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Insecticidal and fungicidal activity of *Cymbopogon citratus* (Poaceae) essential oil on *Atta sexdens* colonies

Atividade inseticida e fungicida do óleo essencial de *Cymbopogon citratus* (Poaceae) em colônias de *Atta sexdens*

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Leafcutter ants are one of the most destructive and economically important herbivores in both forest and agricultural crops, due to their abundance and wide distribution. The most used method for controlling this pest is still through synthetic formicides, due to their high efficiency. However, essential oils represent an alternative, as they are biodegradable and selective and have a toxic effect on a wide range of organisms, including leafcutter ants. The combination of insecticidal activity of a product and possible fungicidal activity constitutes an effective strategy against these insect pests, since both agents of this mutualistic interaction (both organisms are benefited) are affected, ensuring quicker collapse of the colony. The aim of this study was to evaluate the insecticidal and fungicidal activity of *Cymbopogon citratus* essential oil applied on *Atta sexdens* colonies under laboratory conditions. A nebulized spray system with three concentrations (15, 30 and 60%) and a control treatment were used to evaluate the effect of *C. citratus* on *A. sexdens* colonies. The *C. citratus* essential oils showed both insecticidal activity against the leafcutter ants (*A. sexdens*) and fungicidal activity against their symbiotic fungi. Colonies exposed to this essential oil showed considerable reduction in the weight of fungal mass, and mortality of both the queen and worker ants, which proves to be promising for the development of new insecticides for management of this insect pest.
Keywords: bioinseticide, forest crops, massal rearing.

As formigas cortadeiras são um dos herbívoros mais destrutivos e importantes economicamente nas produções florestais e agrícolas devido a sua abundância e ampla distribuição. Os formicidas sintéticos ainda é o método mais utilizado para controlar essa praga em virtude da sua alta eficiência, porém os óleos essenciais representam uma alternativa por serem biodegradáveis, seletivos e apresentarem efeitos tóxicos a uma ampla diversidade de organismos, incluindo as formigas cortadeiras. Combinar a ação inseticida com uma possível ação fungicida de um produto constitui uma ótima estratégia contra esses insetos-praga, uma vez que ambos os agentes mutualistas (ambos os organismos são beneficiados) são afetados, garantindo assim um colapso mais rápido da colônia. O objetivo dessa pesquisa foi avaliar o potencial inseticida e fungicida do óleo essencial de *Cymbopogon citratus* aplicado em colônias de *Atta sexdens* mantidas em condições de laboratório. Um sistema de nebulização com três concentrações (15, 30 e 60%) e um controle foi utilizado para avaliar o efeito do óleo essencial de *C. citratus* sobre colônias de *A. sexdens*. O óleo essencial de *C. citratus* apresentou atividade inseticida para o controle de formigas cortadeiras e fungicida para o controle do fungo simbionte. Colônias expostas a este óleo apresentaram perdas significativas no peso da massa fúngica das colônias e mortalidade das rainhas e operárias, evidenciando ser promissor para o desenvolvimento de novos inseticidas para o manejo dessa espécie-praga.
Palavras-chave: bioinseticida, plantações florestais, criação massal.

1. INTRODUCTION

Leafcutter ants in the genera *Atta* and *Acromyrmex* (Hymenoptera: Formicidae) are known in both forest and agricultural production systems as one of the most destructive herbivores, due to their abundance, wide distribution, and potential for damage [1-3]. In Brazil, *Atta* and *Acromyrmex* species are the main pests that affect *Eucalyptus* spp. and *Pinus* spp. crops [4].

Species from these genera occur in the whole Neotropical realm; however, the largest number of colonies and diversity are found in Brazil [5]. These ants infest regions from the southern United States (USA) to northern Argentina [6]. The symbiotic relation of these ants with the basidiomycete *Leucoagaricus gongylophorus* (Möller) Singer involves intense foraging and the cutting of fresh parts of plants, which serve as a substrate for the fungi, kept as the main nutritional source and habitat for the ant descendants [7].

