

*Full Length Research Paper*

## **Chemical composition and control of *Sclerotium rolfsii* Sacc by essential oils**

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Essential oils of medicinal plants show potential to control phytopathogens. Essential oils of *Lippia sidoides* Cham, *Lippia lasiocalycina* Cham, *Lippia organoides* Kunth - Teresina, *Lippia organoides* Kunth - Jatobá, *Mesosphaerum suaveolens* (L.) Kuntze, *Croton sonderianus* Muell. Arg. And *Croton zehntneri* Pax et Hoffm. were evaluated regarding their chemical composition and in controlling the *Sclerotium rolfsii* Sacc. The experiments were carried out in a completely randomized design with five concentrations and the control (without oil application): 0.0313; 0.0625; 0.1250; 0.2500 and 0.500 mL kg<sup>-1</sup> in Petri dishes with PDA medium (potato-dextrose-agar). The evaluations consisted of daily measurements of the colony diameter in diametrically opposite directions, 24 h after the experiment installation and maintained until the radial reach of the colony on the edges of the Petri dish, in one of the treatments. The chemical composition of the essential oils were evaluated by GC-MS and the following compounds were identified: Thymol (33.5%, *L. sidoides*); piperitenone oxide (67.7%, *L. lasiocalycina*); borneol (19.2%, *L. organoides*); borneol carvacrol (34.4%, *L. organoides* – Jatobá); sabinene (30.3%, *M. suaveolens*); β-sabinene (30.5%, *C. sonderianus*); and estragole (90.1%, *C. zehntneri*). The *S. rolfsii* fungus is highly sensitive to *L. sidoides*, *L. organoides* - Jatobá and *C. zehntneri* essential oils, suggesting its use in the management of Sclerotium wilt in cowpea.

**Key words:** Antifungal activity, alternative control, Sclerotium wilt, *Vigna unguiculata*.

### **INTRODUCTION**

The cowpea (*Vigna unguiculata* (L.)Walp.) is very important in human nutrition because it is a natural source of proteins, calories, vitamins and minerals (Freire

Filho et al., 2011). However, although rustic, *V. unguiculata* is affected by some diseases, among them, the sclerotium wilt. This disease is caused by the

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polyphagous and cosmopolitan fungus *Sclerotium rolfsii* Sacc, that causes high yield loss in infected plants (Bedendo, 2011).

In cowpea, the disease occurs in the stem, and present as a more representative symptom a tangle of white mycelium, which later develops into yellow tones and, under this tangle, there is an intense disruption of the parasitic plant tissue (Athayde et al., 2005). The disease can be lethal, regardless of the phenological stage of the crop, leading to the reduction of the stand, with direct effects on grain yield (Cardoso, 1994).

Among the management practices aiming the disease control, the use of resistant cultivars (Adandonon et al., 2006; Silva et al., 2014); crop rotation (Bedendo, 2011); and the use of biocontrolling microorganisms (Punja et al., 1985; Singh et al., 2002; Adandonon et al., 2006; Pacheco et al., 2016); the use of alternative products based on plant extracts and vegetable oils (Brum et al., 2014) and the use of chemical fungicides (Punja et al., 1985; Sales Júnior et al., 2005; Athayde et al., 2005; Bedendo, 2011) can be highlighted.

The lack of registered fungicides for the crop has stimulated the search for alternative products for the management of *Sclerotium* wilt. Substances such as essential oils, crude extracts and tinctures from plants have been studied because they have in their composition molecules with fungicidal properties (Matos, 1997). In addition to being characterized as secondary plant metabolites and of low toxicity to humans, they are extensively tested in *in vitro* and *in vivo* control of phytopathogens and seed treatment (Rodrigues et al., 2006).

The studies have reported biological properties for essential oil, rich in thymol and carvacrol, as antimicrobial, anti-inflammatory, antioxidant and larvicidal properties (Damasceno et al., 2011; Carvalho et al., 2013; Guimarães et al., 2014).

Considering the studies that report the antimicrobial activity of essential oils of plants existing in the ecosystems of the Mid-North region of Brazil, associated with the sensitivity of *S. rolfsii* to essential oils, the objective of this research was to identify the chemical composition of essential oils of native plants of the Mid-North region of Brazil, evaluating the mycelial sensitivity and its potential use in the control of *S. rolfsii*, agent of *Sclerotium* wilt in cowpea.

