-shoot ratio was consistent across nitrogen levels. Mix I plants had significantly lower root-to-shoot ratios than NI controls across both nitrogen treatments (Mix I vs NI difference: - 0.138, $p = 0.023$), while Mix II showed a similar but marginally non-significant trend (Mix II vs NI difference: - 0.108, $p = 0.093$). The two consortia did not differ from each other ($p = 0.823$; Fig. 7). Under low nitrogen, NI plants had the highest root-to-shoot ratios (1.06 ± 0.04), consistent with greater root investment under nutrient limitation, whereas Mix I and Mix II plants maintained lower ratios (0.90 ± 0.06 and 0.95 ± 0.06, respectively), indicating that inoculation partially relieved the need for increased root foraging under nitrogen limitation.

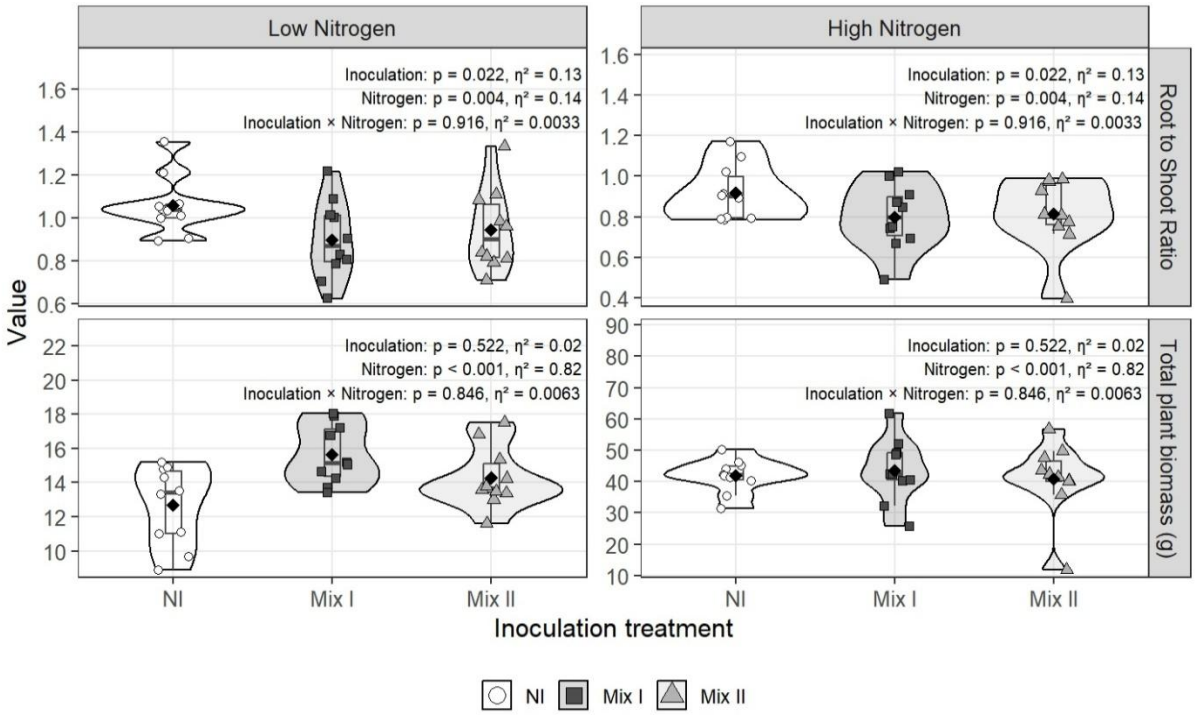

Fig. 7. Effect of endophyte inoculation and nitrogen availability on root-to-shoot ratio and total plant biomass of *Populus* cv. H11-11 at final harvest in Experiment II (Greenhouse-grown experiment). Plants were mock-inoculated (NI, white circles), inoculated with a Salicaceae endophyte consortium (Mix I, dark gray squares), or inoculated with Mix II endophyte consortium (Mix II, light gray triangles) under high nitrogen (HN) or low nitrogen (LN) conditions. Violins show the data distribution, boxes indicate the interquartile range, horizontal lines indicate the median, and filled diamonds indicate the treatment mean. Individual data points are shown as jittered symbols (n = 10 per treatment combination). P-values and partial η^2 shown in right panel are for the inoculation and nitrogen main effects from two-way ANOVA with block as a covariate. No significant inoculation × nitrogen interaction was detected for either trait (root-to-shoot ratio: p = 0.916; total biomass: p = 0.846).

3.3 Experiment III: Rhizobox experiment

Root system architectural traits were examined across two independent experimental rounds combined into a single analysis, with round included as a fixed covariables. Round effect explained a large proportion of variation for most size-related traits ($\eta^2 = 0.42–0.74$), controlling for between-round differences in absolute root size. After accounting for round and block effects, endophyte inoculation significantly affected six of the twelve architectural traits examined (Table 3; Fig. S1, Fig. 8).

