UTILIZATION OF RHIZOSPHERE FUNGI TO CONTROL *Fusarium oxysporum* f.sp. *capsici IN VITRO*

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ABSTRACT

Fusarium caused wilt disease in chilli pepper and destroyed some farmer crops. Results of preliminary research has been discovered that the disease caused by *Fusarium oxysporum* f.sp. *capsici.* The alternative environmental friendly method is to find antagonist microbes which is located in the rhizosphere of healthy pepper plants. This study aims to find out potentially antagonistic fungi to control *Fusarium* wilt disease on pepper plants. The fungi were isolated by soil dilution method or viable plate count method on Potato Dextrose Agar medium with antibiotic Livoplaxacin (25%, w/v). Rhizosphere fungi from healthy pepper plants had been identified. A total of 63 spesies belong to 4 genera included *Penicillium* (45 species), *Aspergillus* (6 species), *Trichoderma* (9 species) and *Candida* (3 species). The highest percentage of distribution of rhizosphere fungi are *P. digitatum* (47.63%), *P. expansum* (19,05%), *T. harzianum* (9,53%), *A. nidulans*, *A. niger*, *Penicillium* sp., *Candida albicans*, and *T. vitrens* i.e 4,76% respectively. All of rhizosphere fungi colonies were found to inhibit *Fusarium oxysporum* f.sp. *capsici in vitro*. The best inhibition was found in *Aspergillus niger* at 88.89 ± 2.2% followed by *A. nidulans* of 85,56 ± 1,6 %, *T. harzianum* at 84,45 ± 1,58% , and *T. virens* by 83,33 ± 1,2%, five days after inoculation. All of them have a very high inhibition criteria.

Keywords: Rhizosphere fungi, *Fusarium oxysporum* f.sp. *capsici*, inhibiting ability, persentage contribution, and antagonistic.

INTRODUCTION

Soil fungi, especially living in the rhizosphere was one of all microorganisms that plays an important role in soil fertility and plant health. Soil was a harbor that has a very high biological diversity and were not yet fully capable studied (Chandrashekar *et al.*,, 2014; Berg *et al.*,, 2015).

Rhizosphere role was inseparable from the issuance exudates. Root exudates of some plants such as peppers (*Capsicum annuum* L.) issued detected 12 amino acid and 7 of sugar. On resistant cultivars root exudates containing mithionine, d-1-β phenylalanine, citrulline and D-xylose. Root exudates that originated from resistant varieties can inhibit germination of pathogen (*Fusarium oxysporum* f.sp. *capsici)*, but susceptible varieties increase spore germination same pathogens. Germination of fungal spore antagonistic (*Trichoderma viride* and *Aspergillus sydowi*) is also influenced by root exudates of resistant and susceptible varieties, but the effect is different ((Naqvi and Chauhan, 1980).

The fungal rhizosphere that antagonistic gave contribution to controlling soil borne pathogen. That was such as *Penicillium* spp., *Aspergillus* spp., *Gliocladium* sp., and *Trichoderma* spp., be able to suppress mycelium of *F. oxysporum*

f.sp. *cubense* (cause Fusarium wilt disease of banana) *in vitro* (Sudarma and Suprapta, 2011). Some interaction happened in rhizosphere related with soil borne pathogen suppress. The interaction included competition, parasitism, and induced plant resistant (Wipps, 2001).

Plant root disease with wilt symptom in pepper plant can caused by several fungus such as *Phytophthora capsici* (Sranghellini *et al.,*, 1996), and Fusarium wilt disease caused by *F. oxysporum* f.sp. *capsici* (Wongpla and Lomthalsong, 2010). Pepper plant that attacked by two pathogen like mentioned above found similar symptom, where the plant wilt result the vascular tissue plugged, then water and nutrient not be able transported to apart of plant from root and stem. Result work of Sudarma and Puspawati (2013) (unpublish data) has identification the pathogen of wilt disease in plant pepper, at Banjarangkan district, Klungkung regency, was *Fusarium oxysporum* f,sp. *capsici* with disease incidence was 76,67%. Disease epidemic of Fusarium wilt in plant pepper was 0.44 per unit per day, then decreased 0.23, 0.12 and 0.11 per unit per day. Therefore, potential of fungal antagonistic that originated from soil rhizosphere (suppressive soil) should be used as biological control agent to controlling soil borne pathogen such as pathogen that cause Fusarium disease in plant pepper.

