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Plant Growth-Promoting Potency of Heavy Metal-Resistant Rhizobacteria from Gold and Copper Mine

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Abstract – **The Growth-promoting effects of heavy metal-tolerant microbes from the rhizosphere of indigenous plant growing in mine waste-degraded soil could have potency for their biotechnological applications in plant-based remedia--tion strategies. Heavy metal-resistant rhizobacteria isolated from gold mine in Pongkor, Indonesia and copper mine in Marinduque, Philippine were tested the potency of plant growth-promoting characteristics *in vitro* such as N_2 fixation (acetylene reduction activity, *nifH* detection), P solubilization ($Ca(PO_4)_3$), $AlPO_4$, $FePO_4$), indole acetic acid (IAA) and ACC deaminase production. Three rhizobacteria (PbSM 2.1, MGR 334, and CuNFbM 4.1) which had multiple heavy metals (Cu, Cd, Pb) - resistant and biofilm-forming rhizobacteria like other 32 rhizobacteria, they showed one or more than one plant growth promotion traits such as N_2 fixation, phosphate solubilization, IAA and ACC deaminase production. The isolated rhizobacteria could be used as biofertilizer in phytoremediation of mine-wasted, as well as, environment-friendly sustainable agriculture which is linked with food security.**

Keywords – **Gold and Copper Mine, Heavy Metal - Resistant Rhizobacteria, Plant Growth - Promoting Potency and Phytoremediation.**

I. INTRODUCTION

The reduction in productive agricultural land is caused by land conversion without balanced the new land acquisition and widespread expansion of degraded land due to mismanagement such as environment-unfriendly industrial activities threatening worldwide food security. These environmental issues have demanded the implementation of environment-friendly and sustainable agriculture. The use of plant growth promoting rhizobacteria (PGPR) holds promise for environment-friendly sustainable agriculture and in phytoremediation which is linked with food security.

Mine tailings - contaminated soil are extreme environments, containing high concentrations of heavy metals and deficient in nutrient and organic matter with extremely low pH where most plants could not grow (1). However, some plant species tolerant to heavy metals are capable of growing on the mine tailing-contaminated soil (2). Some native plant species growing in heavy metal contaminated sites were most efficient in accumulating Cu and Zn in its shoots (3). Such soil, wherein some plants tolerant to heavy metal grow, is expected to harbor heavy metal-resistant plant growth-promoting bacteria which have potential for use in or phytoremediation of mine-degraded soil or environment-friendly sustainable agricult--ure.

The study was intended to characterize the growth promotion potency of heavy metal-resistant rhizobacteria from plant rhizosphere growing in gold mine tailing site of PT. ANEKA TAMBANG (ANTAM) in Pongkor, Bogor, West Java, Indonesia, and copper mine site at Barangays Ino and Capayang in Mogpog, Marinduque, Philippines.

II. MATERIALS AND METHODS

A. *Growth in a Nitrogen-Free Medium, Acetylene Reduction Activity, and Nif H detection*

All isolated rhizobacteria were originated from the rhizosphere growing in gold mine in Pongkor, Indonesia and a copper mine-wasted soil in Marinduque, Philippines. The soil of gold mine-wasted soil contained 30,95 ppm Cu; 0,93 ppm Pb; 39,55 ppm Cd; and 0,240 ppm Hg, and had soil pH (H_2O) about 7.95 while copper mine-wasted soil contained 446,3 ppm Cu; 0,5 ppm Pb; 0,2 ppm Cd; 0,0 ppm Hg; and had soil pH (H_2O) about 4.5; 97 ppm P; 0,23 mg. 100 g^{-1} K_2O; 0,3% organic carbon; as well as classified as sandy loam soil (4).

All heavy metal-resistant rhizobacteria were screened for their ability to grow in a semisolid nitrogen-free bromthymol blue (NFb) medium. The positive growth was showed by pellicle formation below the surface of the medium after 3-5 days incubation at 28°C (5).

The ability of heavy metal-resistant rhizobacteria to fix nitrogen gas was tested using the acetylene reduction assay (6). Bacterial nutrient broth cultures (10^9 CFU/mL) were grown 20 mL of semisolid NFb agar medium. Five days after inoculation, the cotton plugs of the tubes were replaced by rubber septa, a 10% volume (air in the tube) was removed, and a 1/10 volume (air volume) of acetylene was added. The amount of ethylene was measured after 2 hours incubation. All incubations were done at 28°C in the dark, avoiding any movement of the vials. Ethylene was measured using a Hewlett Packard 5890A gas chromato--graph equipped with the flame-ionization detector and a packed column (1.83 m long, 0.318 cm i.e., stainless steel, packed with HayeSepN; Supelco). Calculations were based on peak area. *Azospirillum brasilense* as bacterial standard (collection of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development/ ICABIOGRAD) served as control.