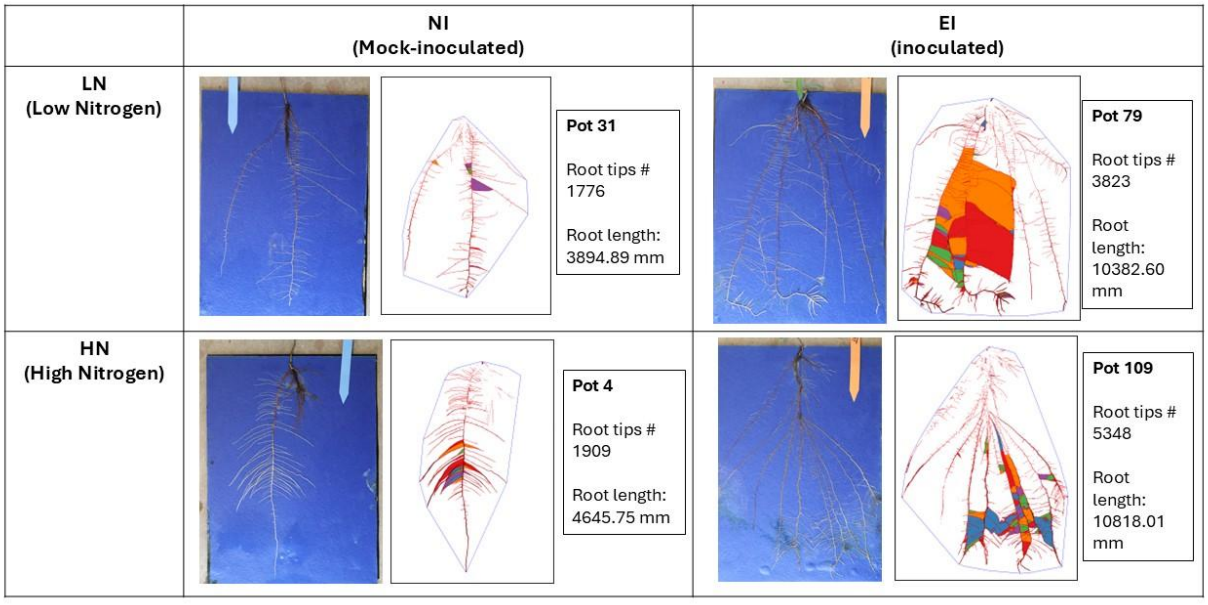

Fig. 8. Representative root images in *Populus trichocarpa* for each treatment combination [mock-inoculated (NI) and endophyte-inoculated (EI) plants under high (HN) and low (LN) nitrogen] from the Rhizobox experiment III. All panels are displayed at the same scale and aligned to a common margin to allow direct visual comparison of rooting depth and extent.

3.3.1 Rooting depth

Rooting depth was the most strongly affected trait, with EI plants rooting substantially deeper than NI controls ($p = 0.0001$, $\eta^2 = 0.31$; EI HN: 361 ± 26 mm, EI LN: 388 ± 11 mm; NI HN: 222 ± 33 mm, NI LN: 351 ± 17 mm; EI vs NI difference: 101 mm, $p = 0.0001$) (Fig. 9). Nitrogen availability also significantly affected rooting depth ($p = 0.005$, $\eta^2 = 0.17$), with low-nitrogen plants rooting deeper than high-nitrogen plants, and a marginally non-significant inoculation × nitrogen interaction ($p = 0.056$, $\eta^2 = 0.09$) driven by the shallower root systems of NI plants under high nitrogen relative to all other treatment combinations..

3.3.2 Root size and extent traits

Root tip number was significantly greater in EI plants than NI controls ($p = 0.029$, $\eta^2 = 0.11$; EI HN: 2,339 ± 692, EI LN: 2,107 ± 585; NI HN: 1,443 ± 519, NI LN: 1,479 ± 755; EI vs NI difference: 761, $p = 0.029$), with no significant interaction with nitrogen ($p = 0.463$) and no significant nitrogen main effect ($p = 0.722$, $\eta^2 = 0.003$) (Fig. 9). Root perimeter was significantly greater in EI plants ($p = 0.025$, $\eta^2 = 0.12$; EI HN: 14,798 ± 3,498 mm, EI LN: 12,281 ± 2,195 mm; NI HN: 9,013 ± 2,953 mm, NI LN: 8,991 ± 3,012 mm; EI vs NI difference: 4,489 mm), with no significant inoculation × nitrogen interaction ($p = 0.459$) and no significant nitrogen main effect ($p = 0.446$, $\eta^2 = 0.014$). Total root length showed a marginally non-significant trend toward greater values in EI plants ($p = 0.089$, $\eta^2 = 0.07$; EI HN: 8,044 ± 2,135 mm, EI LN: 6,464 ± 1,151 mm; NI HN: 5,191 ± 1,901 mm, NI LN: 4,717 ± 1,694 mm; EI vs NI difference: 2,223 mm), while both main nitrogen effect ($p=0.385$) and interaction effects ($p=0.597$) were non-significant. Network area showed a non-significant trend toward greater values in EI plants ($p = 0.141$, $\eta^2 = 0.05$), and surface area did not differ significantly between inoculation treatments ($p = 0.312$, η^2

= 0.03). Across these size and extent traits, significant or near-significant effects were associated with inoculation rather than nitrogen availability, with neither significant nitrogen main effects nor inoculation × nitrogen interactions detected.

3.3.3 Root orientation and angle distribution

Average root orientation differed significantly between inoculation treatments ($p = 0.030$, $\eta^2 = 0.11$), with EI plants maintaining more vertically oriented roots than NI controls (EI HN: 42.2 ± 0.8°, EI LN: 43.0 ± 1.2°; NI HN: 47.7 ± 2.9°, NI LN: 44.2 ± 1.7°; EI vs NI difference: −3.7°, $p = 0.030$). Steep angle frequency was significantly lower in EI plants ($p = 0.032$, $\eta^2 = 0.11$; EI: 0.33 ± 0.02; NI HN: 0.41 ± 0.05, NI LN: 0.37 ± 0.03; EI vs NI difference: −0.07, $p = 0.032$), while shallow angle frequency showed a non-significant trend toward greater values in EI plants ($p = 0.111$, $\eta^2 = 0.06$) (Fig. 9).

3.3.4 Solidity and remaining traits

Solidity differed significantly between inoculation treatments ($p = 0.038$, $\eta^2 = 0.10$), with a significant inoculation × nitrogen interaction ($p = 0.046$, $\eta^2 = 0.09$) and a significant nitrogen main effect ($p = 0.003$, $\eta^2 = 0.20$). NI plants under high nitrogen had substantially greater solidity than all other treatment combinations (NI HN: 0.12 ± 0.02; NI LN: 0.04 ± 0.01; EI HN: 0.07 ± 0.01; EI LN: 0.05 ± 0.01), with NI plants under high nitrogen showing the highest solidity values, indicating a more compact, less spatially distributed root network.. Median root number and width-to-depth ratio were not significantly affected by inoculation, nitrogen, or their interaction (all $p > 0.17$; Table 3; Appendix Fig. S1).

