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The role of indigenous Rhizobia on *Paraserianthes falcataria* (L) Nielsen seedlings in nickel post mining lands

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Abstract. Application of indigenous Rhizobia on degraded land reclamation, especially post mining land allegedly can promote plant growth and development.. The research was to determine effectiveness of indigenous Rhizobia isolates from nickel post mining land to the growth of *P. falcataria* (L) Nielsen seedlings. Three types of native Rhizobia isolates (R1, R2, R3) and their combination (R4, R5, R6, R7) were applied as treatments in randomized completely design. All data were analyzed using Analysis of Variance (ANOVA) and Kruskal Wallis. Significant differences between treatments were analyzed using Duncan's Multiple Range Test (DMRT) and Mann Whitney. The result showed most of growth variables of seedlings was not significantly influenced by Rhizobia inoculation, except stem diameter and biomass. This research also indicated correlation between N content in plant tissue with number of nodules, diameter and biomass although not significant. Single inoculation of Rhizobia did not show optimal results on *P. falcataria* (L) Nielsen seedlings so double inoculation using other potential microbes or input organic fertilizers at certain levels allegedly can increase the potential of Rhizobia on plants, especially in nickel post mining reclamation.

1. Introduction

PGPR (*Plant Growth Promoting Rhizobacteria*) is a beneficial bacteria group that live in the rhizosphere actively and play an important role in plant growth. This group is able to synthesize growth hormones, vitamins, and various organic acids, thus increase nutrients uptake for plants. In generally, PGPR capable to produce enzymes and hormones that promote plant growth, increase nutrient availability, control the microbial activity of pathogens for plants and protects them in environmental stresses [1]. *Rhizobium* strains, one of soil microbe, that colonize legume and non-legume roots (such as Gramineae and Cruciferae) are able to encourage the plant growth by fixing N₂ from the atmosphere and producing phytohormones so that belong to the PGPR [2].

Paraserianthes falcataria (L) Nielsen is a legume and symbiont of Rhizobia. This plant is categorized as one of pioneer species who has fast growth and ability to grow on various types of soil as long as good drainage is sufficient [3]. Moreover, *P. falcataria* (L) Nielsen has good wood quality so it used in the panel and plywood industries [4]. The symbiosis between *P. falcataria* (L) Nielsen and Rhizobia makes this plant known as nitrogen-fixing tree. When the nitrogen-fixing trees drop their leaves and small twigs or the trees die back, the nitrogen and other organic matter accumulated in their tissues can cause the soil fertility [4]. Native plant species, such as *P. falcataria* (L) Nielsen, is able to accelerate the succession of land cover on degraded land [5]. Therefore, *P. falcataria* (L) Nielsen is widely used in reforestation programs to increase soil fertility. However, the initial growth of *P.*



falcataria (L) Nielsen seedlings sometimes have problems on marginal sites so that input fertilizer is needed to stimulate and accelerate initiation in the field.

Indigenous Rhizobia inoculation, as a biofertilizer, on the *P. falcataria* (L) Nielsen seedlings is allegedly able to stimulate plant growth in the former nickel mines soil. Symbiotic association between legumes with nitrogen-fixing microorganisms can overcome nutrient deficiencies on critical lands, such as found on volcanic sites [6]. Rhizobia can improve the growth of legumes in poor soil presented in degraded soils [7]. Furthermore, the ability of legumes-Rhizobia to fix nitrogen makes this symbiosis is very important in the restoration of ex-mining lands, such as ex-bauxite mining land [8]. Rhizobia is reported to be able to tolerate in environmental stresses as is commonly the case during bauxite mining [9].

The aim of the research was to determine effectiveness of indigenous Rhizobia isolates from nickel post-mining land to the growth of *P. falcataria* (L) Nielsen seedlings. The chosen inoculum who has the best effect for the seedlings will be applicated in the restoration of former nickel mining land.

