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Use of Exophytic Microbial on the Control of Fruit Rot Disease of Mango (*Lesiodiplodia theobromae*)

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A B S T R A C T

Post-harvest mango rot is the main cause of yield loss caused by this disease in Bali. Until now, no environmentally friendly control methods have been found such as finding exophyte microbes that are antagonistic to pathogens. The pathogen found to cause fruit rot disease in mangoes is *Lesiodiplodia theobromae*. Exophytic fungi found in healthy mangoes include: *Rhizopus* sp. the number of colonies 24 x 10^2 cfu, while *Nucordia* sp., *A. flavus*, and *A. niger* with colonies 18 x 10^2 cfu each, and Streptomyces sp. with a colony of 12 x 10^2 cfu. The highest in vitro microbial inhibitory test results of *L. theobromae* were obtained from *Rhizopus* sp. 1 and *Rhizopus* sp. 4 when 4 hsi and 7 hsi. The results of antagonistic inhibition test on pathogens (*L. theobromae*) in vivo obtained the highest by the treatment of C (*Rhizopus* sp. 3) which was very significantly different from K + P (control with pathogens).

Introduction

Post-harvest fruit rot is often found during marketing, storage, when consumption is very disturbing in the appearance of damaged fruit and affects the taste of the fruit. Mangoes are very important for tropical countries and subtropics (Prakash *et al.,* 2011), moreover for Indonesia, although mangoes are affected by a number of diseases but are some important diseases and are responsible for yield loss in mango production. There are several diseases that attack post-harvest mangoes, among others: anthracnose, fruit base rot, black rot.

Botryosphaeria rot, stem rot and soft rot, chocolate rot, Pestalotiopsis rot, Charcoal rot, Phoma rot, Alternaria rot, Macrophoma rot, Macrophoma rot, Rhizopus rot, Cladosporium rot, Fusarium rot, Canariomyces rot, Mucor rot, Alternate rot, Macrophoma rot, Ash rot,

Rhizocia ash, Rhizocia rot, Ash rot Hendersonia rot, blue mold, runny soft rot, Sclerotium fruit rot and Yeasty rot (Prakash *et al.,* 2011). Many cases are found in the field of disease but the method of control until now has not been known to be environmentally friendly.

An exophytic fungus is a surface fungus that can live saprophytic but does not cause disease in plants. Phyloplan fungi are mycota that grow on plant surfaces (Langvard, 1980). There are groups of phyloplan mushrooms: resident (stay silent) and casual (coincidence). Resident can multiply on the surface of healthy leaves without being noted to affect the host while casual landed on the surface of the leaf but cannot grow (Leben 1965). The results of Sudarma *et al.,* (2019) stated that exophytic and endophytic fungi can suppress the ability of pathogens in red grape both *in vitro* and *in vivo.*

Materials and Methods

Place and time of research

The study was conducted in two places: 1) looking for sick and healthy fruit specimens from the Batubulan and Supermarkets markets. 2) Plant Disease Laboratory and Agricultural Biotechnology Laboratory. The study was conducted in January to March 2020.

Microbial isolation of exophytes

Isolation of exophyte microbial can be done by dipping the mango into 250 ml of water, then shaking it and rinsing it evenly. This washing water as much as 250 ml is used as a dilution of the microbial population found. Furthermore, 1 ml is taken poured into a Petri dish which is first filled with a PDA media PDA and added anti-bacterial livoploxacin at a dose of 0.1% (w / v).

Identification of exophytic microbes

The stored exophyticmicrobes were then grown on a Petri dish containing a PDA and repeated 5 times. Culture is incubated in a dark room at room temperature (± 27°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 and sporangiophores. Fungal identification using the reference book Samson *et al.,* 1981; Pitt and Hocking, 1997; Barnett and Hunter, 1998; and Indrawati *et al.,* 1999.

