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Biological control of seedling blight of wheat caused by *Fusarium graminearum* with beneficial rhizosphere microorganisms

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Summary

Fusarium graminearum is associated with the cereal damping-off complex which reduces germination, seedling stand and yield. Fifty-two bacterial strains and six *Trichoderma* spp. isolated from the wheat rhizosphere were evaluated for biocontrol of seedling blight of wheat caused by *F. graminearum*. Their potential as biocontrol agents was tested *in vitro* and in the greenhouse. Isolates varied in their ability to inhibit the mycelial growth of *F. graminearum* in agar plate bioassays by 0–79%. This parameter was not related with biocontrol efficacy of *in vivo* assays. In greenhouse trials, all isolates were initially evaluated for reducing disease on wheat cultivars Klein Centauro (moderately resistant to *F. graminearum*) and Pro INTA Oasis (susceptible) planted in sterilized soil artificially infested with the pathogen. Among the 25 bacteria and six fungal isolates that exhibited a pronounced suppressive effect, the most efficient 10 for both cultivars were further assayed on eight cultivars (Buck Candil, Buck Catriel, Buck Chambergo, Buck Poncho, Buck Topacio, Klein Cacique, Klein Centauro and Pro INTA Oasis) potted in cultivated–inoculated soil. Three weeks after sowing, plant stand, percentage of diseased emerging seedlings, plant height and dry weight were evaluated. Among the antagonists only *Stenotrophomonas maltophilia* was significantly better than the control for the average of the eight cultivars for plant stand, height and dry weight. *Stenotrophomonas maltophilia* also caused a non-significant decrease in the percentage of diseased plants. Three strains of *Bacillus cereus* and one isolate of *Trichoderma harzianum* gave also a good control in some cultivars. The ability of these isolates to affect the infection of wheat seedlings by *F. graminearum* may be of potential value in field trials.

Introduction

Cereal damping-off is a disease complex which reduces seed germination, seedling stand and yield. (Cook 1968; Silo-Suh 1994; Luz *et al.* 1998). Many different species of *Fusarium* have been associated with wheat seedling blight, but *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) was the second most predominant after *F. culmorum* (Cook 1968). The pathogen, in addition to seedling blight, causes different seed and soil-borne diseases of seedlings (Sutton 1982). Damage caused by the fungus is expressed in loss of germinability, reduced emergence, and a postemergence blight of seedlings. Râdulescu & Negru (1965) stated that the presence of *F. graminearum* completely inhibited seed germination of maize, oats, barley, sugar beet and sainfoin.

Resistance, chemical treatments, and agricultural practices such as crop rotation are the main strategies for disease management. Fungicides are used extensively to control many soil-borne diseases, but their effectiveness is variable. Furthermore, secondary infections from soil-borne inocula as well as from inocula on plant debris are difficult to control by chemical seed treatments. Public concern with fungicide residues, as well as pathogen resistance to some pesticides, have increased the need to find alternative methods for protection against crop diseases (Harman 1992; Mao *et al.* 1997). In addition, there are few crop varieties that are resistant to Fusarium seedling blight. Thus, biological control using antagonistic microbes alone, or as supplements to minimize the use of chemical pesticides in a system of integrated plant disease management, has become more important in recent years (Daamen *et al.* 1989).

For field crops such as corn, soybean, wheat and rice, seed treatment with biocontrol agents is one of the most suitable application methods for biocontrol of soil-borne pathogens in the spermosphere and rhizosphere

(Harman 1992; Mao *et al*. 1998). Beneficial bacteria and fungi applied as seed treatments provide unique opportunities for protection against soil-borne fungal pathogens and are a potentially feasible alternative to the use of fungicides. Considerable research has been done to investigate antagonistic microorganisms for use in seed treatments (Baird *et al*. 1994; Mathre & Johnston, 1995; Mao *et al*. 1997), but few have been reported on the use of microbes to control damping-off of wheat (Hering *et al*. 1987; De Freitas & Germida 1991). There is information on the use of microbes to control cereal damping-off (Knudsen *et al*. 1995; Mao *et al*. 1997) including *F. graminearum* (Harman *et al*. 1989; Lazzaretti *et al*. 1994; Hedegus & Farkas 1997).

Most fungi used for biological control of damping-off are Hyphomycetes, and among these genera, *Trichoderma* and *Gliocladium* have received the most attention. In addition, the most extensively studied bacterial agents for reducing soil-borne diseases, have been strains of *Pseudomonas* spp., *Bacillus* spp. and *Enterobacter cloacae* (Bernard *et al*. 1995).

The purpose of this study was to select rhizosphere-colonizing bacteria and *Trichoderma* spp., to evaluate their antagonistic ability against *F. graminearum*. Furthermore, the effectiveness of the most inhibitory isolates for control of damping-off in different wheat cultivars was evaluated. Antagonistic bacteria and fungi were isolated from wheat growing in soils collected from agricultural sites in Argentina. *In vitro* tests and plant bioassays were then used to select isolates for testing in the greenhouse under environment conditions conducive to expression of damping-off.

