IMPROVEMENT OF ANTIFUNGAL ACTIVITY OF CITRONELLA OIL AGAINST *ASPERGILLUS FLAVUS* ON RUBBERWOOD (*HEVEA BRASILIENSIS*) USING HEAT CURING

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Received May 2014

JANTAMAS S, MATAN N, MATAN N & AEWSIRI T. 2016. Improvement of antifungal activity of citronella oil against *Aspergillus flavus* on rubberwood (*Hevea brasiliensis*) using heat curing. Optimisation of the inhibitory effect of citronella oil (10, 30 and 50 µg mL^{-1}) with heat curing (30, 90 and 150 °C) and drying periods (1, 12 and 24 hours) against a major mould (*Aspergillus flavus*) found on the surface of rubberwood was investigated using response surface methodology. Specimens were incubated at 25 °C in 100% relative humidity for 90 days and individually rated for the period it took to achieve zero mould growth on rubberwood. Citronella oil components were analysed by gas chromatography–mass spectrometry. Citronella oil (50 µg mL^{-1}) with heat curing (30 and 90 °C) and drying periods (1 to 24 hours) completely inhibited spore germination for at least 90 days. Microscopy investigation confirmed that no spore germination was found in treated rubberwood. Citronellal (27.5%), geraniol (20.4%), citronellol (13.4%) were major constituents of citronella oil. Heat curing may be important for transformation of components and enhancement of antifungal activity of citronella oil. This study showed that a combination of citronella oil and heat curing could protect rubberwood.

Keywords: Optimisation, drying, response surface methodology, enhancement

INTRODUCTION

Although chemical protection from boron, borate, vapour boron, chromated copper arsenate and volatile borate ester (Cameron & Pizzi 1985, Tsunoda 2001) is found to be suitable for preventing mould growth, decay fungi and insects on wood and wood products because of their broad spectrum (Williams & Amburgey 1985), chemical leaching of treated wood under wet conditions (Grace et al. 2006) can cause contamination. This is a serious environmental problem as contaminated wood can affect soil and drinking water and may be found in children playground (Stilwell & Graetz 2001, Townsend et al. 2005, Lesar et al. 2012). The use of essential oils or other natural compounds from plants for wood preservation has been recommended because they are less harmful (Matan & Matan 2012, Mohareb et al. 2013). Another benefit of essential oils is termite control (Saeki et al. 1971, Zhu et al. 2001) and the development of new chemicals for termite prevention with reduced environmental impact (Roszaini et al. 2013). For this research, citronella oil was selected to be applied on rubberwood (*Hevea brasiliensis*) to reduce mould growth.

Citronella oil has white to yellow colour, good flavour and can be produced by steam distillation of *Cymbopogon* plants (Wany et al. 2013). Citronellol, geraniol (Nhu-Trang et al. 2006) and d-limonene (Jaroenkit et al. 2011) were reported to be main components. Antifungal activity of citronella oil against *Aspergillus niger* (Li et al. 2013), *A. flavus*, *Asparagus racemosus* (Singh et al. 2010) and *Colletotrichum gloeosporioides* (Sellamuthu et al. 2013) has been reported. Insect protection with citronella oil has also been noted (De La Puente et al. 2009, Songkro et al. 2012). Citronellol can also be used against the formosan subterranean termite, *Coptotermes formosanus* (Zhu et al. 2001).

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Due to its strong flavour, citronella oil should not be added directly into products or on rubberwood surface. Therefore, to reduce the amount of citronella oil used and to improve its antifungal activity, heat curing using response surface methodology was selected to determine optimal conditions against the growth of *A. flavus* on rubberwood.

MATERIALS AND METHODS

Essential oil

The citronella oil (*Cymbopogon nardus*) used in this study was provided by Thai China Flavors and Fragrances Industry Company, Thailand.

Gas chromatography–mass spectrometry (GC–MS) analysis

This analysis was carried out on trace GC. The average helium carrier gas flow rate was 1 mL min^{-1}. The split ratio of the column was 150:1. Injector and detector temperatures were set at 250 and 260 °C respectively. The column oven temperature was held at 60 °C for 1 min and then programmed at 150 °C for 15 °C min^{-1} up to 300 °C. After that, it was changed to 2 °C min^{-1} for 10 min. Citronella oil (1.0 µL) was injected manually. Constituent identification was based on comparisons of retention times with those of authentic samples comparing their Kovats indices and also by computer matching with the NIST 08.L (database/chem-station data system).

