

The Effect of Photoperiod on the Growth of *Stevia rebaudiana* In Vitro

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Abstract

Stevia rebaudiana, the source of non-caloric natural sweeteners in the form of steviol glycosides, is a plant with a poor germination rate. Therefore, micropropagation is a potential alternative method to propagate the plants in a large number. Light is an important factor for photosynthesis, so changing the intensity, quality, and duration of lighting can affect plant growth. Photoperiod, the duration of light within 24 hour period, has been known to influence the growth of *S. rebaudiana* grown in ex vitro conditions. The purpose of this study is to investigate the effect of various photoperiod (8, 12, and 16 hours) on the growth of *S. rebaudiana* in vitro. The node segments from ex vitro grown *S. rebaudiana* plants were cultured on solid MS media supplemented with 1.13 mg/L BA and 0.35 mg/L IAA. The shoots were rooted on solid half-strength MS media containing 0.1 mg/L IAA. For acclimatization, the rooted shoots were grown on a mixture of fertile soil, burnt rice husk, cocopeat, and manure. The photoperiod treatment was applied from the beginning to the end of the experiment. Our results showed that the highest shoot length was found under 16 hour photoperiod. On the other hand, root number and root length were not affected by photoperiod. Additionally, a 16 hour photoperiod increased shoot length (5.9 cm) compared to a 12-hour (3.48 cm) and 8-hour photoperiod (3.08 cm) after 5 weeks of acclimatization. A 16 hour photoperiod also produced highest total leaf fresh weight (0.2 g). However, different photoperiods did not significantly affect leaf number and leaf area. In conclusion, 16 hour photoperiod is the best condition for *S. rebaudiana* micropropagation.

Keywords: *Stevia rebaudiana*, photoperiod, growth

1. Introduction

The increasing interest in non-sugar sweeteners in recent years has led to the growing popularity of stevia as a sweetener. Stevia is derived from the leaves of *Stevia rebaudiana*, a flowering plant native to Paraguay and belonging to the Asteraceae family [1]. The sweetness of stevia is attributed to a group of diterpene compounds called steviol glycosides, including stevioside, rebaudioside A, rebaudioside M, and others [2]. Steviol glycosides are calorie-free, up to 400 times sweeter than sucrose, and have positive health effects such as decreasing insulin levels and reducing inflammation [3-5].

S. rebaudiana is not extensively cultivated from seeds due to their low germination rate [6]. Instead, *S. rebaudiana* are commonly propagated using stem cuttings. However, it produces limited number of new individual plants. Another meth-

od employed for the propagation of *S. rebaudiana* is micropropagation. This method involves growing small parts from a plant into new individuals in a sterile nutrient medium, often supplemented with plant growth regulators, which enables the generation of a larger quantity of plants in a relatively short time [7].

Photoperiod is an important light factor influencing the growth and development of *S. rebaudiana*, in addition to light quality and intensity [8-10]. It is well established that extending the photoperiod can lengthen the photosynthesis period and enhance dry matter accumulation in numerous plant species [11]. Studies on the effect of photoperiod on the growth of *S. rebaudiana* in ex vitro conditions have been conducted previously [8, 12-13]. However, the impact of photoperiod on the in vitro growth of *S. rebaudiana* has not been extensively explored. Therefore, the aim of this study is to evaluate the

influence of photoperiod on the growth *S. rebaudiana* shoot and root in vitro, as well as the growth of in vitro grown *S. rebaudiana* after acclimatization.

2. Methodology

2.1. Explants Source

The source of explants used in this study were 3-week-old *S. rebaudiana* BL clone obtained from the Biotechnology and Bioindustry Research Center, Bogor, Indonesia. Stem node segments were used as explants for in vitro shoot initiation.

2.2. Treatment and Growth Conditions

This study employed three treatments: 8-hour, 12-hour, and 16-hour photoperiods. The light source used was fluorescent lamps, with an intensity of 48 W/m². The room temperature was maintained at 23 ± 2°C. These treatment and growth conditions were applied during the shoot initiation, rooting, and acclimatization phases.

2.3. Explant Sterilization

The cut node segments were washed with soap in running water for 30 minutes, then immersed in a 0.1% fungicide solution for 5 minutes. Under aseptic conditions in a laminar air-flow cabinet, the explants were immersed in 70% alcohol for 1 minute, then washed in a 0.79% NaClO solution with two drops of Tween 20 for 10 minutes, and finally rinsed three times with sterile distilled water. The sterilized node segments were cut into 1-2 cm sizes.

