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Aromatic plants essential oils activity on *Fusarium verticillioides* Fumonisin B_1 production in corn grain

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Abstract

The minimum inhibitory concentration (MIC) of *Origanum vulgare, Aloysia triphylla, Aloysia polystachya* and *Mentha piperita* essential oils (EOs) against *Fusarium verticillioides* M 7075 (*F. moniliforme*, Sheldon) were assessed, using the semisolid agar antifungal susceptibility (SAAS) technique. *O. vulgare, A. triphylla, A. polystachya* and *M. piperita* EOs were evaluated at final concentrations of 10, 20, 40, 50, 100, 200, 250, 500, 1000 and 1500 μl per litre (μl/l) of culture medium. *A. triphylla* and *O. vulgare* EOs showed the highest inhibitory effects on *F. verticillioides* mycelial development. This inhibition was observed at 250 and 500 μl/l for EOs coming from *Aloysia triphylla* and *O. vulgare*, respectively. Thus, the effects of EOs on FB_1 production were evaluated using corn grain (*Zea mays*) as substrate. The EOs were inserted on the 5th, 10th, 15th and 20th day of maize post-inoculation with a conidia suspension of *F. verticillioides*. *O. vulgare* and *A. triphylla* were applied to give final concentrations of 30 ppm and 45 ppm, respectively. Different effects were observed in the toxicogenicity at the 20th day treatment. The *O. vulgare* EO decreased the production level of FB_1 ($P < 0.01$) while *A. triphyla* EO increased it ($P < 0.001$) with respect to those obtained in the inoculated maize, not EOs treated. Results obtained in the present work indicate that fumonisin production could be inhibited or stimulated by some constituents of EOs coming from aromatic plants. Further studies should be performed to identify the components of EOs with modulatory activity on the growth and fumonisins production of *Fusarium verticillioides*.

Key words: Essential oils, *Fusarium verticillioides*, fumonisin B_1, maize, toxicogenesis.

Introduction

After isolation and characterization of fumonisin B1 (FB_1) and fumonisin B2 (FB_2) from cultures of *Fusarium verticillioides* (*F. moniliforme*, *Gibberella fujikuroi* mating population A) strain MRC 826, interest in these toxins has risen [1]. Diseases induced by mycotoxins cause acute, subchronic and chronic toxicity, depending on different factors such as animal species, age, sex, strain, doses and administration route [2–4]. Contamination with mycotoxins has been detected in different countries in products such as cereals and corn-based food products [5–6]. As regards fumonisins, only FB_1, FB_2 and fumonisin B_3 (FB_3) produce high levels of contamination in naturally contaminated products [5]. During the last years, different groups have reported the infection levels produced by toxicogenic strains of *Fusarium*, *Aspergillus* and *Penicillium* in cereals and grain-based food produced in Argentina. In these studies, *Fusarium* was found in a high percentage of the analyzed samples. *F. nygamai* (*G. fujikuroi* mating population G) and *F. verticillioides*, both fumonisins producers, were the main species found [7–8], and FB_1 was the toxin present in major concentration [5]. Antifungal chemicals, mainly low molecular weight organic acids, have been used for the preservation of stored grains. However, many disadvantages are associated with the use of acids [9], and there is a worldwide trend towards limiting their use in grains and foodstuffs. Natural aromatic plants

may provide an alternative to these preservatives. Over the past years, much effort has been required to search for new antifungal materials from natural sources in order to preserve food and grains, and many antimicrobial compounds coming from plants have been identified [10, 11]. *Origanum vulgare* and *Mentha piperita* have been previously evaluated [12–14]. *In vivo* studies have shown that *Origanum vulgare* essential oil was highly effective in controlling internal wheat fungi [14; 15]. Volatile compounds, generated from corn silks of individual maize genotypes, exhibited different biological activities after their administration to solid cultures of *Aspergillus species* [16]. The inhibitory activity was demonstrated in essential oils from 10 Indian plants against growth of genus *Fusarium* [14, 15, 17, 18]. Furthermore, it was also shown that some constituents of aromatic plants EOs like cineolle, citral, geraniol, linalool, thymol and menthol have antifungal actitvity. The main objective of this work was to characterise the *in vitro* antifungal activities of essential oils coming from four aromatic plants: *Origanum vulgare*, *Aloysia triphylla*, *Aloysia polystachya* and *Mentha piperita*, and their effects on the FB_1 production in corn grain by a toxicogenic strain of *F. verticillioides*.

