



Effects of husk charcoal and watering on the biochemical and physiological properties of coix millet (*Coix lacryma jobi* L.) during vegetative phase

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Article Info

Received : 18th November 2022

Revised : 29th May 2023

Accepted: 24th July 2023

Keywords:

coix millet, drought stress, husk charcoal doses

Abstract

Continuous climate change increases the number of droughts in some areas, thereby affecting agricultural production. Cultivation of coix millet (Jali plant in Indonesian) is profitable because of its high adaptability to drought stress, and its seeds have the potential to be used as food due to their benefits for body health. The application of organic matter, namely husk charcoal, was chosen to increase agricultural production under drought stress. Therefore, this study aimed to determine the effects of husk charcoal on the biochemical and physiological properties of coix millet during vegetative phase under drought stress and to determine the longest watering interval the plant can tolerate. Research was arranged in a split-plot design with three replications. The main plot consisted of three watering intervals (every two, four, and six days). The sub-plots consisted of three doses of husk charcoal, namely 0 g polybag⁻¹ (0 tons ha⁻¹), 32 g polybag⁻¹ (10 tons ha⁻¹), and 64 g polybag⁻¹ (20 tons ha⁻¹). The data were analyzed using analysis of variance (ANOVA) and tested using Duncan's Multiple Distance Test (DMRT) at a significant level of 5%. The results showed that husk charcoal could increase the chlorophyll content in plants experiencing drought stress. The longest watering interval that could be tolerated by coix millet given husk charcoal at the optimal dose (64 g polybag⁻¹) was once every four days.

INTRODUCTION

Constantly changing climatic conditions affect agricultural production and threaten many livelihoods in developing countries. According to Mulwa and Visser (2020), an important strategy to create resilience to climate shocks in agricultural production is to increase food diversification. One of the potential staple food sources is coix millet (*Coix lacryma jobi* L.) (Juhaeti, 2015), which is known as jali plant in Indonesian. Coix millet is adaptive to various types of soil, including marginal ones indicated by low nutrient availability and dry condition (Irawanto et al., 2017). Another advantage of the coix millet is that their seeds are rich in carbohydrates and other beneficial compounds, such as coixol (Amen et al., 2017), polysaccharides (Yao et al., 2015), and phenolic compounds (Wang et al.,

2016). These functional compounds can be used to treat the symptoms of warts, cracked skin, rheumatism, neuralgia, and inflammatory diseases. Therefore, coix millet seeds have the potential to be used as substitute to rice.

One of the problems in dry areas is the limited availability of water, causing plants to experience drought stress. The effect of drought stress is a decrease in plant growth and yield (Xu et al., 2022). One of the efforts to increase plant resistance to drought stress is to add soil organic matter. Soil organic matter has a physical role in increasing the ability to hold water, chemicals to add plant nutrients and biological role in improving soil respiration and the number of soil microorganisms (Margolang et al., 2015). Organic matter is usually given by farmers in the form of livestock manure. Wet tropical climate conditions with high

How to cite: Fadillatunnisa, Indradewa, D., and Putra, E.T.S. (2023). Effects of husk charcoal and watering on the biochemical and physiological properties of coix millet (*Coix lacryma jobi* L.) during vegetative phase. *Ilmu Pertanian (Agricultural Science)*, 8(2), pp. 113–120.

ISSN 0126-4214 (print) ISSN 2527-7162 (online)

temperatures cause the manure to decompose quickly. There is a form of organic matter that is more resistant to decomposition, including husk charcoal. Porosity and aeration of the soil can be increased by adding rice husk charcoal to the media. The large number of cavities causes good aeration and drainage, thus facilitating the movement of plant roots in absorbing nutrients. In addition, rice husk charcoal also has the ability to decay slowly and can last for about one year, so it can be used several times.

