Bacterial endophytes: ecological and practical implications*

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It has long been known that tissues of healthy plants can be colonized internally by microorganisms. The term "endophyte" is commonly used to describe such microorganisms. The best-characterized microbial endophytes are nonpathogenic fungi, for which much compelling evidence of plant/microbe mutualism has been provided. Some endophytic fungi are thought to produce compounds that render plant tissues less attractive to herbivores, while other strains may increase host plant drought resistance. In return, fungal endophytes are thought benefit from the comparatively nutrient rich, buffered environment inside plants. However, endophytic fungi comprise only part of the nonpathogenic microflora found naturally inside plant tissues. Bacterial populations exceeding 10^7 colony forming units (cfu) g^{-1} plant matter have been reported within tissues of various plant species. Notwithstanding their discovery more than four decades ago, much less is known about bacterial endophytes compared to their fungal counterparts. Work with plant species of agricultural and horticultural importance indicates that some endophytic bacterial strains stimulate host plant growth by acting as biocontrol agents, either through direct antagonism of microbial pathogens or by inducing systemic resistance to disease-causing organisms. Other endophytic bacterial strains may protect crops from plant parasitic nematodes and insects. In Brazil, the nitrogen-fixing bacterial endophytes of sugarcane (*Saccharum officinarum* L.), *Acetobacter diazotrophicus* and *Herbaspirillum* spp., colonize internal root, stem and leaf tissues, and are thought to provide up to 80% of the host plant's nitrogen requirement. Other endophytic bacteria stimulate plant growth through mechanisms yet to be elucidated.

In contrast to agricultural crop species, almost nothing is known about bacterial endophytes of trees. There have been occasional reports of endophytic bacteria in asymptomatic angiosperm and gymnosperm species, but little is known about their influence on plant growth. We have found that lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) and white x Engelmann hybrid spruce *(Picea glauca* x *engelmannii)* support bacterial endophyte populations naturally, and that such endophytes colonize internal root and stem tissues with up to 10^5 cfu g^{-1} plant tissue. Furthermore, some of these strains have been found to promote gymnosperm seedling growth. While the precise mechanism by which these bacterial endophytes enhance tree seedling growth is not completely understood, initial re-

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sults suggest that biocontrol of indigenous soil microorganisms that inhibit plant growth is at least partly involved. In addition, an endophytic *Bacillus* strain (Pw2), which was originally isolated from inside surface-sterilized pine root tissues, possesses nitrogenase activity and can colonize pine seedlings systemically after soil inoculation. These observations lead to the intriguing possibility that lodgepole pine harbors an endophytic nitrogen-fixing bacterial population similar to that of sugarcane, which would explain its ability to grow, and even thrive, under nitrogen deficient conditions in the absence of significant rhizospheric nitrogen fixation. Bacterial endophytes may also be important in forest ecosystems by effectively increasing phenotypic plasticity of their long-lived tree hosts under variable or deleterious environmental conditions (*e.g.*, during periods of drought, nutrient deprivation, or pathogen attack). Regardless of the mechanism(s) involved, bacterial endophytes appear to represent another type of mutualistic plant x microorganism symbiosis that warrants further study. In addition to the intriguing ecological questions regarding the diversity, evolution and effects on plant population biology of bacterial endophytes, it may be fruitful to investigate their possible practical applications in agriculture and forestry.

Keywords: endophytes, bacteria, physiology, ecology.

It has long been known that tissues of healthy plants can be colonized internally by microorganisms (Petrini, 1986; 1991; Hallmann & al., 1997). The term "endophyte" is commonly used to describe such microorganisms *i.e.*, bacteria and fungi that live inside plant tissues without causing disease (Wilson, 1995), notwithstanding earlier confusion regarding its precise definition (Wennstrom, 1994; Chanway, 1996).

The best-characterized microbial endophytes are nonpathogenic fungi, for which much compelling evidence of plant/microbe mutualism has been provided (Carroll, 1988; Clay, 1988). Some endophytic fungi are thought to produce compounds that render plant tissues less attractive to herbivores, while other strains may increase host plant drought resistance. In return, fungal endophytes are thought to benefit from the comparatively nutrient rich, buffered environment inside plants. However, endophytic fungi comprise only part of the nonpathogenic microflora found naturally inside plant tissues.

Hollis (1951) detected bacteria inside healthy potato tissues nearly fifty years ago, but considerably less is known about bacterial endophytes compared to their fungal counterparts. From studies of plant species of agricultural and horticultural importance, we know that a wide range of bacterial genera can be isolated from within tissues of healthy plants [Tab. 1; see Baldani & al., (1997); James & Olivares, (1997); and Kirchhof & al., (1997) for additional examples of plant species known to contain endophytic bacteria]. Bacteria have also been isolated from within fruits and seeds of many cereal, vegetable and woody plant species (Samish & al., 1961; 1963; Mundt & Hinkle, 1976). Internal bacterial populations as large as 10^7 colony forming units (cfu) g^{-1} of plant matter (wet weight) have been reported for some plant species (Sturz & al., 1997), but population

sizes between 10^2 and 10^6 cfu g^{-1} (wet weight) of root, stem or leaf tissue are more commonly observed (Hallmann & al., 1997).

