RESEARCH ARTICLE

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In vitro assay of antagonistic activities of endophytic fungi from Calabash tree leaves against pathogenic *Fusarium oxysporum*

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ABSTRACT

Endophytic fungi are microorganisms known as plant-associated fungi and are typically found asymptomatically inside plant tissue. By directly generating secondary metabolites, endophytic fungi help their host plants grow and become more resistant to plant diseases. Furthermore, they can also biosynthesize bioactive compounds used for antimicrobials, which were previously believed to be produced only by the host plant. Medicinal plants as host plants for endophytic fungi which produce secondary metabolites and their ability as biological agents against pathogens. Calabash tree is a medicinal plant that contains active compounds that function as antifungal and antibacterial. This study aims to determine the antagonistic activity of endophytic fungi from calabash tree leaves against *F. oxysporum* Fos, the pathogen that causes fusarium wilt in shallot plants. Twelve endophytic fungi were obtained from isolation from the leaves of the calabash tree, namely EnM_6C_{22}, $EnM_{11}P_2$, EnM_6A_2, EnM_5H_{21}, $EnM_{10}H_{22}$, $EnM_{12}P$, $EnM_{11}P$, EnM_{111}, EnM_5H_{22}, EnM_9Pt_{22}, EnM_5H_1 and EnM_5K. Five of the twelve endophytic fungi of calabash tree leaves have been identified, as *Fusarium sp*., *Rhizoctonia sp*., *Pestalotia sp*., *Colletotrichum sp*., and *Bipolaris sp*. with inhibition percentages of 36.43%, 32.19%, 25.41%, 32.19%, and 27.53% respectively at 7 days after inoculation.

Key words: endophytic fungi, fusarium wilt, plant defense, biological control agent

Introduction

The cultivation of plants in agriculture is inseparable from the attack of plant disease pathogens. Shallot plants are plants that are often attacked by plant pathogens. *F. oxysporum* is a pathogen that often attacks shallot plants and causes Fusarium wilt disease. Fusarium wilt disease is characterized by fast wilting of plants, rotting roots, drooping plants, and visible white or reddish-purple fungus colonies on tubers. Fusarium wilt disease causes cultivation losses in shallot plants by 50% and even crop failure (Juwanda *et al.*, 2016).

Most of the control of pathogenic fungi uses synthetic fungicides because they are considered the easiest and most effective, but many studies have shown that the use of synthetic fungicides has caused harm to humans and agroecosystems, such as polluting the soil and water environment. One of the safe and environmentally friendly controls is to use endophytic fungi as biological control agents. Endophytic fungi can produce secondary metabolites as potential bioactive compounds to be developed into antimicrobial, anti-insect, anticancer, and even biocontrol agents in agriculture (Gouda *et al*., 2016; Posthangbam *et al*., 2017).

The results of several studies stated that endophytic antimicrobials have an effect on suppressing the development of pathogenic fungi such as *Rhizoctonia cerealis*, *R. solani*, and *F. oxysporum* in vitro (Sunariasih *et al*., 2014). The types of endophytic fungi tested in vitro could suppress *F. oxysporum* by 60% (Pinem *et al.*, 2015). Medicine plants have been known as good host plants for various endophytic fungi. The potential endophytic fungi isolated from medicinal plants will be obtained as biological control agents that can also produce potential active compounds that can be used to develop sustainable agriculture to build plant defense to inhibit diseases. One of the medicinal plants that has good properties is the calabash tree.

The calabash tree (*Crescentia cujete* L.) is a broad-headed, spreading-branch, evergreen tropical tree that grows to a height of 25– 40 feet. Its leaves are simple, and its bark is rough. The cauliflorus blooms of the calabash tree sprout straight from the trunk and branches' nodes. The flowers have a yellowish corolla with crimson or purple lines, and they are two inches long. The calabash plant yields enormous, smooth, green fruits with a strong, thin shell that resemble

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gourds. The huge, globulose fruits have a diameter of up to 12 or 14 inches (Gilman, 1993). Calabash contains chemical compounds that act as antimicrobials which are located on leaves, stems, and fruits such as alkaloids, saponins, flavonoids, and tannins (Hutapea, 1993; Lien, 2001; Rismayani, 2013). A previous study revealed that the leaves have the highest antimicrobial (Sari *et al*., 2020). This study aims to determine the percent inhibition of the pathogenic fungus *F. oxysporum* by the endophytic fungi from the leaves of the calabash tree.