2.4. Effect of Photoperiod on In Vitro Shoot Initiation

The node segments were cultured on MS medium (Murashige and Skoog, 1962) containing 1.13 mg/L benzyladenine (BA), 0.35 mg/L indole-3-acetic acid (IAA), 30 g/L sucrose, and 8.5 g/L agar. The medium's pH was adjusted to 5.8 ± 0.2. The medium was autoclaved at 121 °C and 124 kPa for 15 minutes before use. After four weeks, the shoot length, number of nodes per shoot, and number of branches per shoot were measured.

2.5. Effect of Photoperiod on In Vitro Rooting

The 1-3 cm shoots obtained from the initiation phase were cut and cultured on half-strength MS medium containing 0.1 mg/L IAA, 30 g/L sucrose, and 8.5 g/L agar. The medium's pH was adjusted to 5.8 ± 0.2. The medium was autoclaved at 121 °C and 124 kPa for 15 minutes before use. After four weeks, the number of roots per shoot and root length were measured.

2.6. Effect of photoperiod on acclimatization

The in vitro shoots with developed roots were rinsed with sterile distilled water and planted in a growth medium composed of a mixture of fertile soil, burnt rice husk, cocopeat, and manure. Liquid NPK fertilizer and 0.1% fungicide were sprayed at the beginning of acclimatization. Watering was

done initially and every two weeks. After five weeks, the change in shoot length, change in nodes number, total area as well as total fresh weight of the leaves that emerged during acclimatization per shoot were measured.

2.7. Statistical analysis

The experiment was conducted using a completely randomized design (CRD). The biological replicates used for in vitro shoot initiation, in vitro rooting, and acclimatization were 14, 11, and 8 replicates, respectively. Significant differences were analyzed using one-way ANOVA, followed by Tukey's HSD test ($p < 0.05$) using IBM Statistics SPSS 25 software.

3. Result and Discussion

3.1. Effect of Photoperiod on In Vitro Shoot Initiation

Ideal in vitro shoot growth is important for *S. rebaudiana* micropropagation because a higher number of nodes or branches produced leads to a higher rate of plantlet multiplication. Regulating growth conditions such as lighting duration is one way to obtain optimal in vitro shoot growth. *S. rebaudiana* explants treated with a 16 hours photoperiod for four weeks resulted in taller shoots (5.93 cm) compared to the 8-hour (3.08 cm) and 12-hour (3.48 cm) photoperiods (Figure 1a). However, photoperiod did not have a significant effect on the number of nodes per shoot and the number of branches per shoot (Figure 1b and c).

These results support the findings of the positive effect of long photoperiod on plant's growth [14-17]. It has been demonstrated that longer photoperiod increased the daily availability of light for photosynthesis which improved the growth performance of plants [15,18]. This might explain the increase of in vitro *S. rebaudiana* shoot's length in our result.

3.2. Effect of Photoperiod on In Vitro Rooting

In vitro rooting is a crucial stage prior to acclimatization. Plantlets with roots usually have a greater chance of survival during acclimatization. In this study, photoperiod does not affect the number of roots per shoot and root length. The average number of roots per shoot for the 8-hour, 12-hour, and 16-hour photoperiods were 2.36, 2.55, and 2.64, respectively (Figure 2a). Meanwhile, the average root length for the 8-hour, 12-hour, and 16-hour lighting treatments were 1.13 cm, 0.84 cm, and 0.92 cm, respectively (Figure 2b).

There have been several reports suggesting that longer photoperiod can also increase root growth by increasing photosynthesis duration [19-21]. However, our research did not yield the same results as there was no impact of photoperiod on rooting. It probably was not due to the sample size, as our sample size was not too small (30 plants per treatment). The photosynthetic products from the shoots reaching the roots might be not substantial enough to make a significant difference.

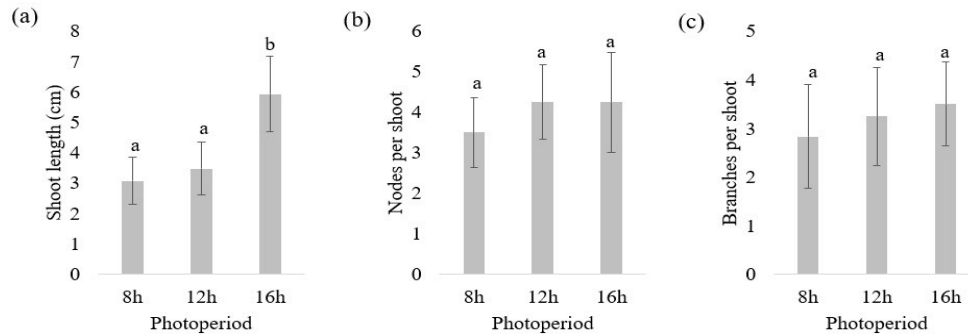


Figure 1. The effect of different photoperiod treatment on (a) in vitro shoot length, (b) the number of nodes per in vitro shoot, and (c) the number of branches per in vitro shoot after four weeks. Data represents mean $\pm$ standard deviation ($n = 14$). Different letters indicate significant differences using Tukey HSD test ($p < 0.05$).

