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Characterization of cassava (*Manihot esculenta* Crantz) resistant to drought stress as a result of selection using PEG 6000

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Abstract

Cassava is a source of carbohydrates that grows in tropical areas. The largest cassava producing province in Indonesia is Lampung, and Central Lampung Regency is the main cassava production center in Lampung Province. Plant responses in response to drought vary, such as plants wilting, stomata closing, and chlorophyll content decreasing. The drought stress inducer used in this research was polyethylene glycol. Drought-sensitive cassava will show reduced growth, while tolerant plants will obtain new genotypes that are resistant to drought stress conditions. The aim of this research was to determine the drought tolerant concentration of PEG 6000; distinguish between chlorophyll a, b, and total; and determine the differences in stomata density and stress tolerance index in cassava treated with PEG 6000 and the control. The PEG 6000 treatment consisted of 5 concentration levels, namely 0%, 10%, 20%, 30%, and 40% with each treatment repeated 5 times. The tolerable concentration of PEG 6000 is 40%. The higher the concentration of PEG 6000, the lower the chlorophyll a, b and total content. As the concentration of PEG 6000 increases, the stomata density increases. The stress tolerance index of cassava is categorized into medium tolerant at concentrations of 10% and 20%, and tolerant at concentrations of 30% and 40%.

Keywords: Cassava; Stomata Density; Chlorophyll; Stress Tolerance Index; PEG 6000

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a plant that grows in tropical regions of Asia, Africa and Latin America. Cassava is also used as a source of carbohydrates besides rice, wheat and corn [1]. Manihot is included in the Euphorbiaceae family which consists of 300 genera and 8000 species [2]. The largest cassava producing country in the world is Nigeria with production reaching 57.9 million metric tons, as well as the country with the second largest consumption of cassava [3]. Indonesia is the third largest cassava producing country after Nigeria and Thailand with an average production of 23.90 million tons [4]. Lampung Province is the largest cassava producing province in Indonesia, while Central Lampung Regency is the main cassava production center in Lampung Province [5].

An obstacle in cassava cultivation is drought which can reduce productivity. The uncertain global climate will have a negative impact on the agricultural sector, such as low rainfall patterns, increasing temperatures and continuous extreme weather [6]. If this continues for a long period of time, it can result in plants lacking water supplies due to

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excessive evapotranspiration so that the water absorbed by plants is not enough to meet the required levels [7]. Drought can trigger plants to become stressed so that productivity and quality decrease [8], especially in the first 3 months after planting [9]. Under the same drought conditions, cassava experiences a comparable decrease in yield with potatoes as a drought sensitive crop [10].

Plant responses in response to drought vary, from plants wilting to chlorophyll content decreasing [11]. The mechanism of cassava resistance to limited water conditions in the atmosphere and in the soil is the closing of the stomata to protect the plant from drought by minimizing the water that is still available during periods of drought [12]. Stomata that close will inhibit the rate of photosynthesis in a plant [13]. An alternative to dealing with an ever-changing climate is to use cassava varieties that are drought resistant. The drought stress inducer used is polyethylene glycol (PEG) as a high molecular weight compound [14]. Plants that are sensitive to drought will show reduced growth, while resistant plants will produce new genotypes that are resistant to drought stress conditions [15]. PEG 6000 was also used to induce drought stress in moon orchid plantlets [Phalaenopsis amabilis (L.) BI.] [16]; Dendrobium sp. orchid plantlets. [17]; peas (Pisum sativum L.) [18]. The aim of this research was to determine the drought tolerant concentration of PEG 6000; distinguish between chlorophyll a, b, and total; and determine differences in stomata density and stress tolerance index in cassava treated with PEG 6000 compared to the control.

2. Material and methods

The tools and materials used consist of cassava (*Manihot esculenta* Crantz), aluminum foil, analytical balance, measuring cup, centrifuge, object glass, cover glass, microscope, optilab, razor blade, 70% ethanol, safranin, spectrophotometer, mortar and pestle, funnel, test tubes, test tube rack, Whatman paper No. 1, and 96% ethanol. The PEG 6000 treatment consisted of 5 different concentration levels, namely 0%, 10%, 20%, 30% and 40% with each treatment repeated 5 times.

2.1. Percentage of Live Plants and Cassava Visualization

Calculation of the number of living cassava plants uses the formula according to Nurcahyani *et al*. [19] are:

$$\text{Number of living plants} = \frac{number\ of\ living\ plants}{total\ number\ of\ plants} \times 100\%$$

Plant visualization includes leaf color after being treated with PEG 6000 with the following classification: green, green with certain parts brown, and brown [19].

