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Actinomycetes, promising tools to control plant diseases and to promote plant growth
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Résumé de l'article

Les actinomycètes représentent une grande proportion de la biomasse microbienne du sol et ont la capacité de produire une large variété d'antibiotiques et d'enzymes extracellulaires. Plusieurs souches d'actinomycètes s'avèrent capables de protéger les plantes contre des maladies. Cette revue se penche sur le potentiel des actinomycètes comme (a) source de composés agronomiques, (b) agents stimulant la croissance des plantes et (c) agents de lutte biologique. La revue donne aussi des exemples de lutte biologique contre les agents phytopathogènes bactériens et fongiques par l'utilisation d'espèces d'actinomycetes déjà disponibles sur le marché ou qui le seront probablement au cours des prochaines années.

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Actinomycetes, promising tools to control plant diseases and to promote plant growth

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Actinomycetes represent a high proportion of the soil microbial biomass and have the capacity to produce a wide variety of antibiotics and of extracellular enzymes. Several strains of actinomycetes have been found to protect plants against plant diseases. This review focuses on the potential of actinomycetes as (a) source of agroactive compounds, (b) plant growth promoting organisms, and (c) biocontrol tools of plant diseases. This review also addresses examples of biological control of fungal and bacterial plant pathogens by actinomycetes species which have already reached the market or are likely to be exploited commercially within the next few years.

[Les actinomycètes, outils prometteurs pour lutter contre les maladies des plantes et stimuler leur croissance]

Les actinomycètes représentent une grande proportion de la biomasse microbienne du sol et ont la capacité de produire une large variété d'antibiotiques et d'enzymes extracellulaires. Plusieurs souches d'actinomycètes s'avèrent capables de protéger les plantes contre des maladies. Cette revue se penche sur le potentiel des actinomycètes comme (a) source de composés agronomiques, (b) agents stimulant la croissance des plantes et (c) agents de lutte biologique. La revue donne aussi des exemples de lutte biologique contre les agents phytopathogènes bactériens et fongiques par l'utilisation d'espèces d'actinomycetes déjà disponibles sur le marché ou qui le seront probablement au cours des prochaines années.

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INTRODUCTION

Bacteria shown to have potential for biocontrol of plant diseases occur in many genera, including *Actinoplanes* (Filonow and Lockwood 1985), *Agrobacterium* (Kerr 1987; Moore and Warren 1979), *Alcaligenes* (Elad and Chet 1987), *Amorphosporangium* (Filonow and Lockwood 1985), *Arthrobacter* (Mitchell and Hurwitz 1965), *Azotobacter* (Meshram and Jager 1983), *Bacillus* (Anwar 1949; Broadbent *et al.* 1977; Burr *et al.* 1978; Capper and Campbell 1986; Hadar *et al.* 1983; Schmiedeknecht *et al.* 1998), *Cellulomonas* (Wadi and Easton 1985), *Enterobacter* (Hadar *et al.* 1983; Kwok *et al.* 1987; Sneh *et al.* 1984), *Erwinia* (Sneh *et al.* 1984), *Flavobacterium* (Chen *et al.* 1987; Leben *et al.* 1987), *Hafnia* (Sneh 1981), *Micromonospora* (Filonow and Lockwood 1985), *Pasteuria* (Brown *et al.* 1985; Stirling 1984), *Pseudomonas* (Burr *et al.* 1978; Caesar and Burr 1987; Chen and Echandi 1984; Colyer and Mount 1984; Elad and Baker 1985; Ganesan and Gnanamanickman 1987), *Rhizobum* and *Bradyrhizobacterium* (Chakraborty and Purkayastha 1984), *Serratia* (Sneh 1981; Sneh *et al.* 1985), *Streptomyces* (Doumbou *et al.* 1998; Emmert and Handelsman 1999; Liu *et al.* 1995; Tahvonen 1982a, 1982b) and *Xanthomonas* (Chen *et al.* 1987; Kwok *et al.* 1987). As shown here, biocontrol agents are not limited to a specific bacterial group; however, given the microbial diversity of agroecosystems, it is probable that the full spectrum of potentially effective strains has barely been explored.

A number of rhizobacteria that were shown to act as effective biocontrol agents by suppressing a variety of economically important phytopathogens often promote overall plant vigor and yield, either when applied to crop seed or incorporated into the soil (Bashan 1998; Brown and Surgeoner 1991; Burr *et al.* 1978; Kloepper *et al.* 1980a, 1980b; Kloepper *et al.* 1989; Turner and Backman 1991). It was in the course of biocontrol studies on plant disease suppression that some bacterial strains were found to promote plant growth even in the absence of pathogen antagonism (Harris *et al.* 1994) and under gnotobiotic conditions (Lifshitz *et al.* 1987). These beneficial bacteria are known as plant growth promoting rhizobacteria, or PGPR (Kloepper *et al.* 1989). The term biocontrol is rather specific and does not always imply a PGPR. However, it can be difficult to separate plant growth promotion from biological control in agricultural systems. Yet, from a product development standpoint, it may be beneficial to have both traits in the same organism (Kloepper 1996).

