4.58 Plant and Endophyte Relationships: Nutrient Management

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Glossary

arbuscular mycorrhiza (AM) The major type of mycorrhizal association found in angiosperm plants. The fungal partner is able to grow into roots and form an intimate endophytic relationship with the host.

biofertilizer A broad term used to describe any biological fertilizer, but usually refers to soil microorganisms which improve plant growth. Examples may include rock phosphate solubilizing fungi or nitrogen-fixing bacteria.

biological nitrogen fixation (BNF) The incorporation of gaseous dinitrogen into ammonia by diazotrophic prokaryotes.

endophyte Usually refers to nonpathogenic bacteria and fungi found in plant tissue, and sometimes is also used to refer to mycorrhizal fungi found in plant roots.

inoculant A microbial input used in agricultural settings to improve plant productivity. Can be applied as a spray or powder to soil or coated onto seeds prior to planting.

mycorrhiza A soil fungus having the ability to form a symbiotic association with the roots of a vascular plant.

N Nitrogen as it is found in a form available for plant absorption.

nutrient-use efficiency (NUE) Plant biomass produced per unit of available nutrient input.

P Phosphorous as it is found in a form available for plant absorption.

rhizobia Nitrogen-fixing soil bacteria that can establish themselves within specialized organs called nodules within legume roots.

4.58.1 Introduction: 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 Dowswell [1] to have been responsible for up to 50% of increases in crop yields worldwide during the twentieth century. There is a strong positive correlation between N and P application from 1960 to 2000 and global cereal production WN and P fertilization increasing 7 and 3.5 times, respectively, while cereal production has doubled [2]. Such increases in agricultural productivity were absolutely necessary to feed an exponentially growing world population that quadrupled to 6 billion during the twentieth 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 (NUE) [2]. Required as it may be, further growth in productivity presents a problem for agriculture for multiple reasons: most of the world's best farmland is already under cultivation; the remaining land is being eroded or marginalized; there are concerns about climate change; water in some regions is becoming scarce; yield potential from Green Revolution technologies has either stagnated or been reached in many regions of the world; and nutrient pollution from agricultural practices has reached unacceptable levels. Nutrient pollution from fertilizer comes mainly from runoff on farms that is the result of overfertilization and poor uptake by plants: only 30–50% of applied N and about 45% of P are taken up by crops [2]. Work to improve 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 chapter discusses the role that bacteria and fungi residing within crop plants may play in improving NUE and may help to 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 will allow development of biotechnologies to increase NUE in modern plant agriculture.

The term 'endophyte' was coined by Heinrich Anton de Bary in 1866 and is derived from the Greek words *endon* (within) and *phyte* (plant)[3]. Endophyte usually refers to nonpathogenic 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 (mya) 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 lichens already growing in this new environment. Ancient fungi (and likely 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 that increased 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 dessication with the help from its endophyte, *Mycosphaerella ascophylli* [6]. It has also been hypothesized that mycorrhiza evolved from established fungal endophytes that developed external hyphae which enhanced host fitness through mineral scavenging in exchange for fixed carbon [7]. This ancient vesicular 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 mya already show extensive colonization by fungal mycorrhiza [6]. Mycorrhizal associations are found 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 nonmycorrhizal (e.g., *Brassicaceae*) [6]. Interestingly, 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 mya 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 thousands, if not millions of endophytes, are believed to still exist undiscovered or are poorly understood in nature, and thus pose a huge potential source of novel mechanisms and genetics, which may be co-opted for the improvement of plant agricultural practices. The rest of this chapter is 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 focus on endophytic mechanisms that increase their procurement. Where possible, the chapter highlights instances of successful application of endophytes for improved plant nutrient management in agriculture.

4.58.2   Endophyte Nutrient Uptake

Early land plants had to evolve the ability to absorb mineral nutrients from soil in contrast to aquatic plants that absorb nutrients by diffusion from water. Early bryophyte-like plants (e.g., mosses) did not have true root systems, and thus it is possible that ancient mycorrhizal hyphae served this function in early land plant evolution [7]. The ancient fungal partner was almost certainly in the phylum Glomeromycota as the AM partner in 90% of modern land plants are *Glomus* species. There are 200 species of this fungus 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, nonphotosynthesizing plants. In exchange for up to 20% of the plant's carbon, AMs are able to increase the plant's supply of P, N, Zn, Cu, Ni, S, Mn, B, Fe, Ca, and K [9], 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 [10]. This is especially valuable for scavenging immobile minerals such as P and Zn, considered the most important macro- and micronutrients provided to plants by AM fungi.

Plant species 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 [11]. For example, uptake of P per root length was fourfold [12] higher in maize compared to nonAM 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 nonAM plants. In a different experiment, *Linum usitatissimum* (flax) inoculated with either *Gigaspora rosea* or *Glomus intraradices* AM accumulated greater biomass and took up more P, while *Solanum lycopersicum* (tomato) showed a decrease in biomass and lower P uptake [13].

AMs are relatively unimportant for increasing N uptake of plant, as most nitrogen in soils is found as diffusible water-soluble nitrate that is easy for plants to absorb through roots, and which AMs appear unable to transfer to the plant [14]. On the other hand, ammonium is the most common form of synthetic nitrogen fertilizer. As the breakdown product of organic decomposition, ammonium is fairly immobile in soils and has been shown to be readily absorbed and translocated to maize through AM association [14]. There is evidence that AMs form below ground hyphal networks connecting neighboring roots [15], allowing the transfer of N from plant to plant, but how this affects plant communities in agricultural settings is not well understood. Although AMs are generally thought to have no saprophytic abilities, it has been shown that at least some species of AM are able to enhance decomposition of organics and transfer liberated ammonium to their plant host [16].

