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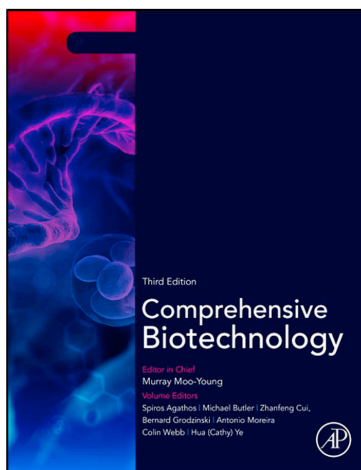
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4.64 Paying the Rent: How Endophytic Microorganisms Help Plant Hosts Obtain Nutrients[☆]

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4.64.1 Plant Nutrient Management and Agricultural Productivity

Application of the principles of soil fertility and plant nutrition in combination with irrigation, farm mechanization, and genetic improvement of crop plants has allowed farmers to coax greater yields from their plants than ever before in what has been termed the Green Revolution. Chemical fertilizer is believed by Borlaug and Dowsell¹ to have been responsible for up to 50% of increases in crop yields worldwide during the 20th century. There is a strong positive correlation between N and P application and global cereal production, where from 1960 to 2000 N and P fertilization increased 7 and 3.5 times, respectively, while cereal production doubled.² Such increases in agricultural productivity were absolutely necessary to feed an exponentially growing world population which quadrupled to 6 billion during the 20th century. Further increases in agricultural productivity are now needed as world population is expected to reach 9 billion by 2050, with N and P use expected to increase another threefold unless there are increases in plant nutrient use efficiency.² Required as it may be, further increases in agricultural productivity will be difficult for several reasons: most of the world's best farmland is already under cultivation; the remaining land is being eroded or marginal; there are concerns about climate change; water in some regions is becoming scarce; yield potential from Green Revolution technologies has either stagnated or been achieved in many regions of the world; and nutrient pollution from agricultural practices has reached unacceptable levels. Nutrient pollution from fertilizer comes mainly from run-off on farms that is the result of overfertilization and poor uptake by plants: only 30%–50% of applied N and about 45% of P is taken up by crops.² Work to improve nutrient use efficiency (NUE) has included development of precision fertilization techniques, alternate land management, the breeding of more efficient crop plants, and research into microbial nutrient cycling. This article will discuss the role that bacteria and fungi residing within crop plants may play in improving NUE and help address humanity's need to sustainably increase agricultural production. Because of the crucial role microbes appear to have played in land plant evolution, improved understanding of their function and potential will allow development of biotechnologies to increase nutrient-use efficiency in modern plant agriculture.

Microbes living inside plants are called endophytes, a term that was coined by Heinrich Anton de Bary in 1866 and is derived from the Greek words *endon* (within) and *phyte* (plant).³ The term usually refers to non-pathogenic bacteria and fungi found in plant tissue, but sometimes is also used to refer to mycorrhizal fungi found in plant roots.⁴ The first endophytes may have already been present in early algae colonizing intertidal zones, or may have begun to colonize plants 500–700 million years ago when molecular studies estimate the first plants came on land.⁵ These early plants were subject to conditions of strong solar radiation, temperature fluctuations, dehydration, and poor mineral nutrition, but they found well-adapted photosynthetic prokaryotes, fungi, and possibly

[☆] *Change History:* March 2018. David Johnston-Monje. Since the last version of this article was written 10 years ago, the title has been changed, the first author's affiliations have changed, two additional authors were added, about 4000 words and 70 additional references were included, an extra section was added just before the conclusions (New Developments and Future Directions), the Website section was erased (they were no longer current or very helpful), and one additional figure was added which brings the total number to 2.

This is an update of D. Johnston-Monje, M.N. Raizada, 4.58 - Plant and Endophyte Relationships: Nutrient Management, Editor(s): Murray Moo-Young, Comprehensive Biotechnology (Second Edition), Academic Press, 2011, Pages 713–727.

lichens already growing in this new environment. Ancient fungi and bacteria would have found the internal spaces of these large, multicellular phototrophs an attractive habitat providing them nourishment and sheltering them from abiotic stress, competition and predation. Beneficial effects on the host would likely have evolved later as endophytes increasing host fitness, size and survival would have had a richer and more stable environment in which to live and reproduce. An example of such an early relationship may be seen in the extant, tidal zone-inhabiting brown alga *Ascophyllum nodosum*, which resists desiccation with help from its endophyte *Mycosphaerella ascophylli*.⁶ Similarly, it has also been hypothesized that mycorrhiza evolved from established fungal endophytes that developed external hyphae that enhanced host fitness through mineral scavenging in exchange for fixed carbon.⁷ This ancient arbuscular mycorrhiza (AM)-plant symbiosis appears to have been very important in early land plant evolution, as the earliest convincing fossil evidence of plants from 435 million years ago already show extensive colonization by fungal mycorrhiza and is still observed today in over 90% of plant species.⁶ Plants eventually developed their own dedicated nutrient absorbing organs (roots); specialized root systems including extended root hairs are thought to be the reason why 10% of plants today are non-mycorrhizal (e.g., Brassicaceae).⁶ Whatever their relationship to mycorrhiza, it is believed that all plants are still host to at least one type of endophyte. Root nodule-forming bacteria are the best understood prokaryotic endophytes, which fix atmospheric nitrogen into ammonia in dedicated symbiosis organs called nodules in exchange for plant supplied sugars and micro-aerobic conditions. This is believed to have evolved 59 million years ago in ancestral legumes which may have co-opted genes involved in the mycorrhizal symbiosis to help control bacterial root infections at the site of wounding or lateral root emergence.⁸ Countless endophytes are believed to still exist undiscovered or poorly understood in nature, and thus pose a huge potential source of inoculants, phytostimulatory mechanisms and genetics which may be co-opted for the improvement of plant agricultural practices. The rest of this article will be concerned with known mechanisms that endophytes use to improve plant NUE and potential avenues of future research. As N and P are the most limiting nutrients for plant growth, we will focus on endophytic mechanisms that increase their procurement. Where possible, the article will highlight instances of successful application of endophytes for improved plant nutrient management in agriculture.

4.64.2 Endophyte Nutrient Uptake

Early land plants had to evolve the ability to absorb mineral nutrients from soil in contrast to aquatic plants which absorb nutrients by diffusion from water. Early bryophyte-like plants (e.g., mosses) did not have true root systems and it is thus possible that ancient mycorrhizal hyphae served this function in early land plant evolution⁷ enhancing access to soil mineral nutrients in exchange for plant photosynthates.⁹ Although recent evidence suggests the most ancient mycorrhizal partners belonged to the order Endogonales of the subdivision Mucoromycotina,¹⁰ fungi of the phylum *Glomeromycota* have since evolved to dominate plant-microbe symbiotic relationships with over 90% of higher plants possessing the necessary genes and biochemical signaling pathways to form mycorrhizal relationships with *Glomeromycetes*.⁹ There are over 200 species of this group of fungi today with features that include obligate biotrophy, propagation via soil spores and inability to be cultured independently of compatible host roots. The angiosperm species involved in these symbioses range from trees to domesticated grasses to parasitic, non-photosynthesizing plants. In exchange for up to 20% of the plant's carbon, AM are able to increase the plant's supply of P, N, Zn, Cu, Ni, S, Mn, B, Fe, Ca, and K,¹¹ primarily by exploring the soil volume more efficiently than roots because hyphae grow faster, are thinner, and branch more extensively. AM associations can increase the nutrient absorptive area up to 100 times relative to root length, with external mycelia weighing as much as 3% of the total root.¹² This is especially valuable for scavenging immobile minerals such as P¹³ and Zn,¹⁴ considered the most important macro and micro nutrients provided to plants by these fungi. Increased nutrient absorption can lead to higher quality fruit in addition to healthier plants: for example, tomato mineral concentration (particularly N, P, and Cu), antioxidant capacity, carotenoids and volatile compounds have been shown to increase when plants have been colonized by AM fungi.¹⁵

Plant species and cultivars vary in their dependence on AM fungi for assistance in nutrient acquisition and also vary in the extent to which their growth visibly responds to AM colonization in soils.¹⁶ For example, uptake of P per root length was fourfold higher in maize compared to non-AM plants when grown in two tropical soils at different soil pH, whereas under similar conditions soybean had nearly a threefold increase in P uptake compared to non-AM plants.¹⁷ Landraces of maize in Mexico have been shown to be able to achieve higher mycorrhizal colonization rates and P absorption compared to modern hybrids,¹⁸ while studies of hundreds of different genotypes in Japan have shown that AM colonization of maize varies with germplasm type (hybrid, inbred or landrace), origin and year of release.¹⁹ AM colonization of onion led to significant increases in the concentrations of N, P and Cu, and significant decreases in Ca, K, Na, Fe, Mn and Zn, with significant differences in responses based on plant genotype and species of fungal inoculant.²⁰ In a different experiment, *Linum usitatissimum* (flax) inoculated with either the AM *Gigaspora rosea* or *Glomus intraradices* accumulated greater biomass and took up more P, while *Solanum lycopersicum* (tomato) showed a decrease in biomass and lower P uptake.²¹ Indeed, there appears to be tremendous variation within modern varieties of a crop species with respect to the impact of AM²²; the beneficial effects of AM are likely inversely correlated with a plant's root branching and ability to absorb P from the soil itself.

