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Antifungal activity and inhibition of fumonisin production by *Rosmarinus officinalis* L. essential oil in *Fusarium verticillioides* (Sacc.) Nirenberg

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ABSTRACT

The chemical composition of *Rosmarinus officinalis* L. essential oil (REO) was analysed by gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy. The main compounds of the REO were 1.8 cineole (52.2%), camphor (15.2%) and α-pinene (12.4%). The mycelial growth of *Fusarium verticillioides* (Sacc.) Nirenberg was reduced significantly by 150 μg/mL of REO. Significant microscopic morphological changes were visualised, such as the rupture of the cell wall and the leakage of cytoplasm at 300 μg/mL of REO. At lower concentrations of REO, the effects on the production of ergosterol and the biomass of mycelium varied, as did the effects on the production of fumonisins, but at ⩾300 μg/mL of REO, these processes were significantly inhibited, showing the effectiveness of the REO as an antifungal agent. The results suggested that the REO acts against *F. verticillioides* by disrupting the cell wall and causing the loss of cellular components, subsequently inhibiting the production of fumonisins and ergosterol.

1. Introduction

Fusarium species are widely distributed grain contaminants. They are economically relevant because of their ability to infect important crops, such as corn, wheat and other grains, in the field (Munkvold, 2003). Fumonisins are mycotoxins produced mainly by the fungus *Fusarium verticillioides*. These mycotoxins have been divided into four groups: fumonisins A, B, C, and P. Analogues of the B series compounds are the most abundant and are subdivided into fumonisin B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3). FB_1, which is the most toxic of compound in this subdivision, is responsible for 70–80% of food contamination. FB_2 and FB_3 are responsible for 15–25% and 3–8% of food contamination, respectively (Rheeder, Marasas, & Vismer, 2002). Fumonisins have been classified by the International Agency for Research on Cancer (2002) as Class 2B substances, i.e., possibly carcinogenic to humans (IARC, 2002).

Fumonisins have been associated with several animal diseases, including equine leucoencephalomalacia and porcine pulmonary oedema, and with the alteration of some immunological parameters in mice and rats (Theumer, Lopez, Masih, Chulze, & Rubinstein, 2002). In humans, the consumption of products contaminated with fumonisins has been associated with an increased risk of developing oesophageal cancer and defective formation of the embryonic neural tube (IARC, 2002; Missmer et al., 2006).

Several strategies for controlling the growth of toxigenic fungi and mycotoxin synthesis employ chemical treatments. However, many problems related to the development of fungus resistance and the emergence of secondary pests have emerging due owing to the indiscriminate use of the chemicals (Cabral, Pinto, & Patriarca, 2013). This fact has increased the risk of high toxic residue levels in food and the environment, which damages the environment and puts human and animal health at risk. Plant essential oils may be an alternative to the employment of these synthetic agents because they have antifungal and antimycotoxigenic activities. Plant essential oils may be easily acquired, have low costs and lack the problems inherent in synthetic chemical products (Soliman & Badeaa, 2002).

Rosemary (*Rosmarinus officinalis* L.) is widely used in the food industry as a flavouring and preservative, due to the presence of phenolic diterpenes, which have antioxidant and antimicrobial properties (Flamini, Cioni, Morelli, Macchia, & Ceccarini, 2002).

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However, few studies have reported its use as an antifungal agent. Thus, the aim of this study was to evaluate the efficacy of *R. officinalis* L. essential oil (REO) to control the growth of *F. verticillioides* in vitro and consequently its fumonisin production.

2. Materials and methods

2.1. Extraction of the essential oil

The essential oil was obtained from rosemary leaves by hydrodistillation using a Clevenger-type apparatus in accordance with the method recommended by the European Pharmacopoeia (Council of Europe, 1997). The extraction was performed for 150 min while maintaining the boiling point at a constant temperature and using 70 g of dried rosemary leaves (Nutricrock®, Maringa, Brazil) and 500 mL of distilled water. The yield of the REO extract was 1500 μL. The oil obtained was stored at 4 °C and protected from light prior to chemical analysis and use.

