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To cite this article: S Purwaningsih et al 2020 IOP Conf. Ser.: Earth Environ. Sci. 572 012011

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Characterization and symbiotic evalution of rhizobium bacteria from various plants on soybean (Glycine Max L) plants in green house

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Abstract. Rhizobium bacteria are bacteria including Plant Growth Promoting Rhizobacteria (PGPR) that are able to fix nitrogen, solubilise phosphate, produce enzymes of protease, IAA hormone and siderophore so that it plays, an important role in increasing agricultural productivity. Several rhizobia have been successfully isolated from 8 legume plants from various regions, with the aim of obtaining Rhizobium isolates which are potential, effective and efficient as biological fertilizer agents. A total of 8 isolates were successfully isolated using selective media for Rhizobium bacteria, namely YEMA, following their activities were tested qualitatively N fixing, solubilise phosphate, protease, IAA hormone and siderophore production. All isolates thrive on YEMA+CR media and showed fast growth in YEMA + BTB.. A total of 11 inoculation treatments were tested on soybean plants consisting of 1. EKP (3), 2. EKP (4), 3. i (1), 4 (combined 1-3), 5. 1 (2), 6. B (1), 7. H (2), 8 (combined 5-7), 9.A (2), 10.2 (1), 11 (combined 9-10). As a control plant without inoculation and without N fertilizer (K_1) and plants without inoculation and with N fertilizer equivalent to 100 kg/ha (K_2) . The planting media used for greenhouse experiments were sterile sand media. The experimental design used were a completely randomized design with 3 replications. Plants growth were observed with parameters included plant height and number of leaves (at 1,2,3,4,5,6 and 7 weeks). Soybean plants werw harvested at 50 days after planting, following measurement of dry weight of canopy, roots, root nodules, total plants and chlorophyll content. The results showed that 8 individual isolates and 3 combined isolates inoculated to soybean plants varied, all of which were able to increase growth. Isolates number H(2) and 1(2) (isolates of nodules root of Peanut plants) gave the highest yields on the growth of soybean plants.

1. Introduction

Soybean (Glycine max L) is a legume crop as a very important agricultural crop commodity because it contains a lot of protein. High protein content requires a lot of nitrogen for growth. During this time to increase plant growth by using chemical fertilizers. The continous application of chemical fertilizers will will poison the soil and pollute the environment, so it is necessary to look for alternative fertilizers that have no side effects and have a negative impact on the environment. One of them is the use of biological fertilizers. One type of biological fertilizer is one of them is the use of Rhizobium bacteria that are able to nitrogen-fixing from the air. Some of the benefits that can be obtained by utilizing nitrogen-fixing bacteria groups as biological fertilizer are that it has no danger or side effects, the efficiency of use can be improved without causing the danger of pollution to the environment



relatively cheap prices, and technology that is quite simple, able to improve soil structure, so able to increase crop yields.

Rhizobium is a bacterium that is able to nitrogen-fixing from the air that is symbiotic with legume plants, Rhizobium in nodules can nitrogen-fixing (N_2) from the air and then convert it into ammonium (NH⁺⁴) in the presence of nitrogenase activity. High and low nitrogenase activity determines the amount of ammonium supply given by Rhizobium to plants, if the activity is high, it will produce a high amount of N that is tethered, so it can improve plant growth. Instead, Rhizobium bacteria take carbohydrates, proteins, and oxygen produced by plants to live and breed [1]. The life of Rhizobium bacteria is highly dependent on host plants, microsymbiont and environmental factors, these three factors determine the results of nitrogen fixation if the relationship between host plants and synergistic Rhizobium bacteria will produce high nitrogen fixation, so nitrogen fixation results can be used by plants for their growth [2]. Soybean growth is strongly influenced by texture, structure, consistency, porosity, density and soil temperature [3]. In addition, there must be a match between Rhizobium bacteria with their host plants, soil physical and chemical conditions, humidity, soil pH, temperature, and environmental conditions that determine the success of inoculation [4]. Nitrogen inhibition by Rhizobium bacteria is able to meet about 50% of the nitrogen requirements used by plants for their growth [5]. [6] stated that the application of biological fertilizers or appropriate biological fertilizers can significantly increase the yield of various plants and reduce the use of artificial fertilizers. Research result [7] showed that compound biofertilizers along with organic matter could increase soybean nodulation and yield in Ultisol soils, while [8] states that effective Rhizobium inoculation can meet nitrogen needs by 50-70% so as to increase plant growth which can ultimately increase crop yields, so Rhizobium has a significant contribution in the growth and increase of agricultural productivity, especially legume crops, but in its life Rhizobium bacteria are strongly influenced by soil conditions, especially soil pH, soil physical, chemical and biological soil characteristics, humidity, environmental factors, if these factors are met will produce maximum tethering [9]. In addition, the success of an inoculation depends on its ability to synergy with indigenous Rhizobium in the soil and its the ability to adapt to the environment [10].

