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Growth, histochemical and physiological responses of non-edible oil producing plant (*Reutealis trisperma*) to gold mine tailings

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Abstract. *Hilmi M, Hamim H, Sulistyaningsih YC, Taufikurahman. 2018. Growth, histochemical and physiological responses of non-edible oil producing plant (*Reutealis trisperma*) to gold mine tailings. Biodiversitas 19: 1294-1302. Reutealis trisperma* (Blanco) Airy Shaw is a non-edible biodiesel producing plant that is able to grow well in various unfavorable environmental conditions. The study aimed to analyze the growth, physiological, and anatomical responses of *R. trisperma* to gold mine tailings. Three-month-old of *R. trisperma* were grown in 8 kg of polybags contained with mixed soil-compost medium treated with 0, 25, 50 and 100% of gold mine tailings for 3 months. Root and shoot growth, physiological and anatomical characters, and histochemical analysis of Pb inside the roots and leaves were examined. The root and shoot growth as well as chlorophyll a and b contents of *R. trisperma* grown in sole gold mine tailing at 100% significantly decreased, while at the lower concentration of gold mine tailings, the decrease of the growth performances was not significant, or even increased shown in that of 25% of tailing treatment. The treatment of gold mine tailing at 100% also induced lipid peroxidation, indicated by the significant increase in malondialdehyde (MDA) contents in the root as well as the leaves. Histochemical analysis showed that accumulation of Pb occurred both in roots as well as in leaves of *R. trisperma* treated with 100% of tailings. High-level tailing treatment also induced anatomical alteration in roots as well as leaves of the species. These results indicated that gold mine tailings induced oxidative stress in roots and leaves of *R. trisperma* resulted in growth inhibition.

Keywords: Gold mine tailings, heavy metals, histochemical analysis, MDA, *Reutealis trisperma*

INTRODUCTION

Utilization of renewable energy resources is encouraged to prevent energy scarcity since the consumption of energy from fossil fuel is increasing every year, while its availability is limited. Biodiesel is one of most prospective renewable energy resources (Manzanera et al. 2008), because there are many species that produce high content of oil including from edible as well as non-edible oil-producing plants (Karmee et al. 2005; Haldar et al. 2009; Kumar and Sharma 2011). *Reutealis trisperma* (Blanco) Airy Shaw, is one of non-edible oil-producing plants which has a good prospect as biodiesel feedstock in Indonesia due to some superior characteristics including high seeds production and oil content, large canopy, deep root system, and able to grow on critical lands such as sloping land, acidic, dry, and even infertile land, which very useful for reclamation of critical lands (Herman et al. 2013).

Indonesia has about 24.3 million ha of critical land (BPS 2013), one of which was due to mineral mining activities. The operation of industrial and small-scale mining disrupted soil horizons and structure, soil microbe populations, and nutrient cycles (Kundu and Ghose 1997). In addition, mining activities usually produces a large amounts of waste (tailings) contains fine rocks, sand, and dust, with very low organic matter, and in many cases especially for gold mining it also contains heavy metal components (Mensah et al. 2015) such as As, Cd, Ni, Pb, Cu, Zn, Co and Hg (Hidayati et al. 2009; Fashola et al. 2016; Setyaningsih et al. 2017). Mining wastes can cover large areas of mine land, which reduce soil productivity (Mensah et al. 2015). Therefore, land reclamation is strongly required to restore the quality and productivity of degraded land due to mining activities. *R. trisperma* is a good plant candidate to be used for reclamation planning, because of its characteristics (Herman et al. 2013). It has deep roots systems and produces high amount of organic matter, which is necessary to accelerate the reclamation process (Sheoran et al. 2010). Organic matters can improve soil fertility through microbe activities (Mimmo et al. 2004) by decomposing mineral and organic matters and also promoting plant growth (Beneduzi et al. 2012; Altuhaish et al. 2014; Grobelak et al. 2015).

The elevation of heavy metals content which normally common in gold mine lands generally produces common toxic effects on plants, such as inhibition of growth and photosynthesis, chlorosis, the alteration of water balance and nutrient assimilation, and senescence induction, which ultimately caused plant death (Singh et al. 2016). All these effects are related to the disturbance of biochemical, and molecular changes in plant tissues and cells, as well as ultrastructure alterations because of the presence of heavy metals (Gamalero et al. 2009).

Previous research showed that *R. trisperma* is able to grow well on critical land including sloping land, dry-acid land, and even degraded lands in the area of post tin-mining (Herman et al. 2013). In addition, this plant was also able to grow well in water culture containing high concentration of cyanide from gold-mine wastewater (Hamim et al. 2017[a]). However, this plants has never been grown in the area of gold mine tailing which normally has many obstacles especially due to higher heavy metal content. Therefore this study was aimed to investigate the effect of gold mine tailing on growth, histochemical and physiological properties of *Reutealis trisperma* (Blanco) Airy Shaw.