In addition to predominantly containing calcium, husk charcoal also has a high magnesium content. Adequate magnesium increases leaf greenness, carbohydrates, fats, and oils. Besides, magnesium also has an important role in the transport of phosphate in plants. According to Guo (2017), reduction of Mg interferes with phloem sucrose production from leaves to the phloem tissue, resulting in carbon accumulation in the leaf source. Accumulation of carbohydrates in leaf sources and withdrawal of root growth are thought to be the early responses to Mg deficiency, as they are involved in biomass formation and carbohydrate partitioning. High magnesium increases phosphate in plants, stimulating root growth and promoting flowering, seed maturation, and fruit ripening (Anonymous, 2015). Koyama and Hayashi (2019) proved that husk charcoal given at a dose of 20 mg ha⁻¹ could improve the panicle weight, straw, and number of grains. Lolomsait (2016) also provides information that husk charcoal increases the stem diameter and fruit length of red chilies. Sembiring et al. (2018) reported that on robusta coffee seedlings, the interaction of top soil and charcoal husk treatment (2:1) and watering once a day and the interaction of top soil and charcoal husk (2:1) and watering every 7 days had a significant effect on the stem diameter and fresh weight shoot. In the water sufficiency treatment, namely watering once a day, the excess water can be stored in the plants. On the other hand, in conditions of limited water, the treatment of watering every 7 days is able to utilize water efficiency for the process of growth and development. This indicates that by combining the rice husk charcoal treatment and watering intervals, it is possible to determine the longest watering interval that is tolerated by the plants, so that the use of water volume and watering can be more efficient. Qian et al. (2019) added that biochar application to the media could increase the activity of sucrose phosphate synthase and sucrose synthase, both of which are involved in the main products of photosynthesis.

This study aimed to determine the effects of husk charcoal doses on the biochemical and physiological properties of coix millet during the vegetative phase under drought stress and to determine watering interval the plant can tolerate.

MATERIALS AND METHODS

Pot experiment was carried out from January to April 2021 in a plastic house in the Citra Tiara Maguwoharjo Housing area, Yogyakarta, and was dominated by the type of soil entisol. During germination, watering was carried out every two days in the afternoon until the plants entered the early vegetative phase. Watering interval treatments were given when the plants entered the initial vegetative phase (4 weeks after planting), and the treatment was stopped in the flowering stage (14 weeks after planting). Biochemical and physiological observations were made at the maximum vegetative growth phase (14 weeks after planting). The leaves used are mature leaves. The vegetative stage is the maximum stage of plant growth so it is carried out during the maximum vegetative period of the plant. Soil observation was carried out at the Yogyakarta Agricultural Technology Study Center (BPTP), and observation of sample plants was carried out at the Laboratory of Plant Production Management, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta.

The materials used in this study were coix millet seeds, husk charcoal, entisol soil, inorganic fertilizers (Urea, TSP, and KCl), and irrigation water. The tools used were hoe, stationery, scissors, an analytical scale, a test tube/venotube, a cuvette, a spatula, a vortex, a spectrophotometer, a micrometer, and an oven. The experiment was arranged in a split-plot environmental design. The main plot was watering interval, consisting of three levels (every two, four, and six days). The subplot was the husk charcoal weight, which consisted of three levels, namely 0 g polybag⁻¹ (0 tons ha⁻¹), 32 g polybag⁻¹ (10 tons ha⁻¹), and 64 g polybag⁻¹ (20 tons ha⁻¹). There were three replications of each treatment combination. ANOVA was used to determine the variance in the observational data before Duncan Multiple Range Test (DMRT) was used ($\alpha = 0.05$). SPSS was used for data analysis, and Microsoft Excel was used for data visualization.

