Effect of *Zingiber officinale* essential oil on *Fusarium verticillioides* and fumonisin production

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**Abstract**

The antifungal activity of ginger essential oil (GEO; *Zingiber officinale* Roscoe) was evaluated against *Fusarium verticillioides* (Saccardo) Nirenberg. The minimum inhibitory concentration (MIC) of GEO was determined by micro-broth dilution. The effects of GEO on fumonisin and ergosterol production were evaluated at concentrations of 500–5000 μg/mL in liquid medium with a 5 mm diameter mycelial disc of *F. verticillioides*. Gas chromatography–mass spectrometry showed that the predominant components of GEO were α-zingiberene (23.9%) and citral (21.7%). GEO exhibited inhibitory activity, with a MIC of 2500 μg/mL, and 4000 and 5000 μg/mL reduced ergosterol biosynthesis by 57% and 100%, respectively. The inhibitory effect on fumonisin B₁ (FB₁) and fumonisin B₂ (FB₂) production was significant at GEO concentrations of 4000 and 2000 μg/mL, respectively. Thus, the inhibition of fungal biomass and fumonisin production was dependent on the concentration of GEO. These results suggest that GEO was able to control the growth of *F. verticillioides* and subsequent fumonisin production.

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1. Introduction

Food contaminated with *Fusarium verticillioides* (Saccardo) Nirenberg and its subsequent fumonisin production is a serious worldwide problem (Martins et al., 2012). Since the discovery of fumonisins, 28 molecules have been characterised and divided into four groups: fumonisins A, B, C, and P. Analogues of series B are the most abundant and subdivided into fumonisin B₁ (FB₁), B₂ (FB₂), and B₃ (FB₃). FB₁, which is the most toxic, is found at high levels. It is responsible for 70%–80% of food contamination. FB₂ and FB₃ are responsible for 15%–25% and 3%–8%, respectively (Rheeder, Marasas, & Vismer, 2002). Fumonisins are associated with various toxic effects in animals, such as equine leukoencephalomalacia, porcine pulmonary edema syndrome in pigs, kidney and liver in rats, and testicular morphological changes and reduced fertility in rabbits (Ewuola & Egbonike, 2010). In humans, the consumption of contaminated products appears to be related to an increased risk of developing esophageal cancer and defects in the formation of embryonic neural tubes (IARC, 2002). Fumonisins have been classified by the International Agency for Research on Cancer (IARC, 2002) as Class 2B substances, i.e., possibly carcinogenic to humans. Therefore, the control of fungal growth in agricultural products is necessary to reduce food-borne illness.

In this context, essential oils have been studied as an alternative to control pathogenic microorganisms because they have been shown to have effects on fungal and bacterial growth (Agarwal, Walia, Dhingra, & Khababay, 2001; Ferreira et al., 2013; Singh et al., 2008). The antimicrobial activity of ginger essential oil (GEO), *Zingiber officinale* Roscoe, has been demonstrated against various microorganisms, including *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Candida albicans*, *Aspergillus flavus*, *A. solani*, *A. oryzae*, *A. niger*, and *Rhizoctonia solani* (Agarwal et al., 2001; Singh et al., 2008). Furthermore, Singh et al. (2008) found that GEO had inhibitory effects against the growth of *F. moniliforme* Sheldon, also known as *F. verticillioides*.

Ficker et al. (2003) evaluated the effects of 36 extracts from 29 plants against various fungal species, and the alcoholic extract of *Z. officinale* showed high antifungal activity, including strains resistant to ketoconazole and amphotericin B. According to these authors, the further exploration of the properties of *Z. officinale* as an antifungal agent is justified because this species is considered safe for human consumption. Although GEO has been shown to be...
effective against several microorganisms, no studies of which we are aware have reported the effects of GEO on fumonisin production. Therefore, the present study evaluated the effects of GEO against the growth of *F. verticillioides* and subsequent fumonisin production.

2. Materials and methods

2.1. Extraction of essential oil

The essential oil was obtained from fresh ginger rhizome 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 180 min, maintaining the boiling point at a constant temperature and using 300 g of the ginger rhizome and 1000 mL of distilled water. The yield of the GEO extraction was 650 µL. The oil obtained was stored at 4 °C and protected from light for subsequent use and chemical analysis.

