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Short Communication

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Effect of Microencapsulated 2(3)-Tert-Butyl-4-Hydroxyanisole (BHA) at Sub-Lethal Dose on the Growth of *Aspergillus flavus* and Production of Aflatoxin in Peanut Food Model System

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Abstract

The need to ensure the microbiological quality and safety of food products has stimulated interest for new strategies avoiding the use of chemical preservatives in foods and feeds. Microencapsulated food grade antioxidants could be an alternative for control fungal contamination in foods. In this sense, the aim of our study was to examine the effect of a sub-lethal dose of microencapsulated butylated hydroxyanisole (BHA) (0.65 mM) on physiological behavior of *Aspergillus flavus,* an aflatoxin B_1 producer fungi. In this sense, we evaluated growth rate, time to growth, biomass and aflatoxin B_1 accumulation of an isolate of *A. flavus* (RCP08108) with two water activities (0.99 and 0.96 a_w) and two growth substrates (Peanut meal extract agar and peanut kernels). All fungal physiological features studied in this work (growth rate, time to growth, biomass and AFB_1 accumulation), were modified by the application of the sub-lethal dose of BHA formulation, for both growth substrate and water availability. Presence of the encapsulated antioxidant showed significant change in Pearson coefficients respect to the controls for all studied parameters. As conclusion, evaluated sub-lethal dose of BHA formulation, resulted in a reduction on growth and toxin accumulation. Results of this work indicate the need to consider both fungal primary and secondary metabolism to determine the effect of food grade antioxidant formulation, in order to improve the production of microcapsules for application in a future in the food system.

Keywords

Peanut, *Aspergillus flavus*, Aflatoxin B_1, Microcapsules, Butylated hydroxyanisole (BHA)

Introduction

Peanut (*Arachis hypogaea*) is a very important dried fruit considered as one of the most widely used nuts due to their nutritional properties and taste. Particularly, the peanut produced in Argentina is known worldwide for its quality. Due to the economic importance of peanut, its quality and safety is essential for their marketing. However, this dried fruit is considered to be a high-risk product for contamination with aflatoxins (AFs) due that frequent contamination with moulds, specially by *Aspergillus flavus* and *Aspergillus parasiticus* [1], long peanut drying times, and occurrence of rainy periods after uprooting [2]. Toxic secondary metabolites such as aflatoxins, specially aflatoxin B_1 (AFB_1), are considered the most carcinogenic, mutagenic, and teratogenic substances found naturally in foods and feeds [3].

Antifungal chemicals, mainly low molecular weight organic acids, have been generally used for the preservation of grains [4]. However, continuous and indiscriminate use of these chemical preservatives in foods and feeds, could lead to toxic effects for consumers and to the development of resistances in microorganisms [5]. Besides, nowadays, consumers prefer low levels of preservatives in foods or preservative-free products. As a counter measure, we propose the use of food grade antioxidants for control moulds development and aflatoxin production in food as one alternative to the synthetic fungicides. The phenolic antioxidant 2(3)-tert-butyl-4-hydroyanisol (BHA) has shown effect against moulds and insect, vectors of aflatoxigenic fungi, on stored peanuts [6-10]. In these last publications, the doses applied were always below those allowed by the Codex Alimentarius [11] and the US Food and Drug Administration (FDA) (maximum usage level of 200 mg/g of peanut oil). Besides, the effect of pure BHA at sub-lethal doses on *A. flavus* was evaluated by Passone et al. [12, 13]. In both studies, concluded that it is necessary to apply the pure food-grade antioxidant into the peanut storage system at levels higher than 5 mM. On the other hand, another work done by the same author, showed that levels of BHA decreased quickly with time when it was applied in peanut food system due to the interaction with physical and biological factors [9]. In order to avoid the quick decrease of this antioxidant, Girardi et al. [14] applied microencapsulation technology to protect BHA from the action of environmental factors. After that, Garcia et al. [15] determined the lethal doses microencapsulated BHA against a *A. flavus* and *A. parasiticus*, and *O. surinamensis* (L.) on peanuts in microcosm. They conclude that 20 mM of this formulation completely inhibited *Aspergillus* section *Flavi* development and aflatoxin accumulation. Nevertheless, the sub-lethal effect of microencapsulated BHA on the development of aflatoxigenic fungi in the peanut food system has not been studied. The behavior of sub-lethals concentrations of the formulation on growth mould and mycotoxin accumulation is of great importance to know if there is stimulation of the growth and mycotoxins production in this alimentary system. For this, our interest is focused on the growth and mycotoxin accumulation produced by *A. flavus* affected by the exposure to a sub-lethal dose of microencapsulated BHA. In this sense we evaluated: i) growth parameters based on radial growth and biomass dry weight and ii) AFB_1 accumulation in presence of sub-lethal inhibitory concentrations (0.65 mM) of antioxidant formulation, at two different water activities (a_w) in peanut meal extract agar (PMEA) and peanut grains.

