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Original Research Paper

Biological control of fumonisins production in maize at field level

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The control of *Fusarium verticillioides* (Sacc.) Niremberg colonization in maize has become an area of interest in crop safety production. In the present study, biological control of *F. verticillioides* with a formulation of *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* was evaluated. Maize seeds were inoculated with a freeze-dried formulation of biocontrol agents and grown in the field. The pathogen incidence and fumonisin accumulation in harvested grain was determined. The number of viable propagules of *F. verticillioides* obtained from the analysis of kernels of treatments with both biocontrol agents did not differ from values obtained from kernels of naturally infected plants and from plants obtained from seeds inoculated with the pathogen. Fumonisin B_1 concentrations in maize were significantly reduced by biocontrol agents. Treatments with *M. oleovorans* produced a reduction of more than 70% and *B. amyloliquefaciens* a reduction of more than 50%. Therefore, the addition of these formulations could significantly improve the quality of maize and also offers the advantage of working with a biological product that does not harm the environment.

Key words. Biological control, maize, *Fusarium verticillioides,* fumonisin, *Bacillus amyloliquefaciens*, *Microbacterium oleovorans*.

INTRODUCTION

Maize (*Zea mays* L.), together with wheat and rice, are staple foods for the world population. Maize is a crop in expansion in temperate climates, where it is used not only for the production of grains and products, but also as a forage crop for silage (Liendo and Martín, 2004). The forecast for world production published by INAI (2009) continues to show the United States as the largest producer, the second place is occupied by Brazil and Argentina is in the third place. Over the sixty percent of maize produced in Argentina is exported. To maintain or increase this percentage, we must ensure importer countries good quality grains that are free of contaminants. Some of the substances that can contaminate maize and maize-based products are a group of mycotoxins called fumonisins. The consumption of fumonisins, such as fumonisin B_1 and B_2, cause harmful effects on animal and human health (Shephard et al., 1996). Fumonisins were catalogued by the International Agency for Research on Cancer as possible human carcinogens (IARC, 2002). These mycotoxins are produced mainly by *Fusarium verticillioides* (Sacc.) Nirenberg, which has been reported as the most frequent pathogen of maize in Argentina (Sydenham et al., 1993; González et al., 1995; Chulze et al., 1996). Because contamination with *Fusarium* and fumonisins begins in the field (Bacon et al., 2008), to be effective control strategies are needed at this level. Biological control, which could be used in crop protection, represents a healthy strategy for the environment. Several studies in biological control have used different microorganisms including bacteria, because of their antagonistic effect on a pathogen (Mc Spadden Gardener and Fravel, 2002). Despite the great diversity of antagonistic bacteria that have been identified, the genus

Bacillus is one of those that contains more antagonistic species than other microorganisms (Sessitsch et al., 2004; Nesci et al., 2005). In previous studies conducted at field level, we have shown that maize seeds treated with *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* showed a significant reduction of *F. verticillioides* incidence and fumonisins level (Pereira et al., 2009; 2010; 2011). In these initial studies, the inocula of *Bacillus* and *Microbacterium* were applied by immersing of the seeds in the same nutrient broth in which the bacteria developed. After that, research was done to improve the efficiency, shelf life, environmental tolerance and ease of handling in order to obtain a bacterial formulation that can be applied successfully in the field. We have demonstrated that *B. amyloliquefaciens* and *M. oleovorans* were more tolerant to ionic and non-ionic potential stress than matric potential stress. These bacteria showed tolerance to heat-shock at 45°C in physiological adaptation assays (Sartori et al., 2010). Previous studies have also shown that growth in MSB medium (molasses soy powder broth) and subsequent freeze drying of *B. amyloliquefaciens* and *M. oleovorans* cells had good viability (Sartori et al., 2012a). Both bacteria had the ability to synthesize betaine and ectoine under high-osmolality conditions (Sartori et al., 2012b); the accumulation of these compatible solutes enables cell proliferation under unfavorable environmental stress conditions (Record et al., 1998).

Therefore, the purpose of the present study is to evaluate the ability of *B. amyloliquefaciens* and *M. oleovorans* formulations to reduce *F. verticillioides* incidence and fumonisin B_1 accumulation in maize grains at harvest during a maize growing season in Argentina.

