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Exophytic and Endophytic Fungus that Potential as Biocontrol Agents on *Lasiodiplodia theobromae* caused Fruit Rot at Sugar-Apple

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ABSTRACT

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Fruit rot disease of sugar apple (*Annona squamosa* L.) caused by *Lasiodiplodia theobromae*. The exophytic fungus found on leaves, fruits and twigs is *Aspergillus* sp. *A. niger*, *Fusarium* sp., *Mycelia sterillia*, *Neurospora* sp., and *Rhizopus* sp. whereas in the endophytes of the leaves, fruits and twigs are *Fusarium* sp., *Penicillium* sp., *Neurosporas* sp., and *Mycelia sterillia*. The diversity and dominance index of the exophytic fungi are 2,3742 and 0.8667, while the diversity and dominance index of endophytic fungi is 2.6356 and 0.6489. Ability inhibitory of antagonistic against *Lasiodiplodia theobromae in vitro*, from exophthalic and endophytic fungi ranged from $65.68 \pm 0.82\%$ to $88.35 \pm 0.46\%$. The highest was obtained from *Aspergillus* sp. fungi of $88.35 \pm 0.46\%$ and lowest by *Aspergillus* sp. of $65.68 \pm 0.82\%$. The results of *in vivo* inhibitory tests exophytic and endophytic fungus against the *Lasiodiplodia theobromae* highest obtained from *Aspergillus* sp. and *A. niger* fungi each pressed by 100%.

Introduction

Sugar-apple fruit rot disease caused by *Lasiodiplodia theobromae* was a very dangerous fruit disease. Approximately 60% of fruits are attacked by pathogens and when it was attacked it was very difficult to control (Sudarma, and Suniti, 2018).

Exophytic or Phyloplane fungus was a fungus that grows on the leaf surface (Langvad, 1980). There are two groups of Phyloplane fungus; resident and causal (Norse, 1972).

Resident may multiply on the surface of healthy leaves without affecting the host, whereas the causal lands on the surface but not be able grow (Leben, 1965). Phyloplane fungus is poorly studied compared to endophytes, saprobe, and pathogenic fungi. Within a few years microbial phyloplane studied there appeared to be interactions with plants, herbivores and leafy pathogens, possibly related to the immune system, organic reabsorption and mineral materials from leachetes, the main redistribution of nutrients to falling leaves and participation in

primary degradation of plant tissue (Saha *et al.*, 2013). Yadav *et al.*, (2011) found that growing phytoplane mushrooms such as *Trichoderma viride* and *Aspegillus flavus* can suppress the maximum of *Alternaria brassicae* on cabbage leaves.

There is now evidence to suggest that in some cases endophytic fungi restrict the growth of cacao pathogens or *in vitro* and *in vivo* destruction (Arnold *et al.*, 2003), this result is a bright light for development as a new source of biocontrol agents to combat cacao pathogens. Endophytic fungi are taxonomically and biologically diverse but all share a character colonizing inner plant tissue without causing visible harm to its host (Wilson, 1995). The beneficial effects for the host include increased tolerance to drought, protecting from eating insects, protecting against nematodes and resistance to pathogenic fungi (Gwinn and Gavin, 1992). Last also found true endophytic on tropical grass. Endophytic-mediated anti-pathogen protection has been observed in host plants rather than gramineae. Examples of endophytic fungi are found to protect tomatoes (Hallman and Sikora, 1995) and bananas (Pocasangre *et al.*, 2001) from nematodes, and green beans and berries from pathogenic fungi. Mejfa *et al.*, (2008) states that endophytic fungi can decrease pathogenic attacks on grasses and other host plants, little is known about the role in natural systems and whether they can be exploited as biocontrol strategies in crop protection. Therefore the authors are interested to examine the parasitic fungus exophytic and endophytic as biocontrol agents against *L. theobromae* causes fruit rot disease in sugar-apple plants.

Materials and Methods

Place and time of research

The research was conducted in two places: 1) looking for sick, healthy plant specimens

from cocoa planted in Bukit Jimbaran area. 2) Laboratory of Plant Disease Science and Agricultural Biotechnology Laboratory. The study was conducted from April to August 2018.

