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Research in Microbiology 156 (2005) 748–754
Research in Microbiology
www.elsevier.com/locate/resmic

Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* in vitro and at the maize root level

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Received 30 July 2004; accepted 1 March 2005

Available online 29 March 2005

Abstract

Bacillus species as a group offer several advantages over other bacteria for protection against root pathogens because of their ability to form endospores, and because of the broad-spectrum activity of their antibiotics. The objectives of this work were to determine the ability of strains of *Bacillus* to inhibit *Fusarium verticillioides* growth and fumonisin B_1 accumulation in vitro, and to evaluate the ability of the best bacterium for preventing rhizosphere and endorhizosphere colonization by *F. verticillioides*. Bacterial populations from the maize rhizoplane were obtained, and the capacity of ten *Bacillus* strains to inhibit fungal growth and fumonisin B_1 accumulation in vitro was assayed. According to these results, *B. subtilis* CE1 was selected as the best antagonist for testing maize root colonization of *F. verticillioides*. *Bacillus subtilis* CE1 at 10^8 and 10^7 CFU ml^{-1} inocula was able to reduce rhizoplane and endorhizosphere colonization of *F. verticillioides* in greenhouse trials. The strain *B. subtilis* CE1 could be a potential biological control agent against *F. verticillioides* at the root level.

Keywords: *Bacillus subtilis*; *Fusarium verticillioides*; Biocontrol; Growth; Fumonisin B_1

1. Introduction

Maize (*Zea mays* L.) is one of the most important cereal grains grown worldwide. *Fusarium verticillioides* is one of the most commonly reported soil-borne fungal pathogens infecting maize [1,2,36] and the most prevalent *Fusarium* isolated in freshly harvested maize in Argentina [11,18]. This microorganism not only causes severe reductions in yields and quality, but also produces secondary metabolites such as fumonisins (FB), especially fumonisin B_1 (FB_1), which affect human and animal health [10,19,31]. *F. verticillioides* can be found in maize fields at different stages of maize ear development [23]. The disease symptoms vary from asymptomatic infection to severe rotting of all plant parts. Movement of the fungus inside the young plant from the roots to the stalk and finally to the cob and kernels has been shown in a number of studies [23,38]. Since *F. verticillioides* is endophytic in maize [2] and is almost universally associated with maize and maize products [43,44], it is very important to control this species in this agriculturally important commodity. Furthermore, root colonization by *F. verticillioides* has been considered the initiator of systemic infection that eventually results in the fungus producing fumonisins in kernels. Seed treatment with biocontrol agents is an appropriate method for biocontrol of soil-borne plant pathogens in the spermosphere and rhizosphere [4,24,30,35]. *Bacillus* species, as a group, offer several advantages over other bacteria for protection against root pathogens because of their ability to form endospores and the broad-spectrum activity of their antibiotics. There are numerous reports of *Bacillus* spp. which repress pathogens [3,13,15]. Rhizosphere colonization and root disease control with *Bacillus* species introduced as seed inoculants have been reported [20,27,29]. The influence of biological control agents that protect seeds and roots has been determined elsewhere [3,28,48], but the

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0923-2508/$ – see front matter
doi:10.1016/j.resmic.2005.03.001

ability of *Bacillus strains* to prevent *F. verticillioides* colonization of maize roots in Argentina has not been evaluated.

The objectives of this work were: (1) to determine the ability of strains of *Bacillus* to inhibit *F. verticillioides* growth and fumonisin B_1 accumulation in vitro, and (2) to evaluate the ability of the most effective bacterium for preventing rhizosphere and endorhizosphere colonization by *F. verticillioides*.

2. Materials and methods

2.1. Sampling

Samples from a commercial maize field (Morgan M401, Argentina SA, Buenos Aires, Argentina) were collected during seedling stage (15 days). The field soil was loamy sand. Twenty plants were randomly chosen and removed. Plants were lifted together with adherent soil into plastic bags, transported to the laboratory within 12 h and analyzed the same day.

2.2. Microorganisms

To obtain bacterial populations from the maize rhizoplane, roots were gently washed in phosphate-buffered saline (PBS, Oxoid, Ltd., London, UK) for 2 min to remove the adhering soil and shaken for 5 min in PBS containing 0.025% Tween 20. A threefold dilution of the homogenates was plated on tryptic soy broth plus 2% agar (TSBA). Plates were incubated for 24–48 h at 25 °C. Total counts and counts per colony type were made. One colony per colony type was isolated and purified on TSBA [25,26,47].

