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Research Article
Effect of Shade Level on Two Stevia (*Stevia rebaudiana* B.) Planting Materials

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Abstract

Background and Objective: Stevia is a sweetener from plants that can be propagated through cuttings and micro-propagation. Shade level is one of the limiting factors that have a relationship with the growth microenvironment such as immediately receiving high light intensity (4000-12000 lux) with temperatures that can reach 35°C, resulting in an increase in evapotranspiration which causes the plants to wilt, resulting in plant vigor won't be good and yield of stevia cuttings and plantlet planting material. The target of this research was to find out and study the response of two stevia (*Stevia rebaudiana* B.) planting materials at shade levels on plant vigor and stevioside content. **Materials and Methods:** The study was conducted from June to November, 2022, using a split-plot design with 4 replications. The first factor is planting material (B), namely cuttings (B1) and plantlets (B2). The second factor is shading (N) which is 25% (N1), 50% (N2) and 75% (N3). The analysis of variance (F-test) on test level 5% was used to determine the effect of treatment and if a significant effect was obtained, the analysis was continued with the least significance difference (LSD) at level 5%. **Results:** The research results obtained were optimum growth (length gain, number of new leaves, chlorophyll content and stomata density) found in cuttings planting material with 50% shade treatment. While the optimal quality of stevia planting materials (stevioside) is found in plantlets with 25% shade treatment. **Conclusion:** Found in this study, 50% shade level using cuttings planting materials, gave better growth and 25% shade level gave better stevioside using plantlet planting materials.

Key words: Stevia, cuttings, plantlets, shade levels, stevioside, micro-propagation

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

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

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a plant used as a sweetener for food and beverages. The part of the stevia plant that is used as a sweetener is the leaf. Propagation of planting material in stevia can be done generatively through seeds or vegetatively through cuttings. Seeds produced by the stevia plant have a low germination percentage and tend to be dormant so the use of cuttings, both shoots and stems, is more widely used. However, the preparation of cuttings planting material is also quite difficult. This is due to the characteristics of the stevia plant which is susceptible to weather conditions. Therefore efforts are needed to overcome it, namely stevia multiplication with tissue culture.

The adaptation of the plantlet environment from heterotrophic (*in vitro*) to autotrophic conditions as a result of tissue culture is known as acclimatization (*in vivo*) Irsyadi[1]. Acclimatization is required for plantlets to grow and develop properly. Plantlets that have good vigor are characterized by large stem diameter, a large number of leaves, large leaf width, long roots and higher biomass fresh weight. According to Lavanya *et al.*[2], when plantlets are transferred to the outside environment, they will immediately receive high light intensity (4000-12000 lux) with temperatures that can reach 35°C, resulting in an increase in evapotranspiration which causes the plants to wilt, resulting in plant vigor won't be good. In this condition, too high a light intensity will cause a change in the orientation of the leaves, closing the stomata (rolling up the leaves) investigated by Mathur *et al.*[3]. This does not only apply to plantlets, but also to cuttings planting material. Based on the problems above, research was needed on the vigor resistance of two stevia planting materials to various types of shade.

MATERIALS AND METHODS

Study area: The research was conducted in the Laboratory of Tissue Culture, Plant Physiology and Gardens, Faculty of Agriculture, Brawijaya University (506 masl, temperature 21-28°C, humidity 70-80%). The experiment began on June, 2022 and ended in November, 2022.

Equipment: The tools used were paranet 25, 50 and 75%, lux meter (LUTRON LX-113S Light Meter, Sydney, Australia), thermohygrometer (HTC-1), analytical balance (0.01 g), oven spectrophotometer (Bio-Rad, SmartSpec Plus Spectrophotometer, California, USA), HPLC (Shimadzu, LC-20AD), water bath (P Selecta) and vortex (Labnet).

