Evidence for Nitrogen-Fixation in the Salicaceae Family

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Abstract

It has been shown that poplars and willows are able to produce high amounts of biomass even at low soil nutrient levels and that the application of nitrogen (N) fertilizers typically results in little or no increase in growth. Poplars growing in rocks and gravel in their native riparian habitat were well supplied with N despite low soil N availability. In different poplar and willow individuals, diverse endophytic bacteria were identified, including a diazotrophic species in which molecular nitrogen-fixation (N_2-fixation) could be verified. Most fast-growing *Populus* and *Salix* species will fix N_2. These findings provide a greater understanding on the Salicaceae family with respect to sustainability of biomass production at low-input energy levels.

Key words: *Populus*, *Salix*, nitrogen-fixation, endophytic bacteria, diazotrophic bacteria

Introduction

Poplar (*Populus* spp.) and willow (*Salix* spp.) species are early successional trees with rapid growth, deep roots, and the ability to grow fast, even in nutrient-poor environments. Because of their fast growth, poplar cultivars are grown widely in plantations, mostly in temperate zones (figure 1). About 25 million acres (10 million hectares) of poplar plantations exist worldwide. Many trials have examined the factors influencing biomass production of poplar cultivars. These trials show that N fertilization usually has little or no effect (Heilman and Xie 1993, Jug and others 1999, Liesebach and others 1999, Coleman and others 2004, DesRochers and others 2006, Booth 2008, Mao and others 2010). Free-air carbon dioxide (CO_2) enrichment experiments (FACE) showed that higher CO_2 levels also require higher soil-N. Poplar was able to increase biomass production under elevated CO_2, however, without additional N (Pregitzer and others 2000, Luo and others 2006). Also, no yield response curves and few detailed fertilizer recommendations exist for poplar or willow.

Poplars growing on rocks and gravel in their native riparian habitat (figure 2) were found to have sufficient amounts of N in their tissues despite low soil N availability (Coleman and others 1994, Lawrence and others 1997). The explanation for the indifference of poplar towards soil N availability has been studied recently. The purpose of this paper is to summarize these findings and discuss the associated opportunities and implications.

Diazotrophic Bacteria

It is well known that a large endophytic community resides in the stem tissue of poplar and willow species, the function of which is still mostly unknown. Ulrich and others (2008b) found a total of 53 genera including *Proteobacteria*, *Actinobacteria*, *Firmicutes,* and *Bacteroidetes*. In poplar and willow grown in contaminated soil, Taghavi and others (2009) identified 78 endophytic strains, of which 71 percent belonged to Gammaproteobacteria, with others from *Serratia* spp.,

Figure 1. Stand of black poplar (*Populus nigra* L.) (Photo source: *Populus nigra* Network, EUFORGEN, Bioversitiy International, Rome).

Figure 2. Seedlings of black poplar from natural regeneration growing in its native habitat on gravel poor in nutrients (Photo source: *Populus nigra* Network, EUFORGEN, Bioversitiy International, Rome).

Rahnella spp., *Pseudomonas* spp., and *Enterobacter* spp. Among these endophytes, several diazotrophic (nitrogen-fixing) bacteria were identified. They remained undiscovered because of their inconspicuous occurrence in the living tissues of the stem and branches and not in root nodules like the legume family (Fabaceae).

Legumes form a symbiosis with *Rhizobia*, a genus of soil bacteria capable of biological N_2-fixation of atmospheric N_2, where the plant exchanges its carbohydrates from photosynthesis for the combined N from its root nodule inhabitants. In this process, N_2 becomes accessible to the plant by conversion into ammonia (NH_3). This conversion requires a high amount of energy in the form of adenosine triphosphate (ATP). Through a particular interaction, a specific bacterium associates with a specific legume, resulting in the familiar root nodules, in which N_2-fixation occurs. In addition to the legumes, woody plant species of nine families (Betulaceae, Cannabaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae, and Rosaceae) are known to associate with other N_2-fixing microbes living in specialized root nodules. Well known are *Frankia* bacteria found in *Alnus* spp. of the Betulaceae family.

Verification of N_2-fixation in *Salicaceae*

In poplars, endophytic bacteria were found inside stem tissues. These endophytes do not cause disease but rather are beneficial to the host by providing hormones, peptide antibiotics, enzymes, and other beneficial substances, thus classified as plant-growth promoting bacteria (Doty and others 2005, 2009; Ulrich and others 2008b; Scherling and others 2009). Plant-growth promoting bacteria were found in poplar and willow species (table 1). Among this array of growth-promoting substances, ammonia is also present in several other plant species without root nodules such as sugar cane, rice, coffee, and sweet potato (Reinhold-Hurek and Hurek 1998, Xin and others 2009). Thus, the common conclusion that plant species without root nodules are not associated with N_2-fixing bacteria has been proven incorrect.

