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FUNGICIDAL EFFECT OF *LIPPIA ALBA* ESSENTIAL OIL ON A WHITE-ROT FUNGUS

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ABSTRACT

Lippia alba is a plant that has antifungal activity against *Aspergillus, Penicillium* and *Trichoderma* genera as well as against human pathogenic microorganisms; however, there are no records on its effect on basidiomycetes which are responsible for white rot of wood. The objective of this study was to evaluate the antifungal activity of *L. alba* for the control of the white-rot fungus *Pleurotus ostreatus*. From *L. alba* leaves, essential oil (EO) was extracted by hydrodistillation, alcoholic extract (AE) was obtained through alcoholic maceration, and aqueous extract (QE) from aqueous infusion. Each extract was added to several culture media to evaluate the fungicidal effect on *P. ostreatus*. AE and QE do not have fungicidal activity. *P. ostreatus* does not survive EO when concentrations are higher than 1,0 mL L^{-1} in malt extract liquid culture medium, or higher than 9,0 mL L^{-1} in particulate sawdust solid culture medium. The physical state of the cultivation medium affects the fungicidal action of EO. In surfaces subject to greater volatility, the minimum fungicidal concentration of the EO is 25,0 mL L^{-1}. Altogether, *L. alba* EO is a potential alternative of biological control of basidiomycetes of white rottenness in wood.

Keywords: Alcoholic maceration, antifungal activity, aqueous infusion, hydrodistillation, *Pleurotus ostreatus*.

INTRODUCTION

Wood has been used as building material because of its properties such as aesthetic appearance, low density, low thermal expansion, and desirable mechanical strength, with indoor and outdoor applications (Wang *et al.* 2005). Biodegradation of wood by fungi is a problem for wooden structures and forest management. Durability is one of the most important considerations for the use of wood in construction. Poor durability has often been recognized as one of the disadvantages of wood (Wang *et al.* 2005, Cheng *et al.* 2008).

Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. (Pleurotaceae) is a white-rot basidiomycete worldwide distributed and usually grows on hardwood. *P. ostreatus* ability to degrade lignocelluloses efficiently is related with mycelial growth that allows nutrient transportation like nitrogen and iron from nutrient-rich areas to nutrient-poor areas (Hammel 1997). Also, for this fungus two types of extracellular enzymatic systems are described: the hydrolytic system and the oxidative and extracellular ligninolytic system. The first comprises hemicellulases and cellulases, that degrades polysaccharide and the last comprises lignin peroxidases, manganese peroxidases and laccases which degrades lignin and opens phenyl rings (Sánchez 2009).

P. ostreatus has been extensively studied mostly about the biochemistry of its mushroom and mycelium productions (Cohen *et al.* 2002). Furthermore, this fungus grows on different substrates without changing the morphology of the mycelium. Because of this, *P. ostreatus* is a good model to test new methodologies and prospect new compounds to control basidiomycetes.

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Synthetic fungicides are used to control basidiomycetes in the wood industry, however, they may damage the environment, reduce air quality, must not be used for indoor and can affect animals' and/or humans' health (Tascioglu *et al.* 2013). Therefore, the search for safer phytochemicals with lower environmental damage and animal toxicity is a primordial demand (Schultz and Nicolas 2000, Wedge *et al.* 2000).

One alternative of biological control is the utilization of extracts of plants that have antifungal activity (Şen and Yalçın 2010). *Lippia alba* is an aromatic plant, broadly cultivated and commercialized in Latin America such as in Mexico, Colombia, Paraguay, Brazil, Uruguay and Argentina (UNCTD, 2005). The leaves of this bush are used mainly because of its sedative, antidepressant, digestive, antihemorrhoid, anti-asthmatic and stomach-relieving properties (Hennebelle *et al.* 2008).

