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Shyam Lal Kandel

Salicaceae Endophytes: Growth Promotion Potential in Rice (*Oryza sativa* L.) and Maize (*Zea mays* L.) and Bio-Control of Plant Pathogen

Shyam L. Kandel

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Reading Committee:
Sharon L. Doty, Chair
Soo-Hyung Kim
Russell Rodriguez

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Abstract

Salicaceae endophytes: Growth promotion potential in rice (*Oryza sativa* L.) and maize (*Zea mays* L.) and bio-control of plant pathogen

Shyam L. Kandel

Chair of the Supervisory Committee:

Professor Sharon L. Doty

School of Environmental and Forest Sciences

Salicaceae plants; poplar (*Populus trichocarpa*) and willow (*Salix sitchensis*) are hosts of many endophyte species. Salicaceae endophytes colonize the plant endosphere and confer various growth benefits to host plants. First three studies were conducted focusing on how these endophytes colonize and support the growth of major food crops; rice and maize under nutrient limited conditions. Fourth study was conducted to investigate the biocontrol and other growth promoting traits of these endophytes.

The first study was conducted to determine the growth promoting potential of Salicaceae endophytes to rice under nitrogen (N) limited conditions. Rice seedlings were inoculated with endophytes and grown in the N limited conditions in the greenhouse for about four months. Endophyte inoculated rice plants were taller, and had higher biomass and tiller numbers over mock inoculated control plants. Furthermore, colonizing performance of these endophytes in rice seedlings was verified through fluorescent microscopy, and counting *in planta* endophyte density. Rice seedlings were considerably colonized by these endophytes.

The second study was conducted to determine the growth potential of Salicaceae endophytes in maize and rice plants in N limited conditions. Endophyte inoculated plants were grown in the greenhouse, and plant physical characters such as plant height and biomass were recorded as growth response. Endophyte inoculated plants outperformed the mock inoculated plants but response was variable depending on crop genotypes or inoculated endophytes. In addition, through ^{15}N dilution assay, evidence of N fixing activity was observed in rice.

The third study was conducted to determine the colonization performance of poplar bacterial and yeast endophytes in rice and maize. Bacterial strains; WP5 (*Rahnella* sp.), and WP9 (*Burkholderia* sp.) labeled with green fluorescent protein, and yeast strain, WP1 were introduced in rice and maize seedlings aseptically. The *in planta* density of endophytes were determined by counting colony forming units and colonization pattern was observed using microscopy. These endophytes were found competent to colonize both rice and maize seedlings. They were observed in leaves and roots, and localized mostly in the intercellular spaces of root cortex and leaf mesophyll tissues. Higher *in planta* population of endophytes were observed in leaves and stems in majority of the colonization assays. Positive growth response was observed in endophytes inoculated rice and maize plants as compared to mock-inoculated control plants.

The fourth study was conducted to investigate the biocontrol potential of Salicaceae endophytes over a soil borne plant pathogen, *Rhizoctonia solani* AG-8. These endophytes were also examined to delineate their other plant growth promoting features including N fixing activity, indole-3- acetic acid (IAA) and siderophore biosynthesis, and phosphate solubulization. Endophyte strains; *Burkholderia*, *Rahnella*, *Pseudomonas*, and *Curtobacterium* displayed antagonistic activity against *R. solani* AG-8. *Burkholderia* spp. showed relatively stronger antagonistic effect than other endophytes, perhaps very useful to explore as biocontrol measures

to manage different soil borne plant pathogens. From nucleotide sequence analysis of *Burkholderia* spp., a 56-kb *ofc* gene cluster responsible for biosynthesis of anti-fungal glycolipopeptide, occidiofungin was detected in all species. Furthermore, these endophytes were found potential to support plant growth through multiple mechanisms such as N fixation, IAA and siderophore production, and phosphate solubilization besides protection from invading plant pathogens.

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Literature Review

1. INTRODUCTION

It is projected that the global human population will reach 9.1 billion, 34 percent greater than the current population by the middle of the 21st century. Moreover, urbanization and per capita income will continue to increase with the increasing population. The increasing population with better income will inevitably demand more food, and that demand can only be met by increasing food production. Annual cereal production is required to increase by nearly 3 billion tonnes, 0.9 billion higher than current production (Alexandratos, 2009). Cereal grains, primarily rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and maize (*Zea mays* L.) are the staple foods for most of the world's population. The production and productivity of cereal crops have improved and increased significantly during the past-half century. The main driving factor for higher cereal productivity is the use of synthetic chemical fertilizers, mainly for nitrogen (N), phosphorus, and potassium. Among these plant nutrients, N is the most limiting in soil for cereal crops. A strong positive relationship has been observed between cereal crop yields and N-fertilizers applied to crop plants (Curatti and Rubio, 2014). The use of N-fertilizers in soil has greatly increased the crop yields worldwide but significantly impacts the natural environment and is costly to manufacture, transport, and apply (Erisman et al., 2008; Beatty and Good, 2011).

There are several assessments showing that a significant portion of N used in agriculture is lost to the environment; as estimated only 17 Tg N consumed by humans out of 100 Tg N used in global agriculture (Erisman et al., 2008; Howarth, 2008; Reay et al., 2012). The N lost from farm lands eventually accumulates in the water bodies such as lakes, rivers or marine systems

which causes eutrophication (excessive growth of algae) creating lethal environments for aquatic animals. The incidence of greater atmospheric N (ammonia or ammonium) coincident with areas of eutrophication is reported in the downwind regions of farm land. Also, elevated concentrations of N (ammonium or nitrate or nitric acid vapor) in the atmosphere can reduce air quality, minimize visibility and impact plant growth (Robarge et al., 2002; Driscoll et al., 2003). In addition, microorganisms convert excess ammonium or nitrate in soil into nitrous oxide, a potent greenhouse gas.

Presently, global agriculture is facing many challenges from climate change, increased population, rapid urbanization, environmental pollution, migration, etc. Among others, climate change is the biggest concern for food production to meet the dietary requirement of increasing population. Climate change makes agriculture vulnerable through rapid changes in weather variables including temperature and precipitation, elevating greenhouse gases such as carbon dioxide (CO_2), methane, nitrous oxide etc. in the atmosphere, and outbreak of new diseases and pests (Tubiello et al., 2007; Fedoroff et al., 2010). Thus in the climate change scenario, even with elevated atmospheric CO_2, growth and development of crop plants is limited by various other stresses such as N, drought, salt, heat or infectious plant diseases. Crop cultivation largely depending on chemical fertilizers contradicts with the climate change mitigating purposes since manufacturing of fertilizers requires high amounts of energy and fossil fuels. Development of new technologies for N management based on biological N fixation (BNF) and use of optimized agronomic practices will help to minimize the dependency on fossil fuel and boost the effectiveness of N used in agriculture and will ultimately support sustainable agriculture (Erisman et al., 2008; Fedoroff et al., 2010; Curatti and Rubio, 2014).

Many recent studies have discovered strains of diazotrophic (N-fixing) microorganisms within different plant species (Hardoim et al., 2015; Carvalho et al., 2016). The use of these so-called diazotrophic endophytes in agriculture has immense potential to minimize the environmental pollution caused by chemical fertilizers and pesticides. Furthermore, the utilization of these natural symbionts in agriculture can impress the general public to appreciate the natural way of farming. This environmentally sustainable approach to grow crop plants through novel plant-microbe associations seems doable to mitigate the undesirable consequences of climate change in global agriculture.

2. Endophytes

The term ‘endophyte’ is derived from the Greek words ‘endon’ meaning within, and ‘phyton’ meaning plant. Endophytes are microorganisms such as bacteria and fungi that inhabit plant tissues through all or part of their life cycle without causing any apparent harm to the host plants (Wilson, 1995). However, the definition of endophytes has been revised multiple times by different authors (Wilson, 1995; Schulz and Boyle, 2006; Hardoim et al., 2015). More recently, Hardoim et al. (2015) reviewed the endophyte definitions with diversity, life cycles, functions, and colonization strategy of endophytes. In this review article, endophytes are defined as various microbial communities including bacterial, archeal, fungal, and protist which contribute to the growth, development, fitness, and diversification of the host plant. Many past studies showed that endophytes can support plant growth promotion through BNF, phytohormone modulation and production, nutrient acquisition, and protection from biotic and abiotic stresses. Several bacterial species were discovered using culture dependent and independent techniques as endophytes from a variety of plant species. The major bacterial endophyte phyla were

Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes, and genera were *Burkholderia*, *Pseudomonas*, *Enterobater*, *Pantoea*, *Stenotrophomonas*, *Acinetobacter*, *Methylobacterium* and *Sphingomonas* (Rosenblueth and Martínez-Romero, 2006; Hardoim et al., 2015). The major fungal endophyte phyla were Glomeromycota, Ascomycota, Basidiomycota, and Zygomycota (Schulz and Boyle, 2005; Hardoim et al., 2015). The most important fungal endophyte that is associated with many terrestrial plants is arbuscular mycorrhizal fungi which help host plants for nutrient acquisition and tolerance from biotic and abiotic stresses. More importantly, some bacterial and fungal endophytes have been commercialized to use in agriculture. For example, endophytes; *Epichloe* have been commercialized to use in grass production in the USA, Australia, and New Zealand (Kauppinen et al., 2016), and *Azospirillum* in different parts of the world (de Souza et al., 2015).

3. Genomics of endophytes/ Molecular understating of endophytes

The recent advances of genetic tools and resources are providing an opportunity to improve our understanding about plant-endophyte interactions. The next generation sequencing technologies and bioinformatics tools are allowing the characterization of many endophytes from a variety of plant species (Taghavi et al., 2009; Weilharter et al., 2011; Firrincieli et al., 2015; Megías et al., 2016; Vincent et al., 2016). More and more genome sequences are being continuously added to the genome repository database. Whole genome sequences of endophytes can provide a detailed insight into the mechanisms that are necessary for the endophytic lifestyle and sharing of symbiotic benefits between host and endophytes. Multiple endophyte species are often reported from the same plant organs or tissues, and the functional genome analysis would help to deduce the role of individual species in a community or consortium

(Nikolic et al., 2011; Sessitsch et al., 2012; Turner et al., 2013). Genome sequencing of poplar endophytes revealed the possible mechanism of carbon source utilization for their survival in the plant endosphere, possibly a necessary symbiotic trait to improve the growth and development of their host plants growing in marginal lands (Taghavi et al., 2009). In addition, whole genome sequencing and comparative genomics of different endophyte species discovered the genes that are responsible for adhesion, colonization, nutrient/substrate utilization, degradation of toxic compounds, phytohormone production, mitigation of biotic and abiotic stresses etc. (Krause et al., 2006; Fouts et al., 2008; Sessitsch et al., 2012; Mitter et al., 2013; Firrincieli et al., 2015). Furthermore, sequence analysis allows us to know the presence of a particular gene in a microbial population such as a *nifH* across the endophytic communities (Reiter et al., 2003; Knauth et al., 2005; Doty et al., 2016). Yet limited studies have been carried out to delineate the potentiality of these genes experimentally.

4. Mechanisms used by endophytes for plant growth promotion

Plant-endophyte interactions promote the host plant growth, especially through N fixation, phytohormone production, phosphorus and iron acquisition, and conferring tolerance to abiotic and biotic stresses (Rosenblueth and Martínez-Romero, 2006; Gaiero et al., 2013; Lebeis, 2014). Diazotrophic endophytes have the potential to convert dinitrogen gas into usable forms such as ammonium and nitrate in the host plants, probably contributing to the N demand of plant growth (Bhattacharjee et al. 2008; Santi et al. 2013). Positive growth response on the physical characteristics of host plants such as biomass or plant height has been reported as a result of plant-endophyte interactions in many diazotrophic endophytes such as *Azoarcus, Burkholderia, Gluconobacter, Klebsiella, Pantoea, Herbaspirillum,, Rahnella* etc. (Elbeltagy et al., 2001;

Hurek et al., 2002; Iniguez et al., 2004; Feng et al., 2006; Momose et al., 2009; He et al., 2013; Botta et al., 2013). Moreover, growth promotion through plant-endophyte interactions has been observed in a variety of crop plants such as rice, wheat, maize, sugar cane, tomato (Elbeltagy et al., 2001; Riggs et al., 2001; Hurek et al., 2002; Iniguez et al., 2004; Momose et al., 2009; Khan et al., 2012; Knoth et al., 2012; Botta et al., 2013; Kandel et al., 2015).

4.1 Biological nitrogen fixation

Nitrogen is one of the major macro-nutrients required for plant survival. The earth's atmosphere consists of 78% dinitrogen gas but it is biologically inert meaning organisms do not have direct access to the atmospheric N. The N is available to the plant from supplied chemical fertilizers, through BNF by microbes, and decomposition of organic matter such as crop residues, farm manure, or green manure (Andrews and Lea, 2013). Few species of bacteria and archaea have the ability to convert the atmospheric N into the ammonium, thereby making it available for plants to utilize. The overall process of conversion of dinitrogen gas into ammonium is called BNF and is catalyzed by the nitrogenase enzyme system. The chemical equation of N fixation is represented as $N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$ (Rees et al., 2005). N fixation requires a high amount of energy to reduce the triple bonds of two N molecules. It is considered that free-living N fixers have relatively limited application in agriculture than plant associated N fixers, which can maintain their energy requirements from the host plants (Olivares et al., 2013). Since early agriculture, farmers generally depended on biologically fixed N that is derived from legume-*Rhizobium* symbiosis. *Rhizobium* associated N fixation in legume plants or *Frankia* associated in actinorhizal plants have been investigated for a long time. The discovery of N-fixing endophytes in sugarcane plants, a non-legume plant species during the late 1980's

has expanded the area of BNF research (Gillis et al., 1989; Dong et al., 1994). Diazotrophic endophytes such as *Azoarcus, Burkholderia, Gluconobacter, Herbaspirillum, Klebsiella, Pantoea, Rahnella* etc. were reported in different plants; supporting the growth of the host plants in nutrient poor conditions (Reinhold-hurek et al., 1993; Riggs et al., 2001; Rosenblueth and Martínez-Romero, 2006; Doty et al., 2009). These endophytes reside in the internal plant tissues which may offer a favorable environment for N fixation, minimizing the competition from other microbes that exist in the rhizosphere or phyllosphere and possibly providing the necessary microaerobic environment (Reinhold-Hurek and Hurek, 1998; Doty et al., 2016).

The most frequently used procedures to estimate the BNF via endophytes in plants are the acetylene reduction assay (ARA), ^{15}N dilution/enrichment assay, and ^{15}N and ^{13}N tracer assays (Momose et al., 2009; Knoth et al., 2014; Pankievicz et al., 2015; Chalk, 2016; Moyes et al., 2016; Puri et al., 2016). ARA has been commonly used to assess the BNF since the discovery of the catalytic ability of nitrogenase enzymes to reduce acetylene (C_2H_2) into ethylene (C_2H_4) equivalent as elemental N into ammonia (Dilworth, 1966; Hardy et al., 1973; Zhang et al., 2016). In this technique, BNF is estimated by observing the ethylene released by the activity of nitrogenase enzyme present in the diazotrophic microbes. Two stable isotopic forms of N; ^{15}N and ^{14}N are found in nature where ^{14}N is available at much higher concentrations than ^{15}N. The technique of ^{15}N dilution is frequently used to estimate the biologically fixed N, and utilization of fixed N in the plant (Iniguez et al., 2004; Montañez et al., 2008; Knoth et al., 2014; Chalk, 2016). The $^{15}N/^{13}N$ tracer method is used to estimate the biologically fixed N in different plant tissues, and the potential translocation pathway of fixed N. In contrast to the ^{15}N dilution method, it gives the direct estimation of fixed N in the plant (Momose et al., 2009; Pankievicz et al., 2015; Doty et al., 2016).

4.2 Phytohormone production and modulation

It has been reported that endophytes can promote plant growth by synthesizing phytohormones like indole-3-acetic acid (IAA) and/or regulating the internal hormonal level in the plant body (Santoyo et al., 2016; Spaepen, and Vanderleyden, 2011). Phytohormones not only modulate the growth and developmental process in plants but also are involved in the plant responses to the challenge of both biotic and abiotic stresses (Depuydt and Hardtke, 2011). The IAA produced by endophytes in plants increases the number of lateral and adventitious roots, facilitating better access for nutrients, and improves root exudation, offering resources for soil microbes to interact with roots (Gamalero and Glick, 2011; Spaepen, and Vanderleyden, 2011). Growth enhancement by improving plant height and/or biomass have been reported in several experiments when plants were inoculated with bacterial endophytes capable of producing IAA (Shi et al., 2009; Xin et al., 2009b; Barra et al., 2016; Khan et al., 2016; Santoyo et al., 2016). Cytokinins and Gibberellins are other groups of plant hormones regulating plant growth reportedly produced by different bacterial endophytes (Bastian et al., 1998; Hardoim et al., 2015; Santoyo et al., 2016). Plant hormones related to plant defense such as jasmonic acid, ethylene, and salicyclic acid have been demonstrated that they can regulate the microbial community in the rhizosphere (Doornbos et al., 2011). A recent study showed that salicyclic acid can modify the structure of bacterial community in the rhizosphere and colonization process within roots (Lebeis et al., 2015).

4.3 Siderophore production

Siderophores are organic compounds secreted by microorganisms and plants in iron limited conditions. Since iron is one of the essential nutrients for both microorganisms and

plants, siderophores are used to chelate the iron from the environment for microbial and plant cells to uptake (Ahmed and Holmström, 2014; Hardoim et al., 2015). Under iron-deficient conditions, microorganisms release siderophores into their surroundings, siderophores form a complex with iron (Fe^{3+}) molecules, and the Fe^{3+}-siderophore complex is taken up. After releasing the iron, siderophores get degraded or exit out from the cells (Saha et al., 2015). In recent years, siderophores gained much attention because of their various possible applications in many areas of research like soil genesis, microbial ecology, agriculture, bioremediation, and medical science. Many recent studies showed that bacterial endophytes have the ability to secrete siderophores and considered it as one of the early steps to be involved in initiating the symbiotic interactions with host plants (Hardoim et al., 2015).

4.4 Phosphate solubilization

Phosphorus (P) is one of the essential macro-nutrients required for plant growth and survival. Plants take up P from soil, however, a large portion of soil P is not readily available to plants. Added P through fertilizers or soluble P available in soil rapidly forms complexes with metal cations such as Ca^{2+}, Fe^{3+} or Al^{3+} and becomes fixed in soil which is not accessible to plants (Gamalero and Glick, 2011). The phosphate solubilizing microorganisms including many endophyte strains have the ability to solubilize the fixed P in soil. Many recent reviews highlighted the mechanisms and importance of P solubilizing microorganisms in agriculture (Khan et al., 2014; Hardoim et al., 2015). The major biochemical pathway used by endophytic bacteria or actinomycetes for phosphate solubilization involves the secretion of gluconic acid and citric acid. The hydroxyl and carboxyl groups of these organic acids interact with phosphates and free the cations, thus releasing the soluble phosphates (Gamalero and Glick, 2011; Oteino et al.,

2015; Passari et al., 2015). Several bacterial endophytes isolated from different plants showed P solubilizing activity which is potentially an important trait for plant growth promotion (Dias et al., 2009; Oteino et al., 2015; Passari et al., 2015; Joe et al., 2016; Rezgui et al., 2016).

4.5 Biocontrol of plant pathogens

Biocontrol is the natural way of suppressing growth and activities of plant pathogens by utilizing introduced or resident microorganisms of host plants. Biocontrol offers an environment friendly cost effective alternative over synthetic pesticides to manage several plant pathogens (Pal and Gardener, 2006). It has been reported that endophyte species of ascomycetous fungi like *Chaetomium, Alternaria, Trichoderma, Fusarium, Penicillium, Paecilomyces* have antagonistic activities against different plant pathogens (Larran et al., 2016; Martínez-Álvarez et al., 2016). A recent review authored by Busby et al. (2016) described the role of diverse fungal endophytes to the expression of plant disease severity. They reported that fungal endophytes can influence the host plant health either by inhibiting or facilitating the invading plant pathogens. Endophytes may use various strategies to protect the host plant from disease damage, including parasitizing the invading pathogen, releasing of different fungistatic compounds, or outcompeting the intruders by pre-occupying the space and resources. However, endophytes mediated disease suppression is referred as a conditional with the spectrum of abiotic and biotic factors, and existing host and pathogen genotypes. The resident fungal endophytes (*Stachybotrys* sp., *Trichoderma atroviride*, *Ulocladium atrum*, and *Truncatella angustata*) of *Populus* leaves can antagonize the rust pathogen, *Melampsora*. Endophytes locally decreased the number of rust spores without displaying any hypersensitive and systemic response (Raghavendra and

Newcombe, 2013). However, little has been known about the potential use of *Populus* endophytes as a biocontrol agent for pathogens of non-host plant species.

Bacterial endophytes can confer resistance or tolerance to the host plant from pathogen damage by releasing antimicrobial compounds, producing siderophores, competing for space and nutrients, and modulating plant resistance response (Friesen et al., 2011; Mercado-Blanco and Lugtenberg, 2014; Hardoim et al., 2015; Santoyo et al., 2016). In addition, many bacterial endophytes can relieve the plant stress by blocking the pathway of ethylene synthesis in plants. Endophytic strains of *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas,* and *Serratia* were found effective to suppress the growth of pathogenic microorganisms on different crop plants (Mercado-Blanco and Lugtenberg, 2014; Esmaeel et al., 2016; Larran et al., 2016). Moreover, some bacterial endophytes have antagonistic effects on broad host range soil borne plant pathogens such as *Rhizoctonia solani. R. solani* can infect a variety of crop plants including rice, maize, wheat, vegetables, lawn grasses etc. and caused a significant loss by collapsing the young seedlings, or damaging the leaves and stem. It has been reported that bacterial endophytes, rhizobacteria or actinomycetes have inhibitory effects on *Rhizoctonia* infection in these crop plants (Kai et al., 2007; Huang et al., 2012; Goudjal et al., 2014).

Several bacterial and fungal endophytes reduce infection of plant roots by root-knot nematodes (*Meloidogyne* spp.), root-lesion nematodes (*Pratylenchus* spp.), burrowing nematode (*Radhopholus similis*), and cyst nematodes (*Globodera* and *Heterodera*) in various crop plants (Sikora et al., 2007, 2008). Endophytic fungal growth either excludes the migration of nematodes to the roots or kills the nematodes during post-infection (Dababat and Sikora, 2007). But it is still not fully understood how fungal or bacterial endophytes evolve, and improve the fitness of the host plants in contrast to saprophytic or plant pathogenic microbes.

4.6 Mitigation of abiotic stress

Abiotic stresses such as cold, drought, salt or heat interfere with plant growth and developmental processes. These stresses may inhibit many physiological and biochemical processes in the plant such as photosynthesis, respiration, water potential, membrane integrity, hormonal balance, etc. (Wahid et al. 2007, Naveed 2014). Studies determined that specific filamentous fungal endophyte strains of *Fusarium culmorum* and *Curvularia protuberate* are key players enabling plants to colonize the high stress habitats (reference their review): survival of panic grass (*Dichanthelium lanuginosum*) in geothermal soil in Yellow Stone National Park, WY, and dunegrass (*Leymus mollis*) in coastal saline habitats, WA (Rodriguez et al., 2008). In addition, the manipulative greenhouse experiments showed that rice plants inoculated with these endophytes maintained the considerable growth under cold, drought, and saline conditions with increased biomass and grain yield (Redman et al. 2011). Based on these studies, it was concluded that fungal endophytes allow host plants to perceive the stress promptly so that the host plant can instantly activate the strong stress response mitigating the effects of stress (Redman et al. 2002; Rodriguez et al., 2009).

Reactive oxygen species (ROS), for examples, perhydroxyl radical, hydrogen peroxide, hydroxyl radical etc. are generated in the plant and animal body when they confront stresses. But accumulation of ROS is undesirable in the living cells as they damage carbohydrates, proteins, lipids, and DNA. Symbiotic plants harboring the fungal endophytes have a higher capacity to either quench or modulate the ROS (Rodriguez et al., 2008). Alternatively, minimum activities of antioxidants such as reduced glutathione, catalase, peroxidase, and polyphenol oxidase were observed in inoculated cucumber plants with endophyte strains *Phoma glomerata* and *Penicillium* sp. growing under the saline or drought condition suggesting

minimum reactive oxygen species were generated due to the presence of endophytes (Waqas et al., 2012). Additionally, it has been reported that many fungal endophytes produce melanin which has a significant role for the survival of fungi in extreme environments providing strong antioxidant activity (Suryanarayanan et al., 2004; Eisenman and Casadevall, 2012). Several bacterial endophyte strains including *Bacillus, Enterobacter, Pseudomonas, Azotobacter, Arthrobacter, Streptomyces,* and *Isoptericola* were successful in alleviating the drought, heat, and salt stress in different crop plants. More importantly, symbiotic plants with these endophytes were not only capable of avoid the stress response but also had significantly increased biomass and height (Rojas-Tapias et al., 2012; Ali et al., 2014; Naveed et al., 2014; Qin et al., 2014; Yaish et al., 2015). However, at present, information about the mechanism of how bacterial endophytes mitigate the abiotic stress is scant.

5. Plant colonization

A plethora of diverse microbial communities reside in the plant system either epiphytically or endophytically. Endophytic colonization refers to the growth and multiplication of endophyte communities in the host plant. Florescence *in situ* hybridization, green fluorescent protein tagging, fluorescence resonance energy transfer (FRET), β-glucuronidase (GUS) staining, and fluorogenic dye staining are common techniques to investigate the colonization of inoculated endophytes in plants (Compant et al., 2005, 2010; Nassar et al., 2005; Banik et al., 2015; Chen et al., 2015; Kandel et al., 2015). With the help of these techniques, many studies reported that endophytes inhabit intercellularly in root and leaf apoplasts, and systemically in the vascular tissues. In addition, the colony counts of bacterial endophytes from surface sterilized above ground and below ground tissues were often estimated as internally colonized populations

of endophytes in the host plants (Germaine et al., 2004; Govindarajan et al., 2008; Rouws et al., 2010; Kandel et al., 2015). Natural openings where root hairs or lateral roots emerge in the root systems, and stomata, wounds and hydathodes in the shoot system are considered as the main entry points for the endophytes into the host plant (Hardoim et al., 2015). The signaling pathways of plant endophyte interactions, and molecular mechanisms of host specificity and endophytic life style in plants remain to be fully understood.

6. Salicaceae endophytes

Poplar (*Populus* spp.) and willow (*Salix* spp.) are members of the Salicaceae family which are early colonizers of nutrient poor habitats particularly wetlands, riparian, or other disturbed areas. They can rapidly grow and cover the open space that is soon available after disturbances. (Isebrands, and Richardson, 2014). The rapid and hardy growth habit make them important for biomass production for bioenergy. According to International Poplar Commission, it is estimated that more than 95 million hectares of land is covered by poplar and willow plants worldwide. Doty et al. (2005, 2009) and Xin et al. (2009a) isolated and characterized several diazotrophic endophytes from poplar and willow plants growing in the riparian environments of western Washington. It has been demonstrated that these endophytes have vital roles to establish the community of poplar and willow as a pioneer species in a marginal site (Knoth et al. 2013, Firrincieli et al. 2015, and Doty et al. 2016). Ulrich et al. (2008), and Taghavi et al. (2009) also described several endophyte communities from poplar and willow plants growing in a nutrient poor sandy or polluted soil. Hacquard and Schadt (2015) recently reviewed the microbial communities of above ground and below ground tissues of *Populus* trees. They highlighted the

contribution of microbial communities residing in the endosphere or on the phyllosphere to the host plant health.

7. Salicaceae endophytes in crop production

Previous studies demonstrated that Salicaceae endophytes can maintain their growth in N-limited media, and can relieve the symptoms of N deficiency in the host plant (Doty et al., 2009, 2016; Knoth et al., 2014). In addition, some of the poplar endophytes can produce significant amounts of the phytohormone, indole-3-acetic acid in *in-vitro* conditions (Xin et al., 2009b; a). An earlier study conducted by Khan *et al.* (2012) showed that inoculation of various crop plants with endophytes from Salicaceae resulted in early flowering and greater fruit yield in tomato and pepper, and higher biomass in maize, tomato, pepper and squash in N limited conditions. Also, sweet maize plants inoculated with endophytes showed increased plant biomass and improvement in photosynthetic capacity (higher CO_2 assimilation rate) of leaves (Knoth et al., 2012). Moreover, it has been shown that these endophytes colonized rice (var M206) plants effectively and resulted in higher biomass and bigger plant stature under nutrient limited conditions upon endophytes inoculation (Kandel et al., 2015).

Objectives of the present study

Since rice and maize are globally consumed major food crops, it is critically important to investigate sustainable ways of their production using microbial symbionts to minimize the negative impact on environment. Understanding the mechanism of endophyte/rice and maize interactions, and utilization of this technology for crop production with minimum supply of N fertilizers or chemicals in general can contribute for climate change mitigation purposes. Therefore, we proposed to execute the experiments to determine the mechanisms of how Salicaceae endophytes can influence the growth and health of rice and maize plants under nutrient limited conditions.

This dissertation research consists of three major objectives.

Objective 1: To determine the impact of Salicaceae endophytes on maize and rice growth under nutrient-limited conditions.

Objective 2: To determine the colonization pattern of Salicaceae endophytes in rice and maize.

Objective 3: To determine the biocontrol potential of Salicaceae endophytes to manage soil borne plant pathogenic fungi of cereals.

REFERENCES

Ahmed, E., and S.J.M. Holmström. 2014. Siderophores in environmental research: roles and applications. Microb. Biotechnol. 7(3): 196–208.

Alexandratos, N. 2009. World food and agriculture to 2030/50: Highlights and views from mid-2009. Rome, Italy: FAO. Expert meeting on how to feed the world in 2050 (12-13 October 2009): 1–35.

Ali, S., T.C. Charles, and B.R. Glick. 2014. Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. Plant Physiol. Biochem. 80(2014): 160–167.

Andrews, M., and P.J. Lea. 2013. Our nitrogen "footprint": the need for increased crop nitrogen use efficiency. Ann. Appl. Biol. 163(2): 165–169.

Banik, A., S.K. Mukhopadhaya, A. Sahana, D. Das, and T.K. Dangar. 2015. Fluorescence resonance energy transfer (FRET)-based technique for tracking of endophytic bacteria in rice roots. Biol. Fertil. Soils 52(2016): 277–282.

Barra, P.J., N.G. Inostroza, J.J. Acuña, M.L. Mora, D.E. Crowley, and M.A. Jorquera. 2016. Formulation of bacterial consortia from avocado (*Persea americana* Mill.) and their effect on growth, biomass and superoxide dismutase activity of wheat seedlings under salt stress. Appl. Soil Ecol. 102(2016): 80–91.

Bastian, F., A. Cohen, P. Piccoli, V. Luna, R. Baraldi, and R. Bottini. 1998. Production of indole-3-acetic acid and gibberellins A1 and A3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically-defined culture media. Plant Growth Regul. 24(1): 7–11.

Beatty, P.H., and A.G. Good. 2011. Future prospects for cereals that fix nitrogen. Science

333(6041): 416–7.

Bhattacharjee, R.B., A. Singh, and S.N. Mukhopadhyay. 2008. Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. Appl. Microbiol. Biotechnol. 80(2): 199–209.

Botta, A.L., A. Santacecilia, C. Ercole, P. Cacchio, and M. Del Gallo. 2013. *In vitro* and *in vivo* inoculation of four endophytic bacteria on *Lycopersicon esculentum*. N. Biotechnol. 30(6): 666–74.

Busby, P.E., M. Ridout, and G. Newcombe. 2016. Fungal endophytes: modifiers of plant disease. Plant Mol. Biol. 90: 645–655.

Carvalho, T.L.G., H.G.F. Ballesteros, F. Thiebaut, P.C.G. Ferreira, and A.S. Hemerly. 2016. Nice to meet you: genetic, epigenetic and metabolic controls of plant perception of beneficial associative and endophytic diazotrophic bacteria in non-leguminous plants. Plant Mol. Biol. 90(6): 561–574.

Chalk, P.M. 2016. The strategic role of 15N in quantifying the contribution of endophytic N2 fixation to the N nutrition of non-legumes. Symbiosis. 69(2): 63-80

Chen, X., L. Miche, S. Sachs, Q. Wang, A. Buschart, H. Yang, C.M. Vera Cruz, T. Hurek, and B. Reinhold-Hurek. 2015. Rice responds to endophytic colonization which is independent of the common symbiotic signaling pathway. New Phytol. 208(2): 531–543.

Compant, S., C. Clément, and A. Sessitsch. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42(5): 669–678.

Compant, S., B. Reiter, A. Sessitsch, J. Nowak, C. Clément, and E.A. Barka. 2005. Endophytic colonization of *Vitis vinifera* L . by plant growth-promoting bacterium *Burkholderia* sp .

strain PsJN. Appl. Environ. Microbiol. 71(4): 1685–1693.

Curatti, L., and L.M. Rubio. 2014. Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. Plant Sci. 225: 130–7.

Dababat, A.E.A., and R.A. Sikora. 2007. Influence of the mutualistic endophyte *Fusarium oxysporum* 162 on *Meloidogyne incognita* attraction and invasion. Nematology 9(6): 771–776.

Depuydt, S., and C.S. Hardtke. 2011. Hormone signalling crosstalk in plant growth regulation. Curr. Biol. 21(9): R365–R373.

Dias, A.C.F., F.E.C. Costa, F.D. Andreote, P.T. Lacava, M. a. Teixeira, L.C. Assumpção, W.L. Araújo, J.L. Azevedo, and I.S. Melo. 2009. Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World J. Microbiol. Biotechnol. 25(2): 189–195.

Dilworth, M.J. 1966. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. Biochim. Biophys. Acta 127(2): 285–294.

Dong, Z., M.J. Canny, M.E. McCully, M.R. Roboredo, C.F. Cabadilla, E. Ortega, and R. Rodes. 1994. A Nitrogen-fixing endophyte of sugarcane stems (A new role for the apoplast). Plant Physiol. 105(4): 1139–1147.

Doornbos, R.F., B.P.J. Geraats, E.E. Kuramae, L.C. Van Loon, and P. a H.M. Bakker. 2011. Effects of jasmonic acid, ethylene, and salicylic acid signaling on the rhizosphere bacterial community of *Arabidopsis thaliana*. Mol. Plant. Microbe. Interact. 24(4): 395–407.

Doty, S.L., B. Oakley, G. Xin, J.W. Kang, G. Singleton, Z. Khan, A. Vajzovic, and J.T. Staley. 2009. Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47: 23–33.

Doty, S.L., A.W. Sher, N.D. Fleck, M. Khorasani, R.E. Bumgarner, Z. Khan, A.W.K. Ko, S.-H. Kim, and T.H. DeLuca. 2016. Variable nitrogen fixation in wild *Populus*. PLoS One 11(5): e0155979.

Driscoll, C.T., D. Whitall, J. Aber, E. Boyer, M. Castro, C. Cronan, C.L. Goodale, P. Groffman, C. Hopkinson, K. Lambert, G. Lawrence, and S. Ollinger. 2003. Nitrogen pollution in the Northeastern United States: sources, effects, and management options. Bioscience 53(April): 357.

Eisenman, H.C., and A. Casadevall. 2012. Synthesis and assembly of fungal melanin. Appl. Microbiol. Biotechnol. 93(3): 931–40.

Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, and H. Mitsui. 2001. Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. 67(11): 5285–5293.

Erisman, J.W., M.A. Sutton, J. Galloway, Z. Klimont, and W. Winiwarter. 2008. How a century of ammonia synthesis changed the world. Nat. Geosci. 1(10): 636–639.

Esmaeel, Q., M. Pupin, N.P. Kieu, G. Chataigné, M. Béchet, J. Deravel, F. Krier, M. Höfte, P. Jacques, and V. Leclère. 2016. *Burkholderia* genome mining for nonribosomal peptide synthetases reveals a great potential for novel siderophores and lipopeptides synthesis. MicrobiologyOpen 5 (3): 512–526.

Fedoroff, N.V., D.S. Battisti, R.N. Beachy, P.J.M. Cooper, D.A. Fischhoff, C.N. Hodges, V.C. Knauf, D. Lobell, B.J. Mazur, D. Molden, and M.P. Reynolds. 2010. Radically rethinking agriculture for the 21st century. Science 327: 833–835.

Feng, Y., D. Shen, and W. Song. 2006. Rice endophyte *Pantoea agglomerans* YS19 promotes host plant growth and affects allocations of host photosynthates. J. Appl. Microbiol. 100(5):

938–45.

Firrincieli, A., R. Otillar, A. Salamov, J. Schmutz, Z. Khan, R.S. Redman, N.D. Fleck, E. Lindquist, I. V. Grigoriev, and S.L. Doty. 2015. Genome sequence of the plant growth promoting endophytic yeast *Rhodotorula graminis* WP1. Front. Microbiol. 6: 1-6.

Fouts, D.E., H.L. Tyler, R.T. DeBoy, S. Daugherty, Q. Ren, J.H. Badger, A.S. Durkin, H. Huot, S. Shrivastava, S. Kothari, R.J. Dodson, Y. Mohamoud, H. Khouri, L.F.W. Roesch, K.A. Krogfelt, C. Struve, E.W. Triplett, and B.A. Methé. 2008. Complete genome sequence of the N2-fixing broad host range endophyte *Klebsiella pneumoniae* 342 and virulence predictions verified in mice. PLoS Genet. 4(7).

Friesen, M.L., S.S. Porter, S.C. Stark, E.J. Von Wettberg, and J.L. Sachs. 2011. Microbially mediated plant functional traits. Annu. Rev. Ecol. Evol. Syst. 42: 23–46.

Gaiero, J.R., C. a McCall, K. a Thompson, N.J. Day, A.S. Best, and K.E. Dunfield. 2013. Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am. J. Bot. 100(9): 1738–50.

Gamalero, E., and B.R. Glick. 2011. Mechanisms used by plant growth-promoting bacteria. p. 17–47. *In* Bacteria in Agrobiology: Plant Nutrient Management.

Germaine, K., E. Keogh, G. Garcia-Cabellos, B. Borremans, D. Lelie, T. Barac, L. Oeyen, J. Vangronsveld, F.P. Moore, E.R.B. Moore, C.D. Campbell, D. Ryan, and D.N. Dowling. 2004. Colonisation of poplar trees by gfp expressing bacterial endophytes. FEMS Microbiol. Ecol. 48(1): 109–18.

Gillis, M., K. Kersters, B. Hoste, D. Janssens, R.M. Kroppenstedt, and M.P. Stephan. 1989. *Acetobactev diazotrophicus* sp . nov ., a nitrogen-fixing acetic acid bacterium associated with sugarcane. Int. J. Systamatic Bacteriol. 39(3): 361–364.

Goudjal, Y., O. Toumatia, A. Yekkour, N. Sabaou, F. Mathieu, and A. Zitouni. 2014. Biocontrol of *Rhizoctonia solani* damping-off and promotion of tomato plant growth by endophytic actinomycetes isolated from native plants of Algerian Sahara. Microbiol. Res. 169(1): 59–65.

Govindarajan, M., J. Balandreau, S.-W. Kwon, H.-Y. Weon, and C. Lakshminarasimhan. 2008. Effects of the inoculation of *Burkholderia vietnamensis* and related endophytic diazotrophic bacteria on grain yield of rice. Microb. Ecol. 55(1): 21–37.

Hacquard, S., and C.W. Schadt. 2015. Towards a holistic understanding of the beneficial interactions across the *Populus* microbiome. New Phytol. 205(4): 1424–1430.

Hardoim, P.R., L.S. van Overbeek, G. Berg, A.M. Pirttilä, S. Compant, A. Campisano, M. Döring, and A. Sessitsch. 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. 79(3): 293–320.

Hardy, R.W.F., R.C. Burns, and R.D. Holsten. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. 5(1): 47–81.

He, H., Z. Ye, D. Yang, J. Yan, L. Xiao, T. Zhong, M. Yuan, X. Cai, Z. Fang, and Y. Jing. 2013. Characterization of endophytic *Rahnella sp*. JN6 from *Polygonum pubescens* and its potential in promoting growth and Cd, Pb, Zn uptake by *Brassica napus*. Chemosphere 90(6): 1960–5.

Howarth, R.W. 2008. Coastal nitrogen pollution: A review of sources and trends globally and regionally. Harmful Algae 8(1): 14–20.

Huang, X., N. Zhang, X. Yong, X. Yang, and Q. Shen. 2012. Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. Microbiol. Res. 167(3):

135–143.

Hurek, T., L.L. Handley, B. Reinhold-Hurek, and Y. Piché. 2002. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol. Plant. Microbe. Interact. 15(3): 233–42.

Iniguez, A.L., Y. Dong, and E.W. Triplett. 2004. Nitrogen fixation in wheat provided by *Klebsiella pneumoniae* 342. Mol. Plant. Microbe. Interact. 17(10): 1078–85.

Joe, M.M., S. Devaraj, A. Benson, and T. Sa. 2016. Isolation of phosphate solubilizing endophytic bacteria from *Phyllanthus amarus* Schum & Thonn: Evaluation of plant growth promotion and antioxidant activity under salt stress. J. Appl. Res. Med. Aromat. Plants. 3(2): 71-77.

Kai, M., U. Effmert, G. Berg, and B. Piechulla. 2007. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. Arch. Microbiol. 187(5): 351–360.

Kandel, S.L., N. Herschberger, S.H. Kim, and S.L. Doty. 2015. Diazotrophic endophytes of poplar and willow for growth promotion of rice plants in nitrogen-limited conditions. Crop Sci. 55(4): 1765–1772.

Kauppinen, M., K. Saikkonen, M. Helander, A.M. Pirttilä, and P.R. Wäli. 2016. Epichloë grass endophytes in sustainable agriculture. Nat. Plants 2: 1–7.

Khan, Z., G. Guelich, H. Phan, R. Redman, and S. Doty. 2012. Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. ISRN Agron.: 1–11.

Khan, A.L., B.A. Halo, A. Elyassi, S. Ali, K. Al-Hosni, J. Hussain, A. Al-Harrasi, and I.-J. Lee. 2016. Indole acetic acid and ACC deaminase from endophytic bacteria improves the growth of *Solanum lycopersicum*. Electron. J. Biotechnol. (2015): 1–7.

Khan, M.S., A. Zaidi, and E. Ahmad. 2014. Mechanism of phosphate solubilization and physiological functions of phosphate-solubilizing microorganisms (MS et al. (eds. . Khan, Ed.). Springer International Publishing Switzerland 2014.

Knauth, S., T. Hurek, D. Brar, and B. Reinhold-Hurek. 2005. Influence of different *Oryza* cultivars on expression of nifH gene pools in roots of rice. Environ. Microbiol. 7(11): 1725–1733.

Knoth, J.L., S.-H. Kim, G.J. Ettl, and S.L. Doty. 2012. Effects of cross host species inoculation of nitrogen-fixing endophytes on growth and leaf physiology of maize. GCB Bioenergy 5(4): 408–418.

Knoth, J.L., S. Kim, G.J. Ettl, and S.L. Doty. 2014. Biological nitrogen fixation and biomass accumulation within poplar clones as a result of inoculations with diazotrophic endophyte consortia. New Phytol. 201(2): 599–609.

Krause, A., A. Ramakumar, D. Bartels, F. Battistoni, T. Bekel, J. Boch, M. Böhm, F. Friedrich, T. Hurek, L. Krause, B. Linke, A.C. McHardy, A. Sarkar, S. Schneiker, A.A. Syed, R. Thauer, F.-J. Vorhölter, S. Weidner, A. Pühler, B. Reinhold-Hurek, O. Kaiser, and A. Goesmann. 2006. Complete genome of the mutualistic, N2-fixing grass endophyte Azoarcus sp. strain BH72. Nat. Biotechnol. 24(11): 1385–91.

Larran, S., M.R. Simon, M. V. Moreno, M.P.S. Siurana, and A. Perell. 2016. Endophytes from wheat as biocontrol agents against tan spot disease. Biol. Control 92(2016): 17–23.

