Analysis of the Potential for Diazotrophic Endophytes to Increase Efficiency of Bioenergy Crop Production

Growth promotion effects of the endophytes isolated from *Populus trichocarpa and Salix sitchensis*

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Abstract

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Sustainable production of biomass for bioenergy relies on low input crop production. Inoculation of bioenergy crops with plant growth promoting endophytes has the potential to reduce the fertilizer inputs through the enhancement of biological nitrogen fixation. While nitrogen fixing (diazotrophic) endophytes colonize many wild plants, these natural relationships may be disrupted in cultivated crop species where breeding and genotype selection often occur under conditions of intensive fertilization and irrigation. A selection of diazotrophic endophytes isolated from willow (*Salix sitchensis,* Sitka willow) and poplar (*Populus trichocarpa,* black cottonwood) growing in nutrient poor river sides were used as inoculum in a series of greenhouse and field trials designed to test the overall hypothesis that naturally occurring diazotrophic endophytes impart plant growth promotion to their host plants. Endophyte inoculations contributed to increased biomass over uninoculated control plants. Biological nitrogen fixation calculated from ^{15}N isotope dilution ranged from 18 – 65%. No significant

effect on leaf physiology was observed for plants inoculated with endophytes previously isolated from similar host plant species; however, consistently higher rates of net CO_2 assimilation was observed as a result of cross species endophyte inoculations. Additionally, phenotypic plasticity in biomass allocation and branch production as a result of endophyte inoculations were observed and may be useful in bioenergy crop breeding and engineering programs.

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Chapter 1

Introduction

Plant-Microbe Symbiosis

Defining the Endophyte

Microorganisms living within plant tissue are collectively referred to as endophytes. The German botanist and founding father of plant pathology, A. de Bary (1866) is credited for the introduction of the term "endophyte" meaning "within plant" as a description for pathogenic fungi living within leaf tissue. Following de Bary's description of endophytes, reports of microorganisms living within healthy plants were made by several other early nineteenth century scientists (Compant *et al.*, 2012). Credit is given to M.L.V. Galippe as the first to demonstrate that all plants can potentially host microorganisms leading to the hypothesis that these microorganisms were derived from the soil (Galippe, 1887a; Galippe, 1887b) as cited by (Compant *et al.*, 2012). The term endophyte has since evolved; at one time including all microorganisms entering plant tissues regardless of pathogenicity to the current definition of asymptomatic infections. Recently the definition of endophyte as proposed by Wilson (1995) somewhat narrowed the term to include only those microbes, bacterial and fungal species, who at any point in their life cycle asymptomatically invade and reside exclusively within plant tissue without causing disease. Wilson acknowledges this definition excludes mycorrhizal fungi while including pathogenic species with a latent phase or that are known pathogens that never exhibit disease symptoms. In this way the term endophyte strictly refers to the location of infection by the microorganism.

Within the last two decades the body of literature referring to endophytic microorganisms has implied that these microorganisms not only reside asymptomatically within plants but provide some benefit to the host plant in return. Indeed endophytic microorganisms are believed to be important in the evolution and the survival of terrestrial plants. While it is unclear of the specifics of the early relationships, plant-microbe interactions have been observed in pre-Creataceous leaf fossils (Taylor & Krings, 2005; Krings *et al.*, 2009). Given the vast body of research describing the ecology of the endosphere, Partida-Martinez and Heil (2011) postulate that a plant completely free of microbes is the exception and should not be considered the biological rule. It is believed that in exchange for a constant supply of energy derived from photosynthates (Dong *et al.*, 1994) and safe shelter provided by the host plant, endophytes reciprocate by delivering enhanced survival mechanisms to the host. These benefits include growth stimulation through the production of growth hormones, nitrogen fixation, enhanced nutrient uptake, and protection from would be pathogenic colonizers. Dispensation of abiotic stress tolerance is another commonly observed benefit; e.g. many naturally occurring endophytic microbes possess the ability to metabolize and detoxify pollutants (Kang *et al.*, 2012; Lee *et al.*, 2012).

Diversity of Endophytes

The identification of endophytic microbes occurs through both culture-dependent and culture-independent methods. The procedure for endophyte cultivation varies widely in terms of surface sterilization technique, tissue preparation, and media selection; e.g. non-selective or selective media inoculated with sectioned or macerated plant tissue. An additional consideration is the sometimes recalcitrant culturability of individual microbial species. Cultivation method, therefore, is likely to bias the observed endophytic microbial community (Tabacchioni *et al.*,

2000). Culture independent methods have been increasingly employed to investigate microbial communities *in planta*. Chelius and Triplett (2001) report only 48% overlap between the culturable component of microbial community associated with maize roots and that directly identified through PCR amplification of the 16S rDNA. An additional two bacterial divisions and 44 phylotypes were revealed using the culture-independent method. PCR amplification of the 16S rDNA also produced six archaeal sequence types previously not observed through culture based isolation methods.

Endophytes span a diverse phylogenic range including bacteria, archaea and fungi. Bacterial species identified as endophytes are largely members of the phyla *Proteobacteria, Firmicutes, Actinobacteria,* and *Bacteroidetes.* The majority of the endophytic bacteria identified belong to the γ-*Proteobacteria* group (Rosenblueth & Martinez-Romero, 2006; Roesch *et al.*, 2008; Doty *et al.*, 2009; Khan & Doty, 2009; Taghavi *et al.*, 2009; Gottel *et al.*, 2011). Fungal endophytes are classified as clavicipitaceous and nonclavicipitaceous based on host specificity and life history traits and are in general members of the Ascomycota and Basidiomycota divisions. An in-depth review and further classification of fungal endophytes is provided by Rodriquez et al. (2009).

Endosphere community composition is determined by the dynamic interactions between plant traits and the soil microbial community. Geography (Roesch *et al.*, 2010; Rout & Callaway, 2012), cultivation practices (Seghers *et al.*, 2004), and plant species composition (Rozycki *et al.*, 1999; Schweitzer *et al.*, 2008) all influence the composition of soil microbial communities. Sturz and Nowak (2000) review the interactive relationship between plant species and rhizospheric bacteria citing evidence for specificity in both epiphytic and endophytic colonization of root tissue. Data demonstrating up to 78% of the variation within the soil microbial biomass is explained by plant genotype supports the hypothesis that feedback and

signaling specific to plant genotype are important factors affecting soil microbial community composition (Schweitzer *et al.*, 2008). A common speculation that endosphere microbial communities are subsets of the rhizosphere population is supported by data comparing the sequence diversity of the marker gene for nitrogen fixation (*nif*H) isolated from the rhizosphere, root, and stem tissue of maize (Roesch *et al.*, 2008). However, in a culture-independent assay of rhizosphere and endosphere microbial communities within *Populus deltoides* two distinct patterns of dominant phylogenetic groups and operational taxonomic units (OTUs) are presented (Gottel *et al.*, 2011). The two populations did not demonstrate similar patterns of dominant phylogenetic groups indicating successful endosphere colonization is not a passive process. While some level of stochasticity (Ulrich *et al.*, 2008) cannot be ignored, competition among potential endophytic microbes (Oliveira *et al.*, 2009) and genetic determinants from both microbes and plants (Dong *et al.*, 2003) combine to ultimately influence the endophytic community composition.

Colonization

Colonization of the host plant by endophytic bacteria likely begins in the rhizosphere with the recognition of plant exudate. The molecular signals produced by the plant include sugars and a complex array of phytochemicals including organic acids and amino acids (Lugtenberg *et al.*, 1999). Chemotaxis is generally considered a competitive advantage for microbes residing in the rhizosphere and may also provide a similar advantage for endophytic microbes which tend to lack competitive vigor outside the endosphere (de Weert *et al.*, 2002; Compant *et al.*, 2010). Attachment to the root surface and subsequent colonization occurs quite rapidly. Inoculation studies conducted in our laboratory demonstrate both yeast and bacterial endophytes attached to the root surface within the first 30 minutes after inoculation (results not

published). Evidence of endospheric penetration by green fluorescent protein labeled (GFP) bacteria was subsequently observed within 2 hours and colonization observed within the cortex and xylem tissue after 24 hours. These results are in line with those reported by Tharek *et al.* (2011) who traced a GFP labeled Enterobacter strain USML2 and reported observing attachment after 12 hours at the lateral root junction

Microorganisms gain entry to host plant tissue through both passive and active mechanisms. Details of reported entry sites and a comprehensive overview of colonization mechanisms are given in several reviews (Hardoim *et al.*, 2008; Compant *et al.*, 2010; Reinhold-Hurek & Hurek, 2011; Monteiro *et al.*, 2012). Briefly, entry generally occurs through lateral root cracks or discontinuities in epidermal tissue. Endophytic microbes must then continue beyond the endodermis and pericycle before reaching the xylem. Once in the vascular system, endophytes can easily migrate to aerial tissue as well as into reproductive organs (Rijavec *et al.*, 2007; Compant *et al.*, 2011). The distribution of endophytic colonization of plant tissue as observed mainly through studies of culturable bacteria shows not only a larger concentration of endophytes in the roots but a greater diversity (Compant *et al.*, 2011). Colonization patterns of *Pseudomonas* species within poplar reported by Germaine *et al.* (2004) support the concept of specific niche colonization. Woody cuttings were inoculated with three separate species of *Pseudomonas* tagged with green fluorescent protein (*gfp*). All three species were re-isolated from the rhizosphere and root tissue, two of three were observed in the sap and stem, and only one species was re-isolated from leaf tissue. In addition to tissue specific colonization, there is evidence that host plant phenology influences the composition and location of the endophytic microbial community (Pirttila *et al.*, 2005; Mishra *et al.*, 2012).

Plant Growth Promotion

The concept that endophytes have a key role in plant vitality is well accepted; however, the specific mechanism(s) remain an area of continued research. Associations with endophytic microbes allow for plant growth to occur in otherwise harsh or hostile environments (Redman *et al.*, 2011; Lopez *et al.*, 2012). Plant growth promoting activities are classified as indirect and direct (Long *et al.*, 2008). Examples of indirect plant growth enhancement include nutrient acquisition through siderophore production (Boyer *et al.*, 1999), phosphate solubilization (Malinowski *et al.*, 2000; Vassilev *et al.*, 2012), abiotic stress tolerance (Boyer *et al.*, 1999; Rodriguez *et al.*, 2008; Weyens *et al.*, 2010; Redman *et al.*, 2011), biotic stress tolerance (Latch, 1993) and prevention of infection by pathogenic species (Waller *et al.*, 2005; Weyens *et al.*, 2009). Direct plant growth promotion activities include nitrogen fixation (Dobereiner, 1992; Baldani *et al.*, 2002) and phytohormone production (Long *et al.*, 2008; Han *et al.*, 2011; Khan *et al.*, 2012). The possibility that plants derive nutrients directly from the decomposition of microbes *in planta* cannot be ignored (Paungfoo-Lonhienne *et al.*, 2010).

Endophytes are adept at manipulating plant cellular response as observed in the well-studied legume-rhizobia symbiosis (Wang *et al.*, 2012). The expression of phytohormones such as gibberellins (Han *et al.*, 2011) and auxins (e.g. indol-3-acetic acid (IAA)) are just two examples of how endophytes influence the host plant physiology. Modulation of plant stress through the control of defense related protein expression (Brusamarello-Santos *et al.*, 2012) and ethylene levels (Glick *et al.*, 1998; Cavalcante *et al.*, 2007) appears to be a key component in maintaining a balanced and beneficial plant-microbe relationship (Hardoim *et al.*, 2008).

Plant growth promoting activities are responsive to environmental cues. Sevilla et al. (2001) published a comprehensive study of the benefit to sugarcane following inoculations with

the wild type diazotroph *Gluconacetobacter diazotrophicus* PA15 (formerly *Acetobacter diazotrophicus* PA15) and a nitrogenase deficient mutant termed Mad3A. Growth promotion was observed only for plants inoculated with wild type G. *diazotrophicus* PA15 under nitrogen limiting conditions. When nitrogen was not a limiting factor the inoculated plants had no significant difference between them, however, both out-performed the uninoculated control group. These results provide evidence of nitrogen fixation and indicate that more than one mechanism is responsible for plant growth promotion.

Plant growth promotion is affected by genotype specific interaction within the plant-microbe symbiosis and mixed community inoculations appear to have greater positive impact on plant vitality. Riggs *et al*. (2001) demonstrate specific interactions between endophytes and host plant genotype are responsible for significant differences in yield between different combinations of endophyte species and maize cultivars. Govindarajan *et al.* (2008) reported the inoculation of rice with the endophytic isolate *Burkholderia vietnamiensis* MGK3 and other diazotrophic isolates increased yield by 5.6 to 12.16% over the uninoculated control treatment. They investigated the effect of the isolates as single culture inocula as well as in a mixed culture inoculum. Increased yield and plant biomass was reported for all inocula but the greatest gain appeared to come from the mixed strain inoculum. It is of interest to note that the uninoculated plants were not sterile and even contained a small community of diazotrophic endophytes in the root tissue. These results indicate that the natural endosphere community can be manipulated.

Role of Endophyte Inoculations for Bioenergy Crop Production

Bioenergy Crops

As the price of fossil fuel derived energy and petroleum based transportation fuels continue to rise, more attention has been focused on renewable bioenergy crop production. The great demand for transportation fuel is driving the push for drop-in ready alternatives to

petroleum based gasoline and diesel fuel. Biodiesel and ethanol are two such fuels. Biodiesel is derived from vegetable oil and animal fats and can be used in its pure form, 100% (B100), or blended with petroleum diesel, e.g. 20% (B20). Biodiesel has many advantages over petroleum diesel: renewable and local production, non-toxic, safer to handle, biodegradable, lowered greenhouse emissions and contribution to air pollution over petroleum diesel. The primary disadvantage of biodiesel is the cost of production combined with lower fuel economy and power (D.O.E., 2012). Biodiesel crops include soybean, canola, peanuts, jojoba, sesame, coconut, oil palm, and jatropha. Ethanol is produced through the fermentation of plant sugars and can also be used in its pure, undiluted form or blended with petroleum based gasoline. Sugars from any plant can be fermented into ethanol however at this time the major crops for ethanol production are sugarcane, sorghum, and maize.

Nitrogen Fixation

All life on Earth is connected to the process of nitrogen fixation. Nitrogen (N) is a necessary component of nearly all biological molecules. In the form of dinitrogen gas (N_2), it makes up close to 80% of the atmosphere. However, atmospheric nitrogen is biologically unavailable due to the strength of the triple bond. A few organisms are capable of reducing dinitrogen to ammonia. They are generally referred to as diazotrophs. The enzymatic process of nitrogen fixation relies on the products of the *nif*-gene complex, the nitrogenase enzyme system. Energetically the reaction is expensive and is commonly represented with this equation:

$$N2 + 8H+ + 8e- +16ATP \rightarrow 2NH3 +H2 + 16\ ADP + 16Pi.$$

Howard and Rees (1996) provide a comprehensive review of biological nitrogen fixation (BNF) in which they examine the structure and properties of the nitrogenase gene complex as well as the functionality of the nitrogenase proteins and accompanying molecules that serve as

coenzymes; notably the Fe protein and the MoFe protein referred to as metallocenters. All known nitrogenase protein complexes are of prokaryotic origin and inactivated by oxygen. One mechanism by which free-living and endophytic diazotrophs shield the nitrogenase complex from oxygen deactivation is the creation of a steep concentration gradient whereby the oxygen is rapidly consumed through respiration near the nitrogenase activity. This gradient can be formed in symbiotic relationships with the aid of specialized plant tissue as in nodulation (Pawlowski, 2008). Another possible mechanism is through the formation of biofilms, microcolonies bound by an extracellular polymeric matrix, as observed in soil surrounding the rhizosphere as well as within the apoplast of aerial plant tissue (Lin *et al.*, 2012). Further strategies for oxygen avoidance or deactivation, described in detail by Montagna and Torres (2008), are: metabolic separation, physical barriers, creation of a strict anaerobic environment, and balancing nitrogenase expression with the inactivation of the enzyme. Qi Cheng (2008) provides a succinct history of nitrogenase research and proposes the possibility of the existence or eventual evolution of a light utilizing nitrogenase complex.

Diazotrophic microorganisms have been isolated from nearly all environments. Obtaining an accurate measurement of biological nitrogen fixation from free-living and associative diazotrophs is problematic due to inadequate quantitative methods (Unkovich & Baldock, 2008) and biological nitrogen fixation from symbiotic systems is often underestimated (Danso *et al.*, 1993). Nonetheless, the contribution of ammonia to the nitrogen cycle through biological fixation is estimated at 5×10^{11} Kg per year (Cheng, 2008).

Relationship between Rhizospheric and Endophytic Diazotrophic Communities

The question of how the microbial community living in the soil compares to the diazotrophic community structure living inside maize plants was investigated by Roesch *et al.*

(2008). They compared the diversity *nif*H genes isolated from rhizosphere soil and root and stem tissue of maize grown in six geographically and environmentally diverse locations. They report differences in the diversity indices between geographic locations as well as among the rhizosphere soil, roots and stem samples. Given the differences in specific community make up there are several bacterial genera that appear to be common in maize. They are *Azospirillum, Bradyrhizobium, Herbaspirillum, Ideonella, Klebsiella* and *Raoultella*: of these *Herbaspirillum, Ideonella,* and *Klebsiella* were dominate in the interior of the plant and detected at much lower incidence in the soil. The study concluded with the observation that the diversity of the diazotrophic bacterial community was decreased from the rhizosphere soil within the root tissue and was the least diverse within stem tissue. They further hypothesize that the plant is the predominant selective agent in determining the endophytic community.

In a more recent examination of diazotrophic community diversity, Roesch *et al*. (2010) investigated four different geographic locations using a similar molecular biology approach. In this study, DNA was extracted from only the soil and the 16sRNA fragments of diazotrophic genera were compared to assess the biodiversity. They again report that while there is a difference in species dominance between the geographic locations, there is a set of common genera present at all four locations in both North and South America. One location however contained a group of genera uniquely different than from the other sites. This not only demonstrates an environmental selective pressure on the microbial community, it indicates that the *nif*H types are limited to one region or another.

