

***In vitro* antifungal activity of *Ocimum selloi* essential oil and methylchavicol against phytopathogenic fungi¹**

Atividade antifúngica *in vitro* do óleo essencial de *Ocimum selloi* e metilchavicol contra fungos fitopatogênicos

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ABSTRACT - The efficacy of *Ocimum selloi* essential oil was evaluated for controlling the growth of mycelia and spores germination *Moniliophthora perniciosa*. Six compounds (99.89%) of the total oil were identified by GC-MS, of which methyl chavicol, methyl eugenol, β -caryophyllene, germacrene-D, bicyclogermacrene and spathulenol. Essential oil was tested for anti-fungal activity, which was determined by disc diffusion and minimum inhibitory concentration (MIC) determination methods. Application of the oil reduced mycelial growth in a dose dependent manner, with maximum inhibition being observed at concentration of 1,000 ppm. Such antifungal activity could be attributed to methyl chavicol since the pure compound was shown to be similarly effective against *Moniliophthora perniciosa* at 1,000 ppm. The oil when applied at a concentration of 1,000 ppm, reduced the spore germination of *Colletotrichum gloeosporioides* and *M. perniciosa* by 93 and 87%, respectively, but had no effect on the *Alternaria alternata*. It is concluded that the oil from *O. selloi* and its major constituent, methyl chavicol, are efficient in inhibiting *M. perniciosa*, 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.

Key words: Antifungal Activity. Methyl Chavicol. *Moniliophthora perniciosa*. *Theobroma cacao*.

RESUMO - A eficácia do óleo essencial de *Ocimum selloi* na inibição do crescimento micelial e na germinação de esporos foi avaliada *in vitro* para os fungos *Moniliophthora perniciosa*, *Colletotrichum gloeosporioides* e *Alternaria alternata*. Seis constituintes (99,89%) do total do óleo foram identificados por CG-EM metil chavicol, metil eugenol, β -cariofileno, germacreno-D, biclogermacreno e espatulenol. O óleo essencial foi testado para atividade antifúngica, o qual foi determinado pelos métodos de difusão e concentração mínima inibitória (MIC). A aplicação do óleo reduziu o crescimento micelial dependente da dose, com máxima inibição na concentração de 1.000 ppm. A atividade antifúngica pode ser atribuída ao composto puro metil chavicol. Este mostrou ser efetivo contra *M. perniciosa* em 1.000 ppm. O óleo quando aplicado na concentração de 1.000 ppm, reduziu a germinação do esporo de *Colletotrichum gloeosporioides* e *M. perniciosa* em 93 e 87%, respectivamente, mas não teve efeito em *Alternaria alternata*. Conclui-se que o óleo essencial e seu constituinte majoritário, metil chavicol, são eficientes na inibição de *M. perniciosa*, mas menos efetivo contra *C. gloeosporioides* e *A. alternata*. Os resultados obtidos nesta pesquisa pode contribuir para o desenvolvimento de um agente alternativo para proteger a cultura do cacau contra esta doença fúngica.

Palavras-chave: Atividade Antifúngica. Metil chavicol. *Moniliophthora perniciosa*, *Theobroma cacao*.

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INTRODUCTION

The essential oils of numerous aromatic and medicinal plants are known to possess antimicrobial activity (CAROVIĆ-STANKO *et al.*, 2010). Indeed, a wide range of plant secondary metabolites exhibit antimicrobial properties, and these are often associated with the natural defense mechanism against attack by phytopathogenic bacteria and fungi (JANSSEN; SCHEFFER; BAERHEIM-SVENDSEN, 1987). In this context, a number of studies have shown that crude plant extracts and essential oils are potentially useful for controlling various types of phytopathogens (CARVALHO *et al.*, 2013; RAHMAN; HOSSAIN; KANG, 2010; SILVA *et al.*, 2012a; SILVA *et al.*, 2012b; SILVA; BASTOS 2007; VIGO-SCHULTZ *et al.*, 2006).

Alternaria alternata (Fr.) Keissl., *Colletotrichum* spp. and *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora [syn. *Crinipellis perniciosa* (Stahel) Singer] are representative of phytopathogens that cause significant economic losses both in Brazil and in many other areas of the world. The mould *A. alternata* is an opportunist pathogen that is responsible for leaf spot and blight disease in more than 380 plant species, while fungi of the genus *Colletotrichum* cause economically significant diseases (e.g. anthracnose) in cereals, grasses, fruit and ornamental plants, mainly in tropical and subtropical regions (SOUZA JÚNIOR; SALES; MARTINS, 2009). Of particular interest, however, is the hemibiotrophic fungus *M. perniciosa*, the causal agent of witches' broom disease in *Theobroma cacao* L. (GRIFFITH 2004; PURDY; SCHMIDT, 1996), since this pathogen is responsible for up to 70% of losses in commercial cocoa plantations in northern Brazil, and especially in the State of Rondônia.

