

Physiological and ultrastructural studies of *Jatropha curcas* and *Reutealis trisperma* in response to gold-mine tailings

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Abstract. Prasetya DN, Hamim, Sulistyaningsih YC. 2022. Physiological and ultrastructural studies of *Jatropha curcas* and *Reutealis trisperma* in response to gold-mine tailings. *Biodiversitas* 23: 3471-3479. Heavy metal contamination through tailings from mining pollutes the environment and harms human health. This study aimed to analyze physiological, especially ultrastructural changes and the photosynthetic activities of *Jatropha curcas* and *Reutealis trisperma* in response to gold mine tailings. *J. curcas* and *R. trisperma* were grown in 8 kg of polybags and treated with 0%, 50%, and 100% gold mine tailings for three months before examining their morphological, physiological, and leaf ultrastructure. The results showed that gold mine tailings with 50% and 100% concentrations affected the growth of both species. This was indicated by reduced plant height, total leaf number, shoot, root dry weight, and decreased chlorophyll and carotenoid content. Furthermore, treatment of gold mine tailing at 50% and 100% induced lipid peroxidation, indicated by the significant increase in leaf malondialdehyde content. The photosynthetic and transpiration rates at 90 days after treatment decreased with the increase in gold mine tailings concentration. The leaf ultrastructure analysis showed high-level tailings of treatment-induced ultrastructure alteration of *J. curcas* and *R. trisperma*. However, these changes did not significantly affect the photosynthesis rate, hence the plants survived until the end of the observation period. The overall effect of tailings on *R. trisperma* was lower than on *J. curcas*, suggesting that *R. trisperma* had higher adaptability than *J. curcas*.

Keywords: Gold mine tailings, *J. curcas*, photosynthesis, *R. trisperma*, ultrastructure

INTRODUCTION

In recent decades, urbanization and industrialization have led to several disturbances in the environment and among them heavy metal pollution is one of the serious problem. Industrial activities are playing a relevant role in pollution, such as producing waste and increasing pollution by the release of potentially toxic elements (Fiorentino et al. 2016). There are different sources of heavy metals in the environment such as natural, agricultural, industrial, domestic effluent, atmospheric, and other sources. The composition and concentration of heavy metals mainly depended on the rock type and environmental conditions. One source of environmental pollution that needs handling is heavy metals produced from gold mining industry activities because of the increasing demand for gold. The main process of gold mining activity is the separation of gold ores by crushing the rock. Generally, this activity produces a high amount of waste that contains rocks, sand, and dust known as tailings, with lower content of organic matter, and even in many cases gold mining is also contained various heavy metals (Mensah et al. 2015) such as Cu, Zn, Pb, Ag, Cd and Hg (Fashola et al. 2016; Hilmi et al. 2018).

Heavy metals can cause harmful effects because they can cause poisoning in living cells such as plants, animals, and even humans via food chain (Dziubanek et al. 2015; Augustsson et al. 2015). In plant, some heavy metals are

highly toxic because they inhibit plant growth, photosynthesis, and chlorophyll biosynthesis. Moreover, they alter water balance, nutrient assimilation, and senescence induction, causing plant death (Daud 2016; Khan 2016; Singh et al. 2016; Zhang et al. 2018). Many experiments found that heavy metals caused the levels of chlorophyll decreased (Sheetal et al. 2016), reduced plant growth, leaves damage, reduce dry weight (Kiran and Prasad 2017). Heavy metals have also been recognized to induce ROS production which caused cell membrane damage, decreased membrane fluidity, also inactivation of receptor, enzyme and ion channel in plant (Shahid et al. 2015). Variation in heavy metal toxicity depends on plant species, specific metal, concentration, chemical form and composition. The entry of metals into roots is considered the first and primary mechanism used by plants against the harmful effects of these metals (Jiang and Liu 2010). Therefore, these heavy metals should be handled and managed properly to reduce negative impact on environment.

Phytoremediation is a potential alternative method to remove contaminants from land or water (Hanks et al. 2015; Sarwar et al. 2017) using plants. Phytoremediation is specially used to remove metal contamination (Ali et al. 2013; Llugany et al. 2012; Fiorentino et al. 2016; Sorrentino et al. 2018). Heavy metals (HMs) are becoming a complex and challenging problem, since HMs cannot be degraded and can interfere with many metabolic processes, inactivating key enzymes. This method has been approved

as an effective and efficient method to reduce various contaminants, including several heavy metal pollutants (Edao 2017). There are several plants with certain characteristics that can be used as phytoremediator of heavy metals. According to Capuana (2011), plants useful as phytoremediation agents have several characteristics, such as fast growth, easy to harvest, and a deep rooting system. Some under-utilized biodiesel-producing plants such as *Jatropha* (*Jatropha curcas*) and candlenut (*Reutealis trisperma*) could be used in the phytoremediation process because they grow well on marginal lands and gold mine tailings (Hilmi et al. 2018; Andriya et al. 2019).

