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Research Article

Characterization of Endophytic-rhizobacteria from Areca Nut Rhizosphere to Dissolve Phosphates, Nitrogen Fixation of IAA Hormone Synthesis

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Abstract

Background and Objective: The ability of rhizobacteria to dissolve phosphate, fix nitrogen and synthesize growth hormones has a direct or indirect effect on increasing the plant growth including seed growth. The rhizobacteria which are inoculated in seeds through biopriming are proven to increase seed growth. The study aims to obtain the potential indigenous rhizobacteria from areca nut plant rhizosphere on marginal soil of Southeast Sulawesi. **Materials and Methods:** Research was arranged in a completely randomized design (CRD) consisting of 10 isolates. The isolates were evaluated their ability to solubilize phosphate, fix nitrogen and synthesize IAA growth hormone. Also evaluated the effect of these isolates in increasing the root length of rice seedlings. Before tested, rice seeds were treated with rhizobacterium isolates of endo-rhizobacteria and germinated using standard germination procedures. Root length was observed on the 7th day after planting. At the same time all isolates were evaluated for their ability to solubilize phosphate, fix N and synthesize IAA. **Results:** The results showed that seed treatment using endo-rhizobacteria significantly increased the root length of treated rice seeds (percentage increase compared to untreated seeds at root length up to 140%). All rhizobacteria isolates tested were able to solubilize phosphate, fix N and synthesize IAA. Although all of the rhizobacteria synthesized IAA, growth promoting effects of the rhizobacteria may not only be due to the synthesized growth regulator. Other factors may have involved in the positive effects of the endo-rhizobacteria on rice seed germination. **Conclusion:** The endophytic and rhizobacterial dominant from the areca nut rhizosphere are the *Pseudomonas* spp. and the *Bacillus* spp. and have ability to solubilize phosphate, fix nitrogen and synthesize IAA and also could increase rice seed growth.

Key words: Areca nut, *Pseudomonas, Bacillus*, solubilize phosphate, seed dormancy

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In line with agricultural development trends that prioritize the cultivation of plants based on environmentally friendly and sustainable technologies, this study is one solution. Every inch of soil rhizosphere has millions of potential rhizobacteria that can be utilized as PGPR (Plant Growth Promoting Rhizobacteria). The difference in host plants where rhizobacteria is active is also thought to be able to find rhizobacteria which have different biochemical characteristics, in this case biochemical characteristics related to their ability to stimulate plant growth through their ability to solubilize phosphate, fix nitrogen and synthesize IAA growth hormone. Similar studies have been carried out, not only in Indonesia, but also throughout the world. The results also vary, depending on the type of commodity and the environmental conditions in which the rhizobacteria are explored. Some of the results of previous studies indicate that the PGPR group's rhizobacteria are generally derived from the types of *Bacillus* spp., *Pseudomonas* spp., *Serratia* spp.[1,2], *Azospirillum* spp., *Azotobacter* spp., *Enterobacter* spp.[3]. In general, these types of bacteria have the ability to increase plant growth and yield and protect plants from various diseases[4-7].

The ability of rhizobacteria to dissolve phosphate, fix nitrogen and synthesize growth hormones has a direct or indirect effect on increasing plant growth and yield[8]. The role of PGPR in enhancing plant growth and yield is related to the ability to synthesize growth hormones[9], nutrient mobilization through P solubilization and nitrogen fixation or indirectly related through the reduction of biotic stress pressures such as through activation of plant resistance mechanisms to disease-causing pathogens.

Rhizobacteria PGPR which are inoculated in seeds through biopriming are proven to improve plant growth and suppress the growth of pathogenic colonies[4,10]. Study on rice seeds[11], showed that rice seed inoculation using *Bacillus* sp. proven to be able to increase growth and yield of rice reaching 22-49%. It also reported that rice seed inoculation with *Pseudomonas fluorescens* was able to increase production[12] by 47%.

Correlated with this study, 200 rhizobacterial isolates from the areca nut plant rhizosphere have been selected for marginal land (Ultisol soil) without fertilization application. Based on the results of the initial selection, 10 best potential isolates were selected for testing the next stage. This study activity is an advanced selection to get the best isolates that will be used for PGPR especially in agricultural commodities which are cultivated on marginal lands.

MATERIALS AND METHODS

Study area: The study was conducted at the Agronomy Laboratory, Agriculture Faculty, Universitas Halu Oleo from March to June, 2019. The study consisted of several series of experiments, namely (1) Preparation of endo-rhizobacterial isolates, (2) IAA synthesis test, (3) Solubilization P test, (4) Nitrogen fixation test, (5) Seed treatment with endophytic-rhizo bacterial isolates, (6) Observation of seedling root length.

