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Assay of Antagonistic Bacteria of Single Isolate and Combination to Control Seedling-off in Chili Seed caused by *Fusarium oxysporum*

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Single isolate and combination of chitinolytic and non-chitinolytic bacteria were assayed to know their ability in reducing chili seedling-off caused by *Fusarium oxysporum*. Eight antagonistic bacterial isolates i.e. *Bacillus* sp. BK13, *Alcaligenes* sp. BK14, *Enterobacter* sp. BK15, *Citrobacter* sp. BK16, and *Bacillus* sp. BK17 showing chitinolytic activity, and *Pseudomonas* sp. KM01, *Alcaligenes* sp. KM02, and *Serratia* sp. KM04 with no chitinolytic activity were assayed to inhibit *F. oxysporum* and *Candida albicans* growth in vitro. All isolates showed varied ability in inhibiting the fungal growth, in which *Enterobacter* sp. BK15 and *Bacillus* sp. BK13 inhibited the most. Inhibition assay on *C. albicans* indicated that non-chitinolytic bacterial isolates is likely to produce other toxic metabolic compounds. Single isolate of *Enterobacter* sp. BK15 and combination of *Bacillus* sp. BK13+*Serratia* sp. KM04 as well showed to reduce more chili seedling-off. All treatments produce higher seedling height and seedling dry-weight than that of isolate-free seeds planted in fungus-inoculated soil but one, *Enterobacter* sp. BK15+*Pseudomonas* sp. KM01.

Key words: Antagonistic bacteria, *Candida albicans*, chili seed, *Fusarium oxysporum*, seedling-off.

Utilization of bacterial isolates as biological control of plant disease remains an important potential alternative to the use of pesticides, since chemical control may harm to animal and human, develop resistance in target populations, and cause environmetal polution. By their interactions with various soil-borne plant pathogens, antagonistic microorganisms serve as powerful biological control agents of plant diseases. The antagonism may operate through antibiosis, competition, predation, or parasitism (Alabouvette *et al*., 2006; Ozbay and Newman, 2004). To inhibit plant disease growth biological control agent may produce enzymes such as chitinase, glucanase and protease to breakdown fungal cell wall (Anitha and Rabeeth, 2010; Patel *et al.*, 2007; Gohel *et al*., 2006), or release secondary metabolites toxic to fungal disease (Ganesan *et al*., 2007; Alabouvette *et al*., 2006).

Many studies in examining bacterial isolates to control plant disease have relied on single bacterial isolate (Raupach and Kloepper, 1998). It has demonstrated inconsistent result. One possible approach to overcoming this inconsistent performance is to include a combination of biocontrol agents in a single preparation (Roberts *et al*., 2005; Raupach and Kloepper, 1998). An increased performance in inhibiting disease growth

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by combination of biocontrol agents has been reported (Ganesan *et al*., 2007; Rini and Sulochana, 2007; Roberts *et al*., 2005; Raupach and Kloepper, 1998). This might due to an expression of greater variety of responsible traits for suppression of one or more pathogens over a wide range of environmental conditions (de Boer *et al*., 2003).

For the purpose of applying such bacterial isolates for biological control of fungal diseases of plant, assay on their ability to inhibit fungal growth either in single isolate or combination of the isolates with different control mechanisms is one of the important steps to be done prior other steps. By combining specific strains of microorganisms, it can be assumed at least one biocontrol mechanism work under the conditions faced by the isolates and may result in a higher level of protection rather than by single isolate (Roberts *et al*., 2005; de Boer *et al*., 2003; Raupach and Kloepper, 1998). In this study single isolate and combination of non-cihitinolytic and chitinolityc bacterial isolates were evaluated against *Fusarium oxysporum* a causal agent of chili seedling-off. Seedling-off is one important soilborne disease in many crops of Solanaceae including chili, tomato, potato, eggplant, and tobacco (Miller *et al*., 1986; Gajbhiye *et al*., 2010) since it causes serious economic loss (Farhan *et al*., 2010; Gangadara *et al*., 2010; Anitha and Rabeeth 2009; Okeniyi, 2007).

