Anatomical and Histochemical Responses of Vetiver Grass (*Chrysopogon zizanioides* L. Roberty) to Phytoremediation Ability of Liquid Batik Waste

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ARTICLE INFO

Received: 9 Dec 2021
Received in revised: 8 Mar 2022
Accepted: 24 Mar 2022
Published online: 3 May 2022
DOI: 10.32526/ennrj/20/202100232

Keywords:
Anatomy/ Batik waste/ Histochemical responses/ Phytoremediation/ Vetiver grass

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ABSTRACT

Due to poor management of Indonesian batik waste, pollutants are discharged directly into rivers and absorbed from soil, causing environmental pollution. A phytoremediation strategy was chosen as one of the environmentally friendly and community-implementable solutions. Vetiver grass (*Chrysopogon zizanioides* L. Roberty) is a type of Poaceae plant that is suitable for the phytoremediation process of batik waste. This study analyzed the anatomical responses, distribution of secondary metabolites as defense compounds, and the ability to absorb heavy metals contained in liquid batik waste. Liquid batik waste was applied as plants irrigation at various concentration (0%, 25%, 50%, 75%, and 100%) for 60 days. Vetiver grass was able to grow well in the applied concentrations range. The results showed that vetiver grass roots could absorb Cu metal better than in the leaves. This plant can be stated to be able to absorb Cu better than Al. Liquid batik waste significantly ($p<0.05$) affected most of the observed anatomical parameters, where concentrations of 75% and 100% were the most influential concentrations according to the DMRT test with a 95% confidence level. The histochemical analysis found that there was an increase in the distribution of lignin, phenolic compound, and terpenoids in the tissues composed of roots and leaves along with the increase in the concentration of the waste applied.

1. INTRODUCTION

Liquid batik waste is one type of textile waste that has an impact on the environment. This is due to the growth of batik industries in Indonesia, especially Java, after Batik was designated as a UNESCO World Heritage (Nurainun et al., 2008). However, this is incompatible with the environmentally friendly disposal of batik waste. According to Fajar et al. (2019), only about 0.6% of the batik industry in Pekalongan has a sewage treatment plant (IPAL), while the remaining liquid batik waste is discharged into water bodies (drainage channels or rivers). In the Special Region of Yogyakarta, especially in Kampung Batik Giriloyo, most batik producers do not dump waste into wells or directly into rivers without filtering (Muliasari and Widiastuti, 2010). This condition certainly causes risks to the environment and humans because the complexity of the molecules used in the batik production process, which consists of heavy metals, makes batik waste difficult to degrade (Forgacs et al., 2004). Liquid batik waste has specific characteristic, thick color, alkaline pH, low in COD and BOD content (Mukimin et al., 2018), and also several heavy metals such as, Chromium (Cr), Copper (Cu), Iron (Fe), Cadmium (Cd), and Alumunium (Al) (Murti and Maryani, 2020).

There are several strategies to reduce the environmental impact of liquid batik waste, one of which is phytoremediation. Phytoremediation is an attempt to use plants to break down, stabilize, or remove pollutants from contaminated soil by leveraging the physiological properties of plants (Escoto et al., 2019). Plants that act as phytoremediation agents must show stronger growth properties than other plants: biomass, non-edible nutritional properties, complex root systems, ability to accumulate excess target pollutants, and specific mechanisms of tolerant stress (Prabakaran et al.,

Citation: Linggawati A, Maryani, Nugroho AP, Rachmawati D. Anatomical and histochemical responses of vetiver grass (*Chrysopogon zizanioides* L. Roberty) to phytoremediation ability of liquid batik waste. Environ. Nat. Resour. J. 2022;20(4):359-368. (https://doi.org/10.32526/ennrj/20/202100232)

2019). Adaptation mechanisms that occur in plants, especially phytoremediator plants are due to the ability of plants to develop a series of self-defense mechanisms, namely increased production of reactive oxygen species (ROS) and antioxidant compounds, physical defense through anatomical structural changes, and increased production of secondary metabolite defense compounds (Loix et al., 2017; He et al., 2018; Isah, 2019). Therefore, it is important to observe the response shown by plants after being stressed by liquid batik waste in phytoremediation efforts.

Vetiver grass is a gramineous plant species that meets these criteria. Vetiver grass is a non-invasive terrestrial plant species that has high biomass and has potential to grow on contaminated lands (Effendi et al., 2017; Effendi et al., 2020). Due to its strong root composition, this plant is intentionally planted with the aim of stabilizing the soil to prevent soil erosion (Mickovski et al., 2005). Vetiver grass is usually grown on the banks of rivers in border areas or in culture to extract the essential oils produced by this plant. Various studies have shown that vetiver grass can function as a phytoremediation agent in a variety of contaminated soils, including land contaminated with crude oil, heavy metals, tofu waste, and wood waste (Effendi et al., 2017; Gautam and Agrawal, 2017; Seroja et al., 2018; Rahmawan et al., 2019).

