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Fusarium Rot Biological Control of Citrus caused by *Fusarium oxysporum*

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KEYWORDS

Fusarium oxysporum;
Trichoderma spp.;
In vitro;
In vivo;
Citrus.

Abstract *Fusarium oxysporum* is a pathogen that causes Fusarium rot disease on citrus plants. The F. *oxysporum* is a soil-borne pathogen whose fungicide is not effective against it and difficult to cure. Thus, the use of *Trichoderma* spp. as one of the most effective and well-known biocontrol agents against many plant diseases in agriculture is needed. To test *Trichoderma* capabilities on the specific pathogen, method of this study consisted of isolation and rejuvenation of F. *oxysporum* and *Trichoderma*, morphological identification of the fungus, *in vitro* test of *Trichoderma* antagonistic ability against F. *oxysporum* on PDA medium, and *in vivo* test conducted in a green house on Rough Lemon (RL) and Japansche Citroen (JC) rootstock seeds to calculate the total disease incidence using a formula. The result of *in vitro* test of this study shows that 3 *Trichoderma* isolates (TJ, TKH, and TST) were able to inhibit the growth of F. *oxysporum* on PDA medium by 65.56%, 62.99%, and 61.19%, respectively. While *in vivo* test shows that the treatment of TJ isolates on RL seeds and TKH on JC seeds shows lowest disease incidence percentage of 3.33%. Therefore, this study proves that *Trichoderma* can be used as a biocontrol agent in controlling Fusarium rot disease in citrus plants. However, further research is needed to detect which *Trichoderma* species TJ and TKH isolates contain.

Introduction

Citrus plants consist of 2 parts, scion and rootstock. The scion is part of the plant which stays above the ground. Meanwhile, rootstock stays under the ground and provides the plant with a root system (Ahsen et al., 2019). The root system is important for absorbing nutrients from the ground. Thus, diseases attacking the rootstock of citrus plants such as Fusarium rot is devastating. *Fusarium* is one of the most devastating soil-borne pathogens that can affect citrus. One of the diseases is Fusarium rot caused by the *Fusarium oxysporum*. The F. *oxysporum* caused long-term infection with two symptoms, namely vascular wilting and root crown rot. The F. *oxysporum* can penetrate host roots via the xylem arteries and colonize upward. The fungus also invades the cortical layer and shows significant brown to black necrotic areas, causing damage to the basal plate (Lecomte et al., 2016). The F. *oxysporum* is a soil-borne pathogen whose fungicide is no longer effective and difficult to cure (Venkataramanamma et al., 2022). Thus, a biocontrol agent that is capable and effective in solving the problem is needed.

Biocontrol agent most found in the form of fungi and bacteria. Fungus and bacteria have been proven as biocontrol agent in previous studies (Nugroho et al., 2022; Triasih et al.,

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2021) to suppress the symptoms of plant pathogens. *Trichoderma* spp. is one of the most effective and well-known biocontrol agents against many plant diseases in agriculture. In a previous study, *Trichoderma* has been used in various cases such as biocontrol agents against cacao black pod rot (BPR) caused by *Phytophthora megakarya* while boosting cocoa growth and decreasing disease incidence (Mbarga et al., 2020). Another previous study inoculated shallot plants with *Trichoderma* as a potential alternative to synthetic fungicides for the protection of shallot plants against *Stemphylium vesicarium* infection (Zapata-Sarmiento et al., 2020). Hence, the purpose of this study is to select the best *Trichoderma* isolate based on its *in vitro* and *in vivo* antagonistic activity on F. *oxysporum.*

Material and methods

Research Design

The design method we used for the *in vitro* test of the antagonistic activity of *Trichoderma* against F. *oxysporum* was a Completely Randomized Design (CRD). The tests were done in a laboratory with four different treatments. The treatments were done by planting *Trichoderma* sp. and F. *oxysporum* in a 9 cm Petri dish with a distance of 3 cm from each other. For control treatments, only F. *oxysporum* that are planted on a side of a petri dish. Each treatment was repeated three times. For *in vivo* tests, we used the Randomized Block Design (RBD) method. The tests were done in a greenhouse using eight treatments consisting of 2 controls and 6 by applying *Trichoderma* suspension to the rootstock seeds of Rough Lemon (RL) and Japansche Citroen (JC) citrus plants. Control treatments were done by applying F. *oxysporum* suspension without *Trichoderma* suspension. Meanwhile, for the other treatments we applied both F. *oxysporum* and different *Trichoderma* suspension. After we applied the suspension, we planted rootstock seeds on sterile soil. Each treatment consisted of three groups for each treatment.

