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# ABILITY OF AMMONIUM EXCRETION, *Indol Acetic Acid* PRODUCTION, AND PHOSPHATE SOLUBILIZATION OF NITROGEN-FIXING BACTERIA ISOLATED FROM CROP RHIZOSPHERE AND THEIR EFFECT ON PLANT GROWTH

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**ABSTRACT**

The aim of this research is to investigate the ability of nitrogen fixing bacteria isolates obtained from crop rhizosphere in the District of Maros, South Sulawesi province, Indonesia to excrete ammonium, to produce growth promoting substance called *Indol Acetic Acid* (IAA), to solubilize phosphate and their effect on plant growth. There were 43 previously isolated nitrogen fixing bacterial isolates inoculated in Burk's medium. Ammonium released was quantified using spectrophotometry method. The isolates with the ability to excrete high amount of ammonium were selected to IAA production and phosphate solubilization analysis. IAA concentration was measured by spectrophotometry method and Phosphate solubilization assay was done by inoculating bacterial isolates on Pikovskaya medium. The ability to solubilize phosphate was marked by a clear zone around bacterial colonies. Selected isolates were then analysed for their effects on growth of *Amaranthus tricolor* L with pot trials conducted under green-house conditions. Among 43 nitrogen fixing bacterial isolates, 14 isolates could excrete ammonium in the concentration of 256, 7 µM until 1027, 77 µM. Those isolates also could produce IAA in the concentration between 5, 59 ppm to 41, 30 ppm. There were 6 isolates that could solubilize phospate with clear zone measure of 1, 5 cm to 3, 1 cm. From the pot trial to evaluate the effect of selected bacterial isolate to plant growth, it was found that inoculated plants have better growth than un-inoculated plants. In summary, all selected nitrogen fixing bacterial isolates in this study promoted the plant growth well.

**Keywords:** ammonium excretion, IAA, nitrogen fixing bacteria, phosphate solubilisation, plant growth.

## 1. INTRODUCTION

Biofertilizers are living microorganism applied to the soil as inoculants to support plant growth by providing nitrogen. Hence biofertilizer is often called as microbial fertilizer (Simanungkalit, 2001). The microorganism has an important effect to the plant growth especially to increase plant productivity and maintain the soil fertility**.** They could affect the plant growth by either promoting or inhibiting it.

Nitrogen fixing bacteria have been selected as inoculants for biofertilizer since they have nitrogenase enzyme, which plays important role to fix stabile nitrogen from the athmosphere to ammonium ($NH_4^+$) (Zahran, 1999). Fixed ammonium is assimilated into glutamate and glutamine which are important to form organic nitrogen compounds in the cells such as amino acid, nucleic acid, protein, etc (Zahran, 1999). Following bacterial death and lysis, organic nitrogen compounds are released to the environment and utilized as nitrogen sources by other organism such as plants.

One of the problems in application of nitrogen fixing bacteria as biofertilizer is the little amount of nitrogen that is released to the environment (Dobbelaere *et al*., 200; Rao *et al*., 1999). This is due to the fact that accumulated fixed nitrogen in the cells causes feedback inhibition to synthesis and activity of nitrogenase (Zhang *et al*., 1997). In addition, ammonium produced from fixed nitrogen is used by the bacterial compounds in the cells. (Bali *et al*., 1992; Boussiba *et al*, 199; Martinez, 1984; Thoma *et al*., 1990).

However, several studies reported that some strains of nitrogen fixing bacteria have unique nitrogen regulation in which the ammonium is excreted out of the cell through simple diffusion rather than being accumulated in the cell. (Brewin *et al*., 1999; Castorph and Kleiner, 1984; Day *et al*., 2001; Kleiner, 1992). This phenomenon has important implication in the utilization of nitrogen fixing bacteria as biofertilizer. Excretion of ammonium omits the negative feedback to nitrogenase so that ammonium is continuously produced, excreted and eventually directly utilized by the plants (Castorph and Kleiner, 1984).

