Combined use of *Enterobacter cloacae* MB20 and the microelements of copper and manganese to control damping-off of tomato

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Abstract. Biocontrol activity of *Enterobacter cloacae* alone and in combination with copper and mangan on the suppression of tomato damping off was assessed. *Rhizoctonia solani* damping-off is difficult to control, since its dormant organ sclerotia survive for long periods under various environmental condition. The availability of micronutrients copper and mangan may play important roles in controlling of plant diseases. Results showed that the application of *E. cloacae* alone decreased damping-off of tomato significantly (P<0.05) by 85%. Combination application of *E. cloacae* with 10µg ml⁻¹ of CuSO₄ showed the highest suppression of the disease, followed by the treatment of *E. cloacae* with 5µg ml⁻¹, as high as 95% and 90% respectively, although the differences was not significant compared to other treatments. Degree of the disease suppression did not differ between application of *E. cloacae* alone and in combination with both concentration of MnSO₄ (5µg ml⁻¹ and 10µg ml⁻¹), the disease decreased about 85%. Inoculation of *R. solani* alone into soil resulted significantly highest (P<0.05) of fungal population, it was about 33.7 x 10⁻³ CFU g⁻¹ soil, and inoculation of *R. solani* and *E. cloacae* reduced significantly fungal population to about 19.0 x 10⁻³ CFU g⁻¹ soil. *E. cloacae* produced chitinase and protease, its chitinolytic index was about 0.84, and proteolytic index was 0.61.

1. Introduction
Applying pesticides for plant disease management raises serious concern on environmental quality, pesticides resistance, and food safety, which have dictated need for alternative plant diseases protection. In particular combination of biocontrol agent with nutrients could affect the disease tolerance or resistance of plants to pathogens [1].

Damping-off is one of serious problem of disease infecting many economically important agricultural and horticultural crops as well as trees worldwide [2]. A pathogen agent of the damping-off is *Rhizoctonia solani* caused yield losses in more than 200 crops globally [3]. High yield losses were reported, up to 50% for sugar beet [4], up to 70% for field-grown lettuce [5] and about 20% for potato [6]. Strategies to control *Rhizoctonia* diseases are limited because of its ecological behaviour. It is extremely broad host range and the high survival rate of sclerotia under various environmental condition [2]. Besides cultivars with complete resistance are not available at present [7]. Therefore, efficient strategies to control the pathogen are urgently required. In the other hand, increasing use of
chemical inputs causes several negative effects, such as the development of pathogen resistance to the applied agents and their non-target environmental impacts [8]. A growing awareness of agricultural practices in using chemical have a great impact on human health and on the environment has spawned research into the development of effective biocontrol agents to protect crop against diseases. Wang et al. [9] reported that the use of an antagonistic microorganisms of a Bacillus sp. strain N antagonized R. solani on horsebean (Vicia faba), and Kumar et al.[10] who also reported a Bacillus sp. strain N antagonized Rhizoctonia solani, Fusarium oxysporum, and Penicillium expansum.

Effective use of biological control agents (BCAs) is a potentially important component of sustainable agriculture. Nowadays, there has been an increasing interest among researchers in using combinations of BCAs with others control methods, such as integrated methods of BCAs with organic matter, fungicides or macro and micro elements. To exploit potential synergistic effects between BCAs and its combinations. Noble and Coventry [11] reviewed the various combinations biological control agents to control soil-borne plant pathogens, including Trichoderma harzianum and organic amendment reduced significantly the disease caused by R. solani. Barkat [12] also reported that a combination of Trichoderma and manure amendment at rate 6 and 10% reduced damping off by 33 and 50% respectively. In addition Peng et al.[13]mentioned that disease control obtained with a combination of Bacillus subtilis NJ-18 and fungicide better than single application of the bacterium or fungicide.

In this study we combined a biocontrol agent of Enterobacter cloacae with microelement manganese (Mn) or copper (Cu) to explore potential synergistic effects among them. E. cloacae is a gram-negative Proteobacterium belonging to the family of Enterobacteriaceae [14].