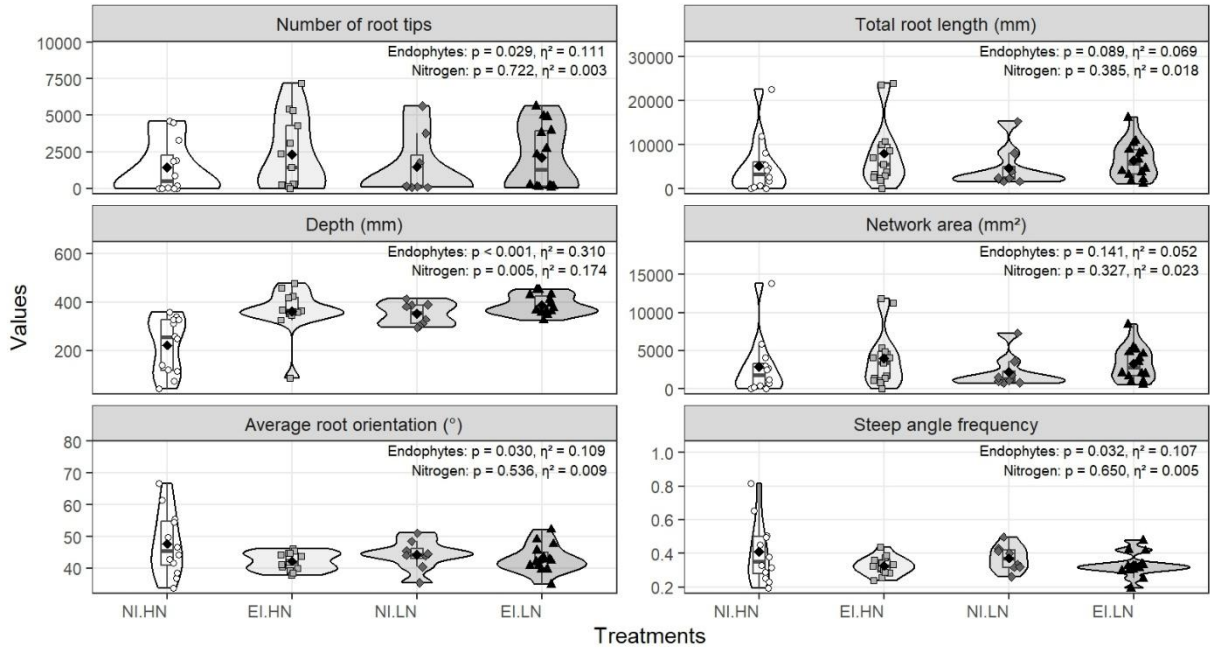

Fig. 9 Effect of Mix III endophyte inoculation on root system architectural traits of *Populus trichocarpa* grown under high nitrogen (HN) and low nitrogen (LN) conditions. Plants were either mock-inoculated (NI; open white circles = NI.HN, gray-filled diamonds = NI.LN) or inoculated with Mix II consortium (EI; gray-filled squares = EI.HN, black-filled triangles = EI.LN). Violins show the data distribution, boxes indicate the interquartile range, horizontal lines indicate the median, and diamonds indicate the treatment mean. Individual data points are shown as jittered symbols. P-values and η^2 effect sizes shown in each panel are for the endophyte inoculation (Endophytes) and nitrogen availability (Nitrogen) main effects from two-way ANOVA.

4 Discussion

4.1 Endophyte inoculation promotes whole-plant growth and biomass accumulation under nitrogen limitation

Salicaceae endophytes have attracted considerable interest as candidates for sustainable nitrogen management in bioenergy systems, yet their capacity to reshape root functional biology in woody hosts remains poorly characterized. This study demonstrates that inoculation of *Populus* with Salicaceae endophyte consortia produced significant increase in whole-plant growth, net photosynthetic rate, biomass accumulation, and altered root functional classes and architectural traits. In Experiment I, both endophyte consortia increased the rate of height growth over the 280-day measurement period, as reflected in the significant inoculation × time interaction, even though the main effect of inoculation on final height was not itself significant. Beyond height, the effects of inoculation on physiology and biomass production were both consistent. Total plant biomass differed strongly among inoculation treatments ($\eta^2 = 0.85$), with both consortia more than doubling biomass relative to mock-inoculated controls under low nitrogen. Net photosynthetic rate was likewise significantly higher in inoculated plants ($\eta^2 = 0.41$), indicating that the biomass gains were accompanied by, and likely supported by, enhanced carbon assimilation capacity rather than by morphological change alone. The magnitude of these responses among the largest observed across all traits measured in this study is consistent with previous work demonstrating growth promotion of *Populus* by these and related Salicaceae endophyte consortia under nitrogen-limited conditions (Knoth *et al*., 2014; Kandel *et al*., 2015). Specific leaf area showed a marginally non-significant tendency toward lower values in inoculated plants ($\eta^2 = 0.23$), a pattern that, while

not statistically robust on its own, is directionally consistent with the broader shift in resource strategy described below.

The role of nitrogen availability in shaping these responses became clear in Experiment II, where contrasting high and low nitrogen treatments were applied alongside inoculation. Here nitrogen availability dominated the total biomass response ($\eta^2 = 0.82$), and the main effect of inoculation on biomass was not significant. This contrast between experiments is itself an important result. In Experiment I, conducted under low nitrogen only, inoculation effects on biomass were large; in Experiment II, the presence of a high nitrogen treatment revealed that the nitrogen supply is a far stronger driver of biomass than inoculation. This pattern is consistent with the broader endophyte literature, in which the benefits of Salicaceae associations tend to be most pronounced under nutrient limitation and diminish as exogenous nitrogen supply increases (Hardoim *et al*., 2015). Taken together, these results support the interpretation that the growth-promoting capacity of these consortia is most functionally relevant under the low-nitrogen conditions characteristic of degraded, riparian, or nutrient-poor soils, the environments in which nitrogen-fixing plant-microbe associations are most agronomically and ecologically valuable, and in which *Populus* is increasingly considered for bioenergy and restoration plantings.