On the other hand, several studies reported that nitrogen fixing bacteria also produce growth promoting substances such as IAA and solubilize phosphate (Chitraselvi *et al*., 2015; Gupta *et al*., 2014; Inui-Kishi *et al*., 2012). Those findings are significantly meaningful since plant growth also needs growth factor such as phythohormone and some minerale such as phosphate.

To increase the plant growth supporting capability of biofertilizer, the bacterial inoculants should have all the properties to fix nitrogen, produce growth promoting substance, and solubilize phosphate (Glick *et al*., 1999; Kloepper *et al*, 1989). Thus, this study aims to obtain isolates with such properties to be utilized as biofertilizer inoculant candidates.

## 2. MATERIALS AND METHODS

### 2.1 Soil sampling

Nitrogen-fixing bacteria were isolated from rhizosphere of maize (*Zea mays* L.), rice (*Oryza sativa*), Shorgum (*Shorgum* sp) in Maros District of South Sulawesi. Rhizosphere of soil samples were collected carefully by uprooting the root and sieved through 2.00 mm mesh. One gram of the each crops soil sample was added into 250 ml Erlenmeyer flask containing 100 ml of Burks or Ashby-nitrogen free media as preliminary screening as well as enrichments growth of nitrogen fixation bacteria, and each of soil samples was inoculated triplicate. The flasks were incubated for 14 days at 27°C.

One ml of those growth medium was collected and transferred into glasses dilution vial which containing 9 ml sterile distilled water, shaken gently for homogeneity. Serial dilutions were made as 0.1 ml of aliquots from dilution $10^{-1}$ until $10^{-3}$ were inoculated by spread method on Burk's and Ashby nitrogen-free solid media. The plates were incubated at 27 ºC for 7 days. Pure colonies were obtained by repeated streaking three times on both nitrogen free solid media. Morphologically different colonies were isolated and subculture for further analysis.

### 2.2 Bacterial strain storage medium

The nitrogen-fixing bacteria were stored in Burk's N-free medium with following composition in $l^{-1}$, Sucrose, 20.0 g; $MgSO_4.7H_2O$, 0.2 g; $K_2HPO_4$, 0.8 g; $KH_2PO_4$, 0.25 g; $CaSO_4$, 0.13 g; $FeCl_3$, 1.45 mg; $Na_2MoO_3$, 0.253 mg. While, Ashby medium in $l^{-1}$as mannitol, 15 g; $CuCl_2.2H_2O$, 0.2 g; $K_2HPO_4$, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; $MoO_3$ (10%), 0.1 ml; $FeCl_3$ (10%), 0.1 ml. The pH was adjusted to 7.0 and autoclaved at 121ºC for 15 min (Atlas, 2009). Those media composition were used as same as previous media at enrichment and isolation stage above.

### 2.3 Quantification of ammonium release in medium

To quantify the ability of all bacteria isolate to release ammonium, one ml of well grown pure bacterial strains with different morphology were inoculated in 30 ml of Burks or Ashby broth at 100 ml Erlenmeyer flasks and incubated in an orbital shaker at 100 rpm in 27ºC for 24h. This incubation condition was an exponential growth phase of the bacteria strain isolates. After 24h incubation samples were taken and centrifuged at 13,000 rpm speed for 15 minutes. Three ml of the supernatant was taken and the pH set to 11 with the addition of NaOH 1N. Then added 0.07 ml EDTA, 0.07 ml sodium potassium tartrate, and 0.13 ml Nessler reagent were homogenized and incubated for 30 min at 25ºC. The absorbance determined at a wavelength of 435 nm by spectrophotometer (Leonard, 1961; Silva et al., 2002).

