

# *In Vitro and In Vivo Inhibition of *Cylindrocladium reteaudii* by Essential Oils of *Acorus calamus* Rhizomes*

Phanin Sintawarak<sup>1</sup>, Suwimon Uthairatsamee<sup>2</sup>, and Tharnrat Keawgrajang<sup>2\*</sup>

<sup>1</sup>Department of National Parks, Wildlife and Plant Conservation, Chatuchak, Bangkok 10900, Thailand

<sup>2</sup>Department of Forest Biology, Faculty of Forestry, Kasetsart University, Bangkok 10900, Thailand

## ARTICLE INFO

Received: 29 Apr 2020  
Received in revised: 23 Aug 2020  
Accepted: 23 Sep 2020  
Published online: 5 Nov 2020  
DOI: 10.32526/ennrj/19/2020076

### Keywords:

*Acorus calamus*/ Leaf blight/  
Biocontrol/ *Eucalyptus*/  
Antifungal activity

### \* Corresponding author:

E-mail: ffortrk@ku.ac.th

## ABSTRACT

*Cylindrocladium reteaudii* (Bugnic.) Boesew. is a severe pathogen which can cause leaf blight disease in *Eucalyptus* seedlings in tropical countries. This study investigated the antifungal activity of essential oils extracted from *Acorus calamus* L. rhizomes in inhibiting the growth of *C. reteaudii*, both in *in vitro* and *in vivo* experiments. The extraction of essential oils from rhizomes was carried out by hydro-distillation technique and the *in vitro* antifungal testing was done by using the poisoned food technique. The results indicated that an essential oil concentration of 2,000 ppm can completely inhibit the fungal growth with a 50% inhibitory concentration value of 54.76 ppm. For the *in vivo* experiment, it was found that an essential oil concentration of 500 ppm and Captan<sup>®</sup> of 1,000 ppm were not significantly different in inhibiting the growth of *C. reteaudii*. However, these two treatments significantly inhibited the fungal growth ( $p < 0.05$ ) when compared with the control treatments. Physiological and anatomical characteristics were investigated to check for the antifungal activity after the application of essential oils. Results showed that essential oil spraying had no effect on the leaf transpiration rate and temperature of the *Eucalyptus* seedlings, but the incident disease ratio was high when an essential oil concentration of more than 1,500 ppm was applied. Therefore, it can be inferred that the essential oils from *A. calamus* rhizomes at an optimum concentration can be an efficient antifungal compound with a potential to control leaf and shoot blight diseases in *Eucalyptus* seedlings in a nursery.

## 1. INTRODUCTION

Owing to a growing demand for forest products and environmental services, the area under planted forests is likely to increase continuously all the world (FAO, 2016). *Acacia* and *Eucalyptus* are short rotation cycle trees which constitute the main planted species in forests around the South and Southeast Asia. Such planted forests are distributed in approximately 7 million ha in these regions (Harwood and Nambiar, 2014). The area under *Eucalyptus* plantations worldwide is about 20 million ha, out of which 0.5 million ha is located in Thailand (Manavakun, 2014). As the plantation area is increasing dramatically every year, so is the threat from pests and pathogens.

Leaf and shoot blight caused by *Cylindrocladium* - an anamorph of *Calonectria* species, is one of the most severe fungal diseases in regions

experiencing high precipitation in Southeast Asia and South America and is a particularly serious problem in *Eucalyptus* plantations and nurseries (Kang et al., 2001; Crous, 2002; Rodas et al., 2005; Thu et al., 2010; Devi, 2011). Previous reports indicate that the mortality rate in *Eucalyptus* plantations were between 60-100% in Vietnam (Old et al., 2003). Resulting from this fungal pathogen, hybrid clonal *Eucalyptus* cutting production in commercial forest nurseries in China decreased drastically (Zhou et al., 2008). Pongpanich (1998) reported that *Cylindrocladium reteaudii* causes leaf blight and damping-off disease in *Eucalyptus* seedlings in Thailand with frequent outbreaks in nurseries. Consequently, this fungus has become a serious disease, especially plaguing the commercial *Eucalyptus* nurseries (Pongpanich et al., 2010). However, this fungus affects a broad range of host