DNA Extraction. To isolate the genomic DNA of the isolates, bacterial cells (~mid log phase) with a population

approximately 10^9 cell/mL were harvested by centrifugati--on and subjected to DNA extraction kit methods (Wizard Genomic DNA Purification kit, Promega). Agarose gel electrophoresis was used to confirm the presence and quality of extracted DNA.

nifH gene Amplification. PCR runs were carried out on DNA extracted from bacterial culture, in a final volume of 25 µL containing 2 µl of DNA preparation, 250 mM each deoxynucleoside triphosphate, 8.5 mL of nuclease-free water, 0.4 µM of each *nifH* primer, and 1x GoTaq® Green Master Mix (Promega) containing final concentration of 1.5 mM $MgCl_2$ and, 0.2 mM each dNTP. The following amplification program was run in the same thermal cycler: 3 min at 95 °C (1 cycle); 3 sec at 94 °C, 30 sec min at 55 °C, and 30 sec at 72 °C (30 cycles); and a final elongation at 72 °C for 5 min. Oligonucleotide primers used in PCR amplification of n*ifH* gene detection was *nifHf* (5'-GGCAAGGGCGGTATCGGCAAGTC-3') and nifHr (5'-CCATCGTGATCGGGTCGGGATG-3') (7).

B. *Mineral Phosphate Solubilizing Activity*

Bacterial isolates were tested by plate assay using Pikovskaya medium (8) containing per liter: 10 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.5 g yeast extract, 0.2 g KCl, 0.2 g NaCl, 0.002 g $FeSO_4.7H_2O$, 0.002 g $MnSO4.H_2O$, 5 g $Ca_3(PO_4)_2$ and 1000 mL distilled water (pH 7). Phosphate-solubilizing bacterial colonies are recognized by clear halos after 5 days of incubation at 28°C and the activity was indexed as the diameter of the colony and halo divided by the diameter (9). The test was also done on agar plates on media supplemented with either $FePO_4$ or AlPO4. For seven rhizobacteria which were copper resistant (CuNFbM 4.1, CdTM 2.1, CdTM 2.2, PbSM 2.1, PbSM 2.2, MGR 334, MGR 335), they were subjected to phosphate solubilizing activity test with presence of 125 ppm Cu.

C. *Indole Acetic Acid (IAA) Production*

IAA was detected by the colorimetric method in the supernatants of the bacterial cultures using Gordon and Weber's reagent (10). All isolates were grown in minimal medium containing 5 mM tryptophan. All isolates were grown overnight in modified nutrient broth M26, respectively. Then, each the nutrient broth culture (100 µl) was added to 10 ml of minimal salt medium containing 5 mM tryptophan (11). After further incubation for 44 h, IAA was quantified as follows: Bacterial cells were removed from the culture medium by centrifugation, and then 2 ml of Gordon and Weber's reagent (1 ml of 0.5 M $FeCl_3$ dissolved with 50 mL of 35% $HClO_4$ in a dark bottle) were mixed with 1 mL of culture supernatant, followed by incubation at room temperature for 25 min. *Azospirillum brasilense* from ICABIOGRAD was used as a positive control. The absorbance at 535 nm was read with a spectrophotometer. For three rhizobacteria which were copper resistant (PbSM 1.1, CuNFbM 4.1, MGR 334), they were subjected to IAA production with presence of 125 ppm Cu.

D. *1-Aminocyclopropane 3-Carboxylate* (ACC) *Deaminase Production*

The ability of bacterial isolates to produce ACC deaminase followed the method of Penrose and Glick (12). All the bacterial isolates were grown in 5 ml of TSB (tryticase soy broth) medium for 24 h with shaking (150 rpm) at 28 °C. Cell pellet collected by centrifugation at 8000 g for 10 min was washed with sterile dH_2O and dissolved in 1 ml of sterile dH_2O and spot inoculated on Petri plates containing DF salts minimal medium + 3 mM ACC (13). Plates containing DF minimal medium without ACC served as negative control while $(NH4)_2SO_4$ (2.0 g^{-1}) as nitrogen source served as a positive control. The plates were incubated for three days at 28 °C. The growth of isolates on ACC-supplemented plates was compared to the positive and negative control plates. Isolates growing well on ACC plates were selected.