2.2. Analysis of the essential oil

The chemical composition of the REO was investigated using gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) spectroscopy. The GC analysis was performed using a Thermo Electron Corporation Focus GC model under the following conditions: a DB-5 capillary column (30 m × 0.32 mm × 0.50 mm); column temperature, 60 °C (1 min), rising to 180 °C at 3 °C/min; injector temperature, 220 °C; detector temperature, 220 °C; split ratio, 1:10; carrier gas, He; flow rate, 1.0 mL/min. The injected volume was 1 μL, diluted in acetone (1:10). The GC–MS analysis was performed using a Quadrupole Mass Spectrometer (Thermo Electron Corporation, DSQ II model) operating at 70 eV. The identification of individual components was based on comparisons of their GC retention indices on nonpolar columns and comparisons with the mass spectra of authentic standards purchased from Sigma–Aldrich (Adams, 2001). For the NMR analysis, the ^{1}H (300.06 MHz) and ^{13}C NMR (75.45 MHz) spectra were obtained using a deuterated chloroform ($CDCl_3$) solution using a Mercury-300BB spectrometer with the δ (ppm) value and the spectra referred to those of $CDCl_3$ (δ: 7.27 for ^{1}H and 77.00 for ^{13}C) as an internal standard.

2.3. Microorganism

F. verticillioides (103 F) was isolated from corn residue used in animal feed, which was implicated in an intoxication (horse) in 1991 by Dr. Elisa Yoko Hirooka, Department of Food Science and Technology, State University of Londrina. The strain was identified at the Science University of Tokyo by Dr. Y. Sugiura, a mycologist, and Dr. Ichinoe, a plant pathologist with expertise in the field of *Fusarium* sp. This isolate had previously been shown to be a highly effective fumonisin producer in liquid culture (Falcão et al., 2011) and was deposited in a culture collection of the University of Londrina (Brazil).

2.4. Culture conditions

F. verticillioides was cultured on PDA (Neogen, Lansing, MI, USA) in a 90 mm diameter Petri dish for 7 days at 25 °C in the dark in an incubator (Model 347G, FANEM, Sao Paulo, Brazil) for the determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) and for the evaluation of the effects of REO on ergosterol and fumonisin production and microconidial morphology.

2.5. Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The MIC of REO was determined using the broth-dilution method in accordance with standard M38-A of the National Committee for Clinical Laboratory Standards (Pfaller, 2002) adapted for broth macrodilution. The assay used 12 tubes, 10 of the test tubes contained RPMI-1640 medium, the REO diluted in a sterile solution of 0.1% Tween 80 (Vetec, Rio de Janeiro, Brazil) and the fungus inoculum to be tested, and the other two tubes contained the positive (medium and inoculum) and negative controls (only medium). An aliquot of 500 μL of RPMI was added to tubes 2–11, and 1000 μL was added to tube 12 (negative control). A 500 μL sample of REO at the concentration of 19,200 μg/mL was added to tubes 1 and 2. Starting with tube 2, a 1:2 serial dilution was performed up to tube 10 by transferring 500 μL from each test tube and then discarding 500 μL from tube 10. A 500 μL sample of the suspension of *F. verticillioides* at 10^4/CFU was placed in tubes 1–11, which reduced the oil concentration in each tube to half of its initial value. The final concentrations of REO in tubes 1–10 were 18.75–9,600 μg/mL. The MIC was considered the lowest concentration of REO that inhibited the visually observable growth of *F. verticillioides*. The positive control was performed using medium that contained only the suspension of *F. verticillioides*. To determine the MFC, 10 μL from each test tube was added to Sabouraud Dextrose Agar. The tubes were incubated at 35 °C for 24 h. The MFC was considered the lowest concentration of REO that prevented visually observable growth of *F. verticillioides*.

2.6. Effect of essential oil on F. verticillioides

An 8-mm diameter mycelial disc of *F. verticillioides* was taken from the PDA culture and was inoculated onto a Petri dish that contained culture medium established by Jiménez, Mateo, Hinojo, and Mateo (2003) and 2% agar. Rosemary essential oil was diluted in a sterile solution of 0.1% Tween-80 and added to the medium to obtain final concentrations of 35, 75, 150, 300 and 600 μg/mL. These concentrations were defined according to Shukla, Singh, Prakash, and Dubey (2012), who proposed using the MIC and two concentrations below and above it. The control for fungal growth (FC) was performed using medium that contained only the inoculum. The plates were incubated at 25 °C for 7 days in the dark. For a positive control (PC), the synthetic anti-fungal nystatin was added to the medium to obtain a final concentration of 1000 μg/mL (Shukla et al., 2012). These plates were used to evaluate mycelial growth and for scanning electron microscopy. Four replications were performed for each of the experimental groups and the control groups.

