BIOEFFICACY AND CHARACTERIZATION OF PLANT GROWTH-PROMOTING BACTERIA TO CONTROL THE BACTERIAL WILT DISEASE OF PEANUT IN INDONESIA

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ABSTRACT

The use of bactericides and resistant varieties to control the bacterial wilt disease have been explored by Indonesian farmers but the pathogen is still difficult to control. The application of biocontrol agents (BCA) is an alternative measure to control the bacterial wilt disease of peanut. The experiments were conducted to study the effectiveness of Pseudomonas fluorescens RH4003 and Bacillus subtilis AB89 to control the bacterial wilt disease of peanut, and to detect some characters of several BCA. Six candidate BCA were evaluated for their effects on seed viability and the effectiveness to control the bacterial wilt disease of peanut in the greenhouse. Disease index of the plants treated with the BCA was significantly different compared with the control but there were no differences among the BCA. Index suppression on six weeks after planting caused by 0.02% streptomycin sulphate, P. fluorescens RH4003, Bacillus sp. KS2, P. fluorescens CK5, B. subtilis AB89, P. fluorescens ES32 was up to 85.7, 83.5, 77.6, 75.3, 67.5, and 58.8 %, respectively. The percentage of seed viability up to 14 days was not significantly different compared with control. Seedling height and number of branches on the seeds treated with Bacillus sp. KS2 were significantly higher compared with those in control and another BCA treatments. Height of the plants treated with BCA were significantly higher compared with those in control treatment.

Key words: Biocontrol, P. fluorescens, B. subtilis, siderophore, Ralstonia solanacearum

INTRODUCTION

Peanut (Arachis hypogea L.) is one of the important crops in Indonesia as a source of protein and oil. The crops are grown on the low land up to 500 m above sea level. The optimum temperature for growing peanut is 28 – 32 °C with RH 65 -75% with full sunlight. The government always seeks to increase peanut production but one of the most important constraints is the occurrence of the disease that causes both qualitative and quantitative losses.

Several pathogens attack peanut in the field and one of the important pathogens is Ralstonia solanacearum that causes bacterial wilt disease. Machmud (1986) reported that the bacterial wilt disease has spread widely in North Sumatra, West Sumatra, Lampung, West Java, Central Java, Bali, and South Sulawesi causing yield loss of 15 – 35% and 60 – 90% in case of susceptibility.
The bacterial wilt disease still caused the reduction of peanut production despite the use of bactericides, resistant varieties, field sanitation and cultural practices. Continuous application of bactericides causes the development of resistant pathogens and may cause the build up of new races, in addition to risks for human as well as environmental health (Machmud and Hayward, 1993).

The use of resistant variety is one of the alternatives as a control method of this disease, but breeding the resistant variety may also cause the build up of new virulent race that can cause the breaking of the resistance. Disease control by cultural practices with crop rotation could suppress the disease inoculums in the field, but according to Machmud and Hayward (1993) this method was ineffective because it needs longer time to decrease the population of the inoculums. Another alternative to control this disease is the application of biocontrol agents isolated from the field. By means of biological control technique, the negative effects caused by the other control methods stated above will be reduced.

*Pseudomonas fluorescens* RH4003 and *Bacillus subtilis* AB89 has been successfully tested to control bacterial wilt disease of tomato (Nawangsih et al. 2005). *P. fluorescens* RH4003 was isolated from the tomato rhizosphere while *B. subtilis* AB89 from rice leaves. The biocontrol agents also have the probability to be applied as broad spectrum agents. It will be more profitable if the biocontrol agents could suppress *R. solanacearum* in the host other than tomato.

This experiment was conducted to investigate some characters related to disease suppression and the bio-efficacies of *P. fluorescens* RH4003, *B. subtilis* AB89 and other bacteria isolated from the rhizosphere of peanut against the bacterial wilt disease of peanut.