III. Results

It has been reported by some researchers that copper-resistant bacteria isolated from copper mine waste-degraded soil revealed characteristics of plant growth promotion. These characteristics, functional redundancies such as nutrient provider, phytostimulator, and stress controllers have been proven to assist pioneer plants at the degraded site to grow and withstand stress condition (2, 14). All isolated rhizobacteria, copper resistant or sensitive, were studied based on their ability for plant growth promotion because the most rhizobacteria were Pb and Cd heavy metal resistant.

A. *Growth in Nitrogen-Free Medium, Acetylene Reduction Activity (ARA), and nifH Detection of Selected Bacteria*

Thirteen (13) rhizobacteria were able to form a pellicle on semisolid NFb media while three rhizobacteria were able to grow on solid nitrogen-free media. Seven of those isolates (CuNFbM 4.1, MGR 5.3, CdNFbA 1.1, MGR 331, MGR 333, MGR 334, and MGR 335) were originally isolated using semisolid NFb media while the other isolates (CdTM 2.1, CdTM 2.2, PbSM 2.1, PbSM 2.2, CuTM 2.1, CdSA 2.1, CdSM 2.1, PbTA 1.1, CdSEM 1.3) were obtained using trypticase soy agar (TSA), sucrose-minimal salts low-phosphate (SLP), and soil extract agar (SEM) media, respectively**.** The 13 pellicle-forming rhizobacteria and three rhizobacteria growing on solid nitrogen-free media showed acetylene reduction activity ranging from 12.98 µmol ethylene h^{-1} $culture^{-1}$ to 3407.72 µmol ethylene h^{-1} $culture^{-1}$. MGR CuNFbM 4.1 and CuTM 2.1 showed the highest and lowest acetylene reduction activity, respectively (Table 1). The lower acetylene reduction activity was reported in other studies (13). Their study showed that rhizobacterial strains isolated from the rhizosphere of pioneer plants growing on heavy-metal contaminated soils had acetylene reduction activities with values ranging from 0.1 - 169.4 nmol ethylene h^{-1} $culture^{-1}$. Park *et al*. (15), on the other hand, found the isolate PM-24 (*Bacillus fusiformis*), one of the rhizobacteria from agricultural crops, exhibiting highest nitrogenase activity value of 3677.81 nmol ethylene h^{-1}.

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Table 1. N_2 -fixing characteristic of heavy metal-resistant rhizobacteria from the rhizosphere growing in gold mine in Pongkor, Indonesia and copper mined-out site in Marinduque, Philippines.

No	Rhizobacteria	Acetylene Reduction Activity (µmol C_2H_4. $hour^{-1}$. $Culture^{-1}$)	*Nif H*
1	CuTM 2.1	12.98	Negative
2	CdNFbA 1.1	16.24	Positive
3	CdTM 2.1	19.28	Negative
4	MGR 5.3	21.16	Negative
5	PbSM 2.2	24.33	Positive
6	*K. pneumonia*	29.49	Positive
7	CdTM 2.2	37.47	Positive
8	PbSM 2.1	48.48	Positive
9	MGR 333	226.24	Negative
10	MGR 334	983.64	Negative
11	CdSA 2.1	797.01	Negative
12	MGR 331	653.64	Negative
13	*A.brasilense*	1341.35	Positive
14	PbTA 1.1	1428.61	Negative
15	CdSM 2.1	2222.52	NT
16	MGR 335	2662.64	Negative
17	CdSEM 1.3	2474.15	NT
18	CuNFbM 4.1	3407.72	Positive
19	CuSEM 3.5	NT	Negative
20	CuTA 2.1	NT	Negative
21	CdSEM 1.1	NT	Negative
22	MGR 2.2	NT	NT
23	PbTA 1.2	NT	Positive
24	CdTA 1.1	NT	NT
25	CuTA 2.2	NT	NT
26	CdTA 2.4	NT	NT
27	CdTA 3.1	NT	NT
28	CdTA 3.2	NT	NT
29	CdTA 3.4	NT	NT
30	CdSA 4.2	NT	NT
31	PbTM 2.2	NT	NT
32	CuNFbM 4.2	NT	NT
33	CuSEM 3.8	NT	NT
34	CdSEM 1.5	NT	NT
35	PbSEM 3.1	NT	NT
36	HMCTA 2.2	NT	NT
37	HMCTA 2.3	NT	NT
38	PbSM 2.3	NT	NT

The six pellicle-forming rhizobacteria on semisolid NFb media (Pb TA 1.2, CuNFbM 4.1, CdTM 2.1, CdTM 2.2, PbSM 2.1, PbSM 2.2) and had ARA, they revealed *nifH* gene positive (*nifH*$^+$). On the contrary, the rest of pellicle-forming bacteria (PbTA 1.1, CuTM 2.1, MGR 335, MGR 334, MGR 5.3), two non-pellicle forming bacteria but could grow in solid nitrogen-free media (MGR 333, MGR 331), and three non-pellicle forming bacteria and could not grow in solid nitrogen-free media (CuTA 2.1, CdSEM 1.1, CuSEM 3.5) were *nifH* gene negative (*nifH*$^-$) (Table 1).

