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CERTIFICATE OF ACCEPTANCE

This is to certify that the paper of **Suryaminarsih Penta, Kusriningrum, Ni'matuzahroh, Tini Surtiningsih** and **Tri Mujoko** entitled "*The Antibiosis of Biological Agents Streptomyces sp., Gliocladium sp., and Trichoderma harzianum From East Java Indonesia To Fusarium Oxysporum"* is accepted for publication in The Philippine Journal of Crop Science, and will be included in our Volume 42: Supplement No.1, June 2017.

Sincerely yours,

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THE ANTIBIOSIS OF BIOLOGICAL AGENTS *Streptomyces* sp. , *Gliocladium* sp., and *Trichoderma harzianum* FROM EAST JAVA INDONESIA TO *FUSARIUM OXYSPORUM*

by

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ABSTRACT

This study was undertaken to determine the species of biological agents *Streptomyces* sp. from Pare-Kediri tomatoes land, *Gliocladium* sp., collection of Pandaan Food Crops and Horticulture Plant Protection, and to know their antagonistic to *F. oxysporum* f.sp. *lycopersici* soil borne pathogens from Wajak village - East Java- Indonesia. Completely randomized design was used and each treatment was repeated four times. Biological agents were identified by morphology characteristics and DNA sequensing. *Streptomyces* sp. wich was found, was identified as *Streptomyces griseorubens* and *Gliocladium* sp. as *Gliocladium virens*. The results also showed that *S. griseurubens G. virens* and *T. harzianum* were hiperparasit of *F. oxysporum* hyphae, food and space competition potentialy to *F. oxysporum* in rhizosphere and induced through the formation of pheripher roots. The third mixture of these biological agents also produced antibiosis in the rhizosphere that could inhibit *F. oxysporum* f.sp*. lycopersici* growth.

Keywords: antagonism, biological agents, rhizosphere.

1. Introduction

Fusarium oxysporum f.sp*. lycopersici* is a fungal pathogen of tomato plants wilting. This fungi lives as a saprophyte and organic matter residual in soil. Combination of several microbial soil saprophyte with multiantagonis mechanism were more efektifly to pressure the population and activity of pathogen. Some biological agents were decomposers and growth hormone producer. Biological agents *Streptomyces* sp., degrades carbon from crop residues and decomposes recalcitrat protein to proteolysis(Doi, *et al*, 2008). *Streptomyces* sp. also can enhance the growth of plant height , fruit produsedd, count of flowers and tomato plants (Sastrahidayat, I.R, 1994). *Trichoderma* sp. and *Gliocladium* sp. as biological agents, also serves as biofertilizer that packed in compost as solvent P and K elements. They were able to promote plant growth . *Gliocladium* sp., *Streptomyces* sp., and *Trichoderma harzianum*, are used as biological agents to control the pathogen population (Pal and Gardener, 2006)

Several studies have shown the relationship mechanisms between microbial pathogens and biological agents that support the process of biological control. *Trichoderma* sp. produces lytik enzymes as a chitin cell wall degradation in mycoparasit proces to get nutrients and improve their own cell wall at the division process (Chater and Chandra, 2006). *S. griseus* produces lytic enzymes that capable degrading of fungal cell walls (Anitha and Rabeeth, 2010). Competition between *Fusarium* sp. pathogenic strains and *Fusarium* sp. non-pathogenic on roots of the host plant is the food competition, Where the two Fusarium grow and develop on the same side of the root (Olivain *et al*., 2006). *T. harzianum* produces indole-3-acetic acid (IAA), which induces the formation of pheripheres roots, increasing the content of IAA and root dry weight, induces plant resistance with xylanase inducer components, and colonizes plant roots (Gruber and Saiboth, 2012: Semangoen, 2000). The toxic metabolic resulting by *Trichoderma virens* against *Phytium* sp. is gliovirin, while *T. harzianum* produces pyrone antibiotics against *Geomannomyces graminis* (Anitha and Rabeth, 2010 [b]). Relationships microbes in the soil can be described as the process of

recycling that is going very complex, complicated, and involves biochemical reactions that the mechanism are still not able to understand (Benitez *et al,* 2004). Fusarium wilt disease can be controlled by using a combination of *Streptomyces* sp., *T. harzianum* and *Gliocladium* sp. The success of this control is determined by the relationship between biological agents such antagonism against Fusarium wilt disease of tomato *(L. esculentum*). *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* . The aim of this study was determine the species of biological agents *Streptomyces* sp. from Pare-Kediri tomatoes land, *Gliocladium* sp., collection of Pandaan Food Crops and Horticulture Plant Protection, and to know their antibiosis to *F. oxysporum* f.sp. *lycopersici* soil borne pathogens from Wajak village - East Java- Indonesia.