Fungal Isolation

This study was conducted from March to October 2022. *F. oxysporum* and 3 *Trichoderma* spp. isolates were taken from Indonesian Citrus and Subtropical Fruits Research, East Java, Indonesia. The *Trichoderma* spp. was taken from three different plant rhizospheres namely, citrus (TJ), mung bean (TKH), and strawberry (TST). Then, the isolation of *F. oxysporum* and *Trichoderma* spp. was done on Potato Dextrose Agar (PDA) medium and at room temperature (28 °C).

Morphological Identification

The fungi were purified and maintained on the PDA medium and then identified based on macroscopic characteristics under a 100x magnification microscope (Mazrou et al., 2020; Watanabe, 2002).

Antagonistic activity of Trichoderma spp. against Fusarium oxysporum

The *in vitro* test for *Trichoderma* spp. with F. *oxysporum* was carried out by a dual culture test on a PDA medium based on a previous study (Zhang et al., 2016). The antagonistic ability of *Trichoderma* was determined based on the percentage of inhibitory ability assessed from the presence or absence of an inhibition zone (Mazrou et al., 2020; Sri Hastuti & Rahmawati, 2016; Triasih et al., 2021). Equation (1) shows the formula for calculating the inhibitory ability.

$$Inhibitory\ Ability\ (\%) = (R1 - R2)/R1\ x\ 100\%$$

notes,

$R1$: radius of the pathogenic fungus away from the antagonist fungal isolate

$R2$: radius of pathogenic fungus close to antagonistic fungal isolate

In the *in vivo* test, direct application of T*richoderma* sp. on rootstock seeds of RL and JC citrus varieties was done. The activities carried out were visually observing the seeds of citrus

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plants attacked by *Fusarium oxysporum*, then calculating the incidence of the disease based on the formula in Equation (2) (Groth et al., 1999).

$$Disease\ Occurence\ =\ a/b\ x\ 100\%$$

notes
a = number of infected plants
b = Number of healthy plants

Data Analysis

To analyze the variance of the data, we used a one-way analysis of variance, using IBM SPSS version 22. Then, the Duncan Multiple Range Test was used to evaluate the distances between treatments based on a previous study (Mazrou *et al*., 2020). The value of p<0.005 in terms of inhibition percentage shows that the treatment shows a significant statistical difference.

Results and Discussion

Morphological Identification of Trichoderma spp.

The isolation and rejuvenation results of 3 *Trichoderma* spp. acquired from Indonesian Citrus and Subtropical Fruits Research, East Java, Indonesia, isolates were grown on PDA medium for 7 days at room temperature (28°C). The morphological appearance of 3 *Trichoderma* spp. after 7 days displayed unique development and sporulation patterns. On previous study (Montes Vergara et al., 2022) *Trichoderma* spp. rapid growth allows them to compete for space and nutrients in their environment.

TJ isolate macroscopically has a dark green colony color and is ring-shaped in the middle. Microscopically, the isolate has hyphae and hyaline, green round conidia measuring 3.73 µm-5.62 µm microscopically. The hyaline sectional conidiophores are 40.07 µm in size and 10.87 µm, hyaline-colored 3-6 bulging phialides (Figure1).

TKH isolate macroscopically has greenish-white colour. Microscopically, the isolate has hyaline sectioned hyphae, round hyaline conidia with the size of 3.04 µm-3.52 µm, hyaline conidiophore with a size of 7.35 µm, and hyaline coloured 3-6 bulging phialides with the size of 7.35 µm (Figure 2).

