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Research

Biological nitrogen fixation and biomass accumulation within poplar clones as a result of inoculations with diazotrophic endophyte consortia

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Summary

- Sustainable production of biomass for bioenergy relies on low-input crop production. Inoculation of bioenergy crops with plant growth-promoting endophytes has the potential to reduce fertilizer inputs through the enhancement of biological nitrogen fixation (BNF).
- Endophytes isolated from native poplar growing in nutrient-poor conditions were selected for a series of glasshouse and field trials designed to test the overall hypothesis that naturally occurring diazotrophic endophytes impart growth promotion of the host plants.
- Endophyte inoculations contributed to increased biomass over uninoculated control plants. This growth promotion was more pronounced with multi-strain consortia than with single-strain inocula. Biological nitrogen fixation was estimated through ^{15}N isotope dilution to be 65% nitrogen derived from air (Ndfa).
- Phenotypic plasticity in biomass allocation and branch production observed as a result of endophyte inoculations may be useful in bioenergy crop breeding and engineering programs.

Introduction

Diazotrophic microorganisms, those that fix nitrogen, have been isolated as free living in the soil (Rozycki *et al.*, 1999) and water (Short *et al.*, 2004), in the rhizosphere and as symbionts in both leguminous and nonleguminous plants (Gallon, 1992). Microbes living asymptomatically within plant tissue are known as endophytes (Wilson, 1995). Diazotrophic endophytes are recognized for having a variety of plant growth-promoting properties. Additionally, many diazotrophic endophytes produce plant hormones and have been shown to alter the plant's own stress response systems; a tactic that likely allows colonization with the apparent side effect of inducing enhanced abiotic stress tolerance (Doty, 2011).

Khan *et al.* (2012) demonstrated an overall positive growth response in several commercially important food crops inoculated with diazotrophic endophytes originally isolated from poplar and willow trees thriving in nutrient-poor substrate. Profound increases in plant growth including earlier flowering and increased fruit yields were seen. For example, cherry tomato *Lycopersicon lycopericum* cv 'Glacier', when inoculated with *Rahnella* strain WP5, had significantly more root and shoot biomass, more flowers (108 vs 65), and more fruits (95 vs 43) than controls. In this same study, a 203% increase of total nitrogen content of root tissue was reported for 'Brightstar SLT' perennial rye grass (*Lolum perenne*) inoculated with a multi-strain consortium (PTD1, WPB, WP19, WP1 and WW6) indicating nitrogen fixation. Similarly, Xin *et al.* (2009b) reported that the inoculation of Kentucky bluegrass (*Poa pratensis* L.) with the poplar endophyte *Burkholderia vietnamiensis* WPB increased biomass by 42% and total plant nitrogen content by 37% over the uninoculated control treatment. Nitrogenase activity of the poplar endophyte *B. vietnamiensis* WPB was previously demonstrated through both an acetylene reduction assay and ^{15}N incorporation (Xin *et al.*, 2009b).

While the majority of research has focused on single-strain isolates from a plant system, Oliveira *et al.* (2002) recognized the importance of the naturally existing endophytic community in sugarcane production, noting that the highest nitrogen content from biological fixation occurred for a consortia, over inoculation with a single endophytic isolate. Govindarajan *et al.* (2008) also reported increased yield and plant biomass with endophyte inoculations where the greatest gain was observed for treatments with mixed strain inoculum. Interestingly the uninoculated plants were not sterile and even contained a small community of diazotrophic endophytes in the root tissue, and the number of colony forming units of bacteria was greater in the inoculated plants. These results suggest that experimental additions of diazotrophs may improve on the natural relationship between plants and the endophytic community. While the observed growth enhancement in plants inoculated with diazotrophic endophytes is well accepted, the specific mechanism(s) remain an area of continued investigation (Sevilla *et al.*, 2001).

The increasing cost of fossil fuels, along with concern for the environmental impact of extraction and use, has positioned renewable energy to be the fastest growing source of primary energy in the near future. To adequately feed biofuel refineries,

biomass must come from a variety of sources maximizing the current production of agricultural land, as well as utilizing marginal land. Marginal land can be described as any nonarable, nutrient-deprived or polluted lands unfit for crop production. Such marginal lands may be well suited for woody biomass production. Forest tree species are attractive as a sustainable source for cellulosic biomass. Particular attention has been given to *Populus* (poplar) tree species due to their capacity for a high rate of growth and ease of propagation (Stettler *et al.*, 1996). These tree species are well suited for growth on marginal lands as they are capable of establishment on nutrient-poor substrate. The purpose of this body of work is to test the hypothesis that naturally occurring diazotrophic endophytes isolated from perennial tree species that thrive in nutrient-limited conditions impart plant growth promotion to their host plants.

Two separate glasshouse experiments were conducted using clonal ramets inoculated with diazotrophic endophytes. The first experiment investigated the hypothesis that inoculations with multi-strain consortia have a larger effect on plant growth promotion than single-strain endophyte inoculations. The second experiment was an isotope dilution assay to test the hypothesis that growth promotion is due at least in part to increased nitrogen availability as a result of biological nitrogen fixation. A long-term field experiment was established to test the effect of endophyte inoculations on biomass production in a more operationally relevant environment. Leaf physiology measurements were taken mid-growing season for the first 2 yr to test the hypothesis that endophyte inoculations alter biomass production by influencing the net CO_2 assimilation rate.

Materials and Methods

Endophytes and inoculations

Eleven bacterial and two yeast strains were chosen from a collection of diazotrophic endophytes isolated from cottonwood and willow species native to the Snoqualmie River in Washington State (Doty *et al.*, 2005, 2009; Xin *et al.*, 2009a,b) for use as single species inocula or as members of multi-strain consortia (Table 1). Isolates were chosen for investigation based on culturability, IAA production, strong growth on nitrogen-free media (Doty *et al.*, 2009), the presence of the *nif*H gene (Doty *et al.*, 2005, 2009), and nitrogenase activity (Xin *et al.*, 2009b) indicating their capacity to fix nitrogen and proven plant growth promotion in other plant species (Xin *et al.*, 2009b; Khan *et al.*, 2012; Knoth *et al.*, 2013).

Inoculation suspensions were prepared as previously described by Knoth *et al.* (2013). The endophyte treatments were delivered to plants in rooting media by injecting the endophyte suspension 3–6 cm below the surface and in close proximity to the roots. Each inoculation contained the equivalent optical density at 600 nm of 0.1 for 25 ml. Uninoculated control groups received injections with the equivalent volume of sterile liquid media.

In order to verify colonization, fresh root, stem and leaf tissues of equal size were removed from each tree for the purpose of endophyte isolations. All tissue was washed with detergent to remove soil and rinsed several times with clear tap water. Root and stem tissue was surface sterilized for 5 min in 1% sodium hypochlorite, then washed 4 times with sterile distilled water. Leaf tissue was surface sterilized for 2 min in 1% sodium hypochlorite, and then washed 4 times with sterile distilled water. Approximately 100 mg of each sample was then macerated in 500 ml of isotonic solution and serially diluted using aseptic technique in a sterile hood, then 100 µl of each of the final dilution (10^{-4}) was plated on nonselective manitol glutamate/Luria-Betani (MG/L) agar and nitrogen-free NFCCM (Rennie, 1981) agar. Colony counts were assessed after 24 and 72 h incubation at room temperature. In addition, freshly cut explants of the surface sterilized tissue were plated directly on both MG/L agar and NFCCM agar. Many of the endophytes produce prodigious exopolysaccharides making accurate enumeration difficult, therefore those results are not reported.