2. Materials and Methods

Material research consists of: *Streptomyces* sp. isolates (from Pare-Kediri land tomatoes) *Gliocladium* sp., *Trichoderma harzianum* (from the collection of Food and Horticulture Crops Plants Protection - Pandaan) and *F. oxysporum* from fusarium wilt deseases tomato plants (from Wajak-Malang village), 80% sand soil and Potato Dextrose Agar medium (SAP Chemical), Malt extrac medium (Citroen). Primary tools used were Vortex, Centrifuge (the 8-place E8 centrifuge is an economical fixed speed centrifuge), Sekker (Yellowline RS 10 Basic Horizontal Shaker).

Descriptive study was conducted to identify the species of biological agents,. Experimental study was conducted to determine the inhibition of antibiosis of biological agents against *F. oxysporum*. It used a completely randomized design with five treatment types of biological agents, namely: single biological agents *Streptomyces* sp. (S), a mixture of *Streptomyces* sp. and *Gliocladium* sp. (SG), a mixture of *Streptomyces* sp. and *T. harzianum* (ST), a mixture of *Streptomyces* sp., *Gliocladium* sp., and *T. harzianum* (SGT) as well as the control treatment (without biological agents), each treatment was repeated four times.

Identification of microorganisms (Sastrahidayar, 1994; , Singh *et al*, 2002*.;* Singletown *et al*, 1993)

Biological agents were isolated by using Dhingra and Sinclair soil plating method: 1 gram soil of chilli tomato land was weighed with analytical balance, then made the suspension by dilution 10^{-4}. Furthermore *Streptomyces* sp. was isolated by preparing of 1 mL suspension and was taken aseptically, it was spreaded on GNA medium. *T. harzianum* and *Gliocladium* sp. from PPFHC - Pandaan was isolated like *Streptomyces* isolated but the isolation was done on PDA media. Biological agents obtained, then purified, and propagated on PDA in Petri dishes.

F. oxysporum were isolated by the excitation of fresh ingredients method. Parts of the plant stems of fusarium wilt tomato plants infections was cleaned, then sterilized with 70% alcohol, then wind dried, then sliced plant skin with a scalpel. The incision was inoculated on PDA medium. Pathogenic fungi that grew was isolated and purified. Fungus *F. oxysporum* which purely was propagated on PDA. *Streptomyces* sp., was Identified in the microbiology laboratory Tropical Diseases Center (TDC) Airlangga University with 16S rRNA DNA sequencing method. *Gliocladium* sp. was identified in Laboratory Culture Collection of Institut Pertanian Bogor by 18S rRNA squensing method. Colonies of *T. harzianum* was identified by macroscopic and microscopic observation in Plant Health Laboratory of the National Development "Veteran" East Java University.

Competition and antibiosis test in the rhizosphere (Olivain *et al*. 2006)

Ruby varieties of tomato seeds was soaked in a solution of 1.25% sodium hypochorit for 20 minutes and washed three times with sterile water. Seeds was germinated on malt extract agar medium (10 g / liter) in Petri dish. These seeds was incubated in the dark at 22 º C for 3 days. 1 cm sprouts / seedlings of the same size was used for treatment. 5 mL suspension of biological agents mixture of *Streptomyces* sp. *Gliocladium* sp, and *T. harzianum* according to treatment and 5 mL suspension of fungal pathogens *F. oxysporum* were inoculated on sandy soil that has been prepared. 1 cm sprouts grown on sandy soil that had been inoculated with biological agents and pathogens. Biological agents and pathogens colony that grown on root sproud were observed by microscope on $1^{st,}$,$3^{3th}5^{th}$ days after inoculation.