Materials and Methods

Isolating of endophytic fungi from Calabash tree

The leaves have been collected from the site Surabaya City, located at Sepuluh Nopember Institute of Technology. The leaves of the plant are cut into small pieces. The leaf pieces were sterilized using 3% NaOCl for 60 seconds and 70% alcohol for 30 seconds and then rinsed twice using sterile distilled water. After the pieces have been sterilized and rinsed, the cut leaves are dried on sterile filter paper, and then cut again with a size of approximately 0.5 × 0.5 cm. The leaf pieces measuring 0.5 × 0.5 cm were placed in a petri dish containing PDA media. As many as four pieces of leaf are placed in one petri dish. Endophytic fungi that grow from within the plant tissue and have gone through the isolation stage then proceed to the purification stage. The fungal mycelia that grew were then re-cultured on new PDA media and incubated for 7 days.

Culture of pathogenic Fusarium oxysporum Fos

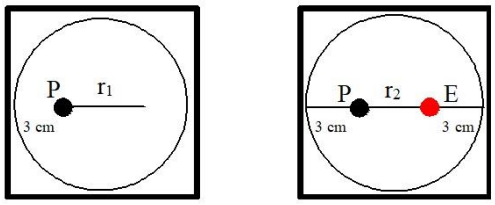
F. oxysporum Fos was collected from the shallot that had the wilt symptoms in Banjarbaru city, isolation and identification were done in a previous study (Rizali *et al*., 2021). Culturing of *F. oxysporum* Fos was carried out by taking a needle of pure culture and inoculated on PDA sterile then incubated for 7 days.

Endophytic fungi of Calabash tree identification

Identification was carried out by matching the characteristics of the fungus obtained such as microscopic and macroscopic characters. The variables observed in this identification were spore variations, shapes, colors, and types of distribution on PDA. Identification of fungi was carried out up to the genus level. Manual book identification refers to Barnett & Hunter (1998) and Watanabe (2002).

Dual culture assay of endophytic fungi against pathogen F. oxysporum Fos

The Antagonism test was carried out using the dual culture method test. One petri dish was used for the Fos pathogen without treatment and another for the two colonies grown side by side (Figure 1). The inhibition power can be calculated using the formula introduced by Skidmore & Dickinson (1973) (1):



Control dish (a) Dual culture dish (b)

Figure 1. *Dual culture method, control dish (a) and dual culture dish (b), where P is pathogen and E is antagonist agent (endophytic fungi).*

$$\text{Inhibition power } (\%) = \frac{r1-r2}{r1} \times 100\,, \quad (1)$$

r1 – colony distance of pathogen on control dish,

r2 – colony distance of pathogen on a dual culture dish.

Results and Discussion

Macroscopic colony and radial growth of endophytic fungi from Calabash tree

The results of the isolation of endophytic fungi from healthy calabash tree leaves produced twelve endophytic fungal isolates, each of which had a different color, surface colony, and colony margin characteristics (Figure 2).

The twelve endophytic fungi isolates had different colony diameters. The difference in colony diameter in the twelve isolates was due to the different growth abilities of each type of isolate. The colony diameter of the endophytic fungus on the leaves of the calabash tree at 3, 5, and 7 DAI (the day after inoculation) can be seen in Figure 3.

Isolate $EnM_{11}P_2$ had the highest colony diameter among the twelve isolates of endophytic fungi isolated during 7 DAI, namely 9.3 cm. Thus, the isolates $EnM_{12}P$, $EnM_{11}P$, EnM_5H_1, and EnM_5K had a total colony diameter ranging from 8.1-8.7 cm. EnM_9Pt_{22} isolate had a total colony diameter of 7.9 cm. The isolates EnM_6A_2, $EnM_{10}H_{22}$, EnM_{111}, and EnM_5H_{22}, the total colony diameter ranged from 6.4-6.7 cm. Otherwise, EnM_6C_{22} and EnM_5H_{21} isolates had a total colony diameter ranging from 5.5-5.9 cm, while EnM_5H_{21} had the lowest colony diameter of 5.5 cm.

