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Screening for The Antifungal Activity of Essential Oils from Bergamot oil (*Citrus hystrix* DC.) and Tea tree oil (*Melaleuca alternifolia*) Against Economically Rice Pathogenic Fungi: A Driving Force of Organic Rice cv. KDML 105 Production

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Abstract

An *in vitro* study was conducted to evaluate the mycelium growth and spore germination inhibition properties of Thai medicinal essential oils. Two Thai medicinal plants, Bergamot oil (*Citrus hystrix* DC.) and Tea tree oil (*Melaleuca alternifolia*) were steam extracted for this study. They were applied *in vitro* to determine antagonistic activity against seven species of economically important rice pathogenic fungi: *Alternaria brassicicola*, *Aspergillus flavus*, *Bipolaris oryzae*, *Fusarium moniliforme*, *Fusarium proliferatum*, *Pyricularia arisea* and *Rhizoctonia solani*. Mycelium growth and spore germination inhibition techniques were applied to evaluate the efficiency of those essential oils at 0.4, 0.6, 0.8, 1.0 and 2.0 % (v/v), with oil-free treatments used as controls. The experiment was conducted *in vitro* on potato dextrose agar (PDA) using a Complete Randomize Design with 3 replications. Mycelium growth inhibition data recorded at 7 days after inoculation at 25±20 C treated with Tea tree oil at a 2.0% v/v concentration showed the strongest mycelium growth inhibition of *F. moniliforme* (45.00%), *F. proliferatum* (42.59%), *B. oryzae* (66.66%), *A. brassicicola* (64.44%) and *A. flavus* (37.03%). Bergamot oil inhibited mycelium growth of all economically pathogenic fungi when applied at a 0.8 % v/v concentration. However, it may not effectively control *P. grisea* and *R. solani*. The data of spore germination inhibition recorded at 24 hr after inoculation at 25±20 C showed that, at 2.0% v/v; Tea tree oil controlled *F. moniliforme* (77.85%), *F. proliferatum* (66.85%), *P. grisea* (57.64%), *B. oryzae* (63.35%), *R. solani*, (71.61%), *A. brassicicola* (99.26%) and *A. flavus* (71.79%). Bergamot oil controlled *F. moniliforme* (80.66%), *P. grisea* (84.12%), *B. oryzae* (84.68%), *R. solani*, (100%), *A. brassicicola* (100%), *A. flavus* (85.14%) and *F. proliferatum* (59.11%). Thus, the experimental results suggested that both essential oils which were extracted from Thai medical plants provided antifungal properties, with respect to restriction of mycelium growth and spore germination, on economically important rice pathogenic fungi. Inhibitory properties of these oils for fungal

inhibition were dependant on the plant and pathogenic fungi species, inoculum concentration and on the testing environmental conditions.

Keywords: Bergamot oil, Tea tree oil, spore germination and essential oil

Introduction

According to Agrawal *et al.* (1989), over 90% of the field crops grown in the world are propagated through seeds, and all of them are attacked by devastating seed borne pathogens. The rice crop (*Oryza sativa*) is attacked by many pathogenic fungi, e.g. *Bipolaris* sp. (brown spot), *Alternaria* sp. (stackburn), *Fusarium* sp. (bakanae), *Rhizoctonia* sp. (sheath blight), *Nigrospora* sp. (kernel smut), *Curvularia* sp (blast). Important storage fungi are *Aspergillus flavus* and *Aspergillus niger*. Fungi contamination is a major cause leading to seed deterioration and loss of rice grain quality (Kaiser, 1987).

Today, fungicide seed treatments are the most used traditional application to protect seeds and young-seedlings from seed- and soil-borne pathogens. However, chemical seed treatments have raised concerns due to their potential negative impact on the environment, human health and non-target organisms. The reliance on fungicide applications may also lead to fungicide resistance problems (Ester *et al.*, 2003). Moreover, the fungal pathogen is normally attached to the seed, so it is difficult to find chemical substances that will destroy the fungus without harming the seeds. Because many fungicides were developed from bromine, iodine, sulfur, copper, and chloride compounds, their overuse may affect seed viability and vigor. The toxicity of fungicides has also been ascribed to produce phytotoxic compounds which may result in seed deterioration (Han, 2000).).

