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Research Papers

In vitro impact on growth, fumonisins and aflatoxins production by *Fusarium verticillioides* and *Aspergillus flavus* using anti-fungal compounds and a biological control agent

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Summary. The temporal efficacy of three different chemical fungicides (Folicur®, Proline®, Sportak 45EW®) and a biocontrol bacterium (Serenade, *B. subtilis*) in reducing growth and toxin production by isolates of *F. verticillioides* and *A. flavus* was studied *in vitro* under different water activity regimes (0.99, 0.98 and 0.95). All the fungicides significantly inhibited mycelial growth compared with the control; the most effective treatment, both against *F. verticillioides* and *A. flavus*, was Sportak 45EW® (approx. 99%). The inhibitory effect of all fungicides generally improved with increasing concentration. Serenade always decreased fungal growth, with optimal results at concentrations of 10^4 and 10^6 (70–75% reduction). All the fungicide treatments resulted in a significant reduction in both FB_1+FB_2 and AFB_1 production when compared to the control, at the end of the incubation period and with the 2 concentrations used (approx. 99%). A threshold concentration inoculum of at least 10^4 CFUs of *B. subtilis* per g was required to achieve a significant control of mycotoxin production. Sportak 45EW® and Serenade gave the best control of mycotoxin production with a reduction of 95% compared to the controls. Use of Serenade in the field should include due consideration to its sensitivity to low water activities, when compared to the target pathogens.

Key words: biological control agent, chemical control, mycotoxin, maize.

Introduction

Maize (*Zea mays* L.) is susceptible to fungal contamination that can occur in the field or during storage. In particular, *Fusarium* spp. and *Aspergillus* section *Flavi* are relevant maize pathogens able to produce several mycotoxins in kernels (Bottalico, 1998; Giorni *et al*., 2007; Samapundo *et al*., 2007). In maize grown in temperate regions, *F. verticillioides* and *F. proliferatum* are commonly the dominant fungi associated with the ripening ear and contamination with fumonisins (FBs), known to cause human and animal toxicoses, are often detected (Samapundo *et al*., 2005; Battilani *et al*., 2008).

Aspergillus section *Flavi*, especially *A. flavus* and *A. parasiticus*, produce aflatoxins (AFs), the most toxic naturally occurring fungal compounds, which represent a significant health hazard for humans and animals (Raper and Fennell, 1965). *Aspergilli* are dominant in tropical regions, but they are also considered an emerging problem in Europe (Piva *et al*., 2006).

To reduce the intake of FBs, the European Commission set action limits of 4000 μg fumonisin B_1 (FB_1)+FB_2 kg^{-1} for unprocessed maize (European Commission Regulation 1126/2007) and maximum levels were also fixed for aflatoxin B_1 (AFB_1) and total aflatoxins (sum of AFB_1, AFB_2, AFG_1, AFG_2) in unprocessed maize (5.0 μg kg^{-1} and 10 μg kg^{-1} re-

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spectively) (European Commission Regulation (EC) 1881/2006). International developments in mycotoxin regulation have increased the pressure to find strategies for the mitigation of mycotoxins in maize (Munkvold and Desjardins, 1997). Fumonisins and AFs are produced during maize cultivation and their mitigation has been approached with a focus on the cropping system (Munkvold and Desjardins, 1997).

Generally, a minimisation of plant stress reduces mycotoxin contamination, in particular AFs (Payne, 1998). Evidence of the specific role of each practice is limited (Lisker and Lillehoj, 1991), although the management of residues, (Cotten and Munkvold, 1998) tillage and crop rotation (Marocco *et al*., 2008) and fertilisation (Jones and Duncan, 1981) have been studied. Sowing time also has an impact on fumonisin contamination (Blandino *et al*., 2004). The use of new hybrids and the control of the European corn borer (ECB) can contribute to reduce fumonisin contamination (Scandolara *et al*., 2008; Folcher *et al*., 2009).