Pathogen identification by PCR and sequencing

Detection was carried out through the stages of extraction of the total DNA of the fungus using the DNeasy Plant Mini Kit (Qiagen/Germany).

Stages of DNA extraction

0.1 gram of sample was crushed using pistil and mortar until smooth then put in 1.5 ml micro tubes and added 400 µl of AP1 buffer and 4 µl of RNase A stock solution then on vortex to homogenize the solution, then the tube containing the mixed solution was incubated for 10 minutes in a water bath with a temperature of 65ºC, and the tubes are turned upside down every 5 minutes, 130 ml of AP2 buffer is added to the mixed solution and then it is vortexed and incubated in the refrigerator for 5 minutes.

After that, centrifugation was carried out at 14,000 rpm for 5 minutes. Supernatant (top phase) produced at this stage was then pipetted and put into DNeasy Mini spin column (white color), and centrifuged at 14,000 rpm for 2 minutes, the fraction in the lower tube (collection tube) moved into a new tube (2 ml) without including the formed

pellet, then added 1.5 AP3/E buffer volume and mixed using a pipette (by sucking and removing the mixture using a micropipette), after that piping as much as 650 µl the mixture, including when a precipitate formed, was put into a DNeasy mini spin column (white in color) and centrifuged for 1 minute at 8000 rpm. The liquid in the 2 ml collection tube is discarded. This stage can be repeated for the remainder of the mixture, then the collection tube is discarded with the liquid inside.

Next DNeasy mini spin column (white) is placed on a new micro tube that is already available, added 500 ul AW AW buffer and centrifuged at 8000 rpm for 1 minute. The solution in the tube was discarded. Another 500 µl AW buffer was added to the DNeasy Mini Spin Column, then centrifuged for 2 minutes at 14,000 rpm, then transferred the DNeasy Mini Spin Column to a new 1.5 ml tube, added 100 ul of AE buffer and put directly into the DNeasy membrane, incubated at room temperature for 5 minutes, then centrifuged for 1 minute at 8000 rpm, the resulting DNA can be directly used or stored at -20ºC until it will be used. Furthermore, the DNA produced is used as a template for PCR. The composition of the PCR reaction is: 1 µl DNA template added to the PCR reaction mixture consisting of: 12.5 µl 2x Dream Taq Green PCR Master Mix (Thermo Scientific), each 1 µl Forward and Reverse 10 mM primers, and water so that the total volume of 25 µl. The primers used are the primary pair ITS1 (5 'TCCTCCGCTTATTGATATGC 3') and IT S4 (5 'TCCGTAGGTGAACCTGCGG 3') which will amplify the internal transcribed spacer (ITS) DNA ribosome (rDNA) area (White at al. 1990). PCR conditions are: 94°C for 5 minutes 1 time, then 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes, repeated 35 times, last 72°C for 10 minutes .The amplification results were electrophoresed using 1.2% agarose gel with 1x TAE buffer at 50 volt for 30 minutes. The DNA band is seen on the UV transilluminator. Generate DNA fragments measuring ± 600bp. Furthermore, DNA fragments are sent to PT Macrogen Inc. Korea) to trace the nucleotide bases to determine the identity of the fungus. (on going).

Inhibitory microbial inhibition test against pathogens

The exophyte microbes found were each tested for their inhibition on the growth of pathogenic fungi by the dual culture technique (in one Petri dish each pathogenic fungus was flanked with two endophyticmicrobes). The inhibition ability 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 pathogenic colonies in a single culture (mm)
B = Pathogenic colony diameter in dual culture (mm)

Prevalence of exophytic microbes

Determining the prevalence of exophytic microbes is based on the frequency of exophytic microbial isolates found in healthy fruit per Petri dish, divided by all isolates found 100 times. The high prevalence of isolates will determine the dominance of exophyticmicrobes in the healthy mango.