2.6.1. Mycelial growth

The mycelial growth of *F. verticillioides* was evaluated according to Tian et al. (2011), with modifications. After incubation for 7 days, the colony diameter was measured using a ruler. The percentage of mycelial growth inhibition (MGI) was calculated according to the following formula: MGI (%) = [(dc − dt)/dc] × 100, where dc (cm) is the mean colony diameter for the controls (FC) and dt (cm) is the mean colony diameter for each group treated with REO.

2.6.2. Microscopy and image capture

The technique for the microcultivation of filamentous fungi was used to prepare samples for microscopy. Two blocks of 1 cm × 1 cm were cut from Petri dishes containing solidified medium established by Jiménez et al. (2003) and *F. verticillioides* that was treated or not treated with REO, and the fungus was seeded on each side of a piece of culture medium. Each block was placed on a sterile lamina and stored in a Petri dish in an incubator for

7 days. After this period, laminae containing the growing fungi were removed and stained using lactophenol cotton blue. Images were captured using an Olympus BX 41 microscope (Shinjuku-ku, Tokyo, Japan) in the Histology Laboratory of the State University of Maringa.

2.6.3. Scanning electron microscopy (SEM)

Seven-day-old fungal cultures treated with the REO at different concentrations (0, 35, 75, 150, 300 and 600 μg/mL) were observed by SEM. Approximately 5 × 10 mm segments were cut from cultures growing on plates. The segments were washed according to Endo, Cortez, Ueda-Nakamura, Nakamura, and Dias-Filho (2010) in 0.1 M phosphate-buffered saline (PBS), pH 7.2, and fixed using 2.5% glutaraldehyde (Sigma Chemical, St. Louis, MO, USA) in 0.1 M sodium cacodylate buffer (EM Sciences, Philadelphia, PA, USA). The material was applied to a poly-L-lysine-coated chip coverslip (Sigma–Aldrich, St. Louis, MO, USA) for 2 h at room temperature. The material was washed using 0.1 M sodium cacodylate buffer and dehydrated using an ethanol series (15–100%). The samples were subjected to critical-point drying in CO_2 (White Martins, Rio de Janeiro, Brazil) and sputter-coated with gold (IC-50, Shimadzu, Kyoto, Japan). The morphological characteristics of the microconidia were determined using a scanning electron microscope (SEM 550 SS, Shimadzu, Kyoto, Japan) operating at 10.0 kV.

2.6.4. Determination of the wet weight of the mycelia

REO was diluted using a sterile solution of 0.1% Tween 80 and added to 24 mL of a liquid medium described by Jiménez et al. (2003) to obtain final concentrations of 35, 75, 150, 300 and 600 μg/mL. *F. verticillioides* conidial suspensions were diluted in 0.1% Tween 80 to obtain approximately 10^6 conidia/mL and 1000 μL of the suspension was added to each sample of medium. The control for fungal growth (FC) was performed using medium that contained only the inoculum. The positive control (PC) contained nystatin (1000 μg/mL) in the medium (Shukla et al., 2012). The flasks were incubated at 25 °C for 14 days with agitation in an MA 830 incubator (Marconi®, Piracicaba, Brazil). After the incubation period, the medium was filtered through pre-weighed filter paper. The wet weight of the mycelia was determined (Dikbas, Kotan, Dadasoglu, & Sahin, 2008). The mycelia were used for determination of the ergosterol content and the filtrates were used for determination of the fumonisin content. Four replications were performed for each of the experimental groups, the FC and PC.

2.6.5. Analytical determination of ergosterol content

The ergosterol extraction procedure was performed according to Silva, Corso, and Matheus (2010) and consisted of transferring the mycelia to a Falcon tube that contained 20 mL of methanol, 5 mL of absolute ethanol PA, and 2 g of potassium hydroxide (Merck, Darmstadt, Germany). This solution was stirred for 5 min in a mixer pipe (KMC 1300 V, Bucheon, Gyeoggi-Do, South Korea) and heated in a water bath (BM Evlab EV:015, Londrina, Brazil) at 70 °C for 40 min. After cooling at room temperature, 5 mL of distilled water was added and the sample was centrifuged (Hettich Universal 320R, Tuttingen, Baden-Wurttemberg, Germany) at 1.735×*g* for 24 min. For the final extraction, n-hexane was added (FMaia, Cotia, Brazil) in a volume equal to that of the supernatant, and the organic fraction was collected in an amber glass vial and evaporated in a water bath at 65 °C. The residue obtained was stored at −18 °C until it was analysed.

