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In Vitro Evaluation of The Inhibitory Power of *Trichoderma harzianum* Against Pathogens that Cause Anthracnose in Chili

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Abstract. This study aims to determine the inhibition of *Trichoderma harzianum* Tc-Jjr-02 isolates against two *Colletotrichum* isolates that cause anthracnose in chili in vitro. The first stage of the test was carried out by a double culture method on the agar dextrose-chloramphenicol medium with five replications. Inhibition of biocontrol agents was determined by comparing the difference between growth of pathogenic colonies in multiple cultures and monoculture with growth of monoculture pathogenic colonies measured every 24 hours for six days. At two weeks after the end of the Observation Test the second stage of research was carried out. Mycelium biocontrol agents that overlap with pathogenic mycelium isolates are sampled and observed under a microscope with 400 times magnification. In vitro test results showed that *T. harzianum* Tc-Jjr-02 isolates were able to inhibit the growth of *C. capsici* and *C. gloeosporioides* colonies with an average of 64.2 ± 3.54 and 65.0 ± 3.93%, respectively. On microscopic observations of overlapping mycelium it appears that *T. harzianum* Tc-Jjr-02 damages the *C. capsici* and *C. gloeosporioides* hyphae cell walls. *T. harzianum* has the potential to be an effective biocontrol agent against pathogens that cause chili anthracnose.

1. Introduction

Chili is one of the strategic commodities in Indonesia which often a causes social upheaval when there is an imbalance between supply and demand.

Efforts to increase production continue to be carried out by all stakeholders in Indonesia, but constraints are often encountered, especially in plantations. Anthracnose attack on chilies is one obstacle that can be detrimental and even lead to crop failure. *Colletotrichum* spp. is a pathogenic fungus that causes anthracnose disease which is very damaging in Indonesia. Anthracnose disease in chilies has caused economic losses in various countries in the tropics and subtropics [1]. There are three species of *Colletotrichum* in Indonesia that cause anthracnose, namely: *C. acutatum*, *C. capsici*, *C. gloeosporioides* [2].

So far the use of chemical pesticides has been carried out to control anthracnose disease in chilies [3]; however, most fail or at least unable to save production optimally and cause *Coletotrichum* pathogenic resistance to fungicides [4]. Pesticide chemicals often fail to control pathogens that are deposited or as a soil borne in the soil due to adsorption by colloidal soils and degraded by soil microbes [5]. In addition, the use of fungicide chemicals in addition to reducing efficiency in cultivation can also have a negative impact on the environment [6] as well as the threat of poisoning to field operators and public health [7], [8].

Many biocontrol agency functions have been used to control diseases caused by pathogenic fungi. One of the potential effective fungi as biopesticides is *Trichoderma harzianum*. *Trichoderma* is able to directly parasitize pathogens, produce enzymes that degrade living cell pathogens [9], and dead fungi cell walls [10], as well as produce antimicrobial compounds and compete for nutrients and space. with pathogens [11]. Secondary metabolites produced by these fungi play a role in helping plant growth and suppress pathogens [12]. One of the *T. harzinum* isolates has been tested for its effectiveness and among them is able to control damping-off on soybeans [13] and leaf blight on potatoes [14].

As in general, experiments in the application of biocontrol agents in vivo are always preceded by in vitro experiments. But so far it has not been studied how the mechanism of direct inhibition, especially in conditions where the mycelium fungi agent control and mycelium fungi pathogens are close to one another and overlap. In vitro isolates of *T. harzianum* Tc-Jjr-02 which tested their inhibitory ability against *Fusarium oxysporum* which causes stem rot of red chilies [15] only showed inhibitory performance against pathogens. For this reason, it is necessary to deepen studies on the performance of biocontrol agents inhibition of pathogens, especially when the two mycelia are touching or overlapping. This information is also important in preparing further in vivo testing for the control of pathogens in the plant canopy.

This study aims to determine the inhibition of *T. harzianum* Tc-Jjr-02 isolates against two *Colletotrichum* isolates that cause anthracnose disease in chilies in vitro and the suppression mechanism of *T, harzianum* in the mycelium that overlaps with the pathogen mycelium.

2. Methodes

2.1 In vitro inhibition test

Pathogenic fungi and biological agents fungi are a collection of Microbiology and Biotechnology Laboratories, Muhammadiyah University, Sidoarjo. *T. harzianum* Tc-Jjr-02 isolates as a potential biological fungi agent has been proven effective in controlling in vitro and in vivo on potato plants [14]. The pathogen isolate used in this experiment were the cause of anthracnose disease, namely *C. capsici* and *C. gloeosporioides* isolated from the red critical chili had passed the Postulate Koch test.

All isolates were grown in PDA-m media [10] for 10 days of incubation. Each culture was sampled to a 5.0 mm diameter and placed opposite each other as a dual culture with a 50 mm distance between the center of the propagules and 25 mm each from the edge of the petri dish (Figure 1, left). This method was repeated five times with each monoculture accompanied by a 5 mm propagule placed in the middle of a petri dish (Figure 1, right).