In order to minimize the undesirable side effects caused by synthetic fungicide applications, researchers have focused on the evaluation of alternative natural bio-fungicides (Anonymus, 2004). Several antifungal compounds of plant origin are known to control seed borne infection (El-Ghaouth, 1997). The use of coated seeds with chemicals or alternative bioactive substances may reduce significantly the percentage of plants damage by pests and the level of pesticide applications (Badei *et al.*, 1996). Another advantage of seed coatings is the preservation of seed quality (Ester *et al.*, 2003). Thus, the present *in vitro* study was conducted to evaluate the mycelium growth and spore germination inhibition property of Thai medical essential oils.

Two Thai medical plants, Bergamot oil (*Citrus hystrix* DC.) and Tea tree oil (*Melaleuca alternifolia*), were steam extracted. They were applied to evaluate their antagonistic activity against seven species of economically important rice pathogenic fungi; *Alternaria brassicicola*, *Aspergillus flavus*, *Bipolaris oryzae*, *Fusarium moniliforme*, *Fusarium proliferatum*, *Pyricularia arisea* and *Rhizoctonia solani*.

Materials and Methods

The experiment was conducted at the Faculty of Agricultural Technology, Department of Technology, Maha Sarakham University, Thailand in the year 2009. The effectiveness of essential oil from Bergamot oil (*Citrus hystrix* DC.) and Tea tree oil (*Melaleuca alternifolia*) against mycelium and spore germination inhibition at different concentration was studied. The experiment consisted of a CRD design with 3 replications. The essential oils were evaluated *in vitro* at concentrations of 0, 0.4, 0.6, 0.8, 1.0, and 2.0 %v/v, on a PDA medium. The agar diffusion method was applied to evaluate their effectiveness. The data was analyzed

by the Statistix program (version 8.0) and mean differences reported at the 95% significance level ($LSD \leq 0.05$).

Mycelium growth inhibition analysis

The experiments were conducted by agar overlay technique (Pitipong, 2008). The essential oil from Bergamot oil (*Citrus hystrix* DC.) and Tea tree oil (*Melaleuca alternifolia*) at different concentrations (0, 0.4, 0.6, 0.8, 1.0, and 2.0 % v/v) on PDA medium was *in vitro* tested against the fungi mycelium growth. The colony diameter was measured and the mycelium inhibition percentage was calculated by following the method of Deans and Svoboda (1990) (Eq 1). Three replications of each treatment were tested and the average was calculated. Control sets were simultaneously run without using the essential oil.

$$\text{Inhibition (\%)} = [(C-T)/C] \times 100 \quad (\text{Eq. 1})$$

Where: C was the colony diameter of the mycelium on the control plate (mm), and T was the colony diameter of the mycelium on the treatment plate (mm).

Spores inhibition analysis

Spores of *Alternaria brassicicola*, *Aspergillus flavus*, *Bipolaris oryzae*, *Fusarium moniliforme*, *Fusarium proliferatum*, *Pyricularia arisea* and *Rhizoctonia solani* were used as the teste fungi. The spore concentration of those fungi was adjusted to approximately 10^6 cfu ml⁻¹ by the hemacytometer. Sterile microscope slides were dropped to 10 μ L of PDA aqueous medium to obtain a thin agar layer on the slide, then a 10 μ L of spores suspension sample was gently spread on each slide. An uncovered watch glass containing either 5 μ L of sterile water as control, or 5 μ L of plant essential oils at concentrations 0.4, 0.6, 0.8, 1.0 and 2.0 % (v/v) were dropped into each slide, then incubated for 24 hrs at 25 ± 2 °C (Pitipong, 2008) and spores inhibition was calculated as described for Eq. 1.