Materials and methods

Pathogen culture

Fusarium graminearum was isolated from a field soil cultivated with wheat in the Buenos Aires province (Argentina). The culture was grown on potato dextrose agar (PDA) at 20 °C and maintained at 4 °C until used. Preparation of *F. graminearum* inoculum was made as follows: oat kernels were autoclaved (150 g/500 ml Erlenmeyer flask plus 100 ml of distilled water) twice on subsequent days at 121 °C for 20 min, and then were inoculated with one 6-mm-diameter PDA plug excised from an actively growing *F. graminearum* culture. Flasks were then placed in a growth chamber at 20 °C with alternating fluorescent light (3500 lux-dark cycles of 12 h) plus the addition of NUV light (365 nm) to induce sporulation. The cultures were shaken every 2 days for 3 weeks to promote uniform colonization. Inoculum was then air-dried for 7 days at room temperature, milled in a blender to pass through a 4-mm sieve, and kept at 4 °C in darkness until used. The inoculum contained 1×10^5 c.f.u./g as determined by serial dilution on a peptone pentachloronitrobenzene (PCNB) medium.

Isolation of rhizosphere antagonists

Trichoderma spp. and bacteria were isolated from soils collected from four wheat fields and one corn field in Argentina. Soil samples were stored in polyethylene bags at room temperature for no more than 15 days before use. Each soil was amended with fragmented oat-kernel inoculum (10% wt/wt) and added (380 g) to 400 ml plastic pots. Seeds of wheat, *Triticum aestivum* L. cv. Pro INTA Oasis (susceptible to *F. graminearum*) and cv. Klein Centauro (moderately resistant), were sown in pots (8 cm in diameter and 12 cm high) with the different soil samples, 50 seeds/cultivar in five pots for each soil sample. After planting, each pot received 150 ml of tap water. Thereafter, pots were transferred to a growth chamber with alternating fluorescent light (20 °C for a 12-h photoperiod) and the soil was watered twice weekly with 200 ml/pot of tap water. The isolation strategy is based in the capacity of some seedlings to survive due to root colonization by antagonists that prevented or reduced infection by *F. graminearum*. After 3 weeks the surviving wheat seedlings were dug up with their roots, and the excess soil was shaken off. Roots from each pot were excised and then gently washed for 10 min with 50 ml of sterile water in a 250-ml flask. Washed root fragments with adhering soil were placed in a mortar with 10 ml of 0.2 M phosphate buffer (13.80 g of NaH_2PO_4, 26.82 g of Na_2HPO_4, and 1 l of sterile water; pH 7.4), macerated and resuspended in 5 ml of phosphate buffer. Each original suspension was serially diluted four times in 1:10 increments in sterile distilled water, and 0.5 ml of 10^{-8} diluted suspension was spread in duplicate onto each of two different agar media: 0.4% oxgall-PDA (Dal Bello, 1982) or nutrient agar (NA) contained in 9 cm diameter Petri dishes. Bacteria were isolated on NA amended with 0.05 g of cycloheximide and fungi were isolated on oxgall-PDA amended with 0.05 g of chloramphenicol. After 2–5 days at 25 °C, all morphologically distinct colonies from each cultivar-soil sample combination were sub-cultured on PDA to obtain single colonies. The selection was based on the following colony characteristics: shape, size, color, edge, surface and pigment. Six isolates of *Trichoderma* spp. and 52 unidentified bacterial colonies were selected for testing. Fungal isolates were stored by subculturing on PDA at 4 °C and bacteria were maintained in nutrient broth (NB) with 40% glycerol at −20 °C.

Inhibition of mycelial growth

All microorganisms isolated were initially screened *in vitro* for antagonism against *F. graminearum* by the dual culture technique. To test for ability of bacteria to inhibit the pathogen, a mycelial disk of *F. graminearum* (6-mm diameter) from a 5-day-old PDA culture was placed off-center on each PDA plate (Liang *et al*. 1996). After 24 h incubation at 25 °C, plates were streak-inoculated 4 cm from the fungal disk with the particular bacterial isolate grown in NA slants for 24 h at 25 °C.

Each bacterial isolate was tested five times. Petri dishes were incubated in the dark at 20 °C and antagonistic effects were determined after 1 week by measuring the percentage inhibition, and the width of the inhibition zone: 0 = no inhibition, <2 mm = slight inhibition, 2–5 mm = moderate inhibition and >5 mm = strong inhibition (Liang *et al.* 1996). Percent growth inhibition was calculated by the following equation: $100(R_1-R_2)/R_1$, where R_1 represents the radius of the colony of *F. graminearum* in the direction with no bacterial colony and R_2 is the radius of the fungal colony in the direction of the bacterial colony.

Trichoderma spp. were screened to assess their antagonistic activity to *F. graminearum* based on the modified method of Camporota (1985). Dual cultures were started with each fungal isolate and the pathogen placed 4 cm apart on PDA plates (9-cm diameter). Agar plugs (6-mm diameter) were removed from 5-day-old PDA cultures of the fungi growing at 25 °C. Plates were incubated in a growth chamber at 20 °C with a 12-h photoperiod under fluorescent and NUV (365 nm) lighting. There were five replicate plates for each dual culture. After 7 days, the inhibitory effect of *Trichoderma* spp. was evaluated through the percentage inhibition of the radial growth of *F. graminearum*. The colonization percentage was determined according to the formula: 100 − [100 (DT/DE)], where DT is the distance of *Trichoderma* growth from the point of inoculation to the colony margin, measured on the straight line that joins both fungal plugs, and DE is the distance between plugs (4 cm). Percent growth inhibition was categorized on a scale from 0 to 4, where 0 = no growth inhibition, 1 = 1–25% growth inhibition, 2 = 26–50% growth inhibition, 3 = 51–75% growth inhibition, and 4 = 76 to 100% growth inhibition.