Ergosterol quantification was performed according to Salmanowicz, Nylund, and Wallander (1990), with modifications. The extract was resuspended in 1 mL of absolute ethanol (Panreac, Barcelona, Spain) and filtered using an LCR Millex filter fitted with a 13-mm diameter PTFE-modified membrane with 0.45-μm pores (Millipore Corporation, Billerica, MA, USA). 100 μL was injected into the chromatographic system.

High-performance liquid chromatography (HPLC) was performed using a Finnigan Surveyor Plus (Thermo Scientific, San Jose, CA, USA) instrument with an ultraviolet/visible spectrum Finnigan Surveyor detection system at a wavelength of 282 nm. For the mobile phase, methanol was used at a flow rate of 1.5 mL min. Reverse-phase HPLC separation was performed on a Spherisorb C18 (Waters, Wexford, Ireland) column with a 5 μm particle size column (150 × 4.6 mm). For quantification, an intermediary solution of 1000 μg/mL of ergosterol (Sigma Chemical, St. Louis, MO, USA) was prepared in absolute ethanol PA (Merck, Sao Paulo, Brazil) and stored in an amber vial at 4 °C. A five-point calibration curve covering the range of 10–100 μg/mL of ergosterol was used to calibrate the HPLC system. The retention time was 4.6 min. The detection limit and quantification limit were 0.15 and 10 μg/mL, respectively. The percentage of ergosterol recovered was 77.4 ± 7.2 with the used method of extraction.

2.7. Effect of rosemary essential oil on fumonisin production by F. verticillioides

2.7.1. Fumonisin extraction

The fumonisins (FB_1 and FB_2) extraction process was performed according to the method of Camargos, Machinski, and Valente Soares (1999), with modifications. The cell-free extract (10 mL was mixed with 10 mL of methanol:water (3:1, v/v; Honeywell Burdick and Jackson, Muskegon, MI, USA) with stirring in shaker tubes (Vortex KMC 1300 V mixer, Bucheon, Gyeoggi-Do, South Korea) for 1 min. The cleaning step was performed in the solid phase using a strong anion-exchange column (Sep Pak Vac Accel Plus QMA, Waters, Wexford, Ireland). The column was conditioned with 10 mL of methanol, followed by 10 mL of methanol:water (3:1, v/v). The filtrate (20 mL) was then applied to the extract on the column and was washed with 10 mL of methanol:water (3:1, v/v) and with 6 mL of methanol. The fumonisins were eluted using 20 mL of methanol:glacial acetic acid (95:5, v/v; Mallinckrodt Baker, Xalostoc, Mexico). The eluate was evaporated in a 99.9% nitrogen flow in a sample concentrator at 60 °C. The residue obtained was stored at −18 °C until it was analysed.

2.7.2. LC–MS/MS analysis

The samples were analysed using a HPLC instrument coupled to a Quadrupole Mass Spectrometer, with electrospray source operated in the positive mode (HPLC-MS/MS API 5000, AB Sciex Pte. Ltd., CA, USA); Zorbax Eclipse XDB-C8 column (5 μm, 4.6 × 150 mm; Agilent Technologies, CA, USA) with a column temperature of 40 °C. The analytical separation was performed using gradient elution with water as the mobile phase A and acetonitrile as mobile phase B, both containing 0.5% formic acid. The gradient was segmented into phases A and B, as follows: 0.0 min, A/B (65:35); 3.5 min (30:70); 8.0 min (65:35); 10.0 min (65:35) with a constant flow of 0.8 mL/min. The analysis time was 10 min, consisting of approximately 3.40 min for the retention of FB_1 and 7.20 min for the retention of FB_2. The limits of detection and quantification of the samples were 0.125 and 0.312 ng/L, respectively. The average recovery rates were 94% for FB_1 and FB_2 and the correlation coefficients obtained were 8.1% and 6.2% for FB_1 and FB_2, respectively. The parameters of the mass spectrometer and the ionisation source were optimised as follows: declustering potential (DP) of 160 for FB_1, 250 and 96 for FB_2; collision energy (CE) 57 and 43 V for FB_1, 51 and 41 V for FB_2; entrance potential (EP) 10; collision cell exit potential (CXP) 28; curtain gas (CUR) 10 psi; collision gas (CAD) medium; temperature 5500 °C; ion source gas 1 (GS1) 10 psi; ion source gas 2 (GS2) 50 psi; and ion spray voltage (IS) 50 V.

