INTERNATIONAL JOURNAL OF
RESEARCH AND CURRENT DEVELOPMENT
Available Online at http://www.journalijrcd.com
VOL. 2, ISSUE, 3, PP.107-111, MARCH, 2017

IJRCD

ISSN: XXX-XXXX RESEARCH ARTICLE

DIVERSITY AND POTENTIAL OF EXOPHYTIC FUNGI AS BIOLOGICAL AGENT TO CONTROL COCOABLACK POD DISEASE CAUSED BY [*PHYTOPHTHORA PALMIVORA* (BUTLER) BUTLER] IN VITRO

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ARTICLE INFO

Article History:

Received 5th December, 2016
Received in revised form 12th January, 2016
Accepted 16th February, 2017
Published online 28th March, 2017

Keywords:
Exophytic fungi, inhibition ability, diversity index, dominance index, and prevalence.

ABSTRACT

The aims of the research to know exophytic fungus which has ability as a biological agent to control the cocoa black pod disease caused by *Phytophthora palmivora*. The results showed that exophytic fungus on the leaves was faund *Aspergillus flavus* (3 isolates), *A. niger* (6), *Aspergillus* spp. (3) and *Mortierella* spp. (9). Exophytic fungus on rind were *Penicillium* spp. (3), *Neurospora* spp. (12) and *Trichotecium* spp. (3), while the exophytic fungus on stem were *Trichoderma* spp. (27), *Nigrospora* spp. (3), *A. niger* (3), *Mortierella* spp. (9) and *Neurospora* spp. (15). Inhibition test the exophytic fungus against *P. palmivora* showed that *Aspergillus* spp. (96.67±2.36%), *A.flavus* (96.67±5.93%), *A. niger* (91.67±8.50% to leaf exophytic fungi, and 98.33±2.5% to stem exophytic fungi), *Neurospora* spp. (89,33±5,01% to rind exophytic fungi and 93.33±2.32% to stem exophytic fungi), and *Trichoderma* spp. (88.33±10.12%). The diversity index showed that leaves exophytic fungal at 1.7675, to rind exophytic fungusat 2,02281, and to stem exophytic fungusat 1.31894. Only to leaf and stem exophytic showed dominance index were 0.6939 and 0.67590 respectively. While the dominance index to rind exophytic funguswas 0,5000, means diversity evenly. Prevalence of leaves exophitic funguswas *Mortierella* spp at 42.86%, followed by *Trichoderma* spp. (47.37%) on stem exophytic, and the achieved the highest by *Neurospora* spp. (66.67%) on rind exophytic.

INTRODUCTION

Cocoa grown in Indonesian 532.000 ha in 1999. More than 70% of cocoa farmers are sharecroppers in Indonesian. Indonesian cocoa exporter number three in the world, the production of 335.000 tons/year, which is equivalent to 294 million US dollars (Purwantara *et al.*, 2004). The success of cocoa production has now been challenged in the form cocoa black pod disease outbreak. Pod rot disease is very dangerous disease for cocoa plants today (Vanegtern *et al.*, 2015), in fact this is the most important disease in most cocoa producing countries (Semangun, 1991). The disease is an average 20-30% cocoa crop damage worldwide each year. In some cases, such as American Semoa, cocoa is not grown commercially for this disease (USDA, 2012).

Cocoa is influenced by several fungal diseases and viruses as well as black pod disease which is a major cause of failure of most of the cocoa plant in State of Sri Lanka. According to the researchers history black pod disease caused by *Phytophthora palmivora* (De Silva *et al.*, 2005). There are least four species of *Phytophthora* include *P. palmivora, P. capsici, P. citropthora* and *P. megakarya* has been recorded as the major species causing black pod disease in India and Africa. Besides, there are other species, namely *P. katsurae* that can be isolated from the cocoa in Ivory Coast (De Silva *et al*., 2005).

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Fungal exophytic is a fungus that can live saprophytic surface but does not cause disease in plant. Fungal phylloplane is a fungus that grows on the plants surface (Langvard, 1980). There is a group of fungal phylloplane: resident and causal. Resident can multiply on the surface of healthy leave without affecting the host while causal is landed on the surface but cannot grow (Leben, 1965).

MATERIALS AND METHODS

Pace and Time Research

The research was conducted in two places: 1) search for diseased plant specimens, and healthy from cocoa plants grown in the area Tabanan, 2) laboratory of Plant Pathology and Laboratory of Agricultural Biotechnology. The study was conducted from April until August 2017.