Treatment of wheat seeds with antagonists

Seeds of wheat, cultivars Klein Centauro (moderately resistant to *F. graminearum*) and Pro INTA Oasis (susceptible), were surface disinfected and coated with antagonists by a method similar to that described by Kim *et al.* (1997). Twenty four hours before sowing, seeds were immersed in 5% sodium hypochlorite solution for 5 min and rinsed for 30 min under running tap water with a final rinse in sterile water. The moistened seeds were stored at room temperature (20–22 °C) for 24 h to promote germination. Each test bacterium was spread over the entire surface of NA slants and incubated for 24 h in darkness at 25 °C to achieve confluent growth over the surface. Five milliliter of sterile water was added to each culture and the bacterial lawn was then scraped from the agar surface with a spatula into a test tube, vortexed for 30 s and resuspended in 15 ml of a 1% (wt/vol) solution of sodium–carboxymethyl cellulose (CMC) in sterile water. The CMC suspension with each bacterium was mixed with 50 g/cv. of surface-sterilized wheat seeds for 20 min. This procedure consistently yielded final populations of approximately 1×10^8 c.f.u. per seed as determined by dilution-plating. Colonies were counted after 2–3 days of incubation at 25 °C. Fungal isolates were grown on PDA slants for 7 days in a growth chamber at 20 °C, with 12 h of light (combined fluorescent-NUV) per day, and conidia were harvested by pouring a few milliliters of sterile water into the tube. The surface colony was scraped off into a tube, the conidial suspension was vortexed for 30 s and filtered through cheesecloth to remove mycelial debris. Spore concentration was counted using a hemocytometer and adjusted to 1×10^6 conidia/ml by the addition of a 1% CMC solution. Twenty milliliter of each fungal suspension were then mixed with 50 g/cv. of disinfected wheat seeds for 20 min. Seeds treated with CMC without antagonists were used as control. After 20 min excess liquid was poured off and the coated seeds were dried for 3 h under a stream of sterile air.

Greenhouse assay for biocontrol activity against damping-off

The 58 isolates progressed from *in vitro* tests to a initial screening for antagonism. This bioassay was designed to allow a rapid assessment of antagonistic effects and was conducted on wheat seedlings growing in sterile soil. Greenhouse mean temperatures were 18 °C (12–20 °C) with 80% RH (70–90% RH). Most effective isolates were further tested on wheat plants growing in field soil.

Sterile soil assay

Inoculum of *F. graminearum* was prepared on oat kernels as described above. *Fusarium graminearum* oat-kernel inoculum was ground in a blender just prior to use, and particles of uniform size (5-mm diameter) were added to the soil. Bacteria and fungi were applied to seeds as described above. The experiment was planted in a factorial design with two cultivars, 58 isolates plus control and six blocks. The coated seeds and control were planted in pots (replicates) of 5 seeds/plastic pot (8 cm in diameter and 12 cm high). Sterile light sandy soil (350 g) was added to each pot. Soil had been sterilized by autoclaving at 120 °C for 30 min on three consecutive days. Seeds were placed on the soil surface, 25 g of the same soil were placed above the seeds and 1 g of inoculum of *F. graminearum* were added to cover the surface of the soil. An additional 25 g of soil was placed above the inoculum. After sowing each pot received 100 ml of tap water. Pots were maintained in a greenhouse at 14–21 °C and watered daily for 21 days. After plants had been harvested and the roots were washed free of soil, disease was assessed on roots and subcrown internodes. Severity of disease was evaluated on a scale of 0–5 in which 0 = no lesion evident, 1 = <25% roots and subcrowns with necrosis, 2 = 26–50% roots and subcrowns with necrosis, 3 = 51–75% roots and subcrowns with necrosis, 4 = 76–100% roots and subcrowns with necrosis, and 5 = no plant emergence.

Data were analyzed by ANOVA and treatment means separated by LSD test ($P \leq 0.05$) to select isolates for a new assay with several cultivars.