Material and Methods

Fungal isolate and preparation of inoculum

A mycotoxigenic isolate (*A. flavus*, RCP08108), obtained from peanut in our lab was included in this research. Reference in brackets are the code of cultures held in the Microbial Ecology Laboratory Collection, Department of Microbiology and Immunology of the National University of Río Cuarto, Córdoba, Argentina. Isolate was sub-cultured on malt extract agar (MEA) plate and incubated at 25 °C for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from MEA plates and it was suspended in 5 mL of distilled peptone water solution (0.1%). After homogenization, the suspension was adjusted using a Neubauer counting chamber to achieve final concentration of $1–5\times10^4$ spores/mL.

Preparation of antioxidant formulation

The antioxidant 2(3)-tert-butyl-4 hydroxyanisole (BHA) microcapsules were made under the methodology applied by Girardi et al. [14]. The antioxidant used for the production of these capsules has industrial grade obtained from Eastman Chemical Company (Kingsport, Tennessee, United State). As wall material, solutions 5% p/v of gelatin (type A, gel strength 240 bloom) and gum Arabic (pH 6) were prepared and maintained at 50 °C in a thermostatic bath (Decalab SRL). On the other hand, a solution of BHA 70% p/v in peanut oil was prepared and added (450 µL) into to gum Arabic solution (50 mL), forming an emulsion by magnetic stirring (Auto Science, AM-5250B). After 10 minutes, the gelatin solution (50 mL) was added and the mix was stirred at 400 rpm during 10 minutes at 50 °C. Then, hydrochloric acid 1M (HCl) was added to adjust the pH to 4 and stirring another 10 minutes. After that the pH was elevated at 9 with NaOH 1M and the stirring continued 10 minutes. Finally, temperature was lowered until 10 °C in an ice bath and 5 mL of formaldehyde was added for 10 minutes, to firm the gelatin-gum arabic coating. The formulation obtained was washed twice with distilled water and frozen at -80 °C for 3 hours. Then a lyophilization process was carried out with a freeze-dried chamber (L-T8-A-B3-CT, RIFICOR) pressure <0.05 mbar and -45 °C for 72 h. Finally, lyophilized microcapsules were ground with a mill CT 193 Cyclotec™ to obtain a fine powder.

Culture medium and peanut kernels preparation

Peanut meal extract agar (PMEA) was prepared at 2% (w/v) with a final pH of 6.5 according to Passone et al. [16]. Water activity (a_w) of the basic medium (0.99) was adjusted to 0.96 a_w, with known amounts of glycerol [17]. Microcapsules were added to culture medium or sterile peanut grains poured in Petri dishes to reach 0.65 mM concentration. Water activity of representative samples of each treatment was checked after autoclaving with an AquaLab Water Activity Meter 4TE with an accuracy of ± 0.001. On the other hand, peanut kernels were sterilized by autoclaving at 120 °C for 20 minutes twice. Water amounts of sterile peanut was adjusted by aseptic addition of distilled water to seeds inside sealed containers which were kept at 4 °C for 48 hours with periodic handshaking during this time. The amount of water necessary to reach the different a_w levels was determined by a calibration curve (water activity-mL vs. water to be added/g substrate) previously made [15] and checked with the AquaLab Water Activity Meter 4TE (Decagon Devices, Inc.). Peanut grains (25 g) were poured in Petri plates forming a single layer and the formulation with or without antioxidant was added. Plates with empty capsules (CEC) (without the antioxidant) and with no treatment (CWC) were used as control.

