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Stimulation of Stevioside Accumulation on *Stevia rebaudiana* (Bertoni) Shoot Culture Induced with Red LED Light in TIS RITA® Bioreactor System

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***Abstract*—**Leaves of *Stevia rebaudiana* contain steviol glycoside which mainly comprise of stevioside, a natural sweetener compound that is 100-300 times sweeter than sucrose. Current cultivation method of *Stevia rebaudiana* in Indonesia has yet to reach its optimum efficiency and productivity to produce stevioside as a safe sugar substitute sweetener for people with diabetes. An alternative method that is not limited by environmental factor is *in vitro* temporary immersion system (TIS) culture method using recipient for automated immersion (RITA®) bioreactor. The aim of this research was to evaluate the effect of red LED light induction towards shoot growth and stevioside accumulation in TIS RITA® bioreactor system, as an endeavour to increase the secondary metabolite synthesis. The result showed that the stevioside accumulation in TIS RITA® bioreactor system induced with red LED light for one hour during night was higher than that in TIS RITA® bioreactor system without red LED light induction, i.e. 71.04 ± 5.36 µg/g and 42.92 ± 5.40 µg/g respectively. Biomass growth rate reached as high as 0.072 ± 0.015/day for red LED light induced TIS RITA® bioreactor system, whereas TIS RITA® bioreactor system without induction was only 0.046 ± 0.003/day. Productivity of *Stevia rebaudiana* shoots induced with red LED light was 0.065 g/L medium/day, whilst shoots without any induction was 0.041 g/L medium/day. Sucrose, salt, and inorganic consumption in both bioreactor media increased as biomass increased. It can be concluded that *Stevia rebaudiana* shoot in TIS RITA® bioreactor induced with red LED light produces biomass and accumulates higher stevioside concentration, in comparison to bioreactor without any light induction.

***Keywords*—**LED, *Stevia rebaudiana*, Stevioside, TIS RITA

I. Introduction

DIABETES mellitus is a metabolic disease, demonstrated by hyperglycemic due to the malfunction of insulin secretion in the body [1]. According to International Diabetes Federation, in 2015 alone, among 161 million adult population in Indonesia, 10 million of them suffer from diabetes. Indonesia as a developing country has a great need to decrease diabetic epidemic [1]. Stevia (*Stevia rebaudiana* Bertoni) is an Asteraceae family plant from the high grounds of Southern America with particular contents of natural sweetener in its leaves, which is estimated to be 100-300 times sweeter than sucrose, with virtually zero calories [2]. The compound responsible is a diterpene glycoside secondary metabolite of stevia, which mainly comprised of stevioside and rebaudioside A. Both compounds are approved by FAO and BPPOM of Indonesia to substitute sugar sweetener in food and beverage products, thus making it safe for people with diabetic issue [3].

Stevia rebaudiana is commonly cultivated using *in vivo* method in Indonesia; however, it is known to have a low productivity and efficiency [4], [5]. Cultivation method using stevia seedlings has a considerably low rate of success, just below 50% and rather high variation of content and amount of stevioside, varying over 5-15% of dry leaf weight, while stem cuttings method causes damage on donor plant [6]-[8]. One alternative method to produce stevia plantlet without environmental restriction is *in vitro* shoot culture method using bioreactor as an automated system.

In vitro shoot culture of *Stevia rebaudiana* has been successfully applied by several researchers using Murashige and Skoog (MS) in a semi-solid medium [9], [10]. Extensive research on shoot multiplication by *in vitro* method has been done previously, one of the research focuses on automatization using TIS bioreactor, which allows the culture to be immersed in a specific time interval. Employing TIS bioreactor is reported to prevent hyperhidricity on the culture, increases the effectiveness of shoot proliferation by optimizing medium and reactor volume in use [11], and yields a better mass transfer [12]. Recipient for automated immersion (RITA®) bioreactor is one of several TIS type bioreactor that consist of two containers, upper container for cultures and lower container for storage of liquid medium [12].

