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Research Article

Azotobacter population, soil nitrogen and groundnut growth in mercury-contaminated tailing inoculated with *Azotobacter*

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Abstract: Gold mine tailing in Buru island, Maluku Province, Indonesia contains high level of mercury but low in carbon and plant nutrients. Revegetation in such condition needs certain soil treatment which is suitable for plant growth. The objective of pot trials was to study the effect of indigenous Plant Growth Promoting Rhizobacteria *Azotobacter* and organic matter on bacterial survival as well as growth of groundnut grown in mine tailing. The experimental design was a Split Plot Design which tested three types of *Azotobacter* liquid inoculant and three soil total organic carbon (TOC) contents. Results showed that *Azotobacter* inoculation increased *Azotobacter* population in tailing at the end of vegetative growth of groundnut. Total nitrogen content in soil decreased when TOC level increased. However, nitrogen uptake and growth of groundnut did not change after *Azotobacter* inoculation or manure amendment. These experiments provided information that *Azotobacter* inoculation on groundnut maintain its proliferation in Hg-contaminated mine tailing.

***Keywords**: Azotobacter, mercury-contaminated tailing, PGPR, revegetation*

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Introduction

Illegal gold mining in Botak Mountain located in Buru District, Maluku Province, Indonesia used amalgamation method by using mercury (Hg) in gold extraction. After mining, other materials are generated as tailings, mine waste containing Hg. Tailing contains very low organic matter and plant macronutrients. Organic carbon content in tailing only increased when vegetation covered the tailing (Shen et al., 2014). Tailings contain low plant macronutrients especially nitrogen, but have micronutrients (Pepper et al., 2012; Swaroop et al., 2013). The texture is usually dominated by coarse grains which cause low tailing capability to hold water (Kujawa, 2011). Another problem of tailing revegetation is its low acidity (Pepper et al., 2012). The management of tailings in Botak Mountain mining area was inappropriate. Tailings are overburden on agricultural land caused deterioration of soil quality; and become recent soil, Entisols, which have exhibited no soil development. Mercury level in such agricultural area increased up to 30.66 mg/kg (Hindersah et al., 2018). The average Hg level in uncontaminated soil was 0.01-0.3 mg/kg (Steinnes, 2004).

To achieve fast tailing deposit revegetation, fast-growing and deep-rooted plants such as Legume might be grown. All legumes are well known pioneer plants which form nodules in which rhizobia fix dinitrogen (N_2) to ammonium (Sulieman and Tran, 2014). Inoculation of Plant Growth Promoting Rhizobacteria (PGPR) such as non-symbiotic N_2 fixer *Azotobacter* is suggested to increase the availability of essential macro nutrients, nitrogen.

Azotobacter are heterotrophic rhizobacteria enable to fix N2 between 2-15 mg N/g carbon source (Tilak et al., 2005). They produce growth regulators auxin, cytokinins and gibberelins (Hindersah and Simarmata, 2004; Patil, 2011; Vikhe, 2014) that stimulate cell extension and division which promote plant growth (Takatsuka and Umeda, 2014). Exopolysacchride production by *Azotobacter* has been proved (Vermani et al., 1997; Janecka et al., 2002; Hindersah et al., 2006). This extracellular structure plays an important role in improving soil porosity and transport of heavy metal pollutants in the soil (Janecka et al., 2002).

Azotobacter resistance to 20 mg/L of $HgCl_2$ has been isolated from tailings in Buru Island although lower cell viability was evident (Hindersah et al., 2017). In the presence of Hg, low cell proliferation was coupled with lipid peroxidation and protein product in the presence of Hg (Onwurah et al., 1999). Organic matter maintains viability of heterotrophic *Azotobacter*, their activity and is also important for improving tailing physical properties and root zone system. So that it is important to add organic matter prior to tailing revegetation.

Legume inoculation by using *Azotobacter* to increase plant growth has been widely studied. *Bardyrhizobium-Azotobacter* dual inoculation showed positive effect on legume nodulation and crop yields (Tilak et al., 2006; Koziel et al., 201). However, inoculation of legume with *Azotobacter* in order to induce mercury-contaminated tailing revegetation is rarely studied. Therefore, the objective of this study was to verify ability of indigenous and non-indigenous *Azotobacter* and its consortium on the availability of soil N and the growth of peanuts, at several level of total organic carbon (TOC) content in tailing.

Materials and Methods

Green house experiment and soil as well as plant analysis has been done from June to August 2015 in Faculty of Agriculture, Universitas Padjadjaran, West Java, Indonesia that is located in tropical region at 732 m above sea level.