Inoculation and incubation

Petri dishes were inoculated centrally with 2 µL of a 1-5 × 10^4 spore's/mL suspension. Plates with the same a_w were enclosed in sealed containers along with beakers containing water glycerol at the same water condition [18]. One hundred PMEA Petri dishes and 90 peanut kernels Petri dishes were inoculated and incubated at 25 °C.

Growth assessment

Colony diameter (mm) and biomass (mg dry weight) were measured at different time periods. Colony radius was daily examined for an overall period of 30 days by measurements at right angles with the aid of a ruler and a binocular magnifier, on PMEA and peanut Petri dishes. Mycelium dry weight was measured as [19] on culture medium. Colonies were cut from the medium, transferred to a beaker containing distilled water (100 ml approximately), then heated in a streamer for 30 min to melt agar. Mycelium remained intact was collected and transferred to a dried, weighed filter paper and dried at 80 °C for 18 hrs. Then the filter paper was weighed, and the dry weight of biomass was calculated by difference.

Extraction of AFB_1 from PMEA and peanut kernels

Extraction of AFB_1 from PMEA

Extraction of AFB_1 in culture medium was made using the methodology proposed by Garcia et al. [20]. From each colony, three agar plugs (diameter 4 mm) were removed from inner, middle and outer part of the colonies at 21 days of plates incubations and placed together in a vial. One mL of methanol was added, and the vials were shaken for 5 s. After 60 min, the extracts were shaken and filtered (Millex-HV0.45 µm 25 mm, Millipore Corporation, Bedford, USA) into another vial and stored at 4 °C until analysis by HPLC instrument (Waters, Mildford, MA, USA).

Extraction of AFB_1 from peanut kernels

Determination of AFB_1 in peanut kernels was performed according to AOAC´s official method 994.08 [21] with some modifications. Total AFs were extracted at 21 days of plates incubation 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. 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 under nitrogen stream at 60 °C and stored at 4 °C until to the high-performance liquid chromatography (HPLC) analysis.

Detection and quantification of aflatoxin B_1

Aflatoxin B_1 was detected and quantified by using a HPLC system (Waters 2696 separations module, Waters, Milford, USA). Toxin quantification was performed according to Trucksess et al. [22] 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 inject in HPLC system (Waters 2696 separation module, Waters, Milford, MA, USA) and chromatographic separations were performed on a stainless steel C18 reverse phase column (150×4.6 mm i.d., 5 um particle size, Phenomenex, USA). Water: methanol: acetonitrile (66.6:16.7:16.7) mixture was used as the mobile phase at a flow rate of 1.5 mL/min. A Waters 2475 module was used for fluorescence detection (λ_{exc} 360 nm; λ_{em} 440 nm). The detection limit (LOD) of the analysis was about 1.5 ng AFB_1/g of peanut based on a signal-to-noise ratio of 3:1. Quantification was achieved with a software integrator (Empower, Milford, MA, USA). AFB_1 was quantified on the basis of the HPLC fluorimetric response compared with that of a range of AFB_1 standards.

Statistical analyses

Analysis of variance of growth rates, time to growth and AFB_1 accumulation was used in order to assess significant differences due to growth conditions and BHA formulation assayed. LSD test was used to establish the differences among mean values of the variables under the different levels of factors at $p<0.05$. Pearson correlation coefficient was used to evaluate correlations between studied factors. Statistical analyses were carried out with Statgraphics® Plus version 5.1 (Manugistics, Inc., MD, USA).