Based on the results of observing the growth of endophytic fungi from calabash tree leaves, five isolates of the endophytic fungi were taken to test the antagonism with the pathogenic *F. oxysporum* Fos. The endophytic fungi taken were based on the color and different surfaces of the colony. The different color and surface of the colony assume that the isolated endophytic fungi are of different species

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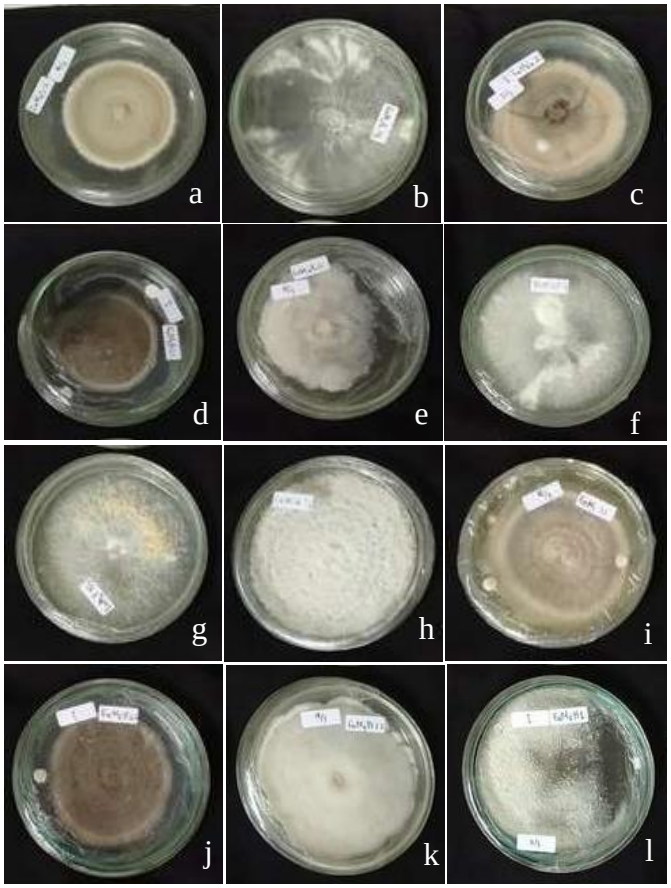

Figure 2. *Macroscopic colonies of 12 endophytic fungi of calabash tree: (a)EnM$_6$C$_{22}$, (b)EnM$_{11}$P$_2$, (c)EnM$_6$A$_2$, (d) EnM$_5$H$_{21}$, (e) EnM$_{10}$H$_{22}$, (f) EnM$_{12}$P, (g) EnM$_{11}$P,(h) EnM$_5$K, (i) EnM$_{111}$,(j) EnM$_5$H$_{22}$, (k) EnM$_9$Pt$_{22}$, (l)EnM$_5$H$_1$.*

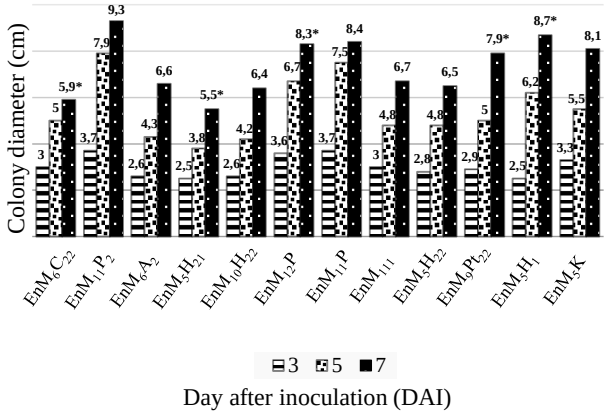

Figure 3. *Diameter colonies of endophytic fungi from calabash tree on 3, 5, and 7 DAI.*

from one another. Different isolates based on the color and surface of these colonies have different growth diameters of the colonies as well. One of the important factors in determining the potential of endophytic fungi as biological agents to suppress the growth of pathogenic fungi is the growth rate of the endophytic fungus colonies themselves (Djafaruddin, 2000).

The endophytic fungi that tested to antagonistic test were fungi with the isolate codes EnM$_9$Pt$_{22}$, EnM$_{12}$P, EnM$_6$C$_{22}$, EnM$_5$H$_1$, and EnM$_5$H$_{21}$ because they had different colors and colony diameters from one another, so they were assumed to represent different species or species. Then the isolate code will be changed to EnMA, EnMB, EnMC, EnMD, and EnME respectively (Table 1). At 7 DAI, EnMA isolate had a colony diameter of 7.9 cm with a yellowish-brown central color surrounded by white, and a thick cotton-coated surface. If the surface of the EnMA isolate colony is like thick cotton, the surface of the EnMB isolate is thin cotton which is bright white and the colony diameter is 8.3 cm.

