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Utilization Exophytic and Endophytic Fungi to Control Banana Fruit Rot (*Lasiodiplodia theobromae*)

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Abstract
Post-harvest disease of bananas, is often found in bananas in the market and fruit consumed as well as fruit after being used as offerings in temples or in storage. There is rot that starts from the base of the bunch that is attached to the fruit stalk and there is from the skin of the fruit. Diseases found in Ambon banana rot were Lasiodiplodia theobromaewith DNA fragments measuring 650 bp successfully amplified using universal primers ITS1/ITS4.The diversity and dominance index of exophytic fungi were as follows: The diversity index (H) found in healthy bananas in exophytic fungi was 2.2067, with a dominance index (D) of 0.7355. While the endophytic fungi found a diversity index (H) of 1.5171 with a dominance index (D) of 0.6875. The inhibition of exophytic fungi against pathogen (L. theobromae) showed that the highest was achieved by the fungus Rhizopus sp. which began to be seen after 3 days after inoculation (DAI) of 100% each, as well as the fungus Neurospora sp. already seen from 2 HSI to 5 HSI each 100%. While the inhibition of endophytic fungi against pathogen is still Rhizopus sp. showed the best with 100% inhibition ranging from 2 HSI to 4 DAI.The results of the inhibition test of selected exophytic and endophytic fungi in vivo against pathogen A (L. theobrimae), the highest inhibition was obtained from treatment D (Neurospora sp. 1) of 80±7.07%, followed by treatment E (Rhizopus sp. 2) was 66±5.48%, treatment B (A. niger 2) was 30±7.01%, treatment C (Rhizopus sp.1) was 24±5.48% and the last one was the smallest of treatment A (A. niger 1) by 14±5.48%.

Key Words:*Lsiodiplodia theobromae, inhibition, exophyric, endophytic, and postharvest diseases.*

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I. Introduction

Banana fruit rot disease (*Musa paradisiaca* L.) is often found on fruit in the market, at home during consumption, fruit after offerings and fruit during storage. Postharvest banana fruit diseases consist of crown rot, which is dominantly caused by the fungus *Colletotrichum musae* and *Fusarium* spp. [1], as well as that the survey results of sick bananas in India were anthracnose (*Colletototrichum musae*), crown rot (*Fusarium semitechum*), finger rot. rot (*Lasiodiplodia theobromae*), and cigar end rot (*Verticillium theobromae*) [2]. Diseases of postharvest bananas as a result of research [3] namely: Alternara rot (*Alternaria tenuissima*), Corynesppra rot (*Corynespora cassicola*), Dresclera soft rot (*Bresclera halodes*), brown streak (*Fusarium chlamidosporum*), Fusarium rot (*Fusarium pallidosoreum*), brown rot (*Fusarium poae*), Macrophoma rot (*Macropomae musae*) and Charcoal rot (*Macrosphomina phaseolina*).

In the development of current research, there is new hope that exophytic and endophytic fungi isolated from healthy bananas can be used as biological agents in controlling banana fruit rot disease. Endophytic fungi are fungi that grow in plant tissues while exophytic fungi are surface fungi that can live saprophytically but do not cause disease in plants. Filoplan fungus is a mycotic fungus that grows on plant surfaces [4]. There are groups of phylloplan mushrooms: resident (stay silent) and casual (coincidentally). Residents can reproduce on healthy leaf surfaces without being noted to affect the host while casuals land on leaf surfaces but cannot grow [5].There were groups of phylloplan fungi: resident (stay silent) and casual (coincidentally). Residents can reproduce on healthy leaf surfaces without being noted to affect the host while casuals land on leaf surfaces but cannot grow [5]. The results of the research that exophytic and endophytic fungi can suppress the pathogenic ability of red wine both in vitro and in vivo [6]. The exophytic fungi found such as *Aspergillus flavus, A. niger* and *Rhizopus* sp. can suppress manganese rot disease caused by *Lasiodiplodia theobromae* both *in vitro* and *in vivo.*Therefore, it is deemed necessary to look for exophytic and endophytic fungi which can later be used as biological agents in controlling postharvest banana rot disease [7].

II. Methodology

A. Place and time of research

The study was conducted in two places: 1) looking for specimens of sick and healthy fruit from the Supermarkets and Batubulan markets. 2) Plant Disease Science Laboratory and Agricultural Biotechnology Laboratory. The research was carried out from January to March 2021.

