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Original Article

Characterization of biocontrol agents isolated from the leaves of mangroves grown in Sampiran Beach, North Sulawesi, Indonesia againts damping-off of cucumber

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Abstract

Bacterial biocontrol isolates MND61, MND63, and MND65 were originally isolated from mangrove leaves of *Derris trifoliata* Lour, *Sonneratia alba* J. Smith, and *Avicennia marina* (Forsk) respectively grown in Sampiran beach, North Manado, North Sulawesi island, Indonesia. They were evaluated on suppression of cucumber damping-off. Identification results showed that based on 16S rRNA sequence, MND61 isolate had 99% similarity to *Kleibsiella pneumoniae* ATTCC13884T, MND63 isolate had 98% similarity to *Bacillus cereus* NENA11, and MND65 isolate had 98% similarity to *Bacillus subtilis* jllsy. Results of greenhouse test showed that MND61 and MND63 suppressed *Pythium aphanidermatum* damping-off of cucumber significantly ($P < 0.005$) by 73-80%. Cocultures of these two biocontrol agent in potato dextrose broth also suppressed the pathogens population about 5-46 %. MND61 isolate produced indole acetic acid, in contrary to MND63 and MND65 isolates did not. The inhibitory assay effect of broth culture of MND61, MND63, and MND65 isolates to phytopatogenic fungi *Fusarium oxysporum*, *P. aphanidermatum*, *P. ultimum*, *Rhizoctonia solani*, *Pyricularia oryzae* and *Xanthomonas oryzae* in PDA plate indicated that two strains (MND63 and MND65) inhibited the growth of *F. oxysporum*, and *R. solani* growth, otherwise MND61 isolate did not inhibit the growth of any phytopatogenic assay.

Keywords: biocontrol agents, cucumber, plant mangroves, *Pythium aphanidermatum*

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Introduction

Production of cucumber in Indonesia (tones) during 2010 to 2014 decreased, i.e; 547.141, 521.535, 511.525, 491.636, and 477.976., respectively (BPS, 2014). Diseases, insects and weeds decreased the production of the all crops produced worldwide by 36%, and that diseases contribute to 14% of the decrease (Agrios, 2005).

Damping-off is one of the most common horticultural disease in greenhouse or nursery affecting germinating seeds and young seedlings (Hulvey *et al*., 2010). In preemergence damping-off, the seeds wither or decay result in a sparse and patchy germination. In postemergence damping-off, the pathogen infects the rootcollar tissue and within days the seedlings become dark, root nearly to the soil surface at the base of the stem, and then wilt and die. These diseases can spread quickly in a few days depending on the environmental conditions of the greenhouse or nursery. *Pythium* sp., *Phytophthora* sp., *Fusarium* sp., *Rhizoctonia* sp., *Sclerotinia* sp., *Botrytis* sp., and *Alternaria* sp. are common soil saprophytic fungi that can cause damping-off diseases when the environmental plant conditions are suitable, such as excess moisture, low light, low temperature, and low pH of the growing medium (Zad and Koshnevice, 2001).

Pythium damping-off cucumber has been reported to occur in most greenhouse in Oman and result in mortality of over 75% of cucumber seedlings and plants in some greenhouse (Al-Kiyumi, 2006). *Pythium* species are considered limiting factors to cucumber production in other parts of the world (Stanghellini and Philips, 1975; Howard *et al*., 1994). In most cases pre- and post-emergence damping-off in cucumber is caused by *P. aphanidermatum* and other *Pythium* sp. (Abbasi and Lazarovits, 2006). In major *Pyhtium* cause economic losses in both greenhouse and field production systems under favorable conditions for disease development (Georgakopoulos *et al*., 2002; Abbasi and Lazarovits, 2006)

Control of phytopathogens by biological means is environmentally advantageous in comparison to chemical control (Nautiyal, 2001). There are several species of microorganisms have been tested as potential biocontrol agent of damping-off, including bacteria such as *Bacillus* sp., *Penicillium* sp., *Pseudomonas fluorescens*, *Pythium oligandrum*, *Serratia plymuthica*, *Streptomyces griseoviridis*, *Trichoderma* spp., and *Gliocladium* sp. (Howell and Stipanovic, 1980; Benhamou *et al*., 2000; Georgakopoulos *et al*., 2002; Nicoletti *et al*., 2004; Huang *et al*., 2012). Production of antibiotics, siderophores and a variety of enzymes have been implicated as mechanisms used by biocontrol agents to limit the damage to plants by phytopathogens (Glick and Bashan, 1990; Bowen and Rovira, 1999). Beneficial rhizosphere organisms are generally classified into two groups based on their primary effects; (1) microorganisms with direct effects on plant growth promotion (plant growth promoting microorganisms (*PGPM*), and (2) microorganisms that indirectly assist with plant productivity through the control of plant pathogens (Whipps, 2004 ; Vassilev *et al*., 2006). Numbers of different free-living N-fixing bacteria are considered to be plant growth promoting rhizobacteria (*PGPR*), including *Azospirillum* and

