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In Vitro Regeneration of *Stevia Rebaudiana* Bertoni from internode and leaf explants using different concentrations of BAP (6-Benzyl Amino Purine)

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Abstract. The aim of this study was to determine the response of the growth of internode explants and leaves of Stevia (*Stevia Rebaudiana* Bertoni), which were cultured at several BAP concentrations. This study was designed using a non Factorial Complete Randomized Design that is by testing the internode and leaf explants at 3 different BAP concentration levels of 2, 3 and 4 ppm and will be repeated 10 times. The parameters observed related to callus growth include: emergence, type, color and percentage of callus formation. In addition, observations were also made on the parameters of bud growth including the appearance of shoots, number and length of shoots. The results of this study indicate that 100% of leaf and internode explants are able to form callus. The color of the callus explants on average leaves are yellowish green while the internode explants are yellowish white and all have compact textures. In shoot growth parameters, only internode explants were able to grow shoots while leaf explants were not. The emergence of shoots (3 DAP), number of shoots (8.70), and shoot length (2.81cm) were produced by MS media with the addition of 3ppm BAP.

1. Introduction

Stevia (*Stevia rebaudiana* Bertoni) is a natural sweetener plant which has a sweet taste reaching 200-300 times higher than sugar [1]. The sweet taste produced from stevia plants comes from the content of steviosida and rebaudiosida [2].Sweeteners from stevia are also low in calories [3] and contain high antioxidants, so it is also good for diabetics. The use of stevia can be mixed with food or drinks such as tea and coffee. Therefore Stevia plant has the potential to be developed on a large scale in Indonesia [4].

Plant propagation of Stevia can be done through the method of multiplication of shoots in vitro. Through this method, the resulting shoots are more uniform and can produce large quantities of seedlings in a relatively short time [5]. Technically, the successful multiplication of shoots in vitro is influenced by several factors such as the use of appropriate growth regulators and also the type of explants used. From some previous studies, the use of explant types can also affect the rate of bud formation [6]. Besides the use of growth regulators cytokinins, especially BAP (6-*Benzyl Amino Pourine*) also proved able to stimulate the formation of shoots in vitro stevia [7], [8]. In previous studies researchers have also tested using several types of cytokines and proven the use of BAP is more influential on budding formation than other types of cytokinins, but have not found the optimal BAP concentration and the type of explants that are appropriate to further stimulate multiplication [9].

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Therefore, further testing is needed in the use of BAP from different types of explants to find out what is the optimal BAP concentration and the right type of explant for the formation of stevia shoots.

2. Methods

2.1Collection of plant material

The plants were collected from green house of Plant Tissue Culture Laboratory, Department of Agricultural Production, Politeknik Negeri Jember-Indonesia.

2.2 Surface sterilization

Stem explants were washed thoroughly under running tap water, followed by treatment with 2g.L^{-1} bactericide and fungicide for 15 min. Then, the explants were sterilized in 70% ethanol for a minute, and finally with 5% Clorox for 2 min and washed 5 times with sterile distilled water.

2.3Culture medium

MS medium containing 3% sucrose, gelled with 0.7% agar and 0.25 mg.L^{-1} IAA supplementation is used as basic media in this study. In addition, different concentration of BAP (2, 3 and 4 ppm) is added as treatment .

2.4 Sub culturing

Stem explant were cultured are cultured on MS 0 media first for 2 weeks. Then new stem and leaves that appear as planting material on the treatment media for 4 weeks.

2.5 Parameters and data analysis

This research tested the response of growing leaf explants and internodes at different BAP concentrations using a non factorial complete randomized design (CRD) with 10 replications. The data obtained were analyzed by Analysis of Variance (ANOVA) and DMRT test at ($p<0.05$). The parameters observed related to callus growth include: emergence, type, color and percentage of callus formation. Besides the parameters related to the growth of shoots that include emergence, number and length of shoots.

3. Results and discussion

3.1 Percentage of callus and shoot formation

Observation of the percentage of shoot and callus formation was carried out at the end of the research activity (30 DAP).The observations showed that all leaf and segment explants were able to form a callus with a percentage of 100%. In leaf explants, callus is formed on the entire surface of explants, whereas in internal explants, callus is formed at the base of the stem.

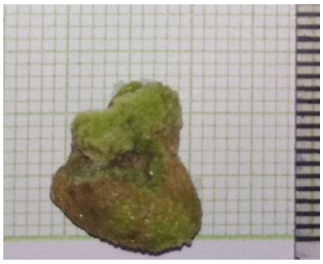

Figure 1. Callus formation in leaf explant

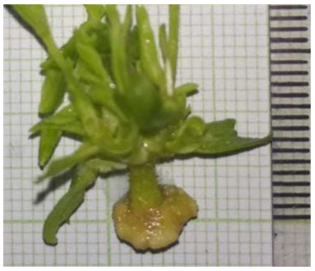

Figure 2. Callus is formed on the base of internode (wounding area)

Callus formation is preceded by cell division and enlargement by auxin hormones and exogenous cytokinins [10].. The addition of BAP at concentrations of 2, 3, and 4 ppm combined with 0.25 ppm IAA on the media proved capable of stimulating cell proliferation and then forming callus. Experiments using the hypocotyl explant of *Physalis angulata* L. plant showed that the combination of BAP and several types of auxin stimulated the formation of compact textured callus [11].

3.2 Day of callus emergence, callus type and color

Table 1. Data on callus emergence, callus type and color from internodes and leaf explants at several levels concentration of BAP.

