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Fungistatic activity of essential oils for the control of bipolaris leaf spot in maize

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Research institutions have emphasized alternative disease control agents because they represent a great option with beneficial effects on human and animal health and ecosystem balance. Despite the studies already done on botanical fungicides, their effective use requires knowledge about the applicability of natural products to different production systems. The main objective of this study is to evaluate the efficacy of essential oils of *Ocimum pupuraceus* L., *Cymbopogon nardus* (L.) Rendle, *Cymbopogon citratus* (DC.) Stapf, and *Lippia sidoides* Cham. on the inhibition of mycelial growth and germination of conidia of *B. maydis*. The other objectives of this study were to perform gas chromatographic and phytotoxicity analyses, and test the control of bipolaris leaf spot using essential oils of *L. sidoides* applied as a preventive and curative agent. Among the treatments studied, the essential oil of *L. sidoides* is effective in inhibiting mycelial growth and conidial germination at the concentrations of 5 and 1%, respectively. The main constituent of the oil is thymol (92.68%). The concentration range 0.75-3% of *L. sidoides* essential oil is phytotoxic to maize plants. Lower values of the area under the progress curve of bipolaris leaf spot are observed at concentrations 0.1-0.5%, when the essential oil was applied as a preventive agent prior to the colonization of plant tissues by the pathogen. The application of the oil as a curative to plants with the disease also shows efficacy at the concentration 0.1%, reducing the severity by more than 54%. These results demonstrate the potential effects of *L. sidoides* essential oil on preventive and curative control of bipolaris leaf spot in maize and mycelial growth.

Key words: Medicinal plants, alternative control, phytopathogens, *Zea mays*, *Bipolaris maydis, Lippia sidoides*.

INTRODUCTION

Brazil is classified as the third largest producer and the second largest exporter of Maize (*Zea mays* L.) in the world. Brazil is relevantly involved with the agricultural scenario (Peixoto, 2014; USDA, 2016; FAO, 2018);

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however, in Brazil, several factors have hindered maize production, among them, diseases. Diseases significantly affect the plant's productive potential (Conab, 2016), and may reduce the productive potential in whole or in part (Manfroi et al., 2016).

Maize diseases can be grouped according to plant organ and the resulting symptoms. They can be divided into leaf diseases, stem and root rot, and spike and grain rot (Ferrari and Possamai, 2015). In disease monitoring, mappings conducted by the agency Embrapa Milho and Sorgo, a high severity of bipolaris leaf spot has been detected in some Brazilian states, such as Rondônia, Mato Grosso, Goiás and Tocantins (Costa et al., 2014).

Because most cultivars are susceptible to bipolaris leaf spot, fungicides have been widely used, especially in tropical Brazilian areas, where the disease meets favorable conditions for its development (hot and humid climate). The indiscriminate use of agrochemicals has raised the resistance to phytopathogens and negatively affected the environment and human health through contamination of food, soil microbiota, water and the ecosystem. Seeking to reduce the problems generated by agrochemicals, natural products have been considered an alternative for disease control (Benvenuti, 2012; Ootani et al., 2013). Currently, research institutions have been emphasizing alternative disease control agents as they are an option with beneficial effects on human and animal health. They also promote ecosystem equilibrium. There are numerous alternatives to conventional chemical control of phytopathogens as a way to reduce the damages associated with their use.

One alternative is to use agents capable of inducing resistance in treated plants, thus activating their own natural defense mechanisms (Walters et al., 2013). Several authors have demonstrated the efficacy of some essential oils applied at certain concentrations for disease control (Diniz et al., 2008; Dalcin et al., 2017).

However, the issue is complex since leaf spot bipolaris is a disease that causes great damage to the corn crop due to the partial defoliation of the plants, and the current control of this disease is still made mainly by the use of fungicides and, thus, requires collective efforts by conducting studies for the purpose of creating knowledge and applicability of natural products to different production systems and patosystems.