MATERIALS AND METHODS

Bacterial- and fungal isolates

Eight isolates of antagonistic bacteria were used in this study. *Bacillus* sp. BK13, *Alcaligenes* sp. BK14, *Enterobacter* sp. BK15, *Citrobacter* sp. BK16, and *Bacillus* sp. BK17 were antagonistic isolates with chitinolytic activity, while *Pseudomonas* sp. KM01, *Alcaligenes* sp. KM02, and *Serratia* sp. KM04 were antagonistic isolates with no chitinolytic activity. *Fusarium oxysporum* and *Candida albicans* was of collection of Laboratory of Microbiology, Department of Biology, University of Sumatera Utara, Medan. All microbial isolates were kept in refrigerator in proper media.

In vitro examination of bacterial-fungal antagonism

The ability of the bacterial isolates to inhibit Fusarium growth was evaluated in vitro. Two pieces of paper disc immersed with H" 10^8 cells/ml of suspension of 3-days old bacterial culture were placed in the opposite direction about 3.5 cm from the center toward the edge of plate. An agar plug (Ø 5-mm) of Fusarium from the margin of an actively growing mycellium was inoculated in the center of plate. Antagonistic assay of chitinolytic isolates was performed in modified salt medium (0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.001 g $ZnSO_4$, and 0.001 g $MnCl_2$ in 1.000 ml) containing 2% (w/v) chitin colloidal agar and non-chitinolytic antagonistic was assayed on potato dextrose agar with 0.5% yeast extract + 0.5% thryptone. Antagonistic assay of *C. albicans* was performed in Mueller Hinton agar by putting the immersed paper disc on the yeast lawn. The assay was conducted to see if the bacterial isolates indicate to produce other antifungal metabolites instead of chitinase. All plates were incubated at 30°C. Inhibitory activity was determined based on the inhibition zone formed around bacterial colonies, measured from 4 to 7 days of incubation for *F. oxysporum* and 2 days for *C. albicans* as the radius of the normal fungal growth subtracted to the radius of the inhibited fungal growth. Two isolates of chitinolytic (BK13 and BK15) and 2 isolates of non-chitinolytic bacteria (KM01 and KM04) showing higher inhibition zone were chosen for further study.

Antagonistic assay of bacterial isolates

In this study both single isolate and combination of two were assayed to reduce seedling-off. Therefore, antagonistic assay between chosen isolates was necessary. Paper disc (Oxoid) of 10-µl of each bacterial isolate suspension (H"10^8cells/ml) was placed on bacterial lawn of tested against-isolate on Mueller Hinton agar. Cultures were incubated for overnight at 30°C. If any, inhibitory activity was determined as diameter of clear zone around the disc. No inhibition was showed between the test isolates.

Control of Fusarium wilt of chili seeds

Conidial suspension was prepared by growing *F. oxysporum* in a flask containing potato dextrose broth for 7 days at 30°C. Fifteen ml of steril destiled water and 5 ml of 0.13 % Tween 80 were added into flask. Flask was shaked for 1 min. Suspension was diluted to get H" 10^7 conidia/ml.

A-100 ml fungal suspension was blended with 500 g sterilized soil mixed with sterilized compost (3:1) in a 30x22x10 cm tray. Chili seeds were surface sterilized with 2% aqueous sodium hypochlorite for 60 minutes and rinsed thoroughly with sterile distilled water. The chili seeds were soaked with 2-days old culture (H"10^6cell/ml) of single culture of BK13, BK15, KM01, and KM04, and cultures combined of BK13+KM01, BK13+KM04, BK15+KM01, and BK15+KM04 for 30 min. The seeds were then planted for 30 days in the trays covered with plastic wrap. Controls were isolate-free seeds planted in fungus-free soil (IFFF) and isolate-free seeds planted in fungus-inoculated soil (IFF). Thirty seeds were used for each treatment.