Preparation of endo-rhizobacterial isolates: The endo-rhizobacterial isolate used was the result of selection from the previous research stage, consisting of 10 isolates. Pure culture of endo-rhizobacterial isolates were propagated using TSA media for the *Bacillus* sp. and for the *Pseudomonas* sp. King's B media was used. The growing bacterial colonies were suspended in a suitable liquid medium to reach a population density[13] of 10^9 CFU mL^{-1}.

IAA synthesis test: The ability of endo-rhizobacteria isolate to synthesize IAA was analyzed using the method Glickman and Dessaux[14]. The IAA content in the sample is calculated by regression made from pure IAA with concentrations of 0, 6.25, 12.5, 25, 50, 75, 100, 150 and 200 µg mL^{-1}.

Solubilization P test: The ability of bacterial endo-rhizo to dissolve phosphate was tested using an insoluble dicalcium phosphate (DCP) medium. The test procedure refers to the Goldstein[15]. Rhizobacterial isolates which have the ability to solubilize phosphate are shown by the presence of halo (clear zone) around the hole which contains a bacterial suspension.

Nitrogen fixation test: Endo-rhizo bacterial isolates which have the ability to fix free nitrogen from the air were analyzed qualitatively using Burk Salt media as follows: (1) $MgSO_4$ 2 g, K_2HPO_4 8 g, KH_2PO_4 2 g and $CaSO_4$ 1.3 g mixed into 1 and used as one and used as Burk Salt media stock, (2) $FeCl_3$ 0.145 and 0.0235 g Na_2MoO_4 were dissolved in 100 mL of distilled water and made as stock of Fe-Mo solution. The 1.3 g of Burk Salt media were mixed with 1 mL of stock of Fe-Mo solution and then added 10 g of sucrose, all the ingredients were solubilize in 1000 mL of sterile distilled water and then sterilized with autoclave at 121°C pressure of 1 atm for 15 min. A total of 20 μL of salt media was put into a sterile test tube. A total of 1 oz of rhizobacteria isolates tested were inserted into the solution and then incubated in an incubator shaker for 48 h using 150 rpm. Positive isolates as nitrogen fixation if the bacteria are able to grow in Burk salt solution which is characterized by media turbidity in the test tube. Isolates which grow are given a + sign (positive), while those who do not grow are coded-(negative).

Seed treatment with endophytic-rhizo bacterial isolates: Before being used, rice seeds were disinfected with 2% sodium hypochlorite for 5 min, washed 3 times with sterile water and dried in a laminar air flow cabinet for 1 h. Dried seeds (10 g) were soaked for 24 h in suspension of each bacterial endo-rhizo isolate (50 mL) at 28°C. After treatment, the seeds are again dried in the laminar air flow cabinet until they reach the initial weight, the seeds are ready for use.

Observation of seedling root length: Rice seeds which have been treated with endo-rhizo bacterial are germinated in a plastic tub measuring 20×15×10 cm (length×width×height) containing sterile husk charcoal as a germination medium. In each treatment 50 seeds were planted, with 3 replications. The measurement of the length of seedling is done at the age of 7 days after planting.

Statistical analysis: Statistical analysis were performed using two-ways of analysis of variances (ANOVA) by means using the Statistical Package of Social Sciences (SPSS) program version 20 for Windows (Chicago, IL, USA). If the test result showed a significant difference, then tests of treatment differences were performed using Duncan's multiple range test (DMRT) at $\alpha = 0.05$.

RESULTS

Ability of endophytic-rhizobacterial to solubilize phosphate and nitrogen fixation: The endophytic and rhizobacterial isolates from the areca nut tested were able to solubilize phosphate and fix nitrogen free from the air. Both endophytic bacteria and rhizobacteria, generally dominated by the *Pseudomonas* spp. group, while the *Bacillus* spp. group, each contained only 1 isolate. Rhizobacteria of the *Pseudomonas* spp. able to solubilize phosphate with a diameter range of halo 1.75-2.00 cm, while the *Bacillus* spp. able to solubilize phosphate with a halo diameter of 2.25 cm. Meanwhile, endophytic bacteria of the *Pseudomonas* spp. able to solubilize phosphate with a diameter range of halo 2.00-2.40 cm, while the *Bacillus* spp. able to solubilize phosphate with a halo diameter of 2.15 cm. On the other hand, rhizobacteria of the *Pseudomonas* spp. able to fix N with categories from slightly turbid to very turbid, while the *Bacillus* spp. able to fix N with very turbid categories. Meanwhile, endophytic bacteria of the *Pseudomonas* spp. able to fix N with categories from slightly turbid to very turbid, while the *Bacillus* spp. able to fix N with turbid categories (Table 1). Differences in exploration areas also affect the ability of endophytic and rhizobacterial to solubilize phosphate and fix N (Table 1).