Microorganisms

All bacteria belong to Soil Biology Laboratory, Faculty of Agriculture, Universitas Padjadjaran. *Azotobacter* sp. was isolated from wild kale rhizosphere grown in tailings of Waekerta Village, Waeapo Subdistrict, Buru Regency, Maluku. Either *Azotobacter chroococcum* or *Bradyrhizobium* sp. were isolated from the food crops rhizosphere.

Growth medium for *Azotobacter* pure culture was Nitrogen free Ashby's medium (mannitol 10 g; K_2HPO_4 0.2 g; $MgSO_4.7H_2O$ 0.2 g; $CaSO_4.2H_2O$ 0.1 g; NaCl 0.2 g; $CaCO_3$ 5 g) while that for *Bradyrhizobium* was yeast mannitol agar (Yeast extract 1 g; Mannitol 10 g; K_2HPO_4 0.5 g; $MgSO_4$ 0.1 g, NaCl 0.1 g, Congo red 0.025 g). Molasses based liquid medium (molasses 1% and NH_4Cl 0.1%) was used to produce *Azotobacter's* liquid inoculant and keep up the viability of *Azotobacter* for 4 months up to 10^8cfu/mL.

Tailing properties

Tailings taken from the Anhoni river basin on Botak Mountain in Buru Regency was very acid, pH 2.7 and contained0.64 mg/kg Hg. Very low fertility of tailings was characterized by 0.1% total organic carbon (TOC), 0.04% N-total, C to N ratio of 2.27; 0.55%total P_2O_5, 11.03%total K_2O, low cation exchange capacity, and very low base saturation. *Azotobacter* population in tailing was only $1.23x10^2$ cfu/g.

Experimental set up

The experimental design of pot trial was a Split Plot Design with three replicates. The main plot was manure dose treatments which adjusted total organic carbon (TOC) to 1.5%, 3% and 4.5%, while the subplot was *Azotobacter* inoculation treatments consisting of Azotobacter sp., *A. chroococcum*, and mixed of both *Azotobacter* species.

Acidity of tailings was adjusted to 6.4 by using agricultural limestone. The weight of growth media in each polybag was 1.5 kg. Tailing was mixed with cow manure and incubated for 3 days before *Azotobacter* application. As much as 100 mL of *Azotobacter* liquid inoculant with concentration of $3.6x10^7$cfu/mL was sprayed into the tailings surface and mixed thoroughly before incubated for 3 days.

Groundnut seeds were soaked in the liquid inoculant of *Bradyrhizobium s*p. of $2.3x10^7$ cfu/mL for 30 minutes before sowing. Two seeds were sowed in the adjacent 2 cm-deep hole in tailing; after seedlings emergence, only seeding was cut off. Plants were kept in greenhouses for 35 days. Fertilization was taken place at one week after sowing which was equal to 50 kg/ha Urea, 100 kg/ha SP-36 and 100 kg/ha KCl.

Plant height was measured once a week from week two to five. At week five, the population of *Azotobacter* in soil was counted by using Dilution Plate Method in free-N Ashby's agar. Effective nodule number is supposed being counted. Soil acidity, soil total N, and N content in plant were analyzed. Soil and plant N were determined by using Kjeldahl method.

Statistical analysis

All data were subjected to analysis of variance (5% F Test). If the results of F test showed a significant effect of the treatments on the parameters, then 5% Duncan's multiple range Test was performed.

Results and Discussion

Plant growth in tailings is retarded since the low nutrient availability. The most influential nutrient elements in plant growth were N and P, while tailings contained only low total nitrogen of 0.04% and phosphorus of 11.004 mg/kg. Coarse texture might induce rapid water loss in root zone although manure was added. Fortunately, no diseases attack was found during the experiment.

Azotobacter population

The effect of manure doses on *Azotobacter* population did not depend on *Azotobacter* inoculation. *Azotobacter* inoculation increased *Azotobacter* population in tailing clearly. Surprisingly, *Azotobacter* count did not alter by increased organic-C in tailing (Table 1).

Table 1. Effect of *Azotobacter* and soil total organic carbon on *Azotobacter* population in tailing at 5 weeks after groundnut sowing.

Treatments	***Azotobacter* Population (10^4cfu/g)***	**Soil acidity***
Azotobacter		
Without*Azotobacter*	4.62 a	7.17 a
Azotobacter sp.	6.09 b	7.13 a
A. chroococcum	5.87 b	7.09 a
*Azotobacter*concortium	7.53 c	7.09 a
Soil total organic carbon		
1.5%	5.33 a	7.12 a
3.0%	6.47 a	7.09 a
4.5%	6.28 a	7.14 a

*Numbers followed by the same letters were not significant based on 5% Duncan's multiple range Test.