Antagonistic test *in vivo*

Antagonistic test in vivo exophytic microbes found by pricking fresh fruit with a spelden needle 20 times, then smeared with antagonistic fungal spores (spores of one Petri

dish in 250 ml of sterile aquadest), then dipped in a suspension of pathogenic fungal spores. Exophytic microbes found include:

A = antagonistic treatment 1 (suspense spore 5x107)
B = antagonistic treatment 1 (5x107 spore suspension)
C = antagonistic treatment 2 (5x107 spore suspension)
D = antagonistic treatment 3 (5x107 spore suspension)
E = antagonistic treatment 4 (5x107 spore suspension)

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 (RCBD), and after analysis of variance (ANOVA) was continued with the smallest real difference test (LSD) at 5% level. Attack parameters measured by formulation: how many punctures attacked by the fungus are divided by the whole prick (20 x) times 100%.

Results and Discussion

Pathogen identification

Based on the results of the isolation of mango rot, two symptoms were obtained, including black symptoms at the tip of the fruit (Figure 1). The results of identification of pathogens that cause fruit rot at the ends are *Lesiodiplodia theobromae*, According to Prakash *et al.,* (2011) diseases that interfere with pathogenic stem end rot are [*Lasiodiplodia theobromae* (Pat.) Griffon & Moubl., *Phomopsis mangiferae* Ahmad, *Dothiorella dominicana* Sydo.] (Figure 1).

The results of the identification of pathogens by molecular techniques obtained by gene transcription of the internal transcribed spacer (ITS) DNA ribosome (rDNA) using 1.2% agarose gel with 1x TAE buffer at 50 volt voltage for 30 minutes. The DNA band is seen on the UV transilluminator as seen on the electropherogram, producing a DNA fragment of 600 bp size for *L. theobromae* (Figure 2).

Based on the results of the alignment of internal transcribed spacer (ITS) DNA rebosome (rDNA) gene sequences with the GenBank database using BalstN, fungus isolate 1 with DNA sequences as follows:

Sequence of pathogenic fungus *L. theobromae*

TGCGGAAGGATCATTACCGAGTTTTCG
AGCTCCGGCTCGACTCTCCCACCCTTT
GTGAACGTACCTCTGTTGCTTTGGCGG
CTCCGGCCGCCAAAGGACCTTCAAACT
CCAGTCAGTAAACGCAGACGTCTGATA
AACAAGTTAATAAACTAAAACTTTCAA
CAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGC
CCCTTGGTATTCCGGGGGGCATGCCTG
TTCGAGCGTCATTACAACCCTCAAGCT
CTGCTTGGAATTGGGCACCGTCCTCAC
TGCGGACGCGCCTCAAAGACCTCGGC
GGTGGCTGTTCAGCCCTCAAGCGTAGT
AGAATACACCTCGCTTTGGAGCGGTTG
GCGTCGCCCGCCGGACGAACCTTCTGA
ACTTTTCTCAAGGTTGACCTCGGATCA
GGTAGGGATACCCGCTGAACTTAAGC
ATATCAATAAGGCGGA

Comparison of the percentage similarity of 18S rRNA gene in patsirisolar fungi with some DNA sequences in GenBank using the BLAST program (Table 1).

Lasiodiplodia theobromae is a common pathogen in a large number of hosts in the

tropics and subtropics. Collection of isolates identified as *L. theobromae* which have been studied on the basis of sequential data from the ITS region and the EF1-α gene (Alves *et al.,* 2008). This fungus secretes several types of enzymes, usually including cell wall degradation and pathogenesis. An increase in global temperatures can increase fungi, such as *L. theobromae* to change their properties. Temperature modulation expresses enzymes, and this affects more markedly when fungi are grown at 37°C than below temperatures (Felix *et al.,* 2018). Pathogens have been collected from 225 *L. theobromae* isolates from 52 plants and from many parts of the world (Mehl *et al.,* 2017).