MATERIALS AND METHODS

The experiment was carried out in field laboratory of Department of Biology, Bogor Agricultural University, Indonesia using 8 kg of polybag from July 2016 until February 2016. The analysis of physiology, anatomy and histochemical was conducted at Laboratory of Plant Physiology and Molecular, and Laboratory of Plant Anatomy of the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, Indonesia.

Materials

Plant materials used in this experiment were 3-month old of *Reutealis trisperma* obtained from Research Institute for Industrial and Refreshment Crops, Ministry of Agriculture, Republic of Indonesia. The media used in this experiment consisted of a mixture of soil and compost (4: 1). Goldmine tailing was obtained from tailing dam of Indonesian gold-mine industry Aneka Tambang Inc. (PT ANTAM) UPBE Pongkor, Bogor, Indonesia.

Analysis of tailing content

The metal content in the tailings was analyzed using atomic absorption spectrophotometry method (AAS). Tailings samples were dried at 105°C for 1 day. The dried samples were then crushed using porcelain mortar and pestles. Five grams of samples were fed into 250 mL Erlenmeyer, followed by the addition of 5 mL of concentrated HNO_3 and 50 mL of distilled water. The samples were destructed using a heat mantle to form a clear solution and their volume became 10 mL, which were then filtrated using Whatman paper No.41. The filtrate of the samples was diluted to 50 mL, and the concentration of metals in the samples were analyzed by atomic absorption spectrophotometer (AAS) AA7000 (Shimadzu, Japan).

Plant growing and treatment experiment

Three-month-old of *Reutealis trisperma* seedlings were transplanted to 8 kg capacity of polybags filled with media contained a combination of soil and different concentrations of gold mine tailings. The composition of each treatment was as follow: (i) 100% soil (S) and 0% tailings (T) (as control), (ii) 25% S and 75% T, (iii) 50% S and 50% T, and (iv) 100% T and 0% S.

During the planting, each polybag was supplemented with 0.5 kg of compost and 0.5 g of NPK fertilizer (6: 6: 6). The plants were then well maintained and watered every 3 days when there was no rain. *R. trisperma* plants were grown for 3 months for further growth, anatomical and physiological analysis.

Growth parameters analysis

For growth parameters, the measurement of root and shoot dry weight, root length, shoot height, and leaf area was carried out. Dry weight was calculated by weighing separately the roots and shoot after drying for 3 days using the oven at temperature of 70°C. Root length and shoot height were measured using 50 cm ruler. Leaf area measurements were performed using a digital image analysis method according to Schneider et al. (2012), where the whole leaves were scanned using a printer scanner HP 1050 (Hewlett Packard-USA) with a resolution of 300 dpi and the area of images then were measured using ImageJ (National Institute of Health, USA).

Determination of photosynthetic pigment contents

After 3 months, the upper fully expanded leaves were sampled for analysis of photosynthetic pigments contents according to Quinet et al. (2012) to quantify chlorophyll (Chl *a* and Chl *b*) and total carotenoid (xanthophyll + b-carotene). The 0.1 g of fresh weight frozen samples were ground in a pre-chilled mortar in the presence of 10 mL cold acetone 80% (Merck, Germany). After the extraction was completed, the mixture was centrifuged (3000*g*) for 10 min at 4°C. The absorbance of the supernatant was read at 663, 646 and 470 nm using Thermospectronic Genesys 20 spectrophotometer (Thermo, USA), and pigment concentrations were calculated according to formula from Lichtenthaler (1987).

$$\text{Chl } a = 12.25\ A663 - 2.79\ A646$$
$$\text{Chl } b = 21.50\ A646 - 5.10\ A663$$
$$Cx{+}c = (1000\ A470 - 1.82\ Ca - 85.02\ Cb) / 198$$

Where :
Chl *a* = Chlorophyll a
Chl *b* = Chlorophyll b
Cx+c = Total carotenoids
A663 = The absorbance at the λ of 663 nm
A646 = The absorbance at the λ of 646 nm
A470 = The absorbance at the λ of 470 nm

Determination of malondialdehyde (MDA)

MDA content was estimated based on the corrected TBA method by Hodges et al., (1999) with a little modification. Fresh functional leaves (0.5 g) were taken and ground in 5 mL 5% TCA (Merck, Germany) extraction solution, followed by centrifugation at 2500*g* for 30 min at 4°C (Heraeus Labofuge 400R, Germany), and the supernatant is the MDA extract solution. Two milliliters of extract solution and 3 mL 0.5% TBA (Merck, Germany) including 5% TCA were mixed vigorously. The mixture was heated at 80°C in constant temperature water bath for 30 min and was then cooled to room temperature. Cooled mixture was centrifuged at 2500*g* for 30 min at 4°C, and finally the supernatant of the mixture was detected at 450, 532 and 600 nm. The concentration of MDA was determined using the formula:

$$\text{CMDA } (\mu\text{mol mL}^{-1}) = 6.45 \times (\text{D532-D600}) - 0.56 \times \text{D450}$$

Where: D450, D532, and D600 are the absorbencies at 450, 532 and 600 nm.

