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Eucalyptus (Corymbia Citriodora) Essential Oil and Biofertilizer Present Fungistatic Effect on Fusarium guttiforme

Evaldo Morais da Silva[a], Justino José Dias Neto[b], Elonha Rodrigues dos Santos[c], Viviane Fernandes Moreira[d], Ronice Alves Veloso[e], Clovis Maurilio de Souza[f], Ildon Rodrigues do Nascimento[g], Raimundo Wagner de Souza Aguiar[h], Moab Diany Dias[i], Evelynne Urzêdo Leão[j], Gentil Cavalheiro Adorian[k], Magno De Oliveira[l], Susana Cristine Siebeneichler[m*]

[a]*Federal University of Pará. Tocantins/Cametá Campus, Rua Padre Antônio Franco - MATINHA, Cametá - PA, 68400-000. Eng. Ag MSc Vegetal Production, PhD student in Biotechnology and Biodiversity*

[b]*SQN 116, block K, CEP 70.773-110, Asa Norte Brasília DF Agronomist, MSc Plant Production, Dr Phytopathology, Post-doctorate in Phytopathology*

[c]*Faculty of the Amazon (FAMA) Address: Rua Walisson Junior Arrigo, 2043 - Bairro Cristo Rei, Vilhena - RO, 76983-496. Agronomist, MSc Plant Production, Dr Agronomia*

[d]*Federal University of Tocantins, Palmas Campus, Avenida Ns 15 Al C no 14, 109, Norte Palmas, Palmas, TO, 77001, Palmas-TO. Eng. Ag MSc Agronomy - Soil Science, Dr. Soil Science*

[e]*INTEGRA - Integrated Colleges of South America. Address: Av. Presidente Geisel, Quadra 180, Lots 01 and 02, Neighborhood: Lagoa Quente, Caldas Novas-GO; CEP: 75.690-000. Eng. Ag Me and Dr in Plant Production with an emphasis on Plant Health*

Federal University of Tocantins, Gurupi Campus, Rua Badejós lt6 7 s/n farms 69/72, rural area, CP. 66 zip code 77402970, Gurupi TO. Eng Agr. MSc in Phytotechnics, Dr. Temperate Climate Fruit

[g]*Federal University of Tocantins, Gurupi Campus, Rua Badejós, Lote 7, Chácaras 69/72. Countryside | PO Box 66. 77402-970, Gurupi-TO. Eng. Ag Dr. in Genetics and Plant Breeding*

[h]*Federal University of Tocantins, Gurupi Campus, Rua Badejós lt6 7 s/n farms 69/72, rural area, CP. 66 zip code 77402970, Gurupi TO. Eng Agr.MSc Agricultural Entomology, Dr.Molecular Biology. pockets of Prod Design. Tech and Innovative Extension of CNPq - Level 2 - CA 94 - Technology and Innovation Program for Agriculture*

[i]*Federal University of Tocantins, Gurupi Campus, Rua Badejós, Lote 7, Chácaras 69/72. Countryside | PO Box 66. 77402-970, Gurupi-TO. Eng. Ag Dr.Phytopathology*

[j]*Tocantins State University, Palmas Campus, Quadra 109 Norte, NS 15 Avenue, North Master Plan Lot, 09.77001-090 Palmas-TO. Eng. Ag MSc Plant Production, Dr. Agronomy, Post doc in Phytopathology*

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* Corresponding author.