Hennebelle *et al.* (2008) reported that the main antimicrobial activities of *L. alba* essential oil are against *Bacillus, Lactobacillus, Enterococcus, Staphylococcus, Streptococcus, Enterobacter, Escherichia, Klebsiella, Pseudomonas, Cryptococcus* and *Trichophyton* genera. The antifungal activity against human pathogens was reported for genera such as *Candida* and *Trichophyton* for the citral and myrcene-citral chemotypes (Oliveira *et al.* 2006). Glamočlija *et al.* (2011) reported antifungal activity against *Aspergillus, Fusarium, Penicillium* and *Trichoderma* genera. Although several studies analyzed the extracts of *L. alba*, specifically the essential oil, for microbiological control in plants (Rozwalka *et al.* 2008), human health (Pessini *et al.* 2003, Tempone *et al.* 2008) and green molds (Glamočlija *et al.* 2011), although the use of this plant or its extracts have not been reported for the control of basidiomycetes. Thus, the objective of this study was to evaluate the antifungal activity of *L. alba* on the mycelium of *P. ostreatus* for the control of wood white rottenness.

MATERIALS AND METHODS

Leaves of *L. alba* (Mill.) N. E. Br. ex Britton & P. Wilson (Verbenaceae), were collected in the morning at nine from March to May, 2009 in the Medicinal Plant Garden of the *Paranaense* University - Campus of Umuarama - Brazil. Dirt was removed with tap water and the leaves were stored in polyethylene plastic bags at -20 ºC. *P. ostreatus* U2-9 from the Laboratory of Molecular Biology of the *Paranaense* University was used.

Extracts of *L. alba* leaves

L. alba leaves were submitted to hydrodistillation to obtain essential oil (OE), to alcoholic maceration to obtain alcoholic extract (AE), and to aqueous infusion to obtain aqueous extract (QE). Defrosted leaves (100 g) were transferred to a Clevenger apparatus for hydrodistillation for one hour in order to extract EO that was filtered (0,22 µm) and stored at -20 ºC. EO yield was determined by considering the EO volume extracted from 100 g of *L. alba* leaves, expressed in percentage of oil volume (mL) per leave mass (100 g) multiplied by 100. AE was prepared by macerating defrosted leaves (1:2) in alcohol 950 mL L^{-1}. The mixture was agitated manually for 15 min and was kept undisturbed for three days. AE was filtered (14 µm), filtered again (0,22 µm) and stored at ambient temperature (25 ºC) in the dark. QE was produced by infusion of defrosted leaves in water (1:10) at 100 ºC for 15 min. The infusion was filtered (14 µm), filtered again (0,22 µm) and immediately used.

Antifungal activity in liquid or solid medium

P. ostreatus mycelium was transferred to 20 g L^{-1} malt agar extract (MAE) previously autoclaved at 121 ºC for 20 min. The growth was carried out at 28 ºC during seven days. Dishes containing homogenous mycelium without sectioning were selected as inoculum.

One MAE disk (6 mm diameter) containing mycelium was transferred to the center of a Petri dish with MAE or to the well of a microplate containg 1,5 mL of 20 g L^{-1} malt extract (ME). Both culture media were previously autoclaved at 121 ºC for 20 min and cooled at ambient temperature. After five days of mycelial growth at 28 ºC, 500 µL of an aqueous mixture containing 0,1; 2,5 or 25,0 mL L^{-1} of EO or AE or QE of *L. alba* leaves was added. The aqueous mixture was manually homogenized for 15 s in liquid medium and for

the Petri dish it was distributed all over the surface on the solid medium. The negative control was done with 500 µL of autoclaved ultrapure water.

After five days, the mycelium of each treatment was transferred to MAE and kept at 28 ºC for 20 days to verify the mycelial viability. The mycelium that grew visually was considered viable.

Addition of emulsifier to the essential oil mixture

For the liquid culture medium, it was observed that for the highest concentrations of EO there was a separation of phases with oil concentration on the surface. Thus, the use of an emulsifier was evaluated to keep the water-oil mixture stable in the liquid culture medium. Therefore, a MAE disk (6 mm diameter) containing the mycelium was transferred to the well of a microplate containing 1,5 mL of ME. After five days of mycelial growth at 28 ºC, 500 µL of an aqueous mixture of EO of *L. alba* leaves with polysorbate-80 (P80) was added to obtain the final EO concentration of 0; 0,1; 0,5; 1; 2,5; 5; 10 or 25 mL L^{-1} in the liquid culture medium. For each EO concentration, the final concentrations of P80 of 0; 1; 10; 40 or 60 mL L^{-1} were tested, totalizing 40 treatments (combination of EO and P80). After five days of contact of the mixture, the mycelium in the liquid culture medium was transferred to MAE and kept at 28 ºC for 20 days, with record of mycelial viability by visual growth.