AM were traditionally thought to be unimportant for increasing plant N uptake because these symbiotic associations are most common in nitrate-rich soils where N is easy for roots to absorb without help from endosymbionts.²³ Ammonium is the most common form of synthetic nitrogen fertilizer, is the breakdown product of organic decomposition, is fairly immobile in soils, and has indeed been shown to be absorbed and translocated to maize through AM association.²³ There is also evidence of absorption and transmission to the plant of organic forms of N (i.e., amino acids) by AM, although they have not been shown to be able to directly degrade it into bioavailable molecules themselves.²⁴ AM are able to form below ground hyphal networks

connecting neighboring roots,²⁵ allowing the transfer of N from plant to plant, but how this affects plant communities in agricultural settings is not well understood. Although AM are generally thought to have no saprophytic abilities, they have been shown that at least some species of AM are able to enhance decomposition of organics and transfer liberated ammonium to their plant host²⁶ perhaps by absorption of mineral nitrogen waste excreted by soil protozoa that have grazed on soil bacteria digesting the organic nitrogen.²⁷

The ability of AM to improve NUE in production agriculture seems to be dependent on a number of factors, but in general they give the best response when colonizing plants with low root-shoot biomass ratios, slow root growth rates, and/or poor root hair development.¹³ When grown in soils with abundant P and Zn, AM appear to be unimportant or even detrimental to plant growth as they become drains on plant carbon without benefitting the plant.²⁸ This might predict that plants adapted to high nutrient soils would be less responsive to AM than varieties adapted to low nutrient soils and a recent comparison of 14 domesticated crops to their wild ancestors do show this convincingly for P, suggesting symbiosis with AM has been selected against by modern breeding.²⁹ A contrasting study testing AM colonization on European maize (high fertilizer input) and African maize (low fertilizer input) showed that although the African variety grew better at low P, it was nearly insensitive to AM infection,³⁰ while European variety greatly benefited from inoculation with AM. The fungal genotype can also influence the outcome of the relationship, with local adaptation of the mycorrhiza to its soil environment being an important predictor of successful symbiosis.^{31,32} For example, African panicum grasses planted in European soil inoculated with European strains of mycorrhiza resulted in stunted plants with lower levels of N and P than those grown in the same soil without mycorrhiza.³³ Due to this variation, experiments should be conducted to validate how a particular variety will respond to AM colonization under field conditions. As elite crops varieties have been bred to respond to high fertilization levels and to resist fungal pathogens, the genetic potential of AM to improve high production agriculture may be limited²⁸ and could likely be improved by targeting symbiosis breeding programs to improve plant nutrient uptake via AM activity under low input conditions.³⁴

As early as 50 million years ago three other major types of mycorrhizal relationships developed in addition to AM, again increasing host nutrient absorption area, but also allowing access to different organic sources of N and P that septate fungi can digest and absorb. Ectomycorrhizal plants, including many tree and shrub species (e.g., pines), associate with 6000 different species of septate basidiomycetes, ascomycetes, and zygomycetes. Secondly, orchids associate with basidiomycete mycorrhiza and, interestingly, can parasitize the fungus for carbon. Finally, ericoid plants (e.g., tea) associate with ascomycete mycorrhiza. These three types of mycorrhizae are able to digest organic compounds by secreting extracellular enzymes including carboxylases and phosphatases to attack and liberate N and P from dead plant cells and other soil organisms.³⁵ Carboxylases have also been shown to associate with Al in the soil to form acidic complexes that weather calcium-rich rock, thereby releasing Ca that is taken up by the mycorrhiza.³⁵ Although these microbes are important in enhancing nutrient uptake by some plants, they will not be considered further in this article due to their non-endophytic lifestyle.

Besides mycorrhiza, fungal endophytes are classified as belonging to the Clavicipitaceae family (class 1) or as nonclavicipitaceous (class 2, 3, 4).³⁶ Clavicipitaceae are a large fungal group consisting of obligate biotrophs and necrotrophs that colonize plants, insects and other fungi; however, they do not display a mycorrhizal habit. Class 4 endophytes, also known as dark septate endophytes (DSE), are fungi that intra- and intercellularly colonize roots with dark, melanized hyphae that have been shown in one study to exit the root and explore soil to aid in plant mineral nutrition, specifically P.³⁷ DSE have been reported in over 600 different plant species and are found worldwide, often coexisting with mycorrhizal fungi, although there are also numerous non-mycorrhizal plant roots which have been found to contain DSE. Inoculation of plants on sterile soil with access to only organic N can increase shoot N and P content by 26%–103%³⁸ and seems to help explain the role of DSE when they replace AM and ectomycorrhizal fungi at sites with extreme environmental conditions.³⁷ While some DSE are able to manipulate phytohormone levels to improve plant nutrition,³⁹ it is likely that their mycorrhizal habit, combined with their saprophytic capabilities, are the main mechanism of DSE-enhanced nutrient uptake. Although not all plants harboring DSE are observed to have enhanced NUE, there are several studies showing DSE are able to improve nutrient levels in plants. For example, DSE isolated from roots of a *Carex* species (sedge) were used to re-inoculate DSE-free plants of that same species and resulted in increased biomass and P content.⁴⁰ Similar results were found studying DSE isolates from Antarctic hair grass (*Deschampsia antarctica*); surface sterilized seeds re-inoculated with DSE of the genera *Oculimacula*, *Mollisia* or *Tapesia* and supplied with organic nitrogen had elevated N and P content in their shoots/roots along with 51%–247% higher biomass, while the same inoculated plants supplied with inorganic nitrogen enjoyed no biomass increase, nor elevated accumulation of nutrients.⁴¹ Inoculation of *Pinus contorta* with the DSE *Phialocephala fortinii* resulted in increased levels of leaf P, enhanced N uptake from soil, and higher plant biomass,⁴² while the endemic (to Spain) *Arabidopsis thaliana* endophyte *Colletotrichum tofieldiae* is able to colonize plants through roots increasing seed production, plant growth and P translocation to shoots, but only under P limiting conditions.⁴³ Inoculation of *Pleosporeales* sp. A103 (a DSE isolated from *Oryza glumaepatula* growing wild in the Brazilian Amazon) onto rice seeds was observed to increase plant biomass, the number of tillers, and uptake of N, P, K, Mg and S.⁴⁴ Approximately 99% of soil P is tied up in organic sources, and DSE have been shown to be able to solubilize and mineralize it for uptake and use by roots and AM.⁴⁵ These fungal endophytes have been shown to secrete a number of degrading enzymes including cellulases, laccases, amylases, lipases, pectinases, xylanases, proteolytic enzymes, tyrosinases, and polyphenol oxidases,³⁷ any of which may allow solubilization or mineralization of organic pools of nutrients for plant hosts. Enzymatic solubilization of insoluble P may explain how *Aspergillus ustus* was also able to increase plant biomass and raise the P content of its host, *Arctostaphylos canescens* (fourwing saltbush), grown on rock- and tri-calcium phosphate sources which are insoluble in soil and unavailable to plant roots for uptake.³⁷

4.64.3 Enhancing Root Growth

A key mechanism underlying endophytic improvement of NUE is modulation of root growth. There are two key root system traits which can increase NUE if they are enhanced: root branching and root hair production, the latter which can account for up to 70% of total root length. Though root development is genetically programmed, many endophytes are able to modulate root size and structure.^{46,47} Specifically, plant growth promoting rhizobacteria (PGPR) and endophytes, including *Gluconacetobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Herbaspirillum* and *Pseudomonas* species, have been observed to improve plant growth through stimulation of root development.⁴⁸ Class 1 fungal endophytes like *Neotyphodium coenophialum* stimulate the development of extensive root systems and longer root hairs, resulting in more efficient absorption of soil P.⁴⁹ There are also several examples of class 2 and 4 endophytes stimulating plant root development, while the hyper-diverse class 3 group usually exists within plant tissues without affecting plant growth.³⁶ In addition to scavenging for nutrients, AM are able to increase root growth and branching through secretion of lipochitooligosaccharides known as "myc factors".⁵⁰⁻⁵² The most common mechanisms by which endophytes are known to modify root development are by producing or blocking the activity of plant hormones.

Among the plant hormones, the most common one used by endophytes to stimulate root growth is secreted auxin.⁵³ Indole-3-acetic acid (IAA) is the most common plant auxin and can affect almost every aspect of plant development including cell enlargement and division, tissue differentiation, and responses to light and gravity. IAA is known to stimulate the elongation of primary roots when applied at low concentrations and at higher concentrations it induces ethylene production, inhibiting primary root elongation while simultaneously inducing initiation of lateral and adventitious roots and root hairs that results in higher root surface area. These phenomena are illustrated in auxin-resistant *Arabidopsis* mutants *axr1* and *axr2* which produce fewer lateral roots than the wild type, while in *Arabidopsis* auxin over-producing mutants, *rooty* and *superroot*, there is a dramatic increase in the formation of lateral roots and root hairs.^{54,55} Microbial biosynthesis of auxin was initially discovered in *Agrobacterium* where it plays a role in gall formation and pathogenesis, but since then many non-pathogenic bacteria and fungi have been shown to synthesize auxin using up to six different genetic pathways.⁵³ In fact, it has been hypothesized that land plants evolved tryptophan-dependent IAA biosynthesis in response to interactions with auxin production and often pathogenic microbes, allowing plants to counteract diseases and control their own auxin signaling.⁵⁶

Although endophytes producing IAA are often found to promote root growth in an auxin-dependent manner, the interaction varies because of the amount of auxin produced, the presence of other interacting hormones, and plant sensitivity to IAA. For example, *Pseudomonas putida* GR12-2 is able to stimulate up to 50% greater root elongation and adventitious root formation in mung bean, but this trait was reduced in *ipdc* insertion mutants deficient in auxin production.⁵⁷ The broad host range bacterial endophyte, *Burkholderia phytofirmans* PsNJ, can greatly stimulate primary root and shoot development in *Arabidopsis*, but auxin metabolism mutants lose most of their capacity to promote plant growth.⁵⁸ In a study of root-promoting endophytic bacteria from *Solanum nigrum* and *Nicotiana attenuate* in Germany, most positive isolates were found to produce IAA,⁵⁹ but high levels of IAA production by certain strains, or external supplementation, resulted in root inhibition. In poplar cuttings, root initiation, branching, and biomass were increased after inoculation with poplar endophytes, *Enterobacter* spp. strain 638, *P. putida* W619, and *Serratia proteamaculans* 568, all of which were found to secrete IAA in vitro, albeit at different levels.⁶⁰ An endophytic yeast that colonizes maize roots, *Williopsis saturnus*, was shown to produce large amounts of IAA as well as indole-3-pyruvic acid (another auxin), stimulating both shoot biomass and root elongation in inoculated corn plants.⁶¹ Another fungal endophyte, *Piriformospora indica*, isolated from a low nutrient desert soil in Rajasthan, India, has been shown to stimulate growth of a wide range of hosts, including *Arabidopsis*, *Zea mays*, *Nicotiana tabacum*, *Populus tremula*, *Oryza sativa*, *Glycine max*, and even several species of terrestrial orchids.⁶² *P. indica* colonized roots have higher biomass and are highly branched, resulting in plants with increased yield and nutrient use efficiency; effects which were attributed to its ability to produce IAA,⁶³ although the fungus has also been shown to inactivate auxin at high concentrations.⁶⁴ Infection of tall fescue with *N. coenophialum* in P-deficient soils resulted in roots with a smaller diameter (11%) and increased root hair length (17%) when compared to uninfected plants, which the authors believe is explained by its production of IAA.⁶⁵ This is echoed in the results of field trials with the auxin and gibberellin secreting endophyte inoculant, *Rhizobium leguminosarum* bv. trifolii E11, which was able to significantly increase rice grain yield, agronomic fertilizer N-use efficiency and harvest index in certain varieties due to root system optimization.⁶⁶