Materials: The material used was cuttings from the stevia plant (*Stevia rebaudiana* B.), obtained from Malang, stevia plantlets using 1 composition of Murashige and Skoog media, 2 mL L^{-1} 6-benzylaminopurine with micro-environmental conditions (23-25°C, humidity 70%), ethanol absolute for analysis (Merck), acetonitrile and water for HPLC, acetone for analysis (Merck).

Methodology: The study used a split-plot design with 4 replications. The first factor (main plot) is planting material (B), namely cuttings (B1) and plantlets (B2). The second factor (subplot) is shading (N), namely 25% (N1), 50% (N2) and 75% (N3).

Total chlorophyll content was calculated using Arnon[4]. The leaves were weighed as much as 2 g and then crushed with a mortar and pestle. Then, the leaf paste was put in a vial of the film (30 mL) and mixed with 10 mL of acetone and then closed. The solution was allowed to stand for 24 hrs in the fridge. After 24 hrs, the solution was filtered through paper Whatman 42. The filter results were then pipetted 1 mL and put into a test tube for dilution. Dilution is done by adding 9 mL of acetone and homogenize. The extract solution obtained was then put in a cuvette and the absorbance level in the spectrophotometer with a wavelength of 645 and 663 nm.

$$\text{Total chlorophyll (Ct, } \mu g\ mL^{-1}) = Ca+Cb$$

$$\text{Total chlorophyll (Ct, } \mu g\ mL^{-1}) = 20.2.\ Abs\ 645+8.02.\ Abs\ 663$$

Then the value of total chlorophyll (µg mL^{-1}) is converted based on the number of samples to in units of mg mL^{-1}.

Observation of stomata was carried out by taking one strand of each treatment, then the epidermis of the lower leaves was covered with clear nail polish then covered with tape, wait until dry, then peeled. Then observed under the microscope Olympus was connected to an optilab photomicroscope with magnification 400 times with an area of view of 0.19625 mm^2. Observations were made by measuring the length, width, number and density of stomata.

Stevioside: The levels of stevioside analyzed using a method from Abd Razak *et al.*[5], with HPLC (Shimadzu, UV detector) with the condition setting of the instrument, including using a C-18 column (4.6×150 mm, 5 m), flow 1 mL min^{-1} using acetonitrile: Water 80:20 mobile phase (v/v). An injection volume of 20 µm and measured at a wavelength of 210 nm.

Statistical analysis: Data analysis was used to determine the impact of the treatment using analysis of variance (F-test) at a test level of 5%. The least significant difference (LSD) at level 5% will be used if a significant effect is obtained.

RESULTS

Plant length: The most noticeable parameter was the increase in plant length. Plant length increased at 2, 4, 6 and 8 WAT (a week after transplanting). The obtained data was shown in Table 1 (2 WAT), Table 2 (4 WAT), Table 3 (6 WAT) and Table 4 (8 WAT).

According to the Table 1-4, the treatment of cuttings with 50% shading produced better results than the other treatments.

Number of leaves: Table 5 showed that, there was a separate effect between the treatment of planting material and shade level. The results showed that cuttings had a higher average number of leaves than plantlets.

Chlorophyll content: The next growth parameter is chlorophyll content. The obtained data shown in Table 6.

Observational data of chlorophyll content showed that cuttings planting material at 50% shade gave better results than other treatments.

Stomatal density: Stomatal density shows the number of stomata in a certain area. Data of this parameter, shown in Table 7 supported with Fig. 1a-f.

Table 1: Average stem length gain on two different planting materials and various levels of shade at 2 WAT

	Increase in plant length (cm/plant)		
	Shade levels (%)		
Treatment	25	50	75
Planting material			
Cutting	1.40^{d}	2.10^{e}	0.80^{c}
Planlet	0.30^{a}	0.63^{b}	0.25^{a}
CV B (%)		30.64	
CV N (%)		27.37	
LSD (p = 0.05%)		0.150	

Numbers followed by different letters in the same column are significantly different based on the 5% LSD test

Table 2: Average stem length gain on two different planting materials and various levels of shade at 4 WAT

