Selection of Rhizosphere Bacterial Isolates and Development of Antagonistic Bacterial-Based Formulation to Control Fusarium Wilt on Shallot

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ABSTRACT

Wilt disease caused by Fusarium spp. is a major disease in shallot-producing areas particularly in the North Coast of West Java. The research was aimed to select rhizosphere bacteria and develop an antagonistic bacterial-based formulation for its effectiveness to suppress wilt disease caused by Fusarium spp. on shallot plant under greenhouse and field trials. The field trials were carried out using randomized complete block design with four replications at the shallot farmer condition in Subang and Indramayu during the 2019 planting season. Antagonistic test of bacterial isolates from the rhizosphere showed that most of the bacterial isolates can be used as a biocontrol to Fusarium spp. pathogen. The result revealed that bacterial suspension formulation (E-76 + DBS-2 isolates) in combination with phytohormone (1:1 [v/v]) at Subang and Indramayu tended to suppress Fusarium wilt disease intensity with the disease inhibition ranging from 73.54–93.39% and 66.3–95.65%, respectively. The spraying application of formulation 2.5 ml/l was obtained as the best formula to suppress the disease. The growth and production of shallot were also affected by the application of antagonistic-bacterial formula.

Keywords: Disease suppression, formulation, Fusarium spp., shallot, wilt.
INTRODUCTION

 Shallots are an important vegetable crop worldwide (Hanci 2018), including in Indonesia. Some production center areas strongly support shallot farming production, i.e. Cirebon, Brebes, Tegal, Kuningan, Wates, East Lombok, and Samosir. In 2012, with a planting area of about 99,519 ha and production of 964,221 t (BPS 2018), Indonesia still had to import shallots of 119,505 t with a value of 53.25 million US dollars. The national productivity is still low (10.22 t/ha) (Rusono et al. 2014) compared to its potential yield that reaches up to 20 t/ha (Basuki et al. 2004). The gap between consumption and production as well as the downward trend in shallot production are both challenges and business opportunities for developing shallot agribusiness.

Plant pathogen attack is one of the constraints that often faced in the shallot cultivation (Havey 1999). Wilt or basal rot disease was caused by Fusarium oxysporum f.sp. cepae, initially reported not as a major disease in Indonesia (Triwidodo et al. 1998). However, the incidence of the disease has increased and made it become a major disease on shallots as well as on garlic production center areas (Wiyatiningsih 2003). Symptoms of the disease were observed both at early and late growing stage by twisting on stems and pale green color on the leaves. The tubers appear to be smaller and fewer than the healthy ones. In certain heavy severe conditions, the plant becomes dry and dies. In Sri Lanka, the disease was first reported between 1992 and 1993 with the main symptoms of leaf wilting or twisting (Kuruppu 1999). In addition to infecting crops, this disease can also infect crop tubers during storage (Widodo et al. 2008). The yield loss caused by Fusarium wilt disease is not known with certainty due to limited information about the disease. Fusarium wilt is detrimental to shallot cultivation because it can cause production to decrease by 50% and even fail to harvest (Wiyatiningsih 2003).

Currently, the effort to control Fusarium wilt disease is still emphasized on control techniques using fungicides that are exposed to food and environmental safety risks. Control the disease through biological suppression is an alternative control that is safe, inexpensive, and environmentally friendly. Therefore, it is necessary to make appropriate biological control efforts to suppress Fusarium wilt disease on shallots.

Some strains of rhizosphere bacteria that are known as Plant Growth Promoting Rhizobacteria (PGPR), can stimulate growth and increase plant resistance. Bloemberg and Lugtenberg (2001) classified PGPR bacteria based on their ability to use as biofertilizer agents (increasing N and P uptake), growth stimulator agents (producing hormones), and biological agents (able to protect plants from pathogenic infections). Rhizosphere bacteria of biological agents may produce antifungal compounds and able to secrete the chitinase and glucanase enzymes (Haedar et al. 2017). Hydrolysis of the fungal cell wall by biocontrol bacterial agents may reduce cell wall integrity, thus the fungus is unable to infect plants (Pratiwi et al. 2015). Kholida and Zulaika (2015) reported that indole acetic acid (IAA) produced by bacteria i.e. Pseudomonas sp. and Azotobacter sp. will stimulate plants for better growth because the plants absorb the bacterial hormone through the roots.

