Antagonistic activity of *Trichoderma harzianum* against *Aspergillus parasiticus* and *Mucor circinelloides* in corn plant (*Zea mays* L.)

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ABSTRACT

Aspergillus parasiticus, the causative agent of *Aspergillus* cob rot, and *Mucor circinelloides*, which causes *Mucor* stem rot, are types of pathogenic fungi that have the potential to reduce the quality of corn (*Zea mays* L) harvests. An effective and environmentally friendly approach to control these pathogens is to use biological agents, such as *Trichoderma harzianum*. This study aims to evaluate the inhibitory potential of *T. harzianum* against *A. Parasiticus* and *M. circinelloides* using antagonism tests with dual culture methods in vitro. The parameters used in this study were measurements of the percentage of the inhibition rate of the biological agent *T. harzianum* against pathogenic fungi. The results of the antagonism test showed that the biological agent *T. harzianum* had moderate inhibition against *A. parasiticus* and *M. circinelloides* with the percentage of each inhibition during the five days incubation period are 32.5% and 42.38%. This indicates that *T. harzianum* has the potential as a biological agent in controlling *A. parasiticus* and *M. circinelloides* in corn plants.

Keywords: Biocontrol; Cob rot; Fungal disease; Stem rot

INTRODUCTION

Corn is a staple food and has the potential to become a strategic commodity that plays an important role in increasing income in Indonesia. Plant Disturbing Organisms (PESTs) are one of the main obstacles in efforts to maintain and improve the quality of corn crop productivity (Lestari et al., 2021). One of the pests that often attack are disease-causing microbes that can affect the production of corn plants, such as pathogenic fungi (Suriani et al., 2021). Pathogenic fungi such as *Aspergillus parasiticus* and *Mucor circinelloides* are major fungal pathogens that decrease corn productivity (Sari et al., 2024).

A. parasiticus is a type of fungus that often contaminates cereals such as corn kernels, growing well at 80% humidity with a temperature of 25-40°C (Bagus et al., 2017). Another symptom of the pathogenic fungus *Aspergillus* that attacks corn seeds is the growth of green mycelium, which gradually covers the seeds (Fitria et al., 2020). *Aspergillus* pathogens on corn kernels can produce several types of mycotoxins, including aflatoxins, ochratoxins, patulin, and sterigmatocystin (Hanif et al., 2019).

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M. circinelloides is a type of fungus that can cause rot from puncture wounds in the cuticle on stems and fruit petals, with symptoms of white or gray colonies appearing with dark spore heads, also irregular fungi colonies and gray-white thread-like strands of mycelia appear (Figure 3) (Ling et al., 2023). *Mucor circinelloides* can cause rot disease in plants with symptoms of skin on the slices containing fine granules with irregular white-gray colonies (Simamora et al., 2022).

Figure 3. Corn stem infected by *Mucor circinelloides* (1) The symptoms that arise are indicated by the formation of thin grayish-black colonies that form spots

Controlling pathogenic fungi in agricultural land have been widely practiced using chemical fungicides that reported to be more effective and faster in inhibiting the growth of these pathogenic fungi (Umboh & Rampe, 2019). However, chemical fungicides on agricultural land have a negative impact for environment if used continuously which is used intensively increase the risk of poisoning in consumers from the harvest of agricultural land (Kusumaningtyas et al., 2021). Fungicide application can also cause decreased soil quality, reduced biomass and soil microbial activity, and increased potential pathogen resistance to fungicides (Wu et al., 2023). As for the economic side, using fungicides tends to be expensive. Chemical fungicides also have the potential to kill microorganisms that are not their targets and can trigger the emergence of pathogen groups with new strains that are more resistant to the fungicides themselves. Therefore, a biologically environmentally friendly (eco-friendly) control effort is needed, namely using antagonistic endophytic microorganisms that can spur growth and increase plant resistance (Rotasouw et al., 2020).