Materials and methods

Preparation of essential oils

EOs were obtained from four regional aromatic plants: *Origanum vulgare* (L.), *Aloysia triphylla* (L'Herit.) Britton, *Aloysia polystachya* (Griseb.) Mold. and *Mentha piperita* L. After identification a voucher specimens was preserved in the Herbarium of the Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba. On the other hand, fresh leaves of *O. vulgare*, *A. triphylla*, *A. polystachya* and *M. piperita* were hydrodistilled in a Clevenger-type apparatus. The oils obtained were dried over anhydrous sodium sulphate and stored at -18 °C until analysis, to avoid oxidation.

Essential oils gas chromatography (GC) analyses

Analyses were carried out using a Shimadzu GC-R1A (FID) gas-chromatograph, fitted with a 30 m $\times$ 0.25 mm (0.25 μm film thickness) fused silica capillary column DB-5 (J&W). The GC operating conditions were as follows: Oven temperature programmed from 60 °C (3 min.) to 240 °C at 5 °C/min., nitrogen as the carrier gas at a constant flow of 0.9 ml/min., and source 70 eV. Identification of the components was accomplished by comparisson of their retention times with those of pure authentic samples: thymol (-Schering-Kahlbaum-Germany), α-pinene (Fluka AG. Buchssg-Switzerland) and anisol (E. Merck AG Dermstadt-Germany).

GC/MS analyses were performed with a Perkin Elmer Q-700 equipped with a SE-30 capillary column (30 m $\times$ 0.25 mm; coating thickness 0.25 μm film). The analytical conditions were: oven temperature from 60 °C (3 min.) to 240 °C at 5 °C/min.; injector and detector temperatures were 250 °C, helium as the carrier gas at a constant flow of 0.9 ml/ min., source 70 eV. The oil components were identified by two libraries MS search-computer using retention indicators as a preselection routine, and then data were confirmed by comparison with the mass spectra available in literature [19, 20].

Fungal strain

A wild-type toxicogenic isolate of *Fusarium verticillioides* (*F. moniliforme* M 7075) obtained from carnation leaves-agar by monosporic isolation, was used in all experiments. This isolate has previously been shown to be a highly fumonisin producer in culture and in maize grain [21]. The isolates were stored in the Departamento de Microbiología e Immunología, Universidad Nacional de Río Cuarto, Córdoba, Argentina. [22, 23].

Testing for antifungal activity. Minimum inhibitory concentration (MIC)

For the evaluation of antifungal activities, experiments were performed according to the semisolid agar antifungal susceptibility method (SAAS) [24] modified. Briefly, five-milliliter aliquots of semisolid brain-heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 0.5% agar, w/v (Bacto Agar, Difco Laboratories), pH $\sim$ 7.4 (without dextrose, buffer or indicator) were prepared in sterility, in 16 by 125 mm glass tubes with and without the addition of EOs. These EOs were dissolved with dimethyl sulfoxide (DMSO), and then added to the different tubes in order to obtain concentrations of 10, 20, 40, 50, 100, 200, 250, 500, 1000 and 1500 μl/l of culture medium. The final concentration of DMSO was adjusted to 4.5 μl/ml in all the tubes. As control, a free EOs-medium with a 4.5

μl/ml final concentration of DMSO was used. Volatile compounds were mixed with the medium at 45 °C, and then media were stored at 4 °C until solidification. In addition, 1 tube with uninoculated EOs-free medium, was included as a sterility control. A conidia suspension (4×10^5 /ml) prepared with a *F. verticillioides* culture grown in V-8 juice agar for 2 weeks and Tween 20 at 2.5% (v/v) in sterile water, were used as inoculums. A standard loopful (~0.001 ml) of this conidia suspension was inserted deeply into each tube of medium containing a known concentration of EOs, as well as EOs-free medium, by a centered down-up motion to form a two dimensional inoculum. Sterile mineral oil (~0.5 ml) was layered on the inoculated medium to inhibit sporulation, and then the tubes were tightly capped. Thus, in order to check the suspension purity and the conidia viability, a loopful of the inoculum suspension was streaked onto Sabouraud dextrose agar. All cultures were incubated for 48 h at 35 °C or until good growth was apparent in the EOs-free control. Within 48 h, when by visual inspection a good growth of the *F. verticillioides* in the EOs-free medium was detected, the growth in all tubes was visually compared with that of the EOs-free control in order to determine inhibition. The growth was scored in the following manner: 4+, growth comparable to that of the EOs-free control; 3+, growth approximately 75% that of the control; 2+, growth approximately 50% that of the control; 1+, growth 25% or less that of the control; and 0, no visible growth. Each treatment had 5 replications whose average gave degree of mycelial development. The experiment was performed three times.

