Research article

Antagonıst Test of Endophyte Fungi Isolated from Leaves of Mangrove (*Rhizophora* sp.) as Antıfungi against Sanca Snakes (*Malayopython* sp.) Disease

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Abstract

Keywords

antagonist test;
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tick

Mangrove plants have many benefits, such as the ability to produce bioactive compounds. Bioactive compounds can be produced by endophytic fungi found on mangrove leaves. Endophytic fungi are also known to produce secondary metabolites that are the same as their hosts produce for plant defence. Endophytic fungi in mangrove leaves (*Rhizophora* sp.) have antagonistic potential against pathogenic fungi. One of the pathogenic fungi was shown to cause a skin disease in pythons (*Malayopython* sp.) through the transmission via tick saliva. Pathogenic fungi found in tick saliva were *Fusarium oxysporum* and *Aspergillus niger*. This study examined the potential of endophytic fungi in mangrove leaves (*Rhizophora* sp.) as anti-fungal pathogens in pythons. Endophytic fungi were isolated from mangrove leaves at the Angke Kapuk Mangrove Nature Park, Pantai Indah Kapuk, DKI Jakarta, and an antagonist test of endophytic fungi against pathogenic fungi was performed using the dual culture assay method. This study obtained 16

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isolates, and nine isolates had antagonistic potential. These nine isolates produced a clear zone in the antagonist test. The inhibition zone indicates endophytic fungi inhibit the growth of pathogenic fungi.

1. Introduction

Mangrove forests are tropical coastal communities dominated by several types of mangrove trees or shrubs that can grow in salty waters. The mangrove forests are inundated by seawater, and their growth is affected by sea tides [1]. Jakarta has three mangrove ecotourism destinations open to the public, one of which is the Angke Kapuk Mangrove Nature Park, which has become a favourite tourist attraction in Jakarta. Angke Kapuk Natural Tourism Park (TWA) is a wetland ecosystem dominated by mangrove trees. Several types of mangroves that dominate the Angke Kapuk TWA area include pidada (*Sonneratia caseolaris*), warakas (*Acrosticum areum*), api-api (*Avicennia marina*), cantinggi (*Ceriops* sp.), blind-buta (*Excoecaria agallocha*), and mangrove (*Rhizophora mucronata* and *Rhizophora stylosa*). Mangrove plants are known to have many benefits for human life, ranging from ecological benefits as a food source to a source of traditional medicine [2]. Poncowati *et al.* [3] reported that mangrove plants contain alkaloids, flavonoids, phenols, steroids, terpenoids, and quinones. These compounds are often used as antioxidants, antimicrobials, and anticancer agents, and they are commonly referred to as bioactive compounds. Recently, popular bioactive compounds have been derived from microbes. One type of microbe that contain bioactive compounds is endophytic microbe [4].

Endophytic fungi can grow in plant tissue without endangering their host plant [5]. Endophytic fungi can also produce secondary metabolites that are the same as those produced by their host plants [6]. Endophytic fungi isolated from host plants have the potential to be developed as secondary metabolite producers. Endophytic microbes, such as endophytic fungi from mangrove plants, are rarely fully utilized. Several studies on mangrove plants have shown that these plants produce a range of natural ingredients including active compounds that act as antimicrobial agents [7]. The endophytic fungi isolated from the leaves of the mangrove (*Rhizophora* sp.) have antagonistic potential against the pathogenic fungus *Fusarium solani* [8]. Hidayat *et al.* [9] reported that endophytic fungi display antagonistic properties against pathogenic fungi. This is shown by the formation of inhibition zone between endophytic fungi and pathogenic microorganisms [9]. Endophytic fungi can produce antibiotic compounds that inhibit pathogen growth by lysing the cell walls of pathogenic microorganisms [9, 10].

