Antagonistic activity of endophytic fungi from Maize plants (*Zea mays* L.) against *Fusarium oxysporum* Schltdl.

Oktira Roka Aji[1*], and *Dilla Rofiyanti*[1]

[1]Biology Department, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan, Bantul, Daerah Istimewa Yogyakarta, Indonesia

Abstract. Endophytic fungi were microorganisms that resided within plant tissues without harming their host plants. These fungi acted as biological agents against harmful fungi by utilizing mechanisms like competing for nutrients, parasitism, and antibiosis. This research aimed to isolate and identify the genus groups of endophytic fungi from maize plants (*Zea mays* L.) and understand the antagonistic mechanisms between endophytic fungi and *Fusarium oxysporum* Schltdl. Through dual culture tests, the highest inhibitory percentage of endophytic fungi against Fusarium oxysporum Schltdl is determined. in the filtrate and volatile compound tests. Antagonistic activity against pathogenic fungi was determined in vitro through dual culture methods, filtrate culture methods, and volatile compound production. Data obtained from the antagonistic activity of endophytic fungi were analyzed based on the calculation of inhibition percentages. The dual culture method revealed eight isolates effectively restraining the growth of *Fusarium oxysporum* Schltdl. Among the filtrate culture experiments, isolate B1.1.1 exhibited the most promising outcomes with an inhibition rate of 39.83%. Notably, isolate D2.2.1 displayed the highest inhibition rate of 44.38% in the antagonism test employing the volatile compound production method. Identification was conducted through ITS gene amplification, confirming that isolate D2.2.1 corresponded to *Trichoderma harzianum*. This research aimed to improve productivity and food security by managing plant diseases, thereby contributing to the achievement of SDG 2 (Zero Hunger).

1 Introduction

Plants are vulnerable to numerous diseases, and fusarium wilt is one of them. It typically affects the roots or the base of the plant stem. Fusarium wilt disease can be identified by the symptoms on the top of the plant [1]. The affected plants exhibit symptoms such as rotten roots and are prone to falling over, making them easy to uproot [2]. Fusarium wilt disease is caused by a pathogenic fungus called *Fusarium oxysporum* Schltdl. This soil-borne pathogen can survive in unfavorable conditions and even form chlamydospores without host plants [3].

* Corresponding author: oktira.aji@bio.uad.ac.id

The fungus is known for attacking various plants such as corn, sugarcane, rice, and sorghum. It causes rot in the stems, cobs, and seeds of corn [4].

Farmers typically rely on fungicides to manage plant diseases. However, the recurrent use of these chemicals can negatively impact human health and the environment due to the residual effects they leave behind. Consequently, there is a need to explore alternative eco-friendly approaches. One such strategy involves using endophytic fungi as biological agents to control diseases caused by plant pathogens [5]. This approach may offer a promising solution to the problem of fungicide overuse and its harmful consequences. By controlling plant diseases, this research helps increase productivity and enhance food security, reducing crop losses and ensuring a more stable food supply. Consequently, this research is crucial for ensuring food security and mitigating global hunger, contributing to the achievement of SDG 2 (Zero Hunger).

2 Material and Methods

2.1 Sample preparation

Maize plant samples were collected from Pathuk District, Gunung Kidul. The samples were chosen due to the typical cultivation environment and relevance to the study's focus on endophytic fungi interactions with *Fusarium oxysporum*. The selected plants were approximately 80 days old, healthy, and devoid of deformities to ensure that the samples were at a developmental stage relevant to the study, free from potential confounding factors, and capable of providing reliable data for analysis. Samples were taken from the roots, stems, and leaves to isolate endophytic fungi.

2.2 Endophytic fungal isolation

The isolation of endophytic fungi was done using a direct seed-planting method. Maize plants' leaves, roots, and stems were washed with running water until they were utterly soil-free. Corn plant organ samples were sterilized by placing them in 70% (v/v) alcohol for 1 minute, then putting in a 0.5% (v/v) NaClO solution for 3 minutes, then placing them in 70% (v/v) alcohol for 30 seconds, and rinsing them with sterile distilled water twice and drying them on sterile Petri dishes [6,7].

Organ samples were cut using a sterile scalpel into 1-2 cm sizes aseptically. The organ samples were placed on PDA media and added with 100 µg/mL chloramphenicol aseptically. A total of 0.1 mL of sterile distilled water from the last rinse was poured on the PDA media by the spreading method to ensure that the surface sterilization process on the organ samples was successful. The culture was incubated at room temperature (25 - 28°C) for seven days [6,7].

