Ciência Rural, Santa Maria, v.47: 05, e20160780, 2017 http://dx.doi.org/10.1590/0103-8478cr20160780
ISSNe 1678-4596
CROP PROTECTION

Essential oils in the control of dry bubble disease in white button mushroom

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ABSTRACT: *Lecanicillium fungicola, which causes Dry bubble disease, induces infections and inflicts major losses in champignon production. The control can be managed through measures of hygiene and use of fungicides; however, in Brazil there are no registered products. This study aimed to estimate the influence of various essential oils extracted from Melissa officinalis, Thymus vulgaris, Origanum vulgare, Eucalyptus globulus, Cinnamomum zeylanicum and Syzygium aromaticum on the* in vitro *development and their uses. Therefore, analysis was performed of the L. fungicola isolates* in vitro *and the best oils were tested* in vivo*. Besides, the Agaricus bisporus - L. fungicola interaction was confirmed by scanning electron microscopy (SEM). Cinnamon and clove oils in concentrations of 0.4% and thyme oil of 0.8% were identified as good growth inhibitors of the pathogenic mycelium. Effective inhibition of the conidial germination was seen in all concentrations by cinnamon oil, and by clove and thyme oils only at 0.4% and 0.8%, respectively. When the essential oils were applied post-infestation in the* in vivo *experiments the incidence of the disease in the mushrooms was much lower. From the SEM it was clear that 19 hours after the inoculation of A. bisporus with L. fungicola, the spores had already completely germinated, revealing the presence of the infection. Therefore, the findings of this study indicated that the oil extracts of cinnamon, clove and thyme are potential and efficient alternatives in the control of dry bubble disease.*
Key words: *dry bubble disease, Lecanicillium fungicola, essential oils, SEM, alternative control.*

Óleos essenciais no controle da doença bolha seca em champignon

RESUMO: *A doença bolha seca, causada pelo fungo Lecanicillium fungicola, provoca infecções graves que resultam em perdas significativas na produção de champignon. O controle se dá através de práticas sanitárias e do uso de fungicidas, porém no Brasil não há produtos registrados. Desta forma, este estudo objetivou avaliar o efeito de óleos essenciais extraídos de Melissa officinalis, Thymus vulgaris, Origanum vulgare, Eucalyptus globulus, Cinnamomum zeylanicum e Syzygium aromaticum sobre o desenvolvimento* in vitro *de isolados de L. fungicola e aplicação dos melhores óleos em teste* in vivo*. Além disso, a interação entre Agaricus bisporus e L. fungicola foi verificada por meio de microscopia eletrônica de varredura (MEV). Os óleos de canela e cravo a partir da concentração 0.4% e tomilho a 0.8% inibiram o crescimento micelial do patógeno. Já a germinação de conídios foi inibida pelo óleo de canela em todas as concentrações, bem como pelos óleos de cravo e tomilho, a 0.4% e 0.8%, respectivamente. No experimento* in vivo*, a aplicação dos óleos essenciais pós-infestação apresentou menor incidência da doença nos cogumelos produzidos. A MEV revelou que após 19 horas da inoculação de L. fungicola em A. bisporus, os esporos já estavam completamente germinados, o que indicava a ocorrência da infecção. Assim, os resultados deste trabalho sugerem que os óleos de canela, cravo e tomilho são alternativas potenciais para o controle da doença bolha seca.*
Palavras-chave: *bolha seca, Lecanicillium fungicola, óleos essenciais, MEV, controle alternativo.*

INTRODUCTION

Dry bubble disease, among the infections affect most severely white button mushroom (*Agaricus bisporus*) production, and is caused by a fungus, *Lecanicillium fungicola* (Preuss) Zare & W. Gams (ZARE & GAMS, 2008), inflicting an estimated 2 – 4% loss per year (BERENDSEN et al., 2012). Control measures that appear effective against dry bubble disease include implementing sanitary practices and spraying fungicides (GEA et al., 2005). However, to control the pests and diseases in mushrooms, while stringent specifications have been laid down regarding the use of chemical products, reports on tolerance to fungicides have triggered the urgency to develop disease resistant strains via genetic enhancement, as an environmentally sustainable and successful strategy (LARGETEAU & SAVOIE, 2010).