MATERIALS AND METHODS

Bacterial strains

Formulation 1: the biological component is *Bacillus amyloliquefaciens* (GenBank accession EU164542). Deposited in the National Bank of microorganisms (BNM), of the Research Institute in Agricultural and Environmental Bioscience (CONICET), BNM N° 531. The formulation was registered in the National Institute of Industrial property, patent N° 20100204256. The formulation consisted of physiologically improved freeze-dried cells of *B. amyloliquefaciens*. This formulation was applied to the maize seeds during presowing at inoculum level of 10^9 cells/g of seeds.

Formulation 2: the biological component is *Microbacterium oleovorans* (GenBank accession EU164543), BNM N° 0532, INPI registration patent N° 20100104257. The formulation consisted of physiologically improved freeze-dried cells of *M. oleovorans.* This formulation was applied to the maize seeds during presowing at inoculum level of 10^9 cells/g of seeds.

Fungal spore preparation

Erlenmeyer flasks (500 ml) containing 100 ml of sporulation medium (Cappellini and Peterson, 1965) were inoculated with a 6 mm disk cut from a single spore isolate of *F. verticillioides* cultured on carnation leaf agar (CLA) (Nelson et al., 1983) using a sterile cork borer. *F. verticillioides* strain M7075, a fumonsin B_1 producer (Collection of Pennsylvania State University, University Park, PA, USA) was used. Culture was incubated at 25°C for 7 days on an oscillatory shaker (200 rpm). After this period, spore concentration was determined by standard plate count method on Nash-Snyder agar (Nelson et al., 1983). Suspensions were diluted in 0.8 % NaCl w/v to obtain 10^7 spores ml^{-1} (Pereira et al., 2009; 2010).

Seeds inoculation

Commercial maize seeds cultivar DK 747 MG RR2 (Monsanto, Argentina) were used. This cultivar is resistant to insects and to the herbicide glyphosate.

Seeds were mixed with the freeze-dried powder (1×10^9 CFU ml^{-1}) of the biocontrol agents, in a plastic bag. Seeds were soaked for 5 min with the corresponding treatment. The seeds were dried over night at room temperature (Bardin and Huang, 2003). In treatments with *F. verticillioides* before sowing, a spore suspension of the pathogen was added to seeds (100 µl / 20 seeds). Treatments performed in field were: 1. Control ; 2. Formulation 1 (*B. amyloliquefaciens) ;* 3. Formulation 1 + *F. verticillioides* ; 4. Formulation 2 (*M. oleovorans) ;* 5. Formulation 2 + *F. verticillioides ;* 6. *F. verticillioides.*

Field planting

The field trials were carried out 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 the maize growing season 2009-2010. Treatments were planted in three randomized blocks with three replications for each treatment. Individual plots were 7 m long, 3 m wide separated by borders of 1.5 m, and consisted of four rows with 25 seeds per row, with a total of 100 seeds per plot. 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.

Cumulative monthly rainfalls (mm) were recorded in field during the whole period of crop growth and development. These data were collected from the Agrometeorological Station, UNRC Experimental Field Station, Río Cuarto, Córdoba, Argentina. The Agrometeorological station is Located within the experimental field station near the site

where the experiment was performed.

Sampling procedures and processing of maize kernels

Physiologically mature cobs were collected 150 days after sowing, when samples had reached the R6 phenological stage (Ritchie and Hanway, 1982). All cobs in each individual plot were removed from the plants and separated from their husks. Peeled cobs from each plot were placed together into plastic bags and immediately transported to the Experimental Station facilities, where the 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). 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 fumonisin contents were stored at -20°C.

Influence of treatments on maize agronomic parameters at harvest

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 in our country (SAGyP, 1994).

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

To determine the incidence of *F. verticillioides* in harvested grain, 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^{-2}, 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 25ºC and after incubation, total number of CFUs of *Fusarium* species was obtained. Colonies were transferred to carnation leaf agar (CLA) and incubated at 25ºC with alternating cycles of 12 hours of white light/black light during 7 days. Fungal identifications were performed according to Nelson et al. (1983) and Leslie and Summerell (2006). Fungal count was expressed as $\log_{10}$ per g of maize.

