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RESEARCH

Diazotrophic Endophytes of Poplar and Willow for Growth Promotion of Rice Plants in Nitrogen-Limited Conditions

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ABSTRACT

Rice (*Oryza sativa* L.) is one of the most important staple food crops. Its cultivation requires a relatively high input of N fertilizers; however, rice plants do not absorb a significant proportion of added fertilizers, resulting in soil and water pollution. The use of diazotrophic (N-fixing) endophytes can provide benefits for rice cultivation by reducing the demand of N fertilizers. Diazotrophic endophytes from the early successional plant species poplar (*Populus trichocarpa* Torr. & A. Gray) and willow (*Salix sitchensis* C. A. Sanson ex Bong.) were added to rice seedlings. Inoculated rice plants were grown in N-limited conditions in the greenhouse, and plant physical characteristics were assessed. Endophyte-inoculated rice plants had greater biomass, higher tiller numbers, and taller plant stature than mock-inoculated controls. Endophyte populations were quantified and visualized in planta within rice plants using fluorescent microscopy. The endophytes colonized rice plants effectively in both roots and foliage. These results demonstrated that diazotrophic endophytes of the eudicots poplar and willow can colonize rice plants and enhance plant growth in N-limited conditions.

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Abbreviations: BNF, biological N fixation; GFP, green fluorescent protein; IAA, indole-3-acetic acid; MG/L, Mannitol Glutamate/Luria; MS, Murashige–Skoog; NL-CCM, N-limited combined C medium.

Along with wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.), rice is one of the most important food grains worldwide with approximately half of the world's population relying on it for more than one-fifth of its daily calorie intake. While the human population continues to increase, the extent of land available for rice production in many countries is decreasing because of urbanization and industrialization (Khush, 2013). To feed the increasing global population, it is critical to maximize the production potential of rice. With the advent of modern agriculture, there has been much focus on breeding for superior plant genotypes to enhance productivity. Many improved and hybrid rice varieties require comparatively large amounts of N to achieve maximum grain yield. In many rice-growing countries, more than 100 kg N ha^{-1} is applied to rice cultivation (Huang et al., 2008; Wang et al., 2012; Roberts et al., 2013; Singh et al., 2014). However, only a small proportion of the applied N is actually utilized by the crop plants, with a significant portion lost to the environment through denitrification, leaching, and ammonia volatilization (Cassman et al., 2002; Choudhury and Kennedy, 2005). In addition, the cultivation of many food crops is facing unprecedented challenges because of climate change through weather extremes such as storms, intense rain, drought, and heat waves (Tubiello et

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al., 2007). Therefore, it is essential to find environmentally sustainable crop production methods that reduce the demand for N fertilizers in cultivation.

Endophytes are microbial symbionts that colonize the interior of plant tissues without displaying any disease symptoms (Wilson, 1995). They establish an association with a host plant that benefits the health of the plant in several ways including providing biotic and abiotic stress resistance and tolerance, enhancing nutrient availability, degrading toxic substances, and producing plant hormones (Doty, 2011). Many endophyte species use intercellular spaces of the cortical tissue or vascular bundles as the primary colonization sites (James and Olivares, 1997; Gyaneshwar et al., 2001; Germaine et al., 2004; Prieto et al., 2011). The symbiotic association of *Rhizobium* in legumes and mycorrhiza in many plant species and their importance to plant health is well understood. Only recently, the variety of significant benefits of endophytes has begun to come to attention (Bulgarelli et al., 2013), especially in terms of increased nutrient acquisition through biological N fixation (BNF) (Olivares et al., 2013; Santi et al., 2013).

