Sharon Lafferty Doty *Editor*

Functional Importance of the Plant Microbiome

Implications for Agriculture, Forestry and Bioenergy

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Chapter 1

Functional Importance of the Plant Endophytic Microbiome: Implications for Agriculture, Forestry, and Bioenergy

Sharon Lafferty Doty

Just as the human microbiome is important for our health [1], so too the plant microbiome is necessary for plant health, but perhaps more so. Since plants cannot move, they face more challenges in acquiring sufficient nutrients from a given site, defending against herbivores and pathogens, and tolerating abiotic stresses including drought, salinity, and pollutants. The plant microbiome may help plants overcome these challenges. Since genetic adaptation is relatively slow in plants, there is a distinct advantage to acquiring an effective microbiome able to more rapidly adapt to a changing environment. Although rhizospheric microorganisms have been extensively studied for decades, the more intimate associations of plants with endophytes, the microorganisms living fully within plants, have been only recently studied. It is now clear, though, that the plant microbiome can have profound impacts on plant growth and health. Comprising an ecosystem within plants, endophytes are involved in nutrient acquisition and cycling, interacting with each other in complex ways. The specific members of the microbiome can vary depending on the environment, plant genotype, and abiotic or biotic stresses [2–6]. The microbiome is so integral to plant survival that the microorganisms within plants can explain as much or more of the phenotypic variation as the plant genotype [7]. In plant biology research, an individual plant should thus be viewed as a whole, the plant along with intimately associated microbiota (a "holobiont"), with the microbiome playing a fundamental role in the adaptation of the plant to environmental challenges [8–10].

Intensive agriculture has stripped away many of the natural partnerships that plants in their native environments would have depended upon. Consequently, the services once provided through symbiosis have been replaced with chemical fertilizers, pesticides, and other inputs. However, as the functional significance of the

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microbiome has been revealed [11], the importance of restoring these relationships to optimize plant growth and yields in an environmentally sustainable manner has been recognized [12]. Plant microbiome sequencing has been performed for several plant species which is an important first step; however, the resulting data often provide only the species-level organization, not the necessary functional clues gained from metagenomic sequencing. For example, a metagenomic analysis of the full endophyte community of roots of field-grown rice provided extensive information on genes associated with the endophytic lifestyle [13]. Genes involved in N-fixation, phytohormone production, ROS detoxification, ACC deaminase, transport systems, signaling, colonization, and other putative symbiosis-related genes were identified, providing information on the functional attributes of the plant microbiome [13]. As sequencing technologies progress, it will be increasingly more feasible to gather this crucial information [14], more crucial since choice of the members of the plant microbiome is likely to be microbial strain specific, not species specific, with plants selecting for particular attributes important for the particular environmental condition [15, 16].

Through a better understanding of beneficial plant-microbiome interactions, improvements in the economic and environmental sustainability of agriculture, forestry, and bioenergy can be achieved. In all three of these industries, a reduction in inputs, whether it be fertilizer, water, or chemical pesticides, would lead to significant cost savings. The cost of using N-fixing microbes is estimated to be only 1% as much as that of using chemical fertilizers [17]. Chemical N fertilizers are produced using fossil fuels, high temperatures, and high pressure. Since diazotrophic endophytes use plant sugars produced from solar energy, the dinitrogen gas abundant in the atmosphere is fixed by the bacteria, providing essentially continuous fertilizer for the crops at little financial cost. In addition to nitrogen, the other main component of chemical fertilizer is phosphate. Since the majority of phosphate in soils is inaccessible to plants, there is rising demand, and there is a finite supply of rock phosphate; the cost of this key macronutrient is rising. Seedling mortality due to drought also results in major financial losses. With the increased frequency and duration of drought, the cost of freshwater rights can become a determining factor in deciding which crops to grow, as it incentivizes the cultivation of only the highest value crops to make up for the high cost of water. Specific endophyte strains can defend the host plant against pathogens [18, 19], potentially reducing the need for chemical pesticides. Through increasing plant growth and crop yields, and decreasing the amounts of inputs including fertilizers, water, and pesticides, endophytes have the potential to increase profit margins. The use of biostimulants has recently gained popularity among agricultural biotechnology companies, with the global market for bio-stimulants for plants estimated to rise to USD 3.6 billion by 2022 [20]. In addition to the economic benefits of appropriate endophyte inoculations, substantial improvements in the environmental sustainability of these industries can be made by lessening the impacts to aquatic ecosystems from chemical run-off, reducing greenhouse gas emissions, and lowering the depletion rate of groundwater reserves.