2. Materials and Methods

2.1. Preparation of symbiotic nitrogen-fixing bacteria inoculums

Rhizobia inoculums used were the best inoculums based on the ability of growth in YEMA (*Yeast Extract Mannitol Agar*) medium [10][11], analysis of IAA (*Indole Acetic Acid*) hormone production, ARA (*Acetylen Reduction Assay*) test, and bacterial viability test [12]. There were 3 best inoculums from the selection results. They were BF A2.1.4, HL 4.1 and A2.1.T5 inoculums. The inoculum of BF A2.1.4 was a Rhizobia strain obtained from former nickel mining land on the backfilling sites. Whereas HL 4.1 inoculum was obtained from natural forest sites and A2.1.T5 came from succession sites. Multiplication of Rhizobia inoculum was done in July 2017 at microbiology laboratory, Environment and Forestry Research and Development Institute of Makassar.

2.2. Preparation of seeds, sprouts media, and plantation media

The strain of legume used in effectiveness test of indigenous Rhizobia isolates was *Paraserianthes falcataria* (L) Nielsen. The seeds were soaked in water for 24 hours before being sown on sprouts media. The composition of sprouts media was sand and compost (1 : 1). Former nickel mine soil used as seedlings media that was previously sterilized by fumigation method. The fumigant used was dazomet 98%.

2.3. Planting and maintenance seedlings

Weaning when the sprout had two leaves for the first time. Seedlings media perforated with a depth of 3 cm in pots. The application of Rhizobia inoculum by giving 2 grams inoculum in each planting hole. The inoculum has met the standard to be used as biological fertilizer, which has 10^8 - 10^9 cells/gr soil [13]. Watering seedlings was carried out every day using sterile water. Moreover, seedlings fertilized using hyponex 1g/L.

2.4. Observation variables

Seedlings were maintained in the glass house in August-November 2017. Air temperature and relative humidity in the glass house during observation were 27-34°C/46-92% (morning), 32-37°C/46-72% (afternoon) and 28-37°C/42-88% (evening). Measuring the height of seedlings was done by using a ruler starting from the base of the stem to the shoot of seedlings. Measurement of the initial height of seedlings began for 2 weeks after inoculation and subsequent measurement was carried out every month for 16 weeks. Stem diameter measured using calipers at an altitude of about 1 cm above the base of the stem. Seedlings biomass was based on the dry weight of seedlings. Seedlings was put in to the oven at 60°C and weighed until it reached a constant weight. Analysis of seed quality index based on Roller method. The number of nodules was observed by counting all the number of nodules formed in the roots of *P. falcataria* (L) Nielsen seedlings. The observation was made on 10 replications for

each treatment randomly selected. Measurement of stem diameter, seedlings biomass, seed quality index, and the total of nodules were made once when the seedlings was 4 months old.

2.5. Treatments, experimental design, and statistical analysis

The design used in this research was a completely randomized design, with the treatments were a type of indigenous Rhizobia isolates. The treatments were 1) seedlings non-inoculated of Rhizobia (R0); 2) seedlings inoculated with BF A2.1.4 inoculum (R1); 3) seedlings inoculated with HL 4.1 inoculum (R2); 4) seedlings inoculated with A2.1.T5 inoculum (R3); 5) seedlings inoculated with combination of BF A2.1.4 and HL 4.1 inoculum (R4); seedlings inoculated with combination of BF A2.1.4 and A2.1.T5 (R5); seedlings inoculated with combination of HL 4.1 and A2.1.T5 inoculum (R6); and 7) seedlings inoculated with combination of BF A2.1.4, HL 4.1 and A2.1.T5 inoculum (R7). Each treatments had 20 replications, so there was 160 experimental units were obtained. All data were analyzed using Analysis of Variance (ANOVA) and Kruskal Wallis. Significant differences between treatments were analyzed using Duncan's Multiple Range Test (DMRT) and Mann Whitney.

3. Results and Discussion

The damaged condition of the former nickel mine causes the vegetation and soil microflora are not capable or only a few to survive. Hence, to improve soil quality, it is necessary to inoculate biofertilizer from the outside. Input of indigenous potential microbes can increase nutrient uptake by plants [14], increase resistance to abiotic stresses [15], and prevent diseases [16]. The application of Rhizobia inoculum on *P. falcataria* (L) Nielsen seedlings gives varied results on several observation variables.

