ENDOPHYTIC BACTERIA: PROSPECTS AND OPPORTUNITIES FOR THE BIOLOGICAL CONTROL OF PLANT-PARASITIC NEMATODES

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Summary. Endophytic bacteria have the capability of colonizing internal host tissues and this makes them a valuable tool to improve crop performance. By systemically colonising roots they have potential to develop into biological control agents of plant-parasitic nematodes. This is because i) they are easy to culture *in vitro*; ii) can be applied as seed treatments; iii) reduce initial root damage; iv) escape microbial competition and also capable of influencing host's response to pathogen attack; v) do not produce any phytotoxic symptoms instead, promote plant growth and; vi) depend on root exudates for multiplication.

In the past two decades, three developments have occurred which have had significant effects on the prospects and opportunities for the biological control of plant-parasitic nematodes. Firstly, the use of agrochemicals, although decreasing the attack of insects and phytopathogenic microorganisms, still represents a high risk to field workers. Concern over these chemicals has led to an increased interest in biological control in its widest sense, in order to obtain more environmentally benign methods of reducing nematode damage. Secondly, it has been demonstrated in several soils that nematophagous fungi and bacteria increase under some perennial crops, and under those grown in monocultures, and so may control some nematode pests, including cyst and root-knot nematodes (Stirling, 1991). Such nematode-suppressive soils have been reported from around the world and include some of the best-documented cases of effective biological control of nematode pests. Thirdly, a number of nematophagous fungi and bacteria based products have been developed commercially for the control of plant-parasitic nematodes, but none has been used widely because control has tended to be erratic at practical application rates. Most research has been empirical and concerned relatively few organisms; there is a need for detailed, quantitative studies on the wide range of potential agents with different modes of action (Kerry, 1990).

ENDOPHYTIC MIROORGANISMS

All microorganisms that inhabit, at least for one period of their life cycle, the interior of a plant, may be considered as an endophyte. The endophytes, epiphytes (those that live on the surface of plants) and phytopathogens (those that cause diseases to plants), differ gradually from each other and it is difficult to draw sharp limits to discriminate each category. Whereas phytopathogens have economic importance, the endophytes and epiphytes may not have such significance. Some endophytic fungi from citrus have inhibited the growth of some endophytic bacteria from the genus *Bacillus*, present in the same host. In this case, fungi and bacteria do not colonize the same regions in the interior of the host or some of the *Bacillus* species are in fact epiphytic, only entering the plant occasionally (Araujo, 1996). These relationships may be important to distinguish endophytes from epiphytes and to understand the maintenance of a necessary equilibrium between endophytes and latent pathogens, avoiding the emergence of diseases.

The existence of endophytes inside different plant tissues is a well-documented phenomenon (Gardner *et al.*, 1982; Patriquin *et al.*, 1983; Gagne *et al.*, 1987; McInroy and Kloepper, 1995; Quadt-Hallmann *et al.*, 1997). In general, endophytic microorganisms are those that inhabit the interior of plants, especially leaves, branches and stems, showing no apparent harm to the host (Azevedo, 1998). In the 70's, endophytes were initially considered neutral, neither causing benefits nor showing detrimental influence on plants, but from the results of more recent studies it has been possible to show that in many cases, they have an important role in host protection against pathogens. Several studies have now shown that the interaction between plants and some endophytic bacteria is associated with beneficial effects such as plant growth promotion and biocontrol potential against plant pathogens (Lalande *et al.*, 1989; Bashan *et al.*, 1990; Chen *et al.*, 1995; Hallmann *et al.*, 1998).

Webber (1981) was probably the first researcher to report an example of plant protection given by an endophytic fungus, in which the endophyte *Phomopsis oblonga* protected elm trees against the beetle *Physocnemum brevilineum*. It was suggested that the endophytic fungus *P. oblonga* was responsible for reducing the spread of the Dutch elm disease causal agent *Ceratocystis ulmi* by controlling its vector, the beetle *P. brevilineum*. The author associated the repellent effect observed towards

the insect to toxic compounds produced by the fungus. Since then several endophytic fungi and bacteria have been used for the control of plant pathogens.

