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Potential use of *Siparuna guianensis* essential oil for the control of *Moniliophthora roreri* in cacao

Uso potencial del aceite esencial de *Siparuna guianensis* para el control de *Moniliophthora roreri* en cacao

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Abstract

Cacao (*Theobroma cacao* L.) is a crop of globally importance on whose production around 20 million people depend directly. The crop is threatened due to the incidence of diseases caused by phytopathogenic fungi such as *Moniliophthora roreri*, causing losses of more than 80 % of the annual production. For this reason, in this study, a natural product such as the essential oil (EO) obtained from *Siparuna guianensis* was evaluated as a control alternative. The chemical composition of the EO, as well as the antifungal effect *in vitro* and *in vivo* on *M. roreri*, the causal agent of moniliasis frosty pod rot, were determined in the cacao pods located in a commercial plantation in southern Huila, Colombia. 28 compounds were detected, predominating: D-Germacrene (26.5 %), (E)-nerolidol (21.5 %), β-caryophyllene (9.3 %), elemol (8.0 %), bicyclogermacrene (7.5 %), δ-elemene (3.5 %), β-elemene (3.0 %), and α-pineno (2.4 %). At a concentration of 1000 µg ml^{-1} of EO *in vitro*, an inhibitory effect of 98 % on the mycelial growth of *M. roreri* was obtained. In the *in vivo* test at concentrations of 1000 µg ml^{-1} and 750 µg ml^{-1}, a direct relationship in the decrease of the incidence, external gravity, and internal gravity of *M. roreri* in cocoa pods was observed, using EO as fungicide. It was concluded that *S. guianensis* EO represents a great potential for the control of frosty pod rot.

Keywords: chemical composition, (D)-germacrene, (E)-nerolidol, *Theobroma cacao* L.

Resumen

El cacao (*Theobroma cacao* L.) es un cultivo de importancia mundial del que dependen directamente alrededor de 20 millones de personas. El cultivo se encuentra en peligro debido a la incidencia de enfermedades causadas por hongos fitopatógenos como *Moniliophthora roreri*, causando pérdidas superiores al 80 % de la producción anual. Por esto, en este estudio se evaluó un producto natural como el aceite esencial (AE) obtenido de *Siparuna guianensis* como alternativa de control. Se determinó la composición química del AE, así como el efecto antifúngico *in vitro* e *in vivo* sobre *M. roreri*, el agente causal de la enfermedad de moniliasis, "Frosty Pod Rot" en mazorcas de cacao ubicadas en una plantación comercial en Huila, Colombia. Se detectaron 28 compuestos, de los cuales predominaron: germacreno D (26.5 %), (E)-nerolidol (21.5 %), β-cariofileno (9.3 %), elemol (8.0 %), bicyclogermacreno (7.5 %), δ-elemeno (3.5 %) y β-elemeno (3.0 %). A una concentración de 1000 µg ml^{-1} de AE *in vitro*, se obtuvo un efecto inhibitorio del 98 % sobre el crecimiento micelial de *M. roreri*. En la prueba *in vivo* a concentraciones de 1000 µg ml^{-1} y 750 µg ml^{-1}, se observó una relación directa en la disminución de la incidencia, severidad externa y severidad interna de *M. roreri* en las mazorcas de cacao usando (AE) como fungicida. Se concluyó que *S. guianensis* (AE) representa un gran potencial en el control de la enfermedad de moniliasis.

Palabras clave: composición química, (D)-germacreno, (E)-nerolidol, *Theobroma cacao* L.