The worldwide efforts in the search of natural products for the crop protection market have progressed significantly and actinomycetes, especially those belonging to the genus *Streptomyces*, appear to be good candidates to find new approaches to control plant diseases (Behal 2000). Actinomycetes belong to the order *Actinomycetales*, a division of the Gram-positive bacteria characterized by a high genomic G+C content (means 74 mol %) (Fox and Stackebrandt 1987; Goodfellow and Cross 1984; Goodfellow *et al.* 1992; Stackebrandt and Woese 1981). Actinomycete species are well-known saprophytic bacteria that decompose organic matter, especially biopolymers such as lignocellulose, starch, and chitin in soil (Crawford *et al.* 1993). Several actinomycetes have characteristic biological features such as a mycelial growth that culminates in sporulation. They also possess the ability to biosynthesize a wide variety of antibiotics as secondary metabolites (Franklin *et al.* 1989; Lechevalier and Waksman 1962). The agroindustry shows a marked interest for actinomycetes as a source of agroactive compounds, of PGPR, and of biocontrol tools (Behal 2000; Tanaka and Omura 1993).

ACTINOMYCETES AS SOURCE OF AGROACTIVE COMPOUNDS

Actinomycetes have been and remain the most fruitful source of microorganisms for all types of bioactive metabolites, including agroactive type. Over one thousand secondary metabolites

from actinomycetes were discovered during 1988-1992. Most of these compounds are produced by various species of the genus *Streptomyces*. In fact, about 60% of the new insecticides and herbicides reported in the past 5 yr originate from *Streptomyces* (Tanaka and Omura 1993). It is also estimated that as many as three-quarters of all streptomycete species are capable of antibiotic production (Alexander 1977). Actinomycetes produce a variety of antibiotics with diverse chemical structures such as polyketides, b-lactams and peptides in addition to a variety of other secondary metabolites that have antifungal, anti-tumor and immunosuppressive activities (Behal 2000).

Kasugamycin is a bactericidal and fungicidal metabolite discovered by Umezawa and coworkers in *Streptomyces kasugaensis* Hamada *et al.* (Umezawa *et al.* 1965). This antibiotic acts as an inhibitor of protein biosynthesis in microorganisms but not in mammals, and its toxicological properties are excellent. Hokko Chemical Industries developed a production process to market the systemically active kasugamycin for control of rice blast *Pyricularia oryzae* Cavara and bacterial *Pseudomonas* diseases in several crops.

Polyoxin B and D were isolated as metabolites of *Streptomyces cacaoi* var. *asoensis* in 1965 by Isono *et al.* (1965) as a new class of natural fungicides. The mode of action of the polyoxins makes them very acceptable with regard to environmental considerations. They interfere with the fungal cell wall synthesis by specifically inhibiting chitin synthase (Endo and Misato 1969). Polyoxin B found application against a number of fungal pathogens in fruits, vegetables and ornamentals. Polyoxin D is marketed by several companies to control rice sheath blight caused by *Rhizoctonia solani* Kühn.

The validamycin family was detected by Takeda researchers in 1968 in a greenhouse assay when screening streptomycete extracts for activity against rice sheath blight. Validamycin A was found to be a prodrug which is converted within the fungal cell to validoxylamine A, an extremely strong inhibitor of trehalase (Kameda *et al.* 1987). This mode of action gives validamycin A a favorable biological selectivity because vertebrates do not depend on the hydrolysis of the disaccharide trehalose for their metabolism.

The isolation of the antifungal metabolite mildiomycin from a culture of *Streptoverticillium rimofaciens* Niida was reported in 1978, also by Takeda scientists (Iwasa *et al.* 1978). Mildiomycin is strongly active against several powdery mildews on various crops (Harada and Kishi 1978), acting as an inhibitor of the fungal protein biosynthesis (Feduchi *et al.* 1985). Its low toxicity in vertebrates would make it an environmentally sound crop protection agent (Harada and Kishi 1978), but the fact that this product never appeared in recent publications would indicate, however, that Takeda's efforts to develop mildiomycin might not be successful yet.

The compounds mentioned above are a few examples of agroactive compounds isolated from actinomycetes. Microbial screening and chemistry procedures were until recently the main tools to discover new agroactive compounds. However, genomic technologies that allow rapid characterization of microbial genomes will certainly become the method of choice for the discovery of new bioactive molecules in the coming years. Furthermore, molecular techniques such as combinatorial biosynthesis (Hutchinson 1999) may lead to the discovery of drugs that cannot be found in nature. Indeed, genetic domains, modules and clusters involved in the microbial biosynthesis of known secondary metabolites can be interchanged and modified to produce bioactive products with unique properties.

ACTINOMYCETES AS PGPR

In attempts to develop commercial biocontrol and plant growth promoting products using rhizobacteria, it is important to recognize the specific challenges they present. To begin with, the interaction between PGPR species and their plant symbionts appears to be specific, even within a crop or cultivar

(Chanway *et al.* 1988; Glick 1995; Kloepper 1996; Lazarovits and Nowak 1997). While a rhizobacterium screened for growth promotion may reveal positive effects on one crop, it may have no effect, or even retard growth of another crop (Gardner *et al.* 1984; O'Neill *et al.* 1992).

Although rhizobacteria may present unique challenges to our attempts to harness their beneficial attributes, the prospects for improved agriculture by the use of biocontrol-PGPR seem excellent. Advances in our understanding of the PGPR systems responsible for plant growth improvement is a first logical step in opening the way to improving these bacterial strains through genetic engineering, and generating more interest in their development for widespread commercial use for both biocontrol and plant growth promotion. As will be discussed later, we believe that actinomycetes are among the most promising biocontrol-PGPR in need of study in future research.

