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Evaluation of food grade antioxidant formulation for sustained antifungal, antiaflatoxigenic and insecticidal activities on peanut conditioned at different water activities

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ABSTRACT

The aim of this study was to investigate antifungal and insecticidal activity of two microencapsulated antioxidants: 2(3)-tert-butyl-4 hydroxyanisole (BHA) and 2,6-di(tert-butyl)-p-cresol (BHT) against *Aspergillus* section *Flavi* and *Oryzaephilus surinamensis* (L.), a vector carrier of aflatoxigenic fungi on stored peanuts. Susceptibility of *Aspergillus* section *Flavi*, insects, and aflatoxin B_1 accumulation in sterile peanut kernels conditioned at two different water activities (a_w) (0.83 a_w and 0.95 a_w) was determined with different doses of antioxidant formulations (10, 20 and 30 mM) during 45 days. Moreover, *Aspergillus* section *Flavi* isolation frequency from live and dead insects was evaluated. The BHA formulation completely inhibited *Aspergillus* section *Flavi* development regardless of a_w and doses assayed. Antifungal effect of microencapsulated BHT was highly dependent on a_w, with 86–100% fungal inhibition at 20 and 30 mM, at the lowest a_w (0.83 a_w) and at the end of the experiment. No aflatoxin accumulation was detected in samples treated with the BHA formulation. In general, low levels of *Aspergillus* section *Flavi* were detected in dead insects. Our results show efficacy for 45 days, in addition microencapsulated BHT could be an alternative to control peanut pests in dry kernels.

1. Introduction

Peanuts (*Arachis hypogaea* L.) are an important food and feed commodity in Argentina. This product is important for the Argentinean economy: (i) by having a total production of 1.16 million tons in 2014/15 harvest season with an increase of 13% of the total production with respect to the last crop year; (ii) by ranking among the world's largest producers of peanuts; (iii) as the leading peanut exporter since 2012 with a fluctuation between 0.44 and 0.68 million tons (SIIA, 2015). Peanuts are considered to be a high-risk product for contamination with aflatoxins (AFs) since it is frequently contaminated with fungi, particularly *Aspergillus flavus* and *Aspergillus parasiticus*, and for the long peanut drying times and occurrence of rainy periods after uprooting (Fonseca, 2012). Many studies reported that *A. flavus* and *A. parasiticus* are among the major storage fungi found regularly in stored peanuts (Atayde et al., 2012; Asis et al., 2005; Horn and Dorner, 1998; Horn, 2005; Jubeen et al., 2012; Passone et al., 2014). Passone et al. (2010) reported the prevalence of *Aspergillus* section *Flavi* aflatoxin producing strains (65 and 75%) on stored peanut in big bags with four different a_w levels. In addition, mycotoxins can be produced in grains in the field, and also during transport and storage where conditions are suitable for their production. Moreover, postharvest losses of agricultural food commodities due to the deterioration by different storage insect pest, is a serious problem in peanuts (Muggleton et al., 1991).

One of the main insect pests in the ecosystem of stored grain is *Oryzaephilus surinamensis* (L.). Constant migration of insect populations within a granary ecosystem efficiently contributes to dispersion of viable fungal spores of several species, including *Aspergillus* spp., which are carried on the vector's body surface or are deposited with its feces (Saint Geroges-Gridelet, 1984). Due to the constant interactions among substrates, biological and non-biotic factors may promote a moldy substrate and toxin accumulation in stored grains (Barra et al., 2013). Development of moulds and insects is commonly controlled using synthetic products, but continuous and indiscriminate use of chemical preservatives in foods and feeds could lead to toxic effects for consumers and

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generation of resistant microorganisms (Daborn et al., 2007; López-Malo et al., 2000). The increasing knowledge of persistent residues, along with the carcinogenic and toxic effects of some synthetic insecticides and fungicides, has resulted in the need to obtain alternatives for control growth of mycotoxigenic fungi and insect pests. Alternatives include the use food grade antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (phenolic antioxidants), which have shown insecticidal and antifungal effects on stored peanuts (Nesci et al., 2011a; Passone et al., 2007, 2008a,b, 2009a). Effective insecticide concentrations of these substances ranged from 20 to 30 mM (Nesci et al., 2011a), which were similar to those used for *Aspergillus* section *Flavi* and aflatoxins inhibition (Passone et al., 2009b). However, the analysis of antioxidant residual levels in stored peanuts shows a fast reduction of these substances, probably due to the environmental and biological factor interactions (Passone et al., 2008c). One option is to apply microencapsulation technology in order to protect food grade antioxidants from the action of physicochemical and technological agents and to slow the release (Shahidi and Han, 1993). Thus, the aim of this study was to investigate the fungicidal and insecticidal activity of microencapsulated BHA and BHT against *A. flavus*, *A. parasiticus*, aflatoxin B_1 accumulation and *O. surinamensis* (L.) on peanuts.