**MATERIALS AND METHODS**

**Biocontrol agents**

Heat tolerant bacteria and fluorescent pseudomonads bacteria were isolated from the rhizosphere of healthy peanut plants among the infected plants as candidate biocontrol agents. Rhizosphere samples were collected from the peanut fields in Bogor, West Java.

To isolate fluorescent pseudomonads, each 10 grams of soil samples were suspended into 100 ml sterilized distilled water and then placed on the rotary shaker 161 rpm for 15 minutes. After a serial dilution, each 50 µl of 10^6, 10^7, and 10^8 diluted samples were spread on to the King’s B agar plate. To isolate the heat tolerance bacteria, the suspension was heated up to 80°C for 10 minutes and after a serial dilution, each 50 µl of diluted samples were spread on to the Tryptic Soy Agar (TSA) plate. The plates were incubated in room temperature (about 28 °C) for 24 hours. Bacterial colonies emerged were separated each other to get the pure cultures. The other biocontrol agents used in this experiment were *P. fluorescens* RH4003 and *B. subtilis* AB89. *P. fluorescens* RH4003 was isolated from tomato rhizosphere while *B. subtilis* AB89 was isolated from the leaf of rice. Both bacteria belong to the collection of Laboratory of Plant Bacteriology, Faculty of Agriculture, Bogor Agricultural University, Indonesia (Nawangsih et al. 2005).

**Hypersensitive Reaction (HR) test**

The fluorescent pseudomonads and heat tolerant bacteria isolated from the rhizosphere were tested for their hypersensitive reaction (HR) on the tobacco leaves. Tobacco leaves were used as indicator of hypersensitive reaction because tobacco is not the host of the tested plant growth-promoting bacteria (PGPB) and the leaves are easily inoculated (Klement et al. 1990). Each isolate was suspended in sterilized distilled water to adjust the concentration 10^6-10^9 cfu ml^-1. The bacterial suspension was injected into the tobacco leaf. Bacteria with positive reaction causing the necrotic area development 24 hours after injection were eliminated from the candidates of biocontrol agents.
**Antibiosis activity test**

The peanut’s rhizosphere bacteria were detected their ability to produce inhibition zone to *R. solanacearum* on King’s B agar medium. The isolate of *R. solanacearum* was suspended in sterilized distilled water and the population density was adjusted to $10^8$ - $10^9$ cfu ml$^{-1}$. One milliliter of the suspension was spreaded onto the surface of King’s B agar plate. A piece of sterile blotter paper (diameter 0.5 cm) was dipped into the biocontrol suspension ($10^8$ – $10^9$ cfu ml$^{-1}$) and put on the center of the plate containing one biocontrol agent each other. The treatments were replicated three times. Development and the diameter of the inhibition zone were observed daily.

**Effects on the viability and the growth of peanut seeds**

The effects of biocontrol agents on peanut seed viability, plant height and number of branches were investigated by dipping the seeds into the suspension of biocontrol agent ($10^{10} – 10^{11}$ cfu ml$^{-1}$) added with 0.01% xanthan gum for 10 hours. Seeds were dipped into the sterilized distilled water with 0.01% xanthan gum as a control.

Seeds were air dried in sterilized Petri dishes and then grown in plastic pots (diameter 10cm) filled with sterilized soil. Each pot was filled with three seeds and they were replicated 3 times. Seed’s viability, height of the seedlings, and number of the branches were counted daily. Seed viability was determined by counting the successfully emergence seedling, shown by fully opened cotyledones and raising of main bud, divided by 100 planted seeds.

