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Mycorrhiza stimulates *Rhizobium* infection in *Paraserianthes falcataria* (L.) I.C. Nielson under Hg contamination

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Abstract

Symbiosis of and AMF increase soybean production, but the information on the association between these microbes in *Paraserianthes falcataria* (L.) I.C. Nielson or 'sengon' under Hg contamination is limited. We observed *P. falcataria* inoculated with arbuscular mycorrhiza fungi (*Glomus* sp.) stimulate nodule formation under Hg contamination. The study was set up in a pot experiment in the growth chamber a the Hg concentration was adjusted to 50 ppm in soil medium. Inoculation of AMF stimulates nodule formation and increase *P. falcataria* tolerance to Hg. We isolated several bacteria from the rhizosphere belonging to *Rhizobium* group and others. Understanding the ecology of soil bacteria is important for Hg bioremediation using *P. falcataria*.

Keywords: AMF, Hg-contamination, *Paraserianthes falcataria* (L.) I.C. Nielson, *Rhizobium*.

Introduction

Soil microorganism consists of rhizosphere microbes contribute greatly to the mineralization of organic including mineralization of macro and microelement for encouraging plant growth (Philippot *et al.* 2013). *Paraserianthes falcataria* (L.) I.C. Nielson or 'sengon' belongs to the Leguminosae group and therefore forms a symbiotic association with *Rhizobium* (Clapp *et al.* 2001). This economically and ecologically important plant is widely used for revegetation of marginal land and phytoremediation of post-mining areas (Sari & Prayudyaningsih 2019). Post-mining in Indonesia resulted from a critical land increase that could eventually become untreated for agricultural land. The marginal land in Indonesia is about 14.006.450 ha (BPS 2018). Mining activities, especially gold mines, contribute to the formation of critical or marginal land. Mercury's effect is particularly damaging to the environment, and ecosystem. Increasing critical land make a low soil fertility and affects plant growth (Von Cossel *et al.* 2019). The issue of several technologies introduced for effective metal removal contamination from soil and phytoremediation is an

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effective technology to clean up heavy metal contaminated areas (Awa & Hadibarata 2020). The phytoremediation of a heavy metal contaminated sites is basically based on absorption and transformation of heavy metal in plant tissue, and therefore stimulation of plant growth will enhance phytoremediation effectiveness. Contaminated sites are mostly poor in nutrient, toxic hence only selected plant grows well. Under such conditions, plant require mechanism that supports their metal tolerance under nutrient deficiency through symbiotic microorganism endosymbiont and rhizosphere microbes. *Paraserianthes falcataria* grows faster due to their associated microbes, especially *Rhizobium* and arbuscular mycorrhizal fungi (AMF) (Juwarkar & Singh 2010). The biological interaction between *P. falcataria* and their associated microbes is not much known. Understanding the physiological association of rhizosphere microbes interact and support each other is critical to stimulate host plant growth. Plant produce substances such as phenolic acids that trigger rhizosphere microbes to infect and form a symbiotic associations (Mandal *et al.* 2010). Phenolic acids are the main polyphenols made by plants during their growth. These compounds act as a signal to initiate legume-rhizobia symbioses. While arbuscular mycorrhizal symbioses accelerate mineralization of phosphate, and colonize root surface and penetrate root tissue to enhance phosphate absorption by host plant. This symbiotic association also benefited the host plant to survive under biotic and abiotic stress (Abiala *et al.* 2013). Mercury pollution occurs all over the world. Both in developed and developing worlds. Up to now, there is no effective technology to remove Hg from contaminated sites. Yet, we observed plants that have the capacity to survive in extreme environments, including *P. falcataria* (Latifah *et al.* 2021). Introducing plants that can grow in marginal land could be used as an alternative technology for decontamination of polluted sites. Though their potential use for phytoremediation of Hg-contaminated sites, our understanding of their microbial symbiont, especially *Rhizobium* and AMF, is still limited. The aims of this study are to analyse the association between *Rhizobium* and AMF under Hg-contamination and analyse of the effect *Rhizobium* and AMF on growth stage of *P. falcataria* in Hg-contaminated conditions.