Introduction

Cacao (*Theobroma cacao* L.) is a crop with a high commercial value throughout the world, and on which many families depend on (International Cocoa *Organization*, [ICCO], 2017). In Colombia alone, 65,341 families depend on this activity, which is carried out by small-scale producers using low-level technology, especially when it comes to treating plant health issues that may arise in the crop (Minagricultura, 2021). One of the main phytosanitary issues is moniliasis frosty pod rot, caused by *Moniliophthora roreri* (*Cif.*) (Evans *et al.*, 1978), a disease that is widely spread geographically and causes losses that can exceed 80 % of the annual production, and that is considered a destructive disease difficult to control due to its pathogenicity (Gómez-López *et al.*, 2020; Lozada *et al.*, 2012). It is considered the most important cause of low production levels of cocoa crop not only in Colombia, but also in countries of Central and South America (Phillips-Mora *et al.*, 2005).

There are different integrated management strategies to control this disease, including cultural practices (Gómez-López *et al.*, 2020); biological control (Bolaños *et al.*, 2016); genetic strategies (Phillips-Mora *et al.*, 2005); as well as the use of synthetic and organic mineral preparations and synthetic chemical fungicides (González-López *et al.*, 2018; Torres-de-la-Cruz *et al.*, 2019). However, the latter, together with other agrochemicals, are not recommended due to their residuality in the plant, soil and water sources, and their danger to human health (Ferreira *et al.*, 2017) especially when the strength of bee colonies is weak. However, toxicity to bees and contamination of their products has been considered to be consequences of insecticide residues, increasing the risk of hazards to human health and to the environment. Here, we evaluated whether the application of Negramina, Siparuna guianensis Aubl., essential oil would be selective against the honey bees Apis mellifera L. without compromising the control of the wax moths Galleria mellonella L. and Achroia grisella F. The Negramina essential oil was chemically characterized and tested for insecticidal and repellent activities against A. mellifera as well as against both moth pests. The chemical composition of the essential oil revealed β-myrcene (79.7%.

Other strategies for the control of moniliasis are evaluating the use of essential oils (EO), which turn out to be an ecological option with great potential that should be tested in commercial plantations (Gómez-López *et al.*, 2020; Dávila & López 2015; Lozada *et al.*, 2012). The biosynthesis of EO occurs in aromatic plants through enzymatic reactions and can be present in leaves, roots, seeds, stems, flowers, and fruits (Rehman *et al.*, 2016), whose liquid volatile compounds are responsible for plant aromas. One family of aromatic and medicinal plants is *Siparunaceae* (de Souza *et al.*, 2022; Silva *et al.*, 2021; Lourenço *et al.*, 2018), which consists of two genera: *Glossocalyx* and *Siparuna*, both of which produce EO. The species *Siparuna guianensis* Aublet stands out within the *Siparunaceae* family, whose quantification of the components of the essential oil has already been reported (Diniz *et al.*, 2022; Souza *et al.*, 2022), as well as its various uses as insecticide and repellent (Ferreira *et al.*, 2019; Toledo *et al.*, 2019; Lourenço *et al.*, 2018), and its acaricidal (Diniz *et al.*, 2022), pharmacological (anthelmintic, anti-inflammatory, antinociceptive, anticariogenic, antimycobacterial activities) and antifungal properties (Conegundes *et al.*, 2021; Silva *et al.*, 2021; Santos *et al.*, 2020). However, there are no reports of the use of *S. guianensis* EO for the control of moniliasis frosty pod rot in cocoa. Consequently, the aim of this study was to determine the main components of *S. guianensis* leaves EO using gas chromatography coupled with mass spectrometry (GC-MS), as well as to evaluate the potential of the EO as a fungicide against *M. roreri*, both through *in vitro* and *in vivo* tests.

Materials and methods

Collection of plant material

The *S. guianensis* specimen was collected in Las Lajas ecological park (1°24'48.01"N and 75°52'51.87"O), located in the municipality of Belén de los Andaquíes (Caquetá, Colombia). The specimen was certified by the botanist Marco Correa of the herbarium Enrique Forero of the Universidad de la Amazonia (HUAZ) and registered under the voucher number 14266.

