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Efficacy of 2, 6-di (*t*-butyl)-p-cresol (BHT) and the entomopathogenic fungus *Purpureocillium lilacinum*, to control *Tribolium confusum* and to reduce aflatoxin B_1 in stored maize

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ABSTRACT

The objective of this study was to evaluate the effectiveness of the treatment with the entomopathogenic fungus *Purpureocillium lilacinum* and the antioxidant BHT alone or combined in sublethal doses and at different water activity (a_W) levels, as insecticides and/or fungicide in sterile maize. Effect on accumulation of aflatoxin B_1 (AFB_1) was also evaluated. In addition, we studied the resilience of *P. lilacinum* in maize grains and the effect of the treatments on *Aspergillus flavus* populations and on *Tribolium confusum*, an insect pest vector of aflatoxigenic fungi. The combined treatment showed approximately 80% of insecticidal efficacy at all of the tested a_W levels. A low incidence and prevention of contamination with *A. flavus* was observed in the live insects under all of the tested conditions. The *A. flavus* populations increased significantly at the end of the incubation period in all of the treatments. However, *P. lilacinum* populations showed no significant differences, when compared to the control treatment, in the presence of the toxigenic fungus. Maximum levels of AFB_1 reduction (of around 90%) were observed after treating maize with the combination of *P. lilacinum* + BHT. Thus the obtained results prove that the treatment of combination was the most effective and show that it is a promising strategy for an integrated management of pests in stored maize.

1. Introduction

Maize, wheat and rice are the most important cereals for human nutrition, according to the Food and Agriculture Organization of the United Nations (FAO). Maize is considered the main cereal of the future, because of its nutritional value and the potential uses of its by-products (Lee, 1999). In Argentina, maize is a key crop in terms of the national production of grains (SAGPyA, 2009) for international export (INAI, 2009).

Aspergillus section *Flavi* species such as *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare have the ability to invade several agricultural commodities during maturation in the field or after harvest. The saprophytic activity of species from this genus may promote a mouldy substrate (Cotty, 1994; Etcheverry et al., 1999; Nesci and Etcheverry, 2002; Nesci et al., 2008) and toxin accumulation in stored grains (Chulze et al., 1989; Resnik et al., 1996; Torres et al., 1997; Garrido et al., 2012). Aflatoxins, especially aflatoxin B_1 (AFB_1), are considered the most carcinogenic, mutagenic, and teratogenic substances naturally found in food and feed (IARC, 1993).

Growth and survival of fungi are highly affected by water availability (water activity, a_W), which is a limiting factor in the functioning of ecosystems (Ramos et al., 1999). Like other stored products, maize is hygroscopic in nature and tends to absorb or release moisture. Even if the grains are properly dried after harvest, exposure to high humidity conditions during storage will cause the grains to absorb water (Devereau et al., 2002). Appropriate storage conditions at all stages in terms of moisture and temperature control, the general maintenance and effective hygiene of storage facilities allow prevention of pests and water ingress (Magan and Aldred, 2007). The moisture content above a certain safe limit, which depends on the type of grain, is conducive to infestation by

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fungi and insects (Gwinner et al., 1996). In maize, for instance, it was determined that a storage moisture content of 13%, a water activity level below 0.65, is sufficiently low to prevent fungus development and mycotoxin production (Castellari et al., 2010).

Harvested maize containing mycelia and spores from aflatoxigenic fungi and insects may cause a significant reduction in grain quality and yield as well as economic losses in livestock feed, which leads to decreases in animal health and production due to toxicity of mycotoxins (Charmley and Prelusky, 1994). Moreover, several insects infesting stored grains are destructive (Loschiavo, 1984). Maize can be colonized by insects such as *Sitophilus zeamais* (Motschulsky), *Rhyzopertha dominica* (Fabricius) and *Tribolium confusum* (Jacquelin du Val), that cause significant damage to stored maize (Mejia, 2007). Insects found in storage systems can break the coat off grains, which is a natural barrier to prevent fungal infection, and facilitate the spread of fungi (Setamau et al., 1998). In fact, previous studies showed that certain insects that attack stored grains have the ability to disperse toxigenic *A. flavus* among those grains (Nesci et al., 2011a, b). The respiration of insects increases the temperature and moisture content of grains, producing favorable conditions for fungal growth (Sinha, 1971). Warming resulting from the metabolic activities of insects and molds may continue even after the insects die (Christensen and Kaufmann, 1965; Sauer et al., 1992). Thus knowledge of the key critical control points during harvesting, drying and storage in the cereal production chain are essential in developing effective post-harvest prevention strategies (Magan and Aldred, 2007). Besides, an obvious need to assess the efficacy of hygiene procedures and structural treatments before storage for maintaining grain quality is essential (Abd-El-Aziz, 2011). Because of all of these reasons it is necessary to develop an integrated management of insects that serve as vectors of aflatoxigenic fungi in stored maize.

The storage stage after harvest represents an essential period for the control of fungi and pests of grains, as it is possible to control some environmental parameters in this agro-ecosystem (Wilson and Pusey, 1985).

