

In vitro activity of essential oil of *Ocimum selloi* and its major chemical compound against *Moniliophthora pernicios*, causal agent of witches' broom disease in cacao

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Abstract

The efficacy of *Ocimum selloi* essential oil was evaluated for controlling the growth of mycelia and spore germination of phytopathogens. Six compounds (99.89%) of the total oil were identified by GC-MS, methyl chavicol, methyl eugenol, β -caryophyllene, germacrene-D, bicyclogermacrene and spathulenol. Essential oil was tested for anti-fungal activity against *Moniliophthora pernicios*, *Colletotrichum gloeosporioides* and *Alternaria alternata*, which was determined by disc diffusion and minimum inhibitory concentration (MIC) determination methods. Application of the oil reduced mycelial growth in all three fungi in a dose dependent manner, with maximum inhibition being observed at a concentration of 1000 ppm. Such antifungal activity could be attributed to methyl chavicol since the pure compound was shown to be similarly effective against *M. pernicios* at 1000 ppm. The oil, when applied at a concentration of 1000 ppm, reduced the spore germination of *C. gloeosporioides* and *M. pernicios* by 93 and 87%, respectively, but had no effect on the *A. alternata*. It is concluded that the oil from *O. selloi* and its major constituent, methyl chavicol, are efficient in inhibiting *M. pernicios*, but less effective against *C. gloeosporioides* and *A. alternata*. The results obtained from this work may contribute to the development of alternative anti-fungal agents to protect the cacao crop from fungal disease.

Keywords: medicinal plant, methyl chavicol, phytopathogens, *Theobroma cacao*, antifungal activity

INTRODUCTION

The essential oils of numerous aromatic and medicinal plants are known to possess antimicrobial activity (Carović-Stanko et al., 2010). Studies have shown that crude plant extracts and essential oils are potentially useful for controlling various types of phytopathogens (Rahman et al., 2010; Silva et al., 2012; Carvalho et al., 2013).

Moniliophthora pernicios, the causal agent of witches' broom disease in *Theobroma cacao* L., mainly infects meristematic vegetative shoots, inflorescences and fruits resulting in hypertrophied damaged tissues that are unsuitable for cultivation or consumption. The application of bioactive natural products, in the form of a crude plant extract or an essential oil, could be more advantageous than the use of synthetic chemicals since they are likely to be cheaper to produce, less harmful to the environment and less toxic to humans (Rahman et al., 2010).

Our research group undertook the task of investigating the in vitro effects of the essential oil from *O. selloi* on the growth of mycelia and the germination of spores of *Alternaria alternata*, *Colletotrichum gloeosporioides* and *M. pernicios*.



MATERIALS AND METHODS

Plant material and extraction of the essential oil

The voucher specimens (number 7474) were deposited in the herbarium of the Department of Biology at Universidad of Lavras (UFLA). Fresh leaves and inflorescences (100 g) of *O. selloi* were subjected to hydrodistillation for 90 min using a Clevenger type apparatus. The essential oil was dried over anhydrous magnesium sulphate and preserved in a sealed vial at 4°C until further analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

The essential oil (EO) was analyzed using a SHIMADZU GC-MS (GC-17A and MS-QP5050A). The oil constituents were expressed as percentage by peak area normalization.

Identification procedure

The linear retention indices (RI) for all of the compounds were determined by co-injection of an oil sample with a solution containing the homologous series of C₈-C₃₂ n-alkanes (Sigma, St Louis, MO, USA) and application of the equation of Vandendool and Kratz (1963). Individual identification of constituents was based by: (a) comparing RI values and key mass spectral data with the literature; (b) with NIST/EPA/NHI library (National Institute of Standards and Technology 1998); (c) co-injection of an oil sample with authentic standards (Adams, 2007).

Fungal pathogens

The fungal species used in the experiment were *Alternaria alternata* (CML184), *Colletotrichum gloeosporioides* (CML459) and *Moniliophthora perniciosa* (CEPLAC1188).