Despite the well-documented history of *Streptomyces* in biocontrol and preliminary evidence of their capacity to enhance plant growth (Aldesuquy *et al.* 1998), *Streptomyces* species have been poorly investigated specifically for their potential as PGPR. This is surprising as streptomycetes, generally accounting for an abundant percentage of the soil microflora, are particularly effective colonizers of plant root systems and are able to endure unfavorable growth conditions by forming spores (Alexander 1977). While the beneficial effect of some strains of PGPR on particular crops is certain, the mechanisms employed by PGPR are unclear (Glick 1995). PGPR can affect plant growth in two general ways, either directly or indirectly. Indirect promotion occurs when PGPR lessen or prevent the harmful effects of one or more deleterious microorganisms. This is chiefly attained through biocontrol, or the antagonism of soil plant pathogens. Specifically, colonization or the biosynthesis of antibiotics (Fenton *et al.* 1992) and other secondary metabolites can prevent pathogen invasion and establishment. Direct promotion of plant growth by PGPR occurs when the plant is supplied with a compound that is synthesized by the bacteria, or when PGPR otherwise facilitates plant uptake of soil nutrients. Possibilities include nitrogen fixation, siderophore synthesis, phytohormone synthesis, and solubilization of minerals to make them available for plant uptake and use (Glick 1995).

Merriman *et al.* (1974) reported the use of a *Streptomyces griseus* (Krainsky) Waksman and Henrici isolate as a seed treatment of barley, oat, wheat and carrot, in order to increase their growth. The isolate was originally selected for the biological control of *Rhizoctonia solani*. Though the *S. griseus* isolate did increase the average grain yield, dry foliage weight, tiller number, and advanced head emergence for both wheat and oat over controls, the differences were not statistically significant. As a seed treatment for carrot, the isolate was more successful. Marketable yields were increased over controls by 17% and 15% in two separate field trials. Specifically, both trials also indicated an increased yield of large and very large grade carrots over controls (Merriman *et al.* 1974). Nearly 20 yr later, El-Abyad *et al.* (1993) described the use of three *Streptomyces* spp. in the control of bacterial, *Fusarium* and *Verticillium* wilts, early blight, and bacterial canker of tomato. The isolates used were *S. pulcher* Rao *et al.*, *S. canescens* Waksman, and *S. citreofluorescens* (Korenyako *et al.*) Pridham. As seed-coating, all three of the strains were effective at variable levels in controlling the test pathogens. In addition, tomato growth was observed to be significantly improved with the antagonistic *Streptomyces* spp. as a seed-coating. An increased availability of growth regulators produced by the inoculum was the reason proposed for the improvement in tomato growth, although this was not formally tested (El-Abyad *et al.* 1993). While these studies by El-Abyad *et al.* (1993) and Merriman *et al.* (1974) reported plant growth enhancement as a function of inoculation with *Streptomyces*, neither they investigated the inoculum under gnotobiotic conditions, nor the possible mechanisms of streptomycete-mediated growth promotion.

Later, the culture filtrates alone of two different *Streptomyces* spp. *(S. olivaceoviridis* (Preobrazhenskaya and Ryabova) Pridham *et al.* and *S. rochei* Berger *et al.)* were found to significantly increase the shoot length and shoot fresh mass, respectively, of wheat plants. Hormone extraction, purification, and bioassay showed that both species produced substantial amounts of growth-regulating substances, including auxins, gibberellins, and cytokinins (Aldesuquy *et al.* 1998). This demonstrated that selected *Streptomyces* spp. produce at least one class of compounds that directly influence plant growth. However, some other putative mechanisms of plant growth promotion (siderophore biosynthesis and phosphorous solubilization) were not assessed in that study.

A commercial biocontrol product, Arzent™, a mixture of four separate, yet compatible strains of *S. hygroscopicus* (Jensen) Waksman and Henrici (Innovative Biosystems, Inc., Moscow, ID), was also tested for its ability to promote radish growth in the greenhouse (Hamby and Crawford 2000). Radish wet weight was found to be 12% greater with Arzent™ treated radishes as compared to sterile talc carrier treatment, and 13% greater than the untreated controls. Arzent™-treated carrot seeds had wet weights that were 18% greater than those with sterile talc carrier treatments, and 19% greater than untreated controls. Under gnotobiotic growth chamber conditions, carrot seeds treated with *Streptomyces lydicus* De Boer *et al.* WYEC108 had also an increase in carrot wet weight of 20% over those with sterile talc carrier treatments and 21% over those with untreated controls. These results suggest the widespread ability of *Streptomyces* to promote plant growth, independent of its characteristics as pathogen antagonism. Recent work in our laboratory has examined the ability of *S. violaceusniger* (Waksman and Curtis) Pridham *et al.* YCED9 to specifically enhance overall plant growth and yield as an activity not associated with biocontrol. To assess this, radishes were grown in greenhouse trials under non-sterile conditions. Spore-containing formulations of YCED 9 were prepared in a sterile talc carrier ($1x10^8$ cfu g^{-1}). Sterile radish seeds were inoculated with $1x10^7$ cfu of YCED9 in the sterile talc carrier, while controls received the sterile talc carrier alone. The plants were germinated in potting soil, and grown for 30 d. At harvest, radishes treated with YCED9 and grown under greenhouse conditions exhibited statistically significant increases in total plant wet weight over the sterile talc carrier control treatments (18%), and over the untreated controls (19%) (Hamby and Crawford 2000).

