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Control of Papaya Rot Disease by Using Exophytic and Endophytic Fungi Which is Environmentally Friendly

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A B S T R A C T

Papaya fruit disease has not been known with certainty the cause of the disease, and until now the appropriate control strategy has not been determined. The results of microscopic identification of rot disease in papaya fruit caused by the fungus *Lasiodiplodia theobromae.* The most exophytic fungi found in the study were Rhizopus sp. as many as 69 isolates, followed by *Aspergillus niger* as many as 6 isolates, only *Actinomyces israelii* only 6 isolates while the other Actinomycetes (*Actinomadura cremea*, *Streptomeces* sp., and *Micromonospora* sp.) each had one isolate. While the endophytic fungi were found Rhizopus sp. as many as 30 isolates, followed by *A. niger* with 18 isolates, and finally *Agromyces ramosus* (Actinomycetes) and *Trichoderma* sp, each with 3 isolates. The highest prevalence was obtained from the fungus Rhizopus sp. The diversity index and the dominance index on exophytic microbes were 2.45 and 0.4078, respectively. The index of diversity and dominance of endophytic microbes were 1.876 and 0.58, respectively. The results of the analysis of the inhibitory power of exophytic and endophytic microbes in vitro, it turns out that almost all have competitive inhibition as well as *Trichoderma* sp. which has a zone of inhibition means that it is antibiotic and also competitive. Most Actinomycetes have no inhibitory power against pathogens (*L.theobromae*). The results of the in vivo inhibition test showed that the highest and best inhibitory power was obtained from treatment E (*Trichoderma* sp.).

Introduction

Papaya postharvest diseases are often found in traditional markets and super markets, which in principle can originate from the garden (field), and can also come from storage and transportation. All diseases cause the appearance of the fruit that is not good and quickly rots so that the aroma is unpleasant, even difficult to consume. There are many known fungal diseases on papaya fruits, including: Rhizopus (*Rhizopus stolonifer* Lind.) or black rot, Anthracnose (*Colletotrichum gloeosporioides* (Penz) Sacc), and Phytophthora (*P. parasitica*) fruit diseases (Ventura *el al.*, 2004; Indriyani *et al.*, 2008).

However, Misra and Prakash (2016) stated that postharvest diseases that have damaged papaya fruit are as follows, Rhizopus (watery fruit rot) disease, Fusarium fruit rot, Fusarium soft rot, Fusarium white rot, Ceratocystis black rot, and Hyalodendron soft rot. Efforts to control fungal diseases can be done by utilizing exophytic and endophytic fungi. 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. Phylloplan fungus is a mycota fungus that grows on plant surfaces (Langvard, 1980). 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 whereas casuals land on leaf surfaces but are unable to grow. The results of the research by Sudarma *et al.*, (2019) stated that exophytic and endophytic fungi can suppress the pathogenic ability of red wine both in vitro and *in vivo*. The results of the latest research from Sudarma *et al.*, (2020) stated that 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*.

Materials and Methods

Place and time of research

The research was carried out in two places: 1) looking for sick and healthy fruit specimens from the Batubulan market and supermarkets. 2) Laboratory of Plant Diseases and Agricultural Biotechnology Laboratory. The research was carried out from April to August 2021.

Disease Study

Diseased fruit taken from traditional markets and supermarkets, first studied about the symptoms of the disease, then isolated the pathogen by slicing the part between the symptomatic and asymptomatic fruit and put it in a Petri dish. After two to three days of observing Petri dishes, the growing mycelia were then transferred to new Petri dishes that already contained PDA with the antifungal Livoplaxacin. After growing, it was tested for pathogenicity and tested microscopically.

Isolation of Endophytic and Escophyte Fungi

Isolation of endophytic fungi, health fruit parts, 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 the media. PDA (which was first given an anti-bacterial antibiotic, namely livoploxacin with a concentration of 0.1% (w/v). The fungus that emerged from the fruit partswas transferred to a test tube containing PDA to be stored and classified by morphospecies. While exophytic fungi can be done by spraying fruit parts. 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).

Identification of Endophytic and Exophytic Fungi

The stored endophytic and exophytic fungi were then grown in Petri dishes containing PDA and repeated 5 times. Cultures were incubated in the dark at room temperature ($\pm 27^{o}$C).

