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CHAPTER 3

The Use of Endophytes and Mycorrhizae in Switchgrass Biomass Production

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Introduction

Switchgrass (*Panicum virgatum* L.), a native warm-season perennial grass found throughout the US, characteristically produces high biomass yields annually with low inputs, and can grow on marginal land. Since the introduction of the Department of Energy's Bioenergy Feedstock Development Program over 3 decades ago, switchgrass has been the subject of intensive study, yielding a plethora of data regarding plant growth and stress resistance. As a C4 species, switchgrass is efficient at converting the sun's energy into carbohydrate compounds, and combined with being

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perennial, the plant offers much promise for future biomass production on a large scale, helping offset the use of fossil fuels. In fact, switchgrass yielded 504% of the energy consumed in a large, multi-farm study in the Central Plains (Schmer et al. 2008), and stands can produce for more than a decade. Furthermore, compared with other bioenergy crops, switchgrass cultivation is relatively simple and requires no specialized equipment by the producer. While yields are high, much more could further be improved for bioenergy purposes. Beneficial plant-microbe interactions, a field of study generating much interest in the past two decades, offer new solutions to improve switchgrass biomass yields, stress tolerance, first-year establishment, and sustainability.

Both bacterial and fungal microorganisms form ancient and mutually beneficial symbiosis with plants, and mycorrhizal fungi in particular are associated with the initial colonization of land by plants (Wang and Qiu 2006; Ryan et al. 2008). A cultivated field of plants represents a complex community of microbes, interacting, competing, and often assisting with plant growth promotion and stress resistance. Generally, beneficial plant-microbe interactions provide plant growth promotion via production of plant hormones, such as auxin, aiding in stress resistance to abiotic stresses including drought and salinity, production of antimicrobial compounds against plant pathogens, and nutrient acquisition such as atmospheric nitrogen fixation and solubilization of phosphorus in soil. These interactions are intricate and multifaceted, often dependent on time of development, genotype, environmental conditions, and native soil communities. Although mycorrhizal fungi and switchgrass interactions have been intensively studied (Parrish and Fike 2005), only a few articles have been published focusing on endophytes in switchgrass and their influence on growth promotion (Ghimire et al. 2009; Kim et al. 2012). Together, beneficial microorganisms could have the potential to help in the development of a low input and sustainable switchgrass production system (Nowak et al. 2011) and offer a practical way to improve plant growth and disease resistance.

Nomenclature, Diversity and Classification

The term 'endophyte' is derived from the Greek term 'endo' (within) and 'phyte' (plant), and may apply to both fungi and bacteria that reside in plant tissues during all or part of their life cycle and cause no apparent harm (Wilson 1995). It is estimated that every plant species has at least one associated bacterial endophyte (Strobel et al. 2004), and they belong to diverse classes of bacteria including alpha, beta, and gamma subdivisions of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria (Rosenblueth and Martinez-Romero 2006). These bacteria thrive within plants where

they successfully colonize roots, translocate to leaves, stems, and even to reproductive organs where they may be vertically transmitted to the next generation, ensuring a stable interaction with its host plant.

The number of microorganisms present in natural ecosystems is tremendous. In fact, estimates of the number of bacterial endophytes in the Brazilian Atlantic forest indicate the possibility of 2–13 million species in the aboveground plant parts alone (Lambais et al. 2006). Of the bacterial species identified, 97% were previously not described. A single plant species may also have a wide range of different bacterial genera associated. In wheat, culture based studies have shown that 88 bacterial species representing 37 genera inhabit the aboveground plant tissue (Legard et al. 1994), which likely underestimate the number of microorganisms as molecular studies yield much larger population numbers (Rasche et al. 2006). Both culture based and molecular based analyses indicate that alpha and beta Proteobacteria are the most numerous colonizers of the phyllosphere (Thompson et al. 1993). In total, 853 bacterial endophytes were isolated from aboveground parts of four agronomic crops and 27 prairie plants including switchgrass. *Cellulomonas, Clavibacter, Curtobacterium,* and *Microbacterium* isolates showed high levels of colonization and had the ability to persist in host plants (Zinniel et al. 2002).

Diazotrophs, or atmospheric nitrogen-fixing bacteria have been isolated from bioenergy crops, including *Miscanthus* spp. and *Pennisetum purpureum*, where *Herbaspirillum frisingense* sp. nov. (Kirchhof et al. 2001), *Azospirillum doebereinerae* (Eckert et al. 2001), and *Herbaspirillum frisingense* (Rothballer et al. 2008) were found. Similarly, different nitrogen-fixing bacteria belonging to genera *Stenotrophomona, Pseudomonas* and *Burkholderia* were isolated from sand dune grasses (*Ammophila arenaria* and *Elymus mollis*) in Oregon, which may biologically fix nitrogen and promote the growth of these plants under poor soil conditions (Dalton et al. 2004). Nitrogen-fixing bacteria have also been isolated from different plant species, such as Kallar grass (*Leptochoa fusa*) (Reinhold-Hurek et al. 1993), lodgepole pine (*Pinus contorta*), western red cedar (*Thuja plicata*) (Bal et al. 2012), and hybrid poplar (*Populous trichocarpa)* (Taghavi et al. 2010). While general surveys of endophytic populations in switchgrass have been undertaken (Zinniel et al. 2002), there are no detailed analysis on native bacterial endophytic interactions in switchgrass.

Fungal endophytic populations may also be substantial, particularly in longer lived plants, as 340 genetically distinct taxa were recovered from two tropical understory plant species (Arnold et al. 2000). Endophytic fungi can also have a significant beneficial impact on switchgrass performance (Kleczewski et al. 2012). While much emphasis has been placed on the study of clavicipitaceous fungal endophytes (*Neotyphodium/Epichloë*) with cool- and warm-season grasses (Rodriguez et al. 2009), two recent

surveys of switchgrass endophytes have failed to identify members of the Clavicipitaceae family (Ghimire et al. 2011; Kleczewski et al. 2012), suggesting that the major endophytic fungi inhabiting switchgrass are of the non-clavicipitaceous type, representing primarily ascomycetous fungi (Kleczewski et al. 2012). These endophytes may be found colonizing tissues above- and/or below-ground (Rodriguez et al. 2009). Recently, 18 taxonomic orders of fungal endophytes were isolated from switchgrass plants in northern Oklahoma belonging to the genera *Alternaria, Codinaeopsis, Fusarium, Gibberella, Hypoerea* and *Periconia*, and switchgrass shoot tissues showed a significantly higher diversity of fungal endophytic species compared to the root tissues (Ghimire et al. 2011). Similar fungal endophytic genera were isolated from switchgrass plants growing in a range of habitats across Indiana and Illinois, such as *Alternaria*, *Epicoccum*, *Phoma*, *Phaeosphaeria* and *Stagonospora* (Kleczewski et al. 2012). Since switchgrass is one of the most promising bioenergy crops, several laboratories in the US have been working on isolation and characterization of bacterial and fungal endophytes from switchgrass. Identifying and harnessing beneficial endophytic microorganisms that have a broad spectrum of plant growth promotion traits and possess various mechanisms for stress tolerance may aid in the development of a low input and sustainable switchgrass feedstock production system, particularly on marginal land.

Mycorrhizae are symbiotic fungi that interact with the roots of vascular plants. These fungi are typically divided into two groups: ectomycorrhizas which have hyphae that do not penetrate individual cells within the root and endomycorrhizas which, as the name implies, have hyphae that penetrate the cell wall and invaginate the cell membrane. Eighty to 92% of land plant species surveyed are associated with mycorrhizal fungi, among them, arbuscular mycorrhizal (AM) fungi are the predominant type (Wang and Qiu 2006), and are placed in the phylum (division) Glomeromycota. AM fungus is characterized by highly branched fungal structures located within the plant root cortical cells. Generally, AM fungi comprise 130 species of fungi classified as Zygomycotina (Simon et al. 1993). AM fungi from the order Glomales (Glomeromycota) are associated with most plant species including angiosperms, gymnosperms, pteridophytes, lycopods, and mosses (reviewed in Hause and Fester 2005). The fungi involved in the AM interaction are obligate biotrophs and reproduce asexually. As obligate biotrophs, AM fungi are not culturable without their host plant, making the study of these organisms difficult. AM fungal associations are important to help switchgrass tolerate unfavorable soil conditions (Parrish and Fike 2005). It has been reported that AM fungi play essential roles in switchgrass growth in acidic soil, which has high levels of exchangeable aluminum and immobile minerals, such as phosphorus (Koslowsky and Boerner 1989; Brejda et al. 1993; Johnson 1998). AM fungal associations

may be more critical in warm-season grasses, such as switchgrass because from an evolutionary perspective, both are of tropical origin (Hetrick et al. 1988).

Infection and Colonization

A focus on endophyte and mycorrhizal enhancement of switchgrass growth and stress tolerance, as well as other plants, requires the establishment of a stable plant-microbe interaction. Hence, the initial microbial infection and subsequent colonization of the plant is requisite for the eventual beneficial impact of the microbe on plant performance. While the focus of this chapter is on switchgrass, little work has been carried out to describe microbial infection and colonization processes with switchgrass systems. Therefore, literature relating to the mechanisms of infection and colonization of other plant species forms the bulk of this section, with the assumption that similar mechanisms are operational during switchgrass-beneficial microbe interactions.