Materials and methods

Soil source

The study was conducted in an environmental microbiology laboratory of LIPI Cibinong, Bogor, from October 2019 to February 2020. The research samples were collected from the coordinates of point 6°29’34”S 106°50’58”E. Then proceed to be analyzed of soil physicochemical properties.

Experimental design

The research was performed in two experiments. The first experiment was conducted to determine the success of mycorrhiza in infecting *P. falcataria.* And the second experiment followed the research experiment of Latifah *et al.* (2021). The experiment was conducted to investigate the toxic concentration of Hg for the effect of AMF and *Rhizobium* on *P. falcataria* growth. The experiment was set up in a Factorial Completely Randomized Design with three factors, namely mycorrhiza, soil condition, and compost treatments. A total of eight treatments were arranged with three replicates for each sample. The same arrangement was used without Hg contamination as controls.

Paraserianthesfalcataria seedling preparation

Seeds of *P. falcataria* were surface-sterilized before being sown into the sterile compost media in the seedling tray. The seedlings were grown until two weeks in a growth chamber. Then, seedlings were inoculated with spores of AMF. Spore of AMF was prepared

from commercial product organic fertilizer in the vermiculite propagule by choosing uniform spores followed the wet sieving method (Brundrett *et al.* 1996). Each seedling was inoculated with ten spores of AMF by attaching the spore on the seedling root using tweezers under microscope. After that, the inoculated seedlings were grown in sterilized compost media, and the trays were placed in the growth chamber for three weeks. The seedlings were irrigated with sterile water daily. The un-inoculated seedlings were also prepared as a control.

Mycorrhizal germination observation

Spore of AMF that have been inoculated into plant roots was placed in a petridish for easy microscopic observation. Observations were conducted every 24 hours, using a compound microscope (OLYMPUS® BX50, Japan). and photographs were taken under 100× magnifications.

Hg-toxicity on *Paraserianthes falcataria* seedling growth and its symbiont

The preparation of Hg-contaminated soil was followed a method of Ambarsari & Qisthi (2017). Briefly, 50 ml of 200 mg L^{-1} $HgCl_2$ was mixed with 200 g dried soil to obtain/ achieved a final concentration of 50 mg kg^{-1} Hg in soil. The Hg-contaminated soil was used after being stabilized for fivedays. After that, the seedling (inoculated and un-inoculated) were planted to the Hg-contaminated soil. Before the seedlings were transferred to the greenhouse, the seedlings were placed in the growth chamber for two weeks for acclimatization. The seedlings were irrigated daily with sterile water. The response of *P. falcataria* under Hg-toxicity was observed by monitoring root and shoot biomass, nodule number, and microorganism characteristic after eight weeks.

Isolation of rhizosphere bacteria

Bacteria were isolated from the soil around the roots (rhizosphere). Isolation was carried out by following the method of Sari *et al.* (2019) using the spread method, and the dilution was carried out ten times. The method of dilution is to take 0.1 g of soil and then dilute it in 0.9 ml of water ten times. The medium used is sodium agar medium. Bacterial observations were made from a dilution of 10^{-5}. The samples were isolated from P-MiRb Hg and P-MiRb control. Observations were made under a microscope by looking at the shape, color, and surface. Bacteria identification was conducted following Bergeys manual Bacteriology (Buchanan *et al.* 1975).

Data analyses

Data presented are mean values ± standard error (SE). All data gathered were subjected to analyses of variance (ANOVA). In addition, the significance of the difference between exposed and control plants was tested by Duncan's Multiple Range Test ($P<0.01$).

