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Antagonistic Compatibility of *Streptomyces griseorubens, Gliocladium virens,* and *Trichoderma harzianum* Againts *Fusarium oxysporum* Cause of Tomato Wilt Deseases

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Authors' contributions

This work was carried out in collaboration between all authors. Author PS designed the study, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches, author Kusriningrum performed the statical analysis. Authors Ni'matuzaroh and TS managed the analysis of the study. All authors read and approved the final manuscript.

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ABSTRACT

This research was intended to discover the compatibility of biological control agents (BCAs) *Streptomyces griseorubens, Gliocladium virens,* and *Trichoderma harzianum* againts *Fusarium oxysporum* f.sp. *capsici in vitro* and *in vivo.* Study was done in rainy season 2012-2013 at East Java-Indonesia. Study was a true experiment designed, using a completely randomized design (CRD), consist of three stages research. These stages were the compatibility of three biological agents in Potato Dextrose Agar (PDA) medium, antagonistic test of biological agents combinations of *S. griseorubens, G. virens, and T. harzianum* capable inhibited microbial pathogens *F. oxysporum* f.sp. *lycopersici* in the laboratory and at the screen house. Mixed of BCAs *S.*

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griseorubens, G. virens, and T. harzianum were compatible and effectively against *F. oxysporum* in Petri dishes and at screen house. Clear zona avarage of antibiosis of BCAs filtrat in Potato Glucose extract shown that the antibiosis from mixed of *S. griseorubens* and *T. harzianum* was higher than the antibiosis of other BCAs treatment. Plant infested with mixed of BCAs significantly protected plant tomato from *F. oxysporum* compared to the untreated control plants. Plant protection by BCAs mixed of *T. harzianum* with *S. griseorubens* was more pronounced than plant protection by mixed of *S. griseorubens* with *G. virens* and single BCAs.

Keywords: Wilt disease; antagonistict; mixed of BCAs.

1. INTRODUCTION

Fusarium oxysporum f.sp. lycopersici (FOL) is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease caused by this fungus is characterized by wilted plants, yellowed leaves and minimal or absent crop yield [1,2]. Based on our survey in East Java (Malang, Pare, Kediri) there was a 10 to 12% yield loss.

F. oxysporum is soil-borne plant pathogens and the most difficult to control. Chemical control effect negative to enviroment, there is renewed interest in biological control based on application of populations of antagonistic micro-organisms. [3,4]. Soil microorganism challenge to be BCAs, the advance of technology these days has come to the application on field, by hoping to be able to make efficient of natural resources, conservation and the everlasting environment, also to produce cheaper and healthier agriculture products [5]. Some saprophyte soil microorganism have been used as comercial biological agents, It can be single antagonistic or multi antagonistic. In the presence of flourencent *Pseudomonas* reduced the affect of the *Fusarium oxysporum* on *L. esculentum* growth [6]. Compatibility of flourencent *Pseudomonas* and *Streptomyces* couse of the adaptation ability to soil humidity [7].

The research and the usage of *S. griseorubens* as biological agent is rarely conducted in agriculture field especially in Indonesia, meanwhile the bacteria, fungus and virus had been through many researches. *T. harzianum* is a soil saprophytic fungus able to become hyperparasitic to several species of fungal pathogen. The growth of *T. harzianum* is very rapid and none pathogenic. The hyphae threads of pathogenic fungus will be cut to pieces because it winded by *Trichoderma* hyphae (as antagonist fungus). *Trichoderma* eventually release antibiotic to phatogenic fungus which is glicotoxin [8,9,10,11,12]. *G. virens* control plant pathogen by several mechanisms such as parasitism, antibiosis, competition and cell destruction. *Gliocladium* will grow around the pathogen and release enzyme that can destroy chitin of pathogen. *G. virens* also producing glicotoxin antibiotics [13] *S. griseorubens, G. virens* and *T. harzianum* as the single antagonist is able to control fusarium wilt disease on tomato and melon in the greenhouse scale [1,14]. This research was intended to discover the biological agents *S. griseorubens, G. virens,* and *T. harzianum* capable inhibited microbial pathogens *F. oxysporum* f.sp. *lycopersici* in the laboratory and in the screen house.

2. METHODS AND MATERIALS

2.1 Isolation of Biological Agents

Isolation of biological agents used soil platting method by Dhingra and Sinclair [15]: 1 gram of Pare-Kediri chilli and tomato soil was made suspension by dilution 10^4. Subsequently 1 mL of soil suspension was spreaded on Glucose Nutrienth Agar (GNA) to get *S. griseorubens* [16] and 1 mL of *T. harzianum* and *G. virens* suspension (BPTPH Pandaan) also spreaded on Potato Dextrose Agar (PDA). Biological agents obtained was purified and propagated on PDA in Petri dishes.