Analysis of roots and leaves anatomy

Anatomical studies were carried out to observe the transversal section of roots and leaves taken from *R. trisperma,* which were grown in media without tailings (as control) and with 100% tailing treatment. Samples for observation were prepared using standard freehand sectioning (Ruzin 1999). The sections were cut with smooth stokes and transferred from the blade into a microscope slide and stained with 1% safranin solution. Stained samples were observed using Olympus CX-23 light microscope and the picture of the samples was taken using Optilab® camera.

Histochemical analysis of lead (Pb)

The observation of lead accumulated by plant tissues was carried out using sodium rhodizonate according to the method proposed by Tung and Temple (1996) with some modification. Samples for observation were prepared with standard freehand sectioning according to Ruzin (1999). Freshly cut thin sections of the samples were soaked in sodium rhodizonate staining solution for 60 minutes. To improve the staining effects, the cut thin sections were soaked in light green 0.1% for 2 minutes. Stained samples were observed using Olympus CX-23 light microscope and the picture of the samples was taken using Optilab® camera.

Analysis of root and leaves ultrastructure

The analysis of ultrastructure of the samples was carried out using transmission electron microscopy (TEM) with the preparation methods according to Cortadellas et al. (2010). Root segments of about 2 mm in length were cut for about 1 cm above the root tip, and leaf rectangular segments (1 × 2 mm) were sectioned from a fully expanded leaf, which was then immediately transferred to 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for overnight fixation. After rinsing three times with the same buffer for 15 min each, the samples were post-fixed in 1% OsO_4 in the same buffer for 2 hours, followed by washing three times in the same buffer. Segments were dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and 100%) and were then infiltrated with resin overnight. Ultrathin (80 nm) sections were cut using ultra-microtome Reichert S Ultracut (Leica, Austria). Ultrathin sections were mounted on copper grids and then observed with transmission electron microscope (TEM) JEM 1010 (JEOL, Japan).

Statistical analysis

Statistical analysis was carried out by one-way ANOVA using SPSS 19.0 statistical software and the means were compared by Duncan's Multi Range Test (DMRT) and independent-samples T (non-factorial) test at the 5% probability level.

RESULTS AND DISCUSSION

The tailings used in this experiment contained very low organic matter (<1%) and low macro elements such as N, P, and K with the pH of 7.0. The tailings also contained a variety of metals classified as both essential and non-essential elements for the plants (Table 1). Some essential elements were abundant in the tailings with the concentration were listed according to the highest as follow: Fe > Mg > Mn > Zn > Co > Cu > Mo. In addition, some non-essential (heavy metals) elements were also contained in the tailings including Pb, Ag, Cd, and Hg (Table 1).

Tailing was derived from the processing of mineral rocks and ores in gold-mine industry involving milling, washing and other processes, which causes the lower content of organic matter and higher mineral elements. The high content of heavy metals in the tailings is caused by leaching of minerals and chemical compounds that are normally used to separate gold from their rocks and other minerals. In addition, during the extraction process of gold, heavy metals that are naturally contained in the rock minerals were washed (Fashola et al. 2016).

After 90 days of treatment using gold mine tailings with different concentration, the response of *R. trisperma* was varied depended on the tailing concentration and the type of parameters that were analyzed. This result will present and discuss the response of the plant to the tailings treatment started from morphological (growth) followed by physiological and anatomical characters, and finalized by histochemical analysis.

Table 1. The content of essential and non-essential elements of gold mine tailing

Essential elements	**Mg**	**Fe**	**Mn**	**Zn**	**Co**	**Cu**	**Mo**
Concentration (ppm)	3962.71	10348.15	1791.46	22.64	3.59	1.18	<0.005
Non-essential elements	**Pb**	**Ag**	**Cd**	**Hg**			
Concentration (ppm)	93.59	13.36	1.26	0.064			

Growth inhibition is a common response of the plants subjected to abiotic stress including heavy metals stress (Malar et al. 2014). The results of this study showed that plant growth was affected by gold mine tailings for 90 days, especially at high concentration (100% of tailings) (Table 2). All growth parameters except for plant height apparently increased at the 25% of tailings treatment, and at 50% of tailing, while at 100% of tailing treatment, all parameters were decreased significantly, except for plant height (Table 2). The decrease of root and shoot dry weight was 59.6% and 45% respectively, root length was 41.7%, and leaf area was 28%, while plant height was only 19.6%. The decrease of growth in 100% of tailing treatment may associate to the characteristics of gold mine tailing that has lower organic compounds and other major nutrient elements such as N, P and K, which are required to support plant growth (Setyaningsih et al. 2017). For the plants treated with 100% of tailings, the source of macronutrient compounds was obtained only from the compost (0.5 kg) provided at the beginning of the treatment to support the growth for 90 days, while the other treatments the plants also got support from the media (mixed soil and compost). In addition, nutrition shortage may happen during 90 days of growth. This is because the tailings also contained heavy metal especially Pb at moderate level (Table 1), which may have negative effect to the plant growth. Heavy metals such as Pb have negative affects to the plant metabolisms particularly photosynthesis, through inhibition of photosynthetic enzymes as well as chlorophyll biosynthesis, and caused the damage of chloroplast membrane, which interfered photosynthetic electron transport (Anggarwal et al. 2011). Heavy metals toxicity also reduced roots ability for nutrients and water uptake (Poschenrieder and Barceló 2004), reduced enzyme activities due to metal binding to the activation site of enzyme (Asati et al. 2016), which ultimately lead to growth inhibition (Pendias 2001).