TST isolate macroscopically has whitish-green colour. Microscopically, the isolate has hyaline sectioned hyphae, green round hyaline conidia with the size of 3.28 µm-3.45 µm, hyaline conidiophore with a size of 30.64 µm, and hyaline coloured 3-6 bulging phialides with the size of 7.33 µm (Figure 3).

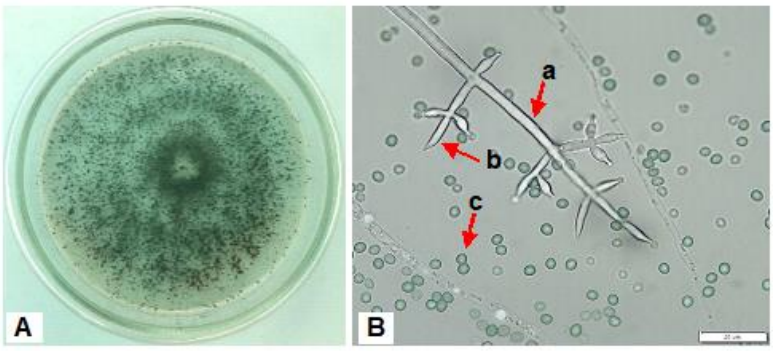

Figure 1. TJ isolates were (A) Macroscopic and (B) Microscopic in appearance. (a) conidiophore, (b) phialide, and (c) conidia.

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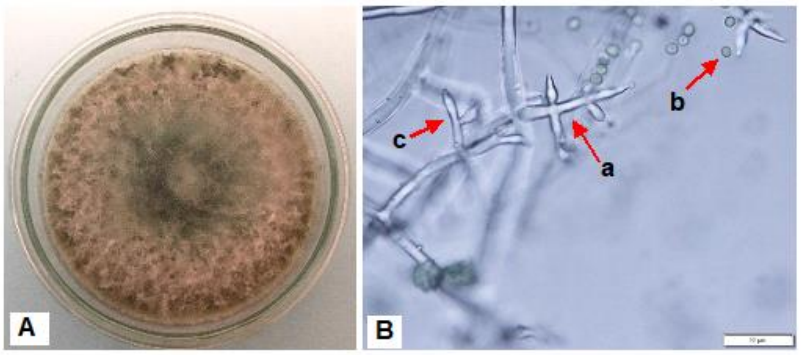

Figure 2. TKH isolates were (A) Macroscopic and (B) Microscopic in appearance. (a) conidiophore, (b) conidia, and (c) phialide.

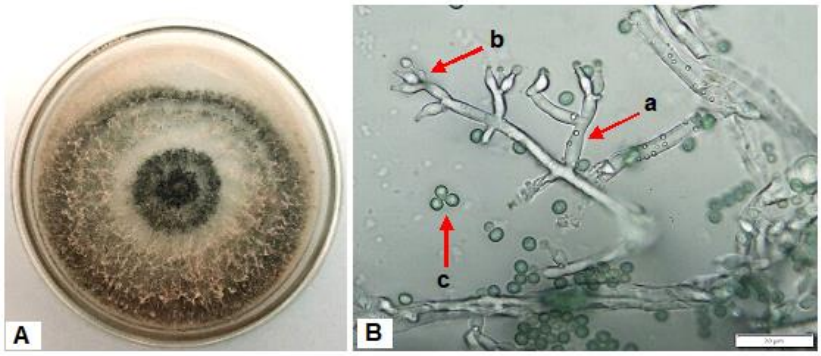

Figure 3. TST isolates were (A) Macroscopic and (B) Microscopic in appearance. (a) conidiophore, (b) phialide, and (c) conidia.

