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The growth responses and antioxidant capabilities of melinjo (*Gnetum gnemon* L.) in different durations of drought stress

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Abstract

Melinjo (*Gnetum gnemon* L.) is a beneficial agroforestry plant that contains antioxidant compounds broadly studied as a natural source in pharmaceutical and nutraceutical uses. The antioxidant compoundsbenefit human health and plant to develop defense mechanisms under environmental stresses, such as drought stress.This study aims to evaluatethe defense mechanism of melinjounder drought stress. Polyethylene glycol (PEG) was used to simulate drought stress to melinjo seedlings for 5, 10, 20, and 40 days compared to zero-day control without PEG. The inhibition and detoxification activities of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, superoxide radical, and superoxide dismutase (SOD) were determined to evaluate antioxidant capabilities during drought stress. Lipid peroxidation and hydrogen peroxide (H_2O_2) contents were also analyzed to evaluate the level of cellular damage during drought stress. The seedling's growth was significantly reduced as a response to the longer duration of drought stress treatment. On the contrary, the level of lipid peroxidation and H_2O_2 wereincreased, followed by the escalation of antioxidant activities. This result indicates that many antioxidant compounds act together to develop a defense mechanism in melinjo seedlings, and the different duration of drought stresses influences their capabilities.
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Keywords: Aantioxidant, Drought stress, Melinjo, Radical scavenging

1. Introduction

As one of the Gnetaceae families, melinjo (*G. gnemon*L.) is one of the evergreen plants widely cultivated in several countries, and its use as a traditional drug to elixir numerous diseases (Rachmawati et al., 2017).Its fruits, leaves, seeds, and flowers are mostly consumable by Indonesian, whether raw or processed. Melinjo contains resveratrols, like gnetin C, gnemonoside A, and gnemonoside B, which does not have side effects for human health and safe to consume directly or after processed (Tatefuji et al., 2014). Melinjo also has a protein content of 9 to 10% with prosperous high antioxidant activity to prevent free radicals from the environment (Siswoyo et al., 2011). Manner and Elevitch (2006) mentioned that melinjo could grow in the dry season for several months. It is indicating that melinjohas a tolerance ability to drought stress.

Drought stress is one of the environmental factors that crucially inhibit plant growth and development. The changes in physiology, morphology, and biochemical properties may occur in plants after drought stress exposure. Similar to salinity stress, drought stress triggers a high accumulation of reactive oxygen (ROS) in plant cells (Hasanuzzaman et al., 2018)like singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), H_2O_2, and hydroxyl radical (Caverzan, 2013). As the defense mechanism to protect cellularly damaged and keep osmotic pressure in balance, antioxidant activity increases when ROS accumulation is mounted (More and Makola, 2020). Plant performs two groups of antioxidants, enzymatic and non-enzymatic antioxidants, which reduce the negative impacts of ROS accumulation (Sharma et al., 2012). There are two major pathways as the action of antioxidant enzymes to prevent the effects of ROS, once is by reducing the formation of superoxide anion radical into H_2O_2 and oxygen (O_2) and the second is by detoxifying the production of H_2O_2 by using the photorespiration pathway (Aydin et al., 2014). SOD is the key antioxidant enzyme that eliminates ROS (Kobayashi et al., 2019) by diminishing the formation of superoxide radicals (Miller, 2012), which are detoxified by other antioxidant enzymes.

The antioxidants capabilities against ROS accumulation are correlated to plant tolerance potency under drought stress (Laxa et al., 2019). The level of environmental stress will affect the ability to gain adaptation and defense mechanisms in plant cells (Malik et al., 2020). The ability of PEG to stimulating drought stress had been broadly known because of its ability to inhibit water and nutrients absorption without causing toxicity in a plant cell. This study aimed to evaluate the growth response and antioxidant capabilities of melinjo seedlings under different durations of drought stress induced by PEG.