Another important mechanism of endophyte-dependent root growth promotion is through the reduction of the volatile plant hormone, ethylene. When synthesized by plants upon exposure to abiotic or biotic environmental stress, it is called stress ethylene and is thought to initiate many plant stress symptoms including senescence, chlorosis and organ abscission which reduce productivity and survival.⁶⁷ Ethylene affects roots by inhibiting elongation, promoting lateral root growth and stimulating root hair formation⁶⁸ and is one of the ways that high concentrations of auxin function to alter root growth. For example, the endophytic fungus *Sebacina vermifera* promotes root and shoot growth in *N. attenuate* by inhibiting plant ethylene signaling via an unknown mechanism.⁶⁹ Rhizosphere-inhabiting bacteria can also affect plant ethylene through the activity of 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) which breaks down the ethylene precursor ACC into α -ketobutyrate and ammonia, the latter which is then used as a reduced nitrogen source by these soil-inhabiting microbes.⁶⁸

Endophytic examples of ACC deaminase have only recently begun to be found but it also appears to be able to enhance plant growth promotion through activity in the endosphere. An important example was observed through the study of *B. phytofirmans* PsNJ which is a strongly growth promoting endophytic bacteria, first isolated from pathogen infected onion⁷⁰; when the ACC deaminase gene in *B. phytofirmans* was mutated, the microbe's ability to promote canola root elongation was reduced.⁷¹ Similarly, isolation of root endophytes from *S. nigrum* (black nightshade) in Germany showed most strains were able to produce IAA and ACC

deaminase, increasing root length and reducing seedling emission of ethylene.⁵⁹ These same isolates show the importance of host species, as they were not able to promote root growth in *N. attenuate*. Many other examples exist of endophytic bacteria possessing ACC deaminase, but their effects of root growth have not yet been demonstrated. For example, in an experiment with potatoes, all shoot endophyte communities in several different potato genotypes contained a high proportion of ACC deaminase-producing endophytes (e.g., *Klebsiella* spp., *Pseudomonas fluorescens*, *Pantoea* spp., and *Erwinia persicina*) of unknown ecological function.⁷² ACC deaminase activity appears to be a widespread trait among rhizobia, as a survey of 233 strains from Saskatchewan, Canada, yielded 27 possessing this gene, mostly *Rhizobium leguminosarum*.⁷³ Whether rhizobia possessing this enzyme are able to stimulate root growth directly has not been shown, but *Rhizobium* expressing ACC deaminase are more effective at forming root nodules on legumes. *Rhizobium leguminosarum* bv. *viciae* contains one copy of ACC deaminase which when mutated reduces its ability to nodulate *Pisum sativum* L. cv. Sparkle (pea)⁷⁴ and when transformed into *Sinorhizobium meliloti*, which does not produce this enzyme, it helped the endophyte gain 35%–40% greater efficiency in nodulating *Medicago sativa* (alfalfa).⁷⁵ Similarly *Mesorhizobium ciceri* expressing a transgenic copy of ACC Deaminase resulted in significantly increased nodulation efficiency, nodule size and plant biomass of two chickpea cultivars.⁷⁶

Plants growing in stress-inducing substrate, including water-logged or polluted soils, have been shown to be inhibited in root growth due to stress ethylene.⁶⁷ In some of these environments, ACC deaminase-containing endophytes have been shown to prevent plant growth inhibition. Root flooding can induce production of large amounts of ethylene in stressed roots, and here it has been shown that tomato plants treated with the rhizobacteria, *Enterobacter cloacae* UW4, *E. cloacae* CAL2, and *P. putida* ATCC17399/pRKACC, all were substantially more tolerant to 9 days of flooding than untreated plants.⁷⁷ Tomatoes transformed with a constitutively expressed bacterial ACC deaminase gene were also more tolerant to flooding stress,⁷⁸ while poplar trees transformed with ACC deaminase isolated from the biocontrol fungus *Trichoderma asperellum* ACCC30536 had increased tolerance to salt stress.⁷⁹ Soil contaminated with heavy metals can also cause significant plant stress leading to overproduction of ethylene; a condition which can be ameliorated by ACC deaminase expressing bacteria or fungi. For example, root growth promoting endophytes were isolated from *Brassica napus* roots growing in lead-contaminated soil, including *P. fluorescens* G10 and *Microbacterium* spp. G16.⁸⁰ These strains could both enhance plant lead tolerance and produce ACC deaminase. A similar study of the halophyte plant *Prosopis strombulifera* growing under high salt conditions yielded 6 of 29 strains bearing ACC deaminase activity.⁸¹ Though ACC deaminase-expressing endophytes are very important when roots are producing high levels of ethylene, they may not confer much benefit to plants growing under ideal conditions.⁶⁸

Other microbially produced phytohormones, including cytokinins and gibberellins (GA), can alter growth but have not been widely reported as root-specific growth-promoting mechanisms by endophytes.⁸² GA is involved in seed germination, seedling emergence, stem and leaf growth, floral induction, flower and fruit growth, and most importantly, promotion of root growth and root hair abundance.⁸³ Though subsequently discovered in plants and bacteria, GA was first discovered in culture filtrates of the fungal pathogen *Fusarium moniliforme* which causes rice shoots to elongate and lodge.⁸³ Two endophytic *Fusarium* spp. isolated from the medicinal plant, *Euphorbia pекinensis*, were able to enhance root development of this plant via external secretion of GA and auxin.⁸⁴ Likewise, it has been found that indigenous *Rhizobium leguminosarum* bv. *trifoli* promotes root and shoot growth by producing auxin and GA, improving seedling vigor and increasing grain yield in rice grown in the Egyptian Nile delta.⁶⁶ Another example of root enhancement through GA comes from two growth-promoting strains of *Bacillus*; *Bacillus pumilus* and *Bacillus licheniformis* were isolated from the rhizosphere of alder (*Alnus glutinosa* L. Gaertn) and shown to stimulate root growth and plant yield through production of GA.⁸⁵ Increased root biomass of maize seedlings coincided with elevated levels of GA3 produced by endophytic *Azospirillum brasilense* and *A. lipoferum* inoculants.⁸⁶ This result was repeated in maize and soybean seedlings with strains of GA-secreting *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, although the authors note that root biomass increased without increases in root elongation or branching.⁸⁷ *Porostereum spadiceum* AGH786 is the first reported Basidiomycete endophyte that has been shown to produce gibberellins which appear to be how it helps soybeans resist salt stress.⁸⁸ Many types of nodule-forming *Rhizobium* have been shown to produce IAA, cytokinin, and GA, as well as ethylene and abscisic acid, but the exact purpose of these substances in their exudates and the importance of their ratios, are not yet well understood.⁸⁹

Cytokinins are known to stimulate cell division, trigger cell expansion, promote stomatal opening, enhance shoot growth and decrease root growth. They have been reported in the exudates of many bacteria, but their importance as a mechanism of microbe-induced plant growth promotion has only been demonstrated in a few examples.⁹⁰ With rhizobial bacteria, cytokinins have been shown to be important in nodule formation.⁹¹ One study of a *Bacillus megaterium* strain that promoted growth of *A. thaliana* and *Phaseolus vulgaris* seedlings showed that plant cytokinin receptor mutants were defective in the root growth promotion response.⁹² *Arabidopsis* growth promotion assays after inoculation with the endophytic fungus *P. indica* (noted above) suggest that cytokinin secretion, possibly in addition to auxin secretion, is responsible for its root growth promotion abilities in plants.⁶⁴ A more recent study shows that another endophytic fungus, *Phomopsis liquidambari* is able to increase plant production of auxin, cytokinin, and ethylene which results in significant increases of N content in rice.³⁹

A few non-hormone metabolites secreted by microbes appear to modify root architecture as well. Under low oxygen conditions, rhizobacteria emitting the volatile glucose metabolites, acetoin and 2,3 butanediol, have been shown to stimulate *Arabidopsis* root growth,⁹³ and this mechanism seems to act through ethylene and cytokinin pathways as indicated by studies using *Arabidopsis* mutants *ein2* and *cre1*, respectively.⁹⁴ On the other hand, microarray analysis of the effects of acetoin and 2,3 butanediol on *Arabidopsis* plants suggests these volatiles involve a wide range of mechanisms including cell wall modifications, primary and secondary metabolism, stress responses, and hormone regulation.⁹⁵ One of these responses appears to be enhanced basipetal auxin transport, resulting in elevated root auxin concentrations responsible for root growth promotion. Although acetoin or butanediol production

within plant tissues by endophytes should elicit these responses, and though genome sequencing projects have shown that some endophytic bacteria (poplar endophytes *Rhizobium tropici*, *Rhodotorula graminis*, *Acinetobacter calcoaceticus* and *Enterobacter asburiae*) do possess the capacity to produce these molecules,⁹⁶ there are still no reports of these mechanisms aiding in root growth from within the endophytic niche.

Another important endophyte produced non-hormone metabolite that can influence plant growth is N-acyl-homoserine lactones (AHLs), bacterial population size-dependent signals that have been shown to be important for cell-to-cell communication in many microbes. It has been found that a range of different AHLs are able to affect *Arabidopsis* primary root growth, stimulating lateral root formation and root hair development in an auxin-like manner, but through an auxin-independent mechanism.⁹⁷ Because only purified AHLs were used in the above study, we can only speculate that endophytes producing AHLs may similarly affect root development. Case in point, a recent set of experiments comparing plant growth promoting effects of endophytic *Acidovorax radialis* and its AHL mutant found no significant difference between inoculants, although mutant bacteria were impaired at root colonization.⁹⁸

AM fungi are known to stimulate root branching, possibly as a way to increase colonisable root area, and are thought to do so by secreting a diffusible 'myc factor'.⁹⁹ This factor was discovered by growing *Gigaspora margarita* and *Gigaspora intraradices* together with *Medicago truncatula*, but separated from physical contact with roots by cellophane membranes. The 'myc factor' was able to stimulate plant nodulation genes as well as significant lateral root formation, without inhibiting primary root elongation as would be expected in response to an increase in auxin.¹⁰⁰ Recent work has identified one example of the factor as sulfated and non-sulfated lipochitoooligosaccharides secreted by *Glomus intraradice*.⁵² Root morphology changes⁵⁰ triggered by AM colonization can correlate to changes in levels of plant auxin,¹⁰¹ abscisic acid¹⁰² and jasmonic acid¹⁰³, but how these changes are triggered by myc factors is still being elucidated.¹⁰⁴