Before experiment, the mine tailing was very acid, pH 2.7; the increase of pH through liming induced *Azotobacter* proliferation. The initial population of *Azotobacter* after inoculation was $1.23x10^3$ cfu/g. This bacterium proliferates optimally at neutral pH and is slightly sensitive to alkaline but not resistance to very acidic soils (Carpa et al., 2010; Jimenez et al*.,* 2011).

Azotobacter consortium was more supported *Azotobacter* growth in mine tailing; when the two species of *Azotobacter* were inoculated separately, *Azotobacter* population slightly decreased (Table 1). Synergistic association between *Azotobacter* sp. and *A. chroococcum* to support indigenous or exogenous *Azotobacter* proliferation might have occurred. The microbial consortium will become more effective because of the complementary metabolic activity of one another (Jadhav et al., 2008). Bacterial consortium is widely used as a biological fertilizer to increase the availability of nutrients in the soil.

Any dose of organic matter has no significant effect on *Azotobacter* population. Since mine tailing contains low N, organic amendment of up to 1.5%, 3% and 4.5% increased C/N up to 34.05; 68.1 and 102.15 respectively. The soil C to N ratio determines whether microbes have limited C or N for growth (Bengtson, 2004). Microbial activity needs C/N ranging from 25 to 35 (Akratos et al., 2017). In the environment with wider C/N ratio, bacteria need longer time to breakdown organic matter. When soil has higher C content, microbe competes with the plant roots for available soil N (Bengtson, 2004). Longer period of revegetation in mine tiling is needed to increase root zone system and alter bacterial population at root zone.

Soil nitrogen and their uptake

Analysis of variance showed that there was no interaction effect between *Azotobacter* species and dose of manure and on total nitrogen in soil as well as nitrogen uptake at 5 week after sowing. Total N and N uptake were similar irrespective of *Azotobacter* treatment but effect of manure application on total nitrogen was significant although higher TOC decreased total N (Table 2).

Total nitrogen is the total of all nitrogen in soil which are available N (NH_4^+ and NO_3^-) for plant uptake, other inorganic nitrogen as well as organic form including soil organisms. Tailing TOC was 0.1%, increased TOC through manure amendment improved root zone system and hence biological processes that play a key role in nitrogen availability.

Before experiment, total nitrogen was very low 0.04%, increased in nitrogen at the end of experiment might be caused by manure which contained 1.8% nitrogen and increased soil microbes. Organic matter contains significant amount of microbes that enable to proliferate in the presence of organic matter. In rhizosphere, plant exudates, mucilage and root slough off serve as nutrient source for nitrogen cycling microbes

(Hinsinger et al., 2006). Although total N increased but N in tailing received any treatment remained low.

Table 2. Effect of *Azotobacter* and soil total organic carbon on total nitrogen content in soil and nitrogen uptake at 5 weeks after sowing.

Treatments	Total Nitrogen (%)*	Nitrogen uptake (g/plant)*
Azotobacter		
Without *Azotobacter*	0.067 a	1.68 a
Azotobacter sp.	0.051 a	1.42 a
Azotobacter chroococcum	0.057 a	1.71 a
Azotobacter consortium	0.053 a	1.86 a
Soil total organic carbon		
1.5%	0.062 b	1.81 a
3.0%	0.057 b	1.70 a
4.5%	0.051 a	1.48 a

*Numbers followed by the same letters were not significant based on 5% Duncan's multiple range Test.

Nitrogenase activity is determined by the availability of Molybdenum (Mo) an iron (Fe) as cofactor (Seefeldt et al., 2009). Mine tailings contained Mo of less than 0.1 mg/kg and Fe of 80.02 mg/kg. Molybdenum (Mo) deficiency affects nitrogenase functional activity since Mo has high affinity for binding substrate (Bell et al., 2003). Increased manure doses as well as *Azotobacter* inoculation did not increase nitrogen uptake by individual groundnut plant (Table 2). All plant received any treatment had similar plant height (Figure 1) and all plants had only 5-6 trifoliate (5.1 in average). Statistically, total nitrogen in mine tailing with 1.5% and 3.0% C-organic was significantly different with that in mine tailing containing4.5% but nominally the increased was very slight (Table 2) so that nitrogen uptake of all plants were not different.

Plant growth

Based on analysis of variance, interaction between manure doses and *Azotobacter* inoculation on plant height was not significant. Statistically, both main treatments had no significant effect on plant height. Vegetative growth characteristics of groundnut received any manure or *Azotobacter* treatments were similar (Figure 1). Although the plant height of groundnut grown in medium contained 3.0% C organic was higher, but the difference was statistically not significant. Another growth parameter observed in our experiment was the number of trifoliate leaves. Based on analysis of variance, there was no interaction effect between manure doses and *Azotobacter* inoculation on peanut leaf number. At 5 weeks after planting the trifoliate leaves was 5.0-5.4 (Table 3).

