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Isolation, physiological characters and effectivity of bacterial isolates of root nodules from various plants on the growth of *Vigna radiata* L

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Abstract. Mung bean (*Vigna radiata* L.) are legume plants that can establish symbiosis with Rhizobacteria to fix N_2 from air. Research on isolation, physiological characters and effectiveness of bacteria isolates of root nodules from various plants on the growth of *Vigna radiata* L has been carried out. The objective of study was to get effective Rhizobacteria isolates which can be potentially used as biological fertilizer agents. Characterization of 16 isolates were carried out and all were included in the fast growing group. For physiological characters of 16 isolates showed that 11 isolates were able to produce IAA, 7 isolates were able to solubilize phosphate, 13 isolates were able to produce siderophore, and 16 isolates had protease enzyme and catalase activities. Ten isolates that have growth support activities: AD (1), AD (2), AD (3), AD (4), AD (7), AD (10), LK (2), EKP (1), EKP (2), and KcKP (1) were tested for its effectiveness on *Vigna radiata* L. As a control plant without inoculation on soil media (K_1) and plants without being inoculated in plants in soil and compost media (K_2). The design used was Completely Randomized Design. The plants were harvested at the age of 55 days. The parameters which were observed were: height of plant, number of leaves, dry weight of canopy, roots, root nodules, total of plants, wet weight of pods, number of pods and chlorophyll content. Inoculation of isolate AD (7) had increased the growth of *Vigna radiata* L compared to control.

1. Introduction

Mung bean (*Vigna radiata* L.) are agricultural commodities which are very potential to meet the nutritional needs of Indonesian people. The demand for these commodities from year to year continues to increase, so productivity needs to be increased. Currently, chemical fertilizers were mostly used to improve productivity. However, with the increasingly high production costs of chemical fertilizers, it is necessary to look for alternatives that are cheaper simple in technology and does not cause environmental pollution. One of the solutions is using biological organic fertilizer (biofertilizer) which is expected to reduce dependance on chemical fertilizers in increasing crop production [1,2,3]. In order to increase the yield of plants, one strategy that can be done is by inoculation. Inoculation with microbes is an effort to improve crop productivity and also improve soil conditions. One of the soil fertilizing microbes group that is expected to increase growth and yield of plants is Rhizobacteria. Inoculation with this bacteria is highly recommended because the cheap cost, the technology is simple and environmentally friendly [4].



Rhizobacteria are bacteria that live in plant root nodules that are capable of fixing nitrogen from air, thus this bacteria can supply nutrients especially N for plants. The availability of Rhizobacteria which are effective, efficient and compatible for plants will be able to increase the growth and yield of crop [5]. In general, inoculation is done by giving Rhizobacteria isolates to the soil so that these bacteria can be associated with legume plants to bind free nitrogen from the air [6]. Rhizobacteria inoculant can reduce the need for N by 70% [7]. Rhizobacteria inoculant could increase dry weight of plant, and effective nodule formation could increase weight of soybean seed [8].

However, there are problems that need to be considered in inoculating Rhizobacteria, which are efficiency, effectiveness and the compatibility of Rhizobacteria inoculants for certain types of plants. In addition, plants response is very dependent on soil conditions and the effectiveness of indigenous Rhizobacteria populations and environmental factors greatly determine the success of an inoculation [9].

Therefore, this research was conducted to obtain potential and effective pure isolates which can increase the production of plant, so that it can be developed as a biological organic fertilizer agent.

2. Materials and Methods

2.1. Materials

Root nodules obtained from *Desmodium* plants were collected from Adaro, South Kalimantan, *Phaseolus vulgaris* and *Canavalis gladiata* were from Lombok, and Edamame and Peanuts were from Cibinong Experimental Garden.

2.2. Isolation of bacterial root nodules from various plants

Root nodules were separated from the root, then washed with 70% alcohol and rinsed with aquadest 5 times. The root nodules were then transferred to the cup and pulverized with a spatula. Each one of the pulverized root nodules was then scraped on petridishes containing YEMA (Yeast Extract Mannitol Agar) medium with the composition: 0.5 g K_2HPO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 3 g $CaCO_3$, 10 g Mannitol, 3 g Yeast extract, 20 g Agar, 1000 mL Aquadest, pH 6.8 [10] using the spread plate method. The YEMA plates were incubated at room temperature (27-28⁰ C) for 2-3 day. Single growing colonies transferred to YEMA media tilted in small reaction tube as pure isolate.