High Yield Agriculture Inputs and Consequences

Along with sunlight and water the two most important elements for high yield crop production are nitrogen and phosphorus. The development of high yielding crop cultivars in

combination with synthetic fertilizers derived from fossil fuels has powered the Green Revolution (Borlaug, 2000). The reduction of dinitrogen gas to the biologically available form of ammonia has been accomplished through what is known as the Haber-Bosch process. This requires the combination of high temperature and high pressure in the presence of an iron crystal catalyst (Howard & Rees 1996). Between the years 1960 and 2000, grain crop yields increased by 94%. During this same time the worldwide use of nitrogen fertilizer increased by an estimated 78 Tg per year and phosphorus fertilizer increased by 31 Tg per year (Vance, 2001).

Natural gas is the primary feedstock used in the production of nitrogen fertilizers in the United States. An economic analysis of the impact of rising natural gas prices on domestic ammonia production highlights the consequences to agricultural costs. In the span of only six years from 2000 to 2006 the cost of ammonia to US farmers rose by 130% (Huang, 2007). Current high intensity agriculture relies on fertilizer inputs applied at rates far greater than plants can consume. Excess nitrogen and phosphorus remaining in the soil have many negative consequences. Soil N is converted through microbial activity to N_2O, a powerful greenhouse gas, and to gaseous NO_x, a compound toxic to plants and destructive to the Ozone layer in the stratosphere (Aneja *et al.*, 2012). Excess nitrate runoff along with excess phosphorus runoff have negative consequences as drinking water pollutants and the cause of eutrophication of lakes and marine estuaries (Socolow, 1999; Vance, 2001).

Increasing Demand for Bioenergy Crops

Advancements in technology and world trade have opened economies of many underdeveloped nations, as the wealth of these nations increases so does their demand for energy. The International Energy Outlook 2011 released by the US Department of Energy predicts a 53% increase in energy consumption from 2008 to 2035 where the majority of

consumption is predicted to occur in nations outside the Organization for Economic Cooperation and Development (non-OECD). Eighty-five percent of this increased demand is attributed to population growth along with strong and long term economic development of many non-OECD nations. By contrast, OECD nations are predicted to increase energy demands by just 18%. Worldwide, transportation fuels remain integral for economic growth depending on stable pricing and reliable sourcing. The increasing cost of fossil fuels along with concern for the environmental impact of extraction has positioned renewable energy to be the fastest growing source of primary energy in the near future.

The United States Congress recognized the need for national energy security and made a commitment to push the development of renewable energy and transportation fuels technology by passing the Energy Independence and Security Act of 2007 (EISA). A major component of the EISA is the expansion of the Renewable Fuels Standards (RFS) set in 2005 from 7.5 billion gallons of renewable fuel to 36 billion gallons by 2022. As a result, advanced pyrolysis technologies have emerged that utilize cellulosic biomass for the production of drop in replacements for diesel and jet fuel (Mettler *et al.*, 2012). Further, the economic feasibility of second generation bioethanol production is becoming more of a reality with research focused on the fermentation of both the 5 and 6- carbon sugars prevalent in cellulosic biomass (Bura *et al.*, 2012). Biofuel derived from cellulosic agricultural and forest residues are referred to as second generation biofuels. Residual agricultural biomass will not sustainably provide adequate feedstock to second generation biofuel refineries alone; dedicated bioenergy crops are necessary (Perlack & Stokes, 2011).

Endophyte Inoculations for Sustainable Bioenergy Crop Production

For the bioenergy crop production to be truly sustainable, current demand for biomass must be met while not compromising resources for the future. To sustainably feed these second generation refineries biomass must come from a variety of sources maximizing the current productivity of agricultural lands as well as utilizing non-arable lands. By definition, non-arable land is naturally unproductive, nutrient poor, contaminated or otherwise unsuitable for food crop production. It follows that sustainable production of biomass crops on marginal lands cannot rely on overly generous inputs of synthetic fertilizers or irrigation that would take away resources from future food crop production.

Throughout history, efforts to improve crop yield and vigor have explored methods to naturally enhance BNF (Herridge & Rose, 2000; Cocking, 2005; Doty, 2011). Direct soil inoculations with endophytic microorganisms have produced mixed results. Unkovich and Baldock (2008) point to data suggesting that field inoculations are not likely to provide a significant source of nitrogen for agriculture. However, Okon and Labandera-Gonzales (1994) report yield increases of 5 – 30% after field inoculations with various strains of *Azospirillum*. Strategies for crop improvement through inoculations with plant growth promoting microbes will therefore need to be holistic including knowledge of plant microbe-interactions both within plant tissues and in the surrounding soils (Sturz & Nowak, 2000).

Poplar as a Bioenergy Crop

Woody biomass is a well understood feedstock for the production of heat and electricity. In 2008 slightly more than 2.1 quadrillion (10^5) BTUs of energy were produced from woody biomass (White, 2010) acquired from short rotation woody crops, such as poplar, or from the

residues of timber harvest and milling. In fact hybrid poplar species are recognized as important bioenergy crops for five out of the seven geographical regions in the United States (DOE, 2006).

Members of the genus *Populus* are attractive as bioenergy crops not only because of their ability to rapidly produce biomass but because of their ability to grow on marginal lands unsuitable for agriculture and without displacing more valuable timber tree species. Poplar tree improvement programs have focused breeding for biomass gain, disease resistance, and lignin content (Heilman & Stettler, 1985; Tuskan, 1998; Dinus *et al.*, 2001). More recently, Populus has been identified as an effective species for phytoremediation. Thus opening the possibility for meeting both the need for biomass production and waste reduction (Zalesny *et al.*, 2009).

Research Objectives

Overarching Research Questions

The overarching question is whether diazotrophic endophytes isolated from native poplar and willow trees provide plant growth promotion to bioenergy crop plants. Two, multifaceted projects were designed to answer the following specific questions:

Question I: Is there a quantifiable plant growth promoting effect of individual species or a consortium of putative diazotrophic endophytes previously isolated from native poplar and willow species when applied as inoculum to crop species important for bioresource based products?

Question II: How much of the growth promotion can be ascribed to *in planta* nitrogen fixation by putative diazotrophic endophytes, both as individual inocula and as part of a consortium, in controlled laboratory conditions?

Question III: Where in the plant tissue do the endophytes reside?

Methodology

To assess growth promotion (Question I), the effect of endophyte inoculation on biomass production was measured through a series of greenhouse and field studies. Plants were inoculated with selected putative diazotrophic endophytes either as individual inoculum or as part of a larger consortium. The inoculated plants were grown alongside a control group of uninoculated plants of the same species. Both growth and physiological data were collected throughout the length of each study and compared to identify any linkage with enhanced growth to the endophyte inocula. Question II was assessed through a ^{15}N (isotopic nitrogen) dilution assay conducted in a greenhouse under controlled conditions. This assay was used to determine the amount of biologically fixed nitrogen the plant utilized compared to the amount of nitrogen it received from the labeled fertilizer. Question III was assessed through epi-fluorescent

microscopy. Endophytes were labeled with the constitutively expressed green fluorescent protein (GFP). Plants were inoculated with the GFP-labeled endophytes and the subsequent colonization of plant tissue was visually recorded.

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Chapter 2

Phenotypic Plasticity within Poplar Clones as a Result of Inoculations with Diazotrophic Endophytes

Introduction

The increasing cost of fossil fuels along with concern for the environmental impact of extraction has positioned renewable energy to be the fastest growing source of primary energy in the near future. To adequately feed biofuel refineries biomass must come from a variety of sources maximizing the current production of agricultural land as well as utilizing marginal land. Marginal land can be described as any non-arable, nutrient deprived, or polluted lands unfit for crop production. Such marginal lands may be well suited for woody biomass production. Forest tree species are attractive as a sustainable source for cellulosic biomass. Particular attention has been given to poplar tree species due to their capacity for high rate of growth and ease of propagation. These tree species are also well suited for growth on marginal lands as they are naturally pioneer species capable of establishment on nutrient poor substrate.

The very same concern over the environmental impact of fossil fuel consumption that has led to the development of renewable energy technology is echoed in the public scrutiny of biomass production for bioenergy. The goal of renewable energy is not only to alleviate dependency of foreign oil but to establish environmentally sustainable energy resources. One concern is the use of petroleum based fertilizers to improve crop production.

Diazotrophic microbes, those that fix nitrogen, have been isolated as free-living in the soil (Rozycki *et al.*, 1999) and water (Short *et al.*, 2004), in the rhizosphere and as symbionts in both leguminous and non-leguminous plants (Gallon, 1992). Diazotrophic endophytes are also

recognized for having a variety of plant growth promoting properties. Many endophytes are capable of solubilizing phosphorus (Vance, 2001) and have been shown to produce siderophores (Boyer *et al.*, 1999), molecules important in the sequestration/uptake of iron. Additionally, many diazotrophic endophytes produce plant hormones and have been shown to alter the plant's own stress response systems; a tactic that likely allows colonization with the apparent side effect of inducing enhanced abiotic stress tolerance (Doty, 2011). Microbes are extremely adept at thriving in challenging environments. A plant in an environment under stress is likely to host bacteria that help it cope with that stress (Rodriguez *et al.*, 2008).

Diazotrophic bacteria increase crop production and health over a range of environmental conditions. A review of nitrogen fixing bacteria presented by Franche *et al.* (2009) describes the interactions of both leguminous and non-leguminous plants and their associated diazotrophic bacteria. Efforts to enhance crop productivity by the addition of diazotrophic bacteria have gained in popularity as energy costs rise thereby raising the cost of manufactured fertilizers. A biological approach to nitrogen fixation is also regarded as a more environmentally favorable and sustainable practice. Broad scale implementation of enhanced biological nitrogen fixation will require multiple species or groups of bacteria to address locations and multiple crops.

While the majority of research has focused on single strain isolates from a plant system, Oliveira *et al.* (2002) recognized the importance of the naturally existing endophytic community in sugarcane production noting that the highest nitrogen from biological fixation occurred for the consortia, over inoculation with a single endophytic isolate. They investigated competition for colonization in a mixed bacterial inoculum. The individual bacterial species colonized the sugarcane with differing success when inoculated as a mixture than when inoculated as single isolate cultures, suggesting competition and inhibition among the bacteria. The competitive

advantages were presumed to result from better adaptation to the *in vitro* conditions by one bacterial strain over another.

Govindarajan *et al.* (2008) reported the inoculation of rice with the endophytic isolate *Burkholderia vietnamiensis* MGK3 and other diazotrophic isolates increased yield by 5.6 to 12.16% over the uninoculated control treatment. They investigated the effect of the isolates as single culture inocula as well as in a mixed culture inoculum. Increased yield and plant biomass were reported for all inocula but the greatest gain came as a result of the mixed strain inoculum. Interestingly the uninoculated plants were not sterile and even contained a small community of diazotrophic endophytes in the root tissue and that the number of colony forming units of bacteria was greater in the inoculated plants. These results suggest that experimental additions of diazotrophs may improve on the natural relationship between plants and the endophytic community. While the observed growth enhancement in plants inoculated with diazotrophic endophytes is well accepted, the specific mechanism(s) remain an area of continued research (Sevilla *et al.*, 2001).

The overarching hypothesis driving this body of work is that naturally occurring diazotrophic endophytes isolated from perennial tree species that thrive in nutrient limited conditions impart plant growth promotion to their host plants. Two separate greenhouse experiments were conducted using clonal ramets inoculated with diazotrophic endophytes. The first experiment investigated the hypothesis that inoculations with multi-strain consortia have a larger effect on plant growth promotion than single strain endophyte inoculations. The second experiment was an isotope dilution assay to test the hypothesis that growth promotion is due to increased nitrogen availability as a result of biological nitrogen fixation. A long-term field experiment was established to test the effect of endophyte inoculations on biomass production in a more operationally relevant environment. Leaf physiology measurements were taken mid-

growing season for the first two years to test the hypothesis that endophyte inoculations alter biomass production by influencing the net CO_2 assimilation rate.

Materials and Methods

Endophytes and Inocula Preparation

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

Inoculation suspensions were prepared by first growing the individual endophytes on nutrient rich media from stock collections held at -80°C. Bacteria were grown on MG/L agar (grams 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 $MgSO_4*7H_2O$, 2ug biotin at pH 7.0 and 15 grams agar) and yeast were grown on yeast extract-peptone-dextrose (YPD) medium (Difco Laboratories, Detroit, MI), then washed three times in nitrogen free Murashige and Skoog (NFMS, MSPOO7 Caisson Labs, UT) and resuspended in liquid media. Endophyte consortia were constructed by mixing equal concentrations (as determined by optical density measured at 600nm) of the individual strains just prior to inoculations. The endophyte treatments were delivered to plants in soil by injecting the endophyte suspension into the soil 3 – 6 cm below the surface and in close proximity to the roots. Each inoculation contained the equivalent optical density at 600nm of 0.1 for 25mL. Uninoculated control groups received soil injections with the equivalent volume of sterile liquid media.

Table 1: Description of diazotrophic endophytes included in the inoculation experiments. The presence or absence of the *nif*H gene was unable to be definitively determined in several isolated this is indicated with n.d..

Isolate Name	Closest 16S rDNA Match	IAA	*nif*H	Included in Inoculum Mix	Experiment
PTD-1	*Rhizobium tropici* bv *populus*	+	+	Consortium A Poplar Mix A Poplar Mix #1 PoplarMix #2	GreenHousePoplar Isotope Dilution
WPB	*Burkholderia vietnamiensis*	+	*nif*H, D, K	PoplarMix #2	Field Poplar Isotope Dilution
WP5	*Rahnella sp.CDC* 2987-79	+	+	Consortium A Poplar Mix A Poplar Mix #1 PoplarMix #2	GreenHousePoplar Field Poplar Isotope Dilution
WP7	*Enterobacter sp* YRL01	+	n.d.	Consortium A Poplar Mix A Poplar Mix #1 PoplarMix #2	GreenHousePoplar Field Poplar Isotope Dilution
WP8	*Pseudomonas graminis* PDD-13b-3	+	n.d.	Consortium A Poplar Mix A Poplar Mix #1 PoplarMix #2	GreenHousePoplar Field Poplar Isotope Dilution
WP19	*Acinetobacter*	-	-	Consortium A Poplar Mix A Poplar Mix #1 PoplarMix #2	GreenHousePoplar Field Poplar Isotope Dilution
WW2	*Herbaspirillum sp*	+	n.d.	Consortium A Willow Bacteria Mix	GreenHousePoplar
WW5	*Sphingomonas yanoikuyae*	+	+	Consortium A Willow Bacteria Mix	GreenHousePoplar
WW6	*Pseudomonas putida*	+	+	Consortium A Willow Bacteria Mix	GreenHousePoplar
WW7	*Spingomonas* ZnH-1	+	+	Consortium A Willow Bacteria Mix	GreenHousePoplar
WW11	*Sphingomonas yanoikuyae*	+	+	Consortium A Willow Bacteria Mix	GreenHousePoplar
WP1	*Rhodotorula graminis*	+	+	Consortium A Poplar Mix A Yeast Mix	GreenHousePoplar Field Poplar Isotope Dilution
PTD3	*Rhodotorula mucilaginosa*	+	n.d.	Consortium A Poplar Mix A Yeast Mix	GreenHousePoplar

Plant Materials

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

Plant Growth and Physiology

Experiment 1: Greenhouse single strain endophyte and consortia inoculation trial

Internally sterile Nisqually-1 ramets were transferred from tissue culture into individual 4-inch square pots (2.17 L "The Sqaure One" McConkey, Sumner, WA) containing Sunshine Mix #2 (SunGro, Bellevue, WA), a low nitrogen soil mixture. Plants were removed from the tissue culture media and rinsed with sterile distilled water to remove any remaining agar, then patted dry prior to weighing and potting. Transplanted cuttings were then watered with distilled water until the soil was completely saturated. The cuttings were allowed to acclimatize to the greenhouse conditions of average temperature 21°C and 14 hour photoperiod for 31 days. During this time the plants received only tap water irrigation. Inoculation occurred as described above.

A total of 63 pots, 7 replications of each of 9 endophyte treatments (listed in Table 1), were arranged in a randomized complete block design on one greenhouse bench. Drip trays were placed under each individual pot to prevent cross contamination through run-off. Each pot was

individually irrigated with tap water through an automated system. Irrigation frequency and duration was adjusted such that the pots were not allowed to flood nor dry out between watering. Additionally, each ramet received 100 mLs of ½ strength, nitrogen free modified Hoagland's media containing (g L^{-1}): 0.22 $CaCl_2(2H_2O)$, 0.17 K_2SO_4, 0.26 $MgSO_4(7H_2O)$, 0.136 KH_2PO_4, 0.015 NaFeDTPA(10% Fe) with 1 ml L^{-1} micronutrient solution containing (gL^{-1}: 0.773 H_3BO_3, 0.169 $MnSO_4$, 0.288 $ZnSO_4(7H_2O)$, 0.062 $CuSO_4(5H_2O)$ and 0.04 H_2MoO_4 (83% MoO_3)) weekly for the first two months post inoculation then 250 mLs twice monthly until the end of the experiment.

Initial green weight was measured at the time of transplantation from tissue culture to the greenhouse pots. Leaf chlorophyll was measured throughout the study and recorded as SPAD units using the nondestructive, handheld Konica Minolta SPAD 502 (Konica Minolta, Ramsey, NJ) chlorophyll meter. The SPAD units recorded are an average of three readings taken from the 2^{nd} leaf from the top of each plant. Plant height was measured as the distance between the root collar and the main shoot apex. Height measurements were recorded at 97 days after inoculation (dai) and again at 135 dai. Final measurements of total green weight, fresh root weight, and green leaf area were made 135 days after inoculation; calculated measurements include the root:shoot ratio, green weight gain, and change in height. Leaf tissue from plants inoculated with 3 of the endophyte consortia were sampled for dimethyl sulphoxide (DMSO) chlorophyll extraction as described by Richardson et al. (2002). Root, stem and leaf tissue samples were taken from each plant and oven dried at 65°C, combined and ground to a fine powder before being analyzed for total nitrogen and carbon content using a PE 2400 Series II CHN elemental analyzer (Perkin Elmer, Waltham, MA; CHN work carried out at the UW SEFS Chemical Analysis Center).