Antifungal activity of *L. alba* EO in particulate solid culture medium

Sawdust of *Eucalyptus* spp was kept in water at 90 ºC for 120 min. Next, the water excess was removed and 1,5 g of sawdust was transferred to 15 mL Falcon tube with lid and autoclaved at 121 ºC for 60 min. Then, the culture medium was inoculated with a MAE disk (6 mm diameter) containing mycelium and kept at 28 ºC for 14 days.

After the total colonization of the particulate culture medium, 1 mL of P80 solution 60 mL L^{-1} containing 0; 0,5; 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 12,5 or 15 mL L^{-1} of EO was added. The particulate culture medium was kept with EO for 1; 1,5; 3; 6; 12; 24; 48; 120 or 240 h. After each period of time, part of the colonized medium was transferred to MAE and kept at 28 ºC for 30 days to verify visually the mycelium growth viability. The mycelium that grew was considered viable. All experiments were replicated ten times.

RESULTS AND DISCUSSION

L. alba EO extraction yield was 1,5 mL kg^{-1} of leaves. This value is within the range of EO extraction yield for this plant that varies from 0,8 mL kg^{-1} (Shukla *et al.* 2009) to 16,0 mL kg^{-1} (Hennebelle *et al.* 2006). *L. alba* EO can vary in quantity and in composition according to climate, soil composition, plant organ, age and vegetative cycle stage (Hennebelle *et al.* 2006). Hennebelle *et al.* (2008) reported as well that yield and composition of *L. alba* EO are highly influenced by chemotypes. This variability of yield and chemical composition of EO causes difficulty for the cultivation and commercialization of this plant (Manica-Cattani *et al.* 2009). One way to minimize this problem would be the use of micropropagation of *L. alba* clones, standardization of cultivation and extraction methods to obtain chemical uniformity (Gupta *et al.* 2001, Biasi and Costa 2003).

The addition of 1,0 mL L^{-1} of EO in ME had a fungicidal effect on *P. ostreatus* and in MAE, the inhibition only occurred with the addition of 25,0 mL L^{-1} of EO (Table 1). EO are easily volatilized (Guenther 1948) and the difference of the fungus inhibition in function of the physical state of the culture medium may be related to the greater evaporation area in the Petri dishes with consequent reduction of EO concentration and fungicidal activity.

Table 1. Mycelial viability of *Pleurotus ostreatus* grown on liquid or solid malt extract culture medium for five days at 28 ºC, followed by the addition of different concentrations (mL L^{-1}) of essential oil (EO), alcoholic extract (AE) or aqueous extract (QE) of *Lippia alba* leaf extract.

Extract concentration in culture medium (mL L^{-1})	Solid culture medium with			Liquid culture medium with		
	EO	AE	QE	EO	AE	QE
0	+	+	+	+	+	+
1	+	+	+	-	+	+
2,5	+	+	+	-	+	+
25	-	+	+	-	+	+

Absence (-) or presence (+) of mycelial growth in the viability test.

Table 1 shows the results of fungicidal effect on *P. ostreatus* in MAE or ME with addition of different concentrations of EO, AE or QE of *L. alba* leaves. AE and QE did not have any fungicidal activity at any concentration of culture medium. However *L. alba* EO presented fungicide activity for the fungus.

Alcoholic and aqueous extract of *Acacia mollissima, Schinopsis lorentzii* and *Pinus brutia* are described as fungicidal against rot-wood fungi (Tascioglu *et al.* 2013). The main compounds in these extracts are tannins, flavanoids, lignans, stilbens, terpenes and terpenoids also described as fungicidal. In our work, based on the lack of fungicidal activity of AE and QE, the extracts composition was undetermined. For the non-volatile compounds of *L. alba* leaves are described three iridoids, geniposidic acid, caryoptoside, 8-epi-loganin and mussaenoside. Also are described phenyletanoid/phenylpropanoid, flavonoid glycosides and biflavonoids (Hennebelle *et al.* 2008). Thus, future studies should investigate the composition of the extracts and even different ways of extraction. Moreover, other plant parts - as roots and stems - could also be evaluated. Because only EO showed a fungicidal effect (Table 1), it was selected for the subsequent experiments.