Preparation of rubberwood

Rubberwood was obtained from a local rubberwood plantation located in Krabi province of southern Thailand. It was cut into 2 cm (width) × 7 cm (length) × 0.5 cm (thick) specimens. Specimens were kept in a conditioned room (20 °C and 65% relative humidity (RH)) for 1 month or until moisture content was 12%.

Mould strain

Aspergillus flavus (WU 0813) was isolated from the surface of rubberwood. The strain was from the culture of Walailak University's Cellulose Protection Technology Laboratory. Aqueous spore suspension of the mould was obtained from 7-day-old potato dextrose broth after incubation at 25 °C. Spore suspension was counted using heamacytometer and adjusted to 10^6 spores mL^{-1} with sterile distilled water. The viability of mould was checked using quantitative colony counts at 10^6 CFU mL^{-1}.

Inhibition of mould spore germination on rubberwood

The variables (concentration, drying period and heat curing) used to determine the period in which zero mould growth (PZMG) is found on the surface of rubberwood are shown in Table 1. Five rubberwood specimens were used and each was immersed in citronella oil at concentrations of 10, 30 and 50 µg mL^{-1} for 10 min. Specimens were dried at different heat curing temperatures (30, 90 and 150 °C) and drying periods (1, 12 and 24 hours) in an oven. *Aspergillus flavus* spores were inoculated by spraying on treated specimens and specimens were stored at 25 °C and 100% RH in an environmental chamber. The rubberwood specimens were individually rated for spore germination on a scale of 0 to 5, with '0' denoting clean specimen and '5' representing heavy mould growth (0 = clean, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80% and 5=100% of mould growth) according to ASTM D4445-91 (ASTM 2003). Observations were carried out until each specimen reached

Table 1 Maximum and minimum levels of variables used in full factorial design

Factor	Parameter	Actual level of code factor		
		-1	0	1
X_1	Concentration (µg ml^{-1})	10	30	50
X_2	Drying time (hours)	1	12	24
X_3	Heat curing temperature (°C)	30	90	150

score 0. PZMG on the rubberwood surface (in days) was also reported.

The independent variables used in this study were X_1, X_2 and X_3 where X_1 = concentration of citronella oil (µg mL^{-1}), X_2 = drying periods (hours) and X_3 = heat curing temperature (°C). Other variables include Y (PZMG), b_0 (intercept), b_1, b_2 and b_3 (linear coefficients), b_{11}, b_{22} and b_{33} (squared coefficients) and b_{12}, b_{13} and b_{23} (interaction coefficients). The model equation for a 3-factor system is:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

Statistical analysis was performed using Statistica software.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of treated specimens (50 µg mL^{-1}, 1 hour, 90 °C) were analysed using a Spectrum One FTIR spectrometer. The spectra were acquired at resolution of 4 cm^{-1} and measurements ranged from 800 to 4000 cm^{-1} at room temperature. The horizontal attenuated total reflectance accessory was mounted into the sample compartment. The internal reflection crystal, which was made of zinc selenide, had 45 °C angle of incidence to the infrared beam. Analysis of spectral data was carried out using Spectrum One software program.

Wettability measurement

Rubberwood treated with 50 µg mL^{-1} of citronella oil with heat curing at 90 °C for 1 hour (n = 3) was prepared. Wettability measurement was done by contact angle goniometer. Distilled water was dropped on each specimen surface. The angle made between the droplet and the specimen surface was measured after 5 s. Measurements were repeated five times at different locations for each sample and average values were used as contact angles.

Microscopy

A microscope was used to observe mould growth on rubberwood surface after being treated with citronella oil at 50 µg mL^{-1} and heat curing at 90 °C for 1 hour on the 90th day and on control treatment (tested with methanol for 3 days).

RESULTS AND DISCUSSION

GC–MS analysis of citronella oil

The components of citronella oil are shown in Table 2. Citronellal (27.5%), geraniol (20.4%) and citronellol (13.4%) were the major components of citronella oil. These main components of citronella oil agreed with other findings (Zhu et al. 2001, Solomon et al. 2012).

