



# The Use of Mercury-Resistant Bacteria to Enhance Phytoremediation of Soil Contaminated with Small-scale Gold Mine Tailing

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## ABSTRACT

In the phytoremediation process, there is an interaction between plants and microorganisms in the soil. The objective of this study was to explore the effect of mercury-resistant bacteria on phytoremediation of soil contaminated with small-scale gold mine tailings that contain mercury. Two isolates of mercury-resistant bacteria (*Brevundimonas vesicularis* and *Nitrococcus mobilis*) were applied to *Paspalum conjugatum* as a mercury accumulator plant that was grown for 70 days on gold mine tailing-contaminated soil. Ammonium thiosulfate was used as a chelating agent to stimulate mercury extraction by the plant. After *P. conjugatum* was harvested (60 days), the remaining soil in the pot was planted with maize until the maximum vegetative period. The results showed that the application of mercury-resistant bacteria and ammonium thiosulfate increased 157-162% of *P. conjugatum* biomass compared to that without the application of mercury-resistant bacteria. The application of mercury-resistant bacteria with ammonium thiosulfate in soil phytoremediation with *P. conjugatum* reduced 18% and 20% mercury content in the soil contaminated with small-scale gold mine tailings containing mercury. The decrease in mercury content in the soil due to the application of *B. vesicularis* and *N. mobilis* in soil phytoremediation with *P. conjugatum* increased biomass production of a maize plant by 131% and 145%, respectively.

## INTRODUCTION

The declining production of crops due to contamination of tailings from traditional gold processing to agricultural land is one of the negative effects of small-scale gold mining activities in Lombok, Indonesia. The tailings contain mercury and several other heavy metals that are toxic to plant growth. The results of a survey conducted by Krisnayanti et al. (2012a) at small-scale gold mining sites in West Lombok showed that tailings of the gold amalgamation process contained 3002 mg Hg/kg which caused high Hg content (25-40 mg/kg) in soil contaminated with the tailings (Krisnayanti et al. 2012b). Mercury ion ( $Hg^{2+}$ ) in the soil can be taken up by plants causing toxicity (Su et al. 2009, Tangahu et al. 2011). The presence of mercury in planting media inhibits the growth and biochemical composition of maize seeds (Muddarisna et al. 2013a). Hyperaccumulator plants can be used to extract or eliminate metals polluting the soil (Tangahu et al. 2011). Muddarisna et al. (2013b) reported that *Paspalum conjugatum*, *Cyperus kylingia*, and *Lindernia crustacea* are three local plant species having the potential to be used as mercury accumulator plants on agricultural soils contaminated with small-scale gold mine tailings containing

mercury in West Lombok, Indonesia. Phytoremediation of heavy metals can be driven through the addition of chelating agents (Wang et al. 2013). Ammonium thiosulfate has been used as a chelating agent to stimulate the accumulation of Hg in a plant grown in gold mine tailings contaminated with Hg (Moreno et al. 2004). Handayanto et al. (2016) reported that the high mercury accumulation (30.09 mg/kg) was found in the *P. conjugatum* shoot with the addition of ammonium thiosulfate. The addition of ammonium thiosulfate increased the accumulation of Hg in the shoot by 71% compared to the treatment without the addition of ammonium thiosulfate.

Although phytoremediation of heavy metals can be stimulated through the addition of chelating agents, phytoremediation is not only carried out by plants because there is always an interaction between plants and microorganisms in the soil which causes increased activity associated with remediation (Compant et al. 2010). Some bacteria can reduce enzymatically  $Hg^{2+}$  to Hg with mercuric reductase, MerA. Another enzyme, organomercurial lyase (MerB), which is present in several bacteria, catalyses the release of Hg-carbon bonds from several forms of organic mercury (Wang 2004). Therefore, efforts to find an application of heavy metal ac-

cumulator plants combined with soil microbial communities that are resistant to mercury are thought to further increase the efficiency of phytoremediation of heavy metal contaminated soils. Some bacteria can survive in an environment contaminated with mercury even though the mechanism of resistance to mercury is not known (Nascimento & Char-tone-Souza 2003). Chasanah et al. (2018) who isolated and identified mercury-resistant bacteria from small-scale gold mine tailings in Central Lombok obtained four isolates of mercury-resistant bacteria that were indicated as *Brevundimonas vesicularis*, *Nitrococcus mobilis*, *Fusobacterium aquatile* and *Fusobacterium necrogenes*. Tests for mercury accumulation in the laboratory showed that these four species were able to accumulate more than 70% of mercury from small scale gold mine tailings.