Direct control of mycotoxin-producing fungi has recently been included among good agricultural practices in small cereals in order to control *Fusarium* head blight (Blandino and Reyneri, 2009), but little information is available on the effects of synthetic fungicides on *Fusarium* ear rot and fumonisin contamination in maize (De Curtis *et al*., 2008). Triazole fungicides, in particular prothioconazole and tebuconazole, were confirmed to be the most effective against *Fusarium* species on wheat in the field (Simpson *et al*., 1986; Vanova *et al*., 2004; Pascale *et al*., 2008).

The use of chemical fungicides is a controversial practice that entails undesirable environmental side effects. An alternative strategy to reduce aflatoxin and fumonisin accumulation in maize ears involves the biological interaction among toxigenic fungi and natural bio-competitive agents. The use of certain bacteria or yeasts to control pre- and post-harvest pathogens and pests of agricultural commodities has been studied *in vitro* with encouraging results (Cavaglieri *et al*., 2005; Etcheverry *et al*., 2009). However, there is little detailed knowledge on the interactions of *F. verticillioides* and *A. flavus* with biocontrol agents such as *Bacillus subtilis* used pre-harvest.

The aim of this study was to determine *in vitro* the temporal efficacy of commercial chemical fungicides known to effectively control Fusaria in small cereals and a biocontrol bacterium, *B. subtilis* (available as a commercial product for field use), in reducing growth and toxin production by isolates of *F. verticillioides* and *A. flavus* under different water activity (a_w) regimes relevant for mycotoxin production.

Materials and methods

Fungal strains

Two fumonisin-producing strains of *F. verticillioides* (MPVP 294, MPVP 289) (Etcheverry *et al*., 2009) and one aflatoxin-producing strain of *A. flavus* (MPVP A 2092) (Giorni *et al*., 2007) were used in the experiments, all carried out in duplicate. The strains were isolated from maize kernels grown in northern Italy and stored in the fungal collection of the Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore in Piacenza and ISPA-CNR (ITEM 10027, ITEM 10026 and ITEM 8069, respectively).

These fungal strains were inoculated in 9 cm Petri dishes containing Potato Dextrose Agar (PDA, Oxoid®, Basingstoke, Hampshire, England) incubated at 25°C for 7 days and used as an inoculum. Two kinds of inoculum were prepared: (1) colonies were washed with 10 mL of sterile water and the fungal suspension was adjusted to a concentration of 10^4 spores per mL and (2) agar discs were cut from the margin of the fungal colony (Ø 2mm) using a sterile cork borer.

Fungicides

Fungicides available according to European legislation and reported by the producing companies as effective against *Fusarium* head blight of small cereals were included in these studies: Folicur SE® (43.1 g L^{-1} of active ingredient (ai) tebuconazole); Proline® (250 g ai L^{-1} prothioconazole) and Sportak 45EW® (450 g ai L^{-1} prochloraz). Media were modified by the addition of 0.1, 0.5, 1.0 and 5.0 mg Kg^{-1} of Folicur SE® and Proline® and 0.01, 0.05, 0.1 and 0.5 mg Kg^{-1} of Sportak 45EW®; these dosages were obtained from the preparation of stock solutions (1000 mg Kg^{-1}) based on the recommended dosage for field use (tebuconazole 215.5 g ha^{-1}; prothioconazole 200 g ha^{-1}; prochloraz 585 g ha^{-1}).

Inoculation and measurement

Petri dishes (Ø 9 cm) with PDA were used for the *in vitro* studies. The media were modified with fun-

gicides, shaken vigorously for mixing before being poured into Petri dishes (approx. 45°C). Three a_w levels (0.99, 0.98 and 0.95) were considered in the study; these treatments were obtained by the addition of 0, 9.2 and 23.0 g of glycerol per 100 mL of distilled water (Dallyn and Fox, 1980).

Inoculum, in the form of a spore suspension or mycelial plug, was placed in the centre of each Petri dish, with all treatments having three replicates. All dishes were incubated at 25°C for 21 days for *F. verticillioides* and 14 days for *A. flavus*.