A final non-hormone metabolite important for plant growth promotion by bacteria is the antibiotic 2,4-diacetylphloroglucinol (DAPG) which is produced by *P. fluorescens* isolates containing the *phlD* gene.¹⁰⁵ DAPG has been shown to inhibit primary root growth, while stimulating lateral root production in tomato seedlings through alteration of auxin signaling.¹⁰⁵ Treatment of corn seed in acidic soil with DAPG producing *Pseudomonads* results in enhanced vigor and increased absorption of P and Mg while reducing Al accumulation.¹⁰⁶

Other endophyte-encoded root growth enhancing mechanisms undoubtedly exist but have yet to be identified or fully understood. For example, by using auxin and ethylene *Arabidopsis* mutants, it has been shown that root growth promotion of *A. thaliana* by a strain of *Bacillus megaterium* is accomplished by a hormone independent pathway.¹⁰⁷ Although there is some evidence that growth promotion induced by *B. phytofirmans* is auxin mediated, *nadC* mutants defective in quinolinate phosphoribosyltransferase (an enzyme of the pyridine nucleotide pathway) lose their growth promotion ability, perhaps pointing to another set of unknown molecules involved in root growth stimulation.¹⁰⁸

4.64.4 Nitrogen Fixation

Even though 78% of the Earth's atmosphere is made up of nitrogen in the form of N₂, this element is generally the most limiting nutrient for plant growth and can only be converted into biologically accessible forms by a few groups of Bacteria and Archaea. These microbes (diazotrophs) generally use fixed carbon from plants to drive the energy-intensive, oxygen-sensitive process of breaking the N₂ triple bond, creating ammonia in the process known as biological nitrogen fixation (BNF). Because of the process's energetic requirements, it is not surprising to find elevated numbers of diazotrophic bacteria in carbon-rich plant rhizospheres. In the soil, ammonia can be oxidized by microbes into nitrates and assimilated into amino acids, but it can also be leached away in groundwater or reduced back into nitrogen gas; thus a closer physical interaction between plant and diazotroph benefits the host by aiding in capture of more bioavailable nitrogen. Endophytic nitrogen fixation is a well-known phenomenon in plants that form nodules which are basically bacterial fermentation organs on roots or stems. These structures are the result of an infection and signaling process between endophyte and plant, and get the plant more nitrogen in exchange for a low oxygen/high sugar environment for the bacteria.³⁵ This association is found in thousands of plant species within four related orders of the Eurosid angiosperms, and is believed to have evolved once about 59 million years ago during a time of elevated atmospheric CO₂.⁸ Interestingly, it has been shown that there are several genes shared between mycorrhizal and root nodule associations, suggesting this evolutionary event co-opted the mechanisms responsible for the ancestral mycorrhizal-root association to establish the first nodules.⁸

Depending on host plant, nodules are infected with Gram positive (actinorhizal) or Gram negative (rhizobial) bacteria, but formation and function involve a similar set of plant-microbe signals coded for by genes that appear to have been transferred horizontally between many different proteobacterial strains.¹⁰⁹ Legumes are the second largest/agronomically important group of crop plants, largely due to the ability of 88% of the 19,000 species described to form nitrogen fixing nodules making them largely nitrogen independent in agricultural settings.¹¹⁰ Soybeans are the world's most grown legume and can be nodulated by *Bradyrhizobium japonicum*, *B. elkanii* and *B. liaoningense*, *Mesorhizobium tianshanense*, *Sinorhizobium fredii* and *S. xinjiangense*, with different growth rates and N fixing capacity; worldwide average N fixation in soybeans by these microbes is estimated at 142 kg N/ha.¹¹¹ Legume nitrogen fixation has been reviewed extensively¹¹² so the focus here will be on the lesser known associative BNF by non-nodule forming endophytes.

There is fossil evidence suggesting that the earliest endophytic nitrogen fixing associations began 400 million years ago between a filamentous cyanobacteria capable of colonizing an early plant through its stomata.¹¹³ As it is believed all plants contain endophytes, the potential for transferring endophytic BNF into crop plants may be underexploited; almost all cultigens except legumes require large inputs of synthetic N, so setting up artificial symbioses with these plants and diazotrophic bacteria would be a great economic and environmental boon for humanity. In the case of the world's three most important cereals, wheat (*Triticum aestivum*), rice (*O. sativa*) and maize (*Z. mays*), they require 20–40 kg N/ha to be taken up for each tonne of grain produced.¹¹⁴ It has been said by some that creating nitrogen fixing cereals is the “Holy Grail” of agricultural research, and there have been serious attempts to make progress toward that goal; one example was the New Frontier Project (1994) of the International Rice Research Institute (IRRI) to coordinate worldwide research into rice–bacteria associations for increasing the BNF and N use efficiency. Much of this type of research has been focused on transferring nodule formation to cereals with very limited success; more promising, the nodulating bacteria can exist endophytically in non-legume plants where they may fix nitrogen.¹¹⁵ For example, *Rhizobium etli*, which normally forms nitrogen-fixing nodules on *P. vulgaris* (common bean), has been found to be an effective endophytic colonizer of maize plants¹¹⁶ and can increase the dry weight of Mexican landraces of maize, perhaps by endophytic BNF.¹¹⁷ Wheat has also been shown to benefit from inoculation with *Rhizobium leguminosarum*, and ¹⁵N tracer techniques show that under low nitrogen fertilization (50 kg N/ha), inoculated plants were able to fix 29% of the nitrogen they accumulated in shoots, whereas uninoculated plants fixed none.¹¹⁸ In addition to *Rhizobium*, it is interesting to realize that cereals have been found to contain many other diazotrophic endophytes including *Azospirillum*, *Azotobacter*, *Brevundimonas*, *Herbaspirillum*, *Ideonella*, *Klebsiella*, *Methylosinus*, *Pantoea*, *Pseudomonas*, *Raoultella*, and *Rhanella*, in maize,¹¹⁹ and *Azospirillum Brevundimonas*, *Enterobacter*, *Herbaspirillum*, *Ideonella*, *Pantoea*, *Pseudomonas*, and *Rheinheimeria*, in rice.¹²⁰ Indirect studies of BNF as a percentage of total plant N have estimated a contribution by endophytic bacteria of up to 33% in maize,¹²¹ up to 21% in some Asian rice varieties,¹²² and up to 49% in wheat inoculated with maize endophyte *Klebsiella pneumoniae* 342.¹²³ A study conducted on the role of endophytic *Bacillus tequilensis* on the nitrogen economy of agave plants showed that plants accumulate twice the N from endophytic bacteria when they are located within the endosphere compared to the rhizosphere.¹²⁴ Yet another study showed that inoculation of poplar clones with a mixture of diazotrophic endophytes resulted in growth promotion and 65% of the trees' N coming from BNF.¹²⁵ These estimates do show that endophytic N-fixation can occur within plants in the absence of nodules; however, the surprisingly large percentages may reflect technical challenges in making such assessments: these studies involved radiolabeling of nitrogen fertilizer, subsequently quantifying the radiolabel in the plant as a percentage of the total nitrogen, and then extrapolating the non-radiolabeled portion as coming from N₂-derived BNF. Furthermore, studies which show high endophyte BNF are typically conducted in pots with considerable inoculum, whereas in the field, endophyte BNF is typically considerably less.¹²³ Many experiments fail to use nitrogen fixation mutants (*nif*⁻) as controls to verify that BNF could be the mechanism of improved plant growth. In one exceptional study, *nif*-mutants of *K. pneumoniae* 342 inoculated onto wheat failed to duplicate the plant growth promotion observed when testing the wild-type endophyte, demonstrating that endophytic BNF may indeed be an important process in cereals.¹²³

There are many other important non-nodule plant-endophyte relationships which result in high levels of BNF and may provide agriculture with novel diazotrophs. Nitrogen fixing cyanobacterial endophytes are believed to have been much more common in ancient plants, with amazing fossil evidence suggesting they were able to colonize through stomata and co-existed with mycorrhiza as early as 400 million years ago.¹¹³ Among extant angiosperms, only the plant *Gunnera* (giant rhubarb, dinosaur food) still maintains cyanobacterial endophytes which colonize the plant through glands on its stem.¹²⁶ These bacteria invade plant cells where they become intracellular symbionts that are oxygen resistant and N fixing, yielding up to 72 kg N/ha/year as estimated for *G. arenaria*.¹²⁷

Another source of promising diazotrophs for agricultural applications might be from angiosperms growing in soils with very low natural levels of nitrogen. Dune grasses such as *Ammophila arenaria* and *Elymus mollis* grow effectively on nitrogen poor sand and have been shown to enjoy high levels of endophytic nitrogen fixation from diazotrophic endophytes including *Pseudomonas* spp., *Stenotrophomonas maltophilia*, *Enterobacter* spp., *Pantoea* spp., *Burkholderia* spp., *Xanthomonas* spp., *Agrobacterium* spp. and *Sphingomonas* spp.^{128,129} Cacti can grow without soil on rocky cliffs, large rocks, and ancient lava flows in hot desert areas of the Baja California Peninsula of Mexico; they have been shown to depend on endophytic bacteria for their mineral nutrition including nitrogen fixation.¹³⁰ Some types of bamboo are also able to grow under low nitrogen conditions and may depend on large populations of diazotrophic endophytes for nitrogen.¹³¹ *Miscanthus sinensis*, a promising second-generation biofuel crop, is a widespread perennial grass that is often the primary colonist on fresh volcanic mudflows possessing very low levels of soil nitrogen; *Miscanthus* has been found to contain a community of nitrogen-fixing endophytes dominated by *Clostridium* spp.¹³² Poplar trees are also colonists of disturbed and low nutrient soils, so there is much interest in their ability to gain fixed nitrogen from diazotrophic endophytes including *Burkholderia* spp., *Rahnella* spp., *Enterobacter* spp., *Acinetobacter*, *Herbaspirillum*, *Stenotrophomonas*, *Sphingomonas*, and *Pseudomonas*.^{125,133} Interestingly, it has been shown that poplar-derived nitrogen-fixing endophytes are able to increase maize¹³⁴ and rice¹³⁵ biomass and stature, although it wasn't clear that this was because of BNF.

Among endophytic plant-diazotroph relationships, the kallar grass–*Azoarcus* spp. strain BH72 interaction has attracted a lot of study. Kallar grass (*Leptochloa fusca* L. Kunth) is a pioneer plant on salty, alkaline, low nutrient soils in the Punjab of Pakistan.¹³⁶ *Azoarcus* spp. strain BH72 was isolated as the predominant nitrogen-fixing endophyte in kallar grass, estimated at 10⁹ bacteria per gram of root dry weight; interestingly these microbes are not found in surrounding soils suggesting they are dependent on the kallar grass host for survival and propagation. By studying patterns of *nifH* expression in kallar and wild rice, it has also been shown that the majority of the actively fixing population exists in a non-culturable state. This activity can be substantial and is estimated to account for up to 34 kg of N/ha/year, equivalent to 50% of what is generally applied to traditional rice farms.