**Citation:** Sintawarak P, Uthairatsamee S, Keawgrajang T. *In Vitro and In Vivo inhibition of *Cylindrocladium reteaudii* by essential oils of *Acorus calamus* Rhizomes*. Environ. Nat. Resour. J. 2021;19(1):34-42. (<https://doi.org/10.32526/ennrj/19/2020076>)

plants and is an important pathogen found in agricultural and forest plants (Kang et al., 2001; Crous, 2002; Old et al., 2003; Lombard et al., 2010a). Recently, subtropical and tropical regions became a hot spot for *Calonectria* (= *Cylindrocladium*), with several new species having been reported (Lombard et al., 2010a; Lombard et al., 2010b; Alfenas et al., 2015; Lombard et al., 2015; Lombard et al., 2016; Liu and Chen, 2017; Pham et al., 2019; Wang et al., 2019). However, the disease control and management of these fungi is still poorly understood.

Many fungicides, like carbendazim, benomyl, and captafol, have been used to control the extent and severity of the disease in nurseries (Mohanani, 2014). However, such fungicides can have negative impacts on people's health and the surrounding environment (Rouabhi, 2010). Recently, biological control has been used instead of chemical control for disease management. Biocontrol agents have been used to control this disease which is plaguing *Eucalyptus* plantations, especially by *Cylindrocladium* spp., in which *Trichoderma hazianum* can be used as a biocontrol agent to inhibit the growth of *C. quinqueseptatum*, which is responsible for the damping-off disease (Mohanani, 2007). The rhizobacterium *Pseudomonas fulva* has been effective in inhibiting the growth of *C. candelabrum* while promoting rooting and growth of *Eucalyptus* (Mafia et al., 2009). Moreover, *Streptomyces ramulosus* has been considered as a potential biocontrol agent in controlling *Eucalyptus* leaf and shoot blight caused by *Cylindrocladium* sp. (Himaman et al., 2016).

Essential oils, like *Cinnamomum verum*, *Curcuma longa*, *Cymbopogon martini*, *Pimpinella anisum*, *Vetiveria zizanioides*, and *Acorus calamus*, are plant extracts, and can be another form of biological agents effective against fungi causing such diseases (Bansod and Rai, 2008; Sharma et al., 2009). Tabassum and Vidyasagar (2013) and Uma et al. (2017) reported that essential oils have a comparable efficiency to fungicides but have no side effects on the users. *Acorus calamus* (Acoraceae), commonly known as sweet flag, is a semi-aquatic plant. It is a perennial monocot with a creeping and highly branched aromatic rhizome (Kamil et al., 2017). The rhizome of *A. calamus* and its essential oils are widely used in the flavoring industry. In addition, it was found that the essential oils extracted from *A. calamus* are efficient in combating many plant fungal pathogens such as *Curvularia lunata*, *Rhizoctonia bataticola*, *Sclerotium rofskii*, and *Uromyces* spp. (Radušienė and Pečiulytė,

2008; Sharma et al., 2009; Kithan and Daiho, 2014; Rita et al., 2017; Roy and Yonzune, 2018). The essential oil extracted from the rhizome of *A. calamus* contains  $\beta$ -asarone and  $\alpha$ -asarone, which are the dominant antimicrobial components in *A. calamus* (Phongpaichit et al., 2005; Devi and Ganjewala, 2009). However, the knowledge about the antifungal activity of *A. calamus* against *C. reteaudii* is still limited. Moreover, most of previous studies have reported on the efficacy under laboratory conditions. Therefore, this study aimed to evaluate the efficiency of essential oils extracted from the rhizome of *A. calamus* to inhibit the growth of *C. reteaudii*, when used in both the *in vitro* and *in vivo* techniques. This study also compared the physiological effects of using rhizome extracted essential oils with an efficient and widely used commercial fungicide to the *Eucalyptus*.

## 2. METHODOLOGY

### 2.1 Essential oil extraction

One year old rhizomes of *A. calamus* were collected from the Bang Bua Thong District, Nonthaburi Province, central Thailand (13°58'22.8"N 100°22'10.2"E). They were air dried to a constant weight. The dried rhizomes were then extracted for their essential oils through hydro-distillation for 3 h. The distilled oils were dried by adding anhydrous sodium sulfate to remove water and kept in sealed glass tubes at 4°C. Finally, the essential oils were obtained at a concentration between 3.67-5.00 (%v/w).