[k]*Catholic University Center of Tocantins, Highway TO-050, Lote 7 s/n Loteamento Coqueirinho, TO, 77000-000, Palmas-TO. Eng. Ag MSc Plant Production,Dr. Sciences (Phytotechnics)*

[l]*Doctoral student in Biotechnology at the University of Vale do Taquari- Univates, Av. Avelino Talini, 171 - Bairro Universitário Lajeado/RS | Brazil | CEP 95914-014*

[m]*Federal University of Tocantins, Campus de Gurupi, Rua Badejós, Lote 7, Chácaras 69/72. Countryside | PO Box 66. 77402-970, Gurupi-TO. Eng. Ag MSc Plant Physiology, Dr. Plant Production, Post doc in Plant Microbiology and Physiology*

Abstract

The objective of this work was to verify the *in vitro* effect of eucalyptus essential oil and of the biofertilizer BIOAGRO on the mycelial growth of *Fusarium guttiforme*, the causative agent of pineapple *fusarium* disease. The experiment was carried out in Laboratories at the Federal University of Tocantins—Brazil. The bioassays were conducted in Petri dishes containing Potato-Dextrose and Agar (PDA) medium. The design used was completely randomized with 13 treatments and five replications. The treatments used were: control, containing BDA; eucalyptus oil in the following doses: 5, 10, 15 and 20 µL; the biofertilizer in doses: 0.75; 1.5; 2.25 and 3.0 mL and the mixture (biofertilizer + eucalyptus essential oil) in doses: 0.75 mL + 20 µL; 1.5 mL + 15 µL; 2.25 mL + 10 µL, and 3.0 mL + 5.0 µL. The treatments were applied in an 8 mm diameter colony of *Fusarium guttiforme* in Petri dishes, later incubated in B.O.D for fourteen days. The average colony growth per Petri dish was calculated. To perform the statistical analysis, software R version 3.5.0 (2019) was used. The isolated comparison of treatment effects was performed by contrasts, with significance compared by the Scheffé test using a 5% probability. The essential oil of eucalyptus (*Corymbia citriodora*) and the biofertilizer BIOAGRO inhibits the mycelial *in vitro* growth of pineapple fusariosis. The concentrations of 15 and 20 µL / 30mL (equivalent to 0.5 and 0.66 mL. L^{-1}) of the eucalyptus essential oil inhibit the mycelial growth of *Fusarium guttiforme*. The concentrations of 2.25 and 3.0 / 30 mL (equivalent to 75 $mL.L^{-1}$ and 100 $mL.L^{-1}$, respectively) of the biofertilizer BIOAGRO promote mycelial growth reduction. The mixture is more efficient than the isolated use of the biofertilizer and of the eucalyptus essential oil in the phytopathogen control.

Keywords: Fusariosis; Alternative control; Integrated Disease Management; Tocantins.

1. Introduction

Fusarium disease caused by the fungus *Fusarium guttiforme* Nirenberg & O'Donnell is one of the main diseases of economic importance for pineapple in Brazil. This disease promotes lesions formation in seedlings, adult plants, and fruits, presenting gummy-like exudation and causing plant death and fruit quality losses [1; 2; 3]. The estimated losses are between 30 and 40% of the marketable fruits yield, in addition to causing 20% of damage in propagation materials [4; 3]. Thus, pineapple cropping by small farmers can become difficult, as often they lack technology to control the disease. The adoption of natural products to control plant diseases is a promising alternative to reduce the indiscriminate use of pesticides [5; 6]. Plant-based products are important alternative sources for the control of fusariosis in pineapple. Among these products, essential oils present low

toxicity for mammals, being widely tested for phytopathogens control [7; 8].

According to author [1], plant extracts with antibiotic action, such as garlic (*Allium sativum*), ginger (*Zenziber officinale*), and barbatimão (*Stryphnodendron barbadetiman*), promoted the control of the pineapple fusariosis with equivalent efficiency compared to the application of chemical fungicide.

Antifungal activity of essential oils of *Cymbopogon nardus* and *Eucalypto cytriodora* on seed-associated microflora [9]. The authors argued that it may be linked to major components of the plants used, such as cintronelal, gernaniol, and citronellol, which present lipophilic character that would interact with the membranes of the microorganisms. Authors reported fungitoxic potential of copaíba and eucalyptus essential oils on phytopathogens, wherein the alternative control was as efficient as chemical control [10].

