Effect of essential oils from *Eucalyptus* on the growth of aflatoxigenic species

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Abstract

In Brazil, *Eucalyptus* species has been cultivated as source of energy and cellulose. They represent the most important cultivated forest in the country. In production areas, the leaves from the trees decay on the soil as green fertilizer. In this study were evaluated pure and blends of essential oils from different species of eucalyptus trees grown in Brazil for antifungal activity against aflatoxigenic species *Aspergillus flavus* and *A. parasiticus.* These fungal species can grow and contaminate grains during the storage period under high r.h. conditions, with an eventual production of aflatoxins. Antifungal activity was evaluated by the radial growth measurement of the fungi inoculated on maize meal extract agar basic medium. The eucalyptus oils were evaluated in a contact assay and a fumigant assay using pure and blended oils. Six concentrations of pure and blended oils were evaluated at the following doses: 0, 2, 4, 16, 32 and 84 µL per 20 mL of fungi culture medium. Fungal inocula from conidia suspensions containing 10^6 spores/mL was inoculated by a needle. Glass Petri dishes were incubated for 9 days at 28^0C (± 0.3°C) in the dark. Antifungal activity was observed in all pure and blended oils, in different concentrations of contact and fumigant assay, for both fungi. *Eucalyptus stageiriana* oil and *E. stageiriana* + the hybrid *E. grandis x E. urophylla* oils blend controlled the total fungal growth at the lowest dose (20 µL).

Keywords: Essential oil; *Eucalyptus* spp.; *Aspergillus flavus*; *Aspergillus parasiticus*; Antifungal activity.

1. Introduction

Combating pests and diseases that affect the pre and post-harvest crops of interest to man has been performed almost exclusively by the use of synthetic pesticide. However, due to all the risks for the environment and human health alternative methods of control have been researched and studies have been conducted with extracts of medicinal plants to control plant pathogens (Salvadori et al., 2003). Some studies have demonstrated the effect of essential oils from plants with several biological effects and the antifungal activity has been demonstrated (Bakkali et al., 2008). In Brazil, *Eucalyptus* species has been cultivated as source of energy and cellulose. They represent the most important cultivated forest in the country. In the production areas the leaves from the trees decay on the soil as green fertilizer. One possible destination for those leaves can be the essential oil extraction, but the *Eucalyptus* essential oil market is small, so new potential uses for this oil can help to increase the market. Essential oils of eucalyptus have several functions and have been considered as an insect repellent, inhibiting germination and growth of other plants, controlling microbial activity of some fungi and bacteria, among others (Boland et al., 1991).

In this study pure and blends of essential oils from different species of eucalyptus trees grown in Brazil were evaluated for antifungal activity against aflatoxigenic species *Aspergillus flavus* and *A. parasiticus.*

2. Materials and methods

2.1. Essential oil extraction

Essential oils used in this study were from *E. grandis, E. staigeiriana, E. citriodora*, and the hybrid *E. grandis x E. urophylla.* Volatile compounds were isolated from 200 g of fresh leaves by hydrodistillation in 2 L of water for 4 hours using Clevenger-type equipment. The oil was dried over anhydrous sodium sulfate and stored under refrigeration.

2.2. Essential oil composition

Four essential oils were characterized for their composition using a gas chromatograph (Shimadzu™ 17A) fitted with a capillary column (Atm 54 ms). Temperature was programmed initially at 50ºC for 3.5 min, and then increased at a rate of 7°C min up to 100^0C. Then the rate was increased at 10°C min up to 250°C, this latter temperature maintained for 3.5 min, and helium gas as carrier gas at a flow rate of 1 mL min. Injection was in split mode at 280°C. The chromatograph was coupled to a Shimadzu™ QP5000 mass selective detector - Electron impact mass spectrometry (EIMS) and the mass spectrum was recorded in the range of 70 eV and a mass / charge ratio (m / z) of 50 to 500.