Even more efficient than kallar grass, endophytic BNF in sugarcane can be as high as 150 kg of N ha/year (or about 60% of its total N) as estimated by ^{15}N isotope and N balance studies.¹³⁷ Sugarcane (*Saccharum officinarum*) is believed to have originated in New Guinea, but since 327 BC has spread throughout the worldwide tropics where it is usually propagated vegetatively in ratoon farming for production of sugar.¹³⁸ It is generally unresponsive to external nitrogen fertilization and has been cropped in Brazil for centuries without any significant input of nitrogen either in chemical or organic forms. A number of diazotrophic endophytes have been isolated from sugarcane, including *Burkholderia brasilensis*, *Burkholderia tropicalis*, *Herbaspirillum rubrisubalbicans* and *Gluconacetobacter diazotrophicus*, and these together seem to work together to synergistically fix the nitrogen in micropropagated sugarcane.¹³⁹ Among these diazotrophs, *G. diazotrophicus* is considered the most important. This bacteria does not survive well in soil and grows optimally at conditions of 10% sucrose and pH 5.5 which are similar to conditions inside cane stems, although it has also been found in many different tropical crops including sweet potato, coffee, pineapple, finger millet, tea and mango.¹⁴⁰ Its nitrogen-fixing activity is largely insensitive to nitrate and ammonium concentrations at 10% sucrose, suggesting it continues to fix nitrogen in stems even while the plant may be taking N up from the soil. Most interestingly, in vitro growth of *G. diazotrophicus* with yeast shows that more than half the fixed N_2 is secreted and usable by the fungus, suggesting that this fixed nitrogen would also be bioavailable to a plant host.¹⁴¹ Experiments investigating the influence of genotype and soil nutrient influence on BNF in sugarcane have confirmed here again that different plant genotypes respond variably to diazotrophic endophyte colonization, and that the bacterial communities and BNF are reduced in response to N fertilizer application, elevated oxygen levels, and lower concentrations of sucrose.

4.64.5 Other Endophytic Mechanisms Affecting Plant Nutrient Status

Some endophytes possess the ability to manipulate host plant metabolism to increase nutrient uptake and alter nutrient homeostasis. When the AM fungus *G. intraradices* colonizes maize roots, one host response is to downregulate its own nitrate reductase, allowing the more efficient fungus to reduce all of the N assimilate.¹⁴² On the other hand, infection of tomato roots with *G. intraradices* Sy167 upregulates expression of the high affinity nitrate transporter LeNRT2.3 in roots, stimulating greater plant uptake of nitrate.¹⁴³ Broomsedge (*Andropogon virginicus* L.) grass infected by two AM fungi and growing in low P sand has higher phosphorus use efficiency at low Pi concentrations and maintains constant levels of tissue P not only by enhancing P uptake, but also by altering plant patterns of P allocation and use.¹⁴⁴ How mycorrhiza induce these changes in plant nutrient homeostasis is not known. The fungal endophyte *P. indica* can colonize the interior of a number of different plant species roots and alter their nutrient metabolism; in *Arabidopsis* and tobacco, *P. indica* stimulates N uptake/nitrate reduction/accumulation by activating a host transcription factor which upregulates *P. indica*-responsive nitrate reductase and the starch-degrading enzyme glucan-water dikinase (SEX1).¹⁴⁵ Tall fescue (*Festuca arundinacea*) grown with the fungal endophyte *N. coenophialum* is able to take up greater amounts of soil nitrate and accumulate more amino acids in its shoot, especially arginine in leaves.¹⁴⁶ Faced with competition for N between the plant and its endophyte, it appears that the plant increases its own sink demand for N by upregulating shoot glutamine synthetase activity by 32%. Similarly, tall fescue (*F. arundinacea* Schreb.) infected with the fungal endophyte *N. coenophialum* (Morgan-Jones and Gams) experiences elevated plant growth and increased tissue P content, suggesting that *N. coenophialum* is an additional P sink and stimulates the plant to increase its P uptake.¹⁴⁷ In poplar infected with a nitrogen-fixing endophytic *Paenibacillus* strain, the plant's metabolic signature was altered, increasing asparagine and urea levels while reducing TCA sugars and organic acids.¹⁴⁸ Opposite to the above strategies, reduced levels of nitrate and amino acids in plant tissues have been reported in *Lolium perenne* infected with *Neotyphodium lolii*¹⁴⁹; it is hypothesized that these nutrient reductions make the plant less attractive for herbivores.

Some of the signals used by endophytes to affect their hosts are starting to be discovered: *Epichloë festucae* releases reactive oxygen species (ROS) to communicate with its grass host *L. perenne*; when ROS levels are altered, the relationship switches from mutualistic to antagonistic, resulting in sickness and death of the plant.¹⁵⁰ A different elicitor was found in culture filtrates of growth-promoting microbe *Bacillus thuringiensis* NEB17, which contain a novel bacteriocin protein called thurigen which enhances both soybean and corn biomass.¹⁵¹ *Pseudomonas fluorescens* B16 is a growth-promoting rhizobacteria that produces pyrroloquinoline quinone under low-nutrient conditions; bacterial mutant studies using a cucumber seedling bioassay showed this compound to be responsible for the observed plant growth promotion.¹⁵² It has long been known that Nod factors secreted by rhizobia are important in nodule formation, but these metabolites are also able to affect other changes in the plant such as increased uptake of calcium in soybean roots through unknown mechanisms.¹⁵³ Treatment of seeds of *Z. mays* (corn), *O. sativa* (rice), *Beta vulgaris* (sugarbeet), *G. max* (soybean), *P. vulgaris* (bean) and *Gossypium hirsutum* (cotton), with Nod factor BjV of *Bradyrhizobium japonicum* 532C resulted in enhanced seed germination and early growth under lab and field conditions which can allow the developing seedlings optimal access to nutrients in the rhizosphere.¹⁵⁴ Transgenic rice overexpressing an early nodulin gene ortholog, OsENOD93-1, had higher shoot dry biomass, seed yield, total amino acids, and total N in roots.¹⁵⁵ While the function of this gene is unknown, given its homology to legume genes involved in nodule formation, it is interesting to speculate that non-legume plants may preserve an ancient bacterial-dependent plant growth promotion pathway.

Modification of soil chemistry is an important way that plants may increase the availability of nutrients for absorption by roots. Plants have been shown to secrete up to 40% of their fixed carbon through their root systems as amino acids, organic acids, sugars, phenolics, mucilage, proteins and an array of additional secondary metabolites which may aid in optimizing their rhizospheres chemically and microbially.¹⁵⁶ Endophytes that help optimize root exudates may indirectly help it absorb nutrients – for example, by infecting tall fescue var DN2 (*F. arundinacea* Shreb.) with *N. coenophialum* (Morgan-Jones and Gams) acid root exudate patterns

were altered which correlated with increased uptake and transport of P, Ca, Zn and Cu in roots grown in low P nutrient solution.⁴⁹ *Neotyphodium coenophialum* infection has been shown to increase and alter fescue release of lipids, carbohydrates and carboxylic acids from its roots,¹⁵⁷ resulting in higher microbial activity and respiration in the rhizosphere.¹⁵⁸ P-deprived tall fescue infected by this endophyte can also increase root exudation of phenolics by 7%, which results in a 375% increase in the rate of soil Fe³⁺ reduction, a necessary step in iron uptake (Fe²⁺) by plants.¹⁴⁷ Under specific conditions, soil C and N pools can also be increased by endophyte infection of tall fescue, effected by either a reduction in soil microbial respiration¹⁵⁹ and/or a reduction in specific species of carbon-consuming rhizobacteria.¹⁶⁰ Endophytes may accomplish this indirectly by altering patterns of root exudation, or perhaps directly by producing alkaloids that are then secreted by roots into the soil.¹⁶¹ In addition to *Neotyphodium*, AM fungi have also been shown to alter plant exudates into the soil, including reducing the levels of total sugars exuded from roots, altering the proportions of exuded amino acids, reducing K⁺ and P leakage, and increasing the release of nitrogen, phenolics and gibberellins.¹⁶²

In contrast to *Neotyphodium* endophytes which modify soils from within their plant hosts, AM-like fungi are able to grow out from roots and able to directly modify the soil themselves. As mentioned previously, at least one species of AM seems to be able to enhance the degradation of organic N^{26,163} but how it does this is not known. Some AM are able to affect the behavior of other soil microbes: *Glomus mosseae* inoculation on diverse plant species resulted in the increase of rhizosphere bacterial respiration, likely by altering exudate patterns into soil.¹⁶⁴ AM fungi themselves directly release large amounts of glycoprotein called glomalin into the soil, which may serve to aggregate soil particles, increase water retention, chelate iron, or serve as an energy source for soil microbes.¹⁶² A different study on the effect of mycelial exudates from the AM fungus *Glomus* spp. MUCI 43205 showed it induced increases in soil populations of several Gammaproteobacteria, including a group of Enterobacteriaceae, although what functional changes resulted in the rhizosphere are not clear.¹⁶⁵ Likewise, the nearly ubiquitous dark septate endophytes have been shown to produce hyphae that exit the plant root and absorb organically bound mineral nutrients. These fungi have been shown to produce cellulases, laccases, amylases, lipases, pectinases, xylanases, proteolytic enzymes, tyrosinases, and polyphenol oxidases, but it is not yet known whether these enzymes are secreted into the soil to aid in plant nutrient absorption.³⁷