At the present time, post-harvest pest control is performed mainly with synthetic chemicals. However, there is a search for safer methods to humans and the environment, with the objective of achieving a healthier grain protection system. Thus, alternatives to conventional pesticides are needed. One of these alternatives is the use of synthetic antioxidants. According to JECFA (1996), butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) are permitted antioxidants in food and animal feed products. BHA and BHT showed effective control of major insect pests of stored maize and of *Aspergillus* section *Flavi* growth and reduction of aflatoxin production (Nesci et al., 2003, 2007, 2008, 2009, 2011a). A second alternative that deserves to be under investigation is the biological control of insects using entomopathogenic fungi. Entomopathogenic fungi are natural enemies of a wide range of insects and some species are used as microbial bio-pesticides since they are considered to offer an environmentally friendly alternative to chemical pesticides (James and Elzen, 2001; Leemon and Jonsson, 2008; Bukhari et al., 2011). In a previous study, different isolates of *Purpureocillium lilacinum* (Thom) Samson showed pathogenicity against insect vectors of aflatoxigenic *A. flavus* in stored maize (Barra et al., 2013a). It is known that entomopathogenic fungi cannot completely replace the need for chemical pesticides in all agro-ecosystems since insecticides are required to suppress the rapid expansion of pest populations. Recently, we performed a study of compatibility between food grade antioxidants and spores of *P. lilacinum* (Barra et al., 2013b). Inglis et al. (2001) suggested that sublethal doses of synthetic chemical insecticides may act as physiologic stressors and predispose the insects to attacks by entomopathogenic fungi. Previously, we observed that food grade antioxidants such as BHA and BHT, in a concentration range between 0.6 and 7 mM did not affect the viability of *P. lilacinum* spores (Barra et al., 2013b). This range is lower than the concentrations used (10–30 mM) when studying their fungicidal and insecticidal activities (Nesci et al., 2003, 2007, 2008, 2009, 2011a). Consequently, in order to assess the level of suppression of the insect and the aflatoxigenic fungus by a combined treatment, we:

a) Evaluated the insecticidal and/or fungicidal activity of *P. lilacinum* + BHT against the *T. confusum* pest and *A. flavus* populations in sterile maize, at different a_W levels (0.95, 0.97 and 0.99).
b) Analyzed the effects of the combined treatment on contamination by aflatoxin B_1 in stored maize.

2. Materials and methods

2.1. Entomopathogenic fungus

The isolate identified as *P. lilacinum* was used in the experiments. This strain was originally isolated from soil samples collected from the Experimental Field Station of the University of Río Cuarto, Córdoba, Argentina and was identified and deposited in the GenBank with the following accession number: JQ926223. This strain showed the lowest TL_{50} against *T. confusum*, an insect pest vector of aflatoxigenic fungi in stored maize (Barra et al., 2013a).

2.2. Aflatoxigenic fungus

The fungus *A. flavus* RCM89 was used in the experiments. It was isolated from stored maize (Nesci et al., 2008) and identified according to Pitt and Hocking (1997), Klich and Pitt (1988) and Pitt (1988). This strain produces aflatoxin B_1 in liquid medium (110.32 ng g^{-1}). The entomopathogenic and aflatoxigenic fungi are held at the Microbial Ecology Laboratory Collection, in the Microbiology and Immunology Department of the National University of Río Cuarto, Córdoba (Argentina).

2.3. Synthetic antioxidant

The 2, 6-di (*t*-butyl)-p-cresol (BHT), an effective synthetic antioxidant that also controls insect vectors of *A. flavus* in the microcosm of maize (Nesci et al., 2011a), was used for the experiments. This compound also showed fungicide effect on *Aspergillus* section *Flavi* growth parameters and aflatoxin production in culture medium, sterilized grains and naturally contaminated grains (Nesci et al., 2003, 2007, 2009; Nesci and Etcheverry, 2006).

The industrial grade antioxidant was obtained from Eastman Chemical Company. BHT had a purity of 99% and contained contaminants such as ash <0.02%, arsenic <3 mg g^{-1} and heavy metals <10 mg g^{-1}. Contaminants from industrial grade antioxidants do not exceed the levels allowed by the Expert Committee on Food Additives (JECFA, 1996). A stock solution of BHT (0.22 g ml^{-1}) was prepared in 95% ethyl alcohol. It was used at a 7 mM (1.48 mg g^{-1}) concentration. The equivalent amount of ethyl alcohol was added to maize in the control treatments.

2.4. Insect

Cultures of one strain of the confused flour beetle *T. confusum* (Jacquelin du Val) were obtained from the Agricultural Zoology Department, Faculty of Agronomy, at the University of Buenos Aires, Argentina. Mixed-sex adults 1–3 weeks old were used in the experiments. Insects were reared on a diet of wheat flour, maize

starch, and yeast (10:10:1.5) in plastic containers. The insects were reared at 27 ± 1 °C, 70 ± 5% relative humidity (r.h.) and a photoperiod of 12:12 h light:dark cycle.