Isolates were identified macroscopically after 3 days of age to determine colony color and growth rate, and microscopic identification to identify septa on hyphae, spore/conidia shape and sporangiophores. Fungal identification

using reference book Samson *et al.,* 1981; Pitt and Hocking, 1997; Barnett and Hunter, 1998; Indrawati *et al.,* (1999).

Inhibition Ability Test of Endophytic and Exophytic Fungi against Pathogens

The endophytic and exophytic fungi that were found were tested for their inhibition against the growth of pathogenic fungi using the dual culture technique (in one Petri dish, one pathogenic fungus was grown each flanked with two endophytic 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 = pathogen colony diameter in single culture (mm)

B = pathogen colony diameter in dual culture (mm)

Endophytic and Exophytic Fungal Prevalence

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

Determining Diversity and Dominance Index

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

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

Where:

H' = Shannon-Wiener diversity index

S = Number of genus

Pi = ni/N as the proportion of the ith species (ni = the total number of individuals of the total microbial species i, N = the total number of individuals in the 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 is classified as tall.

Dominance Index

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

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

Where

C = Simpson's index

S = Number of genus

Pi = ni/N i.e. the proportion of individuals of type i and all individuals (ni = total number of individuals of species i, N = number of all individuals in total n).

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

The criteria used to interpret the dominance of soil microbial species are: close to 0 = low index or lower dominance by one microbial species or no species that extremely dominates other species, close to 1 = large index or tend to be dominated by several microbial species (Pirzan and Pong-Cook, 2008). The dominance index was classified into three groups, namely 0 < D 0.5 (low dominance), 0.5 < D 0.75 (moderate dominance), and 0.75 < D 1.0 (high dominance) (Rahmawati *et al.,* 2020).

Invivo Antagonist Test

In vivo antagonistic test of endophytic and exophytic fungi found by pricking fresh fruit with a spelden needle 10 times, then smeared with antagonistic fungal spores (spores of one Petri dish in 250 ml of sterile distilled water), then immersed in fungal spore suspension. pathogens. Endophytic and exophytic fungi found include:

A = antagonist treatment 1 (spore suspension $5x10^7$)

B = antagonist treatment 2 (spore suspension $5x10^7$)

C = antagonist treatment 3 (spore suspension $5x10^7$)

D = antagonist treatment 4 (spore suspension $5x10^7$)

E = antagonist treatment 5 (spore suspension $5x10^7$)

K-P = control without pathogen

K+P = control with pathogen

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

Results and Discussion

Disease Study

Symptoms of the disease that can be observed are rot on the skin of the fruit accompanied by the growth of mycelia, white color that covers the surface of the fruit, the rot expands over time (Figure 1A), and when compared with healthy fruit, the skin looks smooth and fresh (Figure 1B). After being isolated, it turned out that the mycelia grew rapidly, three days after the inoculation had covered the Petri dish (Figure 1C). After being observed under a microscope and compared with several existing references, it turned out that the pathogen was *Lasiodiplodia theobromae* (Figure 1D). *Lesiodiplodia theobromae* is a pathogen that mostly attacks the fruit of postharvest crops such as mango (Sudarma *et al.,* 2020), and sugar apple (Sudarma *et al.,* 2018).

Exophytic and Endophytic Fungi

The most exophytic fungi found in the study were *Rhizopus* sp. as many as 69 isolates, followed by *Aspergillus niger* as many as 6 isolates, only *Actinomyces israelii* only 6 isolates while the other Actinomycetes (*Actinomadura cremea*, *Streptomeces* sp., and *Micromonospora* sp.) each had three isolates. While the endophytic fungi were found *Rhizopus* sp. as many as 30 isolates, followed by *A. niger* with 18 isolates, and finally

Agromyces ramosus (Actinomycetes) and *Trichoderma*sp, each with 3 isolates (Table 1; Figure 2, 3).

Exophytic and Endophytic Microbial Diversity and Dominance Index

The diversity index and the dominance index on exophytic microbes were 3.5487 and 0.4078, respectively. According to Table 2 (Tauruslina *et al.,* 2015 and Ferianita-Fachrul *et al.,* 2005) stated that with the criteria as mentioned above, the scale of 5 is in the very good category (Rahmawati *et al.,* 2020). However, the highest prevalence was produced by the fungus *Rhizopus* sp. of 69 isolates (Table 3).

The index of diversity and dominance of endophytic microbes were 3.103 and 0.574, respectively (Table 3). This means that the diversity index is included on a scale of 5, with a very good category and the condition of the community structure is very stable (Tauruslina *et al.,* 2015). While the dominance index of 0.574 means the medium category (0.5 < D 0.75), only dominated by the fungus *Rhizopus* sp. of 30 isolates.