2. Materials and methods

2.1. Substrate

Natural peanuts collected during the harvest season 2013–2014 from Córdoba, Argentina, with an initial water content of 0.67 a_w and aflatoxin B_1 (AFB_1)-free were used throughout this study. The water activity (a_w) of sterile peanuts was adjusted by aseptic addition of distilled water to kernels placed inside sealed containers, which were kept at 4 °C for 48 h with periodic hand-shaking during this time. The amount of water necessary to reach the different a_w levels was determined by calibration curves (water activity-mL vs. water to be added/g substrate) previously constructed (Table 1). Therefore, a_w of kernels was modified at 0.83 and 0.95 by the addition of 35 and 150 μL/g of sterile water, respectively and checked with an AquaLab Water Activity Meter 4TE (Decagon Devices, Inc.) with an accuracy of ±0.001.

2.2. Insects

Cultures of one strain of the saw-toothed grain beetle *O. surinamensis* (L.) (Order *Coleoptera*, Family Cucujidae) were obtained from the Laboratory of Agricultural Zoology, Faculty of Agronomy, University of Buenos Aires, Argentina. Mixed-sex adults 1–3 weeks old were used in the assays. Insects were reared on a diet of wheat flour, corn starch and yeast (10:10:1.5) in plastic containers containing 200 g of the mixture. Insects were reared at 27 ± 1 °C and 70 ± 5% relative humidity (RH).

Table 1
Amount of water necessary to reach the different a_w levels in peanut kernels.

a_w	Water (mL/100 g)
0.67	0.0
0.75	0.7
0.77	1.3
0.81	2.5
0.87	5.0
0.92	10.0
0.93	12.5
0.95	15.0

2.3. Fungal isolates and preparation of spore suspension

Two mycotoxigenic isolates were included in this study: *A. flavus* (RCP08108) and *A. parasiticus* (RCP08299). The references in parentheses are the codes of cultures held in the Microbial Ecology Laboratory Collection, Department of Microbiology and Immunology, National University of Río Cuarto, Córdoba, Argentina. Isolates were sub-cultured on malt extract agar (MEA) plates and incubated at 25 °C for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia of each mould from MEA plates and they were suspended in 5 mL of peptone water solution (0.1%). After homogenization, suspensions were adjusted using a Neubauer counting chamber to achieve final concentrations of $1–5 \times 10^4$ spores/mL.

2.4. Preparation of antioxidant formulations

Industrial grade antioxidants, 2(3)-tert-butyl-4 hydroxyanisole (BHA) and 2,6-di(tert-butyl)-*p*-cresol (BHT), obtained from Eastman Chemical Company (Kingsport, Tennessee, United State) were used as core material. BHA had a purity of 98.5% containing as trace elements sulphated ash 100 μg/g, citric acid 2.5 μg/g, arsenic 3 μg/g, and heavy metals 10 μg/g. BHT had a purity of 99% containing as contaminants ash 100 μg/g, arsenic 3 μg/g and heavy metals 10 μg/g. Contaminant compounds of industrial grade antioxidants did not exceed allowed levels by JECFA (1996). Gelatin (type A, gel strength 240 bloom) and gum arabic were used as the wall material. All other chemicals used in this work were of analytical grade. Microcapsules were made by complex coacervation following the methodology proposed by Girardi et al. (2015). Twenty five mL of gelatin and gum arabic solution 5% p/v were prepared at 50 °C in a thermostatic bath (Decalab SRL). The pH of gum arabic solution was adjust to 6 with sodium hydroxide 1 M (NaOH). Four hundred and fifty μL of core material (BHA or BHT 70% and 50% p/v in peanut oil, respectively) were added into the gum arabic solution, forming an emulsion by magnetic stirring (Auto Science, AM-5250B). Then, a gelatin solution was added and the mix was stirred at 400 rpm during 10 min at 50 °C. After that, pH was adjusted to 4 with hydrochloric acid 1 M (HCl) solution and the stirring was continued for 10 min. Subsequently, pH was adjusted to 9 with NaOH 1 M and stirring another 10 min. Then, temperature was lowered until 10 °C in an ice bath and 5 mL of formaldehyde was added during 10 min, to firm the gelatin-gum arabic coating. Microcapsules obtained were washed twice with distilled water and frozen at −80 °C during 3 h and freeze-dried with a chamber (L-T8-A-B3-CT, RIFICOR) pressure <0.05 mbar and −45 °C for 72 h. Finally, samples were ground using a mill CT 193 Cyclotec™ to obtain a fine powder. Empty capsules were performed with the same methodology but without the addition of BHA or BHT, in order to be used as control by replacing the core material with peanut oil.