2.2 Isolation of *F. oxysporum f.sp. lycopersici*

Cut into the base of the stem of a diseased plant lengthwise to reveal the xylem just below the epidermis. Turn off all the leaves and secondary roots, leaving only the main stem and the hypocotyls and main root. Stem sterilization was done by soaking in 10% bleach solution for 5 minutes. Dry the stem on paper towels. Using sterile technique, cut thin: 2-4 mm wedges out of one side of the stem near the root/stem junction making sure to include xylem tissue with each wedge. Placed 5-6 wedges on PDA

plates. Incubate the plates under fluorescent lights. Once the fungus has grown sufficiently from the pieces, transfer isolates onto fresh PDA plates. Incubate the plates for 10-14 days. Colonies of *F. oxysporum* are pigmented with a reddish purple color and surrounding by a pinkish white aerial mycelium [17].

2.3 Compatibility Test

Compatibility test was done in Microbiology laboratorium of Agricultural Faculty by watching the type of biological agents growth in discriptive and colony diameter, Every treatment was repeated five times. Colony diameter average were analyze by t-test [18].

Preparing 0.5 cm colony diameter of 10^{th} day biological agents, *S. Griseorubens T. harzianum, G. virens*. Compatibility tests on the PDA media plating in 20 diameter cm Petri dishes were three types of biological agents colony *S. griseorubens, G. virens,* and *T. harzianum* (SGT) were placed on PDA medium, each with the same distance of 5 cm, and were incubated for 14 days. Along with the treatment of compatibility, also it was prepared control treatment as a comparison.

2.4 Antagonistic *in vitro* test

Antagonistic *in vitro* test was done by completely randomized design (CRD) with seven treatment and every treatment was repeated three times. Data of percentage inhibition was analyzed by Duncan test suspension of biological agents treatment was made: 6 mL was mixed up in 44 mL of sterile water, 0.33 cc of this each suspension biological agents treatment was taken into holes (wells). Then put the 0.5 cm colony of *F. oxysporum* 7 days age, in the presence of biological agents suspension that has been inoculated in the hole at a distance of 5 cm. Each treatment was randomly stored at room temperature for 8 days. The inhibition was calculated by the formula [19]:

$$DI = \frac{Dc - Dt}{Dt} \times 100$$

DI is the percentage inhibition;
Dc is the colony diameter of the control treatment;
Dt is the colony diameter of BCAs treatment.

2.5 Antibiosis in *Potato Glucose Extract* Medium

Antibiosis test consist of 7 treatment placed in randomly, every treatment was done in triplicate. Six mili liter of BCAs (every BCA 38-42 spore/cc) treatment entered into 44 mL Potato Glucose Extract solid medium in Erlenmeyer flasks. BCAs suspension was shaken. Inhibition ability of antibiosis filtrate of BCAs againts *F. oxysporum* was done by Steinkelner method. 0.55 cc antibiosis was given into 0.5 cm Whatman paper disc, then air drying them. These paper disc was inoculated on PDA medium in Petri dish contains *F. oxysporum* suspension. Inhibition ability of antibiosis was done by counting of clear zone diameter [20].

2.6 Antagonism *In vivo* Test

This research was done by Completely Randomized Design (CRD) with seven treatmen and every treatment was done in triplicate. Data of percentage inhibition was analyzed by Duncan's t-test.

Seedling was inoculated by soaking a solution of inoculum combination of biological agents that have been prepared before. Furthermore, the seed was planted in the soil that had been inoculated *F. oxysporum* suspenstion (10^9 spore/mL) for 14 days. It was prepared by filled 3 liters of sterile soil in polybags and inoculated with spore inoculum mass suspension of *F. oxysporum* has been sprayed with a hand sprayer at ground level [15]. Stored for 14 days in the screen house and watering with sterile water every day. Data were collected for: [21] Phase of incubation, performed daily until symptoms of disease were yellowing of the leaves from the bottom, where the control healthy plants not showing symptoms [3]. Disease severity, was conducted with most of the leaves are yellow, wilt and dry every 7 days until harvest [22].

$$KP = \frac{\text{count of yellowing leaf per plant}}{\text{count of all leaf of plant}} \times 100\%$$

KP is the percentage of disease severity per plant [3]

3. RESULTS AND DISCUSSION

3.1 Compatibility Test

Performance of three biological agents *S. griseorubens* (S) *G. virens* (G) and *T. harzianum*

(T) showed none antagonistic, grew thicker than single biological agent (Fig. 1).