2.3. Purification and Characterization of Rhizobacteria

Purification of the isolates was carried out by taking the sample colonies with a loop. The sample was put in sterile 5 mL aquadest and mixed with a vortex. About 0.1 mL of the resultant suspension was poured in a petridishes containing YEMA media, leveled with a spatula and incubated at room temperature (27-28⁰ C) for 2-5 days. Isolated single colonies were grown in a slanted medium in a test tube (as a pure culture).

2.4. Nitrogen-fixing activity test

The ability of the bacteria to fixing nitrogen was tested using semi solid NFB (Nitrogen Free Medium), which consisted of: 5.0 g malic acid, 4.0 g KOH, 0.5 g K_2HPO_4 , 0.5 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2$, $Na_2MoO_4 \cdot 2H_2O$ 0.002 g, 4 mL 1.64% Fe-EDTA, 4 g KOH, 1 mL vit solution, 2 mL microelements, 2 mL BTB (0.5 % alcoholic solution), 22 g agar and 1000 mL aquadest. Bacterial isolates were grown in the semi solid NFB media in a small test tube, incubated at room temperature (28-30⁰ C) for 2-7 days. A positive result was characterized by the formation of a white ring on the surface of the media indicating that these isolates were able to fixing nitrogen [11].

2.5. Test of protease production

Protease production activity was tested qualitatively by using skim milk agar (SMA) as in [12], which consisted of 10.0 g skim milk, 1.0 g D-glucose, 2.5 g yeast extract, 22.0 g agar, 1000 mL aquadest.

Bacterial isolates to be tested were inoculated at the center of a petridishes that had been filled with the media mentioned above, then incubated at room temperature (28-30⁰ C) for 2-5 days. A positive result was a clear zone around the colony, indicating that these isolates were able to produce a protease enzyme.

2.6. Test of siderophores production

The production of siderophores was tested qualitatively by using chrome sulfate azurol (CAS) selective media that consists of blue dye (0.06 g chrome azurol in 50 mL aquabides), 0.0027 g FeCl₃ - 6H₂O (in 10 mM HCl), 0.073 g HDTMA (in 40 mL aquabides), D-glucose (20 g in 100 mL aquabides), casamino acid (5 g in 45 mL aquabides + 1.35 g hydroxyquinoline in 45 mL chloroform), MM9 Medium (5 g KH₂PO₄, 25 g NaCl, 50 g NH₄Cl in 500 mL aquabides). CAS medium (75 mL aquabides + 10 mL MM9 Medium + 3.024 g PIPES + 1.5 g bacto agar in autoclave) + 3 mL casamino + 1 mL glucose + 10 mL blue dye). Bacterial isolates were tested by inoculating a petridishes already containing CAS media agar with the bacteria and then incubated at room temperature (27-28⁰ C) for 7 days. A positive result was the formation of clear zones in the blue media around the bacterial colony, indicating that these isolates were able to produce siderophores [13].

2.7. Test of IAA production

The IAA production activity test was done by using TSB (tryptone soya broth) agar, consisting of 10 g peptone, 2.5 g NaCl, 22 g Agar, and 1000 mL aquadest. Bacterial isolates to be tested were inoculated at the center of a petridishes which has been filled with the media mentioned above, then incubated at room temperature (28-30⁰ C) for 2-5 days. Approximately 1 mL drops of Salkowsky solution (consisting of 1 mL 0.5 M FeCl₃ + 50 mL 50% HClO₄) were administered on top of growing colonies and incubated in the dark for approximately 1 hour. A positive result was noted by a change in color to pink indicating that these isolates were able to produce IAA. Positive isolates were then quantitatively assessed further for their ability to produce IAA using a spectrophotometer [14].

2.8. Phosphate solubilize activity test

Phosphate solubilize was tested qualitatively by using Pycosvkaya media as used in [15], which consisted of 10.0 g glucose, 0.2 g NaCl, 5.0 g Ca₃(PO₄)₂, 0.5 g (NH₄)₂SO₄, 0.2 g KCl, 0.1 g MgSO₄·7H₂O, 0.25 g MnSO₄, 0.25 g FeSO₄, 0.5 g yeast extract, 28 g agar, and 1000 mL aquadest. Bacterial isolates to be tested were inoculated at the center of a well made in petridishes already containing the media mentioned above, then incubated at room temperature (28-30⁰ C) for 2-7 days. A positive result was a colony that produces a halo zone, indicating that the bacteria can solubilize phosphate.

