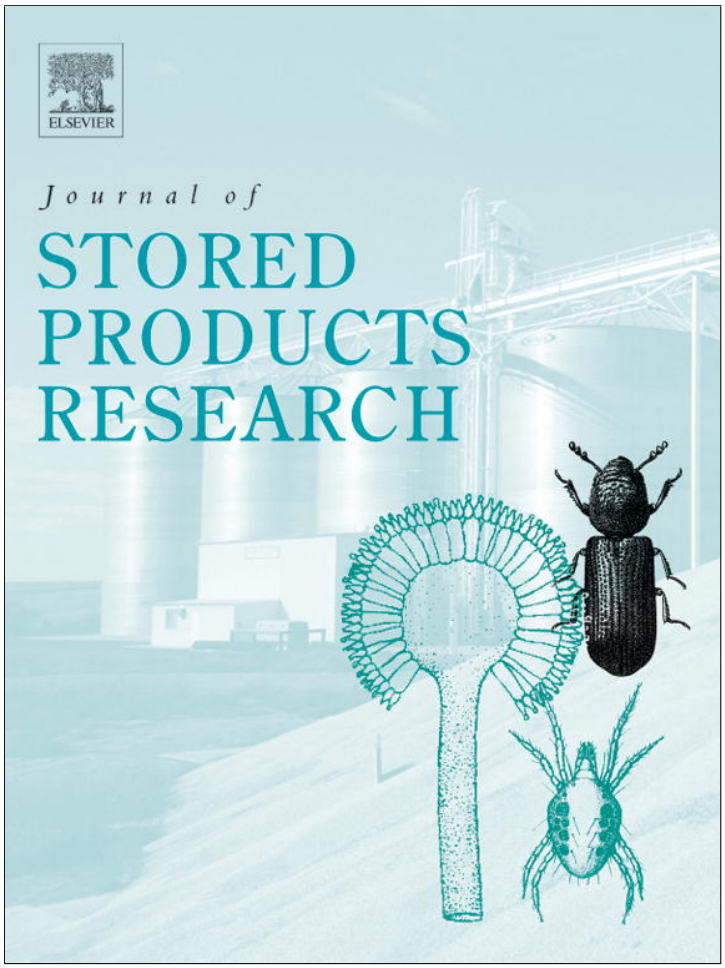

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In vitro compatibility of natural and food grade fungicide and insecticide substances with *Purpureocillium lilacinum* and their effect against *Aspergillus flavus*

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ABSTRACT

The objective of our study was to evaluate the compatibility of 21 *Purpureocillium lilacinum* strains with two benzoic acids 2(3)-tert-butyl-4 hydroxyanisole (BHA) and 2,6-di(tert-butyl)-p-cresol (BHT) and two phenolic acids 3-phenyl-2-propenoic acid (CA) and trans-4-hydroxy-3-methoxycinnamic acid (FA) applied alone or in combination, at different water activities. The impact of chemical substances and entomopathogenic fungi combination on growth parameters and toxin production of *Aspergillus* section *Flavi in vitro* was analyzed. BHT, the mixture BHA + BHT, CA and BHA did not significantly influence the count of viable spores of the 21 *P. lilacinum* strains. The strains JQ926202 and JQ926222 showed compatibility with the four treatments evaluated. The 21 *P. lilacinum* strains and the mixture of these entomopathogenic fungi with BHA (1 mM) increased the lag phase and inhibited the growth rate and aflatoxin B_1 production of *Aspergillus flavus* at three a_w assayed.

1. Introduction

The maize agroecosystem can be colonized by fungal communities at harvest and storage (Etcheverry et al., 1999; Nesci et al., 2008). Many species of fungi, such as *Aspergillus* section *Flavi*, potentially toxicogenic, are widely distributed in the non-rhizospheric soil, debris and insect of maize agroecosystem (Nesci and Etcheverry, 2002). Insects are involved on fungal grain colonization and can cause significant damage to stored maize. Major insects pest of stored maize include *Sitophilus zeamais* (Motschulsky), *Rhyzopertha dominica* (Fabricius) and *Tribolium confusum* (Jacquelin du Val) (Mejía, 2007). The constant migration of insect populations within the maize agroecosystem contributes to the dispersal of viable fungal spores of several species (Saint Geroges-Gridelet, 1984). A previous study conducted in stored maize showed a high percentage of live *S. zeamais* and *R. dominica* contaminated with *Aspergillus* section *Flavi* (Nesci et al., 2011). Therefore, insects could facilitate the dispersion of these fungi. The interaction between the substrate, and the biological and abiotic factors, may promote a moldy substrate and toxin accumulation in stored grains. The main toxins produced by same species of *Aspergillus* section *Flavi* are aflatoxins. These toxins are potent carcinogenic, teratogenic, mutagenic, hepatotoxic and immunosuppressive metabolites (IARC, 1993).

Since many insects carry *Aspergillus* section *Flavi* internally, they are important agents that increase the risk of aflatoxin contamination. Therefore strategies to control insects are needed. Current control measures for these pests rely heavily on the use of synthetic pesticides (Gurusubramanian et al., 2008). However, the indiscriminate use of these chemicals has given rise to a number of problems including resurgence of primary pests (Hazarika et al., 2009), secondary pest outbreak (Cranham, 1966), and resistance development (Kawai, 1997; Gurusubramanian et al., 2008; Roy et al., 2009). One alternative is the use of harmless substances like food grade antioxidants and natural phytochemicals. The effective insecticide concentrations range for these substances was from 20 to 30 mM (Nesci et al., 2011), similar to the effective fungicide concentrations of these chemical against *Aspergillus* section *Flavi* growth and aflatoxin production (Nesci et al., 2003; Nesci and Etcheverry, 2006; Nesci et al., 2007, 2008, 2009).

Another strategy in research is biological control of insect pests. Among the entomopathogenic organisms, fungi are the most

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widely distributed in agriculture (Fiedler and Sosnowska, 2007). Isolation of indigenous entomopathogenic fungi is essential to provide a pool of potential biological control agents. The isolation from the same ecosystem in which they must be effective is an advantage for pest control purposes (Lacey et al., 1996; Chandler et al., 1997). Therefore, we isolated and identified native entomopathogenic fungi from different components of maize agroecosystem. The main entomopathogenic genus isolated was *Purpureocillium*. All *Purpureocillium lilacinum* strains showed virulence against the most important insects pest in maize (Barra et al., 2013). These insects are vectors of aflatoxigenic fungi in this cereal (Nesci et al., 2011). *Purpureocillium lilacinum* (Thom) Samson is a soil saprophyte and has shown promise for use as a biocontrol agent (Morgan-Jones et al., 1984; Jatala, 1986; Dube and Smart, 1987; Khan et al., 2006).