EnMC isolate was taken as one of the isolates to be tested for antagonism because there were acervuli on the surface of the colony, although the diameter of the colony was relatively low, namely 5.9 cm at 7 DAI. The EnMD isolate had a colony diameter of 8.7 cm at 7 DAI which was the highest among the twelve isolates. Another reason this isolate was taken was because it had a black central part of the colony surrounded by white, while the surface of the colony was white cotton. EnME isolate had the lowest colony diameter among the twelve isolates, namely 5.5 cm, however, this isolate had a black colony color and a hairy surface, the color and colony surface of the EnME isolate were the reasons why the EnME isolate was chosen even though it had the lowest growth diameter.

Macroscopic and microscopic characteristics of endophytic fungi from calabash tree

EnM$_A$ isolate shows mycelia branched, unseptate hyphae, and hyalin. Macroconidia, slender crescent-shaped, and has three to six septa, while for macroscopic morphology, EnM$_A$ isolate is the center of the colony is yellowish brown surrounded by yellowish brown color. white, the surface of the colony is like thick cotton and the periphery of the colony is also like thick cotton (Table 1, Figure 4). The microscopic morphology identified belongs to the genus *Fusarium sp*. (Watanabe, 2002).

Fusarium sp. is dominated by colonial surfaces such as cotton. Each isolate from the genus *Fusarium sp*. sporodochium is present, and most have white, purple, or pink colonies at the center of the colony. Macroconidium *Fusarium sp*. is very abundant and has a slender crescent shape, walls thick and smooth, with tapered apical cells and foot-shaped cells below (Sutejo *et al*., 2008; Sari *et al*., 2018).

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Table 1. *Identification of endophytic fungi from the calabash tree.*

Isolate	Macroscopic characteristics	Microscopic characteristics	Identification results
EnM_A	- The center of the colony is yellowish brown, surrounded by white - Thick cotton surface - Thick cotton edging	- Hyphae branched, not septate - Hyaline macroconidia, slender crescents, septa 3-6	*Fusarium sp.*
EnM_B	- Colonies are white - The surface of the cotton colony is thin - The rim of the colony is thin	- Hyphae branched angular, not septate - The branching point has an indentation - Sclerotia form lumps	*Rhizoctonia sp.*
EnM_C	- Colonies are grayish green - The surface of the colony is velvety or hairy - The margins of the colony are serrated - Asservuli	- Hyphae are unbranched, septate - Conidia cylindrical (elliptical), 4-celled - Short conidiophore - Hair (set) 3	*Pestalotia sp.*
EnM_D	- The center of the colony is black, surrounded by white - Cotton colony surface - Cotton colony fringe	- Hyphae branched, septa - Conidia cylindrical (elliptical), the ends of the conidia blunt	*Colletotrichum sp.*
EnM_E	- Colonies are black - The surface of the colony is hairy - The margins of the colony are serrated	- Hyphae are branched and septate - 90° angle branch - Cell nucleus 2 - Conidia cylindrical (elliptical), 4-celled	*Bipolaris sp.*

Identification references: Barnett & Hunter (1972); Dickman (1993); Nag Rag (1993); Alexopoulos *et al*. (1996); Semangun (2000); Watanabe (2002); National Quarantine Service (2004); Semangun (2008); Sutejo *et al*. (2008); Moustafa *et al*. (2015); Madhi (2016); Sari *et al*. (2018).

The microscopic morphology of the EnM_B shows that the hyphae were branched, angular, and without septa and had indentations on the branches (Figure 5). While the macroscopic morphology is a white colony, the surface of the colony is like thin cotton and the edges of the colony are also like thin cotton. Based on macroscopic and microscopic identification, EnM_B isolates were identified as belonging to the genus *Rhizoctonia sp*. (Watanabe, 2002).

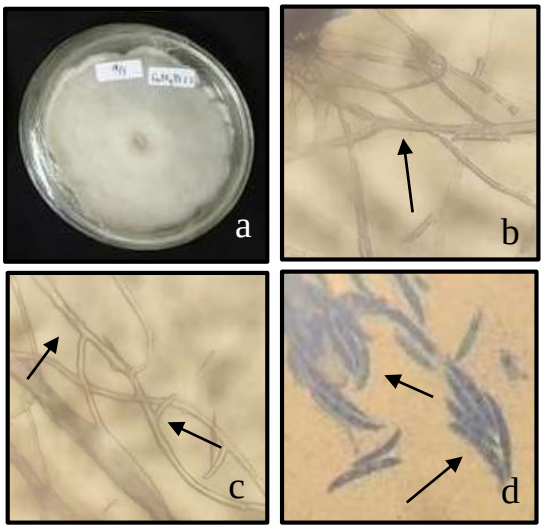

Figure 4. *(a) EnMA (Fusarium sp.) on PDA culture (b) hyaline hyphae (c) mycelium branched unseptate, (d) macroconidia.*