Chemical methods are still most used for control of leafcutter ants, due to their higher efficiency. Some formicides are registered for managing leafcutter ants in Brazil, and they are applied in four distinct ways: dried powder formulations, formulations in thermal nebulization, formicides of liquid formulation, and the application of toxic baits [8]. In planted forests, toxic baits are most used for control as this method is considered safe, practical, and economical [1]. This method overcomes the challenges of ant colony architecture since the bait is transported in and uniformly distributed in the chambers by the ants themselves [2].

Botanical insecticides have been studied as an alternative to synthetic formicides, as these botanical agents are considered environmentally safer [6, 9-11], therefore, there is a constant search for alternatives that are efficient and do not cause negative impacts [12]. Essential oils represent an alternative for pest control because they have certain desirable properties, such as biodegradability, selectivity to pest targets [13-14], and toxic effects on a wide diversity of organisms [10, 15-16], including leafcutter ants [17-20].

The *Cymbopogon* genus belongs to the monocotyledon gramineous plant group, which produces the most important essential oils of the Poaceae (Graminae) family. This family contains around 180 species, subspecies, varieties, and subvarieties widely distributed across temperate and tropical regions of the world [21]. *Cymbopogon citratus* is native to Sri Lanka and the south of India. It is widely grown in tropical areas of America and Asia and contains several phytoconstituents, such as flavonoids, phenolic compounds, and terpenoids, responsible for different biological activities [22]. Among these activities, *C. citratus* essential oil has been used for pathogen and insect control [23-25].

Several studies have reported insecticidal potential for *C. citratus* essential oil agaisnt pests, such as *Trogoderma granarium* [26], *Ulomoides dermestoides* [27], *Sitophilus granarius* [28], *Anticarsia gemmatalis* [29], *Trichoplusia ni* [30], *Aphis citricola* [31], *Bemisia tabaci* [32] and *Rhipicephalus microplus* [33]. Besides the essential oil's bioactivity, which has been previously reported in phytopathogenic fungi such as *Rhizoctonia solani* and *Sclerotium rolfsii* [34].

For that reason, combining this insecticide activity with a possible fungicide activity of a product for leafcutter ant control constitutes a great strategy against these insects, since both mutualistic agents are affected, ensuring quicker collapse of the colony [35]. Therefore, the purpose of this study was to evaluate the insecticide and fungicide potential of *C. citratus* (Poaceae) essential oil applied on *Atta sexdens* (Hymenoptera: Formicidae) colonies under laboratory conditions.

2. MATERIAL AND METHODS

2.1 Essential Oil

The *C. citratus* essential oil was extracted through hydrodistillation on industrial scale and obtained from the Destilaria Bauru Ltda. company (Catanduva, São Paulo, Brazil).

2.2 Essential oil analysis

The analysis of the chemical constituents of the essential oil was carried out in the Organic Chemistry Research Laboratory of the Department of Chemistry at the Universidade Federal de Sergipe, SE, Brazil.

Chemical constituents were analyzed using a GC-FID-MS (GCMSQP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) equipped with an AOC-20i (Shimadzu) automatic injection sampler. Separations were made through Rtx®-5MS Restek a fused-silica capillary column (5% diphenyl / 95% dimethyl polysiloxane) (30 m × 0.25 mm) with 0.25 µm of film thickness in a helium (5.0) constant flow rate of 1.2 $mL.min^{-1}$. The samples (1.0 µL of the ethyl acetate solution) were injected with a split ratio of 1:10. Oven temperature began at 60°C (for 4 min) and increased at a rate of 3 $°C.min^{-1}$ up to 220°C, and then increased to 280°C at a rate of 20 $°C.min^{-1}$. The injector and interface temperatures were both at 280°C.

The mass spectrometry (MS) data (total ion chromatogram, TIC) were obtained in the full scan mode (m/z 40–550) with a scan rate of 0.3 scan/s by electron ionization (EI) at 70 eV. The temperature of the ion source was 200°C. The percentage composition of each constituent was estimated by the area of each component divided by the total area of all components in the sample. The percentage of compounds was calculated through the peak areas of GC-MS and arranged in the GC elution order.