Plant defense strategies in the form of anatomical changes and distribution of secondary metabolites through histochemical techniques are the focus of this study. Anatomical changes that occur in plants are an excellent indicator for determining the effects of environmental stress on plants (Darmanti, 2015). This is the follow-up response that plants exhibit after the physiological response of the plant (Gratani, 2014). Detection of secondary metabolites by histochemical observations can be used to determine the distribution and localization of secondary metabolites that act as plant defense compounds from liquid batik waste stress. According to Badria and Aboelmaaty (2020), histochemical methods describe and track plant growth in ultra-fine-structured ways so that they can further explain the genetic basis of plant physiological and biochemical processes. Anatomical observations of changes in the structure and localization of secondary metabolites in tissues are considered indicators for assessing the ability of these plants to withstand environmental stress. Through this research, the effectiveness of vetiver grass in reducing liquid batik waste and the responses exhibited by the plant are known, and it is hope that this plant can be used as an alternative in the phytoremediation process carried out by the community.

2. METHODOLOGY

2.1 Preparation and planting procedure

Vetiver grass (*Chrysopogon zizanioides* L. Roberty) is obtained from the Center for Agrotechnology Innovation (PIAT) UGM with a plant age of approximately 24 months and was cut to a leaf length of 20 cm to rejuvenate the plant. Geographically, the sampling location is at the coordinates of 7°7’47’1.3”S 110°27’48.8”E. The plant was planted in 2.5 kg of soil in the Greenhouse of the Sawitsari Research Station, Faculty of Biology, Universitas Gadjah Mada. The experimental design used a completely randomized design with five treatments and six replications. The liquid batik waste used in this study was obtained from the PC Batik Production House GKBI Medari, Sleman. Liquid batik waste was applied by watering for 60 days with as much as 200 ml with different waste concentrations, namely 0%, 25%, 50%, 75%, and 100% waste (P_0, P1, P_2, P_3, and P_4). The concentrations of heavy metals Cu, Cr, and Al in the liquid batik waste and planting media used were first measured in the laboratory of the Center for Environmental Health Engineering and Disease Control (BBTKLPP) in Yogyakarta. The results of measuring the concentration of batik waste applied in this study are presented in Table 1.

Table 1. Measurement results on the batik waste

Parameters	Unit	Results
Total Chrome (Cr)	mg/L	<0.0095
Copper (Cu)	mg/L	0.0130
Aluminium (Al)	mg/L	9.8040

2.2 Evaluation of the accumulation ability of Cu and Al metals in batik waste by vetiver grass

The process of observing the ability to accumulate metal in batik waste was carried out by measuring the metal concentrations of vetiver soil samples, roots and leaves. The plant samples and growth medium used for phytoremediation were 3 samples in each treatment group. Sample preparation was carried out by wet digestion method using (HNO_3 and $HClO_4$) (Ratnawati et al., 2019). The metal concentration in the filtrate was measured using AAS (Atomic Absorption Spectrophotometry) at BPTP

Yogyakarta. The efficiency level of heavy metal accumulation in the tissues was assessed using formula (1), and the rate of heavy metal transfer from roots to leaves (TF) was evaluated by the formula (2) (Takarina and Pin, 2017).

$$\text{BCF} = \frac{\text{Metal concentration in plant organ}}{\text{Metal concentration in soil}} \quad (1)$$

$$\text{TF} = \frac{\text{Root BCF value}}{\text{Leaf BCF value}} \quad (2)$$

2.3 Observation of anatomical and histochemical responses

The anatomical response data of vetiver grass was obtained through quantitative and qualitative observations on the anatomical structure of the roots and leaves of the plant after being treated with batik waste. Samples were prepared using the Embedding method according to Sutikno (2014) (Figure 1). Each root and leaf sample that had been cut approximately 0.5 cm long was fixed using FAA (Formaldehyde Alcohol Acetic Acid) solution for 24 h, then the sample was rinsed using 70% alcohol, followed by graded dehydration using alcohol with a concentration of 80%, 95%, and 100% each for 30 min, then followed by the dealcoholization stage using alcohol, alcohol:xylol with different ratios, then carried out the infiltration stage using pure paraffin in an oven at 57°C for 24 h. The samples were then made into blocks using freshly pure paraffin for about one hour. The samples were then sliced using a rotary microtome with a thickness of 16 µm. The samples were then stained with a single stain of 1% safranin in 70% alcohol. The prepared anatomical slides were observed using a microscope equipped with Optilab and measured using Image Raster 3 software. The anatomical parameters of the roots observed in this study were root diameter, appearance of epidermal cells, exodermis thickness, cortex thickness, aerenchyma area (μm^2), endodermis thickness, endodermal cell wall thickness, stele diameter, metaxilem diameter, and number of metaxilem, while the leaf anatomical parameters observed were the thickness of upper and lower epidermis, mesophyll thickness, vascular bundle area (μm^2), number of bulliform cells, and bulliform cells area (μm^2).