The authors described inhibitory effect on the mycelial growth of F. guttiforme in pineapple using lemongrass essential oil. In addition to the use of oils and plant extracts, the use of biofertilizers from anaerobic fermentation of cattle manure stands out as an alternative for the control of phytopathogens [11]. As reported in the literature, the use of biofertilizers inhibited the mycelial growth *of Pythium aphanidermatum, Alternaria solani, Stemphylium solani, Septoria licopersici, Sclerotinia sclerotiorum, Botrytis cinerea, Rhizoctonia solani,* and *Fusarium oxysporum* f.sp. *phaseoli* [12].

According to authors [13], the biofertilizer efficiency can be associated to its chemical and microbiological composition, because as the microbial community existing in the biofertilizer is rich and diverse, one can suggest that all the mechanisms of action of microorganisms on the pathogen can occur simultaneously, with positive effects on pathogen activity suspension.

The objective of this work was to verify the *in vitro* effect of eucalyptus essential oil and of the biofertilizer BIOAGRO on the mycelial growth of *Fusarium guttiforme*, the causative agent of pineapple fusariosis.

2. Material and methods

The experiment was conducted at the Plant Ecophysiology and Integrated Pest Management Laboratories, at the Gurupi University Campus, Federal University of Tocantins - UFT, Brazil, in 2013. The essential oil and the biofertilizer were produced at the University, and the *F. guttiforme* isolate was obtained from Embrapa Mandioca and Fruticultura from Cruz das Almas-BA.

The essential oil was extracted from eucalyptus leaves (*C. citriodora*) supplied by the forestry company Jamp. Adapted from [14], the essential oil extraction methodology was carried out by hydrodistillation in Clevenger apparatus attached to a distiller. For the extraction, 300 g of chopped leaves plus 500 ml of distilled water were deposited in a round-bottom flask with a volume of 1000 ml, for a period of 2.5 hours. The hydrolate was collected and subsequently subjected to centrifugation at 1000 RPM for 5 minutes. Then, the essential oil was collected with the aid of a micropipette, stored in an amber bottle wrapped in aluminum foil and placed under refrigeration at 4ºC to avoid its volatilization and photo-oxidation due to the terpenoid compounds. Table 1 shows the chemical composition of the essential oil of eucalyptus leaves.

Table 1: Chemical composition, concentrations (%) and Kováts index (calculated and tabulated) for the essential oil of C. citriodora.

Chemical compound	Concentration (percentage)	Kovats index	
		IKtab	IKcalc
β-pineno	2.83	980	980
1,8-Cineol	3.44	1033	1027
Isopulegol	15.54	1146	1144
Citronelal	61.78	1153	1152
β-citronelol	7.90	1228	1229
(Z)-Carriofileno	2.13	1404	1399
Total	93.62	-	-

IKtab = tabulated Kovats index / IKcal = calculated Kovats index [15]

The biofertilizer AGROBIO was prepared according to the methodology proposed of the authors [16]. Table 2 shows the analysis of the biofertilizer AGROBIO.

Table 2: Chemical analysis of the biofertilizer as reference [16].

pH	MO	Ca	K	P	N	Mg	C
	$g.L^{-1}$			$mg.L^{-1}$			%
5-6	34.69	1.59	1.2	170	631	480	0.8

The bioassays were conducted in 90x15 mm Petri dishes containing 30 mL of PDA medium (Potato-Dextrose-Agar). The design used was completely randomized with 13 treatments and five replications. The treatments used were: 1) Control containing only PDA; 2) eucalyptus oil at a concentration of 5 µl; 3) eucalyptus oil at 10 µl; 4) eucalyptus oil at 15 µl; 5) eucalyptus oil at 20 µl; 6) Biofertilizer at 0.75 ml; 7) Biofertilizer at 1.5 ml; 8) Biofertilizer at 2.25 ml; 9) Biofertilizer at 3 ml; 10) Biofertilizer + Eucalyptus oil (3 ml + 5µl, respectively); 11) Biofertilizer + Eucalyptus oil (2.25 ml + 10µl, respectively); 12) Biofertilizer + Eucalyptus oil (1.5 ml + 15µl, respectively); and 13) Biofertilizer + Eucalyptus oil (0.75 ml + 20µl, respectively).