Tab. 1. Examples of plant species and internal tissues from which nonpathogenic bacteria have been isolated[1]

Plant species and tissue	Bacterial genera	References
Alfalfa (*Medicago sativa* L.) root	*Erwinia*-like, *Pseudomonas*	Gagné & al., 1987
Coffee (*Coffea arabica* L.) root and stem	*Acetobacter*	Jimenez-Salgado & al., 1997
Cameroon grass (*Pennisetum purpureum* Schumach)	*Acetobacter*	Reis & al., 1994
Corn (*Zea mays* L.) root and stem	*Bacillus, Burkholderia, Corynebacterium, Enterobacter, Klebsiella, Pseudomonas*	Lalande & al., 1989; Fisher & al., 1992; McInroy & Kloepper, 1995; Palus & al., 1996
Cotton (*Gossypium hirsutum* L.) root and stem	*Agrobacterium, Bacillus, Burkholderia, Clavibacter Erwinia, Serratia, Xanthomonas*	Misaghi & Donndelinger, 1990; McInroy & Kloepper, 1995
Cucumber (*Cucumis sativis* L.), root	*Agrobacterium, Arthrobacter, Bacillus, Burkholderia, Chryseobacterium, Enterobacter, Pseudomonas, Stenotrophomonas*	McInroy & Kloepper, 1995
Grapevine (*Vitis* spp.)	*Bacillus, Clavibacter, Comamonas, Curtobacterium, Enterobacter, Klebsiella, Moraxella, Pantoea, Pseudomonas, Rahnella, Rhodococcus, Staphylococcus, Xanthomonas*	Bell & al., 1995a; 1995b
Hybrid spruce (*Picea glauca* × *Engelmannii*) root	*Bacillus, Pseudomonas, Phyllobacterium, actinomycetes, Staphylococcus*	O'Neill & al., 1992; Chanway & al., 1994
Kallar grass (*Leptochloa fusca* [L.] Kunth) root	*Azoarcus*	Reinhold & al., 1986; Reinhold-Hurek & al., 1993
Lodgepole pine (*Pinus contorta* Dougl. Ex Loud) root	*Bacillus*	Shishido & al., 1995
Potato (*Solanum tuberosum* L.) tuber	*Acidovorax, Acinetobacter, Actinomyces, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Capnocytophaga, Cellulomonas, Clavibacter, Comamonas, Corynebacterium, Curtobacterium, Deleya, Enterobacter, Erwinia, Flavobacterium, Kingella, Klebsiella, Leuconostoc, Micrococcus, Pantoea, Pasteurella, Photobacterium, Pseudomonas, Psychrobacter, Serratia, Shewanella, Sphingomonas, Vibrio, Xanthomonas*	Hollis, 1951; de Boer & Copeman 1974; Sturz, 1995; Sturz & Matheson, 1996; Sturz & al., 1998

Plant species and tissue	Bacterial genera	References
Red clover (*Trifolium pratense* L.) leaves, stem and root	*Acidovorax, Agrobacterium,*	Sturz & al., 1997
	Arthrobacter, Bacillus, Bordetella, Cellulomonas, Comamonas, Curtobacterium, Deleya, Enterobacter, Escherichia, Klebsiella, Methylobacterium, Micrococcus, Pantoea, Pasteurella, Phyllobacterium, Pseudomonas, Psychrobacter, Rhizobium, Serratia, Sphingomonas, Variovorax, Xanthomonas	Sturz & al., 1998
Rice (*Oryza sativa* L.) root and stem	*Agrobacterium, Azorhizobium, Azospirillum, Bacillus, Pseudomonas, Rhizobium*	Reddy & al., 1997; Stoltzfus & al., 1997; Yanni & al., 1997
Rough lemon (*Citrus jambhiri* Lush.) root	*Achromobacter, Alcaligenes Moraxella, Acinetobacter, Actinomyces, Arthrobacter, Bacillus, Citrobacter, Corynebacterium, Enterobacter, Flavobacterium, Klebsiella, Providencia, Pseudomonas, Serratia, Vibrio, Yersinia, Rickettsia*-like	Feldman & al., 1977; Gardner & al., 1982
Sorghum bicolor L. Moench shoot	*Herbaspirillum*	James & al., 1997
Sugar beet (*Beta vulgaris* L.) root	*Bacillus, Corynebacterium, Erwinia, Lactobacillus; Pseudomonas, Xanthomonas*	Jacobs & al., 1985
Sugar cane (*Saccharum officinarum* L.) root and stem	*Acetobacter, Herbaspirillum*	Cavalcante & Döbereiner 1988; Gillis & al., 1989; Boddey & al., 1991; Dong & al., 1994; Olivares & al., 1997
Teosinte (*Zea luxurians* Itins and Doebley) stem	*Klebsiella*	Palus & al., 1996