Received 08.20.16 Approved 01.18.17 Returned by the author 03.06.17
CR-2016-0780

Brazilian Ministry of Agriculture has no registered disease control products for the commercial cultivation of *A. bisporus* (MAPA - IN 18, 2009); however, the producers apply fungicides daily, following the recommendations made in other countries, particularly the United States. Some modern facilities permitting prophylactic measures are considered onerous; therefore, their implementation is limited only to those mushroom producers possessing high investment capacities (ZIED et al., 2015).

In light of this, essential oils appear to potentially hold out promise as a feasible alternative to fungicides that can control both pests and diseases, and can safely be used to cultivate organic products (MAPA-IN 18, 2009; SEIXAS et al., 2011). Besides, essential oils are easily obtainable and inexpensive, as well as highly advantageous as they involve minimal issues of toxicity to humans and the environment as against synthetic products (PERINI et al., 2013).

The aim of this study was to determine the effects of the essential oils of *Cinnamomum zeylanicum* (cinnamon), *Eucalyptus globulus* (eucalyptus), *Melissa officinalis* (lemon balm), *Origanum vulgare* (oregano), *Syzygium aromaticum* (clove) and *Thymus vulgaris* (thyme) on the development of *in vitro* and *in vivo* *L. fungicola* isolates and evaluate the ultrastructural pathogen-host interaction.

MATERIALS AND METHODS

The *L. fungicola* isolates, termed LF.1 and LF.2, were drawn from the culture collection of the Laboratory of Edible Mushrooms, Biology Department of the UFLA. They were maintained in the PDA (potato-dextrose-agar) medium at 25ºC. The essential oils extractions were done using dry vegetable materials through hydrodistillation employing a modified Clevenger apparatus adapted to a round bottom flask (4 liters) for 2 hours (FARMACOPEIA BRASILEIRA, 2010).

Evaluation of the fungicidal activity of the essential oils on the mycelial development of the LF.1 and LF.2 strains was done with 0.2%, 0.4%, 0.8% and 1.6% v/v concentrations (TANOVIĆ, et al., 2009). Each essential oil was pre-diluted in a sterile commercial milk powder solution ($10g.L^{-1}$). The 1ml of each concentration was added to 9mL of PDA culture medium at 45°C mean temperature. At the center of each plate a 5-mm diameter disk was placed with mycelium of the monosporic culture grown prior for 7 days in PDA. The plates were then incubated at 25°C (AQUINO et al., 2014). Estimations were conducted through daily measurements of the colony diameters until day ten, after which, the Mycelial Growth Index (MGI) was determined, applying the equation adopted by ABREU et al. (2008).

Antifungal effect evaluation of the essential oils on the conidial germination of the LF.1 and LF.2 strains were done in the same concentrations of the six oils as in the prior assay. Thus, to the plates containing 9mL of 2% agar-water medium a mixture of 1mL of sterile commercial milk powder solution ($10g.L^{-1}$) with essential oil was added. The spore assay was obtained earlier by the addition of 10mL of sterile water to the monosporic culture of each strain. The mycelium was scraped with the Drigalski loop, after which it was filtered and quantified in the Neubauer chamber. Spore suspension was then adjusted to a concentration of 10^6ml^{-1} conidia, and 100µL of the *L. fungicola* spore suspension was used to inoculate each plate. Plates were then incubated at 25°C. Next, under a light microscope 50 spores per area ($2cm^2$) were counted, as well as the percentage of conidia which germinated after 20 hours of incubation. The assay involved three replicates per treatment, for a total of 150 spores. Positive antifungal activity was achieved when there was no evidence of growth; spores showing germ tube emission were regarded as having germinated, indicating negative antifungal activity (ITAKO et al., 2008).

Besides the treatments involving the six essential oils, an inhibition standard constituted by the Sportak fungicide ($160µL.mL^{-1}$ for LF.1 and $320µL.mL^{-1}$ for LF.2) was used, a control made up of the culture medium and 1mL of powdered milk solution ($10g.L^{-1}$) and an absolute control.

