Food-Grade Antioxidants and Antimicrobials To Control Growth and Ochratoxin A Production by *Aspergillus* Section *Nigri* on Peanut Kernels

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MS 09-539: Received 30 December 2009/Accepted 19 March 2010

**ABSTRACT**

Each year, a significant portion of the peanuts produced cannot be marketed because of fungal disease at the postharvest stage and mycotoxin contamination. Antioxidants could be used as an alternative to fungicides to control ochratoxigenic fungi in peanuts during storage. This study was carried out to determine the effect of the antioxidant butylated hydroxyanisole (BHA) and the antimicrobial propyl paraben (PP) on the lag phase before growth, growth rate, and ochratoxin A (OTA) production by *Aspergillus* section *Nigri* strains in peanut kernels under different conditions of water activity (a_w) and temperature. At 20 mM/g BHA, 18°C, and 0.93 a_w, complete inhibition of growth occurred. For PP, there was no growth at 20 mM/g, 18°C, and 0.93, 0.95, and 0.98 a_w. BHA at 20 mM/g inhibited OTA production in peanuts by *Aspergillus carbonarius* and *Aspergillus niger* aggregate strains at 0.93 a_w and 18°C. PP at 20 mM/g completely inhibited OTA production at 18°C. The results of this work suggest that PP is more appropriate than BHA for controlling growth and OTA production by *Aspergillus* section *Nigri* species in peanut kernels.

Mycotoxin contamination is a serious problem in developing countries where climatic conditions and agricultural and storage practices are conducive to fungal growth and toxin production. At present, Argentina does not control either the storage conditions or fungal contamination in cereals, oil seeds, and other raw materials.

Peanut (*Arachis hypogaea* L.) is one of the most important agricultural products in Argentina. The central-south region of Córdoba province produces 94% of the country’s peanuts. The peanut industry exports 90% of its product, with Argentina second in the world in peanut exports (5). Each year, a significant portion of the peanuts produced cannot be marketed because of fungal disease at the postharvest stage and mycotoxin contamination (24).

Ochratoxin A (OTA) is an important mycotoxin for human and domesticated animals with a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity, and immunotoxicity (15). OTA has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (7, 19).

In previous studies, 27 to 32% of Argentinean strains in the *Aspergillus* section *Nigri* produced OTA, and 32 to 50% of the stored peanut samples were contaminated with OTA in the first (2003) and second (2004) year of sampling, respectively (10, 11).

Control of environmental conditions such as water activity (a_w) and temperature during storage could prevent *Aspergillus* section *Nigri* growth and OTA production in several agricultural products. For example, an a_w of less than 0.93 and a temperature of less than 15°C prevented *Aspergillus* section *Nigri* strains from growing in peanuts, maize kernels, and dried grapes (3).

Storage fungi are often managed by use of synthetic chemical products (fungicides), but these products may have adverse effects such as carcinogenicity, teratogenicity, and residual toxicity (14). Food-grade antioxidants, e.g., butylated hydroxyanisole (BHA) and butylated hydroxytoluene, and the antimicrobial agent as propyl paraben (PP) have been used safely as alternatives to fungicides to control fungal species in various food and agricultural products (2, 22). These additives can limit fungal growth and the production of fumonisin and aflatoxin on natural substrates (6, 16, 17, 25). These alternative treatments also have antifungal effects against *Aspergillus* section *Flavi* strains on peanuts (17, 18).

Under some conditions, BHA and PP can inhibit the growth of strains from *Aspergillus* section *Nigri* species and inhibit OTA accumulation on peanut-based medium (4, 12). No data are available on the impact of these food-grade compounds on fungal growth and OTA production on peanuts. These additives could be used as alternative treatments for the control ochratoxigenic fungi in peanuts during storage.

