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RESEARCH ARTICLE

ANTIFUNGAL ACTIVITY OF TEA TREE ESSENTIAL OILS (MELALEUCA ALTERNIFOLIA) AGAINST PHYTOPATHOGENIC FUNGI.

Thanh Khang LE [1], Thi Thu Huong Nguyen [2] and Thi Thuy Tien LE[3].

1. Department of Biotechnology, Research Institute for Oil and Oil Plants - IOOP, Ministy for Industry and Trade, 171 - 175 Ham Nghi street, Nguyen Thai Binh ward, district 1, Ho Chi Minh city, Vietnam.
2. Department of Pharmacology and Biochemistry, Research Center of Ginseng and Medicinal Materials, National Institute of Medicinal Materials (NIMM), 41 Dinh Tien Hoang street, Ben Nghe ward, district 1, Ho Chi Minh city, Vietnam.
3. Department of Biotechnology, Chemical Engineering Faculty, Ho Chi Minh University of Technology, Vietnam National University - Ho Chi Minh City (VNU-HCM), 268 Ly Thuong Kiet street, district 10, Ho Chi Minh city, Vietnam.

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Abstract

The chemical composition and plant pathogenic antifungal activity of tea tree essential oils (Melaleuca alternifolia) collected in Long An, Vietnam was surveyed. By gas chromatography and mass spectrometry (GC-MS), the main chemical composition in tea tree essential oils was identified to be eucalyptol with a concentration of 31.54%. Plant antifungal activity of tea tree essential oils was evaluated with many kinds of dissolvent to determine the minimum inhibitory concentration (MIC). The results showed that tea tree essential oils had the ability to inhibit the growth of five phytopathogenic fungal strains: Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Pyricularia oryzae with the minimum inhibitory concentrations (MICs) were in the range of 6 – 8 µL mL^{-1}.

Introduction:-

Melaleuca alternifolia, the English name "tea tree" has long been studied in the world as a valuable source of essential oils and medicinal herbs. In 1924, this plant was officially described by Maiden and Betche Chee (Maiden and Betche , 1924). The official description of science has set the stage for research on this plant and especially on its essential oils. Tea tree essential oils has been determined more and more specific and accurate in both ingredients and content. From 12 substances (groups) of substances discovered in 1968 (Guenther , 1968), a decade later, about 48 components in tea tree essential oils have been discovered (Swords and Hunter, 1978). With the development of gas chromatography, Brophy and their research group investigated over 800 tea tree essential oils samples and identified about 100 components present in tea tree essential oils (Brophy et al., 1989). In general, the above studies have clarified the composition and content of tea tree essential oils. Thereby, it showed that tea tree essential oils contains terpinen-4-ol (≥ 30%); ter-terpinene (10 - 28%); α- terpinene (5 - 13%); 1.8-cineole (≤ 15%) (Brophy et al., 1989; International Organization for Standardisation, 2004), with high content, promisingly, this is an essential oils with many biological activities and high application potential.

Corresponding Author:-Thanh Khang LE.
Address:-Department of Biotechnology, Research Institute for Oil and Oil Plants - IOOP, Ministy for Industry and Trade, 171 - 175 Ham Nghi street, Nguyen Thai Binh ward, district 1, Ho Chi Minh city, Vietnam.

Tea tree essential oils has been proved to have antifungal activity against Alternaria spp., Aspergillus flavus, A. fumigatus, A. niger, Blastoschizomyces capitatus, Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis, Cladosporium spp., Cryptococcus neoformans, Epidermophyton floccosum, Fusarium spp., Malassezia furfur, M. sympodialis, Microsporum canis, M. gypseum, Penicillium spp., Rhodotorula rubra, Saccharomyces cerevisiae, Trichophyton mentagrophytes, T. rubrum, T. tonsurans, Trichosporon spp. (Bassett et al., 1990); (Southwell et al**.**, 1993; Nenoff et al., 1996; Rushton et al., 1997; Christoph et al., 2000; Griffin et al., 2000; D' Auria et al., 2001), antivirus Nicotiana glutinosa (Schnitzler et al., 2001).

The studies in Vietnam are all in the field, though they have contributed to the planting of tea tree and adapting to the conditions of Vietnam. However, this studies have not specified the essential oils components as well as the extraction methods and directions of the application of tea tree oils, so has not yet improved the oils value of these plant in Vietnam. Controlling plant diseases caused by fungi are mainly based on chemical substances; however, to avoid pathogens forming medicine resistant strains as well as reducing environmental pollution, ecological imbalance, it is necessary to have effective control measures. Therefore, using plant extracts is considered as a new trend to be studied and applied by scientists, especially in preventing plant diseases caused by fungi by essential oils.

