RESEARCH PAPERS - FOCUS ISSUE ON PLANT HEALTH SUSTAINING MEDITERRANEAN ECOSYSTEMS

Effective chemical management for prevention of aflatoxins in maize

Christina S. LAGOGIANNI and Dimitrios I. TSITSIGIANNIS

Agricultural University of Athens, School of Agricultural Production, Infrastructure and Environment, Department of Crop Science, Laboratory of Plant Pathology, Iera Odos 75, 118 55, Athens, Greece

Summary. The presence of aflatoxins in maize grain has been an increasing problem in the Mediterranean area, possibly due to climate change such as increased temperatures and extended drought periods. It is therefore important to prevent the growth of aflatoxigenic *Aspergillus* species in the field. There are no fungicides registered for control *A. flavus* in maize, so this study investigated the efficacy of azoxystrobin, boscalid, cyprodinil, fludioxonil and cyprodinil + fludioxonil to reduce *A. flavus* growth, sporulation and aflatoxin production in *in vitro*, and in maize field studies. Based on *in vitro* inhibition of mycelial growth, the most effective fungicides were cyprodinil (EC_{50} < 0.05 µg mL^{-1}) and fludioxonil (EC_{50} <0.11 µg mL^{-1}), while the least effective was boscalid (EC_{50} 4.35-4.50 µg mL^{-1}). Azoxystrobin almost completely inhibited the conidium germination at > 0.5 µg mL^{-1}. Further evaluation of the fungicides on maize seeds infected with *A. flavus* demonstrated that all the fungicides reduced conidium production by 76 to 94%, and reduced aflatoxin contamination. In a 2-year field study, application of cyprodinil + fludioxonil reduced *A. flavus* ear rot severity by 40%, and was the most effective formulation for reducing aflatoxin contamination, by 83%. The other four single ingredient fungicides also decreased aflatoxin production on maize kernels (fludioxonil by 80%, cyprodinil by 75%, boscalid by 74% and azoxystrobin by 67%). Field data from this study provide farmers with a new effective chemical approach to control *A. flavus* and aflatoxin production in maize within an integrated strategy for management of aflatoxins in maize.

Keywords: fungicides, *Aspergillus flavus*, mycotoxins, plant pathogen, corn.

Introduction

Mycotoxins are toxic and carcinogenic natural metabolites of low molecular weight that are produced by particular species of fungi. These metabolites pose severe threats for the safety and quality of food and feed worldwide, and have considerable social and agro-economic importance. Hundreds of mycotoxins differing in chemical structure can contaminate food and animal feed, causing acute or chronic toxicity to humans and animals (Bennett and Klich, 2003). Mycotoxins are classified as high, medium or low risk, according to the different health risk potential, from illness to death. Food losses due to mycotoxins, and the costs for mycotoxin, management have increased dramatically worldwide. More than 25% of agricul-tural products are contaminated with mycotoxins worldwide (CAST, 2003).

Aspergillus spp. are the most common and ubiquitous fungal species producing mycotoxins in food and feedstuff, and aflatoxins are one of the most economically important mycotoxin groups. These are polyketide secondary metabolites produced predominantly by *A. flavus* and *A. parasiticus*. These fungal species are distributed worldwide infecting many agricultural products in the field, such as maize, cereal grains, tree nuts, peanuts, oily seeds, cottonseed, coffee beans, dried fruits and spices (Smith, 1997; Chu, 2002). Toxicological studies have shown that aflatoxins are among the most potent carcinogenic, teratogenic and mutagenic substances in nature. Aflatoxins are introduced into food chains by pre-harvest and post-harvest contamination of foods and feeds. *Aspergillus flavus* is of great toxicological and economic importance because it is the major aflatoxin-producing

Corresponding author: D. Tsitsigiannis
E-mail: dimtsi@aua.gr

species in crops, and is able to produce many other secondary metabolites with deleterious properties including aspergillic acid, cyclopiazonic acid, kojic acid and helvulic acid (Bennett and Klich, 2003; Horn, 2007; Abbas, 2009). Among aflatoxins, AFB1 is classified as a group I human carcinogen by the International Agency for Research on Cancer (IARC), and is the possible cause of human primary hepatocellular carcinoma (IARC, 1993). The annual reports of the European Union's Rapid Alert System for Food and Feed (RASFF) published from 2002 to 2016 show that most alerts concerning mycotoxins in food or feed concern the AFs.

Maize (*Zea mays* L.) is a staple crop and an important source of human food, forage, flour, and other processed products for industry in many countries. It is very susceptible to contamination by *Aspergillus* species in the field or, as grain, during storage. Fungal spoilage of maize seeds by *A. flavus* and aflatoxin contamination are of major concern. *Aspergillus flavus* can infect maize in warm climates at pre-harvest stages, during crop growth and harvesting, and at post-harvest during storage, transport and processing. Increase in aflatoxin content can occur if the phases of grain drying and storage are poorly managed (Smith, 1997; Chulze, 2010). Climate change phenomena, with alterations of wet and dry cycles, can influence the life and disease cycle of plant pathogens and provide advantages to colonization of crops by xerotolerant fungi and/or those adapted to elevated temperatures, such as aflatoxigenic species. Severe droughts in Balkan countries in 2012 resulted in significant contamination of maize crops with aflatoxins (>70%), and the use of this maize as feed for dairy cattle led to high levels of aflatoxin M1 in milk (up to twice the EU legal limit), and to the well-known "milk scandal" (Popovic *et al.*, 2017). Additionally, during 2003–04, the very hot periods that occurred in parts of northern Italy led to severe contamination of maize with AFB1, causing high concentrations of AFM1 in the milk of animals fed with this cereal. Water and thermal stress can give significant ecological advantages to aflatoxigenic species over other natural pre-harvest species such as *F. verticillioides* in maize in Mediterranean region (Giorni *et al.*, 2007; Battilani *et al.*, 2008).

The prevention of mycotoxin contamination of agricultural products is an important priority in human and animal safety. Strategies to control mycotoxin-producing fungi at pre-harvest stages include improved crop management and agronomic practices, control of insects that favour fungal infection, host plant resistance and biological control (e.g. non-aflatoxigenic antagonistic strains) (Smith, 1997; Abbas *et al.*, 2007; Mauro *et al.*, 2018). Chemical management of several plant diseases remains the main strategy to reduce the incidence of many plant pathogens in most crops. The use of fungicides has become an essential part of modern agriculture (Brent and Hollomon, 2007). However, control of mycotoxin-producing fungi with fungicides is not widely used, because of the cost, the difficulty of applying agrochemicals at the right crop stage to restrict colonization and dispersal by mycotoxigenic fungi to avoid mycotoxin contamination, and because extensive field evaluation has not been carried out. However, if fungicides are applied as pre-harvest treatments at the appropriate crop growth stages (i.e. during anthesis), these can be an efficient, cost-effective strategy for preventing mould growth, and consequent mycotoxin production (Santos, 2011). Several previous studies have shown that fungicides from several groups (including anilinopyrimidines, benzimidazoles, dicarboximides, phenylpyrroles, strobilurins and triazoles) can inhibit the growth and the mycotoxin production in *Aspergillus*, *Fusarium* and *Penicillium* species, although most of these studies report *in vitro* results. Field studies that report effective chemicals for control of aflatoxins in maize are lacking (Badii and Moss, 1988; D'Mello *et al.*, 1998; Matthies and Buchenauer, 2000; Pirgozliev *et al.*, 2002; Ioos *et al.*, 2005; Markoglou *et al.*, 2008; 2011; Schmale and Munkvold, 2009; Doukas *et al.*, 2012; Mateo *et al.*, 2017).