Inhibition Ability of Exophytic and Endophytic Microbes In vitro

The results of the analysis of the inhibition ability of exophytic and endophytic microbes turned out that almost all of them had competitive inhibition as well as *Trichoderma* sp. which has a zone of inhibition means that it is antibiotic and also competitive. Most of the Actinomycetes that do not have inhibitory power against pathogens (*L. theobromae*) (Table 4). The highest inhibition ability on exophytic microbes was isolate *Rhizopus* sp. 22 of 96.30 ± 6.4%, while the smallest by isolate *Rhizopus* sp. 15 and *Rhizopus* sp. 17 respectively 79.20±3.2%. Meanwhile, the highest endophytic microbes were achieved by isolates of *Trichoderma* sp. by 96.30±6.4%, and the smallest by isolates of *Rhizopus* sp. 2 with an inhibition ability of 82.59±6.4% (Table 4, Figure 4).

According to Diana (2018) stated that *Rhizopus* sp. is a pathogen in post-harvest papaya where the fungus contaminates or is dominated by orange papaya fruit (*Carica papaya* L.) with a percentage of half being attacked by the fungus *Rhizopus* sp.

The number of *Rhizopus* sp. both exophytic and endophytic ones indicated that the papaya fruit taken as samples was contaminated with the fungus *Rhizopus* sp. According to Adriani (2010) stated that *Rhizopus stolonifer* can attack strawberries originating from Jorong Taratak Baru, Kanagarian Salimpek, Solok Regency and Pasar Raya Padang with an attack percentage of 24%.

The fungus *Rhizopus* sp. Besides attacking the commodities mentioned above, it also attacks apples (*Malus sylvestris*), avocados (*Parsa americana*), grapes (*Vitis vinivera*), longan (*Nephelium* sp.), Duku (*Lansium domesticum*), Sawo (*Manilkara archras*), Oranges (*Citrus auranticum*) and watermelon (*Citrylus lanatus*) (Aminah and Supraptini, 2003).

There are several types of Rhizopus fungi that cause damage in the form of fruit rot which has many variants (Hurban Hydroponics, ny).

This fungus *Rhizopus nigricans* causes fruit rot and is known in all citrus production centers in the world. Early symptoms of this disease are pale yellow or slightly wrinkled patches. The spots develop into rot then the fruit is covered with mycelia and other fungal fruiting bodies. The rotten fruit is finally coated with black and gray thin flour.

Rhizopus stolonifer is the fungus that causes papaya rot. Only attacks ripe fruit that has

been injured. Whole, healthy and unharmed unripe fruit will not be attacked. Unlike the Phytophthora fungus which can attack fruit at all levels of maturity and unharmed fruit.

Rhizopus cirxinans and *R. arrchizus* both of these fungi cause hull rot on almonds in California and Australia. Infection occurs when the skin of the fruit breaks and cracks. This cracked skin becomes a patchwork of irregular and slimy patches. This tissue then dies causing crimped groove defects in the fruit.

Table.1 Population of exophytic and endophytic fungi

No.	Name of exophytic microbe	Number of isolate	Nameof endophytic microbe	Number of isolate
1	*Rhizopus* sp.	69	*Aspergillus niger*	18
3	*Aspergillus niger*	6	*Rhizopus* sp.	30
4	*Actinomyces israelii*	6	*Agromyces ramosus*	3
5	*Actinomadura cremea*	3	*Trichoderma* sp.	3
6	*Streptomeces* sp.	3		
7	*Micromonospora* sp.	3		

Table.2 Environmental quality weighting criteria (Tauruslina *et al.*, 2015)

Diversity index	Condition of community structure	Category	Scale
>2,41	Very stable	Very good	5
-2,4	More stable	good	4
1,21 – 1,8	Stable enough	Currently	3
0,61 – 1,2	Less stable	Bad	2
<0,6	Unstable	Very bad	1

Table.3 Diversity and dominance index of exophytic and endophytic microbe

Criteria	Exophytic	Endophytic
Diversity index (H)	3,549	0,408
Dominance index (D)	3,103	0,574