2.2. Analysis of essential oil

The chemical composition of GEO was investigated using gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR). The GC analysis was performed with a Thermo Electron Corporation Focus GC model under the following conditions: DB-5 capillary column (30 m × 0.32 mm × 0.50 mm); column temperature, 60 °C (1 min) 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) that operated at 70 eV. The identification of individual components was based on comparisons of their GC retention indices on non-polar columns and comparisons with the mass spectra of authentic standards purchased from Sigma–Aldrich (Adams, 2001).

For NMR, 1H (300.06 MHz) and 13C NMR (75.45 MHz) spectra were recorded in deuterated chloroform (CDCl3) solution in a Mercury-300BB spectrometer with μ × 6 (ppm) and spectra referred to CDCl3 (δ 7.27 for 1H and 77.00 for 13C) as an internal standard.

2.3. Microorganism

*F. verticillioides* (103 F) was isolated from feed samples involved in animal intoxication by Dr. Elisa Yoko Hirooka, Department of Food Science and Technology, State University of Londrina, morphologically identified at Science University, Tokyo, Japan, maintained in potato dextrose agar (PDA), and stored at 4 °C. This isolate had previously been shown to be a highly fumonisin producer in liquid culture (Falcão et al., 2011).

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 oven (Model 347G, FANEM, Sao Paulo, Brazil) to determine the minimum inhibitory concentration (MIC) and evaluate the effects of GEO on ergosterol and fumonisin production and microconidia morphology.

2.5. Determination of the minimum inhibitory concentration

The MIC of GEO was determined by the broth dilution method in accordance with standard M38-A of the National Committee for Clinical Laboratory Standards (Pfaller, 2002). Ginger essential oil was diluted in a sterile solution of 0.001% Tween-80 (Vetec, Rio de Janeiro, Brazil) and tested at final concentrations of 39–40,000 µg/mL. For each concentration of GEO, 500 µL of a suspension of 4 × 10^5 CFU/mL of the *F. verticillioides* conidial was added in a synthetic RPMI-1640 medium (0.5 mL). The tubes were incubated at 35 °C for 72 h. The MIC was considered the lowest concentration of GEO that inhibited the visual growth of *F. verticillioides*. The positive control was performed in medium that contained only the suspension.

2.6. Effect of GEO on ergosterol biosynthesis and fumonisin production

Ginger essential oil was diluted in a sterile solution of 0.001% Tween-80 and added to 50 mL of liquid medium described by Jimenez, Mateo, Hinojo, and Mateo (2003) to obtain final concentrations of 500, 1000, 2000, 4000, and 5000 µg/mL. From the culture in PDA, a 5 mm diameter mycelial disc of *F. verticillioides* was inoculated into each medium. The control for fungal growth and fumonisin production was performed in medium that contained only inoculum. The flasks were incubated at 28 °C for 14 days under agitation in an MA830 incubator (Marconi, Piracicaba, Brazil). After the incubation period, the medium was filtered through joint filter paper. The mycelium was used for ergosterol determination and filtered for fumonisin determination. Four replications were performed for each of the experimental groups and the control group.

2.6.1. Ergosterol and fumonisin standards

Ergosterol (Sigma Chemical, St. Louis, MO, USA) solutions (20 µg/mL) were prepared in absolute ethanol PA (Merck, Sao Paulo, Brazil) and 100 µg/mL of each toxin (i.e., FB1 and FB2; Acros Organics, Geel, Belgium) in acetonitrile:water (50:50, v/v) and stored in amber vials at −18 °C.

2.6.2. Ergosterol extraction and determination by high-performance liquid chromatography

The ergosterol extraction procedure was performed according to Silva, Corso, and Matheus (2010) and consisted of weighing the mycelium and transferring it to a Falcon tube that contained 20 mL methanol, 5 mL absolute ethanol PA, and 2 g 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 subjected to a water bath (BM Evlab EV:015, Londrina, Brazil) at 70 °C/40 min. After cooling at room temperature, 5 mL of distilled water was added and centrifuged (Hettich Universal 320R, Tuttingen, Baden-Wurttemberg, Germany) at 1735 × g for 12 min. For the final extraction, n-hexane was added (FMAia, Cotia, Brazil) in a volume equal to the supernatant, and the organic fraction was collected in an amber glass vial and evaporated in a 99.9% nitrogen flow (Praxair, Rio de Janeiro, Brazil) at 60 °C in a TECVAP TE-0194 sample concentrator (Tecnal, Piracicaba, Brazil). The residue obtained was stored at −18 °C until it was analyzed.