Antibiosis test (Brown *et al*. 2011: Buchanan and Gibon, 1974; Benitez *et al*, 2004; Cook and Baker 1974)

Antibiosisi test was done on 2^{nd},4^{th},6^{th},8^{th}, and 10^{th} days after transplanting. 1 gram soil was taken from the treatment, and was dissolved in 10 mL of sterile water for 1 minute, further was been vortex with high speed 200 rpm, then 4 mL of this suspension was added to 46 mL of sterile water. The suspension obtained was filtered with Whatman paper no 44 and Zeis filter (5G) by using vacuum pressure. The resulting filtrate was centrifuged at 150 rpm for 30 min. This solution containing antibiosis ingredients was stored in a refrigerator (4 ° C) for 24 hours. Antibiosis obtained dripped on the filter paper disk (0.5 cm diameter Whatman paper) until saturated (0.55 cc), then wind dried. This paper disks antibiosis containing was inoculated on PDA medium in Petri dishes that had been inoculated with *F.oxysporum* spore suspension. Inhibition zones caused by the filter paper disk on *F. oxysporum* was an indicator of inhibition.

Result

Identification BCAs

Streptomyces sp. isolat had yellow, bright red, white alike tissue cotton, unshiny colony and Gram positive respons. The hiphae morphology was 11 µm diametre, branch without septae. The spore was on a long, circular chain shape 17,61 x41,8 µm lenght, Spore was hialin, with 11,67 – 12,10 µm diameter. The DNA gen isolation result from supernatan and sediment by PCR showed *Streptomyces* sp. area on *gel elektroporesis* in 1,2 kb, as the same as *Streptomyces griseus* area, the primary common area used for 16S rRNA (Fig 1).

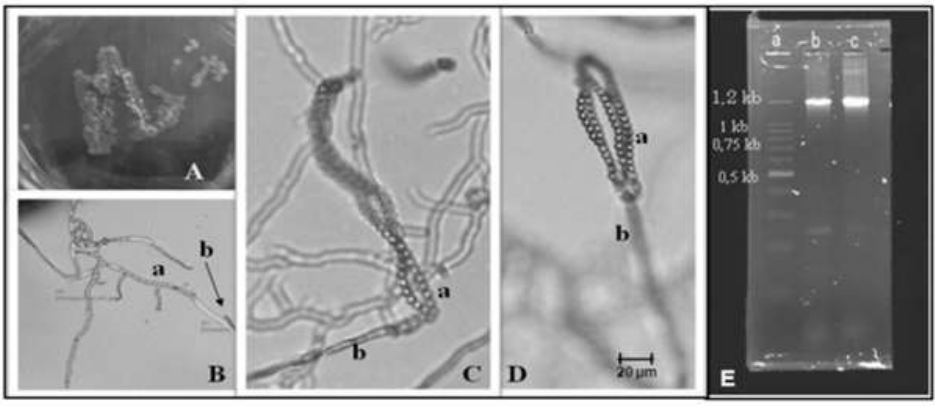

Fig 1. A. Colonies of Streptomyces sp. on PDA at 14 days **B**.Morfology microscopys research *Streptomyces* sp. **D**.Circular spore mycroscopis research, The enlargement 10 x 40. **E**.*Streptomyces* sp DNA electroforesis result on gel agarose (TDC UNAIR)

Basic Local Alignment Tool (BLAST) analysis , shows the majority of Actinomycetes, the result of DNA Squensing, was on the *Streptomyces griseorubens* species area, with the total number 603 approximately similar to level 97%, and level 98% with *Actinobacterium.*

Gliocladium sp isolat had light green colour, circular , solid, soft middle surface and rather white colony, and growed up hiphae.This hifa was branch, hialin and the diameter 28,50 µm. Konidium was 13,75 – 15,56 µm, circular, hialin, branch and stand on konidiofor. The result of *Gliocladium* sp. DNA *squensing* on the agarose gel showed on1,5 kb area and

was not different from *liocladium virens* the primary used (Fig 2). Phylogenetik analysis using BLAST submit Gen-Bank, has identified the similiarity level of 99.608% with strain *Trichoderma* sp. *INBio 3018F.*