## MATERIALS AND METHODS

### Experiment management, isolation and pathogen maintenance

The experiments were carried out in the Plant Pathology Laboratory at Embrapa Mid-North, where the *S. rolfsii* fungus was isolated from cowpea plants with typical symptoms of the disease. Disinfestation was performed with 70% alcohol for 30 s and 1.5% sodium hypochlorite for 2 min. To remove excess hypochlorite, the material was immersed in two consecutive portions of distilled and sterilized water. The tissue fragments were then transferred to Petri

dishes containing PDA medium (Potato, Dextrose and Agar) and subsequently incubated at 25°C for 7 days. After this period the maintenance was performed through re-ricing in Petri dishes with PDA medium.

### Extraction and analysis of the chemical composition of essential oils

The essential oils of six botanical species belonging to the Brazilian Mid-North region (Table 1) were obtained. All studied species are spontaneously occurring shrubs or sub-bushes; none of them are still cultivated, being all vegetatively propagated.

The extraction of the essential oils was carried out by the hydrodistillation method, using the Clevenger apparatus, coupled to the heating mantle used as heat source for the system (Gomes et al., 2014). In each extraction, 200g of dehydrated and previously crushed leaves were used. The inflorescences were also used for *L. origanoides*.

The gas chromatography coupled to mass spectrometry (GC-MS) was performed on a Shimadzu GC-17A / MS QP5050A apparatus with electron impact ionization at 70 and V. Mass spectra were obtained from 43 to 350 m/z. The temperature of the injector / detector and the thermal program were maintained at 240°C. The carrier gas used was helium. Identification was made by comparison with standard spectra of the internal data library and retention times based on the linear retention index. The GC-FID chromatogram was used to determine the relative concentration using the peak areas in the Agilent 5975C system, which method of analysis was similar to the GC-MS system, previously described. It was used a DB-5 capillary column (30m 0.25mm di, 0.25µm film; J & W Scientific, Folsom, CA, USA) and hydrogen was used as carrier gas.

### Evaluation of fungal sensitivity to oils

The experiment was conducted in a completely randomized design, with five concentrations and one control (without oil): 0.0313; 0.0625; 0.125; 0.2500 and; 0.5000 ml kg<sup>-1</sup> in PDA culture medium with four replicates, each replicate being represented by three Petri dishes with 90 mm in diameter.

The essential oil was deposited in the center of the Petri dishes on the solidified medium and evenly distributed with Drigalsky's Spatula. Immediately after the oil distribution, a 5 mm diameter disk containing fragments of the four-day old *S. rolfsii* culture grown in PDA (Veloso et al., 2012) was deposited in the center of each plate. The plates were then sealed with plastic film and incubated in BOD at 25°C and photoperiod of 12h. The control group received the disc with fungal structures. The evaluation consisted in the daily measurement of the diameter of the colonies in diametrically opposite directions, with the aid of a millimeter rule, obtaining an average for each repetition. Evaluations were initiated 24 h after the experiment set up and maintained until the radial growth of the colony reached, in one of the treatments, the total diameter of the Petri dish. With the obtained data, the growth rate of the fungus was determined in the different concentrations tested, using the formula adapted from Lilly and Barnet (1951).  $T_x = (C_n + 1 - C_n) / T$ , where:  $T_x$  = growth rate,  $C_n$  = growth at incubation time "n",  $C_n + 1$  = growth at incubation time n + 1 and T = time interval considered, in this case, 24 h.

The mycelial growth values were transformed into percentage of inhibition, and the inhibitory concentrations of 50% of the mycelial growth (IC 50) were obtained from the logarithmic regressions for each oil / species. For that, the statistical software Costat (Tonin et al., 2013) was used. From the IC 50 data it was possible to classify the sensitivity of the fungus to the oils in: highly sensitive (HS) when  $IC_{50} < 1 \text{ mg L}^{-1}$ ; (1 mL kg<sup>-1</sup> ≤ IC50 ≤ 40 mL kg<sup>-1</sup>) and non-sensitive

**Table 1.** Species, origin, altitude, registration numbers and part of the plant used to obtain the tested essential oils. Teresina, PI, 2018.

Specie	Origin	Altitude (m)	Register	Part of the plant
<i>Lippia sidoides</i>	Teresina	72	CEN 92438	Leaf
<i>Lippia lasiocalycina</i>	São João do Piauí	228	CEN 92437	Leaf
<i>Lippia organoides</i>	Gilbués	481	CEN 92436	Leaf and inflorescence
<i>Lippia organoides</i> - Jatobá	Jatobá do Piauí	240	CESJ 70120	Leaf
<i>Mesosphaerum suaveolens</i>	Parnaíba	5	IPA 57264	Leaf
<i>Croton sonderianus</i>	Parnaíba	5	CEN 92500	Leaf
<i>Croton zehntneri</i>	Valença	208	TEPB 30944	Leaf

(NS) ( $IC_{50} \geq 40 \text{ ml kg}^{-1}$ ), defining the fungitoxicity (Edgington et al., 1971; Tonin et al., 2013). The data obtained for mycelial growth of *S. rolfsii* were submitted to variance analysis and polynomial regression to define the model that best explains the behavior of the studied variable, with the aid of the Assistat software, version 7.7 beta (Assis and Silva, 2013).