Controlling plant diseases caused by fungi are mainly based on chemical substances; however, to avoid pathogens forming medicine resistant strains as well as reducing environmental pollution, ecological imbalance, it is necessary to have effective control measures. Therefore, using plant extracts is considered as a new trend to be studied and applied by scientists, especially in preventing plant diseases caused by fungi.

Materials and methods:-

Materials

Branches and leaves of tea tree were collected at tea tree garden in Long An province, Vietnam. Fresh materials were purified and washed before extracting essential oils.

All fungal strains were obtained from and provided by the Department of Biotechnology, Chemical Engineering Faculty, Ho Chi Minh University of Technology, Vietnam National University - Ho Chi Minh City (VNU-HCM) and Department of Biology, College of Natural Sciences, Can Tho University (CTU). For the present study, the following strains were used: Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Pyricularia oryzae.

Method of extracting essential oils

The materials were directly extracted by steam distillation, with the distillation set of Clevenger essential oils, NaCl was used to support the diffusion of essential oils during the attraction of essential oils following the steam.

Analysis of chemical composition of essential oils

The composition and concentration of the constituents in the essential oils were analyzed by gas chromatography with mass spectrometry (GC - MS), performed on the device GC - MS of THERMO SCIENTIFIC Trace GC Ultra - ISQ, column type was the TG column - SQC (15 mx 0.25 mm x 0.25 µm), the gas carried helium. Temperature program for gas chromatograph was set as follows:

Initial temperature 50°C, keep 1 minute —(2°C/minute)→ 70°C, keep 2 minutes —(2°C/minute)→ 150°C, keep 2 minutes —(10°C/minute)→ 250°C, keep 10 minutes

Test of antifungal activities of tea tree essential oils against phytopathogenic fungi

Five fungal strains were cultured on PDA (Potato Dextrose Agar): Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Pyricularia oryzae. Tea tree essential oils was diluted with Tween 80 for the concentrations of 2, 4, 6, 8 and 10 $\mu L\ mL^{-1}$. The diameter of colonies were notes after six days of incubation.

The inhibitory ability (performance) of essential oils with the growth of fungi (%) was according to the following equation:

$$H(\%) = \frac{R - r}{R} \times 100$$

R: Fungus radius developed in the control (cm). r: Fungus radius in essential oils (cm). In this study, we used carbendazim (97%) (Sigma-Aldrich, USA) (an antifungal drug) as posity control.

Evaluating the morphology of mycelium

To evaluate the microstructural geometry of the mycelium, morphological characterization was carried out using a SEM scanning electron microscope (SEM).

test of activity against phytopathogenic fungi in combination with carbendazim

Carbendazim was mixed with tea tree essential oils at different ratios (Table 1) to test the antifungal activity.

Table 1:-Ratio of tea tree oils concentration: carbendazim

Tea tree essential oils (ml)	80	160	240	320
Carbendazim (mg)	320	240	160	80
Ratio of tea tree oils concentration: carbendazim	1:4	2:3	3:2	4:1

Each experiment was conducted in triplicate.

Results:-

Chemical composition of essential oils

Table 2:-Main chemical composition of tea tree essential oils in Vietnam

No.	Retention time	Ingredients	Content (%)
1	2.35	2 – pentanone, 4 – hydroxyl – 4 – methyl -	6.67
2	3.57	α - pinene	2.71
3	4.54	β - pinene	2
4	5.06	β - myrcene	3.68
5	6.43	Eucalyptol	31.54
6	7.71	1, 4 – cyclohexadiene, 1 - methyl – 4 – (1 – methylethyl) -	8.23
7	8.62	Cyclohexene, 1 - methyl – 4 – (1 – methylethylidene) -	1.73
8	13.83	3 – cyclohexen – 1 – ol, 4 – methyl – 1 – (1 – methylethyl) -	22.47
9	14.49	3 – cyclohexen – 1 – methanol, α, α, 4 – trimethyl - , (S) -	12.16
10	20.02	1H – cycloprop[e]azulene, 1a, 2, 3, 5, 6, 7, 7a, 7b – octahydro – 1, 1, 4, 7 – tetramethyl -, [1aR – (1aα, 7α, 7aβ, 7bα)] -	1.61
11	20.4	Napthalene, 1, 2, 3, 5, 6, 8a – hexahydro – 4, 7 – dimethyl – 1 – (1 – methylethyl) -, (S – cis) -	1.13
12	21.22	Globulol	1.86
13	21.34	1H – cycloprop[e]azulene – 4 – ol, decahydro - 1, 1, 4, 7 – tetramethyl -, [1ar – (1aα, 4β, 4aβ, 7α, 7aβ, 7bα)] -	1.21

