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Research Article
Morpho-physiological Changes of Biodiesel Producer Plants *Reutealis trisperma* (Blanco) in Response to Gold-Mining Wastewater

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Abstract
Background and Objective: *Reutealis trisperma* (Blanco) is a non-edible biodiesel producer plant that has a good prospect due to the higher seed production capacity and oil content. In addition, it also able to grow well under unfavourable environment. The study aimed to analyze the response of *Reutealis trisperma* (*R. trisperma*) to gold mined wastewater. **Materials and Methods:** Five varieties of *R. trisperma* i.e., Kemiri Minyak-1 (KM1), Kemiri Minyak-2 (KM2), Kermindo-1 (KD1), Kermindo-2 (KD2) and Harapan (HR) grown in water culture, were treated with wastewater from gold mining industry with concentration of 0, 125, 250, 500 and 1000 mL for 2 weeks. Shoot and root growth, anatomy as well as some physiological characters were analyzed during the treatment. **Results:** Wastewater treatments for 14 days caused decrease of shoot growth and induced leaf yellowing of the plants. The treatments caused increase in malondialdehyde content up to 4 fold, while chlorophyll a, chlorophyll b and total chlorophyll of the plants decreased significantly. TEM analysis indicated that the root cell of plants exposed to highest concentration of wastewater started to degenerate and had higher number of mitochondria and peroxisome vesicles suggesting that the cellular respiration and anti-oxidative mechanism presumably became more active due to the accumulation of reactive oxygen species. **Conclusion:** Gold mine wastewater treatment caused all the plants to undergo stress characterized by the increase of malondialdehyde and the decrease of chlorophyll content and leaf growth of *R. trisperma*, even though there was variation among the varieties. KM2 and KD2 had the best performance among all varieties in response to gold-mine wastewater.

Key words: Tailing, malondialdehyde, ROS, *Reutealis trisperma*, biofuel, gold mining-wastewater, leaf anatomy, root anatomy

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In response to the depletion of oil reserves and food price volatility due to the growth of biofuel industries, the development of biofuel from non-edible products is strongly required. During the last two decades, several non-edible plants have been widely studied including *Jatropha curcas*[1-3], *Pongamia pinnata*, *Madhuca indica* and *Calophyllum inophyllum*[4]. The latest is *Reutealis trisperma* (Blanco) Airy Shaw, a type of tree that produces seed with high content of oil that also known as Philippines tung or Kemiri Sunan (Indonesian)[5,6].

Among the non-edible oil-producing plants, *Reutealis trisperma* has been considered to have excellent prospects in Indonesia[7] due to the higher seed production capacity and oil content[8]. In addition, *R. trisperma* has also been verified to grow well under unfavourable environment, thus allowing the cultivation of the plant on marginal land to avoid competition of arable land use required for food production[9]. Therefore *R. trisperma* development as a source of renewable energy crops in Indonesia especially in critical land including post mining land is highly potential, since Indonesia has about 59 million ha of critical land[9]. Moreover, utilizing *R. trisperma* as reforestation plant to post-mined lands has many advantages such as: (1) *R. trisperma* is a kind of tree that has deep roots and heavy canopy and resistant to environmental conditions such as erosion and drought[8], (2) The products (seeds) are not used as food or feed, thus the risk of heavy metals contaminant in the food chain can be avoided, (3) *R. trisperma* plantation significantly absorbs atmospheric CO_2 and (4) The biofuel produced by *R. trisperma* can support the economy of the communities around the mining area[10].

On the other hand, post-mined lands especially gold-mine land are critical for plant growth, because it has low nutrient content and organic matter as well as in many cases has high concentration of cyanide[11] and sometimes also heavy metals such as lead (Pb) and mercury (Hg) in high concentrations[12-14]. This is due to the use of sodium cyanide (NaCN) as a solvent in the gold mining system[11], while some heavy metals may exist from activities in relation to the extraction methods as well as from other industrial activities[12,15]. The conditions impedes plants' growth and reproduction on such lands, especially during the early stage of plant establishment.

Cyanide is a strong inhibitor for cytochrome C oxidase of the mitochondrial electron transport chain and some enzymes such as catalase, peroxidase, nitrate/nitrite reductase, superoxide dismutase and Rubisco[16]. Consequently high concentration of cyanide perturbs metabolic function of respiration in the plants[17] and it also has been recognize to cause high accumulation of free radicals[18] which caused inhibition of growth and transpiration, some damage of plant tissues, bleaching of leaves and eventually death[19-21].

Meanwhile, some vascular plants have been identified to have enzyme β-cyanoalanine synthase, which is able to convert free cyanide to the amino acid asparagine[22]. In addition, many higher plants including some grasses and woody plants (such as willow and poplar) have also been identified to tolerate higher cyanide concentration and may be potential for phytoremediation[12,23-25]. The latest experiment using β-cyanoalanine enzymes also suggested that Arabidopsis overexpressing a β-cyanoalanine nitrilase from Pseudomonas fluorescence displayed increased tolerance to toxic levels of exogenous cyanide[26].

Some *R. trisperma* varieties have been tested on their adaptability to several degraded lands including on tin post-mined land but the plants have never been tested on their adaptability to gold post-mined land. In this experiment *R. trisperma* was projected to be used as phytoremediator and efforts were made to find the varieties that are adaptable to higher cyanide contamination of post gold mined land to support phytoremediation program. The study aimed to analyze the response of *R. trisperma* grown in water culture exposed to gold mine wastewater based on morphological, anatomical and physiological characteristics.