ENDOPHYTES AND PLANT-PARASITIC NEMATODES

The use of endophytic bacteria for the management of plant-parasitic nematodes is a relatively new approach. Endophytes colonize the same root tissues as sedentary plant-parasitic nematodes. Therefore, this association of endophytic bacteria with nematodes throughout the nematode life cycle makes these bacteria excellent candidates for biocontrol strategies. There is some evidence that endophytes may contribute to the control of plant-parasitic nematodes (Hallmann *et al.,* 1995). However, control of these parasites seems to be more complex and difficult than for fungal or bacterial pathogens, since damage from nematodes occurs as a result of their feeding habit and internal migration thus limiting the efficacy of bacterial antagonism. Nevertheless, sedentary plant-parasitic nematodes might be an interesting target for antagonistic endophytes, since they stay localized within the plant for several weeks and feed from a single feeding site. Whereas most research on the interaction of endophytic bacteria with nematodes has been conducted on root knot nematode (*Meloidogyne* spp.) (Table I), the association of other nematode species (cyst nematodes) with the endophytic bacteria is also of great interest. In our previous studies, *Pseudomonas aeruginosa* strain IE-6 and its streptomycin- resistant derivative IE-6S^{+} colonized inner root tissues of tomato and significantly reduced *Meloidogyne javanica* population densities under glasshouse and field conditions (Siddiqui *et al.,* 2000; Siddiqui and Ehteshamul-Haque, 2000 a, b; Siddiqui and Ehteshamul-Haque 2001). Some endophytic bacterial strains from cotton significantly reduced galling of cotton roots by the root-knot nematode, *M. incognita* (Hallmann *et al.,* 1998). In that same study (Hallmann *et al.,* 1998), it was reported that nematode populations were correlated with the establishment of endophytic bacteria within roots, suggesting that root-penetrating nematodes provide increased entry sites for, or reduce plant resistance to, endophytic bacteria. Furthermore, excessive leakage of root exudates from damaged roots or photosynthates directly excreted by the developing nematodes presumably also increase bacterial populations in the inner root and shoot tissues. However, Hallmann *et al.* (1999) recovered larger microbial populations from within roots grown in chitin-amended soil than in non-amended soil. Roots grown in the amended soils were less damaged by nematodes. These authors proposed that either the initial nematode inoculum potential in the non-amended soil was too low to significantly increase internal populations of microorganisms or, due to the larger bacterial populations in chitin-amended soils than in non-amended soils, more microorganisms penetrated the root tissue. Although endophytic bacteria prefer to colonize galled tissues of nematode-infested plants (Hallmann *et al.,* 2001), they do not provide consistent control which does not exceed more than 50% (Hallmann *et al.,* 1997 a; Siddiqui and Ehteshamul-Haque 2001; Hallmann *et al.,* 2001). Consistent and reproducible control of *M. incognita* on cotton was provided by only a few bacterial isolates such as *P. fluorescens* 89B-61 (Hallmann *et al.,* 1998), *Brevundimonas vesicularis* IN884 and *Serratia marcescens* (Hallmann *et al.,* 1997 b). Recently *Rhizobium etli* G12, a potential endophyte of potato and its genetically modified strain G12(pGT-trp) containing green fluorescent protein (GFP) significantly decreased the number of galls formed by *M. incognita* on potato (Hallmann *et al.,* 2001).

Table I. Interactions of endophytic bacteria with plant-parasitic nematodes on different plant species.

Plant species	Bacterial species	Nematode species	Reference
Cotton	*Brevundimonas vesicularis*	*Meloidogyne incognita*	Hallmann *et al.*, 1997 b; Hallmann *et al.*, 1998; Hallmann *et al.*, 1999
	Serratia marcescens		
	Pseudomonas fluorescens		
	Burkholderia cepacia		
	Phyllobacterium rubiacearum		
Tomato	*Pseudomonas aeruginosa*	*Meloidogyne javanica*	Siddiqui and Ehteshamul-Haque, 2000; 2001; Siddiqui and Shaukat, 2002 b; Siddiqui and Shaukat, 2002 c
	P. fluorescens		
Mungbean, Tomato, Soybean	*Pseudomonas aeruginosa*	*M. javanica*	Shaukat and Siddiqui, (unpbl.)
		M. incognita	
Beans	*Rhizobium etli*	*M. incognita*	Hallmann *et al.*, 2001
Potatoes			
Arabidposis thaliana			
Cucumber	*Pseudomonas fluorescens*	*M. incognita*	Hallmann *et al.*, 1998

MECHANISM OF ACTION

Although some literature is available on endophytic bacteria controlling plant-parasitic nematodes, little work has been done examining their potential mode-of-action. In general, it is presumed that endophytic bacteria control nematodes by either direct antagonism by means of metabolites or by induced systemic resistance. On the basis of modes-of-action, Hallmann *et al.* (2001) divided endophytic bacterial biocontrol agents into two groups: (i) strains that extensively colonize the internal plant tissues and reduce nematode invasion by niche occupation, antibiosis, or both, and (ii) strains that primarily colonize the root cortex where they stimulate general plant defence/resistance mechanisms. More extensive and continuous colonization of plants might be required for endophytes of the first type because coincidence with nematodes in the same tissue would be necessary for antagonism. In the case of induced resistance, total endophytic population numbers might be less important as long as they exceed a certain threshold level necessary to initiate the plant defence mechanisms (Hallmann, 2001).