Lebeis, S.L. 2014. The potential for give and take in plant-microbiome relationships. Front. Plant Sci. 5 (June): 1-6.

Lebeis, S.L., S.H. Paredes, D.S. Lundberg, N. Breakfield, J. Gehring, M. McDonald, S. Malfatti, T. Glavina del Rio, C.D. Jones, S.G. Tringe, and J.L. Dangl. 2015. Salicylic acid modulates

colonization of the root microbiome by specific bacterial taxa. Science 349(6250): 860–864.

Martínez-Álvarez, P., R.A. Fernández-González, A.V. Sanz-Ros, V. Pando, and J. Javier Diez. 2016. Two fungal endophytes reduce the severity of pitch canker disease in *Pinus radiata* seedlings. Biol. Control 94: 1–10.

Megías, E., M. Megías, F.J. Ollero, and M. Hungria. 2016. Draft genome sequence of *Pantoea ananatis* strain AMG521, a rice plant growth-promoting bacterial endophyte isolated from the Guadalquivir Marshes in Southern Spain. Genome Announc. 4(1): 1–2.

Mercado-Blanco, J., and B. Lugtenberg. 2014. Biotechnological applications of bacterial endophytes. Curr. Biotechnol. 3: 60–75.

Mitter, B., A. Petric, M.W. Shin, P.S.G. Chain, L. Hauberg-Lotte, B. Reinhold-Hurek, J. Nowak, and A. Sessitsch. 2013. Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. Front. Plant Sci. 4(April): 1–15.

Momose, A., N. Ohtake, K. Sueyoshi, T. Sato, Y. Nakanishi, S. Akao, and T. Ohyama. 2009. Nitrogen fixation and translocation in young sugarcane (*Saccharum officinarum* L.) plants associated with endophytic nitrogen-fixing bacteria. Microbes Environ. 24(3): 224–230.

Montañez, A., C. Abreu, P.R. Gill, G. Hardarson, and M. Sicardi. 2008. Biological nitrogen fixation in maize (*Zea mays* L.) by 15N isotope-dilution and identification of associated culturable diazotrophs. Biol. Fertil. Soils 45(3): 253–263.

Moyes, A.B., L.M. Kueppers, J. Pett-ridge, D.L. Carper, N. Vandehey, J.O. Neil, and A.C. Frank. 2016. Evidence for foliar endophytic nitrogen fixation in a widely distributed subalpine conifer. New Phytol. 210: 657–668.

Nassar, A.H., K.A. El-Tarabily, and K. Sivasithamparam. 2005. Promotion of plant growth by an

auxin-producing isolate of the yeast *Williopsis saturnus* endophytic in maize (*Zea mays* L.) roots. Biol. Fertil. Soils 42(2): 97–108.

Naveed, M., B. Mitter, T.G. Reichenauer, K. Wieczorek, and A. Sessitsch. 2014. Increased drought stress resilience of maize through endophytic colonization by *Burkholderia phytofirmans* PsJN and Enterobacter sp. FD17. Environ. Exp. Bot. 97(2014): 30–39.

Nikolic, B., H. Schwab, and A. Sessitsch. 2011. Metagenomic analysis of the 1-aminocyclopropane-1-carboxylate deaminase gene (acdS) operon of an uncultured bacterial endophyte colonizing *Solanum tuberosum* L. Arch. Microbiol. 193(9): 665–676.

Olivares, J., E.J. Bedmar, and J. Sanjuán. 2013. Biological nitrogen fixation in the context of global change. Mol. Plant. Microbe. Interact. 26(5): 486–94.

Oteino, N., R.D. Lally, S. Kiwanuka, A. Lloyd, D. Ryan, K.J. Germaine, and D.N. Dowling. 2015. Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. Front. Microbiol. 6(July): 1–9.

Pal, K.K., and B.M. Gardener. 2006. Biological control of plant pathogens. Plant Heal. Instr.: 1–25.

Pankievicz, V.C.S., F.P. Do Amaral, K.F.D.N. Santos, B. Agtuca, Y. Xu, M.J. Schueller, A.C.M. Arisi, M.B.R. Steffens, E.M. De Souza, F.O. Pedrosa, G. Stacey, and R.A. Ferrieri. 2015. Robust biological nitrogen fixation in a model grass-bacterial association. Plant J. 81(6): 907–919.

Passari, A.K., V.K. Mishra, V.K. Gupta, M.K. Yadav, R. Saikia, and B.P. Singh. 2015. *In vitro* and *in vivo* plant growth promoting activities and DNA fingerprinting of antagonistic endophytic actinomycetes associates with medicinal plants. PLoS One 10(9): e0139468.

Puri, A., K.P. Padda, and C.P. Chanway. 2016. Seedling growth promotion and nitrogen fixation

by a bacterial endophyte *Paenibacillus polymyxa* P2b-2R and its GFP derivative in corn in a long-term trial. Symbiosis. 69(2): 123-129.

Qin, S., Y.J. Zhang, B. Yuan, P.Y. Xu, K. Xing, J. Wang, and J.H. Jiang. 2014. Isolation of ACC deaminase-producing habitat-adapted symbiotic bacteria associated with halophyte *Limonium sinense* (Girard) Kuntze and evaluating their plant growth-promoting activity under salt stress. Plant Soil 374(1-2): 753–766.

Raghavendra, A.K.H., and G. Newcombe. 2013. The contribution of foliar endophytes to quantitative resistance to Melampsora rust. New Phytol. 197(3): 909–18.

Reay, D.S., E. a. Davidson, K. a. Smith, P. Smith, J.M. Melillo, F. Dentener, and P.J. Crutzen. 2012. Global agriculture and nitrous oxide emissions. Nat. Clim. Chang. 2(6): 410–416.

Rees, D.C., F. Akif Tezcan, C. a Haynes, M.Y. Walton, S. Andrade, O. Einsle, and J.B. Howard. 2005. Structural basis of biological nitrogen fixation. Phil. Trans. R. Soc. 363(1829): 971–84.

Reinhold-Hurek, B., and T. Hurek. 1998. Life in grasses: Diazotrophic endophytes. Trends Microbiol. 6(4): 139–144.

Reinhold-hurek, B., T. Hurek, M. Gillis, B. Hoste, M. Vancanneyt, K. Kersters, and J.D.E. Ley. 1993. Associated with roots of Kallar Grass (*Leptochloa fusca* (L .) Kunth), and description of two species , *Azoarcus indigens* sp . nov . and *Azoarcus communis* sp . nov . Int. J. Systamatic Bacteriol. 43(3): 574–584.

Reiter, B., H. Bürgmann, K. Burg, and A. Sessitsch. 2003. Endophytic nifH gene diversity in African sweet potato. Can. J. Microbiol. 49(9): 549–555.

Rezgui , A., A. Ben Ghnaya-Chakroun, J. Vallance, E. Bruez, M.R. Hajlaoui, N. Sadfi-Zouaoui, and P. Rey. 2016. Endophytic bacteria with antagonistic traits inhabit the wood tissues of

grapevines from Tunisian vineyards. Biol. Control (99): 28-37.

Riggs, P.J., M.K. Chelius, a L. Iniguez, S.M. Kaeppler, and E.W. Triplett. 2001. Enhanced maize productivity by inoculation with diazotrophic bacteria. Aust. J. Plant Physiol. 28(9): 829–836.

Robarge, W.P., J.T. Walker, R.B. McCulloch, and G. Murray. 2002. Atmospheric concentrations of ammonia and ammonium at an agricultural site in the southeast United States. Atmos. Environ. 36: 1661–1674.

Rodriguez, R.J., J. Henson, E. Van Volkenburgh, M. Hoy, L. Wright, F. Beckwith, Y.-O. Kim, and R.S. Redman. 2008. Stress tolerance in plants via habitat-adapted symbiosis. ISME J. 2(4): 404–16.

Rojas-Tapias, D., A. Moreno-Galvan, S. Pardo-Diaz, M. Obando, D. Rivera, and R. Bonilla. 2012. Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (*Zea mays*). Appl. Soil Ecol. 61(2012): 264–272.

Rosenblueth, M., and E. Martínez-Romero. 2006. Bacterial endophytes and their interactions with hosts. Mol. Plant. Microbe. Interact. 19(8): 827–37.

Rouws, L.F.M., C.H.S.G. Meneses, H. V. Guedes, M.S. Vidal, J.I. Baldani, and S. Schwab. 2010. Monitoring the colonization of sugarcane and rice plants by the endophytic diazotrophic bacterium *Gluconacetobacter diazotrophicus* marked with gfp and gusA reporter genes. Lett. Appl. Microbiol. 51(3): 325–330.

Saha, M., S. Sarkar, B. Sarkar, B.K. Sharma, S. Bhattacharjee, and P. Tribedi. 2015. Microbial siderophores and their potential applications: a review. Environ. Sci. Pollut. Res. 22: 1–16.

Santi, C., D. Bogusz, and C. Franche. 2013. Biological nitrogen fixation in non-legume plants. Ann. Bot. 111(5): 743–67.

Santoyo, G., G. Moreno-Hagelsieb, M. del Carmen Orozco-Mosqueda, and B.R. Glick. 2016. Plant growth-promoting bacterial endophytes. Microbiol. Res. 183: 92–99.

Schulz, B., and C. Boyle. 2005. The endophytic continuum. Mycol. Res. 109: 661–686.

Schulz, B., and C. Boyle. 2006. What are endophytes? p. 1–14. *In* Microbial Root Endophytes.

Sessitsch, a, P. Hardoim, J. Döring, A. Weilharter, A. Krause, T. Woyke, B. Mitter, L. Hauberg-Lotte, F. Friedrich, M. Rahalkar, T. Hurek, A. Sarkar, L. Bodrossy, L. van Overbeek, D. Brar, J.D. van Elsas, and B. Reinhold-Hurek. 2012. Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol. Plant. Microbe. Interact. 25(1): 28–36.

Shi, Y., K. Lou, and C. Li. 2009. Promotion of plant growth by phytohormone-producing endophytic microbes of sugar beet. Biol. Fertil. Soils 45: 645–653.

Sikora, R.A., L. Pocasangre, A. zum Felde, B. Niere, T.T. Vu, and A.A. Dababat. 2008. Mutualistic endophytic fungi and *in-planta* suppressiveness to plant parasitic nematodes. Biol. Control 46(1): 15–23.

Sikora, R.A., K. Schäfer, and A.A. Dababat. 2007. Modes of action associated with microbially induced *in planta* suppression of plant-parasitic nematodes. Australas. Plant Pathol. 36(2): 124–134.

de Souza, R., A. Ambrosini, and L.M.P. Passaglia. 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. Genet. Mol. Biol. 38(4): 401–419.

Spaepen, Stijn, and Vanderleyden, J. 2011. Auxin and plant-microbe interactions. Cold Spring Harb. Perspect. Biol. 3:a001438: 1–13.

Suryanarayanan, T.S., J.P. Ravishankar, G. Venkatesan, and T.S. Murali. 2004. Characterization of the melanin pigment of a cosmopolitan fungal endophyte. Mycol. Res. 108(8): 974–978.

Taghavi, S., C. Garafola, S. Monchy, L. Newman, A. Hoffman, N. Weyens, T. Barac, J. Vangronsveld, and D.D. Van Der Lelie. 2009. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl. Environ. Microbiol. 75(3): 748–757.

Tubiello, F.N., J.-F. Soussana, and S.M. Howden. 2007. Crop and pasture response to climate change. Proc. Natl. Acad. Sci. U. S. A. 104(50): 19686–19690.

Turner, T.R., E.K. James, and P.S. Poole. 2013. The plant microbiome. Genome Biol. 14(6): 209.

Ulrich, K., A. Ulrich, and D. Ewald. 2008. Diversity of endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiol. Ecol. 63(2): 169–180.

Vincent, J.B., G.D. Weiblen, and G. May. 2016. Host associations and beta diversity of fungal endophyte communities in New Guinea rainforest trees. Mol. Ecol. 25(3): 825–841.

Wahid, a, S. Gelani, M. Ashraf, and M. Foolad. 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 61(3): 199–223.

Waqas, M., A.L. Khan, M. Kamran, M. Hamayun, S.M. Kang, Y.H. Kim, and I.J. Lee. 2012. Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. Molecules 17(9): 10754–10773.

Weilharter, A., B. Mitter, M. V. Shin, P.S.G. Chain, J. Nowak, and A. Sessitsch. 2011. Complete genome sequence of the plant growth-promoting endophyte *Burkholderia phytofirmans* strain PsJN. J. Bacteriol. 193(13): 3383–3384.

Wilson, D. 1995. Endophyte: the evolution of a term, and clarification of its use and definition. Oikos 73: 274–276.

Xin, G., D. Glawe, and S.L. Doty. 2009a. Characterization of three endophytic, indole-3-acetic

acid-producing yeasts occurring in *Populus* trees. Mycol. Res. 113(9): 973–80.

Xin, G., G. Zhang, J.W. Kang, J.T. Staley, and S.L. Doty. 2009b. A diazotrophic, indole-3-acetic acid-producing endophyte from wild cottonwood. Biol. Fertil. Soils 45(6): 669–674.

Yaish, M.W., I. Antony, and B.R. Glick. 2015. Isolation and characterization of endophytic plant growth-promoting bacteria from date palm tree (*Phoenix dactylifera* L.) and their potential role in salinity tolerance. Antonie Van Leeuwenhoek 107(6): 1519–1532.

Zhang, X., D.L. McRose, R. Darnajoux, J.P. Bellenger, F.M.M. Morel, and A.M.L. Kraepiel. 2016. Alternative nitrogenase activity in the environment and nitrogen cycle implications. Biogeochemistry 127(2-3): 189–198.

CHAPTER TWO

DIAZOTROPHIC ENDOPHYTES OF POPLAR AND WILLOW FOR GROWTH PROMOTION OF RICE PLANTS IN NITROGEN-LIMITED CONDITIONS

Shyam L. Kandel, Nicholas Herschberger, Soo-Hyung Kim, and Sharon L. Doty*

School of Environmental and Forest Sciences, College of the Environment, University of Washington, Seattle, WA 98195-2100.

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* Corresponding author: Sharon L. Doty (sldoty@uw.edu)

Author's contribution:

The greenhouse growth assay was conducted by Nick Herschberger; fluorescent microscopy and *in planta* endophyte populations assays were conducted by Shyam L. Kandel. The manuscript was written by Shyam L. Kandel with review and guidance from Soo-Hyung Kim, and Sharon L. Doty.

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.

ABSTRACT

Rice (*Oryza sativa* L.) is one of the most important staple food crops. Its cultivation requires a relatively high input of nitrogen (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.

INTRODUCTION

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 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 X *P. deltoids* W. Bartram ex Marshall) plants inoculated with endophytes of wild poplar and willow had higher biomass as 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.

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^{O}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 $Na_2MoO_4.2H_2O$, 0.028 $Na_2FeEDTA$, 0.1 yeast extract; and Solution 2 [g L^{-1}]: 0.2 $MgSO_4.7H_2O$, 0.06 $CaCl_2$) (Rennie, 1981) and cultured overnight in a rotatory shaker at 160 rpm and 30^{O}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 Carlose 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^{O}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 $30^{O}C$. 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.

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 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 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 noninoculated 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 (Doty et al. 2016) 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., 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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REFERENCES

Amaral, F.P., J.C.F. Bueno, V.S. Hermes, and A.C.M. Arisi. 2014. Gene expression analysis of maize seedlings (DKB240 variety) inoculated with plant growth promoting bacterium *Herbaspirillum seropedicae*. Symbiosis 62:41–50. doi:10.1007/s13199-014-0270-6

Apine, O.A., and J.P. Jadhav. 2011. Optimization of medium for indole-3-acetic acid production using *Pantoea agglomerans* strain PVM. J. Appl. Microbiol. 110:1235–1244. doi:10.1111/j.1365-2672.2011.04976.x

Bhattacharjee, R.B., A. Singh, and S.N. Mukhopadhyay. 2008. Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: Prospects and challenges. Appl. Microbiol. Biotechnol. 80:199–209. doi:10.1007/s00253-008-1567-2

Bulgarelli, D.K., S. Schlaeppi, Spaepen, E.V.L. van Themaat, and P. Schulze-Lefert. 2013. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 64:807–838. doi:10.1146/annurev-arplant-050312-120106

Cassman, K.G., A. Dobermann, and D.T. Walters. 2002. Agroecosystems, nitrogen-use efficiency, and nitrogen management. Ambio 31:132–140.

Choudhury, A.T.M.A., and I.R. Kennedy. 2005. Nitrogen fertilizer losses from rice soils and control of environmental pollution problems. Commun. Soil Sci. Plan. 36:1625–1639. doi:10.1081/CSS-200059104

Doty, S. L. 2011. Nitrogen-fixing endophytic bacteria for improved plant growth. In: D. K. Maheshwari, editor, Bacteria in agrobiology: plant growth responses. Springer, Berlin, Germany. p. 183-199.

Doty, S. L., Dosher, M. R., Singleton, G. L., Moore, A. L., van Aken, B., Stettler, R. F., Strand, S. E., and Gordon, M. P. 2005. Identification of an endophytic rhizobium in stems of *Populus*. Symbiosis 39 (1):27-35.

Doty, S.L., Sher, A.W., Fleck, N.D., Khorasani, M., Bumgarner, R.E., Khan, Z., Ko, A.W., Kim, S.H., and DeLuca, T.H. 2016. Variable nitrogen fixation in wild *Populus*. PloS one, 11(5), p.e0155979.

Doty, S.L., B. Oakley, G. Xin, J.W. Kang, G. Singleton, Z. Khan, A. Vajzovic, and J.T. Staley. 2009. Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47:27–33. doi:10.1007/BF03179967

Egener, T., T. Hurek, and B. Reinhold-Hurek. 1999. Endophytic expression of *nif* genes of *Azoarcus* sp. strain BH72 in rice roots. Mol. Plant Microbe Interact. 12:813–819. doi:10.1094/MPMI.1999.12.9.813

Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, H. Mitsui, and K. Minamisawa. 2001. Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. 67:5285–5293. doi:10.1128/AEM.67.11.5285-5293.2001

Germaine, K., E. Keogh, G. Garcia-Cabellos, B. Borremans, D. Lelie, T. Barac, L. Oeyen, J. Vangronsveld, F.M. Moore, E.R.B. Moore, C.D. Campbell, D. Ryan, and D.N. Dowling. 2004. Colonisation of poplar trees by *gfp* expressing bacterial endophytes. FEMS Microbiol. Ecol. 48:109–118. doi:10.1016/j.femsec.2003.12.009

Govindarajan, M., J. Balandreau, S.W. Kwon, H.Y. Weon, and C. Lakshminarasimhan. 2008. Effects of the inoculation of *Burkholderia vietnamensis* and related endophytic

diazotrophic bacteria on grain yield of rice. Microb. Ecol. 55:21–37. doi:10.1007/s00248-007-9247-9

Gyaneshwar, P., E.K. James, N. Mathan, P.M. Reddy, B. Reinhold-Hurek, and J.K. Ladha. 2001. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. J. Bacteriol. 183:2634–2645. doi:10.1128/JB.183.8.2634-2645.2001

Howarth, R.W. 2008. Coastal nitrogen pollution: A review of sources and trends globally and regionally. Harmful Algae 8:14–20. doi:10.1016/j.hal.2008.08.015

Huang, J., F. He, K. Cui, R.J. Buresh, B. Xu, W. Gong, and S. Peng. 2008. Determination of optimal nitrogen rate for rice varieties using a chlorophyll meter. Field Crops Res. 105:70–80. doi:10.1016/j.fcr.2007.07.006

Iniguez, A.L., Y. Dong, and E.W. Triplett. 2004. Nitrogen fixation in wheat provided by *Klebsiella pneumoniae* 342. Mol. Plant Microbe Interact. 17:1078–1085. doi:10.1094/MPMI.2004.17.10.1078

James, E.K., P. Gyaneshwar, N. Mathan, W.L. Barraquio, P.M. Reddy, P.P.M. Iannetta, F.L. Olivares, and J.K. Ladha. 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Mol. Plant Microbe Interact. 15:894–906. doi:10.1094/MPMI.2002.15.9.894

James, E.K., and F.L. Olivares. 1997. Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. Crit. Rev. Plant Sci. 17:77–119. doi:10.1016/S0735-2689(98)00357-8

Johnson, C.W. 2005. Rice cultivar ‘M-206’. U.S. Patent 6911,589. Date issued: 28 June.

Khan, Z., G. Guelich, H. Phan, R. Redman, and S. Doty. 2012. Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. ISRN Agron. 2012:890280. doi:10.5402/2012/890280.

Khush, G.S. 2013. Strategies for increasing the yield potential of cereals: Case of rice as an example. Plant Breed. 132:433–436.

Kim, S., S. Lowman, G. Hou, J. Nowak, B. Flinn, and C. Mei. 2012. Growth promotion and colonization of switchgrass (*Panicum virgatum*) cv. Alamo by bacterial endophyte *Burkholderia phytofirmans* strain PsJN. Biotechnol. Biofuels 5:37. doi:10.1186/1754-6834-5-37

Knoth, J.L., S.H. Kim, G.J. Ettl, and S.L. Doty. 2013. Effects of cross host species inoculation of nitrogen-fixing endophytes on growth and leaf physiology of maize. GCB Bioenergy 5:408–418. doi:10.1111/gcbb.12006

Knoth, J.L., S.H. Kim, G.J. Ettl, and S.L. Doty. 2014. Biological nitrogen fixation and biomass accumulation within poplar clones as a result of inoculations with diazotrophic endophyte consortia. New Phytol. 201:599–609. doi:10.1111/nph.12536

Lata, H., X.C. Li, B. Silva, R.M. Moraes, and L. Halda-Alija. 2006. Identification of IAA-producing endophytic bacteria from micropropagated *Echinacea* plants using 16S rRNA sequencing. Plant Cell Tiss. Org. 85:353–359.

Long, H.H., D.D. Schmidt, and I.T. Baldwin. 2008. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PLoS ONE 3:e2702. doi:10.1371/journal.pone.0002702

Merzaeva, O.V., and I.G. Shirokikh. 2010. The production of auxins by the endophytic bacteria of winter rye. Appl. Biochem. Microbiol. 46:44–50. doi:10.1134/S0003683810010072

Momose, A., N. Ohtake, K. Sueyoshi, T. Sato, Y. Nakanishi, S. Akao, and T. Ohyama. 2009. Nitrogen fixation and translocation in young sugarcane (*Saccharum officinarum* L.) plants associated with endophytic nitrogen-fixing bacteria. Microbes Environ. 24:224–230. doi:10.1264/jsme2.ME09105

Montanez, A., C. Abreu, P.R. Gill, G. Hardarson, and M. Sicardi. 2009. Biological nitrogen fixation in maize (*Zea mays* L.) by ^{15}N isotope-dilution and identification of associated culturable diazotrophs. Biol. Fertil. Soils 45:253–263. doi:10.1007/s00374-008-0322-2

Olivares, J., E.J. Bedmar, and J. Sanjuan. 2013. Biological nitrogen fixation in the context of global change. Mol. Plant Microbe Interact. 26:486–494. doi:10.1094/MPMI-12-12-0293-CR

Prieto, P., E. Schiliro, M.M. Maldonado-Gonzalez, R. Valderrama, J.B. Barroso-Albarracin, and J. Mercado-Blanco. 2011. Root hairs play a key role in the endophytic colonization of olive roots by *Pseudomonas* spp. with biocontrol activity. Microb. Ecol. 62:435–445. doi:10.1007/s00248-011-9827-6

Quecine, M.C., W.L. Araujo, P.B. Rossetto, A. Ferreira, S. Tsui, P.T. Lacava, M. Mondin, J.L. Azevedo, and A.A. Pizzirani-Kleiner. 2012. Sugarcane growth promotion by the endophytic bacterium *Pantoea agglomerans* 33.1. Appl. Environ. Microbiol. 78:7511–7518. doi:10.1128/AEM.00836-12

R Development Core Team. 2013. R: A language and environment for statistical computing, version 3.0.1. R Foundation for Stat. Comput., Vienna, Austria.

Reinhold-hurek, B., T. Hurek, M. Gillis, B. Hoste, M. Vancanneyt, K. Kersters, and J.D.E. Ley. 1993. Associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int. J. Syst. Bacteriol. 43:574–584. doi:10.1099/00207713-43-3-574

Rennie, R.J. 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. 27:8–14. doi:10.1139/m81-002

Riggs, P.J., M.K. Chelius, A.L. Iniguez, M.K. Shawn, and E.W. Triplett. 2001. Enhanced maize productivity by inoculation with diazotrophic bacteria. Aust. J. Plant Physiol. 28:829–836.

Roberts, T. L., Hardke, J. T., and Jr. Wilson, C. E. 2013. Recommended nitrogen rates and distribution for rice varieties in Arkansas. Division of Agriculture, Research and Extension, University of Arkansas, Fayetteville.

Rodriguez, R.J., D.C. Freeman, E.D. McArthur, Y.O. Kim, and R.S. Redman. 2009. Symbiotic regulation of plant growth, development and reproduction. Commun. Integr. Biol. 2:141–143. doi:10.4161/cib.7821

Roncato-Maccari, L.D.B., H.J.O. Ramos, F.O. Pedrosa, Y. Alquini, L.S. Chubatsu, M.G. Yates, L.U. Rigo, M.B.R. Steffens, and E.M. Souza. 2003. Endophytic *Herbaspirillum seropedicae* expresses *nif* genes in gramineous plants. FEMS Microbiol. Ecol. 45:39–47. doi:10.1016/S0168-6496(03)00108-9

Rosenblueth, M., and E. Martínez-Romero. 2006. Bacterial endophytes and their interactions with hosts. Mol. Plant Microbe Interact. 19:827–837. doi:10.1094/MPMI-19-0827

Santi, C., D. Bogusz, and C. Franche. 2013. Biological nitrogen fixation in non-legume plants. Ann. Bot. (Lond.) 111:743–767. doi:10.1093/aob/mct048

Singh, H., A. Verma, M.W. Ansari, and A. Shukla. 2014. Physiological response of rice (*Oryza sativa* L.) genotypes to elevated nitrogen applied under field conditions. Plant Signal. Behav. 9:e2901. doi:10.4161/psb.29015

Singh, M.K., C. Kushwaha, and R.K. Singh. 2009. Studies on endophytic colonization ability of two upland rice endophytes, *Rhizobium* sp. and *Burkholderia* sp., using green fluorescent protein reporter. Curr. Microbiol. 59:240–243. doi:10.1007/s00284-009-9419-6

Stevens, J.M., R.L. Ulrich, L.A. Taylor, M.W. Wood, D. Deshazer, M.P. Stevens, and E.E. Galyov. 2005. Actin-binding proteins from *Burkholderia mallei* and *Burkholderia thailandensis* can functionally compensate for the actin-based motility defect of a *Burkholderia pseudomallei bimA* mutant. J. Bacteriol. 187:7857–7862. doi:10.1128/JB.187.22.7857-7862.2005

Thomas, P., and K.M. Reddy. 2013. Microscopic elucidation of abundant endophytic bacteria colonizing the cell wall–plasma membrane peri-space in the shoot-tip tissue of banana. AoB Plants 5:Plt011. doi:10.1093/aobpla/plt011

Tubiello, F.N., J.F. Soussana, and S.M. Howden. 2007. Crop and pasture response to climate change. Proc. Natl. Acad. Sci. USA 104:19686–19690. doi:10.1073/pnas.0701728104

Verma, S.C., J.K. Ladha, and A.K. Tripathi. 2001. Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. J. Bacteriol. 91:127–141.

Wang, Q., Huang, J., He, F., Cui, K., Zeng, J., Nie, L., and Peng, S. 2012. Head rice yield of "super" hybrid rice Liangyoupeijiu grown under different nitrogen rates. Field Crops Res. 134:71-79.

Wilson, D. 1995. Endophyte: The evolution of a term, and clarification of its use and definition. Oikos 73:274–276. doi:10.2307/3545919

Wright, K.M., S. Chapman, K. McGeachy, S. Humphris, E. Campbell, I.K. Toth, and N.J. Holden. 2013. The endophytic lifestyle of *Escherichia coli* O157: H7: Quantification and internal localization in roots. Phytopathology 103:333–340. doi:10.1094/PHYTO-08-12-0209-FI

Xin, G., D. Glawe, and S.L. Doty. 2009a. Characterization of three endophytic indole-3-acetic acid-producing yeasts occurring in *Populus* trees. Mycol. Res. 113:973–980. doi:10.1016/j.mycres.2009.06.001

Xin, G., G. Zhang, J.W. Kang, J.T. Staley, and S.L. Doty. 2009b. A diazotrophic, indole-3-acetic acid-producing endophyte from wild cottonwood. Biol. Fertil. Soils 45:669–674. doi:10.1007/s00374-009-0377-8

Xing, Y., and Zhang, Q. 2010. Genetic and molecular bases of rice yield. Annu. Rev. Plant Biol. 61:421–442. doi:10.1146/annurev-arplant-042809-112209

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	*Curtobacterium* sp.
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).

Table 2. Root to 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 Numbers per plant**
Endophyte inoculation	0.84 ± 0.025^{a}	2.83 ± 0.196^{a}
Mock inoculation	0.69 ± 0.027^{b}	1.50 ± 0.150^{b}

Fig 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 ($P < 0.01$).

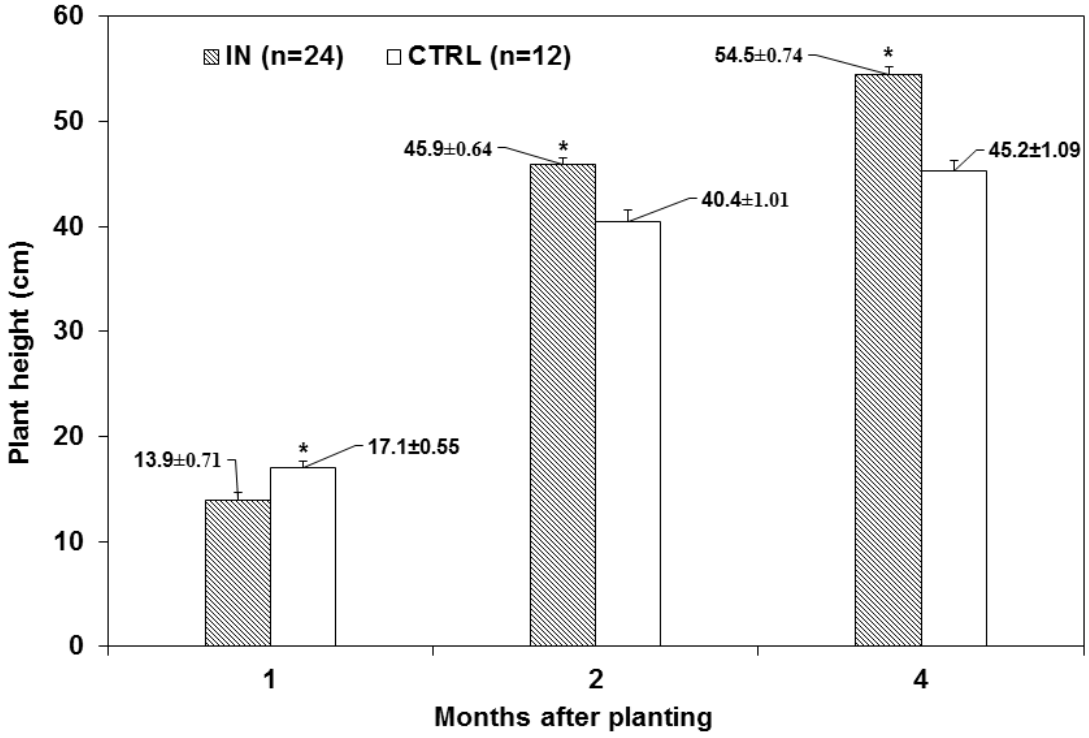


Fig 2. Root, shoot, and total plant biomass in endophyte inoculated (IN) and control (CTRL) plants at 4 months after planting. The bars represent the standard errors of mean. Histograms with asterisk indicate significant differences ($P < 0.01$).

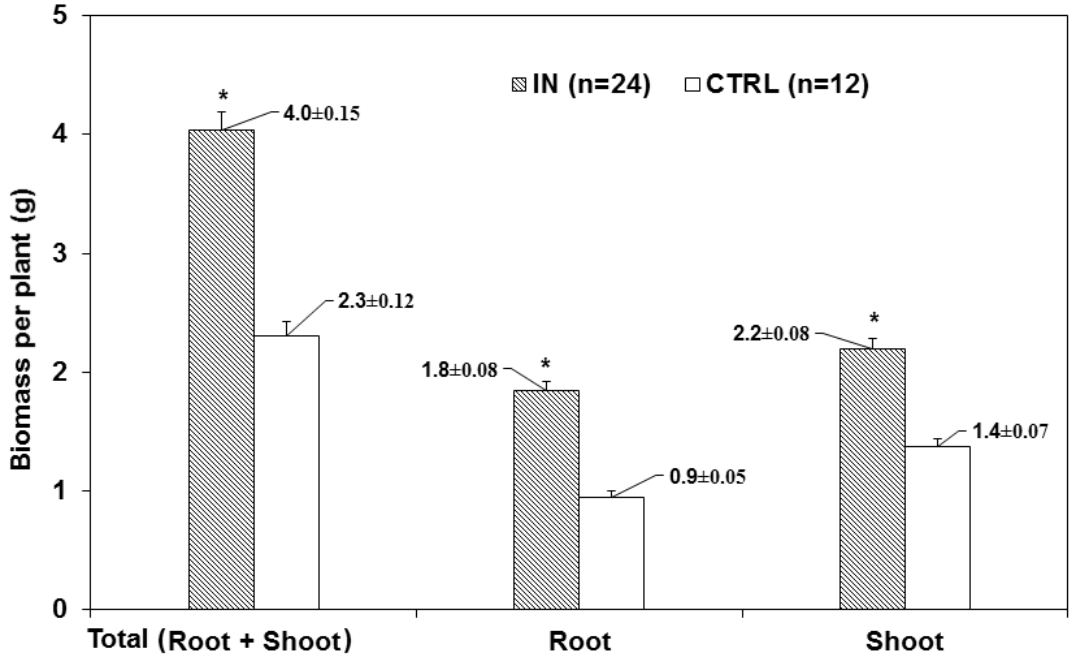


Fig 3. Rice var. M206 root colonized by GFP labeled endophytes WP9*gfp*: A and B, PTD1*gfp*: C and D, and WP5*gfp*: E and F at 20 days after inoculation. Images on the left (A, C, and E) were taken with GFP filter, and on the right (B, D, and F) were taken without GFP filter at 630 magnifications.

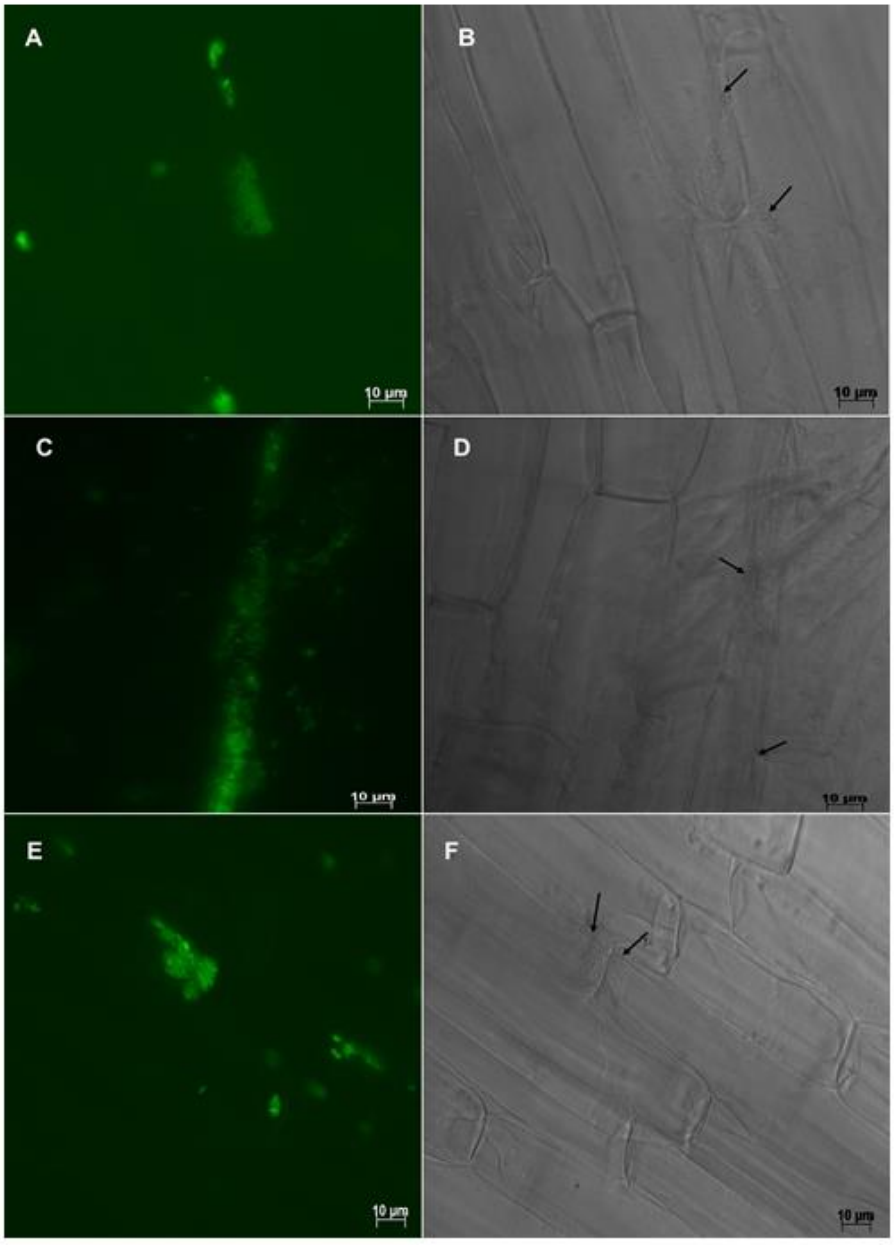
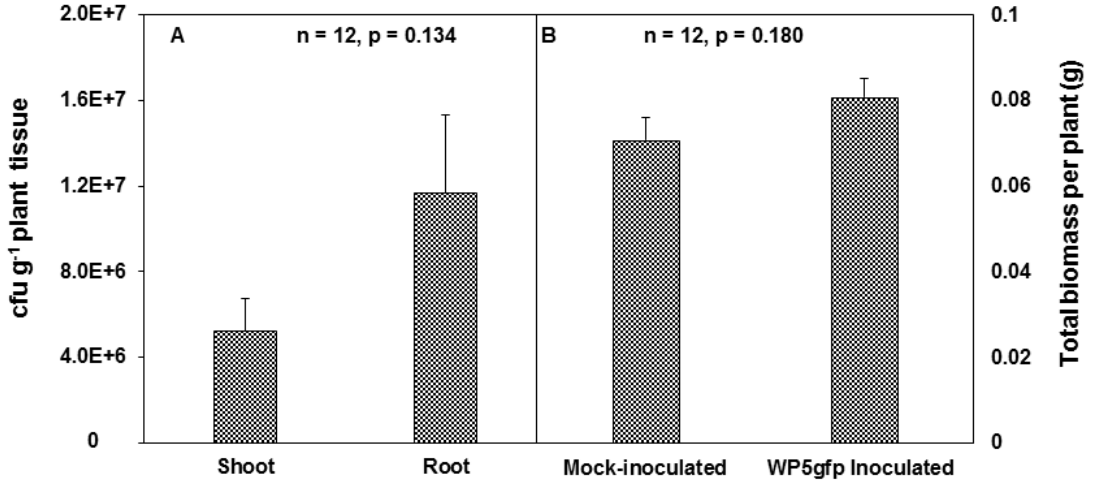


Fig 4. Quantification of endophytic populations, *gfp* expressing colonies isolated from plant tissues and total plant biomass at 20 days after inoculation. (A) cfu per gram in shoot including leaves and root; (B) Total biomass per plant (g) in mock-inoculated and WP5*gfp* inoculated rice plants. The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



CHAPTER THREE

GROWTH RESPONSE IN MAIZE AND RICE PLANTS BY THE INOCULATION OF SALICACEAE ENDOPHYTES UNDER NUTRIENT LIMITED CONDITIONS

ABSTRACT

In recent years, the contribution of the microbiome to plant growth and development has received considerable attention both in agriculture and natural ecosystems. A Plethora of microbial communities colonize above ground or below ground plant parts both on the surface (i.e. epiphytes), and in the internal tissues (i.e. endophytes). Perhaps endophytes have more intimate interactions with specific colonizing strategies to the host plant than nomadic life styles of epiphytes on the surface. Dozens of endophyte strains of Salicaceae plants; poplar (*Populus trichocarpa* Torr. & A. Gray) and willow (*Salix sitchensis* C. A. Sanson ex Bong.), with potential for growth promotion, reduction of biotic and abiotic stresses, and degradation of toxic substances have been characterized. This study aimed to investigate the growth response in endophyte treated maize and rice plants under nitrogen (N) depleted conditions. Maize and rice seedlings were inoculated with Salicaceae endophytes and grown in the greenhouse at the University of Washington, Seattle, WA. Additionally, endophyte inoculated maize and rice plants were grown with a supply of ^{15}N labelled N to observe the growth response, and to estimate the biologically fixed N resulting from plant-endophyte interactions. Both maize and rice plants showed variable responses upon treatment with endophytes. However, the majority of the inoculated maize and rice plants were superior in growth and had higher root or shoot biomass as compared to mock-inoculated control plants. In rice shoots, added $d^{15}N$ was found diluted by N fixed through inoculated endophytes.

INTRODUCTION

Rice, wheat, and maize are important food grains in the world. The global human population acquires a significant portion of its calorie mainly from these three crops (IRRI). While these crops have been grown for thousands of years in different parts of the world, the production and productivity of these crops have been significantly improved in the past century due to the introduction of chemical fertilizers, notably N fertilizer, agrochemicals including pesticides and herbicides, superior crop genotypes, and farm mechanization (Edgerton, 2009; Grassini et al., 2013; Uphoff and Dazzo, 2016). For example, the productivity of maize in the U.S. has increased from nearly 1.6 to 9.5 tonnes ha^{-1} during the early 20^{th} century to now (Edgerton, 2009). At present, U.S. is the number one producer of maize grains accounting for more than one third of the global maize production (FAOSTAT, 2012). But the existing practices in agriculture are considered unsustainable as they are resulting in undesirable consequences to our human health and natural environment. The increased use of N and phosphorus fertilizers can pollute the soil and water systems, release the potent greenhouse gases such as nitrous oxide, and expand the areas of eutrophication. Furthermore, many other challenges including declining areas of arable land, depleting of fresh water, insurgence of new pest and disease, and changing global weather patterns are making agriculture a daunting enterprise (Foley et al., 2005; Reay et al., 2012; Uphoff, 2012; Srivastava et al., 2016).

There is global demand for more food to feed every child born not only of today but tomorrow despite the challenges that agriculture is currently facing. Yet there is potential to increase the crop yield without damaging our environment by integrating different approaches. It is suggested that using conservation tillage, organic farming, crop diversification, biocontrol of pest and

pathogens, and microbial growth enhancers like endophytes would bring resilience in agriculture (Edgerton, 2009; Lin, 2011; Reganold and Wachter, 2016; Uphoff and Dazzo, 2016). More recently, many studies have been showing that the human microbiome, the sum of microbial genomes, has profound effects in our growth and development (Lloyd-Price et al., 2016). The microbial communities residing in and on our body are treated as benign partners, not the malignant invaders. Likewise, microbiomes of plants provide many benefits to host plants including nutrients and water acquisition, and tolerance to biotic and abiotic stresses (Gaiero et al., 2013; Berg et al., 2014; Lebeis, 2014). The importance of the plant microbiome is highlighted as a "new frontier" in agriculture to achieve the optimum crop yields without damaging our environment. By modulating plant microbiomes, there is potential to improve the crop yields in a sustainable way by minimizing the environmental footprints of agriculture (Turner et al., 2013; Lebeis, 2014; Schlaeppi and Bulgarelli, 2015).