The identification of the components was made by determination of their retention indices related to those of a homologous series of n-alkanes (Dool and Kratz, 1963), and fragmentation patterns in mass spectra with those stored on the spectrometer database and the bibliography (Adams, 2001).

2.3. Antifungal assays

To evaluate the effect of essential oils against aflatoxigenic species, *Aspergillus flavus* and *A. parasiticus,* the oils were evaluated in a contact assay and a headspace volatile exposure assay for fumigant activity determination (Villela et al., 2009) with three replicates. Six different doses (0, 2, 4, 16, 32 and 84 µL per 20 mL basic medium) of the pure and blend of two essential oils from *E. grandis, E. staigeiriana, E. citriodora*, and the hybrid *E. grandis x E. urophylla* were tested. All doses of oils and blend were dissolved with 200 µL of acetone, and acetone alone was used in the control.

The basic medium was the maize meal extract agar (MMEA) as used by Marin et al. (1995). Twenty mL of this medium was placed in 9 cm diameter glass Petri dishes. The fungal species inoculum was obtained from colonies of *A. flavus* and *A. parasiticus* maintained at constant temperature of 28°C for 7 days. The inocula in water suspension were adjusted to 10^6 spores mL in water with 1.0% DMSO.

In contact assay, the doses of essential oil were added to the autoclaved and cooled but still liquid basal medium. The inoculation was performed after basal medium solidification by a needle that was immersed in the inoculum stock solution and applied in one point of the basal medium. In the headspace volatile assay the oil was applied over a round filter paper dish of approximately 2 cm diameter placed in the inner of plate lids. The inoculation was performed similarly as in the contact assay and Petri dish was incubated with the lid upside down. For both assays, the Petri dishes were sealed and incubate in the dark at constant temperature of 28°C (± 0.3°C) and the mycelial growth was evaluated after 9 d by colony diameter measurements.

Mycelium growth means of each treatment were compared by Tukey's test with probability level < 0.05 using Statistical Analysis System software (SAS, 2004).

3. Results and discussion

Several peaks were detected in the oil chromatograms but only for *E. staigeiriana* oil was possible to identify most of them (Table 1). The great number of observed compounds in this oil probably was due to the hydrodestilation time of 4 h that allowed the extraction of monoterpenes and sesquiterpenes from leaves (Viturro et al., 2003; Franco et al., 2005).

Table 1 Number of peaks detected and identified in oils extracted from Eucalyptus leaves.

Species	Peaks detected	Peaks identified (%)
Hybrid *E. grandis* x *E. urophylla*	47	21 (44.6)
Eucalyptus grandis	37	20 (54.0)
Eucalyptus staigeiriana	39	38 (97.4)
Eucalyptus citriodora	40	21(52.5)

Citronellal, α-pinene and limonene were the major components observed in the oils from *E. citriodora*, *E. grandis* and *E. staigeiriana*, respectively. In a previous study, Boland (1991) observed the same major compounds in oils of *Eucalyptus* spp. leaves but with different percentages for major components. The hybrid *E. urograndis* showed α-cimene as a major compound (Table 2).

Table 2 Volatile compounds identified and their percentage in the oils of *E. urograndis, E. grandis, E. staigeiriana* and *E. citriodora.*