The biological control agent *B. subtilis* (Serenade, strain QST713, 5×10^9 CFU g^{-1}, Agraquest, powder formulate) was also included in this study. An aliquot of 10 g of the powder formulation was blended with 90 mL of PDA medium, maintained at 45°C for the time necessary to homogenize the solution to obtain a suspension of 10^8 cells mL^{-1}. Serial dilutions were carried out between 10^{-3} until 10^{-8} and finally poured into Petri dishes.

The diameter of the fungal colonies was measured along two perpendicular diagonals crossing the inoculum point after 7, 14 and 21 days for *F. verticillioides* and after 7 and 14 days for *A. flavus*.

Mycotoxin analysis

Mycotoxins were analysed from selected sample sets. Only dishes inoculated with a spore suspension as inoculum, the colonies grown on unmodified control media and the following treatments were considered: 0.5 and 5 mg Kg^{-1} for Folicur SE® and Proline®, 0.05 and 0.5 mg Kg^{-1} for Sportak 45EW® and 10^4 and 10^8 CFU g^{-1} of Serenade for *F. verticillioides* MPVP 294 and *A. flavus*. The selected concentrations were chosen as they are considered the most representative of the data set.

Fumonisins

An aliquot of the content of Petri dishes (1.8 g) was weighed and transferred to a flask. FBs were extracted with 10 mL of methanol for 45 min using a magnetic stirrer; then the solution was poured into a glass vial and centrifuged at 3000 g for 5 min; the solution was diluted (0.1 mL brought to 1 mL) with acetonitrile+water (30+70 by volume) and filtered (Millex HV 0.45 μm, Millipore Corporation, Bedford, MA, USA) before HPLC analysis. The analysis was carried out using a LC-MS/MS system,. as described elsewhere (Pietri *et al*., 2010). Fumonisin production was quantified in ng g^{-1} of culture medium. The limit of detection was 20 ng g^{-1} for both FB_1 and FB_2. Average recovery values were 97.1±1.8 and 95.6±2.2 % for FB_1 and FB_2, respectively.

Aflatoxins

An aliquot of the content of Petri dishes (1.8 g), was weighed and transferred to a flask. Aflatoxins were extracted for 60 min with 20 mL of methanol using a magnetic stirrer; then, the solution was poured into a glass vial and centrifuged at 3000 g, for 5 min; the solution was diluted (0.1 mL brought to 1 mL) with acetonitrile+water (25+75 by volume) and filtered (Millex HV 0.45 μm) before HPLC analysis. The analysis was performed using an HPLC instrument, as described elsewhere (Pietri *et al*., 2010). AFs production was quantified in ng g^{-1} of culture medium. The limit of detection was 0.5 ng g^{-1} for each AF. The average recovery value for AFB_1 was 96.9±1.7.

Data analysis

Data on fumonisin and aflatoxin production (values+1) were logarithmically transformed before statistical analysis. This was required because of the wide variability of the data (Clewer and Scarisbrick, 2001). Analysis of variance was performed considering all factors (fungicide types and dosage, a_w); the ANOVA 1 of the statistical package SPSS was applied to data collected on fungal growth and mycotoxin production (Statistical Package for Social Science, ver.15.0.1, 2006. SPSS Inc., Chicago, IL, USA). The analysis was conducted separately for Sportak 45EW® and Serenade due to the difference in active dosage and kind of product, respectively. Means were compared using the Tukey test. The ED_{50} values, amount of active ingredient required to produce a specific effect to half reduce the fungal growth rate, was computed by linear regression analysis (Marin *et al*., 1998).

Results

Effect of fungicides on fungal growth

The results obtained in the duplicate trials, for all the fungi considered, were very similar and data from the first trial were used for statistical

analysis. All products used, their dosage, and media a_w significantly influenced fungal growth while the inoculum type had no significant effect (Tables 1 and 2). All products used significantly reduced fungal growth.

The fungicide concentration explained 18 and 47% of total variance for *F. verticillioides* (MPVP 294 and MPVP 289, respectively) and 39% for *A. flavus*. The factor interactions (a_w, fungicides and concentration) significantly influenced the fungal growth, but explained <1% of the total variance.

