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Evaluation of the control ability of five essential oils against *Aspergillus* section *Nigri* growth and ochratoxin A accumulation in peanut meal extract agar conditioned at different water activities levels

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ABSTRACT

Essential oils (EOs) from boldo [*Pëumus boldus* Mol.], poleo [*Lippia turbinata* var. *integrifolia* (Griseb.)], clove [*Syzygium aromaticum* L.], anise [*Pimpinella anisum*] and thyme [*Thymus vulgaris*]) obtained by hydrodistillation were evaluated for their effectiveness against the growth of *Aspergillus niger* aggregate and *A. carbonarius* and accumulation of ochratoxin A (OTA). The evaluation was performed by compound dissolution at the doses of 0, 500, 1500 and 2500 μL/L in peanut meal extract agar (PMEA) and exposure to volatiles of boldo, poleo (0, 1000, 2000 and 3000 μL/L) and clove oils (0, 1000, 3000 and 5000 μL/L), taking into account the levels of the water activity of the medium (a_W 0.98, 0.95, 0.93). Statistical analyses on growth of *Aspergillus* strains indicated that the major effect was produced by oil concentrations followed by substrate a_W, and that reductions in antifungal efficiency of the oils tested were observed in vapor exposure assay. At all a_W levels, complete fungal growth inhibition was achieved with boldo EO at doses of 1500 and 2000 μL/L by contact and volatile assays, respectively. Contact exposure by poleo and clove EOs showed total fungal inhibition at the middle level tested of 1500 μL/L, regardless of a_W, while their antifungal effects in headspace volatile assay were closely dependent on medium a_W. The fumigant activity of poleo (2000 μL/L) and clove oils (3000 μL/L) inhibited growth rate by 66.0% and 80.6% at a_W 0.98 and 0.93, respectively. OTA accumulation was closely dependent on a_W conditions. The antiochratoxigenic property of the volatile fractions of boldo, poleo and clove EOs (1000 μL/L) was more significant at low a_W levels, inhibition percentages were estimated at 14.7, 41.7 and 78.5% at a_W 0.98, 0.95 and 0.93, respectively. Our results suggest that boldo, poleo and clove oils affect the OTA biosynthesis pathway of both *Aspergillus* species. This finding leaves open the possibility of their use by vapor exposure as effective non-toxic biopreservatives against OTA contamination in stored peanuts.

1. Introduction

Argentina's climate provides conditions favorable for the production of a wide range of agricultural products including cereals, oil seeds, fruits and nuts. Climatic conditions at harvest time affect the ability to commercialize peanuts. Export of peanut increased 0.55 million tons consolidating Argentina as the primary worldwide exporter; only a small amount is used for internal consumption (SIIA, 2012). This oily substrate is subjected to severe sanitary controls during export. During storage, the climatic conditions and the mycoflora contamination will determine mycotoxin occurrence.

The contamination of different agricultural commodities with ochratoxin A (OTA) occurs predominantly postharvest. *Aspergillus niger* aggregate and *A. carbonarius* are among the major storage fungi found regularly in important grains stored throughout the world, such as peanuts (Engel, 2000; Silvério et al., 2010; Visconti et al., 2008). In previous studies in Argentina, Magnoli et al. (2006, 2007) reported, in two successive years of sampling, the prevalence of *Aspergillus* section *Nigri* OTA producing strains (27 and 32%) and variable percentage of storage peanut seed samples contaminated with OTA (32 and 50%) with levels that ranged from 0.5 to 170 ng/g and 5.6 to 130 ng/g, for the first and second year of sampling, respectively. OTA is one of the most important mycotoxins, based on the observed teratogenic, embryotoxic, genotoxic, neurotoxic, immunosuppressive, carcinogenic (IARC, 1993 group 2B), and nephrotoxic effects (JECFA, 2008). Therefore, the final regulations proposed by the European Union for maximum levels of OTA allowed in cereal grains was 5 μg/kg (European Commission, 2006).

A. niger aggregate and *A. carbonarius* growth is markedly affected by environmental factors, especially a_W (0.90–0.99) and temperature (15–30 °C) which are limiting factors in the functioning of ecosystems (Astoreca et al., 2009; Bellí et al., 2005; Esteban et al., 2005; Romero et al., 2007). Several studies also report the involvement of abiotic stress on OTA accumulation and on the activation of its biosynthetic pathways

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(Astoreca et al., 2009; Kapetanakou et al., 2011; Leong et al., 2006; Schmidt-Heydt et al., 2008).

Chemical control remains the main measure to reduce the incidence of post-harvest diseases in various foods. Antimicrobial chemicals belonging to the groups of benzimidazoles, aromatic hydrocarbons, and inhibitors of sterol biosynthesis are often used as post-harvest treatments; however, the application of high concentrations increases the risk of toxic residues in the products (Al-Omair and Helaleh, 2004; Baird et al., 1991; Chilvers et al., 2006; Šimko, 2005). Therefore, there has been increased interest in the research in using natural antimicrobial or antifungal substances, which may replace synthetic pesticides or contribute to the development of new pest control agents. During the past 22 years, essential oils (EO) have been shown to possess a broad spectrum of antifungal activity (Thompson, 1989; Tian et al., 2011). Screening experiments with 41 aqueous and ethanolic extracts and 22 EOs carried out in our laboratory against *Aspergillus* section *Flavi* strains have shown boldo, poleo, clove, anise and thyme oils as potential antifungal candidates (Bluma et al., 2008). In that study, EOs were screened for antifungal effect by direct addition and diffusion in the media. However recent studies have shown that smaller compounds such as monoterpenes are most efficient when used as headspace volatiles (Avila-Sosa et al., 2012). This characteristic makes EOs attractive as possible fumigants for the protection of stored products.

Thus, the objectives of this study were: (a) to examine the efficacy of boldo, poleo, clove, anise and thyme oils against *A. niger* aggregate and *A. carbonarius* growth and OTA accumulation on peanut meal extract agar under different environmental conditions (a_W 0.98, 0.95, 0.93); (b) to compare the antifungal effects of these essential oils in contact and headspace volatile assays.

2. Materials and methods

2.1. Essential oils and chemical characterization

The plant species *Pëumus boldus* Mol. (boldo), *Lippia turbinata* var. *integrifolia* (Griseb.) (poleo), *Syzygium aromaticum* L. (clove), *Pimpinella anisum* (anise) and *Thymus vulgaris* (thyme) used in this study were purchased from the local market. The plant species were stored at 4 °C after harvest. The plant materials were obtained from dried leaves of *P. boldus*, dried leaves and stems of *L. turbinata* var. *integrifolia*, dried flower buds of *S. aromaticum*, dried seeds of *P. anisum* and dried leaves and stems of *T. vulgaris*. A portion (100 g) of each plant material parts was submitted to water-distillation for 3 h, using an extractor of essential oils (EOs) by steam distillation at laboratory scale (Figmay S.R.L.) (yield 2.0%, 1.02%, 10.0%, 6.0% and 1.0% v/w for boldo, poleo, clove, anise and thyme, respectively). The obtained 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).