Observation of number of seedling-off, seedling height and seedling dry-weight

Number of seedling damping-off was measured as: (total seedling off of IFF seedling/ total of IFFF seedling) x 100%. damping off reduction (%) was measured as: [(number of IFF seedling off - number of seedling off of treated seeds) / (number of IFFF seedlings)] x 100%. To examine seedling height and seedling dry-weight, 3 seedlings of each treatment were randomly chosen.

RESULTS

In vitro assay of bacterial-fungal antagonism

Many antagonistic bacteria are assayed in controlling plant diseases caused by fungal pathogens. In controlling the disease bacteria may employ antibiosis, competition, predation, or parasitism (Alabouvette *et al*., 2006; Ozbay and Newman, 2004). In this study bacterial antagonism was assayed to know their ability to inhibit *F. oxysporum* growth in vitro prior seed treatment using single isolate and combination of chitinolytic and non-chitinolytic bacterial preparation. It was shown that bacterial ability to inhibit growth of hyphae was varied (Figure 1), both of single isolate and combination. Several study demonstrated chitinolytic bacterial isolates in contolling spesific fungi (Kim *et al*., 2008; Mahadtanapuk *et al*., 2007; Alabouvette *et al*., 2006; Getha and Vikineswary, 2002. Our previous study showed that single isolate of chitinolytic bacteria inhibited growth of different fungi such as *F. oxysporum*, *Ganoderma boninense*, and *Penicillium semitectum* (Farhan *et al*., 2010; de Boer *et al*., 2003). Several non-chitinolytic soil bacterial isolates inhibited growth of *F. oxysporum* in vitro to some extent (Suryanto *et al*., 2013?).

Not all isolates might produce chitinase. To examine non-chitinolityc bacterial isolates, simple test of *C. albicans* was performed since the yeast wall consists of more glucan. Data showed that BK17 that inhibited less on *F. oxysporum* but more on *C. albicans*. It is more likely that other isolates might excrete other mycolytic enzyme such as glucanase and other toxic secondary metabolites.

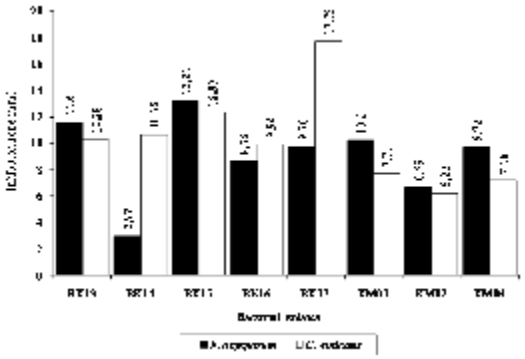

Fig. 1. Inhibition zone produced by antagonistic bacterial isolates in vitro

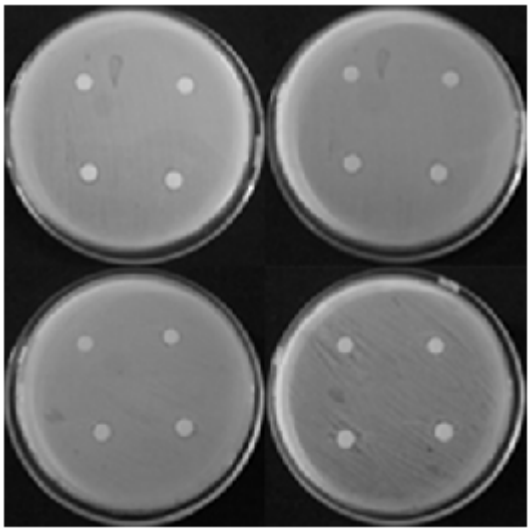

Fig. 2. Antagonistic assay between bacterial isolates. (a). others on BK13, (b). others on BK15, (c). others on KM01, and (d). others on KM04