The microbial communities living inside the plant body without eliciting any disease symptom or adverse impact to the host plant are called endophytes (Hardoim et al., 2008; Rodriguez et al., 2009; Reinhold-Hurek and Hurek, 2011). Endophytes are the most influential component of plant microbiome and have the potential to promote the host plant growth, especially through phytohormone production, N fixation, phosphorus and iron acquisition, and conferring tolerance to pathogens or drought (Rosenblueth and Martínez-Romero, 2006; Gaiero et al., 2013; Berg et al., 2014; Lebeis, 2014; Hardoim et al., 2015). N-fixing endophytes (also called diazotrophic endophytes) carry genes encoding for the nitrogenase that is responsible for biological N fixation, converting the atmospheric N into ammonium which is utilized in plant metabolism (Olivares et al., 2013). Several plant endophyte strains have been investigated during recent

years to explore the mechanisms they used for plant growth promotion, and potential use in agriculture (Lucero et al., 2014; Hamilton et al., 2016; Santoyo et al., 2016). Positive growth response on physical characteristics of host plants such as biomass or plant height has been reported in a variety of crop plants such as rice, wheat, maize, sugar cane, tomato (Elbeltagy et al., 2001; Riggs et al., 2001; Hurek et al., 2002; Iniguez et al., 2004; Momose et al., 2009; Khan et al., 2012; Knoth et al., 2012; Botta et al., 2013; Kandel et al., 2015).

Doty et al. (2009) and Xin et al. (2009) isolated and characterized several diazotrophic endophytes from Salicaceae plants; poplar and willow growing in the riparian environments. Past studies demonstrated that endophytes of Salicaceae plants can maintain their growth in N-limited medium, and potential to relieve the N stress in the host plant (Doty et al., 2009, 2016; Knoth et al., 2014; Kandel et al., 2015). In addition, some of the poplar endophytes can produce significant amounts of phytohormone, indole-3-acetic acid (IAA) in *in-vitro* condition (Xin et al., 2009). The previous studies showed that these endophytes have a broad host range, and improved the host plant growth by increasing biomass, height, leaf area etc. in maize, rice, tomato, pepper, squash, and Douglas fir in N limited conditions (Khan et al., 2012, 2015; Knoth et al., 2012; Kandel et al., 2015). Although past studies demonstrated that these endophytes have a broad host range, Khan et al. 2012 showed that growth benefit on host plants is host/cultivar specific that would limit its use by farmers. In this study, we proposed to investigate the growth response in various endophyte treated genotypes of maize and rice under N limited conditions. Moreover, possible biologically fixed N by endophytes was monitored in maize and rice. Understanding the growth response of diazotrophic endophytes of Salicaceae plants in rice and

maize can contribute for sustainable food production with the minimum application of chemical inputs.

MATERIALS AND METHODS

Endophyte strains and growth conditions

Salicaceae endophytes; Table 1 (Doty et al., 2009; Firrincieli et al., 2015), isolated from wild poplar and wild willow plants were used in this study. These endophytes as an individual strain or consortium: a collection of multiple strains, were used in inoculation experiments. These endophytes were chosen because of their ability to grow in the N limited culture medium. They were grown at 30°C in N-limited combined carbon medium (NLCCM) for 2 days (Rennie, 1981). Few isolated colonies from 2-days old culture were then transferred to NLCCM broth, and grown overnight in a shaking incubator at 30^{o}C. The optical density of endophyte growth in NLCCM broth was determined using diode array spectrophotometer (Biochrom US, Holliston, MA, USA). The endophyte culture was adjusted to 0.1 OD_{600} (approximate 2 x 10^7 colony forming units, CFU mL^{-1}) in the N free MS (Murashige-Skoog) medium (Caisson Inc., Logan, UT, USA).

Table 1: List of Salicaceae endophytes included in the inoculation experiments.

Endophyte strains	**Best BLAST match**	**Included Consortia #**	**GenBank Accession #**
PTD-1	*Rhizobium tropici* bv *populus*	Consortium # 1, # 2	KT962907
WP1	*Rhodotorula graminis*	Consortium # 1, # 2	EU563924
WPB	*Burkholderia vietnamiensis*	Consortium # 1	EU563933–EU563935
WP5	*Rahnella sp.*CDC 2987-79	Consortium # 1, # 2	KU497675
WP9	*Burkholderia* sp.	Consortium # 1, # 2	KU523562
WP19	*Acinetobacter calcoaceticus.*	Consortium # 1	KU523563
WW5	*Sphingomonas yanoikuyae*	Consortium # 1	KT984987
WW6	*Pseudomonas putida*	Consortium # 1	KU557506
WW7	*Curtobacterium* sp.	Consortium # 1	KU523564
WP40	*Burkholderia* sp.	Consortium # 2	KF597274
WP41	*Burkholderia* sp.	Consortium # 2	KF597275
WP42	*Burkholderia* sp.	Consortium # 2	KF597275
WP25	*Burkholderia vietnamiensis*	Consortium # 2	KF750606
WW17	*Burkholderia vietnamiensis*	Consortium # 2	KF597278
Snoq. 117.2	*Pseudomonas* species	Consortium # 2	KF597277

Plant materials

Several maize and rice genotypes were used in different inoculation experiments.

For rice, varieties; M206 (Johnson, 2005) and Presidio (Imai et al., 2013), and hybrids; XL745 and XL723 (http://www.ricetec.com/) were used. For maize, hybrids XR1404 and XR1634 (http://www.ruppseeds.com/), organic hybrids; 07M91, 14A91, 29B17, and 23A71 (http://www.blueriverorgseed.com/), and CIMMYT maize inbred lines; CML539 and CML444 (Wu et al., 2016) were used.

Endophyte inoculation

Maize and rice seeds were surface sterilized in NaClO (3% v/v) for up to 20 to 30 minutes (maize), or 4 hours (rice) followed by rinsing five to eight times with sterile, deionized water. The surface sterilized maize and rice seeds were then placed on sterilized filter paper or water agar plates (0.5%) and allowed to germinate at room temperature. Young germinated seedlings were inoculated with endophyte cultures by co-cultivating in endophyte culture in a glass beaker and held overnight to facilitate the ingress of endophyte cells in young plant tissues with gentle shaking (50 rpm). Mock-inoculated control plants were co-cultivated similarly in N free MS (Caisson Inc.) without endophyte culture. In soil inoculation experiments, the area around the 7-10 days old seedlings was drenched with endophyte culture. Mock inoculated plants were drenched with medium without culture.

Plant growth and measurements

Inoculated maize and rice seedlings were grown in the Douglas Research Conservatory greenhouse at the University of Washington, Seattle, WA. Both inoculated maize and rice were

grown either for a month or several months in the low nutrient sunshine mix (SunGro, Bellevue, WA, USA). Treatments in each experiment were either randomized completely within each replicated block or on the entire greenhouse bench. In all 1 month experiments, small pots supported with flat bottom trays, and in the long duration experiments, bigger pots with saucers were used to prevent cross contamination. Trays or pots were rotated every other week to minimize any biased effects of light or temperature. For 1 month experiments, both maize and rice plants received water every week without any supplemental plant nutrients. For long duration experiments, they received plant nutrients (g L^{-1}): KH_2PO_4, 6.996; K_2HPO_4, 1.986; K_2SO_4, 17.497; $MgSO_4.7H_2O$, 11.978; $MgCl_2.6H_2O$, 10.002; $CaCl_2.H_2O$, 19.298; $MnSO_4.H_2O$, 0.338; $CuSO_4.5H_2O$, 0.050; $ZnSO_4.7H_2O$, 0.058; H_3BO_3, 0.383; $Na_2MoO_4.2H_2O$, 0.024; $CoSO_4.7H_2O$, 0.011; Fe Sequestrine, 3.5 supplemented with 0.22 g L^{-1} ammonium nitrate once per week, and additional water as needed for entire growth period.

In N split experiment, five different N doses were used, and corresponding treatments were designated as N0- No N, N1- 25% N (0.16 g L^{-1}), N2- 50% N (0.32 g L^{-1}), N3- 75% N (0.48 g L^{-1}), N4- 100% N (0.64 g L^{-1}). Plant physical characters such as root length, shoot length, biomass, and specific leaf area were observed at the end of each experiment, and physiological observations such as chlorophyll fluorescence and SPAD readings were observed in some experiments during the plant growth period. All 1 month experiments were repeated twice and data were combined for analysis.

The chlorophyll fluorescence of maize leaf was observed by using a portable fluorometer OS-30P+ (Opti-Sciences, Inc., Hudson, NH, USA). The targeted leaf portion was darkened for 30 minutes before taking minimal fluorescence, Fo, followed by illuminating a saturating light beam to gain maximal fluorescence, Fm. The variable fluorescence, Fv (= Fm - Fo), was computed by a built-in program to calculate maximal photochemical efficiency of Photosystem II (Fv/Fm) (Baker, 2008). SPAD was used as an indirect estimation of leaf chlorophyll content or "leaf greenness". The *in vivo* SPAD readings (leaf greenness) was found to be strongly correlated with the *in vitro* chlorophyll content of samples (Cerovic et al., 2012). A portable chlorophyll meter (SPAD-502; Konica Minolta Sensing Americas, Inc., Ramsey, NJ, USA) was used for SPAD readings. The youngest fully expanded leaf was used for both observations.

Total N and C analysis in plant tissues

For total N and C analysis, root and leaf samples were collected before final harvesting and used for analysis. Samples were oven dried, ground, and delivered to the Analytical Service Center, University of Washington for analysis.

^{15}N isotope dilution assay in maize and rice

A ^{15}N isotope dilution assay was used to monitor the biologically fixed N in maize and rice by endophytes. In this technique, endophyte-inoculated plants and mock-inoculated plants received the same amount of ^{15}N-labelled N fertilizer. Four inoculation treatments were used in this experiment including; mock-inoculated control, WP5, WP5 *nifH mutant*, and consortium #1. The randomized complete block design with 6 replications was used. Ammonium sulfate, $(NH_4)_2SO_4$ with 10% excess ^{15}N was added to enrich the sunshine mix (SunGro) with ^{15}N. Ten mg of

$(NH_4)_2SO_4$ was used for 1 kilogram of wet perlite mix. To facilitate uniform distribution of ^{15}N, soil was mixed thoroughly daily for two weeks before planting inoculated maize and rice seedlings. Maize and rice plants were grown for about 7 and 14 weeks respectively with the supplement of 1x Qubit NFM until harvest. Additional water was supplied if soil moisture was not enough. Leaf, stem and root tissues were harvested, ground in liquid N, and oven dried at 70^oC for three days. Root and shoot dry weight were recorded, and about 10 mg of plant tissue was delivered to the Alaska Stable Isotope Facility, University of Alaska (http://ine.uaf.edu/werc/asif/) for elemental analysis isotope-ratio mass spectrometry. The $^{15}N_{sample}$ atom%, and total N content per unit plant biomass was calculated for each sample.

Statistical analysis

Analysis of variance, and Tukey posthoc procedure was used to compare the growth response between inoculated and control groups. Means with standard errors were presented with histograms in figures or listed in the tables. Data were analyzed using Minitab 17 (Minitab Inc., State College, PA, USA) or R statistical version 3.0.1 (http://www.r-project.org/).

RESULTS

Experiment 1: 1 month single-strain inoculation trial in maize and rice

Root and shoot length (cm plant^{-1}), and root and shoot biomass (g plant^{-1}) were recorded at 30 days after inoculation (DAI) in both maize (hybrid XR1634) and rice (var. Presidio). In maize XR1634, root and shoot length, and root and total (root+shoot) biomass were significantly higher in endophyte inoculated plants as compared to mock inoculated control plants (Table 2). Shoot biomass was also higher in inoculated plants but only endophyte strain, WPB inoculated plants

had significantly higher biomass than control plants. In rice Presido, inoculated plants had higher biomass and longer root and shoot but only root length was significantly higher in WPB inoculated plants than corresponding controls. The highest biomass was observed in WPB and WP1 inoculation in XR1634, and WP1 inoculation in Presidio (Table 2).

Table 2. Endophyte-inoculated maize hybrid XR1634, and rice var. Presidio grown in the greenhouse. Means (standard error) of biomass and root and shoot length (n = 10-12). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

Endophyte strain	**Length (cm plant^{-1})**		**Dry weight (g plant^{-1})**		
	Root	**Shoot**	**Root**	**Shoot**	**Total**
Maize: hybrid XR1634					
WP5	44.24(0.85)a	34.31(0.38)a	0.23(<0.01)a	0.24(<0.01)ab	0.47(0.01)a
WPB	41.76(1.21)a	34.62(0.30)a	0.24(<0.01)a	0.26(0.01)a	0.50(0.01)a
WP1	41.63(1.15)a	33.75(0.58)a	0.25(0.01)a	0.24(<0.01)ab	0.50(0.01)a
Control	34.66(1.32)b	30.13(1.25)b	0.18(0.01)b	0.21(0.01)b	0.40(0.02)b
p-value	<0.01	<0.01	<0.01	0.020	<0.01
Rice: var. Presidio					
WP5	10.87(0.56)ab	19.26(1.26)	0.053(0.003)	0.064(0.005)	0.118(0.003)
WPB	11.50(0.57)a	20.47(1.21)	0.060(0.003)	0.063(0.004)	0.123(0.003)
WP1	10.62(0.48)ab	19.13(1.57)	0.057(0.005)	0.069(0.004)	0.127(0.005)
Control	9.49(0.39)b	18.21(0.89)	0.055(0.003)	0.060(0.010)	0.115(0.008)
P-value	0.025	0.591	0.658	0.826	0.515

Experiment 2: 1 month single strain inoculation trial in organic maize and rice hybrids.

Plant length (cm plant^{-1}), and biomass (g plant^{-1}) were observed at 30 DAI in both maize and rice hybrids. In maize hybrid 07M91, root and shoot length were significantly higher in WP42 inoculated plants as compared to control plants (Table 3). In 29B17 and 23A71, shoot length was significantly higher in WP42 inoculated plants than control plants. The shoot biomass was significantly higher in WP1 inoculated plants in hybrid 29B17. In rice, the total biomass was higher in all inoculated plants in hybrid XL723 but only with WP5, WP40, and WP41 inoculation in hybrid XL745. In general, the majority of the inoculated plants had longer root and/or shoot, and higher biomass as compared to control plants (Table 3). The highest biomass was observed in WP5 inoculation in 07M91, WP1 inoculation in 14A91, WP42 inoculation in 29B17 and 23A71. Similarly, biomass was highest in WP41 inoculation in XL745, and WP42 inoculation in XL723 (Table 3).

Table 3. Endophyte-inoculated organic maize hybrids; 07M91, 14A91, 29B17, and 23A71, and rice hybrids, XL745, and XL723 grown in the greenhouse. Means (standard error) of biomass and plant height (n = 10-12). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

	Endophyte strain	**Length (cm plant^{-1})**		**Dry weight (g plant^{-1})**		
		Root	**Shoot**	**Root**	**Shoot**	**Total**
Maize hybrids: 07M91, 14A91, 29B17, and 23A71						
07M91	WP1	44.80(2.55)ab	39.16(0.96)b	0.25(0.02)	0.30(0.02)	0.56(0.03)
	WP5	37.53(2.26)b	40.13(0.67)ab	0.30a(0.01)	0.33(0.02)	0.63(0.03)
	WP40	38.26(2.74)ab	41.94(0.57)ab	0.25a(0.01)	0.32(0.02)	0.57(0.02)
	WP41	38.61(1.44)ab	39.41(0.98)b	0.26a(0.02)	0.19(0.03)	0.54(0.02)

	WP42	48.09(3.69)a	43.94(0.91)a	0.28a(0.03)	0.29(0.03)	0.57(0.05)
	Control	36.33(1.80)b	39.53(1.17)b	0.24a(0.02)	0.29(0.02)	0.53(0.03)
	p-value	0.006	0.007	0.161	0.557	0.371
14A91	WP1	38.46(1.74)	40.26(1.25)a	0.32(0.04)	0.35(0.04)	0.67(0.07)
	WP5	41.34(3.08)	39.41(0.67)a	0.22(0.02)	0.31(0.02)	0.53(0.04)
	WP40	40.24(3.41)	37.15(2.60)a	0.20(0.04)	0.33(0.04)	0.54(0.07)
	WP41	47.37(3.31)	39.79(0.57)a	0.25(0.04)	0.35(0.02)	0.59(.05)
	WP42	37.63(1.54)	36.88(1.27)a	0.20(0.03)	0.25(0.05)	0.45(0.07)
	Control	42.37(3.26)	34.77(0.76)a	0.27(0.02)	0.31(0.04)	0.58(0.04)
	p-value	0.238	0.077	0.141	0.501	0.355
29B17	WP1	39.33(4.02)	36.91(1.61)ab	0.23(0.02)	0.29(0.02)ab	0.52(0.04)
	WP5	36.46(3.14)	33.50(2.08)b	0.16(0.04)	0.27(0.06)ab	0.43(0.08)
	WP40	36.79(2.67)	39.29(1.10)ab	0.23(0.04)	0.25(0.04)ab	0.48(0.06)
	WP41	36.48(1.17)	35.44(1.29)b	0.26(0.02)	0.19(0.03)b	0.44(0.03)
	WP42	38.71(3.21)	42.52(1.73)a	0.28(0.02)	0.37(0.02)a	0.65(0.02)
	Control	36.77(3.16)	33.78(2.17)b	0.27(0.04)	0.21(0.04)ab	0.47(0.08)
	p-value	0.976	0.001	0.150	0.012	0.098
23A71	WP1	34.99(2.60)ab	42.81(0.78)ab	0.27(0.02)	0.33(0.02)	0.60(0.04)
	WP5	32.92(1.48)b	42.16(1.16)ab	0.27(0.02)	0.32(0.03)	0.59(0.04)
	WP40	35.47(2.11)ab	43.78(0.55)ab	0.27(0.03)	0.36(0.05)	0.62(0.04)
	WP41	34.25(3.91)ab	38.20(2.73)ab	0.33(0.02)	0.29(0.03)	0.62(0.04)
	WP42	42.76(1.09)a	46.05(0.72)a	0.33(0.03)	0.36(0.02)	0.69(0.04)
	Control	41.80(2.50)ab	40.34(1.59)b	0.30(0.03)	0.31(0.03)	0.61(0.05)
	p-value	0.023	0.007	0.305	0.703	0.764
Rice hybrids: XL745, and XL723						
XL745	WP1	17.70(0.84)	24.30(2.02)b	0.11(0.01)	0.14(0.02)	0.25(0.04)
	WP5	18.43(1.17)	27.66(1.47)ab	0.16(0.03)	0.15(0.01)	0.31(0.05)
	WP40	16.78(0.90)	27.88(1.58)ab	0.16(0.03)	0.15(0.01)	0.31(0.05)
	WP41	17.32(0.67)	29.99(1.94)ab	0.15(0.01)	0.16(0.02)	0.31(0.03)
	WP42	16.52(1.41)	26.50(2.00)ab	0.12(<0.01)	0.12(<0.01	0.24(0.01)
	Control	17.13(0.78)	31.46(1.03)a	0.14(<0.01)	0.16(0.01)	0.30(0.01)

	p-value	0.846	0.035	0.348	0.103	0.209
XL723	WP1	20.60(061)	29.93(1.07)ab	0.16(0.01)	0.16(0.01)	0.32(0.02)
	WP5	19.77(0.25)	31.83(0.74)ab	0.19(0.01)	0.17(0.01)	0.36(0.02)
	WP40	19.38(0.84)	30.68(1.71)ab	0.17(0.01)	0.16(0.01)	0.33(0.03)
	WP41	18.96(0.66)	27.47(0.94)b	0.15(0.01)	0.17(<0.01)	0.32(0.02)
	WP42	20.48(1.46)	31.97(0.67)a	0.15(0.01)	0.23(0.05)	0.38(0.05)
	Control	18.93(0.81)	31.35(0.91)ab	0.15(0.01)	0.16(<0.01)	0.31(0.01)
	p-value	0.586	0.038	0.584	0.308	0.643

Experiment 3: Long-term single strain inoculation trial in maize and rice

In maize, root biomass in WP1 inoculated plants, and SPAD in Snoq. 117.2 inoculated plants were significantly higher as compared to control plants (Table 4). Total biomass, specific leaf area (SLA), and chlorophyll fluorescence were all higher in WP1 inoculated plants but not statistically significantly different than control plants at the 95% level of significance. Total N content in root and leaf tissues were also analyzed. Root N content was significantly higher in WP1 inoculated plants. In overall, N content in both root and leaf tissues was higher in endophyte inoculated plants as compared to in control plants. In rice, root and shoot biomass, plant height, and SLA were higher in WP5 and Snoq. 117.2 inoculated plants (Table 6). The highest biomass was observed in WP1 inoculation in 29B17, and WP5 inoculation in M206 (Table 4 and 6).

Table 4**.** Plant dry weight, chlorophyll fluorescence, SPAD, SLA, and plant height per inoculated and mock-inoculated control treatments in maize hybrid 29B17 at 110 DAI. Means (standard error) of biomass and plant height (n = 7). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

Endophyte strain	**Dry weight (g plant^{-1})**			**SLA (cm^2g^{-1})**	**Plant ht. (cm)**	**SPAD**	**Fv/Fm**
	Root	**Shoot**	**Total**				
	6.70	23.59	30.28	228.72	158.26	42.50	0.756
WP1	(0.89)a	(1.00)	(0.88)	(7.31)	(9.33)	(0.79)ab	(<0.01)
	4.57	23.72	28.29	229.70	145.56	40.85	0.757
WP5	(0.30)b	(1.69)	(1.94)	(10.1)	(6.24)	(0.87)ab	(<0.01)
	4.47	21.90	26.37	247.80	135.16	41.60	0.762
WP42	(0.33)b	(1.07)	(1.19)	(12.7)	(5.76)	(0.69)ab	(<0.01)
	4.44	23.26	27.70	220.87	137.54	40.28	0.757
WW17	(0.40)b	(1.53)	(1.81)	(6.18)	(6.37)	(0.92)ab	(<0.01)
Snoq.	3.99	22.56	26.57	243.43	150.31	42.87	0.754
117.2	(0.32)b	(0.99)	(1.29)	(5.32)	(8.20)	(0.32)a	(<0.01)
	4.17	24.54	28.71	220.85	138.80	39.09	0.752
Control	(0.40)b	(0.70)	(0.86)	(7.92)	(6.96)	(1.33)b	(<0.01)
p-value	0.005	0.711	0.378	0.153	0.217	0.040	0.678

Table 5. Nitrogen (N) and Carbon (C) concentration in root and leaf tissues per inoculated and mock-inoculated control plants in maize hybrid 29B17 at 110 DAI.

Endophyte strain	**Root**		**Leaf**	
	N %	**C %**	**N %**	**C %**
WP1	0.47(0.06)a	42.78(0.79)	1.27(0.07)	42.45(0.07)
WP5	0.28(0.04)b	43.82(0.30)	1.32(0.11)	42.25(0.07)

WP42	0.23(0.31)b	43.86(0.28)	1.13(0.07)	42.65(0.09)
WW17	0.33(0.04)ab	44.13(0.40)	1.45(0.17)	42.42(0.27)
Snoq. 117.2	0.32(0.45)ab	43.99(0.11)	1.52(0.04)	42.55(0.18)
Control	0.23(0.03)b	43.97(0.23)	1.16(0.03)	42.74(0.14)
p-value	0.010	0.258	0.082	0.369

Table 6**.** Plant dry weight, SPAD, SLA, and plant height per inoculated and mock-inoculated control treatments in rice (var. M206) at 110 DAI. Means (standard error) of biomass and plant height (n = 5). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

Endophyte strain	**Dry weight (g plant^{-1})**			**SPAD**	**SLA (cm^2g^{-1})**	**Plant ht. (cm)**
	Root	**Shoot**	**Total**			
WP1	2.49(0.13)	3.85(0.10)	6.35(0.17)	35.79 (0.72)	21.71(1.34)	50.77(0.93)
WP5	3.48(0.11)	4.28(0.16)	7.77(0.20)	36.34 (0.75)	23.41(1.78)	53.04(1.52)
WP42	2.60(0.36)	3.49(0.39)	6.10(0.75)	34.15 (1.44)	22.54(1.58)	50.31(1.31)
WW17	2.61(0.25)	3.37(0.34)	5.99(0.57)	35.09 (1.11)	20.36(1.49)	49.67(1.31)
Snoq. 117.2	3.12(0.28)	4.29(0.28)	7.42(0.54)	36.01 (1.66)	20.54(1.31)	51.57(1.32)
Control	2.67(0.40)	3.81(0.55)	6.48(0.95)	35.66 (2.15)	20.30(0.79)	51.03(2.17)
p-value	0.131	0.316	0.232	0.901	0.527	0.688

Experiment 4: Consortia inoculation trial in maize with split dose of N.

Two maize hybrids; XR1404 and XR1634 were inoculated with Consortia #1 and #2, and grown under 5 different N regimes (Fig 1). A subset of inoculated plants were harvested at 45 DAI, and the remaining plants were transferred to bigger pots and grown for about 4 months. Leaf area and leaf CO_2 exchange measurements were taken in these trials but no any significant results were produced pertaining to the inoculations and N regimes.

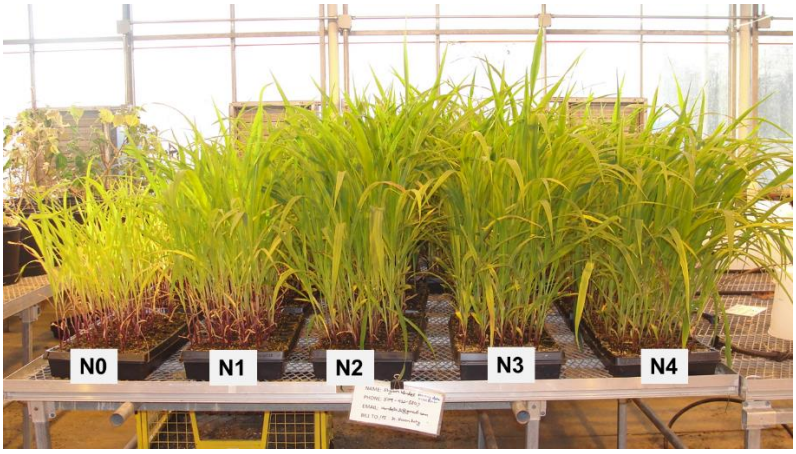


Fig 1. Maize plants 5 weeks after inoculation with endophytes**.**

Total biomass was significantly higher in consortium #1 inoculated plants in no N regime, however it was significantly higher in mock inoculated control plants under full dose of N. In other N regimes, biomass was not significantly different among three inoculations. Interestingly, total biomass in both consortia inoculation at 50% and 75% N regimes were smaller than at 100% N regime.

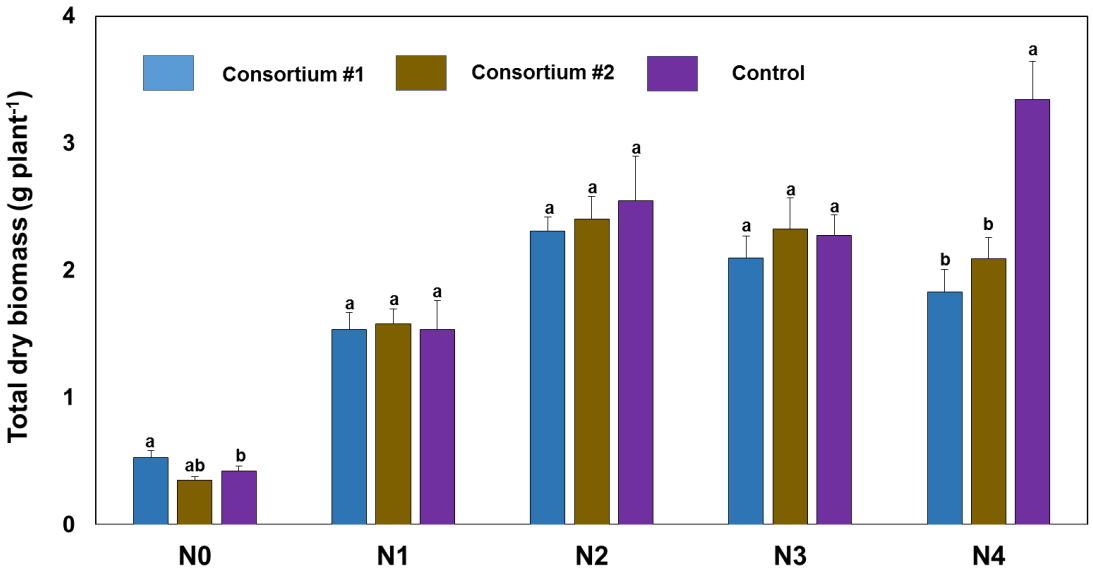


Fig 2. Total biomass of maize hybrid XR1404 in different N regimes at 45 DAI. Histograms followed by different letters within each N levels are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

In XR1404, in long duration experiment (110 DAI), total biomass was significantly higher in consortium #1 inoculated plants in no N regime. In 25% N regime, biomass was significantly higher in both consortium #1, and consortium #2 inoculated plants over mock inoculated plants. Under 50% N regime, biomass was also higher in inoculated plants but not statistically significantly different. But under full dose of N, biomass was significantly higher in consortium #2 and mock inoculated plants than consortium #1 inoculated plants.

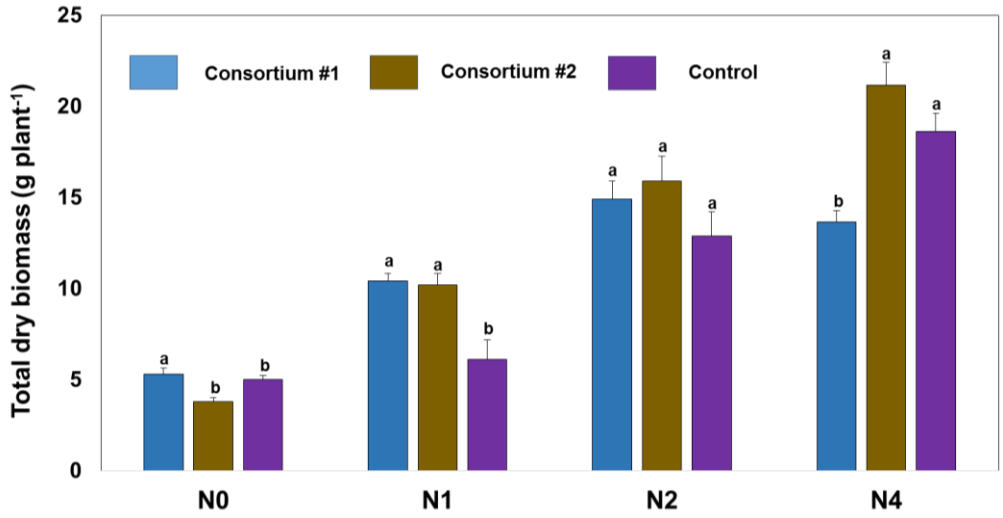


Fig 3. Total biomass of maize hybrid XR1404 in different N regimes at 110 DAI. Histograms followed by different letters within each N levels are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

In XR1634, total biomass was significantly higher in consortium #1 inoculated plants under 100% N regime, and consortium #2 inoculated plants under 50% N regime. In other N regimes, biomass was not significantly different among three inoculations.

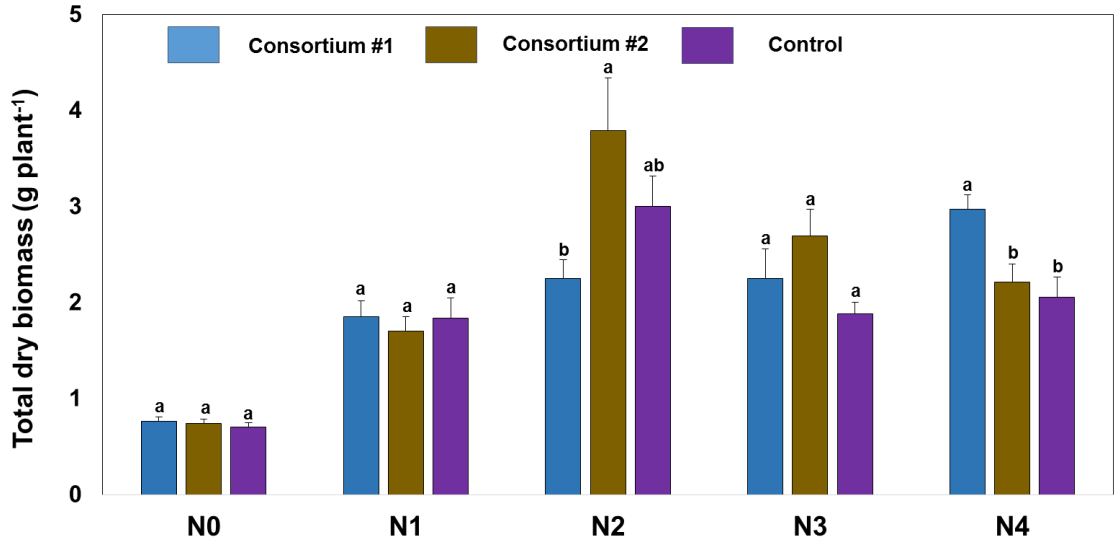


Fig 4. Total biomass of maize hybrid XR1634 in different N regimes at 45 DAI. Histograms followed by different letters within each N levels are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

In XR1634, in long duration experiment (110 DAI), total biomass was significantly higher in consortium #2 inoculated plants in no N regime. In 25% and 50% N regime, biomass was significantly higher in both consortium #1 and consortium #2, and consortium #2 inoculated plants respectively but under full dose of N, it was not significantly different among there inoculations. In 50% N regime, biomass gained in consortium #2 inoculated plants was nearly equal with biomass gained in control plants under full dose of N.

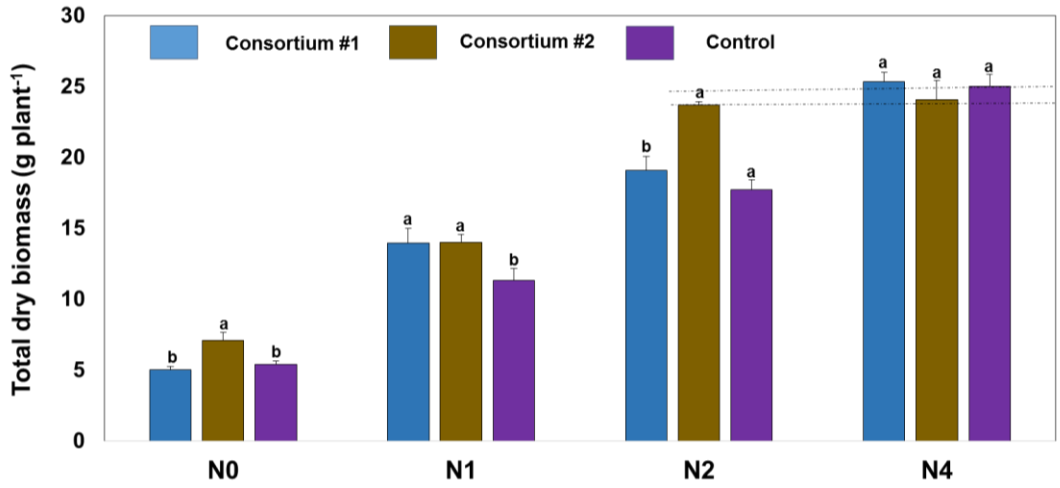


Fig 5. Total biomass of maize hybrid XR1634 in different N regimes at 110 DAI. Histograms followed by different letters within each N levels are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

Experiment 5: Consortia inoculation trial in maize inbred lines.

Two CIMMYT maize inbred lines (CML); CML539 and CML444 were used in this consortia inoculation experiment. In maize inbred line CML539, root and shoot biomass were higher in both consortia inoculated plants as compared to control plants (Table 7). However, it was not consistent with inbred line CML444. In CML444, root and shoot biomass was only higher in consortium #2 inoculated plants not the consortium #1. In addition surprisingly, SPAD readings were significantly higher in mock inoculated plants as opposed to the endophyte inoculated plants in CML444. The highest biomass was observed in consortium #2 inoculation in both inbred lines; CML539, and CML444 (Table 7).

Table 7. Endophyte-inoculated maize inbred lines; CML539, and CML444 grown in the greenhouse. Means (standard error) of biomass and plant height (n = 8-15). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

	Endophyte treatment	**Dry weight (g plant^{-1})**			**SPAD**	
		Root	**Shoot**	**Total**	**90 DAI**	**105 DAI**
CML539	Const #1	17.09(1.48)	17.84(0.52)b	34.94(1.74)	24.03(0.75)	20.86(0.48)
	Const #2	15.54(1.14)	21.03(1.27)a	36.57(1.99)	24.26(0.65)	22.33(0.41)
	Control	13.84(1.05)	17.35(0.89)b	31.19(1.62)	23.62(0.58)	21.7(0.48)
	p-value	0.260	0.014	0.181	0.863	0.160
CML444	Const #1	9.92(0.56)b	12.56(0.57)b	22.50(0.93)b	17.63(0.80)b	17.62(0.60)b
	Const #2	14.92(1.64)a	18.16(1.56)a	33.08(3.07)a	16.78(0.45)b	17.12(0.85)b
	Control	15.99(0.96)a	12.26(0.30)b	28.26(1.14)a	20.38(1.02)a	20.10(0.93)a
	p-value	<0.001	<0.001	<0.001	0.032	0.033

Experiment 6: 1 month consortia inoculation trial in organic maize hybrids.

In organic maize hybrids 23A71, and 29B17, total (root+shoot) biomass was significantly higher in inoculated plants as compared to control plants (Table 8). It was also higher in other two hybrids 07M91 and 14A91 but not statistically significantly higher than mock inoculated plants. Unexpectedly, SPAD was significantly higher in mock inoculated plants in hybrids 23A71 and 07M91, it seems SPAD readings were not much different between endophyte versus mock inoculated plants (Table 8).

Table 8. Endophyte-inoculated organic maize hybrids; 07M91, 14A91, 29B17, and 23A71 grown in the greenhouse. Means (standard error) of biomass and plant height (n = 10-12). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

	Endophyte treatment	**Length (cm plant^{-1})**		**Dry weight (g plant^{-1})**			**SPAD at 25 DAI**
		Root	**Shoot**	**Root**	**Shoot**	**Total**	
23A71	Const #2	44.68 (1.97)	32.71 (0.85)b	0.30 (0.01)a	0.37 (0.01)	0.67 (0.02)a	29.292 (0.478)b
	control	46.68 (1.57)	43.52 (1.02)a	0.26 (0.01)b	0.34 (0.02)	0.60 (0.02)b	31.483 (0.734)a
	P-value	0.42	<0.001	0.003	0.100	0.004	0.020
29B17	Const #2	51.24 (3.53)	26.88 (1.17)b	0.21 (0.02)	0.34 (0.01)a	0.56 (0.03)a	31.908 (0.546)
	control	45.69 (1.94)	34.24 (1.04)a	0.20 (0.01)	0.24 (0.02)b	0.44 (0.03)b	32.575 (0.963)
	P-value	0.147	<0.001	0.424	<0.001	<0.001	0.553

07M91	Const #2	49.86 (2.48)a	33.11 (1.18)b	0.30 (0.01)	0.31 (0.01)	0.61 (0.03)	31.292 (0.478)b
	control	44.01 (1.42)b	40.90 (0.40)a	0.27 (0.02)	0.32 (0.01)	0.59 (0.03)	33.442 (0.611)a
	P-value	0.037	<0.001	0.183	0.480	0.500	0.011
14A91	Const #2	43.64 (2.10)	30.15 (1.69)b	0.22 (0.01)	0.30 (0.03)	0.52 (0.05)	34.283 (0.743)
	control	46.92 (1.15)	35.62 (0.40)a	0.21 (0.02)	0.27 (0.01)	0.48 (0.03)	33.567 (0.679)
	P-value	0.148	0.001	0.670	0.243	0.288	0.484

Experiment 7: 1 month consortia inoculation trial in rice hybrids.

In rice hybrid XL745, inoculated plants were significantly taller, and total biomass was higher than control plants. In hybrid XL723, there was no significant difference in root and shoot length in inoculated versus control plants. However, shoot and total biomass were higher in inoculated plants (Table 9).

Table 9. Endophyte-inoculated rice hybrids; XL745, and XL723 grown in the greenhouse. Means (standard error) of biomass and plant height (n = 5-7). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

	Endophyte	**Length (cm plant^{-1})**		**Dry weight (g plant^{-1})**		
	treatment	**Root**	**Shoot**	**Root**	**Shoot**	**Total**
XL745	Const #2	16.78(0.63)	29.40(0.60)a	0.10(0.026)	0.08(0.005)	0.18(0.026)
	control	17.9(0.52a)	26.62(0.57)b	0.09(0.026)	0.09(0.005)	0.18(0.025)
	P-value	0.179	0.002	0.809	0.469	0.920

XL723	Const #2	12.94(0.83)	21.97(1.05)	0.04(0.006)	0.07(0.009)	0.11(0.012)
	control	13.8(0.74)	23.05(0.74)	0.05(0.005)	0.06(0.006)	0.10(<0.01)
	P-value	0.441	0.401	0.602	0.452	0.778

Experiment 8: 1 month consortia inoculation in soil in organic maize hybrids.

In organic maize hybrids 29B17 and 07M91, roots were significantly longer in inoculated plants as compared to control plants (Table 10). In hybrid 14A91, inoculated plants were significantly taller and total biomass was higher than control plants. In all 4 hybrids, root and shoot biomass was higher in inoculated plant but not statistically significantly higher than mock inoculated plants.

Table 10. Endophyte-inoculated organic maize hybrids; 07M91, 14A91, 29B17, and 23A71 grown in the greenhouse. Means (standard error) of biomass and plant height (n = 10-12). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

	Endophyte	**Length (cm plant^{-1})**		**Dry weight (g plant^{-1})**		
	treatment	**Root**	**Shoot**	**Root**	**Shoot**	**Total**
23A71	Const #2	46.14(1.67)	34.48(0.68)a	0.44(0.16)	0.55(0.19)	0.99(0.36)
	control	46.14(2.27)	37.28(0.55)a	0.22(0.07)	0.25(0.08)	0.47(0.14)
	P-value	0.999	0.004	0.214	0.159	0.181
29B17	Const #2	51.05(1.71)a	33.53(1.24)	0.31(0.06)	0.38(0.09)	0.69(0.15)
	control	39.00(1.65)b	34.95(0.95)	0.27(0.07)	0.36(0.10)	0.63(0.16)
	P-value	<0.01	0.389	0.679	0.882	0.796
07M91	Const #2	48.84(1.87)a	35.94(1.09)	0.49(0.22)	0.62(0.25)	1.11(0.46)
	control	39.34(1.05)b	36.99(0.67)	0.42(0.14)	0.53(0.18)	0.95(0.33)
	P-value	<0.01	0.437	0.776	0.774	0.774

14A91	Const #2	46.00(2.12)	34.06(1.38)a	0.23(0.06)	0.33(0.07)	0.56(0.12)
	control	47.66(2.08)	30.13(0.61)b	0.23(0.05)	0.30(0.07)	0.53(0.12)
	P-value	0.579	0.012	0.981	0.767	0.875

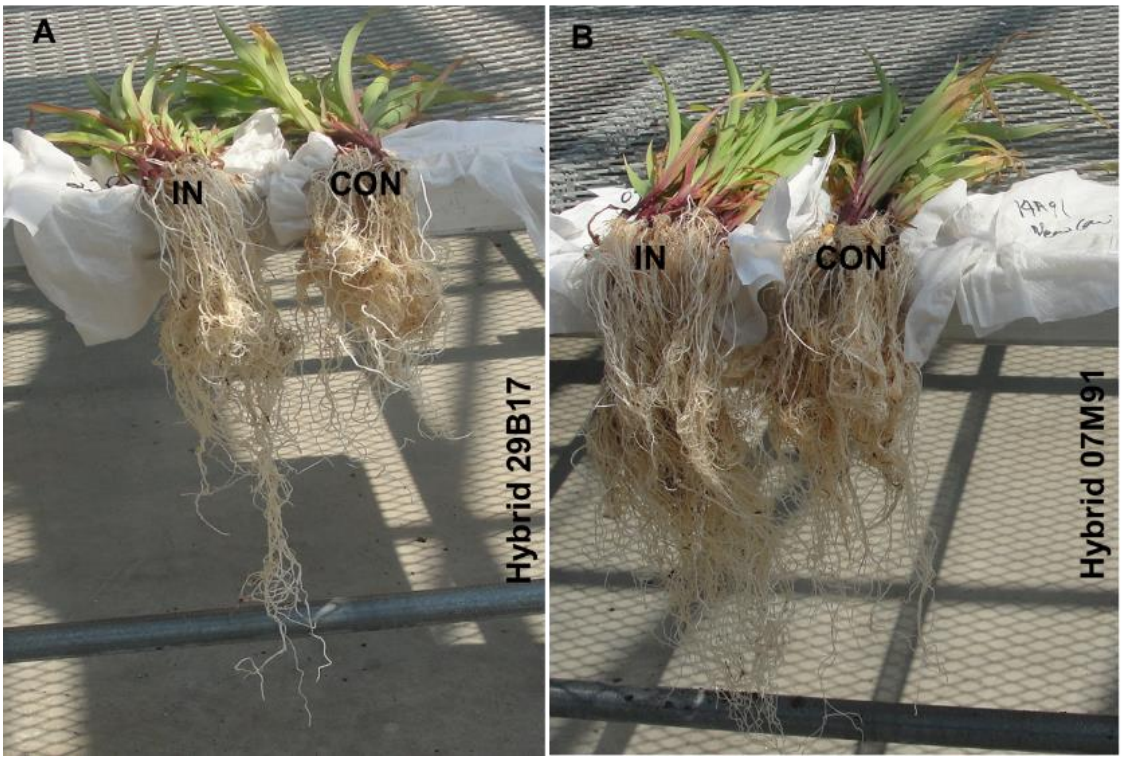


Fig 6. A month old endophyte inoculated maize seedlings; (A) hybrid 29B17, and (B) hybrid 07M91. In= inoculated, and CON= mock-inoculated control plants.

