Biological Control 53 (2010) 258–266

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Impact of bacterial biological control agents on fumonisin B_1 content and *Fusarium verticillioides* infection of field-grown maize

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ARTICLE INFO

Article history:
Received 6 April 2009
Accepted 5 February 2010
Available online 11 February 2010

Keywords:
Biocontrol agents
Fusarium verticillioides
Fumonisin B_1
Maize

ABSTRACT

The effects of four bacterial biocontrol agents on *Fusarium verticillioides* infection and fumonisin accumulation in the maize agroecosystem were evaluated in a 2-year field study. The antagonistic abilities of the four agents were examined following two application techniques consisting of inoculating seeds during pre-sowing and maize ears at flowering. Seed inoculation with *F. verticillioides* and co-inoculation with this fungus and each of the four agents were also examined. Treatment effects on maize performance were also assessed through determination of the number of plants per hectare, kernels yield (kg ha^{-1}) and kernel–cob relations. *F. verticillioides* infection and fumonisin B_1 contents were determined in kernels of physiologically mature maize plants at harvest time. Maize yield remained unchanged with respect to controls in both field surveys; however significantly higher FB_1 contents were obtained after inoculation of seeds with *F. verticillioides* M7075. Seed treatment with *Bacillus amyloliquefaciens* and *Enterobacter hormaechei* reduced the infection by the fungus and FB_1 contents both years of the study while ear inoculation produced highly variable results. The number of colony forming units of *F. verticillioides* obtained from harvested maize kernels was positively correlated with fumonisin B_1 content; however none of these parameters showed significant correlation with kernel yield. The overall results suggest that in years conducive for *F. verticillioides* infection and fumonisin production, seed treatment with *Bacillus amyloliquefaciens* and *Enterobacter hormaechei* may improve quality of maize grains obtained at harvest by reducing toxin content.

1. Introduction

Maize (*Zea mays* L.) constitutes a key crop in terms of production throughout the world. In Argentina, maize represents, together with soy (*Glycine max* [L.] Merrill) and wheat (*Triticum aestivum* L.), the basis of national grain production (SAGPyA, 2009). The total area under maize cultivation in Argentina has been increasing in many temperate zones where the crop is used not only as a source of grains intended for human consumption and derivatives production, but also as forage (Liendo and Martín, 2004). One of the main factors related to this crop was the introduction of hybrids, starting in 1933, that have improved its yield worldwide in different soil types (Miller, 1999). In spite of hybrid and transgenic crop technologies, maize remains susceptible to infection and contamination with *Fusarium* species and its mycotoxins. *Fusarium verticillioides* (Sacc.) Nirenberg [G] (synonym: *F. moniliforme* Sheld.) infects maize during the different growth and developmental stages presented by the crop (Bacon et al., 2008). The fungus could be traced from maize seed, to recovery from roots of plants produced from these seed, from the stems, and finally isolated from kernels produced on these plants (Bacon et al., 2001). Thus, while this species is disseminated vertically within plants, horizontal dissemination also can occur between plants via wounds due to the activity of insects, from soil to roots and other injured plant parts, via soil or aerially borne spores (Bacon et al., 2008).

Under favorable conditions, infection may lead to kernel decay (Munkvold and Desjardins, 1997; Bacon et al., 2001). Infected kernels usually contain high levels of fumonisins, mycotoxins with acute toxic effects to certain livestock, especially horses and swine, and with carcinogenic properties in rats (Duvick, 2001).

Fumonisins were catalogued by the International Agency for Research on Cancer as possible human carcinogens (IARC, 2002). Fumonisin B_1 (FB_1) is the major fumonisin present in maize and maize base products across the five continents and its accidental consumption has been positively correlated with the incidence of human esophageal cancer (Shephard et al., 1996; Fandohan et al., 2005a). *Fusarium verticillioides* has been reported as the most prevalent pathogen of maize in Argentina (Sydenham et al., 1993; González et al., 1995; Chulze et al., 1996) and FB_1 levels up to

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doi:10.1016/j.biocontrol.2010.02.001

15.5 ppm were found in kernels from Argentinian maize producing provinces prior to storage (Pacin et al., 2009).

Seed treatment with biocontrol agents has been shown to be an effective method for introducing the agents into a plant—soil borne pathogen system and to facilitate their antagonistic activities (Mao et al., 1998; Duijff et al., 1999; Batson et al., 2000; Pal et al., 2001; Dawar et al., 2008; Correa et al., 2009). In a previous study conducted under greenhouse conditions we found that maize seed treatment with the bacterial agents used in the present survey allowed the bacteria to associate with the roots of developing seedlings and to exert antagonism against *Fusarium verticillioides* (Pereira et al., 2009). *Bacillus amyloliquefaciens* isolate 1 and *Microbacterium oleovorans* isolate 2 were shown to effectively reduce *F. verticillioides* propagules and fumonisin content in maize kernels at harvest when applied as seed coatings (Pereira et al., 2007).

The present study was conducted to evaluate the ability of four bacterial isolates to reduce *F. verticillioides* infection and fumonisin content in kernels of physiologically mature maize plants following application to seeds prior to sowing and to maize ears at flowering. The effects of the treatments on maize performance at harvest were also evaluated in this field assessment over two consecutive maize growing seasons in Argentina.

2. Materials and methods

2.1. Microorganisms

2.1.1. Bacteria

Bacillus amyloliquefaciens isolate 1 (Genbank Accession No. EU164542), *Microbacterium oleovorans* isolate 2 (Genbank Accession No. EU164543), *Microbacterium oleovorans* isolate 3 (Genbank Accession No. EU164544) and *Enterobacter hormaechei* isolate 4 (Genbank Accession No. EU164545) were used in this study. Strains were originally isolated from the roots of field-grown maize in Córdoba province, Argentina, and identified from axenic cultures based on morpho-physiological characteristics (Slepecky and Hemphill, 1992; Holt et al., 1994) and 16S rDNA gene sequence similarity. These four isolates were selected on the basis of *in vitro* antagonism tests against *F. verticillioides* (Cavaglieri et al., 2005).