In our investigation, based on the efficacy of various fungicides against *Aspergillus* spp. from previously published *in vitro* and some field studies, several agrochemicals were selected and evaluated for effects on *A. flavus* growth and aflatoxin production in *in vitro*, and in *in situ* studies in a maize field. The aims of the study were: a) to evaluate the effects of the fungicides azoxystrobin, boscalid, cyprodinil, fludioxonil and a cyprodinil + fludioxonil mixture on growth and sporulation of different toxigenic isolates of *A. flavus in vitro*, either on growth media or on maize seeds; b) to determine the ability of these fungicides to reduce the aflatoxin production in maize seeds; and c) to test the efficacy of selected fungicides for control *A. flavus* and aflatoxin contamination in maize in field experiments.

Materials and methods

Fungus strains and culture conditions

Aspergillus flavus strains A6.10, D1.3 and 12S were used in these experiments. Strain A6.10 originated from Greek maize fields and D1.3 from pistachio fields, and are held in the culture collection of the Laboratory of Plant Pathology, Department of Crop Science, Agricultural University of Athens. Isolate 12S originated from a cotton field in the USA. To verify the identity of *Aspergillus* isolates at the species level, their genomic DNA was extracted, and the calmodulin gene was PCR-amplified using the primer pair CL1- 5' (GARTWCCAAGGAGGCCTTCTC) 3' and CL2A- 5' (TTCCGTACCCCGATCTTCGGAA) 3' following the procedure of O'Donnell *et al.* (2000). PCR products were sequenced and identified as *A. flavus* by BLAST analysis with 100% identity compared to published *A. flavus* sequences. The ability of the three *A. flavus* isolates to produce AFB1 was confirmed using thin layer chromatography (TLC) (Scott, 1995). The isolates were stored at -80°C, as conidium suspensions in 25% (v:v) glycerol solution.

Fungicide formulations

Table 1 shows the active ingredients (a.i.), concentrations, commercial product names and manufacturers of the fungicide formulations used in this study. All fungicides (azoxystrobin, boscalid, cyprodinil and fludioxonil) used in *in vitro* mycelium growth tests were pure technical grades, supplied by Syngenta Crop Protection (Greece), BASF (Greece), or purchased from Sigma-Aldrich. Standard stock solutions of the fungicides were made in appropriate

organic solvents at various concentrations and stored at -20°C. All fungicides were added aseptically from the stock solutions to sterilized growth media prior to inoculation with fungus strains, and the final amount of solvent did not exceed 1% (v:v) in treated samples.

The commercial fungicide products were applied to maize seeds and plants at the greatest dosages recommended by the respective manufacturers. These were 1.2 g L^{-1} for Cantus® (carboxamide group), 1 g L^{-1} for Geoxe® (phenylpyrrole), 0.5 g L^{-1} for Chorus® (annilopyrimidine), 1 g L^{-1} for Quadris (strobilurin) and e) 1 g L^{-1} for Switch (formulated mixture cyprodinil + fludioxonil).

Mycelium growth and conidium germination of *Aspergillus flavus* as affected by fungicides

Inhibition of the three aflatoxigenic *A. flavus* strains was determined using discriminatory concentrations of the four tested fungicide active ingredients, and was based on calculation of effective concentration of each fungicide causing 50% inhibition of mycelium growth or conidium germination (EC_{50}s). Boscalid was tested at 1, 10 or 50 µg mL^{-1}; cyprodinil at 0.01, 0.1, 1, 10 or 50 µg mL^{-1}; fludioxonil at 0.1, 10 or 50 µg mL^{-1}; and azoxystrobin at 0.05, 0.1, 0.5, 1 or 5 µg mL^{-1}. Czapek-dox agar (CZA) amended with these fungicide amounts was used for fungitoxicity tests. Inoculum, consisting of 2 mm diam. mycelium plugs, cut from the edges of actively growing colonies of 5-d-old *A. flavus*, grown on water agar, was transferred to the centres of prepared fungicide-amended CZA Petri plates for radial growth measurements. Cultures were then incubated at 28°C in the dark for 3 d. Assessments of colony growth were carried out

Table 1. Fungicides used in this study, their active ingredients, formulations, applied doses and manufacturers.

Commercial fungicide name	Active ingredient, and formulation	Applied dose[a]	Manufacturer
Cantus®	Boscalid, 50% wettable granule (WG)	1.2 g L^{-1}	BASF
Geoxe®	Fludioxonil, 50% WG	1 g L^{-1}	Syngenta
Chorus®	Cyprodinil, 50% WG	0.5 g L^{-1}	Syngenta
Quadris®	Azoxystrobin, 25% Suspension Concentrate	1 g L^{-1}	Syngenta
Switch®	Cyprodinil, 37.5% + Fludioxonil 25%	1 g L^{-1}	Syngenta

[a] Greatest manufacturer's recommended dose.

daily by measuring two diameters of each growing colony until the fungi in the untreated control treatments covered the medium surfaces. The mean colony diameter was expressed as percentage of the mean diameter of the untreated experimental controls. This colony growth experiment was conducted in triplicate for each *A. flavus* strain, and was repeated at least twice to detect variations in the sensitivity responses of the fungal isolates to the fungicides boscalid, cyprodinil or fludioxonil.

For the evaluation of the effect of azoxystrobin on conidium germination, 40 µL of inoculum (2×10^5 conidia mL^{-1}) was spread on CZA in Petri dishes, which were then incubated at 28°C in the dark. After 15h incubation, conidium germination was measured and expressed as percentage of the mean germination of the untreated experimental controls. The experiment was conducted in triplicate for each *A. flavus* strain.