2.5. Rehydration, inoculation and incubation of sterile maize

The assay was carried out with subsamples of sterile maize. The grains were autoclaved at 121 °C for 15 min. Samples had an initial water activity level of 0.76. Samples of 100 g of maize were weighed into sterile 250 ml flasks and rehydrated to the required a_W by addition of sterile distilled water using a moisture absorption curve and a synthetic antioxidant solution. Then, flasks were stored at 4 °C for 72 h to modify the water activity of grains to the required levels (0.99, 0.97 and 0.95). They were regularly shaken to obtain a uniform distribution of water and the antioxidant. The flasks containing the rehydrated maize were inoculated with 1 ml of a spore suspension (10^4 spores ml^{-1}) of *A. flavus* and/or 1 ml of a spore suspension (10^7 spores ml^{-1}) of *P. lilacinum*. This volume of water had already been subtracted from the initial amount of water added for the rehydration to achieve the grains' a_W level. Twenty *T. confusum* adults were placed per flask. Tests with three replicates were performed. Flasks were placed in a chamber under controlled conditions (27 ± 1 °C, 70 ± 5% r.h., with a photoperiod of 12:12 h light: dark cycle) (Wicklow et al., 1998). Flasks were incubated for 11 days. The assayed treatments were designed according to the scheme shown in Table 1.

The colonization of maize grains by *A. flavus* and *P. lilacinum* was assessed. Subsamples of 10 g were taken from each treatment, ground and homogenized with a 0.1% peptone-water solution. Serial dilutions were performed and 0.1 ml was spread on dichloran rose bengal chloramphenicol agar (DRBC) medium (Pitt and Hocking, 1997) and semi-selective isolation medium (MS; Barra et al., 2013a) for isolation of aflatoxigenic and entomopathogenic fungi, respectively. Plates were incubated at 25 ± 1 °C for 7 and 10 days to allow growth of *A. flavus* and *P. lilacinum*, respectively. The counting of fungal propagules was recorded as CFU g^{-1} for each treatment. The fungal count was expressed as $\log_{10}$ g^{-1} of maize.

Insect mortality was analyzed and compared with the untreated control samples. All dead insects were placed directly on plates containing MS medium, which were incubated at 25 °C for 7 days to confirm that the inoculated fungus was the causal agent of insects' mortality. The insects that survived the experiment were killed by freezing at −20 °C. All insects were plated directly on malt extract agar medium (MEA) with 10% NaCl and incubated at 25 °C for 7 days. The number of insects from which *A. flavus* colonies developed was counted. The experiment was repeated three times.

2.6. Aflatoxin B_1 (AFB_1) analysis

After 11 days of incubation, 40 g of all of the maize samples were frozen for a later extraction and quantification of AFB_1. The toxin determination was performed according to the AOAC Official Method 994.08, with modifications. Aflatoxin was extracted from ground maize (25 g) with 100 ml of acetonitrile:water (84:16) during 30 min in an orbital shaker. The supernatant was filtered through Whatman N°4 filter paper and a 5 ml aliquot of the extract was applied to a multifunctional cleaned column (MycoSep® 224 AflaZon column, Romer Labs, Inc. America). The filtrate (2 ml) was evaporated to dryness and redissolved in 400 ml of mobile phase until analysis by high-performance liquid chromatography (HPLC). Aflatoxin quantification was performed by HPLC according to Trucksess et al. (1994) with some modifications. A 200 ml aliquot was derivatized with 700 ml of trifluoroacetic acid:acetic acid:-water (20:10:70). The derivatized aflatoxins (50 μl solution) were analyzed by a reverse-phase HPLC/fluorescence detection system. The HPLC system consisted of a Hewlett–Packard workstation. Chromatographic separations were performed in a stainless steel C_{18} reversed-phase column (150 × 4.6 mm i.d. 5 μm particle size) (Luna-Phenomenex, Torrance, CA, USA). Water:-methanol:acetonitrile (4:4:1) was used as mobile phase at a flow rate of 1.5 ml min^{-1}. Fluorescence of the aflatoxin derivative was recorded at excitation and emission wavelengths of 360 and 440 nm, respectively. Standard curves were constructed with different concentrations of AFB_1. The toxin was quantified by correlating the peak heights of sample extracts with the calibration curves. The detection limit under these conditions was 1 ng g^{-1}.

2.7. Statistical analyses

Analyses of variance of completely randomized designs were used to compare the survival of *P. lilacinum*, the frequency of *A. flavus*, the number of dead and live insects and the percentage of insects contaminated with *A. flavus*. Means were compared using the Tukey test ($P < 0.05$). All statistical analyses were performed using the InfoStat program (for Windows 2008, InfoStat group FCA, National University of Córdoba, Argentina).

3. Results

3.1. Assessment of P. lilacinum viability in maize grains. Effect of treatments on the population of A. flavus

Statistical analyses on the survival of the entomopathogenic fungus and the effect of treatments on *A. flavus* population, water activity and their interactions were statistically significant. The

Table 1
Treatments assayed in maize at water activity values of 0.99, 0.97 and 0.95.

