

Biomass Enhancement of *Stevia rebaudiana* Bertoni Shoot Culture in Temporary Immersion System (TIS) RITA® Bioreactor Optimized in Two Different Immersion Periods

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Abstract. Stevia plant contains steviol glycosides, which are estimated to be 300 times sweeter than sucrose. However, conventional (*in vivo*) propagation of *Stevia rebaudiana* in Indonesia was not effective due to poor results. Therefore, an alternative method to propagate stevia plants is needed. One of them is by using *in vitro* method. Multiplication with a large quantity of stevia biomass in a relatively short period can be conducted by using TIS RITA® (Recipient for Automated Temporary Immersion System). This study aimed to evaluate the effect of the immersion period of the medium on growth and the medium bioconversion into the production of shoot biomass. The bioreactors were set up with 15 min and 30 min immersions periods, scheduled every 6 h, and incubated for 21 d. The result indicated that the immersion period affected the biomass and growth rate (μ). Amount of 30 min immersion showed a greater percentage of shoot multiplication, higher biomass, percentage of leaf growth, growth rate, and productivity compared to 15 min immersion. The pattern of sucrose, mineral, and inorganic compounds consumption followed the growth of plant biomass for both systems. In conclusion, 30 min immersion gave a greater efficiency medium bioconversion to plant biomass compared with 15 min immersion.

Key words: Diabetes prevention, micropropagation, natural sweetener, sweetener plant, tissue culture

1 Introduction

Indonesia has been classified as one of the top 10 countries worldwide with a high number of who has diabetes in 2013. The same pattern is predicted to continue unless diabetes prevention and management interventions are taken [1]. The rising prevalence of diabetes is

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highly caused by the high rate of foods and drink consumption that contains high sugar and artificial sweeteners, such as aspartame, saccharin, and cyclamate [2]. Therefore exploration of natural sweetener that contains low calorie is needed. Stevia (*S. rebaudiana*) is a herbaceous perennial shrub in Indonesia that contains several sweet-tasting compounds in its leaves. The main components that provide a sweet taste are stevioside, which have been estimated to be 300 times as sweet as sucrose [3]. Stevioside has been shown to possess beneficial effects on human health and can be used as a natural sweetener, especially for diabetic patients [4].

Stevia is commonly propagated using in vivo method, but this method was not effective. Seed propagation was not efficient due to low fertility [5], great variability on the stevioside level and composition [6], and poor germination rates (below 10 %) [7]. *In vitro* culture provide the best method to overcome those problems and has the potential to produce a large number of stevia plantlets in a short time.

In vitro cultivation method has been widely applied in various plants. This method requires a closed sterile container, where culture is given certain nutrients, either in solid or liquid form. Various studies on the use of in vitro culture methods for biomass propagation has been done, including in vitro culture methods in TIS (Temporary Immersion System) bioreactors [8]. The bioreactor is a modified liquid culture system where the immersion of explant happens periodically. In culture systems with temporary tissue immersion, it is clear that the immersion time is very important and decisive [9] since this factor determines nutrient uptake, which affects the biomass enhancement.

A previous study on the cultivation of stevia on TIS RITA® with immersion periods 15 min for 6 h had been conducted and proven significantly increased stevia biomass. Shoot multiplication was greatly increased, with 30 new healthy shoots produced from each bud clump, resulted in approximately 90 new stevia shoots per vessel. In comparison with semi-solid media with the same supplemented PGR, TIS produced 2.00 times more biomass production [10]. Referring to the previous research, this study was conducted to determine the potential of *S. rebaudiana* cultivation in the TIS RITA® bioreactor system, by evaluating the effect of immersion period of the medium on the growth rate, productivity, the rate of nutrient uptake by *S. rebaudiana*, and bioconversion medium into biomass during cultivation.

2 Material and methods

2.1 Culture medium preparation

The medium was prepared for the initiation, subculture and acclimatization and also bioreactors. The initiation medium was consisted of MS salts, 30 g L⁻¹ sucrose, and added with several plant growth regulators (PGRs) for optimization, i.e: 0.5 mg kg⁻¹ Benzyl Amino Purine (BAP); 0.6 mg kg⁻¹ BAP, 0.7 mg kg⁻¹ BAP; 1 mg kg⁻¹ Kinetin; and 2 mg kg⁻¹ Kinetin. Subculture medium has a similar composition to the initiation medium but were added with 1 mg kg⁻¹ Kinetin. Acclimatization and bioreactors medium as the best initiation medium consisted of half-strength MS salts, 1 mg kg⁻¹ Kinetin, and 30 g L⁻¹ sucrose. The pH of each medium was adjusted to 5.8 and autoclaved at 121°C for 15 min. Then, the sterilized media was added by 200 mg kg⁻¹ Augmentin.