2. Materials and methods

2.1. Plant materials and treatments

The experiment was accomplished in August 2019 at the greenhouse and Plant Analysis Laboratory University of Jember, Jember, East-Java, Indonesia. Melinjo seed planted in the 4 kg mixed medium containing sand, soil, and organic compost (1:1:1, w/w). Homogenous and healthy four-month melinjo seedlings were used as plant materials. All seedlings were well watered every two days (with and without PEG applications). The field capacity of the medium had been measured to determine the watering volume. The experiment was designed in a randomized block with five replicates. The drought stress was performed using 15% PEG with five different durations of application (0, 5, 10, 20, and 40 days). Leaf samples were taken, and growth parameters were also measured two days after the last application.

2.2 Growth parameters

Morphological parameters, including shoot length, root length, number of leaves, shoot diameter, fresh weight, and dry weight, were measured to perform the growth response of each melinjo seedling after drought stress treatment. These parameters were recorded two days after the last application.

2.3 Total chlorophyll analysis

The chlorophyll concentration was assayed using 80% acetone to extract 0.1 g leaf samples of each treatment. The supernatant was taken after centrifugation at 4500 rpm for 10 min. The chlorophyll concentration was calculated based on the absorbance (Abs) recorded at

663, 710, and 646 nm (Holm-Hansen and Riemann, 1978). The results were quantitatively determined using equations:

Total chlorophyll (mg/L) = 7.18 ($Abs_{663} - Abs_{710}$) + 17.32 ($Abs_{646} - Abs_{710}$)

2.4. H_2O_2 analysis

The assay of H_2O_2 content was done using the protocolof Graf and Penniston (1980). As much as 0.1 g of leaf samples were homogenized in 1 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 12000 rpm for 5 min. The assay consisted of 0.5 ml supernatant, 0.5 ml of 10 mM phosphate buffer (pH 7.0), and 1 ml of 1M potassium iodide. The absorbance was measured at 390 nm after 30 min incubation at room temperature. H_2O_2 content was estimated using a standard calibration curve of H_2O_2 without the addition of the sample.

2.5. Malondialdehyde (MDA) concentration

Estimation of MDA contentwas done by following the protocol of Cakmak and Horst (1991). The leaf sample for 0.2 g was homogenized in 5 ml of 0.1% TCA, centrifuged at 12000 rpm for 5 min. The assay was carried out by adding 0.5% thiobarbituric acid (dissolved in 20% TCA) to the supernatant. The mixture was incubated at 90°C for 30 min, and the absorbance was measured at 532 and 600 nm.

2.6.Protein extraction

The leaf samples taken from each seedling were homogenized in phosphate buffer (1:3, w/v) to extract the soluble protein, then ground on mortar (Makkar et al., 1993). The homogenate was then centrifuged at 10000 rpm in 4°C for 15 min. The supernatant was taken and used to determine the total soluble protein and antioxidants activity.

2.6.1. Total soluble protein determination

Total soluble protein was determined by the protocol of Bradford (1976). The assay mixtureconsisted of 5 µl supernatant, 45 µl H_2O, and 950 µl Bradford's solution. The Abs measured at 595 nm after vortex the mixture. The protein content of bovine serum albumin (BSA) was used as a standard.

2.7. Free radical scavenging activity

2.7.1.ABTS scavenging activity

The antioxidant activity based on the ABTS assay was done using the Re et al. (1999) method. The ABTS stock solution (7 mM ABTS with 2.45 mM potassium persulfate) was incubated for 12-16 h in the dark before used, which then diluted with 0.2 M sodium phosphate buffer saline (pH 7.4) to reach the absorbance 0.7-0.75 at 734 nm. As much as 950µl of ABTS solution was added into 20 µl of supernatant to perform the assay. The absorbance (A) measured at 734 nm, and the activity was calculated based on the equation:

Scavenging activity of ABTS (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100\%$

2.7.2. DPPH scavenging activity

The assay of DPPH scavenging activity was performed by adding 800 µl of 0.5mM DPPH solutions and 100 µl of 96% methanol to 100 µl supernatant(Blois, 1958).

