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Isolation of Mercury-Resistant Endophytic and Rhizosphere Microorganisms from Grasses in Abandoned Gold Mining Area

Isolasi Mikroorganisme Endofit dan Rhizosfer Resisten Merkuri dari Rumput di Areal Bekas Tambang Emas

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ABSTRACT

There were about 900 hotspots of artisanal and small scale gold mining (ASGM) in Indonesia that recovered gold through amalgamation and cyanidation techniques. Amalgamation technique causes mercury (Hg) pollution to the soil. This study was a preliminary study that aimed to isolate Hg-resistant endophytic and rhizosphere microorganisms from pioneer grasses in the Hg-polluted soil. The most potential microorganism will be used for Hg phytoremediation in the future study. Pioneer grasses were collected from the abandoned gold mining area in Central Lombok Regency, West Nusa Tenggara. Total microorganisms were counted using Colony Forming Unit (CFU) or Standard Plate Count. The microorganism colony was characterized based on morphological characteristics. Hg-resistant endophytic and rhizosphere microorganisms were successfully isolated from pioneer grass (Cynodon dactylon and Eleusine indica) in the study site. The colonies of rhizosphere microorganisms were diverse morphologically compared to endophytic microorganisms based on the number of isolated microorganisms, 20 isolates and 17 isolates, respectively. The density of rhizosphere microorganisms was higher (96%) than endophytic microorganisms (4%). The density of rhizosphere bacteria and fungi were $47x10^3$ and $2x10^3$ CFU g^{-1}, respectively. However, the density of endophytic bacteria and fungi were only $2x10^3$ and $1x10^3$ CFU g^{-1}, respectively.

Keywords: endophytic microorganism, Hg-resistant, microorganism density, rhizosphere microorganism

ABSTRAK

Terdapat sekitar 900 titik pertambangan emas skala kecil (PESK) di Indonesia yang memperoleh emas melalui teknik amalgamasi dan sianidasi. Teknik amalgamasi menyebabkan pencemaran merkuri (Hg) di tanah. Penelitian ini merupakan penelitian pendahuluan (preliminary study) yang bertujuan untuk mengisolasi mikroorganisme endofit dan rhizosfer resisten Hg dari rumput pionir yang tumbuh di tanah yang tercemar Hg. Mikroorganisme paling berpotensi akan diaplikasikan pada fitoremediasi merkuri di penelitian selanjutnya. Sampel rumput pionir diambil dari lahan pertanian bekas kawasan pertambangan emas dengan teknik amalgamasi di Desa Bonjeruk, Kecamatan Jonggat, Kabupaten Lombok Tengah, Nusa Tenggara Barat. Total mikroorganisme dihitung menggunakan Colony Forming Unit (CFU) atau Standard Plate Count. Koloni mikroorganisme dikarakterisasi berdasarkan ciri morfologi. Mikroorganisme endofit dan rizosfer yang resisten Hg berhasil diisolasi dari rumput pionir (Cynodon dactylon dan Eleusine indica) di lokasi penelitian. Koloni mikroorganisme rizosfer sangat beragam secara morfologi dibandingkan dengan mikroorganisme endofit berdasarkan jumlah mikroorganisme terisolasi, berturut-turut 20 isolat dan 17 isolat. Kepadatan mikroorganisme rizosfer lebih tinggi (96%) dibandingkan mikroorganisme endofit (4%). Kepadatan bakteri dan jamur rizosfer masing-masing adalah $47x10^3$ dan $2x10^3$ CFU g^{-1} sedangkan kepadatan bakteri endofit dan jamur masing-masing hanya $2x10^3$ dan $1x10^3$ CFU g^{-1}.

Kata kunci: kepadatan mikroorganisme, mikroorganisme endofit, mikroorganisme rizosfer, resisten Hg

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INTRODUCTION

Indonesia lies in the 7th ranked of gold-producing countries in 2017, with total production is 154.3 tonnes of gold per year ('O'Connell *et al.,* 2018). Indonesia's gold production is supplied by large scale, medium scale, and artisanal and small scale gold mining (ASGM). There are about 900 identified hotspots of ASGM activity in Indonesia and they are found in 32 provinces and 197 cities/ regencies all over Indonesia (BaliFokus Foundation, 2012). Artisanal and small-scale gold mining recovers gold through amalgamation and cyanidation techniques (Handayanto *et al.,* 2014). Amalgamation technique (adding mercury to extract gold from the ore) causes mercury (Hg) pollution; the tailing (solid waste) that contains Hg might pollute soil and water (Gonçalves *et al*., 2017).