2.2. Fungal strains

Six *Aspergillus* section *Nigri* strains were evaluated: *A. niger* aggregate (RCP42, RCP176, and RCP191) and *A. carbonarius* (RCPG, RCP4 and RCP203). All the strains were isolated from peanut seeds in Argentina (Magnoli et al., 2006) and were identified by classic taxonomy according to the methodology proposed by Klich (2002). The OTA production ability was assayed on YES broth (2% yeast extract, 15% sucrose, 0.5% $MgSO_4 \cdot 7H_2O$) (YES broth; a_W 0.99; 14 days of incubation at 25 °C; 8.4, 88.7, 125.3 and 71.4 ng/g OTA for *A. niger* aggr. RCP42, RCP191, and *A. carbonarius*, RCP4, RCP203, respectively). The strains were maintained in glycerol (15%, Sigma-Aldrich) at −80 °C and kept in the culture collection at the Department of Microbiology and Immunology, National University of Río Cuarto, Córdoba, Argentina.

2.3. Culture medium

Peanut meal extract agar (PMEA) was prepared at 2% (w/v) with a final pH of 6.5. Thirty grams of ground peanut per liter was boiled for 45 min and the resultant mixture filtered through a double layer of muslin. The volume was made up to 1 L and agar-agar at 2% (w/v) was added (Passone et al., 2005). The water activity (a_W) of the basic medium (0.99) was adjusted to 0.98, 0.95 and 0.93 with known amounts of glycerol (Dallyn and Fox, 1980). The basic medium was autoclaved at 121 °C for 20 min before cooling at 50 °C and then poured into 9 cm and 5 cm sterile Petri dishes for contact and volatile assays, respectively. Water activity of representative samples of each treatment was checked after autoclaving with an AquaLab Water Activity Meter 4TE (Decagon Devices, Inc.).

2.4. Growth studies

2.4.1. Contact assay

The effect of five EOs (boldo, poleo, clove, anise and thyme) on the growth of six *Aspergillus* section *Nigri* isolates in PMEA at three a_W levels and 25 °C was studied. Medium Petri dishes (9 cm diameter) containing 20 mL PMEA were prepared. The appropriate amount (0, 500, 1500, 2500 μL/L) of each EO was added to 20 mL of the autoclaved and cooled medium. Spores of 5-day-old cultures obtained from each test fungus grown on malt extract agar (MEA) plate were suspended in 0.2% soft agar and 2 μL of 10^5 spores/mL were spot inoculated into the center of each Petri plate (Pitt, 1979). The plates were sealed with polyethylene film and incubated at 25 °C for 14 days. Those treatments in which no fungal development was observed at 14 days were incubated for a maximum of 35 days. Tests were undertaken in quadruplicate and each colony was measured daily in two directions at right angles to each other to obtain the mean diameter.

2.4.2. Volatile assay

The effect of three EOs (boldo, poleo and clove) on the growth of four ochratoxigenic isolates (*A. niger* aggr. RCP42 and RCP191, and *A. carbonarius* RCP4 and RCP203) in PMEA at three a_W levels and at 25 °C was studied. Two small Petri dishes (5 cm diameter) each containing 10 mL PMEA were prepared and placed, without a cover, into a large Petri dish (14 cm diameter). The plates were spot inoculated with 2 μL of 10^5 spores/mL suspended in 0.2% soft agar into the center of each small Petri dish (Pitt, 1979). Sterilized cotton was placed in the center of the large 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. Boldo and poleo oil doses were 1000, 2000 and 3000 μL per liter of PMEA, while clove EO doses were 1000, 3000 and 5000 μL per liter of PMEA. The control plates (without essential oil) were inoculated following the same procedure. The plates were sealed with polyethylene film and incubated at 25 °C for 14 days. Those treatments in which no fungal development was observed at 14 days were incubated for a maximum of 35 days. Tests were undertaken in quadruplicate and each colony was measured daily in two directions at right angles to each other to obtain the mean diameter.

After growth was evaluated, all samples were frozen for later extraction and OTA quantification.

2.5. Ochratoxin A accumulation

At the end of the incubation period (14 days), OTA was determined following the methodology of Bragulat et al. (2001), slightly modified. A piece of PMEA with mycelium, approximately 1 cm × 1 cm, was taken from the center of the colony (ochratoxin producers + different treatments with EOs from the volatile assay), weighed and transferred to an Eppendorf tube, into which 1 mL of methanol was added. The mixture was centrifuged at 14,000 rpm for 10 min. The solutions were

Table 1
ANOVA test. Effects of essential oils (EO) and their concentrations (C) on growth rate and ochratoxin A production by *Aspergillus* section *Nigri* isolates (I) grown on peanut meal extract agar (PMEA) at various water activity (a_W) levels.

Source of variation	Contact assay			Volatile assay					
	Growth rate			Growth rate			Ochratoxin A		
	DF	MS	*F* value[a]	DF	MS	*F* value	DF	MS	*F* value
a_W	2	0.615	656.57**	2	0.293	427.21**	2	15,264,326.69	13.74**
I	5	0.144	153.50**	3	0.020	29.39**	3	7,350,486.80	6.62**
EO	4	0.466	497.97**	2	0.108	157.29**	2	2,677,763.23	2.51*
C	3	5.224	5580.45**	3	1.657	2419.33**	3	8,550,869.06	7.70**
$a_W \times I$	10	0.049	52.06**	6	0.009	13.35**	6	4,433,803.79	3.99**
$a_W \times EO$	8	0.036	38.53**	4	0.012	17.97**	4	328,249.28	0.30
$a_W \times C$	6	0.057	60.69**	6	0.013	19.39**	6	1,404,279.70	1.26
$I \times EO$	20	0.006	6.22**	6	0.005	6.70**	6	3,404,006.71	3.06*
$I \times C$	15	0.070	75.09**	9	0.006	9.33**	9	4,171,994.39	3.76**
$EO \times C$	12	0.168	179.59**	6	0.024	35.71**	6	708,400.90	0.64
$a_W \times I \times EO$	40	0.004	4.52**	12	0.002	3.47**	12	1,784,829.48	1.61
$a_W \times I \times C$	30	0.013	14.24**	18	0.014	21.15**	18	2,178,006.86	1.96*
$a_W \times EO \times C$	24	0.036	38.91**	12	0.007	10.78**	12	1,209,645.07	1.09
$I \times EO \times C$	60	0.009	9.45**	18	0.002	3.27**	18	2,017,116.75	1.82*
$a_W \times I \times EO \times C$	120	0.004	4.35**	36	0.002	2.28**	36	1,399,746.90	1.26
Error	1080	0.001		432	0.001		432	1,110,872.00	

[a] Snedecor's *F* test.
** Highly significant at $p<0.001$.
* Significant at $p<0.05$.