	Insect number (*T. confusum*)	Chemical compound	Fungal inoculum
T1	0	–	–
T2	0	–	*P. lilacinum*
T3	0	–	*A. flavus*
T4	0	BHT	*P. lilacinum*
T5	0	BHT	*A. flavus*
T6	20	–	–
T7	20	–	*P. lilacinum*
T8	20	–	*A. flavus*
T9	20	–	*P. lilacinum* and *A. flavus*
T10	20	BHT	*P. lilacinum*
T11	20	BHT	*A. flavus*
T12	20	BHT	*P. lilacinum* and *A. flavus*
T13	20	BHT	–

most significant effect was that of treatments. Therefore, Fig. 1a shows the average of 3 a_W with the entomopathogenic fungus and Fig. 1b with *A. flavus*. In the entomopathogenic fungus control treatment (T2), the *P. lilacinum* count increased from a log of 7 to a log of 9.1 after 11 days of incubation. A similar count was observed in the other treatments throughout the experience. In presence of the toxigenic fungus (T9), the *P. lilacinum* count showed no significant differences with the control (T2). The food grade antioxidant BHT, the presence of *T. confusum*, the toxigenic *A. flavus* and the different combinations of these treatments did not significantly influence the stability of the entomopathogenic fungus population.

In the toxigenic fungus control treatment (T3), the *A. flavus* population increased significantly at the end of the incubation period (Fig. 1b). Counts increased from a log of 4 to a log of 10.2. The populational size of *A. flavus* increased twice or more at 11 days in all of the treatments, compared to the initial inoculum. In treatments with BHT and/or the entomopathogenic fungus (T5, T11 and T12), significant reductions in the *A. flavus* populations were observed when comparing with the control at the end of the incubation period. The greatest reduction in the toxigenic fungus count was obtained with the combined treatment of *P. lilacinum* + BHT (T12).

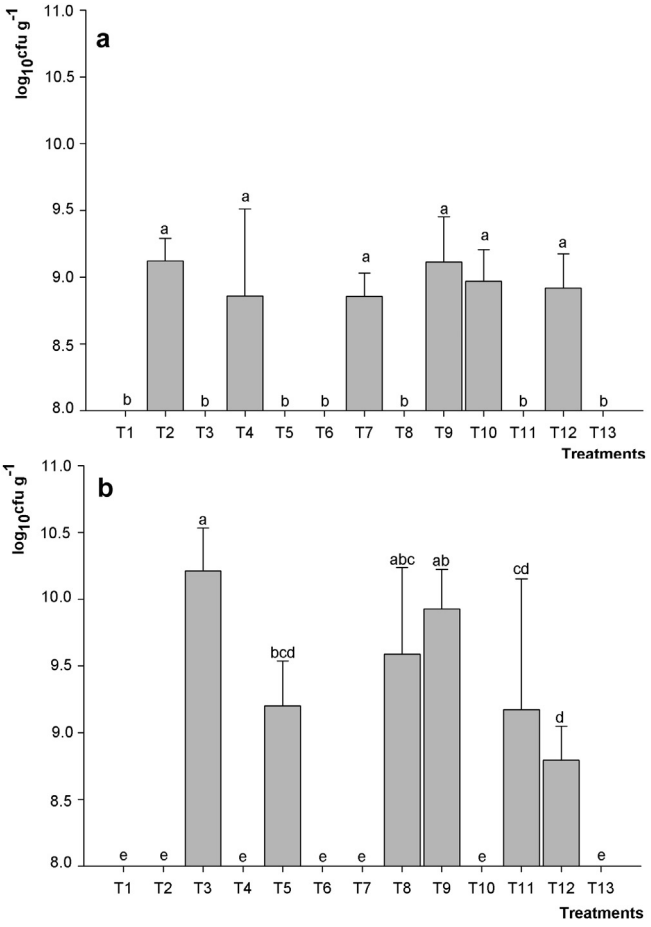

Fig. 1. *P. lilacinum* (a) and *A. flavus* (b) population ($\log_{10}$ CFU g^{-1}) isolated from maize grains with different treatments. Bars represent means and standard deviation for each treatment. Different letters above each bar indicate a significant difference between treatments based on Tukey's test ($P < 0.05$). T1: control; T2: *P. lilacinum*; T3: *A. flavus*; T4: *P. lilacinum* + BHT; T5: *A. flavus* + BHT; T6: *T. confusum*; T7: *P. lilacinum* + *T. confusum*; T8: *A. flavus* + *T. confusum*; T9: *P. lilacinum* + *A. flavus* + *T. confusum*; T10: *P. lilacinum* + *T. confusum* + BHT; T11: *A. flavus* + *T. confusum* + BHT; T12: *P. lilacinum* + *A. flavus* + *T. confusum* + BHT; T13: *T. confusum* + BHT.

3.2. Insecticidal activity of treatments

The mortality in the control treatment was low (20%) and no mycosis was detected on insects. All treatments showed significant differences in insecticidal activity after 11 days of exposure ($P < 0.0001$). In general, the insecticidal effect was lower when the a_W decreased (Fig. 2 a,b,c). A reduction in insecticidal activity was also observed when the same treatment was applied to maize with an additional inoculum of *A. flavus*. Treatment with *P. lilacinum* (T7) showed mortality rates of 75, 65 and 70% at a_W levels of 0.99, 0.97 and 0.95, respectively. In addition, when *P. lilacinum* was applied to maize inoculated with *A. flavus* (T9), mortality rates of 70, 75 and 55% were observed at a_W levels of 0.99, 0.97 and 0.95, respectively. The same effect was observed when comparing T13 (BHT) with T11 (BHT + *A. flavus*) and T10 (BHT + *P. lilacinum*) with T12 (BHT + *P. lilacinum* + *A. flavus*).