Preparation of spore suspension and standard methyl chavicol

The spore suspensions were of 7-day-old cultures and mixed with sterile distilled water (22×10⁴ spore mL⁻¹). EO and standard methyl chavicol (MC) (≥98.9% purity by GC; Riedel-de Haën, Seelze, Germany) were dissolved in 1% propylene glycol (PG) separately to prepare the stock solutions.

Mycelial growth assay

The EO and MC were tested at 125, 250, 500 or 1000 ppm. Two different sets of control (0 ppm of EO and PG and 0 ppm of EO) were used. The plates were incubated in a chamber at 25±1°C under a 12-h photoperiod. The growth inhibition was calculated by mycelial growth index [MGI=(D₁/N₁)+(D₂/N₂)+...+(D_n/N_n)], where D₁, D₂ and D_n represent the diameters of the mycelia at the first, second and last measurements, and N₁, N₂ and N_n represent the number of days after inoculation. The percentage inhibition of mycelial growth [IMG=[(D_c-D_t)×100]/D_c, in which D_c and D_t represent the diameters of the mycelia in the control and in the treatment, respectively. The minimum concentration at which no visible growth was observed was defined as the MIC, which was expressed in ppm and the concentration to kill half of the population was determined to be the lethal dose (LD₅₀).

Spore germination assay

Glass depression slides (containing three wells each) were placed inside petri dishes that had been lined with wet filter paper. Each well received an aliquot (100 µL) of the essential oil solubilized in 1% PG to give a final concentration of 125, 250, 500 or 1000 ppm. Control without EO was tested in the same way. Subsequently, a 30-µL aliquot of a suspension of spores of *A. alternata* (22×10⁴ spores mL⁻¹), *C. gloeosporioides* (20×10⁴ spores mL⁻¹) or *M. perniciosa* (24×10⁴ spores mL⁻¹) was added to each well and the glass slides were incubated at 25±1°C for 24 h. At the end of the incubation period, each well received a few drops of lacto-phenol-cotton blue stain and the slides were observed under the light microscope. All spores exhibiting germ tubes, independent of their length, were considered to be germinating spores. Each treatment involved three repetitions, each one comprising

three wells, and 200 spores were counted per well to give a total of 600 spores counted per treatment. The results were expressed as percentage of spore germination.

Statistical analysis

All experiments were conducted according to a completely randomized design. Mean values were compared using Tukey's test at $P \leq 0.05$ and regression analyses.

RESULTS AND DISCUSSION

GC/MS analysis of the essential oil from the leaves and inflorescences of *O. selloi* led to the identification of six main components, namely, two phenylpropanoids and four sesquiterpenes, representing 93.86 and 6.03% of the total oil, respectively. The identified compounds are listed in Table 1 according to their elution order on a DB-5 MS capillary column. The major constituent was methyl chavicol, and this component accounted for more than 93% of the total oil. The other compounds were methyl eugenol (0.62%), β -caryophyllene (2.22%), germacrene-D (1.33%), bicyclogermacrene (1.21%) and spathulenol (1.27%).

Table 1. The principal chemical constituents of the essential oil from leaves and inflorescences of *Ocimum selloi*.

Compound	RI ¹	Composition (%)
Methyl chavicol	1198	93.24
Methyl eugenol	1397	0.62
β -Caryophyllene	1418	2.22
Germacrene-D	1480	1.33
Bicyclogermacrene	1494	1.21
Spathulenol	1575	1.27
Phenylpropanoids		93.86
Sesquiterpenes		6.03
Total		99.89

¹Retention indices relative to n-alkanes on DB-5 MS capillary column.

The essential oil from *O. selloi* was able to inhibit mycelia growth in all three of the species of fungi assayed. The toxic effect was observed in a dosage response manner, i.e., the higher the concentration of essential oil the greater was the reduction in the growth of mycelia (Figures 1 and 2). The essential oil inhibited the mycelial growth of *M. pernicioso* by 100% at oil concentrations of 500 and 1000 ppm (Figure 2c), and low MIC and LD₅₀ values were obtained (Table 2). As shown in Figure 3, pure methyl chavicol or estragole also reduced the growth of mycelia of *M. pernicioso*, and completely inhibited growth at 1000 ppm.