The eucalyptus oil, the biofertilizer and the mixture of both were distributed on the surface of the culture medium with the aid of a Drigalski spatula. Then, 8 mm diameter discs of *F. guttiforme* colony were cut with the aid of a disc cutter and transferred to the center of each Petri dish containing the treatments. The Petri dishes were sealed with plastic film, identified and incubated in BOD adjusted with a 12-hour photoperiod at a temperature of 25 ± 2ºC for fourteen days [17].

Before the beginning of the experiment, the fungus *F. guttiforme* was propagated in the laboratory at the Federal University of Tocantins in PDA culture medium for 10 days in B.O.D at 25 ± 2 ºC and photoperiod of 12 hours.

To evaluate the effect of treatments on pathogen development, measurements of the radial expansion of the colony were made on two orthogonal axes, using a 20 cm millimeter rule. The average growth per plate was calculated by adding the value of the two axes divided by two. Seven evaluations were carried out at 48-hour intervals, starting on the second day and ending 14 days after the installation of the experiment.

The data were manually and electronically summarized and presented in tabular and graphical form for analysis and interpretation. The mean values of the fungal cultures' diameter were used to plot the growth curves according to the evaluation dates, and submitted to regression analysis to select the best fit model, based on the highest value of the determination coefficient. Microsoft Excel spreadsheets (2007) and software R version 3.5.0 (2019) were used to perform the statistical analyzes. Contrasts were adopted to study the isolated comparison of treatment effects, with significance compared by the Scheffé test using a 5% probability, using the software SISVAR, 2000 [18].

3. Results and Discussion

The distribution of the mean values of the fungal cultures diameter did not show adherence to normality. The data normality rejected the hypothesis of nullity by asymmetry (0.28276), kurtosis (1.0802) - based on moments of the empirical data distribution function and by the Shapiro-Wilk test (0.55569) - based on regression and correlation, all significant at $p<0.001$. Even after successive attempts to transfor the data ($X=x+c$; $X=x/k$; $X=1/(\sqrt{x})$; $X=\sqrt{x}$; $X=\log_{f_0}(x)$; $X=\text{sen}(x)$; $X=\text{arcsen}(x)$; $X=\text{arcsen}\ \sqrt{(x/100)}$), there was only an approximation to normality, not leading to variance stabilization, remaining significant and not adhering to normality.

A positive asymmetry coefficient was generated in the histogram of the frequencies of the fungal mycelium diameters (Figure 1). The distribution is heavy-tailed or is deformed in relation to the normal distribution.

Figure 1: Histogram of the frequency of the fungal mycelium diameters at fourteen days. Bars indicate the frequency of diameters in millimeters. The red curve represents the positive asymmetry coefficient.

According to author [19], the distributions have heavy tails, in the sense that they present values that are far from the location measures, with greater probability than the normal distribution, and these occur when the variance is very large. The author also reports that they may be associated with the occurrence of outliers - as

observed in Figure 2, understood as values that are very distant from those of the other observations. According to the author, although a discrepant value can result from a measurement error, it can also be a genuine result, indicating an extreme behavior of the variable, which deserves to be studied, and not removed. Kurtosis with values greater than zero indicates a tapered or centrally concentrated distribution than normal, and with tails heavier than this, in the sense that values that are very far from the average can be obtained. Asymmetric distributions, obviously, deviate from normality, with positive values indicating asymmetry on the right. These explanations are consistent with what was found in the statistical analyzes performed (Fig. 1 and 2).