The importance of diazotrophic (N-fixing) endophytes to crop growth is primarily due to their ability to convert dinitrogen gas into usable forms such as ammonium and nitrate (Iniguez et al., 2004; Bhattacharjee et al., 2008; Montanez et al., 2009). Some endophytes also produce significant amounts of plant hormones such as indole-3-acetic acid (IAA) that are crucial for plant growth and development (Lata et al., 2006; Xin et al., 2009a,b; Merzaeva and Shirokikh, 2010; Apine and Jadhav, 2011). Several diazotrophic endophyte species were discovered in poplar and willow plants that were growing in nutrient-limited sandy and rocky riparian environments (Doty et al., 2005, 2009). These endophytic strains have been shown to promote growth in both monocots and eudicots (Xin et al., 2009a; Khan et al., 2012; Knoth et al., 2013) and produce phytohormones and fix atmospheric N thereby stimulating plant growth (Xin et al., 2009a,b; Knoth et al., 2014).

Hybrid cottonwood (*Populus trichocarpa* Torr. & A. Gray × *P. deltoides* W. Bartram ex Marshall) plants inoculated with endophytes of wild poplar and willow had higher biomass than uninoculated control plants (Knoth et al., 2014). It was shown by the ^{15}N isotopic dilution technique that about 65% of the total N in the leaves of inoculated cottonwood was contributed from biological N fixation. We hypothesized that poplar and willow diazotrophic endophytes can similarly colonize rice plants and support plant growth under N-deficient conditions. Our objectives were to quantify the growth promotion conferred by poplar and willow endophytes in rice grown in N-limited medium and to visualize and quantify the endophyte population in rice plant to determine colonization effectiveness.

Table 1. Endophytes previously isolated from poplar and willow plants used in this study.

Poplar endophytes	Closest match†	Willow endophytes	Closest match†
WP1	*Rhodotorula graminis*	WW5	*Sphingomonas yanoikuyae*
WPB	*Burkholderia vietnamiensis*	WW6	*Pseudomonas* sp. H9zhy
PTD1	*Rhizobium tropici*	WW7	*Sphingomonas* sp. ZnH-1
WP5	*Rahnella* sp. CDC 2987-79		
WP9	*Burkholderia* sp. H801		
WP19	*Acinetobacter calcoaceticus*		

† The 16S rRNA gene for each strain was sequenced and identified by using BLAST on NCBI database (Doty et al., 2009).

MATERIALS AND METHODS

Endophyte Strains and Growth Conditions

Previously isolated poplar and willow endophytes (Doty et al., 2009; Table 1) were used for this study. They were originally isolated by selection on a N-free medium and were confirmed to have the nitrogenase subunit gene (*nifH*) by polymerase chain reaction (Doty et al., 2009). They grow efficiently on a N-free growth medium. All endophytes used in this study were cultured at 30°C in Mannitol Glutamate/Luria (MG/L) agar medium (g L^{-1}: 5.0 tryptone, 2.5 yeast extract, 5.2 NaCl, 10.0 mannitol, 1.32 sodium glutamate, 0.50 KH_2PO_4, 0.2 $MgSO_4.7H_2O$, and 10 bacto-agar). The endophytes were transferred to N-limited combined C broth (Solution 1 [g L^{-1}]: 5.0 sucrose, 5.0 mannitol, 0.5 mL L^{-1} sodium lactate, 0.8 K_2HPO_4, 0.2 KH_2PO_4, 0.1 NaCl, 0.025 $Na_2MoO_4 \times 2H_2O$, 0.028 $Na_2FeEDTA$, 0.1 yeast extract; and Solution 2 [g L^{-1}]: 0.2 $MgSO_4 \times 7H_2O$, 0.06 $CaCl_2$) (Rennie, 1981) and cultured overnight in a rotatory shaker at 160 rpm and 30°C. Each endophyte strain was added to N-free Murashige–Skoog (MS) medium (Caisson Inc.) to an optical density (OD_{600}) of approximately 0.01 to make a consortium of 0.1 OD_{600} for inoculation.

The use of endophytes expressing green or red fluorescent protein has become a useful technique to visualize their population and microhabitat in the host plant (Prieto et al., 2011; Quecine et al., 2012; Wright et al., 2013; Thomas and Reddy, 2013). To verify the colonization of endophytes in rice plants, *Burkholderia* sp. strain WPB, *Rhizobium tropici* strain PTD1, and *Rahnella* sp. strain WP5 labeled with green fluorescent protein were used. The broad host range plasmid pBHR1-GFP (Stevens et al., 2005) with constitutive expression of the green fluorescent protein was introduced through electroporation (Knoth et al., 2013).