The ability of AM to improve NUE in production agriculture seems to be dependent on a number of factors. When grown in soils with abundant P and Zn, AMs appear to be unimportant or even detrimental to plant growth as they become drains on plant carbon without benefits to the plant [17]. This might predict that a crop variety adapted to high soil nutrients would be less responsive to AM than a variety adapted to low soil nutrients. However, in a test of AM colonization on European maize (high fertilizer input) and African maize (low fertilizer input) it was shown that although the African variety grew better at low P, it was almost insensitive to AM infection [18], while it greatly benefited the European variety. Indeed, there appears to be tremendous variation within modern

varieties of a crop species with respect to the impact of AM [19]; the beneficial effects of AM are likely inversely correlated with the root branching and ability to absorb P from soil itself. Due to this variation, experiments should be conducted to validate how a particular variety will respond to AM colonization under field conditions. As improved varieties of crops have been bred to respond to high fertilization levels and resist fungal pathogens, the genetic potential of AM to improve high-production agriculture may be limited [17] unless new breeding programs develop plants with improved AM response [20].

As early as 50 mya, three other major types of mycorrhizal relationships developed in addition to AM, again increasing host nutrient absorption area, and 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., pine trees), associate with 6000 different species of septate basidiomycetes, ascomycetes, and zygomycetes. Second, orchids associate with basidiomycete mycorrhiza and interestingly can parasitize the fungus as a carbon source. 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 [11]. Carboxylases have also been shown to associate with aluminium (Al) in the soil to form acidic complexes that weather calcium-rich rock, thereby releasing Ca that is taken up by the mycorrhiza [11]. Although these microbes are important in enhancing nutrient uptake by some plants, they will not be considered further in this article due to their nonendophytic origin.

Besides mycorrhiza, fungal endophytes are classified as belonging to the Clavicipitaceae family (class 1) or as nonclavicipitaceous (class 2, 3, and 4) [21]. Clavicipitaceae are a large fungal group consisting of obligate biotrophs and necrotrophs that colonize plants, insects, and other fungi. However, they do not have a mycorrhizal habit. Class 4 endophytes, also known as dark septate endophytes (DSEs), 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 [22]. DSEs have been reported in over 600 different plant species and are found worldwide, often coexisting with mycorrhizal fungi, although there are numerous nonmycorrhizal plant roots that have been found to contain DSEs. DSEs may improve phosphorous supply to the host and appear to replace AMs and ectomycorrhizal fungi at sites with extreme environmental conditions [22]. Although some DSEs are able to secrete phytohormones that elicit plant growth responses, it is likely that their newly discovered mycorrhizal habit, combined with their saprophytic capabilities, is the main mechanism of DSE-enhanced nutrient uptake. Although all plants harboring DSEs are not observed to have enhanced NUE, there are several studies showing that DSEs are able to improve nutrient levels in plants. For example, DSEs isolated from roots of a *Carex* species (sedge) were used to reinoculate DSE-free plants of that same species and resulted in increased biomass and P content [23]. Inoculation of *Pinus contorta* by the DSE *Phialocephala fortinii* resulted in increased levels of leaf P, enhanced N uptake from soil, and higher plant biomass [24]. Inoculation of *Phialophora graminicola* onto the grass *Vulpia ciliate* likewise was observed to increase plant biomass and elevate levels of N and P [25]. Approximately 99% of soil P is tied up in organic sources, and DSEs may be able to mineralize it for uptake and use by plants, but their ability to do this has not yet been explicitly demonstrated. DSEs have, however, been shown to secrete a number of degrading enzymes that may allow these fungi to access organic pools of nutrients for plant hosts. Among these enzymes are cellulases, laccases, amylases, lipases, pectinases, xylanases, proteolytic enzymes, tyrosinases, and polyphenol oxidases [22]. 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 saltbrush), grown on rock- and tricalcium phosphate sources that are less soluble in soil and poorly available to plant roots for uptake [22].

4.58.3 Enhancing Root Growth

A key mechanism underlying endophytic improvement of NUE is modulation of root growth. Among key root system traits which can increase NUE if they are enhanced are root branching and root hair production; the latter 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 [26]. 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 [27]. Class 1 fungal endophytes such as *Neotyphodium coenophialum* stimulate the development of extensive root systems and longer root hairs, resulting in more efficient absorption of soil P [28]. There are also several examples of class 2 and 4 endophytes stimulating plant root development, while the hyperdiverse class 3 group usually exists within plant tissues without affecting plant growth [21]. In addition to scavenging for nutrients, AMs are able to increase lateral root formation through as-yet unknown diffusible signals [29]. There are different mechanisms by which endophytes modify root development, but most appear to produce or block plant hormones including auxin, ethylene, cytokinin, and gibberellin (GA).

By far the most common mechanism that endophytes use to stimulate root growth is through secretion of auxin within the plant [30]. 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, but at higher concentrations induces ethylene production and inhibits primary root elongation reducing root depth; IAA simultaneously induces initiation of lateral and adventitious roots and root hairs, which serve to increase 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 overproducing mutants, *rooty* and *superroot*, there is a dramatic increase in the formation of lateral roots and root hairs [31, 32]. Microbial biosynthesis of auxin was initially discovered in

Agrobacterium where it plays a role in gall formation and pathogenesis, but since then many nonpathogenic bacteria and fungi have been shown to synthesize auxin using up to six different genetic pathways [30].

Endophytes producing IAA are often found to promote root growth in an auxin-dependent manner, although exact results on host roots depend on the amount of auxin produced, the presence of other interacting hormones, and plant sensitivity to IAA. For example, *Pseudomonas putida* GR12-2 was 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 [33]. The broad host range bacterial endophyte, *Burkholderia phytofirmans* PsNJ, can greatly stimulate root and shoot development in *Arabidopsis*, but plant auxin insensitive mutants showed no growth response to inoculation with the strain [34]. In a study of root-promoting endophytic bacteria from *S. nigrum* and *Nicotiana attenuate* in Germany, most positive isolates were found to produce IAA [35], 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 [36]. An endophytic yeast that colonizes maize roots, *Williopsis saturnus*, was shown to produce large amounts of IAA as well as another auxin, indole-3-pyruvic acid, stimulating both shoot biomass and root elongation in inoculated corn plants. 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*, *N. tabacum*, *Populus tremula*, *Oryza sativa*, *Glycine max*, and even several species of terrestrial orchids [37]. *P. indica* colonized roots have higher biomass and are highly branched, resulting in plants with increased yield and NUE – effects which were attributed to its ability to produce IAA [38], though the fungus has also been shown to inactivate (conjugate) auxin at high concentrations [39]. Infection by 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 [40]. This is echoed in the results of field trials with the auxin- and GA-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 [41].