The morphological characteristics of *Rhizoctonia sp*. include having a thin mycelium and white thread fibers (Alexopoulos *et al*., 1996). Mycelium *Rhizoctonia sp*. wide and consists of long cells and produces curved branches from the main hyphae. *Rhizoctonia sp*. itself does not produce spores, and the color of the mycelium will change if the age of the mycelium increases. Under certain conditions, *Rhizoctonia sp*. will produce sclerotia in the form of enlarged lumps (Semangun, 2008). Sclerotia are dense and massive collections of monoloid cells that form granules of various sizes. Sclerotia serves as a food reserve, especially when the nutritional conditions where it grows are depleted.

The macroscopic characteristics of EnM_C isolate are grayish-green colonies, the surface of the colony is velvety or hairy, the edges of the colony are serrated and on the surface of the colony there are acervuli, while the microscopic characteristics at 10x magnification show unbranched and unseptated hyphae, and conidia at 40x magnification

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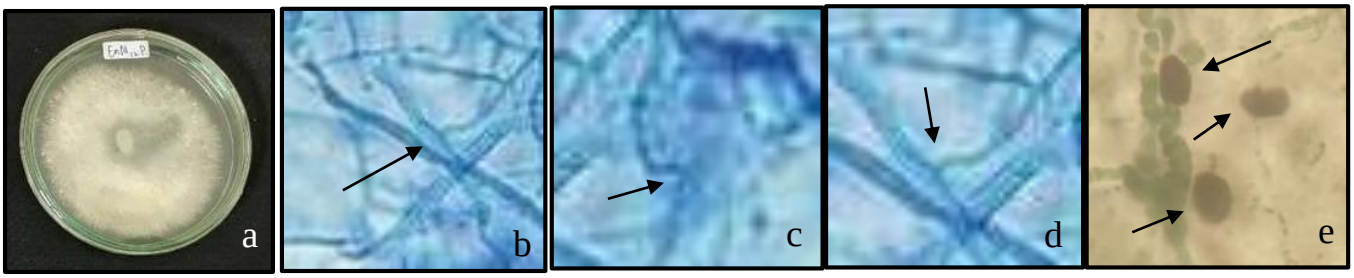

Figure 5. *(a) Isolate EnM_B (Rhizoctonia sp.) on PDA culture, (b) mycelium branching (b) pale brown hyphae, (c-d) rectanguler hyphae branching, (e) sclerotia.*

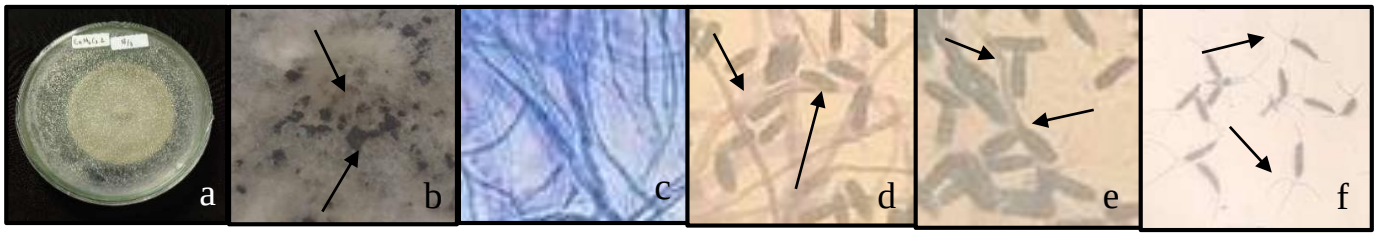

Figure 6. *(a) Isolate EnM_C (Pestalotia sp.) on PDA culture: (a) macroscopic (colony), (b) aservuli, (c) hyphae (d) conidia, (e) conidiofor, (f) setae of conidia.*

cylindrical (elliptical) with four cells, has hair or whip hair (setae) and conidiophores (Figure 6). These results were identified as belonging to *Pestalotia sp*. (Watanabe, 2002; Nag Rag, 1993).