One method to enhance stevioside content in stevia leaves is to induce the culture using particular light. It is known from previous researches that red LED induction on stevia plant shows an increase in stevioside content [13]. Presently, there has not been any comprehensive research regarding the effect of red LED light induction on stevioside content in stevia propagated by *in vitro* shoot culture. Correspondingly, this research aims to evaluate the response of red LED light stimulation on shoots of stevia cultured in RITA® bioreactor system towards its stevioside accumulation, biomass changes, growth rate, and nutrition rate of intake by biomass of *Stevia rebaudiana* during period of cultivation.

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II. Materials and Methods

A. Preparation of Medium Culture

Medium was prepared for initiation culture, subculture, acclimatization culture, and bioreactor culture. Initiation culture medium consisted of agar, 4.4 g/L MS, 30 g/L sucrose, and a variety of plant growth hormone (PGR) concentration: 0.5 ppm 6-benzylaminopurine (BAP); 0.6 ppm BAP; 0.7 BAP; 1 ppm Kinetin; and 2 ppm Kinetin [14]. Subculture medium composed of the exact initiation culture medium, differed in the types of PGR used, in which all medium was supplemented with 1 ppm Kinetin. Acclimatization and bioreactor medium composed of 2.2 g/L MS, 30 g/L sucrose, and 1 ppm Kinetin [14]. The pH of all medium was adjusted to 5.6-5.8, then sterilized using autoclave at 121°C for 15 minutes. Sterile mediums were then supplemented with 200 ppm of Augmentin.

B. Shoot Culture Initiation and Subculture

Stevia rebaudiana used in this research was obtained from Research Center of Tea and Quinine, Gambung. West Java, Indonesia. Young shoots up to 2nd and 3rd node explants were cut and washed with water before submerged in a 3 ppm of 5% hexaconazole solution for 3 minutes, then rinsed with running water. In an aseptic condition inside laminar air flow (LAF), shoot and node explants of stevia were sterilized with 70% ethanol for 1 minute. Shoot explants were transferred into Erlenmeyer that contained 5% of sodium hypochlorite (NaClO) and 2 drops of 20% tween, and shook gently for 5 minutes. Node explants went through similar process of sterile washing, although for node explants, 10% of NaClO was used instead and shook for 10 minutes. Explants were then rinsed with sterile aquades 3 times, each lasted 5 minutes. Explants were further cut in petri dish, removing parts of leaves that remained. Dried explants were transferred into culture bottles, filled with semi-solid medium, aseptically. After 14 days of cultivation, shoot and node explants were transferred into subculture medium for another 14 days. Cultures were cultivated in 25 °C temperature, photoperiod of 16 h, and light intensity of 2.000-2.500 lux.

C. Acclimatization of Stevia rebaudiana Shoots

Shoot culture of *Stevia rebaudiana* were transferred from semi-solid medium into liquid thin layer medium aseptically in LAF. Shoots were initially cut to achieve homogenous size. The culture was agitated using a 40 rpm shaker for 14 days. Cultivation conditions were similar to the previous conditions.

D. Shoot Culture in RITA® Bioreactor

Shoot explants of *Stevia rebaudiana* for cultivation in bioreactor originated from shoots that were acclimatized in thin layer medium. Explants were weighed to 1 gram before carefully transferred into sterile bioreactor in LAF. The inlet and outlet of bioreactor were attached to microfilter to prevent air contamination. Air flow was regulated using automatic timer with immersion intensity of 15 minutes every 6 hour period [15]. Light induction was varied: (1) red LED interruption during the night for 1 hour; (2) no interruption during the night. Shoots were cultivated for 21 days in room temperature, photoperiod of 16 h, and light intensity of 2.000-2.500 lux for both fluorescent and LED light.

E. Shoot and Medium Analysis

Cultivated shoots were weighed and the final number of shoots was counted. Shoots were dried in oven to attain the dry weight. Conductivity of bioreactor medium was measured using conductivity meter (Eutech Instruments Con-110), whilst sucrose content was measured using refractometer (Milwaukee MA871).