filtered (Titan 2 HPLC Filter Green; 17 mm and 0.45 μm), evaporated to dryness, redissolved in mobile phase and the extract injected into the HPLC. The OTA production was detected and quantified by the methodology proposed by Scudamore and MacDonald (1998) with some modifications. The reversed phase high performance liquid chromatography (HPLC) with fluorescence detection (λ_{exc} 330 nm; λ_{em} 460 nm) was applied. The C18 column (Supelcosil LC-ABZ, Supelco; 150×4.6 mm, 5 μm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20×4.6 mm, 5 μm particle size) was used. The mobile phase (acetonitrile/water/acetic acid, 99:99:2) was pumped at 1.0 mL/min. The injection volume was 50 μL and the retention time was around 6 min. An ochratoxin A standard was obtained from Biopure (Tullin, Austria) and standard curves were constructed with different levels of OTA (5 to 100 ng/g). This toxin was quantified by correlating peak areas of sample extracts and those of standard curves. The mean recovery of the method used was calculated by spiking PMEA at different levels, ranging from 5 to 100 ng/g, and was estimated at 89.2 ± 9.7%. The lowest detection limit was 1 ng/g.

2.6. Data analysis

The radii of the colonies were plotted against time, and a linear regression applied, in order to obtain the growth rate as the slope of the line. Lag phase for growth was calculated mathematically and defined as the time (days) for a colony to reach diameter of 5 mm (Marín et al., 1995). In all cases, the experiments were carried out at least in four replicates per treatment. The growth of fungal cultures containing different concentrations of all EOs was compared with that of the control culture with no EOs.

Table 2
Effect of EOs on the lag phase of *Aspergillus* section *Nigri* strains at different a_W.

Strains	a_W	Lag phase (h)									
		C	Boldo	Poleo	Clove	Anise			Thyme		
		0	500	500	500	500	1500	2500	500	1500	2500
RCP42	0.98	41.8^{b}	84.0^{d}	53.6^{c}	186.5^{f}	52.6^{c}	21.7^{a}	–	14.5^{a}	169.1^{e}	–
	0.95	52.8^{a}	92.1^{c}	79.8^{b}	137.1^{d}	46.2^{a}	91.8^{c}	–	51.4^{a}	–	–
	0.93	67.9^{b}	113.8^{d}	98.1^{c}	154.5^{e}	24.0^{a}	–	–	61.8^{b}	–	–
RCP176	0.98	41.8^{c}	55.7^{d}	82.4^{e}	176.8^{g}	52.7^{d}	25.5^{b}	–	20.3^{a}	154.4^{f}	–
	0.95	53.3^{b}	101.4^{e}	85.4^{d}	162.6^{f}	40.4^{a}	62.5^{c}	–	33.5^{a}	–	–
	0.93	51.8^{b}	227.4^{f}	112.7^{d}	158.4^{e}	19.3^{a}	–	–	71.2^{c}	–	–
RCP191	0.98	29.4^{a}	54.7^{b}	136.3^{d}	159.9^{e}	88.9^{c}	18.3^{a}	–	16.6^{a}	134.1^{d}	–
	0.95	47.7^{a}	146.8^{d}	89.6^{b}	113.5^{c}	46.1^{a}	44.2^{a}	–	45.9^{a}	311.8^{e}	–
	0.93	64.0^{b}	111.4^{c}	143.7^{e}	130.0^{d}	20.5^{a}	–	–	63.6^{b}	–	–
RCPG	0.98	56.8^{b}	81.2^{c}	85.3^{c}	–	20.6^{a}	94.9^{c}	–	22.1^{a}	270.0^{d}	–
	0.95	71.8^{b}	–	255.3^{c}	–	51.6^{ab}	59.0^{b}	–	45.9^{a}	–	–
	0.93	151.3^{c}	–	–	–	43.5^{a}	–	–	96.2^{b}	–	–
RCP4	0.98	45.8^{bc}	73.0^{d}	69.8^{d}	109.4^{f}	39.1^{b}	15.7^{a}	123.4^{g}	48.9^{c}	87.8^{e}	–
	0.95	55.0^{b}	150.2^{d}	–	165.6^{d}	64.1^{b}	129.6^{c}	–	15.1^{a}	7.9^{a}	–
	0.93	77.9^{a}	206.2^{d}	–	–	93.1^{ab}	–	–	103.8^{b}	150.5^{c}	–
RCP203	0.98	30.4^{ab}	57.8^{bc}	96.9^{c}	–	35.1^{ab}	16.9^{a}	66.4^{bc}	66.7^{bc}	139.3^{d}	219.3^{e}
	0.95	59.9^{a}	–	150.4^{d}	–	60.4^{a}	121.9^{c}	–	114.6^{b}	268.2^{e}	–
	0.93	79.1^{a}	–	–	–	132.7^{b}	251.3^{c}	–	–	–	–

Mean values based on quadruplicated data. Mean in a row with a letter in common is not significantly different according to Duncan New Multiple Range Test ($p<0.001$). *A. niger* aggregate: RCP176, RCP42, and RCP191. *A. carbonarius*: RCPG, RCP4, and RCP203. (–) Under these conditions, the strains were not able to reach the exponential phase.

All data analyses were performed by ANOVA. *F*-value was found in ANOVA using SAS program 6.1 SAS Institute, Cary, NC (1998). A posteriori tests were performed using SigmaStat program Version 3.10. (Systat Software, Inc.). The significant differences for lag phase and growth rate were determined using Duncan's New Multiple Range Test at $p<0.05$ level. Fisher's LSD test ($\alpha = 0.05$) was employed to determine significant differences between treatments and control for OTA accumulation.

3. Results

3.1. Contact assay

3.1.1. Growth studies

Statistical analyses of growth of *Aspergillus* section *Nigri*; water activity (a_W), isolates (I), essential oils (EOs), concentrations (C) and two-; three-; and four-way interactions indicated that they were statistically significant (Table 1). The major effect was produced by C, followed by a_W of the medium and EO treatments.