## RESULTS AND DISCUSSION

The chemical composition of essential oils obtained by gas chromatography coupled with mass spectrometry (CG-EM) identified 73 compounds in all evaluated oils. For some species, it was not possible to identify all the constituents present in the essential oil. The results of this study revealed that only the oils of *L. organoides* - Jatobá and *C. zehntneri*, had 100% of their constituents identified. The major compounds identified were thymol, piperitenone oxide, borneol, carvacrol, sabinene,  $\beta$ -sabinene and estragol, for *L. sidoides*, *L. lasiocalycina*, *L. organoides* - Teresina, *L. organoides* - Jatobá, *H. suaveolens*, *C. sonderianus* and *C. zehntneri*, respectively. The essential oils of *L. sidoides*, *L. organoides* and *Croton zehntneri* have demonstrated properties of epidemiological interest, with potential to control plant diseases. Such characteristics are usually associated with the presence of major compounds in their chemical composition.

As highlighted in Table 2, the main constituent of the *L. sidoides* essential oil, thymol (33.2%), is associated with its excellent level of control (Lemos et al., 1990; Gonçalves et al., 2015; Athayde et al., 2005) observed in the control of the fungus *S. rolfsii* (Figure 1). In addition, a representative example of the GC chromatogram with peak assignments is shown in Figure 2. For *L. organoides* - Jatobá, the main constituent detected was carvacrol (54.4%), which also showed exceptional bioactivity on the mentioned fungus, the result of which is in addition to those obtained by other authors who had demonstrated bioactivity of this compound on some microorganisms (Lorenzi and Matos, 2002; Queiroz et al., 2014).

The chemical composition variability of each essential oil is of extreme importance for its effective action, since

the antimicrobial activity is directly related to the synergism of its chemical composition, where compounds like alcohols, phenols, terpenes and ketones are pointed out as the main responsible ones by toxic properties (Sellamuthu et al., 2013). Terpenes (Table 3) are considered to be the secondary metabolites most produced by plants with applications in different areas, such as in the pharmaceutical and solvent industries (Wang et al., 2016). According to Sangwan et al. (2001), the genotypic characteristics associated with the environmental factors can be determinant in the biosynthesis of the secondary metabolites, including the essential oils. For example, the climatic conditions may influence the enzymatic activities of a particular plant species (Barros et al., 2009).

Regarding this species (*L. organoides* - Jatobá), no records were found in the researched literature of any effect of its essential oil on filamentous fungi - this being the first record. However, some authors have demonstrated bioactivity of this essential oil against *Staphylococcus aureus* (Queiroz et al., 2014) and *S. aureus* and *Escherichiacoli* (Sarrazin et al., 2015).

In this work, the results obtained by essential oils extracted from plants of the genus *Croton* also deserve to be highlighted, especially *C. zehntneri*, which showed excellent fungicidal action on *S. rolfsii*, similar to those revealed by *L. sidoides* and *L. organoides* - Jatobá (Figures 3 and 4). Although there are no results in the researched literature showing the effect of essential oils extracted from *Croton* on *S. rolfsii*, there are reports that demonstrate the action of *C. conduplicatus* in the control of *Lasiodiplodia theobromae* (Peixinho et al., 2017) and *C. rhamnifolioides* acting against fungi of the genus *Candida* (Vidal et al., 2016). In view of the bioactivity demonstrated by the essential oils studied against the fungus *S. rolfsii*, it proved reasonable to evaluate its efficiency individually.

The essential oil of *M. suaveolens* has sabinene (3.0%) and 1.8-cineole (12.0%) as the main constituents with different composition from those observed by other authors (Martins et al., 2006; Branquinho, 2015). The sensitivity of fungi to a particular toxic substance can be expressed, according to Tonin et al. (2013) by  $ED_{50}$

**Table 2.** Retention index (RI) and chemical composition of essential oils of *Lippia sidoides* (Ls), *Lippia lasiocalycina* (Ll), *Lippia organoides* (Lo), *Lippia organoides* Jatobá (Lo-J), *Mesosphaerum suaveolens* (Ms), *Croton sonderianus* (Cs) and *Croton zehntneri* (Cz).