Isolation of endophytic and exophytic fungus

Isolation of endophytic fungi, plant parts such as fruit, leaves and stems were washed with sterile water flowing, then the plant part was strawed with 0.525% sodium hypochlorite for 3 minutes, 70% alcohol for 2 minutes, then sprinkled with sterile water for 1 minute and subsequently placed on PDA media (firstly given antibiotic antibiotics ielivoploxasin with a concentration of 0.1% (w/v).

Mushrooms emerging from leaf fragments are transferred to test tubes containing PDA media to be stored and classified through morphospesies. While eksofit mushrooms can be done by spraying the plant (fruit, leaves and stems). The wash water is collected, then in the tube, then taken, from a 1 ml tube grown into a PDA previously filled with livoploxasin with a concentration of 0.1% (w / v).

Identification of Endophytic and Exophytic Fungus

The endophytic and exophytic fungus are exfused then grown on a Petri dish containing the PDA and repeated 5 times. The culture is cubed in a dark room at room temperature ($\pm 27^{\circ}\text{C}$).

Isolates were identified macroscopically after 3 days to determine colony color and growth rate, and microscopic identification to determine septa in hyphae, spore/conidia and sporangiophore. Fungal identification using reference book Samson *et al.*, (1981), Pitt and Hocking (1997), Barnett and Hunter (1998), and Indrawati *et al.*, (1999).

Inhibitory test of endophytic and exophytic fungus against pathogens

The endophytic and exophytic fungi found respectively were tested for their inhibitory resistance to the growth of pathogenic fungi with dual culture techniques (in one Petri dish grown each of a single pathogenic fungus flanked by two endophytic or exophytic fungi).

The inhibitory power can be calculated as follows (Dollar, 2001; Mojica-Marin *et al.*, 2008):

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

Where:

A = Diameter of *P. palmivora* colony in single culture (mm)

B = Diameter of *P. palmivora* colony in dual culture (mm)

Prevalence of endophytic and exophytic fungus

Determining the prevalence of endophytic and exophytic fungus was based on the frequency of endophytic and exophytic fungal isolates found (leaves, stems, flowers and fruit) per Petri dish, divided by all isolates found 100% times. The magnitude of the prevalence of isolates will determine the dominance of endophytic and exophytic fungi present in healthy sugar-apple plant parts.

Determining Diversity and Domination Indices

The diversity and dominance of contaminant fungi can be determined by calculating the Shannon-Wiener diversity index (Odum, 1971) and soil microbial dominance calculated by calculating the Simpson index (Pirzan and Pong-Masak, 2008).

Index of microbial diversity

The soil microbial diversity index is determined by the Shannon-Wiener diversity index by the formula (Odum, 1971):

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

Where:

H' = Diversity index of Shannon-Wiener

S = Number of genera

P_i = n_i/N as the proportion of species to i (n_i = total number of individuals total microbial type i, N = total number of individuals in total n)

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

Dominance index

The soil microbial dominance index was calculated by calculating Simpson index (Pirzan and Pong-Masak, 2008), with the following formula:

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

Where:

C = Simpson index

S = Number of genera

P_i = n_i/N as the proportion of species to i (n_i = total number of individuals total microbial type i, N = total number of individuals in total n)

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

The criteria used to interpret the dominance of the soil microbial type are: close to 0 = low index or lower domination by one microbial species or no species that extreme dominates other species, close to 1 = large index or tends to be dominated by some microbial species (Pirzan and Pong-Cook, 2008).

***In vivo* antagonist test**

An *in vivo* antagonistic test of endophytic and exophytic fungi was found by piercing fresh fruit with spelden needles 20 times, then smeared with antagonistic fungal spores (spore one Petri dish in 250 ml sterile aquades), then dipped into mushroom spore suspension pathogens.