**Effects on the development of peanut bacterial wilt disease and height of the plants**

The experiment was arranged as randomized complete block design with six replications. The biocontrol agents used in this experiment were *P. fluorescens* RH4003, *B. subtilis* AB89, fluorescent pseudomonads isolate CK5, heat tolerant bacterial isolate KS2 and *P. fluorescens* ES32. Two peanut seeds were grown in each polyethylene bag filled with 1.5 kg soil infested with 200 ml of *R. solanacearum* suspension ($10^8$ – $10^9$ cfu ml$^{-1}$). At two weeks after planting, 200 ml of each biocontrol agent suspension was poured into the soil. Sterilized distilled water or streptomycin sulphate 0.02% were used as a control treatment. Plant height and disease incidence were calculated every week. Disease index was estimated using the following formula:

$$\text{Disease Severity (DS)} = \frac{\sum (n_i \times v_i)}{N \times V} \times 100\%$$

- $n_i$ = number of plants with certain disease severity scale
- $v_i$ = numerical number of disease severity scale
- $i$ = disease severity scale
- $N$ = total number of sample plants
- $V$ = the highest scale of disease severity

Disease severity was recorded using the scale: 0 = no symptom, 1 = only one leaf wilted, 2 = 2 – 4 leaves wilted, 3 = 5 or all leaves wilted but the plant is still alive, and 4 = the whole plant died.

Index suppression caused by biocontrol agents was calculated using formula:

$$\text{Index Suppression} = \frac{\text{DS control} - \text{DS treatment}}{\text{DS control}} \times 100\%$$

Data were statistically compared using Statistical Analysis System (SAS) and the significance was measured using Duncan Multiple Range Test (DMRT) with $\alpha = 0.05$.  

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RESULTS AND DISCUSSION

Hypersensitive reaction of biocontrol agents isolated from peanut rhizosphere

There were 18 isolates of fluorescent pseudomonads and 21 isolates of heat tolerant bacteria which were successfully isolated from the rhizosphere of peanut. Among these, six isolates of fluorescent pseudomonads and two isolates of heat tolerant bacteria caused hypersensitive reaction (HR) positively on tobacco plant. Those isolates were fluorescent pseudomonads CK1, CK2, CK3, CK4, CK9, CK10 and heat tolerant bacteria isolates KS1 and KS7. Positive results in the hypersensitivity reaction showed that the bacteria has the potential as a plant pathogen and was therefore not eligible as a candidate biocontrol agent.

Detection of antibiosis mechanism (antagonistic activity)

Based on the antibiosis mechanism (antagonistic activity) test toward ten isolates of fluorescent pseudomonads, 16 isolates of heat tolerant bacteria, both from the rhizosphere of peanut, and 6 isolates belonging to the collection of the Laboratory of Plant Bacteriology, Faculty of Agriculture, Bogor Agricultural University, five isolates positively produced inhibition zones, i.e. fluorescent pseudomonads CK5, P. fluorescens ES32, P. fluorescens RH4003, B. subtilis AB89, and heat tolerant bacteria KS2. Among these, at 4 days after treatment, P. fluorescens ES32 isolated from the rhizosphere of Graminae plant produced the largest inhibition zone with 21 mm diameter, followed by B. subtilis AB89, fluorescence pseudomonads CK5, heat tolerant bacteria KS2, and P. fluorescens RH4003 and control with the inhibition zone diameter of 13 mm, 8 mm, 8 mm, 3 mm, and 0 mm, respectively.

One of the antibiotic compounds produced by plant growth-promoting bacteria is a siderophore, which is a low molecular weight (< 10,000 D), ferric specific ligands produced by microbes in order to combat Fe insolubility (Sayyed et al. 2005; Crowley 2001). From the rhizosphere of groundnut (Arachis hypogaea), Sayyed et al. (2010) found that Alcaligenes faecalis BCCM 2374 produced siderophore in modified succinic acid medium (SM). The bacterium enhanced seed germination, root length, shoots length, and chlorophyll content.

Effects on seed viability and number of branches

The viability of peanut seeds dipped into the suspension of B. subtilis AB89 and P. fluorescens ES32 decreased compared with those dipped in sterilized distilled water (control) while P. fluorescens RH4003, fluorescent pseudomonads isolate CK5 and heat tolerant bacterial isolate KS2 increased the viability of the seeds (Table 1). Notably, KS2, the heat tolerant bacterium, showed the best seed viability among the isolates tested, however, the percentage seedling emergence was not significantly different among the treatments.

