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INHIBITION OF *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.CAUSE ANTHTRACNOSE DISEASE IN LIME [*Citrus Aurantifolia* (Christm.) Swingle] PLANT USING ENDOPHYTIC AND EXOPHYTIC FUNGI *IN VITRO*

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ABSTRACT

The aim of the research to determine in vitro inhibitory of endophytic and exophytic (phylloplane) fungi from healthy leaves of lime plants against *Colletotrichum gloeosporioides* that sauses anthracnose disease in the lime plants on Kertalangu village, District of East Denpasar, Bali. The research was conducted from January to July 2015. The study used a survey method on three plots lime plants belonging to local farmers and plant samples used were taken by diagonal method. Each plot was taken five healthy plants. *Colletotrichum gloeosporioides* isolats obtained results of identification based on microscopic morphology. The results showed that endophytic fungi can be isolated as much as three types include *Aspergillus flavus*, *Cylindrocladium* sp., and *Phytophthora* sp. Antagonistic test results from three species only *A. flavus* against *C. gloeosporioides* amounted to 83.93±7.39%. While the exophytic fungus which can be isolated as much as six types included Aspergillus flavus with inhibition 92,86±1,33; *Aspergillus niger* with inhibition of 88.87±3.23%, *Aspergillus* spp. (78,57±10,10%), *Culvularia* sp. (66,67±2,41%), *Fusarium* sp. (85±2,34), *Nigrospora* spp. (66,45±2,48), and *Penicillium* spp. (89,27±9,05%). The highest prevalence of endophytic was A. flavus as big as 80%, and exophytic fungi found in *Nigrospora* spp. amounted to 43,48%. Based on the inhibition ability of fungi that have potential as biological agents to control *C. gloeosporioides* was *Aspergillus flavus*, dan *Penicillium* spp., *A, niger*, dan *Fusarium* sp.

Keywords: Endophytic and exophytic fungi, *Colletotrichum gloeosporiodes*, inhibition ability, prevalence, and lime [*Citrus aurantifolia* (Christm.)

INTRODUCTION

Failure of lime plant cultivation can not be separated from the attack of pests and pathogens. One of the diseases that are now found is the anthracnose disease. This disease has damaged the lime plant leaves at Kertalangu vellage, East Denpasar. According to USDA (2013) the pathogen of anthracnose disease in lime survive from year to year on dead twigs and leaves in mature lesions. Pathogens infect only young

tissue, than spores spread by rain splash. Along with the leaves dry up on lime along with a large amount of inoculums that can be making it difficult to control athracnose.

The possibility of disease dissemination through conidia, although appresoria, fragments of hyphae, thick-walled cells resemble appresorium can also play a part. Local dispersal discovered more or less by rain splash, with propagules sometimes passing season in the soil so that it can affect the plant the following year. Dissemination distance due to human influences that allow a spread, and contribute to the rapid spread of this fungus in the current year (CABI and EPPO, 1994).

Anthacnose caused by *Colletotrichum acutatum* is one of the most frequently reported diseases, species of the genus cause disease in a wide range of plants in the world. Initially described the disease comes from *Carica papaya, Capsicum frutescens*, and *Delphinium ajacis* in Australia by Simmonds (1965). Now the disease was found in lime plant at Kertalangu village, East Denpasar. The disease has never been indentified pathogen, and how to control. Inhibition testing of endophytic and exophytic fungi against pathogen is needed as a first step to finding potential biological agents that can be used as a alternative control is effective, efficient and environmentally friendly.

Endophytic fungi in taxonomy and biology of diverse but all colonize plant tissues without causing danger inside looks against its host (Wilson, 1995). Beneficial effects for the host include improved tolerance to drought, protect from insects, protects against nematodes (especially the roots) and resistance faced pathogenic fungi (Gwinn and Gavin, 1992). While the exophytic (phylloplane) fungus very greatly in size, density and type. Depending on a number of biotic and abiotic factors that affect the growth and vitality. Exophytic fungus can be as pathogenic fungi growth barrier (Bakker *et al.,* 2002).

MATERIALS AND METHODS

Place and Time Research

Research carried out with two stages: the first stage survey at Kertalangu vellage, District of East Denpasar. The second stage, doing research in the laboratory. Laboratory of Plant Pathology, and laboratory of Biotechnology, Faculty of Agriculture, Udayana University. The study was conducted from January to June 2015.