4.2 Endophyte inoculation shifts biomass partitioning independently of nitrogen availability

Our first hypothesis predicted that endophyte inoculation would reduce the root-to-shoot ratio by alleviating nitrogen limitation and thereby reducing the plant's reliance on extensive root foraging. This hypothesis was partially supported. Inoculated plants consistently showed lower root-to-shoot ratios than mock-inoculated controls across both pot experiments, indicating that

inoculation does redirect a greater proportion of biomass toward shoot tissue. However, the mechanism we originally proposed that this reallocation is driven by relief from nitrogen limitation through fixation was not fully borne out by the data. In Experiment II, the inoculation × nitrogen interaction for root-to-shoot ratio was far from significant ($p = 0.916$), while the main effect of inoculation remained significant ($p = 0.022$, $\eta^2 = 0.13$) and operated in the same direction under both high and low nitrogen. If the reduction in root-to-shoot ratio were driven solely by endophyte-mediated nitrogen supplementation, the effect would be expected to be strongest under low nitrogen, where the relative contribution of fixed nitrogen to the plant's budget should be greatest, and weak or absent under high nitrogen, where the plant is already nitrogen-replete. The observation that inoculation reduced root-to-shoot ratio equally across both nitrogen levels argues against a strictly fixation-dependent mechanism.

An additional consideration is that the relatively small pot volumes used in these experiments may have physically constrained root expansion. For the larger, faster-growing inoculated plants, which could have become pot-bound earlier or more severely than the smaller mock-inoculated controls. Such root restriction can itself reduce root-to-shoot ratio independently of treatment, and may have interacted with inoculation effects on biomass allocation. Because root confinement was not quantified, its contribution to the observed reduction in root-to-shoot ratio cannot be fully separated from treatment effects, and this should be considered when interpreting the allocation results.

A notable contrast between the two pot experiments warrants discussion. In the indoor-grown experiment (Experiment I), inoculation produced large increases in total biomass, photosynthesis, and root biomass. Whereas in the greenhouse-grown experiment (Experiment II), inoculation had no significant effect on total biomass, which was instead dominated by nitrogen availability.

Several non-exclusive factors may underlie this difference, including the different growth environments, pot sizes, and durations of the two experiments. One additional possibility relates to the unintended water stress experienced during the indoor-grown experiment. Because some of the plants in that experiment were inadvertently exposed to drought, the surviving low-nitrogen plants were grown under conditions that may have included episodic water limitation. Salicaceae endophytes of the type used here are well documented to improve the water-use efficiency and drought tolerance of *Populus*, lowering stomatal conductance while maintaining carbon assimilation and, in some cases, increasing biomass and root-to-shoot ratio under water deficit (Banan *et al*., 2024; Hendrickson *et al*., 2025). It is therefore plausible that the pronounced inoculation benefits observed in the indoor-grown experiment reflect, in part, endophyte-mediated alleviation of water stress in addition to effects on nitrogen and root growth. On the other hand, the well-watered greenhouse-grown experiment did not impose the conditions under which such water-related benefits would be expressed. This interpretation is necessarily tentative, as water status was not a controlled variable and the relevant physiological responses (e.g., stomatal conductance, water-use efficiency) were not measured in the present study. Therefore, it represents a testable hypothesis for the differing magnitude of endophyte effects between experiments and a clear direction for future work in which water availability is explicitly manipulated.

This nitrogen-independence points instead toward a regulatory pathway that does not depend on the plant's nitrogen status. Salicaceae endophytes, including auxin-producing strains in the consortia used here, have been documented to produce phytohormones that regulate source–sink relationships and carbon allocation between root and shoot tissues (Xin *et al*., 2009, Compant *et al*., 2010; Khan *et al*., 2012, Sukumar *et al*., 2013; Kandel *et al*., 2017). A framework that

combines nitrogen fixation with phytohormone-mediated regulation therefore accounts for the data more completely than nitrogen fixation alone: fixation may contribute to the overall growth promotion observed under low nitrogen in Experiment I, while a hormonally mediated shift in carbon partitioning could operate independently of nitrogen status and thereby explain the consistent reduction in root-to-shoot ratio across nitrogen environments in Experiment II. It is important to emphasize that the present study did not measure phytohormone production or quantify nitrogen fixation directly, and this interpretation therefore remains inferential. Distinguishing the relative contributions of these two pathways will require direct measurement for example, ^{15}N isotope dilution to quantify the proportion of plant nitrogen derived from fixation, paired with targeted profiling of endophyte-derived phytohormones in planta.

4.3 Endophyte inoculation drives a shift in root functional class allocation

Our second hypothesis predicted that endophyte inoculation would reduce fine root biomass and increase coarse root investment, on the reasoning that relief from nitrogen stress downregulates the fine root proliferation that plants deploy as a foraging response. This hypothesis was tested only in the indoor-grown experiment (Experiment I), where roots were separated into fine and coarse fractions, and was strongly supported.. Inoculated plants showed significantly greater coarse root biomass ($\eta^2 = 0.83$) and total root biomass ($\eta^2 = 0.85$), together with a substantially reduced fine-to-coarse root ratio ($\eta^2 = 0.65$), relative to mock-inoculated controls. The direction and magnitude of these effects are consistent with a reallocation of carbon away from fine root proliferation and toward structural, transport-oriented coarse root development. Fine roots are the primary organs of nitrogen and water acquisition, and their proliferation is characteristically upregulated under nitrogen limitation as part of a foraging strategy (Fitter, 1991; Pregitzer *et al*.,

2002). The reduction in fine-to-coarse root ratio observed in inoculated plants is consistent with a relaxation of this foraging response under improved nitrogen status. Notably, fine root biomass itself was also higher in inoculated plants in absolute terms, but coarse root biomass increased proportionally more, so that the balance of allocation shifted toward coarse roots. This distinction matters: inoculation did not simply suppress fine root growth, but rather rebalanced the relative investment among root classes in favor of structural tissue. It is worth noting that a relaxation of nitrogen foraging alone would be expected to reduce fine root biomass, yet fine root biomass increased in absolute terms under inoculation. This shift in allocation toward coarse roots, combined with an overall increase in root biomass, is difficult to explain solely by relief from nitrogen limitation. Instead, the pattern is consistent with a direct, nitrogen-independent stimulation of root growth, such as that associated with endophyte-derived auxin production (Xin *et al*., 2009a; Xin *et al*., 2009b; Khan *et al*., 2012). As with the root-to-shoot results, the data are best explained by the combined action of nitrogen-related and growth-stimulation pathways rather than by foraging relaxation alone.