All the fungicides significantly ($P< 0.05$) inhibited mycelial growth compared to the control and Sportak 45EW® was also the most effective at the lowest dosages applied, both against *F. verticillioides* and *A. flavus*. The inhibitory effect of all fungicides generally improved with increasing concentration. The fungicides tested at the highest concentrations resulted in a 79, 47 and 61% decrease in fungal growth compared to the controls, respectively for *F. verticillioides* MPVP 294, MPVP 289 and *A. flavus* (Table 1), while no growth was observed with Sportak 45EW® application (Table 2).

Mycelial growth of both fungal species was slower with decreasing a_w and increased with time. Fungal growth was significantly influenced by all factors considered in the experiments carried out with Serenade. Fungal growth was lower with spore suspension inoculum for *F. verticillioides* MPVP 294 and *A. flavus*. All the concentrations of Serenade decreased fungal growth (Table 3); the lowest concentration (10^3) limited growth to 48, 71 and 65% of the control for *F. verticillioides* (MPVP 294 and 289) and *A. flavus*, respectively. Concentrations from 10^6 and 10^8 gave the most significant effects, decreasing fungal growth of both mycotoxigenic species by 70–75%.

Table 1. Results of ANOVA regarding the effects of inoculation method, commercial fungicide products (Folicur® and Proline®) and water activity (0.99, 0.98 and 0.95) on *in vitro* growth of *Fusarium verticillioides* (MPVP 294 and MPVP 289) and *Aspergillus flavus* incubated at 25°C for 14 days, measured as colony radius (mm).

Factor	*F. verticillioides* (MPVP 294) radius (mm)		*F. verticillioides* (MPVP 289) radius (mm)		*A. flavus* radius (mm)	
Commercial products						
Folicur®	33.4	b [a]	23.3	b	28.5	b
Proline®	37.2	a	24.7	a	31.7	a
Inoculum type						
Spore suspension	38.5	n.s.	24.6	n.s.	24.9	n.s.
Agar disc	38.4	n.s.	23.9	n.s.	25.0	n.s.
Dosage (mg kg^{-1})						
0	41.4	a	33.0	a	37.1	a
0.1	40.4	ab	26.6	b	33.1	b
0.5	39.8	b	24.4	c	31.7	c
1	38.4	c	20.3	d	28.8	d
5	32.8	d	15.7	e	22.7	e
Water activity						
0.99	40.2	b	28.4	a	30.2	b
0.98	41.6	a	28.0	a	36.2	a
0.95	33.8	c	15.7	b	25.7	c

[a] Different letters indicate significantly different growth of fungi ($P\leq 0.05$). n.s., not significant.

Table 2. Results of ANOVA regarding the effect of inoculation method, the fungicide Sportak 45EW®, and water activity (0.99, 0.98 and 0.95) on *in vitro* growth of *Fusarium verticillioides* (MPVP 294 and MPVP 289) and *Aspergillus flavus* incubated at 25°C for 14 days, measured as colony radius (mm).

Factor	*F. verticillioides* (MPVP 294) radius (mm)		*F. verticillioides* (MPVP 289) radius (mm)		*A.flavus* radius (mm)	
Inoculum type						
Spore suspension	20.7	n.s.	19.5	n.s.	17.8	n.s.
Agar disc	19.4	n.s.	19.3	n.s.	17.1	n.s.
Dosage (mg kg^{-1})						
0	41.4	a [a]	33.0	a	38.7	a
0.01	2.2	b	9.8	b	4.3	b
0.05	0.0	c	7.6	c	1.7	c
0.1	0.0	c	1.2	d	0.0	c
0.5	0.0	c	0.0	e	0.0	c
Water activity						
0.99	9.1	a	12.3	a	9.8	a
0.98	8.8	b	12.2	a	10.7	a
0.95	8.5	c	6.3	b	6.2	b

[a] See Table 1.

Interestingly, the inhibitory effect of Serenade decreased with a decrease of media a_w.