Table 1. Percentage of inhibition rate of F. *oxysporum* by three isolated of *Trichoderma* spp. from two to seven days of incubation

Isolate	Percentace of Inhibition rate at each incubation day											
	2 days ($\bar{x}$ ± SE)		3 days ($\bar{x}$ ± SE)		4 days ($\bar{x}$ ± SE)		5 days ($\bar{x}$ ± SE)		6 days ($\bar{x}$ ± SE)		7 days ($\bar{x}$ ± SE)	
Control	0.0±0.0	a	0.0±0.0	a	0.0±0.0	a	0.0±0.0	a	0.0±0.0	a	0.0±0.0	a
TJ	10.0±8.2	ab	21.6±7.3	abc	42.8±7.6	cd	51.8±4.4	cd	61.6±2.3	cd	65.6±2.4	c
TKH	11.1±8.2	ab	30.8±12.1	bc	52.6±12.0	e	61.5±1.4	d	63.7±0.4	cd	63.0±0.7	c
TST	15.0±6.2	ab	12.0±7.6	abc	29.6±1.4	bc	42.7±5.0	bc	54.5±2.7	cd	61.2±1.7	c

Note: SE: Standat Error. Each treatment was done in three repetitions. The above data has been evaluated using the Duncan Multiple Range Test at $\alpha < 0.05$. The column following the inhibition percentage shows that the same letter indicates the treatment dose not show a statistical difference.

The colony of *Trichoderma* is yellowish green coloured and on PDA medium. Branched conidiophore carrying spore mass in each phialides. The form of the phialides is mostly vertical, short, and thick with the size of 8.5–11 × 2.4–2.7 µm. The phialosphorous conidia characteristics are hyaline-coloured, round, and single-celled with the size of 2.4–2.7 × 2.1–2.5 µm. For the chlamydospores, the colour is pale chocolate, sub globose, and grained with the size of 5.2–7.5 µm (Watanabe, 2002).

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Antagonistic activity of Trichoderma spp. against Fusarium oxysporum

Based on the result of *in vitro* antagonistic activity from three *Trichoderma* spp. for 3-7 days, we got variations of the result. The results are shown in Table 1

Based on Table 1, the test control has an inhibition rate of 0%. Meanwhile, on the sixth day, TKH isolate has a higher inhibition rate than TJ and TST isolate. However, on the seventh day, TJ isolate has a higher inhibition rate than TKH and TST isolate with an inhibition rate of 65.6%, 63.0% dan 61.2%, respectively. The result of *in vitro* test shows that the three isolates can inhibit the growth of F. *oxysporum* pathogen, as shown by the rapid growth of *Trichoderma* compared to F. *oxysporum*. These results comply with the findings of the previous research (Larran et al., 2020) which shows faster growth of T. *harzianum* compared to F. *sudanense*. Thus, shows the potential of T. *harzianum* as a biocontrol agent. *Trichoderma* sp. is the most commonly used biocontrol agent for a broad spectrum of root, shoot and postharvest pathogens. The known biological control mechanisms of *Trichoderma* are direct antagonism of phytopathogenic fungi consisting of antibiosis, competition, direct attack with hydrolytic enzymes (Woo et al., 2014), mycoparasitism, and induced systemic resistance (ISR) (Gorai et al., 2020). On previous study (Tchameni et al., 2017) T. *asperellum* can inhibit the in vitro growth of *Phytophthora megakarya* by means of mycoparasitism and necrotrophy by destroying the cells in the hyphae and colling the pathogenic hyphae.

In vivo an antagonistic activity test of the three *Trichoderma* spp. was done by applying the three *Trichoderma* on the rootstock seeds of RL and JC citrus varieties for 12 weeks. The disease occurrence is increasing every week as shown in Table 2.