B. Molecular Identification

a. DNA Extraction

DNA extraction followed the procedure of [8], 0.2 g of pathogenic fungal mycelium samples were ground with liquid nitrogen and powdered fungal pathogens were put into Eppendorf tubes. Then 500 µL of CTAB buffer and 50 µL of -mercaptoethanol were added, then mixed until homogeneous with a vortex. To lyse the cell wall, heating is carried out at a temperature of 70ºC for 60 minutes where every 10 minutes it is back and forth to speed up the lysis process. Then cooled down to room temperature. Then 500 µL of chloroform isoamylalcohol (24:1) was added to the tube and mixed until homogeneous by vortex and centrifuged at 12,000 rpm for 15 minutes. The supernatant obtained was transferred to a new Eppendorf tube by adding 500 µL of sodium acetate, mixed until homogeneous by vortex and centrifuged again at 12,000 rpm for 10 minutes.The supernatant was transferred to an eppendorf tube and then 500 µL of sodium acetate and isopropanol were added, mixed until homogeneous by vortex and centrifuged again at 12,000 rpm for 10 minutes. The tube was shaken gently to bind DNA and incubated at -20 C for 30 minutes. The DNA threads obtained were precipitated by centrifugation for 10 minutes. The supernatant was discarded, the pellet was washed with ethanol (70%) then centrifuged at 8,000 rpm for 5 minutes. The ethanol was removed and the pellets were dried. The pellet was resuspended with 50 µL of TE buffer and stored at -20ºC for further use in the DNA amplification process.

C. Isolation of endophytic and exophytic fungi

Isolation of endophytic fungi, plant parts such as fruit, leaves and stems were washed with sterile running water, then the plant parts were sterilized with 0.525% sodium hypochlorite for 3 minutes, and 70% alcohol for 2 minutes, then rinsed with sterile water for 1 minute. and then placed on PDA media (which was first given an anti-bacterial antibiotic, namely livoploxacin with a concentration of 0.1% (w/v). The fungus that emerged from the leaf pieces was transferred to a test tube containing PDA to be stored and classified by morphospecies. Exophytes can be carried out by spraying plant parts (fruit, leaves and stems).The washing water is collected, then in a tube, then taken, from a 1 ml tube it is grown into a PDA which has previously been filled with livoploxacin with a concentration of 0.1% (w/v).

D. Identification of endophytic and exophytic fungi

The stored endophytic and exophytic fungi were then grown in a Petri dish containing PDA and repeated 5 times. The cultures were incubated in a dark room at room temperature (± 27^{o}C). Isolates were identified macroscopically after 3 days of age to determine colony color and growth rate, and microscopic identification to determine septa in hyphae, spore/conidia shape and sporangiophores. Fungal identification using the reference book [9], [10], [11], [12], and identification of Actinomycetes using references [13].

E. Test of inhibition of exophytic microbes against pathogens

Each of the microbes found for their inhibition was tested for their inhibition against the growth of pathogenic fungi using a dual culture technique (each one of the pathogenic fungi was grown in one Petri dish, sandwiched with two exophytic fungi). The inhibition power can be calculated as follows [14], [15]:

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

Where:

A = Diameter of pathogenic colonies in a single culture (mm)

B = Pathogenic colony diameter in dual culture (mm)

F. Prevalence of endophytic and exophytic fungi

Determining the prevalence of endophytic and exophytic fungi was based on the frequency of the exophytic microbial isolates found in healthy fruit per Petri dish, divided by all isolates found times 100%. The magnitude of the prevalence of the isolates will determine the dominance of the exophytic fungi that are present in the healthy mango fruit.

G. In Vivo antagonistic test

In vivo antagonistic test of the endophytic and exophytic fungi was found by stabbing fresh fruit with a spore needle 20 times, then smearing it with antagonistic fungal spores (spores of one Petri dish in 250 ml of sterile distilled water), then immersed in a suspension of pathogenic fungal spores. Exophytic microbes found include:

A = antagonist treatment 1 (spore suspension $5x10^7$)
B = antagonist treatment 1 (spore suspension $5x10^7$)
C = antagonist treatment 2 (spore suspension $5x10^7$)
D = antagonist treatment 3 (spore suspension $5x10^7$)
E = antagonist treatment 4 (spore suspension $5x10^7$)
K-P = control without pathogens
K + P = control with pathogens

All treatments were repeated 5 times. The experiment was designed with a randomized block design (RBD), and after analysis of variance (ANOVA) was carried out, it was followed by the least significant difference test (LSD) at the 5% level. Attack parameters measured by formulation: how many punctures were attacked by the fungus divided by all punctures (20 x) times 100%.