Pathogenic microorganisms can infect various living things, such as pythons and can cause skin diseases. Skin diseases in pythons are caused by obligate blood-sucking ectoparasites such as ticks [10]. Ticks can transmit various pathogens, including viruses, bacteria, protozoa, fungi, and nematodes. There are two families of ticks, Ixodidae (hard ticks) and Argasidae (soft ticks) [11]. Vertebrate skin consists of two layers, namely the epidermis and the dermis. These layers have immune and effector cells that are inflammatory immune defence mechanisms. To reach blood vessels, ticks generate food pools by cutting into their host's skin with chelicerae that extend and tear the epidermis, followed by inserting their hypostome into the dermis, resulting in significant damage. Transmission of pathogens from ticks is assisted by their saliva, which creates a local immune environment at the bite site. The transmission of pathogens from ticks via their saliva secretions can enhance the formation and infection [12]. Zivkovic *et al.* [13] reported that pathogenic fungi in ticks were *Aspergillus parasiticus, Penicillium steckii* and *Scopulariopsis brevicaulis*.

This study examined the potential of endophytic fungi from mangrove leaves (*Rhizophora* sp.) as anti-fungal pathogens in pythons. *Fusarium oxysporum* UNJCC F-132 and *Aspergillus niger* UNJCC F-133 as pathogenic fungi were used in this study. The antagonistic test

of mangrove plant microorganisms was examined to identify the potential of endophytic fungi on mangrove leaves to act as antifungal pathogens. The antagonist test is a method used to prove that an antagonistic microorganism can inhibit the activity of other microorganisms in adjacent areas. Gao *et al.* [14] conducted an antagonism test of pathogenic and endophytic fungi using the dual culture assay method. Therefore, the dual culture assay method which refers to Gao *et al.* [14] was used in this study.

2. Materials and Methods

2.1 Sampling

Sampling was carried out in the mangrove ecosystem in the Angke Kapuk Natural Tourism Park (TWA) located in the Kamal Muara sub-district, Pantai Indah Kapuk, North Jakarta, with three repetitions at each different station. The leaves of the mangrove plant (*Rhizophora* sp.) were used as isolated tissue. This was because the leaves contained endophytic microbes (fungi) which produce secondary metabolites. The endophytic fungi were isolated from leaf tissue and then used for identification and antagonist tests.

2.2 Isolation of endophytic fungi

Fungi isolation was carried out following Aparna *et al.* [15]. Mangrove leaf samples were sterilized for 1 min using 70% alcohol and then immersed in 99% sodium hypochlorite (NaOCl) for 2 min. Then, the samples were rinsed with sterile distilled water three times and dried with sterile tissue paper. After that, the leaf samples were placed onto PDA (Potato Dextrose Agar) and YMA (Yeast Malt Agar) media which had previously been added 200 mg/L chloramphenicol to prevent contamination from bacteria in the media. The mangrove leaf samples were then incubated for approximately three days at room temperature. The isolates that grew were separated and activated until pure culture in PDA and YMA media were attained. They were then incubated for seven days at room temperature, after which purification was carried out [16].

2.3 Pure cultue of endophytic fungi

Colonies obtained from the isolation were then purified by taking some of the mycelia in the growing zone and transferring them aseptically to PDA culture media. Colonies were incubated at room temperature for five days upside down. Several types of endophytic fungi can only grow optimally if there is nutrition from the host [15, 17]. Therefore, several endophytic fungi obtained from the isolation were transfered into PDA culture media and suplemented with 10% mangrove leaf extract following the method described by Ilmi *et al*. [17].

2.4 Observation of fungal morphology

Fungi identification was conducted using macroscopic and microscopic observations. According to Arnold *et al.* [18], macroscopic identification based on the characteristics of the colonies formed, such as colony shape, texture, growing zone, colony colour, radial lines, and exudate drops were used for identification. Meanwhile, microscopic observations were done by observing the presence or absence of sexual and asexual spores, the shape of the spores, the shape of the surface of the conidia, the septum on the hyphae, and the growth of the hyphae, and the observations were used for identification [18, 19].