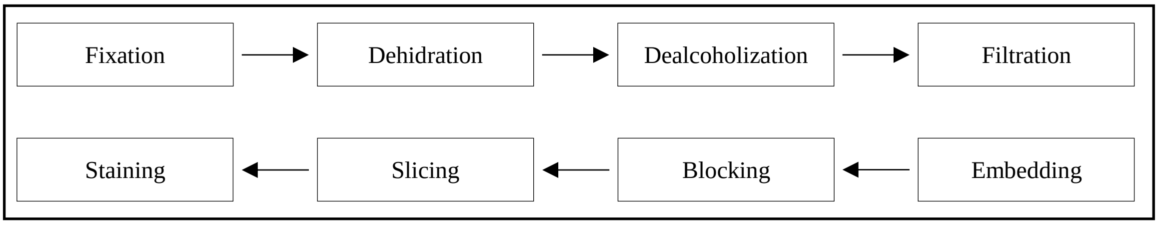


Figure 1. Flowchart of the embedding process carried out in this study.

The secondary metabolites of lignin, phenolic compounds, and terpenoids in the root and leaf tissues were observed histochemically using the free-hand section method. Phloroglucin-HCl reagent was used to observe lignin (Saulle et al., 2018), potassium dichromate (K_2CrO_4) to observe phenolic compounds (Badria and Aboelmaaty, 2020), and $CuSO_4$ 5% to detect terpenoids (Rahayu et al., 2021).

2.4 Data analysis

The observed data were tabulated using the Microsoft Excel application, then homogeneity and normality test were performed using the SPSS application for further analysis of variation (ANOVA) with a 95% confidence level. ANOVA was conducted to determine the effect of liquid batik waste in this study, then Duncan's Multiple Range Test (DMRT) further test was conducted to determine the most influential treatment in this study.

3. RESULTS AND DISCUSSION

3.1 Heavy metals accumulation on soil and plant organ after application of liquid batik waste

The liquid batik waste used in this study did not significantly affect the Cu metal concentration in the vetiver grass soil and roots ($p>0.05$). However, the difference in concentration of liquid batik waste treatment affected the plant's ability to translocate Cu from roots to leaves, where the increase in concentration of liquid batik waste was not accompanied by the ability to accumulate Cu in the leaves (Table 2). Vetiver can accumulate Cu much better in the roots than in the leaves. The higher BCF_{root} value than BCF_{leaf} in this study proves this.

The findings in the study Ghadiri et al. (2018) also showed that the roots of vetiver grass planted in polluted land accumulated higher Cu than in their leaves. The ability of Cu accumulation by vetiver grass was influenced by the Cu content in the soil and the duration of treatment, where the older plants were able to retain Cu in the roots better than the younger plants (Danh et al., 2012).

Table 2. Concentration of Cu and Al after batik waste treatment

Metal concentration in sample	Treatment				
	P_0	P_1	P_2	P_3	P_4
Cu					
Soil (mg/kg)	17.1±3.6[a]	18.0±1.6[a]	17.7±0.9[a]	18.3±1.4[a]	18.3±1.4[a]
Root (mg/kg)	17.4±3.5[a]	17.2±0.9[a]	17.7±1.1[a]	20.4±2.3[a]	20.3±4.7[a]
Leaf (mg/kg)	16.9±4.1[c]	12.7±0.9[b]	17.4±2.2[c]	6.3±0.8[a]	7.6±1.2[a]
BCF_{root} value	1.06	0.95	1.00	1.11	1.10
BCF_{leaf} value	1.05	0.71	0.99	0.35	0.42
TF value	0.99	0.75	0.99	0.32	0.38
Al					
Soil (mg/kg)	4475.1±167.4[c]	4286.6±113.1[bc]	4112.6±87.9[ab]	4004.1±144.3[a]	3912.1±33.3[a]
Root (mg/kg)	957.0±64.4[a]	1086.8±152.1[ab]	1194.4±102.6[ab]	1054.0±30.7[ab]	1418.9±451.3[b]
Leaf (mg/kg)	445.8±50.4[a]	453.1±40.2[a]	456.5±23.0[a]	489.6±35.0[a]	481.5±24.1[a]
BCF_{root} value	0.21	0.25	0.29	0.26	0.36
BCF_{leaf} value	0.10	0.11	0.11	0.12	0.12
TF value	0.48	0.44	0.38	0.46	0.33