Compounds	*E. urograndis*	*E. grandis*	*E. staigeiriana*	*E. citirodora*
isopropyl butanoate	0.24	-	-	-
alpha-pinene	0.66	26.97	2.7	0.04
p-cimene	41.32	0.84	1	-
limonene	2.27	-	17.66	-
1.8-cineole	4.78	10.92	3.89	0.33
gamma-terpinene	3.56	0.32	-	-
exo-fenchol	0.47	-	-	-
trans-limonene oxide	0.23	-	-	-
trans-pinocarveol	0.94	-	-	-
borneol	1.16	-	-	-
terpinen-4-ol	2.6	-	-	-
alfa-terpineol	2.82	3.58	-	-
piperitona	0.34	-	-	-
thymol	0.65	-	-	-
carvacrol	0.39	-	-	-
drima-7.9-(11)-diene	0.14	-	-	-
flavesona	4	3.8	-	-
globulol	1.59	-	-	-
sesquithuriferol	0.99	-	-	-
leptospermone	9.96	5.14	-	-
beta-eudesmol	0.83	-	-	-
campen	-	0.74	-	-
beta-pinene	-	0.06	5.56	0.05
α-cimene	-	5.91	-	-
trans-verbenol	-	0.75	-	-
camphene hydrate	-	0.4	-	-
pinocarvona	-	0.53	-	-
bornyl acetate	-	0.17	-	-
trans-beta-guaiane	-	0.1	-	-
beta-atlantol	-	0.3	-	-
khusimore	-	0.88	-	-
mirane	-	-	0.59	-
alpha-phellandrene	-	-	4.37	-
alfa-terpinene	-	-	0.18	-
beta-e-ocimene	-	-	0.21	-
gama-terpinene	-	-	1.53	-
trans-oxide linanol	-	-	0.08	-
p-mentha-2nd. 4 (8)-diene	-	-	6.68	-
linalool	-	-	1.72	-

Compounds	*E. urograndis*	*E. grandis*	*E. staigeiriana*	*E. citirodora*
1-terpineol	-	-	0.13	-
trans-p-mentha-2-en-1-ol	-	-	0.17	0.02
neoiso-3-tujanol	-	-	0.18	-
iso-isopulegol	-	-	0.23	-
neoiso-isopulegol	-	-	0.11	-
p-mentha-1 .5-dien-8-ol	-	-	1.02	-
terpinen-4-ol	-	-	1.65	-
meta-cymene-8-ol	-	-	0.28	-
alfa-terpineol	-	-	5.39	0.26
trans-piperitol	-	-	0.17	-
citronellol	-	-	2.36	14.81
neral	-	-	9.34	-
geraniol	-	-	4.31	-
geranial	-	-	10.84	-
lavandulil acetate	-	-	0.4	-
methyl geranato	-	-	5.62	-
acetate citronellyl	-	-	1.02	0.02
neril acetate	-	-	2.81	-
geranyl acetate	-	-	5.05	-
beta-z-farnesene	-	-	0.14	2.99
germacrene a	-	-	0.07	-
alfa-e.e-farnesene	-	-	0.12	-
globulol	-	-	0.17	-
espatulenol	-	-	0.43	-
z-sesquilavandulol	-	-	0.4	-
gamma-pinene	-	-	-	0.09
p-mentha-3.8-diene	-	-	-	0.04
terpinolene	-	-	-	0.07
trans-rose oxide	-	-	-	0.03
citronella	-	-	-	19.45
3-tujanol	-	-	-	6.25
format citronellyl	-	-	-	0.02
dehidro-anomadendrano	-	-	-	0.2
alfa-muuroleno	-	-	-	0.17
trans-beta-guaiane	-	-	-	0.16
alfa-cardineno	-	-	-	0.29
7-epi-alfa-eudesmol	-	-	-	0.51

Results observed in headspace volatile assay could be considered statistically the same observed for contact assay for all oils and blends (Table 3). Exceptions to this similarity were observed with *E. grandis* oil for both fungi and with *E. urograndis* oil for *A. parasiticus*. For *E. grandis* oil, the headspace volatile assay offered antifungal activity at lower doses than contact assay and for *E. urograndis* oil, contact assay showed lower efficient doses than in volatile assay.

All pure oils and blends showed some antifungal activity against *A. flavus* and *A. parasiticus* in both types of assays with different doses. The total fungal growth control for both fungi was achieved for all oils and blends with exception of *E. grandis* oil and the blend of E*. grandis* and the hybrid *E. grandis x E. urophylla* oil for *A. flavus* in the contact assay. The oil of *E. staigeiriana,* the blend of oils from *E. staigeiriana* and the hybrid *E. grandis x E. urophylla* showed an antifungal activity at the lowest dose (20 µL) (Tables 4-7).

Table 3 Effect of antifungal activity for contact or volatile activity of *Eucalyptus* spp leaves extracts against the fungi *A. flavus* and *A. parasiticus*.