One effective solution in controlling diseases caused by pathogenic fungi in plants is using biological agents, such as *Trichoderma* sp. (Hamidson et al., 2020). The *Trichoderma* sp. fungus is the most promising technology for sustainable agriculture because it can break down organic matter well, increase plant growth, and control diseases that attack plants (Nawaal et al., 2022). In its natural habitat, *Trichoderma* sp. has antagonistic activities that can attack and inhibit the growth of pathogenic fungi that cause disease in plants by releasing toxins in the form of cellulase and chitinase enzymes (Ruswandari et al., 2020). *Trichoderma* sp. can live and develop in healthy plant tissue and form colonies well. Although colony formation occurs in the host plant tissue, this does not harm the host plant because of its nature as an endophytic fungus. This type of fungus can be found in

plants, such as roots, stems, leaves, and flowers (Rotasouw et al., 2020). *Trichoderma harzianum* is classified as antagonistic fungi because these fungi have an antagonistic antibiosis mechanism against pathogenic fungi by producing secondary metabolite compounds, such as peptaibol, pyrone, and viridol (Singh et al., 2018). Despite the well-documented efficacy of *Trichoderma harzianum* as a biocontrol agent, research on its antagonistic effects against *Aspergillus parasiticus* and *Mucor circinelloides*, the causative agents of corn cob rot and stem rot, remains limited.

This study aims to fill this research gap by evaluating the inhibitory potential of *T. harzianum* against these fungal pathogens using the dual culture in vitro method. The dual culture method involved co-cultivation of *T. harzianum* with each pathogen in a single petri dish to assess competitive interactions. Diseased corn plants were used as a source for fungal isolation, followed by morphological and growth characterization. The antagonistic potential of *Trichoderma harzianum* was determined by measuring the inhibiting rate of fungal growth. The findings of this study will contribute to the advancement of sustainable and eco-friendly disease management strategies in corn production.

MATERIALS AND METHODS

Research Design

The study was experimental research conducted at the Biotechnology Laboratory of the Department of Biology, Diponegoro University, from October 2nd to November 20th, 2023. The analysis of antagonistic fungi against pathogens in corn was performed using the dual culture method in the antagonistic test. The research was arranged in a Completely Randomized Design (CRD), and the results were analyzed based on the inhibition category of the data on the inhibition of antagonistic fungi against pathogenic fungi obtained.

Corn Sampling

Corn seed samples suspected of being infected with pathogenic fungi were taken from a corn field in Brumbung Village, Mranggen Sub-District, Demak Regency, Central Java, Indonesia. Plant samples were taken from the infected organs, the corn seed cobs. Plant samples were then put in plastic for further testing in the laboratory.

Preparation of PDA Media

The PDA media were prepared using 3.9 g PDA powder and 100 ml distilled water put in an Erlenmeyer flask, which was then homogenized, heated in microwave for 3 minutes until clear PDA media was obtained in the Erlenmeyer flask, and covered for further sterilization in autoclave 121^{o}C 1 atm for 15 minutes. Sterile PDA media added with Chloramphenicol were poured into sterile Petri dishes and allowed to solidify.

Isolation and Koch's Postulate Test

Pathogenic fungi were isolated from infected corn parts (seed and stem) by soaking them in 70% alcohol for 15-30 seconds, which were rinsed in distilled water, dried, and placed on sterile PDA media to be incubated for 7 days, then purified by culturing them on new sterile PDA media (Wakhidah

et al., 2021) and incubating them for 5 days (Safitri et al., 2023). Koch's Postulate Test was carried out, in which healthy corn cobs were peeled off, sterilized with 70% alcohol, pierced and filled with purified fungi suspension using sterile ose, and then incubated until the symptoms were seen, and the corn cob decayed. The grown colonies were isolated and incubated for 5 days to identify the fungi (Safitri et al., 2023). The *Trichoderma* sp. isolates were obtained by rejuvenating the cultures from the Laboratory of Pest and Disease Observation Temanggung, Central Java, by culturing them on a PDA medium in a new Petri dish and incubating them for 3 days.

