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Mechanisms involved in biocontrol by microbial inoculants

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Summary — Biological control offers alternative environmentally friendly strategies for the control of phytopathogens in agriculture and horticulture. Biocontrol metabolites are designed so that they do not have any adverse effects on host plants or on indigenous microflora and, in addition, resistance to these metabolites does not appear to develop. As promising alternatives to chemical pesticides, some biocontrol agents have been found to produce a variety of antifungal secondary metabolites and lytic enzymes. The 2,4-diacetylphloroglucinol is a secondary metabolite produced by *Pseudomonas fluorescens* F113, a strain capable of protecting sugar beet against the causal agent of 'damping off', *Pythium ultimum;* environmental and genetic factors involved in 2,4-diacetylphloroglucinol production are discussed. *Stenotrophomonas maltophilia* strain W81(P) produces chitinase and protease enzymes and is capable of conferring plant protection against the disease-causing activity of *Pythium ultimum* in vitro; transposon mutagenesis and subsequent in vivo assays have demonstrated that the biocontrol ability of W81(P) is mediated by lytic enzyme production.

Pseudomonas fluorescens* / 2,4-diacetylphloroglucinol / *Stenotrophomonas maltophilia* / hydrolytic enzymes / *Pythium ultimum

Résumé — Mécanismes impliqués dans le biocontrôle par des inoculants microbiens. Le contrôle biologique offre une stratégie alternative et respectueuse de l'environnement pour le contrôle d'agents phytopathogènes. Les métabolites de biocontrôle sont conçus de façon à ne pas avoir des effets négatifs sur les plantes hôtes et sur la microflore indigène ; de plus, ces métabolites ne semblent pas développer des phénomènes de résistance à leur action. En tant qu'alternative encourageante aux pesticides chimiques, des agents de biocontrôle peuvent produire une variété importante de métabolites secondaires et d'enzymes lytiques. Le 2,4 diacéthylfluoroglucinol est un métabolite secondaire produit par *Pseudomonas fluorescens* F113, une souche capable de protéger la betterave à sucre vis-à-vis de *Pythium ultimum*, agent de fonte de semis ; les facteurs environnementaux et génétiques conditionnant la production du 2,4 diacéthylfluoroglucinol sont discutés. *Stenotrophomonas maltophilia* strain W81(P) produit des chitinases et des protéases capables de conférer une protection vis-à-vis des maladies induites par le *Pythium ultimum.* Après mutagenèse par transposon et des essais in vivo, il a été démontré que l'activité de biocontrôle de *Stenotrophomonas maltophilia* se fait effectivement via la production d'enzymes lytiques.

Pseudomonas fluorescens* / 2,4-diacétylphloroglucinol / *Stenotrophomonas maltophilia* / enzymes hydrolytiques / *Pythium ultimum

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INTRODUCTION

The fact that consumers of agricultural produce are now demanding foods which are free from chemical contamination, in particular pesticide residues, has been recognized both by food producers and legislators. As a direct result, the agrifood industry is now facing directives limiting the use of and, in some cases, banning certain agrichemicals. Therefore, in order to maintain current food production levels, alternatives must be exploited for the safe control of crop pathogens and pests. Presently, the control of soil-borne diseases of food crops is mediated through the application of fungicides and soil fumigants such as methyl bromide (MeBr). However, fungicides traditionally used for the control of plant pests, for example metalaxyl and benomyl, are now widely perceived as environmentally detrimental. MeBr, used as a soil fumigation agent, is a significant contributor to stratospheric ozone depletion as greater than 60% of the applied pesticide may be released from the plant bed and enter the atmosphere (Noling and Becker, 1994). These chemical pesticides may, in some cases, have also lost their antifungal effectiveness due to the emergence of resistant pathogenic strains. Furthermore, the application of chemical fungicides and fumigation of soil may be lethal to many soil insects and to key microorganisms (eg, mycorrhizal fungi) that underpin biological mechanisms that ensure soil fertility. The resulting agricultural produce may often contain higher levels of pesticide residue than the legal limit set by several countries. In an attempt to overcome these problems the EU Commission has limited the production and use of MeBr to 1991 levels, with an aim to further decreases within a few years. An international treaty (USEPA 1993) further restricts MeBr use.