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Klebsiella species (Glick, 1995). An important step in disease control is a selection of effective biocontrol agents. The present research work was performed to explore possible bacterial control agents isolated from mangrove plants for protection cucumber from *Pythium* damping-off and to study the mechanisms of biocontrol agents on suppression of a fungal pathogen.

Method

Evaluation of Bacterial Strains on Suppression of Cucumber Damping-off Caused by *P. aphanidermatum* (greenhouse test)

Each of fifteen bacterial strains (MND58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, and 72) were isolated from mangrove plants. The isolates were inoculated from NA slant agar medium to LB medium and were incubated for 24 h at room temperature. Then 0.5 mL of each culture were inoculated into 50 mL medium No.3 (10 g polypeptone, 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, and one liter distilled water) and were incubated for five days. No.3 medium was used because it is known as a biological active compound producing medium (Asaka and Shoda, 1996). 100 g of soil was transfered into a plastic pot (Hammy production, Indonesia: 9.5 cm in diameter, 6.5 cm in height), then the soil was inoculated with 1/12 part of 2-3 days of *P. aphanidermatum* grown in petri dish with 9 cm in diameter containing 10^{-1} PDA at room temperature. After that the soil was inoculated with 10 mL of each isolates tested by using micropipet. Five cucumber seeds pre-germinated for two-days were transplanted to the pot using forceps. The pots were kept in green house for three weeks, and watered everyday with NaCl 0.3%, to know the grow ability in saline soil condition of the tested isolates. In this experiment, negative control (no inoculation of BCAs and pathogen) and positive control (inoculation with pathogen only) were also prepared. This plant tested was performed in triplicate. After three weeks of planting, the percentage of damping-off of cucumber were recorded.

Identification of Selected Bacterial Strain

Three of the best of performing bacterial isolates (MND61, MND63, and MND65) were selected for identification based on the 16s rRNA analyses. The bacterial strains were identified by Indonesian culture collection (InaCC), Research Center for Biology, Indonesian Institute of Sciences, at Cibinong Science Center, Indonesia.

Interaction Between Strain MND61, MND63, MND65 and *P. aphanidermatum* in Liquid Medium

A pathogen of *P. aphanidermatum* were single and co-culture cultivated with each of the biocontrol isolates of MND61, MND63, and MND65. For single culture of *P.aphanidermatum*, its actively growing mycelium was inoculated into 20 mL of a sterile potato dextrose broth (24 g PDB Himedia, 1 L distilled water). Whereas for co-culture pathogen and MND61, MND63, and MND65 isolates. 20 mL of a sterile PDB medium were inoculated with 2% (v/v) of each fresh bacterial suspension (MND61, MND63, and MND65) and the actively growing mycelium of *P. aphanidermatum*. These single and cocultures were incubated at room temperature, the population of the pathogen were counted on the third, sixth, ninth and thirteenth days of incubation time.

Production of IAA by MND61 and MND63 Isolate

Twenty mL *Tryptic Soy Broth* (TSB) medium containing 200 ppm L-tryptophan was prepared. One loop of the bacterial tested were inoculated to it, and they were incubated on a rotary shaker for 72 h. IAA production was measured at incubation time 24 h, 48 h, and 72 h as described by Gravel *et al*. (2007). After the incubation periods, bacterial cells were centrifuged (8000 rpm, 10 min). One mL of each bacterial supernatant was mixed vigorously with 2 mL Salkowski's reagent (50 mL $HClO_4$, 25 mL distilled water, and 1mL $FeCl_3$ 0.5M). The mixture was incubated at room temperature for 20 minutes and the absorbance was measured at 535 nm. The concentration of IAA was evaluated by comparison with standard curved prepared using serial dilution of a 50 mg ml^{-1} IAA (Sigma-Aldrich solution) in half-strength TSB. Four replicates were used for each treatment