Selected *Bacillus* strains were examined for Gram reaction, oxygen requirement, spore formation and catalase reaction [47]. Ten out of 74 strains of *Bacillus* spp. were selected according to exclusionary principles criteria proposed by Cavaglieri et al. [8]. These bacteria obtained higher niche overlap indices (NOIs).

Strain CE1, the best in vitro competitor, was identified on the basis of 16S rDNA gene sequence similarity analysis conducted by Macrogen (Pathfinder in Genomics Research–Macrogen Inc., Korea). *Bacillus subtilis* CE1 (RCRL 1289 demonstrated 100% similarity with *B. subtilis* 1413, MO4; AY553097).

F. verticilloides M7075, a fumonisin B_1 producer, was isolated from maize in Argentina. This strain is deposited in the *Fusarium* Research Center Collection (Pennsylvania State University, University Park, PA, USA).

2.3. Bacillus *inoculum preparation*

Two loopfuls of each bacterium from 3 day-old cultures on triptic soy agar (AS) were transferred separately to 50 ml tryptic soy broth (TSB) medium and incubated overnight at 25 °C. Bacterial cells were centrifuged (4500*g*, 15 min) and re-suspended in spore suspension (sodium lauryl sulfate 0.01%). Serial decimal dilutions were done to obtain 10^9 cells ml^{-1} from each bacterium homogenate. The concentration of cell suspension was determined using a hemocytometer. Viability was confirmed by standard plate count method using TSBA.

2.4. Fungal spore preparation

Erlenmeyer flasks (500 ml) containing 100 ml of a sporulation medium [7] were inoculated with a 6 mm disk cut from a single spore isolate of *F. verticillioides* cultured on carnation leaf agar (CLA) [37] using a sterile cork borer. Culture was incubated at 25 °C for 7 days on an oscillatory shaker (200 rpm). At the end of the incubation period, spores were harvested by filtration through sterile gauze. The spore suspension was prepared in sodium-lauryl sulfate (0.01%, w/v) and NaCl (5%). The spore concentration was determined using a hemocytometer and suspensions were diluted to obtain 10^6 spores ml^{-1}. Conidial viability was confirmed by the standard plate method using Nash and Snyder agar [37].

2.5. In vitro studies

2.5.1. Determination of antibiosis

Culture medium was 3% maize meal extract agar (MMEA) [32]. Petri dishes were inoculated with 10 µl of 10^6 spores ml^{-1} of *F. verticillioides* in the center of each plate. Ten microliters of the different bacterial antagonists to a final concentration of 10^9 CFU ml^{-1} were inoculated in 2.5 mm diameter wells around the fungal inoculum. Treatments were incubated for 10 days at 25 °C in polyethylene bags. The inhibitory effects of *Bacillus* strains on the linear growth of *F. verticillioides* were determined. For growth of dual cultures (*Bacillus* spp.–*F. verticillioides*), complete growth inhibition was considered to be 100% inhibition. The percent of inhibited *F. verticillioides* was calculated.

2.5.2. Determination of inhibition of fumonisin B_1 production

Before cooling, MMEA (10 ml) was combined with 100 µl (10^{10} cells ml^{-1}) of each antagonist and poured into Petri dishes (60 × 10 mm). Ten microliters of *F. verticillioides* (10^6 cells ml^{-1}) were inoculated into the center of each plate. Cultures were incubated at 25 °C for 20 days in polyethylene bags. Toxins were extracted with acetonitrile–water (1:1, v/v) by shaking the culture media and mycelia from co-inoculated and control cultures with the solvent for 30 min on an orbital shaker (150 rev min^{-1}) and then filtering the extracts through filter paper no. 4; Whatman) [17]. The extracts were frozen and stored at –20 °C until analyzed. An aliquot of the extract (1000 µl) was taken and diluted with acetonitrile–water as necessary for high performance liquid

chromatography. The fumonisin content of the OPA derivative was evaluated by HPLC, as described by Shephard et al. [46] and modified by Doko et al. [14].