Based on growth parameters, *R. trisperma* treated with a low concentration of tailings had an increased growth (Table 2). This could happen because gold mine tailings used in the experiment contained higher concentration of some essential nutrients such as Fe, Mg, Mn, Zn, Co, Cu, Mo (Table 1). These metals are involved in essential biological functions, and therefore have positive biological effects in terms of growth and crop productivity, even though the excessive amount of these metals both in the growth media or plant body can generate toxic effects to the plants (Shahid et al. 2015). The other metals such as Pb, Cd, Hg and Ag which are non-essential heavy metals (Asati et al. 2016) and also contained in the tailings may not have negative impact to *R. trisperma* plants at lower (25%) tailing treatments. This data suggested that *R. trisperma* was tolerant to gold-mine tailings indicated by favor growth under 25 and 50% of gold mine tailings without any toxicity symptom such as chlorosis or necrosis in their leaves.

In agreement with this experiment, many studies also reported that low concentration of heavy metals in the growth media increased plant growth, while the higher concentration caused the decrease of plant growth. Jadia and Fulekar (2008) who worked with sunflower, for example, showed that that the treatment of heavy metals (Pb, Cd, Ni, and Cu) at 10 and 20 ppm improved root and shoot dry weight of sunflower, however the dry weight of the plants decreased gradually as the concentration of heavy metals in the media increased up to 40 and 50 ppm. In *Jatropha curcas,* Shu et al. (2011) also found that the treatment with low concentration of Pb treatment (0.5 mM) increased leaves area of the plants, while higher concentrations (1, 2, 3 and 4 mM) resulted in significant reduction of leaves. The reduction of roots and shoot due to heavy metals application has been reported in many other studies (such as Surjendu et al. 2007; Daud et al. 2009; Chaves et al. 2011; Li et al. 2004).

To analyze the effect of tailing treatment to physiological parameters, photosynthesis pigments of the leaves and malondialdehyde content of roots and leaves were investigated. As presented in Figure 1, photosynthetic pigment contents of leaves especially chlorophyll a and b decreased significantly in the tailing treatment at 100%, while carotenoid content did not change in response to the treatments. The decrease of chlorophyll b due to 100% tailing treatment was more (46,3%) than that of chlorophyll a (20.5%). This result was in agreement with the previous study conducted by Singh and Tewari (2003) who described that the content of chlorophyll b in *Brassica juncea* treated with Cd was lower than the content of chlorophyll a due to the higher degradation of chlorophyll b and lower interconversion of chlorophyll b from chlorophyll a. Interestingly, the treatment with 25% of tailing significantly increased chlorophyll a content, while chlorophyll b content was slightly higher as compared to the control but not significantly different (Figure 1). The alteration in pigment composition, especially in the lower level of light-harvesting chlorophyll proteins (LHCPS), is one of the defense mechanism of plants to help them survive under adverse conditions, including heavy metal stress (Malar et al. 2014).

The decrease of chlorophyll content is among the parameters when the plants undergo abiotic stress, including heavy metal stress and to some extent, it caused leaf chlorosis. Some previous studies described that heavy metals can inhibit two key enzymes involved in chlorophyll biosynthesis, i.e., δ-aminolevulinic acid (ALA)-dehydratase (EC 4.2.1.24) and protochlorophyllide reductase (Van Assche and Clijsters 1990; De Filippis and Pallaghy 1994).

Table 2. Effect of tailings application on growth of Reutealis trisperma after 90 days treatment

Tailings conc. (%)	Growth parameters				
	Root dry weight (g)	**Shoot dry weight (g)**	**Root length (cm)**	**Shoot height (cm)**	**Leaf area (cm^2)**
0	11.4^b	27.9^b	28.3^b	36.8^a	204.40^b
25	13.5^b	35.5^c	33.0^c	38.3^a	252.18^c
50	14.9^b	32.4^{bc}	27.3^b	34.3^a	202.56^b
100	4.6^a	15.2^a	16.5^a	32.0^a	147.18^a

Note: The numbers within the same columns followed by the same uppercase letters are not significantly different at P ≤ 0.05, as determined by Duncan test

In addition, excessive amount of heavy metals in plants generates oxygen radicals, which may induce lipid peroxidation and cause the damage of thylakoid membrane and photosynthetic pigments (Droppa and Horvath 1990). Different from chlorophyll, carotenoid contents was not significant affected by tailings treatments of 100% (Figure 1). The biggest decrease of carotenoid content occur at 100% tailings treatment by 8.5%.The less effect on carotenoid content in *R. trisperma* might represent supportive role of carotenoid compounds against oxidative stress caused by heavy metals (Candan and Tarhan 2003).