This study aims to obtain effective, efficient and potential Rhizobium isolates as biological fertilizer agents to increase plant growth and ultimately be able to increase Soybean yields.

2. Materials and Methods

2.1. Materials

A total of 8 samples from root nodules of plants name were isolated, using standard media for Rhizobium bacteria, namely Yeast Extract Mannitol Agar (YEMA) consisting of K_2 HPO₄ 0.5 g, MgSO₄7H₂O 0.2 g, NaCl 0.1 g, CaCO₃ 3 g, Mannitol 10 g, Yeast extract 3 g, Agar 20 g, Aquadest 1000 ml with pH 6.8 [11].

2.2. Rhizobacterial isolation

The isolation was performed by diluting 1 g of soil samples in 9 ml of physiological saline solution (NaCl 0.85%) in a small reaction tube then was shaken using a vortex. Serial dilution was prepared by diluting 1 ml solution into 9 ml of NaCl 0, 85% until $10^{-1}-10^{-5}$ dilution series were acquired. About 0.1 ml solution was poured into a medium in a petridish, containing YEMA media, spread out with a spatula and incubated at room temperature (27-28^o C) for 2-5 days. The colonies formed were observed and counted daily, by plate count method [12]. The isolate obtained was removed to a slanted medium, then purified to obtain a pure isolate (1 colony).

2.3. Purification of Rhizobium bacteria

Purification was carried out by means of colonies growing in the sloping YEMA media in a small test tube taken using loop inserted into a sterile aquadest (5 ml), then in a vortex, in a 0.1 ml pipette inserted in a petridish containing media, leveled with a spatula then incubated at room temperature

(27-28[°] C), single colonies grown in YEMA media tilted in small test tubes as pure culture. single colonies that grow are planted in YEMA media obliquely in small test tubes as a pure culture.

2.4. Characterization of Rhizobium bacteria

Characterization of Rhizobium bacterial isolates were grown in selective media made by modifying the base media (YEMA) with the addition of several types of dyes as indicators namely Congo Red, the color of the colonies was observed, the pink colonies indicated the Rhizobium bacteria, and Brom Thymol Blue, then observed growth and color change, blue bacterial colonies included in the slow-growing group and the yellow ones belong to the fast-growing group [13].

2.5. Test of siderophores production

The production of siderophores was tested qualitatively by using selective media that Chrome Sulfate Azurol (CAS) Agar, consisting of: Blue dye (Chome Azurol 0.06 g in 50 ml aquabides), FeCl₃6H₂0 0.0027 g (in 10 mM HCl), HDTMA 0.073 g (in 40 ml aquabides), Glucose (D Glucose 20 g in 100 ml aquabides), Casamino Acid (Casamino Acid 5 g in 45 aquabides + Hydroxyquinoline 1.35 g in 45 Chloroform), MM9 Medium (KH₂PO₄ 5 g, NaCl 25 g, NH₄Cl 50 g in 500 ml aquabides). Medium CAS (aquabides 75 ml + MM 9 Medium 10 ml + PIPES 3.024 g + Bactoagar 1.5) in autoclave) + Casamino 3 ml+ Glucose 1 ml + Blue Dye 10 ml). Bacterial isolates were tested on plates inoculated on petridish already contains CAS media Agar, then incubated at room temperature (27-28⁰ C) for 7 days, a positive result will form clear zones around the bacterial colony formation indicating that these isolates were able to produce siderophores [14].