Minor pathogens, while not typically manifesting disease in a plant, may impede plant growth, either by sequestering nutrients or through excretion of products detrimental to the plant (Davison 1988). To assess this possibility, we grew sterile carrot seeds *in vitro* on sterile agar-water. Seeds were aseptically inoculated with spores ($1x10^7$ cfu) of YCED9 in a sterile talc carrier. Control seeds received sterile talc carrier alone. The seeds were allowed to germinate and grown for 30 d. At harvest, carrot seedlings treated with YCED9 and grown in gnotobiotic growth chamber conditions had a statistically higher average wet weight over that of plants from the sterile talc treatments (17%), as well as over untreated controls (19%) (Hamby and Crawford 2000). Even though *S. violaceusniger* YCED9 does produce antibiotics and other antifungal metabolites, the results suggested that there were also more direct means of plant growth promotion occurring, in part because the parameters of this experiment were gnotobiotic. Growth promotion was not limited to YCED9. When we tested other species of *Streptomyces*, similar results were observed. In greenhouse assays, radishes grown from seed treated with *Streptomyces lydicus* WYEC108, also isolated by Crawford *et al.* (1993) had a wet weight 8% greater than that of sterile talc treatments, and 10% greater than untreated controls.

Direct and indirect interactions between actinomycetes and other nonpathogenic soil microorganisms also influence plant growth. For example, Abdel-Fattah and Mohamedin (2000),

Ames (1989), Becker *et al.* (1999) and Tylka *et al.* (1991) reported that actinomycetes stimulated the intensity of mycorrhizal formation and that resulted in improved plant growth.

The information available on streptomycetes as plant growth promoters is limited, so is the information describing the possibility of their direct growth promotion mechanisms. Like most rhizobacteria, it seems highly probable that streptomycetes are capable of directly enhancing plant growth. Manulis *et al.* (1994) described the production of the plant hormone indole-3-acetic acid (IAA) and the pathways of its synthesis by various *Streptomyces* spp. including, *S. violaceus* (Rossi Doria) Waksman, *S. scabies* (Thaxter) Waksman and Henrici, *S. griseus, S. exfoliatus* (Waksman and Curtis) Waksman and Henrici, *S. coelicolor* (Müller) Waksman and Henrici, and *S. lividans* Krasil'nikov *et al.* While prior works had reported IAA synthesis in *Streptomyces* spp. (El-Sayed *et al.* 1987; El-Shanshoury 1991), this was the first confirmation of its production using modern analytical methods such as HPLC and GC-MS, and Manulis *et al.* (1994) provided a detailed description of the IAA biosynthetic pathways in *Streptomyces*. There is very little understanding about microbial phytohormones and even less so about their effect on plant growth. Aldesuquy *et al.* (1998) studied the effect of streptomycete culture filtrates on the growth of wheat plants. Shoot fresh mass, dry mass, length, and diameter all displayed statistically significantly increases with certain strains at varying sample times. *S. olivaceoviridis* had a pronounced effect on yield components (spikelet number, spike length, and fresh and dry mass of the developing grain) of wheat plants. The culture filtrates of all three strains appear to enhance the growth and crop yield of wheat plants (Aldesuquy *et al.* 1998). This study lent further credence to the possibility that certain rhizobacteria, including the actinomycetes, may act as plant growth enhancers. This activity may be due to, at least in part, an increase in bioavailable phytohormones that are PGPR-produced since all PGPR strains (*Streptomyces rimosus* Sobin *et al.*, *Streptomyces rochei*, and *Streptomyces olivaceoviridis*) produced substantial amounts of exogenous auxins (IAA), as well as gibberellins and cytokinins.

The endogenous (Muller and Raymond 1984) and exogenous (Muller *et al.* 1984) production of siderophore-mediated iron transport have been characterized in a strain of *Streptomyces pilosus* Ettlinger *et al.* by Muller *et al.* (1984). Both the endogenous (ferrioxamine) and exogenous (ferrichrome) siderophores of this species are classified as the hydroxamate-type and appear to be cross-reactive, as they share a common transport system (Muller *et al.* 1984). We are investigating streptomycete production of siderophores as a mechanism facilitating plant iron uptake. Preliminary results have shown strain YCED9 to be positive for iron chelation under iron-limited conditions. This was shown using a modification (Buyer *et al.* 1989) of Schwyn and Neilands CAS assay (Schwyn and Neilands 1987) in both solid and liquid culture. The specific nature of these siderophores is still under investigation. Though the microbial production of phytohormones and siderophore is as yet not fully understood, the widespread ability of PGPR to produce IAA indicates a putatively important mode of interaction with plants (Brown 1972; Kloepper *et al.* 1989; Tien *et al.* 1979).