3.1. Effect of Rhizobia inoculation on height and stem diameter of *P. falcataria* (L) Nielsen seedlings

Rhizobia inoculation did not have a significant effect generally. The seedlings leaves are yellow and fall out, plants look dwarfed (Figure 1). This color change is caused by the lack of chlorophyll pigment in the seedlings leaves. Chlorophyll is an essential pigment that plays a role in photosynthesis to convert light energy to stored chemical energy [17]. Photosynthesis process that does not take place properly causes nutrients (photosynthates) to be limited so that it can not support plant growth. Chlorophyll deficiency can cause stress on plants and die quickly [18].



Figure 1. (A) The growth of *P. falcataria* (L) Nielsen seedlings in several treatments; (B) Nodules formed are small relatively; (C) Nodules that have been split with black fill.

Measurement of the height and stem diameter of seedlings was done after 16 weeks of observations. Indigenous Rhizobia inoculation did not have a significant effect on the high addition of *P. falcataria* (L) Nielsen seedlings. Isolate infection in seedlings roots was not effective in spurring plant growth. This was presumably due to low P levels in the planting medium. Chemical analysis on former nickel mine indicated that available phosphorus was low [19]. Phosphorus has an important

role in plants even though it is needed at low levels. Phosphorus does not only increase seed yields but also affect nodulation at legume roots, thus N fixation [20]. However, there was a difference in growth between seedlings inoculated with Rhizobia and controls (non-inoculated Rhizobia). The treatments of R1 (inoculum BF A2.1.4) gave the best results compared to other treatments, which was 10.89 cm, while the R0 treatment (control) gave the lowest yield with a height increase of 9.81 cm. Measurement results can be seen in Figure 2.

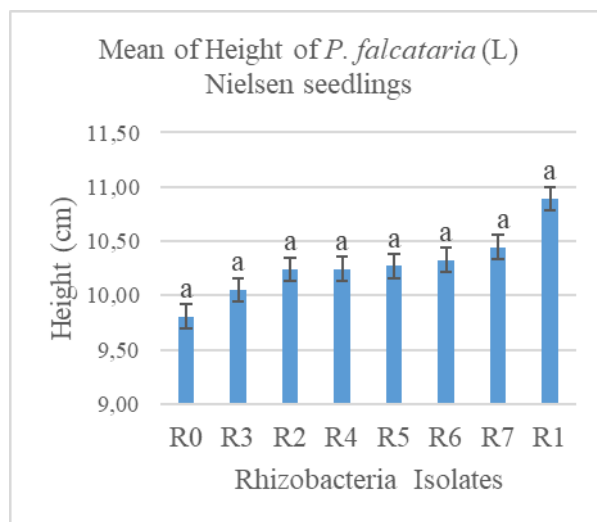


Figure 2. The effect of Indigenous Rhizobacteria Isolates to height of *P. falcataria* (L) Nielsen seedlings on 16th weeks after planted

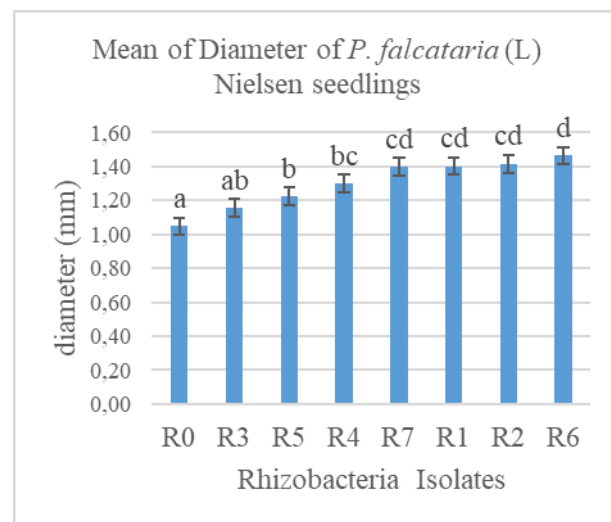


Figure 3. The effect of Indigenous Rhizobacteria Isolates to stem diameter of *P. falcataria* (L) Nielsen seedlings on 16th weeks after planted