2.2 Shoot culture initiation of Stevia

Stevia rebaudiana Bertoni plants were collected from the Research Center for Tea and Quinine, Gambung, Indonesia. The tips of young, actively growing shoot tips and nodal

segments were cut and washed three times with tap water to remove the dust particles and other contaminants. The explants were drenched with 3 mg kg⁻¹ fungicides HEKSA (Heksakonazol 5 %) for 3 min and followed by water. In a laminar airflow chamber (LAF), the washed explants were subjected to surface sterilization with ethanol 70 % (v/v) for 1 min. Shoots were dipped in NaClO 5 % and Tween-20 for 5 min, but nodes were dipped in NaClO 10 % and Tween-20 for 10 min. Both explants were rinsed three times with sterile distilled water, placed in Petri dishes lined with filter paper, and transferred into a solid medium. The cultures were incubated at 23 °C ± 1 °C with a 16 h photoperiod. Subcultures were done after 30 d intervals. Nodal segments from the proliferated shoots were subcultures for further multiplication of shoots. The regenerated multiple shoots were cut, and individual shoots were placed in semi-solid MS medium supplemented with 1 mg kg⁻¹ Kinetin.

2.3 Stevia shoots acclimatization in the liquid medium

S. rebaudiana shoots in the solid medium were sub-cultured into thin layer liquid medium culture aseptically. Approximately 10 shoots of stevia were cultivated in 5 mL of medium and incubated on a shaker and shaken at 40 rpm at room temperature (25 °C) for 3 d, under 2 000 lx to 2 500 lx ordinary white fluorescent lamp for 16/8 h daily photoperiod.

2.4 Shoot culture in RITA® bioreactor

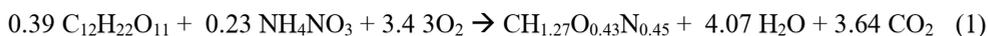
Shoots were selected from thin layer liquid medium culture and ready to be cultivated in 250 mL media. Bioreactors were assembled and the airflow was adjusted by an automatic timer with two variations, which gave two different immersion periods: (i) 15 min immersion time; (ii) 30 min immersion time, scheduled every 6 h.

2.5 Plantlet and medium analysis

After 21 d of cultivation, plantlets were harvested, counted (number of shoots and leaves), weighed, and then dried to obtain data of the dry weight data. Final sucrose content and conductivity of the culture medium were also tested. The conductivity was measured by using a conductivity meter (Eutech Instruments Con-110) and the sucrose content in the medium was measured by using a refractometer (Milwaukee MA871).

2.6 Data analysis

Data of dry weight were transferred into the growth curve. Data of sucrose content and conductivity of medium were used to construct a mass distribution model of *Stevia rebaudiana* shoot culture in TIS RITA® bioreactor. The mass balance modeling was based on the following reaction in Formula (1) [11]:



The growth and sucrose consumption curves were developed based on the following Equation (2) to Equation (4):

a. Growth kinetics
Logistic equation [10]:

$$\frac{d}{dt} [X] = \mu X \left(1 - \frac{X}{X_{max}}\right) \quad (2)$$

$$\mu = \mu_{max} * \left(\frac{[C_{12}H_{22}O_{11}]}{k_{C_{12}H_{22}O_{11}} + [C_{12}H_{22}O_{11}]} \right) \quad (3)$$

b. Sucrose consumption [12]:

$$\begin{aligned} \frac{d}{dt} [C_{12}H_{22}O_{11}] &= -q_{C_{12}H_{22}O_{11}} * X \\ q_{C_{12}H_{22}O_{11}} &= \frac{\mu}{Y_{x/C_{12}H_{22}O_{11}}} \\ Y_{x/C_{12}H_{22}O_{11}} &= \Delta X / \Delta [C_{12}H_{22}O_{11}] \end{aligned} \quad (4)$$

S. rebaudiana productivity in RITA® system was determined by dividing the addition of dry biomass weight (g) with the volume of medium (L) and cultivation time (d).

