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Endophytic fungi isolated from *Heliotropium indicum* and their antagonism activity toward *Fusarium solani* and *F. oxysporum*

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Abstract. *Mustofa A, Hastuti US, Susanto H. 2024. Endophytic fungi isolated from* Heliotropium indicum *and their antagonism activity toward* Fusarium solani *and* F. oxysporum. *Biodiversitas 25: 5063-5073.* Endophytic fungi have great potential as biological control agents due to their ability to produce various bioactive compounds that can inhibit the growth of plant pathogens. One genus of pathogens that is often a problem in agriculture is *Fusarium*, which causes wilt disease in various types of plants. This study aimed to identify endophytic fungi from the leaves and stems of *Heliotropium indicum*, analyzed the content of secondary metabolites in plant extracts and endophytic fungal isolates, and evaluated the antagonistic potential of endophytic fungal isolates against pathogenic fungi. This study consisted of observing the location of endophytic fungi in plant tissues, identification of endophytic fungi, detection of secondary metabolite in fluid culture of each endophytic fungi and *H. indicum*, and antagonism test. The histological observations showed that endophytic fungi are found in the leaf epidermal, sponge, and parenchyma tissue. There were ten endophytic fungi isolates found in leaves and stems, namely *Nigrospora gorlenkoana, N. guilinensis, N. musae, N. oryzae, N. rubi, Nigrospora* sp. 1, *Nigrospora* sp. 2, *Nigrospora* sp. 3, *Nigrospora* sp. 4, and *Penicillium oxalicum.* The 10 endophytic fungal isolates including leaf and stem extracts of *H. indicum* contained secondary metabolite compounds; however, the species *Nigrospora* sp. 2 had high potential metabolite compounds. These metabolite compounds were able to inhibit the mycelial growth of *Fusarium oxysporum*, indicating their potential as biocontrol agents. The antagonism test results showed that the ten fungi isolates have antagonism effect toward *F. solani* and *F. oxysporum*. The highest antagonism effect against *F. oxysporum* was 77.2%, the lowest antagonism effect was 46.5%, the highest antagonism effect against *F. solani* was 64%, and the lowest was 28.2%. The antagonism mechanism of micoparasitism can be seen from the microscopic observations.

Keywords: Biocontrol agents, micoparasitism, *Nigrospora*, plant pathogens, secondary metabolites

INTRODUCTION

The presence of resistance to *Fusarium* causes several problems in the agricultural sector, one of which is in banana plants. BPS (2024) shows that national banana production reached 8.7 million tons in 2021. This production increased from 8.1 million tons in previous years. In recent years, many banana plants have wilted due to *Fusarium oxysporum* infection (Siamak and Zheng 2018). Research by Dong et al. (2015) reported a 22% reduction in plant height and a substantial decrease in dry weight of leaves, false stems and roots. The disease also caused a reduction in the moisture content of leaves and roots, which further impacted the overall yield of the crop (Carmona et al. 2018).

Fusarium wilt disease, caused by various species of the *Fusarium*, significantly affects yields of economically important crops in Indonesia, particularly in bananas. The disease is widespread in all banana-producing provinces, with incidence rates ranging from 0.08% to 100%, with an average of about 24%. These high incidence rates lead to production losses, as the disease affects local banana cultivars such as '*Barangan*', *'Raja'*, and *'Ambon Hijau'* (Hermanto et al. 2011). *Fusarium* wilt on banana in Banyumas, Indonesia, caused by *Fusarium* disease incidence reached 50% in all districts surveyed. *F. oxysporum* isolates attacking banana in the same area showed similar characteristics and pathogenic, indicating a widespread and consistent disease in this region (Rahayuniati et al. 2024).

Disease control caused by pathogenic fungi is often difficult, so an environmentally friendly strategy is needed to reduces dependence on chemical fungicides that can cause resistance. One effective method to control fungal pathogens is through the use of antagonistic fungi, one of them is endophytic fungi which act as biocontrol agents. These fungi can manage plant diseases by employing various mechanisms like antibiosis, mycoparasitism, induced resistance and competition (Heydari and Pessarakli 2010; Islam et al. 2023).

Endophytic fungi have shown significant potential in controlling *Fusarium* wilt, a wilt disease caused by *F. oxysporum*, which affects a variety of crops. Several studies have shown that endophytic fungi can inhibit the growth of *F. oxysporum*. For example, *Trichoderma asperellum* isolated from shallots inhibited pathogen by 72.52% in a dual culture assay (Trizelia et al. 2023). Similarly, *Trichoderma* spp. isolated from banana plants effectively suppressed *F. oxysporum* and *F. solani* (Ningsih et al. 2016; Sudantha 2021).

The endophytic fungi have a symbiotic mutualism relationship with the host plant, such as the endophytic fungi species that live in *Heliotropium indicum* as the host

plant. Biological control using endophytic fungi has been proven to be an environmentally friendly alternative to control the *Fusarium* wilt disease caused by *Fusarium* sp. (Ajilogba and Babalola 2013). *H. indicum*, a traditional medicinal plant, has great potential as an under-explored source of natural antibiotics. *H. indicum* has traditionally been used to treat conditions such as stomachache, hypertension, skin rashes, and wounds caused by infections (Koffuor et al. 2012; Sivajothi et al. 2015; Idowu and Tolulope 2023). However, research on the diversity of endophytic fungi associated with *H. indicum* has not been identified and the potential of endophytic fungi in this plant has not been studied.

The interaction of endophytic fungi with the host plants is essential to the study because the endophytic fungi and the host plant can be used to study the fungi isolate to inhibit the pathogenic fungi growth. Antagonistic molds can inhibit the growth of pathogenic fungi that cause disease in plants. The use of antagonistic molds as biological agents helps reduce dependence on synthetic chemicals that potentially can damage the environment, so antagonistic molds are a more environmentally friendly alternative than chemical fungicides. This can help maintain the balance of the soil ecosystem (Chen et al. 2021). The endophytic fungi can be used as antagonistic fungi because the fungi could produce some enzymes that potentially cause damage to the hyphae of pathogenic fungi and inhibit the colony growth. This study aimed to identify endophytic fungi from the leaves and stems of *H. indicum*, analyzed the content of secondary metabolites in plant extracts and endophytic fungal isolates, and evaluated the antagonistic potential of endophytic fungal isolates against pathogenic fungi.