4.4 Principal component analysis

Principal component analysis integrated these individual trait responses into a single multivariate picture and confirmed the interpretation at the whole-plant level. Mock-inoculated plants clustered in the negative PC1 space, characterized by high root-to-shoot ratio, high fine-to-coarse root ratio, and high specific leaf area. This combination is consistent with a nitrogen-foraging strategy under nitrogen limitation, in which carbon is disproportionately allocated to fine root proliferation at the expense of structural growth. The co-loading of high SLA in this cluster is consistent with the production of thin, low-cost leaves under nutrient stress, an indicator of a

conservative position on the leaf and whole-plant economics spectrum (Wright *et al*., 2004; Reich, 2014). In contrast, endophyte-inoculated plants clustered in the positive PC1 space, anchored by coarse root weight, total root weight, net photosynthetic rate, and multiple shoot biomass traits. This separation reflects a shift toward a growth-oriented, biomass-accumulation phenotype, in which carbon freed from extensive nitrogen foraging is redirected toward structural root development and shoot growth. We interpret this as a transition consistent with endophyte-mediated relief from nitrogen limitation, rather than as an intensification of resource acquisition per se, the inoculated plants are not foraging more aggressively, but rather are released from the need to forage and are therefore able to invest in growth.

The first principal component explained 60.9% of total variance, a strikingly high proportion that indicates the inoculation effect was not confined to one or two traits but instead expressed as a coherent, whole-plant reorganization. In other words, the primary axis of phenotypic variation in this experiment was itself the contrast between a nitrogen-foraging strategy and a nitrogen-supplemented, biomass-accumulation strategy. This coordinated, multi-trait nature of the response is one of the central findings of the study, because it suggests that inoculation acts on an integrated plant-level program of resource allocation rather than on isolated organs or processes.

4.4.1 Consortium-level differences between Mix I and II

While the two consortia (MIX I and Mix II) produced broadly similar effects relative to the mock-inoculated control, they were not identical, and the secondary axis of the ordination captured the differences between them. Secondary separation along PC2, which explained 15.4% of total variance, was driven by coarse root-to-shoot ratio loading positively against fine-to-coarse root

ratio and SLA. This pattern indicates that the two consortia differed primarily in the details of their root biomass allocation rather than in the overall direction of their effect on the plant. Such differences could plausibly arise from variation among the constituent strains in the identity and quantity of phytohormones produced, in colonization success, and persistence within the host. Because both consortia were applied as multi-strain mixtures rather than as individual isolates, the present study cannot attribute these differences to particular strains, nor can it determine whether interactions among strains within a consortium were additive, synergistic, or antagonistic. The consortium-level differences observed here therefore identify a clear and tractable direction for follow-up work at the strain level, but should be interpreted cautiously given the design of the present experiments.

4.5 Endophyte inoculation promotes deeper, more extensive root system architecture

Root system architectural responses to endophyte inoculation were investigated in the hydroponic rhizobox experiment, where image-based phenotyping across two independent experimental rounds provided sufficient statistical power to detect treatment effects after accounting for between-round variation. Rooting depth emerged as the most clear and consistent architectural response. It was significant in both experimental rounds analyzed independently, was confirmed by analysis of covariance following adjustment for initial plant size, and showed an increasing effect size from the first round ($\eta^2 = 0.45$) to the second ($\eta^2 = 0.61$). The consistency of the depth response across rounds, and covariate adjustment distinguishes it from the more variable responses of other traits and gives us the greatest confidence in its biological reality. The shallower root systems of mock-inoculated plants under high nitrogen (222 ± 33 mm), relative to all other treatment combinations, are consistent with classical root foraging theory, in which vertical root

penetration is reduced when surface nitrogen supplies are adequate and increased under nitrogen limitation as the plant searches for mobile nitrate at depth (Hodge, 2004; Gruber *et al*., 2013). Another important finding is that endophyte inoculation overrode this plastic foraging response, with inoculated plants maintaining deep rooting regardless of nitrogen status.

The combined analysis also detected significantly greater root tip number, greater root system perimeter, and a more vertically oriented root system in inoculated plants. Together these traits describe a root system that is not only deeper but also more extensive and more directionally organized, that is, inoculation shifted root architecture toward a phenotype that explores a greater soil volume in the vertical dimension. By contrast, nitrogen availability significantly affected only depth and solidity among the architectural traits examined, and even for these it influenced the magnitude rather than the direction of the inoculation effect. The general absence of significant inoculation × nitrogen interactions across the architectural traits mirrors the pattern seen for biomass partitioning in Experiment II, and reinforces the conclusion that an important component of endophyte-mediated root modification operates independently of the plant's nitrogen status. As with the biomass partitioning results, this nitrogen-independence is consistent with a phytohormone-mediated contribution to root growth promotion acting in parallel with nitrogen fixation, though the present study cannot demonstrate this mechanism directly.

The convergence of evidence across the three experiments is worth emphasizing. The pot experiments showed that inoculation rebalances biomass among organs and root classes; the hydroponic rhizobox experiment III showed that inoculation also reorganizes the architectural traits of the root system; and in both cases a substantial part of the effect was independent of

nitrogen supply. That these findings, generated in different growth systems and with separately propagated plant material, point in the same direction supports the interpretation that Salicaceae endophyte consortia exert coordinated, multi-scale effects on *Populus* root functional biology.