Theabsorbance was measured at 517 nm after incubation of the mixture at room temperature for 20 min. The following formula calculated the DPPH scavenging activity:

DPPH scavenging activity (%) = $[(A_{control} - A_{sample}) / A_{control}]$ x 100%

2.7.3.Superoxide radical scavenging activity

Superoxide radical scavenging activity assay was done by adding 100 μl 10 mM pyrogallol to the solution containing 1.8 ml of 50 mM Tris-hydrochloride(Tris-HCl) pH 8.2 and 10 μl supernatant, then incubated for 10 min (Tang et al., 2010). The absorbance was measured for 4 min at 320 nm. The formula calculated the scavenging activity:

Inhibition percentage (%) = $[(S_0-S_1)/S_0)$ x 100%

Where S_0 is the slope of control, and S_1 is the slope of the sample.

2.8. SOD activity

SOD activity was done based on the autoxidation of pyrogallol(Marklund and Marklund, 1974). 100μl of 0.2 mM pyrogallol was added to the mixture containing 50 mM Tris-HCl pH 8.2 and 1 mM ethylenediamine tetraacetic acid (EDTA) with 50 μl supernatant. The absorbance was measured at 420 nm. The SOD activity was calculated as unit/mg protein, where one unit is equal to the 50% inhibition of pyrogallol auto-oxidation.

2.9 Ribonucleic acid (RNA) isolation and Polymerase Chain Reaction (PCR)analysis

0.1 g leaf was ground in liquid nitrogen until the powdery texture was formed, which then homogenized in 1 ml of AccuZol^{TM}ready use reagent. Then, 200μl of chloroform was added into the solution and shaked vigorously for 15 sec, continued with incubation on ice for 5 min. The mixture was centrifuged at 12000 rpm for 15 min at 4^{o}C. The supernatant was then added with isopropyl alcohol in equal volume, followed by centrifugation at 12000 rpm for 10 min at 4^{o}C. The supernatant was discarded, and 80% ethanol was added into the pellet, re-centrifuged at 12.000 rpm for 5 min at 4^{o}C. The pellet was collected then dissolved in RNAase-free water. The RNA concentration was measured based on the Abs result at 260 and 280 nm. As much as 1 μg of RNA template was used for reverse transcription by using the Hyperscript^{TM}RT master mix (GeneAll®) to synthesis the complementary DNA (cDNA). The reverse transcription was performed by adding 10 μl master-mix and sterilized ddH_2O up to 20 μl as the final volume. The reaction began after incubation at 95^{o}C for 5 min. The cDNA product was then amplified using PCR with the following condition: 94^{o}C for 3 min, 94^{o}C for 30 sec, 56^{o}C for 30 min, 72^{o}C for 30 sec, and 72^{o}C for 7 min as the final elongation. The primer used to performed the expression of gene encoding SOD, Copper/Zinc Superoxide Dismutase 1 (*CSD1*)is used as the target gene (forward: GAACTGCCACCTTCACAA and reverse: TGGTAGGGCTGTTGTTGTC, accession number: NM_100757.4), and *β-tubulin* (forward: ATCGATTCCGTTCTCGATGT and reverse: ATCCAGTTCCTCCTCCCAA, accession number: 125664.4)was used as the housekeeping gene.

2.10. Statistical analysis

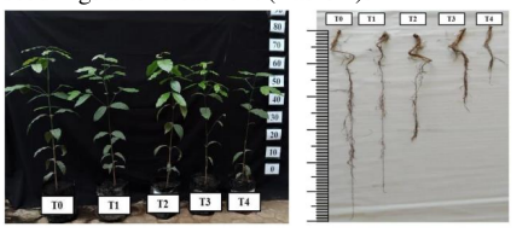
All figures and statistical analyses were performed using SPSS Statistics 17.0 software. The data were expressed as the mean ± standard deviation (SD) of five replications.

The data was collected and analyzed using Analysis of Variance (ANOVA), which then continued with Duncan multiple range tests in $P \geq .05$ as statistically significant.