Extraction of essential oils

The leaves of the material collected from *S. guianensis* were air dried and, subsequently, the steam was distilled (8 kg of fresh material 100 L^{-1} of H_2O) for two hours in a steam distillation and extraction equipment made of stainless steel (Company JM Estrada S.A., Colombia). The EO obtained was dried in anhydrous sodium sulfate and conserved in a sealed amber bottle at 4 °C for further analysis.

GC-MS analysis

The identification of the constituents of *S. guianensis* EO was performed by gas chromatography coupled to mass spectrometry (GC-MS) using an Agilent 7890A-5975C by Agilent Technologies Inc. (Wilmington, CA) equipped with an HP-5ms capillary column (60 m × 25 mm internal diameter × 0.25 m film thickness), in stationary phase ((5 %-phenyl)-methylpolysiloxane). The oven temperature was set at 40° C for 5 minutes up to 160 °C in increments of 3 °C min^{-1}, and then at 2.5 °C min^{-1} up to 280 °C and maintained at this temperature for 10 min. The injection port temperature was 280 °C and the detector temperature, 300 °C. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}, split 1:20. Mass

spectrometry detection was performed under electron impact (EI) mode with an ionization energy of 70 eV, ionization current 60 µA, mass range 40-400 uma, and scan time 1 s. The injection volume of the essential oil was diluted in hexane (2 µL mL^{-1}), 1 µL.

A standard mixture SUPELCO™ of C8-C20 paraffins was used, ~40 mg L^{-1} for each component in hexane. The compounds present in the EO were identified by comparison with the Kovats Experimental Index (IK Exp) (Adams, 2007), obtained using the formula given by Zellner *et al*. (2008), which was contrasted with the Kovats Index reported in the literature (IK Lit), by comparing mass spectra obtained from the chromatographic profile of the EO of *S. guianenis* leaves with literature, and the spectral similarity index of the MS fragmentation pattern using the libraries NIST 2.0 and WILEY 7N, through the MSD Chem NIST 08 database and literature (Adams, 2007). A semi-quantification of each EO component was performed using the area under the curve to establish the respective relative abundance (%).

In vitro antifungal activity test

The inoculum source was a native isolate of *M. roreri* obtained from the husk of *T. cacao* with symptoms and signs of moniliasis, collected in the municipality of Belén de los Andaquíes (Caquetá, Colombia). With the isolate of *M. roreri* previously obtained according to Phillips-Mora *et al*. (2005), the antifungal activity of *S. guianensis* EO was evaluated. For this, pre-colonized agar discs of 8 mm in diameter were extracted and inoculated in Petri dishes (90 × 18 mm) containing malt extract agar enriched with V8 vegetable juice (Campbell Soup Company) in concentrations of 100, 250, 500, 750 and 1000 µg ml^{-1} of EO, and then incubated at 25 °C to allow growth of *M. roreri*. This assay was done by duplicate. The radial growth was measured daily over a period of eleven days (at which time the control colonies had covered the totality of the area of the Petri dish) by recording two perpendicular diameters of the fungal colony. Growth inhibition was calculated using the following formula, expressed as a percentage: Inhibition (%) = (R-r)/(R × 100), where r is the radius of the *M. roreri* colony in the control treatment and R is the radius of the *M. roreri* colony of the treatment under different concentrations of *S. guianensis*. In order to verify this, a completely randomized plot design with five repetitions was used, where the treatments were characterized by the concentrations of *S. guianensis* EO mentioned before and compared to negative control composed of copper oxychloride (58 % in the form of wettable powder diluted to 3 %), and a positive control (without EO).