However, entomopathogenic fungi cannot completely replace chemical insecticides in all agroecosystems. Insecticides generally are required to suppress the rapid expansion of pest populations. Strategies have been used to increase efficiency and accelerate insect mortality by combining entomopathogenic fungi with sublethal doses of chemical and botanical insecticides. A chemical substance and an entomopathogenic fungi are compatible when the doses of the chemical substance do not affect the viability, development and virulence of the fungi (Alves et al., 1998; Schumacher and Poehling, 2012). The application of selective insecticides has improved efficiency of the entomopathogenic fungi against insects pest (Quintela and McCoy, 1998; Dayakar et al., 2000; Serebrov et al., 2005; Purwari and Sachan, 2006). There are numerous examples where the application of sublethal doses of chemical pesticides has enhanced the efficacy of entomopathogens against insects pest. Sublethal doses of synthetic chemical insecticides may act as physiologic stressors and so predispose the insect to entomopathogenic fungus attack (Inglis et al., 2001).

The effective establishment of entomopathogenic fungi in the natural environment for control of pests must be considered as a practical problem. Ignorance of this knowledge could be a crucial factor, limiting the consistency of control in the real ecosystem. The fluctuation of water activity and temperature could have an impact on biocontrol effect. Hence, biological control fungi must be stable to fluctuating abiotic factors (Magan, 2001). Moreover, tolerance of entomopathogenic fungi to sublethal doses of synthetic chemical insecticides would allow us to produce entomopathogenic fungi that could compete in a natural storage environment.

The triple effect of losses due to insects, fungi, and mycotoxins can be devastating when conditions are favorable for maize deterioration. The combined use of different methods such as the use of biological control agents of insect vectors of aflatoxigenic fungi, plus the use of food grade antioxidants in sublethal doses, could help reduce aflatoxins in stored maize. The objective of this study was to evaluate the compatibility of *P. lilacinum* strains with different food grade antioxidants and natural phytochemicals at different water activities. The impact of the treatments with chemical substances and entomopathogenic fungi on the parameters of growth and toxin production of *Aspergillus* section *Flavi in vitro* was also evaluated.

2. Materials and methods

2.1. Entomopathogenic fungus

Twenty-one isolates identified as *P. lilacinum* were used in these experiments. These strains were originally isolated from soil samples collected from the University of Río Cuarto Experimental Field Station in Río Cuarto, Córdoba, Argentina and were identified and deposited in GenBank. All strains showed virulence against *T. confusum*, *R. dominica* and *S. zeamais*, three insect pest vectors of aflatoxigenic fungi (Barra et al., 2013). These strains are held in the Microbiology and Immunology Department Collection of the National University of Río Cuarto, Córdoba (Argentina).

2.2. Aflatoxigenic fungi

One isolates belonging to *Aspergillus* section *Flavi*, aflatoxigenic *Aspergillus flavus* (RCM89) was used in these experiments. This isolate was recovered from stored maize (Nesci et al., 2008) and identified according to Pitt and Hocking (1997), Klich and Pitt (1988) and Pitt (1988). The strain is held in the Microbiology and Immunology Department Collection of the National University of Río Cuarto, Córdoba (Argentina).

2.3. Insecticides and aflatoxigenic fungicides

Effective synthetic antioxidants and phenolic acids against insect vectors of *A. flavus* in maize microcosm (Nesci et al., 2011) were used at different doses to check that their not affect the spores viability of the entomopathogenic fungus. Moreover, these substances showed fungicide effect on *Aspergillus* section *Flavi* growth parameters and aflatoxin production in culture medium, sterilized grain and naturally contaminated grain (Nesci et al., 2003, 2007, 2009; Nesci and Etcheverry, 2006).

Industrial grade antioxidants, obtained from Eastman Chemical Company, were benzoic acid, 2(3)-tert-butyl-4 hydroxyanisole (BHA) and 2,6-di(t-butyl)-p-cresol (BHT). BHA had a purity of 98.5% containing as trace elements sulfated ash <0.01%, citric acid <2500 $\mu g\ g^{-1}$, arsenic <3 $\mu g\ g^{-1}$ and heavy metals <10 $\mu g\ g^{-1}$. BHT had a purity of 99% containing contaminants such as ash <0.02%, arsenic <3 $\mu g\ g^{-1}$ and heavy metals <10 $\mu g\ g^{-1}$. Contaminants from industrial grade antioxidants do not exceed levels allowed by the Expert Committee on Food Additives (JECFA) (1996). Both compounds were added alone (BHA: 1 mM [1.8 mg ml^{-1}], BHT: 7 mM [1.48 mg ml^{-1}]), and mixed (BHA: 0.7 mM + BHT: 6 mM [0.126 mg ml^{-1} + 1.27 mg ml^{-1}]). Stock solutions of BHA (0.18 g ml^{-1}) and BHT (0.22 g ml^{-1}) were prepared in 95% ethyl alcohol and the appropriate amount was added to maize meal extract agar (MMEA).

The phenolic acids used were CA: trans-cinnamic acid (3-phenyl-2-propenoic acid) and FA: ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid) and were obtained from Aldrich Chemical, Steinheim, Germany. Both compounds were added to medium at different doses alone (CA: 1 mM [0.14 mg ml^{-1}], FA: 7 mM [1.35 mg ml^{-1}]) and mixed (CA: 0.6 mM + FA: 2 mM [0.08 mg ml^{-1} + 0.39 mg ml^{-1}]). Stock solutions of CA (0.14 mg ml^{-1}) and FA (0.19 mg ml^{-1}) were prepared in 80% ethyl alcohol, and the appropriate amount was added to MMEA.