[1] Adapted from Tab. 1 in Hallmann et al. (1997)

Furthermore, some bacterial endophytes have been shown capable of stimulating host plant growth (Chanway, 1997; Hallmann & al., 1997). The primary mechanisms by which bacterial endophytes are thought to enhance plant growth are nitrogen fixation (Boddey & Döbereiner, 1995), and biocontrol of disease-causing or yield-reducing microorganisms, either through direct antagonism of pathogens or by inducing systemic resistance to such organisms (Hallmann & al., 1997). However, plant growth stimulating endophytic bacteria may exert positive effects on plant performance in other ways, possibly by producing phytohormones or causing enhanced nutrient and

water uptake (*e.g., Azoarcus*) (Triplett, 1996; Hallmann & al., 1997, Lazarovits & Nowak, 1997).

In contrast to agricultural and horticultural crop species, almost nothing is known about bacterial endophytes of trees. There have been occasional reports of endophytic bacteria in asymptomatic angiosperm and gymnosperm species, but little is known about their diversity and influence on plant growth (Chanway, 1997). Results from my laboratory indicate that lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) and white × Engelmann hybrid spruce (*Picea glauca* × *engelmannii*) support bacterial endophyte populations naturally, and that such endophytes colonize internal root and stem tissues with up to 10^5 cfu g^{-1} of plant tissue. We have not yet examined gymnosperm leaf tissues in detail for the presence of these microorganisms, but it is likely that they will be found in leaves as well.

It is of great interest that some of these strains promote gymnosperm seedling growth (Chanway & al., 1994). The precise mechanism by which these bacterial endophytes enhance seedling growth has not yet been elucidated, but initial results suggest that biocontrol of indigenous soil microorganisms that inhibit plant growth, is at least partly involved. In addition, an endophytic *Bacillus* strain (Pw2) (Shishido & al., 1995), originally isolated from inside surface-sterilized pine root tissue, possesses nitrogenase activity and can colonize pine seedlings systemically after soil inoculation (C. Chanway, unpubl. data).

The ecology of bacterial endophytes in agricultural crops as well as methodology for studying such microorganisms have been comprehensively reviewed by Hallmann & al. (1997). In this paper, I would like to complement the information provided by Hallmann & al. (1997) by reviewing mechanisms of plant growth enhancement by endophytic bacteria, emphasizing research on diazotrophic bacterial endophytes, and conclude by highlighting recent developments in research of bacterial endophytes of tree species, a topic on which little has been written.

Mechanisms of plant growth promotion by bacterial endophytes

(i) N_2 Fixation by diazotrophic endophytes

In some parts of Brazil, sugar cane (*Saccharum officinarum* L.) has been grown continuously for over 100 years without addition of nitrogen fertilizers (Neyra & Döbereiner, 1977). ^{15}N-based evaluation of nitrogen balance in pot experiments indicated that certain sugar cane varieties derived 50%–80% of plant N from biological nitrogen fixation (BNF), equivalent to 150-170 kg N ha^{-1} y^{-1} (Lima & al.,

1987; Boddey & al., 1991; Urquiaga & al., 1992; Boddey & Döbereiner, 1995). In contrast, no BNF can be detected in sugar cane cultivars that are routinely fertilized with mineral N (Triplett, 1996).

Rhizospheric BNF is known to occur in many plant species (Vose & Ruschel, 1981), and diazotrophic *Beijerinckia* have been found in the sugar cane rhizosphere (Baldani & al., 1997). However, the amount of BNF that results from rhizospheric associations is generally an order of magnitude less than the estimates for Brazilian sugar cane (Postgate, 1982). In addition, no one has been able to demonstrate that the rhizosphere diazotroph, *Beijerinckia*, is of any significance in the N nutrition of sugar cane (Baldani & al., 1997). These observations led Cavalcante & Döbereiner (1988) to look for the causative agent(s) of BNF in sugar cane in a less conventional habitat, *within* surface sterilized plant tissues. Their work resulted in the discovery of a new, gram negative, microaerobic, acid-producing N_2-fixing bacterium inside sugar cane root and stem tissues, later named *Acetobacter diazotrophicus* (Gillis & al., 1989).

Since the discovery of *A. diazotrophicus* in Brazilian sugar cane in the late 1980's, endophytic diazotrophs have been reported in several other plant species (Tab. 1), most notably rice (*Agrobacterium, Azospirillum, Bacillus, Pseudomonas* and *Rhizobium*) and Kallar grass *(Azoarcus)*. Diazotrophic endophytes participating in associations with the latter two plant species have been examined in some detail, and readers are referred to recent review articles by Triplett (1996); Baldani & al. (1997); Barraquio & al. (1997); James & Olivares (1997); Reinhold-Hurek & Hurek (1997) and Stolzfus & al. (1997) for further information. I will focus on the *A. diazotrophicus* × sugar cane interaction in this article because the endophyte appears to be uniquely well-adapted to fix N_2 while inside the host plant, and much information has been published on this plant/microbe association.