Thus, the objective of this work is to evaluate the efficacy of essential oils for the control of leaf spot bipolaris in maize.

MATERIAL AND METHODS

Plant species

The plants used for the extraction of essential oil were *Ocimum pupuraceus* L. (dark opal basil) - Lamiaceae, *Cymbopogon nardus* (L.) Rendle (citronella) – Poaceae, *Cymbopogon citratus* (DC.) Stapf. (lemon grass) - Poaceae, and *Lippia sidoides* Cham. (rosemary-pepper) – verbenaceae.

The extraction of essential oils was carried out using hydro-distillation in a modified Clevenger equipment (Guimarães et al., 2008). 200 g of leaves of each test substance, dehydrated and chopped, were placed in a round bottom flask and covered with distilled water. The extraction time was two hours of boiling. Subsequently, the supernatant was collected using a micropipette, and stored in amber bottles covered with aluminum foil (protection from light), and then stored in a refrigerator at 4°C until the bioassays were conducted.

Isolation of *Bipolaris maydis*

The phytopathogen was obtained from maize plants presenting typical symptoms of bipolaris leaf spot. The isolation was performed on Petri dishes containing PDA culture medium (potato, dextrose, agar). The fungus was identified using optical microscopy according to the characterization of its vegetative and reproductive structures, which was made by consulting specialized literature (Barnett and Hunter, 1972; Ellis, 1971).

Inhibition of mycelial growth

The fungitoxic activity was evaluated *in vitro* using increasing concentrations of essential oils (1, 2, 3, 4 and 5%). The experiment was completely randomized in a factorial design of four replications and five evaluation periods (2, 4, 6, 8 and 10 days of incubation).

In order to evaluate the effects of essential oils on the mycelial growth of *B. maydis*, the methodology described by Seixa et al. (2008) and Ferreira et al. (2018) was used.

Chromatographic analyses of essential oils

Qualitative and quantitative analyses of the essential oils were performed using gas chromatography together with GC-MS mass spectrometry. The chromatograph used was the Shimadzu GC-210, equipped with a QP2010 Plus mass selective detector. The equipment was operated with the following setup: RTX-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness), temperature programming in the column: 60-240°C (3°C/min), injector temperature: 220°C, carrier gas: Helium, and splitless injection with injected volume of 1 µl of a 1:1000 solution in hexane. For the mass spectrometer (MS), the following setup was used: Impact energy of 70 eV, and temperature of the ion source and the interface: 200°C. The spectra obtained were compared with the library database Nist and Wiley 229, and the retention index calculated for each constituent was compared with tabulated levels according to Adams (2007). The quantification of the contents of the compounds was expressed as percentage.

Phytotoxicity of essential oil in maize plants

The phytotoxicity test was performed with the most promising essential oil for inhibition of mycelial growth *in vitro*. The experimental design was completely randomized with four replications. The treatments consisted of one control (water) and nine oil concentrations. Maize seeds were sown in plastic pots containing soil manure, soil and commercial Plantmax® substrate at a ratio of 1:2:1. Each vase was sown with four seeds of the cultivar Tractor® because it is susceptible to the disease and widely cultivated in the region. Irrigation was performed daily by hand. Twenty days after sowing, different concentrations (0.01, 0.1, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0%) of the essential oil were applied. The solutions were prepared as described above for the *in vitro* assay. The application on the leaves was carried out using a spray

Table 1. Effects of the essential oil of *Lippia sidoides* at increasing concentrations on mycelial growth (mm) of *B. maydis*.