Quantitative Evaluation of Growth Inhibition of MND61, MND63, and MND65 Isolates on Bacterial and Fungal Phytopathogenic

Mycelium of each fungal pathogens (*F. oxysporum*, *P. aphanidermatum*, *P. ultimum*, *Pyricularia oryzae*) and one loop of *X. oryzae* were inoculated onto the center of a sterile PDA plate, three holes (diameter 9 mm) were made using a cork borer (the position of the holes were at the same distance from the center of the medium, where at each fungal pathogen was placed). Seventy µl of a 7-day culture of strain MND61, MND63, and MND65 in No.3S medium (30 g polypeptoneS, 10 g glucose, 1 g KH2PO4, 0.5 g MgSO4, and one liter distilled water) was put into each hole. For the negative control distilled water was used. The plates were incubated for 5 days and growth inhibition area was observed.

Data Analysis

Data were statistically assayed using analysis of variance (ANOVA) with Minitab 16 software. Significance of mean differences was determined using the Duncan's test.

Results

Evaluation of Bacterial Strains on Suppression of Cucumber Damping-off Caused by *P. aphanidermatum* (greenhouse test)

Among fifteen bacterial isolated tested, showed that the highest damping-off suppression observed in MND63, followed by MND61 and MND65 (Fig. 1). Their suppressiveness was about 73-80% higher than that of positive control. MND63 isolate decreased damping-off of cucumber significantly by 80%, and degree of damping-off suppression was not different between MND61 and MND65, the disease decreased was about

73%. Their 16S rRNA sequence analysis showed that the closest species of the strains were *Klebsiella pneumoniae*, *Bacillus cereus*, and *Bacillus subtilis*, respectively (Table 1).

The lowest suppression of the damping-off was observed when the culture broth of MND60, 64, 70, 71, and 72 isolate was applied into the soil, the disease decreased was about 0-7%.

Table 1. Sequencing result of Isolates MND61, MND63, and MND65

No	Isolate	Closest species	Similarity
1	MND61	*Klebsiella pneumoniae* ATTCC13884T	99%
2	MND63	*Bacillus cereus* NENA11	98%
3	MND65	*Bacillus subtilis* jllsy	98%

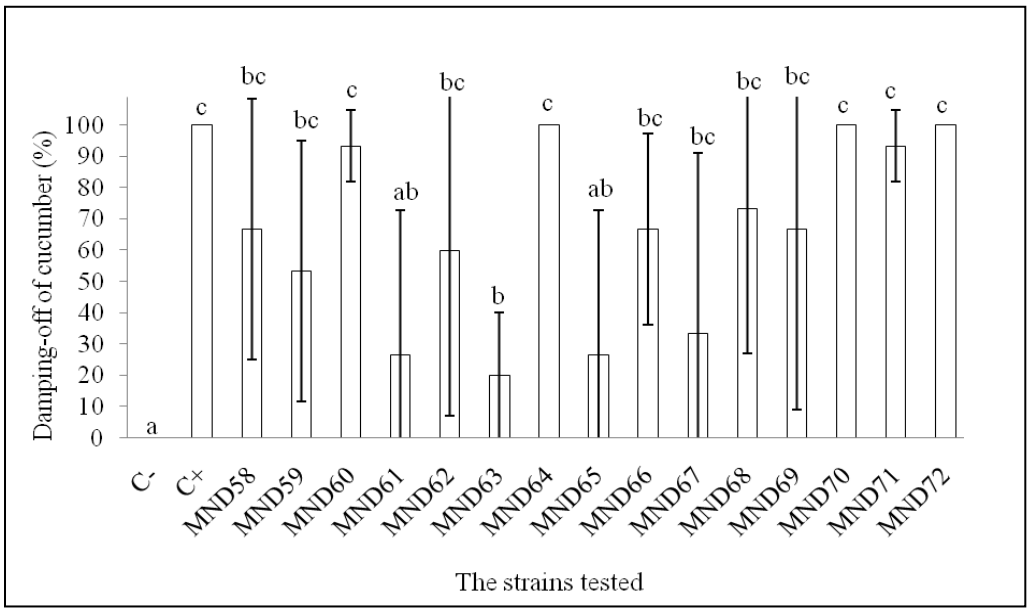

Figure 1. The abality of bacterial strains to suppress cucumber damping off caused by *P. aphanidermatum*

Interaction Between Strain MND61, MND63, MND65 Isolates and *P. aphanidermatum* in Liquid Medium

The population of *P. aphanidermatum* in single culture increased, compared with in its coculture. MND63, MND65, and MND61 isolates) reduced population of the pathogen in PDB medium by 5-46% (Fig.2). MND61 isolate produced IAA extracellular for about 168 mg.L^{-1} at 48 h incubation time (Fig.3).