A. diazotrophicus is unique physiologically in that it typically can tolerate high sucrose concentrations (10–30%) (Cavalcante & Döbereiner, 1988; Stephan & al., 1991), though it does not transport or metabolize sucrose *per se* (Alvarez & Martinez-Drets, 1995). It is thought to rely on an extracellular saccharolytic enzyme to provide monosaccharides (*i.e.*, glucose and fructose) for growth (Alvarez & Martinez-Drets, 1995). The bacterium is acid-producing (2-ketogluconic acid and 2,5-diketogluconic acid from glucose) with a pH optimum for growth of 5.5 (Stephan & al., 1991). It can grow and fix N_2 at pH's as low as 2.5 and has an upper pH limit for growth *in vitro* of 7.5 (Gillis & al., 1989; Stephan & al., 1991). *A. diazotrophicus* is also quite tolerant of O_2, even when fixing N_2, with an optimal dissolved O_2 concentration of 0.2 kPa when growing on 10% sucrose, and de-

tectable nitrogenase activity occurs in the presence of 4.0 kPa O_2 (Boddey & Döbereiner, 1995).

Perhaps the most exciting characteristic of this diazotroph is its apparent insensitivity to mineral N while fixing N_2: *A. diazotrophicus* possesses no nitrate reductase so its nitrogenase is active even in the presence of 80 mM NO_3^- (Li & MacRae, 1991). Furthermore, nitrogenase activity is only partially inhibited by NH_4^+ and amino acids, especially when bacteria grow in the presence of high sucrose levels (*i.e.,* 10%) (Boddey & al., 1991). In addition, using an amylolytic yeast, Cojho & al. (1993) demonstrated, at least in principle, that *A. diazotrophicus* is capable of excreting some of its newly fixed nitrogen into its surrounding medium for uptake by other organisms. These characteristics led Boddey & al. (1991) to conclude that *A. diazotrophicus* is very well-adapted to complement plant assimilation of mineral N with N_2 fixation. From the evidence available, this conclusion seems warranted.

Transimission of *A. diazotrophicus* in sugar cane is thought to occur primarily through vegetative propagation of setts (James & Olivares, 1997). Indeed, the microorganism does not persist in soil, even between rows of sugar cane plants or in association with weeds in sugar cane fields (Baldani & al., 1997; James & Olivares, 1997). These observations are consistent with Baldani & al. 's (1997) classification of *A. diazotrophicus* as an obligate endophyte, incapable of persisting in the absence of its plant host.

While unable to persist on its own in the soil, this microorganism can maintain detectable populations in the sugar cane rhizosphere and especially in sugar cane trash (Li & MacRae, 1992; Reis & al., 1994). Because sugar cane setts are not invariably infected with *A. diazotrophicus,* sugar cane rhizosphere- and trash-associated populations may also play an important role as an inoculum source in transmission of the endophyte. This is underscored by the work of James & al. (1994), who demonstrated that plant infection by *A. diazotrophicus* can occur through young root tips, where vascular tissue is not fully differentiated, as well as at points of lateral root emergence. Bellone & al. (1997) provided interesting evidence that *A. diazotrophicus* may actually enter roots via infection threads in root hairs, similar to the infection pathway of *Rhizobium* in legumes. James & al. (1994) also observed structures in root epidermal cells that resembled infection threads, but confirmation of their existence will require a more detailed study performed under gnotobiotic conditions (James & Olivares, 1997). The issues of specific pathways of infection and the possible development of infection threads still need to be resolved, but there is little doubt that that infection of plant hosts by soil-associated *A. diazotrophicus* may also occur via root systems.

Paula & al. (1991; 1992) provided evidence that other plant-associated organisms such as mycorrhizal fungi may act as vectors for transmission of *A. diazotrophicus* to sugar cane. Inoculation of sweet potato (*Ipomoea batatas* L. Lam.) with spores of the mycorrhizal fungus, *Glomus clarum* containing *A. diazotrophicus* and other bacteria, resulted in infection of the host plant by the bacterial endophyte (Paula & al., 1991). In fact, colonization of stem tissue was greater as a result of the spore-containing bacteria treatment compared to co-inoculation with *Glomus* spores and pure cultures of *A. diazotrophicus*. Plant to plant transmission of *A. diazotrophicus* may also occur via phloem feeding insects such as the pink sugar cane mealy bug (*Saccharococcus sacchari*), which has been shown to contain *Acetobacter* after feeding on sugar cane (Ashbolt & Inkerman, 1990). Not surprisingly, *Acetobacter* within the mealy bug body were not actively fixing N_2. These results indicate that *A. diazotrophicus* is not only endophytic, but that it is also adapted to survive in plant-associated fungi and insects.