MATERIALS AND METHODS

Plant materials: The experiment was carried out in the glass house of Department of Biology, Bogor Agricultural University, Bogor, Indonesia from 7 March until 25 November, 2016. In this experiment, four varieties of *Reutealis trisperma* (Kemiri Minyak-1, Kemiri Minyak-2, Kermindo-1, Kermindo-2) and one provenance (Harapan) were provided by Research Institute for Industrial and Refreshment Crops, Ministry of Agriculture, Republic of Indonesia.

Water culture preparation: Hoagland's stock solution was prepared which consisted of macro nutrients [KNO_3, $Ca(NO_3)_2.4H_2O$, $NH_4H_2PO_4$, $MgSO_4.7H_2O$] and micronutrients (KCl, H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, H_2MoO_4 and NaFeEDTA). Media solution then was prepared in a big container before distribution to experiment box contained 8 L of solution.

Plant preparation: One month old *R. trisperma* plants were removed carefully from the polybag, the roots were cleaned with water to remove soil and other solid media and then were planted in the box contained Hoagland's solution. To stand properly, the plants were equipped with perforated stereo foam and supported by fine sponge. To ensure air supply, each box was equipped with aerator. At the beginning, all the plants were grown under half strength Hoagland's solution for 1 week and then the plants were grown under full strength solution for 3 weeks.

Treatment with gold mining wastewater: Gold-mining wastewater was collected directly from liquid municipal of Gold-mining industry (Aneka Tambang Inc. UBPE Pongkor, Bogor, Indonesia). The treatment of gold-mining wastewater was given to the plants after 4 weeks establishment in the culture by adding wastewater to the solution with different concentrations [0 mL (without liquid waste), 125 mL (equivalent with 0.2 ppm of CN), 250 mL (equivalent with 0.4 ppm of CN), 500 mL (equivalent with 0.8 ppm of CN) and 1000 mL (equivalent with 1.6 ppm of CN)]. To keep the volume of the solution inside the box similar, distilled water was added to each box so that the total volume of all media were similar. The treatment of wastewater was given for 14 days to see the response of the treated plants.

The experiment was conducted using a completely randomized design with two factors, the first factor was 5 varieties of *R. trisperma* namely: (a) Kemiri Minyak-1 (KM1), (b) Kemiri Minyak-2 (KM2), (c) Kermindo-1 (KD1), (d) Kermindo-2 (KD2) and (e) Harapan provenance (HR). The second factor was liquid waste treatment which comprised (W1) 0 mL (without liquid waste), (W2) 125 mL, (W3) 250 mL, (W4) 500 mL and (W5) 1000 mL. Each experiment unit was repeated 5 times with 4 plants box (unit experiment).

Observations were made by measuring the growth and development of the shoot and roots during the treatment. Many changes such as wilting, necrosis, discoloration of the leaves and roots were recorded every day. After 14 days of the treatment, the plants were harvested for observation of morphological, anatomical and physiological parameters. For morphological analysis, root length, stem length, leaf number and leaf area were measured. At the end of treatment period, chlorophyll content was observed and the accumulation of lipid peroxidation to view the content of ROS was calculated by measuring the malondialdehyde content. Anatomical analysis using light microscope and TEM was carried out to observe different structure of leaves and roots of all the varieties in response to the wastewater treatments.

Analysis of leaves and roots anatomy: For anatomical analysis, roots and leaf samples were preserved in fixative solution of 70% of ethanol (p.a. Merck KGaA, Darmstadt, Germany) until the time of measurement. Leaves and roots were cut using microtome and then were analyzed to observe the epidermis, cortical and vascular tissues. After cutting, leaves were dipped for 10 min in sodium hypochlorite to remove debris and chlorophyll and then were rinsed for 10 min using distilled water. Finally the leaves were dipped into safranin for 15 min for coloring. After coloring, the tissues were placed in the object glass which had been dripped with glycerin 10% for observation.

The analysis of root cell using transmission electron microscope (TEM) was carried out in Eijkman Institute for Molecular Biology, Jakarta, Indonesia using method developed by Cortadellas *et al.*[27]. Root samples of $1\times1\times1$ mm^3 were placed into eppendorf containing 0.1 M sodium cacodylate buffer. After rinsed several times, the samples were dipped into 2.5% glutaraldehyde for 24 h at 4°C. The samples then were ready for fixation with 2% osmium tetroxide and 2.5% $K_3Fe(CN)_6$ in buffer and shaken at 4°C for 2 h. The samples then were dehydrated using a series of increased ethanol concentrations (p.a. Merck KGaA, Darmstadt, Germany) starting from 30, 50, 70 and 95% respectively for 15 min. Finally they were dehydrated with absolute ethanol twice for 15 min and embedded in Spurr's ERL resin. For ultrastructural observations, ultrathin sections of 70 nm thickness were cut with an ultra-microtome (Leica Reichert Ultracut S, Vienna, Austria) and were mounted on copper grids with 300 square meshes. The sections were stained with 1% uranyl acetate. Observation and photography were done by transmission electron microscopy (JEOL JEM 1010, Tokyo, Japan).

