



## USE OF EXOPHYTIC FUNGI IN SUPPRESSING ANTHRACNOSE PATHOGENS ON RAMBUTAN FRUIT (*Nephelium lappaceum* L.) *IN VITRO*

By

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### Abstract:

Anthracnose disease in rambutan fruit is caused by *Colletotrichum fructicola* which is found both in the harvest and post-harvest. The diversity index of exophytic fungi ranges from 0.4634 - 1.3143 with structural conditions from less stable to very rotten to moderate categories and with a score of 1-3. The dominance index of 0.643 - 0.773 is close to 1, this is supported by the uniformity index of 0.0816 - 1.1379, meaning that there are dominant species in the fruit exophyte, namely *Neurospora* sp, in the leaf exophyte *Aspergillus flavus* and in the stem exophyte *Aspergillus niger*. The highest in vitro inhibition of exophytic microbes on fruit was achieved by *A. niger* 4 of  $92.22 \pm 0.3\%$ . In the leaf exophyte, the highest inhibition was achieved by *A. niger* 1 of  $83.33 \pm 0.2\%$ . The highest inhibition on stem exophytes was achieved by *Neurospora* sp. 3 at  $94.44 \pm 0.1\%$ .

### Keywords:

Anthracnose, diversity index and dominance index, in vitro inhibition, exophytic fungi.



## INTRODUCTION

Anthrachnose disease caused by *Colletotrichum fructicola* has damaged rambutan fruit in Bali [1]. Symptoms of anthracnose in rambutan appear as brown spots on the skin of the fruit which gradually turn black and rot into the inside of the fruit so that the fruit looks unsightly. Rambutan fruit in Bali has become popular and is used during religious ceremonies. Rambutan fruit production in Bali in 2019 was 21,445 tons, in 2010 it was 15,943 tons and in 2021 it was 23,367 tons [2]. The increase in rambutan fruit in Bali Province in 2021 requires serious attention and handling regarding the problem of pest attacks and disease disturbances that infect rambutan fruit both before and after harvest (post-harvest).

Fruit rot disease (anthracnose) in rambutan fruit is a disease that is quite serious in disturbing rambutan fruit both freshly harvested and in storage (post-harvest). During the early stage, symptoms develop similar to brown spot disease. Furthermore, the infected area is widely overgrown with aerial mycelium that cannot be observed in the next stage of infection. The spots appear black, circular lesions with increasing size and becoming large, the spots settle, with orange to pink conidial masses observed in the infected pericarp under humid conditions after 5-6 days [3].

Rambutan fruit rot disease was first reported in 2017 attacking rambutan fruit in Thailand and Sri Lanka. Two pathogens have been reported, namely *C. fructicola* and *C. queenslandicum* [4]. In tropical fruit crops, anthracnose is mainly caused by species belonging to the genus *Colletotrichum*. This phytopathogen can infect several parts of the fruit plant; however, infection during post-harvest or ripening stages is responsible for major economic losses. Due to the formation of black to dark brown sunken lesions on the fruit surface, anthracnose reduces fruit quality and marketability. Among the most common tropical fruit crops susceptible to anthracnose are mango, papaya, banana, avocado, guava, and dragon fruit; these are economically relevant products in many developing countries.

It is important to document newly recorded *Colletotrichum* spp. associated with fruit anthracnose as they can infect multiple hosts, but some species may be host-specific. Through the use of multiple markers, many phylogenetic species of *Colletotrichum* have been found to be reported as anthracnose pathogens. Considering that disease management strategies rely heavily on adequate knowledge of the causative agents, up-to-date information on *Colletotrichum* species and the hazards posed by the most recently identified species in tropical fruit plantations and harvested fruits is vital. In addition, newly recorded species may be important for biosecurity and should be listed as quarantine pathogens, given that tropical fruits are traded worldwide [5].

The use of exophytic fungi in controlling plant diseases has not been fully studied, but there are several studies that have found that leaf surfaces called phylloplanes provide a suitable habitat for the growth of antagonistic microorganisms that can compete with pathogens for nutrients and inhibit pathogen multiplication by secreting antibiotics or toxins. The plant phyllosphere is a dynamic ecosystem inhabited by certain bacteria, yeasts and fungi. Their activities are related to various interactions between biotic and abiotic environmental factors. The interactions of microorganisms living on the aboveground plant surface are based on antibiosis, competition and parasitism that protect plants from pathogens and improve plant health [6].