Previous studies found that *J. curcas* could be used for phytoremediation of soil or land contaminated with heavy metals (Edao 2017). *R. trisperma* has good adaptability and grows in marginal lands such as post-tin-mining soil containing heavy metals (Pranowo et al. 2015) and adapts to gold mine's liquid waste (Hamim et al. 2017). There are many studies on the physiological effect of heavy metals on *J. curcas* and *R. trisperma*. However, studies on physiology, especially photosynthesis and ultrastructural changes, are limited. Therefore, this study aimed to investigate the effect of gold mine tailing on physiology, especially photosynthesis and ultrastructural changes in *J. curcas* and *R. trisperma* under heavy metal stress.

MATERIALS AND METHODS

The experiment was conducted in the greenhouse of the Department of Biology, IPB University from October 2020 to March 2021. The observation was conducted in the field, while physiology analysis was conducted at the Laboratory of Plant Physiology and Molecular of the Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University. Moreover, the ultrastructural analysis was performed at TEM and Histology Laboratory, Eijkman Institute.

Materials

The plant materials used in the experiments were *J. curcas* and *R. trisperma* provided by Indonesian Crops and Refreshing Plant Research Institute, Parung Kuda, Sukabumi, Indonesia. Gold mine solid tailings were obtained from the tailing dam of Indonesian gold mining industry Aneka Tambang Inc. (PT. ANTAM) UBPE Pongkor, Bogor, Indonesia.

Procedures

Plant growing and treatment experiment

The experiment was conducted using a completely random design with two factors. The first factor was *J. curcas* and *R. trisperma*, while the second factor was three tailing concentrations in the growth medium of 0% as control, 50%, and 100% tailing. The young seedlings (one week old) were then transferred into polybags (10×15 cm) which had been filled with planting media of mixed soil and compost 1:1 (v/v) (Hamim et al. 2020b). The seedlings were watered every day to keep the soil moist. The seedlings of *J. curcas* and *R. trisperma* were grown for one

month before they were moved to larger polybag (20×30 cm) for approximately 8 kg of soil, then filled with soil and treated with different concentrations of gold mine tailings. At the beginning of treatment, each polybag was supplemented with 0.5 kg of compost and 0.5 g of NPK fertilizer (Hilmi et al. 2018), and their growth was observed for 3 months.

Plants growth parameters analysis

Shoot height, number of leaves, roots, and shoot dry weight were measured for growth parameters. The plant height was measured with a ruler from above the soil medium to the growing point. The number of leaves was measured by counting the leaves formed from the beginning of the treatment to the end of the observation period. Additionally, dry weight was measured by separating the roots and shoots and oven-drying for five days at 70°C.

Chlorophyll and carotenoid analysis

The upper fully expanded leaves were sampled to analyze photosynthetic pigment contents according to Quinet et al. (2012) to quantify chlorophyll and carotenoid. The 0.1 g of fresh leaves samples were pounded with mortar and given 10 mL acetone 80%. After extraction, the mixture was centrifuged (3000 g) for 10 min at 4°C. Absorbance was measured using a spectrophotometer with 470, 646, and 663 nm wavelengths. The calculations are according to Lichtenthaler's formula (1987).

$$\text{Chl a} = 12.25 (A_{663}) - 2.79 (A_{646})$$

$$\text{Chl b} = 21.50 (A_{646}) - 5.10 (A_{663})$$

$$\text{Chl total} = 7.15 (A_{663}) + 18.71 (A_{646})$$

$$\text{Carotenoid} = \frac{(1000)[A_{470} - 1.82 (\text{Chl a}) - 85.02 (\text{Chl b})]}{198}$$

Where:

A_{470,646,663} = the absorbance at the λ of 470, 646, and 663 nm

Lipid peroxidation analysis (MDA content)

Malondialdehyde (MDA) was carried out according to the method by Wang et al. (2013), with a few modifications. The 0.5 g of fresh leaves were pounded with mortar in 10 ml of 5% trichloroacetic acid (TCA), followed by centrifuged with speed of 3000 rpm for 25 min, at 4°C of temperature. Two milliliters of extract solution were taken and added with 3 mL of 0.5% thiobarbituric acid (TBA) in 5% TCA then mixed vigorously. The mixture was heated using a water bath at 80°C, 30 min. The cooled mixture was centrifuged for 25 min at 3000 rpm of speed and at 4°C. Absorbance of supernatant was measured at 450, 532, and 600 nm. The concentration of MDA was determined using the equation:

$$\text{CMDA} (\mu\text{mol/g BB}) = [6.45 \times (A_{532} - A_{600})] - [0.56 \times A_{450}]$$

Where:

A_{450,532,600} = the absorbance at the λ 450, 532, and 600 nm

Gas exchange analysis

Gas exchange was measured using the LI-COR EX4800 on fully expanded leaves with Photosynthetic Active Reaction (PAR) from 50, 100, 250, 750, dan 1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The parameters measured included photosynthesis and transpiration rates (Zhou et al. 2018).