Another important mechanism of endophyte-dependent root growth promotion is through the reduction of the volatile plant hormone, ethylene. Ethylene that is synthesized by plants upon exposure to abiotic or biotic environmental stress is called stress ethylene and is thought to initiate many plant stress symptoms including senescence, chlorosis, and organ abscission that reduce plant productivity and survival [42]. Ethylene affects roots by inhibiting elongation, promoting lateral root growth, and stimulating root hair formation [43]. In this way, ethylene in roots antagonizes auxin function and reduces root surface area available for nutrient absorption. For example, the endophytic fungus *Sebacina vermifera* promotes root and shoot growth in *N. attenuate* by inhibiting plant ethylene signaling via an unknown mechanism [44]. Rhizosphere-inhabiting bacteria can also affect plant ethylene by secreting 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which breaks down the ethylene precursor ACC into α-ketobutyrate and ammonia, the latter is then used as a reduced nitrogen source by these soil-inhabiting microbes [43].

Endophytic examples of ACC deaminase have only recently begun to be found but it appears to be an important mechanism in plant growth promotion similar to rhizosphere bacteria. *B. phytofirmans* PsJN is a strong growth-promoting endophytic bacteria isolated from pathogen-infected onion [45]. When the ACC deaminase gene in *B. phytofirmans* was mutated, the microbe's ability to promote the elongation of the roots of canola seedlings was reduced [46]. 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 [35]. 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 on root growth have not 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 such as *Klebsiella* spp., *P. fluorescens*, *Pantoea* spp., and *Erwinia persicina* [47]. ACC deaminase activity appears to be a widespread trait among *Rhizobia*, as a survey of 233 strains from Saskatchewan, Canada, yielded 27 isolates possessing this gene, mostly *R. leguminosarum* [48]. Whether rhizobia possessing this enzyme are able to stimuli root growth directly has not been shown, but Rhizobia expressing ACC deaminase are more effective at forming root nodules on legumes. *R. leguminosarum* bv. *viciae* contains one copy of ACC deaminase, which when mutated reduces its ability to nodulate *Pisum sativum* L. cv. Sparkle (pea) [49]. This ACC deaminase gene from *R. leguminosarum* bv. *viciae* was introduced into *Sinorhizobium meliloti* which does not have this enzyme; transgenic bacteria showed 35–40% greater efficiency in nodulating *Medicago sativa* (alfalfa) [50].

Plants growing in stress-inducing soils, including water-logged or polluted soils, have been shown to be inhibited in root growth due to stress ethylene [42]. 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 [51]. Tomatoes transformed with a constitutively expressed bacterial ACC deaminase gene were also more tolerant to flooding stress, than untransformed plants [52]. 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. 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 [53]. 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 [54]. 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 [43].

Other microbially produced phytohormones, including cytokinins and GAs, can alter growth but have not been widely reported as root-specific growth-promoting mechanisms of endophytes [55]. 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 [56]. 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 [56]. Two endophytic *Fusarium* spp. isolated from the medicinal plant, *Euphorbia pekinensis*, were able to enhance root development of this plant via external secretion of GA and auxin [57]. Likewise, it has been found that indigenous *R. 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. Another example of root enhancement through GA comes from two growth-promoting strains of *Bacillus, Bacillus pumilus* and *B. licheniformis*, which were isolated from the rhizosphere of alder (*Alnus glutinosa* L. Gaertn) and shown to stimulate root growth and plant yield through production of GA [58]. Increased root biomass of maize seedlings coincided with elevated levels of GA3 (as measured by capillary gas chromatography) inoculated with different strains of endophytic *Azospirillum brazilensis* and *A. lipoferum* [59]. This result was repeated in maize and soybean seedlings using strains of GA-secreting *A. brasilense* Az39 and *Brayrhizobium japonicum* E109, although the authors note that root biomass increased without increases in root elongation or branching [60]. Many types of nodule-forming *Rhizobium* have been shown to produce IAA, cytokinin, and GA, as well as ethylene and abscissic acid, but the exact purpose of these substances in their exudates, and the importance of their ratios, are not yet well understood [61].

Cytokinins are known to stimulate cell division, trigger cell expansion, promote stomatal opening, stimulate shoot growth, and decrease root growth. They have been reported as an exudate of many bacteria, but their importance as a mechanism of plant growth promotion by bacteria has only been demonstrated in a few examples [62]. In Rhizobial strains, cytokinins have been shown to be important in nodule formation [63]. One study of a *B. megaterium* strain that promoted growth of *Arabidopsis thaliana* and *Phaseolus vulgaris* seedlings showed that plant cytokinin-receptor mutants were defective in the root growth-promotion response [64]. *Arabidopsis* growth-promotion assays involving inoculation with the endophytic fungus *P. indica* (noted above) suggest that fungal cytokinin secretion, possibly in addition to auxin secretion, is responsible for its root growth promotion abilities in plants [39].

A few nonhormone 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 [65], and this mechanism seems to act through ethylene and cytokinin pathways as indicated by mutant studies using *Arabidopsis* mutants *ein2* and *cre1*, respectively [66]. On the other hand, microarray analysis of the effects of acetoin and 2,3 butanediol on *Arabidopsis* plants suggests that these volatiles involve a wide range of mechanisms including cell-wall modifications, primary and secondary metabolism, stress responses, and hormone regulation [67]. 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 may elicit these responses, there are still no reports of these mechanisms at work within the endophytic niche.

Another important nonhormone metabolite class important for plant growth by microbes are the 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 including endophytes. It was found that a range of different AHLs were able to affect *Arabidopsis* primary root growth, increase lateral root formation, and stimulate root hair development, in a manner similar to that observed for auxin, but in an auxin-independent manner [68]. Because only purified AHLs were used in the above study, we can only speculate that endophytes producing AHLs may similarly affect root development.