Ergosterol quantification was performed according to Salm-anowicz, Nylund, and Wallander (1990) with modifications. High-performance liquid chromatography (HPLC) was performed with a Finnigan Surveyor Plus (Thermo Scientific, San Jose, CA, USA) with an ultraviolet/visible spectrum Finnigan Surveyor detection system at a wavelength of 282 nm. The extract was resuspended in 1 mL of absolute ethanol, and 100 µL was injected into the chromatographic system. 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) with a 5 µm particle size column (150 × 4.6 mm). The retention time was 4.6 min. The detection limit was 0.15 µg/mL.
2.6.3. Fumonisin extraction and determination by high-performance liquid chromatography

The FB1 and FB2 extraction process was performed according to the method of Camargos, Machinski, and Valente Soares (1999) with modifications. Cell-free extract (10 mL) was mixed with 10 mL of methanol:water (3:1, v/v; Honeywell Burdick and Jackson, Muskegon, MI, USA) under 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 with a strong anion exchange column (Sep Pak Vac Accel Plus QMA, Waters, Wexford, Ireland). The column was packed with 10 mL methanol, followed by 10 mL methanol:water (3:1, v/v). The filtrate (20 mL) was then applied to the extract on the column, washed with 10 mL methanol:water (3:1, v/v), and 6 mL methanol. Fumonisins were eluted with 20 mL methanol:glacial acetic acid (95:5, v/v; Mallinckrodt Baker, Xalostoc, Mexico). The eluate was evaporated in a 99.9% nitrogen flow by the concentrator samples at 60 °C. The residue obtained was stored at −18 °C until it was analyzed.

For the fumonisins, chromatographic quantification was coupled to fluorescent detection (Finnigan Surveyor) with an excitation wavelength of 330 nm, emission wavelength of 465 nm, coupled to fluorescent detection (Finnigan Surveyor) with an excitation wavelength of 330 nm, emission wavelength of 465 nm, using an isocratic mobile phase composed of methanol-phosphate buffer, pH 3.35 (70:30), flow rate of 1 mL/min, 100 °C reaction temperature of 65 °C, a retention time of 5.4 min for FB1 and 11 min for FB2. The detection limits were 0.12 µg/mL for FB1 and 0.05 µg/mL for FB2. Quantification was performed by measuring the peak area that corresponded to fumonisin or ergosterol in the sample compared with the standard peak area.

2.7. Scanning electron microscopy

From the F. verticillioides culture in PDA, a 5 mm diameter disc was inoculated into a Petri dish that contained culture medium established by Jimenez et al. (2003) and 2% agar. GEO was added to the medium to obtain final concentrations of 3000 and 5000 µg/mL. The plates were incubated at 25 °C for 10 days under a 12/12 h photoperiod. The control was performed in medium that contained only inoculum. Four replications were performed for each experimental and control group. The conidia was washed according to Endo, Cortez, Ueda-Nakamura, Nakamura, and Dias-Filho (2010) in 0.01 M phosphate-buffer saline (PBS), pH 7.2, and fixed with 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 1 h at room temperature. The material was washed in 0.1 M sodium cacodylate buffer and dried in ethanol (50–100%). The samples were subjected to critical-point drying in CO2 (White Martins, Rio de Janeiro, Brazil) and sputter-coated with gold (IC-50, Shimadzu, Kyoto, Japan). The morphological characteristics of the microconidia were determined with a scanning electron microscope (SEM 550 SS, Shimadzu, Kyoto, Japan) that operated at 10.0 kV.

2.8. Statistical analysis

All experiments were repeated four times, and the treatment results were statistically evaluated using analysis of variance (ANOVA) for multiple comparisons followed by the Tukey test. Differences were considered significant at values of p < 0.05. The data were analyzed using the statistical program R (R Development Core Team, 2006).