Leaves ultrastructure analysis

The samples' ultrastructure was analyzed using transmission electron microscopy (TEM) with the preparation methods according to Cortadellas et al. (2012). Small pieces (1×1 mm) were taken from the fully expanded leaves and fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), then rinsed in the same buffer and post-fixed in 1% OsO₄ and then rinsed again with the same buffer. The fixed leaf segments were dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, and 100%) and followed by infiltrated with Spurr's resin under vacuum and room temperature. Ultrathin (70 nm) sections were cut using an ultramicrotome. Ultrathin sections were placed in a grid, coated with formvar in chloroform, and then stained. The sample was observed with the transmission electron microscope (TEM) 1010 (JEOL, Japan), 80.0 KV.

Data analysis

Data were analyzed using Analysis of Variance, and the means were compared by Duncan's Multi Range Test (DMRT) with $\alpha = 5\%$.

RESULTS AND DISCUSSION

The results showed that tailings significantly decreased plant height and the number of leaves in *J. curcas* but were not significant in *R. trisperma* (Table 1). Reduction of the plant height has occurred in all species ranging from 15.60% to 28.79%, except in *J. curcas*, where the decrease was only 9.50% due to 50% tailing. Furthermore, the

number of leaves decreased in all species ranging from 26.6% to 47.88%, except in *R. trisperma*, where the reduction was only 14.6% due to 50% tailing. The dry weight of root and shoot also decreased in the range of 16.2% to 35.9% for root, except in *J. curcas*, which showed a decrease of 15.1% due to 50% tailing. Shoot dry weight decreased by between 17.4% and 35.0%, except in *R. trisperma*, which showed a decrease of 16.6% due to 50% tailing. Gold mine tailing at 100% significantly reduced the growth of all the species, as shown in Figure 1. However, all the species grew well till the end of the observations, suggesting their adaptability. The reduction of growth in 100% of tailing treatment may be because gold mine tailing has a low content of organic compounds and important nutrient which support plant growth such as N, P, and K (Setyaningsih et al. 2017).

Table 1. Parameters of growth *J. curcas* and *R. trisperma* grew at different tailing concentrations

Parameters	Tailing conc.	Species	
		<i>J. curcas</i>	<i>R. trisperma</i>
Plant height (cm)	0%	70.50 ^a	17.30 ^c
	50%	63.80 ^a	14.60 ^c
	100%	50.20 ^b	12.84 ^c
Total leaves number (blade)	0%	82.60 ^a	15.00 ^c
	50%	60.60 ^b	12.80 ^c
	100%	46.40 ^b	12.40 ^c
Root dry weights (g)	0%	33.90 ^a	13.58 ^d
	50%	28.78 ^b	11.38 ^e
	100%	21.70 ^c	10.30 ^e
Shoot dry weights (g)	0%	92.41 ^a	31.18 ^d
	50%	76.25 ^b	26.00 ^e
	100%	60.02 ^c	20.58 ^f

Note: The numbers followed by the same uppercase letters at every column are not significantly different at $P \leq 0.05$, as determined by DMRT