Relative water content (%)

Sample weighing 10 g was taken and calculated as fresh weight (FW). The sample was submerged in water

in a polythene bag for 24 hours at room temperature. Within 24 hours, a fixed weight was achieved, which was the saturated weight (SW). Samples were put at the oven at 80°C for 48 hours to obtain a fixed weight, which was dry weight (DW) (Hasthanasombut and Ntui, 2010). Measurements were made at the maximum vegetative growth phase (14 weeks after planting). The relative water content was calculated by equation (1).

$$RWC = [(FW-DW)/(SW-DW)] \times 100 \dots\dots\dots (1)$$

Hydrogen peroxide (ppm)

1 g sample was taken and crushed with liquid N₂. Then, added with 1000 mm TCA 0.1%, 0.5 ml supernatant, 500 µl potassium phosphate buffer, and 2 mL KI. The materials were placed in a cuvette, closed, and incubated for 1 hour in a dark place. A spectrophotometer with a 390 nm wavelength was being used to read it (Alexieva et al., 2001). The leaves used are mature leaves.

Superoxide dismutase (unit/ml)

0.2 g of leaves were taken and crushed with liquid N₂. Then, 50 mM potassium phosphate buffer (pH 7), 1% PVP, 0.2 mM ascorbic acid, 50 nM potassium phosphate buffer (pH 7), 0.1 mM EDTA, 0.3 mM riboflavin and 0.3 mM nitroblue tetrazolium (NBT) were put into the cuvettes. Furthermore, measurements were made with a spectrophotometer at a wavelength of 560 nm. Enzymes activity is expressed in units of mg⁻¹ protein (Giannopolitis and Ries, 1977). SOD calculation was calculated based on equation (2).

$$SOD \text{ activity} = (\text{control} + \text{sample}) / 0.5 \times \text{sample result} \dots(2)$$

Ascorbic Acid (ppm)

The measurement of vitamin C levels was carried out by the Iod titration method (Cresna et al., 2014), namely by taking 10 grams of crushed leaf samples, which were then diluted to 250 ml and filtered. Then 25 ml of filtrate was taken and added with 2 ml of 1% starch solution as an indicator. Titration was carried out with 0.01 N iodine solution until a blue color was formed, and the amount of iodine used was recorded in milliliter units. Ascorbic acid test results are expressed in percent (%). Vitamin C content was calculated using equation (3).

$$\% \text{ Vit C} = (a.\text{ml Iod. } 0.88. \text{ FP. } 100\%) / (\text{mg}) \dots\dots\dots(3)$$

Proline level (µmol/g)

0.5 g of leaves was taken and pounded using liquid N₂. Then 10 ml of 3% sulfosalicylic acid solution was dissolved to separate the filtrate and added with 2 ml of nindrihidin acid solution (1.25 g in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid with shaking and heating) and 2 ml of glacial acetic acid in a test tube. The absorbance was measured at a wavelength of 520 nm (Bates et al., 1973). The proline concentration was determined by the proline standard curve (Sigma).

Malondialdehyde (µmol/g)

0.5 g of leaves were taken and crushed with liquid N₂. Each leaf was put into a centrifuge tube by adding 0.1% TCA. Then it was centrifuged at 1500 rpm 10 minutes 4°C, and the supernatant was taken. A total of 0.5% TBA in 1.5 ml of 20% TCA was added to 500 µl of supernatant and vortexed. After that, it was incubated at 90°C for 20 minutes and soaked in cold water, followed by putting it to centrifuge at 10,000 rpm for 5 minutes. The supernatant was then put into the spectrophotometer cuvette at a wavelength of 532 & 600 nm.

Stomatal opening width (µm)

The observation was made when the plant entered the maximum vegetative phase during the day by applying clear nail polish under the leaves. Once dried, the nail polish was removed using clear tape. The stomatal prints of the nail polish were attached to a glass object and observed under a microscope. An eyepiece with micrometer in the form of a fence with a magnification of 420 X was used to observe the stomatal opening width (Haryanti, 2010). The ocular micrometer was calibrated with an objective micrometer.