Identification

The fungi were identified macroscopically in pure colonies on petri dishes (Sholihah et al., 2019). The observed characteristics include color, shape, symmetry/asymmetry, and direction of colony growth. The identification was also carried out microscopically in object glass with Lactophenol Cotton Blue (LCB) under a microscope at 400 times magnification with observed characteristics including the form of shape, color, concentrated/unconcentrated spores or conidia, hyphae (Safitri et al., 2023), conidiospores, and phialids (Sholihah et al., 2019).

Antagonistic Test using Dual Culture Method

The antagonistic test was performed using a dual culture method carried out with the inoculum of pathogenic fungal isolates and *Trichoderma* sp. grown at a distance of 3 cm on the same PDA growth medium with a diameter of 9 cm. The inoculum of both fungi was taken with a 4-mm-diameter cork borer (Safitri et al., 2023). The test was conducted with 3 replications and 2 treatments: control and antagonist. The culture was then incubated at room temperature, and the growth colonies and inhibition zones between the two fungal colonies were observed.

The final result of the antagonistic test in this study was to determine the ability of *Trichoderma harzianum* to inhibit the growth of pathogenic fungi. The inhibitory rate of antagonistic fungi was calculated based on the following formula (Halwiyah et al., 2019):

$$I = \frac{(r1-r2)}{r1} \times 100\% \qquad (1)$$

Remarks:
I : Inhibitory rate (%)
r1 : Radius of pathogenic fungi colonies that move away from antagonistic fungi colonies
r2 : Radius of pathogenic fungi colonies approaching antagonistic fungi colonies

According to Win et al. (2021), the antagonistic mechanism of *Trichoderma* sp. against phytopathogens studied in vitro can be grouped into 4 categories based on the inhibitory rate:

1 (low) = 1-25%,
2 (medium) = 26-50%
3 (high) = 51-75%
4 (extremely high) = 76-100%

RESULTS AND DISCUSSION

Symptoms of *Aspergillus* Cob Rot in Corn

Corn cob rot in this study was indicated by the appearance of fungi colonies on the corn cob samples used. The kernels on the corn samples showed a blackened color, with the cobs starting to soften and rot (Figure 1). Common symptoms of *Aspergillus* infection on corn kernels include discoloration of the kernels and increased mycotoxin content. Morphologically, corn kernels infected with this fungus can be identified by green to black sections with a granular texture. *Aspergillus* infection of corn kernels results from conditions suitable for this fungus to grow on corn. Several factors can influence the growth of this fungus, such as temperature, humidity, and the use of chemicals in corn cultivation. The growth medium most often contaminated with *Aspergillus* is agricultural food. High organic content in agricultural products, suitable humidity, and the ease of *Aspergillus* spores growth and development. *Aspergillus* morphology is generally characterized by green to dark green or yellowish-green colonies with a granular colony texture. The young colonies will be white and turn yellowish-green after the conidia are formed (Kapli et al., 2022). *Aspergillus* in infected corn will cause the accumulation of mycotoxins that are harmful when consumed. This will affect the yield and production quality of the corn kernels. If the infected corn is consumed, the kernels will affect human health due to harmful mycotoxins, such as carcinogenic aflatoxins. Aflatoxin compounds are secondary metabolite compounds produced by *Aspergillus* that produce aflatoxin toxins of AFB1, AFB2, AFG1, and AFG2. This compound can be lethal when consumed in high concentrations. In addition, even if consumed in low concentrations over a long period, it can also cause liver cancer and kidney cancer (Sasongkowati et al., 2024). *A*flatoxin is a secondary metabolite produced by *Aspergillus parasiticus, w*hich is toxic, carcinogenic, mutagenic, stable, and resistant to degradation. The commonly found groups of aflatoxins are AFB2, AFG1, and AFG2, and the most toxic are AFB1 (Abdelaziz et al., 2022). Aflatoxins are often contaminants in agricultural products from tropical countries, such as nuts, rice, wheat, cotton seeds, and corn seeds (Rajarajan et al., 2021). This compound has the potential to cause several serious diseases in humans, such as liver cancer, immunosuppression, and stunting (Navale et al., 2021).