### 2.4 In Vitro IAA production

The production of IAA was evaluated by the colorimetric method described by Gordon and Weber (1951), with some modifications. 3 ml of bacterial isolate suspension (containing $10^7$ CFU/ml) was transferred into 12 ml of Minimal Salt Medium supplemented with tryptophan (1mg/ml) and then incubated at $28^0$C with continuous shaking for 6 days.

To quantify the IAA, treated samples were centrifuged 10 min at 5500 rpm every 48 hours. 1 ml of the clear supernatant was mixed with 4 ml of the Salkowski's reagent (50 ml of 35% perchloric acid and 1 ml of 0.05 M FeCl3 solution). The mixture was incubated in the dark at 37°C for 30 min. Development of pink color indicates the IAA production and optical density was measured at 535 nm using UV spectrophotometer (Shimadzu UV -VIS).

### 2.5 In vitro screening of phosphate solubilizing activity

Bacterial strains were evaluated for their ability to solubilize inorganic phosphate described by Deswal and Kumar (2013). The phosphate solubilization by the isolates from inorganic sources was screened by using Pikovskaya agar which contained $1^{-l}$: 10 g glucose; 5 g $Ca_3(PO_4)2$; 0.2 g KCl; 0.5 g $(NH_4)2SO_4$; 0.2 g NaCl; 0.1 g $MgSO_4$ $7H_2O$;0.002 g $MnSO_4H_2O$; 0.002 g $FeSO_47H_2O$ and 15 g Bacto agar. Briefly, the test isolates were inoculated in Pikovskaya agar and incubated for 3 days at 28ºC. Solubilization of phosphate was indicated by the zone of clearance around the bacterial colony. The solubilizing activity was calculated by the ratio between the halo diameter and the colony diameter. According to Silva and Vidor (2000), the solubilization process can be classified into low solubilization (E<2), average solubilization (2 < E <3) or high solubilization (E > 3).

### 2.6 Plant inoculation assay

Based on the capability to excrete ammonium, 14 isolates were selected for the pot trials according to modified Walpola (2012) Method. Initially, the bacterial strains were aerobically grown on burk's agar medium by transferring a single colony into 50 ml flasks containing burk's broth medium on a rotating shaker (100 pm) for 48 h at 30ºC. The bacterial suspension was then diluted in sterile distilled water to a final concentration of $10^7$CFU/ml, and resulting suspensions were used to treat eleven-day-old *Amaranthus tricolor* L. seedlings. The *Amaranthus tricolor* L. seeds was sterilized using 'surface sterilization' method before grown in a grown medium consisting of 2 Kg of sterile pure sand in a polybag. The experiment was carried out in a green house located at the Biology department, State University of Makassar.

Bacteria applications were following the syringing method in which 1, 5 ml of bacterial suspension was inoculated into the middle part of the seedling roots. Control plants received 1, 5 ml of diluted Burk's broth medium with no bacteria. Bacteria applications were grouped into 14 treatment groups. There were three replications for each treatment. The pots were arranged in a completely randomized block design. During the study period, *Amaranthus tricolor* L seedlings were watered daily by Fahreus (N-free) medium to maintain the water holding capacity and nutrient availability of the soil. Growth promoting effects of bacterial treatments were assessed by measuring the plant height and dry weight of

each plant. Dry weight were analysed by weighing parts of the plant that had been oven-dried at 70ºC for 2 days to a constant weight on an analytical balance. The plat height was measered from the soil surface to the highest leaf tip.

## 3. RESULTS AND DISCUSSIONS

We successfully obtained 43 nitrogen fixing bacterial isolates from rhizospheric soil in Maros District of South Sulawesi Province. More isolates were obtained from rhizospheric soil of rice compared to those from maize or shorgum (23 isolates from rice, 17 isolates from rice, and 3 isolates from shorgum). This might be due to the fact that the rice field had been fertilized longer and more intesively than the others. Agustian *et al*, (2010) concluded that changes in the rhizospheric environment especially in bacterial population could be caused by land cultivation, fertilizer application, and calcification. The changes in the environment not only affect the number of microorganism in soils but also the variety of microorganism.