Endophytic microbes and mycorrhizal fungi can inhabit various parts of the plant, such as the root, stem and leaves, and can also be found in flowers, fruits and seeds (Zakria et al. 2008; Rodriguez et al. 2009; Compant et al. 2011; Kim et al. 2012). However, most efforts have focused on microbes located within the soil compartment, and more specifically the beneficial bacteria living in this region and their interactions with the root system of the plant. As we explore the steps associated with bacterial infection and colonization, it is worth noting that many studies have followed these processes through the use of readily visible tags in the bacteria of interest, such as GFP (Compant et al. 2008; Prieto and Mercado-Blanco 2008; Kim et al. 2012; Weyens et al. 2012). However, care must be taken when using such tagged microbes, as it has been shown that GFP-tagging can modify the natural behavior of the microbe (Weyens et al. 2012).

Initial Bacterial Endophyte Root Infection

The first interaction of soil bacteria with the plant occurs at the rhizoplane, and a sufficient titer of robust bacteria are required in the soil region in close proximity to the root surface (rhizosphere). An experiment demonstrated that sustained, high rhizosphere soil populations of the endophyte *Bacillus subtilis* GY-IVI were required for efficient endophytic colonization of the root (Zhao et al. 2011). It has also been suggested that these robust and high titer levels of the colonizing bacteria in the soils help bacterial endophyte competition, indicating that these bacteria are highly competent at rhizosphere/rhizoplane colonization (Whipps 2001; Compant et al. 2005a).

A variety of bacterial traits are known to be required for rhizosphere and/or rhizoplane colonization competence (Compant et al. 2010). Numerous studies have shown that bacterial colonization of the rhizoplane occurs initially with localization across various regions of the root, including root tips, sites of lateral root emergence, and root hair zones (Compant et al. 2008; Prieto and Mercado-Blanco 2008; Zhang et al. 2010). During rhizoplane colonization, single cells have been observed, leading to the development of colonies along the root surface, and to the establishment of biofilms (Hansen et al. 1997; Benizri et al. 2001). However, rhizoplane colonization does not occur uniformly (Compant et al. 2010). For example, *Pseudomonas fluorescens* PICF7 predominantly colonized the root differentiation zone (Prieto and Mercado-Blanco 2008), and the more mature parts of the root exhibited little colonization by *Pantoea agglomerans* YS19 (Zhang et al. 2010). This variation on rhizoplane colonization distribution may be due to differences in root exudate production (Lugtenberg and Dekkers 1999), the protective microenvironment of different regions of the root (Prieto and Mercado-Blanco 2008), and/or the presence of specific or preferential cell surface binding sites for the bacteria (Miao et al. 2008). Regardless of the sites of rhizoplane colonization, it has been reported that the population densities of bacteria in the soil are approximately 2 orders of magnitude higher (10^7–10^9 CFU per g of rhizosphere soil) than are found on the root surface (Benizri et al. 2001; Bais et al. 2006).

Bacterial Root Internalization and Colonization as Endophytes

Following rhizoplane colonization, internalization of the bacteria and their development as endophytes can occur quite rapidly, within days of inoculation/rhizoplane contact (Compant et al. 2008; Prieto and Mercado-Blanco 2008; Zakria et al. 2008). In order to colonize the plant interior, bacteria must make their way past the root surface. This can happen through the presence of surface openings, such as cracks produced during lateral root emergence (James and Olivares 1998), or other wounds. Furthermore, other root areas, such as the elongation and differentiation regions may contain cells that are more fragile or less differentiated, and more susceptible to bacterial penetration (Reinhold-Hurek and Hurek 2011). As with rhizosphere and rhizoplane colonization competence, a variety of bacterial traits are associated with competence for endophytic colonization. These include flagella, nod genes, type IV pili and twitching motility (Compant et al. 2008). Many of these traits are associated with bacterial adherence and movement, or bio-control of other surrounding microorganisms, providing a competitive advantage for the colonizing bacteria. In addition, bacterially-secreted, cell wall-degrading enzymes are important for bacterial penetration (Quadt-Hallmann et al. 1997) and

internal colonization, including cellulolytic and pectinolytic enzymes (Quadt-Hallmann et al. 1997; Kovtunovych et al. 1999). The endophyte *Burkholderia phytofirmans* strain PsJN, known to colonize switchgrass (Kim et al. 2012), produces endogluconase and polygalacturonase (Compant et al. 2005b), to aid in cell wall degradation.

Following initial root penetration, bacterial colonization proceeds within the root cortex, and can extend into the central vascular cylinder xylem vessels (Compant et al. 2008; Priedo and Mercado-Blanco 2008; Zakria et al. 2008). However, not all bacterial endophytes colonize the xylem. For example, Priedo and Mercado-Blanco (2008) noted that *Pseudomonas fluorescens* PICF7 remained in the root cortex region and was never found in the xylem, with no subsequent translocation elsewhere. The inability of some endophytes to colonize the xylem and move past the root may be due to the presence of filters formed at branch root junctions (Shane et al. 2000), which may limit bacterial movement (Zakria et al. 2008). In addition, as endophytes are aided in their penetration through the root endodermis and pericycle by cell wall-degrading enzymes (James et al. 2002), it may be that some endophytes produce sub-optimal levels of enzymes to allow penetration into the vascular tissue.

Colonization within regions like the root cortex occurs within the intercellular spaces, outside of living cells (Reinhold-Hurek and Hurek 1998; Priedo and Mercado-Blanco 2008), which is not surprising as these are rich in minerals (potassium, calcium, sulfur, phosphorus, chlorine), sugars (Madore and Webb 1981) and non-carbohydrate metabolites, such as various amino acids and organic acids (Canny and McCully 1988; Canny and Huang 1993). Endophyte alterations of apoplastic pH can alter enzyme activities, sugar uptake of host cells, and sugar concentrations for the colonizing microbes (Bacon and Hinton 2002). Hence, this environment is supportive of endophyte growth, promoting compound cycling between the endophyte and the plant.

Bacterial Endophyte Colonization of Plant Aerial Tissues

The ability of some endophytes to colonize the xylem provides the opportunity for their systemic spread throughout the rest of the plant, via the transpirational stream in the xylem lumen. However, not all endophytes are capable of colonizing the aerial parts of plants. This may reflect the inability of some to adapt and survive the different niches represented by aerial tissues and organs (Compant et al. 2010). In switchgrass, *B. phytofirmans* strain PsJN titers were higher in the root than in the leaves 7 days post-inoculation of the roots. However, by 14 days post-inoculation, titers were higher in leaves and sheaths than in the roots, indicating translocation to these tissues (Kim et al. 2012). Generally, bacterial endophyte titers in the

aerial plant tissues are reported to be lower than in the root (Rosenbleuth and Martínez-Romero 2006; Compant et al. 2008). In addition, a fair amount of variation can be observed in these tissues. Compant et al. (2008) reported that PsJN could be found in only 10–60% of grape inflorescence stalks and grape berries following initial inoculation of roots. These were localized to xylem vessels, and only a single or few cells were observed. These results further indicated the importance of the xylem for systemic spread of endophytes, allowing them to reach as far as the reproductive tissues. However, this spread was very slow, taking 5 weeks to reach inflorescence tissues. The very low titers of PsJN that ended up in these tissues was attributed to competition with other co-localized endophytes, which can inhabit different tissues and organs, reflecting different niches of colonization (Compant et al. 2011). This report of endophytic bacteria being low or absent in flowers and fruits echoes other comments (Hallman 2001), suggesting low vertical transmission. Bacterial colonization, in general, varies from one cultivar to another and depends on many factors. For example, in soybean, plant genotype, tissue age, season of isolation, and herbicide application, all affected colonization (Kuklinsky-Sobral et al. 2004).

Fungal Endophyte and Mycorrhizal Colonization

In addition to beneficial bacterial endophytes, beneficial fungi also exist with the potential to enhance switchgrass performance. These beneficial fungi represent both mycorrhizae and endophytes. As with the bacterial endophyte interaction, root exudates, as well as CO_2 release, play a role in stimulating development of the initial interaction, enhancing fungal spore germination, hyphal growth towards the root and hyphal branching (Giovannetti et al. 1993). The key exudate molecules are the strigolactones (Akiyama et al. 2005; Besserer et al. 2006), which form a concentration gradient helping the fungus to assess closeness of the host root.