While the positive implications of endophyte inoculations for agriculture have been well reviewed [20–25], less attention has been given to potential impacts on forestry [26, 27]. Successful inoculation of widely used conifer species with endophytes could increase forest productivity and reduce reforestation costs, particularly through reducing tending costs during early stand management. Major advantages of using natural plant-microbe symbioses are that (1) they are easily applied at the greenhouse stage prior to out-planting, (2) the increased drought tolerance can occur just weeks after inoculation, (3) the microorganisms are easy and inexpensive to grow, and (4) they provide multiple benefits including increased nutrient acquisition, drought tolerance, growth, and overall health. By augmenting the microbiome of nursery stock at the greenhouse stage, foresters and restoration practitioners may be able to reduce the mortality rate during establishment. With the increased frequency and duration of drought, and the increased cost of fertilizers, the improved resilience and growth of trees from bio-inoculants would be an economic advantage for the forestry industry.

With limited arable lands and resources for both agriculture and bioenergy production, biomass for bioenergy should ideally be produced with fewer inputs and on marginal lands without competing with agriculture. Symbiosis with microorganisms can allow plants to overcome the challenges faced in these environments, including low-nutrient soils with limited water. Overcoming such challenges will be even more crucial when they are confronted with the increased temperatures and re-localization of precipitation seen with climate change. By understanding the natural plant-microbe interactions at work to increase plant stress tolerance in biomass crops, symbiosis-based technologies may be developed to increase biomass production. *Populus* is a flagship genus for the production of environmentally sustainable biomass for cellulosic ethanol and biochemicals. Endophytes from hybrid poplar have also been shown to increase growth of this important bioenergy crop [28–30]. Endophytes from native poplar applied to hybrid poplar can increase photosynthetic efficiency [31], drought tolerance [32], N_2-fixation [33], and a doubling of root mass accumulation [33]. This increased rooting could improve below-ground carbon storage and may also help with drought tolerance. Plants can increase photosynthetic rates under elevated CO_2 conditions only until other factors such as nutrients and water become limiting. Diazotrophic endophytes that also increase drought tolerance could therefore be used to improve the growth and sustainability of biomass production.

The focus of this book is on the functional importance of endophytes to plant growth and health. Endophytes can increase nutrient availability for plants through nitrogen fixation (Chap. 2), phosphate solubilization, and siderophore production (Chap. 3). The phytobiome can improve photosynthetic efficiency and water use efficiency (Chap. 4). Specific endophytes can increase tolerance to abiotic stresses including temperature, drought, and salinity (Chap. 5). Many endophyte strains are capable of producing hormones or modulating the host phytohormones, improving both plant growth and stress tolerance (Chap. 6).

To maximize the benefits of these symbioses, further research is required to understand at the mechanistic level how endophytes perform all of these integral roles for the host plant. It is time for a greener revolution, not based on chemical applications but on natural plant-microbe partnerships.

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Chapter 2
Endophytic N-Fixation: Controversy and a Path Forward

Sharon Lafferty Doty

Nitrogen (N) is an essential macronutrient due to its being a component of proteins, nucleic acids, and the energy currency of cells, ATP. While nearly 80% of our atmosphere is comprised of N, it is in an inert form, inaccessible to most life forms. Lightning strikes convert N_2 gas into ammonia or nitrate that is deposited in soils through rainfall, accounting for approximately 10% of available N [1]. Biological reduction of the triple bond of N_2 gas, however, requires the nitrogenase complex, a multi-subunit enzyme found in a subset of archaeal and bacterial species. This oxygen-sensitive complex "fixes" the dinitrogen gas into ammonia through an energy-intensive process, requiring 20–30 molecules of ATP per molecule of dinitrogen gas under normal physiological conditions [2]. Plants acquire N from soils rich in organic matter where previously fixed N is made available through decomposition, but where soils are nutrient poor, N is the key nutrient limiting growth.