Transpiration rate (mg hour⁻¹cm⁻²)

Measurements were done from 11 AM to 2 PM on the 2nd-4th leaf that was fully opened and exposed to the sun. The underside of the leaf was covered with transparent plastic and cobalt chloride paper and then secured with paper clips. The cobalt chloride paper took the same amount of time as the standard to transform from blue to pink. Temperature, light intensity, and humidity were also recorded. The transpiration rate was calculated by equation (4).

$$\text{Transpiration rate} = 1/L. 60/T. CF (\text{mg}/\text{hour}/\text{cm}^2) \dots(4)$$

(Bailey et al., 1951)

Chlorophyll content (mg/g/leaf fresh weight)

Leaf chlorophyll filtrate was made by weighing 1 g of leaf sample, which was then cleaned, cut into small pieces, added with 20 mL of 80% acetone using a measuring pipette, and put into the mortar. The sample was filtered with filter paper in a glass beaker and put into a test tube. Chlorophyll content was measured using a spectrophotometer. With existing materials, the absorbance values were observed and recorded (Arnon, 1949). After obtaining the absorbance value, the chlorophyll content was calculated using equation (5).

$$\text{Total chlorophyll} = 20.2 D_{645} + 8.02 D_{663} \text{ (mg/l) } \dots(5)$$

Photosynthesis rate (mg.CO2.dm⁻².hour)

The photosynthesis rate was measured by covering the leaves for approximately three hours. The gas in the hood was put into a 10 ml tube containing 10 ml of BTB (1:1) and shaken for 1–2 minutes. The transmittance of the BTB solution was measured using a spectrophotometer at a wavelength of 615 nm. The photosynthesis rate was calculated by equation (6).

$$\text{Photosynthesis} = (X. \text{ BM CO}_2. \text{ Vas}) / (\text{CO}_2 \text{ weight. LD. Time}) \dots\dots\dots(6) \text{ (Pratt and Mendoza, 1979)}$$

RESULTS AND DISCUSSION

There was no interaction effect of husk charcoal doses and watering interval on the relative water

content, H₂O₂, SOD, ascorbic acid, proline, MDA, stomatal opening width, transpiration rate, and photosynthesis rate. However, there was an interaction effect of husk charcoal doses and watering interval on the leaf chlorophyll content.

Watering interval had a significant effect on the relative water content. Compared to coix millet watered every four and two days, those watered every six days had a lower relative water content (Table 1). The decrease in soil moisture content due to drought stress causes the groundwater potential to be lower than the plant water potential, making it difficult for plants to absorb water. One indicator that shows the status of plant water potential is the relative water content. Therefore, the low relative water content indicates that the plant is experiencing drought stress. The provision of husk charcoal in the planting media was carried out to increase the water holding capacity, so that coix millet could be watered at longer intervals without causing drought stress.

Increased levels of proline in the leaves can increase the content of ABA, which regulates stomata opening. This opinion is in accordance with the research conducted by Bandurska et al. (2017), reporting that barley plants cv. maresi experienced an increase in the free proline level in the leaves by increasing the ABA content, thus affecting stomata closure. Treatment of watering interval every six days caused the width of the stomatal opening to be smaller than that of those watered every four and two days (Table 1). This is because the decrease in moisture content by drought

Table 1. Relative water content (RWC), stomatal opening width, transpiration, and photosynthetic rate of coix millet leaves

Treatments	Relative water content (%)	Stomata opening width (µm)	Transpiration (mg hour ⁻¹ cm ⁻²)	Photosynthetic (mg.CO ₂ .dm ⁻² .hour)
Husk Charcoal				
0 g polybag ⁻¹	77.55 a	0.11 b	6.93 a	52.94 b
32 g polybag ⁻¹	76.91 a	0.12 ab	6.57 a	89.44 a
64 g polybag ⁻¹	76.43 a	0.12 a	4.49 a	76.02 ab
CV (%)	0.72	7.05	9.71	13.07
Watering Interval				
Every 2 days	77.79 q	0.13 p	9.60 p	92.59 p
Every 4 days	82.62 p	0.11 q	4.88 q	71.19 pq
Every 6 days	70.47 r	0.10 r	3.51 q	54.61 q
CV (%)	7.94	13.01	22.52	13.16
Interaction	(-)	(-)	(-)	(-)

Remarks: Based on (α = 0.05), the values with the same letters in one column are not significantly different, (-) interaction effects are not significant.