Biological control offers significant advantages over the use of such chemical treatments. Biocontrol strategies, based on the negative interactions among microbial communities and between microbes and higher organisms (Weller, 1988; Atlas et al, 1993), tend to be highly selective. These strategies do not have any adverse effects on the host plant or on the indigenous beneficial microbial population and, in addition, resistance to biopesticides does not appear to develop.

Biocontrol strategy

Suppressive soils in which the development of soil-borne plant diseases are impeded are well documented (Weller, 1988). Such soils may provide a rich and valuable reserve of plant-beneficial microorganisms. These natural examples of biological control are often due to the presence of health-promoting rhizosphere microflora. In particular, it is recognized that some pseudomonads, especially strains of fluorescent *Pseudomonas*, play a positive role in pest inhibition in established suppressive soils. This observed protection of plants against attack by fungal and other pests is often due to the production of a variety of secondary metabolites (reviewed in O'Sullivan and O'Gara, 1992). Plant protection against pathogen attack conferred by application of strains of fluorescent *Pseudomonas* has been shown to be mediated by compounds such as phenazines, pyrrol-type antibiotics and 2,4-diacetylphloroglucinol (Phl) produced by these microbes (O'Sullivan and O'Gara, 1992; Thomashow and Weller, 1992; Dowling and O'Gara, 1994; Voisard et al, 1994; Cronin et al, unpublished results). In addition, niche exclusion, competition for nutrients, production of iron scavenging siderophores, cyanide and lytic enzymes have all been implicated directly in plant protection by pseudomonad isolates (O'Sullivan and O'Gara, 1988, 1992; Lorito et al, 1994; Kobayashi et al, 1995; Cronin et al, unpublished results; Dunne et al, unpublished results). A more direct mode of action involves the stimulation of the plant's own defence mechanisms through the induction of systemic acquired resistance (Smith et al, 1991; Van Peer et al, 1991; Leeman et al, 1995; Pieterse et al, 1996).

In an effort to improve or create novel biocontrol agents as an effective alternative to chemical treatments, extensive studies have resulted in the isolation and identification of a number of the secondary metabolites involved in plant protection. Many effective biocontrol strains produce hydrogen cyanide (HCN) and Phl which is largely responsible for the protection of sugar beet against 'damping off' caused by the fungus *Pythium ultimum* (reviewed in Dowling and O'Gara, 1994; Shanahan et al, 1992). The role of Phl in the protection of agronomically important crops has been demonstrated against a wide

variety of fungal pathogens. However, the control of certain diseases may require the production of more than one antifungal metabolite. For instance, the control of black root rot of tobacco, caused by *Thielaviopsis basicola*, requires the production of both Phl and HCN by the *Pseudomonas fluorescens* strain CHAO (Keel et al, 1992).

Alternatively, the inhibition of phytopathogenic fungi in soil by lytic enzyme producing bacteria has previously been proven as a relatively successful biocontrol strategy. Chitinases and glucanases have a proven ability to degrade the chitin and glucan matrix integral to the structure of many fungal cell walls (Lorito et al, 1994). Successful cloning of the biosynthetic genes responsible for the production of these enzymes has resulted in the development of transgenic microbes and plants with improved abilities to combat fungal disease due to the production and export of microbial enzymes.

The role of 2,4-diacetylphloroglucinol (Phl) in biocontrol

While Phl is seen to have a broad inhibitory spectrum and may effectively inhibit such phytopathogenic fungi as *Pythium*, *Gaeumannomyces* and *Thielaviopsis* (Keel et al, 1990, 1992; Haas et al, 1991; Vincent et al, 1991; Fenton et al, 1992), the exact mechanism of action of this compound has yet to be fully elucidated.

P fluorescens strain F113 is a soil isolate that originated from the rhizosphere of sugar beet plants grown in a suppressive soil capable of conferring protection against the causal agent of 'damping off', *P ultimum*, both in the laboratory and in vivo. This strain produces a number of secondary metabolites including protease, HCN and Phl (Shanahan et al, 1992). Current studies involving F113 are concentrating largely on regulation and synthesis on Phl through biochemical and genetic techniques.

Factors affecting 2,4-diacetylphloroglucinol production

A high performance liquid chromatography (HPLC) assay has been developed for the determination of Phl and its monoacetylated precursor, monoacetylphloroglucinol (MAPG), in growth culture media (Shanahan et al, 1993). This assay is used to monitor production of the two secondary metabolites by the *Pseudomonas* strain F113 in both qualitative and quantitative fashion. This facilitates the investigation of physiological as well as regulatory parameters influencing their production.