At 14 days after treatment the percentage of seedling emergence after application of KS2, RH4003, control, and AB89 was up to 93.3, 86.7, 73.3, and 53.3%, respectively. Biocontrol agent isolate KS2 and RH4003 tended to increase the percentage of seedling emergence while isolate AB89 decreased it compared with control. Isolates KS2 and RH4003 are rhizosphere bacteria while AB89 is a phyllosphere bacteria. Even though both isolates KS2 and AB89 belong to the genus Bacillus, their effects were different on the peanut seedling.

Seedling height was affected by the isolate of biocontrol agents (Table 2). Seeds treated with Bacillus sp. KS-2 grew faster compared with those treated with the other biocontrol agents or control. Seedling stems produced by those seeds on 14 days after treatment was the highest, up to 3.7 cm, and significantly different compared with those on control, i.e. 2.7 cm.
Table 1. Percentage of seed viability after dipping into the biocontrol agents suspension.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2 DAP*</th>
<th>4 DAP*</th>
<th>6 DAP*</th>
<th>8 DAP*</th>
<th>10 DAP*</th>
<th>12 DAP*</th>
<th>14 DAP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis AB89</td>
<td>0 d**</td>
<td>46.67 c</td>
<td>46.67 c</td>
<td>46.67 c</td>
<td>46.67 c</td>
<td>46.67 c</td>
<td>53.33 c</td>
</tr>
<tr>
<td>Bacillus sp. KS-2</td>
<td>40.0 ab</td>
<td>93.33 a</td>
<td>93.33 a</td>
<td>93.33 a</td>
<td>93.33 a</td>
<td>93.33 a</td>
<td>93.33 a</td>
</tr>
<tr>
<td>P. fluorescens RH4003</td>
<td>60.0 a</td>
<td>80.00 ab</td>
<td>86.67 a</td>
<td>86.67 a</td>
<td>86.67 ab</td>
<td>86.67 ab</td>
<td>86.67 ab</td>
</tr>
<tr>
<td>P. fluorescens ES32</td>
<td>26.66 bcd</td>
<td>53.33 abc</td>
<td>53.33 bc</td>
<td>53.33 bc</td>
<td>60.00 bc</td>
<td>60.00 bc</td>
<td>60.00 bc</td>
</tr>
<tr>
<td>P. fluorescens CK-5</td>
<td>33.33 abc</td>
<td>80.0 ab</td>
<td>80.00 ab</td>
<td>80.00 ab</td>
<td>80.00 ab</td>
<td>80.00 ab</td>
<td>80.00 ab</td>
</tr>
<tr>
<td>Control</td>
<td>6.67 cd</td>
<td>73.33 abc</td>
<td>73.33 abc</td>
<td>73.33 abc</td>
<td>73.33 abc</td>
<td>73.33 abc</td>
<td>73.33 abc</td>
</tr>
</tbody>
</table>

* Days after planting of the seeds
** Means of the same column, followed by the same letter do not differ according to Duncan Multiple Range Test (P<0.05)

The number of branches of seedlings from seeds treated with B. subtilis AB89 was lower compared with those in control (Table 3). Fourteen days after planting, the average branch number of peanut plant treated with AB89 was 3.7 while 4.8 in the non-treated control. The highest number of branches, 6.7 branches was from KS2, the heat tolerant bacterium.