Evaluation of fungicides for effects on *Aspergillus flavus* aflatoxin production and conidium production in maize seeds

The efficacy of the different commercial fungicides for inhibiting aflatoxin production on maize seeds was evaluated. Forty g of aflatoxin-free maize seeds (maize line N9, House of Agriculture Spirou, Athens, Greece) for each chemical compound were sterilized with 10% NaClO for 10 min, washed with distilled water, placed in 70% ethanol for 3 min, and then washed with ddH$_2$O. The seeds were then placed into 250 mL flasks containing each commercial fungicide at concentrations according to the manufacturer's recommendations (Table 1). Twenty-four h after the application of the fungicides, the seed lots were inoculated by dipping in inoculum of 10^6 conidia mL^{-1} of each *A. flavus* strain (the conidium suspensions were prepared in sterile ddH$_2$O containing 0.05 g L^{-1} Tween 80). The flasks were placed at 28°C in the dark for 13 d, to let the inoculated strains grow and produce aflatoxin B1 (AFB1). The flasks were shaken every 2–3 d to redistribute inoculum. The evaluation of fungicides for inhibition of AFB1 production was qualitatively determined by thin layer chromatography (TLC). The inoculated seeds were ground and homogenized using a grinder. Three g of each resulting fine powder was transferred into a 50 mL Falcon tube to which 5 mL of water amended with Tween 80 (0.01%) and 5 mL of acetone were consecutively added. The samples were shaken at 150 rpm for 10 min

and they kept still for 5 min at room temperature. Five mL of chloroform was then added to each sample and shaken at 150 rpm for 10 min. The samples were each passed through a filter paper and the resulting supernatant was collected into a new tube. The supernatant was then centrifuged at 3,000 rpm for 10 min and the lower phase was transferred into a new glass tube and kept under a fume hood to dry. The resulting extract was then re-dissolved with 100 µL of methanol, and 10 µL of the sample was spotted and separated on a TLC plate (TLC Silica gel 60, Merck) with diethylether–methanol–water (96:3:1, v/v), and determined using UV light at 254 nm (Scott, 1995). The AFB1 used as standard was purchased from Sigma-Aldrich.

For evaluation of fungicides for prevention of *A. flavus* conidium production, ten maize seeds from each treatment were sterilized (as above), and 24h later, were inoculated with a droplet of 10 µL of a suspension of 10^6 conidia mL^{-1} (in sterile ddH$_2$O containing 0.05 g L^{-1} Tween 80) of each *A. flavus* strain. After 5 d incubation at 28°C with 14 h light, conidium production was measured by counting numbers of conidia using a Neubauer haemocytometer. The experiment was repeated three times for each fungicide, with 30 replicated seeds for each *A. flavus* isolate.

Field experiments

Field experiments were carried out in experimental plots of the Agricultural University of Athens, Greece, during the summers 2014 and 2015. The maize line N9 was planted into three replicates, and each replicate included plots to which seven different treatments were applied. These were the fungicides azoxystrobin, boscalid, cyprodinil, fludioxonil and cyprodinil + fludioxonil, a positive experimental control (Control +; infected plants with no fungicide treatment), and a negative control (Control -; non-infected plants with no fungicide treatment). Ten maize plants were used for each treatment. Maize seeds were planted into each plot as 0.4 m long by 0.4 m wide strips, in completely randomized block designs, in irrigated fields. The fungicide treatments were applied twice to each trial, using a nozzle sprayer across the rows. The first application was carried out at full flowering and the second 1 week later. The inoculation of maize ears was performed with *A. flavus* strain A6.10, by injecting conidial suspensions (10^6 conidia mL^{-1} in sterile ddH$_2$O containing 0.05 g L^{-1} Tween 80), 2 d after the second fungicide application. *Aspergillus*

flavus inoculum was prepared by growing the strain on Petri plates containing malt extract agar for 6 d. All maize ears, except those treated with ddH$_2$O, were inoculated with 5 mL of conidium suspension, using a 10 mL capacity syringe with a needle. Three mL of the inoculum was injected through the silk into the top of each maize ear and two mL through the husk into the middle of the ear at each of four points. In Control - maize plants, sterile ddH$_2$0 was injected into the ears using the same method. After inoculation, the ears were each covered with a paper bag for 48h to maintain high humidity (Zummo and Scott, 1989).

Assessments of maize ear rot and aflatoxin analyses

Ear rot was evaluated at the end of each growing season (70–80 d after A. flavus inoculation), by estimating the percentage of the rotted kernels in each maize ear using a scale of 1 to 7, where 1 = 0%, 2 = 1–3%, 3 = 4–10%, 4 = 11–25%, 5 = 26–50%, 6 = 51–75%, and 7 = 76–100% of the kernels with fungal signs and spoilage symptoms (Reid et al., 1999). After harvest, maize ears were placed in separate bags and kernels were removed. Maize seeds were placed in a dryer so that the relative humidity reached 15–18%. The kernels were then homogenized using a grinder. Total aflatoxin content was determined for 40 g of each seed sample using the Agra-Quant® ELISA Aflatoxin kit, 4–40 ppb (Romer-Labs).

Statistical analyses

All experimental data were analyzed with SPSS statistical software (SPSS Inc.). For the laboratory experiments, analysis of variance (ANOVA) was used to determine the effects of replication, treatment and A. flavus strain, on conidium production on maize seeds. For the field experiments, ANOVA was used to determine the effects of replication, treatment, year and their interactions, on disease severity and aflatoxin production. When significant F-tests were obtained for treatments (P≤0.05), data were subjected to means separation by Tukey's honestly significant difference (HSD) test.

Results

Effects of fungicides on *in vitro* growth inhibition of *Aspergillus flavus* strains

Since no fungicides are available to control Aspergillus ear rot in maize, we assessed the effects of three active ingredients (boscalid, cyprodinil or fludioxonil), firstly on A. flavus mycelium growth on CZA, and secondly, of azoxystrobin on A. flavus conidium germination on water agar, since the azoxystrobin mode of action is mainly due to inhibition of conidium production. The effects of the three fungicides treatments on mycelium growth are shown in Table 2. Most of the fungicide rate treatments significantly reduced growth of the A. flavus strains. Cyprodinil and fludioxonil showed similar mycelium growth inhibition for all the three A. flavus strains, with cyprodinil being the most effective active ingredient. At the concentration of 0.1 μg mL^{-1}, cyprodinil gave 87–89% growth inhibition, and 90% inhibition at 10 μg mL^{-1}. Fludioxonil gave growth inhibition of 73–85% at 1 μg mL^{-1}and 80–86% at 10 μg mL^{-1}, for all three strains. On the other hand, boscalid was the least effective fungicide for inhibiting A. flavus mycelium growth. For this fungicide, growth inhibition percentages for all A. flavus strains were from 1–5% at 1 μg mL^{-1} and from 16–23% at 10 μg mL^{-1}. Azoxystrobin almost completely inhibited (98–100%) conidium germination at 1 and 5 μg mL^{-1}, at 0.5 μg mL^{-1} inhibition was 93–95%, at 0.1 μg mL^{-1} from 57–67%, and at 0.05 μg mL^{-1} from 33–43% (Table 3). Figure 1 shows that EC$_{50}$ values were low for cyprodinil (0.03–0.05), fludioxonil (0.06–0.11) and azoxystrobin (0.07–0.12), but much greater for boscalid (4.35-4.50), for the three A. flavus strains.

