Original Research Article

ANTAGONISTIC COMPATIBILITY OF *Streptomyces griseorubens, Gliocladium virens,* and *Trichoderma harzianum* AGAINTS *Fusarium oxysporum* CAUSE OF TOMATO WILT DESEASES.

ABSTRACT

This research was intended to discover the compatibility of biological control agents (BCAs) *Strepromyces 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 tree stages research. First stage of study was the compatibility of the three biological agents in sintetis medium (PDA). In the next research phase, were antagonistic test of biological agents combinations *S. griseorubens, Gliocladium virens, and Trichoderma harzianum* capable inhibited microbial pathogens *F. oxysporum* f.sp. *lycopersici* in the laboratory and in the screenhouse. Mixture of BCAs *S. griseorubens, G. virens, and T. harzianum* were compatibel and effectively against *F. oxysporum* on Petri dishes and at screenhouse. Clear zona avarage of antibiosis of BCAs filtrat in Potato Glocose extrac shown the antibiosis of *S. griseorubens* and *T. harzianum* mixtured higher than the antibiosis of other BCAs treatment. Morewere plant infested with mixture of BCAs significantly protected plant tomato from *F. oxysporum* compared to the untreated control plants. Plant protection by BCAs mixture of *T. harzianum* with *S. griseorubens* was more pronounced than plant protection by mixture of *S. griseorubens* with *G. virens* and single BCAs. Soil filtrate that was inoculation with mixtured of biological agents *G. virens* (G), *T. harzianum* (T), and *S. griseurubens* (S), resulted antibiosis which could inhibit the growth of fungal pathogens *F. oxysporum.*

Keywords: Wilt deseases, Antagonitic, mixture of Biological Agents (BCAs)

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. There may be a 30 to 40% yield loss (5**).** Fungal phytopathogens are cause of many plant diseases and much loss of crop yields, especially in tropical and subtropical regions**.** *Fusarium oxysporum* is major soilborne fungal pathogens of both greenhouse and field grown tomatoes in the warm vegetable growing areas of the world (14).

Plant diseases induced by soil-borne plant pathogens are among the most difficult to control. In the absence of effective chemical control methods, there is renewed interest in biological control based on application of populations of antagonistic micro-organisms. (2). Although lots of challenge occurs, the advance of soil microorganism 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 (7). The research and the usage of *Actinomycetes* as an biological agent is rarely conducted in agriculture field especially in Indonesia, meanwhile

the bacteria, fungus and virus had been through many researches. *Trichoderma sp.* fungus is a saprofyt fungus that lives in the ground and able to become hyperparasite to several species of pathogen fungus. The growth of *Trichoderma sp.* is very rapid and not becoming disease for high level plants. The hypha threads of pathogenic fungus will be cut to pieces because it winded by *Trichoderma* hypa (as antagonist fungus). *Trichoderma* eventually release deadly antibiotics to harmful fungus which is Glicotoxin (9). *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 pathogen cuticle. *G. virens* also producing glicotoxin antibiotics (11) *S. griseorubens, G. virens* and *Trichoderma sp.* as the single antagonist is able to control *fusarium* wilt disease on tomato and melon in the greenhouse scale (10,20). This Research was intended to discover the biological agents *S. griseorubens* f.sp, *capsicum, Gliocladium virens, and Trichoderma harzianum* capable inhibited microbial pathogens *F. oxysporum* f.sp. *lycopersici* in the laboratory and in the screenhouse. *in vitro* and *in vivo.*

Isolation of biological agents used soil plating method by Dhingra and Sinclair (19): 1 gram of chilli and tomato soil weigh with an analytical balance, then made the suspension by dilution 10^4. Subsequently 1 mL of *Streptomyces griseorubens* suspension was spreaded on Glucose Nutrienth Agar (GNA) 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.

2.1 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. Trim off all the leaves and secondary roots leaving only the main stem and the hypocotyls and main root. Surface sterilize the stem by soaking in 10% bleach solution for 5 minutes. Dry the stem on paper towels. Using sterile technique, cut thin (2-4 mm thick) wedges out of one side of the stem near the root/stem junction making sure to include xylem tissue with each wedge. Place 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 surmounted by a pinkish white aerial mycelium (16).

2.2 Compatibility test

Preparing 0,5 cm diameter colony of 10^{th} days biological agents, *Streptomyces 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 Trichoderma 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.

2.3 Antagonism compatibility *in vitro* test

6 mL suspension biological agents treatment was mixed up in 44 mL of sterile water , 0.33 cc of this each suspension biological agents treatment was taken into holes (wells) wich have been made before. 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 (8):

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

DI is the percentage inhibition;
Dc is the diameter of the control;
Dc is the diameter of inhibition.