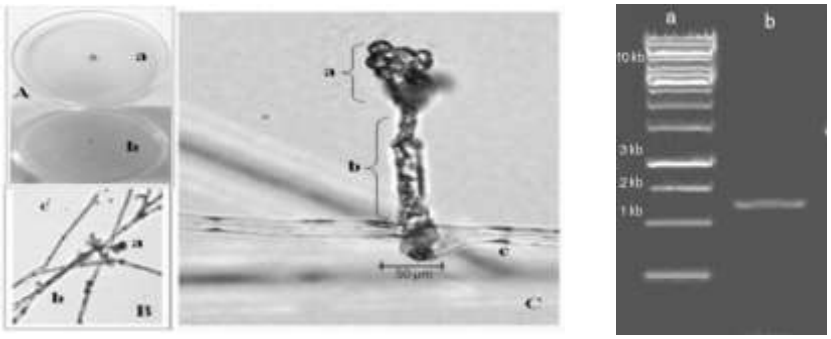

Fig 2. **A.** Colonies of *Gliocladium* sp. on PDA age of 4 days, **B**. Microscopic observation of *Gliocladium* sp **C**. Microscopic observation *Gliocladium*Magnification 10 X 40.**D**. DNA electrophoresis results *Gliocladium* sp. (IPBCC).

Macroscopic observations on *Trichoderma harzianum* colonies showed green, velvet shaped, and there was a circular zone. Conidia was round, 14.65 µm measuring, and hyaline. It had filamentous hyphae with septae, branched, hyaline, and 35.75 m in diameter (Fig 3).

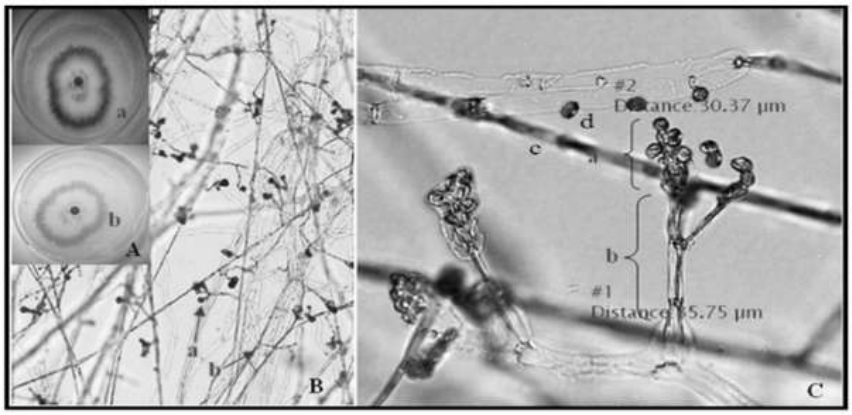

Fig 3. A. *Gliocladium* sp. colony on PDA media on 4 days. B. *Gliocladium* sp. colony mycroscopis observation **C.***Gliocladium* sp. konidium mycroscopis observation Enlargement 10 x 40D. *Gliocladiums*p. DNA electroporesis result (IPBCC).

The result of obstacle on the antibiosis soil filtrat

The average inhibition zone from soil filtrat that consists of singular biological *S. griseorubens,* the mixed of biological agensia *S. griseorubens, G. virens, T. harzianum* and control toward the development of *F. oxysporum* was different from the 2nd ,4th, 6th, 8th and 10th days after inoculation. The average inhibition zone of soil filtrat consists of *Streptomyces* sp, *Gliocladium* sp*,* *T. harzianum* mixture was taller than soil filtrat that consists of singular *S. griseurubens* (table 1).

Table 1. The inhibitions zone average of antibiosis soil filtrat to *F.oxysporum*.