Note: Similar letter notation shows no significant difference in DMRT's test with a significance level 5%. (P_0=control (0% concentration); P_1=25% concentration; P_2=50% concentration; P_3=75% concentration; P_4=100% concentration)

On the other hand, the soil and root Al concentrations of vetiver were significantly affected by the treatment of liquid batik waste ($p<0.05$). As the concentration of batik waste increased, the concentration of Al in the roots increased. Increasing the concentration of batik waste lowers the pH of the soil, leading to the release of toxic Al^{3+} by H^+, making this form available to plants (Kinraide, 1997). The addition of complex metal species (Cd, Co, Cu, Pb, and Zn) with high concentrations in maize has been shown to reduce pH, therefore this condition affects the availability of Zn^{2+} and other metals dissolved in the soil so that their availability increases (Romdhane et al., 2021).

Plants can accumulate Al in high concentrations. Rahman et al. (2018), explained that woody plants were able to accumulate <1,000 mg/kg, while monocotyledonous plants such as *Oryza sativa*, *Glycine max*, and *Zea mays* were able to accumulate Al at high concentrations of <500 mg/kg. Likewise in this study, Al in the roots and leaves of the vetiver grass showed a high concentration compared to the Cu concentration (Table 2). Nevertheless, the ability of roots and leaves to accumulate Al, represented by BCF values, was very low, less than 1. Therefore, vetiver grass are excluders of Al metal in this study.

3.2 Anatomical responses

In general, the vetiver grass root anatomy is composed of single-cell epidermal tissue (Figure 2(a-e)), the cortex is composed of the exodermis, aerenchyma, and ordinary parenchyma cells, and the endodermis with 'U' thickening on the longitudinal wall. The central cylinder is composed of a layer of perisikel cells next to the endodermis, polyarch xylem type, phloem arranged between xylem elements, and pith cells which are isodiametrically shaped. The leaf anatomy (Figure 2(f-j)) consists of the abaxial epidermis tissue that has a smaller size and a more homogeneous/uniform shape compared to the adaxial epidermis. Bulliform cells are arranged in the center of the adaxial side of the leaf midrib. Mesophyll tissue is composed of parenchyma cells, where in the older leaves, the parenchyma tissue in the mesophyll forms a wide intercellular space so that aerenchyma is formed. Sclerenchyma tissue is located inside direction of the epidermis which is directly opposite the vascular tissue. Sclerenchyma also surrounds the transport tissue. Leaf stomata are scattered on the adaxial and abaxial surfaces.

The concentration of Cu and Al that accumulates more in the roots causes more pronounced anatomical changes than in the leaves.

The results of ANOVA showed that the applied liquid batik waste had a significant effect ($p<0.05$) on most of the root anatomical parameters except for the number of metaxylem parameters, as well as on most of the leaf anatomy parameters except for the number and bulliform cells area (Table 3). DMRT's further test with a significance value of 95% showed that the high concentration of waste treatment, namely 75% and 100%, was the concentration that had the most effect on increasing the measurement results of each anatomical parameter. Similar discoveries were found in the plant *Phragmites australis* (Cav.) Trin. Ex Steud. namely that the toxicity of various heavy metals (Mn, Zn, Cu, Pb, Cr, Ni, and Cd) causes degradation of the root epidermis, significant reduction in air space, and an increase in cell size in the endodermal tissue of these plants (Minkina et al., 2018).

The anatomical response indicated by the structure of vetiver grass roots is the form of defense to block the metal movement to the central cylinder and translocated to leaves, as also found in research by Gomes et al. (2011) in *Brachiaria decumbens*. The apoplastic barrier in the roots consisting of the epidermis, exodermis, and endodermis is the structure most affected by the occurrence of liquid batik waste stress in this study. Figure 3 shows the occurrence of damage to the cells that make up the epidermis accompanied by an increase in the thickness of the root exodermis. Figure 3(a) shows that at control treatment epidermal tissue is still exist, while in plants with high treatment concentrations (75% and 100%) it appears that there is a thickening of the exodermis as a compensation for the damage to the epidermal cells (Figure 3(b-c)). The location of the epidermis which is in direct contact with the environment allows the epidermal cells to experience oxidative stress, resulting in a thin cell wall that has the potential to damage the tissue (Gomes et al., 2011). Damage to epidermal cells is accompanied by thickening of the epidermal and endoderm cells, which act as a root apoplast barrier. Restricting the movement of toxic ions occurs by thickening these two structures (Lıska et al., 2016).