Retention rates were obtained by injecting a mix of linear hydrocarbons (C_7-C_{30}), and compounds were identified by comparing the retention rates [36] through computerized comparison of the mass spectra obtained with those registered in the mass spectra database of three libraries of the equipment: WILEY8, NIST107, and NIST21, along with the mass spectra from literature [37].

2.3 Insects

The *Atta sexdens* colonies were obtained from mass rearing at the Forest Entomology Laboratory of the Department of Forestry Sciences at the Universidade Federal de Sergipe, São Cristóvão, SE, where they were kept under a controlled environment (25 ± 10 ºC and 70 ± 10% RH). Artificial colonies consisted of three chambers: fungus garden (1000 mL), waste chamber (500 mL), and feeding chamber (500 mL). Food was provided periodically: corn flakes and *Hibiscus* spp. flowers. The colonies were kept in the laboratory until the fungus garden achieved a volume of 1000 mL, ideal for performing bioassays.

2.4 Nebulization bioassay

The experiment was carried out at the Forest Entomology Laboratory of the Department of Forestry Sciences at the Universidade Federal de Sergipe.

A nebulization system containing three concentrations (15, 30, and 60 %) and a control treatment, with three repetitions, was used to evaluate the effect of *C. citratus* essential oil on *Atta sexdens* colonies [38].

For each colony, 5 mL of the mixed product was used, the commercial standard. The control treatment consisted of 5 mL of mineral oil alone. The concentrations were prepared from an essential oil solution in proportions of 15, 30 and 60% and an oil adjuvant (mineral oil) that were manually homogenized to form a mixture. They were then placed in the nebulization unit I-205®.

All colonies were weighed before nebulization and after each evaluation to verify change in fungus weight after the application. Evaluations occurred at 1, 2, 3, 7, 14, 28, 35, 42, 50, and 60 days after application of the treatments through the following parameters: change in fungus weight; ant intoxication; ant mortality; ant activities, including food transport; chamber shifts of the fungus; fungus in the waste chamber; presence of filamentous fungi in the symbiotic fungus; queen mortality; and general aspects of the colony [39].

2.5 Statistical analysis

All of the parameters were submitted to an analysis of variance, then the means on the colony weight were compared by the Tukey test at 5% probability and mortality data to regression analysis, being the models selected, according to the significance of the regression coefficients (t, $P < 0.05$) and the coefficient of determination (R^2). The statistical analyses were performed by the usage of SISVAR 5.6 software [40].

3. RESULTS

3.1 Essential oil Characterization

Twenty-three components were identified in description of the profile of the volatile organic compounds in *C. citratus* essential oil, corresponding to 99.4% of the total composition (Table 1). The major constituents were monoterpenes: geranial (51.9%) and neral (41.1%), the structures are presented in Figure 1.

Table 1: Chemical composition (%) of essential oil of Cymbopogon citratus.

Compounds	RI exp.*	RI lit.**	% peak area[a]
tricyclene	921	921	0.1
α-pinene	932	932	0.2
camphene	946	946	1.2
6-methyl-5-hepten-2-one	985	981	0.7
myrcene	989	988	tr
limonene	1027	1024	0.2
NI (aliphatic ketone)	1070	-	0.3
linalool	1098	1095	0.3
NI	1147	-	0.2
citronellal	1151	1148	0.2
(*Z*)-isocitral	1163	1160	0.2
rosefuran epoxide	1173	1173	tr
(*E*)-isocitral	1181	1177	0.7
neral	1240	1235	**41.1**
piperitone	1257	1249	tr
NI	1259	-	tr
geranial	1271	1264	**51.9**
isobornyl acetate	1288	1283	tr
α-copaene	1375	1374	0.2
geranyl acetate	1381	1379	0.4
(*E*)-caryophyllene	1420	1417	0.8
α-humulene	1454	1452	tr
caryophyllene oxide	1585	1582	0.4
Total			99.4%

* Experimental retention index calculated according to van den Dool & Kratz (1963).
** Retention index in the literature (Adams, 2007).
[a] NI = not identified.
[a] tr = trace, < 0.1%; % of the peak area calculated by GC-MS.