MATERIALS AND METHOD

Soil samples

Soil samples taken from rhizosphere of pepper plant healthy with diagonal method for one field, and replicated three times. For each hole for a pepper plant healthy was taken 100 g soil, and mixed before taking place in plastic bag, than put in an ice box. Before the analyzed soil samples were placed in refrigerator for 24 hours.

Isolation of fungal rhizosphere

Each soil samples was taken 10 grams of soil was diluted with 90 ml of sterile water, dilution was continued 10^{-3}- 10^{-6}. One milliliter was placed in a Petri dish had previously been filled with potato dextrose agar (PDA) medium and supplemented with antibiotics (antibacterial) levoplaxasin 250 mg/l (w/v). Colonies of rhizosphere fungi will grow after two days, then count the number colonies by colony forming unit (cfu). Furthermore, each colony purified and transferred to a new Petri dish.

The percentage of distribution

The percentage contribution can be calculated as follows, total number of colony forming unit (cfu) of a particular species divided by the total number of cfu entire species times 100% (Chandrashekar *et al.,*, 2014). The percentage contribution may illustrate that there are certain species that dominate and contribute in rhizosphere of pepper plant healthy, it can be seen with the highest percentage value contribution.

Inhibition test of fungal rhizosphere

Each rhizosphere fungal tested for inhibitory against the growth of *F. oxysporum* f.sp. *capsici* with a dual culture technique. Percent inhibition can be calculated using the following formula (Dolar, 2001; Jayalal and Adikaram, 2007; Mojica-Martin *et al.,*, 2008):

$$\% \text{ inhibition} = \frac{A - B}{A} \times 100$$

A = Diameter of *Fusarium oxysporum* f.sp. *capsici* colony in the control (mm)

B = Diameter of rhizosphere fungal colony in treated (mm).

Inhibition ability of rhizosphere fungal against pathogen was calculated every day until the control (*F. oxysporum* f.sp. *capsici*, single culture) and observation halted until control have met Petri dishes. Antagonistic inhibition mechanism against pathogen can be known whether it competition or antibiosis by observing the presence and absence inhibition zones in Petri dish. If the any clear zone (zone of inhibition) mean inhibition by antibiosis mechanism. Inhibition criteria can be grouped as follows: inhibition of 0-20% = very low, 21-40% = lower, 41-60% = moderate, 61-80% = high, and 81-100% = very high.

Identification of rhizosphere fungal of pepper plant healthy

Rhizosphere fungal have been successfully purified and gown in culture medium PDA in Petri dish, subsequently indentified microscopic morphology included shape and color of conidia (spores), conidiophores, hyphae structures. The fungal rhizosphere documented by using the tool OPTILAB that relate directly to a laptop. The observation was matched with a compatible reference, such as Samson *et al.*,, 1981; Pitt dan Hocking, 1997; Barnett dan Hunter, 1998; dan Indrawati *et al.*,, 1999. Diba *et al.*,, 2007; Rahman *et al.*,, 2011; Samson *et al.*,, 2011).

RESULTS AND DISCUSSION

Identification of fungal rhizosphere

A total 63 isolates of fungal origin rhizosphere of pepper plant healthy have been identified which are included in the four genera such as *Aspergillus, Candida, Penicillium* and *Trichoderma*. In the *Aspergillus* genera were found six species such as *A. nidulans* and *A niger* three species, respectively. Same with genera of *Candida* only three species, namely *C. albicans*. In the genera of *Penicillium* found 45 species (30 species of *P. digitatum*, 12 species of *P. expansum*, and 3 species of *Penicillium* sp). In the genera of *Trichoderma* was found 9 species (six species of *T. harzianum*, and three species of *T. virens*) (Table 1).