The growth of the fungi at the ED_{50} concentrations (Figure 1) was compared. In general, all the antifungal agents inhibited fungal growth of *F. verticillioides* more effectively than that of *A. flavus*. In particular, *F. verticillioides* (MPVP 289) mycelial growth was reduced more than that of MPVP 294 by all the products. Sportak 45EW® was the most active antifungal agent (ED_{50} value was 0.0025 μg Kg^{-1}) and its efficacy was very similar to Serenade (ED_{50}=10^4 CFU g^{-1}). Triazoles, with an ED_{50} value of 5.5 μg Kg^{-1}, inhibited fungal growth with more efficacy against *F. verticillioides* MPVP 289 than MPVP 294 and the *A. flavus* strain (Figure 1).

Effects of fungicides on FBs and AFs

All the treatments significantly ($P \leq 0.01$) inhibited mycotoxin production when compared to the control at the end of the incubation period (data not shown). Sportak 45EW® and Serenade gave the best control of FB_1+FB_2 and AFB_1 production with a reduction of 95% compared to the control. The control (12590 μg kg^{-1} FB_1+FB_2 and 631 μg kg^{-1} AFB_1) was confirmed at ED_{50} dose, with 98 and 83% reduction in FBs and AFB_1, respectively, with Folicur® and Proline® and 100 and 99.6% with Sportak 45EW® and Serenade.

Discussion

In this study all the compounds tested significantly reduced fungal development when compared with the control, but Sportak 45EW® was more effective than the triazoles. This is in agreement with Doohan *et al*. (1996) who reported that prochloraz significantly reduced *F. culmorum* on small grains in the field and Mateo *et al*. (2011), which showed prochloraz to be the most active antifungal agent against *F. langsethiae in vitro*.

Very good results were also obtained with the biocontrol agent Serenade. All the compounds had an inhibitory effect on mycelial growth and mycotoxin production at all the concentrations used, against

Table 3. Results of ANOVA regarding the effect of Serenade (different concentrations), inoculation method, and water activity on *in vitro* growth at 25°C of *Fusarium verticillioides* (MPVP 294 and MPVP 289) and *Aspergillus flavus* after 14 days of incubation.

Factors	*F. verticillioides* (MPVP 294) radius (mm)		*F. verticillioides* (MPVP 289) radius (mm		*A. flavus* radius (mm)	
Inoculum type						
Spore suspension	15.4	b [a]	18.1	n.s.	14.9	b
Agar disc	22.2	a	18.7	n.s.	19.4	a
Concentration (CFU)						
0	41.1	a	37.9	a	42.4	a
10^3	18.4	c	27.0	b	29.3	b
10^4	20.5	c	18.3	c	15.1	c
10^5	27.3	b	15.8	d	11.4	c
10^6	11.0	d	12.2	e	12.7	c
10^7	10.3	d	9.9	f	12.6	c
10^8	9.5	d	7.8	g	13.4	c
Water activity						
0.99	17.2	b	15.2	c	12.7	b
0.98	18.0	b	22.0	a	13.4	b
0.95	24.3	a	18.0	b	32.5	a

[a] See Table 1.

both *F. verticillioides* and *A. flavus*; a decrease in fungal growth of approx. 40 and 70–75% was observed with chemical and biological control, respectively, and this result is very positive because triazoles were previously considered active only against *Fusaria*.

Considering *Aspergillus* section *Flavi*, conventional methods of plant disease control with the use of fungicides (benomyl, thiabendazole, carboxine) were reported as ineffective in maize when applied at environmentally safe concentrations (Bhatnagar *et al*., 1993). However, in some *in vitro* studies prochloraz and imazalil seemed to be effective in reducing growth and aflatoxin formation by *A. flavus* and *A. parasiticus* (Delen and Tosun, 1999); this finding is supported by the results of this study.