Plant Material

Rice 'M-206', a commonly grown Calrose medium grain variety in Northern California (Johnson, 2005), was used for this experiment. Before germination, seeds were surface-sterilized in NaClO (2–3% v/v) for up to 4 h and rinsed five to eight times with sterile, deionized water. Aliquots of water from the final rinse were plated on MG/L agar to confirm the sterilization

protocol. The rice seeds were then plated on either water agar plates (0.5%) or sterilized filter paper and allowed to germinate at room temperature.

Endophyte Colonization

For greenhouse experiments, surface-sterilized rice seeds were germinated on water agar plates for 3 to 5 d. The rice seedlings were removed from the agar plates and inoculated with an endophyte consortium in 50 mL conical tubes by placing on a 160 rpm shaker at room temperature for 4 h. For the microscopy and population count studies, seedlings were inoculated with labeled endophytes and incubated overnight to facilitate the colonization of young plant tissues. Inoculated seedlings were grown in N-free MS agar for twenty days in the growth chamber. For both greenhouse and laboratory studies, control plants were mock inoculated with endophyte-free medium.

Plant Growth and Measurement

Inoculated plants were transferred to low-nutrient moss–perlite mix (Sunshine Mix #2; SunGro) and allowed to grow for 1 mo before transplanting to 10.16-cm square pots (McConkey, Sumner) where they were allowed to grow for another 3 mo. They were grown under a 14-hour photoperiod in the Douglas Research Conservatory greenhouse at the University of Washington, Seattle, WA. A completely randomized design with 24 replications for endophyte-inoculated plants and 12 replications for mock-inoculated control plants was used. The plants were rotated weekly to prevent any localized effects of light or airflow. The plants were supplemented with N-free Hoagland's nutrient solution once per week with an additional water supply if required (Knoth et al., 2013).

Plant height was recorded monthly by measuring from the crown to the tip of the highest leaf. Biomass measurements were taken after the plants were harvested and dried at 80°C for a few days. The number of tillers was determined by checking each plant for individual tillers while the plants were still growing in pots.

Fluorescent Microscopy and Enumeration of In Planta Endophyte Populations

Rice plants inoculated with PTD1, WP9, and WP5 labeled with green fluorescent protein were analyzed by fluorescent microscopy. Endophyte colonized specimens were observed using the compound microscope equipped with Axio Imager 2 (Karl Zeiss, LLC). Plant tissues colonized by endophytes were photographed with Zeiss AxioVision Software. For negative controls, mock-inoculated plant samples were used. The images were taken at 630 magnifications with and without a green fluorescent protein (GFP) filter.

Rahnella sp. (WP5) labeled with green fluorescent protein was used to quantify the in planta endophyte populations in rice plants. After 10 d of inoculation, rice plants were harvested and root and shoot, including leaves, were weighed. Plant tissues were ground in the N-limited combined C medium (NL-CCM) using sterile technique and serially diluted from 10^{-1} to 10^{-3} dilutions in the NL-CCM. Aliquots of 100 μL from 10^{-2} and 10^{-3} dilutions were used to plate on selective medium (MG/L with 100 μg mL^{-1} of gentamycin and carbenicillin) using flame-sterilized spreading glass rods. Plates were incubated overnight at 30°C. Following incubation, total colony-forming units were observed and counted on each plate.

Statistical Analysis

Analysis of variance was used to identify the significance of endophyte inoculation on rice growth. Tukey multiple comparison of means was used to compare the growth response between inoculated and control groups. Data were analyzed using R statistical software version 3.0.1 (R Development Core Team, 2013).