RI <sup>a</sup>	RI <sup>b</sup>	Compound	Ls	Ll	Lo	Lo-J	Ms	Cs	Cz
850	866	Z-3-Hexaneol	0.5	0.3	0.4	-	-	-	-
924	921	$\alpha$ -Thujene	1.1	-	-	1.1	0.9	-	-
932	928	$\alpha$ -Pinene	0.5	0.5	0.6	0.3	2.8	0.6	-
946	943	Camphene	-	-	1.8	-	-	-	-
969	967	Sabinene	-	-	0.8	-	30.0	-	0.2
974	971	Octen-3-OL	0.7	-	1.1	-	7.0	-	-
988	984	$\beta$ -Myrcene	2.2	0.2	0.5	2.0	0.2	-	-
1002	1001	$\alpha$ -Phellandrene	-	-	-	0.1	-	1.2	-
1014	1011	$\alpha$ -Terpinene	1.6	-	-	1.7	0.2	-	-
1020	1019	Para-Cymene	13.1	0.5	-	13.3	3.3	0.5	-
1024	1023	Limonene	0.8	11.9	1.4	0.4	1.2	-	-
1025	1023	$\beta$ -Phellandrene	-	-	-	-	-	1.3	-
1026	1025	1,8-Cineole	2.3	-	6.9	0.2	11.3	2.9	2.9
1032	1030	Z-Ocimene	0.2	0.5	-	-	-	-	-
1044	1040	E- $\beta$ -Ocimene	-	0.2	-	-	0.2	-	0.6
1054	1052	$\gamma$ -Terpinene	4.3	0.5	-	7.8	0.3	0.3	-
1065	1060	Z SABinene Hidrate	0.4	-	-	-	-	-	-
1083	1083	Fenchone	-	-	-	-	1.0	-	-
1086	1083	Terpinolene	-	-	0.4	-	-	0.5	-
1095	1093	Linalool	0.6	0.4	-	-	0.3	0.7	-
1114	1109	Fenchol	-	-	-	-	0.5	-	-
1119	1115	p-Mentha-E-2,8-Dien-1-OL	-	0.2	-	-	-	-	-
1133	1129	Z-p-Mentha-2,8-Dien-1-OL	-	0.2	-	-	-	-	-
1140	1144	E-Verbenol	-	0.2	-	-	-	-	-
1165	1161	Borneol	-	-	19.2	-	-	0.4	-
1174	1172	4-Terpineol	1.6	-	0.6	0.8	2.6	1.1	-
1186	1185	$\alpha$ -Terpineol	0.8	0.5	0.7	-	0.3	0.3	0.8
1195	1195	Estragolee	-	-	-	-	-	-	90.1
1232	1229	Thymol Methyl Ether	2.8	-	-	5.5	-	-	-
1241	1239	Carvacrol, Methyl Eter	-	-	-	0.4	-	-	-
1289	1290	Thymol	33.2	1.2	-	3.0	-	-	-
1298	1297	Carvacrol	0.6	-	-	54.5	-	-	-
1335	1331	$\delta$ -Elemene	-	-	-	-	-	0.6	-
1339	1332	E-Carvil Acetate	-	0.2	-	-	-	-	-
1340	1336	Piperitenone	-	2.2	-	-	-	-	-
1349	1348	Thymol Acetate	0.3	-	-	0.5	-	-	-