An increase in the number of branches was expected to increase the number of flowers. One of the factors affecting the number and development of branches, caused by plant growth-promoting bacteria, is the production of growth regulators. Khakipour et al. (2008) reported based on some literatures there are five groups of plant regulators, i.e. auxins, gibberellins, cytokinines, abscisic acids and ethylene. Auxins are a group of herbal hormones where IAA (indole-3-acetic acid) is the most important. IAA is a natural auxin with vast physiological effects that plays an important role in growth and distinction (Glick 1995). Among the plant-growth microorganisms which are capable of producing herbal hormones are Azotobacter, Pseudomonas, Azospirillum, Rhizobium, Bacillus, Enterobacter, and Mycorrhiza fungus (Khakipour et al. 2008). Karnwal (2009) reported that P. fluorescens AK1 and P. aeruginosa AK2 produced indole acetic acid in the presence of L-tryptophan.

Based on the data shown in Table 1, Table 2, and Table 3, it appears that B. subtilis AB89 caused negative effects on peanut plants. These bacteria decreased the number of branches, seed viability, and seedling height. Because IAA levels were not analyzed, there are two possibilities...
related with the production of IAA by *B. subtilis* AB89, i.e. the bacteria did not produce IAA or they produced IAA in higher concentration beyond the tolerance of peanut plants.

**Table 3.** Number of branches after the application of biocontrol agents.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of branches</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 DAP</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> AB89</td>
<td>0 c**</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. KS-2</td>
<td>1.2 a</td>
</tr>
<tr>
<td><em>P. fluorescens</em> RH4003</td>
<td>0.8 a</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ES32</td>
<td>0.7 ab</td>
</tr>
<tr>
<td><em>P. fluorescens</em> CK-5</td>
<td>0.7 ab</td>
</tr>
<tr>
<td>Control</td>
<td>0.1 bc</td>
</tr>
</tbody>
</table>

**Means of the same column, followed by the same letter do not differ according to Duncan Multiple Range Test (*P*<0.05).**

**Effects on plant height and disease development**

The effects of the biocontrol agents on plant height show that plants treated with *Bacillus* sp. KS-2, *P. fluorescens* RH4003, or *P. fluorescens* CK-5 were taller than the control at five and six weeks after planting the seeds (Table 4). Plant height, i.e. 34.2 cm, 33.6 cm, and 36.5 cm, respectively, were not significantly different compared with plants treated with streptomycin sulphate 0.02%, i.e. 37.1 cm. The results show that the biocontrol agents can possibly be used as alternative control to substitute synthetic antibiotics. Plant growth promotion by the biocontrol agents might be advantageous because the young plants could avoid pathogen attack, such as, damping off.

**Table 4.** Effects of the biocontrol agents on plant height.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plant Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 WAP *</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> AB89</td>
<td>8.00 a**</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. KS-2</td>
<td>8.20 a</td>
</tr>
<tr>
<td><em>P. fluorescens</em> RH4003</td>
<td>6.40 a</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ES32</td>
<td>8.90 a</td>
</tr>
<tr>
<td><em>P. fluorescens</em> CK-5</td>
<td>9.10 a</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>8.90 a</td>
</tr>
<tr>
<td>Control</td>
<td>8.90 a</td>
</tr>
</tbody>
</table>

**Weeks after planting of the seeds**

**Means of the same column, followed by the same letter do not differ according to Duncan Multiple Range Test (**P**<0.05)**

Disease severity of bacterial wilt at 6 weeks after planting is shown in Table 5. All of the biocontrol agents tested were able to suppress the disease index, with 0.02% streptomycin sulphate as the most effective. Among the biocontrol agents, *P. fluorescens* RH4003 showed the most suppressive effect on the disease index. Disease severity on the plants treated with biocontrol agents at 6 weeks after planting were varied from 5 to 15% while up to 35% in control.

Disease severity observed six weeks after planting on the plants treated with 0.02% of streptomycin sulphate, *P. fluorescens* RH4003, *Bacillus* sp. KS2, *P. fluorescens* CK5, *B. subtilis* AB89, *P. fluorescens* ES32 and non treated was up to 5.0, 5.8, 7.9, 9.2, 10.8, 14.58 and 35.4%,
respectively. However, *P. fluorescens* RH4003 and *B. subtilis* AB89 were able to control the disease up to this time. Index suppression on six weeks after planting caused by 0.02% streptomycin sulphate, *P. fluorescens* RH4003, *Bacillus* sp. KS2, *P. fluorescens* CK5, *B. subtilis* AB89, *P. fluorescens* ES32 was up to 85.7, 83.5, 77.6, 75.3, 67.5, and 58.8%, respectively.