The purpose of this study was, therefore, to study the effect of the combined application of mercury-resistant bacteria and mercury accumulator plant in the phytoremediation of soil contaminated by gold mine tailings containing mercury.

## MATERIALS AND METHODS

### Site Description

A pot experimental study was conducted in a 4 m × 5 m screen house situated at the Bonjeruk Village, Jonggat District of Central Lombok Regency, Indonesia (8°24' - 8°57' S and 116°05' - 116°24' E). The study was carried out from November 2018 to March 2019. The location of the screen house was at the farmers' land that had been buried/contaminated with gold amalgamation tailings containing mercury since 2011. Soil contaminated with the tailings used for this study had the following characteristics: pH = 6.4, C = 0.95%, N = 0.10%, P = 0.98 mg/kg, S = 8.92 mg/kg, cation exchange capacity = 14.25 cmol/kg, K = 3.25 cmol/kg, Ca = 3.04 cmol/kg, Mg = 1.26 cmol/kg, Na = 0.89 cmol/kg, and Hg = 41.37 mg/kg. The total value of Hg exceeded the threshold of the Indonesian Government Regulation for Hg in solid waste of 0.03 mg/kg. The research materials used were two isolates of mercury-resistant bacteria (*Brevundimonas vesicularis* and *Nitrococcus mobilis*) (Chasanah et al. 2018), *Paspalum conjugatum* P.J. Bergius, ammonium thiosulfate ( $[\text{NH}_4]_2\text{S}_2\text{O}_3$ ) as a chelating agent, and maize seeds.

### Experiment 1: Mercury Extraction by *P. conjugatum*

The research treatments consisted of a combination of applications of mercury-resistant bacterial isolates (*B. vesicularis* and *N. mobilis*), ammonium thiosulfate with doses of 0 and 2 g/kg that were applied together with the use of *P. conjugatum* as a mercury accumulator plant. The treatment combinations were (1) B1K0 (*B. vesicularis* isolate without

ammonium thiosulfate), (2) B2K0 (*N. mobilis* isolate without ammonium thiosulfate), (3) B1K1 (*B. vesicularis* isolate with ammonium thiosulfate), (4) B2K1 (*N. mobilis* isolate with ammonium thiosulfate), (5) B0K0 (without isolates of mercury-resistant bacteria, and ammonium thiosulfate), and (6) B0K1 (without isolates of mercury-resistant bacteria, and with ammonium thiosulfate).

Each bacterial isolate of 12.5 mL ( $10^3$  cfu/mL) (Chasanah et al. 2018) was applied to each pot containing small-scale gold mine tailing-contaminated soil. The six treatments were arranged in a randomized block design with four replications. Each pot received NPK fertilizer of 100kg/ha and organic matter of 10t/ha (Handayanto et al. 2016). The NPK fertilizer is produced by PT. Petrokimia Gresik, Indonesia, as Phonska, which contains 15% N, 15%  $\text{P}_2\text{O}_5$ , 15%  $\text{K}_2\text{O}$  and 10% S. The organic matter used for this study was local cow manure containing 1.46% N, 0.98%  $\text{P}_2\text{O}_5$  and 0.65%  $\text{K}_2\text{O}$ . Before being planted, the 2-week-old *P. conjugatum* seedling was acclimatized for one week in a polybag containing soil contaminated with small-scale gold mine tailings.