As the fungal hyphae grow and then contact the root epidermis, each contacting hypha produces an appressorium (hypophodium), which flattens out and adheres to the epidermal cell surface. Through the production of localized cell wall-degrading enzymes, and the turgor pressure exerted by the contacting hyphal tip, the fungus is able to penetrate the epidermal cell wall (Bonfante and Perotto 1995). Once across the cell wall, the root host cell membranes invaginate to accommodate the fungus, resulting in the development of an apoplastic space between the fungus and plant cell, providing the interface for exchanges between both organisms (Vierheilig 2004). In order to extend into the inner cortical tissues, a novel structure, the prepenetration apparatus (PPA) develops within the root, which helps direct the course of hyphal movement through the root (Genre et al. 2008). The PPA formation represents a tunnel or bridge through the cortical cells,

with microtubules, microfilaments and rich in ER-cisternae. In addition, a reorientation of the plant cell nucleus occurs, with its movement to the site of fungal attachment, and it then leads and serves as a guide for the elongating PPA (Genre et al. 2008), which provides a tunnel for the growing hyphae as they colonize and grow towards the inner root cortical cells (Parniske 2008). The hyphae may grow along and between cells and eventually colonize the internal cortical cells, including longitudinally into adjacent cells, still under the guidance of the PPA (Genre et al. 2008). It is evident that key changes in growth and behavior of both plant and fungal cells take place to allow this process to occur, and a variety of genes/traits from both partners are involved in the success of this process (Gadkar et al. 2001; Genre et al. 2008; Parniske 2008).

Studies with various endophytic fungi have suggested that fungal entry can occur in the leaf through hyphae in wound sites, stomata, or penetration via appressoria (Ernst et al. 2003). Fungal growth tends to be primarily intercellular, having little effect on the surrounding host cells (Ernst et al. 2003; Gao and Mendgen 2006). As some non-clavicipitaceous fungi can be transferred vertically (Rodriguez et al. 2009), fungal growth may extend into inflorescence primordia, and eventually into the ovules with colonization of the scutellum and embryo axis of the seed (Rodriguez et al. 2009). In the case of root-colonizing fungal endophytes, root surface colonization was followed by direct hyphal penetration or through appressorium formation, and subsequent growth through epidermal and cortical cell walls (Gao and Mendgen 2006).

Plant Growth Promotion

Endophytes, including bacteria and fungi, and arbuscular mycorrhizal (AM) fungi, directly or indirectly affect plant growth. In general, these microorganisms promote host plant growth, enhance nutrient uptake and stress tolerance, and inhibit plant pathogen growth. These three plant growth-promoting microorganisms have been studied in a broad range of plants including switchgrass, as will be detailed below.

Bacterial Endophytes

Plant growth-promoting bacterial endophytes can affect growth directly by providing bacterium-synthesized compounds, often plant hormones, and by facilitating the acquisition of compounds from the environment, including atmospheric nitrogen fixation. Endophytes may also act indirectly by decreasing or preventing the colonization or the deleterious effects of pathogenic organisms (Lodewyckx et al. 2002) by producing antibiotics to outcompete plant pathogens (Bibi et al. 2012).

One of the most well-studied bacterial endophyte associations is atmospheric nitrogen fixation by specific endophytes. This symbiosis is well known in leguminous plants (Stacey et al. 2006) where the soil bacteria *Rhizobia* infect the roots of the host plants, inducing the formation of nodules where they fix atmospheric nitrogen and provide it to the host plant in exchange for carbon compounds (Lodewyckx et al. 2002). Additionally mutualistic associations through the fixation of nitrogen can also be observed in non-leguminous plants, such as rice (Mattos et al. 2008), maize (Montañez et al. 2009), sugarcane (Oliveira et al. 2009), wheat (Webster et al. 1997), strawberries (de Melo Pereira et al. 2012), and grasses (Reinhold-Hurek et al. 1993; Kirchhof et al. 2001).

Nitrogen-fixing bacteria have been studied extensively in the bioenergy crop sugarcane, and include *Gluconacetobacter* spp., *Azospirillum* spp., *Herbaspirillum* spp. and *Burkholderia* spp. (James and Olivares 1998; James et al. 2001; Suman et al. 2005; de Carvalho et al. 2011). In fact, in Brazil, the cultivation of sugarcane uses only a small amount of fertilizer (de Carvalho et al. 2011) without showing nitrogen deficiency symptoms (Rosenblueth and Martinez-Romero 2006), and there is evidence that a significant amount of nitrogen is obtained from plants associated with bacterial endophytes (de Carvalho et al. 2011). To date, there are no reports on nitrogen-fixing bacterial endophytes in switchgrass, and screening for diazotrophic bacteria that inhabit switchgrass is under way in our laboratory.

There have been numerous publications on plant growth promotion by bacterial endophytes (see review in Berg 2009; Mei and Flinn 2010). In switchgrass, young seedlings of the cultivar Alamo inoculated with *Burkholderia phytofirmans* strain PsJN, isolated from onion roots (Frommel et al. 1991), showed significant growth promotion with an increase of root and shoot length of 35.6% and 32.8%, respectively, as well as an increase of fresh weight of 83.6% compared with control plants (non-inoculated) after one month under *in vitro* conditions (Kim et al. 2012). The same pattern was observed under growth chamber and greenhouse conditions, where plants inoculated with the *B. phytofirmans* strain PsJN showed persistent growth vigor with significant increases in fresh and dry weights, and an increase in the number of early tillers (Kim et al. 2012). Also, results showed that *B. phytofirmans* strain PsJN has potential in the development of a low input and sustainable switchgrass feedstock production system on marginal lands as higher biomass yields were observed under sub-optimal growth conditions with PsJN inoculated plants over control (Kim et al. 2012). However, PsJN growth promotion is genotype specific in switchgrass as the upland cultivar Cave-in-Rock did not respond to inoculation. We are currently isolating bacterial endophytes from switchgrass tissues and have made progress in screening and selecting beneficial bacterial endophytes which have a broad spectrum of growth promotion in various switchgrass cultivars.

Fungal Endophytes

Fungal endophytes are most commonly found living in aboveground plant tissues and occasionally in roots (Saikkonen et al. 1998). Plants infected with fungal endophytes gain growth promotion, stress tolerance, water use efficiency, and protection against vertebrate herbivores and root nematodes (Schardl et al. 2004; Rodriguez and Redman 2008; Rodriguez et al. 2009). During the interactions, endophytes obtain shelter, nutrition and dissemination through propagules of the host plants (Schardl et al. 2004). Like bacterial endophytes, fungal endophytes also promote host plant growth, such as increased root growth and longer root hairs (Malinowski et al. 1999), which may contribute to enhanced nutrient uptake. For instance, the root and shoot biomass of poplar, maize, tobacco, bacopa, *Artemisia*, and parsley was doubled compared with their respective controls after four weeks of *Piriformospora indica* inoculation (Varma et al. 1999).

Fungal endophytes of the genus *Neotyphodium* (an asexual form of *Epichloë* spp.) have been well studied for their symbiotic associations with different grass species, especially the family Pooideae, which includes many important species of forage and turf grasses (Clay 1990; Schardl et al. 2004; Sugawara 2011). Through this symbiosis, grasses have exhibited increased growth, tolerance to stress and resistance to herbivores (Schardl et al. 2004; Faeth et al. 2010). For instance, plant growth, biomass yield and tiller number increased when ryegrass (*Lolium perenne*) was inoculated with *N. lolii* (Spiering et al. 2006), and Dahurian wild rye *(Elymus dahuricus)* with *Neotyphodium* spp. (Zhang and Nan 2007). Endophyte-infected plants showed a higher survival rate, regrowth rate, and more biomass seed production compared to non-infected plants after a year in the field (Iannone et al. 2012).

In switchgrass, NF/GA-993 (a synthetic lowland switchgrass cultivar) inoculated with six strains of *Sebacina vermifera* fungal endophytes showed increased plant growth, root length, and biomass production (Ghimire et al. 2009). Recently, Sasan and Bidochka (2012) found that the fungal endophyte *Metarhizium robertsii* was able to endophytically colonize the roots of switchgrass and promoted growth and increased the density of root hairs (Sasan and Bidochka 2012). However, fungal endophytes recently isolated from switchgrass plants had both beneficial and detrimental effects on switchgrass biomass yields in greenhouse conditions. *Phaeosphaeria pontiformis*, *Epicoccum nigrum, Alternaria* spp. and *Colletotrichum* spp. increased total biomass by 25–33%, *Stagonospora* spp. increased shoot biomass by 22%, and *Colletotrichum* sp. increased root biomass by 45%, but over 60% of isolates tested reduced switchgrass growth (Kleczewski et al. 2012).

Arbuscular Mycorrhizal (AM) Fungi

AM fungi can enhance a plant's ability to acquire nutrients like phosphorus and nitrogen (Clark 2002; Parrish and Fike 2005; Leigh et al. 2009; Schroeder-Moreno et al. 2011), phytoremediate contaminated soil (Entry et al. 1999), and withstand acidic soil (Clark 2002). AM hyphae have the ability to extend beyond the usual nutrient absorption zone of plant roots, therefore reaching additional essential nutrients and transporting them to the plant (Clark 2002).