AM fungi are known to stimulate root branching, possibly as a way to increase colonizable root area, and are thought to do so by secreting a diffusible myc factor, which has yet to be identified [69]. This factor was discovered by growing *G. margarita* and *G. intraradices* together with *M. truculata*, 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 [70]. Root morphology changes [29] triggered by AM colonization can correlate to changes in levels of plant auxin [71], abscissic acid [72], ethylene, and jasmonic acid [73] but how these changes are triggered is still not known.

A final nonhormone 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 [74]. DAPG has been shown to inhibit primary root growth, while stimulating lateral root production in tomato seedlings through alteration of auxin signaling [74]. 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 [75].

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 *B. megaterium* is accomplished by a hormone-independent pathway [76]. Although there is some evidence that growth promotion induced by *B. phytofirmans* is auxin mediated, *nadC* mutants defective in quinolinate phosphoribosyltransferase (QAPRTase), an enzyme of the pyridine nucleotide pathway, lose their growth-promotion ability and this may point to another set of unknown molecules involved in root growth stimulation [77].

4.58.4   Nitrogen Fixation

Even though 78% of the Earth's atmosphere is made up of nitrogen in the form of N_2, nitrogen is generally the most limiting soil nutrient for plant growth and cannot be converted into biologically accessible forms except by a few groups of prokaryotes, including eubacteria, cyanobacteria, and actinomycetes. These microbes (diazotropohs) use fixed carbon from plants to drive the

energy-intensive, oxygen-sensitive process of breaking the N_2 triple bond, creating ammonia (biological nitrogen fixation, BNF). Because of the requirement for fixed carbon, it is not surprising to find elevated numbers of diazotrophic bacteria in carbon-rich plant rhizospheres. In the rhizosphere, ammonia can be oxidized by soil microbes into nitrates and assimilated into amino acids, but it can also be leached or reduced back into nitrogen gas and lost. Thus, a closer physical interaction between plant and diazotroph would likely benefit the host by capturing more of the fixed nitrogen. Endophytic nitrogen fixation is a well-known phenomenon in plants that form nodules which are basically specialized microbial fermentation organs on roots or stems. These structures are the result of an infection and signaling process between endophyte and plant, which results in increased nitrogen to the plant by creating a low-oxygen/high-sugar environment to permit N fixation [11]. This association is found in four related orders within the Eurosid angiosperms, which includes thousands of plant species, and is believed to have evolved once (and been lost in many groups since then) about 59 mya during a time of elevated atmospheric CO_2 [8]. Interestingly, it has been shown that there are several genes shared between mycorrhizal and root-nodule associations, including receptors, suggesting this evolutionary event co-opted the mechanisms responsible for the ancestral mycorhizal–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 nodule 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 [78]. Next to graminaceous grasses like maize, legumes are the largest and most agronomically important group of 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 [79]. Soybeans are the world's most grown legume and can be nodulated by *Bradyrhizobium japonicum*, *B. elkanii*, *B. liaoningense*, *Mesorhizobium tianshanense*, *S. 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^{-1} [80]. Legume nitrogen fixation has been reviewed extensively [81], so the focus here is 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 mya between a filamentous, stomatal colonizing cyanobacteria and an early plant [82]. As it is believed that endophytes exist within every plant species, the potential for endophytic BNF may be underexploited; almost all cultivated crops except for legumes require large inputs of synthetic N. 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^{-1} to be taken up for each tonne of grain produced [83]. 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 is 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 of rice. Much of this related research has been focused on transferring nodule formation to cereals with very limited success; more promising, the nodulating bacteria can exist endophytically in nonlegume plants where they may fix nitrogen [84]. For example, *R. etli*, which normally forms nitrogen-fixing nodules on *P. vulgaris* (common bean), has been found to be an effective endophytic colonizer of maize plants [85], where it has been shown to increase the dry weight of some Mexican landraces possibly by nitrogen fixation [86]. Wheat has also been shown to benefit from inoculation with *R. leguminosarum*, and ^{15}N tracer techniques show that under low-nitrogen fertilization (50 kg N ha^{-1}), inoculated plants were able to fix 29% of the nitrogen they accumulated in shoots, whereas uninoculated plants fixed none [87]. In addition to rhizobia, it is interesting to realize that cereals have been found to contain many other diazotrophic endophytes as well: these include *Pantoea*, *Pseudomonas*, *Rhanella*, *Azospirillum*, *Brevundimonas*, *Klebsiella*, *Ideonella*, and *Herbaspirrilum* in maize [88]; and *Enterobacter*, *Brevundimonas*, *Pantoea*, *Pseudomonas*, *Herbaspirillum*, *Ideonella*, *Azospirillum*, and *Rheinheimera* in rice [89]. Indirect studies have estimated a contribution by endophytic bacteria to plant BNF as a percentage of total plant N, as being up to 33% in maize [90], up to 21% in some Asian rice varieties [91], and up to 49% in wheat inoculated with maize endophyte *Klebsiella pneumoniae* 342 [92]. These estimates show that endophtyic N fixation does occur in these cereals 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 nonradiolabeled portion as coming from N_2-derived BNF. Furthermore, studies that show high endophyte BNF are typically conducted in pots with considerable inoculum, whereas in the field endophyte BNF is typically considerably less [92]. Many experiments fail to use the best control to verify BNF, which is inoculating with nitrogen-fixation mutants (*nif*$^-$). However, in the *K. pneumoniae* 342 experiment above, *nif*-mutants failed to cause plant growth promotion, demonstrating that BNF can be an important process in cereals [92].

