

Diversity of endophytic fungi from shallots as a *Fusarium oxysporum* biological control agent

Trizelia*, Haliatur Rahma, Martinius, Edo Rahman and Santia Marhamah

ABSTRACT

Fusarium oxysporum is the primary cause of fusarium wilt, one of the major diseases that harm shallots. 50% of yield can be lost due to fusarium wilt. It is anticipated that endophytic fungus will inhibit the growth of Fusarium wilt disease in shallots. The endophytic fungi from shallot plants that have the potential to act as biological control agents for the fungal disease *F. oxysporum* are suggested to be chosen in this study. Using Malt Extract Agar (MEA) medium, endophytic fungi were isolated from healthy shallot plant components (roots, tubers, stems, and leaves). Using dual culture and volatile techniques, the antagonistic potential of endophytic fungi against *F. oxysporum* was examined. Using shallot seeds, pathogenicity studies for endophytic fungus were conducted in vitro. Twelve endophytic fungal isolates were able to impede the growth of *F. oxysporum* using the dual culture method; the percentage of inhibition reached 72.52% at seven days post-inoculation. The endophytic fungus was also able to stop *F. oxysporum* mycelial growth using the volatile compound test, with a percentage inhibition ranging from 4.08 - 27.37%. The findings of the pathogenicity test demonstrated that the four isolates posed a risk to the shallot seeds. The most successful isolate in preventing the growth of *F. oxysporum* was the endophytic fungus AB4D1, which is a member of the *Trichoderma asperellum* species.

Key word: Antagonist; DNA; dual culture; *Fusarium oxysporum*; shallot; volatile

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INTRODUCTION

Allium ascalonicum L., or scallions, are a popular vegetable in Indonesia and offer a number of health advantages. The spice family, which includes onions, is used as both traditional medicinal components and food seasoning. With an average national shallot productivity of 9.24 tonnes/ha, Indonesia's shallot productivity remains low and well below its production potential of around 20 tonnes/ha (Ministry of Agriculture, 2014). Low shallot production of *F. oxysporum* wilt disease can be attributed to a number of factors, including the disease itself.

Fusarium oxysporum f. sp. *cepae* (Foc) is the culprit behind the deadly Fusarium wilt disease

that affects shallots in Indonesia. This disease reduces tuber dry weight by up to 30% during storage, results in yield losses of up to 50% in some Indonesian shallot production centers (Wiyatiningsih, 2010), and reduces the generation of shallot seeds (Misra *et al.*, 2014). The symptoms of fusarium wilt illness include fast plant wilting, root rot, drooping plants that appear ready to collapse, and visible white fungal colonies at the bulb's base (Juwanda *et al.*, 2016). Because *F. oxysporum cepae* can adapt to a wide range of harsh climatic conditions, it has proven to be a challenging fungus to control up until this point. The ability of this fungus to produce chlamydospores allows it to persist in the soil for

extended periods of time in the absence of a host plant (Sudantha, 2013). Using uncertified seeds and diseased shallot seeds can both hasten the spread of fusarium wilt disease (Sudantha *et al.*, 2018). A soil-borne fungus known as *F. oxysporum* f. sp. *cepae* is capable of infecting nearly every stage of plant growth (Cramer, 2000). Generally, crop rotation, sanitation, and insecticides are used to control this disease. But up to now, particularly in endemic areas, this control has proven challenging (Wiyatiningsih, 2010).

As controls, synthetic fungicides have been employed. The environment is negatively impacted by the ongoing use of pesticides, which makes pathogens more resilient to pesticides and leaves residues behind. This is why using natural materials requires environmentally responsible control. Using endophytic fungi that are hostile to diseases is the first.

The use of chemical fungicides is one method of disease management for shallot plants. Djafarudin (2004) and Soesanto (2008) claim that the regular application of chemical fungicides results in plant residues and the death of nontarget species. Only around 20% of chemical fungicides that are used actually reach their target; the other 80% fall to the ground and can pollute surrounding areas. Finding safer and more ecologically friendly alternatives to traditional pest control techniques is therefore essential. One is the application of pathogen-opposing endophytic fungus (Sinaga, 2009).

