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Activity of natural compounds from peanut skins on *Fusarium verticillioides* growth and fumonisin B_1 production

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ABSTRACT

The objectives of this investigation were to evaluate the antifungal and antimycotoxigenic properties of peanut skin extracts (PSE) against *Fusarium verticillioides*. The PSE were prepared by a multisolvent extraction procedure, and the activity on growth parameters and fumonisin B_1 (FB_1) production of the three PSE was explored at different concentrations on potato dextrose agar and in artificially infected maize kernels, respectively. The results demonstrated that all PSE had a significant influence on growth rate or lag phase of *F. verticillioides*. The yellow and purple (250–500 $\mu g\,ml^{-1}$) extracts decreased growth rate, whereas brown extract extended the lag phase. Only the yellow extract at 62.5 $\mu g\,ml^{-1}$ was able to affect both growth rate and lag phase. With respect to mycotoxin production a significant stimulation on FB_1 production was observed with purple (62.5 $\mu g\,ml^{-1}$) and brown (250 $\mu g\,ml^{-1}$) extracts. In contrast, a significant decreased in FB_1 was observed at 62.5 $\mu g\,ml^{-1}$ of yellow extract. These findings showed that natural compounds from PSE possess inhibitory effects on *F. verticillioides* growth and mycotoxin production. Thus, PSE could be use as an alternative to minimize fungal contamination.

1. Introduction

Fusarium is a large complex genus with species adapted to a wide range of habitats. They are worldwide in distribution and many are important plant pathogens. However, many species are soil borne and exist as saprophytes important in breaking down plant residues. Fumonisins are fungal secondary metabolites produced by species of *Fusarium*, mainly *F. verticillioides* and *F. proliferatum* (Krska et al., 2007). There are several identified fumonisins, but fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) are the most important and constitute up to 70% of the fumonisins found in naturally contaminated foods (Niderkorn et al., 2009). Fumonisin B_1 and FB_2 are phytotoxic to corn (Lamprecht et al., 1994), cytotoxic to various mammalian cell lines (Abbas et al., 1993) and FB_1 is carcinogenic in rat liver and kidney (IARC, 2002). The occurrence of these analogs in home-grown corn has been associated with an increased risk of esophageal cancer in humans (Shephard et al., 2000). Fumonisin B_1 is the most toxic fumonisin analog and it is considered possible carcinogens to human and classified as class 2B (IARC, 2002). Considering the high incidence of *Fusarium* species on crops and the impact of fumonisin on human and animal health, the application of strategies to prevent their formation in foods, as well as, to eliminate, inactivate or reduce their presence in food products, becomes necessary. For many years, synthetic fungicides have been used for control plant pathogenic fungi. However, the extensive used of these chemicals led to the development of resistance in many areas around the world and also increases the risk of toxic residues in the products (Marei et al., 2012). Thus, the exploitation of natural substances with bioactivity against fungi has been the target of interest in the search for ecologically safe products (Dambolena et al., 2012). Agricultural wastes represent a largely ignored source of high-value phytochemicals and value-added industrial products that could contribute to sustainability objectives (Das and Singh, 2004). Peanut skin is a by-product of the peanut blanching operation that has low economic value despite its high content of active components including flavonoids, phenolic acids, phytosterols, alkaloids, and stilbenes. Some therapeutic effects have been reported for peanut seed extracts, such as antioxidative, antibacterial, antifungal, and anti-inflammatory activities

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(Lopes et al., 2011; Yu et al., 2005). In previous studies peanut skin compounds have been reported as antimicrobial agents (Sarnoski et al., 2012); however, to our best knowledge there is no information of the effects of peanut skin extracts (PSE) on FB_1 production and fungal growth. Thus, the objective of this investigation was to evaluate the antifungal and antifumonisin property of PSE against *Fusarium verticillioides.*

2. Materials and methods

2.1. Materials

Peanut skins from Argentine peanuts (*Arachis hypogaea*) (cv Runner, 2010 crop year) were obtained by a blanching process and were provided for the Company "Lorenzati, Ruesch y Cia", Ticino, Córdoba, Argentina in March, 2010. The peanut skins were kept in a sealed plastic bag and stored at 4 °C until used.