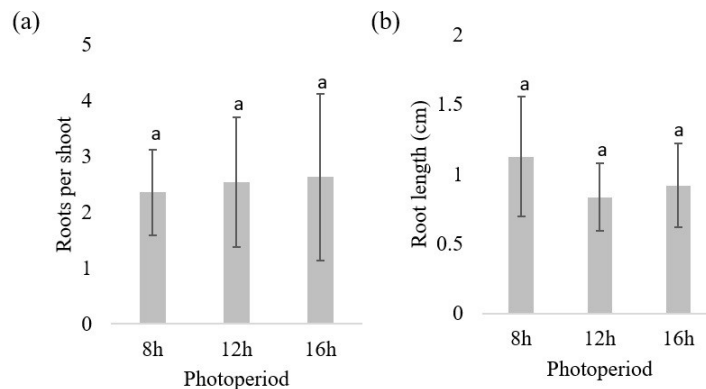


Figure 2. The effect of different photoperiod treatment on (a) the number of in vitro roots per shoot and (b) in vitro root length after four weeks. Data represents mean $\pm$ standard deviation ($n = 11$). Different letters indicate significant differences using Tukey HSD test ($p < 0.05$).

3.3. Effect of Photoperiod on Acclimatization

Acclimatization is a stage carried out before the micro-propagated plantlets are exposed to ex vitro conditions. After 5 weeks of acclimatization, the plantlets become more vigorous in all treatments (Figure 3). Photoperiod did not significantly affect the number of nodes (Figure 4a). However, there was a trend where longer photoperiods resulted in higher shoot length (Figure 4b), which might be due to increase in photosynthesis duration [15,18]. The number of nodes and

total leaf area per shoot were also not influenced by photoperiod (Figure 4c-d). The highest total leaf fresh weight was obtained with a 16-hour photoperiod treatment (0.2 g) (Figure 4e), consistent with previous findings. This also might be due to increase in photosynthesis duration, which in turn causes the accumulation of biomass in the leaves [22-23]. Thus, the most effective photoperiod for the growth enhancement of *S. rebaudiana* plantlets during the acclimatization process is 16 hours.

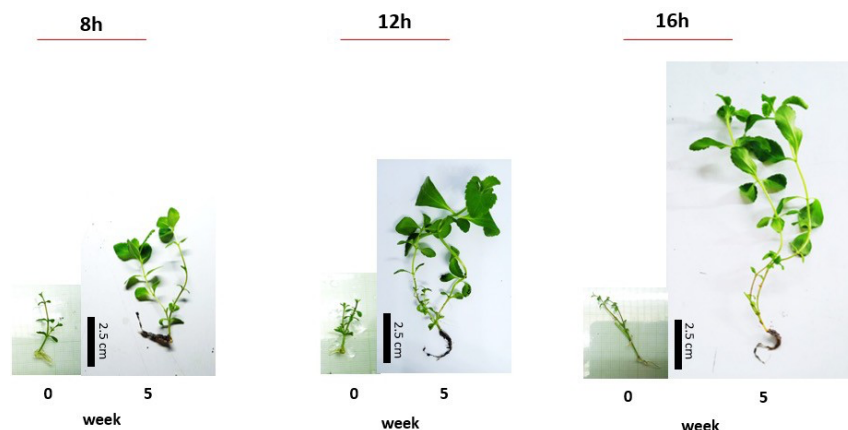


Figure 3. *S. rebaudiana* plantlets morphology before and after five weeks acclimatization under all photoperiod treatments.