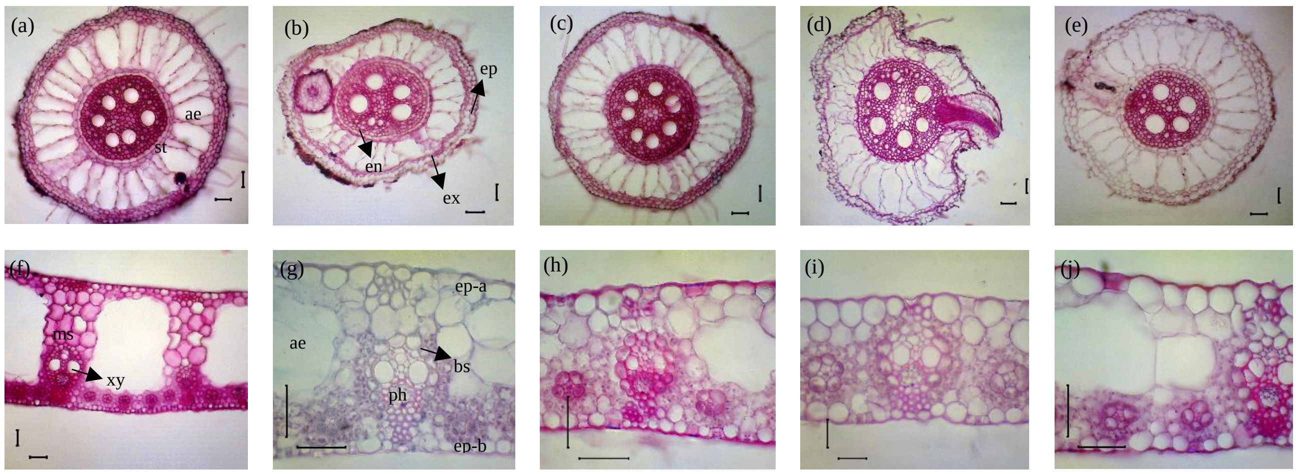


Figure 2. Cross section of roots (a-e) and leaves (f-j) of vetiver grass after treatment of batik waste for 60 days of observation. Desc: (a) P_0 (Control), (b) P_1 (Concentration 25%), (c) P_2 (Concentration 50%), (d) P_3 (Concentration 75%), and (e) P_4 (Concentration 100%). [ae=aerenchyma; st=stele; ep=epidermis; ex=exodermis; en=endodermis; ms=mesophyll; xy=xylem; ep-a=adaxial epidermis; ep-b=abaxial epidermis; ph=phloem; bs=bundle sheath] Bar=50 µm

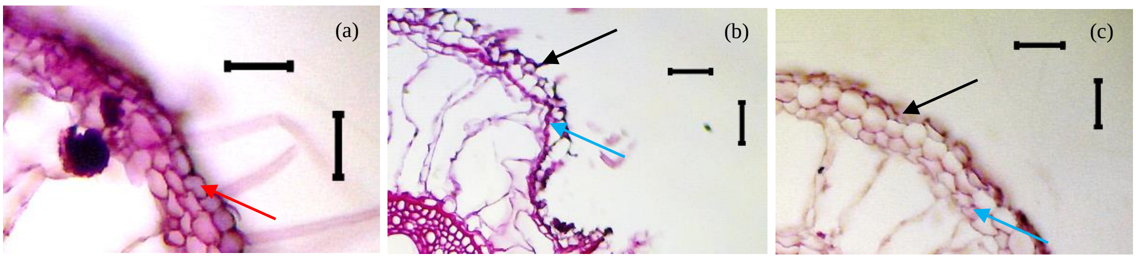


Figure 3. Comparison of appearance of epidermal cells after batik waste treatment. (a) P_0 (Control), (b) P_3 (Concentration 75%), and (c) P_4 (Concentration 100%). [Red arrow=epidermal cells; Blue arrow=exodermis cells; Black arrow=markers of epidermal cells have been degraded] Bar=50 µm

Table 3. Measurement results on the anatomical parameters of vetiver grass roots after batik waste treatment