Determination of fumonisin B_1

The concentration of this toxin was determined by high performance liquid chromatography (HPLC), according to the AOAC official method N° 995.15, based on Shephard et al. (1990) with modifications of Doko et al. (1995). Two sub-samples of 15 g of ground maize from each treatment were analyzed. Fumonisin was extracted from each sample with 50 ml of a mixture of acetonitrile/water 1:1(v/v) and the mixture was shaken during 1 hour on an orbital shaker at 120 rpm. Then the extracts were filtered through a filter paper Whatman N° 4. Ten milliliters of the filtered extract were cleaned through Bond-Elut cartridges (SAX 500 mg, strong anion exchange cartridges, VARIAN), which were previously conditioned by successive passage of methanol (5 ml) and methanol:water (5 ml) 3:1 (v/v). Cleaning procedures were performed at atmospheric pressure. The cartridges were then washed with 5 ml of methanol:water 3:1 (v/v) and finally with 5 ml of methanol. Fumonisin was eluted from the cartridges with 1 % acetic acid in methanol (10 ml). The eluants were evaporated to dryness at 40°C in a rotary evaporator. For HPLC analysis the residues were redissolved in 500 µl of acetonitrile: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-phtaldialdehyde 40 mg, methanol 1 ml, 2-mercaptoethanol 50 µl, o.1 M sodium tetraborate 5 ml). The mixture was protected from light by covering the tubes with aluminum paper and then it was manually shaken for 30 s. The samples were left in the dark for 3 min and after this time they were injected in duplicate into the HPLC equipment. 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 C18 reversed-phase column (150 x 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 fumonisin B_1 standard (Romer Labs) diluted in acetonitrile-water 1:1 (v/v). Two injections were made per sample. Data are presented as mean values of triplicates for each treatment. Fumonisin was quantified by correlating peak height of samples with that of standard curves. The detection limit under these conditions was 20 ng g^{-1}.

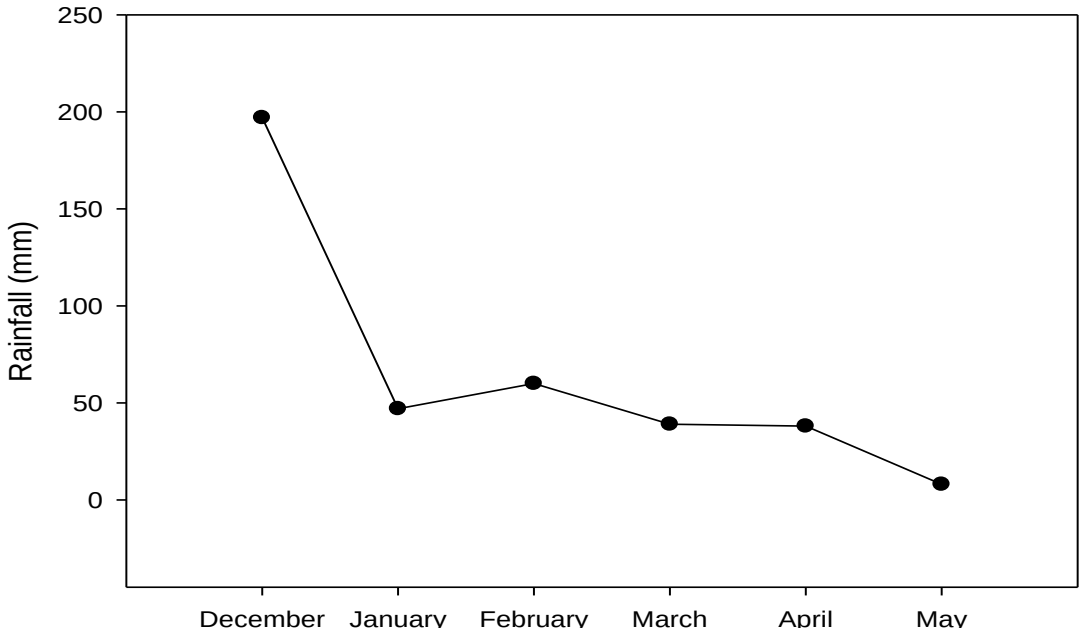

Figure 1: Monthly rainfall (mm) recorded in the experimental area in 2009-2010. Flowering date: February

Table 1. Effects of treatments at harvest during the 2009/2010 maize growing season