Experiment 9: ^{15}N isotope dilution assay in maize and rice

In both maize and rice plants, the abundance of d^{15}N in root and shoot tissues was higher in consortium #1 inoculated plants as compared to other inoculations (Table 11) but the difference was not statistically significant at alpha= 0.05. In maize, N content per unit plant biomass was higher in WP5, WP5 *nifH*, and Consortium #1 as compared to mock inoculated control plants but it is not statistically significantly different. The maximum biomass was observed in WP5 *nifh*

inoculated plants as compared to other treatments. In rice, the ratio of $d^{15}N$ in shoot to total of root and shoot was significantly smaller, and the ratio of $d^{15}N$ in root to total of root and shoot was significantly higher in consortium #1 inoculated plants as compared to other treatments (Table 11). Total biomass, and N content per unit plant biomass were higher in WP5 and Consortium #1 treatments as compared to control and WP5 *nifh* but they are not statistically different.

Table 11. Endophyte-inoculated organic maize hybrid 29B17, and rice var. M206 grown in the greenhouse. Means (standard error) of biomass, $d^{15}N$ in root and shoot, and N mass (n = 4 for $d^{15}N$ analysis, n= 6 (rice) or 7(maize) for biomass analysis). Means followed by different letters in each column within are significantly different at alpha = 0.05 using Tukey's multiple comparison test.

Endophyte strains	**Total (root+ shoot) $d^{15}N$**	**$d^{15}N$ in shoot / Total $d^{15}N$**	**$d^{15}N$ in root / Total $d^{15}N$**	**$d^{15}N$ in shoot / $d^{15}N$ in root**	**N (µg plant $^{-1}$)**	**Biomass (g plant^{-1})**
Maize: hybrid 29B17						
Control	1852.5(82)	0.560(0.002)	0.439 (0.002)	1.274 (0.014)	85.8 (23.1)	8.23(2.27)
WP5 *nifH*	1872.7(68.3)	0.557(0.003)	0.442 (0.003)	1.260 (0.017)	118.39 (5.71)	12.15(0.618)
WP5	1855.4(10)	0.566(0.011)	0.433 (0.011)	1.309 (0.058)	112.24 (8.08)	11.04(0.84)
Const #1	1921.5(18.5)	0.549(0.003)	0.450 (0.003)	1.220 (0.016)	101.4 (12.8)	9.18(1.14)
P-value	0.794	0.356	0.356	0.347	0.431	0.251

Rice: var. M206

Control	785.9(26.6)	0.701(0.016)a	0.298 (0.016)b	2.37 (0.197)ab	31.77 (5.97)	3.864(0.757)
WP5 *nifh*	772.41(3.38)	0.730(0.009)a	0.269 (0.009)b	2.72 (0.143)a	33.9 (4.43)	3.778(0.627)
WP5	768.42(2.57)	0.696(0.012)a	0.303 (0.012)b	2.307 (0.141)ab	39.03 (4.16)	4.959(0.602)
Const #1	851.5(36.7)	0.637(0.011)b	0.362 (0.011)a	1.762 (0.087)b	39.19 (9.76)	4.89(1.25)
P-value	0.102	0.00593	0.00593	0.0115	0.804	0.652

DISCUSSION

In this study, all inoculation experiments except N split experiments were carried out in N limited conditions with minimum added nutrients through fertigation. Despite the marginal growth conditions, Salicaceae endophyte-inoculated plants showed positive growth response in major food crops; maize and rice. The variable growth responses observed in different maize and rice genotypes were host specific or endophyte inoculum specific. The mechanism of host specificity in legume-rhizobia symbiosis have been studied for many years. It is generally accepted that host specificity begins at the very early stage of communication between the two symbiotic partners (Wang et al., 2012). However, very little research has been accomplished in order to understand the complexity of host specificity in plant-endophyte interactions.

In 1-month single strain inoculation experiment in maize hybrid XR1634, the highest biomass was observed in WPB and WP1 inoculated plants. However in a similar experiment in organic maize hybrids; 07M91, 14A91, 29B17, and 23A71; biomass was highest in WP5 inoculation in

07M91, WP1 inoculation in 14A91, WP42 inoculation in 29B17 and 23A71. In rice var. Presidio, and hybrids XL745 and XL723, biomass was highest in WP1 inoculated Presidio plants but it was maximum in WP41 inoculation in XL745, and WP42 inoculation in XL723 plants. This clearly showed the different strain specific growth response in different host genotypes. Host specific interaction was observed in fungal endophytes of different grasses and other plants (Arnold and Lutzoni, 2007; Takach and Young, 2014; Schirrmann and Leuchtmann, 2015). But very limiting literature is currently available studying the host specificity pattern in bacterial endophytes (Dong et al., 2001). Early signaling molecules exchanged between plant and bacterial endophytes followed by a multitude of feedbacks could distinguish the mutualistic interaction between two symbiotic partners leading to the observed host specific interaction.

In a long duration experiment in maize hybrid 29B17, significantly higher root biomass with largest total biomass was observed in WP1 inoculated plants along with significantly higher N content in root tissues. There was no large difference in chlorophyll fluorescence and SPAD readings in WP1 inoculated versus control or other strain inoculated plants. It indicates that WP1 may have more potential to modify the root systems as compared to above ground systems. The vigorous root systems offer the better environments for plants to access the nutrients and water which could contribute more total biomass and potentially more crop yield (Rich et al., 2016). In addition, vigorous root systems have better access to the soil moisture in dry conditions especially in rain-fed agriculture, which would be desirable traits in crops in climate change scenario to adapt the drought (Manschadi et al., 2008; Palta et al., 2011; Rich et al., 2016). In a long term experiment in rice var. M206, highest root and total biomass was observed in WP5 inoculated plants, in addition to taller plant stature, and highest SLA and SPAD. In contrast to

maize plants, rice plants may respond differently by maintaining relatively similar growth in above ground and below ground systems. Alternatively, there could be different growth response in between yeast (WP1) and bacterial (WP5) endophytes. WP1 is an effective IAA producer (Xin et al., 2009) thus potential to influence more in the root systems than above ground systems which was noticed in maize. In an earlier study, similar result of highest root biomass was observed in a sweet corn inoculated with WP1 over other bacterial or consortium inoculations.

In N split experiment, consortium #2 inoculated plants produced highest biomass at 50% N regime at 45 DAI in both maize hybrids XR1404, and XR1634. Since consortium #2 had more endophyte strains than consortium #1, there might be additive effects of additional strains. Recent studies showed that consortia or multiple endophyte strains outperformed the individual strain regarding the growth response in endophyte inoculated plants. It is suggested that the growth enhancement in host plants is most likely the additive effect of individual strain in a community (Knoth et al., 2012; Timm et al., 2016). In addition, the higher biomass in both consortium #1 and consortium #2 inoculation in XR1404 at 45 DAI under 50% and 75% N regimes than at 100% N regime may indicate that endophytes perhaps perceive the signal of excess N in host plants especially in early stage of plant growth thus slackening their capabilities. It has been shown that metabolic potential of endophytes depends upon the requirement of host plants as they grow (Lundberg et al., 2012). The significantly higher biomass in control plants at 100% N regime corroborates this phenomena. However this was not replicated in hybrid XR1634and could be the result of a host specific response. In a long duration experiment in hybrid 1404, biomass was linearly increased in consortium #2 and mock inoculated plants with increasing dose of N. But in consortium #1 inoculation, biomass was highest in 50% N not in the

100% N. This may indicate that a successful plant-endophyte interaction is not only influenced by host genotype but also the endophyte strains present in the consortia. A detailed study of the root microbiome of *A. thaliana* showed that a narrow range of bacterial communities were able to colonize and establish in the root endosphere. This study suggested that endophytes are competent to circumvent the plant defense system, and rapidly build up the population inside the plant body in contrast to rhizosphere communities (Lundberg et al., 2012). In a long duration experiment in XR1634, total biomass was linearly increased in all cases with increasing dose of N. More importantly in this experiment, consortium #2 inoculated plants produced nearly the same amount of biomass at 50% N that was in the mock inoculated control plants under full dose of N. Probably it is not overstated to infer that endophyte inoculation can reduce the N demand by 50% particularly in hybrid XR1634.

Inbred lines are important genetic materials to develop a new crop cultivar or variety. Host specific biomass response was observed upon inoculation with Salicaceae endophytes in maize inbred lines; CML539, and CML444. Higher total biomass was observed in endophyte inoculated CML539 plants than control plants which was not repeatable in CML444. But as consistent with previous experiments, consortium #2 performed better than consortium #1 in both lines. Since consortium #2 performed better in earlier experiments, consortium #2 was used to inoculate organic maize hybrids: 07M91, 14A91, 29B17, and 23A71, and rice hybrids: XL745 and XL723 in two separate 1 month long experiments. Seedling inoculation, and soil inoculation method was used to introduce endophytes in host plants. Total biomass was significantly higher or higher in endophyte inoculated in all maize and rice genotypes compared to in control plants. In seedling inoculation experiments, hybrids 23A71 and 29B17 had significantly higher biomass

than controls but there was no significant difference in hybrids 07M91 and 14A91. This is potentially a host specific response as observed similarly in the earlier experiments.

In soil inoculation experiments, there was no significant different in biomass between endophytes inoculated versus control groups though there was higher biomass in inoculated groups. By comparing these two inoculation methods, seedling inoculation could be better than soil inoculation to introduce the endophytes into host plants. Seedling inoculation has been used in many past studies dominantly for the growth chamber or greenhouse conditions (Knoth et al., 2012; Amaral et al., 2014; Kandel et al., 2015; Pankievicz et al., 2015; Rangel de Souza et al., 2016). However, this technique may not be realistic for widespread application in the field which is mainly due to delicacy of the technique. Instead, soil inoculation is a more convenient way to introduce the endophytes to plant rhizosphere and consequently into the plant tissues. This technique may have potential to apply endophytes in *in-situ* condition of plant growth in the field. However, very few studies have examined the effectiveness and large scale commercial application of soil inoculation procedure especially for bacterial endophytes (de Souza et al., 2015). With the soil inoculation method, there may be interference of the strains in the inoculum by the soil bacteria. Some rhizobacteria produce different antibiotic compounds which have lethal effects on other microorganisms. The biocontrol properties of these compounds have extensively studied in the context of soil borne plant pathogens (Haas and Keel, 2003; Berendsen et al., 2012). Though, the significance of these compounds is far less discussed against the activities of endophytes and their colonization process. Furthermore, in the seedling inoculation procedure, the microbes do not need to detect and use chemotaxis to get to the plant since they are coating the seedling. Furthermore, in the seedling inoculation procedure, the microbes do not

need to detect and use chemotaxis to get to the plant since they are coating the seedling. But with the soil drench method, the Salicaceae endophytes would need to detect the suitable host plant and swim to the roots. These extra challenges could explain the reduced benefit. Development of foliar application methods or seed coating methods could circumvent these problems.

In the ^{15}N isotope dilution assay in maize, none of the differences were statistically significant. In rice, higher amount of $d^{15}N$ was observed in root tissues of WP5 and Consortium #1 inoculated plants as compared to WP5 *nifH* and mock inoculations. However, the pattern was opposite regarding the amount of $d^{15}N$ in shoot tissues. In shoots, $d^{15}N$ is diluted by BNF but in roots, they were not. Similar results were reported in an earlier study where 65% of total N in leaves and 45% in stems were contributed through BNF in endophytes inoculated poplar plants (Knoth et al., 2014). Though ^{15}N isotope dilution has been widely used to quantify biologically fixed N in legumes and non-legumes, it has been noted that this technique is not efficient in estimating low levels of N fixation (Witty, 1983; Chalk, 1985; Montañez et al., 2008). Maize and rice were grown for 7 and 14 weeks respectively in our ^{15}N isotope dilution assay. The growth period used for this assay in maize might not be long enough to observe the quantifiable amount of N fixation by endophytes. The smaller values of total ^{15}N in rice tissues support this hypothesis. It may have been too long considering that the greenhouse condition is not sterile and so any contaminating diazotrophs in the greenhouse would interfere with the values for the control and nifH mutant inoculated plants.

This study revealed the very simple but useful information regarding the phenotypic response of different maize and rice genotypes resulting from plant-endophyte interactions. The results from

this study can be utilized to understand the mechanism of host specificity further. Advanced molecular techniques such as genomics, proteomics, and metabolomics would be helpful to elucidate the components of host specificity including early signaling processes, and host specific metabolic compounds generated during plant-endophyte interactions. By understanding the mechanism of host specificity, we can improve the broader applicability of endophyte-enhanced growth promotion in major food crops like maize and rice.

REFERENCES

Amaral, F.P., J.C.F. Bueno, V.S. Hermes, and A.C.M. Arisi. 2014. Gene expression analysis of maize seedlings (DKB240 variety) inoculated with plant growth promoting bacterium *Herbaspirillum seropedicae*. Symbiosis 62(1): 41–50.

Arnold, A.E., and F. Lutzoni. 2007. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology 88(3): 541–549.

Berendsen, R.L., C.M.J. Pieterse, and P. a H.M. Bakker. 2012. The rhizosphere microbiome and plant health. Trends Plant Sci. 17(8): 478–86.

Berg, G., M. Grube, M. Schloter, and K. Smalla. 2014. Unraveling the plant microbiome: Looking back and future perspectives. Front. Microbiol. 5(June): 1–7.

Botta, A.L., A. Santacecilia, C. Ercole, P. Cacchio, and M. Del Gallo. 2013. *In vitro* and *in vivo* inoculation of four endophytic bacteria on *Lycopersicon esculentum*. N. Biotechnol. 30(6): 666–74.

Chalk, P.M. 1985. Review - Estimation of N2 fixation by isotope dilution. Soil Biol. Biochem. 17(4): 389 – 410.

Dong, Y., A.L. Iniguez, and E.W. Triplett. 2003. Quantitative assessments of the host range and

strain specificity of endophytic colonization by *Klebsiella pneumoniae* 342.Plant and soil. 257(1): 49–59.

Doty, S.L., B. Oakley, G. Xin, J.W. Kang, G. Singleton, Z. Khan, A. Vajzovic, and J.T. Staley. 2009. Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47: 23–33.

Doty, S.L., A.W. Sher, N.D. Fleck, M. Khorasani, R.E. Bumgarner, Z. Khan, A.W.K. Ko, S.-H. Kim, and T.H. DeLuca. 2016. Variable nitrogen fixation in wild *Populus*. PLoS One 11(5): e0155979.

Edgerton, M.D. 2009. Increasing crop productivity to meet global needs for feed, food, and fuel. Plant Physiol. 149(1): 7–13.

Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, and H. Mitsui. 2001. Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp . isolated from wild rice species endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp . isolated from wild rice species. Appl. Environ. Microbiol. 67(11): 5285–5293.

Firrincieli, A., R. Otillar, A. Salamov, J. Schmutz, Z. Khan, R.S. Redman, N.D. Fleck, E. Lindquist, I. V. Grigoriev, and S.L. Doty. 2015. Genome sequence of the plant growth promoting endophytic yeast Rhodotorula graminis WP1. Front. Microbiol. 6(September): 6–11.

Foley, J. a, R. Defries, G.P. Asner, C. Barford, G. Bonan, S.R. Carpenter, F.S. Chapin, M.T. Coe, G.C. Daily, H.K. Gibbs, J.H. Helkowski, T. Holloway, E. a Howard, C.J. Kucharik, C. Monfreda, J. a Patz, I.C. Prentice, N. Ramankutty, and P.K. Snyder. 2005. Global consequences of land use. Science 309(5734): 570–4.

Gaiero, J.R., C. a McCall, K. a Thompson, N.J. Day, A.S. Best, and K.E. Dunfield. 2013. Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am. J. Bot. 100(9): 1738–50.

Grassini, P., K.M. Eskridge, and K.G. Cassman. 2013. Distinguishing between yield advances and yield plateaus in historical crop production trends. Nat. Commun. 4: 2918.

Haas, D., and C. Keel. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annu. Rev. Phytopathol. 41(1): 117–153.

Hamilton, C.E., J.D. Bever, J. Labbé, X. Yang, and H. Yin. 2016. Mitigating climate change through managing constructed-microbial communities in agriculture. Agric. Ecosyst. Environ. 216(2016): 304–308.

Hardoim, P.R., L.S. van Overbeek, G. Berg, A.M. Pirttilä, S. Compant, A. Campisano, M. Döring, and A. Sessitsch. 2015. The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. 79(3): 293–320.

Hardoim, P.R., L.S. van Overbeek, and J.D. Van Elsas. 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. 16(10): 463–71.

Hurek, T., L.L. Handley, B. Reinhold-hurek, Y. Piché, C. De, C. Pavillon, U. Laval, and C. Gk-p. 2002. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol. Plant-Microbe Interact. 15(3): 233–242.

Imai, I., J.A. Kimball, B. Conway, K.M. Yeater, S.R. McCouch, and A. McClung. 2013. Validation of yield-enhancing quantitative trait loci from a low-yielding wild ancestor of rice. Mol. Breed. 32(1): 101–120.

Iniguez, A.L., Y. Dong, and E.W. Triplett. 2004. Nitrogen fixation in wheat provided by *Klebsiella pneumoniae* 342. Mol. Plant. Microbe. Interact. 17(10): 1078–85.

Kandel, S.L., N. Herschberger, S.H. Kim, and S.L. Doty. 2015. Diazotrophic endophytes of poplar and willow for growth promotion of rice plants in nitrogen-limited conditions. Crop Sci. 55(4): 1765–1772.

Khan, Z., G. Guelich, H. Phan, R. Redman, and S. Doty. 2012. Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. ISRN Agron.: 1–11.

Khan, Z., S.L. Kandel, D.N. Ramos, G.J. Ettl, S. Kim, and S.L. Doty. 2015. Increased biomass of nursery-grown douglas-fir seedlings upon inoculation with diazotrophic endophytic consortia. Forests 6: 3582–3593.

Knoth, J.L., S.-H. Kim, G.J. Ettl, and S.L. Doty. 2012. Effects of cross host species inoculation of nitrogen-fixing endophytes on growth and leaf physiology of maize. GCB Bioenergy 5(4): 408–418.

Knoth, J.L., S. Kim, G.J. Ettl, and S.L. Doty. 2014. Biological nitrogen fixation and biomass accumulation within poplar clones as a result of inoculations with diazotrophic endophyte consortia. New Phytol. 201(2): 599–609.

Lebeis, S.L. 2014. The potential for give and take in plant-microbiome relationships. Front. Plant Sci. 5(June): 287.

Lin, B.B. 2011. Resilience in agriculture through crop diversification: Adaptive management for environmental change. Bioscience 61(3): 183–193.

Lloyd-Price, J., G. Abu-Ali, and C. Huttenhower. 2016. The healthy human microbiome. Genome Med. 8: 51.

Lucero, M.E., S. Debolt, A. Unc, L. V Reyes, and R.L. Mcculley. 2014. Using microbial

community interactions within plant microbiomes to advance an evergreen agricultural revolution. p. 183–202. *In* Sustainable agroecosystems in climate change mitigation.

Lundberg, D.S., S.L. Lebeis, S.H. Paredes, S. Yourstone, J. Gehring, S. Malfatti, J. Tremblay, A. Engelbrektson, V. Kunin, T.G. del Rio, R.C. Edgar, T. Eickhorst, R.E. Ley, P. Hugenholtz, S.G. Tringe, and J.L. Dangl. 2012. Defining the core *Arabidopsis thaliana* root microbiome. Nature 488(7409): 86–90.

Manschadi, A.M., G.L. Hammer, J.T. Christopher, and P. DeVoil. 2008. Genotypic variation in seedling root architectural traits and implications for drought adaptation in wheat (*Triticum aestivum* L.). Plant Soil. 303(1-2): 115-129.

Momose, A., N. Ohtake, K. Sueyoshi, T. Sato, Y. Nakanishi, S. Akao, and T. Ohyama. 2009. Nitrogen fixation and translocation in young sugarcane (*Saccharum officinarum* L.) Plants associated with endophytic nitrogen-fixing bacteria. Microbes Environ. 24(3): 224–230.

Montañez, A., C. Abreu, P.R. Gill, G. Hardarson, and M. Sicardi. 2008. Biological nitrogen fixation in maize (*Zea mays* L.) by 15N isotope-dilution and identification of associated culturable diazotrophs. Biol. Fertil. Soils 45(3): 253–263.

Olivares, J., E.J. Bedmar, and J. Sanjuán. 2013. Biological nitrogen fixation in the context of global change. Mol. Plant. Microbe. Interact. 26(5): 486–94.

Palta, J.A., X. Chen, S.P. Milroy, G.J. Rebetzke, M.F. Dreccer, and M. Watt. 2011. Large root systems: are they useful in adapting wheat to dry environments? Funct. Plant Biol. 38(5): 347-354.

Pankievicz, V.C.S., F.P. Do Amaral, K.F.D.N. Santos, B. Agtuca, Y. Xu, M.J. Schueller, A.C.M. Arisi, M.B.R. Steffens, E.M. De Souza, F.O. Pedrosa, G. Stacey, and R.A. Ferrieri. 2015. Robust biological nitrogen fixation in a model grass-bacterial association. Plant J. 81(6):

907–919.

Rangel de Souza, A.L.S., S.A. De Souza, M.V. V De Oliveira, T.M. Ferraz, F.A.M.M.A. Figueiredo, N.D. Da Silva, P.L. Rangel, C.R.S. Panisset, F.L. Olivares, E. Campostrini, and G.A. De Souza Filho. 2016. Endophytic colonization of *Arabidopsis thaliana* by *Gluconacetobacter diazotrophicus* and its effect on plant growth promotion, plant physiology, and activation of plant defense. Plant Soil 399(1-2): 257–270.

Reay, D.S., E. a. Davidson, K. a. Smith, P. Smith, J.M. Melillo, F. Dentener, and P.J. Crutzen. 2012. Global agriculture and nitrous oxide emissions. Nat. Clim. Chang. 2(6): 410–416.

Reganold, J.P., and J.M. Wachter. 2016. Organic agriculture in the twenty-first century. Nat. Plants 2(February): 15221.

Reinhold-Hurek, B., and T. Hurek. 2011. Living inside plants: bacterial endophytes. Curr. Opin. Plant Biol. 14(4): 435–43.

Rennie, R.J. 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. 27(1): 8–14.

Rich, S.M., A.P. Wasson, R.A. Richards, T. Katore, R. Prashar, R. Chowdhary, D.C. Saxena, H.M. Mamrutha, A. Zwart, S.C. Misra, S. V. Sai Prasad, R. Chatrath, J. Christopher, and M. Watt. 2016. Wheats developed for high yield on stored soil moisture have deep vigorous root systems. Funct. Plant Biol. 43(2): 173-188.

Riggs, P.J., M.K. Chelius, a L. Iniguez, S.M. Kaeppler, and E.W. Triplett. 2001. Enhanced maize productivity by inoculation with diazotrophic bacteria. Aust. J. Plant Physiol. 28(9): 829–836.

Rodriguez, R.J., J.F. White, A.E. Arnold, and R.S. Redman. 2009. Fungal endophytes: diversity and functional roles. New Phytol. 182(2): 314–30.

Rosenblueth, M., and E. Martínez-Romero. 2006. Bacterial endophytes and their interactions with hosts. Mol. Plant. Microbe. Interact. 19(8): 827–37.

Santoyo, G., G. Moreno-Hagelsieb, M. del Carmen Orozco-Mosqueda, and B.R. Glick. 2016. Plant growth-promoting bacterial endophytes. Microbiol. Res. 183(2016): 92–99.

Schirrmann, M.K., and A. Leuchtmann. 2015. The role of host-specificity in the reproductive isolation of Epichloe endophytes revealed by reciprocal infections. Fungal Ecol. 15: 29-38.

Schlaeppi, K., and D. Bulgarelli. 2015 The plant microbiome at work. Molecular Plant-Microbe Interactions. 28(3): 212-217.

de Souza, R., A. Ambrosini, and L.M.P. Passaglia. 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. Genet. Mol. Biol. 38(4): 401–419.

Srivastava, P., R. Singh, S. Tripathi, and A.S. Raghubanshi. 2016. An urgent need for sustainable thinking in agriculture – An Indian scenario. Ecol. Indic. 67(2016): 611–622.

Takach, J.E., and C.A. Young. 2014. Alkaloid genotype diversity of tall fescue endophytes. Crop Sci. 54(2): 667–678.

Timm, C.M., D.A. Pelletier, S.S. Jawdy, L.E. Gunter, J.A. Henning, N. Engle, J. Aufrecht, E. Gee, I. Nookaew, Z. Yang, T.-Y. Lu, T.J. Tschaplinski, M.J. Doktycz, G.A. Tuskan, and D.J. Weston. 2016. Two Poplar-associated bacterial isolates induce additive favorable responses in a constructed plant-microbiome system. Front. Plant Sci 7: 497.

Turner, T.R., E.K. James, and P.S. Poole. 2013. The plant microbiome. Genome Biol. 14(6): 209.

Uphoff, N. 2012. Supporting food security in the 21st century through resource-conserving increases in agricultural production. Agric. Food Secur. 1(1): 1–18.

Uphoff, N., and F. Dazzo. 2016. Making rice production more environmentally-friendly.

Environments 3(2): 12.

Wang, D., S. Yang, F. Tang, and H. Zhu. 2012. Symbiosis specificity in the legume - rhizobial mutualism. Cell. Microbiol. 14(February): 334–342.

Witty, J.F. 1983. Estimating N2-fixation in the field using 15N -labeled fertilizer: some problems and solutions. Soil Biol. Biochem. 15(6): 631–639.

Wu, Y., F. San Vicente, K. Huang, T. Dhliwayo, D.E. Costich, K. Semagn, N. Sudha, M. Olsen, B.M. Prasanna, X. Zhang, and R. Babu. 2016. Molecular characterization of CIMMYT maize inbred lines with genotyping-by-sequencing SNPs. Theor. Appl. Genet. 129(4): 1–13.

Xin, G., D. Glawe, and S.L. Doty. 2009. Characterization of three endophytic, indole-3-acetic acid-producing yeasts occurring in *Populus* trees. Mycol. Res. 113(9): 973–80.

CHAPTER FOUR

COLONIZATION OF RICE AND MAIZE SEEDLINGS BY POPLAR ENDOPHYTES

ABSTRACT

Endophytes, microbial communities that reside within the plant body, have various growth promoting traits that are crucial for plant growth and development. Endophytes of wild poplar (*Populus trichocarpa*) improve plant growth not only in their native host but also in many other crop plants such as cereal grains and vegetables under conditions of limited nitrogen (N) supply. However, the colonization efficiency of these endophytes is considered critical to observe their activities in response to inoculation. This is the first report that is showing the specific density and colonization patterns of poplar endophytes including both bacterial and yeast strains in different rice and organic maize genotypes. Green fluorescent protein-labeled bacterial strains, WP5*gfp* (*Rahnella* sp.), and WP9*gfp* (*Burkholderia* sp.), and yeast strain WP1 (*Rhodotorula graminis*) were used to inoculate young maize and rice seedlings. Inoculated seedlings were grown aseptically in the growth chamber in N free growth medium. Both maize and rice seedlings were robustly colonized by WP5*gfp*, WP9*gfp* and WP1 in roots as well as in leaves. They were often observed in the intercellular spaces in the root cortex or leaf mesophyll tissues. The density of endophytic populations of individual strains was higher in shoot (leaf+stem) tissues than roots in nearly all colonization assays. The growth response to WP5 and WP1 inoculation in maize and rice was variable, though in the majority of the experiments, inoculated plants had higher root or shoot biomass as compared to mock-inoculated control plants.

INTRODUCTION

Poplars (*Populus* spp.) are hosts of many endophyte strains. The majority of the strains are members of α-, β- and γ-classes of Proteobacteria, while some of them are yeast strains. The major endophyte strains isolated from wild poplar from its native habitat in western Washington were identified as *Burkholderia*, *Curtobacterium*, *Rahnella*, *Pseudomonas*, *Acinetobacter*, *Pantoea*, *Rhodotorula*, and *Rhizobium* species (Doty et al., 2005, 2009, 2016). Many of them can grow in N limited medium, possess a *nifH* gene, and are positive in the acetylene reduction assay: a common assay used for nitrogenase activity (Doty et al., 2005, 2009, 2016; Xin et al., 2009b). In addition, these strains produce substantial amounts of the growth hormone, indole-3-acetic acid (Xin et al., 2009b; a). Using the ^{15}N dilution assay, it has been shown that inoculated endophytes in poplar plants can contribute about 65% of the total N in the leaves and increased plant biomass through biological N fixation (Knoth et al., 2014). Furthermore, a superior phenotypic performance of endophyte treated plants was reported in maize, rice, and vegetables under N limited conditions (Khan et al., 2012; Knoth et al., 2012; Kandel et al., 2015). A few of the endophytes from cultivated poplaralso assist poplar and other plant species to metabolize specific hazardous toxic compounds, otherwise these compounds could be lethal for plant survival (Kang et al., 2012; Lee et al., 2012; Khan et al., 2014). From comparative genomics, it has been reported that endophytic strains of *Burkholderia* have several genes, for example, glutathione-S-transferase genes related to the break down and reclamation of toxic substances (Mitter et al., 2013).

The colonization ability of endophytes is considered as a crucial criterion to provide the growth benefits to the host plant. For endophytic colonization, bacteria in the vicinity of plant roots

probably swim towards the roots through chemotactic activity. Bacteria then adhere and anchor firmly on the root surface. These bacterial communities potentially interact with the host plants in a specific manner to subdue the plant defense system, and ingress inside the plant tissues through natural openings (van der Lelie et al., 2009; Compant et al., 2010). The genome survey and comparative genomics of endophyte strains showed that they have various genes including biofilm production, adhesion, motility etc. that are contributing to the endophytic life style and mobility within a host plant (Krause et al., 2006; Taghavi et al., 2009; Mitter et al., 2013; Ali et al., 2014). Endophytes utilize intercellular spaces to reside and multiply since the area has an abundance of carbohydrates, amino acids, and inorganic nutrients (Dong et al., 1995; Elbeltagy et al., 2001; Hardoim et al., 2015). They may exclusively colonize the intercellular spaces of the plant tissue, or potentiality to enter into the vascular tissue and spread systemically into different plant parts (Germaine et al., 2004; Iniguez et al., 2004; Compant et al., 2005; Reinhold-Hurek and Hurek, 2011). Colonization can be localized at the tissue level or extended throughout the plant body. The ruptured areas created during the emergence of lateral roots or root hairs serve as an entry point for many endophytes. As an early event of endophytic colonization, inoculated endophytes were first observed in root hairs, and subsequently in the root cortex (McCully, 2001; Compant et al., 2005; Prieto et al., 2011).

Reporter genes, such as, GUS (β-glucuronidase) and green fluorescence protein (GFP) have been commonly used to track the colonization of different endophyte species inside the plant tissue (Elbeltagy et al., 2001; James et al., 2002; Germaine et al., 2004; Iniguez et al., 2004; Prieto et al., 2011; Botta et al., 2013; Kandel et al., 2015). Bacterial endophytes tagged with GFP constitutively express the fluorescence proteins *in situ* which allows entire bacterial cells to glow

in the presence of ultraviolet light or blue light, and oxygen (Rollins et al., 2001; Reinhold-Hurek and Hurek, 2011). *In situ* glowing of bacterial cells in plant tissue allows one to localize and follow the dynamics of colonization in different plant parts. The use of GFP tagged endophytes helps to determine the success in colonization, sites for ingress, and the micro-habitat in the plant (Elbeltagy et al., 2001). Isolation and enumeration of GFP tagged endophytes from an inoculated host plant provides an estimation of endophyte populations in a particular plant part (Elbeltagy et al., 2001; Germaine et al., 2004; Kandel et al., 2015). In addition, GFP fused in-frame to a gene of interest can be used to visualize and quantify the expression of the gene. Egener et al. (1999) observed high level expression of nitrogenase in the rice root using the GFP and immunogold labeling in Kallar grass endopyte *Azoarcus* sp. BH72. Using fluorescent microscopy, live bacterial cells can be observed in the dissected or intact plant tissues. However, auto fluorescence produced from plant cell wall or cell organelles particularly in the leaf tissues may limit the usage of this technique. Little information is available regarding the colonization pattern of yeast endophytes. Light or electron microscopy is used to observe the yeast cells stained by Toluidine blue or calcofluor white (Sigma-Aldrich, St. Louis) (Nassar et al., 2005).

Endophytes act as growth enhancers to the host plants (Gaiero et al., 2013; Lebeis, 2014; Hardoim et al., 2015). The positive but variable growth response was observed among different plant genotypes grown with added endophytes (Khan et al., 2012; Vargas et al., 2012; Neiverth et al., 2014; do Amaral et al., 2016). The mechanism of host specificity during plant-endophyte interaction is not known yet, but this is an important issue faced by endophyte researchers for the application of this technology. The evidence of colonization of crop plants by poplar endophytes was previously reported (Knoth et al., 2012; Kandel et al., 2015). But the colonization patterns,

and relative density of an individual strain including yeast strain in various rice and organic maize genotypes under N limited environment is not well understood. Rice and maize are among the major staple food crops worldwide. The information related to the host specificity in these food crops would be beneficial to explore the potentiality of poplar endophytes for crop production. By assessing the colonization pattern, and *in-planta* endophyte populations in some maize and rice genotypes, we may observe the discrimination at colonization level, if at all. The objectives of this research were: (1) to enumerate *in-planta* endophyte populations in whole maize and rice seedlings; (2) To visualize the communities of inoculated endophytes in maize and rice tissues through fluorescent or bright field microscopy; (3) to determine the biomass gained in rice and maize plants with the addition of endophytes.

MATERIALS AND METHODS

Endophyte strains and growth conditions

Green fluorescence protein (GFP) labelled bacterial strains; WP5*gfp* (*Rahnella* sp.), and WP9*gfp* (*Burkholderia* sp.) (Doty et al., 2009), and yeast strain WP1 (*Rhodotorula graminis*) (Firrincieli et al., 2015) isolated from wild poplar were used in this study (Fig 1). They were grown at 30°C in N-limited combined carbon medium (NLCCM) for 2 days (Rennie, 1981). A few colonies from 2-days old culture were then transferred to NLCCM broth, and incubated overnight at 30ºC in a rotating shaker. The optical density of endophyte culture was determined using diode array spectrophotometer (Biochrom US, Holliston, MA, USA). The endophyte culture was adjusted to 0.1 OD_{600} for inoculation dispensing in the N free MS (Murashige-Skoog) medium (Caisson Inc., Logan, UT, USA).

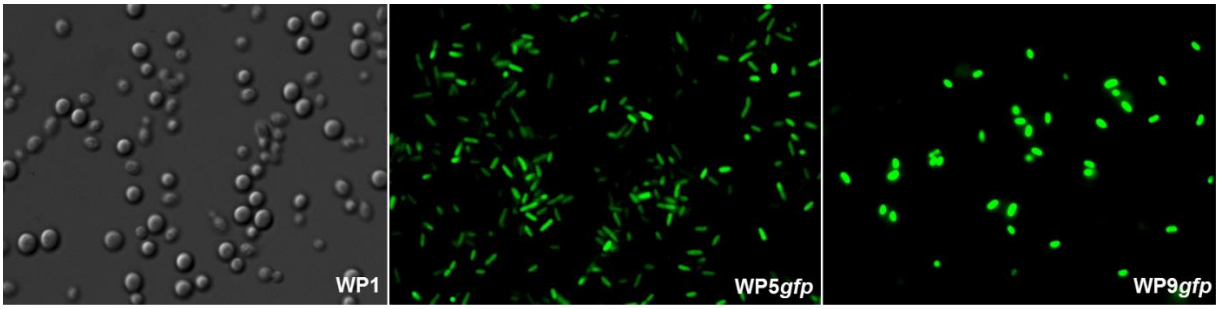


Fig 1. Microphotographs of yeast strain, WP1; and bacterial strains WP5*gfp*, and WP9*gfp*.

Plant materials

Two organic maize hybrids; 29B17 and 14A91 (http://www.blueriverorgseed.com/), and two rice genotypes; var. M206 (Johnson, 2005), and hybrid XL745 (http://www.ricetec.com/) were used for microscopy and colonization assays.

Endophyte inoculation

Maize and rice seedlings were prepared in the growth chamber using surface sterilized seeds. Maize seeds were surface sterilized by incubating up to 20 to 30 minutes in NaClO (3% v/v), and rice seeds for 4 hours followed by rinsing five to eight times with sterile, deionized water. About one week old sterile seedlings were inoculated by co-cultivating overnight in endophyte culture on rotating shaker with gentle agitation (50 rpm). For mock inoculation, sterile seedlings were co-cultivated similarly in N free MS (Caisson Inc.) without endophyte culture. In the root inoculation assay, maize roots were restrictedly inoculated with WP1 with no exposure to stem or leaves. Mock inoculated plants were similarly inoculated with N free MS broth. 50 ml centrifuge tubes were cut into about half size (Fig 2; A), hold upright in a container, and filled 2/3rd with water agar. Seeds were placed on the agar and allowed to germinate for few days. The tubes were uplifted about ½ inch from container, and flooded with WP1 inoculum enough to

submerge young roots grown through agar and spread out. After overnight co-cultivation, inoculated seedlings were gently moved to the magenta boxes with N free MS and placed by pushing roots slightly into the MS (Fig 2; B).

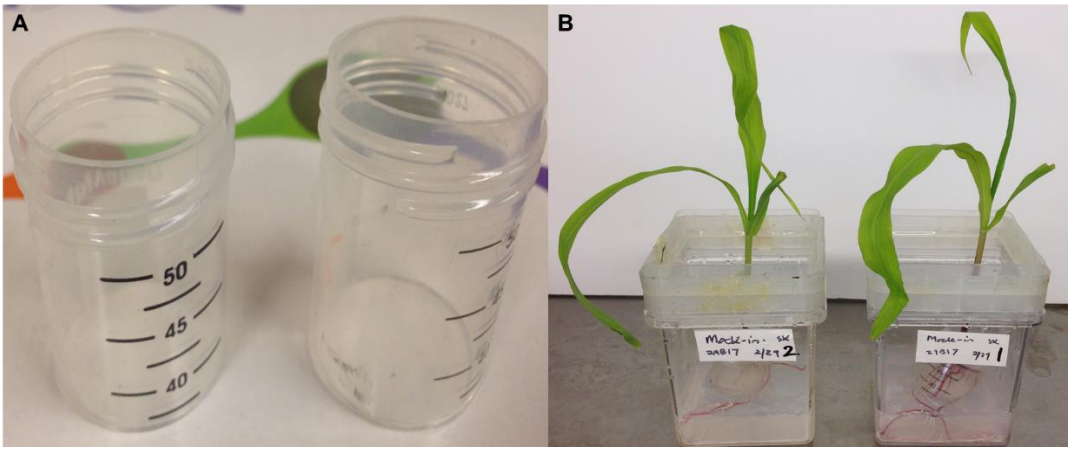


Fig 2. Root inoculation of maize (hybrid 29B17) seedlings by WP1. (A) About half size centrifuge tubes to set up seed germination; (B) Inoculated seedlings growing in the magenta boxes.

Plant growth and measurement

Inoculated seedlings were grown in the N free MS agar in glass tubes and two tier sterile magenta boxes for 11, and 25 or 15 days respectively in the growth chamber (Fig 3). At the end of the experiments, inoculated seedlings were harvested, and rinsed multiple times with sterile water. Fresh root and shoot biomass were recorded and subsequently were used for colony forming units (cfu) count or microscopy.

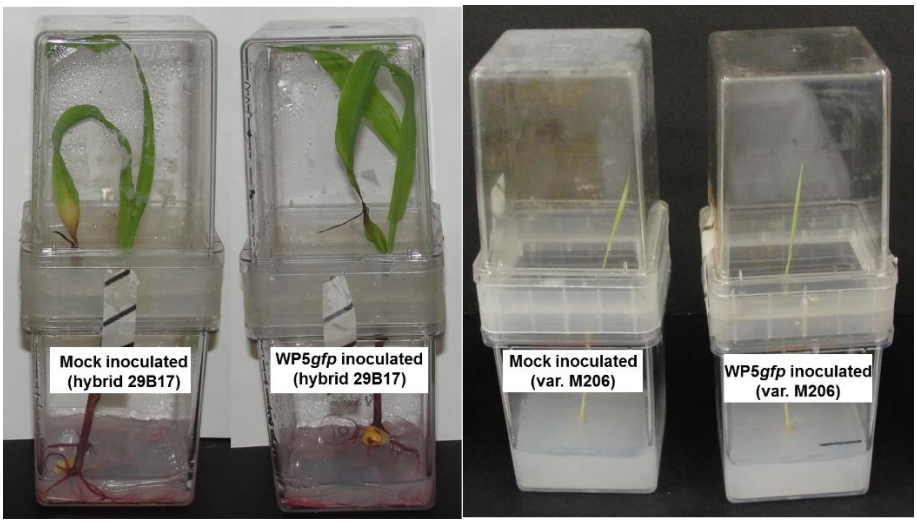


Fig 3. Two tier magenta boxes carrying inoculated seedlings of maize (hybrid 29B17), and rice (var. M206).

Visualization of *in-planta* endophyte populations using microscopy

Fluorescent and bright field microscopy were used to observe *in-planta* population of bacterial (WP5*gfp*, and WP9*gfp*), and yeast (WP1) strains respectively (Fig 1). The reporter gene GFP was transferred into strains, WP5, and WP9 to visualize the endophytic population in maize and rice plants. Broad host range plasmid pBHR1-GFP was used to label these strains with GFP. Transformation was performed through electroporation (Stevens et al., 2005). Root systems including root hairs, lateral seminal roots, and leaf lamina were used to observe under the microscope to detect the colonization pattern of endophytes in maize (Fig 4), and rice. The compound microscope (Zeiss, Boston, MA, USA) equipped with Axio Imager 2 (Zeiss Microscopy, LLC, Thornwood, NY, USA) was used to observe specimens. Specimens with individual bacterial cells or microcolonies inside the plant tissues were photographed using Zeiss AxioVision Software. The photographs were taken at 400 or 630 magnifications with blue light

(GFP filter), or transmission light. For negative controls, mock-inoculated plant tissues were used.