Table 1. The number of species, colonies and percentage contribution of rhizosphere fungal of pepper plant healthy

No.	Spesies of rhizosphere fungal	Number of colony (CFU/g of soil) x 10^3	% contribution
1	*Aspergillus nidulans*	3	4.76
2.	*Aspergillus niger*	3	4.76
3	*Candida albican*	3	4.76
4	*Penicillium digitatum*	30	47.62
5.	*Penicillium expansum*	12	19.05
6	*Penicillium sp.*	3	4.76
7	*Trichoderma harzianum*	6	9.52
8	*Trichoderma virens*	3	4.76
	Total	63	100

Contribution percentage of rhizosphere fungal found was highest 47.62% by *P. digitatum,* followed by *P. expansum* was 19.05%, 9.52% of *T. harzianum,* and *A. nidulans, A. niger, Candida albican*, and *T. virens* were 4.76%, respectively (Table 1)

Aspergillus genera has conidiophores upright, simple, terminating in a glubosa or clavate swelling, bearing phialides at the apex or radiating from the apex or the entire surface; conidia (phialospores) 1-celled, globose, often variously colored in mass, in dry basipetal chains (Barnett dan Hunter, 1998; Gautam and Bhadauria, 2012). *Aspergillus* genera consists of hundreds od species of mold that was faound throughout the world. First discovered in 1729 by biologist Pier Antonio Micheli of Italy. *Aspergillus* name was also the name of the structure formation of asexual spores generally to all species of *Aspergillus*. About a third of species are also known to have a sexual stage. *Aspergillus* can be classified as follows : kingdom of Fungi, Division of Ascomycota, classes of Eurotiomycetes, Order of Eurotiales, family of Trichocomaceae, and the genera of *Aspergillus* (Bennett, 2010).

Candida albicans, can grow in at least three different morphologies; yeast, pseudohyphae and hyphae (Sudbery *et al.,*, 2004). *Candida albicans* is a fungus species are pathogenic to humans from devisio Deuteromycota . This fungus is the cause oppotunistic infection called candidiasis of the skin, mucosa, and organs in humans. Some characteristics of this species are egg-shaped (ovoid) or spherical with a diameter of 3-5 μm and can produce pseudohyphae. Rose (1990) states that *Candida albicans* has two types of morphology, namely forms such as yeast and hyphae form. Additionally, phenotype or appearance of these microorganisms can also be changed from white and flat to become wrinkle irregular, star-shaped, circular, shapes such as coffee, and opaque . This fungi have the ability to attach to host cells and colonize.

Candida albicans was included in the phyllum Ascomycota, upphyllum of Saccharomycetes, class of Saccharomycetes, order of Sacharomycetales, family of Sachasromycetadae, genera of *Candida*, and species of *Candida albicans* (CP Robin) Berkhout. 1923. synonyms: *Candida stellatitreae*, and *Oidium albicans* (Sudbery, 2011).

Colonies of *Penicillium* sp. usually green, sometimes white, mostly have conidiophores. Single conidiophores (mononematus) or compound (synematous), consisting of a single trunk dividing some phialide (simple/monoverticillata). Phialide was a structure that sustains conidia, cylindrical basal part narrowed neck, or lancoelate (approximately partially embedded in the basal part of the end pieces). Conidia form long chains, divergent or column, globular, elliptical or fusiform, transparent or greenish, with walls smooth or bumpy (Gam *et al.,*, 1987). Conidia form long chains, divergent or column, globular, elliptical or fusiform, transparent or greenish, with walls smooth or bumpy (Figure 3) (Alexander, 1930).

Trichoderma spp. was a cosmopolitan fungus , often exist in all types of soil, manure and decaying plant tissue. Dominance in the ground equipped with a diversity of metabolic capabilities and competitive nature aggressive. These characteristics make this fungus on wood decomposers significant and herbs. *Trichoderma* spp. also able to degrade waste relatively quickly without removing the stench (Rahman *et al.,*, 2011). *Trichoderma* is included in the kingdom Fungi, Ascomycota devisio, subdevisio

Pezizomycotina, class of Sordariomycetes, order of Hypocreales, family of Hypocreaceae familia, and genera of *Hypocrea* (perfect stage) or *Trichoderma* (imperfect stage) (Druzhinina *et al.,*, 2005).