Pot/cultivated soil assay

Three *Trichoderma* spp. and seven bacterial isolates that demonstrated the greatest biocontrol activity against *F. graminearum* for the evaluated cultivars in sterilized soil, were selected to test their ability to reduce damping-off in eight wheat cultivars (Buck Candil, Buck Catriel, Buck Chambergo, Buck Poncho, Buck Topacio, Klein Cacique, Klein Centauro and Pro INTA Oasis). A seed-treatment method in non-sterilized, cultivated soil was used. Soil (sandy clay loam, pH 7.4) was collected from a field near La Plata (Buenos Aires Province) from the top 10 to 15-cm layer of the soil profile. Soil was prepared 1 week prior to the experiment and air-dried at room temperature on a bench in the greenhouse. Air-dried soil was placed in plastic pots (15 cm diameter and 15 cm high) at 1 kg/pot. For screenings, surface sterilized seeds of wheat (cvs. Pro INTA Oasis and Klein Centauro) were artificially infested with the pathogen prior to bacterial or fungal treatment. The pathogen was cultured in 250-ml Erlenmeyer flasks containing 100 ml of carboxymethylcellulose medium (Cappellini & Peterson 1965) for stimulating sporulation. After 7 days shake incubation (rotary shaker, 100 rev/min) at 23 °C, the resulting slurry was centrifuged at 3700 rev/min for 20 min. The supernatant was discarded by filtration through filter paper (Whatman no. 1), the resulting mycelial pellets were washed twice with sterile water and resuspended in 10 ml of sterile water. Spore concentration was adjusted to 2.5×10^5 conidia/ml by the addition of a 1% CMC solution. Bacteria and *Trichoderma* suspensions were prepared as previously described. The seeds were mixed with the pathogen suspension for 20 min, the surplus liquid was poured off and the seeds were air-dried for 1 h under a stream of sterile air. After a second treatment with the antagonists for 20 min as previously described, the coated seeds were air-dried for 30 min. Control seeds were processed as above without antagonists. The initial population for each bacterial or fungal isolate on the seeds, was determined by dilution-plating immediately following the treatment. To enumerate bacterial cells and *Trichoderma* spores, seeds without *F. graminearum* were used. Colonies were counted after 2–3 days of incubation at 25 °C. Treatments were arranged in a factorial design with three replications and two factors: eight cultivars and 12 treatments (10 antagonists and controls with and without *F. graminearum*). Five seeds were sown about 2 cm deep in each pot replication. The soil surface was levelled and watered (200 ml/pot) to approximate field capacity. Pots were maintained in the greenhouse under controlled environment conditions, with a maximum daylight temperature of 26 °C, falling to a minimum of 9 °C at night. On alternate days, tap water (150–200 ml/pot) was added to each pot. Three weeks after sowing, plants were harvested and the roots were washed free of soil. Seedling stand, percentage of diseased emerging seedlings and plant height were evaluated; then seedlings were oven-dried at 60 °C for 24 h and the dry weight was calculated. Dry weight was recorded based on individual plants from each pot. The experiment was conducted twice and data were analyzed by ANOVA. Mean separation was accomplished using the LSD test ($P \leq 0.05$). Since the results from repeated experiments were similar, the average of both trials was presented.

Results

Inhibition of mycelial growth

In total 58 microorganisms (52 bacteria and six *Trichoderma* spp.) were isolated from wheat rhizosphere. From these 58 isolates, 57 microorganisms (51 bacteria and six fungi) showed inhibition of mycelial growth of *F. graminearum* (data not shown), but 31 isolates were the only strains capable of inhibiting the disease in the sterile soil assay (Table 1). The growth inhibition ranged from 0% (1 bacterium) to amounts between 1 and 25% (10 bacteria), 26–50% (15 bacteria), 51–75% (23 bacteria and six *Trichoderma*) and 76–100% (three bacteria). From these 52 bacterial isolates, 29 cultures produced some inhibition zone on PDA plates, with 16 microorganisms producing an inhibition zone greater than 5 mm, six produced inhibition zones between 2 and 5 mm and seven formed an inhibition zone less than 2 mm. Most of the bacterial isolates which inhibited growth of *F. graminearum* also produced an inhibition halo, but there was no relationship between the extent of growth reduction and the size of the inhibition zone. Therefore, some of the bacterial isolates (e.g., 8–12–27) that markedly inhibited *F. graminearum* growth did not produce an inhibition zone, and *vice versa* (i.e., 15). Among the 58 rhizosphere isolates, only the six *Trichoderma* spp. were initially identified. They included one isolate of *Trichoderma aureoviride*, two isolates of *T. hamatum* and three isolates of *T. harzianum*. *In vitro* inhibitory effects varied with isolates, even among those belonging to the same species. For instance, among the three strains of *T. harzianum*, organism 16 strongly inhibited *F. graminearum*, whereas organisms 3 and 11 showed moderate inhibition to the pathogen. There was no relationship between the reduction in the mycelial growth of the pathogen and the size of the inhibition zone caused by a particular antagonist.

Sterile soil assay

In greenhouse experiments, of 58 original rhizosphere isolates initially screened for ability to suppress damping-off of wheat, 31 isolates had a beneficial effect in any of both cultivars when applied to wheat seeds in sterilized soils (Table 1). Nevertheless, all plants sustained disease

Table 1. In vitro antagonism against *F. graminearum* and cultivar reaction to the inhibition of damping-off after treatment with selected bacteria and *Trichoderma* spp. isolated from the wheat rhizosphere and added to sterile soils.