2.4. Entomopathogenic fungus spores viability assay

The compatibility of 21 *P. lilacinum* strains with the best dose of synthetic antioxidants and natural phytochemicals was evaluated according to the methodology described by Nesci et al. (2004). The basic medium used in this study was 3% MMEA. These media were made by boiling 30 g dry maize meal in 1 l distilled water for 60 min. The resulting mixture was filtering through a double layer of muslin. The volume was made up to 1 l with distilled water and the appropriate amount of agar was added (Marín et al., 1998). The water activity (a_W) of the basic medium (0.99) was adjusted to 0.97 and 0.95 by addition of glycerol (Dallyn and Fox, 1980). MMEA was autoclaved at 121 °C for 15 min before cooling to 50 °C and pouring into sterile plastic Petri dishes. The solutions of synthetic antioxidants and natural phytochemicals were added at different doses

alone and mixed to the MMEA. In the control treatment, the equivalent amount of ethyl alcohol was added, while in each treatment the amount of water was maintained.

Entomopathogenic fungi were grown on Potato Dextrose Agar (PDA) (dextrose 10 g l^{-1}, potato 200 g l^{-1}, agar 15 g l^{-1}) for 7 days at 25 °C to obtain heavily sporulating cultures. Fungal count was determined using serial dilutions from a spore suspension of 10^7 spores ml^{-1} and 0.1 ml aliquots of ten-fold dilutions were spread on the plates of different treatments. Petri plates of the same a_W values were sealed in polyethylene bags, and incubated for 25 days at 25 ± 1 °C. The fungal count as CFU ml^{-1} was determined in each treatment.

To assess the effect of chemical insecticides on the fungal count the viability of the entomopathogenic fungi spores, *in vitro* on solid culture media, was determined. The data collected were compared to controls to determine effect on spores viability. The treatments were grouped as compatible or non-compatible. In the compatible group, an enhancement of spore viability (>100%) over control was recorded, whereas that in non-compatible category, reduction in viability (<100%) over control was observed (Ganga Visalakshy et al., 2005).

2.5. Interaction between chemical substances and P. lilacinum strains on aflatoxigenic A. flavus growth and aflatoxin B_1 accumulation

The inhibitory activity on lag phase and growth rate of the synthetic antioxidants, natural phytochemicals and entomopathogenic fungus against *A. flavus* was tested. *A. flavus* strain was grown on malt extract agar (MEA) for 5 days at 30 °C to obtain heavily sporulating cultures. Entomopathogenic fungal suspensions were prepared following the procedure used for spores viability assay. The basic medium used in this assay was MMEA at three different a_w (0.99, 0.97 and 0.95). The assay consisted of evaluating the following interactions: *A. flavus* RCM89 + *P. lilacinum* strains; *A. flavus* RCM89 + BHA (1 mM) and *A. flavus* RCM89 + *P. lilacinum* strains + BHA (1 mM). For the first interaction each entomopathogenic fungal suspension with 10^7 spores ml^{-1} was pour-plated in 20 ml of MMEA. In the second interaction, MMEA medium was amended with BHA 1 mM, and in the last interaction the medium with BHA was poured into the Petri dishes which contained the suspension of *P. lilacinum*. In all treatments, after medium solidification, *A. flavus* RCM89 was spot inoculated with spores suspended in semi-solid agar (Pitt, 1979).

Petri plates of the same a_w values were sealed in polyethylene bags. The inoculated plates were incubated at 25 °C and the colony radii were measured daily during 11 days. For each colony, two radii measured at right angles to one another were averaged to find the mean radius for that colony. All colony radii were determined by using three replicates for each test interaction. A linear model was used to described mycelial fungal growth. The radial growth rate (mm day^{-1}) was subsequently calculated by linear regression of the linear phase for growth and the time at which the line intercept the *x*-axis was used to calculated the lag phase in relation to *Aspergillus* RCM89, entomopathogenic fungal strain, synthetic antioxidant and water activity. Lag phase is referred to a delay before the visual fungal growth occurred. During lag phase begins the germination of the spores and the initial hyphal formation (Meletiadis et al., 2001). Once germination has taken place, hyphae elongate leading to mycelium spreading and macroscopic colonies formation.

After 11 days of incubation at 25 °C, a plug of agar medium and biomass (1 × 1 cm) was taken from each colony of each treatment for aflatoxin B_1 determination. It was transferred to an Eppendorf tube, and 500 µl of chloroform was added. The mixture was centrifuged at 850 × *g* for 20 min, and the solvent was removed from the chloroform extract under nitrogen gas. The residue was redissolved in 10 µl of chloroform for screening by TLC (Geisen, 1996). Samples were quantitatively determined by HPLC following the methodology of detection of Trucksess et al. (1994). Pure AFB_1 solution (Sigma, St. Louis, MO, USA) was used as standard. The limit of detection of the analytical method was 1 ng g^{-1}.

2.6. Statistical analysis

The effect of treatment and water activity on spores viability of entomopathogenic fungus was analyzed with ANOVA. To establish significant differences, Waller–Duncan k-ratio *t*-test ($P < 0.05$) was performed. Also, the ANOVA was done for lag phase and growth rate; and the Tukey test ($P < 0.001$) was performed to establish significant differences. For all statistical analysis SAS (version 9.1, SAS Institute Inc, Cary NC, USA.) was used.

3. Results

3.1. Compatibility of different antioxidants and natural phytochemicals with P. lilacinum strains

Statistical analyses of the effects of antioxidants and natural phytochemicals and the interaction between *P. lilacinum* strains and treatments on spores viability of each strain showed significant differences ($P < 0.001$). The major effect was that of chemical substances ($F = 39.15$). No significant differences between strains ($F = 1.87$, $P > 0.0116$) and a_w ($F = 0.10$, $P > 0.9015$) were observed; thus, Fig. 1 summarizes the effect of treatments on the 21 fungal strains and the three a_w tested. The food grade antioxidants BHA, BHT, the mixture BHA + BHT and the natural phytochemical CA did not significantly influence the count of viable spores of the 21 *P. lilacinum* strains. FA and the mixture FA + CA caused a significant inhibition of the count of viable spores relative to the untreated control. The entomopathogenic viable spores count was significantly affected with highest concentrations of BHA and CA (2, 3, 4, 5, 7 and 10 mM) and BHT and FA (10 mM).