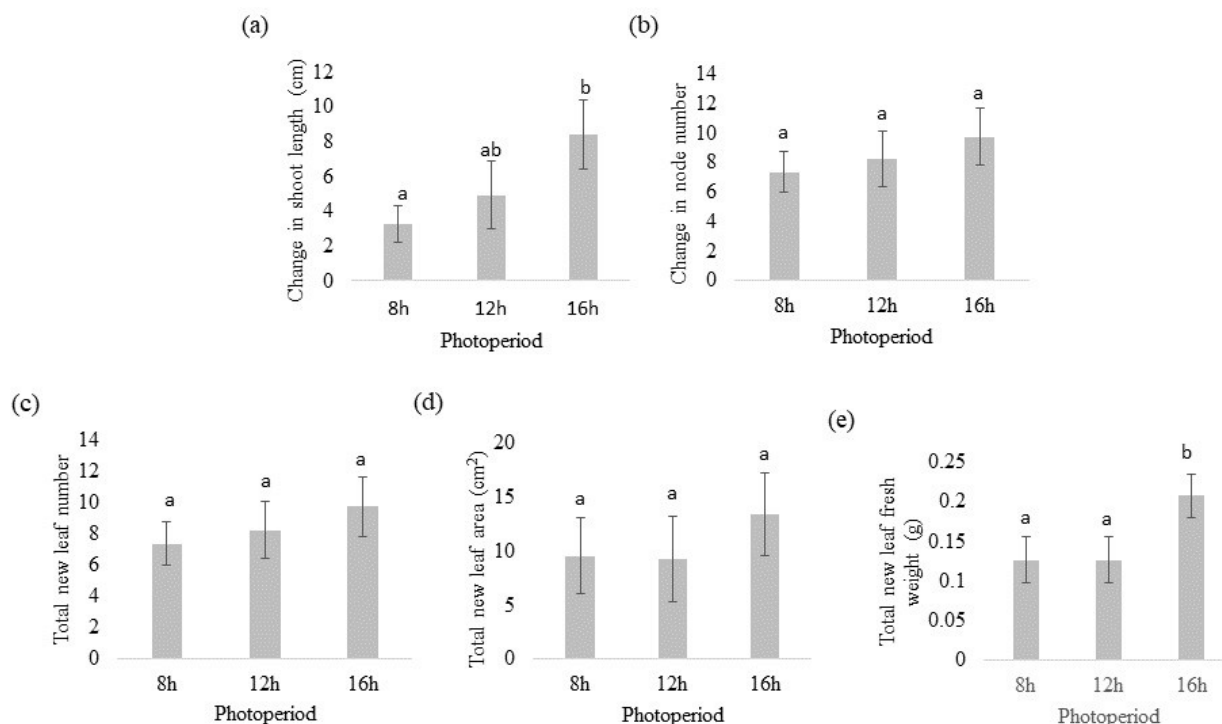


Figure 4. The effect of different photoperiod treatment on (a) the change in shoot length, (b) the change in node number, (c) total number of the new leaves, (d) total area of the new leaves, and (e) total fresh weight of the new leaves of *S. rebaudiana* plantlets after five weeks acclimatization. Data represents mean $\pm$ standard deviation (n = 8). Different letters indicate significant differences using Tukey HSD test ($p < 0.05$).

4. Conclusion

A 16-hour photoperiod increases shoot length during the in vitro shoot initiation phase, making this photoperiod most suitable for shoot multiplication. On the other hand, a 16-hour photoperiod also increases shoot length and leaf fresh weight during acclimatization. The increase in leaf biomass will enhance the total steviol glycoside production in *S. rebaudiana*. The results of this study indicate that photoperiod has an impact on the in vitro growth of *S. rebaudiana*.

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References

- [1.] Gupta E, Purwar S, Sundaram S, Rai G. Journal of Medicinal Plants Research Review Nutritional and therapeutic values of *Stevia rebaudiana*: A review. Journal of Medicinal Plants Research [Internet]. 2013;7(46):3343–53. Available from: <https://www.essentialnutrition.com.br/media/artigos/sweetlift/Sweet-Lift-16.pdf>
- [2.] Ohta M, Sasa S, Inoue A, Tamai T, Fujita I, Morita K, et al. Characterization of Novel Steviol Glycosides from Leaves of *Stevia rebaudiana* Morita. Journal of Applied Glycoscience. 2010;57(3):199–209.
- [3.] Ashwell M. Stevia, Nature's Zero-Calorie Sustainable Sweetener. Nutrition Today. 2015 May;50(3):129–34.
- [4.] Anton SD, Martin CK, Han H, Coulon S, Cefalu WT, Geiselman P, et al. Effects of stevia, aspartame, and sucrose on food intake, satiety, and postprandial glucose and insulin levels. Appetite. 2010 Aug;55(1):37–43.
- [5.] Choi DH, Cho UM, Hwang HS. Anti-inflammation effect of rebaudioside A by inhibition of the MAPK and NF- κ B signal pathway in RAW264. 7 macrophage. Journal of applied biological chemistry. 2018;61(2):205–11.
- [6.] Goettemoeller J, Ching A. Seed germination in *Stevia rebaudiana*. Perspective on new crops and new uses. ASHS Press. 1999 Jan 1; 510–511.
- [7.] Khalil SA, Zamir R, Ahmad N. Selection of suitable propagation method for consistent plantlets production in *Stevia rebaudiana* (Bertoni). Saudi Journal of Biological Sciences. 2014 Dec;21(6):566–73.
- [8.] Ceunen S, Geuns JM. Influence of photoperiodism on the spatio-temporal accumulation of steviol glycosides in *Stevia rebaudiana* (Bertoni). Plant Science. 2013 Jan 1;198:72–82.
- [9.] Ramírez-Mosqueda MA, Iglesias-Andreu LG, Bautista-Aguilar JR. The effect of light quality on growth and development of in vitro plantlet of *Stevia rebaudiana* Bertoni. Sugar Tech. 2017 Jun;19:331–6.
- [10.] Nakonechnaya OV, Gafitskaya IV, Burkovskaya EV, Khrolen-