Exophytic fungi or phylloplanes are fungi that grow on the surface of leaves [7]. There are two groups of phylloplanes fungi; resident and causal [8]. Residents can multiply on the surface of healthy leaves without affecting the host, while causals land on the surface but cannot grow [9]. Phylloplanes fungi are less studied compared to endophytes, saprobes, and pathogenic fungi. In recent years,

phylloplane microbes have been studied, it turns out that there are interactions with plants, herbivores and pathogens that live on leaves, possibly related to the immune system, reabsorption of organic and mineral materials from leachetes, redistribution of nutrients until the leaves full and participation in the primary degradation of plant tissue. There is a finding [10] that phylloplanes fungi that grow such as *Trchoderma viride* and *Aspegillus flavus* can suppress the maximum *Alternaria brassicae* on cabbage leaves.

## MATERIALS AND METHODS

### Place and Time of Research

The research was conducted in two places: 1) looking for sick and healthy panicle specimens from Farmer Plantations in Paku Dui Village, Tegalalang, Gianyar. 2) Plant Disease Laboratory and Agricultural Biotechnology Laboratory. The research was conducted from April to August 2024.

### Isolation of Exophytic Fungi

Isolation of exophytic fungi, plant parts such as fruits, leaves and stems from healthy plants are washed with sterile running water, then the washing water is collected, then 1 ml of the collected water is taken and placed on PDA media (which has been previously given an antibacterial antibiotic, namely livoploxacin with a concentration of 0.1% (w / v). The fungi that appear after 2 days are counted as colony forming units. Furthermore, the fungi that grow are transferred to a new Petri dish as many colonies as grow (can be separated).

### Identification of Exophytic Fungi

Exophytic fungi were then grown on Petri dishes containing PDA and repeated 3 times. The cultures were incubated in a dark room at room temperature ( $\pm 27^{\circ}\text{C}$ ). Isolates were identified macroscopically after 3 days to determine colony colour and growth rate, and microscopic identification to determine septa in hyphae, spore/conidia shape and sporangiophores. Identification of fungi using reference books [11, 12, 13, 14, and 15).

### Inhibitory Test of Exophytic Fungi against Pathogens

The endophytic and exophytic fungi found were each tested for their inhibitory power against the growth of pathogenic fungi using the dual culture technique (in one Petri dish, one pathogenic fungus was grown flanked by two endophytic fungi). The inhibitory power can be calculated as follows [16; 17].

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

Where:

A = Diameter of pathogen colonies in single culture (mm)

B = Diameter of pathogen colonies in dual culture (mm)

## Prevalence of Exophytic Fungi

Determining the prevalence of endophytic and exophytic fungi is 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 magnitude of the isolate prevalence will determine the dominance of endophytic fungi in healthy mango fruit.

## Determining Diversity and Dominance Index

The diversity and dominance of contaminant fungi can be determined by calculating the Shannon-Wiener diversity index [18] and the dominance of soil microbes is calculated by calculating the Simpson index [19].

### (1) Microbial diversity index

The soil microbial diversity index is determined by the Shannon-Wiener diversity index, namely by the formula [18].

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

Where:  $H'$  = Shannon-Wiener diversity index

$S$  = Number of genera

$P_i = n_i/N$  as a proportion of type  $i$  ( $n_i$  = Total number of individuals of microbial type

$i$ ,  $N$  = Total number of individuals in total  $n$ )

The criteria used to interpret Shannon-Wiener diversity [20] are:  $H'$  value  $< 1$ , meaning low diversity,  $H'$  value  $1 - 3$  meaning moderate diversity and  $H'$  value  $> 3$  meaning high diversity or (Table 1).

**Table 1. Environmental quality weighting assessment criteria [21]**

Diversity index	Condition of community structure	Category	Scale
$>2.41$	Very stable	Very good	5
$1.21 - 2.4$	More stable	Good	4
$0.61 - 1.2$	Quite stable	Average	3
$<0.6$	Less stable	Bad	2
	Not stable	Very bad	1

### (1) Dominance index

The pest and natural enemy dominance index is calculated by calculating the Simpson index [22] with the following formula:

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

Where:

$C$  = Simpson index

$S$  = Number of genera

$P_i = n_i/N$ , namely the proportion of individuals of species  $i$  and all individuals ( $n_i$  = Total number of individuals of species  $i$ ,  $N$  = Total number of individuals in total  $n$ )

Furthermore, the species dominance index (D) can be calculated using the 1-C formulation [23].

The criteria used to interpret the dominance of soil microbial species are: approaching 0 = low index or increasingly low dominance by one microbial species or no species that extremely dominates other species, approaching 1 = large index or tends to be dominated by several microbial species [22].