Several report have demonstrated that micro elements are able to control plant diseases, such as fungal and bacterial diseases decreased with manganese. Wadhwa et al. [15] revealed that application of 5 ppm Mn and Cu 10 ppm increased resistance of clusterbean against root rot diseases. Liew et al. [16] reported that foliar applications of Cu reduced fungal disease and increased yield in MR219 rice cultivar.

The present research work aimed to evaluate the effectiveness of a combination of E. cloacae with manganese or copper in controlling of tomato damping off caused by Rhizoctonia solani.

2. Materials and Methods

2.1. Biocontrol agent and fungal pathogen
Bacterial biocontrol agents used in this study was Enterobacter cloacae MB20 was isolated from leaves of a mangrove plant, Avicennia alba Blume, and fungal pathogen used was Rhizoctonia solani Khün.

2.2. Plant material and soil
Tomato as a model plant was used tomato seed (Solanum lycopersicum ) variety Palupi, produced by Benih Unggul Jawara, Indonesia. Soil was collected from the top layer (0 to 10 cm) in the experimental area of Research Center for Biology, Indonesian Institute of Sciences, Cibinong, West Java, Indonesia. The characteristics of the soil was as follows; pH 5.7; total C and N 22.6 and 2.1g kg$^{-1}$; texture silty clay (sand 7%, silt 50%, and clay 43%); soil taxonomy was Latosol. The soil was dried and sieved through a 2-mm mesh sieve and then used for the experiment.

2.3. Plant assay
Ninety grams of dried soil was mixed with 20 mL of sterile distilled water containing 1% of NaCl and inoculated with a 1/10 part of 3-4 days of R. solani mycelia grown in a petri dish with 9 cm in diameter containing 10$^{1}$ PDA at room temperature, and then transferred to a plastic pot (Hammy production, Indonesia: 9.5 cm in diameter, 6.5 cm in height), and incubated for about 1 to 2 days. After incubation, 15 mL of a 5 day-culture of Enterobacter cloacae MB20 in No.3 medium was added to the pot and mixed, and supplemented with 1mL of Mn or Cu 5ppm, according to the treatments. Five
tomato seed pre-germinated for two days were transplanted to the pot using forceps. The seed were grown for 3 weeks in greenhouse. After three weeks of planting, damping-off incidence in tomato was recorded. Plants were watered everyday to adjust the moisture content to about 60% of the maximum water holding capacity (MWHC).

The experiments were performed using a completely randomized design (CRD) with seven treatments and four replications as follows; 1) negative control (no inoculated of E. cloacae or R. solani and without supplementation of Cu or Mn; 2) positive control (plant inoculated with R.solani only); 3) plant infected with R.solani and E. cloacae; 4) plant infected with R. solani and E. cloacae +Mn 5 ppm; 5) plant infected with R.solani and E. cloacae +Mn 10 ppm; 6) plant infected with R.solani and E. cloacae +Cu 5ppm; and 7) plant infected with R.solani and E. cloacae +Cu 10 ppm.

2.4. Preparation of colloidal chitin
Colloidal chitin was prepared according to Hood [17] with minor modification. Briefly 20 g of powder chitin (Hi Media) was dissolved with 400 mL of concentrated HCl, and incubated at 4°C overnight. After the incubation, it was filtered using a glass woll. The filtrate obtained was added with a sterile cold distilled water and NaOH 10 N slowly to reach up 500 mL and pH 7.00. The mixed solution was centrifuged at 7,000 rpm for 10 minutes, and collected pellet. Afterwards the collected pellet centrifuged again at the same rate and times.