2.5. Microcosm assays. Inoculation and incubation conditions

To determine antifungal, antiaflatoxigenic and insecticidal activity of formulations, 300 g of peanut kernels were distributed into plastic jars of 500 mL capacity and microencapsulated antioxidants were added at different doses (10, 20, 30 mM) and mixed to obtain an homogeneous distribution. Plastic jars containing both control and treated peanut kernels were inoculated with 1 mL of *A. flavus* and *A. parasiticus* spores suspension (500 uL of 10^4 spores/mL, of each fungal isolate). Twenty adults of *O. surinamensis* (L.) were introduced into each jar, then the jars were put in the chamber under controlled conditions (25 ± 1 °C, 70 ± 5% r.h.). The assay was done using three replicates per treatment. Antifungal, antiaflatoxigenic and insecticidal effects were assayed at different times

during a 45 day holding period. Treatments with empty capsules (without BHA or BHT) and without capsules were used as control.

2.5.1. Antifungal activity

Colonization of kernels was assessed as propagules per gram of peanuts after 12, 20, 27, 35 and 45 days of incubation. A sample of 10 g was taken from each treatment, milled and shaken for 30 min with 90 mL of 1 g/l peptone: distilled water plus 0.06 g/l of Triton X-100. Serial decimal dilutions until 10^{10} for control samples and until 10^{7} for treated samples were done. An aliquot of 0.1 mL of three last serial dilutions of each treatment was spread on the surface of solid medium (Pitt and Hocking, 1997), Dichloran Rose Bengal Chloramphenicol Saline (DRBCS) (Horn and Dorner, 1998) by triplicate. Plates were incubated in darkness at 25 °C for 5 days. Characteristic colonies were counted and average was reported as colony forming units of *Aspergillus* section *Flavi* per gram (CFU/g) of peanut.

2.5.2. Aflatoxin B_1 analysis

Determination of AFB_1 was performed according to AOAC's official method 994.08 with some modifications. After 27 and 35 days of incubation, total AFs were extracted from a representative sample (25 g) of ground peanut with 100 mL of acetonitrile: water (84:14 v/v) for 30 min using an orbital shaker, and the supernatant was filtered through Whatman N° 4 filter paper. Then, 5 mL of the extract was applied to a multifunctional cleaned column (R-BIOPHARM Rhone LTD). The filtrate (2 mL) was evaporated to dryness until to high-performance liquid chromatography (HPLC) analysis. Aflatoxin B_1 quantification was performed according to Trucksess et al. (1994), with some modifications. Dry extracts were dissolved in 200 μL of acetonitrile: water (9:1) and derivatized with 700 μL of trifluoroacetic acid: acetic acid: water (20: 10: 70). One hundred μL of the derivatized solutions were injected in HPLC system (Waters 2696 separation module, Waters, Milford, USA). Chromatographic separations were performed on a stainless steel C18 reverse phase column (150 × 4.6 mm i.d., 5 um particle size, Phenomenex, Torrance, California, USA). Water: methanol: acetonitrile (66.6: 16.7: 16.7) mixture was used as mobile phase at a flow rate of 1.5 mL/min. A Waters 2475 module was used for fluorescence detection (λexc 360 nm; λem 440 nm). Detection (LOD) and quantification (LOQ) limits of the analytical method were 1.5 ng/mL and 4.5 ng/mL, respectively.

2.5.3. Insecticidal activity of BHA and BHT microencapsulated

Insecticidal activity was checked daily during 45 days from the beginning of exposure. Percentage of insect mortality was calculated using the Abbott correction formula (Abbott, 1925).

2.5.4. Determination of fungal contamination in insects

During 45 days of incubation, peanut kernels from each plastic jar were separated and all insects were collected and counted. Insects that survived the period of the experiment were killed by freezing at −20 °C. Live and dead insects were plated directly on DRBCS and incubated at 25 °C for 7 days. Number of insects from who developed colonies of *Aspergillus* section *Flavi* was determined.

2.6. Data analysis

Analysis of variance of fungal count, mycotoxins and insect mortality was used in order to assess significant differences due to effect of different variables assayed (a_w, formulation concentration and incubation time). Fisher's least significant difference (LSD) tests were performed to establish differences among mean values of CFU/g and insect mortality variables at $p < 0.05$.

Probit analysis (Finney, 1971) was performed to estimate lethal time 50 (LT_{50}) and lethal doses 50 (LD_{50}) with a confidence limit of 95%. Statistical analyses were carried out with Statgraphics® Plus version 5.1 (Manugistics, Inc, Maryland,USA).