2.9. Test of catalase activity

The qualitative catalase activity test was performed by placing one drop of 3% H₂O₂ on the glass object. One loop of 24 hours culture was placed on H₂O₂ then mixed. Positive catalase is indicated by the formation of gas bubbles as a result of the reaction.

2.10. Effect of Rhizobacteria on *Vigna radiata* L plant in soil and compost medium

The study was conducted in greenhouse of Microbiology, Research Center for Biology, Indonesian Institute of Sciences by using a mixture of soil and compost (equivalent to 10 tons / ha) in polybags which are each filled with 2 kg as a planting medium. Ten isolates with high activities: AD (1), AD (2), AD (3), AD (4), AD (7), AD (10), LK (2), EKP (1), EKP (2) and KcKP (1) were tested for its effectiveness on *Vigna radiata* L plants growth. As control plant without inoculation on soil media (K₁) and plants without being inoculated in plants in soil and compost media (K₂). The design used was Completely Randomized Design with each treatment three replication. To maintain moisture every day the plants are doused with rain water. The plants were harvested at the age of 55 days. The

parameters which were observed: height of plant, number of leaves, dry weight of canopy, roots, root nodules, total of plants, wet weight of pods, number of pods and chlorophyll content.

3. Results and Discussion

Sixteen pure isolates were obtained from root nodules of various plants. The isolates were characterized by growing on selective YEMA media added with Congo Red (CR) and Brom Thymol Blue (BTB) as an indicator. After characterization, it was shown that in YEMA media, which was added with Congo Red, everything was pink which mean that it did not absorb the red color from the Congo Red indicator, this condition showed the characteristic of Rhizobacteria. As stated by [16] that Rhizobacteria isolates grown on media containing CR which were incubated for 3 days grew rapidly and did not absorb indicator solutions. Whereas in the YEMA medium which was added with Brom Thymol Blue, all isolates belonged to the group of fast growing Rhizobacteria which was characterized by changes in color to yellow, due to the production of acids or bases in the media. YEMA + BTB media is used to classify bacteria that have fast growth (yellow) and slow growth (blue) based on the production of acids and bases in the media [17].

Based on protein decomposition test results using skim milk media, it was found that all sixteen isolates could break down the protein which was marked by the formation of clear zones (Table 1). Phosphate solubilizing ability test results showed that 9 isolates have the ability to solubilize phosphate. The dissolution capacity of P from inorganic minerals is highly dependent on the concentration and type of organic acids which are excreted, while the dissolution of P from organic compounds depends on the catalytic capacity of the phosphatase enzyme produced by the bacteria [18]. Phosphatase activity is strongly influenced by nitrogen media content, increasing nitrogen media content can increase phosphatase activity in the media [19]. Based on the results of siderophore detection test on sixteen isolates using CAS media, 13 isolates were able to form a clear zone. According to [20] if a clear orange zone is formed around the colony, it shows that the colony is capable of producing siderophore.

Table 1. Characterization and qualitative test of nitrogen fixation (nf), protease, siderophore, IAA (Indole Acetic Acid), phosphate solubilizing (ps), and catalase of root nodule isolate from various plants.

Isolat number	Plant origin	YEMA +CR	YEMA +BTB	nf	Prote-ase	Sidero-phore	IAA	ps	Catalase
AD (1)	Desmodium	+pink	+yellow	+	+	+	+	-	+
AD (2)	Desmodium	+pink	+yellow	+	+	+	-	-	+
AD (3)	Desmodium	+pink	+yellow	+	+	+	+	+	+
AD (4)	Desmodium	+pink	+yellow	+	+	-	-	-	+
AD (5)	Desmodium	+pink	+yellow	-	+	+	-	-	+
AD (6)	Desmodium	+pink	+yellow	+	+	+	+	+	+
AD (7)	Desmodium	+pink	+yellow	+	+	+	-	-	+
AD (8)	Desmodium	+pink	+yellow	+	+	+	+	+	+
AD (9)	Desmodium	+pink	+yellow	-	+	+	+	+	+
AD (10)	Desmodium	+pink	+yellow	-	+	+	+	+	+
LB (1)	<i>P. vulgaris</i>	+pink	+yellow	-	+	-	+	-	+
LB (2)	<i>P. vulgaris</i>	+pink	+yellow	+	+	+	+	+	+
LK (2)	<i>C. gladiata</i>	+pink	+yellow	+	+	+	-	-	+
EKP (1)	<i>C. gladiata</i>	+pink	+yellow	-	+	+	+	+	+
EKP (2)	Edamame	+pink	+yellow	+	+	-	+	+	+
KcKP (1)	Peanut	+pink	+kuning	+	+	+	+	-	+

NB: *Phaseolus vulgaris* and *Canavalis gladiata*

According to [21] the production of siderophore can also be a major factor in the host's resilience system from disease attacks. Siderophore is classified by ligands used for iron rods. According [22] siderophore is a complex compound of Fe^{3+} or specific iron chelating produced by several types of microbes to hide iron elements in the rhizosphere, so that it is not available for the development of pathogenic microbes [23].