M. perniciosa mainly infects meristematic vegetative shoots, inflorescences and fruits resulting in hypertrophied damaged tissues that are unsuitable for cultivation or consumption. Although an integrated disease control management program, including agricultural, chemical, biologic and molecular strategies, is recommended for *M. perniciosa*, a great deal of research in Brazil has focused on cheap and environmentally friendly methods that could be suitable for local farmers. Within this context, 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; HOSSAIN; KANG, 2010; SILVA; BASTOS 2007; STANGARLIN *et al.*, 1999).

Many of the 100 or so species of the genus *Ocimum* (Lamiaceae) represent valuable sources of essential oils that have been used not only in folk medicine, but also as insect repellents, food flavors and aromatizing agents

in fragrances and perfumes. Paula, Gomes-Carneiro, Paumgarten (2003), for example, reported that the oil from *Ocimum selloi* Benth. was effective against mosquitoes, showed low acute toxicity, posed no mutagenic risk and did not cause irritation to human skin. The main compounds present in the essential oil of *O. selloi* are methyl chavicol (estragole; 1-allyl-4-methoxybenzene), *trans*-anethole, *cis*-anethole and caryophyllene (FRANCA *et al.*, 2008; MORAES *et al.*, 2002; VIEIRA; SIMON 2000).

Considering the small number and high cost of agrochemicals available for the eradication of phytopathogens, the emerging signs of resistance towards traditional fungicides, and the need to reduce the environmental impact of synthetic agrochemicals, 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 *A. alternata*, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and *M. perniciosa*.

MATERIALS AND METHODS

Plant material

The leaves and flowers of *O. selloi* were collected from the Lavras county of the Republic of Brazil. The plant was identified on the basis of morphological features and a voucher specimens (number 7474) were deposited in the herbarium of the Department of Biology at Universidade Federal de Lavras (UFLA).

Extraction of the essential oil

The fresh leaves and inflorescences portions (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 for further analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis of the essential oil was performed using a SHIMADZU GC-MS (GC-17A and MS-QP5050A with quadrupole detector) equipped with DB-5 MS fused silica capillary column (300 × 0.25 mm i.d.; 0.25 µm film thickness). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. The carrier gas helium was used at a constant flow rate of 1.2 ml min⁻¹. Injector and MS transfer line temperature were set at 250 °C and 280 °C, respectively. The oven temperature was programmed isothermal at 50 °C for 2 min, increased at 4 °C min⁻¹ to 200 °C, then raised to 300 °C at 10 °C min⁻¹ and finally held isothermal for 10 min. Diluted samples (20%, v/v, in dichloromethane) of 0.5 µL was injected in the split mode ratio 1:100.

The relative percentage of the oil constituents was 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 Van den Dool and Kratz (1963). Individual identification of constituents were based by: (a) comparing RI values and key mass spectral features with those published in the literature; (b) comparing individual mass spectra with spectral data from the 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 plant pathogenic fungi were obtained from the collection of the Department of Phytopathology at UFPA. Cultures of each fungal species were maintained on potato-dextrose agar (PDA) slants and stored at 4 °C. The fungal species used in the experiment were *A. alternata* (CML 184), *C. gloeosporioides* (CML 459) and *M. perniciosa* (CEPLAC 1188; CML).

Preparation of spore suspension and standard methyl chavicol

The spore suspension of *A. alternata*, *C. gloeosporioides* and *M. perniciosa* were obtained from their respective 7 days old cultures, mixed with sterile distilled water to obtain a homogenous spore suspension of 22 x 10⁴ spore mL⁻¹. Essential oil and standard methyl chavicol (≥ 98.9% purity by GC; Riedel-de Haën, Seelze, Germany) were dissolved in 1% propylene glycol (PG) separately to prepare the stock solutions, which were further diluted to prepare test samples.