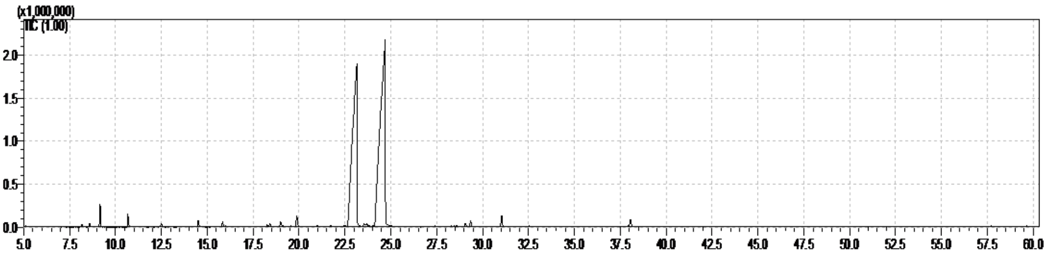

Figure 1: Representative Total Ion Chromatogram (TIC) of Cymbopogon citratus essential oil.

3.2 Nebulization Bioassay

The colony weight data did not differ between treatments and the control ($F_{3,11}$ = 0.549; df = 3; *P* > 0.663) before nebulization (Table 2). After the nebulization process, the treatments differed from the control, affecting the colonies' weight ($F_{3,11}$ = 10.788; df = 3; *P* < 0.0035) (Table 2).

Table 2: Weights of Atta sexdens (Hymenoptera: Formicidae) colonies maintained in the laboratory before and after application of Cymbopogon citratus essential oil at different concentrations.

Concentration (%)	Initial weight (g)	Weight after 60 days (g)
0	129.48 ± 10.07 Aa	118.81 ± 8.06 Aa
15	111.66 ± 10.07 Aa	056.16 ± 8.06 Bb
30	120.68 ± 10.07 Aa	076.82 ± 8.06 Bb
60	117.29 ± 10.07 Aa	074.83 ± 8.06 Bb

The treatments (mean ± standard error of the mean) followed by the same letter did not differ significantly from each other by the Tukey test (*P* < 0.05).

The *C. citratus* essential oil concentration evaluated on the *Atta sexdens* colonies was effective, since it caused colony and queen mortality (Figure 2).

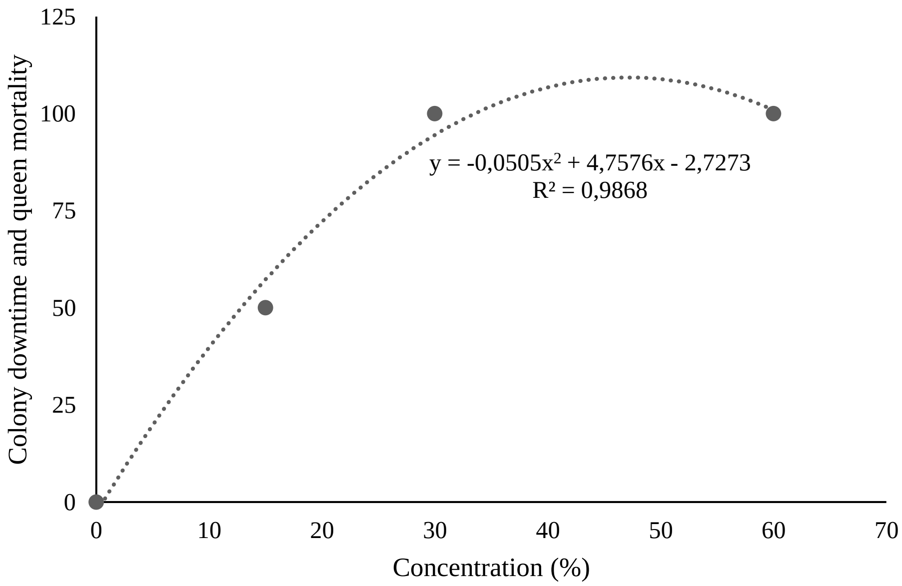

Figure 2: Insecticidal and fungicidal activity of Cymbopogon citratus essential oil at different concentrations in Atta sexdens colonies maintained in the laboratory.