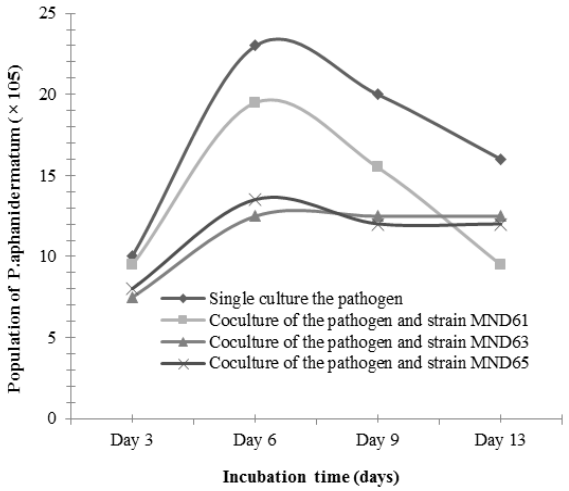

Figure 2. Influence of strain MND61, MND63, and MND65 on the population of *P. aphanidermatum*

Quantitative Evaluation of Growth Inhibition of MND61, MND63 Isolates and MND65 on Bacterial and Fungal Phytopathogenic

A table indicated that two of the three isolates tested (MND63, and MND65) inhibited to *F. oxysporum* and *R. solani* growth. Whereas MND61 isolate inhibited to *P. aphanidermatum* growth, and did not inhibit to *F. oxysporum*, *P. ultimum*, *P. oryzae*, *R. solani*, and *X. oryzae* (Table 2).

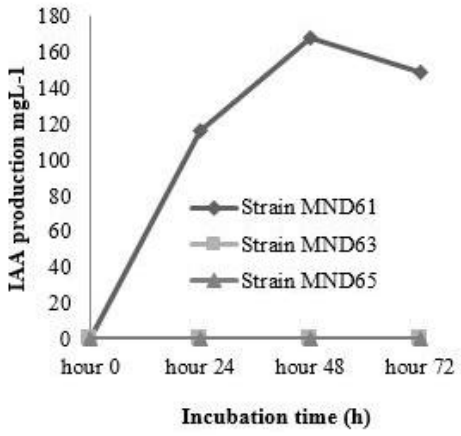

Figure 3. IAA production by strain MND61, MND63, and MND65

Table 2. Antagonistic of MND61, MND63, and MND65 isolates on bacterial and fungal phytopathogenic growth

No	Phytophatogenic	MND61	MND63	MND65
1	*F. oxysporum*	-	+	+
2	*P. aphanidermatum*	+	-	-
3	*P. ultimum*	-	-	-
4	*P. oryzae*	-	-	-
5	*R. solani*	-	+	+
6	*X. oryzae*	-	-	-

Discussions

Klebsiella pneumoniae have been reported as plant growth-promoting bacteria (Kuan *et al*., 2016) and nitrogen fixing bacteria (Iniguez *et al*., 2004). In our research work, we found *K. pneumoniae* MND61 as a biocontrol agent of *P. aphanidematum* damping-off in cucumber (Fig 1), and produced IAA (Fig 3). The following strains of *Klebsiella* have been reported produce IAA, like *K. pneumoniae* ATCC13883 produced IAA 35 µg mL^{-1} at 36 and 48 h incubation time (El-Khawas and Adachi, 1999). *K. pneumoniae* strain K8 produced a maximum IAA (27.5 mg L^{-1}) in the presence of tryphtophan 1 ppm. Results of pot experiment indicated that IAA producing *K. pneumoniae* significantly increased the root length and shoot height of wheat inoculated seedling over the control (Sachdev *et al*., 2009). Nitrogen fixation by *K. pneumoniae* 342 in wheat, resulted taller, more robust, and greener wheat leaves than the control. It also significantly increased the dry weight of roots and shoots compared with controls (Iniguez *et al*., 2004). *K. pneumoniae* strain as had a profound effect on root morphology of *Poa pratensis*. Roots of the inoculated plants were larger, and more numerous root hairs than those of the control plants (Haahtela *et al*., 1986). *K. pneumoniae* MND61 inhibited to *P. aphanidermatum* growth, and did not inhibit to *F. oxysporum*, *P. ultimum*, *P. oryzae*, *R. solani*, and *X. oryzae* (Table 1).