2.2. Chlorophyll Content Analysis

Analysis of chlorophyll content used the Miazek and Ledakowicz method [20]. 0.1 gram of uniform cassava plant leaves were crushed with a mortar and pestle and 10 ml of 96% ethanol was added. The solution was filtered with Whatman paper No. 1 and put it in the flacon and then close it tightly. The sample solution and standard solution (96% ethanol) were taken as 1 mL and put into a cuvette. After that, absorption readings were carried out with a UV spectrophotometer at wavelengths of 665 nm and 649 nm with three repetitions for each sample. Chlorophyll content is calculated using the following formula:

Chlorophyll a = $(12{,}21 \times A_{665} - 2{,}81 \times A_{649})$ mg/l
Chlorophyll b = $(20{,}13 \times A_{649} - 5{,}03 \times A_{665})$ mg/l
Total chlorophyll = $(17{,}3 \times A_{649} + 7{,}18 \times A_{665})$ mg/l

Note:
A665= Absorbance at a wavelength of 665 nm
A649= Absorbance at a wavelength of 649 nm

2.3. Stomata Density

Stomata density was determined by taking cassava leaves and fixing them with 70% ethanol. Then, make a preparation by cutting the bottom surface of the leaf using a razor blade, and placing it on a glass object. The preparations were given 1 drop of safranin as a coloring, and covered with a cover glass. Observations were made using a microscope with a magnification of 400 times and photographed using an optilab. Stomata density is calculated using the formula according to Lestari [21] as follows:

$$\boldsymbol{S}tomata\ density = \frac{Number\ of\ stomata}{Area\ of\ field\ of\ view}$$

2.4. Stress Tolerance Index

Calculation of the stress tolerance index (STI) can be calculated using the formula according to Fernandez [22] as follows:

$$STI = \frac{Ypi\ x\ Ysi}{(Yp)^2} \text{ x } 100\%$$

Note:

STI: Stress tolerance index
Ypi: Plant wet weight under normal conditions
Ysi: The wet weight of each plant under stress conditions
Yp: Average wet weight of plants under normal conditions

Determining the criteria for plant tolerance levels to drought stress include STI < 0.5 is a very sensitive plant, STI > 0.5 is a sensitive plant, STI > 0.75 is a medium tolerant plant, and STI > 1.0 is a plant that is drought stress tolerant.

3. Results and discussion

Higher levels of drought cause decreased plant growth, as indicated by significant changes in plant growth, leaf number, and biomass [23]. One way to obtain cassava that is resistant to drought stress is to use PEG 6000. The application of PEG 6000 is one of the biological control methods used as a selection agent to produce varieties that are drought resistant [24].

3.1. Percentage of Number of Live Plants and Cassava Visualization

Observations of live cassava and visualization of plants were carried out on days 3 to 15. Observations of cassava selection with PEG treatment at five concentration levels, namely 0% (control), 10%, 20%, 30%, and 40% showed that cassava plants could survive until the 15th day. The percentage of live plants resulting from selection with PEG 6000 is presented in Table 1.

Table 1 Percentage of Number of Live Cassava Plants Selection Results with PEG 6000

PEG 6000 Concentration (%)	**Percentage of Live Cassava Plants on days 3,7,11,15 (%)**			
	3	7	11	15
0	100	100	100	100
10	100	100	100	100
20	100	100	100	100
30	100	100	100	100
40	100	100	100	100

Table 1 shows that on days 1 to 15 of observation in the PEG 6000 treatment with concentration levels of 0%, 10%, 20%, 30% and 40% there were no deaths or 100% survival, but at concentrations 30% and 40% are characterized by yellowing to brown stems and wilted leaves, which can be seen in Figure 1. Observations regarding the visualization of cassava plants at various concentrations are presented in Table 2.