3. Results

3.1. Effects of antioxidant formulations on Aspergillus section Flavi populations in peanut kernels

Effects of single factors as well as their two and three way interactions on Log_{10} of CFU/g levels for both types of controls (control without capsules, CWC and with empty capsules, CEC) were determined by ANOVA ($p < 0.1$). This fungal parameter was highly significantly affected by the type of control ($p = 0.0$, $DF = 1$, f-value = 25.2) and a_w ($p = 0.0$, $DF = 1$, f-value = 46.2).

An ANOVA test for Log_{10} CFU/g between peanut treated with microencapsulated BHA or BHT compared with CEC was also performed. For treatments with BHA microcapsules, the fungal count was highly significantly affected ($p < 0.1$) by all factors: treatment (T), water activity (a_w) and time (t) (T: $p = 0.0$, $DF = 3$, f-value = 22.5; a_w: $p = 0.0$, $DF = 1$, f-value = 21; t: $p = 0.0$, $DF = 4$, f-value = 4.6) and by T*a_w interaction ($p = 0.0$, $DF = 3$, f-value = 23.5). The major effect was produced by T*a_w followed by T, a_w and t. In addition, *Aspergillus* section *Flavi* levels in peanut treated with BHT formulation was also significantly affected by all individual factors (T: $p = 0.0$, $DF = 3$, f-value = 13.4; a_w: $p = 0.0$, $DF = 1$, f-value = 207.7; t: $p = 0.0$, $DF = 4$, f-value = 11.4) and by the three way interaction (T*a_w*t: $p = 0.0$, $DF = 12$, f-value = 2). The major effect was due to peanut a_w condition followed by T and t.

Fig. 1(A–D) shows *Aspergillus* section *Flavi* counts at different a_w levels (0.83 and 0.95), incubation periods (12, 20, 27, 35 and 45 days) and in presence of BHA and BHT formulations (10, 20 and 30 mM). Peanut kernels without capsules (CWC) and treated with empty capsules (CEC) were used as the controls in this study.

Aspergillus section *Flavi* counts in untreated peanuts (CWC) increased throughout the incubation period, regardless of a_w condition. Fungal levels at the first sampling time were 4 and 6.5 log_{10} CFU/g at 0.83 and 0.95 a_w, respectively reaching around 8 log_{10} CFU/g at the end of the assay. A similar result was observed when *Aspergillus* section *Flavi* counts were estimated in the CEC conditioned at the highest a_w studied (0.95). However, at 0.83 a_W fungal level remained near to 2 log_{10} CFU/g during 27 days, while *Aspergillus* counts were lower than 1×10^2 CFU/g at the two last incubation times.

Antifungal effects of chemical formulations were dose, a_w and t dependent. The two highest doses (20 and 30 mM) of microencapsulated antioxidants were able to prolong the fungal growth control during 45 days when they were applied on peanuts conditioned at 0.83 a_w. Formulation based on BHA produced significant ($p < 0.05$) *Aspergillus* count decreases (around 99%) during the first 20 days of incubation at all concentrations tested (Fig. 1A,B). Meanwhile, levels of *Aspergillus* section *Flavi* in presence of this antioxidant increased between 1.5 and 2.5 log_{10} CFU/g at 27 and 35 days, respectively. Though, total suppression of *A. flavus* and *A. parasiticus* growth was observed at the end of the incubation period, except in peanut treated with 10 mM at 0.95 a_w where fungal level remained around of 1×10^2 CFU/g. Antifungal effectiveness of the formulation based on BHT was highly affected by a_w. Fungal counts were significantly decreased ($p < 0.05$) between 90 and 75% by the presence of 20 and 30 mM of microencapsulated BHT throughout the incubation period but only at 0.83 a_w (Fig. 1C,D). Meanwhile, at the highest a_w, *Aspergillus* section *Flavi* levels in peanut treated with 10 mM were similar to CWC at all sampling done. Finally, when antifungal activity of microencapsulated BHT was evaluated at 0.95 a_w, formulation was highly effective only until the first 12 days at 30 mM where *Aspergillus* counts were lower than 1×10^2 CFU/g.