	Increase in plant length (cm/plant)		
	Shade levels (%)		
Treatment	25	50	25
Planting material			
Cutting	2.850^{d}	3.550^{e}	2.250^{c}
Planlet	1.750^{a}	2.075^{b}	1.700^{a}
CV B (%)		11.84	
CV N (%)		10.57	
LSD (p = 0.05%)		0.150	

Numbers followed by different letters in the same column are significantly different based on the 5% LSD test

Table 3: Average stem length gain on two different planting materials and various levels of shade at 6 WAT

	Increase in plant length (cm/plant)		
	Shade levels (%)		
Treatment	25	50	25
Planting material			
Cutting	3.063^{d}	3.763^{e}	2.463^{c}
Planlet	1.963^{a}	2.288^{b}	1.913^{a}
CV B (%)		10.86	
CV N (%)		9.700	
LSD (p = 0.05%)		0.150	

Numbers followed by different letters in the same column and row are significantly different based on the 5% LSD test

Table 4: Average stem length gain on two different planting materials and various levels of shade at 8 WAT

	Increase in plant length (cm/plant)		
	Shade levels (%)		
Treatment	25	50	25
Planting material			
Cutting	3.150[d]	3.850[e]	2.550[c]
Planlet	2.050[a]	2.375[b]	2.000[a]
CV B (%)		10.50	
CV N (%)		9.380	
LSD (p = 0.05%)		0.150	

Numbers followed by different letters in the same column and row are significantly different based on the 5% LSD test

Table 5: Average number of new leaves on two different planting materials and various percentages of shade at various ages of observation

	Number of new leaves (sheet/plant)			
	Observation (WAT)			
Treatment	1	2	3	4
Planting material				
Cutting	4.670	8.500[b]	12.25[b]	14.00[b]
Planlet	3.170	4.750[a]	7.830[a]	9.580[a]
CV B (%)	49.99	37.78	27.25	23.20
LSD (p = 0.5%)	NS	2.300	2.510	2.510
Shade levels (%)				
25	3.100[a]	6.375	9.875	11.63
50	5.400[b]	6.375	9.875	11.63
75	3.300[a]	7.125	10.37	12.13
CV N (%)	22.72	21.93	12.16	14.28
LSD (p = 0.5%)	0.56	NS	NS	NS

Numbers followed by different letters in the same column were significantly different based on the 5% LSD test

Table 6: Average of chlorophyll content of two different planting materials and various percentages of shade at various ages of observation

	Chlorophyll content (μg g^{-1})		
	Shade levels (%)		
Treatment	25	50	75
Planting material			
Cutting	14.87[c]	17.74[d]	12.29[b]
Planlet	0.300[a]	0.630[a]	0.250[a]
CV B (%)		24.00	
CV N (%)		8.670	
LSD (p = 0.05%)		0.710	

Numbers followed by different letters in the same column and row are significantly different based on the 5% LSD test

Table 7: Average stomatal density of two different planting materials and various percentages of shade at various ages of observation

	Stomatal density		
	Shade levels (%)		
Treatment	25	50	75
Planting material			
Cutting	57.99[d]	61.53[e]	43.14[b]
Planlet	40.31[a]	55.16[c]	44.55[b]
CV B (%)		7.710	
CV N (%)		8.620	
LSD (p = 0.05%)		2.730	

Numbers followed by different letters in the same column are significantly different based on the 5% LSD test

Based on Table 7 and supported by Fig. 1a-f, it was found that the stomatal density was densest in the cutting's treatment with 50% shade.

Dry weight of leaves: The data on the dry weight of leaves, showed that there was a separate effect of planting materials and shade levels (Table 8).

Table 8 showed that there were significant differences in the average leaf dry weight separately. Cuttings planting material had a higher leaf dry weight compared to plantlet leaf dry weight.

Stevioside content: The stevioside content index is a parameter of stevia plant quality. The stevioside index was observed using HPLC at the last observation by comparing the resulting area on the chromatography chart. Stevioside area data was presented in Table 9.