ACTINOMYCETES AS BIOCONTROL TOOLS

A prime example of *Streptomyces* biocontrol agent is *Streptomyces griseoviridis* Anderson *et al.* strain K61. This strain, originally isolated from light coloured *Sphagnum* peat (Tahvonen 1982a, 1982b), has been reported to be antagonistic to a variety of plant pathogens including *Alternaria brassicola* (Schw.) Wiltsh., *Botrytis cinerea* Pers.:Fr., *Fusarium avenaceum* (Fr.:Fr.) Sacc., *F. culmorum* (Wm. G. Smith) Sacc., *F. oxysporum* Schlechtend.:Fr. f.sp. *dianthi* (Prill. & Delac.) W.C. Snyder & H.N. Hans, *Pythium debaryanum* Hesse sensu Middleton, *Phomopsis sclerotioides* Kesterens, *Rhizoctonia*

solani and *Sclerotinia sclerotiorum* (Lib.) de Bary (Tahvonen 1982a, 1982b; Tahvonen and Avikainen 1987). *Streptomyces griseoviridis* strain K61 is used in root dipping or growth nutrient treatment of cut flowers, potted plants, greenhouse cucumbers, and various other vegetables (Mohammadi and Lahdenpera 1992). Mycostop™ (developed by Kemira Oy) is a biofungicide that contains *S. griseoviridis* as the active ingredient. This product is available in United States (Cross and Polonenko 1996) and Europe (Tahvonen 1982a).

Several properties associated with actinomycetes might explain the ability of sereval of them to act as biocontrol tools. Those properties are the ability to colonize plant surface, the antibiosis against plant pathogens, the synthesis of particular extracellular proteins, and the degradation of phytotoxins.

Plant colonization and biocontrol

Evidence indicates that actinomycetes are quantitatively and qualitatively important in the rhizosphere (Barakate *et al.* 2002; Crawford *et al.*1993; Doumbou *et al.* 2001; Miller *et al.* 1989, 1990), where they may influence plant growth and protect plant roots against invasion by root pathogenic fungi (Lechevalier 1988). However root-microorganism interactions have been extensively studied only for the nitrogen-fixing *Frankia* species (Sardi *et al.* 1992) and a few species of the genus *Streptomyces* that are phytopathogens (Loria *et al.* 1997).

It is generally assumed that root colonization by introduced bacteria is essential for the biocontrol of root pathogens and that increasing the population of such an introduced bacteria on roots should enhance disease control (Suslow and Schroth 1982). The key to the concept of root colonization is that root-colonizing bacteria grow on roots in the presence of the indigenous microflora (Schroth and Hancock 1982) and thus root colonists are competitive with soil bacteria and fungi. While we prefer to use the term root colonization, other terms have been proposed. Rhizosphere competence was used by Schmidt (1979) in relation to rhizobia, to describe soil microorganisms that show enhanced growth in response to developing plant roots. In this context, rhizosphere competent microorganisms are those that show the classical rhizosphere effect. The term rhizosphere competence has been used in relation to biological control agents, and Baker (1991) redefined it as the ability of a microorganism, applied by seed treatment, to colonize the rhizosphere of developing roots, a definition that does not differ substantially from that proposed by Schmidt (1979). Several reports have used rhizosphere competence and root colonization interchangeably as synonyms (Hozore and Alexander 1991; Suslow and Schroth 1982). While each definition differs from the others, there is general agreement that root colonization is an active process involving growth of the introduced bacteria on or around roots and is not simply a passive chance encounter between a soil bacterium and a root. We consider true colonists to be those bacteria that colonize plant surfaces in competitive conditions, in natural field soils.

A microorganism that colonizes roots is ideal for use as a biocontrol agent against soil-borne diseases (Weller 1988). *Streptomyces griseoviridis* is a good example for colonization of plant rhizosphere by actinomycetes. *S. griseoviridis* is an antagonistic microorganism effective in biocontrol of plant diseases such as the *Fusarium* wilt of carnation, the damping-off of *Brassica* and the root rot disease of cucumber (Tahvonen and Lahdenpera 1988). The active root-colonization ability of the biocontrol agent *S. griseoviridis* was tested on turnip rape and carrot in non-sterile soil and by plate test (Kortemaa *et al.* 1994). Plate test and successful root-colonization, root-colonization frequencies and population densities for sand-tube method all indicate that *S. griseoviridis* colonizes, at least at the seedling stage, turnip rape better than carrot root. Since the responses of *S. griseoviridis* to root colonization of two plant species in standard conditions were clearly different, the mechanism of root colonization must be affected by some property that varies between dif-

ferent plant species. Plant species are known to produce various types and quantities of root exudates (Curl and Truelove 1986), which influence root colonization (Weller 1988). It is possible that the root exudates of carrot lack some characteristics necessary for the proliferation of *S. griseoviridis*. The efficacy of *S. griseoviridis* seed dressing on barley and spring wheat against foot rot disease was investigated by Tahvonen *et al.* (1994) who demonstrated that wheat yields can be increased by seed dressings more efficiently than those of barley.