Microorganisms often display a preference for specific carbon sources for the production of particular secondary metabolites. It has previously been reported that carbon and nitrogen sources can influence such metabolite production (Feng et al, 1994). *P fluorescens* strain HV37a pro-

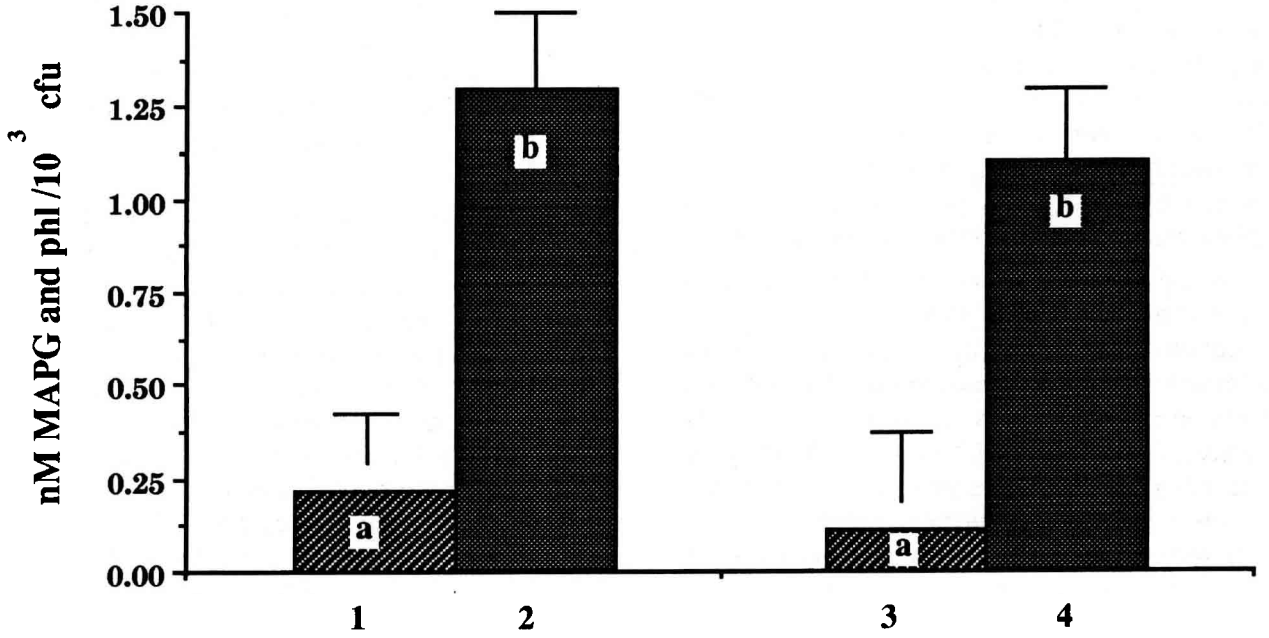

Fig 1. Effect of carbon source availability on the production of monoacetylphloroglucinol (MAPG) and diacetylphloroglucinol (Phl) by *Pseudomonas fluorescens* F113. 1) MAPG production in 0.1 M succinate; 2) MAPG production in 0.1 M sucrose; 3) Phl production in 0.1 M succinate; 4) Phl production in 0.1 M sucrose. Columns containing the same letter are not significantly different at the $P < 0.05$ level by analysis of variance.

duces three distinct antifungal metabolites, and the biosynthesis of these was seen to be differentially regulated by glucose (Douglas and Gutterson, 1986). The production of Phl and MAPG by F113 is greatly dependent on the carbon and nitrogen source on which it is grown. This effect is most apparent when the strain is grown with either sucrose or succinate as the sole carbon source (fig 1). This holds major implications for effective biological control as antifungal secondary metabolite production will be dependent on the components of seed and root exudates. A knowledge of the nutrient components of these plant exudates will help predict the production of Phl in situ.

Phl production in F113 is also influenced by varying growth conditions. We have previously reported that the optimum temperature for Phl production is 12 °C and that F113 only produces Phl in small quantities unless the ratio of surface area to volume was increased. This is an indication that the physical parameters of soil could be conducive to maximum metabolite production (Shanahan et al, 1992).