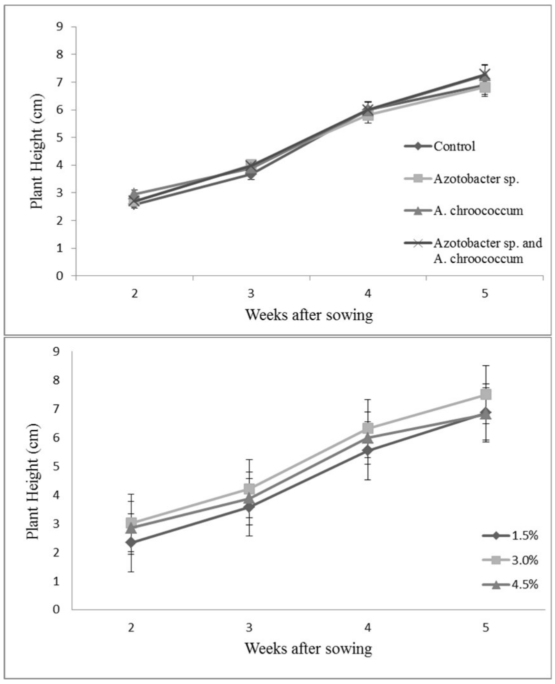

Figure 1.Effect of *Azotobacter* inoculation (above) and soil total organic carbon (below) on plant height at 2 weeks to 5 weeks after sowing.

Plant growth in mine tailings is worst due to the low available nutrient and too loose tailing structure. Tailings contain N and P only 0.04% and 11.04 mg/kg respectively. Nitrogen is a major constituent of protein, nucleic acid and chlorophyll while P is one of the elements that influences root development, strengthening stems, the formation of flowers and seeds (Taiz and Zeiger, 2003). Manure amendment and *Azotobacter* inoculation did not increase nutrient supply to groundnut. *Azotobacter* proliferation in mine tailing occurred (Table 1) but their activity in nitrogen fixation might be inhibited by chemical and physical characteristics of mine tailing.

Groundnut grown in mine tailing formed no nodules Nodule formation is related to soil nutrition, temperature, soil acidity and temperature. Nutrients needed for the formation of the nodules are P, K, Ca, Fe and Mo and less than 5 mM N (Weisany et al., 2013; Downie, 2014). Mine tailings were very poor in those nutrients and nodulation was inhibited although all groundnut plant was fertilized by 37.5 mg Urea, 75 mg TSP and 75 mg KCl. Plant establishment in tailing is not only the function of nutrient. Development of root zone system needs long period and comprehensive reconstruction which consider physical structure and hydraulic function that are needed to sustain plant growth (Huang et al., 2012) and finally tailing revegetation.

Table 3. Effect of *Azotobacter* and soil total organic carbon on number of groundnut trifoliates at 2-5 weeks after sowing.

Treatments	Weeks after sowing*			
	2	3	4	5
Azotobacter				
Without *Azotobacter*	2.2	3.2	4.4	5.2
Azotobacter sp.	2.0	3.0	4.1	5.1
Azotobacter chroococcum	2.1	3.3	4.3	5.3
Azotobacter consortium	2.4	3.5	4.4	5.0
Soil total organic carbon				
1.5%	2.0	3.0	4.1	5.1
3.0%	2.2	3.6	4.6	5.4
4.5%	2.3	3.2	4.3	5.0

*All numbers in the same column were not significantly different based on 5%Duncan's multiple range Test.

Conclusion

There was no interaction effect between *Azotobacter* inoculation and TOC on *Azotobacter* population, soil acidity, total nitrogen in tailing and nitrogen uptake by groundnut. Inoculating *Azotobacter* sp., *A. chroococcum* and its consortium enhanced *Azotobacter* population in tailing up to 7.53 x 10^4cfu/g but *Azotobacter* population in tailing did not change by increasing soil TOC from 1.5% to 3%.

Total nitrogen decreased after increasing TOC of tailing to 4.5% but compared to 1.5% and 3% C-organic but the decreased was very slight so that nitrogen uptake of all plants were not different. Either *Azotobacter* or TOC content did not influence plant growth.

At five weeks after sowing all plants showed retarded growth due to physical and chemical mine tailing characteristics although inorganic nitrogen, phosphorous and potassium fertilizer has been added to growth medium. Regardless the treatment, plant height and trifoliate number of groundnut at 5 weeks after sowing were only 6.82-7.28 cm and 5.0-5.4 respectively. Improving tailing physical and chemical characteristics is suggested in the next pot experiment in order to increase pioneer plant growth.

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