III. Results And Discussion

A. Pathogen identification

Symptoms of disease on the fruit began to look black rot at the base of the fruit until it spread to the fruit fingers, fungal mycelium growth quickly covered the Petri dish, for 3 days the Petri dish was full. Mycelium growth on banana slices with symptoms of illness was then isolated again showing white mycelium. Observations under the microscope revealed that the conidia were oval in shape, with one insulated shape, the symptoms of which were similar to those caused by the pathogenic *Lesiodiplodia thoebromae*. The results were identified based on the reference obtained by pathogen according to [16], [17], [18].

The results of molecular identification showed that 650 bp DNA fragments were successfully amplified from 2 fungal samples using ITS1/ITS4 universal primer (Figure 1). The amplified DNA sample was then used for the sequencing stage to determine the fungal species. Sequencing analysis confirmed that the identity of the fungal sample was *L. theobromae* with 99-100% homology (Table 1; Figure 2).

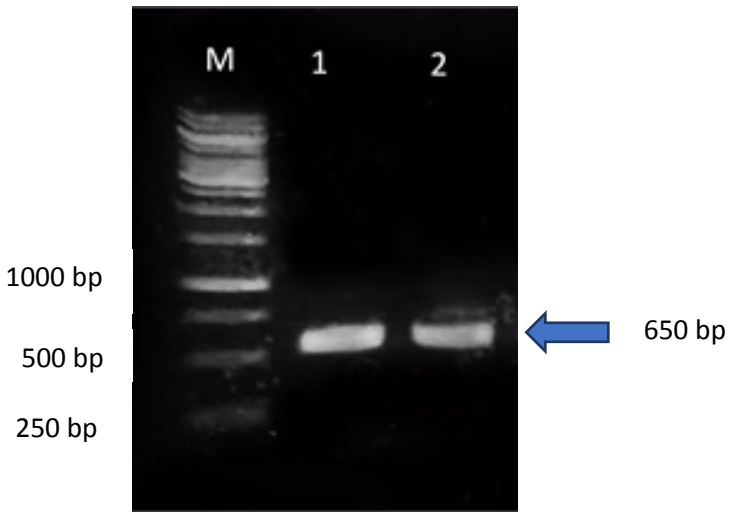

Figure 1. Visualization of amplified fungal pathogenic DNA using ITS1/ITS4 universal primer on 1% agarose gel. M: DNA marker (1kb ladder); Sample no. 1 (pathogen isolate), repeat 2 (pathogen isolate)

Table1. Homology (%) of nucleotide sequences of *L. theobromae* isolates with severalisolates that have been reported in GenBank

Sikuen	L theobromae	LC490867_IDN	MK530072_MYS	MK530070_MYS	KP998517_MYS	MF176233_PAK	MW138058_BGD
Lasiodiplodia_theobromae	ID	100.0%	99.8%	99.8%	99.8%	99.8%	99.8%
LC490867_L_theobromae_IDN	100.0%	ID	99.8%	99.8%	99.8%	99.8%	99.8%
MK530072_L_theobromae_MYS	99.8%	99.8%	ID	100.0%	100.0%	100.0%	100.0%
MK530070_L_theobromae_MYS	99.8%	99.8%	100.0%	ID	100.0%	100.0%	100.0%
KP998517_L_theobromae_MYS	99.8%	99.8%	100.0%	100.0%	ID	100.0%	100.0%
MF176233_L_theobromae_PAK	99.8%	99.8%	100.0%	100.0%	100.0%	ID	100.0%
MW138058_L_theobromae_BGD	99.8%	99.8%	100.0%	100.0%	100.0%	100.0%	ID
FJ904841_L_theobromae_KEN	99.8%	99.8%	100.0%	100.0%	100.0%	100.0%	100.0%
KR867699_L_theobromae_IRN	99.8%	99.8%	100.0%	100.0%	100.0%	100.0%	100.0%
EF622068_L_theobromae_TZA	99.8%	99.8%	100.0%	100.0%	100.0%	100.0%	100.0%
KU877345_L_theobromae_CHN	99.6%	99.6%	99.8%	99.8%	99.8%	99.8%	99.8%
MK530036_L_theobromae_MYS	99.8%	99.8%	100.0%	100.0%	100.0%	100.0%	100.0%
DQ103554_L_rubropurpurea	91.5%	91.5%	91.7%	91.7%	91.7%	91.7%	91.7%