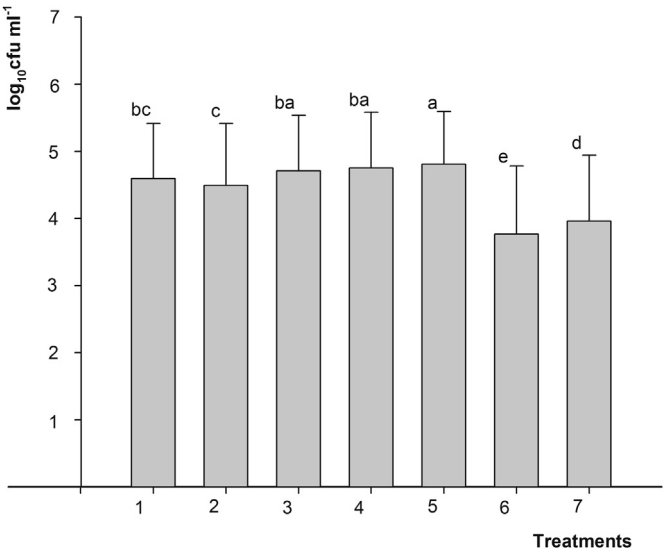

Fig. 1. Effect of synthetic antioxidants and natural phytochemicals on 21 *P. lilacinum* strains viable spores count at three a_w after 25 days of incubation at 25 °C. Bars represent means and standard errors for different treatment. Different letters above each bar indicate a significant difference based on Waller–Duncan k-ratio *t*-test (k-ratio = 100, Critical value of $T = 1.75688$, Minimum significant difference = 0.1634). (1) Control, (2) BHA: 1 mM, (3) BHT: 7 mM, (4) BHA + BHT: 0.7 + 6 mM, (5) CA: 1 mM, (6) FA: 7 mM and (7) CA + FA: 0.6 + 2 mM.

The compatibility between the treatments which showed the least effect on spores viability fungal count is shown in Table 1. Fourteen, 18, 14 and 8 strains showed spores viability with BHT, CA, the mixture BHA + BHT and BHA respectively. In these treatments spore viability was significantly higher than in the control. The strain JQ926212 with BHA had the least number of viable spores, while the strain JQ926202 showed the greatest spore viability (117.8%) with the mixture BHA + BHT. The strains JQ926202 and JQ926222 showed compatibility with the four treatments evaluated.

3.2. Interactions of A. flavus with BHA and P. lilacinum strains

The lag phase (days) of *A. flavus* RCM89 at three water activities, with BHA, the 21 *P. lilacinum* strains and the combination of BHA with the entomopathogenic fungus, are shown in Table 2. In untreated controls similar lag phases were observed for *A. flavus* RCM89 at the three water activities evaluated. They varied from 3.36 to 3.21 days. The lag phase increased by 17 days by interaction with the 21 *P. lilacinum* strains. According to the Tukey test the lag phase of the aflatoxigenic strain was significantly influenced by the BHA and BHA + *P. lilacinum* mixture.

Paired cultures of *A. flavus* with the 21 *P. lilacinum* strains caused complete inhibition of growth of the aflatoxigenic strain at all a_w values tested, while BHA gave greater than 70% control at the same conditions. As well, the mixture BHA + *P. lilacinum* strains completely inhibited growth of *A. flavus* at 0.99, 0.97 and 0.95 a_w. Thus, the 21 *P. lilacinum* strains and the mixture of these entomopathogenic fungi with BHA (1 mM) inhibited the growth rate and increased the lag phase of *A. flavus* RCM89 at 0.99, 0.97 and 0.95 a_w (Table 3).

We examined the aflatoxin B_1 accumulation in paired cultures between *A. flavus* RCM89 in interaction with 21 *P. lilacinum* strains and the presence of BHA in MMEA at 0.99, 0.97 and 0.95 a_w. In control treatments, *A. flavus* strain accumulated different levels of aflatoxin B_1. The higher level was detected at 0.97 a_w (5.2 ng g^{-1}). At 0.99 and 0.95 a_w AFB_1 levels were lower (1.27 and 2.06 ng g^{-1}, respectively). No AFB_1 production was detected in the interaction of entomopathogenic fungus, BHA and the mixture of entomopathogenic fungus + BHA with the *Aspergillus* strain assayed at all water activities.

Table 1
Percentage of viable spores of *P. lilacinum* strains at different treatments.

P. lilacinum strains (GenBank accession N°)	Treatments			
	BHT	BHA + BHT	CA	BHA
JQ926202	104.5 (C)	117.8 (C)	111.5 (C)	102.1 (C)
JQ926203	91.5	112.4 (C)	84.1	93.0
JQ926204	107.8 (C)	113.5 (C)	96.2	101.9 (C)
JQ926205	101.1 (C)	96.1	105.8 (C)	104.7 (C)
JQ926206	108.4 (C)	114.8 (C)	103.0 (C)	95.8
JQ926207	104.3 (C)	101.9 (C)	103.2 (C)	95.6
JQ926208	100.4 (C)	99.1	106.1 (C)	96.5
JQ926210	96.5	100.6 (C)	99.3	98.7
JQ926211	108.9 (C)	102.8 (C)	105.0 (C)	98.2
JQ926212	104.5 (C)	113.2 (C)	102.3 (C)	83.2
JQ926214	103.9 (C)	95.6	110.0 (C)	97.4
JQ926216	93.4	86.9	111.5 (C)	102.4 (C)
JQ926217	98.2	112.8 (C)	105.6 (C)	105.2 (C)
JQ926218	96.1	101.1 (C)	103.2 (C)	93.7
JQ926220	97.3	105.8 (C)	107.6 (C)	86.5
JQ926222	107.1 (C)	112.2 (C)	102.6 (C)	101.7 (C)
JQ926223	103.9 (C)	102.3 (C)	110.6 (C)	98.7
JQ926225	100.2 (C)	90.4	116.1 (C)	101.7 (C)
JQ926226	101.7 (C)	93.8	108.4 (C)	101.7 (C)
JQ926227	110.4 (C)	101.5 (C)	102.6 (C)	99.3
JQ926228	85.8	99.1	104.1 (C)	87.4

BHT: 7 mM; BHA + BHT: 0.7 + 6 mM; CA: 1 mM; BHA: 1 mM.
Compatible (C): an enhancement of spores viability (>100%) over control.
Non-compatible: reduction in viability (<100%) over control.

Table 2
Effect of *P. lilacinum*–synthetic antioxidant–*A. flavus* interactions on the lag phase (days) under different water activity conditions.