As a final mechanism that microbes could employ to improve plant NUE, bacteria and fungi living within the root can completely exit the endosphere and colonize the rhizosphere where they can scavenge and mineralize organic, gaseous or insoluble forms of N and P and release it for absorption for the plant as they die. Most studies in this area will show that the endophytes in question have a mechanism to mineralize nutrients in the soil, but not that the microbes can colonize the rhizosphere. For example, in a study of soybean endophytes, it was found that 49% were able to solubilize mineral phosphate, as compared to 52% of the leaf epiphytic bacteria, but it was not shown that the endophytes traveled to the rhizosphere where phosphate solubilization would be important.¹⁶⁶ In lettuce and maize, seed inoculation with phosphate solubilizing strains of *Rhizobium leguminosarum* bv. phaseoli was shown under field conditions to increase their P content by 6% and 8%, respectively, although it was not shown that this effect was caused by bacterial activity in the soil.¹⁶⁷ In order to conclusively demonstrate that endophytes can exit the plant to colonize the rhizosphere, we used electroporation to tag a seed transmitted species of phosphate solubilizing *Enterobacter* with antibiotic resistance and GFP, injected it into maize stems, and detected these bacteria 5 days later in the rhizosphere (Fig. 1); after inoculation onto seeds this bacteria has also been shown to intracellularly colonize root hairs.¹⁶⁸ In further studies we have observed that the most abundant bacteria in maize rhizospheres are in fact seed transmitted *Burkholderia* and *Enterobacter*¹⁶⁹ suggesting that plants have evolutionary incentives (i.e., enhancing soil nutrition) to auto-inoculate their microbiome with pre-adapted seed endophytes, rather than leave the microbial makeup of their rhizospheres open to random sampling of the surrounding soil. Similarly, orchid seeds, which are small and nutrient-poor for embryo development, possess endophytic *Rhizoctonia* fungi that grow out of the seed and enzymatically degrade the surrounding substrate to provide the embryo with nutrients for growth.¹⁷⁰ In another example of this phenomenon, the cardon cactus *Pachycereus pringlei* can grow on bare rock in Northern Mexico with help from its seed transmitted endophytes (mostly *Bacillus* spp., *Klebsiella* spp., *Staphylococcus* spp.) which appear to exit the seed to colonize and weather rock, liberating vital minerals for the developing seedling.¹³⁰ These cactus-associated bacteria likely solubilize inorganic phosphates by releasing organic acids such as gluconic acid or 2-ketogluconic acid, or may mineralize organic phosphates by secreting extracellular phosphatases.¹⁷¹ Another recent and fascinating discovery is that seed transmitted bacteria colonizing grass rhizospheres can fix and scavenge nitrogen, secreting exoenzymes and eventually dying in the process (or being killed by plant rhizosecretions) and releasing organic nitrogen that is then bioavailable for absorption by roots or mycorrhizae, or for further mineralization by other rhizospheric microbial fauna.^{172,173} Root cells have even been shown to phagocytize, digest and absorb soil inhabiting microbes from what the authors speculate is a “microbe nursery maintained by plants through exudation of photosynthates facilitating direct nutrient supply”.¹⁷⁴ In summary, it seems that plants are capable of inoculating their own rhizospheres with seed transmitted microbes that they then feed using rhizosecretions and exploit for nutrients by scavenging their remains or even phagocytizing and digesting them inside root cells.

4.64.6 Application of Endophytes to Agriculture

Endophyte inoculants have traditionally been applied as soil or seed inoculants, assuming that they will find a way to enter their hosts either through specialized signaling as do rhizobia and mycorrhiza to form symbiosomes, or via more basic crack entry such as that observed by *K. pneumoniae* 342 at lateral root junctions.¹⁷⁶ To be consistently effective, inoculations should be properly

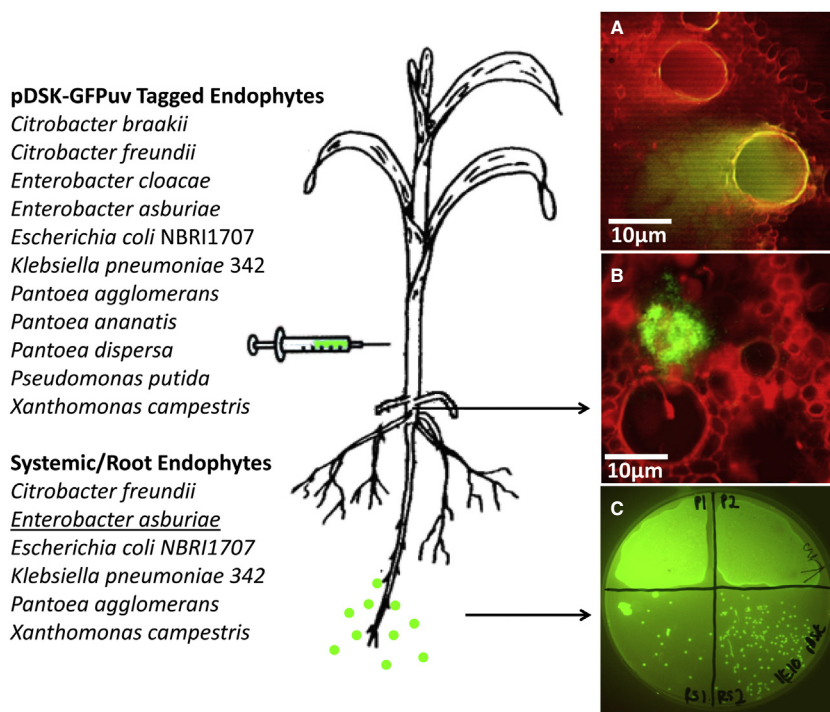


Figure 1 Persistence and migration of *Zea* seed endophytes in stems, roots and the rhizosphere. The 11 endophytes indicated were successfully tagged with pDSK-GFPuv (out of 124 isolates attempted) and injected into maize stems. The six indicated endophytes migrated to roots and persisted for >5 days as shown by fluorescence microscopy and culturing from macerated root tissues onto R2A-Kanamycin media (A) *Pantoea agglomerans* shown spilling out of a metaxylem vessel (B) *Enterobacter asburiae* spilling out of root vascular tissue (C) Culturing confirmed that *E. asburiae* was present in the roots of two plants (top two quadrants) as well as in their rhizospheres (bottom two quadrants). Adapted from [175].

researched and steps should be taken to assess whether there are already beneficial endophytes present in the soil, how the microbes of interest might persist and colonize the plant, and whether these microbes will be able to promote improved plant nutrition under the expected growing conditions. Early attempts at soil inoculation followed these principles, but were usually as simple as taking “infected soil” from fields with well-nodulated legumes to fields where legumes had not been grown before. Commercial production and sale of microbial inoculants became possible in 1895, when Nobbe and Hiltner were awarded the first patents for the application of pure rhizobial cultures to legumes, which was developed into the microbial inoculant ‘Nitragin’ and sold in the United States.¹⁷⁷ US and Brazilian agriculture have benefited immensely from BNF through inoculation with efficient strains of rhizobia in soybean production; in 2016 these nations were the first and second largest soybean producers in the world, also enjoying some of the highest yields (~3500 and 2900 kg/ha, respectively - FAOSTAT) that remain relatively independent of nitrogen fertilization thanks to stable, efficient nodule forming strains of *Bradyrhizobium japonicum* and *B. elkanii*. In Brazil, where soybeans are usually grown without N inputs, imported inoculants of *B. japonicum* have evolved in soils to become super inoculating strains; one such example is CPAC7 which persists well in soils, competes well to form nodules, and re-uptakes hydrogen gas (Hup+), a waste product of nitrogen fixation.¹¹¹ Meanwhile in China, where the soy-*Bradyrhizobium* symbiosis evolved and the crop was domesticated, average soybean yields are only 50% of that in the USA (1800 kg/ha - FAOSTAT). These lower yields are mostly the result of poor BNF caused by nodule formation with ineffective bacteria, with 22% of plants in some areas forming nodules full of cheaters (i.e., strains of bacteria that form nodules but don’t fix N).¹¹¹ Introduction of efficient new strains into Chinese soils might be expected to be difficult because of competition with large native rhizobial populations in the range of 10^4 bacteria/g of soil; an idea confirmed by a recent meta-analysis of 28 soybean seed inoculation experiments, that found a strongly inverse correlation between successful nodulation and abundance of indigenous soil rhizobia.¹⁷⁸ Nevertheless, experiments using *B. japonicum* strains as soil inoculants in different parts of China show it is possible to displace native rhizobia from soybean nodules and produce yield increases of 6%–33%.¹¹¹ Contrary to the situation of soy in China, natural inoculation of non-host plants by rhizobia can sometimes boost agricultural productivity, as, for example, does nodule-forming *Rhizobium trifolii* that colonizes rice when it is grown in rotation with Egyptian clover, reducing N application to one third of the recommended dose without decreasing grain yields.⁶⁶ Rhizobial inoculants for legumes are well established and widely sold as an agricultural product, with formulations such as Monsanto’s TAGTEAM taking the known nitrogen fixation potential of *Rhizobium* and combining it with the effects of the phosphate solubilizing soil fungus, *Penicillium bilaii*. A clear direction for the future application of endophytes to inoculants will be this kind of microbial synergism; for example, it was shown that mixing *Bacillus thuringiensis* KR1 (isolated from nodules on kudzu vines) with *Bradyrhizobium japonicum* SB1 yielded significantly greater nodule number and plant biomass than inoculation

with the *Bradyrhizobium* alone¹⁷⁹. A different approach used by EMD Crop Bioscience (since purchased by Monsanto as part of its BioAg Alliance) is to include Nod factors in their OPTIMIZE seed inoculant, mixing live *Bradyrhizobium japonicum* endophytes with their patented formulation of lipo-chitooligosaccharide, which stimulates early seed germination and enhances root branching and development. Interestingly, this same rhizobium-derived compound is registered as TORQUE for application to maize where it is able to increase seedling vigor even though this giant grass does not produce symbiotic nodules.

AM fungi have obviously played an important role in plant nutrition over evolutionary time and can stimulate crop plant growth, boost yield and reduce the need for phosphate fertilization by up to 50%,¹⁸⁰ but several factors have limited their usefulness in production agriculture: plant cultivars respond differently to AM colonization; AM species show variation in ability to provide nutrients to plants; high soil nutrient levels (especially P) can inhibit AM colonization and functioning; and certain agricultural practices such as fungicide treatment and tillage can disrupt AM populations in soils.²⁸ Natural populations of AM can be found almost everywhere, but have likely evolved to colonize specific varieties of plants over millions of years, with some taxa being restricted by soil factors such as pH and temperature, while others appear to have cosmopolitan distribution and physiological flexibility. AM form hyphal networks that can function to distribute nutrients and carbon between plants, buffering plant communities against nutrient stresses that affect individuals, although these networks are slow growing and are susceptible to disruption by tillage, chemical application, extended fallow periods, or planting with fungicide-exuding plants (such as Brassicaceae). As AM are obligate biotrophs, production of inocula is not possible without plant material, meaning that it has traditionally been made as a simple mixture of soil, roots, hyphae, AM spores, and any number of contaminating microbes from pot cultures, while newer techniques allow the growth of root cell cultures to support the growth of pure AM cultures. There are a number of AM mycorrhizal inoculants on the market such as BuRIZE (containing *G. intraradices*) made by BioScientific, Bio-organic's Endomycorrhizal Inoculant containing a blend of eight *Glomus* species, or from Mycorrhizal Applications, a range of mycorrhizal inoculants blended with ectomycorrhiza, PGPR, and trichoderma and available as powder, granular, liquid, gel or tablet forms. The effect of combining the enhanced nutrient absorption capabilities of AM with root growth promotion and N fixation of rhizobia can result in synergistic NUE^{181–183} which so far only seems to have been developed commercially by Premier Tech Agriculture with their product AGTIV, which is a blend of *G. intraradices*, *Rhizobium leguminosarum* biovar viciae, and *Bradyrhizobium japonicum*.