MATERIALS AND METHODS

Plant material and microorganisms

Heliotropium indicum leaves and stems were collected from medical plants collection at Boncong Village, Bancar Sub-district, Tuban District, Indonesia, with coordinates 6.771437014325708 North Latitude, 111.74337657028175 South Latitude. The samples used were six months old plants. The research was conducted in the period of June-September 2024. Antagonism test using pathogenic fungal species i.e.: *F. oxysporum* f. sp. *lycopersici* and *F. solani* f. sp. *capsici*, originated from the Microbiology Laboratory of Universitas Malang, Malang, Indonesia.

Histologic observation on endophytic fungi position

Heliotropium indicum leaves and branches were washed with distilled water, then cut paradermally and longitudinally to make microscopic preparations. Then, histological observations were made on the position of endophytic fungi in *H. Indicum* tissue.

Isolation of endophytic fungi

Heliotropium indicum leaves and branches were washed and submerged in 1% NaOCl for 30 sec. Then rinsed with distilled water and submerged in 70% alcohol for 30 sec, then rinsed again with distilled water. Leaf parts were cut of 1×1 cm^2 in size, the branches were cut with a thickness of 0.5 cm. Then, each sample was inoculated on Potato Dextrose Agar (PDA) media added with chloramphenicol (100 mg/L) and incubated at 25-27°C for one week. Each species of endophytic fungi was grown and then inoculated on PDA slant medium and incubated at 27°C for 3 days for morphological observation. Culture slides of each endophytic fungal isolate were prepared for microscopic observation and fungal characteristics were described for identification purposes.

Identification of endophytic fungi

The identification of fungi based on macroscopic observations includes: the colony color, the color of the colony characters, the colony diameters, and the color of the of the colony base. The microscopic observations include: the spores morphology, sporangium, hyphae, and conidia shape (Mulyadi et al. 2021).

The endophytic fungi liquid culture preparation

Secondary metabolites were obtained from endophytic fungi liquid cultures. Each colony of endophytic fungi species on PDA media was cut into 5×1 cm^2 in size, then inoculated on potato dextrose liquid medium and incubated at 27°C at 120 rpm in rate for 3 days. After that, the liquid culture was centrifuged at 3000 rpm for 10 min. The supernatant was used to detect the content of secondary metabolite compounds, namely: alkaloids, flavonoids, phenolic, tannins, and terpenoids produced by each endophytic fungal isolate.

Detection of secondary metabolites

The contents of alkaloids, flavonoids, phenolic, tannins, and terpenoids in each endophytic fungal species liquid culture and also the extracts in the leaves and stems extract were analyzed by spectrophotometry method.

Quantitative alkaloid determination

Quinine was used as a standard solution; the concentrations were 0, 0.1, 0.5, 1, 2.5, and 5 (mg/L). Then, 10 mg of atropine was dissolved in chloroform to 100 mL. Then the solid sample was crushed and weighed as much as 0.5 g then dissolved in DMSO as much as 10 mL and added with 1 mL of HCl 2N 5 mL of Brom cresol green solution and 5 mL of phosphate buffer, homogenized, and allowed to stand for 1 hour. Transfer the solution to a separatory funnel, then mix with 10 mL chloroform. Shake the solution and allow it to form a layer. For the standard, take 5 mL of standard, add 1 mL of 2 N HCl, 5 mL of Brom cresol green, and 5 mL of phosphate buffer, homogenize, and let stand. The solution can be used for the following process. In alkaloid determination, take 1 mL of the standard solution that has been prepared (orange-yellow solution). The solution is then diluted with chloroform to a volume of 5 mL. Measure the absorbance at λ 470 nm and the alkaloid concentration was calculated using the standard regression equation (Tambe and Bhambar 2014).

Quantitative flavonoid determination

To prepare the standard solution, 100 mg of quercetin was dissolved in distilled water to reach a volume of 100 mL, resulting in various concentrations such as 0, 0.5, 1, 10, 25, and 50 mg/L. In sample preparation, solid samples were crushed, weighed 1 g, and dissolved in 10 mL of methanol homogenized for 30 min, then filtered using a vacuum filter and centrifuged at 3000 rpm for 10 min. The supernatant was taken for further processing. For flavonoid content determination, 0.1 mL of sample or standard solution was mixed with 0.1 mL of 2% Al_2Cl_3 solution, homogenized, and allowed to stand for 60 min. After that, 1 mL of distilled water is added. If the solution turns red, the sample contains flavonoids. The absorbance was measured at λ=420 nm, and the flavonoid concentration was calculated using the standard regression equation.

Quantitative phenolics determination

The standard used is gallic acid and is made with a concentration of 0; 2; 5; 10; 25; 50 (mg/L). For sample preparation, the sample was crushed and then weighed as much as 0.5-2 g, then dissolved in methanol up to 25 mL in a volumetric flask, then homogenized and allowed to stand for about 30 min, then filtered and if necessary centrifuged at 3000 rpm for 10 min and the supernatant was taken. For liquid samples, take 1 mL of sample, add 5 mL of methanol, put in a test tube, and homogenize using a vortex for 5 min. Filter the solution with a vacuum filter, and take the filtrate. Determination of phenol is done by means of 1 mL of sample or standard plus 0.2 mL folin c, 0.6 mL Na_2CO_3 0.2 mM. Homogenize with vortex for 5 min and let stand for 120 min. Measure the absorbance at λ 765 nm and the phenolic concentration was calculated using the standard regression equation (Alara et al. 2018).