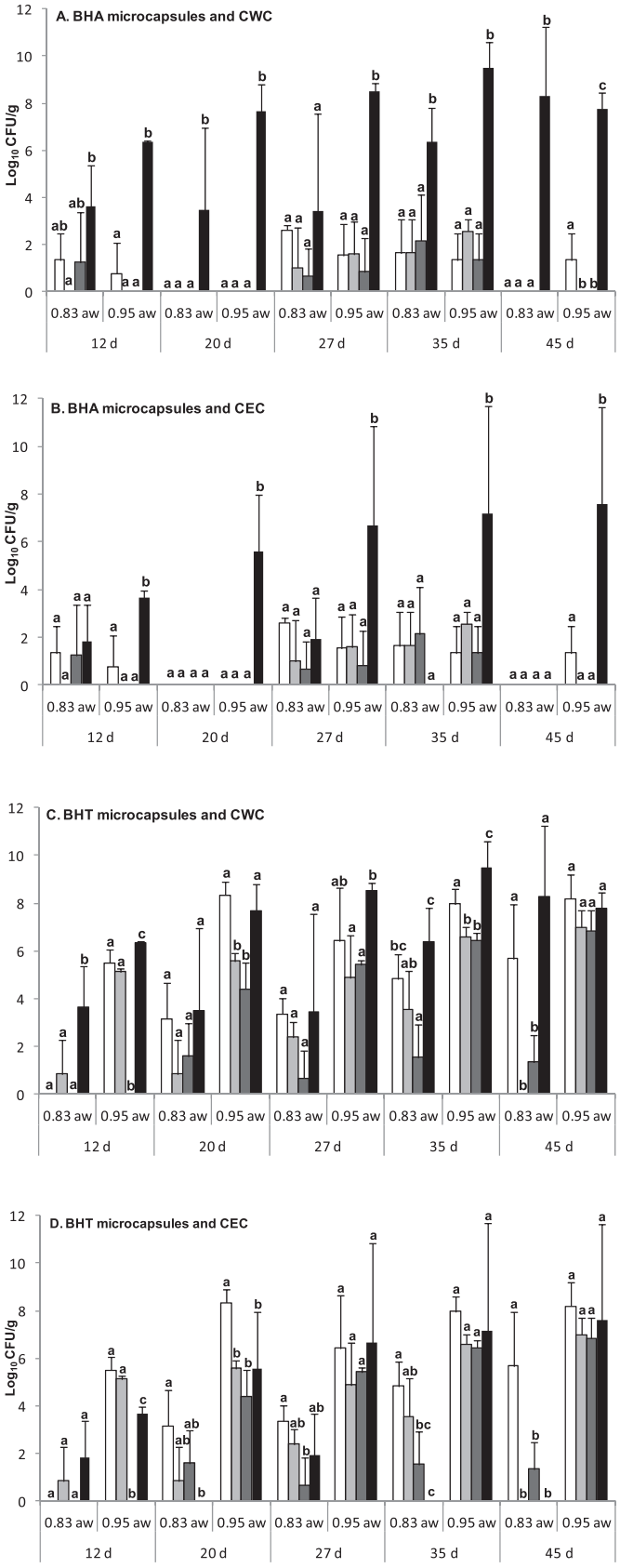

Fig. 1. Mean levels of *Aspergillus* section *Flavi* population (Log CFU/g) isolated from peanut kernels treated with different formulations [A) BHA microcapsules compared with CWC; B) BHA microcapsules compared with CEC; C) BHT microcapsules compared with CWC and D) BHT microcapsules compared with CEC] during 12, 20, 27, 35 and 45 days and conditioned at 0.83 and 0.95 a_w. □ 10 mM; ▫ 20 mM; ■ 30 mM; ▪ corresponding control. Data with the same letter for the same a_w between CEC or CWC and treatments with formulations are not significantly different according to LSD test ($p < 0.05$).

3.2. Effects of BHA formulation on aflatoxin B_1 accumulation in peanut kernels

Aflatoxin B_1 (AFB_1) accumulation was evaluated in peanut samples from CWC, CEC and treated with BHA microcapsules at two sampling periods (27 and 35 days), due to the increase of *Aspergillus* section *Flavi* population at these times. ANOVA test showed significant differences ($p < 0.1$, $DF = 1$, f-value $= 2.89$) in AFB_1 accumulations by *A. flavus* and *A. parasiticus* in the control treatments.

Aqueous stress condition of the CWC peanut kernels aided with incubation time increased accumulation of AFB_1 by *Aspergillus* section *Flavi* strains. At 0.83 a_w, the levels of this metabolite were 72% higher than at 0.95 a_w, while their levels increased around of 50% between 27 and 35 days of incubation. As *Aspergillus* section *Flavi* growth assays, presence of empty capsules in peanut kernels affected AFB_1 levels with reductions between 92.4 and 99.9% compared with CWC, regardless of studied factors (a_w and t). No toxin values were detected in peanut samples treated with BHA formulation, regardless of concentration, a_w and time tested.

3.3. Insecticidal activity of microencapsulated BHA and BHT

Mortality percentages of *O. surinamensis* (L.) estimated in peanut samples from both CWC and CEC controls were 35 and 55%, respectively. CEC showed greater number of dead insects compared to the CWC.