Remarks : R0 = no inoculation, R1 = inoculation of BF A2.1.4 inoculum, R2 = inoculation of HL 4.1 inoculum , R3 = inoculation of A2.1.T5 inoculum, R4 = inoculation of combination BF A2.1.4 and HL 4.1 inoculums, R5 = inoculation of combination BF A2.1.4 and A2.1.T5 inoculums, R6 = inoculation of combination HL 4.1 and A2.1.T5 inoculums, R7 = inoculation of combination BF A2.1.4, HL 4.1 and A2.1.T5 inoculums
Bar that followed by the same letters are not significant at 5% level according to Duncan Test

Association of Rhizobia inoculum and *P. falcataria* (L) Nielsen seedlings nodules increased stem diameter better than controls (Figure 3). The combination of HL 4.1 and A2.1.T5 inoculums (R6) gave the best yield, which was 1.46 mm. It meaned if the treatment was more effective in giving a significant effect on the increase in stem diameter at 16 weeks seedlings age. Non-inoculate Rhizobia seedlings shown low increase in stem diameter, which was 1.05 grams. *Rhizobium* inoculation on *Dalbergia sissoo* seedlings showed significant effect in improving growth, biomass production and nodulation [21].

3.2. Effect of indigenous Rhizobia inoculation on biomass and the number of nodules formed in *P. falcataria* (L) Nielsen seedlings

Inoculation of indigenous Rhizobia to *P. falcataria* (L) Nielsen seedlings was able to increase seedlings biomass. Observations showed that Rhizobia inoculation had a significant effect at 16 weeks. BF A2.1.4 was single inoculum who gave the best results in seedlings biomass, which was 0.55 grams. Inoculation of Rhizobium can increase the total of biomass plants, as in *Albizia procera*, effectively [22][23]. The lowest diameter increase indicated by the seedlings inoculated A2.1.T5 inoculum (R3), which was 0.21 grams, lower than control (0.22 grams). In addition to seedlings biomass variables, A2.1.T5 inoculum did not give good results too in the height and diameter of

seedlings. Single inoculum A2.1.T5 was not effective in infecting *P. falcataria* (L) Nielsen seedlings. Observation results can be seen in Figure 4.

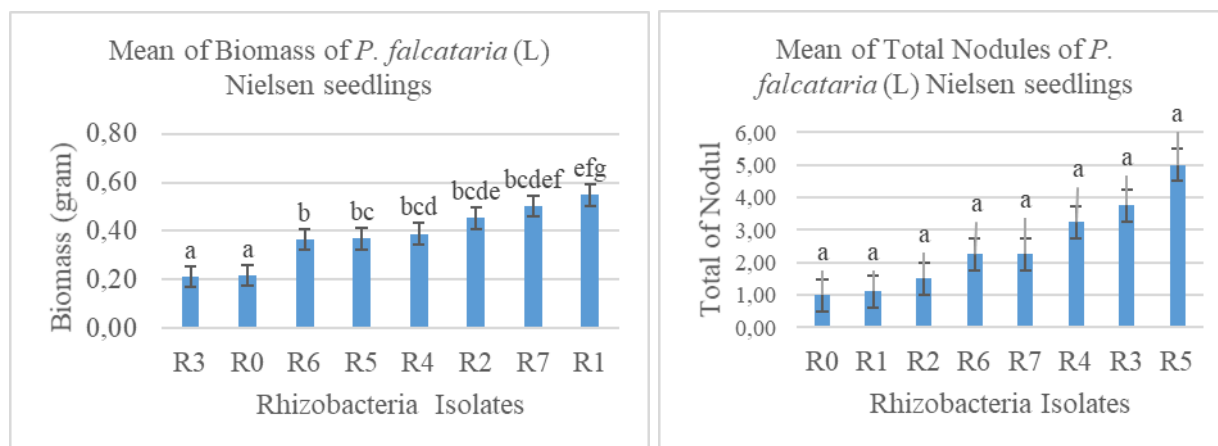


Figure 4. The effect of Indigenous Rhizobacteria isolates to biomass of *P. falcataria* (L) Nielsen seedlings on 16th weeks after planted