Endophytic fungi are able to survive by building colonies in plant tissues without damaging their hosts. They can be found in plant tissues such as leaves, flowers, fruits, or plant roots at specific times of the year (Clay, 1988). In host plant tissues that display mutualistic interactions—that is, positive interactions with their hosts and negative interactions with pests—endophytic fungi are major players, according to Azevedo (2000). Plant growth hormones, antiviral, antifungal, anticancer, and other useful chemicals are produced by endophytic fungus (Noverita *et al.*, 2009). According to Motaal *et al.* (2010), a variety of endophytic fungi generate bioactive substances that strengthen host defenses against pathogen

invasion. According to Photita *et al.* (2004), pathogen-controlling enzymes are produced with great activity by antagonistic endophytic fungi.

Endophytic fungi can defend plants against diseases through direct or indirect processes. The synthesis of antibiotic chemicals and enzymes, such as mycoparasitism, competition for nutrients and space, and antibiotics themselves, all contribute to the direct reduction of pathogen growth. The production of secondary metabolites like ethylene, salicylic acid, and jasmonic acid, which act as antimicrobials like phytoalexins or boost plant resistance to pathogen attack, is one way that indirect inhibition takes place (Gao *et al.*, 2010; Adeleke *et al.*, 2022).

Numerous researchers have documented the function of endophytic fungus in shielding plant hosts against illness. According to Suswanto *et al.* (2018), six isolates of endophytic fungus, including *Gliocladium* sp., were obtained from pepper plants (*Piper nigrum*). With regard to *Septobasidium* spp., *Aspergillus flavus*, *Aspergillus niger*, *Fusarium* spp., *Trichoderma* spp., and *T. harzianum* exhibited the maximum inhibition (59–75%) and showed no detrimental impacts on plants. According to Tondok *et al.* (2012), the endophytic fungus *Xylariaceae* and *Calocybe gambosa* that were separated from cocoa pods were able to decrease the severity of *Phytophthora palmivora*-caused cocoa pod disease by 38.8 and 33.8%, respectively. In order to determine the diversity of endophytic fungi isolated from shallots as a biological control agent for *F. oxysporum*, this study was carried out.

MATERIALS AND METHODS

How to Prepare *Fusarium oxysporum*

The *F. oxysporum* fungus that was employed was a collection from Andalas University's Faculty of Agriculture's Biocontrol Laboratory. The shallot roots yielded the fungus that was isolated. The fungus was grown for seven days in an incubator using PDA media.

Endophytic fungal isolation and purification

The shallot plantations in Air Batumbuak Village, Alahan Panjang, West Sumatra, Indonesia,

provided the shallot plants used in the isolation of endophytic fungus. Five healthy shallot plant samples, along with their soil, were collected from the field, sealed in plastic bags, and sent to the lab for additional analysis. We isolated endophytic fungi by employing the technique outlined by Hazalin *et al.* (2009). To get rid of dirt, the roots, tubers, and leaves of the shallot plant were first cleaned by running water. Next, in laminar air flow, each plant portion was chopped into five pieces at a distance of ± 1 cm. After immersing the sample pieces in 70% alcohol, 2% NaOCl, and 70% alcohol for one minute each, and rinsing them three times with distilled water, the process was repeated for one minute. Following air drying on sterile tissue, the sliced samples were put one piece at a time into a petri plate with five pieces of MEA media. The process of purification involved moving the endophytic fungi that were cultivating on MEA in a Petri dish to a Petri plate that contained PDA.

Test for antagonists

Methods involving dual cultures

A dual culture approach was used to test shallot endophytic fungi against *F. oxysporum* as antagonists. Seven-day-old endophytic and pathogenic fungi were gathered using a cork borer (0.7 cm) and arranged in pairs 3 cm apart on the PDA medium surface. Petri dishes were incubated at room temperature for seven days. The percentage of inhibition was used to assess the capacity of endophytic fungi to limit the growth of *F. oxysporum*. Using the formula below, the percentage growth inhibition (I) was determined:

$$I = [(C-T)/C] \times 100\%$$

where T is the radial growth of pathogens with endophytic fungi, C is the radial growth of the pathogen without endophytic fungi, and I is the percentage suppression of the pathogen by antagonists.