ANTIBIOSIS

Antibiosis is an important mechanism used by plant-beneficial microorganisms for the suppression of soil-borne plant pathogens including plant-parasitic nematodes. An endophytic *P. aeruginosa* strain IE-6 produced toxic compounds *in vitro* that caused substantial mortality of *M. javanica* juveniles. Partial characterization of the active compounds revealed that active principal(s) was soluble in ethyl acetate and proteinaceous or glycoproteinaceous in nature (Siddiqui *et al.*, 2000). Similarly, Ali *et. al.* (2002) demonstrated that active nematicidal principal(s) by *P. aeruginosa* strain-78 were heat labile, sensitive to extreme pH values, polar in nature and having a molecular weight of less than 8000 Da. In the subsequent studies this strain has been frequently isolated from the root tissues of several crops including mungbean, tomato and soybean (Shaukat and Siddiqui unpbl.). Several other plant-associated bacteria and facultative-parasitic fungi produce metabolites *in vitro* that may inhibit hatch of eggs and the mobility of the second-stage juveniles, but whether these compounds are produced at effective concentrations within a cyst or egg mass and, the rhizosphere and roots is unknown. Experiments that demonstrate antagonism *in vitro* are followed by test in soils and, if applications of the microorganism reduce nematode infections, it is assumed that the same mechanism(s) is responsible. However, there is no justification for such an assumption unless the metabolite is detected at effective concentrations in the rhizosphere and roots (Kerry, 2000). Direct isolation of the potential nematicidal compounds from the crop rhizosphere and roots are technically difficult and laborious. As an alternative, marker genes can be introduced into bacteria to give the organisms a unique physiological property. The assumption is that the background organisms do not possess this activity and that the marker gene does not alter the fitness of the bacterium. These markers can also be used as reporter genes, when they lack their own promoter, but are placed downstream from an exogenous promoter, so they can report on the activity of that promoter. These promoter genes can regulate important biocontrol functions such as siderophore or antibiotic production (Paulitz, 2000).

ENHANCEMENT OF HOST DEFENCE MECHANISMS

The mode-of-action presently gaining the most interest is related to induction of plant defence mechanisms. Endophytic colonizers might be better inducers of plant defence mechanisms since they establish a much closer relationship over an extended period of time with the plants as compared to rhizosphere bacteria, which can be inhibited by competition with other microorganisms on the root surface. However, induction of plant defence mechanism always requires some kind of plant-endophyte recognition. Application of *P. aeruginosa* IE-6S^{+} to one half of the split-root system of tomato caused a significant (42%) systemic reduction in nematode penetration in the other half of the split-root system (Siddiqui and Shaukat, 2002 c). The use of bacteria-mediated induced systemic resistance for the control of plant-parasitic nematodes is still a new research area, and studies on the mode-of-action have only just started to be explored. Gene products on the surface of the bacterial endophyte such as lipopolysaccharides (LPSs) may function as elicitors to specifically bind to receptors on or near the plant cell surface (Hallmann, 2001). Reitz *et al.* (2000), identified purified LPS as the causal mechanism of systemic resistance induced by an endophytic bacterium *Rhizobium etli* G12 (Hallmann *et al.*, 2001) against the potato cyst nematode *Globodera pallida* in potato. Similar mechanisms might also be involved in the control of root-knot nematode (*Meloidogyne* spp.). Reitz *et al.* (2001) further demonstrated that the resistant reaction triggered by *R. etli* G12 was not accompanied by enhanced accumulation of pathogenesis-related proteins such as chitinase and β-1,3-glucanase. In general, pathogen or chemical induced resistance is associated with local and systemic accumulation of pathogenesis-related proteins in plant tissue (van Loon, 1997), increased peroxidase activity and enhanced lignin synthesis (Hammerschmidt *et al.*, 1982; Schneider and Ullrich, 1994). Similar processes are also known to be commonly expressed by the plant in response to nematode invasion (Zacheo and Bleve-Zacheo, 1995; Rahimi *et al.*, 1998).

HOW ENDOPHYTES ENTER THEIR HOSTS? SOURCE AND AVENUE OF ENTRANCE

The source of endophytic colonization is diverse and can range from transmission via seeds and vegetative planting material to entrance from the surrounding environment such as rhizosphere and phyllosphere. While the importance of seeds as a source of endophytic bacteria is still controversial, several observations favor the rhizosphere soil as the primary source for endophytic colonization.