The successfully isolated fungi were then subcultured on PDA media and incubated for three days at room temperature (25 - 28°C). Macroscopic observations were made by observing the morphology of the fungal mycelium, which included the color of the upper surface colony, the color of the reverse colony, and the texture of the colony surface. Microscopic observations were carried out with a pure culture of endophytic fungi isolated aseptically using a sterile ose, transferred to the surface of a glass object, and then dripped with Lactophenol cotton blue. The preparations were observed with a light microscope. Observations include the structure of hyphae (concentrated or non-concentrated) and the structure of spores [8].

2.3 Dual Culture Assay

To carry out an antagonism test, small pieces of pure culture of the fungus *Fusarium oxysporum* Schltdl. (± 5 mm) and each endophytic fungal isolate (± 5 mm) was inoculated in one petri dish containing PDA media at a distance of 3 cm. As a negative control, small pieces of *F. oxysporum*. (± 5 mm) was planted in the center of the Petri dish containing PDA media. Isolates were incubated for seven days [9].

2.4 Antifungal non-volatile compounds test

Pure cultures of endophytic fungi were inoculated in 10 mL of PDB media. The endophytic fungal culture was incubated for ten days at room temperature (25-28°C). The fungal culture was filtered using filter paper, and the filtrate was filtered again using a 0.22 µm sterile syringe filter. The cell-free filtrate was mixed with sterile PDA media in petri dishes. Pieces of fungal culture of *Fusarium oxysporum* Schltdl. (± 5 mm) were inoculated at three points of the petri dish. As a negative control, the fungus *F. oxysporum*. It was inoculated on a petri dish containing PDA medium only. The isolates were incubated for five days [10,11].

2.5 Antifungal volatile compounds test

Pure culture of *Fusarium oxysporum* Schltdl. (± 5 mm) and endophytic fungi (± 5 mm) were inoculated in the center of a petri dish containing PDA media separately. Both Petri dishes were cupped facing each other (*F. oxysporum*. fungus on top, endophytic fungus on bottom). As a negative control, the petri dish containing the fungus *Fusarium oxysporum* Schltdl. It was cupped with a petri dish containing PDA medium only. The fungus was incubated for six days [12]. The percentage of inhibition in the antifungal non-volatile compounds test and antifungal volatile compounds test were measured using the formula:

$$Inhibition\ (\%) = \left(\frac{C-T}{T}\right) \times 100 \quad (1)$$

Where:
C = Diameter of the fungal colony in the control plate
T = Diameter of the fungal colony in the treated plate

2.6 Fungal identification

Molecular identification of fungal strains was completed by DNA amplification and sequencing of internal transcribed spacer (ITS) regions. Fungal mycelia (20 mg) were frozen overnight. Fungal DNA isolation was performed according to the manufacturer's protocol using the Wizard® Genomic DNA Purification Kit Protocol (Promega). Then, the isolated DNA was amplified by polymerase chain reaction (PCR). PCR was performed using GoTaq® Master Mix (Promega). The primers used, namely ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATGC) (references?), were mixed with the Master Mix Kit and DNA template with a total volume of 50 µL. The mixture was then added into a thermal cycler using programmed PCR (BioRad, USA). The amplified fungal DNA (PCR products) was then sent to a commercial service for sequencing, and base sequences were compared using the BLAST Algorithm with publicly accessible databases, including GenBank.

3 Results and Discussion

The results of the isolation of endophytic fungi from corn plants (*Zea mays* L.) obtained 28 endophytic fungi, with 16 isolates from leaf organ parts, one isolate from stem organ parts, and 11 isolates from root organ parts. Fig. 1 shows the isolation results of endophytic fungi from maize plants (*Zea mays* L.), highlighting the diversity and distribution of fungal isolates obtained. More endophytic fungi were found in the leaf organ. The isolation results of endophytic fungi were found more in the leaf organs [13]. The corn leaves used were old leaves, so the results of endophytic fungi isolation were more widely obtained in leaf organs. More endophytic fungal isolates were found in leaf organs, especially in older leaves than young leaves [14]. Fewer endophytic fungi were found in the stem organ than in the leaf organ because the epidermis layer of corn stems has a dense structure that functions as a protector called silica cells, making it difficult for endophytic fungi to penetrate plant tissue. The epidermis has a dense structure and can thicken because it contains silica to strengthen plant tissue structure and function as a protector [15]. Eight-eight isolates are known to inhibit the growth of the pathogenic fungus *Fusarium oxysporum* Schltdl. Through the mechanism of nutrient competition, the diameter of the development of endophytic fungi was more significant than the diameter of the fungus *Fusarium oxysporum* Schltdl so that the growth of endophytic fungi can fill the media and suppress the growth of *Fusarium oxysporum* Schltdl fungus.