Results

Mycorrhiza on germination seed

Mycorrhizal inoculation was intended to study the effect of microbial symbiont on the *P. falcataria* seedlings growth for Hg-phytoremediation and also to verify the speed of spore germination and infect these seedlings (Figure 1). Hyphae growth rates were conducted to see germination spores in infecting sengon seedlings. Observations were conducted daily by looking at the growth of hyphae that appear through the media. The hyphae appeared after three days of inoculation. While on the seventh day, the hyphae have spread in compost media. We found spore of AMF quickly infect the *P. falcataria* seedlings. Observations of germination spores were also performed on the agronomic character after three weeks. The

effect of mycorrhiza on seedling of *P. falcataria* is shown in Table 1, i.e., mycorrhizae treatment (P1) and without mycorrhizae (P0). Mycorrhiza did significantly affect height of shoot and stem diameter. Treatment with mycorrhizae showed higher than without mycorrhizae, with a difference of height of shoot and diameter stem of 28.72% and 30%, respectively.

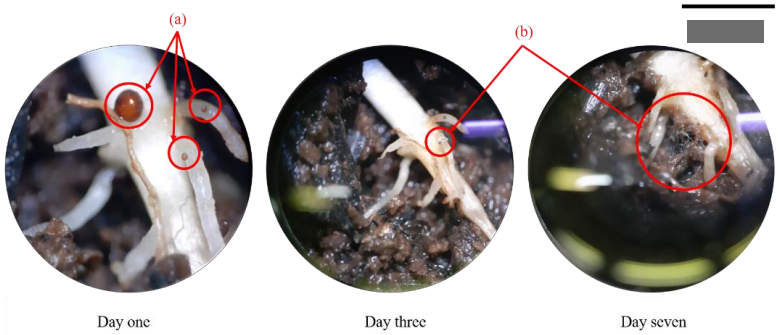

Figure 1. Mycorrhiza spore germination as observed during hyphae penetration on the root of *P. falcataria* (Bar 2 mm): (a) Mycorrhiza spore; (b) Hyphae. Bar = 100 µm.

Table 1. Agronomic characters of seedling inoculated with Mycorrhizae within three weeks

Treatment	Mean± SD		
	Plant Height (cm)	Number of Leaves	Diameter (mm)
P1	6,37±0,09 b	3,91±0,67 tn	0,50±0,01 b
P0	4,54±0,13 a	3,17±0,58 tn	0,35±0,02 a

Notes: P1= *P. falcataria* with AMF, P0= *P. falcataria* without AMF. Value are means±SD obtained from 12 independent replication of the experiment. Value with different characters indicates significant difference (P<0.01, ANOVA).

Seedling growth (plant height, number of leaves and stem diameter) with AMF inoculation showed better results than seedlings without AMF inoculation (Figure 2). Visual differences are quite clear between P1 and P0. These data were also proven by anatomical analysis of the mycorrhizal-infected roots of *P. falcataria* (Figure 3), which was observed at eight weeks age. On the seventh day, AMF hyphae grew, which demonstrated the existence of an infection process. In the third week, we found out arbuscules and vesicles as a form of mycorrhizal infection in infecting plant roots, resulting in very significant growth differences.

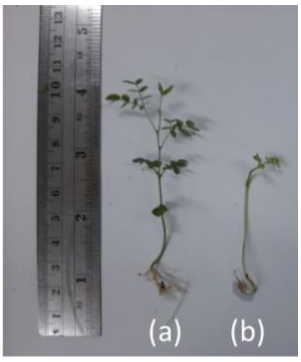

Figure 2. Morphological characteristics of *P. falcataria* seedlings after three weeks. (a) AMF inoculated seeds, and (b) without AMF.