Results and Discussion

In the present article we were focused to assess the physiological behavior of *A. flavus* (RCP08108) in presence of a sub-lethal dose (0.65 mM) of microencapsulated BHA. With this respect, our results showed that all studied factors [growth substrate (Gs), treatment (T), water activity (a_w)] and their interactions, affected significantly ($p<0.05$) the evaluated growth parameters ("growth rate" [µ (mm/d)] and "time to growth" [λ (days)]). For measure of the colony, growth rate was highly affected by a_w ($p<0.1$ and $F = 71.23$) followed by Gs ($p<0.1$ and $F = 19.56$) and T ($p<0.1$ and $F = 3.4$). While λ was greatly affected by Gs ($p<0.1$ and $F = 150.50$) and T ($p<0.1$ and $F = 26.5$). On the other hand, both growth parameters obtained from biomass dry weight also were affected by a_w and T (a_w, $p<0.1$; $F = 104.93$ and T, $p<0.1$; $F = 5.50$ for µ and a_w, $p<0.1$; $F = 12.12$ and T, $p<0.1$; $F = 119.03$ for λ). Our research showed a reduction of µ (mm/d) and increase in λ (days) in presence of microencapsulated BHA being growth higher on peanut kernels than on culture medium (data not shown). This reduction in mould growth due to the addition of a low dose of microencapsulated BHA is agree with Passone et al. [13] which showed that sub-lethal doses of a mix of pure BHA : BHT (1+1 mm and 5+5 mm) caused a higher and significant ($p<0.001$) reduction in growth rate of *A. flavus* on PMEA culture medium.

On the other side, the percentages of inhibition for radial growth of *A. flavus* from culture medium and in plates with the kernels are shown in figure 1A and B. Mould growth inhibition was calculated as follow:

$$\%GI = (1-Dt/Dc)*100$$

Where *Dt*: is the diameter of growth zone in the treated plate and *Dc*: is the diameter of growth zone in the control plate. A decreased of the percentage of inhibition for the

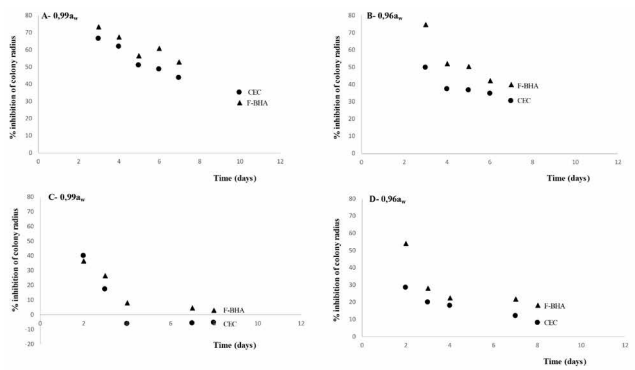

Figure 1: Percentage inhibition of colony diameter of *Aspergillus flavus* in PMEA (A and B) and in peanut grains (B and D) affected by F-BHA (triangles) and by CEC (circles) at two aw (0.99: figures A and C; 0.96: figures B and D).

mould with time is observed for both growth substrates and a_w. The highest inhibition of the radial growth was observed on PMEA with the addition of 0.65 mM of microencapsulated BHA at 0.99 a_w. However, this effect is occurring in the "worst case" scenario when high amounts of water are available to mould behavior. In the case of peanut grains, the highest growth inhibition was obtained also in treated plates with F-BHA, but at the lowest a_w (0.96). This result confirms the importance of working with real substrates due that data obtained from culture medium could not be extrapolated to real situations in natural ecosystems or food system when other factors might interact with mould development [23]. There are many works that studied the effect of a_w on *A. flavus* under different culture media and grains [7, 16, 23-26]. Most of these works found that low levels of a_w produce a decrease of growth mould and the growth parameters were different between culture medium and kernels. For this, it is necessary to evaluate eco physiological behavior of aflatoxigenic strains in food system where exist other factors such as kernel components: fat, carbohydrate, protein, salt and pH which could influence in the effectiveness of the antimicrobial potency [27]. On the other hand, negatives percentages in growth inhibition in grains with empty capsules at 0.99 $_{aw}$ was observed. This means the occurrence of a stimulation in growth of *A. flavus* in CEC. May be, the components of the formulation could be used by fungi as a substrate for their growth. Nevertheless, it is almost unlikely that peanut storage grains in real situation reach this water condition (0.99 $_{aw}$). This result is not agreeing with Garcia et al. [15] which showed that empty capsules seems to enhance antifungal effect the authors conclude that application of formaldehyde as crosslinking agent, could act as antifungal and anti-aflatoxigenic agent together with antioxidants. However, the doses of the BHA formulation applied to peanuts in the last work were 15, 30 and 46 time higher than those used in our work (10, 20 and 30 mM).