RESULTS

Plant Physical Characteristics

Plant height was measured three times during the plant growth period. Mock-inoculated control plants were 23% taller ($p = 0.007$) than their respective inoculated plants 1 mo after planting. Two and four months after planting, the inoculated plants were significantly taller than the control plants ($p \leq 0.001$; Fig. 1) at 14 and 21% height, respectively. Endophyte-inoculated plants had significantly greater total root and shoot biomass than the control plants (Fig. 2).

Root to shoot biomass ratio was calculated using the root dry biomass and shoot dry biomass. The endophyte-inoculated plants had a significantly higher root to shoot ratio than the mock-inoculated control plants (Table 2). Similarly, endophyte-inoculated plants had significantly more tillers, 89% more than respective control plants at harvest (Table 2; $p = 0.01$).

Visualization, Enumeration of *gfp*-Expressing WP5 Endophyte, and Total Plant Biomass

Three endophytes labeled with green fluorescent protein (PTD1, WP5, and WP9) were used to visualize their colonization ability in rice plants. As shown by fluorescent microscopy (Fig. 3), all three strains (PTD1, WP5, and WP9) successfully colonized the rice plants. Endophytes tended to congregate and grow in the intercellular spaces between the plant cells. The mock-inoculated control plants did not show any fluorescent bacteria (data not shown).

Using the selectable marker for kanamycin resistance encoded by the fluorescence plasmid and the natural resistance of WP5 to carbenicillin, we were able to quantify the total number of colony-forming units in the inoculated plants. WP5 labeled with green fluorescent protein efficiently colonized all of the rice plants used in this assay. Higher endophyte populations (colony-forming units per gram of plant tissue) were observed in the roots when compared with the stem and leaves ($p = 0.134$; Fig. 4). Higher plant biomass was observed in the endophyte-inoculated plants than mock-inoculated controls, though it was not statistically significant in this 20-d experiment ($p = 0.18$; Fig. 4). Extracts of the mock-inoculated control plants did not show any growth in the culture plates with antibiotics.

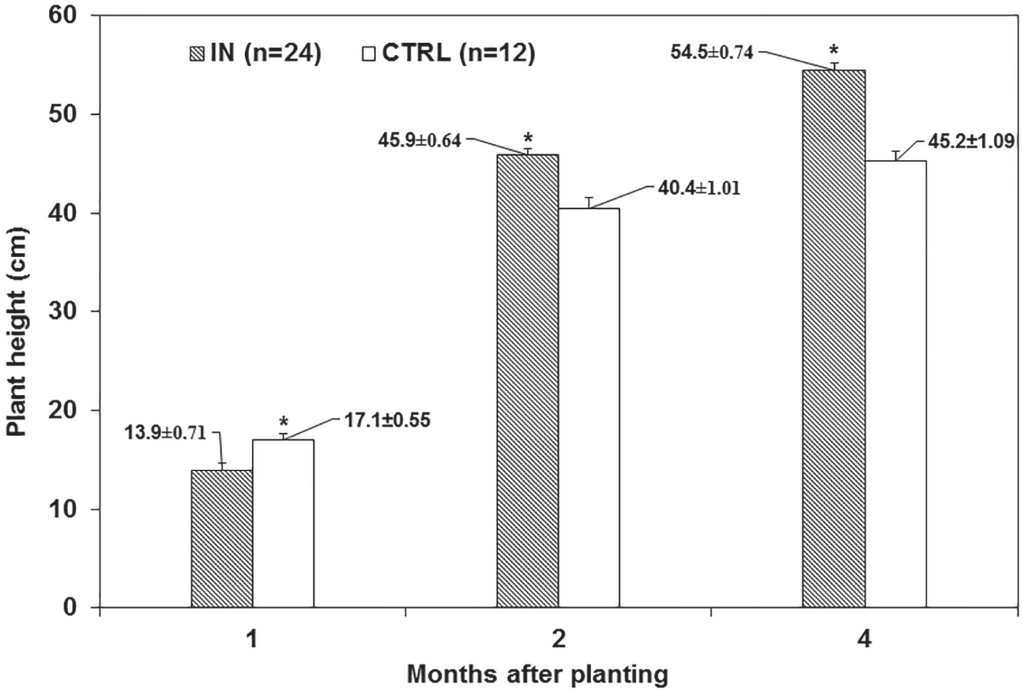

Figure 1. Plant height of endophyte-inoculated (IN) and control (CTRL) plants at different periods of growth stages. The bars represent the standard errors of mean. Histograms with asterisk indicate significant differences ($p < 0.01$).

