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*Full Length Article*

# Efficacy of *Enterobacter cloacae* KtB3 to Control Damping-Off Disease on Soybean Caused by *Sclerotium rolfsii*

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## Abstract

Damping-off caused by *Sclerotium rolfsii* is one of important disease on soybean in Indonesia; may cause the yield loose up to 59% when no control measure is applied. An isolate of rhizobacterium (KtB3) was tested for its potential to suppress the growth of *S. rolfsii* and to control damping-off disease on soybean. This isolate significantly ($P<0.05$) suppressed the growth of *S. rolfsii* on PDA medium with inhibitory activity by 96.97%. The cell-free cultural filtrate of isolate KtB3 showed obvious antifungal activity against *S. rolfsii* indicated by the formation of inhibition zone around diffusion wells. This result indicated that there is an antifungal substance produced and released by KtB3 into the cultural medium. The isolate KtB3 was identified as *Enterobacter cloacae* based on 16S rRNA gene analysis. Application of compost formula of *E. cloacae* KtB3 on soybean seed prior to planting significantly ($P<0.05$) suppressed damping off disease. Percentage of disease incidence of post emergence damping-off on soybean without formula treatment (control) was 100%, while on soybean treated with compost formula of *E. cloacae* KtB3 was only 12.59%. The results of this study suggested that *E. cloacae* KtB3 is a potential bio-agent to control damping-off disease on soybean. © 2018 Friends Science Publishers

**Keywords:** Damping-off disease; *Enterobacter cloacae* KtB3; Bio-agent

## Introduction

Soybean (*Glycine max*) is the third important food crops in Indonesia after rice and corn (Danapriatna, 2007; Sastrahidayat *et al*., 2011). The demand for soybean in Indonesia is continually increased due to its increased use in food industry. According to the data presented by Indonesian Statistical Agency (2014), the total production of soybean in 2009 was 974,512 ton, obviously decreased to 779,992 ton in 2013. The decline of soybean production is partly due to the occurrence of plant disease. One of important disease on soybean in Indonesia is damping-off disease caused by *Sclerotium rolfsii* (Semangun, 2004; Sastrahidayat *et al*., 2011). Damping-off disease on soybean can also be caused by *Pythium ultimum* (Hudge, 2015). Damping off disease commonly occurs on the early stage of soybean growth which is characterized by damping-off symptom or seedling rot. The infected plant will turn to be wilting and gradually yellowing and finally the plant dies. The yield losses resulted from the damping off disease may reach 59% (Akem and Dashiell, 1991).

Synthetic fungicides are generally used by the farmers to control damping-off disease, however this measure is not always effective to reduce the disease incidence. In addition, the use of synthetic pesticides may result in adverse effect to the environment as well as the development of resistance against fungicide. This phenomenon was reported by Soesanto (2008), where the increase of disease incidence occurred caused by *Pythium*, *Fusarium* and *Phytopthora* as a result of the use of pentachlorobenzene (PCNB).

The use of bio-agents to control plant fungal diseases may reduce the use of synthetic chemical fungicides, hence reduce the adverse effect to the environment. One of bio-agents that potentially be used for plant fungal disease control is rhizobacteria. Rhizobacteria are bacteria that colonize plant root and give beneficial effect to the plant through growth promotion (Saharan and Nehra, 2011). Rhizobateria are able to produce phyto-hormones such as *indole acetic acid* (IAA), ACC deaminase, to fix atmospheric nitrogen, act as antagonistic microbes against plant pathogen through the production of siderophores, β-1,3-glucanase, chitinase, cellulase, antibiotics and are able to dissolve phosphate and other nutrients in the soil (Soesanto, 2008; Guo *et al*., 2015; Saleem *et al*., 2015). For example *Pseudomonas fluorescens* produced 2,4-*diacetyl phloroglucinol* that effectively suppress plant fungal pathogens (Nowak-Thompson *et al*., 1994). *Pseudomonas stutzeri* produced extracellular chitinase enzyme and laminarinase that able to decompose the mycelia of *Fusarium solani* (Mauch *et al*., 1988).