Plant materials

All growth and physiology experiments were carried out using ramets of *Populus trichocarpa* (Torr. & A. Gray) clone Nisqually-1. A line of internally sterile ramets was established and maintained under antibiotic pressure in tissue culture for use in Expt 1. The antibiotic treatment was rotated to prevent microbial resistance and periodic checks were performed to ensure no culturable endophytes were prevalent in the tissue culture population. Rooted cuttings from the cottonwood hybrid, *Populus trichocarpa* × *Populus deltoides* (Bartr.), clone H11-11, were used in Expt 2, the ^{15}N isotope dilution assay. The ramets for Expt 3, the field trial, originated as cuttings taken from dormant ramets of Nisqually-1 clones maintained by the School of Environmental and Forest Sciences (SEFS) at the University of Washington in the field.

Experiment 1: glasshouse single-strain endophyte and consortia inoculation trial

Internally sterile Nisqually-1 ramets were transferred from tissue culture into individual 4-inch square pots (2.17 l 'The Square One'; McConkey, Sumner, WA, USA) containing Sunshine Mix B2 (SunGro, Bellevue, WA, USA), a low-nitrogen soil mixture. Plants were removed from the tissue culture media and rinsed with sterile distilled water to remove any remaining agar. Transplanted cuttings were then watered with filtered deionized water until the soil was completely saturated. The cuttings were allowed to acclimatize to the glasshouse conditions of average temperature 21°C and 14 h photoperiod for 31 d. During this time the plants received only tap water irrigation. Inoculation was performed as described above.

A total of 63 pots, seven replications of each of nine endophyte treatments, were arranged in a randomized complete block design on one glasshouse bench. Drip trays were placed under each individual pot to prevent cross-contamination through run-off. Each pot was individually irrigated with tap water through an automated system. Irrigation frequency and duration was adjusted such that the pots were not allowed to flood nor dry out between watering. Additionally, each ramet received 100 ml of ½ strength,

Table 1 Description of the diazotrophic endophytes and consortia composition

Isolate name	Closest 16S rDNA match	IAA	*nif*H	Reference	Included in inoculum mix
PTD-1	*Rhizobium tropici* bv *populus*	+	+	1	Consortium A Poplar Mix A Poplar Mix B1 Poplar Mix B2
WPB	*Burkholderia vietnamiensis*	+	*nif*H, D, K	2, 3	Poplar Mix B2
WP5	*Rahnella* sp.*CDC* 2987-79	+	+	2	Consortium A Poplar Mix A Poplar Mix B1 Poplar Mix B2
WP7	*Enterobacter* sp YRL01	+	nd	2	Consortium A Poplar Mix A Poplar Mix B1 Poplar Mix B2
WP8	*Pseudomonas graminis* PDD-13b-3	+	nd	2	Consortium A Poplar Mix A Poplar Mix B1 Poplar Mix B2
WP19	*Acinetobacter*	–	–	2	Consortium A Poplar Mix A Poplar Mix B1 Poplar Mix B2
WW2	*Herbaspirillum* sp	+	nd	2	Consortium A Willow Mix
WW5	*Sphingomonas yanoikuyae*	+	+	2	Consortium A Willow Mix
WW6	*Pseudomonas putida*	+	+	2	Consortium A Willow Mix
WW7	*Spingomonas* ZnH-1	+	+	2	Consortium A Willow Mix
WW11	*Sphingomonas yanoikuyae*	+	+	2	Consortium A Willow Mix
WP1	*Rhodotorula graminis*	+	+	4	Consortium A Poplar Mix A Yeast Mix
PTD3	*Rhodotorula mucilaginosa*	+	nd	4	Consortium A Poplar Mix A Yeast Mix

Details of the molecular characterization of the endophytes are found in the original research reports as noted: 1, Doty *et al.* (2005); 2, Doty *et al.* (2009); 3, Xin *et al.* (2009b); 4, Xin *et al.* (2009a).
nd, not determined.

nitrogen-free modified Hoagland's media containing ($g\,l^{-1}$): 0.22 $CaCl_2(2H_2O)$, 0.17 K_2SO_4, 0.26 $MgSO_4(7H_2O)$, 0.136 KH_2PO_4, 0.015 NaFeDTPA(10% Fe) with 1 $ml\,l^{-1}$ micronutrient solution containing ($g\,l^{-1}$: 0.773 H_3BO_3, 0.169 $MnSO_4$, 0.288 $ZnSO_4(7H_2O)$, 0.062 $CuSO_4(5H_2O)$ and 0.04 H_2MoO_4 (83% MoO_3)) weekly for the first 2 months post inoculation then 250 ml twice monthly until the end of the experiment.

Initial weight was measured at the time of transplanting from tissue culture to the glasshouse pots. Leaf greenness due to chlorophyll was measured throughout the study and recorded as SPAD units using the nondestructive, handheld Konica Minolta SPAD 502 (Konica Minolta, Ramsey, NJ, USA) chlorophyll meter. The SPAD units recorded are an average of three readings taken from the second leaf from the top of each plant. Plant height was measured as the distance between the root collar and the main shoot apex. Height measurements were recorded at 97 d after inoculation (dai) and again at 135 dai. Final measurements of total weight, fresh root weight and green leaf area were made 135 d after inoculation; calculated measurements include the root:shoot ratio, green weight gain and change in height. Leaf tissue samples from plants inoculated with three of the endophyte consortia were sampled for dimethyl sulphoxide (DMSO) chlorophyll extraction as described by Richardson *et al.* (2002). Root, stem and leaf tissue samples were taken from each plant and oven dried at 65°C, combined and ground to a fine powder before being analyzed for total nitrogen and carbon content using a PE 2400 Series II CHN elemental analyzer (Perkin Elmer, Waltham, MA, USA; CHN work was carried out at the University of Washington's School of Environmental and Forest Sciences Chemical Analysis Center).