Effectiveness of the fungicides on *Aspergillus flavus* sporulation and aflatoxin B1 production in maize seeds

The next experiment evaluated the commercial fungicide formulations of azoxystrobin, boscalid, cyprodinil or fludioxonil on A. flavus *in vitro* growth, and conidium and aflatoxin production in maize seeds. All the tested fungicides inhibited conidium production of the three A. flavus strains, with statistically significant differences compared to Control+ treatment (Figure 2). Specifically, cyprodinil treatment reduced A. flavus conidium production (on average for the three strains) by 83.4%, fludioxinil by 75.8%, azoxystrobin by 79.6%, boscalid by 85.4% and cyprodinil + fludioxinil by 93.8%. Among the three isolates, there was no difference after the application of each chemical formulation, except for the Control+ where isolate D1.3 sporulated more profusely compared to the other two isolates (Figure 2).

Table 2. Mean colony radial growth inhibition (%) for the fungicides fludioxonil, cyprodinil or boscalid at different concentrations for three toxigenic *Aspergillus flavus* strains.

Fungicide	Concentration (µg mL^{-1})	Growth inhibition (%)[b]		
		A 6.10[a]	12S[a]	D 1.3[a]
Boscalid	1	1.09 ± 0.11 a	4.55 ± 0.08 a	2.20 ± 0.02 a
	10	1.10 ± 0.14 a	22.73 ± 0.05 b	16.48 ± 0.02 b
	50	21.98 ± 0.01 ab	31.82 ± 0.06 b	17.58 ± 0.03 b
Fludioxonil	0.1	59.34 ± 0.06 a	48.86 ± 0.06 a	59.34 ± 0.03 a
	1	84.62 ± 0.03 b	72.73 ± 0.03 b	79.12 ± 0.02 b
	10	83.52 ± 0.03 b	80.68 ± 0.03 b	80.22 ± 0.01 b
	50	87.91 ± 0.08 b	77.78 ± 0.05 b	87.91 ± 0.01 b
Cyprodinil	0.01	49.45 ± 0.03 a	65.91 ± 0.03 a	65.93 ± 0.04 a
	0.1	89.01 ± 0.03 b	87.64 ± 0.03 b	86.81 ± 0.02 b
	1	89.05 ± 0.03 b	88.50 ± 0.01 b	89.01 ± 0.01 b
	10	90.11 ± 0.04 b	89.51 ± 0.02 b	90.02 ± 0.01 b
	50	91.82 ± 0.06 b	90.57 ± 0.03 b	90.21 ± 0.02 b

[a] Aflatoxigenic strains of *A. flavus*.
[b] Statistically significant differences for each condition are indicated with different lower-case letters, by analysis of variance (ANOVA) and Tukey's test ($P \leq 0.05$).

Table 3. Mean inhibition proportions (%, ± standard errors) of conidium germination for three toxigenic strains of *Aspergillus flavus*, resulting from treatments with different concentrations of azoxystrobin.

Fungicide	Concentration (µg mL^{-1})	Conidium germination inhibition (%)[b]		
		A6.10[a]	12S[a]	D1.3[a]
Azozystrobin	0.05	34.54 ± 5.63 a	43.23 ± 6.81 a	32.57 ± 5.24 a
	0.1	67.23 ± 4.63 b	59.25 ± 4.57 b	57.12 ± 2.94 b
	0.5	93.28 ± 2.01 c	93.17 ± 1.23 c	95.36 ± 1.68 c
	1	99.27 ± 0.72 c	100.00 ± 0.00 c	100.00 ± 0.00 c
	5	100.00 ± 0.00 c	98.03 ± 1.17 c	100.00 ± 0.00 c

[a] Aflatoxigenic strains of *A. flavus*
[b] Statistically significant differences for each condition are indicated with different letters, as shown by analysis of variance and Tukey's test ($P \leq 0.05$).

Evaluation of the different fungicides for effects on AFB1 mycotoxin production, using TLC, showed that mycotoxin extracted from maize seeds was reduced by the fungicides. Cyprodinil + fludioxinil completely inhibited ABF1 production by all three *A. flavus* strains compared to the Control+ treatment (Figure 3). Azoxystrobin greatly reduced AFB1 production by strains D1.3 and A6.10, and inhibited production by strain 12S. Fludioxinil inhibited AFB1 production by strains D1.3 and A6.10 and greatly reduced production by strain 12S. Cyprodinil inhibited AFB1 production by strains 12S and A6.10, but not by
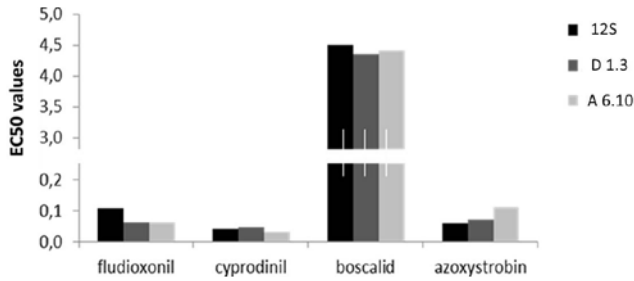
Figure 1. EC$_{50}$ values for fludioxonil, cyprodinil or boscalid for inhibition of mycelium growth, and for azoyzstrobin for inhibition of conidium germination, of *Aspergillus flavus* strains 12S, D1.3 and A6.10.

Figure 2. Mean numbers of conidia produced by *Aspergillus flavus* strains D1.3, A6.10 and 12S in maize seeds treated with different fungicides. Within treatments, columns accompanied by different lower case letters are significantly different ($P \leq 0.05$) among strains, and upper case letters indicate differences ($P \leq 0.05$) between the fungicides. Vertical bars indicate standard errors of the means.

strain D1.3. Boscalid inhibited AFB1 production by strains A6.10 and 12S, and slightly decreased production by strain D1.3. Cyprodinil + fludioxinil strongly inhibited production of the mycotoxin by all three *A. flavus* strains.

Of the three *A. flavus* strains, growth of D1.3 (isolated from pistachios) was the least sensitive to the fungicides, and mycotoxin production by this strain was also the least affected by the fungicides, except for cyprodinil + fludioxinil.