3 Results and discussions

The effect of various PGRs on the in vitro growth of *S. rebaudiana* was evaluated in terms of shoot number and leaf number (Figure 1 and Table 1). Kinetin 2 mg kg⁻¹ produced the highest number of shoot multiplication but not followed with the highest multiplication of stevia leaves. PGR BAP produced high multiplication of shoots and leaves but the explants morphology was not normal and not healthy. The explants cultured with the addition of BAP had small brownish stems (Figure 1 (C–E)). BAP is widely used to increase the number of shoots in stevia multiplication [13]. However, a large number of shoots in the BAP medium showed abnormal phenotypes such as brownish and non-vigor stems. Meanwhile, 1.0 mg kg⁻¹ Kinetin showed quite a high number of shoots and leaves multiplication with vigor stem and normal looking leaves. Kinetin 1 mg kg⁻¹ was decided to become the PGR used in further experiment.

Table 1. Differences in the number of stevia shoots and leaves in various types of PGR.

PGR	∑ Number of Shoots ± SD	∑ Number of Leaves ± SD	Visual Description
1 mg kg ⁻¹ Kinetin	46 ± 2.243	195 ± 4.219	Greenish stems and leaves
2 mg kg ⁻¹ Kinetin	70 ± 2.360	156 ± 2.984	Greenish stems and leaves
0.5 mg kg ⁻¹ BAP	64 ± 1.569	201 ± 3.087	Brownish stems
0.6 mg kg ⁻¹ BAP	48 ± 1.621	210 ± 5.368	Brownish stems
0.7 mg kg ⁻¹ BAP	48 ± 1.581	154 ± 3.399	Brownish stems



Fig. 1. Growth of *Stevia* shoots cultivated in a solid medium with different concentrations of PGRs: (A) 1 mg kg⁻¹ Kinectin, (B) 2 mg kg⁻¹ Kinectin, (C) 0.5 mg kg⁻¹ BAP, (D) 0.6 mg kg⁻¹ BAP, and (E) 0.7 mg kg⁻¹ BAP.

The result of the initiation process showed that 70 shoots meristem (either apical shoots or lateral shoots) could proliferate into 243 shoots with 89.74 % percentage of success. Multiplication percentage of initiation reached 352.86 %, or in other words, one explant could proliferate into three new shoots. The subculture phase showed that 247 shoots could proliferate into 610 shoots with a success rate of 98.34 %. Multiplication percentage of subculture was 246.96 %, or in other words, an average of one shoot could proliferate into two new shoots. These shoots were then acclimatized in thin layer culture.

S. rebaudiana shoots were then cultivated in RITA[®] bioreactor for 3 wk. According to Table 2, RITA[®] 30 (intensity periods for 30 min) gave the highest multiplication of stevia shoots compared with RITA[®] 15 (intensity periods for 15 min), with an average percentage increase of shoots for RITA[®] 30 was 89.71 % ± 5.83 %, while RITA[®] 15 only 76.90 % ± 4.85 %. Percentage of leaf growth in RITA[®] 30 reached 85.24 % ± 5.99 % while in RITA[®] 15 only 79.73 % ± 7.76 %.

Table 2. The increasing number of shoots and leaves produced bioreactor RITA[®] with immersion periods 15 min and 30 min

TIS RITA [®]	Average Initial Number of Shoots ± SD	Average Number of Shoots Produced ± SD	Increasing percentage number of shoots ± SD	Average Initial Number of Leaves ± SD	Average Number of Leaves Produced ± SD	Increasing percentage number of leaves ± SD
15	1.53 ± 0.46	6.55 ± 0.64	76.90 ± 4.85	3.93 ± 1.31	19.58 ± 1.02	79.73 ± 7.76
30	1.30 ± 0.02	12.61 ± 1.88	89.71 ± 5.83	3.15 ± 0.22	21.37 ± 1.04	85.24 ± 5.99

TIS RITA[®] 30 did not only produce the highest number of shoots and leaves, but this system also gave a better culture quality (Figure 2C). After incubation of 21 d in the bioreactor, the stems in TIS RITA[®] 15 were purplish-brown and the leaves turned to yellow (Figure 2B). This symptom developed presumably due to RITA[®] 15 had shorter and less frequent immersion regime than RITA[®] 30, as they reduce nutrient and water uptake and consequently limited the rate of nitrogen uptake.