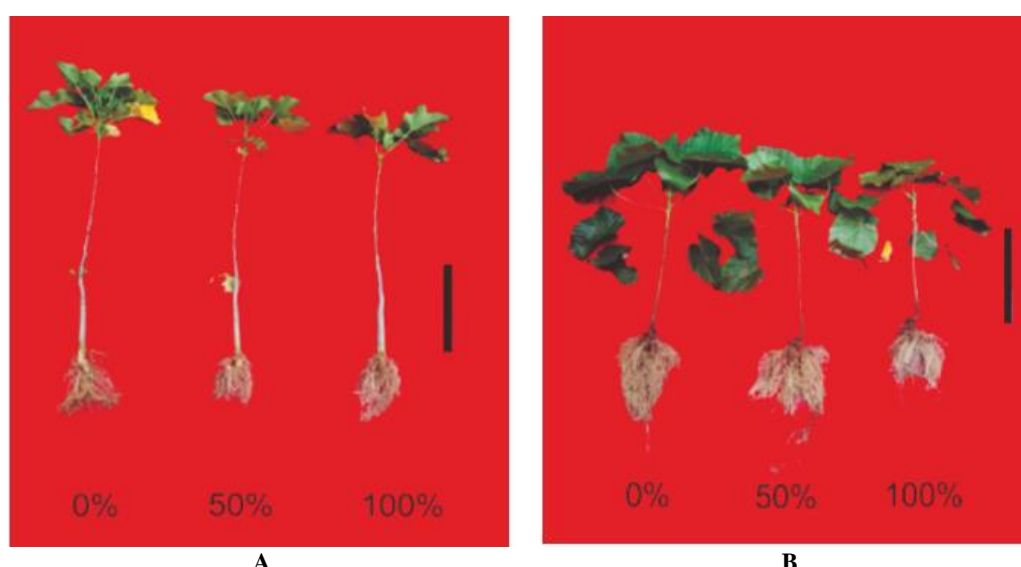


Figure 1. Morphology of *J. curcas* (A) and *R. trisperma* (B) after 90 days of gold-mine tailing treatments with different concentrations from left to right (0, 50 and 100% of tailing). Bar scale: 30 cm

Many studies also found that a low concentration of heavy metals in the growth medium decreased plant growth. According to Zhou et al. (2018), privet plant height decreased with increasing concentrations of given heavy metals. Guala et al. (2010), heavy metals are toxic to legumes because they can cause chlorosis, reduced plant growth, decreased productivity, and limited nutrient uptake. Andriya et al. (2019) also found that root and shoot dry weight decreased significantly in the four plants used, including *J. curcas* and *R. trisperma*, after treatment with 100% tailings.

Growth inhibition is a major common response when plants are under abiotic stress, including heavy metal stress (Malar et al. 2014). The presence of high amounts of non-essential heavy metals affect plant growth and development, damage leaves, and reduce the plants' dry weight (Kiran and Prasad 2017). Heavy metal toxicity also decreased enzyme activities due to metal binding to the enzyme's activation site, which ultimately led to growth inhibition (Asati et al. 2016). Nevertheless, both plants still survived until the end of the observations, which showed that these plants had good adaptability to gold mine tailings.

Chlorophyll and carotenoid content

Decrease in chlorophyll content is one of the most common responses to heavy metal stress in plants. Heavy metals cause the formation of oxygen radicals (ROS) (Najeeb et al. 2017) that inhibit the biosynthesis of photosynthetic pigment, cause damage to the membrane, and also reduce the amount of chlorophyll pigment (Aransiola et al. 2013). There are three main types of photosynthetic pigment, namely chlorophyll a, chlorophyll b and carotenoids. Chlorophyll a is an electron donor in the electron transport chain, and chlorophyll b functions to absorb higher frequency blue light for use in photosynthesis. Carotenoids are also present in chloroplast and serve as accessory pigment, regulating the interactions between plants and the environment by trapping solar energy and passing it to chlorophyll (Xie et al. 2019). The decrease of chlorophyll and carotenoids in *J. curcas* than *R. trisperma* occurred with increasing in gold mine tailings concentration, as shown in Figure 2. This suggests that the gold mine tailings treatment reduced chlorophyll and carotenoid content.

The decrease of chlorophyll a content occur in all plants by between 26.98% and 43.50%, except in *R. trisperma*, where the reduction was 20.08% due to 50% tailing, as shown in Figure 2. Chlorophyll b decreased by between 24.39% and 50.55% except in *R. trisperma*, where it only decreased by 16.02% due to 50% tailing. Consequently, gold mine tailings reduced all plants' total chlorophyll by 26.77% and 38.32%, except for *R. trisperma*, where the decrease was only by about 19.61% due to 50% tailing. Carotenoid content also decreased in the range of 19.11% to 34.50%, except in *R. trisperma*, which showed a reduction of only 15.77% due to 50% tailing. This result supports Sheetal et al. (2016), which found a decrease in chlorophyll a and b in *Brassica juncea* var. Pusa Jaikisan with 50 ppm heavy metal.

Chlorophyll and carotenoids have been shown to have major functions in photosynthesis, they act as conversion agent to change solar energy to become chemical energy. These pigments guarantee that plants are able to synthesize their own substances (Rai et al. 2016). Heavy metals such as Hg, Cu, Cr, Cd, and Zn reduce chlorophyll content in various plants (Aggarwal et al. 2012). This is because heavy metals replace the essential element, Mg^{2+} , in the chlorophyll molecule, causing excess ROS production and lipid peroxidation in the chlorophyll membrane (Kuzminov et al. 2013). Furthermore, some previous studies found that heavy metals could inhibit δ -aminolevulinic acid (ALA)-dehydratase and protochlorophyllide reductase, the two critical enzymes involved in chlorophyll biosynthesis (De Filippis and Pallaghy 1994). In line with this, Rascio and Navarie-Izzo (2011) showed that heavy metal poisoning disrupted electron transport in the chloroplast membrane.