### 2.2 Fungal isolation and the preparation of fungal pure culture

*Eucalyptus* leaf samples, affected by blight disease, were collected at the nursery managed by the Siam forestry Co. Ltd, Kanchanaburi Province, western Thailand (14°15'25.9"N 99°44'56.0"E). *C. reteaudii* was isolated by using the tissue transplanting method on Potato Dextrose Agar (PDA) media and sub cultured on PDA to obtain a pure culture. They were then identified at the species level according to the procedure outlined in Crous (2002). Additionally, the pure culture of *C. reteaudii* was checked for its pathogenicity according to Koch's postulates.

### 2.3 *In vitro* antifungal activity of the essential oils

The efficiency of *A. calamus* essential oil in inhibiting the growth of *C. reteaudii* was examined by the poisoned food technique (Sharma et al., 2009). Cultured media were prepared by dissolving *A. calamus* essential oil in a 2% dimethyl sulfoxide

(DMSO) solution. Different concentrations of essential oil were used, viz. 31.25, 62.5, 125, 250, 500, 1,000, and 2,000 parts per million (ppm). PDA medium and 2% DMSO incorporated PDA medium were used as control treatments. Mycelium plugs having 8 mm diameter were obtained from four day old cultures and were placed at the center of the plate. Five replications were maintained for each simulation. The cultures were incubated at room temperature ( $30\pm 2^{\circ}\text{C}$ ). When the *C. reteaudii* colony on control treatments (only PDA and 2% DMSO) grew to the size of a Petri-dish, the colony diameter was measured and the percent inhibition was calculated according to the formula:

$$\% \text{ Inhibition} = [(A - B) / A] \times 100$$

Where; A is the growth of colony fungus (cm) in the PDA medium and B is the growth of the colony fungus (cm) in treatment media.

The percent inhibition was compared by a one-way ANOVA and the means were compared using the Duncan's new multiple range test (DMRT) at a confidence level of 95%.

## 2.4 In vivo antifungal activity of the essential oils

### 2.4.1 Plant material

Shoots of *Eucalyptus* hybrid clone H4 (*Eucalyptus camaldulensis* x *E. urophylla*), of 10 cm length, were cut and shoots with two mature leaves, leaf and branch skin, without any lesions caused by diseases and insects, were selected. The bottom 1 cm of the selected shoots was cut and dipped in 500 ppm of Indole-3-butyric acid (IBA) and talcum powder. Each shoot was then placed in a seedling pot containing sterilized coconut fiber and osmocote fertilizer. The seedling pots were placed in the nursery and a constant humidity (air humidity about 80-90%) was maintained by spraying water three times a day until the *Eucalyptus* cuttings were two months old. Subsequently, the antifungal activity of the derived essential oils was examined.

### 2.4.2 Experimental design and data measurement

The experimental setup was a completely randomized design (CRD) consisting of eight treatments: sterile water, 2% DMSO, Captan® 1,000 ppm (fungicide) and essential oil at 50 ppm, 500 ppm, 1,000 ppm, 1,500 ppm, 2,000 ppm. Three replicates were used for each treatment.

Before the experiment was conducted, each simulation was covered with a polypropylene bag to protect any cross-contamination from other replicates. In each seedling, photos of *Eucalyptus* leaves were taken by a digital camera (Cannon EOS 5D Mark III) and the leaf surface area was determined by the Image J software (Aboukarima et al., 2017). Ten mL of *C. reteaudii* spore suspension ( $5 \times 10^5$  spore/mL) was then sprayed onto the seedling. After an incubation time of 24 h, each treatment was sprayed with 30 mL of different solutions according to the experimental design at 7 p.m. and was sprayed every 3 days and continuously sprayed 3 times. The total duration was 11 days since the spore suspension of *C. reteaudii* was sprayed. After the last spray, the leaf surface area of the *Eucalyptus* seedling was measured again and the disease ratio calculated as per the formula below:

$$\text{Disease ratio} = \text{diseased leaf area} / \text{total leaf area (cm}^2\text{)}$$

Finally, the disease ratio determined during each treatment was analyzed by One-way ANOVA and means were compared by DMRT at a significance level of 95%.