Concentration	Evaluation time (days)					Inhibition*	Regression equation	R^2
(%)	1	2	3	4	5	(%)		
T (water)	15.06±1.52	20.81±0.34	26.48±0.87	33.85±0.68	39.28±0.58	0	y = 6.148x + 8.652	0.99
1	7.41±1.01	11±1.78	15.87±1.73	19.83±2.08	24.95±0.20	36	y = 4.391x + 2.639	0.99
2	-	4.91±0.20	12.07±2.08	21.08±3.79	25.44±4.10	35	y = 6.705x – 7.415	0.98
3	-	5.1±0.24	7.51±0.46	15.56±1.86	20.17±1.09	48	y = 5.08x – 5.572	0.98
4	-	3.79±0.51	7.34±0.84	15.24±0.53	19.82±1.11	49	y = 5.109x – 6.089	0.98
5	-	-	-	-	-	100	-	-

(-): no growth, (*): evaluation considered until the tenth day of incubation (mean ± standard deviation).

(capacity of 500 ml) until the point of flowing from leaves. 24 h after the application, the evaluations were carried out using a scale adapted from Freitas et al. (2009) and Cogliatti et al. (2011), where; 0% = absence of phytotoxicity, 1-25% = mild leaf necrosis or mild plant chlorosis, 26-50% = moderate leaf necrosis or moderate plant chlorosis, 51-75% = high leaf necrosis or high plant chlorosis, 76-100% = wilting and plant dryness.

Inhibition of *Bipolaris maydis* conidial germination

This assay was conducted in a completely randomized design with three replicates. An 1 mL aliquot of the conidial suspension of *B. maydis* (10^4 conidia mL^{-1}) and another 1 mL conidia suspension at different concentrations (0.0, 0.05, 0.1, 0.25, 0.5 and 1%) of the essential oil containing tween 80 (2%) was applied to each container (small glass) (Balbi-Peña et al., 2006). They were incubated in a humid chamber with a 14 h photoperiod. 300 conidia were counted per treatment by observing germinated and not germinated conidia using an optical microscope (Aguiar et al., 2014). After this period, the percentage of germination of conidia inhibition was calculated according to an adapted methodology (Aguiar et al., 2014; Balbi-Peña et al., 2006).

Preventive and curative control of bipolaris leaf spot using the essential oil

From the data obtained in the *in vitro* and phytotoxicity bioassays, the tests of preventive and curative control of *B. maydis* leaf spot at different essential oil concentrations (0.01, 0.05, 0.1, 0.25 and 0.5%) and the control using only distilled water were conducted. The experiment was completely randomized with three replications. The solutions were prepared as described for the *in vitro* assay.

For the preventive control test, three completely healthy maize plants were used containing five leaves each, to which the essential oil solution was previously applied at the concentrations already described. The application was carried out using a spray until the point of flowing from leaves. After one hour of oil application, the maize plants were inoculated with 5 mL of 1 x 10^4 mL^{-1} of solution of *B. maydis* conidia. Then, the plants were transferred to the humid chamber for 48 h to provide adequate conditions for pathogen development.

After 48 h, five evaluations of disease severity were performed at a two-day interval adopting the score developed by Santos et al. (2005) as follow; 0: Healthy plant, 1: Less than 1% of diseased leaf area, 3: 1-5% of diseased leaf area, 5: 6-25% of diseased leaf area, 7: 26-50% of diseased leaf area, and 9: more than 50% of diseased leaf area. In the curative assay, maize plants were initially inoculated with 5 mL of 1 x 10^4 mL^{-1} of solution of *B. maydis* conidia. Then, the plants were transferred to the humid chamber for 48 h to provide adequate conditions for pathogen development. After the appearance of the first lesions characteristic of the disease, the different concentrations of essential oil were applied. Five evaluations of disease severity were carried out after the application of the essential oil at intervals of two days using the grading scale previously described.

Statistical analysis

The results were expressed as mean ± standard deviation for the *in vitro* mycorrhizal growth inhibition and conidial germination assays. The *in vivo* curative and preventive control was subjected to linear regression. The area under the disease progression curve (AUDPC) was calculated according to Schneider et al. (1976). Regression equations were adjusted using the Excel® software.