Effect of heat curing on growth of mould on rubberwood

The surface plots in Figures 1a, b and c suggested that the optimum points were within the design limits. All surface plots showed that, as the concentration of the citronella oil increased, the PZMG on rubberwood surface increased up to a certain level. For drying periods, no PZMG difference was found when it increased from 1 to 24 hours. The PZMG decreased when temperature increased (> 90 °C). Maximum PZMG was obtained at approximately 90 days after (1) using concentration of 50 µg mL^{-1}, (2) setting the temperature at 30 or 90 °C and (3) drying periods from 1 to 24 hours.

Results of full factorial experiments to determine effects of concentration (X_1), drying period (X_2) and heat curing temperature (X_3) are shown in Table 2. The PZMG is best predicted by the following equation:

$$Y = -3.91 - 1.84X_1 + 0.64X_3 + 0.07X_1^2 - 0.01X_1X_3 - 0.002X_3^2 \quad (2)$$

where Y predicted PZMG via concentration (X_1), drying period (X_2) and heat curing temperature (X_3).

The coefficient of determination (r^2) was 0.94, which indicated that the equation adequately fitted the data. As seen from Table 3, when the temperature increased from 30 to 90 °C, the efficacy of citronella oil against *A. flavus* was not affected. Therefore, this confirmed that heat curing (30–90 °C) could be used to enhance the antifungal activity of citronella

Table 2 Chemical composition of citronella oil

Component	% Area
Eugenol	1.1
Limonene	4.7
Citronellal	27.5
Linalool	1.0
Citronellol	13.4
Geraniol	20.4
Citronellyl acetate	4.8
Geranyl acetate	4.6
Germacrene D	3.6
Muurolene	1.3
Cadinene	4.4
Sum	86.8

