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Evaluation of *Stevia rebaudiana* Leaf-Axillary Shoot Formation, Cultured in MS Medium Supplemented with IAA-BAP and MS Medium Supplemented with Kinetin

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Abstract

Stevia rebaudiana leaves can be used as a sweetener alternatives because they contain steviol glycoside derivative compounds, including steviosides and rebaudioside-A. Propagation of *Stevia* is more optimally carried out using *in vitro* culture when compared to conventional propagation through seeds or cuttings. This study aimed to evaluate the formation and growth of *Stevia* shoots and leaves in MS medium containing a mixture of IAA and BAP with MS medium containing kinetin only, as well as evaluating the use of a liquid medium containing kinetin. *Stevia* was initiated from apical shoot then grown in MS medium containing a mixture of IAA and BAP with MS medium containing kinetin only. *Stevia* was subcultured every 4 weeks. Several parameters measured were number of axillary shoots and number of leaves. It was transferred into a liquid medium for 7 days. The results showed that the formation and growth of axillary buds and leaves at the initiation stage were better in medium containing IAA and BAP compared to medium containing single hormone kinetin. At the stage of shoot multiplication and maintenance, cultivation in semi-solid medium containing kinetin showed more leaves and axillary shoots compared to that cultivated in semi-solid medium with the addition of IAA and BAP. Plants acclimatized in liquid medium supplemented with 1 ppm kinetin showed fast plant growth but were not accompanied by sturdy stem growth. The presence of brownish color on certain parts of the plant such as in some leaves and stems was also observed.

Keywords: *axillary shoot, BAP, IAA, kinetin,* Stevia rebaudiana

1. Introduction

Diabetes is one of the biggest health problems in the world. In 2017, it was estimated that around 4 million people died from diabetes and its complications [1]. The main cause of diabetes is the unhealthy lifestyle of modern society, including consuming too much high-energy dietary [1]. *Stevia rebaudiana* is one of the plants that has a great potential as a producer of alternative sweetener compounds. The leaves of this plant produce steviol glycoside compounds which are known to be about 250 to 300 times sweeter than sucrose [2]. In addition, *Stevia* extract can replace the sugar and be marketed as a non-caloric sweetener. The most abundant steviol glycosides are stevioside and rebaudioside-A. Since 1995, many of food industries used *Stevia* extracts to sweeten their food products, such as in Japan, Brazil, and other countries [3].

One of the problems in the regeneration of *Stevia* is the non-uniform quality of seed. Regeneration of *Stevia* by *in vivo* production is not an ideal propagation method because the germination rate is low and the variety of offspring is high [4]. Therefore, to overcome that problem, in this study, *Stevia* propagation was developed using tissue culture technique.

Tissue culture or micropropagation is a method of plant propagation using an aseptic culture of cells, tissues, organs, and their components under in vitro aseptic conditions with predetermined chemical and physical conditions [5]. The principle of tissue culture is the totipotency of plants, where plant cells can grow into new individuals identical to their parents. Tissue culture has many advantages over conventional plant propagation methods. With this method, large numbers of new plants can be produced in a short time and without requiring a lot of space. The properties of the tillers produced by the

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tissue culture method will also resemble the properties of the parents. Plants resulting from micropropagation can also be given improved characteristics through manipulation of physical and chemical conditions, such as diseases-free plant and more secondary metabolites production [6].

In vitro culture of *Stevia* has been carried out by several researchers [7, 8]. Previous studies used a different composition of plant growth regulators (PGRs) to produce optimal growth, including 1.13 ppm BAP and 0.35 ppm IAA in the study by Sumaryono and Sinta [8] and 1 ppm kinetin in the study by Melviana et al. [7]. The purposes of this study were to evaluate the formation and growth of shoots and leaves of Stevia in semi solid medium containing a mixture of IAA and BAP or kinetin solely, and to evaluate the use of a liquid medium containing kinetin for shoot maturation.

2. Methodology

2.1 Stevia Explant

Explants were obtained from the Indonesian Center for Biotechnology and Bioindustry Research, Bogor, West Java. Apical shoot containing three nodes from 4 weeks-old of *S. rebaudiana* plants, with a height about 30-50 cm, were used as an explant.

2.2 Shoot Initiation

Stevia shoots (Figure 1) were washed in running tap water for 3 minutes, soaked in a solution containing 0.5 % AntracolTM fungicide, then rinsed twice with distilled water and dried using filter paper. Explants were then sterilized using 70% alcohol, followed by 0,27% (v/v) NaClO of commercial bleach solution added with 2 drops of Tween-20 for 5 minutes. Subsequently, the explants were rinsed three times with sterile distilled water and dried using sterilized filter paper. The explants were then transferred into a petri dish lined with sterile filter paper.