Experiment 2: Long term field trial

The experimental field site is located in the Charles L. Pack Experimental Forest, Pierce County Washington. The soil at this site is characterized as sandy, glacial outwash of the Indianola series (mixed, mesic Dystric Xeropsamments) (Gaulke *et al.*, 2006). This site is excessively drained, nutrient limited, and receives full sun. Prior to the installation of this study half of the site was covered in blackberry bushes and half of the site had been used the previous summer for a short term field trial with an annual crop described in Chapter 3. Site prep included removal of any existing vegetation along with the top layer of soil (approx. 30 centimeters inches) of soil followed by disk tilling. The 26 meters x 54 meters site is surrounded by a two meter tall exclosure to protect the poplar trees from deer and elk browse. Individual tree plots are arranged in eight rows of 18 plots with 2.5 meters between row plots and within rows. Available soil nitrogen analysis was performed for each plot two weeks prior to planting. Three evenly spaced soil cores from each plot were taken from the top 40 cm, mixed and bagged on site. The soil samples were held on ice during transportation from the field to limit any effects from biological nitrification. Nitrogen analysis was performed by the University of Washington's School of Environmental and Forest Sciences Chemical Analysis Center.

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

Cuttings from the lateral branches of two ramets of Nisqually-1 were collected in the weeks prior to the break of winter dormancy. The cuttings were planted to a depth of approximately 12 cm in separate 2.26 liter pots containing commercially available, low nutrient, potting mix, Sunshine Mix #2 (SunGro, Bellevue, WA). Total stem length and weight were

recorded prior to planting. The cuttings were allowed to root for 3 weeks and received irrigation with tap water as needed to maintain moist soil. Inoculations were carried out as described above using two single strain inocula, WP1 and WPB, one multi-strain consortium, Poplar Mix #2, and sterile media as a control. Pots containing cuttings that received the same endophyte inocula were grouped together 8 to a tray to help maintain soil moisture and avoid cross contamination after inoculation. The plants were inoculated two times, one week apart to ensure sufficient exposure. Fresh endophyte preparations were made for each inoculation. The inoculated poplar ramets were relocated to a glass house at the Charles L. Pack Experimental Forest and allowed to acclimatize for two weeks prior to planting in the field site. The ramets were carefully transplanted along with the potting soil so that the fragile root zone remained as undisturbed as possible. The transplantation of all ramets was done in a single day. This location receives substantial rainfall in the spring and fall months. Manual irrigation was applied during the first growing season to protect against excessive drought. Other site maintenance included the removal of competing plants, most grasses, within a 40 cm radius of each tree and manually trimming between rows. The second growing season received continued overgrowth maintenance but no further irrigation.

Tree height and diameter at 10 cm above the root collar were collected five times at nearly monthly intervals. Soil samples were gathered from each tree plot and the total available soil nitrogen content was measured to correspond with the growth measurements. Soil analysis was conducted at the SEFS Soil Analysis Laboratory. Dominant stem height was measured twice in the second growing season. Biomass was estimated non-destructively by calculating volume (V) assuming a paraboloid shape (Bruce & Schumacher, 1950) for the major stem and branches measured over 5 cm in length using the equation: $V = \frac{1}{2} AL$; where A is the sum of the

area from each stem and branch at 10 cm from its base and L is sum of the height of the major stem and the length of each branch from where it meets the stem.

Ramets from each inoculation treatment group were randomly chosen to investigate the effect of inoculations on leaf level physiology. Ten ramets were chosen from each inoculation treatment group for instantaneous CO_2 assimilation rate measurements in the first growing season. Gas exchange was measured using the LI-6400XT portable photosynthesis system (LI-COR, Lincoln, NE) from the second most developed leaf from the top of each ramet. In the first growing season instantaneous photosynthesis was measured at ambient and saturating CO2 concentrations, 380 ppm and 700 ppm respectively, with the block temperature of 25^{o}C, and the photosynthetically active radiation (PAR) set to 1500 µmol photons $m^{-2}_{leaf\ area}\ s^{-1}$. Leaf CO_2 assimilation rate (A_{max}), transpiration rate (E), and stomatal conductance (g_s) were recorded after a minimum stabilization time of four minutes. The photosynthetic response to carbon dioxide concentration (A/C_i) was measured in the second season growing from five ramets per inoculation treatment group selected from the same group measured the previous year. The CO_2 assimilation is controlled by the carboxylation rate of ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco), the rate of RuBP regeneration, and triose phosphate use (TPU) (Farquhar *et al.*, 1980; Sharkey *et al.*, 2007). A/Ci measurements were used to estimate the Rubisco-limited response (V_{cmax}), the rate of electron transport (J_{max}), and TPU using the estimator utility provided by Sharkey et al. (2007). These measurements were made at 25°C and 1500 PAR. In both years, the leaf used for gas exchange measurement was collected immediately following the final data recording. The leaf tissue was then transported on ice and held at -80°C. Leaf nitrogen (leaf N) content and total chlorophyll content was quantified as described above. Photosynthetic nitrogen use efficiency (PNUE) was calculated as net photosynthesis per leaf nitrogen content.

In August of the second growing season, three trees from each inoculation treatment that had previously been measured for gas exchange data were sacrificed to measure the dry above ground and root biomass. Grassy ground cover along with the corresponding thin layer of roots was removed from each plot. For the determination of the above ground biomass each of the selected trees was cut at the root collar bagged and placed in a 75^{o}C oven for one week. The above ground biomass reported is the total dry mass of all stems and leaves of each ramet. For the determination of the below ground, root, biomass of a 50 cm^{3} section of soil surrounding each tree was removed and passed through a ¼" screen on site. The root ball and remaining root tissue collected from the soil was bagged and placed in the 75^{o}C oven along with the above ground biomass for one week. Prior to weighing the dry root tissue, contents of each bag were passed through an $^{1}/_{8}$" inch screen to remove any persistent soil. Collection of root mass from field specimens is inherently difficult (Vogt *et al.*, 1998). The current method of comparing total root mass per volume soil was chosen as the most practical for this experiment.

Prior to drying, fresh root, stem and leaf tissues of equal size were removed from each tree for the purpose of endophyte isolations. All tissue was washed with detergent to remove soil and rinsed several times with clear tap water. Root and stem tissue was surface sterilized for 5 minutes in 1% sodium hypochlorite, then washed 4 times with sterile distilled water. Leaf tissue was surface sterilized for 2 minutes in 1% sodium hypochlorite, and then washed 4 times with sterile distilled water. Approximately 100 mg of each sample was then macerated in 500 ml of isotonic solution and serially diluted using aseptic technique in a sterile hood then 100 µl of each of the final dilution (10^{-4}) was plated on non-selective MG/L agar and nitrogen free NFCCM (Rennie, 1981) agar. Colony counts were performed after 72 hours incubation at room temperature. In addition, freshly cut explants of the surface sterilized tissue were plated directly on both MG/L and NFCCM.

Experiment 3: Estimation of Biological Nitrogen Fixation through Isotope Dilution

A ^{15}N isotope dilution assay was conducted with the objective to estimate the amount of nitrogen (N) gained through biological fixation within poplar cuttings inoculated with the diazotrophic endophyte consortium Poplar Mix #2. Nitrogen occurs naturally in two isotopic forms, ^{15}N and ^{14}N with ^{14}N is in much greater abundance. The ^{15}N content in nitrogen fixing plant tissue is the result of the native N acquired through soil, N_2 from the atmosphere and N from the applied ^{15}N fertilizer (Danso et al, 1993). To estimate the amount of nitrogen acquired through fixation, nitrogen derived from air (Ndfa), the ^{15}N content of non-fixing reference plants is compared to that of the nitrogen fixer. In this assay the uninoculated control plants serve as the non-fixing reference for the inoculated plants (Oliveira *et al.*, 2002). Thus the equation used to estimate the %Ndfa is as follows:

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

Rooted cuttings of the cottonwood hybrid, *Populus trichocarpa* x *P. deltoides* clone H11-11 were weighed prior to transplanting to 4-inch square pots, with individual drip pans, containing low nutrient Sunshine Mix #2. The pots were prepared by adding 0.35 Kg of root media per pot followed by 700 ml of tap water to wet the soil. The cuttings were each given 200 ml of ½ strength modified Hoagland's solution containing 4mM NH_4NO_3 and then maintained with tap water for a two month acclimation period. The greenhouse temperature was kept at an average of 21°C with a 14 hour photoperiod. Five clonal ramets were randomly chosen for each of the three inoculation treatments; live endophyte consortium, heat killed endophyte consortium, or uninoculated control. The endophyte suspensions were prepared as described above for a total OD_{600} of 0.064 in 30 ml for the multi-strain consortium where each strain is added in equal proportions. The multi-strained consortium consisted of the endophytic bacteria

WPB, WP5, WP7, WP8, WP19, PTD-1 and the yeast WP1 (described in Table 1). The heat killed endophyte suspension was prepared from a portion of the live consortium by subjecting it to an autoclave cycle of 15 minutes at 121°C and 15psi. Plants were inoculated with 30 ml of endophyte suspension; either live, heat killed, or 30 ml of sterile NFMS. To each pot 200 ml of ½ strength Hoagland's solution containing 23.78 mg of $(NH_4)_2SO_4$ with 10% excess ^{15}N was added 13 days after inoculation. Plants were fertigated with 250 ml nitrogen free ½ strength Hoagland's solutions every two weeks and watered with distilled water as needed. Sixty-four days after receiving the ^{15}N the plants were harvested and cleaned of root media using distilled water and green weight measurements of the whole plant were taken. Equal portions of root tissue were removed and set aside for endophyte re-isolation as described above. Root, stem and leaf tissue was then separated and oven dried at 70^{o}C for 3 days. The dry tissue was again weighed and then ground to a fine powder with mortar and pestle. Dried tissue samples were sent to the Alaska Stable Isotope Facility (ASIF) at the Water & Environmental Research Center at the University of Alaska Fairbanks. Stable isotope data was obtained using continuous-flow isotope ratio mass spectrometry (CFIRMS). Stable isotope ratios were reported as parts per thousand (‰) deviation from the international standards PDB Air (nitrogen).

Statistical Analysis

All experiments were designed for contrast of treatment means to the corresponding uninoculated control group. Data was analyzed using the SAS statistical software version 9.3 (SAS Institute Inc., Cary, NC). Where appropriate, the means of final weight or weight gain over time were adjusted with the "weight" statement by initial green weight and the initial total available N of the soil in the field trial was used as a covariate. Duncan's multiple range test (DMRT) and the CONTRAST statement within PROC GLM were used to compare endophyte

treatment means between the endophyte inoculation treatments. Unless otherwise stated, all tests were performed with alpha = 0.05.

Results

Experiment 1: Greenhouse single strain endophyte and consortia inoculation trial

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

Plant height at 97 days after inoculation (dai) was significantly taller between the three consortia inoculation treatments, Consortium A, Poplar Mix A, and Poplar Mix #1 and the uninoculated control group with 90% confidence (Table 2). This difference, however, was no longer statistically significant at 135 dai. The calculated plant height change between the two measurement dates (height at 135 – 97 dai) resulted in negative values for individual ramets and an overall (*i.e.* group mean) loss of height for WW7 inoculated ramets. This suggests that the rate of growth for stem height was even among inoculation treatments and not sufficient to overcome the influence of media settling or disturbance from irrigation on the variability of data collection for this metric. Ramets inoculated with the Poplar Mix #1 had significantly more total

leaf area at 97 dai and 135 dai than the control group at the 99% and 95% level of confidence respectively. Ramets inoculated with Consortium A had significantly more leaf area 97 dai at the 90% confidence level, however difference in leaf area was no longer significant 135 dai (Table 2).

Table 2 Greenhouse gnotobiotic experiment. Growth parameters of ramets of *P. trichocarpa* clone Nisqually-1 inoculated with multi- strain or single strain diazotrophic endophytes. Unweighted Means of growth variables (standard error). Biomass gain and allocation measured 135 days after inoculation (dai) with diazotrophic endophyte consortia or single strain isolates as indicated. Asterisks denote significant differences from the Control for *alpha = 0.05 and ** alpha=0. 01; n = 7

Endophyte Treatment	Final Green Weight (g)	Weight Gain (g)	Root Weight (g)	Root/Shoot Ratio	Height Change (cm)	Height (cm) 97dai	Height (cm) 135 dai
Consortium A	14.69 (4.00)**	13.88 (3.87)**	13.40 (3.32)**	6.54 (0.629)**	0.22 (0.15)	16.23 (2.51)**	16.22 (2.62)
Poplar Mix A	11.34 (2.37)	10.69 (2.26)	9.99(1.64)	6.69 (0.49)**	0.31(0.21)	16.51 (1.58)*	16.27 (1.52)
Willow	15.47 (2.09)*	14.74 (1.96)*	13.02 (1.66)**	7.9 (0.39)**	0.33 (0.13)	15.46 (1.23)	15.79 (1.18)
Poplar Mix #1	17.88 (3.46)**	16.89 (3.20)**	14.95 (2.97)**	5.71 (0.53)*	1.04 (0.72)	16.14 (1.22)*	17.19 (1.04)
Yeast Mix	10.64 (1.83)	9.86 (1.74)	9.16 (1.55)	6.58 (0.65)	0.27 (0.144)	13.97 (1.66)	14.24 (1.09)
WP5	9.78 (1.20)	9.11 (1.14)	8.35 (1.00)	6.11 (0.56)	0.30 (0.22)	14.62 (1.37)	14.92 (1.26)
WW7	9.66 (1.48)	8.96 (1.39)	8.077 (1.22)	5.32 (0.43)	-0.13 (0.27)	13.37 (1.10)	13.24 (1.55)
WP1	9.37 (2.16)	8.67 (2.03)	7.61 (1.78)	4.64 (0.45)	0.37 (0.48)	14.84 (1.59)	15.21 (1.56)
Control	8.81 (1.59)	8.03 (1.55)	6.57 (1.08)	4.35 (0.44)	1.08 (1.05)	14.19 (0.76)	16.4 (1.02)

Endophyte inoculation had a significant effect on plant carbon (p =0.0243). Total plant carbon content was higher for the control group ramets than those inoculated by all endophyte treatments except for those inoculated with Consortium A (Fig. 1a). On the whole, endophyte inoculation increased total nitrogen content (p = 0.1731). An increase in total plant nitrogen of up to 25% (Poplar Mix A) was observed for inoculated plants; however not all endophyte treatments increased the total plant nitrogen content over the control group (Fig. 1b).

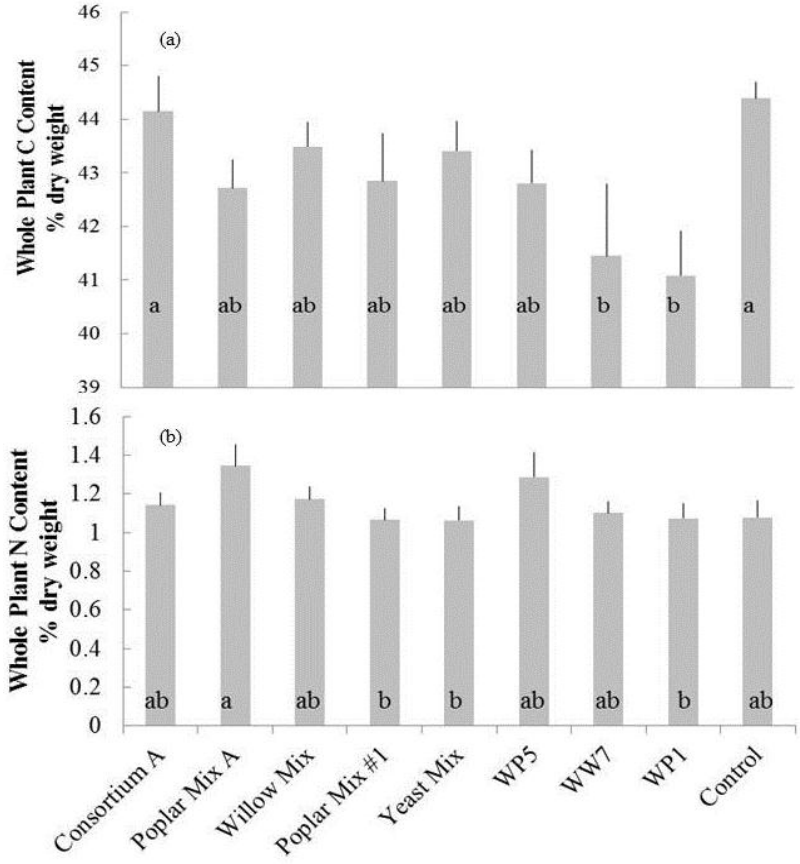

Figure 1: Plant Carbon and Nitrogen Content for Greenhouse Gnotobiotic Experiment. a.) Whole plant carbon (C) content. b.) Whole plant nitrogen (N) content. Treatment group means for multi-strain endophyte consortia and single strain inocula compared to uninoculated, internally sterile control. Bars indicate group mean with standard error lines with different letters indicate means are significantly different at alpha = 0.05 using DMRT, n =7

Total leaf area and leaf SPAD response to endophyte inoculations was varied (Table 3). Significant differences in leaf SPAD measurements compared to the control group (24.33) were

observed for Poplar Mix A (26.87, $p \leq 0.01$) and Willow Mix (26. 53, $p \leq 0.05$) inoculated ramets 97 dai. The total leaf area was significantly larger 97 dai for Consortium A (89.65cm^2, $p \leq 0.05$), and Poplar Mix #1(105.51cm^2 $p \leq 0.01$) treatments over the control (76.33cm^2). Poplar Mix A (25.79, $p \leq 0.01$), Willow Mix (23.24, $p \leq 0.01$), Poplar Mix #1 (21.56, $p \leq 0.05$), and the