3. Results

3.1. Growth responses

The application of 15% PEG was significantly decreased the shoot and root length, fresh and dry weight, as shown in Fig. 1. Comparing to the control, 40 days PEG application decreased up to 32.37%, 53.8%, 42.34%, and 42.37% for shoot length, root length, fresh and dry weight of melinjo seedlings after treatment (Table 1).



(a) (b)

Fig. 1. Effect of drought stress on melinjo seedling; (A) plant growth and (B) root system after 40 days application.T0) 0% PEG, T1) 15% PEG for 5 days, T2) 15% PEG for 10 days, T3) 15% PEG for 20 days, and T4) 15% PEG for 40 days. PEG: polyethylene glycol.

Table 1 Growth response of melinjo seedlings after 5 to 40 days 15% PEG application

	Duration of Stress (days)				
Growth Parameters	0	5	10	20	40
Shoot length (cm)	65.50^{a}	56,90^{ab}	48,06^{bc}	47,08^{c}	44,30^{c}
Number of leaves	14.60^{a}	10.20^{a}	11.40^{a}	17.40^{a}	18.00^{a}
Shoot diameter (cm)	0.59^{a}	0.56^{a}	0.58^{a}	0.65^{a}	0.52^{a}
Root length (cm)	55.06^{a}	51.26^{ab}	39.66^{bc}	27.84^{cd}	25.42^{d}
Fresh weight (g)	23.19^{a}	22.67^{a}	18.83^{ab}	17.81^{bc}	13.37^{c}
Dry weight (g)	9.04^{a}	8.84^{ab}	7.34^{ab}	6.95^{bc}	5.21^{c}

The same alphabet in the same row is not significantly different in Duncan's Test ($P \leq .05$). PEG: polyethylene glycol.

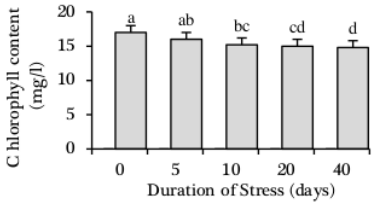


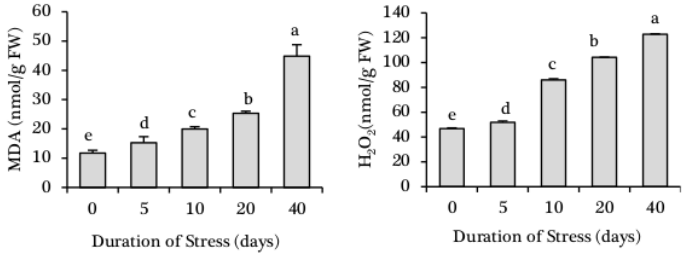
Fig. 2. Chlorophyll content of melinjo seedlings after PEG application for 0 to 40 days. Values are means of 5 replications ± SD followed with Duncan's test at $P \leq .05$ (means with

the same letter are not significantly different). PEG: polyethylene glycol, SD: standard deviation.

The longer duration of stress led to more suppression of thegrowth of the seedlings. Applications of 15% PEG for 10-40 days to the melinjo seedlings were significantly decreased the chlorophyll content, whereas the PEG application for 5 days was not significantly decreased chlorophyll content. Compared to the control treatment, chlorophyll content with 15% PEG application for 10 days was decreased up to 6.86% and decreased more up to 13.78% for 40 days stress than the control (Fig. 2).

3.2 MDA and H_2O_2

Different durations of drought stress induced by 15% PEG from 5 to 40 days to the melinjo seedlings were significantly increased the amount of MDA and H_2O_2 contents (Fig. 3).



(a) (b)

Fig. 3. Concentration ofMDA (a) and H_2O_2 (b) in melinjo subjected to drought stress treatment for 0 to 40 days. Values are means of 5 replications ± SD followed with Duncan's test at $P \leq .05$ (means with the same letter are not significantly different).Malondialdehyde:MDA,hydrogen peroxide: H_2O_2, SD: standard deviation.