2.5.3. Maize seed sterilization and inoculation

Maize seeds were sterilized by gamma irradiation at 25 °C in a ^{60}Co source at the National Commission on Atomic Energy (Buenos Aires, Argentina); a dose of 1200 krad was used. This treatment was used to eliminate the natural microflora of seeds without affecting their germination, viability and nutritive value [16]. Sterility tests were performed to determine the efficacy of irradiation treatment. Maize seeds were placed in dichloran–rose-bengal–chloramphenicol agar (DRBC) [39] and plates were incubated for 7 days at 25 °C.

To determine the initial water content, 2 g of maize seeds (five replicates) were dried using an oven with forced air circulation for 3 h at 130 ± 1 °C. Samples were then weighed until they reached constant weight and the initial water content was determined. Twenty grams of irradiated maize seeds were placed in borosilicate Petri dishes and adjusted with sterile distilled water to achieve 0.982 water activity. The water content of maize was allowed to equilibrate for 7 days at 5 °C to avoid seed germination.

Petri dishes containing 70 g of maize grains were inoculated with 1 ml (10^6 spores ml^{-1}) of *F. verticillioides* and 1 ml (10^9 cells ml^{-1}) of *B. subtilis*. Control treatment consisted in 70 g of maize grains with 1 ml (10^6 cells ml^{-1}) of *F. verticillioides*. Cultures were incubated for 35 days at 25 °C in a chamber under controlled relative humidity (97%) achieved using a K_2SO_4 saturated solution. To avoid any changes in relative humidity, the saline solution was replaced daily with fresh solution over a 35-day period. Three replicates per treatment were removed at 10, 20 and 35 interval days for analysis of ergosterol content, fumonisin B_1 content and *F. verticillioides* colony-forming units (CFUs).

2.5.4. Ergosterol analysis

Ergosterol was determined according to the method proposed by Seitz et al. [45] and modified by Bermingham et al. [5]. A 10 g sample of ground maize was extracted with 50 ml of methanol for 30 min on a shaker. The extract was filtered and 25 ml were added to a screw-capped test tube containing 3 g of KOH. The mixture was agitated on a vortex mixer to dissolve the KOH and 10 ml of *n*-hexane was added. The mixture was incubated at 75 °C in a water bath for 30 min and then it was allowed to cool to room temperature. The mixture was allowed to settle and 25 ml of the extract was obtained. Five ml of distilled water were added to the mixture. It was shaken and then the hexane layer removed and transferred to a 50 ml beaker. The mixture was extracted a second time and combined with the first hexane extract. The pooled hexane extracts were evaporated on a hot-water bath (65 °C). The residue was redissolved in 5 ml of methanol (HPLC grade), filtered through a Millipore filter of 0.45 μm and the filtrate subject to HPLC analysis. Twenty ml of the filtrate were analyzed using the method proposed by Bermingham et al. [5]. The extract was loaded on a reverse phase column (Phenomenex IB-SIL 5 C18, 150 × 4.6 mm, 00F-0082-E0) with a UV detector (Hewlett–Packard). The absorbance of ergosterol was detected at 282 nm. The mobile phase was methanol–water (96:4) at a flow rate of 1.2 ml min^{-1}. A standard ergosterol (Sigma) retention time of 8.3 min was used to develop a calibration curve using ergosterol solutions of 1, 2, 3, 5 and 6 mg ml^{-1}. A 20 μl injection was used for each level. The precision of the ergosterol extraction method was determined by analysis of ergosterol concentration. The mean ergosterol concentration for 5 experimental samples ranged from 92.6 to 102 μg g^{-1} and the percentage coefficient of variation by the ergosterol concentrations were: 1 μg, 8.1%; 5 μg, 3.9%; 10 μg, 2.6%; 25 μg, 2.7%. The ergosterol recovery from spiked samples with 100 μg had a mean of 96 μg (n = 10, range = 89–100) and a coefficient of variation of 3.6%.

2.5.5. Fumonisin B_1 analysis

The fumonisin B_1 concentration was determined using the method proposed by Shephard et al. [46]. Ground maize seeds (25 g) were extracted with 50 ml of methanol:water (3:1, v/v) and shaken for 30 min on an orbital shaker. The extract was filtered through filter paper (no. 4, Whatman, Inc., Clifton, NJ). The extract was quantitatively analyzed for FB_1 by HPLC as described above.