The production of various secondary metabolites, including siderophore, HCN and protease, has been seen to be affected by the iron status of the cell (Adams et al, 1994). The concentration of available Fe^{3+} ions enhances Phl production in F113. In a plate inhibition assay, in which overnight cultures of F113 were grown on SA media amended with increasing concentrations of ferric chloride, increased Phl production resulted in increased inhibition of the *Bacillus* test culture (Dowling et al, 1996) (fig 2).

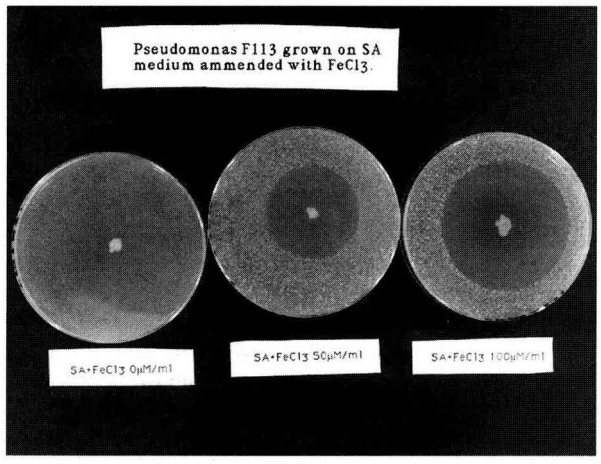

Fig 2. Effect of iron availability on the biocontrol ability of *Pseudomonas fluorescens* F113 against a diacetylphloroglucinol (Phl) sensitive *Bacillus* strain: *Pseudomonas fluorescens* F113 grown in the presence of 0 μM $FeCl_3$, *Pseudomonas fluorescens* F113 grown in the presence of 50 μM $FeCl_3$, *Pseudomonas fluorescens* F113 grown in the presence of 100 μM $FeCl_3$.

Genetic factors in 2,4-diacetylphloroglucinol synthesis

Mutants of *P fluorescens* strain F113 defective in Phl production have been isolated and characterized. These mutants were shown to be incapable of inhibiting *Pythium* using an in vitro plate bioassay and through HPLC analysis demonstrated as unable to produce Phl or MAPG. Further phenotypic characterization allowed the mutants to be classed as either biosynthetic or regulatory mutants. G22 has been previously reported as a member of the Phl biosynthetic class of mutants of F113 and is a near isogenic mutant defective only in Phl synthesis (Fenton et al, 1992). The second class of mutants belong to the regulatory class and exhibit pleiotropic phenotypes in that they are unable to produce Phl, protease or HCN.

Through complementation analysis three distinct loci were implicated as necessary for Phl production in F113. These loci include a biosynthetic locus consisting of 6kb of DNA, and two regulatory loci which correspond to the *gacA* and *lemA* two-component sensor/regulator system (Hrabak and Willis, 1992; Laville et al, 1992). In addition, a third distinct regulatory region has been identified which can complement either of the regulatory mutations. Further detailed analysis of other regulatory components has also implicated two sigma factors, sigma 38 and sigma 70, either directly or indirectly and a loci-linked regulator in the regulation of Phl production (Sarniguet et al, 1994; Cook et al, 1995; Schnider et al, 1995). A complete structured model for the complex regulation of Phl production in *P fluorescens* F113 has yet to be fully elucidated.

Genes involved in the biosynthesis of Phl have been cloned from different strains (Fenton et al, 1992; Bangera et al, 1996). Sequence analysis has resulted in the identification of a number of putative genes involved in the biosynthetic pathway (Cook et al, 1995; Delany et al, unpublished data). These genes show similarity to chalcone and stilbene sythases of plants; sterol carrier proteins of eukaryotes; drug and sugar transporters and transcriptional repressors (Cook et al, 1995; Delany et al, unpublished). To date, at least six putative open reading frames have been implicated in Phl biosynthesis. However, the precise role of each in the biosynthetic pathway remains unclear.