On the other hand, among 43 isolates we found 14 isolates which released high concentrated ammonium in the ammonium release quantification assay (Table-1). This assay analyzed the concentration of ammonium excreted by the bacterial isolates to the growth medium when cultured in a free nitrogen medium by batch culture system. It was found that the concentration of ammonium release ranged from 256, 7 µM to 1027,77 µM. Highest concentrated ammonium was released by AMS421 isolate.

**Table-1.** Ammonium concentration released by nitrogen fixing bacteria isolates.

| Isolate code | Ammonium concentration (µM) |
|---|---|
| AMP141 | 433,33 |
| AMP143 | 313,3 |
| AMP151 | 256,7 |
| AMP251 | 603,3 |
| AMJ341 | 643,3 |
| AMS421 | 1027,77 |
| AMS423 | 690,74 |
| BMP131 | 264,81 |
| BMP212 | 277,77 |
| BMP221 | 272,22 |
| BMJ112 | 596,29 |
| BMJ132 | 346,29 |
| BMJ212 | 442,59 |
| BMJ221 | 575,92 |

Previous studies reported that several nitrogen fixing bacteria which release ammonium are wild type strain such as *Azotobacter vinelandii* strain OP, *Azotobacter vinelandii* strain UW136 and *Azotobacter vinelandii* strain ISSDS-428 which releases 260,251 µM and 600 µM, 200 µM, 1107,692 µMol of ammonium respectively (Bali *et al*., 1992; Hartono *et al*., 2009; Gordon and Weber, 1951; Kleiner and Kleinschmidt, 1956). Narula *et al*. (1981) also reported that A. chrococcum released 2, 61 mM ammonium after fermentative culture for 18 days.

In this study, ammonium release was quantified after 24 h incubation assuming that the bacteria have been in the exponential growth phase. Our previous study showed that nitrogen fixing bacteria started to release ammonium at the initial phase of exponential growth until reaching stationary growth (Hartono et al., 2009). Studies on *A. Chrococcum, A. vinelandii*, and *Klebsiella pneumoniae* have shown that ammonium release reaches its highest concentration when the bacteria were in their exponential growth condition. It is clear that in this phase, the bacteria fix huge amount of nitrogen to fulfill the nitrogen need for fast growth. Accumulated nitrogen in the cells diffuses in to growth media based on different concentration gradient.

We also evaluated the ability to produce IAA among 14 isolates. IAA concentration produced ranged from 5, 59 up to 41, 3 ppm. The highest concentration of IAA was produced by AMP143 isolate (Figure-1).

Previous studies successfully evaluated IAA production in several wild type strain of nitrogen fixing bacteria such as *Enterobacter* sp. (FJ890899), *E. Homaechei* subsp. steigerwaltii (FJ890898), Labrys portucalensis (FJ890891), and *Burkholderia sp*. (FJ890895) which produce 16-140 µg/ml of IAA (Agustian *et al*., 2010) and *Pseudomonas nitroreducens* which produce 13.83-90.17 µg/ml of IAA (Chitraselvi *et al*., 2015).

Figure-1 shows that IAA production started at day 2 (48 h incubation) and continued until day 6 (144 hr). This result goes along with the statement of Chitraselvi *et al*, (2015) and Inui-Kishi *et al*., (2012) that IAA is produced in the stationary phase of bacterial growth as secondary metabolite. Differences in IAA production periods as shown in Figure-1 could be due to differences in bacterial growth periods, particularly the start and and the duration of stationary phase.

Microorganism isolated from rhizosferic soil of plants potentially produce IAA because the plant root excrete exudates containing tryptopan. Tryptopan is a precursor in the biosynthesis of IAA in plants and microorganism (Khalid, 2004).