When tested *in vivo*, the lowest oil concentrations that successfully achieved total inhibition in the *in vitro* pathogen tests were used. "Sítio dos Micélios", a company in Barbacena-MG provided the commercial compound for the mushroom production. For treatment with the essential oil, 2kg blocks were assembled with 5 replicates, besides the controls made up of the Sportak fungicide and absolute control. A covering layer of dystroferric red latosol and calcitic limestone 2:1 (v/v) was added after the substrate was completely colonized by *A. bisporus*. The soil surface was ruffled seven days after the cover layer was added. After total colonization, temperature adjustments to 18±1°C were made and the relative humidity was maintained at 80%, throughout the mushroom growth cycle. After two days, a spore suspension (10^6mL^{-1} conidia) from each strain of *L. fungicola* was sprayed (5mL per block) onto the surface of the compound cover layer, as a

post-infestation treatment measure. The treatment was completed by adding 5ml of a spray solution of the essential oils by block (TANOVIĆ et al., 2009). Pre-infestation treatment involved application of the essential oils on day one and the spore suspension on day two (REGNIER & COMBRINCK, 2010). Mushrooms were then harvested and weighed. They were distinguished as healthy or diseased for further calculation of the incidence of the disease and productivity of the mushroom.

The pathogen-inoculated basidiocarps were hand-chopped into pieces ($2cm^2$) using a scalpel and studied under a scanning electron microscope. Sample collection was done at 13, 16, 19 and 23 hours post inoculation. Fixing of these samples was then done in a modified Karnovsky's solution (2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.05M sodium cacodylate buffer, 0.001M CaCl2, pH 7.2) and treated according to the method of ALVES et al. (2008). Images produced were recorded and studied using the Photopaint software of the Corel Draw X6 package.

For both the *in vitro* tests, a completely randomized experimental design was adopted, in a factorial scheme (oils x concentrations x strains) involving three replicates. Data were submitted to the analysis of variance and the Scott-Knott test was used to compare the means ($P<0.05$). For the *in vivo* test a randomized complete block design was selected for the experiment, in a factorial scheme (oils x strains x mode of application) which included five replicates. Data were submitted to the analysis of variance and the Tukey test was used to compare the means ($P<0.05$). The R Core Development Team software was employed for the data analysis (VENABLES & SMITH, 2007).

RESULTS AND DISCUSSION

On analysis of all the treatments, the essential oils of lemon balm, eucalyptus and oregano induced a decrease in the variables analyzed, based on the concentration used; unfortunately, these oils were inadequate to control the *L. fungicola* (Table 1 and 2). In this context, BARRERA-NECHA et al (2008) verified that eucalyptus oil could not inhibit the mycelial growth of *Colletotrichum gloeosporioides* in various concentrations. However, SOYLU et al (2006) reported contrasting results, as they confirmed the antifungal activity of the oregano essential oil, evident from the complete inhibition of the *Phytophthora infestans* mycelial growth. Similarly, TAGAMI et al (2009) revealed that the raw extract of lemon balm

Table 1 - Treatments means of Mycelial Growth Index (MGI) of *Lecanicillium fungicola*.

Treatments	Means MGI (mm)	Treatments	Means MGI (mm)	Treatments	Means MGI (mm)
FUN (1)	0 a	EUC (1.6%/1)	2.86 b	THY (0.4%/2)	7.30 e
FUN (2)	0 a	CLO (0.2%/1)	3.93 c	EUC (0.2%/1)	7.33 e
CIN (0.4%/1)	0 a	EUC (0.8%/1)	4.33 c	ORE (0.8%/2)	7.43 e
CIN (0.4%/2)	0 a	EUC (1.6%/2)	4.43 c	LEM (0.4%/1)	7.56 e
CIN (0.8%/1)	0 a	LEM (1.6%/1)	4.90 c	ORE (0.2%/1)	7.66 e
CIN (0.8%/2)	0 a	THY (0.2%/1)	5.76 d	LEM (0.2%/1)	7.70 e
CIN (1.6%/1)	0 a	THY (0.4%/1)	5.83 d	ORE (0.4%/1)	7.90 e
CIN (1.6%/2)	0 a	CIN (0.2%/2)	5.90 d	CON (1)	8.23 f
CLO (0.4%/1)	0 a	CLO (0.2%/2)	6.06 d	ORE (0.4%/2)	8.60 f
CLO (0.4%/2)	0 a	EUC (0.4%/1)	6.36 d	EUC (0.4%/2)	8.60 f
CLO (0.8%/1)	0 a	ORE (0.8%/1)	6.46 d	CON (2)	8.63 f
CLO (0.8%/2)	0 a	CIN (0.2%/1)	6.66 d	LEM (0.2%/2)	8.76 f
CLO (1.6%/1)	0 a	LEM (0.8%/1)	6.70 d	LEM (0.4%/2)	8.76 f
CLO (1.6%/2)	0 a	ORE (1.6%/1)	6.70 d	LEM (0.8%/2)	8.83 f
THY (0.8%/1)	0 a	THY (0.2%/2)	6.73 d	EUC (0.2%/2)	8.83 f
THY (0.8%/2)	0 a	ORE (1.6%/2)	6.83 d	ORE (0.2%/2)	8.90 f
THY (1.6%/1)	0 a	EUC (0.8%/2)	6.93 d	MILK (2)	9.30 f
THY (1.6%/2)	0 a	LEM (1.6%/2)	7.30 e	MILK (1)	9.43 f