Effect of EOs on FB_1 production

The FB_1 production was determined using healthy maize as substratum. Corn grain free from FB_1 (300 g), was placed in 1.000-ml dark Erlenmeyer flasks at 35% humidity and sterilized for two consecutive days in autoclave for 15 minutes at 121 °C. Autocleaved maize was inoculated with 200 μl of a conidia suspension of *F. verticillioides* prepared as described in Testing for antifungal activity. Incubation lasted 28 days in the dark at 25 °C, with manual stirring the first 5 days. The *A. triphylla* and *O. vulgare* EOs were applied on a sterilized paper disk Whatman No. 3 (14 mm diameter), which was placed over corn grain on the 5th, 10th, 15th and 20th day post-inoculation, in order to evaluated their effects on the different stages of the fungus development. The EOs concentrations used were 30 μl/kg of maize (30 ppm) for *O. vulgare* and 45 μl/kg of maize (45 ppm) for *A. triphylla*; corresponding to the average of 3 and 4 scores of SAAS, respectively. Control flasks were prepared following the same procedure, however, no EOs were added on paper disk. Five replications of each treatment were done. The experiment was performed twice. Separation and purification of the toxin were performed in the fermented maize following the methodology of Voss et al. [25] modified.

Fumonisin B_1 quantification

Samples (100 μl) from the watered extracts were diluted with acetonitrile (100 μl). Before the quantification assays, the samples were diluted 1/50 with acetonitrile/water (1:1). The quantification of the diluted extracts was performed following the methodology proposed by Shephard et al. [26]. 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 μl of 2-mercaptoethanol to 1 ml of methanol containing 40 mg of *o*-phthaldialdehyde. The derivatized samples were analyzed by means of a Hewlett Packard HPLC equipped with fluorescence detector. The wavelengths used were 335 nm and 440 nm for excitation and emission, respectively. An analytical reversal phase column C_{18} (150 mm $\times$ 4.6 mm internal diameter and 5 μm particle size) connected to a precolumn C_{18} (20 mm $\times$ 4.6 mm and 5 μm particle size) was also used. The mobile phase was methanol, NaH_2PO_4 0.1 M (75:25), the pH was set at 3.35 $\pm$ 0.2 with orthophosphoric acid, and a flow rate of 1.5 ml/min. was used. The quantification of fumonisin B1 was carried out by comparing the peak areas obtained from watered extracts with those corresponding to the standards of 10.5; 5.25 and 2.625 μg/ml FB_1 (PROMEC, Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg; Republic of South Africa).

Statistical evaluation

Data from these studies were analyzed by one-way analysis of variance (ANOVA) and Bonferroni Multiple Comparison test.

Results giving P values < 0.05 were considered significantly different.

Results

Essential oils Gas-Chromatograph (GC) analyses

The qualitative and quantitative components of the four essential oils analyzed are shown in Table 1. Substances present in concentrations lower than 1% are not included in the table. The essential oil obtained from *O. vulgare* plants is characterized by a high content of alcoholic and phenolic compounds (terpineol+thymol, 42.3% of the total oil). This is a characteristic observed in essential oils coming from all *Origanum* types [27–28]. The major components of *A. triphyla* EO were myrcenona, α-thujone, lippifoli – 1 (6) -ene-5-one, limonene, (65.4% of the total oil). Carvone, α-thujone and limonene (82.8% of the total oil) were the main compounds found in *A. polystachya* EO, while the *M. piperita* EO presented the highest menthol amount (at least 95% of the total oil) from the four essential oils analyzed.

Testing for antifungal activity. Minimum inhibitory concentration (MIC)

The effects of EOs on *F. verticillioides* growth in semisolid brain-heart infusion broth are shown in Table 2. All the EOs at low concentrations (10 and 20 μl/l) did not significantly alter the fungal growth, neither did *A. triphylla* or *A. polystachya* at 40 μl/l or *M. piperita* at 40, 50, 100, and 200 μl/l. On the other hand, *O. vulgare* EO at 40, 50, 100, 200, 250, 500, 1000 y 1500 μl/l reduced *F. verticillioides* growth by 3, 3, 3, 3, 2, 1, 1 and 0 score, respectively. *A. polystachya* exhibited similar influence to *O. vulgare* on *F. verticillioides* development. Conversely, at 1500 μl/l, *A. polystachya* did not completely inhibit growth. *A. triphyla* and *M. piperita* EOs completely prevented mold growth at concentrations of 1000 and 1500 μl/l respectively. In general, the inhibitory effect of EOs on *F. verticillioides* growth followed the sequence: *A. triphyla* > *O. vulgare* > *A. polystachya* > *M. piperita*. Having into account the results obtained in the SAAS assays, *A. triphyla* and *O. vulgare* EOs were used in order to know their effects on *F. verticillioides* toxicogenesis.