There are many other important non-nodule plant–endophyte relationships that result in high amounts of BNF and may provide agriculture with novel diazotrophs. Diazotrophic cyanobacterial endophytes are believed to have been much more common in ancient plants, with amazing fossil evidence suggesting that they colonized plants through stomata and coexisted with mycorhiza as early as 400 mya [82]. Among extant angiosperms, only the giant *Gunnera* (giant rhubarb, dinosaur food) still maintains cyanobacterial endophytes that colonize the plant through glands on its stem [93] and invade cells where they become intracellular symbionts that are oxygen resistant and N fixing, yielding up to 72 kg N ha^{-1} yr^{-1} as estimated for *G. Arenaria* [94].

More promising diaztrophs for agricultural applications are culturable nitrogen-fixing endosymbionts from angiosperms growing under 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. 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 [95]. Some types of bamboo are also able to grow under low-nitrogen conditions and may

depend on large populations of diazatrophic endophytes for nitrogen [96]. *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 [97]. Poplar trees are also colonists of disturbed and low-nutrient soils, so there is much interest in their ability to gain fixed nitrogen from diazatrophic endophtyes including *Burkholderia, Rahnella* sp., *Enterobacter* sp., *Acinetobacter, Herbaspirillum, Stenotrophomonas, Sphingomonas, and Pseudomonas* spp [98].

Among endophytic plant–diazotroph relationships, the kallar grass–*Azoarcus* spp. strain BH72 interaction and the sugarcane–*Gluconacetobacter diazotrophicus* interaction have attracted the most attention as well as full genome sequencing projects. Kallar grass (*Leptochloa fusca* L. Kunth) is a pioneer plant on salty, alkaline, low-nutrient soils in the Punjab of Pakistan [99]. *Azoarcus* spp. strain BH72 was isolated as the predominant nitrogen-fixing endophyte in kallar grass, estimated at 10^9 bacteria per gram of root dry weight; interestingly, these microbes are not found in surrounding soils suggesting that 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 these microbes exist in different grass hosts, but that the majority of the actively fixing population exists in a nonculturable state. This activity can be substantial and is estimated to account for up to 34 kg-N ha^{-1} yr^{-1}, equivalent to 50% of what is generally applied to traditional rice farms. However, the most effective example of non-nodule-based endophytic nitrogen fixation occurs in sugarcane, at a rate of 150 kg-N ha^{-1} yr^{-1} (or about 60% of its total N) as estimated by N^{15} isotope and N balance studies [100]. *Saccharum officinarum* is believed to have originated in New Guinea, but since 327 BC has spread throughout the worldwide tropics where it is often grown vegetatively in ratoon farming for production of sugar [101]. 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 in organic forms. A number of diazotrophic endophytes have been isolated from sugarcane, including *B. brasilensis, B. tropicalis, Herbaspirillum rubrisubalbicans, and G. diazotrophicus,* and these seem to work together to fix the greatest amount of nitrogen in micropropagated sugarcane [102]. *G. diazotrophicus* has been found in many different tropical plant species including sweet potato, coffee, pineapple, *Eleusine coracana grass*, tea, mango, and finger millet where it is assumed to also play an important role in host BNF [103]. *G. diazotrophicus* does not survive well in soil and grows optimally at conditions of 10% sucrose and at pH 5.5, which are similar to conditions inside cane stems. Its nitrogen-fixing activity is insensitive to nitrate and is poorly responsive to ammonium at 10% sucrose, suggesting that it continues to fix nitrogen in canes 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 yeast, suggesting that this fixed nitrogen is bioavailable to its plant host as well [104]. 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.

4.58.5 Other Endophytic Mechanisms Affecting Plant Nutrient Status

Some endophytes appear to possess the ability to manipulate host plant metabolism to increase nutrient uptake and change 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 [105]. 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 [106]. 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 [107]. How these mycorrhizae 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, where it promotes plant growth. In *Arabidopsis* and tobacco roots, *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) [108]. 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 [109]. 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 the shoot glutamine synthetase activity (by 32%). Similarly, tall fescue (*F. arundinacea* Schreb.) plants infected with the fungal endophyte *N. coenophialum* (Morgan-Jones and Gams) experience 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 [110]. 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 [111]. Opposite to the above strategies, reduced levels of nitrate and amino acids in plant tissues have been reported in *Lolium perenne* infected with *N. lolii* [112]; 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 [113]. A different elicitor was found in culture filtrates of growth-promoting microbe *B. thuringiensis* NEB17, which contain a novel bacteriocin protein called thurigen that enhances both soybean and corn biomass [114]. *P. fluorescens* B16 is a growth-promoting rhizobacteria that produces pyrroloquinoline quinine under low-nutrient conditions;

bacterial mutant studies using a cucumber-seedling bioassay showed this compound to be responsible for the observed plant growth promotion [115]. It has long been known that Nod factors secreted by rhizobia are important in nodule formation, but these compounds are also able to affect other changes in the plant such as increased uptake of calcium in soybean roots through unknown mechanisms [116]. Treatment of seeds of *Z. mays* (corn), *O. sativa* (rice), *Beta vulgaris* (sugarbeet), *G. max* (soybean), *P. vulgaris* (bean), and *Gossipium hirsutum* (cotton), with Nod factor BjV of *B. japonicum* 532C, resulted in enhanced seed germination and early growth under lab and field conditions that can allow the developing seedlings optimal access to nutrients in the rhizosphere [117]. 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 [118]. Although the function of this gene is unknown, given its homology to legume genes involved in nodule formation, it is interesting to speculate that nonlegume plants may have an ancient bacterial-dependent plant growth-promotion pathway.

Modification of the soil via exudates is an important way that roots may increase the availability of nutrients. 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 that may aid in optimizing their rhizospheres chemically and microbially [119]. An example of altered exudates affecting plant nutrition was seen by infecting tall fescue (*F. arundinacea* Shreb.) with *N. coenophialum* (Morgan-Jones and Gams) fungus, which was observed to stimulate uptake and transport of greater P, Ca, Zn, and Cu in roots grown in low-P nutrient solution [28]. This effect was specific to the plant DN2 genotype and appeared to be related to root growth reprogramming and an altered pattern of acid exudation by roots. *N. coenophialum* infection has been shown to increase fescue release of organic carbon from its roots, resulting in higher microbial activity and respiration stimulated by changes in the rhizodeposits [120]. 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 Fe^{3+} reduction, a necessary step in iron uptake (Fe^{2+}) by plants [110]. Under specific conditions, soil C and N pools can also be increased by endophyte infection of tall fescue, caused by either a reduction in soil microbial respiration [121] and/or a reduction in specific species of carbon-consuming rhizobacteria [122]. This may be caused by altered patterns of root exudation, or it may be caused by a buildup of endophyte-derived alkaloids in the soil [123]. 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 GAs [124].