F. Stevioside Content Analysis

Stevioside content was analyzed using high performance liquid chromatography (HPLC) [16]. A 100 mg dry stevia biomass was grinded into powder before extracted with 10 mL of 70% ethanol solution in waterbath condition of 70 °C for 30 minutes [17]. Extracts were filtrated and dried thoroughly. Dried extracts were dissolved with 2 mL of acetonitrile. Sample was then analyzed using C18 column HPLC with 80% acetonitrile eluent, UV detector was 210 nm, and rate of elution was 1 mL/minute. Stevioside standard was 98% in stevioside purity obtained from Shananxi Sinuote Biotech Co. Ltd.

G. Data Analysis

Dry weight data of *Stevia rebaudiana* shoots were used to form growth rate curve. The result of medium analysis on sucrose content was utilized to develop mass balance model of *Stevia rebaudiana in vitro* shoot culture in RITA® bioreactor system. The model developed was based on respiration reaction of the following [18]: $0.39\ C_{12}H_{22}O_{11} + 0.23\ NH_4NO_3 + 3.43\ O_2 \rightarrow CH_{1.27}O_{0.43}N_{0.45} + 4.07\ H_2O + 3.64\ CO_2$.

Molecular formula of *Stevia rebaudiana* used was the empirical form of *Atropa belladonna* hairy root culture molecular formula. Productivity of stevia shoot culture was determined by the ratio of weight accumulated (g) by the volume of medium absorbed (L) and period of cultivation (days) [19].

III. Result and Discussion

Stevia shoot produced after the initiation was shown in Fig. 1. Shoot regeneration (apical shoot and lateral shoot) was formed on initiation medium with 2 ppm Kinetin (b), with a total of 70 ± 2.23 shoots. Meanwhile, the most shoots that produced leaves were cultivated using the medium supplemented with 0.5 ppm BAP (c), with the total of 201 ± 2.97 leaves (Table I). The number of leaf produced in various mediums slightly differ with the average of 11.86-13 leaves per bottle. Although the selection of subculture medium was based on the highest regenerating shoots in initiation stage, another crucial factor to consider was the phenotype of the shoots. In Fig. 1, stevia shoots in medium supplemented with BAP were brownish (red circles). Shoots with abnormal morphology is known to be prone of dying during next stage of subculture into a new medium [20]. Phenotype of shoots that formed in medium supplemented with 1 ppm Kinetin (a); however, showed a promising quality with visually green and

fresh leaves and stems, and more leaves in comparison with 2 ppm Kinetin medium, yielded 195 ± 4.55 leaves in the course of 14 days of cultivation. Other research indicated that the best stevia shoots were produced with 1 ppm Kinetin, instead of 0.5 ppm, 1.5 ppm, or 2 ppm Kinetin [10]. Therefore, medium with 1 ppm Kinetin was used for further experiment.

(a) (b) (c) (d) (e)

Fig. 1 *Stevia rebaudiana* shoots in various initiation medium: (a) 1 ppm Kinetin; (b) 2 ppm Kinetin; (c) 0.5 ppm BAP; (d) 0.6 ppm BAP; (e) 0.7 ppm BAP

TABLE I
NUMBER OF SHOOTS AND LEAVES OF *STEVIA REBAUDIANA* IN A VARIETY OF INITIATION MEDIUMS

Medium	Σ Final Shoots ± SD	Σ Final Leaves ± SD	Visual Description
1 ppm Kinetin	46 ± 1,54	195 ± 4,55	Green shoots and leaves
2 ppm Kinetin	70 ± 2,23	156 ± 4,00	Green shoots and leaves
0.5 ppm BAP	64 ±1,86	201 ± 2,97	Green leaves, brown shoots
0.6 ppm BAP	48 ± 2,00	192 ± 6,13	Green leaves, brown shoots
0.7 ppm BAP	48 ± 0,94	154 ± 4,20	Green leaves, brown shoots

During shoot initiation period, 70 shoots, either apical or lateral, were proliferated into 247 new shoots, thus giving a 352.86% multiplication. Furthermore, after shoot was subcultured, 610 new shoots were produced, giving a 246.93% multiplication. The percentage of success for initiation and subculture stage were 89.74% and 98.38% respectively.

Acclimatized *Stevia rebaudiana* shoots in thin layer medium were used to cultivate in RITA® bioreactor for 21 days. RITA® 1 bioreactor system represented as control, without any light irradiation during the night. In contrast, RITA® 2 bioreactor system represented the treatment variable, in which red LED light was applied in the culture.