Experiment 2: estimation of biological nitrogen fixation through isotope dilution

A ^{15}N isotope dilution assay was conducted with the objective to estimate the amount of nitrogen gained through biological

fixation within poplar cuttings inoculated with the diazotrophic endophyte consortium Poplar Mix B2 (see Table 1). Nitrogen occurs naturally in two isotopic forms, ^{15}N and ^{14}N, where ^{14}N is in much greater abundance. The ^{15}N content in nitrogen-fixing plant tissue is the result of the native nitrogen acquired through soil, N_2 from the atmosphere and nitrogen from the applied ^{15}N fertilizer (Danso *et al.*, 1993). To estimate the amount of nitrogen acquired through fixation, nitrogen derived from air (Ndfa), the ^{15}N content of nonfixing reference plants is compared to that of the nitrogen fixer. In this assay the uninoculated control plants served as the nonfixing reference for the inoculated plants (Oliveira *et al.*, 2002). However, the uninoculated plants were not internally sterile and therefore might have contained some native endophytes. The equation used to estimate the %Ndfa is as follows:

$$\%\text{Ndfa} = 100 \times \left(1 - \frac{\delta‰15\text{N atm. excess Inoculated Plant}}{\delta‰15\text{N atm. excess Uninoculated Control}}\right).$$

Rooted cuttings of the cottonwood hybrid, *Populus trichocarpa* × *P. deltoides* clone H11-11 were weighed before transplanting to 4-inch square pots, with individual drip pans, containing low-nutrient Sunshine Mix B2. The pots were prepared by adding 0.35 kg of root media per pot followed by 700 ml of tap water to wet the soil. The cuttings were each given 200 ml of ½ strength modified Hoagland's solution containing 4 mM NH_4NO_3 and then maintained with tap water for a 2-month acclimation period. The glasshouse temperature was kept at an average of 21°C with a 14 h photoperiod. The multi-strained consortium (Poplar Mix B2) consisted of the endophytic bacteria WPB, WP5, WP7, WP8, WP19, PTD-1 and the yeast WP1 (described in Table 1). Before inoculating the plants, 100 µl of the endophyte solution and the sterile media were spread on nutrient-rich, MG/L agar to check the colony count. The live mix contained 3.9×10^8 colony forming units (CFU) per milliliter with multiple colony types of the expected morphology. The sterile media grew no colonies. Five ramets were inoculated with 30 ml of endophyte suspension and five ramets were inoculated with 30 ml of sterile nitrogen-free Murashige and Skoog. To each pot 200 ml of ½ strength Hoagland's solution containing 23.78 mg of $(NH_4)_2SO_4$ with 10% excess ^{15}N was added 13 d after inoculation. Plants were fertigated with 250 ml nitrogen-free ½ strength Hoagland's solution every 2 wk and watered with distilled water as needed. Sixty-four days after receiving the ^{15}N, the plants were harvested and cleaned of root media using distilled water, and weight measurements of the whole plant were taken. Equal portions of root tissue were removed and set aside for endophyte re-isolation. Root, stem and leaf tissue were then separated and oven dried at 70°C for 3 d. The dry tissue was again weighed and then ground to a fine powder with mortar and pestle. Dried tissue samples were sent to the Alaska Stable Isotope Facility (ASIF) at the Water & Environmental Research Center at the University of Alaska Fairbanks. Stable isotope data was obtained using continuous-flow isotope ratio mass spectrometry (CFIRMS). Stable isotope ratios were reported as parts per thousand (‰) deviations from the international standards PDB Air (nitrogen).

Experiment 3: long-term field trial

The experimental field site is located in the Charles L. Pack Experimental Forest, Pierce County, Washington State. The soil at this site is characterized as sandy, glacial outwash of the Indianola series (mixed, mesic Dystric Xeropsamments) (Gaulke *et al.*, 2006). This site is excessively drained, nutrient limited and receives full sun. Before the installation of this study half of the site was covered in blackberry bushes and half of the site had been used the previous summer for a short-term field trial with an annual crop (Knoth *et al.*, 2013). Site preparation included removal of any existing vegetation along with the top layer of soil (*c.* 30 cm) of soil followed by disk tilling. The 26 m × 54 m site was surrounded by a 2-m tall exclosure to protect the poplar trees from deer and elk browse. Individual tree plots were arranged in eight rows of 18 plots with 2.5 m between row plots and within rows. Available soil nitrogen analysis was performed for each plot 2 wk before planting. Three evenly spaced soil cores from each plot were taken from the top 40 cm, mixed and bagged on site. The soil samples were held on ice during transportation from the field to limit any effects from biological nitrification. Nitrogen analysis was performed by the UW SEFS Chemical Analysis Center.

A total of 144 ramets representing 36 replications of each of the four endophyte treatments were arranged to conform to a randomized complete block design. A statistical analysis of the randomized plot assignments was made to ensure similar initial soil nitrogen concentrations were represented between the endophyte treatment groups.

Before planting at the field location, the cuttings were allowed to root for 3 wk receiving irrigation with tap water as needed to maintain soil moisture. Inoculations were carried out as described above using two single-strain inocula, WP1 and WPB, one multi-strain consortium, Poplar Mix B2, and sterile medium as a control. The plants were inoculated two times – 1 wk apart – to ensure sufficient exposure. Fresh endophyte preparations were made for each inoculation. The inoculated poplar ramets were relocated to a glass house at the Charles L. Pack Experimental Forest and allowed to acclimatize for 2 wk before planting in the field site. The ramets were carefully transplanted along with the rooting media so that the fragile root zone remained as undisturbed as possible. All ramets were transplanted in the same day. Manual irrigation was applied during the first growing season to protect against drought. Other site maintenance included weed removal within a 40-cm radius of each tree and manual trimming between rows. The second growing season received continued overgrowth maintenance but no further irrigation.

Tree height and diameter at 10 cm above the root collar measurements were collected five times at nearly monthly intervals during the first growing season. Soil samples were gathered from each tree plot and the total available soil nitrogen content was measured to correspond with the growth measurements. Dominant stem height was measured twice in the second growing

season. Biomass was estimated nondestructively by calculating volume (V) assuming a paraboloid shape (Bruce & Schumacher, 1950) for the major stem and branches measured over 5 cm in length using the equation: $V = ½\ AL$ (A, sum of the area from each stem and branch at 10 cm from its base; L, sum of the height of the major stem and the length of each branch from where it meets the stem).

Ramets from each inoculation treatment group were randomly chosen to investigate the effect of inoculations on leaf level physiology. Ten ramets were chosen from each inoculation treatment group for instantaneous CO_2 assimilation rate measurements in the first growing season. Gas exchange was measured using the LI-6400XT portable photosynthesis system (LI-COR, Lincoln, NE, USA) from the second most developed leaf from the top of each ramet. In the first growing season instantaneous photosynthesis was measured at ambient and saturating CO_2 concentrations, 380 and 700 ppm, respectively, with the block temperature of 25°C, and the photosynthetically active radiation (PAR) set to 1500 µmol photons $m^{-2}_{leaf\ area}\ s^{-1}$. Leaf CO_2 assimilation rate (A_{max}), transpiration rate (E) and stomatal conductance (g_s) were recorded after a minimum stabilization time of four minutes. The photosynthetic response to carbon dioxide concentration at the intercellular air space inside leaf (A/C_i) was measured in the second growing season from five ramets per inoculation treatment group selected from the same group measured the previous year. A/C_i measurements were used to estimate the Rubisco capacity (V_{cmax}), the potential rate of electron transport (J_{max}), and triose phosphate utilization (TPU) rate using the estimator utility provided by Sharkey *et al.* (2007). These measurements were made at 25°C and 1500 µmol $m^{-2}\ s^{-1}$ PAR. In both years, the leaf used for gas exchange measurement was collected immediately following the final data recording. The leaf tissue was then transported on ice and held at −80°C. Leaf nitrogen (leaf N) content and total chlorophyll content was quantified as described above. Photosynthetic nitrogen use efficiency (PNUE) was calculated as net photosynthesis per leaf N content.