Table 3: Phenotypic Response Greenhouse Gnotobiotic Experiment. *P. trichocarpa* clone Nisqually-1 ramets inoculated with multi- strain or single strain diazotrophic endophytes. Unweighted Means of Leaf physiology variables (standard error). SPAD is a nondestructive measurement of leaf chlorophyll reported in relative units, higher SPAD value corresponds to higher leaf chlorophyll. Asterisks denote significant differences from the Control for •alpha = 0.1, * alpha = 0.05 and ** alpha=0.01. dai: days after inoculation, n = 7

Endophyte Treatment	Leaf Area (cm^2) 97 dai	Leaf Area (cm^2) 135 dai	SPAD 97 dai	SPAD 135 dai
Consortium A	89.65 (23.06)*	68.21 (19.45)	20.69 (2.49)	19.81(2.66)
Poplar Mix A	69.53 (11.32)	53.50 (8.90)	26.87 (2.08)**	25.79 (1.78)**
Willow	72.36 (7.92)	72.84 (20.50)	26.53 (1.11)*	23.24 (1.06)**
Poplar Mix #1	105.51 (18.77)**	89.97 (14.62)	21.43 (0.59)	21.56 (1.13)*
Yeast Mix	67.09 (10.94)	56.79 (10.66)	23.7 (1.60)	21.33 (1.73)•
WP5	66.26 (7.30)	55.64 (13.47)	24.22 (2.18)	17.08 (1.36)
WW7	66.64 (9.53)	55.07 (8.95)	22.29 (1.57)	19.66 (2.24)
WP1	69.64 (15.04)	68.47 (14.33)	23.17 (2.58)	18.40 (1.82)
Control	76.33 (12.20)	61.11 (14.24)	24.33 (2.06)	17.09 (1.67)

Yeast Mix (21.33, $p \leq 0.1$), treatment groups had significantly higher SPAD measurements than the uninoculated control (17.09) 135 dai. The overall trend in SPAD readings for the remaining inocula was significantly different from the uninoculated control group. Total leaf area demonstrated a slight overall decline at 135 dai but the differences between treatment groups were not statistically significant. Four treatment groups, Consortium A, Willow Mix, Poplar Mix #1, and the uninoculated Control were analyzed for total extracted chlorophyll content. Endophyte inoculations had an overall significantly positive impact on total extractable chlorophyll ($p = 0.073$). Ramets inoculated with Willow Mix and Poplar Mix #1 each contained

15% more and ramets inoculated with Consortium A had 4% more extracted total chlorophyll than the Control group (Fig. 2).

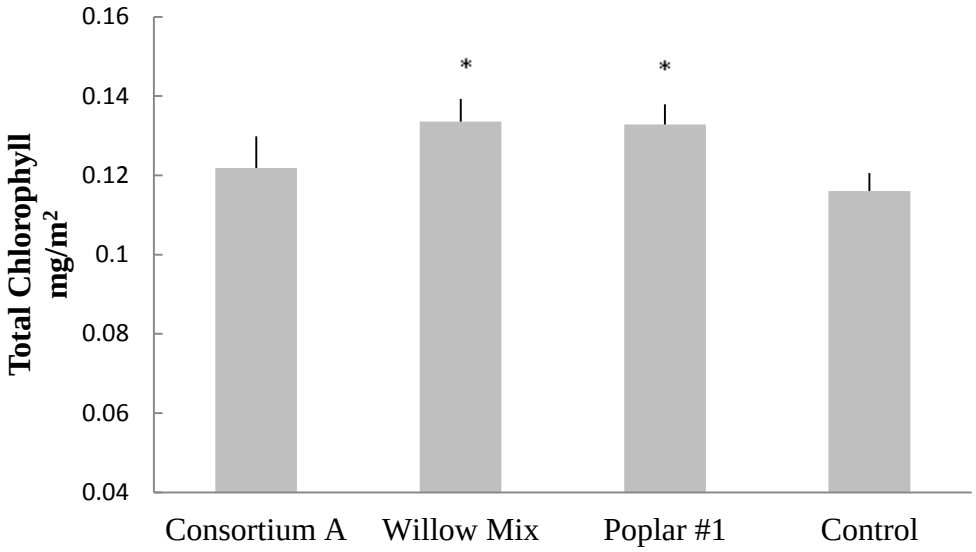

Figure 2: Total Chlorophyll Content of Select Endophyte Treatments. Comparison of total DMSO extracted chlorophyll between clonal ramets inoculated with multi-strain endophyte consortia and the internally sterile, uninoculated control. Bars indicate group means with standard error lines, * indicates significant difference from Control at alpha = 0.05, n = 7

Experiment 2: Long term field trial

Endophyte inoculation had a significant effect on main stem height ($p < 0.001$) and above ground volume ($p = 0.0023$). The inoculation treatment WP1 produced the tallest ramets and the most above ground biomass gain as measured by increase in total volume over the course of the first growing season. Total increase in main stem height and above ground volume accumulated through July of 2012 for WP1 inoculated ramets were 35% and 38.4% more than the uninoculated control group (Fig. 3). Height gain and volume gain were most negatively affected by inoculation with WPB while plant growth was nearly equal between the Poplar Mix #2 consortium inoculated ramets and the uninoculated control group. Endophyte inoculation also had a significant effect on the total number of branches, sylleptic and proleptic, measured mid-

growing season in year two ($p = 0.060$). The difference between inoculation treatments that

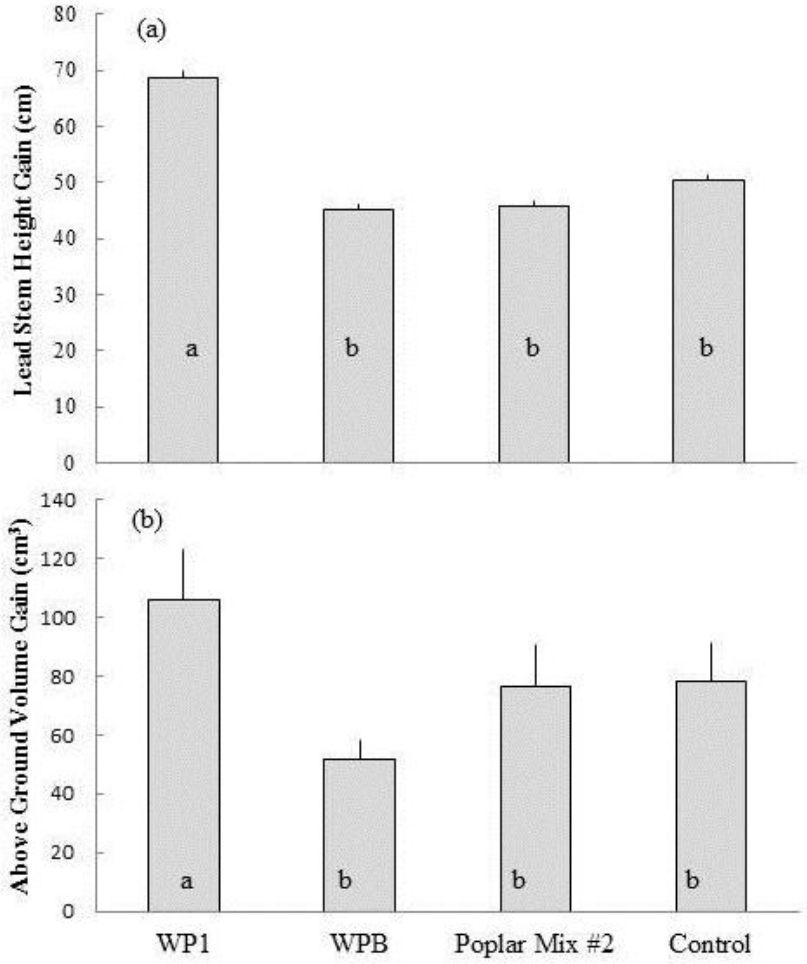

Figure 3: Field Trial Comparison of Biomass Gain. a) Comparison of height gain and b) above ground volume gain for a 12 month period. Treatment means for endophyte inoculated poplar Nisqually-1 ramets compared to the uninoculated control group. Bars with different letters indicate means are significantly different at alpha = 0.05 using DMRT n = 36.

emerged corresponds with above ground volume and height gains. Ramets inoculated with WP1 have the most branches (9.5) while those inoculated with WPB (6.4) have the least with no difference between the Poplar Mix #2 (7.4) inoculated ramets and the control group (7.6) (Fig. 4).

The nitrogen content available for plant uptake was measured at several intervals during the first growing season and again in the fall as trees transitioned to dormancy. There was no statistically significant difference between the endophyte inoculation treatment groups for NO_3N,

NH_4H or the total available N content of the soil from April to September. Soil nitrogen content increased for all treatment groups in November with the largest increase occurring in the soil within plots of the endophyte treatment group WP1 (Fig. 5). Soil nitrogen was measured again in July of the second growing season. No statistical differences were observed between inoculation treatments for soil NO_3N, NH_4H, or the total available N measured in the second season. Within the endophyte inoculation treatment groups, the NH_4H content increased slightly from the previous July. However, the decreases of NO_3N within all treatment groups led to a decrease in the total available N from the previous July (data for NH_4H and NO_3N not shown).

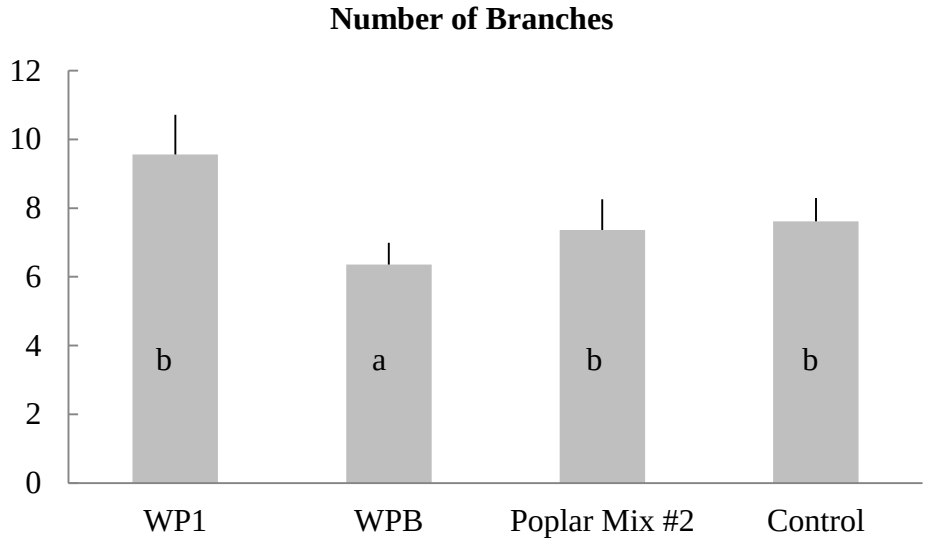

Figure 4: Endophyte Inoculation Effect on Branch Production. Treatment means for endophyte inoculated poplar Nisqually-1 ramets compared to the uninoculated control group. Bars represent treatment group means with standard error lines for total branch count. All branches measuring over 5cm in length were included in count. Asterisk indicates significant difference from Control. Bars with different letters indicate means are significantly different at alpha = 0.05 using DMRT, n=36

The net CO_2 assimilation rate recorded in both the first and second years was not significantly different at the ambient (380 ppm) or saturated (700 ppm) CO_2 concentrations between inoculation treatment groups. No significant differences were observed for stomatal conductance (g_s) or transpiration rate (E) CO_2 (380 ppm). At saturating CO_2 (700 ppm) there were no significant differences between inoculation groups for g_s, however WP1 inoculated

ramets recorded a significantly increased rate of transpiration (Table 4). The calculated photosynthetic nitrogen use efficiency (PNUE) was not significantly different between treatments at either concentration of CO_2 (data not presented).

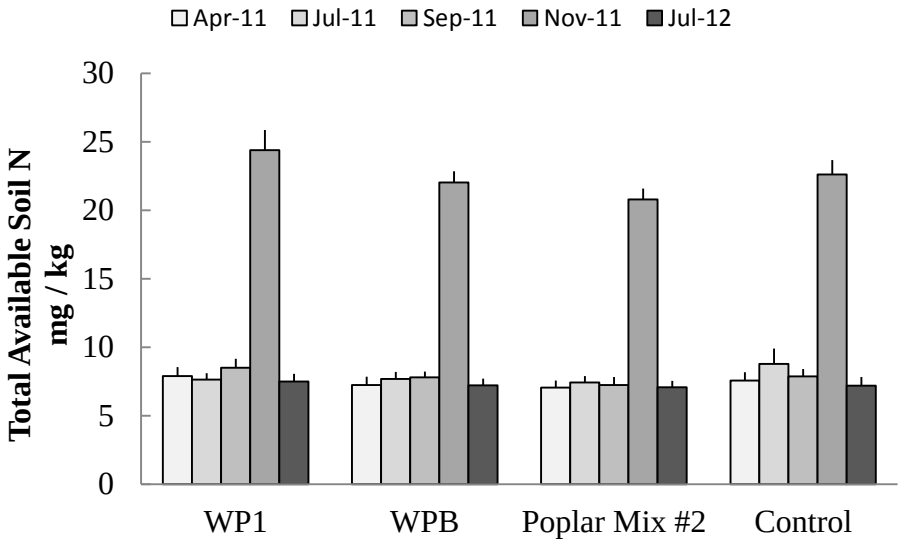

Figure 5: Change in Soil Available Nitrogen (N). Mean soil N over time where Total Available N is the sum of the NO_3N and NH_4H soil content. Bars represent treatment group means with standard error lines. n = 36.

Table 4: First Year Leaf Physiology Data. Inoculation group means ± standard error, n= 36. Leaf physiology is not significantly affected by endophyte inoculation at the ambient CO_2 (A_{380}) concentration. At saturating CO_2 (A_{700}) concentration inoculation with WP1 significantly increases the transpiration rate. Total Chlorophyll content was significantly affected by endophyte inoculation (ANOVA p = 0.03). Symbols indicate significant difference between the means of treatment group and control: • alpha = 0.1 and ** alpha = 0.01.

Inoculum	A_{380} µmol CO_2 m^{-2} s^{-1}	Transpiration (E) mmol H_2O m^{-2} s^{-1}	Conductance (g_s) mol H_2O m^{-2} s^{-1}	LeafN % dry weight	Total Chlorophyll mg/cm^2
WP1	15.38 ±0.70	3.83 ±0.17	0.23 ±0.0084	2.49 ±0.18	0.028 ±0.0015•
WPB	14.98 ±1.31	3.79 ±0.16	0.23 ±0.0096	2.51 ±0.29	0.032 ±0.0020
Poplar Mix #2	14.51 ±0.77	3.83 ±0.25	0.23 ±0.0113	2.40 ±0.25	0.025 ±0.0023**
Control	15.54 ±0.65	3.50 ±0.13	0.21 ±0.0064	2.79 ±0.20	0.033 ±0.0026

Inoculum	A_{700} µmol CO_2 m^{-2} s^{-1}	(E) mmol H_2O m^{-2} s^{-1}	(g_s) mol H_2O m^{-2} s^{-1}
WP1	22.36 ±3.2	6.48 ±0.45•	0.24 ±0.0246
WPB	24.59 ±6.6	5.80 ±1.35	0.21 ±0.0764
Poplar Mix #2	21.11 ±4.5	5.87 ±0.53	0.22 ±0.0317
Control	23.18 ±2.9	6.16 ±0.42	0.22 ±0.0230

Endophyte inoculation treatments did not have a significant effect on the photosynthetic response to changes in CO_2 concentration (Fig. 6). There was also no significant difference for the estimated rates of Vcmax, J_{max}, and TPU. Leaf nitrogen content was not found to be significantly different between treatment groups at any time. The total chlorophyll content recorded in the first growing season however was significantly affected by endophyte inoculation (ANOVA, p = 0.03). Poplar Mix #2 inoculated plants had the lowest chlorophyll of all treatments and remained lowered in the second season but the difference was no longer statistically significant.

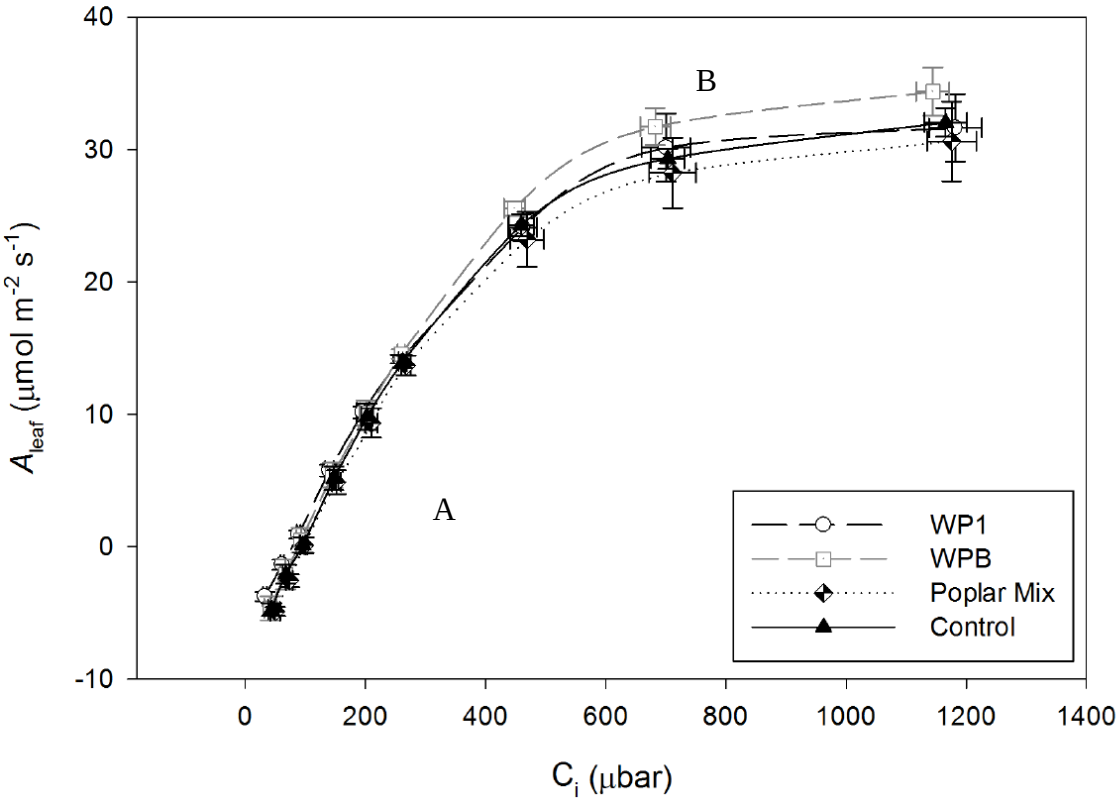

Figure 6: Second Year Carbon Assimilation Data. A/Ci curves for each treatment represent the treatment average. No change in slope between treatments at A indicates the Rubisco activity is not altered between the treatment groups. Some effect on the rate of RuBP regeneration is observed between endophyte treatments, B, however this difference is not statistically significant; n = 5.

