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Antifungal activity of ethyl acetate extract of *Trichoderma harzianum* against *Fusarium* sp.

Aktivitas antijamur ekstrak etil asetat *Trichoderma harzianum* terhadap *Fusarium* sp.

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ABSTRACT

Fusarium sp. is one of the main pathogens causing wilt disease in chili plants (*Capsicum annuum* L.) which can cause a significant decrease in production. Control of this disease still relies heavily on synthetic fungicides that have the potential to cause pathogen resistance and negative impacts on the environment. Therefore, an alternative biological control is needed, namely with *Trichoderma harzianum*. This study aims to test the antifungal activity of ethyl acetate extract of *T. harzianum* against Fusarium sp. in vitro. *T. harzianum* isolates were incubated with Potato Dextrose Broth (PDB) medium for nine days, then their secondary metabolites were extracted using ethyl acetate solvent and evaporated to obtain a crude extract. Antifungal activity was tested using the disc diffusion method against *Fusarium* sp. suspensions with a concentration of 1×10^7 spores/mL. The treatments used included ethyl acetate extract of *T. harzianum* (10 mg/mL), natacen as a positive control, aquadest as a negative control, and ethyl acetate as a solvent control. The results showed that the ethyl acetate extract of *T. harzianum* was able to inhibit the growth of *Fusarium* sp. with an average inhibition zone diameter of 17 mm. Meanwhile, natacen produced an inhibition zone of 35.5 mm, while the negative control and solvent control showed no inhibitory activity. These results indicate that the secondary metabolites contained in the ethyl acetate extract of *T. harzianum* possess antifungal activity against *Fusarium* sp. and have the potential to be developed as a safer and more sustainable biological control agent..

Keywords: antifungal activity, biological control, ethyl acetate, Fusarium *sp.,* Trichoderma harzianum

INTISARI

Fusarium sp. adalah salah satu patogen utama yang menyebabkan penyakit layu pada tanaman cabai (*Capsicum annuum* L.) yang dapat menyebabkan penurunan produksi yang signifikan. Pengendalian penyakit ini masih sangat bergantung pada fungisida sintetis yang berpotensi menyebabkan resistensi patogen dan dampak negatif terhadap lingkungan. Oleh karena itu, diperlukan pengendalian biologis alternatif, yaitu dengan *Trichoderma harzianum*. Studi ini bertujuan untuk menguji aktivitas antijamur ekstrak etil asetat *T . harzianum* terhadap *Fusarium* sp. secara in vitro. Isolat *T. harzianum* diinkubasi dengan medium *Potato Dextrose Broth* (PDB) selama sembilan hari, kemudian metabolit sekundernya diekstraksi menggunakan pelarut etil asetat dan dievaporasi untuk mendapatkan ekstrak kasar. Aktivitas antijamur diuji menggunakan metode difusi cakram terhadap suspensi *Fusarium* sp. dengan konsentrasi 1×10^7 spora/mL. Perlakuan yang digunakan meliputi ekstrak etil asetat dari *T. harzianum* (10 mg/mL), natacen sebagai kontrol positif, aquadest sebagai kontrol negatif, dan etil asetat sebagai kontrol pelarut. Hasilnya menunjukkan bahwa ekstrak etil asetat dari *T. harzianum* mampu menghambat pertumbuhan *Fusarium* sp. dengan diameter zona hambatan rata-rata 17 mm. Sedangkan,

natacen menghasilkan zona penghambatan sebesar 35,5 mm, kontrol negatif dan kontrol pelarut tidak menunjukkan aktivitas penghambatan. Hasil ini menunjukkan bahwa metabolit sekunder yang terkandung dalam ekstrak etil asetat *T . harzianum* memiliki aktivitas antijamur terhadap *Fusarium* sp. dan berpotensi dikembangkan sebagai agen pengendalian hayati yang lebih aman dan berkelanjutan.

Kata kunci: aktivitas antijamur, pengendalian biologis, etil asetat, Fusarium sp., Trichoderma harzianum

INTRODUCTION

Chili Peppers (*Capsicum annuum* L.) is one of the strategic horticultural commodities that has high economic value and plays an important role in meeting the food needs of the Indonesian people. Chili production in Indonesia has shown a fluctuating trend in recent years. Based on data from the Badan Pusat Statistik (BPS) Indonesia, Indonesia's large red chili production in 2023 will reach 675.02 tons, decreasing to 600.42 tons (BPS, 2025). In 2024, inflation of red chili commodities is recorded to increase to more than 20% in several consumption center areas due to a decline in crop supply that affects the stability of national food prices and people's purchasing power (Bank Indonesia, 2024). This decline in chili production indicates a serious problem in chili cultivation, one of which is caused by plant disease attacks.