Oils	*A. flavus* - P Value[1]	*A. parasiticus* - P Value
G[2]	0.0049	0.0138
U	0.3455	0.0500
S	0.1783	0.3506
C	0.1828	0.7065
G+U	0.4116	0.3506
S+G	0.8449	0.2932
C+G	0.9008	0.3910
S+U	0.3632	0.3632
S+C	0.3434	0.8449
C+U	0.2534	0.2048

[1]Level of significance 5% to compare contact versus votalite.[2] = G = *E. grandis*; C = *E. citriodora*; U= the hybrid *E. grandis x E. urophylla*; S=*E. staigeiriana*

Table 4 Mycelial growth in antifungal test for contact activity of *Eucalyptus* spp leaves extracts against *A. flavus.*

	Colony diameter (mm)[2] Dose (µL)						
Oils[1]	**0**	**10**	**20**	**80**	**160**	**420**	**Mean**
G	37. Aa	37 Aa	31 Aa	30 Aa	10 Bb	6 Ca	25.2 a
C	37 Aa	34 Aa	33 Aa	28 Aa	24 Ba	2 Cb	26.6 a
C+G	37 Aa	31 Aa	21 Bb	19 Bb	13 Bb	0 Cb	20.3 b
G+U	37 Aa	23 Bb	19 Bb	14 Cb	13 Cb	5 Da	18.4 b
U	37 Aa	26 Bb	20 Bb	17 Cb	4 Dc	2 Db	18.4 b
C+U	37 Aa	30 Ba	25 Bb	21 Bb	0 Cc	0 Cb	18.7 b
S+G	37 Aa	40 Aa	33 Aa	4 Bc	0 Bc	0 Bb	17.8 b
S+C	37 Aa	36 Aa	33 Aa	0 Bc	0 Bc	0 Bb	19.0 b
S	37 Aa	19 Bc	17 Bc	0 Cc	0 Cc	0 Cb	12.1 c
S+U	37.Aa	12 Bc	4.Cd	0 Cc	0 Cc	0 Cb	8.8 d

[1]See Table 3 for description of oils. [2]Means followed by same capital letter in the row or the same letter in column do not differ, by Tukeys multiple range test at 5% significance.

Table 5 Turkey test for treatments (oils) x dose (µl) in antifungal test for contact activity of *Eucalyptus* spp leaves extracts against *A. parasiticus*

	Colony diameter (mm)[2] Dose (µL)						
Oils[1]	**0**	**10**	**20**	**80**	**160**	**420**	**Mean**
C	36 Aa	34 Aa	31 Aa	25.Aa	23 Aa	5 Ba	25.6 a
G	36 Aa	36 Aa	29 Aa	25 Aa	10 Bb	6 Ba	23.6 a
G+U	36 Aa	22 Bb	16 Bb	12 Bb	12 Bb	5 Ca	17.1 bc
U	36 Aa	24 Bb	17 Bb	14 Bb	4 Cc	3 Ca	14.9 c
C+G	36 Aa	22 Bb	12 Cb	12 Cb	7 CDc	0 Da	18.5 b
C+U	36 Aa	26 Bb	25 Ba	21 Ba	0 Cc	0 Ca	17.7 b
S+G	36 Aa	39 Aa	35 Aa	0 Bc	0 Bc	0 Ba	17.9 b
S+C	36 Aa	39 Aa	32 Aa	0 Bc	0 Bc	0 Ba	16.5 bc
S	36 Aa	17 Bb	16 Bb	4 Cc	0 Cc	0 Ca	12.2 d
S+U	36 Aa	14 Bb	5 Cc	5 Cc	0 Cc	0 Ca	10.0 d

[1]See Table 3 for description of oils. [2]Means followed by same capital letter in the row or the same letter in column do not differ, by Tukeys multiple range test at 5% significance.