### (1) Evenness index (E)

To determine the balance of the community, the evenness index is used, namely a measure of the similarity of the number of individuals between species in a community. The more similar the number of individuals between species (the more even the distribution), the greater the degree of balance. The formula for the evenness index (e) is obtained from [24].

$$E = H' / \ln S$$

Where:

H' = diversity index

S = Number of species

E = Evenness evenness index

The smaller the value of the diversity index (H'), the smaller the uniformity index (E) will be, which indicates the dominance of one species over another species.

Here are the ranges:

E < 0.4: small population uniformity

0.4 < E < 0.6: medium population uniformity E

> 0.6: high population uniformity.

## RESULTS AND DISCUSSION

### Exophytic and Endophytic Microbial Population

#### a. Exophytic microbial population

The largest exophytic microbial population in rambutan fruit was obtained from *A.niger* as much as  $33 \times 10^3$  cfu, followed by *A. flavus* as much as  $6 \times 10^3$  cfu and *Neurospora* sp.  $3 \times 10^3$  cfu, exophytes in leaves were obtained from *A. flavus* as much as  $18 \times 10^3$  cfu, *A. niger* as much as 9 cfu and *Micromonospora* sp. (Actinomycetes) as much as  $3 \times 10^3$  cfu, *Miselia sterilia* as much as  $3 \times 10^3$  cfu and *Streptomyces* sp. (Actinomycetes) as much as  $3 \times 10^3$  cfu. While the stem exophytes were obtained from *A. niger* as much as  $9 \times 10^3$  cfu, *Neurospora* sp. as much as  $36 \times 10^3$  cfu and *Trichoderma* sp. as much as  $3 \times 10^3$  cfu (Table 2, Figure 1).

**Table 2. Population of exophytic microbes in healthy rambutan fruit, leaves and stems**

No.	Fruit		Leaf		Stem	
	Name of microbes	Population x10 <sup>3</sup> (cfu/ml)	Name of microbes	Population x10 <sup>3</sup> cfu/ml	Name of microbes	Population x 10 <sup>3</sup> cfu/ml
1	<i>Aspergillus flavus</i>	6 (14%)*	<i>Aspergillus flavus</i>	18 (50%)	<i>A. niger</i>	9 (19%)
2	<i>A. niger</i>	33 (79%)	<i>A. niger</i>	9 (25%)	<i>Neurospora sp.</i>	36 (75%)
3	<i>Neurospora sp.</i>	3 (7%)	<i>Micromonospora (Actinomycetes)</i>	3 (8%)	<i>Trichoderma sp.</i>	3 (6%)
4			<i>Miselia sterilia</i>	3 (8%)		
5			<i>Streptomyces sp. (Actinomycetes)</i>	3 (8%)		
Total		42		36		48

\* The numbers in brackets are the prevalence of microbes found.