Growth average of colony diameter *S. griseorubens* (S) *G. virens* (G) and *T. harzianum* (T) in compatibility test (SGT) on 2nd day after inoculation showed none significantly different when compared with controls (SSS, GGG, TTT). However, on 4th and 6th day after inoculations, diameter growth of biological agents such colony in compatibility testing (SGT) was larger and significantly different than the control. On 8th day, the growth of biological agents on a compatibility test was not different from the control of biological agents (Fig. 2).

S. griseorubens, G. virens and *T. harzianum* as biological agents could grow on PDA media and formed an association that does not harm each other or not produced secondary metabolites that could inhibit the growth of biological agents each other. The three biological agents are saprophyte soil microbes, and produce antibiosis to mycoparasite only affect microbial pathogens. This opinion is supported by the results of some other research, plant pathogenic fungi cell walls composed of chitinase which is a key enzyme and responsible for the lysis of the cell wall. *S. griseus* degrade fungal cell walls by lytic enzymes [19]. *Trichoderma* sp. produced lytic enzyme that degraded chitin, interfungus parasitism and can improve the cell wall itself on the division process [23]. *Gliocladium* sp. and *Trichoderma* sp. produce chitinase enzymes that can cause parasitic on plant pathogens, whereas less effective antibiosis produced degrades *F. oxysporum* [13]. The existence of microbes of different biological agents also induce the microbes to grow faster. As noted by some researchers that *Trichoderma* sp. and *Gliocladium* sp. produce fungal pathogen that work in synergy with the intracellular enzyme produced by *G. virens*. Both of these biological agents was synergy in controlling pathogenic tomatoes until 57% [11,24].

3.2 Antagonism Compatibility *In vitro*

Giving a single biological agents *S. griseorubens (S), G. virens (G), T. harzianum (T)*, a mix of two biological agents (SG, ST, GT) and a mix of three biological agents (SGT) to tomato seed, significantly inhibited the development of the colony diameter of *F. oxysporum.* Giving only *G. virens* to tomato seed shown small and significantly diffrent average inhibition than the other single of biological agents *S. griseorubens* and *T. harzianum* and the combination treatment (Table 1).

Table 1. Inhibitors average of biological agents *S. griseorubens (S), G. virens (G), T. harzianum* (T) against *F. oxysporum*

Treatment of BCAs	Inhibitor average±SE(%)
S. griseorubens, *T. harzianum* (ST)	63.97±7.27[a]
G. virens, *T. harzianum* (GT)	59.29±3.87[ab]
S. griseorubens, *G. virens,* *T. harzianum* (SGT)	59.07±1.82[ab]
S. griseorubens, *G. virens* (SG)	58.41±1.93[ab]
T. harzianum (T)	49.05±3.41 [ab]
S. griseorubens (S)	45.11±5.37[b]
G. virens (G)	40.24 ±7.27[c]

Explanation: The same letters which added behind the numbers indicate none significant difference in the Duncan's t-test (p < 0.05)

Single biological agent *G. virens* (G), have ability to inhibit microbial pathogens *F. oxysporum* (F) lower than the inhibition ability of single biological agents *T. harzianum, S. griseorubens* (T .S), and mix of biological agents (TS, GS, TG and TGS). *G. virens* serves only as competitors and parasitic. They did not produce antibiosis on PDA media (*in vitro*) [13,4,15]. Antibiosis of biological agents *G.virens*, *T. harzianum* and *S. griseorubens* on Potato Glucose Extract (PGE) also proved that *G. virens* did not produce antibiosis on observations 2nd, 4th, 6th day after the inoculation of biological agents. Single BCA *T. harzianum* and *S. griseorubens* and combination of BCAs on observation produced antibiosis (Table 2).

This suggests that a mix of all three biological agents produced antibiosis derived from *T. harzianum* and *S. griseorubens f.sp. capsicum*. Single biological agents *T. harzianum* (T) and a mix of biological agents (TG, TS, GS and SGT) can result inhibition ability higher than inhibition ability of single biological agents *S. griseorubens* (S) and *G. virens* (G). Several studies have shown that the fungus *Trichoderma* sp. is a saprophyte fungus that lives in the soil and becomes hyper parasite on some pathogenic fungi. *T. harzianum* also inhibit the growth of *F. oxysporum* colonies growing very rapidly, and producing antifungal namely Glicotoxin. *Gliocladium* sp., *Streptomyces* sp. *Trichoderma* sp. clasified as soil saprophyte fungi and used as biological agents have multi antagonistic mechanism and compatible againts *F. oxysporum* [20].