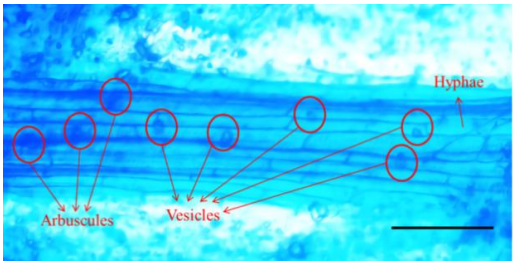

Figure 3. Infection of AMF on root showed morphological characteristics in *P. falcataria* with mycorrhiza. Bar = 100 µm.

***Paraserianthes falcataria* growth under Hg-contamination**

Paraserianthes falcataria grown on soil contaminated Hg-was conducted to verify the effectiveness of introduced mycorrhizae (P-Mi) and un-introduced mycorrhiza with un-sterile soil (P-Rb) to improve plant growth (Table 2).

Table 2. Root dry weight, shoot dry weight, and total dry biomass of *P. falcataria* seedlings aged eight weeks under Hg contaminated conditions.

Treatment	Dry Weight Root (g)		Dry Weight Shoot (g)		Total Dry Plant Biomass (g)	
	Mercury	Control	Mercury	Control	Mercury	Control
P-Mi	0.23±0.07 a	0.14±0.02 a	0.54±0.31 a*	0.27±0.05 a	0.77±0.33 ab*	0.47±0.06 ab
P-Rb	0.21±0.02 a	0.15±0.06 a	0.60±0.13 a*	0.35±0.27 a	0.80±0.10 ab*	0.50±0.32 ab
P-MiRb	0.55±0.26 ab*	0.25±0.13 a	0.99±0.40 a*	0.55±0.31 ab	1.33±0.41 ab*	0.79±0.44 ab
P-0	0.24±0.06 a	0.15±0.09 a	0.39±0.22 a	0.33±0.11 a	0.62±0.30 a	0.41±0.19 a
P-Mi C	0.23±0.16 a	0.32±0.16 a	0.74±0.35 a	0.74±0.24 ab	0.96±0.51 ab	1.05±0.43 ab
P-Rb C	0.65±0.19 b*	0.26±0.13 a	0.81±0.43 a	1.00±0.24 b	1.46±0.24 ab	1.26±0.37 b
P-MiRb C	0.34±0.11 ab	0.28±0.10 a	0.99±0.44 a	0.84±0.31 ab	1.53±0.54 b	1.52±0.42 b
P-0 C	0.20±0.12 a	0.19±0.13 a	0.61±0.52 a	0.56±0.25 ab	0.81±0.64 ab	0.75±0.38 ab

Notes: Value are means±SD obtained from 3 independent replication of the experiment. Value with different characters indicates significant difference ($P<0.01$, ANOVA). *indicates significant differences ($P<0.01$, ANOVA) between each treatment and control. P-Mi = *P. falcataria* with AMF in sterile soil, P-Rb = *P. falcataria* without AMF in unsterile soil, P-MiRb = *P. falcataria* with AMF in unsterile soil, P-0 = *P. falcataria* without AMF in sterile soil, P-Mi C = *P. falcataria* with AMF in sterile soil + compost, P-Rb C = *P. falcataria* without AMF in unsterile soil + compost, P-MiRb C = *P. falcataria* with AMF in unsterile soil + compost, P-0 C= *P. falcataria* without AMF in sterile soil + compost.

The average growth of plants in media contaminated with Hg was 2-fold higher compared to plants not contaminated. The highest growth was obtained from the treatment P-RbC contaminated with Hg with a value of 69.64%. Compost gives nutrient to growing plant, so that compost treatment is higher than without compost. The increase in plant biomass (dry weight) is based on nutrient absorption and symbiont's factor. Data shows that the growth of *P. falcataria* plants is not only from nutrients (compost). The symbionts affect the growth of *P. falcataria*, because compost in the pot experiment can be presumed that plants or symbionts absorb carbon to form compounds to help sustain plant intake in Hg conditions. A significant difference was also shown by the cross-section of the plant image, the color of the leaves on *P. falcataria*. The treatment with P-Rb results in brighter leaf color compared to P-Mi (Figure 4). Based on the color chart for plant tissues, the P-Rb treatment has a 5GY 5/8 color, while the P-Mi has a 5GY 7/8. The difference in the number value on the color chart results in difference in the brightness of the leaf color.