Further phylogenetic analysis showed that *L. theobromae* isolates formed two groups. *L. theobromae* isolates from Bali formed a group with 5 isolates from the genebank among them. While the second group consisted of 6 isolates from the genebank. As an out group isolate, *L. rubropurpurea* was used.

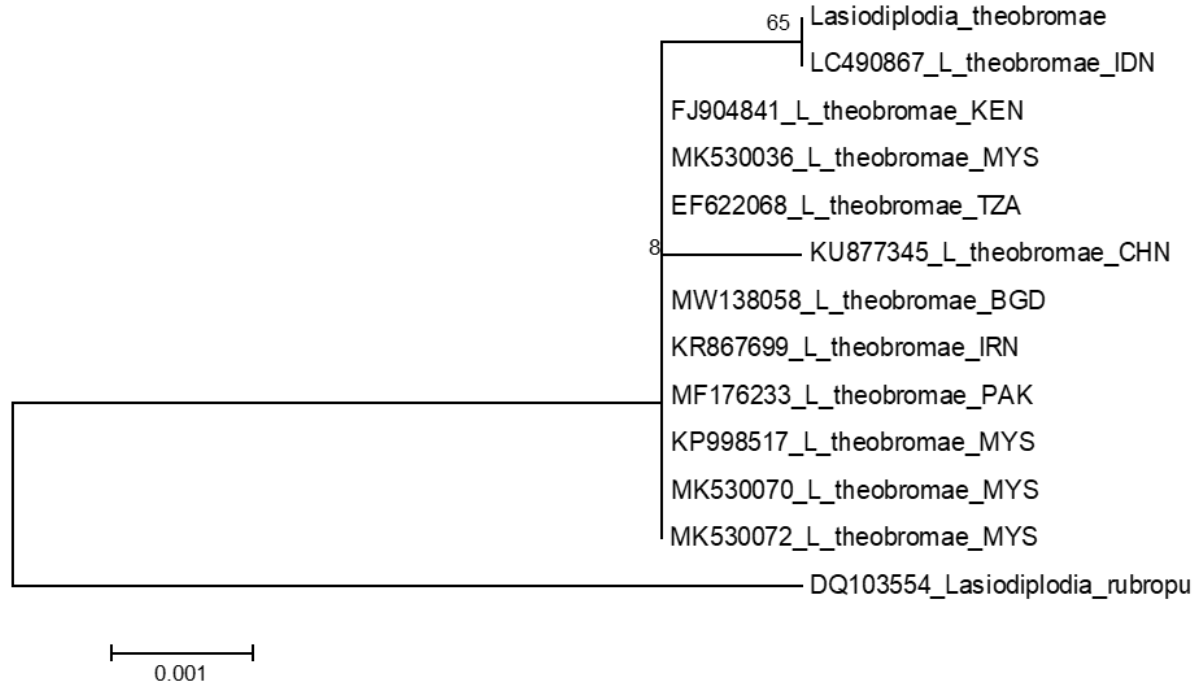

Figure 2. Phylogenetic analysis of *L. theobromae* isolate based on partial nucleotide sequence alignment of DNA-A using Mega 6.06 (Neighbor Joining Algorithm with 1,000 bootstraps replicates)