Tabel 2 Percentage of Cassava Visualization of Selection Results with PEG 6000

PEG 6000 Concentration (%)	**Percentage of Cassava Plant Visualization on Day of Observation (%)**			
	3	7	11	15
0	G: 100	G: 100	G: 100	G: 100
10	G: 100	G: 100	G: 100	G: 100
20	G: 100	G: 100	G: 100	G: 100
30	G: 100	G: 100	G: 96 GB: 4	G: 92 GB: 8
40	G: 100	G: 96 GB: 4	G: 92 GB: 8	G: 84 GB: 12

Note: G= Green; GB= Green Brown; B= Brown

The results of plant visualization observations showed that there was an effect of giving PEG 6000 to cassava after 15 days of giving PEG 6000, which was indicated by the color of the leaves changing to green-brown or yellow and the plants starting to show wilting. Based on Table 2 at PEG concentrations of 0%, 10% and 20% the leaf color did not change (green) 100%, while at 30% PEG concentration on the 11th day the green leaf color changed to brown green by 4% and on the 11th day 15 leaf colors changed to brown green by 8%. A concentration of 40% on the 7th day showed 4% brown green leaf color and 96% green leaf color. On the 15th day, the concentration of 40% experienced a decrease in visualization, namely the leaves experienced wilting symptoms with a percentage of 84%. In this study, there were no brown leaves which meant that the plant was dead.

Higher concentrations of PEG 6000 produce more water-binding ethylene subunits. This prevents water from entering the plant tissue, making it more difficult for plant roots to absorb water and causing dryness in the plant [25]. Cassava plants after 6 weeks of age are presented in Figure 1.

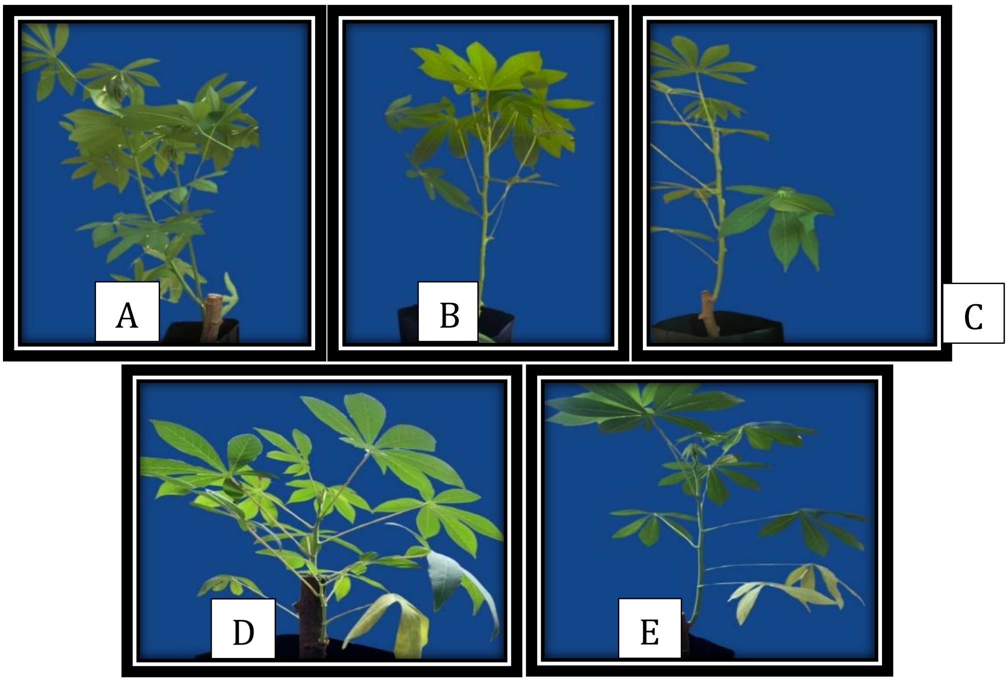

Figure 1 Cassava plants aged 4 weeks after being given PEG for 15 days with PEG concentrations: A = 0%, B = 10%, C= 20%, D = 30%, E= 40%

Based on Figure 1, it is known that at PEG concentrations of 30% and 40% there are changes in the leaves which start to turn yellow and brown, whereas at concentrations of 0% to 20% there are no changes. Several studies say that the higher the concentration of PEG 6000, the higher the drought stress. Plants that experience drought stress due to administration of PEG 6000 will show visual changes, the leaves will curl and dry out (Figure 1).

The results of this study are supported by research conducted by Song et al. [26] who stated that the appearance of curled and dry leaves indicates that the leaves are unable to carry out metabolism. Rolling and drying indicates that the leaves are no longer able to absorb nutrients from the medium, so they cannot continue metabolic processes, including the process of forming leaf chlorophyll. The browning of cassava plants occurs due to the oxidation of phenolic compounds by the enzyme polyphenol oxidase. Phenolic compounds which have an aromatic benzene group will be converted into quinone compounds which are brown in color. Plants that remain green can increase production and transpiration efficiency when plants experience water shortages, such as sorghum, corn and wheat [27].