For all the treatments, each plant seedling was grown in a 30 cm diameter plastic pot filled with fifteen kilograms of soil contaminated with small-scale gold mine tailings (air-dried and sieved to pass through a 2 mm sieve) and placed in the screen house. During the experiment, well water was supplied daily to each pot in order to keep the moisture content of the soil at the approximate water holding capacity so as not to inhibit the growth of *P. conjugatum*. The ammonium thiosulfate (2 g/kg) was supplied at 8 weeks after planting (Wang et al. 2013). At harvest (70 days after planting), *P. conjugatum* shoots and roots were separated manually from the soil by sieving and rinsing with water. The shoots and roots were then oven-dried at 60°C for 48 hours, weighed and ground to pass through a 1 mm sieve for analysis of mercury content. Measurement of Hg content in plant samples was carried out following the method of Moreno et al. (2005). A total of 0.2 g of ground plant sample was put into a 50mL borosilicate beaker, added with 10 mL of nitric acid, and then left for 10-15 hours. The next day the mixture of plant sample and nitric acid was heated for 2 hours at 120°C and then added with 20 mL of distilled water. Standard determination of 0.5, 1, 2, 4, 7.5 and 10 ppb Hg referred to the standard of tomato leaves containing 0.034 ppm Hg. Mercury concentration was measured by a Cold Vapour Atomic Absorption Spectrometer F732-S (Shanghai Huaguang Instrument Company). The ability of *P. conjugatum* for mercury translocation upwards from root to shoot was evaluated by determining the translocation factor (TF) (Yoon et al. 2006). TF = concentration of mercury in shoots/ concentration of mercury in roots.

The data obtained were analysed by performing the

one-way analysis of variance, and further statistical test for significant differences among treatment means was conducted by employing the least significant difference test at the 95% level of confidence with the aid of Microsoft Excel Office 2016 software.

### Experiment 2: Growth and Yield of Maize Biomass

After harvesting *P. conjugatum*, the remaining soil in the pot was used for the growing a local variety of maize. Six treatments similar to experiment 1 and one control (soil contaminated with small-scale gold mine tailings without phytoremediation treatment), were arranged in a randomized block design with four replicates. Each pot was supplied with the Phonska NPK fertilizer with a dose equivalent to 100 kg/ha. Three pre-germinated maize seeds were planted in each pot and thinned to one after one week. During the experiment, water was supplied daily to each pot to maintain soil moisture in the condition of the water holding capacity so as not to inhibit the growth of maize. Maize plant was harvested at the maximum vegetative period (60 days). Maize shoots and roots were separated, washed, weighed, and dried in an oven at 40°C for 48 hours for mercury analysis. Mercury concentrations in maize shoots and roots and the planting media were analysed using the same method as in Experiment 1. The data obtained were also subjected to statistical analysis as Experiment 1.

## RESULTS AND DISCUSSION

### *P. conjugatum* Biomass

The application of mercury-resistant bacteria combined with ammonium thiosulfate significantly ( $p < 0.05$ ) improved the growth of *P. conjugatum* as a mercury accumulator plant (Fig. 1). Compared with the B0K0 treatment (without application of mercury-resistant bacteria and ammonium thiosulfate), the highest increase (162%) in total biomass (shoot + root) of *P. conjugatum* was obtained in the B1K1 treatment (application of *B. vesicularis* with ammonium thiosulfate). However, the increase in total biomass of *P. conjugatum* in the B1K1 treatment was not significantly different from that of the B2K1 treatment (application of *N. mobilis* with ammonium thiosulfate) of 157%. The lowest increase in *P. conjugatum* biomass (50%) was obtained in the B2K0 treatment (application of *N. mobilis* without ammonium thiosulfate).

Data presented in Fig. 1 show that the addition of ammonium thiosulfate increased plant biomass even without application of mercury-resistant bacteria. Muddarisna et al. (2013a) reported that addition of ammonium thiosulfate had a significant effect on the dry weight of the shoots of *P. conjugatum*, *Cyperus kyllingia* and *Lindernia crustacea* used for phytoremediation of mercury-polluted soil. The increase in plant biomass due to the addition of ammonium thiosulfate was probably because of the addition of plant