Chlorophyll content analysis: Chlorophyll content was analyzed using method developed by Yoshida *et al.*[28]. Two grams of fresh leaves were ground using 80% of acetone (p.a. Merck KGaA, Darmstadt, Germany) and then were filtered using Whatman paper No. 1 into 100 mL of volumetric flask until all the chlorophyll were dissolved into the acetone solution. Acetone was added to the volumetric flask to reach exactly 100 mL in volume of chlorophyll in acetone solution. A 5 mL of chlorophyll solution was taken from 100 mL volumetric flask then put into 50 mL of volumetric flask and diluted using 80% of acetone until 50 mL. The absorbance of chlorophyll solution was measured using spectrophotometer (Shimadzu, UV-1700, Kyoto, Japan) at the 645 and 663 nm wavelength (λ). Chlorophyll content was measured using the formula as follow[28]:

$$\text{Chl a} = 0.0127.\ A663 - 0.00269.\ A645$$

$$\text{Chl b} = 0.0229.\ A645 - 0.00468.\ A663$$

$$\text{Total Chl} = \text{Chl a} + \text{Chl b} = 0.0202.\ A645 + 0.00802.\ A663$$

Where:

Chl a = Chlorophyll a
Chl b = Chlorophyll b
A663 = The absorbance at the λ of 663 nm
A645 = The absorbance at the λ of 645 nm

Lipid peroxidation analysis: Lipid peroxidation was estimated as described in Mihara *et al.*[29] with some modifications based on Ono *et al.*[30]. Fresh roots (0.2 g) were ground in 0.5 mL of 0.1% (w/v) trichloracetic acid (TCA) at 4°C. The root extract then was added to 3 mL of 1% H_3PO_4 and 1 mL of 0.6% of TBA that was dissolved in 20% of TCA. The solution then was incubated in the oven at 100°C for 30 min. After cooling at the room temperature, 4 mL n-butanol was added to the solution and then followed by centrifugation at 4200 rpm at 28°C for 20 min. The absorbance of the supernatant then was measured using a UV-VIS spectrophotometer (Shimadzu, UV-1700, Kyoto, Japan) at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 520 nm. The concentration of MDA was calculated from its extinction coefficient (ε =155 L mmol^{-1} cm^{-1}).

RESULTS

Content of liquid from gold mining waste: Chemical analysis of wastewater showed that the gold mine wastewater contained relatively low nutrients including K, Ca, Mg, Fe, Zn, Mn and Co (Table 1) with the acidity value of 7.5. On the other hand, the cyanide (CN) content was very high (Table 1) i.e., almost 13 times of that contained in the solid tailings. In addition, the heavy metal content particularly of Pb, Cd, Ag and Hg elements was also very low (Table 1). During the treatment, Hoagland's solution was stabilized to maintain the pH solution of 6.5-7.

Growth and morphological analysis: The analysis of overall plant growth showed that all five varieties used in the experiment had relatively uniformed growth. The plant growth rate measured based on the observation of average leaf area and new leaves development was affected by liquid waste treatment given for 14 days. The average leaf area of *R. trisperma* was strongly affected by the liquid waste treatments. Plant treated with higher liquid waste concentration had significantly ($p<0.05$) lower leaf area (Fig. 1). The development of new leaves also tended to decrease in line with the increase of liquid waste concentration in the media, even though the difference was not statistically significant (Fig. 1). Difference in gradient of reductions in response to increased wastewater concentration between leaf area and leaf development was found, in which leaf area showed steeper decrease compared to leaf development (Fig. 1).

Each variety also developed different response in regards to treatment, Kemiri Minyak-2 (KM2) had the biggest leaf area, while Kemiri Minyak-1 (KM1) and Harapan line (HR) had the lowest leaf area (Fig. 2a). For leaf development, Kermindo-2 (KD2) still had the highest rate of leaf development, while Kemiri Minyak-1 (KM1) and Harapan line (HR) had the lowest rate of leaf development compared to other varieties (Fig. 2b).

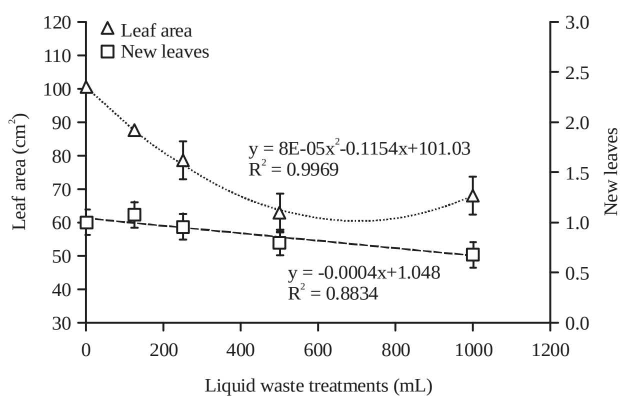

Fig. 1: Average of leaf area and new leaves growth of *R. trisperma* treated with 0, 125, 250, 500 and 1000 mL of liquid waste for 14 days

Bars represent standard error of the means. The equation of leaf area: $y = 8E\text{-}05x^2\text{-}0.1154x+101.03$ ($R^2 = 0.9969$). The equation of leave development (growth): $y = -0.0004x+1.048$ ($R^2 = 0.8834$)