In August of the second growing season, three trees from each inoculation treatment that had previously been measured for gas exchange data were sacrificed to measure biomass allocation. The top 10 cm of soil containing roots from the grassy ground cover was removed from each plot. Trees were cut at the root collar and all aboveground biomass was bagged and placed in a 75°C oven for 1 wk. The aboveground biomass was reported as the total dry mass of all stems and leaves of each ramet. For the determination of the root biomass, a 50 cm^3 section of soil surrounding each tree was removed and passed through a ¼" screen on site. The root ball and remaining root tissue collected from the soil was bagged and placed in the 75°C oven along with the aboveground biomass for 1 wk. Before weighing the dry root tissue, contents of each bag were passed through a one-eighth-inch screen to remove any persistent soil.

Statistical analysis

All experiments were designed for contrast of treatment means to the corresponding uninoculated control group. Data were analyzed using the SAS statistical software v9.3 (SAS Institute Inc., Cary, NC, USA). Initial green weight of the individual trees and initial total available N of the soil in the field trial were used as covariates. Duncan's multiple range test (DMRT) and the CONTRAST statement within PROC GLM were used to compare endophyte treatment means between the endophyte inoculation treatments. Unless otherwise stated, all tests were performed with alpha = 0.05.

Results

Experiment 1: glasshouse single-strain endophyte and consortia inoculation trial

Endophyte-inoculated ramets of internally sterile *Populus trichocarpa* clone Nisqually-1 demonstrated no signs of pathogenesis; rather, all inoculation treatments exhibited some positive affect on growth (Table 2). Consortia inoculations demonstrated a more positive growth effect than single-strain inoculations. Inoculation with the Poplar Mix B1, Willow Bacteria Mix and Consortium A produced significantly more biomass than the uninoculated control group; 110% ($P < 0.001$), 84% ($P = 0.02$) and 73% ($P < 0.01$), respectively (Table 2). Single-strain inoculations resulted in less biomass gain over the control with 13%, 11% and 8% gain from inoculation with WP5, WW7 and WP1, respectively. Biomass allocation for all treatment groups favored root production (Table 2).

Plant height at 97 d after inoculation (dai) was significantly greater between the three consortia inoculation treatments, Consortium A, Poplar Mix A and Poplar Mix B1, and the uninoculated control group with 90% confidence (Table 2). This difference, however, was no longer statistically significant at 135 dai. Ramets inoculated with the Poplar Mix B1 had significantly greater total leaf area at 97 dai and 135 dai than the control group at the 99% and 95% level of confidence, respectively. Ramets inoculated with Consortium A had significantly greater leaf area 97 dai at the 90% confidence level; however, difference in leaf area was no longer significant 135 dai (Table 2).

Endophyte inoculation showed an increase in total nitrogen content, with up to a 25% increase observed for Poplar Mix A inoculated ramets. While there was a notable response (Table 3), not all endophyte treatments increased the total plant nitrogen content over the control group ($P = 0.1731$). Total plant carbon content was significantly higher for the control group ramets than those inoculated by all endophyte treatments except for those inoculated with Consortium A ($P = 0.0243$) (Table 3).

Total leaf area and leaf SPAD response to endophyte inoculations were varied (Table 3). Significant increases in leaf SPAD measurements compared to the control group (24.33) were observed for Poplar Mix A (26.87, $P \leq 0.01$) and Willow Mix (26. 53, $P \leq 0.05$) inoculated ramets 97 dai. The total leaf area was significantly larger 97 dai for Consortium A (89.65 cm^2, $P \leq 0.05$), and Poplar Mix B1(105.51 cm^2, $P \leq 0.01$) treatments than for the control (76.33 cm^2). Poplar Mix A (25.79, $P \leq 0.01$), Willow Mix (23.24, $P \leq 0.01$), Poplar Mix B1 (21.56, $P \leq 0.05$) and the Yeast Mix (21.33, $P \leq 0.1$), treatment groups

Table 2 Glasshouse gnotobiotic experiment

Endophyte treatment	Final green weight (g)	Weight gain (g)	Root weight (g)	Root/shoot ratio	Height change (cm)	Height (cm) 97 dai	Height (cm) 135 dai
Consortium A	14.69 (4.00)**	13.88 (3.87)**	13.40 (3.32)**	6.54 (0.629)**	0.22 (0.15)	16.23 (2.51)**	16.22 (2.62)
Poplar Mix A	11.34 (2.37)	10.69 (2.26)	9.99 (1.64)	6.69 (0.49)**	0.31 (0.21)	16.51 (1.58)*	16.27 (1.52)
Willow Mix	15.47 (2.09)*	14.74 (1.96)*	13.02 (1.66)**	7.9 (0.39)**	0.33 (0.13)	15.46 (1.23)	15.79 (1.18)
Poplar Mix B1	17.88 (3.46)**	16.89 (3.20)**	14.95 (2.97)**	5.71 (0.53)*	1.04 (0.72)	16.14 (1.22)*	17.19 (1.04)
Yeast Mix	10.64 (1.83)	9.86 (1.74)	9.16 (1.55)	6.58 (0.65)	0.27 (0.144)	13.97 (1.66)	14.24 (1.09)
WP5	9.78 (1.20)	9.11 (1.14)	8.35 (1.00)	6.11 (0.56)	0.30 (0.22)	14.62 (1.37)	14.92 (1.26)
WW7	9.66 (1.48)	8.96 (1.39)	8.077 (1.22)	5.32 (0.43)	-0.13 (0.27)	13.37 (1.10)	13.24 (1.55)
WP1	9.37 (2.16)	8.67 (2.03)	7.61 (1.78)	4.64 (0.45)	0.37 (0.48)	14.84 (1.59)	15.21 (1.56)
Control	8.81 (1.59)	8.03 (1.55)	6.57 (1.08)	4.35 (0.44)	1.08 (1.05)	14.19 (0.76)	16.4 (1.02)
Contrasts $P > F$							
Inoculated vs control	0.0895	0.0801	0.0325	0.0063	0.0467	0.2517	0.5639
Consortia vs single isolate	0.0025	0.0020	0.0012	0.0011	0.0392	0.0754	0.0449

Growth parameters of ramets of *Populus trichocarpa* clone Nisqually-1 inoculated with multi-strain or single-strain diazotrophic endophytes. Unweighted means of growth variables (SE). Biomass gain and allocation measured 135 d after inoculation (dai) with diazotrophic endophyte consortia or single-strain isolates as indicated. Asterisks denote significant differences from the control: *, alpha = 0.05; **, alpha = 0.01; $n = 7$.