The specific isolates of the biocontrol agents used were resistant to commonly used seed treatments (Carboxin–Thiram–Metalaxil) and to antibiotics at 5 μg ml^{-1} (*B. amyloliquefaciens* was resistant to nalidixic acid, *M. oleovorans* to streptomycin and *E. hormaechei* to chloramphenicol). All isolates were stored at −20 °C in glycerol and, when required for experimental use, they were transferred to nutrient agar (meat extract 3 g, soy peptone 5 g, NaCl 8 g, agar 15 g, distilled water 1000 ml).

Inoculum used to prepare seed coatings or ear sprays was grown on nutrient broth (meat extract 3 g, soy peptone 5 g, NaCl 8 g, distilled water 1000 ml) incubated at 28 °C with shaking (100 rpm) until late log phase. The total numbers of viable cells of the liquid cultures were determined on nutrient agar amended to contain the corresponding antibiotic by 10-fold serial dilutions and standard plate count methods. Culture suspensions (CS) of 10^9 colony forming units (CFUs) ml^{-1} were used to treat maize seeds. Maize ears were treated during flowering, at R1 phenological stage (Ritchie and Hanway, 1982), with CS of 10^8 CFUs ml^{-1}. CS of each of the four BCAs were individually placed into sterile plastic sprayers with a volume of 1 ml per spray and each ear was sprayed five times directly in the silk channel without removing the husks. All the ears on every plant in the treatment were sprayed.

2.1.2. Fungi

Fusarium verticillioides strain M7075, a fumonisin B_1 producer strain isolated from maize in Argentina and deposited in the *Fusarium* Research Center Collection (Pennsylvania State University, University Park, PA, USA), was included when required in some of the treatments. To obtain fungal conidia, Cappellini Peterson (CP) (1965) sterile broth was inoculated with mycelia from a monosporic *F. verticillioides* culture grown on carnation leaf agar medium (CLA) (Nelson et al., 1983). Cultures were incubated at 25 °C for 7 days on an orbital shaker at 200 rpm. Spore concentrations of the cultures were determined using a Neubauer camera and viability was assessed and confirmed by standard plate count methods on Nash–Snyder agar (Nelson et al. 1983). Suspensions were diluted in 0.8% NaCl w/v to obtain 10^7 spores ml^{-1}. The final concentration of fungal inoculum used in this study was previously shown to be within the range for infection (Bacon et al., 1994).

2.2. Treatments—soil characteristics

Seeds of maize cultivar DK684RR2 (Monsanto, Argentina) were used in both evaluated maize growing seasons. This cultivar is resistant to the herbicide glyphosate.

All treatments are detailed in Table 1. In seed coat treatments, BCAs were applied to maize seeds alone (T8 to T11) and in combination with *F. verticillioides* M7075 (T4 to T7). In the treatments in which the BCAs were applied to the ears of maize at the beginning of flowering (T3a to T3d), the plants were grown from *F. verticillioides* treated seeds. Field experiments were performed at the University of Río Cuarto Experimental Field Station in Río Cuarto, Córdoba, Argentina (30°57'S latitude, 64°50'W longitude, 562 m altitude) during two consecutive maize growing seasons (2006–2007 and 2007–2008). Treatments in both years were planted in a randomized complete block design with three replications per treatment. Individual plots were 7 m long, 3 m wide and consisted of four rows with 25 seeds per row. Treatments were separated from each other with three rows of maize planted with untreated seeds. The entire experiment was bordered on all four sides with three rows of maize planted to the same cultivar (DK684RR2, Monsanto). One application of the herbicide glyphosate was performed on the entire parcel 1 week after sowing according to common agricultural practices used for RR cultivars. Soil consisted of a sandy loam texture (pH 6.1 in water 1:1 w/v, 1.4% organic matter, 86 ppm of nitrates).

One hundred maize seeds were sown per individual plot (300 per treatment). Seeds were soaked in Erlenmeyer flasks containing either sterile water (control), *F. verticillioides* suspension (fungal control), BCA suspensions (seed coatings with each of the four agents), or BCA-*F. verticillioides* 1:1 (v/v) suspensions (seed coatings with each of the four agents in combination with the fungus). Afterwards, flasks were incubated for one hour at 28 °C with shaking (100 rpm) to facilitate adhesion of microorganisms to the seeds. After incubation, viable cell numbers maintained the same ranges described in the previous section. Immediately before sowing, three replicates of 10 g of seeds from each treatment were placed in flasks with 90 ml of sterile phosphate-buffered saline solution (PBS) to estimate the size of microbial inoculum (CFUs per ml) that remained associated with the seeds.

2.3. Climatic conditions

Air and soil daily maximum and minimum temperatures were recorded. Soil temperatures were determined at a depth of 5 cm. Cumulative monthly rainfalls (mm) were also registered during the whole period of crop growth and development at field. Climatic data were from the Agrometeorology Chair, UNRC Experimental Field Station, Río Cuarto, Córdoba, Argentina. Agrometeorological station is located within the Experimental Field Station, 250 m away from the site where treatments were performed.

Table 1
Description of treatments in experiments evaluating the effectiveness of *B. amyloliquefaciens*, *M. oleovorans*, and *E. hormaechei* performed in fields tests conducted during the 2006–2007 and 2007–2008 maize growing seasons on control of *F. verticilloides*.

Treatment	Inoculation procedures*	
	At sowing Seeds treated with	At flowering Ears treated with
T1	Sterile water	–
T2	Suspension of *F. verticillioides* M7075	–
T3a	Suspension of *F. verticillioides* M7075	Spray of *B. amyloliquefaciens*
T3b	Suspension of *F. verticillioides* M7075	Spray of *M. oleovorans* isolate 2
T3c	Suspension of *F. verticillioides* M7075	Spray of *M. oleovorans* isolate 3
T3d	Suspension of *F. verticillioides* M7075	Spray of *E. hormaechei*
T4	Suspension of *F. verticillioides* M7075 + *B. amyloliquefaciens*	–
T5	Suspension of *F. verticillioides* M7075 + *M. oleovorans* isolate 2	–
T6	Suspension of *F. verticillioides* M7075 + *M. oleovorans* isolate 3	–
T7	Suspension of *F. verticillioides* M7075 + *E. hormaechei*	–
T8	Suspension of *B. amyloliquefaciens*	–
T9	Suspension of *M. oleovorans* isolate 2	–
T10	Suspension of *M. oleovorans* isolate 3	–
T11	Suspension of *E. hormaechei*	–