Treatment	*A. flavus* RCM89		
	0.99	0.97	0.95
Control	3.21 ± 0.09a	3.44 ± 0.16 a	3.36 ± 0.49a
BHA 1 mM	16.23 ± 2.32b	12.30 ± 1.77b	15.35 ± 0.21b
BHA + *P. lilacinum*[a]	>c	>c	>c
P. lilacinum[a]	>c	>c	>c

Values are the mean of three replicates. Means in a column with a letter in common are not significantly different ($P < 0.05$, Tukey test).
>17 days.
[a] Effect of 21 *P. lilacinum* strains on *A. flavus* lag phase.

4. Discussion

The present study was done to evaluate the compatibility of food grade antioxidants and natural phytochemicals with different strains of *P. lilacinum*, isolated from maize agroecosystem. The results revealed that BHT, the mixture BHA + BHT, CA and BHA were the most compatible substances with the 21 entomopathogenic fungi strains. Moreover the growth of some strains was stimulated in the presence of some treatments.

Previously, we performed a study of the insecticidal activity of synthetic antioxidants and natural phytochemicals against insect vectors of *A. flavus* in stored maize, and we observed that BHA, BHT, the mixture BHA + BHT, FA and the mixture FA + CA, in the concentration range of 20–30 mM, had the highest insecticidal activity against *S. zeamais*, *T. confusum* and *R. dominica* after 120 days of incubation (Nesci et al., 2011). Moreover, we previously evaluated the effect of these substances on *Aspergillus* section *Flavi* growth parameters and aflatoxin production in culture medium, sterilized grain and naturally contaminated grain (Nesci et al., 2003; Nesci and Etcheverry, 2006; Nesci et al., 2007; Nesci et al., 2008; Nesci and Etcheverry, 2009; Nesci et al., 2009). The results of these studies showed that the effective fungicide concentration range for these substances was from 10 to 30 mM, similar to the insecticide concentration range. However, in the present study we observed that the treatments in the concentration range of 0.6–7 mM did not affect the count of viable spores. This range is lower than those effective fungicides and insecticides concentrations. Therefore, the combination of low doses of these chemical substances with the entomopathogenic fungus promote a level of insect and aflatoxigenic fungi suppression similar to that achieved with full chemical treatment.

This outcome of insecticides on the growth of fungi can be different due to the chemical nature of products and the fungal species that are interacting with it (Antonio et al., 2001; Kumar

Table 3
Effect of synthetic antioxidant and *P. lilacinum* on growth rate (mm d^{-1}) of *A. flavus* RCM89 under different water activity conditions.

Treatment	*A. flavus* RCM89		
	0.99	0.97	0.95
Control	0.93 ± 0.03a	0.87 ± 0.04a	0.94 ± 0.15a
BHA 1 mM	0.19 ± 0.02b	0.25 ± 0.03b	0.19 ± 0.01b
BHA + *P. lilacinum*[a]	NGc	NGc	NGc
P. lilacinum[a]	NGc	NGc	NGc

mm d^{-1} = radial growth rate.
Values are the mean of three replicates. Means in a column with a letter in common are not significantly different ($P < 0.05$, Tukey test).
NG: no growth.
[a] Effect of 21 *P. lilacinum* strains on *A. flavus* growth rate.

et al., 2000). The use of incompatible insecticides and fungal agents may lead to suppression of growth and reproduction of the agent, and thus limit its effect on integrated pest management (Anderson and Roberts, 1983; Duarte et al., 1992; Malo, 1993). Akbar et al. (2012) showed that isolates of *Metarhizium anisopliae* were badly affected by chlorpyrifos, match, profenofos and metalaxyl. Huang et al. (2008) showed that the inhibitory effects on *Paecilomyces fumosoroseus* conidia germination increased when the triadimefon, chlorothalonil and abamectin concentrations were increased.

The inhibitory effects of chemical substances on the germination and growth of entomopathogenic fungi often vary among taxa and strains. Therefore, it may be possible to select genotypes which are naturally less susceptible (Inglis et al., 2001). Nevertheless, in this study no significant differences were observed between *P. lilacinum* strains. There are different studies regarding the effect of compatibility of substances that are commonly used as pesticides and entomopathogenic fungi. However, this study is the first report of compatibility between natural phytochemicals present in maize and food grade antioxidants on viability of *P. lilacinum* spores. Inglis et al. (2001) showed that all classes of agrochemicals are potentially inhibitory to entomopathogenic Hyphomycetes, including herbicides, insecticides and fungicides. More recent research performed a comprehensive study on the effect of chemicals on the development of entomopathogenic fungi and concluded that insecticides and herbicides are generally not harmful to the growth of these kinds of fungi (Klingen and Haukeland, 2006). Alizadeh et al. (2007) tested the effect of several pesticides on entomopathogenic fungal development and showed relatively little fungal inhibition of imidacloprid. Some authors showed that the addition of plant oils with insecticide effects, such as coconut, neem and sunflower oil could accelerate mycelia growth and sporulation of *Paecilomyces farinosus*, thereby enhancing its infectivity against target pests (Ganga Vishalakshy et al., 2005). Nana et al. (2012) demonstrated that combination of emulsificable extract from *Calpurnia aurea* with *M. anisopliae* did not affect fungal growth parameters, namely, germination, radial growth, mycelial dried weight and conidial yield regardless of the concentrations tested. Alves et al. (1998) and Moino Jr. and Alves (1998) suggested possible explanations for this compatibility: The fungi, as a physiological mechanism of insecticide resistance, would be able to metabolize and use it as secondary nutrient. Conversely, in a toxic environment, the fungus could increase the production of conidia.

The mechanisms of action of antioxidants and natural phytochemicals as insecticides have not been studied. Only one study showed the antioxidant BHT as a protective agent of synthetic sex pheromone of *Acleris fimbriana*, increasing the catching efficacy (Liu and Meng, 2003). The combination of entomopathogenic fungi and sublethal concentrations of insecticides can exhibit synergistic (Anderson et al., 1989) or additive (Delgado et al., 1999) effect. The enhanced efficacy (i.e. synergistic effect) of *Beauveria bassiana* applied in combination with imidacloprid against *Diaprepes abbreviatus* was due to the effect of imidacloprid as a neural toxin that can affect the behavior of various insects promoting fungal colonization (Quintela and McCoy, 1998; Boucias et al., 1996).