Endophytic and exophytic fungi are found, among others:

K+P = control without pathogen

A = antagonistic treatment 1 (spore suspension 5×10^7)

B = antagonistic treatment 2 (spore suspension 5×10^7)

C = antagonistic treatment 3 (spore suspension 5×10^7)

D = antagonistic treatment 4 (spore suspension 5×10^7)

E = antagonistic treatment 5 (spore suspension 5×10^7)

F = antagonistic treatment 6 (spore suspension 5×10^7)

K-P = control with pathogen

All treatments were repeated 4 times. The experiments were designed with randomized block design (RAK), and after variance

analysis (ANOVA) followed by the least significant difference test (LSD) at 5% level.

Results and Discussion

Exophytic and endophytic fungus

Exophytic and endophytic fungus derived from fruit, leaves and twigs isolated using a material of 1 g. The types of fungi found are *Aspergillus* sp., *Aspergillus niger*, *Neurospora* sp., *Fusarium* sp., *Rhizopus* sp., *Penicillium* sp., And Mycelia sterillia (Table 1; Fig. 1 and 2).

Fungi that are found to dominate the type exophytic is the fungus *A. niger* and *Rhizopus* sp. with 9 isolates, while at the endophytic fungi that predominates are *Fusarium* sp. and myceliasterillia with 9 isolates. The diversity of exophytic fungi in the phyloplane is the surface above the plant part, and the endophytes in the inner tissues. Endophytes are known to be microbes that live in plants that are neutral or beneficial to host plants. In particular bacteria or fungi, and there may be 3 types: 1) other host pathogens that are not pathogenic in their endophytic affiliation, 2) nonpathogenic microbes, and 3) non-pathogenic pathogens but still able to colonize via selection or genetic alteration (Backman and Sikora, 2008). Endophytic fungi are important and useful as a source of natural bioactive compounds with their potential applications in agriculture, medicine and food industry. Many useful bioactive compounds with antimicrobial, insectidal, cytotoxic and anti-cancer, have been successfully investigated from endophytic fungi. During the long period of co-evolution, friendly relationships have been established between each endophytic and its host.

Some endophytic fungi have the ability to produce some or similar bioactive compounds such as those originating from the host plant.

The bioactive compounds are paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin and diosgenin (Zhao *et al.*, 2010). Phyloplane fungus that exist on the leaf surface, among these fungi are selected to be antagonistic tested facing *Alternaria brassicae* that cause rickshaw leaves on cabbage. Colony interactions were demonstrated by *Trichoderma viride* and *Aspergillus flavus* with the maximum inhibition of *A. brassicae* (Yadav *et al.*, 2011). According to Borgohain *et al.*, (2014) states that there are 11 fungi found and 5 species of fungi that dominate one that

corresponds to the fungus found in this study are *Aspergillus fumigatus* and *Fusarium* sp.

Diversity and dominance index, and Prevalence

The diversity and dominance index of the eco-fungus is 2.374 and 0.8667 respectively. The diversity index with a value of <2.4 means the fungi population is more stable with good category, the dominance index is close to 1, it means there is a dominant *A. niger* mushroom with prevalence of 18% (Table 2).

Table.1 Exophytic and endohytic fungus derived from fruit, leaves and twigs

No.	Exophytic fungus	Number of isolates	Endophytic fungus	Number of isolates
Fruit			Fruit	
1	<i>Aspergillus</i> sp.	3	<i>Fusarium</i> sp.	6
2	<i>Aspergillus niger</i>	9	<i>Penicillium</i> sp.	3
3	<i>Mycelia sterillia</i>	3	<i>Neurospora</i> sp.	3
4			<i>Mycelia sterillia</i>	3
Leaf			Leaf	
1	<i>Aspergillus</i> sp.	6	<i>Fusarium</i> sp.	9
2	<i>Aspergillus niger</i>	6	<i>Neurospora</i> sp.	3
3	<i>Neurospora</i> sp.	3	<i>Aspergillus</i> sp.	3
Twig			Twig	
1	<i>Aspergillus niger</i>	3	<i>Fusarium</i> sp.	6
2	<i>Fusarium</i> sp.	3	<i>Mycelia sterillia</i>	9
3	<i>Rhizopussp</i>	9		