Biochemical approaches to studying the biosynthesis of 2,4-diacetylphloroglucinol

A putative pathway for the synthesis of MAPG has been documented by Mann (1987). MAPG is also produced by the *P fluorescens* strain F113 and has been implicated as a Phl precursor. The activity of a transacetylase enzyme, responsible for the conversion of MAPG to Phl, is proposed as the final step in the biosynthetic pathway. In a biochemical assay investigating the conversion of MAPG to Phl, the MAPG acetyltransferase activity of whole cell extracts of F113 can be demonstrated and quantified. On boiling the cell extracts this activity was lost, an indication that this activity is enzymatic (Shanahan et al, 1993). G22, the biosynthetic mutant of F113, has no such activity. However, on introduction of the pCU203 biosynthetic clone, MAPG acetyltransferase activity is restored. This demonstrates that the MAPG acetyltransferase activity is encoded on the pCU203 biosynthetic clone. The precise gene responsible for this activity has yet to be identified in F113. However, the combination of biochemical and genetic approaches used in our study permits the assignation of enzymatic activity to specific genetic loci (Dowling et al, 1996).

Ecological implications of 2,4-diacetylphloroglucinol in biocontrol

The cloning and characterization of genes involved in the production of a biocontrol metabolite offers possibilities for the development of improved biocontrol agents. We have previously shown that the introduction of the Phl biosynthetic genes directly confers biocontrol ability on *P fluorescens* strain M114 (Fenton et al, 1992).

However, the large-scale use of soil inoculants as biopesticides poses important ecological questions. The direct inhibition or antagonism of target pathogens is beneficial but the disruption of indigenous microbiota in the rhizosphere could result in long-term deleterious effects. In an experiment monitoring the persistence of F113 and G22 following ten resowings over a period of 270 days, there was no significant deleterious effect to G22 under the experimental conditions (Carroll et al, 1995). In addition, this experiment has demonstrated that there was no lasting perturbation of the resident bacterial microflora, although the biocontrol ability of the applied strain is dependent on its establishment in the rhizosphere.

In order to monitor the fate of genetically modified microorganisms following release into the environment, a chromosomally integratable *lacZY* cassette was developed in our laboratory (Fedi et al, 1996). This facilitates the marking of potential biocontrol pseudomonads without the use of antibiotic resistance markers that are generally perceived as undesirable in organisms designed for release in large quantities. In microcosm experiments, a *lac*ZY marked strain of F113 was unaltered with respect to ecological fitness and enabled monitoring of its in situ performance. Interestingly, lateral transfer of the *lacZY* marker, although detected in vitro, could not be detected in rhizosphere microcosms.

Crop protection using lytic enzyme producing inoculants

The isolation of potential biocontrol bacterial strains from the environment in which they would be required to function may help to ensure that the selected strains are suited for delivery as biopesticide inoculants into that environment (Weller, 1988; Cook, 1993). Many successful studies have focused primarily on the isolation and identification of members of the fluorescent pseudomonad population present in the rhizosphere due to their abilities to confer plant protection through production of a range of secondary metabolites (Fenton et al, 1992; Shanahan et al, 1992; Dowling and O'Gara 1994). Therefore, a screening strategy was initiated with the objective of isolating plant-beneficial microbes from the rhizosphere of crop plants grown in a fungal disease suppressive soil.

Of the isolated bacterial strains, *Stenotrophomonas maltophilia* strain W81(P) proved most effective against the causal agent of 'damping off' in sugar beet, *P ultimum*, when assayed under in vitro and in soil microcosm conditions (fig 3). Frequently isolated from polluted environments, strains of *S maltophilia* have been relatively well studied with regard to their molecular microbial ecology and the exploitation of their potential for bioremediation (Blake et al, 1993). However, *S maltophilia* strains have also been isolated from the rhizosphere of a range of crop plants (Milus and Rothrock, 1993; Kobayashi et al, 1995), and these almost exclusively root-associated strains (McInroy and Kloepper, 1994) have been evaluated as potential biopesticides (Kobayashi et al, 1995).

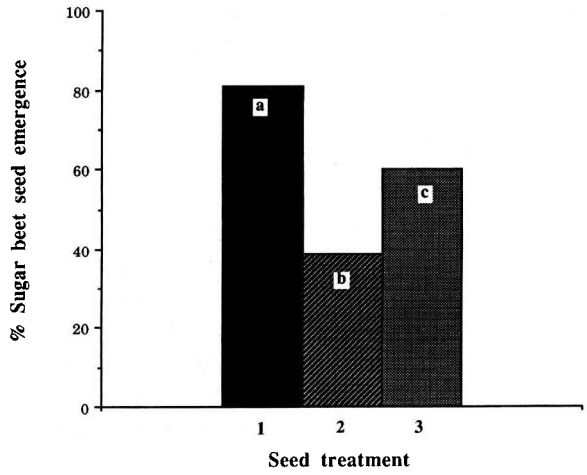

Fig 3. Emergence of sugar beet seeds inoculated with 1) negative control (untreated); 2) fungicide-treated seeds; 3) *Stenotrophomonas maltophilia* strain W81(P) in a soil subject to 'damping-off' by *Pythium ultimum* following 18 days incubation. Columns containing the same letter are not significantly different at the $P < 0.05$ level by analysis of variance.