2.5 Antagonist test

Endophytic fungi isolates were tested using the dual culture assay method following the method of Sukmawati *et al.* [20] with modifications. Pieces of endophytic and pathogenic fungi were grown in the same petri dish. Pathogenic fungi isolates (test pathogens) were placed at a distance of 3 cm from the endophytic fungi (antagonists). PDA were used as the culture media. Samples were incubated at 37ºC for 24 h. Calculation of the percentage of inhibition rate (IR) of antagonistic fungi, as reported by Sukmawati *et al.* [20], was used.

$$\text{IR } (\%) = \frac{\text{Rk}-\text{R1}}{\text{Rk}} \times 100\% \quad (1)$$

Notes :
IR = Percentage of inhibition rate (%)
Rk = Growth distance of pathogenic fungi control from the point of inoculation to the edge of the colony
R1 = The radius of the pathogenic fungi colony whose growth direction approaches the antagonist fungi colony

3. Results and Discussion

Sixteen isolates were obtained from the leaves of the mangrove plant (*Rhizophora* sp). There were 3 isolates from station 1, 6 isolates from station 2, and 7 isolates from station 3. The temperatures at stations 1, 2, and 3 were 26°C, 25ºC, and 25.5ºC, respectively. Humidity values at stations 1, 2, and 3 were 75%, 67%, and 67%, respectively. Temperature and humidity are factors that affect the growth of endophytic fungi. Station 3 was in a more open location and was directly exposed to sunlight, in contrast to stations 2 and 1, which were in a protected location and were not too exposed to sunlight. The growth of endophytic fungi is highly dependent on the host's nutrition [21]. According to Sarkar *et al.* [22], light intensity is an external factor that determines the amount and components produced by endophytic fungi. This resulted in more endophytic fungi being obtained from station 3 due to its location, which was directly exposed to sunlight. The exposure to sunlight optimized the photosynthetic process, resulting in more nutrients being produced to support the growth of endophytic fungi. From the results of macroscopic observations, the characteristics and morphology of the endophytic fungi varied greatly. The macroscopic morphology of the studied endophytic fungi is shown in Table 1 and Figure 1. The microscopic morphology of the studied endophytic fungi is shown in Table 2 and Figure 2.

The pathogenic fungi used in this study were *Fusarium oxysporum* UNJCC F-132 isolate and *Aspergillus niger* UNJCC F-133 isolate from python skin. There were several types of endophytic fungi that had antagonistic potential as determined through the antagonist tests of the endophytic fungi on pathogenic fungi, namely S1.3.1, S1.3.2, S3.2.4, S2.2.5, S3.3.6, S2.2.8, S2.1.9, S2.3.10, and S3.1.12. Potential antagonists were verified by the formation of an inhibition zone. The results of calculating the percentage of inhibition zones by endophytic fungi against the pathogen *Aspergillus niger* are shown in Table 3 and Figure 3. The endophytic fungi against the pathogenic fungi *Fusarium oxysporum* are shown in Table 4 and Figure 4.

Fusarium oxysporum and *Aspergillus niger* are pathogenic fungi isolated from python skin. From the antagonist test experiment results, there were five isolates of endophytic fungi with antagonistic potential against the fungi *Aspergillus niger*. There were four isolates of endophytic

Table 1. The results of macroscopic morphological observations of endophytic fungi isolates from the leaves of the mangrove plant (*Rhizophora* sp.)

Isolate No.	Colony Color	Colony Texture	Radial Furrow	Exudate Drop	Growing Zone	Zonation
S1.3.1	(Front) white; (Back) white	*Cottony*	-	-	✓	✓
S1.3.2	(Front) white; (Back) ivory	*Cottony*	-	-	✓	-
S1.2.3	(Front) Paynes's grey, light flesh; (Back) light flesh	*Velvet*	-	-	✓	-
S3.2.4	(Front) ivory; (Back) ivory	*Cottony*	-	-	-	-
S2.2.5	(Front) cold grey, (Back) cream	*Cottony*	-	✓	-	-
S3.3.6	(Front) warm grey; (Back) warm grey	*Cottony*	-	-	-	-
S3.1.7	(Front) white; (Back) crem, burnt ochre	*Cottony*	✓	✓	✓	✓
S2.2.8	(Front) ivory; (Back) ivory	*Cottony*	-	-	✓	✓
S2.1.9	(Front) medium flesh; (Back) white flesh	*Cottony*	-	-	✓	-
S2.3.10	(Front) medium flesh; (Back) light flesh	*Cottony*	-	-	-	-
S3.3.11	(Front) paynes's grey, ivory, chrome green opaque; (Back) walnut brown	*Velvet*	✓	-	✓	✓
S3.1.12	(Front) white; (Back) cream	*Cottony*	-	-	-	-
S3.2.13	(Front) white, cold grey; (Back) ivory	*Cottony*	-	-	✓	-
S2.3.14	(Front) light flesh, medium flesh; (Back) burnt ochre	*Cottony*	-	-	-	-
S2.3.15	(Front) Caput mortuum violet, cold grey; (Back) burnt sienna	*Cottony*	-	-	-	✓
S3.3.16	(Front) Payne's grey; (Back) black	*Velvet*	-	-	-	-

