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Antifungal and antiaflatoxigenic activity by vapor contact of three essential oils, and effects of environmental factors on their efficacy

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ABSTRACT

The present investigation reports the antifungal and antiaflatoxigenic properties of essential oils (EOs) from boldo (*Pëumus boldus* Mol.), poleo (*Lippia turbinata* var. *integrifolia* Griseb., clove (*Syzygium aromaticum* L.) and from boldo and poleo mixtures present in the headspace of peanut extract medium at three water activity levels (a_W) (0.98, 0.95 and 0.93). Moreover, the ability of boldo and poleo oils to maintain their antifungal activity was evaluated after subjecting them to environmental variations. Boldo EO at doses $\geq$1500 μL/L showed a highly significant effect on *Aspergillus* section *Flavi* lag phase (>300 h), growth rate (93–100% of inhibition) and aflatoxin B_1 (AFB_1) accumulation (100% of inhibition) at all a_W levels assayed. The antifungal and antiaflatoxigenic effects of poleo and clove EOs were highly dependent on a_W. In general boldo–poleo oil mixtures showed less inhibitory activity on *Aspergillus* strains than the boldo pure oil. The antifungal ability of volatile components released by boldo and poleo EOs was stable against temperature changes; while it was reduced when poleo was stored during six months and when boldo was exposed to sunlight and UV.

Boldo and poleo EO volatile fractions can be used as effective non-toxic biopreservatives in stored peanut industry against AF contamination.

1. Introduction

Among different mycotoxins, aflatoxins (AFs), a difuran-containing polyketide derived *Aspergillus* toxin, are the most significant mycotoxins in peanuts during postharvest processing (Ding, Li, Bai, & Zhou, 2012; Ezekiel et al., 2012; Kamika & Takoy, 2011). Aflatoxins (AFs) in general and specially aflatoxin B_1 (AFB_1) is a genotoxic, immunotoxic and hepatocarcinogenic secondary metabolite (group 1) (IARC, 1993, 2002) produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* and *Aspergillus pseudotamarii* (Varga, Frisvad, & Samson, 2011). There are reports that hepatic carcinoma and other serious diseases may be induced by consuming food or using raw materials for food processing contaminated with AFs (Li, Yoshizawa, Kawamura, Luo, & Li, 2001; McKean et al., 2006; Turner, Moore, Hall, Prentice, & Wild, 2003). Nearly 5 billion people are exposed to AFs in different developing countries and aflatoxicosis is ranked 6th among the 10 most severe health risks identified by WHO and have ability to accumulate in the organism (Galvano, Ritieni, Piva, & Pietri, 2005). Considering, further the impact on international market is necessary remembering that mycotoxin contamination of agricultural commodities has important economic implications. The losses due to rejection of shipments and lower prices for lower quality can be devastating to developing countries typically grain exporters. For these reasons, AFs levels are highly regulated in peanut and peanut derived products in most countries. Consequently, the European Union and Food and Drug Administration U.S. instituted legislation to protect the health of consumers and set limits for total AFs and AFB_1 in peanuts of 4 and 2 ng/g, respectively (European Commission (EC) Commission Regulation, No 165/2010; FDA U.S. Regulations, CPG Sec. 570.375/09).

The world peanut production obtained during the period 2010/2011 was estimated at 33 million tons, Argentina ranked 14th, contributing with 0.95% of total world production (USDA, 2012). Argentina has established as the world's largest exporter of peanuts and the excellence in this product has given it international prestige to this industry becoming the largest peanut supplier in the European Union (MAGyP, 2012). However, we have studied toxigenic and sclerotial characteristics of *Aspergillus* section *Flavi* populations in the peanut agroecosystem and the 100% of the

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samples were contaminated with potentially toxigenic species (Passone, Rosso, Ciancio, & Etcheverry, 2010).

Although, different synthetic antimicrobials have been successfully commercialized in recent years to minimize such losses, they encounter major problems not only due to their adverse side effects on consumers but also for the development of resistance by microorganisms (Tolouee et al., 2010). Hence, there must be optimization of alternative methods for pest and disease control that produce minimal damage to the environment and human health and with different action mechanisms on the target cell to avoid the development of resistance by microorganisms. Currently, different plant products have been formulated for large scale application in eco-friendly and biorational management of storage pests and are being used as botanical antimicrobials. Amongst plant products the essential oils (EOs) derived from aromatic plants have been well studied during the last two to three decades as a potential candidates against different microbes (Abdollahi, Hassani, Ghosta, Meshkatalsada, & Shabani, 2011; Bullerman, Lieu, & Seiler, 1977; Mallozzi, Correa, Haraguchi, & Brignani, 1996; Marandi et al., 2011; Prakash, Singh, Kedia, Singh, & Dubey, 2012).

The vegetative growth and subsequent aflatoxin by *Aspergillus* section *Flavi* were found to be sensitive to twenty EOs extracted from some medicinal plants (Dubey, Shukla, Kumar, Singh, & Prakash, 2010; Soliman & Badeaa, 2002). Recently, oil extracted from *Hibiscus sabdariffa*, *Nigella sativa*, *Eucalyptus globulus* Labill, *Callistemon lanceolatus* (Sm.) Sweet showed variable fungistatic and fungicidal properties against *Aspergillus* section *Flavi* (El-Nagerabi, Al-Bahry, Elshafie, & AlHilali, 2012; Rocha Vilela et al., 2009; Shukla, Singh, Prakash, & Dubey, 2012). In a contact assay, we demonstrated that the application of high concentrations (2500 μL/L) of *Pëumus boldus* Mol. (boldo) and *Lippia turbinata* var. *integrifolia* (Griseb.) (poleo) oils completely inhibited fungal development. The antiaflatoxigenic property of these EOs (500 μL/L) was more marked with the medium a_W reduction (Passone, Girardi, Ferrand, & Etcheverry, 2012). Besides, oil from the dried flower buds of *Syzygium aromaticum* L. (clove) at the dose of 1500 μL/L completely inhibited the growth of *A. flavus* and *A. parasiticus* strains. Some EOs and their components are bioactive compounds in commercial use as food additives through encapsulation technologies (Ávila-Sosa et al., 2012; Leimann, Gonçalves, Machado, & Bolzan, 2009).