Analysis results of GC - MS showed that there were 13 components in tea tree essential oils of Vietnam, belonging to seven groups of compounds which were groups etyl butanoat such as 2 – pentanone, 4 – hydroxyl – 4 – methyl - , pinene such as α - pinene and β – pinene, β – myrcene, terpinene such as 1, 4 – cyclohexadiene, 1 - methyl – 4 – (1 – methylethyl) -, cyclohexene, 1 - methyl – 4 – (1 – methylethylidene) -, eucalyptol, terpineol such as 3 – cyclohexen – 1 – ol, 4 – methyl – 1 – (1-methylethyl) – and 3 – cyclohexen – 1 – methanol, α, α, 4 – trimethyl - , (S) -, aristolochene such as 1H – cycloprop[e]azulene, 1a, 2, 3, 5, 6, 7, 7a, 7b – octahydro – 1, 1, 4, 7 – tetramethyl -, [1aR – (1aα, 7α, 7aβ, 7bα)] – and napthalene, 1, 2, 3, 5, 6, 8a – hexahydro – 4, 7 – dimethyl-1 – (1 – methylethyl) -, (S – cis) – and compounds ledol such as globulol and 1H – cycloprop[e]azulene – 4 – ol, decahydro - 1, 1, 4, 7 – tetramethyl -, [1ar – (1aα, 4β, 4aβ, 7α, 7aβ, 7bα)] -. The total content of all constituents accounts for more than 90% of the extracted oils. The constituents with the highest content in tea tree essential oils were eucalyptol and terpineol compounds, so tea tree essential oils had a fairly high boiling point, density and extraction, in accordance with other studies on tea tree essential oils that have been announced. These were eucalyptol and terpineol compounds that created biological activities to tea tree essential oils such as antimicrobial, antifungal, antioxidant and cytotoxic activities. (Roy and Vijayalaxmi, 2013).

Eucalyptol is a natural organic compound, is a colorless liquid at room temperature. It has a ring ether and is a monotecenoid. Terpineol is a monoterpene that has been isolated from a variety of sources such as tea tree oils, pine oils and petitgrain oils. There are four isomers , α -, β-, ᵞ - terpineol and terpinen – 4 - ol. β- and ᵞ - terpineol only different by the position of the double link. Terpineol usually a mixture of these isomers with α - terpineol is the main ingredient.

Antifungal activity against phytopathogenesis fungi

With an initial concentration of 10 µL mL^{-1}, the tea tree essential oils was diluted with lower concentration scales, thereby determining the MIC value. Test results were presented in Table 3 and Figure 1.

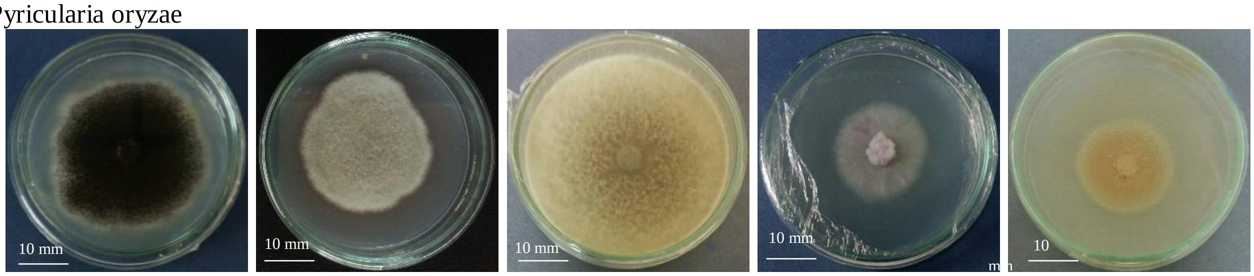
Table 3:-Antifungal performance of tea tree essential oils

I. Tested fungi specie	Active test sample	Antifungal performance (%)					
		0 µL mL^{-1}	2 µL mL^{-1}	4 µL mL^{-1}	6 µL mL^{-1}	8 µL mL^{-1}	10 µL mL^{-1}
Aspergillus niger	Tea tree essential oils	-	44.3^{e} ± 1.24	68.6^{d} ± 1.27	76.0^{c} ± 1.78	85.4^{b} ± 0.77	100^{a} ± 0
	Carbendazim	-	45.5^{e} ± 0.1	72.8^{d} ± 1.75	79.1^{c} ± 1.83	94^{b} ± 0.66	100^{a} ± 0
Corynespora cassiicola	Tea tree essential oils	-	43.8^{d} ± 0.83	70.4^{c} ± 0.82	91.4^{b} ± 0.92	100^{a} ± 0	100^{a} ± 0
	Carbendazim	-	77.8^{d} ± 1.08	82.4^{c} ± 0.29	98.5^{b} ± 0.37	100^{a} ± 0	100^{a} ± 0
Colletotrichum sp.	Tea tree essential oils	-	55.3^{c} ± 1.02	76.7^{b} ± 0.78	100^{a} ± 0	100^{a} ± 0	100^{a} ± 0
	Carbendazim	-	61.1^{c} ± 4.6	83.5^{b} ± 0.11	100^{a} ± 0	100^{a} ± 0	100^{a} ± 0
Fusarium oxysporum	Tea tree essential oils	-	15.0^{e} ± 1.78	37.6^{d} ± 1.91	52.7^{c} ± 2.43	68.5^{b} ± 11.83	100^{a} ± 0
	Carbendazim	-	39.2^{e} ± 4.74	70.7^{d} ± 1.4	74.4^{c} ± 2.7	90.6^{b} ± 11.35	100^{a} ± 0
Pyricularia oryzae	Tea tree essential oils	-	14.5^{c} ± 2.32	41.5^{b} ± 1.81	100^{a} ± 0	100^{a} ± 0	100^{a} ± 0
	Carbendazim	-	20.7^{c} ± 1.59	51.4^{b} ± 1.04	100^{a} ± 0	100^{a} ± 0	100^{a} ± 0