The higher plants belong to the Family Leguminosae are known to be associated with a diverse of soil microbes

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and benefited to the plant through their antagonistic activities to the soil borne diseases. According to Sugiyama and Yazaki (2012), Leguminosae plants develop symbiotic interaction with rhizobacteria and arbuscular mycorhiza to get nutrients such as phosphate and nitrogen. Hynes *et al*. (2008) screened 563 bacteria isolated from the root of *Pisum sativum*, *Lens culinaris*, and *Cicer arietinum* for their antagonistic activities. Among them 76% produced siderophores, 5% showed ACC deamines and 7% isolates produced indole. Other isolates could suppress the growth of *Pythium* sp. strain P88-p3, *Fusarium avenaceum* and *Rhizoctonia solani* CKP7. Solichatun *et al*. (2013) reported that rhizobacteria isolated from the rhizosphere of groundnut could inhibit the growth of *Fusarium oxysporum* f.sp. *lycopersici* with inhibitory activity by 89−98%. Three isolates of rhizobacteria viz. *Staphylococcus xylosus* BAC-JAG15, *Serratia* sp. BAC-JAG4, and *Stenotrophomonas* sp. BAC-JAG1 showed 90% inhibitory activity against *Fusarium oxysforum* under *in vitro* condition (Adame-Garcia *et al*., 2016). This study was carried out to evaluate the efficacy of compost formulation of *Enterobacter cloacae* KtB3 to control damping-off disease on soybean under green house condition.

## Materials and Methods

### Evaluation of KtB3 for Antifungal Activity

Isolate KtB3 was isolated from the rhizospehere of groundnut (*Arachis hypogaea*) and maintained in the Laboratory of Biopesticide Udayana University. Inhibitory activity against *S. rolfsii* was done *in vitro* on potato dextrose agar (PDA). Isolate of *S. rolfsii* was provided by the Laboratory of Plant Pathology, Faculty of Agriculture, Udayana University and has been confirmed for its virulence against soybean plant. The isolate KtB3 was tested for their antagonistic activities against *S. rolfsii*. A method developed by Parwati *et al*. (2014) was applied in this test. A mycelial plug of *S. rolfsii* was put in the center of PDA medium on a Petri dish and then each isolate of rhizobacteria was inoculated in four sides of fungal colony at 2 cm distance. For control, a fungal colony without inoculation of rhizobacteria was prepared. Ten Petri dishes were prepared each for treatment with isolate KtB3 and control. The culture was incubated in the dark at an incubator with temperature 28±2°C. The size of fungal colony was measured at the third day of incubation using millimeter block paper. Inhibitory activity was calculated according to the following formula:

$$I = \frac{C-T}{C} \times 100\%$$

Where:

I = Inhibitory activity (%)
C = Size of fungal colony on control
T = Size of fungal colony with KtB3 treatment.

### Evaluation of Cultural Filtrate for Antifungal Activity

Evaluation of the antifungal activity of cultural filtrate of isolate KtB3 against *S. rolfsii* was done based on method developed by Parwati *et al*. (2014), and Ambaradewi (2012). The isolate KtB3 was cultured in potato dextrose broth (PDB) medium. Three hundred milliliter PDB medium was put in 500-mL Erlenmeyer flask and sterilized in an autoclave for 20 min. One milliliter of KtB3 suspension (containing $10^7$ CFU/mL) was inoculated into PDB medium and incubated in an orbital shaker at 200 rpm for a month. This culture was then subjected to the centrifugation at 1000xg for 15 min. The pellet was discarded and the supernatant was collected and passed through Millipore membrane with pore size 0.45 µm (Yonezawa Ltd, Japan).

Ten milliliter of melted PDA medium was put into Petri dish and mixed with 30 sclerotia of *S. rolfsii*. After the culture become solid, two diffusion wells were made per Petri dish using cork borer (5 mm diameter). Into each well, 20 µL cell free filtrate was added. Sterile PDB broth at the same volume was added into wells of control. Ten Petri dishes were prepared each for cell-free filtrate treatment and control. The cultures were incubated in the dark at room temperature (28±2°C). The size of inhibition zone around the diffusion well was measured using millimeter block paper.

### Molecular Identification

Isolate KtB3 was subjected to the molecular identification to determine its species through analysis of 16S rRNA gene sequence. Isolate KtB3 was grown in tryptic soy broth medium (casein peptone: 17 g, soya peptone: 3 g, NaCl: 5 g, 2.5 g $K_2HPO_4$: 2.5 g, glucose: 2.5 g and distilled water to make 1 liter) and incubated in a orbital shaker the dark under room temperature (28±2°C) for 24 h. One milliliter of culture was transferred into a 2-mL Eppendorf tube and centrifuged for 5 min at 5.000xg. Extraction and purification of DNA was done using GeneJET Genomic DNA Purification Kit procedure (Thermo Fisher Scientific Inc., USA).