Mycorrhizal fungi and other rhizosphere microflora have played significant roles in switchgrass growth in nature (Parrish and Fike 2005). In field conditions, switchgrass plants are commonly associated with AM fungi and have shown growth stimulation under different conditions (Brejda et al. 1998; Parrish and Fike 2005; Schroeder-Moreno et al. 2011). Under acidic soil conditions, the inoculation of AM fungi (*Glomus, Gigaspora* and *Acaulospora*) increased the root length of switchgrass plants, as well as the uptake of minerals such as phosphorus, nitrogen, sulfur, potassium, calcium, magnesium, zinc, and copper but reduced the uptake of manganese, iron, boron, and aluminum (Clark 2002). Inoculation with the AM fungi *Gisgospora margarita*, *Gi. Rosea*, *Glomus clarum*, and *Scutellospora heterogama* significantly increased nitrogen in shoots (Schroeder-Moreno et al. 2011), which implies AM fungi play an important role in N cycling from the soil to switchgrass plants.

Microorganism diversities affect plant growth promotion because plants exist in a community of bacteria, fungi, algae and/or viruses (Rodriguez and Redman 2008), and plants could be associated with more than one microorganism. Inoculation of switchgrass seedlings with multiple types of rhizosphere microflora increased the yield of shoots and roots up to 15-fold and also increased nitrogen uptake 6-fold and phosphorus uptake 37-fold, compared with the control plants infected with rhizosphere bacteria only (Brejda et al. 1998). Environmental factors, such as nutrients and stress, also influence symbiosis between host plants and endophytes as well as AM fungi. Under high nutrient availability, symbiotic *Neotyphodium occultans-Lolium multiflorum* association showed higher seed weight than that of non-symbiotic plants (Gundel et al. 2012). Under greenhouse conditions, the combination of AM fungus and the fungal endophyte *Epichloe elymi* on growth promotion in the grass *Elymus hystrix* was found to be additive (Larimer et al. 2012). However, the presence and specificity of the fungal endophyte altered the interaction of AM fungus with the host plant as endophyte infection increased *Glomus mosseae* colonization while decreasing *G. claroideums* colonization (Bibi et al. 2012).

Stress Tolerance

Plant growth is usually limited by biotic and abiotic stresses. Abiotic stress includes various environmental stresses, such as drought, temperature, salinity, air pollution, heavy metals, pesticides and soil pH. Biotic refers to living organisms that cause diseases, such as bacterial and fungal pathogens, pests, insects, viruses, and nematodes. Symbiotic relationships with endophytes and mycorrhizal fungi have been shown to increase stress tolerance in host plants (Gibert and Hazard 2011).

Abiotic Stress Tolerance

Drought is one of the most wide spread and common abiotic stresses and causes economically important losses in agriculture and forestry crops every year. The mutualistic symbiosis between bacterial or fungal endophytes or AM fungi and host plants could enhance host plant drought tolerance. For example, Japanese Bitter Orange (*Poncirus trifoliate*) seedlings inoculated with AM fungus *Glomus mosseae* enhanced plant height and increased relative water and chlorophyll contents when seedlings were subjected to three days of water depletion (Fan and Liu 2011). Similar results were observed when AM fungus inoculated rice plants were under drought conditions, with increased levels of protective compounds, such as ascorbate and proline, produced in the plants (Ruíz-Sánchez et al. 2011). The evergreen tree *Theobroma cacao* infected with the endophytic fungus *Trichoderma hamatum* isolate DIS 219b exhibited delayed drought stress by changes in stomatal conductance, water potential, and net photosynthesis (Bae et al. 2009).

In grasses, endophytic associations also increased drought tolerance as some accessions of the perennial ryegrass (*Lolium perenne*) infected by *N. lolli* showed more tillers, greater tiller length and higher biomass than non-infected plants (Kane 2011). Endophytic inoculation of *Epichloë festucae* in *Fetusca eskia* enhanced seedling survival under drought conditions (Gibert and Hazard 2011). A perennial bunchgrass *Achnatherum sibiricum* infected with endophytic fungi showed a higher root/shoot ratio and net photosynthetic rate than non-inoculated plants under drought conditions (Han et al. 2011). The symbiosis between *Agrotis hyemalis* and *Epichloe amarillans* produced 40% more inflorescences, earlier flowering and greater seed mass than non-inoculated plants under drought conditions (Davitt et al. 2011). However, when *Panicum rigidulum* plants were subjected to drought conditions, endophyte *Balansia benningsiana* infected plants did not show any advantages over control plants during drought stress but endophyte infection helped rapid leaf regrowth during recovery (Ren and Clay 2009).

Cultivated soils are becoming more saline due to excessive fertilizer use, the use of wastewater from urban and peri-urban areas and agricultural drainage as well as the desertification processes (Bashan and de-Bashan 2010). Plant growth promoting bacteria offer the potential to reduce the impact of this stress. For instance, cucumber plants inoculated with *Paecilomyces formosus* showed increased shoot length compared with that of non-inoculated plants under high salinity conditions (Khan et al. 2012). In studies with *Salicornia brachiata*, the most salt-tolerant plant species among *Salicornia* spp., *Brachybacterium saurashtrense* and *Pseudomonas* sp. bacterial endophytes significantly increased plant growth under salt stress conditions. The bacteria *Pseudomonas putida* and *P. pseudialcaligens* inoculation increased plant growth of chickpeas under saline conditions in pot experiments (Patel et al. 2012). Inoculation of AM fungi *Glomus mosseae, G. deserticola* and *Gigaspora gergaria* enhanced the growth of wheat (*Triticum aestivium*) under high salinity conditions as well as increased nutrient uptake (potassium, nitrogen, phosphorus and magnesium), proline levels, acid and alkaline phosphatase activities, and total soluble protein content (Abdel-Fattah and Asrar 2012).

Phytoremediation is the process by which plants can uptake, accumulate, or metabolize toxic compounds, such as heavy metals, from contaminated soil (Kumar et al. 1995). The plant-endophyte association has been used at phytoremediation sites to degrade toxic compounds for practical use (Van Aken et al. 2004). *Brassica juncea* inoculated with a plant growth promoting bacterium strain A3R3 showed increased plant growth when grown in soil at different concentrations of nickel, with the increases of fresh and dry weights by 50 and 45%, respectively at 450 mg nickel/kg soil compared with non-inoculated plants (Ma et al. 2011). Many plant growth-promoting endophytes could alleviate plant stress from contaminants by degrading such contaminants, and in return, could provide the products for plant use (Weyens et al. 2009a, b). For phytoremediation of toxic metals, endophytes may have a metal-resistant or sequestration system and could reduce metal toxicity and influence metal translocation to the aboveground plant parts. Metal-resistant endophytic bacteria have been found in the genera *Pseudomonas, Methylobacterium, Microbacterium* and *Burkholderia*. In tall fescue (*Lolium arundinaceum*) grown under greenhouse conditions in a solution contaminated with cadmium, endophytic fungus (*Neotyphodium coenophialum*) infection enhanced cadmium accumulation and increased cadmium transport from roots to the shoots (Ren and Gao 2011). In *Festuca arundinacea* and *Festuca pratensis* grasses, grown under high cadmium conditions, results showed higher biomass production and higher levels of cadmium accumulation in the roots and shoots of endophyte-infected plants versus uninfected plants (Soleimani et al. 2010). Under greenhouse conditions, the seedlings of guinea grass (*Panicum maximum*) cultivars

inoculated with *Pantoea* spp. Jp3-3 exhibited significant alleviation from the negative effect caused by the stress of 300 μM copper (Huo et al. 2012). Switchgrass and two other grasses, bahia grass (*Paspalum notatum*) and Johnson grass (*Sorghum halepenese*), were inoculated with two AM fungi, *Glomus mosseae* and *G. intraradices*, and results showed that the aboveground biomass of these three grasses contained 26.3 to 71.7% of the total amount of ^{137}Cs, and 23.8–88.7% of the total amount of ^{90}Sr (Entry et al. 1999). The proportion of contaminant removal from the soils by these plant species was significantly increased, possibly due to root colonization by mycorrhizal fungi and the high density of roots (Entry et al. 1999).

AM fungi also have the ability to boost switchgrass plant growth under acidic soils. Of the AM fungi tested, *Glomus clarum* and *G. diaphanum* aided to increase the dry matter of plants on soils at pHca 4 and pHca 5 compared with the non-inoculated plants (Clark et al. 1999a). The benefits of AM fungi could be attributed to an increase in acquisition of mineral nutrients such as phosphorus and a decrease of the toxic elements ferrous, boron, aluminum and manganese (Clark et al. 1999b), which are present in acidic soils.

Biotic Stress Tolerance

Endophytes inhibit plant pathogen growth and prevent or reduce disease development through the production of toxic alkaloids or by occupying the same ecological niche as the pathogen (Clay 1990). Studies found that three *Bacillus* strains and two *Pseudomonas fluorescens* strains decreased up to 60% of the disease symptoms caused by *Pseudomonas syringae*, a powdery mildew and angular leaf spot, and increased the fresh weight of inoculated melon plants compared with non-inoculated controls (García-Gutiérrez et al. 2012). In tomato plants, bio-control of *Bacillus subtillis* S499 was tested for antagonism against *Fusarium* spp. by treating the seeds with a formulated powder containing different concentrations of viable spores of *B. subtillis* S499, and results showed that all treatments significantly reduced disease severity up to 65–70% compared with control plants (non-inoculated seeds) (Nihorimbere et al. 2010).