The letters that follow the values in the same row are statistically significant at 5%. (-) is not antifungal.

Aspergillus niger Corynespora cassiicola Colletotrichum sp. Fusarium oxysporum Pyricularia oryzae

0 µl/ml



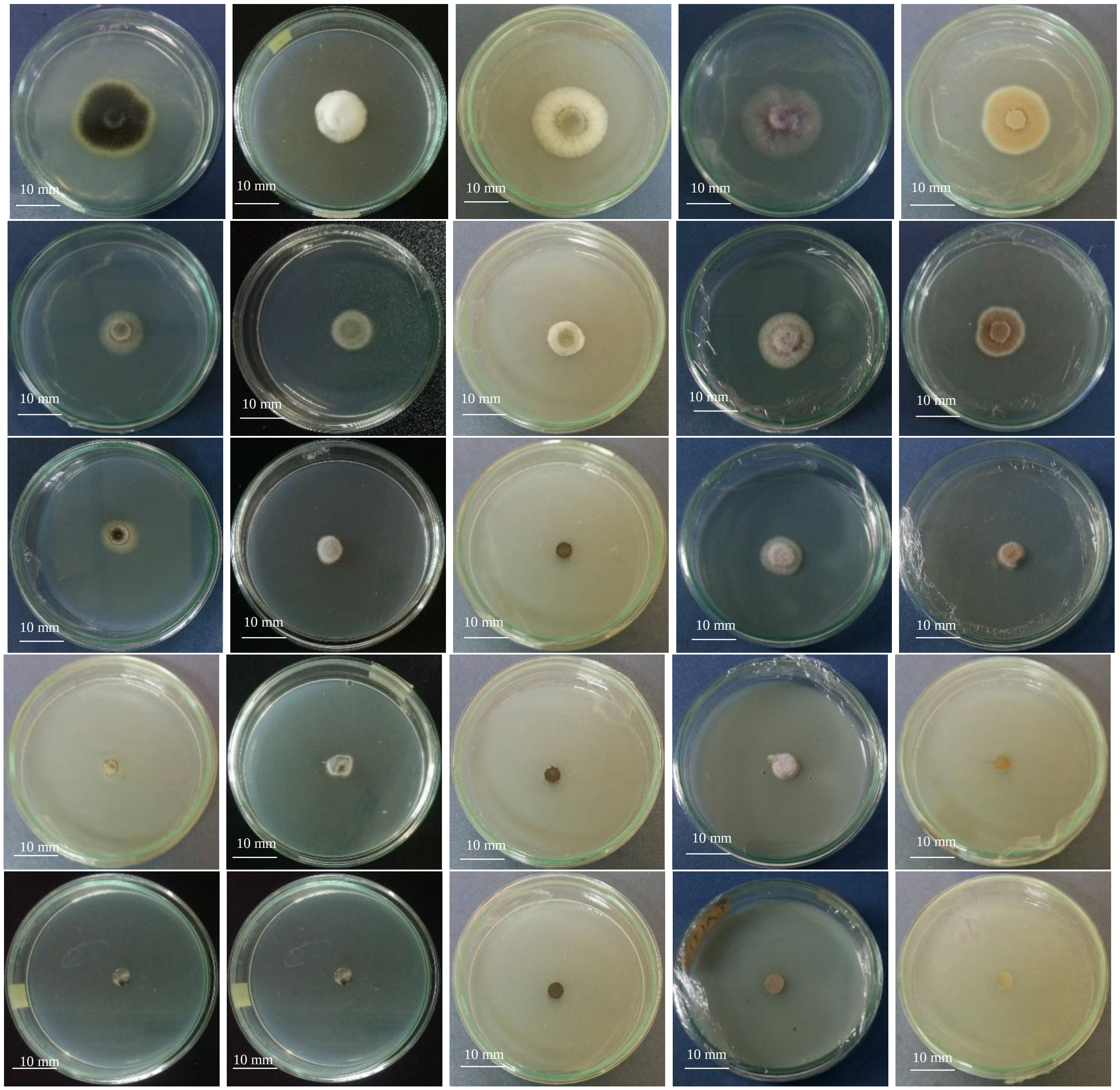


Figure 1:-Diameter of colonies at different concentrations of tea tree essential oils