The highest insecticidal effect was observed in the *P. lilacinum* + BHT treatment (T10), which showed a mortality rate above 80% that was leveled when the a_W decreased. Treatment with *P. lilacinum* + BHT in the presence of an additional inoculum of *A. flavus* (T12) caused a reduction of the *T. confusum* population of around 60% in the 3 a_W evaluated. A similar percentage reduction was observed with T11 (65%), when the chemical treatment was used alone.

3.3. Effects of treatments on infection of T. confusum by A. flavus

The frequency of *A. flavus* isolation from collected insects is shown in Fig. 3. The isolation of *A. flavus* from dead and live insects exposed to different treatments showed significant differences ($P < 0.0001$). *A. flavus* was isolated from dead and live insects from all of the treatments. The control treatment (T8) showed a 90% of *T. confusum* contamination by *A. flavus* with a_W at 0.99 and a 100% with a_W at 0.97 and 0.95. In this treatment, live insects showed more contamination by *A. flavus* than dead insects. In the treatment with *P. lilacinum* (T9), a 100% of *T. confusum* contamination with *A. flavus* was observed with a_W at 0.99, a 40% with a_W at 0.97 and a 95% with a_W at 0.95. The lowest percentage (30%) of contamination by *A. flavus* with T9 was observed in live insects with a_W at 0.99, while the highest percentage was observed with a_W at 0.97 and 0.95. A 100% of live and dead insects showed contamination by *A. flavus* in the treatments with BHT (T11) and combination of *P. lilacinum* + BHT (T12), in the 3 a_W evaluated. Both treatments showed a low contamination by *A. flavus* in live insects with a_W at 0.99 and 0.97.

3.4. Effects of treatments on accumulation of aflatoxin B_1 (AFB_1)

Accumulation of AFB_1 in maize grains after 11 days of incubation was determined. Table 2 shows accumulation of AFB_1 in maize without the chemical compound and the fungal inoculum (T1), in maize with the *A. flavus* inoculum (T3) and in different treatments where *A. flavus* was added. The toxigenic fungus accumulated AFB_1 at the 3 a_W assayed (T3). The highest level was detected with a_W at 0.95 (495 ng g^{-1}). On the other hand, AFB_1 levels were lower with a_W at 0.97 and 0.99 (235 and 115 ng g^{-1}, respectively).

The lowest reduction effect was observed with the entomopathogenic fungus alone (T9), with inhibition percentages of 10.9 and 40.7% with a_W at 0.97 and 0.95, respectively. No reduction was observed with a_W at 0.99. The treatment with the antioxidant BHT (T5), showed percentages of inhibition of approximately 90% with a_W at 0.99 and 0.97. In contrast, this effect was lower (49.5%) with a_W at 0.95. When BHT was applied to maize infested with