Table 6 Turkey test for treatments (oils) x dose (µl) in antifungal test for volatile activity of *Eucalyptus* spp leaves extracts against *A. flavus*

	Colony diameter (mm)[2] Dose (µL)						
Oils[1]	**0**	**10**	**20**	**80**	**160**	**420**	**Mean**
C+G	34 Aa	32 Aa	29 Aa	21 Aab	4 Bab	2 Ba	20.3 bc
S+U	34 Aa	34. Aa	36 Aa	34 Aa	2 Bb	0 Ba	23.4 a
G+U	34 Aa	41 Aa	35 Aa	26 Aa	11 Ba	0 Ca	24.5 a
C+U	34 Aa	32 Aa	31 Aa	18 Bb	4 Cab	0 Ca	19.5 cd
S+G	34 Aa	31 Aa	30 Aa	21 Bab	3 Cab	0 Ca	19.8 bcd
S+C	34 Aa	38 Aa	38 Aa	11 Bb	0 Cb	0 Ca	20.0 bcd
C	34 Aa	46 Aa	38 Aa	17 Bb	0 Cb	0 Ca	22.6 ab
G	34 Aa	30 Aa	29 Aa	16 Bb	1 Cb	0 Ca	18.5 cd
U	34 Aa	30 Aa	24 Aa	10 Bb	8 BCa	0 Ca	17.6 cd
S	34 Aa	39 Aa	30 Aa	0 Bc	0 Bb	0 Ba	172 d

[1]See Table 3 for description of oils. [2]Means followed by same capital letter in the row or the same letter in column do not differ, by Tukeys multiple range test at 5% significance.

Table 7 Turkey test for treatments (oils) x dose (µl) in antifungal test for volatile activity of *Eucalyptus* spp leaves extracts against *A. parasiticus.*

	Colony diameter (mm) [2] Dose (µL)						
Oils[1]	**0**	**10**	**20**	**80**	**160**	**420**	**Mean**
G+U	31 Aa	40 Aa	33 Aa	18 Ba	8 Cab	0 Ca	21.6 a
C+G	31 Aa	20 Bab	19 Bab	20 Ba	15 Ba	0 Ca	17.4 cde
S+U	31 Aa	36 Aa	25 Aa	28 Aa	5 Bb	0 Ba	20.9ab
U	31 Aa	28 Aa	15 Bab	7 Cb	7 Cab	0 Ca	14.8 def
C+U	31 Aa	23 ABab	18 Bab	18 Ba	2 Cb	0 Ca	15.5 cdef
G	31 Aa	29 Aa	25 Aa	19 Aa	3 Bb	0 Ba	17.8 cd
S+G	31 Aa	17 Bab	16 Bab	20 Ba	1 Cb	0 Ca	18.6 bc
C	31 Aa	30 Aa	30 Aa	9 Bb	0 Bb	0 Ba	16.6 cde
S+C	31 Aa	41 Aa	40 Aa	0 Bb	0 Bb	0 Ba	14.3 f
S	31 Aa	34 Aa	3 Bb	0 Bb	0 Bb	0 Ba	14.4 f

[1]See Table 3 for description of oils. [2]Means followed by same capital letter in the row or the same letter in column do not differ, by Tukeys multiple range test at 5% significance.

The blends of oil from different *Eucalyptus* species or hybrid showed different antifungal activity in comparison to pure oils. In some blends the antifungal activity was much lower than the one of pure oils for one of the tested fungus and better than the one of pure oils for the other. In some blends the antifungal activity was the same as observed for pure oils. Only *E. citriodora* + *E. grandis* blend in contact assay showed better antifungal activity than the both pure oils.

Even if all *Eucalyptus* oils and blends have showed antifungal activity in laboratory bioassay there is a need for further studies in the future. The oil extracted from *E. staigeiriana* was the one that showed the best potential. The major components of this oil were limonene and geranial that have confirmed the antifungal activity already observed (Adegoke et al., 2000; Lee et al., 2008). However, antifungal activity could be due to other minor compounds, individually or with a synergistic effect of several of them, as already observed by Vilela et al. (2009) and Burt (2004).

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