Test for volatile compounds

The ability of volatile produced by antagonistic fungi as volatile chemicals to inhibit the growth of infections was tested through the use of the volatile test method. Seven-day-old pathogenic and antagonistic fungi were separated by 0.5 cm

and positioned in the center of a petri plate with different PDA media. In addition, the two Petri dishes were positioned so that their faces were facing one another. The antagonistic fungus is displayed below, while the pathogenic fungus, *F. oxysporum*, appears at the top. The cultures were kept at room temperature until the petri dish (7 hsi) was filled with pathogenic fungi from the control.

Test for pathogenicity

An *in vitro* test of endophytic fungi's impact on shallot seeds was used to establish the fungi's non-pathogenic character. Shallot seeds were surface sterilized by soaking them in 70% ethanol for 30 seconds, 1% NaOCl for two minutes, and then three times in sterile distilled water. Next, the seeds were sown in PDA media that had been covered in pure endophytic fungal isolates that had been incubated for seven days. One week was spent incubating a total of twenty-five shallot seeds that were placed in a petri dish. On the seventh day of germination, healthy shallot seeds exhibiting necrosis were seen. Endophytic fungal isolates were gathered for additional study from endophytic fungal cultures that had no effect on shallot seed germination.

Identification of endophytic fungus

Using DNA sequences in the ITS rDNA region, endophytic fungi that possess the ability to impede the growth of the pathogenic fungus *F. oxysporum* by over 60% were subsequently identified through morphological and molecular characteristics. We acquired fungal isolates from the Jakarta Science Genetics Laboratory. Using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005), DNA was extracted from fungal isolates. Internal Transcribed Spacer (ITS) 1 (5'TCCGTAGGTGAACCTGCGG 3') and ITS-4 (5'TCCCTCCGCTTATTGATATGC 3') were the molecular markers that were employed (White *et al.* 1990). Using MyTaq HS Red Mix (Bioline BIO-25047), PCR amplification was carried out to separate the ITS 1 and ITS 4 fragments from each isolate. The ITS sequence has a 600 bp length. After being purified with a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, D4001), the

PCR products were forwarded to a Malaysian FIRST BASE sequencing company. The Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>) was used to analyze DNA sequences (Altschul *et al.*, 1997). Using phylogenetic trees made with the MEGA 5 tool, relationships between sequences and outgroup sequences were examined (Tamura *et al.*, 2011).

RESULTS

Eleven endophytic fungus isolates were shown to be able to prevent the growth of *F. oxysporum* colonies in the antagonist test conducted by the shallot endophytic fungus utilizing the multiple culture method against the pathogenic fungus *Fusarium oxysporum*. The analysis of variance revealed significant differences between the isolates in the suppression of *F. oxysporum* by endophytic isolates from shallot plants ($F = 10.33$; $df = 10.37$, $P < 0.0000$). Table 1 displays the average percentage of inhibition of 11 endophytic fungal isolates against *F. oxysporum*.

Table 1. Inhibition of 11 endophytic fungi isolates against *F. oxysporum* 7 days after incubation

Isolates	Percentage of inhibition (%) \pm SE
AB4D1	72.52 \pm 2.81 a
AB2B3	67.73 \pm 2.45 ab
AB3U4	64.62 \pm 0.78 abc
AB3U3	58.55 \pm 2.08 bcd
AB5A1	56.67 \pm 5.45 bcde
AB5U2	55.48 \pm 0.74 bcde
AB2D1	53.68 \pm 1.13 cde
AB5A2	53.07 \pm 1.63 cde
AB2B1	48.23 \pm 2.60 de
AB1B6	48.13 \pm 1.62 de
AB1A1	45.03 \pm 3.71 e

*Means of inhibition at the same time, followed by the same letter are not significantly different, according to Tukey Test ($P \leq 0.05$).