Field assessments of fungicides for control of ear rot symptoms and aflatoxin production

Analysis of variance revealed that neither year nor interactions between year and other experimental factors significantly affected ear rot severity or aflatoxin production, so data from the two years (2014 and 2015) were combined and are presented here (Figure 4). Maize plants treated with cyprodinil + fludioxinil showed significantly less ear rot severity (by 40%) compared to the Control + plants. In contrast, the other four fungicides were not very effective in reducing ear rot severity, which ranged for both years between 5–10% of infected corn kernels/ear (Figure 4). For these four fungicides, the lack of substantial decrease in ear rot symptoms was not correlated with the reductions of total aflatoxins produced in maize kernels (Figure 5). Boscalid decreased grain aflatoxin content by 74%, azoxystrobin by 67%, cyprodinil by 75%, fludioxonil by 80% and cyprodinil + fludioxinil by 83%.

In conclusion, cyprodinil + fludioxinil was able to significantly lower ear rot severity and total aflatoxin contamination in maize under a high *A. flavus* conidium inoculum pressure per plant. These field results
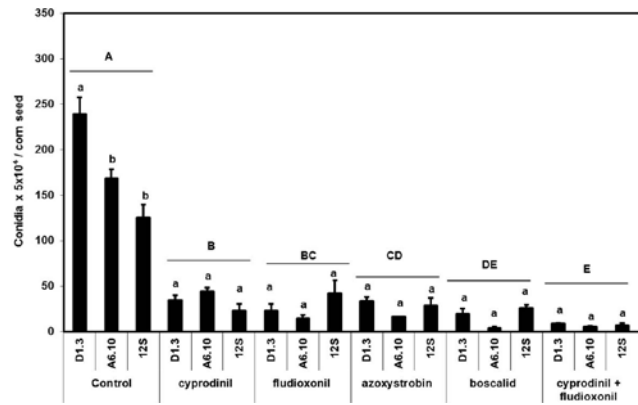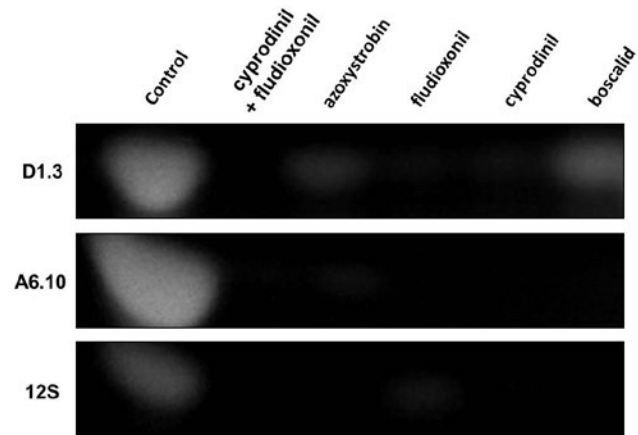

Figure 3. Thin layer chromtography assays of the effectiveness of five fungicides on aflatoxin production by *Aspergillus flavus* strains D1.3, A6.10 and 12S.

are in agreement with the reductions in aflatoxin contamination that the four fungicides caused in the *in vitro* maize seed tests. The data from this study can potentially provide farmers with a new chemical approach to control of *A. flavus* and aflatoxin production in the framework of an Integrated Pest Management (IPM) strategy.
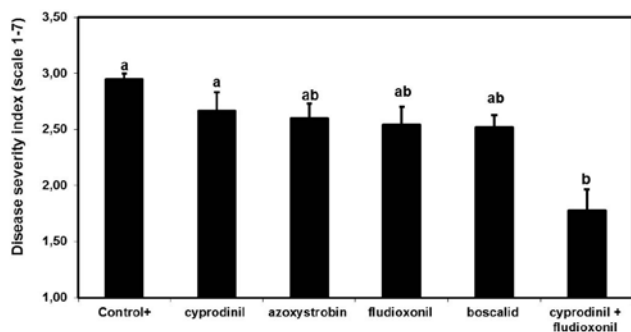
Figure 4. Mean Aspergillus ear rot severity indices on field-grown maize plants artificially inoculated with *Aspergillus flavus* strain A6.10, and treated with different fungicides. Columns followed by different letters are significantly different ($P \leq 0.05$), according to Tukey's HSD test. Vertical bars indicate standard errors of the means.
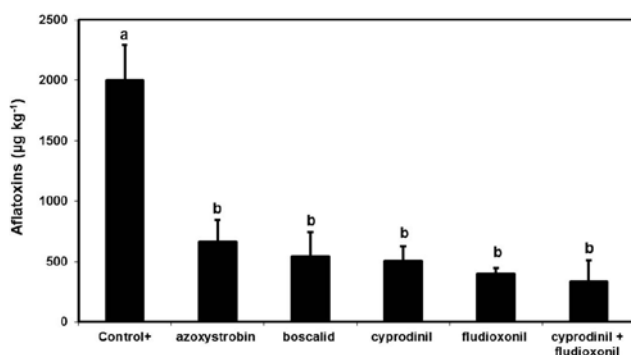

Figure 5. Mean aflatoxins contents ($\mu g\ kg^{-1}$) in maize kernels from field grown plants that were artificially inoculated with *Aspergillus flavus* strain A6.10 and treated with different fungicides. Columns accompanied by different letters are significantly different ($P \leq 0.05$) according to Tukey's HSD test. Vertical bars indicate standard errors of the means.

Discussion

Mycotoxins, and particularly aflatoxins, are one of the major threats to food quality and safety of the human population worldwide. There is continuous risk of mycotoxins "from the farm to the fork". Accumulation of mycotoxins in crops is increasing worldwide possibly due to climatic changes, the use of plant varieties with high yield but which are susceptible to mycotoxin accumulation, and new agricultural practices such as reduced tillage (Chulze, 2010;

Battilani *et al.*, 2012). European Union legislation is very strict regarding the presence of mycotoxins in various products, and emphasizes the importance of development of effective methods for reducing mycotoxin contamination. Economically effective solutions are those that, with the help of agricultural precision technology and sustainable IPM strategies, may contribute to the exclusion of mycotoxigenic fungi from plant hosts and/or the restriction of mycotoxin contamination of crop products either at pre- or post-harvest. It is also foreseen that climate conditions and/or production practices may have impacts, by favouring growth of mycotoxigenic fungi and consequently mycotoxin contamination (Eldayne *et al.*, 2009; Russell *et al.*, 2010).