Notes: (-) absent, (✓) present

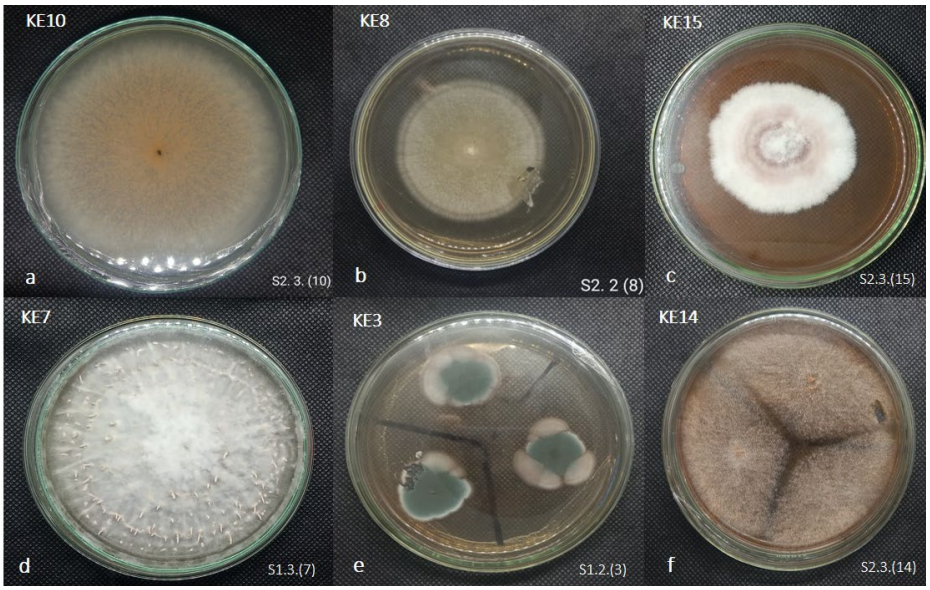

Figure 1. The results of macroscopic observations of endophytic fungi on mangrove leaves. a) KE10=S2.3.10, b) KE8=S2.2.8, c) KE15=S2.3.15, d) KE7=S1.3.7, e) KE3=S1.2.3, and f) KE14 = S2.3.14

Table 2. The results of microscopic morphological observations of endophytic fungi isolates from the leaves of the mangrove plant (*Rhizophora sp.*) at 400-1000x magnification.

Isolate No.	Hypha Segment	Hypha Growth	Conidia	Conidia Form
S1.3.1	✓	Branched	✓	Round
S1.3.2	✓	Branched	✓	Oval
S1.2.3	✓	Branched	-	-
S3.2.4	✓	Branched	✓	Round
S2.2.5	✓	Branched	✓	Round
S3.3.6	✓	Branched	✓	Round
S3.1.7	✓	Branched	✓	Round
S2.2.8	✓	Branched	✓	Oval
S2.1.9	✓	Branched	-	-
S2.3.10	✓	Branched	-	-
S3.3.11	✓	Branched	✓	Round
S3.1.12	✓	Branched	✓	Round
S3.2.13	✓	Branched	-	-
S2.3.14	✓	Branched	-	-
S2.3.15	✓	Branched	✓	Round
S3.3.16	✓	Branched	✓	Round