Toxicity of antioxidant formulations against insects are summarized in Fig. 2. In general, the maximum dose (30 mM) of both microencapsulated antioxidants significantly affected the survival of insect population after 45 days of exposure. At this concentration, BHA demonstrated the highest insecticidal activity (100%) at the two a_w studied. Meanwhile, significant mortality percentages ($p < 0.05$) of 80 and 97% were reached with the dose of 20 mM at 0.83 and 0.95 a_w, respectively. Furthermore, results from Probit analyses showed that lethal dose 50 (LC_{50}) and lethal time 50 (LT_{50}) were found to be 9.95 ± 1.2 mM and 2.6 and 10.4 days (30 mM) and 3.9 and 20.9 days (20 mM) at 0.83 and 0.95 a_w, respectively. In relation to insecticidal effects produced by BHT formulation, the two highest doses (20 and 30 mM) killed about 60 and 75% of the pests at 0.83 and 0.95 a_w, respectively. The lowest dose (10 mM) produced 30% of mortality at 0.83 a_w, but 95% of mortality was observed at 0.95 a_w. For this microencapsulated antioxidant, Probit analysis showed LC_{50} and LT_{50} of 29.3 ± 17.9 mM and between 19.4 and 31.2 days, respectively, regardless of peanut water condition.

3.4. Effect of BHA and BHT microcapsules on infection of *O. surinamensis* (L.) with *Aspergillus* section *Flavi*

The isolation of *Aspergillus* section *Flavi* from dead and live insects showed significant differences ($p < 0.05$) in that peanut samples treated with 10 mM of microencapsulated BHA at both a_w levels (0.85 and 0.95), in which live insects presented the highest levels of fungal contamination (Fig. 3). Dead insects (99%) obtained from the sample treated with 20 mM of BHA formulation presented a low percentage of infection (12%) with *Aspergillus* section *Flavi* at 0.83 a_w, while at 0.95 a_w only surviving insects (18%) presented fungal contamination. *Aspergillus* section *Flavi* was not isolated from insects collected from peanut treated with 30 mM of BHA formulation at both a_w assayed, coinciding with the low fungal incidence.

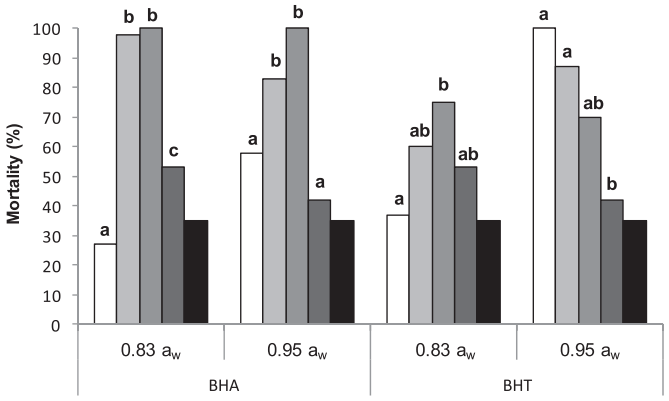

Fig. 2. Effect of BHA and BHT microcapsules on *O. surinamensis* mortality, evaluated in peanut kernels conditioned at two different water activities. □ 10 mM; ▒ 20 mM; ▒ 30 mM, ■ CEC and ■ CWC. Bars with different letters for each a_w between CEC and treatments with formulations are significantly different according to LSD test ($p < 0.05$).

Regarding the effect of the BHT microcapsules, a high percentage of contaminated insects (70%) was found in peanut samples treated with 10 mM and significant differences ($p < 0.05$) between live (60%) and dead insects (8%) were observed at 0.83 a_w. Meanwhile, at this a_w condition, low levels of contaminated insects (13 and 15%) were observed in peanut treated with the higher doses (20 and 30 mM). At 30 mM only live insects presented *Aspergillus* contamination. Finally, high *Aspergillus* section *Flavi* levels were presented in insects isolated from peanut kernels conditioned at 0.95 a_w, regardless of BHT concentrations assayed, agreeing with the high fungal incidence.