- ko YA, Grishchenko OV, Zhuravlev YN, Subbotin EP, Kulchin YN. Effect of light intensity on the morphogenesis of *Stevia rebaudiana* under in vitro conditions. *Russian Journal of Plant Physiology*. 2019 Jul;66:656-63.
- [11.] Adams SR, Langton FA. Photoperiod and plant growth: a review. *The Journal of Horticultural Science and Biotechnology*. 2005 Jan 1;80(1):2-10.
- [12.] Yoneda Y, Shimizu H, Nakashima H, Miyasaka J, Ohdoi K. Effects of light intensity and photoperiod on improving steviol glycosides content in *Stevia rebaudiana* (Bertoni) Bertoni while conserving light energy consumption. *Journal of applied research on medicinal and aromatic plants*. 2017 Dec 1;7:64-73.
- [13.] de Andrade MV, de Castro RD, da Silva Cunha D, Neto VG, Carosio MG, Ferreira AG, de Souza-Neta LC, Fernandez LG, Ribeiro PR. *Stevia rebaudiana* (Bert.) Bertoni cultivated under different photoperiod conditions: Improving physiological and biochemical traits for industrial applications. *Industrial Crops and Products*. 2021 Sep 15;168:113595.
- [14.] Kumar S, Singh MC. Effect of photoperiod on growth characteristics in *Chrysanthemum morifolium* Ramat. cv. Zembla. *Research on Crops*. 2017 Jan 1;18(1):110.
- [15.] Kelly N, Choe D, Meng Q, Runkle ES. Promotion of lettuce growth under an increasing daily light integral depends on the combination of the photosynthetic photon flux density and photoperiod. *Scientia Horticulturae*. 2020 Oct 15;272:109565.
- [16.] Lam VP, Choi J, Park J. Enhancing growth and glucosinolate accumulation in watercress (*Nasturtium officinale* L.) by regulating light intensity and photoperiod in plant factories. *Agriculture*. 2021 Jul 30;11(8):723.
- [17.] Kristina N, Herawati N, Resigia E. Shoots and Roots Induction of Garlic on Different Composition of Plant Growth Regulators and Photoperiod. In IOP Conference Series: Earth and Environmental Science 2023 May 1 (Vol. 1177, No. 1, p. 012025). IOP Publishing.
- [18.] Elkins C, van Iersel MW. Longer photoperiods with the same daily light integral increase daily electron transport through photosystem II in lettuce. *Plants*. 2020 Sep 10;9(9):1172.
- [19.] Lopez RG, Runkle ES. Photosynthetic daily light integral during propagation influences rooting and growth of cuttings and subsequent development of New Guinea impatiens and petunia. *HortScience*. 2008 Dec 1;43(7):2052-9.
- [20.] Xu Y, Yang M, Cheng F, Liu S, Liang Y. Effects of LED photoperiods and light qualities on in vitro growth and chlorophyll fluorescence of *Cunninghamia lanceolata*. *BMC Plant Biology*. 2020 Dec;20:1-2.
- [21.] Xia, J. and Mattson, N., 2022. Response of common ice plant (*Mesembryanthemum crystallinum* L.) to photoperiod/daily light integral in vertical hydroponic production. *Horticulturae*, 8(7), p.653.
- [22.] Kozai T, Watanabe K, Jeong BR. Stem elongation and growth of *Solanum tuberosum* L. in vitro in response to photosynthetic photon flux, photoperiod and difference in photoperiod and dark period temperatures. *Scientia horticulturae*. 1995 Oct 1;64(1-2):1-9.
- [23.] Yan Z, He D, Niu G, Zhai H. Evaluation of growth and quality of hydroponic lettuce at harvest as affected by the light intensity, photoperiod and light quality at seedling stage. *Scientia horticulturae*. 2019 Apr 5;248:138-44.