Table 3 Glasshouse gnotobiotic experiment

Endophyte treatment	Leaf area (cm^2) 97 dai	Leaf area (cm^2) 135 dai	SPAD 97 dai	SPAD 135 dai	Nitrogen% DW 135 dai	Carbon% DW 135 dai
Consortium A	89.65 (23.06)*	68.21 (19.45)	20.69 (2.49)	19.81 (2.66)	1.14 (0.04)	44.15 (0.66)
Poplar Mix A	69.53 (11.32)	53.50 (8.90)	26.87 (2.08)**	25.79 (1.78)**	1.35 (0.11)*	42.72 (0.54)
Willow Mix	72.36 (7.92)	72.84 (20.50)	26.53 (1.11)*	23.24 (1.06)**	1.17 (0.07)	43.49 (0.45)
Poplar Mix B1	105.51 (18.77)**	89.97 (14.62)	21.43 (0.59)	21.56 (1.13)*	1.07 (0.06)	42.84 (0.89)
Yeast Mix	67.09 (10.94)	56.79 (10.66)	23.7 (1.60)	21.33 (1.73)•	1.06 (0.07)	43.41 (0.56)
WP5	66.26 (7.30)	55.64 (13.47)	24.22 (2.18)	17.08 (1.36)	1.29 (0.13)•	42.81 (0.62)
WW7	66.64 (9.53)	55.07 (8.95)	22.29 (1.57)	19.66 (2.24)	1.10 (0.06)	41.45 (1.34)**
WP1	69.64 (15.04)	68.47 (14.33)	23.17 (2.58)	18.40 (1.82)	1.07 (0.08)	41.09 (0.83)**
Control	76.33 (12.20)	61.11 (14.24)	24.33 (2.06)	17.09 (1.67)	1.08 (0.09)	44.39 (0.30)
Contrasts $P > F$						
Inoculated vs control	0.6049	0.4921	0.3960	0.1882	0.5833	0.0324
Consortia vs single isolate	0.0512	0.1295	0.6133	0.0059	0.8695	0.0016

Phenotypic response of ramets of *Populus trichocarpa* clone Nisqually-1 inoculated with multi-strain or single-strain diazotrophic endophytes. Unweighted means of leaf physiology variables (SE) 97 and 135 d after inoculation (dai). SPAD is a nondestructive measurement of leaf chlorophyll reported in relative units; higher SPAD value corresponds to higher leaf chlorophyll. Total plant %N and %C were measured 135 dai. Symbols denote significant differences from the control: •, alpha = 0.1; *, alpha = 0.05; **, alpha = 0.01; $n = 7$.

had significantly higher SPAD measurements than the uninoculated control (17.09) 135 dai. At 135 dai, however, total leaf area demonstrated a slight overall decline, but the differences between treatment groups were not statistically significant. Four treatment groups – Consortium A, Willow Mix, Poplar Mix B1 and the uninoculated control – were analyzed for total extracted chlorophyll content. Endophyte inoculations had an overall marginally significantly positive impact on total extractable chlorophyll ($P = 0.073$). Ramets inoculated with Willow Mix and Poplar Mix B1 each contained 15% more extracted total chlorophyll and ramets inoculated with Consortium A had 4% more extracted total chlorophyll than the Control group (Fig. 1). Overall, the endophytes increased greenness (SPAD), leaf area and total chlorophyll content compared to the uninoculated trees.

Experiment 2: estimation of biological nitrogen fixation through isotope dilution

This experiment was designed to test nitrogen fixation by diazotrophic endophytes under very limited nitrogen conditions and differs from Expt 1 in that a different poplar clone (H11-11) was used and that the ramets were not internally sterile. Plant growth promotion was not observed for endophyte-inoculated ramets compared to the uninoculated controls. Though not statistically significant, biomass gain was less for the endophyte-inoculated ramets than the control ramets (Fig. 2a). Although total nitrogen content was not significantly affected by endophyte inoculation, inoculated ramets had higher total nitrogen content in root tissue and lower nitrogen content in stem tissue. The total nitrogen content of the leaf tissue was

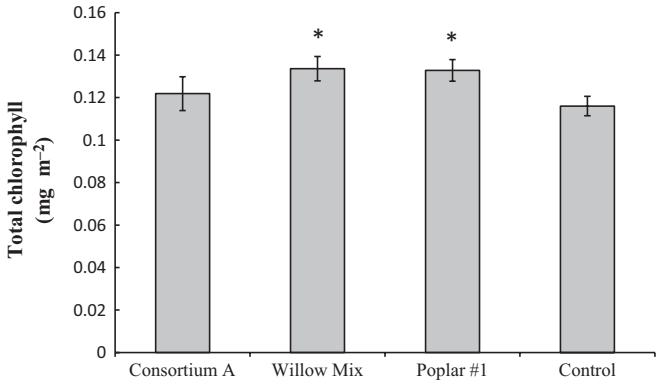

Fig. 1 Total chlorophyll content of select endophyte treatments of *Populus trichocarpa* clone Nisqually-1 inoculated with multi-strain or single-strain diazotrophic endophytes. Comparison of total dimethyl sulphoxide (DMSO) extracted chlorophyll between clonal ramets inoculated with multi-strain endophyte consortia and the internally sterile, uninoculated control. Bars indicate group means ± SE, * indicates significant difference from control at alpha = 0.05 ($n = 7$).

the same for inoculated ramets as the uninoculated controls (Fig. 2b). In terms of isotope dilution, the ‰ ^{15}N atmospheric excess was lower in the stem tissue ($P = 0.216$) and significantly less in leaf tissue ($P = 0.031$) of the inoculated ramets (Fig. 2c), suggesting that nitrogen fixation occurred in these tissues. No difference in isotope concentration was observed in the root tissues of inoculated ramets suggesting that the biological nitrogen fixation occurred in the aboveground tissues only. BNF was calculated as 65% of total nitrogen based on isotope dilution values in leaf tissue and 45% based on the combined isotope dilution observed in the leaf and stem tissues. Calculations for %Ndfa are based on the uninoculated control plants as the nonfixing reference. However, bacterial growth was recovered on nitrogen-free media from all ramets; therefore, the %Ndfa calculation is an estimate of the biological nitrogen fixation contribution from inoculations over that which occurred in the glasshouse-grown uninoculated poplar.

Experiment 3: long term field trial

The effects of endophytes on poplar clone performance seen in the glasshouse and lab experiments also occurred in an operational field setting, although the response was less consistent in a heterogeneous environment. Endophyte inoculation had a significant effect on main stem height ($P < 0.001$) and aboveground volume ($P = 0.0023$). The inoculation treatment WP1 produced the tallest ramets and the most aboveground biomass gain as measured by increase in total volume over the course of the first growing season. Total increase in main stem height and aboveground volume accumulated over 12 months for WP1-inoculated ramets were 35% and 38.4% more than the uninoculated control group (Fig. 3a,b), respectively. Height gain and volume gain were most negatively affected by inoculation with WPB, while plant growth was nearly equal between the Poplar Mix B2 consortium inoculated ramets and the uninoculated control group. Endophyte inoculation also had a marginally significant