Some bacterial isolates that produce extracellular enzymes and growth hormones can be applied as biopesticides. The alternative use of biopesticides in modern agriculture is currently needed to replace the use of inorganics pesticides which have a negative impact to soil and environments. Some biological agents such as Trichoderma spp. and Pseudomonas fluorescens P60 were reported to be able to suppress F. oxysporum f.sp. gladioli and F. oxysporum f.sp. zingiberi causes wilting in gladiolus and zingiber (Prabowo et al. 2006). Previous studies reported that rhizosphere bacteria (DBS-2 isolates) have potential as biological control agents (Suryadi et al. unpublished data).

This study was aimed to select, characterize the rhizosphere bacterial isolates, and develop bacterial antagonist-based formulation for their activity to suppress Fusarium wilt disease on shallot.
MATERIAL AND METHOD

Selection, Morphological Observation, and Gram Tests of Bacterial Isolates

The rhizospheric plant samples were collected from shallot tuber plant from Patrol–Indramayu (West Java). A single colony was taken and inoculated into nutrient agar (NA) medium (0.3% beef extract, 0.5% peptone, and 1.5% agar) (Kurm et al. 2019) then incubated for 24 hours at 30°C until bacteria grew. One loopful of bacterial cells was transferred from the NA slant medium into a liquid NB medium in a screw cap tube and then incubated for 24 hours until cloudy. The absorbance was measured at a wavelength of 620 nm. Morphological and gram test observations were done using a microscope at magnifications of 40× and 100×.

Characterization of Extracellular Enzymes (Chitinase, Glucanase)/IAA-Producing Bacteria

The chitinase assay was carried out by growing bacterial solution through centrifugation at 10,000 rpm and 4°C for 10 min. Supernatant as a crude extract (300 μl) was then mixed with 300 μl PBS pH 7 and 600 μl of colloidal chitin in a screw tube. It was vortexed and incubated at 37°C water bath for 30 min. The mixture was poured into an Eppendorf tube and centrifuged at 5,000 rpm for 5 min. The supernatant was taken 500 μl then added with 500 μl distilled water and 1,000 μl Schales reagent. The mixture was boiled at 100°C for 10 min, then cooled and the absorbance was measured at a wavelength of 420 nm (Soeka and Sulistiani 2011).

The glucanase assay was done by culturing bacteria through centrifugation at a speed of 10,000 rpm for 15 min. Supernatant (1 ml) as a crude extract was added with 1 ml of glucan pellet solution. The mixture was vortexed and incubated at 37°C for 60 min. Then, 1 ml of DNS solution was added for 15 min in a water bath. The mixture was measured for absorbance at a wavelength of 540 nm (Goncalves et al. 2010). For qualitative enzymes assay, a total of each 3 μl of suspension was dropped into solid chitin/glucan medium in Petri dish which had been divided into two quadrants. Each quadrant was dropped with 3 μl of suspension and allowed to dry for one day in laminar flow. The Petri dish was incubated for 3–6 days, then 0.3% Congo red solution was dropped to cover the entire surface of the medium and left for 5 min, then 0.1% NaCl solution was dropped to cover the entire surface of the solid chitin medium. The medium was then observed for a clear zone formation (Suryadi et al. 2013; Murtiyaningsih and Hazmi 2017). The clear zone was measured using formula as follows:

\[ I = \frac{d_1}{d_2} \]

where, \( I \) = chitinolytic/glucanolytic index, \( d_1 \) = clear zone diameter, \( d_2 \) = colony diameter.

For IAA assay, the bacterial culture was grown on an NB medium which enriched with 2% tryptophan, and then incubated for 2 days at room temperature and centrifuged at a speed of 10,000 rpm of 4°C for 10 min. The supernatant (2 ml) then was added with 4 ml of Salkowsky reagents (2% 0.5M FeCl₃ in 35% HClO₄ solution) (Glickmann and Dessaux 1995). The solution was homogeneous using vortex and then incubated for 1 hour. The absorbance was measured using a spectrophotometer at a wavelength of 530 nm (Karnwal 2009).