Explant Type	BAP Level	Parameters		
		Day of callus emergence (DAP)	Callus Type	Callus Color
Internode	2 ppm	20,70 a	Compact	yellowish white
	3 ppm	19,50 a	Compact	yellowish white
	4 ppm	22,00 b	Compact	yellowish white
Leaf	2 ppm	9,20 a	Compact	greenish yellow
	3ppm	4,20 b	Compact	greenish yellow
	4 ppm	3,00 b	Compact	greenish yellow

The number followed by the same letter are not significantly different at ($p<0.05$) level of Duncan's test.

The results of the analysis using ANOVA, show that the day of callus appearance on internode explants and leaves from several BAP concentrations showed very significant different results. In internode explants, the fastest callus appearance was at 19.5 DAP at a concentration of 3 ppm BAP. Whereas in leaf explants, the appearance of callus started from 3 DAP at a concentration of 4 ppm, and was not significantly different from the concentration of 3 ppm. In both explants tested, the slowest response in the emergence of shoots was at a concentration of 2 ppm BAP **(Table 1).**

The appearance of callus in explode internode was faster on the media with the addition of 3 ppm BAP. Allegedly the role of the concentration of 3 ppm spurs cell proliferation from the base of explants in contact with the media. Previous studies on the *Mucuna pruriens* L. concentration of 3 ppm BAP were able to stimulate callus heavier than at other concentrations. Scaling at the base of the explant can also occur in the area where the cuttings are opening, which is the initial preparation for root formation [12].

In leaf explants **(Table 1),** the fastest callus appears at 3-4 DAP. It also showed that the callus from the leaves appeared faster than the formation of internode explant callus. The fastest appearance of callus was also seen at a concentration of 4 ppm BAP and was not significantly different from 3 ppm BAP. In the process of callus formation, cytokines play a role in stimulating cell proliferation. Cytokinins drive the G2 phase to the M phase so that there is an increase in the rate of protein synthesis and eventually the cell divides more quickly and there is an accumulation of cells called callus [13].

The type of callus formed from explants internodes and leaf explants is compact callus. This callus is hard-textured and forms lumps. Whereas in the callus color parameters, the two explants have different color effects. Scoring data show that on explants internodes the average callus is yellowish white, whereas the average leaf explant is greenish yellow **(Figure 1 and 2).** The compact callus texture has cells that bond together more densely and are difficult to separate. The use of cytokines can stimulate cells to be compact and green. The water potential in the cells formed is influenced by the

balance of cytokinins. Water, nutrients and sucrose from diffuse media in cells causes an increase in cell osmotic pressure and impacts on cell walls becoming stiff [5], [14].

The color of the callus at the base of the internodes differs from that of the greenish yellow leaves. Callus formed from leaves can indicate that the green color is chlorophyll found in leaf tissue. The presence of cytokinin in the treatment media also stimulates the formation of chlorophyll from the callus that is formed so that it is greenish yellow [15].

3.3 Day of shoots emergence, shoots number and length

Table 2. Data on shoots emergence, shoots number and length from internodes explants at several levels concentration of BAP.

Explant Type	BAP Level	Parameters		
		Day of shoots emergence (DAP)	Shoots number	Shoots length
Internode	2 ppm	3,20 a	4,70 a	2,53 a
	3 ppm	3,50 a	8,70 b	2,81 a
	4 ppm	3,00 a	5,80 a	1,61 b

Figure 2. *Stevia rebaudiana* Bertoni planlet from internode explant (30 DAP) grown on (a) MS+0,25 ppm IAA+2 ppm BAP; (b) MS+0,25 ppm IAA + 3 ppm BAP; (c) MS+0,25 ppm IAA+ 4 ppm BAP

The number followed by the same letter are not significantly different at ($p<0.05$) level of Duncan's test.

Anova analysis results on the emergence of shoots showed no significant different effect, while the number and length of the shoot parameters BAP concentration level had a very significant effect. In this study, shoot formation only existed in explants internodes only **(Figure 2.)**, whereas in leaf explants no shoots grew, only callus was formed.

From several BAP concentrations tested, the average shoots appeared at 3 DAP. But at the end of the observation (30 DAP) there are differences in the number and length of shoots. At a concentration of 3 ppm, 8.7 buds were formed. Whereas at concentrations of 2 and 4 ppm only 4.7 and 5.8 buds were formed. These results indicate that the concentration of 3 ppm is the optimal concentration in driving the formation of stevia through explant internode. In several previous studies, the use of internode stevia as an explant with the addition of the BAP hormone further spurred bud multiplication [16].

Observation data on shoot lengths in **Table 2** shows that BAP at concentrations of 2 and 3 ppm showed lengths that were not significantly different, but were very different from BAP concentrations of 4 ppm which had the shortest shoots. The length of shoots from 2 ppm concentration is 2.53 cm and 3 ppm is 2.81 ppm, while 4 ppm has the shortest shoots 1.61 cm. These data indicate that the higher the BAP concentration will suppress the elongation of the shoots. Conversely at lower BAP concentrations, elongation of the stem by the auxin hormone becomes stimulated. In other studies, the

combination of BAP and IAA growth regulators is best for the formation and elongation of stevia shoots [17]. In its function BAP stimulates the number of shoots while auxin stimulates stem elongation [18].

4. Conclusions

From this study it can be concluded that the use of BAP provides a different growth response between internode and leaf explants. Callus was able to appear in both explants but the fastest speed of callus appeared in leaf explants was 3 DAP. Internode explants have compact explants and yellowish white while leaf explants have compact greenish yellow callus. In shoot growth parameters, only internode explants can grow shoots, whereas leaf explants only grow callus. Concentration of 3 ppm BAP is able to grow more shoots, namely 8.7 shoots than other concentrations. For the parameters of shoot length, at a concentration of 3 ppm it also has the longest average shoot growth of 2.81 cm.

Acknowledgements

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