With respect to AFB_1 accumulation, ANOVA test showed that toxin levels at the final of incubation period (21 days) was statistically affected by all studied parameters: Gs ($p<0.05$ and $F = 134.2$), a_w ($p<0.05$ and $F = 52.2$) and T ($p<0.05$ and $F = 28.9$) and their two and three-way interaction [(Gs*aw; $p<0.05$ and $F = 24.8$); (Gs*T; $p<0.05$ and $F = 12.6$); (a_w*T; $p<0.05$ and $F = 14.01$) and (Gs*aw*T; $p<0.05$ and $F = 7.04$)]. For the two a_w assayed, AFB_1 levels detected in controls were significantly higher respect to BHA treatment according to LSD test ($p<0.05$), regardless of substrate evaluated (data not shown). After 21 days of incubation, toxin accumulation evaluated on PMEA and peanut plates at different growth substrate and a_w is represented in figure 2. Aflatoxin B_1 levels were between 1-6.4 times higher in peanut kernels than in culture medium. Besides, for the two a_w assayed, the amounts of toxin detected in controls were significantly higher respect to F-BHA treatment according to LSD test ($p<0.05$), regardless of substrate evaluated. Particularly, on PMEA, microencapsulated BHA significantly affected ($p<0.05$) toxin levels, with reductions around 82 and 90% for 0.96 and 0.99 a_w, respectively, compared with CWC. Also, presence of CEC on the culture medium showed significant reductions in AFB_1 accumulation, regardless water condition. As in Garcia et al. [15], components of microcapsules together with antioxidant have an inhibitory effect for the toxin accumulation. As we said before, formaldehyde used as crosslinking substance could act anti-aflatoxigenic agent. Agu and Palmer [28] revealed that low doses (0.1%) of formaldehyde had a good antifungal effect on sorghum seeds. Besides, Codifer et al. [29] and Mann et al. [30] 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. On the other hand, Passone et al. [13] showed that peanut treated with a mix of pure BHA : BHT at sub-lethal dose (<5 mM) can lead to fungal growth, increase resistance structures, and stimulate aflD gene expression and AFB_1 accumulation on PMEA. In the case of peanut kernels, effect of the formulation against toxin accumulation was clearly observed for the lowest a_w (0.96) with reduction of 53%. No statistical differences ($p<0.05$) between AFB_1 levels at 0.99 a_w was observed between treatment and controls. However, at real conditions, peanut is not stored with this high degree of wet.

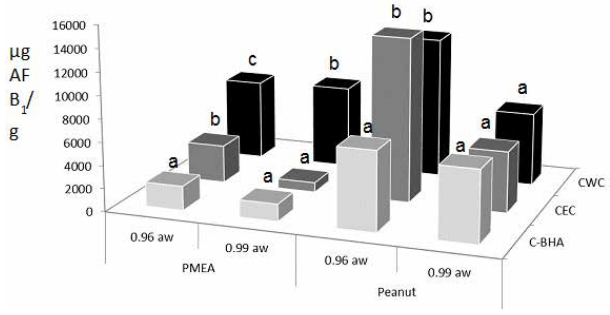

Figure 2: Aflatoxin B_1 levels detected at 21 days of incubation on PMEA and peanut grains treated with F-BHA, CEC and CWC at the two different water activities (0.96 and 0.99).

Finally, Pearson correlation coefficients of the experiments were performed on PMEA and peanut kernels for colony radius and AFB_1 accumulation through experimental time to evaluate toxin evolution together growth (Table 1). Respect to PMEA assay, colony radius (mm) and AFB_1 accumulation, showed significant positive correlation ($p<0.05$) only for CWC at 0.96 a_w. However, for the highest water condition

Table 1: Correlation among aflatoxin B_1 and growth diameter on PMEA and peanut grains at different water activities (Pearson coefficients and P-values).

	a_w		Pearson Correlation Coefficients	P-values
PMEA assay	**0.96**	**C-BHA[1]**	0.68	0.20
		CEC[2]	0.55	0.34
		CWC[3]	0.95	0.01
	0.99	**C-BHA**	0.97	0.00
		CEC	0.91	0.03
		CWC	0.87	0.04
Peanut grains	**0.96**	**C-BHA**	0.91	0.27
		CEC	0.88	0.31
		CWC	0.99	0.01
	0.99	**C-BHA**	0.57	0.62
		CEC	0.48	0.68
		CWC	0.99	0.00

[1]C-BHA: BHA microcapsules.