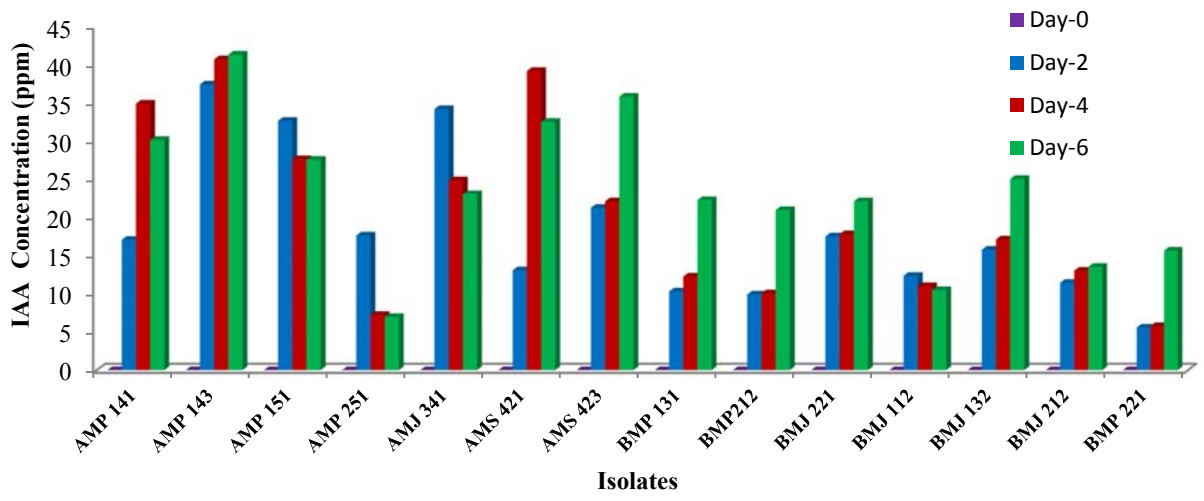


**Figure 1.** IAA production of nitrogen fixing bacteria Isolates.

We then evaluated the ability to solubilize phosphate among IAA producing isolates using Pikovskaya medium with 3 day-incubation. Six from 14 isolates could solubilize phosphate by showing clear zone around bacterial colonies on the medium (Table-2 and Figure-2).

**Table-2.** Phosphate solubilization of nitrogen fixing bacteria Isolates.

| **Isolat code** | **Clear zone ratio** (cm) |
|---|---|
| AMP141 | 1,5 |
| AMP143 | 1,6 |
| AMP151 | 2,5 |
| AMP251 | 2,2 |
| AMJ341 | ND |
| AMS421 | ND |
| AMS423 | 1,7 |
| BMP131 | 3,1 |
| BMP212 | ND |
| BMP221 | ND |
| BMJ112 | ND |
| BMJ132 | ND |
| BMJ212 | ND |
| BMJ221 | ND |

ND = no detectable

Among 6 isolates, only 1 has high ability to solubilize phosphate with clear zone ratio (E)>3 cm (BMP131). The other 2 isolates with 2< E < 3 are categorized mid-ability while the other 3 isolates with E<2 are categorized as low ability.

This study shows higher ability of phosphate solubilization compared to previous study by Chitraselvi et al, (2015) which reported several bacteria with the ability to produce clear zone up to 0.9 cm. Yet Gupta et al (2014) reported isolates with the ability to produce clear zone 61, 7 cm.

Several strains of nitrogen fixing bacteria that could solubilize phosphate are *Pseudomonas putida, P. cepacia and P. fluorescens* (Deshwal and Kumar, 2013). Bacillus altitudinis, *Pseudomonas monteilii*, and *Pseudomonas mandelii*. All those species form associations with rice plants and fix nitrogen (Habibi et al., 2014).