4.6 Nitrogen fixation and auxin production as parallel, counteracting mechanisms

The pattern of root responses observed across our experiments is most coherently explained by the simultaneous action of two endophyte-associated mechanisms that influence root growth in opposing directions. On one hand, the reduction in root-to-shoot ratio with inoculation, consistent across both pot experiments and independent of nitrogen supply, is consistent with relief from nitrogen limitation reducing the plant's reliance on foraging root growth and permitting proportionally greater investment in shoot tissue. On the other hand, the concurrent increases in absolute total root biomass, coarse root biomass, rooting depth, root tip number, and perimeter cannot be explained by relaxed foraging traits alone, since relief from nitrogen stress would be expected to reduce, not increase, root proliferation. These increases in absolute root growth are instead consistent with direct stimulation of root development by endophyte-derived auxin. Many of the strains in these consortia are documented IAA producers, and WP1, one of the strongest auxin-producing strains in the collection from which these consortia were assembled, is present in both the Mix I and Mix II consortia (Xin *et al*., 2009b; Khan *et al*., 2012). Auxin is a central regulator of lateral root initiation, root elongation, and gravitropic growth, providing a plausible mechanism for the greater rooting depth, root tip number, and more vertically oriented root systems observed in inoculated plants, including under high nitrogen where a foraging-driven increase in rooting would not be expected. Viewed together, these observations suggest that the

net root phenotype of endophyte-inoculated *Populus* may reflect the integration of two counteracting processes: a nitrogen-fixation-driven reduction in foraging root traits that lowers the root-to-shoot ratio, and an auxin-driven stimulation of root proliferation and elongation that increases absolute root size and reorganizes root architecture. Considering both nitrogen fixation and auxin production provides a more comprehensive explanation for the observed responses. This perspective reconciles the reduction in root-to-shoot ratio with the increase in absolute root growth and may also explain why changes in root architecture were not strongly dependent on nitrogen availability, as auxin production by the endophytes can occur regardless of the host's nitrogen status. Because phytohormone production was not quantified in the present study, this interpretation remains inferential. A direct measurement of endophyte-derived auxin in planta, alongside quantification of nitrogen fixation represents an important next step for distinguishing the relative contributions of these two mechanisms.

4.2 Limitations and future directions

4.2.1 Verification of endophyte colonization and persistance

Several limitations of the present study should be acknowledged when interpreting these findings. First, endophyte colonization of *Populus* roots was not directly verified following inoculation. Although the consistency and magnitude of the phenotypic responses strongly suggest that the endophytes established and exerted their effects, we cannot exclude the possibility that some portion of the response reflects factors associated with the inoculation procedure rather than colonization itself, and we cannot characterize how colonization density or distribution varied among plants or treatments. Future studies should incorporate direct verification of colonization

to confirm endophyte persistence and abundance over the course of the experiment and to relate colonization to the magnitude of host response.

4.2.2 Quantification of biological nitrogen fixation

Second, while the growth and architectural responses observed are consistent with biological nitrogen fixation as a contributing mechanism, this study did not directly quantify nitrogen fixation rates or the transfer of fixed nitrogen to host tissue. The nitrogen-independent component of the biomass partitioning and architectural responses, in particular, indicates that fixation alone cannot account for all of the observed effects, but the present design cannot partition the response into fixation-dependent and fixation-independent components. Incorporation of ^{15}N isotope dilution or natural abundance approaches in future experiments would provide direct evidence of Salicaceae activity in planta and allow this partitioning to be made. Recent work has shown that nitrogenase gene expression and nitrogen fixation activity in *Populus* endophytes can be highly dynamic and spatially heterogeneous, and is not necessarily fully repressed even under nitrogen-replete conditions (Sher *et al*., 2024a). This underscores both the complexity of in planta fixation and the value of direct measurement approaches in future work.

4.2.3 Differences in growth environments

Third, all three experiments were conducted in controlled greenhouse environments using pot- and rhizobox-based systems. These systems impose spatial constraints on root development that may not fully reproduce the architectural plasticity expressed in field soil. The rhizobox system in particular constrains root growth toward a largely two-dimensional plane against the imaging

surface, which may have limited the expression of foraging behaviors that operate in three dimensions and may have influenced the absolute values of the architectural traits measured. The architectural responses documented here should therefore be regarded as evidence of an endophyte effect under controlled conditions, but their magnitude and form in field soil remain to be established. Field-based assessments using approaches such as soil coring or excavation-based root phenotyping would provide a more ecologically realistic test of whether the responses observed translate to plantation conditions.

4.2.4 Exclusion of high nitrogen plants in Experiment I

A limitation of Experiment I is that the exclusion of high-nitrogen plants reduced the original factorial design to a single-factor comparison and prevented evaluation of nitrogen effects or inoculation × nitrogen interactions. More importantly, the event that necessitated this exclusion, an unintended water deficit, may itself have influenced the inoculation responses measured in this experiment. Water stress triggers a broad cascade of physiological and metabolic responses in *Populus*, and Salicaceae endophytes of the type used here are known to modulate these responses, improving water-use efficiency and drought tolerance by altering stomatal behavior and root-zone metabolism (Banan *et al*., 2024; Khan *et al*., 2016; Hendrickson *et al*., 2025). It is therefore possible that part of the pronounced inoculation benefit observed in Experiment I reflects endophyte-mediated alleviation of water stress, in addition to effects on nitrogen status and root growth. Because water availability was not a controlled variable and water-related physiological traits were not measured, this possibility cannot be evaluated directly, but it represents a plausible contributor to the strong responses seen here and a clear motivation for future experiments in which water status is explicitly manipulated. A further consideration is that all plants shared a

common aboveground environment before exclusion, so interactions between stressed and non-stressed plants may have influenced microenvironmental conditions. For these reasons, findings from Experiment I are interpreted as inoculation responses under low nitrogen and water-variable conditions, and are considered alongside Experiments II and III rather than in isolation.

4.2.5 Strain specific effects and phytohormone contributions

Finally, the use of multi-strain consortia, while appropriate for testing the practical potential of these inoculants, precludes attribution of specific effects to individual strains and does not reveal whether interactions among consortium members shaped the responses observed. The consortium-level differences detected along PC2 indicate that strain composition does matter. Strain-level dissection of consortium effects combined with characterization of the nitrogen fixation and phytohormone biosynthesis capacities of the constituent strains represents a productive direction for mechanistic follow-up.

Together, these limitations underscores clear research needs which are- the verification of colonization, quantification of biological nitrogen fixation and phytohormone production, assessment of responses under field conditions, and resolution of the contributions of individual strains.. Addressing these questions will be essential to translating the consistent greenhouse responses documented here into reliable, predictable outcomes in *Populus* production systems under variable nitrogen environments.