The growth of the fungus *Pestalotia sp*. on PDA media tends to be slow. The surface of the mycelium colony of the fungus Pestalotia sp. old ones appear to form like small black fruiting bodies which are commonly called aservuli (Moustafa *et al*., 2015; Alexopoulos *et al*., 1996). Microscopic characteristics of *Pestalotia sp.* are hyphae that do not branch and are not insulated, hyphae are slightly dark in color, conidiophores are short and barely visible (hyaline), conidia are cylindrical (elliptical) with slightly tapered ends of the conidia. On the conidia of the fungus *Pestalotia* sp. there are structures such as hair or whip bristles (setae) which number 3 and are located at one end of the conidia (Barnett & Hunter, 1972; Madhi, 2016).

The EnM_D isolate showed microscopic characteristics with 100x magnification, branched and septate hyphae, and cylindrical conidia (ellipses) with blunt conidial ends. The macroscopic characteristics are the center of the colony is black and surrounded by grayish white, the colony's surface is like cotton, and the edges of the colony are also like cotton (Figure 7). Based on the macroscopic and microscopic characteristics obtained, EnM_5H_1 isolate was identified as belonging to *Colletotrichum sp*. (Watanabe, 2002).

General parts of the fungus *Colletotrichum sp*. have hyphae that are insulated and branched and produce conidia that are 1-celled and look transparent (hyaline) and elongated with rounded or tapered ends. Conidia mass of the fungus *Colletotrichum sp*. black and many (Dickman, 1993; Semangun, 2000).

The results of the microscopic characteristics of the EnM_E isolate at 40x magnification were branched and septate hyphae, the branching hyphae forming an angle of 90°. Meanwhile, at 100x magnification, it was seen that there were two cell nuclei (binucleated) in each hyphal septate. At 100x magnification, conidia are cylindrical (ellipse) in which the conidia from this EnM_E isolate are four-celled.

The macroscopic characteristics of EnME isolates are black colonies, the surface of the colonies is hairy, and the edges of the colonies are serrated (Figure 8). Based on the microscopic and macroscopic results, EnME isolates were

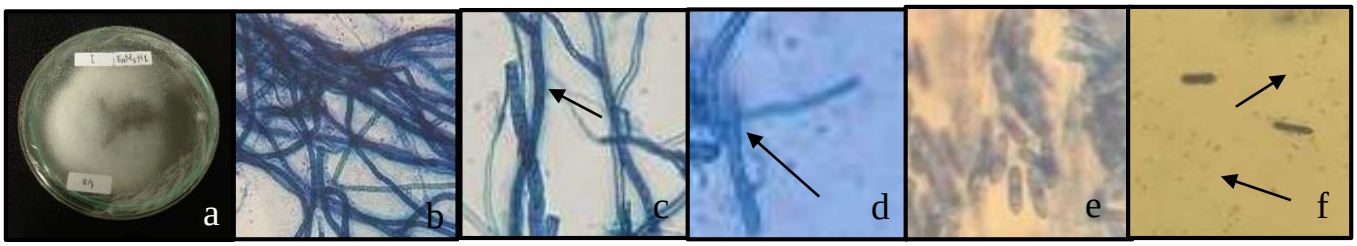

Figure 7. *(a) Isolate EnM_D (Colletotrichum sp.) on PDA culture, (b) mycelium, (c) septate hyphae, (d) branching hyphae, (e) conidia, (f) microconidia.*

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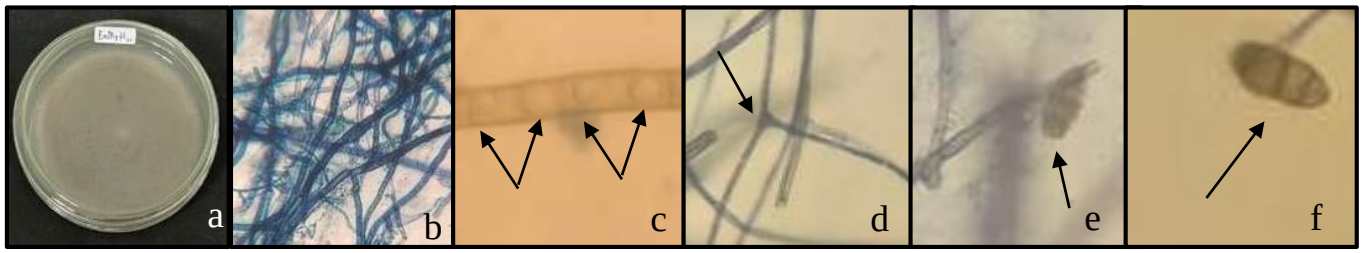

Figure 8. *(a) Isolate EnME (Bipolaris sp.) on PDA, (b) mycelium,(c) nucleus, (d) branching hyphae, (e-f) conidia.*

identified as belonging to the genus *Bipolaris* sp. (Watanabe, 2002).