RESULTS

Inhibition of mycelial growth

The essential oils of *O. pupuraceus*, *C. nardus* and *C. citratus* showed no inhibitory effects on the mycelial growth of the pathogen *in vitro*. Only the essential oil of *L. sidoides* had a fungitoxic effect at the concentrations tested as shown in Table 1 and Figure 1. There was inhibition of mycelial growth at the concentrations 2-4% of essential oil until two days of incubation in relation to the control (water). Thus, these same concentrations initially inhibited the mycelial growth of the pathogen, and maintained a decelerated growth in relation to the control (water) until the tenth day of evaluation. The 5% concentration of the oil completely inhibited the mycelial growth of *B. maydis* at the evaluated times.

Chemical constituents of *Lippia sidoides* essential oil

The chromatographic analysis of *L. sidoides* essential oil revealed, in quality and quantity, its chemical constituents. Thymol (92.6%) was the main component, followed by caryophyllene (2.2%) and ρ-cymene (1.1%), among other constituents that form this compound as indicated in

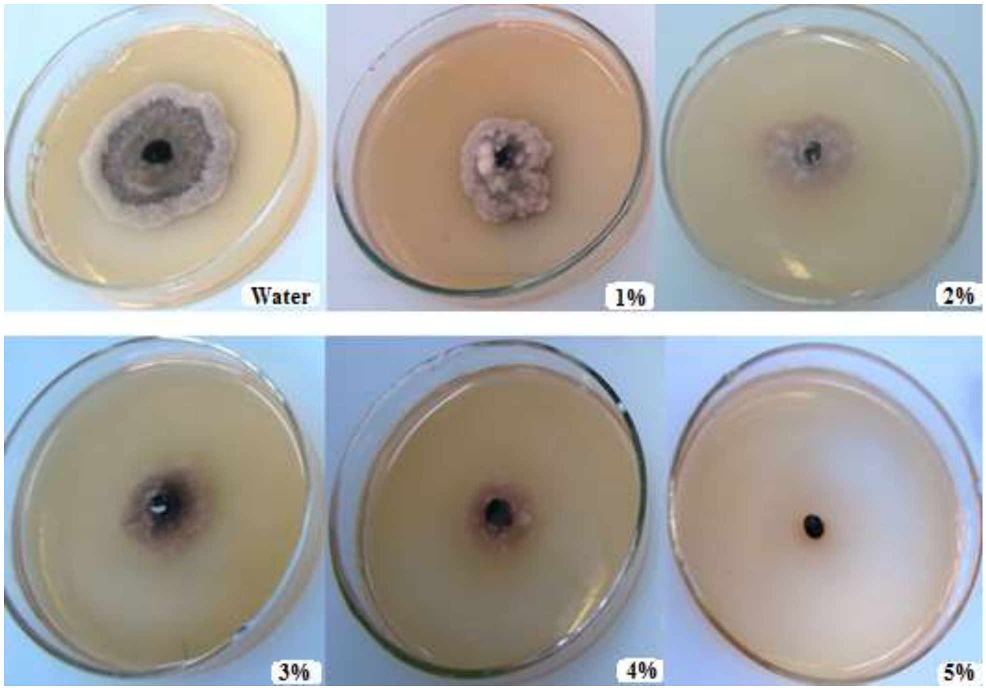

Figure 1. Inhibition of mycelial growth of *Bipolaris maydis* subjected to different concentrations of the essential oil of *Lippia sidoides* six days after the incubation.

Table 2. Chemical constituents of *Lippia sidoides* essential oil identified by GC/MS and their respective contents expressed as percentage.

[a]NC	Compound	[b]RT	[c]CRI	(%)
1	α-thujeno	5.915	927	0.051
2	α-terpinene	8.680	1018	0.091
3	ρ-cimene	8.944	1025	1.162
4	γ-terpinene	10.176	1058	0.250
5	cis-sabinene hidrate	10.656	1071	0.102
6	4-terpineol	15.19	1182	0.453
7	Thymol methyl ether	17.264	1230	0.430
8	Thymol	20.075	1294	92.684
9	(E)-caryophyllene	25.369	1419	2.235
10	α-humulene	26.849	1456	0.134
11	caryophyllene	31.878	1582	0.617
	Total (%)	-	-	98.179

[a]NC = Number of compounds; [b]RT = Retention time; [c]CRI = Calculated retention index.