2.8. Statistical analysis

All of the experiments were repeated four times. The results were expressed as the mean value ± standard deviation and were compared using an analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's test for comparisons with control groups using GraphPad Prism 5.0 software (GraphPad Software, Inc.).

3. Results and discussion

The results of the GC–MS and NMR analysis of the volatile profile of REO are listed in Table 1. The major compounds in the essential oil were 1.8 cineole (52.2%), camphor (15.2%) and α-pinene (12.4%). These results were consistent with previous findings. Jalali-Heravi, Moazeni, and Sereshti (2011) and Jiang et al. (2011) also found that 1.8 cineole was the major component of REO.

In the present study, REO inhibited the growth of *F. verticillioides* with a MIC and MFC of 150 μg/mL. This activity is attributable to the hydrophobic nature of essential oils causing the loss of membrane integrity and leakage of cellular material. The antifungal activity of REO was exploited by Sacchetti et al. (2005), who demonstrated a MIC of 90 μg/mL for *Candida albicans*, 120 μg/mL for *Rhodotorula glutinis*, 60 μg/mL for *Schizosaccharomyces pombe*, 180 μg/mL for *Saccharomyces cerevisiae* and 120 μg/mL for *Yarrowia lipolytica*; in this study the major component of REO was verbenone, the microorganism concentration was 10^5 conidia/mL and the technique used was different from that used in our study. Jiang et al. (2011) described that REO had a MIC of 1000 μg/mL and a MFC of 4000 μg/mL for *Aspergillus niger*; in this study the major component of the REO, the concentration of the microorganism and techniques utilised were similar to ours.

REO inhibited the mycelial growth of *F. verticillioides* in a dose-dependent manner (Table 2). High concentrations of essential oils (⩾150 μg/mL) exhibited significantly different fungal control activities ($p < 0.05$), whereas the levels of activities of relatively low concentrations (⩽75 μg/mL) was not significantly different ($p > 0.05$). Daferera, Ziogas, and Polissiou (2003) observed 72% inhibition of the mycelial growth of *Fusarium* sp. at 1000 μg/mL of REO. In the present study, the same result was obtained using a lower concentration (600 μg/mL).

With regard to the morphological structure of *F. verticillioides* that was determined using SEM (Fig. 1), the FC showed intact structures with long monophialides, homogeneous hyphae of consistent diameter and large numbers of microconidia (Fig. 1(A and B)). According to Pitt and Hocking (1997) *F. verticillioides* characteristically produce slightly curved fusiform microconidia that form chains of long monophialides. Reduced amounts of microconidia were observed in samples grown in high concentrations (⩾150 μg/mL) of REO, and they were smaller than normal. SEM imaging showed that the length of the microconidia had increased to 10.7 μm (Fig. 1(C)) in samples grown in 75 μg/mL of REO in relation to that of the FC, for which the lengths ranged from 7.15 to 8.24 μm (Fig. 1(A and B)). Fig. 1(D and E) shows the reduced length of the microconidia, of 7.03 and 5.72 μm at 150 and 300 μg/mL of REO, respectively. In the PC (Fig. 1(F)), the length of the microconidia was 6.17 μm. According to the studies of Li et al. (2006), changes in the size of microconidia may be related to the REO suppressing the expression of the velvet homologous gene *veA* of *F. verticillioides* (FvVE1). This activity can affect colony morphology by increasing the size of conidia due to reducing the turgor pressure on the cell wall and by the modification of the surface properties of the cell by osmotic stabilisers in an attempt to restore the osmotic balance.

The appearance of the microconidia was changed when increased concentrations of REO were used. The microconidia appeared wrinkled due to the reduction in cytoplasmic contents, as was verified by Yamamoto-Ribeiro et al. (2013) in a study that evaluated the effects of ginger essential oil on *F. verticillioides*. At the concentration of 300 μg/mL REO (Fig. 1(D)), the apparent rupture of the cell wall and leakage of the cytoplasmic contents was observed, and due to these effects, the hyphae appeared flattened and wrinkled. This result indicated that the antifungal property of REO against *F. verticillioides* occurs through the loss of membrane integrity and thus the blockage of cell growth. This effect of REO on the integrity and rigidity of the cell wall is consistent with the antifungal properties described by Carmo, Lima, Souza, and Sousa (2008).