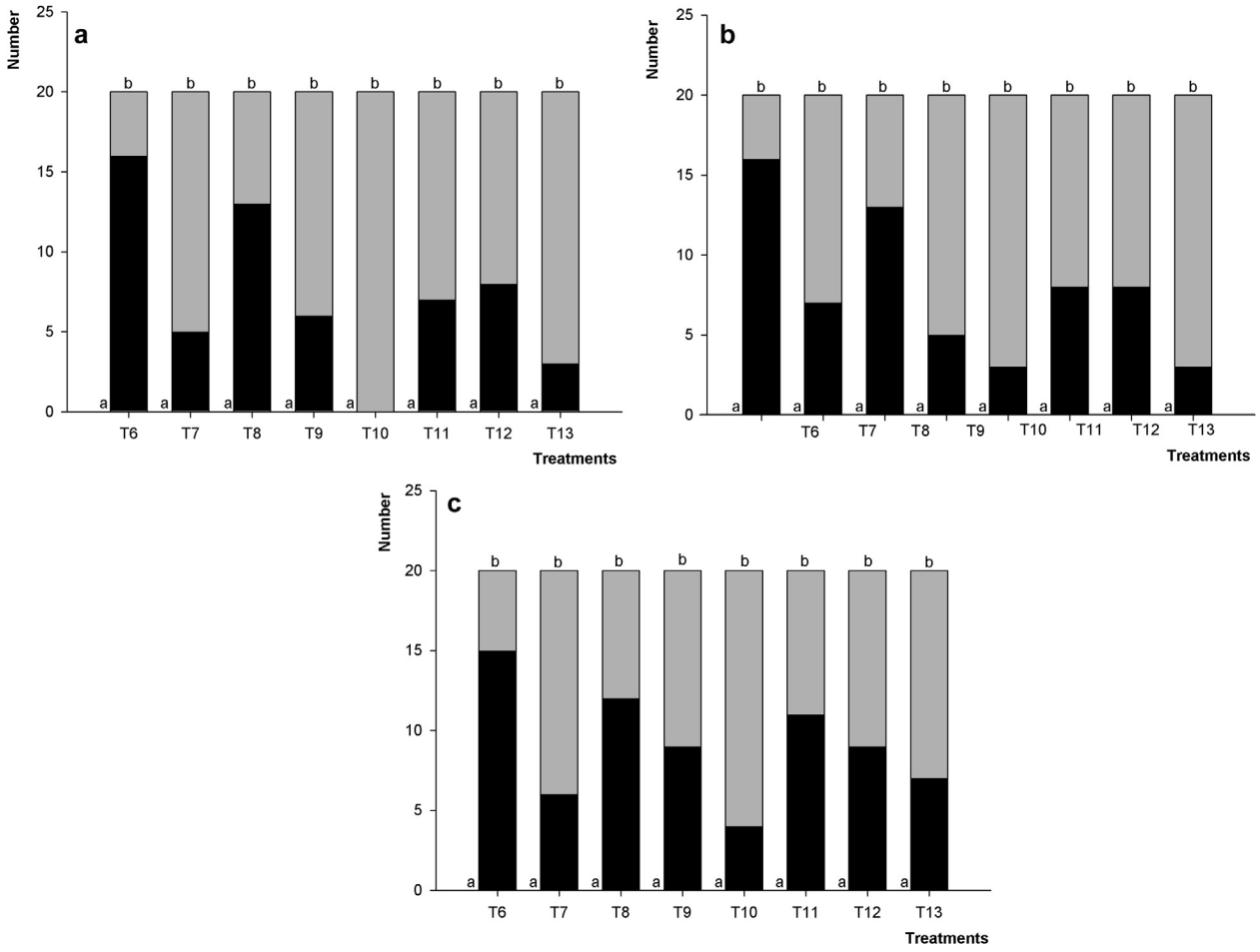

Fig. 2. Insecticidal activity of different treatments against *Tribolium confusum* with a_W at three levels, 0.99 (a); 0.97 (b); 0.95 (c). Black: live insects, Grey: dead insects. Values for live and dead insects from the same treatment with different letters are significantly different according to Tukey's test ($P < 0.05$). T6: *T. confusum*; T7: *P. lilacinum* + *T. confusum*; T8: *A. flavus* + *T. confusum*; T9: *P. lilacinum* + *A. flavus* + *T. confusum*; T10: *P. lilacinum* + *T. confusum* + BHT; T11: *A. flavus* + *T. confusum* + BHT; T12: *P. lilacinum* + *A. flavus* + *T. confusum* + BHT; T13: *T. confusum* + BHT.

T. confusum (T11), the percentages of AFB_1 reduction were similar to those obtained when the antioxidant was applied without the presence of insects (T5), with a_W at 0.99 and 0.97. However, when a_W decreased to 0.95 we observed the highest effect of AFB_1 reduction (85.6%) with T11. The reduction effect was highly significant in grains treated with the combination of *P. lilacinum* + BHT (T12). In this treatment, the lowest level of AFB_1 (1.6 ng g^{-1}) was detected with a_W at 0.97 (which implies a percentage of reduction of 99.3%). On the other hand, the percentages of reduction were of 98.4% and 87.8% with a_W at 0.99 and 0.95, respectively.

4. Discussion

The entomopathogenic fungus and the synthetic antioxidant used in this study showed their potential as insecticides against insect vectors of *Aspergillus* section *Flavi* for the preservation of stored maize. The combined treatment consisting in the chemical compound (BHT) and the fungal inoculum (*P. lilacinum*) showed up to 80% of insecticidal activity at all of the evaluated water activities. The food grade antioxidant alone and combined with *P. lilacinum* caused the lowest percentages of living insects contaminated with *A. flavus*, in all of the tested conditions. Thus, both treatments decreased the dispersion of aflatoxigenic fungi in maize grains. Furthermore, the highest levels of AFB_1 reduction (approximately 90%) were observed in maize treated with the combined treatment. Therefore, application of BHT + *P. lilacinum* causes an increase in the insecticidal activity against *T. confusum*, a decrease in the dispersion of *A. flavus* and higher percentages of AFB_1 reduction in maize grains.

Previously, we performed a compatibility study of natural and food grade fungicidal and insecticidal substances with *P. lilacinum* in maize meal extract agar medium. The obtained results showed that BHT, the mixture of BHA (2(3)-tert-butyl-4-hydroxyanisole) + BHT, CA (3-phenyl-2-propenoic acid) and BHA alone at a concentration range of 0.6–7 mM were the most compatible substances with 21 strains of *P. lilacinum* (Barra et al., 2013a). Subsequently, a study was performed *in vitro* to evaluate the effect of treatments based on the combination of these substances with spores of *P. lilacinum* on natural maize grains (Barra et al., 2013c). Results revealed that the combined treatment of BHT (7 mM) + *P. lilacinum* (10^7 spores mL^{-1}) caused a 100% mortality of *T. confusum*, *S. zeamais* and *R. dominica*. Moreover, we observed that only this treatment caused a significant reduction of the natural mycoflora of maize. Therefore, we selected this treatment for further studies in order to reduce the number of treatments.