Isolation of Endophytic and Exophytic Fungi

Isolation of endophytic fungi with cut lime leaves healthy covering an area of 1 cm^2 each five pieces to a Petri dish, repeated three time. Before the healthy leaf cut, the leaves are cleaned with a solution Natriumhypochloride/NaClO (Bayclin) 10%, then rinsed with sterile water, placed in a 70% alcohol after it is rinsed with sterile water. Healthy leaf pieces were placed on PDA medium (previously plus antibacterial Livofloxacin 25% w/v) in a Petri dish. Endophytic fungal mycelium will grow after two

to three days. Father purified by placing each fungal colony on the new PDA medium. The same was done for the isolation of fungi exophytic, but leaves healthy without washing with a solution as mentioned above. Furthermore endophytic and exophytic fungi grown at room temperature (27±2^{o}C).

Identification of Pathogen, Endofitic and Exophytic Fungi

The pathogen, endophytic and exophytic fungi further identified the shape and color of the colony, and microscopic morphology, covering conidiophores, conidia (spores) forms and fruiting bodies. Pictures of pathogen, endophythic and exophytic fungi that obtained, subsequently matched with corresponding reference books (Samson *et al*., 1981; Pitt dan Hocking, 1997; Barnett dan Hunter, 1998; Indrawati *et al*., 1999; Gams dan Bissett, 2002; Samson *et al*., 2011; Visagie *et al*., 2014).

Pathogenicity Test

Pathogenicity test was required for certain microbes found true as plant pathogens, healthy fresh leaves of lime plant placed in a closed Petri dish containing wet tissue paper pads. Leaves first cleaned and then stabbed with a needle spelden then dipped into a spore suspension with a density of 10^{7}, and healthy leaf without treatment was also placed into different Petri dish as a control.

Inhibition Test

All endophytic and exophytic fungi te4sted for inhibitory against pathogens with a dual culture method (in a Petri dish there was more than one isolate) were grown in a Petri dish. Inhibition can be calculated using the following formula ((Dolar, 2001; Mojica-Marin *et al*., 2008):

$$(\%)\ \text{inhibition} = \frac{A - B}{A} \times 100$$

Where:

A = Colony diameter of pathogen in single culture (mm)

B = Colony diameter of pathogen in dual culture (mm)

Inhibiting ability of endophytic and exophytic fungi against pathogen was calculated every day, until the control (single culture of pathogen) and observation halted until single culture of pathogen has fulfilled the Petri dish. Inhibition mechanism of antagonistic fungi against pathogenic fungi can be known whether it competition or antibiosis, by observing the presence and absence of inhibition zones in Petri dish. If the was any clear zone (zone of inhibition) mean inhibition by antibiosis mechanism.

The Prevalence (Frequency of Isolates)

The prevalence (frequency of isolates) can be calculated by the formulation that was the number of a certain type of fungus isolates were found to be shared with the rest of the fungus were found time 100%. The highest prevalence would illustrate the dominance of certain fungi in certain places.

RESULTS AND DISCUSSION

Symptom and Pathogen of Anthracnose

The observation of symptoms of anthracnose disease on lime plants showed that the symptoms occur on leaves that have been ahead of the old, this was due to pathogen infection process takes place very slowly. Infections have aoccured in young leaves but symptoms have not appeared (symptomless). With age, the leaves began to appear as chlorosis symptoms, which develop into necrosis, severe attack causes the leaves turn brown and fall (Figure 1A). White mycelium of pathogens growing such as cotton on PDA in Petri dishes.

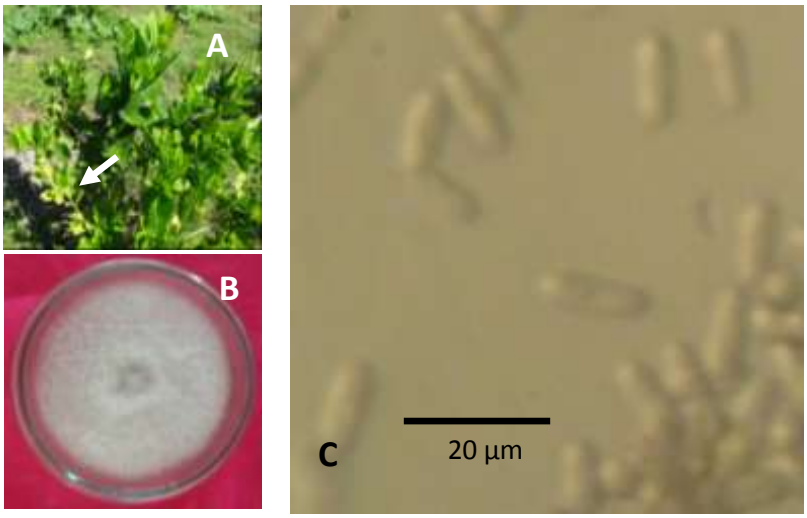


Figure 1. (A) Symptom of anthracnose in lime plant (arrow), (B) colony in Petri dish, and (C) conidia of *Colletotrichum gloeosporioides*