Although, the rhizosphere is a major source of bacteria and fungi for endophytic colonization, not all soil microorganisms can become endophytes. Two dominant genera in the soil and rhizosphere, *Bacillus* and *Arthrobacter,* were not detected as endophytes (Hallmann *et al.*, 1998). The internal environment of plant tissue is different from the soil and might not meet the requirements of those non-colonizing bacteria. The plant itself also seems to control internal colonization by excluding potential endophytic colonizers from penetration under certain circumstances. Huang (1986) summarized the avenues of entry for different plant pathogenic bacteria. Such pathways included stomata (Roos and Hattingh, 1983), lenticels (Fox *et al.*, 1971), wounds including broken trichomes, areas of emerging lateral roots (Jacobs *et al.*, 1984) and the germinating radicle (Gagne *et al.*, 1987). Bacteria may enter intact plant tissue by invagination of the root hair cell wall, by penetration of the junction between root hair and adjacent epidermal cells, or by enzymatic processes involving degradation of cell wall bound polysaccharides (Huang 1986). Studies of *in planta* enzymatic activity demonstrated hydrolysis of wall-bound cellulose in the vicinity of an endophytic *Enterobacter asburiae* JM22 (Quadt-Hallmann *et al.*, 1997). Plant wounding induced either by biotic (fungi, plant-parasitic nematodes, insects) or abiotic factors (tillage, extreme temperature fluctuations, grafting, root pruning) is ubiquitous in any agroecosystem and is probably a major factor for bacterial entrance. For example, wounds artificially induced by either adding nematodes to the soil (Hallmann *et al.,* 1998) or carborundum to the bacterial inoculum suspension prior to leaf application (Quadt-Hallmann and Kloepper, 1996) increased internal population densities of applied endophytic bacteria. Besides causing wounds that serve as entry points for the bacteria, the nematode juveniles also carried individual bacterial cells adhering to their cuticle (Hallmann *et al.,* 1998).

FACTORS INFLUENCING ENDOPHYTIC COLONIZATION

The total endophyte density within a given type of plant tissue is not constant and changes over time. Understanding factors affecting bacterial colonization of the rhizosphere and plant tissues is necessary to harness the beneficial potential of endophytic bacteria. Major factors influencing total population densities include plant age as well as various biotic and abiotic environmental factors. The effects of some of these factors have been investigated, as discussed below.

Abiotic Factors

The internal plant tissues provide a protective environment for endophytic bacteria. However, several factors (e.g., temperature, rainfall, edaphic factors, UV radiation) that affect the colonization of bacteria in the phylloplane and rhizosphere will also likely influence the colonization and survival of bacterial endophytes, though indirectly, since these factors have a direct effect on the endophyte's host (Hallmann *et al.*, 1997a).

The bacterium *Acetobacter diazotrophicus*, isolated for the first time from sugarcane was detected inside the cortical cells of stems and xylem vessels. However, bacterial quantification has shown that in plants fertilized with high nitrogen levels, there was a severe decrease in the bacterial numbers compared to the numbers found inside plants with low nitrogen levels. From a practical perspective, high nitrogen fertilization of the fields that is normally required for the control of plant-parasitic nematodes (Shaukat *et al.*, 2002), might also be a threat to the maintenance of endophytic associations occurring naturally (Fuentes-Ramires *et al.*, 1999).

Agriculture by its own nature is antiecological and, with the use of implements, fertilizers, insecticides, nematicides, fungicides, herbicides and, antibiotics, profound biological modifications have occurred. The products, such as nematicides and fungicides, aim at the control of plant-parasitic nematode and phytopathogenic fungi. However, they may be responsible for eliminating important species of bacteria that control other pests and microorganisms that are performing a crucial role in the environment, inhibiting the growth and the multiplication of other microorganisms. One group of microorganisms that is affected by these anthropogenic modifications is the microbial endophytes. Bavistin fungicide was highly toxic to endophytic strain IE-6 of *P. aeruginosa* as compared to other pesticides used (this strain is also an inner root and shoot colonizer of tomato). Captan and bavistin had no additive effect on the bacterium suppressing root-knot development whereas benlate and furadan (a frequently used nematicide), both nematicidal, enhanced control (Siddiqui *et al.*, 2001). Since a pesticide tolerant strain of a biocontrol agent could become more rhizosphere competent by the addition of chemical to the soil, use of bacterial strains naturally tolerant to pesticide may increase their root colonization and biocontrol potential against nematode.

Soil physical and chemical factors, including pH, salinity, and soil texture are likely to affect endophytic bacteria indirectly by altering the saprophytic bacterial community in the rhizosphere and, therefore, preselecting the source of potential endophytes (Hallmann *et al.*, 1997 a). Soil moisture also interferes with the bacterial populations in

the rhizosphere and inner root tissues. Siddiqui and Ehteshamul-Haque (2001) demonstrated that nematode biocontrol and growth promoting potential of *P. aeruginosa* IE-6S$^+$ was enhanced when soil was kept at 50% or 75% of the maximum moisture holding capacity (MHC), whereas a 25% MHC reduced bacterial efficacy. Furthermore, endophytic populations of IE-6S$^+$ were greatest when the bacterium was kept at 75% MHC in nematode-infested soil compared with 25% or 50% MHC.