Preparation of Fusarium Culture, Antagonistic Test (In Vitro)

Fusarium spp. isolates were obtained by isolating pathogens from plant samples that showed symptoms of Fusarium wilt disease from shallot-producing areas (Cirebon, West Java). The shallot diseased-tubers were disinfected by rubbing them with cotton soaked with 76% alcohol and dried. The tuber boundary of the diseased and healthy part was cut into small pieces and then incubated for 7 days to grow in a Petri dish containing PDA media plus 100 ppm streptomycin.

The fungus was incubated in PDA Medium which was divided into two parts with a distance of 3 cm and at one side was perforated using a sterilized cork borer. Fusarium fungi were put to the hollow part of the medium. The other side of
the medium was streaked with antagonistic bacterial isolates. The culture was incubated for 3–7 days. Furthermore, the diameter of fungi was measured (Khaeruni and Rahman 2012) following the formula of disease suppression:

$$P = \frac{(r_1 - r_2)}{r_1} \times 100\%$$

where, $P =$ disease suppression, $r_1 =$ the radius of a pathogenic colony that grows in the opposite direction to the antagonist, $r_2 =$ radius of pathogenic colony that grows closer to pathogen isolates.

**Formula Preparation**

Selected bacteria were isolated from the rhizosphere of shallot plants and cultured following standard procedures of previous works (Suryadi et al. unpublished). Bacterial isolates were selected based on enzymes (chitinase and glucanase) and IAA production. The selected bacterial isolate E-76 and DBS-2 was mass propagated in Erlenmeyer tube containing nutrient broth with 75 rpm agitation for 2 days. The rate of bacterial growth was observed every 12 hours to determine the time of harvest of bacterial cells carried out in the log phase. Furthermore, the bacterial cells were harvested by centrifugation, and then washed with sterile distilled water to remove old culture residues. Cells were suspended with new media with a cell density of $10^9$ cfu/ml and induced with 2% tryptophan to produce IAA (Ghosh and Basu 2002) for 24 hours before being added to the carrier for the formulation process. Multiplication of selected bacteria for the production of formulas was carried out on a 10 l scale using nutrient broth enriched with yeast extract and inducer 0.2% chitosan.

**Green House (GH) Trial**

The efficacy test in the greenhouse (Bogor) was carried out using shallots cv. “Bima Curut” by performing artificial inoculation. Isolate *Fusarium* spp. was propagated on a medium mixture of sand/corn which moistened with 400 ml of water/kg media and then packaged in a heat-resistant plastic bag. The bags were sterilized at 120°C for 20 min for two days and each bag was inoculated with two *Fusarium* spp. culture disks that were actively growing on a Petri dish (diameter 9 cm) containing PDA media. The bag containing inoculation media was incubated at 28 ± 2°C for two weeks and used as a source of inoculum.

A 30 cm × 60 cm polybag filled with 5 kg of soil, and then manure was added 10 days before seedling. Five tubers of shallot were planted in each polybag with three replications. The control treatment (without inoculation) was prepared, while Carbendazyme fungicide (0.1%) was used as chemical control.

At the seedling stage, a suspension-based formulation of antagonistic bacteria (containing $10^9$ CFU/ml) was applied into a polybag @ 5 g/kg of soil, and then inoculated with *Fusarium* spp. Plants were watered as needed and maintained by fertilizing urea, SP-36, and KCl at doze of 1.6 g, 1.2 g, and 0.8 g per polybag, respectively. After 45 days of planting, the plants were carefully removed and observed.