2.6. Phosphate solubilizing activity test

The phosphate-solubilizing was tested qualitatively by using Pycosvkaya media as used [13]. consisting of: Glucose 10 g, NaCl 0.2 g, $Ca_3(PO_4)_2 5$ g, $(NH_4)_2SO_4 0.5$ g, KCl 0.2 g, MgSO₄7H₂O 0.1 g, MnSO₄ 0.25 g, FeSO₄ 0.25 g, Yeast Extract 0.5 g, Agar 28 g, aquadest 1000 ml. Bacterial isolates to be tested was inoculated at the center of the cup that petridish already contain the above-mentioned media, then incubated at room temperature (28-30⁰ C) for 2- 7 days, a positive result is marked with a colony that produces a halo zone, indicating that the bacteria have the ability to phosphate solubilizing.

2.7. Test of protease production

The protease production activity was tested qualitatively by using the media Skin Milk Agar (SMA) as used by [16]. Consisting of: Skim milk 10 g, D-Glucose 1 g, Yeast Extract 2.5 g Agar 22 g, aquadest 1000 ml. Bacterial isolates to be tested was inoculated at the center of the petridish which has been filled with the above-mentioned media, then incubated at room temperature $(28-30^{\circ} C)$ for 2-5 days, a positive result formed a clear zone, indicating that these isolates were able to produce a protease enzyme.

2.8. Test of IAA production

The IAA production activity was tested has qualitatively grown on media Tryptone Soya Broth (TSB) consisting of Peptone 10 g, NaCl 2.5 g, Agar 22 g, aquadest 1000 ml. Bacterial isolates to be tested were inoculated at the center of the petridish which has been filled with the above-mentioned media, then incubated at room temperature ($28-30^{\circ}$ C) for 2-5 days. Colonies that grow later drops solution Salkowsky (consisting of 1 ml 0.5 M FeCl₃ + 50 mL HClO₄ 50%) approximately 1 ml, incubated in a dark place approximately 1 hour, a positive result was marked by a change in color to pink indicating that these isolates were able to produce IAA (positive)[17].

2.9. Nitrogen-fixing activity test

The test capabilities in nitrogen fixation by using media Nitrogen Free Medium (NFB). Consisting of: Malic Acid 5 g, KOH 4 g, K_2 HPO₄ 0.5 g, FeSO₄7H₂O 0.5 g, MnSO₄H₂O 0.01 g, MgSO₄7H₂O 0.01 g, NaCl 0.1 g, CaCl₂ 0.02 g, Na₂MoO₄2H₂O 0.002 g, Fe-EDTA 1.64% 4 ml, KOH 4 g, Vit solution 1 ml, Micro Elemen 2 ml, BTB (0.5% alcoholic sol.) 2 ml, Agar 22 g and aquadest 1000 ml. Bacterial isolates were grown in semi-solid media NFB in a small test tube, incubated at room temperature (28- 30^{0} C) for 2-7 days, which is characterized by the formation of a white ring on the surface of the media indicating that these isolates were able to nitrogen-fixing [18].