Maize meal extract agar (MMEA) was specifically chosen because entomopathogenic fungi and *A. flavus* will be in interaction in stored maize. The successful development of *P. lilacinum* strains in MMEA was supported by other authors who noted that the ideal substrate for optimal development of entomopathogenic fungi is a cereal, because carbon and nitrogen, supplied by this cereal, are the main nutrients required for growth and sporulation. Maltose, one of the best carbon sources present in maize, release by the action of starch hydrolyzing enzymes present in the fungus induces sporulation (Coudron et al., 1985).

In the current study, spore viability of *P. lilacinum* strains was not affected by the range of a_w evaluated. Moreover, in the MMEA medium *P. lilacinum* strains showed good growth at the water activities and temperature appropriate for growth and toxin production by *Aspergillus* Section *Flavi* (Gqaleni et al., 1996). To select potential biocontrol agents, it is important to consider the relationship between biological interactions and stressful environmental factors (Nesci et al., 2005). Many fungal interactions are influenced by water activity, temperature and substrate (Lacey and Magan, 1991). Changes in these environmental factors have an impact that could be crucial in determining the level of co-existence and dominance of species in a particular ecological niche (Marín et al., 1998; Nesci et al., 2005). In this study we observed a total inhibition of *A. flavus* growth rate when this toxigenic strain interacts with all *P. lilacinum* strains. However, BHA produced an increase of the lag phase and a reduction of *A. flavus* growth rate. A total *A. flavus* growth inhibition was observed when this fungus interacts with the entomopathogens and the antioxidant. It is important to know when the germination of the spores begins, because spoilage occurs as soon as visible hyphae can be observed. Therefore, an effective treatment must affect the germination and elongation rates of spores, resulting in longer lag phases.

The entomopathogenic fungi and the mixture of these with the antioxidant BHA showed higher inhibitory effect on the growth of the pathogen that when the antioxidant was evaluated alone. The ability of a microorganism to compete and exclude toxigenic species during the colonization of a particular niche, increase the possibility to establish the potential biocontrol agent in the specific niches and contribute to reduce the undesirable inoculums in the specific ecosystem (Etcheverry et al., 2009).

Aflatoxin B_1 production only occurred in control treatments at all water activities evaluated. Total inhibition of aflatoxin production was observed in the presence of BHA 1 mM. This result coincided with a previous study in which we evaluated the effect of antioxidants on aflatoxin B_1 accumulation in MMEA at different water activities (Nesci et al., 2003). In this previous study we observed stimulation of toxin production in the presence of BHT 1, 10 and 20 mM at 0.98 a_w. The same effect of stimulation was observed in the presence of cinnamic acid 1 and 5 mM at different water activities (Nesci and Etcheverry, 2006). The BHA used at sublethal doses did not show stimulation on aflatoxin production, therefore the compatibility of this substance with the entomopathogenic fungi suggests that it is possible to perform this combination to achieve an integrated pest management.

From a human health perspective, the Codex Alimentarius (2006) and the US Food and Drug Administration (FDA) allow the use of phenolic antioxidants in foods because they are regarded as safe (GRAS) chemicals. Furthermore, the large differentiation in host infection between strains of *Paecilomyces lilacinus* (Fiedler and Sosnowska, 2007), recently rename as *P. lilacinum* (Luangsa-ard et al., 2011), consider the use of this biological control agent safe for the environment. Therefore the rational use of reduced level of these chemicals in combination with the biocontrol agent is a promising strategy of integrated pest management. Research to further study and the evaluation of the implementation in the real ecosystem are now in progress.

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References

Akbar, S., Freed, S., Hameed, A., Tahira Gul, H., Akmal, M., Naem Malik, M., Naeem, M., Khan, M., 2012. Compatibility of *Metarhizium anisopliae* with different insecticides and fungicides. African Journal of Microbiology Research 17, 3956–3962.

Alizadeh, A., Samih, M.A., Khezri, M., Riseh, R.S., 2007. Compatibility of *Beauveria bassiana* (Bals.) Vuill. with several pesticides. International Journal of Agriculture and Biology 9, 31–34.

Alves, S.B., Moino Jr., A., Almeida, J.E.M., 1998. Produtos fitosanitários e entomopatógenos. In: Alves, S.B. (Ed.), Controle microbiano de insetos. Fealq, São Paulo.

Anderson, T.E., Hajek, A.E., Roberts, D.W., Preisler, K., Robertson, J.L., 1989. Colorado potato beetle (Coleoptera: Chrysomelidae): effects of combinations of *Beauveria bassiana* with insecticides. Journal of Economic Entomology 82 (1), 83–89.

Anderson, T.E., Roberts, D.W., 1983. Compatibility of *Beauveria bassiana* strain with formulations used in Colorado Potato Beetle (Coleoptera: Chrysomelidae) control. Journal of Economic Entomology 76, 1437–1441.

Antonio, B.F., Almeida, J.E.M., Clovis, L., 2001. Effect of Thiamethoxam on entomopathogenic microorganisms. Neotropical Entomology 30, 437–447.

Barra, P., Rosso, L., Nesci, A., Etcheverry, M., 2013. Isolation and identification of entomopathogenic fungi and their evaluation against *Tribolium confusum*, *Sitophilus zeamais*, and *Rhyzopertha dominica* in stored maize. Journal of Pest Science 86, 217–226.

Boucias, D.G., Stokes, C., Storey, G., Pendland, J.C., 1996. The effect of imidacloprid on the termite *Reticulitermes flavipes* and its interaction with the mycopahtogen *Beauveria bassiana*. *Pflanzenchutz-Nachr* Bayer 49, 103–145.

Chandler, D., Hay, D., Reid, A.P., 1997. Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils. Applied Soil Ecology 5, 133–141.

Codex Alimentarius, 2006. Food Additive Details. Update up to the 29th session of the Codex Alimentarius Commission. The Joint FAO/WHO Committee on Food Additives. http: www.codexalimentarius.net/web/jecfa.

Coudron, J.A., Kroha, M.J., El-Sayed, G.N., 1985. A novel semi-liquid for propagating entomopathogenic fungi. Journal of Invertebrated Pathology 46, 335–336.

Cranham, J.E., 1966. Tea pests and their control. Annual Review of Entomology 11, 491–514.

Dallyn, H., Fox, A., 1980. Spoilage of material of reduced water activity by xerophilic fungi. In: Gould, G.H., Corry, E.L. (Eds.), Microbial Growth and Survival in Extreme Environments. London and New York.