Since endophytes have the ability to inhibit or prevent pathogen growth, they have been considered as biological control agents. In the interaction of Italian ryegrass (*Lolium multiflorum* Lam) with the fungal endophyte *Neotyphodium*, the ryegrass exhibited increased resistance to *Trigonotylus caelestialium* (Shiba et al. 2011). Additionally, the bird cherry oat-aphid (*Rhopalosiphum padi*), a notorious pest of forage and cereal grasses, showed a preference to non-infected plants of Alpine timothy (*Pleum alpinum*) over the plants infected with *Neotyphodium* spp. (Clément et al. 2011). Perennial ryegrass (*L. perenne*) plants colonized by *N. lolii* exhibited reduced aphid populations and in some cases the aphids exhibited reduced adult life-

span and fecundity (Meister et al. 2006). Tall fescue plants inoculated with *Neotyphodium coenophialum* decreased the survival rate and feeding of the corn flea beetle, *Chaetocnema pilucaria* (Ball et al. 2011). Similar preferences were observed in *Achnatherum inebrians* (drunken horse grass) where *Neotyphodium gansuense*-infected plants decreased the preference of herbivores such as bird cherry-oat aphid, carmine spider mite (*Tetranychus cinnabarius*), grasshopper (*Oedaleus decorus*) and seed-harvesting ant (*Messor aciculatus*) due to high levels of ergine, ergonovine and ergoit alkaloids produced by the fungal endophyte (Zhang et al. 2011). Recently, endophytic bacteria isolated from root tissue of six plants growing in a tidal flat area of Korea showed antagonistic potential toward the pathogenic oomycete fungi *Phytophtore capsici* and *Pythium ultimim*, and some of them were able to degrade biopolymers, such as cellulose and β-1,3-glucan, which are major components of the cell wall of oomycetes (Bibi et al. 2012).

In switchgrass production, it was found that large-scale planting of switchgrass could be devastated by *Puccinia emaculata* Schwein, a rust fungus (Zhao B. http: //hayandforage.com/biofuels/rust-resistant-switchgrass-research-goal-0323). In the future, it may be possible to identify endophytes which produce antifungal compounds to help offset losses caused by the rust fungus.

Mechanisms

As plants are sessile organisms, the wide diversity of mutually beneficial plant-microbe interactions represents an ancient evolutionary partnership, helping the host plant survive and thrive, even in some of the harshest environments on the planet. Mechanisms of growth promotion by bacterial and fungal endophytes as well as AM fungi have been investigated in grasses for decades, and various mechanisms play roles in promoting plant growth and development. Bacterial endophytes are capable of producing or regulating plant hormones, helping acquire vital nutrients, and bio-control of pathogens (Sturz et al. 2000). Plant associated fungi, both endophytic and mycorrhizal, also confer a range of growth promotion benefits to their host plant including nutrient acquisition. Furthermore, a particular bacterial or fungal endophyte may utilize one or more mechanisms to promote plant growth and may even utilize different mechanisms at various points during the life cycle of plants. While it is clear that endophytes can benefit the host plant in many ways, establishing clear-cut growth promotion in the field can be difficult due to a number of factors including the diversity of native microorganisms in the soil and soil conditions. A more profound understanding of these mechanisms is allowing scientists to discover new ways to integrate their use into increasing yields of bioenergy crops like

switchgrass. Also, by utilizing tools of modern molecular biology and functional genomics to understand the complexity of growth promotion at the genetic level, additional light will be shed on these complex interactions. As more is learned about the biochemistry, molecular biology, and physiology of microbe-plant interactions, it is evident that bacterial and fungal microorganisms will be important components for sustainable bioenergy feedstock production in the future.

Plant growth promotion can generally be achieved directly by interactions between the microorganism and host and/or indirectly through antagonistic activity against plant and environmental pathogens (Berg 2009). In this section, we will discuss both mechanisms and how different beneficial microbes may work together to benefit the host plant simultaneously (Muller et al. 2009), as well as how microorganisms, especially bacteria, may share mechanisms of actions genetically through horizontal gene transfer.

Phytohormone Production and Regulation

Plant tissues produce or regulate different hormones to respond to internal and external cues during practically every aspect of plant growth and development. Bacterial endophytes have the ability to produce plant hormones and regulate their balance as well. Auxin, a hormone associated with plant growth promotion, influences many plant cellular functions and is an important regulator of growth and development. Bacterial endophytes are commonly capable of producing auxin which, at the genetic level, may either be constituently expressed or inducible (Mattos et al. 2008). Auxin producing bacterial endophytes increased the number and length of lateral roots in wheat (Barbieri and Galli 1993). Increased root length, root surface area and the number of root tips were observed in hybrid poplar inoculated with auxin producing bacteria, resulting in enhanced uptake of nitrate and phosphorus and boosting biomass by 60% compared with non-inoculated plantlets (Taghavi et al. 2009). Furthermore, *Pseudomona flourescen* significantly increased the growth of maize plant radicles under laboratory conditions via the production of auxin (Montañez et al. 2012). To date, multiple auxin biosynthesis pathways have been identified in bacteria, and their regulation is controlled by several different genetic and environmental factors (Bertalan et al. 2009). The production of native auxin, indole-3-acetic acid (IAA) by bacteria has been documented in species such as *Rhizobium*, *Pseudomonas*, *Azospirillum*, *Azotobacter* and *Bacillus* (Hayat et al. 2010).

Cytokinins are a diverse range of compounds that, like other plant hormones, are involved in many activities of plant growth and development. As a group, they have been shown to regulate cell division, seed dormancy

and germination, senescence, new bud formation, and leaf expansion. They also play roles in controlling plant organ development, mediating responses to various extrinsic factors and the response to biotic and abiotic stresses (Spichal 2012). Researchers have demonstrated that certain endophytic bacteria are able to produce cytokinins and promote lateral root growth (Senthilkumar et al. 2009). Zeatin, a native plant growth promotive hormone, belonging to the cytokinin family, has been found in significantly higher levels in the beneficial bacteria *B. subtilis* and *P. putida* (Sgroy et al. 2009).

Gibberellins are native plant growth promotive hormones. Many plant growth promoting endophytes also produce gibberellins to enhance host plant growth (Joo et al. 2009; Fernando et al. 2010). For example, one *Penicillium citrinum* isolate, IR-3-3 from the sand dune flora, produced higher physiologically active gibberellins and stimulated Waito-c rice and *Atriplex gemelinii* seedling growth (Khan et al. 2008). Gibberellic acid levels were also high in the plant associated bacteria *Lysinibacillus fusiformis, Achromobacter xylosoxidans, Brevibacterium halotolerans,* and *Bacillus licheniformis* (Sgroy et al. 2009).

Ethylene, a simple organic molecule (CH_2=CH_2), is commonly thought to be a growth inhibitive hormone. It is typically produced when plants are exposed to environmental stress, repressing plant growth and development until the stress disappears or the levels of ethylene decrease (Gamalero and Glick 2012). Ethylene inhibits stem elongation, promotes lateral swelling of stems, and causes stems to lose their sensitivity to gravi-trophic stimulation (Glick 2005). In biomass production as in agriculture generally, it is important to keep ethylene low in order to maximize yields. An enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase produced by bacteria, interferes with the physiological processes of the host plant by decreasing ethylene levels (Hardoim et al. 2008) via metabolizing ACC, a precursor to ethylene so ethylene levels are reduced in plants, and plant growth is promoted. Activity of ACC deaminase is a common feature found in plant-growth promoting bacteria such as *Enterobacter, Pseudomonas* and *Burkholderia* (Shah et al. 1998; Sessitsch et al. 2005; Govindasamy et al. 2008). *Burkholderia phytofirmans* strain PsJN stimulates growth of many plant species, including potato, tomato, grapevine, and switchgrass (Pillay and Nowak 1997; Nowak et al. 1998; Barka et al. 2002; Kim et al. 2012) and was reported to have a high activity of ACC deaminase (Sessitsch et al. 2005). Endophytes that produce ACC deaminase have also been shown to increase host plant growth in soils with high salinity (Egamberdieva 2012; Siddikee et al. 2012) and increase drought tolerance (Arshad et al. 2008; Belimov et al. 2009). *Pseudomonas* sp. strain A3R3 showed higher ACC deaminase activity and increased plant growth in nickel contaminated soil (Ma et al. 2011).

Abscisic acid (ABA) is involved in responses to environmental stresses such as heat, drought, and salt, and is also produced by endophytes.

Endophytic bacterial strains SF2, SF3, and SF4 isolated from sunflowers (*Helianthus annuus*) had the ability to produce ABA and jasmonic acid, which increased under drought conditions (Forchetti et al. 2007), implying these endophytes enhance stress tolerance of host plants. Two strains of *Azospirillum brasilensis*, successfully used to increase the yield of maize and wheat in field conditions, were both able to produce different plant growth regulators such as IAA, gibberellic acid, zeatin and ABA (Perrig et al. 2007), highlighting the ability of endophytes to confer multiple mechanisms of growth promotion.