Table 2. Percentage inhibition of mycelial growth (IMG) of different concentrations of the essential oil from *Ocimum selloi* tested against three fungal isolates, minimum inhibitory concentration (MIC) and lethal dose (LD₅₀)

Fungal strains	IMG (%)				MIC (ppm)	LD ₅₀ (ppm)
	Essential oil concentration (ppm)					
	125	250	500	1000		
<i>Alternaria alternata</i>	0.6	1.7	19.2	45.7	>1000	585.17
<i>Colletotrichum gloeosporioides</i>	0.0	4.0	3.9	13.2	>1000	752.23
<i>Moniliophthora pernicioso</i>	5.4	3.2	100.0	100.0	250-500	415.25

The essential oil affected the germination of spores of *C. gloeosporioides* and *M. pernicioso* at all concentrations tested, although the largest reductions in germination (93

and 87%, respectively) were observed at 1000 ppm (Figure 4). However, *A. alternata* spores were not affected by any of the treatments applied.

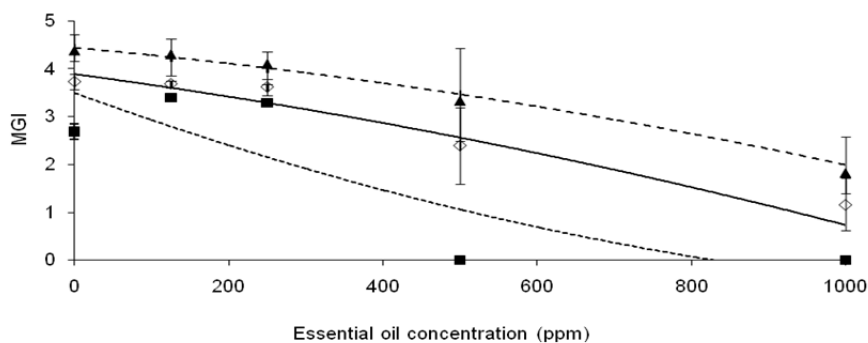


Figure 1. Effects of different concentrations of essential oil from *Ocimum selloi* on the mycelial growth index (MGI) of \diamond *Alternaria alternata* ($y = -0.000001x^2 - 0.002150x + 3.885923$; $R^2 = 96.18$), \blacktriangle *Colletotrichum gloeosporioides* ($y = -0.000001x^2 - 0.001445x + 4.437385$; $R^2 = 99.48$) and \blacksquare *Moniliophthora perniciosa* ($y = -0.000002x^2 - 0.005871x + 3.496410$; $R^2 = 70.43$).