Table 2.

Phytotoxicity of *Lippia sidoides* essential oil to maize plants

Among the oils tested *in vitro*, only *L. sidoides* was efficient to inhibit mycelial growth of *B. maydis*, and thus was selected for the phytotoxicity test. This essential oil causes phytotoxicity from the concentration 0.75%. It causes burning or necrosis in 60% of the leaf area, besides yellowing or plant chlorosis. The concentration 3.0% causes a high degree of phytotoxicity, and irreversible lesions were verified in all plants, with burning in 96.6% of the leaf area. Because the concentration 0.5% did not cause any phytotoxicity to maize plants, it was chosen as the maximum dosage to be used in the preventive and curative control of bipolaris leaf spot.

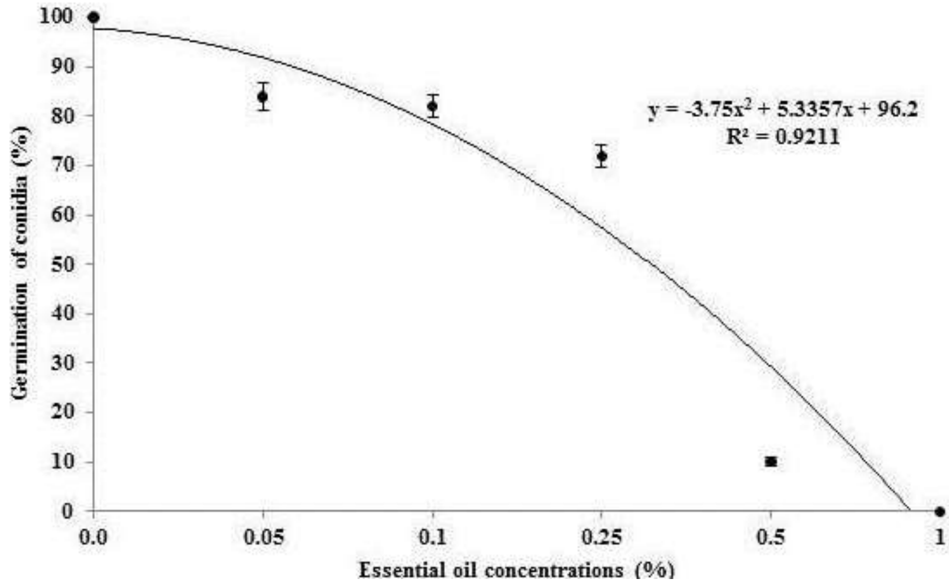

Figure 2. Inhibition of germination of conidia of *Bipolaris maydis* in function of increasing concentrations of *Lippia sidoides* essential oil (mean ± standard deviation).