Isolate	Organism	Origin		*In vitro* antagonism on PDA		Cultivar reaction[a]	
		Cultivar	Field soil	Growth[b] inhibition	Inhibition zone[c]	Pro INTA Oasis	Klein Centauro
1	Bacterium	Pro INTA Oasis	Vegetable (Gorina)	57.8	*	0.15	1.28
2	Bacterium	Pro INTA Oasis	Wheat (La Plata)	51.5	**	0.35	1.0
3	*T. harzianum*	Pro INTA Oasis	Wheat (La Plata)	62.5	–	0.35	1.55
4	Bacterium	Pro INTA Oasis	Wheat (La Plata)	63.6	**	0.42	1.83
5	Bacterium	Klein Centauro	Corn (Los Hornos)	53.0	**	0.43	1.23
6	Bacterium	Pro INTA Oasis	Wheat (La Plata)	55.6	**	0.52	1.52
7	Bacterium	Klein Centauro	Wheat (La Plata)	79.5	*	0.53	1.03
8	Bacterium	Klein Centauro	Wheat (La Plata)	62.5	–	0.55	1.52
9	Bacterium	Klein Centauro	Wheat (La Plata)	3.40	**	0.60	1.47
10	Bacterium	Pro INTA Oasis	Corn (Los Hornos)	37.5	–	0.63	1.72
11	*T. harzianum*	Pro INTA Oasis	Wheat (La Plata)	58.7	–	0.64	1.21
12	Bacterium	Klein Centauro	Wheat (Los Hornos)	75.0	–	0.68	1.47
13	Bacterium	Klein Centauro	Wheat (La Plata)	19.3	***	0.70	1.20
14	Bacterium	Klein Centauro	Wheat (La Plata)	27.2	***	0.70	0.95
15	Bacterium	Klein Centauro	Wheat (La Plata)	17.0	***	0.72	1.45
16	*T. harzianum*	Klein Centauro	Wheat (La Plata)	74.0	–	0.88	1.03
17	*T. hamatum*	Klein Centauro	Wheat (La Plata)	58.7	–	0.92	1.22
18	Bacterium	Pro INTA Oasis	Wheat (La Plata)	57.9	***	0.93	0.53
19	Bacterium	Pro INTA Oasis	Wheat (La Plata)	29.5	***	1	1.92
20	Bacterium	Klein Centauro	Wheat (La Plata)	57.9	–	1.05	1.95
21	*T. hamatum*	Pro INTA Oasis	Wheat (La Plata)	58.2	–	1.15	3.02
22	Bacterium	Pro INTA Oasis	Corn (Los Hornos)	23.8	***	1.15	2.08
23	Bacterium	Klein Centauro	Wheat (Los Hornos)	68.1	***	1.35	2.93
24	Bacterium	Pro INTA Oasis	Corn (Los Hornos)	77.2	*	1.37	3.23
25	Bacterium	Pro INTA Oasis	Wheat (Los Hornos)	42.0	–	1.38	3.1
26	Bacterium	Pro INTA Oasis	Wheat (Los Hornos)	60.2	**	1.50	4.15
27	Bacterium	Pro INTA Oasis	Wheat (Tres Arroyos)	55.6	–	1.53	3.13
28	*T. aureoviride*	Pro INTA Oasis	Wheat (La Plata)	70.0	–	1.53	2
29	Bacterium	Klein Centauro	Wheat (La Plata)	56.8	***	1.55	1.62
30	Bacterium	Pro INTA Oasis	Corn (Los Hornos)	67.0	**	1.60	1.55
43	Bacterium	Pro INTA Oasis	Corn (Los Hornos)	52.2	–	2.95	1.17
Control						2.33	2.47

[a] Seedling reaction of cultivars Pro INTA Oasis (susceptible to *F. graminearum*) and Klein Centauro (moderately resistant) to suppression of damping-off by effect of seed treatments with biocontrol agents in sterilized soil infested with *F. graminearum* at 14 – 21 °C. Evaluations were made at harvest (21 days after planting) and only results of six *Trichoderma* and 25 bacterial isolates that reduced diseases as compared with the nontreated controls, are presented. Rating of necrosis in roots and subcrown internodes is from 0 to 5 scale, where 0 = completely healthy plant; 1 = less than 25% necrosis; 2 = 26–50% necrosis; 3 = 51–75% necrosis; 4 = 76–100% necrosis, and 5 = no plant emergency. Values were the results of means of measurements from six replicates. LSD = 0.63.

[b] Percent growth inhibition was determined after 7 days. Numbers are the means of the percent growth inhibition of *F. graminearum* of five replications.

[c] Width of bacterial inhibition zone was measured after 7 days. Values were categorized on a scale from – to ***, where – is 0 mm which denotes no inhibition; * is <2 mm standing for slight inhibition; ** is 2–5 mm standing for moderate inhibition; *** is >5 mm standing for strong inhibition (Liang *et al.* 1996).

symptoms in one or more tissues of seedlings. Disease developed extensively in roots and less in subcrown internodes and leaf sheaths. Treatments with six *Trichoderma* spp. and 25 bacterial isolates significantly reduced severity of symptoms of *F. graminearum* damping-off as compared with the non-treated controls. Wheat cultivars showed differences in the reaction of the disease to the biocontrol agents. Some of them were only effective in one cultivar, but the four *Trichoderma* (3–11–16 and 17) and 10 bacterial isolates (strains 1–2–6–7–8–9–10–12–13 and 26), reduced severity of damping-off in both Pro INTA Oasis and Klein Centauro (Table 1). The best 10 (strains 1, 2, 5, 7, 11, 13, 14, 16–18) for both cultivars were choosen for the following assay.

Pot/cultivated soil assay

The results obtained for the pot/soil experiment are given in Tables 2–5. Data showed that there were significant differences between isolates, cultivars and the interaction of cultivars × antagonists for all traits. Among the antagonists only *S. maltophilia* (strain 18) was significantly better than the control for the average of the eight cultivars for plant stand, height and dry weight. *Stenotrophomonas maltophilia* also caused a decrease in the percentage of diseased plants but it was not statistically different from the control. Three strains of *B. cereus* (strains 2, 5 and 7) and one isolate of *T. harzianum* (strain 16) gave also a good behaviour in

Table 2. Effect of 10 selected isolates from the rhizosphere of wheat on plant stand of eight wheat cvs. grown in pots with cultivated soil artificially infested with *F. graminearum*.