Microscopic morphology observations showed that conidia appear oval with rounded edges each blunt. Based on the search reference that containing a variety of *Colletotrichum* species, such as *C. capsici* conidia curved shape (McKenzie, 2013), *C. acutatum* conidia tapered at both ends; *C. candidum* conidia ovate, slightly shorter, and *C. coccodes* conidia oval and long (Schnabel et al., 2006); whereas *C. gloeosporioides* conidia tapering at the ends of each round (dam et al., 2010; Weir et al., 2012), exactly the same as the cause anthracnose pathogen observations on lime leaves. Conidia size range of 5-10 x 10-15 µm (Figure 1C and 1D). Based on the search reference (Weir et al.,

2012) and matched with the discovery of the pathogen from the isolation it can be concluded pathogen causes anthracnose disease on the leaves of lime comes from Kertelangu village, East Denpasar was a *Colletotrichum gloeosporioides*.

Inhibition of Endophytic Fungi

The results showed that there were three types of endophytic fungi were found to include, *Cylindrocladium* sp., *Phytophthora* sp., and *Aspergillus flavus.* From third endophytic fungi only *A. flavus* that have inhibitory effect on *C. gloeosporioides* in vitro (Figure 2), amounting to 83.93±7.39%. So does the prevalence of the fungus *A. flavus* as endophytic fungi was the highest at 80% (Table 1; Figure 3).

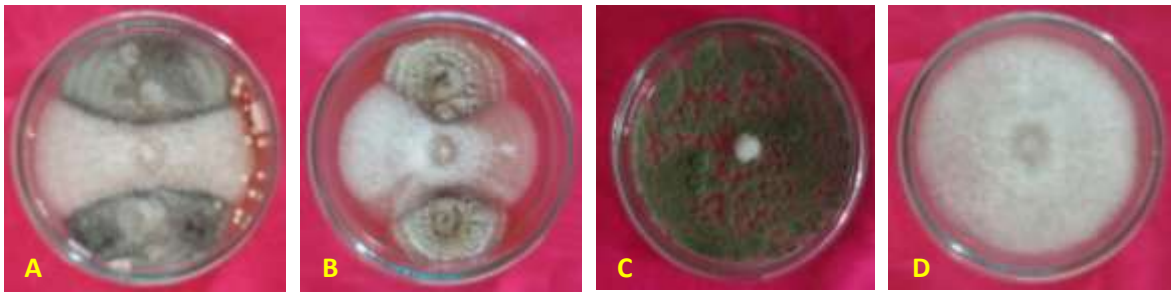


Figure 2. Inhibition of endophytic fungi (A) *Cylindrocladium* sp., (B) *Phytophthora* sp., and (C) *Aspergillus flavus*, and (D) control (*Colletotrichum gloeosporioides*), 5 days age after inoculation.

Table 1. Prevalence and inhibition of endophytic fungi on *C. gloeosporioides* in vitro

Endophytic fungi	Prevalensce (%)	Inhibition (%)
Cylindrocladium sp.,	10	-
Phytophthora sp.	10	-
Aspergillus flavus	80	83,93±7,39

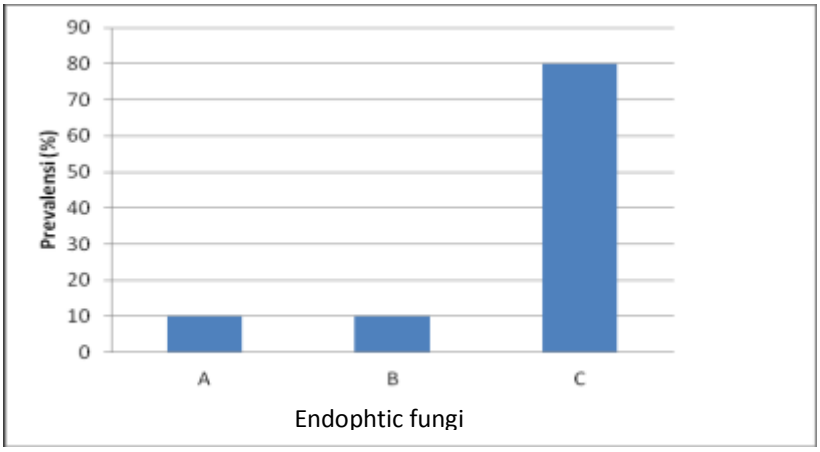


Figure 3. Prevalence of endophytic fungi (A = *Cylindrocladium* sp., B = *Phytophthora* sp., and C = *Aspergillus flavus*)