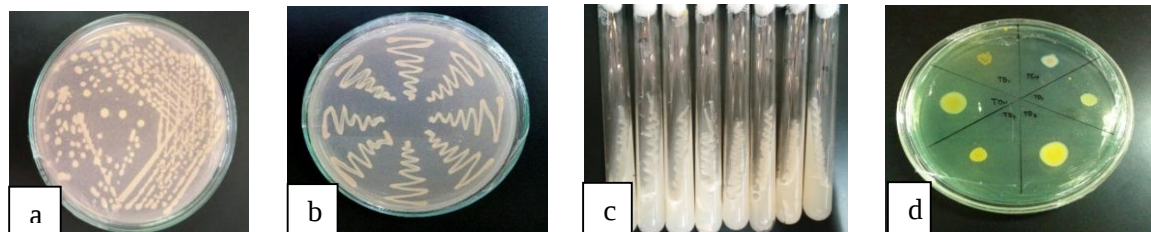


Figure 1. Rhizobacteria isolates in YEMA+CR medium (a) + (b), in tube culture (c), in media YEMA+BTB (d)

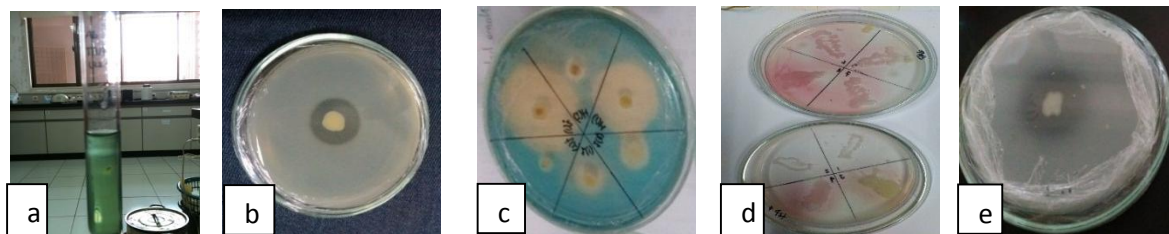


Figure 2. Qualitative tests results of the ability in N fixation (formed felicel)(a), Protease (b), Siderophore (c), IAA production (d), Phosphate solubilization (d) of Rhizobacteria isolates.

For catalase test, it was found that sixteen positive isolates can remodel hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) which is indicated by the formation of bubbles when the isolate is added with 3 % H_2O_2 . Bubbles formed due to the molecular structure of H_2O_2 are unstable, so when catalase is present, H_2O_2 breaks down into water and oxygen, so the isolates are aerobic which means that they need oxygen in the metabolic process [24,25]. IAA production test in the sixteen isolates showed that 11 isolates were capable of producing IAA hormone, which was marked by the color change of isolates to faded pink. IAA is a fitohormon which has an important role as a regulator of plants development [26]. IAA production capability of each microbe is very different, depend on the conditions of microbial culture, growing media and environment [27, 28].

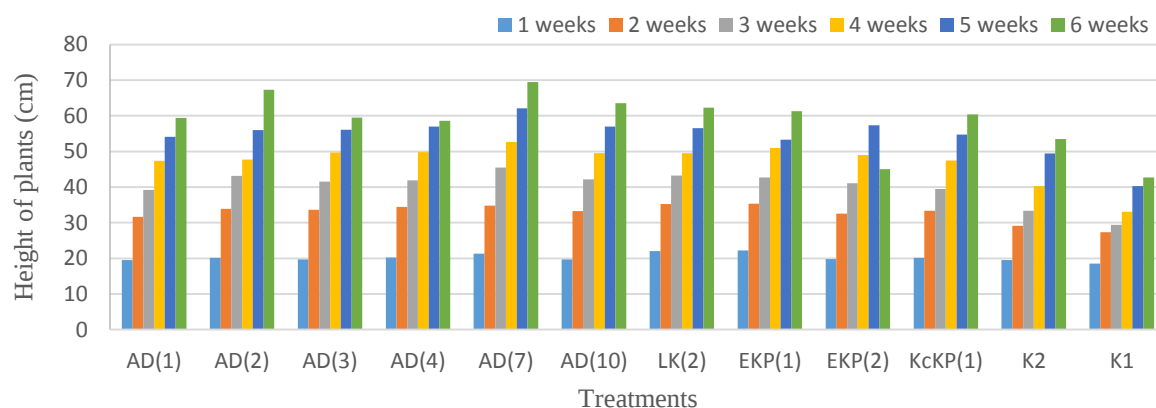


Figure 3. The average of plant height of *Vigna radiata* L with rhizobacteria inoculant (cm)