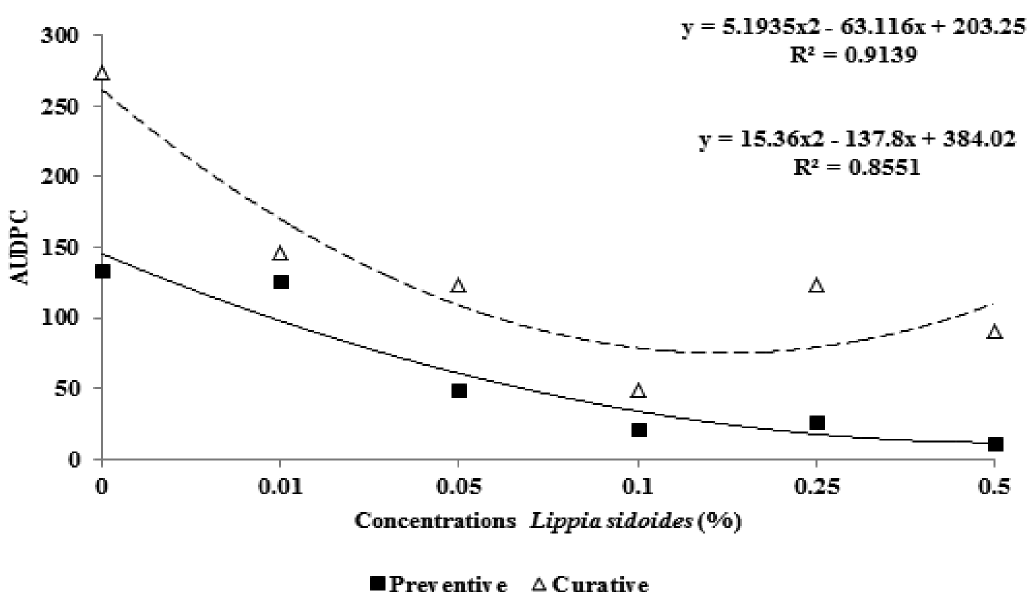

Figure 3. Area under the disease progression curve (AUDPC) of bipolaris leaf spot in maize leaves in function of increasing concentrations of essential oil of *Lippia sidoides* applied in a preventive and curative manner.

Inhibition of *Bipolaris maydis* conidial germination

Inhibition of conidial germination was proportional to the increase in concentrations as presented in Figure 2.

Considering the absolute control with 100% germination of conidia after 14 h of incubation, and comparing it with the germination of conidia subjected to the different concentrations of the essential oil of *L. sidoides*, the concentrations 0.05, 0.01 and 0.25% decreased the germination of *B. maydis* by 16, 18 and 28%, respectively as depicted in Figure 3. The concentration 0.5% inhibited 90% of conidial germination. At the concentration 1% of the essential oil of *L. sidoides*, there was total inhibition of conidia (100%), evidencing a great potential of pathogen inhibition by the oil. In spite of the efficiency and due to the phytotoxicity verified up to the oil dose 1%, doses at the concentrations up to 0.5% were tested for the *in vivo* control of the disease.

Preventive and curative control of bipolaris leaf spot in maize

Concerning the preventive effects of *L. sidoides* essential oil on the progress of bipolaris leaf spot as indicated in Figure 3, the severity of the disease decreased from the concentration 0.05. A good control level was observed at the concentrations 0.1-0.25 when compared to the absolute control. At the highest concentration tested (0.5%), there was a decrease in disease severity up to 91% when compared to the absolute control.

Concerning the curative application on the presence of the disease, although it did not have the same effectiveness as the preventive control, there was a greater control level at the concentration 0.1%, with an 82% reduction in disease severity. This same concentration also demonstrated an efficacy in preventive application when there was an 84% reduction of leaf spot. The essential oil of *L. sidoides* presents a great potential as an alternative preventive and curative control that is, before or after the occurrence of pathogen infection in maize leaves.

DISCUSSION

In relation to the benefits of using the essential oil of *L. sidoides* in maize plants, emphasizing the control of bipolaris leaf spot, we proved that there is a technical feasibility. However, its active principles are chemical compounds that may cause phytotoxicity or irreversible damage to plant leaves. Thus, recommendation of use should only be made after tests to verify the sensitivity of the plant and the maximum oil concentration, taking into account the cultivar. The definition of doses represents an economically interesting practice for researchers and for future use by farmers.

The effects of *L. sidoides* essential oil on bipolaris leaf spot result from the effects of both the main compound (thymol) and the interaction of minor compounds. Moreira et al. (2011) isolated two antifungal proteins from flowers of *L. solides* that were able to inhibit the development of *Botrytis cinera*. Ferreira et al. (2018), testing the fungitoxic activity of the essential oil of *L. sidoides* on *Curvularia lunata*, found a total pathogen inhibition at the concentration 50 mg/mL. Oliveira et al. (2008) evaluated the effects of essential oils of plants of the genus Lippia on contaminating fungi during plant propagation. The authors showed that the essential oil of *L. sidoides* was effective in inhibiting the mycelial growth of all evaluated fungi (*Aspergillus niger*, *Penicillum* sp., *Fusarium* sp., and *Fusarium oxysporum*).