Figure 1. Corn seeds cobs infected by *Aspergillus parasiticus* shown in blue circle

Koch's Postulate Test of Aspergillus sp.

Koch's Postulate test was carried out on healthy corn cobs, and the inoculation results showed the same symptoms between the seeds on the inoculated corn cobs and the symptoms on the plants from which the fungi isolates were isolated. The results of re-isolation of inoculated corn seeds also showed the same type of fungi. Koch's Postulate Test aims to prove and ensure that the isolated microbes are pathogenic by looking at the similarity of symptoms (Kumala et al., 2023). Colonies that grow in the Koch Postulate Test results showed a granular green color, both in the seeds in the inoculated healthy corn cobs and the results of their re-isolation. The color and texture of the colonies formed were similar to the symptoms on the original plant. If the results of pathogen isolation show the same type of microbe, it can be said that the microbe has met the requirements of Koch's Postulate Test so that it can be known that the microbe is indeed pathogenic to the original plant (Budi et al., 2022).

Identification of Aspergillus sp.

Macroscopic observations on PDA media showed that the growing colonies were green with a colony diameter of 5.2 cm, granular colony texture, white inverted colonies, growth zones and radial grooves, and no exudate drops (Figure 2). Microscopically, this fungus was characterized by apparent, transparent hyphae, septate hyphae (hyphae with partitions), clear, transparent conidiophores without branches, vesicles, metula, fialid, and round (globose) conidia, and no ascus or ascospores. According to the identification book by Watanabe (2002), the species was identified as *A. parasiticus*, with the key determination of *Aspergillus* Mich : Fr. is 1a - 2b, with morphological characteristics in the form of erect conidiophores, simple, rough surface, having foot cells at the base, expanding at the apex to form round vesicles, and bearing conidia heads made up of uniseriate or, in rare cases, biseriate phialides, which were yellowish green, radiate, columnar, and concentrated, with pale green colonies. *A. parasiticus* is characterized by a dark green colony color and microscopic features in globular vesicles with a set of sterigmata (Nikolić et al., 2021).

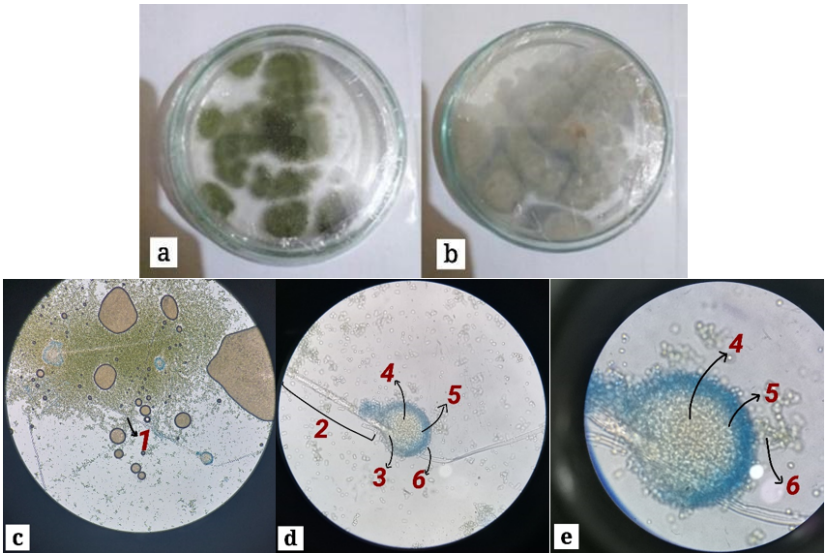

Figure 2. Characteristic Appearance of Pathogenic Fungi Isolates (*Aspergillus parasiticus*). a. upper colony, b. reverse colony, c. 40X microscopy, d. 100X microscopy, e. 400X microscopy (1: foot cell, 2: conidiophore, 3: vesicle, 4: metula, 5: fialid, 6: conidia)

Symptoms of Mucor Stem Rot in Corn

Mucor stem rot is characterized by symptoms of light brown to golden tissue. In addition, gray colonies with a cotton-like texture also appear. The pathogenic fungus *M. circinelloides* causes rot with symptoms of white or gray colonies with dark spore heads and cottony texture (Ali & Samosir 2021).