Antagonist	Cultivars[a]								
	B. Topacio	K. Cacique	P. INTA Oasis	B. Chambergo	K. Centauro	B. Poncho	B. Candil	B. Catriel	Average
17	6.66 ab[b]	13.33 a	6.66 a	20 ab	6.66 ab	0 a	6.66 a	0 a	7.5 a
11	0 a	26.66 a	26.66 ab	60 cd	26.66 ab	40 ab	13.33 a	20 ab	26.67 b
16	13.33 ab	13.33 a	40 ab	26.66 ab	26.66 ab	20 ab	0 a	60 cd	25 b
2	40 b	26.66 a	20 ab	20 ab	0 a	26.66 ab	13.33 a	46.66 bcd	24.2 b
5	20 ab	20 a	53.33 b	26.66 abc	20 ab	33.33 ab	6.66 a	40 bc	27.5 b
1	13.33 ab	26.66 a	26.66 ab	13.33 a	40 ab	15 ab	6.66 a	46.66 bcd	23.5 ab
7	26.66 ab	46.66 ab	46.66 b	66.66 de	20 ab	33.33 ab	0 a	33.33 a	34.2 bc
13	26.66 ab	40 ab	26.66 ab	26.66 abc	43.33 ab	0 a	13.33 a	40 bc	23.3 ab
14	20 ab	26.66 a	26.66 ab	40 abcd	33.33 ab	20 ab	26.66 a	26.66 a	27.5 b
18	33.33 ab	66.66 b	46.66 b	66.66 de	46.66 b	46.66 b	26.66 a	60 bcd	49.2 c
Control + *F. gram.*	0 a	46.66 ab	40 ab	53.33 bcd	20 a	46.66 b	6.66 a	6.66 a	25 b
Control	86.66 c	100 c	100 c	100 e	86.66 c	100 c	93.33 b	80 d	93.3 d
Average[c]	23.89 ab	37.78 cd	38.33 cd	43.33 d	26.67 abc	31.81 bcd	17.78 a	38.33 cd	
Cultivars	***								
Antagonists	***								
Interaction cv. × ant.	**								

[a] Values were the results of means of measurements from five replicates for percentage of diseased emerging seedlings in pot/cultivated soil assays. Plants were evaluated 21 days after sowing.
[b] Values within the same column followed by the same letter do not differ significantly ($P \leq 0.05$) according to LSD test.
[c] For the average of the cultivars values within the same row followed by the same letter do not differ significantly ($P \leq 0.05$) according to LSD test.
[d] Significant at $P \leq 0.001$ (***) or $P \leq 0.01$ (**).

Table 3. Effect of 10 selected isolates from the rhizosphere of wheat on the percent of diseased emerging plants of eight wheat cvs. grown in pots with cultivated soil artificially infested with *F. graminearum*.

Antagonist	Cultivars[a]								
	B. Topacio	K. Cacique	P. INTA Oasis	B.Chambergo	K. Centauro	B. Poncho	B. Candil	B. Catriel	Average
17	93.33 b[b]	100 c	100 c	93.33 b	93.33 b	100 c	93.33 b	100 d	96.67 d
11	100 b	86.66 ab	93.33 bc	86.66 b	93.33 b	60 b	86.66 b	80 cd	85.83 cd
16	93.33 b	86.66 bc	80 bc	80 b	86.66 b	93.33 c	100 b	60 bc	85 cd
2	73.33 b	100 c	80 bc	93.33 b	100 b	100 c	100 b	53 b	87.5 cd
5	93.33 b	80 bc	53.33 b	73.33 b	93.33 b	80 bc	100 b	73.33 bc	80.83 c
1	93.33 b	86.66 bc	80 bc	86.66 b	80 b	85 bc	93.33 b	60 bc	83.12 cd
7	86.66 b	80 bc	73.33 bc	66.66 b	80 b	66.6 bc	100 b	80 cd	79.17 bc
13	80 b	66.66 bc	73.33 bc	73.33 b	86.66 b	100 c	86.66 b	66.66 bc	79.17 bc
14	86.66 b	73.33 bc	66.66 bc	60 b	66.66 b	93.33 bc	73.33 b	80 cd	75 bc
18	80 b	60 b	80 b	60 b	66.66 b	76.66 bc	73.33 b	40 b	65.83 b
Control + *F. gram.*	100 b	60 b	73.33 b	53.33 b	100 b	73.33 bc	80 b	93.3 cd	79.17 bc
Control	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
Average[c]	81.67 cd	73.33 abcd	71.11 abc	68.89 ab	78.89 bcd	76.53 abcd	82.22 d	65.56 a	
Cultivars	***								
Antagonists	***								
Interaction cv. × ant.	**								

[a] Values were the results of means of measurements from five replicates for percentage of seedling stand in pot/cultivated soil assays. Plants were evaluated 21 days after sowing.
[b] Values within the same column followed by the same letter do not differ significantly ($P \leq 0.05$) according to LSD test.
[c] For the average of the cultivars values within the same row followed by the same letter do not differ significantly ($P \leq 0.05$) according to LSD test.
[d] Significant at $P \leq 0.001$ (***) or $P \leq 0.01$ (**).

some cultivars, in spite of its average for all the cultivars was not significantly different than the control. The results showed that cultivars Klein Cacique, Pro INTA Oasis, Buck Chambergo and Buck Poncho had the higher plant stand average. Buck Catriel also had a good average of emerging plants due to its better response to the effect of the antagonists, compared with the low plant stand of the control in infested soil. To demonstrate the effect of the antagonists respect to *F. graminearum* in this cultivar, the average was calculated

Table 4. Effect of 10 selected isolates from the rhizosphere of wheat on the plant height of eight wheat cvs. grown in pots with cultivated soil artificially infested with *F. graminearum*.