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TABLE 1. Effect of butylated hydroxyanisole (BHA) concentrations on lag phase of Aspergillus section Nigri strains at different a_w levels and temperatures

<table>
<thead>
<tr>
<th>Aspergillus strain</th>
<th>18°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM/g</td>
<td>1 mM/g</td>
</tr>
<tr>
<td>A. carbonarius</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCPG</td>
<td>0.98</td>
<td>38 HI</td>
</tr>
<tr>
<td>A. carbonarius</td>
<td>0.95</td>
<td>44 EFGHI</td>
</tr>
<tr>
<td>RCP203</td>
<td>0.93</td>
<td>56 DEFGHI</td>
</tr>
<tr>
<td>A. niger RCP42</td>
<td>0.98</td>
<td>43 EFGHI</td>
</tr>
<tr>
<td>A. niger RCP191</td>
<td>0.98</td>
<td>48 GH</td>
</tr>
<tr>
<td></td>
<td>0.93</td>
<td>50 DEFGHI</td>
</tr>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

* Hour values followed by the same letter are not significantly different according to the LSD test (P > 0.001).

b —, under the experimental conditions, strains were not able to reach the exponential phase.

The aim of the present study was to evaluate the effect of the best food-grade antioxidant, BHA, and the antimicrobial PP on (i) lag phase before growth, (ii) growth rate, and (iii) OTA production by strains of *Aspergillus* section *Nigri* on peanut kernels under different environmental conditions.

**MATERIALS AND METHODS**

**Fungal strains.** Four *Aspergillus* section *Nigri* strains were evaluated: *A. carbonarius* strains RCPG and RCP203 and *A. niger* aggregates RCP42 and RCP191. All strains were isolated from peanut kernels in Argentina and were identified based on morphological characters (8). OTA production was assayed on YES medium (2% yeast extract and 15% sucrose) (11). Strains were maintained in glycerol (15%) at −80°C and kept in the culture collection at the National University of Río Cuarto (Córdoba, Argentina).

**Substrate.** Peanut kernels (Runner variety) that had been irradiated (7 kGy) and retained their germinative capacity were used. Kernels were checked for sterility and the absence of OTA before use. Irradiated peanut kernels were rehydrated to 0.93, 0.95, and 0.98 a_w by adding sterile distilled water. The a_w of representative samples of each treatment was checked at the beginning of the experiment with an AquA Lab Series 3 water activity meter (Decagon Devices, Inc., Pullman, WA).

**Antioxidants.** The antioxidant BHA (2(3)-tert-butyl-4-hydroxyanisole) and antimicrobial PP (n-propyl 4-hydroxybenzoate) were obtained from Sigma-Aldrich Chemical Co. (Dorset, UK). Stock solutions of BHA and PP (1 M) were prepared by dissolving 18 g in 100 ml of 95% ethyl alcohol in water (vol/vol).

**Inoculation and incubation conditions.** Peanut kernels in bottles (500 g) were conditioned with an appropriate amount of water for the a_w levels. From stock solutions of BHA or PP, 0.5, 2.5, 5, and 10 ml were added to peanuts to reach final concentrations of 1, 5, 10, and 20 mM/g. The peanut mixtures were kept at 4°C for 48 h with manual periodic shaking to allow absorption and to reach equilibrium. Twenty grams of peanuts was placed as a monolayer into sterile petri dishes and inoculated centrally with 5 μl of a fungal spore suspension (1 × 10^6 spores per ml) from a 7-day-old culture growing on 2% malt extract agar (20). Aqueous spore suspensions were prepared by adding various amounts of glycerol to reach the desired a_w (0.93, 0.95, and 0.98). Inoculated peanuts in plates with the same a_w were sealed in plastic containers. Each container held beakers with a glycerol-water solution at the same a_w as the peanuts to maintain a constant relative humidity. Four replicate plates per treatment were used and incubated at 18 and 25°C for 4 weeks. The entire experiment was conducted twice (n = 8). Control plates without BHA or PP also were prepared.