Clavicipitaceous endophytes, especially species of *Epichloë* and *Neotyphodium*, are able to promote the growth and stress resistance of grasses.¹⁸⁴ They intercellularly colonize shoot portions of plants where they can help protect their host from predation by producing toxic secondary metabolites, and can increase plant abiotic stress tolerance through multiple mechanisms. The fact that Clavicipitaceous endophytes are naturally transmitted through grass seeds has provided a convenient way for distribution, resulting in a wide range of commercially important plant species that can be bought as “endophyte enhanced” seed. One of the most widely distributed endophyte-infected plants in the US is Kentucky 31. This variety was obtained from a vigorous stand of tall fescue on a Kentucky farm in 1931 and widely planted for livestock forage and erosion control until it was recognized to cause toxicity problems in grazing livestock.¹⁸⁵ Seeds of this grass were found to contain ergot-alkaloid producing *N. coenophialum* endophytes, which are also responsible for much of the stress resistance. For forage applications, this grass variety has been replaced by Jesup tall fescue infected by non-ergot-alkaloid producing MaxQ endophytes (strains AR502 and AR542 of *N. coenophialum*), which maintain endophyte-conferred growth promotion, without the problem of toxicity to grazing livestock.¹⁸⁶ As many grasses are used for turf and not animal forage, however, there are still many seeds sold containing ergot-alkaloid endophytes. This includes grasses like Turf-Type Perennial Ryegrass, Chewings, Creeping Red, Hard and other fine fescue species. While some of these species of fungal endophytes are known to occur in other agriculturally important grasses like rice, wheat, and corn, there has been little effort to develop these for improving NUE.

A few other examples of endophyte inoculants exist in tropical countries where biofertilizers have been viewed for a long time as economically attractive alternatives to chemical fertilizers.¹⁸⁷ *Azospirillum* spp. are rhizosphere inhabitants that are also sometimes found as endophytes in plant roots where they can fix nitrogen and secrete root growth promoting hormones aiding the nutrient uptake of a wide variety of plants¹⁸⁸ – the average benefit from soil inoculation has been reported to be equivalent to 15–20 kg/ha of applied N, improving grain yield of cereals by 5%–20%, millets by 30% and forage crops by over 50%.¹⁸⁹ Examples of commercially available *Azospirillum* inoculants include BIOPROMOTER sold by Manidharma Biotech; NITROFIX sold by Ruchi Biochemicals; BIO N sold by the National Institute of Molecular Biology & Biotech in the Philippines; and SYMBION-N which is a synergistic blend of *Azospirillum*, *Rhizobium*, *Acetobacter* and *Azotobacter* sold by T. Stanes Company in India. *Pseudomonas fluorescens* is also a rhizosphere inhabitant that can be found as an endophyte, where it can promote root growth and antagonize pathogens through production of antibiotics and siderophores. Examples of *Pseudomonas* endophyte products that promote root growth are sold in India by Mani Dharma Biotech, while FOSFORINA is a Cuban strain of *P. fluorescens* that reduces the need for P fertilization by solubilizing phosphate in rhizospheres. BIOGRO is a biofertilizer mixture containing a strain of *P. fluorescens*, two bacilli and a soil yeast isolated from rice rhizospheres in Vietnam; they were selected for their ability to fix nitrogen, solubilize mineral phosphate, and secrete antibiotic compounds.¹⁹⁰ This blend has been shown to significantly increase grain and straw yields, total N uptake, and grain concentration of N: farmers using this product in Vietnam were able to reduce their N application by up to 52% while increasing rice yields.¹⁹¹ A different blend of unidentified rhizosphere and endophytic bacteria is marketed in Pakistan under the name BIOPOWER, with claims that use of the product in combination with half the recommended N-fertilizer will result in considerable yield increase.¹⁹² While there is much lab, greenhouse and field evidence that other endophytes can increase the NUE of important crops like rice, corn, sugarcane and wheat, mature commercial products and inoculation practices have yet to be developed.

4.64.7 New Developments and Future Directions

To continue the development of endophytes for crop application, discovery and screening of novel strains will be critical to find candidates for new inoculant products, then formulation and field testing of these inoculants will be critical to show they can perform under agricultural conditions. Fortunately, the microbiome fever that began in 2007 with the declaration of the human microbiome project¹⁹³ has also spread to North American and European agriculture, with many startup companies since coming into existence and raising over \$2 billion USD to patent and commercialize plant and soil microbes.¹⁹⁴ The majority of these companies are focusing on bioprospecting microbes from rhizospheres and soils, likely theorizing that inoculants that colonize and mineralize soils or help roots grow will improve plant nutrition. For example, Novozymes bioprospects microbes from soils, screens for those that help roots uptake nitrogen and phosphorus, then re-applies candidate microbes onto seeds reasoning that as plants germinate the inoculants can become part of the rhizosphere and aid in nutrient acquisition.¹⁹⁵ Bioconsortia takes this approach to a more sophisticated level, screening plants for phenotypes of interest and isolating and reformulating entire plant associated microbial communities into seed treatments, drenches, or granule products that can aid fertilizer use efficiency, growth improvement and abiotic stress tolerance. Although companies sourcing microbes from soils and rhizospheres may inadvertently discover endophytic microbes, a few are explicitly mining plant endospheres in their search for product candidates. New Leaf Symbiotics has a strong focus on cytokinin producing and nitrogen fixing *Methylobacteria* endophytes, while Adaptive Symbiotic Technologies bioprospects for fungal endophytes in plants growing in stressful habitats which can then theoretically be transferred to crop plants. Indigo Agriculture is a Boston-based, endophyte focused, startup that aims to deploy these microbes as seed inoculants to improve crop NUE and resistance to abiotic stress—commercial scale field trials in cotton, wheat and maize suggest their microbes are able to help protect plants from drought. Because of their reliance on seed inoculation, the company has apparently focused their bioprospecting strategy on seed endospheres in an effort to find winning product candidates that can survive on seeds long enough to colonize the germinating plant.^{196–198} Other examples of companies that are currently releasing endophyte products include Plant Response (from Spain) whose strain PRB110 is an endophytic *Colletotrichum* that mobilizes phosphorus and transfers it to shoots; Azotic Technologies (from England) introduces the nitrogen fixing sugar cane endophyte *G. diazotrophicus* into other crop plants; MicroGen Biotech in Ireland offers a product called MBOOSTER which contains endophytic *P. fluorescens* that uses ACC deaminase to reduce plant stress and secretes gluconic acid to solubilize phosphorus; the Canadian multinational Lallemande that has acquired endophyte products called ENDO-RICE (*Herbaspirillum*), GRAMINOSOIL (*Azospirillum*) and AZOS (*Azospirillum brasilense* abv-5) by buying Uruguayan company Lage y Cía S.A. and Brazilian company Farroupilha Laboratory.

Besides soil and seeds, further bioprospecting for agriculturally useful endophytes may strike gold by studying the microbiomes of wild plants growing in stressful environments. Agricultural researchers are echoing the call, suggesting we need to “go back to the roots to assess and access the microbiome of indigenous plants in their native habitats, which represent a yet untapped avenue to further exploit microbes and plant traits in modern agriculture.”¹⁹⁹ Evidence supporting these ideas is that plant genotypes do control endophyte selection, infection process and symbiotic outcome,^{29,200} and that plants growing in stressful environments (i.e., salt, drought, disease) do select different fungal endophytes that confer stress resistance.²⁰¹ An example of using plant genotype to help guide a productive bioprospecting strategy, we have searched for beneficial endophytes in seeds of wild relatives and ancient landraces of maize which yielded 124 different bacterial morphotypes, of which many displayed plant beneficial activities such as antagonism to pathogens, phosphate solubilization (Fig. 2A), ACC deaminase activity (Fig. 2B), and plant growth promotion

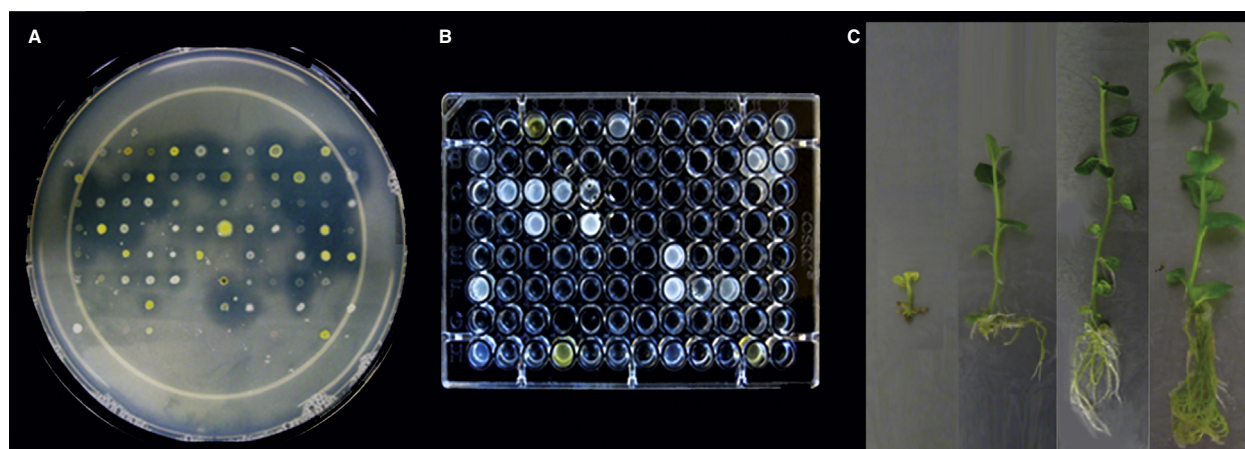


Figure 2 Assays performed on maize seed endophytes. (A) Phosphate solubilization was checked by plating isolates on tricalcium phosphate media and looking for clear halo production. (B) Ability to metabolize ACC was determined by growing isolates in nitrogen-free LGI, supplemented with 50-mM ACC as the sole nitrogen source, and screening for growth. (C) Ability to promote root and shoot growth was determined by inoculating gnotobiotically grown potatoes with isolates followed by measurements of growth promotion for 1 month. From left to right, potatoes were inoculated with isolates of *Enterobacter cloacae*, *Cellulomonas denverensis*, sterile buffer, and *Methylobacterium brachiatum*. Adapted from [175].