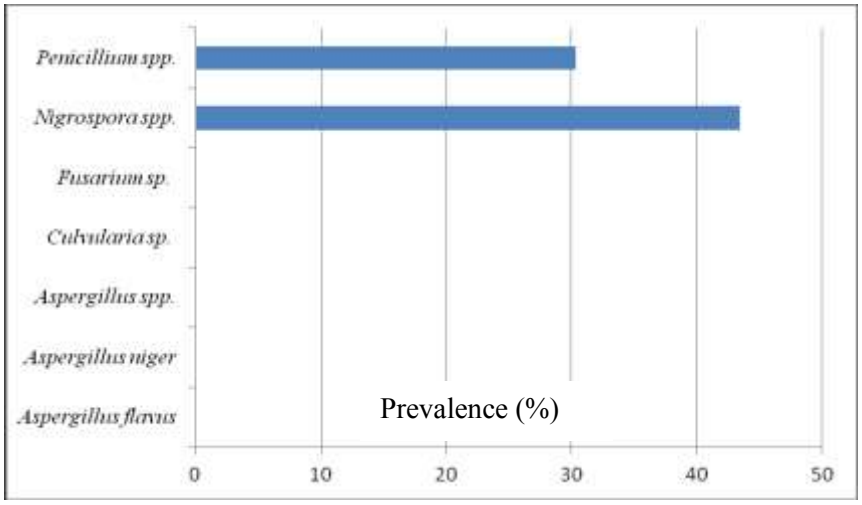
Aspergillus flavus, a fungus commonly found in seeds of walnut plant (*Tetracarpidium conophorum* (Mull.Arg.) Hutch. and Diaz.) in Nigeria (Africa) (Amadi, 2005). The results of the research by Basha *et al.* (2010) states the *A. flavus* as a endophytic fungus was able to inhibit *C. gloeosporioides* (the cause anthracnose disease in pepper) amounted to 70.2%. Based on inhibition test and prevalence of A flavus against pathogens, gives an indication that the endophytic fungus have great potential to be developed as biological control agents.

Inhibition of Exophytic Fungi

A total of seven kinds of exophytic fungi ie, *A. flavus, A. niger, Aspergillus* spp., *Culvularia* sp. *Fusarium* sp., *Nigrospora* spp., and *Penicillium* spp. Prevalence and inhibition of exophytic fungi against *C. gloeosporioides* more as shown in Table 2; Figure 4 and 5).

Table 2. Prevalence and inhibition of exophytic fungi against *C. gloeosporioides* in vitro

Endophytic fungi	Prevalence (%)	Inhibition (%)
Aspergillus flavus	0,04	92,86±1,33
Aspergillus niger	0,04	88,87±3,23
Aspergillus spp.	0,09	78,57±10,10
Culvularia sp.	0,04	66,67±2,41
Fusarium sp.	0,04	85±2,34
Nigrospora spp.	43,48	66,45±2,48
Penicillium spp.	30,34	89,27±9,05



Gambar 4. Prevalence of exophytic fungi

The highest prevalence was found in *Nigrospora* spp. amounting to 43.48%, followed by *Penicillium* spp. amounting to 30.34%, while other fungi prevalence was very small in phylloplane lime. Fungi as described above was also found in phylloplane of medical plants (Prabaskaran *et al*., 2011). Inhibition of exophytic fungi against *C.*

gloeosporioides, as well as the potentially very good as biological agents were *Aspergillus flavus, Penicillium* spp., *A. niger*, and *Fusarium* sp. with percentage inhibition 92.86±1.33%, 89.27±9,05%, 88.87%, and 85±2.34%, respectively (Table 2; Figure 5).