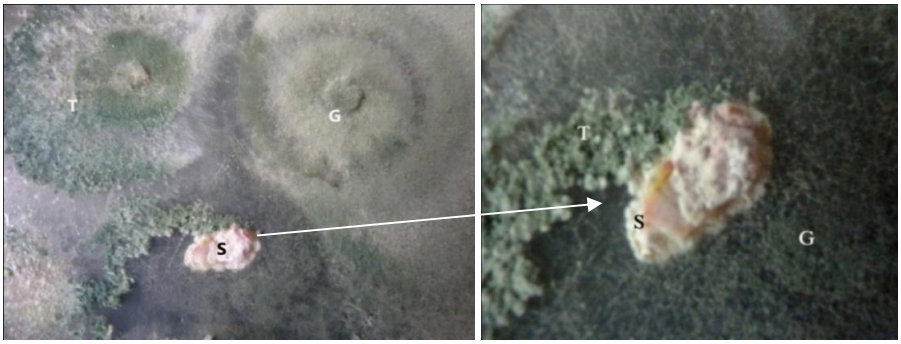

Fig. 1. *T, harzianum* colony(T), *S. griseorubens* colony (S), *G. virens* colony (G) on PDA medium, 14th day after inoculations BCAs

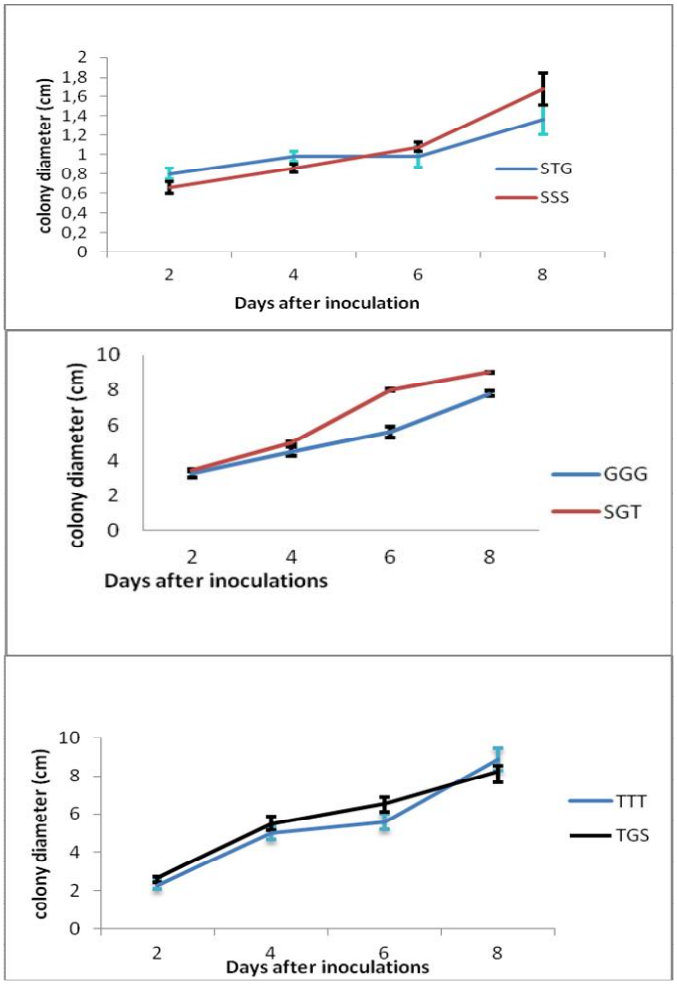

Fig. 2. Graphic of biological agents colony diameter on compatibility test. SGT/GTS/TGS are the same treatment, consist of *S. griseorubens, G.viren, T. harzianum,* Control consist of three colony of GGG (*G.viren*), TTT (*T. harzianum*), SSS (*S. griseorubens*.)