The results showed that the antifungal activity of tea tree essential oils was depended on concentration. Tea tree essential oils inhibited mycelial growth of fungi strains: Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Pyricularia oryzae. In all the test fungi strains, complete mycelial growth inhibition was observed at a tea tree essential oils concentration of 10 µL mL^{-1} (Figure 1). Tea tree essential oils had a significantly ($p \leq 0.05$) higher inhibitory effect on Colletotrichum sp. and Pyricularia oryzae than on Corynespora cassiicola, Aspergillus niger and Fusarium oxysporum at a concentration of 6 µL mL^{-1}after six days of incubation. The range of mycelial growth inhibition was between 14.5 % and 100%. At a concentration of 6 µL mL^{-1}, the tea tree essential oils completely inhibited the mycelial growth of Colletotrichum sp. and Pyricularia oryzae. However, complete inhibition of Corynespora cassiicola was observed at a concentration of 8 µL mL^{-1}; Aspergillus niger and Fusarium oxysporum was observed at a concentration of 10 µL mL^{-1} (Table 3).

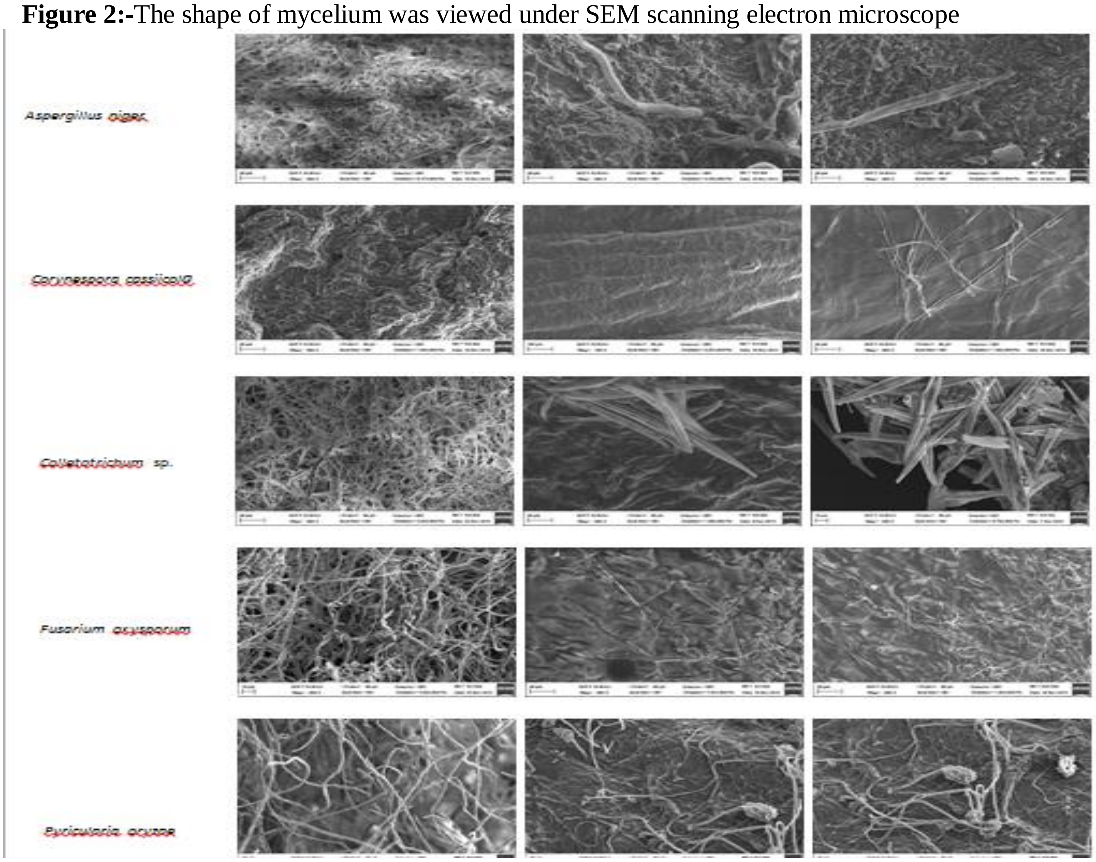
The highest and lowest rates of mycelial growth inhibition by the tea tree essential oils at a concentration of 2 µL mL^{-1}were observed in Colletotrichum sp. (55.31 %) and Pyricularia oryzae (14.52 %), respectively. The inhibition of mycelial growth in the five test fungi strains at tea tree essential oils concentration of 2 µl/ml was significantly (p