The Table 1 displays those eight endophytic fungal isolates exhibited an inhibition of greater than 50%, whereas three isolates exhibited an inhibition

of less than 50%. The isolates from AB4D1 exhibited the maximum inhibition (72.52 %), whereas the isolates from AB1A1 exhibited the lowest inhibition (45.03 %). Figure 1 shows the outcomes of monitoring the inhibition of every endophytic fungal isolate (7 days).

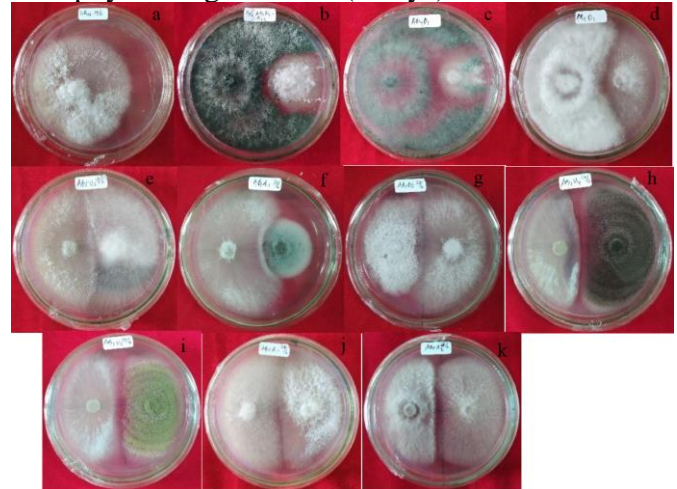


Figure 1. Antagonistic test of endophytic fungal isolates against *F. oxysporum* in dual culture methods at 7 DAI (a= control (without endophytic fungus), b-k= using endophytic fungus)

Test for volatile compounds

The volatile compounds produced by 11 isolates of endophytic fungi were found to be able to inhibit the growth of pathogenic fungus in the antagonist test of shallot endophytic fungi against *F. oxysporum*, as determined by the volatile compound test method. Different isolates showed different levels of suppression of volatile chemicals produced by endophytic fungi ($F = 10.33$; $df = 8.73$, $P < 0.0000$). When *F. oxysporum* colonies were observed seven days after incubation, the growth inhibition ranged from 4.8 to 27.38 (Table 2). Figure 2 depicts the mycelial growth of *F. oxysporum* in the treatment and control groups.

Test for pathogenicity

The pathogenicity test of endophytic fungi on shallot seed germination revealed a range of responses, some of which were necrotic in the sprouts, some of which were fatal to the seeds, and some of which were infected after the seeds germinated. Only three of the 11 endophytic fungal isolates were found to be

non-pathogenic, whereas eight of the isolates were pathogenic to shallot seeds according to the pathogenicity test.

Table 2. Percent of inhibition activity on mycelial growth of *F. oxysporum* produced by volatile compounds of endophytic fungi from shallot

Isolate	Mycelial growth inhibition (%)± SE
AB4D1	27.38± 3.86 a
AB2B3	22.32± 3.87 ab
AB2B1	22.05± 3.77 ab
AB5A1	15.55± 1.30 abc
AB5A2	13.65± 1.45 bc
AB3U4	10.38± 0.94 bc
AB2D1	10.30± 2.18 bc
AB3U3	8.85± 3.25 c
AB5U2	7.33±0.99 c
AB1B6	6.34± 0.55c
AB1A1	4.80± 3.16 c

The results in Table 2 show that isolate AB4D1 had the highest inhibitory ability (27.38%), while isolate AB1A1 produced the lowest inhibition of only 4.80%.

The three isolates, AB3U3, AB2B3, and AB4D1, did not produce necrosis in shallot sprouts or inhabit the germination of shallot seeds. Figure 2 shows onion seed germination on medium harboring endophytic fungi.