Hg pollution in the soil is harmful to humans and the environment (Marrugo-Negrete *et al.*, 2016). Hg in the soil is persistent (Fan *et al*., 2018). Mercury concentration in the soil of an ASGM, with amalgamation technique, location in Lombok (Sekarbela, near Mataram city) is approximately 741-7,874 mg kg^{-1} (Krisnayanti *et al.*, 2012). This concentration exceeds the minimum concentration of Hg in solid waste based on Indonesian Government Regulation (2014), which is 0.3 mg kg^{1}. Also, it exceeds the maximum concentration of Hg in the soil for agricultural land based on the Canadian Council of Ministers Environment (1999), which is 12 mg kg^{-1}. The high Hg concentration in the soil is a major limiting factor for plant growth (Hodson, 2012). The primary symptoms of Hg toxicity in maize are chlorosis (Muddarisna *et al.,* 2013). Plants generally exhibit symptoms of heavy metal toxicity when it is exposed to a high concentration in the soil, except for hyperaccumulator plants that are resistant and able to accumulate heavy metals (Idris *et al.,* 2004).

Some pioneer grass in gold-mine polluted areas such as *Digitaria radicosa*, *Paspalum conjugatum*, *Cyperus kyllingia*, *Cynodon dactylon*, and *Eleusine indica* exhibit Hg resistant (Hidayati *et al.,* 2009; Muddarisna *et al.,* 2013). The ability of plants to survive in heavy metal polluted soil is inseparable from plant association with beneficial microorganisms, either within plant body or rhizosphere niche (Thijs *et al.,* 2017). These microorganisms produce plant growth-promoting substances such as indole acetic acid (IAA) and siderophore that help plants to survive in harsh conditions (Rajkumar *et al.,* 2010; Tirry *et al.,* 2018). Microorganisms that are isolated from heavy metal polluted soil also exhibit heavy metal resistance (Lodewyckx *et al.,* 2002). This study aimed to isolate Hg-resistant endophytic and rhizosphere microorganisms (bacteria and fungi) from pioneer grasses in the abandoned gold mining area.

MATERIALS AND METHODS

Sampling Site

Pioneer grasses were collected from the abandoned gold mining area in Bonjeruk Village, Jonggat Sub-Regency, Central Lombok Regency, West Nusa Tenggara, Indonesia (8° 24' - 8° 57' S and 116° 05' - 116° 24' E, Figure 1). Grass samples were collected in April 2019. Mercury concentration in the soil was 41.37 mg kg^{-1} (Ustiatik *et al.*, 2020). Grass samples in the same length size were collected in triplicates (grass length 15-20 cm). Grass species were *Cynodon dactylon* and *Eleusine indica*. Soil in the rhizosphere of these grasses was also collected for rhizosphere microorganism isolation. Grass and soil samples were kept in the polyethylene plastics bag and stored in the cooling box for laboratory analysis.