Inhibition ability test of fungal rhizosphere

Inhibition ability of fungal rhizosphere against *F. oxysporum* f.sp. *capsici* was found highest in *Aspergillus niger* by 88.89 ± 2.2%, followed by *A. nidulans* of 85.56 ± 1.6%, *Trichoderma harzianum* of 84.45 ± 1.58%, *P. expansum* of 84.44 % ± 2.22%, *T. virens* of 83.33 ± 1.2%, *P. digitatum* of 79.35 ± 14.29%, *Penicillium* sp. of 78.89 ± 1.3%, and *Candida albicans* by 69 ± 1.3% at age 6 days after inoculation (Table 2). Inhibitory mechanism fungi in the rhizosphere against pathogen were of antibiotics and competition. Antibiosis mechanism that found in the Petri dish there was a yellowish translucent zone issued by antagonist fungi (*Aspergillus nidulans*, and *Penicillium* sp.) (Figure 2).

Others show the mechanism of inhibition of the competition, both space and nutrient competition. Fungal antagonists were found to justify the theory that the suppressive soil (healthy plant habitats) will contain many types and density of fungal antagonist which protects the roots of healthy plants from pathogens infection in the rhizosphere. This fungus has the potential to be developed and applied *in vivo* (Sudarma and Suprapta, 2011).

Aspergillus spp. has been tried *in vitro*, can suppress the growth of *P. palmivora* (cause fruit rot disease of cocoa). *Aspergillus niger* has the highest inhibition than both the other fungus (*A. fumigatus* and *A. repens*), which amounted to 54% (Adebola and Amadi, 2010). *Aspergillus niger* can improve biological control of inoculant bacteria (*P. fluorescens*) against the disease of root knot nematodes, as well as against *Fusarium* spp. (Siddiqui *et al.,*, 2004; Dawar *et al.,*, 2008). *Aspergillus nidulans* can be antagonistic to *Colletotrichum gloeosporioides* (causes antraknose in vanilla plant). The result of Fakhrunnisa *et al.,* (2006) research found that *A. niger*, can inhibit the growth of *Fusarium* spp. through antibiosis mechanism *in vitro*. Bosah *et al.,* (2010) has also been found that *Aspergillus* spp. can inhibit the growth of pathogenic fungi *Sclerotium rolfsii* with inhibition of 73.12 to 88.35%. The process of inhibition caused by *Aspergillus* spp. produce the enzyme chitinase and β-1 , 3-glucanase (Laminarinase) that has the ability to break down the components of fungal cell walls of pathogens such as chitin and β-1,3- glucan.

Candida albicans was a form of yeast that is found in all humans. This fungus was usually harmless, but if the population exceeds controls. *Candida albicans* reproduce themselves by forming buds that will continue pseudohyphae elongated shape. Pseudohyphae were formed with many groups blastospore round or oval around the septum.

In some strains, blastospores large, round or like bottles, in small amount these cells can develop into thick-walled chlamydospores and a diameter of about 8-12 µm. Morphology of *C. albicans* colonies on solid medium in Sabouraud Dextrose Agar, generally round in shape with a slightly convex surface, smooth, slippery and sometimes a little convoluted, especially in colonies that have been older. Age culture affect the small colony.