While capable of fungal inhibition, *S maltophilia* W81(P) is unable to produce secondary metabolites such as HCN and Phl frequently implicated in plant protection against pathogens by pseudomonad species. However, using substrate-containing solid media W81(P) was seen to produce the extracellular lytic enzymes chitinase and protease (Dunne et al, unpublished results).

Protection of plants against multiple pests

W81(P) produces the enzymes chitinase and protease, both of which have been demonstrated as being directly involved in the biocontrol of some pathogenic fungi (Mitchell and Hurwitz, 1965; Haran et al, 1996). Further studies completed by a number of groups have demonstrated antifungal plant protection mediated through multi-enzyme activities, including chitinase and protease production, by biocontrol agents (Mitchell and Hurwitz, 1965; Oppenheim and Chet, 1992; Kobayashi et al, 1995). Extensive research has elucidated the involvement of the mycolytic properties of microbial chitinases and other enzymes so completely that genes encoding for bacterial enzyme production have been utilized in the development of transgenic plants with enhanced pest resistance (Oppenheim and Chet, 1992).

Other potential targets for biological control are plant parasitic nematodes. Cyst nematodes are major agricultural pests causing yield losses that vary according to soil composition and pathogen densities. Traditional control protocols have involved crop rotation and the use of environmentally detrimental and often toxic chemical nematicides. However, the use of many of these compounds has been limited by international agreement and so biopesticides are emerging as potential alternatives for crop protection (Sikora and Hoffman-Hergarten, 1994; Cronin et al, unpublished results).

Globodera rostochiensis is a cyst nematode that effects potato production and is capable of causing economic crop failure due to the stunting of plants and promotion of early plant senescence when present in high numbers. The egg shell of the nematode, *G rostochiensis*, contains a chitin and protein matrix. Previous studies have shown that commercially available purified microbial enzymes, including chitinases and proteases, can significantly reduce the hatch of *G rostochiensis* in vitro (Cronin et al, unpublished results). The most effective inhibition of nematode hatch was observed when the chitin-protein matrix of the egg wall was damaged through incubation of cysts in buffers containing combinations of both chitinase and protease enzymes (fig 4). A further study has recently demonstrated that the use of chitinolytic bacterial inoculants may provide an effective alternative to chemical treatments for the control of potato cyst nematodes (Cronin et al, unpublished results).

In order to evaluate the effectiveness of W81(P) enzyme production in the control of *G rostochiensis*, in vitro assays analogous to the purified enzyme assays were completed. These assays involved the incubation of nematode cysts in the presence of W81(P), followed by periodic sampling. The preliminary results obtained show that W81(P) treatment of cysts may provide the basis for an environmentally friendly strategy for the control of cyst nematodes which is easily integrated into current agricultural practices and which is compatible with biocontrol strategies targeting phytopathogenic fungi.

Elucidation of mechanisms involved in biocontrol by Stenotrophomonas maltophilia *strain W81(P)*

To determine whether lytic enzyme production by *S maltophilia* strain W81(P) is directly responsi-

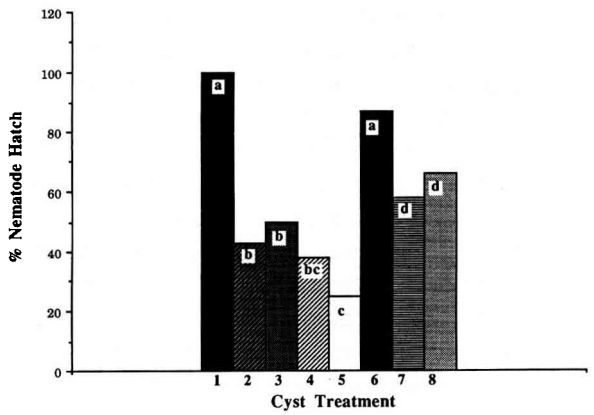

Fig 4. Effects of commercially available purified microbial enzymes on the hatch of *Globodera rostociensis* in vitro. 1) Control; 2) chitinase; 3) collaginase; 4) protease; 5) chitinase and protease; 6) chitinase and collaginase; 7) collaginase and protease; 8) chitinase, collaginase and protease. Columns containing the same letter are not significantly different at the $P < 0.05$ level analysis of variance.