≤ 0.05) different. The minimum inhibitory concentrations (MICs) of the tea tree essential oils was on the test pathogens were in the range of 6 – 8 $\mu L\ mL^{-1}$. The lowest MIC value (6 $\mu L\ mL^{-1}$) was observed in Colletotrichum sp. and Pyricularia oryzae while the other Corynespora cassiicola isolates had MICs value of $\mu L\ mL^{-1}$and 10 $\mu L\ mL^{-1}$for Aspergillus niger and Fusarium oxysporum.

The tea tree essential oils exhibited a concentration - dependent activity against the test fungi. Overall, as the concentration of the tea tree essential oils increased, the activity against the test fungi increased represented by an increase in the diameter of the inhibition zones. However, there were some instances where more dilute tea tree essential oils produced larger inhibition zones than the less dilute oils. Thus, as the tea tree essential oils concentration increased, the antifungal activity against the test fungi increased.

Evaluate morphology of mycelium

Figure 2:-The shape of mycelium was viewed under SEM scanning electron microscope



SEM was used to examine the morphological variations of five phytopathogenic fungal strains: Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Pyricularia oryzae (Figure 2). We found remarkable morphological and structural alterations of the hypha. The shape of mycelium of Aspergillus niger was sparse, irregular mycelium and there were places where the mycelium was larger than the control sample. The mycelium of Corynespora cassiicola growed sparse, long, branched fungal hyphae with horizontal walls and unusual deformed hyphae. Fungal hyphae of Colletotrichum sp. were poorly developed, forming a cluster of flat disk spore stalks, sickle, curved spore stalks (with a noticeable difference) and spores were broken horizontally. The number of fungus hyphae of Fusarium oxysporum was less, mycelium was long, faint and poorly branched. There were microconidia, no macroconidia and thick walled spores were observed in Fusarium oxysporum. The number of fungi mycelium of Pyricularia oryzae was less, ungus hyphae were long, smooth and poor branching. Spores of Pyricularia oryzae growed into clusters and there were many partitions forming many compartments. The SEM result also showed that the effect on fungi was similar between the tea tree essential oils and carbendazim.

Synergistic activity of tea tree essential oils with carbendazim against phytopathogenic fungi

Results of evaluation of plant pathogen resistance activity of tea tree essential oils and carbendazim are recorded in Table 4.

Table 4:-Test results of synergistic activity of tea tree essential oils with carbendazim against phytopathogenic fungi

Ratio of tea tree essential oils concentration: carbendazim	Antifungal performance (%)				
	Aspergillus niger	**Corynespora cassiicola**	**Colletotrichum sp.**	**Fusarium oxysporum**	**Pyricularia oryzae**
1:4	$92.5^{b} \pm 0.56$	$81.0^{c} \pm 1.19$	$61.5^{bc} \pm 11.85$	$57.1^{c} \pm 13.65$	$42.7^{d} \pm 9.84$
2:3	$92.5^{b} \pm 1.26$	$97.9^{b} \pm 0.56$	$75.7^{bc} \pm 4.84$	$72.3^{bc} \pm 1.89$	$65.3^{c} \pm 8.62$
3:2	$98.3^{a} \pm 1.54$	$100^{a} \pm 0$	$100^{a} \pm 0$	$100^{a} \pm 0$	$85.1^{b} \pm 6.26$
4:1	$100^{a} \pm 0$	$100^{a} \pm 0$	$100^{a} \pm 0$	$100^{a} \pm 0$	$100^{a} \pm 0$

The letters that follow values in the same column are statistically significant at the level 5%.

Aspergillus niger Corynespora cassiicola Colletotrichum sp. Fusarium oxysporum Pyricularia oryzae