Mean lag phases (h) of *Aspergillus* strains at three a_W, with boldo, poleo, clove, anise and thyme, are shown in Table 2. In control treatments, the mean lag phase increased from 37.7 to 61.2 h and from 44.3 to 102.8 h when the water availability ranged from optimal to marginal conditions (a_W 0.98 to 0.93) for both *A. niger* aggregate (RCP42, RCP176, RCP191) and *A. carbonarius* (RCPG, RCP4, RCP203), respectively. At 1500 and 2500 μL/L of boldo, poleo and clove EOs none of the strains developed visible mycelium at any a_W. The lag phase of all species tested was significantly increased ($p<0.05$) for 500 μL/L of boldo, poleo and clove EOs, regardless of a_W. When compared to the control treatment the lag phase of *A. niger* aggregate strains increased between 13.9 and 175.6 h, 11.8 and 106.9 h and 65.8 and 144.7 h when grown with 500 μL/L of boldo, poleo and

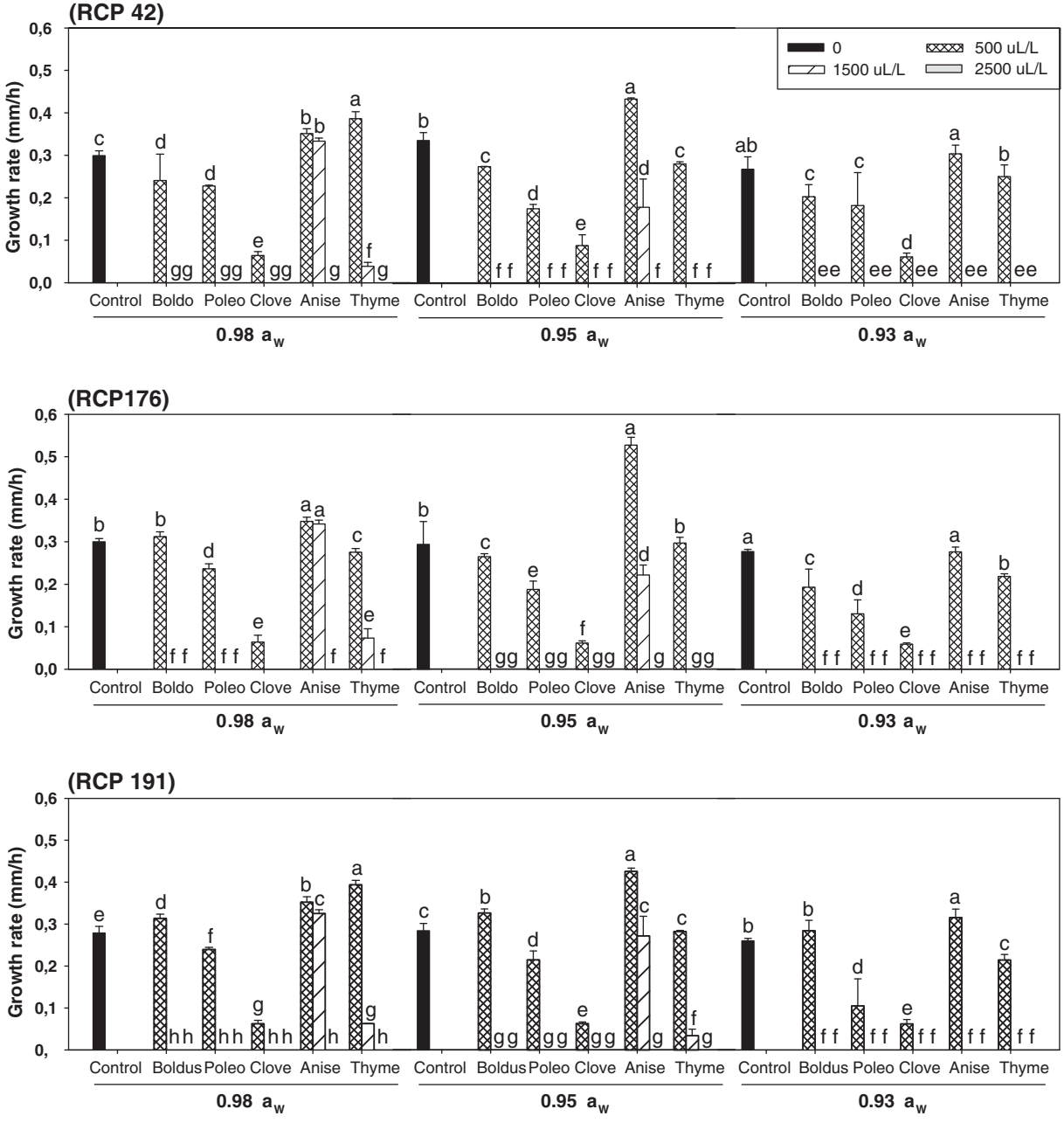

Fig. 1. Effect of EOs on growth rate of *A. niger* aggregate strains at different a_W levels on PMEA. Mean values based on quadruplicate data with letters in common for each a_W are not significantly different according to Duncan New Multiple Range Test ($p<0.05$).

clove EOs respectively, regardless of the a_W. *Aspergillus carbonarius* strains appear to be more sensitive to these treatments (500 μL/L of boldo, poleo and clove EOs) with increases in lag phases estimated between 24.0 and >183.5 h. In general, reductions of *Aspergillus* section *Nigri* lag phases were observed with the addition of anise (500 and 1500 μL/L) and thyme EOs (500 μL/L) to peanut medium modified at a_W 0.98. However, thyme EO (1500 μL/L) in peanut medium modified at a_W 0.98 increased the lag phases of *A. niger* aggregate and *A. carbonarius* strains between 104.7 and 127.3 h and 42.0 and 108.9 h, respectively, whereas, at the highest dose (2500 μL/L) of these EOs (anise and thyme) the growth of all *A. niger* aggregate strains and *A. carbonarius* RCPG was totally inhibited.

Growth rates of *Aspergillus* section *Nigri* strains at different a_W levels in the presence of EOs (0–2500 μL/L) at 25 °C are shown in Figs. 1 and 2. Medium water availability affected growth rates of control cultures; reduction percentages were estimated at 16.4 and 30.4% at a_W 0.95 and 0.93, respectively. The profiles of growth showed that poleo and clove EOs, at the lowest concentration used (500 μL/L), significantly reduced ($p<0.05$) the growth rate of the fungal isolates. When fungal isolates grew in the presence of poleo EO (500 μL/L), the inhibition percentages ranged between 14.0 and 54.1%, 24.3 and 100%, and 32.2 and 100% at a_W 0.98, 0.95 and 0.93, respectively, whereas clove EO produced the major inhibitory effects on fungal growth rates in the order of 48.8 to 100%. The sensitivity of *A. carbonarius* isolates to 500 μL/L of boldo EO was demonstrated (except for RCP203; a_W 0.98); however, this inhibitory effect was not reflected in growth rates of the *A. niger* aggregate strains (RCP176 and RCP191). Fungal growth was not statistically influenced by 500 μL/L of thyme EO, but reductions in the order of 42.4 to 100% were achieved with the dose of 1500 μL/L. The decrease of the medium a_W, added to the incorporation of sub-inhibitory doses of anise EO (500 and 1500 μL/L) stimulated the growth rate in most of the strains studied.