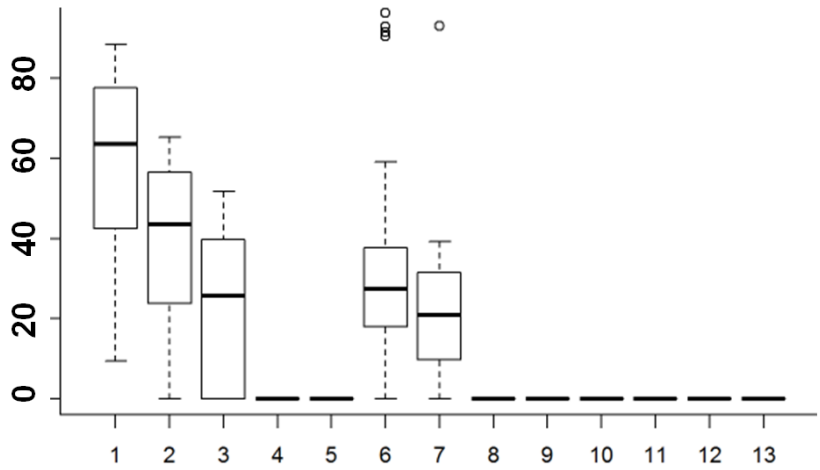

Figure 2: Box plot of the mycelial diameter of F. guttiforme at 14 days. 1 (Control); 2 (Eucalyptus oil at 5 µl) 3 (Eucalyptus oil at 10 µl); 4 (Eucalyptus oil at 15 µl); 5 (Eucalyptus oil at 20 µl); 6 (Biofertilizer at 0.75 ml); 7 (Biofertilizer at 1.5 ml); 8 (Biofertilizer at 2.25 ml); 9 (Biofertilizer at 3 ml); 10 (Biofertilizer + Eucalyptus oil at 3.0 mL + 5.0 µL, respectively); 11 (Biofertilizer + Eucalyptus oil at 2.25 mL + 10 µL, respectively); 12 (Biofertilizer + Eucalyptus oil at 1.5 mL + 15 µL, respectively) and 13 (Biofertilizer + Eucalyptus oil at 0.75 mL + 20 µL, respectively).

Still in an attempt to verify the data normality, an exploratory analysis was performed using descriptive statistics, and through this, the construction of a box plot. Among other interpretations, the use of the box plot (Fig. 2) corroborated with the statistical tests, demonstrating that the data do not fit within a normal distribution. When different from zero, the data amplitude, in the vast majority of treatments, was greater than the interquartile distance, showing that there is a strong indication of positive asymmetry, leading to non-adherence to normality.

As normality tests were performed and all data sets resulted in different distributions than normal, it was necessary to continue the investigation of data sets using non-parametric methods, as recommended by [20]. These methods are used in inference problems in which the distributions of the populations involved do not need to belong to a specific family of probability distributions, such as normal. A non-parametric statistical test is based on a model that specifies only very general conditions, and not on a specific form of the distribution, from which the sample was extracted.

The control differed significantly from all treatments (Table 3), demonstrating the effects of the treatments used.

Table 3: Statistical analysis of mycelial diameter of F. guttiforme at 14 days. The "code" sequentially identifies the treatments used (reference group). The numbers in the line to the left (comparison group) list the treatments that are statistically different from the reference group compared pairwise. For significantly different groups (p-value <0.05). For the non-significant groups, the calculated exact p-value was used.