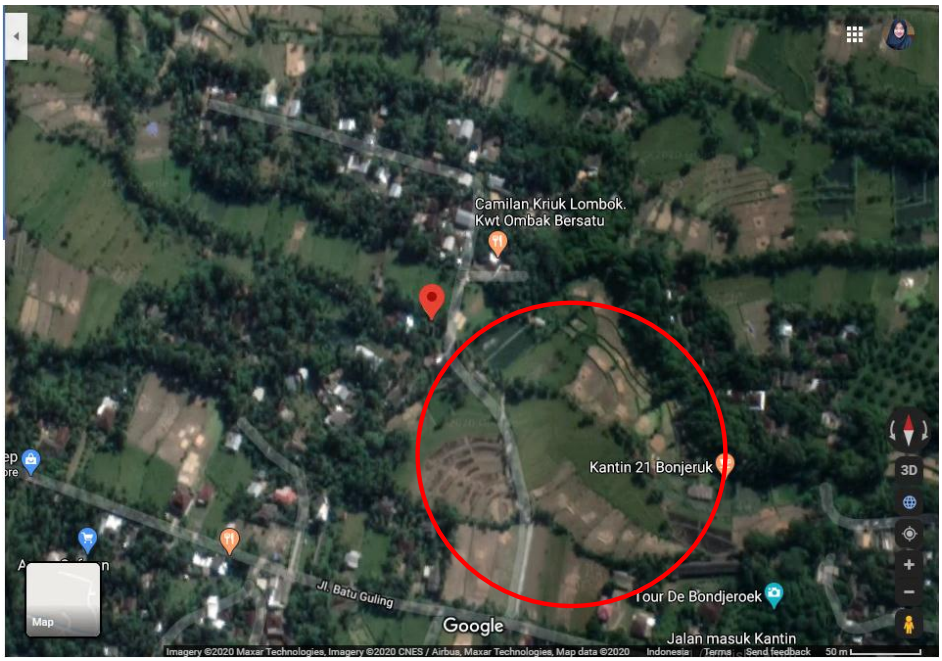

Figure 1. Sampling site of Hg-resistant rhizosphere and endophytic microorganism from local grasses (*Cynodon dactylon* and *Eleusine indica*) in abandoned gold mining area, Central Lombok Regency, West Nusa Tenggara

Endophytic Microorganism Isolation

Grass samples were washed with running tap water to remove all the debris. Grass samples were surface sterilized with 70% ethanol for 3 minutes, sodium hypochlorite (NaClO) 2.5% for 5 minutes, and rinsed several times with sterile deionized water (Xu *et al.,* 2014; Qian *et al.,* 2018). The last rinsed water was cultured in the nutrient agar (OXOID CM0003B) to confirm the success of surface sterilization (Anjum and Chandra, 2015). Five grams of each grass samples were mashed with sterile mortar and pestle in the Laminar Air Flow (LAF). The mashed sample was suspended with 45 mL of sodium chloride (NaCl) solution 0.86% (v/v) and mixed it with Vortex. One milliliter of aliquot was diluted in 9 mL of NaCl to make ten folds of serial dilution (up to 10^{-5}). One hundred microliters of aliquot in each serial dilution was cultured in Nutrient Agar (NA) for bacteria isolation and Potato Dextrose Agar (OXOID CM0139B) for fungi isolation. The cultured media were added with 10 mg L^{-1} of mercury chloride ($HgCl_2$) for Hg-resistant microorganism isolation (Xu *et al.,* 2014; Anjum and Chandra, 2015; Chasanah *et al.,* 2018). The inoculated media were incubated at 28 °C (room temperature) for 48 hours for microorganism enumeration and characterization (Nemati *et al.,* 2016).

Rhizosphere Microorganism Isolation

Soil rhizosphere samples were cleaned from roots and all debris. Five grams of soil sample were suspended in 45 mL NaCl solution 0.86% (v/v) then mixed it with Vortex. One milliliter of aliquot was diluted in 9 mL of NaCl to make ten folds of serial dilution (up to 10^{-8}). One hundred microliters of aliquot in 10^{-6}, 10^{-7}, and 10^{-8} serial dilution were cultured in NA for bacteria isolation and PDA for fungi isolation. The cultured media were added with 10 mg/L of $HgCl_2$ for Hg-resistant microorganism isolation (Xu *et al.,* 2014; Anjum and Chandra, 2015; Chasanah *et al.,* 2018). The inoculated media were incubated at room temperature (28 °C) for 48 hours for microorganism characterization and enumeration (Nemati *et al.,* 2016).

Microbial Enumeration and Characterization

Total microorganisms were counted using Colony Forming Unit (CFU) or Standard Plate Count (Nemati *et al.,* 2016). Microorganism colony diversity was characterized based on morphological characteristics such as colony size, form, margin, chromogenesis (pigmentation), elevation, and texture. A colony was determined as a single distinct colony when it was different among other colonies on the same Petri dish.

Data Analysis

Data were analyzed using One Way ANOVA at 5% significant level using Genstat software. The relationships among treatment grouping were determined with Tukey's test at 5% significance level.

RESULTS AND DISCUSSION

This study successfully isolated Hg-resistant endophytic and rhizosphere microorganisms from pioneer grass (*Cynodon dactylon* and *Eleusine indica*) in the study site. These microorganisms survived on media containing 10 mg L^{-1} $HgCl_2$, which is only Hg-resistant microorganisms that able to survive on those media (Maiti and Bhattacharyya, 2013; Xu *et al.,* 2014; Anjum and Chandra, 2015; Chasanah *et al.,* 2018).