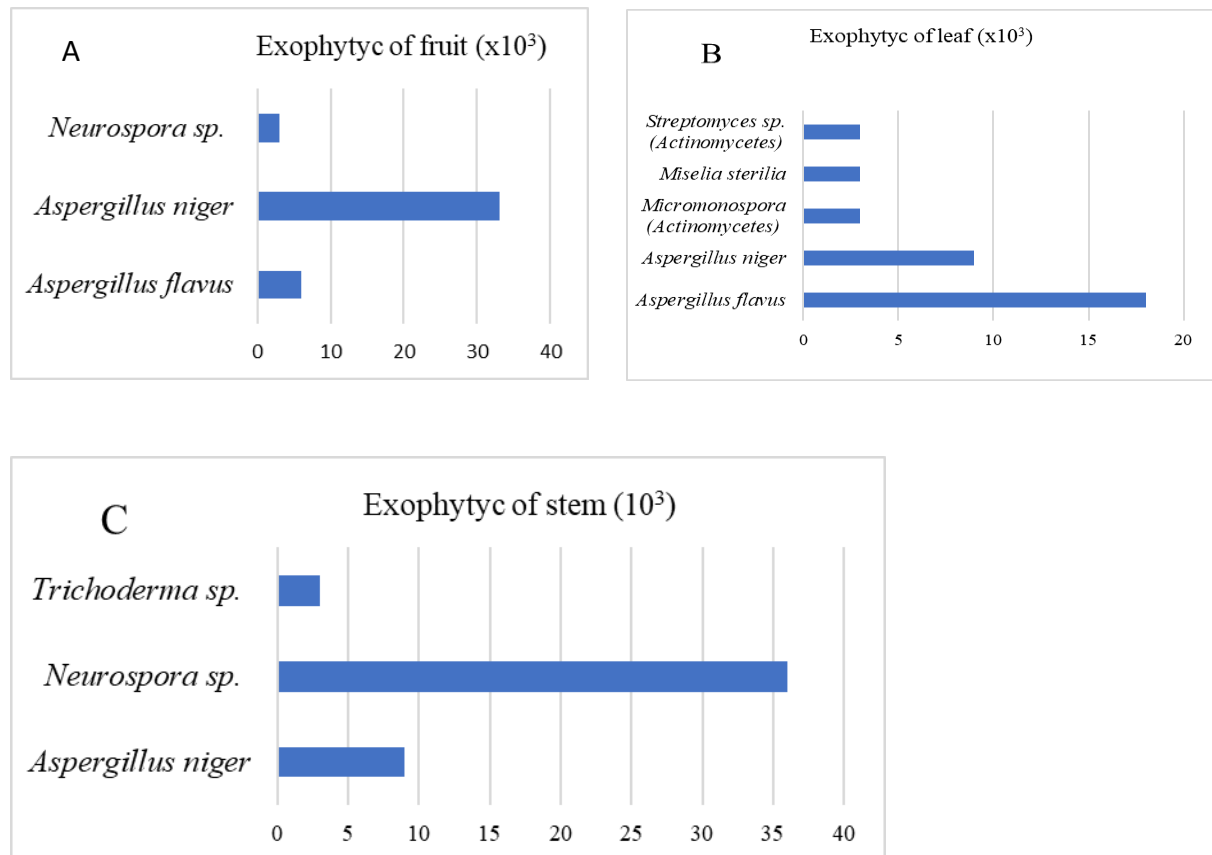


Figure 1. Population of exophytic microbes in fruit (A), leaves (B) and stems (C) (x 10<sup>3</sup>) of healthy rambutan.

The highest prevalence in fruit exophytes was found to be *A. niger* at 79%, in leaf exophytes the highest prevalence was *A. flavus* at 50% and in stem exophytes the highest prevalence was *Neurospora sp.* at 75%.

In line with this study, the population of exophytic microbes found in rice leaves, fruits and stems were  $7.1 \times 10^5$  cfu/ml,  $3.8 \times 10^5$  cfu/ml, and  $3.1 \times 10^5$  cfu/ml, respectively, and the highest prevalence in leaf exophytes was *Streptomyces* sp. and *Aspergillus* sp. at 20% each, the highest prevalence in fruit exophytes was *Phytophthora* sp. at 33%, and in stem exophytes was

*A. flavus* at 33% [25]. While the highest exophyte in apple fruit was obtained from *Lasiodiplodia theobromae* at 22% [26].

### Diversity and Dominance Index

The index of exophytic microbial diversity of fruit is 0.4634, meaning that with the assessment criteria it is unstable with a very bad category, its dominance index with a Simpson value (C) of 0.773, meaning that there is a dominance of microbes found in the rambutan niche, namely *Neurospora* sp. fungus, of 88% with an evenness value (E) of 0.082, the small value of E means that there is a species that dominates. The index of exophytic microbial diversity on leaves is 1.3143, meaning that with the assessment criteria the condition of the community structure is quite stable with a moderate category (scale 3), the dominance index (D) of 0.667 means that there is a species that dominates, namely *A. flavus* of 50% with a uniformity value

(E) of 0.1379. The diversity index of stem exophytes was 0.656, meaning that the community structure was less stable with a poor category (scale 2), the dominance index (Simpson index) was 0.6429, with a uniformity value (E) of 0.137, and the dominance index by the *A. niger* species was 79% (Table 3).

**Table 3. Diversity, dominance and uniformity indices of healthy rambutan exophytes**

Plant parts	Diversity index (H)	Dominance/Simpson index (D/C)	Uniformity index (E)	Dominant species
Fruit	0.4634	0.773	0.0816	<i>Neurospora</i> sp.
Leaves	1.3143	0.667	0.1379	<i>A. flavus</i>
Stem	0.6429	0.643	0.1359	<i>A. niger</i>

The diversity index of pineapple fruit exophytes was obtained at 2.2067 with a dominance index of 0.7355, meaning that there is a dominant species, namely *Rhizopus* sp. at 43% [27]. While the results of the study [28] proved that the diversity index of apple fruit exophytes with a diversity index of 1.249, and a dominance index of 0.885, the dominant microbe is *L. theobromae* at 22%.

### Inhibitory Ability of Exophytic Fungi *In Vitro*

The highest in vitro inhibition power of exophytic microbes on fruit was achieved by *A. niger* 4 at  $92.22 \pm 0.3\%$  and the lowest was achieved by *A. niger* 3 at  $66.67 \pm 0.5\%$ . In leaf exophytes, the highest inhibition power was achieved by *A. niger* 1 at  $83.33 \pm 0.2\%$  and the lowest was achieved by *A. niger* 3 at  $72.22 \pm 0.5\%$ , the highest inhibition power on stem endophytes was achieved by *Neurospora* sp. 3 at  $94.44 \pm 0.1\%$  and the lowest was achieved by *Trichoderma* sp. at  $72.22 \pm 0.4\%$  (Table 4).