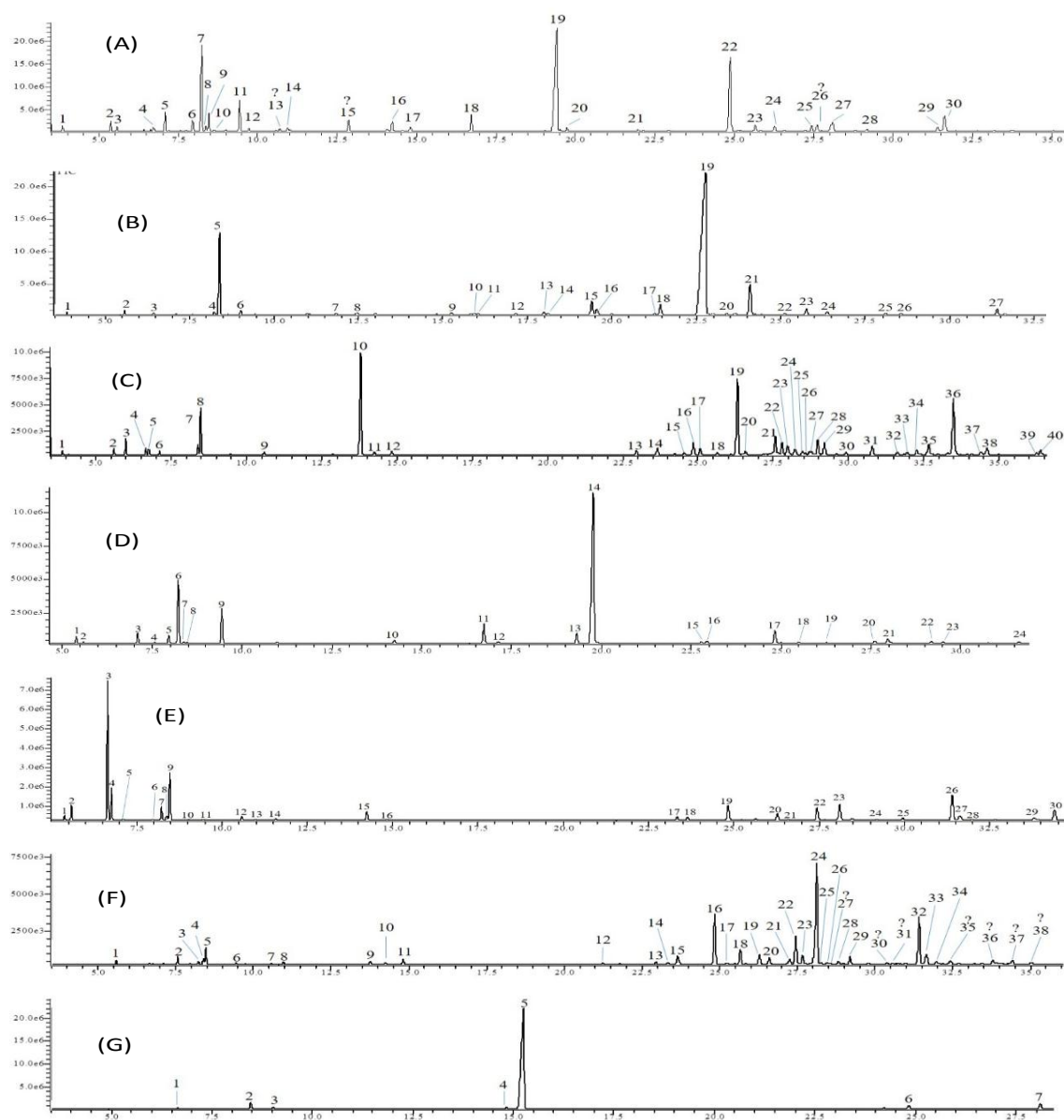
Table 2. Contd.

1366	1366	Piperitenone Oxide	-	67.2	-	-	-	-	-
1374	1370	$\alpha$ -Copaene	-	-	0.9	0.6	-	0.4	-
1387	1379	$\beta$ -Bourbonene	-	-	-	-	1.1	2.3	-
1388	1396	Iso Jasnone	-	0.2	-	-	-	-	-
1389	1386	$\beta$ -Elemene	-	-	1.4	-	1.0	12.7	-
1410	1407	$\alpha$ -Cedrene	-	-	0.4	-	-	-	-
1417	1414	Trans-Caryophyllene	17.4	-	2.6	4.0	5.1	0.4	2.1
1419	1419	$\beta$ -Cedrene	-	-	1.3	-	-	-	-
1431	1423	$\beta$ -Gurjinene	-	-	-	-	-	3.8	-
1432	1430	$\alpha$ -Bergamotene	-	-	-	0.3	-	-	-
1437	1433	$\alpha$ -Guaiene	-	-	0.4	-	2.2	-	-
1439	1433	Aromadendrene	1.2	-	-	-	-	2.3	-
1452	1448	$\alpha$ -Humulene	0.9	-	16.0	0.1	0.3	1.7	-
1453	1446	Geranyl Acetone	-	6.4	-	-	-	-	-
1454	1450	$\beta$ -Farnesene	-	0.2	-	-	-	-	-
1458	1356	Alloaromadendrene	-	-	-	-	4.3	1.3	-
1464	1455	$\alpha$ -Acoradiene	-	-	0.6	-	-	-	-
1483	1471	$\alpha$ -Amorphene	-	-	-	-	-	7.2	-
1484	1476	Germacrene-D	1.1	-	3.9	-	5.9	2.0	-
1489	1481	$\beta$ -Sabineno	-	-	2.4	0.8	-	30.5	-
1494	1491	Biciclogermacrene	2.8	-	-	-	-	0.4	-
1496	1488	Valencene	-	-	2.2	1.6	-	-	-
1498	1494	$\alpha$ -Selinene	-	-	1.5	-	-	-	-
1500	1491	Bicyclogermacrene	-	1.3	-	-	0.3	0.5	3.3
1505	1502	$\beta$ -Bisabolene	-	0.5	0.3	-	-	-	-
1508	1500	Germacrene A	-	-	0.7	-	-	0.3	-
1513	1509	$\gamma$ -Cadinene	-	-	-	-	-	2.1	-
1522	1518	$\delta$ -Cadinene	0.4	-	2.6	0.6	0.8	0.3	-
1539	1536	$\alpha$ -Copaene-11-Ol	-	-	0.5	-	-	-	-
1577	1573	Spathulenol	0.8	0.2	-	0.3	9.0	12.7	-
1578	1579	Epiglobulol	-	-	-	-	-	3.4	-
1582	1578	Caryophyllene Oxide	3.7	0.2	0.5	0.4	2.2	-	-
1586	1586	Isospathulenol	-	1.2	-	-	-	-	-
1592	1587	Viridiflorol	-	-	-	-	0.4	0.7	-
1606	1604	Humulene Epoxide	-	-	2.5	-	-	-	-
1651	1649	Pogostol	-	-	-	-	3.9	-	-