Enumeration of *in-planta* endophyte populations through colony forming unit counts

WP5*gfp* and WP1 inoculated maize plants, and WP1 inoculated rice plants were harvested at 25 or 15 DAI to estimate the endophytic population of WP5*gfp* or WP1. Root and shoot tissues including leaves were weighed, surface sterilized, ground in the NLCCM broth, and used to quantify *in-planta* endophyte populations of WP5*gfp* and WP1 in maize, and WP1 in rice. Surface sterilization was performed by rinsing plant tissues in 70% ethanol for few seconds followed by dipping in NaClO (2% v/v) for about a minute, and then washed five times with sterile water. The surface sterilized tissues were ground in the NLCCM broth using sterile mortar and pestle. The ground samples then serially diluted from 10^{-1} to 10^{-4} dilutions. Aliquots of 100 µl from 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on Mannitol Glutamate/Luria (MG/L) agar (WP1) or MG/L agar with 100 µg ml^{-1} of gentamycin and carbenicillin (WP5*gfp*), using a flame-sterilized spreading glass rod (Cangelosi et al. 1991). Dilution plates were incubated overnight at 30^{o}C. Following incubation, CFU were count, and recorded from every plate. The WP1 CFU were enumerated by counting pink yeast cells in MG/L plates. To verify the surface sterilization process, one hundred microliter water from final rinse was plated on MG/L agar, and incubated overnight as mentioned earlier.

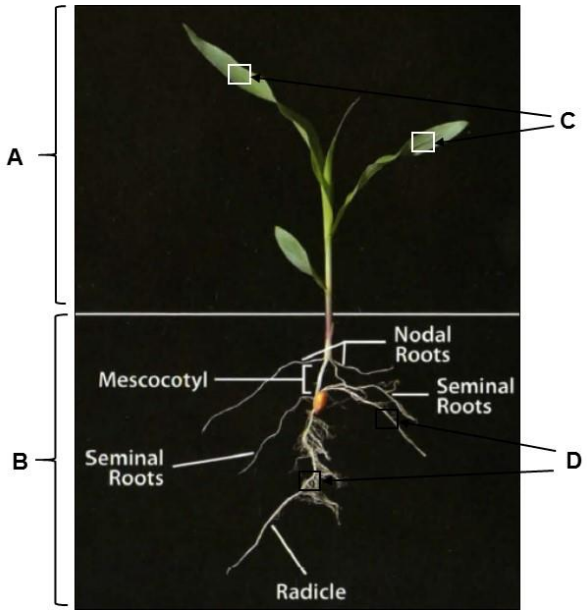


Fig 4. Schematic representation of a maize plant. Adapted from Iowa State University Extension Bulletin, Abendroth et al. (Abendroth et al., 2011). (A) and (B) Whole plant body (root and shoot systems) was used to enumerate colonized populations of WP5*gfp* and WP1; (C) and (D) Tissues used in fluorescent microscopy (WP9*gfp*, WP5*gfp*), and bright field microscopy (WP1).

Statistical analysis

Analysis of variance, and Tukey posthoc procedure was used to compare the growth response between inoculated and control groups. Data were analyzed using Minitab 17 (Minitab Inc., State College, PA, USA).

RESULTS

Colonization of maize (hybrids 29B17 and 14A91) roots at 11 DAI

The localization and distribution of endophytic population of WP5g*fp* and WP9*gfp* in young maize roots was demonstrated through visualization of GFP-labeled bacterial cells under fluorescent microscopy. When multiple samples were thoroughly checked under the microscope,

nearly all inoculated plants were found colonized by WP5g*fp* and WP9*gfp*. WP5*gfp* and WP9*gfp* populations were observed repeatedly in elongation and differentiation zones of lateral seminal roots. In both hybrids (29B17 and 14A91), they were observed in between cells probably in the intercellular spaces of cell layers in longitudinal direction (Figs 6 and 9; A and B, and Figs 7, 8, 10), and middle lamella areas of transverse wall between two adjacent cells (Fig 5 and Fig 9; A and B). Aggregates or individual bacterial cells were mostly present in the cortical cells but also detected in the xylem vessels (Fig 6; C and D).

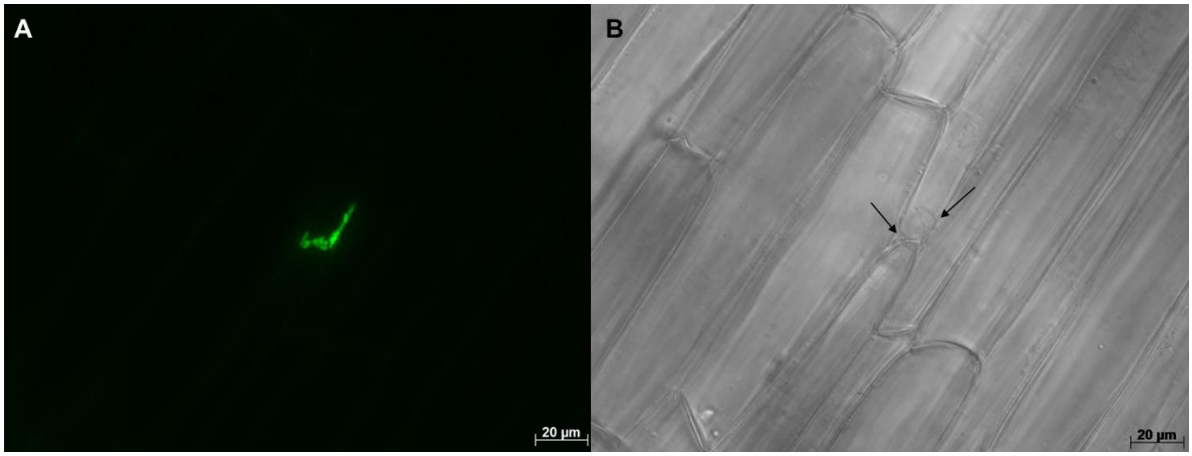


Fig 5. Maize (hybrid 29B17) roots colonized by WP5*gfp* visualized under 630x magnification. Image on the left (A) was taken with GFP filter, and on the right (B) was taken without GFP filter.

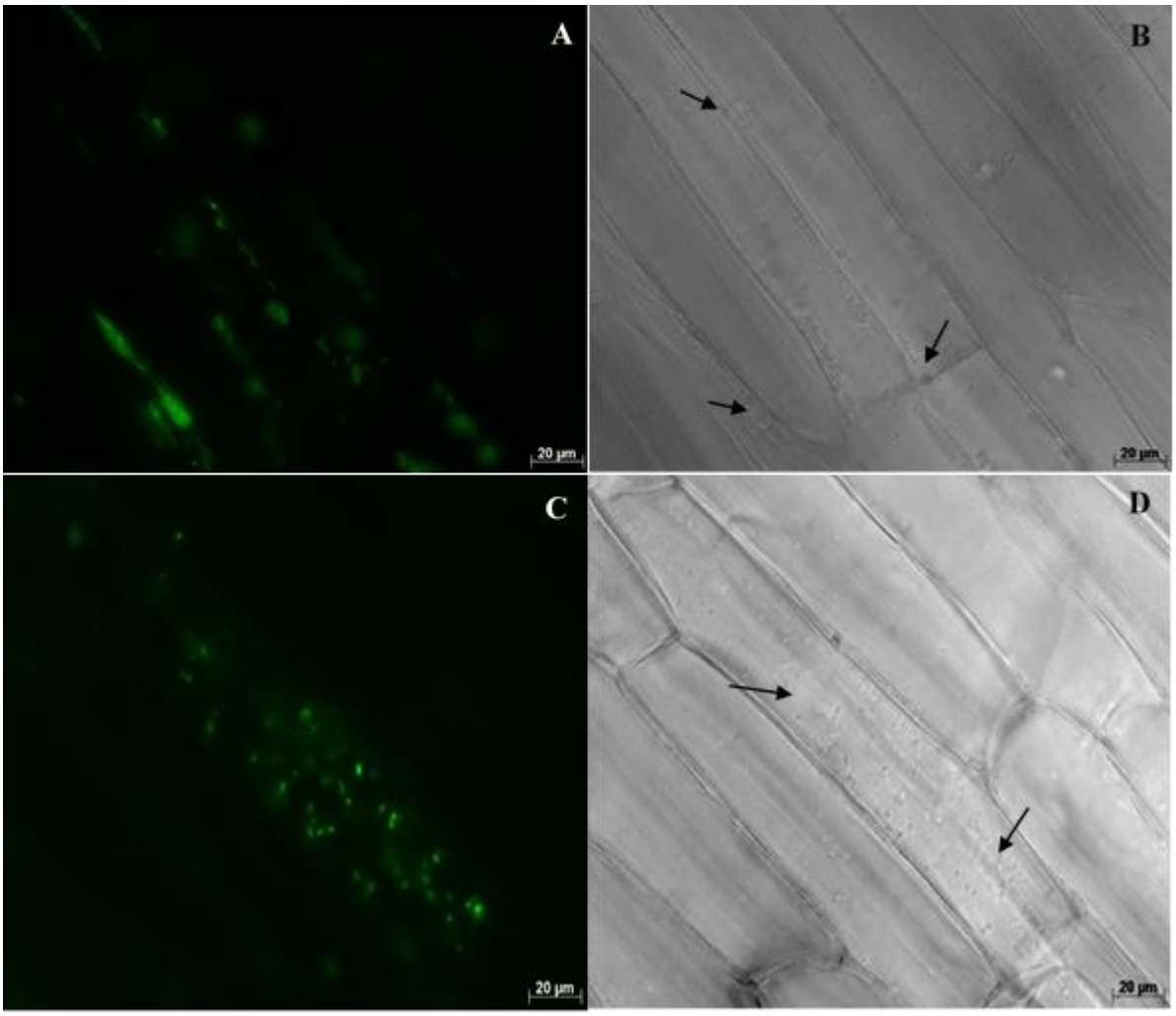


Fig 6. Maize (hybrid 29B17) roots colonized by WP5*gfp* visualized under 630x magnification. Images on the left (A and C) were taken with GFP filter, and those on the right (B and D) were taken without GFP filter.

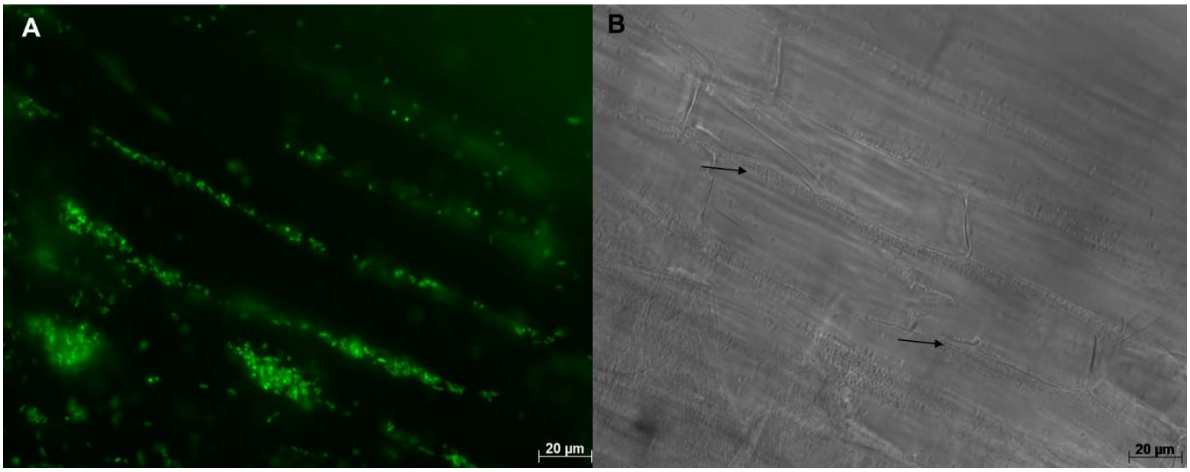


Fig 7. Maize (hybrid 29B17) roots colonized by WP9*gfp* visualized under 630x magnification. Image on the left (A) was taken with GFP filter, and on the right (B) was taken without GFP filter.

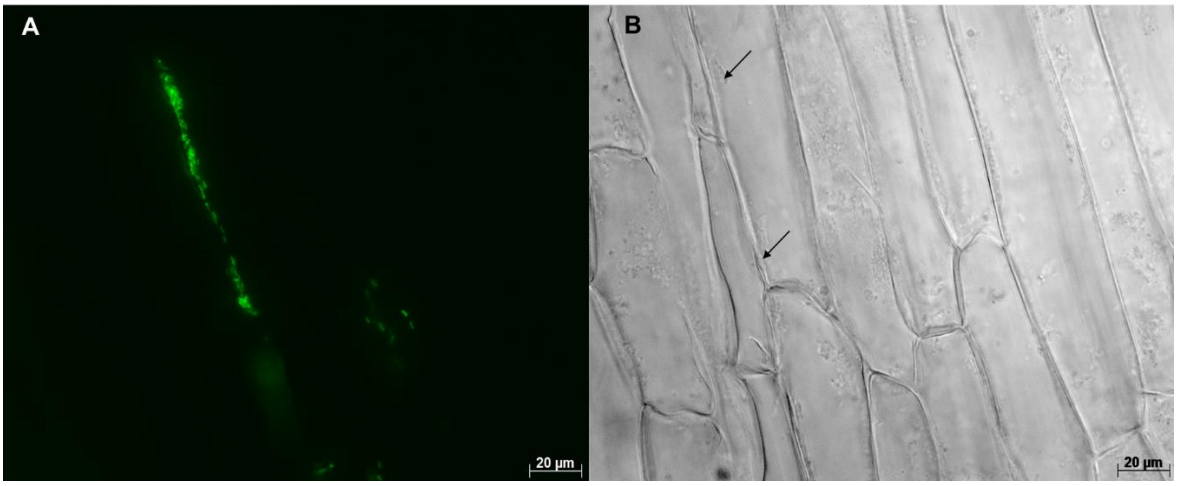


Fig 8. Maize (hybrid 14A91) roots colonized by WP5*gfp* visualized under 630x magnification. Image on the left (A) was taken with GFP filter, and on the right (B) was taken without GFP filter.

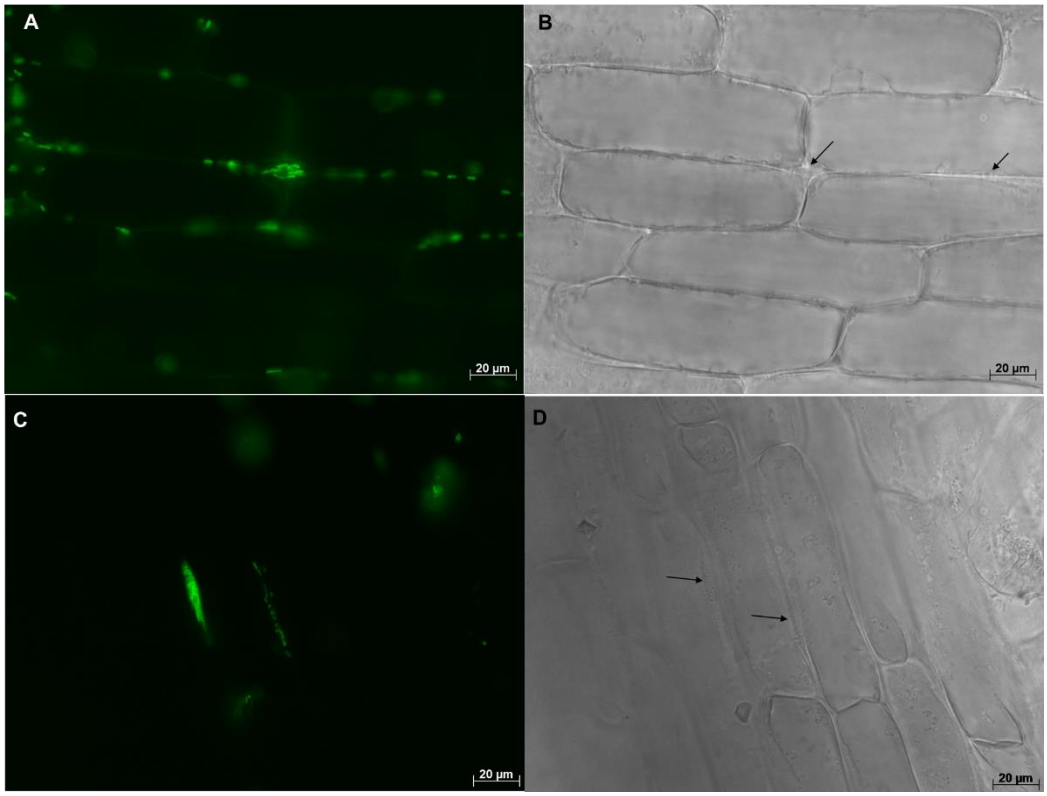


Fig 9. Maize (hybrid 14A91) roots colonized by WP5*gfp* visualized under 630x magnification. Images on the left (A and C) were taken with GFP filter, and those on the right (B and D) were taken without GFP filter.

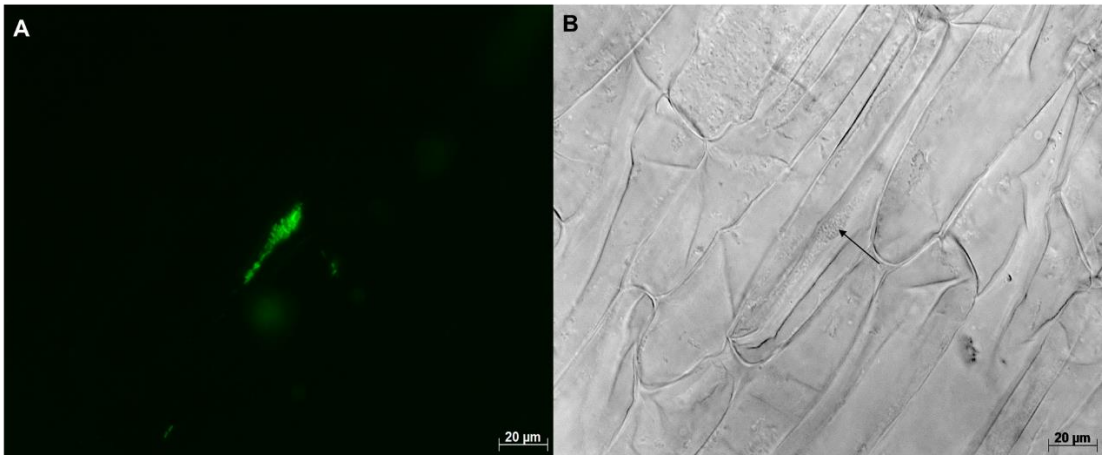


Fig 10. Maize (hybrid 14A91) roots colonized by WP9*gfp* visualized under 630x magnification. Image on the left (A) was taken with GFP filter, and on the right (B) was taken without GFP filter.

Biomass of WP5*gfp* and WP9*gfp* inoculated maize (hybrids 29B17 and 14A91) plants at 11 DAI

Fresh root and shoot biomass (g plant^{-1}) was recorded at 11 DAI. In hybrid 29B17, root biomass was significantly (p-valve=0.042) higher in WP9*gfp* inoculated plants, and shoot biomass was in WP5*gfp* inoculated plants (p-valve=0.038) than mock-inoculated control plants (Fig 11). In hybrid 14A91, there was higher biomass in WP5*gfp* and WP9*gfp* inoculated plants but the difference was not statistically different.

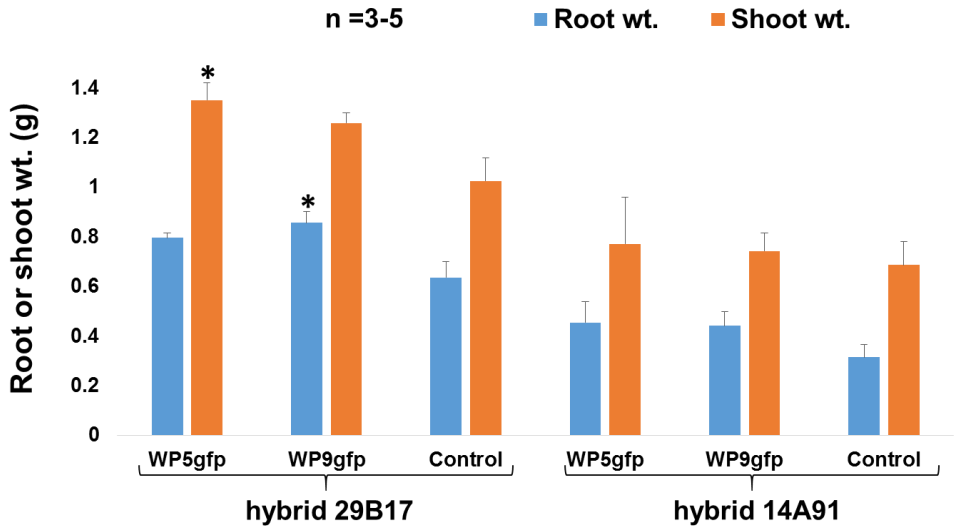


Fig 11. Root and shoot biomass of WP5*gfp*, and WP9*gfp* inoculated and mock-inoculated control plants in maize hybrid 29B17 and 14A91 at 11 DAI. The bars represent the standard errors of mean. Histograms with asterisk indicate the significant difference ($P < 0.05$) between inoculated and corresponding mock inoculated control.

Colonization of rice (var. M206 and hybrid XL745) roots at 11 DAI

The colonization of roots by WP5*gfp* and WP9*gfp* was observed in rice (var. M206 and hybrid XL745) plants using fluorescent microscopy. In both rice plants, they were observed in intercellular spaces of cell layers (Figs 12, 14, and 15), and in the vicinity of the cross wall between two neighboring cells (Fig 13). Individual cells or microcolonies of WP5*gfp* and WP9*gfp* were mostly present in the cortical region of young lateral roots.

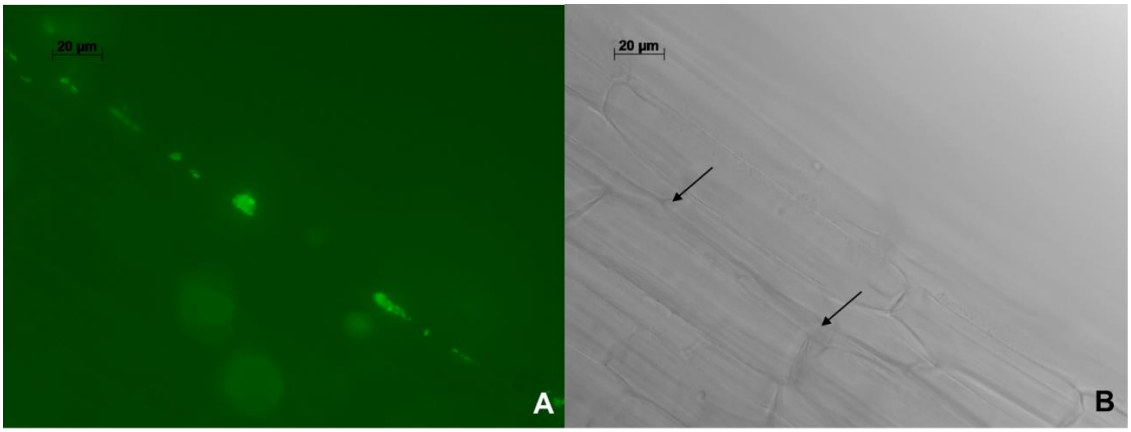


Fig 12. Rice (var. M206) roots colonized by WP5*gfp* visualized under 630x magnification. Image on the left (A) was taken with GFP filter, and on the right (B) was taken without GFP filter.

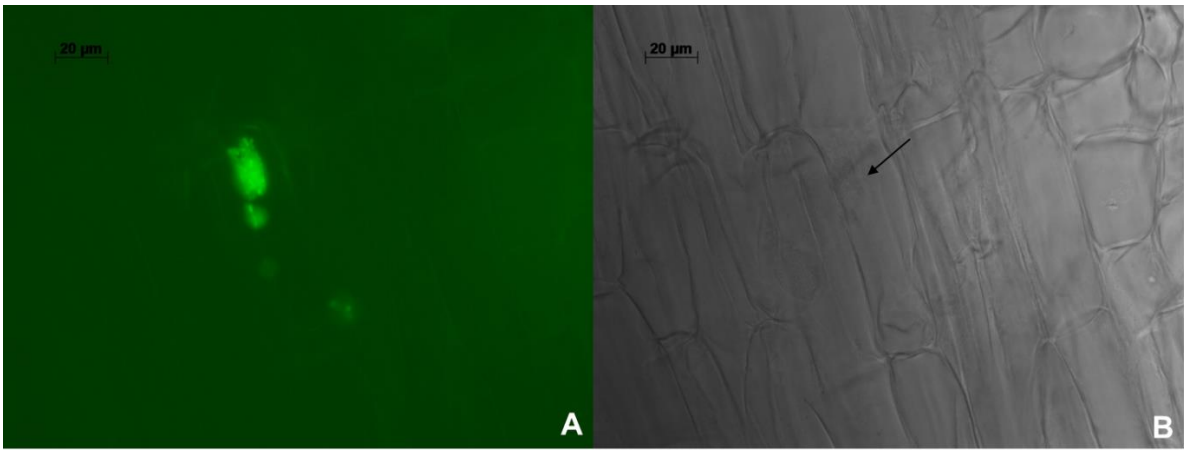


Fig 13. Rice (var. M206) roots colonized by WP9*gfp* visualized under 630x magnification. Image on the left (A) was taken with GFP filter, and on the right (B) was taken without GFP filter.

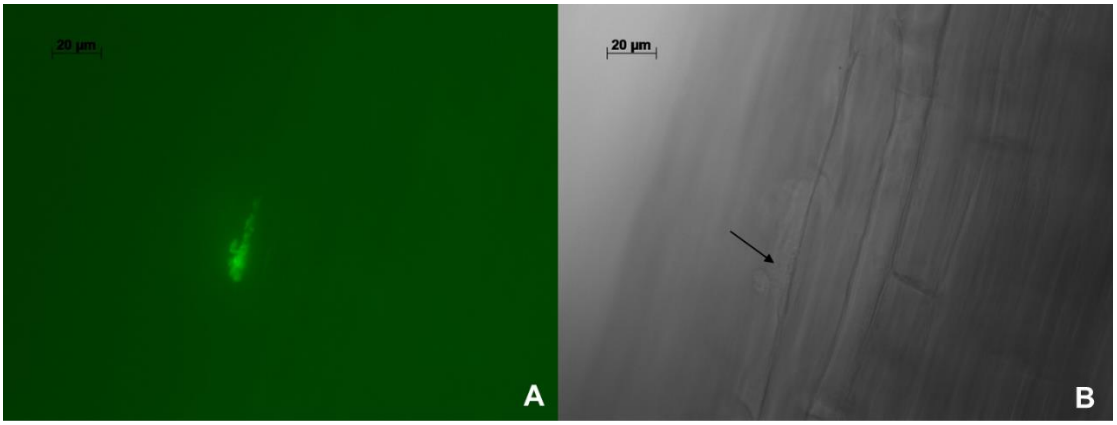


Fig 14. Rice (hybrid XL745) roots colonized by WP5*gfp* visualized under 630x magnification. Image on the left (A) was taken with GFP filter, and on the right (B) was taken without GFP filter.

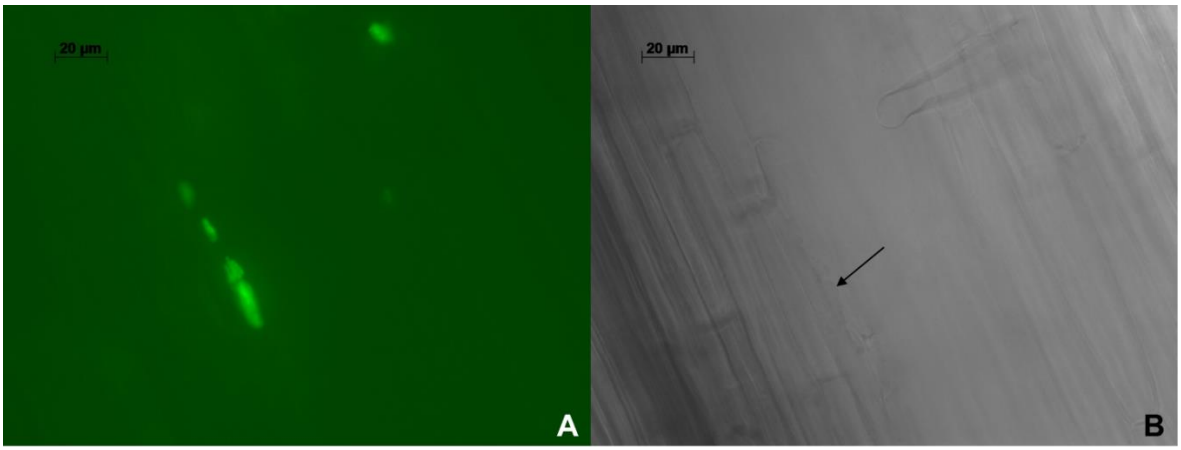


Fig 15. Rice (hybrid XL745) roots colonized by WP9*gfp* visualized under 630x magnification. Image on the left (A) was taken with GFP filter, and on the right (B) was taken without GFP filter.

Biomass of WP5*gfp* and WP9*gfp* inoculated rice (var. M206 and hybrid XL745) plants at 11 DAI

In var. M206, root and shoot biomass was higher in WP9*gfp* inoculated plants but in hybrid Xl745, biomass was higher in WP5*gfp* inoculated plants than mock-inoculated control plants (Fig 16). However, the biomass difference was not statistically different.

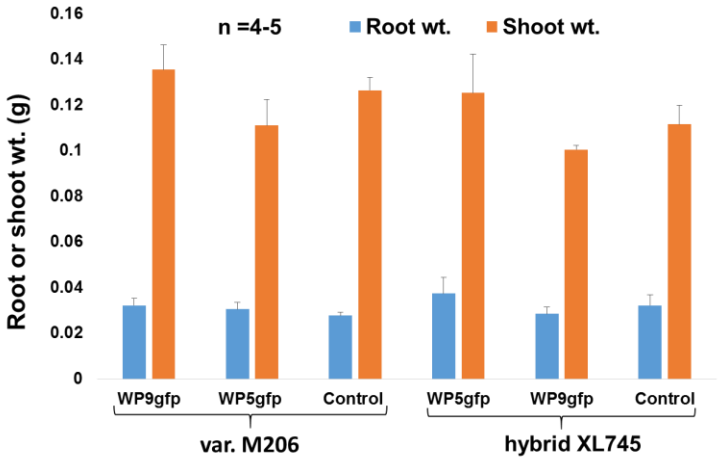


Fig 16. Root and shoot biomass of WP5*gfp*, WP9*gfp*, and mock-inoculated control plants in rice var. M206 and hybrid XL745 at 11 DAI. The bars represent the standard errors of mean.

Colonization of maize (hybrid 29B17) plants at 25 DAI

The colonization pattern in both root and leaf tissues were observed under the compound microscope. In root system, colonization was observed both in radicle, and seminal roots (Figs 17 and 18). Microcolonies (Fig 17; A and B) or individual or aggregations of bacterial communities were observed in root cortex (Figs 17 and 18). They extensively used intercellular spaces and cell junctures as microhabitats for colonization in both roots and leaves (Figs 17, 18, and 19). The growing of bacterial cells adjoining to the plant cell wall was ubiquitous in all observed samples. In leaves, no colonization was detected in the midrib area but efficient

colonization was observed in the intercellular spaces of mesophyll cells (Fig 19; A and B), and around the guard cells (Fig 19; C and D).

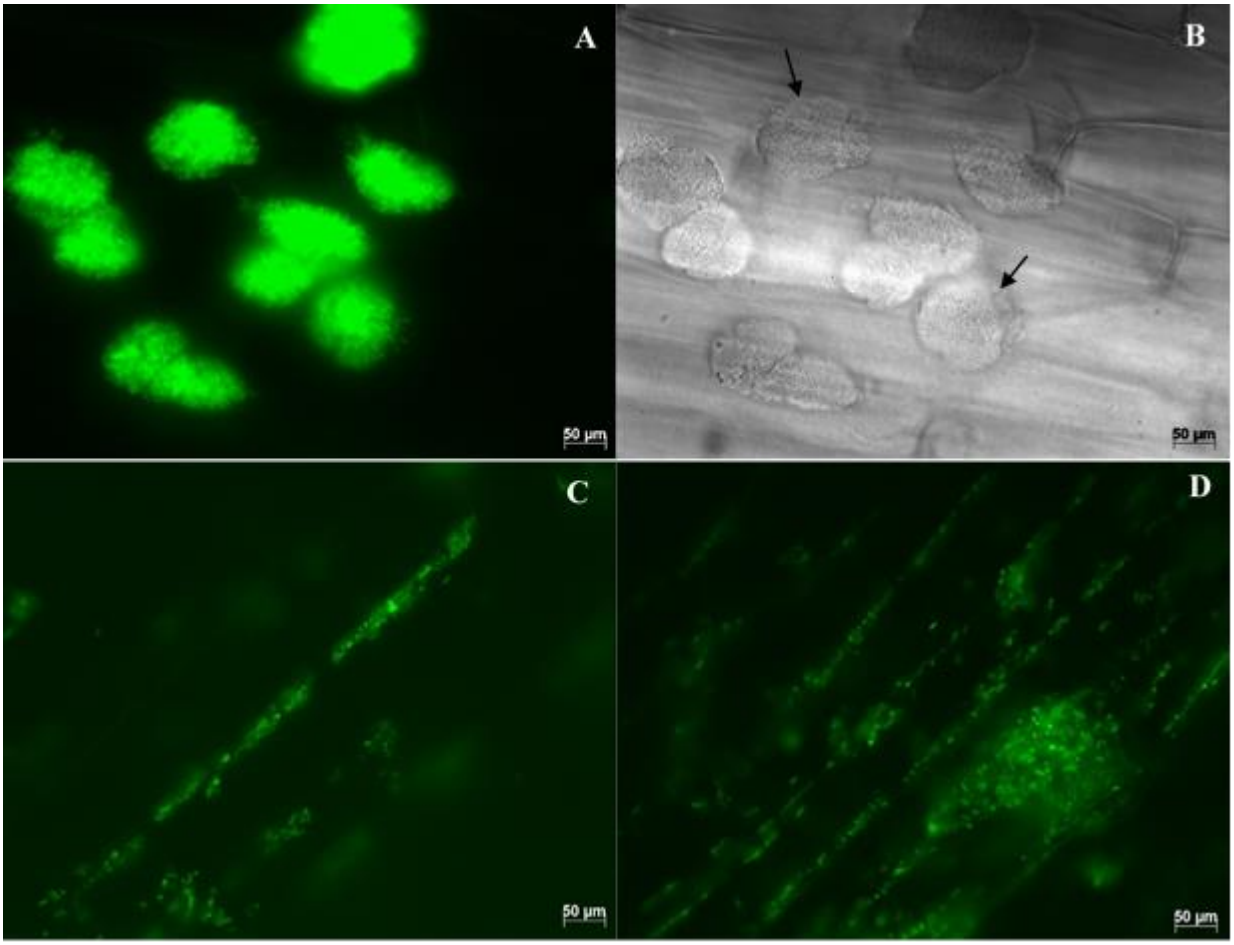


Fig 17. Maize (hybrid 29B17) seminal roots colonized by WP5*gfp*, visualized under 63x magnification. Images; A, C, and D were taken with GFP filter, and image B was taken without GFP filter.

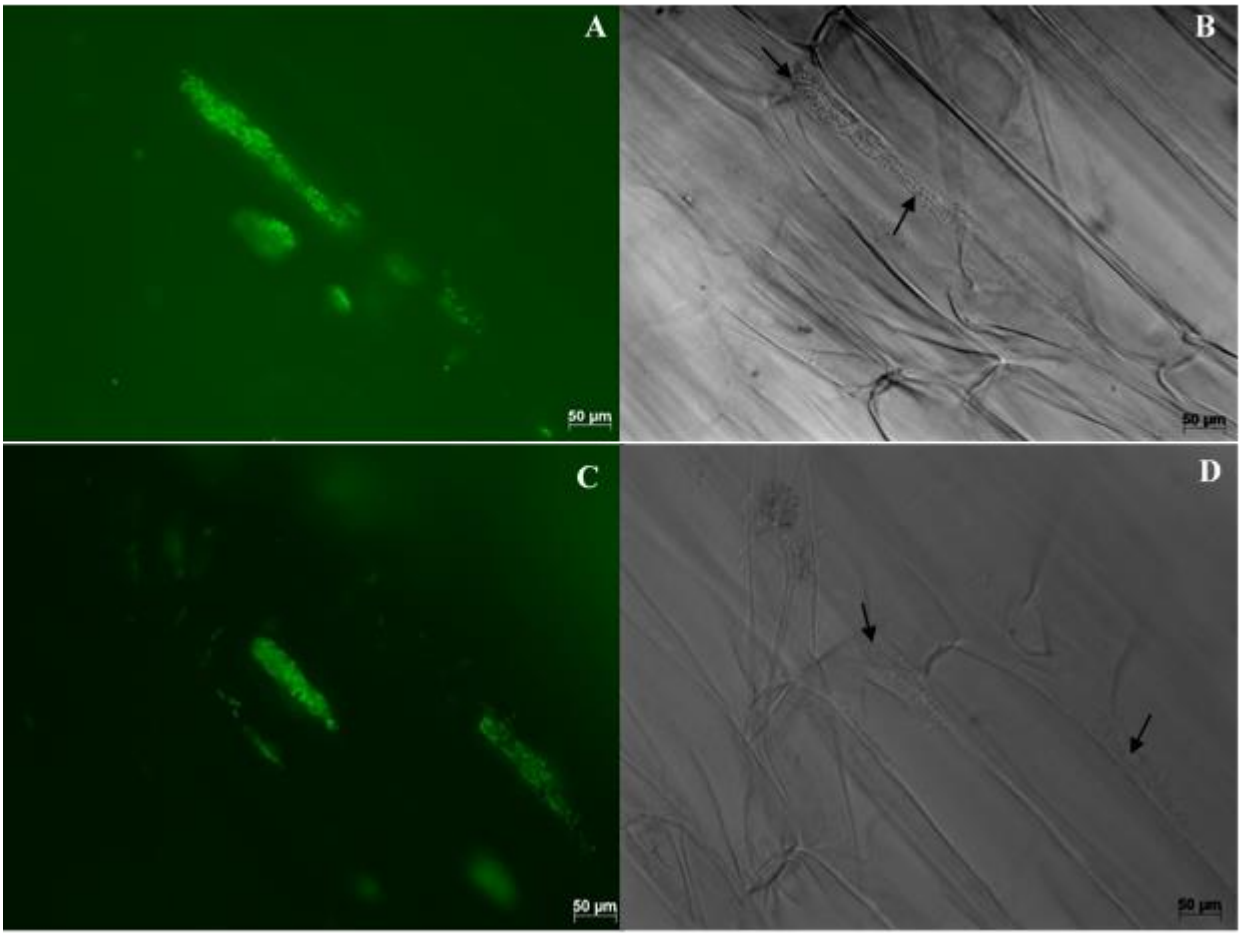


Fig 18. Maize (hybrid 29B17) radicle roots colonized by WP5*gfp*, visualized under 63x magnification. Images on the left (A and C) were taken with GFP filter, and on the right (B and D) were taken without GFP filter.

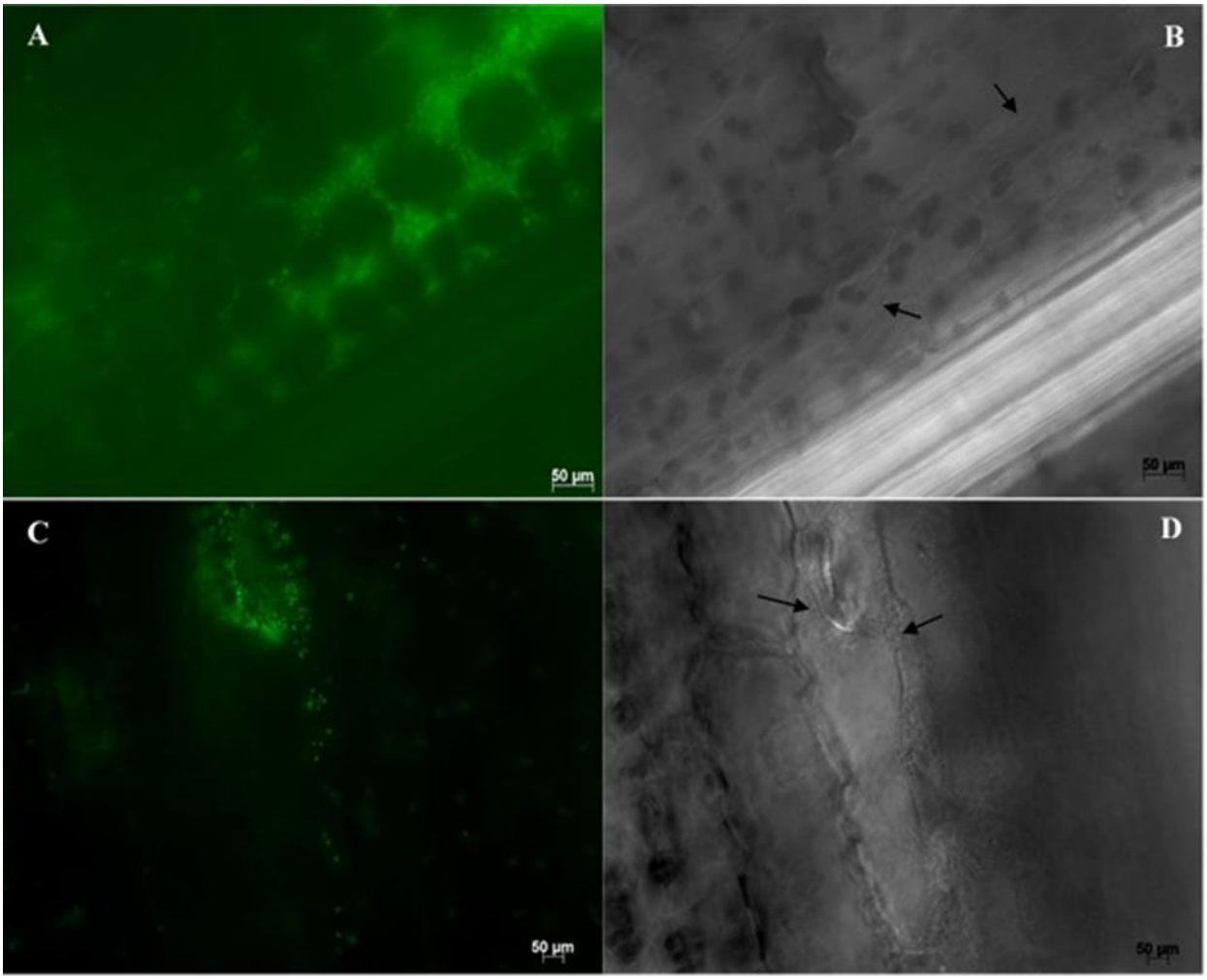


Fig 19. Maize (hybrid 29B17) leaves colonized by WP5*gfp*, visualized under 63x magnification. Images on the left (A and C) were taken with GFP filter, and images on the right (B and D) were taken without GFP filter.

Biomass of WP5*gfp* inoculated maize (hybrid 29B17) plants at 25 DAI

Growth benefit and colonization pattern in host plant by WP5*gfp* was observed in a month-old maize hybrid 29B17. Fresh root, root and shoot biomass (g plant^{-1}) were significantly higher in inoculated plants as compared to their respective control groups ($p= 0.011$) (root), and ($p= 0.021$ (root and shoot). Root weight was 20%, and root and shoot weight was 16% higher in inoculated

plants (Fig 20). WP5*gfp* inoculated plants had higher root bulk and bigger plant stature as compared to mock inoculated plants (Fig 21).