**Growth parameters.** Assessment of growth was made daily during the incubation period by examination of peanut kernel cultures with a binocular magnifier (×10). Two diameters of the growing colonies were measured at right angles from each other until the colony reached the edge of the plate. The radii of the colonies were plotted against time, and a linear regression analysis was applied to obtain the growth rate (millimeters per day) as the slope of the line.

The length of the lag phase before growth also was determined, with lag phase defined as the time (hours) required for each colony to reach 2.5 mm in diameter. Growth analyses were conducted for 3 a_w levels × 2 temperatures × 4 strains × 4 replicates × 1 antioxidant and 1 antimicrobial × 5 treatments per antioxidant and antimicrobial.

**OTA determination.** At the end of the incubation period, three replicates per treatment for *A. carbonarius* RCPG and *A. niger* aggregate RCP191 were destructively sampled, dried at 50°C for 24 h, and stored at −20°C until analyzed for OTA. Five grams of a finely ground peanut sample was added to a 250-ml Erlenmeyer flask with 50 ml of an acetonitrile-water mixture (84:16). The contents were shaken in an orbital shaker for 30 min and filtered through filter paper (Whatman no. 1, Whatman, Clifton, NJ). The extract (7 ml) was added to a clean up column (MycoSep 229 Ochra column, MFC, Romer Labs, Inc., Union, MO) and acified with 70 μl of acetic acid. Four milliliters of the
purified extract was removed, evaporated to dryness, redissolved in 200 μl of the mobile phase (acetonitrile–water–acetic acid, 57:41:2), and injected into the high-performance liquid chromatography (HPLC) system, which consisted of an HP model 1100 pump (Hewlett Packard, Palo Alto, CA) connected to a Hewlett Packard 1100 Series variable wavelength detector and a data module Hewlett Packard Kayak XA (HP ChemStation revision A.06.01).

OTA levels in each treatment were measured as previously described (23) with minor modifications. HPLC with a fluorescence detector (λex 330 nm, λem 460 nm) was applied. A C18 column (150 by 4.6 mm inside diameter, 5-μm particle size; Supelcosil LC-ABZ, Supelco, Bellefonte, PA) was used. The mobile phase was pumped at 1.0 ml/min, the injection volume was 100 μl, and the retention time was 4 min. The detection limit was 1 ng/g. Toxin analyses were conducted for 3 αw levels × 2 temperatures × 2 replicates × 4 replicates × 1 antioxidant and 1 antimicrobial × 5 treatments per antioxidant and antimicrobial.

**Assay of spiking and recovery of OTA.** A stock solution of 4 ml of OTA in methanol with at 5 μg/ml was prepared for recovery determination. Each OTA-free finely ground peanut sample (5 g) contained in a 250-ml Erlenmeyer flask was spiked with OTA at an equivalent of 0.1, 0.5, 1.0, and 1.8 μg/g. Spiking was carried out in triplicate, and a single analysis of the blank sample was carried out. After 16 h for the solvent to evaporate, extraction solvent (acetonitrile–water, 84:16, vol/vol) was added, and the OTA concentration was determined using the protocol previously described.

**Statistical analysis.** Data were analyzed with an analysis of variance. All data were transformed to log(x + 1) to homogenize the variance. Means were compared using Fisher’s protected least significant difference (LSD) test to determine the influence of the abiotic factors (water activity, temperature, antioxidant and antimicrobial concentrations, and strains), growth rate, length of lag phase, and OTA concentration produced by the species tested. The analysis was conducted using the PROC GLM in SAS (SAS Institute, Cary, NC) (21).

**RESULTS**

**Effect of BHA and PP treatments on lag phase and growth rate.** In control treatments, the lag-phase length increased as αw and the temperature decreased for all strains assayed. In general, all strains behaved similarly at a given temperature (Tables 1 and 2). From 10 mM/g BHA or PP, the increase in the length of the lag phase was significant for both BHA and PP (P < 0.001) under all conditions assayed with respect to the control. At 20 mM/g BHA and 18°C, the strains did not reach the exponential phase at 0.93 αw, whereas at 0.95 and 0.98 αw, the lag phase increased significantly in comparison to the control assay (63%) (Table 1). With the PP treatment, at 18°C and 20 mM/g, the strains were not able to reach the exponential phase at any αw assayed, whereas at 25°C and 20 mM/g, longer lag phases at 0.93 and 0.95 αw were observed (Table 2).