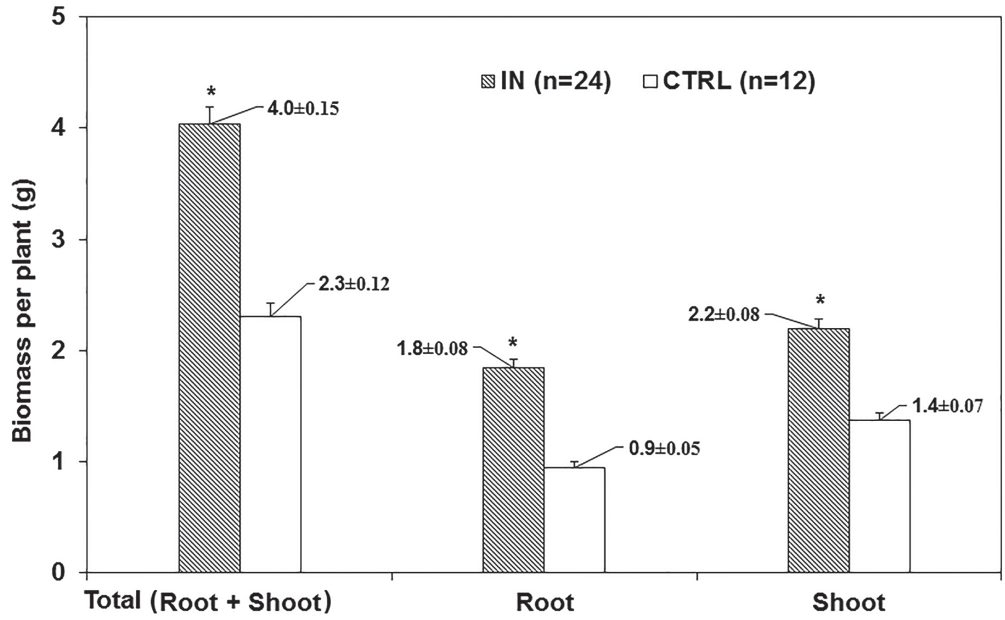

Figure 2. Root, shoot, and total plant biomass in endophyte-inoculated (IN) and control (CTRL) plants at 4 mo after planting. The bars represent the standard errors of mean. Histograms with asterisk indicate significant differences ($p < 0.01$).

Table 2. Root/shoot ratio and tiller numbers per plant in endophyte-inoculated and mock-inoculated plants. Treatment means were compared using Tukey multiple comparison statistic; means not sharing a letter are significantly different ($p \leq 0.01$).

Treatments	Root/shoot ratio	Tiller no. per plant
Endophyte inoculation	0.84 ± 0.025a	2.83 ± 0.196a
Mock inoculation	0.69 ± 0.027b	1.50 ± 0.150b

DISCUSSION

We have shown for the first time that diazotrophic endophytes from poplar and willow plants can colonize and promote growth and development in rice. Since rice panicles were not fully emerged during harvesting, plant biomass and tillering capacity were used to assess the growth stimulation facilitated by endophytes. Tiller numbers are an important yield trait and were considered as a