Two primers viz. 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') were used to amplify the 16S rRNA gene. The reaction was performed in PCR machine GeneAmp® PCR system 2700 (Applied Biosystem, California USA) under the following conditions: pre-denaturation at temperature 94°C (2 min 2 sec) followed by 30 cycles each of denaturation at 94°C (15 sec), annealing at 50°C (30 sec), extension at 72°C (1 min 30 sec), and final extension at 72°C for 10 min.

Pure DNA of KtB3 was sequenced using Genetic Analyzer machine (Applied Biosystem ABI PRISM310, California, USA). The sequence was aligned with GenBank using BLAST-N program from the National Center for

Biotechnology Information (NCBI) to determine the similarity of isolate KtB3 with other previously identified isolates. Phylogeny analysis was performed using MEGA 4.0 neighbor joining method with a bootstrap 1000x.

**Formula Preparation**

Formula of KtB3 was prepared in form of compost made of leaves of rain tree (*Samanea saman*). The fresh leaves of rain tree (1 kg) was chopped off into small pieces and mixed with 10 g sucrose and sterilized in autoclave for 20 min. This mixture was cooled down and added with 10 mL suspension of KtB3 containing $10^7$ CFU per mL and was incubated for a month in the dark at 29±2°C.

**Greenhouse Experiment**

Randomized block design was applied in this experiment with four treatments namely: control (without formula treatment), treatment with 2 g formula per pot, 5 g formula per pot, and 6 g formula per pot. Each treatment was replicated 6 times, thus there are 24 experiment units respectively consisting of 10 pots. Cultural medium for growing soybean consisting of fertile soil and compost (2:1) was previously sterilized in an autoclave for 20 min. This medium was put in a polyethylene bag (1 kg/bag). Inoculation with *S. rolfsii* was done by inoculating 50 sclerotia/polybag in the planting hole (5 cm depth). Formula of KtB3 was put above the sclerotia respectively at the dose of 2 g; 4 g and 6 g per polybag after which the soybean seed was planted. The planting hole was then covered with cultural medium. Several parameters were measured in this experiment namely pre-emergence damping-off incidence, post-emergence damping-off incidence and density of sclerotia in the soil. The incidence of pre-emergence damping-off was calculated based on formula developed by Yulfida and Rustam (2003) as follows:

$$S = \left(\frac{A+B}{B}\ x\ 100\%\right) - (100\% - D)$$

Where:

S = Incidence of pre-emergence damping-off (%)
A = number of planted seed
B = number of emerged seedlings
D = germination rate (%).

Incidence of post-emergence damping-off was calculated based on the formula developed by Yulfida and Rustam (2003) as follows:

$$K = \frac{n}{N} \times 100\%$$

Where:

K = incidence of post-emergence damping-off (%)
n = number of infected seedlings
N = number of emerged seedlings.

## Results

**Antifungal Activities of KtB3 against the Growth of *S. rolfsii***

Rhizobacterium Isolate KtB3 effectively suppressed the growth of *S. rolfsiii* on PDA medium. The average size of *S. rolfsii* colonies on control was 5,020 $mm^2$, while the average size of fungal colonies treated with KtB3 was only 152 $mm^2$. This data indicated that the inhibitory activity of KtB3 against the radial growth of *S. rolfsii* was 96.97%. The appearance of *S. rolfsii* colony on control and colony treated with KtB3 is presented in Fig. 1. There is a clear zone between fungal colony and KtB3 indicated that the KtB3 released antifungal substances into the medium.

Cell free cultural filtrate of KtB3 obviously suppressed the growth of *S. rolfsii* indicated by the formation of inhibition zone around diffusion wells as presented in Fig. 2. Average size of inhibition zone on cultures treated with cell-free filtrate of KtB3 was 237 $mm^2$ while no inhibition zone was observed on cultures of control (Fig. 2). This result revealed that there is an antifungal substance in the cell-free filtrate suggested that rhizobacterium isolate KtB3 produced and released antifungal substance into the medium. It is clear that the mechanism by which isolate KtB3 suppresses the growth of *S. rolfsii* is through antibiosis.