B. Exophytic and Endophytic Fungi

Exophytic fungi found in healthy fruit were *Aspergillus niger* as many as 2 isolates, *A. flavus* as many as 4 isolates, *Oidium* sp. a total of 4 isolates, *Nocardia asteroids* (Actinomycetes) as many as 2 isolates, *Nocardia* sp. (Actinomycetes) as many as 2 isolates, *Neurospora* sp. a total of 12 isolates and *Rhizopus* sp. a total of 18 isolates (Table 2; Figure 3). While the endophytic fungi found in healthy banana fruit were 6 isolates of *A. niger*, *Colletotrichum* sp. as many as 2 isolates, *A. flavus* as many as 2 isolates and *Rhizopus* sp. a total of 6 isolates (Table 2; Figure 4).Like the research of [7] which states that exophytic and endophytic fungi found in healthy grapes are exophytic fungi, including *Neurospora* sp. as many as 2 isolates, Actinomycetes as many as 12 isolates, and *A. flavus* as many as 15 isolates, while the endophytic fungi were *A. flavus* as many as 5 isolates and *Aspergillus* sp. as many as 2 isolates. Likewise, in healthy mangoes, 6 isolates of the exophytic *A.*

*flavus*were found, 6 isolates of *A. niger*, *Nucordia* sp. (Actinomycetes) as many as 6 isolates, *Rhizopus* sp. as many as 8 silat and *Streptomyces* (Actinomycetes) as many as 4 isolates [20].

Table 2. Types and populations of exophytic and endophytic fungi found on bananas healthy

No.	Exophytic fungi	Number of isolate	Endophytic fungi	Number of isolate
1	*A. niger*	2	*A. niger*	6
2	*A. flavus*	4	*Colletotrichum*sp.	2
3	*Oidium* sp.	4	*A. flavus*	2
4	*Nocardia asteroids* (Actinomycetes)	2	*Rhizopus* sp.	6
5	*Nocardia* sp. (Actinomycetes)	2		
6	*Neurospora* sp.	12		
7	*Rhizopus* sp.	18		
	Jumlah	44		16

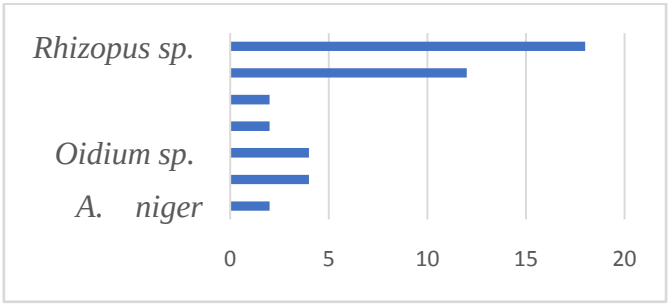

Figure 3. Types and populations of exophytic fungi on healthy bananas

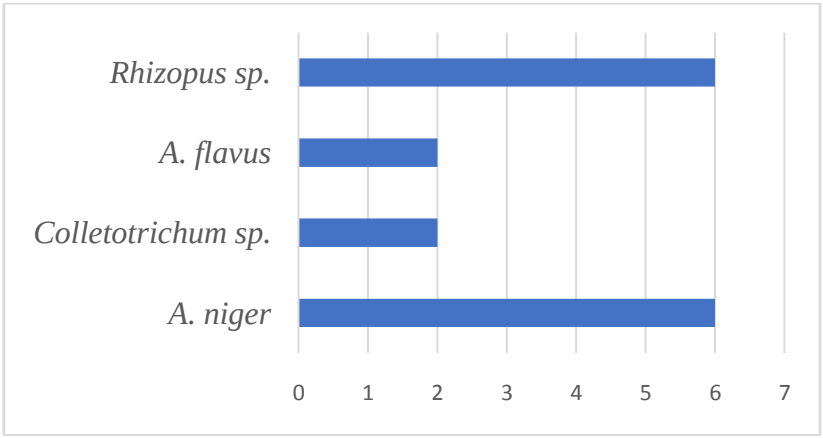

Table 4. Types and populations of exophytic and endophytic fungi found on bananas healthy

C. Exophytic and Endophytic Fungi Diversity and Dominance Index

The diversity index (H) found in healthy bananas in exophytic fungi was 2.2067, with a dominance index (D) of 0.7355. It means that the good category with scale 4 with the condition of the community structure is more stable, and is dominated by the fungus Rhizopus sp. as many as 18 isolates. While the endophytic fungi found a diversity index (H) of 1.5171 with a dominance index (D) of 0.6875. This means that the medium category with a scale of 3 where the condition of the community structure is quite stable, which dominates is the fungus *A. niger* and *Rhizopus* sp. each of 6 isolates. The more diverse fungal species, the more stable they are in the environment, this is in accordance with the research results of [21] which proves that in healthy sugar apple plants, the diversity index is found to reach 2.374 and the dominance index reaches 0.8667 which is dominated by the fungus *A. niger*.