Several previous field trails have demonstrated that fungicides, including organophoshorus fungicides, thiabendazole, triadimefon, propiconazole, tolclofos-methyl can be used to control mainly *Fusarium* mycotoxin formation, with the timing of pesticide treatments being particularly important for controlling mycotoxin production (D'Mello *et al.*, 1998; EC report 1999; Cowger *et al.*, 2016). Our studies examined the effects of four fungicide active ingredients for control of aflatoxigenic *A. flavus* strains. The fungicides used in this study are extensively used antifungal compounds applied in agriculture to control different fungal pathogens in different crops. Most of the fungicides tested reduced the growth of all three *A. flavus* strains. The exception was boscalid that did not inhibit growth of the strains. Previous studies have also shown that pyrimidine and phenylpyrrole fungicides can inhibit mycelial growth rates of mycotoxigenic aspergilli such as *A. parasiticus* and *A. carbonarius* (Tjamos *et al.,* 2004; Belli *et al.,* 2006; Markoglou *et al.,* 2008). Those results agree with those obtained in the present study, where $0.1\ \mu g\ mL^{-1}$ of cyprodinil reduced growth of the three *A. flavus* strains by 87–89%, and $1\ \mu g\ mL^{-1}$ fludioxonil reduced growth by 73–85%. These substances were also effective against *Penicillium digitatum* (Kanetis *et al.,* 2008). Sakuda *et al.,* (2014) demonstrated that strobilurins were effective for reducing AFB1 accumulation, also verified in the present study, where azoxystrobin at $0.5\ \mu g\ mL^{-1}$ inhibited conidium germination of *A. flavus* by 93–95%.

All of the tested fungicide products effectively inhibited conidium production (by 83-94%) and aflatoxin development for all three *A. flavus* strains in maize seeds. The product containing cyprodinil + fludioxinil gave strong inhibition of aflatoxin contamination

of maize seeds for all the tested strains (Figure 3), and no statistically significant difference was detected for reduction due to this product for conidium production in maize seeds by all three strains. In contrast, the three strains demonstrated different responses in aflatoxin inhibition resulting from the four fungicides, indicating the possible involvement of differential genetic factors among the three strains that may be involved in aflatoxin regulation. The boscalid-based product inhibited conidium and aflatoxin production in maize seeds by all three toxigenic strains, but did not affect *in vitro* mycelium growth. The succinate dehydrogenase inhibitor (SDHI) action of boscalid has direct effects on mould fungi by interfering with conidium production, inhibiting mitrochondrial respiration and the subsequent production of ATP in fungal cells (Sierotzki and Scalliet, 2013).

In the field experiments, maize plants treated with cyprodinil + fludioxinil had 40% less severe ear rot severity from *A. flavus* infection compared with the Control+ plants. The other four single ingredient fungicides were not very effective for reducing maize ear rot. However, all the tested chemicals decreased aflatoxin contamination in maize seeds from 67% (from azoxystrobin treatments) to 83% (from cyprodinil + fludioxinil). The two single ingredient products containing cyprodinil (Chorus®) or fludioxonil (Geoxe®) (fludioxonil) also decreased aflatoxin production in maize ears by, respectively, 75% and 80%. Boscalid also decreased aflatoxin content in the maize seed by 74%. Aflatoxin contamination levels of maize kernels were high in these field trials because of the artificial inoculation of *A. flavus* into maize ears, and the optimum warm and dry weather conditions prevailing during the field trials. Although ear rot severity in most treatments was between 1 and 10%, aflatoxin contamination levels were high (200–2000 µg kg^{-1}). To our knowledge, this is the first study that demonstrates efficient chemical control of *A. flavus* growth and aflatoxin accumulation in maize fields.

Previous 3-year field studies in the Mississippi Delta (USA), indicated that none of the fungicides azoxystrobin, pyraclostrobin, propiconazole, tetraconazole, dithiocarbamate or fungicide mixtures of trioxystrobin + propiconazole and azoxystrobin + propiconazole, applied to maize at the mid-silking growth stage, were effective for reducing aflatoxin contamination (Abbas *et al.*, 2009). These data indicate that the number of fungicide applications, as well as the plant growth stage or other agronomic or environmental factors can, play crucial roles in the chemical control of aflatoxins. Tjamos *et al.* (2004) demonstrated that two spray applications of cyprodinil + fludioxinil (the first 1 week before veraison and 2 weeks before harvesting) effectively reduced the incidence of Aspergillus sour rot in raisin- and wine-producing vineyards in Greece. In other experiments on grapevine, a mixture of cyprodinil (37.5%) and fludioxonil (25%) was the most effective fungicide formulation for control of *A. carbonarius* growth and ochratoxin mycotoxin production, together with penconazole (10%) and azoxystrobin (25%). Molot and Solanet (2003), demonstrated that the fungicide products Switch®, Scala® (pyrimethanil) and Mikal® (fosetyl-Al + folpet) were the most effective for reducing fungal colonization and OTA content of wines. Considering *Aspergillus* section *Flavi*, conventional methods of plant disease control using fungicides (benomyl, thiabendazole, carboxin) were reported to be ineffective in maize when applied at environmentally safe concentrations (Bhatnagar *et al.*, 1993). However, in some *in vitro* studies, prochloraz, prothioconazole, tebuconazole or imazalil were effective for reducing growth and aflatoxin formation by *A. flavus* and / or *A. parasiticus* (Delen and Tosun, 1999; Formenti *et al.*, 2012).

In our study, the strobilurin fungicide azoxystrobin also decreased aflatoxin content in maize kernels by 67%. In the USA, azoxystrobin is a registered fungicide for use in maize crops. According to the manufacturer, early-season applications of azoxystrobin provide preventive control of several maize diseases (rusts, anthracnose, leaf blight, gray leaf spot, Northern corn leaf blight, Northern corn leaf spot, Southern corn leaf blight), and enhanced plant performance, while the maize leaf and ear shoots are developing at the early vegetative growth stages. Strobilurins or quinone-outside inhibitor fungicides (Qols) are widely marketed for maize production, for the management of biotic stresses, with the suggestion that these fungicides can increase yields even in the absence of disease (Hershman *et al.*, 2011; Wise and Mueller, 2011). In plants, Qol fungicides may increase water and nitrogen use efficiency, improve chlorophyll retention and delay senescence, thus lengthening crop growth periods (Janse van Rensburg *et al.*, 2016). Based on the data from the present study, azoxystrobin could also contribute to *A. flavus* and aflatoxin control in countries where this fungicide is officially registered for use in maize.

Results obtained from *in vitro* experiments with different fungicides are not always similar to those obtained in field trials, due to the influence of several factors. It is essential to consider that the most efficient fungicide to reduce the ear rot severity caused by *A. flavus* is not always the optimum choice because it can promote aflatoxin production. Even if fungal growth is reduced, aflatoxin production could still be promoted. Thus, the best fungicides are those that prevent or reduce fungal growth and mycotoxin production at the same time (Santos *et al.*, 2011).