IR<sup>a</sup>: relative retention index, calculated by the Van den dool equation. %: Compound percentage. The compounds of the table are in ascending order of column Rxi-5HT, 30 m x 0,25mm elution. IR<sup>b</sup>: experimental retention indexes.



**Figure 1.**(A) Cowpea plant with disease symptom; (B) *S. rolfsii* in PDA medium; (C) Visualization with optical microscope.

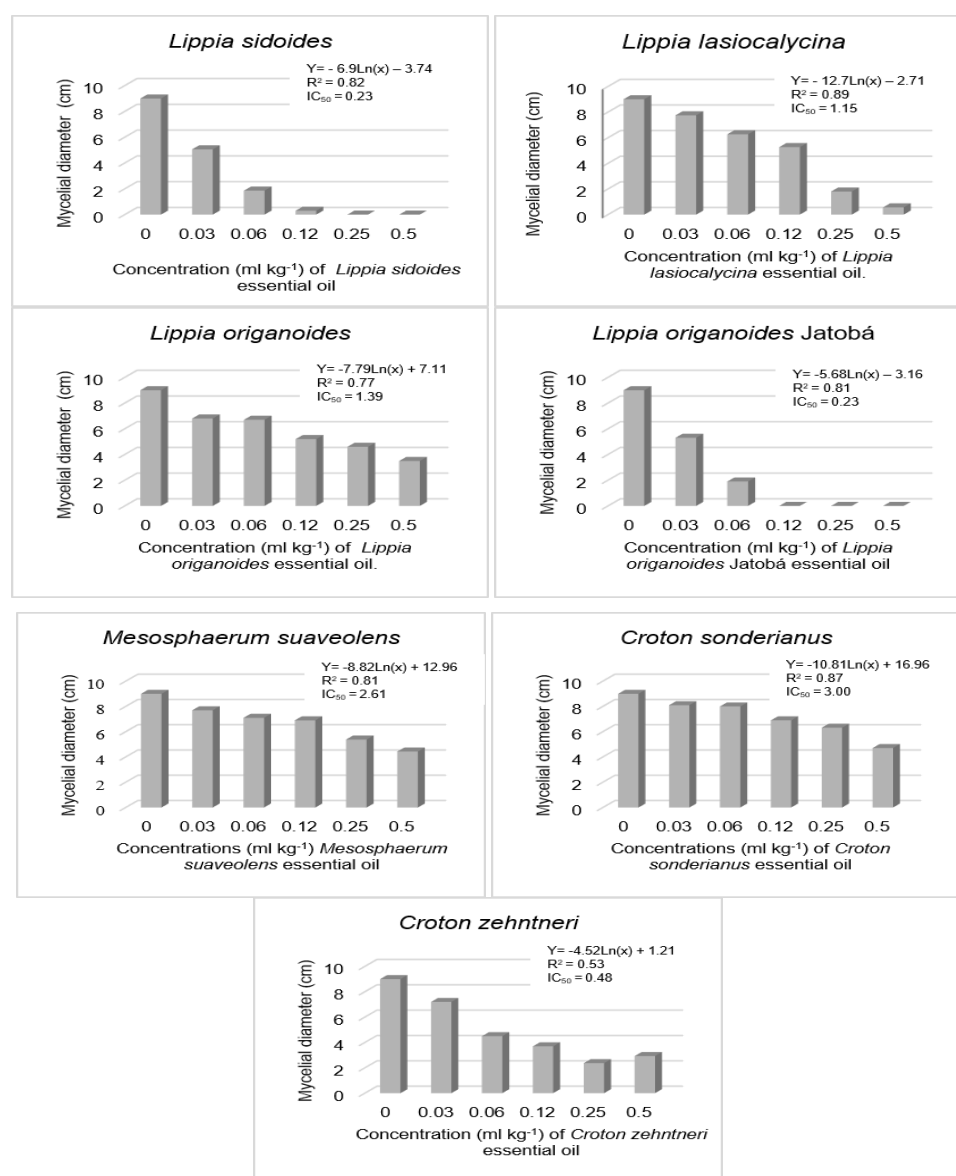


**Figure 2.** GC chromatogram of essential oils of *L. sidoides* (A), *L. lasiocalycina* (B), *L. origanoides* (C), *L. origanoides* Jatobá (D), *M. suaveolens* (E), *C. sonderianus* (F) and *C. zehntneri* (G) with peak assignments.