Fusarium sp. is one of the most destructive pathogens in *Capsicum* spp. cultivation that causes fusarium wilt disease with yield losses that can reach 10% to 80% depending on the variety, environmental conditions, and control strategies used (Tilahun et al*.,* 2026). Research by Febriana et al. (2024) reported that the intensity of *Fusarium* attacks on chili plants in several Indonesian agricultural centers reached more than 40%, especially in the rainy season. The high rate of attacks shows that fusarium wilt disease is still one of the main limiting factors in chili production.

Fusarium wilt disease control has so far relied heavily on synthetic fungicides. However, the continuous use of fungicides can cause various problems, such as chemical residues in soil and water, the emergence of resistant pathogenic strains, and negative impacts on human health and ecosystem balance (Febriana et al., 2024). In addition, *Fusarium* sp. has the ability to survive in the soil for a long period of time, so control using chemical fungicides is often less effective if done repeatedly. Therefore, the development of a more environmentally friendly and sustainable control strategy is very important.

One of the widely developed alternatives is the utilization of biological agents from the group of antagonistic fungi, specifically *Trichoderma harzianum* (Haque et al., 2023). This fungus has been widely reported to be able to suppress *Fusarium* growth through various mechanisms, such as space and nutrient competition, antibiotics, hyperparasitism, and the induction of plant resistance (Sharma & Singh, 2020). In addition, *T. harzianum* has the ability to produce a variety of secondary metabolites with high antimicrobial and antifungal activity. Research by Guzmán-Guzmán et al. (2023) shows that the secondary metabolites produced *by T. harzianum* include terpenoids, polyketidades, alkaloids, peptaibols, steroids, and volatile compounds that play a role in inhibiting the growth of plant pathogens.

Trichoderma harzianum is one of the most effective biocontrol agents against various plant diseases caused by pathogenic fungi, especially *Fussarium* sp. (Petrucci et al., 2023). Research by Ihkwanisa *et al*. (2023) shows that *Trichoderma* spp. is able to inhibit the growth of *Fusarium oxysporum* by up to 58.33% using the dual culture method. However, most previous studies have

focused on filtrate culture tests or dual culture methods, while studies on the antifungal activity of secondary metabolites extracted using organic solvents against *Fusarium* spp. are still relatively limited. In fact, the secondary metabolites produced by each *T. harzianum* isolate can differ depending on the strain, fermentation conditions, and extraction method used.

Ethyl acetate is a semi-polar solvent that is widely used in the extraction of secondary metabolites of fungi because it is able to dissolve various bioactive compounds with a wide polarity range and is easily separated through the process (Wakhidah & Anggarani, 2021). Research by Woo et al. (2023) shows that *Trichoderma* ethyl acetate extract contains a variety of bioactive compounds, such as peptaibol, terpenoids, and other volatile compounds that have the potential to be antifungal agents. These compounds are known to be able to interfere with cell membrane permeability, inhibit the growth of hyphae, and interfere with the physiological processes of pathogenic fungi. In addition, Vinale et al. (2014) reported that ethyl acetate extract *of T. harzianum* contains the compounds 6-pentyl-2H-pyran-2-one (6-PP), anthraquinone, and sterols that have antifungal activity against various plant pathogens.

Although the potential of secondary metabolites of *T. harzianum* has been widely reported, studies on the antifungal activity of *T. harzianum* ethyl acetate extract against *Fusarium* sp., particularly using the disc diffusion method as an approach to evaluate the activity of its secondary metabolites directly, are limited. Therefore, this study was conducted to test the antifungal activity of *T. harzianum* ethyl acetate extract against *Fusarium* sp. in *vitro*. The results of this study are expected to provide scientific information on the potential of secondary metabolites *of T. harzianum* as a safer, more effective, and sustainable biological control agent to support environmentally friendly chili cultivation.

MATERIALS AND METHOD

Materials

The tools used in this study include aluminum foil, analytical scale, autoclave, beaker glass, caliper, centrifugal tube, centrifuge, filter paper, hemocytometer, hotplate magnetic stirrer, laminar air flow (LAF), micropipette, microscope, microtube, paper disk, petri dish, plastic wrap, rotary evaporator, vortex.