The total colonies of isolated microorganisms were 17 colonies of endophytic microorganisms and 20 colonies of rhizosphere microorganisms (Figure 2). The microorganism was distinguished based on the colonies' morphological characteristics such as colonies' form, size, pigmentation, margin, elevation, and texture. It also showed that endophytic bacteria colonies' appearance was more diverse (Table 1 and Figure 3A) than endophytic fungi (Table 2). However, the rhizosphere fungi colonies were more diverse (Table 3) than the rhizosphere bacteria colonies (Table 4 and Figure 3B). Based on the colony morphological characteristics analysis, there were only two distinct colonies of endophytic fungi (Figure 4A). But, there were eight distinct colonies of rhizosphere fungi (Figure 4B). The colonies of rhizosphere microorganisms were morphologically more diverse than the colonies of endophytic microorganisms. The number of colonies that were isolated implies the diversity of microorganisms. A colony was counted as a single distinct colony when it was different among other colonies on the same growth medium. This study revealed significantly different isolated microorganism densities in the study site (Figure 5). The density of rhizosphere microorganisms in the study site was higher than endophytic microorganisms ($P < 0.05$), approximately 96% of total isolated microorganisms. However, endophytic microorganisms were only 4% of the total isolated microorganisms. The density of rhizosphere bacteria and fungi were $47x10^3$ and $2x10^3$ CFU g^{-1}, respectively. However, the density of endophytic bacteria and fungi were $2x10^3$ and $1x10^3$ CFU g^{-1}, respectively. Bacteria (endophytic and rhizosphere) had a higher density than fungi. The density of endophytic microorganisms varies depending on the plant part and species (Elmagzob *et*

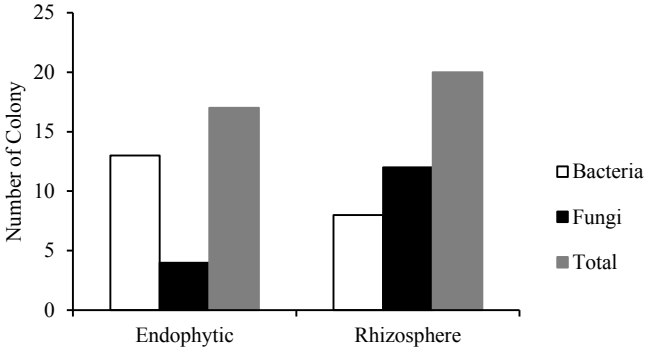

Figure 2. The number of different Hg-resistant microorganism colonies based on colony morphology in the inoculated media (Endophytic bacteria = 13 isolates; Rhizosphere bacteria = 4 isolates; Endophytic fungi = 8 isolates; Rhizosphere fungi = 12 isolates)

Table 1. Colony morphology characterization of isolated endophytic bacteria from local grasses

Isolate code	Colony morphology					
	Size (mm)	Form	Margin	Chromogenesis	Elevation	Texture
BA	6.0	Circular	Undulate	Cream	Convex	Smooth
BB	11.0	Circular	Undulate	Orange	Umbonate	Smooth
BC	5.0	Circular	Undulate	Orange	Convex	Smooth
BD	10.0	Circular	Entire	Cream	Umbonate	Smooth
BE	8.0	Circular	Undulate	Cream	Flat	Dry
BF	6.5	Circular	Lobate	Cream	Umbonate	Rough
BG	13.25	Irregular	Lobate	Cream	Umbonate	Rough
GA	3.5	Circular	Lobate	Cream	Flat	Dry
GB	14.0	Circular	Entire	Cream	Flat	Contain concentric rings
GC	31.5	Rhizoid	Lobate	Cream	Flat	Dry
GD	14.0	Circular	Entire	Cream	Flat	Contain concentric rings
GE	14.0	Circular	Entire	Cream	Flat	Contain concentric rings
GF	31.5	Rhizoid	Lobate	Cream	Flat	Dry

Note: B = *Cynodon dactylon*; G = *Eleusine indica*

al., 2019). It varies from $4.5x10^2$ to $2.8x10^3$ CFU g^{-1} of fresh weight (Costa *et al.,* 2012). Under stress conditions, the density of microorganisms, either endophytic or rhizosphere, will decrease. The total bioactivity, density, and diversity of microorganisms decreased with the increase of heavy metal concentrations but enhanced the development of metal-resistant microbial populations (Xie *et al.,* 2016; Elmagzob *et al.,* 2019).