THY – essential oil of thyme; CLO – essential oil of clove; CIN – essential oil of cinnamon; FUN – Sportak fungicide; EUC – essential oil of eucalyptus; LEM – essential oil of lemon balm; ORE – essential oil of oregano; CON – absolute control; MILK – control of milk solution; 1 – strain LF.1; 2 – strain LF.2. *Means followed by the same letter do not differ significantly by Scott–Knott test ($P<0.05$).

Table 2 - Treatments means (%) of conidial germination of *Lecanicillium fungicola*.

Treatments	Means (%)	Treatments	Means (%)	Treatments	Means (%)
FUN (1)	0 a	THY (1.6%/1)	0 a	EUC (0.8%/1)	30 g
FUN (2)	0 a	THY (1.6%/2)	0 a	CLO (0.2%/2)	30 g
CIN (0.2%/1)	0 a	LEM (1.6%/2)	10 b	ORE (0.8%/1)	30 g
CIN (0.2%/2)	0 a	EUC (1.6%/2)	12 b	ORE (0.8%/2)	32 h
CIN (0.4%/1)	0 a	THY (0.4%/1)	12 b	THY (0.2%/2)	32 h
CIN (0.4%/2)	0 a	LEM (1.6%/1)	14 c	EUC (0.4%/1)	34 h
CIN (0.8%/1)	0 a	LEM (0.4%/1)	16 c	EUC (0.4%/2)	34 h
CIN (0.8%/2)	0 a	CLO (0.2%/1)	16 c	ORE (0.2%/1)	34 h
CIN (1.6%/1)	0 a	THY (0.4%/2)	16 c	EUC (0.2%/2)	36 h
CIN (1.6%/2)	0 a	EUC (1.6%/1)	18 d	EUC (0.2%/1)	40 i
CLO (0.4%/1)	0 a	LEM (0.2%/2)	20 d	LEM (0.2%/1)	40 i
CLO (0.4%/2)	0 a	LEM (0.4%/2)	20 d	ORE (0.4%/1)	44 j
CLO (0.8%/1)	0 a	LEM (0.8%/1)	20 d	ORE (0.2%/2)	46 j
CLO (0.8%/2)	0 a	ORE (1.6%/1)	24 e	ORE (0.4%/2)	46 j
CLO (1.6%/1)	0 a	THY (0.2%/1)	24 e	CON (1)	100 k
CLO (1.6%/2)	0 a	EUC (0.8%/2)	26 f	CON (2)	100 k
THY (0.8%/1)	0 a	ORE (1.6%/2)	26 f	MILK (1)	100 k
THY (0.8%/2)	0 a	LEM (0.8%/2)	30 g	MILK (2)	100 k

THY – essential oil of thyme; CLO – essential oil of clove; CIN – essential oil of cinnamon; FUN – Sportak fungicide; EUC – essential oil of eucalyptus; LEM – essential oil of lemon balm; ORE – essential oil of oregano; CON – absolute control; MILK – control of milk solution; 1 – strain LF.1; 2 – strain LF.2. *Means followed by the same letter do not differ significantly by Scott–Knott test ($P<0.05$).

exhibited a fungitoxic effect on the mycelial growth of fungus *Alternaria alternata* and *C. graminicola*.