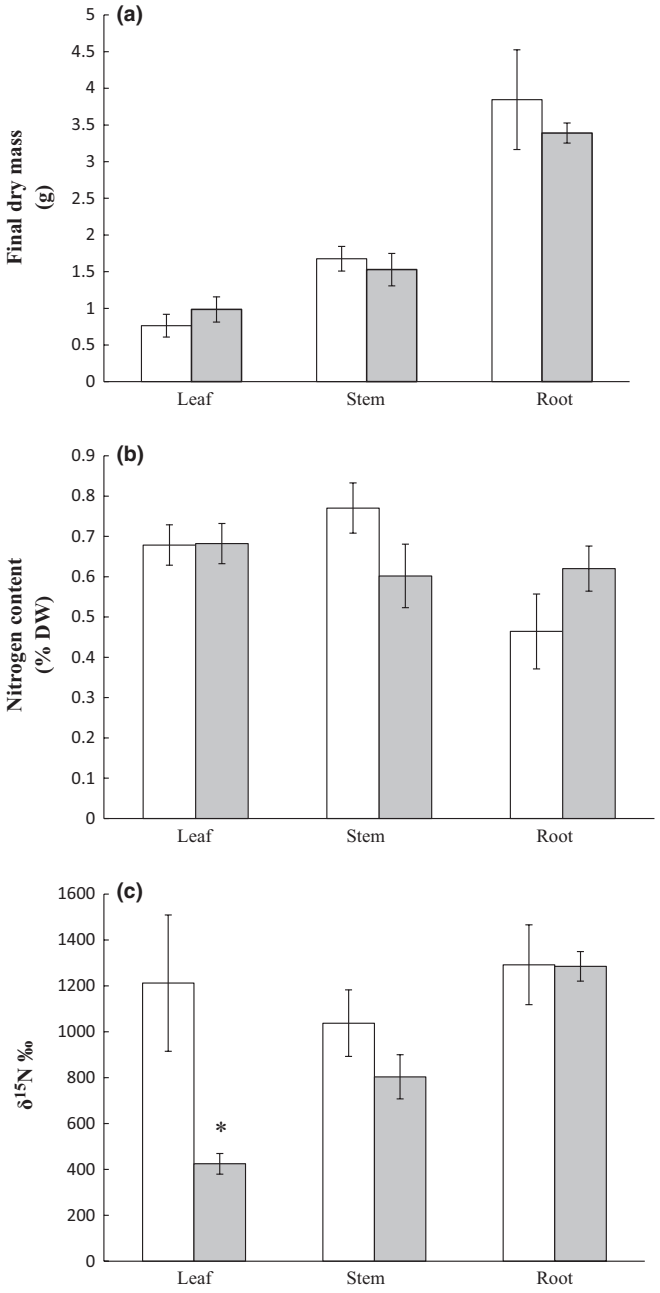

Fig. 2 Glasshouse isotope dilution assay to estimate BNF (biological nitrogen fixation) within ramets of hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) clone H11-11 inoculated with endophyte consortium Poplar Mix B2. Endophyte-inoculated plants were grown in ^{15}N enriched soil for 64 d; (a) final dry biomass by tissue type, (b) total nitrogen content by plant tissue and (c) atmospheric excess ^{15}N concentration by plant tissue. *indicates significant difference from control at alpha = 0.05 ($n = 3$). Bars indicate group means ± SE; open bars, uninoculated control; closed bars, Poplar Mix B2 inoculation group.

effect on the total number of branches, sylleptic and proleptic, measured mid-growing season in year two ($P = 0.060$). Ramets inoculated with WP1 had the most branches (9.5), while those inoculated with WPB (6.4) had the least with no difference between the Poplar Mix B2-inoculated ramets (7.4) and the control group (7.6) (Fig. 3c).

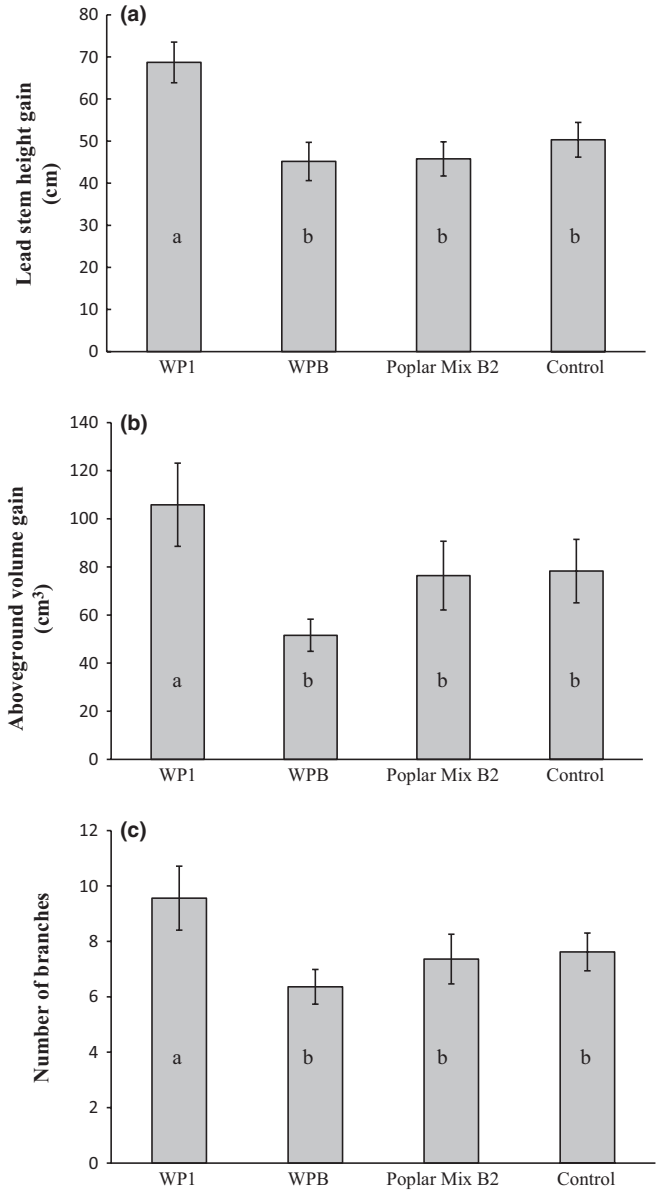

Fig. 3 Field trial comparison of biomass gain between ramets of *Populus trichocarpa* clone Nisqually-1 inoculated with multi-strain or single-strain diazotrophic endophytes. Bars represent treatment means ± SE for endophyte-inoculated poplar Nisqually-1 ramets compared to the uninoculated control group. (a) Height gain, (b) aboveground volume gain and (c) number of branches formed for a 12-month period. Bars indicate group means ± SE. Different letters indicate means are significantly different at alpha = 0.05 using Duncan's multiple range test (DMRT) ($n = 36$).

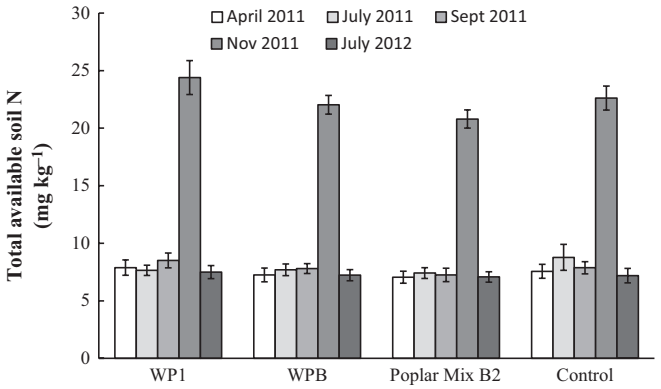

Fig. 4 Change in soil available nitrogen (N). Mean soil N over time where total available N is the sum of the NO_3N and NH_4N soil content. Soil N concentrations are not depleted throughout the growing season. The large N deposit at the onset of dormancy suggests that excess N acquired through biological nitrogen fixation is being redistributed to the roots of *Populus trichocarpa*. Bars represent treatment group means ± SE ($n = 36$).