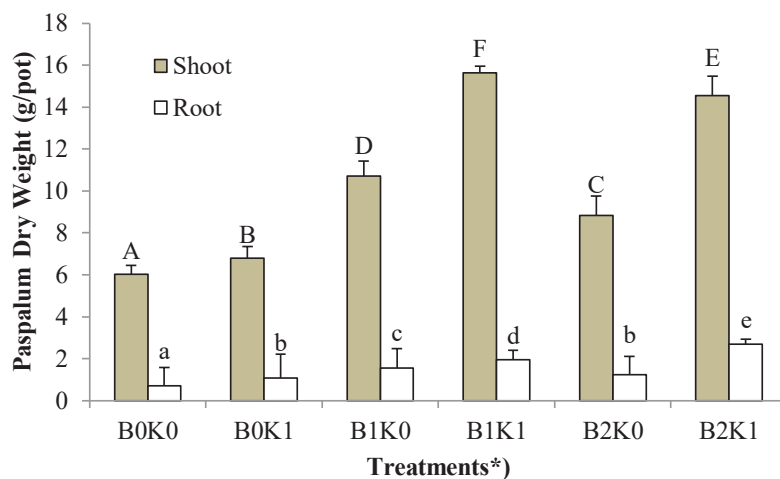


Fig. 1: Effect of application of mercury-resistant bacteria and ammonium thiosulfate on the biomass of *P. conjugatum* at 70 days. Vertical bars represent standard deviation while the same capital and small letters are not significantly different for each treatment mean at 0.05 level of probability. \*) B0 = no bacterial isolate, B1 = *B. vesicularis* isolate, B2 = *N. mobilis* isolate, K0 = without ammonium thiosulfate, K1 = with ammonium thiosulfate 2 g/kg.

nutrients (nitrogen and sulphur) supplied through ammonium thiosulfate at the 8-week old plant that was able to enhance plant growth (Handayanto et al. 2016). Chelating agents, such as ammonium thiosulfate, have been used as soil extractants, a source of micronutrient fertilizers and to maintain solubility of micronutrient in hydroponic solutions (Salt et al. 1995). If the addition of ammonium thiosulfate was combined with the application of mercury-resistant bacteria, it further enhanced the improvement of accumulator plant growth, which in turn improved the absorption of mercury by the mercury accumulator plant.

Liagat & Eltem (2016) reported that *B. vesicularis* is an endophytic bacterium that can produce indole acetic acid (IAA) phytohormone to stimulate plant growth (Ying et al. 2015), and produce more nutrient uptake from the soil (Li et al. 2008). Endophytic bacteria can supply nutrients for plant growth by dissolving phosphate and fixing nitrogen (Marra et al. 2012). In addition, endophytic bacteria can also protect plants against pathogenic microorganisms (Khalifa et al. 2015) and degradation of toxic compounds (Sheng et al. 2008). *N. mobilis* is known as an ammonia-oxidizing bacterium which can oxidize nitrite to nitrate (Yu et al. 2012, Koide et al. 2014). This bacterium can derive energy from the oxidation of ammonia and nitrite in the degradation of organic wastes and recycling of nutrients in subterranean habitat (Koilaraj et al. 2012). *N. mobilis* is also an important and dominant ammonia-oxidizing bacterium in various

wastewater treatment systems (Thandar et al. 2016).

### Accumulation of Mercury by *P. conjugatum*

Referring to the *P. conjugatum* biomass and mercury concentration in the *P. conjugatum* plant, mercury accumulation in *P. conjugatum* plant can be calculated. The highest mercury accumulation (8.29 mg/kg dry weight) was found in the B1K1 treatment (*B. vesicularis* with ammonium thiosulfate), and the lowest was found in the B0K0 treatment (without bacterial isolates and ammonium thiosulfate) (Fig. 2). The application of mercury-resistant bacteria with ammonium thiosulfate to *P. conjugatum* reduced the mercury content in the soil contaminated by small-scale gold mine tailings which was 41.73 mg/kg, reduced by 3% (B0K0 treatment) up to 20% (B1K1 treatment). Microbes have developed a mechanism for detoxifying mercury based on intracellular reduction of  $Hg^{2+}$  to  $Hg^0$  which is non-toxic by mercury reductase enzymes and the loss of diffusional  $Hg^0$  from cells (Wagner-Dobler 2003).