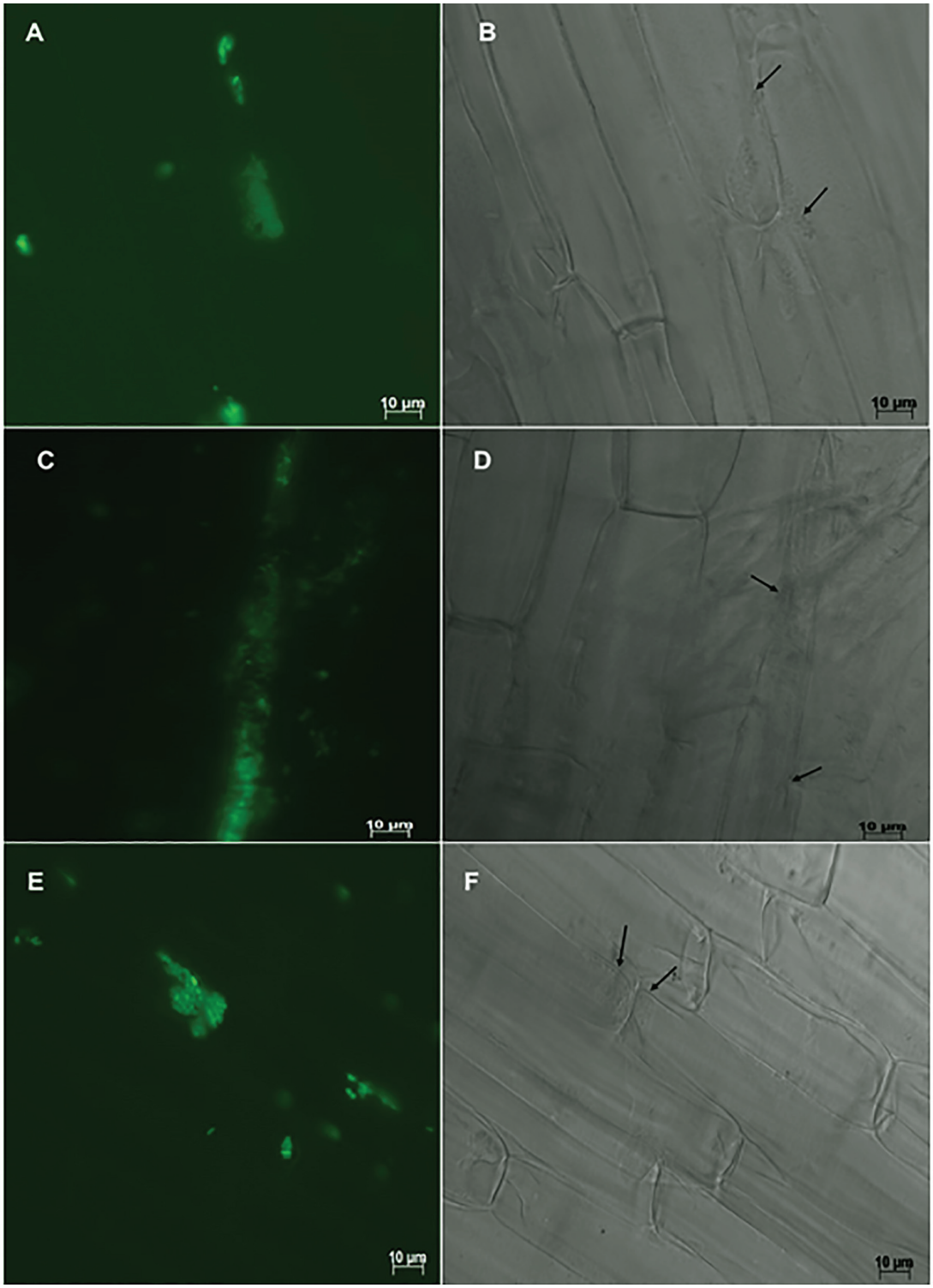

Figure 3. Rice 'M-206' root colonized by green fluorescent protein (GFP)-labeled endophytes WP9*gfp*; (A) and (B), PTD1*gfp*; (C) and (D), and WP5*gfp*; (E) and (F) at 20 d after inoculation. Images on the left (A, C, and E) were taken with GFP filter and images on the right (B, D, and F) were taken without GFP filter at 630 magnification.

general approximation of crop yield attribution (Xing and Zhang, 2010). We found that endophyte-inoculated plants had significantly more tillers at 3 mo than the noninoculated control plants (Table 2).