Figure 2. Onion seed germination on media containing endophytic fungi A=AB3U3, B=AB4D1 and C= AB2B3)

Identification

Using the ITS1/ITS4 primer pair, endophytic fungi that are not harmful to shallot seeds but have the ability to limit the growth of the pathogenic fungus *F. oxysporum* by more than 60% were further identified molecularly. A band measuring roughly 600 bp is produced by the endophytic fungal DNA amplification (Figure 3).

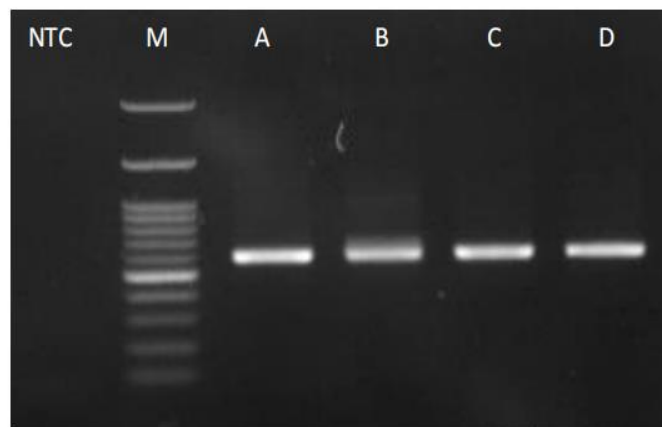


Figure 3. Bands of purified PCR products at 600 bp. A. AB4D1, B. ABAU3, C. AB2B3, D. PC21. M = Marker ladder 100 bp

Following ITS region amplification with the universal primer ITS1/ITS4, all isolates produced 600 bp DNA fragments. Table 5 displays the outcomes of the nucleotide base alignment with the NCBI website database. Three isolates (AB4D1, AB2B3, and PC21) have a similarity level of 98.37 - 98.68% with the *Trichoderma asperellum* fungus group, whereas one isolate (ABAU3) has a similarity rate of 98.43% with the *Aspergillus flavus* fungus.

Figures 4 and 5 illustrate the phylogenetic relationship among the collected isolates. AB4D1, AB2B3, and PC21 constitute a distinct group and have a tight relationship with the *T. asperellum* group in comparison to *T. viride* and *T. hamatum*. Schuster and Schmoll (2010) claim that *Trichoderma* sp. is a soilborne fungus that is globally distributed, resulting in close family relationships between isolates from different nations. Comparing *Apergillus niger*, *A. tamarrii*, and *A. nomius* to *Apergillus flavus*, however, reveals a tight link between the isolate ABAU3.

Discussion

Petrini (1991) defined endophytic fungi as those that reside in plant organs and either fully or partially invade plant tissues internally, all without harming their hosts. According to Stone *et al.* (2004), higher plants have multiple layers that make up the body structure of the plant.

Table 5. Level of similarity of endophytic fungi isolates from shallot plants based on BLAST analysis

Isolate Code	Sequence Length (bp)	Padanan Species	Similarity (%)	No Accession
AB4D1	610	<i>Trichoderma asperellum</i> strain PK1J2	98.7	OK336355.1
AB2B3	616	<i>Trichoderma asperellum</i> isolate T20	98.52	MK210235.1
PC21	604	<i>Trichoderma asperellum</i> strain IIPRCPT-100	98.68	MK841027.1
AB3U3	889	<i>Aspergillus flavus</i> strain 20	98.43	MK120614.1