Bacterial isolates with the ability to excrete ammonium and to produce IAA were then selected for pot trials to investigate their ability to stimulate growth of *Amaranthus tricolor* L. The plant growth indicators were plant height and dry weight as shown in Figures 2 and 3. All *Amaranthus tricolor* in inoculated groups grew higher than that in the control group (Figure-2). Besides, *Amaranthus tricolor* L. in the inoculated groups had greater biomass compared to that in control group (Figure-3). Hence, all selected bacterial isolates contributed to better plant growth though their effects are different from each other.

It can be shown that the best isolates in terms of their effect to plant growth were isolate AMS421, BMP131, BMJ 212 and BMP 221. This result is almost similar with the data of plant dry weight of which inoculated plants with isolate AMS421, BMP131, and BMP 221 have the greatest dry weight among other plants. However isolate BMJ 212 showed inconsistency in terms of their effect to plant dry weight compared to their effect to plant height.

Isolate AMS421 had the greatest amount of ammonium excretion, while isolate BMP131 had the best ability to solubilize phosphate. Although having low

ability to produce growth hormone and solubilize phosphate, isolate BMJ 212 and BMP 221 have moderate ability in ammonium excretion.

In this study, how the ability to excrete ammonium, solubilize phosphate and produce growth hormone affected the plant growth has not been analyzed. It is suggested that in the future study this connection might be revealed.