Data on the area of the chromatogram (Table 9) obtained showed that there was an interaction between treatments. The index of stevioside levels in plantlet planting material at 25% shade was higher than the other treatments.

Table 8: Average dry weight of leaves of two different planting materials and various percentages of shade at various ages of observation

Treatment	Dry weight of leaves (g/plant)
Planting material	
Cutting	18.45[b]
Planlet	12.37[a]
CV B (%)	25.33
LSD (p = 0.5%)	2.440
Shade levels (%)	
25	25.00
50	50.00
75	75.00
CV N (%)	22.72
BNT (p = 0.5%)	NS

Numbers followed by different letters in the same column are significantly different based on the 5% LSD test

Table 9: Average stevioside chromatogram area of two different planting materials and various percentages of shade at various ages of observation

	Stevioside index content (area)		
	Shade levels (%)		
Treatment	25	50	75
Planting material			
Cutting	51973939.37[d]	38369822.03[a]	46206377.96[b]
Planlet	66553647.16[f]	48309163.95[c]	60564897.19[e]
CV B (%)		4.330	
CV N (%)		2.220	
LSD (p = 0.05%)		727630.108	

Numbers followed by different letters in the same column are significantly different based on the 5% LSD test

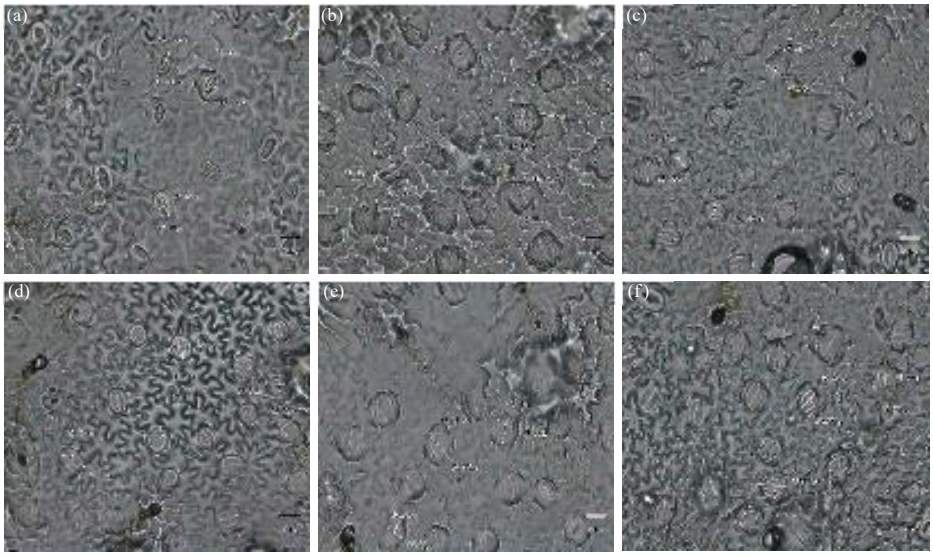

Fig. 1(a-f): Stomatal density, (a) Cutting, shading 25%, (b) Cutting, shading 50%, (c) Cutting, shading 75%, (d) Planlet, shading 25%, (e) Plantlets, shading 50% and (f) Plantlets, shading 75%

DISCUSSION

This research implies that providing planting material using cuttings and plantlets is easier, more efficient and faster than using seeds. Providing planting material requires an appropriate acclimatization method based on the growth parameters and results obtained. In the variable plant length experiment, cuttings with 50% shade provided greater plant length, chlorophyll content and stomata density than other treatments. Planting material derived from cuttings has the advantage of producing perfect plants with roots, leaves and stems in a relatively short period and is similar in nature. Stevia plants propagated vegetatively will have the same properties as their parents, with the exception that the resulting plants grow faster than generative propagation.