Results and Discussion

Table 1 demonstrates that most of the studied fungi, *P. grisea*, *B. oryzae*, *F. moniliforme*, *F. proliferatum*, *A. brassicicola*, *A. flavus*, and *R. solani* were inhibited at 2.0% v/v of bergamot essential oil. The strongest significant mycelial inhibitory effect was observed at those concentrations. On the other hand, *B. oryzae*, *F. moniliforme*, *F. proliferatum*, *A. brassicicola*, and *A. flavus* were inhibited by the Tree Tea essential oil, while *P. grisea* and *R. solani* were not effectively controlled. The data on the spore germination inhibitory effects showed that all species of economically rice pathogenic fungi were susceptible to both essential oils, bergamot and tree tea. Both essential oils controlled all fungals species when treated at a concentration of 1.0% (v/v), especially on *A. brassicicola* and *R. solani*, which were completely inhibited (100 % inhibition) by both essential oils (Table 2).

The experiment results suggest that both the concentration and type of active compound are important factors that determine their potential antifungal activity. Moreover, the antifungal effectiveness of Bergamot and Tree Tea essential oils is probably affected by the different oil extraction methods, and by the different sensitivity of the test strains used (Saikia *et al.*, 2001).

The plant extracts showed clear antimicrobial properties, although the mechanism of action are poorly understood. However, it must be pointed out that the intrinsic activity of a compound is very important for its effectiveness. The mode of action of antifungal agents also depends on the type of target microorganisms and is mainly related to their cell wall structure and to their outer membrane arrangement (Dorman and Deans, 2000). These observations suggest that the physical and chemical properties (solubility and volatility) might have considerable effects on

the *in vitro* antimicrobial activity (Inouye *et al.*, 2000) of the oils we tested. High hydrophobic compounds are generally reported to be very effective on the primary site at the cytoplasmic membrane (Sikkema *et al.*, 1995). The effects of essential oils when separated from the fungal membranes suggests that their activity is based on their lipophilic properties. The interactions between antimicrobial compounds and cell membranes affect both the lipid ordering and the bilayer stability (Ben Arfa *et al.*, 2006). Their mode of action appeared to be at the phospholipid bilayer, caused by biochemical mechanisms, catalyzed by the phospholipid bilayers of the cell, and related to the cell membrane disruption. These processes include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions (Knobloch *et al.*, 1988). Eugenol is able to inhibit the respiration and ion transport processes, increase membrane permeability and the release of cellular content (Uribe *et al.*, 1985). Moreover, it is able to inhibit the respiration of cell suspensions and to disrupt the permeability barrier of microbial membrane structure (Cox *et al.*, 2000). Morris *et al.*, (1979) reported that the fungi treated with essential oils showed a decrease in size, had an irregular shape with cell wall modifications and that the cell surface had depressions. Such modifications on cell morphology may be related to the interference of the oil components with enzymatic reactions of cell wall synthesis, which affects fungal morphogenesis and inhibited growth

Table 1: The mycelium growth effect of leech lime oil (*Citrus hystrix* DC.) and tea tree oil (*Melaleuca alternifolia*) against Economically important Rice Pathogenic Fungi