MDA increased to 30.29% in 5 days of stress treatment, whereas its level increased to 282.59% after 40 days. Also, the concentrations of H_2O_2 were increased after 5, 10, 20, and 40 days application by 10.71%, 83.78%, 122.48%, and 162.23% over control. Both MDA and H_2O_2 showed the highest value at the most extended stress (40 days). The result indicates that a higher level of cellular damage followed the more extended period of stress in melinjo seedlings by accumulating ROS and lipid peroxidation during drought stress.

3.3 Antioxidant capabilities

The antioxidant capabilities in melinjo seedlings increased during the long duration of drought stress, which is shown in their inhibition activity to some radicals (Fig. 4). In control, melinjo seedlings have 15.26%, 2.86%, and 15.71% scavenging activity to the ABTS, DPPH, and superoxide radicals. After 15% PEG application for 5, 10, 20, and 40 days, the activities were increased up to 43.81%, 44.17%, and 236.47% for ABTS, DPPH, and superoxide radicals scavenging compare to the control. Similar to the DPPH, ABTS, and superoxide radical scavenging activity, SOD as one of the antioxidant enzymes, exhibit a similar trend (Fig. 5). The application of 15% PEG for 5 to 40 days was significantly affected the SOD

activity. Melinjo seedling without stress treatment has SOD activity for 0.87 unit/mg protein, increasing along with the longer duration of stress up to 1.02 unit/mg protein. Subjected the plants to 5 days of drought stress led to an increase in SOD activity by 3.44%, then rising for up to 16.09% after 40 days of stress.

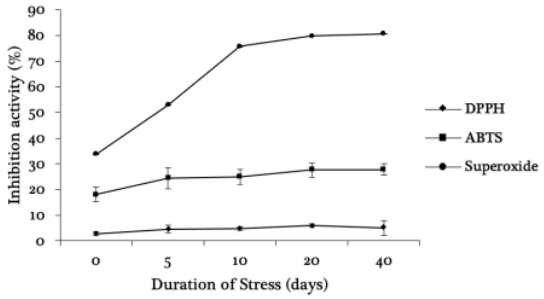


Fig. 4. ABTS, DPPH, superoxide radical scavenging activities of melinjo seedlings after 0 to 40 days PEG application. Values are means of 5 replications ± SD, PEG: polyethylene glycol.

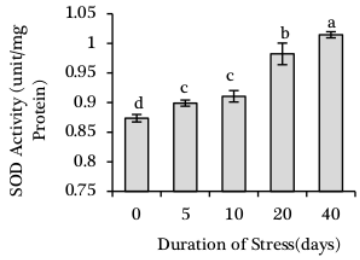


Fig. 5. SOD activity of melinjo after 0-40 days PEG application. Values are mean of 5 replications ± SD followed with Duncan's test at $P \leq .05$ (means with the same letter are not significantly different).SOD: superoxide dismutase, PEG: polyethylene glycol, SD: standard deviation.

3.4 CSD1 expression

The increasing antioxidant capabilities of melinjo after drought stress treatment were correlated with the activation of genes encoding antioxidants. Once is the gene encoding SOD as the antioxidant enzymes that play a pivotal role in ROS elimination. It has been known that several genes encode SOD, once is *CSD1*. In this study, the expression of *CSD1* was identified based on the band thickness of cDNA from each sample by using *CSD1* primer and *β-tubulin* as gene reference to confirm that this cDNA in the same concentration. The result implies that the expression of *CSD1* in melinjo seedlings tends to increase after PEG application for 5 to 40 days, shown in the thickest cDNA band in the Fig. 6. The longer duration of drought stress up to 40 days by 15% PEG application in melinjo was followed with the increased expression level of *CSD1*, shown in the thickest cDNA band in the result (Fig. 6).