*Suspensions of biocontrol agents consisted of nutrient broths with 10^9 CFUs ml^{-1} of each agent when used for inoculation of seeds during pre-sowing and with 10^8 CFUs ml^{-1} of each agent when used for inoculation of maize ears during flowering. Suspensions of *F. verticillioides* consisted of Cappellini Peterson broths with 10^7 spores ml^{-1}. For co-treatment of seeds with *F. verticillioides* and each of the four agents (T4 to T7) 1:1 (v/v) suspensions were used. Once seeds were distributed in the flasks with the different suspensions incubation for 2 h at 28 °C with shaking (100 rpm) was performed to facilitate adhesion of microorganisms, without altering described cell densities. Sprays of each of the four BCAs were applied directly in the silk channel of maize ears at R1 phenological stage by using manual sprayers. Five shoots were performed per ear without removing the husks, all the ears of every treated plant were sprayed. Sown was performed at the University of Río Cuarto Experimental Field Station in Río Cuarto, Córdoba, Argentina (30°57'S latitude, 64°50'W longitude, 562 m altitude) during two consecutive maize growing seasons (2006–2007 and 2007–2008). DK684RR2 (Monsanto) seeds were used. Treatments were planted in a randomized complete block design with three replications per treatment. Individual plots were 7 m long, 3 m wide and consisted of four rows with 25 seeds per row. Treatments were separated from each other with three rows sown with untreated seeds. In the first field assay (2006–2007 season) sowing was performed on November 30, sprays were applied at flowering (R1 stage), 41 days after sowing, and harvest was performed on March 20. During 2007–2008 maize growing season sowing was performed on November 15, sprays were applied at flowering (R1 stage), 45 days after sowing, and harvest was performed on March 12.

2.4. Sampling procedures and processing of the samples

Physiologically mature cobs were collected 150 days after sowing, when samples had reached the R6 phenological stage (Ritchie and Hanway, 1982). All cobs present in each individual plot were removed from the plants and separated from its husks. Peeled cobs from each plot were placed together into plastic bags and immediately transported to the experimental station workplace were kernels were separated from the cobs with a static threshing machine (Forti MA, Buenos Aires, Argentina). After threshing, kernels and cobs were weighed separately to determine kernel yield and kernel–cob relations. Afterwards, all kernels from the same plot were milled together and homogenized with an electric miller (RAS Mill, Romer Labs, USA). The primary milled samples from each plot weighed around 7 kg, and two sub-samples from each primary sample were immediately taken to be used for determination of *F. verticillioides* colony forming units. Milled sub-samples used for the analysis of fumonisin contents were stored at −20 °C for 2 weeks.

2.5. Influence of treatments on maize agronomic parameters

The effects of the different treatments on maize plants and yield at harvest were evaluated through analysis of the number of plants per hectare (ha), total yield (kg ha^{-1}) and the kernel–cob relations (percentage of the total weight of the whole maize cob represented by the kernels and by the cob). Moisture content of harvested kernels was measured with a Delver HD1000D Hygrometer. Maize yield was adjusted to 14.5% kernel moisture content according to current regulations for maize commercialization (SAGyP, 1994).

2.6. Isolation and quantification of F. verticillioides from maize kernels

Two milled sub-samples of 10 g from each individual plot were separately added to Erlenmeyer flasks with 90 ml of sterile phosphate-buffered saline solution (PBS: NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, distilled water 1000 ml, pH 7.3) to obtain a 1/10 dilution. Serial decimal dilutions were performed in sterile PBS up to 10^{-3} and 0.1 ml from each dilution was spread plated in duplicate on Nash-Snyder solid medium (Nelson et al., 1983) for selective isolation of *Fusarium* species. Plates were incubated 7 days at 28 °C and after incubation total number of CFUs of *Fusarium* species was performed. Colonies were purified on carnation leaf agar (CLA) according to the single-spore method and axenic cultures were incubated at 25 °C with a day length of 12 h. Fungal identifications were performed according to Nelson et al. (1983) and Leslie and Summerell (2006). The numbers of CFUs of *F. verticillioides* are presented as the mean values for each individual plot or as mean values of treatments whenever no significant block effects were obtained after statistical analysis.

2.7. Determination of FB_1 content in maize kernels at harvest

Two sub-samples of 15 g from the milled samples from each individual plot were used to perform fumonisin quantification. Total fumonisin content of each sample was determined by high performance liquid chromatography (HPLC) according to the official method AOAC number 995.15 based on Shephard et al. (1990) with modifications of Doko et al. (1995). The toxin was extracted from each milled sample with 50 ml of acetonitrile and water (1:1, v/v) with shaking (100 rpm) for 1 h on an orbital shaker. Afterward, the extract was filtered through filter paper Whatman No. 4. Ten milliliters of the filtered extract were cleaned through Bond–Elut cartridges (SAX 500 mg, strong anion exchange cartridges, VARIAN) that were previously conditioned by successive passage of methanol (5 ml) and methanol and water (5 ml, 3:1 v/v). Cleaning procedures were performed at atmospheric pressure. The cartridges were then washed with 5 ml of methanol and water (3:1 v/v) and finally with 5 ml of methanol. Fumonisins were eluted from the cartridges with 1% acetic acid in methanol (10 ml). The eluants were collected and evaporated to dryness at 40 °C in a rotary evaporator. For HPLC analysis the residues were redissolved in 500 µl of acetonitrile and water (1:1, v/v). Aliquots of 50 µl of this solution were added to eppendorf tubes and mixed with 200 µl of derivatizing solution (*o*-phthaldialdehyde 40 mg, methanol 1 ml,

2-mercaptoethanol 50 μl, 0.1 M sodium tetraborate 5 ml). The mixture was protected from light by covering the tubes with aluminum paper and manually shaken for 30 s. The samples were left in the dark for 3 min and after this time were injected in duplicate into the HPLC apparatus. A reversed phase high-pressure liquid chromatography/fluorescence detection system consisting of a HP 1100 pump (Hewlett Packard, Palo Alto, CA, USA) connected to a programmable HP 1046A fluorescence detector, and to a HP workstation was used. Chromatographic separations were performed on a stainless steel C_{18} reversed-phase column (150 × 4.6 mm i.d., 5 μm particle size, Luna-Phenomenex, Torrance, CA, USA). A methanol–sodium dihydrogen phosphate 0.1 M (75:25 v/v) solution adjusted to pH 3.35 with orthophosphoric acid was used as isocratic mobile phase, at a flow rate of 1.5 ml min^{-1}. Calibration curves were constructed with FB_1 standard (Romer Labs) diluted in acetonitrile–water (1:1 v/v). Two injections were made per sample. Data are presented as the mean values of each individual plot or as mean values of treatment replicates if no significant block effects were obtained after statistical analysis.