The results from this study have demonstrated that chemical control can be an important tool for the management of aflatoxins in maize. Prevention of growth of mycotoxin-producing fungi can be the most effective strategy for controlling the presence of fungi and mycotoxins in crops, as was suggested by the Codex Alimentarius Commission in Good Agricultural Practices (GAP) (CAC/RCP 51, 2003). However, the greatest recommended field application rates by the manufacturers may be required (as used in the present study), especially under abnormal climatic conditions of water or heat stress to maize crops. Optimization of fungicide doses in field treatments is necessary to ensure efficacy of fungicides, which can be influenced by many agronomic (e.g. plant nutrition) and environmental variables (e.g. temperature and humidity), cultivar response, sensitivity of conidia to antifungal compounds, persistence of fungicides on plant tissues, dynamics and extent of translocation of different systemic fungicide compounds, therapeutic effects, and fungicide resistance in relation to mycotoxin production (Subhani *et al.*, 2011; Ivic *et al.*, 2012). Drought and heat stress during the time that maize crops mature are the principle contributing factors to aflatoxin contamination of grain. Climate change is causing insecurity about future temperature and rainfall regimes. Southern Europe and the Mediterranean basin are the most vulnerable areas in Europe, because of the combined climate change effects of increased temperature and reduced precipitation. The European Commission suggests that, in southern Europe, climate changes may lead to temperature increases of 4–5°C, in combination with increased drought periods (Garcia Cela *et al.*, 2011; Battilani *et al.*, 2012).

In conclusion, reliance on fungicides will not eliminate aflatoxin production in different agro-climatic regions. Fungicides, biological disease control, insect control and novel disease resistant germplasm, in combination with disease forecasting models and decision support systems will create an Integrated Pest Management strategy likely to give greatest promise for mitigating *Aspergillus* infection and aflatoxin contamination in different crops.

Acknowledgements

Literature cited

Abbas H.K., J.R. Wilkinson, R.M. Zablotowicz, C. Accinelli, C.A. Abel, H.A. Bruns and M.A. Weaver, 2009. Ecology of *Aspergillus flavus*, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. *Toxin Reviews*, 28, 142–153.

Badii F. and M.O. Moss, 1988. The effect of the fungicides tridemorph, fenpropimorph and fenarimol on growth and aflatoxin production by *Aspergillus parasiticus* Speare. *Letters in Applied Microbiology*, 7, 37–39.

Battilani P., C. Barbano and G. Piva, 2008. Aflatoxin B1 contamination in maize related to the aridity index in North Italy. *World Mycotoxin Journal*, 1, 449–456.

Battilani P., V. Rossi, P. Giorni, A. Pietri, A. Gualla, H.J. Van der Fels-Klerx, C.J.H. Booij, A. Moretti, A. Logrieco, F. Miglietta, P. Toscano, M. Miraglia, B. De Santis and C. Brera, 2012. *Modelling, predicting and mapping the emergence of aflatoxins in cereals in the EU due to climate change*. EFSA Scientific Report, EFSA Supporting Publications.

Belli N., S. Marin, V. Sanchis and A.J. Ramos, 2006. Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes. Food *Additives and Contaminants*, 23, 1021–1029.

Bennett J.W. and M. Klich, 2003. Mycotoxins. *Clinical Microbiology Reviews*, 16, 497–516.

Bhatnagar D., P.J. Cotty and T.E. Cleveland, 1993. Pre-harvest aflatoxin contamination: molecular strategies for its control. In: *Flavour and Safety: Molecular Analysis and Design* (A.M. Spanier, N. Okai, N. Tamura, ed.), American Chemical Society, Washington D.C., 272–292.

Brent K.J. and D.W. Hollomon, 2007. *Fungicide resistance in crop pathogens: how it can be managed?* 2nd ed. Fungicide Resistance Action Committee, Brussels, Belgium, 1–56 pp.

CAC/RCP 51-2003. Code of practice for the prevention and reduction of mycotoxin contamination in cereals.

CAST, 2003. *Mycotoxins: Risk in Plant, Animal and Human Systems*. Ames, Iowa, USA

Chu F.S., 2002. Mycotoxins. In: *Foodborne Diseases*, 2nd edition (D.O. Cliver, H.P. Riemann, ed.), Academic Press, London, UK, 271–303.

Chulze S.N., 2010. Strategies to reduce mycotoxin levels in maize during storage: a review. *Food Additives and Contaminants,* 27, 651–657.

Cowger C., R. Weisz, C. Arellano and P. Murphy, 2016. Profitability of integrated management of Fusarium Head Blight in North Carolina winter wheat. *Phytopathology*, 106, 814–823.

D'Mello J.P.F., A.M.C. MacDonald, D. Postel, W.T.P. Dijksma, A. Dujardin and C.M. Placinta, 1998. Pesticide use and mycotoxin production in *Fusarium* and *Aspergillus* phytopathogens. *European Journal of Plant Pathology*, 104, 741–751.

Delen N. and N. Tosun, 1999. Effects of some DMI's on fungal growth and aflatoxin production in aflatoxigenic fungi. *Journal of Turkish Phytopathology*, 28, 35–43.

Doukas E.G., A. N. Markoglou, J.G. Vontas and B.N. Ziogas, 2012. Effect of DMI-resistance mechanisms on cross-resistance patterns, fitness parameters and aflatoxin production in *Aspergillus parasiticus* Speare. *Fungal Genetics and Biology,* 49, 792–801.

EC report (European Commission report) 1999. Opinion on the relationship between the use of plant protection products on food plants and the occurrence of mycotoxins in foods, European Commission SCP/RESI/063, Belgium.

Edlayne G., A. Simone and J.D. Felicio, 2009. Chemical and biological approaches for mycotoxin control: a review. *Recent Patents on Food, Nutrition & Agriculture,* 1, 155–161.

Formenti S., N. Magan, A. Pietri and P. Battilani, 2012. *In vitro* impact on growth, fumonisins and aflatoxins production by *Fusarium verticillioides* and *Aspergillus flavus* using antifungal compounds and a biological control agent. *Phytopathologia Mediterranea*, 51, 247–256.

García-Cela E., A.J. Ramos, V. Sanchis and S. Marin, 2012. Ochratoxigenic moulds and effectiveness of grape field antifungals in a climatic change scenario. *Journal of the Science of Food and Agriculture*, 92, 1455–1461.

Giorni P., N. Magan, P.A. Bertuzzi and P. Battilani, 2007. Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. *International Journal of Food Microbiology*, 113, 330–333.

Hershman D.E., P. Vincelli and C.A. Kaiser 2011. *Foliar Fungicide Use in Corn and Soybeans.* University of Kentucky College of Agriculture, Food and Environment, Plant pathology fact sheet. PPFS-MISC-05. https://plantpathology.ca.uky.edu/files/ppfs-gen-12.pdf.

Horn B.W., 2007. Biodiversity of *Aspergillus* section *Flavi* in the United States: a review. *Food Additives & Contaminants*, 24, 1088–1101.

IARC, 1993. Monographs on the evaluation of carcinogenic risks to humans. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins, International Agency for Research on Cancer, Lyon, France

Ioos R., A. Belhadj, M. Menez and A. Faure, 2005. The effects of fungicides on *Fusarium* spp. and *Microdochium nivale* and their associated trichothecene mycotoxins in French naturally-infected cereal grains. *Crop Protection*, 24, 894–902.