Mercury concentration in the study site (41.37 mg kg^{-1}) exceeded Hg's permissible concentration in the soil based on Indonesian Government Regulation (2014). It was also exceeded the minimum recommended level for agricultural land (Canadian Council of Ministers Environment, 1999; Ustiatik *et al.*, 2020). Hg concentration in the previous study by Ustiatik *et al.* (2020) was 17 times lower than the study by Krisnayanti *et al.* (2012). After eight years, Hg concentration in the abandoned gold mining area decreased. This location is currently used as agricultural land and fish ponds. Some grass species vegetated in this area, such as *Cynodon dactylon* and *Eleusine indica*. These grasses were pioneer vegetation in the study site.

Plants' ability to grow in polluted areas cannot be separated from beneficial microorganisms and plants' interaction. These microorganisms reside within the plant's body or in the rhizosphere (Thijs *et al.,* 2017). These microorganisms help plants survive in heavy metal stress conditions by producing plant growth hormones such as IAA, siderophore production, and nitrogen fixation (Rajkumar *et al.*, 2009; Montalban *et al.,* 2016).

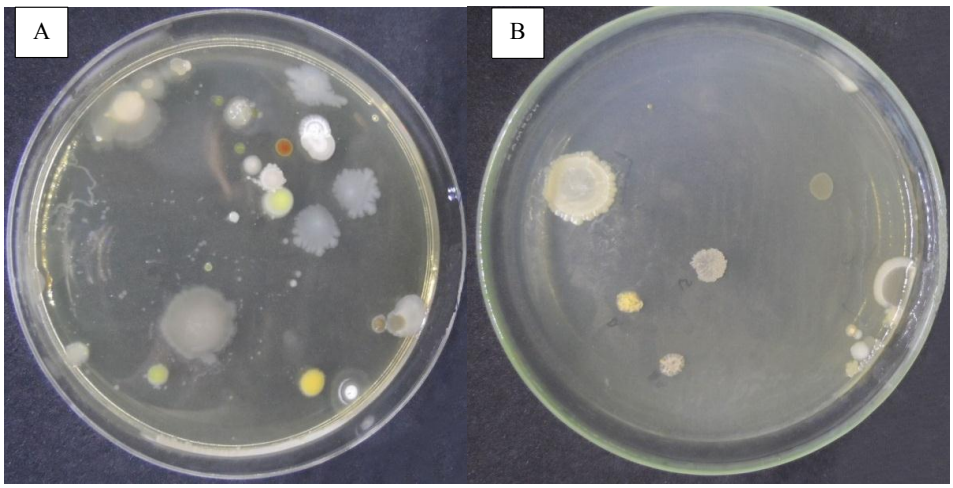

Figure 3. Endophytic bacteria colonies (A) and rhizosphere bacteria colonies (B)

Table 2. Colony morphology characterization of isolated endophytic fungi from local grasses

Isolate code	Colony characteristics					
	Size (mm)	Form	Margin	Pigmentation		Elevation
				Front	Back	
B1	47.75	Filamentous	Lobate	Black-greenish	Grey (center) White (margin)	Raised
B2	48.00	Irregular	Undulate	White (margin) Goldish-yellow (center)	White-yellowish (margin) Goldish-yellow (center)	Raised
G1	4.25	Rhizoid	Lobate	White	White-yellowish	Umbonate
G2	4.25	Rhizoid	Lobate	White	White-yellowish	Umbonate

Note: B = *Cynodon dactylon*; G = *Eleusine indica*

Table 3. Colony morphology characterization of isolated rhizosphere bacteria from the rootzone of local grasses

Isolate	Colony morphology					
	Size (mm)	Form	Margin	Chromogenesis	Elevation	Texture
RHI A	10.5	Circular	Entire	Yellow	Raised	Contain concentric rings radial
RHI B	6.0	Circular	Entire	Cream	Raised	Smooth and shiny
RHI C	13.25	Rhizoid	Lobate	Cream	Crateriform	Contain concentric rings
RHI D	1.5	Circular	Entire	Yellowish	Convex	Shiny
RHI E	11.0	Circular	Lobate	Cream	Umbonate	Smooth and shiny
RHI F	14.0	Irregular	Lobate	Cream	Raised	Contain concentric rings
RHI G	5.5	Irregular	Entire	Cream	Flat	Dry
RHI H	3.5	Irregular	Entire	Yellowish	Umbonate	Smooth and shiny

Table 4. Colony morphology characterization of isolated rhizosphere fungi from the rootzone of local grasses