5 Conclusion

This study provides a multi-experiment characterization of the effects of Salicaceae endophyte consortia on root functional traits in *Populus*, integrating two pot-based biomass allocation experiments with image-based root architectural phenotyping across two independent hydroponic experimental rounds. The three experiments were designed to test whether endophyte inoculation reduces root-to-shoot ratio, shifts the balance of biomass between fine and coarse roots, and alters root system architecture.

Our first hypothesis, that inoculation would reduce the root-to-shoot ratio, was supported across both pot experiments. Inoculated plants had lower root-to-shoot ratios than mock-inoculated controls in both experiments; this reduction was statistically significant in Experiment II ($p = 0.022$) and showed the same direction, though marginally non-significant, in Experiment I ($p = 0.064$). The consistency of the direction of this effect across two independent experiments indicates that endophyte inoculation shifts a greater proportion of biomass toward shoot tissue in *Populus*. However, the mechanism originally proposed for this effect, that it would arise from nitrogen-fixation-mediated relief of nitrogen limitation, was not supported. The reduction in root-to-shoot ratio was independent of nitrogen availability in Experiment II, and nitrogen fixation was not directly measured. The data therefore indicate that endophyte effects on biomass partitioning are not attributable to nitrogen supplementation alone and are consistent with the involvement of additional, nitrogen-independent mechanisms such as phytohormone-mediated regulation of root growth.

Our second hypothesis, that inoculation would shift root biomass allocation toward coarse roots, was tested in Experiment I and was supported at the level of relative allocation. Inoculated plants had significantly greater coarse root biomass and total root biomass, and a substantially lower fine-

to-coarse root ratio, than mock-inoculated controls. Fine root biomass was not reduced in absolute terms; rather, coarse root biomass increased proportionally more than fine root biomass, so that the balance of investment among root classes shifted toward coarse, structural roots. Principal component analysis confirmed that these shifts were part of a coordinated and whole-plant response. The first principal component, which explained 60.9% of total trait variance, separated mock-inoculated plants characterized by high root-to-shoot ratio, high fine-to-coarse root ratio, and high specific leaf area from inoculated plants, which were associated with greater coarse and total root biomass, net photosynthetic rate, and shoot biomass.

Our third hypothesis, that inoculation would alter root system architecture, was supported in the hydroponic rhizobox experiment III. Inoculated plants developed significantly deeper root systems than mock-inoculated controls, and rooting depth was the most consistent architectural response, significant in both experimental rounds and confirmed after adjustment for initial plant size. Inoculation was also associated with greater root tip number, greater root system perimeter, and a more vertically oriented root system, indicating that inoculation produced a deeper and altered root system architecture overall rather than a change in depth alone.

Taken together, these results show that Salicaceae endophyte consortia alter root functional traits and biomass partitioning in *Populus* in a coordinated manner that is consistent across independent experiments and separately propagated plant batches. From an applied perspective, the consistency and magnitude of these effects suggest that Salicaceae endophyte consortia merit further evaluation as biological inoculants for improving below-ground performance in *Populus* production systems where nitrogen inputs are limited. The shift toward greater coarse structural root biomass may be of particular relevance to perennial bioenergy and restoration plantings,

although this study did not measure field performance or soil conditions, and those links remain to be established. Translating these greenhouse-based responses into field-scale outcomes will require verification of endophyte colonization persistence under ambient soil conditions, direct quantification of biological nitrogen fixation in planta, and assessment of root architectural responses in three-dimensional soil environments.