Giving of Biological contol	Inhibitions zone average									
	2 hsi		4 hsi		6 hsi		8 hsi		10 hsi	
	(cm)	Transf. $\sqrt{(x+0,5)}$	(cm)	Transf. $\sqrt{(x+0,5)}$	(cm)	Transf. $\sqrt{(x+0,5)}$	(cm)	Transf. $\sqrt{(x+0,5)}$	(cm)	Transf. $\sqrt{(x+0,5)}$
SGT	0,15	0.80 b	0,33	0.91 a	0,23	0.85 a	0,60	0,85 a	0,95	1,05 a
SG	0,00	0.71 c	0,00	0,71 c	0,00	0.71 b	0,10	0,78 b	0,40	0.78 a
ST	0,00	0.71 c	0, 08	0.76 b	0,10	0.79 ab	0,08	0.76 b	0,13	0.76 a
S	0,48	0,99 a	0,10	0.79 b	0,08	0.76 b	0.00	0.71 b	0,18	0.71 a
Kontrol	0,00	0.71 c	0,00	0,71 c	0,00	0.71 b	0,00	0,71 b	0,00	0,71 a

Discussion

The result of squensing DNA *Streptomyces* sp, that was uploaded from the world gen bank, had similarity description 98 % with *Actinobacterium ZXY004* and with *S . griseorubens strain 2418.* Based on the morphology colony character, the size and the spore shape, the isolat S*treptomyces* sp had the similarity with strain *S. griseorubens* sp. was filament bacterium, small sprouted colony like cotton, and also produces *coccus* spore (Brown *et al,* 2012*). Actinobacterium ZXY* belongs to *Actynomycetes* Genus and another name of *Streptomyces griseorubens* was *Actinomyces griseorubens* (Cook and Baker, 1974).

BLAST analysis result of seqeunsing DNA *Gliocladium* sp with 18S rRNA showed the similarity 90 % with *Trichoderma* sp. Morphology of *Gliocladium* sp. was almost the same as *T. harzianum.* The upload result of BLAST analysis showed the similarity with the pimary standard used , that was *Gliocladium virens.* Skreekanth*, et al.*

(2011) also shows the differences on the colony surface on PDA media between *Gliocladium* sp. with *T. harzianum.*

Trichoderma harzianum Isolat from BPPHPTPH Pandaan that had the same morphology features with isolat *T harzianum* as is publised by BPTPH Bogor, such as light green colony to dark green , like wool, produces konodia aseksual with globul shape and the konodia forms like grape and grows quickly. (Akladious and Abbas, 2012; Alam *et al*. 2003; Buchanan and Gibbon, 1974)

Filtrat from the soil observation showed there was antibiosis that can inhibit the *F. oxysporum*. Antibiosis on rizofer was antibiosis from *G. virens* and *T.harzianum*. This statement was supported by the observations result of biological agensia population and the fungus patogen population. While in the 30th days populations of *S.griseorubens* was not found and the inhibition zone of soil filtrat consists of *S.griseorubens* on the 8th, 10th day was 0 cm .

Biological agensia *T. harzianum* and *G. virens* could grow and develop around the root faster than *F. oxysporum*, in the 1st day after planting. The competition between biological agensia fungus and patogen fungus is a nutritious source competition because in the 3rd day, *F. oxysporum* grows and develops in the same root of biological agensia *T.harzianum* and *G. virens*. Some researches found that *T. harzianum* and *G. virens* are soil saprofit fungus that can develop quickly. In 48 hours both biological agensia had formed colony and twists the root and penetrates on the interselluler root (Agrios, 1994; Allexopoulus 1996; Singh *et al.*, 2002). In vitro observation showed that both biological agensia grew quickly and can obstruct the development of *F. oxysporum*, and also forms colony in the root plant (Cook and Baker, 1974). The competition between Fusarium patogenik and non patogenik is nutritious competition because both hifa grows on the same side of the sprouted root (Olivain *et al , 2006*).

Conclusion

Streptomyces sp. is *Streptomyces griseorubens f.sp. capsicum* and *Gliocladium* sp. is *Gliocladium virens*. Multi antagonist relationship *S. griseorubens f.sp.capsicum*, *G.virens* and *T. harzianum* on *Fusarium oxysporum F.sp lycopersici* in rizofer is : Antagonism of antibiosis relationship produced by the mixed of biological agensia, *S. griseorubens* f.sp.*capsicum, G.virens* and *T.harzianum*. Indirect antagonism is the competition and plant resistance induction of *G. virens* and *T.harzianum*.

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