2.5.6. F. verticillioides *CFUs*

Ground maize (10 g) was blended with 90 ml of 0.1% peptone water solution. Serial dilutions from 10^{-2} to 10^{-4} were made and 0.1 ml aliquots were inoculated in triplicate on Nash–Snyder agar. Plates were incubated for 7 days at 28 °C. Total counts were made. *F. verticillioides* was confirmed according to Nelson et al. [37].

2.6. Greenhouse studies

According to results obtained in in vitro assays *B. subtilis* CE1 was selected as the best antagonist to test maize root colonization of *F. verticillioides*. *B. subtilis* CE1 inoculum was prepared as described above. Serial decimal dilutions were done to obtain 10^6, 10^7 and 10^8 cells ml^{-1}. Antagonistic treatments were prepared: (1) *B. subtilis* CE1 at 10^6 cells ml^{-1}; (2) *B. subtilis* CE1 at 10^7 cells ml^{-1}; and (3) *B. subtilis* CE1 at 10^6 cells ml^{-1}.

Seventy-five seeds of maize were submerged in 100 ml of bacterial inoculum in 250 ml Erlenmeyer flasks. Control seeds were submerged in 100 ml of TSB. Flasks (250 ml Erlenmeyer flasks) were incubated at 25 °C on a rotatory shaker at 70 rpm for 2 h to allow bacterial cells to adhere to seeds. After incubation, excess inoculum was removed and seeds were immediately planted for greenhouse studies.

B. subtilis CE1 was tested for its ability to inhibit *F. verticillioides* root colonization using a modification of the tube assay described by Weller and Cook [49]. Plastic tubes

2.5 mm in diameter and 16.5 cm long were filled with 2 cm of field maize soil. One treated seed was placed on top of the soil and covered with 2 cm of sand–vermiculite (1:1, v/v). Non-treated seeds were planted as controls in each assay. Water was added to each tube on alternate days after planting, racks of tubes were covered with aluminum foil, kept in the dark for 5 days at 20 °C, then uncovered and kept at 20 °C with a 14 h photoperiod. After 20 days, all plants were collected and CFU counts of *F. verticillioides* from rhizoplane and endorhizosphere levels were made according to Cavaglieri et al. [8]. This assay was done twice.

2.7. Statistical analysis

Antibiosis, ergosterol and fumonisin B_1 content and CFU counts were analyzed by analysis of variance. Means were compared by Fisher's LSD test to determine the significant difference between control and co-cultures except for fumonisin B_1 content, for which the Scheffé test was performed. The Pearson correlation coefficient was used to evaluate the strength of the relationships between in vitro and in-greenhouse conditions [41]. The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

3. Results

3.1. Antibiosis and reduction of fumonisin B_1 production

Antibiosis produced by 10 *Bacillus* strains on *F. verticillioides* M7075 and their influence on fumonisin B_1 production in vitro are shown in Table 1. All strains significantly inhibited *F. verticillioides* growth. Antibiosis ranged between 28 and 78%. *Bacillus* sp. 3 and *Bacillus* sp. CE1 produced the greatest antifungal activity. Mean values of fumonisin B_1 production were compared. Only *Bacillus* sp. CE1 and *Bacillus* sp. 86, were able to reduce toxin production, 50 and 29%, respectively. *Bacillus* sp. CE1 showed the most consistent *F. verticillioides* inhibition of both growth and fumonisin production in vitro.

3.2. Influence of B. subtilis CE1 on F. verticillioides ergosterol content, fumonisin B_1 accumulation and CFUs

The influence of *B. subtilis* CE1 on *F. verticillioides* M7075 ergosterol content, fumonisin production and CFUs on irradiated maize at different incubation periods is shown in Table 2. Ergosterol content was reduced 68, 44 and 42% after 10, 20 and 35 incubation days, respectively, when it