The queens and colonies subjected to the treatment died in the course of 19 days, in contrast with the colonies exposed only to the control treatment, which showed no change in the observed parameters after nebulization, with no intoxication or mortality of worker ants.

After nebulization, all treatments with *C. citratus* essential oil concentrations showed intoxication and ant mortality, absence of flower-cutting and incorporation in the symbiotic fungi, reduction in the symbiotic fungal volume, and growth of filamentous fungi.

Major signs of intoxication included fungal oxidation, reduction in walking activity of the ants, transfer of immature pupae to the upper part of the fungus garden, transfer of dead ants to the waste chamber or leaving dead ants in the fungus chamber, absence of flower-cutting behavior, absence of handling the corn flakes and their further incorporation in the fungi, and increase in humidity in the fungus chamber. In addition, filamentous fungi grew in the fungus chamber. All the colonies were weighed at the end of evaluations to confirm reduction in the symbiotic fungi. There was a significant difference between the control and the other treatments ($F_{3,11} = 10.788$; df = 3; $P > 0.003$) (Table 2 and Figure 3).

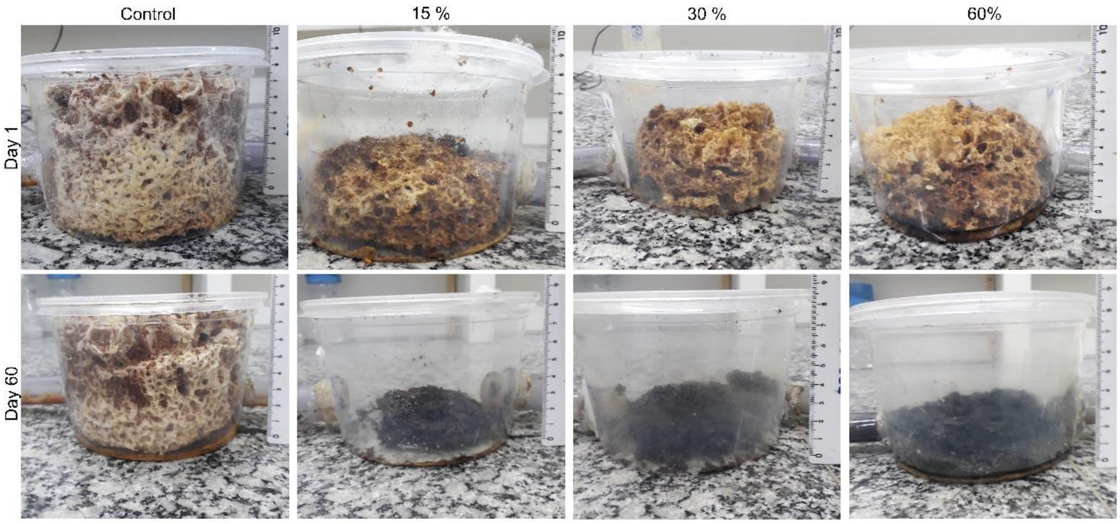

Figure 3: Colonies of Atta sexdens (Hymenoptera: Formicidae) subjected to mist application of Cymbopogon citratus essential oil, 1 and 60 days after application.

4. DISCUSSION

The chemical composition of *C. citratus* essential oil and its insecticidal and fungicidal potential on *A. sexdens* colonies under laboratory conditions were investigated in this study. The main compounds found in the essential oil were geranial and neral, compounds that have been reported in studies conducted by several researchers as the most abundant, even in plants from different regions [31, 33, 41-42]. *Cymbopogon* is an important essential oil genus of aromatic grasses of the Poaceae family [43]. *Cymbopogon citratus* (D.C.) Stapf is one of most widely distributed species, with proven larvicidal [33] and insecticidal [26] activity and antibacterial, antioxidant, antifungal, and anticarcinogenic properties [44]. These characteristics are attributed to the constituents of the essential oil extracted from the leaves, especially citral, an isometric mixture of neral and geranial [45]. Most of the insecticidal properties of the *C. citratus* essential oil are due to citral, as already shown in other studies [46-48].