B. *Phosphate Solubilization Activity*

Thirty-two (32) rhizobacteria were tested for phosphate solubilizing activity using Ca_3 $(PO_4)_2$, $Ca_3(PO_4)_2$, $AlPO_4$, or FePO4 as insoluble phosphate sources. Phosphate solubilizing activity is (Fig.1) indicated by phosphate solubilization index (SI) expressed in cm on medium with $Ca_3(PO_4)_2$ ranging from 1.1 in CdNFbA 1.1, MGR 5.3, MGR 335, CdSEM 1.1, CuTA 2.2, and CdTA 3.4 to 2.85 in PbSM 2.2. On medium with $AlPO_4$, rhizobacteria showed phosphate solubilizing activity ranging from 1.1 in MGR 331, PbTA 1.1, and CdSEM 1.1 up to 1.5 in MGR 335. Ten rhizobacteria (CuTM 2.1, CdTM 2.1, CdTM 2.2, PbSM 2.1, MGR 334, PbTA 1.1, CuNFbM 4.1, MGR 335, CuTA 2.1 and CuSEM 3.8) revealed phosphate solubilizing activity of 1.1 in PbTA 1.1 to 1.5 in CdTM 2.1, CdTM 2.2 and MGR 335 on media with FePO4 (Table 2a). Navarro-Noya *et al*. (14) found that heterotrophic bacteria from rhizosphere of pioneer plants growing in copper mined-out site indicated phosphate solubilization activity from $Ca_3(PO_4)_2$ with solubilization indexes which ranged from 2-5.

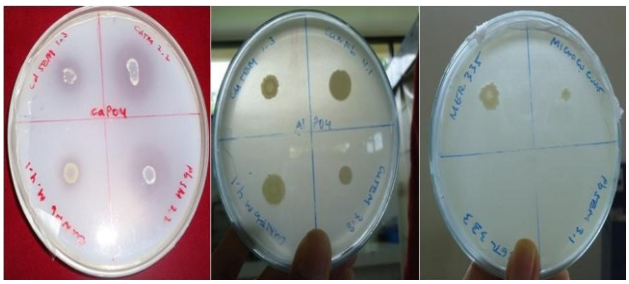

Fig. 1. Plates showing phosphate solubilization on Pikovskaya's medium with $Ca_3(PO4)_2$ (right), AlPO4 (Center), FePO4 (left) as phosphate source.

Table 2a. Phosphate solubilization activity of heavy metal-resistant rhizobacteria from the rhizosphere growing in gold mine in Pongkor, Indonesia and copper mined-out site in Marinduque, Philippines.

No	Isolates	Solubilization Index		
		$Ca_3(PO_4)_2$	AlPO4	FePO4
1	CuTM 2.1	1.25	1.3	1.2
2	CdNFbA 1.1	1.1	Negative	Negative
3	CdTM 2.1	1.4	1.3	1.5
4	MGR 5.3	1.1	Negative	Negative
5	MGR 331	1.3	1.1	Negative
6	PbSM 2.2	2.85	1.28	Negative
7	*K. pneumonia*	NT	NT	Negative
8	CdTM 2.2	1.7	1.3	1.5
9	PbSM 2.1	1.7	1.4	1.4
10	MGR 333	1.1	1.2	Negative
11	MGR 334	1.3	1.2	1.4
12	*A. brasilense*	NT	NT	Negative
13	PbTA 1.1	1.4	1.1	1.1
14	CdSA 2.1	1.4	Negative	Negative
15	CuNFbM 4.1	1.10	1.4	1.4
16	CdSM 2.1	2.8	Negative	Negative
17	CdSEM 1.3	1.8	1.3	Negative
18	MGR 335	1.1	1.5	1.5
19	CuSEM 3.5	Negative	Negative	Negative
20	CuTA 2.1	1.3	Negative	1.4
21	CdSEM 1.1	1.1	1.1	Negative
22	MGR 2.2	1.2	1.3	Negative
23	PbTA 1.2	1.6	Negative	Negative