The strains of *L. fungicola* revealed different responses to treatments. Most frequently, the LF.1 strain showed a higher degree of success in inhibiting mycelial growth, in terms of the LF.2 strain. However, for the spore germination variable, the inhibitory effect exerted by the treatments differed between the two strains in such a manner that, for a specific oil concentration, one strain experienced a higher degree of mycelial growth inhibition, while the other experienced a higher degree of spore germination inhibition. For instance, for lemon balm 1.6%, the LF.1 strain showed a lower MGI, while in the LF.2 strain the percentage of conidia germination was lower. From these results it is evident that the different effects are possible for the two variables within the same strain. This implies an interesting possibility of combining the oils to guarantee disease control in a situation in which different lineages of the pathogen may possibly be present.

In the same context, it must be noted that for treatments which did not show a completely inhibition of the variables analyzed, a huge variation in the inhibition rate was evident in the *L. fungicola* spores, ranging between 54 and 90% inhibition (Table 2). From these treatments could be pointed out 1.6% lemon balm, 1.6% eucalyptus and 0.4% thyme which provided percentage of conidia germination from 10 to 18%, considering the two strains. Therefore, although strong inhibition of the mycelial growth is not visible, a few of these treatments could exert a high spore germination inhibition rate in *L. fungicola*, which could effectively control the disease.

Besides the positive control (Sportak fungicide), cinnamon and clove oils at 0.4%, 0.8% and 1.6% concentrations and thyme oil at 0.8% and 1.6% exhibited 100% inhibition of mycelial growth and conidial germination for the two strains (Table 1 and 2). TEIXEIRA et al, (2013) reported similar results, which confirmed that the thyme, clove and cinnamon oils in concentrations above 0.025% could totally inhibit the mycelial growth and spore germination in the fungus *Stenocarpella maydis*. Therefore, these oils offered a greater potential of use in *L. fungicola* control, as a viable alternative, superior to utilizing chemical fungicides in *A. bisporus* cultivation.

Thus, for the *in vivo* test, clove and cinnamon oils (0.4%) and thyme (0.8%) were chosen and applied prior to (pre-infestation) or after (post-infestation) inoculation of the pathogen; the findings are listed in table 3. In the case of the LF.1 strain, when post-infestation application of the oils was done,

Table 3 - Dry bubble disease incidence means (%) according to strains and period of essential oils application.

	LF.1			LF.2		
	Cinnamon	Thyme	Clove	Cinnamon	Thyme	Clove
Post-Infestation	18.66 Ab	19.56 Ab	33.4 Ab	23.36 Aa	5.94 Ab	17.70 Aa
Pre-Infestation	91.34 Aa	62.94 Ba	54.92 Ba	31.64 Ba	52.07 Aa	19.99 Ba

*Means followed by the same capital letter in the row and lower case in the column, do not differ by Tukey test (P<0.05).

all the treatments exhibited lower incidence of the disease. However, for the LF.2 strain, only the thyme oil exhibited this effect, because for cinnamon and clove, the time of the oil application seemed to have no significant effect on the incidence of the disease. These results imply that the effectiveness of any treatment is dependent on the genetic variability of the pathogenic strains. Therefore, the findings of this study showed greater evidence that using a combination of essential oils is a safer way to explore treatment effectiveness.

Using the scanning electron microscope, an increase in the number of *L. fungicola* spores and the higher prevalence of germination was evident over time, implying the presence of the infection within approximately 19 hours (Figure 1). This finding concurs with the results of ZIED et al. (2015), who reported conidial germination after 15 hours of inoculation. Most likely, occurrence of the disease is higher when the oils are applied pre-infestation, which occurs because of the volatility of the oils