D. *In Vitro* Inhibitory Test

The inhibition of exophytic fungi against pathogen (*Lesiodiplodia theobromae*) showed that the highest was achieved by the fungus *Rhizopus* sp. which began to be seen after 3 days after inoculation (DAI) of 100% each, as well as the fungus *Neurospora* sp. it was seen from 2 DAI to 5 DAI at 100% each (Table 3). While the inhibition of endophytic fungi against pathogen is still *Rhizopus* sp. showed the best with 100% inhibition ranging from 2 DAI to 4 DAI (Table 4).

Table 3. Inhibition of exophytic fungi against pathogen (*Lesiodiplodia theobromae*)

No.	Nama jamur eksofit	2 DAI (%)	3 DAI (%)	4 DAI (%)	5 DAI (%)
1	*A. flavus* 1	-	-	-	-
2	*A. flavus* 2	-	-	-	-
3	*A. flavus* 3	-	-	-	-
4	*A. flavus* 4	-	-	-	-
5	*A. niger* 1	-	-	-	-
6	*A. niger* 2		61,54	100	100
7	*Neurospora* sp.1	52	100	100	100
8	*Neurospora* sp.2	40	100	100	100
9	*Neurospora* sp.3	28	74,29	100	100
10	*Neurospora* sp.4	20	100	100	100
11	*Neurospora* sp. 5	28	100	100	100
12	*Neurospora* sp. 6	20	71,43	77,78	77,78
13	*Neurospora* sp.7-12	100	100	100	100
19	*Oidium* sp. 1	-	-	-	-
20	*Oidium* sp. *2*	40	74,29	77,78	77,78
21	*Oidium* sp. 3	-	-	-	-
22	*Oidium* sp. 4	100	100	100	100
23	*Nocardia asteroids* 1 (Actinomycetes)	-	-	-	-
24	*Nocardia asteroids* 2 (Actinomycetes)	-	-	-	-
25	*Nocardia* sp.1 (Actinomycetes)	-	-	-	-
26	*Nocardia* sp.2 (Actinomycetes)	-	-	-	-
27	*Rhizopus* sp. 1-18	-	100	100	100

Table 4. Inhibition of endophytic fungi against pathogen (*Lesiodiplodia theobromae*)

No.	Nama jamur endofit	1 DAI (%)	2 DAI (%)	3 DAI (%)	4 DAI (%)
1	*A. niger* 1	-	78,57	86,67	86,67
3	*A. niger* 2	-	-	-	-
2	*Colletotrichum* sp. 1	20	71,43	77,78	77,78
3	*A. niger* 3	-	-	-	-
4	*A. flavus* 1	-	-	-	-
5	*A. niger* 4	-	-	-	-
6	*A. niger* 5	40	58,85	70	95
7	*Colletotrichum* sp. 2	50	100	100	100
8	*A. niger* 6	10	-	-	-
9	*A. flavus* 2	-	-	-	-
10	*A. niger* 7	50	53,85	62,25	70
11	*Rhizopus* sp. 1-6	-	100	100	100

DAI = days after inoculation

E. *In Vivo* Inhibitory Test

The results of the inhibition test of selected exophytic and endophytic fungi *in vivo*, the highest inhibition was obtained from treatment D (*Neurospora* sp. 1) of 80±7.07%, followed by treatment E (*Rhizopus* sp. 2) of 66±5.48 %, treatment B (*A. niger* 2) was 30±7.01%, treatment C (*Rhizopus* sp.1) was 24±5.48% and finally the smallest of treatment A (*A. niger* 1) was 14± 5.48% (Table 5).