To verify the ability to fix N_2, a first screening is efficient by employing the polymerase chain reaction to look for the presence of *nifH*, a gene encoding for one of the subunits of nitrogenase, the enzyme facilitating N-fixation (Doty and others 2009). Conclusive is also the acetylene reduction assay in which positive N_2-fixation activity of bacterial cultures is demonstrated by increased ethylene concentration over time (Doty and others 2009). Xin and others (2009) analyzed incorporation of the rare isotope $^{15}N_2$ instead of the common $^{14}N_2$ and showed that a strain of the endophytic bacteria *Burkholderia vietnamensis* isolated from a wild-grown *Populus trichocarpa* tree was able to fix $^{15}N_2$ by a 20-fold higher concentration of this isotope when compared with normal air. This endophyte was then inoculated onto Kentucky bluegrass (*Poa pratensis* L.) cultured on an N-free medium. After 50 days, the inoculated plants had increased 42 percent in weight and 37 percent in N when compared with the uninoculated control plants—showing that inoculation of N-fixing endophytes may enhance plant growth under N-limiting conditions. This particular *B. vietnamensis* strain is also able to provide IAA, a growth promoting hormone to the hosting plant, which may also have played a role in the biomass gain.

Another example of growth enhancement was shown by Ulrich and others (2008a) using an endophytic strain P22 of *Paenibacillus humicus* isolated from poplar. It caused a pronounced increase in root number and root length in poplar compared with uninoculated controls. The same effect was found when rooting macro cuttings of this poplar clone (Ulrich and others 2010). An analysis of the metabolites produced by the inoculated poplar showed that the poplar reacted pronouncedly to the presence of this endophyte by producing much higher amounts of asparagines and plant accessible urea (CH_4N_2O), but reduced amounts of organic acids of the tricarboxylic acid cycle. This effect on the metabolite profiles reflects remarkable changes in N assimilation in the plant (Scherling and others 2009).

Table 1. Bacteria isolated from poplar and willow host tree species showing nitrogenase activity.

Tree species	Bacterial strain	Method of verification	Reference
P. trichocarpa × *P. deltoides*	*Rhizobium tropici*	Culture on N-free medium	Doty and others 2005
[Populus alba × *(Populus davidiana + Populus simonii)* × *Populus tomentosa]*	*Paenibacillus humicus strain P22*	Metabolite analysis (urea)	Scherling and others 2009
P. trichocarpa, Salix sitchensis	*Burkholderia, Rahnella, Enterobacter, Acinetobacter,*	Culture on N-free medium; PCR with nifH primer; acetylene reduction assay	Doty and others 2009
P. trichocarpa	*Burkholderia vietnamensis*	Culture on N-free medium; PCR with nifH primer; acetylene reduction assay; $^{15}N_2$ incorporation assay; inoculation on other organism	Xin and others 2009

PCR = polymerase chain reaction.

Thus far, it is unknown if diazotrophic bacteria are present in all Salicaceae species. It can be expected that fast growing poplar and willow species adapted to riparian habitats with sandy soils poor in N availability are able to fix N. With respect to potential uses of plant-growth promoting bacteria, the best approach will be to quantify growth enhancement because of the symbiotic interactions between specific poplar and willow genotypes with specific bacterial strains.

Opportunities for Practical Use

The technical fixation process of plant-accessible ammonia from molecular N_2 requires an energy-input of 946 kJ $mole^{-1}$ and is thus highly energy consumptive. For this reason, plants favored for renewable energy crops are those able to produce high amounts of biomass with low requirements for synthetic fertilizer. Furthermore, negative influences of excessive N on the environment (e.g., groundwater leaching and emission of detrimental N_2O) can be avoided when growing N_2-fixing plants.

The energy source for the biological N_2-fixation is ATP of which an equivalent of 16 moles is hydrolyzed in the process. Biological N_2-fixation is more energy efficient than the inorganic process because it is enzyme supported and because the N is produced in the required amount and location. N_2-fixing plant species have therefore received much attention for both soil improvement and for reducing fertilizer usage. For those reasons, methods to initiate N_2-fixation in crop species by inoculation of diazotrophic endophytes have been investigated (Cocking 2005).

Because N_2-fixation is an energy-intensive process, N_2-fixing plants make ready use of freely available N in the soil (Cooke and others 2005). They can therefore be used to sequester surplus N in N-rich sites. For example, poplars and willows are being used to sequester N from sewage sludge (Dimitriou and Aronsson 2004). Other species, however, may be able to sequester higher amounts of N.

In agroforestry systems, poplar is being grown admixed with numerous crop plants (Yadava 2010). These systems have become common in many places and yield high-quality crops and high monetary returns for both the poplars and the crop plants (Bangarwa and von Wuehlisch 2009). In another study, poplars were grown in agroforestry systems with N_2-fixing plants; e.g., *Hippophae rhamnoides* (Mao and others 2010). Although the soil N increased, no biomass increase in the poplars occurred. This unexpected result is easily explainable when considering the N_2-fixing ability of poplar.

Implications for Tree Improvement

Analyses of endophytic bacteria in poplar and willow individuals showed that the bacterial communities differed considerably between trees (Ulrich and others 2008a, 2008b; Scherling and others 2009) indicating that the tree and bacteria interact in such a way that a certain bacteria community evolves within a particular tree genotype. The tree can thus acquire supplementary adaptive characteristics, which are not encoded by its genes. This adaptation may offset predicted gene expressions; e.g., in marker-assisted selections. The success of artificial inoculations with growth-promoting bacteria depends on the harmony of the bacterial strain and the genotype of the hosting tree.

It would be of practical importance to know the extent to which species or genotypes within the Salicaceae family vary in their ability to fix N_2. The special spectrum of bacteria found in different host genotypes suggests considerable variability. There may even be species that are unable to fix N_2. This inability could apply to species having evolved on sites where N was at or above sufficiency. Further research is warranted to better understand differences among genotypes and the potential for tree improvement on sites where N is limiting.

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