Table.2 Diversity and dominance index, and prevalence in exophytic fungus

No.	Name of fungi	pi	pi/P	LN pi	(pi/P) x ln(pi)	(pi/P)2
1	<i>Aspergillus</i> sp.	9	0,2	2,197224577	0,439444915	0,04
2	<i>Aspergillus niger</i>	18	0,4	2,890371758	1,156148703	0,16
3	<i>Mycelia sterillia</i>	3	0,066667	2,890371758	0,192691451	0,004444444
4	<i>Neurospora</i> sp.	3	0,066667	1,098612289	0,073240819	0,004444444
5	<i>Fusarium</i> sp.	3	0,066667	1,098612289	0,073240819	0,004444444
6	<i>Rhizopussp</i>	9	0,2	2,197224577	0,439444915	0,04
		45		H'= D = 1-0,2533 = 0,8667	2,374211623	0,253333333

Table.3 Diversity and dominance index, and prevalence in endophytic fungus

No	Name of fungi	pi	pi/P	Ln pi	(pi/p) x ln (pi)	(pi/P)2
1	<i>Fusarium</i> sp.	21	0,46667	3,04452244	1,420777138	0,21777778
2	<i>Penicillium</i> sp.	3	0,06667	1,09861229	0,073240819	0,00444444
3	<i>Neurospora</i> sp.	6	0,13333	1,79175947	0,238901263	0,01777778
4	Mesiliasterilia	15	0,33333	2,708050201	0,9026834	0,11111111
		45			2,63560262	0,35111111
	H' = 2,6356, D = 1-0,35111 = 0,6489					

Table.4 The criteria for assessment of environmental quality weighting (Tauruslina *et al.*, 2015)

Diversity index	Community structure conditions	Catagory	Scale
>2,41	Very stable	Very good	5
-2,4	More stable	Good	4
1,21 – 1,8	Quite stable	Medium	3
0,61 – 1,2	Less stable	Bad	2
<0,6	Unstable	Very bad	1

Table.5 Inhibition ability test of exophytic and endophytic fungi in vitro

Origin of fungi	Name of fungi	Inhibion ability (%)
1. Leaf exophytic3	<i>Aspergillus niger</i>	68,64±1,59
2. Leaf exophytic 4	<i>Aspergillus niger</i>	75,15±2,24
3. Leaf exophytic 5	<i>Neurospora</i> sp.	74,69±0,72
4. Fruit exophytic 1	<i>Aspergillus</i> sp.	65,68±0,82
5. Fruit exophytic 3	<i>Aspergillus niger</i>	72,00±0,31
6. Fruit exophytic 5	<i>Aspergillus niger</i>	80,71±1,07*
7. Twig exophytic 2	<i>Aspergillus niger</i>	71,31±0,68
8. Twig exophytic 3	<i>Rhizopus</i> sp.	82,92±0,50*
9. Twig exophytic 4	<i>Rhizopus</i> sp.	76,67±3,27
10. Twig exophytic 5	<i>Rhizopus</i> sp.	82,22±3,27*
11. Leaf endophytic 1	<i>Fusarium</i> sp.	81,85±0,52*
12. Leaf endophytic 2	<i>Neurospora</i> sp.	86,67±3,14*
13. Leaf endophytic 3	<i>Fusarium</i> sp.	78,15±4,19
14. Leaf endophytic 4	<i>Aspergillus</i> sp.	88,35±0,46*
15. Leaf endophytic 5	<i>Fusarium</i> sp.	78,26±1,22
16. Twig endophytic 1	<i>Mycelia sterillia</i>	68,20±1,49
17. Twig endophytic 2	<i>Mycelia sterillia</i>	75,92±2,62
18. Twig endophytic 4	<i>Mycelia sterillia</i>	71,85±0,52