In Table II and Fig. 2, stevia shoot culture in RITA® 2 bioreactor system had a higher dry weight than that of shoot culture in RITA® 1 bioreactor system, ergo, a higher growth rate. Red light induction has a particular function as energy inducer in light reaction of photosynthesis, where red light induces excitation of electron to activate the hydrolysis of water, reducing ADP^+ into ATP and $NADP^+$ into NADPH, generating chemical energy for further metabolism of plant [21]. The process itself requires nutrition from liquid medium. The increment of nutrition consumption indirectly increases the rate of photosynthesis that constructs biomass, which was used for the growth of stevia shoot [21]. These results were similarly reported in [13]. A system with red light induction had a higher biomass in comparison with a system without red light induction (Table II) [13]. Therefore, RITA® 2 bioreactor system had a higher biomass growth rate and lower doubling time than RITA® 1 bioreactor system.

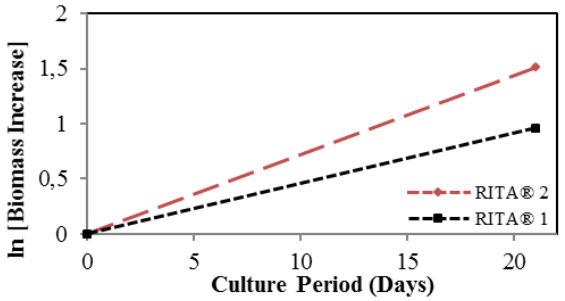
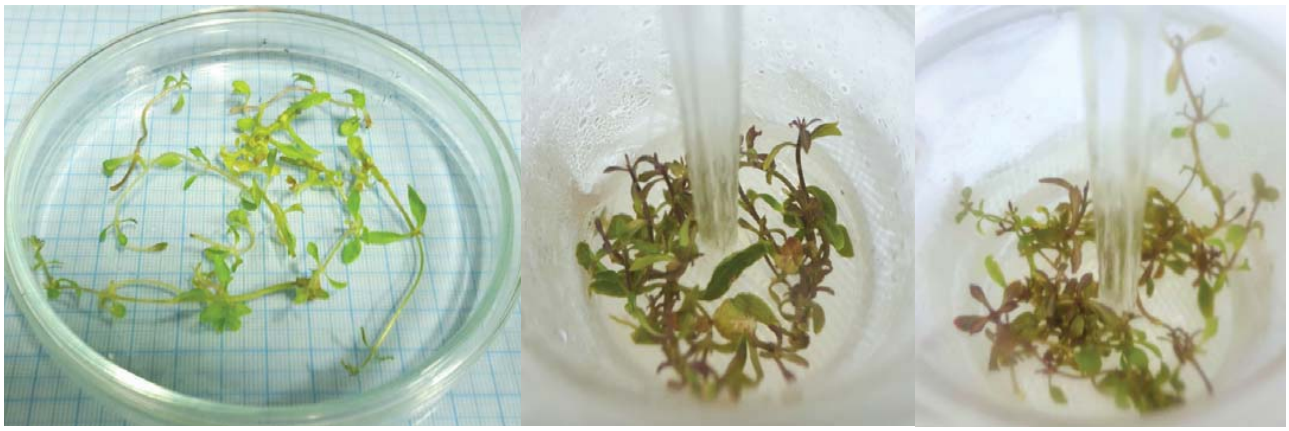


Fig. 2 Average growth rate of biomass increase in RITA® bioreactor systems

Cultivation result in Fig. 3 showed a partly reddish-purple color in leaves and stems of *Stevia rebaudiana*, moreover the stems became hardened. Such color might be the result of somewhat low consumption rate of nitrogen in RITA® bioreactor system, which in effect, produced reddish-purple colored pigment, instead of green pigment. In a system of low nitrogen intake, plant will seem thinner with hardened stem, as a result of over-produced carbohydrate, which cannot be used to synthesize amino acid or other related nitrogen compounds [21]. The excess of carbohydrate production is hence utilized in anthocyanin synthesis, producing pigment of slight reddish-brown color. This is commonly observed in plants with nitrogen deficiency [21]. A comparable phenomenon occurred in a nitrogen deprived *Arabidopsis thaliana* and cineraria *in vitro* culture that displayed an increase in anthocyanin content [22]. Red LED light irradiation on grapes also increased anthocyanin content, as a result of endogenous plant hormone induction, abscisic acid, as observed in the previous research [23]. Similar process might have happened in stevia leaves [23].