Leaf Malondialdehyde (MDA) content

Lipid peroxidation in plants was determined by measuring MDA levels in the leaves. The results showed that the MDA content generally increased with tailings. The leaves treated with 100% tailings were found to have a higher MDA content, which increased by 29.09% and 25.00% in *J. curcas* and *R. trisperma*, respectively, as shown in Table 2. Hilmi et al. (2018) found that the MDA content in *R. trisperma* increased gradually with tailings in the growth medium.

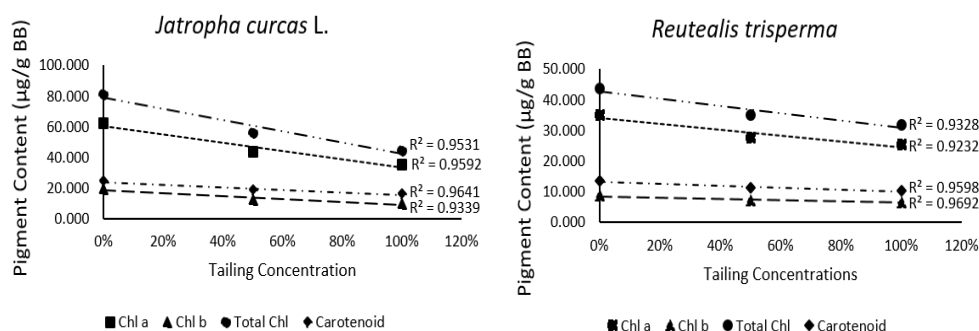


Figure 2. The effect of gold mine tailing treatment on photosynthetic pigments after 90 days. Chl-a: Chlorophyll a; Chl-b: Chlorophyll b; Total Chl: total chlorophyll

MDA is the cell's lipid peroxidation product when a plant undergoes abiotic stress, and it indicates the extent of oxidative stress (Hu et al. 2012). Toxic heavy metals inhibit the absorption of water and nutrients in plants, interfere metabolism, inhibit seed germination, and trigger the formation of Reactive Oxygen Species (ROS) due to increased oxidative stress (Gjorgieva et al. 2010).

Gas exchange parameters

Photosynthesis is an essential physiological aspect strongly influenced by temperature, drought, and heavy metal stresses. When plants are under stress, the diffusion of CO₂ decreases, the carboxylation reaction will be low, and the rate of photosynthesis will decrease. The plants' photosynthetic rate decreased 45 days after treatment (DAT) with PAR 1500 mol m⁻²s⁻¹. However, the 90 DAT measurements showed a significant decrease in the photosynthetic rate in both plants, as shown in Table 3.

Many plant species under heavy metal stress have shown decreased photosynthetic rates. Sheetal et al. (2016) showed that treatment of 200 mg/kg Pb could decrease the photosynthetic rate of *Brassica juncea* var. Pusa Jaikisan. Moreover, 0.5 mM Hg treatment reduced the rate of photosynthesis in *Paspalum conjugatum*, *Mikania micrantha*, *Cyperus kyllingia*, and *Brachiaria mutica* (Hamim et al. 2020a). Giannakoula et al. (2021) showed that treatment of 800 µM Pb, 800 µM Cu, 500 µM Cu+ Pb, also 800 µM Cu + Pb significantly decreased photosynthetic rate. Meanwhile, treatment of Cu and Cu+Pb with a concentration of 800 µM caused a significant reduction in transpiration rate.

The light curve analysis of the photosynthetic rate of plants with PAR 50, 100, 250, 750, and 1500 mol m⁻²s⁻¹ resulted in three groups with the lowest in plants with 100% tailings treatment. The second and third groups were 50% tailings and control treatment, respectively. The three groups on this curve indicate that the photosynthesis rate is likely to increase with PAR at a PAR of 1500 mol m⁻²s⁻¹, as shown in Figure 3.

The hormone abscisic acid (ABA) in the roots is transported through the xylem vessels to signal that the plant is under stress. The accumulation of ABA in the leaves causes the stomata closure, reducing the diffusion of CO₂, carboxylation efficiency, the rates of photosynthesis, and transpiration (Grant 2012; Zlatev and Lidon 2012). The results at 45 DAT with PAR of 1500 mol m⁻²s⁻¹ showed a decrease in the transpiration rate of plants treated with tailings 50% and 100%. In contrast, the results at 90 DAT observations showed a significant decrease in transpiration rate in both plants, as shown in Table 4.

Vassilev et al. (2011) found that the cause of inhibition of photosynthesis and transpiration rates in metal-treated legumes is due to a decrease in the intercellular space in the leaf mesophyll. These changes inhibit the diffusion of CO₂ to the chloroplasts and cause the low carboxylase activity of rubisco, limiting CO₂ absorption (Dias et al. 2013). Heavy metal content that exceeds the optimal limit inhibits the rate of photosynthesis, chlorophyll synthesis, water, and nutrient absorption, and enzyme activity (Asati et al. 2016). According to Aggarwal et al. (2011), the toxic effects of heavy metals affect the photosynthesis process directly or

indirectly. The direct impact is inhibition of the light reaction and decreased NADP production, while the indirect effect is the inhibition of chlorophyll synthesis.