Bacterial Nitrogen-fixation

Endophytic bacteria that live freely in the internal tissues of plants and cause no apparent harm have a diverse range of growth promotion mechanisms including nitrogen fixation. Although 78% of the earth's atmosphere is nitrogen, nitrogen is often a limiting factor in agriculture since it is not readily available to plants. Bacteria and Archea are the only organisms that can fix atmospheric di-nitrogen, thereby making it available for plant growth. This activity is termed biological nitrogen fixation (BNF) and is catalyzed by the oxygen sensitive nitrogenase enzyme to convert N_2 to bio-available NH_3. Nitrogenases are complex metalloenzymes with highly conserved structural and mechanistic features (reviewed in Alberty 1994; Burgess and Lowe 1996; Rees and Howard 2000). The enzyme is oxygen sensitive, which imposes physiological constraints on the organism. Additionally, the enzyme has a relatively slow turnover time (Thorneley and Lowe 1985), which requires the microbe to synthesize large quantities of the protein, up to twenty percent of protein in the cell (reviewed in Dixon and Khan 2004). Also, the conversion of atmospheric di-nitrogen to a form that can be used by plants requires 16 ATP to reduce one molecule of N_2, making it one of the most energy demanding reactions identified in bacterial organisms (Thorneley and Lowe 1985). Together, the amount of energy, the low oxygen requirement, and the amount of protein required to create the nitrogenase enzyme, place a large burden on a nitrogen fixing endophyte. As a result, the synthesis of the nitrogenase complex is stringently regulated at the genetic level (Dixon and Khan 2004). It has been suggested that bacterial endophytes are placed in a more favorable environment compared to rhizospheric bacteria because they are less vulnerable to competition from native soil bacteria and are shielded from various biotic and abiotic stresses (Reinhold-Hurek and Hurek 1998). Perhaps the most-studied grass inoculated with free living nitrogen-fixing endophytes is sugarcane. *Burkholderia* MG43 inoculated sugarcane plantlets produced a 20% increase in yield over un-inoculated control (Govindarajan et al. 2006), and it was demonstrated that 60 to 80% of nitrogen accumulated

in sugarcane came from atmospheric nitrogen fixation (Boddey et al. 1995). The authors also noted that farmers in Brazil have observed some varieties of sugarcane grown in fields for decades, even up to a century without showing any decline in soil N reserve or yield, despite the supply deficit of nitrogen (Boddey et al. 1995). Rice has also been studied in the context of its relationship with free-living nitrogen-fixing *Burkholderia* spp. In one field experiment, 31% of plant nitrogen was derived from BNF and inoculation resulted in as high as a 69% increase in biomass compared to the un-inoculated control (Baldani et al. 2000). Researchers also found *Burkholderia vietnamiensis* inoculated rice seedlings increased yield by 5.6 to 12.16%, and 42% of nitrogen found in the inoculated plants came from atmospheric nitrogen fixation (Govindarajan et al. 2008). In addition to rice, *Burkholderia* were found to be among the most common nitrogen-fixing isolates from maize plants cultivated in Mexico, and many were reported to be new species (Estrada et al. 2002). These findings support the use of free-living nitrogen-fixing endophytes in the effort to reduce the use of synthetic nitrogen fertilizer and offer hope in creating high-yielding, low-input agricultural production systems.

Bio-Control of Pathogens

Another mechanism of plant growth promotion by endophytes is bio-control of pathogens. Endophytes have evolved a diverse range of bio-control mechanisms including production of antibiotics, both antifungal and antibacterial, siderophore secretion, and enzyme production (reviewed by Compant et al. 2005b). Together, these bio-control properties enable endophytes to outcompete pathogens for their niche and limit damages caused by plant pathogens as well as protect their host plant, resulting in increased survival and growth.

Fungal endophytic colonization confers a positive impact on resistance to pests, mites, and nematodes in grasses (Schardl et al. 2004). Perennial ryegrass (*L. perenne*) plants colonized by *N. lolii* reduced aphid populations, adult life span and fecundity (Meister et al. 2006). *Neotyphodium* spp. form mutualistic associations with several grass genera and produce a range of bio-control agents, some of which have insecticidal properties whereas others are associated with health and welfare issues for grazing animals. Through selection, several novel endophytes that produce predominantly insecticidal bio-control agents have now been successfully commercialized in many temperate grassland areas in New Zealand, Australia, USA, and South America (Easton 2007).

One of the most commonly recognized bio-control mechanisms associated with endophytic plant growth promoting bacteria and fungi is the production of antibiotics. Agents produced include but are not limited

to pyrrolnitrin, phenazines, herbicolin, and oomycin. Furthermore, many endophytic organisms are able to produce multiple agents, which have bio-cidal properties towards various organisms. Pyrrolnitrin, a secondary metabolite isolated from *B. cepacia*, was shown to have activities against both phytopathogenic fungi and bacteria (El-Banna and Winkelmann 1998). The gene cluster regulating the production of pyrrolnitrin is similar to the gene cluster in *Pseudomonas* and was suggested to have been acquired by horizontal gene transfer (de Souza and Raaijmakers 2003). Other strains of *Burkholderia* were reported to produce a large variety of anti-fungal agents such as occidiofungin and burkholdinesn (Lu et al. 2009; Tawfik et al. 2010). *Burkholderia* MP-1 produces at least four anti-fungal compounds including phenylacetic acid, hydrocinnamic acid, 4-hydroxyphenylacetic acid, and 4-hydroxyphenylacetate methyl ester (Mao et al. 2006). The small size of genes encoding antibacterial agents and the relatively small number of genes in bacteria and fungi may allow genes encoding antibiotic agents to be transformed to various growth promoting endophytes.

Siderophore Secretion

Iron, one of the most abundant minerals on the planet, is not readily available to bacteria because its most commonly found form, ferric iron (Fe^{+3}), is only slightly soluble and tightly bound to many particles in the soil. To gather iron needed for growth, bacteria and fungi secrete low molecular weight compounds called siderophores. Bacterial siderophores generally act to inhibit pathogenic fungi as a result of having higher affinity to iron than fungal siderophores (Ordentlich et al. 1988). Like many mechanisms of action in bacteria and fungi, environmental factors such as pH, nutrient levels including iron may affect synthesis of siderophores. Siderophore secretion has been confirmed in a number of bacterial taxa including *Bacillus, Pseudomonas, Rhodococcus, Serratia, Obesumbacterium* and *Lysinibacillus* (Czajkowski et al. 2012) as well as the fungal endophyte actinomycetes (Nimnoi et al. 2010). Genes encoding siderophores may be more difficult to introduce to other plant growth promoting endophytes since studies have shown that they are located in multiple loci (Osullivan et al. 1990) and have complex control mechanisms (Ovadis et al. 2004).

Mechanisms for Abiotic Stress Tolerance

Abiotic stresses include various environmental factors such as hot and cold extremes, drought, salinity, metal contamination and synthetic chemicals, among others, and all may decrease performance of bioenergy crops like switchgrass in the field. To help the host plant tolerate abiotic stresses,

endophytes and AM fungi have evolved a number of mechanisms that improve plant growth and health.

Symbiotic microorganisms help with drought tolerance through the production of protective compounds such as peroxidase, ascorbate, and proline (Fan and Liu 2011; Ruíz-Sánchez et al. 2011). Plant associated microbes may also benefit the host plant by changing stomatal conductance, water potential, and net photosynthesis during drought (Bae et al. 2009).

Endophytes and AM fungi may modify carbohydrate metabolism and photosynthesis, or produce beneficial compounds to enhance cold tolerance in the host plant. When grapevine plants were exposed for five days to chilling conditions, net photosynthesis was higher compared with the levels of the control plants, helping them to withstand long periods of cold exposure (Fernandez et al. 2012a). Recently, it was found that *B. phytofirmans* PsJN modified trehalose metabolism, which may be a part of the mechanism under which *B. phytofirmans* PsJN increased chilling tolerance to grapevine (Fernandez et al. 2012b). In tomato plants, the AM fungus *Glomus mosseae* reduced membrane lipid peroxidation, increased photosynthetic pigments, accumulated osmotic adjustment compounds, and increased antioxidant enzyme activities (superoxide dismutase, catalase, peroxidase and ascorbate peroxidase), which lead to alleviating the damage caused by cold temperatures (Abdel Latef and He 2011). Chemical compounds produced by fungal endophytes may play important roles in host plant tolerance to cold temperatures. For example, a native grass *Anchnatherun robustum* (sleepygrass) infected with *Neotyphodium* spp. produced high levels of ergot alkaloids and demonstrated higher overwintering survival compared with non-infected plants, or even plants infected with *Neotyphodium* spp. with no alkaloid production (Faeth et al. 2010). These results indicate that alkaloids may protect plants against winter conditions.