Quantitative tannin determination

The material used in the standard solution is tannic acid with a concentration of 0, 0.1, 0.5, 1.0, 2.5, 5.0 (mg/L). The standard (50 mg/L) was prepared by dissolving 5 mg of tannic acid in 20% ethanol to 100 mL). In the preparation of samples and standard solutions, solid samples were crushed and then weighed as much as 0.1 g, then dissolved in methanol up to 10 mL in a volumetric flask, then homogenized and allowed to stand for about 30 min, then vacuum filtered and centrifuged at 3000 rpm for 10 min and the supernatant was taken. To determine tannin content, 5 mL of sample or standard was added with 0.5 mL $FeCl_3$ 0.1 M and 0.5 mL $K_3Fe(CN)$ 6 0.008 M, then homogenized and allowed to stand for 30 min. The solution was then diluted with distilled water to a volume of 10 mL. Measure the absorbance at λ=620 nm and the tannin concentration was calculated using the standard regression equation.

Quantitative terpenoid determination

The standard solution was prepared by dissolving 0.5 mg of linalool in chloroform up to 100 mL and made several concentrations, namely 0, 0.01, 0.05, 0.1, 0.25, and 0.5 mg/L. For the preparation of samples and standard solutions, solid samples were crushed and weighed as much as 2-10 g, dissolved in methanol up to 25 mL, homogenized, and allowed to stand for 30 min. After that, it was filtered and centrifuged at 3000 rpm for 10 min. Then, the supernatant was taken and added with chloroform (1 mL of supernatant was added with 1 mL of chloroform each). After that, let stand and take the top phase (non-polar) as much as 5 mL. Terpenoid content is determined by taking 5 mL of the sample or standard solution and adding 3 mL of concentrated H_2SO_4, then homogenizing and letting it stand for a while. If a brownish-red color is formed in the solution, it is proven that the sample contains terpenoids. Afterward, dilute the solution with chloroform to 10 mL in volume. Then, the absorbance at λ=538 nm. The terpenoid content concentration is determined by the Regresi standard equation (Indumathi et al. 2014).

Fungal antagonism test

The interaction between endophytic fungi species and pathogenic fungi was evaluated by using the dual culture technique. Each fungal isolate on a PDA plate medium was incubated at 25°C for 3×24 h. Each pathogenic fungi colony and each endophytic fungus colony was cut with a 5 mm diameter cork borer aseptically and placed at two points and placed on opposite sides, then incubated at 25°C for 3×24 h. After that, the antagonism activity of the fungi was observed. The antagonism ability of endophytic fungi against pathogenic fungi was calculated using the antagonism ability formula.

$$\text{The antagonism ability } (\%) = \frac{R1 - R2}{R1}\ x\ 100\%$$

Where:

R1: The radius of the pathogenic mold away from the endophytic fungus colony.

R2: The radius of the pathogenic mold colony approaching the endophytic fungus colony.

RESULTS AND DISCUSSION

Histology observation of endophytic fungi in *H. indicum*

This histology observation result proved the endophytic fungi location in *H. indicum* plant tissues was in the epidermal tissue, sponge tissue, stomata guard cells, leaves and branch parenchymal tissues. In addition, microconidia were found on the branches epidermal tissue surface of branches. Conidiophores are also found in the leaf epidermal tissue (Figure 1). Although mycelium is found in both leave and branch tissues, mycelium does not cause damage to the plant tissues, there is no mycelium that penetrates the cell wall or cytoplasm.

Endophytic fungi are found in various parts of plants, including leaves, stems, and roots (Mazaro et al. 2022). Endophytic fungi are also found in the intercellular spaces and vascular vessels of leaves and stems (Mishra et al. 2017; Hastuti et al. 2019). Although endophytic fungi live in the host plant tissues, endophytic fungi do not cause any damage (Hastuti et al. 2018). Endophytic fungi take only a small amount of nutrients to live and reproduce, so they do not cause the death of the host plants (Mukti et al. 2018).