Bioassays

Mycelial growth assay

In order to evaluate the effects on mycelial growth, essential oil from *O. selloi* and standard methyl chavicol were solubilized separately in 1% propylene glycol (PG) so as to ease its incorporation into the agar medium. The essential oil and standard methyl chavicol were tested at 125; 250; 500; or 1,000 ppm. Two different sets of control (0 ppm of essential oil and PG + 0 ppm of essential oil) were used. The oil and standard were incorporated to the autoclaved and cooled (40 °C) potato dextrose agar medium (PDA). The medium amended with oil or standard was then poured into sterilized Petri dishes (9 cm diameter). A mycelial disc of 7 mm diameter of the test pathogens taken from a 7 days old culture, using a sterilized cork borer was placed at the centre of the Petri dish containing

the medium. The plates were then sealed with parafilm™ and incubated at chamber room at 25 ± 1 °C under a 12 h photoperiod. The diameter of the growing mycelia was performed every two days, time by which the growth of control would have reached 2/3 of the Petri dish. The plates were used in triplicates for each treatment.

The growth inhibition of oil and standard trails compared to control was calculated by mycelial growth index (MGI), using the following formula: $MGI = (D_1 / N_1) + (D_2 / N_2) + \dots + (D_n / N_n)$, 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.

Additionally, the percentage inhibition of mycelial growth (IMG) was estimated using the equation: $IMG = [(D_c - D_t) \times 100] / D_c$, in which D_c and D_t represent the diameters of the mycelia in the control and in the treatment, respectively. Bioassays were repeated six times for each of the oil concentrations.

The minimum concentration at which no visible growth was observed was defined as the MIC, which was expressed in ppm and the lethal dose (LD₅₀) defined as the concentration that is able to inhibit 50% of mycelial growth, which was expressed in ppm were determined.

Spore germination assay

The second bioassay was designed to evaluate the effect of different concentrations of essential oil on the spore germination of the fungal isolates. 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% propylene glycol to give a final concentration of 125; 250; 500; or 1,000 ppm. Control without essential oil was tested in the same way. Subsequently, a 30 µL aliquot of a suspension of spores of *A. alternata* (22 x 10⁴ spores.mL⁻¹), *C. gloeosporioides* (20 x 10⁴ spores.mL⁻¹) or *M. perniciosa* (24 x10⁴ 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 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 $\rho \leq 0.05$ 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 others compounds were methyl eugenol (0.62%), β -caryophyllene (2.22%), germacrene-D (1.33%), bicyclogermacrene (1.21%) and spathulenol (1.27%).

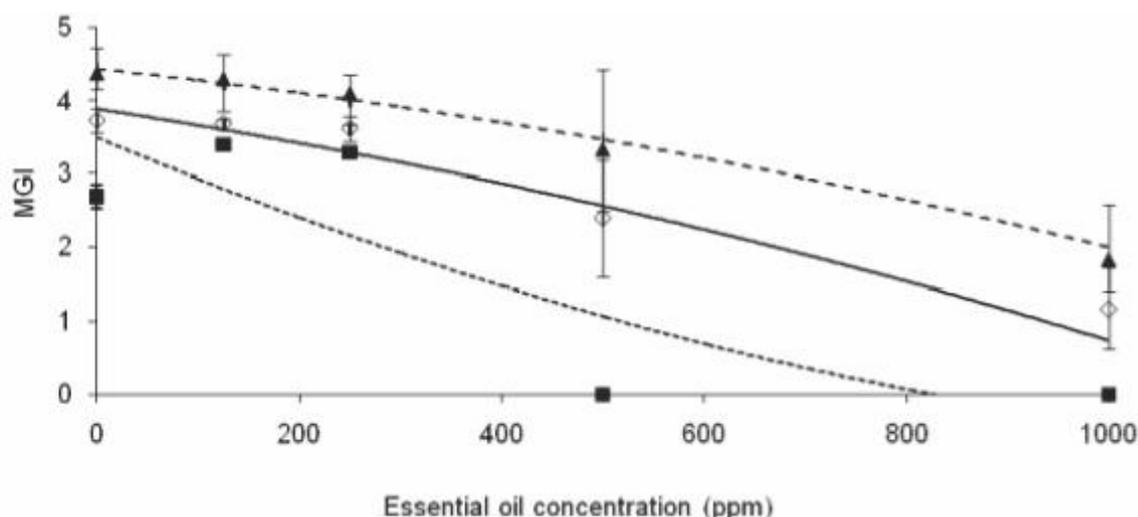
The essential oil from *O. selloi* was able to inhibit mycelial growth in all three fungal species assayed. The toxic effect was in a dosage response manner, i.e. the higher the concentration of essential oil the greater was the reduction in the growth of mycelia (Fig. 1 and Fig.2). For *A. alternata* and *C. gloeosporioides*, maximum IMG values of 45.7 and 13.2%, respectively, were attained when the concentration of essential oil was 1,000 ppm (Table 2). Of more interest, however, was the effect of the essential oil on the mycelial growth of *M. perniciosa*, which was inhibited by 100% at oil concentrations of 500 and 1,000 ppm (Fig. 2c), and the relative low MIC and LD₅₀ values obtained (Table 2). As shown in Fig. 3, pure methyl