In addition, according to Devi and Prasad (2004), high concentrations of heavy metals can affect photosynthesis by inhibiting electron transport in PS I and PSII. PS I is a membrane-bound protein complex that catalyzes the oxidation of plastocyanin and reduction of ferredoxin under light conditions. A site of heavy metal toxicity was found on the reducing side of PS I, such as mercury interacting with the donor side of PS I and with plastocyanin (Myśliwa-Kurczel et al. 2013) and also Ferredoxin was found to be the place of Hg²⁺ and Cu²⁺ action (Šeršeň and Kráľová 2013). PS II is a multisubunit pigment-protein complex and that leads to the release of electrons, protons, and molecular oxygen, and most heavy metals inhibit PS II activity (Fodor 2013). The photosynthetic electron transport at the level of PS II both at oxidizing and reducing sides is effectively inhibited by different heavy metals. Heavy metals may impair the functions of PS II directly via the plastoquinone pool or indirectly via feedback regulation by inhibition of the photosynthetic carbon reduction cycle enzymes and changes in ATP level (Rai et al. 2016).

Table 2. Levels of malondialdehyde (MDA) *J. curcas* and *R. trisperma* grew at different tailing concentrations

Parameters	Tailing concentrations	Species	
		<i>J. curcas</i>	<i>R. trisperma</i>
Malondialdehyde (µmol/g BB)	0 %	0.39 ^d	0.84 ^b
	50 %	0.45 ^d	1.06 ^a
	100 %	0.55 ^c	1.12 ^a

Note: The numbers followed by the same uppercase letters at every column are not significantly different at P≤0.05, as determined by DMRT

Table 3. The photosynthetic rate of *J. curcas* and *R. trisperma* grew at different tailing concentrations with PAR 1500 mol m⁻²s⁻¹

Tailing concentration	Photosynthetic rate (µmol CO ₂ m ⁻² s ⁻¹)			
	<i>J. curcas</i>		<i>R. trisperma</i>	
	45 HSP	90 HSP	45 HSP	90 HSP
0%	28.10 ^a	29.57 ^a	23.34 ^a	27.75 ^b
50%	27.47 ^a	27.31 ^b	22.59 ^a	26.45 ^c
100%	26.62 ^a	25.44 ^d	22.35 ^a	25.79 ^d

Note: The numbers followed by the same uppercase letters at every column are not significantly different at P≤0.05, as determined by DMRT

Table 4. The transpiration rates of *J. curcas* and *R. trisperma* grew at different concentrations of tailing with PAR 1500 mol m⁻²s⁻¹

Tailing concentration	Transpiration rate (mmol H ₂ O m ⁻² s ⁻¹)			
	<i>J. curcas</i>		<i>R. trisperma</i>	
	45 HSP	90 HSP	45 HSP	90 HSP
0%	5.49 ^a	5.79 ^a	5.86 ^a	5.75 ^a
50%	5.13 ^a	4.46 ^d	4.90 ^a	5.07 ^b
100%	4.81 ^a	3.31 ^e	4.74 ^a	4.68 ^c

Note: The numbers followed by the same uppercase letters at every column are not significantly different at P≤0.05, as determined by DMRT

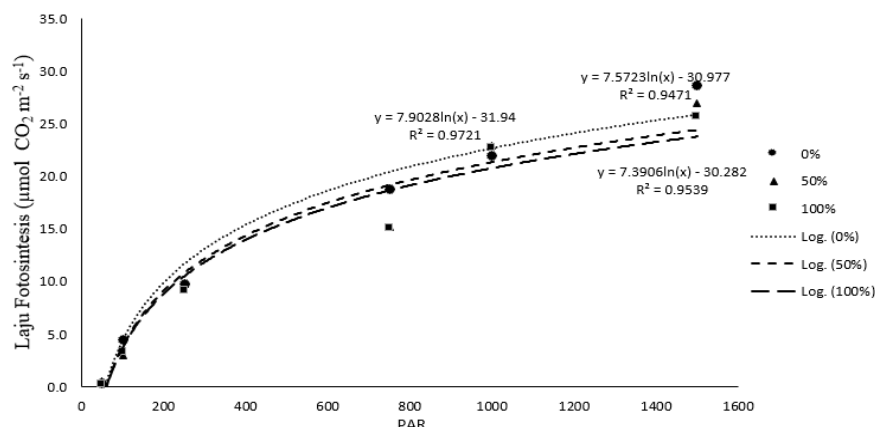


Figure 3. The light curve of the photosynthetic rate of *J. curcas* and *R. trisperma* treated by gold mine tailings with concentrations 0%, 50%, and 100% for 90 days with different PAR (from 50 to 1500 mol m⁻²s⁻¹)