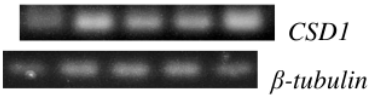


Fig. 6. Expression of *CSD1* encoded SOD and β-tubulin of melinjo seedlings after 0 - 40 days PEG application. The differences in band thickness among each treatment indicate the different expression levels of *CSD1* and *β-tubulin* expressions. *CSD1*: Copper/Zinc Superoxide Dismutase 1, SOD: superoxide dismutase, PEG: polyethylene glycol.

4. Discussion

Li et al. (2011) and Zheng et al. (2016) reported that drought stress reduces plant growth by affecting various physiological and biochemical processes such as plant height, leaf area, photosynthesis, chlorophyll synthesis, nutrient, and carbohydrate metabolism. At the cellular level, division and expansion in a plant's growth zones determine organ and plant-level growth responses to drought (Avramova et al., 2015). PEG addition in plant medium may block water absorption and plant nutrients, resulting in the loss of plant biomass and organ development, including leaf. Leaf function, organ development, root distribution are changed after exposure to drought stress, followed by other modifications to perform defense mechanisms in plants (Kapoor et al., 2020). Suppression of leaf area enlargement and the leaf's number in limiting light-harvesting used for the photosynthetic process (Anjum et al., 2011). Nitrogen is one of the important nutrients used in plant metabolism for growth and development. When nitrogen is blocked, the plant will experience a deficiency, which can have an impact on growth inhibition, including by causing an imbalance of physiological and biochemical processes, one of which is a decrease in the ability to synthesize chlorophyll, which then caused chlorosis on leaf and turn to have an impact on the photosynthesis process (Ahanger et al., 2016). With that, drought stress induced by 15% PEG impacts the lower amount of chlorophyll content of melinjo seedling than without PEG. Chlorophyll is one of the major parts of the plant photosynthetic system in light absorption used for energy in carbohydrate synthesis from water and carbon dioxide(Xu et al., 2020). Reducing the amount of chlorophyll content may reduce the photosynthetic process and result in the decreasing plant biomass, as shown in the fresh and dry weight. Sivakumar et al. (2018) mentioned thatthe degradation of chlorophyll content is correlated with the high accumulation of ROS, which then affects the lipid peroxidation and follows with chlorophyll degradation.

Hydrogen peroxide is the most stable ROS that reactively oxidized other molecules (Michelet et al., 2013). The excess of H_2O_2 content in plant cells triggers the Heiber-Weiss reaction to become faster and affects hydroxyl radical formation, which causing lipid peroxidation (Smirnoff and Arnaud, 2019). The level of lipid peroxidation in a plant cell is detected based on the amount of MDA, indicating the degree of cellular damage. On the other hand, H_2O_2 plays a role as a signaling component that can induce plant responses to the environmental (Sharma et al., 2012). Hydrogen peroxide is one of the precursors in forming a plant defense system by increasing the antioxidant activities (Mittler, 2002).

The antioxidant capabilities in inhibiting free radicals can be known based on the assay of ABTS, DPPH, and superoxide radicals. Plant abilities to cope with ABTS and DPPH have

been known for understanding stress tolerance (Cui et al., 2010). Ilyasov et al. (2020) explained that ABTS assays are the most abundant antioxidant capacity assays based on the electron transfer from antioxidant to $ABTS^+$ radical. The same principle is to obtain in the DPPH method. DPPH adsorption ability will decrease after accepting an electron donor that also results in a color change of the solution (Frezzini et al., 2019). Compare to the DPPH method, antioxidant activity based on the ABTS showed a higher percentage. It was supported by the result from Lee et al. (2015) that antioxidant activity assay using the ABTS method has higher sensitivity than the DPPH method.