Figure 5. The effect of Indigenous Rhizobacteria isolates to total of nodules of *P. falcataria* (L) Nielsen seedlings on 16th weeks after planted

Remarks : R0 = no inoculation, R1 = inoculation of BF A2.1.4 inoculum, R2 = inoculation of HL 4.1 inoculum, R3 = inoculation of A2.1.T5 inoculum, R4 = inoculation of combination BF A2.1.4 and HL 4.1 inoculum, R5 = inoculation of combination BF A2.1.4 and A2.1.T5 inoculum, R6 = inoculation of combination HL 4.1 and A2.1.T5 inoculum, R7 = inoculation of combination BF A2.1.4, HL 4.1 and A2.1.T5 inoculum
Bar that followed by the same letters are not significant at 5% level according to Mann Whitney Test

The seedlings which was inoculated by indigenous Rhizobia produced a few and small nodules. However, treatments with inoculums showed better results than control. The combination of BF A2.1.4 and A2.1.T5 inoculums gave the mean of total nodules better (5.00 nodules), greater than seedlings with non-inoculated Rhizobia (1.00 nodul) (Figure 5). In other studies, inoculation of Rhizobia strains on *Retama sphaerocarpa* can increase nodules formation [24]. The results of the observation showed nodules blackish generally which indicated that the nodules did not contain leghemoglobin (Figure 1). Pink color on the inside of the nodules indicates the presence of leghemoglobin pigment which plays a role in the nitrogen fixation process [6].

3.3. Effect of Rhizobia inoculation on N content in plant tissues

Nitrogen plays an important role in plant growth and development. Nitrogen is used in the synthesize of enzymes, proteins, chlorophylls, DNA and RNA. In symbiosis between Rhizobia and legumes, nitrogen obtained from symbiotic fixation of atmospheric N₂ by nitrogenase enzyme in rhizobial bacteroids [25]. Analysis of nitrogen content showed that *P. falcataria* (L) Nielsen seedlings gave similar results in all treatments. Rhizobia inoculation on seedlings was not able to increase nitrogen content in plant tissues significantly. However, the combination of HL 4.1 and A2.1.T5 inoculums caused greater nitrogen content in the plant tissue than the other treatments, it was 3.16%. Rhizobia inoculation can increase N levels in plants [24]. The different results was shown by inoculation of single Rhizobia with HL 4.1 inoculum. It was the lowest mean of the nitrogen content, which was 2.84%, lower than control (Figure 6). It was allegedly due to the lack of potential nutrients needed by bacteria in the growing medium so as to fulfill their life needs into parasites for plants. Therefore, nutrients was not used properly by plants but bacteria used it to stay alive.=

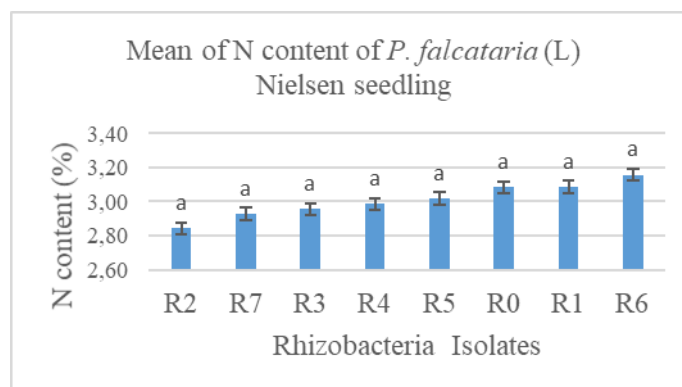


Figure 6. The effect of Indigenous Rhizobacteria Isolates to N content of *P. falcataria* (L) Nielsen seedling on 16th weeks after planted

Remarks : R0 =no inoculation, R1 = inoculation of BF A2.1.4 inoculum, R2 = inoculation of HL 4.1 inoculum , R3 = inoculation of A2.1.T5 inoculum, R4 = inoculation of combination BF A2.1.4 and HL 4.1 inoculum, R5 = inoculation of combination BF A2.1.4 and A2.1.T5 inoculum, R6 = inoculation of combination HL 4.1 and A2.1.T5 inoculum, R7 = inoculation of combination BF A2.1.4, HL 4.1 and A2.1.T5 inoculum
Bar that followed by the same letters are not significant at 5% level according to Duncan Test

3.4. The relationship between N content and number of nodules, height, stem diameter, and biomass of *P. falcataria* (L) Nielsen.