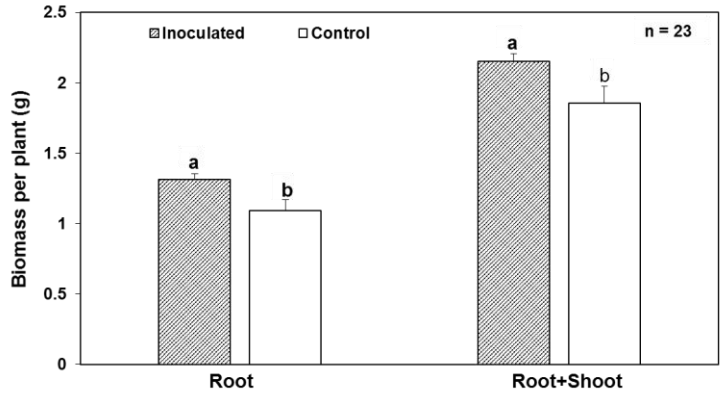


Fig 20. Root, and root and shoot biomass of WP5*gfp* inoculated and mock-inoculated control plants in maize hybrid 29B17 at 25 DAI. The bars represent the standard errors of mean. Histograms with asterisk indicate significant differences ($P < 0.05$).

Fig 21. WP5*gfp* inoculated (B) and mock-inoculated control (A) maize (hybrid 29B17) plants at 25 DAI.

Enumeration of *gfp* expressing WP5 populations in maize (hybrid 29B17) plants

Endophytic populations of WP5*gfp* were recovered from surface sterilized maize root, and leaf and stem samples. Higher WP5*gfp* populations (cfu per gram of plant tissue) were observed in leaf and stem in contrast to root samples (Fig 22). Average WP5*gfp* population was 2.9 X 10^7 cfu per gram of root, and 3.9 X 10^7 cfu per gram of leaf and stem. No growth was observed in the mock-inoculated control plants. Also, no growth of any microbes were observed on MG/L plates that were used to validate our surface sterilization protocol.

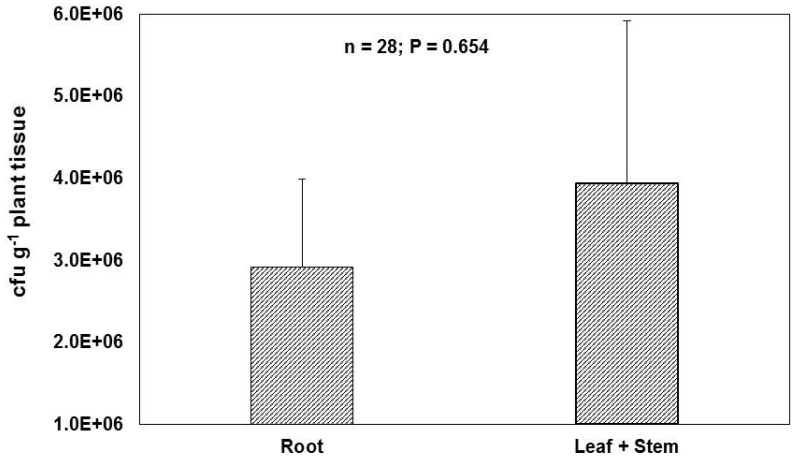


Fig 22. Quantification of endophytic populations, *gfp* expressing colonies (cfu) of WP5 per gram in shoot including leaves and stem, and root. The bars represent the standard errors of mean.

Colonization of maize (hybrid 29B17) plants by WP1 at 25 DAI

The colonization evidence of maize roots and leaves by endophytic yeast, WP1 was confirmed through microscopy. Individual yeast cells were observed in the cortical region of root tissues (Fig 23). Yeast cells were observed as congregated onto the plant cell wall in roots, and in both upper (adaxial), and the bottom (abaxial) leaf surfaces (Fig 24). No colonization evidence was observed in the mock inoculated control plants (figures not shown).

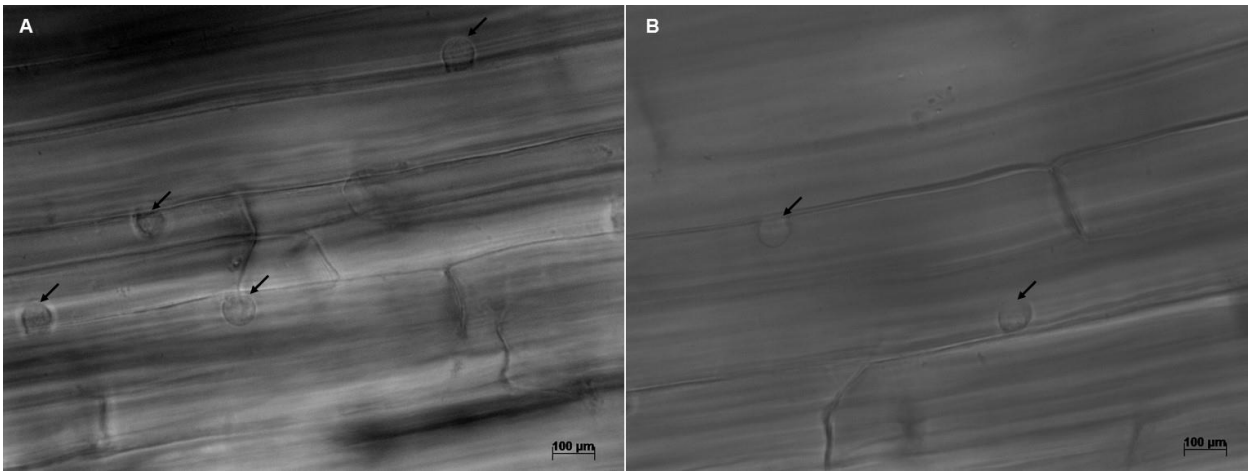


Fig 23. Maize (hybrid 29B17) roots colonized by yeast endophyte, WP1 visualized under 40x magnification. (A) seminal roots, and (B) radicle roots. Arrows showed the yeast cells (WP1) in cortical tissues of roots.

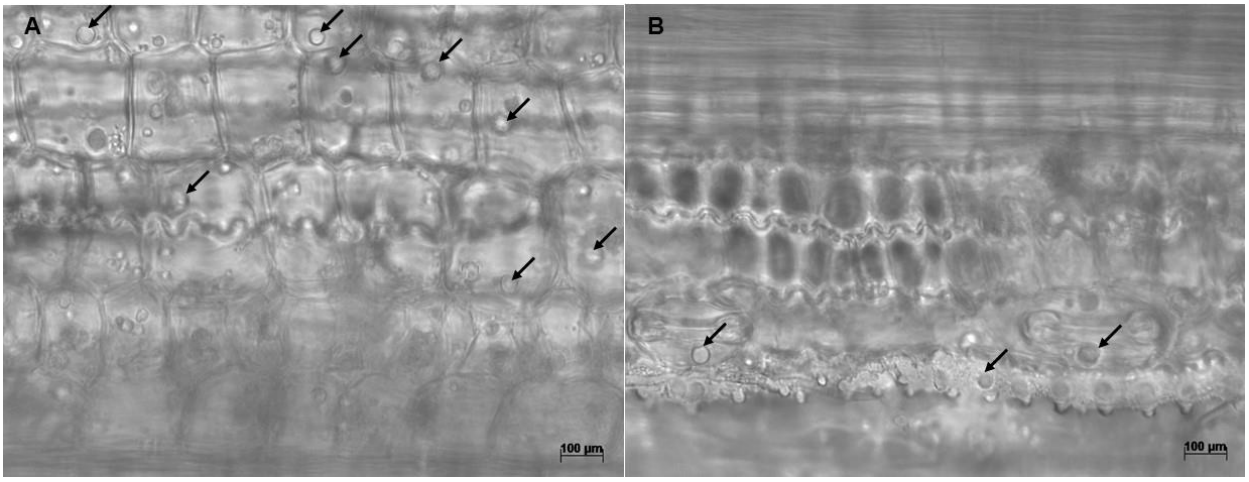


Fig 24. Maize (hybrid 29B17) leaves colonized by yeast endophyte, WP1. (A) WP1 cells in upper or Adaxial leaf surface, and (B) in the bottom or Abaxial leaf surface. Arrows showed the yeast cells (WP1) in mesophyll tissues of leaf.

Enumeration of WP1 population in maize (hybrid 29B17) plants

Both root and above ground tissues of maize seedlings were colonized by WP1. A higher population of WP1 was observed in per unit of root biomass than shoot biomass. Total cfu g^{-1} of root was 1.05 X 10^6, and 4.9 X 10^5 in shoot (leaf and stem) (Fig 25).

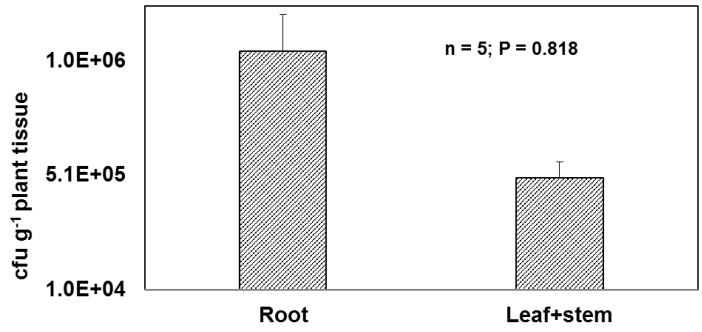


Fig 25. Quantification of endophytic populations; pink colonies (cfu) of WP1 per gram in shoot including leaves and stem, and root. The bars represent the standard errors of mean.

Biomass of WP1 inoculated maize (hybrid 29B17) plants

Total (root+shoot) fresh biomass was higher (2.17 g $plant^{-1}$) in WP1 inoculated plants as compared to mock inoculated controlled plants (2.10 g $plant^{-1}$) but the difference was not statistically significant (Fig 26).

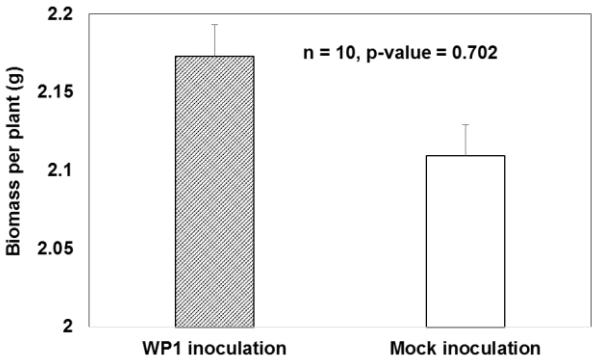


Fig 26. Total (root+shoot) biomass of WP1 inoculated and mock-inoculated control plants in maize hybrid 29B17 at 25 DAI. The bars represent the standard errors of mean.

Root inoculation assay in maize (hybrid 29B17): CFU per unit biomass at 15 DAI

Although only roots of maize seedlings were co-cultivated with WP1 culture for inoculation, colonization was observed in both root and above ground tissues (Fig 28). The WP1 population was higher in per unit biomass of leaf and stem tissues than per unit of root biomass but the difference was not statistically different (Fig 27).

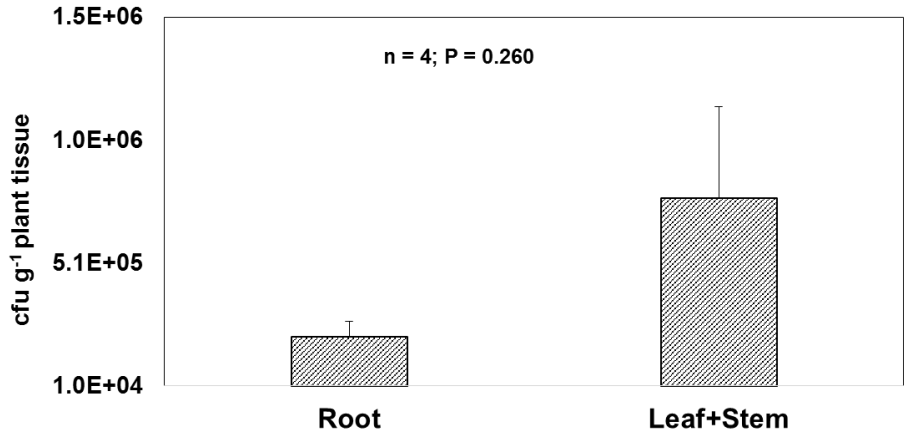


Fig 27. Quantification of endophytic populations; pink colonies (cfu) of WP1 per gram in shoot including leaves and stem, and root. The bars represent the standard errors of mean.

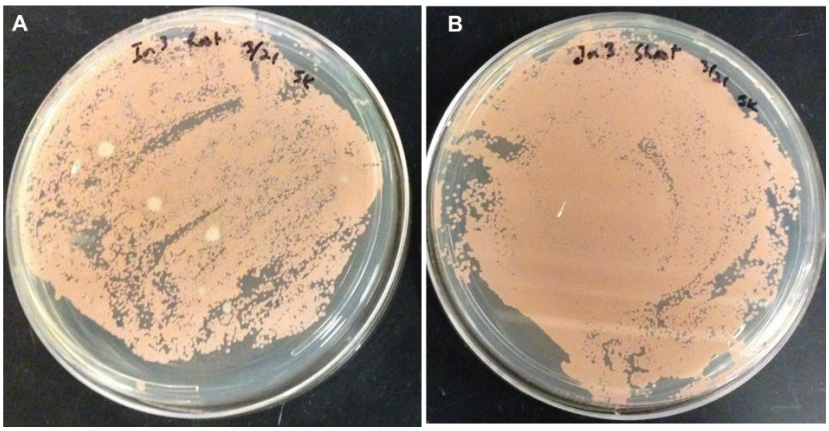


Fig 28. Two days growth of WP1 in MG/L plates from root and above ground tissues (leaf+stem) of same individual maize seedling. Both plates were from original ground suspension prior to dilution.

Root inoculation assay in maize (hybrid 29B17): Biomass per plant at 15 DAI

Shoot biomass per plant was higher in WP1 inoculated plants than mock inoculated control plants but root biomass was higher in control plants (Fig 29). The total biomass of inoculated plants (2.46 g plant^{-1}) was nearly as equal as control plants (2.50 g plant^{-1}).

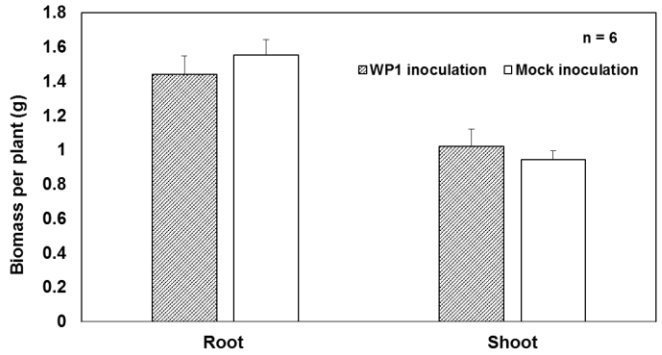


Fig 29. Root and shoot biomass of WP1 inoculated and mock-inoculated control plants in maize hybrid 29B17 at 15 DAI. The bars represent the standard errors of mean.

Colonization of rice (var. M206) plants by WP1 at 25 DAI

WP1 colonized rice (var. M206) plants in both root and above ground tissues. WP1 population was higher in per unit biomass of leaf and stem tissues than per unit of root biomass but the difference was not statistically different (Fig 30).

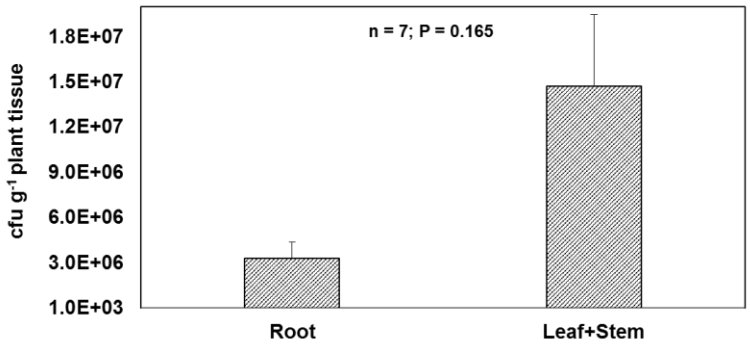


Fig 30. Quantification of endophytic populations; pink colonies (cfu) of WP1 per gram in shoot including leaves and stem, and root. The bars represent the standard errors of mean.

Biomass of WP1 inoculated rice (var. M206) plants at 25 DAI

Shoot biomass per plant was significantly higher in WP1 inoculated plants than mock inoculated control plants (Fig 31). The total biomass was also higher in inoculated plants (0.195 g plant^{-1}) than control plants (0.158 g plant^{-1}) but the difference was not statistically different.

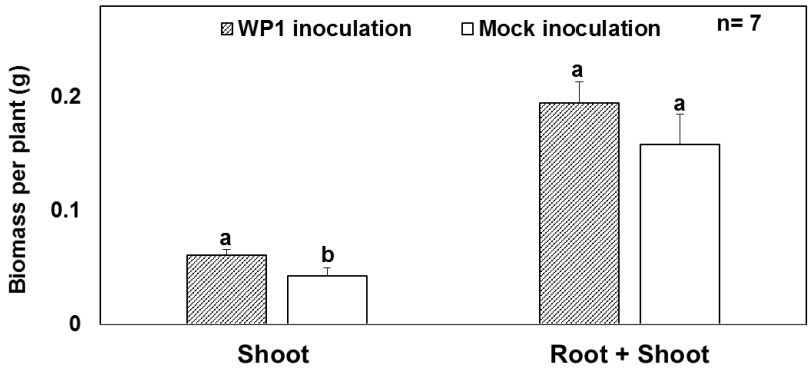


Fig 31. Root and shoot biomass of WP1 inoculated and mock-inoculated control plants in rice at 25 DAI. The bars represent the standard errors of mean. Histograms with different letter indicate the significant difference ($P < 0.05$) between inoculated and corresponding mock inoculated control.

DISCUSSION

In this study, both organic maize and rice seedlings were colonized by poplar endophyte strains. The endophytic population of inoculated WP5*gfp*, WP9*gfp*, and WP1 were observed through microscopy, and estimated *in planta* density by counting CFU in each plant. Inoculated maize and rice seedlings were grown in N free growth medium since these endophyte strains grow abundantly in N limited medium, and showed N-fixing activity in vivo and in vitro (Xin et al., 2009b; Knoth et al., 2014; Doty et al., 2016). The results showed that inoculated maize, and rice (var. M206) plants had more biomass than controls under N deprived conditions indicating the

positive growth response of these endophytes to the maize and rice plants. Plant growth promoting activities of endophyte strains, *Rahnella* and *Burkholderia* have been described in many native and inoculated distant host plants (Taghavi et al., 2009; Kim et al., 2012; Knoth et al., 2012; Ker et al., 2012; He et al., 2013; Lowman and Kim-Dura, 2016). The increased biomass has been reported in inoculated plants without added N or limited N supply.

Bacterial strains, both WP5*gfp* and WP9*gfp,* were observed in all four inoculated maize and rice genotypes at 11 DAI. In maize hybrids 29B17 and 14A91, colonization was detected in root cortex and xylem tissues (Figs 5-10). Individual cell or communities of WP5*gfp* and WP9*gfp* were distributed in between plants cells or intercellular spaces. In rice; var. M206, and hybrid Xl745, mostly colonization was observed in the root cortex. The colonization pattern in rice was also intercellular as mentioned in maize. Though, it seems colonization was relatively higher in maize seedlings than rice from visual assessment of colonization pictures. Previous studies have also shown the intercellular colonization pattern of different bacterial endophytes in the host plant (Germaine et al., 2004; Iniguez et al., 2004; Prieto et al., 2011; Wei et al., 2013). In both rice and maize, endophyte inoculated plants had significantly higher or higher root and shoot biomass than mock inoculated control plants except WP9*gfp* in rice hybrid XL745 (Figs 15 and 16). Similar early response of inoculation is reported in endophyte inoculated *Arabidopsis thaliana*, and rice seedlings where higher root growth and biomass was observed in inoculated seedlings than controls (Redman et al., 2011; Abbamondi et al., 2016).

In another colonization experiment, inoculated maize (hybrid 29B17) seedlings were incubated for a longer time; about four weeks (25 DAI). WP5*gfp* inoculated maize plants showed

colonization evidences on both roots and leaves. Several microcolonies of WP5*gfp* were observed in cortical root tissues (Fig 17, A and B). WP5*gfp* colonized mesophyll tissue and surrounding of guard cells in maize leaves (Fig 6). Mesophyll cells are the active site of photosynthesis in plant leaves. Demand of N in rubisco and other photosynthetic enzymes may have a connection to the diazotrophic activity of bacterial endophytes in leaves. In past studies, it has been also shown that bacterial diazotrophic endophytes such as *Klebsiella variicola* colonized the mesophyll cells of sugarcane leaves, *Herbaspirillum* sp. colonized young leaves and shoots of wild rice, *Herbaspirillum eropedicae* Z67 colonized leaf vein, mesophyll cells, and substomatal cavities of rice leaves, and *Serratia marcescens* colonized the leaf sheaths and leaf aerenchyma of rice plants (Elbeltagy et al., 2001; Gyaneshwar et al., 2001; James et al., 2002; Wei et al., 2013). In addition, an earlier study showed that higher N fixation was occurred in leaves and stems than roots in endophytes inoculated poplar plants (Knoth et al., 2014). WP5*gfp* inoculated maize plants had significantly higher root and total (root+shoot) biomass over mock inoculated control plants (Fig 20). Similar results of higher biomass was observed in endophyte inoculated canola, maize and *Arabidopsis* plants at about a month after inoculation (Knoth et al., 2012; Puri et al., 2016b; Rangel de Souza et al., 2016; Sheoran et al., 2016). Furthermore, higher density of *in planta* WP5*gfp* was observed in shoot tissues than roots (Fig 22). This is in contrast to a recent study that showed higher endophytic population of *Paenibacillus polymyxa* strain P2b-2R*gfp* in roots versus stems in maize (Puri et al., 2016a).

Multiple endophytic yeast genera potential to promote plant growth were reported before. But the colonization process used by endophytic yeasts to colonize the plant body is not well understood yet. Yeast cells are devoid of flagella, and it seems they do not have the evident

colonization strategy as used by other filamentous fungus (Doty, 2013). In this study, the colonization property of a *Populus* yeast endophyte; WP1 was verified in inoculated maize and rice seedlings through microscopy and *in planta* cfu count method. The roots and shoot tissues of both maize and rice seedlings were colonized by WP1, and significant *in planta* endophytic population was recovered from roots and shoots (stems and leaves) in both crops. In these assays, seedlings were co-cultivated overnight with WP1 culture for inoculation. However, in the root colonization assay, roots were restrictedly inoculated with WP1 culture to examine whether WP1 could migrate to the shoots of inoculated seedlings. High *in planta* WP1 population was recovered from inoculated seedlings but we do not have any knowledge how endophytic yeast can migrate from roots to leaves. Bacterial endophytes colonize vascular tissues especially xylem vessels, and spread systemically through these tissues in the plant body (James and Olivares, 1997; McCully, 2001; Compant et al., 2005; Naveed et al., 2014). It is plausible to suppose that WP1 can also use vascular tissues to spread from roots to leaves and stems.

A recent genome analysis showed that WP1 genome contains genes that are related to plant growth promotion including phytohormone production, adhesion and plant colonization, nutrient acquisition, and stress alleviation (Firrincieli et al., 2015). The detail process of bacterial attachment to plant cells have been reported in plant growth enhancers such as *Rhizobium*, *Azospirillum*, *Pseudomonas*, and in a plant pathogen, *Agrobacterium*. Biofilm produced by these bacteria play major role in early process of adhesion and plant colonization (Rodríguez-Navarro et al., 2007; Bogino et al., 2013). WP1 also produces biofilm which may potentially facilitate the yeast cells to attach on plant surface and subsequent colonization. It is possible that WP1 might passively ingress through crack entry process in roots, multiply shortly, invade the xylem

vessels, and ascend to the above ground parts through transpiration stream. Under the microscope, we sometimes observed that yeast cells were surrounded by bacterial cells, so it could be possible that bacteria may facilitate the movement of the yeast cells for short distances. Both maize and rice plants inoculated with WP1 had superior biomass over mock inoculated plants. Prior studies showed that endophytic or epiphytic yeasts isolated from different host plants are known to have plant growth promoting potentials as other plant endophytes (Nassar et al., 2005; Xin et al., 2009a; Limtong et al., 2014; Nutaratat et al., 2014; Sun et al., 2014). Further research would ascertain the importance of yeasts in plant growth promotion.

In this study, a high level of colonization was observed in all the lines of both rice and maize plants through microscopy and by enumerating *in planta* densities of endophytes: WP5*gfp*, WP9*gfp*, and WP1. These results indicated that endophytes were not restricted at the colonization level suggesting that the lack of response has more to do with the interaction while within rather than in early signaling or early defense. The lack of strong impact of endophytes on certain maize lines (chapter 3) was therefore not due to an inability to colonize these lines. The symbiotic microorganisms including endophytes use a subtle approach to colonize the host plants without eliciting defense responses or to a lesser degree than pathogens (Compant et al., 2010; Zamioudis and Pieterse, 2012; Alquéres et al., 2013). These microorganisms can also modulate the host defense responses that would facilitate the entry and colonization process (Brusamarello-Santos et al., 2012; Lebeis, 2014). Several plant endophyte strains are investigated during recent decades to explore the mechanisms they used for plant growth promotion, colonization strategy, and potential use in agriculture (Lucero et al., 2014; Hamilton et al., 2016; Santoyo et al., 2016). A detailed study of the interacting process of endophytes with

host plants by both bacterial and yeast endophytes in different crop genotypes in diverse biophysical environments would provide more specific knowledge about mechanisms of host specificity, and their potential use in agriculture.

REFERENCES

Abbamondi, G.R., G. Tommonaro, N. Weyens, S. Thijs, W. Sillen, P. Gkorezis, C. Iodice, W. de Melo Rangel, B. Nicolaus, and J. Vangronsveld. 2016. Plant growth-promoting effects of rhizospheric and endophytic bacteria associated with different tomato cultivars and new tomato hybrids. Chem. Biol. Technol. Agric. 3(1): 1.

Abendroth, L.J., R.W. Elmore, M.J. Boyer, and S.K. Marlay. 2011. Corn growth and development. Iowa State University, Ames, IA.

Ali, S., J. Duan, T.C. Charles, and B.R. Glick. 2014. A bioinformatics approach to the determination of genes involved in endophytic behavior in *Burkholderia* spp. J. Theor. Biol. 343: 193–8.

Alquéres, S., C. Meneses, L. Rouws, M. Rothballer, I. Baldani, M. Schmid, and A. Hartmann. 2013. The bacterial superoxide dismutase and glutathione reductase are crucial for endophytic colonization of rice roots by *Gluconacetobacter diazotrophicus* PAL5. Mol. Plant. Microbe. Interact. 26: 937–945.

do Amaral, F.P., V.C.S. Pankievicz, A.C.M. Arisi, E.M. de Souza, F. Pedrosa, and G. Stacey. 2016. Differential growth responses of *Brachypodium distachyon* genotypes to inoculation with plant growth promoting rhizobacteria. Plant Mol. Biol. 90(6): 689–697.

Bogino, P.C., M. de las M. Oliva, F.G. Sorroche, and W. Giordano. 2013. The role of bacterial biofilms and surface components in plant-bacterial associations. Int. J. Mol. Sci. 14(8):

15838–15859.

Botta, A.L., A. Santacecilia, C. Ercole, P. Cacchio, and M. Del Gallo. 2013. In vitro and in vivo inoculation of four endophytic bacteria on *Lycopersicon esculentum*. N. Biotechnol. 30(6): 666–74.

Brusamarello-Santos, L.C.C., F. Pacheco, S.M.M. Aljanabi, R. a. Monteiro, L.M. Cruz, V. a. Baura, F.O. Pedrosa, E.M. Souza, and R. Wassem. 2012. Differential gene expression of rice roots inoculated with the diazotroph *Herbaspirillum seropedicae*. Plant Soil 356(2012): 113–125.

Cangelosi, G.A., E.A. Best, G. Martinettii, and N.E. W. 1991. Genetic analysis of *Agrobacterium*. Methods Enzymol. 204: 384–397.

Compant, S., C. Clément, and A. Sessitsch. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42(5): 669–678.

Compant, S., B. Reiter, A. Sessitsch, J. Nowak, C. Clément, and E.A. Barka. 2005. Endophytic colonization of *Vitis vinifera* L . by plant growth-promoting bacterium *Burkholderia* sp . strain PsJN. Appl. Environ. Microbiol. 71(4): 1685–1693.

Dong, Z., M. Heydrich, K. Bernard, and M.E. McCully. 1995. Further evidence that the N2-fixing endophytic bacterium from the intercellular spaces of sugarcane stems is *Acetobacter diazotrophicus*. Appl. Environ. Microbiol. 61(5): 1843–1846.

Doty, S.L. 2013. Endophytic yeasts: biology and applications. In Symbiotic Endophytes. p. 335–343. Springer Berlin Heidelberg.

Doty, S.L., M.R. Dosher, G.L. Singleton, A.L. Moore, B. Van Aken, R.F. Stettler, S.E. Strand, and M.P. Gordon. 2005. Identification of an endophytic *Rhizobium* in stems of *Populus*.

Symbiosis (2005): 27–35.

Doty, S.L., B. Oakley, G. Xin, J.W. Kang, G. Singleton, Z. Khan, A. Vajzovic, and J.T. Staley. 2009. Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47: 23–33.

Doty, S.L., A.W. Sher, N.D. Fleck, M. Khorasani, R.E. Bumgarner, Z. Khan, A.W.K. Ko, S.-H. Kim, and T.H. DeLuca. 2016. Variable nitrogen fixation in wild *Populus*. PLoS One 11(5): e0155979.

Egener, T., T. Hurek, B. Reinhold-hurek, M. Mikrobiologie, and A. Symbioseforschung. 1999. Endophytic expression of nif genes of *Azoarcus* sp . strain BH72 in rice roots. Mol. Plant-Microbe Interact. 12(9): 813–819.

Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, and H. Mitsui. 2001. Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. 67(11): 5285–5293.

Firrincieli, A., R. Otillar, A. Salamov, J. Schmutz, Z. Khan, R.S. Redman, N.D. Fleck, E. Lindquist, I. V. Grigoriev, and S.L. Doty. 2015. Genome sequence of the plant growth promoting endophytic yeast *Rhodotorula graminis* WP1. Front. Microbiol. 6(September): 6–11.

Gaiero, J.R., C. a McCall, K. a Thompson, N.J. Day, A.S. Best, and K.E. Dunfield. 2013. Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am. J. Bot. 100(9): 1738–50.

Germaine, K., E. Keogh, G. Garcia-Cabellos, B. Borremans, D. Lelie, T. Barac, L. Oeyen, J. Vangronsveld, F.P. Moore, E.R.B. Moore, C.D. Campbell, D. Ryan, and D.N. Dowling. 2004. Colonisation of poplar trees by gfp expressing bacterial endophytes. FEMS

Microbiol. Ecol. 48(1): 109–18.

Gyaneshwar, P., E.K. James, N. Mathan, P.M. Reddy, B. Reinhold-hurek, and K. Jagdish. 2001. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. J. Bacteriol. 183(8): 2634–2645.

Hamilton, C.E., J.D. Bever, J. Labbé, X. Yang, and H. Yin. 2016. Mitigating climate change through managing constructed-microbial communities in agriculture. Agric. Ecosyst. Environ. 216(2016): 304–308.

Hardoim, P.R., L.S. van Overbeek, G. Berg, A.M. Pirttilä, S. Compant, A. Campisano, M. Döring, and A. Sessitsch. 2015. The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. 79(3): 293–320.

He, H., Z. Ye, D. Yang, J. Yan, L. Xiao, T. Zhong, M. Yuan, X. Cai, Z. Fang, and Y. Jing. 2013. Characterization of endophytic *Rahnella* sp. JN6 from *Polygonum pubescens* and its potential in promoting growth and Cd, Pb, Zn uptake by *Brassica napus*. Chemosphere 90(6): 1960–5.

Iniguez, A.L., Y. Dong, and E.W. Triplett. 2004. Nitrogen fixation in wheat provided by *Klebsiella pneumoniae* 342. Mol. Plant. Microbe. Interact. 17(10): 1078–85.

James, E.K., P. Gyaneshwar, N. Mathan, W.L. Barraquio, P.M. Reddy, P.P.M. Iannetta, F.L. Olivares, and J.K. Ladha. 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Mol. Plant. Microbe. Interact. 15(9): 894–906.

James, E.K., and F.L. Olivares. 1997. Infection and colonization of sugarcane and other graminaceous plants by endophytic diazotrophs. CRC. Crit. Rev. Plant Sci. 17(1): 77–119.

Kandel, S.L., N. Herschberger, S.H. Kim, and S.L. Doty. 2015. Diazotrophic endophytes of poplar and willow for growth promotion of rice plants in nitrogen-limited conditions. Crop Sci. 55(4): 1765–1772.

Kang, J.W., Z. Khan, and S.L. Doty. 2012. Biodegradation of trichloroethylene by an endophyte of hybrid poplar. Appl. Environ. Microbiol. 78(9): 3504–3507.

Ker, K., P. Seguin, B.T. Driscoll, J.W. Fyles, and D.L. Smith. 2012. Switchgrass establishment and seeding year production can be improved by inoculation with rhizosphere endophytes. Biomass and Bioenergy 47(1): 295–301.

Khan, Z., G. Guelich, H. Phan, R. Redman, and S. Doty. 2012. Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. ISRN Agron.: 1–11.

Khan, Z., D. Roman, T. Kintz, M.D. Alas, R. Yap, and S. Doty. 2014. Degradation, phytoprotection and phytoremediation of phenanthrene by endophyte *Pseudomonas putida*, PD1. Environ. Sci. Technol. 48(20): 12221–12228.

Kim, S., S. Lowman, G. Hou, J. Nowak, B. Flinn, and C. Mei. 2012. Growth promotion and colonization of switchgrass (*Panicum virgatum*) cv. Alamo by bacterial endophyte *Burkholderia phytofirmans* strain PsJN. Biotechnol. Biofuels 5(1): 37.

Knoth, J.L., S.-H. Kim, G.J. Ettl, and S.L. Doty. 2012. Effects of cross host species inoculation of nitrogen-fixing endophytes on growth and leaf physiology of maize. GCB Bioenergy 5(4): 408–418.

Knoth, J.L., S. Kim, G.J. Ettl, and S.L. Doty. 2014. Biological nitrogen fixation and biomass accumulation within poplar clones as a result of inoculations with diazotrophic endophyte consortia. New Phytol. 201(2): 599–609.

Krause, A., A. Ramakumar, D. Bartels, F. Battistoni, T. Bekel, J. Boch, M. Böhm, F. Friedrich,

T. Hurek, L. Krause, B. Linke, A.C. McHardy, A. Sarkar, S. Schneiker, A.A. Syed, R. Thauer, F.-J. Vorhölter, S. Weidner, A. Pühler, B. Reinhold-Hurek, O. Kaiser, and A. Goesmann. 2006. Complete genome of the mutualistic, N2-fixing grass endophyte *Azoarcus* sp. strain BH72. Nat. Biotechnol. 24(11): 1385–91.

Lebeis, S.L. 2014. The potential for give and take in plant-microbiome relationships. Front. Plant Sci. 5(June): 287.

Lee, K.Y., S.E. Strand, and S.L. Doty. 2012. Phytoremediation of chlorpyrifos by *Populus* and *Salix*. Int. J. phytoremediatio 14(1): 48–61.

van der Lelie, D., S. Taghavi, S. Monchy, J. Schwender, L. Miller, R. Ferrieri, A. Rogers, X. Wu, W. Zhu, N. Weyens, J. Vangronsveld, and L. Newman. 2009. Poplar and its bacterial endophytes: coexistence and harmony. CRC. Crit. Rev. Plant Sci. 28(5): 346–358.

Limtong, S., R. Kaewwichian, W. Yongmanitchai, and H. Kawasaki. 2014. Diversity of culturable yeasts in phylloplane of sugarcane in Thailand and their capability to produce indole-3-acetic acid. World J. Microbiol. Biotechnol. 30(6): 1785–1796.

Lowman, S., and S. Kim-Dura. 2016. Strategies for enhancement of switchgrass (*Panicum virgatum* L .) performance under limited nitrogen supply based on utilization of N-fixing bacterial endophytes. Plant Soil: 1–17.

Lucero, M.E., S. Debolt, A. Unc, L. V Reyes, and R.L. Mcculley. 2014. Using microbial community interactions within plant microbiomes to advance an evergreen agricultural revolution. p. 183–202. *In* Sustainable agroecosystems in climate change mitigation.

McCully, M.E. 2001. Niches for bacterial endophytes in crop plants: a plant biologist's view. Funct. Plant Biol. 28(9): 983–990.

Mitter, B., A. Petric, M.W. Shin, P.S.G. Chain, L. Hauberg-Lotte, B. Reinhold-Hurek, J. Nowak,

and A. Sessitsch. 2013. Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. Front. Plant Sci. 4(April): 1–15.

Nassar, A.H., K.A. El-Tarabily, and K. Sivasithamparam. 2005. Promotion of plant growth by an auxin-producing isolate of the yeast *Williopsis saturnus* endophytic in maize (*Zea mays* L.) roots. Biol. Fertil. Soils 42(2): 97–108.

Naveed, M., B. Mitter, T.G. Reichenauer, K. Wieczorek, and A. Sessitsch. 2014. Increased drought stress resilience of maize through endophytic colonization by *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17. Environ. Exp. Bot. 97(2014): 30–39.

Neiverth, A., S. Delai, D.M. Garcia, K. Saatkamp, E.M. de Souza, F. de O. Pedrosa, V.F. Guimarães, M.F. dos Santos, E.C.G. Vendruscolo, and A.C.T. da Costa. 2014. Performance of different wheat genotypes inoculated with the plant growth promoting bacterium *Herbaspirillum seropedicae*. Eur. J. Soil Biol. 64: 1–5.

Nutaratat, P., N. Srisuk, P. Arunrattiyakorn, and S. Limtong. 2014. Plant growth-promoting traits of epiphytic and endophytic yeasts isolated from rice and sugar cane leaves in Thailand. Fungal Biol. 118(8): 683–694.

Prieto, P., E. Schilirò, M.M. Maldonado-González, R. Valderrama, J.B. Barroso-Albarracín, and J. Mercado-Blanco. 2011. Root hairs play a key role in the endophytic colonization of olive roots by *Pseudomonas* spp. with biocontrol activity. Microb. Ecol. 62(2): 435–45.

Puri, A., K.P. Padda, and C.P. Chanway. 2016b. Evidence of nitrogen fixation and growth promotion in canola (*Brassica napus* L.) by an endophytic diazotroph *Paenibacillus polymyxa* P2b-2R. Biol. Fertil. Soils 52(1): 119–125.

Puri, A., K.P. Padda, and C.P. Chanway. 2016a. Seedling growth promotion and nitrogen

fixation by a bacterial endophyte *Paenibacillus polymyxa* P2b-2R and its GFP derivative in corn in a long-term trial. Symbiosis. 69(2): 123-129.

Rangel de Souza, A.L.S., S.A. De Souza, M.V. V De Oliveira, T.M. Ferraz, F.A.M.M.A. Figueiredo, N.D. Da Silva, P.L. Rangel, C.R.S. Panisset, F.L. Olivares, E. Campostrini, and G.A. De Souza Filho. 2016. Endophytic colonization of *Arabidopsis thaliana* by *Gluconacetobacter diazotrophicus* and its effect on plant growth promotion, plant physiology, and activation of plant defense. Plant Soil 399(1-2): 257–270.

Redman, R.S., Y.O. Kim, C.J.D. a Woodward, C. Greer, L. Espino, S.L. Doty, R.J. Rodriguez, and L. Sharon. 2011. Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS One 6(7): e14823.

Reinhold-Hurek, B., and T. Hurek. 2011. Living inside plants: bacterial endophytes. Curr. Opin. Plant Biol. 14(4): 435–43.

Rennie, R.J. 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. 27(1): 8–14.

Rodríguez-Navarro, D.N., M.S. Dardanelli, and J.E. Ruíz-Saínz. 2007. Attachment of bacteria to the roots of higher plants. FEMS Microbiol. Lett. 272(2): 127–136.

Rollins, J.A., T.J. Wolpert, K.B. Johnson, M.B. Dickman, L.M. Ciuffetti, J.M. Lorang, R.P. Tuori, J.P. Martinez, T.L. Sawyer, and R.S. Redman. 2001. Green fluorescent protein is lighting up fungal biology. Appl. Environ. Microbiol. 67(5): 1987–1994.

Santoyo, G., G. Moreno-Hagelsieb, M. del Carmen Orozco-Mosqueda, and B.R. Glick. 2016. Plant growth-promoting bacterial endophytes. Microbiol. Res. 183(2016): 92–99.

Sheoran, N., A. Kumar, V. Munjal, A.V. Nadakkakath, and S.J. Eapen. 2016. *Pseudomonas putida* BP25 alters root phenotype and triggers salicylic acid signaling as a feedback loop in

regulating endophytic colonization in *Arabidopsis thaliana*. Physiol. Mol. Plant Pathol. 93(2016): 99–111.

Sun, P.F., W.T. Fang, L.Y. Shin, J.Y. Wei, S.F. Fu, and J.Y. Chou. 2014. Indole-3-acetic acid-producing yeasts in the phyllosphere of the carnivorous plant *Drosera indica* L. PLoS One 9(12): 1–22.

Taghavi, S., C. Garafola, S. Monchy, L. Newman, A. Hoffman, N. Weyens, T. Barac, J. Vangronsveld, D.D. Van Der Lelie, and D. Van Der Lelie. 2009. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl. Environ. Microbiol. 75(3): 748–757.

Vargas, L., T.L.G. de Carvalho, P.C.G. Ferreira, V.L.D. Baldani, J.I. Baldani, and A.S. Hemerly. 2012. Early responses of rice (*Oryza sativa* L.) seedlings to inoculation with beneficial diazotrophic bacteria are dependent on plant and bacterial genotypes. Plant Soil 356(2012): 127–137.

Wei, C.-Y., L. Lin, L.-J. Luo, Y.-X. Xing, C.-J. Hu, L.-T. Yang, Y.-R. Li, and Q. An. 2013. Endophytic nitrogen-fixing *Klebsiella variicola* strain DX120E promotes sugarcane growth. Biol. Fertil. Soils 50(4): 657–666.

Xin, G., D. Glawe, and S.L. Doty. 2009a. Characterization of three endophytic, indole-3-acetic acid-producing yeasts occurring in *Populus* trees. Mycol. Res. 113(Pt 9): 973–80.

Xin, G., G. Zhang, J.W. Kang, J.T. Staley, and S.L. Doty. 2009b. A diazotrophic, indole-3-acetic acid-producing endophyte from wild cottonwood. Biol. Fertil. Soils 45(6): 669–674.

Zamioudis, C., and C.M.J. Pieterse. 2012. Modulation of host immunity by beneficial microbes. Mol. Plant. Microbe. Interact. 25(2): 139–50.

CHAPTER FIVE

BIO-CONTROL AND PLANT GROWTH PROMOTING POTENTIAL OF SALICACEAE ENDOPHYTES

Shyam L. Kandel[1], Andrea Firrincieli[2], Giuseppe Scarascia Mugnozza[2], Antoine Harfouche[2], Soo-Hyung Kim[1], and Sharon L. Doty[1]*

[1]School of Environmental and Forest Sciences, College of the Environment, University of Washington, Seattle, WA 98195-2100, and [2]Department for Innovation Biological, Agro-Food and Forest System, University of Tuscia, Italy.

Author's contribution:

All experiments and analysis except comparative genomic analysis was conducted by Shyam L. Kandel with review and guidance from Soo-Hyung Kim, and Sharon L. Doty. All comparative genomic analysis was conducted by Andrea Firrincieli. The manuscript was written by Shyam L. Kandel.