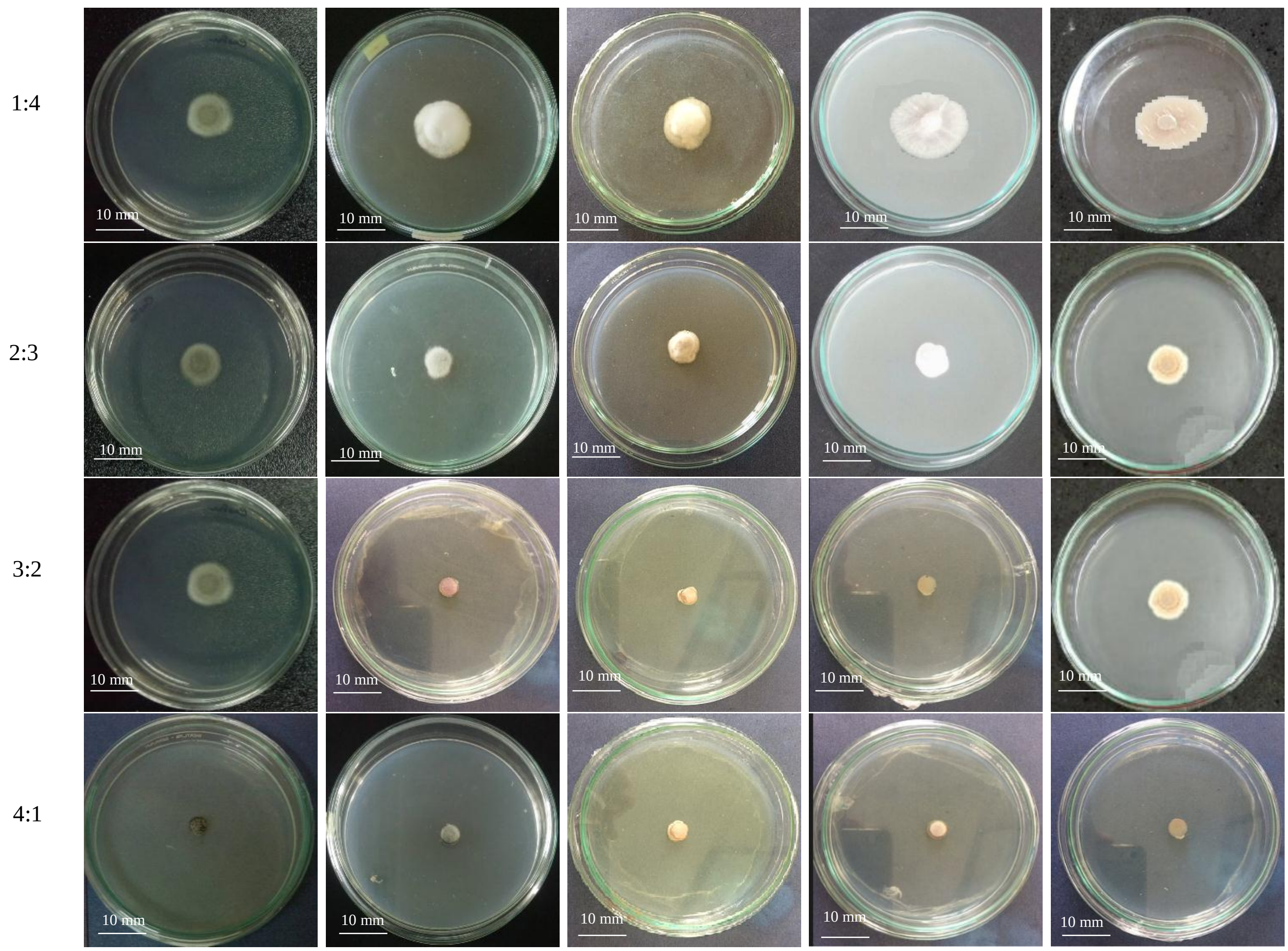


Figure 3:-Diameter of colonies at different tea tree essential oils concentration: carbendazim

Table 4 and Figure 3, depict the effect of combination treatment of tea tree essential oils with antifungal drug (carbendazim) on five phytopathogenic fungal strains: Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Pyricularia oryzae. There was a significant antagonistic association between the tea tree essential oils and carbendazim in comparison with carbendazim alone (Table 3, Table 4, Figure 1, and Figure 3). Interestingly, the addition of the tea tree essential oils to the growth medium in the presence of carbendazim showed synergistic action against five phytopathogenic fungal strains: Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum, and Pyricularia oryzae. This was evidenced by growth inhibition at lower concentration in comparison with that of the antifungal drug carbendazim. Recorded diameters of dermatophyte growth inhibition zones indicated that the fungicidal efficacy of tea tree essential oils with carbendazim mixtures against Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Pyricularia oryzae was higher than that of pure tea tree essential oils extracts. Table 3 and Table 4 demonstrated that among tested pure and mixed tea tree essential oils with carbendazim, tea tree essential oils with carbendazim mixture registered the higher than pure tea tree essential oils at concentration 2 μL mL^{-1}. Moreover, at concentration 2 μL $mL^{-1}$with tea tree essential oils concentration: carbendazim was 3:2 resistanced 100 % with three studied fungi strains (Aspergillus niger, Corynespora cassiicola, Colletotrichum sp. and Fusarium oxysporum) and tea tree essential oils concentration: carbendazim was 4:1 with Pyricularia oryzae. Pure extracted tea tree essential oils recorded high antifungal indices against all the investigated fungal strains. Nevertheless, antifungal indices were equaled to 100 % at concentrations of 2 μL $mL^{-1}$of pure tea tree essential oils and tea tree essential oils with carbendazim mixtures was higher than pure extracted tea tree essential oils. Through 4 concentration levels, the effect of pathogenic fungus resistance on plants with the concentration of tea tree oils: carbendazim with synthetic origin are 4:1 for Pyricularia oryzae, 3:2 for Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Aspergillus niger (although with the concentration of tea tree oils: carbendazim of synthetic origin are 4:1 for 100% antifungal performance for Aspergillus niger but statistically, there was no significant differences with the concentration of tea tree oils: fungicides of synthetic origin were 3:2).