Treatment	Number of plants per hectare	Kernel-cob Relation	Yield ± SE (kg ha^{-1})
T1	47619 a	79%-21%	5924 ± 1333 a
T2	38572 a	77%-23%	4283 ± 2899 a
T3	45238 a	78%-22%	4369 ± 1128 a
T4	43333 a	78%-22%	6657 ± 214 a
T5	40238 a	80%-20%	5969 ± 1031 a
T6	45238 a	79%-21%	5641 ± 690 a

Same letters indicate no significant differences between treatments according to Duncan's multiple range test (P<0.05). T1: Control; T2: formulation 1; T3: formulation 1 + *F. verticillioides;* T4: formulation 2; T5 : formulation 2 + *F. verticillioides*; T6 : *F. verticillioides.*

Statistical analysis

Analysis of variance (ANOVA) was made for *F. verticillioides* incidence and fumonisin B_1 and B_2 concentration. The software used was SAS System for Windows 6.11 (SAS Institute, Cary, NC). To determine statistical significance, Duncan's multiple range test (P<0.05) was performed.

RESULTS

Maize agronomic parameters

Figure 1 shows the cumulative monthly rainfall recorded in the experimental field from December 2009 to May 2010. Cumulative rainfall values (420.4 mm) were below average and occurred mainly in December and were scarce in the other months. This may have affected average yield (5474 kg ha^{-1}). Table 1 shows the effects of treatments at harvest. Treatments of *M. oleovorans* alone (T4) and *M. oleovorans* and *B. amyloliquefaciens* coinoculated with the pathogen (T3 and T5) did not show differences with control treatment (T1). The addition of biocontrol agents did not significantly affect the number of plants per hectare obtained for control treatment (T1). Further, treatment of seeds with *F. verticillioides* (T6) also did not affect the number of plants per hectare with respect to T1 values. Kernel-cob relations presented no significant differences

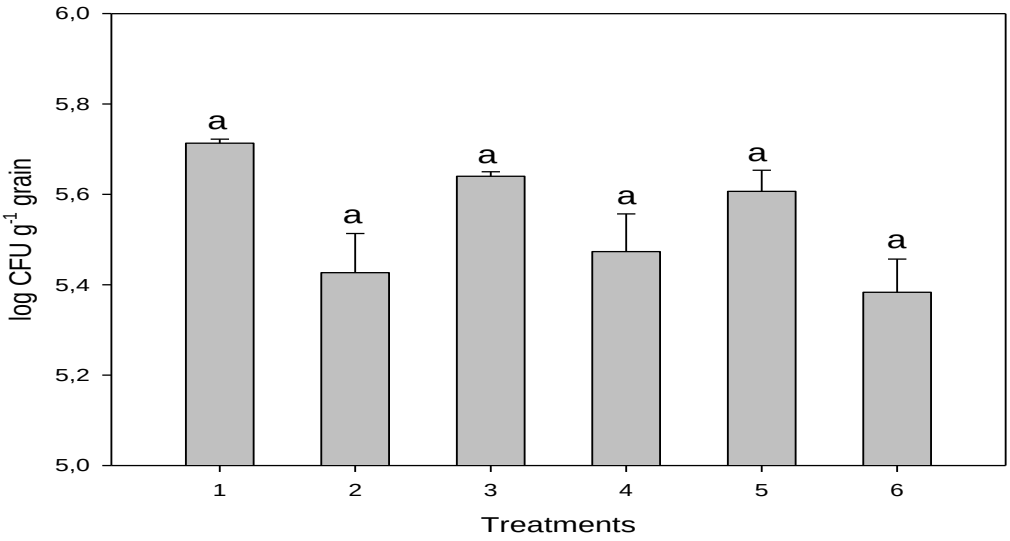

Figure 2: Number of colony forming units of *F. verticillioides* in harvested maize kernels during 2009-2010. T1: Control; T2: formulation 1; T3: formulation 1 + *F. verticillioides;* T4: formulation 2 ; T5 : formulation 2 + *F. verticillioides*, T6 : *F. verticillioides.* Different letters indicate significant differences between treatments according to Duncan's multiple range test (P<0.05).