Beneficial microbes could offer host plant tolerance to high salinity to aid in plant growth. To achieve increased tolerance to high salinity soils, beneficial organisms, both bacterial and fungal, may display a combination of traits such as the production of IAA, phosphate solubilisation, siderophore production, and ACC deaminase activity (Jha et al. 2012). The salt-tolerant *Azospirillum brasilenses* isolate NH produced IAA under salt-stress conditions, and it is believed that the production of this plant growth regulator may contribute to the increase in salt tolerance of inoculated wheat plants (Nabti et al. 2010). Under similar conditions, the endophytic strains, *B. subtilis*, *B. pumilus*, and *P. putida* isolated from the roots of *Prosopis strombulifera* (Argentine screwbean) produced significantly higher IAA (Sgroy et al. 2009).

Genetic Modifications and Functional Genomics

Both bacterial and fungal endophyte-plant interactions involve modifications of plant gene expression and overall plant physiology/biochemistry to beneficially impact growth and stress tolerance. While monitoring specific gene expression during beneficial endophyte-sugarcane interactions, Arencibia et al. (2006) identified 47 differentially expressed sequence tags (EST) using cDNA-AFLP analysis. The transcripts showed significant genetic homologies to major signaling pathways such as the ethylene signaling pathway. For example, PYK10 encodes for a root- and hypocotyl-specific β-glucosidase/myrosinase and is important during the endophyte *P. indica* and *Arabidopsis* beneficial bio-control against herbivores and pathogens (Sherameti et al. 2008). NoxA was found to be crucial in regulating hyphal morphogenesis and growth in the mutualistic symbiotic interaction between the fungal endophyte *Epichloë festucae* and perennial ryegrass (Tanaka et al. 2008). Functional genomics research will help scientists understand and elucidate mechanisms under which beneficial microorganisms promote host plant growth and enhance stress tolerance. Currently we are carrying out studies of mechanisms of plant growth promotion by bacterial endophytes using the responsive switchgrass cultivar Alamo and non-responsive cultivar Cave-in-Rock to *Burkholderia phytofirmans* strain PsJN (Kim et al. 2012). Comparative global gene expression profiling is being conducted using both cultivars following *B. phytofirmans* strain PsJN inoculation with DOE-funded switchgrass EST microarray chips by Genomics Core Facility in the Noble Foundation. Approximately 35,200 switchgrass ID probes were identified to show significant differences between switchgrass cultivars Alamo and Cave-In-Rock after *B. phytofirmans* strain PsJN inoculation. Using the rice genome as a model for the analysis of the data along with the MapMan (Usadel et al. 2005) and the PageMap (Usadel et al. 2006) software, we are currently analyzing this large data set. Results showed that in Alamo almost 2000 genes were unique up-regulated at 0.5 day. On the other hand, in Cave-in-Rock, the number of unique up-regulated genes for 0.5 day was only 901. The significant changes are found in transcription factor genes, plant hormone and cell wall metabolism (unpublished data).

Bacterial and Fungal endophytes exhibit a diverse range of growth promoting mechanisms. In many cases, endophytes, primarily bacteria, possess multiple mechanisms of action and differentially express these traits at different stages of plant growth and development. Under stress conditions, endophytes help the host plant survive and flourish, as in the case of ACC deaminase activity and bio-control compound production. Under normal conditions, endophytes help fix atmospheric di-nitrogen and produce plant hormones to help the plant grow to its maximum potential.

Together, under both stress and normal conditions, endophytes ensure its host plant thrives, and its nutrient rich environment is maintained.

Isolation and Identification

In order to classify and study functions of endophytes and mycorrhizal fungi, endophytes first need to be isolated from host plants and mycorrhizal fungi from soil samples containing host plant roots. Next, the organisms need to be purified before they are identified and finally characterized using molecular tools.

Endophyte Isolation

In general, for bacterial and fungal endophyte isolation, the samples, including any host plant tissue, are collected and brought to laboratories where they can be stored in plastic bags at 4°C for a few days prior to surface sterilization.

Surface Sterilization

Root samples should first be washed with tap water to remove any soil from the root surface before sterilization. Aboveground plant tissue can be directly washed with 70% ethanol for one minute, sterilized with 20–50% bleach solution for 10–20 min depending on the type of tissue; for tender tissues, a lower concentration of bleach and shorter duration of time should be used. The tissue is finally rinsed with sterile water 3–5 times under aseptic conditions. After sterilization, the tissue surface should be clean and free of microorganisms. To ensure the efficacy of surface-sterilization, 50 µl of the final wash should be plated, and surface-sterilized tissues can be rolled onto culture media and incubated at 27°C for a few days to see if any remaining microorganisms were present (Coombs and Franco 2003).

Bacterial Endophyte Growth

Surface-sterilized samples containing endophytic bacteria are ground with sterile water, and serial dilutions are prepared and plated on Luria-Bertani (LB) medium or other bacterial media and grown at 28–37°C for a few days. It is advantageous to add a fungicide such as Benomyl (DuPont) at 50 µg/ml to the bacterial culture media to prevent fungal growth, particularly if there are fungal contaminations (Coombs and Franco 2003). Observations should be taken every day in order to isolate individual colonies for further

identification. Individual colonies are streaked and re-grown two additional times to obtain pure bacterial strains.

Fungal Endophyte Growth

For endophytic fungal growth, surface-sterilized tissues are cut into small pieces or homogenized using a homogenizer and plated on Potato dextrose agar (PDA) or other fungal culture media, such as MEA medium (2% malt extract and 1.8% agar) (Vallino et al. 2009) supplemented with several antibiotics, such as ampicillin (100 mg/L), chloramphenicol (50 mg/L) and streptomycin (50 mg/L) to prevent bacterial growth (Ghimire et al. 2011; Craven K, personal communication). Plates containing tissues are incubated at 25–28°C for up to one month. Observations should be taken every day in order to isolate individual strains for further identification. The growing fungal colonies are then re-plated on fresh medium to obtain individual colonies.

Mycorrhizal Fungus Isolation

Arbuscular mycorrhizal (AM) fungi are the majority of mycorrhizal fungi and are the focus of this section. AM fungi penetrate the cortical cells of roots and form arbuscules (tree-like structures) and vesicles within host plant cells, and their hyphae penetrate into the soil to aid in absorption of nutrients, extending the area of nutrient acquisition. AM spores can be isolated from soil samples containing roots by the wet sieving method, which is widely used and works well with sandy soil samples (Utobo et al. 2011). Briefly, after soil samples are collected, they are suspended in water (approximately 15–30 ml/g), and mixed vigorously. If spores form in the interior of roots, soil and root samples are blended and the suspension solution is left to settle for a while, and then the supernatant is decanted through standard sieves, which should capture the spores of interest. The procedure should then be repeated, particularly with soil containing large amounts of clay. Spores isolated can be further purified by sucrose centrifugation particularly if the soil is rich in organic debris because it may be difficult to isolate spores hidden in organic matter (Utobo et al. 2011).

AM Fungus Growth

AM fungi are obligate and must be grown with their host plant. Soil collected or spores isolated from soil can be used as inoculum, which is called a soil trap culture. To create a plant trap culture, plants containing mycorrhizal fungi are collected from the field and are transplanted to a potting medium

(sterile soil or soil-sand mixture, which should have low available P and not rich in organic matter) (http: //www.scribd.com/doc/58675784/4-3-Mycorrhiza0403). More details can be found in the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) (http: //invam.caf.wvu.edu).

Identification

Several methods can be used to identify endophytic bacteria and fungi as well as AM fungi, such as morphological characterization, genomic sequencing, and staining (Zinniel et al. 2002).

Bacterial Endophyte Identification

Individual bacterial colonies can be identified by morphology, or the observation of colony colors and physical shapes observed under a microscope. Gram positive or negative cultures can be distinguished with staining. More recently, 16S rRNA gene sequences have widely been used to identify bacterial species and to construct a phenogram. Bacterial genomic DNA needs to be isolated in order to amplify specific 16S rRNA gene sequences using a standard bacterial DNA isolation protocol (Sambrook et al. 1989). For general bacterial endophyte classification, universal PCR primers F27 (5′-AGA GTT TAT CMT GGC TCA G-3′) and R1492 (5′-GRT ACC TTG TTA CGA CTT-3′) are used to amplify partial bacterial 16S rDNA sequences (Diallo et al. 2004). The ability of bacterial endophytes to fix atmospheric nitrogen can be tested by growing bacteria in nitrogen-free medium for several cycles of cultures or PCR can be used to amplify the nifH gene, which is a conserved region in the dinitrogenase reductase gene complex. Fatty acid analysis, carbon source utilization, and antibiotic resistance (hygromycin, chloramphenicol, gentamycin, kanamycin, ampicillin, streptomycin, tetracycline, and rifampin) could be done for further identification.

Fungal Endophyte Identification

Fungal morphology can be observed under a microscope, and chitin, a specific fungal cell wall component, can be stained with dyes to generally identify fungal species. For a more specific identification, fungal genomic DNA can be isolated using a standard bacterial DNA isolation protocol (Sambrook et al. 1989) or commercial kits, such as the DNeasy Plant Mini Kit (Qiagen).