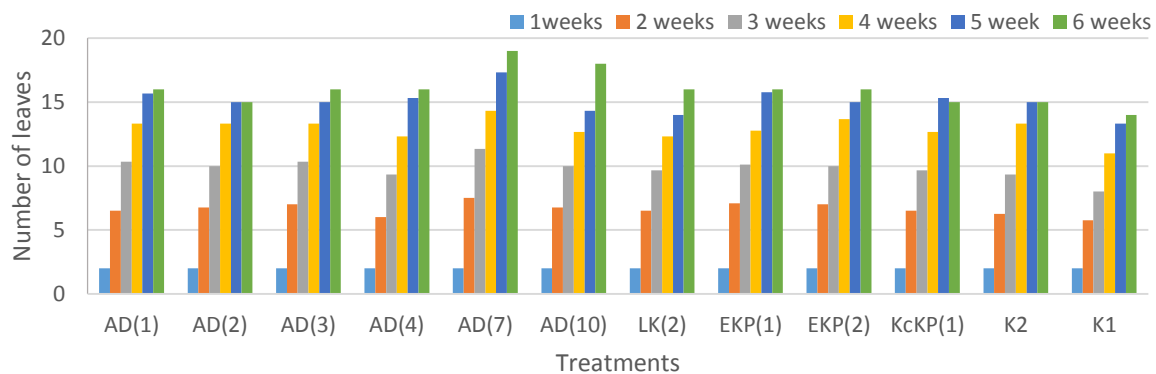


Figure 4. The average of leaves number of *Vigna radiata* L with rhizobacteria inoculant

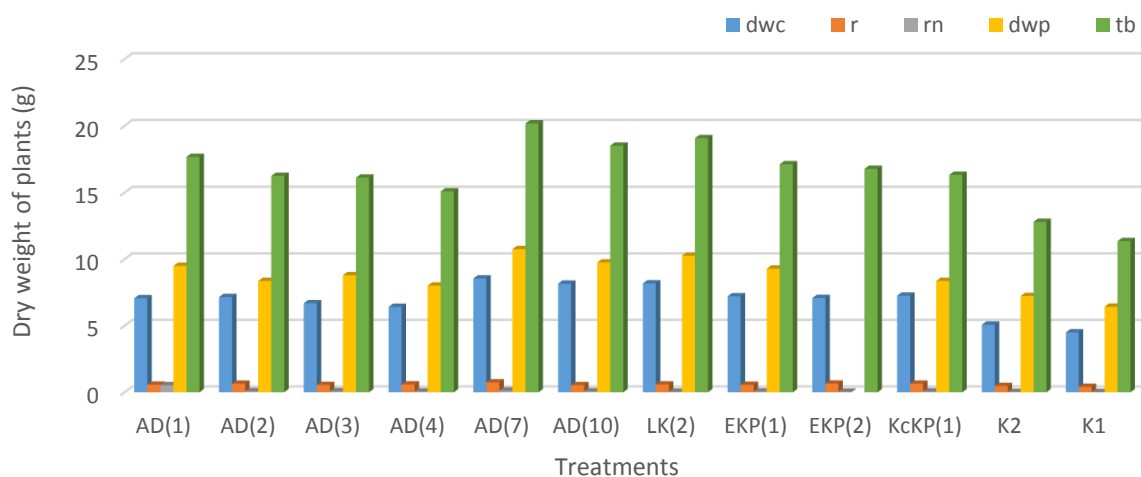


Figure 5. The average of above ground dry weight of canopy (dwc), roots (r), root nodules (rn), pods (dwp) and total biomass (tb) of *Vigna radiata* L plants with Rhizobacteria isolates at harvest (55 days) (gram)