Juhnke *et al.* (1987) tested the root-colonization ability of bacteria on wheat in fields and found that compared to other rhizobacteria, *Streptomyces* species were poor colonizers. In contrast, studies of Petrolini *et al.* (1996) and Sardi *et al.* (1992) provided evidence that streptomycetes are constantly present in cortical tissues of roots and that, despite heterogeneity in individual features, they can be regarded as a population that is reasonably consistent, having some common, well defined physiological peculiarities. Actinomycetes are also isolated from other plant organs: seeds and ovules (Mundt and Hinkle 1976), leaves (de Araujo *et al.* 2000; Matsukuma *et al.* 1995), tubers (Faucher *et al.* 1992). Endophytic streptomycetes (Gurney and Mantle 1993) colonize an ecological niche similar to that of pathogens, especially vascular wilt pathogens, which might favor them as candidates for biocontrol agents. Some intensive work on rhizosphere biocontrol agents has shown that five of six rhizobacteria, which induced systemic resistance in cucumber, exhibited both external and internal root colonization (Kloepper and Beauchamp 1992). In our laboratory, we isolated several actinomycetes from potato tuber, that protected raspberry and potato against different plant pathogens (Doumbou *et al.* 1998; Faucher *et al.* 1992; Valois *et al.* 1996). Proteomic studies are currently in progress on some of these biocontrol agents. The purpose of our work is to identify actinomycetal proteins that are specifically induced in the presence of plants as well as the genetic determinants involved in plant root colonization.

Proteins involved in biocontrol

Actinomycetes have the ability to produce a wide variety of extracellular enzymes that allow them to degrade various biopolymers in soil. The capacity of actinomycetes to produce extracellular enzymes gained renewed attention due to their important role in biocontrol. In particular, numerous correlations between fungal antagonism and bacterial production of chitinases or glucanases have been noted (Fayad *et al.* 2001; Lim *et al.* 1991; Valois *et al.* 1996). Chitin and b-1,3-glucans are major constituents of many fungal cell walls (Sietsma and Wessels 1979), and various workers have demonstrated *in vitro* lysis of fungal cell walls either by bacterial chitinases or glucanases alone or by a combination of both enzymes (Fiske *et al.* 1990; Ordentlich *et al.* 1988). These studies have lent support to the hypothesis that these hydrolytic enzymes may contribute to biocontrol efficacy. Several attempts to test this hypothesis using both genetic and molecular approaches have been undertaken recently.

The amendment of soil with certain organic substrates, particularly chitin, has been shown to increase the proliferation of *Streptomyces* (Kutzner 1981), as well as their production of chitinases (Trejo-Estrada *et al.* 1998). We also showed that mature composts amended with chitin residues acquired suppressive properties against several plant pathogens (Côté *et al.* 2001; Labrie *et al.* 2001; Roy *et al.* 1997). In these suppressive composts, the microbial population is characterized by a proliferation of Gram-positive bacteria belonging mainly to the bacterial group of actinomycetes (Labrie *et al.* 2001). Chitin is found in the cuticle of insects and the shell of crustaceans and mollusks as well as in the cell walls of most taxonomic groups of fungi. Chitin in fungal cell walls is normally present in a highly rigid, crystalline state. In the hyphal apex, however, the chitin is sensitive to treatments with dilute HCl or chitinase (Wessels *et al.* 1990). The sensitivity of the fungal cell wall to lytic enzymes has

been exploited by using chitinase-producing bacteria to control plant-pathogenic fungi in the rhizosphere. During the last decade, chitinases have received increased attention because of their wide range of applications. Since some cell walls are rich in chitin, the potential application of chitinase for biocontrol of fungal phytopathogens is promising. The chitinase-producing strains, including actinomycetes, could be used directly in biocontrol of fungi or indirectly by using purified proteins or through gene manipulation. Yuan and Crawford (1995) investigated mechanisms involved in the control of fungal root and seed diseases by an antifungal biocontrol agent, *Streptomyces lydicus* WYEC108, an organism which was isolated from the rhizosphere of linseed plants, and produces both antifungal antibiotics and extracellular chitinase. Results obtained showed that WYEC108 was capable not only of destroying germinating oospores of *Pythium ultimum* Trow but also of damaging the cell walls of the fungal hyphae. These results show that *S. lydicus* WYEC108 is a potentially potent biocontrol agent for use in controlling *Pythium* seed and root rot.

Valois *et al.* (1996) screened a collection of actinomycetes for their ability to grow on fungal cell wall of pathogenic *Phytophthora* species. Thirteen strains were selected and shown to produce extracellular β-1,3-, b-1,4-and b-1,6-glucanases. These enzymes can hydrolyze glucans from *Phytophthora* cell walls and cause lysis of living *Phytophthora* cells. From these 13 strains, 11 were able to protect raspberry plants against *Phytophthora fragariae* C.J. Hickman var. *rubi.* Nevertheless, no strict correlation has been established between production of these hydrolytic enzymes and the biocontrol ability of actinomycetes in our study. However, such a correlation between enzyme production and the capacity to suppress plant diseases, has been suggested for another biocontrol agent by Chernin *et al.* (1995). They showed that the ability of *Enterobacter agglomerans* Ewing and Fife, a gram-negative biocontrol agent, to protect plants against diseases caused by *Rhizoctonia solani*, a fungus with chitinous cell walls, was lost by making it defective in chitinase production.

Many *Streptomyces* species are lignocellulose decomposers (Chamberlain and Crawford 2000) and are sources of antibiotics (Tanaka and Omura 1993). Strains with both the abilities to degrade lignocellulose and antagonize fungal root pathogens should have good potential for development into a biocontrol product, which could be useful to turfgrass growers or managers, to control both thatch accumulation and fungal diseases of turf (Chamberlain and Crawford 2000). The ability to degrade complex substrates could also be an asset in biocontrol. Doumbou *et al.* (1998) showed that actinomycetes degrading thaxtomin A, a phytotoxin produced by the plant pathogenic *S. scabies*, protected growing potato plants against common scab.