The addition of P80 to the liquid culture medium did not cause any change in fungal growth surviving (Table 2). However, the EO antifungal activity was affected by the concentration of emulsifier added. When EO was added to 0,1 mL L^{-1} of liquid culture medium, there was no inhibition, except for the treatment without P80 addition (Table 2). This indicates that P80 provided higher homogeneity to the liquid culture medium with EO, decreasing the anaerobic condition caused by the EO accumulation on top of the culture medium. To obtain a higher emulsifying effect in the liquid culture medium the concentration of 60,0 mL L^{-1} of P80 was chosen for the subsequent experiments

Table 2. Mycelial viability of *Pleurotus ostreatus* grown on liquid malt extract culture medium (20 g L^{-1}) for five days at 28 ºC, followed by the addition of different concentrations of *Lippia alba* essential oil with different concentrations of polysorbate-80.

Essential oil concentration in liquid culture medium (mL L^{-1})	Polysorbate-80 concentration in liquid culture medium (mL L^{-1})				
	0	1	10	40	60
0	+	+	+	+	+
0,1	-	+	+	+	+
0,5	-	-	-	+	+
1	-	-	-	-	-
2,5	-	-	-	-	-
5	-	-	-	-	-
10	-	-	-	-	-
25	-	-	-	-	-

Absence (-) or presence (+) of mycelial growth in the viability test.

P. ostreatus is mainly a saprophyte that decomposes wood and, therefore, simulating the development of this fungus on sawdust is appropriate to evaluate the antifungal activity of *L. alba* EO under conditions close to the natural ones. In this culture medium, the fungicide effect occurred at EO concentration over 8 mL L^{-1} with 60 mL L^{-1} of P80 exposed for any time (Table 3).

Even the minimal exposure of one hour of the EO caused a fungicide effect which is promising for the use on the wood industry because there was no temporary fungistatic effect. It is verified that the EO concentration is the main factor in the fungicidal effect because lower or equal EO concentrations to 8,0 mL L^{-1} had neither fungicidal nor fungistatic effect, even after 240 h of exposure (Table 3).

Table 3. Mycelia viability of *Pleurotus ostreatus* grown on particulate sawdust culture medium for 14 days at 28 ºC followed by the addition of different concentrations of *Lippia alba* essential oil with 60 mL L^{-1} polysorbate-80 for different periods.

Essential oil concentration in the culture medium (mL L^{-1})	Time (h)								
	1	**1,5**	**3**	**6**	**12**	**24**	**48**	**120**	**240**
0	+	+	+	+	+	+	+	+	+
0,5	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+
9	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
12,5	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-

Absence (-) or presence (+) of mycelial growth.

The chemical analysis of *L. alba* EO used in this work was characterized by gas chromatography and mass spectrometry analysis, and the structure of the main compounds were confirmed by hydrogen-1 and carbon-13 nuclear magnetic resonance spectroscopy and published on Glamočlija *et al.* (2011). The main found components were geranial (50,94%) and neral (33,32%) and 97,69% of the EO chemical constituents were identified and classified as citral chemotype (Glamočlija *et al.* 2011). Geranial seems to be the main fungicidal component of *L. alba* EO. According to Shukla *et al.* (2009), geranial from *L. alba* caused inhibition of growth on 76% of 17 tested fungi, whereas, at the same concentration, neral component presented inhibition only on 22%. It indicates that the high geranial concentration of *L. alba* EO may explain the EO antifungal activity.

As reported by Burt (2004) an important characteristic of EO and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and making them more permeable (Sikkema *et al.* 1994). Leakage of ions and other cell contents can then occur. The EO mechanism of action is similar to other phenolics; this is generally considered to be a disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport and coagulation of cell contents (Denyer and Hugo 1991, Sikkema *et al.* 1995, Davidson 1997). EO components also appear to act on cell proteins as ATPases enzymes embedded in the cytoplasmic membrane (Knobloch *et al.* 1989). Therefore, different EO compounds can act differently and affect the antifungal activity. Thus, it is important to develop studies that evaluate the antifungal activity of isolated EO compounds. Such prospects may direct the synthesis of less harmful compounds to health and environment. Moreover, the EO composition may vary according to the genotype and phenotype of the plant (Silva *et al.* 2006), directing the control of culture conditions to maintain the composition and antifungal activity of *L. alba* EO.