## 2.5 Physiological and anatomical characteristics of *Eucalyptus* seedlings

The transpiration rate and mean leaf surface temperature of the *Eucalyptus* seedlings were investigated to check for the antifungal activity after the application of essential oils. To measure the transpiration rate, water was added to each potted seedling until saturation was reached and was then wrapped with polythene. Pots containing seedlings were weighed at 6.00 a.m. and 6.00 p.m. After determining the loss in weight, an amount equal to the water used during the day was added. The transpiration rate was calculated following the formula below:

$$\text{Transpiration rate} = (W_s - W_d) / \text{LSA},$$

Where;  $W_s$  is the weight of the pots at 6.00 a.m.,  $W_d$  is weight of the pots at 6.00 p.m. and LSA is leaf surface area. To determine the average leaf temperature, three leaves were randomly selected to measure the temperature by using a Testo 830-T1 infrared thermometer at 6:00 a.m., 9:00 a.m., noon (12:00), 3:00 p.m., and 6:00 p.m. Individual leaf temperatures measured each day were used to obtain an averaged leaf temperature. All the data were

analyzed by a One-way ANOVA and the means were compared using DMRT at a significance level of 95%.

## 2.6 Anatomical investigation of leaf characters

The effect of essential oils on the anatomy of *Eucalyptus* leaf was also conducted. Three leaf specimens of each treatment were randomly selected from three treatment groups, i.e., sterile water, 500 ppm, and 2,000 ppm. Thus, nine leaves were prepared in total. The cross section of leaves was prepared by using a freezing microtome. The thin cross-section leaf was selected under stereo microscope (Zeiss Stemi 508) for preparing the semi-permanent slide. The observation of leaf anatomical characteristic was done under the compound microscope (Zeiss Axioskop 40).

## 3. RESULTS

### 3.1 *In vitro* antifungal activity of the essential oils

All concentrations of essential oils derived from *A. calamus* were able to inhibit the growth of *C. reteaudii* *in vitro* (Table 1). When the concentration of essential oil was high, the percentage of growth

inhibition increased (Table 1). However, at 2,000 ppm, the growth of *C. reteaudii* was completely inhibited with the corresponding IC<sub>50</sub> value (50% inhibition concentration) being 54.76 ppm.

**Table 1.** Percentage growth inhibition of *C. reteaudii* and 50% inhibition concentration (IC<sub>50</sub>) by *A. calamus* essential oils at various concentrations

Treatment		Percentage of growth inhibition (mean±SD)
<i>A. calamus</i> essential oil	31.25 ppm	36.44 <sup>a</sup> ±2.14
	62.50 ppm	50.22 <sup>e</sup> ±0.50
	125 ppm	52.22 <sup>e</sup> ±0.79
	250 ppm	56.00 <sup>d</sup> ±2.02
	500 ppm	76.56 <sup>c</sup> ±3.25
	1,000 ppm	79.22 <sup>b</sup> ±1.01
	2,000 ppm	100.00 <sup>a</sup> ±0.00
Control	Only PDA	0.00 <sup>h</sup> ±0.00
	2% DMSO	0.00 <sup>h</sup> ±0.00
IC <sub>50</sub> (ppm)		54.76

Note: SD=standard deviation; means in the column with similar letters are not statistically significant from each other at  $p>0.05$ .



**Figure 1.** *In vivo* evaluation of the essential oil extracted from *A. calamus* rhizomes against *C. reteaudii*, *Eucalyptus* leaf and shoot blight pathogen. (A=sterile water, B=2% DMSO, C=50 ppm, D=500 ppm, E=1,000 ppm, F=1,500 ppm, G=2,000 ppm, H=Captan® 1,000 ppm)

### 3.2 *In vivo* antifungal activity of the essential oils

The disease ratio was significantly different among treatments ( $p<0.05$ , Table 2). Spraying essential oil at 500 ppm was not significantly different in inhibiting the growth of *C. reteaudii* when compared to spraying Captan® at 1,000 ppm. These

two treatments had a leaf disease ratio which was less than the control treatments. In addition, the results showed that spraying the essential oil at a concentration of 1,500 and 2,000 ppm increased the *C. reteaudii* diseases in the *Eucalyptus* seedlings, with a disease ratio 0.40 and 0.41, respectively (Figure 1).