Actinomycetal proteins other than hydrolases might also be involved in biocontrol. For example, Vernekar *et al.* (1999) discovered an alkaline protease inhibitor (API) as a novel class of antifungal proteins against phytopathogenic fungi such as *Alternaria, Fusarium*, and *Rhizoctonia*. The activity of API appears to be associated with its ability to inhibit the fungal serine alkaline protease, which is indispensable for their growth.

Antibiosis and biocontrol

Over one thousand secondary metabolites from actinomycetes were discovered during the years 1988-1992. Actinomycetes produce a variety of antibiotics with diverse chemical structures such as polyketides, β-lactams and peptides in addition to a variety of other secondary metabolites that have antifungal, anti-tumor and immunosuppressive activities (Behal 2000). Antibiotics are generally considered to be organic compounds of low molecular weight produced by microbes. It is proposed that the production of antibiotics increases an organism aptitude for survival in the former case by acting as an alternative (chemical) defense mechanism (Maplestone *et al.* 1992). Several studies have reported antagonism between actinomycetes and a diversity of

phytopathogens such as *Alternaria, Fusarium, Macrophomina, Phytophthora, Pythium, Rhizoctonia, Verticillium* (Chattopadhyay and Nandi 1982; Heisey and Putnam 1986; Hussain *et al.* 1990; Iwasa *et al.* 1970; Merriman *et al.* 1974; Rothrock and Gottlieb 1981, 1984; Sabaou and Bounaga 1987; Trejo-Estrada *et al.* 1998; Valois *et al.* 1996; Wadi and Easton 1985).

Antibiosis as a mechanism of biological control of plant disease has been studied in several systems (Chamberlain and Crawford 1999; Crawford *et al.* 1993; El-Abyad *et al.* 1993; Kortemaa *et al.* 1994). Gottlieb (1976) has reviewed the evidence that antibiotics may be produced by members of soil microflora in their natural environment, and function there in an antagonistic capacity. Numerous experiments have demonstrated the difficulty in introducing a new organism into normal soil which already has an established indigenous population and, in contrast, the ease with which organisms can be introduced into sterile soil (Gottlieb 1976). Such experiments clearly demonstrate that microorganisms in soil are in direct competition, so that, any factor which kills other organisms would certainly be advantageous to the producer. The evidence does not prove that antibiotics are responsible for the competitive antagonism between species since, as yet, antibiotics have not been physically isolated from soil. However, there is evidence for the analytical detection of antibiotics in soil; in particular, Zviagintsev *et al.* (1976) have demonstrated that antibiotic heliomycin is produced by *Actinomyces olivocinereus* Vinogradova in unsterilised, unsupplemented soil. They used the method of fluorescent microscopy, utilizing the intrinsic fluorescence of the actinomycete, to observe the development of precursor and synthesis of the antibiotic directly in soil.

Demain (1980, 1989) has presented evidence that antibiotics are indeed produced in nature and are implicated in competition between bacteria, fungi and amoeba and between microorganisms and higher plants, insects or large animals. For example, mutants of *Gliocladium virens* Miller *et al.* show a strong correlation between the ability to produce the antibiotic gliovirin and the ability to protect cotton seedlings from disease caused by the phytopathogen, *Pythium ultimum* (Howell and Stipanovic 1983). This indicates that antibiotics do indeed act in antagonistic capacities in nature.

Rothrock and Gottlieb (1984) presented evidence that the antibiotic geldanamycin is produced in soil by *Streptomyces hygroscopicus* var. *geldanus*, and that the antibiotic accounts for the antagonism of *S. hygroscopicus* var. *geldanus* to *Rhizoctonia solani* in soil. *Streptomyces hygroscopicus* var. *geldanus* inhibited the growth of *R. solani* and controlled *Rhizoctonia* root rot of pea in previously sterilized soil if incubated for 2 or more d prior to infesting soil with *R. solani* and planting. Methanol extracts of soils in which the antagonist was incubated for 2 or more d inhibited growth of *R. solani*. The geldanamycin concentration was present at 88 μg gram^{-1} of soil on average, after 7 d of incubation. The period of incubation necessary for antibiotic production and disease control was similar, with no disease control occurring where no antibiotic was detected. Amending the soil with geldanamycin in amounts equivalent to that produced after two or seven days of incubation controlled disease and reduced saprophytic growth of the pathogen. *Streptomyces hygroscopicus* var. *geldanus* strain EF-76 protected potato against common scab. Mutants of *S. hygroscopicus* strain EF-76 defective in the production of geldanamycin lost the ability to control the disease (Beausé-jour *et al.* 2001).

Control of potato scab by two other *Streptomyces* strains (*S. diastatochromonogenes* (Krainsky) Waksman and Henrici strain PonSSII and *S. scabies* strain PonR) was also demonstrated in a 4-yr field-plot experiment (Liu *et al.* 1995). These two strains produced antibiotics lethal to pathogenic strains of *S. scabies* in antibiosis tests. Both strains significantly decreased the appearance of scab on potato tubers as compared to the nonamended control treatment. Strain PonSSII had numeri-

cally fewer scab lesions than strain PonR every yr. This is in direct correlation with the more vigorous growth and larger inhibition zones of PonSSII against pathogenic strains than those of PonR in *in vitro* tests. Becker *et al.* (1997) showed that antibiotic production by suppressive strain PonSSII could be stimulated or repressed by the presence of other *Streptomyces* strains including pathogenic ones. Interspecies communication could thus be of significance in pathogen suppression.