2.10. Effectiveness of Rhizobium bacteria on the growth on Glycine max L plants

The results of isolation which have high activity are carried out to test the effectiveness of the growth of *Glycine max* L. The research was carried out in a Microbiology greenhouse, Research Center for Biology by using sterile sand media, in 0.5 gallon plastic pots. As much as 1.5 kg of sterile sand as a growing medium, then after the seeds have been planted on it, added sterile sand has been mixed with paraffin and benzol (sterile) as high as 2 cm as a cover for the seeds planted. And the seeds used are *Glycine max* L var. Anjasmoro. Inoculation with Rhizobium isolates used were: 1. EKP (3), 2. EKP (4), 3. i (1), 4 (combined 1-3), 5. 1(2), 6. B (1), 7. H(2), 8 (combined 5-7), 9. A(2), 10. 2(1), 11 (combined 9-10). As a control plant without inoculation and without N fertilizer (K₁) and plants without inoculation and with N fertilizer equivalent to 100 kg/ha (K₂). The experimental design used was a Completely Randomized Design with 3 replications for each treatment. Plants are harvested at the age of 40 days. Parameters observed included a plant of height, number of leaves, chlorophyll content using SPAD-502 Plus [19], canopy dry weight, roots, root nodules and total plants. To maintain moisture (24%) watering is carried out every single day using the nutrient solution without N bound as was done by [20].

| Solution | The element of solution | Quantity | |
|---|--------------------------------------|------------|--|
| A (Standard solution of Calcium Sulfur) | CaSO ₄ 2H ₂ O | 2,5 grams | |
| | MgSO ₄ 7H ₂ O | 2,5 grams | |
| | Sterile distilled water | 10 liters | |
| B (Standard solution of Ferric Citrate) | Ferric Citrate | 30 grams | |
| | Sterile distilled water | 1 litres | |
| C (Standard solution of Fosfat) | KH ₂ PO ₄ | 34 grams | |
| | КОН | 1,96 grams | |
| | Sterile distilled water | 1 liter | |
| D (Standard solution of Trice element) | MnSO ₄ H ₂ O | 1 grams | |
| | $ZnSO_45H_2O$ | 0,25 grams | |
| | CuSO ₄ 5H ₂ O | 0,25 grams | |
| | NaMoO ₂ 2H ₂ O | 0,06 grams | |
| | H_3BO_3 | 0,50 grams | |
| | CaCl ₂ 6H ₂ O | 0,05 grams | |
| | Sterile distilled water | 1 liter | |

Table 1: The composition of nutrient solution (10 liters of solution A + 10 ml of solution B + 10 ml of solution C + 10 ml solution of D)

3. Results and Discussion

3.1 Characterization test

The results showed that in the qualitative testing of the isolates grown in YEMA + Congo Red media all thrived and pink (table 2), this showed that the isolates did not absorb the red color from the Congo Red indicator that was on the media, this indicated that the isolate is a Rhizobium bacterium, as [21] reported that one of the cash characteristics of the Rhizobium bacteria is that it does not absorb red on media containing Congo red, which is characterized by a pink colony.

3.2 *Qualitative test*

The next qualitative test to find out the growth was grown in YEMA media added Brom Thymol Blue all isolates included in the fast-growing group marked with a yellow colony (table 2). As said by [22] that there are two growth groups of Rhizobium bacteria, they are slow-growing groups that are marked

by blue colonies and fast-growing groups are marked with yellow colonies. For qualitative tests in protease media, all isolates from clear zones which indicate that the isolate is able to produce protease enzymes (table 2), this shows that the gram is able to break down proteins into peptides and amino acids which are characterized by the formation of clear zones. The clear zone is an indicator that the isolate is able to utilize protein in the media as its nutritional source [23]. Qualitative test to find out the isolate is able to produce IAA hormone is done by growing isolates on Trypton Soya Broth (TSB) media, all isolates are able to produce IAA hormones which are marked by colony to red (Table 2), this indicates that the isolate is able to produce IAA hormones, the more concentrated the red color the more IAA hormone is produced. As reported by [24] that the red color that occurs due to the interaction of IAA hormones with Fe³⁺ ions in the Salkowaski reagent that forms a tris (indole 3 aceto) iron complex through complex and redox reactions, the higher the concentrated red color produces IAA hormone. For tests in producing siderophore, isolates were grown in Chrome Azurol Sulfate (CAS agar) media. All isolates were able to produce the siderophore hormone, which is characterized by the formation of clear zones which are orange (table 2), the greater the zones the stronger the production of siderophore, siderophore functions to inhibit the growth of pathogens caused by the deficiency of Fe^{3+} needed by pathogens because Fe^{3+} is already bound by siderophore, besides that iron is an important element in disease development so that by binding to iron by siderophore the pathogen is less able infecting plants, there by inhibiting the