Table 2. Chlorophyll content of coix millet leaves

Watering Interval	Husk Charcoal			Average
	0 g polybag ⁻¹	32 g polybag ⁻¹	64 g polybag ⁻¹	
Every 2 days	1.16 ab	1.18 ab	1.24 a	1.19
Every 4 days	1.02 c	1.23 a	1.07 bc	1.11
Every 6 days	0.90 d	1.21 a	1.15 ab	1.09
Average	1.03	1.16	1.21	(+)
CV (%)	9.94			

Remarks: Based on ($\alpha = 0.05$), the values with the same letters in one column are not significantly different, (-) interaction effects are not significant.

Table 3. H₂O₂, SOD, ascorbic acid, proline, and MDA of coix millet

Treatments	H ₂ O ₂ (ppm)	SOD (unit/ml)	Ascorbic acid (ppm)	Proline ($\mu\text{mol/g}$)	MDA ($\mu\text{mol/g}$)
Husk Charcoal					
0 g polybag ⁻¹	17.08 a	1.05 a	57.68 a	5.86 a	2.39 a
32 g polybag ⁻¹	17.66 a	0.34 a	46.93 a	6.59 a	2.29 a
64 g polybag ⁻¹	19.58 a	0.59 a	49.86 a	5.23 a	3.07 a
CV (%)	7.22	23.76	10.79	11.53	16.31
Watering Interval					
Every 2 days	19.07 p	0.75 p	60.62 p	3.80 q	2.56 p
Every 4 days	18.45 p	0.52 p	47.91 q	5.79 pq	2.55 p
Every 6 days	16.81 p	0.71 p	45.95 q	8.08 p	2.64 p
CV (%)	6.46	18.05	15.46	18.62	1.96
Interaction	(-)	(-)	(-)	(-)	(-)

Remarks: Based on ($\alpha = 0.05$), the values with the same letters in one column are not significantly different, (-) interaction effects are not significant.

stress causes plants to close their stomata to reduce excessive transpiration. Giving husk charcoal to the media was able to increase the width of the stomatal opening under drought stress. Giving 64 g polybag⁻¹ (20 tons ha⁻¹) and 32 g polybag⁻¹ (10 tons ha⁻¹) of husk charcoal was able to increase the maximum stomatal opening width compared to without husk charcoal (Table 1). This is due to the fact that giving husk charcoal has a significant effect on plant physiology because the given medium can reduce temperature and increase soil moisture. Increased soil moisture will affect the width of the stomatal opening and allow the plants to carry out photosynthesis.

The decrease in the width of the stomatal opening can reduce the transpiration rate in coix millet. The decrease in the width of the stomatal opening is a plant strategy to avoid excessive transpiration. Meanwhile, the closure of the stomata for too long will interfere with the photosynthesis due to the lack of incoming

CO₂ supply. Watering interval of six and four days resulted in a lower transpiration rate than watering every two days (Table 1). The provision of husk charcoal is expected to increase the transpiration rate under drought stress. Nevertheless, the provision of husk charcoal had no significant effect on the transpiration rate in coix millet (Table 1). This is due to the lack of effort by the rice husk charcoal to store enough water so that the plants respond by reducing the width of their stomata openings and lowering the rate of transpiration.

There was an interaction effect of husk charcoal doses and watering interval on the leaf chlorophyll content. Combined with medium husk charcoal application, which is 32 g polybag⁻¹ (10 tons ha⁻¹), the increase in watering interval did not significantly reduce leaf chlorophyll content. Meanwhile, in plants that were not given husk charcoal, increasing watering interval significantly decreased leaf chlorophyll content

(Table 2). The decrease in relative water content due to drought stress will slowly reduce the CO₂ concentration in the leaves so that it can reduce the chlorophyll content in plants. The decrease in chlorophyll content will reduce the rate of photosynthesis in plants. This is because the lack of water will affect the content and organization of chlorophyll in chloroplasts in tissues. In addition, the treatment without husk charcoal reduced the chlorophyll content in the leaves due to the leaching of nutrients in the soil, thereby reducing the uptake of nitrogen nutrients for the leaves and reducing the constituent material of leaf chlorophyll in plants. On the other hand, plants that experience sufficient water have increased relative water content, thereby increasing the chlorophyll content in the leaves, which will increase the rate of photosynthesis in plants. The application of husk charcoal to plants causes a decrease in nutrient leaching, and it improves the availability of nutrients such as nitrogen, which is an essential nutrient that functions as a constituent of chlorophyll.