4. Discussion

In the present work, *Aspergillus* section *Flavi* and *O. surinamensis* (L.) were exposed to different concentrations of microencapsulated BHA and BHT in peanut kernels incubated during 45 days in microcosms. Peanut treated with empty capsules produced *Aspergillus* section *Flavi* growth, AFB_1 accumulation and *O. surinamensis* (L.) development reductions compared to CWC. Hence, wall material seems to enhance antifungal and insecticidal effect and impart

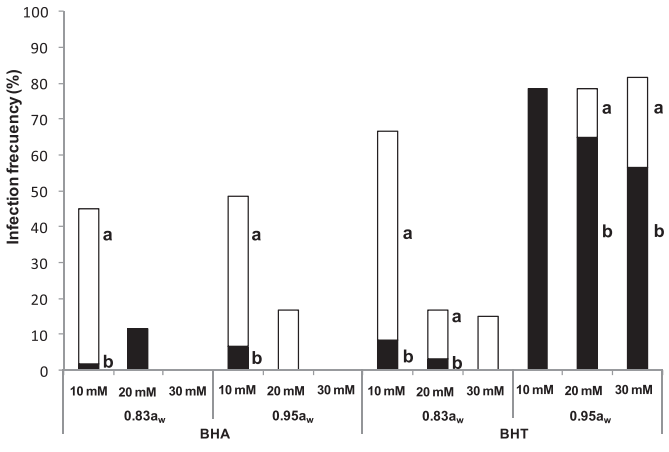

Fig. 3. Infection percentages of *O. surinamensis* with *Aspergillus* section *Flavi* at different treatments. Bars in dark are dead insects and bars in white are live insects contaminated with *Aspergillus*. Live and dead insects with *Aspergillus* section *Flavi* for each treatment with the same letter are not significantly different according to LSD test ($p < 0.05$).

specific properties of the coating. This result is in accordance with Shtykova et al. (2008), which studied the effect of different BHT microcapsules against pine weevil (*Hylobious abietis*). They founded an increase of antifeedant effect when BHT formulations contained thickener and an alkylglucoside based nonionic surfactant as components of their wall. In our case, formaldehyde used as crosslinking agent could act as antifungal and antiaflatoxigenic agent together with antioxidants. Agu and Palmer (1999) revealed that low doses (0.1%) of formaldehyde had a good antifungal effect on sorghum seeds. Besides, Codifer et al. (1976) and Mann et al. (1970) applied formaldehyde on peanut and peanut meal in order to inactivate AFs present in these substrates. These authors observed high toxin inhibitions at the end of the assay. Conversely, activity against fungi, AF accumulation and insect pests present in peanut storage system has been previously evaluated under the effect of free BHA and BHT showing that concentrations ≥20 mM are needed for prevention of grain deterioration (Etcheverry et al., 2011). The screening test can be considered as a guideline for the application of capsules containing these pesticide antioxidants.

In this research, decrease of biological activity demonstrated that antioxidants were trapped in the microparticles in an active form. Recently, both BHA and BHT showed a sustained released rate from capsules (Girardi et al., 2015). Our results showed that these encapsulated preservatives could inhibit the growth of *Aspergillus* section *Flavi* over at least 45 days, compared to less than 35 days for free BHA and BHT in peanut system (Passone et al., 2008a). Similarly, bioactivity of free nisin and nisin-loaded solid lipid nanoparticles (SLN) was compared by Prombutara et al. (2012). Free nisin displayed antimicrobial activity against *Listeria monocytogenes* DMST2871 and *Lactobacillus plantarum* MSTR850 for three and one day, respectively. After that, bacterial growth it recovered suggesting that significant antibacterial effects no persisted beyond these times. In contrast, the inhibitory effect of nisin against *L. monocytogenes* DMST2871 and *L. plantarum* from loaded SLN was prolonged more than 20 and 15 days, respectively. Negative control of unloaded SLN did not show any antibacterial activity.

High antifungal action (75–100%) by BHA formulation was found in this assay. This behavior was independent of dose and a_w tested. Meanwhile, antifungal activity of BHT was highly dependent of peanut a_w. The last preservative formulation only was effective against mould at the lowest a_w (0.83) and at the two higher doses applied, by decreasing *Aspergillus* growth in the order of 80 and 90% at 20 and 30 mM respectively. In addition, BHA microcapsules reduced their inhibitory effects against *Aspergillus* section *Flavi* development between 27 and 35 days of incubation, but fungal levels never exceeded 2.5 $\log_{10}$ CFU/g. This behavior could be due to encapsulation efficiency that was around of 80.4% (Girardi et al., 2015). Therefore, free antioxidants could be responsible for the antifungal effect during the first 20 days of incubation and antioxidants doses released from the microparticles were still insufficient to completely inhibit the fungal growth during this period. These results are according to Girardi et al. (2015) who analyzed the antioxidant permanence inside of microcapsules. They founded that BHA and BHT were stable with time and still remained in the order of 464–1624 ng/g and 71–857 ng/g after 30 and 80 days of incubation for BHA and BHT, respectively. Previously, it has been observed a short time of antifungal action of free BHA and BHT (10 and 20 mM) applied on peanut (Passone et al., 2008a), leading to a loss of activity by insufficient concentrations (no detected-30%) at 35 days of incubation (Passone et al., 2007). Probably, this is due to volatilization (Dziezak, 1986; Rajalakshmi and Narasimhan, 1995) or biological degradation (Passone et al., 2008a).