The materials used include 70% alcohol, ethyl acetate, *Fusarium* sp., Potato Dextrose Agar (PDA) media, Potato Dextrose Broth (PDB) media, natacen, streptomycin, sterile aqueducts, and *Trichoderma harzianum*.

Preparation of PDA and PDB Media

Potato Dextrose Agar (PDA) media is made by dissolving 1.95 g of PDA powder into 50 mL of aquades, then heated and homogenized using a magnetic stirrer. The media is sterilized using an autoclave at 121°C for 15 minutes. After the medium has cooled, the antibiotic streptomycin is added aseptically to prevent bacterial contamination. Next, the media is poured into a sterile petri dish and allowed to solidify in the laminar air flow (LAF). Potato Dextrose Broth (PDB) media is made by dissolving 1.2 g of PDB powder into 50 mL of aquades, then sterilized using an autoclave at 121°C for 15 minutes.

Rejuvenation and Characterization of Isolates of *T. harzianum* and *Fusarium* sp.

Isolates *of T. harzianum* and *Fusarium sp.* placed in the center of a petri dish containing PDA media using an ose needle. This process is carried out

inside the LAF to prevent contamination. Next, the petri dish is closed and wrapped, then incubated at 25°C-28°C for 4-7 days. Morphological characterization was carried out macroscopically based on colony color, texture, growth pattern, and pigmentation to confirm the characteristics of each isolate.

Fermentation of *T. harzianum* Isolate

Mycelium *T. harzianum* that had grown on PDA media was inoculated into 5 mL of PDB media and incubated for 3 days as a starter culture. Furthermore, the starter culture was transferred into a sterile 45 mL PDB media and re-incubated statically for 6 days at room temperature so that the total fermentation lasted for 9 days. The fermentation process is carried out to obtain secondary metabolites produced during the growth of the fungus.

Extraction of *T. harzianum* Isolate

Extraction of secondary metabolites is carried out using ethyl acetate solvents referring to Philip et al. (2025) with some modifications. Fermented cultures *T. harzianum* filtered using filter paper to separate the mushroom biomass from the filtrate containing extracellular metabolites. The filtrate is then centrified at a speed of 3500 rpm for 5 minutes to remove the remaining biomass and obtain a clearer supernatant. A total of 100 mL of supernatant was extracted using 100 mL of ethyl acetate and homogenized using magnetic stirrer for 10 minutes until two phases are formed, namely the water phase and the organic phase. The organic phase is then separated, while the water phase is extracted again using 70 mL of ethyl acetate with the same procedure. All organic phases obtained from the two extraction stages were combined to obtain a liquid extract of 151.5 mL. The extract is then evaporated using rotary ovaporator at 42°C and 80 rpm for 30 minutes until a coarse extract of ethyl acetate is obtained.

Manufacture of *Fusarium* sp. Isolate Spore Suspension.

Fusarium sp. suspension It is made by diluting mushroom cultures using sterile aqueducts and then homogenizing using vortex. The density of the spores was calculated using a hemocytometer under a microscope based on the method of Gabriel and Riyatno (1989). The concentration of spores is calculated using the formula:

$$C = \frac{t}{n} \times \frac{1}{4} \times 10^1 \times 10^6$$

Information:
C: Density of spores per ml of solution
t: Total number of spores in the observed box
n: Number of sample boxes
1/4: Volume of space
10^1: Dilution
10^6: Constant

The suspension was then diluted using sterile aqueducts until it reached a working concentration of 1 x 10^7 spores/mL before being used in antifungal activity testing.

Antifungal Activity Test *Fusarium* sp.

Antifungal activity of ethyl acetate extract of *T. harzianum* against *Fusarium* sp. tested using the disc diffusion method referring to Pranatayana &

Khalimi (2023). A total of 1 mL of *Fusarium* sp. suspension with a concentration of 1 x 10^7 spores/mL was inoculated evenly on PDA media using the spread plate method. Sterile 8 mm diameter paper discs were impregnated with 20 µL of each treatment consisting of ethyl acetate extract of *T. harzianum* (10 mg/mL), commercial natacen as positive control, aquades as negative control, and ethyl acetate as solvent control. Next, the paper disk is placed on the surface of the PDA media that has been inoculated with *Fusarium* sp. and incubated at 25–28°C for 72 hours. Antifungal activity is determined based on the diameter of the barrier zone formed around the paper disk and measured using a caliper. Measurements were made against the total diameter of the clear zone including the diameter of the paper disk.