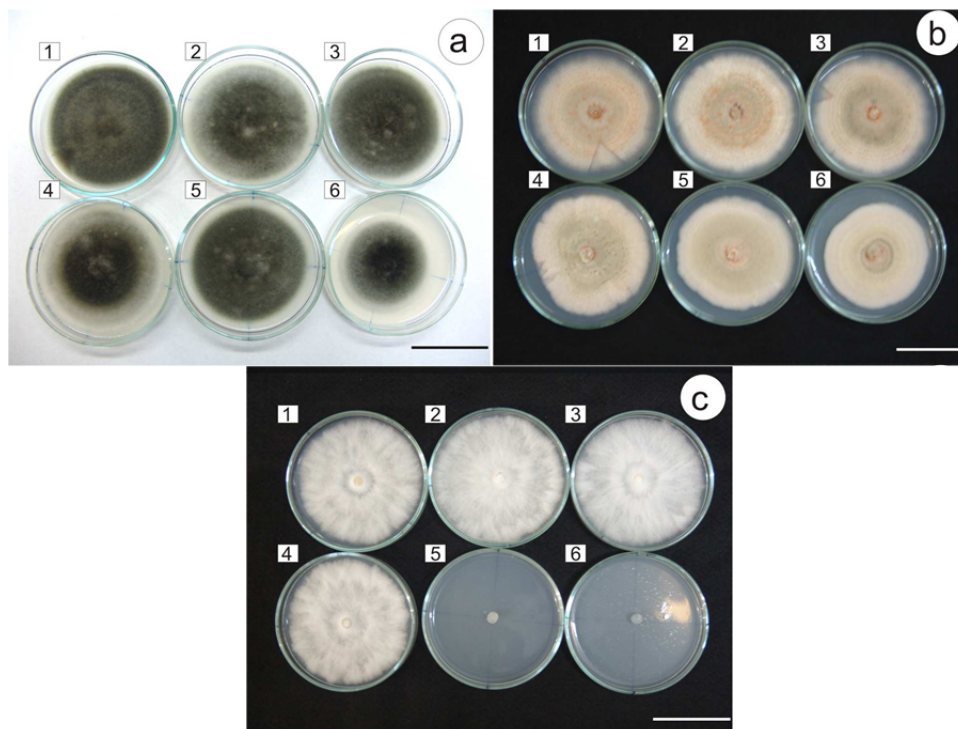


Figure 2. Effects of different concentrations of essential oil from *Ocimum selloi* on the growth of mycelia of *Alternaria alternata* (a), *Colletotrichum gloeosporioides* (b) and *Moniliophthora perniciosa* (c). Each panel shows (1) 0 ppm of essential oil (control), (2) PG + 0 ppm of essential oil (control), (3) 125 ppm, (4) 250 ppm, (5) 500 ppm, and (6) 1000 ppm. Bar = 5 cm.

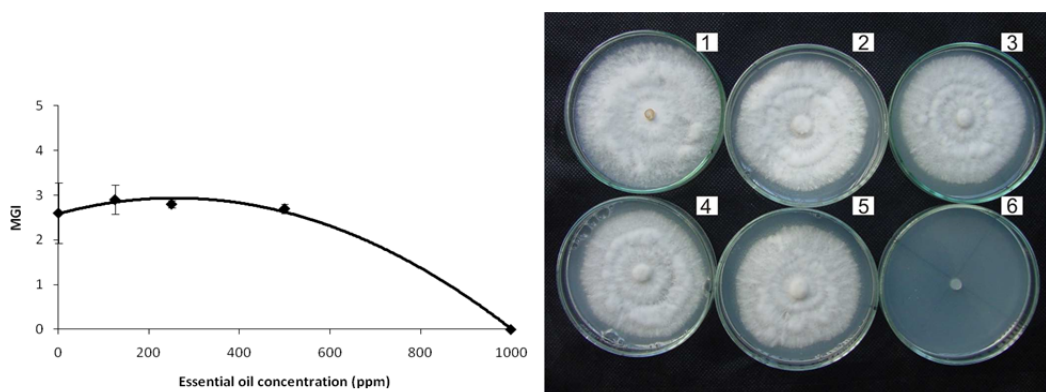


Figure 3. Effects of different concentrations of methyl chavicol (standard) on the mycelial growth index (MGI) ($y = -5E - 06X^2 + 0.0027x + 2.5862$; $R^2 = 99.54^{**}$) and mycelia growth of *Moniliophthora perniciosa*. Each panel shows (1) 0 ppm of methyl chavicol (control), (2) PG + 0 ppm of methyl chavicol (control), (3) 125 ppm, (4) 250 ppm, (5) 500 ppm, and (6) 1000 ppm of methyl chavicol.