From the accumulation of mercury in the *P. conjugatum* plant, more than 80% of Hg accumulation was found in the shoot of the plant. In line with the dry weight of plant biomass, the use of ammonium thiosulfate stimulated Hg uptake by plants. This is thought to occur because mercury has a strong affinity with thiol groups, especially sulphide and bisulphide complexes (Moreno et al. 2004, Selin 2009). Moreno et al. (2005) reported that *Bracia juncea* could ac-

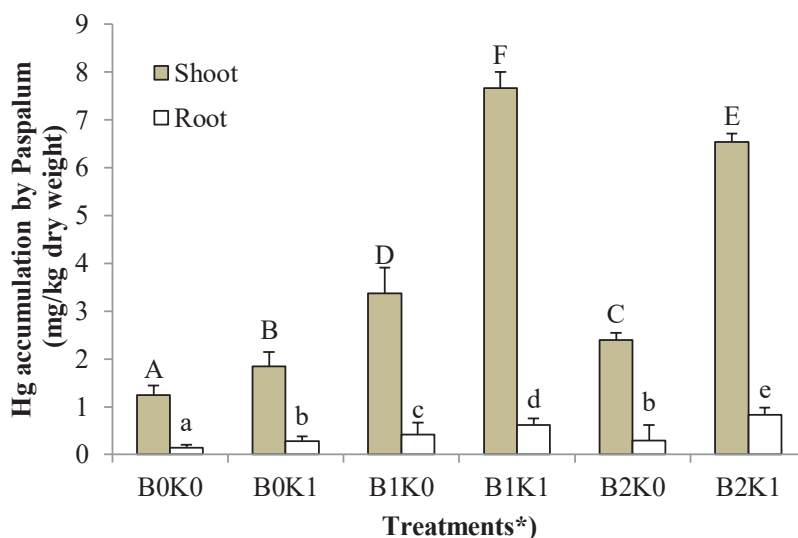


Fig. 2: Effect of application of mercury-resistant bacteria and ammonium thiosulfate on the accumulation of mercury by *P. conjugatum* at 70 days. Vertical bars represent standard deviation while the same capital and small letters are not significantly different for each treatment mean at 0.05 level of probability. \*) B0 = no bacterial isolate, B1 = *B. vesicularis* isolate, B2 = *N. mobilis* isolate, K0 = without ammonium thiosulfate, K1 = with ammonium thiosulfate 2 g/kg.

accumulate Hg to 40 mg/kg in plant tissue after the application of ammonium thiosulfate to mine tailings contaminated with 2.8 mg Hg/kg. However, the use of ammonium thiosulfate can also create the risk of underground water pollution by Hg which is mobilized by the thiosulfate.

The increase in mercury accumulation by *P. conjugatum* in the B1PK1 treatment (*B. vesicularis* and ammonium thiosulfate) was probably due to the symbiotic relationship between microorganisms and plants that increased heavy metal extraction from the soil (Tangahu et al. 2011). In this study, the symbiosis of mercury-resistant bacteria and mercury accumulator plant increased mercury accumulation up to 459% (B1PK1 treatment) compared to treatments without the application of mercury-resistant bacteria. High mercury accumulation in the *P. conjugatum* plant caused mercury content in the soil to decrease up to 20% (B1PK1 treatment) from the original mercury content in the soil of 41.37 mg/kg. This technology can be used to extract mercury from agricultural soil before it is used to grow food crops. However, this technology still raises the problem of processing the biomass of extracting plants containing mercury so as not to re-pollute the environment.

### Translocation Factor of Mercury

As the effect on Hg accumulation of shoot and root, the application of mercury-resistant bacteria and ammonium thiosulfate also had a significant effect on the *P. conjugatum*

translocation factor (TF) values for mercury which were all greater than 1 (Fig. 3). The highest translocation factor value (1.54) was found in the B1K1 treatment (*B. vesicularis* and ammonium thiosulfate) which was not significantly different from the TF value in the B2K1 treatment (*N. mobilis* and ammonium thiosulfate) of 1.47 (Fig. 3). The lowest TF value (1.02) in the B0K0 treatment (without mercury-resistant bacteria and ammonium thiosulfate). Differences in TF values in the treatments showed differences in the effectiveness of each treatment in the transport of mercury from the root system to the shoot as a place of accumulation (Selin 2009). This difference is also thought to be related to the modification of plant growth under conditions of heavy metal stress as a result of the absence of certain amino acids in plants (Ashraf et al. 2011).