Mucor circinelloides

Microscopic identification was carried out on corn plants showing stem rot symptoms to ensure that they were in accordance with the characteristics of the fungus *M. circinelloides*. The hyphae are non-concentrated based on the identification results (Figure 4). It also has sporangium and sporangiophore. Sporangiophore of *M. circinelloides* is transparent and has a smooth surface. In addition, there are also spherical, single or branched sporangiums. The sporangium of *M. circinelloides* is located at the top of the sporangiophore. The microscopic characteristics of *M. circinelloides* are that the hyphae are not insulating, have single sporangiophores and no rhizoids are visible. In addition, *M. circinelloides* also has sporangium and columella, which are round with round and smooth spores (Izzatinnisa' et al., 2020). Macroscopic identification showed the characteristics of gray-white colonies (Figure 5). *M. circinelloides* have white colonies, in which the increasing age of the fungus will cause the color changes to gray. The colonies grow densely with cotton-like mycelium. In addition, *M. circinelloides* has a flat colony surface and no concentric radial lines. *M. circinelloides* has no exudate points, and the colony can grow to a diameter of 9 cm. The genus *Mucor* is a Zygomycetes fungus, which is white with a cotton texture without exudates, and its colonies can grow to fill Petri dishes.

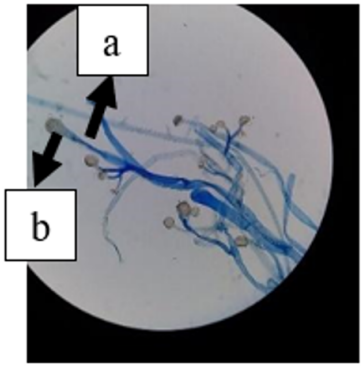

Figure 4. Microscopic *Mucor circinelloides* (a) sporangiophore, (b) sporangium

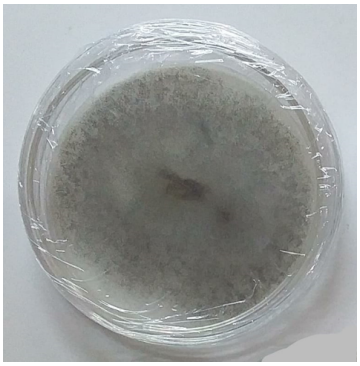

Figure 5. Macroscopic *Mucor circinelloides*

Antagonistic Fungi (*Trichoderma harzianum)*

Based on the microscopic observation, *Trichoderma* sp. isolates on PDA growth media (Figure 6) showed a dark green colony color with a colony diameter of 8.3 cm. The texture was granular with a whitish-green reverse colony without a growing zone, radial furrows, or exudate drop. The microscopic characteristics obtained include septate apparent transparent hyphae without foot cells or Hulle cells, branched conidiophores with mono-verticillate branching type, no vesicles, round conidia of bright yellow color, phialids, and no metula, ascus, or ascospores. Identification was carried out using the identification book "Pictorial Atlas Of Soil And Seed Fungi: Morphologies Of Cultured Fungi And Key To Species" by Watanabe (2002), and the results confirm that the type of *Trichoderma* sp. is *T. harzianum* with the key determination of *Trichoderma* Pers. : Fr. 1b - 2a. Its morphological characteristics include hyaline, upright, branched conidiophores, with a mass of spores at the apical end in verticillate short and thick phialids; insulated conidia, hyaline, round, spherical, subglobose, or ovoid, single-celled, and brown chlamydospores that are subglobose. The initial growth of *T. harzianum* on the culture media was indicated by the characteristics of dense and thick mycelium, greenish white, then developed into green in the middle and white at the edges. White and green circles were formed with clear boundaries. The green color looked more significant and more intense, and the color looked clearer. Colony color is influenced by phial phosphorus pigment, the number of spores, and the pH of the medium. Colonies appear transparent on media with limited nutrients, while on media with more nutrients, colonies appear whiter. In the growth media, color changes also occur due to *T. harzianum* degrading the media as nutrients for its growth (Suharni et al., 2023).

