

Nitrogen fixation in *Populus*:

Identification and localization of the key diazotrophs *in planta* (Doty&Kim)

Relevant Categories: Bacterial; microbial single cell; microbial function; plant

Specific Aims: Poplar (*Populus*) trees are an early successional pioneer plant species able to colonize nutrient-limited, cobble-dominated riparian zones. Native poplar plants have a diverse microbiota including strains that can fix dinitrogen gas, and promote plant growth and health under abiotic stresses including nutrient limitation and drought. Our lab demonstrated using the $^{15}\text{N}_2$ incorporation assay that N_2 is fixed at high levels in wild poplar by endophytes, the microorganisms that live within plants. Although we have cultured and characterized dozens of N_2 -fixing (diazotrophic) endophytes from poplar and willow (*I*), it is unknown which strains are the most active *in planta*, where they are located within the plant, and if a specific consortium of strains is required. In order to optimize an inoculum of endophyte strains for poplar plantations for bioenergy, we propose to use the strengths of the EMSL and JGI to identify and characterize the key diazotrophic strains from wild poplar.

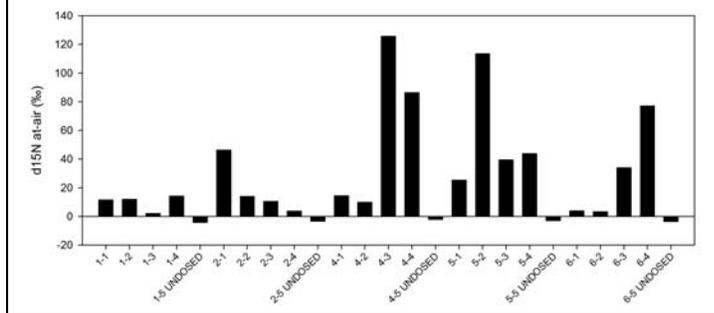
DOE Mission Relevance: Just as research on the human microbiome has demonstrated the profound importance of our microbiota on human health, plants are also strongly influenced by the ecosystem within them. To improve the environmental and economic sustainability of biomass production, it is essential that the biological interactions between plants and associated beneficial microbiota be more fully understood. Nitrogen is an essential macronutrient for plant growth. The use of N fertilizer for bioenergy plantations, however, is incompatible with the goals of climate change mitigation since its production requires fossil fuels, and soil bacteria convert the excess N fertilizer into nitrous oxide, a potent greenhouse gas. It has long been assumed that only legumes and actinorhizal plants can benefit from symbiotic N_2 -fixation; however, it has become clear that endophytic N_2 -fixation can provide substantial N to the host plant (2). Through research on this process, a more accurate model of global N cycling can be produced (3). This proposal addresses the JECISI call for proposals on “Plant-microbe interactions that impact climate.” We propose to research the exchange of N between the microorganisms within plants. We will identify the key species for N transfer in *Populus* and then ultimately test how this plant-microbe interaction impacts plant responses to drought and elevated CO_2 and temperature.

Background/Introduction: In naturally occurring low-nutrient environments, some plants associate with N-fixing microorganisms to acquire this essential nutrient for life. In the well-known symbioses of rhizobia with legumes, as well as *Frankia* with actinorhizal plants, diazotrophic bacteria inhabit root nodules and fix atmospheric N_2 gas into NH_3 and other N sources that can be used by the host plant. However, a wide variety of other plant species that are incapable of such nodule formation are also able to thrive in low-nutrient settings. In the last decade, there has been a proliferation in research on endophytes, the microorganisms living fully within plants (reviewed in (4-7)). These discoveries point to a relatively unexplored diversity of microbial life that is especially critical to the growth of plants in low-nutrient areas. Although many of the studies confirmed Koch’s postulates, demonstrating that specific diazotrophs could be isolated and then added back for increased plant growth in N-limiting conditions, they have not determined which microbial species were the most biologically relevant in the field, where profound levels of biological N_2 -fixation were seen. Clearly, a genomics-oriented approach is required to address this question. Recently, two groups studying diazotrophic N_2 -fixation in