2.8. Data analysis

The numbers of propagules of *Fusarium verticillioides* in maize kernels, FB_1 contents of harvested kernels as well as maize agronomic parameters were analyzed with ANOVA for randomized block design (SAS for Windows 6.11, SAS Institute, Cary, NC). The Tukey test was used for posteriori comparisons between treatments. When no significant block effects were observed, values from the different treatment replications were grouped together and averaged per treatment. Pearson product moment correlation coefficients (SigmaStat for Windows Version 3.5, Systat Software Inc., Germany) were used to analyze correlations between kernel yield, fumonisin B_1 content and the number of CFUs of *Fusarium verticillioides* in kernels at harvest. A $P < 0.05$ significance level was used throughout. Data were $\log_{10}$ transformed prior to statistical analyses.

3. Results

3.1. Maize agronomic parameters

The addition of BCAs to both maize seeds and maize ears did not significantly affect the number of plants per hectare obtained for the control (T1) during the two evaluated seasons (Table 2). Further, treatment of seeds with *F. verticillioides* (T2) also did not affect the number of plants per hectare with respect to T1 values. Plants grown from seeds coated with *F. verticillioides* (T2 and T3) grew well without disease symptoms during the entire season in the field. Kernel–cob relations determined in both seasons were consistent with previous reports for the cultivar used (Dekalb, 2008). Kernel–cob relations, as well as kernel yields (kg ha^{-1}), presented no significant differences when compared to control values (T1) for both seasons. Mean soil and air temperatures were similar in both seasons (Fig. 1). Air temperatures varied between 20 and 23 °C during warmer months. Rainfall patterns markedly differed between 2006–2007 and 2007–2008 seasons (Fig. 1a and b, respectively). According to rainfall data, a cumulative value of 90 mm was obtained during the harvest month (March) in the 2006–2007 season, while 150 mm were obtained during the same period during the 2007–2008 season.

3.2. Colony forming units of Fusarium verticillioides in harvested maize kernels

As shown in Fig. 2, during both seasons the number of CFUs of *F. verticillioides* obtained from T2 kernels (plants grown from *F. verticillioides* coated seeds) was significantly higher than control values (T1). The number of CFUs of *F. verticillioides* was significantly reduced after spraying with *B. amyloliquefaciens, M. oleovorans* isolate 2 and *E. hormaechei*, (T3a, T3b and T3d, respectively), when compared to T2 values (fungal control) during the last field assay. The number of CFUs of *F. verticillioides* was significantly lower in the kernels of plants grown from *F. verticillioides*-BCA co-treated seeds (T4 to T7) in relation to T2 (fungal control) values during both maize growing seasons (Fig. 2). During the 2006–2007 growing season, the number of viable propagules of *F. verticillioides* obtained from kernels of T8, T9, T10 and T11 plants (grown from seeds treated with each of the four BCAs) did not differ from values obtained from kernels of naturally infected plants (T1) (Fig. 2a). The number of CFUs of *F. verticillioides* was significantly reduced in T6 (*F. verticilliodes* and *M. oleovorans* isolate 3 co-inoculation) when compared to T1 (control) and T2 (fungal control) values during 2007–2008 assay (Fig. 2b). During this last season, seed treatments with *B. amyloliquefaciens* or *M. oleovorans* isolate 2 (T8 and T9, respectively) significantly reduced the number of CFUs of *F. verticillioides* with respect to T1 and T2 values.

Table 2
Effects of treatments on selected maize agronomic parameters at harvest during the (a) 2006–2007 and (b) 2007–2008 maize growing seasons.

Treatment	Number of plants per hectare	Kernel–cob relation	Yield (kg ha^{-1})
(a)			
T1	40000 ± 4971 ba*	80%–20% a	6429 ± 1367 a
T2	33174 ± 6500 ba	83%–17% a	5598 ± 1106 a
T3a	36190 ± 3299 ba	75%–25% a	7569 ± 837 a
T3b	38095 ± 155 ba	83%–17% a	9056 ± 1421 a
T3c	38095 ± 201 ba	82%–18% a	8585 ± 1995 a
T3d	41635 ± 1962 a	84%–16% a	9184 ± 1424 a
T4	34603 ± 5819 ba	81%–19% a	7933 ± 1254 a
T5	40476 ± 2076 ba	82%–18% a	8739 ± 1157 a
T6	34286 ± 2474 ba	83%–17% a	7607 ± 266 a
T7	33968 ± 4268 ba	80%–20% a	6988 ± 1014 a
T8	33492 ± 3024 ba	80%–20% a	7030 ± 1295 a
T9	27778 ± 7148 b	77%–23% a	6475 ± 2166 a
T10	28095 ± 5851 ba	79%–21% a	5525 ± 1418 a
T11	35080 ± 5415 ba	82%–18% a	7503 ± 1155 a
(b)			
T1	43810 ± 0 a*	78%–22% a	4081 ± 765 a
T2	42064 ± 3024 a	73%–27% a	2706 ± 608 a
T3a	39365 ± 2200 a	75%–25% a	4477 ± 1162 a
T3b	39335 ± 2200 a	81%–19% a	4385 ± 729 a
T3c	40635 ± 2910 a	73%–27% a	4451 ± 1027 a
T3d	40635 ± 2910 a	75%–25% a	4297 ± 721 a
T4	39048 ± 4762 a	77%–23% a	3073 ± 60 a
T5	37143 ± 4972 a	79%–21% a	3824 ± 425 a
T6	37460 ± 2147 a	75%–25% a	3251 ± 1511a
T7	38095 ± 5040 a	75%–25% a	3051 ± 205 a
T8	43016 ± 728 a	73%–27% a	3452 ± 152 a
T9	43175 ± 1100 a	73%–27% a	2638 ± 494 a
T10	42540 ± 1455 a	76%–24% a	3434 ± 1149 a
T11	42857 ± 1650 a	76%–24% a	4107 ± 2369 a