For identification, the internal transcribed spacer (ITS) regions of fungal ribosomal DNA are widely used because the regions are highly variable (Ghimire et al. 2011). The specific primers ITS1 (5′-TCC GTA GGT GAA CCT TGC GG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) are used to PCR-amplify highly variable ITS1 and ITS2 regions surrounding the 5.8S coding region (Martin and Rygiewicz 2005). However, the primers do not effectively exclude host plant sequences in mixed samples so ITS1-F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4-B (5′-CAG GAG ACT TGT ACA CGG TCC AG-3′) were designed to amplify fungal ITS regions, and a pair of ITS1F and ITS4 resulted in strong PCR amplification from both ascomycetes and basidiomycetes (Gardes and Bruns 1993).

In general, PCR reactions should include 1X reaction buffer containing Mg^{++}, 1 µl of 10 µM of each primer, 1 µl of 10 mM dNTPs, 0.5–1.0 unit Taq DNA polymerase, and 50–200 ng genomic DNA to total 25–50 µl. PCR can be performed in any thermal cycler with program like 95°C for 2–4 min, then 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec for 35 cycles, finally 72°C for 10 min. PCR products are checked in agarose gel first to make sure only one clear band exists, then the product is either cloned into pGEM-T vector (Promega) or similar kits, such as TA cloning kits for sequencing. Direct sequencing of the PCR product after purification using Qiagen's PCR purification kit is also an option.

Once PCR product sequences are obtained, BLASTN searches can be performed to compare similar sequences from gene bank to identify the species of the target microorganism. A phylogenetic tree can also be constructed to further clarify its evolutionary relationship among other species. In addition, PCR-RFLP, Length Heterogeneity PCR, and Terminal Restriction Fragment Length Polymorphism can be used to characterize microbial communities (Martin and Rygiewicz 2005).

AM Fungal Identification

Total DNA was extracted from about 20–50 mg of a trap culture of mycorrhizal fungal mycelium using the DNeasy PlantMini Kit (Qiagen). Partial ribosomal SSU DNA fragments were then amplified using a universal eukaryotic primer NS31 (5′-TTG GAG GGC AAG TCT GGT GCC-3′) (Simon et al. 1992) and the primer AM1 (5′-GTT TCC CGT AAG GCG CCG AA-3′), which only amplifies AM fungal SSU sequences but not plant sequences (Helgason et al. 1998). Basically, the PCR reaction follows the protocol described above. PCR products were run on agarose gel to ensure that only one band amplified, and then they were purified with Qiagen PCR

purification kit for direct sequencing with either NS31 or AM1 primer. Also, PCR products can be cloned to the pGEM-T vector and then sequenced with T7 and /or SP6 primers.

Visual identification can be carried out on AM fungal spores, as they are larger than other fungal spores; most spores are between 100 to 200 μm in diameter and can be easily observed under a dissecting microscope (Jarstfer and Sylvia 2002). AM fungi in roots can also be observed after chemical staining by using the staining agent 0.05% trypan blue in lactophenol reported by Phillips and Hayman (1970). The chemical trypan blue is considered a carcinogen but is still used in some laboratories (Utobo et al. 2011). Alternatively, a simple and inexpensive method has been developed (Vierheilig et al. 1998) with ink and vinegar as the staining agent, which is not toxic. Although it is very easy to use, cheap, quick, and can be used for large number of samples, not all inks stain all AM fungi. In general, almost all black inks give good staining, and the structures are clearest under a dark field illumination with a stereomicroscope (Vierheilig et al. 2005).

Future and Perspective

Bioenergy production will become increasingly important in the future to relieve dependence on fossil fuels and lower greenhouse gas emissions because fossil-based energy is limited and its demand is continually increasing due to economic and population growth around the world. Switchgrass is one of the most promising bioenergy crops due to persistent high yields and its ability to grow on marginal land. Development of a low input and sustainable switchgrass feedstock production system is imperative as the use of chemical fertilizers causes deleterious environmental effects, such as water pollution and N_2O release to atmosphere, a potential greenhouse gas. Endophytes and AM fungi have the potential to help address these challenges due to their enhancement of nutrient acquisition, including nitrogen fixation and mobilization of mineral nutrients as well as increased biotic and abiotic stress tolerance, which together will reduce the amount of fertilizer application and/or pesticide and fungicide use. It will also open a door to growing potential bioenergy crops, such as switchgrass on marginal land or achieving the same yield while reducing fertilizer use, resulting in lower cost and contributing to sustainable rural development.

Plants live in complex environmental conditions containing various microorganisms, both beneficial and detrimental. Although endophytes and AM fungi could benefit plant growth, other microorganisms may have negative effects, and different endophytes and AM fungi may not be compatible, therefore the specific functional compatibility of endophytes and AM fungi needs to be further investigated to develop multi-functional

bio-inoculants (Podile and Kishore 2007) in switchgrass production. Additionally, while studies with endophytes as well as other plant growth promoting microorganisms in laboratories have been encouraging, there have also been reports of a general decrease in performance from the laboratory to the field (Riggs et al. 2001; Gyaneshwar et al. 2002). As with any ecosystem, the variables of field conditions and native microbial populations will have to be addressed to maximize the beneficial effects of bacteria and fungi. Therefore, screening endophytes having a broad spectrum of growth promotion that continues throughout the life of the plant will be another topic for endophyte application.

Genotype specific responses of host plants to endophytes are also a large barrier in application. For example, in poplar, different cultivars had different responses to different endophytes (Taghavi et al. 2009). One of the most studied plant growth promoting bacterium, *B. phytofirmans* strain PsJN, has a beneficial effect on many species, such as potato, tomato, and grape. However, PsJN is also genotype specific. In switchgrass, PsJN promoted growth of the lowland cultivar Alamo but not the upland cultivar Cave-in-Rock (Kim et al. 2012). Understanding these differences will also help in developing a more reliable, stable, and broad spectrum of growth promotion in plants.

Complete understanding of the mechanisms of various beneficial symbioses is the foundation for effectively applying these microorganisms in a sustainable switchgrass feedstock production and to achieve their synergistic activities (Podile and Kishore 2007). As more is learned from functional genomics of endophytic microorganisms in growth promotion, it may be possible to share these important genes between similar microorganisms through horizontal gene transfer via transformation, conjugation, or transduction, all common occurrences in the bacterial world. Researchers first reported *in planta* horizontal gene transfer in the bioenergy crop hybrid poplar when they found *Burkholderia cepacia* VM1468 transferred its toluene degradation gene to other endophytes (Taghavi et al. 2005). This suggests that such transfer may be used to modify and improve the growth-promoting effects of other endophytes via gene sharing. The phenomenon of horizontal gene transfer may also occur in nature between different genera as the gene encoding the anti-fungal agent pyrrolnitrin in *Burkholderia* was likely horizontally transferred from *Pseudomonas* (de Souza and Raaijmakers 2003). Since AM fungi are coenocytic (many nuclei coexist in a common cytoplasm), genetic exchange was recently reported in different AM fungus *Glomus intraradices* strains (Colard et al. 2011), which could be beneficial for host plant growth. Generating novel AM fungus genotypes through genetic exchange will be a powerful tool in developing AM fungi that are more beneficial in bioenergy crop production.

Compared with plant genetic engineering, it is much easier for microorganisms to be genetically modified. One could easily transform some useful foreign genes into bacteria or fungi. For instance, the *Bacillus thuringiensis cry1Ac7* and *Serratia marcescens chiA* genes were transformed to sugarcane-associated endophytic bacteria, which helped increase the tolerance of sugarcane plant to the sugarcane borer *Eldana saccharina* (Downing et al. 2000). These applications indicate that we may be able to genetically engineer endophytes with useful genes, such as the *Bacillus thuringiensis* toxin gene, to protect host plants against herbivorous insects, herbicide resistance genes to impart host plant resistance to herbicides, and genes related to abiotic stress tolerance to enhance host plant tolerance to abiotic stresses. An efficient endophyte transformation method by *Agrobacterium* was developed by Abello et al. (2008), which will help in the transfer and expression of agronomically important genes in host plants via endophytes. As functional genomics research is continually advanced, scientists will better understand the mechanisms under which beneficial microorganisms promote host plant growth and enhance stress tolerance to effectively utilize these microbes in bioenergy crop production. For example, endophytes having the ability to fix atmospheric nitrogen could be combined with endophytes having the ability to enhance host plant tolerance to abiotic stresses or endophytes inhibiting pathogen growth or with an AM fungus to improve nutrient uptake or, possibly, all could be combined.

Since 1999, over 15 new patents have been registered for microbial endophytes (Mei and Flinn 2010). The worldwide market for microbial inoculants is experiencing an annual growth rate of approximately 10% (Berg 2009). As world population demand for food is continually increasing, bioenergy crops should be grown on poor or marginal lands or contaminated soil, not competing with food crops for fertile lands. The use of endophytes and AM fungi may help bioenergy crops, such as switchgrass, grow on these lands via their normal mechanisms of action or genetic modification by introducing nitrogen fixation genes, heavy metal accumulation genes, or contaminated compound degradation genes.

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