Based on differences in plant heights (Fig. 1), biomass (Fig. 2), and number of tillers (Table 2) in the greenhouse experiment, we showed that these *nifH*-harboring endophytes benefit overall rice growth. Control plants were slightly taller at 1 mo, but this pattern was reversed by the second month (Fig. 1). As has been postulated before with both fungal (Rodriguez et al., 2009) and bacterial endophytes (Knoth et al., 2014), this pattern may be due to a

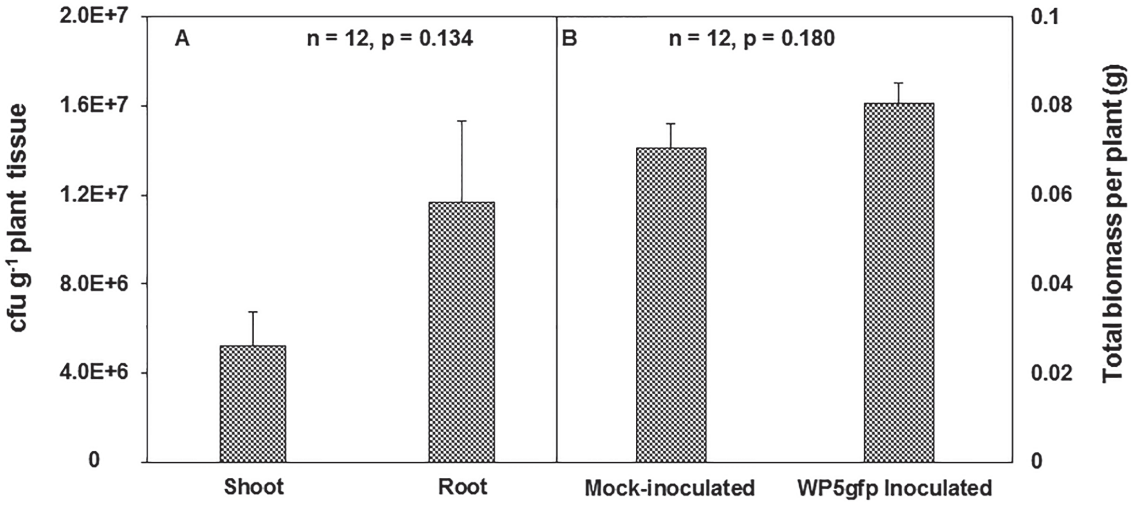

Figure 4. Quantification of endophytic populations, *gfp*-expressing colonies isolated from plant tissues, and total plant biomass at 20 d after inoculation. (A) Colony-forming units (cfu) per gram in shoot including leaves and root; (B) total biomass per plant (g) in mock-inoculated and WP5*gfp*-inoculated rice plants. The bars represent the standard errors of mean.

differential allocation of photosynthates in endophyte-colonized plants to the below-ground biomass. Early development of a strong root system may give plants an advantage in nutrient acquisition over time, and this could be beneficial adaptation for plants that are symbiotic with endophytes.

The increase in biomass in the inoculated plants in nutrient-poor medium compared with mock-inoculated control plants is presumed to be due to multiple beneficial traits of the endophytes. With the presence of nitrogenase genes, the ability of these endophytes to grow in vitro in N-limited media (Doty et al., 2009) and based on the results from ^{15}N dilution (Knoth et al., 2014) and ^{15}N incorporation studies (S. L. Doty, unpublished data, 2015) that demonstrated N fixation in poplar, we can postulate that the growth effect may have been due to in planta endophytic N fixation. Past studies have shown that diazotrophic endophytes isolated from plants adapted to N-poor soil (Doty et al., 2009; Reinhold-hurek et al., 1993; Riggs et al., 2001; Rosenblueth and Martínez-Romero, 2006) promoted plant growth in N- limited conditions (Gyaneshwar et al., 2001; Iniguez et al., 2004; Khan et al., 2012). The growth promotion reported here may also be due to the synergistic effects of phytohormone production, BNF, or other mechanisms or unknown processes. Other proposed mechanisms by which bacterial endophytes can benefit host plants include increased nutrient availability (P, Fe, and other microelements) or 1-aminocyclopropane-1-carboxylate deaminase activity that lessens plant ethylene levels (Kim et al., 2012; Long et al., 2008; Quecine et al., 2012). Quecine et al. (2012) reported that the higher plant biomass in endophyte-inoculated sugarcane (*Saccharum officinarum* L.) plants over controls was primarily due to phosphate solubilization and IAA production by the endophytes. The majority of the poplar and willow endophytes used in this study also have the ability to solubilize phosphate and to produce phytohormones and siderophores (data not shown).