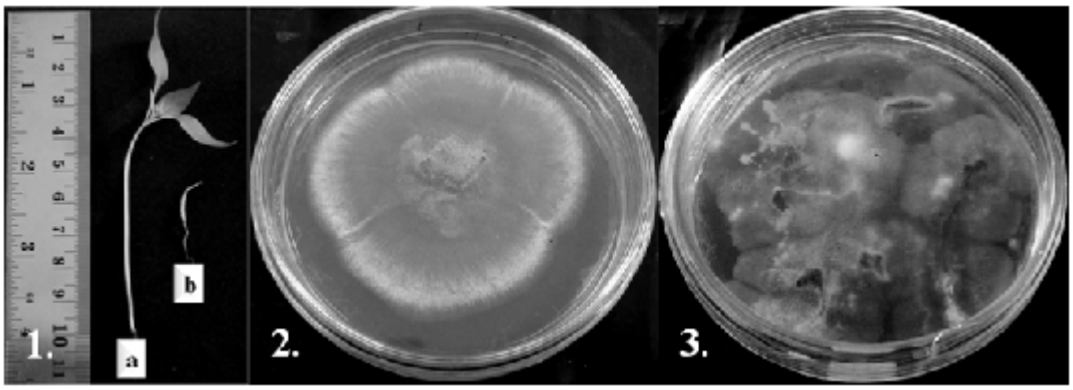

Fig. 3. Chili seedling growth: (1). a. chili seedling of unaffected, and b. death seedling caused by Fusarium wilt; (2). Colony of *F. oxysporum* and (3). growth of *F. oxysporum* from infected seedlings

Assay of antagonistic effect between bacterial isolates

Before examining bacterial isolate ability to control chili seedling-off, antagonistic assay was conducted to know if there is an antagonistic effect between combined isolates as a treatment to control the disease. No antagonistic behaviour indicated as no inhibition zone between bacterial isolates was observed in the test (Fig. 2.). Compatibility test was also done by other researcher to know if the biological control agents using as single preparation did not suppress each other (Ganesan *et al*. 2007; Rini and Sulochana 2007; Roberts *et al*. 2005).

Control of chili seedling-off

Seedling-off caused by Fusarium was observed after planting the seed in fungal inoculated soil (Fig. 3). Disease manifestation was observed as seedling wilted with yellowing leaf followed by seedling slunted and desiccated stem, collars appeared slunted and discolored with yellowish gray and then leaf turning to brown and brittle. *Fusarium* also causes plant to grow abnormally, or uses the plant as agent of the pathogen transmission to other host plants. The pathogen infects young root, growing, developing and spreading in root and stem vessel, inhibiting water and nutrient transport (Miller *et al*., 1986). Reisolation of infected seedling showed the same disease agent *F. oxysporum* (Fig. 3). This clearly confirmed that *F. oxysporum* used was pathogen to chili.

Chili seeds were treated by soaking them into single or combination of antagonistic bacterial solution prior planting in soil inoculated with *F. oxysporum*. Most of seedlings planted in soil inoculated with *F. oxysporum* were succeptible to seedling-off (IFF). No infected plant were observed

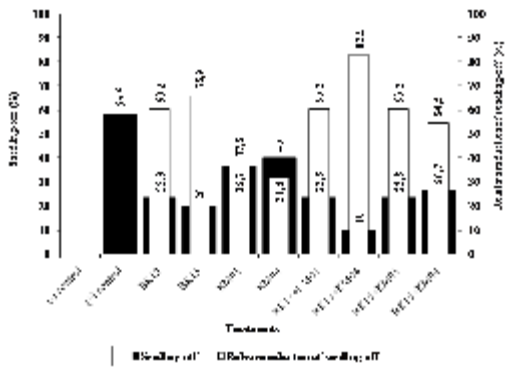

Fig. 4. Effect of chili seed soaking treatment with single and combination of bacterial isolates on percentage of seedling-off