The *C. citratus* concentrations applied on the *A. sexdens* colonies caused queen and colony deaths. This may be attributed to the mechanisms of essential oil activity, which may cause neurotoxic effects in insects due to terpenoids, involving several mechanisms, notably through gamma-aminobutyric acid (GABA), octopamine synapses, and acetylcholinesterase (AChE) inibition [49]. Several constituents of essential oils have been identified as inhibitors of AChE, including monoterpenes like neral, geranial, and linalool [50-52] present in the *C. citratus* essential oil used. AChE inhibition triggers an imbalance because hydrolysis of the ACh in the synapses does not occur. This causes an abnormally large accumulation of ACh, resulting in large nerve stimulation, which leads to behavioral shifts, asphyxiation, hyperactivity, and finally death [53].

Exposure to lethal doses of *C. citratus* essential oil in *Ulomoides dermestoides* Fairmaire (Coleoptera: Tenebrionidae) adults caused effects such as muscular contractions and altered movement, followed by irreparable paralysis, indicative of neurotoxic effects [27]. The effects on the nervous system of insect pests have been reported for other species, such as *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) [54], *Callosobruchus maculatus* Fabricius (Coleoptera: Chrysomelidae) [42], *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) [30], and *Sitophilus granarius* Linnaeus (Coleoptera: Curculionidae) [28] after exposure to the *C. citratus* essential oil (by contact or fumigation).

Atta sexdens colonies exposed to *Lippia sidoides* (Verbenaceae) essential oil through nebulization showed several behavioral changes, such as: fungal oxidation, reduction in ant movement, transfer of dead ants to the waste chamber, absence of flower-cutting behavior, increase in humidity in the fungus chamber, growth of filamentous fungi, just like the inactivity of the colonies and survival of the queens, through nearly the entire period of evaluation [38]. These changes were observed in this study as well. The transfer of immature pupae to the upper part of the fungus garden to avoid a contaminated environment from the essential oil and from filamentous fungi showed a disturb in equilibrium of the treated colonies from essential oils [55].

The volume of symbiotic fungi was reduced after exposure to *C. citratus*, indicating its fungicidal activity. The citral applied by fumigation on the symbiotic fungus *Leucoagaricus gongylophorus* had higher fungistatic activity. The concentration of this compound necessary to inhibit 50% of mycelial growth was around 3.3 times less when compared to the concentration of the essential oil of chemotype citral of *Myrcia lundiana*; the citral compound also showed a fungicidal effect in the 123.21 μL^{-1} concentration by fumigation [19]. In vitro experiments with the *L. gongylophorus* fungus in a growth medium containing several concentrations of azadirachtin showed less growth in fungus weight, even under low concentrations of this compound [35].

The reduction in the final weight of the fungi observed in this study and others occurs through low production of hyphae, with direct effects on the colony, since hyphae make nutrients available for queens and worker ants [35, 56]. The leafcutter ants feed on liquid nutrients found on the gongylidia, which are rich in carbohydrates and necessary to feed the queen [57].

In general, the antifungal activity of citral may be due to its capacity to penetrate fungal cells, changing mitochondria morphologically and modifying respiratory rates [58-59]. The bioactivity of the essential oil has been reported on phytopathogenic fungi [34].

Mortality of the colonies and their queens occurred in the period of 19 days in this study. In *A. sexdens* colonies treated with different indoxacarb concentrations, also under laboratory conditions, mortality rates of around 60% occurred; however, sulfluramid caused 100% mortality in the period of 21 days after application [60]. In the field, *Atta sexdens rubropilosa* treated with commercial sulfluramid products showed a reduction in nest activities three days after application of the bait. The average total paralysis of activity in the treated nests occurred in 20.55 days, while untreated nests showed normal activity [61], similar to the results found in this study.

5. CONCLUSION

This study highlights the potential of *Cymbopogon citratus* essential oil as an insecticide for control of leafcutter ants and as a fungicide for control of symbiotic fungi. This essential oil caused significant losses in colony weight and mortality of the queen and worker ants, showing promise for development of new insecticides for leafcutter ant management.

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