Notes: (-) absent, (✓) present

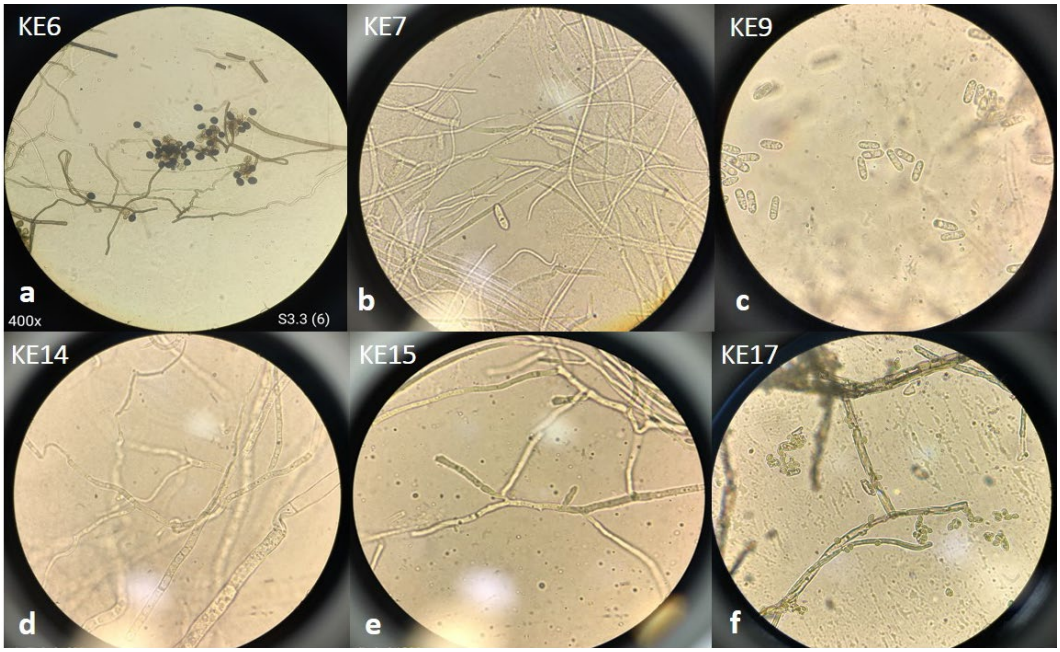

Figure 2. The results of microscopic observations of endophytic fungi on mangrove leaves. a) KE6=S3.3.6, b) KE7=S3.1.7, c) KE9=S2.1.9, d) KE14=S2.13.14, e) KE15=S2.3.15, and f) KE17= S3.3.17

Table 3. Percentage of inhibition rate (IR%) by endophytic fungi against *Aspergillus niger* pathogenic fungi

Isolate No.	Rk (cm)	R1 (cm)	PGI (%)
S1.3.1	4.5	1	77.7
S3.2.4	4.5	1	77.7
S3.3.6	4.5	1.5	66.6
S2.1.9	4.5	1	77.7
S3.1.12	4.5	1.5	66.6

Notes: Rk = radius of control pathogenic fungi, R1= radius of growth of pathogenic fungi towards endophytic fungi