2.2. Peanut skin extraction

The ethanolic extract (EE) from peanut skin was obtained according to the method published by Nepote et al. (2004) with slight modifications. Briefly, to obtain the EE, the peanut skins were previously defatted by two extractions with n-hexane (50 ml each 10 g peanut skins) during 12 h each one at room temperature. The dry defatted peanut skins (20 g) were extracted by solid–liquid extraction using ethanol 70%, during 24 h by maceration in darkness at room temperature. The extract was filtered and the residue was extracted again under the same conditions. The combined filtrate was evaporated to dryness in a rotary evaporator at 35 °C.

2.3. Separation of the ethanolic extract

The crude EE was purified by partition with 90 ml hexane, 300 ml ethyl acetate (EtOAc) and 55 ml water. The EtOAc fraction was evaporated in a rotary evaporator and separated with ethanol in a column packed with Sephadex LH-20 (internal diameter: 10 mm, length: 33.5 cm, elution flow 0.5 ± 0.05 ml min^{-1}). Fractions with different colors in visible light were separated from the column and identified as: yellow, purple and brown.

2.4. Fungal culture

The culture of *F. verticillioides* M3125, isolated from maize in California is a fumonisin-producing strain (Leslie et al., 1992). The strain obtained from carnation leaves-agar by monosporic isolation, was used in all experiments.

2.5. Antifungal activity of peanut skin extracts

Inoculum was prepared by growing on PDA agar for 7 days at 25 °C to obtained heavily sporulating cultures. A conidial suspension was placed in aqueous solution, after homogenizing, the suspension was counted using a Neubauer chamber and adjusted to 10^6 conidia ml^{-1}.

The PSE were tested at different concentrations of 500, 250, 125 and 62.5 μg ml^{-1} of PDA agar to control growth of *F. verticillioides* M 3125. Peanut skin extracts were dissolved in 70% ethyl alcohol and added to the autoclaved based medium. PDA plates were inoculated centrally with 10 μl of the conidia suspension and were incubated for 8 days at 25 °C.

The radial mycelial growth was determined by periodical measurement of two right-angled diameters of the colonies. Colony diameters versus time were plotted and radial growth rates (mm day^{-1}) were evaluated from the slope by linear regression. Lag phase was determined as the abscissa from growth rate curves. All the experiments were performed in triplicate.

2.6. Effect of peanut skin extracts on FB_1 biosynthesis on maize

Fumonisin B_1 biosynthesis was determined by using healthy maize as substratum. Corn grain free from FB_1 (25 g), was placed in 250 ml dark Erlenmeyer flasks and sterilized for 2 consecutive days in an autoclave for 15 min at 121 °C. The PSE first dissolved in 70% ethyl alcohol and then mixed with water. Water solution (8 ml) was added on autoclaved maize in order to reach 35% humidity. The used concentrations were 62.5 and 250 μg ml^{-1}. Maize was then inoculated with 50 μl of a conidial suspension (10^6 conidia ml^{-1}) of *F. verticillioides* prepared as described above. Immediately, in order to obtain good homogenization, flasks with inoculated maize were shaken vigorously. Treatments were incubated 28 days in dark at 25 °C, with manual stirring the first 5 days. Control flasks were prepared following the same procedure; however, no PSE were added on water. Four replications of each treatment were done.

2.7. FB_1 quantification

Briefly, after incubation, fermented maize was sterilized in an autoclave for 15 min at 121 °C and dried in a vacuum oven at 60 °C until constant weight was achieved. Later, 10 g of dried maize was finely ground. The FB_1 was extracted with ultrapure water by shaking the powder and water for 2 h in an orbital shaker. The aqueous extracts were centrifuged at 9000 × *g*, and filtered through filter paper (Whatman no. 4, Whatman International, Maidstone, UK). Samples (500 μl) from the aqueous extracts were diluted with acetonitrile (500 μl). The quantification of the diluted extracts was performed following the methodology proposed by Shephard et al. (1990). Briefly, an aliquot (50 μl) of this solution was derivatized with 200 μl of o-phthaldialdehyde. This solution was obtained by adding 5 ml of 0.1 M sodium tetraborate and 50 ml of 2-mercaptoethanol to 1 ml of methanol containing 40 mg of o-phthaldialdehyde. The derivatized samples were analyzed by Hewlett Packard HPLC equipped with a fluorescence detector. The wavelengths used were 335 and 440 nm for excitation and emission, respectively. An analytical reversed-phase column C18 (150 mm × 4.6 mm internal diameter and 5 mm particle size) was connected to a precolumn C18 (20 mm × 4.6 mm and 5 mm particle size). The mobile phase was methanol, NaH_2PO_4 0.1 M (75:25); the pH was set at 3.35 ± 0.2 with orthophosphoric acid, and a flow rate of 1.5 ml min^{-1}. The quantification of FB_1 was carried out by comparing the peak areas obtained from aqueous extracts with those corresponding to the standards of 10.544, 5.135 and 2.567 μg ml^{-1} of FB_1 (PROMEC, Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, Republic of South Africa).