24	CdTA 1.1	NT	NT	NT
25	CuTA 2.2	1.1	Negative	Negative
26	CdTA 2.4	Negative	Negative	Negative
27	CdTA 3.1	Negative	1.25	Negative
28	CdTA 3.2	1.4	Negative	Negative
29	CdTA 3.4	1.1	Negative	Negative
30	CdSA 4.2	1.2	Negative	Negative
31	PbTM 2.2	2.4	Negative	Negative
32	CuNFbM 4.2	Negative	Negative	Negative
33	CuSEM 3.8	1.2	1.33	1.20
34	CdSEM 1.5	Negative	1.2	Negative
35	PbSEM 3.1	Negative	Negative	Negative
36	HMCTA 2.2	NT	NT	NT
37	HMCTA 2.3	NT	NT	NT
38	PbSM 2.3	Negative	Negative	Negative

Eight (8) rhizobacteria (CuTM 2.1, CdTM 2.1, CdTM 2.2, PbSM 2.1, MGR 334, CuNFbM4.1, MGR 335, and CuSEM 3.8) from Marinduque and 1 isolate (PbTA 1.1) from Bogor were able to solubilize three forms of phosphate bond (Table 2). On Pikovskaya's media + $Ca_3(PO_4)_2$ supplemented with Cu 125 ppm, seven isolates still showed growth and phosphate solubilization activity. Meanwhile, in Pikovskaya's media + AlPO4 or $FePO_4$ supplemented with Cu 125, those isolates were not able to grow and solubilize phosphate from $AlPO_4$ and $FePO_4$. The solubilization activity of those seven rhizobacteria on medium supplemented with $Ca_3(PO_4)_2$ and 125 ppm Cu are presented in Table 2b. The pressure of 125 ppm Cu on the selected rhizobacteria led to enhancement and reduction in phosphate solubilizing activity from 1.10 – 1.7 to 1.5 – 2.7 and 1.3 – 2.85 to 1.20 -2.00, respectively.

Table 2b. Phosphate solubilization activity (cm) of the copper-resistant isolates from Cu mined-out site in Marinduque, Philippines.

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No	ISOLATE CODES	PHOSPHATE SOLUBILIZATION INDEX	
		$Ca_3(PO4)_2$	$Ca_3(PO4)_2$ + Cu 125 ppm
1	CuNFbM 4.1	1.10	1.50
2	CdTM 2 1	1.4	1.20
3	CdTM 2 2	1.7	2.7
4	PbSM 2.1	1.7	2.3
5	PbSM 2.2	2.85	2.00
6	MGR 334	1.3	1.25
7	MGR 335	1.1	1.7

C. Indole Acetic Acid (IAA) Production

Most of the tested rhizobacteria were IAA producer with IAA concentration ranging from 2.92 µg mL^{-1} in HMCTA 2.2 to 129.0 µg mL^{-1} in CdNFbA 1.1. The IAA extract using Gordon and Weber's reagent ($FeCl_3$ + $HClO_4$) showed IAA oxidation which was marked with pink up to red color (Fig 2). The rhizobacteria can be divided into three groups based on the amount of IAA: 1 - 50 µg mL^{-1} (30 isolates), 50 - 100 µg mL^{-1} (three isolates), and >100 µg mL^{-1} (four isolates) (Table 3). IAA production of five selected bacterial isolates (CdTM 2.1, CdTM 2.2, CuNFbM4.1, PbSM 2.1, and PbSM 2.2) decreased to about 50% when they were grown under 125 ppm copper in the media ranging from 4.72-11.32 ppm to 2.26-3.29. Yu *et al* (16) reported that bacteria isolated from V-Ti magnetite mine tailing soil (67% of 93 isolates) produced IAA and the concentration of IAA produced by 53 isolates was higher than 20 µg mL^{-1}. On the other hand, He *et al*. (2) reported that culturable Cu-resistant bacteria from rhizosphere growing on a copper mine wasteland produced IAA ranging from 0.7 µg mL^{-1} to 50.5 µg mL^{-1}. The study of Navarro-Noya *et al*. (14) revealed that bacterial strains isolated from the rhizosphere of pioneer plants growing on heavy-metal contaminated soils produced IAA in the amount of 3.25 – 20.64 µg mL^{-}