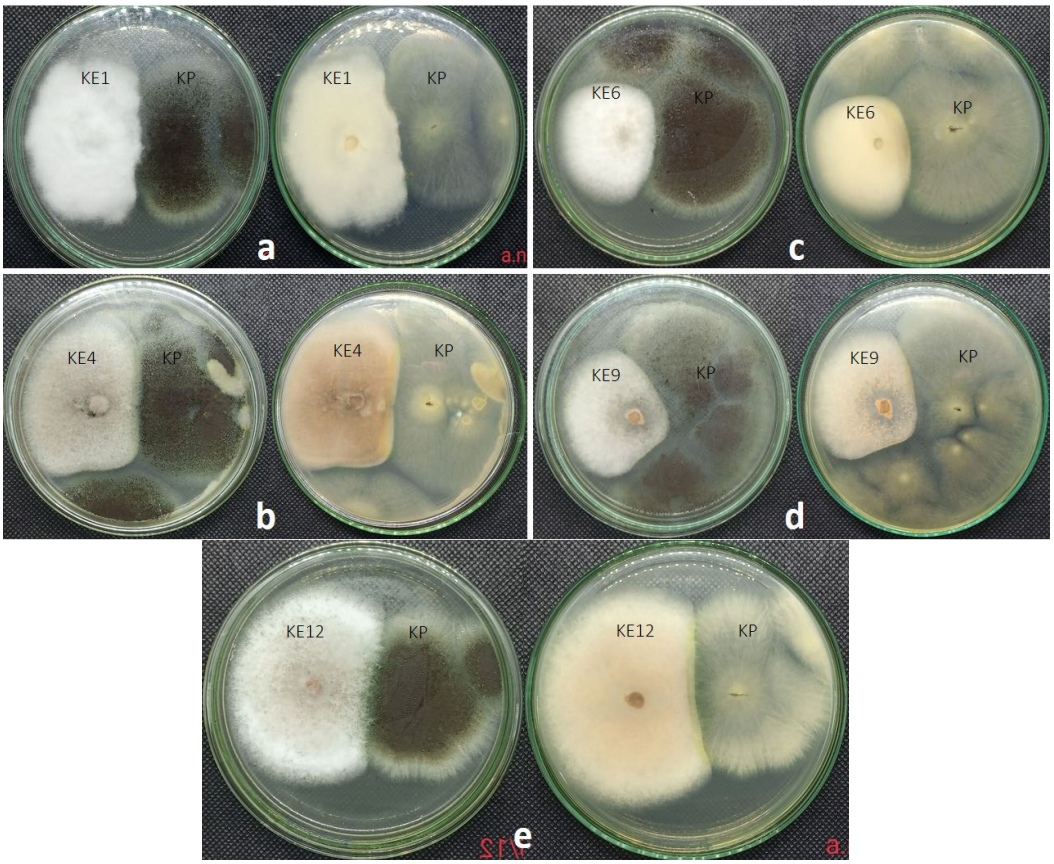

Figure 3. The results of antagonist test of endophytic fungi against the pathogenic fungi *Aspergillus niger.* a) KE1=S1.3.1 and KP = *Aspergillus niger*, b) KE4=S3.2.4 and KP = *Aspergillus niger*, c) KE6=S3.3.6 and KP = *Aspergillus niger*, d) KE9= S2.1.9 and KP = *Aspergillus niger*, and e) KE12=S3.1.12 and KP=*Aspergillus niger*

Table 4. Percentage of Inhibition Rate (IR%) by endophytic fungi against the pathogenic fungi *Fusarium oxysporum*

Isolate No.	Rk (cm)	R1 (cm)	PGI (%)
S1.3.2	4.5	1	77.7
S2.2.5	4.5	1	77.7
S2.2.8	4.5	1.5	66.6
S2.3.10	4.5	2	55.5

Notes: Rk = radius of control pathogenic fungi, R1= radius of growth of pathogenic fungi towards endophytic fungi

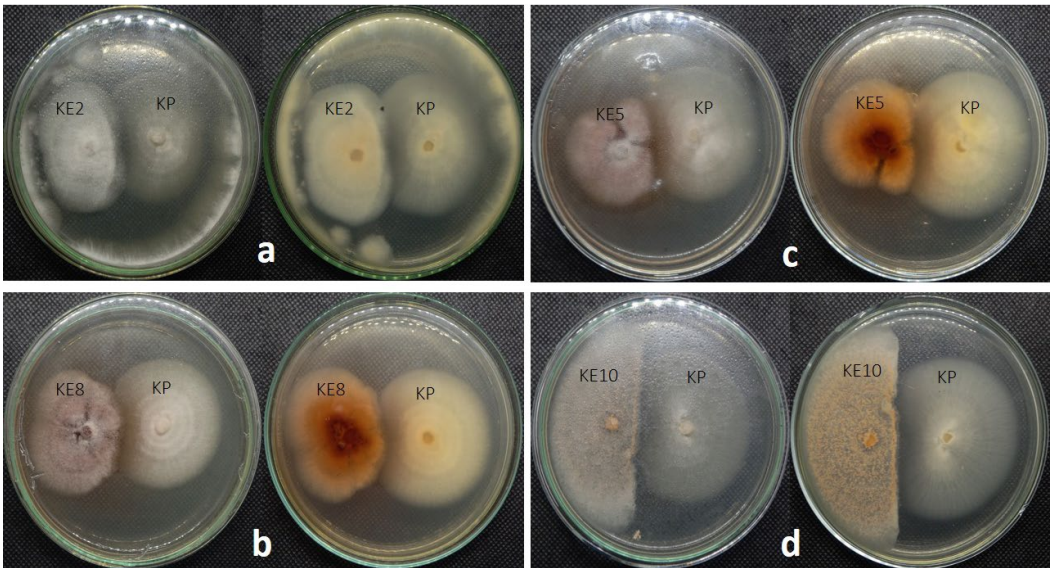

Figure 4. Endophytic fungi antagonist test results against the pathogenic fungi *Fusarium oxysporum.* a) KE2=S1.3.2 and KP = *Fusarium oxysporum*, b) KE5=S2.2.5 and KP = *Fusarium oxysporum*, c) KE8=S2.2.8 and KP = *Fusarium oxysporum*, and d) KE10= S2.3.10 and KP = *Fusarium oxysporum*