**Land Preparation, Seedling, Planting, and Efficacy Test in the Field Trials**

Field experiments (total of 518.4 m² plot size) were conducted to evaluate the efficacy of biocontrol agents against Fusarium wilt disease. Before application in the field, the formula containing bacterial isolates E-76 and DBS-2 was combined with phytohormone 2.5% at a ratio of 1:1 (v/v). The experiment was carried out at two locations of farmers’ field trials at the district of Pusakajaya Subang (6°19’35.918” N, 107°54’47.8332” E, 18 masl) and Patrol Indramayu (6°16’55.7076” N, 107°59’13.038” E, 6 masl), West Java. Shallot seeds cv “Bima Curut” was planted in each plot following farmer culture technique recommendations. The shallot was cut off at the edges with a uniform size of 5 g each. Shallots were planted in each 1.2 × 18 m² plot size with a spacing of 15 cm × 10 cm. The number of plants was 1,100 plants per plot. Artificial inoculation was carried out by spraying the *Fusarium* spp. (10⁶) spore solution evenly on the seedlings with the addition of Tween 80 as an
adjuvant. The control treatment was prepared without formula application, while the control fungicide treatment was used following farmers' recommendation. Bacterial formulation (containing \(10^9\) CFU/ml) plus phytohormone (IAA) \((1:1 \ [v/v])\) was applied as seed treatment (immersion) for 24 h at a dose of @ 2.5 l per ha. Furthermore, plant seedlings were applied by spraying bacterial antagonist formula prepared at concentrations \((2.5\ \text{ml/l, 1.25 ml/l, 0.625 ml/l, and 0.3125 ml/l})\). The shallot plants were applied by spraying formula since the nursery until pre-harvest stage with interval once a week. Spraying was done evenly throughout the surface of the plant. The treatments were arranged in a randomized block design (RBD) with four replications. All cultivation practices including irrigation, weeding, and fertilizing applications were carried out periodically following the farmer cultivation conditions. Fertilizer application was done with inorganic fertilizer \((P\ 200\ \text{kg/ha, NPK \[15:15:15\], 100 \text{kg/ha and KNO}_3\ \text{150 kg/ha}})\) and organic compost (rate of 5 t/ha) to support the growth of shallot plants.

**Data Analysis**

In the greenhouse trial, the growth variables i.e. length of the shoot/root and number of tubers were observed. Disease severity was assessed using five scale basis, where 1 = no symptoms in plants (0%), 2 = rotted roots (<10% rotted basal plates), 3 = 10–30% rotted roots (10% rotted basal plates), 4 = completely rotted roots (10–30% rotted basal plates), 5 = completely rotted roots and (>30% rotted basal plates) (Rengwalska and Simon 1986). Vigour index was determined according to formula Abdul-Baki and Anderson (Sharma 2018). Vigour index is calculated by multiplying germination (%) and seedling length (mm).

In the field trials, sampling of plants was carried out using 10 plants per plot. Observations were made based on the data variable of incidence of Fusarium wilt disease, plant height, and tuber weight yield. Disease incidence was counted once a week for five observations starting at two weeks after planting. Disease incidence was calculated using the formula (Rosmahani et al. 2002):

\[
P = \frac{A}{N} \times 100\%
\]

where, \(P\) = level of disease damage, \(A\) = number of plants attacked, \(N\) = number of plants observed.

The area under disease progress curve (AUDPC) was determined using the formula Simko and Piepho (2012) as follows:

\[
\text{AUDPC} = \sum_{i=1}^{n-1} [(t_i+1 - t_i) (y_i + y_{i+1})/2]
\]

where, \(t\) = disease observation time (days), \(y\) = percentage of disease intensity at the \(i\)th observation, \(n\) = total number of observations.

Data were analyzed with analysis of variance (Anova). If the F test in the treatment is significant, then mean value is determined by Duncan's multiple range test (DMRT) \((p = 0.05\%\) for further analysis.

**RESULT AND DISCUSSION**

**Selection and Characterization of Rhizosphere Bacterial Isolates (Chitinolytic, Glucanolytic, and IAA Assays)**

The rhizosphere is an excellent habitat for microbial growth, so plant roots provide organic material which in general can stimulate microbial growth (Hardestyariki et al. 2013). The rhizosphere is rich in exudates released by plants in the process of root secretion. The exudate content is in the form of carbohydrates, amino acids, organic acids, enzymes, and other compounds. Rhizosphere bacteria can be found in large quantities in the root surface area, where nutrients are provided by exudates and lysates of plants (Lugtenberg et al. 1999). If the microbial population around the rhizosphere is dominated by beneficial microbes, plants will benefit greatly from the presence of these microbes.