2.4 Antibiosis in Potato Glucose Extrac medium

6 mL of BCAs treatment entered into 44 mL Potato Glucose Extrac solid medium in Erlenmeyer tubes. BCAs suspension was shaken. Inhibition ability of antibiosis of BCAs filtrat 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 diametre (24).

2.5 Antagonism in vivo 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* 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 (19). Stored for 14 days in the screen house and watering with sterile water every day. Data were collected for: (1) The period of incubation, performed daily until symptoms of disease were yellowing of the leaves from the bottom, where the control healthy plants not showing symptoms. (2) The severity of the disease, was conducted with most of the leaves are yellow, wilt and dry every 7 days until harvest (3).

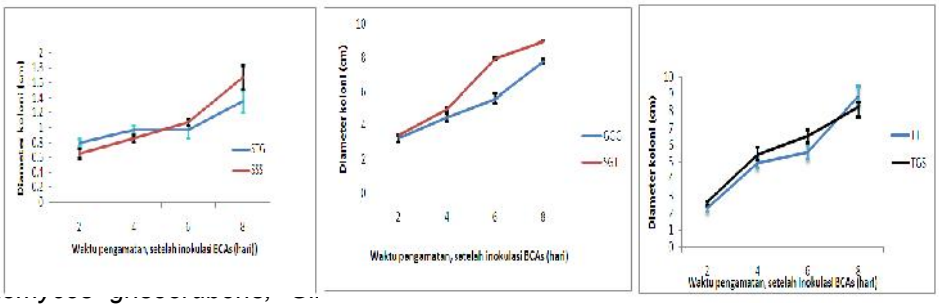
$$KP = \frac{\text{count of yellowing leaf}}{\text{count of all leaf of plant}} \times 100\%$$

KP is the percentage of disease severity(3)

3. RESULTS AND DISCUSSION

3.1 Compatibility test

Growth average of diameter colony *S. griseorubens* (S) *G.virens* (G)and *T. harzianum* (T) in compatibility test (SGT) on 2 nd days after inoculation showed no significantly different when compared with controls (SSS, GGG, TTT). However, on 4 th and 6th days after inoculations, diameter growth of biological agents such colony in compatibility testing (SGT) was larger and significantly different than the control. On 8 thdays, the growth of biological agents on a compatibility test was not different from the control of biological agents (Fig.1)



Figu

Strep biological agents could grow together on PDA 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 is a saprophyte soil microbes, and produce antibiosis mycoparasite only affect microbial pathogens. This opinion is supported by the results of research, plant pathogenic fungi cell walls composed of chitinase which is a key

enzyme and responsible for the lysis of the cell wall. *Streptomyces griseus* degrade fungal cell walls by lytic enzymes (8). *Trichoderma* sp. produced lytic enzyme that degraded chitin, mycoparasit process and can improve the cell wall itself on the division process (15). *Gliocladium* sp. and *Trichoderma* sp. produce chitinase enzymes that can cause parasitic on plant pathogens, whereas less effective antibiosis produced degrades *F. oxysporum* (11). 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% (21,22).

3.2 Antagonism compatibility in vitro

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

Tabel 1. Inhibitors average of biological agents *S. griseorubens (S), G. virens (G)* dan *T. harzianum* (T) againts *F. oxysporum*

Treatment of BCAs	Inhibitor average (%)
S. griseorubens, T. harzianum (ST)	63,97 [a]
G. virens, T. harzianum (GT)	59,29 [ab]
S. griseorubens,G. virens, T. harzianum (SGT)	59,07 [ab]
S. griseorubens, G. virens (SG)	58 ,41 [ab]
T. harzianum (T)	49,05 [ab]
S. griseorubens (S)	45,11 [b]
G. virens (G)	40,24 [c]

Explanation : The same letters in addition to numbers showed no significant difference in the Duncan level test.

Single biological agents *S. griseurubens* (S), *G.virens* (G), and *T. harzianum* have Inhibition ability of microbial pathogens *F. oxysporum* (F) lower than the inhibition ability of biological agents combination (TS, GS, TG and TGS). This is because *G.virens* serves only as competitors and parasites,and did not produce antibiosis on PDA (11,15,21). Antibiosis biological agents *G.virens*, *T. harzianum* and *S. griseorubens f.sp. capsicum* on Potato Glucose Extrac (PGE) also proved that *G.virens* not generate antibiosis on observations 2^{nd},4^{th},6^{th} days after inoculation biological agents (Table 2).