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Appendix

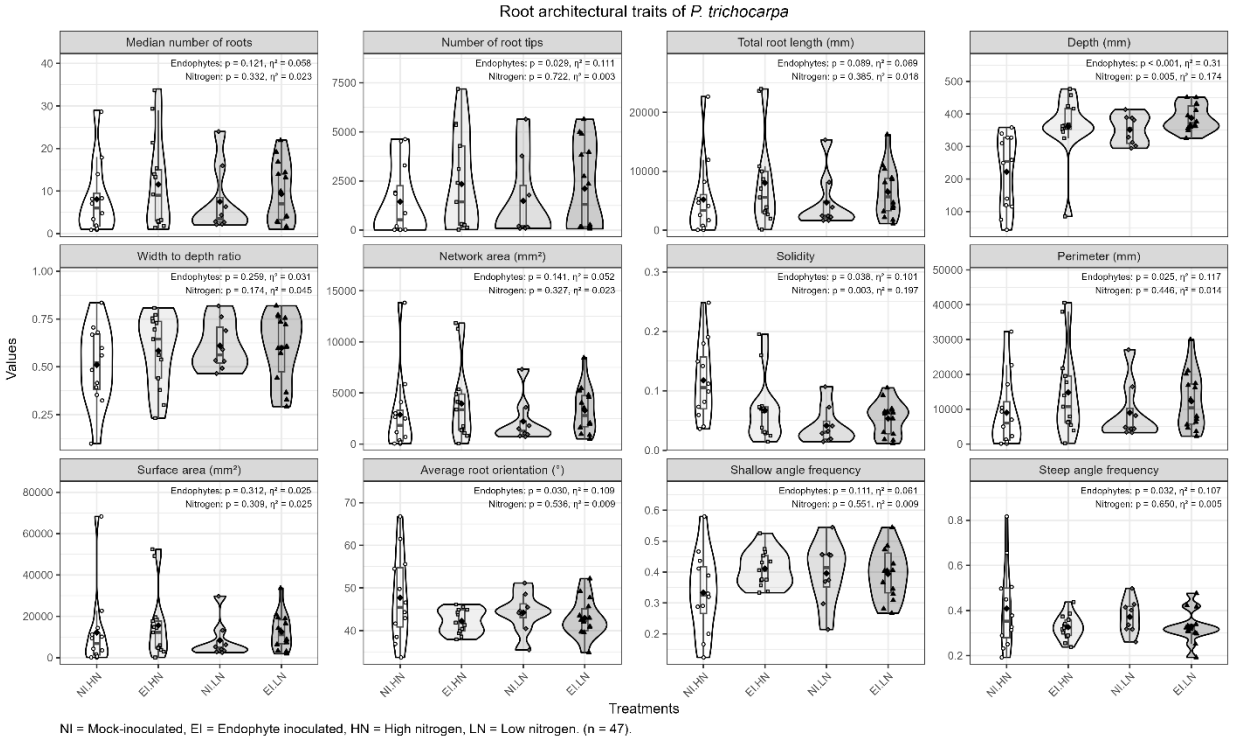

Fig. S1 Main effect of endophyte inoculation on root system architectural traits of *Populus trichocarpa* grown under high nitrogen (HN) and low nitrogen (LN) conditions. Plants were either mock-inoculated (NI; open white circles = NI.HN, gray-filled diamonds = NI.LN) or inoculated with a diazotrophic endophyte consortium (EI; gray-filled squares = EI.HN, black-filled triangles = EI.LN). Data were combined from two independent experimental rounds using separate tissue culture-propagated plant batches (n = 47; NI.HN = 12, EI.HN = 13, NI.LN = 8, EI.LN = 14). Violins show the data distribution, boxes indicate the interquartile range, horizontal lines indicate the median, and diamonds indicate the treatment mean. Individual data points are shown as jittered symbols. P-values and η^2 effect sizes shown in each panel are for the endophyte inoculation (Endophytes) and nitrogen availability (Nitrogen) main effects from two-way ANOVA.

Table 3. Root system architectural traits of *Populus trichocarpa* (Nisqually-1) measured from experiment III (Rhizobox experiment**)** images at final harvest using RhizoVision Explorer (Seethepalli *et al*., 2021). Values are treatment group means ± SE from the combined analysis of two independent experimental rounds (NI HN = 12, EI HN = 13, NI LN = 8, EI LN = 14). P-values and η² effect sizes are from two-way ANOVA

Traits	**NI HN (mean ± SE)**	**EI HN (mean ± SE)**	**NI LN (mean ± SE)**	**EI LN (mean ± SE)**	**Inoculation p-value**	**Inoculation η²**	**Nitrogen p-value**	**Nitrogen η²**	**Interaction p-value**
Median number of roots	11.0 ± 2.6	15.0 ± 3.5	6.0 ± 1.8	10.0 ± 2.1	0.121	0.058	0.332	0.023	0.741
Number of root tips	1,443 ± 519	2,339 ± 692	1,479 ± 755	2,107 ± 585	**0.029***	0.111	0.722	0.003	0.463
Total root length (mm)	5,191 ± 1,901	8,044 ± 2,135	4,717 ± 1,694	6,464 ± 1,151	0.089	0.069	0.385	0.018	0.597
Depth (mm)	222 ± 33	361 ± 26	351 ± 17	388 ± 11	**<0.001***	0.310	**0.005***	0.174	0.056
Width-to-depth ratio	0.49 ± 0.07	0.40 ± 0.06	0.32 ± 0.05	0.35 ± 0.04	0.259	0.031	0.174	0.045	0.319
Network area (mm²)	3,298 ± 1,319	5,592 ± 1,606	2,731 ± 1,254	3,843 ± 699	0.141	0.052	0.327	0.023	0.696
Solidity	0.12 ± 0.02	0.07 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	**0.038***	0.101	**0.003***	0.197	**0.046***
Perimeter (mm)	9,013 ± 2,953	14,798 ± 3,498	8,991 ± 3,012	12,281 ± 2,195	**0.025***	0.117	0.446	0.014	0.459
Surface area (mm²)	4,648 ± 2,598	7,124 ± 2,477	3,264 ± 1,731	4,494 ± 871	0.312	0.025	0.309	0.025	0.798
Average root orientation (°)	47.7 ± 2.9	42.2 ± 0.8	44.2 ± 1.7	43.0 ± 1.2	**0.030***	0.109	0.536	0.009	0.120
Shallow angle frequency	0.31 ± 0.04	0.38 ± 0.02	0.34 ± 0.02	0.37 ± 0.02	0.111	0.061	0.551	0.009	0.321
Steep angle frequency	0.41 ± 0.05	0.33 ± 0.02	0.37 ± 0.03	0.33 ± 0.02	**0.032***	0.107	0.650	0.005	0.322

*NI = mock-inoculated; EI = endophyte-inoculated; HN = high nitrogen; LN = low nitrogen. * p < 0.05.*

Table 4. Root system architectural traits of *Populus trichocarpa* (Nisqually-1) measured from experiment III (Rhizobox experiment**)** and their biological description (Seethepalli *et al*., 2021).

Traits	**Physical / biological description**
Median number of roots	Median count of roots intersected by horizontal line scans across all rows of the segmented image; reflects the typical number of roots present along the width of the system.
Number of root tips	Count of tip pixels in the skeletonized image; indexes the number of root apices, the primary sites of nutrient and water uptake and meristematic activity.
Total root length (mm)	Summed length of the root skeleton; represents the overall linear extent of the root system and its potential for soil exploration.
Depth (mm)	Vertical extent of the root system within the imaging plane; indicates the degree of vertical exploration of the substrate.
Width-to-depth ratio	Maximum width divided by depth; lower values indicate a deeper, narrower root system, higher values a shallower, more laterally spreading system.
Network area (mm²)	Total number of root pixels in the segmented image; provides an integrated measure of overall root system size in the imaging plane.
Solidity	Ratio of network area to convex hull area; higher values indicate a more compact, space-filling root architecture, lower values a more open, exploratory system.
Perimeter (mm)	Total length of the root boundary in the segmented image; indexes the extent of the root-medium interface.
Surface area (mm²)	Sum of cross-sectional perimeters across all medial axis pixels; estimates the total root surface available for uptake.
Average root orientation (°)	Mean orientation of medial axis pixels computed over local 40×40 pixel neighbourhoods; lower values indicate more vertically directed root growth.
Shallow angle frequency	Proportion of local medial axis orientations falling below 30°; reflects the frequency of more horizontally directed root segments.
Steep angle frequency	Proportion of local medial axis orientations falling between 60° and 90°; reflects the frequency of more vertically directed root segments.