The characteristics of the colony of the fungus *Bipolaris sp*. are fast growth, hairy surface texture, and black on the surface and at the base (Sobanbabu *et al*., 2018). Microscopic characteristics of *Bipolaris sp*. have hyphae branched and septum. Conidia from the fungus *Bipolaris sp*. has an oval shape with a blunt tip and has 2-4 distoseptate septa (National Plant Quarantine Service, 2004).

Inhibition growth of pathogenic F.oxysporum Fos by endophytic fungi from Calabash tree leaves

Based on the results of observations at 7 DAI, the five genera of endophytic fungi of calabash tree leaves identified can inhibit the growth of *F.oxysporum* Fos (Figure 9).

At 7 DAI, the lowest percentage of inhibition is showed by EnM_C (*Pestalotia sp*.) 25.41%otherwise the highest percent inhibition is showed by EnM_A 36.43% (*Fusarium sp*.).

The EnMA endophyte (*Fusarium sp*.) showed the highest percentage among the five endophytic fungi, a value is 36.43%. In dual control with EnMA (*Fusarium sp*.), the growth of pathogenic fungi is slower than that of endophytic fungi and it appears that an antibiosis mechanism occurs.

The mechanism of antibiosis is indicated by the formation of a clear zone at the junction between the endophytic fungi and the pathogenic fungi (Figure 10). The compounds produced by endophytic fungi of the genus *Fusarium sp*.

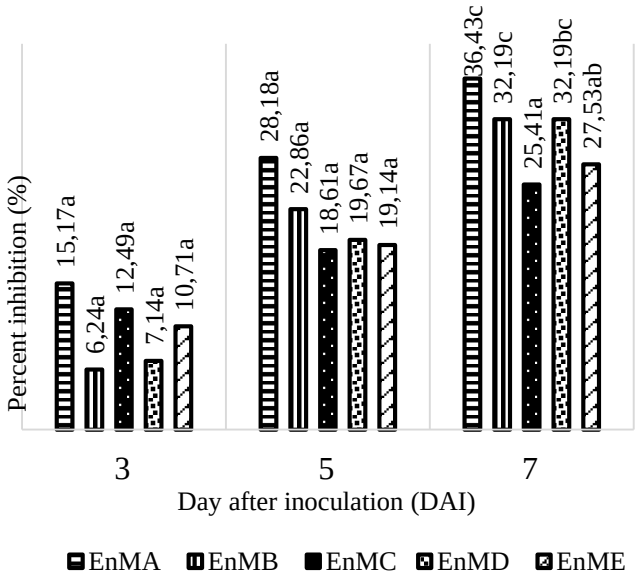

Figure 9. *The percentage inhibition of F.oxysporum Fos by endophytic fungi of calabash tree on 3, 5, and 7 DAI.*
n.b: DMRT 5% (n = 4)

exhibit various activities, such as antibacterial, antifungal, and cytotoxic activity. Endophytic fungi in the genus *Fusarium sp*. produce a variety of bioactive secondary metabolites including naphtoquinones, for example, javanicin, fusarubin, solanine, martisin, and nectraiafurone (Luo & Xing Zhao, 2021).

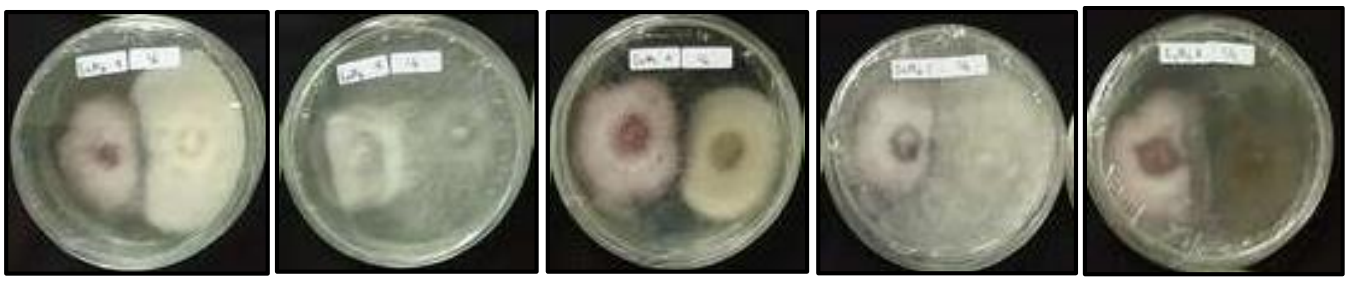

Figure 10. *Antagonism test in vitro F.oxysporum against endophytic fungi of calabash tree on 7 DAI plants.*