Thus, the objectives of this study were: (a) to examine the efficacy of boldo, poleo and clove oils by vapor contact assay against *A. flavus* and *A. parasiticus* growth and AFB_1 accumulation on peanut meal extract agar under different environmental conditions (0.98, 0.95, 0.93 a_W); (b) to evaluate the antifungal and antiaflatoxigenic properties of boldo and poleo oil mixtures present in the headspace of peanut extract medium at three water activity levels; (c) to determine if boldo and poleo oils have the ability to maintain their antifungal activity after subjecting them to environmental variations that occur in the peanut storage agroecosystem.

2. Materials and methods

2.1. Fungal isolates

Two *A. flavus* Link (RCP08270 and RCP08108) and two *A. parasiticus* Speare (RCP08299 and RCP08300) were used in this study. These strains were originally isolated from stored peanut in Córdoba, Argentina, in August/December 2008 period (Passone et al., 2010), and it was previously demonstrated to be aflatoxin producers in peanut meal extract agar (PMEA; 0.99 a_W; 11 days of incubation at 25 °C; 30.3 ± 4.6 ng/g, 33.0 ± 12.1 ng/g, 953.3 ± 23.1 ng/g and 49.7 ± 39.3 ng/g AFB_1 for RCP08270, RCP08108, RCP08299, RCP08300, respectively). These isolates were deposited in the *Aspergillus* section *Flavi* culture collection, Microbiology and Immunology Department of the National University of Río Cuarto. The fungi were maintained on slants of malt extract agar (MEA) at 4 °C and stored as spore suspensions in 1.5 g/L glycerol at −20 °C.

2.2. Essential oils and chemical characterization

The plant species *P. boldus* Mol. (boldo), *L. turbinata* var. *integrifolia* (Griseb.) (poleo) and *S. aromaticum* L. (clove) used in this study were purchased from a local market. The plant species were stored at −20 °C after harvest (Usai et al., 2011). The plant materials were obtained from dried leaves of *P. boldus*, dried leaves and stems of *L. turbinata* var. *integrifolia* and dried flower buds of *S. aromaticum*. A portion (100 g) of each plant material parts was submitted for 3 h to water-distillation, using an extractor of EOs by steam distillation at laboratory scale (Figmay S.R.L.) (yield 2.0; 1.02 and 10.0 mL/g for boldo, poleo and clove, respectively). The EO was dried over anhydrous sodium sulfate and, after filtration, stored in sterilized vial at 4 °C for up to 1 week until tested. Chemical characterizations of these EOs were previously determined in our laboratory by Bluma and Etcheverry (2008).

2.3. Culture medium

The basic medium used in this study was peanut meal extract agar (PMEA), made by boiling 30 g of dried peanut meal in 1 L water for 60 min. The resulting mixture was filtered through a double layer of muslin and 15 g/L agar was added (Passone, Resnik, & Etcheverry, 2005). The water activity (a_W) of the basic medium (0.99) was adjusted to 0.98, 0.95 and 0.93 by addition of a non-ionic solute, glycerol, according to Dallyn and Fox (1980). PMEA was autoclaved at 121 °C for 20 min, and poured into sterile Petri dishes for volatile assays. The water activity of the medium was checked after autoclaving with an AquaLab Water Activity Meter 4TE (Decagon Devices, Inc.).

2.4. Vapor contact assay

The effect of three EOs (boldo, poleo and clove) on the growth of four aflatoxigenic isolates in PMEA at three a_W levels and 25 °C was studied. Two small Petri dishes (5 cm diameter) containing each one 10 mL PMEA were prepared and placed, without cover, into a big Petri dish (14 cm diameter). The plates were spot inoculated with 2 μL into center of each small Petri dish, with 10^5 spores/mL suspended in 0.2 g/100 mL soft agar (Pitt, 1979). Sterilized cotton was placed in the center of the big Petri dish; that is between the two small PMEA agar plates. Essential oil was added to the cotton, having no direct contact with the PMEA agar plates. In a separate assay, boldo and poleo oil doses were 1000, 1500, 2000, 2500 and 3000 μL per liter of PMEA, while clove EO doses were 1000, 2000, 3000, 4000 and 5000 μL per liter of PMEA. In a mixture assay, boldo and poleo oil doses were 250, 500 and 750 μL/L and 500, 1000 and 1500 μL/L, respectively to obtain the following treatments: B–P mixtures (250 + 500 μL/L), (250 + 1000 μL/L), (250 + 1500 μL/L), (500 + 500 μL/L), (500 + 1000 μL/L), (500 + 1500 μL/L), (750 + 500 μL/L), (750 + 1000 μL/L), (750 + 1500 μL/L). The control plates (without essential oil) were inoculated following the same procedure. The plates were sealed with polyethylene film and incubated at a temperature of 25 °C during 11 days. Those treatments in which no fungal development was observed at 11 days were incubated for a maximum of 35 days. Tests were undertaken in quadruplicate and each colony was daily measured in two directions at right angles to each other to obtain the mean radii. The radii of the colonies were plotted against time, and a linear