* Data are means and standard deviations of treatment replications since no significant block effects were observed. Different letters indicate significant differences between treatments ($P < 0.05$ ANOVA complete randomized blocks). T1: control, T2: *F. verticillioides* M7075 seed treatment, T3a: *F. verticillioides* M7075 seed treatment + *Bacillus amyloliquefaciens* isolate 1 sprayed during flowering, T3b: *F. verticillioides* M7075 seed treatment + *Microbacterium oleovorans* isolate 2 sprayed during flowering, T3c: *F. verticillioides* M7075 seed treatment + *Microbacterium oleovorans* isolate 3 sprayed during flowering, T3d: *F. verticillioides* M7075 seed treatment + *Enterobacter hormaechei* isolate 4 sprayed during flowering, T4: *F. verticillioides* M7075 + *B. amyloliquefaciens* isolate 1 co-treatment of seeds, T5: *F. verticillioides* M7075 + *M. oleovorans* isolate 2 co-treatment of seeds, T6: *F. verticillioides* M7075 + *M. oleovorans* isolate 3 co-treatment of seeds, T7: *F. verticillioides* M7075 + *E. hormaechei* isolate 4 co-treatment of seeds, T8: *B. amyloliquefaciens* isolate 1 seed treatment, T9: *M. oleovorans* isolate 2 seed treatment, T10: *M. oleovorans* isolate 3 seed treatment, T11: *E. hormaechei* isolate 4 seed treatment.

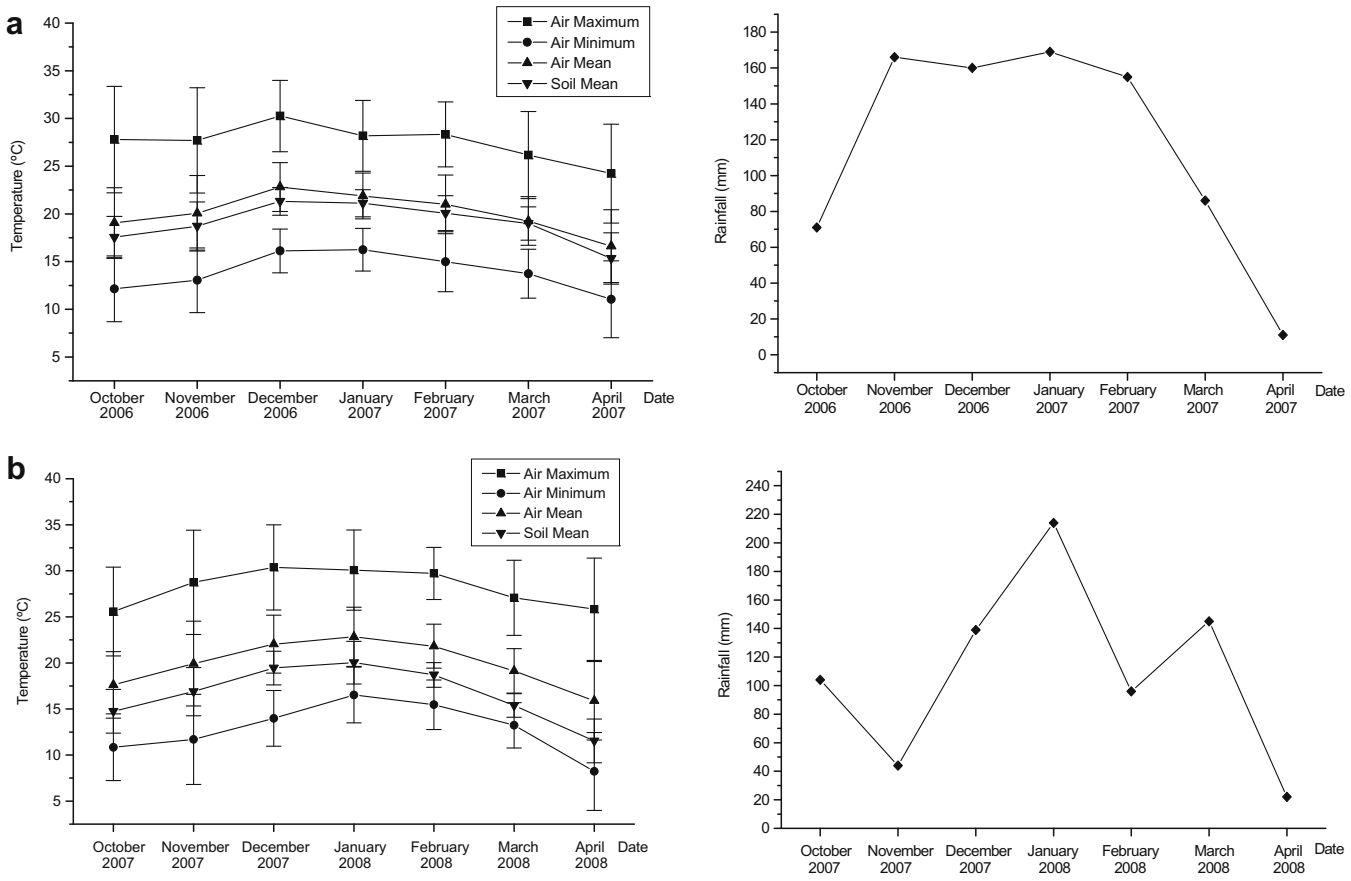

Fig. 1. Soil and air temperatures (°C) and cumulative monthly rainfall (mm) registered in the experimental area used in (a) 2006–2007 and (b) 2007–2008 maize growing seasons.