Streptomyces violaceusniger YCED9 is a good model as an example of the potential of a streptomycete as a biological control agent. It was isolated in 1990 from rhizosphere soil and originally selected for its potential to suppress damping-off in lettuce caused by *Pythium ultimum* (Crawford *et al.* 1993). Subsequent studies of YCED9 revealed that it produced three antimicrobial compounds. These included nigericin, geldanamycin, and a fungicidal complex of polyene-like compounds termed AFA (Anti-*Fusarium* Activity) that included guanidylfungin A (Trejo-Estrada *et al.* 1998). The composition of the media in which YCED9 was grown was found to play a key role in the production of each of these metabolites.

In plate bioassays, YCED9 inhibited *in vitro* growth of several fungal pathogens. In greenhouse trials, YCED9 provided significant disease suppression of four significant turfgrass pathogens, including *Colletotrichum graminicola* (Ces.) G.W. Wils., *Sclerotinia homeocarpa* F.T. Bennett, *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier FL-151, and *Rhizoctonia solani* MSU (Trejo-Estrada *et al.* 1998). In a related study, YCED9 was used to actively degrade thatch (12-16%) in a solid-state thatch-culture system that investigated the potential of YCED9 to enhance turfgrass thatch degradation (Chamberlain and Crawford 2000). Though not the focus of the study, YCED9 spore formulations were also observed to enhance growth (observable biomass yield) of Kentucky bluegrass as compared to untreated control plant cultures in growth chamber experiments. The study also found that for all but one of six fungal pathogens tested, the YCED9 formulation cultures reduced disease incidence, as shown by observable plant biomass yields in comparison to pathogen-treated controls (Chamberlain and Crawford 2000).

Although purified antibiotics are applied as pesticides to control plant diseases, for many years it was questioned whether or not antibiotics were produced in nonsterile soil in sufficient quantity to play any role in interactions among microorganisms. There is now sufficient evidence, mostly obtained by genetic methods, to indicate that antibiotics do function in biocontrol in nature.

PERSPECTIVES

Several scientific publications indicate that actinomycete species are capable of effectively controlling fungal and bacterial plant pathogens. In most cases, the levels of biocontrol achieved by the various actinomycetes in laboratory or controlled-environment studies are sufficient to suggest that they could provide reliable, effective, alternative to or complement chemical pesticides. Therefore, a few plant pathogens have been controlled successfully by actinomycetes species, but many attempts to develop biocontrol formulations have met with problems in practice. In order to develop actinomycete biocontrol agents for commercial use, the consistency of their performance must be improved. Accomplishing this will require research in many diverse areas, because biological control is the culmination of complex interactions among host, pathogen, antagonist, and environment.

New research to identify actinomycete traits that function in plant colonization and pathogen antagonism is critically important, and molecular genetics offers the best approach to such studies. For example, transposon mutagenesis can be used to generate mutants of actinomycetes deficient in single traits of interest. The mutants can then be evaluated to establish the importance of those traits for the bio-

control ability of that actinomycete strain. Identifying important traits allows more efficient selection of new strains. Such traits can further be altered to make a strain more effective. Ultimately, the possibility exists of genetically engineering superior biocontrol agents by moving genes from one strain to another. The lux technology (White *et al.* 1996), which is based on transformation of environmental bacteria with genes of the lux operon from the marine bacteria *Vibrio fischeri* (Beijerinck) Lehmann and Neumann and *V. harveyi* (Johnson and Shunk) Baumann *et al.*, can be used for that purpose. The transformed strains are bioluminescent and thus provide a rapid and very accurate tool for the study of population dynamics, metabolic activity and spatial distribution of specific actinomycetes in environmental samples. Isolation of genes likely to enhance ecological performance on basis of their elevated level of expression in rhizosphere can be used. Additional research is also needed on soil physical and chemical factors that influence both root colonization and the expression of traits important to antagonism in the rhizosphere. By identifying these factors, it may be possible to manipulate these in the field so as to enhance root colonization. More research is also needed on the formulation and delivery of the preparations so they remain viable under less than optimal conditions. It must be kept in mind that growers cannot be expected to buy new equipment or to modify equipment or farming practices substantially to accommodate a biological treatment.

Research teams should not overlook the possible negative impacts linked to the use of biocontrol tools. Since biocontrol is often associated with antibiosis, pathogens might develop resistance to antibiotics produced by antagonistic actinomycetes. In fact, Neeno-Eckwall and Schottel (1999, 2001) established that spontaneous mutants of the pathogenic *Streptomyces scabies* strain RB4 exhibiting resistance to an antibiotic produced by a potato scab-suppressive isolate arose at a frequency of 10^{-4}. Antibiotics produced by actinomycetes often have a large spectrum of action. Effect of introduced actinomycetes on non-target organisms has thus to be evaluated. Evidence for horizontal transfer of virulence genes between common scab-inducing streptomycetes and saprophytic species has been recently presented (Bukhalid *et al.* 2002). Horizontal transfer of genes between introduced actinomycetes and indigenous microorganisms is another risk associated with biocontrol that needs to be assessed.

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