Regarding the weight of the mycelia, there was an increase of 14.5 and 18.1% in cultures treated with 35 and 75 μg/mL REO, respectively, compared to that of the FC. At the concentration of 150 μg/mL of REO, a reduction of 12.0% in the weight of the

Table 1
Chemical composition of *Rosmarinus officinalis* L. essential oil (REO).

No.	Compounds	RI[a]	Percentage (%)	Identification[b]
1	α-Pinene	930	12.4	CG/MS, NMR
2	Canfene	945	3.7	CG/MS, NMR
3	β-Pinene	974	1.8	CG/MS, NMR
4	β-Myrcene	989	0.7	CG/MS, NMR
5	α-Phellandrene	1002	0.1	CG/MS
6	3-Carene	1008	0.2	CG/MS
7	α-Terpinene	1013	0.4	CG/MS, NMR
8	p-Cimene	1020	2.1	CG/MS, NMR
9	Limonene	1024	3.5	CG/MS, NMR
10	1.8-cineole	1027	52.2	CG/MS, RMN
11	*Trans*-β-ocimene	1041	0.1	CG/MS
12	γ-Terpinene	1055	0.4	CG/MS, NMR
13	Linalol	1098	0.4	CG/MS, NMR
14	6-Canfenol	1111	0.1	CG/MS
15	Camphor	1142	15.2	CG/MS, NMR
16	Isoborneol	1154	0.1	CG/MS
17	Borneol	1163	3.0	CG/MS, NMR
18	4-Terpineol	1175	0.5	CG/MS, NMR
19	α-Terpineol	1189	2.3	CG/MS, NMR
20	Verbenone	1209	0.1	CG/MS
21	Isobornyl acetate	1285	0.3	CG/MS, NMR
22	Eugenol	1355	0.1	CG/MS, NMR
23	β-Caryophyllene	1420	0.2	CG/MS, NMR
24	*Cis*-guaia-3,9-dien-11-ol	1644	0.1	CG/MS
	Total		100.0	

[a] RI = Retention indices obtained with reference to an *n*-alkane series C_8H_{18}–$C_{20}H_{42}$ using DB-5 column.
[b] GC/MS – gas chromatography/mass spectrometry and NMR – nuclear magnetic resonance spectroscopy.

Table 2
Inhibitory effect of Rosemary essential oil (ROE) on the mycelial growth of *Fusarium verticillioides*. The liquid medium was prepared according to Jiménez et al. (2003) with the addition of 2% agar. The cultures were incubated for 7 days at 25 °C in the dark ($n = 4$).

REO concentration (μg/mL)	Growth inhibition[b] (%)
FC[c]	0 ± 0.0
35	7.7 ± 1.4
75	10.7 ± 0.0
150	17.0 ± 2.4[a]
300	29.7 ± 7.0[a]
600	67.0 ± 7.0[a]
PC[d]	79.3 ± 3.7[a]

[a] Significant differences at $p < 0.05$.
[b] Values are average ± standard deviation of replicates.
[c] FC = Fungal control (medium containing only the inoculum of an 8-mm diameter disc of *F. verticillioides* mycelia).
[d] PC = Positive control (medium containing the inoculum and the antifungal nystatin at 1000 μg/mL).