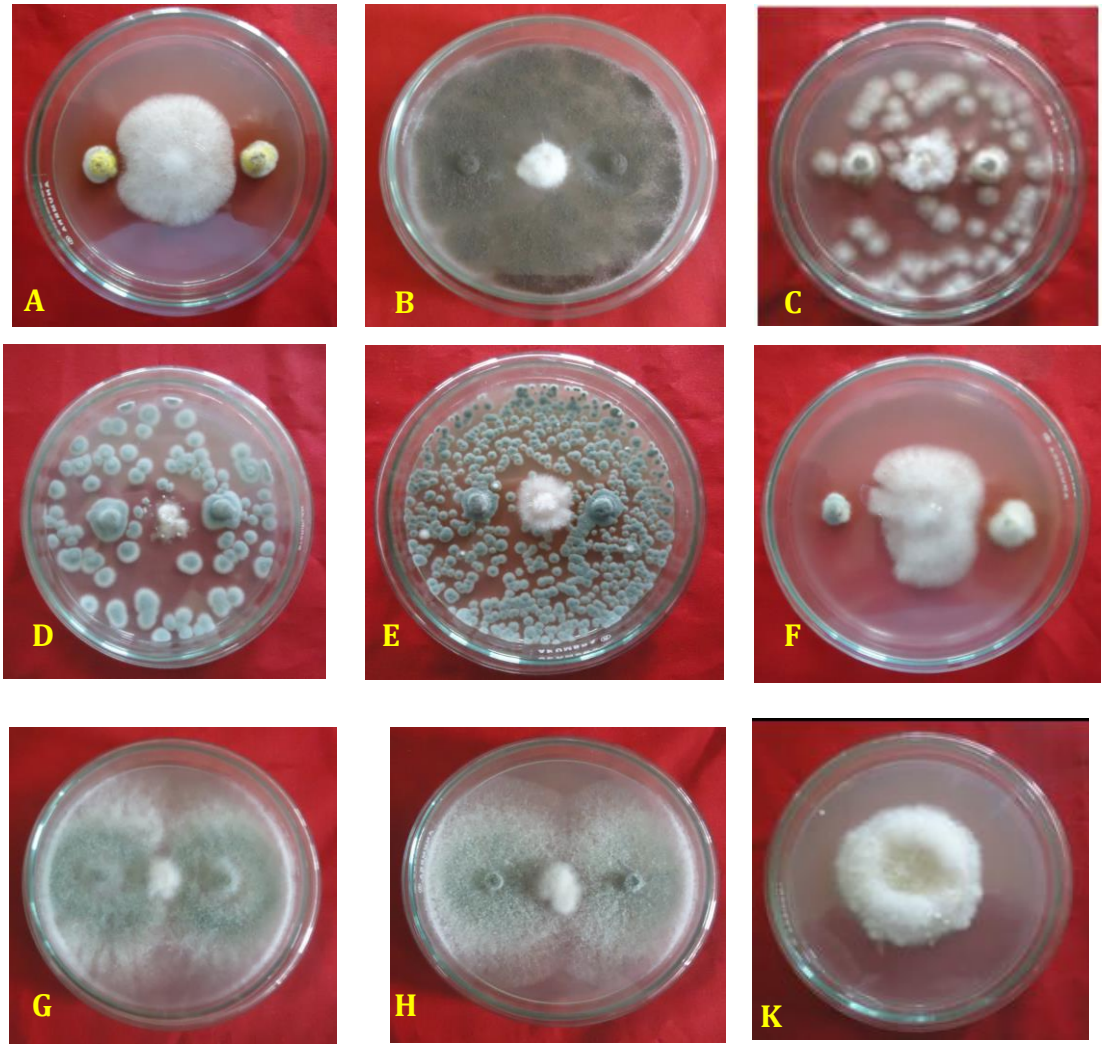

Fig.* 2. Inhibition ability of fungal rhizosphere against *Fusarium oxysporum* f.sp. *capsici* *in vitro. (A = *A. nidulans*, B = *A. niger*, C = *Candida albicans*, D = *P. digitatum*, E = *P. expansum*, F = *Pinicillium* sp., G = *T. harzianum*, H = *T. virens*, dan K = kontrol or *F. oxysporum* f.sp. *capsici*), 3 days after inoculation.