Analysis of the relationship on N content to seedlings vegetative organ formation showed that there was a close relationship between N stored in plant tissues with the number of nodules formed, stem diameter and biomass of *P. falcataria* (L) Nielsen seedlings. This closeness was based on the correlation coefficient that approaches 1 or -1 although it was not significant. Different result occur in the relationship between N content and seedlings height. The correlation test results indicate an unrelated relationship between the two variables (Table 1).

Table 1. Correlation between N content with Total of Nodules, Height, Stem Diameter and Biomass of *P. falcataria* (L) Nielsen seedlings

		N content	Total of nodul
N content	Pearson Correlation	1	.055
	Sig. (2-tailed)		.797
	N	24	24
Total of nodules	Pearson Correlation	.055	1
	Sig. (2-tailed)	.797	
	N	24	24
		N content	Height
N content	Pearson Correlation	1	.030
	Sig. (2-tailed)		.888
	N	24	24
Height	Pearson Correlation	.030	1
	Sig. (2-tailed)	.888	
	N	24	24
		N content	Diameter
N content	Pearson Correlation	1	.097
	Sig. (2-tailed)		.651
	N	24	24

Stem Diameter	Pearson Correlation	.097	1
	Sig. (2-tailed)	.651	
	N	24	24
		N content	Biomass
N content	Pearson Correlation	1	-.065
	Sig. (2-tailed)		.764
	N	24	24
Biomass	Pearson Correlation	-.065	1
	Sig. (2-tailed)	.764	
	N	24	24

Inoculation of Rhizobia bacteria in *P. falcataria* (L) Nielsen seedlings did not show optimal results. Low quality planting media, especially low P levels in the soil are thought to be the cause of bacteria did not effective in infecting the plant roots. Double inoculation can increase the formation of vegetative organ and crop production, especially in native species [26]. The combination of nitrogen fixing bacteria and phosphate solubilizing bacteria is more effective than single inoculation [27]. Therefore, double inoculation using FMA, phosphate solubilizing bacteria or input of organic fertilizers at certain levels allegedly can increase the potential of Rhizobia as a biofertilizer in plants. This technology may improve survival and juvenile growth of plants on marginal site, like on post mining land. Some previous studies have shown that a single inoculation of Rhizobia can improve the success of restoration on degraded land. Some legume trees inoculated Rhizobia were widely applied for live fencing, landscape stabilization and land reclamation in Brazil [28]. In certain soil conditions that do not allow the role of Rhizobia optimally, it may be necessary to use double inoculation using other potential microbes. Inoculation of Acacia with Rhizobia (single inoculation) had only lower effect on total N and P of the plant compared with inoculation with FMA that increased plant P content by up to 48 times and plant N content till 30 times [29].

4. Conclusion

Rhizobia inoculation on *P. falcataria* (L) Nielsen seedlings using former nickel mine soil did not show a significant effects, except for the stem diameter and seedlings biomass. The low content of phosphorus in the soil was thought to be an obstacle for Rhizobia in infecting the plant roots. However, treatment with the addition of Rhizobia inoculums gave better results than controls. Moreover, there was a close relationship between N stored in plant tissues with the number of nodules formed, stem diameter and biomass of *P. falcataria* (L) Nielsen seedlings, although not significant. Inoculum of BF A2.1.4 and HL 4.1 gave better growth effect for all observation variables. The combination of Rhizobia with other potential microbes, such as phosphate solubilizing bacteria and arbuscular mycorrhizal fungi, as well as input of organic matter is thought to increase potential of Rhizobia as a biofertilizer.

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