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Biological control of phytopathogens by phloroglucinol and hydrolytic enzyme producing bacterial inoculants

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Abstract

Environmental and consumer concerns have focused interest on the development of biological control as an alternative, environmentally-friendly strategy for the protection of agricultural and horticultural crops against phytopathogens. Biological control agents, producing a variety of secondary metabolites and hydrolytic enzymes, have been identified among fungi, actinomycetes and bacteria. *Pseudomonas fluorescens* F113 and *Stenotrophomonas maltophilia* W81 inhibit growth of the phytopathogenic fungus *Pythium ultimum* in vitro and are capable of protecting sugar beet against the effects of damping-off under soil conditions. Transposon mutagenesis of strains F113 and W81 has demonstrated that the biocontrol abilities of these strains are mediated by 2,4-diacetylphloroglucinol (PHL) or lytic enzyme production, respectively. *Globodera rostochiensis* is a phytopathogenic cyst nematode of major agronomic importance. Purified PHL, lytic enzymes, and chitinolytic or phloroglucinol-producing bacterial inoculants negatively influence hatch of *G. rostochiensis* eggs and decrease subsequent viability of juvenile cyst nematodes in vitro. Similar results were obtained under soil conditions.

Introduction

Biological control exploits the negative interactions that occur between disease-suppressive microorganisms and plant pathogens for the improvement of plant health in an environmentally friendly manner (Cook et al. 1995, Handelsman and Stabb 1996). Potential biocontrol agents, mediating plant protection through the production of a variety of hydrolytic enzymes and secondary metabolites (Chernin et al. 1995, Dowling and O'Gara 1994, Howell and Stipanovic 1980, O'Sullivan and O'Gara 1992), have been identified among fungi, actinomycetes and bacteria (Becker and Schwinn 1993, Cook 1993, Dunne et al. 1997a, Keel and Défago 1997). Bacterial strains *Pseudomonas fluorescens* F113 and *Stenotrophomonas maltophilia* W81 were previously isolated from the rhizosphere of field grown sugar beet due to their ability *in vitro* to inhibit growth of the causal agent of damping-off, *Pythium ultimum* (Dunne et al. 1996 and 1997a, Fenton et al. 1992). Transposon mutagenesis of F113 and W81 resulted in the isolation of mutants F113G22 and W81A1 which are unable to antagonize *Pythium ultimum* due to their inability to produce 2,4-diacetylphloroglucinol (PHL) or chitinolytic and proteolytic enzymes, respectively (Cronin et al. 1997a and 1997b, Dunne et al. 1997b, Fenton et al. 1992).

Globodera rostochiensis is a cyst nematode capable of causing extensive damage to potato crops. Traditional protection measures against such damage have included crop rotation and the use of toxic chemical treatments. However, biological control of parasitic nematodes using fungal or bacterial strains has been proposed as an alternative crop protection strategy (Mankau, 1980, Spiegel et al. 1991). Experimental results presented here demonstrate

the importance of PHL and hydrolytic enzyme production in the anti-fungal biocontrol abilities of *P. fluorescens* F113 and *S. maltophilia* W81, and further implicate production of the secondary metabolite PHL in the ability of F113 to negatively influence the life-cycle of *G. rostochiensis* in vitro and in soil. Assessment of the chitinolytic bacterial strains *S. maltophilia* M1-12 and *Chromobacterium* spp. UP1, previously isolated from a soil suppressive to potato cyst nematode infestation (Cronin et al. 1997c), demonstrate the potential of hydrolytic enzyme-producing bacterial inoculants for the control of nematode-mediated disease.

Materials and Methods

Microorganisms and growth conditions. Bacterial strains were routinely grown shaking in Luria Bertani broth (LB) (Sambrook et al. 1989) or Sucrose Asparagine (SA) (Scher and Baker 1982) at 28 °C. *Pythium ultimum* was obtained from the Commonwealth Mycological Institute (Surrey, England) and was maintained on corn meal agar (Difco, Detroit, MI, USA). Cysts of the nematode *Globodera rostochiensis* (virulence group Ro1) were stored dry at 25 °C for four to five months to ensure that the diapause was completed. Prior to performing experiments, the cysts were incubated on filter paper soaking in distilled water for seven days at 20 °C to promote homogenous hatching rates.