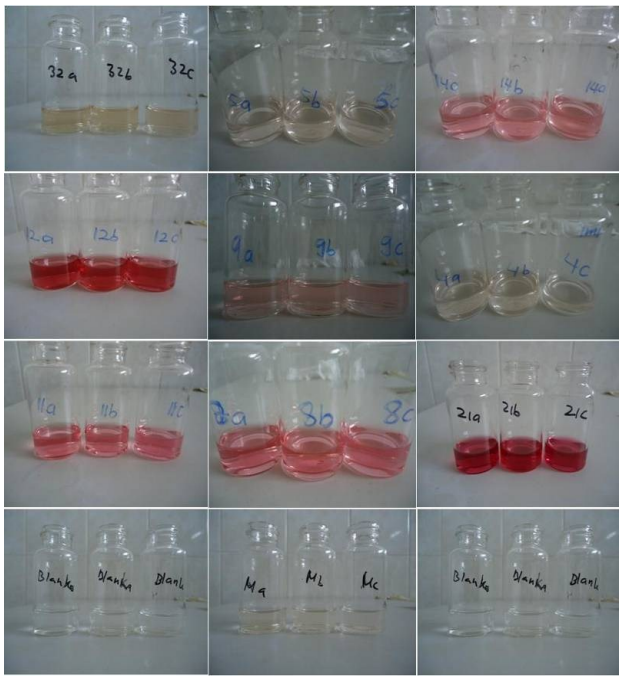

Fig. 2. *Indole acetic acid* (IAA) production of heavy metal-resistant rhizobacteria isolates from the copper mine site in Marinduque Philippines. IAA oxidation using Gordon and Weber's reagent ($FeCl_3$+$HClO_4$) generate pink up to red color after 25 min of incubation. Positive control: *Azospirillum brasilensis*. Negative control: dH_2O (Blank) or media (M).

Table 3. IAA and ACC deaminase production of heavy metal-resistant rhizobacteria from rhizosphere growing in gold mine in Pongkor, Indonesia and copper mined-out site in Marinduque, Philippines.

No	Isolates	IAA (µg.ml^{-1})	ACC Deaminase
1	CuTM 2.1	3.60*	Negative
2	CdNFbA 1.1	129.00***	Negative
3	CdTM 2.1	4.72*	Negative
4	MGR 5.3	14.32*	Positive
5	MGR 331	125.42***	Negative
6	PbSM 2.2	6.86*	Positive
7	*K. pneumonia*	NT	Negative

8	CdTM 2.2	6.56*	Positive
9	PbSM 2.1	7.49*	Positive
10	MGR 333	62.54**	Negative
11	MGR 334	46.22*	Negative
12	*A. brasilense*	114.96***	Positive
13	PbTA 1.1	7.32*	Positive
14	CdSA 2.1	71.36**	Negative
15	CuNFbM 4.1	11.32*	Negative
16	CdSM 2.1	49.5*	Positive
17	CdSEM 1.3	7.54*	Negative
18	MGR 335	126.79***	Negative
19	CuSEM 3.5	3.18*	Negative
20	CuTA 2.1	3.55*	Negative
21	CdSEM 1.1	4.32*	Negative
22	MGR 2.2	15.25*	Negative
23	PbTA 1.2	3.36*	Negative
24	CdTA 1.1	3.19*	NT
25	CuTA 2.2	3.24*	Negative
26	CdTA 2.4	8.22*	NT
27	CdTA 3.1	8.74*	NT
28	CdTA 3.2	3.22*	NT
29	CdTA 3.4	3.07*	NT
30	CdSA 4.2	5.38*	NT
31	PbTM 2.2	32.00*	Negative
32	CuNFbM 4.2	7.81*	Negative
33	CuSEM 3.8	96.67**	Negative
34	CdSEM 1.5	3.18*	Negative
35	PbSEM 3.1	15.35*	Negative
36	HMCTA 2.2	2.92*	NT
37	HMCTA 2.3	8.28*	Negative
38	PbSM 2.3	4.54*	Negative

*1 – 50 ppm;** 50- 100 ppm; ***>100 ppm; - NT: Not tested

D. *1 - Aminocyclopropane 3 - Carboxylate (ACC) Deaminase Production.*

The capability of bacteria to grow on a minimal medium with ACC as the sole N source is indicative of ACC deaminase present in the bacteria (17). Six (6) rhizobacteria MGR 5.3, PbSM 2.2, CdTM 2.2, PbSM 2.1, PbTA 1.1, and CdSM 2.1 are ACC deaminase producers and except MGR 5.3 MGR 334 are copper resistant (Figure 3 and Table 3). According to De Poel *et al*. (18), ACC deaminase of plant growth promoting bacteria could metabolize ACC which, in turn, reduces ethylene producti--on. The ethylene concentration brings about favorable plant growth and alleviating stress susceptibility. ACC deaminase-producing rhizobacteria obtained from Cu mined-out site in Mogpog, Marinduque might have contributed to the tolerance of *talahib* plants toward copper on the copper-contaminated soil and in the functioning of IAA growth promotion.