Isolate	Colony morphology					
	Size (mm)	Form	Margin	Pigmentation		Elevation
				Front	Back	
RHI 1	32.5	Irregular	Undulate	Yellow	Yellow	Raised
RHI 2	40.0	Rhizoid	Filamentous	Gray	Black	Flat
RHI 3	33.0	Circular	Filamentous	Gray	White-pinkish	Flat
RHI 4	18.0	Irregular	Filamentous	White	White-yellowish	Flat
RHI 5	42.5	Irregular	Lobate	White-Yellowish	White-yellowish	Umbonate
RHI 6	15.0	Circular	Lobate	White	White-brownish	Umbonate
RHI 7	20.0	Circular	Undulate	Green	Green-brownish	Umbonate
RHI 8	19.0	Circular	Entire	Green	Black	Raised
RHI 9	8.5	Circular	Filamentous	White	Brown	Umbonate
RHI 10	9.5	Circular	Lobate	White	White-yellowish	Crateriform
RHI 11	52.0	Circular	Undulate	Pink-goldish	White-brownish	Crateriform
RHI 12	35.5	Circular	Filamentous	Green-whitish	Green-blackish	Umbonate

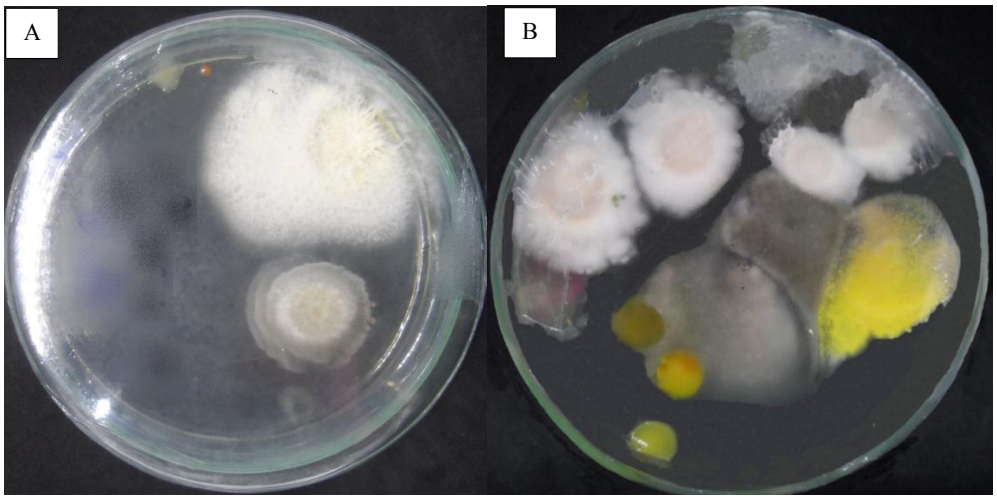

Figure 4. Endophytic fungi colonies (A) and rhizosphere fungi colonies (B)

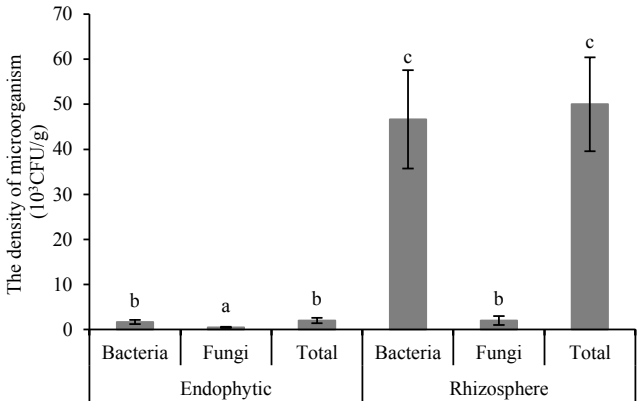

Figure 5. The mean (± standard deviation) of density of Hg-resistant microorganisms in the study site. The means with the same letter are not significantly different (P > 0.05) as determined by Tukey's test

CONCLUSION

The colonies of rhizosphere microorganisms were morphologically more diverse than the colonies of endophytic microorganisms. The density of rhizosphere microorganisms in the study site was higher than endophytic microorganisms. The density of rhizosphere bacteria and fungi were $47x10^3$ and $2x10^3$ CFU g^{-1}, respectively. However, the density of endophytic bacteria and fungi were only $2x10^3$ and $1x10^3$ CFU g^{-1}, respectively.

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