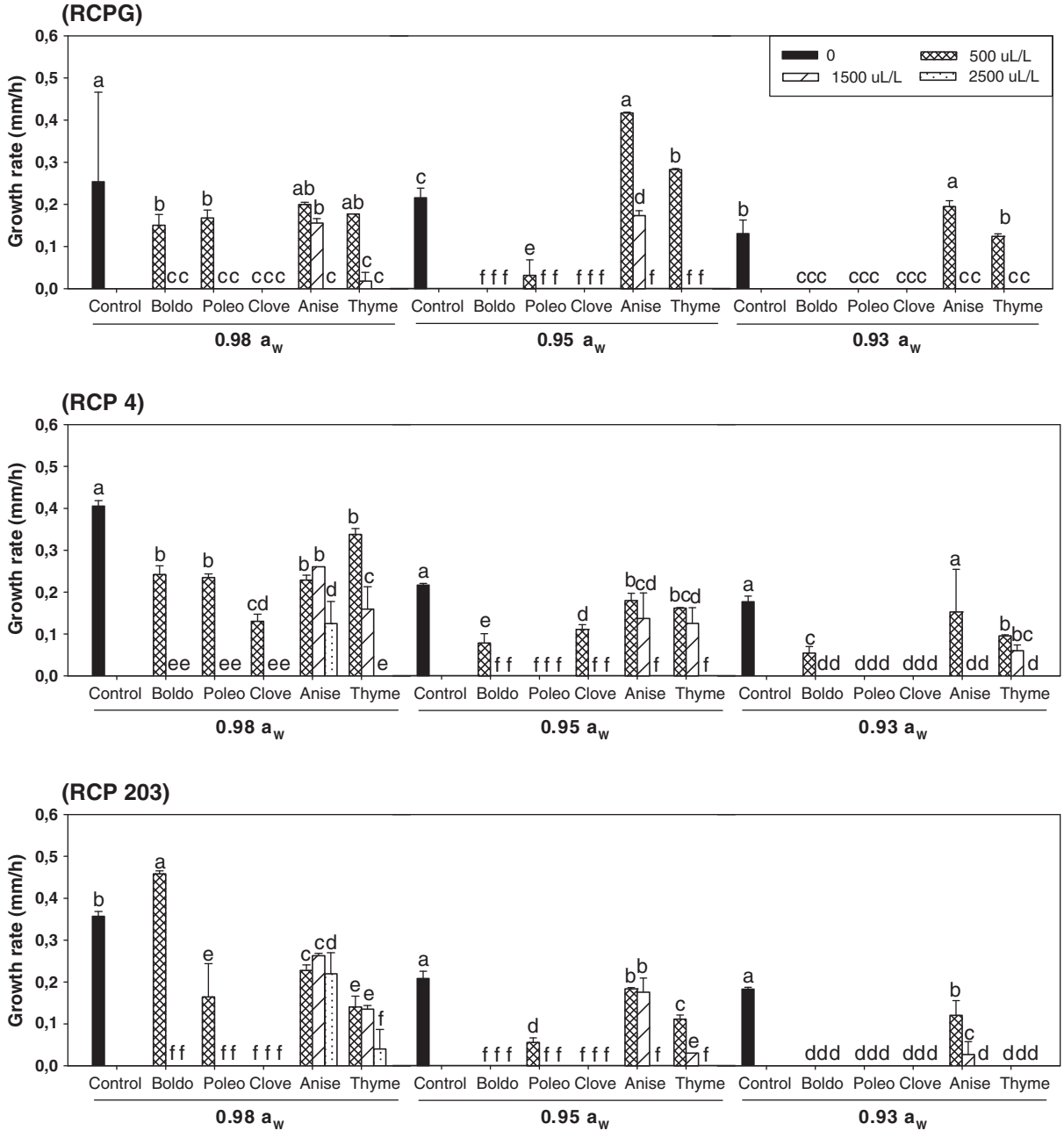

Fig. 2. Effect of EOs on growth rate of *A. carbonarius* strains at different a_W levels on PMEA. Mean values based on quadruplicated data with letters in common for each a_W are not significantly different according to Duncan New Multiple Range Test ($p<0.05$).

Table 3
Effect of EO volatile fractions on the lag phase of *Aspergillus* section *Nigri* strains at different a_W.

Strains	a_W	Lag phase (h)								
		C	Boldo		Poleo			Clove		
		0	1000	2000	1000	2000	3000	1000	3000	5000
RCP42	0.98	41.8^{a}	174.0^{b}	–	23.6^{a}	21.3^{a}	23.3^{a}	29.1^{a}	23.2^{a}	31.9^{a}
	0.95	52.8^{a}	–	–	83.0^{c}	66.5^{b}	108.1^{d}	56.4^{ab}	40.9^{a}	45.6^{a}
	0.93	67.9^{a}	–	–	91.4^{a}	–	–	57.1^{a}	170.6^{ab}	123.8^{ab}
RCP191	0.98	29.4^{a}	69.6^{a}	187.0^{b}	24.8^{a}	33.5^{a}	27.5^{a}	11.2^{a}	11.8^{a}	13.8^{a}
	0.95	47.7^{a}	198.4^{b}	–	59.6^{a}	86.6^{a}	57.2^{a}	39.1^{a}	43.7^{a}	48.2^{a}
	0.93	64.0^{abc}	–	–	73.9^{abc}	162.2^{bc}	178.0^{c}	48.0^{ab}	49.8^{ab}	8.3^{a}
RCP4	0.98	45.8^{b}	118.9^{c}	–	21.0^{ab}	22.8^{ab}	27.8^{ab}	2.2^{a}	13.7^{ab}	10.6^{a}
	0.95	55.0^{a}	122.6^{a}	–	65.6^{a}	89.8^{a}	158.9^{a}	36.7^{a}	38.8^{a}	62.2^{a}
	0.93	77.9^{a}	–	–	107.9^{a}	202.0^{b}	–	31.3^{a}	52.6^{a}	159.2^{b}
RCP203	0.98	30.4^{a}	170.1^{b}	–	33.9^{a}	35.5^{a}	36.4^{a}	31.7^{a}	31.5^{a}	26.1^{a}
	0.95	59.9^{a}	214.5^{bc}	–	82.6^{b}	173.8^{b}	–	42.8^{a}	37.8^{a}	33.7^{a}
	0.93	79.0^{a}	–	–	181.3^{b}	273.7^{c}	–	47.9^{a}	104.4^{a}	172.5^{b}

Mean values based on quadruplicated data. Mean in a row with a letter in common is not significantly different according to Duncan New Multiple Range Test ($p<0.001$). *A. niger* aggregate: RCP42, and RCP191. *A. carbonarius*: RCP4, and RCP203. (–) Under these conditions, the strains were not able to reach the exponential phase.

Results obtained in the contact assay indicate the possibility of exploiting boldo, poleo and clove EOs as possible fumigants at doses >500 μL/L for control of strains of *Aspergillus* section *Nigri*.

3.2. Volatile assay

3.2.1. Growth studies

ANOVA results are shown in Table 1. The single factors a_W, I, EOs, C as well as their two-; three-; and four-way interaction had a significant impact on the growth of *Aspergillus* section *Nigri*. Similar to the contact assays, the major effect was produced by C, followed by medium a_W levels and EO treatments.