Biocontrol efficacy of bacterial inoculants in vitro. In vitro antagonism of *Pythium ultimum* by *P. fluorescens* F113, *S. maltophilia* W81 and their mutant derivatives was determined using the solid medium bioassay previously described by Fenton et al. (1992).

To determine the effects of purified chitinase, protease or collagenase on egg hatch of *G. rostochiensis* in vitro one milliliter volumes of enzyme solution (approximately 0.5 units enzyme/ml) were added to 20 ml of 50 mM sodium phosphate buffer, pH 6.4 in 100 ml Erlenmeyer flasks. Twenty-five cysts were introduced into the flasks and incubated at 12 °C for 8 weeks. At fortnightly intervals six cysts were removed aseptically and placed in 0.3 mM picrolonic acid, an artificial hatching factor, for 28 days. During this period weekly visual counts of hatched eggs per cyst were made and fresh picrolonic acid was added. At the end of the 4 week period the total number of observed eggs hatched was calculated. Lytic enzyme-free buffer was used as a control. The experiment was carried out in triplicate with two groups of three cysts assayed per replicate.

To investigate the ability of chitinolytic or phloroglucinol-producing bacterial inoculants to influence hatch of *G. rostochiensis*, inoculated phosphate buffer containing nematode cysts was incubated at 12 °C for at least 3 weeks, with gentle shaking on alternate days. Cysts were then sampled and exposed to picrolonic acid prior to enumeration of hatched juveniles. Strains tested included wild type *P. fluorescens* F113, PHL-deficient mutant F113G22, *S. maltophilia* M1-12 and *Chromobacterium* UP1. The experiment was performed in duplicate and two groups of three cysts were assayed for egg hatch per replicate.

The influence of *P. fluorescens* F113 and F113G22 on the viability of *G. rostochiensis* juveniles was studied as follows. Approximately 130 nematodes were added to phosphate buffer in the wells of a 96-well titration plate. The plate was incubated at 12 °C for 48 h, with gentle shaking every 12 h, and the ratio of mobile to motionless juveniles determined. Four replicates were studied per treatment.

Biocontrol efficacy of bacterial inoculants in soil. The ability of wild type F113, F113G22, wild type W81 and W81A1 to protect sugar beet against damping-off disease was investigated in natural soil microcosms using procedures previously described by Fenton et al.

(1992). Sugar beet seeds (cv. Accord) were inoculated with approximately 6 log CFU/seed of the bacteria studied. Controls consisted of seeds dipped in quarter-strength Ringer's solution (untreated control) or a solution containing the synthetic fungicides Previcur N (i.e., propamocarb; Schering AG, Germany) at 20 ml/kg seed and thiram at 7.5 g/kg seed (commercial control). Infection of seeds by *Pythium* spp. was assessed daily for the first seven days of the experiment. Plant emergence was determined at 28 days. The experiment was repeated three times.

The influence of bacterial inoculants on cyst nematode egg hatch was evaluated by co-inoculating bacterial inoculants and cysts of *G. rostochiensis* in soil which was then mixed thoroughly. The water content of the soil was maintained at 60% saturation of the soil porosity. The pots were incubated in a growth chamber (12 °C, 16 h photoperiod). Strains tested included wild type F113, mutant F113G22, *S. maltophilia* M1-12 and *Chromobacterium* UP1. Cysts were extracted from the soil as described by Winfield et al. (1987) before incubation in picrolonic acid. Hatched juveniles were counted, as described above. Five replicates were used per treatment and three groups of two cysts were assayed per replicate. The effect of PHL-producing bacterial inoculants on juvenile nematode viability was also evaluated in soil microcosms. The experimental procedures were similar to those described above, except that the soil also contained approximately 200 juveniles per 150 g soil. Juvenile nematodes were reisolated from soil using a Baermann funnel (Van Gundy 1983), counted microscopically and the ratio of mobile to motionless nematodes determined. The experiment was performed in triplicate.