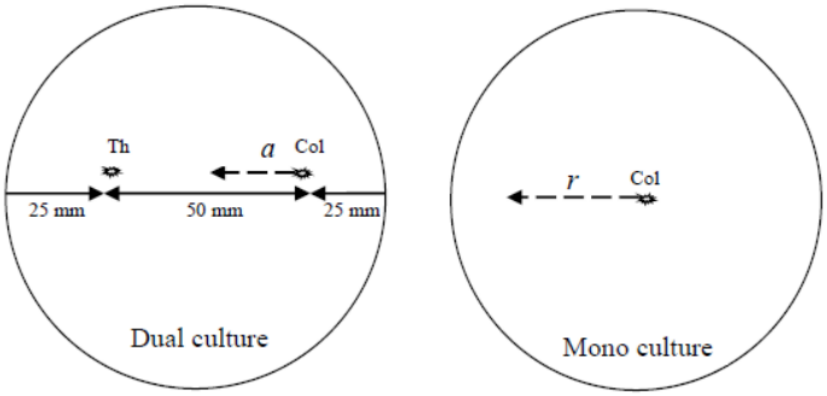

Figure 1. Placement of biocontrol fungi and pathogenic fungi in vitro inhibition tests; dual culture (left) and monoculture (right) as controls. Th: initial propagules of *Trichoderma*, Col: initial propagules of *Colletotrichum*, *a*: the distance to the pathogen mycelium growth direction from the

center of the colony perpendicular to the center of the *Trichoderma* colony, *r*: the distance of the growth of pathogenic mycelium perpendicular to the center of the colony in the monoculture.

The inhibitory capacity of biological agents against pathogens is calculated using the following formula:

$$\text{IC} = ((r - a)/r) \text{ x } 100\%$$

Provided that r is the diameter of a pathogenic fungal colony monoculture, *a* is the distance between the center of the colony and the edge of the pathogenic colony that leads perpendicular to the biological agent colony.

2.2 Microscopic observation

Two weeks after the last observation (144 HAI), from the area where there was an overlap between the pathogenic mycelium fungi and the biocontrol fungi mycelium was sampled with an ose needle and placed on top of the glass object in exactly one drop that had distilled water; after being pressed gently and flattened, the object is covered with a glass cover. Observations were carried out under a microscope with a magnification of 400 times to determine hyphal conditions that overlap between pathogens and biological agents.

3. Results and Discussion

3.1 In vitro inhibition test

Based on observations of the growth of the biocontrol agent colonies and both pathogenic isolates both monoculture and dual culture, the percentage of *T. harzianum* inhibition was obtained for both pathogens as shown in Table 1.

Table 1. Inhibition of *T. harzanum* against *C. capsici* and *C. gloeosporioides* in vitro 24-168 hours after after inoculation (HAI)

	24 HAI	48 HAI	72 HAI	96 HAI	144 HAI	168 HAI
Inhibition of *T. harzianum* against *C. capsici*	-4.4%	10.4%	28.0%	47.2%	63.7%	64.2%
Stdev	0.06%	6.07%	10.18%	6.34%	4.00%	3.54%
Inhibition of *T. harzianum* against *C. gloeosporioides*	10.0%	16.1%	19.8%	43.9%	64.7%	65.0%
Stdev	0.05 %	4.56%	4.86%	4.70%	3.97%	3.93%

The inhibition of *T. harzianum* against *C. capsici* has begun to appear at 48 HAI and has grown to reach 64.2 ± 3.54% at 168 HAI, while the inhibition of this biocontrol agent against *C. gloeosporioides* starts from 24 HAI with the value of inhibition relative to the inhibitory value against *C. capsici* which is 10.0 ± 0.05% and becomes 65.0 ± 3,593% at 168 HAI.

Appearance of inhibition of colonies of both pathogens by their respective biocontrol agencies is shown in Figure 2 on 168 HAI.

Figure 2. Appearance of inhibition of pathogenic colonies by *T. harzianum* against *C. capsici* (left) and *C. gloeosporioides* (right) on 168 HAI

3.2 Microscopic observation

The results of observations of overlapping hyphae appear to be that the consistency of *T. harzianum* hyphae forms, while both hyphae *G. capsici* (Fig. 2) and *G. gloeospoiroides* (Fig. 3) appear to be inconsistent. The second hypha isolate pathogen is not intact and looks like it is broken or cut into pieces.

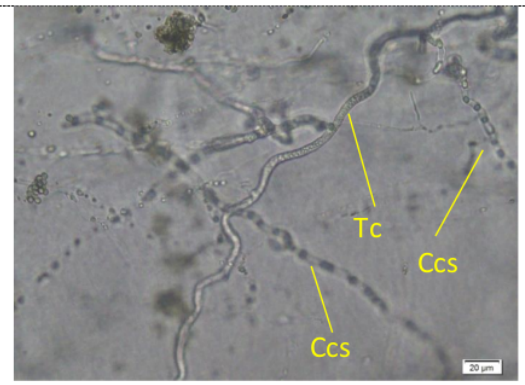

Figure 3. Destruction of *C. capsici* hypha by *T. harzianum* Tc-Jjr-02; Tc is hyphae *T. harzianum*, Ccs is hyphae *C. capsici*.