In vivo antifungal activity test

The *in vivo* antifungal activity test was carried out in a commercial plantation in southern Huila (Colombia) (75º46´55,5 E - 1º56´55,7 W), that was composed of cacao trees grafted with clone CCN-51 in two seasons (i. August to November 2014; ii. February to May 2015). The pods selected for testing exhibited healthy conditions, were under 10 cm long (less than 2 months of growth), and in growth stage BBCH 75 (Niemenak *et al*., 2010). On the surface of each pod, the study area was previously demarcated (4 cm^2) using varnish, and the area was then inoculated with spores of *M. roreri* which was duly sprayed with distilled water, covering the ear with a paper bag inside a plastic bag, to stimulate the germination process of the pathogen (Ochoa-Fonseca *et al*., 2017). The cocoa pods were inoculated four times (one inoculation every 15 days) with the two treatments that had exhibited the greatest inhibition percentages during the *in vitro* test, using an aqueous solution composed by Tween 80 (BDH Ltd., Poole, United Kingdom) at 10 % as a solvent of the *S. guianensis* EO, through directed sprays in the demarcated area, mainly with a 300 ml high-pressure sprayer (generic).

A completely randomized plot design composed of five treatments was used: a) 1000 µg ml^{-1} EO, b) 750 µg ml^{-1} EO, c) copper oxychloride 58 % WP at 3 % as a positive control (control+), d) aqueous dilution of Tween 80 at 10 %, and d) without application of EO as a negative control (control-) with four repetitions per treatment. Each treatment consisted of 20 pods for a total of 800 pods for the *in vivo* antifungal activity test of the essential oil (20 per treatment × 5 treatments × 4 repetitions × 2 evaluation periods).

To measure the antifungal effect of *S. guianensis* EO on the artificial inoculation of *M. roreri*, destructive sampling was performed 15 days after the last application of the treatments, which coincided with the months of November 2014 and May 2015. There, the following variables were measured: a) Disease incidence (DI), defined as the percentage of diseased fruit in relation to the total inoculated fruit; b) External severity (ES), based on the external appearance of the fruit and signs of the pathogen, where: 0 = healthy fruit, 1 = oily spots, 2 = swelling and/or premature ripening, 3 = spot (necrosis), 4 = mycelium up to 25 % of the necrotic spot, and 5 = mycelium covering more than 25 % of the necrotic spot; and c) Internal severity (IS), measured as the percentage of internal necrosis in each fruit when sectioned longitudinally using the following scale: 0 = 0 %; 1 = 1-20 %; 2 = 21-40 %; 3 = 41-60 %; 4 = 61-80 %, and 5 > 80 % (Phillips-Mora *et al*., 2005).

Statistical analysis

A generalized linear model (GLM) was adjusted to analyze the effect of the fixed factor (essential oil concentration) in each *in vitro* and *in vivo* test (% inhibition, DI, ES and IS). Blocks nested during the monitoring period (temporal repetition under both laboratory and field conditions) and plots associated with the concentration of the essential

oil within blocks were included as random effects. The assumptions of normality and homogeneity of variance were evaluated using an exploratory residual analysis. Differences between concentrations of the essential oil were analyzed with Fisher's LSD post-hoc test with a significance of $\alpha = 0.05$. Statistical analyses were carried out using the statistical program InfoStat (Di Rienzo *et al.*, 2020).

Results

Chemical composition of *S. guianensis* EO

The chemical composition of *S. guianensis* EO was analyzed using GC-MS, obtaining the chromatogram observed in Figure 1 and the results presented in Table 1. In total, 28 compounds were detected, the predominant of which were D-germacrene (26.5 %); (E)-nerolidol (21.5 %); β-caryophyllene (9.3 %); elemol (8.0 %); bicyclogermacrene (7.5 %); δ-elemene (3.5 %); β-elemene (3.0 %) and α-pinene (2.6 %). In general, a predominance of sesquiterpenes (94 %) and a lesser proportion of monoterpenes (6 %) were found.

In vitro antifungal activity test

The growth of the fungus was completely inhibited during the entire incubation period at a concentration of 1000 µg ml^{-1} EO ($p < 0.001$), a similar result to that achieved with the control treatment, which in the *in vitro* experiment corresponded to Copper oxychloride (Figure 2, $p < 0.001$). However, treatments with an EO concentration of 100 to 250 µg ml^{-1} had no effect on the inhibition of *M. roreri* ($p < 0.001$).