Synthesis of IAA by endophytic-rhizo bacterial: Like the previous explanation which all types of bacteria tested were able to solubilize phosphate and fix nitrogen free from the air, the results of this study also showed which all bacteria tested both endophytic and rhizobacterial had the ability to synthesize IAA growth hormone on tryptophan containing media. The results showed that rhizobacteria had a wider IAA concentration range compared to endophytic bacteria at 5.45-71.27 μg mL^{-1} filtrate, whereas, endophytic bacteria had a narrower concentration range of 26.36-67.00 μg mL^{-1} filtrate (Fig. 1). Meanwhile *Pseudomonas* spp. have the ability to synthesize IAA higher than the *Bacillus* spp. (Fig. 1).

Table 1: Ability of endophytic-rhizobacteria *Bacillus* spp. or *Psedomonas* spp. In solubilize phosphate and fixing N

Bacteria group	Isolates code	Phosphate solubilizing (halo diameter = cm)	N fixation	Isolate origin (Sub-district)	Types of bacteria
Pseudomonas spp.	RJ6	2.00	Turbid	West Ranomeeto	Rhizo
Pseudomonas spp.	M5	1.95	Very turbid	Mowila	Rhizo
Pseudomonas spp.	W2	1.75	Slightly turbid	Palangga	Rhizo
Pseudomonas spp.	L1	1.95	Turbid	Benua	Rhizo
Bacillus spp.	LA6	2.25	Very turbid	Lainea	Rhizo
Pseudomonas spp.	WSE15	2.20	Very turbid	Landono	Endophytic
Pseudomonas spp.	ME4	2.40	Very turbid	Mowila	Endophytic
Pseudomonas spp.	LAE2	2.00	Slightly turbid	Lainea	Endophytic
Pseudomonas spp.	PE4	2.20	Slightly turbid	Benua	Endophytic
Bacillus spp.	LE6	2.15	Turbid	Benua	Endophytic

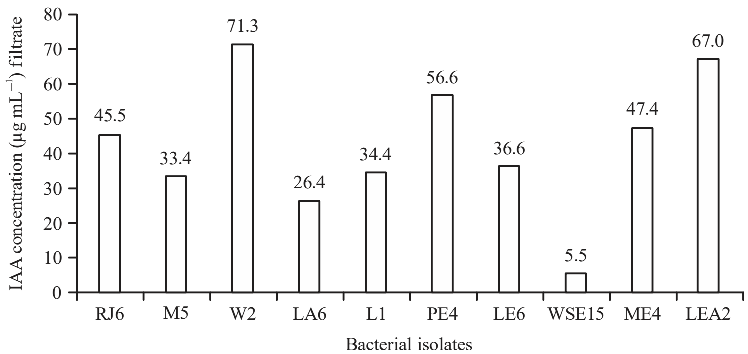

Fig. 1: Ability of endophytic-rhizo bacteria to synthesize IAA growth hormone in media containing tryptophan

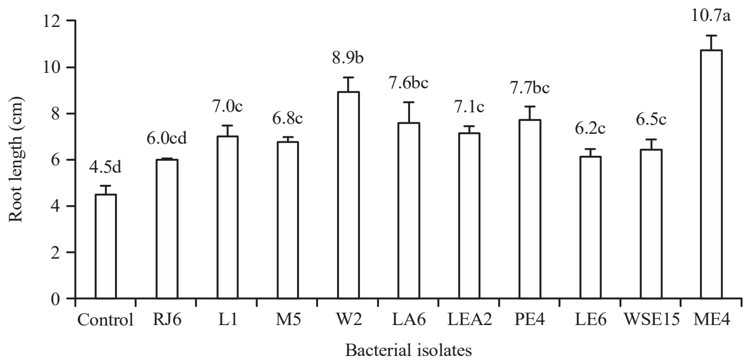

Fig. 2: Ability of endophytic-rhizo bacteria to increase the root length of rice seedling
Different letters on bars show significant difference

Effect of endophytic-rhizo bacterial treatment on root length of rice seedling: Seed inoculation with endophytic and rhizobacterial isolates from both the *Pseudomonas* spp. and or *Bacillus* spp. generally able to increase the root length of rice seeds compared to without bacterial treatment (Fig. 2). Of the 2 types of bacteria namely endophytic bacteria and rhizobacteria, it appears that rhizobacteria are more able to increase root length compared to endophytic bacteria. Based on the bacterial group tested, the *Pseudomonas* spp. more able to increase the root length of rice seeds compared with the *Bacillus* spp. group, with the best treatment found in ME4 rhizobacterial isolates (Fig. 2).