Ivić D., Z. Sever and B. Kuzmanovska, 2012. *In vitro* sensitivity of *Fusarium graminearum*, *F. avenaceum* and *F. verticillioides* to carbendazim, tebuconazole, flutriafol, metconazole and prochloraz. *Pesticides and Phytomedicine*, 26, 35–42.

Janse van Rensburg B., N.W. McLaren, A. Schoeman and B.C. Flett, 2016. The effects of cultivar and prophylactic fungicide spray for leaf diseases on colonisation of maize ears by fumonisin producing *Fusarium s*pp. and fumonisin synthesis in South Africa. *Crop Protection*, 79, 56–63.

Kanetis L., H. Förster, C.A. Jones, K.A. Borkovich and J.E. Adaskaveg, 2008. Characterization of genetic and biochemical mechanisms of fludioxonil and pyrimethanil resistance in field isolates of *Penicillium digitatum*. *Phytopathology*, 98, 205–214.

Markoglou A.N., E.G. Doukas and B.N. Ziogas, , 2008. Phenylpyrrole-resistance and aflatoxin production in *Aspergillus parasiticus* Speare. *International Journal of Food Microbiology*, 127, 268–275.

Markoglou A.N., E.G. Doukas and B.N. Ziogas, 2011. Effect of anilinopyrimidine resistance on aflatoxin production and fitness parameters in *Aspergillus parasiticus* Speare. *International Journal of Food Microbiology*, 146, 130–136.

Mateo E.M., J.V. Gómeza, J.V. Gimeno-Adelantado, D. Romera, R. Mateo- Castro and M. Jiménez, 2017. Assessment of azole fungicides as a tool to control growth of *Aspergillus flavus* and aflatoxin B1 and B2 production in maize. *Food Additives & Contaminants*, 34, 1039–1051.

Matthies A. and H. Buchenauer, 2000. Effect of tebuconazole (Folicur®) and prochloraz (Sportale®) treatments on Fusarium head scab development, yield and deoxynivalenol (DON) content in grains of wheat following artificial inoculation with *Fusarium culmorum*. *Journal of Plant Diseases and Protection*, 107, 33–52.

Mauro A., E. Garcia-Cela, A. Pietri, P.J. Cotty and P. Battilani, 2018. Biological control products for aflatoxin prevention in Italy: commercial field evaluation of atoxigenic *Aspergillus flavus* active ingredients. *Toxins,* 10(1), 30; https://doi.org/10.3390/toxins10010030.

Molot B. and D. Solanet, 2003. Ochratoxine: Prevention du risque. Etude au vignoble de fongicides actifs contre *Aspergillus carbonarius* incidences sur la presence aux vendanges. *Proceedings of Les Entretiens Viti-Vinicoles Rhone-Mediterranée*, Nimes, France, 18–21.

O'Donnell K., 2000. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia*, 92, 919–938.

Popovic R., B. Radovanov and J.W. Dunn, 2017. Food scare crisis: the effect on Serbian dairy market. *International Food and Agribusiness Management Review,* 20, 113–127.

Pirgozliev, S.R., S.G. Edwards, M.C. Hare and P. Jenkinson, 2002. Effect of dose rate of azoxystrobin and metconazole on the development of Fusarium head blight and the accumulation of deoxynivalenol (DON) in wheat grain. *European Journal of Plant Pathology,* 108, 469–478.

Reid L.M., R.W. Nicol, T. Ouellet, M. Savard, J.D. Miller, J.C. Young, D.W. Stewart and A.W. Schaafsma, 1999. Interaction of *Fusarium graminearum* and *F. moniliforme* in maize ears: Disease progress, fungal biomass, and mycotoxin accumulation. *Phytopathology,* 89, 1028–1037.

Russell R., M. Paterson and N. Lima, 2010. How will climate change affect mycotoxins in food? *Food Research International,* 43, 1902–1914.

Sakuda S., D.F. Prabowo, K. Takagi, K. Shiomi, M. Mori, S. Omura and H. Nagasawa, 2014. Inhibitory effects of res-

piration inhibitors on aflatoxin production. *Toxins*, 6, 1193–1200.

Santos L., S. Marin, V. Sanchis and A.J. Ramos, 2011. *In vitro* effect of some fungicides on growth and aflatoxins production by *Aspergillus flavus* isolated from *Capsicum powder*. *Food Additives and Contaminants,* 28, 98–106.

Schmale D.G. and G.P. Munkvold, 2009. Mycotoxins in Crops. The Plant Health Instructor. DOI: 10.1094/PHI-I-2009-0715-01.

Scott P.M., 1995. Natural toxins. In: *The Official Methods of Analysis of AOAC International* (P.A. Cunniff ed.), Gaithersburg, Maryland, USA, 1–51.

Shin J.H., J.H. Han, J.K. Lee and K.S. Kim, 2014. Characterization of the maize stalk rot pathogens *Fusarium subglutinans* and *F. temperatum* and the effect of fungicides on their mycelial growth and colony formation. *Plant Pathology Journal*, 30, 397–406.

Sierotzki H. and G. Scalliet, 2013. A review of current knowledge of resistance aspects for the next-generation Succinate Dehydrogenase Inhibitor fungicides. *Phytopathology*, 103, 880–887

Smith J.E., 1997. Aflatoxins. In: *Handbook of Plant and Fungal Toxicants* (J.F.P. D'Mello ed.), CRC Press, Boca Raton, NY, 269–285.

Subhani M.N., S.T. Sahi, S. Hussain, A. Ali, J. Iqbal and K. Hameed, 2011. Evaluation of various fungicides for the control of gram wilt caused by *Fusarium oxysporum* f. sp. *ciceris*. *African Journal of Agricultural Research*, 6, 4555–4559.

Tjamos S.E., P.P. Antoniou, A. Kazantzidou, D.F. Antonopoulos, I. Papageorgiou and E.C. Tjamos, 2004. *Aspergillus niger* and *Aspergillus carbonarius* in Corinth raisin and wine-producing vineyards in Greece: Population, composition, ochratoxin A production and chemical control. *Journal of Phytopathology,* 152, 250–255.

Wise K. and D. Mueller, 2011. Are fungicides no longer just for fungi? An analysis of foliar fungicide use in corn. APSnet Features. DOI: 10.1094/APSnetFeature-2011-0531.

Yoneyama K., S. Sekido and T. Misato, 1978. Studies on the fungicidal action of dithiocarbamates 2. Effect of sodium dimethyldithiocarbamate on the fatty acid synthesis of *Xanthomonas oryzae*. *Annals of the Phytopathological Society of Japan*, 44, 313–320.

Zummo N., and G.E. Scott, 1989. Evaluation of field inoculation techniques for screening maize genotypes against kernel infection by *Aspergillus flavus* in Mississippi. *Plant Disease*, 73, 313–316.