3. Results and discussion

The results of the GC–MS analysis of GEO are presented in Table 1. The major compounds in the essential oil were α-zingiberene (23.9%) and citral (a mixture of neral and geranial isomers; 21.7%). Several studies have reported the composition of the essential oil from Z. officinale and demonstrated a wide variation in the chemical composition of GEO. Wohlmutl, Smith, Brooks, Myers,

Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Percentual (%)</th>
<th>RIa</th>
<th>Identification</th>
</tr>
</thead>
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<tr>
<td>Camphene</td>
<td>8.43</td>
<td>967</td>
<td>GC/MS, NMR</td>
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<tr>
<td>β-pinene</td>
<td>0.03</td>
<td>994</td>
<td>GC/MS, NMR</td>
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<tr>
<td>Myrcene</td>
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<td>1005</td>
<td>GC/MS, NMR</td>
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<td>α-phellandrene</td>
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<td>1017</td>
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<tr>
<td>β-cymene</td>
<td>0.61</td>
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</tr>
<tr>
<td>β-phellandrene</td>
<td>7.73</td>
<td>1036</td>
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</tr>
<tr>
<td>1,8-cineole</td>
<td>5.62</td>
<td>1039</td>
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</tr>
<tr>
<td>γ-terpinene</td>
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<td>1062</td>
<td>GC/MS, NMR</td>
</tr>
<tr>
<td>Linalool</td>
<td>0.79</td>
<td>1100</td>
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<tr>
<td>Borneol</td>
<td>0.50</td>
<td>1165</td>
<td>GC/MS, NMR</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>1.10</td>
<td>1190</td>
<td>GC/MS, NMR</td>
</tr>
<tr>
<td>Citronellol</td>
<td>0.92</td>
<td>1228</td>
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</tr>
<tr>
<td>Neral</td>
<td>7.47</td>
<td>1240</td>
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<td>0.80</td>
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<td>Geraniol</td>
<td>14.16</td>
<td>1270</td>
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<tr>
<td>α-copaene</td>
<td>0.29</td>
<td>1374</td>
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</tr>
<tr>
<td>Geranylacetate</td>
<td>0.32</td>
<td>1383</td>
<td>GC/MS, NMR</td>
</tr>
<tr>
<td>Germacrene D</td>
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<td>1479</td>
<td>GC/MS, NMR</td>
</tr>
<tr>
<td>α-curcumene</td>
<td>6.09</td>
<td>1481</td>
<td>GC/MS, NMR</td>
</tr>
<tr>
<td>α-zingiberene</td>
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<td>1494</td>
<td>GC/MS, NMR</td>
</tr>
<tr>
<td>(E,E)-α-farnesene</td>
<td>9.98</td>
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<td>GC/MS, NMR</td>
</tr>
<tr>
<td>β-sesquiphellandrene</td>
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<tr>
<td>Z-nerolidol</td>
<td>0.50</td>
<td>1555</td>
<td>GC/MS</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>99.25</td>
<td></td>
</tr>
</tbody>
</table>

GC/MS – gas chromatography/mass spectrometry.
NMR – nuclear magnetic resonance.
a Retention indices obtained with reference to n-alkane series C14H28–C20H42 on DB-5 column.
by at 2000

It is responsible for regulating the flow and activity of many membrane-bound enzymes (Bendaha et al., 2011). Some antifungal agents inhibit cell growth by interrupting ergosterol biosynthesis that results from the binding of antifungals to ergosterol on the cellular membrane. Thus, this affects the integrity and function of some membrane-bound proteins and leads to osmotic disturbances, fungal cell growth, and proliferation (Bendaha et al., 2011). Therefore, the determination of ergosterol produced by *F. verticillioides* exposed to different concentrations of GEO was used to quantify the fungal biomass. As shown in Fig. 1, GEO at lower concentrations (500–3000 µg/mL) caused oscillations in ergosterol production and effectively inhibited ergosterol production in *F. verticillioides* at 4000 and 5000 µg/mL, with inhibition ranging from 57% to 100%. This oscillation was reflected by an increase in the ergosterol biosynthesis of *F. verticillioides* at 1000 µg/mL GEO compared with the control group and a reduction and complete inhibition at higher concentrations. Dambolena et al. (2008) and Lucini, Zunino, López, and Zygadlo (2006) also observed oscillations in ergosterol biosynthesis. Lucini et al. (2006) studied monoterpenes and found that the presence of these compounds at low concentrations leads to lipid peroxidation in fungi, which induces an adaptive response, results in the reprogramming of genomic expression to protect the cellular wall structure, and consequently increases ergosterol biosynthesis. Dambolena et al. (2008) found that the components limonene, menthol, menthone, and thymol (75 ppm) were able to increase ergosterol production by *F. verticillioides*.