Dayakar, S., Kanaujia, K.R., Rathore, R.R.S., 2000. Compatibility of entomogenous fungi with commonly used insecticides for management of *Spodoptera litura* (Fab.). In: Ignacimuthu, S., Sen, A. (Eds.), Microbials in Insect Pest Management. Oxford and IBH Publishing Co. Pvt. Ltd, M. Delhi, Kolkata.

Delgado, F.X., Britton, J.H., Onsager, J.A., Swearingen, W., 1999. Field assessment of *Beauveria bassiana* (Balsamo) Vuillemin and potential synergism with diflubenzuron for control of savana grasshopper complex (orthoptera) in Mali. Journal of Invertebrated Pathology 73, 34–39.

Duarte, A., Menendez, J.M., Triguero, N., 1992. Estudio preliminar sobre la compatibilidad de *Metarhizium anisopliae* con algunos plaguicidas químicos. Revista Forestal Baracoa 22, 31–39.

Dube, B., Smart, G.C., 1987. Biological control of *Meloidogyne* by *Paecilomyces lilacinus* and *Pasteuria penetrans*. Journal of Nematology 19, 222–227.

Etcheverry, M., Nesci, A., Barros, G., Torres, A., Chulze, S., 1999. Occurrence of *Aspergillus* section *Flavi* and aflatoxin B_1 in corn genotypes and corn meal in Argentina. Mycopathologia 147, 37–41.

Etcheverry, M., Scandolara, A., Nesci, A., Vilas Boas Ribeiro, M.S., Pereira, P., Battilani, P., 2009. Biological interactions to select biocontrol agents against toxigenic strains of *Aspergillus flavus* and *Fusarium verticillioides* from maize. Mycopathologia 167, 287–295.

Fiedler, Z., Sosnowska, D., 2007. Nematophagous fungus *Paecilomyces lilacinus* (Thom.) Samson is also a biological agent for control of greenhouse insects and mite pests. BioControl 52, 547–558.

Ganga Visalakshy, P.N., Krishnamoorthy, A., Manoj Kumar, A., 2005. Compatibility of plant oils and additives with *Paecilomyces farinosus*, a potential entomopathogenic fungus. Journal of Food Agriculture and Environment 4, 333–335.

Geisen, R., 1996. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. Applied and Environmental Microbiology 19, 388–392.

Gqaleni, N., Smith, J.E., Gettinby, G., 1996. The production of cyclopiazonic acid by *Penicillium* commune and cyclopiazonic acid and aflatoxin by *Aspergillus flavus* as affected by water activity and temperature on maize grains. Mycopathologia 136, 103–108.

Gurusubramanian, G., Rahman, A., Sarmah, M., Roy, S., Bora, S., 2008. Pesticide usage pattern in tea ecosystem, their retrospects and alternative measures. Journal of Environmental Biology 29, 813–826.

Hazarika, L.K., Bhuyan, M., Hazarika, B.N., 2009. Insect pests of tea and their management. Annual Review of Entomology 54, 267–284.

Huang, Z., Shun-xiang, R., Jian-hui, W.U., Huang, T., 2008. Effect of pesticides on infectious activity of *Paecilomyces fumosoroseus*. Journal of South China Agricultural University, 03.

IARC International Agency for Research on Cancer, 1993. Monograph on the Evaluation of Carcinogenic Risk to Humans. IARC, Lyon, France.

Inglis, G.D., Goettel, M.S., Butt, T.M., Strasser, H., 2001. Use of Hyphomycetos fungi for managing insect pests. In: Butt, T.M., Jackson, C., Magan, N. (Eds.), Fungi as Biological Agents. Progress, Problems and Potential. CAB Publishing, Wallingford, UK.

Jatala, P., 1986. Biological control of plant parasitic nematodes. Annual Review of Phytopathology 24, 453–489.

Joint FAO/WHO. Expert Committee on Food Additives (JECFA), 1996. Monograph on the Evaluation of Carcinogenic Risk to Human, vol. 56, pp. 257–263. Lyon, France.

Kawai, A., 1997. Prospect for integrated pest management in tea cultivation in Japan. JARQ 31, 213–217.

Khan, A., Williams, K.L., Nevalainen, H.K.M., 2006. Control of plant-parasitic nematodes by *Paecilomyces lilacinus* and *Monacrosporium lysipagum* in pot trials. BioControl 51, 643–658.

Klich, M.A., Pitt, J.I., 1988. Differentiation of *Aspergillus flavus* from *Aspergillus parasiticus* and other closely related species. Transaction of the British Mycological Society 91, 99–108.

Klingen, I., Haukeland, S., 2006. The soil as a reservoir for natural enemies of pest insects and mites with emphasis on fungi and nematodes. In: Eilenberg, J., Hokkanen, H.M.T. (Eds.), An Ecological and Societal Approach to Biological Control. Springer, Dordrecht, The Netherlands.

Kumar, A., Mukherjee, P., Guha, A., Adyantaya, S.D., Mandale, A.B., Kumar, R., Sastry, M., 2000. Amphoterization of colloidal gold particles by capping with valine molecules and their phase transfer from water to toluene by electrostatic complexation with fatty amine molecules. Langmuir 16, 9775–9783.

Lacey, J., Magan, N., 1991. Fungi in cereal grains: their occurrence and water and temperature relationships. In: Chelkowski, J. (Ed.), Cereal Grain, Mycotoxins, Fungi and Quality in Drying and Storage. Elsevier Science Publisher BV, Amsterdam.

Lacey, L.A., Fransen, J.J., Carruthers, R., 1996. Global distribution of naturally occurring fungi of *Bemisia*, their biology and use as biological control agents. In: Gerling, D., Mayer, R. (Eds.), Taxonomy, Biology, Damage, Control and Management. Andover.

Liu, Y.X., Meng, X.Z., 2003. Trapping effect of synthetic sex pheromone of *Acleris fimbriana* (Lepidoptera: Tortricidae) in Chinese northern orchard. Journal of Chemical Sciences 58, 421–425.

Luangsa-ard, J., Houbrakern, J., Doom, T., Hong, S.-B., Borman, A.M., Hywel-Jones, N.L., Samson, R., 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiology Letters 321, 141–149.

Magan, N., 2001. Physiological approaches to improving the ecological fitness of fungal biocontrol agents. In: Butt, T.M., Jackson, C., Magan, N. (Eds.), Fungi as Biocontrol Agents. Progress, Problems and Potential. CAB Publishing, Wallingford, UK.