In the control treatments, the mean lag phases increased from 36.9 to 72.2 h when medium water availability ranged from optimal to marginal conditions (a_W 0.98 to 0.93) (Table 3). Boldo EO was the most effective; at all a_W levels assayed, it increased the lag phase to more than 40.2 and 157.6 h at doses of 1000 and 2000 μL/L, respectively. In the presence of boldo EO volatile fraction at the dose of 3000 μL/L, none of the ochratoxigenic isolates formed visible mycelium, regardless of medium a_W. At a_W 0.95 and 0.93, poleo EO extended the lag phases of all *Aspergillus* isolates between 9.9 and 102.3 h, 13.7 and >194.7 h, and

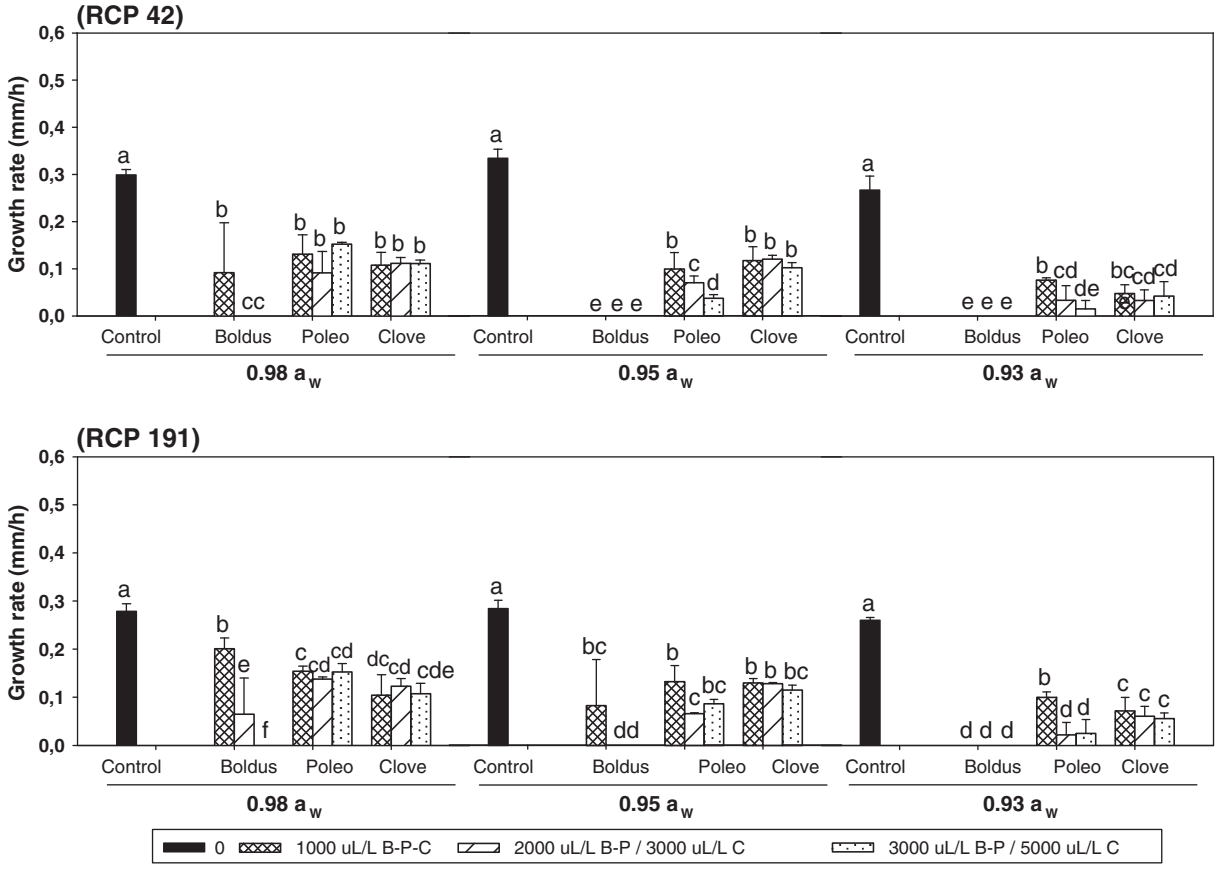

Fig. 3. Effect of EO volatile fractions on growth rate of *A. niger* aggregate strains at different a_W levels on PMEA. Mean values based on triplicate data with letters in common for each a_W are not significantly different according to Duncan New Multiple Range Test ($p<0.05$).

9.5 and >103.9 h at the doses of 1000, 2000 and 3000 μL/L, respectively; while at a_W 0.98 this oil could only modify the lag phase of one isolate (RCP203). When a_W was reduced to 0.93, the highest dose of clove EO extended all fungal lag phases, while no antifungal effect was observed in the rest of the conditions assayed.

Figs. 3 and 4 show the effect of EO volatile fractions (0–3000 μL/L for boldo and poleo; 0–5000 μL/L for clove) on *Aspergillus* section *Nigri* growth rate in PMEA at three a_W levels. The growth rate of control treatments was affected by medium a_W modifications; *A. carbonarius* strains were the most affected, showing a reduction in the order of 52.8% at 0.93 a_W. The behavior of the four isolates was similar: the combination of EO volatile fractions and low a_W increased the antifungal effects on the growth rate of all ochratoxigenic strains. Boldo EO showed the greatest effect on fungal isolates, with significant reductions ($p<0.05$) at 1000 and 2000 μL/L and totally inhibiting the growth at the dose of 3000 μL/L at all a_W levels assayed. The antifungal effect of poleo and clove EOs was highly dependent on a_W. The *Aspergillus* growth rate was reduced by 67.8% when a_W was 0.95, while by 82.1% reduction was observed when a_W was further lowered to 0.93. The lowest antifungal effect was observed at a_W 0.98.

3.2.2. Ochratoxin A accumulation

Table 1 shows the influence of single factors (a_W, I, EOs, C) as well as their interactions on OTA production. OTA accumulation significantly depended on a_W, oil concentrations and fungal isolate.

The water availability of the medium affected OTA accumulation by control treatments: at a_W 0.93 reductions were estimated around 90%, while increases in the order of 397.5% were observed at a_W 0.95 (Table 4). The presence of boldo EO volatile fraction in the surrounding atmosphere of peanut medium had a significant effect ($p<0.001$) on OTA accumulation. All ochratoxigenic isolates showed reduced OTA accumulation between 1.6 and 100% and 85.7 and 100% in the presence of 1000 and 2000 μL/L of boldo EO, respectively at all a_W levels. Poleo EO was less effective, inhibiting OTA accumulation between 18.1 and 100%, and 59.6 and 100% with doses of 2000 and 3000 μL/L, respectively. In fact, at the highest a_W level studied (0.98), *A. niger* aggregate RCP42 and *A. carbonarius* RCP4 and RCP203 tended to show OTA stimulation in the presence of 1000 μL/L of poleo EO. The antiochratoxigenic effect of clove EO was more significant ($p<0.001$) at a_W 0.93: reductions in the order of 64.6, 87.1 and 100% were estimated with doses of 1000, 3000 and 5000 μL/L, respectively.