Statistical analysis. Data were analysed by analysis of variance, using procedures of the Statistical Analysis System (SAS Institute, Cary, NC, USA). When appropriate, treatments were compared using Fisher's least significant difference test. All analyses were conducted at $P = 0.05$.

Results and Discussion

Biocontrol of fungi and cyst nematodes by PHL-producing *P. fluorescens* F113. Antagonistic bacteria have received much attention as potential environmentally friendly agents for the control of fungal and nematode pests (Cook et al. 1993, Dunne et al. 1997a). *Pseudomonas fluorescens* F113 is an effective biocontrol agent isolated from the sugar beet rhizosphere and has been the focus of considerable interest due to its ability to antagonize both the fungal pathogen *P. ultimum* and the bacterial potato pathogen *Erwinia carotovora* (Cronin et al. 1997b, Fenton et al. 1992). Previous studies involving transposon mutagenesis of F113 resulted in the isolation of the near-isogenic mutant F113G22 which is incapable of inhibiting growth of *P. ultimum* in vitro due to its inability to produce PHL (Table 1) (Fenton et al. 1992). The development of a high performance liquid chromatography (HPLC) assay allowed investigation of factors influencing secondary metabolite production by F113, and demonstrated that efficient phloroglucinol production by the strain is dependent upon the presence of suitable carbon sources and the availability of iron (Dowling et al. 1996). These results hold major implications for the successful application of microbial biocontrol agents as effective secondary metabolite production may be dependent on both the components of seed and root exudates and the physical parameters of the soil environment (Dowling et al. 1996). Assessment of the influence *P. fluorescens* F113 may exert on the hatching rates and subsequent viability of the cyst nematode *G. rostochiensis* demonstrated that exposure of cysts to F113 increased the ability of *G. rostochiensis* to hatch both in vitro and in soil

microcosms (Table 2). In contrast, the PHL-deficient mutant F113G22 had no effect on hatching rates (Table 2), directly associating PHL production by F113 with its ability to influence cyst nematode hatch. Further evaluation of the effects of wild type F113 and F113G22, on the viability of exposed juvenile nematodes demonstrated that significant increased mortality occurred only in the presence of the wild type strain (Table 2). As juvenile nematodes of *G. rostochiensis* can persist in soil for a period of only 10 to 12 days in the absence of the host potato plant, a biocontrol strategy exploiting PHL-producing F113 should involve introduction of the microbial inoculant into the soil prior to potato sowing in order to increase hatch. The effects of F113 on nematode juvenile viability may further improve the efficiency of potato plant protection.

Table 1. Evaluation of biological control of *Pythium ultimum* under laboratory and natural microcosm conditions.

Treatment	Inhibition of <i>Pythium ultimum</i> growth (in vitro)	<i>Pythium</i> -free seeds at 24 h in soil (%)	Sugarbeet emergence at 28 days in soil (%)
Commercial control	+	100 a ^{y,z}	72 (4) a
Untreated control	-	5 (5) b	38 (6) c
F113Rif	+	90 (4) a	59 (4) b
F113G22	-	33 (8) b	41 (4) c
W81	+	87 (9) a	64 (7) ab
W81A1	-	30 (11) b	36 (4) c

^y Standard deviations are given in parentheses.

^z Within each column, values with the same letter are not significantly different according to protected LSD test.

Table 2. Effects of 2,4-diacetylphloroglucinol-producing *P. fluorescens* F113 on hatch and juvenile viability of the potato cyst nematode *Globodera rostochiensis* under in vitro and soil conditions.

Treatment	Untreated control	F113 (10 ⁷ CFU/ml)	F113G22 (10 ⁷ CFU/ml)
In vitro			
Hatched juveniles/cyst	120 (9) ^y a ^z	190 (8) b	111 (7) a
Viable juvenile nematodes/cyst (%)	89 (4) a	28 (3) b	84 (9) a
In soil			
Hatched juveniles/cyst	140 (19) a	229 (78) b	130 (38) a
Viable juvenile nematodes/cyst (%)	85 (18) a	26 (11) b	84 (11) b

^y Standard deviations are given in parentheses.