Malo, A.R., 1993. Estudio sobre la compatibilidad del hongo *Beauveria bassiana* (Bals.) Vuill. con formulaciones comerciales de fungicidas e insecticidas. Revista Colombiana de Entomología 19, 151–158.

Marín, S., Sanchis, V., Ramos, A.J., Viñas, I., Magan, N., 1998. Environmental factors *in vitro* interactions and niche overlap between *Fusarium moniliforme*, *F. proliferatum*, and *F. graminearum*, *Aspergillus* and *Penicillium* species from maize grain. Mycological Research 102, 831–837.

Mejía, D., 2007. FAO (Food and Agriculture Organization of the United Nations). http://www.fao.org/inpho/content/compend/text/ch2304.htm.

Meletiadis, J., Meis, J.F., Mouton, J.W., Verweij, P.E., 2001. Analysis of growth characteristics of filamentous fungi in different nutrient media. Journal of Clinical Microbiology 39, 478–484.

Moino Jr., A., Alves, S.B., 1998. Efeito de Imidacloprid e Fipronil sobre *Beauveria bassiana* (Bals.) Vuill. E *Metarhizium anisopliae* (Metsch.) Sorok. e no comportamento de limpeza de Heterotermestenuis (Hagen). Anais da Sociedade Entomológica do Brasil 27, 611–619.

Morgan-Jones, G., White, G.F., Rodriguez-Kabana, R., 1984. Phytonematode pathology: ultrastructural studies. II. Parasitism of *Meloidogynearenaria* eggs and larvae by *Paecilomyces lilacinus*. Nematropica 14, 57–71.

Nana, P., Maniania, N.K., Maranga, R.O., Boga, H.I., Kutima, H.L., Eloff, J.N., 2012. Compatibility between *Calpurnia aurea* leaf extract, attraction aggregation, and attachment pheromone and entomopathogenic fungus *Metarhizium anisopliae* on viability, growth, and virulence of the pathogen. Journal of Pest Sciences 85, 109–115.

Nesci, A., Etcheverry, M., 2002. *Aspergillus* section *Flavi* populations from field maize in Argentina. Letters in Applied Microbiology 34, 343–348.

Nesci, A., Etcheverry, M., 2006. Control of *Aspergillus* growth and aflatoxin production using natural maize phytochemicals under different conditions of water activity. Pest Management Science 62, 775–784.

Nesci, A., Etcheverry, M., 2009. Effect of natural maize phytochemicals on *Aspergillus* Section *Flavi* sclerotia characteristics under different conditions of growth media and water potential. Fungal Ecology 2, 44–51.

Nesci, A., Rodriguez, M., Etcheverry, M., 2003. Control of *Aspergillus* growth and aflatoxin production using antioxidants at different conditions of water activity and pH. Journal of Applied Microbiology 95, 279–287.

Nesci, A., Etcheverry, M., Magan, N., 2004. Osmotic and matric potential effects on growth, sugar alcohol and sugar accumulation by *Aspergillus* Section *Flavi* strains from Argentina. Journal of Applied Microbiology 96, 965–972.

Nesci, A., Bluma, R., Etcheverry, M., 2005. In vitro selection of maize rhizobacteria to study potential biological control of *Aspergillus* section *Flavi*. European Journal of Plant Pathology 113, 1–13.

Nesci, A., Gsponer, N., Etcheverry, M., 2007. Natural maize phenolic acids for control aflatoxigenic fungi on maize. Journal of Food Science 72, 180–185.

Nesci, A., Ferrari, L., Etcvheverry, M., 2008. Effect of synthetic antioxidants on stored maize grain mycoflora *in situ*. Journal of the Science of Food and Agriculture 88, 797–804.

Nesci, A., Marín, S., Etcheverry, M., Sanchis, V., 2009. Natural maize phytochemicals for control of maize mycoflora and aflatoxigenic fungi. World Mycotoxin Journal 3, 305–312.

Nesci, A., Barra, P., Etcheverry, M., 2011. Integrated management of insect vectors of *Aspergillus flavus* in stored maize using synthetic antioxidants and natural phytochemicals. Journal of Stored Products Research 47, 231–237.

Pitt, J.I., 1979. The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*. Academic Press, London.

Pitt, J.I., 1988. A Laboratory Guide to Common *Penicillium* Species. CSIRO Division of Food Research Sydney, North Ryde, Australia.

Pitt, J.I., Hocking, A.D., 1997. In: Pitt, J.I., Hocking, A.D. (Eds.), Fungi and Food Spoilage. Blackie Academic and Professional, London.

Purwari, J.P., Sachan, G.C., 2006. Synergistic effect of entomogenous fungi on some insecticides against Bihar hairy caterpillar *Spilarctia oblique* (Lepidoptera: Arctiidae). Microbiological Research 161, 38–42.

Quintela, E.D., McCoy, C.W., 1998. Synergistic effect of imidacloprid and two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviates* (Coleoptera: Curculionidae) in soil. Journal of Economic Entomology 91, 110–122.

Roy, S., Gurusubramanian, G., Mukhopadhyay, A., 2009. Antifeedant and insecticidal activity of *Clerodendron infortunatum Gaertn*. (Verbinaceae) extract tested on tea mosquito bug, *Helopeltis theivora Waterhouse* (Hetoraptera: miridae). Field Crops Research 10, 152–158.

Saint Geroges-Gridelet, D., 1984. Effects of dietary lipids on the population growth of *Dermatophagoides pteronyssinus*. In: Proceedings of 6th International Congress of Acarology, Chichester.

Schumacher, V., Poehling, H.M., 2012. In vitro effect of pesticides on the germination, vegetative growth, and conidial production of two strains of *Metarhizium anisopliae*. Fungal Biology 116, 121–132.

Serebrov, V.V., Khodyrev, V.P., Gerber, O.N., Tsvetkova, V.P., 2005. Perspectives of combined use of entomopathogenic fungi and chemical insecticides against Colorado beetle (*Leptinotarsa decemlineata*). Mycology and Phytopathology 39, 89–98.

Trucksess, M.W., Stack, M.E., Nesheim, S., Albert, R.H., Romer, T.R., 1994. Multifunctional column coupled with liquid chromatography for determination of aflatoxins B1, B2, G1, G2 in corn, almonds, Brazil nuts, peanuts and pistachio nuts: collaborative study. Journal of AOAC International 6, 1512–1521.