### Growth and Biomass Yield of Maize

At harvest time of the maximum vegetative age (60 days), the height of maize plants varied from 45.7 cm (control) to 105.8 cm (B1K1 treatment) (Fig. 4). Compared with the control, phytoremediation of soil with *P. conjugatum* and application of mercury-resistant bacteria and ammonium thiosulfate increased plant height by 96% (B1K0), 108% (B2K0), 145% (B1K1), 131% (B2K1), 110% (B0K1), and 69% (B0K0). Application of mercury-resistant bacteria in the soil phytoremediation process further enhanced the growth of maize plants. The combination of treatment in the soil

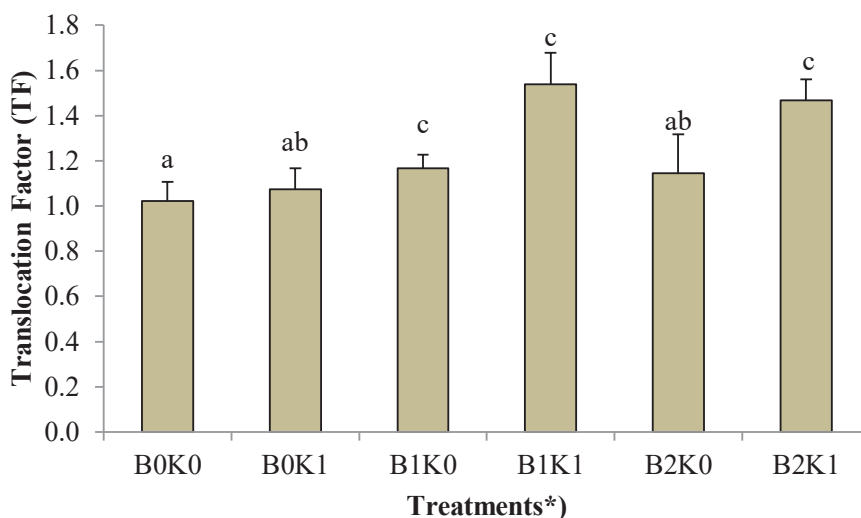


Fig. 3: Effect of application of mercury-resistant bacteria and ammonium thiosulfate on the translocation factor of mercury by *P. conjugatum* at 70 days. Vertical bars represent standard deviation while the same letters are not significantly different for each treatment mean at 0.05 level of probability.

\*) B0 = no bacterial isolate, B1 = *B. vesicularis* isolate, B2 = *N. mobilis* isolate, K0 = without ammonium thiosulfate, K1 = with ammonium thiosulfate 2 g/kg.

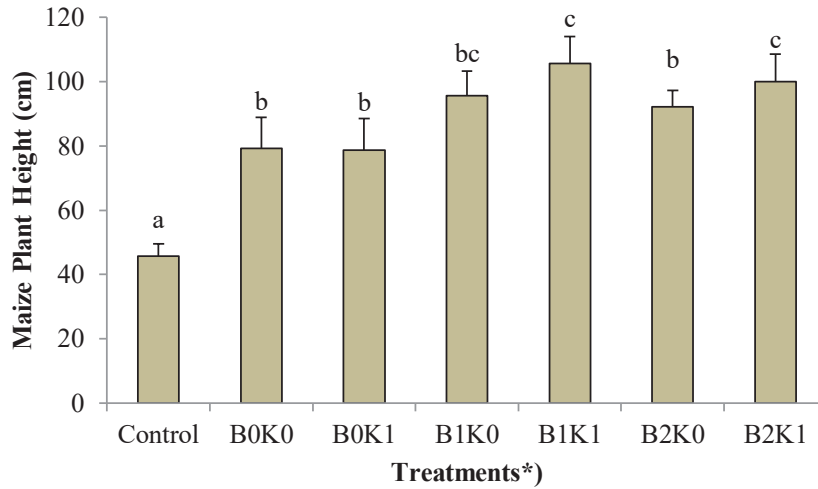


Fig. 4: Height of maize grown on post-phytoremediation soil at 60 days. Vertical bars represent standard deviation while the same letters are not significantly different for each treatment mean at 0.05 level of probability. \*) B0 = no bacterial isolate, B1 = *B. vesicularis* isolate, B2 = *N. mobilis* isolate, K0 = without ammonium thiosulfate, K1 = with ammonium thiosulfate 2 g/kg, Control = soil not subjected to phytoremediation.

phytoremediation process which then resulted in the best growth of maize plant was the B1K1 treatment. This is in line with the results of the highest Hg reduction in soil by the B1K1 treatment (Fig. 2).