The biomass data collected from the destructively harvested samples was only statistically significant for biomass allocation (root:shoot ratio, $p = 0.098$). The results, however, agree with the findings that WP1 inoculated ramets have considerably more above ground biomass than all other treatments, with an increase of dry biomass of 44% over the control group. Ramets inoculated with the Poplar Mix #2 gained only 10% above ground biomass over the control and WPB inoculated ramets measured 33% less above ground biomass than the uninoculated control group (Table 5). Total dry root mass was nearly the same for all endophyte inoculation treatments. While the WPB inoculated ramets gained less overall, the allocation to

the roots was significantly greater than any other endophyte treatment with 44% (95% confidence) of the total dry biomass going to root tissue. WP1 and Poplar Mix #2 inoculation seemed to have an opposite effect on the ramets with the majority of the biomass (72% and 70% respectively) allocated to the above ground tissue. Biomass allocation also favored the above ground tissue for the uninoculated control group with 65% more biomass above ground than below.

Table 5: Biomass Allocation of poplar clone Nisqually-after Endophyte Inoculation. Mean below ground and above ground biomass ±standard error. The root to shoot ratio is indicative of the biomass allocation favoring root growth in WPB inoculated ramets. The asterisk indicates means significantly different than the control group at alpha = 0.05, n =3.

Inoculum	Root Dry Mass (g)	Total Above Ground DryMass (g)	Root:Shoot Ratio
WP1	63.07 ±4.47	161.8 ±34.43	0.41 ±0.06
WPB	59.33 ±10.13	75.37 ±20.56	0.89 ±0.21*
Poplar Mix #2	53.03 ±7.88	124.1 ±12.82	0.44 ±0.10
Control	60.70 ±21.82	112.73 ±60.17	0.67 ±0.12

The endophyte reisolation assay revealed growth on both nutrient rich and nitrogen free media for all samples tested. Growth was also visible near the freshly cut ends of the explant tissue on both types of media for all trees sampled. Colony morphology was noted to be similar to that of the known cultures used for the inoculations. Further identification through the necessary molecular techniques was prohibited by cost.

Experiment 3: Estimation of Biological Nitrogen Fixation through Isotope Dilution

Prior to inoculating the plants, 100 µl of each endophyte solution and the sterile media were spread on nutrient rich, MG/L agar to check for colony count and effectiveness of the autoclave treatment. The live mix contained 3.9×10^8 colony forming units (CFU) per milliliter with multiple colony types of the expected morphology. The heat killed (HK) endophyte

solution was plated without dilution and resulted in 870 CFU per milliliter with the same colony morphology. The sterile media grew no colonies. Therefore, calculations for %Ndfa are based on the uninoculated control plants as the non-fixing reference. The ‰ ^{15}N atmospheric excess did not differ in root tissue between the treatment groups; however it was lower in the stem tissue and significantly less in leaf tissue for live mix and HK inoculated plants (Fig. 7a). BNF was calculated as 65% and 18% based on isotope dilution values in leaf tissue from live and HK inoculated plants respectively. Calculations of BNF based on leaf and stem tissue combined resulted in 45% for live and 43% for HK inoculated plants. Nitrogen content (% dry weight) was higher in the root tissue for live mix inoculated ramets compared to the HK and control groups and lower in the stem tissue (Fig. 7b). The nitrogen content of the leaf tissue was the same for the three treatments. Together, this indicates that nitrogen fixation occurs in the leaf tissue. Plant growth promotion was observed in the HK inoculated plants while the live mix inoculated ramets exhibited less biomass gain over the duration of the 2 month assay than the uninoculated control plants (Fig. 8). Further, bacterial growth was recovered on both the nutrient rich and nitrogen free media from all ramets. Therefore, the calculated %Ndfa is an estimate of the biological nitrogen fixation contribution from inoculations over that which naturally occurs.

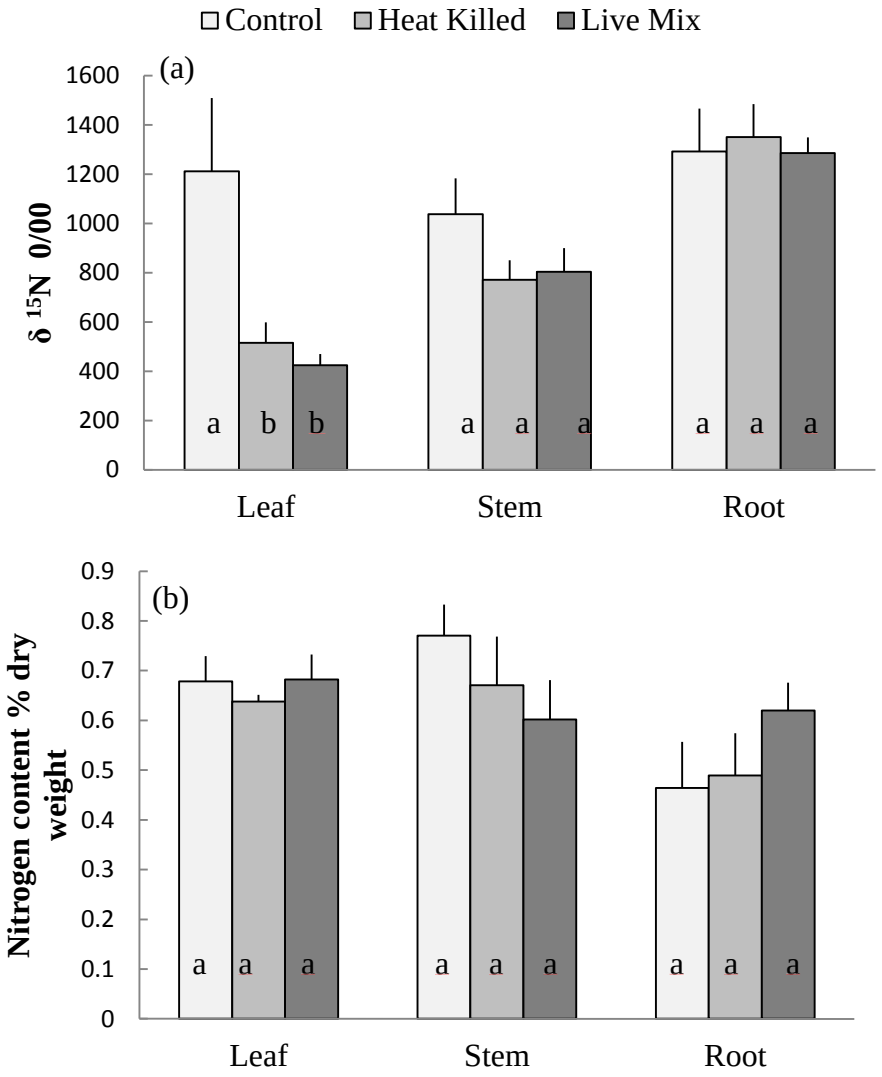

Figure 7: Greenhouse Isotope Dilution Assay to Estimate BNF (biological nitrogen fixation). Endophyte inoculated pants were plants were grown in ^{15}N enriched soil for 64 days; a) Atmospheric excess ^{15}N concentration by plant tissue, b) total nitrogen content by plant tissue. Bars with the same letter indicate no significant difference within plant tissue type between treatment means, alpha = 0.05, n =3.

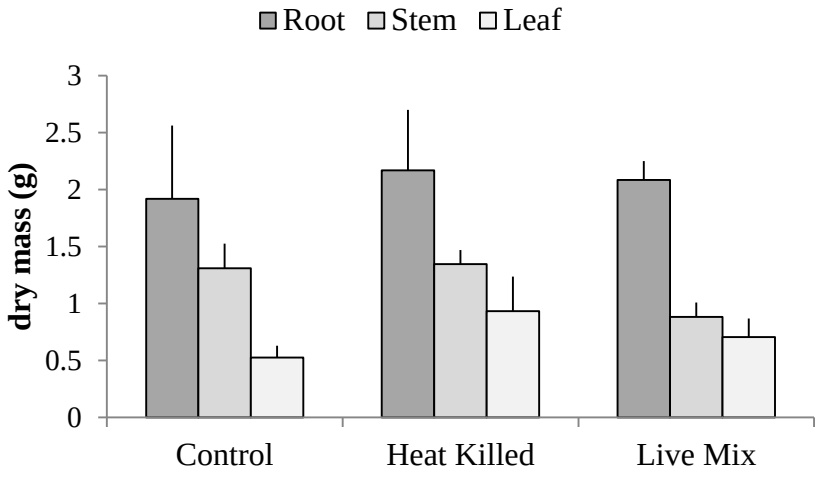

Figure 8: Isotope Dilution Assay Biomass Allocation. Ramets of the cottonwood hybrid clone H11-11 were inoculated with Live Mix (Poplar Mix #2) endophyte consortium, Heat Killed (Poplar Mix #2 – autoclaved prior to inoculation), or sterile media (uninoculated Control). Bars represent treatment means at the end of 64 days. Error bars are one standard error, n =3. No significant differences between means within tissue type.

Discussion

The results of the greenhouse trial support the hypothesis that multi-strain endophytic communities have a greater influence on plant biomass than single strain inoculations. This experiment was conducted on genetically identical ramets of *Populus trichocarpa* clone Nisqually-1 under controlled environmental conditions. Therefore, the observed phenotypic variations in response to nitrogen limitation between the inoculation treatment groups indicate a strong influence on host plant physiology by the interaction between the host plant and the endophytic microbial community (Falconer & Mackay, 1996). Further, different combinations of bacterial and fungal (i.e. yeast) endophytes resulted in differing levels of the promotion of biomass production. The level of variation within treatment groups is also notable suggesting that colonization by members of each consortium may have differed within each host plant.

The isotope dilution assay demonstrated biological nitrogen fixation in endophyte inoculated ramets of the cottonwood hybrid clone H11-11. The nitrogen content measured for

each plant tissue was very low for all treatment groups indicating a nitrogen limited system was achieved. No significant difference in foliar nitrogen content was observed between treatment groups while the live endophyte inoculated plants demonstrated a 65% dilution of the ^{15}N content of the non-fixing control. Similar isotope dilution assays in forest tree species have demonstrated no correlation between foliar nitrogen content and %Ndfa (Anand & Chanway, 2012; Bal & Chanway, 2012). In the current assay, the HK inoculum was found to have sustained some viable cells therefore the 18% dilution of the ^{15}N content of the non-fixing control is considered to be due to the diazotrophic activity of surviving endophytes. No plant growth promotion was observed as a result of inoculation with the live Poplar Mix #2 endophyte consortium after the 64 day assay. Anand and Chanway (2012) demonstrate plant growth promotion due to inoculation with diazotrophic endophytes occurs after an initial suppression of plant growth. They hypothesize the delay is a result of the energy costs for nitrogen fixation and results indicate that plant growth promotion by diazotrophic endophytes in long lived tree species becomes greater over time. Follow up studies of the long-term field trial will allos us to see if this is true in poplar.

Poplar trees are known to quickly colonize nutrient poor soil, and can persist for up to 200 years(Van Pelt *et al.*, 2006). The hypothesis that plant growth promotion is due to in part to biological nitrogen fixation is supported by the data reported here but needs to be investigated further. Data from the ^{15}N isotope dilution assay and the soil nitrogen content data from the field trial provide evidence that Populus has the capability to deposit nitrogen to nutrient limited soils through biological nitrogen fixation. Many of the endophytes used in these experiments are believed to be capable of fixing atmospheric nitrogen as evidenced by the presence of the *nif*H gene and strong growth in nitrogen free media; additionally, some of the strains have been shown to produce and elute nitrate and ammonia in solution (Doty, unpublished data). The endophytes

in this study were originally isolated from low N soils along the Snoqualmie River in Western Washington. The nitrogen fixation observed in this study would support poplar colonization of these nutrient deficient sites.

The soil nitrogen content surrounding the individual trees in the field trial was not remarkably lowered in spite of the rapid gain in biomass over the course of the growing season. There was a pulse of nitrogen in the November sample that was over two times larger than the other sample periods. The substantial deposit of nitrogen observed just as the trees transitioned into dormancy cannot be attributed to the decomposition of leaf litter only and is unlikely to have come from other symbiotic systems in the proximity of the tree plots given the uniformity of the data across the treatment groups. One explanation is that nitrogen is translocated to roots prior to leaf abscission. A pulse-chase of labeled ^{15}N would allow testing of the hypothesis that N fixed in the leaves was then translocated and deposited in the roots. Further investigations including assays that allow for the measurement of the contribution of biological nitrogen fixation from the soil microbial community are needed.

The hypothesis that endophyte inoculations alter biomass production by influencing the net CO_2 assimilation rate was tested by evaluating the influence of endophyte inoculation on leaf level photosynthetic capacity. Net CO_2 assimilation rate was not altered for poplar ramets between the endophyte inoculation treatment groups. This is consistent with the findings of Rogers *et al.* (2011) who reported no significant effect on leaf level physiology after inoculations with the plant growth promoting endophyte *Enterobacter* sp. 638. Endophyte inoculations did, however, contribute to differences in total leaf area and above ground biomass. Total CO_2 assimilation is likely to have increased in plants with more leaf area given the same net CO_2 assimilation rate. Endophyte inoculations do not have a negative impact on the photosynthetic

apparatus while contributing positively to the total net CO_2 assimilation rate by influencing leaf production.

Field experiment data provide evidence that phenotypic plasticity is a result of the interaction between the host plant and the endophytic microbial community. Differences were observed for biomass gain and allocation as well as the occurrence of sylleptic and proleptic branches. Branch formation within poplar species has been well characterized and is known to be under genetic control. Phenotype variance observed within species or the same genotype being the result of the variation of the environment (Wu & Hinckley, 2001; Ma *et al.*, 2008). In other words, phenotypic plasticity is controlled by the interaction between the genotype and the environment (GxE). The role of branching in selection for high biomass production crops is, as yet, not well defined. Traditionally, forest tree breeding programs have focused on wood quality traits and favor pedigrees that produce non-forking, straight stems. More recently, forest trees such as poplar have gained attention as being valuable for rapid biomass production for biofuels. Additional to the short rotation age, poplar species regenerate well after coppicing. This study suggests some endophytes increase branching patterns, and therefore they may be useful in silage systems to enhance biomass production. It may be that shorter, bushier genotypes are favored for biomass production; genotype x environment (in this case endophyte colonization) could enhance this growth form. Given the correlation between growth characteristics and wood chemistry (Novaes *et al.*, 2009) more investigation is needed to determine the potential for endophyte inoculations to tailor the production of biomass for bioenergy.

Together, these experiments support the overall hypothesis that plant growth and vigor is directly related to the composition of the endophytic community within the host plant. Data from the isotope dilution assay provides evidence of biological nitrogen fixation in poplar inoculated with diazotrophic endophytes. Because this trial concerns clonal ramets grown under

common garden conditions; it can be assumed that the observed variation within phenotypic traits is due to a genetic by endophyte interaction (Falconer & Mackay, 1996). Therefore, genetic breeding and engineering programs for biomass crops would be wise to include potential gain through the alteration of the composition of the endophytic microbial population. Important information may be gained from future research focused on the analysis of the colonization patterns within individual ramets. In addition the specific interaction between the host plant and the endophytic community should be analyzed through gene expression and metabolic assays.

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Chapter 3

Effects of Cross Host Species Inoculation of Nitrogen Fixing Endophytes on Growth and Leaf Physiology of Maize

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Introduction

There is a growing emphasis on sustainable food and energy crop production that maintains high productivity while minimizing inputs such as fertilizer, herbicide, and insecticide that are both economically and ecologically costly. Major gains have been made by genetic selection of high yielding varieties and matching genotypes to specific environmental conditions. However much of this improvement has been made within the context of modern agricultural practices that involve the routine application of chemical fertilizers. The improvement of non-leguminous crop species to become self-sufficient in terms of nitrogen fertilizer is not a new concept (Cocking, 2005). Indeed, since the discovery of *Rhizobium* in the early 19th century, scientists have identified numerous bacterial and fungal species with growth promoting effects captured by plants either through rhizospheric or endophytic relationships (Sturz & Nowak, 2000; Bhattacharjee *et al.*, 2008; Hirsch, 2009). The term endophyte, meaning within plant, is given for fungal and bacterial microorganisms living within plant tissue without causing disease Wilson (1995). Symbiotic relationships between endophytes and their plant hosts are likely the result of selective pressure for plant association with specific symbionts, those which provide the maximum benefits to their hosts. The benefits to the plant might include growth stimulation

through the production of growth hormones, nitrogen fixation, enhanced nutrient uptake, and protection from potential pathogenic colonizers (Latch, 1993; Malinowski *et al.*, 2000; Khan, AL *et al.*, 2012; Taule *et al.*, 2012). Endophytic relationships also bestow both abiotic and biotic stress tolerance to plant hosts (Rodriguez *et al.*, 2008; Redman *et al.*, 2011). These interactions suggest endophytes have co-evolved toward symbiosis with plant hosts. The ability of relatively fast adaptation and genetic evolution within microbial communities compared to that of the more complex host plants suggests that it is in part the microbial element allows for timely adaptation to environmental challenges. This rapid adaptation of microbes hints at the potential broad applicability of endophytic relationships; recently reported cross species inoculations with Class 2 fungal endophytes demonstrated their ability to confer abiotic stress resistance to rice (Redman *et al.*, 2011). The findings of Redman et al. (2011) highlight the role environmental pressures play in contributing to the endophytic biome of the host plant; e.g. endophytes that confer salt stress tolerance are likely to be isolated from plants thriving in a high salt environment. Logically, endophytes isolated from plants growing under nutrient limitation (e.g., nitrogen), are likely to contribute to the nutritional demands of the host. Moreover, with successful cross host applications, endophyte inoculations can be a critical tool for adapting crops to climate change that can complement current efforts for crop genetic improvements which may take longer time scales (Kim & Cregg, 2012).