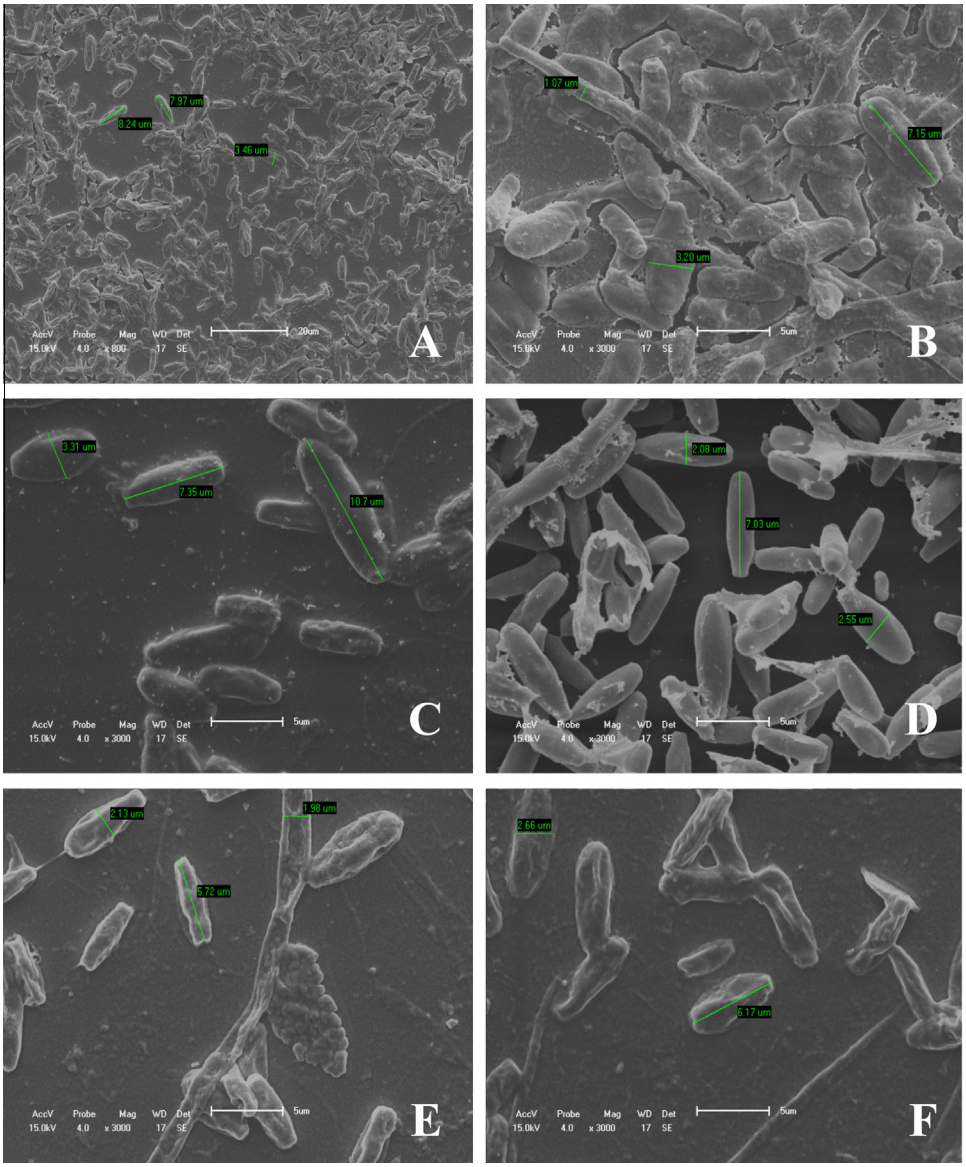

Fig. 1. Scanning electron micrographs of *F. verticillioides* treated with rosemary essential oil (REO). (A and B) untreated microconidia (FC – fungal control). (C–E) Microconidia treated with 75, 150 and 300 μg/mL of REO, respectively. (F) PC – positive control, microconidial treated with nystatin (1000 μg/mL). The liquid medium was prepared according to Jiménez et al. (2003) with the addition of 2% agar. The cultures were incubated for 7 days at 25 °C in the dark ($n = 4$).

mycelia was observed and significant reductions ($p < 0.05$) in this value occurred in cultures treated with REO at 300 μg/mL (50.7%) and 600 μg/mL (100%). The amount of ergosterol produced by *F. verticillioides* exposed to REO at different concentrations was used to quantify the fungal biomass. As shown in Fig. 2, REO at lower concentrations (35–150 μg/mL) caused fluctuations in ergosterol production and at 300 and 600 μg/mL, REO effectively inhibited ergosterol production in *F. verticillioides*, with inhibition of 75.6 and 100%, respectively.