Explants were then cultured in semi-solid Murashige-Skoog (MS) medium [9] containing 30 g/L sucrose and 8 g/L agar, supplemented with 0.35 ppm IAA and 1.13 ppm BAP or 1 ppm kinetin solely. The pH of the medium was adjusted to 5.6 to 5.8. The cultures were then incubated in room temperature under 12/12 hours photoperiod and light and light intensity 1000 lux using 36-watt TLD lamps. Subculture was carried out three times in every 4 weeks using similar initiation medium. The new regenerated shoots were separated and transferred into new medium during subculture.

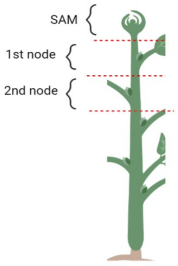

Figure 1. Explant used in this research

2.3 Shoot culture in liquid medium

Regenerated shoot from semi-solid medium were transferred into liquid medium, which consisted of half-strength MS medium, supplemented with 20 g/L sucrose and 1 ppm kinetin. The pH of the medium was adjusted to 5.6 to 5.8. The culture was then incubated on 40-rpm shaker in room temperature with 12/12 photoperiod.

3. Result and Discussion

3.1 Stevia Initiation

At the initiation stage, the medium containing IAA and BAP produced leaves and more axillary shoots, which were developed from the explant's nodes, compared to kinetin (Figures 2 A and B). Explants growing in IAA and BAP has regenerated leaves at 2 weeks after initiation (Figure 3A), meanwhile explants in medium containing kinetin did not show any growth, and some explants indicated yellowing (Figure 3B).

Shoot initiation stage usually need combination of auxin and cytokinin to regenerate new axillary buds. Auxins and cytokinins influence organ regeneration by controlling cell differentiation [7]. Auxins will trigger cell elongation and cell growth, while cytokinins stimulate cell division and shoot formation in culture. At the initiation stage, therefore, concentration of cytokinin in growth medium was usually higher than auxin in order for the explant can grow more shoots [10, 11].

Two weeks after subculture, the formation of axillary shoots and leaves in the medium containing IAA and BAP was slower and less than the number of axillary shoots and leaves regenerated in medium containing kinetin (Figure 4A). During the rest of the subculturing period, faster axillary shoot regeneration occurred in medium containing IAA and BAP. At 4 weeks after subculture, therefore, the number of axillary shoots in both media were same. Leaf formation in medium containing IAA and BAP was slower than in medium containing kinetin. Number of leaves in those medium, therefore, were fewer compared to the leaves in medium containing kinetin. Longer internodes were observed in medium containing kinetin (Figures 5A and B).

The addition of kinetin to Stevia results in higher number of leaves and internodes compared to other PGRs such as IAA and BAP. The higher number of internodes can produce more explant when it is sub-cultured. The higher number of leaves can produce more steviol glycosides in *Stevia*. Kinetin is a cytokinin-type growth regulator, which can increase shoot propagation and cell division or plant biomass and plant cell differentiation [12]. Giving PGR kinetin as much as 1 ppm was the best concentration for the growth of Stevia shoot. Melviana et al. [7] showed that the addition of 1 ppm kinetin to the semi-solid culture of *Stevia* was able to produce a relatively high number of shoot and leaf multiplication compared to other PGRs, and the resulting plantlets were

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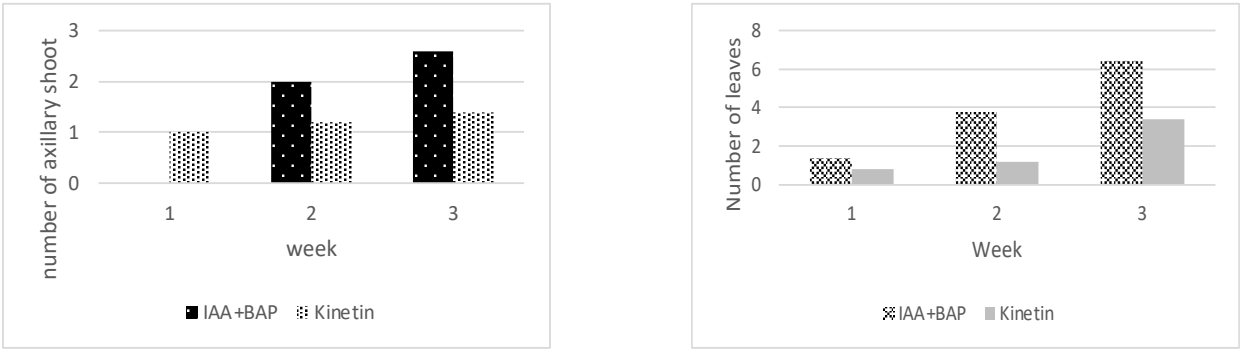
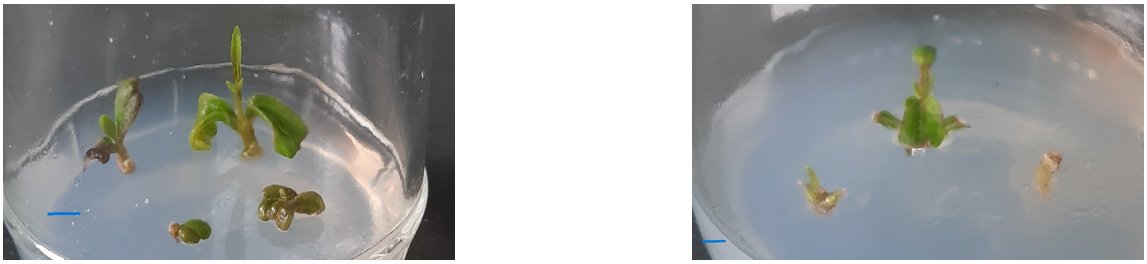
also green, indicating healthy plant growth. Similar result was shown in *Hypericum spectabile* where the highest number of shoot buds was produced in medium containing 1 ppm kinetin. Nevertheless, medium containing BAP was faster to produce shoot bud and leaves than kinetin. Based on Muhammad et al. [16], multiplication rate of shoot bud development was dependent upon cytokinin type, its concentration, and medium used.