Bacillus cereus MND63 and *B. subtilis* MND65 decreased damping-off of cucumber (Fig.1). *Bacillus subtilis* is one of the most extensively used bacteria for biocontrol of plant diseases (Ongena and Jacques, 2008), and together with other *Bacillus* species represent about half of the commercially available biopesticides in the world market (Fravel, 2005). The potential of *Bacillus* species is based on their ability to produce a broad array of lipopeptides showing powerful antifungal properties together with low toxicity, high biodegradability and environmental friendly characteristic compared with chemical pesticides (Chen *et al*., 2008). *B. subtilis* produced a variety of bioactive metabolites that could be involved in antibiosis among which lipopeptides from the surfactin, iturin and fengycin families (Bonmatin *et al*., 2003; Peypoux *et al*., 1999). Other *Bacillus cereus* strains also showed that they have been proven to be a reliable biocontrol agent of *Phytophthora* damping-off and root rot of soybean (Emmert and Handelsman, 1999). In case of *B.cereus* UW 85, which is capable of producing two antibiotics were zwittermicin A, an aminopolyol group and antibiotic B, an aminoglycoside containing disaccharide. These two antibiotics might be responsible for the suppression of damping- off of alfalfa caused by *Phytophthora medicaginis* (Silo-suh *et al*., 1994). *B. cereus* MND63, and *B. subtilis* MND65 inhibited to *F. oxysporum* and *R. solani* growth (Table 1). El-Hamshary *et al*. (2008) stated that four strains of *B. cereus* inhibited to *F. oxysporum* growth in King's medium B, the probably mechanisms was by production of siderophore. Furthermore Romeiro *et al*. (2010) reported that supernatant from a culture of a *B. cereus* strain suppresses the pathogens *Pseudomonas syringae* pv. tomato, *Xanthomonas vesicatoria*, *Alternalia solani*, and *Corynespora cassiicola*. These results strongly suggest that *B. cereus* strains produce antimicrobial compounds.

In this research work, *B. subtilis* MND65 was initially screened on cucumber damping-off *in vivo* test, and its biocontrol effect was tested by antagonistic effect of its culture broth to inhibit *P. aphanidermatum*. It was found that *B. subtilis* MND65 showed potential biocontrol effect to *Pythium* damping-off of cucumber, it suppressed *P. aphanidermatum* population in co-culture (Fig.2), while they did not inhibit the growth the pathogen in PDA plates (Table 1). The results revealed that some potential biocontrol strains that did less antagonistic activity against the pathogen on PDA plates would be overlooked on plants in vivo test. Other strain, such as *Streptomyces rubrolavendule* S4, that belongs to actinomycetes group have also reported controled *P. aphanidermatum* caused seedling damping-off of *Amaranthus tricolor* (Loliam *et al*., 2013).

Some of *B. subtilis* strains, which could biocontrol *Fusarium* wilt of cucumber, were initially screened with the dual culture method (Chung *et al*., 2008; Raza *et al*., 2009; Gajbhiye *et al*., 2010), in which the strains showed clear inhibition zone to pathogen on PDA plates. Then the candidates were tested in pot experiment to verify the ability to control disease. *B. subtilis* EM488 was screened for *in vitro* inhibition of *P. ultimum*, *R. solani*, *F. oxysporum* and *Phytophthora capsici* on PDA plates. Its biocontrol function was possibly attributed to the production of bacilysin and iturin (Chung *et al*., 2008). Similarly to *B. subtilis* B579, screened with the dual culture method, showed a good preventive effect on suppression of *Fusarium* wilt of cucumber in pot experiments (Zhang *et al*., 2008; Chen *et al*., 2010). *B. subtilis* strain NCD-2 produced fengycin lipopeptide that play a primary role in inhibiting the growth of *R. solani in vitro* (Guo *et al*., 2014). *B. subtilis* strain EU07 was selected as the best antagonist and evaluated against *Fusarium oxysporum* f.sp. radicis-lycopersici (FORL). It inhibited by 64% to the growth of FORL *in vitro* test (Baysal *et al*., 2008).

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