The so-called green revolution of the twentieth century was made possible through the Haber-Bosch process for production of chemical N fertilizer. Using high temperature (400–650° C) and pressure (200–400 atm), and approximately 2% of global fossil fuels, this method produces over 450M tons of N fertilizer each year [3]. While this process is effective, its widespread use in commercial agriculture is not environmentally sustainable. Levels of ammonia in the atmosphere have increased significantly as a result of intensive agricultural practices [4]. Only about half of the applied fertilizer is taken up by plants. The excess N is converted by soil microorganisms to nitrous oxide, a potent greenhouse gas, or is leached into aquatic systems, disrupting the natural ecosystems [5].

Another source of fixed N for plants relies on biological N-fixation. Select groups of plants have evolved intimate partnerships with N-fixing (diazotrophic) bacteria harbored in specialized organs, termed nodules, most commonly found on the roots

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of such plants. These nodulated plants include the legumes and *Parasponia* that associate with rhizobia and the actinorhizal plants that associate with *Frankia*. The symbiotic interactions between the N-fixing bacteria and host plants have been extensively studied with a recent focus on the signaling mechanisms that trigger the specific association [6, 7]. Following mutual recognition, a root nodule forms, providing a specialized organ for symbiotic N-fixation in which photosynthate is exchanged for fixed N [8, 9]. Root nodules are generally 2–5 mm in diameter and are occupied by up to 10^9 rhizobia [1]. Since the nitrogenase complex is oxygen labile [10] and yet requires high levels of ATP for the reaction, legumes express a leghemoglobin that maintains a low-oxygen environment in the nodule, while the diazotrophic bacteria use a high-oxygen-affinity cytochrome oxidase for oxidative phosphorylation. The nodule further limits oxygen with an oxygen-diffusion barrier. While providing an apparently ideal environment for N-fixation, legumes tightly control the housed bacteria, sanctioning nutrients to favor the most effective symbionts [11].

There are many natural environments in which organic N is limiting and yet non-nodulating plant species thrive. From where do these plants obtain this essential nutrient? Over the last few decades, studies have demonstrated that N-fixing bacteria can be found throughout the plant body of such plants, tightly bound to the plant surface or within the plant in the apoplastic intercellular spaces or within plant cells. So-called associative and endophytic diazotrophic bacteria (AEDB), these bacteria may be specifically recognized by the host plant [12]. Unlike rhizobia that commonly use an infection thread to enter the plant host, endophytes use crack-entry, colonizing the lateral root junctions, and migrating within the plant. N-fixing endophytic bacteria were first isolated from grasses such as kallar grass [13], sugarcane [14], wild rice [15], and maize [16, 17], but also from a wide variety of plant species including African sweet potato [18], rock-colonizing cactus [19], miscanthus [20], feather mosses [21], dune grasses [22], coffee plants [23], invasive grasses [24], and poplar and willow [25–27]. Significant rates of biological N-fixation (BNF) from AEDB have been recorded [28–34]. Through isolations of culturable endophytic strains from the native hosts and re-inoculation into host or non-host plant species, it has been demonstrated in multiple studies over the last few decades that plants often benefit from these endophytic microorganisms with increased health and growth. Significant N-fixation has been quantified in some of these cases, such as in sugarcane [35], wheat [36], rice [37], lodgepole pine [38], and Western redcedar [39].

Despite several decades of global research on N-fixation in a diversity of non-nodulated plants, it is a widely held belief that only symbiotic N-fixation in root nodules is significant for plant growth. This view has led to the recent focus on systems biology transgenic approaches to attempt to solve the global fertilizer problem [3]. One approach is to engineer non-legumes to express a functional nitrogenase complex [40] while another approach is to engineer them to form root nodules in which rhizobia would fix N [41]. While both approaches are extraordinarily complex and would be major scientific achievements, they suffer from two basic problems: they are not widely applicable to crops other than the specific, engineered lines, and many countries restrict the use of transgenic crops.