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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