| Concentration (%) | Mycelium growth inhibition (%) | | | | | | | | | | | | | |
|-------------------|--------------------------------|----|------------------|----------|-----------------------|----------|------------------------|--------|------------------------|---------|------------------|--------|------------------|----|
| | <i>P. grisea</i> | | <i>B. oryzae</i> | | <i>F. moniliforme</i> | | <i>F. proliferatum</i> | | <i>A. brassicicola</i> | | <i>A. flavus</i> | | <i>R. solani</i> | |
| | B | TT | B | TT | B | TT | B | TT | B | TT | B | TT | B | TT |
| Control | 0d | 0a | 0c | 0c | 0c | 0c | 0c | 0a | 0c | 0d | 0a | 0b | 0b | 0a |
| 0.4 | 24.09c | 0a | 38.88b | 33.33b | 53.33b | 23.33abc | 59.58 b | 25.92a | 36.29b | 35.55c | 0a | 0b | 5.03ab | 0a |
| 0.6 | 67.07b | 0a | 51.10ab | 51.847ab | 62.59ab | 15.00bc | 65.13b | 22.22a | 60.73a | 59.99ab | 11.11b | 0b | 0.00b | 0a |
| 0.8 | 100.00a | 0a | 56.66ab | 66.66a | 72.14ab | 6.66bc | 65.13b | 59.25a | 68.14a | 55.55bc | 11.11b | 0b | 0.00b | 0a |
| 1.0 | 100.00a | 0a | 64.44a | 66.66a | 67.84ab | 30.00ab | 65.13b | 22.22a | 68.14a | 68.88ab | 11.11b | 44.44a | 0.00b | 0a |
| 2.0 | 100.00a | 0a | 73.99a | 66.66a | 81.40a | 45.00 a | 82.56a | 42.59a | 68.14a | 82.220a | 11.11b | 55.55a | 7.40a | 0a |

B: bergamot essential oil, TT: tea tree essential oil

Numbers followed by different letters within each column indicated significant statistically difference by LSD at the 5% level

Table 2: The spore germination inhibition effect of leech lime oil (*Citrus hystrix* DC.) and tea tree oil (*Melaleuca alternifolia*) Against Economically important Rice Pathogenic Fungi

| Concentration (%) | Spore germination inhibition (%) | | | | | | | | | | | | | |
|-------------------|----------------------------------|---------|------------------|--------|-----------------------|--------|------------------------|---------|------------------------|---------|------------------|--------|------------------|--------|
| | <i>P. grisea</i> | | <i>B. oryzae</i> | | <i>F. moniliforme</i> | | <i>F. proliferatum</i> | | <i>A. brassicicola</i> | | <i>A. flavus</i> | | <i>R. solani</i> | |
| | B | TT | B | TT | B | TT | B | TT | B | TT | B | TT | B | TT |
| Control | 0.00e | 0.00d | 0.00e | 0.00c | 0.00d | 0.00e | 0.00e | 0.00d | 0.00c | 0.00e | 0.00d | 0.00c | 0.00d | 0.00d |
| 0.4 | 30.78d | 21.78c | 28.36d | 32.09b | 35.99c | 20.10d | 14.92d | 26.31c | 90.05b | 58.63d | 34.94c | 41.29b | 60.51c | 47.12c |
| 0.6 | 34.00d | 43.04b | 51.70c | 38.71b | 50.94bc | 38.40c | 35.65c | 47.43b | 98.77a | 70.43c | 54.45b | 60.58a | 92.00b | 56.24b |
| 0.8 | 57.19c | 49.20ab | 60.95b | 40.28b | 51.68bc | 58.11b | 42.55bc | 48.30b | 100.00a | 81.32b | 59.40b | 65.00a | 100.00a | 62.01b |
| 1.0 | 67.05b | 56.86a | 78.59a | 53.49a | 61.20b | 63.09b | 59.11a | 57.77ab | 100.00a | 96.29a | 71.51ab | 70.03a | 100.00a | 71.77a |
| 2.0 | 84.12a | 57.64a | 84.68a | 63.35a | 80.66a | 77.85a | 54.82ab | 66.85a | 100.00a | 99.267a | 85.14a | 71.79a | 100.00a | 76.61a |

B: bergamot essential oil, TT: tea tree essential oil

The different letters indicated the statistically difference by LSD at 5% level

Conclusions

The essential oils from Bergamot oil (*Citrus hystrix* DC.) and Tea tree oil (*Melaleuca alternifolia*) provided anti-mycelial growth, and disrupted spore germination activity on economically important rice pathogenic fungi. The mode of action based on the deterioration of the fungal cellular structure lead to complete cell death. These findings increase the opportunity and possibility of exploiting both essential oils as promising candidates for use in crop production systems as alternative safe natural antifungal agents. Considerable follow-up laboratory and field research is necessary prior to the use and commercialization of these products.

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