The dry weight of the shoots and roots of maize plants

increased on the soils which had been remediated with a combination of *P. conjugatum* and mercury-resistant bacteria. Consistent with the capability of the B1K1 treatment (*B. vesicularis* with ammonium thiosulfate) in accumulating the highest Hg, the highest increase in the dry weight of

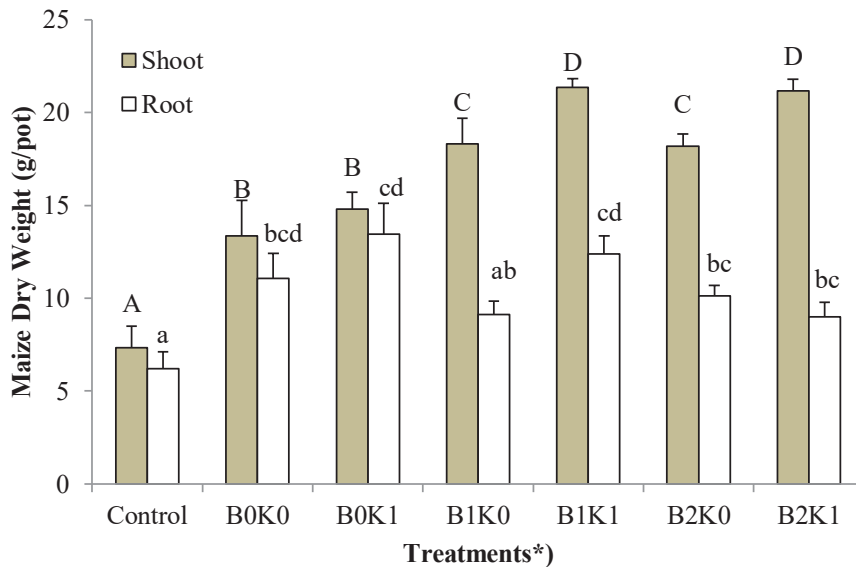


Fig. 5: Shoot and root dry weight of maize grown on post-phytoremediation soil at 60 days. Vertical bars represent standard deviation while the same capital and small letters are not significantly different for each treatment mean at 0.05 level of probability. \*) B0 = no bacterial isolate, B1 = *B. vesicularis* isolate, B2 = *N. mobilis* isolate, K0 = without ammonium thiosulfate, K1 = with ammonium thiosulfate 2 g/kg, Control = soil not subjected to phytoremediation.

shoots and roots were also found in the B1K1 treatment although it was not significantly different from the B2K1 treatment (Fig. 5). Compared to the control, the increase in maize biomass (shoot and root) grown on soil previously remediated through the B1K1 and B2K1 treatments were 145% and 131%, respectively, while those grown on soil previously remediated through the B1K0 and B2K0 treatments were 96% and 108% respectively. Biomass of maize plants grown on the soil remediated with *P. conjugatum* without mercury-resistant bacteria and ammonium thiosulfate were low and not significantly different from the control. The low increase of maize plant growth and yield of maize biomass on post-phytoremediation without the addition of mercury-resistant bacteria and ammonium thiosulfate compared to those grown on post-phytoremediation with the addition of mercury-resistant bacteria and ammonium thiosulfate was associated with mercury uptake by plants (Fig. 6).

The remaining mercury in the growing media without the addition of mercury-resistant bacteria and ammonium thiosulfate was higher than in the growing media with the addition of ammonium thiosulfate, thus inhibiting plant growth. In plants, mercury causes damage to enzymes, polynucleotides, nutrient transport systems, and disrupts cell membrane integrity (Nagajyoti et al. 2010). Root extension is often used as the first indication that plants are poisoned by Hg elements (Moldovan et al. 2013). Symptoms of mercury toxicity, in general, are the inhibition of seed and root

growth, and the inhibition of photosynthesis, which in turn reduces crop production. In addition, mercury accumulated in plant root can inhibit the absorption of K by plants (Hooda 2010). Mercury absorbed by plants can cause some enzymes to be inactive because of the incorporation of mercury into sulfhydryl peroxide through the formation of reactive oxygen compounds, such as superoxide, hydroxyl radicals, and hydrogen peroxide (Chen & Yang 2012).