Relationship between IAA synthesis and root length of rice seedling: Regression analysis between IAA synthesis and root length of rice seedling was positively correlated. But the relationship is relatively small, which is marked by the low value of the correlation coefficient which only reaches 16.71%. This indicates that a significant increase in IAA production by endophytic and rhizobacterial is not always followed by an increase in the root length of rice seedling. Seed treatment with *Pseudomonas* sp. The ME4 which was only able to synthesize IAA 47.27 µg mL^{-1} bacterial filtrate, was actually more able to increase the length of rice roots compared to isolates of *Pseudomonas* sp. W2 which synthesized IAA 71.27 µg mL^{-1} bacterial filtrate. The results of the regression analysis between the synthesis of IAA produced by endophytic bacteria and rhizobacteria on the root length of rice seedling are shown in Fig. 3.

DISCUSSION

The endophytic and rhizobacterial isolates from the betel plant tested were able to dissolve phosphate, which was marked by the presence of clear zones on media containing phosphate sources. This study is in line with previous studies that reported that the class of bacteria can solubilize phosphate both endophytic bacteria[16-19] and rhizobacteria[20-26].

The ability to solubilize phosphate is generally dominated by the *Pseudomonas* spp. group, compared to the *Bacillus* spp. group, both in endophytic and rhizobacterial bacteria. In line with this study, also reported that bacteria from the *Pseudomonas* spp. more effective in dissolving phosphate in TCP media compared to *Bacillus* spp.[1]. Karpagam and Nagalakshmi[22] reported that as many as 37 phosphate solvent microbial isolates on Pikovskaya agar

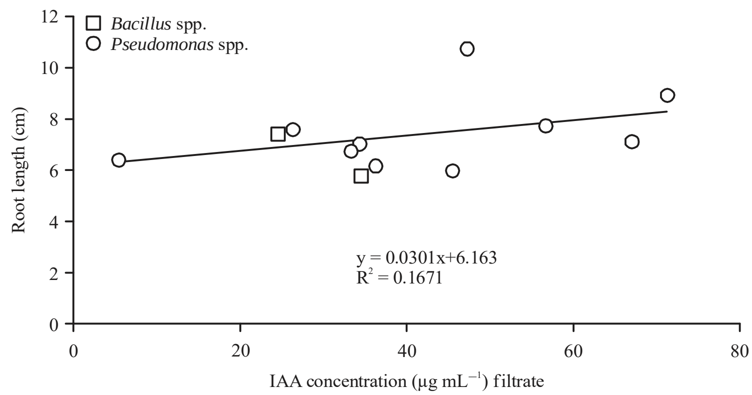

Fig. 3: Results of a regression analysis between the synthesis of indole acetic acid (IAA) produced by endophytic-rhizo bacterial isolates to the root length of rice seedling treated by each endophytic -rhizo bacterial isolate

medium contained insoluble tri-calcium phosphate (TCP) which was marked by the presence of clear zones on the media. The results showed that 8 isolates of the genus *Pseudomonas* sp., *Bacillus* sp. and *Rhizobium* sp. shows the highest phosphate solubilization index ranging from 1.13-3.0. The mechanism of phosphate solvent bacteria (PSB) in dissolving organic and inorganic phosphate into solubilized phosphate[27], so that it plant root can use it and other soil microbes is by producing organic acids which can replace P in bonds with Al or Fe so that the elements P will be released into soluble P which plants can use.

A large number of microorganisms in the rhizosphere are known to solubilize and provide insoluble phosphorus in the forms available to plants[28]. The endophytic and rhizobacterial isolates are also able to fix nitrogen free from the air which is characterized by the degree of turbidity in the bulk salt media. The previous studies reported that bacteria were able to fix nitrogen both endophytic bacteria[17-19,29-31] and rhizobacteria[1,20,23,26]. Endophytic bacterial isolates and rhizobacteria of the *Pseudomonas* spp. able to fix N with categories from slightly turbid to very turbid, while the *Bacillus* spp. able to fix N with very turbid categories. Meanwhile, endophytic bacteria of the *Pseudomonas* spp. able to fix N with categories from slightly turbid to very turbid, while the *Bacillus* spp. able to fix N with turbid categories. Differences in exploration areas also affect the ability of endophytic bacteria and rhizobacteria to fix N.