**Molecular Identity of KtB3**

Amplification of PCR product of sequence 16S rRNA isolate KtB3 by using two primers viz. 16S (63F 5'-CAG GCC TAA CAC ATG CAA GTC-3' and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') resulted in DNA fragment of ± 1,300 bp (Fig. 3). The DNA fragment was then purified and subjected to sequencing to identify isolate KtB3 based on its similarity with other species of bacteria that previously have been identified and deposited in GenBank.

Based on sequence comparison with database of GenBank using BLAST program, isolate KtB3 has a close relationship with *E. cloacae* strain AB2, *E. cloacae* strain AB6, *E. cloacae* strain VIT-PAAJ, *E. cloacae* strain Bio103, *E. cloacae* strain VITPSSJ, *E. cloacae* strain BIBT_VC_H, *E. cloacae* strain PW 113, *E. cloacae* strain PM 84, *E. cloacae* strain FS 14, *E. cloacae* strain FM 27, and *E. cloacae* strain FS 4 as presented in Table 1.

Result of phylogenetic tree analysis using Maximum Parsimony (MP) method with 1.000x replicates of Bootstrap showed that isolate KtB3 is *E. cloacae*, because it is in the same clade with the sequence of *E. cloacae* with 82.2% Bootstrap Support (BS) (Fig. 4).

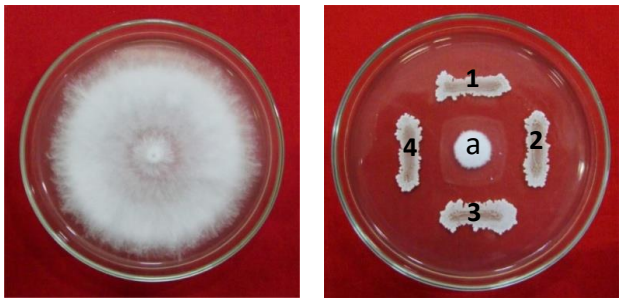


**Fig. 1:** Appearances of colonies of *S. rolfsii* on control (A) and with treatment of KtB3 (B)
a = colony of *S. rolfsii*, 1,2,3,4 = colonies of KtB3

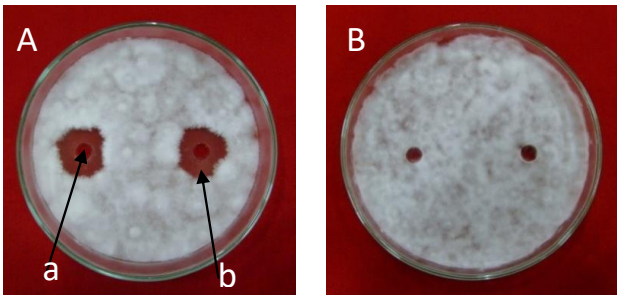


**Fig. 2:** Formation of inhibition zone around the diffusion wells added with cell-free
filtrate of KtB3 (A), and no inhibition zone was developed around the diffusion wells of control (B). a = diffusion well; b = inhibition zone

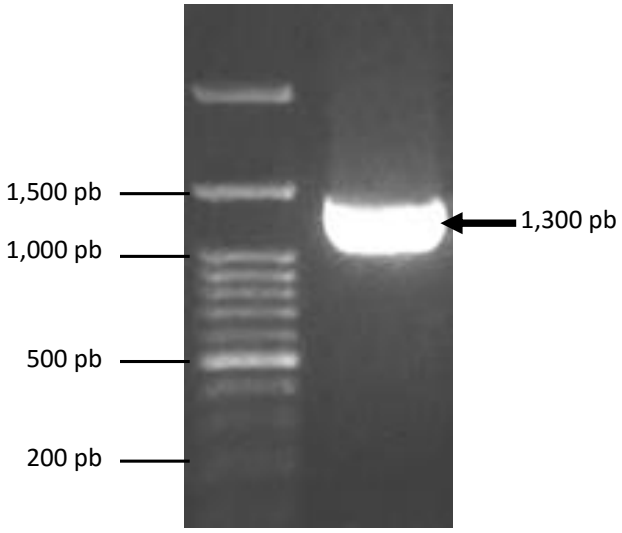


**Fig. 3:** PCR amplification of 16S rRNA gene on agarose gel (M= marker 100 bp; arrow indicates PCR product of isolate KtB3)

**Pre-emergence Damping-off**

Application of compost formulation of KtB3 significantly (P<0.05) suppressed the pre-emergence damping off disease on soybean. The lowest incidence of pre-emergence damping-off was 2.11%, resulted from treatment with the dose of 6 g/pot, followed by treatment with the dose of 4 g/pot and 2 g/pot as presented in Table 2, while on control percentage of pre-emergence damping-off was 29.55%.