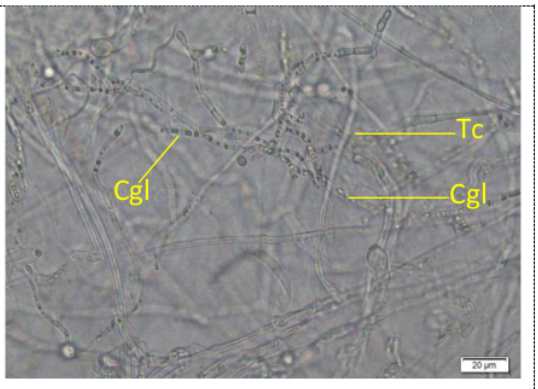

Figure 4. Destruction of *C. gloeosporioides* hyphae by *T. harzianum* Tc-Jjr-02; Tc is *T. harzianum* hypha, Cgl is *C.* gloeosporioides hypha.

3.3 Discussion

The inhibition of *T. harzianum* fungi against *C. capsici* and *C. gloeospoiroides* is not the same based on the initial time but the same at the end of the observation. This shows the response of the two different isolates to the activity of this biocontrol agent fungi colony. The inhibition of *T. harzianum* against *C. capsici* began to appear at 48 HAI or slower than *C. gloeosporioides* which have shown inhibition at 24 HAI (Table 1). Two different isolates showed different resistance performance against contraction fungi in vitro. However, the inhibition that reaches 64-65% in anthracnose pathogens in chilies is potentially effective in controlling pathogens. *T. harzinum* inhibits faster in *C. capsici* than in the pathogen causing damping-off *Rhizoctonia solani* which starts to show inhibition of 7.85% in 36 HAI [13].

Starting 96 HAI isolates *Trichoderma* in this experiment is not only as an antifungal by inhibiting the growth of pathogens but also has invaded pathogenic colonies as shown in Figure 1 (168 HAI). *T.*

harzinum which was tested in vitro against *Botrytis cinerea* also showed characteristics as an antifungal as well as invading pathogenic colonies with high intensity [16]. At the beginning of growth, namely 24-96 HAI, *T. harzianum* dominates the growth space. *Trichoderma* spp. has the ability to more quickly utilize space and nutrients with pathogens so that this biocontrol agent has a competitive advantage [17].

Macroscopically the effect of *T. harzianum* fungal activity in vitro can damage the stability of hyphal cells as indicated by damage to the pathogen cell walls. This shows that the activity of biocontrol agent cells producing extracellular compounds has caused lysis of cell pathogens. *T. harzianum* fungi not only produce chitinolytic compounds that can damage the pathogen cell walls [18] but also produce antibiotic compounds against pathogenic colonies so that they can inhibit the growth of pathogenic colonies [19], [20]. Some extracellular enzymes including endochitinases, b-1,3-glucanases, and proteases play a role in the mechanism of *Trichoderma*'s micoparasitism which causes damage to cell walls of pathogenic fungi [21]–[23]. The fact that the characteristics of the biocontrol fungi agent in this experiment are consistent with the results of testing the administration of filtrate from *T. harzianum* culture with a concentration of 10% can inhibit the growth of *Sclerotinia sclerotiorum* colonies in vitro up to 48.0% [24]. The broken *Colletotrichum* cell wall can be caused by the activity of the extracellular proteinase enzyme produced by *T. harzianum*. The various enzymes produced by *T. harzianum* are L-amino acid oxidase associated with cell wall destruction and 33-KDa endochitinases, 29-KDa b-1,3-glucanase, 36-KDa b-1,3-glucanase, 78- KDa b-1,3-glucanase, b-1,6-glucanase, aspartic proteases, and serine proteases associated with mycoparasitation of pathogenic fungi cells [25].

The biocontrol mechanism of *T. harzianum* in both anthracnose pathogens in this experiment from the beginning to the end of the observation (24-144 HAI) was carried out in the form of competition to gain control over space and nutrition; then starting 144 HAI in vitro tests up to two weeks after 166 HAI, the fungi of the biocontrol agent effectively parasitize the pathogen. Correspondingly, the results of tests on *Colletotrichum dracaenophilum* show that Trichoderma suppresses pathogens indirectly through the competition mechanism for competing for nutrition and space and directly through the mechanism of antibiosis and mycoparasitism [26].

4. Conclution

The biological agent of *Trichoderma harzianum* isolate Tc-Jjr-02 was able to inhibit the growth of *Colletotrihum capsici* colonies by 64.2 ± 3.54% and inhibit *C. gloeosporioides* by 65.0 ± 3.93%. Microscopically it appears that almost all the walls of the *C. capsici* and *C. gloeosporioides* hyphae are damaged as a consequence of the influence of the *T. harzianum* Tc-Jjr-02 hyphae that are overlapping with hyphal pathogens.

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