TABLE II
MASS, SUM OF SHOOTS, GROWTH RATE, DOUBLING TIME, AND PRODUCTIVITY CHANGES OF *STEVIA REBAUDIANA* SHOOTS THROUGHOUT CULTIVATION PERIOD IN RITA® BIOREACTOR SYSTEMS

	Initial Dry Weight (g) ± SD	Initial Sum of Shoots	Final Dry Weight (g) ± SD	Final Sum of Shoots	Growth Rate ($days^{-1}$)	Doubling Time (days)	Productivity ($g \cdot L_{medium}^{-1} \cdot days^{-1}$)
RITA®1	0,134 ± 0,004	13,5 ± 2,12	0,35 ± 0,014	49 ± 2,83	0.0.457 ± 0.003	15,17 ± 1,08	0,041
RITA®2	0,097 ± 0,024	13 ± 1,41	0,44 ± 0,028	61 ± 2,83	0.0721 ± 0.015	9,61 ± 2,02	0,065



(a) (b) (c)

Fig. 3 *Stevia rebaudiana* shoots before and after cultivated in RITA® bioreactor: (a) day-0; (b) day-21 in RITA® 1 bioreactor; (c) day-21 in RITA® 2 bioreactor

In Fig. 4, conductivity value of RITA® bioreactor system medium throughout cultivation period decreased. A decrease in conductivity defined a consumption of salt and inorganic compounds in the medium. Naturally consumed inorganic salts by plants are NH^{4+}, NO^{3-}, K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}, SO_4^{2-}, and PO_4^{3-} [24]. Consumption of inorganic compounds in plant is linear to the rise of plant biomass [25]. Conductivity of RITA® 1 bioreactor system dropped 0.29 mS ± 0.014, whereas RITA® 2 bioreactor system was 0.53 mS ± 0.255. The result of conductivity changes proved that RITA® 2 bioreactor system had a higher consumption rate than RITA® 1 bioreactor system.

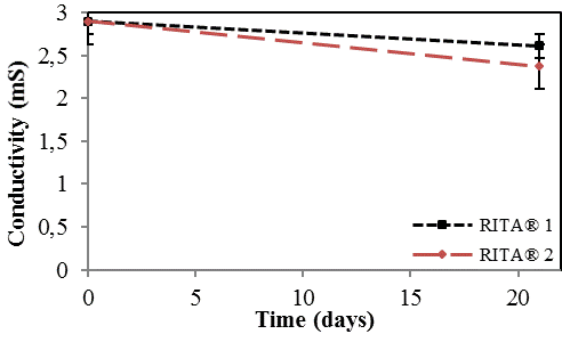


Fig. 4 Changes of conductivity in RITA® bioreactor media

Fig. 5 illustrates the declining sucrose content in both RITA® bioreactors throughout cultivation period. The reduction of sucrose content in RITA® 1 bioreactor system was 1.93 g ± 0, whilst in RITA® 2 bioreactor system was 3.85 g ± 1.36. Sucrose consumption in RITA® 2 bioreactor system was almost 200% more than the consumption in RITA® 1 bioreactor system. Sucrose consumption rate for RITA® 1 bioreactor system and RITA® 2 bioreactor system was 0.092 $g.L^{-1}.day^{-1}$ and 0.184 $g.L^{-1}.day^{-1}$, respectively, which might be correlated with rate of biomass formation and stevioside content.