Table 2. Fumonisin B_1 content in maize kernel at harvest during 2009-2010

Treatment	Fumonisin B_1 (ppb)		
	Median	Min	Max
T1	160.21b	44.13	206.47
T2	78.65d	43.12	420.93
T3	114.26c	24.82	827.25
T4	44.40f	35.94	1071.44
T5	45.33e	<	1009.43
T6	201.94a	73.95	1263.79

n= 3 samples analyzed per treatment
< detection limit (20 ppb)
T1: Control; T2: formulation 1; T3: formulation 1 + *F. verticillioides;* T4: formulation 2 ; T5 : formulation 2 + *F. verticillioides*, T6 : *F. verticillioides.*
Different letters indicate significant differences between treatments according to Duncan's multiple range test (P<0.05).

when compared to control values (T1).

Incidence of *F. verticillioides*

As shown in Figure 2, the number of colony forming units of *F. verticillioides* in harvested maize kernels did not differ between treatments and control (P=0.754). The viable count of the pathogen was of the order of 5 log for all treatments. The number of viable propagules of *F. verticillioides* obtained from kernels of treatments with biocontrol agents did not differ from values obtained from kernels of naturally infected plants (T1) and from plants obtained from seeds inoculated with the pathogen (T6). Treatment caused no stimulation of *Fusarium* propagules growth.

Fumonisin B_1 accumulation

Fumonisin B_1 concentrations in harvested maize are presented in Table 2. The highest content of FB_1 was

Table 3. Pearson correlation coefficients between different variables: FB1, yield and incidence

	Yield	Incidence
FB_1	-0.289 (0.579)	-0.245 (0.640)
Yield	-----	-0.406 (0.424)

FB1: fumonisin B1
(P<0.05) indicates a significant relationship between the two variables

detected in kernels of T6, maize seeds inoculated with *F. verticillioides*. In this treatment, the FB_1 content was 1263.79 ppb. In treatments of seeds inoculated with the formulations (T2 and T4), FB_1 levels were significantly lower than values obtained in T1 (control), with a reduction of 51% and 72 % for the treatments with the formulations of *B. amyloliquefaciens* and *M. oleovorans,* respectively. When the comparison was made between T6 (maize seeds inoculated with the pathogen) and the co-inoculation between the pathogen and the formulations of biocontrol agents (T3 and T5), the reduction percentages were 43% and 77.5 %, respectively.

Correlation between variables

Table 3 shows the Pearson correlation coefficients between FB_1, yield and incidence. A negative correlation was observed between all variables (P > 0.05). A weak correlation was observed between FB_1 with yield and incidence, and a modest correlation between yield and incidence.

DISCUSSION

The aim of this study was to evaluate the effect of the application to seeds of two formulations of biocontrol agents on fumonisin B_1 accumulation and *F. verticillioides* infection on maize grains at harvest. This study also assessed the interactions between different variables that may alter maize agronomic parameters, mainly yield at harvest. This is the first maize growing season (2009-2010) where these biocontrol agents were applied as the active components of two formulations, after four consecutive maize growing seasons (2004-2005, 2005-2006, 2006-2007 and 2007-2008), where *B. amyloliquefaciens* and *M. oleovorans* were applied in liquid cultures.

The application of both formulations produced significant reductions in fumonisin B_1 content in maize grains. The major reduction, 72% to 77.5 %, was observed with the formulation of *M. oleovorans* (T4), while the formulation of *B. amyloliquefaciens* caused a reduction of about 50 %. These results are similar to those we found for the 2007-2008 maize growing season, in which we observed a reduction of 47% and 81% of FB_1 accumulation with the application of inoculum cultures of *B. amyloliquefaciens* and *M. oleovorans,* respectively (Pereira et al., 2011).

Whereas significant reductions were observed in fumonisin B_1 content, none of the two formulations caused a significant reduction in the incidence of *F. verticillioides.* It was observed that inoculum levels of *F. verticillioides* in grains were negatively and not significantly correlated with fumonisin B_1 accumulation. These results are not in agreement with the study by Chulze et al. (1999), where the authors found a positive correlation between FB_1 content and CFUs *F. verticillioides* count. In a previous study, we observed a decrease of *F. verticillioides* incidence in treatments with the addition of these biocontrol agents, and a consequent reduction of fumonisins in grain crops during maize growing seasons 2006-2007 and 2007-2008 (Pereira et al., 2010).