The endophytic and rhizobacterial isolate are also able to produce growth hormones in the form of IAA on media containing tryptophan with different ranges. The results showed that rhizobacteria had a wider IAA concentration range compared to endophytic bacteria which was 5.45-71.27 µg mL^{-1} filtrate, whereas, endophytic bacteria had a narrower concentration range of 26.36-67.00 µg mL^{-1} filtrate. The results of the same study were reported by bacteria from the *Pseudomonas* sp. has the ability to synthesize IAA hormones higher than other groups of bacteria[1]. Reetha *et al*.[32] also reported that IAA production from *Bacillus* spp. lower than the IAA production of *P. fluorescens*.

Inoculation of endophytic and rhizobacterial isolates isolated from areca nut plants applied to rice seeds in general was able to increase the root length of rice seedling which were significantly different from controls. The longest root germination of rice seeds inoculated by bacteria was obtained in the endophytic bacterial isolate of *Pseusomonas* sp. ME4 was then followed by *Pseusomonas* sp. W2 (Fig. 2). The ability of endophytic and rhizobacterial isolates to increase the root length of local rice seedling is directly related to their ability to produce growth hormones in the form of IAA. The mechanism of endophytic microbes in increasing plant growth is by producing IAA[30,33] so that the presence of endophytic bacterial types in plants can help increase plant growth.

The rhizobacterial isolates are also able to produce IAA hormones[34-36]. It was further reported that the isolate *Pseudomonas* sp. inoculated with the seed immersion method can increase root and shoot length, root dry weight and fresh and dry weight of brinjal plants when compared to controls. The best treatment was obtained from inoculated *Pseudomonas* sp. isolate 15 (I15) showed maximum plant growth activity followed[37] by isolate 1-14.

The results of regression analysis of the relationship of IAA synthesis and root length of upland rice seedling were positively correlated, although they did not always show results in accordance with the increase in IAA production affecting root length. The results of previous studies also report the same thing, high IAA production by rhizobacteria does not always correlate with high plant growth[38,39].

Conversely, excessive IAA production is reported to inhibit seed viability[40]. Furthermore, Vacheron *et al*.[41] states that IAA production by rhizobacteria can affect positively or negatively on plant growth depending on the concentration used. At low concentrations they can improve growth, while at high concentrations they can inhibit plant growth. The results of this study are relevant to another research who reported that the exogenous treatment of seeds with IAA at concentrations of 0.5 and 1.0 µM caused similar stunting effects on root and shoot growth compared with controls both at 5 and 10 days of observation[42], whereas, higher IAA concentrations (10.0 µM) could inhibit the growth of seedlings. The role of endophytic and rhizobacterial isolates from the areca nut in triggering plant growth were related to their ability to solubilize phosphate, fix nitrogen and synthesize IAA growth hormone. Both endophytic bacteria and rhizobacteria, generally dominated by the *Pseudomonas* spp. and the *Bacillus* spp. The main contribution of *Pseudomonas* spp. and *Bacillus* spp. were to increase the availability of growth hormones that serve to trigger plant growth and increase the availability of phosphor. The application of endophytic bacteria and rhizobacteria may reduce the needed for plant for fertilizer *P. Pseudomonas* spp. and or *Bacillus* spp. could synthesize IAA growth hormone and increase the root length of rice seeds. Moreover, it could use as breakage of seed dormancy.

CONCLUSION

The endophytic and rhizobacterial isolates from the areca nut rhizosphere can solubilize phosphate, fix nitrogen and synthesize IAA and could trigger plant growth. Pseudomonas spp. and the Bacillus spp found dominant in areca nut rhizosphere. The application of endophytic bacteria and rhizobacteria may reduce the needed source of fertilizer P and have potential as breaking of seed dormancy on rice seed.

SIGNIFICANCE STATEMENT

This study discovers the role of endophytic bacteria and rhizobacterial isolates from the areca nut rhizosphere to solubilize phosphate, fix nitrogen and synthesize IAA that can be beneficial for facilitating the growth of the seed. This study will help the researcher to uncover the critical area relating to finding out the endophytic bacteria and rhizobacterial isolates from various types of plant's environmental conditions that can stimulate growth seed. Thus, a new theory on the role of endophytic bacteria and rhizobacterial from the areca nut rhizosphere may have arrived. The application of endophytic bacteria and rhizobacteria improves seed growth.

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