Morphological observations to bacterial isolates showed that all isolates had the form of coccus. The results of the Gram test were show that most bacterial isolates belong to Gram-positive bacteria and some are Gram-negative bacteria (data not shown). The OD measurements of all isolates...
showed a different speed of growth. There were some isolates showed slower growth and some showed faster growth. The highest OD value was TK 3 isolate (2.18), and it was able to grow faster on liquid chitin medium for 48 hours. The lowest OD value was TK 1 isolate (1.84) which showed slow growth on liquid chitin medium for 48 hours. Bacterial isolates were obtained from soil samples, leeks, and bulbs of shallot plants originating from the Brebes area (13 bacterial isolates). Morphological characteristics of bacterial colonies need to be made to facilitate the process of identifying bacteria. Morphological parameters of cell colonies in the growth medium were observed in the form of color, shape, and size of the colonies in the medium. Gram staining is used to determine the morphology of bacterial cells and to distinguish between Gram-positive and Gram-negative bacteria. Most purple bacteria are classified as Gram-positive bacteria that can absorb violet crystalline dyes because they have thicker cell walls and contain 90% peptidoglycan (Sabdaningsih et al. 2013). Gram-negative bacteria have a relatively thin layer of cell walls (<10 nm). These differences in cell wall layers give different properties to cells (Mai-Prochnow et al. 2016). Quantitative analysis showed that there was an isolate (TK 2) that produced the highest chitinase, with a concentration of 6.32 mg/l, while the lowest chitinase yield was TS 2 isolate with a concentration of 4.02 mg/l (Table 1). DBS-2 showed higher chitinolytic index as well as glucanolytic index. As supported by previous study, the isolates were also secreting chitinolytic enzymes (Suryadi et al. unpublished). Chitin or glucan substrate degradation depends upon enzyme activity, environmental factors such as pH, temperature, and aeration, as well as sources of nutrients used in the medium, and the metabolism of microorganisms.

Protein denaturation due to salt concentrations and pH values will eliminate enzyme activity (Soeka and Sulistiani 2011; Setia and Suharjono 2015). Isolates that showed a clear zone around the colony are considered as chitinolytic producing bacteria because bacteria can break the chitin substrate on the medium (Hao et al. 2012). The clear zone was formed because of the process of chitin/glucan substrate breakdown into simple compounds/glucose after secreting the enzymes (Suryadi et al. 2013). There was an isolate (TS 1) that produced the highest glucanase (7.52 mg/l), while the lowest glucanase was shown by TK 5 isolate (2.28 mg/l). Almost all isolates formed clear zones. The largest glucanolytic index (9) was produced by TB 2 isolate, while the smallest glucanolytic index was produced by TK 4 isolate (1.13) (Table 1). All bacterial isolates showed the formation of clear zone when cultured on solid medium containing 3% colloidal chitin or glucan. The clear zone was formed after the second day and the longer incubation will widen in diameter until the entire substrate was completely degraded by bacteria or it becomes completely

| Isolates code | Chitinase (mg/l) | Chitinolytic index | Glucanase (mg/l) | Glucanolytic index | IAA (mg/l) | Gram test | Morphology |
|---------------|------------------|--------------------|------------------|--------------------|------------|-----------|------------|
| DBS-2         | 6.24             | 1.50               | 7.22             | 2.67               | 0.484      | -         | Milky white, bacillus |
| E-65*         | 6.30             | 1.44               | 6.80             | 4.50               | 0.287      | +         | White, bacillus |
| E-76*         | 6.21             | 1.04               | 3.00             | 1.38               | 0.348      | -         | Yellow, coccus |
| TB 2          | 5.87             | 1.40               | 2.36             | 9.00               | 0.277      | -         | Pale yellow, coccus |
| TK 1          | 6.25             | 1.42               | 7.44             | 5.88               | 0.399      | +         | Milky white, bacillus |
| TK 2          | 6.32             | 1.42               | 6.10             | 5.71               | 0.324      | +         | Yellow, coccus |
| TK 3          | 6.02             | 1.22               | 2.35             | 1.21               | 1.113      | -         | Pale yellow, coccus |
| TK 4          | 6.29             | 1.22               | 6.23             | 1.13               | 0.338      | +         | Pale yellow, coccus |
| TK 5          | 6.26             | 1.10               | 2.28             | 1.50               | 1.047      | +         | Milky white, bacillus |
| TK 6          | 5.35             | 1.33               | 6.94             | 1.18               | 0.301      | +         | Pale yellow, coccus |
| TK 7          | 6.10             | 1.22               | 6.91             | 5.63               | 0.371      | +         | Pale yellow, coccus |
| TS 1          | 6.30             | 1.12               | 7.52             | 4.09               | 0.273      | +         | Pale yellow, coccus |
| TS 2          | 4.02             | 1.30               | 2.60             | 1.31               | 0.681      | +         | Milky white, bacillus |