The physical state of the culture medium is another important factor of the fungicidal activity; therefore, lower EO concentrations (0,5 to 1,0 mL L^{-1}) were needed for the liquid culture medium whereas higher concentrations (9,0 to 25,0 mL L^{-1}) were needed for the solid culture medium (Table 1, 2 and 3). Particulate solid sawdust culture medium has natural wood capillaries (Segerholma and Claessona 2008) where the mycelium can grow. These capillaries provide physical protection to the mycelium and make the contact with EO difficult. Thus, a higher EO concentration is needed to cause the antifungal effect regarding the liquid culture medium. The development of applications for *L. alba* EO is still not well-explored. Rao *et al.* (2000) reported antifungal activity against sugarcane pathogens, Park *et al.* (2007) and Lee *et al.*(2008) reported bioactivity against phytopathogens and dermatophytes, and Glamočlija *et al.* (2011) reported antifungal activity against green moulds (*Trichoderma* ssp., *Fusarium* ssp., *Aspergillus* ssp. and *Penicillium* ssp.) genus Geels *et al.* (1988) reported that *Trichoderama, Aspergillus* and *Penicillium* species can cause mushroom production losses and suggested the use of *L. alba* EO instead broad spectrum systemic fungicides to control those green moulds. Glamočlija *et al.* (2011) indicates *L. alba* EO to control green molds with minimum inhibitory concentration (MIC) in a range of 0,3 to 1,25 mg mL^{-1} and minimum fungicidal concentration (MFC) in a range of 0,6 to 1,25 mg mL^{-1}. Results obtained in our study under similar conditions with liquid media showed that just 1,0 mL L^{-1} (circa de 0,5 mg mL^{-1}) of *L. alba* EO has fungicidal activity on *P. ostreatus.*

In this work we showed that the *L. alba* essential oil may be further explored to develop wood decay preservative agents or fumigants. Some possibilities are described like: isolate the main fungicidal compound and encapsulate the volatile to prevent evaporation and allow the release of the active compound only with humidity, which plays an important role in promoting fungal growth (Voda *et al.* 2003).

The biodegradation of wood by basidiomycetes is a serious problem for increase the use of wood (Cheng *et al.* 2008). The main basidiomycete recognized worldwide as major wood decay fungi are white rot fungi such as *Coriolus versicolor, Lenzites betulina, Pycnoporus coccineus, Trichaptum abietinum, Oligoporus lowei, Schizophyllum commune and Pleurotus ostreatus* (Wang *et al.* 2005, Cheng *et al.* 2008, Yen and Chang 2008, Yen *et al.* 2008, Hsu *et al.* 2009, Tascioglu *et al.* 2013) and brown-rot fungus such as Coniophora puteana (Voda *et al.* 2003), *Fomitopsis palustris, Gloeophyllum trabeum, Laetiporus sulphureus, Antrodia taxa, Fomitopsis pinicola* and *Phaeolus schweinitzii* (Xu and Goodell 2001, Wang *et al.* 2005, Yen and Chang 2008, Cheng *et al.* 2008, Tascioglu *et al.* 2013). In this work we use the white-rot *P. ostreatus* - that prefer hardwoods - as a model for *in vitro* tests. Future experiments should expand the use of *L. alba* EO as fungicidal to other important white-rot fungi and also to brown-rot fungi to protect softwoods.

CONCLUSIONS

The alcoholic and aqueous extracts of *L. alba* leaves have no antifungal activity against *P. ostreatus.* The essential oil has fungicidal effect at concentrations that are higher than 1,0 mL L^{-1} in liquid culture media of malt extract or higher than 8,0 mL L^{-1} in particulate sawdust solid culture media. The physical state of the culture medium affects the fungicidal activity of essential oil. On surfaces where the essential oil is more volatile, the minimum concentration for fungicidal activity is 25,0 mL L^{-1}. *L. alba* essential oil is a potential alternative for biological control against wood white-rot fungus.

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