**Table 2.** Disease ratio of *Eucalyptus* seedlings of various concentrations of essential oil

Treatments		Disease ratio (mean±SD)
<i>A. calamus</i> essential oils	50 ppm	0.24 <sup>b</sup> ±0.01
	500 ppm	0.11 <sup>a</sup> ±0.06
	1,000 ppm	0.25 <sup>b</sup> ±0.07
	1,500 ppm	0.40 <sup>c</sup> ±0.01
	2,000 ppm	0.41 <sup>c</sup> ±0.01
Chemical	Captan® 1,000 ppm	0.11 <sup>a</sup> ±0.02
Control	Sterile water	0.22 <sup>b</sup> ±0.01
	2% DMSO	0.26 <sup>b</sup> ±0.09

Note: SD=standard deviation; means in the column with similar letters are not statistically significant from each other at  $p>0.05$ .

### 3.3 Effect of *A. calamus* essential oil on the physiological and anatomical characteristics of *Eucalyptus* seedlings

The results indicated that the transpiration rate per leaf surface area and the mean leaf temperature were not significantly different across treatments ( $p>0.05$ , Table 3). Although spraying essential oil at a concentration of 1,000 and 1,500 ppm resulted in increasing the disease ratio, the two concentrations had no effect on the transpiration rates and leaf temperatures of the seedlings. Our results showed that spraying the essential oil at a concentration of 2,000 ppm affected the structure of mesophyll layer. The cellular shrinkage in the mesophyll layer of the leaf had lost the intercellular space (Figure 2).

**Table 3.** Comparison the transpiration rate and leaf daily temperature of *Eucalyptus* seedlings between before and after the application in different treatments

Treatments		Transpiration rate (mg/cm <sup>2</sup> )		Leave daily temperature (°C)	
		Before	After	Before	After
<i>A. calamus</i> essential oil	50 ppm	55.57	26.13	27.47	28.80
	500 ppm	56.13	27.65	27.54	28.51
	1,000 ppm	58.87	36.26	27.53	28.85
	1,500 ppm	48.76	33.17	27.62	28.35
	2,000 ppm	53.71	33.16	27.48	28.59
Chemical	Captan® 1,000 ppm	50.81	27.60	27.71	28.47
Control	Sterile water	59.98	28.64	27.51	28.65
	2% DMSO	51.22	24.09	27.70	28.27
		ns	ns	ns	ns

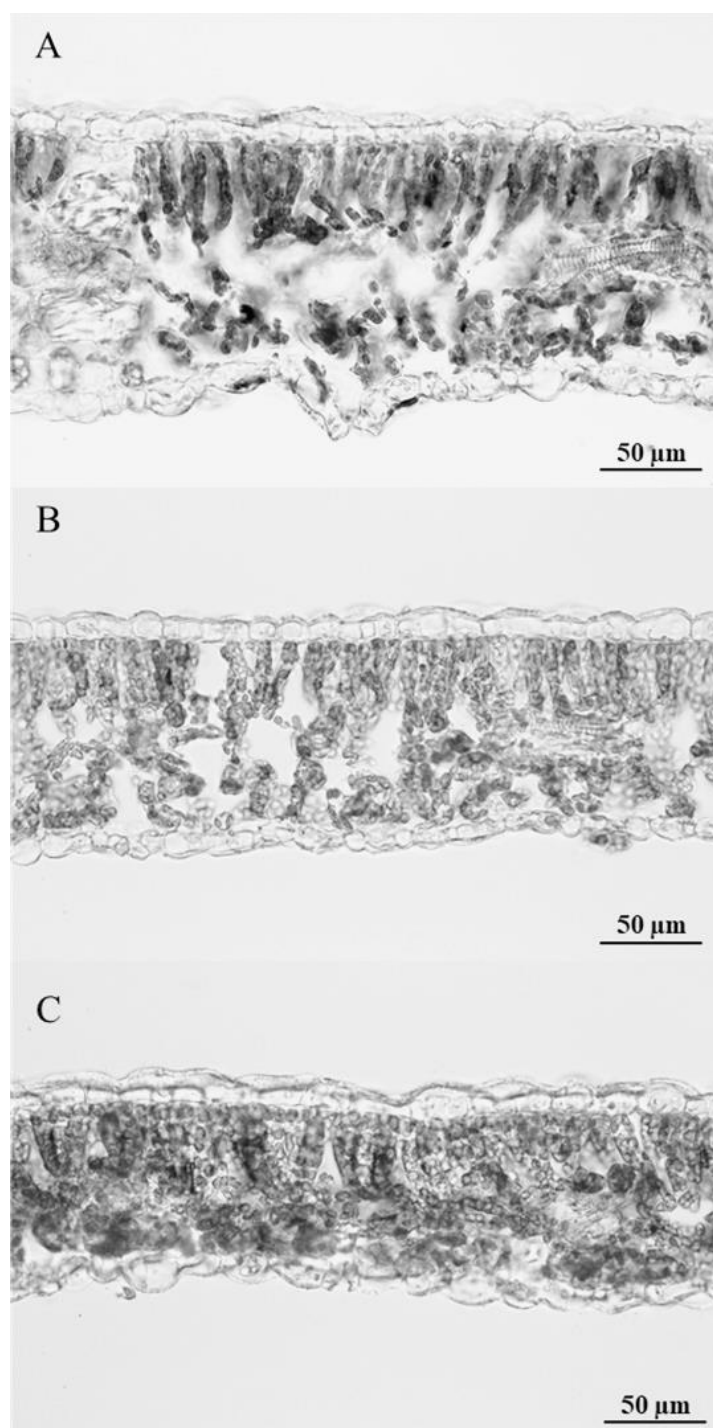
Note: Means (n=3) in column are not statistically significant at  $p>0.05$ .