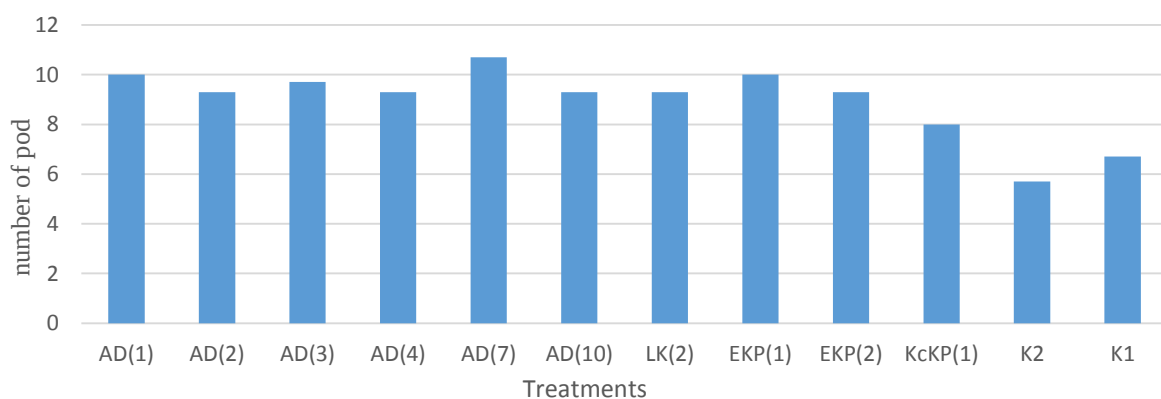


Figure 6: Number of pods of *Vigna radiata* L. plants inoculated with Rhizobacteria isolates at harvest (55 days)

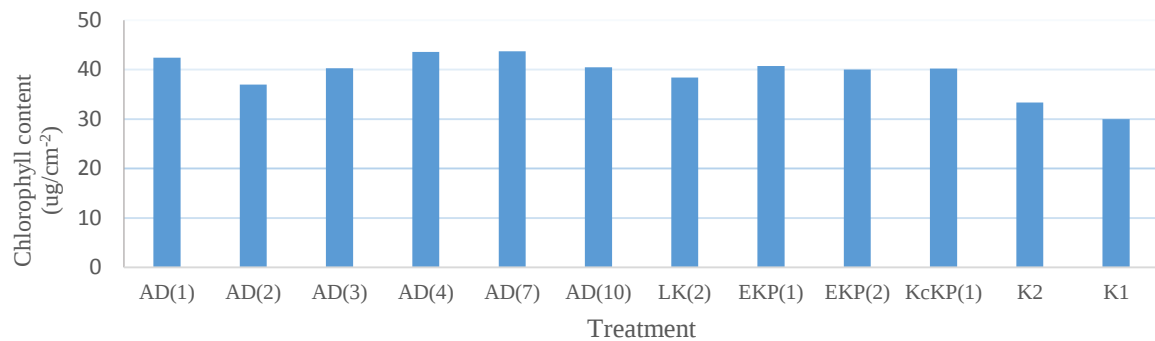


Figure 7. Chlorophyll content of *Vigna radiata* L plants inoculated with Rhizobacteria isolates at the age of 40 days



Figure 8. Growth of *Vigna radiata* L plants inoculated with Rhizobacteria isolates at the age of 40 days

The effectiveness test of Rhizobacteria isolates on the growth and yield of *Vigna radiata* L showed that the plants inoculated with isolate AD (7) gave the highest results on all parameters measured, including: plant height, number of leaves, plant dry weight, root, root nodules, total plants, wet weights of pods, and chlorophyll content. This showed that the isolate have a compatibility and synergy and were able to compete with native microbes in the soil so as to increase the growth and yield of mung bean plants. As [29] that the success of an inoculation depends on if there is a match between the host plant and the isolate given. In addition, the isolate is able to compete with indigenous (indigenous) microbes, and able to adapt to local environmental conditions [30].

4. Conclusions

Sixteen pure isolates were found, the 16 isolates were characterized as Rhizobacteria, and belonged to the fast growing group. Physiological characters of 16 isolates, 11 isolates were able to produce IAA, 7 isolates were able to dissolve phosphate, 13 isolates were able to produce siderophore, and 16 isolates had protease enzyme activity and catalase activity. The effectiveness test for *Vigna radiata* L plants showed isolate AD (7) was able to increase the growth of *Vigna radiata* L plants. These isolates can be developed as biological fertilizer agents, especially for *Vigna radiata* L plants.

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NB: This study is to look at the activity and effectiveness of each isolate, good isolates will do further research as POH formulas, after getting the formula new identification is done.