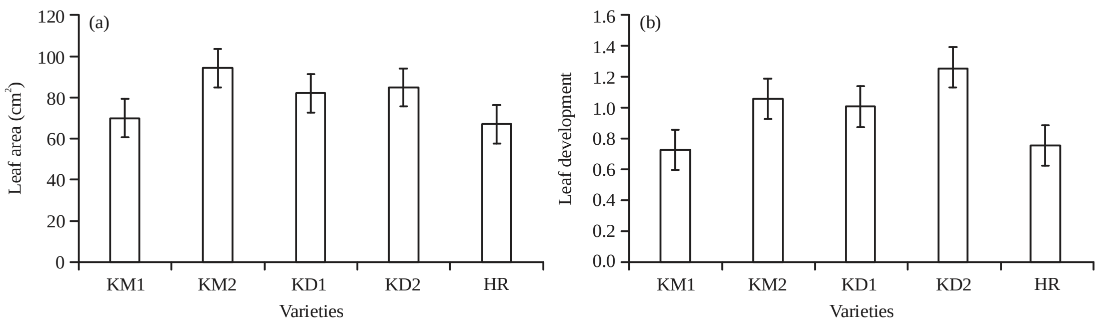

Fig. 2(a-b): Different response of five *R. trisperma* varieties to the treatments of 0, 125, 250, 500 and 1000 mL of liquid waste for 14 days based on the average of (a) Leaf area and (b) New leaf development

Bars represent standard error of the means, KM1: Kemiri Minyak-1, KM2: Kemiri Minyak-2, KD1: Kermindo-1, KD2: Kermindo-2 and HR: Harapan line

Fig. 3(a-b): Changes of leaf color due to gold-mining liquid waste (a) *R. trisperma* leaf variety KM2 without treatment (W1) had dark green color and (b) *R. trisperma* treated with 1000 mL liquid waste (W5) for 14 days had yellowish leaf

Table 1: Content of some macro- and micronutrients, cyanide (CN) and heavy metals of liquid from gold-mining waste

Macro and micro nutrient compounds	Content (ppm)	Cyanide and heavy metals	Content (ppm)
K	22.25	Cyanide (CN)	12.40
Ca	12.02	Pb	<0.004
Mg	1.63	Cd	0.003
Fe	2.17	Ag	0.074
Cu	2.06	Hg	<0.002
Zn	0.87		
Mn	0.08		
Mo	<0.005		
B	<0.020		
Ni	0.046		
Co	<0.005		

In addition to the leaves size and development changes, leaves also underwent changes of color from green to yellowish in response to the treatment (Fig. 3). Green RGB analysis using leaf images scanned by HP J1050 Scanner at

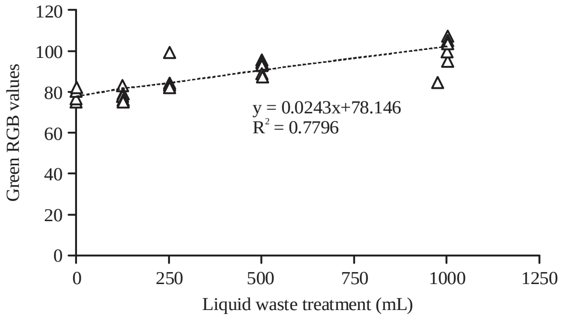

Fig. 4: Changes of RGB color of *R. trisperma* leaf in response to the treatment of gold-mining wastewater from 0-1000 mL

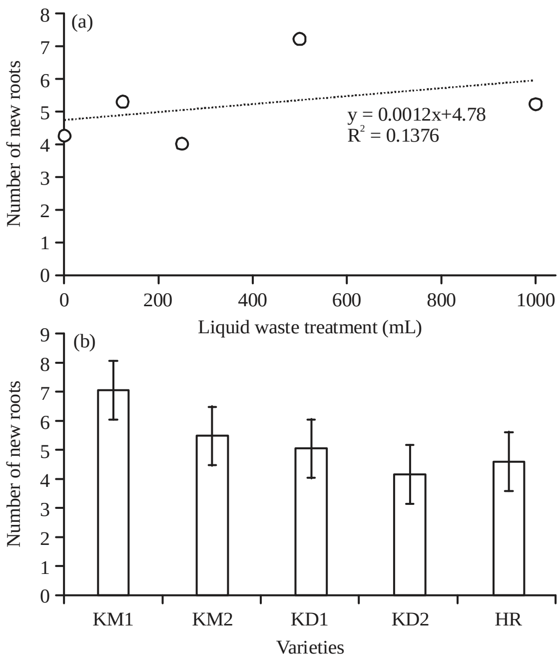

Fig. 5(a-b): (a) Development of new roots of 5 *R. trisperma* varieties in response to the gold-mining liquid waste treatment from 0-1000 mL (b) Each varieties had different response to the treatments
(Bars represent standard error of the means)

300 dpi was conducted to quantify the color changes. The RGB data indicated that the treatment with liquid gold-mine waste had caused gradual changes in leaf color from green to yellowish green and was shown in changes of RGB values from low to high. The graph of the average RGB value in response to the treatment is shown in Fig. 4.

In contrast to the leaves, roots growth of *R. trisperma* did not yield in consistent response in regards to the treatment of gold mine wastewater. There was even a tendency that liquid waste caused an increase in the number of roots, albeit the R^2 value of the trend was very low (Fig. 5a). In reverse to the leaves growth, the roots growth of KM1 had the highest value, whereas KD2 had the lowest (Fig. 5b).