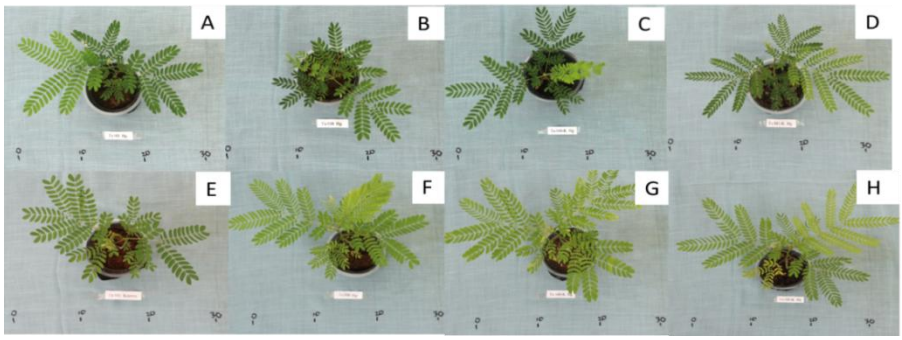

Figure 4. Cross-section of Leaf Color Mercury Treatment. (A) P-Mi, (B) P-0, (C) P-Mi C, (D) P- 0 C, (E) P-MiRb, (F) P-Rb, (G) P-MiRb C, (H) P-Rb C. P-Mi = *P. falcataria* with AMF in sterile soil, P-Rb = *P. falcataria* without AMF in unsterile soil, P-MiRb = *P. falcataria* with AMF in unsterile soil, P-0 = *P. falcataria* without AMF in sterile soil, P-Mi C = *P. falcataria* with AMF in sterile soil + compost, P-Rb C = *P. falcataria* without AMF in unsterile soil + compost, P-MiRb C = *P. falcataria* with AMF in unsterile soil + compost, P-0 C= *P. falcataria* without AMF in sterile soil + compost.

Microorganism on Hg-condition

The difference is seen in the biodiversity number of symbionts involved. Calculation of the number of nodules on *P. falcataria* seedling was conducted to expect *Rhizobium* as affected by the treatment which finally affects plant growth. Mycorrhiza and Hg affected nodule formation and activity. The highest value of P-MiRbC was 14.94% higher, than P-0C in the Hg-condition. The difference was also observed from compost treatment, i.e., 4.95-14.18% than without compost. The amount of nodule was also affected by the Hg-contamination i.e., about 3.71-44.79% decreased. These results indicate that *Rhizobium* was not tolerant to Hg and that their growth depends on the nutrients status of the pot media (Figure 5).