The amount of photosynthesis was significantly impacted by the watering interval treatment. Watering every six days caused the rate of photosynthesis to be lower (Table 1). In the treatment of watering every six days, the width of the stomatal opening was smaller, causing a decrease in the availability of CO₂ and the rate of photosynthesis in plants. When plants experience drought stress, plants carry out a defense mechanism by closing stomata to prevent water loss, thereby limiting the availability of CO₂ in the chloroplast, reducing electron transport, and causing a reduction in ATP and NADPH (Ashraf and Harris, 2013). In this condition, it will disrupt the photosynthesis process and reduce the rate of photosynthesis in plants (Wang et al., 2019). Giving husk charcoal increased the rate of photosynthesis in coix millet under drought stress. Giving husk charcoal at the doses of 32 g polybag⁻¹ (10 tons ha⁻¹) and 64 g polybag⁻¹ (20 tons ha⁻¹) increased the rate of photosynthesis, which was higher than those without husk charcoal (Table 1). According to Qian et al. (2019), biochar application increased leaf chlorophyll in soybean plants. Leaf chlorophyll correlates with nitrogen content (Padilla et al., 2014), in which husk charcoal can increase the nutrient availability and the efficiency of fertilizers.

Watering interval treatment had no significant effect on H₂O₂, SOD, and MDA. Nevertheless, there was a significant effect on ascorbic acid and proline (Table 3). Coix millet treated with watering interval

of six and four days had lower levels of ascorbic acid than those given watering every two days. Meanwhile, plants watered every six and four days had higher proline level than those watered every two days. The longer the watering interval, the lower the ascorbic acid content and the higher the proline content in coix millet. Drought stress at long watering interval causes chlorophyll degradation because there is no effort to fight the emergence of free radicals indicated by decreased ascorbic acid levels. The increase in proline levels in drought-stressed plants was not able to overcome the degradation of chlorophyll. It is suspected that the increase in proline levels in drought-stressed coix millet caused a decrease in ascorbic acid because most of the biomass was used to synthesize proline. The provision of husk charcoal in the media is expected to be able to store sufficient water to reduce the occurrence of dryness, thereby reducing free radicals. However, the administration of husk charcoal was not able to reduce free radicals with no significant effect on H₂O₂, SOD, ascorbic acid, proline, and MDA (Table 3). This is due to the lack of husk charcoal efforts to store enough water to reduce the occurrence of free radicals due to drought. Large amounts of reactive oxygen molecules will be produced when cells lose water under drought conditions, and these oxygen free radicals will destroy the plant cell structure, increasing the relative permeability of the plasma membrane. Continuous electrolyte extravasation reduces plant growth and development because it raises relative conductivity, accumulates a lot of ROS that is bad for lipid membrane peroxidation, increases MDA content, and is toxic to cells and interferes with normal metabolic processes (Marques et al., 2019; Chutipajit, 2016).

CONCLUSIONS

Husk charcoal doses and watering intervals had an interaction effect on the amount of chlorophyll in the leaves. The application of rice husk charcoal at a dose of 32 g polybag⁻¹ (10 tons ha⁻¹) was able to increase stomatal opening width, chlorophyll content, and photosynthesis rate under drought stress. The longest watering interval that could be tolerated by coix millet when the planting medium was given husk charcoal at the optimal dose was once every four days, indicated by higher relative water content, chlorophyll content, and photosynthetic rate.

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