There have been several reports showing growth inhibition of fungal pathogens treated with bacterial strains like *Bacillus amyloliquefaciens*, *Microbacterium oleovorans* (Cavaglieri *et al*., 2005; Pereira *et al*., 2007; Etcheverry *et al*., 2009), *Amphibacillus xylanus* and *Sporolactobacillus inulinus* (Nesci *et al*., 2005; Etcheverry *et al*., 2009). The use of biological control agents with antagonistic effects on the main maize pathogens could represent a promising alternative (Pereira *et al*., 2010). Our results show that *B. subtilis* is competitive and can inhibit *F. verticillioides* growth and fumonisin production. This suggests that the mechanism of action of this bacterium may be a competitive exclusion of the pathogen in maize, as suggested by Motomura *et al*. (1996); therefore, it is supported by Bacon *et al*. (2001) that described *B. subtilis* and *F. verticillioides* as ecological homologues occupying the same ecological niche. Furthermore, *B. subtilis* has been shown to control *A. flavus* and aflatoxin production both in the field and during storage (Kimura and Hirano, 1988; Nesci *et al*., 2005).

Bacillus subtilis has been reported to produce some bioactive metabolites and this may play an important role in the antagonism. A recent study identified fengycin as the prominent lipopeptide in

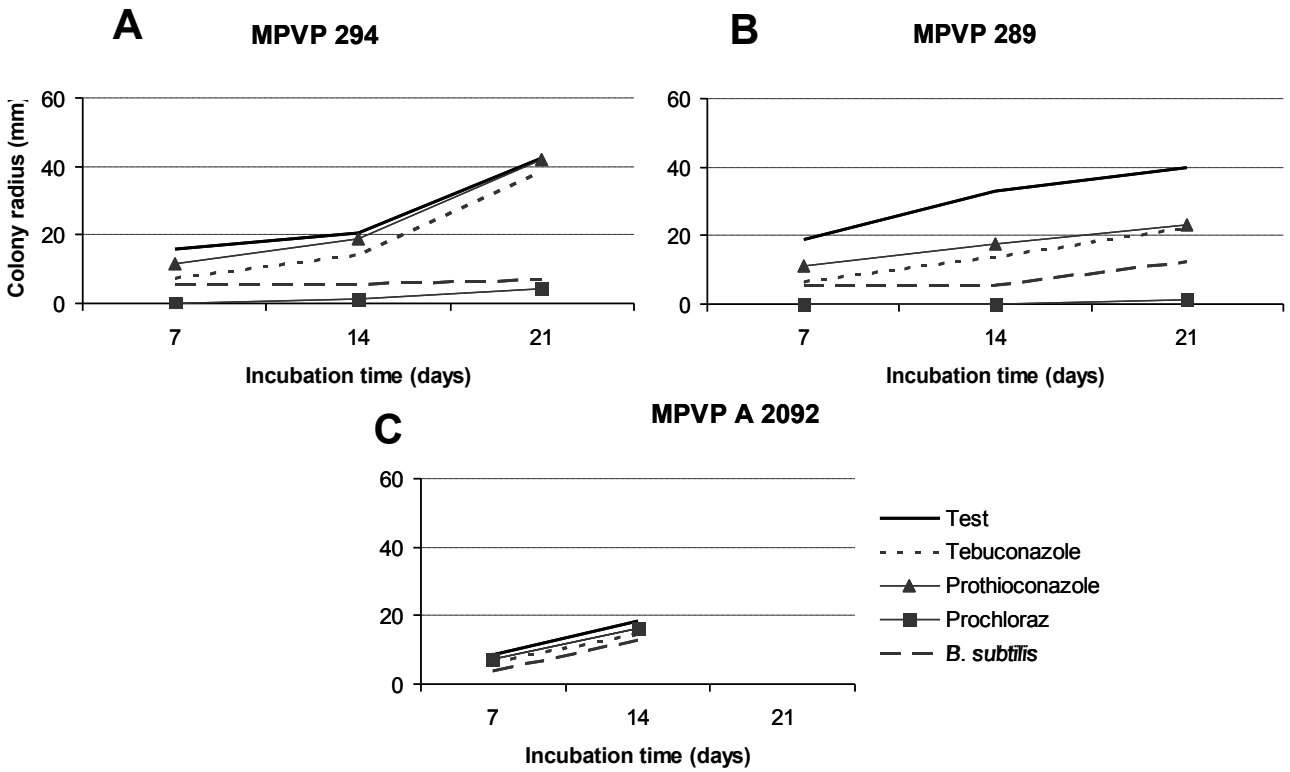

Figure 1. Effect of active ingredients (Folicur®, Proline® and Sportak45EW®) and a biocontol agent (Serenade), applied at their ED_{50}, on *in vitro* growth of *Fusarium verticillioides* (MPVP 294 and MPVP 289) at 25°C after 7, 14 and 21 days of incubation (A and B) and *Aspergillus flavus* at 25°C after 7and14 days of incubation (C), measured as colony radius (mm).

B. subtilis strains which antagonize *G. zeae* (Wang *et al*., 2007) and antimicrobial cyclic lipopeptides were identified also by Dunlap *et al*. (2011). Recent studies with a *Streptomycete* strain showed that the metabolites were more effective than the growth of the bacterial strain in inhibiting growth and aflatoxin contamination *in vitro* and in stored groundnuts (Sultan and Magan, 2011).

Bacillus subtilis is however more sensitive to low a_w than some actinomycetes and fungi. This may be an important factor which needs to be taken into account when examining relative competitiveness and environmental stress factors (Magan, 2006). Environmental stress factors are important because it has been observed that several interactions were influenced by a_w, temperature and substrate. Changes in environmental factors cause an impact that can be decisive in determining the co-existence or dominance of species in a particular ecological niche (Giorni *et al*., 2009).