(Fig. 2C). Some of these bacteria have been sequenced,²⁰² patented,²⁰³ licenced to industry for product development, and gone on to be featured in numerous subsequent publications.^{168,204–206}

Future work to develop endophytes into inoculants has to prioritize their proper formulation and reliable delivery into the endospheres of crops growing in agricultural settings – failure to achieve these important steps are perhaps the most overlooked factors in developing a reliable inoculant product.²⁰⁷ While classical endophyte products such as rhizobia for legumes and mycorrhiza need to be inoculated into soil and survive long enough to be able to colonize roots as the seed germinates, it is less obvious how other types of bacterial or fungal endophytes should be delivered into plant endospheres. Inoculation of tissue cultured plantlets could be the ideal method of delivery for plants such as strawberries and potatoes that are vegetatively propagated,²⁰⁸ but for most other crops that are planted as seeds that are buried into farm soils, seed inoculation remains the obvious and most convenient choice. To develop endophyte seed inoculant formulations, researchers might want to mimic rhizobial seed inoculant products that have been sold for decades with formulations that are planter compatible and that ensure the bacterial populations applied to seed surfaces can survive desiccation, high or low temperatures, and seed coat/pesticide toxicity.²⁰⁹ These formulations usually include extender agents that feed and buffer the microbes on the seed surface, as well as microbe compatible flowability agents like BASF's FLO RITE 1706 Plantability Polymer that coat the bacteria onto the surface while still allowing the seed to drop through the planter without sticking. Even better than formulating endophytes to survive on the seed surface, a recent, revolutionary innovation/patent informs us how to install endophytes inside the seed by spraying them into the plant's flowers where the inoculants are protected alongside the plant embryo.^{210,211} Despite exciting results in the laboratory/greenhouse, Endoseed™ technology has not yet been scaled up/optimized for commercial implementation. Proof of a farm-ready formulation should include evidence of endophyte colonization and plant growth benefit/yield increases, but initial tests are usually conducted in a controlled (and often aseptic) lab or greenhouse environment which may not be predictive of inoculant behavior in the field where variable environmental stresses and competition from soil microbes dramatically reduce endophyte survival and effectiveness.²⁰⁷ This realization has led at least some companies to eschew traditional stages of the traditional agricultural development pipeline altogether: "There is nothing that translates a greenhouse result to a field result. Because the field is so complex, we have to test [seeds] in the field directly." says Thomas Schäfer, vice president of bio-ag research at Novozymes. In their partnership with Monsanto, the Bioag Alliance has eliminated all greenhouse testing from their microbial inoculant product pipeline, instead doing all their screening directly in agricultural fields, for example, coating 2000 different rhizosphere microbe formulations onto seed that were planted on some 500,000 plots in 2015.¹⁹⁵ Such a pragmatic approach to find field ready microbes unfortunately also discards a majority of other beneficial microbes which were not formulated to facilitate their survival and colonization of the germinating seedling. For example, while there are instances where simple formulations for seed inoculation with endophytes have resulted in increases of agricultural yield,²¹² there are also numerous studies where simple seed inoculation has failed to introduce the microbe into the host endosphere. In one experiment, seed inoculated with *Beauveria bassiana* and grown on six different Colombian soils resulted in less than 3% bean plant colonization,²¹³ while in another study of 76,000 field-grown maize plants arising from *Pantoea stewartii* infected seed (without optimized formulation), the seed transmission rate of this vascular pathogen was only 0.14%.²¹⁴ It is a shame to see promising endophytes such as *B. phytofirmans* PsJN fail to impact agriculture because they are not properly formulated and die on seed surfaces before getting the chance to get inside the germinating plant. Further understanding of microbial ecology and innovation in inoculant formulation will be necessary in order to properly leverage microbial diversity to improve world agriculture.

Another important future direction in applied crop microbial ecology will be the dissection, isolation, upregulation and genetic transfer of beneficial mechanisms from endophytes to other microbes or even to plants. A large number of endophyte genomes are now available including the kallar grass endophyte *Azoarcus* sp. BH72,²¹⁵ sugarcane endophytes *G. diazotrophicus* Pal5²¹⁶ and *Herbaspirillum seropedicae* Z67, corn endophyte *K. pneumoniae* 342,²¹⁷ rice endophyte *Azospirillum* sp. B510,²¹⁸ tall fescue endophytes *Epichloe festucae*, *Neotyphodium coenophialum* and *Neotyphodium lolii*, onion endophyte *Burkholderia phytofirmans* PsJN,²¹⁹ *Piriformospora indica*,²²⁰ *AM G. intraradices*,²²¹ and various rhizobial and frankia nodule-forming bacteria. Several poplar endophytes have been also been sequenced including *Enterobacter* sp. strain 638, *P. putida* W619, *S. proteamaculans* 568 and *S. maltophilia* R551-3; their genome sequences show that these microbes possess the ability to produce acetoin, synthesize IAA and metabolize GABA, but they do not have functional ACC deaminase.⁶⁰ In a recent giant feat of sequencing, 484 genomes of bacterial isolates from root endospheres and rhizospheres of Brassicaceae (mostly *Arabidopsis*), poplar, and maize were reported and compared to 3837 other publicly available bacterial genomes to characterize genomic features of endophytes; these plant-associated bacteria possess more genes for carbohydrate metabolism than do free-living bacteria.²²² As some endophytes live within plants in unculturable states, it may also be important to undertake metagenomic approaches to acquire a more complete picture of the endophytic community; this was done in a European project to sequence 100 Mb of endophyte DNA extracted from inside rice plants which showed the bacterial community to be enriched in genes for flagella, carbohydrate metabolic enzymes, protein secretion systems, iron acquisition and storage, quorum sensing, nitrogen metabolism, and detoxification of reactive oxygen species.²²³ Better understanding of the microbial genes involved in these mechanisms may allow for their pyramiding within endophytes or their transfer into plants for enhanced NUE. Transfer of ACC deaminase from *E. cloacae* into rhizospheric *Azospirillum brasilense* increased the root elongation potential of this strain in tomato and canola,²²⁴ suggesting that similar transgenic techniques may increase the root growth promoting ability of endophytic strains. This has also been shown to be an effective technique to improve nodule forming rhizobia: ACC deaminase genes from *Sinorhizobium* sp. BL3 were introduced into *Rhizobium* sp. strain TAL1145, resulting in greater numbers and sizes of nodules, as well as higher root mass in the leguminous tree *Leucaena leucocephala*.²²⁵ Root stimulation by transgenic auxin production in endophytes may also enhance root development: the entire tryptophan monooxygenase pathway was introduced into *Pseudomonas fluorescens* strain CHA0 elevating synthesis of IAA, and stimulating an increase in root fresh weight

of cucumber by 17%–36% in natural soil.²²⁶ Constitutive overexpression of the nitrogen fixing transcriptional regulator *nifA* was shown to significantly increase N fixation by endophytic *Enterobacter gergoviae* 57-7 *in planta*, and may be a useful trait to introduce into other diazotrophic endophytes.²²⁷ Another strategy to increase endophytic N fixation has been to add an additional copy of the *nifHDK* operon under a stronger *nifH* promoter, allowing *Rhizobium etli* to increase its nitrogenase activity by up to 58% which correlated to increases of its host plant's weight by 38%, nitrogen content by 15%, and seed yield by 36%.²²⁸ Novel genes from endophytes may be used to make transgenic plants with improved NUE: tomato plants constitutively expressing bacterial ACC deaminase are able to better tolerate flooding and heavy metal stress,²²⁹ while *N. tabacum* plants expressing a phytase gene from the soil fungus *Aspergillus niger* accumulated up to 52% more P than controls when grown in soils amended with either phytate or phosphate and lime.²³⁰ Many other transferable mechanisms to improve plant NUE must exist in the Earth's countless undiscovered or understudied endophytes and will hopefully lead to genetically enhanced inoculants, perhaps produced by companies such as the joint venture between Bayer and Ginkgo Bioworks which is to be called Joyn Bio. To date, only one genetically modified endophyte has been commercially released for improving NUE: strain RMBPC-2 of *Sinorhizobium meliloti*, sold by the American company, Research Seeds Inc., which was modified with genes to enhance C4-dicarboxylic acid uptake and nitrogen fixation in symbiosis with alfalfa.²³¹

4.64.8 Conclusions

Industrial fertilizer use has permitted large increases in global agricultural production, but further increasing its application is not a sustainable solution to meet future food demands. Endophytes naturally occur in all plants and have significant potential to improve nutrient use efficiency. Endosymbiotic associations that benefited NUE likely evolved as early as 400 million years ago in the form of mycorrhiza that assisted the first rootless land plants to absorb nutrients from the soil. The plant genetic machinery required for this association has persisted in most lineages of modern land plants and been co-opted by nodule-forming plants to enable rhizobial symbioses, while the underlying mechanisms and host control of the countless other endophytic associations are largely unknown. While many endophytes gain access to plants from the environment (i.e., soils), inter-generational transmission can occur via seed (e.g., Clavicipitaceous fungi) or by vegetative plant propagation (e.g., in sugarcane). Endophytes improve plant NUE using a diversity of mechanisms which include formation of extra-root hyphae for nutrient absorption; stimulation of root growth by manipulating levels of phytohormones (e.g., auxin, ethylene) and other metabolites (e.g., acetoin); altering plant metabolism to stimulate higher nitrogen and phosphate uptake; nitrogen fixation by both nodulating and non-nodulating endophytes; colonization and modification of soil directly by endophytes or indirectly by changing root exudates profiles; and even serving as sources of nutrients to be phagocytized by roots. Estimates of the contributions of nitrogen-fixing endophytes to plant nitrogen supply may vary widely in the literature because of methodological differences. The beneficial traits exhibited by an endophyte can change in different hosts or environments which currently limit their utility in agriculture. Several other challenges have prevented wider adoption of endophyte inoculants, including competition from endogenous microbes, host genotype specificity, establishment and persistence, and difficulty in developing formulations to aid in their delivery. Despite the difficulties, recent years have seen the beginning of many new ventures raising billions of dollars to develop plant microbiology for agriculture in North America and Europe, while endophyte-based inoculants other than rhizobia (including AM, *Azospirillum*, *Pseudomonas* and Clavicipitaceous fungi) have already been available commercially for some crops in tropical countries for decades. Countless endophytes, their metabolites and genes still exist out in nature and possess great potential to aid agriculture in the future.

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