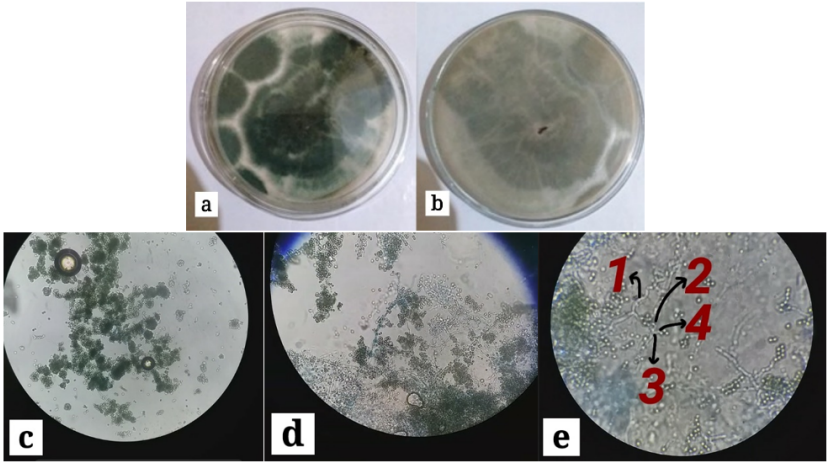

Figure 6. Characteristic appearance of *Trichoderma harzianum* isolates a. upper colony, b. reverse colony, c. 40X microscopy, d. 100X microscopy, e. 1000X microscopy (1: conidiophore, 2: conidiophore branch, 3: phialid, 4: conidia/phialospore)

Growth of Pathogenic Isolates

The growth phase of fungi occurs through several stages. The stages in this phase are often represented on the fungi growth curve. The fungi growth phase consists of the lag, acceleration, exponential (log), stationary, and autolysis phases (Heirina et al., 2020). The lag phase is the growth phase of the fungus adapting to its environmental conditions. The acceleration phase is the phase when cell

division begins. The log or logarithmic phase of fungi growth occurs between days 7 and 14, where biomass increases. During the lag phase, the fungi cells can adapt to their environment and produce enzymes that degrade the substrate. The stationary phase occurs between days 15 and 21, where fungi growth is relatively constant and balanced by the number of dead cells. The autolysis or death phase is when the number of cells decreases or cell death occurs.

Aspergillus parasiticus

The growth of *A. parasiticus* isolates can be seen in the growth curve of the isolates (Figure 7). The curve shows that the three replicates of *A. parasiticus* isolate experienced a consistent increase for 5 days after isolation. *A. parasiticus* isolates after 2 days of isolation experienced a significant increase in colony diameter because cell growth began to enter the logarithmic phase. *A. parasiticus* growth can be influenced by factors such as temperature and relative humidity. The growth of *Aspergillus* sp. on bread showed that the fungus grew faster at room temperature (25 °C-28°C) than in the refrigerator (10 °C-15 °C). The maximum growth of *Aspergillus* sp. on corn and soybean was achieved at 20 °C with 90 % relative humidity or at 40 °C with 70 % relative humidity (Agriopoulou et al., 2020). In addition, *Aspergillus* sp. grew well on various media, such as PDA, and waste products, such as tempeh wastewater and coconut water. The main nutrient required for Aspergillus growth is carbohydrate.