[2]CEC: Control with empty capsules.

[3]CWC: Control without capsules.

(0.99) all treatment presented positive correlation. This result could be due that environmental conditions are the optimal to toxin accumulation. Also, García et al. [20] showed that AFB_1 increased together with growth of *A. flavus* maize and maize agar medium (MAM) with significant Pearson coefficient ($p<0.05$). Regarding experiments performed on peanut, only significant positive correlation ($p<0.05$) between colony radius and AFB_1 accumulation was found for CWC at both a_w. These results show that presence of a sub-inhibitory dose of F-BHA could lead in changes in *A. flavus* toxin evolution with time by modifying final concentrations, regardless of substrate.

As are shown in results, reductions of both growth and mycotoxin accumulation occurs at the lowest a_w (0.96), so in further studies we should confirm that at as water activity decreases, the antimicrobial effect increases, but using the sub-lethal dose on storage real condition. On the other hand, percentage of colony growth inhibition decreased with time. Thus, taking into account the low dose of BHA used in this work, this makes us think that antioxidant is being consumed/oxidized over time. Our results are agreeing with Passone et al. [9] where they observed a 66% of pure BHA reduction peanut kernels after 30 days by the effect of physical and biological factors. Besides, in our work, samples treated with F-BHA presented the lowest levels of the toxin for both growth substrate and a_w. However, previous studies suggest that sub-inhibitory antioxidant levels carried out to aflatoxin production stimulation [13, 31-33]. Passone et al. [13] founded high levels of the toxin accumulation and an induction in aflD gene expression involved in aflatoxin biosynthesis of *A. flavus* in PMEA when a sub-lethal dose of free BHA and butylated hydroxytoluene mixture (1+1 mM) was used. Therefore, it would be necessary to control the dose to be applied when the formulation is tested under real storage conditions to assess residual level over time.

Yeast and moulds are found in a wide range of environments due to their capacity to utilize a variety of substrates and to their relative tolerance to low pH, low water activity, and low temperature [34]. Generally, foods have essential nutrients for fungal growth, thus fungi can appear and spoil different foods and feeds. Despite the absence of direct correlation between mould growth and mycotoxins production, the prevention of fungal growth in raw materials and foods leads invariably to the prevention of mycotoxins presence [35]. Thus, our next step, is add effective doses of microencapsulated BHA to peanut storage in real conditions to assess mould level and mycotoxins accumulation at different time during the storage period in a peanut processing company.

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This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

The authors declare that have no conflict of interest.

References

1. Passone MA, Nesci A, Montemarani A, Etcheverry M. 2014. Incidence of *Aspergillus* section *Flavi* and interrelated mycoflora in peanut agroecosystems in Argentina. In: Faulkner AG, Aflatoxins: Food sources, occurrence and toxicological effects. New York, USA, pp 157-190.
2. Fonseca H. 2012. A aflatoxina e o amendoim. Boletim Técnico no. 13.
3. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. 2010. Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. *IARC Monogr Eval Carcinog Risks Hum* 92: 1-853.
4. López AG, Theumer MG, Zygadlo JA, Rubinstein HR. 2004. Aromatic plants essential oils activity on *Fusarium verticillioides* fumonisin B_1 production in corn grain. *Mycopathologia* 158(3): 343-349. https://doi.org/10.1007/s11046-005-3969-3
5. López-Malo A, Alzadora SM, Guerrero S, 2000. Natural antimicrobials from plants. In: Tapia MS, Lopez-Malo A, Alzadora S (eds) Minimally processed fruit and vegetables. Fundamental aspect and application, Aspen Publishers, Gaithersburg, USA, pp 237-264.
6. Nesci A, Montemarani A, Passone MA, Etcheverry M. 2011. Insecticidal activity of synthetic antioxidants, natural phytochemicals, and essential oils against an *Aspergillus* section *Flavi* vector (*Oryzaephilus surinamensis* L.) in microcosm. *J Pest Sci* 84(1): 107-115. https://doi.org/10.1007/s10340-010-0333-2
7. Passone MA, Resnik S, Etcheverry MG. 2007. Potential use of phenolic antioxidants on peanut to control growth and aflatoxin B1 accumulation by *Aspergillus flavus* and *Aspergillus parasiticus*. *J Sci Food Agric* 87(11): 2121-2130. https://doi.org/10.1002/jsfa.2975
8. Passone MA, Resnik S, Etcheverry MG. 2008. The potential of food grade antioxidants in the control of *Aspergillus* section *Flavi*, interrelated mycoflora and aflatoxin B_1 accumulation on peanut grains. *Food Control* 19(4): 364-371. https://doi.org/10.1016/j.foodcont.2007.04.014
9. Passone MA, Funes GJ, Resnik SL, Etcheverry MG. 2008. Residue