Esophophytic microbes and prevalence

Exophytic microbes found in most mangoes are *Rhizopus* sp. with a population of 24 x10^{2}cfu, followed by *Aspergillus flavus, A. niger* and *Nucordia* sp. (Actinomycetes) each with a population of 18 x 10^{2}cfu, and finally the least is *Streptomyces* sp. (Actinomycetes) as much as 12 x 10^{2} cfu, the highest prevalence held by *Rhizopus* sp. with a value of 26.67%, followed by *Nucordia* sp*., A. flavus* and *A. niger* respectively 20% and the lowest value held by *Streptomyces* sp. with a value of 13.33% (Table 2; Figure 3).

Inhibition ability of exophytic microbes on pathogens *in vitro*

The highest inhibitory microbial inhibition of pathogens (*A. niger*) was achieved by *Rhizopus* sp. 1 at 3 dai (days after inoculation) of 98 ± 0.2% and *Rhizopus* sp. 3 when 7 dai is 99.0 ± 0.1%. The highest inhibitory microbial inhibition against pathogens (*Lasiodiplodia theobromae*) was obtained from *Rhizopus*sp. 1 of 88.89 ± 0.3% at 4 hsi, while at 7 dai the highest was achieved by *Rhizopus* sp. 3 at 98 ± 0.2%, followed by *Rhizopus* sp. 1 by 80 ± 0.5%, *Rhizopus* sp. 2 at 77.78 ± 0.4%, *A. niger* 1 at 77.78 ± 0.3% and finally *Rhizopus* sp.1 at 72.22 ± 0.2% (Table 3).

Inhibition ability of exophytic microbes on pathogen *in vivo*

The observation result of 3 dai (days after inoculation) antagonistic test *in vivo*, the best antagonist with pathogen (*L. theobromae*) obtained treatment A (*Rhizopus* sp. 4) with the highest attack percentage of 95 ± 4.47%, followed by treatment B (*A. niger*) at 67 ± 4%, then D (*Rhizopus* sp. 2) and E (*Rhizopus* sp. 1) each attack percentage 54 ± 3.74% and 52 ± 2.45, K-P treatment (control without pathogen)) the percentage of attacks was 4.17 ± 4% and KP (control with pathogens) the percentage of attacks was 94 ± 3.74%, all differed markedly except for treatments A and K + P (Table 4; Figure 4 and 5). There were as many as 20 species of *A. niger* found potentially used as biological agents against pathogens (*Phytophthora palmivora*) fruit rot pathogens in cocoa. *Aspergillus niger* is directly related to food ingredients in the media. *A. niger* also produces every enzyme such as enzymamase, amyloglucosidase, pectinase, cellulose, glucoside, which breaks down urea into amino acids and CO2 (Wulandari *et al.,* 2016).

In this research isolate actinomycetes, from the rhizosphere of wheat plants (*Triticum aestivum* L.), succeeded in antagonistic activity in certain root rot fungi (*Fusarium culmorum*, *Fusarium graminearum, Fusarium verticilloides* and *Bipolaris sorokiniana*) (Orakci *et al.,* 2010). Streptomyces spp. successfully isolated as endophytic it can be used as a fight against phytopathogenic fungi such as *Aspergillus niger, Aspergillus flavus, Alternaria brassicicola*, *Botrytis cinerea, Penicillium digitatum, Fusarium oxysporum, Penicillium pinophilum*, *Phytophthora dresclea* and *Colletotrichum* falcatum (Gangwar *et al.,* 2011).