Studies have shown the potential of essential oils for the control of phytopathogens by both direct effects on pathogen structures and induction of phytoalexins (Schwan-Estrada and Stangarlin, 2001). Guimarães et al. (2011), evaluating the fungitoxic activity of the essential oil of *C. citratus* and citral, verified that both the oil and its components showed activity against phytopathogens. *Alternaria alternata* and *Bipolaris* sp. were the most sensitive, and *Fusarium oxyporium* was the most resistant to the action of these compounds. Seixas et al. (2011) studied the control of *Fusarium subglutinans* using essential oil of *C. nardus* L. and the compound citronellal, and found a greater inhibitory effect by citronellal.

In this work, the other oils tested did not present any inhibitory effect on *B. maydis*. These results should not be extended to other phytopathogens, which may have a different sensitivity or a greater capacity to metabolize chemical constituents. Different from the results of this work, Gonçalves et al. (2015) analyzed the chemical characterization of *L. sidoides* oil and verified the presence of carvacrol (33.2%) and 1,8-cineol (24.4%) as the main components. However, Ferreira et al. (2018) found results similar to those of this study. The authors demonstrated that primary constituents of *L. sidoides* oil included thymol (92.6%), (E) - cariophilene (2.2%), and ρ-cymene (1.1%). It is worth mentioning that the composition of essential oils may vary according to the location and time of collection, plant age, used substrates, fertilization etc. These requirements may increase or decrease the metabolites present in plants (Pina et al., 2018; Ribeiro et al., 2018).

The concentrations tested for inhibition of conidial germination of *B. maydis* were efficient; a 100% inhibition occurred at the dose 1%. Ferreira et al. (2018) explained that it is necessary to understand the mechanisms of action of essential oils against pathogenic fungi. According to Solórzano-Santos et al., (2012), the potentiality of essential oils can be attributed to their hydrophobicity, which allows them to partition with cell membrane lipids and mitochondria of bacteria, causing disturbance in cellular structures, increasing membrane permeability, which leads to the leakage of molecules essential to survival and consequently kills the bacteria (Miranda et al., 2016).

For fungi, according to Juven et al. (1994), there is a release of cell contents through changes in the permeability of fungal membranes. Therefore, the presence of the -OH group in the molecular structure, a major compound of the essential oil of *L. sidoides*, has the ability to bind to amine and hydroxylamine groups of proteins present in fungal cell membranes.

The effects of the essential oil on bipolaris leaf spot in maize plants is promising, yet preventive applications are better than curative applications. In the scientific literature, there are few reports on the effects of essential oils for the control of bipolaris leaf spot. Oil solutions of *L. sidoides* are preventively applied, forming a protective layer on maize leaves and inhibiting the germination of the conidial germinative tube. Ferreira et al. (2018) observed the same effect regarding the preventive effect of the treatment with essential oil of *Lippia*. The authors showed that the compounds of the substance might have a greater contact with *C. lunata* conidia on the imminence of germ-tube formation and the subsequent development of hyphae. Veloso (2016) concluded that the essential oil of *Morinda citrifolia* has fungitoxic properties, inhibiting the mycelial growth of *B. maydis* and *Exserohilum turcicum*, as well as the decreasing the severity of the disease.

Conclusions

In view of the results, the inhibitory effects of the oil on mycelial growth, as well as on the germination of conidia of *B. maydis*, are confirmed. It is possible to state that the essential oil of *L. sidoides* is a promising antifungal agent due to its mixture of active components for a preventive and curative control of the progress of bipolaris leaf spot.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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