*ICABIOGRAD culture collection.
clear (Liu et al. 2011). This indicates that the isolates secrete extracellular enzymes (chitinolytic/glucanolytic). Different diameters of each isolate showed differences in the activity of each chitinolytic/glucanolytic enzyme (Maggadani et al. 2017). Chitinase plays an important role in bacterial pathogenesis for hosts containing chitin. Chitinase is present in plants along with various protein-related pathogenesis (PRP) as part of the plant's defense mechanism. Chitinase can also be directly used as a biopesticide against various fungi and insects (Rathore and Gupta 2015). β glucans are glucose homopolymers that are bound by β-(1,3) and β-(1,6)-glucosides bonds and are found in many cell walls. Some microorganisms, such as yeast, fungi, and cereals (wheat and barley), have high economic value because they contain large amounts of β-glcans (Robak 2013). Isolates of DBS-2, E-65, TK 2 TK 4, TS 2, and TK 7 showed the potential as a biological control agent which can be further used as biocontrol agents formulation to inhibit fungi due to the potential of producing chitinase and glucanase.

The highest IAA concentration was TK 3 isolate (1.113 mg/l). IAA production will increase under reduced growth conditions, limited carbon availability, and under acidic pH environmental conditions. This condition occurs when bacteria enter the stationary phase (A'ini 2013; Dewi et al. 2015). IAA growth hormone functions as an important molecular signal in the regulation of plant development, increases resistance to pathogens, and promotes plant growth. Exogenous IAA is auxin hormone produced by organisms other than plants and has its function to increase cell elasticity (Joshi and Bhatt 2011). Although some isolates representing well in terms of IAA, enzyme production and antagonistic in vitro test, we selected only DBS-2 isolates (Kosakonia sp. Syn Enterobacter sp) and E-76 (Burkholderia sp.) which well-characterized to produce those characters for the current formulation, whilst other isolates have not yet been further characterized in terms of identity and compatibility test.

**Antagonistic Test to *Fusarium spp.* and Bioassay in GH**

Prior to the test in the GH, *Fusarium spp.* was first cultured for seven days to check for the spore formation. Each bacterial isolate was tested for its inhibition against *Fusarium* fungi. Up to seven days after incubation, all the treatments of the formula tended to inhibit the growth of *Fusarium* spp. compared to control (Figure 1).

Lv et al. (2011) classified the diameter inhibition of ≥20 mm (very strong), 10–20 mm (strong), 5–10 mm (moderate), and <5 mm (less or weak). Based on these criteria, isolates of TK 7, TK 2, TS 2, TK 4, and E-65 have inhibitory properties of 5.7, 5, 5.1, 5.4, and 5.2 mm so that the isolates have moderate inhibition, whereas the other isolates have inhibition of less than 5 mm (weak inhibition). The antagonistic test (*in vitro* assay) to *Fusarium* fungi showed TK 3 isolates less inhibit the pathogen, whereas the highest percentage of inhibition against *Fusarium* spp. was TK 7 isolate (57.14%).

Figure 2 shows that the application of the formula decreases fungal growth or increases inhibition range from 69.13 to 100% in the GH test. The application of the formulation to the shallot plant vigor index is presented in Figure 3. The vigor data is the result of the multiplication of plant growth (shoot length + root length) with plant germination rate. Although still varied, the vigor index of the formula on plant treatment was still better than the control. The severity of the disease ranges from 12 to 64%, where the treatment was still lower than that of the control (Figure 4). In addition to disease severity, all treatments were not significantly different to plant height (cm) and tuber weight yield (g/pot) in the GH (Figure 5).

**Evaluation of Bacterial Formulations Against Fusarium Wilt Intensity in the Field Trials**

The analysis of variance showed that there were significant differences between the plots of application of inoculated bacterial formula treatment as compared with those without application (control) on the intensity of wilt disease (*Fusarium* spp.), both in Subang and Indramayu.
The lowest intensity of Fusarium wilt disease both in Subang and Indramayu was seen in treatment A (2.5 ml/l), which was 1–2.12% or decreased by 93.39–95.65%, respectively compared to controls (without application) (Table 2). The application of bacterial formula which was applied in combination with phytohormone, both in Subang and Indramayu was able to control Fusarium spp. pathogens, with disease suppression (DS) on ranging beetwen 73.54 to 93.39% and 66.3 to 95.65%, respectively.