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 modify soil directly. As mentioned previously, at least one species (http://www.nature.com/nature/journal/v413/n6853/full/413297a0.html) of AM seems to be able to enhance the degradation of organic N [16,125] but how it does this is not known. Some AMs are able to affect the behavior of other soil microbes: *G. mosseae* inoculation on diverse plant species resulted in metabolic stimulation of bacterial rhizosphere populations including various groups of uncultured bacteria and *Paenibacillus* species, likely through altered exudates patterns into soil [126]. 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 [124]. A different study on the effect of mycelial exudates from the AM fungus *Glomus* spp. MUCL 43205 showed that 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 [127]. Likewise, the nearly ubiquitous root-colonizing fungi known as DSEs have been shown to produce hyphae that exit the plant root and absorb organically bound mineral nutrients. These fungi have been shown to secrete 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 nutrient absorption [22].

As a final mechanism, some soil rhizosphere bacteria and fungi that can exist as endophytes within roots are able to mineralize organic or insoluble forms of N and P. As it has rarely been shown that endophytes exit the root to directly affect the soil, it is not clear how such apparent phosphate solubilization or organic compound degradation could occur. 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, although it was not shown whether the endophytes traveled to plant surfaces or soil where phosphate solubilization would be important [128]. In lettuce and maize, seed inoculation with phosphate-solubilizing strains of *R. leguminosarum bv. phaseoli* was shown to increase their P content by 6% and 8%, respectively, under field conditions, although it was not demonstrated that these normally nodule-inhabiting bacteria establish an endophytic lifestyle within these plants [129]. There are a few examples where validated endophytes have been shown to exit the plant to improve nutrient bioavailability in the soil. For example, 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 [130]. Similarly, 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 [95]. The cardon cactus-associated bacteria can either solubilize inorganic phosphates by releasing organic acids, such as gluconic acid and 2-ketogluconic acid, or mineralize organic phosphates by secreting extracellular phosphatases [131].

4.58.6   Application of Endophytes to Agriculture

Applications of endophytes to agriculture have traditionally taken the form of soil or seed inoculation. Externally inhabiting endophytes have the ability to gain entry to their hosts, from the specialized signaling rhizobia and mycorrhiza use to form symbiosomes, to more basic crack entry such as that observed by *K. pneumonii* 342 at lateral root junctions [132]. To be effective, inoculations should be properly planned and steps should first be taken to assess whether there are beneficial endophytes already

present in the soil, how the microbes of interest might persist and compete, and whether these are able to promote improved mineral nutrition of the plant of interest under the expected growing conditions. Early attempts at soil inoculation followed this principle, 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 cultures of rhizobia on legumes, and resulted in the first commercial microbial inoculant called Nitragin™ in the United States [133]. Countries such as the United States and Brazil have benefited immensely from BNF through inoculation with efficient strains of rhizobia for soybean production; these nations are the first and second largest soybean producers in the world, with high yields in 2007 (both ~2800 kg ha^{-1}) (FAOSTAT) that remain relatively independent of nitrogen fertilization, thanks to stable, efficient nodule-forming populations of *B. 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 reuptakes hydrogen gas (Hup+), a waste product of nitrogen fixation [80]. In 2007, average soybeans yields in China (1450 kg ha^{-1}, FAOSTAT) were only 50% of that in Brazil and USA despite being the center of origin for soybeans. The poor production of soybeans in China may be caused by poor BNF, with 22% of nodules in some areas containing ineffective nodules [80]. Many Chinese soils have high-native rhizobial microbe counts (in the range of 10^4 bacteria/g of soil) and would not be expected to allow introduced inoculants to take hold. Nevertheless, experiments using *B. japonicum* strains as soil inoculants in different parts of China show it is possible to displace native rhizobia in soybean nodules and produce yield increases of 6–33% [80]. Natural inoculation of nonhost plants by native rhizobia may also play important roles in agriculture, as rice grown in rotation with Egyptian clover is naturally colonized by nodule forming *R. trifolii*, reducing N application to one-third of the recommended dose without decreasing grain yields. Rhizobial inoculants for legumes are a well established and widely sold agricultural product, and formulations such as Tagteam™ are using the known nitrogen-fixation potential of rhizobium and combining it with the effects of accessory microbes, like 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, mixing *B. thuringiensis* KR1 (isolated from nodules on kudzu vines) with *B. japonicum* SB1 yielded significantly greater nodule number and plant biomass, than inoculation with the rhizobium alone [134]. A different approach used by EMD Crop Bioscience has been to include Nod factors in their Optimize 400™ seed inoculant, mixing live *B. Japonicum* endophytes with their patented formulation of lipo-chitooligosaccharide, which stimulates early seed germination and enhances root branching and development.