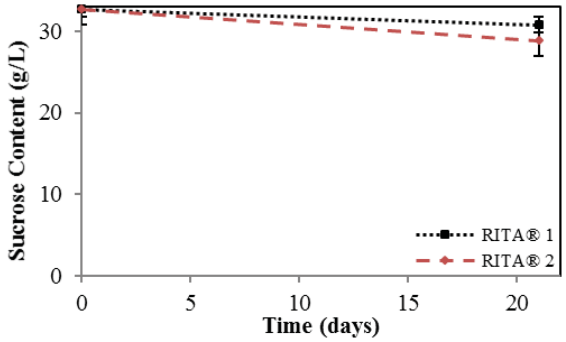


Fig. 5 Changes of sucrose content in RITA® bioreactor media

Sucrose is the carbon source utilized by cells to respire and establish various metabolic activity, so that sucrose content in medium will decrease as biomass increases [26]. The increase of sucrose consumption rate in RITA® 2 bioreactor system might include considerable number of enzyme activity that involve in sugar metabolism [23]. Furthermore, sucrose phosphate synthase enzyme activity in stevia is also regulated by red light, causing a higher accumulation of sugar treated in shoots than shoots without red light induction [27], [28].

Based on mass balance data in Tables III and IV, actual biomass dry weight measured for both RITA® 1 and RITA® 2

bioreactor systems were less than the quantified value in mass balance calculation. The real dry weight of RITA® 1 bioreactor was 32.2% less than the hypothetical calculation, whereas for RITA® 2 bioreactor was 48.9% less.

TABLE III
MASS BALANCE OF RITA® 1 BIOREACTOR SYSTEM

	$C_{12}H_{22}O_{11}$	NH_4NO_3	O_2	$CH_{1.7}O_{0.43}N_{0.45}$	H_2O	CO_2
Initial mole (mol)	0.096			0.005	0.000	0.000
Reacting mole (mol)	0.006	0.003	0.050	0.014	0.059	0.053
Remaining mole (mol)	0.090			0.020	0.059	0.053
Remaining mass (g)	30.788			**0.516**	1.058	2.314
Actual dry weight biomass (g)				**0.35 ± 0.014**		

TABLE IV
MASS BALANCE OF RITA® 2 BIOREACTOR SYSTEM

	$C_{12}H_{22}O_{11}$	NH_4NO_3	O_2	$CH_{1.7}O_{0.43}N_{0.45}$	H_2O	CO_2
Initial mole (mol)	0.096			0.004	0.000	0.000
Reacting mole (mol)	0.011	0.007	0.099	0.029	0.118	0.105
Remaining mole (mol)	0.084			0.033	0.118	0.105
Remaining mass (g)	28.861			**0.861**	2.117	4.627
Actual dry weight biomass (g)				**0.44 ± 0.028**		

Shoots of lower dry weight in RITA® bioreactor systems in comparison with its hypothetical calculation through mass balance reaction was mainly influenced by a two few circumstances. Firstly, the biomass formula used for *Stevia rebaudiana* shoots that serves as the fundamental reaction of mass balance was originated from *in vitro Atropa belladonna* hairy root biomass. Presumably, the biomass formula did not represent the stevia shoot biomass. Cells of different species tend to have a different consumption pattern of nutrition and biomass formation; hence, it forms a distinctive biomass formula that vary among different types of cells. Secondly, is the absence of substrate limiting agent known in this research. The presence of a limiting agent will affect the biomass growth rate in RITA® 2 bioreactor system.

Stevioside accumulation in shoot *in vitro* culture was analyzed through HPLC. Fig. 6 illustrated the stevioside content in RITA® 1 bioreactor system, treated as control without the presence of red LED light induction during night time, contained 42.92 µg/g ± 5.4, while stevioside content in RITA® 2 bioreactor system treated with red LED light induction for 1 hour during the night time was 71.04 µg/g ± 5.36, roughly 40% more than control. Reports in reference [13] conducted a similar condition of *ex vivo* experiment, revealed that red light irradiation during night photoperiod increased stevioside accumulation in each plant up to 200% in comparison with plants without red light induction, additionally, as much as 55% more stevioside accumulation per dry weight basis than in control plants was observed [13].