The results of the study [29] found that the exophytic fungus *A. niger* has the highest inhibitory power against the banana fruit pathogen (*C. muase*) in vitro. Likewise, the results of the study [30] found that the inhibitory power of the antagonistic microbe, namely *A. niger*, in vitro has the highest inhibitory power against the apple fruit rot pathogen (*Fusarium decemcellulare*).



*Neurospora* sp. is an exophytic fungus that has the strongest inhibitory power in controlling post-harvest grape rot disease (*A. niger*) in vitro, namely 88.89% [31].

Tabel. 6.6. Daya hambat mikroba ekofit terhadap patogen (*C. fruticola*)

No.	Buah		Daun		Batang	
	Nama mikroba	Daya hambat (%)	Nama mikroba	Daya hambat (%)	Nama mikroba	Daya hambat (%)
1	<i>Aspergillus niger</i> 1	92.22	<i>Aspergillus flavus</i> 1	83.33±2	<i>A. niger</i> 1	94.44±0.4
2	<i>A. niger</i> 2	72.22±0.8	<i>A. flavus</i> 2	77.78±1	<i>A. niger</i> 2	94.44±0.2
3	<i>A. niger</i> 3	66.67±0.5	<i>A. flavus</i> 3	77.78±3	<i>Neurospora</i> sp.1	94.44±2
4	<i>A. niger</i> 4	72.22±0.9	<i>A. flavus</i> 4	82.22±0.2	<i>Neurospora</i> sp.2	94.44±0.3
5	<i>A. niger</i> 5	74.44±2	<i>A. flavus</i> 5	80±0.9	<i>Neurospora</i> sp.3	94.44±0.1
6	<i>A. niger</i> 3	80±3	<i>A. flavus</i> 6	83.33±2	<i>Neurospora</i> sp.4	94.44±0.5
7	<i>A. niger</i> 4	92.22 ± 0.3	<i>A. niger</i> 1	83.33±0.2	<i>Neurospora</i> sp.5	94.44±0.3
8	<i>A. niger</i> 6	83.33±4	<i>A. niger</i> 2	77.78±0.8	<i>Neurospora</i> sp. 6	83.33±0.2
9	<i>A. niger</i> 7	66.67±2	<i>A. niger</i> 3	72.22±0.5	<i>Neurospora</i> sp.7	94.44±0.2
10	<i>A. niger</i> 8	66.67±4	<i>Micromonospora</i> 1(Actinomycetes) —	-	<i>Neurospora</i> sp. 8	94.44±0.3
11	<i>A. niger</i> 9	91.11±0.6	<i>Miselia sterilia</i> 1	-	<i>Neurospora</i> sp. 9	94.44±0.5
12	<i>A. niger</i> 10	72.22±0.5	<i>Streptomyces</i> sp. (Actinomycetes)	-	<i>Neurospora</i> sp. 10	83.33±0.2
13	<i>A. niger</i> 11	81.11±0.6			<i>Neurospora</i> sp. 11	94.44±0.4
14	<i>A. flavus</i> 1	83.33±3			<i>Neurospopra</i> sp.12	94.44±0.3
15	<i>A. flavus</i> 2	77.78±0.9			<i>Trichoderma</i> sp.1	72.22±0.4
16	<i>Neurospora</i> sp.	83.33±0.6			<i>A. niger</i> 1	94.44±0.2

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## CONCLUSION

Based on the results and discussions above, it can be concluded as follows: the diversity index of exophytic fungi ranges from 0.4634 - 1.3143 with structural conditions ranging from less stable to very rotten to moderate categories and with a score of 1-3. The dominance index of 0.643 - 0.773 is close to 1, this is supported by the uniformity index of 0.0816 - 1.1379, meaning that there are dominant species in the fruit exophyte, namely *Neurospora* sp, in the leaf exophyte *A. flavus* and in the stem exophyte *A. niger*. The highest in vitro inhibition of exophytic microbes on fruit was achieved by *A. niger* 4 of  $92.22 \pm 0.3\%$ . In the leaf exophyte, the highest inhibition was achieved by *A. niger* 1 of  $83.33 \pm 0.2\%$ . The highest inhibition on the stem exophyte was achieved by *Neurospora* sp. 3 of  $94.44 \pm 0.1\%$ .

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