Disease Progression

The intensity of wilt disease in the untreated plot (without application) was higher than that of the treatment plot, at five times observation period (Figure 6). The lowest disease progression in the treatment plot were application A (2.5 ml/l) and B.
where there was an inhibition of the intensity attacks 93.33% and 91.43% (in Subang) and 95.65% and 79.34% (in Indramayu), respectively. Based upon the analysis of the area under disease progress curves (AUDPC), the highest disease values were found in control treatments (without application), with AUDPC values of 402.975 (in Subang) and 448.875 (in Indramayu) (Figure 7).

The higher intensity of Fusarium wilt in the treatment plot is thought to be affected by several factors, including the availability of a high initial inoculum in the soil. This relates to the location of research which is an endemic area of Fusarium wilt disease. This is presumably due to the formula in the treatment has been able to colonize the root surface widely and produce antifungal activity, so that the pathogen is disturbed in its development.

The results of the study are in line with the application using the *P. fluorescens* bacterial formula which is also reported to have the ability to stick strongly to the root surface (Kloeper et al. 1997).

In general, the formulation treatment can suppress the intensity of wilt disease. It is suspected that antagonistic bacteria are able to compete with pathogens in the soil which are affected by soil physical, chemical, and biological factors (Agrios 2005). Formulas containing bacterial colonies require adaptation in the soil. A large number of pathogenic propagules in or near the host plant allow more inoculums to reach the host earlier, so that epidemic changes are greater. If the experimental plot already has an initial pathogen inoculum, indicating that the location of the experiment is an endemic area. *Fusarium* spp.

![Figure 5. Effect of antagonistic bacterial-based formula to plant height (cm) and tuber weight (g/pot) of shallot (GH test).](image)

| Treatment (ml/l) | Wilt disease intensity (%) | Disease suppression (%) |
|-----------------|---------------------------|-------------------------|
|                 | Subang        | Indramayu | Subang       | Indramayu |
| A = 2.5         | 2.12 d        | 1.00 e    | 93.39        | 95.65     |
| B = 1.25        | 2.75 cd       | 4.75 bc   | 91.43        | 79.35     |
| C = 0.625       | 4.12 cd       | 7.25 bc   | 87.17        | 68.47     |
| D = 0.3125      | 8.50 c        | 7.75 bc   | 73.54        | 66.30     |
| E = Control (without application) | 32.12 a | 23.00 a | -            | -         |
| F = Control fungicide | 16.25 b | 14.75 ab | 49.40        | 35.87     |

Means value in column followed by the same letter are not significantly different by DMRT (P < 0.05). Disease suppression (DS) was assessed by formula DS = (C−T)/C × 100%, where C = control, T = treatment.
remains active in the soil as source inoculums, both in the form of thick walls passive mycelium and chlamydospore (Saragih and Silalahi 2006).

Various biotic and abiotic factors greatly influence the various stages of antagonistic colonization on the root surface or around the rhizosphere. Colonization affects the antagonistic inhibition of pathogens, where pathogens need time to adapt to the environment, or the presence of pathogenic fungal virulence factors to develop. Virulent pathogens are able to quickly infect their host and subsequently produce more inoculums than less virulent pathogens (Agrios 2005). The Fusarium is one of the genera of fungi that is very important economically as well as pathogenic species causing vascular wilt disease in various plants. Fusarium spp. can live in a variety of ecosystems including soil and plant roots worldwide (Bennet et al. 2008; Sutrisni and Widodo 2012). Pathogenic fungi that cause rot disease shallot or wilt, can still live longer in the soil without a host (Semangun 2000). Fusarium spp. infects shallots on the body parts of injured shallots, due to activities during cultivation and after harvest.

The speed of the appearance of symptoms of wilting in control occurs due to the aggressiveness of pathogens in causing the disease. In addition, the
suitability of pathogens with shallot plants is thought to cause symptoms to appear earlier (Maryani et al. 2005). The development of the disease may be affected by an endemic area of Fusarium wilt. Other factors supporting the suspected higher disease severities were temperature and relative humidity during the study (temperature 30.5°C and RH 70%). The fastest incubation period occurs in the plot without treatment (control), because it is closely related to the location of the study as an endemic area of wilt disease. This proves that the land used has been contaminated with *Fusarium* spp., which is in line with the high intensity of the disease. The period of disease development in bacterial formulations treatment can inhibit the growth of *Fusarium*. The disease inhibition may be the result of various mechanisms, including competition and antibiosis (Raaijmakers and Weller 1998).