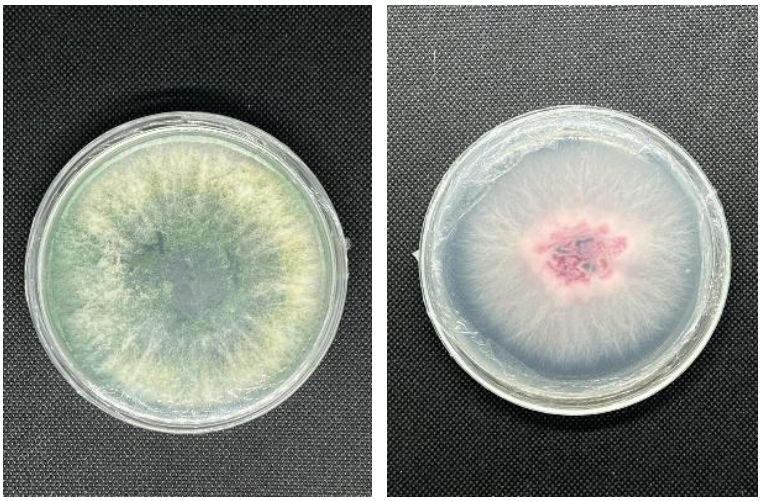
Data Analysis

The study consisted of four treatments with two biological replicas in each treatment. The diameter data of the barrier zone is presented descriptively in the form of an average value (mm).

RESULTS AND DISCUSSION

Morphological Characteristics of Isolate

Morphological characterization of *T. harzianum* and *Fusarium sp. isolates* was performed macroscopically on PDA media for seven days of incubation. The results of the observations showed that the two isolates had different colony characteristics and corresponded to the typical morphology of each genus. The *isolate of T. harzianum* shows rapid colony growth with the initial mycelium white and a cotton-like texture. As the incubation time increases, the middle of the colony turns green due to the formation of conidae, while the yellowish-white mycelium spreads to cover the entire surface of the medium. On the 7th day, the colony forms a concentric pattern resembling a ring with an increasingly intense green color in the center (Figure 1a). These characteristics correspond to the morphological description of *T. harzianum* which generally has a white mycelium in the early stages of growth and produces green conidia when it reaches the sporulation phase Guzmán-Guzmán et al. (2023).



a b

Figure 1. Colony morphology of *Trichoderma harzanium* (a) and *Fusarium* sp. (b) grown on Potato Dextrose Agar (PDA) media after 7 days of incubation.

Meanwhile, *Fusarium* sp. *isolates* form cotton-like textured colonies with a white color that then develop to pink to purplish in the middle of the colony. After seven days of incubation, the growing colony fills the surface of the PDA media and exhibits a distinctive purplish-pink pigmentation (Figure 1b). These characteristics correspond to the macroscopic morphology of *Fusarium* sp. which generally produces white to pink colonies with purple pigmentation on PDA media (Mugisho et al*.,* 2026).

Fermentation Results of *T. harzianum* Isolate

The fermentation of *T. harzianum* isolates aims to obtain secondary metabolites that have the potential to have antifungal activity. The medium used in the fermentation process is Potato Dextrose Broth (PDB), which is a liquid medium that is rich in carbon and nutrient sources to support the growth of fungi. The use of liquid media such as PDB is known to support mycelium growth and secondary metabolite production because nutrients are distributed more homogeneously than solid media l (Guzmán-Guzmán et al., 2023).

The fermentation process is carried out for nine days at room temperature. On days 1 to 3, *T. harzianum* cultures show the beginning of growth which is characterized by the formation of white mycelium. Furthermore, on the 4th to the 7th day, the mycelium grows denser and the color of the culture changes to dark green due to the formation of conidia. The rapid growth of mycelium in this period indicates that *T. harzianum* is in a phase of active growth characterized by high metabolic activity and biomass formation.

On day 8 to day 9, mycelium growth begins to slow down and shows no significant changes compared to the previous day. This condition indicates that the culture is starting to enter the stationary phase due to the reduced availability of nutrients in the media. The stationary phase in fungi is generally associated with increased production of secondary metabolites in response to nutrient limitations and changes in environmental conditions (Guzmán-Guzmán et al., 2023).