4. Discussion

In the present study, lag phase, mycelial growth and OTA accumulation by *Aspergillus* section *Nigri* isolates were found to be significantly influenced by medium a_W. All fungal strains that were examined usually showed optimum a_W level for growth at 0.98, while 0.95 was the favorable a_W for produce OTA. Overall growth and OTA were reduced to 30% and 98% at a_W 0.93. Similar findings were reported in an ecophysiological study by Astoreca et al. (2009) who observed that OTA production occurs in a more restricted range of a_W than the range for fungal growth.

The five oils analyzed had clear dose and a_W-dependent antifungal activity on both fungal species at the concentrations tested. This behavior is consistent with the results obtained by Tolouee et al. (2010) where *Matricaria chamomilla* L. EO was reported as a potent inhibitor of *A. niger* growth in vitro. Growth inhibition started from

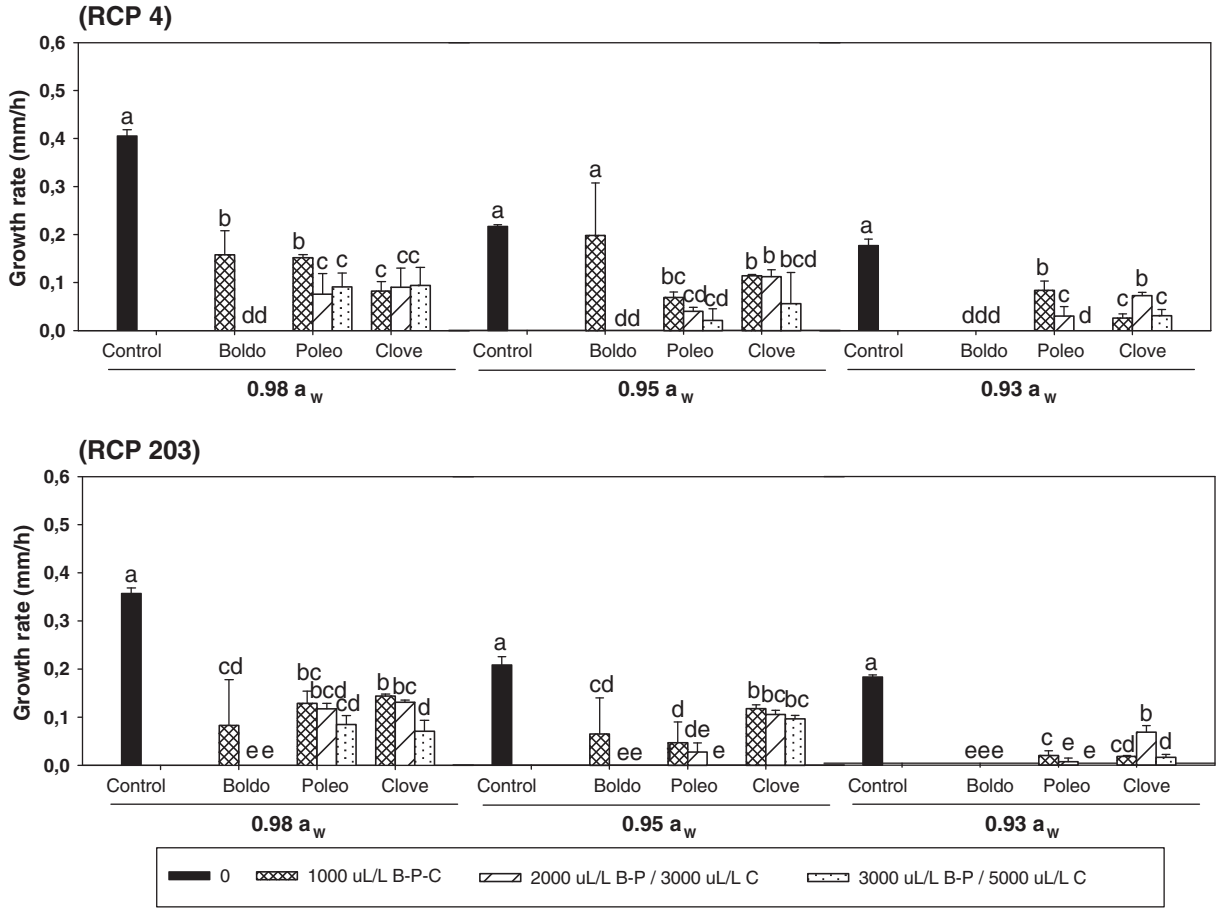

Fig. 4. Effect of EO volatile fractions on growth rate of *A. carbonarius* strains at different a_W levels on PMEA. Mean values based on quadruplicated data with letters in common for each a_W are not significantly different according to Duncan New Multiple Range Test ($p<0.05$).

Table 4
Effect of EO volatile fractions and a_W on ochratoxin A accumulation by *Aspergillus* section *Nigri* strains.

Essential oil	Doses (μL/L)	Ochratoxin A (ng/g)[a]			
		RCP42	RCP191	RCP4	RCP203
0.98 a_W					
Control	0	109.5^{a}	91.4^{a}	169.0^{b}	389.7^{ab}
Boldo	1000	17.2^{b}	47.8^{b}	9.8^{c}	20.9^{c}
	2000	n.d.^{d}	15.7^{cd}	n.d.^{c}	n.d.^{c}
Poleo	1000	134.5^{a}	53.0^{ab}	222.9^{bc}	818.1^{a}
	2000	89.7^{ab}	n.d.^{d}	54.7^{c}	2.1^{c}
	3000	44.2^{ab}	n.d.^{d}	n.d.^{c}	n.d.^{c}
Clove	1000	n.d.^{b}	19.7^{cd}	1894.9^{a}	74.5^{bc}
	3000	n.d.^{b}	n.d.^{d}	26.6^{c}	n.d.^{c}
0.95 a_W					
Control	0	91.1^{a}	1150.3^{a}	758.3^{b}	1779.7^{a}
Boldo	1000	n.d.^{c}	n.d.^{c}	746.2^{b}	1362.4^{a}
Poleo	1000	75.5^{a}	24.1^{c}	59.1^{c}	313.3^{b}
	2000	2.6^{c}	n.d.^{c}	n.d.^{c}	2.1^{c}
	3000	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}
Clove	1000	52.3^{ab}	175.1^{b}	1963.1^{a}	1284.5^{a}
	3000	10.2^{bc}	84.6^{bc}	1895.7^{a}	203.0^{b}
	5000	2.0^{c}	21.4^{c}	37.2^{c}	829.6^{ab}
0.93 a_W					
Control	0	37.5^{a}	22.6^{a}	7.7^{ab}	8.0^{a}
Poleo	1000	n.d.^{b}	n.d.^{c}	2.1^{bc}	n.d.^{b}
Clove	1000	11.3^{b}	10.1^{b}	5.4^{abc}	n.d.^{b}
	3000	n.d.^{b}	n.d.^{c}	9.8^{a}	n.d.^{b}

n.d.: not detected.
Mean in a column for each a_W with a letter in common is not significantly different according to Fisher's LSD Test ($p<0.001$). *A. niger* aggregate: RCP42, RCP191. *A. carbonarius*: RCP4, and RCP203.
[a] Mean of four replicates.