Table.4 Inhibition ability of exophytic and endophytic microbe

No.	Name of exophytic microbe	Inhibition ability (%)	Nameof endophytic microbe	Inhibition ability (%)
1	*Rhizopus* sp. 1	83.33±5.6	*Aspergillus niger*1	-
2	*Rhizopus* sp. 2	85.18±3.2	*Rhizopus* sp. 1	-
3	*Aspergillus niger*1	-	*Rhizopus* sp. 2	82.59±6.4
4	*Rhizopus* sp. 3	85.18±3.2	*Rhizopus* sp. 3	-
5	*Rhizopus* sp. 4	83.33±5.6	*Rhizopus* sp. 4	83.33±5.6
6	*Rhizopus* sp. 5	90.74±8.5	*Rhizopus* sp. 5	92.59±6.4
7	*Rhizopus* sp. 6	92.59±6.4	*Agromyces ramosus*	-
8	*Rhizopus* sp. 7	90.74±8.49	*A. niger*2	-
9	*Rhizopus* sp. 8	-	*A, niger*3	85.18±3.2
10	*Rhizopus* sp. 9	-	*A. niger*4	-
11	*Rhizopus* sp. 10	83.33±5.6	*A. niger*5	-
12	*Rhizopus* sp. 11	85.19±6.4	*A. niger*6	-
13	*Rhizopus* sp. 12	85.19±6.4	*Rhizopus* sp. 6	85.19±6.4
14	*Actinomyces israelii*	-	*Rhizopus* sp. 7	85.18±3.2
15	*Rhizopus* sp. 13	87.03±3.2	*Rhizopus* sp. 8	86.30±2.8
16	*Rhizopus* sp. 14	83.33±5.6	*Trichoderma* sp.	96.30±6.4
17	*Rhizopus* sp. 15	79.20±3.2	*Rhizopus* sp. 9	83.33±5.6
18	*Rhizopus* sp. 16	84.44±1.3	*Rhizopus sp*. 10	92.59±6.4
19	*Rhizopus* sp. 17	79.20±3.2		
20	*Rhizopus* sp. 18	85.40±3.2		
21	*Actinomadura cremea*	-		
22	*Streptomeces* sp.	-		
23	*Micromonospora* sp.	85.19±6.4		
24	*Actinomyces israeli*	-		
25	*Rhizopus* sp. 19	92.59±6.4		
26	*A. niger*2	81.48±6.4		
27	*Rhizopus* sp. 20	92.59±6.4		
28	*Rhizopus* sp. 21	88.89±11.1		
29	*Rhizopus* sp. 22	96.30±6.4		
30	*Rhizopus* sp. 23	92.59±6.4		

Table.5 Inhibition ability of exophytic and endophytic fungi

Treatment	Inhibition ability (%)	LSD 5%	LSD 1%
A (Endo III.4) *Trichoderma* sp.	95±4.47	A	A
B (Exo III.10) *Rhizopus* sp. 10	67±4.00	C	C
C (Endo II.3) *A, niger*	82±2.44	B	B
D (Exo III.9) *Rhizopus* sp. 9	54±3.74	D	D
E (Exo I.7) *Rhizopus* sp. 7	52±2.45	E	E
F (K+P)	17±4.00	F	F
G (K-P)_	94±3.74	A	A

Fig.1 Observations of disease symptoms (A), healthy papaya (B), pathogenic fungal mycelium in Petri dishes (C), and pathogen microscopic test results (k = conidia, and m = mycelium)