Using fluorescent microscopy, we observed that these endophytes reside outside plant cells in the apoplastic spaces and xylem tissue of the rice plant (Fig. 3), a phenomenon that appears widespread in endophyte–host relationships (Egener et al., 1999; Gyaneshwar et al., 2001; Roncato-Maccari et al., 2003; Rosenblueth and Martínez-Romero, 2006; Prieto et al., 2011). Previous colonization studies in rice using different endophytes such as *Serratia marcescens, Rhizobium* sp., *Burkholderia* sp., *B. vietnamiensis, Azoarcus* sp., *Pantoea agglomerans,* and *Herbaspirillum* sp. strain B50 showed the community of endophytes in intercellular spaces in roots, stems, and leaves, sites of lateral root emergence, the cavities of root aerenchyma, and xylem vessels in leaf sheaths and stems (Gyaneshwar et al., 2001; Singh et al., 2009; Govindarajan et al., 2008; Egener et al., 1999; Verma et al., 2001; Elbeltagy et al., 2001). In our study, similar evidence indicated the effective colonization ability of these endophytes. *Rahnella* sp. strain WP5 appeared to be an effective colonizer of both root and shoot tissues with high numbers of colony-forming units per gram of plant tissue (Fig. 4). However, higher populations were observed in the roots. Past studies concentrating on the poplar endophyte, *Pseudomonas* sp. labeled with GFP also showed similar results when it was reinoculated into its native host plant (Germaine et al., 2004). The larger endophyte populations in roots shown in colonization assays seem to support the higher initial root growth resulting in smaller plant stature during the first month of inoculation in the greenhouse experiment. For colonization assays, plants were grown only for 20 d in the growth chamber for the sake of axenic growth and ease of study. It is possible that a longer study in larger vessels would be necessary for more substantial shoot colonization.

The endophytic colonization of rice plants with positive growth responses lends credence to the idea that these endophytes are indeed symbionts. Several studies have shown growth promotion in different crops such as rice, maize, wheat, sugarcane, and switchgrass (*Panicum virgatum* L.) through BNF mediated by diazotrophic endophytes (Amaral et al., 2014; Kim et al., 2012; Iniguez et al., 2004; Momose et al., 2009; James et al., 2002). To our knowledge, this is the first evidence to show growth promotion in rice by poplar and willow endophytes. Studies on the contribution of BNF and other plant growth-promoting properties of poplar and willow endophytes in their native and other host plants are relatively recent (Khan et al., 2012; Knoth et al., 2013, 2014). Further studies are needed to estimate the biologically fixed N and to determine the specific mechanism by which this symbiosis benefits the host plant.

CONCLUSIONS

Results of this study suggest that diazotrophic endophytes of the eudicots poplar and willow can colonize and improve the physical characteristics (biomass, plant height, and tiller numbers) of the monocot rice grown in N-limited conditions. Colonization and superior growth of endophyte-inoculated rice plants without added N fertilizer supports our hypothesis that these endophytes may fix N in other plant hosts as they do in poplar (Knoth et al., 2014; Doty et al., unpublished data, 2015). It is well known that adding chemical fertilizers to crops gives positive biomass results. Yet these positive results come with the unsustainable costs to the environment (Howarth, 2008). This research offers the potential alternative for chemical fertilizers in crop production, thus aiding sustainable agriculture with minimum impacts on the environment.

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