Therefore, it is possible that the decrease in FB_1 content in the grains from seeds treated with both formulations can result from degradation of the toxin by the active components of the formulations and / or from inhibition of the biosynthetic pathway, regardless of inoculum size of *F. verticillioides* in grains at harvest. Benedetti et al. (2006) demonstrated that different bacterial isolates of maize agroecosystem have the ability to degrade FB_1 in *in vitro* assays. Among these bacteria, *Sphingomonas sp.* MTA144, showed strong fumonisin degrading activity (Täubel, 2005; Heinl et al., 2010).

In this field trial as in four previous assays (Pereira et al., 2010), we worked with the plant material $DKRR_2$ (Monsanto). Presello et al. (2008) observed that the effects of inoculation with *F. verticillioides* on maize yield were dependent on the hybrid. The kernel-cob relation for $DKRR_2$ cultivar is around 80-20%, for the agroecological zone of Río Cuarto (Dekalb, 2012), similar to the values found in this work. Despite the low rainfall recorded in the maize growing season 2009-1010, a good average yield was obtained (5474 kg ha^{-1}), and no adverse effects were observed in the maize agronomic parameters of treatments in which the seeds were inoculated with the pathogen and with the formulations.

Many previous studies have shown that bacterial biocontrol agents can be used to reduce the incidence of fungi associated with maize, and *F. verticillioides* toxins production. (Bressan, 2003; Bacon et al., 2001; Bacon and Hinton, 2006). However many of these works were not field studies, where the number of variables involved exceeds those found in laboratory or greenhouse trials. Therefore, the incidence of *F. verticillioides* and fumonisins found in maize grains at harvest may also be influenced by environmental conditions. Warfied and Gilchrist (1999) have determined that growth and toxin production by *F. verticillioides* in the field are strongly affected by substrate moisture content, with grain colonization and toxin

production being higher when the moisture content of grains is lower. In a previous study, during the 2006-2007 maize growing season, the highest production of the toxin was 1623 ppb and responded to treatment in which seeds were inoculated with the pathogen. Using the same treatment, during the 2007-2008 season, where rainfall was higher from maturity to flowering, FB1 showed a maximum of 3800 ppb (Pereira et al., 2010). In the maize growing season 2009-2010, in which cumulative rainfall values were below average, the maximum value of FB_1 obtained in the treatment with the pathogen was 1263.79 ppb. Our results are in agreement with those found by Miller et al. (1995), who reported that the production of fumonisins was limited to fields that exhibited drought stress, suggesting that it is the stress condition the one which favors the production of the toxin and not necessarily the moisture content. High levels of FB are associated with dry and warm climates, although the major FB producing fungus *F. verticillioides* occurs ubiquitously in maize producing areas (Shephard et al., 1996). In contrast, Bush et al. (2004) found high levels of fumonisins and associated this result to late rains in Clayton, North Carolina (USA). Other authors found no correlation between rainfall records and the level of fumonisin in the 2006 and 2007 maize growing seasons produced by subsistence farmers in South Africa (Ncube et al., 2011).

Several studies in different parts of the world showed low levels of fumonisins in Bt hybrids (Munkvold et al., 1999; Pietri and Piva, 2000; Papst et al., 2005; Hammond et al., 2004). A study in Argentina from 1999 to 2005, in different agroecological zones, showed that the median of total fumonisin contamination was 1712 ug / kg (Pacin et al., 2007). Another study conducted in seven locations in Argentina with Bt maize during the growing seasons 2002/2003 and 2003/2004 showed mean values of fumonisins from 0.2 to 0.043 ug / g (Barros et al., 2009). Considering that in the present study, as in our previous field trials we worked with Bt maize, it is possible that this could have been a cause of low levels of fumonisin B_1 in the control treatment. Fumonisin B_1 levels found in this study were lower than the limits recommended by the Official Journal of the European Union (CE, 2007), and total values for fumonisins established by The Center for Food and Drug Administration for maize intended for human consumption (FDA, 2001). Unlike non-Bt maize, in Europe and in other parts of the world, in field trials Bt maize has been shown to have significantly lower fumonisin levels than non-Bt maize. Folcher et al. (2010) found more than 50% of the samples not suitable for consumption according to European Union recommendations (CE, 2007).

However, taking into account that favorable conditions may occur for *F. verticillioides* infection and / or the synthesis of fumonisins, the addition of the two formulations we developed to Bt and non-Bt maize could significantly improve the quality of grains. They also have the advantage of being stable both to prolonged storage and to environmental fluctuations in the field.

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