Table 2: Stomatal density of *R. trisperma* varieties of KM1, KM2, KD1, KD2 and HR

	Stomatal density mm^{-2}	
Varieties	Abaxial	Adaxial
KM1	262.05	0
KM2	257.86	0
KD1	270.43	0
KD2	167.71	0
HR	157.23	0

Analysis of plant anatomy: Observation of leaf anatomy of *R. trisperma* leaf tissue using light microscopy showed a single layer of palisade with a light layer of epidermis. The tissue thickness of all varieties was relatively uniform between 12-17 µm for lower and upper epidermis, where the upper epidermal tissues were slightly thicker than the lower tissues (Fig. 6). Palisade tissue thickness was almost equal to the spongy tissue, which was between 27-36 µm. KM2 had the tallest palisade tissues compared to other varieties (Fig. 6). While there was changes in leaf area and development, the gold-mining wastewater treatment did not affect *R. trisperma* leaf anatomy significantly ($p<0.05$).

Observations on the paradermal leaves showed a difference between the upper and lower leaves, based on the stomata existence. Stomata were only found on the underside of leaves and none was found at the upper side of the leaves (Table 2). There were similarities between stomatal density of KM1, KM2 and KD1 which was in the range 260 sec mm^{-2} while the variety of KD2 almost identical to HR, which had lower density of stomata (around 160 mm^{-2}).

Although leaf anatomy of control and treated plants was not significantly different, the roots of plants treated with gold-mining wastewater in high concentration (1000 mL) for 2 weeks showed dark color which can be observed visually as well as using light microscope. In addition to the differences of the side wall of root cells found under light microscope observation, TEM analysis showed that the cell structure of the root treated with high effluent (1000 mL) for 14 days experienced shrinkage so that the gap between the cell wall was bigger (Fig. 7b). These cells also had higher number of mitochondria (m) and endoplasmic reticulum (ER) compared to the control cells. Moreover, the (Fig. 7b) showes that the root cells treated with highest concentration of gold-mine waste had a large numbers of vesicular body, which is indicative of abundance of peroxisomes (P) (Fig. 7).

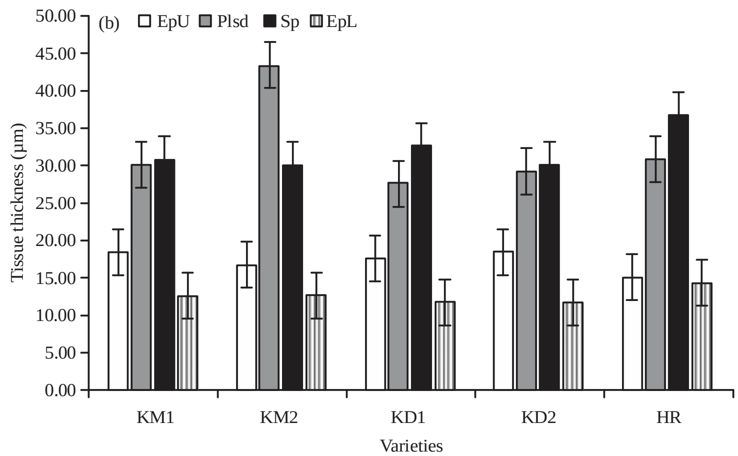

Fig. 6(a-b): (a) Leaf anatomy of *R. trisperma* var. KM1 and (b) The results of measurements on the upper epidermal tissue (EpU), palisade tissue (Plsd), spongy tissue (Sp) and lower epidermal tissue (EpL) *R. trisperma* leaf by using light microscopy
(Bars represent standard error of the means)

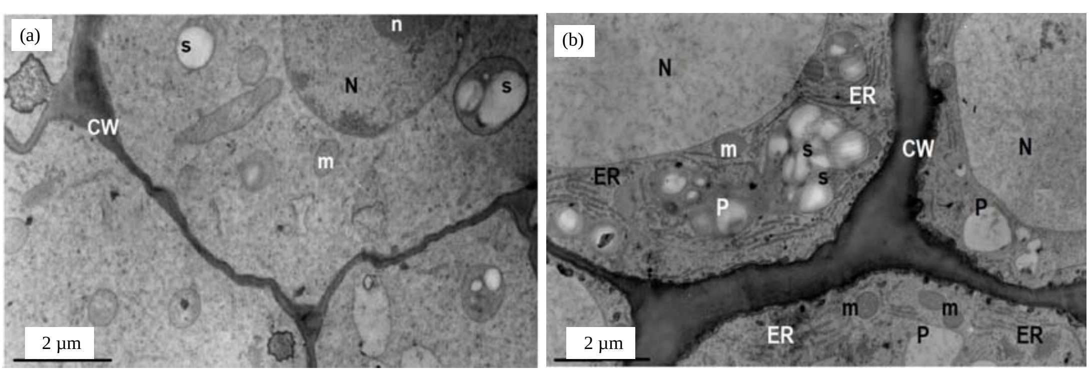

Fig. 7(a-b): Results of the TEM analysis of plant root tissues of *R. trisperma* (KM2) without liquid waste treatment (a) The treated plant with 1000 mL of gold-mining wastewater and (b) The roots of the plants treated with wastewater treatment showed cell shrinkage which resulted in gap formation between cells and formation a lot of vesicles that served as peroxisomes (P), as well as reticulum endoplasmic (ER) and numerous mitochondria (m)
N: Nucleus, CW: Cell wall, S: Starch, ER: Endoplasmic reticulum, m: Mitochondria and P: Peroxisomes