3.3. Fumonisin B_1 content

A significantly higher FB_1 content was obtained in kernels of plants grown from seeds treated with *F. verticillioides* (treatment 2), in kernels of plants grown from seeds treated with *F. verticillioides* and sprayed during flowering with *B. amyloliquefaciens* (treatment T3a) and in kernels from plants grown from seeds treated with *M. oleovorans* isolate 2 (treatment 9) when compared with values obtained from the rest of the treatments during the first evaluated season (Fig. 3a). During the second season, kernels of T3b and T3d treatments (of plants grown from seeds treated with *F. verticillioides* and sprayed during flowering with *M. oleovorans* isolate 2 and *E. hormaechei*, respectively) were significantly higher in FB_1 content with respect to control values (T1) and even when compared with fungal control (T2) (Fig. 3b). In kernels obtained from T4, T5, T6 and T7 plants (grown from seeds co-treated with *F. verticillioides* and the different BCAs) fumonisin B_1 content remained unchanged with respect to controls (T1) during both years of the study. The maximum FB_1 levels obtained during the 2006–2007 season was 1623 ppb and it was obtained from kernels of T2 plants (grown from seeds treated with *F. verticillioides*). In this treatment, the FB_1 content was about 3800 ppb during the second growing season (2007–2008). The highest level of FB1 (7940 ppb) was obtained in T3d kernels (of plants grown from seeds treated with *F. verticillioides* and sprayed during flowering with *E. hormaechei*) during the second growing season. After seed treatment with *B. amyloliquefaciens* and *E. hormaechei* (T8 and T11, respectively) fumonisin levels registered in maize kernels were significantly lower than values obtained in T2 kernels (of plants grown from seeds treated with *F. verticillioides*) and did not differ from levels obtained in naturally infected plants (T1). This result was consistent during both seasons.

Mean number of CFUs of *F. verticillioides* that remained associated with the seeds after treatment with *F. verticillioides* M7075 was 10^4 CFU g^{-1} while the mean number of CFUs of the BCAs that remained associated with treated seeds was between 1×10^5 and 3×10^5 CFU g^{-1} (data not shown).

3.4. Correlations between variables

There was a positive correlation between the number of CFUs of *F. verticillioides* and fumonisin B_1 content in maize kernels at harvest in both years of the study (Table 3). There was a tendency for a negative correlation for these two variables with kernel yield, however, the correlation was not significant ($P > 0.05$). Thus our results indicate that a higher *F. verticillioides* inoculum in kernels of physiologically mature maize plants was correlated with increased fumonisin B_1 contents of harvested grains. This trend was especially clear in the second growing season ($r = 0.694$, $P = 0.026$) rather than during the first season in which the correlation was lower ($r = 0.275$, $P = 0.038$).

4. Discussion

Control strategies, aimed at reducing crop infection by phytopathogens, have been widely based on the use of chemical pesticides (Kanampiu et al., 2002). The massive use of chemical pesticides promotes water pollution, soil degradation and insect resistance and resurgence. The use of biological control agents with

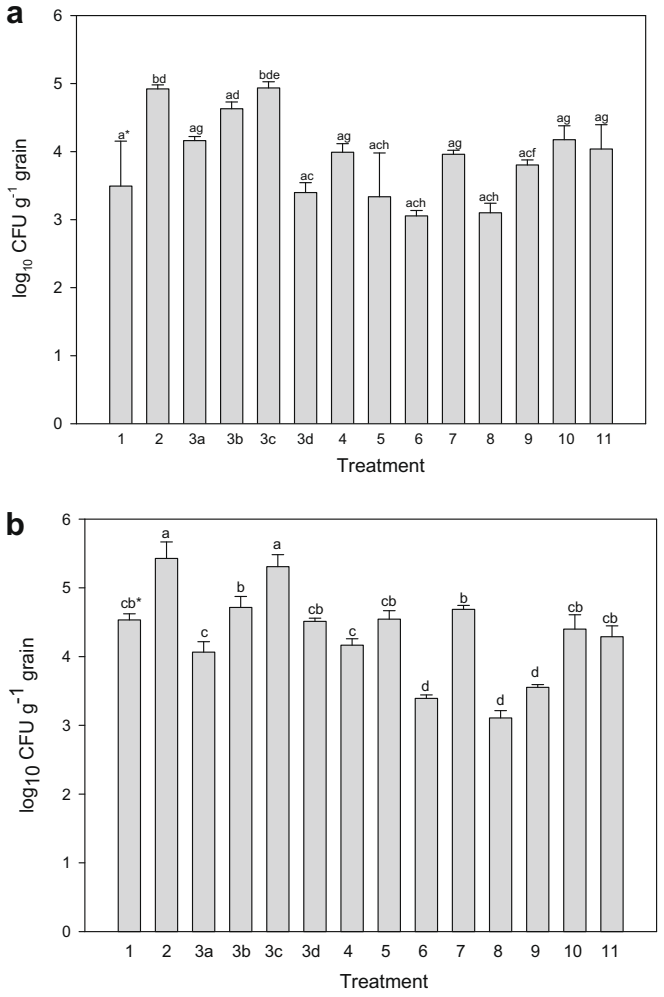

Fig. 2. Number of colony forming units of *Fusarium verticillioides* in maize kernels at harvest during (a) 2006–2007 and (b) 2007–2008 maize growing seasons. *Data are the means and standard deviations of treatment replications (six samples analyzed in duplicate) since no significant block effects were observed. Different letters indicate significant differences between treatments ($P < 0.05$, ANOVA complete randomized blocks). T1: control, T2: *F. verticillioides* M7075 seed treatment, T3a: *F. verticillioides* M7075 seed treatment + *Bacillus amyloliquefaciens* isolate 1 sprayed during flowering, T3b: *F. verticillioides* M7075 seed treatment + *Microbacterium oleovorans* isolate 2 sprayed during flowering, T3c: *F. verticillioides* M7075 seed treatment + *Microbacterium oleovorans* isolate 3 sprayed during flowering, T3d: *F. verticillioides* M7075 seed treatment + *Enterobacter hormaechei* isolate 4 sprayed during flowering, T4: *F. verticillioides* M7075 + *B. amyloliquefaciens* isolate 1 co-treatment of seeds, T5: *F. verticillioides* M7075 + *M. oleovorans* isolate 2 co-treatment of seeds, T6: *F. verticillioides* M7075 + *M. oleovorans* isolate 3 co-treatment of seeds, T7: *F. verticillioides* M7075 + *E. hormaechei* isolate 4 co-treatment of seeds, T8: *B. amyloliquefaciens* isolate 1 seed treatment, T9: *M. oleovorans* isolate 2 seed treatment, T10: *M. oleovorans* isolate 3 seed treatment, T11: *E. hormaechei* isolate 4 seed treatment.