The data of MDA measurement in the roots and leaves showed that MDA content generally increased gradually in accordance to the increase of tailings in growth medium, except MDA content in root with 25% tailings treatment (Figure 2). The increase of MDA content in the roots was significantly higher than that in leaves in response to the tailing concentration. The maximum MDA contents were found in the roots treated with100% of tailings which increased at about 74.4% compared with their control, while in the leaves, it was only 34.2% higher (Figure 2). Malondialdehyde **(**MDA) is the product of lipid peroxidation in the cell when a plant undergoes abiotic stress, and MDA content is often used as an indicator of the extent of oxidative stress in the plant (Hu et al. 2012; Repetto et al. 2010; Hamim et al. 2017[b]). The higher MDA content in the roots suggested that *R. trisperma* underwent more stress due to the treatment of gold-mine tailings than that in the shoot. This was understandable because the first organ exposed to gold mine tailings containing heavy metal such as Pb, Ag, and Cd was the roots.

In many species, the root has ability to prevent or reduce uptake of heavy metals by binding them to the cell wall (Manara 2012). However, not all metal content could be stopped from entering the cell. The accumulation of metal ions in the root cell induced ROS, which may increase lipid peroxidation and lead to the increase of MDA production (Del Rio et al. 2002). The similar results have been reported in wheat seedlings grown at media containing Cd and Pb (Surjendu et al. 2007; Malar et al. 2011), in tomato plant (Borges et al. 2018), and in *Camellia sinensis* exposed to Cd (Mohanpuria et al. 2007).

To understand the response of gold mine tailing to plant anatomy, microscopic analysis of transversal leaves and roots anatomy were carried out to control plants and the plant treated with 100% gold mine tailings. Roots and leaves anatomy data showed that the size of root tissues of the plants treated by 100% tailings treatments was changed. The diameter of vascular bundle of *R. trisperma* roots decreased from 332.14 μm in control plants to 270.88 μm (18.4%) in 100% gold mine tailing treatment (Table 3). In contrast, the thickness of exodermis and endodermis layer increased significantly. The thickness of exodermis layer of treated plant increased from 80.72 μm to 99.00 μm (22.7%), while the thickness of endodermis layer also increased from 17.12 μm to 23.66 μm (55.7%) in response to 100% gold mine tailings. The decrease in vascular bundle diameter is one of effects of heavy metal stress on plants (Poschenrieder and Barceló 2004). The decrease of vascular bundle diameter as an impact of heavy metals toxicity has also been observed in many species such as in *Triticum aestivum* exposed to Cu stress (Atabayeva et al. 2016) and *Salix caprea* treated with combination of Cd and Zn (Vaculík et al. 2012).

Exodermis and endodermis are typical angiosperm tissue, which has function to regulate the flow of water, nutrient, and other material through apoplastic. Both tissues are characterized by specific cell wall modifications with Kasparian strip structure and may develop suberin and thickening tertiary walls. These tissues play important role

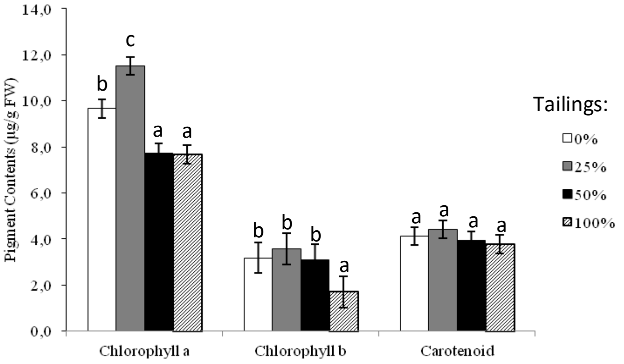

Figure 1. The effect of gold mine tailings treatment on the content of photosynthetic pigment (chlorophyll a, chlorophyll b and carotenoids) of *R. trisperma*. Different letters above the bars indicate significant differences according to Duncan's Multiple Range test at P<0.05. Error bars show SE

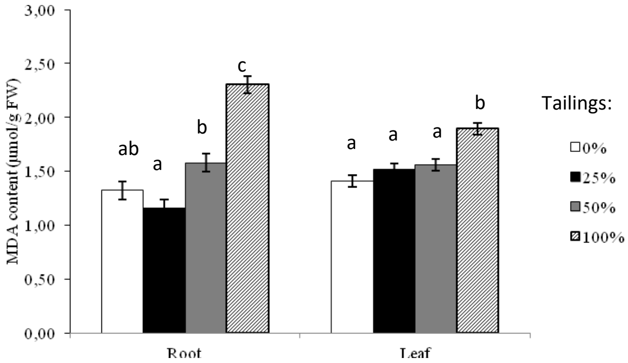

Figure 2. Effect of gold mine tailings treatment on MDA content of *R. trisperma* roots and leaves. Different letters above the bars indicate significant differences according to Duncan's Multiple Range test at P<0.05. Error bars show SE.