Table 1. Mean of each of the variables evaluated in the in vivo experiment

Treatment	Incidence (%)	SE	SI
1000 µg OE ml^{-1}	0	0	0
750 µg OE ml^{-1}	0	0	0
Copper oxychloride	20	0.2±0.1	0.2±0.1
Water dilution at 10 % Tween 80	100	1.7±0.2	2±0.1
Control	100	1.4±0.2	1.3±0.1

SE: external severity, SI: internal severity, n=800 pods

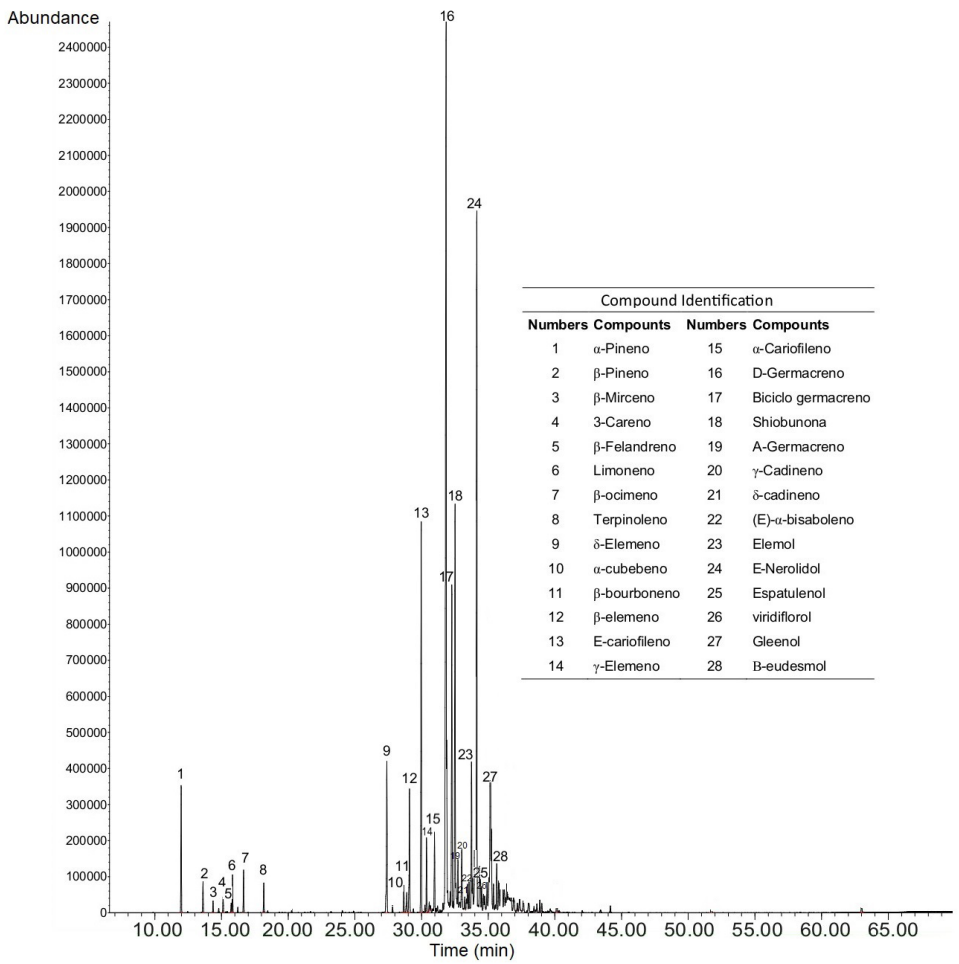

Figure 1. Chromatogram (GC-MS) of *S. guianensis* essential oil.