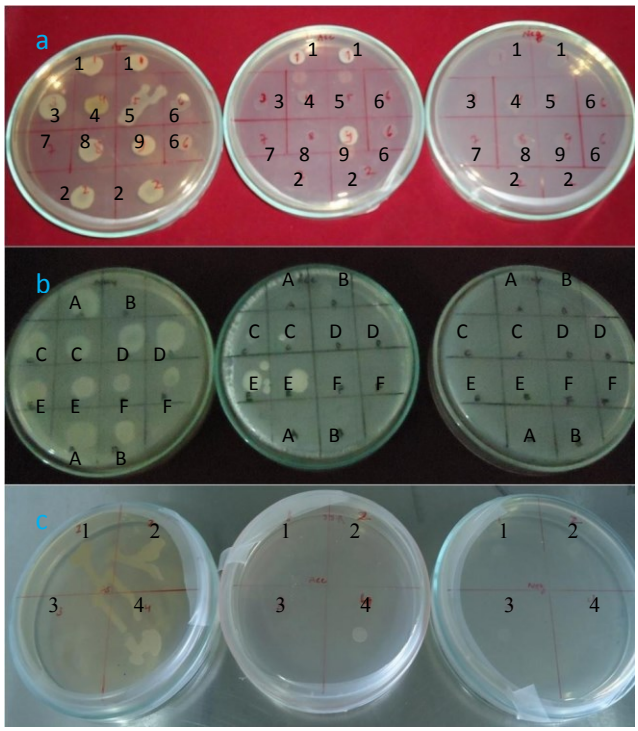

Fig. 3. Growth of heavy metal-resistant rhizobacteria isolates from gold mine site of PT. ANTAM in Pongkor, Bogor, Indonesia and abandoned copper mine site in Marinduque, Philippines on Dworkin and Foster (DF) salts medium with ammonium sulphate (left), with ACC (center), and without ammonium sulphate and ACC (right). (a) PbSM 2.1 (1); *Klebsiella pneumonia* (2); CdTM 2.1 (3); MGR 335 (4); CdSEM 1.3 (5); *Stetrophomonas maltophilia* (6); CuNFbM 4.1 (7); MGR 334 (8); CdTM 2.2 (9). (b) CuTA 2.1 (A); PbTA 1.2 (B); CuTM 2.1 (C); PbTM 2.2 (D); PbSM 2.2 (E); and CuSEM 3.5 (F). (c) CuSEM 3.5 (1); CdNFbA1.1 (2); CuNFbM 4.1 (3); *Azospirillum brasilensis* (4).

IV. Discussion

The nifH gene is the most widely sequenced marker gene used to identify nitrogen-fixing bacteria and archaea (19). The combination of primer pair used in this study was *nifHF* and *nifHR* which is according to Minierdi *et al*. (7) is a specific primer to amplify the 760 bp bacterial DNA fragment of the nifH gene. Amplified products of the rhizobacteria of CdNFbA 1.1, CdTM 2.1, PbSM 2.2, CdTM 2.2, PbSM 2.1, CuNFbM 4.1, PbTA 1.2, and positive control (*Azospirillum brasilense* and *Klebsiella pneumonia*) showed the presence of 760 bp segments indicating the existence of the *nifH* gene. With regard to some rhizobacteria that could grow in semisolid nitrogen-free media and indicated ARA value but did not exhibit DNA fragment 760 bp when its DNA genome was amplified using *nifH* primer, Gaby and Buckley (19) stated that the choice of nifH primer should be based on consideration how the primer could cover existing *nifH variation* in a group of bacteria which will be analyzed. Nevertheless, the rhizobacteria could grow in semisolid or solid nitrogen-free media and showed acetylene reduction activity indicating that the rhizobacteria are an N_2–fixer.

CdNFbA 1.1 was isolated from semisolid NFb medium but it was not able to form a pellicle as seen in the confirmation step using this isolation medium. Instead, it was able to grow in solid nitrogen-free media and the former revealed *nifH*$^+$. The possibility of this event occurred when the pellicle was taken using a loop for purifying in agar NFb + YE + ammonium medium. An aerobic N_2-fixer grew or was present on the surface of the medium which unintentionally was also taken by the loop. This aerobic N_2-fixer therefore persisted during purification and was also used in this study.

CdTM 2.2, PbSM 2.1, CdTM 2.1, and PbSM 2.2 are resistant to high concentration of Cu, Pb, and Cd. In addition, those isolates could form a pellicle on semisolid NFb medium, indicated *nifH*$^+$ and acetylene reduction activity.