## 4. DISCUSSION

*Cylindrocladium* (=Calonectria) is one of the most severe leaf and shoot blight pathogens affecting *Eucalyptus* trees in South and Southeast Asia (Kang et al., 2001; Crous, 2002; Old et al., 2003; Lombard et al., 2010a). Owing to suitable environmental conditions, several new *Cylindrocladium* taxa were reported in these regions (Liu and Chen, 2017; Pham et al., 2019; Wang et al., 2019). Moreover, these fungi have a wide range of hosts, including agricultural and forest plants. Consequently, disease control of these fungi becomes even more difficult. With regards to *C. reteaudii*, it is one of the most virulent fungal diseases affecting *Eucalyptus* trees (Pongpanich et al., 2010; Devi, 2011; Filho et al., 2018). However, disease management of this pathogen is still poorly understood, particularly after the application of biological agents. Several previous studies have indicated that the essential oils of *A. calamus* are effective in inhibiting fungal pathogens, both in

humans and plants (Radušienė and Pečiulytė, 2008; Sharma et al., 2009; Yami and Shukla, 2016; Rita et al., 2017). Accordingly, our results indicated that *A. calamus* essential oil inhibited the growth of *C. reteaudii*, which causes leaf and shoot blight in *Eucalyptus*. This study is the first of its kind to report the growth inhibition of *C. reteaudii* using essential oils of *A. calamus*, as indicated by a  $IC_{50}$  value of 54.76 ppm. Phongpaichit et al. (2005) previously found that hyphae and conidia shrank and collapsed when treated with the levels of  $\beta$ -asarone from *A. calamus* extracts. Essential oils have a highly complex chemical composition with apparently different mechanisms to inhibit fungal growth.

Generally, the inhibitory activity of *A. calamus* extract against several fungal pathogens in laboratory bioassays have been reported previously (Kithan and Daiho, 2014; Rita et al., 2017; Roy and Yonzune, 2018). Contrastingly, the inhibitory activity in nursery bioassay (*in vivo*), where environmental conditions



**Figure 2.** Cross section of *Eucalyptus* leaf under different treatments (A=sterile water, B=essential oil at 500 ppm, and C=essential oil at 2,000 ppm).

are controlled, is still limited. This study firstly concluded that a concentration of 500 ppm essential oil, obtained from *A. calamus* rhizomes, is efficient in inhibiting the leaf blight disease caused by *C. reteaudii* in *Eucalyptus* seedlings. Moreover, this concentration of essential oils was found as effective as double the concentration of Captan® (1,000 ppm) and can be used as an alternative to it. Captan® is one of the most efficient fungicides and is widely used for

controlling *Eucalyptus* diseases in commercial nurseries, especially in Thailand. However, Captan® has many ill-effects on human health and the environment (Gordon, 2010; Rouabhi, 2010). Therefore, the essential oil of *A. calamus* extract can be used as an alternative to control the disease in *Eucalyptus* seedlings without compromising the safety of humans and the environment. Furthermore, the pathogens find it hard to build sufficient resistance

against essential oils, and thus it has a potential as an environmentally friendly biofungicide (Derbalah et al., 2012).