Code	**Treatment (reference grup)**	**Significantly different (< 0,05) (comparison group)**
1	Control	2 3 4 5 8 9 10 11 12 13
2	Eucalyptus oil 5 µl	1 3 4 5 8 9 10 11 12 13
3	Eucalyptus oil 10 µl	1 2 4 5 8 9 10 11 12 13
4	Eucalyptus oil 15 µl	1 2 3 6 7
5	Eucalyptus oil 20 µl	1 2 3 6 7
6	Biofertilizer 0.75ml	4 5 8 9 10 11 12 13
7	Biofertilizer 1.5ml	4 5 8 9 10 11 12 13
8	Biofertilizer 2.25ml	1 2 3 6 7
9	Biofertilizer 3ml	1 2 3 6 7
10	Biofert. + EO 3 ml + 5µl	1 2 3 6 7
11	Biofert. + EO 2.25ml + 10µl	1 2 3 6 7
12	Biofert. + EO 5 ml +15µl	1 2 3 6 7
13	Biofert. + EO 0.75ml + 20µl	1 2 3 6 7
Code	**Treatment (reference grup)**	**Not significant - code (exact p-value) (comparison group)**
1	Control	6 (0.6761); 7 (0.1437)
2	Eucalyptus oil 5 µl	6 (0.6761); 7 (0.1437)
3	Eucalyptus oil 10 µl	6 (0.6761); 7 (0.1437)
4	Eucalyptus oil 15 µl	
5	Eucalyptus oil 20 µl	
6	Biofertilizer 0.75ml	1(0.6761); 2(0.6761); 3(0.6761); 7(0.6761)
7	Biofertilizer 1.5ml	1(0.1437); 2(0.1437); 3(0.1437); 6(0.6761)
8	Biofertilizer 2.25ml	
9	Biofertilizer 3ml	
10	Biofert. + EO 3 ml + 5µl	
11	Biofert. + EO 2.25ml + 10µl	
12	Biofert. + EO 5 ml +15µl	
13	Biofert. + EO 0.75ml + 20µl	

Non-parametric test: Wilcoxon's classification sum test. Biofert = Biofertilizer. EO = Eucalyptus oil.

The tested concentrations of eucalyptus essential oil conditioned the reduction of *in vitro* mycelial growth of *F. guttiforme* at doses 5 and 10 µL, differing significantly between them and between the control. The doses of 15 and 20 µL of eucalyptus essential oil (Treatments 4 and 5) showed 100% inhibition of mycelial growth (Fig. 2, table 3 and fig. 3).