Table 3. Anatomical characteristics of root tissues in R. trisperma control plants and plants treated with 100% tailings

Concentration of tailing	Diameter of vascular bundle (μm)	Exodermis thickness (μm)	Endodermis thickness (μm)	Amount of crystal (mm-2)
0%	332.14b	80.72a	17.12a	8a
100%	270.88a	99.00b	23.66b	92b

Note: Mean within the same columns followed by the same uppercase letter indicates no significant difference at P ≤ 0.05, as determined by an independent-samples t-test in the protection against various types of stress such as drought, pathogens, organic contaminants, salinity and heavy metals (Enstone et al. 2003). The thickening of these tissues in *R. trisperma* under 100% tailing treatment may have important role to minimize the translocation of heavy metals contained in the tailings. By analyzing some tolerant and susceptible varieties of wheat, Atabayeva et al. (2016) considered that the increase of exodermis and endodermis thickening was an important indicator of adaptive mechanism to heavy metal stress. The increase of exodermis and endodermis thickening was observed by Gomes et al. (2011) in the root tissues of *Brachiaria decumbens* due to heavy metals contamination (Cd, Pb, Zn, Cu). High proportions of exodermis and endodermis tissue in roots were also characterized as high tolerance to heavy metals (Lux et al. 2004).

Interestingly, the treatment with100% tailings also caused a significant increase in the amount of crystals observed in cortical tissue of root plants (Table 3).The average number of crystal found in the cortical cell of the plants treated with gold mine tailing 100% increased up to 92 crystals per mm^2, while in the control plants it was only 8 crystals per mm^2 (Table 3). The function of crystals formation inside cells was estimated to detoxify heavy metals in plant tissues (Franceschi and Nakata 2005). Crystals development may part of the mechanism of heavy metals detoxification by binding and immobilization of heavy metals to reduce its toxicity (Punz and Sieghardt 1993). The increasing amount of crystals because of tailings treatment was also observed by Todeschini et al. (2011), who demonstrated that Zn contamination in poplar (*Populus alba*) induced formation of calcium-oxalate crystals. Heavy metals such as Cd, Co, Fe, Pb, Sr, and Zn were also reported to cause an increase in calcium oxalate crystals formation (Punz and Sieghardt 1993). The changes of the root anatomy of plant treated by 100% gold mine tailings and the development of crystal inside the cells were presented in Figure 3.

Tailings treatment was also induced anatomical changes in leaves tissue of *R. trisperma*, which include the decrease of upper and lower epidermis thickness resulted in the decrease of leaf blade (lamina) thickness significantly (Table 4). Almost similar, the thickness of upper and lower epidermis decreased approximately 28% due to gold mine tailing treatment which caused the decrease of lamina by 6.2%. The previous studies conducted by Al-Saadi et al. (2013) also found similar results that heavy metals such as Ag and Cu caused a decrease of leaf blade thickness of Potamogeton plant. In *Pisum sativum*, Cd toxicity also decreased the leaf size and thickened lamina (Tran et al. 2013). Heavy metals also caused growth inhibition of leaf mesophyll tissues, reduced leaf blade thickness, and also reduced the size of epidermal cells (Tang et al. 2013). In addition, heavy metals may affect the leaf size and structure through inhibition of water transport from the root to the shoot, and cause a decrease of osmotic pressure in the leaves. The decrease of osmotic pressure caused by inhibition of water transport affects the leaf cells turgidity and reduces the leaf cell size which results in the decrease of lamina thickness (Rucińska-Sobkowiak 2016).

A histochemical staining technique using sodium rhodizonate was conducted for detecting lead in root and leaf tissues of control plant and treated plant with 100% of tailings. The appearance of red stains was observed in the root and leave tissues from the plant treated with 100% of gold mine tailings, while there was no red stains observed in roots and leaves tissue of control plants (Figure 4 and 5). In the root tissues of the plants treated by 100% of gold mine tailing, lead deposits were detected in epidermal cells, exodermal layer, cortical layer (Figure 4.C), endodermal cells, xylem vessels and phloem elements (Figure 4.D). The large area of lead deposits was found in the surrounding of vascular tissues (Figure 4.D). The deposits of lead were dominant on the cell walls and at the intercellular spaces of the cells.

Table 4. Anatomical characteristics of leaves tissues in *R. trisperma* control plants and plants treated with 100% tailings.

Concentration of tailing	Leaf blade Thickness (µm)	Upper epidermis Thickness (µm)	Lower epidermis thickness (µm)
0%	213.40^b	36.18^b	32.04^b
100%	200.24^a	25.94^a	23.30^a

Note: The numbers within the same columns followed by the same uppercase letter indicates no significant difference at P ≤ 0.05, as determined by an independent-samples t-test.