ABSTRACT

Microbial communities of Salicaceae plants; poplar (*Populus trichocarpa*) and willow (*Salix sitchensis*) endosphere have been demonstrated to be important for plant growth promotion, protection from biotic and abiotic stresses, and degradation of toxic compounds. Our study aimed to investigate bio-control activities of Salicaceae endophytes against the soil borne plant pathogen *Rhizoctonia solani* AG-8. Additionally, different plant growth promoting traits such as biological nitrogen fixation (BNF), indole-3-acetic acid (IAA) biosynthesis, phosphate solubilization, and siderophore production were determined in all bio-control strains. *Burkholderia*, *Rahnella*, *Pseudomonas*, and *Curtobacterium* were major endophyte genera that showed bio-control activities in *in-vitro* assays. The bio-control activities of *Burkholderia* strains was stronger as compared to other stains. Genomes of *B.*strains, WP40 and WP42, were surveyed to identify the putative genes involved in the bio-control activities. A 56-kb *ofc* gene cluster responsible for biosynthesis of the anti-fungal glycolipopeptide, occidiofungin, was present in the genomes of the *B.* strains. Nearly all endophyte strains showing the bio-control activities produced IAA, solubilized tricalcium phosphate, and synthesized siderophores in the culture medium. Moreover, some strains reduced acetylene into ethylene in the acetylene reduction assay, a common assay used for assessing BNF. Salicaceae endophytes can be useful for bio-control of plant pathogens, and also for plant growth promotion possibly through the mechanisms of BNF, IAA production, and nutrient acquisition.

INTRODUCTION

Endophytes are bacterial and fungal communities that colonize the plant interior and contribute to the growth, development, fitness, and adaptation of the host plant (Rodriguez et al., 2008; Hardoim et al., 2008, 2015). Endophytes often confer considerable benefits to the host plants they inhabit. The mechanisms for plant growth promotion may be through nutrient acquisition, phytohormone production, and/or protection of host plants from abiotic and biotic stresses (Chen et al., 1995; Elbeltagy et al., 2001; Riggs et al., 2001; Redman et al., 2011; Hardoim et al., 2015; Kandel et al., 2015). Biotic stress, especially due to pathogenic microorganisms, causes major crop losses worldwide which is equivalent to $220 billion loss every year (Chakraborty and Newton, 2011). In response, growers often rely on a variety of chemicals to control these plant pathogens; however, such widespread use comes at both economic and environmental costs, causing undesirable consequences to human health through air, water, and soil pollution. Alternatively, the use of microbial organisms, termed biocontrol, to manage plant diseases offers an environmentally-friendly and more sustainable replacement to the chemical pesticides. Several past studies demonstrated that endophytes have the potential to control many plant diseases caused by different plant pathogens (Ryan et al., 2008; Compant et al., 2010). Endophytic bacteria including *Aureobactrium, Bacillus, Paenibacillus, Phyllobacterium, Pseudomonans,* and *Burkholderia* recovered from host plants or seeds showed anti-fungal activities against the plant pathogens, *Fusarium oxysporum, Rhizoctonia solani, Sclerotium rolfsii, Verticillium dahlia* and many others (Chen et al., 1995; Pleban et al., 1995; Rybakova et al., 2015).

Growth promotion has been observed in a variety of crop plants such as rice, wheat, maize, sugar cane, tomato through plant-endophyte interactions (Elbeltagy et al., 2001; Riggs et al., 2001; Hurek et al., 2002; Iniguez et al., 2004; Momose et al., 2009; Khan et al., 2012; Knoth et al., 2012; Botta et al., 2013). Many endophyte strains are considered as diazotrophs meaning that they are capable of biological nitrogen fixation (BNF). They carry genes encoding for the enzyme nitrogenase which catalyzes the chemical reactions to convert dinitrogen gas into nitrogen (N) compounds such as ammonium and nitrate which are potentially available for N metabolism by the plant (Bhattacharjee et al., 2008; Santi et al., 2013). Furthermore, endophytes can also produce phytohormones, siderophores, and solubilize insoluble inorganic phosphates (Khan et al., 2015). Siderophores are organic compounds that are produced by organisms during iron limiting conditions. Previous studies showed that plants can utilize microbial siderophores for iron acquisition. Iron deficient tomato plants supplemented with microbial siderophores, for example, produced higher crop yields, and had increased chlorophyll and iron content in the leaves (Radzki et al., 2013). In addition, siderophores are considered to be helpful in the biological control of plant pathogens (Verma et al., 2011; Ahmed and Holmström, 2014). Phosphorus is one of the primary macronutrients required for plant growth. Many plant associated rhizo or endophytic bacteria can solubilize insoluble inorganic phosphates which is potentially available for plants to uptake. Positive growth response has been reported in different crop plants inoculated with phosphate solubilizing endophytes (Dias et al., 2009; Manoel et al., 2015; Oteino et al., 2015; Passari et al., 2015). Endophytes have the potential to synthesize different plant hormones including IAA, gibberellic acid, cytokinin, and abscisic acid (Patten and Glick, 2002; Pirttila et al., 2004; Feng et al., 2006; Sgroy et al., 2009; Shi et al., 2009; Videira et al., 2012; Hardoim et al., 2015).

Dozens of microbial endophyte strains were isolated from poplar and willow plants that may support the host plant growth in the nutrient limited, cobble-dominated riparian ecosystems of the Snoqualmie River in western Washington (Doty et al., 2005, 2009). Several poplar and willow endophytes are diazotrophs with the ability of producing phytohormones and siderophores, and solubilizing the inorganic phosphates (Khan et al., 2015). A recent study showed that inoculated Salicaceae endophytes in hybrid poplar plants can contribute about 65% of the total N in the leaves and increased plant biomass through BNF (Knoth et al., 2014). Additionally, a significant amount of IAA production has been observed by poplar endophytes *in vitro* (Xin et al., 2009b; a). Cross inoculation of poplar and willow endophytes in other plant species (rice, maize, tomato, pepper, grasses, and conifer seedlings) showed substantial growth enhancement in nutrient-poor conditions (Khan et al., 2012, 2015; Kandel et al., 2015). Furthermore, inoculated sweet maize plants with Salicaceae endophytes showed improvement in photosynthetic capacity (higher CO_2 assimilation rate) of leaves with higher biomass, and also resulted in early flowering in tomato and pepper (Khan et al., 2012; Knoth et al., 2012).

Burkholderia, *Pseudomonas*, *Curtobacterium*, and *Sphingomonas* were the most common endophyte genera discovered in poplar and willow plants through culture dependent and independent methods (Doty et al., 2005, 2009, 2016). Previous studies have shown that plant associated endo or rhizospheric *Burkholderia* species can degrade toxic compounds, promote plant growth, fix atmospheric N, and inhibit the growth of plant pathogenic fungi or oomycetes (Perin et al., 2006; Suárez-Moreno et al., 2012; Mitter et al., 2013; Bernabeu et al., 2015). More recent studies suggested the wide application of *Burkholderia* in agriculture for plant growth promotion, and biological disease control (Mattos et al., 2008; Govindarajan et al., 2008;

Paungfoo-Lonhienne et al., 2014; Bernabeu et al., 2015). However some *Burkholderia* species are a potential risk for human health due to the pathogenic effects of some strains in immuno-compromised humans. Recent genome sequencing and analysis of different plant associated, environmental, and pathogenic *Burkholderia* species revealed two phylogenetically distinct groups. Plant associated and saprophytic species belong to one group, and human, plant and animal pathogens or opportunistic pathogens, and some environmental species belong to a separate group (Suárez-Moreno et al., 2012; Estrada-de los Santos et al., 2013). Angus et al. (Angus et al., 2014) also showed that plant associated endophytic *Burkholderia* are deficient in mechanisms that precludes the possibility of human infection. Poplar endophytic *Burkholderia* genomes were recently sequenced but no analysis has been done yet to understand genes that may be involved in plant growth promotion, growth inhibition of plant pathogenic fungi or distinction from pathogenic strains.

The objectives of this study were to explore the capabilities of poplar and willow endophytes in inhibiting the growth of the broad host range plant pathogenic fungus, *Rhizoctonia solani* AG-8; to understand the potential *Burkholderia* genes involved in fungal growth suppression; and to characterize the plant growth promoting properties. *R. solani* AG-8 is a widespread pathogen of many economically important crops including small grain crops such wheat and barley, grain legumes, and brassicas worldwide (Hane et al., 2014). Additionally, comparative genomics was performed with known pathogenic *Burkholderia* strains to observe the distinction between pathogenic and plant associated endophytic life styles.

MATERIALS AND METHODS

Endophyte strains and growth media

A total of 55 poplar and 4 willow endophyte strains (Table 1 and Table S1) were used in this study. Poplar and willow endophyte strains (except PD1) were isolated from plant samples that were collected at the Three Forks Natural Area in King County, WA in the riparian zone of the Snoqualmie River (+47° 31' 14.30", -121° 46' 28.32"). Samples were surface-sterilized with 10% bleach (10 min.) and 1% Iodophor (5 min.), and rinsed three times in sterile deionized water. Several endophyte strains were characterized in previous studies (Doty et al., 2005; Xin et al., 2009a), and others were characterized in this study. Endophyte strains were grown at 30°C in Mannitol Glutamate/Luria (MG/L) agar medium (Cangelosi et al., 1991) for 2 days before being subjected to the antifungal assay. Endophyte strains having antifungal activities were further tested for different growth promoting traits including phosphate solubilization, siderophore production, and IAA production.

Antifungal assay

Antifungal activities of poplar and willow endophytes were tested using the plant pathogenic fungus; *Rhizoctonia solani* AG-8. An agar plug of about 2 cm^2 freshly grown *Rhizoctonia* mycelium was placed at the center of ¼ PDA (Leslie et al., 2006) plates, and allowed to grow at room temperature before introduction of endophytes. A few colonies of each endophyte strain were introduced after 1 week, and placed at the 12, 3, 6, and 9 o'clock positions around the fungal plug. After 7 to 10 days, growth inhibition pattern of *R. solani* AG-8 was observed. The approximate growth arrest by endophytes over *R. solani* AG-8 was denoted as *Rhizoctonia* growth inhibition; REI through subjective ratings: RGI 1: ≥ 70% growth arrest, RG 2: 40-70%

growth arrest RGI 3: ≤ 40% growth arrest. For negative controls, sterile water drops were used instead of endophyte colonies. Only strains that were positive for antifungal activities were used to test further plant growth promoting properties, and investigated for potential molecular mechanisms through genome analyses.

Phosphate solubilization assay

Phosphate solubilizing property of poplar and willow endophytes was determined using National Botanical Research Institute's Phosphate (NBRIP) agar medium (Nautiyal, 1999). A few colonies of individual endophyte strains were introduced per NBRIP plate in quadruplicate positions using sterile inoculating sticks. The clear halo area around an endophyte colony was observed after incubating 1 week at 30^{o}C. Subjective ratings (++; a distinct clear halo, +; a halo only nearby colony, and -; no solubilization) of phosphate solubilization assay were recorded for each endophyte strains per plate. The poplar endophyte WP5 (Doty et al., 2009) was used as a reference strain for comparisons.

Siderophore production assay

M9 minimal medium supplemented with Chrome Azurol S (CAS) was used for the siderophore production assay. Minimal medium (Yun et al., 2000) was prepared, autoclaved, and mixed with filter-sterilized pre-warmed $MgSO_4$ (2 mM final), $CaCl_2$ (0.1 mM final), sucrose (0.2% final), and Casamino acids (0.9% final) (Loewen, 1984). CAS solution was prepared according to the protocol developed by Schwyn and Neilands (Schwyn and Neilands, 1987), autoclaved, and combined with minimal medium prior to the assay. The area of color conversion from blue to

orange around an endophyte colony was measured after incubating 1 week at 30^{o}C for each endophyte strains per plate.

IAA quantification assay

For IAA quantification, endophytes were grown in YEM broth (g L^{-1}: 0.5 yeast extract, and 10 mannitol) with or without 0.1% (w/v) L- tryptophan for 5 days, and pelleted through centrifugation. One ml of the supernatant was incubated with 2 ml of Salkowski reagent (2 ml of 0.5 M $FeCl_3$, and 98 ml of 35% $HClO_4$; (Gordon and Weber, 1951) for about half an hour, and the optical density at a wavelength of 530 nm was observed (Xin et al., 2009a). A standard curve was developed with known amounts of IAA using Salkowski reagent and YEM broth without endophytes, and computed the IAA amount produced by each endophyte strain using the standard curve. In ARA, IAA, and siderophore assays, duplicate samples were assayed and means of duplicates were used.

Acetylene Reduction Assay (ARA)

Endophyte strains were grown over night at 30^{o}C in MG/L or N limited carbon combined medium (NLCCM) on rotatory shaker. The overnight grown strains in MG/L cultures were microfuged at 4^{o}C at 8000 rpm for 10 minutes, combined with NLCCM cultures already in progress, and incubated on shaker for another night. Cell density was determined through spectrophotometry, and OD_{600} was adjusted to 1.0. Sixteen milliliter culture of each strain was transferred into balch tubes, dosed with 500 ul acetylene, and incubated for 24 hours. Three tubes per strain were prepared; two were for the dosed cultures and one was for undosed control. After 24 hours, 5 ml air from autosampler vials was removed and replaced with 5 ml headspace from

the balch tubes. Samples were analyzed in DeLuca Biogeochemistry Lab at University of Washington, WA.

Analysis of the nitrogenase subunit gene, nifH

The nitrogenase subunit gene, nifH, was amplified through PCR using universal nifH, nifH-b1 (Bürgmann et al., 2004), and nifH (Poly et al., 2001) primers. Colony PCR was performed in a same way as mentioned below.

Identification of endophytes through 16S/18S rDNA sequencing

Overnight grown bacterial colonies were used for colony PCR by transferring a single colony into 20 µl of sterile water, vortexed briefly, and using 1 µl as DNA templates for PCR analysis. The universal 16S rRNA primers, 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify 16S rDNA which produced 1.5 kb amplicon products. PCR reactions of 25 µl consisted of 1 µl template DNA, 12.5 µl of PCR premixed Buffer E (EpiCenter, Madison, WI), 0.8 µl of each primer (at 0.2 µg μl^{-1}), 0.3 µl of Taq DNA polymerase (New England BioLabs, Inc., Ipswich, MA), and 9.6 µl of sterile water. Amplified PCR products were incubated with ExoSAP PCR cleanup reagent **(Affymetrix, Inc., Cleveland, OH) at** 37°C for 30 min followed by 80°C for 15 min in P100 Thermal Cycler (Bio-Rad, Inc., Hercules, CA). The ExoSAP cleaned 16S PCR products were sequenced by Sanger sequencing approach (GENEWIZ, South Plainfield, NJ). For yeast strain WP 4-3-1, 18S rDNA was amplified and sequenced as described before (Xin et al., 2009a).

Nucleotide sequence accession numbers

The 16S rDNA sequences of all poplar endophytes were deposited in GeneBank (NCBI) database under accession numbers KF597274, KF597275, KF597276, KU495920, KU500894, KU500893, KU550576, KU500895, KU500892, KU500891, and KU550577 for WP40, WP41, WP42, WP 4-2-2, WP 4-3-1, WP 4-3-2, WP 4-3-3, WP 4-4-2, WP 4-5-3, WP 4-4-6, and WP 4-10-4 respectively.

Comparative analysis of genomic regions related to the synthesis of anti-*Rhizoctonia* compounds in *Burkholderia* species and *Pseudomonas putida* PD1

Genomes of endophyte strains which showed strong antifungal activities, were compared against phylogenetically related strains where the synthesis of anti-*Rhizoctonia* metabolites was functionally assessed through knock-out studies (Gu et al., 2009a). Other gene clusters encoding for putative non-ribosomal peptidase, polyketide synthases, and other enzymes involved in the synthesis of secondary metabolites characterized for a generic antimicrobial and antifungal effect were detected using antiSMAH, a bioinformatics tool for automatic genomic identification and analysis of biosynthetic gene clusters (Weber et al., 2015). We set a minimum ClusterFinder probability of 0.5 and searched for gene clusters with a minimum size of 4 open reading frames (orf) characterized by 5 or more biosynthesis PFAM-related domains. The presence of biosynthetic gene clusters for anti-*Rhizoctonia* metabolites were assessed through a multi-genome alignment approach using a multiple genome alignment and visualization package; Mauve (Darling et al., 2004). Gene clusters encoding for antifungal compounds used in this work as reference, are listed: *occidiofungin* gene cluster from *Burkholderia contaminans* MS14 (EU938698.5); *afc* (AFC-BC11) gene cluster from *B. cepacia* BC11 (AF076477.1); pyrrolnitrin

gene cluster *prnABCD* from *B. pyrrocinia* CH-67 (AF161186.1); the polyketide synthases (PKSs) genomic island (locus tag: Bamb_5918-5933), and the 4-hydroxy-2-alkylquinolines (HAQs) biosynthetic gene luster *hmqABCDEFG* (locus tag: Bamb_5763-5769) from *B. cepacia* AMMD (GCA_000203915.1).

For the analysis of antifungal biosynthetic gene clusters in *Pseudomonas putida* PD1: the following operons are listed: *hcnABC* gene cluster form *P. fluorescens* CHA0 (AF053760); the phenazine-1-carboxylic acid gene cluster *phzABCDEFG* from *P. fluorescens* 2-79 (L48616); the 2,4-diacetylphloroglucinol synthetic gene cluster *phlABCDEF* from *P. fluorescens* Q2-87 (U41818.1); pyrrolnitrin gene cluster *prnABCD* from *P. fluorescens* (U74493).

Assessing the plant growth promoting properties in *Burkholderia* spp. (WPB, WP40 and WP42) genomes

The draft genomes *Burkholderia* sp. WP40, *Burkholderia* sp. WP42, and *Burkholderia vietnamensis* WPB were sequenced and annotated at the Joint Genome Institute as a part of the sequencing project "Defining the functional diversity of the *Populus* root microbiome (Bioproject accession: PRJNA247585)". Genomes are publically available at: https://img.jgi.doe.gov/. The Integrated Microbial Genome platform (Markowitz et al., 2012) was used for data-mining the genome of *Burkholderia* sp. WP40, *Burkholderia* sp. WP42 and *Burkholderia vietnamensis* WPB for genes with beneficial effect for plant fitness.

Assessing the presence of virulence-associated loci in *Burkholderia* sp. WP40, *Burkholderia* sp. WP42, and *Burkholderia vietnamiensis* WPB

To compare the secretion systems between known pathogenic strains such as *Burkholderia mallei* ATCC 2334, *B. thailandensis* E263 and *B. pseudomallei* K96243, and plant endophytic *Burkholderia* spp., Type III Secretion System and Type VI Secretion Systems components were compared between these two groups. The presence of virulence associated loci was assessed through the Integrated Microbial Genomes platform. Type III secretion system gene cluster (BMAA1517 – 1557, NC_006349) and the capsule biosynthetic gene cluster (BMA2286 – 2310; NC_006348) from *Burkholderia mallei* ATCC 2334 were downloaded from Virulence Factor Database (Chen, 2004), aligned against WP40, WP42, and WPB setting a BLAST cutoff at minimum 10% identity and maximum E-value of 0.1. When positive hits were observed, a gene neighborhood analysis, in association with a multi-alignment genome analysis through the Integrated Microbial Genomes Expert Review (IMG-ER) platform (Markowitz et al., 2009), was performed in order to assess if such genes are organized in known virulence related gene cluster in phylogenetically related strains.

For Type VI Secretion System, the cluster 5 (T6SS-5; BTHII_0873 – 0854; (Schwarz et al., 2010)), and 1 (T6SS-1; BPSS1496 – 1511; (Burtnick et al., 2011)) of *B. thailandensis* E263 (NC_007651) and *B. pseudomallei* K96243 respectively, were used. A multi-alignment for amino acid sequence of T3SS and T6SS was performed through ClustalOmega web service (Sievers et al., 2011).

RESULTS

In-vitro screening for anti-fungal (*R. solani* AG-8) activities of poplar and willow endophytes

Among 55 poplar and 4 willow endophyte stains used in the *in-vitro* study of anti-fungal activities, 13 poplar and 1 willow strains showed observable antifungal activities (Table 1). *Burkholderia* species strains, WPB, WP40, WP41, and WP42, from wild poplar plants, showed the strongest antifungal activities (Fig 1). The 16S rRNA gene for each strain was sequenced and identified the best match by using BLAST on NCBI database from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

Table 1. Description of the Salicaceae endophytes having anti-fungal properties.

Endophyte strains	***R. solani* growth inhibition†**	**Best BLAST match**	**GenBank accession #**	**Reference**
WPB	RGI1	*Burkholderia vietnamiensis*	EU563933-EU563935	Xin et al., 2009
WP40	RGI1	*Burkholderia* sp.	KF597274	This study.
WP41	RGI1	*Burkholderia* sp.	KF597275	This study.
WP42	RGI1	*Burkholderia* sp.	KF597275	This study.
PD1	RGI 3	*Pseudomonas putida*	KF443801	Khan et al., 2014
WP 4-2-2	RGI 2	*Burkholderia* sp.	KU495920	This study.
WP 4-3-1	RGI 3	*Rhodotorula graminis*	KU500894	This study.
WP 4-3-2	RGI 3	*Burkholderia* sp.	KU500893	This study.
WP 4-3-3	RGI 2	*Curtobacterium* sp.	KU550576	This study.

WP 4-4-2	RGI 2	*Rahnella aquatilis*	KU500895	This study.
WP 4-5-3	RGI 2	*Rahnella aquatilis*	KU500892	This study.
WP 4-4-6	RGI 3	*Pseudomonas* sp.	KU500891	This study.
WP 4-10-4	RGI 2	*Curtobacterium* sp.	KU550577	This study.
WW7	RGI 3	*Curtobacterium* sp.	KU523564	Doty et al., 2009

†Estimate of inhibition; RGI 1: ≥ 70% growth arrest, RG 2: 40-70% growth arrest RGI 3: ≤ 40% growth arrest.

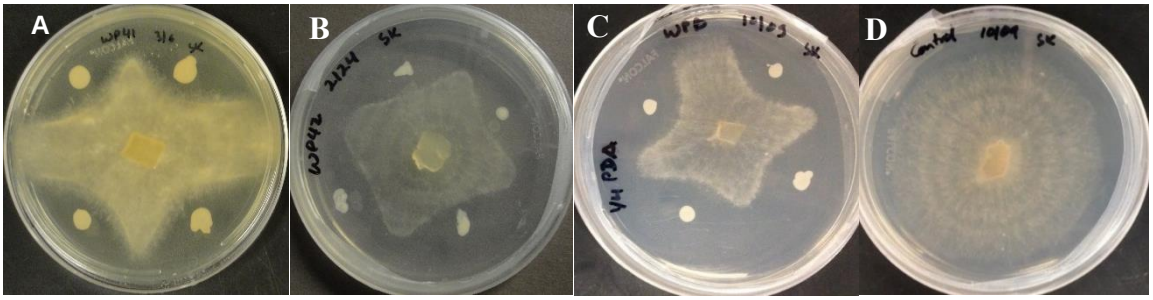


Fig 1. *In-vitro* inhibition of *R. solani* AG-8 (RGI 1) by poplar endophytes; *Burkholderia* species (plates; A to C) compared to the negative control, water alone (plate D).

Identification of endophytes

Poplar endophytes were identified through 16 rDNA sequencing. Endophyte strains WPB (Xin et al., 2009b), WW7 (Doty et al., 2009), and PD1 (Khan et al., 2014), were previously identified, but other strains including WP40, WP41, WP42, WP 4-2-2, WP 4-3-1, WP 4-3-2, WP 4-3-3, WP 4-4-2, WP 4-5-3, WP 4-4-6, WP 4-10-4 were sequenced and identified in this study. As shown in Table 1, the best 16S rDNA match of poplar endophytes through BLAST search (NCBI) were *Burkholderia, Rahnella, Curtobacterium,* and *Pseudomonas.*

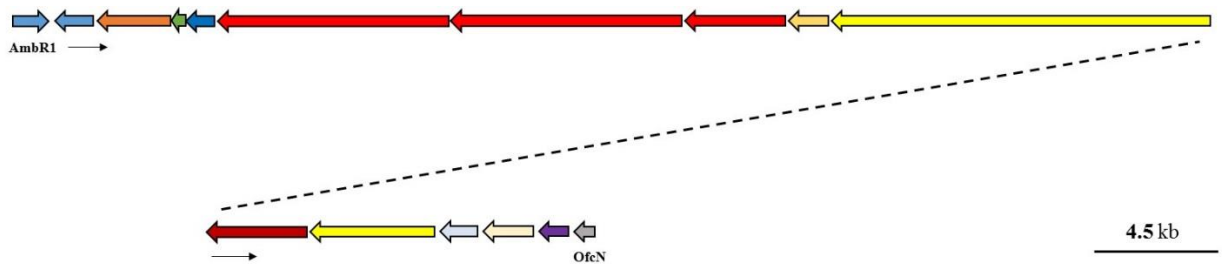
Comparative analysis of genomic regions related to the synthesis of anti-*Rhizoctonia* compounds in *Burkholderia* species (WP40, WP42, and WPB)

Gene clusters such as pyrrolnitrin (*prn*ABCD), HQSs (*hmq*ABCDEFG), AFC-BC11 and PKSs genomic island, involved in the synthesis of known antifungal compounds, are absent in WPB, WP40, and WP42 (Table 2).

Table 2. Analysis for antifungal gene clusters in WPB, WP40 and WP42

Biosynthetic gene cluster	*Burkholderia* strains			Reference
	WPB	WP40	WP42	
occidiofungin gene cluster	+	+	+	(Gu et al., 2009a)
afc gene cluster	-	-	-	(Kang et al., 1998)
prnABCD	-	-	-	(Hammer et al., 1999)
PKSs genomic island	-	-	-	(Mahenthiralingam et al., 2011)
hmqABCDEFG	-	-	-	(Vial et al., 2008)

However, a synteny for the 56-kb *ofc* gene cluster of *Burkholderia contaminans* MS14, a known antifungal strain, was detected (Fig 2). As reported in Gu et al., (Gu et al., 2009a), the *ofc* gene cluster consists of 15 open reading frames (*orf*) involved in the biosynthesis and secretion of *occidiofungin*, a cyclic glycopeptide, which has been reported to inhibit the growth of *R. solani* and other plant and human pathogens (Lu et al., 2009). The *ofc* region was annotated in antiSMASH as hybrid NRPS-Type-1 PKS gene cluster that contains three *orf* each of which encode for a non-ribosomal peptide synthetase (NRPS), and a coding sequence for a Type 1 PKS.



	Protein sequence % identity															
Strain	AmbR1	AmbR2	OfcA	OfcB	OfcC	OfcD	OfcE	OfcF	OfcG	OfcH	OfcI	OfcJ	OfcK	OfcL	OfcM	OfcN
WPB/40/42	82.95	69.62	86.07	69.81	89.91	85.52	85.57	84.07	91.64	86.98	86.72	84.25	82.62	89.93	94.3	80.3

WPB/WP40/WP42 ofc gene cluster:

- AmbR1/2: Transcriptional regulator, LuxR family
- OfcA: Cyclic peptide transporter
- OfcB: Hypothetical protein
- OfcC: Glycosyltransferase
- OfcD/E/F: NRPS
- OfcG: Predicted Zn-dependent hydrolases
- OfcH: putative PKS
- OfcI: Monooxygenase
- OfcJ: putative PKS
- OfcK: Taurine dioxgenase
- OfcL: Aminotransferase
- OfcM: UDP-glucuronate decarboxylase
- OfcN: Thioesterase

Fig 2. Graphical representation of *ofc* gene cluster in *Burkholderia* species (WPB, WP40, and WP42).

Finally, unlike *Pseudomonas* strains belonging to the species *chlororaphis*, *fluorescens*, and other unidentified *Pseudomonas* spp., PD1 lacks genes involved in the biosynthesis of known antimicrobials and antifungal such as hydrogen cyanide (*hcnABC*), phenazine-1-carboxylic acid (*phzABCDEFG*), 2,4-diacetylphloroglucinol (*phlACBD*), and pyrrolnitrin (*prnABCD*). However, genome annotation of PD1 revealed the presence of chitinase and secreted protein annotated as chitin deacetylase which may play a role to inhibit the fungal growth.

IAA production

The amount of IAA produced by different endophytes was relatively higher once supplemented with L-tryptophan, an active compound in plant exudate (Kamilova et al., 2006; Hardoim et al., 2008). However, the produced amount of IAA was variable with different strains (Fig 3).

Endophyte strain WP 4-4-6 produced the highest amount; 46.18 µg ml^{-1}, and strain WP 4-10-4 produced the smallest amount; 1.51 µg ml^{-1} of endophyte culture. Negligible amount of IAA was observed for all endophyte strains without addition of L-tryptophan.

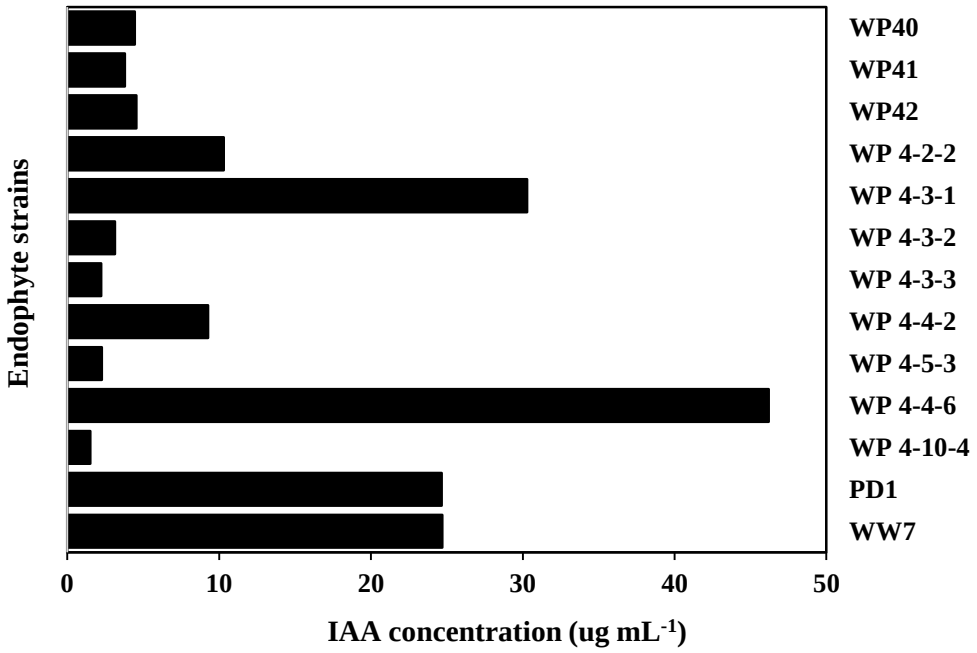


Fig 3. IAA amount produced by different poplar and willow endophytes.

Phosphate solubilization

Phosphate solubilization was monitored for only endophyte strains that were found positive for antifungal activities. Tricalcium phosphate/NBRIP plates were used for the phosphate solubilization assay. The halo area surrounded by endophyte colonies was used as an indicator of phosphate solubilization. Several strains solubilized tricalcium phosphate more effectively than others (Fig 4). The best performing strains were WPB, WP40, WP41, WP42, WP 4-4-2, WP 4-5-3, and developed a very distinct halo (Table 3). Some strains; WP 4-2-2, WP 4-3-2, WP 4-4-6,

PD1, and WW7 showed a relatively smaller halo area. No solubilization was observed by strains WP 4-3-1, WP 4-3-3, and WP 4-10-4.

Table 3. Observation of phosphate solubilization activity in poplar and willow endophytes.

Endophyte strains	**Phosphate solubilization activity†**	**Endophyte strains**	**Phosphate solubilization activity†**
WPB	++	WP 4-3-3	-
WP40	++	WP 4-4-2	++
WP41	++	WP 4-5-3	++
WP42	++	WP 4-4-6	+
WP 4-2-2	+	WP 4-10-4	-
WP 4-3-1	-	PD1	+
WP 4-3-2	+	WW7	+

†Estimate of solubilization; ++: High solubilization, +: Moderate solubilization, and - : No solubilization.

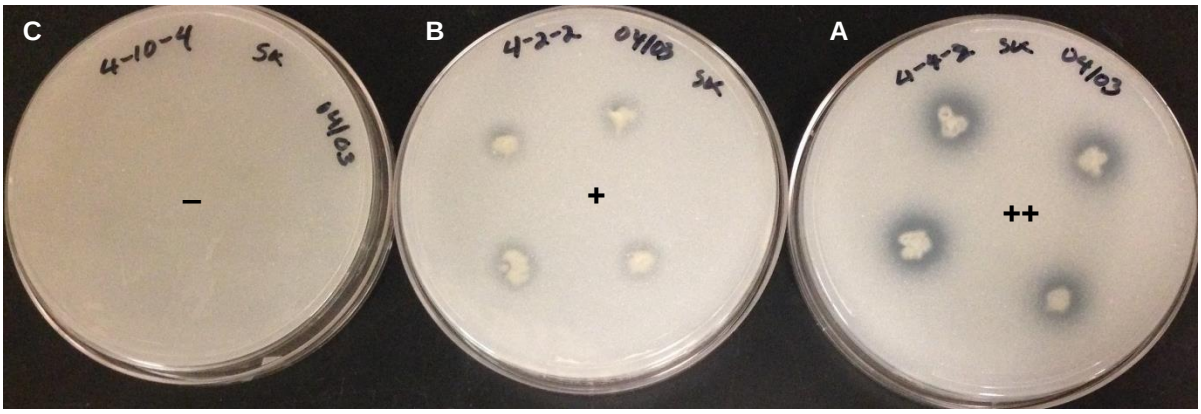


Fig 4. Tricalcium phosphate (NBRIP) plates showing the phosphate solubilization gradient; highly solubilization (Plate A to B) to no solubilization (Plate C).

Siderophore production

CAS agar plates were used to observe the siderophore activity of poplar and willow endophytes. The orange halo area surrounded by endophyte colonies was measured to assess the siderophore production *in-vitro* (Fig 5). Poplar endophytes, WPB, WP40, WP41, WP42, WP 4-2-2, WP 4-3-2, WP 4-4-2 and WP 4-5-3, and willow endophyte WW7 showed siderophore activity creating the orange halo area contiguous with colony growth (Fig 6). No siderophore activity was observed by strains, WP 4-3-1, WP 4-4-6, WP 4-10-4, and PD1. The largest orange halo area; 7.539 cm^2 was observed in willow endophyte, WW7 (Fig 5).

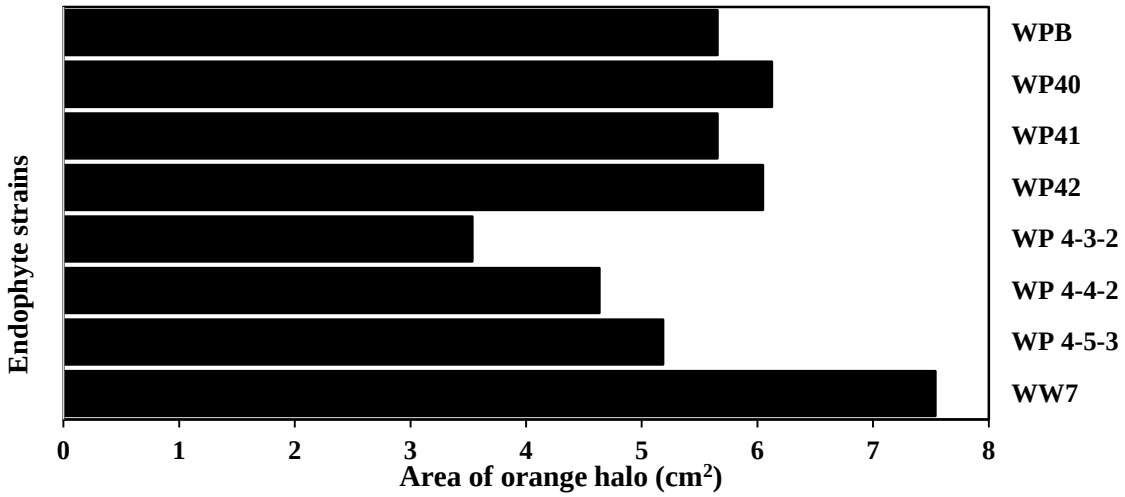


Fig 5. Area of orange halo (cm^2) displayed by different poplar and willow endophytes in CAS agar plates.

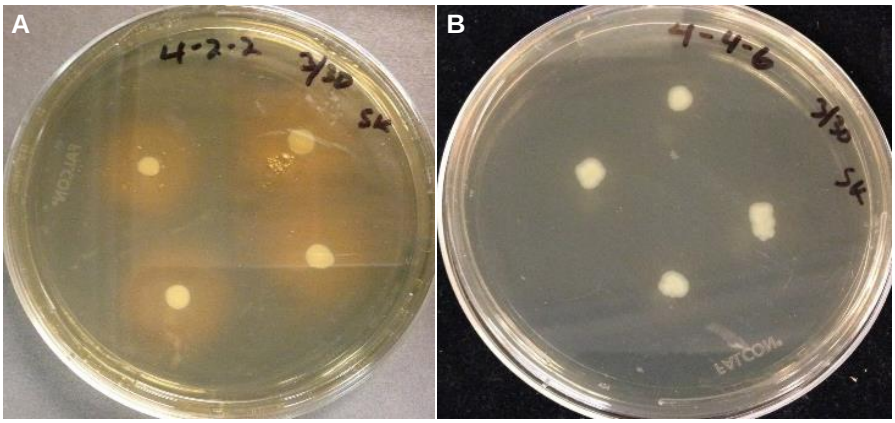


Fig 6. An orange halo contiguous with endophyte colony (Endophyte WP 4-2-2) indicated the presence of siderophore activity (Plate A), and no halo immediate to the endophyte colony (Endophyte WP 4-4-6) indicated the absence of siderophore activity (Plate B).

Acetylene reduction assay

In ARA, the activity of nitrogenase enzyme leads to reduce acetylene gas into ethylene which is monitored through gas chromatography. The reduced amount of ethylene provides the estimate of N-fixed by diazotrophs. More than 300 ppm concentration of ethylene was produced by strains WP9, WP 4-3-2, WP 4-10-4, WP 4-4-6, and positive control *Azotobacter* sp. (Fig 7). Relatively smaller amount of ethylene was produced by endophyte strains WP40, WP41, WP42, WP 4-4-2, and WW7.

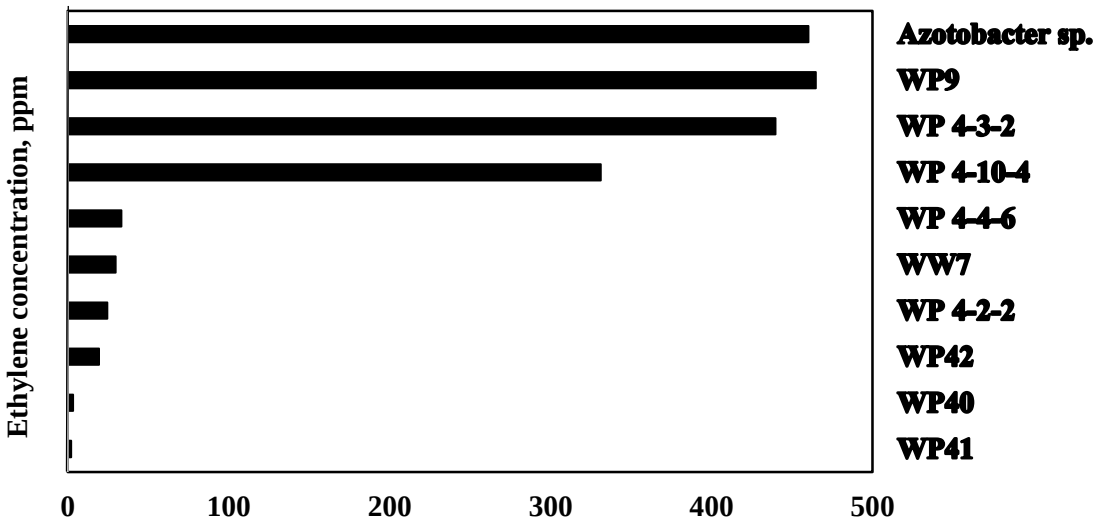


Fig 7. Acetylene reduction assay. Ethylene produced by endophyte strains after 24 hours of exposure to acetylene.

Analysis of the nitrogenase subunit gene, nifH

Endophyte strains WP40, WP41, WP42, WP 4-4-2, and WP 4-5-3 were found positive to universal *nifH*, and *nifH* primers. Strains WP 4-4-2, WP 4-5-3 were positive to *nifH-b1*. The presence of gene cluster *nifHDK* in strain WPB, and its N fixing potentiality through ARA was studied in the past (Xin et al., 2009b).

Plant growth promoting features in poplar *Burkholderia* (WP40, WP42, and WPB) genomes

Plant associated (endophytic or rhizospheric) *Burkholderia* species have promising plant growth promoting properties (BNF, phosphate solubilization, siderophore production, degradation of aromatic compounds, and phytohormone production). From a genome analysis of poplar endophytic *Burkholderia*; WPB, WP40, and WP42, it is revealed that they carry putative genes

that are responsible for the above mentioned characteristics related to plant growth promotion (Table 4). As observed for other *Burkholderia* strains, the *nifHDK* operon was detected in WPB, WP40, and WP42 along with 1-aminocyclopropane-1-carboxylate (ACC) deaminase coding sequence. In addition, all of these *Burkholderia* strains (WPB, WP40, and WP42) have pyrroloquinoline quinone (pqq) operon with multiple genes (*pqqBCDE*) that are essential to solubilize rock phosphates in soil. An interesting feature of WPB, WP40, and WP42 is the presence of a non-canonical ornibactin (orb) gene cluster which encodes for biosynthesis of siderophore compounds. Compared to other *orb* gene clusters, this non-canonical cluster is present in all core genes but lacks N-acetyltransferase coding sequence orbK, which is not essential in the synthesis of ornibactin (Franke et al., 2014). Interestingly, this gene arrangement has never been observed and seems to be unique in the *B. vietnamiensis* species (Fig 8).

Table 4. Genome location of plant growth promoting genes found in WPB, WP40, and WP42.

Gene	Phosphate solubilization	N fixation	Phytohormone production/modulation	
Burkholderia	([1]*pqqBCDE*)	([2]*nifHDK*)	([3]Tryptophan-2-monooxygenase)	([4]ACC deaminase)
WP40	+	+	+	+
WP42	+	+	+	+
WPB	+	+	+	+

[1] pyrroloquinoline quinone biosynthetic gene cluster (*pqqBCDE*): WPB (Ga0008009_1289 – 12812), WP40 (Ga0008008_12747-12744), and WP42 (EX20DRAFT_05687-05684); [2] *nifHDK*: WPB (Ga0008009_108258-108260), WP40 (Ga0008008_108160-108162), and WP42 (EX20DRAFT_01912-01910); [3] Tryptophan-2-monooxygenase: WPB (Ga0008009_10147), WP40 (Ga0008008_10147), and WP42 (EX20DRAFT_00380); [4] 1-aminocyclopropane-1-carboxylate deaminase: WPB (Ga0008009_11518), WP40 (Ga0008008_ 11518), and WP42 (EX20DRAFT_03759).