Table 2. Avarage of Inhibition zone of biological agents crude extract to *F. oxysporum*

No	Biological agents giving	Clear zone (....mm)		
		2 dai	4 dai	6 dai
1	*T. harzianum*	0.2	0.2	0.3
2	*G. virens*	0	0	0
3	*S. griseorubens*	1.3	0.4	0
4	*G. virens, T. harzianum.*	0	0	0
5	*T. harzianum, S. griseorubens*	1.2	0.7	0.2
6	*G. virens. S. griseorubens*	0.3	0.3	0
7	*G. virens, S. griseorubens* and *T. harzianuim*	0	0.4	0.2

Notes: Dai is day after inoculations

3.3 Antagonistic Compatibility *in vivo*

Tomato plants that were not given biological agents showed fusarium wilt symptoms on 39-40th days after planting tomato seedlings. Meanwhile, the plants treated with biological agents, appeared symptoms at 46-50th days after planting. Longer incubation occured because of biological agents mixture could inhibit the development of *F. oxysporum*. Giving of biological agents with the pathogen in the soil for one week, the incubation period occurred after 45 days [25]. Single biological agents *S. griseorubens* (S) and a mix of biological agents, *S. griseorubens* with *G. virens* (SG), *S. griseorubens*, with *T. harzianum* (ST), and a mixed of *S. griseorubens*, *G. virens*, *T. harzianum* (SGT) can inhibit disease severity (Table 3).

Each of these biological agents inhibit the development of pathogens with multiple mechanisms and can develop optimally in soils containing organic matter, so that they can be complement each other. Based on several studies, the *Trichoderma* sp., *Streptomyces* sp., and *Gliocladium* sp., were antagonistic to fungal and bacterial diseases of plant roots [22,25,20]. Three biological agents can flourish together on compost, manure and garden soil . In synthetic medium, *S. griseorubens* did not develop optimal, but developed optimal in field conditions. The study also found that for 4 weeks, the average population of biological agents *S. griseorubens* more higher than the avarage populations of biological agents *T. harzianum* [12].

On 55th and 62 day after planting, combination of biological agents *T. harzianum* and *S. griseorubens* demonstrated lower power resistor ability of severity disease than power resistor ability of other biological agents combinations. Several studies in the screenhouse and in the field proved that inhibit ability of *T. harzianum* to pathogens was lower than inhibit ability of *G. virens* in soil. Otherwise, *Gliocladium virens* grew very fast and produced antibiotics gliovirin. Antibiotics worked in synergy with intracellular enzymes to inhibit the development of fungal plant pathogens [22,26]. Providing a mixed of biological agents *T. harzianum* (T), *G.virens* (G), *S. griseorubens* (S) can also enhance plant growth and provide organic material for plants as planting soil decomposition. Biological agents can also induce the growth of pheriphere new roots more and replaces the root of suffering discoloration, can repair the affected plant roots by *F. oxysporum* also [27,28].

Table 3. Avarage of wilt desease severity of tomato plant at 41th, 48th, 55th and 62th day after planting (dap)

No	Giving of biological agents	Avarage of deseases severity (%)			
		41 dap	48 dap	56 dap	62 dap
1	Control (withaot bcas) (K)	3.80±2.19^{a}	7.60±0^{a}	17.86±1.57^{a}	44.64±3.30^{a}
2	*S. griseorubens, T. harzianum.* (ST)	0.00±0^{a}	5.70± 1.9^{a}	7.14± 5.91^{a}	19.65±3.41^{b}
3	*S. griseorubens* (S)	0.00± 0^{a}	1.90±1.9^{a}	15.42±2.90.^{a}	16.07±7.46^{b}
4	*S. griseorubens, G. virens* (SG)	1.90± 1.9^{a}	3.80±2.19^{a}	7.15± 4.13^{a}	16.07±7.36^{b}
5.	*S. griseorubens, G. virens,T. harzianum* (SGT)	1.52±1.9^{a}	5.77±5.77^{a}	5.77±3.77^{a}	14.29±5.63^{b}

Explanation: The same alphabet beside the number in the same coloum shown insignificant Duncan test ($p < 0.05$)

4. CONCLUSION

S. griseorubens, G. virens and *T. harzianum* as biological agents were compatible grow on PDA media and formed an association that does not harm each other or not produced secondary metabolites that could inhibit the growth of biological agents each other. A single biological agents *S. griseorubens (S), T. harzianum (T)*, a mix of two biological agents (SG, ST, GT) and a mixed of three biological agents (SGT) more inhibited the development of the colony diameter of *F. oxysporum* than a single biological agent *G. virens in vivo.* Giving mix of two biological agents *S. griseorubens* and *G*. *virens* as well as *S*. *griseorubens* and *T. harzianum* as well as three biological agents *S*. *griseorubens*, *G. virens* and *T. harzianum* to inhibit disease severity of tomato fusarium wilt caused by *T. oxysporum f.sp*. *lycopersici.*

CONSENT

All author declare that written informed consent was obtain from the approved of our research parties

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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