Some antifungal agents inhibit cell growth by interrupting ergosterol biosynthesis, which results from the antifungals binding to ergosterol on the cellular membrane. Thus, this process affects the integrity and function of some of the membrane-bound proteins and leads to osmotic disturbances, disruption of cell growth and proliferation (Bendaha et al., 2011). The oscillations observed in the amounts of mycelia and the reduction of ergosterol production may have been due to stresses caused by some components of the REO. These components are capable of activating a compensatory mechanism, which generated an adaptive response of the

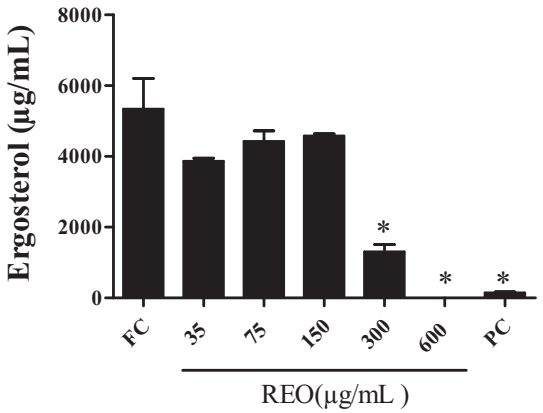

Fig. 2. Effect of *Rosmarinus officinalis* L. essential oil (REO) on the production of ergosterol by *F. verticillioides*, as determined using HPLC-UV. The cultures were incubated for 14 days with agitation at 25 °C ($n = 4$). FC – fungal control (inoculum without REO). PC – positive control (inoculum treated with 1000 μg/mL of nystatin). *$p < 0.05$.

Table 3
Effect of *Rosmarinus officinalis* L. essential oil (REO) on fumonisin production by *F. verticillioides*. The cultures were incubated for 14 days with agitation at 25 °C ($n = 4$). FC – fungal control (inoculum without REO). PC – positive control (inoculum treated with 1000 μg/mL of nystatin).

REO (μg/mL)	Fumonisin B_1		Fumonisin B_2	
	Concentration (ng/L)[b]	Inhibition (%)	Concentration (ng/L)[b]	Inhibition (%)
FC	383.5 ± 62.8	0	92 ± 32.9	0
35	601.7 ± 30.1	0	115.0 ± 24.0	0
75	428.7 ± 222.0	0	98.8 ± 49.8	0
150	380.3 ± 216.2	0.8	76.2 ± 22.0	17.2
300	8.6 ± 7.8[a]	97.7	2.4 ± 2.0[a]	97.4
600	1.7 ± 0.05[a]	99.6	0.6 ± 0.03[a]	99.4
PC	138.00 ± 0.9[a]	64.0	15.0 ± 0.8[a]	83.7

[a] Significantly different ($p < 0.05$) compared with the FC (Fungal Control).
[b] Values obtained by HPLC–MS/MS analysis, expressed as the mean values ± standard deviation.

fungus, resulting in the reprogramming of genomic expression to protect the cell-wall structure (Parveen et al., 2004).

REO significantly inhibited the production of fumonisins B_1 (FB_1) and B_2 (FB_2) by *F. verticillioides* ($p < 0.05$) from the concentration of 300 μg/mL (Table 3). At low concentrations of REO (35 and 75 μg/mL), there was an increase in the production of fumonisins. Prakash et al. (2010) reported that lower concentrations of *Piper betle* L. essential oil stimulated the production of aflatoxin B_1 by *Aspergillus flavus*. The cytotoxic effect of essential oil could induce an adaptive response, resulting in the reprogramming of gene expression to preserve the microorganism, with the subsequent increase in toxin production. Our results do not support such findings because REO did not cause an increase in ergosterol production. The increase in biomass could account for the increase in fumonisin production. Perhaps some compounds present in the essential oil accelerated the toxin metabolism as a response to stress, even though such a stimulus was not sufficient to cause an increase in ergosterol production (Magan, Hope, Colleate, & Baxter, 2002). The REO was effective in inhibiting the production of FB_1 and FB_2 at concentrations of 300 and 600 μg/mL compared with 1000 μg/mL of the antifungal nystatin.

4. Conclusions

This study showed that *R. officinalis* essential oil (REO) has strong antifungal activity against *F. verticillioides*, as indicated by the fluctuations that REO caused in the size of the fungal structures and the weight of the mycelium and the changes in the morphology of the microconidia and by its inhibitory effects on ergosterol and fumonisin production. The fungitoxic effects of REO on *F. verticillioides* may be attributed to the disruption of the cell wall and the loss of cellular components. Therefore, this essential oil can be used as an alternative fungicide that carries less risk to the environment and to humans.

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