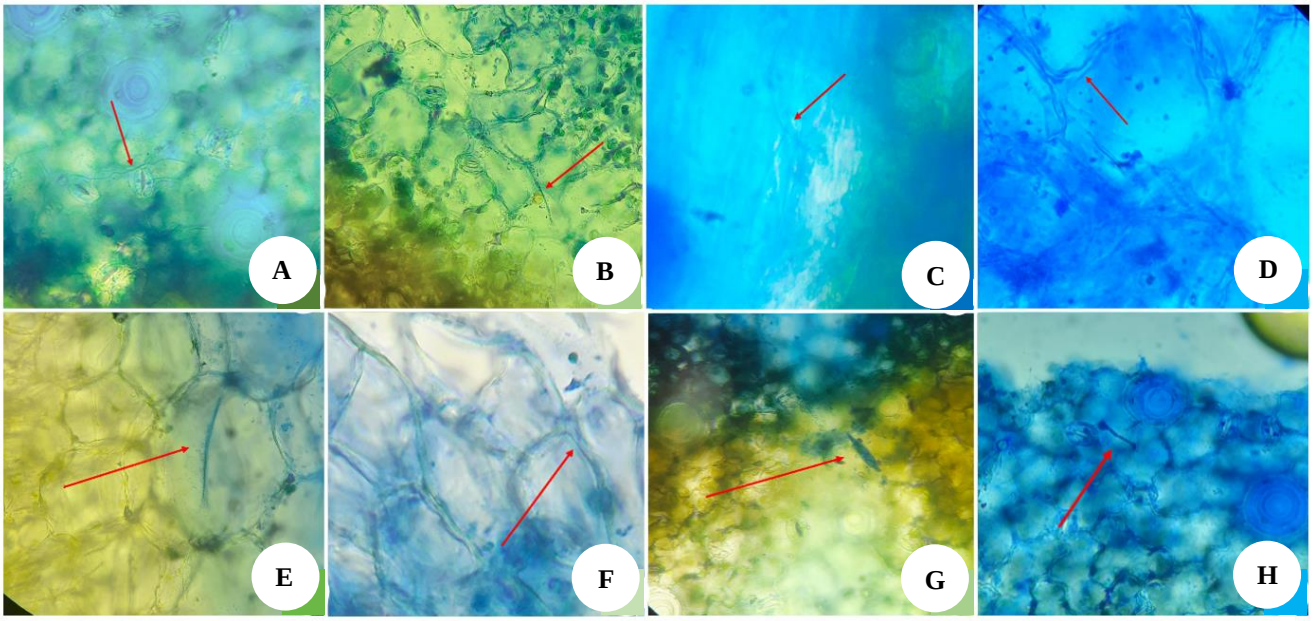

Figure 1. The location of endophytic fungi on *Heliotropium indicum* leaves with 400x microscopic magnification. A. At the epidermal stomata guard cells; B. At the paradermal section, epidermal cells; C. At leaf sponge tissue; D. Transversal section, sponge tissue; E. Transfersal section, at the branches parenchymal cells; F. In the parenchyme intercellular space of branch (transversal section); G. Paradermal section, conidia are found on the leaf epidermis surface; H. Conidiophores, on the leaf epidermis surface

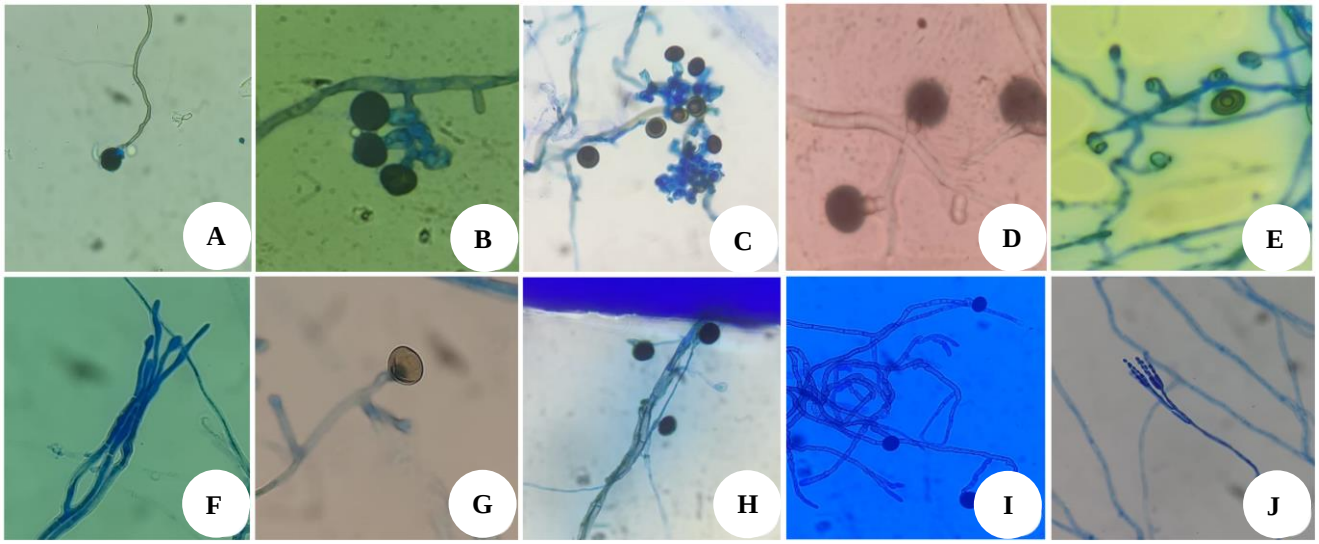

Figure 2. The microscopic observation result of each endophytic fungi species isolated from *Heliotropium indicum*, leaf, and stem with 400x microscopic magnification. A. *N. gorlenkoana*; B. *N. guilinensis*; C. *N. musae*; D. *N. oryzae*; E. *N. rubi*; F. *Nigrospora* sp. 1; G. *Nigrospora* sp. 2; H. *Nigrospora* sp. 3; I. *Nigrospora* sp. 4; J. *P. oxalicum*

Microscopic observation

Based on the results of isolation and identification result of endophytic fungi, 10 species of endophytic fungi were found on leaves and stems (Figure 2). The endophytic fungi isolates were characterized and identified using a fungi determination key (Wang et al. 2017) (Tables 1 and 2). The results of this study found 9 species of *Nigrospora* and 1 species of *Penicillium*, including: *Nigrospora gorlenkoana* (A), *N. guilinensis* (B), *N. musae* (C), *N. oryzae* (D), *N. rubi* (E), *Nigrospora* sp. 1 (F), *Nigrospora* sp. 2 (G), *Nigrospora* sp. 3 (H), *Nigrospora* sp. 4 (I), and *Penicillium oxalicum* (J), The conidia form of *Nigrospora* conidia has the same shape, i.e. globus. However, specifically in the species *Nigrospora* sp. 1, *Nigrospora* sp. 2, *Nigrospora* sp. 3 and *Nigrospora* sp. 4 have a sterile cell structure. The general characteristics of species from *Nigrospora* are that they have hyaline hyphae, separated hyphae and cottony hyphae. There is significant variation in morphological characteristics among *Nigrospora* species. The colony color, colony diameter, conidia shape and size, and hyphae shape all provide important clues for species identification and classification. *P. oxalicum* was

found on the leaves of *H. Indicum* plant, the main characteristic of *P. oxalicum* is the color of green colonies with white edges, the color of colony base is yellow or orange. Colonies on PDA reach 16-mm at 25°C after 5 days, cotton-like, initially white and turning into dark green. The hyphae length is 150 µm, hyphae of 3 µm in diameter. Conidiophores erect with 3µm diameters, hyaline, smooth, mono- or bi-verticulate with 2-3 metulae, metulae up to 5 µm long, 2.5 µm diameter. Phialides 7 µm in size, hyaline and cylindrical. Conidia ellipsoid shape at 2.5 µm in size, chained, subhyaline, dark green in color.