Table 1
In vitro *Bacillus* strain antagonism toward *F. verticillioides* M7075

Bacillus strain	*F. verticillioides* M7075					
	Colony diameter (mm)[a]	Scheffe test	Antibiosis (%)	Fumonisin B_1 production ($\mu g\,g^{-1}$)[b]	Scheffe test	Fumonisin B_1 reduction (%)
C	39.33 ± 13.54	b	–	3.9 ± 1.5	e	–
3	7.66 ± 5.09	i	78	22.1 ± 18.8	a	0
CE1	11.33 ± 4.69	h	60	1.9 ± 0.9	f	50
13	23.66 ± 14.89	f	35	6.5 ± 3.4	c	0
20	19.33 ± 13.11	g	40	4.1 ± 1.5	e	0
24	28.64 ± 18.22	e	28	6.4 ± 3.4	cd	0
56	32.77 ± 12.59	d	42	5.6 ± 2.7	d	0
64	29.66 ± 10.06	c	28	3.8 ± 1.1	e	0
67	35.66 ± 11.80	a	35	7.8 ± 3.3	b	0
80	36.00 ± 18.52	c	43	7.1 ± 3.1	bc	0
86	38.66 ± 14.53	b	31	2.7 ± 1.2	f	29

Values are the mean ± SD (standard deviation of 3 replicates). Letters in common are not significantly different according to [a]Fisher's protected LSD test ($P < 0.001$), [b]Scheffé test ($P < 0.001$).

Table 2
Ergosterol production, fumonisin B_1 production and CFUs of *F. verticillioides* M7075 grown in co-cultures (*F. verticillioides–B. subtilis* CE1) on irradiated maize

Incubation (days)	Treatment	*F. verticillioides* M7075		
		Ergosterol ($\mu g\,g^{-1}$)	Fumonisin B_1 ($\mu g\,g^{-1}$)	$\log_{10}$ CFUs ($\log_{10}$ CFU g^{-1})
10	C	31.6 ± 1.1 c	184.9 ± 1.3 e	10 ± 7.7 b
20	C	37.8 ± 1.1 b	577.1 ± 1.1 a	10.2 ± 8.8 a
35	C	47.4 ± 1.7 a	526.9 ± 1.1 b	10 ± 7.2 a
10	FB	10.8 ± 1.1 f	160.5 ± 1.1 f	6 ± 3.7 bc
20	FB	21.1 ± 0.5 e	499.7 ± 1.1 c	6.9 ± 3.0 cd
35	FB	27.0 ± 1.3 d	244.6 ± 9.2 d	4.9 ± 3.5 d

Values are the mean ± SD (standard deviation) of 5 replicates. C: maize + *F. verticillioides* M7075; FB: dual cultures *F. verticillioides–B. subtilis* CE1 on maize. Means in a column with a letter in common are not significantly different according to the LSD test ($P < 0.001$).

Table 3
F. verticillioides root counts from rhizoplane and endorhizosphere of maize after 20 growing days

Bacterial seed treatments (cells ml^{-1})	$\log_{10}$ *F. verticillioides* CFU g^{-1} of root tissue[a]			
	Rhizoplane	Inhibition (%)	Endorhizosphere	Inhibition (%)
Untreated control	5.064 ± 3.061 a	0	5.756 ± 3.238 a	0
10^6	4.889 ± 3.397 ab	3.45	4.948 ± 3.238 b	14.03
10^7	4.497 ± 4.061 b	11.20	3.695 ± 3.364 c	35.79
10^8	0.649 ± 2.447 c	98.55	0.008 ± 0.384 d	99.86

[a] Values are the mean ± SD (standard deviation) of 15 replicates. The letters in common are not significantly different according to LSD test ($P < 0.001$).

Table 4
Correlation coefficient of antibiosis on maize grains and *F. verticillioides* CFUs in greenhouse experiments

Interaction	Correlation coefficient (r)*
Antibiosis assay vs on grain assay	0.998
Antibiosis assay vs greenhouse assay	0.995
On grains assay vs greenhouse assay	0.995

Significance was defined as any correlation >0.50.
* $P < 0.001$.

was compared with controls. The maximum level detected was 47.5 μg g^{-1} after 35 incubation days. *B. subtilis* CE1 significantly reduced FB_1 accumulation, which reached 53% at 35 incubation days. About 10^{10} cells ml^{-1} of *F. verticillioides* were found in control treatments at all incubation periods assayed. The bacterial treatment reduced the CFUs over 24–23% at 20 and 35 incubation days, respectively (Table 2).