Table 2. Avarage of Inhibitor zone of biological agents crude extrac to *F. oxysporum*

No	Biological agents giving	Clear zone (halo) .....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* dan T. *harzianum.*	0	0	0
5	*T. harzianum* dan *S. griseorubens*	1,2	0,7	0,2
6	*G. virens* dan *S. griseorubens*	0,3	0,3	0
7	*G. virens , S. griseorubens* and *T. harzianuim*	0	0,4	0,2

Notes : Dai is days after inoculations

This suggests that a mixture of all three biological agents still produced antibiosis derived from *T. harzianum* and *S. griseorubens f.sp. capsicum*. Single biological agents *T. harzianum* (T) and a mixture 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 fungi that lives in the soil and becomes hyperparasite 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 used as biological agents and active ingredients of fertilizer biocompos (24).

3.4 Antagonistic compatibility in Screen house (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 (18). Single biological agents *S. griseorubens* (S) and a mixture of biological agents, *S. griseorubens* with *G.virens* (SG), *S. griseorubens*, with *T. harzianum* (ST), and a mixture of *S. griseorubens*, *G.virens*, *T. harzianum* (SGT) can inhibit disease severity (Table 3).

Table 3 Avarage of wilt deseases severity of tomato plant at 41th, 48th, 55th and 62th days 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 [a]	7,60 [a]	17,86 [a]	44,64 [a]
2	*S. griseorubens, T. harzianum.* (st)	0,00 [a]	5,70 [a]	7,14 [a]	19,65 [b]
3	*S. griseorubens* (s)	0,00 [a]	1,90 [a]	15,42 [a]	16,07 [b]
4	*S. griseorubens, G. virens* (sg)	1,90 [a]	3,80 [a]	7,15 [a]	16,07 [b]
5.	*S. griseorubens, G. virens,T. harzianum* (sgt)	1,52 [a]	5,77 [a]	5,77 [a]	14,29 [b]

Explanation: The same alphabet beside the number in the same coloum shown insignificant Duncan test ($p < 0,05$)

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 (3,18,24). This 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* (23).

On 55^{th} and 62^{th} days after planting, combination of biological agents *T. harzianum* and *S. griseorubens f.sp. capsicum* demonstrated lower power resistor ability of (high disease severity) than power resistor ability of other biological agents combinations. Several studies in the screenhouse and in the field proved that *T. harzianum* inhibit ability in soil borne pathogens was lower than *G.virens* inhibit ability. 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 (3,4). Providing a mixture of biological agents *T. harzianum* (T), *G.virens* (G), *S. griseorubens f.sp. capsicum* (S) can also enhance plant growth and provide organic material for plants as planting soil decomposition Giving biological agents can also grow new roots more and replaces the root of suffering discoloration and can repair the affected plant roots by *F. oxysporum* (17).

4. CONCLUSION

Biological agents *S. griseorubens*, *G.virens* and *T. harzianum* were compatible microbes to inhibit the development of *F. oxysporum* in vitro conditions. Mixture 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 f.sp. capsicum* f.sp. *capsicum*, *G.virens* and *T. harzianum* in inhibit ability of disease severity of tomato fusarium wilt caused by *F. oxysporum* f.sp. *lycopersici*.

Identification of *Streptomyces griseorubens* was done at Tropical Deseases Centre (TDC) of Airlangga University by squensing DNA 16 SRNA