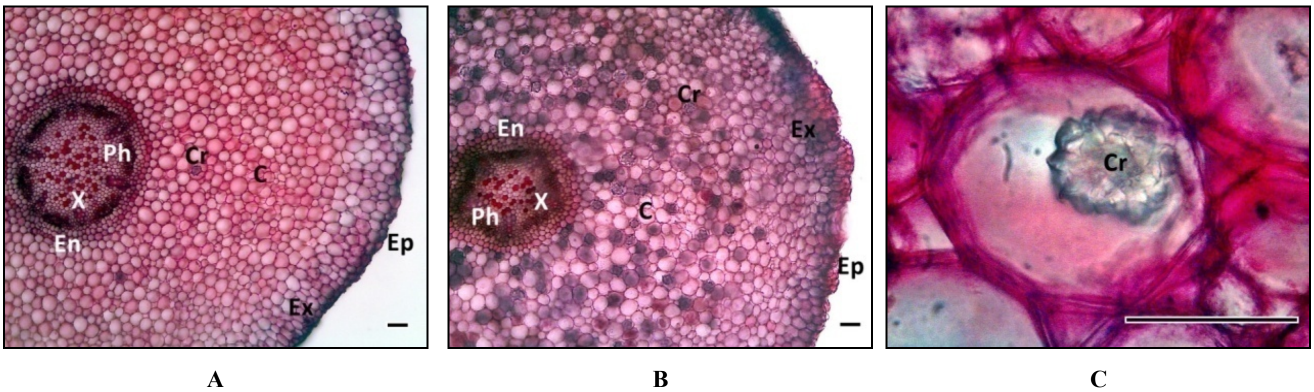

Figure 3. Root anatomical structure of *R. trisperma* without tailings treatment (A) and treated with 100% tailings (B), druce-form crystal (C). Ep: epidermis, Ex: exodermis, C: cortex, Cr: crystal, En: endodermis, Ph: phloem, X: xylem. Bar length: 50 µm.

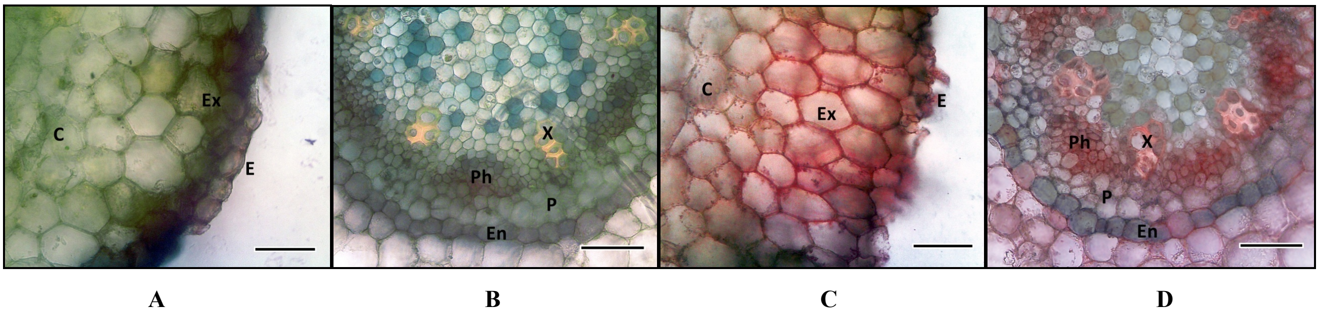

Figure 4. Histochemical analysis of lead using sodium rhodizonate in roots tissue of control plants (A and B) and plants treated with 100% tailings (C and D). E: epidermis, Ex: exodermis, C: cortex, En: endodermis, P: pericycle, X: xylem, Ph: phloem. Red-stained tissues showed deposition of lead. Bar length: 50 µm

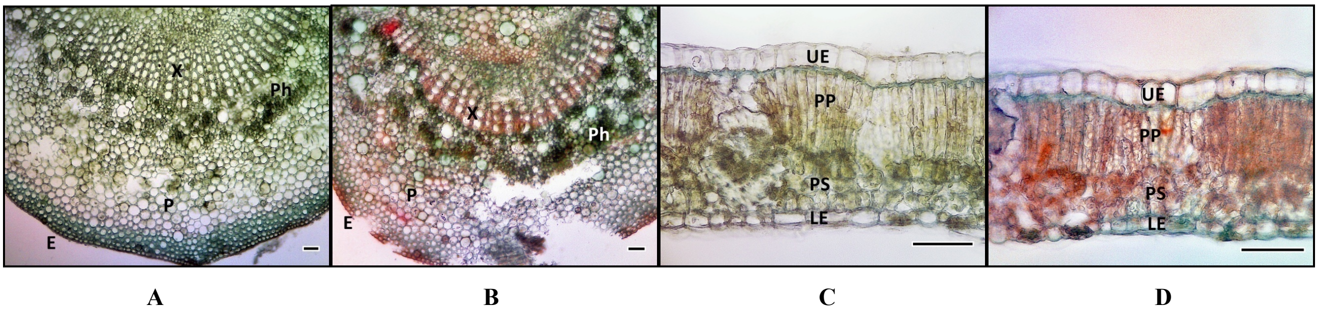

Figure 5. Histochemical analysis of lead using sodium rhodizonate in leaves tissue. Control plants (A and B) and plants treated with 100% tailings (C and D). Red-stained tissues showed accumulation of lead. E: epidermis, UE: upper epidermis, LE: lower epidermis, P: parenchyma, X: xylem, Ph: phloem. Red-stained tissues showed deposition of lead. Bar length: 50 µm