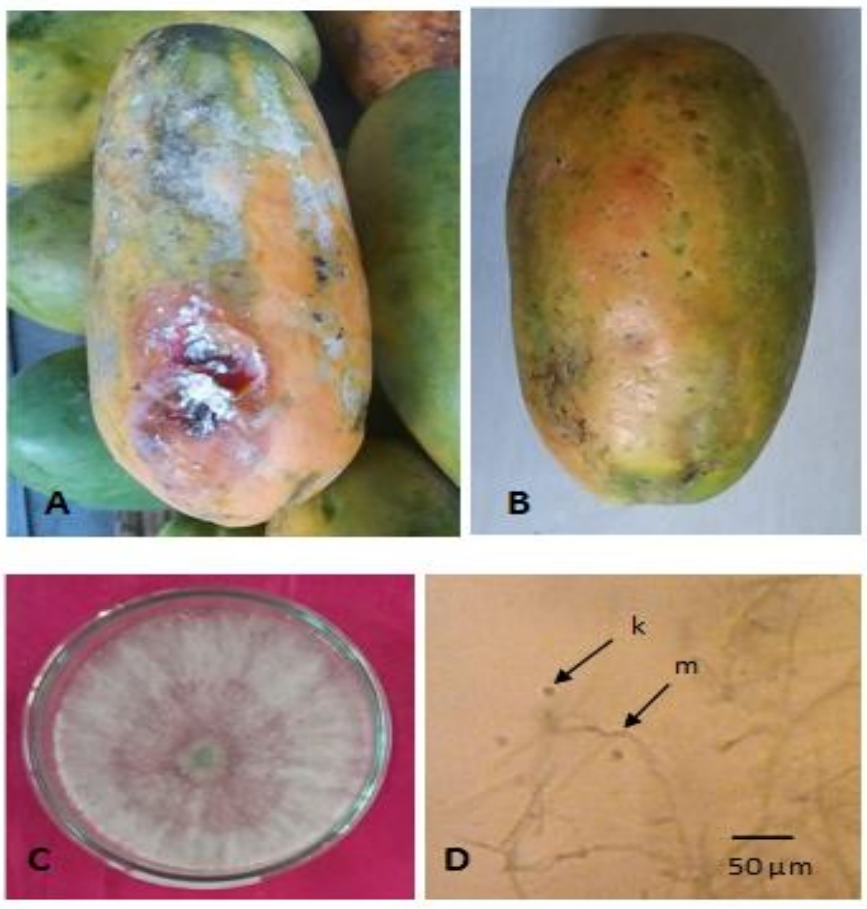

Fig.2 Population of exophytic microbe

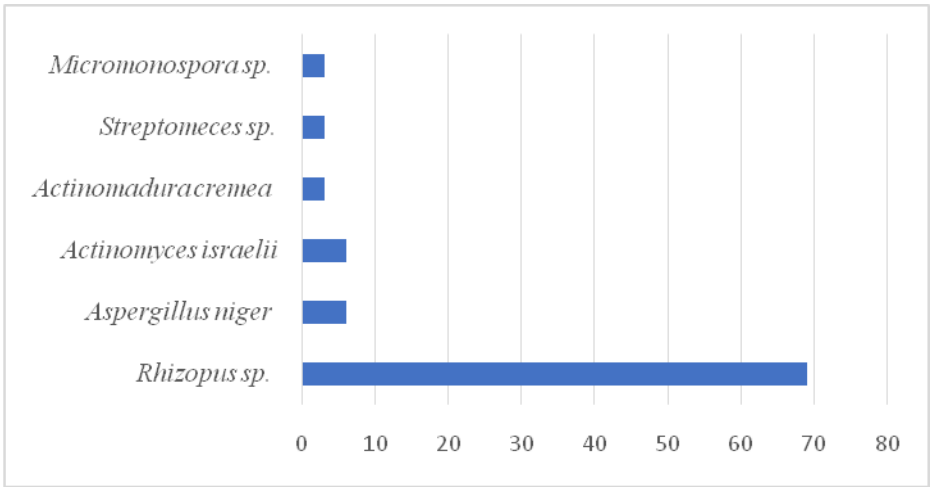

Fig.3 Population of endophytic microbe

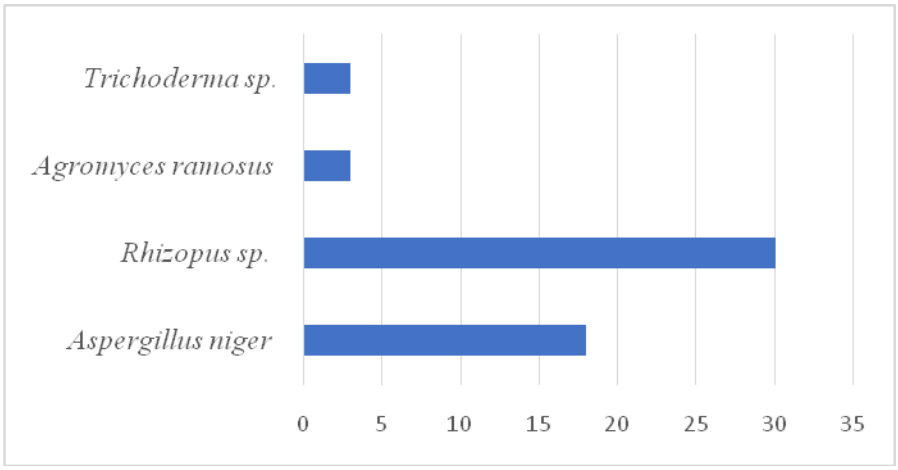

Fig.4 Inhibitory test of some of the best isolates, A = *Trichoderma* sp. (Endo III,4), B = *Rhizopus* sp. (Exo III,9), C = *Rhizopus* sp. (Exo III.10), D = *Rhizopus* sp. 7 (Exo I.7), E = *Aspergillus niger* (Endo II.3) and F = *Lasiodiplodia theobromae* (control), 2 days after inoculation