Stevioside biosynthesis pathway follows a similar pathway as gibberellin hormone, a methylerythritol 4-phospate (MEP) pathway, towards the formation of ent-kaurenoic acid intermediate. Red light treatment activates a distinct phytochrome that might change the pathway to steviol glycoside [13]. Red LED light stimulation appeared to not affect specific glycosyltransferases that catalyze the glycosylation of steviol, rather early steviol glycoside biosynthesis pathway [13].

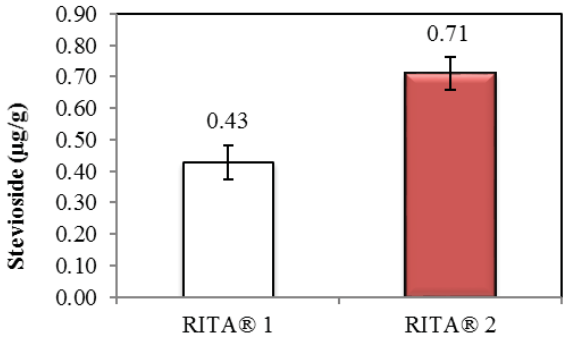


Fig. 6 Stevioside content of *Stevia rebaudiana* in RITA® bioreactor systems

IV. CONCLUSION

Stevia rebaudiana shoot culture cultivated in RITA® bioreactor system treated with red LED light induction showed an increase in regards to stevioside accumulation of 71.04 µg/g ± 5.36 and its biomass growth rate of 0.072 ± 0.015 day^{-1}. Red LED light induction method on Stevia rebaudiana shoots in RITA® bioreactor system was proven capable of increasing biomass and stimulating stevioside to a higher value compared to RITA® bioreactor system without red LED light treatment.

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REFERENCES

[1] D. E. Sentochnik and G. M. Eliopoules, "Infection and diabetes". *Joslins Diabetes Mellitus*, vol. 14, pp. 1022, 2005.
[2] D. Mousumi, "Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*". *J. Med. Plant. Res.*, vol. 2, pp. 45-51, 2008.
[3] M. V. Chalapathi and S. Thimmegowda, "Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bertoni) a future crop of India". *Crop. Res. Hisar*, vol. 14, pp. 347-350, 1997.
[4] J. E. Brandle, A. N. Starratt, and M. Gijzen, "*Stevia rebaudiana*: its agricultural, biological, and chemical properties". *Can. J. Plant Sci.*, vol. 78, pp. 527-536, 1998.
[5] Djajadi, "Pengembangan tanaman pemanis *Stevia rebaudiana* (Bertoni) di Indonesia". *Perspektif*, vol. 13, pp. 25-33, 2014.
[6] Y. Miyazaki and H. Watanabe, "Studies on the cultivation of *Stevia rebaudiana* Bertoni". *J. Trop. Agric.*, vol. 17, pp. 154-157, 1974.
[7] S. Nakamura and Y. Tamura, "Variation in the main glycosides of stevia (*Stevia rebaudiana* (Bertoni))". *Jpn. J. Trop. Agric.*, vol. 29, pp. 109-116, 1985.
[8] U. N. A. A. Razak, C. B. Ong, T. S. Yu, and L. K. Lau, "*In vitro* micropropagation of *Stevia rebaudiana* Bertoni in Malaysia". *Brazilian Archieves of Biology and Technology*, vol. 57, pp. 23-28, 2014.