Table.1 The similarity of isolate 1 with isolates in GenBank

Lasiodiplodia theobromae	Similarity percentage (%)	*Accession Number*
Lasiodiplodia theobromae **isolat BTMA10**	100	KY657465
Lasiodiplodia theobromae **isolat BTMA9**	100	KY657464
Lasiodiplodia theobromae **isolat BTMA8**	100	KY657463
Lasiodiplodia theobromae **isolat BTMA7**	100	KY657462
Lasiodiplodia theobromae **isolat BTMA6**	100	KY657461
Lasiodiplodia theobromae **isolat BTMA5**	100	KY657460
Lasiodiplodia theobromae **isolat BTMA4**	100	KY657459
Lasiodiplodia theobromae **isolat BTMA3**	100	KY657458
Lasiodiplodia theobromae **isolat BTMA2**	100	KY657457

Table.2 Exophytic microbial types and populations found in healthy mangoes

No.	Name of microbes	Population (x 10^2cfu)	Prevalence (%)
1	*Rhizopus* sp.	24	26,67
2	*Nucordia* sp. (Actinomycetes)	18	20
3	*Streptomyces* sp. (Actinomycetes)	12	13,33
4	*Aspergillus flavus*	18	20
5	*Aspergillus niger*	18	20
Total		**90**	**100**

Table.3 Inhibition ablity of exophytic microbes on *L. theobromae*

Number of isolate	Microbes name	*L. theobromae*	
		Inhibition ability 4 dai* (%)	Inhibition ability 7 dai* (%)
1	*Rhizopus* sp.1	88,89±0,3	72,22±0,2
2	Nucordi sp. (Actinomycetes) 1	-	-
3	*Streptomyces* sp. (Actinomycetes) 1	-	-
4	*Aspergillus flavus* 1	-	-
5	*A. niger* 1	83,33±0,4	77,78±0,3
6	*A.niger* 2	-	-
7	*Rhizopus* sp. 2	83,33±0,5	80±0,5
8	*Nucordia* sp. (Actinomycetes) 2	-	-
9	*A.flavus* 2	-	-
10	*Rhizopus* sp.3	77.78±0,6	77,78±0,4
11	*A.flavus*3	-	-
12	*Streptomyces* sp. (Actinomycetes) 2	-	-
13	*Rhizopus* sp.4	83,33±0,3	98±0,2
14	*Nucordia* sp. (Actinomycetes) 3	-	-
15	*Rhizopus* sp. 5	-	-

*dai = days after inoculation

Table.4 The best inhibitory test results in vivo are antogonists against pathogens *(L.theobromae)*

Treatment	Name of microbes	Diseases incidence (%)	Notation	
			5%	1%
A	*Rhizopus* sp. 4	95±4,47	A	A
B	*A. niger* 1	67±4	B	B
C	*Rhizopus* sp. 3	14±3,74	E	E
D	*Rhizopus* sp. 2	54±3,74	C	C
E	*Rhizopus* sp. 1	52±2,45	D	D
K-P	Control without pathogen	4,17±4	F	F
K+P	Control with pathogen	94±3,74	A	A

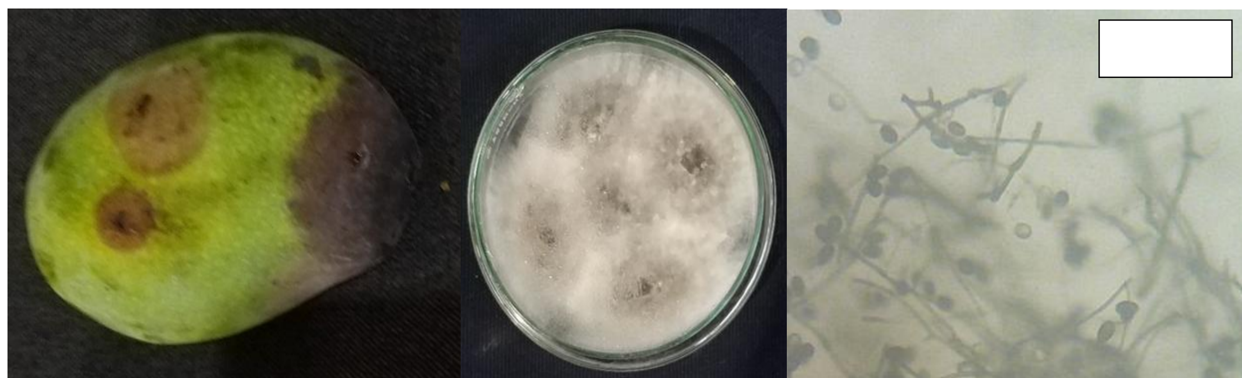

Figure.1 Symptoms of mango rot disease (A), (B) mycelium growth in Petri dishes, and (C) for conidia and conidiophores of fungal pathogens (*Lesiodiplodia theobromae*)