Antagonist	Cultivars[a]								
	B. Topacio	K. Cacique	P. INTA Oasis	B. Chambergo	K. Centauro	B. Poncho	B. Candil	B. Catriel	Average
17	1.21 a[b]	1.09 a	0.91 a	3.58 a	1.93 a	0 a	0.56 a	0 a	1.16 a
11	0 a	0.85 a	5.49 ab	8.47 abc	3.57 a	8.80 ab	2.81 a	4.66 ab	4.33 ab
16	0 a	5.22 ab	6.88 abc	7.70 abc	5.05 a	3.51 ab	0 a	12.19 b	5.07 ab
2	9.66 a	4.11 ab	4.87 ab	6.42 abc	0 a	2.79 a	2.03 a	9.64 ab	4.94 ab
5	3.52 a	6.61 ab	13.72 bc	6.20 ab	3.90 a	6.49 ab	1.66 a	10.65 ab	6.59 b
1	0.67 a	5.68 ab	8.63 abc	3.95 ab	8.51 a	4.41 ab	0.59 a	9.15 ab	5.20 ab
7	2.66 a	8.43 abc	16.18 c	18.99 cd	4.25 a	9.43 ab	0 a	3.57 ab	7.94 bc
13	4.41 a	8.43 abc	8.57 ab	7.96 abc	2 a	0 a	3.57 a	11.08 ab	5.74 b
14	4.63 a	8.27 abc	6.98 ab	10.58 abc	9.9 a	4.47 ab	6.76 a	3.54 ab	6.89 bc
18	4.46 a	17.75 c	6.14 ab	16.66 c	11.52 b	13.24 b	4.56 a	13.57 b	10.99 c
Control + *F. gram.*	0 a	12.19 bc	10.05 ab	14.16 bc	0 a	9.33 ab	1.76 a	1.67 a	6.15 b
Control	21.99 b	26.87 d	32.90 d	28.13 d	26.41 c	28.55 c	22.24 b	21.99 b	26.13 d
Average[c]	4.44 ab	8.78 cde	10.11 de	11.07 e	6.42 abc	7.58 bcd	3.88 a	8.48 cde	
Cultivars	***								
Antagonists	***								
Interaction cv. × ant.	***								

[a] Values were the results of means of measurements from five replicates for plant height in pot/cultivated soil assays. Plants were evaluated 21 days after sowing.
[b] Values within the same column followed by the same letter do not differ significantly ($P \leq 0.05$) according to LSD test.
[c] For the average of the cultivars values within the same row followed by the same letter do not differ significantly ($P \leq 0.05$) according to LSD test.
[d] Significant at $P \leq 0.001$ (***).

Table 5. Effect of 10 selected isolates from the rhizosphere of wheat on the dry weight of eight wheat cvs. grown in pots with cultivated soil artificially infested with *F. graminearum*.

Antagonist	Cultivars[a]								
	B. Topacio	K. Cacique	P. INTA Oasis	B. Chambergo	K. Centauro	B. Poncho	B. Candil	B. Catriel	Average
17	153.33 ab[b]	152.33 a	153 a	164 a	157.33 a	150 a	151.33 a	150 a	153.9 a
11	150 a	161 a	168 ab	181.66 ab	158.33 a	177.33 ab	159 a	164.66 ab	165 ab
16	159 ab	162.66 ab	173.66 ab	174.66 ab	164 a	160.66 ab	150 a	187 b	166.5 ab
2	186.66 b	164.33 b	168 ab	175.33 ab	150 a	160.33 ab	158.66 a	180.66 ab	168 ab
5	164.66 ab	169 ab	192.66 b	171.66 ab	162 a	170.66 ab	156.66 a	187.33 b	171.8 bc
1	152 ab	167.33 ab	176.66 ab	165 a	175.66 a	164.66 ab	153.33 a	177.66 ab	166.5 ab
7	158.66 ab	176 ab	202 ab	222.66 b	163 a	184 ab	150 a	159.66 ab	177 bc
13	167.33 ab	172.66 ab	184 ab	183.66 ab	157.66 a	150 a	161.33 a	185 ab	170.2 ab
14	169 ab	176 b	173.66 ab	191 ab	185.66 a	164 ab	170 a	162.66 ab	174 bc
18	166.33 ab	199 ab	169 ab	213.33 b	189.33 a	194 b	164.33 a	192 bc	185.9 c
Control + *F. gram.*	150 a	184.33 ab	178.66 ab	205.33 b	150 a	182.66 ab	155.66 a	155 a	170.2 b
Control	257.66 c	230.66 c	251.66 c	264 c	249 c	249.33 c	217.33 c	224.66 c	243 d
Average[c]	169.6 a	176.3 a	182.6 ab	192.7 b	176.8 a	175.6 a	162.3 a	177.2 a	
Cultivars	***								
Antagonists	***								
Interaction cv. × ant.	**								

[a] Values were the results of means of measurements from five replicates for dry weight in pot/cultivated soil assays. Plants were evaluated 21 days after sowing.
[b] Values within the same column followed by the same letter do not differ significantly ($P \leq 0.05$) according to LSD test.
[c] For the average of the cultivars values within the same row followed by the same letter do not differ significantly ($P \leq 0.05$) according to LSD test.
[d] Significant at $P \leq 0.001$ (***) or $P \leq 0.01$ (**).

without considering the means of the controls. In this way values of plant stand, percentage of diseased plants, height and dry weight were 37.33%, 54%, 7.80 cm and 174 mg when compared with 6.66%, 93.33%, 1.67 cm and 155 mg of the control, respectively.