BHA at 20 mM/g, 18°C, and 0.93 αw completely inhibited growth of all strains (Fig. 1). At this same temperature with 5 and 10 mM/g BHA, the growth rate was reduced only for the *A. carbonarius* strains at all αw assayed (Fig. 1A and 1B). At 25°C and 0.95 and 0.98 αw, a significant reduction in growth rate was observed with 5 and
10 mM/g BHA in all strains ($P < 0.001$). At the lowest level of BHA (1 mM/g) and 25°C, fungal growth was stimulated at 0.95 $a_w$ in both A. niger aggregate strains (Fig. 1C and 1D) ($P < 0.001$).

PP completely inhibited growth at the highest level used at 18°C under all $a_w$ conditions for all strains (Fig. 2). Complete growth inhibition also occurred with 10 mM/g at 0.93 $a_w$ for both A. carbonarius strains (Fig. 2A and 2B). At 25°C, mycelial growth was significantly reduced at 20 mM/g PP at all $a_w$ levels and for all strains assayed. For A. niger aggregate strains, at 0.98 $a_w$, significant differences in growth rate were observed from 5 to 20 mM/g PP, whereas at 0.95 $a_w$, a significant increase in growth rate occurred at 1 mM/g PP ($P < 0.001$) (Fig. 2C and 2D).

**Effect of BHA and PP treatments on OTA production.** Recovery of the toxin was 97.3% ± 7.4% from the peanuts at different OTA levels tested. In general, OTA production did not follow the same pattern as the growth rate. BHA at 20 mM/g completely inhibited OTA production at 0.93 $a_w$ in both A. carbonarius and A. niger aggregate strains (Fig. 3A and 3C). At the same temperature and 0.95 $a_w$, a reduction to around 94% in toxin production was observed at 5, 10, and 20 mM/g in the A. carbonarius RCPG strain (Fig. 3A). At the same BHA concentrations, a reduction in OTA production in the A. niger aggregate strains was observed only at 0.95 $a_w$ (Fig. 3C). Toxin production at 25°C had a different pattern than that observed at 18°C. In general, at 25°C, BHA did not reduce OTA production under any of the conditions assayed. At 20 mM/g BHA and 0.93 $a_w$, a 300% increase in OTA accumulation occurred for A. carbonarius; whereas for A. niger RCP191, this increase was observed at 5 mM/g and 0.98 $a_w$ (Fig. 3B and 3D).

PP at 20 mM/g and 18°C completely inhibited OTA production in both assayed strains under all $a_w$ conditions (Fig. 4A and 4C). OTA production also was reduced at 10 mM/g and 0.95 $a_w$ in the A. carbonarius strain. For the A. niger aggregate strain at 10 mM/g PP and 18°C, a significant reduction (70%) in OTA production was observed at all $a_w$ levels assayed (Fig. 4A and 4C). At 25°C, the OTA production profile of both strains changed. PP at 20 mM/g and 25°C completely inhibited OTA production at 0.93 $a_w$ in the A. carbonarius strain (Fig. 4B). For the A. niger aggregate strain at all PP concentrations and
levels assayed, OTA production was not reduced (Fig. 4D).

The analysis of variance for the effect of single variables, i.e., strains, $a_w$, temperature, and BHA or PP level, revealed that all variables alone and all interactions were significant ($P < 0.001$) in relation to lag phases, growth rates, and OTA production (Tables 3 and 4).