Moreover, in the present work the effect of microencapsulated BHA on AFB_1 contamination was evaluated after 27 and 35 days of peanut incubation at 0.83 and 0.95 a_w. These times were selected

according to *Aspergillus* section *Flavi* population results, where significant increases occurred of fungal counts. However, accumulation of this metabolite was not detected in any of treated samples. Similarly, previous studies carried out with free BHA on irradiated and natural peanut kernels demonstrated that antiaflatoxigenic effect was higher than antifungal effect (Passone et al., 2007, 2008a). A relationship between increase of AF accumulation and oxidative stress based on chemical induction has been documented (Jayashree and Subramanyam, 2000; Molyneux et al., 2007; Reverberi et al., 2005). Therefore, action mechanism of antiaflatoxigenic activity of BHA appears to be associated with attenuation of oxidative stress response of fungus to organic peroxides (Deshpande et al., 1996).

While there are reports which evaluate the antifungal activity of antioxidants, these studies were performed with the free compounds (Nesci et al., 2011b; Passone et al., 2007, 2008a,b; 2009a,b). Therefore, this is the first work in which encapsulated food grade antioxidants were applied to control of fungi and mycotoxins in a food system. Some researchers have worked on encapsulation of plant extracts and biocide agents with antifungal target (Fernandes et al., 2012; Jämsä et al., 2013; Lam et al., 2013). Nevertheless, these works were made under agar model but in most cases, results obtained on culture media cannot necessarily be extrapolated to natural ecosystems where there are other factors that influence fungal growth (Garcia et al., 2011).

In this work, microencapsulated food grade antioxidants demonstrated a high insecticidal activity against *O. surinamensis* (L.) at 45 days of incubation. Mortality percentages higher than 60% were obtained with the application of 20 and 30 mM of both formulations, while total insect mortality was produced by the presence of high dose of BHA. Insecticidal effects of free food grade antioxidants BHA and BHT and the mixture of them (BHA/BHT) were demonstrated against *O. surinamensis* (L.) in stored peanuts by Nesci et al. (2011a) giving a 100% of mortality at 20 mM of each chemical compound and the mixture 20 mM/20 mM. Also, Barra et al. (2013) showed that free BHA and BHT and the mixture of them (20 mM concentration) produced high insecticidal activities against *Sitophilus zeamais*, *Tribolium confusum* and *Rhyzopertha dominica* (pest and vector carriers of aflatoxigenic fungi in stored maize) after 120 days of incubation. Shtykova et al. (2008), could establish an antifeedant activity close to 1 (complete inhibition of feeding) on pine weevil (*H. abietis*) with BHT microcapsules containing Eudragit copolymer and surfactant. In our work, BHA microcapsules had the best insecticidal effect due that LT_{50} and LD_{50} values were lower than estimated for BHT formulation. Some authors have studied mortality of this insect and they estimated LT_{50} and LD_{50} values by using some commercial insecticides, chemical compounds, and essential oils (Ebadollahi et al., 2010; Pourmirza, 2006; Pourmirza and Tajbakhsh, 2008; Toews and Subramanyam, 2003).

Finally, the highest dose assayed (30 mM) of BHA formulation produced a fungal dispersion decrease during all incubation period, regardless of a_w conditions, where no *Aspergillus* section *Flavi* was isolated from living or dead insects. Although, BHT capsules showed a high effectiveness against insects, most of them showed *Aspergillus* section *Flavi* contamination, except at 0.83 a_w for the highest doses (20 and 30 mM) where low CFU/g levels were observed at the end of the experiment.

In conclusion, microencapsulation methodology not only preserved antifungal, antiaflatoxigenic and insecticidal effectiveness of antioxidants but rather that prolonged for up to 45 days. The BHA formulation was the most effective strategy for achieving development control of both fungi and insects. This formulation at 10 mM was able to inhibit fungal contamination but only it killed about 40% of insects. Therefore, dose of 20 mM could be better for achieve an integrated control strategy in stored peanut. On the other hand, high doses ($\geq$20 mM) of BHT formulation could be an alternative to currently used synthetic fungicides to control peanut contamination in dry grains. BHA and BHT microcapsules could be developed as part of a plan for prevention of the aflatoxigenic mycobiota and insect vectors in stored peanut to increase the shelf life. Therefore, the results of this work open the door for further studies to evaluate the effect of microencapsulated antioxidants in peanut stored in big bags in a storage company and in order to establish the influence of local climatic conditions, biological interactions and storage time.

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