2.5. Qualitative test for chitinase
Chitinolytic enzyme activity of E. cloacae was detected based on appearance of the clear zones around its colonies. E. cloacae was inoculated onto chitin agar plates. Composition of chitian agar media for 1L was 0.7 g K$_{2}$HPO$_{4}$, 0.3 g KH$_{2}$PO$_{4}$, 0.5 g MgSO$_{4}$,5H$_{2}$O, 0.01 g FeSO$_{4}$,7H$_{2}$O, 0.001 g ZnSO$_{4}$, 0.001 g MnCl$_{2}$, 8 g of colloidal chitin, and 20 g of agar. Plates were incubated at 37°C for 2-4 days. This experiment was performed three replicates. A clear zone of chitin agar media gave an indication of chitinase producing organisms. Chitinolytic index was calculated by using the formula

$$\text{Chitinolytic index} = \frac{\text{a diameter of the clear zone}}{\text{a diameter of its colony}}$$  (1)

2.6. Qualitative test for protease
Proteolytic enzyme activity of E. cloacae was detected based on appearance of the clear zones around its colonies. E. cloacae was inoculated onto skim milk agar plates containing tryptone (0.5 % w/v), yeast extract (0.25 % w/v), dextrose (0.1% w/v), agar (2%, w/v), and skim milk (2%, w/v). Plates were incubated at 37°C for 24 h. This experiment was performed three replicates. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms [18].

$$\text{Proteolytic index} = \frac{\text{a diameter of the clear zone}}{\text{a diameter of its colony}}$$  (2)

2.7. Data analysis
Data were subjected to analysis of variance (ANOVA) with Minitab 16 software. The significance of mean differences was determined using the Duncan’s test. The responses were judged significant at 5% level.

3. Results and Discussion

3.1. Plant assay
All of treatments of biocontrol agent of E. cloacae with and without Mn or Cu reduced incidence of tomato damping-off significantly (P<0.05) at level 95-85%. E. cloacae colonized spermosphere and rhizosphere in several plants species including cucumber [19,20]. E. cloacae suppressed damping-off of Pythium ultimum in cucumber and other plants [19,21]. Ability of E. cloacae in suppression of plant
pathogens due its potential in fatty acid competition[22], root colonization [19,20], produced chitinase [23,24] and ammonia production[25].

Our result showed that combined application of E.cloacae with Cu 10 ppm reduced the highest of tomato damping-off from 100% to 5%, although its degree suppression was not significant with application of E. cloacae alone that reduced the disease from 100% to 15 % or with combination of the others concentration of Mn and Cu (Figure 1). Manganese (Mn) play important role in the development of plant resistance to root diseases [26] and foliar disease [27]. Manganese can control a number of diseases, its has an important role in lignin and phenol biosynthesis and several other functions [1]. Manganese also plays an important role in cellular processes of carbohydrate , lipid , and protein metabolisms. It is also as cofactor for some enzymes in bacteria and other organisms, contributes to protection against oxidative stress and may also contribute directly to the catalytic detoxification of reactive oxygen species (28). Copper promotes the formation of lignin and this resulted in decrease fungal diseases in different plant diseases [29]. Copper is also used as catalyzer for electron transfer reactions in some metalloenzymes , such as cytochrome oxidase. It is also worked as a cofactor by copper-detoxifying enzymes. Intracellular copper levels must be finely controlled, due to its toxicity( 30).

The ability of E. cloacae in tomato root colonization to reduce damping-off of tomato was confirmed with the monitoring of R. solani population in the rhizosphere soil . The result showed that the addition of E. cloacae alone or with combination of manganese and or copper significantly reduced population of R. solani (Figure 2). Addition of E. cloacae alone reduced population of R. solani significantly from 33.7 x 10^3 CFU g^-1 soil to about 19.0 x 10^3 CFU g^-1 soil. This results indicated that E. cloacae is potential of bacterial biocontrol agent to reduce of damping-off tomato disease by reduction of R. solani population through root colonization in rhizosphere soil. The combination application of E. cloacae with micronutrient of manganese or copper reduced population of R. solani higher than the application of E. cloacae alone. The most effective combination of treatments to control damping-off of tomato disease was combination of E. cloacae and 10 ppm of Cu, with 5% of the disease incidence. Its might be due copper is toxic to R. solani, that caused inhibit of a fungal pathogen growth. This result is in agreement with Wadhwa et al.[15] who reported that application of 5 and 10 ppm of manganese and copper play role in suppressed R. solani in soil that caused of cluster bean root rot disease. Mechanism of copper in inhibiting pathogen is associated with its ability to bind amine groups and carboxyl in enzymes of R. solani cell wall [31]. Copper ions
will bind to chemical groups contained in proteins, then destroy the function of proteins and enzymes, resulting damage and leakage of cell membrane of the fungal [32]. *E. cloaca* produced metabolite compounds of volatile antifungal of ammonia that may have fungistatic activity in soil [25], its colonization activity in rhizosphere through using of glucose at low concentration of plants exudates as nutrient source [33].