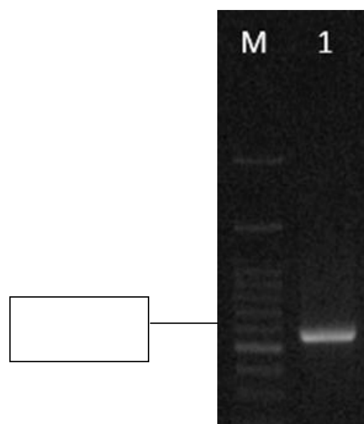

Fig.2 Pathogen *L. Theobromae* Amplikon gen 18S rRNA.
M. DNA Ladder 100 bp

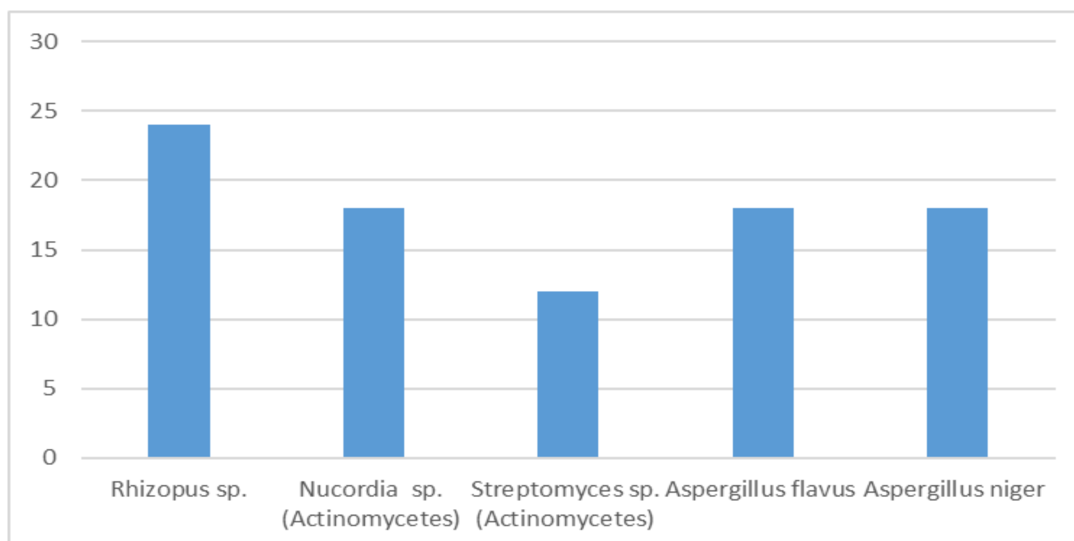

Figure.3 Types of exophytic microbes derived from healthy mangoes

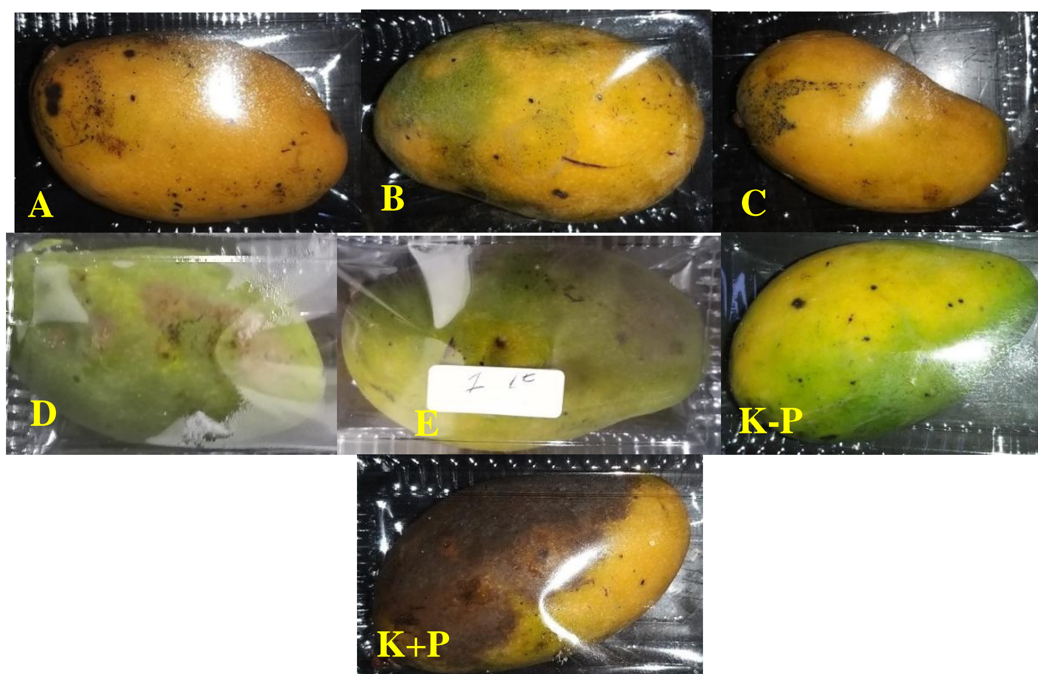

Figure.4 & 5 *In vivo* antagonist test is the best antagonist with pathogen (*L. theobromae*), (A = *Rhizopus*sp. 4, B = *A. niger* 1, C = *Rhizopus* sp. 3, D = *Rhizopus* sp. 2, E = *Rhizopus* sp. 1, K-P = control without pathogen and K+P = control with pathogen)

Rhizopus sp. can suppress the growth of *Aspergillus flavus* toxigenic molds and degrade aflatoxin. *Rhizopus* sp. can also produce compounds that can inhibit pathogenic bacteria and function as antioxidants. *Rhizopus* sp. absorb some mineral elements and convert them into organic minerals so that they can increase the absorption of minerals in the body better. Utilization of fermented feed ingredients by *Rhizopus* sp. in cattle showing better results compared to without fermentation. *Rhizopus* sp. it is also very potential to be applied as supplement feed for livestock (Endrawati and Kusumaningtyas, 2017). Sixteen endophytic fungi have been able to be identified as

Acremonium sp., *Aspergillus* spp., *Cephalosporium* sp., *Fusarium* spp., *Helicocephalum* spp., *Penicillium* spp., *Rhizopus* sp., and 4 species were not able to be identified. Antagonistic test results of the percentage of inhibition ranged from 36.93% - 100%. Statistical analysis shows that endophytic fungi are able to control *P. infestans* (Wulandari *et al.,* 2014).

Based on the results and discussion above, it can be concluded as follows:the pathogen found to cause fruit rot disease in mangoes is *Lesiodiplodia theobromae*. Exophyte microbes found in healthy mangoes include: *Rhizopus* sp. the number of colonies 24 x 10^2cfu, while *Nucordia* sp., *A. flavus*, and *A. niger* with colonies 18 x 10^2cfu each, and Streptomyces sp. with a colony of 12 x 10^2cfu. *Rhizopus* sp. Microbial inhibitory test results: 1 and *Rhizopu*s sp. 4 when 4 dai (days after inoculation) and 7 dai against pathogens (*L. theobromae*). The results of antagonistic inhibition test on pathogens (*L. theobromae*) *in vivo* obtained the highest by the treatment of C (*Rhizopus* sp. 3) which was very significantly different from K + P (control with pathogens).

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