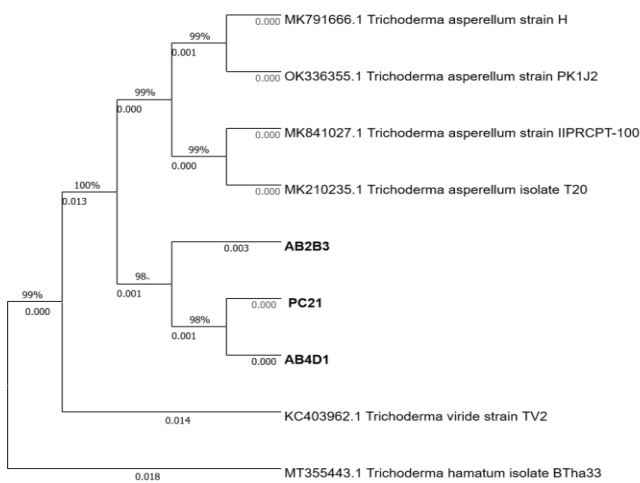


Figure 4. Phylogenetic tree between *Trichoderma asperellum* isolates and relationship species from NCBI

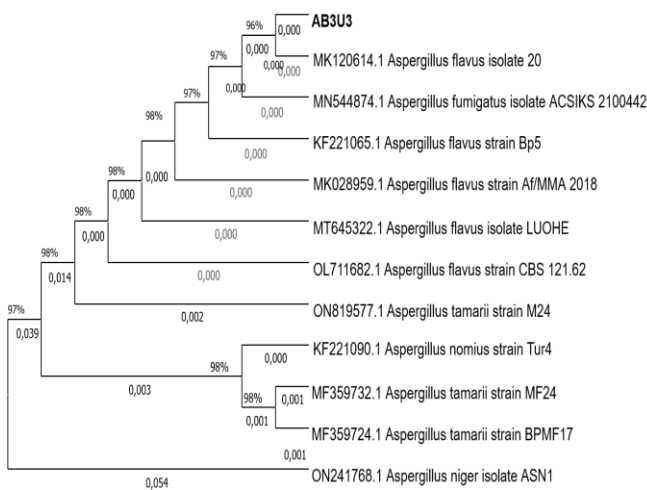


Figure 5. Phylogenetic tree of *Aspergillus flavus* isolates with relationship species from NCBI

These plants have a variety of environments that are home to assemblages of different microorganism species. One of the main elements of the assemblage are fungi, which come in many varieties: epiphytes, which colonize the surface of leaves and twigs; endophytes, which live inside leaves; bark endophytes; and decomposers of wood, which are found in xylem endophytes and wood decomposers. The fact that endophytic fungi have colonized healthy plants' interior tissue provides fresh information, even though the relationship between the two is still unclear. Higher plants are thought to function as these fungi's anchorages, much like harbors. A comparison of the traits of endophytic fungi on broad-leaf and narrow-leaf hosts was also provided by Stone *et al.* (2004). According to Faeth (2002), endophytic fungi have systemic colonization in grass, particularly when they are in the asexual phase. This type of plant mutualism is demonstrated by the alkaloids in endophytic fungal-infected grass and the mycotoxins the fungi produce, which shield the host plants against predators. According to Rodriguez *et al.* (2009), endophytic fungus and all plants share a symbiotic relationship in natural environments. Numerous fungal species have a significant influence on plant communities by enhancing plant health through increased biomass, decreased water consumption, and tolerance to biotic and abiotic challenges. The antagonist test results demonstrated that eleven endophytic fungal isolates reduced the growth of *F. oxysporum* colonies. An inhibitory zone that developed at the meeting point of the

two fungal colonies demonstrated the isolates' antagonistic potential. Eight endophytic fungal isolates showed greater than 50% inhibition. Because antagonistic fungus may create organic acids that pathogenic fungi cannot use, antagonistic mycelia are typically larger than pathogenic mycelia. Furthermore, antagonistic fungi have the capacity to produce antibiosis, which prevents the growth of harmful fungi (Suwahyono, 2008). The degree of inhibition is brought about by its capacity to outcompete other organisms for resources and space, allowing it to develop swiftly and preventing *F. oxysporum* from proliferating. The variations in the inhibition zone values show how differently each isolate may stop the spread of harmful microbes (Suanda and Ratnadi, 2015). The size of the inhibition zone that endophytic fungi isolates form against pathogenic fungi depends on a number of factors, including the interaction between the hydrolytic enzyme production capacity of *Trichoderma* isolates, the age of the fungal culture, the quantity of enzymes produced, the medium composition, and the duration of incubation.

