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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