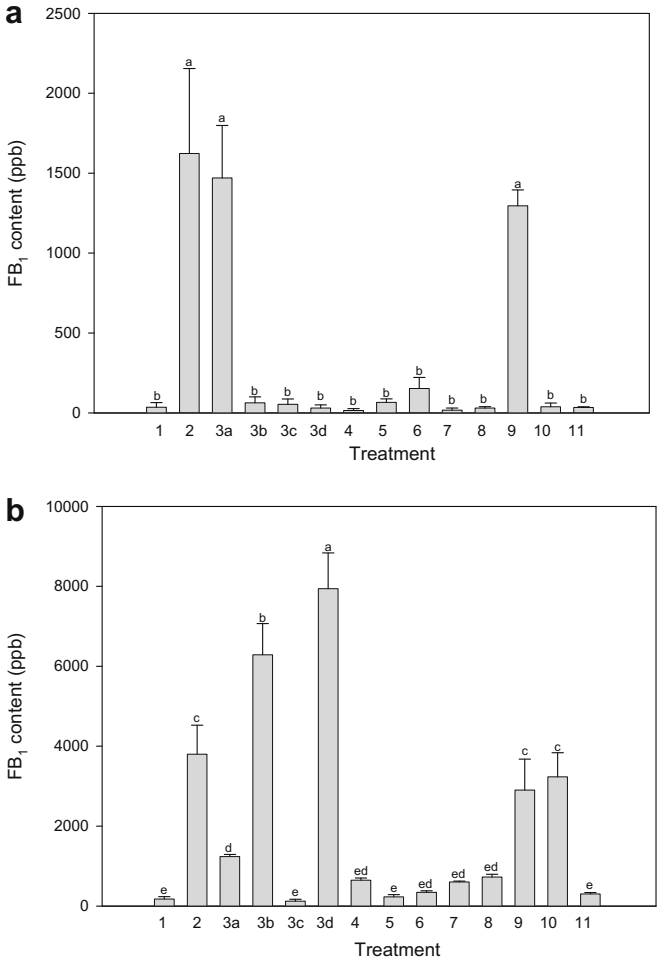

Fig. 3. Fumonisin B_1 content in maize kernels at harvest during 2006–2007 (a) and 2007–2008 (b) maize growing seasons. *Data represent means and standard deviations of treatment replications (six samples analyzed in duplicate) since no significant block effects were observed. Different letters indicate significant differences between treatments ($p < 0.05$, ANOVA complete randomized blocks). T1: control, T2: *F. verticillioides* M7075 seed treatment, T3a: *F. verticillioides* M7075 seed treatment + *Bacillus amyloliquefaciens* isolate 1 sprayed during flowering, T3b: *F. verticillioides* M7075 seed treatment + *Microbacterium oleovorans* isolate 2 sprayed during flowering, T3c: *F. verticillioides* M7075 seed treatment + *Microbacterium oleovorans* isolate 3 sprayed during flowering, T3d: *F. verticillioides* M7075 seed treatment + *Enterobacter hormaechei* isolate 4 sprayed during flowering, T4: *F. verticillioides* M7075 + *B. amyloliquefaciens* isolate 1 co-treatment of seeds, T5: *F. verticillioides* M7075 + *M. oleovorans* isolate 2 co-treatment of seeds, T6: *F. verticillioides* M7075 + *M. oleovorans* isolate 3 co-treatment of seeds, T7: *F. verticillioides* M7075 + *E. hormaechei* isolate 4 co-treatment of seeds, T8: *B. amyloliquefaciens* isolate 1 seed treatment, T9: *M. oleovorans* isolate 2 seed treatment, T10: *M. oleovorans* isolate 3 seed treatment, T11: *E. hormaechei* isolate 4 seed treatment.

antagonistic effects on crop pathogens could represent a promising alternative (Whipps, 1997; Bloemberg and Lugtenberg, 2001).

Results of the present study addressed the field performance of maize grown from seeds treated with a fumonisin producer strain of *F. verticillioides*, with four bacterial biocontrol agents and also co-treated with *F. verticillioides* and each of the agents tested. Two different techniques of BCAs inoculation were evaluated; seed coating and spraying the ears during flowering. And the treatment effects on FB_1 content and the number of CFUs of *F. verticillioides* in maize kernels at harvest were assessed. The infection of maize by *F. verticillioides* can cause highly variable results ranging from systemic asymptomatic infections to severe rotting and wilting. Different studies suggested several possible factors that influenced the relationship between this fungus and maize. These factors included the fungal inoculum sizes in soil and kernels, the nutritional state of the plant, the plant and fungal genotypes and the environmental conditions (Cantalejo et al., 1998; Miller, 1999; Fandohan et al., 2003). Later studies indicated that *F. verticillioides* can remain as an endophyte of maize based on its ability to detoxify antimicrobial compounds (BOA: benzoxazolin-2(3H)-one and MBOA: 6-methoxybenzoxazolin 2(3H)-one as major classes) synthesized by the plant and/or to produce phytohormones analogs (Glenn et al., 2003; Malonek et al., 2004). More recently, data show that maize oxylipins, produced via lipoxygenase (LOX) pathway, constitute a key factor related to fungal mycotoxin biosynthesis and sporogenesis (Gao et al., 2007). Whichever the mode that allows *F. verticillioides* to interact with maize in asymptomatic and symptomatic

Table 3
Correlations between maize yield, fumonisin B_1 content and number of CFUs of *F. verticillioides* in maize kernels at harvest during the (a) 2006–2007 and (b) 2007–2008 maize growing seasons.

	FB_1 content	CFUs of *F. verticillioides*
(a)		
Kernel yield	$r = -0.189$	$r = -0.121$
	$P = 0.230$	$P = 0.445$
FB_1 content	—	$r = 0.275$
		$P = 0.038$
(b)		
Kernel yield	$r = -0.038$	$r = -0.013$
	$P = 0.917$	$P = 0.971$
FB_1 content	—	$r = 0.694$
		$P = 0.026$

infections, fumonisin occurrence constitutes a latent and imminent risk whenever the pathogen is present in the maize agroecosystem. Our results showed that in spite of the maintenance of maize yield, seed treatment with *F. verticillioides* M7075 increased FB_1 content at harvest both years of the study.