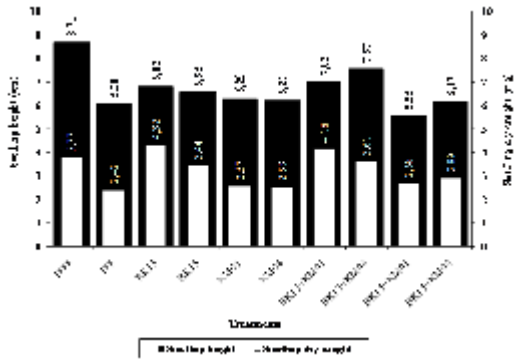

Fig. 5. Effect of chili seed soaking treatment with the bacterial isolates on seedling height and seedling dry-weight

in seed planted in soil without Fusarium inoculation (IFFF). On the other hand the seed treated with antagonistic bacteria showed to reduce seedling-off to some extent (Figure 4). This variation might be due to variation of bacterial mycolytic activity, fungal cell wall composition, the growth rate of the bacterial and the fungi, and other antifungal metabolites.

Single bacterial treatment of BK15 reduced more seedling-off compared to other singles, but combinations of BK13+KM04 showed more in reducing seedling-off. It seems that all combination tended to reduce seedling-off. Unlike KM01, combination of other isolate to KM04 and all single and combination of BK13 is likely to contribute to better seedling performance either to reduce seedling-off or to increase chili seedling stand to some extent compared to that of IFF. However, our previous study showed that BK13 seemed not to much reduce disease of other plant pathogen like *G. boninense* (Suryanto *et al*., 2012).

Manifestation of the disease was observed after 12 days of seedling in IFF and delayed between 13-19 days of seedling in the treated seeds. The numbers of seedling-off were increased rapidly to 4 weeks of observation. Morphological and physiological alteration of seedling may occur caused by *F. oxysporum* as manifested in alteration of seedling height and seedling dry-weight. Inspite of giving different effect, all treated seeds produced seedling higher than that of IFF (Figure 5), but one combination of BK15+KM01. However all treatments showed seedling height shorter than that of IFFF seedling height.

DISCUSSION

Antagonistic microorganisms play an important role in controling other microorganism to growth (Alabouvette *et al*., 2006; Ozbay and Newman, 2004). It is well known that antibiosis, competition, predation, or parasitism were mechanisms used by the antagonistic microorganism in keeping microbial equilibrium in nature. To know bacterial isolates potential of antagonistics, an assay was performanced by growing the bacterial isolates next to fungi. Our isolates indicated to inhibit the fungal growth at least through antibiosis and parasitism, by producing secondary metabolites toxic to others by non-chitinolytic isolates and mycolitic enzymes by chitinoytic isolates, respectively. Antibiosis is a very common phenomenon responsible for the activity of many biological control agents (Alabouvette *et al*., 2006), while several hydrolytic enzymes are reported to degrade cell walls of pathogenic fungi (Alabouvette *et al*., 2006; Ozbay and Newman, 2004).

In preparation of two microorganisms in combination as biological control agents antagonistic behavior of one microorganism toward the other should be considered. Bacterial isolates combined in biocontrol preparations must be compatible so they do not compete in order to increase disease suppression to occur (Ganesan *et al*., 2007; Rini and Sulochana, 2007). Our study showed that bacterial isolates used in this study did not show antagonistic behavior of one isolate toward others.

Combinations of biocontrol isolates are expected to perform better to decrease disease incindence since they may produce multiple antifungal traits combined to suppress multiple plant disease (Rini and Sulochana, 2007; Alabouvette *et al*., 2006; Raupach and Kloepper, 1998). Isolate combined between chitinolytic BK13 and non-chitinolytic KM04 demonstrated to decrease more seedling-off caused by *F. oxysproum*. It seems that other toxic metabolites in addition to chitinase take their role to contribute in inhibiting fungal growth (Getha and Vikineswary, 2002). Combining microorganisms have also been reported to increase plant performance (Ganesan *et al*., 2007; Raupach and Kloepper, 1998).

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