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

Compound	RI ^a	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

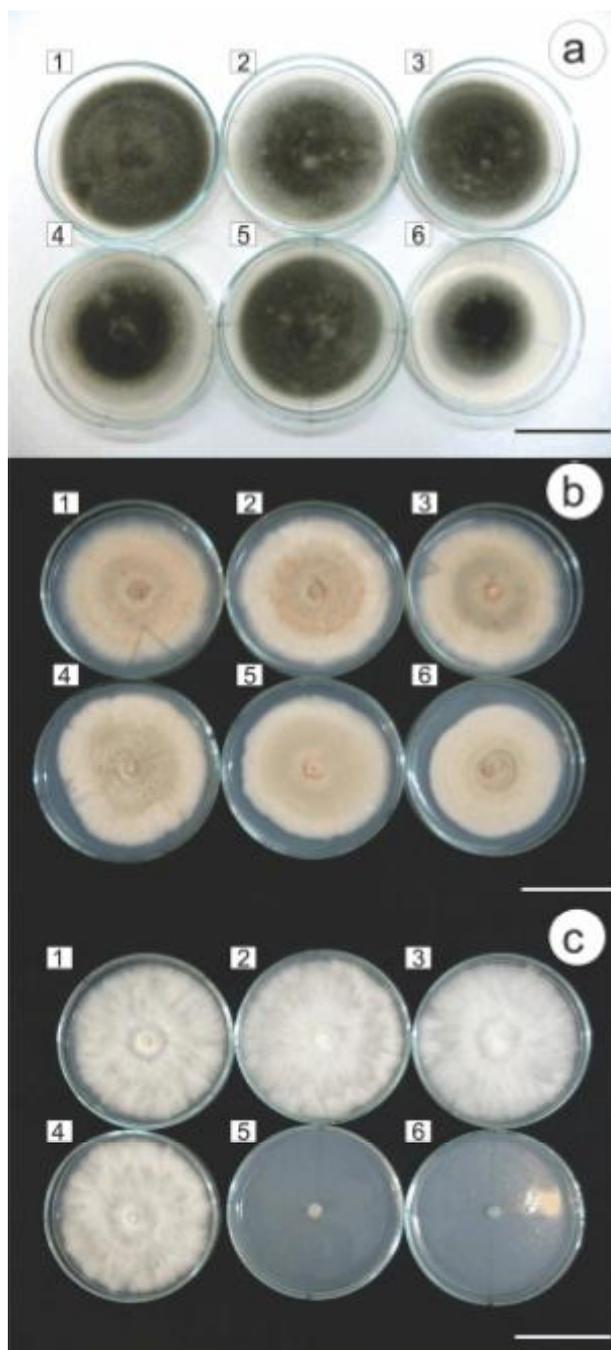
^aRetention indices relative to n-alkanes on DB-5 MS capillary column

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* ($y = -0.000002x^2 - 0.005871x + 3.496410$; $R^2 = 70.43$)



chavicol or estragole also reduced the growth of mycelia of *M. pernicioso*, and complete inhibition of growth was observed at 1,000 ppm.

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 pernicioso* (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