Various studies have been done to verify the absorption of lead by some of terrestrial plants with focus on the roots organ (Tung and Temple 1996; Baranowska-Morek and Wierzbicka 2004). In roots, the lead is primarily accumulated in apoplastic spaces with little lead transport into aboveground portions of the plant (Tanton and Crowdy 1971). Lead was first absorbed by root hairs and accumulated in epidermis cell walls. The metal binding at the epidermis cell wall is part of a strategy to prevent metal from going further into the internal tissues at roots. However, in the plants exposed to high concentration of lead, the substance was also transported apoplastically into the internal tissues such as cortical, endodermal and even vascular tissue (Tung and Temple 1996).

Histochemical analysis of this study showed that *R. trisperma* exposed to high concentration of tailings (100%) apparently accumulated lead in the root tissues. This data indicated that 100% of gold mine tailing may contain high enough Pb which caused the plant absorbed this metal into the internal tissues of the roots. The presence of Pb in the vascular tissues of the roots enabled the plants to transport and distribute this metal to other parts of the tissues including leaves.

Histochemical analysis of leaves was carried out by observing the transverse leave petioles and laminas of control plants and treated plants with 100% of tailing (Figure 5). In the leaf petiole of plants treated with 100% gold mine tailings, lead deposition was found in the epidermal cells, parenchymal cells, and xylem vessels indicated by red color, while it has not appeared in the phloem tissues (Figure 5.B). This data showed that the distribution of Pb in this plant was attributed mostly by water flow through xylem and transpiration from roots to the leaves. In the lamina leaf, lead deposition was also found in almost all the tissues including upper and lower epidermis, parenchymal tissues as well as stomatal openings in the lower part of epidermal tissues (Figure 5.D). From anatomy analysis showed that *R. trisperma* was absorbed and accumulated Pb in the roots and in the leaves suggesting that this plant may have ability, to some extent, to tolerate Pb from gold mine tailings.

Terrestrial plants tend to absorb lead from the soil and retain most of this substance in their roots. However, there is some evidence that plant foliage may also take up lead, and it is possible that this lead is moved to other parts of the plant (Asati et al. 2016). Some accumulator plants such as *Chenopodium rubrum*, *Aster subulatus*, *Brassica chinensis* and *Amaranthus mangostanus* were able to accumulate lead in their above-ground tissues and considered better candidates for phytoremediation (Yuan et al. 2016). Many ultrastructural studies also showed heavy metals accumulation in the cell wall, vacuoles, and intercellular spaces and it is signed with deposition of metals (Almeida et al. 2007; Pourrut et al. 2013; Wierzbicka 1999). Sites of heavy metals deposition depend on the metal and plant species (Kouhi et al. 2016). Positive response of *R. trisperma* on gold mine tailing with better growth under lower concentration (25%) and its capacity to sustain growth under 100% of gold mine tailing for 90 days (Table 2) and the indication of Pb accumulation inside the leaves (Figures 3-5) without any significant toxic symptom suggesting that this plant is tolerant to gold mine tailing and potential to be used for phytoremediation program.

In conclusion, growth, anatomical and physiological characters of *R. trisperma* grown on the media containing various concentration of gold mine tailings were analyzed for 90 days. Low levels of gold mine tailings (25%) improved growth parameters of *R. trisperma*, but the highest concentration of tailings (100%) significantly decreased all growth parameters. Goldmine tailing at 100% caused the decrease of photosynthetic pigments of the leaves, but not carotenoid, with the reduction of chlorophyll b was higher than chlorophyll a. Conversely, 100% of gold mine tailing caused significant increase of malondialdehyde (MDA) content in roots and leaves of *R. trisperma*, suggesting that the plants underwent oxidative stress, even though its increase was higher in the roots than in the leaves. There were significant changes in roots and leaves anatomy in response to gold mine tailings. The treatment of 100% tailing decreased diameter of vascular bundle, increased the thickness of exodermal and epidermal layer of the roots, and induced accumulation of crystals in the root cells. In leaves, tailings caused a decrease of upper and lower epidermis thickness resulted in declining leaf blade thickness. From histochemical analysis, it showed that lead was entered to the roots of *R. trisperma* and distributed to all part of the leaf tissues. Based on present studies, the abilities of *R. trisperma* to sustain growth under gold mine tailing up to 100% for 90 days without any symptom of toxicity suggesting that this plant is tolerant to gold mine tailing and potential to be used for phytoremediation program.

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