Exophytic Fungus Isolation

Fungal exophytic used in this study came from the cocoa plant collected from the area Tabanan. Survey cocoa leaf, stem and fruit from four different locations in the centre of cocoa cultivation Tabanan regency. Leaves, stems and fruit is picked soaked in sterile aqua water and shaken for one hour. The result of the agitation the pipetted 1 ml of water, poured

into a Petri dish had previously been filled with PDA (potato dextrose agar), into a Petri dish added livoploxasin antibiotic with a concentration of 0.1% (w/v). After two to three days colonies were calculated by colony forming unit (cfu) to determine the number of colonies per plant. Furthermore, purified fungal colonies in PDA media for 3 days. Furthermore, the fungus is identified by microscopic morphology. Colonies may be stored in a refrigerator before use to determine inhibition test *in vitro.*

Identification of Exophytic Fungi

Exophytic fungi that stored subsequently grown in Petri dishes containing PDA and repeated 5 times. Culture were incubated in the dark at room temperature (±27^{o}C). Isolates were identified macroscopically after 3 days old to determine the colony colour and growth rate, and the identification of microscopically to determine septa in hyphae, form spores/conidia and sporangiophore. Fungus identification using reference books Samson *et al*., 1981; Pitt and Hocking, 1997, Barnett and Hunter, 1998, and Indrawati *et al*., 1999).

Prevalence of Exophytic Fungi

Determine the prevalence of fungal exophytic based on the frequency of fungal isolates were found (leaves, stems and fruits) per Petri dish and shared with all isolates were found times 100%.

Determine the Diversity and Dominance Index

Diversity and dominance fungal exophytic can be determined by calculating the Shannon-Wiener diversity index (Odum, 1971) and the domination by calculating the Simpson index (Pirzan and Pong-Cook, 2008).

Diversity index

Exophytic fungal diversity index was determined by the Shannon-Wiener diversity index was formula (Odum, 1971).

$$H' = - \sum_{i=1}^{s} Pi \ ln \ Pi.$$

Where:
H' = Shannon-Wiener diversity index
S = number of genera
Pi = pi/P as proportion of all i (pi = total number of individual types of microbes total i, P = total number of individuals in total p)

The criteria used to interpret the diversity of Shannon-Wiener (Ferianita-Fachrul *et al*., 2005), namely: H' value is <1, meaning low diversity, H' value 1-3 means diversity classified as moderate and H' values >3 means diversity classified high.

Dominance index

Dominance index of exophytic fungi was calculated with counting the Simpson index (Pirzan and Pong-Cook, 2008), with the following formula:

$$C = \sum_{i=1}^{S} Pi^2$$

Where:
C = Simpson index
S = number of genera
Pi = pi/P ie, the proportion of an individual type i and all individual (pi = total number of individual types i, P = total number of individual in total p).

Furthermore, dominance index (D) can be calculated by formula 1-C (Rad *et al*, 2009).

The criteria used to interpret the dominance of species of fungi are: near 0 = low or lower index is dominated by a single species of fungus or not there is extreme species dominate other species.

Inhibition Ability of Exophytic Fungi

Exophytic fungi that found each tested for inhibitory to the growth of pathogenic fungi (*P. palmivora*) with dual culture technique (in a Petri dish grown one pathogen flanked by two exophytic fungi). Ability inhibition can be calculated as follows (Dollar, 2001; Mojica-Marin *et al.*, 2008).

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

Where: A = *Phytophthora palmivora* colony diameter in a single culture (mm)

B = *Phytophthora palmivora* colonies diameter in dual culture (mm).

RESULTS AND DISSCUSSION

The Diversity, Dominance and Prevalence of Exophytic Fungi

Exophytic fungal were found on health cocoa leaf include *Aspergillus flavus*(3 isolates), *A. niger*(6), *Aspergillus* spp. (3) and *Mortierella* spp. (9). Rind exophytic fungus were found: *Penicillium* spp. (3), *Neurospora* spp. (12) and *Trichotecium* spp. (3). While stem exophytic fungi were found: *Trichoderma* spp. (27), *Nigrospora* spp. (3), *A. niger* (3), *Mortierella* spp. (9) and *Neurospora* spp. (15) (Table 1).

Table 1 The diversity, dominance. and prevalence of exophytic fungus

Fungi name	Leave exophytic fungus	Rind exophytic fungus	Stem exophytic fungus
Aspergillus spp.	3 (14.29%)*	-	-
Aspergillus flavus	3 (14.29%)	-	-
Aspergillus niger	6 (28.57%)	-	3 (0.05%)
Mortierella spp.	9 (42.86%)	-	9 (15.79%)
Neurospora spp.	-	12 (66.67%)	15 (26.32%)
Nigrospora spp.	-	-	3 (0.05%)
Penicillium spp.	-	3 (16.67%)	-
Trichoderma spp.	-	-	27 (47.37%)
Trichotecium spp.	-	3 (16.67%)	-
Total	21	18	57
Diversity index	1.7675	2.02281	1.31894
Dominance index	0.6939	0.5000	0.67590