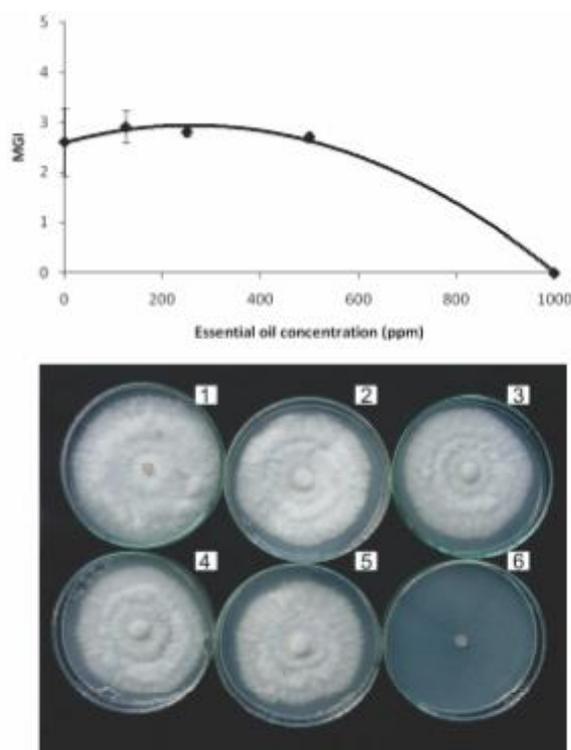


The essential oil from *O. selloi* affected the germination of spores of *C. gloeosporioides* and *M. pernicioso* at all concentrations tested, although the largest reductions in germination (93 and 87%, respectively) were observed at 1,000 ppm (Figure 4). In complete contrast, the germination of *A. alternata* spores was not affected by any of the treatments applied.

The essential oils are promising natural antifungal agents with potential applications in agriculture to control phytopathogenic fungi causing severe destruction of crops. The numerous aromatic and medicinal plants are considered to be non-phytotoxic compounds and potentially effective against plant pathogenic fungi (CAROVIĆ-STANKO *et al.*, 2010).

The hydrodistillation of the leaves and flowers part of *O. selloi* gave light yellowish oil with the major components of the oil having phenylpropanoids and sesquiterpenes. In recent years, several researchers have reported that mono - and sesquiterpenes have

Figure 3 - Effects of different concentrations of methyl chavicol (standard) on the mycelial growth index (MGI) - ($y = -5E-06X^2 + 0.0027x + 2.5862$; $R^2 = 99.54^{**}$) a) and mycelia growth to *Moniliophthora pernicioso* (b) - 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. Bar = 5 cm



enormous potential to inhibit microbial pathogens (CAROVIĆ-STANKO *et al.*, 2010; RAHMAN; HOSSAIN; KANG, 2010). Also, was reported a significant difference in antifungal activity among functional groups, that phenylpropanoids compounds were more toxic than hydrocarbons (KIM *et al.*, 2008).

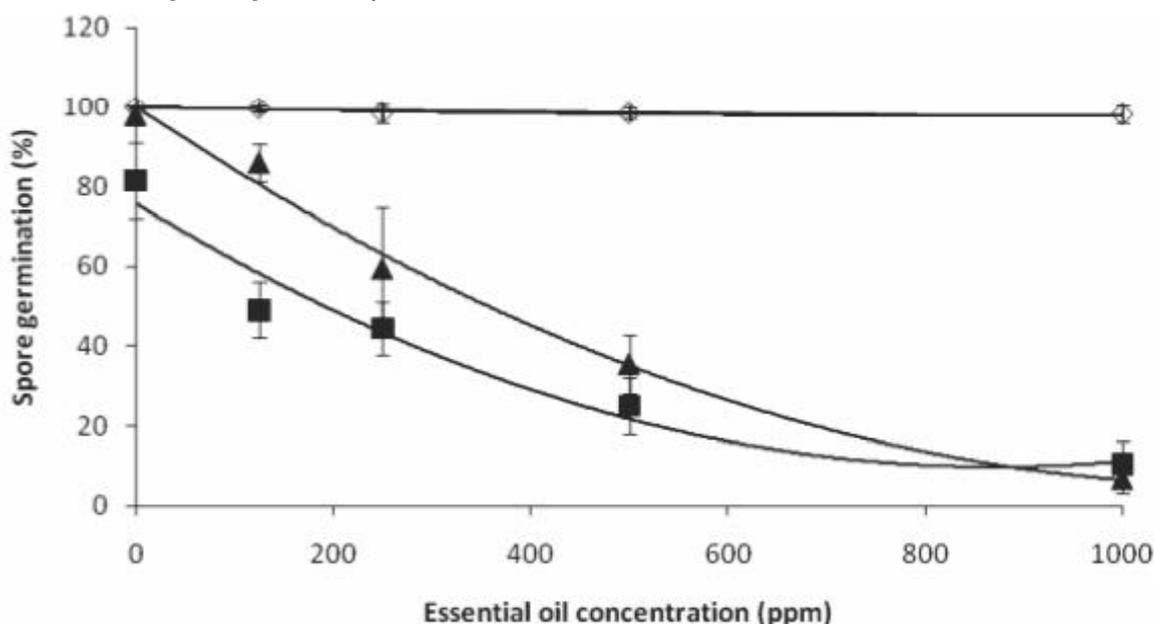
The results show that the essential oil of *O. selloi* was able to inhibit the growth of mycelia of the three fungal isolates studied, with the order of sensitivity to the oil being *M. pernicioso* bigger than *C. gloeosporioides* and *A. alternata*. Furthermore, the essential oil caused a reduction in the germination of spores of *M. pernicioso* 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. pernicioso* was also evaluated. The results revealed that the essential oil and pure methyl chavicol were similarly effective in causing complete inhibition of mycelial growth at a concentration of 1,000 ppm. Previous studies demonstrated that the essential oil from another species of *Ocimum*, namely *O. basilicum* L., as well as the pure phenylpropanoids methyl chavicol and eugenol, were able to reduce significantly the mycelial growth of the phytopathogenic fungus *Botrytis fabae* Sardiña (OXENAHM; SVOBODAL; WALTERS, 2005). Additionally, a number of other phenylpropanoids have been reported to exhibit antifungal activity, including apiole (MEEPAGALA *et al.*, 2005; RAZZAGHI-ABYANEH *et al.*, 2007) and the