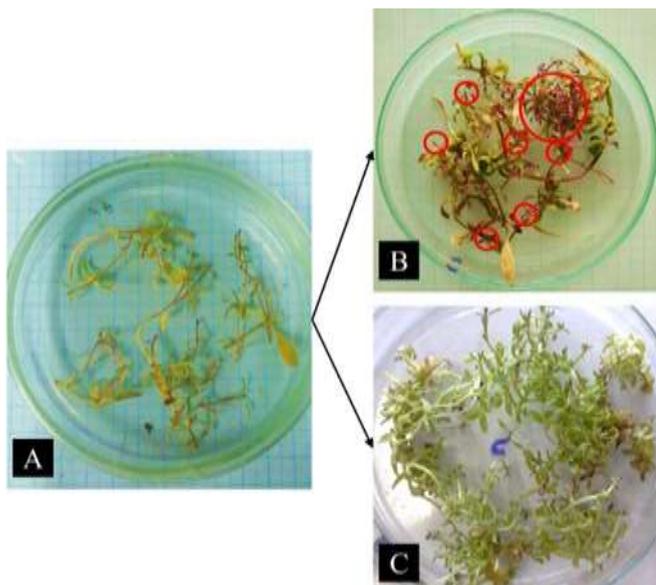


Fig. 2. Stevia shoots before and after cultivated in TIS RITA® bioreactors: (A) Day 0 for RITA® 15 min and 30 min; (B) Day 21 for RITA® 15 min; and (C) Day 21 for RITA® 30 min.

Nitrogen is the mineral element that plants require in greatest amounts. It serves as a constituent of many plant cell components, including amino acids and nucleic acids. Therefore, nitrogen deficiency rapidly inhibits plant growth. If such a deficiency persists, most species show chlorosis (yellowing of the leaves), especially in the older leaves near the base of the plant) [14]. Under severe nitrogen deficiency, these leaves become completely yellow (or tan) and died. Younger leaves may not show these symptoms initially because nitrogen can be mobilized from older leaves. Thus a nitrogen-deficient plant may have light green upper leaves and yellow or tan lower leaves [15].

When nitrogen deficiency develops slowly, plants may have markedly slender and often woody stems. This woodiness may be due to a buildup of excess carbohydrates that cannot be used in the synthesis of amino acids or other nitrogen compounds. Carbohydrates which not used in nitrogen metabolism may also be used in anthocyanin synthesis, leading to the accumulation of pigment. This condition is revealed as a purple coloration in leaves, petioles, and stems [16].

Table 3. The increasing number of shoots and leaves produced bioreactor RITA® with immersion periods 15 min and 30 min

TIS RITA®	Initial mass (g) ± SD		Final mass (g) ± SD		Growth rate (g DW d ⁻¹)	Productivity (g. L medium ⁻¹ d ⁻¹)	Doubling time (d)
	Fresh weight	Dry weight	Fresh weight	Dry weight			
15	0.950 ± 0.071	0.140 ± 0.014	2.490 ± 0.354	0.355 ± 0.007	0.045 ± 0.004	0.041	15.275 ± 1.495
30	0.900 ± 0.000	0.077 ± 0.005	3.115 ± 0.064	0.425 ± 0.021	0.081 ± 0.001	0.066	0.573 ± 8.551

The growth rate of *S.rebaudiana* cultivated in RITA® 30 min was greater than RITA® 15 min. Results in Table 3, showed that the growth rate of *S. rebaudiana* in RITA® 30 min

was $0.081 \text{ g} \pm 0.001 \text{ g DW d}^{-1}$ with doubling time (dt) $0.573 \text{ d} \pm 8.551 \text{ d}$ while in RITA[®] 15 min was $0.045 \text{ g} \pm 0.001 \text{ g DW d}^{-1}$ with a dt $15.275 \text{ d} \pm 1.495 \text{ d}$. The result in Table 3 also indicated two different growth patterns in shoot culture. According to Mordocco et al. [17], the immersion periods in RITA[®] system can determine the productivity of the systems and it has been proven by the increasing number of shoots of *Grandiflora × amelanchier* Rehder (serviceberry) up to 2.6 times with immersion 5 min every 60 min compared with 5 min every 30 min. The observation of the culture in RITA[®] bioreactor showed that the growth culture for RITA[®] 30 system was better than RITA[®] 15. The immersion periods is one of the critical parameters which can influence the growth of the culture, since the period of immersion related to the length of contact between the culture and medium. These conditions determine the absorption of nutrients by explants.