Ethyl Acetate Extraction *T. Harzianum*

The extraction process begins with filtering the fermented *culture of T. harzianum* using filter paper to separate the mycelium biomass from the liquid medium. The filtered filtrate is then centrifuged until a clearer supernatant is obtained. A total of 100 mL of supernatant was extracted using ethyl acetate through two extraction stages so that an organic phase of 151.5 mL was obtained. The formation of the organic phase shows that the secondary metabolites produced by *T. harzianum* are successfully partitioned into ethyl acetate solvents. Ethyl acetate is a semi-polar solvent that is effectively used to extract various secondary metabolites of fungi, such as terpenoids, peptaibols, alkaloids, and other bioactive compounds that have the potential to have antifungal activity (Woo et al., 2023).

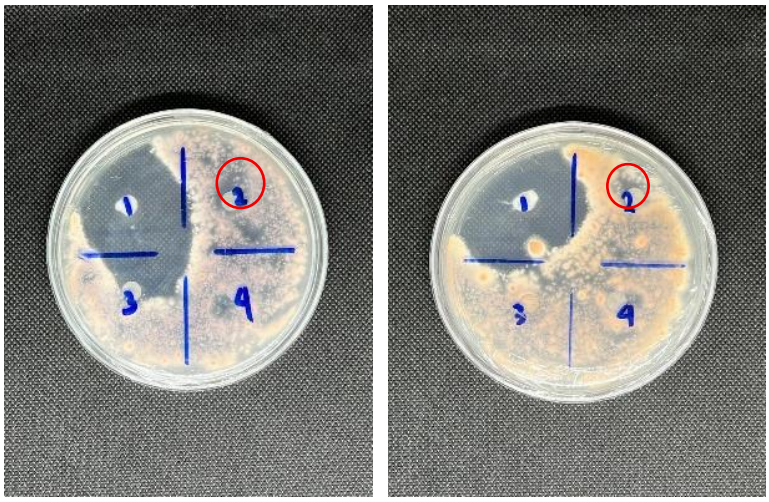
Evaporation of Ethyl Acetate *T. Harzianum*

The evaporation process is carried out to separate the ethyl acetate solvent from the extract so that secondary metabolites are obtained in a more concentrated form. Evaporation using a rotary evaporator at a temperature of 42°C yielded 10.307 g of coarse extract. Subsequently, the extract was dried at room temperature for three days until 10.285 g of dry extract was obtained. The relatively small difference in mass indicates that most of the solvents have been successfully removed during the evaporation process using a rotary evaporator.

Advanced drying aims to obtain a more stable extract and minimize the presence of residual solvents that can affect the results of antifungal activity tests (Sasidharan et al., 2011).

Antifungal Activity Test Results

The results of the test of ethyl acetate extract *of T. harzianum* against *Fusarium* sp. showed the presence of antifungal activity against *Fusarium* sp. This is shown by the formation of a clear zone around the disc paper that has been given the extract can be seen in (Figure).



a b

Figure 2. Antifungal activity of the ethyl acetate extract from *Trichoderma harzianum* against *Fusarium* sp. after 72 hours of incubation. (a) Replicate 1 and (b) Replicate 2.

The results of the measurement of the inhibition diameter of ethyl acetate extract of *T. harzianum* against the fungus *Fusarium* sp. Each can be seen in Table 1.

Table 1. Inhibition diameter of ethyl acetate extract of *T. harzianum*

Treatment	Diameter of clear zone around disc (mm)		
	R1	R2	Average
Natacen (K+)	38	33	35.5
Aquadest (K-)	0	0	0
Ethyl acetate extract *T. harzianum*	17	17	17
Ethyl acetate solvent (K solvent)	0	0	0

Based on the results of the measurement of the inhibition zone diameter, ethyl acetate *extract of T. harzianum* was proven to be able to inhibit the growth of *Fusarium* sp. which was shown by the formation of clear zones around the treatment disc. The average diameter of the inhibition zone produced by ethyl acetate *extract of T. harzianum* was 17 mm, while the positive control of natacen resulted in an inhibition zone of 35.5 mm. Meanwhile, negative control in the

form of aqueduct and ethyl acetate solvent control did not indicate the formation of an inhibition zone. These results showed that the inhibiting activity that occurred came from the secondary metabolites contained in *T. harzianum* extract, not from the solvent used.