Discussion:-

From the results, it showed that the tea tree essential oils in this study had the main active ingredient of eucalyptol 31.54%, meeting the requirements of ISO 4730:2004, similar to the reference ingredients (Brophy et al. , 1989).

From the research results, it showed that the tea tree essential oils showed activity to inhibit five strains of fungal pathogens in plants. Of which, Aspergillus molds not only caused disease in plants but also caused serious diseases in humans such as Aspergillosis (Thomas, 2015). Corynespora cassiicola fungus was known as a pathogen of many agricultural crops, especially cowpeas, cucumbers, papayas, rubbers, soybeans, and tomatoes. Colletotrichum sp. caused very common and serious harm on many crops, from vegetables such as peppers, tomatoes, cucurbits, melons, etc. to fruit trees such as mangoes, durian, papayas, bananas, dragon fruits. F.oxysporum fungus caused yellow wilt in many plants, this fungus harmed many crops on all parts, especially the root and root parts of the plant (Rosado-Álvarez, 2014) and Pyricularia oryzae fungus caused rice blast disease as the most dangerous agent for rice in the world as well as in Vietnam. The result showed that the antibacterial and antifungal potential of tea trea essential oils can be applied in practice.

SEM was used to test morphological variations of active test fungal strains. The results showed remarkable changes in the morphology and structure of mycelium. These changes include sparse, poorly developed hyphae, abnormally deformed mycelia, horizontally broken spores. The SEM result showed that the tea tree essential oils plays an important role in damaging mycelium morphology and structure. The influence of this tea tree essential oils treatment on the growth of five phytopathogenic fungal strains: Aspergillus niger, Corynespora cassiicola, Colletotrichum sp., Fusarium oxysporum and Pyricularia oryzae conforms to some current antifungal drug targets, and thus has the potential to be developed into a future biological pesticide.

In the current study, the tea tree essential oils was tested alone or in combination with standard antifungal against different strains of fungi. The results showed the tea tree essential oils possesses the ability to modulate the actions of against fungi strains. With the increasing incidence of medicine resistant fungi strains, exploration of natural products from plants represents an interesting alternative therapy, since they may modulate the action of medicine resistant, either by increasing or decreasing their activities. Furthermore, the activity of some antifungal agents was potentiated (synergistic effect) upon the addition of the essential oils into the growth medium at inhibitory concentrations. The antimicrobial activity of gentamicin, ciprofloxacin and amikacin against resistant strains of **P. aeruginosa** was significantly modulated by the essential oils when added at a sub-inhibitory concentration. Diterpenes found in the essential oils have been reported as modifiers of antibiotic activity (Nicolson et al. , 1999), suggesting that its use could represent an advance against drug resistance mechanisms (Rajyaguru et al. , 1998).

Hence, combination of essential oils and antibiotics offers a promising alternative strategy for the treatment of infectious diseases.

Tea tree essential oils can be regarded as a new original plant biopesticide that is highly efficient, broad spectrum and safe. The practical application of tea tree essential oils as a biological pesticide should involve consideration of the application modes and time according to the different fungi rates (weather, temperature and other properties). Besides, some additives to the tea tree essential oils also should be developed to reduce its volatilization. Using tea tree essential oils individually or combination of tea tree essential oils and carbendazim are effective in inhibiting the development of fungal pathogens in plants. The study also gave a suitable ratio of the concentration of tea tree essential oils and carbendazim to effectively inhibit the plant pathogenic fungus in the most optimal and cost saving manner by using essential oils, and limiting the use of chemical drugs causing environmental pollution.

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Authors' contributions

Thi Thu Huong NGUYEN and Thi Thuy Tien LE conceived and designed the experiments, discussed the results, and designed the figures with Thanh Khang LE, and was the overall supervisor of the experiments; Thanh Khang LE wrote the paper, managed the fungal strains, did additional experiments with Thi Thu Huong NGUYEN and Thi Thuy Tien LE for manuscript revisions, and contributed ideas in discussing the results. In addition, all authors contributed to the analysis of the paper.

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