the lowest oil concentration of 15.62 μg/mL (~7.5%) and reached a maximum of ~92.5% at the final concentration of 1000 μg/mL. Garcia et al. (2012) observed that the effect of plant extracts (*Equisetum arvense* and *Stevia rebaudiana*) on growth for both *Aspergillus flavus* and *Fusarium verticillioides* was highly dependent on a_W ($p<0.05$), while the inoculum size was less important. Extracts were effective at a_W 0.95 in mold growth inhibition; however, for low a_W, results were not so clear.

Although in this study the effect of boldo, poleo, clove, anise and thyme oils on *Aspergillus* section *Nigri* has been evaluated for the first time, previous studies have reported the use of other spices that can be used as potential antifungal agents. The effects of lemon (*Citrus lemon* L.), mandarin (*C. reticulata* L.), grapefruit (*C. paradisi* L.) and orange (*C. sinensis* L.) EOs were studied on *A. niger*, achieving 100% growth inhibition at the dose of 9400 μL/L in a contact assay (Viuda-Martos et al., 2008). Recently, Tian et al. (2011) reported the efficacy of *Cicuta virosa* L. var. *latisecta* Celak oil applied in a concentration of 4000 μL/L in reducing the growth of four fungal species (*A. niger*, *A. oryzae*, *A. flavus* and *Alternaria alternata*) at high levels of 70.6–87.7%.

Although the concentrations of oils tested in this work were not the same, the contact assay showed higher inhibition than the headspace volatile assay. Complete growth inhibition was verified at concentrations of ≥1500 μL/L for boldo, poleo and clove EOs in the contact assay and headspace volatile exposure to ≥2000 μL/L for boldo, while total fungal inhibition was not reached with doses of 3000 and 5000 μL/L of poleo and clove EOs, respectively. The EO composition was not analyzed in this study, but Mazutti et al. (2008) observed that the composition of boldo oil varies according to different combinations of extraction temperature and pressure. This may explain the increased antifungal activity obtained in the contact assay in relation to the headspace assay. Our results showed that both assays are necessary to evaluate antifungal effects of EOs. In a previous study conducted by Goñi et al. (2009), behavior of clove EO was not the same in direct contact and vapor phase assays: i.e., the inhibitory effect increased by 37.1% in the vapor phase when it was assayed against *Escherichia coli* and *Yersinia enterocolitica*, but decreased by 27.4% when it was evaluated against a range of Gram-positive bacteria. On the one hand, Bluma et al. (2009) demonstrated that the vapor generated by 5000 μL/L of poleo oil significantly reduced growth of *Aspergillus* section *Flavi* in the order of 78.0%, whereas the dose of 3000 μL/L completely inhibited fungal development in the direct contact assay (Bluma and Etcheverry, 2008). The antifungal activity effected by anise and thyme was only observed at the highest concentration tested (2500 μL/L) in the direct contact assay. This approach is consistent with the reports of Bluma and Etcheverry (2008) where a dose of 2000 mg/g of anise EO was not effective in controlling *Aspergillus* section *Flavi* growth. On the other hand, Nesci et al. (2011) observed that 3000 μL/g of thyme oil did not reduce *Aspergillus* section *Flavi* counts in a peanut storage system. For a better evaluation of potential antifungal use of a specific EO, both the availability of EO in a range of media, physical–chemical variations and its degree of antifungal activity should be considered.

The ultrastructural analysis conducted by Ahmad Khan and Ahmad (2011) has highlighted the multiple sites of action of eight EOs in fungal cells, including damage to the cell walls, cell membranes, and cytoplasmic contents of *Aspergillus fumigatus* and *Trichophyton rubrum*. In addition, the authors demonstrated that test oils inhibited elastase and keratinase activities. It is believed that lipophilic properties of oils may assist in the penetration of cell membranes and in the accumulation of polysaccharides under water stress conditions. This may lead to rupture of plasmalemma in fungal cells (Ultee et al., 2002).

Inhibition of OTA required a lower oil dose than was required for inhibition of fungal growth, regardless of medium a_W. The inverse correlation was reported by Pereira et al. (2006) who observed that clove EO completely inhibited the mycelial growth of *A. ochraceus*, but did not have a pronounced antiochratoxigenic action. Our results agree with those obtained by Murthy et al. (2009) who found that growth and OTA production decreased progressively with the increase of Ajowan ethanolic extract (AEE) concentrations: at the dose of 250 μL/g, complete inhibition of fungal growth and OTA production was possible. It is reported that the inhibitory effect of spice oils was mainly due to the most abundant component present in the spice extract (Vilela et al., 2009). Compositional analyses of the most effective EOs (boldo, poleo and clove) were carried out previously in our laboratory (Bluma and Etcheverry, 2008). The study revealed that α-terpinolene (73.8%) and α-terperpine (15.3%) are the main components present in boldo, while peperitenone oxide (48.6%) and limonene (24.5%) are the main phytochemicals of poleo. Eugenol (75.4%) and α-cariofilene (14.7%) were the main components of clove. To date, there is no bibliography on the direct application of α-terpinolene and peperitenone oxide, the main phytochemicals of boldo and poleo, respectively. Studies have been conducted to determine the antimicrobial effect of the main clove EO active component. The effects of clove EO and its principal component, eugenol, on growth and mycotoxin production by some toxigenic fungal genera such as *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. have been reported by Amiri et al. (2008), Nesci et al. (2011), and Pereira et al. (2006). Jayashree and Subramanyam (1999) showed that eugenol inhibited aflatoxin production without any significant effect on growth of the organism up to a concentration of 0.75 mmol/L. These authors reported the ability of eugenol to inhibit the lipid peroxidation involved in aflatoxin production, without any significant constraints on growth or events of primary metabolism.

Boldo EO appears to be more effective than the rest of the EOs evaluated, this suggests that boldo EO volatile fraction at 2000–3000 μL/L is potentially an effective compound for complete inhibition of *Aspergillus* section *Nigri* growth and OTA production over a range of environmental conditions in peanut based medium. However, the results of this work also confirmed that besides the antifungal activity, the highest doses of poleo and clove EOs assayed presented antiochratoxigenic activity. Thus, the effects of the volatile fractions of boldo, poleo and clove EOs

on *Aspergillus* section *Nigri* strains on peanut grain are being studied to establish their abilities as fumigants.

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