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The EnM_B endophyte has the second-highest percentage after the EnM_A, which is 32.19%. EnM_B, identified as *Rhizoctonia sp*. showed the mechanism of parasitism. The mechanism of parasitism is developed when the mycelia of the pathogenic fungus is covered by the mycelia of the endophytic fungus, thus the pathogenic fungi do not have space for their habitat so their growth is stunted. The inhibition of the growth of pathogenic fungal mycelia occurs due to lysis of the pathogenic fungal mycelium, endophytic fungal hyphae entwining pathogenic fungal hyphae so that the growth of pathogenic fungi will be pressured due to running out of space to grow (Nahdah *et al*., 2020).

The EnM_D endophyte has the same percentage as the EnM_B, which is 32.19%. However, the mechanism secreted by endophytic fungi from the genus *Colletotrichum sp*. is different from the mechanism secreted by *Rhizoctonia sp*. If *Rhizoctonia sp*. shows the mechanism of parasitism, *Colletotrichum* sp. secrete an antibiosis mechanism against the pathogen *F. oxysporum*. Antibiosis is one of the potential endophytic mechanisms that can contribute to host protection, especially host defense against pathogens (Herre *et al*., 2007). The clear zone in the antagonism test was formed because the antifungal compounds of endophytic fungi inhibit the growth of pathogenic fungi. Antifungal compounds will work to inhibit the development of pathogenic fungi if there is direct contact with pathogenic fungi.

Otherwise, EnM_E has a percentage of 27.53% which is relatively low, and the mechanism secreted by the fungus *Biporalis sp*. is the same as that released by fungi from *Fusarium* sp. and *Colletotrichum sp*., namely the antibiosis mechanism. The mechanism of antibiosis by endophytic fungi is closely related to the ability of endophytic fungi isolates to produce enzymes, such as chitinase, protease, cellulase, and other secondary compounds which play an important role in inducing plant resistance. Endophytic fungi can produce extracellular enzymes including chitinase, cellulase, protease, and pectinase. The chitinase enzyme is an enzyme produced by antagonistic fungi to control pathogenic fungi, especially soil-borne pathogenic fungi because this enzyme can degrade the cell walls of pathogenic fungi composed of chitin compounds (Backman & Sikora, 2008).

The endophytic EnM_C shows the lowest percentage among the five endophytes, the value is 25.41%. The mechanism released by *Pestalotia sp*. is the same as the mechanism secreted by *Fusarium sp*., *Colletotrichum sp*., and *Biporalis sp*., it showed the antibiosis mechanism, characterized by the presence of a clear zone. The zone of inhibition (clear zone) produced by potential isolates indicates that these microbes secrete chemical substances that inhibit the colonization of other microbes. These chemical substances have antimicrobial activity that contains antibiotics, pigments, toxins, and enzyme inhibitors (Gao et al., 2010). *Pestalotia sp*. has been reported to produce bioactive alkaloids, terpenoids, coumarin isocoumarin derivatives, chromones, quinones, semiquinones, peptides, xanthones, xanthone derivatives, phenols, phenolic acids, and lactones with various antifungal, antimicrobial, and antitumor activities (Xu *et al*., 2010).

Conclusion

Twelve isolates of endophytic fungi were obtained from calabash tree leaves, five isolates are EnM_A identified as genus *Fusarium sp*., EnM_B identified as *Rhizoctonia sp* EnM_C identified as *Pestalotia sp*., EnM_D identified as *Colletotrichum sp*., and EnM_E identified as *Bipolaris sp*. It is shown from the antagonism test that the endophytes can inhibit the mycelial growth of the pathogenic *F. oxysporum* Fos with each percentage of EnM_A is 36.43%, EnM_B is 32.19%, EnM_C is 25.41%, EnM_D is 32.19%, and EnM_E was 27.53% at 7 DAI.

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