There is considerable debate regarding the specific microsites that *A. diazotrophicus* colonizes once inside plant tissues (James & Olivares, 1997). James & al. (1994) observed the endophyte within enlarged, intact epidermal cells up to 15 days after inoculation. Thereafter, it was observed in xylem vessels at the base of the stem, leading these authors to hypothesize that the microorganism spreads systemically to stem and leaf tissues *via* the transpiration stream. However, Dong & al. (1994) observed *A. diazotrophicus* intercellular colonization in sugar cane stem parenchyma, but later argued against its ability to colonize the xylem apoplast on the basis that (i) there is little or no carbon substrate in the stem apoplast, (ii) the endophyte stimulated plant defence mechanisms once in the xylem, and (iii) its movement within xylem lumens would be severely restricted due to limited vessel continuity (Dong & al., 1997).

Thus, there is general agreement that *A. diazotrophicus* colonizes sugar cane systemically, but exactly how this is accomplished is not clear. Part of the problem in determining the pathway(s) of translocation and specific microsites colonized within plants may result from the evaluation of *A. diazotrophicus* in different plant cultivars. For example, Dong & al. (1997) studied a bacterial wilt resistant sugar cane clone (Ja 60-5), with limited xylem continuity, but the degree of wilt resistance associated with the cultivars (NA 56-79 and SP 70-1143) studied by James & al. (1994) was not reported. Because continuity of xylem vessels characterizes sugar cane clones that are susceptible to bacterial wilt (Teakle & al., 1978), there is likely significant variability in disease resistance and hence, in the morphology and continuity of sugar cane xylem vessels. It would be fruitful to examine bacterial wilt resistant and susceptible sugar

cane cultivars in the same experiment to help resolve the question of *A. diazotrophicus* movement via the transpiration stream.

A. diazotrophicus was originally postulated to be a sugar cane specific endophyte (Li & MacRae, 1991), but further examination has revealed its existence in certain other sugar-rich plant species such as Cameroon grass (*Pennisetum purpureum* Schumach), sweet potato and coffee (*Coffea arabica* L.) (Paula & al., 1991; James & Olivares, 1997; Jimenez-Salgado & al., 1997). Notwithstanding its ability to colonize a few other plant species, *A. diazotrophicus* seems to have quite a limited host range, which may explain, at least in part, the limited genetic diversity that also characterizes this bacterial species (Caballero-Mellado & Martinez-Romero, 1994, Caballero- Mellado & al., 1995).

When all the evidence is considered, it seems likely that *A. diazotrophicus* is indeed the causal agent of N_2 fixation in sugar cane (Triplett, 1996; Baldani & al., 1997). However, such a contention has not been proven so far. Indeed, there are other "candidate" endophytes that may be important, as sugar cane is known to harbor a variety of endophytic bacteria, including pathogens such as *Clavibacter xyli* subsp. *xyli* and *Xanthomonas albilineans,* but also other diazotrophs such as *Bacillus* and *Erwinia* (James & Olivares, 1997). Most of these bacterial endophytes are present in such small numbers that they are thought to have no significant negative or positive effect on plant growth (James & Olivares, 1997). However, there is no strong evidence that plant growth responses are well-correlated with population sizes of beneficial bacteria colonizing plant tissues. The bacterial population size needs only to reach a threshold level for growth promotion to occur (Holl & Chanway, 1992), but it is very difficult to know what that threshold population size is. Therefore, even some of the bacteria that colonize internal tissues sparsely may elicit plant growth responses, and it would be worth evaluating their effects on plant growth and nitrogen nutrition as well.

On the other hand, diazotrophic *Herbaspirillum* spp. have been shown to colonize internal sugar cane tissues with populations ten-fold greater than the 10^4–10^6 cell g^{-1} fresh weight of sugar cane tissue *A. diazotrophicus* can reach (Dong & al., 1994; Olivares & al., 1996; 1997; Baldani & al., 1997). If a sizeable population is important for N_2 fixation, then these microorganisms could be responsible for a significant portion of sugar cane BNF in the field. However, unlike *A. diazotrophicus,* diazotrophic *Herbaspirillum* possess nitrate reductase and N_2 fixation is inhibited by the presence of fixed N (Baldani & al., 1992). In addition, it is unclear what substrates endophytic *Herbaspirillum* metabolize in sugar cane, as it, too, is unable to utilize sucrose (James & Olivares, 1997). Further research is required to elucidate the relative contributions of *A. diazotrophicus,*

Herbaspirillum spp., and other endophytic diazotrophs to N nutrition in sugar cane. Even though results so far are somewhat incomplete, they are very interesting from an ecological perspective and may hold significant promise from a practical standpoint.

(ii) Biocontrol of plant pathogens

There are several examples in the literature of biological control of microbial pathogens through inoculation with bacterial endophytes (Hallmann & al., 1997). These include control of *Fusarium oxysporum* on cotton (Chen & al., 1995), *Verticillium albo-atrum* and *Rhizoctonia solani* on potato (Nowak & al., 1995) and cotton (Pleban & al., 1995), and *Clavibacter michinganensis* on potato (Van Buren & al., 1993). As indicated by Hallmann & al. (1997), bacterial endophytes are particularly well-placed physically to antagonize certain plant pathogens. While many endophytic bacteria are capable of inhibiting pathogen growth or activity directly, inoculation of plants with endophytic bacteria also can result in induced systemic resistance (ISR) in the host plant (Hallmann & al., 1997). This, of course, renders the task of determining the precise mechanism by which bacterial endophytes inhibit pathogens *in vivo* challenging. To demonstrate ISR as the mechanism by which bacterial endophytes control disease, it must be shown that no contact occurs between the inducing bacteria and the disease-causing pathogen (Van Loon, 1997).