*Prevalenceis number of isolates divided by total isolates times 100%

Prevalence exophytic fungus on the leaves of cocoa found in *Mortierella* spp. amounting to 42.86%, the skin of the fruit was found in *Neurospora* spp. 66.67%, while on the trunk was found in *Trichoderma* spp. amounted to 47.37% (Table 1). The diversity index that achieved of leaves, rind, and stem exophytic fungi each 1.7675, 2.02281, and 1,31384 respectively. Just on leaves and stem exophytic fungus the dominance index of 0.6939 and 0,67590 respectively. While diversity of rind exophytic fungus is relatively evenly distributed so that the dominance index 0.5000. In rind exophytic fungus dominance looked at *Neurospora* spp. and *Trichotecium* each 66.67%, respectively, so dominance index of 0.5000.

The results were consistent with that found that *Trichoderma viride* and *Aspergillus flavus* have colonies interaction with the percentage of maximum barrier against *Alternaria brassicae* (causes brown spot of the leaves cabbage). Effect of volatile and non-volatile compounds from *T. viride* give maximum emphasis on growth of pathogenic hyphae (Yadav *et al.*, 2011). *Aspergillus fumigatus* was a fungus that is also found in castor phylloplane (Borgohain *et al*., 2014). *Aspergillus flavus, Penicillium janthinellum* and *Aspergillus* sp. was isolated from phylloplane medicinal plants (Prabakaran *et al*., 2011). *Aspergillus niger, Trichoderma* and *Aspergillus* sp. was fungus isolated from medicinal plants, Ficaceae family (Dalal, 2014). Fungus of *T. koningii, A. niger* and *A. flavus* that dominates leaves Dark (*Butea monosperma* (Lamk.) Tabub) (Chauhan *et al.*, 2014).

Based on the diversity index obtained ranges from 1-3, meaning the diversity index medium (Table 2, 3, and 4). While the dominance index ranges from >0.5 in the leaves and stems exophytic fungus means there fungus that dominates so obtained dominance index >0,5 (Table 3), the fungus was *Montierella s*pp. (42.86%) and *Trichoderma* spp. (47.37%). *Trichoderma* highest nomination in stem phylloplane mean fungi exophytic that interact with the surface of the rod so as to protect the stem from pathogenic attack disorder (Table 4).

Aspergillus spp. (96.67±2.36%), *A.flavus*(96.67±5.93%), *A. niger*(91.67±8.50% in leaves phylloplane and 98.33±2.5% in stem phylloplane), *Neurospora* spp. (89.33±5.01% in rind phylloplane and 93.33±2.32% in stem phylloplane), and *Trichoderma* spp. (88.33±10.12%) (Table 6). Exophytic fungal can inhibit pathogen above 75%, it means a very strong inhibition against pathogens *in vitro.*

Aspergillus niger, Aspergillus sp. and *Trichoderma* were a fungus that grows on the leaf surface (phylloplane) medicinal plants of family Ficaceae. This fungus can protect plants from pathogens that cause disease (Dalal, 2014).

Further Thakur and Harsh (2014) states that the fungus *T. harzianum* cause maximum inhibition, while *A. niger* shows the minimum inhibitory against pathogen that cause Alternaria leaf spot disease. According Evueh and Ogbebor (2008) *Aspergillus* sp., was a phylloplane fungus that is able to make lyses cytoplasm *Colletotrichum gloeosporioides* on potato dextrose agar medium. Nayak (2015) states that *Penicillium digitatum, Trichoderma* sp., and *A. niger* were a fungus phylloplane that can be isolated from leaves of the mango (*Mangifera indica*). Thakur and Harsh (2016) suggest that *T. harzianum* ISO-1, *T. harzianum* ISO-2, and *A. niger* was able to inhibit the growth of *Alternaria alternata* causes leaf spot disease on *Rauwolfia serpentine in vitro*. Moreover, the results of research Angela and Sri (2016) found that *Aspergillus, Trichoderma* and *Penicillium* isolated from surface sterilized leaves from four plants (*Datura alba, Curcuma longa, Hibiscus rosasinensis*, and *Rauwolfa serpentine*). Nayak (2015) explains that *A. flavus, A. niger* and *Trichoderma harzianum* were isolated from the leaves of medicinal plants, *Solanum ningrum* with two different techniques.