Nitrogen often limits plant productivity and is made available to plants in the absence of chemical fertilizer by two main sources; 1) the recycling of biologically available nitrogen deposited in the soil through decomposition of organic matter or manure and/or 2) the fixation of atmospheric dinitrogen gas by diazotrophic (nitrogen fixing) microbes. Diazotrophic microbes have been isolated as free-living in the soil (Rozycki *et al.*, 1999) and water (Short *et al.*, 2004),

in the rhizosphere, and as symbionts in both leguminous and non-leguminous plants (Bhattacharjee *et al.*, 2008). Diazotrophic endophytes have been isolated from several non-leguminous energy crops including sugarcane (Dobereiner, 1992), poplar and willow (Doty *et al.*, 2005; Doty *et al.*, 2009), miscanthus (Davis *et al.*, 2010), and corn (Montanez *et al.*, 2009). Diazotrophs seem to be ubiquitous. However, endophytes differ in their colonization capacity for different hosts (Rosenblueth & Martinez-Romero, 2006).

Willow (*Salix sitchensis*) and poplar (*Populus trichocarpa*, black cottonwood) are both fast growing species with the ability to thrive in nutrient limited environments. Interest in these species for efficient biomass production has led to the isolation and characterization of diazotrophic endophytic bacteria (Doty *et al.*, 2005; Doty *et al.*, 2009; Xin *et al.*, 2009b) and yeast (Xin *et al.*, 2009a) from these tree species. Further, Xin et al. (2009b) demonstrated the colonization of Kentucky bluegrass by the poplar endophyte WPB with an overall increase in plant biomass of 42% over the uninoculated controls. Recently, Khan et al.(2012) tested the effectiveness of several willow and poplar endophyte isolates for plant growth promotion of a variety of commercially important crops. Successful colonization and increased biomass were observed for nearly all the crop species tested, but with cultivar-specificity. These reports provide additional evidence for specific interactions between plant host cultivars and endophytic strains.

The current study tested the hypothesis that diazotrophic endophytes isolated from poplar and willow can be employed as cross species inoculum for growth promotion in corn; a bioenergy crop species typically grown with large inputs of fertilizer. Experiment 1 tested poplar and willow endophytes as single strain inoculants in a greenhouse trial with the objective of comparing the main effect of plant growth promotion under two nitrogen fertilizer regimes;

low-N and high-N. The objective of the second greenhouse experiment was to compare the main effect of plant growth promotion between single strain endophyte inoculations and multi-strain consortia. The objective of Experiment 3 was to test whether the plant growth promoting effect of endophyte inoculations would transfer from the greenhouse to the field and if the level of growth promotion would differ at differing levels of applied nitrogen fertilizer.

Materials and Methods

Endophytes

Eight bacterial strains (WPB, WP5, WP19, PTD-1, WW2, WW6, WW7 and WW11) and one yeast strain (WP1) were chosen from a collection of diazotrophic endophytes isolated from cottonwood and willow species native to the Snoqualmie River in Washington State (Doty *et al.*, 2005; Doty *et al.*, 2009; Xin *et al.*, 2009a) for investigation of plant growth promotion in corn (*Zea mays*) as a novel host species. Each strain exhibited strong growth on nitrogen free media and gave positive amplification of the *nif*H marker gene for nitrogenase reductase. Endophytes were tested for production of the growth hormone, indoleacetic acid (IAA) using the colorimetric method described by Gordon and Weber (1951). All endophytes, except for WP19, tested positive for IAA production.

The single strain endophyte inoculation treatments were as follows; WP1 (*Rhodotorula graminis),* WPB (*Burkholderia vietnamiensis*), and WW7 and WW11 (both *Sphingomonas sp).* The two multi-strain consortia were: 1) Poplar Mix (WPB, PTD-1 (*Rhizobium tropici bv populus),* WP5 (*Rahnella sp.*), and WP19 (*Acinetobacter sp.*); and 2) Willow Mix (WW7, WW11, WW2 (*Herbaspirillum* sp), and WW6 (*Pseudomonas* sp).

Plant Materials

The effects of endophyte inoculations on a sweet corn cultivar (Honey & Cream, Territorial Seed Company, OR) that is suitable for Pacific Northwest climate were tested. Seed kernels were surface sterilized by incubation in 1% sodium hypochlorite solution for 30 minutes followed by three washes in sterile distilled water. The sterility of water from the third rinse was verified on MG/L agar (grams 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 $MgSO_4*7H_2O$, 2ug biotin at pH 7.0 and 15 grams agar). The kernels were then allowed to germinate in sterile, sealed containers on a thin layer of water agar. Just as root and shoot tissue emerged, within 4 days, the seedlings were divided equally into groups for inoculation. All endophytes were cultured overnight in nutrient rich MG/L broth or yeast extract-peptone-dextrose (YPD) medium (Difco Laboratories, Detroit, MI) from stock collections held at -80^{o}C. The endophytes were washed three times in nitrogen free Murashige and Skoog (MS) (MSPOO7, Caisson Labs, UT) liquid broth, then suspended to an OD_{600} of 0.05 for the single strain inocula and a total OD_{600} 0.10 for the multi-strain consortia where each strain is added in equal proportion. The corn seedlings were incubated for 3 hours on a gentle shaker (40-60 rpm) submerged in endophyte solution. The control group was incubated in the same manner with sterile nitrogen free media (MS).

Fluorescent microscopy

To verify the effectiveness of the inoculation technique, two of the bacterial isolates were labeled with green fluorescent protein through electroporation with the broad host range plasmid pBHR1-GFP (Stevens *et al.*, 2005). Inoculation occurred as described above with WPB*gfp* and PTD-1*gfp* as separate inocula along with an uninoculated control group. Colonization of root, stem and leaf tissue was verified through fluorescent microscopy using a Zeiss Imager M2

equipped with an AxioCam MRM and recorded with Zeiss AxioVision software (Karl Zeiss, LLC, Thornwood, NY, USA). Colonization was followed from one week after inoculation for two months with samples visualized at one week, one month and two months after inoculation.

Plant growth and physiology

Experiment 1: Greenhouse single strain inoculation trials in sand

Endophytes WP1, WPB, WPB-2, WW7, and WW11 were evaluated as single strain inoculants for plant growth promotion under two nitrogen regimes through fertigation with a modified Hoagland's nutrient solution containing (g L^{-1}): 0.22 $CaCl_2(2H_2O)$, 0.17 K_2SO_4, 0.26 $MgSO_4(7H_2O)$, 0.136 KH_2PO_4, 0.015 NaFeDTPA(10% Fe) with 1 ml L^{-1} micronutrient solution containing (gL^{-1}: 0.773 H_3BO_3, 0.169 $MnSO_4$, 0.288 $ZnSO_4(7H_2O)$, 0.062 $CuSO_4(5H_2O)$ and 0.04 H_2MoO_4 (83% MoO_3)). The concentration of nitrogen was controlled through varied addition of ammonium nitrate ($NH_4(NO_3)$) with a concentration of 0.04 g L^{-1} (Low-N) and 0.322 g L^{-1} (High-N) Inoculated seedlings were sown in a 1:1 sterilized sand and perlite mixture in 4 6-well plastic seedling packs (1.13 L per 6-well pack McConkey, Sumner, WA) per endophyte treatment and maintained in separate drip trays to avoid cross inoculation or contamination of the uninoculated control group. Three weeks after inoculation, 6 plants (n=6) from each treatment (6 endophyte treatments x 2 nitrogen treatments) were transferred to individual 4 inch square pots (2.17 L "The Square One" McConkey, Sumner, WA) containing the same sand:perlite mixture, separated by individual drip trays, and arranged in a complete randomized block design. Plants in the remaining three 6-well packs were harvested 25 days after inoculation (DAI), then pooled by 6-well pack (*n*= 3) and cleaned of the sand mixture. Roots and leaves were separated from the stalk. Plant tissue was placed in brown paper bags and oven dried at 70^{o}C for three days prior to being weighed. Transferred plants continued to receive 200 mL fertigation solution once weekly

and distilled water was applied as needed. The corn plants were maintained in the greenhouse at an average temperature of 21^{o}C with a 14 hour photoperiod for 90 days after inoculation. At harvest, the majority of corn plants completed tasseling and entered the silking stage; cob formation was nascent and is not reported separately than overall stem mass. Plants were harvested and processed for measurements described above for the 25 DAI.

Experiment 2: Greenhouse single strain endophyte and consortia inoculation trial

Growth and physiological responses to inoculation with Poplar Mix and Willow Mix consortia were compared to WP1, WW11 inoculations and an uninoculated control group with or without nitrogen limitation. Square 2.17 L pots were filled with 0.35 kg Sunshine #2 mix (SunGro, Bellevue, WA) and saturated with tap water. The sweet corn was prepared as described above and inoculated seedlings were sown 4 to a pot and then thinned to 2 per pot after one week. A total of 50 pots, 5 replications of each endophyte x nitrogen treatment, were arranged in a randomized complete block design on one greenhouse bench and separated by individual drip trays to avoid cross inoculation. All plants received 200mL of the modified Hoagland's solution once a week; half received no nitrogen (no-N) and the other half received 0.322 g L^{-1} added $NH_4(NO_3)$ (high-N). Plants were irrigated with tap water as needed to avoid drought and maintained at 21^{o}C with a 14 hour photoperiod. Instantaneous photosynthesis data was measured 32 DAI and 36 DAI from the second most developed leaf from the top of the plant using the LI-6400XT portable photosynthesis system (LI-COR, Lincoln, NE) with block temperature set to 25^{o}C, CO_2 concentration of the chamber was set to 1000 µmol CO_2 mol^{-1}_{air} and the photosynthetically active radiation (PAR) set to 1500 µmol photons $m^{-2}_{leaf\ area}$ s^{-1}. Leaf net CO_2 assimilation rate at saturating light (A_{max}) was recorded after 5 minutes stabilization period, and measurements occurred between 11:00 and 15:00 both days. Plants were harvested

39 DAI and cleaned of planting media before roots and leaves were removed from the stalk. Green leaf area was measured immediately using a LI-3100C leaf area meter (LI-COR, Lincoln, NE). Dry plant tissue weight was measured after tissue was allowed to oven dry at 70^{o}C for three days. Specific leaf area (SLA) was calculated as the green leaf area per gram of leaf dry mass, cm^2g^{-1}.

Experiment 3: Field site single strain endophyte and consortia inoculation trial

A field trial was initiated with the sweet corn inoculations as described in experiment 2. Inoculated seedlings were sown in 24-well seedling trays (4.52 L per tray, McConkey, Sumner, WA) containing 1:1 sterilized sand and perlite mix and maintained for one week at 21^{o}C with a photoperiod of 14 hours then relocated to a greenhouse at the Charles L. Pack Experimental Forest where they were allowed to acclimate to ambient temperature for one week prior to outplanting. Up to this point, the corn plants received only irrigation with tap water. This experiment tested five endophyte treatments, Poplar Mix, Willow Mix, WP1, WW11 and uninoculated control, with three levels of nitrogen fertilizer, and was replicated in three blocks (N = 45). The corn was planted in one meter square plots separated by one meter on all sides from any neighboring plot. Each block contained one row of each of the three nitrogen levels; each row contained each of the five endophyte treatments, endophyte treatments were randomized across the entire plot to account for variation in soil condition across the field.

The Willow Mix inoculated plants became contaminated with fungus during transportation and transplantation to the field site and suffered approximately 2/3 mortality. Therefore only one full experimental block was planted with all endophyte treatments. The data acquired from Willow Mix inoculated plants had no statistical power and is not reported here.

The open plots within blocks where Willow Mix plants died had no effect on neighboring plots and were excluded from contributing to any variation in the statistical model.

The experimental site is located in the Charles L. Pack Experimental Forest, Pierce County Washington. The soil is a sandy, glacial outwash of the Indianola series (mixed, mesic Dystric Xeropsamments) (Gaulke *et al.*, 2006). This site is excessively drained, nutrient limited, and receives full sun. Site prep included the removal of approximately 12 inches of the top soil along with any vegetation using a bulldozer, and the entire site was repeatedly disked to reduce variability. The one meter plots were then individually tilled to a depth of at least 24 inches. All plots received 24g of granulated monopotassium phosphate (MPK). Three levels of nitrogen were applied as pelleted urea, 0 gm^{-2}, 6 gm^{-2} and 24 gm^{-2}, with half of the nitrogen applied along with the MPK prior to planting and the remaining nitrogen dose applied as a side dress four weeks after planting. The corn was planted with an initial density of 18 stalks per plot, and then thinned to 9 stalks per plot four weeks after planting.

Plants were harvested 87 days after inoculation. Stalks from each plot were bundled together and wrapped in plastic for transport from the field. All harvested plants were kept refrigerated until final measurements were taken. Leaves were separated from the stalks and total green leaf area was measured using the LI-3100C leaf area meter (LI-COR, Lincoln, NE). Plant height was measured as the distance from the root collar to the top of the stalk. Leaves, stalks, and cobs were dried in a 70^{o}C oven three days prior to determination of dry mass. Leaf N content was obtained from the oven dried leaf tissue using a PE 2400 Series II CHN elemental analyzer (Perkin Elmer, Waltham, MA; CHN work carried out at the UW SEFS Chemical Analysis Center). Specific leaf area (SLA) was calculated as the green leaf area per gram of leaf dry mass, cm^2g^{-1}. Leaf chlorophyll was measured twice with a Konica Minolta SPAD 502

(Konica Minolta, Ramsey, NJ) hand held chlorophyll meter, four (August) and eight (September) weeks after transplanting. Measurements were taken on the second full leaf from the top of the plant for consistency and an average of 5 measurements per plot was recorded. Instantaneous photosynthesis data was measured from the second most developed leaf from the top of the plant using the LI-6400XT portable photosynthesis system (LI-COR, Lincoln, NE). Measurements were taken after plot thinning in the 4th week after transplanting under ambient humidity with a block temperature of 25^{o}C, reference CO_2 concentration of the chamber was set to 440 µmol mol^{-1} and the photosynthetically active radiation (PAR) set to 2000 µmol photons $m^{-2}_{leaf\ area}\ s^{-1}$. Leaf CO_2 assimilation rate at saturating light (A_{max}), transpiration rate (E), and stomatal conductance (g_s) were recorded after a stabilization of 10 minutes. Intrinsic water-use efficiency (iWUE) was calculated as net photosynthesis per unit water lost, A_{max}/g_s. Photosystem II maximum quantum yield was measured through dark adapted fluorescence, Fv/Fm, using the LI-6400XT after a period of 3 hours of natural darkness. The leaf carbon:nitrogen ratio was calculated at the end of the study as the final leaf C (gm^{-2}) per leaf N(gm^{-2}).

Statistical analysis

All experiments were designed for contrast of treatment means to the corresponding uninoculated control group. Data were analyzed using the SAS statistical software version 9.3 (SAS Institute Inc., Cary, NC). Two-way ANOVA was used to determine the significance of the effects of endophyte inoculation and nitrogen treatment levels. Duncan's multiple range test (DMRT) and the CONTRAST statement within PROC GLM were used to compare endophyte treatment means within nitrogen treatment levels.

Results

Fluorescent microscopy

Successful colonization by both WPB*gfp* and PTD-1*gfp* was evident in root and stem tissue when viewed one week following inoculation. The bacteria were also evident in root and stem tissue when viewed one and two months after inoculation (data not shown). A representative micrograph demonstrating the colonization of leaf tissue two months after inoculation by PTD-1*gfp* is shown in Figure 1.