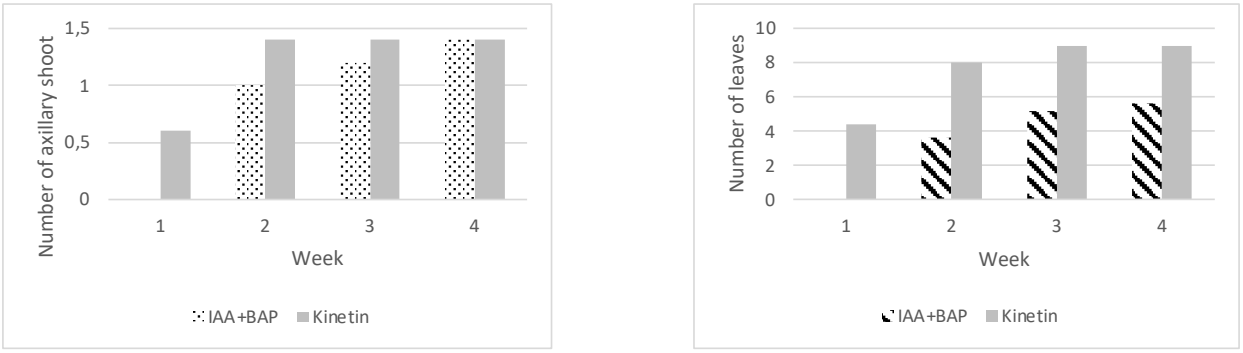
(A) (B)

Figure 2. (A) Number of axillary shoots and (B) Number of leaves at 3 weeks after initiation stage



(A) (B)

Figure 3. (A) *Stevia* culture on IAA and BAP medium and (B) *Stevia* culture on kinetin medium at 2 weeks after initiation. Scale bar indicated 1 cm.



(A) (B)

Figure 4. Number of axillary shoots and (B) number of leaves at 4 weeks after multiplication stage

3.2. Culture on Liquid Medium

Shoot maturation was conducted to strengthen the shoot for further research which included plantlet regeneration and multiplication in bioreactor as well as for increasing secondary metabolite content in plantlets. After the explants were propagated in sufficient quantities, the culture was transferred to MS half-strength liquid medium with 1 ppm kinetin PGR for 7 days [7]. In Figure 6, it can be observed that the cultured plants were brown in some parts. The browning can be caused by stress on plants such as changes in temperature, osmotic pressure, or other changes [13]. These changes can lead to enzymatic oxidation reactions of phenolic compounds that can produce quinones that are brownish-yellow in colour. Quinone compounds can damage plant tissues causing stunted plant growth [11,14]. In this study, stress occurred in plants due to continuous immersion in plants previously cultivated in solid media which was causing the browning and yellowing of tissues.

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(A) (B)

Figure 5. (A) Shoot culture in IAA and BAP medium; (B) Shoot culture in kinetin medium. Scale bar indicated 1 cm.

Figure 6. *Stevia* after being cultured in liquid medium. Circle = brown leaf

4. Conclusion

At the initiation stage, the shoot culture of *Stevia* cultivated in a semi-solid medium containing IAA and BAP showed good growth such as higher leaf number and axillary shoots, while at the shoot multiplication and maintenance stage, cultivation on semi-solid medium with the addition of kinetin showed good growth such as higher leaf number and axillary shoots compared to cultivation in semi-solid medium with the addition of IAA and BAP. Acclimatized plants cultured in a liquid medium with the addition of 1 ppm kinetin showed fairly fast plant growth but was not accompanied by strong stem growth and the presence of brown colour in certain plant parts such as some leaves and stems.

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