2.8. Statistical evaluation

Statistical analyses were conducted using INFOSTAT/Professional 2005p.1 (F.C.A.-Universidad Nacional de Córdoba, Argentina) at $p = 0.05$. Data from these studies were analyzed through two-way analysis of variance (ANOVA). Normality of data was tested using the Shapiro–Wilk test. Comparisons between treatments were performed by the DGC (Di Rienzo, Guzmán and Casanoves) test (Di Rienzo et al., 2002). Results giving *P* values <0.05 were considered significantly different.

3. Results and discussion

The effect of PSE on *F. verticillioides* M3125 growth was evaluated in a range of concentrations between 0 and 500 μg ml^{-1}. The

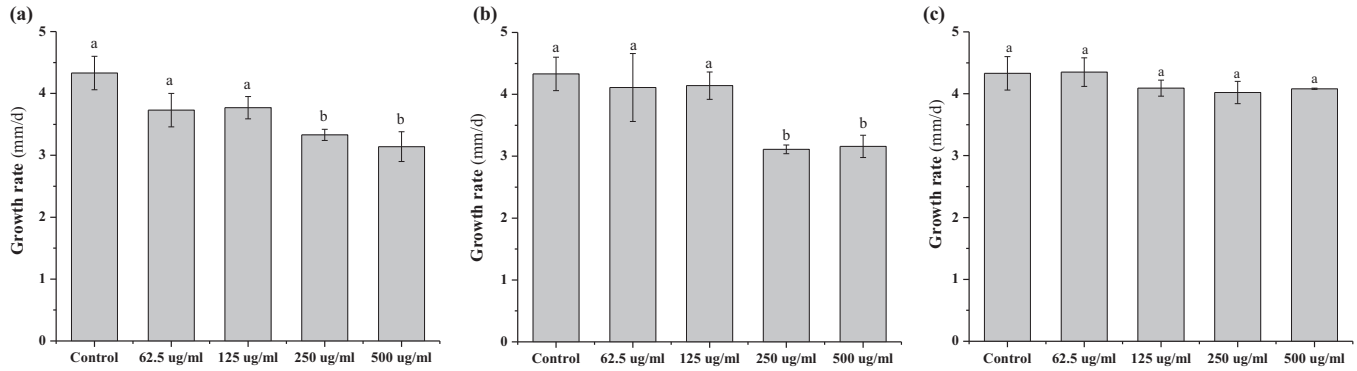

Fig. 1. Effect of different concentrations ($\mu g\,ml^{-1}$) of yellow (a), purple (b) and brown (c) extracts from peanut skin on *Fusarium vertillioides* growth rate on PDA agar at 25 °C. Bars with different letters are statistically different ($p < 0.05$).