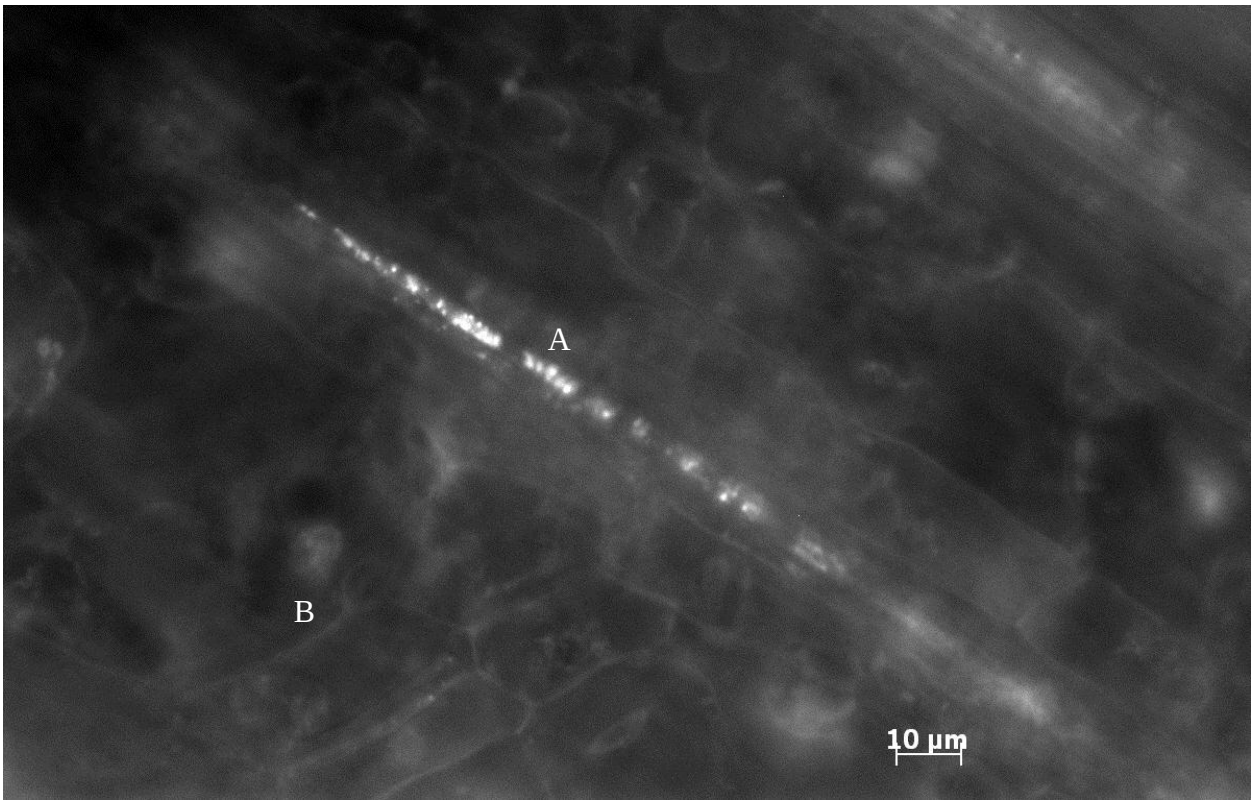

Figure 1: PTD-1*gfp* in sweet corn leaf tissue at 1000x magnification. A) Brightly fluorescing bacteria colonizing the leaf vascular tissue. B) leaf cortex with some autofluorescence from cell wall.

Experiment 1: Greenhouse single strain inoculation trial

Significant early growth promotion was observed for each of the single strain inoculants over the control group within both nitrogen application levels recorded 25 DAI (Table 1). Within the Low-N treatment the largest gain in total biomass of 61% occurred in the WP1 inoculated plants followed by WW7 with 52% gain, WPB with 35% gain and WW11 with 26% gain at 25 DAI. Biomass allocation in this low-N treatment group is pronounced in roots over above ground biomass. Inoculated plants accumulated more root mass than the control group; with 84%, 47%, 41% and 37% gains from WP1, WPB, WW7, and WW11 inoculations respectively. Above ground biomass gains occurred similarly for the inoculated plants with 41% (WP1), 35% (WW7), 23% (WPB), and 16% (WW11). Endophyte inoculations improved early total biomass within the high-N treatment as well; WPB inoculated plants gained 40%, WW11 gained 35%, WW7 gained 34%, and WP1 gained 11% at 25 DAI over the control group. While root mass was improved over the control group, more biomass was allocated to the above ground tissue under the high-N regime (Table 1). Plants inoculated with WPB exhibited a 42% gain followed by WW11 (35% gain), WW7 (30% gain), and WP1 (10% gain) in above ground biomass over the control group.

At 90 DAI, total dry plant weight and the above ground biomass were no longer statistically different between the inoculated plants and the control group for either nitrogen application level. Within the high nitrogen application group the root mass gain was still present for plants inoculated with WW11 (24% gain), WPB (15% gain) and WP1 (13% gain) over the uninoculated control group.

Table 1. Greenhouse Experiment 1. Oven-dry biomass of endophyte inoculated maize plants grown in sand under two nitrogen fertigation regimes. Means (standard error) of biomass for single strain inoculation trial at 25 DAI (n= 3) and 90 DAI (n= 6). Means followed by different letters in each column within a nitrogen treatment are significantly different at alpha = 0.10 using Duncan's multiple range test. *P* - values are given where Endophyte treatment means differ from the Control group for a specific contrast of means within the Nitrogen treatment level.

Nitrogen Treatment	Endophyte Treatment	25 days after inoculation			90 days after inoculation		
		Above Ground Dry Weight (g/plant)	**Root Dry Weight (g/plant)**	**Total Biomass Dry Weight (g/plant)**	**Above Ground Dry Weight (g/plant)**	**Root Dry Weight (g/plant)**	**Total Biomass Dry Weight (g/plant)**
Treatment A Low-N **0.04 g L^{-1} $NH_4(NO_3)$**	Control	0.155 (.005)b	0.140 (0.03)b	0.295 (0.02)c	4.60 (0.24)a	2.00 (0.28)a	6.60 (0.47)a
	WP1	0.220 (0.04)a p=0.09	0.257 (0.04)a p=0.006	0.476 (0.02)a p=0.003	4.77 (0.34)a	2.00 (0.21)a	6.77 (0.52)a
	WPB	0.191 (0.02)ab	0.206 (0.04)a p=0.078	0.397 (0.01)ab p=0.053	4.64 (0.26)a	2.36 (0.30)a	7.00 (0.45)a
	WW11	0.181 (0.01)ab	0.193 (0.02)ab	0.373 (0.02)bc	4.56 (0.30)a	1.93 (0.22)a	6.48 (0.48)a
	WW7	0.210 (0.03)ab	0.238 (0.08)a	0.449 (0.07)ab p=0.007	4.50 (0.21)a	1.89 (0.09)a	6.39 (0.25)a
Treatment B High-N **0.322 g L^{-1} $NH_4(NO_3)$**	Control	0.249 (0.02)b	0.191 (0.03)c	0.439 (0.03)b	32.61 (4.7)a	5.33 (0.34)b	37.94 (4.52)a
	WP1	0.274 (0.04)ab p=0.071	0.214 (0.01)bc	0.487 (0.05)ab	33.01 (4.6)a	6.03 (0.57)ab	39.04 (4.61)a
	WPB	0.355 (0.03)a	0.263 (0.01)a p=0.04	0.617 (0.04)a	27.89 (1.9)a	6.12 (0.40)ab	34.01 (1.98)a
	WW11	0.337 (0.04)ab	0.256 (0.03)ab p=0.06	0.593 (0.05)a	30.16 (1.8)a	6.56 (0.59)ab p=0.06	36.73 (1.64)a
	WW7	0.323 (0.12)ab	0.276 (0.03)a p=0.02	0.590 (0.09)a	32.37 (3.3)a	5.51 (0.33)ab	37.87 (3.39)a

Experiment 2: Greenhouse single strain isolate and consortia inoculation trial

Nitrogen application had a significant effect on plant growth and biomass (Table 2). Though not statistically significant, plants inoculated with the Poplar Mix, Willow Mix, and WW11 gained more total biomass than the uninoculated control group while plants inoculated with WP1 produced the least biomass in the no added nitrogen treatment group. Under added nitrogen, total biomass gain was observed for all inoculation types relative to the control group. Plants inoculated with the Willow Mix consortium gained the most with a 10% gain over the control group under both nitrogen regimes. Consistent with Experiment 1, root gain in inoculated plants was favored over above ground biomass. Willow Mix inoculated plants gained 14% and 11% root mass over the control group under the no-N and high-N regimes respectively.

Changes in biomass allocation and leaf properties were evident for endophyte inoculated plants within nitrogen treatment levels. Green leaf area was increased for plants inoculated with the Poplar Mix, WP1 and WW11 under both of the nitrogen regimes with marginally significant increases over the control group observed in plants inoculated with WP1 ($p=0.091$) and Willow Mix ($p = 0.19$) within the high-N regime. However, specific leaf area was only marginally significant different between WP1 ($p=0.104$) inoculated plants under no-N and WW11 ($p = 0.070$) under the high-N regime.

Table 2. Greenhouse Experiment 2. Endophyte inoculated maize grown in the greenhouse under two nitrogen fertigation regimes. Means (standard error) of biomass and growth parameters 39 DAI (n = 5). Means followed by different letters in each column within a nitrogen treatment are significantly different at alpha = 0.10 using Duncan's multiple range test. P-value is given where specific contrast of means differ from the control group within nitrogen treatment.

Nitrogen Treatment	Endophyte Treatment	Root Dry weight (g/plant)	Total Above Ground Dry weight (g/plant)	Total Biomass Dry weight (g/plant)	Leaf Area (cm^2/plant)	Specific Leaf Area (cm^2/g leaf)
	Control	1.81 (0.22)a	4.41 (0.56)a	6.22 (0.75)a	546.47 (60.84)a	242.60 (6.24)ab
No-N	Poplar Mix	1.64 (0.16)a	4.66 (0.32)a	6.31 (0.47)a	562.86 (39.47)a	238.78 (15.52)ab
	Willow Mix	1.85 (0.21)a	5.01 (0.84)a	6.86 (1.03)a	561.22 (90.52)a	222.62 (14.84)b
	WP1	1.62 (0.13)a	4.30 (0.63)a	5.92 (0.75)a	551.42 (71.97)a	269.92 (8.88)a
	WW11	1.89 (0.44)a	4.68 (.084)a	6.57 (1.25)a	538.10 (89.69)a	248.24 (8.07)ab
High-N **0.322 g L^{-1} $NH_4(NO_3)$**	Control	4.24 (0.64)a	17.01 (1.33)a	21.25 (1.59)a	1430.58 (69.50)ab	212.78 (7.43)b
	Poplar Mix	4.50 (0.40)a	17.29 (1.52)a	21.78 (1.23)a	1539.59 (85.05)ab	218.43 (7.94)b
	Willow Mix	4.47 (0.28)a	18.96 (1.58)a	23.43 (1.69)a	1565.44 (85.09)ab	223.78 (8.05)ab
	WP1	4.58 (0.59)a	17.59 (0.68)a	22.17 (1.11)a	1607.34 (49.91)a $^{p = 0.091}$	217.40 (3.35)b
	WW11	4.40 (0.25)a	17.00 (1.30)a	21.39 (1.19)a	1501.10 (54.26)ab	230.77 (5.23)a $^{p = .070}$
Nitrogen	*P* > F	***	***	***	***	***
Endophyte		ns	ns	ns	ns	ns

***: Nitrogen application is significant at $p < 0.0001$ for all parameters measured.
ns: Endophyte inoculation is not significant as a main effect at alpha = 0.10.

Experiment 3: Field site endophyte consortia inoculation trial

Overall, endophyte inoculated plants produced more biomass and had a higher leaf net CO_2 assimilation rate at saturating light (A_{max}) than the uninoculated control plants. Significant differences were achieved for above ground biomass, average plant height, SLA, leaf N and leaf C measurements between the nitrogen treatments (Table 3). There was no significant interaction between the endophyte inoculation and the applied nitrogen treatments. None of the single species or consortium endophyte inoculation treatments tested in this study were able to produce enough growth enhancement to overcome the nitrogen limitations in the low-N (0 gm^{-2}) and mid-N (6 gm^{-2}) treatments.

The low-N (0 gm^{-2}) treatment group displayed the most variability with 50% more above ground biomass for the Poplar Mix inoculated plants than the uninoculated controls. Additionally, the leaf nitrogen content (N gm^{-2}) measured from endophyte inoculated plants increased over the uninoculated control plants ($P = 0.038$; Table 3). A similar pattern is visible in both the mid-N (6 gm^{-2}) and high-N (24 gm^{-2}) treatment groups. Plants inoculated with Poplar Mix had 24% (mid-N) and 22% (high-N) more biomass, a 15% (mid-N) and 7% (high-N) increase in stem height and a 9% (mid-N) and 3% (high-N) increase in leaf N over the uninoculated controls. Also trending higher in leaf N content, though not statistically significant, were plants inoculated with WW11 with a 14% increase over the uninoculated plants within both the mid-N and high-N regimes. Leaf SPAD was significantly affected by the applied N treatment (August SPAD $p<0.001$ and September SPAD $p<0.001$), however, endophyte inoculation did not significantly alter the August SPAD readings within N treatment levels. The September SPAD readings were similar between inoculation treatments within low-N and mid-N treatment levels with the exception of Poplar Mix and WW11 inoculated plants in the low-N

Table 6. Experiment 3: Field site single strain endophyte and consortia inoculation trial. Plot means (standard error) for biomass, stem height, specific leaf area (SLA), leaf nitrogen (N), and leaf carbon (C) measured at harvest 87 DAI endophyte inoculated maize. Plot means (standard error) for SPAD taken twice during the growing season. Significant difference from control group within nitrogen treatment is indicated by; ° alpha = 0.1, * alpha = 0.05, and ** 0.01.

Nitrogen Treatment	Endophyte Treatment	Above Ground Dry Weight (g/plant)	Stem Height (cm)	SLA (cm^2/g)	Leaf N (g/M^2)	Leaf C (g/M^2)	SPAD August	SPAD Sept.
Low-N	Control	9.35 (2.67)	64.31(7.4)	236.78(9.22)	0.693 (0.031)	17.93 (0.77)	29.6 (3.6)	21.5 (2.05)
0 g/m^2	Poplar Mix	19.29 (7.81) °	90.61(12.22) $^{p=0.25}$	211.89 (24.18)$^{p=0.22}$	1.083 (0.335)	20.44 (2.61)	28.6 (2.3)	30.4 (6.57) °
	WP1	11.45 (0.32)	73.47 (3.59)	236.98 (6.19)	0.756 (0.020)	17.61 (0.35)	26.9 (0.7)	21.5 (1.65)
	WW11	13.83 (0.39)	109.80 (27.2) °	238.54 (3.5)	0.716 (0.076)	17.65 (0.26)	29.5 (0.73)	27.2 (1.18)
Mid-N	Control	17.42 (0.399)	84.24 (5.54)	221.85 (13.06)	0.910 (0.051)	20.44 (2.09)	34.6 (2.5)	27.9 (3.06)
6 g/m^2	Poplar Mix	21.63 (1.73) °	96.21 (0.59) °	219.58 (21.119)	0.994 (0.161)	19.59 (1.89)	36.6 (1.212)	27.9 (1.25)
	WP1	19.66 (1.66)	92.73 (3.8)	209.49 (11.509)	0.977 (0.027)	20.21 (0.93)	30.9 (1.452)	27.8 (2.55)
	WW11	20.58 (4.62)	97.19 (8.2) °	217.72 (1.313)	1.035 (0.161)	19.51 (0.22)	35.3 (2.86)	32.1 (2.21)
High-N	Control	37.93 (6.06)	112.92 (4.19)	193.79 (6.16)	1.280 (0.039)	22.09 (0.56)	36.7 (15)	40.4 (0.78)
24 g/m^2	Poplar Mix	46.65 (6.54) °	121.24 (2.8) °	184.07 (15.08)	1.330 (0.125)$^{p=0.21}$	23.05 (1.86)	37.8 (1.72)	38.0 (0.96) °
	WP1	37.38 (3.02)	117.52 (3.86)	206.57 (6.11)	1.320 (0.026) °	20.93 (0.64)	37.3 (1.12)	37.0 (0.95)*
	WW11	33.78 (4.28)	113.47 (5.07)	199.21 (4.13)	1.460 (0.036) °	21.31 (0.69)	36.6 (0.64)	37.2 (0.82)*
Low-N		13.86 (2.18)c	86.39 (0.91)b	230.53 (7.02)a	0.821 (0.945)b	18.45 (0.737)b	28.8 (0.81)c	25.5 (2.03)b
Mid-N		19.82 (1.20)b	92.59 (0.71)b	217.16 (6.08)a	0.9794 (0.052)b	19.94 (0.64)b	34.4 (1.11)b	28.9 (1.15)b
High-N		38.94 (2.63)a	116.29 (1.13)a	195.91 (4.54)b	1.35 (0.364)a	21.85 (0.52)a	37.10 (0.48)a	38.1 (0.55)a

Nitrogen level means followed by different letters are significantly different at alpha = 0.05 using Duncan's multiple range test.

treatment group. SPAD readings within the high-N treatment for all endophyte inoculated plants were significantly lower than the control group (Table 3).

The photosynthetic rate (A_{max}), measured at saturating light and CO_2 concentration, was significantly affected by nitrogen application ($p = 0.0058$). The average photosynthetic rate among all N treatments was measured at 35.83 µmol CO_2 m^{-2} s^{-1}, with the control = 31.25 µmol CO_2 m^{-2} S^{-1}, Poplar Mix = 36.89 µmol CO_2 m^{-2} S^{-1}, WW11 = 35.97 µmol CO_2 m^{-2} S^{-1}, and WP1 = 34.64 µmol CO_2 m^{-2} S^{-1} (Table 4). Variation within the low-N plots was high and no statistical significance was achieved with the small sample size, however an 18% increase in CO_2 assimilation is observed for endophyte inoculated plants over the uninoculated controls; 31.74 µmol CO_2 m^{-2} S^{-1} and 26.9 µmol CO_2 m^{-2} S^{-1} respectively (Figure 2). Within the mid-N treatment there was an increase of 19% CO_2 assimilation measured from the inoculated plants over that measured from the control plants; 35.4 µmol CO_2 m^{-2} S^{-1} and 29.6 µmol CO_2 m^{-2} S^{-1} respectively. The Poplar Mix inoculated plants had the most significant increase in CO_2 assimilation (38.0 µmol CO_2 m^{-2} S^{-1}, $p=0.028$) followed by the WP1 (34.73 µmol CO_2 m^{-2} S^{-1}, $p= 0.129$) and the WW11 (33.57 µmol CO_2 m^{-2} S^{-1}, $p=0.22$) inoculated plants (Table 4). Within the high-N treatment there was a 13% increase in CO_2 assimilation rate measured from inoculated plants over the uninoculated control plants; 40.32 µmol CO_2 m^{-2} S^{-1} and 35.8 µmol CO_2 m^{-2} S^{-1}, respectively. Again the plants inoculated with the Poplar Mix recorded a significantly higher rate of CO_2 assimilation with 42.16 CO_2 m^{-2} S^{-1} ($p = 0.007$), followed by plants inoculated with WW11 (40.1 µmol CO_2 m^{-2} S^{-1}, $p = 0.036$), and WP1 (38.7 µmol CO_2 m^{-2} S^{-1}, ($p = 0.120$). The leaf carbon to leaf nitrogen ratio data was significantly affected by the nitrogen application regime ($p < 0.001$) and only significantly different between endophyte inoculated treatments in the high-N treatment group ($p = 0.0024$).