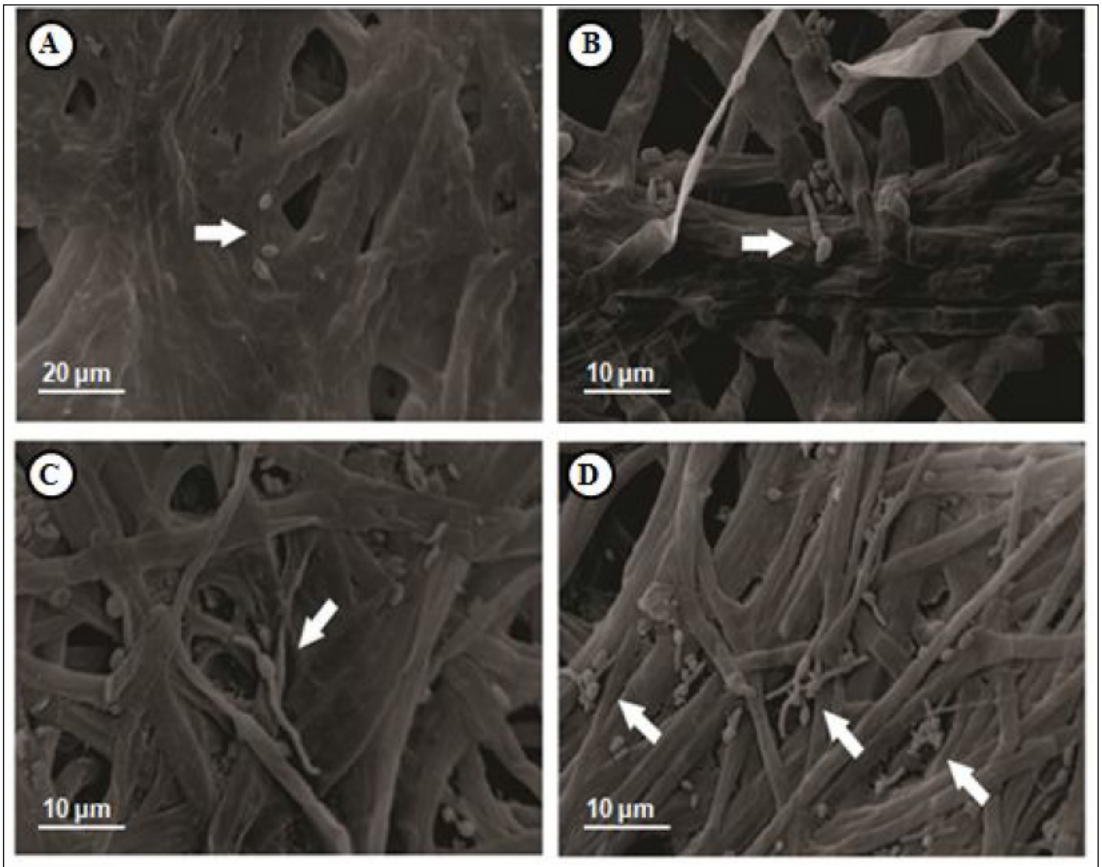

Figure 1 - Scanning electron micrographs of *A. bisporus* inoculated with *L. fungicola*. Sample collect times after inoculation. (A) 13 hours, (B) 16 hours, (C) 19 hours and (D) 23 hours.

employed. As a large percentage of the spores do not germinate within the first hours, it is possible that this dormancy period is long enough for the oil to be at a concentration lower than required to be effective. In light of this, in practice, it is not possible to control the time when the spores will be present; therefore, the findings of this study emphasized the importance of applying the essential oil, or a combination of essential oils, sequentially, through the whole course of the production cycle.

Thus, essential oils are a promising option for the cultivation of pesticide-free mushrooms, avoiding inflicting harm to either man or environment. However, the two aspects of cost-effectiveness and application efficiency must be taken into account, which justify the necessity for greater research into the practical utilization of essential oils in mushroom cultivation.

CONCLUSION

Cinnamon, thyme and clove oils were reported to be most effective in inhibiting the mycelial growth and germination of the *L. fungicola* spores, and hence identified as the most suitable for use in the studies on disease control in the *A. bisporus* cultivation.

Generally, the post-infestation applications of the essential oils were demonstrated to more effectively control the disease, rather than their application during the pre-infestation stage.

ACKNOWLEDGMENTS

The author expresses gratitude for the financial support extended by the Fundação de Amparo à Pesquisa de Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Thanks also to the company Sítio dos Micélios for the champignon compounds provided at concession.

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