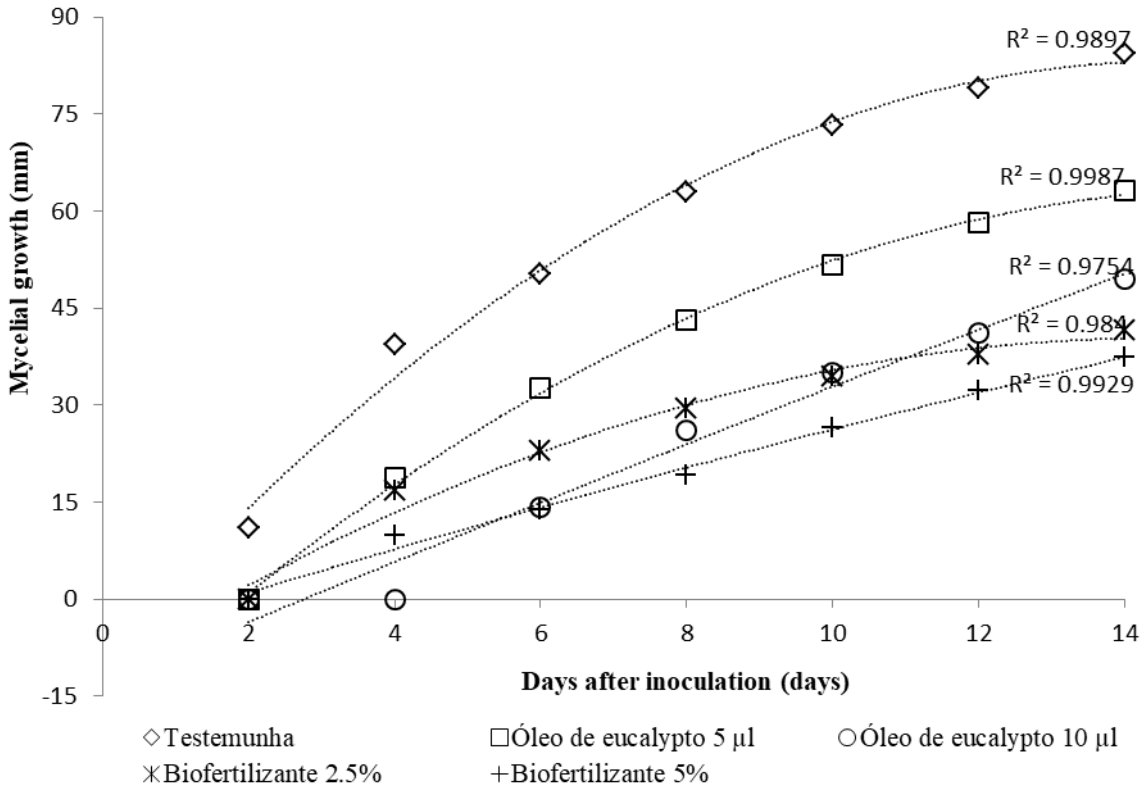

Figura 3: Mycelial growth progress curve of the treatments control, Eucalyptus oil 5 µl, Eucalyptus oil 10 µl, Biofertilizer 0.75 ml (2,5%), and Biofertilizer 1.5 ml (5%).

The adjusted regression equations demonstrate a mycelial growth rate of the fungus F. guttiforme in the control treatment of 5.95 mm day-¹, reaching 84.36 mm after 14 days of evaluation. The 10µL dose showed a mycelial growth rate of 3.62 mm day-¹, reaching 49.41 mm at 14 days (Fig. 3).

The authors [21] evaluated antifungal activity of plant extracts on the inhibition of mycelial growth of the fungus, and reported that essential oils may have the same mechanisms of action as synthetic fungicides, possibly acting on the fungus cell wall, causing leakage of cellular content.

Evaluating the effect of lemongrass essential oil and citronellal compound (C10H18O) in the control of *F. guttiforme* and verified that both essential oil and citronellal compound inhibited the growth of the fungus only in the initial four days of evaluation [11]. Differently, in this study, the doses of 15 and 20 µL (Fig. 2) completely inhibited the mycelial growth. Although lemongrass has the same major compound found in eucalyptus, the difference in results must have another reason not yet known, which gives eucalyptus oil greater potential in controlling *F. guttiforme*.

Assessed the total or partial inhibition of mycelial growth of *Glomerella cingulata* and *Colletotrichum gloeosporioides*, *in vitro*, and demonstrated the existence of biologically active compounds, with fungitoxic effect, in most medicinal and aromatic plants used in the form of aqueous extracts, decoctions and essential oils [22]. The concentration of the major compounds is determinant in the toxicity presented by the essential oil of

C. citriodora. Among the constituents of the essential oils of *C. citriodora*, citronellal stands out, with inhibitory activity previously reported by [23,9].

Eucalyptus oil has a broad spectrum of biological activity, including, antimicrobial, fungicide, and insecticide [24]. The use of eucalyptus oil is an effective alternative to synthetic pesticides as it does not cause damage to the environment [25; 26]. These classes of substances are promising sources of active ingredients due to their compounds, mainly monoterpenes [27; 28]. The mechanism of action of monoterpenes mainly involves a toxic effect on the structure and function of the cell membrane [29; 30].

Fungistatic activity of the biofertilizer strongly inhibited the mycelial growth of *F. guttiforme*, being more expressive at doses of 2.25 and 3.0 mL (Fig. 2 and 3). However, for 0.75 and 1.50 mL, there was only partial inhibition of the mycelial development of the fungus. The inhibitory effect of the mycelial development of the pathogen was directly proportional to the increase in the fertilizer dose. These results are in accordance with authors [31], who reported the effect of biofertilizers on the mycelial development and in the control of the citrus black spot (Guignardia citricarpa) and observed that the inhibitory effect on the fungus was directly proportional to the biofertilizer concentrations.