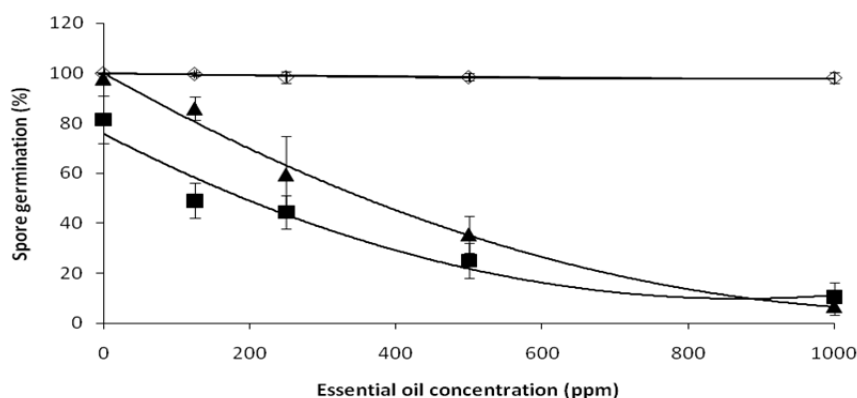


Figure 4. Effects of different concentrations of essential oil from *Ocimum selloi* on the spore germination from \diamond *Alternaria alternata* ($y = 0.000003x^2 - 0.005192x + 100.034615$; $R^2 = 0.87$), \blacktriangle *Coletotrichum gloesporioides* ($y = 0.000073x^2 - 0.166687x + 100.226923$; $R^2 = 0.99$) and \blacksquare *Moniliophthora perniciosa* ($y = 0.000087x^2 - 0.151606x + 75.846154$; $R^2 = 0.99$)

The results demonstrate that the essential oil of *O. selloi* was able to inhibit the growth of mycelia of the three fungal isolates studied, with the order of sensitivity to the oil being *M. perniciosa* > *C. gloesporioides* > *A. alternata*. Furthermore, the essential oil caused a reduction in the germination of spores of *M. perniciosa* at all concentrations tested, thus indicating a possible application of this natural product in the treatment of infected cacao crops.

Since the major constituent (>93%) of the essential oil was methyl chavicol, the fungicidal activity of this phenylpropanoid against *M. perniciosa* was also evaluated. The results revealed that the essential oil and pure methyl chavicol were similarly effective in causing complete inhibition of mycelia growth at a concentration of 1000 ppm. Previous studies demonstrated that the essential oil from another species of *Ocimum*, namely *O. basilicum*, as well as the pure phenylpropanoids methyl chavicol and eugenol, were able to reduce significantly the mycelial growth of the phytopathogenic fungus *Botrytis fabae* (Oxenham et al., 2005). Additionally, a number of other phenylpropanoids have been reported to exhibit antifungal activity, including apiol (Meepagala et al., 2005; Razzaghi-Abyaneh et al., 2007) and the related nothoapiol (Laouer et al., 2009). In this study, the

essential oil showed a remarkable anti-fungal activity against *M. pernicioso*, which could be attributed to the presence of phenylpropanoids, mainly methyl chavicol. This was further supported by our findings, when using pure methyl chavicol at 1000 ppm. Although the essential oil at 500 ppm had an inhibitory effect against *M. pernicioso*, while the methyl chavicol standard at 500 ppm had no effect. It is possible that the minor components might be involved in some type of synergism with the other active compounds. Volatile compounds, such as β -caryophyllene, spathulenol and methyl eugenol contain the strong antifungal properties (Rahman et al., 2010).

With regard to *M. pernicioso*, inhibition of mycelia growth and spore germination by the essential oils and leaf extracts from a number of species of *Piper* has been described (Silva and Bastos, 2007). Thus, the essential oil from *P. aduncum* completely inhibited mycelia growth and spore germination in the phytopathogenic fungus when applied at 50 and 100 ppm, respectively, whilst oils from *P. dilatatum*, *P. callosum* and *P. marginatum* var. *anisatum* inhibited *M. pernicioso* spore germination at concentrations of 400 and 500 ppm, respectively (Silva and Bastos, 2007). Additionally, the essential oils of *P. callosum*, *P. marginatum* var. *anisatum* and *P. enckea* exhibited antifungal activities against *M. pernicioso*, *Phytophthora palmivora* and *P. capsici*, all of which attack cacao plants (Silva and Bastos, 2007). The present study, however, reports for the first time the antifungal activity of an individual component of an essential oil, namely, pure methyl chavicol against *M. pernicioso*. Kim et al. (2008) reported there was a significant difference in antifungal activities among phenylpropanoids, and that the position of the double bond of the propenyl group is important for antifungal activity.

CONCLUSIONS

The essential oil from *O. selloi*, when applied at a concentration of 1000 ppm, is able to inhibit mycelia growth (100%) and spore germination (87%) of *M. pernicioso*. The antifungal activity is attributed to the major constituent methyl chavicol, which showed activity at 1000 ppm similar to that of the essential oil. Although the present findings suggest a possible application of this natural product in the treatment of infected cacao crops, further *in vivo* studies are required in order to confirm the efficacy of this natural fungicide and to determine its safety.

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