Van Peer & al. (1991) were the first to demonstrate ISR using an endophytic bacterium. *Pseudomonas fluorescens* strain (WCS417r) was inoculated onto root systems of carnation (*Dianthus caryophyllus* L.), where it colonized internal root tissues. One week later, plants were challenged with *Fusarium oxysporum* f. sp. *dianthi* on stems, and plants treated with the bacterial endophyte developed disease symptoms less frequently and with less intensity than controls. Because strain WCS417r could not be isolated from stem tissues displaying the protective effect, the biocontrol mechanism was concluded to be ISR.

Since that initial report, there have been several demonstrations of ISR by inoculation with endophytic bacteria (Liu & al., 1995a; 1995b; 1995c; Tuzun & Kloepper, 1995; Benhamou & al., 1996a; 1996b; 1996c). Other studies have eliminated the possibility that biocontrol results from translocation of substances produced by endophytic bacteria that are inhibitory to pathogens. Heat-killed endophytic bacteria as well as purifed lipopolysaccharide also provided effective disease control (Van Peer & Schippers, 1992; Leeman & al., 1995).

There is also some evidence that ISR resulting from inoculation with endophytic bacteria affords a degree of protection from plant-parasitic nematodes (Hallmann & al., 1995), but little work has been done in this area. Recently, Benhamou & al. (1998) have demonstrated that ISR efficacy can be significantly enhanced when plants are co-treated with endophytic bacteria and chemical elicitors of ISR such as chitosan, a chitin derivative that occurs in the cell wall of many fungi. Such an approach, involving biotic and abiotic ISR elicitors, may prove to be an effective adjunct to purely chemical or biological means of pathogen control, and warrants further study.

(iii) Bacterial endophytes of trees

Little is known about the nature and composition of endophytic bacteria in trees. Gardner & al. (1982) isolated representatives of thirteen genera from xylem fluid of rough lemon rootstock (Tab. 1), and found population sizes ranging from 10^2–10^4 cfu g^{-1} of xylem fluid. However, when xylem tissues were aseptically homogenized, up to 10^6 cfu g^{-1} of plant tissue were recovered. Forty-eight of the 850 isolates they obtained were potentially phytopathogenic based on their ability to elicit HR in tobacco (*Nicotiana tabacum* L.), but the role of the other 802 isolates was not determined.

In a subsequent study, Gardner & al. (1984) inoculated rough lemon and sweet orange (*Citrus sinensis* Osbeck) seedlings with pseudomonads isolated from washed, homogenized root tissues, and observed a range of seedling growth responses, from inhibitory to stimulatory. While some of these plant growth altering pseudomonads may have originated from internal root tissues, the methodology employed precluded the authors from separating isolates originating from the root exterior and interior.

In an initial study of gymnosperm root-associated bacteria, O'Neill & al. (1992) isolated 22 strains from surface-sterilized roots of naturally-regenerating white x Engelmann hybrid spruce seedlings. We also found a range of effects on seedling growth in a greenhouse screening assay using spruce: three strains were inhibitory, five strains were stimulatory and the remaining strains had no significant effect on seedling growth (O'Neill & al., 1992). Based on the magnitude and consistency of seedling growth effects, the two best plant growth promoting endophytes were identified and selected for further study: one isolate was *Pseudomonas putida* and the other belonged to *Staphylococcus*. While the positive effect of both of these strains on plant growth was reproducible in the greenhouse, a field trial with two ecotypes of one-year old spruce seedlings planted at three different reforestation sites yielded mixed results (Chanway & Holl, 1993). For example, *P. putida* enhanced

seedling growth of only one of two spruce ecotypes planted at two of three reforestation sites. In addition, it had inhibitory effects in three of the spruce ecotype × planting site treatment combinations.

Evaluation of gymnosperm bacterial endophytes was only a small part of a larger project designed to characterize gymnosperm root-associated bacterial (*i.e.*, external and internal colonists) (O'Neill & al., 1992; Chanway & Holl, 1992; 1994). Therefore, we undertook a subsequent bacterial isolation and screening program emphasizing endophytic bacteria as possible tree seedling growth promoting agents (Chanway & al., 1994; 1997). As seen in our earlier work (O'Neill & al., 1992), several bacterial strains isolated from surface-sterilized roots of white × Engelmann hybrid spruce seedlings caused reproducible spruce seedling biomass increases of up to 36% two months after seed was sown and inoculated in greenhouse trials (Chanway & al., 1994). Three of these strains were *Bacillus*, three were actinomycetes, likely *Streptomyces*, and one was *Phyllobacterium*. An additional strain that performed well in greenhouse assays could not be identified using GC-FAME or Biolog, and may represent a novel species.