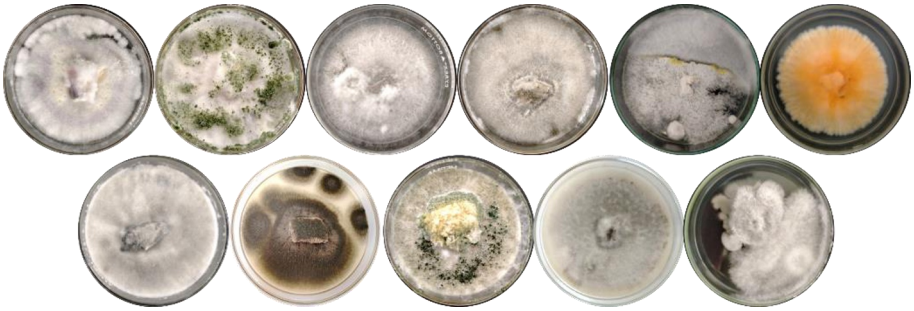

Fig. 1. Isolation results of endophytic fungi in maize plants (*Zea mays* L.).

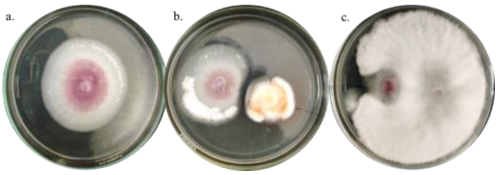

Fig. 2. Antagonistic activity of the endophytic fungus from maize plants (*Zea mays* L.) against *Fusarium oxysporum* Schltdl. (a.) Control (b.) isolate A3.2.1 and (c.) isolate A2.1.1

An isolate with code A3.2.1 isolated from the root organ of corn plants (*Zea mays* L.) has an antibiotic mechanism. Although the results showed that the pathogenic fungus was more significant in diameter than the endophytic fungus, it could still suppress the growth of pathogenic fungi because pathogenic fungi grow slowly and do not fill the space. A clear zone is formed from the results of the inhibition. The mechanism of nutrient competition occurs due to the growth of endophytic fungal mycelium that fills the media and pathogenic

fungi so that the development of pathogenic fungi is suppressed in the media space. In contrast, the antibiosis mechanism occurs due to the antibiotic substances produced by endophytic fungi, characterized by the inhibition zone formed [16]. Fig. 2 shows the antagonistic activity of endophytic fungi from maize plants (*Zea mays* L.) against *Fusarium oxysporum* Schltdl.

Seven isolates were isolates that can inhibit pathogen growth by producing non-volatile compounds. In comparison, six other isolates cannot impede the development of F. *oxysporum*, in this case, proving that endophytic fungi have the effectiveness to inhibit the growth of pathogenic fungi by producing a specific metabolite. The efficacy of the inhibitory activity of endophytic fungi against other pathogens is thought to be that the isolate produces metabolite compounds that have activity as antifungal [17]. Table 1 shows the percentage inhibition of *Fusarium oxysporum* by endophytic fungi, evaluated using antifungal, non-volatile, and volatile compounds tests. Fig 3 shows the results of the antifungal non-volatile compounds test, illustrating the inhibition of *Fusarium oxysporum* growth by endophytic fungal isolates. The isolate with the highest percentage of inhibition is isolate B1.1.1, which amounted to 39.83% inhibition.

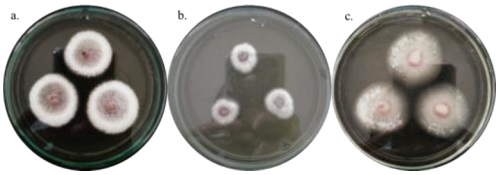

Fig. 3. Antifungal non-volatile compounds test. (a.) Control (b.) isolate B1.1.1 and (c.) isolate D2.2.1.