Anatomical parameters	Measurement result (μm)				
	Treatment				
	P_0	P_1	P_2	P_3	P_4
Root					
Root diameter	604.59±77.80^{b}	465.73±54.80^{a}	518.75±76.00^{a}	678.32±156.40^{c}	683.52±18.00^{c}
Exodermis thickness	11.26±3.20^{a}	14.66±3.70^{b}	15.44±4.20^{bc}	18.35±5.90^{cd}	21.21±3.70^{d}
Cortex thickness	168.24±22.50^{bc}	134.34±13.60^{a}	147.31±17.80^{ab}	195.11±53.30^{d}	188.64±25.20^{cd}
Aerenchyma area (μm^2)	4,689.67^{b}	2,651.33^{a}	4,644.07^{b}	5,333.27^{b}	6,375.40^{c}
Endodermis thickness	14.74±1.50^{bc}	10.84±1.60^{a}	12.94±2.80^{ab}	15.85±5.60^{c}	12.68±4.40^{ab}
Endodermal cell wall thickness	2.59±0.50^{a}	2.54±0.40^{a}	4.08±2.00^{c}	3.44±1.00^{bc}	3.20±0.90^{ab}
Stele diameter	258.65±25.90^{b}	212.37±27.20^{a}	231.58±42.90^{ab}	309.56±62.30^{c}	305.48±26.10^{c}
Metaxylem diameter	44.52±8.10^{bc}	34.51±12.10^{a}	37.82±5.40^{ab}	55.22±11.50^{d}	48.33±10.20^{cd}
Number of metaxylem	9.0±7.2^{a}	6.0±1.7^{a}	6.0±2.8^{a}	6.0±1.0^{a}	6.0±2.6^{a}
Leaf					
Adaxial epidermis thickness	13.32±2.90^{b}	12.27±2.50^{b}	10.89±1.60^{a}	13.77±1.60^{b}	12.13±4.50^{a}
Abaxial epidermis thickness	17.02±6.30^{a}	17.43±5.60^{a}	19.01±2.50^{a}	21.06±3.60^{a}	18.61±7.50^{a}
Mesophyll thickness	252.46±124.50^{c}	167.75±28.34^{b}	107.15±49.40^{a}	111.69±40.20^{a}	180.35±60.90^{b}
Vascular bundle area (μm^2)	7,676.34^{c}	5,313.97^{b}	3,223.30^{ab}	3,115.15^{a}	5,155.85^{ab}
Number of bulliform cells	9.0±0.0^{a}	10.0±1.2^{a}	9.0±1.5^{a}	9.0±1.7^{a}	9.0±1.2^{a}
Bulliform cells area (μm^2)	6,650.76^{a}	11,105.20^{a}	12,493.89^{a}	9,819.17^{a}	10,677.06^{a}

Note: Similar letter notation shows no significant difference in DMRT's test with a significance level 5%. (P_0=control (0% concentration); P_1=25% concentration; P_2=50% concentration; P_3=75% concentration; P_4=100% concentration)

The defense response of vetiver grass from liquid batik waste stress was also demonstrated by the intercellular space of aerenchyma along with the increase in waste water concentration. In vetiver grass, aerenchyma is a common structure found in the root cortex and leaf mesophyll tissue. Even so, stressful conditions allow aerenchyma development to be more intensive in aquatic and semi-aquatic plants (Rajhi and Mhadhbi, 2019). Aerenchyma development occurs influenced by programmed cell death (PCD) in cortical cells that are affected by ROS (Steffens et al., 2011).