There was no statistically significant difference for soil nitrogen content available for plant uptake between the endophyte inoculation treatment groups for samples taken during the first and second growing season. Soil nitrogen content increased for all treatment groups in early November with the largest increase occurring in the soil within plots of the endophyte treatment group WP1 (Fig. 4).

The net CO_2 assimilation rate recorded in both the first and second years was not significantly different at the ambient (380 ppm) or saturated (700 ppm) CO_2 concentrations between inoculation treatment groups. No significant differences were observed for stomatal conductance (g_s) or transpiration rate (E) CO_2 (380 ppm). At saturating CO_2 (700 ppm) there was no significant difference between inoculation groups for g_s; however, WP1-inoculated ramets recorded a significantly increased rate of transpiration (Table 4). The calculated photosynthetic nitrogen use efficiency (PNUE) was not significantly different between treatments at either concentration of CO_2 (data not presented).

Endophyte inoculation treatments did not have a significant effect on the photosynthetic response to changes in CO_2 concentration (Fig. 5). There was also no significant difference for the estimated rates of V_{cmax}, J_{max} and TPU. Leaf nitrogen content was not found to be significantly different between treatment groups at any time. The total chlorophyll content recorded in the first growing season, however, was significantly affected by endophyte inoculation (ANOVA, $P = 0.03$). Poplar Mix B2 inoculated plants had the lowest chlorophyll of all treatments and remained lowered in the second season but the difference was no longer statistically significant.

The biomass data collected from the destructively harvested samples were marginally significant for biomass allocation (root : shoot ratio, $P = 0.098$). The results agree with the findings that WP1-inoculated ramets had considerably more aboveground biomass than all other treatments, with a 44% increase in dry biomass over the control group. Ramets inoculated with the Poplar Mix B2 gained 10% aboveground biomass over the control and WPB inoculated ramets measured 33% less aboveground biomass than the uninoculated control group (Table 5). Total root biomass was nearly the same for all endophyte inoculation treatments. While the WPB-inoculated ramets gained less overall biomass, the allocation to the roots was significantly greater than any other endophyte treatment with 44% ($P < 0.05$) of the total dry biomass going to root tissue. Aboveground biomass accumulation was 72%, 70% and 65% more than belowground biomass accumulation for WP1, Poplar Mix B2 and the control treatment groups, respectively.

Table 4 First-year leaf physiology data for *Populus trichocarpa* clone Nisqually-1 inoculated with multi-strain or single-strain diazotrophic endophytes

Inoculum	Net CO_2 assimilation (A) μmol CO_2 m^{-2} s^{-1}	Transpiration (E) mmol H_2O m^{-2} s^{-1}	Conductance (g_s) mol H_2O m^{-2} s^{-1}	Leaf N% dry weight	Total chlorophyll mg cm^{-2}
A_{380}WP1	15.38 ± 0.70	3.83 ± 0.17	0.23 ± 0.0084	2.49 ± 0.18	0.028 ± 0.0015•
WPB	14.98 ± 1.31	3.79 ± 0.16	0.23 ± 0.0096	2.51 ± 0.29	0.032 ± 0.0020
Poplar Mix B2	14.51 ± 0.77	3.83 ± 0.25	0.23 ± 0.0113	2.40 ± 0.25	0.025 ± 0.0023**
Control	15.54 ± 0.65	3.50 ± 0.13	0.21 ± 0.0064	2.79 ± 0.20	0.033 ± 0.0026
A_{700}					
WP1	22.36 ± 3.2	6.48 ± 0.45•	0.24 ± 0.0246		
WPB	24.59 ± 6.6	5.80 ± 1.35	0.21 ± 0.0764		
Poplar Mix B2	21.11 ± 4.5	5.87 ± 0.53	0.22 ± 0.0317		
Control	23.18 ± 2.9	6.16 ± 0.42	0.22 ± 0.0230		

Inoculation group means ± SE, $n = 36$. Leaf physiology is not significantly affected by endophyte inoculation at the ambient CO_2 (A_{380}) concentration. At saturating CO_2 (A_{700}) concentration inoculation with WP1 significantly increases the transpiration rate. Total Chlorophyll content was significantly affected by endophyte inoculation (ANOVA $P = 0.03$). Symbols indicate significant difference between the means of treatment group and control: •, alpha = 0.1; **, alpha = 0.01.

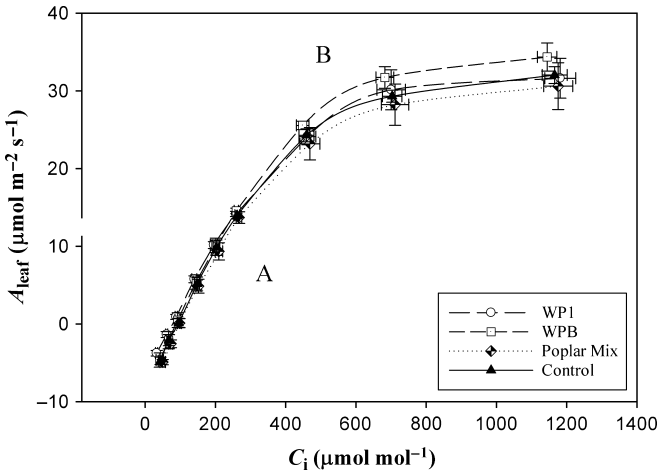

Fig. 5 Second-year leaf CO_2 assimilation data for *Populus trichocarpa* clone Nisqually-1 inoculated with multi-strain or single-strain diazotrophic endophytes. A/C_i curves for each endophyte inoculation treatment represent the treatment average with two-way error bars. No change in slope between treatments at 'A' suggests the Rubisco activity is not altered between the treatment groups. Some effect on the rate of RuBP regeneration is observed between endophyte treatments, 'B', but this difference is not statistically significant ($n = 5$).