[9] M. Anbazhagan, M. Kalpana, R. Rajendran, V. Natarajan, and D. Dhanavel, "*In vitro* production of *Stevia rebaudiana* Bertoni". *Emirates J. Food Agric.*, vol. 22, pp. 216-222, 2010.
[10] S. Deshmukh and R. Ade, *In vitro rapid multiplication of Stevia rebaudiana: an important natural sweetener herb*. Amravati, India: Department of Biotechnology, Sant Gadge Baba Amravati University, 2012.
[11] H. Etienne, and M. Berthouly, "Temporary immersion in plant micropropagation". *Plant Cell Tiss. Org. Cult.*, vol. 69, pp. 215-231, 2002.
[12] P. T. Lyam, M. L. Musa, Z. O. Jamaleddine, U. A. Okere, W. T. Odofin, and A. Carlos, "The potential of temporary immersion bioreactors (TIBs) in meeting crop production demand in Nigeria". *J. Biology and Life Science*, vol. 3, pp. 66-86, 2012.
[13] S. Ceunen, S. Werbrouck, and J. M. C. Geuns, "Stimulation of steviol glycoside accumulation in *Stevia rebaudiana* by red LED light". *Journal of Plant Physiology*, vol. 169, pp. 749-752, 2012.
[14] T. Murashige, and F. Skoog, "A revised medium for rapid growth and bio assays with tobacco tissue cultures". *Physiologia planetarium*, vol. 15, pp. 473 – 497, 1962.
[15] N. Noordin, R. Ibrahim, N. H. Sajahan, S. M. M. Nahar, S. H. M. Nahar, and N. R. A. Rashid, *Micropropagation of Stevia rebaudiana through temporary immersion bioreactor system*. Agrotechnology and Bioscience Division, Malaysian Nuclear Agency, Ministry of Science, Technology and Innovation Malaysia, and Faculty of Applied Sciences, UiTM Shah Alam, Selangor, Malaysia, 2012.
[16] N. A. Samah, A. D. A. Hisham, and S. A. Rahim, "Determination of stevioside and rebaudioside A in *Stevia Rebaudiana* leaves via preparative high performance liquid chromatography (prep-HPLC)". *International Journal of Chemical and Environmental Engineering*, 2012, pp. 332.
[17] N. Kolb, J. L. Herrera, D. J. Ferreyra, and R. F. Uliana, "Analysis of sweet diterpene glycosides from *Stevia rebaudiana*: improved HPLC method". *J. Agric. Food Chem*, vol. 49, pp, 4538-4541, 2001.
[18] A. Saterbak, L. V. McIntire, and K. Y. San, *Bioengineering Fundamentals*. New Jersey, USA: Pearson Prentice Hall, 2007, pp. 159-161.
[19] M. L. Kyriakides, E. S. Tzanakakis, C. Kiparissidis, L. V. Ekaterianiadou, and D. A. Kyriakidis, "Kinetics of xanthan gum production from whey by constructed strains of *Xanthomonas campestris* in batch fermentations". *Chem. Eng. Technol.*, vol. 20, pp. 354-360, 1997.
[20] M. B. Ahmed, M. Salahin, R. Karim, M. A. Razvy, M. M. Hanan, R. Sultan, M. Hosain, and R. Islam, "An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni)". *American- Eurasian Journal of Science Research*, vol. 2, pp, 121 – 125, 2007.
[21] L. Taiz, and E. Zeiger, *Plant Physiology*. Sunderland, UK: Sinauer Associates, 2002, pp. 73.
[22] A. Mujib, *Somatic Embryogenesis in Ornamentals and Its Applications*. New Delhi: India: Springer, 2016, pp. 61.
[23] S. Kondo, H. Tomiyama, A. Rodyung, K. Okawa, H. Ohara, S. Sugaya, N. Terahara, and N. Hirai, "Abscisic acid metabolism and anthocyanin synthesis in grape skin are affected by light emitting diode (LED) irradiation at night". *Journal of Plant Physiology*, vol. 171, pp. 823-829, 2014.
[24] M. Taya, M. Hegglin, J. Prenosil, and J. Bourne, "On-line monitoring of cell growth in plant tissue cultures by conductometry". *Enzyme Microb. Technol.*, vol. 11, pp. 170-176, 1989.
[25] K. Hahlbrock, "Further studies on the relationship between rates of nitrate uptake, growth and conductivity changes in the medium of plant cell suspension cultures". *Planta*. 1975, pp. 311-318.
[26] K. L. Shuler and F. Kargi, *Bioprocess Engineering Basic Concepts*. New Jersey, USA: Prentice Hall PTR, 2002, pp. 231-234.
[27] A. D. Kinghorn, *Stevia: The Genus Stevia*. New York, USA: Taylor & Francis, 2002, pp. 178-180.
[28] Y. Ning, H. H. Deng, Q. M. Li, and X. Z. Ai, "Effects of red and blue light quality on the metabolites and key enzyme activities of carbon-nitrogen metabolism in celery". *Journal of Plant Physiology*, vol. 51, pp. 112-118, 2014.