**Table 3.** Percentage of chemical compounds by group from essential oils of *L. sidoides* (Ls), *L. lasiocalycina* (Ll), *L. origanoides* (Lo), *L. origanoides* Jatobá (Lo-J), *M. suaveolens* (Ms), *C. sonderianus* (Cs) and *C. zehntneri* (Cz).

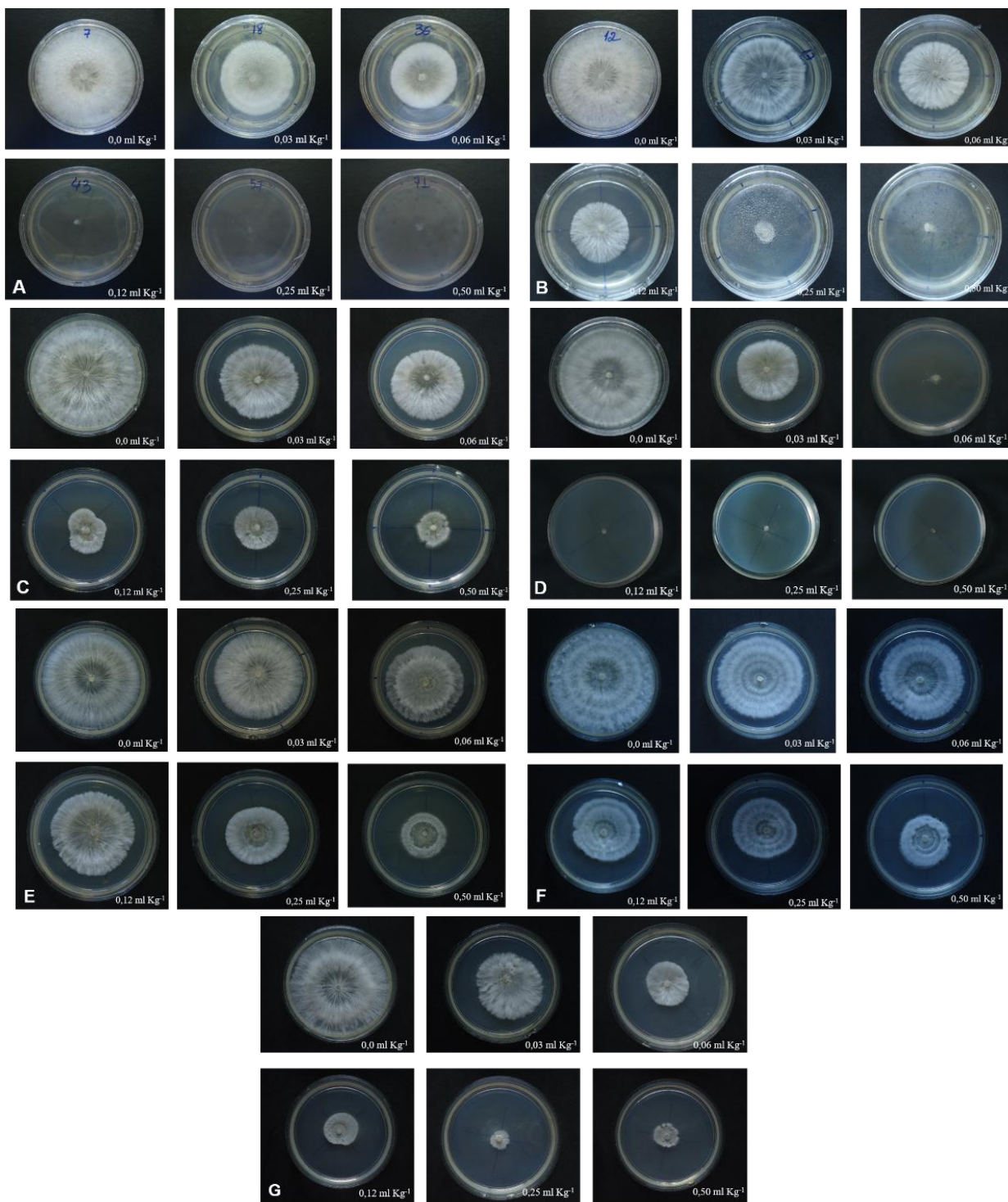
Group of compounds	%						
	Ls	Ll	Lo	Lo - J	Ms	Cs	Cz
Monoterpenes hydrocarbons	57.25	14.52	4.19	26.52	46.14	4.36	0.84
Oxygenated monoterpenes	10.39	72.36	29.86	64.75	16.04	5.37	93.75
Sesquiterpenes hydrocarbons	23.94	2.02	37.24	8	20.91	68.7	5.41
Oxygenated sesquiterpenes	4.55	8.05	3.9	0.73	15.54	16.79	v
Other	0.48	v	0.4	v	v	v	v
Not identified	3.39	3.05	24.41	v	1.37	4.78	v