ble for the conferred plant protection observed against both nematode and fungal pests, an insertional mutant, W81A1, deficient in extracellular enzyme production was created through transposon mutagenesis (Dunne et al, unpublished results). A genomic library was constructed and enzyme production was restored through introduction of a cosmid-borne complementary region, pCU800 (Dunne et al, unpublished results). Evaluation of extracellular chitinase and protease production by wild-type W81(P) and its mutant derivatives involved assessment on substrate-containing solid media and spectrophotometric quantification in colourimetric enzyme assays (McKellar, 1981).

Subsequent evaluation of the biological control abilities of wild-type *S maltophilia* W81(P) and its mutant derivatives initially involved solid media bioassays where inhibition of fungal growth is an indication of bacterial antifungal activity. Using this bioassay it was observed that W81A1 is unable to inhibit *Pythium* growth until the complementing cosmid-borne clone is introduced (Dunne et al, unpublished results). In vivo microcosm assays clearly demonstrated that both wild-type W81(P) and the complemented mutant, W81A1pCU800, when applied to sugar beet seeds at an inoculum level of 10^6 cfu/seed, protect the plant against attack by *P ultimum*. The enzyme deficient insertional mutant, W81A1, however, is unable to confer protection on sugar beet plants which subsequently succumb to the effects of 'damping off' (fig 5).

Similar in vitro and in vivo experiments directly investigating the involvement of the lytic enzymes chitinase and protease in cyst nematode control by W81(P) are currently ongoing.

CONCLUSION

Crop protection through the application of biocontrol agents is compatible with the requirement for the production of quality agricultural products in an environmentally friendly way. Extensive studies, such as those described here, have resulted in the elucidation of many factors mediating biological control in pseudomonads. This knowledge has resulted in the ability to clone biosynthetic or regulatory genes involved in biocontrol and, ultimately, to new strategies for the creation of novel biocontrol agents. However, alternative biotechnological strategies have also focused on the development and evaluation of consortia or mixed microbial inoculants, exhibiting multiple mechanisms of pest control, for enhanced crop protection. The development of microbial inoculants capable of both secondary metabolite and lytic enzyme production, for example, may provide an alternative means of protection for crops vulnerable to attack by multiple pests.

The ability to deliver viable microbial inoculants, or their metabolites, in an active form and at the appropriate time and site of action is essential for effective biological control. Therefore, the development of efficient, environmentally safe delivery systems, and the evaluation of the influences that these inoculants bring to bear on the resident microflora, are important aspects of biological control strategies. The successful development and introduction into agri-

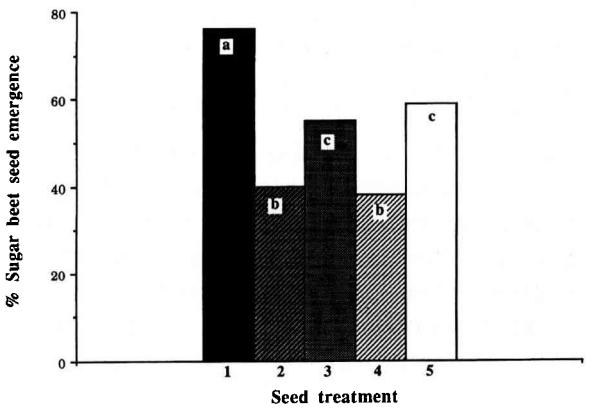

Fig 5. Emergence of sugar beet seeds inoculated with 1) fungicide-treated control; 2) untreated control; 3) *Stenotrophomonas maltophilia* strain W81(P); 4) *S maltophilia* strain W81A1; 5) *S maltophilia* strain W81A1pCU800 in a soil subject to 'damping-off' by *Pythium ultimum*. Columns containing the same letter are not significantly different at the $P < 0.05$ level of analysis of variance.

cultural practice of reliable biocontrol agents will lead to the reduction of harmful pesticide treatment for agricultural produce and a subsequent reduction of chemical pesticide residues in foods.

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