| Table 2: | Characterization | and qualitativ | ve test of proteas | e, Indole Ac | cetic Acid (| (IAA), | siderophore, |
|-----------|---------------------|----------------|---------------------|---------------|--------------|----------|--------------|
| phosphate | solubilizing, nitro | ogen fixation | of isolated root no | dule rhizobia | a from varic | ous plan | nts |

| isolate | | YEMA+CR | | YEMA+BTB | | Prote | | Sidero | o Phosphate | N- |
|---------|--------|---------|-------|----------|--------|-------|-----|--------|--------------|----------|
| No | code | growth | color | growth | color | ase | IAA | -phore | solubilizing | Fixation |
| 1 | EKP(3) | +++ | pink | fast | yellow | + | ++ | + | + | + |
| 2 | EKP(4) | +++ | pink | fast | yellow | + | ++ | + | + | + |
| 3 | i(1) | +++ | pink | fast | yellow | + | ++ | + | + | + |
| 4 | 1(2) | +++ | pink | fast | yellow | + | ++ | + | + | + |
| 5 | B(1) | +++ | pink | fast | yellow | + | ++ | + | + | + |
| 6 | H(2) | +++ | pink | fast | yellow | + | ++ | + | + | + |
| 7 | A(2) | +++ | pink | fast | yellow | + | ++ | + | + | + |
| 8 | 2(1) | +++ | pink | fast | yellow | + | ++ | + | + | + |

Notes: +++=fertile, YEMA = Yeast Extract Mannitol Agar, CR= Congo Red, BTB= Brom Thymol Blue, + = formed felicel, += formed halozone, ++= produce IAA hormone.



Figure 1: Morphological and biochemical characters of the isolates root nodulr rhizobia. Growth performance on YEMA+CR medium(a) and YEMA+BTB medium(b). qualitative tests results of protease (c), IAA hormone(d), ability in N fixation (formed felicel)(e), phosphate solubilization (f) and siderophore(g) of rhizobia isolates

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doi:10.1088/1755-1315/572/1/012011



Figure 2: The average plant height of inoculated Glycine max L with Rhizobia isolates



Figure 3: The average number of leaves of inoculated *Glycine max* L with Rhizobia isolates

development of disease in plants [25]. Qualitative test to determine its ability to phosphate solubilizing is done by growing isolates into Pycovskaya media, all isolates are able to phosphate solubilizing which is marked by the formation of clear zones around the colony (table 1), this shows the isolate produces organic acids (can be in the form of citric acid, glutamic, succinate), lactate, oxalate, glycaxalate, malic, fumalate, tartatat or alpha-ketobutyric acid) extracellular which is able to bind to Ca ions which are bound in the form of $Ca_3(PO_4)_2$ on Pycovskaya media so as to free H₂PO₄ ions to form a more colored area, clear compared to areas that still have P bound [26]. The next qualitative test to determine its ability to nitrogen-fixing was carried out by growing isolates on NFB semi-solid media, isolates capable of nitrogen-fixing were characterized by the formation of a white ring on the surface of the media and a change in the color of the media from greenish beige to greenish-blue, this indicates that the isolate was able to nitrogen-fixing (table 2). The best potential for nitrogen-fixing bacteria is that they can change the color of the media from greenish-yellow to dark blue and can form a pellicle or ring on the surface of the media, this indicates that the isolate is capable of nitrogen-fixing the media from greenish-yellow to dark blue and can form fixing. NFB media does not contain nitrogen in the composition of the meterial, so the isolates that can