These results prove that although some *Nigrospora* species have specific host plants, they can also be found on other plants. For example, *N. gorlenkoana*, *N. oryzae*, *N. osmanthi*, and *N. rubi* were found on bamboo and Chinese rose host plants (Hao et al. 2020). *N. sphaerica* was found on the host plants *Cananga odorata* (Hastuti et al. 2019), *Musa* spp. (Zakaria and Aziz 2018), *Citrullus lanatus*, *Vigna unguiculata* and *Hylocereus polyrhizus* (Hong et al. 2022). This is in accordance with the research of Tong et al (2022) that *Nigrospora* species are found in many plants species as endophytic fungi and are widely distributed in medicinal plants and can produce secondary metabolites.

Nigrospora is a diverse genus of fungi that can colonize plants through symbiotic mutualism. The presence of *Nigrospora* as an endophytic fungus can improve the growth and development of host plants, as well as their resistance to pathogenic microbes (Boban et al. 2023; Dutta et al. 2023; Poorni et al. 2023; Chong and Liu 2024). *Nigrospora* can modulate the expression of genes related to plant defense and phytohormones to establish a balanced mutualistic symbiosis with the host plant (Vahabi et al. 2015; Bastías and Gundel 2023; Peterson et al. 2023). *Nigrospora* is an important mediator in plant-herbivore interactions and can increase the resistance of host plants to herbivorous insects through the production of various alkaloid-based defense compounds in plant tissues (Thakur et al. 2013). In addition, *Nigrospora* also has the potential to be an anti-insect by affecting the survival and development of polyphage pests, such as *Spodoptera litura* (Thakur et al. 2012).

Table 1. The characteristic of *Nigrospora* found in *Heliotropium indicum* plant

Observed characteristics	Genus *Nigrospora*				
	N. gorlenkoana	***N. guilinensis***	***N. musae***	***N. oryzae***	***N. rubi***
Colony color	White	Gray	Grayish white	Black	White
Colony diameter	82 mm	89 mm	84 mm	90 mm	80 mm
Nature of the colony	Cotton	Cotton	Cotton	Cotton	Cotton
The typical color of the base of the colony	Grayish white	Gray, black	Black mottled with creamy edges	Black	Yellow with black spots
Isolated from	Stem	Stem	Leaf	Leaves	Stem
Hyphae color	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline
Bulkheads on hyphae	Available	Available	Available	Available	Available
Diameter of hyphae	3.3 µm	3.75 µm	6 µm	3 µm	3 µm
Color of conidiophores	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline
Diameter of conidiophores	3 µm	7 µm	10 µm*	5.6 µm	5 µm
Conidia present/absent	There are conidia	There are conidia	There are conidia	There are conidia	There are conidia
Conidia diameter	13 µm	12.5 µm	14 µm	15 µm	15 µm
Conidia shape	Globus	Globus	Globus	Globus	Globus
Conidia color	Brown	Brown	Brown	Brown	Brown

Table 2. The characteristic of *Nigrospora* found in *Heliotropium indicum* plant

Observed characteristics	Genus *Nigrospora*			
	***Nigrospora* sp. 1**	***Nigrospora* sp. 2**	***Nigrospora* sp. 3**	***Nigrospora* sp. 4**
Colony color	White	White	White	White
Colony diameter	83 mm	31 mm	86 mm	89 mm
Nature of the colony	Cotton	Cotton	Cotton	Cotton
The typical color of the base of the colony	Yellow	Orange	White to gray	Gray
Isolated from	Leaf	Stem	Stem	Stem
Hyphae color	Hyaline	Hyaline	Hyaline	Hyaline
Bulkheads on hyphae	Available	Available	Available	Available
Diameter of hyphae	3.75 µm	3 µm	3.75 µm	2.5 µm
Color of conidiophores	Hyaline	Hyaline	Hyaline	Hyaline
Diameter of conidiophores	8.75 µm	3 µm	5 µm	7 µm
Conidia	There are conidia	There are conidia	There are conidia	There are conidia
Conidia diameter	10.5 µm	16.2 µm	12.5 µm	15 µm
Conidia shape	Globus	Globus	Globus	Globus
Conidia color	Brown	Brown	Brown	Brown

Table 3. The secondary metabolites content of the endophytic fungi in *Heliotropium indicum* at liquid culture

Isolat code	Species	Secondery metabolite (mg/L)				
		Alkaloid	**Flavonoid**	**Phenolic**	**Tannin**	**Terpenoid**
A	*N. gorlenkoana*	628.529	4400.78	5456.24	1082.09	432.46
B	*N. guilinensis*	884.412	6334.38	7905.22	1576.78	607.02
C	*N. musae*	829.265	6096.09	7599.09	1512.78	570.18
D	*N. oryzae*	896.176	6357.81	7825.85	1583.02	589.47
E	*N. rubi*	805.000	6037.50	7400.68	1515.92	573.68
F	*Nigrospora* sp. 1	705.000	5271.88	6448.30	1331.77	503.51
G	*Nigrospora* sp. 2	1046.912	7596.09	9277.10	1854.56	707.02
H	*Nigrospora* sp. 3	843.971	6037.50	7338.32	1511.24	557.90
I	*Nigrospora* sp. 4	932.941	6533.59	8058.28	1611.11	611.40
J	*P. oxalicum*	1000.588	7350.00	8931.29	1746.88	669.30
Secondary metabolites content from leaves and stem of *H. indicum* (mg/kg)						
H. indicum leaves		152402.344	21776.47	78760.92	59046.05	468956.92
H. indicum stem		190585.938	19541.18	101935.102	51370.61	316462.59

The interaction between endophytic fungi and host plants involves the production of secondary metabolites and hydrolytic enzymes by fungi, which help plants combat phytopathogens, such as pathogenic insects and fungi, and improve tolerance to abiotic stresses, such as heavy metals, drought, salts, and high temperatures (de Carvalho et al. 2020). Nigrosporas are able to produce secondary metabolites that act as antibacterial and antifungal fungi that help maintain a balance of antagonism with competing microbes (Schulz et al. 2015).