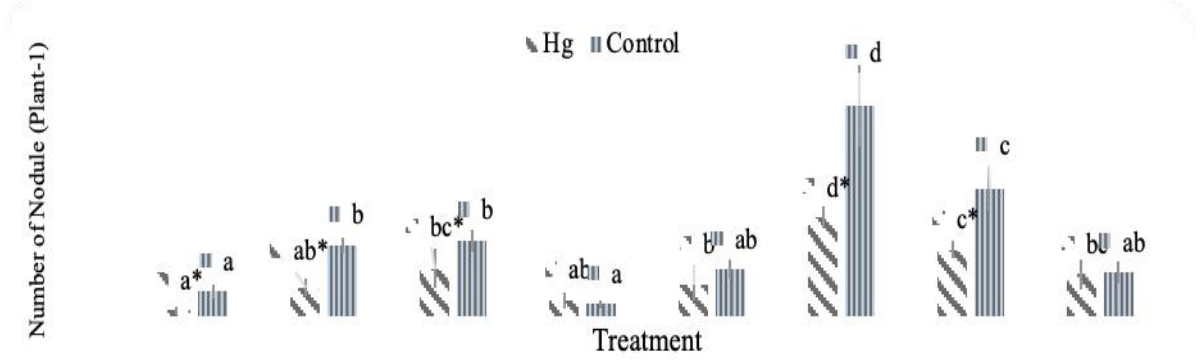

Figure 5. Number of Nodules. Value are means±SD obtained from 3 independent replication of the experiment. *indicates significant differences (P<0,01, ANOVA) of control. P-Mi = *P. falcataria* with AMF in sterile soil, P-Rb = *P. falcataria* without AMF in unsterile soil, P-MiRb = *P. falcataria* with AMF in unsterile soil, P-0 = *P. falcataria* without AMF in sterile soil, P-Mi C = *P. falcataria* with AMF in sterile soil + compost, P-Rb C = *P. falcataria* without AMF in unsterile soil + compost, P-MiRb C = *P. falcataria* with AMF in unsterile soil + compost, P-0 C= *P. falcataria* without AMF in sterile soil + compost.

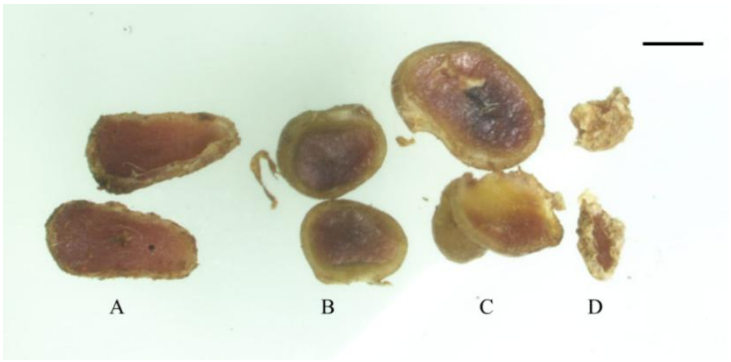

Figure 6. Nodule morphological Characteristic; (A) P-Mi C control, (B) P-Mi Control, (C) P-Mi C Hg, (D) P-Mi Hg. Bar = 0.5 mm. P-Mi = *P. falcataria* with AMF in steril soil, P-Mi C = *P. falcataria*with AMF in steril soil + compost.

Characteristics of nodules have a clear difference. Morphological observation showed that nodule from each treatment is different size and color (Figure 6), wherein the P-Mi Hg has a small shape and wrinkles, while in P-MiC Hg has a larger size than P-Mi Hg. Hg-condition has a color density lowest than the control. Root nodules were found in all treatments. Even though *Rhizobium* was not inoculated but we observed the significant number of the roots nodules, which implies that *P. falcataria* has endophytic bacteria, including *Rhizobium*. The data also showed that the treatment gave significant results ($P<0.05$) on the number of active nodules. We supposed that the bacterial isolate came from soil media because all treatments found active nodules. The bacterial isolates from soil media was differ morphologically. The difference in size and also the distribution of different colors shows a significant difference from each treatment. Nutrition has more influence on nodule growth. Bacteria need nutrients to grow, and when exposed to heavy metals, they have different characteristics. To determine the microorganisms in the roots, isolation was carried out using NA medium (Sodium agar) for the growth of bacteria. A bacterial colony grows from rhizosphere P-RbMi Hg, as shown in Figure 7.

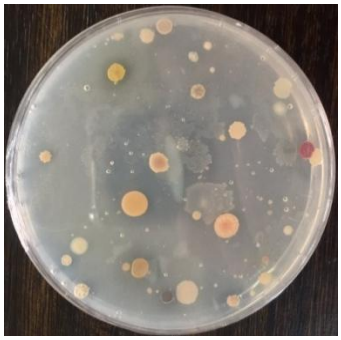

Figure 7. Bacterial Colony Morphology isolated from the rhizosphere of *P. falcataria*. Colonies circled by different colors show different shapes, colors, and surfaces morphology.