The application of bacterial antagonist formulations enriched with growth hormone in this study is thought to cause shallots to be more resistant to pathogenic infections while allowing physiological changes. This is might be due to the formulation of bacteria/growth hormone applied to plants, capable of acting as a biological agent as well as PGPR which stimulates the growth of the root system and inhibits harmful fungi (Raupach and Kloepper 1998).

### Effect of Antagonist formulations on Plant Height and Tuber Weights

Plant height growth measurement in field plots before application and after application in Subang and Indramayu were shown in Table 3 and Table 4. Treatment tends to be higher when compared to plots without application formulation. The application of bacterial formulations significantly affected tubers weight of shallot. The highest tuber weights were obtained by treatment A (2.5 ml/l) and B (1.25 ml/l), compared with the smallest tuber in the control treatment (Table 3, 4).

The low yield of tubers in the F treatment (control fungicide) in this study is in line with the inability of the fungicide to control pathogens, which results in the high intensity of the disease and the effect on plant growth. This is supported by another study that states some efforts to control Fusarium wilt in tomatoes with fungicides also did not produce satisfying results (Semangun 2000).

The highest shallot tuber weight in the treatment was suspected because of PGPR action. The highest tuber weight of the treatment was

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**Table 3. Effect of bacterial formulation to plant height and tuber weight of shallot (Subang).**

| Treatment (ml/l) | Plant height (cm) | Tuber weight (g/plot) |
|------------------|-------------------|-----------------------|
|                  | Before application (4 WAT) | After application (8 WAT) |
| A = 2.5          | 23.25 a            | 30.75 a               | 54.50 a |
| B = 1.25         | 17.50 c            | 29.00 b               | 49.50 a |
| C = 0.625        | 17.75 c            | 26.50 c               | 43.50 b |
| D = 0.3125       | 15.75 ed           | 23.25 d               | 39.87 bc |
| E = Control (without application) | 14.25 d | 18.50 c | 23.00 d |
| F = Control fungicide | 20.50 b  | 26.00 c | 36.75 c |

Means value in column followed by the same letter are not significantly different by DMRT (P < 0.05).

**Table 4. Effect of bacterial formulation to plant height and tuber weight of shallot (Indramayu).**

| Treatment (ml/l) | Plant height (cm) | Tuber weight (g/plot) |
|------------------|-------------------|-----------------------|
|                  | Before application (4 WAT) | After application (8 WAT) |
| A = 2.5          | 18.50 a            | 33.50 a               | 59.25 a |
| B = 1.25         | 15.25 b            | 28.25 b               | 54.75 b |
| C = 0.625        | 12.50 c            | 28.25 b               | 49.05 cd |
| D = 0.3125       | 13.75 bc           | 29.50 b               | 51.37 bc |
| E = Control (without application) | 12.00 c  | 17.75 d | 28.87 e |
| F = Control fungicide | 13.50 bc  | 22.75 c | 46.37 d |

Means value in column followed by the same letter are not significantly different by DMRT (P < 0.05).
presumably due to indirect effect of bacterial formula which was able to suppress pathogens. Thus, PGPR formulation plays a role in stimulating plant growth. In line with other research, PGPR bacteria were reported to produce siderophores, antimicrobial compounds (salicylic acid), and growth hormones (IAA), which can stimulate plant growth (Raupach and Kloeper 1998). Aside to used different potential isolates for mixture formulation, further research is needed to optimize bacterial formula in different agroecological zone.

CONCLUSION

The majority isolates from rhizosphere plants have a round shape and Gram-positive bacteria. The antagonistic test of bacterial isolates shows that most of the bacterial isolates can be used as a biocontrol to Fusarium spp. The used mixture of bacterial formula (isolates of E-76 and DBS-2) combined with growth hormone 1:1 (v/v) using a concentration of 2.5 ml/l is the most effective biological control to Fusarium wilt both in the GH and the field trials. The treatment could reduce the development of Fusarium wilt disease in Subang and Indramayu with the disease intensity ranging from 1–2.12% or disease suppression (DS) value of 93.39–95.65%, compared to control treatment (without application). Application of bacterial formulas in the field affected plant height and yield (tuber weight) production of shallots. The formula might be further used to optimize its efficacy under multi-location sites.

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