Mineral nutrition has been identified as one of the key factors influencing the production of antibiotics by biocontrol bacteria. Recently, these factors have also been found to affect bacterial inner root populations. Siddiqui and Shaukat (2002 b) demonstrated that zinc and glycerol singly or in combination promoted nematode biocontrol potential and endophytic population densities of *P. aeruginosa* IE-6S$^+$ and *P. fluorescens* CHA0 in tomato. In a separate study, Zn at 1.6 mg/kg of soil markedly reduced endophytic population densities of IE-6S$^+$ in tomato. However, biocontrol potential of the bacterium to suppress root-knot nematode was not affected (Siddiqui *et al.*, 2002 a).

Organic amendments to the soil have been studied for the management of several plant-parasitic nematodes in economically important crops. These amendments have also been shown to influence endophytic populations. For instance, the incorporation of 1% chitin to soil modified not only the bacterial spectrum in the rhizosphere but also the endophytic community structure of cotton roots (Hallmann *et al.,* 1999). In their study, the endophytic bacterial community of cotton grown in non-amended soil was dominated by *Phyllobacterium rubiacearum* which accounted for 61% of the total population. Other strains with significant occurrence were *Burkholderia cepacia* (9%), *B. pickettii* (9%) and *Phyllobacterium myrsinacearum* (8%). In contrast, the endophytic population isolated from cotton roots grown in chitin-amended soil was dominated by *B. cepacia,* which made up 73% of the recovered population. Hallmann *et al.* (1999) suggested that bacterial species, especially endophytes, which were exclusively removed or specifically promoted by the chitin amendment, might contribute to the observed suppressiveness of the nematode populations. Similarly, soil amendment with *Argemone mexicana,* a tropical annual weed in conjunction with *P. aeruginosa* IE-6S$^+$ caused soil suppressiveness to nematode population densities in soil and reduced root-knot disease severity in tomato. *A. mexicana* at 10 g/kg of soil enhanced population densities of *P. aeruginosa* IE-6S$^+$ in the rhizosphere and inner root tissues of tomato while a 30 g/kg of soil reduced bacterial populations (Shaukat *et al.*, 2002).

Before application of the endophytes in practical agriculture, these factors should be taken into consideration. The expression of pest resistance may be affected by several factors, i.e. active amounts of allelochemicals, plant genotype (Breen, 1993 a, b), endophyte concentration (Breen, 1992), soil fertility, and endophyte genotype (Bacon, 1988; Christensen *et al.*, 1991; Breen, 1992). Hydric stress, temperature, soil pH, nematode resistance and other factors may also affect the endophyte concentration and toxin production. Breen (1994) idealized a model with all of these interactions affecting the increase or decrease in plant resistance to insects-pests in the presence of endophytes.

Biotic Factors

Endophytic bacteria, fungi, viruses and nematodes are the common inhabitants of a microenvironment where they interact each other by competition, antibiosis, niche exclusion, symbiosis and mutualism. Siddiqui and Ehteshamul-Haque (2001) demonstrated that internal population density of *P. aeruginosa* IE-6S$^+$ in tomato roots was not markedly influenced when the bacterium was co-inoculated with either *Bradyrhizobium japonicum*, the root-nodulating bacterium or *Bacillus subtilis.* Whereas *B. japonicum* drastically reduced bacterial populations in the rhizosphere, *B. subtilis* had no such effect. Since *P. aeruginosa* and *B. japonicum* are the Gram-negative bacteria, it is possible that they compete for the same food source in the rhizosphere while *B. subtilis* being Gram-positive occupies a different ecological niche and avoids competition in the rhizosphere. In a separate study, inner root colonization by IE-6S$^+$ enhanced when the bacterium was applied in conjunction with *P. fluorescens* strain CHA0. However, the colonization pattern of IE-6S$^+$ in tomato roots was not influenced when a mixture of *P. aeruginosa* IE-6S$^+$, *P. fluorescens* CHA0 and *B. japonicum* 569Sm^r was added (Siddiqui and Shaukat 2002 a). Similarly, combined application of the systemic endophyte *Enterobacter asburiae* strain JM22 with a rhizosphere colonist (*Arthrobacter agilis*) did not affect inner root colonization patterns of JM22 when compared with a single application of JM22 (Quadt-Hallmann *et al.,* 1997). These authors further demonstrated that when JM22 was applied together with another endophyte (*Paenibacillus macerans*), the coexistence of both endophytes resulted in reduced population densities of both endophytes compared with their single application (Quadt-Hallmann *et al.,* 1997).