As a result according to Hossain *et al.*[6], plant cuttings will outperform plantlets in terms of growth. Furthermore, Sinta and Amanah[7] reported the survival rate of stevia plantlets two months after being transferred into polybags in full sun was 63.3% of the acclimatized plantlets. This decline may be because stevia is grown in the lowlands, as stevia grows well in the highlands in the tropics.

It was discovered that stomatal conductance decreased primarily due to a decrease in the number of stomata caused by reduced light. Because there are more leaves, longer leaf lengths and lower stem weight in the shade, the leaf-to-stem ratio is 50% higher. According to Kumar *et al.*[8], under certain shade conditions, these conditions indicated that stevia plant growth is optimal. The study by Semchenko *et al.*[9] revealed significant complexity and interspecific variation in the effects of shade on plant growth. Moderate shading can boost plant growth significantly, especially in species with limited morphological plasticity. Lugassi-Ben-Hamo *et al.*[10] reported similar results for Lisianthus flower plants, stating that reducing light to 60% reduced stevia plant growth and biomass. As a result, the type of planting material and the amount of shade have varying effects on the growth of stevia plants. According to Osman *et al.*[11] the 50% shade treatments had more root and leaf numbers and these conditions are critical for potential Stevia propagation. According to Angelini *et al.*[12], they confirmed that shade slowed growth and flowering. Furthermore, a 60% reduction in light-delayed flowering, reduced plant biomass production, significantly reduced the percentage of flowering plants and decreased flowering rate.

The main aim of the research is to obtain planting material that has good vigor. Because of this goal, cutting planting material in 50% shade was the best planting material in this study. Cuttings are a type of plant propagation system that is relatively simple and generates fine-quality plant material with traits similar to those of its parent in a shorter period Istomo *et al.*[13]. As a result of better growth in the use of planting material in the form of stem cuttings, the use of cuttings produces a greater dry weight than the use of plantlets. Besides that, it is possible that plantlet planting material with 50% shade can also be used as planting material based on results.

Steviol glycoside production in plant species is uncommon; only *Stevia rebaudiana* and three other species, *Stevia phlebophylla*, *Rubus suavissimus* and *Angelica keiskei*, possess this trait reported by Evans *et al.*[14]. Steviol glycosides are tetracyclic diterpenes with a precursor similar to gibberellin Brandle and Telmer[15]. The discovery of new diterpene glycosides has increased in recent years, with at least 34 steviol glycosides discovered in stevia described by Ceunen *et al.*[16].

According to the research, the highest levels of stevioside were found in the use of planting material in the form of plantlets with a shade level of 25%, but it is known that the observed growth variables in this treatment have slower growth than in that treatment. Gupta *et al.*[17] discovered that light reduction did not affect the concentrations of steviol glycosides, except during the flower initiation stage of plant development, when the light was reduced by 25%, rebaudioside A levels increased significantly. This condition was also consistent with Kumar *et al.*[8] observation that glycoside synthesis was reduced at or shortly before flowering, allowing for more time for glycoside accumulation. This condition causes stevioside levels to be higher when using plantlet planting material with a 25% shade.

Based on the results obtained, shade treatment using a screen can be used on planting material for stevia cuttings and plantlets. So, it is possible that stevia plants can be planted under shade plants.

CONCLUSION

The stevia growth (increase in plant length, number of new leaves, chlorophyll content and stomata density) which was better than other treatments found in the 50% shade treatment using cuttings planting material. The quality of stevia (stevioside) was better in the 25% shade treatment using plantlet planting material. It means that stevia planting material can be prepared by providing 50% shade treatment. So, it is possible that stevia can be planted under shade plants with a shade limit of 50%.

SIGNIFICANCE STATEMENT

Stevia (*Stevia rebaudiana* Bertoni) is a plant used as a sweetener for food and beverages. This study demonstrates the proper acclimatization method for stevia plants so that they can grow well and vigor and produce optimal stevioside quality. The conclusion from this research is stevia growth (increase in plant length, number of new leaves, chlorophyll content and stomata density) which was better than other treatments found in the 50% shade treatment using cuttings planting material.

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