After three days of incubation, we obtained 11 isolates of rhizosphere microbes. They are having various shapes and colors (Figure 7 and Table 3). Isolation was carried out from two soils from the P-MiRb treatment contaminated and not contaminated with Hg. Both treatments were found to have root nodules. So it is very likely, from 11 types of bacteria,

there was one bacterium that forms an interaction with *P. falcataria*. While other bacteria have the possibility of influencing growth in polluted conditions. However, because the medium is not specific for *Rhizobium,* further analysis is needed to see *Rhizobium* in the soil and *P. falcataria*.

Table 3. Isolated microbes from P-MiRb contaminated and not contaminated Hg.

No.	Isolate code	Shapes	Color	Source	
				P-MiRb Hg	P-MiRb Control
1.	B1	Globular, ultrasmooth, smooth shiny surface	Pink	√	√
2.	B2	Globular, irregular, rough surface	Pink	×	√
3.	B3	Subglobular, ultrasmooth, smooth shiny surface	Yellow	√	√
4.	B4	Globular, irregular, smooth shiny surface	Milky white	√	√
5.	B5	Subglobular, ultrasmooth, smooth shiny surface	Pale brown	√	√
6.	B6	Subglobular, ultrasmooth, smooth shiny surface	Milky white	×	√
7.	B7	Globular, ultrasmooth, rough surface	Red	×	√
8.	B8	Globular, ultrasmooth, smooth shiny surface	Milky white	√	√
9.	B9	Globular, ultrasmooth, rough surface	Milky white	×	√
10.	B10	Subglobular, irregular, rough surface	Black	×	√
11.	B11	Globular, ultrasmooth, rough surface	Yellow	√	√

Discussion

Plants have different tolerance levels, tolerance of plants to mercury is variable (Utomo *et al.* 2014). Microorganisms reduce environmental toxicity levels of pollutants (Azubuike *et al.* 2016). Mycorrhiza form symbiotic associations with various types of plants, providing nutrients, protecting plants from root diseases, and protecting from environmental toxic conditions (Ferlian *et al.*, 2018). Mycorrhizal spores can live in any conditions and have a strong resistance due to a strong cell wall system. Germination of mycorrhizal spores that infect plant is very critical in the plant microbes association system. *Glomus* sp. has the ability to germinate quickly. Within three days, the hyphae have appeared (Figure 1), and on the seventh day mycorrhizal hyphae have spread in the soil. And the distribution of mycorrhizal hyphae on the seventh day, was the starting of intensive interaction between *Glomus* sp. and *P. falcaratia.* This proves that mycorrhizae have the ability to interact with various kinds of plants, including legumes (Budiastuti *et al.* 2021). Plants infected with mycorrhizae had higher height and stem diameters compared to plants that were not inoculated from mycorrhizae (Table 1). This emphasized the effectiveness of mycorrhizae in promoting *P. falcataria* growth. The success of mycorrhiza in infecting plants is also evidenced by arbuscules and vesicles in the root (Figure 3). Earlier study observed that mycorrhizae could increase plant growth within 42 days (Halder *et al.* 2015). The result ~~is~~ proves that *Glomus* sp. used in the pot experiments has fast properties to infecting the plant. This phenomenon is also observed on a plant grown in mercury-contaminated conditions. Plants with mycorrhizae showed higher biomass levels than plants without mycorrhizae (Table 2). Latifah *et al.* (2021) reported that the presence of indigenous bacteria in symbiosis of *Glomus* with *P. falcataria* made a sharp increase of plant growth, and *P. falcataria* has different responses to Hg-contamination. Seedling growth significantly increases when