^z Within each row, values with the same letter are not significantly different according to protected LSD test.

Assessment of biocontrol by hydrolytic enzyme-producing bacterial inoculants
 In addition to antibiosis, biological control of both fungal and nematode pests by bacterial strains may be mediated by the production of hydrolytic enzymes (Chernin et al. 1995). Bacterial strains *S. maltophilia* W81, *S. maltophilia* M1-12 and *Chromobacterium* strain UPI1

P. fluorescens F113, were previously isolated from the soil environment (Cronin et al. 1997c, Dunne et al. 1997a). *S. maltophilia* W81 proved capable of inhibiting growth of *P. ultimum* in vitro and effectively conferred protection on sugar beet seeds against colonization and subsequent damping-off in naturally *Pythium* infested soil microcosms (Table 1). However, a lytic enzyme deficient transposon-induced mutant, W81A1, was unable to antagonize the oomycete under similar conditions (Table 1). Further genetic complementation and biochemical assays have demonstrated that W81 antifungal activity is mediated by the lytic disruption of the structural integrity of the *Pythium* cell wall (*data not shown*). In similar fashion the integrity of the structure of the *G. rostochiensis* egg shell, which is formed from a chitin and protein matrix, can be disrupted by purified commercial hydrolytic enzymes leading to subsequent decreases in egg hatch. However, while chitinase and protease enzymes proved effective when used individually (Table 3) combinations of both hydrolytic enzymes caused significantly greater decreases (Table 3). Evaluation of the chitinolytic bacterial strains *S. maltophilia* M1-12 and *Chromobacterium* strain UPI, under both in vitro and soil conditions, also significantly decreased the ability of *G. rostochiensis* eggs to hatch (Table 3).

Table 3. Effects of purified lytic enzymes and chitinolytic bacteria on hatch of the potato cyst nematode *Globodera rostochiensis*.

Treatment	Egg hatch in vitro (% hatched eggs/cyst)	Egg hatch in soil (% hatched eggs/cyst)
Purified enzymes		
Untreated control	100 a ^x	
Chitinase	43 (6) b ^y	
Protease	39 (7) bc	
Collagenase	51 (9) b	
Chitinase, protease	26 (7) c	
Chitinase, collagenase	87 (7) a	
Collagenase, protease	58 (6) d	
Chitinase, protease, collagenase	68 (5) d	
Bacterial strains		
Untreated control	74 (11) a	65 (9) a
<i>S. maltophilia</i> M1-12 ^z	29 (7) c	38 (4) b
<i>Chromobacterium</i> spp. UPI ^z	19 (9) c	40 (5) b

^x Within each column, values with the same letter are not significantly different according to a protected LSD test

^y Standard deviations are given in parentheses.

^z Inoculum density for both bacterial strains was 10⁷ CFU/ml.

In summary, the results presented here demonstrate the biocontrol efficiency of both hydrolytic enzyme and PHL-producing bacterial inoculants to protect against the effects of phytopathogens. Future strategies may involve evaluation of these biocontrol agents, individually and when used in synergistic combinations, under conditions relevant to current agricultural practices.