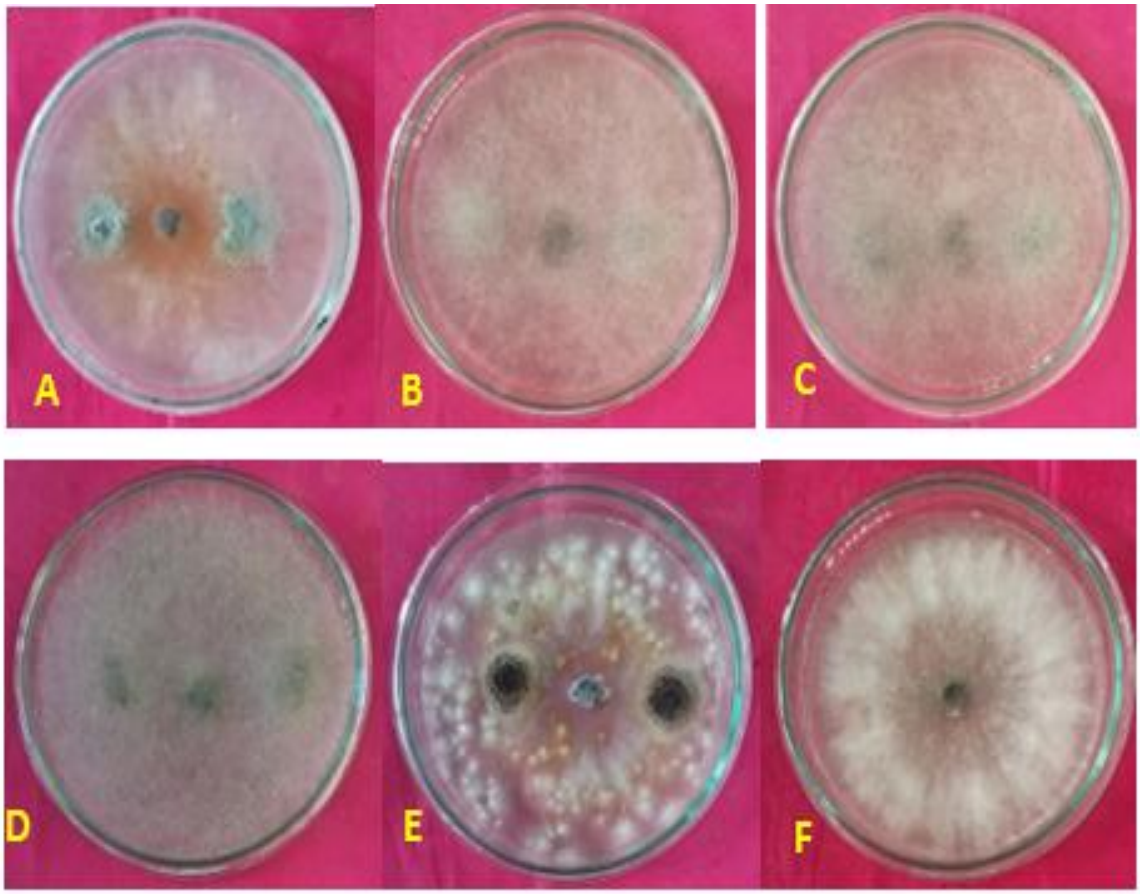

Fig.5 The results of the inhibitory treatment of exophytic and endophytic fungi against pathogens (*Lasiodiplodia theobromoe*) *in vivo*, 4 days after inoculation (A = *Trichoderma* sp., B = *Rhizopus* sp. 10, C = *A. niger*, D = *Rhizopus* sp. 9, E = *Rhizopus* sp.7), F = control with pathogen, and F = control without pathogen

Invivo Inhibition of Exophytic and Endophytic Fungi

The results of the in vivo inhibition of exophytic and endophytic fungi showed that the highest inhibitory power was obtained from treatment E (*Trichoderma* sp.) of 95±4.47 not significantly different from the control (without pathogen) of 94±3.74%, followed by treatment C (*A.niger*) was 82±2.44%, then B (*Rhizopus* sp. 10) was 67±4.00%, treatment D (*Rhizopus* sp. 9) was 54±3.74%, treatment E (*Rhizopus* sp. 7), and finally the smallest control with pathogen treatment (Table 5). It turns out that only Trichoderma sp. which showed the best inhibition *in vivo*. This inhibition occurs either antibiosis (because there is a zone of inhibition) or competitively because it fills the space of the Petri dish.