3.3. Greenhouse studies

Table 3 shows the influence of *B. subtilis* CE1 on native *F. verticillioides* colonization at different inoculum sizes and maize root levels. Seed bacterization with *B. subtilis* CE1 at 10^8, 10^7 and 10^6 cells ml^{-1} inoculum concentrations inhibited *F. verticillioides* counts at the rhizoplane level, whereas all bacterial treatments reduced the fungal CFUs at the endorhizosphere level. The 10^8 cells ml^{-1} dose showed the highest inhibition percentages of *F. verticillioides* inhibition at the two root levels tested.

3.4. Correlation analysis

To analyze the behavior of *B. subtilis* CE1 10^8 cells ml^{-1} between in vitro and greenhouse studies, the Pearson correlation coefficient index was applied. The three experiments showed positive correlations ($r = 0.998$, $r = 0.995$). There was substantial antibiosis (60%), and *F. verticillioides* CFU reduction (40–50%) in in vitro assays. Moreover, a similar behavior in greenhouse trials, in which colonization inhibition reached high values (98.55–99.86%) could be observed. These results showed that in vitro antagonistic ability was maintained in the greenhouse study (Table 4).

4. Discussion

This work reveals that *B. subtilis* CE1 can be used to inhibit *F. verticillioides* maize root colonization. The application of this knowledge should contribute to preventing vertical transmission of endophytic *F. verticillioides* and could help to reduce future maize grain fumonisin contamination.

In previous studies we demonstrated the utility of the niche overlap index (NOI) in choosing the best bacterium as a potential biocontrol agent [8,9]. In this work, inhibitory studies conducted in vitro with *Bacillus* strains, selected according to NOI, demonstrated their ability to inhibit *F. verticillioides* growth. Earlier studies reported *Bacillus* spp. antibiotic properties and potential biological control of plant pathogenic fungi [33,34,40].

One of our *Bacillus* strains stimulated fumonisin B_1 production in co-culture. This is in agreement with Cuero et al. [12] who found *B. amyloliquefaciens* able to increase toxin production of *Aspergillus flavus* in maize grains. It is possible that the *Bacillus* strain under our experimental conditions altered the substrate enhancing growth of *F. verticillioides* and its ability to produce FB_1.

B. subtilis CE1, isolated in this research, was demonstrated to be the most effective in reduction, affecting fungal growth parameters and toxin production in vitro at all incubation periods tested.

In terms of its potential application as a biocontrol agent, seed bacterization studies with *B. subtilis* CE1 were carried out to assess, in a rhizosphere environment, its antagonism toward the indigenous maize *F. verticillioides*. Application of bacteria to seeds has been used for biological control of soil-borne plant pathogens affecting many host plants [4,21,42]. There exist few data concerning inhibition of *F. verticillioides* by bacterial isolates. Hinton and Bacon [22] reported that one isolate of *Enterobacter cloacae* associated, as an endophyte, with corn roots, stems and leaves, is antagonistic toward *F. moniliforme* and other toxic fungi associated with corn. More recently, studies involving *F. verticillioides* and *B. subtilis* showed promising reduction of mycotoxin accumulation during the endophytic growth phase [3]. The results obtained in this study show the ability of *B. subtilis* CE1 to reduce rhizoplane and endorhizosphere *F. verticillioides* colonization at all inoculum and maize root levels tested, with 10^8 cells ml^{-1} being the most effective concentration.

It is generally recognized that expression of antagonism by a microorganism towards a pathogen in culture medium cannot be regarded as evidence that the microorganism will control the pathogen in the field [42]. Bevivino et al. [6] obtained a poor correlation between in vitro and greenhouse studies when assessing *Burkholderia cepacia* antagonism against *F. moniliforme* and *F. proliferatum* on maize. In contrast, results obtained in the present study indicated an important correlation between antagonism recorded in vitro and the effectiveness of *B. subtilis* CE1 at inhibiting colonization of *F. verticillioides* on maize grain and in greenhouse trials.

In conclusion, *B. subtilis* has potential, as a maize inoculant, for inhibiting *F. verticillioides* root colonization. Research is continuing on the development of this bacterium as a biocontrol agent in field trials.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) FONCYT-PICT03/08-7197 and (ANPCYT) FONCYT-PICT03/08-14551.

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