The 9th International Symposium for Sustainable HumanosphereIOP PublishingIOP Conf. Series: Earth and Environmental Science 572 (2020) 012011doi:10.1088/1755-1315/572/1/012011

grow on these media are isolates capable of free nitrogen-fixing, semi-solid NFB media indicate that there is nitrogenase activity carried out by nitrogen-fixing bacteria, bacteria that are suspected to be able to free nitrogen too has the ability to change the pH of the media to be more alkaline, this is characterized by a change in color, color changes in NFB media occur because of the nature of the Bromthymol blue indicator which turns blue at higher pH, it is due to the presence of nitrogenase activity. As said by [27] that white pellicles formed on the surface of media produced by bacteria on NFB media are caused in the media there is no excess oxygen, the rate of oxygen diffusion is equal to the respiration rate of organisms which is a good condition for the activity of the nitrogenase enzyme which helps reduce acetylene to ethylene which can help in the process of nitrogen-fixing.



Figure 4: The average of the dry weight of canopy (DWC), root (DWR), root nodules (DWRN) and dry weight of total plant (DWTP) of inoculated *Glycine max* L with Rhizobia isolates





3.3 Effectiveness of inoculated Glycine max L with Rhizobia isolates

Test the effectiveness of isolates on the growth of soybean plants shows that the results vary, this is because the level of effectiveness of each isolate is different, so there is a difference in growth if the inoculation is effective there will be a maximum nitrogen-fixing, so plants can make maximum use for their growth. [28] stated that the use of Rhizobium inoculation was significantly able to increase soybean yield. Substantial increases in nodulation directly affect growth and yield due to the potential

for $N_2\,$ fixation in soybeans. Rhizobium inoculation application also increases nodulation, growth and yield.

Based on each parameter observed showed that for measurement of plant height the highest value in plants inoculated with isolate B(1) at 1 and 3 weeks, isolate 1(2) at 2 weeks, isolate H(2) at age 4, 6 and 7 weeks and EKP(3) isolate at 6 weeks. (figure 2). For the calculation of the highest number of leaves in plants inoculated with EKP(3) isolates at ages 1, 4 and 6 weeks, isolates 1(2) and H(2) at ages 2, 3 and 5 weeks, isolate A(2) at 7 weeks (figure 3). This results showeds that the highest value of dry matter measurements for upper plants, roots, root nodules and total plants in inoculated plants with isolate 1(2) (figure 4), while for chlorophyll content the highest value was plants that were inoculated with isolate B (1) (figure 5), this shows that inoculation would have a positive effect on plant growth if the isolates that were inoculated were effective, had a match and compatibility with plants as said by [29] that the success an inoculation depends on the effectiveness and efficiency of the isolates that play a role and has a compatibility and harmony with the host plant, besides the technique and time of inoculation is also very influential on the for plants, each isolate has a different ability to adapt to its environment [30]. From all of the parameters observed showed that the highest yield was the plants inoculated with isolate H(2) and 1(2) and, this indicated that the isolates has ability to match with the plants, so they could potensial to developed as biological fertilizer, especially for soybean plants.

4. Conclusion

The qualitative testing of the eight isolates showed that all isolates characterized the Rhizobium bacteria, included in the fast-growing group, were able to produce protease enzymes, produce IAA and siderophore hormones and had the ability to phosphate solubilizing and be able to nitrogen-fixing. Rhizobium inoculation has a positive effect on growth. The success of an inoculation depends on the effectiveness, efficiency, harmony, and compatibility of the isolate inoculated with its host plant. Isolates H(2) and 1(2) (isolates from Peanut root nodules) gave the highest yield, so the isolates could be developed as biological fertilizer agents, especially soybean plants.

5. Acknowledgment

The author would like to thank the Head of the Research Center for Biology - LIPI and the Project Leader for Utilizing Microbial Bioresources for improving the quality of crops, soils, and agricultural productivity as a basis for sustainable farming systems for their support so that the research activities can run well, and would like to thank all colleagues of Agricultural Microbiology laboratory for the technical assistance and in the implementation of this research.

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