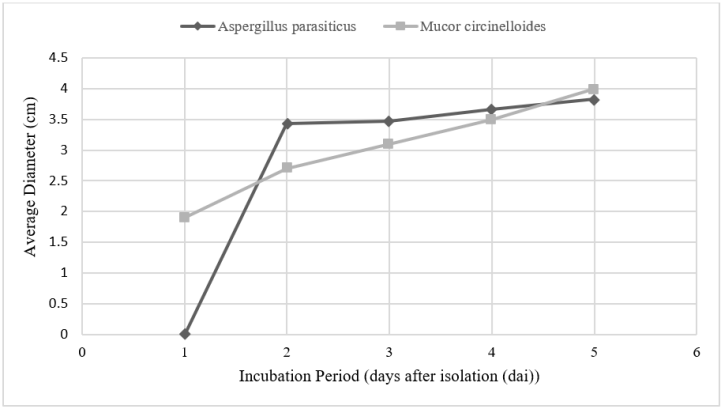

Figure 7. Growth of pathogenic fungi isolates *Aspergillus parasiticus* and *Mucor circinelloides* from corn seed cobs (*Zea mays* L.)

Mucor circinelloides

The results of observations for 5 days regarding the branches of the pathogenic fungus *Mucor circinelloides* (Figure 7) showed that the pathogenic branches continued to increase from day to day. *Mucor circinelloides* growth is influenced by several factors, such as warm temperatures, high humidity, and appropriate soil pH. High humidity will increase the risk of infection in plant tissues because the potential for *Mucor circinelloides* to live is higher. *Mucor circinelloides* is a dimorphic mold genus that requires growth of carbon dioxide and hexose sugar (Widiyantini & Kumoro, 2017).

In Vitro Antagonistic Test

The antagonistic test in this study was carried out in vitro with a double culture method on a 9-cm-diameter petri dish with a distance of 3 cm between isolates. This test aims to determine the amount

of ability of antagonistic fungi to inhibit the growth of pathogenic fungi. The test isolates selected as antagonistic fungi are isolates that can inhibit > 70 % (Safitri et al., 2019).

Trichoderma harzianum with Aspergillus parasiticus

The inhibitory rate of antagonistic fungi from the first day to the seventh day order was 22.86 %, 34.19 %; 36.19 %; 32.78 %; 32.22 %; 33, 73 %; and 32.41 % (Figure 8), with an average value of 32.5%. The inhibitory rate is classified into three categories, namely low (0%-30 %), medium (31 %-40 %), and high (higher than 40 %) (Arti et al., 2021). Based on this, the inhibitory rate of *T. harzianum* against *A. parasiticus* is in the medium category. Fungi with more than 50 % inhibition rate can be used as biological agents to control pathogenic fungi, so *T. harzianum* has no less potential as a biological agent in inhibiting the growth of pathogenic fungi *A. parasiticus growth*. The inhibition of *T. harzianum* against *A. parasiticus* growth can also be seen directly in the petri dish. The antagonistic mechanism formed from the treatment is an antibiosis mechanism, seen from the formation of an inhibition zone around *T. harzianum,* which is marked with an orange circle (Figure 9). The formation of the inhibition zone is thought to be due to the formation of secondary metabolites or active compounds. *T. harzianum* produces metabolites and filtrates that can inhibit the growth of the pathogenic fungus *A. parasiticus* (Rahmadanty et al., 2023).

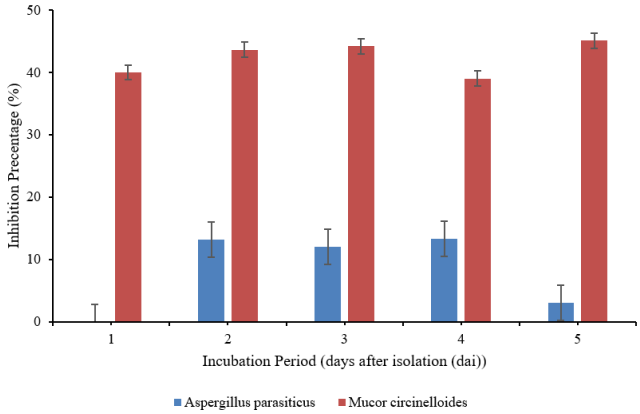

Figure 8. Inhibitory Rate of *Trichoderma harzianum* against *Aspergillus parasiticus* and *Mucor circinelloides* 5 days after incubation (dai)