Visually, it can be seen in Figure 5, after 4 days of inoculation it appears that only treatment A (*Trichoderma* sp.) gave the best results and hopes to be used as an antagonist fungus, namely as a biological agent.

Trichoderma sp. especially asexual fungi which are present in all types of agricultural soil and also in rotting wood. Antagonistic activity of *Trichoderma* species indicates that it can parasitize many soil and foliage borne pathogens. Fungi are also decomposers of cellulose waste materials. Recent discoveries have shown that fungi not only act as biocontrol agents, but also stimulate plant resistance, as well as plant growth and development resulting in increased crop production. Biocontrol activity involving mycoparasitism, antibiotic and nutrient competition, also induces a defense response or systemic resistance response in plants. This response is an important part of *Trichoderma* in the biocontrol program. Currently, *Trichoderma* spp., is used to control plant diseases in sustainable disease management systems (Naher *et al.*, 2014). According to Ghazanfar *et al.*, (2018) stated that new technologies in all fields of agriculture have increased agricultural production, but some modern practices affect the environment. A recent challenge faced by advanced agriculture is to achieve higher yields in an environmentally sound manner. Therefore, it is necessary to immediately find an environmentally friendly solution. Among the various types of species used as biocontrol agents, *Trichoderma* is widely used as a biocontrol agent against various types of plant pathogens. *Trichoderma* sp. recent studies have shown that this fungus not only acts as a biocontrol agent but also stimulates plant resistance, plant growth and development which has an impact on increasing crop production. Antagonistic activity involves mycoparasitism, antibiotics, competition for nutrients and also induces systemic resistance in plants.

Among other biocontrol mechanisms, antibiosis, competition, and mycoparasitism are among the main features by which microorganisms, including *Thrichoderma*, react to the presence of other competitive pathogenic organisms, thereby preventing or inhibiting their development. Stimulation of each process involves the biosynthesis of targeted metabolites such as growth regulators, enzymes, siderophores, antibiotics, etc. The biological control activity of *Trichoderma* spp. and describe recent advances in demonstrating the ecological significance of *Trichoderma* at the biochemical and molecular level in the rhizosphere as well as the benefits of symbiosis with host plants in terms of physiological and biochemical mechanisms. An applicative point of view, given here strongly supports the possibility to use *Trichoderma* as a safe, environmentally friendly and effective biocontrol agent for various plant species (Sood *et al.*, 2020).

Based on the results and discussion above, it can be concluded as follows: The results of microscopic identification of rot disease in papaya fruit are caused by the fungus *Lasiodiplodia theobromae*. The most exophytic fungi found in the study were *Rhizopus* sp. as many as 69 isolates, followed by *Aspergillus niger* as many as 6 isolates, only Actinomyces israelii only 6 isolates while the other Actinomycetes (*Actinomadura cremea*, *Streptomeces* sp., and *Micromonospora* sp.) each had one isolate. While the endophytic fungi were found *Rhizopus* sp. as many as 30 isolates, followed by *A. niger* with 18 isolates, and finally *Agromyces ramosus* (Actinomycetes) and *Trichoderma* sp, each with 3 isolates. The highest prevalence was obtained from the fungus *Rhizopus* sp. The diversity index and dominance index on exophytic microbes were obtained at 3.549 and 0.408, respectively. The index of diversity and dominance of

endophytic microbes were 3.103 and 0.574, respectively. The results of the analysis of the inhibitory power of exophytic and endophytic microbes in vitro, it turns out that almost all have competitive inhibition as well as Trichoderma sp. which has a zone of inhibition means that it is antibiotic and also competitive. Most Actinomycetes have no inhibitory power against pathogens (*L.theobromae*). The results of the in vivo inhibition test showed that the highest and best inhibitory power was obtained from treatment E (*Trichoderma* sp.).

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