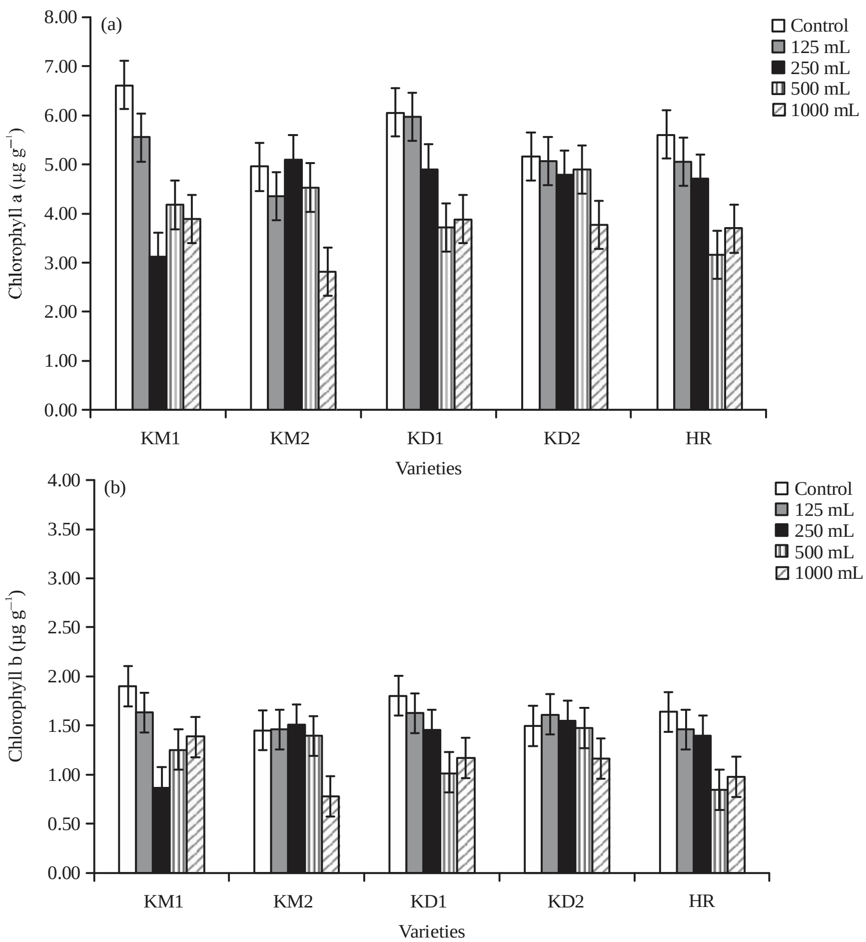

Fig. 8(a-b): Average content of chlorophyll a (Chl a) and chlorophyll b (Chl b) of *R. trisperma* leaves in response to the treatment of gold-mining wastewater from 0 -1000 mL. At the treatment of 1000 mL chlorophyll contents decreased up to 35% as compared to that of control plants
(Bars represent standard error of the means)

Chlorophyll and MDA analysis: The treatment with gold-mining wastewater significantly affected some physiological parameters including concentration of chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll of the leaves as well as malondialdehyde (MDA) content of the roots (Fig. 8-9). In general, Chl a and Chl b decreased significantly in response to wastewater treatment and at the concentration of 1000 mL the decrease reached up to approximately 35%. There were variation of responses among the varieties based on these parameters, where KM2 and KD2 varieties tended to be less sensitive to the treatment using wastewater up to 500 mL (equivalent with 0.8 ppm cyanide) compared to other varieties, even though at the concentration of 1000 mL, Chl a and Chl b of those varieties also decreased substantially (Fig. 8). Among the five varieties, KM1 and HR suffered from decrease in chlorophyll content the most.

Wastewater treatment caused dramatic increase of MDA content in the root of all varieties (Fig. 9). High concentration of MDA was an indication of lipid peroxidation associated with the accumulation of reactive oxygen species (ROS) or commonly known as free radicals in *R. trisperma* tissues. There was no specific pattern of MDA accumulation among the varieties but KM2 tended to have lower MDA content after wastewater treatment up to 250 mL (equivalent to 0.4 ppm cyanide), while KD2 had the highest MDA in response to high (1000 mL) wastewater treatment (Fig. 9).

In average, gold-mine liquid waste treatment for 2 weeks led to gradual increase of MDA content of *R. trisperma* following the curve of $Y=-0.0002x^2+0.441x+59.057$ ($R^2 = 0.98$). The treatment also caused the decrease of total chlorophyll content (Fig. 10). The increase of MDA due to wastewater treatment of 125-500 mL was almost linear . However, at 1000 mL concentration or equivalent to 1.6 ppm CN, increased

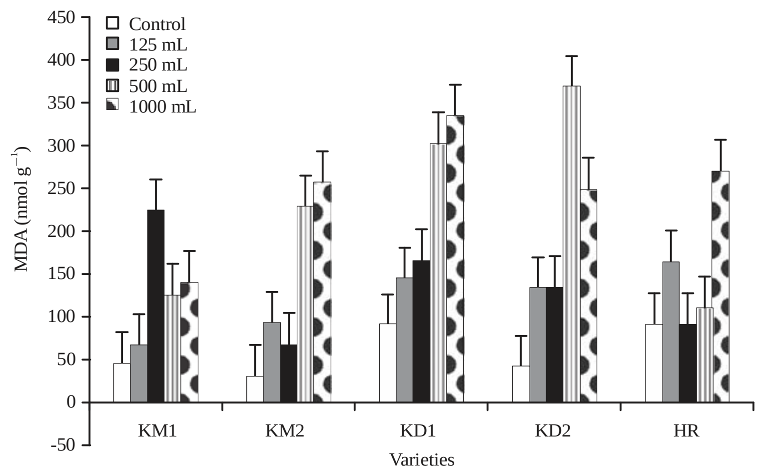

Fig. 9: Average content of malondialdehyde (MDA) of *R. trisperma* root tissue treated with liquid waste of 0 (control), 125, 250, 500 and 1000 mL which increased more than 4 times
(Bars represent standard error of the means)