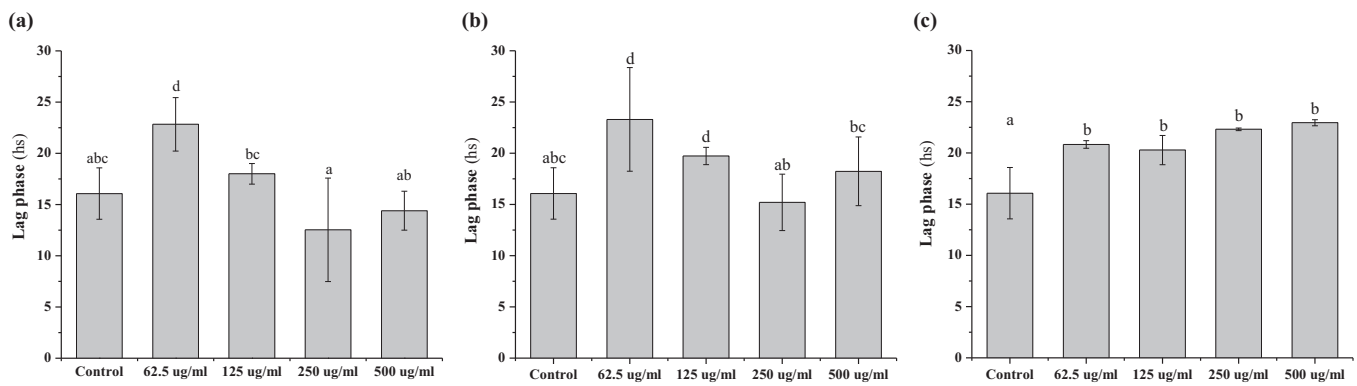

Fig. 2. Effect of different concentrations ($\mu g\,ml^{-1}$) of yellow (a), purple (b) and brown (c) extracts from peanut skin on *Fusarium vertillioides* lag phase on PDA agar at 25 °C. Bars with different letters are statistically different ($p < 0.05$).

results revealed that growth parameters were affected by the presence of these extracts in the medium. The most active inhibitor on growth rate was the purple extract at 250 and 500 $\mu g\,ml^{-1}$, showing a reduction of fungal growth of 28%. The yellow extract inhibited the growth rate around 19% at all concentration assayed, while brown extract did not show effect on growth rate (Fig. 1). Despite the observation that the presence of brown and purple extracts at 62.5 and 125 $\mu g\,ml^{-1}$ had no effect on the growth rate of the *Fusarium* isolate, a marked effect is noted on lag phase. Furthermore, a lengthened lag phase was also observed in the presence of the lowest concentration of yellow extract (62.5 $\mu g\,ml^{-1}$) (Fig. 2). Our results are consistent with those reported by Sarnoski et al. (2012), who informed that proanthocyanidins extracted from peanut skins extended the lag phase growth of yeast strains. Furthermore, our results are in agreement with those reported by other authors related to the effect on growth parameters of natural compounds that are also present in peanut skin. Thus, Chan (2002) reported inhibition of over 75% at 25–50 $\mu g\,ml^{-1}$ of resveratrol, for fungal species of *Trichophyton*, *Epidermophyton* and *Microsporum*. Moreover, Romero et al. (2009) showed that caffeic acid and quercetin had a significant influence on growth rate and lag phase of *Aspergillus carbonarius*. On the other hand, Mandalari et al. (2010) reported that both natural almond skin and blanched almond skin flavonoid-rich fractions had antimicrobial activity in the range 250–500 $\mu g\,ml^{-1}$, similar to the concentrations employed in this study. According to the results obtained in the present study and based on results reported by above mentioned authors who claimed that various phenolic compounds have negative effect on fungal growth and some of that compounds naturally occurring in peanut skin, the antifungal activity could be attributed to the presence of catechin, epicatechin, protocatechuic, caffeic and ferulic acids and/or resveratrol (Francisco and Resurreccion, 2009; Yu et al., 2005).

To our best knowledge, there is no information available that analyzed the effect of natural compounds from PSE on fumonisin biosynthesis. In the present study, FB_1 production was assayed at 62.5 and 250 $\mu g\,g^{-1}$ of PSE. The results shown in Fig. 3 indicated that while the yellow extract at 62.5 $\mu g\,g^{-1}$ was the most active

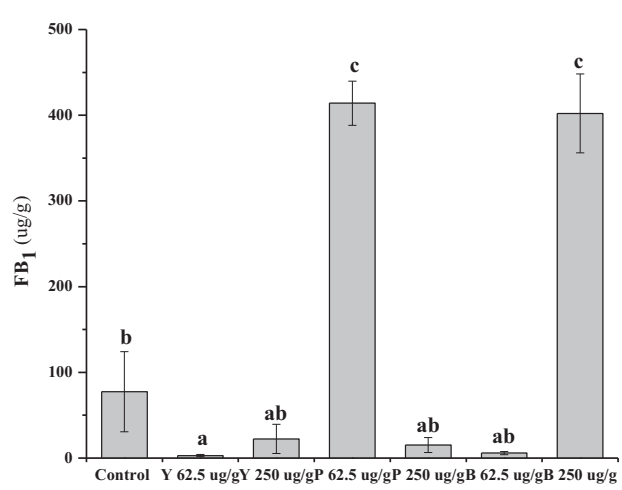

Fig. 3. Effects of different concentrations ($\mu g\,ml^{-1}$) of yellow, purple and brown extracts from peanut skin on FB_1 production ($\mu g\,g^{-1}$) in artificially infected maize kernels after 28 days of incubation at 25 °C.