Similar relationships have been observed for bacteria-fungi associations. *Paecilomyces lilacinus*, an egg parasite of root-knot and cyst nematode did not exert any inhibitory effect on root colonization by *P. aeruginosa* strain IE-6 in tomato under field conditions (Siddiqui *et al.*, 2000). Root infection with *Rhizoctonia solani* promoted colonization of two endophytes *Enterobacter asburiae* JM22 and *P. fluorescens* 89B-27, which extensively colonized the necrotic tissue and extended 2-4 cm into healthy tissue (Mahafee *et al.*, 1997). By contrast, bacterial colonization in the inner root tissues was negatively correlated with *Fusarium oxysporum* infection while positively related with the degree of bacterial establishment in the rhizosphere (Siddiqui and Ehteshamul-Haque 2001). Likewise, Fisher *et al.* (1992) analysing

the combined incidence of bacterial and fungal endophyte in corn found that areas with high fungal colonization greatly reduced bacterial populations. These authors further demonstrated that whereas endophytic fungi colonized greatly in the middle of stem, endophytic bacteria were more prevalent in the root and lower stem parts. This antagonistic association of endophytic bacteria and fungi in plant tissue might be of practical use for developing combinations of biological control agents covering root and vascular pathogens. Recently, Siddiqui *et al.* (2002 b) demonstrated that combined application of *F. solani*, a mutualistic endophyte and *P. aeruginosa* IE-6S$^+$ caused marked reduction of *M. javanica* populations and subsequent root-knot development in tomato. In addition, inner root colonization by *F. solani* was greatly reduced when high inoculum levels of the bacterium (1.3 x 10^8 cfu/ml) and low application rates of the fungus (3.5 x 10^6 cfu/ml) were used together. Similarly, *P. aeruginosa* at low inoculum levels (2.8 x 10^7 cfu/ml) in the presence of high dosages of *F. solani* (2.2 x 10^7 cfu/ml) resulted in complete elimination of the bacterium from the inner root tissues.

Host genotype influences the rate of nematode development and their fecundity. Cultivar selection and inoculum density may also influence bacterial survival and establishment in the internal root tissue and its biocontrol and growth promoting potential. Cultivar influenced the level of biological control of potato cyst nematode caused by *Agrobacterium radiobacter* (Hackenberg and Sikora, 1992). Pillay and Nowak (1997) observed that the number of endophytic *Pseudomonas* sp. was greatest at 10 °C, at the inoculum density of approximately 4 x 10^8 – 7 x 10^8 cfu/ml, and did not vary with the tomato genotype. Bell *et al.* (1995) compared two grapevine cultivars differentiating in the degree of resistance to crown gall to demonstrate that the plant genotype did not selectively affect endophytic bacteria within the xylem. In our studies, *P. aeruginosa* at 2.5 x 10^8 cfu/ml in the presence of 4000 J2/plant, 7.4 x 10^8 cfu/ml in the absence of *M. javanica* and 7.4 x 10^8 cfu/ml in the presence of 500 J2/plant exhibited high internal population densities of the bacterium in tomato (Siddiqui and Ehteshamul-Haque, 2001). In the same study it was observed that an application rate of more than 2.5 x 10^8 cfu/ml did not further promote biocontrol potential of the bacterium but a rate below this level resulted in a significant reduction in the biocontrol efficacy (Siddiqui and Ehteshamul-Haque, 2001).

ADVANTAGES OF THE ENDOPHYTES IN PRACTICAL AGRICULTURE

By colonising the root systemically, endophytes offer several advantages over some other biocontrol agents: i) since these organisms multiply extensively on or in the root tissues that are damaged by the nematodes whereby they reduce their dependence on other environmental factors for their multiplication and survival which gives them added advantage as potential biological control agents. However, exudation from roots differs markedly between plant species and cultivars and alters the efficacy of these agents which may repel the bacteria (Sikora, 1992); ii) once inside the root tissue, these bacteria can escape from microbial competition, extreme environmental conditions and host responses to pathogen attack; iii) most of these bacteria are easy to culture *in vitro* and could be applied as seed treatments that permit targeted application and reduced inoculum; and iv) these organisms do not produce any phytotoxic symptoms instead they increase plant growth. Furthermore, they could also confer other important characteristics to plants, such as greater resistance to stress conditions (i. e. water), alteration in physiological properties, production of phytohormones and other compounds of biotechnological interest (i.e. enzymes and pharmaceutical drugs). In addition to the economical aspects, the study of endophytic microorganisms leads to strong academic interests, concerning the discovery of new microbial species, mainly when tropical hosts are investigated.