Table 7: Experiment 3: Field site single strain endophyte and consortia inoculation trial. Leaf level physiology treatment means (standard error) for leaf CO_2 assimilation rate at saturating light (A_{max}), transpiration rate (E), stomatal conductance (g_s), leaf carbon to nitrogen ratio (C:N), intrinsic water use efficiency, and the dark adapted quantum yield of photosystem II (Fv/Fm). Significant difference from control group within nitrogen treatment is indicated by; ° alpha = 0.1, * alpha = 0.05, and ** 0.01. Differences between the nitrogen levels are indicated by different letters.

Nitrogen Treatment	Endophyte Treatment	A_{max} µmol CO_2 m^{-2} S^{-1}	E mmol H_2O m^{-2} s^{-1}	g_s mol H_2O m^{-2} s^{-1}	Leaf C:N	iWUE (Amax/g_s)	Fv/Fm
Low-N	Control	26.9 (0.03)	1.97 (0.05)	0.287 (0.11)	25.23 (1.62)	152.10 (8.76)	0.737 (.001)
0 g/m^2	Poplar Mix	30.5 (2.63)	2.58 (0.35)	0.224 (0.007)	20.82 (3.18) °	136.61 (12.17)	0.745 (0.006)
	WP1	30.5 (5.22)	2.39 (0.90)	0.204 (0.044)	24.44 (0.64)	144.68 (20.13)	0.757 (.002)
	WW11	34.2 (2.47)	2.48 (0.36)	0.242 (0.021)	23.33 (0.477)	111.49 (44.83)	0.741 (0.011)
Mid-N	Control	29.6 (4.97)	2.15 (0.65)	0.186 (0.042)	20.05(1.0)	106.99 (6.69)	0.769 (0.005)
6 g/m^2	Poplar Mix	38.0 (1.7)**	2.82 (0.37) °	0.228 (0.017)$^{p=0.27}$	20.09 (1.27)	167.63 (8.26)	0.767 (0.008)
	WP1	34.7 (2.44) °	2.86 (0.26) °	0.219 (0.021)	22.32 (1.52)	129.10 (14.16)	0.765 (0.007)
	WW11	33.6 (2.58)$^{p=0.2}$	3.15 (0.53)*	0.264 (0.39) °	19.98 (0.45)	163.17 (12.9)	0.753 (0.753)
High-N	Control	35.8 (2.89)	2.91 (0.44)	0.220 (0.025)	15.05 (0.13)	170.50 (3.36)	0.765 (0.007)
24 g/m^2	Poplar Mix	42.2 (1.7)**	3.86 (0.13)*	0.285 (0.019)*	17.33 (0.26)**	148.38 (5.35) °	0.769 (0.005)
	WP1	38.7 (2.41) °	3.16 (0.18)	0.228 (0.018)	16.36 (0.028)**	152.76 (8.36)	0.753 (0.007)
	WW11	40.1 (1.5)*	3.52 (0.24)*	0.263 (0.01) °	16.13 (0.49)*	164.25 (6.1)$^{p=0.214}$	0.764 (0.107)
Low-N		30.86 (1.67)b	2.39 (0.25)b	0.234 (0.02)a	23.57 (0.99)a	138.47 (9.5)b	0.746 (0.004)b
Mid-N		33.97 (1.9)b	2.74 (0.232)b	0.224 (0.016)a	20.61 (0.57)b	155.20 (6.58)ab	0.765 (0.003)a
High-N		39.19 (1.17)a	3.36 (0.16)a	0.249 (0.11)a	16.22 (0.272)c	158.97 (3.71)a	0.763 (0.004)a

Nitrogen level means followed by different letters are significantly different at alpha = 0.05 using Duncan's multiple range test.

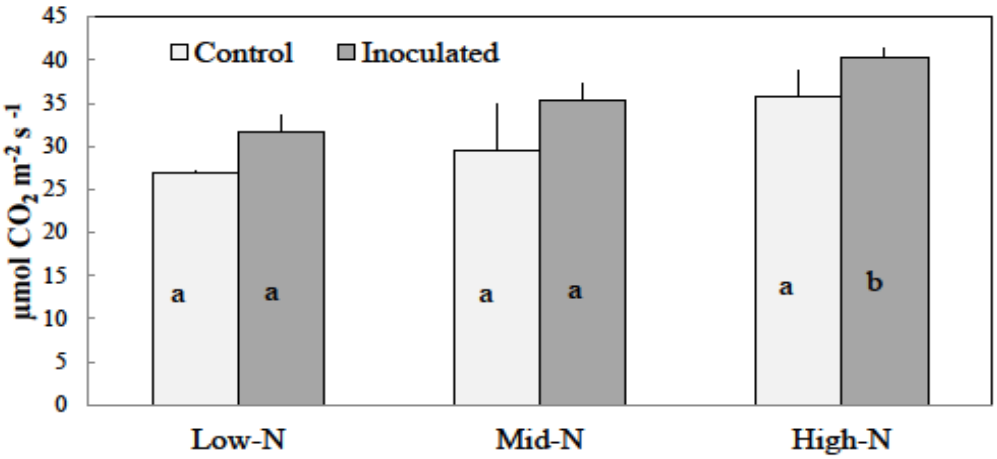

Figure 2: Mean CO_2 assimilation rate of all inoculated plants and the uninoculated control plants at three levels of nitrogen fertilization with standard error. Low-N = 0 g m^{-2}, mid-N = 6 gm^{-2}, and high-N = 24 g m^{-2}. Bars with different letters are different within N treatment group at the 90% confidence level.

Transpiration rate (E) increased with endophyte inoculation in all nitrogen application levels and was significantly higher in the mid-N (p= 0.029) and high-N (p=0.0024) treatment groups for the endophyte inoculated plants (Table 4). Stomatal conductance was not significantly affected by endophyte inoculation in the low-N treatment though inoculated plants recorded lower g_s than the control group. Conductance was increased compared to the control group for endophyte inoculated plants in both the mid-N and high-N treatments; Poplar Mix and WW11 showed the highest increases. The calculated iWUE for the inoculated plants within N application treatment groups was highly variable and no significant differences were found between the control plants and endophyte inoculated plants in both the low and mid-N treatments. Poplar Mix inoculated plants recorded significantly lowered iWUE in the high-N treatment. Changes in maximum quantum yield, dark adapted Fv/Fm, is an indicator of stress

caused to photosystem II (Lambers *et al.*, 2008). Nitrogen treatment had a significant effect on Fv/Fm ($p < 0.01$) whereas the endophyte inoculations did not ($p = 0.39$), suggesting no biological stress resulted from inoculations.

Discussion

Colonization of the monocotyledonous annual grass (*Zea mays*) with diazotrophic endophytes isolated from dicotyledonous perennial trees (*Populus trichocarpa* and *Salix sitchensis*) was verified through fluorescent microscopy. The poplar endophytes PTD-1 and WPB were visualized colonizing root, stem, and leaf tissue and appeared to be primarily located in vascular tissue. Inoculations of the sweet corn variety "Honey and Cream" by both single isolate endophytes and multi-strain consortia had an overall positive growth affect with no indication of pathogenesis. To our knowledge, this report is the first of its kind to describe a significant impact on leaf level physiology attributable to endophytic colonization. This is in contrast to an earlier study where colonization of poplar with the single endophyte, *Enterobacter* sp 638, had no significant impact on leaf physiology (Rogers *et al.*, 2011).

The early significant increase in root formation observed in the single isolate greenhouse trial could help seedlings be more robust against early environmental challenge. For example, longer and more abundant roots will establish a solid foundation for resistance to drought and physical challenge. This early burst of growth did not appear to have an overall negative affect on inoculated plants at harvest. Endophyte inoculation did not significantly decrease biomass nor did endophyte inoculation significantly affect the dark adapted quantum yield suggesting there is little or no metabolic cost for the plant host. Similar early growth response with endophyte inoculation is reported by Redman et al. (2011) where more rapid root development was observed in symbiotic rice seedlings over non-symbionts. While the mechanism for abiotic

stress protection from such symbiosis is not clear, the early and robust root growth is likely to play an important role.

Leaf physiology was significantly affected by diazotrophic endophyte inoculation where a higher rate of light saturated net CO_2 assimilation (A_{max}) was consistently recorded for inoculated plants in the field. A_{max} values recorded within the high-N treatment are close to other reported A_{max} of corn leaves grown in high nutrients (Kim *et al.*, 2007). Increased CO_2 assimilation rate was greatest for the plants inoculated with the Poplar Mix consortium. These plants also showed the greatest increase in above ground biomass, plant height, and leaf N. The increased A_{max} could be a result of increased leaf N through nitrogen fixation by the endophytes that make up the Poplar Mix consortium. A_{max} is influenced by the allocation of N within the leaf to proteins involved in the photosynthetic apparatus. Carbon assimilation occurs in *Zea mays* through the C4 photosynthetic pathway where gaseous CO_2 is fixed in mesophyll cells by phosphoenolpyruvate (PEP) carboxylase to form oxaloacetate and is eventually transported into bundle sheath cells as malate. The CO_2 is then released within the bundle sheath cell chloroplast to be refixed by the enzyme ribulose-1,5,-bisphosphate (RuBP) carboxylase (Rubisco) and assimilated to sucrose or starch through the C3 photosynthetic carbon reduction cycle. Thus, the rate of CO_2 assimilation in maize is regulated by the rate of C4 enzyme activities including PEP carboxylase and NADP-malic enzyme as well as RuBP regeneration; all three require energy derived from the electron transport chain located on the thylakoid membrane (Sage & Monson, 1999). Mathematical modeling of the C4 pathway by von Caemmerer and Furbank (von Caemmerer & Furbank, 1999; 1999) illustrate the relationship between light saturated PEP regeneration rate and the overall CO_2 assimilation rate by Rubisco. Gas exchange measurements of C4 leaves taken under both saturating light and CO_2 should be a reflection of Rubisco capacity

and RuBP regeneration rate (von Caemmerer & Furbank, 1999; von Caemmerer & Furbank, 2003). PEP regeneration rate also affects A_{max} and it is difficult to distinguish between these limiting factors. Increased leaf nitrogen is expected to be correlated with a higher percentage of leaf N allocated to the thylakoid membrane proteins of C4 plants (Makino *et al.*, 2003).

The leaf carbon to leaf nitrogen ratio (C:N) provides an indirect measurement of photosynthetic use efficiency (PNUE). The results of Experiment 3 are consistent with expected higher PNUE for plants under nitrogen limitation. Endophyte inoculated plants grown in the high-N treatment had a significantly higher C:N ratio (higher PNUE) and higher leaf N than the uninoculated control plants. However, the SPAD measurements were higher for the uninoculated control plants suggesting a difference in nitrogen allocation. While the SPAD measurements are effective non-destructive representation of leaf N status in corn and other field crops, a considerable variability can be introduced in correlating the SPAD readings with leaf N or chlorophyll content (Yang *et al.*, 2012). Further study specifically aimed at measuring Rubisco activity and the efficiency of the electron transport chain is needed to better determine the possible contributions of diazotrophic endophyte colonization to N allocation to photosynthetic apparatuses and the regulation of C4 photosynthesis.

Intrinsic water use efficiency (iWUE) was not significantly different between endophyte treatments within applied nitrogen levels. However, the data implies a slight interaction between the nitrogen application rate and endophyte inoculation, where iWUE is lowered in both the low-N and high-N treatment groups and raised within the mid-N for the inoculated plants in comparison to the control plants. Transpiration rate is raised by endophyte inoculation and becomes significantly different from the control group within the mid-N and high-N treatments. The higher transpiration rate is in line with the higher A_{max} for inoculated plants reflecting more

open stomata to meet the increased demand for CO2. This rise in transpiration may also be a reflection of increased water availability through increased root mass.

The results of these trials are consistent with those reported by Mehnaz *et al*. (2010) where a measurable increase in biomass is observed in endophyte inoculated corn after 30 days growth in the greenhouse. Likewise, Mehnaz *et al*. (2010) report endophyte inoculation increases biomass in field trials, however without strong statistical significance citing large variation in field trials. Where the common garden is more readily achieved in the greenhouse, field sites are ripe with natural variations. The native soil microbial community cannot be controlled and cannot be assumed to be evenly distributed within each test plot. Diazotrophic microbes isolated from soil have also been shown to colonize sweet corn and promote growth (Mehnaz & Lazarovits, 2006). Additionally, Montanez *et al.* (2009) present evidence for biological nitrogen fixation capacity of endophytes native to maize cultivars. Their report demonstrates that variation in capacity for biological nitrogen fixation is affected through fertilization, that is, more available nitrogen in the soil leads to less biologically fixed nitrogen. Variation in biological nitrogen fixation is also attributable to variation of colonizing microbes.

This series of trials demonstrated an overall increase in biomass with significant early gains in both root and shoot production for a monocotyledonous grass inoculated with diazotrophic endophytes previously isolated from a dicotyledonous tree species. Furthermore endophyte colonization increased leaf nitrogen content (gm^{-2}) and CO_2 assimilation rate of sweet corn in a field trial under three levels of nitrogen application. Variation observed in the current report could be an effect of multiple, unintended infections or variation of the soil microbial communities between test plots. The larger variation within endophyte treatments, especially within the consortia treatments, may be due to variation in the colonization of the individual

species within the host plant or microbial interactions. Additional investigation is required to assess the location, relative abundance and relationship of these endophytes with the host plant's native microbial community as well as the artificial community used as inoculum.

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Chapter 4

Conclusion

The experiments presented in Chapters 2 and 3 were designed to answer specific questions regarding the mechanisms by which endophytes may impart growth promotion to their host plants in nutrient limited conditions. The overall hypothesis that endophyte inoculations will increase biomass production of bioenergy relevant crop species under nutrient limited conditions was also supported. Indeed, whether the inoculation occurred with a single colony isolate or with a multi-strain consortium, biomass production of inoculated plants was higher than that of the uninoculated controls. While plants inoculated with diazotrophic endophytes trended higher in total nitrogen content and correspondingly higher concentrations of extractable chlorophyll, it is unclear from these studies how much growth promotion can be attributed to biological nitrogen fixation. The ^{15}N isotope dilution assay confirmed biological nitrogen fixation occurred in inoculated plants; however a corresponding increase in biomass gain was only present in the HK inoculation treatment group during the 2 month greenhouse study. A reasonable explanation is that the isotope dilution experiments were conducted with poplar, a long-lived perennial species and the duration of this assay was too short to capture the growth promotion through increased BNF.

Evidence of host plant genetic interaction with specific endophyte inoculants is given in Chapter 2. Internally sterile ramets of the *Populus trichocarpa* clone Nisqually-1 served as the plant host to determine the effectiveness of plant growth promotion from single colony inoculants as compared to multi-strain consortia. Biomass gain was evident for all inoculation treatments when compared to the uninoculated control group, however the greatest gains were observed as a result of inoculation with the multi-strain consortia. Biomass allocation and

branching behavior differed between inoculation treatments suggesting each endophyte or population of endophytes interacts in a unique manner with the host plant. The large variation within each endophyte treatment group may be accounted for by the stochasticity by which the individuals within the consortia colonized the host plant. It must be noted that both inoculated and uninoculated poplar contained N-fxing endophytes.

The cross species inoculation experiments described in Chapter 3 further demonstrate the effectiveness of endophyte inoculations for plant growth promotion under nutrient limitation. Again, data from these trials also did not indicate that endophyte inoculation alone is enough to overcome severe nitrogen limitation. However, a synergistic effect was observed within the mid-N fertilization regime. This provides evidence and encouragement that while nitrogen fertilization may not be completely eliminated, a much lower rate of application in combination with select endophyte inoculations is an effective path forward for sustainable and lower input cultivation of bioenergy crops.

The effect of endophyte inoculation on the net photosynthetic rate was found to differ between the two host species investigated. Chapter 2 focused on the interaction between endophytes isolated from native poplar trees within a poplar tree host of the same species. In this model, no significant differences were recorded for leaf physiology between the endophyte treatment groups. Chapter 3 focused on the interaction between poplar endophytes and the cross species host maize. In these experiments, leaf physiology characteristics were observed to be affected by the endophyte inoculations. There are two possible explanations for the differences observed between these two sets of experiments; distant host species genotypes and the difference between the C_3 and C_4 photosynthetic pathways. This result suggests that larger gains in CO_2 assimilation and biomass production may occur with cross species endophyte

inoculations. What is not known is whether the benefits of cross species inoculations will be robust or merely a short term improvement.

Colonization of host plants was verified by reisolation from surface sterilized tissue and through the microscopic observation of fluorescently (GFP) labeled bacterial endophytes. Microscopic observations of colonization were made over time (not reported within this body of work) and for all plant tissue types. Root colonization with the GFP labeled bacterial endophytes was observed to occur within 30 minutes after inoculation regardless of host species. Colonization was observed within the xylem and leaf cortex for both host species.

Future work should not only investigate colonization by single endophyte species, but rather focus on the colonization patterns of the endophytic community. Nucleic acid probes for fluorescent in situ hybridization (FISH) have been designed specific to many of the endophytes studied in this body of work. This technique will also be useful in the investigation of the interaction between the native endophytic community and the endophyte strains employed as inoculants. Further, probes such as these may provide a cost effective method for investigating the interaction between the endophytic community and the rhizospheric community.

This body of research confirmed that the dynamic interaction between host plants and the endophytic population should be an integral part of bioenergy crop improvement and agronomic management.

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