Based on Table 2, on control RL and JC, disease occurrence starts at the 8th week and increases to the 12th week. The disease occurrence of the control RL in the 12th week is 36.7 %. On RL treatment using TJ and JC using TKH, the disease occurrence starts at week 8th and is stagnant to week 12th with a disease occurrence of 3.33%. RL treatment using TKH and TST as well as JC treatment using TKH and TST disease occurrence starts on week 7th. All four treatments disease occurrence gradually increases until the 12th week with disease occurrence of 13.3 %, 10.0 %, 16.7 %, and 6.7 %, respectively. Treatment of RL using TJ and JC using TKH shows the lowest and the most stagnant percentage of disease occurrence compared to other treatments. Thus, showing that the *Trichoderma* applied successfully inhibits the growth of the pathogen causing Fusarium rot. Based on the previous study (Taufiq, 2012), *Trichoderma* can inhibit the growth pathogen for up to 66.67 % dan 68%. The use of good and resistant citrus rootstock can lessen the loss caused by Fusarium rot disease (Ahsen et al., 2019). Based on previos study (Adnan et al., 2019) the success of *Trichoderma* sp. as an antagonist caused by its ability to survive in various unfavorable conditions, high reproductive capacity, efficient utilization of nutrients, ability to modify the rhizosphere, and strong aggressiveness against plant pathogenic fungi.

Table 2. Percentage of the disease occurrence for eight treatments from 7th to 12th weeks

Treatment	Percentage of disease Occurrence at chosen weeks					
	7th week ($\bar{x}$ ± SE)	8th week ($\bar{x}$ ± SE)	9th week ($\bar{x}$ ± SE)	10th week ($\bar{x}$ ± SE)	11th week ($\bar{x}$ ± SE)	12th week ($\bar{x}$ ± SE)
Control RL	0.0±0.0 a	10.0±0.0 a	20.0±0.0 c	26.7±2.7 b	30.0±4.7 c	36.7±7.2 d

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Treatment	Percentage of disease Occurrence at chosen weeks											
	7th week ($\bar{x}$ ± SE)		8th week ($\bar{x}$ ± SE)		9th week ($\bar{x}$ ± SE)		10th week ($\bar{x}$ ± SE)		11th week ($\bar{x}$ ± SE)		12th week ($\bar{x}$ ± SE)	
Control JC	0.0±0.0	a	6.7±2.7	a	16.7±2.7	bc	26.7±2.7	b	33.3±5.4	c	33.3±5.4	cd
RL TJ	0.0±0.0	a	3.3±2.7	a	3.3±2.7	a	3.3±2.7	a	3.3±2.7	a	3.3±2.7	a
RL TKH	3.3±2.7	a	6.7±2.7	a	10.0±4.7	ab	10.0±4.7	a	13.3±7.2	a	13.3±72	ab
RL TST	6.7±2.7	a	6.7±2.7	a	6.7±2.7	a	10.0±4.7	a	10.0±4.7	ab	10.0±4.7	ab
JC TJ	3.3±2.7	a	0.0±0.0	a	6.7±2.7	a	6.7±2.7	a	10.0±0.0	ab	16.7±2.7	b
JC THK	0.0±0.0	a	3.3±2.7	a	3.3±2.7	a	3.3±2.7	a	3.3±2.7	a	3.3±2.7	a
JC TST	3.3±2.7	a	3.3±2.7	a	3.3±2.7	a	3.3±2.7	a	6.7±2.7	ab	6.7±2.7	ab

Note: SE: Standard Error, RL TJ: seed rootstock Rough Lemon with TJ isolate, RL TKH: seed rootstock Rough Lemon with TKH isolate, RL TST: seed rootstock Rough Lemon with TST isolate, JC TJ: seed rootstock Japansche Citroen with TJ isolate, JC TKH: seed rootstock Japansche Citroen with TKH isolate, and JC TST: seed rootstock Japansche Citroen with TST isolate. Each treatment consists of a group of three. The above data has been evaluated using the DMRT at $\alpha < 0.05$. The column following the inhibition percentage shows that the same letter indicates the treatment does not show a statistical difference.

Conclusion and suggestion

Based on *in vitro*, *Trichoderma* has been proven to inhibit the growth of F. *oxysporum* on PDA medium. Meanwhile, *in vivo* tests shows that the application of *Trichoderma* isolate (TJ and TKH) can suppress the Fusarium rot disease on RL and JC rootstock seeds. Thus, *Trichoderma* can be used as a biocontrol agent to inhibit the Fusarium rot disease on citrus. However, further research is needed to identify the species of *Trichoderma* on TJ and TKH isolates.

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