In addition, the seedling growth promotion efficacy of some of these strains was altered significantly when assays were conducted in the presence of a small amount (2% v/v) of forest soil known to contain seedling growth inhibiting organisms (*i.e.*, minor pathogens). One of the endophytic actinomycetes (isolate W2) as well as the *Phyllobacterium* isolate (W3) clearly stimulated spruce seedling growth only in the absence of forest soil. In its presence, seedling growth was inhibited. These results suggested that growth promotion by W2 and W3 occurred via a mechanism unrelated to biocontrol of minor pathogens, and may have involved one of the direct plant growth promotion mechanisms (Kloepper, 1993; Glick, 1995; Chanway 1997). However, actinomycete isolate N1 and *Bacillus* isolate N4 stimulated seedling growth only in the presence of forest soil, which suggested that these strains acted through a biocontrol mechanism, possibly by inducing systemic resistance in the host plant. Elucidation of this possibility requires further experimentation.

We have also looked for bacterial endophytes in lodgepole pine. After isolation of several bacterial strains and screening trials for effects on seedling growth, we identified a plant growth promoting *Bacillus* strain (Pw2) that originated from internal root tissues of a naturally-regenerating 2–3-year-old pine seedling (Shishido & al., 1995). Our studies indicate that Pw2 can colonize external and internal pine and spruce root tissues after seed or root inoculation. Colonization of internal root tissues may depend on lateral root development, and results in endophytic bacterial population sizes approaching 10^6 cfu g^{-1} root tissue (Shishido & al., 1995; Chanway,

1997; Shishido, 1997). In addition, using a surface-sterilization, dilution plating assay as well as immunofluorescence microscopy, a rifamycin-resistant derivative of this strain, Pw-2-R, was shown to be capable of colonizing internal pine and white × Engelmann hybrid spruce stem tissues after soil or root inoculation (M. Shishido & C. P. Chanway, unpubl. data). Five months after root inoculation, internal stem bacterial populations reached 10^5 cfu g^{-1} of stem tissue (Shishido, 1997).

To ascertain which microbial characteristic(s) facilitate entrance of bacterial endophytes into plant tissues, we compared the biochemical capabilities of the endophytic *Bacillus polymyxa* strain Pw2 with those of another plant-growth promoting, nonendophytic strain, *B. polymyxa* L6-16R. Strain L6-16R is unable to enter plant tissues even when co-inoculated with an endophytic microorganism (Shishido & al., 1995; Bent & Chanway, 1997). According to Biolog, both strains possessed similar metabolic capabilities, but with some potentially important exceptions (Shishido & al., 1995). Strain Pw-2R was able to metabolize sorbitol, but strain L6-16R was not. Mavingui & al. (1992) found that, in general, *Bacillus polymyxa* strains isolated from the rhizoplane of wheat (*Triticum aestivum* L.) were capable of metabolizing sorbitol while rhizosphere and non-rhizosphere isolates were not. They hypothesized that intense competition for oxygen would occur on the root surface due to root respiration, which would result in selection pressure for bacteria capable of anaerobic growth on highly reduced substrates, such as sorbitol. In addition, strain Pw-2 was able to metabolize D-melezitose, a sugar that has been detected in the sap of conifers (Lehninger, 1975). However, the occurrence of sorbitol and D-melezitose in lodgepole pine root tissues and its use by other *Bacillus* root endophytes must be demonstrated before a role for these substrates in internal root colonization by *Bacillus* can be postulated with greater confidence.

To facilitate root colonization, it is logical to suspect that root endophytic bacteria may also possess the ability to metabolize structural components of plant cells. In particular, the ability to metabolize pectin (polygalacturonic acid), the primary component of the middle lamellae of plant cell walls, has been proposed to at least partly explain why bacterial root endophytes are often found in the root cortex intercellularly (Balandreau & Knowles, 1978; Baldani & Döbereiner, 1980). Both strains L6 and Pw-2 possessed pectolytic activity *in vitro*, but only strain Pw-2 was able to metabolize D-galacturonic acid (Shishido & al., 1995), the primary monomeric component of pectin (Paul & Clark, 1989).

It is not clear whether strain Pw-2's capability to metabolize monomeric galacturonic acid after break down of the pectin polymer

was related to its ability to enter root tissues. However, breakdown products of plant cell walls are known to induce systemic disease responses in plants (Brock & al., 1994), which leads to the possibility that Pw2 avoids plant defense mechanisms by metabolising cell wall components before they elicit a defense response by the host plant. This possibility also requires further investigation.