skin fraction in inhibiting mycotoxin biosynthesis in inoculated corn; maize treated with yellow and purple extracts at 250 $\mu g\,g^{-1}$ and brown extract at 62.5 $\mu g\,g^{-1}$ had no significant inhibitory effect compared with the control treatment. On the contrary, both purple extract at 62.5 $\mu g\,g^{-1}$ and brown extract at 250 $\mu g\,g^{-1}$ increased FB_1 concentration to 414 and 402 $\mu g\,g^{-1}$, respectively, comparing to control (77 $\mu g\,g^{-1}$); the results demonstrated a stimulatory effect on the mycotoxin production. Furthermore, in other studies have been reported that phenolic compounds stimulated fumonisin levels (Menniti et al., 2010; Reynoso et al., 2002).

The possible role of alcoholic extracts from vegetal source, in inhibiting growth and toxin production has been of recent interest as an alternative strategy to the use of chemical fungicides (Bouamama et al., 2006; Haouala et al., 2008; Osorio et al., 2010; Talibi et al., 2012). With respect to PSE, Sarnoski et al. (2012) reported that it could be acting as a yeast inhibitory agent through a variety of mechanisms, either synergistically or not. One of the explanations for inhibition of microorganisms due to tannins (proanthocyanidins) is iron deprivation (Wauters et al., 2001). These compounds are known transition metal chelators (Malesev and Kuntic, 2007; Rice-Evans et al., 1997); therefore, PSE inhibition of microorganisms growth could be related to chelation. It was hypothesized that the active components of the extract may bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and membrane bound enzymes. The effects could lead to inhibition of proton motive force, inhibition of the respiratory chain and electron transfer, and inhibition of substrate oxidation. This could result in the uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, protein, lipid, and polysaccharides leading to cell injury or death (Cowan, 1999; Shan et al., 2008). In the present study, we demonstrated that PSE from blanched skins had antifungal and antimycotoxicogenic effects. Dry blanching is the most commonly used method to separate skins from peanut kernels. During this heat treatment, the brown color of peanut increases due primarily to sugar-amino acid reactions with subsequent production of melanins (Sobolev and Cole, 2004). Maillard reaction products especially melanoidins, possess antioxidant capacity through scavenging oxygen radicals or chelating metals (Yilmaz and Toledo, 2005). Heat, therefore, increases the antioxidant capacity of peanut skins (Francisco and Resurreccion, 2009). Literature on the antimicrobial activity of Maillard compounds (Einarsson et al., 1983; Rufián-Henares and Morales, 2006; Wang et al., 2011) reports that these kinds of compounds exert its biological activity by decreasing the microbial growth. Consequently, it seems likely that not only to pure compounds of the ethanolic fractions from blanched peanut skin but also the compounds derived from the heat process, are involved in fungal inhibition.

According to an integrated synthesis of the results reported above, it is clear that yellow extract at 62.5 $\mu g\,ml^{-1}$ from peanut skin showed the most antifungal effect under the growth parameters analyzed as well as FB_1 accumulation in maize kernels. While assays with the other PSE showed that inhibition of growth and mycotoxin production do not always occur together. This behavior could be due to different action mechanisms of the diverse compounds presents in the PSE on fungal cell at diverse target levels. Although PSE have been studied as antimicrobial agents, the mechanism of antifungal and antimycotoxicogenic activity is still poorly understood and needs to be better characterized to optimize the selection of these extracts as an alternative to control fungal spoilage in food. To our best knowledge, the effects of PSE on *F. verticillioides* growth and FB_1 production were investigated, for the first time.

4. Conclusions

The present study showed that PSE are a potential source of natural compounds with activity against *F. verticillioides* growth and FB_1 production. This finding suggest that peanut skin, a by-product of the peanut blanching operation that has low economic value despite its high content of active components including polyphenols, presents an alternative to the use of synthetic fungicides.

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