Among the biocontrol agents tested in this present study, two isolates, i.e. *B. subtilis* AB89 and *P. fluorescens* ES32 decreased seed viability, seedling height, and number of branches. *P. fluorescens* ES32 produced the widest diameter of the inhibition zone, but it decreased the number of branches, height of the seedlings, and seed viability compared with those in control plants. *B. subtilis* AB89 was previously applied as seed treatment on tomato and did not cause negative effects (Nawangsih et al. 2005).

One of the factors affecting the success of biocontrol in the field is the ability of the biocontrol agent to colonize the root system (Lugtenberg et al. 2001; Brimecombe et al. 2001). Inadequate colonization leads to decreased biocontrol activity. The biocontrol agents tested in this experiment were able to control the disease incidence up to 6 weeks after planting of the seeds but the relation with the ability for root colonization must be investigated later.

**Table 5.** Disease severity of the bacterial wilt on the plants treated with the biocontrol agents.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>2 WAP*</th>
<th>3 WAP</th>
<th>4 WAP</th>
<th>5 WAP</th>
<th>6 WAP</th>
<th>Index Suppression at 6 WAP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> AB89</td>
<td>3.75 b</td>
<td>5.42 b</td>
<td>8.33 b</td>
<td>10.00 b</td>
<td>10.83 b</td>
<td>67.5</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. KS2</td>
<td>3.33 b</td>
<td>4.17 b</td>
<td>5.42 b</td>
<td>7.08 b</td>
<td>7.92 b</td>
<td>77.6</td>
</tr>
<tr>
<td><em>P. fluorescens</em> RH4003</td>
<td>2.92 b</td>
<td>3.75 b</td>
<td>5.00 b</td>
<td>5.83 b</td>
<td>5.83 b</td>
<td>83.5</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ES32</td>
<td>5.83 b</td>
<td>7.92 b</td>
<td>12.92 b</td>
<td>14.17 b</td>
<td>14.58 b</td>
<td>58.8</td>
</tr>
<tr>
<td><em>P. fluorescens</em> CK-5</td>
<td>4.58 b</td>
<td>5.83 b</td>
<td>7.50 b</td>
<td>7.92 b</td>
<td>9.17 b</td>
<td>75.3</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>2.50 b</td>
<td>2.92 b</td>
<td>3.75 b</td>
<td>4.58 b</td>
<td>5.00 b</td>
<td>85.7</td>
</tr>
<tr>
<td>Control</td>
<td>16.25 a</td>
<td>23.75 a</td>
<td>28.33 a</td>
<td>33.75 a</td>
<td>35.42 a</td>
<td>0</td>
</tr>
</tbody>
</table>

* Weeks after planting of the seeds

** Mean of the same column, followed by the same letter do not differ according to Duncan test (*P*<0.05)

**CONCLUSIONS**

All of the plant growth-promoting bacteria (PGPB) was able to suppress the bacterial wilt disease of peanut. The application of *P. fluorescens* RH4003 caused the highest value of disease suppression among the PGPB which was up to 83.5%, while the commercial product (0.02% streptomycin sulphate) was up to 85.7%. *P. fluorescens* RH4003, *Bacillus* sp. KS2, and *P. fluorescens* CK5 were able to increase the seed viability, height of the seedlings, and number of branches, but *P. fluorescens* ES32 and *B. subtilis* AB89 suppressed these variables. *P. fluorescens* RH4003 has the potential as a biocontrol agent of *R. solanacearum* in peanut but it requires further study on peanut root colonization.

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