Perhaps the most exciting characteristic of strain Pw2 is that it is diazotrophic (M. Shishido & C. P. Chanway, unpubl. data). This observation leads to the intriguing possibility that lodgepole pine harbors a systemic, endophytic, nitrogen-fixing bacterial population, similar to that found in sugar cane, which would explain its ability to grow, and even thrive, under nitrogen deficient conditions in the absence of significant rhizospheric nitrogen fixation (Binkley, 1995). Indeed, the $^{15}N/^{14}N$ ratio of pine foliage in a central coast forest in British Columbia almost devoid of nitrogen fixing species was observed to be low enough to suggest that BNF supplies plant N (F. B. Holl, pers. commun.). We are currently actively investigating this possibility by looking for diazotrophic endophytes within lodgepole pine tissues and evaluating the contribution of strain Pw2 to the N nutrition of pine seedlings.

We have also conducted initial field trials with *B. polymyxa* strain Pw2-R and *Pseudomonas chloroaphis* strain Sm3-RN, another bacterial endophyte capable of stimulating seedling growth in the greenhouse (Chanway & al., 1997). Two years after bacterial inoculation and planting at nine sites in British Columbia and Alberta, Canada, spruce treated with strain Pw2-R showed mean biomass increases up to 33% above controls at seven of the nine sites. However, due to large "within treatment" variability, biomass increases less than 28% were not statistically significant, rendering all but one of the seven increases statistically not significant. Spruce biomass decreases in response to inoculation with Pw2-R (statistically not significant) were also observed at two of the nine sites. Pw2-R had no significant effect on lodgepole pine biomass at any of the sites, and mean biomass increases of up to 21% were observed at only three sites.

In contrast, *Pseudomonas* strain Sm3-RN caused spruce biomass increases of up to 57% at five of the nine sites, three of which were statistically significant. However, decreases in spruce biomass were observed at the remaining four sites, and in one case, growth inhibition was significant. Sm3-RN was not evaluated on pine.

Population sizes of Pw2-R and Sm3-RN were generally below the assay detection limit of ca. 10^2 cfu g^{-1} plant tissue, which led us to question how effectively internal plant tissues were colonized at the onset of the experiment. Therefore, we also evaluated seedlings that were inoculated with strains Pw2-R and Sm3-RN and grown in

the greenhouse for four months before planting at four of the reforestation sites described above (M. Shishido & C.P. Chanway, unpubl. data). The period of growth in the greenhouse facilitated internal tissue colonization by these microorganisms so that mean internal root populations reached ca. 10^3–10^4 cfu g^{-1} tissue. As expected, mean seedling biomass also increased in the greenhouse due to bacterial inoculation, resulting in seedlings that, on average, were up to 9% heavier than controls. Because seedling growth responses in the field were confounded with those that occurred in the greenhouse, field responses were evaluated using relative growth rates (RGR's) instead of absolute growth rates.

In general, after the first growing season, RGR's of seedlings containing endophytic bacteria were greater than those of control seedlings at all four planting sites. In some cases, RGR's of inoculated plants were double the control value. This was particularly interesting in view of results with seedlings that we inoculated and planted immediately at the same sites without a preplanting, post inoculation growth period in the greenhouse. At two of the four sites, seedlings inoculated at the time of planting (i.e., with no greenhouse growth period) did not respond to bacterial treatment, and in one case, responded negatively. However, shoot and root RGR's of seedlings pretreated in the greenhouse before planting at the same sites were 23%–132% greater than controls, and endophytic populations in root tissues of 10^2–4×10^4 cfu g^{-1} plant tissue were detected in seedlings at three of the four sites. These results suggest that a period of growth under a controlled environment to facilitate establishment of endophytic bacterial populations may be an important step in successful application of plant growth promoting bacterial endophytes in forestry. Future research will elucidate this possibility as well as the mechanism by which bacterial endophytes stimulate gymnosperm growth.

Conclusions

Results from research with endophytic bacteria are significant and exciting. From an ecological perspective, they raise intriguing questions regarding the evolution of the plant × microbe relationships. Are most bacterial endophytes mutualistic, or are they simply opportunists, capable of "fooling" plant defense mechanisms allowing them to live in a nutrient rich environment? Do diazotrophic or plant growth promoting endophytes represent the endpoint of an adaptive process that has resulted in a new symbiosis, or are we observing the ongoing development of a relationship that may ultimately result in a more specialized symbiosis, characterized by

complex infection methods such as root hair infection and infection thread development or compartmentalization of the endophytes?

From a practical perspective, systemically endophytic bacteria such as *A. diazotrophicus* or *B. polymyxa* strain Pw2-R could be used as vectors to deliver specific gene products to plants, such as *Bacillus thuringiensis* toxins. Such an approach may be more feasible than attempting to genetically alter the plant host directly. In addition, diazotrophic endophytic bacteria hold great potential for reducing agricultural inputs, especially mineral N, at least for certain crops such as sugar cane. Inoculation of forest seedlings with effective diazotrophic or plant growth promoting endophytic bacteria could also enhance growth and yield of trees significantly, especially at nutrient poor sites. While results so far are intriguing, there is much more work to be done if we are to understand the role of these plant × microbe associations in nature, and ultimately manage them for more efficient and sustainable plant production with fewer chemical inputs.

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