AM fungi have obviously played an important role in plant nutrition over evolutionary time, but several factors limit their usefulness in production agriculture: different plant cultivars react differently to AM colonization; 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 [17]. Natural populations of AM can be found in almost any soil, and have likely evolved to occupy specific habitats over millions of years with some taxa being restricted by factors such as pH and temperature, while others appear to have cosmopolitan distribution and physiological flexibility. AMs form hyphal networks that can function to distribute nutrients and carbon between plants, buffering plant communities against nutrient stresses that affect individual plants; how this might operate and be exploited in agricultural systems is not well understood. Soils that have had their native AM disrupted by tillage, chemical application, extended fallow periods, or planting with fungicide-exuding plants (such as *Brassicaceae*) will likely have low levels of AM available to colonize crop plants. As AMs are obligate biotrophs, production of inoculum is not possible without plant material, complicating its mass production. AM inocula can be as simple as a 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 mycorhizal inoculants available such as BuRIZE™ (containing *G. intraradices*) made by BioScientific, or Bio-organic's Endomycorrhizal Inoculant™ containing a blend of *G. aggregatum*, *G. clarum*, *G. deserticola*, *G. intraradices*, *G. monosporus*, *G. mosseae*, *G. Margarita*, and *Paraglomus brasilianum*. Mycorrhizal Applications sells a range of mycorrhizal inoculants in powder, granular, liquid, gel, or tablet forms, and in blends of AM plus ectomycorrhiza, PGPR, and trichoderma. The effect of combining the enhanced nutrient absorption capabilities of AM with root growth promotion can result in synergistic NUE, although endophyte/host/environmental interactions seem to greatly affect the outcome of the relationship [135]. There are studies showing co-inoculation of AM and rhizobia can result in higher root biomass, N/P/K uptake, higher nodule formation, and greater N fixation in legumes, than inoculation with either microbe individually [136], but there do not appear to be commercial blends of these microbes yet available.

Clavicipitaceous endophytes, especially *Epichloë* and *Neotyphodium* species, are able to promote the growth and stress resistance of grasses under biotic and abiotic stresses, including low soil nutrients [137]. They intercellularly colonize shoot portions of plants where they are often seen to help in protecting their host from predation through the production of toxic secondary metabolites, as well as aiding in abiotic stress resistance through multiple mechanisms. The fact that they are naturally transmitted to seeds has provided a convenient way for distribution of endophyte-infected plants. A wide range of commercially important grass species 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 [138]. Seeds of this grass were found to contain ergot-alkaloid producing *N. coenophialum* endophytes, which are also responsible for much of the stress resistance in this vigorous grass. For forage applications, this variety has been replaced by Jesup tall fescue infected by non-ergot-alkaloid producing MaxQ™ endophytes (strains AR502 and AR542 of *N. coenophialum*), which maintains endophyte-conferred growth promotion, without the problem of toxicity to grazing livestock [139]. As many grasses are used for turf grass and not forage, there are still many

seeds sold containing ergot-alkaloid endophytes. This includes grasses such as Turf-Type Perennial Ryegrass, Chewings, Creeping Red, Hard, and other fine fescue species. Although some of these species of fungal endophytes are known to occur in other agriculturally important grasses such as rice, wheat, and corn, there has been little effort to develop these for improving NUE.

Azospirillum spp. are rhizosphere inhabitants that are also sometimes found as endophytes in plant roots. Azospirilla are capable of nitrogen fixation and secretion of hormones which stimulate root development, improving nutrient uptake in a wide variety of species [140]. *Azospirillum* inoculants are recommended for maize, wheat, barley, sorghum, pear millet, and forage crops, where their average benefit from soil inoculation is equivalent to 15–20 kg ha^{-1} of applied N, improving grain yield of cereals by 5–20%, millets by 30%, and forage crops by over 50% [141]. Examples of commercially available *Azospirillum* inoculants include Biopromoter™, sold by Manidharma Biotech, Nitrofix™ sold by Ruchi Biochemicals, and Bio N™ sold by FNCA in the Phillippines. *Azospirillum* can also be found blended with other microbes such as phosphate solubilizing rhizosphere microbes: one example is EMAS™ sold by FNCA in Indonesia for inoculation on tea, rubber, cocoa, oil palm, sugarcane, rice, maize, and potato.

A few other examples of endophyte inoculants exist in tropical countries where biofertilizers are viewed as economically attractive alternatives or supplements to chemical fertilizers [142]. Endophytic strains of *P. fluorescens* are sold in India, which promote root growth, while FOSFORINA™ is a strain of *P. fluorescens* distributed in Cuba to reduce the need for mineral phosphorus applications by solubilizing phosphate in plant 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 [143]. 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 43 kg-N ha^{-1} while increasing rice yields [143]. 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 [144]. Although there is much lab, greenhouse, and field evidence that other endophytes can increase the NUE of commercially important crops such as rice, corn, sugarcane, and wheat, commercial products and inoculation practices have not been developed for a large number of endophytes.

To continue the development of endophytes for agricultural application, discovery of novel strains will be a fruitful endeavor, which may result in new species that can be directly used in agriculture, or at least provide us with important new and additional information about mechanisms that endophytes can use to improve plant NUE. If every plant harbors at least one novel endophyte, then there are still about 300 000 undiscovered endophytes in the world! Wild plants may grow in nutrient-poor environments enriched in endophytes that help their hosts to acquire nutrients or resist stresses not found in domesticated crops growing under optimized conditions. For example, plants have been shown to enrich their endophytic communities for hydrocarbon metabolizers when grown in petroleum-contaminated soil [145]. Fungal endophytes conferring specific stress resistance have also been shown to infect hosts in an environmentally determined manner for salt, drought, and disease resistance [146], suggesting that habitat-adapted symbiosis may be a trait which can be selected for. Work with AM infecting genetically diverse wheat has shown that host genetics can affect the benefits conferred by the association [147]. Furthermore, the expression of certain plant ethylene-regulatory genes has been shown to correlate negatively to the success of diazotrophic endophytic associations in sugar cane [148]. Wild crop relatives may have genetic makeups that sponsor residence of a different endophytic population and may be important for plant-breeding programs. Rare or endemic species of plants may also contain novel strains of endophytes that may be transferred to crop plants for agronomic benefit. Our lab has been developing endophyte inoculants for maize by screening a genetically diverse panel of *Zea* (maize) seed for novel endophytes with an ability to promote plant growth (**Figure 1**). Of 124 seed endophyte bacterial isolates, many had beneficial activities such as phosphate solubilization (**Figure 1** (**a**)), ACC deaminase activity (**Figure 1** (**b**)), and 4 were able to significantly promote potato root growth in a gnotobiotic assay (**Figure 1** (**c**)). Further tests should show whether these effects can be reproduced in maize and other plant species under field conditions.