P. oxalicum was found as an endophytic fungus of *H. indicum* and found in various other plant species, such as *Taxus cuspidata* (Li et al. 2021), *Musa paradisiaca* (Zakaria and Aziz 2018) and *Boerhaavia diffusa* (Khalil et al. 2021). This fungus is found in different parts of the plant such as roots, stems, leaves, and seeds. *P. oxalicum* species provide a variety of benefits to host plants, including protection against pathogens, increased growth, and the production of bioactive compounds (Sarma et al. 2020; Toghueo and Boyom 2020) *P. oxalicum* produces bioactive compounds with antiviral, antibacterial, and anti-inflammatory properties, which could be useful in treating neurodegenerative diseases (Kim et al. 2022).

Analysis of secondary metabolite compounds

The results of this study proved that 10 species of endophytic fungi isolated from the stems and leaves of *H. indicum* could produce several secondary metabolites that were useful as producers of antibiotic compounds (Table 3). There are differences in the content of secondary metabolites produced by each species of endophytic fungi. *Nigrospora* sp. 2 is the highest potential to produce secondary metabolite compounds compared to other fungal species isolated from *H. indicum.*

The diversity of secondary metabolites produced by different species of endophytic fungi can be attributed to several factors: each fungal species has a unique genetic makeup that influences the types of secondary metabolites it can produce. The genes responsible for these metabolites are often clustered and located near telomeres, which can vary significantly between endophyt fungi species (Keller et al. 2005). The relationship between endophytic fungi and their host plants can influence the types of secondary metabolites produced. Some fungi produce the same compounds as their host plants, while others produce unique metabolites that help in plant defense and adaptation (Gautam and Avasthi 2019; Rashmi et al. 2019). The ecological roles of secondary metabolites, such as defense against pathogens, herbivores, and competition with other microorganisms, drive the diversity of these compounds. Different species may produce specific metabolites to fulfill these roles in their particular ecological niches (Mohamed and Abd-Elsalam 2023). In summary, the variation in secondary metabolites among endophytic fungi species is a result of genetic differences, host interactions, and ecological roles.

Antagonism analysis

Testing the antagonism of 10 isolates of endophytic fungi on *H. indicum* plants consists of fungal species *N. gorlenkoana, N. guilinensis, N. musae, N. oryzae, N. rubi, Nigrospora* sp. 1, *Nigrospora* sp. 2, *Nigrospora* sp. 3, *Nigrospora* sp. 4, and *P. oxalicum* which are antagonized with *F. solani* and *F. oxysporum*. The study results of the antagonism of endophytic fungi with pathogenic fungi consist of morphological data of antagonism (Figure 3; Figure 4) and measurement of antagonism effect between fungi (Tables 4 and 5).

Table 4. Antagonism strength of 10 endophytic fungal isolates against the pathogenic fungus *Fusarium oxysporum*

Antagonistic fungi	Pathogenic fungi	Antagonism power (%)
N. gorlenkoana	*F. oxysporum*	55.9
N. guilinensis	*F. oxysporum*	51.1
N. musae	*F. oxysporum*	59.1
N. oryzae	*F. oxysporum*	77.2
N. rubi	*F. oxysporum*	64.5
Nigrospora sp. 1	*F. oxysporum*	66.1
Nigrospora sp. 2	*F. oxysporum*	46.5
Nigrospora sp. 3	*F. oxysporum*	63.5
Nigrospora sp. 4	*F. oxysporum*	52.2
P. oxalicum	*F. oxysporum*	51.2

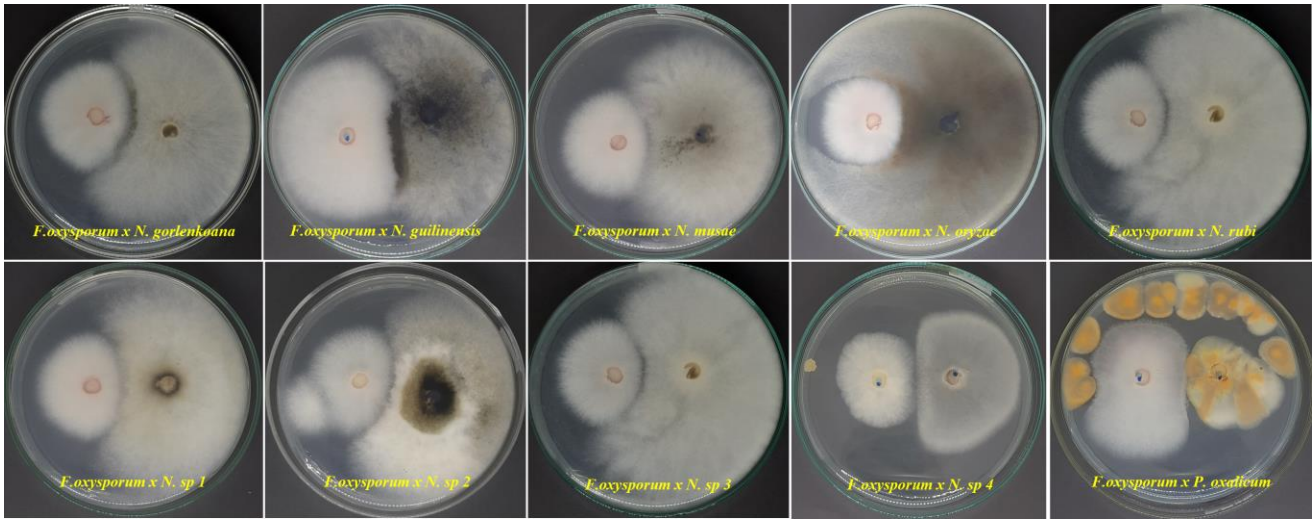

Figure 3. Morphology of antagonism between *Fusarium oxysporum* and ten isolates of the endophytic fungus *Heliotropium indicum*