**DISCUSSION**

This study was conducted to determine whether BHA and PP could inhibit growth and OTA production by *Aspergillus* section *Nigri* strains at 18 and 25°C under different $a_w$ conditions in peanuts. The lag phase, mycelial growth rate, and OTA production by *Aspergillus* section *Nigri* strains were all significantly influenced by BHA and PP treatment, incubation temperature, $a_w$ level, and their interactions. Similar results have been obtained in previous studies with BHA and PP in *Aspergillus* section *Flavi* and *Fusarium* strains on maize and peanuts, respectively (6, 17, 18, 25). BHA at 20 mM/liter on peanut-based medium completely inhibited both growth and OTA production at 25°C at all $a_w$ levels and regardless of the *Aspergillus* section *Nigri* strain assayed (4). In the present study, the complete inhibition of growth on peanuts occurred only at 18°C and 0.93 $a_w$. OTA production did not follow the same general pattern as that observed for growth. OTA production was completely inhibited at the same concentration of BHA (20 mM/g) but under all environmental conditions. In the present study on peanuts, OTA production was observed only at 18°C and at all $a_w$ levels assayed, but the response of strains at 25°C was different.

These results partially agree with those of Passone et al. (17), who evaluated two concentrations of BHA on aflatoxin B$_1$ (AFB$_1$) production by *A. flavus* strains growing on peanuts at 28°C. These researchers observed that 20 mM/g BHA completely inhibited AFB$_1$ production at 0.982 and 0.955 $a_w$ at only 11 days of incubation, whereas stimulation of AFB$_1$ production occurred after 35 days of incubation.

**FIGURE 2.** Effect of propyl paraben (PP) on growth rate (millimeters per day) of *Aspergillus carbonarius* RCPG (A) and RCP203 (B) and *Aspergillus niger* aggregates RCP42 (C) and RCP191 (D) at $a_w$ levels of 0.98 ($\bullet$), 0.95 ($\triangle$), and 0.93 ($\square$) and different temperatures. Mean values with a letter in common are not significantly different according to the LSD test ($P > 0.001$).
Recently, Passone et al. (18) found that BHA at 20 mM/g reduced AFB$_1$ production in peanuts by _A. flavus_ strains in the presence of natural competing mycobiota at 0.982 and 0.955 $a_w$ after both 11 and 35 days of incubation, respectively.

In another study (12) conducted with PP and _Aspergillus_ section _Nigri_ strains on peanut-based medium, when PP was added at 5 mM/liter none of the strains could reach the exponential growth phase and growth inhibition occurred at all $a_w$ levels. OTA production followed the pattern observed for growth parameters. These results partially agree with those of the present study; growth and OTA production were both inhibited at the highest level of PP (20 mM/g) and lower temperature (18°C). Only one previous report was found on the effects of PP on growth parameters and mycotoxin production in _Aspergillus_ species in peanuts. Passone et al. (17) found that PP at 10 and 20 mM/g at 0.955 $a_w$ and 30°C inhibited growth by strains of _Aspergillus_ section _Flavi_ by 53 to 100%, whereas AFB$_1$ production was reduced significantly only at 20 mM/g. These results only partially agree with those of the present study; PP at some $a_w$ levels in the _A. carbonarius_ strain did not inhibit OTA production.

In the present work, different levels of BHA or PP (5 mM/g PP and 5 mM/g BHA at 18°C, and 5, 10, and 20 mM/g PP at 25°C) stimulated OTA production by _A. carbonarius_ and _A. niger_ aggregate. This stimulation was observed previously (4, 12) in BHA and PP in vitro studies but only at the lowest concentration assayed (1 mM/liter), which stimulated both fungal growth and OTA production by some _Aspergillus_ section _Nigri_ strains. This result also was observed by others, with other antioxidants and antimicrobials. Passone et al. (16) evaluated the effect of various antioxidants on growth and AFB$_1$ production by _Aspergillus_ section _Flavi_ strains and found that subinhibitory concentrations (1 mM/liter) of butylated hydroxytoluene and propyl hydroxybenzoate increased the fungal growth rate and butylated hydroxytoluene at the same concentration stimulated AFB$_1$ production. Torres et al. (25) found stimulation of fumonisin production by _Fusarium verticilloides_ on maize with 0.5 and 2.7 mM/g BHA at 0.995 and 0.95 $a_w$, respectively.