Although some countries, including Switzerland and Slovakia, have their own regulations defining acceptable fumonisin levels in maize (Shephard et al., 2000; Labuda et al., 2003), there is no global legislation to regulate that levels. A recommendation of the Official Journal of the European Union (2006) sets these values between 5 and 60 ppm of $FB_1 + FB_{2+}FB_3$ as the recommended levels for these toxins in maize intended for poultry and livestock food. The Center for Food and Drug Administration established total values of $FB_1 + FB_2 + FB_3$ up to 4 ppm for maize intended for human consumption (FDA, 2001).

During the 2007–2008 growing season, the FB_1 contents we obtained after spraying of maize ears with *M. oleovorans* isolate 2 and *E. hormaechei* (T3b and T3d, respectively) did not provide levels recommended for maize intended for human consumption. After seed treatment with *B. amyloliquefaciens* isolate 1 alone or in combination with *F. verticillioides* M7075 strain (T8 and T4, respectively), kernel infection by the fungus at harvest as well as FB_1 contents of harvested grains were significantly diminished with respect to *F. verticillioides* inoculated treatment (T2) during both analyzed maize growing seasons. This indicates that in years conducive for *F. verticillioides* infection or under conditions that favor toxin synthesis the addition of this agent could significantly improve maize kernel quality.

Additionally we observed that climatic variables appeared to have had a role in fumonisin content. Higher fumonisin B_1 contents were obtained during the 2007–2008 growing season in which rainfall was higher from flowering to maturity (January to March). Similar results were reported by Fandohan et al. (2005b) when studying occurrence of *Fusarium* and fumonisin contamination in maize from Benin, West Africa, and also by Bush et al. (2004) who reported an increase in fumonisin levels associated with late-season rainfall in Clayton, North Carolina. In contrast, Miller et al. (1995) found that fumonisin production in maize was limited to drought-stressed fields. It seems that it is stress and not necessarily water availability that induces fumonisin synthesis.

Fumonisin B_1 content and the number of CFUs of *F. verticillioides* in maize kernels at harvest were the only two variables that were significantly positively correlated in both years of the study. Chulze et al. (1999) also found a positive correlation between these two variables under *in vitro* conditions. Kernel yields were not significantly correlated with the number of viable propagules of *F. verticillioides* or with fumonisin B_1 contents although a negative correlation tendency prevailed between these pairs of variables. In agreement with results obtained in the present work, Yates et al. (2005) found no detrimental effects of seed inoculation with *F. verticillioides* on kernel yield of mature plants at harvest under field conditions. Presello et al. (2008) observed that the effects of inoculation with *F. verticillioides* on maize yield were dependent on the hybrid used since yield was reduced in only two of the three types tested.

The numbers of CFUs of *F. verticillioides* and fumonisin B_1 contents were generally reduced in a higher magnitude after seed treatment with BCAs while the addition of these agents as sprays on maize ears resulted in highly variable results in different years of the study. Unexpectedly, FB_1 content was significantly increased after seed treatment with *M. oleovorans* isolate 2 in both years of the study in comparison with uninoculated control (T1). This result is markedly different from the results obtained in a previous field study that evaluated the impact of seed treatment with *M. oleovorans* isolate 2 on fumonisin contents at harvest during 2004–2005 maize growing season (Pereira et al., 2007). The bias observed in results after seed treatment with *M. oleovorans* isolate 2 between these independent studies could have resulted from differences in soil and environmental variables.

Spraying has also been used in field tests as a proper technique to inoculate not only biocontrol agents but also the pathogens in order to test infection processes in agronomic crops (Korsten et al., 1997; Munkvold and Carlton, 1997; Jacobsen et al., 2004; Ji et al., 2006; Jiang et al., 2006). Results of the present study suggest that the plant's phenological stage at which inoculation with BCAs takes place may affect *F. verticillioides* colonization and fumonisin B_1 production. Furthermore, considering that antagonistic effects of used biocontrol agents on *F. verticillioides* are based on competition for the niche that maize roots represent and by antibiotic production, it could be expected that the longer the period in contact with the plant, the greater the chance to exert antagonism. Hallman and Berg (2006) stated that the endophytic community of a particular plant species can be affected by biotic factors such as the plant species itself, plant genotype and age, niche specialization and colonization pathways of associated microorganisms and by abiotic factors such as climatic conditions.

We did not observe extensive kernel rot in either year of the study in any of the treatments (data not shown). However, a relatively high number of CFUs of *F. verticillioides* was obtained in the kernels of *F. verticillioides* inoculated treatment. Desjardins et al. (1998) also reported that maize inoculated with a fumonisin-producing strain presented high kernel infection without visible disease symptoms. So, it has to be considered that apparently asymptomatic kernels and normal yield values may not necessarily be indicative of good grain quality. There are no reports available related to the effects of biocontrol agents used in the field on maize yield and kernel quality parameters. Maize performance in the field was neither affected by seed treatment with *B. amyloliquefaciens*, *M. oleovorans* or *E. hormaechei* when applied alone or in combination with *F. verticillioides*.

Biological control studies have increased over the last years, driven by the interest in alternatives to chemical pesticides. Probably the most important motive for growers to change from chemical control to biocontrol or integrated pest strategies is pesticide resistance. However, as Bolckmans (2002) stated demands from environmental protection authorities and from consumers stimulate the use of natural control strategies. Our data demonstrated that mature maize plants grown from seeds treated with the fumonisin producer strain *F. verticillioides* M7075, although presenting yields equal to naturally infected plants, possessed higher levels of infection with the fungus and also had higher FB_1 content. Seed treatment with *B. amyloliquefaciens* isolate 1 and *E. hormaechei* isolate 4 reduced *F. verticillioides* infection and fumonisin content consistently throughout the 2 years of the test. Results of the present study provide insight into biocontrol abilities of bacterial agents, applied at field scale; and show the influence on such abilities of

the technique used to inoculate plants within the maize agroecosystem. Finally, proper harvest and storage conditions of maize need to be followed to ensure a good quality product with minimum mycotoxin levels.

Acknowledgments

This work was carried out through grants from SECYT-UNRC 2006–2008 and CONICET PIP 5822/05. Authors are also thankful to the members of the Departamento de Producción Vegetal, FAyV-UNRC, for assistance during the set-up of field experiments and to the Cátedra de Agrometeorología, FAyV-UNRC for assessing with climatological data from the experimental field station.

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