Effect of EOs on FB_1 production

The effects of EOs on *F. verticillioides* FB_1 production are shown in Figure 1. When the maize was treated with *O. vulgare* at day 5th, 10th, and 15th post-inoculation, no effects were observed in the FB_1 synthesis. At day 20th, 30 ppm of this EO exhibited a significant inhibitory effect on FB_1 production with respect to control ($P < 0.01$). On the other hand, at a concentration of 45 ppm, *A. triphyla* EO did not affect the *F. verticillioides* toxicogenesis when maize was treated at day 5th, 10th and 15th post-inoculation. Conversely, at day 20th, *A. triphyla* EO showed a strong stimulatory effect on the FB_1 production ($P < 0.001$).

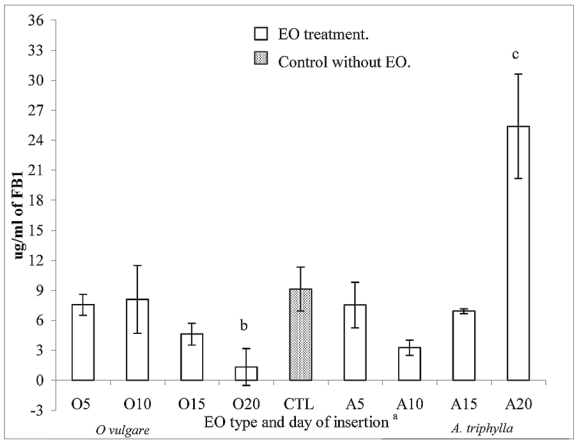

Figure 1. Effects of EO in FB_1 production by using autocleaved maize grain as substratum. Aliquots of 200 μl of a conidia suspension of *Fusarium verticillioides* was added and then maize was cultured in the dark at 25 °C for 28 days. The EOs *O. vulgare* (30 ppm) and *A. triphylla* (45 ppm) were applied at 5th, 10th, 15th and 20th days post-inoculation. The quantification was performed following the methodology proposed by Shephard et al. (1990). Five replications were done of each treatment. [a]The numbers 5, 10, 15 and 20 represent the post-inoculation day in which the different EOs were added. O = treatment with *Origanum vulgare*, A = treatment with *Aloysia triphylla* and CTL = Control without EOs. [b]$P < 0.01$, with respect to the control. [c]$P < 0.001$, with respect to the control. $n \geq 5$.

Discussion

The antifungal activities of essential oils have been studied for a long time [11, 29, 30, 31, 32, 33]. However, their effects on the production of secondary fungal metabolites have been poorly explored [34, 35].

Among the components of essential oils, the oxygenated-containing compounds, substances with a diverse bioactivity, have shown a stronger antifungal activity [15] compared to those of hydrocarbonated ones. However, the oxygen-compounds have diverse bioactivity. Thus, several authors have proposed a scale of potential antifungal activity: phenols > alcohols > aldehydes > ketones > esters > hydrocarbons

Table 1. Main composition of the aromatic plants EOs studied.[a]

Components	Aromatic plants			
	O. vulgare	*A. triphylla*	*A. polystachya*	*M. piperita*
Thymol	21.2			
Terpineol	21.1			
γ-terpinene	9.1			
Myrcenone		36.5		
α-thujone		13.1	30.3	
Limonene		6.9	14.3	
Lippifoli-1(6)-ene-5-one		8.9		
Carvone			38.2	
Menthol				95
Others	48.6	34.6	17.2	5

[a]Results for individual components are expressed as percentages of total EOs.

Table 2. Testing for antifungal activity.

Essential oils	Concentrations[a] ($n = 5$)									
μl/l	1500	1000	500	250	200	100	50	40	20	10
O. vulgare	0	1	1[b]	2	3	3	3	3[c]	4[c]	4
A. triphylla	0	0	1	1[b]	2	2	2[c]	4[c]	4	4
A. polystachya	1	1	1[b]	2	3	3	3	4	4	4
M. piperita	0	1[b]	2	3	4	4	4	4	4	4

0 – No visible growth, 1 – Growth 25% or less than Control, 2 – Growth approximately 50% of the Control, 3 – Growth approximately 75% of the Control, 4 – Growth comparable to the Control.