In the present study, the biocontrol agent *P. lilacinum* remained viable in sterile maize at concentrations of spores higher than those inoculated at first. The viability of the spores from this strain of *P. lilacinum* was not significantly affected by the range of a_W

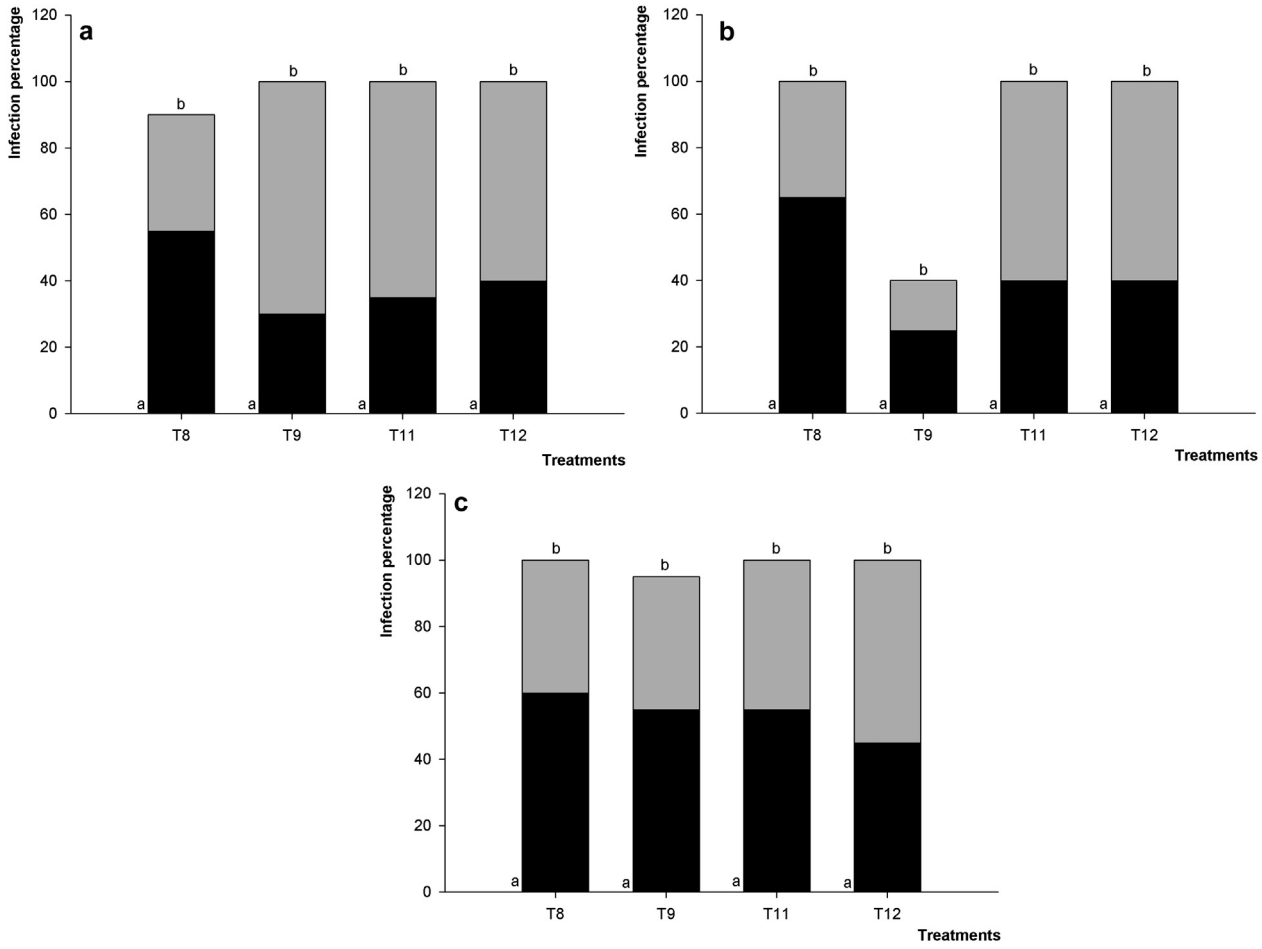

Fig. 3. Effect of treatments on infection (percentage) of *Tribolium confusum* with *Aspergillus flavus* with a_W at three levels, (a) 0.99; (b) 0.97; (c) 0.95. Black: live insects contaminated with *A. flavus*. Grey: dead insects contaminated with *A. flavus*. Live and dead insects contaminated with *A. flavus* from each treatment with the same letter are not significantly different according to Tukey's test ($P > 0.05$). T8: *A. flavus* + *T. confusum*; T9: *P. lilacinum* + *A. flavus* + *T. confusum*; T11: *A. flavus* + *T. confusum* + BHT; T12: *P. lilacinum* + *A. flavus* + *T. confusum* + BHT.