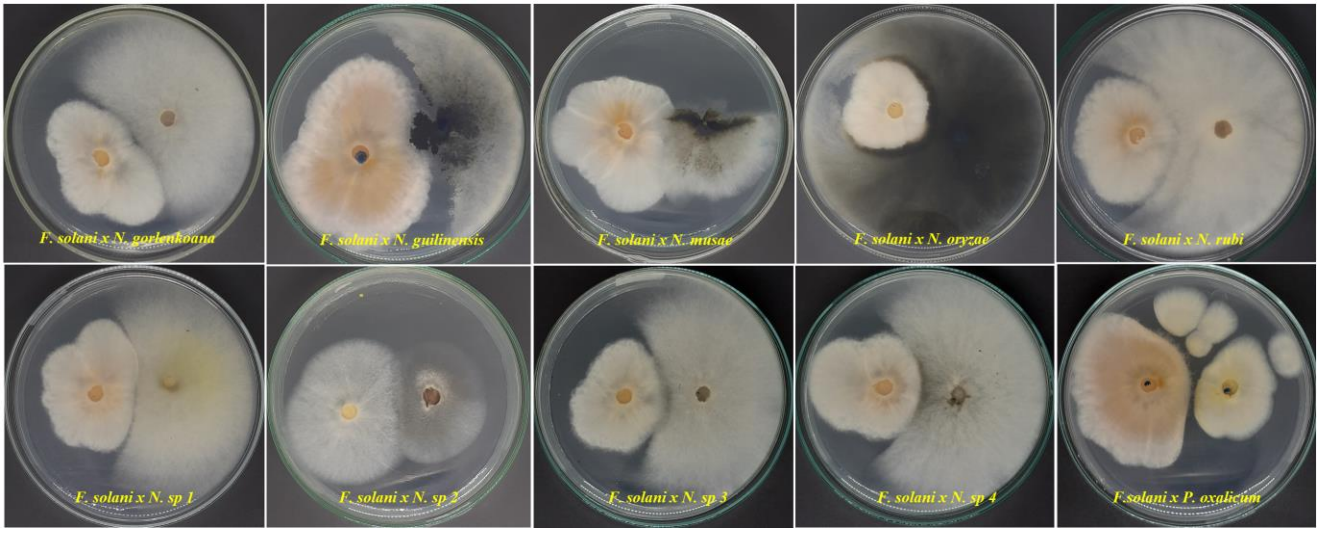

Figure 4. Morphological antagonism between *Fusarium solani* and 10 isolates of the endophytic fungus *Heliotropium indicum*

Table 5. Antagonism strength of 10 endophytic fungal isolates against the pathogenic fungus *Fusarium solani*

Antagonistic fungi	Pathogenic fungi	Antagonism power (%)
N. gorlenkoana	*F. solani*	28.2
N. guilinensis	*F. solani*	40.9
N. musae	*F. solani*	64.0
N. oryzae	*F. solani*	48.2
N. rubi	*F. solani*	39.6
Nigrospora sp. 1	*F. solani*	35.4
Nigrospora sp. 2	*F. solani*	63.6
Nigrospora sp. 3	*F. solani*	42.8
Nigrospora sp. 4	*F. solani*	56.3
P. oxalicum	*F. solani*	53.6

Ten endophytic fungi isolates were able to fight pathogenic fungi with varying antagonistic effects (Tables 3 and 4). The highest antagonistic effect against *F. oxysporum* was 77.2% while the lowest antagonistic effect was 46.5% while the antagonistic power against *F. solani* was the highest was 64% and the lowest was 28.2%. The findings of this study showed that 10 isolates of *H. indicum* endophytic fungi had the capacity to inhibit the growth of pathogenic fungi.

The results of this study are in accordance with the results of the study by Nuraini et al. (2017) that the antagonistic mechanisms used by endophytic fungi against *F. oxysporum* include competition, antibiosis, and parasitism which show strong inhibition through this mechanism. In contrast, the antagonistic mechanism of endophytic fungi against *F. solani*, such as that carried out by *Metarhizium robertsii*, mainly involves competition and enzyme inhibition, which may be less effective (He et al. 2023). In addition, based on the mechanism of inhibition level, it shows that endophytic fungi show a higher level of inhibition against *F. oxysporum* than *F. solani*. For example, in the study of Mengjun et al. (2019), endophytic bacteria from *Carex moorcroftii* showed an inhibition level of more than 60%

against *F. oxysporum*, which is higher than its effect on *F. solani*. Various types of endophytic fungi have been found to be effective against *F. oxysporum*. For example, 403 fungal species showed antagonistic effects against *F. oxysporum* compared to fewer fungal species against *F. solani* (Huang et al. 2020). Endophytic fungi antagonism can affect the growth of *F. oxysporum* through several mechanisms such as *Paraconiothyrium variabile* showed strong antagonism against *F. oxysporum* by causing disorganization of the pathogen's mycelium (Combès et al. 2012). In addition, several *Trichoderma* species produce volatile organic compounds (VOCs) that significantly inhibit the growth of *F. oxysporum* mycelium (Rajani et al. 2021). For example, *Trichoderma asperellum* AB4D1 was able to inhibit the growth of *F. oxysporum* mycelium by up to 72.52% in the dual culture method (Trizelia et al. 2023).

The mechanism of antagonism between 10 isolated mold isolates from *H. indicum* against *F. solani* and *F. oxysporum* can involve various ways, such as nutrient competition, production of antimicrobial compounds, and physical interactions between mold hyphae (Hastuti et al. 2018; Rahmawati et al. 2018). This can be seen in the results of microscopic observations that show the existence of a mycoparatic mechanism, namely the fungal hyphae are twisted, penetrate the pathogenic mold hyphae and grow attached along the pathogenic fungal hyphae (Figure 5). In addition, antagonism with dual culture techniques between phytopathogens and endophytic fungi can produce secondary metabolites, which diffuse to the contact points of pathogenic fungi (Abdelrahem et al. 2023). Fungal co-cultures and endophytic fungi can produce bioactive compounds that are effective for controlling plant pathogens (Abdelrahem et al. 2024). These fungi have antifungal activity and can inhibit plant pathogens' growth, which is one form of fungal antagonism (Hassane et al. 2022a, b).