fungi with potential antagonists against the pathogenic fungi *Fusarium oxysporum*. There was an inhibition zone that indicated the inhibited growth of pathogenic fungi by endophytic fungi [21]. The ability to outgrow pathogenic fungi suggests that endophytic fungi inhibit pathogen growth by competing for space and nutrients. The spread of pathogenic fungi is further restricted when the mycelia of the two fungi come into contact [22]. Biological agents with antifungal properties are known to secrete hydrolytic enzymes that can break down the cell walls of pathogens, thereby limiting their growth [14, 23]. In addition, inhibition can also be due to the production of secondary metabolites that can inhibit the permeability of the cell membrane, with these compounds entering pathogenic fungi cells through holes in the cell membrane and denaturing cell membrane lipids [14].

The presence of an inhibition zone that appears is due to the secretory activity of an antibiotic or alkaloid substance. *Aspergillus* fungi can produce antifungal substances so that they can inhibit pathogenic fungi. Lelana *et al.* [24] added that the formation of the inhibition zone indicated the presence of secondary metabolites and antibiosis compounds secreted by endophytic fungi. The interaction mechanism shown by *Aspergillus* fungi in inhibiting pathogenic fungi, according to Daami-Remadi *et al.* [25], can occur through 3 interactions, namely microparasitism (direct parasitism on pathogenic hyphae), competition for space and nutrition, lysis, and antibiosis. Ibrahim *et al.* [12] reported that microparasitism can be the result of entanglement of hyphae from other fungi. In terms of microscopic characters, the hyphae of endophytic fungi become wrapped around the pathogenic fungi and even penetrate the pathogenic hyphae walls so that the pathogenic fungi's growth is inhibited. This presumably takes place through a process of penetration into the pathogen's cell wall with the help of cell wall degrading enzymes such as chitinase, glucanase and protease. This prevented the pathogenic fungal's mycelium from growing, allowing the isolated endophytic fungi to emit volatile compounds [14].

Penicillium fungi can produce antibiotic substances [26]. The inhibition mechanism carried out by *Penicillium* fungi involves competition and antibiosis (producing antibiotic substances). *Penicillium* spp. are often used as a biological control agent and are one of the largest genera often used as a biological control agent. This was confirmed by Patkowska *et al.* [27], who showed that high antagonistic ability was found in the fungi *Clonostachys* spp., *Myrothecium* spp., *Penicillium* spp., and *Trichoderma* spp. Olivain *et al.* [28] reported that the non-pathogenic *Fusarium* fungi could control other pathogens, both fungi and bacteria, through competition for space and nutrients. Ibrahim *et al*. [12] reported that the endophytic fungus *Syncepalastrum racemosum* derived from the leaves of *Makhamia tomentosa* produces ethyl acetate, methanol and hexane, while Rehman *et al.* [29] found that endophytic isolates of *Neurospora* sp. from the plant *Nothapodytes foetida* produced camptothecin which can function as an anticancer agent.

4. Conclusions

Sixteen isolates of endophytic fungi were obtained from mangrove leaves of *Rhizophora* sp. species. Of the 16 isolates, 9 displayed antagonistic potential against pathogenic fungi. Five endophytic isolates showed antagonistic potential against the pathogenic fungi *Aspergillus niger*, and four isolates of endophytic fungi had potential antagonists against the pathogenic fungus *Fusarium oxysporum* from pythons. There was an inhibition zone in the antagonist test of the nine isolates. The percentage of inhibition zones were in the range of 55.5%-77.7%. Antagonistic activity can occur due to nutrient depletion caused by competition, or by enzymes produced by endophytic fungi, which can break down the cell walls of pathogenic fungi.

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