### Mercury Uptake by Maize

The results of the analysis of mercury in the maize shoots and roots showed that the accumulation of Hg in the maize plant was mostly found in the roots. From the average accumulation of Hg in the maize shoots and roots in all treatments, about 98% of the mercury was accumulated in the roots, while in the shoot was only 2% or an average of 0.008 mg/kg dry weight. This was in line with a study on the potential of maize for phytoremediation of soil contaminated with gold mine tailings conducted in Lombok by Afandi et al. (2019) that 95.7-98.6% of the Hg absorbed by the maize plant was concentrated in the maize root, and no mercury was detected in maize seeds. Therefore, it can be stated that the maize shoot is safe to be used as livestock feed because it is below the threshold of mercury content according to the threshold of the Indonesian Government Regulation of 0.03 mg Hg/kg.

Maize is capable of continuous phytoextraction of metals from contaminated soils by translocating them from roots

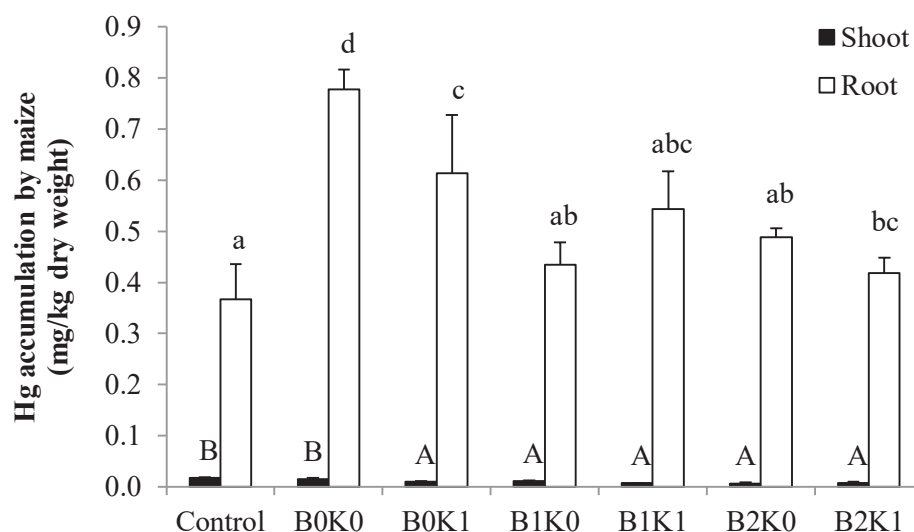


Fig. 6: Accumulation of mercury by maize grown on post-phytoremediation soil at 60 days. Vertical bars represent standard deviation while the same capital and small letters are not significantly different for each treatment mean at 0.05 level of probability. \*) B0 = no bacterial isolate, B1 = *B. vesicularis* isolate, B2 = *N. mobilis* isolate, K0 = without ammonium thiosulfate, K1 = with ammonium thiosulfate 2 g/kg, Control = soil not subjected to phytoremediation.

to shoots (Nascimento & Xing 2006). The maize plant has been even shown to accumulate certain heavy metals such as Cd (Kimenyu et al. 2009) and Pb (Pereira et al. 2007) above levels that define metal hyperaccumulation. Máthé-Gáspár & Anton (2005) have grouped maize as an accumulator and a metal tolerant plant. The potential use of maize in phytoextraction technology is thus advocated, especially for developing countries with scarce funds available for environmental restoration (Wuana & Okieimen 2010).

## CONCLUSION

The application of *B. vesicularis* and *N. mobilis* combined with ammonium thiosulfate as a chelating agent enhanced the growth and biomass of *P. conjugatum* in the phytoremediation process of soil contaminated with small-scale gold mine tailings. This, in turn, increased mercury accumulation in plants so that it reduced 18-20% of mercury content in the soil contaminated with gold mine tailings. The decrease of mercury content in the soil led to improved growth and yield of maize planted after soil phytoremediation through the application of *B. vesicularis* and *N. mobilis*.

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