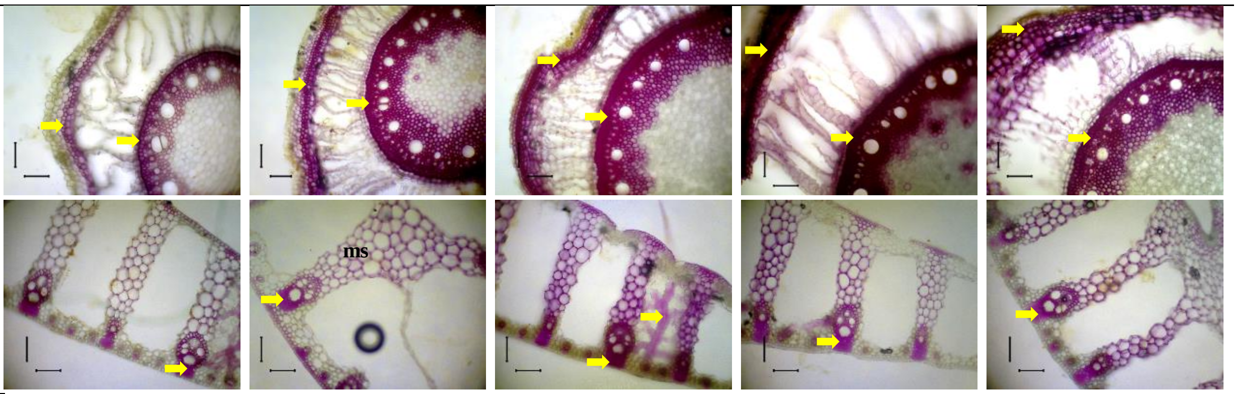
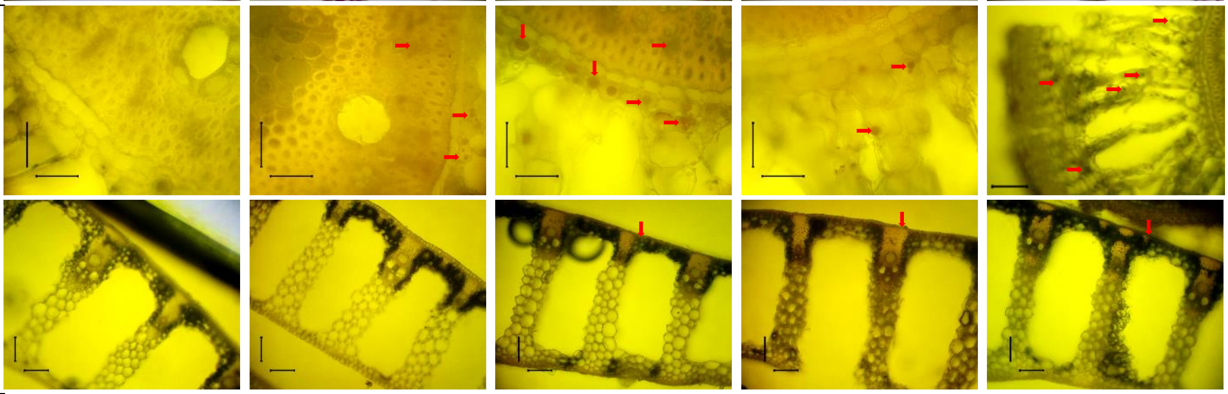
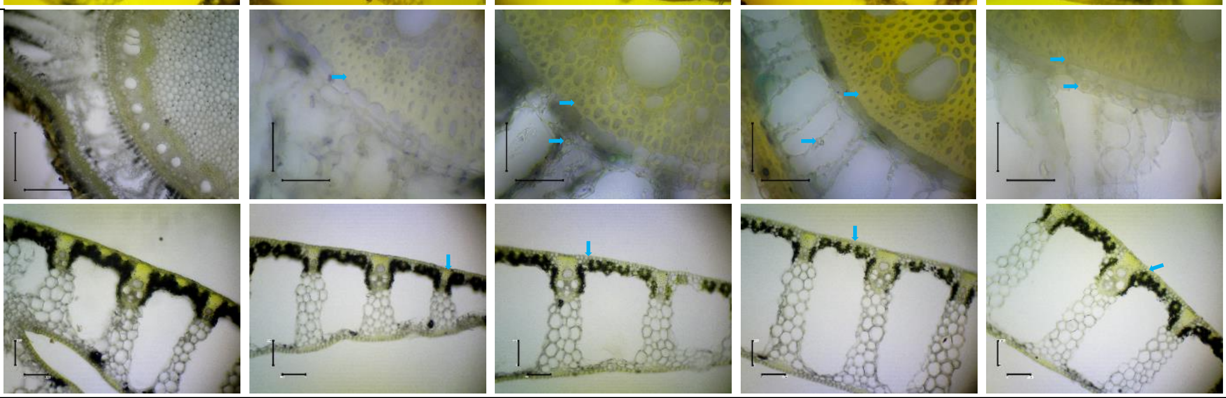
The low concentration of Cu and Al that can accumulate in the leaves causes the response to anatomical changes that occur in the leaves is not so significant, where there is no damage to cells or tissues due to stress (Figure 2(f-j)). Changes occur in the form of an increase in the thickness of the adaxial and abaxial epidermis, as well as an increase in the area of vascular tissue in the leaves, and a decrease in the thickness of the mesophyll (Table 3). Similar findings also occurred in *Potamogeton* plants that were stressed by several heavy metals (Zn, Cu, Pb, and Cd) showing similar results to this study, such as a decrease in leaf thickness, an increase in adaxial and abaxial epidermal cell size (Al-Saadi et al., 2013). According to Thongchai et al. (2021), the increase in epidermal thickness is caused by the effect of growth medium, which increases the amount of heavy metals that can be absorbed into the epidermal layer and then deposited on the epidermal cell wall.

3.3 Histochemical observation

Secondary metabolites are compounds that have a role in plant adaptation to environmental stress. High levels concentrations of secondary metabolites can make plants more resilient to environmental stress (Mazid et al., 2011). Histochemical techniques allow for qualitative analysis of secondary metabolites related to plant tissue deposition. Table 4 shows the difference in the level of color density due to the distribution of secondary metabolites in the tissues that make up the roots and leaves and the reaction results with the reagents used. Liquid batik waste has an effect on the level of reaction color density and distribution of idioblasts as the concentration increases.