Considering our *in vitro* trials as a whole, the chemical treatments and the use of *B. subtilis* efficiently controlled mycotoxin production, both by *F. verticillioides* and *A. flavus*. In contrast, some previous studies reported an increase in mycotoxin production by *Fusarium* spp. in the presence of some fungicides (Magan *et al*., 2002; Falcão *et al*., 2011). In particular, Falcão *et al.* (2011) reported that fludioxonil+metalaxyl-m added to culture medium at the recommended dose (1.5 μL mL^{-1}) increased the mean FB_1 production by three *F. verticillioides* strains. Magan *et al.* (2002) found that sub-optimal concentrations of triazole fungicides stimulated the production of DON by *F. culmorum* isolates from different parts of Europe, especially when combined with reduced a_w. Our results emphasize that chemical and biological control against *Fusarium* did not enhance either *A. flavus* growth or aflatoxin production.

Mitigation of mycotoxins in maize is crucial all over the world, even if the focus on different toxins

depends on the maize growing region. Southern Europe commonly has problems with FBs, but in drier years, contamination with AFs becomes more important (Magan *et al*., 2002; Battilani *et al*., 2005; Pietri *et al*., 2009). Hybrids with genetic resistance towards Fusaria and Aspergilli are still in development and they will not be available commercially in the next 3-5 years (Berardo *et al*., 2005; Lanubile *et al*., 2011). Guidelines for optimising the cropping system to minimise mycotoxin contamination are available, but the direct control of fungi with chemical or biological agents is considered important, mainly in high risk conditions (Rossi *et al*., 2007). Several studies have indicated that ECB control can be a useful indirect action for reducing mycotoxin levels, although there have been variable results in terms of the magnitude of mycotoxin reduction (Blandino *et al*., 2008; Saladini *et al*., 2008; Mazzoni *et al*., 2011).

Few studies have demonstrated the importance of direct chemical control with fungicides or biological control on maize and little information is available on the effects of synthetic fungicides on *Fusarium* ear rot and fumonisin contamination (Folcher *et al*., 2009; Mazzoni *et al*., 2011). More information is available regarding fungal control using fungicides on wheat, where Triazoles have proved to be the most active compounds against *Fusarium* spp. infection, *Fusarium* head blight (FHB) and DON contamination (Edwards *et al*., 2001; Blandino and Reyneri, 2009)

The present study suggests that there are differences in the efficacy of different fungicides against *F. verticillioides* and *A. flavus*. This could have implications for field control and further studies are needed to examine the efficacy in the field under natural infection of maize.

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