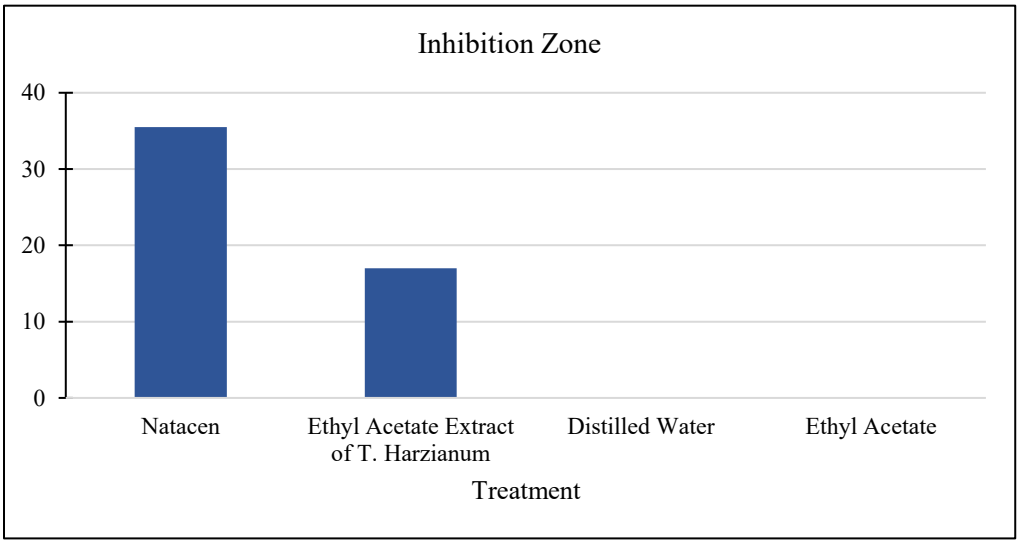


Figure 3. Inhibition zone diameters produced by *Trichoderma harzianum* ethyl acetate extract and control treatments against *Fusarium* sp. after 72 h of incubation.

The ability of *T. harzianum* ethyl acetate extract in inhibiting the growth of *Fusarium* sp. is thought to be related to the content of secondary metabolites that are antifungal. Various studies report that *T. harzianum* ethyl acetate extract contains a variety of bioactive compounds, such as peptaibol, terpenoids, volatile compounds, as well as harzianic acid derivatives that play a role in antagonistic activity against pathogenic fungi (Staropoli et al., 2023). These compounds are known to be able to inhibit the growth of fungi through various mechanisms, including disrupting the integrity of cell membranes, inhibiting the formation of hyphae, and disrupting the metabolic processes necessary for pathogen growth.

The diameter of the inhibition zone of 17 mm indicates that ethyl acetate extract *of T. harzianum* has a fairly strong antifungal activity against *Fusarium* sp. Although these values are still lower than natacen as a commercial fungicide, these results suggest that the secondary metabolites produced *by T. harzianum* have potential as biological control agents. The difference in inhibition between extracts and natacen can be caused by the nature of the extract which is still in the form of a coarse extract so that the content of the active compound has not been optimally concentrated. In addition, the concentration of the extract used in this study of 10 mg/mL may not have been able to produce maximum inhibiting activity.

The results of this study are in line with the research of Yassin et al. (2021) which reported that ethyl acetate extract *of T. harzianum* produced an inhibition zone of 17.56 mm against *Fusarium chlamydosporum*. This value is relatively close to the results of this study which resulted in an inhibition zone of 17 mm against *Fusarium* sp. In addition, Lakhdari et al. (2023) reported that *T. harzianum ethyl acetate extract* was able to inhibit the growth of *Fusarium solani* by up to 51.48%. The similarity of these results suggests that the secondary metabolites produced *by T. harzianum* have consistent antifungal activity against different species of the genus *Fusarium*.

The antifungal activity of ethyl acetate *extract of T. harzianum* in this study shows its potential use as an environmentally friendly biocontrol agent. The use of secondary metabolites from *T. harzianum* has the potential to be an alternative or complement to the use of synthetic fungicides in the control of fusarium wilt disease in chili plants. Further development through optimization of fermentation conditions, increased extract concentrations, and identification of specific active compounds is needed to increase effectiveness and support their use in sustainable farming systems.

CONCLUSION

This study shows that ethyl acetate extract *Trichoderma harzianum* has antifungal activity against *Fusarium* sp. by *In vitro* which is characterized by the formation of an inhibition zone in the disc diffusion method. Ethyl acetate extract *T. harzianum* It produced an average diameter of 17 mm in the inhibition zone, while Natacen as a positive control produced an inhibition zone of 35.5 mm. Meanwhile, negative controls in the form of acuades and ethyl acetate showed no inhibitory activity. The results showed that the secondary metabolites contained in ethyl acetate extract *T. harzianum* potential as a biological control agent against *Fusarium* sp. and has relatively strong antifungal activity.

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