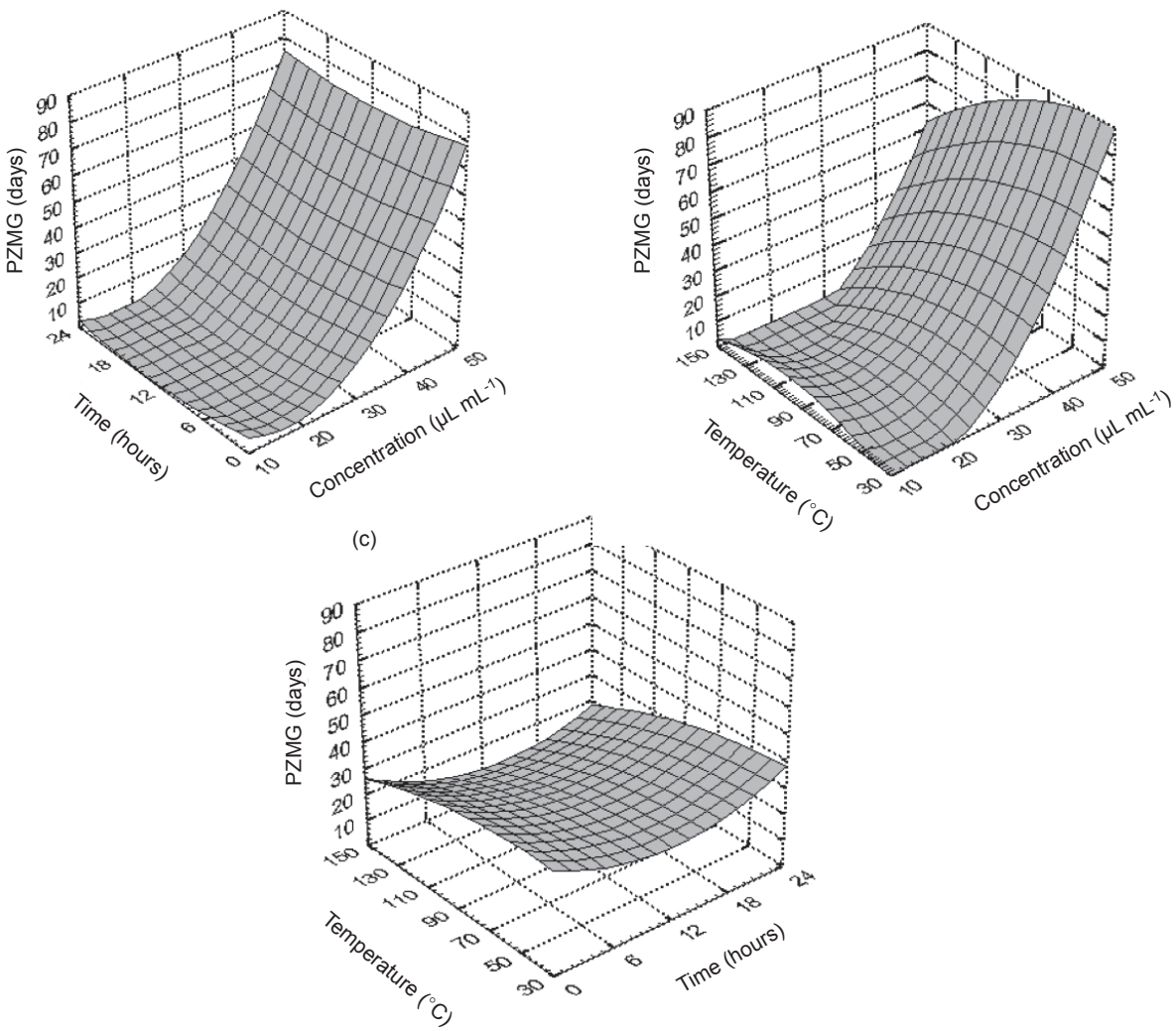

Figure 1 Response surface plots showing effects of citronella oil with different (a) drying times and concentrations, (b) temperatures for heat curing and concentrations and (c) temperatures and drying times on zero mould growth (PZMG) on rubberwood surface

oil. Higher heat curing temperature (> 90 °C), also showed decrease in *A. flavus* (Table 3). Mild temperatures (60–70 °C) were reported to enhance the effect of essential oil (Ait-Ouazzou et al. 2011, Matan et al. 2013). Low concentrations of three commercial citrus fruit essential oils (orange, lemon and mandarin from Spain) at 0.2 µL mL^{-1} in combination with heat treatment for 10 min at 54 °C were reported to show synergistic lethal effects of bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella enteritidis*) (Espina et al. 2011). Therefore, the results from this study were in agreement with other reports. Curing citronella oil between 30–90 °C can be utilised together with wood drying in factories because temperatures in kiln dryers are normally 70–90 °C. On the other hand, higher temperature can decrease the stability of some essential oils and components. For example, the retention of cinnamaldehyde (main component of cinnamon oil) decreased to 17.4% after incubation at 100 °C for 8 hours (Yeh et al. 2013).

Concentration of citronella oil is significant to *A. flavus* inhibition. A concentration of citronella oil at 0.5% (v v^{-1}) was threshold in killing the conidia of *A. niger* (Li et al. 2013). In a medium containing 0.5 to 2.0% citronella oil, it was found that no mould survived after 10 days. In this experiment, at concentration of 50 mg mL^{-1} (and with heat curing), citronella oil reduced the amount of *A. flavus* on rubberwood surface by five times for at least 90 days. Therefore, using a concentration of citronella oil at 50 mg mL^{-1} on rubberwood products may be acceptable.

FTIR spectra

The FTIR spectra of untreated rubberwood and rubberwood treated with citronella oil are shown in Figure 2. Generally, rubberwood without citronella oil (control) showed characteristic absorption of (1) around 3000–3700 cm^{-1}, attributed to the hydroxyl groups in the phenolic and aliphatic structures. Bands at (2) around 2850 and 2920 cm^{-1} predominantly arose from the stretching of methyl and methylene groups of cellulose and lignin. The band at (3) 1740 cm^{-1} was caused by C=O stretching in unconjugated ketones, carbonyls and ester groups of the carbohydrate origin (Bodirlău et al. 2008). At (4) 1656 cm^{-1}, the band was attributed to C-O stretching bands and the bands at (5) 1360 to 1460 cm^{-1} were associated with C-H deformation in lignin, cellulose and hemicelluloses groups (Kartal et al. 2013). The position of (6) 1226 cm^{-1}corresponded to aromatic phenol C-O stretching. Bands in the (7) 900–1180 cm^{-1} region were associated with C-O-C stretching and C-O ester bond stretching (structure of glycosidic linkage) of cellulose, hemicelluloses and lignin groups (Cao & Tan 2004). In addition, after rubberwood was treated with citronella oil (50 µg mL^{-1}, 90 °C, for 1 hour) after both the first day and 90th day of storage, slight changes in absorbance and a pattern of spectra were observed compared with the control. Treated rubberwood had slight decrease in the amplitude of some bands (4 and 7). However, the peaks occurring in citronella oil were not found in treated rubberwood. The disappearance of a dominant FTIR peak of citronella oil on treated rubberwood might be associated with the incubation of treated rubberwood at 50 °C for 1 hour. High temperature affects essential oil content and its components (Argyropoulos & Müller 2014). From these results, it can be hypothesised that treatment of rubberwood using citronella oil with incubation at 90 °C for 1 hour may induce either the incorporation of some citronella oil derivative into rubberwood surface or change the surface structure, resulting in an increase of antifungal activity.