The treatments in which 2.25 and 3.0 mL of biofertilizer were used completely inhibited the development of *F. guttiforme* (Fig. 2), corroborated by results obtained by authors [12], on the effect of biofertilizers in the mycelial growth and spore germination of *P. aphanidermatum, A. solani, S. solani, S. licopersici, S. sclerotiorum, B. cinerea, R. solani, and, F. oxysporum* f. sp. *phaseoli*.

The doses 0.75 and 1.5 mL of biofertilizer (Fig. 3) has a seasonal inhibitory response, being more efficient on the first evaluation dates and less efficient on the last dates. These results demonstrate that the biofertilizer has an antagonistic effect on the development of *F. guttiforme*. Probably, this is associated with the toxic effects of the compounds present in the biofertilizer in interfering with the essential metabolic processes for the mycelial development of *F. guttiforme*. The authors [12] found that the metabolites in biofertilizers were responsible for both inhibiting mycelial expansion and also inhibiting the germination of the evaluated fungi. The authors [32] observed that the biofertilizer Agrobio decreased 61% of the germination of *urediniospores* of *Hemileia vastatrix,* and attributes this effect to the high content of copper and manganese found in this biofertilizer. A synergistic effect between the combined treatments of eucalyptus oil and biofertilizer in the control of *F. guttiforme* was observed (Fig. 2 and 3). In all combinations, there was 100% inhibition of the fungus mycelial growth. Whereas, in the doses of 5 and 10µL of the essential oil alone, there was 25.10 and 41.42% of inhibition, respectively; and for the doses of biofertilizer at 0.75 and 1.25 mL alone, there was 50.78 and 55.67% of *F. guttiforme* mycelial growth inhibition, respectively. Thus, there is a strong synergism effect between the essential oil and the biofertilizer in controlling the pathogen.

The interaction between essential oils may represent an alternative for their potential action, however, much remains to be clarified [33]. The author [11], evaluating the effect of lemongrass essential oil and citronellal compound ($C_{10}H_{18}O$) in the control of *F. guttiforme*, verified that the interaction of these two compounds provided a greater inhibitory effect of the fungus because of the synergism between the compounds. From the

estimates of the analyzed contrasts (Table 4), a significant difference between the treatments used was observed, when comparing the effect of the eucalyptus oil and of the isolated biofertilizer in relation to their combination. Demonstrating that the use of the mixture is more efficient than the isolated use of the biofertilizer and of the eucalyptus oil in the control of the phytopathogen. As the transformation of the data led to an approximation to normality, the Scheffé test was used to make estimates of the contrasts of interest and their respective significance.

Table 4: Estimates of contrasts of interest and their respective significance, for the treatments evaluated in general, Gurupi, Brazil.

Contrasts	Estimates
Eucalyptus oil VS Eucalyptus oil + biofertilizer	12.38 *
Biofertilizer VS Eucalyptus oil	-3.13 ns
Biofertilizer VS Eucalyptus oil + biofertilizer	9.25 *

Ns= not significant at 5% probability. *= significant at 5% probability by the Scheffé test.

4. Conclusions

The essential oil of eucalyptus *C. citriodora* and the biofertilizer BIOAGRO inhibit the *in vitro* mycelial growth of pineapple fusariosis.

The concentrations of 15 and 20 µL/30mL equivalent to 0.5 and 0.66 $mL.L^{-1}$, respectively, of the eucalyptus essential oil *C. citriodora* inhibit the mycelial growth of *Fusarium guttiforme*. The concentrations of 2.25 and 3.0/30mL equivalent to 75 $mL.L^{-1}$ and 100 $mL.L^{-1}$, respectively, of the biofertilizer BIOAGRO reduce the *F. guttiforme* mycelial growth. The use of the mixture is more efficient than the isolated use of the biofertilizer and of the eucalyptus oil (*C. citriodora*) in the phytopathogen control.

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