Lignin is a major compound that forms the secondary cell wall which plays a role in maintaining the strength and impermeability to water in plant tissues (Tao et al., 2009). Therefore, the reddish color change caused by the reaction with phloroglucin-HCl is seen in cells with thick cell walls, such as on root exodermis, endodermis, xylem cell wall in roots and leaves, as well as on sclerenchyma cell walls in leaves. Lignin is one of the product compounds of the phenylpropanoid pathway, which is part of the shikimate pathway in the synthesis of phenolic compounds (Printz et al., 2016). Lignin deposit that have undergone a biosynthetic process are then polymerized on the cell wall by peroxidase and laccase (Alejandro et al., 2012). The production of lignin is strongly influenced by the activity of the enzyme Phenylalanine Ammonia-lyase/PAL, where its activity is strongly influenced by the environment, especially heavy metals. Research on the *Prosopis glandulosa* plant from oxidative stress after exposure to heavy metals Cd and Cu (concentration of Cd^{2+} 0.001 M and Cu^{2+} 0.52 M) for 96 h, showed an increase in PAL enzyme activity in the phenylpropanoid pathway which caused an increase in the production of phenolic compounds and flavonoids which has a role as a non-enzymatic antioxidant and protects the plant (González-Mendoza et al., 2018). This qualitatively illustrates the effect of liquid batik waste on the lignin content of vetiver grass.

Table 4. Distribution of secondary metabolites in root and leaf tissue of vetiver grass after batik waste treatment

Type of secondary metabolites	Reaction on root and leaf tissue[2]				
	P_0	P_1	P_2	P_3	P_4
Lignin					
Phenolic compound					
Terpenoid					

Note: Yellow arrows indicate lignin compounds, red arrows indicate phenolic compounds, and blue arrows indicate terpenoids in plant tissue. (P_0=control (0% concentration); P_1=25% concentration; P_2=50% concentration; P_3=75% concentration; P_4=100% concentration)

The phenolic compounds in this study were observed by the reaction that occurred between these compounds and 5% potassium dichromate (K_2CrO_4) reagent and showed a brownish yellow color (Table 4). As a common reagent, potassium dichromate is capable of detecting phenolic compounds in tissues with different concentrations depending on the concentration of the target compound in the tissue (Badria and Aboelmaaty, 2019). The distribution and localization of phenolic compounds were more as idioblasts in the root cortex tissue and increased along with the increase in the concentration of liquid batik waste that was applied. This is related to the defense mechanism of these plants from liquid batik waste stress in this study. Research conducted by Melato et al. (2012), explained that there was an increase in the content of phenolic compounds in vetiver grass after receiving heavy metal stress Fe, Pb, Ni, As, Zn, Cu, and Cr which were measured quantitatively using spectrophotometry. It was further explained that this occurs because phenolic compounds are related to plant defense mechanisms against environmental stresses in the form of heavy metals and other stresses. Although no quantitative observations were made in this study, increased levels of color density and distribution of idioblast blasts explained high concentrations of phenolic compounds in tissues.

In line with observations on lignin and phenolic compounds, the distribution of terpenoids in vetiver grass was also affected by the applied liquid batik waste treatment. In addition to being found in the cell walls of the xylem constituents in roots and leaves, terpenoid compounds in roots are more commonly found in the form of idioblast structures distributed in the root cortex tissue. The same finding was also found in undifferentiated cells in *Cinchona ledgeriana* Moens culture which was observed for terpenoid compounds using 5% $CuSO_4$ reagent (Pratiwi et al., 2020). In leaves, terpenoids are also found in the leaf cuticle because the cuticle is composed of terpenoid and flavonoid compounds that are involved in defense mechanisms from biotic and abiotic (Singh et al., 2018; Ziv et al., 2018).

4. CONCLUSION

Vetiver grass was able to accumulate Cu metal better than Al contained in liquid batik waste used in this study, and its storage capacity was better in the roots than in the leaves. Therefore, the root structure shows a more pronounced anatomical response than the leaves. ANOVA showed that the treatment of batik waste significantly affected the anatomical response at most of the observed parameters ($p<0.05$). The histochemically observed distribution and localization of secondary metabolites in the roots and leaves of vetiver showed an increase with increasing application concentration.

ACKNOWLEDGEMENTS

I would like to thank all those who have helped to complete this research. In particular, the first author would like to thank Dr. Maryani, M.Sc who has funded research in the laboratory of Plant Structure and Development. The author also expresses deep gratitude to the UGM PIAT staff who have facilitated the procurement of research sample plants.

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