The photosynthesis process in both species kept running, with only a slight decrease, and the plants survived until the end of the observation period. Gold mine tailings have a relatively high Pb content than non-essential heavy metals (Hilmi et al. 2018). Pb levels in the tailings and soil were 63.31 ppm and 13.44 ppm, respectively (Andriya et al. 2019). Therefore, these results are consistent with Hamim et al. (2020a), which found that treatment of Pb up to 0.5 mM in water culture did not significantly affect the photosynthetic rate in *P. conjugatum*, *M. micrantha*, *C. kyllingia*, and *B. mutica*. However, the rate significantly decreased when Hg was treated with the same concentration, indicating the adaptability of the two plant species to gold mine tailings.

Leaves mesophyll ultrastructure

Abnormalities in cell internal structure due to the effects of heavy metal stress on some plant species have been reported. These changes occur due to direct interactions between heavy metals and structural components or as an indirect impact of changes in metabolic processes caused by metal stress (Barceló and Poschenrieder 2004). In this research, some changes in internal cell structure have occurred on both plant species treated with 100% tailing. Tailings treatment seems to have a different effect on the two plant species studied. The level of damage of leaf mesophyll cells in *J. curcas* due to 100% tailings treatment was more severe than that in *R. trisperma* cells. In control plants grown on media without tailings, leaf mesophyll cells appeared normal with cell organelles such as nucleus, vacuole and chloroplast were well developed. Some starch grains were also present in the chloroplast, as shown in Figures 4A and 4B. In *J. curcas* treated with 100% tailings, severe damage was observed. Cells underwent disorganization, plasma membrane rupture occurred to lead to plasmolysis, some organelles including

the nucleus and vacuole disappeared. The chloroplast membrane was severely damaged, most of them shrunk without any starch grains inside. In addition, electron dense deposits suspected as metal accumulation were also detected on the cell wall surfaces, as shown in Figure 4C. In *R. trisperma*, the mesophyll cells were also damaged, but the cell membrane is still intact without any rupture, normal chloroplast containing starch grains were observed. In some cells nucleus was destroyed and the vacuole was swollen. Deposits suspected of metal accumulation were also found on the surface of the cell wall, as shown in Figure 4D.

Tailing is main waste of gold mining process and has a high content of heavy metals (Fashola et al. 2016). Hilmi et al. (2018) found that tailings contained a variety of heavy metals classified as both essential and non-essential elements for the plants. Some essential elements were contained in the tailings such as Fe, Mg, Mn, Zn, Co, Cu, and Mo. Furthermore, non-essential heavy metal elements are also contained in the tailings such as Pb, Ag, Cd, and Hg. Baruah et al. (2016), showed that 15 mg L⁻¹ Cd in *M. hastata* caused the thylakoid structure to become smaller and suffer severe damage and swelling of the chloroplast. Małkowski et al. (2019) showed that Cd metal with concentrations of 1 and 10 mM caused membrane damage and plasmolysis and decreased the number of mitochondria. Daud et al. (2013), showed that 100 M cadmium (Cd) caused changes in cell membrane structure and the nucleus, vacuole, chloroplast, and mitochondria. Samardjieva et al. (2015) also showed that Zn metal stress damages cell structures such as the nucleus, vacuole, and the presence of Zn deposits in the vacuole. The other study, the increase in the number and size of vacuoles, were also detected (Hamim et al. 2018), such as transgenic cotton cultivars exposed to Cd which have greater number of vacuoles and enlarge vacuoles (Daud et al. 2009).