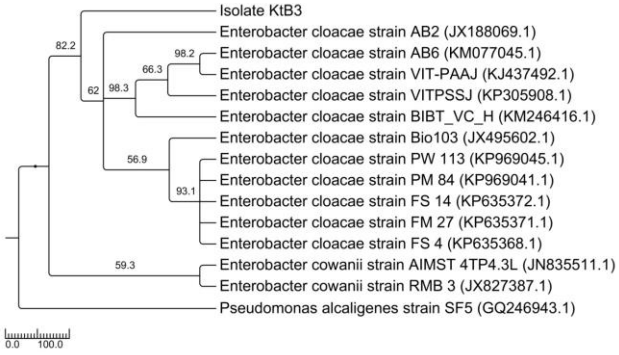


**Fig. 4:** Phylogenetic relationship tree of isolate KtB3 with 14 isolates of bacteria based on 16S rRNA gene using Maximum Parsimony method

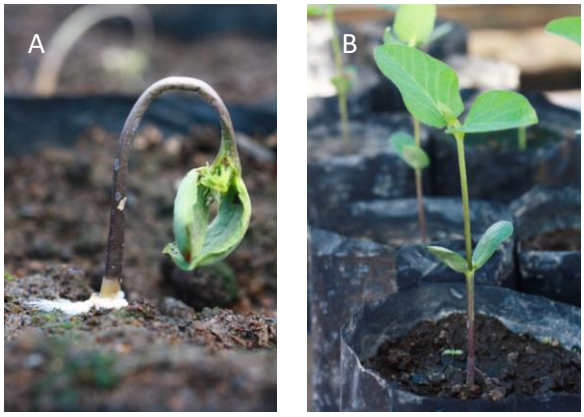


**Fig. 5:** Symptom of post-emergence damping-off on soybean of control (A) and treated with compost formula of KtB3 at a dose of 6 g/pot (B)

**Post Emergence Damping-off**

Treatment with compost formula of KtB3 significantly (P<0.05) suppressed the incidence of post emergence damping-off disease on soybean grown in a green house. Severe symptom of post emergence damping-off was shown by soybean of control, while no symptom appeared on soybean treated with compost formula of KtB3 (Fig. 5). The disease incidence of post emergence damping-off on soybean of control was 100%, while the disease incidences of soybean plants treated with compost formula of KtB3 varied from 12.59 to 35.88% as presented in Table 3. Treatment with compost formula at the dose of 6 g/pot showed the lowest disease incidence of post emergence damping-off when compared with other doses.

**Table 1:** Comparison of percentage of similarities of 16S rRNA gene of isolate KtB3 with several DNA sequent of GenBank using BLAST program

| Isolate | Percentage of similarity (%) | *Accession number* |
|---|---|---|
| *Enterobacter cloacae* strain AB2 | 100 | JX188069.1 |
| *Enterobacter cloacae* strain AB6 | 100 | KM077045.1 |
| *Enterobacter cloacae* strain VIT-PAAJ | 100 | KJ437492.1 |
| *Enterobacter cloacae* strain Bio103 | 100 | JX495602.1 |
| *Enterobacter cloacae* strain VITPSSJ | 99 | KP305908.1 |
| *Enterobacter cloacae* strain BIBT_VC_H | 99 | KM246416.1 |
| *Enterobacter cloacae* strain PW 113 | 99 | KP969045.1 |
| *Enterobacter cloacae* strain PM 84 | 99 | KP969041.1 |
| *Enterobacter cloacae* strain FS 14 | 99 | KP635372.1 |
| *Enterobacter cloacae* strain FM 27 | 99 | KP635371.1 |
| *Enterobacter cloacae* strain FS 4 | 99 | KP635368.1 |

**Table 2:** Effect of compost formulation of KtB3 to the pre-emergence damping off 7 days after planting

| Treatment | Incidence of pre-emergence damping-off (%) |
|---|---|
| Control | 29.55 a* |
| D2 | 6.73 b |
| D4 | 4.41 b |
| D6 | 2.11 b |

*) Values followed by the same letters did not significantly different based on Duncan's Multiple Range Test at 5 % level. Data was transformed into $\sin^{-1}\sqrt{(Y/100)}$ before analysis