The solubilization data revealed that isolates can solubilize phosphate in the form of $Ca_3(PO_4)_2$ stronger than $AlPO_4$ and $FePO_4$. The gold mine site soil where isolates were taken, is a neutral soil. Such neutral soils are rich in calcium phosphate, while aluminum phosphate and ferric phosphate is a major constituent of acidic soils. Since the isolates were adapted to calcium phosphate exposure in their natural habitat, their ability to solubilize calcium phosphate was probably stronger than that of aluminum phosphate or ferric phosphate. It was not the case for isolates from the copper mined-out site which is acidic soil but isolates exhibited stronger $Ca_3(PO_4)_2$ solubilization than in $AlPO_4$ and $FePO_4$.

Soil taken from the Cu mined-out site has low pH (4.5) and textural grade analysis showed that this soil was of the sandy loam soil class. Such soil has low clay percentage (4). Soil which has pH below 5.5 affects solubility (availability) of P in soils and is characterized by cracking clays, where aluminum and iron dominate. Above this level, calcium and magnesium are the dominant ions and fixation is less permanent (20). It seemed that isolates obtained from the soil in Cu mined-out site in Mogpog, Marinduque did not experience $AlPO_4$ and $FePO_4$ exposure so that isolates exhibited stronger $Ca_3(PO_4)_2$ solubilization than in $AlPO_4$ and $FePO_4$.

Six (6) copper-resistant rhizobacteria from Cu mine waste-degraded soil, i.e., CdTM 2.1, CdTM 2.2, PbSM 2.1, CuNFbM4.1, MGR 335, and CuSEM 3.8 were able to solubilize three forms of phosphate bond. Shrivastava et al. (21) reported that phosphate-solubilizing bacteria showed synthesis pyrroloquinoline quinone (PQQ), production of gluconic acid, and release phosphorus from tricalcium phosphate. The bacteria which formed PQQ also showed higher tolerance to ultraviolet C radiation or UVC (100-280 nm) and oxidative stress in PSBs grown under PQQ synthesis inducible conditions, example of which is phosphate starvation. From the results of the present study, it may be suggested that PQQ may have occurred in isolated phosphate-solubilization rhizobacteria and contributed to the survival of these isolates under extreme abiotic stress conditions of excessive copper both in isolation media and when they are associated with roots of *talahib* growing on Cu-contaminated soil. Elguindi *et al*. (22) stated that excessive Cu may generate oxidative stress to bacteria due to the ability of copper to generate reactive oxygen species and act as a strong soft metal, leading to a release of iron from Fe-S cluster. Regarding rhizobacteria that could improve phosphate solubilization activity when the bacteria were exposed to 125 ppm Cu. Further research is needed to reveal oxidative stress of heavy metal Cu could enhance the phosphate solubilizing activity of the rhizobacteria.

As one of the plant growth promotion mechanisms of bacteria, it is known that IAA affects plant cell division, extension, and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and fluorescence; affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions (23). The functioning of IAA in plant growth promotion in ethylene-inducing stress environment needs another bacterial mechanism to ensure that plant could reduce excessive ethylene production to retard senescence. ACC deaminase-containing plant growth-promoting bacteria reduce ethylene production following a wide range of abiotic and biotic stresses (24). Four (4) copper resistant isolates from rhizospheric soil of *talahib* growing on copper mine-degraded soil: PbSM 2.2, CdTM 2.2, PbSM 2.1, and CdSM 2.1 were able to produce IAA and grow on medium with ACC as a nitrogen source. ACC in the plant which acts as the immediate precursor of ethylene is therefore reduced since it is being utilized by the PGPB for ammonia and α-ketoglutarate production.

V. Conclusion

Majority of isolated heavy metal resistant-rhizobacteria from plant growing in gold mine in Pongkor Indonesia and copper mine in Marinduque Philippines were plant growth promoter. Three rhizobacteria from copper mine (CdTM 2.1, CdSM 2.1, and PbSM 2.2) which had the highest MIC value of three heavy metals (Cu, Pb, Cd), they were able to show plant growth promotion traits, namely N_2 fixation, phosphate solubilization, IAA and ACC deaminase production.

Based on the *in vitro* test results, the existence of copper-resistant bacteria with functional redundancy inhabiting the rhizosphere of *talahib* suggests that this community contributes to the tolerance and persistence of the plant under stress condition of copper mine degraded soil. The isolated rhizobacteria have potency as biofertilizer in phytoremediation and sustainable agriculture. However, the effectiveness and efficacy of the non-pathogenic isolated plant growth promoting rhizobacteria have yet to be proven in *vivo* before releasing for use in phytoremediation program of mine waste-degraded soil and in sustainable agriculture.

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