sugarcane used expression analysis of the nitrogenase subunit gene, *nifH*. They identified that rhizobial species, rather than the more prevalent endophytic species, were likely the primary contributors of N (8, 9). A recent metagenomic study of the endophytic community in rice roots revealed that there was a high apparent density of N-fixing endophytes (10). However, expression of *nifH* revealed that the dominant *nifH* expressed was only that of *Rhizobium*. These studies demonstrate that expression analysis and sequencing are essential tools to guide diazotrophic endophyte research. In recent years, several genomes of diazotrophic endophytes have been sequenced, providing a wealth of information on possible mechanisms for successful plant-microbe interactions. In addition to genes involved in N₂-fixation, other putative symbiosis-related genes were for phytohormone production, ROS detoxification, ACC deaminase, transport systems, signaling, and colonization. Eleven of the poplar endophyte strains from Doty's lab are being sequenced by the JGI as part of the ORNL/JGI poplar microbiome study. More of these full genomic sequencing projects are needed for a better understanding of the genes necessary for endophytic colonization, plant growth promotion and also N₂-fixation *in planta*.

In order to quantify N₂-fixation in wild poplar, we used the direct ¹⁵N₂ incorporation assay. Isotopic analysis of the poplar tissues from two independent experiments indicated high levels of N₂ fixation in many of the cuttings from wild plants (11). There was variation in N₂ fixation between cuttings of the same plant (Figure 1), alluding to the intriguing possibility that

Figure 1. ¹⁵N incorporation in wild poplar plants in hydroponics. Cuttings from five independent plants (different genotypes) were collected during the peak growing season. Rooted plants were exposed to 6.17% atom excess of ¹⁵N₂ gas in N-free hydroponic medium for two weeks. Data from samples of the same plant were not averaged since the diazotrophic endophytes are not equally distributed throughout the plants.



there may be microbial social requirements that limit effective N₂ fixation to particular groupings within the plant that have achieved a threshold density (12). Although other research groups have reported on endophytes from poplar (13-16), no others have demonstrated endophytes with the high levels of N₂-fixation (17), broad host range (18), and dramatic growth enhancements under stress (19) that we have seen with endophytes from wild poplar in challenging environments. Building upon the strong body of evidence that endophytes from wild poplar can improve the growth and stress tolerance of a broad range of plant

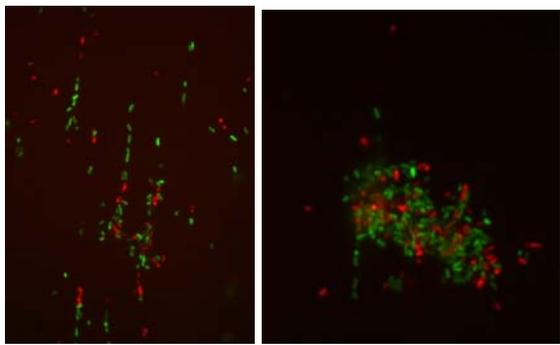
species, this proposal seeks to more fully understand the plant-microbe interactions involved in endophytic symbiosis, specifically in regards to N₂-fixation, with the ultimate goal of optimizing this technology towards increasing biomass yields on marginal lands with reduced nutrient and water inputs. Since poplar was the first tree to have its genome sequenced, it can serve as a model system for studying N₂-fixation not only in non-nodulating trees but also in plants in general.

Approach

Overview: One of the most direct technologies for visualizing N transfer is Nano-SIMS. However, the diffuse nature of endophytes within the plant body creates technical challenges. It will be important to first localize the probable N-fixing cells *in planta* to help overcome this challenge. Combining information from fluorescence microscopy, specific sample areas can be identified to test using Nano-SIMS for symbiotic N₂ fixation. Knowing the location and general diazotrophic species information will guide cell sorting experiments to isolate the active diazotrophic microorganisms from wild poplar tissues for full genomic sequencing and further analysis.