Antagonist fungi hyphae attack pathogenic fungi through a variety of complex mechanisms (Hastuti and Rahmawati 2016, Mustofa and Hastuti 2024), i.e: Mycoparasitism, antagonistic molds directly parasitize and attack pathogenic molds, competing for nutrients and space. Degradation of pathogenic cell walls, antagonistic fungi produce enzymes that degrade pathogenic mold cell walls. Competition for nutrients and space, antagonistic fungi compete with pathogenic fungi for resources. Production of antimicrobial compounds (Antibiotics), the antagonist fungi secretes antimicrobial compounds that inhibit the growth of pathogens (Tyśkiewicz et al. 2022; Saldaña-Mendoza et al. 2023). In addition, some endophytic fungi directly parasitize pathogenic fungi, attacking their spores, mycelium, and cells, thus inhibiting their growth. Endophytic fungi can induce systemic resistance in plants, improving plant defense mechanisms against pathogens. It involves modulating plant gene expression and hormone signaling (Nuraini et al. 2017; Baron and Rigobelo 2022).

In the soil, antagonistic fungi and pathogenic fungi coexist, so naturally, the interaction between the two fungi will produce a balance in soil ecosystems. The results of this study show that ten endophytic mold isolates that are symbiotic with *H. indicum* can be categorized as antagonistic fungi that have mycoparasitic, by attaching, twisting, and penetrating the hyphae of pathogenic fungi. In addition, endophytic fungi isolate contains secondary metabolite compounds to inhibit the growth of pathogenic fungi, so that it can be a biological control agent against various plant pathogens, including *F. oxysporum* and *F. solani*.

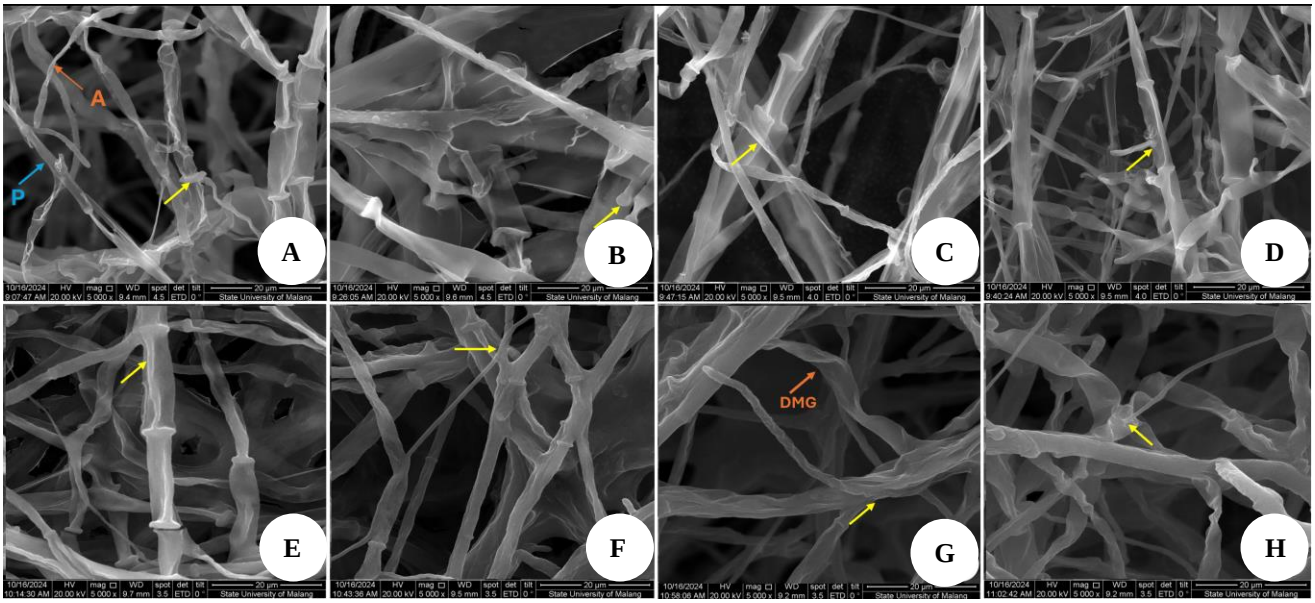

Figure 5. Mechanism of mycoparasitism of *Nigrospora gorlenkoana* against *Fusarium solani*. A. orange arrow: hyphae antagonist, blue arrow: pathogenic fungus hyphae, yellow arrow: twisted hyphae; B. yellow arrow: damaged hyphae *N. gorlenkoana* against *F. oxysporum*; C. yellow arrow: hyphae attached; D. yellow arrow: penetrating hyphae *Nigrospora* sp. 1 against *F. solani*; E-F. yellow arrow: hyphae attached *Nigrospora* sp. 1 against *F. oxysporum*; G. orange arrow: damaged hyphae, yellow arrow: twisted hyphae; H. yellow arrow: penetrating hyphae

Future recommendations from this study are to identify endophytic fungal species, which need to use two methods, namely the independent cultivation method and the dependent cultivation method. Independent cultivation methods include PCR, DNA sequencing, NGS, molecular fingerprinting, and phylogenetic analysis. In contrast, the dependent cultivation method is a conventional method that observes the morphological characteristics of endophytic fungi microscopically and macroscopically, which match the determination key. Molecular techniques provide a more accurate and comprehensive insight into endophytic fungi's diversity and ecological role. In addition, to analyze bioactive compounds of endophytic fungi, it is necessary to use metabolomics methods that include mass spectrometry, nuclear magnetic resonance (NMR), and Lacer-induced fluorescence. These methods will provide advantages of bioactive application possibilities in various fields such as agriculture, foods, biofuels, biomedical, bioremediation, pharmaceutical industry, and textile industry.

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