*Forwarded to inhibition ability *in vivo*

Table.6 Inhibition ability test of exophytic and endophytic in vivo

Code	Origin of fungi	Name of fungi	Disease incidence (%)	Inhibition ability (%)
K-P	Control without pathogen		0 a*	100 a*
A	Endofitdaun 4	<i>Aspergillus</i> sp.	0 a	100 a
B	Eksofitbuah 5	<i>Aspergillus niger</i>	0 a	100 a
C	Endofitdaun 1	<i>Fusarium</i> sp.	3 ab	97 ab
D	Endofitdaun 2	<i>Neurospora</i> sp.	7 ab	93 ab
E	Eksofit ranting 5	<i>Rhizopus</i> sp.	15 ab	85 ab
F	Eksofit ranting 3	<i>Rhizopus</i> sp.	30 b	70 b
K+P	Control with pathogen	<i>Lasiodiplodia theobromae</i>	70 c	30 c

Fig.1 Exophytic fungus found in fruit, leaf, and twig sugar-apple

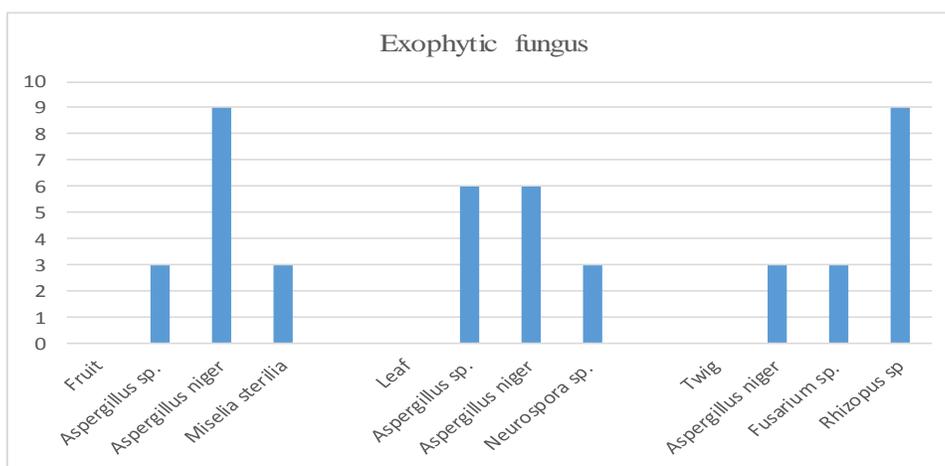


Fig.2 Endophytic fungus found in fruit, leaf, and twig sugar apple

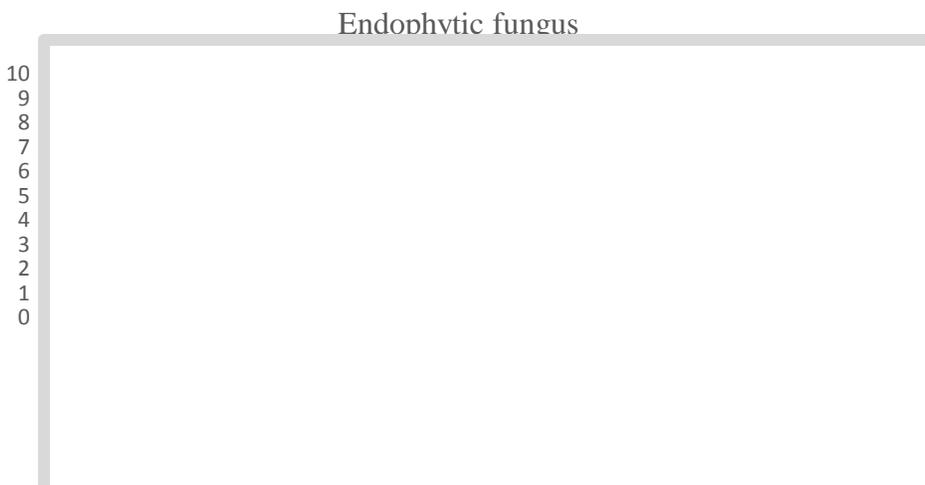


Fig.3 The antagonistic fungus which has the highest inhibition ability against *Lesiodiplodia theobromae*, (A) *Aspergillus* sp., (C) *Rhizopus* sp., (D) *Rhizopus* sp., (E) *Fusarium* sp., (F) *Aspergillus niger*, and (K) control (pathogens) *Lesiodiplodia theobromae*