Aim 1) At EMSL, identify probable sites within poplar plants where N₂-fixation occurs. Using Fluorescent *in vitro* Hybridization (FISH) with *nifH* probes, we will test *in vitro*-propagated *Populus trichocarpa* colonized by Doty's known diazotrophic strains that had been isolated from wild poplar. These strains are labeled with stable plasmids that have constitutively expressed GFP, RFP, and a variety of other fluorescent tags (Figure 2). Samples can be delivered by hand

Figure 2. Colonization of hybrid poplar by fluorescently-labeled (GFP and RFP) endophytes. The endophytes are commonly in the intercellular spaces between plant cells (left) but sometimes in microcolonies (right).



to EMSL approximately two months after the start of project. Using this defined system, we can develop any necessary protocols for optimizing detection of the strains and of diazotrophic activity *in planta*. Further validation can be performed using commercial nitrogenase antibody to complement the FISH data. Using the STORM/PALM Super Resolution Fluorescence Microscope, we can quantify the levels of nitrogenase expression. Successful completion of Aim 1 will then provide important information on the plant organs in which significant N₂ fixation may occur, if *nifH* expression occurs only in microcolonies or is more diffuse by individual cells throughout the plant, and if Doty's

currently studied endophyte isolates express *nifH in planta*.

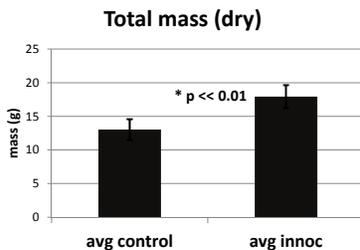
Aim 2) At EMSL, use fluorescent microscopy and Nano-SIMS to test for endophytic N-fixation. In Doty's lab, *in vitro*-propagated *Populus trichocarpa* will be colonized by the strains identified in Aim1 to be potentially fixing N₂ *in planta*. After allowing sufficient time (approximately 2-4 weeks) for full colonization, the plants will be exposed to ¹⁵N₂ gas, and then fixed and sectioned as necessary such that regions identified by fluorescent microscopy to have high levels of nitrogenase expression can be directly used by the Nano-SIMS. With information from these controlled experiments with *in vitro* propagated poplar and a few diazotrophic species, we can optimize the ¹⁵N₂ labeling or other parameters prior to beginning testing of wild poplar samples in early summer 2016. The goal of Aim 2 will be to locate the regions within wild poplar where N₂-fixation occurs strongly to aid isolation of the most influential endophytes in Aim 3.

Aim 3) At JGI using the Raman-microfluidic device currently under development, isolate actively N₂-fixing cells from within wild poplar samples. With information gained from Aims 1

and 2, live wild poplar tissue samples of similar characteristics will be harvested and exposed in Doty's Lab to $^{15}\text{N}_2$ gas, and the endophytic bacteria will be gently extracted. With the JGI technology currently under development, the Raman-microfluidic device can sort microbial cells labeled with ^{15}N . Further testing of the sorted cells by rDNA PCR and sequencing can be used to select away duplicates and insure a broad diazotrophic species sampling for genomic DNA sequencing of approximately twenty-five ^{15}N -positive cells. If the Raman-microfluidic device is not available or in order to complement the sorting done using the new technology, the Influx-Flow Cytometer Cell Sorter and FISH-FACS could be used at EMSL to sort cells expressing *nifH* mRNA or nitrogenase enzyme. These sorted cells would then be sent to JGI for genomic sequencing.

Aim 4) At JGI, perform full genomic sequencing of the active diazotrophic microorganisms. Comparative genomic analysis will provide valuable information for elucidating the genes common to active diazotrophic endophytes. In addition, the genome sequence information will aid in optimizing culture conditions. By knowing the genomic sequences of the active diazotrophs and the locations of *in planta* N_2 -fixation and if communities of different microbial species are required for the N transfer, Doty's plant microbiology lab will optimize culturing of these specific strains from wild poplar samples.

Figure 3. Drought responses in poplar. Plants inoculated with a 9-strain consortium of diazotrophic endophytes (left) and plants mock-inoculated (right) subjected to one month of drought.



Aim 5) Doty's plant microbiology lab and Kim's ecophysiology lab will then test if inoculating *in vitro*-grown poplar with the new isolates leads to increased growth and improved physiology under N-limitation. Our previous results indicated that colonization with diazotrophic endophytes also increases drought tolerance (Figure 3). We will therefore test the effects of the new strains on plant responses to drought as well as to heat and elevated CO_2 .

Future Directions: In a follow-up proposal, we will use LC-MS to identify the nitrogenous compounds released by the endophytes using a time course assay following a pulse of $^{15}\text{N}_2$ gas. We propose to study the exchange of C and N using isotopic analysis under normal conditions and under abiotic stresses to

better understand the plant-endophyte symbiosis and the mechanisms by which endophytes improve plant health and growth.

Appendix 1: References and Notes

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