The growth of harmful fungus is inhibited by antagonistic agents such as parasitism, antibiosis, and competition. The suppression of one organism by the metabolites of another is known as antibiosis. This is because many plant parasite fungi are toxic to antibiotics, which include aldehydes, alcohols, acetones, organic acids, and volatile and non-volatile compounds produced by microbes (Fries, 1973; Fokema, 1976, Bashar and Rai, 1994, and Akter *et al.*, 2014). According to Bashar and Chakma (2014), the growth of *F. oxysporum* was suppressed by volatile compounds produced by *T. viride*, *A. niger*, *A. flavus*, and *A. fumigatus* by 297.5%, 20.15%, 15.78%, and 12.25%, respectively. The growth of the *F. oxysporum* mycelium was suppressed by the volatile metabolites produced by isolates *A. flavus*, *A. niger*, *A. fumigatus*, and *T. viride* by 18.00%, 14.50%, 12.00%, and 10.25%, respectively, according to Aktar *et al.* (2014). Additionally, Aktar *et al.* (2014) observed that the growth of

Curvularia lunata mycelium was suppressed by 20.86 and 14.85%, respectively, by volatile metabolites produced by isolates of *T. viride* and *A. niger*. The variations in the organisms participating in the interactions are most likely the cause of the variation in % inhibition seen in this investigation.

The isolates with codes AB4D1, ABAU3, AB2B3, and PC21 are the results of the identification of endophytic fungi that are not harmful to shallot seeds and have the ability to limit the growth of the pathogenic fungus *F. oxysporum* by more than 60%. One isolates was identified as *A. flavus* and three isolates as *T. asperellum*. The *T. asperellum* fungus has been extensively documented. The fungus *Trichoderma* sp. demonstrated another antagonistic activity, which is the mechanism of antibiosis, at both the macro and microscales. While the microscopic examination of the lysis of *Fusarium* sp. fungal hyphae reveals an antagonistic zone between the harmful and friendly fungi, the macroscopic mechanism entails the formation of an inhibitory zone. β -1,3-glucanase, chitinase, cellulase, and proteinase are among the enzymes that *Trichoderma* spp. manufacture (Kumar *et al.*, 2012). According to Dendang (2015), *Trichoderma* is also known to create chemicals that act as antibiotics, such as enzymes that break down cell walls. Nugroho and Wahyudi's (2000) research revealed that the mechanism underlying this antibiosis is connected to the antagonistic fungus in the *Trichoderma* genus' capacity to generate more potent enzymes, such as *T. asperellum*'s greater volatile chemicals. According to Nugroho and Wahyudi (2000), *Trichoderma* sp. is able to penetrate other fungi's hyphae because it produces the hydrolytic enzymes β -1,3 gluconase, chitinase, and cellulase, which may break down other fungal cells, which are primarily made of β -1,3 glucone and chitin. Many extracellular enzymes, including protease, pectinase, invertase, and amylase, are produced by *A. niger* and have the ability to stop the growth of pathogenic fungus (Soesanto, 2013; Nurbailis *et al.*, 2015). *Aspergillus* species produce mycotoxins, like

aflatoxins and ochratoxins, function as antibiotics by preventing the growth of pathogens. By inhibiting the growth of the pathogenic fungus *F. oxysporum*, varying culture ages can influence the pace of *A. niger* growth (Sarah et al., 2018).

Eleven endophytic fungal isolates were able to inhibit the growth of *F. oxysporum* using the dual culture method. At seven days post-inoculation, the percentage of inhibition reached 72.52%. The endophytic fungus was also able to stop *F. oxysporum*'s mycelial growth using the volatile compound test, with an inhibition percentage ranging from 4.08 to 27.37%. The findings of the pathogenicity test demonstrated that the four isolates posed a risk to the shallot seeds. The most successful isolate in preventing the growth of *F. oxysporum* was the endophytic fungus AB4D1, which is a member of the *Trichoderma asperellum* species.

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