v: trace (<0.05%).



**Figure 3.** In vitro mycelial growth of *Sclerotium rolfsii* in six concentrations (mL kg<sup>-1</sup>) of *L. sidoides*, *L. lasiocalycina*, *L. origanoides*, *L. origanoides* Jatobá, *M. suaveolens*, *C. sonderianus* and *C. zehntneri* essential oils. IC<sub>50</sub> = Concentration that inhibits 50% of the mycelial growth.





**Figure 4.** *In vitro* mycelial growth of *Sclerotium rolfsii* in six concentrations (mL kg<sup>-1</sup>) of *L. sidoides* (A), *L. lasiocalycina* (B), *L. organoides* (C), *L. organoides* Jatobá (D), *M. suaveolens* (E), *C. sonderianus* (F) and *C. zehntneri* (G) essential oils.

(effective dose), EC<sub>50</sub> (effective concentration) or IC<sub>50</sub> (inhibition concentration). If a fungus is sensitive to a given substance, its fungitoxicity becomes apparent;

otherwise it will be innocuous (non-toxic). On the other hand, if a substance does not present fungitoxicity the fungus is considered insensitive (Reis et al., 2007). The



oils that showed high toxicity, demonstrated by the high levels of inhibition ( $IC_{50} < 1 \text{ ml kg}^{-1}$ ) were *L. sidoides*, *L. organoides* - *Jatobá* and *C. zehntneri* (Figure 2).

The antifungal properties of the essential oils are due to their lipophilic characteristics (Bakkali et al., 2008), which occur with the presence of compounds such as monoterpene phenols, especially thymol, carvacrol and eugenol (Barrera-Necha et al., 2008). Thus, the hydrophobicity of the essential oil allows an interaction between it and the lipids of the cell membrane, interfering in its permeability and causing changes in its structure (Costa et al., 2011). Some of these alterations were confirmed by optical microscopy, where the *Syzygium aromaticum* essential oil significantly interfered in the mycelial growth of *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum*. In this study, several morphological alterations were observed, such as the presence of vacuoles, disorganization of cellular contents, decrease in cell wall sharpness, intense fragmentation and less turgidity of hyphae (Costa et al., 2011).

As previously shown (Figure 3), among the tested oils, those of *L. sidoides*, *L. organoides* *Jatobá* and *C. zehntneri* deserve special mention in the control of *S. rolfsii*, for which the fungus showed high sensitivity. These three species presented yields of, respectively, 1.38; 0.4 and; 1.20%, whose results reinforce the possibility of its *in vivo* use. None of these species has been cultivated and its use has now been made by extractivism.

In this way, the great antifungal potential of the essential oils is evident. Its broad spectrum of action encourages further studies, in order to better understand the physiology of plants, especially the factors that influence the synthesis of its compounds. The next stage should focus on the development of an industrial scale production process aiming at obtaining alternative inputs based on essential oils plant diseases control.

In summary, the results show that the chemical composition of the essential oils identified the following major constituents: thymol (*Lippia sidoides*); piperitenone oxide (*Lippia lasiocalicina*); borneol (*Lippia organoides*); carvacrol (*Lippia organoides* - *Jatobá*); sabinene (*Mesosphaerum suaveolens*);  $\beta$ -sabinene (*Croton sonderianus*) and estragole (*Croton zehntneri*), being *Sclerotium rolfsii* highly sensitive to the essential oils of *Lippia sidoides*, *L. organoides* - *Jatobá*, and *Croton zehntneri*.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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