Discussion

Together, the experiments reported in this article support the overall hypothesis that plant growth and vigor are directly related to the composition of the endophytic community within the host plant and provide further evidence of biological nitrogen fixation in nonleguminous forest tree species. Research indicating nitrogen fixation within poplar was reported in the early 1980s. Wetwood samples of *Populus deltoides* (Schink *et al.*, 1981) and *P. trichocarpa* (Kamp, 1986) produced positive results for the acetylene reduction assay (ARA). Recent metabolic profiling of micropropagated hybrid poplar inoculated with a *Paenibacillus* strain displayed that in comparison to uninoculated controls, inoculated plants had eight-fold more asparagine and six-fold more urea (Scherling *et al.*, 2009). From the results of our ^{15}N isotope dilution assay we infer that mineral N from the soil remains in the roots while nitrogen allocated to the leaves and stem is derived largely from BNF and suggests potential signaling mechanisms for optimized nitrogen allocation. The nitrogen fixation observed in this study would support poplar colonization of nutrient-deficient sites. No plant growth promotion was observed as a result of inoculation with the Poplar Mix B2 endophyte consortium after the 64 d assay. This retardation of plant growth is indicative of the carbon cost for establishing endophytic relationships in long-lived trees as compared to in annual plants. These results are similar to those reported by Anand & Chanway (2013) who demonstrated that conifer tree growth promotion due to inoculation with diazotrophic endophytes occurred after an initial suppression of plant growth. They hypothesized that the delay is a result of the energy and carbon costs for nitrogen fixation and results indicate that plant growth promotion by diazotrophic endophytes in long-lived tree species becomes greater over time. Follow-up studies of the long-term field trial will allow us to see if this is true in poplar.

Our data indicate that leaf-level net CO_2 assimilation rate was not altered for poplar ramets between the endophyte inoculation treatment groups. This is consistent with the findings of Rogers *et al.* (2012) who reported no significant effect on leaf-level physiology after inoculations with the plant growth-promoting endophyte *Enterobacter* sp. 638. Endophyte inoculations did, however, contribute to differences in total leaf area and aboveground biomass. Total CO_2 assimilation and subsequent growth is likely to have increased in plants with greater leaf area given the same net CO_2 assimilation rate per leaf area. By contrast the leaf level net CO_2 assimilation rate increased in maize inoculated with the same poplar endophytes employed in the current study (Knoth *et al.*, 2013). This suggests that the mechanism for growth promotion is influenced by the specific interaction of the host plant and the endophytic microbial community.

The results of our glasshouse trial support the hypothesis that multi-strain endophytic communities have a greater influence on

Table 5 Biomass allocation of *Populus trichocarpa* clone Nisqually-1 inoculated with multi-strain or single-strain diazotrophic endophytes. Mean belowground and aboveground biomass ± SE

Inoculum	Root dry mass (g)	Total above ground dry mass (g)	Root: shoot ratio
WP1	63.07 ± 4.47	161.8 ± 34.43	0.41 ± 0.06
WPB	59.33 ± 10.13	75.37 ± 20.56	0.89 ± 0.21*
Poplar Mix B2	53.03 ± 7.88	124.1 ± 12.82	0.44 ± 0.10
Control	60.70 ± 21.82	112.73 ± 60.17	0.67 ± 0.12

The root: shoot ratio is indicative of the biomass allocation favoring root growth in WPB-inoculated ramets. * indicates means significantly different than the control group at alpha = 0.05; $n = 3$.

plant biomass than single-strain inoculations. These results are consistent with previous research indicating greater BNF and biomass gain is attained from multi-strain mixtures of endophytes than single-strain inoculations (Oliveira *et al.*, 2002; Govindarajan *et al.*, 2008; Knoth *et al.*, 2013). Phenotypic variation between the mixed-strain inoculation groups are indicative of the different role each endophytic strain may have in plant growth promotion (Riggs *et al.*, 2001; Oliveira *et al.*, 2002; Govindarajan *et al.*, 2008).

Variation in biomass allocation and branching patterns within a single *P. trichocarpa* genotype were observed between the different endophyte treatments. For example, differences in the occurrence of sylleptic branches in ramets of the *P. trichocarpa* clone Nisqually-1 were affected by endophyte inoculations in both Expts 1 and 3. Branch formation within poplar species has been well characterized and is known to be under strong genetic control (Wu & Hinckley, 2001) where phenotype variance observed within species or the same genotype is the result of environmental variation (Wu & Stettler, 1997, 1998; Ma *et al.*, 2008). The effect of nitrogen fertilization on the genetic regulation of growth and wood chemistry traits was recently reported by Novaes *et al.* (2009). Their findings were consistent with previous research reviewed by Wu & Hinckley (2001) indicating that resource availability is critical for sylleptic branch formation where the vigor of the main stem is positively correlated with sylleptic branching. Both Expts 1 and 3 were conducted within common garden conditions under nitrogen limitation. The phenotypic response of our experimental endophyte inoculations is therefore a result of the interaction between the host plant's own genetic make-up, the environment and the specific endophyte inoculation (i.e. $G \times E \times Endo$). Variation in branch formation is likely due to variations in nutrient uptake, BNF, plant hormone production or some other mechanism facilitated by the endophytic community. Given the correlation between growth characteristics and wood chemistry (Novaes *et al.*, 2009), further investigation is needed to determine the potential for endophyte inoculations to tailor the production of biomass for bioenergy.

The level of variation within multi-strain inoculum treatment groups in this current report is notable, suggesting that colonization by members of each consortium may have differed within each host plant. Colonization patterns of *Pseudomonas* species within poplar reported by Germaine *et al.* (2004) support the concept of specific niche colonization. In addition to tissue specific colonization, there is evidence that host plant phenology influences the composition and location of the endophytic microbial community (Pirttila *et al.*, 2005; Mishra *et al.*, 2012). Future research to characterize the colonization patterns of individual endophytes and multi-strain consortia used in these experiments are needed to explain the variation observed between ramets within treatment groups. In addition, the specific interaction between the host plant and the endophytic community should be analyzed through gene expression and metabolic assays.

A growing body of research focused on land-use change has begun to look more closely at the effects of forest tree plantations on soil nutrient composition. Lafleur *et al.* (2013) reported an increase of soil mineral weathering in hybrid poplar plantations relative to abandoned agricultural sites thus increasing the availability of nutrients for tree growth. Further, a review of the effects of canopy composition on soil properties highlights the significant influence of overstory composition on the physical, chemical and biological characteristics of top soil (Augusto *et al.*, 2002). To begin to understand the effect of endophyte inoculations on the rhizosphere, we monitored soil nitrogen content throughout the first growing season of the long-term field trial. We observed no remarkable depletion of total available nitrogen in spite of the rapid gain in biomass. This is consistent with the findings of Boothroyd-Roberts *et al.* (2013) who noted no observable effect from 10-yr-old hybrid poplar plantations on soil chemistry, also measured at the peak of the growing season, when compared to abandoned fields. In this same study the authors report an increase in understory regeneration within the poplar plantation as compared to the open fields. We noted a substantial deposit of nitrogen in November just as the trees transitioned into dormancy that could not be attributed to the decomposition of leaf litter and is unlikely to have come from other symbiotic systems in the proximity of the tree plots given the uniformity of the data across the treatment groups. One explanation is that nitrogen is translocated to the roots before leaf abscission. Some of the endophyte strains used in this study have been shown to produce and elute nitrate and ammonia in solution (S. L. Doty, unpublished data). A pulse-chase of labeled ^{15}N would allow testing of the hypothesis that nitrogen fixed in the leaves was then translocated and deposited in the roots.

The establishment of the long-term field trial will allow for future complementary studies needed to elucidate the role of these endophyte inoculations in biological nitrogen fixation, biomass gain, soil fertility and the phenotypic plasticity of their plants hosts.

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