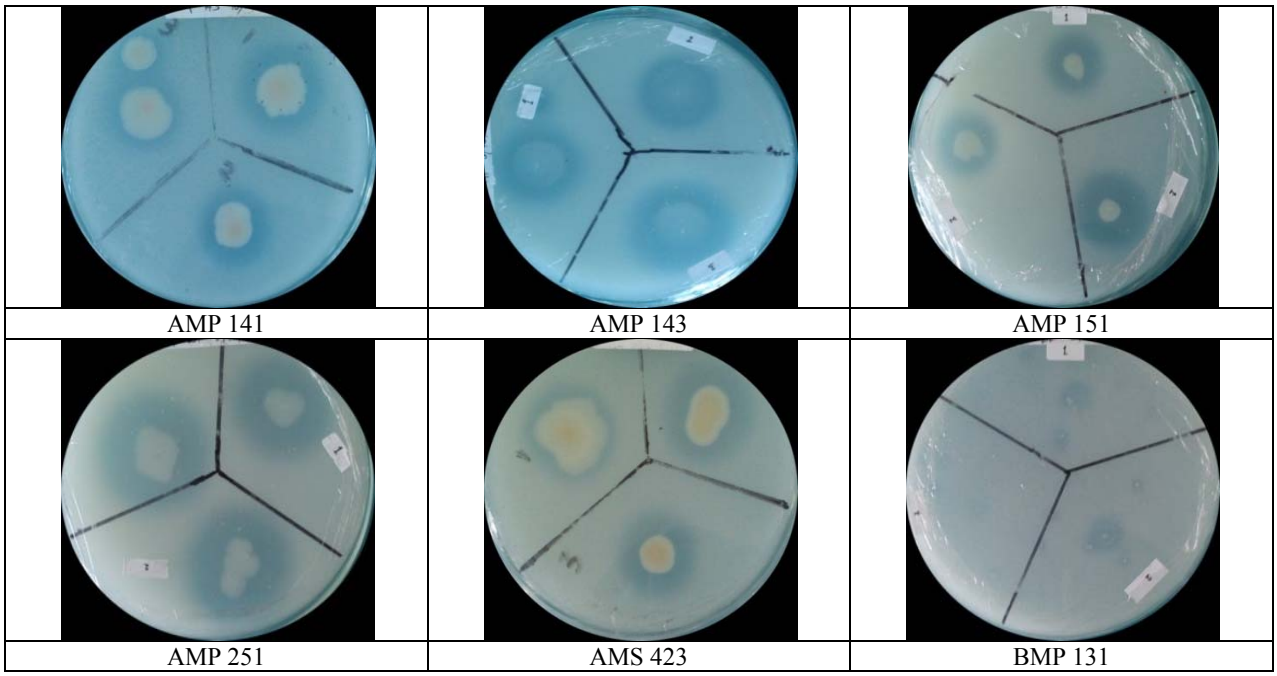


**Figure-2.** Phosphate solubilization on Pikovskaya medium of nitrogen fixing bacteria.

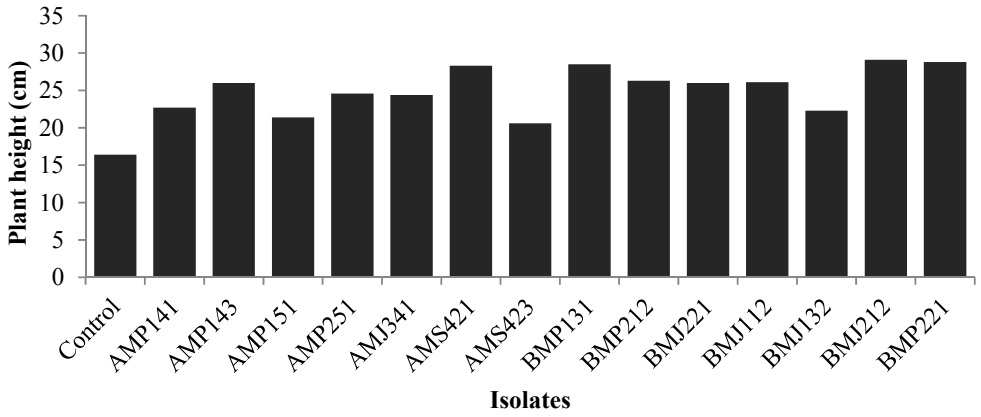


**Figure-3.** Plant height of *Amaranthus tricolor* L. which is inoculated by selected nitrogen fixing bacteria.

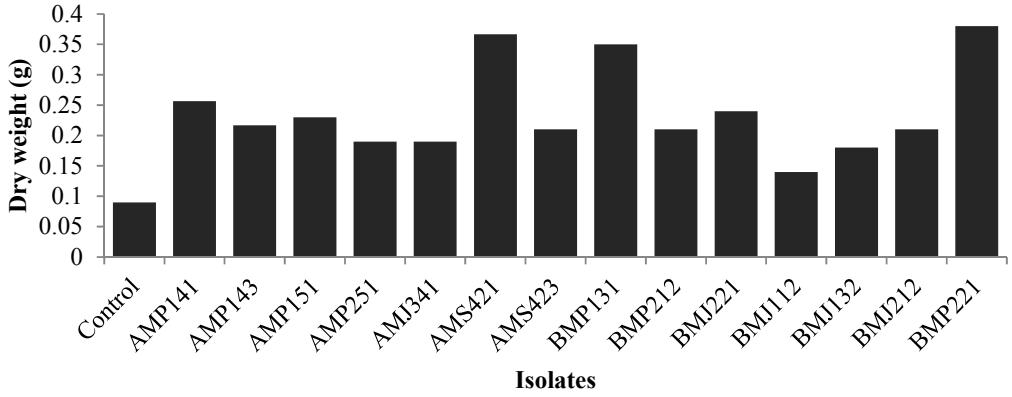


**Figure-4.** Dry weight of Amaranthus tricolor L. which is inoculated by selected nitrogen fixing bacteria.

## 4. CONCLUSIONS

Among 43 isolates of nitrogen fixing bacteria obtained from rhizospheric soils of crops in Maros District, we found 14 isolates that could release high concentrated ammonium of from 256, 7 µM to 1027,77 µM and produce growth promoting substance of 5,59 ppm to 41,30 ppm. Among 14 isolates, 6 isolates could solubilize phosphate with clear zone ration of 1, 5 cm to 3,1 cm. Pot trials demonstrate that all selected isolates in this research promote better *Amaranthus tricolor* L plant growth compared to the growth of un-inoculated plant.

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