levels of food-grade antioxidants in postharvest treated in-pod peanuts during five months of storage. *Food Chem* 106(2): 691-697. https://doi.org/10.1016/j.foodchem.2007.06.032

10. Passone MA, Doprado M, Etcheverry M. 2009. Food-grade antioxidants for control of *Aspergillus* section *Flavi* and interrelated mycoflora of stored peanuts with different water activities. *World Mycotoxin Journal* 2(4): 399-407. https://doi.org/10.3920/WMJ2008.1110
11. Codex Alimentarius. 2006. Food additive details. Update up to the 29th session of the Codex Alimentarius Commission. The Joint FAO/WHO committee on Food Additives.
12. Passone MA, Rosso LC, Varela MC, Ciancio A, Etcheverry MG. 2011. Effects of sub-lethal food grade antioxidant doses and environmental stressors on growth, sclerotia, aflatoxins and aflD (nor-1) expression by *Aspergillus parasiticus* RCP08300. *World Mycotoxin Journal* 4(2): 157-167. https://doi.org/10.3920/WMJ2010.1261
13. Passone MA, Rosso LC, Etcheverry M. 2012. Influence of sub-lethal antioxidant doses, water potential and temperature on growth, sclerotia, aflatoxins and aflD (=nor-1) expression by *Aspergillus flavus* RCP08108. *Microbiol Res* 167(8): 470-477. https://doi.org/10.1016/j.micres.2011.11.004
14. Girardi N, Garcia D, Nesci A, Passon MA, Etcheverry M. 2015. Stability of food grade antioxidants formulation to use as preservatives on stored peanut. *LWT - Food Science and Technology* 65(2): 1019-1026. https://doi.org/10.1016/j.lwt.2015.01.052
15. Garcia D, Girardi NS, Passone MA, Nesci A, Etcheverry M. 2016. Evaluation of food grade antioxidant formulation for sustained antifungal, antiaflatoxigenic and insecticidal activities on peanut conditioned at different water activities. *J Stored Prod Res* 65: 6-12. https://doi.org/10.1016/j.jspr.2015.11.002
16. Passone MA, Resnik SL, Etcheverry MG. 2005. *In vitro* effect of phenolic antioxidants on germination, growth and aflatoxin B1 accumulation by peanut *Aspergillus* section Flavi. *J Appl Microbiol* 99(3): 682-691. https://doi.org/10.1111/j.1365-2672.2005.02661.x
17. Dallyn H, Fox A. 1980. Spoilage of material of reduced water activity by xerophilic fungi. In: Gould G, Corry E (eds) Microbial growth and survival in extreme environments. London and New York, Academic Press, pp 129-139.
18. Dallyn H. 1978. Effect of substrate water activity on growth of certain xerophilic fungi. PhD Thesis, South Bank University, London, UK.
19. Taniwaki MH, Pitt JI, Hocking AD, Fleet GH. 2006. Comparison of hyphal length, ergosterol, mycelium dry weight and colony diameter for quantifying growth of fungi from foods. *Adv Exp Med Biol* 571: 49-67. https://doi.org/10.1007/0-387-28391-9_3
20. Garcia D, Ramos AJ, Sanchis V, Marín S. 2012. Effect of *Equisetum arvense* and *Stevia rebaudiana* extracts on growth and mycotoxin production by *Aspergillus flavus* and *Fusarium verticillioides* in maize seeds as affected by water activity. *Int J Food Microbiol* 153(1-2):21-27. https://doi.org/10.1016/j.ijfoodmicro.2011.10.010
21. AOAC Official Method 994.08. 2000. Aflatoxins in Corn, Almonds, Brazil Nuts, Peanuts, and Pistachio Nuts. Multifunctional column (Mycosep) method: official methods of analysis of AOAC International, 17th edition, Volume 2, AOAC International: Natural toxins- (chapter 49), Gaithersburg, Maryland, USA, pp 26-27.