contaminated with 10-20 ppm $HgCl_2$. The unique results are also obtained from the difference in the color leaves of plants (Figure 4). Data showed that there is a clear difference in the color of leaves. The P-Rb treatment has a 5GY 5/8 color and it has brighter leaf color than the other treatment. Based on the color chart for plant tissues (Wilde & Voigt 1977), the difference in one of the number hue, value, and chroma on the color chart result in very clear differences in the leaf color. It is suspected that there is interference of heavy metal, which makes the color of the leaves change. One of the signs of heavy metal toxicity in the plant is the changes in the color of leaves, hence hinder inhibiting the photosynthesis process, transpiration rate, and changes in chlorophyll (Chandra & Kang, 2016). Generally, mycorrhizae that live under stress will increase malondialdehyde, ion leakage, catalase, peroxidase, proline, and polyphenol oxidase level to break down nutrients bound to heavy metals (Eliaspour *et al.* 2020), especially P. Phosphate is easily immobilized in soil, hence plants cannot absorb soil-P. Mycorrhizae as well as bacteria have the ability to solubilize P in the soil.

Nodules found in the roots of *P. falcataria* prove the success of the bacteria to form symbiotic associations with plants. Naturally, legume plants are associated with *Rhizobium* by forming nodules on plant roots. Symbiosis of *Rhizobium* and AMF was already competing with each other, as we can see in P-Mi treatment reduced the number of nodule formation, while P-Rb treatment increased nodule formation in Hg-condition (Figure 5). Hg contaminated in the soil is expected to disrupt this molecular communication as well as inhibit their growth. Under Hg contamination, the nodule formation was disturbed, indicated by the changing of nodule characteristics (size, shape, and color) (Figure 6). These results implied that *Rhizobium* was not tolerant to mercury, and growth depended on nutrients status in soil. We try to find that difference by doing isolation in the *P. falcataria* rhizosphere (Figure 7). The symbiosis between rhizobia, mycorrhiza, and plant involving sophisticated and complex molecular communication (Chang *et al.* 2017). Understanding the symbiotic association between symbionts and their host is critical for the success of Hg-phytoremediation. Our finding was in line with Rossiana *et al.* (2019) that the combination of *Rhizobium* and AMF increased phytoremediation of heavy metals by *P. falcataria*. Other bacteria associated with *P. falcataria* also contribute to plant growth. The experiment was carried out with only the addition of mycorrhizae (inoculation), there was no addition of bacteria in the experimental pot, but all research samples found nodules.Therefore, these results prove that there were other bacteria in the plant that form the nodules. There are bacteria in the soil, including *Rhizobium* that activate the growth association with *P. falcataria*. Plants have their way of symbiosis with microorganisms. Plants can emit signals that are understood by microorganisms. With this signal, new microorganisms can penetrate plant roots and form a symbiotic mutualism with these plants (Yadav *et al.* 2015). One of the bacteria found has a character similar to *Rhizobium* (Table 3), which is round in shape, white in color, convex elevation, glossy smooth surface, including gram-negative (Sari *et al.* 2019). This similarity proves that of the bacteria found, there is one bacterium that activates a signal from the plant to form symbiosis to form *Rhizobium*, so that root nodules are formed.

Conclusion

Glomus sp. rapidly infect plant root resulted in increase the growth of *P. falcataria* seedlings by 30% higher than the control. Mycorrhizal infection reduces the formation of root nodule including *Rhizobium*. However, the interaction between mycorrhiza and *Rhizobium* benefitted *P. falcataria* growth under Hg-contamination. Understanding microbial ecology of plant microbiome in the rhizosphere of *P. falcataria* will enhance phytoremediation of Hg.

Conflict of interest

The authors state no conflict of interest from this manuscript.

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Author contributions

All authors have reviewed the final version of the manuscript and approved it for publication. IL, IMS, AK, INS designed the study; IL, II performed research and collected the data; IL analysed the data; IL, and IMS wrote - the paper. II, INS, TPN, AZNI, GR, FY, AK is the main contributor of this manuscript.

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