FUTURE PROSPECTS

Some literature is available on the antagonistic behaviour of rhizosphere bacteria against plant-parasitic nematodes attacking several crop plants. The modes-of-action of some of these bacteria towards phytonematodes are also described (Oostendorp and Sikora, 1990; Devidas and Rehberger, 1992; Hasky-Günther *et al.*, 1998). The nature of these bacteria as systemic colonists should be taken into consideration because we believe that once inside, different modes-of-action occur. They can cause hypersensitive reaction in the plant making roots less attractive to nematodes.

Nematode behavior is greatly dependent on the specific components in the root exudates of the host. Sikora and Hoffmann-Hergarten (1993) in a review article suggested that bacterial metabolism of these components can breakdown the chain of command coding for recognition of specific behavioral pathways between parasite and host, pertinent to survival. On the other hand, it is proposed that endophytes can make a plant release toxic exudates (such as phytoalexins). Therefore, exudates emitted from the roots in response to a systemic colonist could be of interest and should be tested for their nematicidal activity. The role of these bacteria inside the giant cell is yet to be determined. Metabolites of bacterial origin inside the cells could be of significant importance because they could reduce the reproductive potential of the female. Soil treatment with *P. aeruginoa* resulted in a significant reduction in egg mass production by *M. javanica* in tomato (Siddiqui and Ehteshamul-Haque, 2000 a).

Population densities of the agent under test should also be monitored to ensure that it has survived in the inner tissues throughout the period that activity against the

nematode target is required. Such monitoring may require the development of selective media, which can be a difficult and time-consuming task. Strains resistant to antibiotics could be developed for this purpose. These strains can either be selected as spontaneous chromosomal mutants or engineered with the antibiotic resistant genes. Rifampicin and nilidixic acid are commonly used because there is little background resistance to these antibiotics in soil bacteria. In our laboratory, we developed a streptomycin-resistant strain (IE-6 S^+) of *Pseudomonas aeruginosa*. Since streptomycin-resistant bacteria rarely occur in nature, reisolation of this strain (from the rhizosphere and inner root and shoot tissues) on King's B medium supplemented with 100 ppm streptomycin inhibit other rhizosphere bacteria and facilitates the isolation of the target bacterium (Siddiqui and Ehteshamul-Haque, 2001). However, antibiotic resistance can have pleiotrophic effects on the growth and competitiveness of the marked strains. During studies on internal plant colonization by bacteria, McInroy *et al.* (1996) observed lack of growth of rifampicin-resistant mutant (rif+) on tryptic soy agar amended with rifampicin (RTSA). However, colonies transferred after primary isolation on tryptic soy agar (TSA) (from plants treated with rif+ mutants) to RTSA grew within 18 hours. Controls, consisting of colonies on TSA from non-treated plants, did not grow after transfer to RTSA. These authors further demonstrated that antibiotic masking was encountered when bacteria were isolated from within roots and stems. Although causes for this antibiotic masking remain to be elucidated, methods for quantifying internal plant colonization by antibiotic resistant bacteria should account for this possibility. Whether induction of antibiotic resistance in an endophytic bacterium affects its ability to colonize inner root tissues and nematode biocontrol is another question, which needs to be answered before its application under field conditions. In our previous study, *P. aeruginosa* IE-6 and its streptomycin-resistant derivative IE-6S^+ did not differ significantly in their potential to reduce *M. javanica* population densities in roots and both bacteria followed same pattern of colonization in the inner root tissues of tomato seedlings (Siddiqui and Shaukat, 2003).

Green fluorescent microscopy is another technique for the quantification of bacteria residing plant tissues. The method utilizes the green fluorescent protein (GFP) produced by a jellyfish *Aequores victoria*. This protein absorbs violet light and fluoresces green. It is amenable to direct *in situ* observation with confocal laser microscopy or epifluorescence microscopy. Fluorescence microscopy of endophytic *Rhizobium etli* strain G12 tagged with a marker gene *gfp* was found as a sensitive and a rapid technique to study external and internal colonization of plant roots by bacteria interacting with *M. incognita* (Hallmann *et al.*, 2001). Other techniques for the quantification of bacteria including viable staining of the cells, electron microscopy, immunological staining and quantification by ELISA, nucleic acid hybridization and autoradiography have been used by several workers dealing with the bacterial endophytes. Each of this technique has its own advantages and disadvantages and are discussed in detail in a review by Hallmann *et al.* (1997).