evaluated, coinciding with previous studies *in vitro* (Barra et al., 2013a). The preservation of entomopathogenic fungi in maize grains was suggested by Coudron et al. (1985), who reported that cereals were optimal substrates for the development and survival of these fungi. Moreover, Mar et al. (2012) proved that *Paecilomyces lilacinus* strains were able to maintain a high germination percentage (of 80%) after 60 days of incubation in rice, wheat, maize and sorghum. It is of great interest that the entomopathogenic fungi have the capacity to remain in the same ecosystem where it interacts with insect vectors of aflatoxigenic fungi and under the same environmental conditions in which *A. flavus* is able to grow and produce AFB_1 (Gqaleni et al., 1996; Nesci et al., 2005). *P. lilacinum* was able to inhibit the growth of *A. flavus* and the accumulation of AFB_1 when interacting with the pathogen in maize meal extract agar (Barra et al., 2013a). However, this behavior was not observed in grains. Thus, it is important to clarify that the *A. flavus* inoculum used in this study was higher than that previously tested *in vitro* (Barra et al., 2013a). Comparing the population sizes of *A. flavus* at the end of the incubation periods, we can conclude that, in the sterile grains used in this study, counts were higher than those observed in non-sterile grains (Nesci et al., 2009). This may have occurred because *A. flavus* must interact with the natural mycoflora in non-sterile grains. These biological interactions would 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, the insecticidal activity of the treatment composed by *P. lilacinum* + BHT showed similar mortality rates at the three a_W conditions assayed. However, percentages were variable when the entomopathogenic fungus and the antioxidant were used alone. The combined treatment showed the highest insecticidal activity against *T. confusum*, even when maize was inoculated with *A. flavus*. Purwar and Sachan (2006) showed that combinations of insecticides such as imidacloprid and oxydemeton methyl with *Beauveria bassiana* were more toxic against *Spilarctia obliqua* than

Table 2
Influence of treatments on AFB_1 accumulation by *A. flavus* strain RCM89 in maize at different water activity levels.

a_W	Treatments					
	T1	T3	T5	T9	T11	T12
0.99	1.1	115	2 (98.4)	175 (0)	10 (92.1)	1.8 (98.4)
0.97	Nd	235	6 (97.4)	210 (10.9)	4 (98.3)	1.6 (99.3)
0.95	Nd	495	250 (49.5)	294 (40.7)	70 (85.6)	60 (87.8)

The percentage of inhibition of AFB_1 accumulation is in parentheses.
AFB_1 values were in ng g^{-1}.
Nd: not detected.
T1: maize without chemical compound and fungal inoculum; T3: maize with *A. flavus*; T5: *A. flavus* + BHT; T9: *P. lilacinum* + *A. flavus* + *T. confusum*; T11: *A. flavus* + *T. confusum* + BHT; T12: *P. lilacinum* + *A. flavus* + *T. confusum* + BHT.

when used alone. *B. bassiana* and *Metarhizium anisopliae* combined with sublethal doses of imidacloprid as a contact or oral treatment synergistically increased mortality of *Diaprepes abbreviates* (Quintela and McCoy, 1998). This synergistic effect was due to a reduction in motility produced by imidacloprid, which increased the adhesion of spores that would normally be removed by friction as larvae move through their tunnels in soil. Neves et al. (2001) and Hirose et al. (2001) suggested that insects that die from contact with chemical products will be quickly colonized by saprophytic bacteria, eliminating the chances for development of entomopathogenic fungi. As a consequence, no conidia formation will occur. In this study, saprophytic microorganisms did not colonize insects. *P. lilacinum* was isolated from all of the dead insects collected from maize of T12. On the other hand, *A. flavus* contamination was found in both dead and live insects collected from all of the treatments. The combined treatment of BHT + *P. lilacinum* was unable to reduce *A. flavus* populations due to pronounced differences in the growth rates of both fungi. Previously, we performed a study to evaluate the growth rates of *A. flavus* and *P. lilacinum in vitro*. *A. flavus* showed a growth rate of approximately 0.9 mm d^{-1} with a_W between 0.95 and 0.99 (Nesci et al., 2011a). In contrast, *P. lilacinum* showed a growth rate not faster than 0.18 mm d^{-1}. Moreover, the growth rate was inhibited when decreasing the a_W (Barra et al., 2015).

BHT applied at sublethal doses did not reduce the rapid growth rate of *A. flavus* in this study. This caused high counts of this fungus at the end of the incubation period. However, an increase in the efficiency of *P. lilacinum* as insecticide was observed.

This result is in contrast with previous results from stored maize with unmodified a_W and treated with high doses of antioxidants. Synthetic antioxidants at concentrations of and above 20 mM had an insecticidal effect and decreased the dispersion of aflatoxigenic Aspergilli during storage (Nesci et al., 2011a).

Reduction of aflatoxin B_1 is not always due to a decrease in the growth of *A. flavus*. In this study, production of aflatoxin B_1 was detected in all of the treatments at 11 days of incubation. The highest level of AFB_1 was produced in control treatments with a_W at 0.95, while the lowest level was produced in the presence of treatment T12 (*P. lilacinum* + *A. flavus* + *T. confusum* + BHT) with a_W at 0.97. These results are not consistent with previous studies in maize meal extract agar (Nesci and Etcheverry, 2006), in irradiated maize (Nesci et al., 2007) and in natural maize (Nesci et al., 2011a). In those works, no aflatoxin B_1 production was detected in most of the treatments after 11 days of incubation. The amount of aflatoxin B_1 produced by *A. flavus* in the presence of *P. lilacinum* and/or the antioxidant was low compared to the control. These findings suggest that stimulation of aflatoxin production did not occur with subinhibitory doses of the antioxidant and are contrary to observations with other inhibitors (Yousef and Marth, 1981; Marshall and Bullerman, 1986).

In conclusion, the combined treatment of *P. lilacinum* + BHT (T12) was the most effective in reducing the total living insect vectors and the AFB_1 levels. Therefore, it represents a promising strategy for an integrated management of pests in stored maize.

Conflict of interest

The authors declare that they have no conflict of interest.

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