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Literature

- Becker, J.O., and Schwinn, F.J. 1993. Control of soil-borne pathogens with living bacteria and fungi: status and outlook. *Pestic. Sci.* 37:355-363.
- Chernin, L., Ismailov, Z., Haran, S., and Chet, I. 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl. Environ. Microbiol.* 61:1720-1726.
- Cook, R.J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. *Annu. Rev. Phytopathol.* 31:53-80.
- Cook, R.J., Thomashow, L.S., Weller, D.M., Fujimoto, D., Mazzola, M., Bangera, G., and Kim, D. 1995. Molecular mechanisms of defence by rhizobacteria against root disease. *Proc. Natl. Acad. Sci. USA* 92:4197-4201.
- Cronin, D., Moënné-Loccoz, Y., Fenton, A., Dunne, C., Dowling, D.N., and O'Gara, F. 1997a. Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol pseudomonad strain F113 with the potato cyst nematode *Globodera rostochiensis*. *Appl. Environ. Microbiol.* 63:1357-1361.
- Cronin, D., Moënné-Loccoz, Y., Fenton, A., Dunne, C., Dowling, D.N., and O'Gara, F. 1997b. Ecological interaction of a biocontrol *Pseudomonas fluorescens* strain producing 2,4-diacetylphloroglucinol with the soft rot potato pathogen *Erwinia carotovora* subsp. *atroseptica*. *FEMS Microbiol. Ecol.* 23:95-106.
- Cronin, D., Moënné-Loccoz, Y., Dunne, C., and O'Gara, F. 1997c. Inhibition of egg hatch of the potato cyst nematode *Globodera rostochiensis* by chitinase-producing bacteria. *Eur. J. Plant Pathol.* 103:433-440.
- Dowling, D.N., Sexton, R., Fenton, A., Delany, I., Fedi, S., McHugh, B., Callanan, M., Moënné-Loccoz, Y., and O'Gara, F. 1996. Iron regulation in plant-associated *Pseudomonas fluorescens* M114. Pages 502-511 in: *Molecular biology of Pseudomonads*. T. Nakazawa, ed. ASM Press, Washington DC.
- Dowling, D.N., and O'Gara, F. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *Trends Biotechnol.* 12:133-141.
- Dunne, C., Delany, I., Fenton, A., Lohrke, S., Moënné-Loccoz, Y., and O'Gara, F. 1996. The biology and application of *Pseudomonas* inoculants for the biocontrol of phytopathogens. Pages 441-448 in: *Biology of Plant-Microbe Interactions*. G. Stacey, B. Mullin and P.M. Gresshoff, eds. IS-MPMI, St. Paul, MI.
- Dunne, C., Delany, I., Fenton, A., and O'Gara, F. 1997a. Mechanisms involved in biocontrol by microbial inoculants. *Agronomie* 16:721-729.
- Dunne, C., Crowley, J., Moënné-Loccoz, Y., Dowling, D.N., De Bruijn, F.J., and O'Gara, F. 1997b. Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by extracellular proteolytic activity. *Microbiology*:143:3921-3931.
- Fenton, A.M., Stephens, P.M., Crowley, J., O'Callaghan, M., and O'Gara, F. 1992. Exploitation of a gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Appl. Environ. Microbiol.* 58:3873-3878.
- Handelsman, J., and Stabb, E.V. 1996. Biocontrol of soilborne plant pathogens. *Plant Cell* 8:1869-1869.

- Keel, C., and Défago, G. 1997. Interactions between beneficial soil bacteria and root pathogens: mechanisms and ecological impact. Pages 27-46 in: Multitrophic interactions in terrestrial systems. A.C. Gange and V.K. Brown, eds. Blackwell Scientific Publisher, London.
- Mankau, R. 1980. Biological control of nematode pests by natural enemies. *Annu. Rev. Phytopathol.* 18:415-440.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning, a Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Scher, F.M., and Baker, R. 1982. Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. *Phytopathology* 72:1567-1573.
- Spiegel, Y., Cohn, E., Galper, S., Sharon, E., and Chet, I. 1991. Evaluation of a newly isolated bacterium, *Pseudomonas chitinolytica* sp. nov., for controlling the root-knot nematode *Meloidogyne javanica*. *Biocon. Sci. Technol.* 1:115-125.
- Van Gundy, S.D. 1982. Nematodes. Pages 1121-1130 in: *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties*, 2nd edition. A.L. Page, ed. American Society of Agronomy, Madison, WI.
- Winfield, A.L., Enfield, M.A., and Foreman, J.H. 1987. A column elutriator for extracting nematodes and other small invertebrates from soil samples. *Ann. Appl. Biol.* 111:223-231.