3.2. Chlorophyll Content Analysis

The effect of giving PEG 6000 to cassava plants can be determined by the chlorophyll content in the plants. The chlorophyll content was observed by comparing cassava without PEG 6000 with cassava treated with PEG 6000 at concentrations of 10%, 20%, 30% and 40%. The chlorophyll content analysis carried out included analysis of the chlorophyll a, chlorophyll b and total chlorophyll content. This analysis can be used as an application in selecting cassava that is resistant to drought stress. The chlorophyll content of cassava given PEG 6000 is presented in Table 3.

Tabel 3 Chlorophyll content of Cassava selected with PEG 6000

Treatment	**Cassava Chlorophyll Content (mg/g tissue) Mean ± St. Dev**		
	Chlorophyll a	Chlorophyll b	Total Chlorophyll
Control (0%)	17,28 ± 1,87[a]	17,49 ± 2,54[a]	34,90 ± 4,12[a]
PEG (10%)	17,56 ± 2,03[a]	15,37± 1,93[a]	33,06 ± 3,96[a]
PEG (20%)	17,31 ± 1,07[b]	16,12 ± 1,12[a]	33,56 ± 1,90[a]
PEG (30%)	12,37 ± 1,68[ab]	15,92 ± 1,00[a]	28,38± 1,58[ab]
PEG (40%)	10,00 ± 1,21[b]	12,96 ± 1,29[a]	23,03 ± 1,43[b]

Note: Numbers followed by the same letter indicate that there is no significant difference between treatments at a confidence level of 95%.

The addition of PEG 6000 to soil media with a concentration of 40% significantly reduced the content of chlorophyll a, chlorophyll b and total chlorophyll in cassava plants. The decrease in chlorophyll content in cassava plants is in line with research conducted by Song [28] which stated that PEG can reduce the chlorophyll a content in rice plants. Chlorophyll a and total chlorophyll can be used as indicators of drought stress in rice. Keyvan [29], suggested that the total chlorophyll content decreased in wheat that experienced drought stress. Decreased chlorophyll content can result in decreased food production and reserves.

The results of research regarding chlorophyll content are also in line with research conducted by Song et al. [26], the results obtained were that there was a decrease in the content of chlorophyll a, chlorophyll b and total chlorophyll in rice plants with increasing concentrations of PEG 6000 given to the growth medium. The decrease in chlorophyll content is caused by aspects of plant growth that are influenced by water. Water content can result in a physiological response, namely a decrease in leaf chlorophyll concentration due to hampered chlorophyll formation and hampered absorption of nutrients, especially nitrogen and magnesium which have an important role in chlorophyll synthesis [30]. Water shortage stress can inhibit photosynthetic activity and the distribution of assimilates into the reproductive organs. Insufficient water availability in plants can also inhibit chlorophyll synthesis in leaves because the rate of photosynthesis decreases [31]. In terms of chlorophyll content, the higher the concentration of PEG 6000, the lower the chlorophyll a, b and total cassava plant content.

3.3. Stomata Density

Plants respond to water shortages by reducing the rate of transpiration in order to maintain the remaining water content by closing the stomata [32]. Stomata density on cassava that has been treated with PEG 6000 with 5 concentration levels is presented in Table 4.

Table 4 Cassava Stomata Density

PEG 6000 Concentration (%)	Stomata Density (mm2) (Mean ± St. Dev)
0	355 ± 50,9^{a}
10	370 ± 71,8^{a}
20	375 ± 34,5^{a}
30	400 ± 20,9^{a}
40	405 ± 28,9^{a}

Note: Numbers followed by the same letter indicate that there is no significant difference between treatments at a confidence level of 95%.

In Table 4, the highest stomata density was found when PEG 6000 was given a 40% concentration, namely 405 mm2, followed by a 30% concentration, namely 400 mm2, and the lowest at a 0% concentration of 355 mm2. The results of the stomata density did not show a significant difference, but the administration of PEG 6000 had a tendency to be different from the control. This is because cassava is still 45 HST old which can be categorized as a young plant. The development of stomata will be in line with the development of a plant's organs, resulting in a lower number of stomata in younger organs than in mature organs [33]. The lower surface of cassava leaves when applied with PEG 6000 concentrations of 0% and 40% is presented in Figure 2.