[a]Expressed as μl of EOs/l of culture medium (Brain-heart semisolid agar).

[b]Score of minimal inhibitory concentration.

[c]To determine the effects of EOs on the FB_1 production in corn grain, an average of both concentrations was used. (30 μl/l and 45 μl/l for *O. vulgare* and *A. triphylla*, respectively).

[36]. The antifungal activity of *O. vulgare* EO against *F. verticillioides* was one of the highest one in that scale (Table 2), then our results corroborate the strong bioactivity of phenolic components rich-EOs (Table 1) [15, 28, 37]. The results obtained in the present work show that carbonilic compounds rich-EOs (Table 1) have different antifungal activities (Table 2). Thus, *A. polystachya* EO has less bioactivity than those from *A. triphyla* EO. These effects could be caused by the differents EOs main component structures, since myrcenone, the main constituent of *A. triphyla* EO, is an acyclic monoterpene, *A. polystachya* EO is mainly constituted by bicyclic monoterpene. In the same sense, the observed growth inhibitory effect of *O. vulgare* could be related with some previously reported properties of acyclic monoterpenes, rather than bicyclic compounds, for binding to lipid phase of fungal membranes [38]. At present, different authors are studying the antifungal activity of aromatic plants EOs against *Fusarium* and *Aspergillus* in order to prevent the attack and damage of grains [12, 13, 14]. In this work, we studied the antifungal activities of four regional aromatic plants EOs, and we also correlated the action of these EOs on the FB_1 production by a toxicogenic strain of *F. verticillioides*. Similar studies have been designed to determine the effects of aromatic plants EOs on the growth of a diversity of microorganisms [11, 33, 39], as well as toxicogenic and pathogenic fungi [34, 35]. In these experimental models, it was observed an *in vitro* inhibitory effects of EOs over the development of such microorganisms, wich was related to the methodology, the strain and the concentrations used, similarly to the findings reported by other authors in similar experimental conditions, and with other kinds of moulds [29, 30, 33]. From the EOs evaluated in the present work, the strongest *in vitro* antifungal activity against *F. verticillioides* was detected in the EO coming from *O. vulgare* (Table

2). Antiaflatoxicogenic properties of some EOs on the mycotoxin-producers *Aspergillus* species [31, 35, 40], and also in the biosynthesis of ochratoxin A [34] have been demonstrated, but little is known about the modulatory actions of plants EOs on the mycotoxins production by toxicogenic strains of *Fusarium*. In our work *O. vulgare* and *A. triphyla* EOs showed the highest inhibitory activity in fungal growth. Previous reports showed a direct relationship between inhibitory effects of essential oils on fungal growth and toxin formation [35, 39, 40]. Then, in order to evaluate possible alterations induced by these EOs on the toxicogenesis of *F. verticillioides*, we applied them in concentrations that allowed the fungus development on maize (Score 3 of SAAS, Table 2).

While *O. vulgare* EO highly inhibited the production of FB_1 upon the grain, we have observed opposite results for *A. triphyla* (Figure 1) EO when applied at day 20-post inoculation. Some monoterpenes such as timol (principal compound in *O. vulgare* EO) mentol and cinnamaldehyde demonstrated antioxidant properties on the lipid peroxidation [37, 41]. Moreover these monoterpenes showed inhitory capacity on toxicogenesis [42] and the esclerotial development [43]. Similar to others EOs compositions, the *A. triphyla* EO main component was myrcenone, and a high content of alpha thujone and isomers of myrcenone was also observed. These components showed oxidant properties [44] Thus, *A. triphylla* EO may have increased the FB_1 levels production by a probable increase of lipid peroxidation. Although antifungal properties have been shown for several EOs, up to date, little is known about their effects on the synthesis of mycotoxins. Moreover, in other studies, EOs are used to observe their effects on the FB_1 production *in vitro* [17, 33] but not using corn grain as in this report [39]. Thus, although some studies have been carried out in *M. piperita*, remains unclear the EO *A. triphylla* action on *F. verticillioides* toxicogenesis. Further research should be focused on the study of aromatic plants EOs, or fractions of them, as modulator substances of the FB_1 biosinthetic pathways used by *F. verticillioides*.

From what it has been observed in this work, we think that a question unfolds as regards the effects of EOs of regional aromatic plants and their possible beneficial mechanisms to prevent or control fungal attack and the presence of mycotoxin upon foodstuff.

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