CONSENT

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

REFERENCES

1. Abeysinghe, S. 2007. Biological control of *Fusarium solani* f.spp. *phaseoli* the causal agents of root rot of bean using *Bacillus subtilis CA 32* and *Trichoderma harzianum* RU01. Ruhuna Journal of Science. 2: 62-88.
2. Alabouvette, C., C. Olivain, Q. Migheli, C. Steinberg. 2009. Microbiological control of soil-borne phytopathogenic fungi with special emphasis on wilt-inducing *Fusarium oxysporum. On Line Journal* DOI: 10.1111/j.1469 - 8137.2009.03014.x
3. Anitha, A. and M. Rabeeth. 2010a. Control of Fusarium wilt tomato by bioformulation of *Streptomyces griseus* in green house condition. African Journal of Basic & Aplied Sciences 1 (1-2): 9 – 14.
4. _____ , 2010 [b]. Degradation of fungal cell walls of phytopatogenic fungi by lytic enzyme of *Streptomyces griseus.* African Journal of Plant Science . 4 (3): 061-066.
5. Asha, B., C. Nayaka, U. Shankar, Srinivas, Nirjana. 2011. Biological control of *F. oxysporum* f. sp. *lycopersici* causing wilt of tomato by *Pseudomonas fluorescens.* International Journal of Microbiology Research. 3(2): 79-84
6. Bollen, G.J. 1974. Fungal recolonization of heat-treated glasshouse soils. Agro Ecosystems I: 139-155
7. Cook J. R. and K. F. Baker. 1996. The nature and practice of biological control of plant pathogens. APS PRESS. The American Phytopathological Society. St. Paul, Minnesota.
8. Fahri, Y. and M. Dikilita. 2007. Control of fusarium wilt of tomato by combination of *Pseudomonas florescent*, non *patogen Fusarium* and *Trichoderma harzianum* T-22 in greenhouse conditions. Plant Pathology Journal 6(2) : 159-163.
9. Gruber, S. and V. Seiboth. 2012. Self versus non-self: fungal cell wall degradation In *Trichoderma*. *Microbiology* 158:26-34.
10. Kaewchai, S., Soytong, K., and Hyde, K.D. 2009. Mycofungicides and fungal biofertilizers. Fungal Diversity. 38: 25-50.
11. Kyeong, S. J., Hong, M. K., and Bong, K. C., 2000. Purification and antifungal activities of an antibiotic produced by *Gliocladium virens* G1 against plant patogen. Plant Patholohy Journal, J.17(1) : 53-56.
12. Larkin, R.P. and D.R. Fravel. 1993. Biocontrol of Fusarium Wilt of tomato. Biocontrol of Plant Deseases Laboratory. Bestvile
13. Menzies JG, Koch C, Seywerd F. 1990. Additions to the host range of *Fusarium oxysporum*f. sp. *radicis-lycopersici.* Plant Diseases. 74: 569–572.
14. Morid, B., S. Hajmansoor, N. Kakvan, 2012. Screening of resistance genes to fusarium root rot and fusarium wilt diseases in tomato (*Lycopersicon esculentum*) cultivars using RAPD and CAPs markers. European Journal of Experimental Biology. 2 (4):931-939

15. Olivain C., C. Humbert, J. Nahalkova, J. Fatehi, FL. Haridon, and C. Alobouvete. 2006. Colonitation of tomato root by phatogenic and non patogenic *Fusarium oxysporums* strains inoculated together and separately into the soil. Aplaid and Enviromental Microbiology. 72 (2): 1523-1531.
16. Reis A, Costa H, Boiteux LS, Lopes CA. 2005. First Report of *Fusarium oxysporum* f. sp. *lycopersici* Race 3 on Tomato in Brazil. Fitopatol. Bras. 30(4): 426-428.
17. Siddiquee, Shafiquzzaman, Soon G. T., and Y. U. Kalsum. 2010. Isozyme Analisis and Relationships Among Three Species in Malaysia *Trichoderma* Isolates. Mycrobial Biotechnol. 20(9): 1266 – 1275
18. Singh R.,B.K. Singh, R.S. Upadhyay, B. Rai and Y. S. Lee. 2002 Biological control of fusarium wilt disease of pigeonpea. Plant Pathology Journal 18(3) : 279-283.
19. Singleton, J.D. Mihail and C.M. Rush. 1993. Methods for research on soilborne phytopatogenik fungi . APS Press. The American Phytophatological Society. St. Paul Minesota.
20. Staniazsek M, Kozik EU, Marczewski W . 2007. A CAPS marker TAO1902 diagnostic for the I-2 gene conferring resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 2 in tomato. Plant Breeding. 126(3): 331-333.
21. Stipanovic, R.D. and C.R. Howell. 1982. The Structure of *Gliovirin*, A new Antibiotic from *Gliocladium virens.* The Journal of Antibioticsicotoxin.
22. Suharjono, Tri Handayani, Soejono, Susanti Dewi. 2008. Antagonis test of *Trichoderma* sp. dan *Gliocladium* sp. againts *Fusarium oxysporum* cause of wilt deseases of some variety of Purwodadi field banana in Vitro (*In Indinesian*). Biologi Study, Mathemathic and Scient Faculty, Unibraw Malang, *e-mail :* calistus@brawijaya.ac.id
23. Suryaminarsih dan Mujoko. 2012. Growth population of multiantagonis *Streptomyces* sp. *Gliocladium* sp and *Trichoderma harzianum* as biological agents of fusarium wilt disease in natural and semi natural package pellet formula (*In Indonesian*). Plumula, 1 (2): 202-210.
24. Steinkellner S., R. Mammerder, and H. Vierhellig. 2008. Germination of *Fusarium oxysporums* in root exudates from tomato plants callenged with diffrent *Fusarium oxysporums* strains. *Plant pathology.* 122: 395-401
24. Titus, A. and G.N. Pereira. 2008. The role of Actinomycetes in coffee plantation ecology. *Ineedcoffee.com*