Several beneficial activities of essential oils from plant extract have been reported, with the potential in controlling various fungal disease species being one of the most intensively published in literature. However, there are few studies focusing on toxicity of the essential oil on the plant, particularly from the *A. calamus* essential oil. According to previous reports, the transpiration rate and photosynthesis of plants is affected when essential oils are applied (Baker, 1970; Frender, 2017). Our results indicated that the seedlings had a high disease ratio after the application of essential oils at concentrations above 1,500 ppm. Moreover, we found that the mesophyll layer of *Eucalyptus* leaves was collapsed in intercellular spaces (Figure 2), which is an important structure for exchanging gases during photosynthesis, and respiration of the plant. Furthermore, the essential oils can cause electrolyte leakage, which can disrupt the cell membrane and result in loss of integrity (Kaur et al., 2010; Poonpaiboonpipat et al., 2013). Baker (1970) reported that plant processes, including photosynthesis, respiration, and transpiration, are disturbed when high concentrations of oils are applied. Therefore, our results indicated that the seedlings might be susceptible to fungal infections causing diseases when applying high concentrations of the essential oil. Moreover, burnt leaf and twig of *Eucalyptus* seedlings was observed 24 h after the foliage was sprayed with *A. calamus* essential oil at a concentration >2,000 ppm (data not published). The essential oils from some plant species have phytotoxicity, which can effect germination and growth of several monocots and dicots (Poonpaiboonpipat et al., 2013; Ibáñez and Blázquez, 2018; Yoshida et al., 2018; Smeriglio et al., 2019). The essential oil of *A. calamus* has been previously shown to inhibit seed germination and growth of seedlings of *Lactuca sativa* and *Lolium perenne* (Satyal et al., 2013). These previous works, however, did not provide information regarding susceptibility to plant disease.

This study indicates the potential use of essential oil from rhizome of *A. calamus* as an effective biocontrol agent, showing strong inhibitory effects on the growth of *C. reteaudii* both in laboratory and nursery bioassays. In laboratory bioassay, the recommended concentration of the *A. calamus* essential oil was 2,000 ppm for inhibiting growth of

*C. reteaudii* *in vitro*. While, 500 ppm of the *A. calamus* essential oil was recommended to control the leaf blight disease caused by *C. reteaudii* in *Eucalyptus* seedlings, the essential oil should be used at recommended concentration every three days and continuously sprayed three times. However, the optimum concentration of essential oil should be determined carefully as a high concentration (more than 1,500 ppm) might damage the leaves making them more prone to the fungal disease. This finding warrants further research on the fungicidal effects of *A. calamus* against other *Cylindrocladium* species and phytotoxicity of the essential oil from *A. calamus* on the growth and physiological process of *Eucalyptus*.

## 5. CONCLUSION

The potential of *A. calamus* essential oil as a biocontrol agent was conducted both in laboratory and nursery conditions. Our results confirmed the fungicidal effects of *A. calamus* prevent *C. reteaudii* from causing leaf and shoot blight in *Eucalyptus*. However, an essential oil concentration of 500 ppm applied every three days and continuously sprayed three times is recommended to control the growth of *C. reteaudii* under nursery conditions. Contrastingly, more than 1,500 ppm of essential oil could damage the seedlings increasing disease incidence.

## ACKNOWLEDGEMENTS

This work was funded by the National Research Council of Thailand (Year 2018). A young researcher scholarship for graduate student was awarded to the first author. We would like to sincerely thank the Siam forestry Co. Ltd, Kanchanaburi Province, western Thailand for all the kind support. We thank the two referees for their invaluable comments and suggestions which helped in improving our manuscript substantially. The manuscript has been thoroughly reviewed with the kind help of Dr. Tushar Andriyas, Center for Material Sciences, Institute of Interdisciplinary Studies, and University of Allahabad, India.

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