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	1,000		
<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>Monilophthora pernicioso</i>	5.4	3.2	100.0	100.0	250 – 500	415.25

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 *Colletotrichum gloeosporioides* ($y = 0.000073x^2 - 0.166687x + 100.226923$; $R^2 = 0.99$) and \blacksquare *Monilophthora pernicioso* ($y = 0.000087x^2 - 0.151606x + 75.846154$; $R^2 = 0.99$)



related nothoapiole (LAOUER *et al.*, 2009). In this study, the essential oil showed a remarkable anti-fungal activity against *M. perniciosa*, which could be attributed to the presence of phenylpropanoids mainly methyl chavicol. This was further supported by our findings, when was used standard methyl chavicol at 1,000 ppm. Although, essential oil at 500 ppm had efficient inhibition effect against *M. perniciosa*, but the standard at 500 ppm had not 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 (RAHMAN; HOSSAIN; KANG, 2010), methyl eugenol (KIM *et al.*, 2008) have been claimed to contain the strong antifungal properties.

Various studies have shown that the essential oils from many aromatic or medicinal plants are active against a range of phytopathogenic fungi. Thus oils from *Pimenta racemosa* (Mill.) J.W.Moore and *Thymus vulgaris* L. were able to inhibit completely the growth of the phytopathogenic fungi *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. and *Cryphonectria parasitica* (Murrill) M.E. Barr (KIM *et al.*, 2008), whilst the essential oil from *Aloysia gratissima* (Gillies & Hook.) Tronc. was active against *C. gloeosporioides* and *Fusarium oxysporum* Schltdl. in a dose-dependent fashion (CARDOSO, 2005). Treatment of *Botrytis cinerea* Pers., *F. oxysporum* and *Bipolaris sorokiniana* (Sacc.) Shoemaker with essential oil derived from *Eucalyptus* spp. at a concentration of 500 ppm resulted in a significant reduction of mycelia growth (SALGADO *et al.*, 2003).

With regard to *M. perniciosa*, 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; BASTOS, 2007). Thus, the essential oil from *P. aduncum* L. completely inhibited mycelia growth and spore germination in the phytopathogenic fungus when applied at 50 and 100 ppm, respectively, whilst oils from *P. dilatatum* Rich., *P. callosum* Opiz and *P. marginatum* var. *anisatum* C.DC. inhibited *M. perniciosa* spore germination at concentrations of 400, 400 and 500 ppm, respectively (SILVA; BASTOS, 2007). Additionally, the essential oils *P. callosum*, *P. marginatum* var. *anisatum* and *P. enckea* C.DC. exhibited antifungal activities against *M. perniciosa*, *Phytophthora palmivora* (E.J. Butler) E.J. Butler and *P. capsici* Leonian, all of which attack cacao plants (SILVA; 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. perniciosa*. 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. Kim *et al.* (2008) reported there was a significant difference in antifungal activities among phenylpropanoids, that the position of the double bond of the propenyl group is also important for antifungal activity.

CONCLUSIONS

1. The essential oil from *O. selloi*, when applied at a concentration of 1,000 ppm, is able to inhibit mycelia growth (100%) and spore germination (87%) of *M. perniciosa*;
2. The antifungal activity is attributed to the major constituent methyl chavicol, which showed activity at 1,000 ppm similar to that of the essential oil.
3. The essential oil from *O. selloi* was more effective to inhibit mycelia growth and spore germination of *M. perniciosa* than *C. gloeosporioides* and *A. alternata*.

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