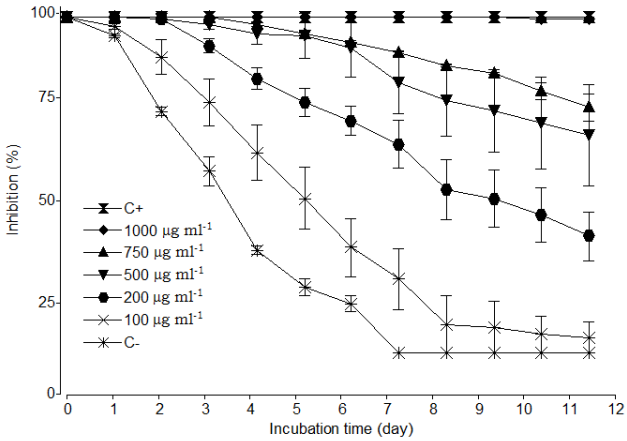

Figure 2. Effects of different concentrations of the *S. guianensis* essential oil on the colony inhibition (%) of *M. roreri* mycelial growth.

Note. C+: Copper oxychloride, C-: Uncontrolled growth. Values are means ± standard deviation (n = 5).

In vivo antifungal activity test

The *S. guianensis* EO was most effective at concentrations of 1000 µg ml^{-1} and 750 µg ml^{-1}, at which point the incidence and external severity of *M. roreri* were negligible (Table 1). As for SI, in treatments with concentrations of 1000 and 750 µg ml^{-1} EO, there were no signs of the presence of *M. roreri*. However, the 1000 µg ml^{-1} EO treatment did exhibit a slight necrosis of the external tissue of the demarcated area on the cacao pod, different from symptoms and signs of disease; this contrasted with what was found in the treatment where a surfactant was used (Tween 80 at 10 %), in which a mycelial growth of *M. roreri* occurred on the pod husk. The copper oxychloride 3 % showed 100 % inhibition of growth of *M. roreri*, while the negative control (without application of EO) did not show any inhibition as described in Figure 2. The information presented in Figure 2 corresponds to average values of the inhibition percentage of EO on *M. roreri* from the *in vivo* evaluation periods (August to November 2013 and February to May 2015) with the aim to decrease the effect of seasonality in this assay.

Discussion

GC-MS analysis and identification of components of *S. guianensis* EO

According to the GC-MS analysis of the *S. guianensis* EO, important compounds were found, for example D-germacrene (26.5 %), which was also reported within the majority of the researchers found, such as by Santana de Oliveira *et al.* (2020) with 7.6 %; Moura et al. (2020), 14.4 %; Ferreira *et al.* (2020), 9.9 %; Melo *et al.* (2017), 10 %; and Andrade *et al.* (2013), 8.7 %. This variability in chemical composition and abundance depends on several factors such as the time of year, the part of the plant and the place of collection (Diniz *et al.*, 2022).

In general, *S. guianensis* EO has a predominance of sesquiterpenes, followed by oxygenated sesquiterpenes and, to a lesser extent, monoterpenes, which is consistent with observations of Diniz *et al.* (2022), Souza *et al.* (2022), and others, who identified them as the main constituents of the volatile oils from *S. guianensis* leaves collected in Zona da Mata, Minas Gerais; Cantá, Roraima state; and Monte do Carmo, Tocantins, in Brazil.

The biological activity of EO from plants often results from the synergistic nature of their components of different polarity (Arokiyaraj *et al.*, 2022), which could probably explain the antifungal activity of *S. guianensis* EO where D-germacrene (26.5 %); (E)-nerolidol (21.5 %); (β)-caryophyllene (9.3 %); elemol (8.0 %); bicyclogermacrene (7.5 %); δ-elemene (3.5 %); β-elemene (3.0 %) and α-pinene (2.4 %) predominated.