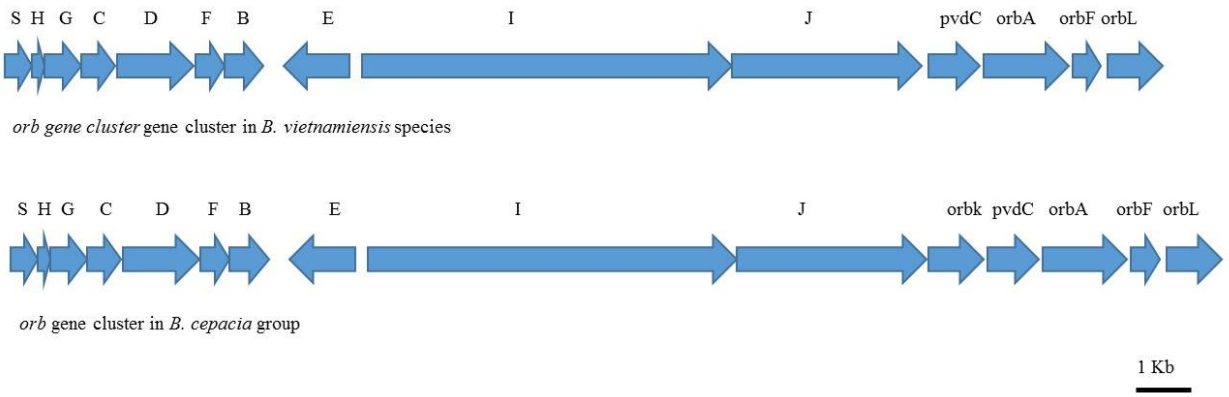


Fig 8. Ornibactin (orb) gene cluster organization in *Burkholderia vietnamensis,* and *B. cepacia* groups.

Assessing the presence of virulence-associated loci in *Burkholderia* sp. WP40, *Burkholderia* sp. WP42, and *Burkholderia vietnamiensis* WPB Capsule polysaccharide biosynthetic genes

A positive hit for the polyketide synthase WcbR of *B. mallei* ATCC2334 genes was detected in WPB (locus_tag: Ga0008009_116100), WP40 (locus tag: Ga0008008_116100), and WP42 (locus_tag: EX20DRAFT_04097) showing a 29.7% sequence similarity. However, no gene cluster related to the synthesis of a polysaccharide capsule was detected in WP40, WP42, and WPB.

Type III secretion system (T3SS) and Type VI secretion system (T6SS)

The T3SS is considered as an important criteria for pathogenic bacteria. However, the presence of gene clusters encoding for T3SS components was also confirmed for the plant endosymbiont *Burkholderia phytofirmans* PsJN and other non-pathogenic *Burkholderia* strains where the gene encoding for needle forming protein seems to be missing (Viallard et al., 1998; Mitter et al., 2013; Zuleta et al., 2014). WPB, WP40, and WP42 carry only one T3SS cluster, which share an

amino acidic sequence similarity of over 90% with the endosymbiont *Burkholderia* sp. *KJ006* and the environmental strains *B. vietnamiensis* G4 and *B. ambifaria* AMMD (File S1). As in G4 strain, AMMD, and PsJN strains, the gene responsible for the needle protein is missing. Similarly, pathogens, non-pathogens, and opportunistic pathogens may have up to six T6SS clusters where each cluster exerts different roles in cell-to-cell interactions and pathogenesis. The best known T6SS gene clusters are the T6SS-5, of *B. thailandensis E264,* and the *B. pseudomallei* K96243 T6SS-1, and were extensively characterized for their critical role in virulence (Schwarz et al., 2010; Burtnick et al., 2011).

Through a multi-alignment genomes analysis, three T6SS clusters are present in WP40, WP42, and WPB, but none of them is in synteny with the T6SS-5 and T6SS-1 of E264 *and* K96243 respectively. A syntenic region was only observed for the non-virulence gene clusters T6SS-1 and T6SS-6 of *B. thailandensis* E264 and *B. pseudomallei* K96243 respectively (Schwarz et al., 2010; Burtnick et al., 2011), which show a protein sequence identity over 73% (File S2).

DISCUSSION

In this study, we characterized antifungal and plant growth promoting characteristics of poplar and willow endophytes. We used *in-vitro* microbiological techniques to observe the phenotypic functionality of these endophytes related to plant growth promotion and suppression of a fungal plant pathogen, and genome analyses to understand the underlying mechanism for these functions. The main objective of this study was to investigate whether any of the poplar or willow endophytes can suppress the growth of broad host range plant pathogenic fungus *R. solani* AG-8. The endophytes used in this study were isolated from poplar or willow plants

colonizing the cobble-dominated riparian environment, a marginal site for plant growth. Previous studies suggested that endophytes have the potential to support the growth of these early successional plants in that adverse environment (Doty et al., 2005, 2009; Knoth et al., 2014). With the large number of available strains from this environment, it is important to focus the research on those with the greatest potential of providing plant health benefits. No previous studies tested for antifungal activities of these endophytes. Among the 55 poplar and 4 willow endophyte strains used in this study, ten poplar and one willow endophytes inhibited *Rhizoctonia* growth in the *in-vitro* condition. These were then further tested for additional growth promoting activities such as phytohormone production, phosphate solubilization, and siderophore production.

From the antifungal assay, the strains of *Burkholderia*, *Rahnella*, *Pseudomonas*, and *Curtobacterium* genera were found to be capable of inhibiting the *Rhizoctonia* growth in the *in-vitro* condition. The inhibition was very distinct and strongest in the case of the *Burkholderia* spp. as compared to other endophyte strains. Previously, *Burkholderia* strains were studied to evaluate their antifungal activities for several plant pathogens including *R. solani* and various mechanisms were hypothesized for the fungal growth suppression (Chiarini et al., 2006; Compant et al., 2008; Gu et al., 2009a; Karki et al., 2012). It was demonstrated that *Burkholderia* produced a glycolipopeptide named occidiofungin which has an inhibitory effect on various plant and human fungal pathogens (Gu et al., 2009a; b). From our genome analysis of poplar endophytes, *Burkholderia* spp. (WP40, WP42, and WPB), an NRPS-Type_1PKS gene cluster responsible for biosynthesis of an occidiofungin-like cyclic peptide was discovered. Among various genes embedded within that 56-kb *ofc* gene cluster, ambR1 was reported as a key

regulatory gene controlling the biosynthesis of occidiofungin (Gu et al., 2009b), which is also conserved in the genomes of poplar *Burkholderia*. Karki et al. (Karki et al., 2012) showed that *Burkholderia* spp. from rice plants released toxoflavin, a phytotoxic antimicrobial compound, responsible for suppression of *Rhizoctonia* growth. In contrary, poplar *Burkholderia* did not show any toxoflavin compounds *in-vitro* (data not shown) indicating no potential risk of virulence in plants. We are currently testing the antifungal activities of these endophytes to other fungal pathogens as well as *Rhizoctonia* damage suppression and growth promotion in wheat seedlings in the green house.

The genomes of several poplar endophytic *Burkholderia* were recently sequenced, allowing molecular approaches to investigate the possible mechanisms that are responsible for *Rhizoctonia* growth suppression. Understanding the molecular mechanisms of microbe- mediated antifungal activities will not only expand the resources for biological control of plant diseases but also provide important information that can be useful to formulate biofungicides excluding any potential health risks. Putative genes responsible for fungal growth suppression in poplar *Burkholderia* were similar to another *Burkholderia* strain that showed the antifungal activities (Gu et al., 2009a). Nevertheless, the presence of any antifungal gene in the genome does not imply that particular endophyte is capable of secreting any antimicrobial compounds. A functional analysis of these genes would be required to confirm the possible role on fungal growth suppression. Although antifungal properties of fungal endophytes of poplar have been investigated (Busby et al., 2016), to our knowledge, our study is the first to analyze the antifungal properties of bacteria recovered from the poplar and willow endosphere. The endophytic lifestyle of *Burkholderia* offers more robust and long-lasting disease control

strategies for many plant pathogens like *Rhizoctonia* since they colonize the entire plant body and stably persist. In addition, they use internal plant tissues for nutrition and multiplication which excludes them from competition with other microbes present in the phyllosphere or rhizosphere.

Historically bacterial strains belonging to the genus *Burkholderia* were reported as mammalian opportunistic pathogens or pathogens (Yabuuchi et al., 1992; Coenye, Tom, Vandamme, Peter, Govan, John R. W., and Lipuma, 2001). Though from the last two decades, many plant associated *Burkholderia* communities were discovered as diazoptrophs or plant growth promoting agents (Estrada-De Los Santos et al., 2001; Perin et al., 2006; Doty et al., 2009; Bernabeu et al., 2015; Walker et al., 2015). For the safe use of *Burkholderia* strains to inoculate crop plants for growth promotion, distinctions should be made between potentially pathogenic strains and the majority of environmental isolates. Using a genome analysis of poplar endophytes (WP40, WP42, and WPB), we tested for the distinction in secretion systems and virulence factors between pathogenic and plant growth promoting *Burkholderia* strains. Various extracellular and cell wall surface structures including capsular polysaccharides involved during the infection caused by pathogen, *B. mallei* were present but no gene cluster that can encode for polysaccharide capsule was detected in poplar *Burkholderia* (WP40, WP42, and WPB). Exopolysaccharide production is common among most plant-associated bacteria and is involved in plant colonization (Danhorn and Fuqua, 2007; Meneses et al., 2011; Serrato et al., 2013). Type III secretion system (T3SS) cluster 3 is required for delivering virulence factors into host cells in pathogenic *Burkholderia,* however only T3SS cluster 1 is present in WP40, WP42, and WPB genomes. In addition, genes that encode for needle-forming proteins to conduit virulence factors

during infection were absent in WP40, WP42, and WPB. More importantly, no shared synteny with the Type VI secretion system gene clusters specifically involved in pathogenesis was observed in WP40, WP42, and WPB. Similar distinctions on secretion systems and virulence factors were observed in different pathogenic and plant associated symbiotic *Burkholderia* or *Pseudomonas* strains in the previous studies (Angus et al., 2014; Mazurier et al., 2015). In addition, Castro-Gonzalez et al. (Castro-González et al., 2011) observed that genes encoding for the transmissibility factors related to bacterial pathogenicity in clinical isolates of *Burkholderia* were missing in plant associated diazotrophic *Burkholderia* isolates. These transmissibility factors, *cbl*A encoding for giant cable pili, and the epidemic marker strain regulator (*esm*R), from *B. cenocepacia* J2315 and *B. cepacia* respectively, were missing in WP40, WP42, and WPB. Furthermore, it has been observed that pathogenic strains often lack the inhibitory effect against bacterial and fungal plant pathogens (Chauhan et al., 2015).

Poplar and willow endophytes including *Burkholderia* are reported to be important for plant growth enhancement in resource poor environments. Cross inoculation of poplar and willow endophytes in other plant species (rice, maize, tomato, conifer seedlings) showed the substantial improvement in growth in green house conditions (Khan et al., 2012; Knoth et al., 2012; Kandel et al., 2015; Khan et al., 2015). It is considered that endophytes promote plant growth through many ways including nutrient acquisition, phytohormone production or protection from abiotic and biotic stresses (Richardson et al., 2009; Gamalero and Glick, 2011). Evidence of microbial synthesis of phytohormones like IAA has been observed in the case of many plant growth promoting microbes including endophytes (Shi et al., 2009; Xin et al., 2009b; Spaepen, 2015). IAA synthesis through different endophytes in host plants can stimulate root growth as well as

influence other developmental processes such as apical dominance, tropic responses, flowering, fruiting etc. An enhanced root growth may provide additional area for endophytes to colonize, possibly increasing the metabolic functions of both plants and microbial communities. L-tryptophan is considered as an important precursor for IAA biosynthesis in microbes (Spaepen and Vanderleyden, 2011; Spaepen, 2015). All endophyte strains positive for antifungal activities were also found positive for IAA biosynthesis when supplemented with L-tryptophan. However, the amount of IAA produced varied with different strains (Fig 3). In contrast, they barely produced any amount of IAA in the absence of L-tryptophan. Since IAA production by Salicaceae endophytes depends on the presence of L-tryptophan in the culture medium, it is speculated that endophytes rely on plant L-tryptophan for IAA biosynthesis. L-tryptophan is commonly available in the plant exudates which may enhance the mutual interactions of plant and endophytes by sharing the resources of reciprocal interests (Kravchenko et al., 2004; Kamilova et al., 2006). Similar findings for IAA bioassay were documented on several endophytes isolated from a variety of plant species (Shi et al., 2009; Xin et al., 2009a; Videira et al., 2012). Furthermore, some strains reduced acetylene into ethylene in ARA, to the quantification of N-fixation process. ARA is useful to monitor the N-fixing activities of microorganisms (Hardy et al., 1973).

The majority of the endophyte strains having antifungal activities were able to create a halo area in NBRIP medium containing insoluble phosphorus (Table 5). The formation of a halo area encircling the endophyte colony indicates the solubilization of insoluble phosphate present in the medium. Many past studies reported that endophytic or growth promoting soil bacteria solubilize inaccessible soil phosphorus into bioavailable forms; potential phosphorus resources for plants to

uptake for their growth and development (Gamalero and Glick, 2011; Oteino et al., 2015). Bacteria use various mechanisms to solubilize phosphate including acidification of soil by releasing gluconic and citric acid or hydrogen ion (H^+), and binding free P in the medium by the action of exopolysaccharides. The details of individual mechanisms has been reviewed elsewhere (Richardson et al., 2009; Gamalero and Glick, 2011). It is suggested that inoculation of phosphate solubilizing bacteria can help host plants to adapt to nutrient limited environments (Dias et al., 2009; Manoel et al., 2015). By understanding the specific mechanism of poplar and willow endophytes responsible for phosphorus solubilization, these endophytes can be utilized for plant growth promotion in addition to biocontrol of plant diseases. Furthermore, many endophyte strains capable of *Rhizoctonia* growth suppression, IAA production, and phosphate solubilization also produced siderophores. Siderophores are iron chelating organic compounds produced by microbes or plants to accrue iron from environments. Plant growth promotion and relief of iron deficiency symptoms have been demonstrated through microbial siderophores in different crop plants (Ahmed and Holmström, 2014; Saha et al., 2015).

Endophyte strains WP40, WP41, WP42, WP 4-4-2, and WP 4-5-3 reduced acetylene into ethylene in ARA, indicating N-fixation, and were found positive for the nifH gene by PCR. However, strains WP 4-3-2, WP 4-10-4, and WP 4-4-6 were ARA positive but none of these had a nifH PCR product. However, although the primers are considered as “universal”, it is often the case that this primer set does not amplify nifH of diazotrophic strains (Bürgmann et al., 2004). Previous studies also reported the discrepancy in relationship between the nifH profiles and ARA results (Deslippe et al., 2005; Patra et al., 2006). Future studies for these strains would be to try other primers, or get the genomes sequenced. Furthermore, strains 4-4-2 and 4-5-3 were nifH

positive but ARA negative. The culture conditions for ARA may not be favorable for all strains to show N fixing activity. It has been advised that bacterial nitrogenase activities would differ with the culture medium, growth conditions, and growth stages of bacteria (Lin et al., 2012). More microaerobic culture conditions would be appropriate or maybe it is only active *in planta*.

The results from genome analyses of WPB, WP40, and WP 42 corroborate the results from ARA and nifH analysis, phosphate solubilization, IAA production, and siderophore production. Previous studies observed in-vitro biological N fixing activity in WPB through an acetylene reduction assay (Xin et al., 2009b). Since we observed the nifHDK gene cluster, encoding for the nitrogenase enzyme in WP40 and WP42 in addition to WPB, we can postulate that these endophytes can fix atmospheric N which is potentially available to the host plants to assimilate. In addition, *pqq* operon with different genes (*pqqBCDE*) encode for phosphate solubilization, and tryptophan-2-monooxygenase and ACC deaminase for phytohormone roduction/modulation were observed in WP40, WP42, and WPB genomes. It has been shown that the expression of *pqqABCDE* genes stimulates the gluconic acid production which solubilizes the insoluble phosphates (Richardson et al., 2009; Oteino et al., 2015). In addition, it is claimed that ACC deaminase producing bacteria help plants to mitigate the biotic and abiotic stresses by lowering the ethylene level in plants that would otherwise interfere with the plant physiological processes and eventually damage the entire plant body (Glick, 2014).

An efficient and sustainable way of crop production is crucial today to feed the growing world population with minimum impact to the environment. The utilization of microbial symbionts in crop cultivation offers the environmentally-friendly and sustainable way of farming.

Endophytes promote plant growth, possibly through the mechanisms of IAA production, phosphate solubilization, and siderophore production but may also protect the host plants from pathogen-induced plant diseases. Recent advances in molecular biology and functional genomics will help to expand the existing understanding of plant-endophyte interactions. Integration of multiple microbial strains as a single consortium can offer different benefits to the crop plants. Further investigations about outcomes of plant-endophyte interactions in different ecological settings and under field conditions would be beneficial to utilize the plant growth promoting potential of endophytes.

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REFERENCES

Ahmed, E., and S.J.M. Holmström. 2014. Siderophores in environmental research: roles and applications. Microb. Biotechnol. 7(3): 196–208.

Angus, A.A., C.M. Agapakis, S. Fong, S. Yerrapragada, P. Estrada-de los Santos, P. Yang, N. Song, S. Kano, J. Caballero-Mellado, S.M. de Faria, F.D. Dakora, G. Weinstock, and A.M. Hirsch. 2014. Plant-associated symbiotic *Burkholderia* species lack hallmark strategies required in mammalian pathogenesis. PLoS One 9(1): e83779.

Bernabeu, P.R., M. Pistorio, G. Torres-tejerizo, P.E.L. Santos, M.L. Galar, J.L. Boiardi, and

M.F. Luna. 2015. Colonization and plant growth-promotion of tomato by *Burkholderia tropica*. Sci. Hortic. (Amsterdam). 191: 113–120.

Bhattacharjee, R.B., A. Singh, and S.N. Mukhopadhyay. 2008. Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. Appl. Microbiol. Biotechnol. 80(2): 199–209.

Botta, A.L., A. Santacecilia, C. Ercole, P. Cacchio, and M. Del Gallo. 2013. *In vitro* and *in vivo* inoculation of four endophytic bacteria on *Lycopersicon esculentum*. N. Biotechnol. 30(6): 666–74.

Bürgmann, H., F. Widmer, W. Von Sigler, and H. Bu. 2004. New molecular screening tools for analysis of free-living diazotrophs in soil. Appl. Environ. Microbiol. 70(1): 240–247.

Burtnick, M.N., P.J. Brett, S. V. Harding, S.A. Ngugi, W.J. Ribot, N. Chantratita, A. Scorpio, T.S. Milne, R.E. Dean, D.L. Fritz, S.J. Peacock, J.L. Prior, T.P. Atkins, and D. DeShazer. 2011. The cluster 1 type VI secretion system is a major virulence determinant in *Burkholderia pseudomallei*. Infect. Immun. 79(4): 1512–1525.

Busby, P.E., M. Ridout, and G. Newcombe. 2016. Fungal endophytes: modifiers of plant disease. Plant Mol. Biol. 90: 645–655.

Cangelosi, G.A., E.A. Best, G. Martinettii, and N.E. W. 1991. Genetic analysis of *Agrobacterium*. Methods Enzymol. 204: 384–397.

Castro-González, R., L. Martínez-Aguilar, A. Ramírez-Trujillo, P. Estrada-de los Santos, and J. Caballero-Mellado. 2011. High diversity of culturable *Burkholderia* species associated with sugarcane. Plant Soil 345(1): 155–169.

Chakraborty, S., and a. C. Newton. 2011. Climate change, plant diseases and food security: An overview. Plant Pathol. 60(1): 2–14.

Chauhan, H., D.J. Bagyaraj, G. Selvakumar, and S.P. Sundaram. 2015. Novel plant growth promoting rhizobacteria- Prospects and potential. Appl. Soil Ecol. 95: 38–53.

Chen, L. 2004. VFDB: a reference database for bacterial virulence factors. Nucleic Acids Res. 33(Database issue): D325–D328.

Chen, C., E.M. Bauske, G. Musson, R. Rodriguezkabana, and J.W. Kloepper. 1995. Biological control of fusarium wilt on cotton by use of endophytic bacteria. Biol. Control 5(1): 83–91.

Chiarini, L., A. Bevivino, C. Dalmastri, S. Tabacchioni, and P. Visca. 2006. *Burkholderia cepacia* complex species: health hazards and biotechnological potential. Trends Microbiol. 14(6): 277–86.

Coenye, Tom, Vandamme, Peter, Govan, John R. W., and Lipuma, J.J. 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. J. Clin. Microbiol. 39(10): 3427–3436.

Compant, S., C. Clément, and A. Sessitsch. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42(5): 669–678.

Compant, S., J. Nowak, T. Coenye, C. Clément, and E. Ait Barka. 2008. Diversity and occurrence of *Burkholderia* spp. in the natural environment. FEMS Microbiol. Rev. 32(4): 607–626.

Danhorn, T., and C. Fuqua. 2007. Biofilm formation by plant-associated bacteria. Annu. Rev. Microbiol. 61: 401–22.

Darling, A.C.E., B. Mau, F.R. Blattner, and N.T. Perna. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. 14(7): 1394–403.

Deslippe, J.R., K.N. Egger, and G.H.R. Henry. 2005. Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian high arctic. FEMS Microbiol. Ecol.

53(1): 41–50.

Dias, A.C.F., F.E.C. Costa, F.D. Andreote, P.T. Lacava, M. a. Teixeira, L.C. Assumpção, W.L. Araújo, J.L. Azevedo, and I.S. Melo. 2009. Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World J. Microbiol. Biotechnol. 25(2): 189–195.

Doty, S.L., M.R. Dosher, G.L. Singleton, A.L. Moore, B. Van Aken, R.F. Stettler, S.E. Strand, and M.P. Gordon. 2005. Identification of an endophytic *Rhizobium* in stems of *Populus*. Symbiosis (2005): 27–35.

Doty, S.L., B. Oakley, G. Xin, J.W. Kang, G. Singleton, Z. Khan, A. Vajzovic, and J.T. Staley. 2009. Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47: 23–33.

Doty, S.L., A.W. Sher, N.D. Fleck, M. Khorasani, R.E. Bumgarner, Z. Khan, A.W.K. Ko, S.-H. Kim, and T.H. DeLuca. 2016. Variable Nitrogen Fixation in Wild *Populus*. PLoS One 11(5): e0155979.

Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, and H. Mitsui. 2001. Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. 67(11): 5285–5293.

Estrada-De Los Santos, P., R. Bustillos-Cristales, and J. Caballero-Mellado. 2001. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl. Environ. Microbiol. 67(6): 2790–2798.

Estrada-de los Santos, P., P. Vinuesa, L. Martínez-Aguilar, A.M. Hirsch, and J. Caballero-Mellado. 2013. Phylogenetic analysis of *Burkholderia* species by multilocus sequence analysis. Curr. Microbiol. 67(1): 51–60.

Feng, Y., D. Shen, and W. Song. 2006. Rice endophyte *Pantoea agglomerans* YS19 promotes host plant growth and affects allocations of host photosynthates. J. Appl. Microbiol. 100(5): 938–45.

Franke, J., K. Ishida, and C. Hertweck. 2014. Evolution of siderophore pathways in human pathogenic bacteria. J. Am. Chem. Soc. 136: 5599–5602.

Gamalero, E., and B.R. Glick. 2011. Mechanisms used by plant growth-promoting bacteria. p. 17–47. *In* Bacteria in Agrobiology: Plant Nutrient Management.

Glick, B.R. 2014. Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol. Res. 169(1): 30–39.

Gordon, S.A., and R.P. Weber. 1951. Colorimetric estimation of inodoleacetic acid. Plant Physiol. 26(1): 192–195.

Govindarajan, M., J. Balandreau, S.-W. Kwon, H.-Y. Weon, and C. Lakshminarasimhan. 2008. Effects of the inoculation of *Burkholderia vietnamensis* and related endophytic diazotrophic bacteria on grain yield of rice. Microb. Ecol. 55(1): 21–37.

Gu, G., L. Smith, N. Wang, H. Wang, and S.E. Lu. 2009a. Biosynthesis of an antifungal oligopeptide in *Burkholderia contaminans* strain MS14. Biochem. Biophys. Res. Commun. 380(2): 328–332.

Gu, G., N. Wang, N. Chaney, L. Smith, and S.-E. Lu. 2009b. AmbR1 is a key transcriptional regulator for production of antifungal activity of *Burkholderia contaminans* strain MS14. FEMS Microbiol. Lett. 297(1): 54–60.

Hammer, P.E., W. Burd, D.S. Hill, J.M. Ligon, and K.H. Van Pée. 1999. Conservation of the pyrrolnitrin biosynthetic gene cluster among six pyrrolnitrin-producing strains. FEMS Microbiol. Lett. 180(1): 39–44.

Hane, J.K., J.P. Anderson, A.H. Williams, J. Sperschneider, and K.B. Singh. 2014. Genome sequencing and comparative genomics of the broad host-range pathogen *Rhizoctonia solani* AG8. PLoS Genet. 10(5): 11–12.

Hardoim, P.R., L.S. van Overbeek, G. Berg, A.M. Pirttilä, S. Compant, A. Campisano, M. Döring, and A. Sessitsch. 2015. The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. 79(3): 293–320.

Hardoim, P.R., L.S. van Overbeek, and J.D. Van Elsas. 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. 16(10): 463–71.

Hardy, R.W.F., R.C. Burns, and R.D. Holsten. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. 5(1): 47–81.

Hurek, T., L.L. Handley, B. Reinhold-Hurek, and Y. Piché. 2002. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol. Plant. Microbe. Interact. 15(3): 233–42.

Iniguez, A.L., Y. Dong, and E.W. Triplett. 2004. Nitrogen fixation in wheat provided by *Klebsiella pneumoniae* 342. Mol. Plant. Microbe. Interact. 17(10): 1078–85.

Kamilova, F., L. V Kravchenko, A.I. Shaposhnikov, T. Azarova, N. Makarova, and B. Lugtenberg. 2006. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol. Plant. Microbe. Interact. 19(3): 250–256.

Kandel, S.L., N. Herschberger, S.H. Kim, and S.L. Doty. 2015. Diazotrophic endophytes of poplar and willow for growth promotion of rice plants in nitrogen-limited conditions. Crop Sci. 55(4): 1765–1772.

Kang, Y., R. Carlson, W. Tharpe, and M. a Schell. 1998. Characterization of genes involved in biosynthesis of a novel antibiotic from *Burkholderia cepacia* BC11 and their role in biological control of *Rhizoctonia solani*. Appl. Environ. Microbiol. 64(10): 3939–47.

Karki, H.S., B.K. Shrestha, J.W. Han, D.E. Groth, I.K. Barphagha, M.C. Rush, R. a. Melanson, B.S. Kim, and J.H. Ham. 2012. Diversities in virulence, antifungal activity, pigmentation and DNA fingerprint among strains of *Burkholderia glumae*. PLoS One 7(9): e45376.

Khan, Z., G. Guelich, H. Phan, R. Redman, and S. Doty. 2012. Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. ISRN Agron.: 1–11.

Khan, Z., S.L. Kandel, D.N. Ramos, G.J. Ettl, S. Kim, and S.L. Doty. 2015. Increased biomass of nursery-grown douglas-fir seedlings upon inoculation with diazotrophic endophytic consortia. Forests 6: 3582–3593.

Khan, Z., D. Roman, T. Kintz, M.D. Alas, R. Yap, and S. Doty. 2014. Degradation, phytoprotection and phytoremediation of phenanthrene by endophyte *Pseudomonas putida*, PD1. Environ. Sci. Technol. 48(20): 12221–12228.

Knoth, J.L., S.-H. Kim, G.J. Ettl, and S.L. Doty. 2012. Effects of cross host species inoculation of nitrogen-fixing endophytes on growth and leaf physiology of maize. GCB Bioenergy 5(4): 408–418.

Knoth, J.L., S. Kim, G.J. Ettl, and S.L. Doty. 2014. Biological nitrogen fixation and biomass accumulation within poplar clones as a result of inoculations with diazotrophic endophyte consortia. New Phytol. 201(2): 599–609.

Kravchenko, L. V, T.S. Azarova, N.M. Makarova, and I. a Tikhonovich. 2004. The effect of tryptophan of plant root metabolites on the phyto stimulating activity of rhizobacteria . Mikrobiologiia 73(2): 195–198.

Leslie, J. F., Summerell, B. A., and Bullock, S. 2006. The Fusarium laboratory manual (Vol. 2, No. 10). Ames, IA, USA: Blackwell Publication.

Lin, L., Z. Li, C. Hu, X. Zhang, S. Chang, L. Yang, Y. Li, and Q. An. 2012. Plant growth-promoting nitrogen-fixing enterobacteria are in association with sugarcane plants growing in Guangxi, China. Microbes Environ. 27(4): 391–398.

Loewen, P.C. 1984. Isolation of catalase-deficient *Escherichia coli* mutants and genetic mapping of katE. a locus that affects catalase activity. J. Bacteriol. 157(2): 622–626.

Lu, S.-E., J. Novak, F.W. Austin, G. Gu, D. Ellis, M. Kirk, S. Wilson-Stanford, M. Tonelli, and L. Smith. 2009. Occidiofungin, a unique antifungal glycopeptide produced by a strain of *Burkholderia contaminans*. Biochemistry 48(35): 8312–8321.

Mahenthiralingam, E., L. Song, A. Sass, J. White, C. Wilmot, A. Marchbank, O. Boaisha, J. Paine, D. Knight, and G.L. Challis. 2011. Enacyloxins are products of an unusual hybrid modular polyketide synthase encoded by a cryptic *Burkholderia ambifaria* genomic island. Chem. Biol. 18(5): 665–677.

Manoel, J., T. Marta, L.S. De Albuquerque, Y.C. Montaldo, J. Ubaldo, L. De Oliveira, S. Geórgia, M.S. Nascimento, and R. Oliveira. 2015. Potential of the endophytic bacteria (*Herbaspirillum* spp . and *Bacillus* spp .) to promote sugarcane growth. Aust. J. Crop Sci. 9(8): 754–760.

Markowitz, V.M., I.-M.A. Chen, K. Palaniappan, K. Chu, E. Szeto, Y. Grechkin, A. Ratner, B. Jacob, J. Huang, P. Williams, M. Huntemann, I. Anderson, K. Mavromatis, N.N. Ivanova, and N.C. Kyrpides. 2012. IMG: the integrated microbial genomes database and comparative analysis system. Nucleic Acids Res. 40(D1): D115–D122.

Markowitz, V.M., K. Mavromatis, N.N. Ivanova, I.-M.A. Chen, K. Chu, and N.C. Kyrpides.

2009. IMG ER: a system for microbial genome annotation expert review and curation. Bioinformatics 25(17): 2271–2278.

Mattos, K. a., V.L.M. Pádua, A. Romeiro, L.F. Hallack, B.C. Neves, T.M.U. Ulisses, C.F. Barros, A.R. Todeschini, J.O. Previato, and L. Mendonça-Previato. 2008. Endophytic colonization of rice (*Oryza sativa* L.) by the diazotrophic bacterium *Burkholderia kururiensis* and its ability to enhance plant growth. An. Acad. Bras. Cienc. 80(3): 477–493.

Mazurier, S., A. Merieau, D. Bergeau, V. Decoin, D. Sperandio, A. Crépin, C. Barbey, K. Jeannot, M. Vicré-Gibouin, P. Plésiat, P. Lemanceau, and X. Latour. 2015. Type three secretion system and virulence markers highlight similarities and differences between human and plant-associated pseudomonads related to *Pseudomonas fluorescens* and *P. putida*. Appl. Environ. Microbiol. 81(7): 2579–2590.

Meneses, C.H.S.G., L.F.M. Rouws, J.L. Simoes-Araujo, M.S. Vidal, and J.I. Baldani. 2011. Exopolysaccharide production is required for biofilm formation and plant colonization by the nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus*. Mol. Plant. Microbe. Interact. 24(12): 1448–58.

Mitter, B., A. Petric, M.W. Shin, P.S.G. Chain, L. Hauberg-Lotte, B. Reinhold-Hurek, J. Nowak, and A. Sessitsch. 2013. Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. Front. Plant Sci. 4(April): 1–15.

Momose, A., N. Ohtake, K. Sueyoshi, T. Sato, Y. Nakanishi, S. Akao, and T. Ohyama. 2009. Nitrogen fixation and translocation in young sugarcane (*Saccharum officinarum* L.) plants associated with endophytic nitrogen-fixing bacteria. Microbes Environ. 24(3): 224–230.

Nautiyal, C.S. 1999. An efficient microbiological growth medium for screening phosphate

solubilizing microorganisms. FEMS Microbiol. Lett. 170(1): 265–270.

Oteino, N., R.D. Lally, S. Kiwanuka, A. Lloyd, D. Ryan, K.J. Germaine, and D.N. Dowling. 2015. Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. Front. Microbiol. 6(July): 1–9.

Passari, A.K., V.K. Mishra, V.K. Gupta, M.K. Yadav, R. Saikia, and B.P. Singh. 2015. *In vitro* and *in vivo* plant growth promoting activities and DNA fingerprinting of antagonistic endophytic actinomycetes associates with medicinal plants. PLoS One 10(9): e0139468.

Patra, A.K., L. Abbadie, A. Clays-Josserand, V. Degrange, S.J. Grayston, N. Guillaumaud, P. Loiseau, F. Louault, S. Mahmood, S. Nazaret, L. Philippot, F. Poly, J.I. Prosser, and X. Le Roux. 2006. Effects of management regime and plant species on the enzyme activity and genetic structure of N-fixing, denitrifying and nitrifying bacterial communities in grassland soils. Environ. Microbiol. 8(6): 1005–1016.

Patten, C.L., and B.R. Glick. 2002. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl. Environ. Microbiol. 68(8): 3795–801.

Paungfoo-Lonhienne, C., T.G. a Lonhienne, Y.K. Yeoh, R.I. Webb, P. Lakshmanan, C.X. Chan, P.E. Lim, M. a. Ragan, S. Schmidt, and P. Hugenholtz. 2014. A new species of *Burkholderia* isolated from sugarcane roots promotes plant growth. Microb. Biotechnol. 7(2): 142–154.

Perin, L., L. Martínez-Aguilar, R. Castro-González, P. Estrada-De Los Santos, T. Cabellos-Avelar, H. V Guedes, V.M. Reis, and J. Caballero-Mellado. 2006. Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. Appl. Environ. Microbiol. 72(5): 3103–3110.

Pirttila, A.M., P. Joensuu, H. Pospiech, J. Jalonen, and A. Hohtola. 2004. Bud endophytes of

Scots pine produce adenine derivatives and other compounds that affect morphology and mitigate browning of callus cultures. Physiol. Plant. 121(2): 305–312.

Pleban, S., F. Ingel, and Ii. Chet. 1995. Control of *Rhizoctonia solani* and *Sclerotium rolfsii* in the greenhouse using endophytic *Bacillus* spp. Eur. J. Plant Pathol. 101: 665–672.

Poly, F., L.J. Monrozier, and R. Bally. 2001. Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. Res. Microbiol. 152(1): 95–103.

Radzki, W., F.J. Gutierrez Mañero, E. Algar, J. a. Lucas García, a. García-Villaraco, and B. Ramos Solano. 2013. Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. Antonie Van Leeuwenhoek 104(3): 321–330.

Redman, R.S., Y.O. Kim, C.J.D. a Woodward, C. Greer, L. Espino, S.L. Doty, R.J. Rodriguez, and L. Sharon. 2011. Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS One 6(7): e14823.

Richardson, A.E., J.M. Barea, A.M. McNeill, and C. Prigent-Combaret. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321(1-2): 305–339.

Riggs, P.J., M.K. Chelius, a L. Iniguez, S.M. Kaeppler, and E.W. Triplett. 2001. Enhanced maize productivity by inoculation with diazotrophic bacteria. Aust. J. Plant Physiol. 28(9): 829–836.

Rodriguez, R.J., J. Henson, E. Van Volkenburgh, M. Hoy, L. Wright, F. Beckwith, Y.-O. Kim, and R.S. Redman. 2008. Stress tolerance in plants via habitat-adapted symbiosis. ISME J. 2(4): 404–16.

Ryan, R.P., K. Germaine, A. Franks, D.J. Ryan, and D.N. Dowling. 2008. Bacterial endophytes:

recent developments and applications. FEMS Microbiol. Lett. 278(1): 1–9.

Rybakova, D., T. Cernava, M. Köberl, S. Liebminger, M. Etemadi, and G. Berg. 2015. Endophytes-assisted biocontrol: novel insights in ecology and the mode of action of *Paenibacillus*. Plant Soil: 1–16.

Saha, M., S. Sarkar, B. Sarkar, B.K. Sharma, S. Bhattacharjee, and P. Tribedi. 2015. Microbial siderophores and their potential applications: a review. Environ. Sci. Pollut. Res. 22: 1–16.

Santi, C., D. Bogusz, and C. Franche. 2013. Biological nitrogen fixation in non-legume plants. Ann. Bot. 111(5): 743–67.

Schwarz, S., T.E. West, F. Boyer, W.-C. Chiang, M.A. Carl, R.D. Hood, L. Rohmer, T. Tolker-Nielsen, S.J. Skerrett, and J.D. Mougous. 2010. *Burkholderia* type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. PLoS Pathog. 6(8): e1001068.

Schwyn, B., and J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160(1): 47–56.

Serrato, R. V, C.H.S.G. Meneses, M.S. Vidal, A.P. Santana-Filho, M. Iacomini, G.L. Sassaki, and J.I. Baldani. 2013. Structural studies of an exopolysaccharide produced by *Gluconacetobacter diazotrophicus* Pal5. Carbohydr. Polym. 98(1): 1153–1159.

Sgroy, V., F. Cassán, O. Masciarelli, M.F. Del Papa, A. Lagares, and V. Luna. 2009. Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte *Prosopis strombulifera*. Appl. Microbiol. Biotechnol. 85(2): 371–381.

Shi, Y., K. Lou, and C. Li. 2009. Promotion of plant growth by phytohormone-producing endophytic microbes of sugar beet. Biol. Fertil. Soils 45(2009): 645–653.

Sievers, F., A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Soding, J.D. Thompson, and D.G. Higgins. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7(1): 539–539.

Spaepen, S. 2015. Plant hormones produced by microbes. p. 247–256. *In* Principles of Plant-Microbe Interactions.

Spaepen, S., and J. Vanderleyden. 2011. Auxin and plant-microbe interactions. Cold Spring Harb. Perspect. Biol. 3:a001438(4): 1–13.

Suárez-Moreno, Z.R., J. Caballero-Mellado, B.G. Coutinho, L. Mendonça-Previato, E.K. James, and V. Venturi. 2012. Common features of environmental and potentially beneficial plant-associated *Burkholderia*. Microb. Ecol. 63(2): 249–266.

Verma, V.C., S.K. Singh, and S. Prakash. 2011. Bio-control and plant growth promotion potential of siderophore producing endophytic *Streptomyces* from *Azadirachta indica* A. Juss. J. Basic Microbiol. 51(5): 550–556.

Vial, L., F. Lépine, S. Milot, M.C. Groleau, V. Dekimpe, D.E. Woods, and E. Déziel. 2008. *Burkholderia pseudomallei*, *B. thailandensis*, and *B. ambifaria* produce 4-hydroxy-2-alkylquinoline analogues with a methyl group at the 3 position that is required for quorum-sensing regulation. J. Bacteriol. 190(15): 5339–5352.

Viallard V, Poirier I, Cournoyer B, Haurat J, Wiebkin S, Ophel-keller K, Balandreaul J. 1998. *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas*] *phenazinium*, [*Pseudomonas*] *pyrrocinia* and [*Pseudomonas*] *glathei* as *Burkholderia.* Int J Systamatic Bacteriol. 48:549–563.

Videira, S.S., D.M. de Oliveira, R.F. de Morais, W.L. Borges, V.L.D. Baldani, and J.I. Baldani.

2012. Genetic diversity and plant growth promoting traits of diazotrophic bacteria isolated from two *Pennisetum purpureum* Schum. genotypes grown in the field. Plant Soil 356(1-2): 51–66.

Walker, R., Agapakis, C. M., Watkin, E., and Hirsch, A. M. 2015. Symbiotic nitrogen fixation in legumes: perspectives on the diversity and evolution of nodulation by *Rhizobium* and *Burkholderia* Species. Biological Nitrogen Fixation 913-925.

Weber, T., K. Blin, S. Duddela, D. Krug, H.U. Kim, R. Bruccoleri, S.Y. Lee, M.A. Fischbach, R. Müller, W. Wohlleben, R. Breitling, E. Takano, and M.H. Medema. 2015. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res. 43(W1): W237–W243.

Xin, G., D. Glawe, and S.L. Doty. 2009a. Characterization of three endophytic, indole-3-acetic acid-producing yeasts occurring in *Populus* trees. Mycol. Res. 113(9): 973–80.

Xin, G., G. Zhang, J.W. Kang, J.T. Staley, and S.L. Doty. 2009b. A diazotrophic, indole-3-acetic acid-producing endophyte from wild cottonwood. Biol. Fertil. Soils 45(6): 669–674.

Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, M. Arakawa, and M. Yabuuchi, E; Kosako, Y; Oyaizu, H; Yano, I; Hotta, H; Hashimoto, Y; Ezaki, T; Arakawa. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. and Immunol. 36(12): 1251–1275.

Yun, M., C.G. Park, J.Y. Kim, C.O. Rock, S. Jackowski, and H.W. Park. 2000. Structural basis for the feedback regulation of *Escherichia coli* pantothenate kinase by coenzyme A. J. Biol. Chem. 275(36): 28093–9.

Zuleta LFG, Cunha CDO, de Carvalho FM, Ciapina LP, Souza RC, Mercante FM, de Faria SM, Baldani JI, Straliotto R, Hungria M, de Vasconcelos ATR. 2014. The complete genome of *Burkholderia phenoliruptrix* strain BR3459a, a symbiont of *Mimosa flocculosa*: highlighting the coexistence of symbiotic and pathogenic genes. BMC Genomics 15:535.

Table S1 Poplar and willow endophytes used to test anti-fungal activities.

Strain names	Closest 16s rDNA match
PTD1	*Rhizobium tropici*
WPB	*Burkholderia vietnamiensis*
WP1	*Rhodotorula graminis*
WP5	*Rahnella* species
WP9	*Burkholderia* species
WP19	*Acinetobacte*r *calcoaceticus*
Snoq 117.2	*Pseudomonas* species
WP40	*Burkholderia* species
WP41	*Burkholderia* species
WP42	*Burkholderia* species
WW17	*Burkholderia vietnamiensis*
WW5	*Sphingomonas* species
WW6	*Pseudomonas putida*
WW7	*Curtobacterium* species
WP 4-1-1	Unidentified
WP 4-1-2	Unidentified
WP 4-1-3	Unidentified
WP 4-1-4	Unidentified
WP 4-2-1	Unidentified
WP 4-2-2	Burkholderia sp.
WP 4-2-3	Unidentified
WP 4-3-1	*Rhodotorula graminis*
WP 4-3-2	*Burkholderia* sp.
WP 4-3-3	*Curtobacterium* sp.
WP 4-3-4	Unidentified
WP 4-3-5	Unidentified
WP 4-3-6	Unidentified

Strain names	Closest 16s rDNA match
WP 4-3-7	Unidentified
WP 4-3-8	Unidentified
WP 4-4-1	Unidentified
WP 4-4-2	*Rahnella aquatilis*
WP 4-4-3	Unidentified
WP 4-4-4	Unidentified
WP 4-4-5	Unidentified
WP 4-4-6	Pseudomonas sp.
WP 4-5-1	Unidentified
WP 4-5-2	Unidentified
WP 4-5-3	*Rahnella aquatilis*
WP 4-6-1	Unidentified
WP 4-6-2	Unidentified
WP 4-6-3	Unidentified
WP 4-6-4	Unidentified
WP 4-7-1	Unidentified
WP 4-7-2	Unidentified
WP 4-7-3	Unidentified
WP 4-7-4	Unidentified
WP 4-8-1	Unidentified
WP 4-8-2	Unidentified
WP 4-8-3	Unidentified
WP 4-8-4	Unidentified
WP 4-8-5	Unidentified
WP 4-8-6	Unidentified
WP 4-9-1	Unidentified
WP 4-9-2	Unidentified
WP 4-9-3	Unidentified
WP 4-9-4	Unidentified

Strain names	**Closest 16s rDNA match**
WP 4-10-1	Unidentified
WP 4-10-2	Unidentified
WP 4-10-3	Unidentified
WP 4-10-4	*Curtobacterium* sp.
WP 4-10-5	Unidentified