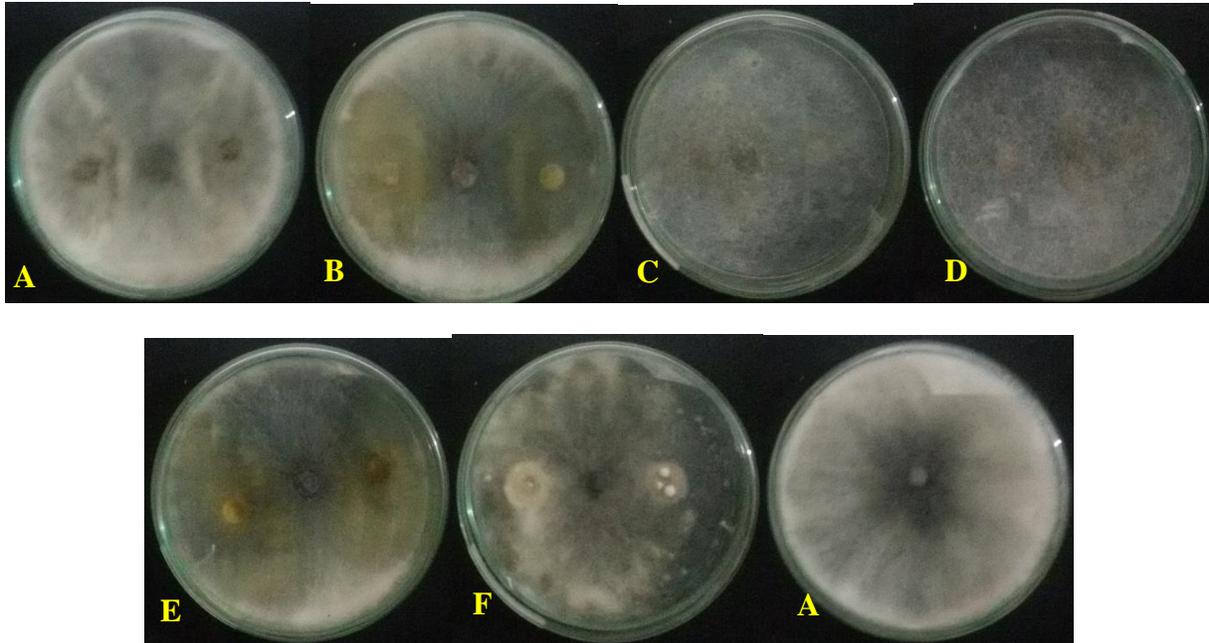


Fig.4 In vivo antagonistic test against *Lasiodiplodia theobromae*, (K-P) control without pathogens, (A) *Aspergillus* sp., (B) *Aspergillus niger*, (C) *Fusarium* sp., (D) *Neurospora* sp., (E) *Rhizopus* sp., (F) *Rhizopus* sp., and (K+P) *Lasiodiplodia theobromae*, 3 days after inoculation



In the endophytic fungi the diversity index reached 2.6356 and the dominance index reached 0.6489 (Table 3). This means that the condition of community structure is very stable with very good category according to Tauruslina *et al.*, (2015) (Table 4). While the dominance index > 0.5 means close to 1, this is due to the dominance of *Fusarium* sp. which reached 46.67% prevalence.

Inhibition Ability of Exophytic and Endophytic Fungi *in Vitro*

The results of inhibition ability *in vitro* experiments of exophytic and endophytic fungi ranged from $65.68 \pm 0.82\%$ to $88.35 \pm 0.46\%$. This fungus will be tested *in vivo*. The fungus was *Aspergillus* sp. highest with inhibition ability of $88.35 \pm 0.46\%$, followed by fungus *Neurospora* sp. amounted to $86.67 \pm 3.14\%$, then the fungus *Rhizopus* sp. respectively $82.92 \pm 0.50\%$ and $82.22 \pm 3.27\%$, then *Fusarium* sp. equal to $81.85 \pm 0.52\%$, and *Aspergillus niger* equal to $80.71 \pm 1.07\%$ (Table 5; Fig. 3). According to Selim *et al.*, (2012) states that one of the fungi found in medicinal plants in China is *Fusarium* sp. and *Aspergillus* sp.