Possible mode of action

Microscopy (Figures 3a and b) and wettability measurement (during storage) results showed that the spores of *A. flavus* could not germinate for at least 90 days on the rubberwood surface after using heat curing at 90 °C for 1 hour with 50 µg mL^{-1} of citronella oil when kept at accelerated conditions (100% RH, 25 °C). Although reversible growth of spores of *A. flavus* on rubberwood surface could be observed, the spores were not able to germinate on treated specimens. Results from the wettability test suggested that there was no significant change in the contact angle between treated (14 ± 2°) and control rubberwood (13 ± 2°). While hydrophobicity is one of the important characteristics of essential oil, results from this study demonstrate that water can be

Table 3 Full factorial design (3^3) matrix with experimental values of period with zero mould growth (PZMG) on rubberwood surface (days)

Run	Uncoded process variable			PZMG (days)
	Concentration (%) (X1)	Time (min) (X2)	Temperature (°C) (X3)	*Aspergillus flavus*
1	10	1	30	3
2	10	12	30	3
3	10	24	30	3
4	10	1	90	3
5	10	12	90	3
6	10	24	90	3
7	10	1	150	3
8	10	12	150	3
9	10	24	150	3
10	30	1	30	3
11	30	12	30	7
12	30	24	30	20
13	30	1	90	45
14	30	12	90	7
15	30	24	90	7
16	30	1	150	7
17	30	12	150	3
18	30	24	150	3
19	50	1	30	90
20	50	12	30	90
21	50	24	30	90
22	50	1	90	90
23	50	12	90	90
24	50	24	90	90
25	50	1	150	45
26	50	12	150	45
27	50	24	150	45
28	30	12	90	7
29	30	12	90	7
30	30	12	90	7
31	30	12	90	7

absorbed into treated specimens. Therefore, no hydrophobic effect from citronella oil was found. Based on the components of citronella oil, citronellal and geraniol were found to be significant. However, in heat curing, both components could be changed according to FTIR results. Furthermore, Argyropoulos and Müller (2014) found that the percentage of citronellal and geranial in lemon balm leaves decreased when using hot-air drying at 60 °C, while citronellol indicated an increase. The change of main citronella oil components in heat curing to create a new compound and/or a minor component synergic effect would be possible to enhance the effect of citronella oil on mould growth. A synergy of the minor essential oil components during heat curing was also reported by Matan et al. (2013). In addition, Li et al. (2013) hypothesised that citronella oil could destroy the *A. niger* hypha