Table 1. Percentage inhibition of *Fusarium oxysporum* by endophytic fungi using antifungal non-volatile and volatile compounds tests

No	Isolate	Inhibition (%)	
		Antifungal non-volatile compounds test	Antifungal volatile compounds test
1	A1. 2. 1	9,72 ± 1,37	25.29 ± 16.92
2	A2. 1. 1	n.d.	43.40 ± 7.95
3	A2. 2. 1	n.d.	30.72 ± 12.02
4	A3. 2. 1	30,74 ± 6,05	18.04 ± 16.08
5	A3. 2. 2	n.d.	33.46 ± 2.96
6	B1. 1. 1	39,83 ± 2,58	39.93 ± 15.62
7	D2. 1. 1	n.d.	19.41 ± 8.60
8	D2. 1. 2	8,79 ± 6,22	41.31 ± 8.13
9	D2. 2. 1	n.d.	44.38 ± 9.34
10	D3. 1. 1	n.d.	19.41 ± 8.60
11	D3. 1. 2	n.d.	21.50 ± 8.41
12	D3. 2. 1	n.d.	18.30 ± 10.17
13	D3. 2. 2	n.d.	17.45 ± 5.82

Figure 4. shows the inhibitory effect of endophytic fungi on the growth of *Fusarium oxysporum*, measured by the reduced diameter of the fungal colony. Antagonism activity test

using the volatile compound production method aims to see whether endophytic fungi can produce a volatile compound that can inhibit the growth of pathogenic fungi. The results of the volatile compound production test showed that endophytic fungi could produce a volatile compound (metabolite) that could inhibit the growth of pathogenic fungi. Endophytic fungi inhibit the growth of pathogenic microbes through competition for space and nutrients and by producing volatile and non-volatile compounds (antibiosis) [18]. The diameter of pathogen growth was measured in the volatile compound production test. The percentage of growth inhibition was then calculated based on the antagonistic activity between endophytic fungi and the pathogenic fungus *Fusarium oxysporum* Schltdl. Figure 5 shows the electrophoresis results depicting the amplified PCR products of the ITS gene, demonstrating bands at approximately 600 base pairs in length. Identification through ITS gene amplification confirmed that isolate D2.2.1 is *Trichoderma harzianum*, with a similarity score of 97.30% based on BLAST results.

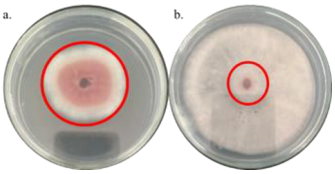

Fig. 4. Antifungal volatile compounds test. (a.) Control (b.) isolate D2.2.1. The red circle indicates the diameter of the fungal growth of *Fusarium oxysporum* Schltdl.

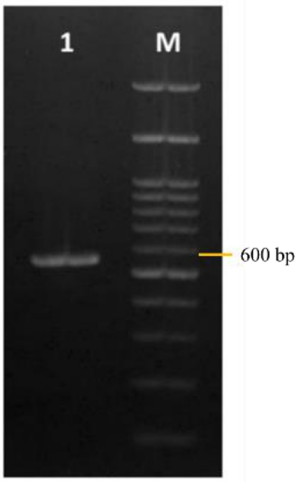

Fig. 5. Electrophoresis Results of PCR Products for ITS Gene (600 bp).

The endophytic fungus genus *Trichoderma harzianum* can inhibit the growth of the pathogenic fungus *Fusarium oxysporum* Schltdl. Because it antagonistically inhibits other pathogenic fungi by producing a volatile metabolite. *Trichoderma harzianum* can make a volatile compound that can inhibit or kill other pathogenic fungi, so *Trichoderma harzianum* fungi include endophytic fungi with high antagonism [19]. *Trichoderma harzianum* produces volatile compounds such as 6-pentyl pyrrole, viri dins, haziarnic acid, gliotoxin, kininginins, and cytosperone [20].

4 Conclusion

Endophytic fungi successfully isolated from maize plants. These fungi can inhibit the growth of the pathogenic fungus *Fusarium oxysporum* Schltdl through multiple mechanisms. Among all the fungi studied, *Trichoderma harzianum* exhibits the most potent inhibition and has the potential to be used as a biological control agent against the fungal pathogen *Fusarium oxysporum* Schltdl.

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