Inhibition ability of exophytic and endophytic fungi *in Vivo*

The six exophytic and endophytic fungi were best tested for inhibition ability to *Lesiodiplodia theobromae* *in vivo* (Fig. 3). The results of repeated observations four times indicate that the endophytic fungi of leaves 4 (*Aspergillus* sp.) and fruit exophytic 5 (*A. niger*) have inhibition ability with percentage of attack 0%, followed by leaf endophytic 1 (*Fusarium* sp.) of 3%, leaf endophytic 2 (*Neurospora* sp.) of 7%, twig exophytic 5 (*Rhizopus* sp.) of 15%, twig exophytic 3 (*Rhizopus* sp.) of 30%, controls plus pathogens with attack rate of 70%, and control without pathogens 0% (Table 6; Fig. 4).

The best fungi protect the fruit from pathogen attack is endophytic of leaves 4 (*Aspergillus* sp.) and fruit exophytic 5 (*Aspergillus niger*) each with 0% attack percentage, followed by leaf endophytic 1 (*Fusarium* sp.), leaf endophytic 2 (*Neurospora* sp.), twig exophytic 3 and 5 (*Rhizopus* sp.) each with a 3%, 7% and 15% disease incidence, whereas the severely affected was twig exophytic 3 (*Rhizopus* sp.) with 30% and different attack percentages manifest with control without pathogens and control with pathogens. Endophytic fungi, especially asexual, for example systemic endophytes in grasses, are commonly seen as mutually beneficial plants primarily through the action of mycotoxins, such as the alkaloids that infect the grass, which protects the plant host from herbivores. Many facts for the mutually beneficial concept of defense derive from agronomic studies of grass cultivars, particularly some endophytic-host interactions (Faeth, 2002).

Aspergillus flavus suppresses the maximum growth of *Alternaria brassicae*, also observed the effect of volatile and non-volatile metabolite compounds released by phyloplane fungus (Yadav *et al.*, 2011). According to Thakur and Harsh (2016) states that the fungus phyloplane *A. niger* can suppress by 50% against *Alternaria alternata* in the Sarpagandha plant (*Rauwolfia serpentina*). Borgohain and Chutia (2014) state that *Aspergillus fumigatus* and *Fusarium* sp. is a phyloplane fungi found in a castor plant (*Ricinus communis* L.). While *Aspergillus fumiguloris*, *Aspergillus* sp. and *F. moniliforme* have been isolated from phyloplane medicinal plants (*Azadirachta indica*). These medicinal plants release phytochemical compounds such as flavonoids, cardiac glycosides and terpenoids (Prabakaran *et al.*, 2011). *Rhizopus* sp. is a phyloplane fungus that dominates adult leaves in host plants Muga (Ray *et al.*, 2014).

In conclusion, the exophytic fungus found on leaves, fruits and twigs is *Aspergillus* sp. *A. niger*, *Fusarium* sp., *Miseliasterillia*, *Neurospora* sp., and *Rhizopus* sp. whereas in the endophytes of the leaves, fruits and twigs are *Fusarium* sp., *Penicillium* sp., *Neurosporas* sp., and *Mycelia sterillia*. The diversity and dominance index of the exophytic fungi are 1.6575 and 0.8667, while the diversity and dominance index of endophytic fungi is 2.6356 and 0.6489. Ability inhibitory of antagonistic against *Lesiodiplodia theobromae* *in vitro*, from exophytic and endophytic fungi ranged from $65.68 \pm 0.82\%$ to $88.35 \pm 0.46\%$. The highest was obtained from *Aspergillus* sp. fungi of $88.35 \pm 0.46\%$ and lowest by *Aspergillus* sp. of $65.68 \pm 0.82\%$. The results of *in vivo* inhibitory tests exophytic and endophytic fungus against the *Lesiodiplodia theobromae* highest obtained from *Aspergillus* sp. and *A. niger* fungi each pressed by 100%.

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