Antioxidant activity based on the inhibition of superoxide radicals is used as the reaction of auto-oxidation of pyrogallol, which results in the production of purpurogallin. The higher inhibition percentage to superoxideradicals may indicate the excessive activity of the antioxidants, especially SOD. Sharma et al. (2012) mentioned that SOD plays a role in the inhibition of free radicals, formed as superoxide anion ($^{\bullet}O_2$). The reaction was done by donating 1 electron and 2 hydrogen ions to the $^{\bullet}O_2$ andresults in H_2O_2 formation. This enzyme acts as a major mediator in intracellular H_2O_2 production that is formed from $^{\bullet}O_2$ conversion. The increasing activity of SOD is pointed out as the enzyme's maximum capability within the complex tolerance mechanism of the plant against oxidative stress (Fu et al., 2016; Wang et al., 2010; Wu et a., 2016).Plant tolerance ability to abiotic stress such as drought is triggered by complex pathways, including the signaling mechanism in the cell to develop cellular homeostasis balance and defense system against stress(Golldack et al., 2014). Osakabe et al. (2014) mentioned that plant response abiotic stress involves a molecular mechanism triggered by a transduction signal that responds to every environmental change. The transduction signal is correlated to the plant defense mechanism in the ROS scavenging process by activating specific genes responsible for the balance of cellular metabolism. *CSD1* is one of Cu/Zn superoxide dismutases (SODs), which encodes SOD in the plant defense system, especially in preventing ROS accumulation caused by environmental stresses (Sunkar et al., 2012). Ma et al. (2015) mentioned that the expression of*CSD1* and *CSD2* induced ROS levels in mRNA levels hadbeen broadly identified in different plants that up-regulated after induction of abiotic stress. Moreover, the overexpression of the gene encoding CuZnSOD in transgenic sweet potato increases after drought stress treatment (Yan et al., 2016).

Drought stress exposure up to 40 days in melinjo seedlings was caused a reducing plant growth, increasing lipid peroxidation and ROS production, which then inducing antioxidant capabilities followed by the upregulating of *CSD1* expression. These results show that antioxidant capabilities in melinjo seedling act for a defense mechanism against drought stress. Besides, antioxidant capabilities found in melinjo seedlings are also potential to further use as a phytomedicine in the field of nutraceuticals and pharmaceuticals. Tili et al. (2013) stated that plants with high antioxidant activities could be used as a source of natural medicines or referred to as medicinal plants. In the previous study, it was reported that melinjo has antioxidant activities to inhibit the ABTS, hydroxyl, superoxide radicals, and the ability to inhibit the activity of α-amylase and α-glucosidase, which perform the antidiabetic potency (Supriyadi et al., 2019).

Drought stress induced by 15% PEG, resulting in inhibition of the growth of melinjo seedlings, by blocking water absorption from growth medium without causing any toxicity. In response to drought stress, melinjo seedlings forming the ROS higher than in normal conditions, which causing cell damage in plants. Melinjo seedlings develop a defense mechanism to mitigate further cell damage, once is through antioxidative pathway by upregulating the antioxidant gene expression (*CSD1*), eincreasing the enzymatic antioxidant activity (SOD), and inhibitory activity against free radicals (DPPH and ABTS). The duration of the melinjo plant being gripped by drought, followed by an increase in its defense mechanism.The growth responses and antioxidant capabilities of melinjo (Gnetum gnemon L.) in different durations of drought stress This study aims to develop a defense mechanism under environmental stress, such as drought stress, which can be used

5. Conclusions

Different durations of drought stress induced by 15% PEG significantly affected the growth and antioxidant capabilities of melinjo seedlings. The longer duration of drought stress caused more reduction in growth but triggered more escalation in cellular damage that followed with higher antioxidant capabilities of melinjo seedlings. The results can also show a way to increase the antioxidant compounds in melinjo plants, which can be used for medicinal purposes. Further correlating studies can be carried out with different molecular approaches and identification to increase the ability of plants to be tolerant of environmental stress conditions. Studies on production technology and the utilization of antioxidant compounds from melinjo also need to be carried out.

Author Contributions

Experimental design and data analysis, Zahela Siti Asyiyah, Laras Sekar Arum, andTri Agus Siswoyo; laboratory assays and writing — original draft preparation and editing, Laras Sekar Arum andBella Rhea Lavifa Sanjaya; Supervision, project administration, and writing—review, Tri Agus Siswoyo.

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Conflicts of Interest

The authors declare no conflict of interest.

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