Antifungal effects of *S. guianensis* EO on *M. roreri*

This study confirms the potential use of the essential oil of *Siparuna guianensis* as a biocontrol that inhibits the growth of *M. roreri* in cacao pods. This conclusion is supported by the results of the *in vitro* and *in vivo* tests, where the EO at concentrations of 1000 µg ml^{-1} and 750 µg ml^{-1} was more efficient, led to lower incidence of moniliasis, and less external severity. Given that the fungus *M. roreri* is a pathogen with a high incidence in cacao farms that can cause losses of over 70 % in Colombian provinces such as Caquetá (Sterling *et al.*, 2015) and Santander (Phillips-Mora *et al.*, 2005), the results achieved in this study using a biocontrol are of high importance, as they represent an opportunity to manage the disease in an integrated way. Furthermore, the results of the biocontrol are very similar to those achieved using commercial fungicides (Torres-de-la-Cruz *et al.*, 2019).

In the *in vitro* experiment, *S. guianensis* was able to control over 90 % of the formation and germination of conidia; these results are similar to those reported by Gómez-López *et al.* (2020), who used EO from *Origanum vulgare* L. (Lamiaceae), *Schinus molle* L. (Anacardiaceae), *Syzygium aromaticum* (L.) (Myrtaceae), *Thymus vulgaris* L. (Lamiaceae), Pimenta dioica (L.), Merr. (Myrtaceae) and *Cinnamomum verum* J.Presl (Lauraceae), obtaining total inhibition of the growth of *M. roreri* at concentrations higher than 500 µl L^{-1}.

In this study, the registered diameters of the mycelial growth were used to calculate the respective inhibition percentages, expressing the inhibitory effect of *S. guianensis* EO in *M. roreri* as a decrease percentage in mycelial growth; it was also observed

that the inhibitive effect of *S. guianensis* EO decreases as its concentration diminishes. This goes in hand with what was found by Lozada *et al.* (2012), who evaluated the effects of five essential oils of *Lipia Lippia origanoides*, *L. citriodora*, and *L. alba* against isolates of moniliasis (*M. roreri*), and found that the essential oils inhibited 100 % of germination and mycelial growth when applied at concentrations of 800 - 1000 µg ml^{-1}.

The results obtained here are also superior to those obtained in a similar study where the agrochemical Azoxystrobin (1250 mg L^{-1}) was used, which led to an inhibition of 78 % of mycelial growth (Torres-de-la-Cruz *et al.*, 2019).

Meanwhile, in the *in vivo* experiment of this study, *S. guianensis* exhibited the same efficiency as that achieved by the use of copper oxychloride in reducing the internal and external severity of the disease in cacao pods. These results are similar to those reported for *in vivo* tests conducted by Ochoa-Fonseca *et al.* (2017), who demonstrated that applying a calcium polysulfide and silicosulphocalcic stock completely inhibits the growth of moniliasis in cacao fruits.

As for the necrosis found in the demarcated area when the surface was impregnated with the concentration of 1000 µg ml^{-1} EO, the results here coincided with those of Aguiar *et al.* (2015): the cells died due to the exposure to the *S. guianensis* EO, a possible effect of the toxicity of the acid.

Conclusion

According to the analysis performed using the GC-MS technique, 28 compounds were found in the *S. guianensis* EO, predominating: D-germacrene (26.5 %); (E)-nerolidol (21.5 %); (β)-caryophyllene (9.3 %); elemol (8.0 %); bikegermacrene (7.5 %); δ-elemene (3.5 %); β-elemene (3.0 %) and α-pineno (2.4 %). A significant effect of *S. guianensis* EO was found for the control of moniliasis frosty pod rot in cocoa, both *in vitro* and *in vivo*, where, at a concentration of more than 750 µg ml^{-1}, an inhibitory effect of 98 % on the growth of *M. roreri* mycelium was obtained, which demonstrates the efficacy of *S. guianensis* EO at these concentrations. Finally, a direct relation between the values of external and internal severity was observed during the *in vivo* experiment on the fungicidal effects of *S. guianensis* EO on the severity of *M. roreri* in *T. cacao* pods. It was concluded that *S. guianensis* EO represents a great potential for the control of frosty pod rot, therefore, the possibility of investigating the development of bioproducts based on this EO is open, as well as its evaluation in integrated management schemes for *M. roreri*.

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