For the purpose of screening, *in vitro* and *in vivo* techniques are used to test for biological control activity towards plant-parasitic nematodes. *In vitro* screening aims at the selection of a potential isolate with toxin(s) production whereas *in vivo* screening provides detailed information about bacteria-nematode-plant interactions. *In vivo* techniques, however, are laborious and time consuming whereas *in vitro* screening reduces the number of active isolates. The bacterial strains that do not show antagonism towards nematodes *in vitro* and *in vivo* should not be discarded but they should be tested for their ability to colonize inner tissues. Isolates with negative antagonism towards plant-parasitic nematodes and positive systemic colonization could be evaluated against other plant pathogens in particular towards wilt-inducing fungi. Vascular wilt fungi have evolved to occupy a selective ecological niche, the xylem vessels of their host (Pennypacker *et al.*, 1990). Biological control of these pathogens might be more effective if the bacterial antagonists also colonized the vascular system (Sharma and Nowak, 1998).

Soil-borne root-infecting fungi alone or in combination with plant-parasitic nematodes cause substantial damage to crop plants and are often targeted in biocontrol. However, the majority of root fungi and nematodes are non-pathogenic, and a large number of them may even be beneficial to plants and/or contribute positively to ecosystem functioning. Indeed non-pathogenic fungi and nematodes play an important role in the decomposition of organic matter, nutrient cycling and natural control of plant pathogens. Common endophytic fungi including *Trichoderma*, *Penicillim* and *Fusarium* are well documented as decomposers of celluloses and hemicelluloses (Domsch *et al.*, 1980). Furthermore, some endophytic fungi (mycorrhizal fungi) form a beneficial symbiotic association with roots that increases the plant's ability to absorb phosphorus, minor elements and water (Gerdtmann, 1968). Regarding non-pathogenic endophytic nematodes, their potential role in the ecology of root environment is not yet clear but possibly some of these species may be fugal feeders, bacterial feeders, and even invertebrate predators and thereby they may reduce plant damage due to these menaces. In this context, it is surprising that non-pathogenic endophytic fungi and nematodes have been largely neglected as non-target species. Therefore, it is suggested that before the application of potential endophytes in practical agriculture, role of such bacteria on non-target endophytic organisms should also be assessed. Recently we demonstrated that the application of endophytic *P. aeruginosa* strain IE-6S^+ into soil not only causes changes in the diversity of rhizosphere fungi and nematodes but also considerably alters the diversity of endophytic fungi and nematodes (Siddiqui and Shaukat, unpbl.).

Appropriate inoculum delivery methods need to be developed and tested for agronomic use of endophytic

bacteria. Though various methods have been successfully tried for the release of endophytic bacteria for their colonization and eventual growth promoting activity (van Peer *et al.,* 1990; Fahey *et al.*, 1991) but they have not been specifically developed in connection with the nematode biocontrol. In general, the methods used for the application of rhizobacteria (seed dressing, soil inoculation, talc powder-carboxymethyl cellulose etc.) for the control of plant- parasitic nematodes may serve the purpose. Similarly, the methods for the application of endophytic bacteria to promote plant growth may also be applicable. Musson *et al.* (1995) evaluated several delivery methods including stab-inoculation of bacteria into stems, soaking seeds in bacterial suspensions, methyl cellulose seed coating, foliar spray, bacteria-impregnated granules applied in furrow, vacuum infiltration and pruned-root dip. It was found that each method was suitable for the specific bacterial strain. Some of these delivery systems would be too labor-intensive for commercial agricultural practices. Therefore, there is an urgent need to develop inexpensive and reliable methods for the introduction of endophytic bacteria with nematode biocontrol potential. The successful development of a formulation for a potential endophyte will be based mostly on the understanding of ecology of these bacteria particularly in the host tissues.

Whilst endophytic bacteria have been successfully used for the control of root-infecting fungi and plant-parasitic nematode, our current experience suggests that in future they will not provide alternative means to chemical nematicides. Their performance is often inconsistent under varied field conditions in different seasons and the appropriate level of protection is slow to achieve. The development and implementation of the endophytic bacterial inoculants in agricultural practice needs: i) improvement of strains by classical or genetic engineering methods to increase the consistency of their effect against nematodes in the field; ii) a better knowledge of the ecological impact of the added endophytes in order to increase their effect and to assess the risk of their deliberate release; iii) a set of protocols and regulations for release approval based on real risk; and iv) a production and commercialization system previously adapted for living organisms.

CONCLUSION

Although first described in the 19th century, endophytic microorganisms were studied in more detail only from the 80's onwards. They have been increasingly recognized as being of great importance for protecting plants against pests, including among others, insects, nematodes and plant pathogenic fungi and bacteria. They also cause physiological modifications in their hosts, such as making them more resistant to moisture stress. Some endophytic microorganisms are able to produce compounds of biotechnological value such as antibiotics and antitumor agents.

ACKNOWLEDGMENTS

We are thankful to Dr. Keith Davies of Rothamsted Experimental Station, UK for his valuable suggestions, which led to the improvement of the manuscript.

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Accepted for publication on 6 March 2003.