![Figure 3](http://meridian.allenpress.com/jfp/article-pdf/73/8/1493/1677039/0362-028x-73_8_1493.pdf)
Because antioxidants and antimicrobials can both promote and inhibit growth and toxin production, determining optimal application doses on natural substrates is very important. High levels of antioxidants or antimicrobials do not assure inhibition of mycotoxin production and could produce undesirable effects on the organoleptic properties of peanuts. In contrast, subinhibitory doses plus inadequate distribution of the additive, especially at low $a_w$ levels, could not assuring inhibition of mycotoxin production and could produce undesirable effects on the organoleptic properties of peanuts. In contrast, subinhibitory doses plus inadequate distribution of the additive, especially at low $a_w$ levels, could

![Figure 4](image-url)

**FIGURE 4.** Effect of propyl paraben (PP) on ochratoxin A production by Aspergillus carbonarius RCPG at 18°C (A), A. carbonarius RCPG at 25°C (B), A. niger aggregate RCP191 at 18°C (C), and A. niger aggregate RCP191 at 25°C (D) under different $a_w$ conditions. Detection limit was 1 ng/g.

| TABLE 3. Analysis of variance of effect of water activity ($a_w$), temperature ($T$), concentration of BHA or PP ($C$), and different isolates ($I$) and their interactions on lag phase and growth rate of Aspergillus section Nigri strains |
|---|---|---|---|---|
| Source of variation | df $^a$ | Lag phase $| MS^b | F $^c$ | Growth rate $| MS | F $^c$ |
| $I$ | 3 | 118,320.07 | 10.17$^*$ | 46.52 | 15,759.66$^*$ |
| $C$ | 4 | 17,221,859.92 | 1,488.66$^*$ | 399.52 | 99,999.99$^*$ |
| $a_w$ | 2 | 1,278,237.00 | 107.70$^*$ | 42.33 | 13,452.28$^*$ |
| $T$ | 1 | 1,399,113.58 | 107.70$^*$ | 1.94 | 644.12$^*$ |
| $I \times C$ | 28 | 60,075.76 | 5.13$^*$ | 34.22 | 10,629.59$^*$ |
| $I \times C \times a_w$ | 78 | 108,995.33 | 9.55$^*$ | 10.99 | 3,523.52$^*$ |
| $I \times C \times a_w \times T$ | 119 | 103,023.12 | 8.95$^*$ | 0.38 | 177.16$^*$ |

$^a$ df, degrees of freedom.

$^b$ MS, mean square.

$^c$ Snedecor $F$; * $P < 0.001$. 
stimulate fungal sporulation, growth, and secondary metabolism and increase mycotoxin contamination (13).

The mechanism of inhibition of fungal growth and mycotoxin production by BHA and PP is not known. Several antifungal activity mechanisms have been proposed for these phenolic compounds, e.g., impairment of enzymatic processes involved in energy production and structural component synthesis due changes in the cell membrane, alteration of fungal physiological status, and changes in nucleic acid biosynthesis (1, 9).

The use of antioxidants and antimicrobials could be an alternative to control ochratoxigenic fungi in peanuts during storage before the product is shipped to internal and external markets and processed. In general, the results of this work suggest that PP is more appropriate than BHA for limiting growth and OTA production by Aspergillus section Nigri strains on peanuts, but the antifungal effect is affected by strain and environmental conditions. Further assays are needed to test the effectiveness of BHA and PP alone or in combination as inhibitors of ochratoxigenic Aspergillus section Nigri strains in peanuts in the presence of natural contaminating fungi.

**ACKNOWLEDGMENTS**

This work was supported by grant FONCYT PICT No. 8-14552 (2006 and 2007) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

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