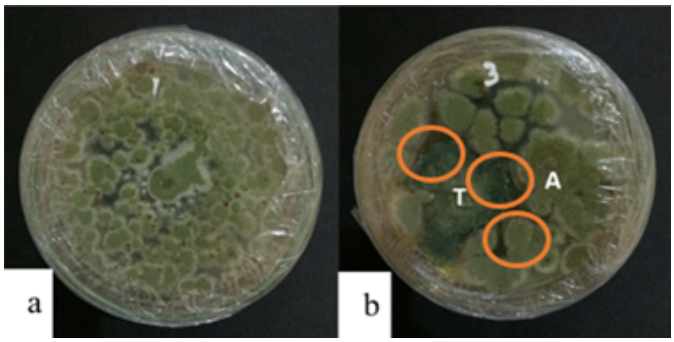

Figure 9. Antagonistic Test of *Trichoderma harzianum* against the pathogenic fungus *Aspergillus parasiticus* at 7 days after isolation shown in orange circle. (a) Control, (b) Antagonistic Test, (A) *Aspergillus parasiticus* (T) *Trichoderma harzianum*; orange circles indicate clear zones.

Trichoderma harzianum with Mucor circinelloides

Trichoderma sp. performed optimal inhibition starting from day 2 after inoculation (Figure 8). The growth on day 2 resulted in an inhibitory rate of 43.60 % and increased on day 3 to 44.20 %. However, on the 4th day, it decreased to 39 %. The decrease can be caused by the growth of filaments that are less than optimal, causing the percentage value to decrease from the previous day. The length of the radius of pathogenic colonies affects the percentage of endophytic fungi inhibition (Pasalo et al., 2022). However, the inhibitory rate started to increase again on day 5, which is enough to prove that the antagonistic fungus *Trichoderma harzianum* is able to inhibit the growth of the fungus *Mucor circinelloides*.

The values of the inhibitory rate of *Trichoderma harzianum* against *Mucor circinelloides* were 39-45.10 % from day 1 to day 5, and the average value was 42.38 %. This inhibitory rate value is included in the medium category. The inhibitory rate values are divided into 4 categories, namely low (1-25 %), medium (26-50 %), high (51-75 %), and extremely high (76-100 %) (Win et al., 2021). Although the inhibitory rate value obtained tended to increase, the inhibitory effect was not significant. The inhibitory effect of *Trichoderma harzianum* is still relatively low but can inhibit the growth of *Mucor circinelloides*. The less than 60 % inhibitory rate value means that the antagonistic fungus only has a minimal inhibitory effect on the development of pathogenic fungi that attack (Halwiyah et al., 2019). Meanwhile, if the inhibitory rate value is more than 60 %, it can be said that antagonistic fungi can inhibit the growth of pathogens to the maximum.

CONCLUSION

Antagonistic test results showed that the application of biological agents *Trichoderma harzianum* was classified as moderate against *Aspergillus parasiticus* and *Mucor circinelloides* with an average inhibitory rate of 32.5 % within five days and 42.38 %, respectively. An antagonist agent can be said to be a biological agent if it can inhibit pathogens > 50 %. Therefore, it can be said that *Trichoderma harzianum* only has a minimal inhibitory effect on the growth of pathogenic fungi that attack corn plants.

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AUTHORS CONTRIBUTIONS

FAN, MFIS, and MC contributed to the design of the topic, methods, and implementation of the research with SP guiding, giving direction during the research, and financing the research needs. FAN and MFIS were responsible for determining the sampling areas, finding corn samples infected with pathogenic fungi, and antagonistic tests against *Aspergillus* cob rot. MC was responsible for the antagonistic test and fungicide mechanism against *Mucor* Stem Rot. This manuscript was primarily

composed by FAN, and all authors provided critical feedback and contributed to the development of the research, analysis, and manuscript.

DECLARATION OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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