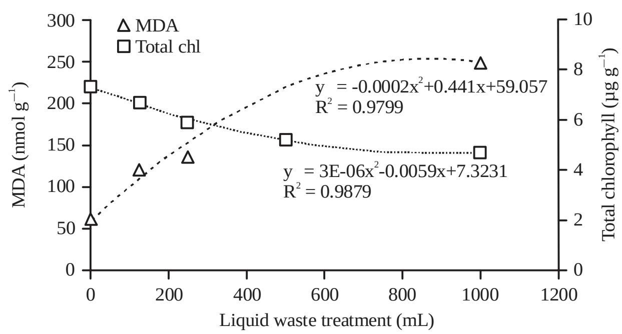

Fig. 10: Average of malondialdehyde (MDA) and total chlorophyll content of *R. trisperma* plant tissue treated with liquid waste of 0, 125, 250, 500 and1000 mL. MDA content increased up to 4 times, while chlorophyll contents decreased up to 35% due to the treatment as compared to that of control plants

in MDA content occurred more than fourfold of the control plants. Whereas for total chlorophyll content. it decreased only approximately 35%.

Principal components analysis of *R. trisperma* varieties: To distinguish the tolerance level of the *R. trisperma* varieties to wastewater treatment, four quantitative data including new leaf development (leaf growth), leaf area, the decrease of total chlorophyll and the increase of MDA content of the plant were analyzed using principal components analysis (PCA) and clustered using K-mean clustering. In this analysis, the calculated data were the differences between control and treated plants. Before calculation, the data were standardized using the average and standard deviation of the data sets. The graph shows that two varieties (KD2 and KM2) were separated from the other three (KD1, KM1 and HR). KM2 and KD2 were favored from other three due to the higher leaf growth and leaf area and the lower decrease of chlorophyll content than the other varieties (Fig. 11). This analysis shows that *R. trisperma* varieties KD2 and KM2 are more tolerant to the gold mining wastewater than the others.

DISCUSSION

Analysis of gold-mining wastewater showed that the cyanide content was classified as very high (12.40 ppm), while other compounds, including heavy metals were in lower concentration (Table 1), hence cyanide was the substance that may have significant effect to the plant. This data were different from that of solid tailing which in many cases contained lower cyanide but with very high heavy metal compunds[12-14]. This is understandable because sodium cyanide (NaCN) is often used as a solvent in the gold mining system[11] and therefore the effluent produced by gold mining

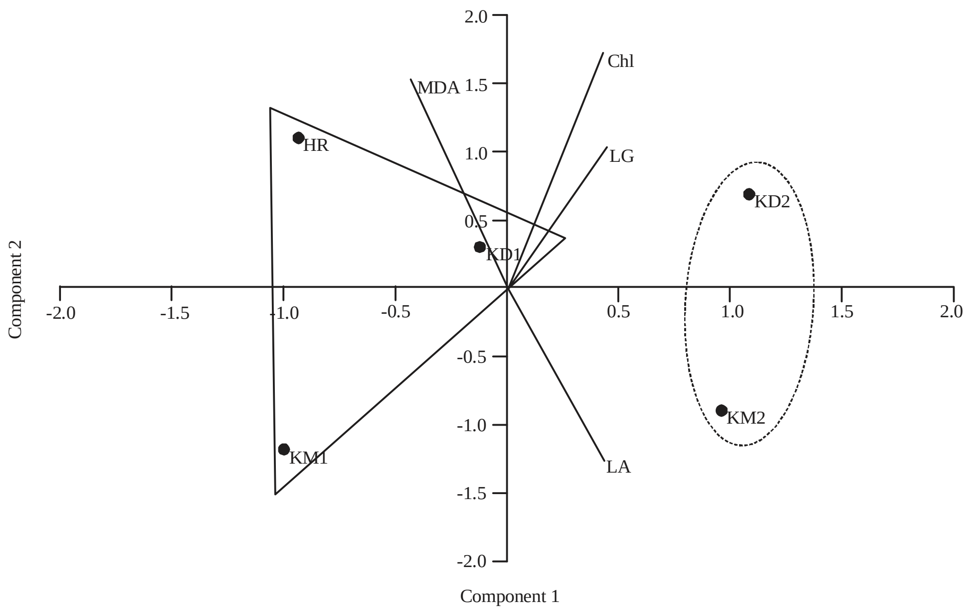

Fig. 11: Biplot graph of *R. trisperma* genotypes based on Principal components analysis (PCA) and K-mean clustering of leaf growth (LG), leaf area (LA), total chlorophyll (Chl) and MDA content (MDA) of the plant in response to wastewater treatment. The graph shows separation of two varieties (KD2 and KM2) from the other three (KD1, KM1 and HR)

industry still has high content of cyanide. The presence of cyanide in wastewater and other compounds partly explained the perturbation of the plants growth and reproduction planted in gold mine areas, therefore recovery or remediation process is required.