CONCLUTIONS

Based on the research results can be concluded that exophytic fungus on the leaves was found *A.flavus* (3 isolates), *A. niger* (6), *Aspergillus* spp. (3) and *Morterella* spp. (9). Rind exophytic fungus found: *Penicillium* spp. (3), *Neurospora* spp. (12) and *Trichotecium* spp. (3).While stem exophytic

Table 2 The Diversity, and dominance index leaves exophytic fungus

Fungi name	pi	pi/P	Ln pi	Pi/ P x Ln pi	$(pi/P)^2$
Aspergillus spp.	3	0.142857143	1.098612289	0.156944613	0.020408163
A. flavus	3	0.142857143	1.098612289	0.156944613	0.020408163
A. niger	6	0.285714286	1.791759469	0.511931277	0.081632653
Mortirella spp.	9	0.428571429	2.197224577	0.941667676	0.183673469
Total	21			1.767488178*	0.306122449**

*Diversity index, and **dominance index = 1-0.306122449

Table 3 The diversity, dominance index rind exophytic fungus

Fungi name	pi	pi/P	Ln pi	pi/P x Ln pi	$(pi/P)^2$
Neurospora spp.	12	0.666666667	2.48490665	1.656604433	0.444444444
Penicillium spp.	3	0.166666667	1.098612289	0.183102048	0.027777778
Trichotecium spp.	3	0.166666667	1.098612289	0.183102048	0.027777778
Total	18			2.022808529*	0.5000**

*Diversity index, and **dominance index = 1- 0.5000

Ability inhibition exophytic fungi on Phytophthora palmivora

Based on the observations found that the exophytic fungus which showed inhibitory effect on *P. palmivora* were

Table 4 The diversity, dominance index stem exophytic fungus

Fungi name	pi	pi/P	Ln pi	pi/P x Ln pi	$(pi/P)^2$
Aspergillus niger	3	0.052631579	1.098612289	0.057821699	0.002770083
Mortierella spp.	9	0.157894737	2.197224577	0.346930196	0.024930748
Neurospora spp.	15	0.263157895	2.708050201	0.71264479	0.069252078
Nigrospora spp.	3	0.052631579	1.098612289	0.057821699	0.002770083
Trichoderma spp.	27	0.473684211	3.295836866	0.143721983	0.224376731
Total	57			1.318940368*	0.324099723**

*Diversity index,and *Dominance index = 1 – 0.0324099723

Table 6 Inhibition ability of exophytic fungus on *Phytophthora palmivora*

Fungi name	Leaves exophytis fungus (%)	Rind exophyutic fungus (%)	Stem exophytic fungus (%)
Aspergillus spp.	96.67±2,36*	-	-
Aspergillus flavus	96.67±5,93	-	-
Aspergillus niger	91,67±8,50	-	98,33±2,5
Mortierella spp.	-	-	-
Neurospora spp.	-	89,33±5,01	93,33±2,32
Nigrospora spp.	-	-	-
Penicillium spp.	-	-	-
Trichoderma spp.	-	-	88,33±10,12
Trichotecium spp.	-	-	-

*inhibition ability >75% it mean very strongly.

fungus found: *Trichoderma* spp. (27), *Nigrospora* spp. (3), *A. niger* (3), *Mortierella* spp. (9) and *Neurospora* spp. (15). Having testing the results of inhibitory power against *P. plamivora* was *Aspergillus* spp. (96.67±2.36%), *A.flavus*(96.67±5.93%), *A. niger*(91.67±8.50% in leaves phylloplane and 98.33±2.5% in stem surface), *Neurospora* spp. (89.33±5.01% in rind surface and 93.33±2.32% in stem surface), and *Trichoderma* spp. (88.33±10.12%). Prevalence exophytic fungus on the leaves of cocoa found in *Mortierella* spp. amounting to 42.86%, the skin of the fruit was found in *Neurospora* spp. 66.67%, while on the trunk was found in *Trichoderma* spp. amounted to 47.37%. The diversity index that achieved of leaves, rind, and stem exophytic fungus each 1.7675, 2.02281, and 1,31384 respectively. Just on leaves and stem exophytic fungi the dominance index of 0.6939 and 0,67590 respectively. While diversity of rind exophytic fungus was relatively evenly distributed so that the dominance index 0.5000. In rind exophytic fungus dominance looked at *Neurospora* spp. and *Trichotecium* each 66.67%, respectively, so dominance index of 0.5000.

Acknowledgements

Authors wish to thank to the Rector of Udayana University for their assistance and the opportunity given so that research can be resolved, Dean of the Faculty of Agriculture, Udayana University, and Chairman of the Institute for Research and Community Service Udayana University, for their help and cooperation so that research can be funded to completion.

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How to cite this article:
I Made Sudarma and Ni Wayan Suniti,*:* Diversity and Potential of Exophytic Fungi As Biological Agent To Control Cocoablack Pod Disease Caused By [Phytophthora Palmivora (Butler) Butler] In Vitro. International Journal of Research and Current Development 2017; 2(3): 107-111.