One important future direction of endophyte research 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 [149], sugarcane endophytes *G. diazotrophicus* Pal5 [150] and *H.seropedicae* Z67, corn endophyte *K. pneumonii* 342 [151], rice endophyte *Azospirillum* sp. B510 [152], tall fescue endophytes *Epichloe festucae*,
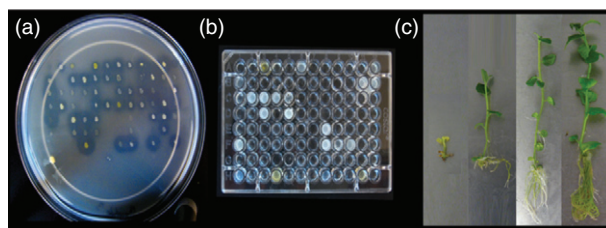

Figure 1   Assays performed on maize seed endophytes. (a) Phosphate solubilization was checked by plating isolates on tricalcium phosphate media and looking for clear halo production. (b) Ability to metabolize ACC was determined by growing isolates in nitrogen-free LGI, supplemented with 50-mM ACC as 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* (D Johnston-Monje and M Raizada, unpublished).

N. coenophialum and *N. lolii*, onion endophyte *B. phytofirmans* PsJN, AM *G. intraradices* [153], and several rhizobial and Frankia nodule-forming bacteria. Several poplar endophytes have also been sequenced including *Enterobacter* sp. strain 638, *P. putida* W619, *S. proteamaculans* 568, and *Stenotrophomonas maltophilia* R551-3.; the genome sequences suggest that these microbes possess the growth-promoting mechanisms of acetoin production, IAA synthesis, and gamma-aminobutyric acid (GABA) metabolism, but no functional ACC deaminase [36]. As some endophytes live within plants in unculturable states, it may be important to undertake metagenomic approaches to acquire genomic information, and that is being done for rice in an ambitious project to understand the entire community of endophytes present by sequencing of 100 Mb of DNA extracted from inside rice plants. Isolation of the 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 *Azospirrilum brasilensis* increased the root-elongation potential of this strain in tomato and canola [154], suggesting that similar transgenic techniques may increase the root growth-promoting ability of endophytic strains. This has been shown to be an effective technique in nodule-forming rhizobia: ACC deaminase genes from *Sinorhizobium* sp. BL3 were introduced into *Rhizobium* sp. strain TAL1145, increasing its ACC deaminase activity, resulting in nodules with greater number and size, and producing higher root mass on the tree legume *Leucaena leucocephala* [155]. Root stimulation by transgenic auxin production in endophytes may also enhance root development: the entire tryptophan monooxygenase pathway was introduced into *P. fluorescncs* strain CHA0 elevating synthesis of IAA, and stimulating an increase in root fresh weight of cucumber by 17–36% in natural soil [156]. Constitutive expression of the nitrogen-fixing transcriptional regulator *nifA* was shown to significantly increase N fixation by corn endophyte *E. gergoviae* 57-7 *in plauta* and may be a useful trait to introduce into other diazotrophic endophytes [157]. Another strategy to increase endophytic N fixation has been to add an additional copy of the *nifHDK* operon under a stronger *nifH* promoter, allowing *R. etli* to have increased nitrogenase activity up to 58%, and which increased *P. vulgaris* weight by 38%, increased plant nitrogen content by 15%, and increased seed yield by 36% [158]. 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 [159], while *N. tabacum* plants expressing a phytase gene from the soil fungus *A. niger* accumulated up to 52% more P than controls when grown in soils amended with either phytate or phosphate and lime [160]. Many other transferable, genetic mechanisms to improve plant NUE by endophytes must exist in the countless undiscovered or understudied endophytes and will hopefully lead to genetically enhanced inoculants in the future. Despite much investment and some promising experiments, only one genetically modified endophyte has thus far been commercially released: strain RMBPC-2 of *S. meliloti*, sold by the American company, Research Seeds Inc., has been modified with genes to enhance C4-dicarboxylic acid uptake and nitrogen fixation in symbiosis with alfalfa.

4.58.7 Conclusions

Industrial fertilizer use has permitted the large increase in global agricultural production but this is not a sustainable solution to meet future food demands. Endophytes naturally occur in plant species and have significant potential to improve NUE. Endophytic associations that benefited NUE likely evolved as early as 400 mya in the form of mycorrhiza that assisted the first rootless land plants to absorb nutrients. The plant genetic machinery required for this association has likely persisted in most lineages of modern land plants. Interestingly, nodule-forming plants co-opted some of these genes to enable rhizobial symbioses. There are many species of endophytic fungi and bacteria where the underlying genetics of the association are not understood. An endophyte can colonize multiple genera yet exhibits genotype host specificity within a plant species. Although many endophytes gain access to plants from the environment (e.g., soils), intergenerational 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 that include formation of extra-root hyphae for nutrient absorption; stimulation of root growth by manipulating levels of phytohormones (e.g., auxin and ethylene) and other metabolites (e.g., acetoin); alteration of plant metabolism to stimulate higher nitrogen and phosphate uptake; nitrogen fixation by both nodulating and non-nodulating endophytes; and modification of soil directly by endophytes or indirectly by changing root exudate profiles. 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 limits the utility of endophytes in agriculture. Nevertheless, many endophyte-based inoculants are popular commercially, primarily in developing nations, including AM, rhizobia and *Azospirillium*; Clavicipitaceous fungi are available as infected seeds. Endophytic metabolites are also commercially available to enhance NUE. Several challenges have prevented wider adoption of endophyte inoculants, including competition from endogenous microbes, host genotype specificity, establishment, and persistence. Though many species of endophyes exist, few have been explored commercially. Endophytes represent a significant, largely untapped genetic resource, to aid future efforts in plant biotechnology; recent whole genome-sequencing efforts may accelerate progress in this area.

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http://www.emdcropbioscience.com/LCOpromoter/index.html – EMD's LCO Promoter Technology in action
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http://www.omafra.gov.on.ca/english/crops/facts/endophyt.htm#role – Debunking endophytes (in turfgrass)