3.2. Qualitative test for chitinase and protease

The result indicated that colony of *E. cloaca* MB20 shown clear zone area in chitin agar plate and skim milk agar plate, therefore this a bacterial biocontrol agent was judged produce chitin and protein, with chitinolytic index was in range 0.81-0.88, and proteinolytic was about 0.60-0.62 (Table 1 and Figure 3). Chitin is major constituent of the cell walls of filamentous fungi. Chitinolytic activity of bacterial biocontrol agent may cause deformation of hyphae of fungal pathogen that result in inhibition growth of *R. solani*. [34] who found that chitinolytic activity of *Enterobacter* sp. against *R. solani* growth. The mechanism of chitin degradation of bacteria through the hydrolysis of β-1,4-glicosidic bond in chitin compound [35].

![Figure 2. Influence of E. cloaca on population of R. solani in the rhizosphere soil](image)

**Table 1.** Chitinolytic activity of *E. cloaca* MB2

| Replication | Diameter of colony (cm) | Diameter of clear zone (cm) | Chitinolytic Index |
|-------------|------------------------|----------------------------|-------------------|
| 1           | 1.4 ± 0.06             | 1.7 ± 0.06                 | 0.82 ± 0.04       |
| 2           | 1.3 ± 0.06             | 1.6 ± 0.06                 | 0.81 ± 0.04       |
| 3           | 1.5 ± 0.06             | 1.7 ± 0.06                 | 0.88 ± 0.04       |
| Means       |                        |                            | 0.84 ± 0.04       |

Proteolytic activity of *E. cloaca* cause hydrolysis of protein of that compose of *R. solani* cell membrane. Polypeptides of protein are hydrolyzed to small peptides and amino acids are used as nutrients source of the bacterial biocontrol agent [36]. The result of proteolytic activity of *E. cloaca* MB20 confirms the previous report of Owoseni and Onilude[37] who reported that genus of *Enterobacter* sp. have proteolytic activity that were able to make clear zone area at the media contained 5% protein.
Table 2. Proteolytic activity of *E. cloacae* MB20

| Replication | Diameter of colony (cm) | Diameter of clear zone (cm) | Proteolytic Index |
|-------------|-------------------------|-----------------------------|-------------------|
| 1           | 1.0 ± 0.06              | 1.6 ± 0.06                  | 0.62 ± 0.01       |
| 2           | 0.9 ± 0.06              | 1.5 ± 0.06                  | 0.60 ± 0.01       |
| 3           | 0.9 ± 0.06              | 1.5 ± 0.06                  | 0.60 ± 0.01       |
| Mean        |                        |                             | 0.61 ± 0.01       |

Figure 3. Chitinolytic activity of *E. cloacae* (a), and proteolytic activity (b)

4. Conclusion
Application of *E. cloacae* alone into soil decreased damping-off of tomato by 85%. Furthermore combination of biological control of *E. cloacae* MB20 with Cu 10 ppm was the most effective in controlling of tomato damping-off caused by *R. solani*, its decreased the disease incidence to about 95%. *E. cloacae* reduced population of *R. solani* significantly from 33.7 x 10³ CFU g⁻¹ soil to about 19.0 x10³ CFU g⁻¹ soil, produced chitinase and protease with chitinolytic and proteolytic index was 0.84 and 0.61 respectively. Colonization of root tomato, production of lytic enzyme of chitinase and protease and possibility of *E. cloacae* to produce antifungal compounds were considered as suppression mechanisms of damping-off of tomato caused by *R. solani*.

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