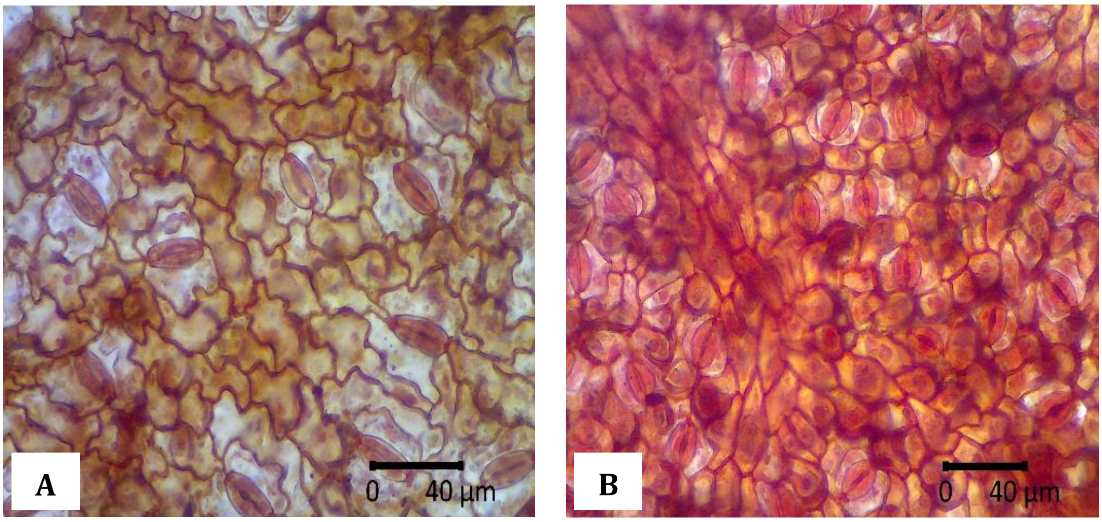

Figure 2 Lower surface of leaves at 400x magnification (A) 0% concentration, and (B) 40% concentration

In Figure 2, the distance between stomata at a PEG concentration of 40% is closer so that the stomatal density is greater than at a concentration of 0%. This result is in line with research by Izza and Laily [34], the number of stomata is directly proportional to the density of stomata, if the number of stomata is greater, the density of stomata is also greater. Apart from that, the small size of the stomata can influence the distance between the stomata to be closer so that in the field of view of the microscope the density of the stomata will be higher, and vice versa, the large size of the stomata will result in the distance between the stomata being further apart so that the density of the stomata will be lower [35].

Stomata density is related to plant resistance to drought stress. Stomata that have a greater density indicate that the plant is not stressed due to changes in environmental conditions, namely drought stress, so that the plant can tolerate conditions of water shortage. If the stomata density is lower, it indicates that the plant is intolerant so that it is able to feel the impact of drought stress [36].

3.4. Stress Tolerance Index

Plant resistance to drought stress can be determined using the Stress Tolerance Index (STI). A tolerant cassava indicates that the cassava is able to maintain life even in stressful conditions. Cassava stress tolerance index of various concentrations is presented in Table 5.

Tabel 5 Stress Tolerance Index

PEG 6000 Concentration (%)	Stress Tolerance Index (Mean ± St. Dev)
10	0,886 ± 0,103[b]
20	0,897 ± 0,099[b]
30	1,477 ± 0,005[a]
40	1,583 ± 0,027[a]

Note: Numbers followed by the same letter indicate that they are not significantly different between treatments at a confidence level of 95%

In Table 5, it can be seen that the cassava stress tolerance index of PEG concentrations of 10% and 20% is included in the category of medium tolerant plants, while PEG concentrations of 30% and 40% are plants that are tolerant to drought stress. A larger ITC result indicates that the plant is increasingly tolerant to drought stress conditions, whereas a smaller ITC indicates that the plant is very sensitive to changes in conditions so that the plant becomes susceptible or intolerant.

Plants that are tolerant in the germination phase are likely to be tolerant in the vegetative and generative phases [37]. Apart from that, plant tolerance to drought stress can also be seen from the higher stomata density, whereas sensitive plants have a lower stomata density [38]. This is in line with this research, that administration of 40% concentration of PEG 6000 has the highest stomata density and is a plant that is tolerant to drought stress.

4. Conclusion

The tolerant PEG 6000 concentration for selecting cassava plants for optimum growth is 40%. The higher the concentration of PEG 6000, the lower the chlorophyll a, b and total content. There was an increase in stomata density as the concentration of PEG 6000 increased. The stress tolerance index for cassava was categorized as medium tolerant at concentrations of 10% and 20%, and tolerant at concentrations of 30% and 40%.

Compliance with ethical standards

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Disclosure of Conflict of interest

All authors have no conflicts of interest

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