22. Trucksess MW, Stack ME, Nesheim S, Albert RH, Romer TR. 1994. Multifunctional column coupled with liquid chromatography for determination of aflatoxins B1, B2, G1, G2, in corn, almonds, Brazil nuts, peanuts and pistachio nuts: collaborative study. *J AOAC Int* 77(6): 1512-1521. https://doi.org/10.1007/0-387-28391-9_3
23. Garcia D, Ramos AJ, Sanchis V, Marín S. 2011. Modelling the effect of temperature and water activity in the growth boundaries of *Aspergillus ochraceus* and *Aspergillus parasiticus*. *Food Microbiol* 28(3): 406-417. https://doi.org/10.1016/j.fm.2010.10.004
24. Faraj MK, Smith JE, Harran G. 1991. Interaction of water activity and temperature on aflatoxin production by *Asperguillus flavus* and *Aspergillus parasiticus* in irradiated maize seeds. *Food Addit Contam* 8(6): 731-736. https://doi.org/10.1080/02652039109374031
25. Garcia D, Ramos AJ, Sanchis V, Marín S. 2013. Modelling kinetics of aflatoxin production by *Aspergillus flavus* in maize-based medium and maize grain. *Int J Food Microbiol* 162(2): 182-189. https://doi.org/10.1016/j.ijfoodmicro.2013.01.004
26. Holmquist GU, Walker HW, Stahr HM. 1983. Influence of temperature, pH, water activity and antifungal agents on growth of *Aspergillus flavus* and *A. parasiticus*. *J Food Sci* 48(3): 778-782. https://doi.org/10.1111/j.1365-2621.1983.tb14897.x
27. Holley RA, Patel D. 2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiol* 22(4): 273-292. https://doi.org/10.1016/j.fm.2004.08.006
28. Agu RC, Palmer GH. 1999. Development of micro-organisms during the malting of sorghum. *J Inst Brew* 105(2): 101-106. https://doi.org/10.1002/j.2050-0416.1999.tb00012.x
29. Codifer LP Jr., Mann GE, Dollear FG. 1976. Aflatoxin inactivation: treatment of peanut meal with formaldehyde and calcium hydroxide. *J Am Oil Chem Soc* 53(5): 204-206. https://doi.org/10.1007/BF02633305
30. Mann GE, Codifer LP Jr., Gardner HK Jr., Koltun SP, Dollear FG. 1970. Chemical inactivation of aflatoxins in peanut and cottonseed meals. *J Am Oil Chem Soc* 47(5): 173-176. https://doi.org/10.1007/BF02638746
31. Marshall DL, Bullerman LB. 1986. Antimicrobial activity of sucrose fatty acid ester emulsifiers. *J Food Sci* 51(2): 468-470. https://doi.org/10.1111/j.1365-2621.1986.tb11157.x
32. Nesci A, Rodriguez M, Etcheverry M. 2003. Control of *Aspergillus* growth and aflatoxin production using antioxidants at different conditions of water activity and pH. *J Appl Microbiol* 95(2): 279-287. https://doi.org/10.1046/j.1365-2672.2003.01973.x
33. Nesci A, Gsponer N, Etcheverry M. 2007. Natural maize phenolic acids for control of aflatoxigenic fungi on maize. *J Food Sci* 72(5): M180-M185. https://doi.org/10.1111/j.1750-3841.2007.00394.x
34. Huis in't Veld JHJ, 1996. Microbial and biochemical spoilage of foods: an overview. *Int J Food Microbiol* 33(1): 1-18. https://doi.org/10.1016/0168-1605(96)01139-7
35. Garcia D, Ramos AJ, Sanchis V, Marín S. 2009. Predicting mycotoxins in foods: a review. *Food Microbiol* 26(8): 757-769. https://doi.org/10.1016/j.fm.2009.05.014