All varieties of *R. trisperma* used in the experiment were relatively adaptable to the treatment of gold-mining wastewater for the short time exposure. However after 14 days of treatment the plants underwent stress especially those treated by 1000 mL of gold-mining wastewater, indicated by the decrease of leaves growth and development as well as chlorophyll content and some leaves became yellowish (Fig. 1, 3, 4 and 8). These responses are indicative of the negative impact of cyanide to *R. trisperma* plants which in many cases have also been known to cause leaf bleaching and growth inhibition[20,21]. There was different reaction between roots and shoots due to the wastewater treatment, where the decrease of leaf growth was higher that root growth in response to the treatment. These responses were almost similar to the general response of plants to environmental stress such as drought and salt stress[31-33] which involves hormonal regulation to control shoot and root growth[34]. There was little variations in plant morphology among the varieties of *R. trisperma*, especially the leaves, in response to wastewater treatments which can be used as an indicator of plant's tolerance to the wastewater treatment.

High concentration of cyanide in the wastewater also caused the accumulation of ROS inside the leaves, indicated by the malondialdehyde content, which increased dramatically (Fig. 9). It has been noted by Oracz *et al.*[18] that in sunflower plant, cyanide induced the formation reactive oxygen species (ROS) and stimulated the production of H_2O_2 on the axis of the embryo through NADH oxidase enzyme stimulation and inhibition of antioxidant enzymes such as catalase. ROS have been known to cause some damage to the membrane and proteins at the cellular level, which caused damage to the plant tissue[35-37]. Figure 10 also shows that the increase of ROS of the leaves was concomitant to the decrease of total chlorophyll content suggesting that ROS accumulation due to wastewater treatment caused lipid peroxidation and cellular degradation. Towill *et al.*[17] also observed that high content of cyanide can impede respiration via the cytochrome iron complex oxidase path. In addition, cyanide also inhibits the formation of ATP so that other processes involving the use of ATP were also hampered. Even at low concentrations, cyanide was able to inhibit the germination and growth of plants[19].

Fourteen days treatment with wastewater did not significantly influence leaf anatomy but at cellular level high concentration of wastewater treatment caused changes of root cellular ultrastructure. From TEM analysis data showed that the root cells of plants treated with highest gold-mine wastewater (1000 mL) underwent shrinkage and had higher number of mitochondria, suggesting that these cells performed respiratory process more intensively than the cells of control plants (Fig. 7). The graphs (Fig. 7b) also showed that the treated plant cells had a large numbers of vesicle, in which presumably the numerous peroxisomes were very active in order to neutralize accumulation of ROS inside the cells. Peroxisomes are organelles which have important role in neutralizing the negative effects of ROS by changing H_2O_2 into H_2O [35] and the accumulation of reactive oxygen species (ROS) were very high in the tissue of treated plants with wastewater up to 1000 mL (Fig. 10).

The increased content of ROS is common in plants facing environmental stress such as drought, high temperatures and other stress including higher cyanide content[18,35,37]. ROS such as H_2O_2 accumulation will result in the destruction of proteins and lipids from cells if not addressed. It showed that the liquid waste had caused *R. trisperma* plants underwent heavy stress especially due to higher cyanide content[17], resulting in high level accumulation of ROS (Fig. 10). The data is consistent to what was found in sunflower plant treated with cyanide from tailings[18]. The duration of the treatment may not be long enough to cause cellular damage but was able to induce cellular scavenging mechanism trough peroxisomes activity.

Tolerant variation among the varieties to gold mining wastewater: There was different response among the five varieties to wastewater treatment with higher cyanide concentration indicated by morphological and physiological parameters. Some research suggest that many higher plants are adaptive to high cyanide concentration[12,24,25], which in many cases even able to convert free cyanide to asparagine[22]. Among the five genotypes analyzed in this experiment, the variation between genotypes was not strong enough to distinguish the varieties that is more tolerant to the treatments. Therefore a principal components analysis (PCA) was applied to find the most tolerant among the varieties. Based on the PCA analysis, KM2 and KD2 are more tolerant to wastewater than others (Fig. 11), based on their capability to sustain leaf growth and chlorophyll content during the treatments (Fig. 2 and 8). Leaf growth is an important parameters that has been used to determine tolerance level of plant to cyanide toxicity such as in vetiver grasses[25]. The KM2 and KD2 had the highest leaves growth, even though the root growth was not significantly different among all varieties, suggesting that leaves growth was more succeptible to cyanide toxicity than root growth. Therefore KM2 and KD2 are potential candidate of *R. trisperma* plant to be cultivated in the area of gold-mining land.

CONCLUSION

Wastewater collected from gold mining factory which contain high cyanide but lower heavy metals were treated to five varieties of *R. trisperma* grown in water culture as a treatment. Gold-mine wastewater treatment for 2 weeks caused decrease in leaf growth and chlorophyll content of all varieties, while it increased malondialdehyde content dramatically. TEM analysis showed that the root cells of plants treated with highest gold-mine wastewater underwent shrinkage and had higher number of mitochondria and peroxisomes. There was variation among varieties in response to the gold-mine wastewater treatment with *R. trisperma* var. KM2 and KD2 considered as the most tolerant based on PCA analysis.

SIGNIFICANCE STATEMENTS

This study discovers the morphological and physiological responses of *Reutealis trisperma* varieties to gold-mine wastewater contained high level of cyanide, which can be beneficial for the development of biofuel producer plants for phytoremediation. This study will help the researchers to uncover critical areas of combining biofuel production and phytoremediation through cultivation of some underutilized species on post mined land that many researchers were not able to explore. Thus a new theory on maximizing the benefit of phytoremediation program may be arrived at.

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