**Table 3:** Effect of treatment with compost formula of KtB3 to the incidence of post emergence damping-off disease at 19$^{th}$ day after planting

| Treatment | Incidence of post emergence damping off (%) |
|---|---|
| Control | 100,00 a* |
| D2 | 35,88 b |
| D4 | 27,78 bc |
| D6 | 12,59 c |

*) Values followed by the same letters did not significantly different based on Duncan's Multiple Range Test at 5 % level. Data was transformed into $\sin^{-1}\sqrt{(Y/100)}$ before analysis

## Discussion

Several species of microbes have been tested and showed antagonistic activities against plant fungal pathogens and some of them have been successfully controlled diseases of important agricultural crops (Suprapta, 2012; Widnyana *et al*., 2013; Parwati *et al*., 2014; Suprapta *et al*., 2014a; Guo *et al*., 2015; Hudge, 2015; Adame-Garcia *et al*., 2016). Our present study confirmed that a rhizobacterium isolate KtB3 effectively inhibited the growth of *S. rolfsii* on PDA medium. This result indicated that rhizobacterium isolate KtB3 act as antagonist against *S. rolfsii*. The cell-free cultural filtrate of isolate KtB3 obviously inhibited the growth of *S. rolfsii* indicated by the formation of inhibition zone around the diffusion wells, suggested that isolate KtB3 produced and released antifungal substance into the medium that suppressed the growth of *S. rolfsiii*. The type of antifungal substance produced by KtB3 has not identified yet. This work would be done for further study.

Antagonism between rhizobacteria and plant fungal pathogens may be happened through antibiosis, parasitism, predation, competition, production of extracellular enzymes, and induced resistance (Zhang, 2004). *E. cloacae* EcCT-501 was reported to be effectively suppressed the damping off disease on cucumber through the production of siderophore hydroxamate, aerobactin and catechol (Costa and Loper, 1993). *E. cloacae* subsp. *cloacae* ENHKU01 was also reported to posses antagonistic activity against *Collectotricum capsici*, *Sclerotinia sclerotiorum*, *Alternaria* sp., *Didymella bryoniae*, and *Fusarium oxysporum* under *in vitro* condition by producing chitinase enzyme, siderophore aerobactin and enterobactin (Liu *et al*., 2013). *Vibrio* sp. R-10 produced siderophore amphibactin that act as antifungal substance (Martinez *et al*., 2003). *Burkholderia* sp. strain MSSP produced hydroxymetyl-chroman-4-one that act as antifungal against *Pythium ultimum*, *Phytophthora capsici*, and *S. sclerotiorum* (Kang *et al*., 2004).

Based on molecular analysis of 16S rRNA gene, the isolate KtB3 was identified as *E. cloacae.* This is the first report on its antifungal activity against *S. rolfsii* the cause of damping-off disease on soybean. Other study showed that *E. agglomerans* Al7Kla effectively reduced the intensity of blast disease on rice caused by *Pyricularia oryzae* (Suprapta *et al*., 2014a), while *E. cloacae* Gg14D was reported to be plant growth promoting rhizobacteria, but it has no antifungal activity (Suprapta *et al*., 2014b).

Compost formula of *E. cloacae* KtB3 containing the leaf of *S. saman* effectively inhibited the growth of *S. rolfsii*, the cause of damping-off disease on soybean. Application of compost formula of KtB3 obviously suppressed the pre-emergence and post emergence damping-off disease on soybean. The highest dose tested in this study was 6 g/pot and resulted in 87.41% reduction of the incidence of post emergence damping-off on soybean. The performance of compost formula of KtB3 in suppressing damping-off disease is relatively high compared with other study done by Parwati *et al*. (2014) who found that compost formula containing the leaf of *S. saman* and suspension of *Pantoea agglomerans* GTA24 could reduce damping-off disease caused by *S. rolfsii* by 53.33%. Although compost formula of KtB3 at a dose of 6 g/pot effectively suppressed the post emergence damping-off, but 12.59% of soybean plants were infected by *S. rolfsii*. This result indicated that the dose of compost formula is needed to be increased (more than 6 g/pot). Field test is necessary to confirm the performance of compost formula of KtB3 under natural complex condition.

## Conclusion

*E. cloacae* KtB3 was confirmed as antagonist against *S. rolfsii* the cause of damping-off disease on soybean. This bacterium effectively suppressed the growth of *S. rolfsii* on PDA medium through releasing antifungal substances. The compost formula of KtB3 effectively reduced the incidence of damping-off disease by 87.41%.

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