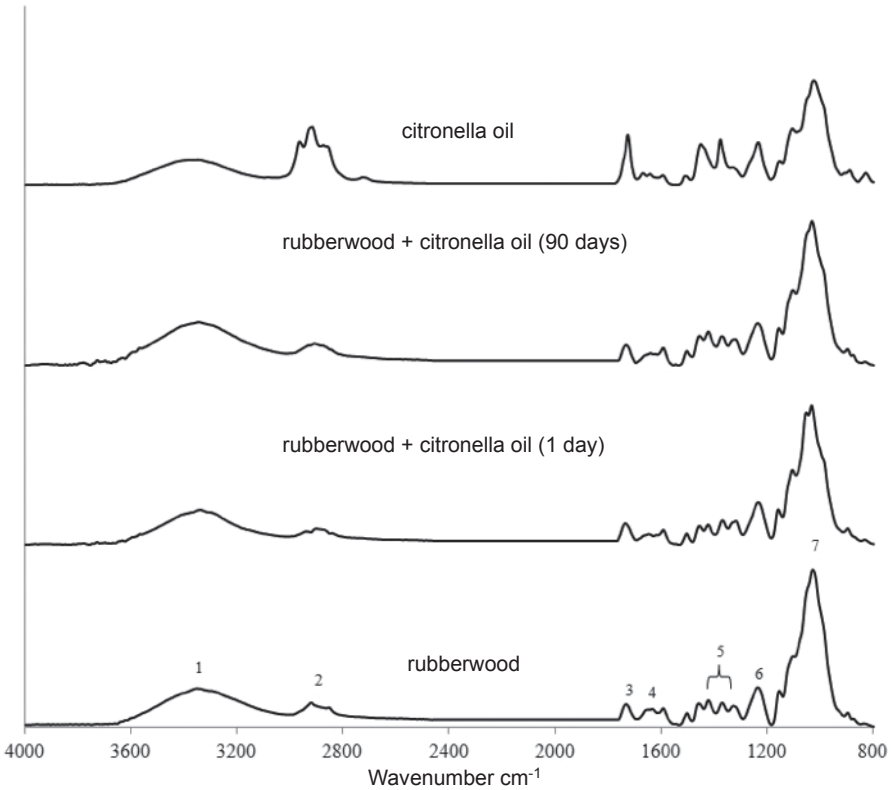

Figure 2 Fourier transform infrared spectra of rubberwood treated with and without citronella oil (50 µg mL^{-1}, 90 °C for 1 hour)

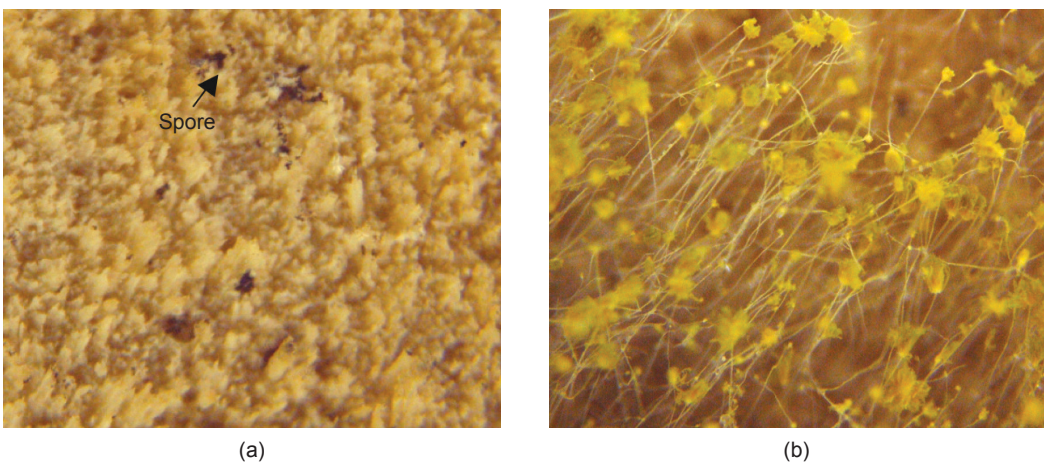

Figure 3 Spores of *Aspergillus flavus* observed on the surface of rubberwood treated with 50 µg mL^{-1} of citronella oil at 90 °C for 1 hour on the (a) 90th day and (b) mycelium of *A. flavus* on rubberwood without citronella oil on the third day

cell walls and then act on the sporoplasm to kill the conidia. This result also confirmed that no spore germination was found after 90 days of storage of treated rubberwood.

CONCLUSIONS

The change of some components in citronella oil during heat curing could have significant impact on germination of *A. flavus*. Citronella oil could be used to increase PZMG under efficient incubation conditions. The increased antifungal activity of citronella oil after heat curing at the concentrations studied had fungistatic effect inhibiting only spore germination.

ACKNOWLEDGEMENTS

This study was supported by the Research Center of Excellence in Wood Science and Engineering, the Institute of Research and Development of Walailak University and the Thailand Research Fund (Grant no. MRG555E090).

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