Ginger essential oil significantly inhibited production of FB1 by *F. verticillioides* (*p < 0.05*) at a concentration of 4000 µg/mL and completely inhibited at 5000 µg/mL (Fig. 2(A)); Ginger essential oil inhibited production of FB2 at 2000 µg/mL and completely inhibited production at 3000 µg/mL (Fig. 2(B)). Thus, we found a
correlation between GEO-induced ergosterol inhibition (Fig. 1) and the reduction of fumonisin production (Fig. 2). Therefore, the decrease in fumonisin production was proportional to the decrease in fungal biomass. A similar result was found by Dambolena et al. (2010). Others studies demonstrated that essential oils and compounds, limonene and thymol, inhibit fumonisin production by F. verticillioides (Dambolena et al., 2008; Fandohan et al., 2004).

Our results showed a difference between the MIC and the inhibition of FB1 and ergosterol biosynthesis. Dambolena et al. (2008) to study the effect of terpenoids in F verticillioides obtained MIC of 75 µg/L for limonene. This same concentration applied to corn seeds was not showed effect on the biosynthesis of ergosterol and was not able to fully reduce FB1. A total inhibition of fungal growth was visualised only in the concentration of 1000 µg/L. Shukla, Singh, Prakash, and Dubey (2012) also observed inhibition of mycelial biomass by Aspergillus flavus in dosages of essential oil excess to MIC and concluded that the mycotoxin inhibiting cannot be attributed entirely to a reduction in fungal growth, but the ability of fungi to produce mycotoxin.

With regard to the morphological structure of F. verticillioides, determined by scanning electron microscopy, microconidia exposed to different concentrations of GEO showed changes in morphology compared with controls (Fig. 3). In the control group, the microconidia were turgid with a smooth cell wall (Fig. 3(A)), but this aspect changed in proportion to the applied concentration of GEO. Fig. 3(B) shows an apparent reduction of cytoplasmic contents at 3000 µg/mL GEO. At 5000 µg/mL GEO, the microconidia exhibited a flat aspect, showing an absence of cytoplasmic content and a reduction of membrane integrity (Fig. 3(C)). Similarly, Tyagi and Malik (2010) found that yeast cells shrank and were apparently devoid of cytoplasmic contents in the presence of Cymbopogon citratus essential oil. These alterations in the cellular wall may be related to the lipophilic properties of the oil’s components, making it permeable to the cell wall and cytoplasmic membrane, thus increasing the possibility of their interaction with the cell wall, affecting enzymatic reactions that regulate cell wall synthesis, and disrupting cell integrity (Maffei, Camusso, & Sacco, 2001; Rasooli, Rezaei, & Allameh, 2006). This mechanism impairs membrane fluidity, leading to leakage of the cytoplasmic contents and changing the structure of several layers of polysaccharides, fatty acids, and phospholipids (Rasooli et al., 2006; Tyagi and Malik, 2010).

4. Conclusion

The results of the present study demonstrate the antifungal effects of GEO on F. verticillioides, reflected by changes in the morphology of microconidia and inhibitory effects on fumonisin and ergosterol production. These data suggest that GEO may be used to control the presence of F. verticillioides and its toxic metabolites. Future studies with other microorganisms should be conducted to evaluate the effectiveness of GEO in food preservation. Moreover, future research in the field should be conducted with different cultivars and storage conditions to generate more effective fungicides or preservatives with less risk to the environment and health.

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