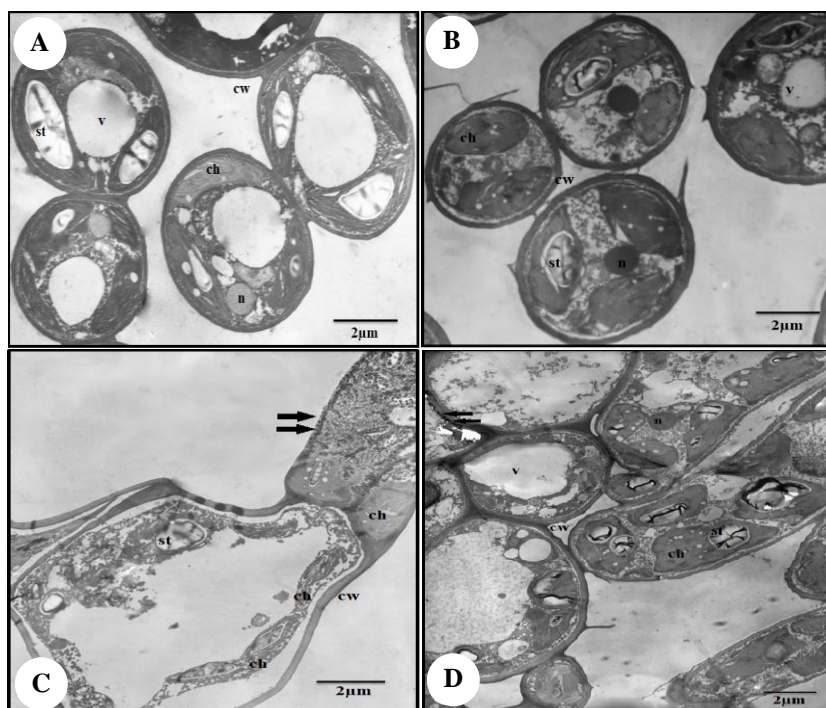


Figure 4. Mesophyll cells of *J. curcas* (A) and *R. trisperma* (B) without tailings, *J. curcas* (C) and *R. trisperma* (D) with 100% tailings. cw: cell wall; st: starch grain; ch: chloroplast; n: nucleus; v: vacuole, (bar length = 2µm). Black arrow shows metal deposits

Heavy metal toxicity caused ultrastructural changes in mesophyll cells of *J. curcas* and *R. trisperma*. Oxidative stress due to excess ROS causes plasma membrane disruption in root cells, the disorganization of thylakoid membranes in foliar mesophyll cells, and the destruction of mitochondria in root and leaf cells, damaging lipids and structural proteins (Anjum et al. 2015). In this study, plant subjected to 100% gold mine tailing underwent cell disorganization and some organelles were damaged. Nucleus and vacuoles disappeared, the structure of chloroplasts shrunk or slightly swollen, as shown in Figure 4C and 4D. In addition, Cd-induced production of ROS promoted oxidative burst in *C. pteridoides* that disrupts chloroplast (Bora et al. 2020). The damage of the chloroplast structure caused the reduction of chlorophyll content in the treated tissues. Chloroplasts are abiotic stress-sensitive organelles with a high level of unsaturated fatty acids, such as linoleic and linolenic acids, in their membranes (Sha et al. 2019). The decrease of chlorophyll when plants undergo abiotic stress, including heavy metal stress, caused chlorosis in leaf.

The ultrastructural analysis showed that both plant species accumulated material suspected to be heavy metals in the cell walls. Cell wall can effectively biosorbed and acts as a suitable storage reservoir for excessively accumulated metals (Parrotta et al. 2015). According to Pourrut et al. (2013), in addition to the cell wall, metal deposits were also found in vacuoles, and intercellular spaces. Small deposits were reported to be detected in other organelles such as mitochondria, nuclei and chloroplasts (Sharma and Dubey 2005). The deposition of heavy metal in cell walls is considered an efficient strategy for plants to

tolerate this metallic element. The compartmentalization and sequestration of heavy metal into the cell wall reduces the level of toxic metal in the cytosol. Additionally, it is a potentially important mechanism for heavy metal detoxification and tolerance. The cell wall acts as a cation exchanger, holding variable metal quantities and excluding others. Therefore, they bind heavy metal ions, inhibiting their diffusion into the cell (Fu et al. 2011). Furthermore, the vacuolization that occurred could have alleviated heavy metal toxicity by sequestering the metal inside the vacuoles. In this case, vacuoles are a major sink for heavy metal accumulation in plants subjected to metal stress (Vaculik et al. 2015; Adil et al. 2020). Storage and localization of Cd as electron-dense precipitates in the vacuoles of root cells of *M. hastata* treated with Cd may help in Cd detoxification (Sharma et al. 2016).

Decreased water absorption affects metal toxicity due to decreased cell wall extensibility or elasticity by cross-linking the pectin carboxyl groups with heavy metals. Metal stress is also suspected to cause the hampering of plant hormones, especially auxins (Ronzan et al. 2018). The decline of photosynthesis indirectly affects cell enlargement, which also has an important role in plant growth because the capacity of plant growth is determined by cell enlargement and expansion. These changes lead to the death of most cells and significantly affect plant growth because the nucleus was disappeared so cell division is disturbed and the growth will slow down.

The tailings treatment on *J. curcas* and *R. trisperma* with 50% and 100% concentrations decreased morphological and physiological parameters and increased malondialdehyde (MDA) levels. The 100% tailings treatment had a more

significant negative impact on the two plant species than the 50% tailings treatment. Specifically, the 100% tailings treatment-induced changes in leaf ultrastructure in *J. curcas* and *R. trisperma*. The negative effect of tailings treatment on *R. trisperma* was lower than on *J. curcas*, suggesting that *R. trisperma* has greater adaptability than *J. curcas*.

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