Discussion

Biological control is becoming an important component of plant disease management. The design and development of techniques for isolating and screening poten-

tial biocontrol agents is crucial for the detection of bioactive microorganisms (Swadling & Jeffries 1996). In this study, antagonists were isolated following a protocol proposed by Cook & Baker (1983), which is to seek effective antagonists in which the target pathogen is present but produces little or no disease in spite of a susceptible host and apparently favorable environment.

The rhizosphere colonists have considerable potential for protection against seed and root rots (Cook & Baker 1983; Liang *et al*. 1996). According to this criterion, we used macerates of washed roots from two cultivars sown in different soils as a source of candidate biocontrol agents.

Kloepper (1991) recommended the use of rapid prescreen techniques allowing large number of strains to be tested. This has led to the widespread use of *in vitro* dual culture techniques as the initial screening procedure for potential biocontrol agents. *In vitro* screening has met varied success. Several reviews indicate organisms selected from *in vitro* tests generally fail as biological control agents under field conditions (Webber & Hedger 1986; Lewis & Papavizas 1991) and on the contrary, there are reports where the method proved useful for preliminary selection of biocontol agents (Campbell 1986; Swadling & Jeffries 1996). Jackson *et al*. (1997) point out that agar techniques should be included because *in vivo* screening can be too severe, rejecting potentially useful candidates. As a result of this approach, 29 bacteria were found to produce detectable inhibition zones on agar, but this parameter was not related to either with growth suppression of *F. graminearum* or biocontrol efficacy in soil trials. Data suggest that *in vitro* screening was a poor predictor of *in vivo* performance. This inconsistency may be due to the parameters measured in each system are different (Fravel 1988). Fokkema (1973) pointed out that the various growth rates of a saprophytic fungi affect the radial growth of the pathogen independent of their antagonistic actions, whereas the inhibition zone remains unaffected. Alternately, the width of the inhibition zone may be affected by the delay of the growth of the test organism. Similar effects could be attributed to some saprophytic bacteria. In relation to the growth inhibition Bharat & Singh (1980) considered insignificant mycelial growth reduction under 30%, but in this study all isolates progressed to greenhouse screening. The purpose was to test the relationship between *in vitro* inhibition of *F. graminearum* and wheat damping-off suppression by rhizosphere antagonists. Our results showed a different behaviour according to each bioassay. In sterilized soil, 31 isolates had a beneficial effect when applied to wheat seeds and in general, there were greater plant stands and lower severity of damping-off compared to controls. Only *S. maltophilia* was capable of suppressing the damping-off on the eight wheat cultivars under greenhouse conditions, demonstrating its biological effect independently on the resistance of the cultivars. Interactions cultivars × antagonists were present. *Trichoderma harzianum* and *B. cereus* improved some traits in some cultivars. The higher plant stand under greenhouse conditions of the cultivars Klein Cacique and Pro INTA Oasis was caused by their higher level of resistance compared to other cultivars. This is shown by the high plant stand of their respective controls with *F. graminearum*. Strains of *T. harzianum* have been effective against *F. graminearum* and other soil-borne pathogens (Kwok *et al*. 1987; Harman *et al*. 1989; Datnoff *et al*. 1995; Knudsen *et al*. 1995; Luz *et al*. 1998). Strains of *B. cereus* and *S. maltophilia* highly reduced the disease severity induced by *F. graminearum* in greenhouse studies. There are reports regarding the antifungal activity of *B. cereus* expressed as inhibition of the disease severity (Silo-Suh *et al*. 1994; Kong *et al*. 1997; Alippi *et al*. 2000) or increasing the plant emergence (Handelsman *et al*. 1990).

In this study, *S. maltophilia* was the most effective of the antagonists in growth-chamber. Not only did it have the best efficacy in suppressing damping-off, which was significantly superior in effectiveness to other antagonists, but also its performance was more consistent for dry weight compared with *Trichoderma* and other bacterial isolates. With regard to the toxicological and environmental risks caused by *S. maltophilia* (Spencer 1995), safety questions need to be addressed before its potential use can be recommended (Zhang & Yuen 1999). There are reports of rhizosphere strains of *S. maltophilia* being used as effective antagonists of the fungal root pathogen *Pythium ultimum* (Dunne *et al*. 1997) and Rhizoctonia damping-off (Kwok *et al*. 1987). Strain C3 of *S. maltophilia* is chitinolytic, and was selected originally as an antagonist of *Rhizoctonia solani* AG 1-IA (Giesler & Yuen 1998). In growth-chamber and field experiments on tall fescue, the bacterial strain suppressed leaf spot caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker (Zhang & Yuen 1999).

Seed bacterization has proven a successful method for enhancing biological control of plant diseases. In this study, seed treatments with a bacterial strain significantly decreased severity of seedling blight and increased seedling stands compared with those from untreated seeds in pathogen-infested soil. Seed coating may be specially effective for controlling preemergence and postemergence diseases. In such cases the mature plant may be immune, so protection is needed only for short period at the seedling stage (Chao *et al*. 1986).

Although there is little information on the biocontrol of damping-off of wheat, these data indicate that the prospects for the biological control of damping-off and related soil-borne diseases of wheat appears to be promising. Our results indicate that treatments with *S. maltophilia* significantly reduced damping-off of wheat and it is a potential biocontrol agent of this disease. Alternatively, compatible chemical fungicides may be required for complete disease control (Lo *et al*. 1996).

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