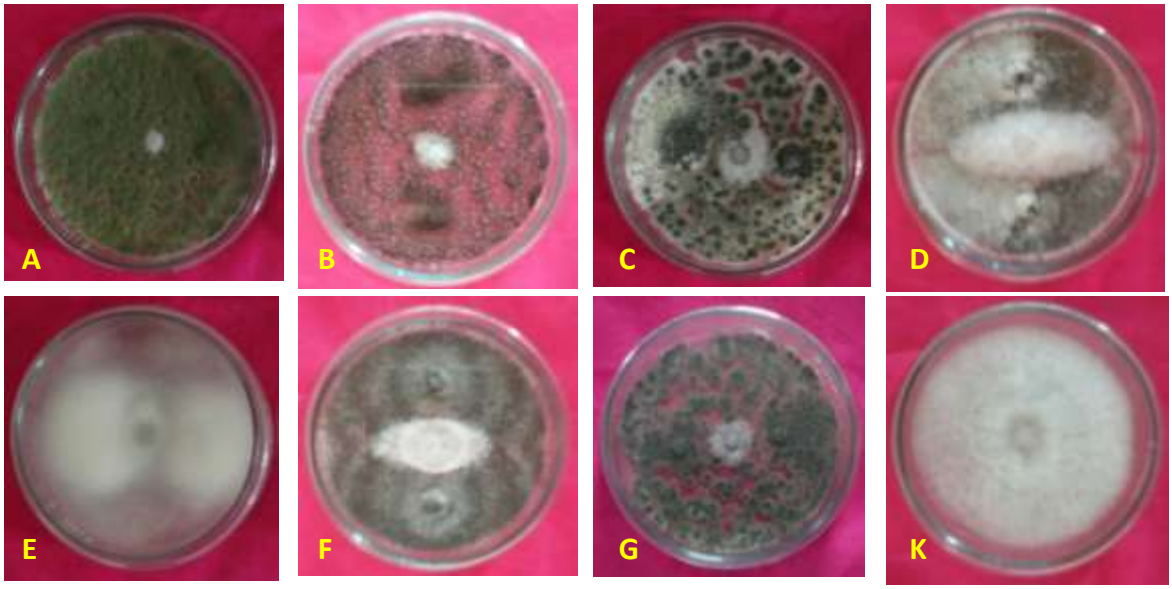


Figure 5. Inhibition of exophytic fungi against *C. gloeosporioides*, (A) *Aspergillus flavus*, (B) *A. niger*, (C) *Aspergillus* sp., (D) *Culvularia* sp., (E) *Fusarium* sp., (F) *Nigrospora* sp., (G) *Penicillium* sp., and (K) control (*C. gloeosporioides*), 5 days after inoculation.

Aspergillus flavus has also been proven as phylloplane can inhibit the growth of *Alternaria brassicae* (causes Alternaria spot on the leaves of cabbage) with inhibition maximum compared to other fungi (Yadav *et al*., 2011). *Aspergillus fumigates*, *Fusarium* sp., and *Culvularia clavata* also found as phylloplane fungi in the castor plant (Ricinus communis L.) silkworm food woody plants (Borgohin et al., 2014). The *Aspergillus niger*, *Aspergillus* sp., and *Culvularia* sp. also been found as phylloplane fungi on medicinal plants family of Ficaceae (Dalai, 2014). Aspergillus niger can maximally inhibit the growth of the pathogen of Alternaria leaf spot (*Alternaria alternata*) Akaskara plant (Spilanthes oleracea) (Thakur and Harsh, 2014). While *Aspergillus* sp. as phylloplane fungi beed used as biological agents to control *C. gloeosporioides* causes anthracnose on rubber tree (*Hevea brasiliensis* Muell. Arg.) (Evueh and Ogbebor, 2008).

CONCLUSION

Based on the results of this study concluded that endophytic fungi can be isolated as much as 3 types included *Aspergillus flavus*, *Cylindrocladium* sp., and *Phytophthora* sp., From three species only *A. flavus* inhibition against *C. gloeosporioides* of 83.93±7.39%. While the exophytic fungi which can be isolated as much as six types

include, *Aspergillus flavus* with inhibitory it gained 92.86±1.33%; *Aspergillus niger* with inhibition of 88.87±3.23%, *Aspergillus* spp. (78.57±10.10%), *Culvularia* sp. (66.67±2.41%), *Fusarium* sp. (85±2,34%), *Nigrospora* spp. (66.45±2.48), and *Penicillium* spp. (89.27±9.05%). The highest prevalence of endophytic fungi were *A. flavus* by 80%, and the highest prevalence of the exophytic fungi were *Nigrospora* spp., amounted to 43,48%, and *Penicillium* spp., amounted to 34.34%. Based on the inhibitory power fungus as a potential biological agents for controlling *C. gloeosporioides* was *A. flavus* and *Penicillium* spp., *A. niger* and *Fusarium* sp.

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