Table 5. Inhibition of selected exophytic and endophytic fungi against pathogen (*L. theobromae*) *in vivo*

Teatment code	Replication					Average	Notation	
	I	II	III	IV	V		5%	1%
K-P	100	100	100	100	100	100±0.0	A	A
K+P	0	0	0	0	0	0±0.00	G	G
A	10	20	10	20	10	14±5.48	F	F
B	30	30	40	20	30	30±7.01	D	D
C	20	20	30	20	30	24±548	E	E
D	80	90	80	70	80	80±7.07	B	B
E	70	70	60	70	60	66±5.48	C	C

Where: A = *Aspergillus niger* 1 (exophytic fungus), B = *A. niger* 2 (exophytic fungus), C = *Rhizopus* sp. 1 (endophytic fungi), D = *Neurospora* sp. 1 (exophytic fungi), and E = *Rhizopus* sp. 2 (endophytic fungi)

Endophytes are asymptomatic fungal or bacterial microorganisms found in almost all reported living plant species. Endophytic microbes are plant-associated microbes that form symbiotic associations with their host plants by living in internal tissues, which makes them useful for agriculture as a tool in improving plant performance. Many endophytic fungi produce secondary metabolites such as auxins, gibberellins and others that

help in the growth and development of their host plants. Some of these compounds are antibiotics that have antifungal, antibacterial and insecticidal properties, which strongly inhibit the growth of other microorganisms, including plant pathogens [22].

Endophytic fungi that live on trees are horizontally transmitted through seeds, and are not known to grow into seeds and infect plants systemically after seed germination, endophytic fungi living in tissues of seeds can produce spores capable of infecting plants grown from seeds. It is certain that the endophytes can be carried with the host if the seeds are carried from the inoculum source of the mature plant. Following infection, endophytes may remain dormant until triggered by natural leaf senescence, abscission or damage to growth or may sporulate. If the host tissue is not immediately available for infection, such as leaves on deciduous trees in winter, the endophytes must overwinter in the cut leaves. In cases where leaves and stems are available all year round, the endophytes may not have to survive outside the host. Abscised plant material can get caught in the canopy or fall to the ground and rain. Insects or wind can spread spores from absorbed plant material to endophytic-free plant tissues such as newly emerging leaves and twigs [23].

The endophytic mechanism mediating plant resistance to disease is as follows, over the past two decades, many researchers have focused on the response of plant resistance to pathogens and parasites of various scales. Systemic resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance. SAR, which is caused by infectious pathogens, is salicylic acid-mediated and is associated with accumulation of pathogenesis-associated (PR) proteins. ISR, induced by several non-pathogenic rhizobacteria, is mediated by jasmonic acid or ethylene and is not associated with accumulation of PR protein [24], [25].

These PR proteins consist of a variety of enzymes, some of which can act directly to lyse invading cells, including chitinase and -1,3-glucanase (Fukuda and Shinshi 1994), strengthen cell wall boundaries to fight infection, or induce cell-mediated cell proliferation. local death. Fungal endophytes that induce ISR may also be associated with the expression of pathogenesis-related genes. *F. solani*, isolated from tomato root tissue exerted a systemic effect induced resistance to the tomato leaf pathogen, *Septoria lycopersici* and triggered PR, PR5 and PR7 gene expression in roots [26].

IV. Conclusions

Diseases found in Ambon banana rot were *Lasiodiplodia theobromae*with DNA fragments measuring 650 bp successfully amplified using universal primers ITS1/ITS4.The diversity and dominance index of exophytic fungi were as follows: The diversity index (H) found in healthy bananas in exophytic fungi was 2.2067, with a dominance index (D) of 0.7355. While the endophytic fungi found a diversity index (H) of 1.5171 with a dominance index (D) of 0.6875. The inhibition of exophytic fungi against pathogen (*L. theobromae*) showed that the highest was achieved by the fungus *Rhizopus* sp. which began to be seen after 3 days after inoculation (HSI) of 100% each, as well as the fungus *Neurospora* sp. already seen from 2 HSI to 5 HSI each 100%. While the inhibition of endophytic fungi against pathogen is still *Rhizopus* sp. showed the best with 100% inhibition ranging from 2 DAI to 4 DAI.The results of the inhibition test of selected exophytic and endophytic fungi *in vivo* against pathogen A (*L. theobrimae*), the highest inhibition was obtained from treatment D (*Neurospora* sp. 1) of 80±7.07%, followed by treatment E (*Rhizopus* sp. 2) was 66±5.48%, treatment B (*A. niger* 2) was 30±7.01%, treatment C (*Rhizopus* sp.1) was 24±5.48% and the last one was the smallest of treatment A (*A. niger* 1) by 14±5.48%.

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