Colonies yellowish white color and smelled like sour aroma tape. In a liquid medium such as glucose yeast extract peptone, *Candida albicans* grows at the base of the tube (Jha *et al.*,, 2006). *Candida albicans* is a dimorphic fungus because of its ability to grow in two different forms, namely as a stem cell will develop into blastospora and produce sprouts that will form a pseudohyphae . Yeast cells (blastospore) is round, oblong or oval with a size of 2-5 x 3-6 µm up to 2-5.5 x 5-28 µm (Tsafrir, 2015).

Penicillium sp. have been tried as microbial antagonists against *Sclerotium rolfsii* which is a soil borne pathogen that damages more than 500 species of plants, but the most inhibitory power lower than *Trichoderma* sp. and *Aspergillus* sp. which amounted to 46.24-56.98% (Bosah *et al.*,, 2010). Haggag and Mohamed (2007), states that *Penicillium* sp. can antagonistic through a mechanism that is issued several alkaloid compounds such as agroklavine and ergometrine which has anti-fungal properties against *Botrytis cinerea*, *Fusarium solani* and

Alternaria tenius. Idris *et al.*,, (2008) have found *Penicillium* sp. as microbial antagonists against *Ganoderma* sp. cause disease in plants Palma. *Penicillium* sp. has also been tried in controlling the Lanas disease in tobacco plants caused by *Phytophthora parasitica* var. *nicotianae* (Roeswitawati, 2007). Mechanism of action of penicillin that has activity against the synthesis of peptidoglycan, which causes cell lysis and death. They inhibit one of the stages required for cross- bonding peptidoglycan, transpeptidation, because of similarities between molecules streriochemical penicillin and D-Ala-D-Ala dipeptide. This enzyme is located in the outer regions of the cytoplasmic membrane: penicillin binding proteins (penicillin-binding protein/PBP) (Bryskier, 2005).

Trichoderma was also known as mycoparasit that can grow and take nutrients in pathogens, so that pathogens can not thrive in soil. *Trichoderma harzianum* has the ability antagonist best compared with other antagonist microbes, such as *Bacillus thuringiensis, Rhizobium meliloti* and *A. niger* to control root rot sunflower crop (Dawar *et al.*,, 2008). The mechanism of inhibition of *Trichoderma* against soil borne pathogens, namely: (1) generate the enzyme chitinase, β-1,3-glucanase (Katatny *et al.*,, 2000), β-1,4 glucanase and lipase compound that can break down chitin, glucan and fats the cell walls of pathogens (Benitez *et al.*,, 2004; Vinale *et al.*, (2008); (2) mycoparasitism (Howell, 2003); (3) antibiosis to produce antibiotic 6-pentyl-a-pyrone (6pp), heptilidic acid and peptaibol (Barea *et al.*,, 2005; Vinale *et al.*,, 2008); (4) competition for nutrients and space (Lo, 1998; Benitez *et al.*,, 2004); (5) the root colonization (Harman *et al.*,, 2004; Vinale *et al.*,, 2008); induce local and systemic resistance (Harman, 2006).

CONCLUSION

Rhizosphere fungal origin from pepper plant healthy were able to be identified was four genus which consists of: *Penicillium* genera includes 30 species of *Penicillium digitatum,* three *Penicillium* sp., and 12 species of *P. expansum*; six genera of *Aspergillus* which consists of each of the three species of *A. nidulans* and *A. niger*; three genera of *Trichoderma* which consists of six species each of the three species of *T. harzianum* and *T. virens* three species; and three species of the *Candida albicans*. The prevalence of rhizosphere fungal as follows: *P. digitatum* highest of 47.63%, subsequently *P. expansum* by 19.05%, 9.53% of *T. harzianum, A. nidulans, A. niger, Penicillium* sp., *Candida albicans* and *T. vitrens* each by 4.76 %. All rhizosphere fungal isolates were found to inhibit pathogen (*Fusarium oxysporum* f.sp. *capsici*) *in vitro*, very high inhibition was found in *Aspergillus niger*, hereinafter *Aspergillus nidulans, Penicillium expansum*, *Trichoderma harzianum*, and *Trichoderma virens*, with a very high inhibition criteria.

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