Conductivity value indicated the number of ions and dissolved salts contained in the bioreactor medium. The study stated that the decrease conductivity level in the culture medium showed the consumption of mineral compounds and inorganic compounds by cells [18], such as NH_4^+ , NO_3^- , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , SO_4^{2-} , and PO_4^{3-} . All treated culture consumed minerals contained in the medium, therefore the conductivity of two bioreactors systems generally decreased. However, consumption of mineral on RITA[®] 30 ($2.925 \text{ mS} \pm 0.033 \text{ mS}$) was relatively higher compared to RITA[®] 15 ($2.520 \text{ mS} \pm 0.014 \text{ mS}$). Consumption of mineral compounds will be directly proportional to the addition of biomass. The higher the consumption of mineral and inorganic compounds, the higher the increase of biomass [19].

The sucrose consumption rate of RITA[®] 30 was higher when compared to RITA[®] 15. RITA[®] 30 had consumption rate of $0.184 \text{ g L}^{-1} \text{ d}^{-1}$ while RITA[®] 15 was only $0.092 \text{ g L}^{-1} \text{ d}^{-1}$. The decrease in mass of sucrose at RITA[®] 15 system amounted to only 5.89 %, whereas in RITA[®] 30 was 11.66 %. The rate of sugar consumption would correlate with the rate of biomass enhancement. Sucrose is used as a carbon source for the cells, both for the structure and metabolites, so the addition of biomass needs more of sucrose. Conversely the lower the consumption of its substrate (sucrose), then the lower the enhancement of biomass [19].

Tables 4 and Table 5 showed the mass balance of culture in RITA[®] 15 and 30. The table showed a hypothetical amount of biomass and the value was different from the actual amount of biomass obtained from the measurement of plantlets weight. The hypothetical amount of biomass in RITA[®] 15 was 0.498 g while the actual was $0.355 \text{ g} \pm 0.007 \text{ g}$. The hypothetical amount of biomass in RITA[®] 30 was 0.023 g while the actual was $0.426 \text{ g} \pm 0.021 \text{ g}$. This imbalance might have resulted from the use of the root biomass formula of *Atropa belladonna* Linn. for the shoot biomass formula of *S. rebaudiana*. Each cell had a different and unique substrate consumption pattern and biomass formation pattern. Another factor that might influence the construction of a mass balance model was the limiting substrate in the reaction, as this was not examined in this study. Knowledge of the limiting substrate will be very helpful in validating the model that has been built.

Table 4. Culture mass balance in TIS RITA[®] 15 system

Reaction Equation	0.39 C ₁₂ H ₂₂ O ₁₁	0.23 NH ₄ NO ₃	3.43 O ₂	CH _{1.27} O _{0.43} N _{0.45}	4.07 H ₂ O	3.64 CO ₂
Beginning (mol)	0.096	-	-	0.004	-	-
Reaction (mol)	0.006	0.003	0.049	0.014	0.058	0.052
Final (mol)	0.090	0.000	-	0.019	0.058	0.052
Final (g)	30.788	-	-	0.498	1.050	2.296

Table 5. Culture mass balance in TIS RITA® 30 system

Reaction Equation	0.39 C ₁₂ H ₂₂ O ₁₁	0.23 NH ₄ NO ₃	3.43 O ₂	CH _{1.27} O _{0.43} N _{0.45}	4.07 H ₂ O	3.64 CO ₂
Beginning (mol)	0.096			0.003		
Reaction (mol)	0.011	0.003	0.004	0.001	0.001	0.001
Final (mol)	0.084			0.001	0.001	0.001
Final (g)	28.861			0.023	0.011	0.064

4 Conclusions

In vitro propagation of *S. rebaudiana* cultured in TIS RITA® bioreactors was potential to be developed as an efficient method of its shoot production. Furthermore, the period of culture immersion was a critical parameter that affected the growth of stevia culture in TIS bioreactor. Immersion of 30 min in medium was an optimal immersion period for growth of the *S. rebaudiana* shoots compared with 15 min immersion. Comparison between the percentage of shoot and leaf growth, growth rate, biomass acquisition, as well as productivity of *S. rebaudiana* with the consumption of sucrose and nutrition showed that the RITA® culture system with 30 min immersion period gave better results of bioconversion medium to become biomass than 15 min immersion period every 6 h.

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