Annals of Tropical Research 43 (2):88-101(2021)
https://doi.org/10.32945/atr4328.2021

Influence of plant growth regulators and UV light exposure on the formation and phenolic content of *Stevia rebaudiana* Bertoni callus: A preliminary study

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ABSTRACT

Received: 4 September 2020 | Accepted: 29 April 2021

This study was conducted to evaluate the effect of different combination and levels of plant growth regulators, 2,4-dichlorophenoxy acetic acid (2,4-D) and 6-benzylaminopurine (BAP), on callus induction of *Stevia rebaudiana* Bertoni leaf and internode explants, and the total phenolic content of Stevia callus as influenced by the length of exposure to ultraviolet (UV) light radiation. Early callus initiation was recorded in leaf explants inoculated in Murashige and Skoog (MS) medium supplemented with 2,4-D and BAP. After a week of incubation, leaf explants showed callus formation while in internode explants, callus formation was observed 2 weeks after inoculation. The culture medium supplemented with 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4) was the best treatment for leaf explants with 95% callus formation and the addition of 1.0mg L^{-1} 2,4-D + 4.0mg L^{-1} BAP (T_3) was the best treatment for internode explant with 90% callus formation. After 4 weeks of incubation, the leaf and internode calli were observed as compact, non-embryogenic, and yellowish green in all treatments except those inoculated in MS medium alone (control). Stevia leaf callus was subjected to UV radiation after 4 weeks at varying time of exposure. The exposed calli turned brown signifying a possible increasing production of secondary metabolites. Furthermore, combination of 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4) and 3h of UV light exposure yielded the highest phenolic content of 87.71mg GAE per g callus. The results further revealed that both medium composition and time of exposure affect the production of phenolic content of Stevia leaf callus.

Keywords: *Stevia rebaudiana* Bertoni, Phenolic Content, UV light exposure, Callus

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INTRODUCTION

Stevia rebaudiana Bertoni or Stevia is a perennial herb of Asteraceae family and is commonly known as the "sweet leaf", "sugar leaf" or "candy leaf". Stevia is native in Paraguay and Brazil but is also widely grown in the South America and East Asia due to its natural sweet content. It is a sweetener of drinks, of various foods, and of traditional medicines -- the reason why it was called as the "sweet herb of Paraguay". The natural sweet properties of Stevia are characterized by the chemical component of diterpene glycoside which includes primarily the stevioside, steviolside, dulcoside and rebaudioside A to E (Gupta et al 2013). Almost all parts of the plant have the sweet taste; however, the sweet glycosides are typically concentrated in the green serrated leaves. Steviol glycosides, stevioside and rebaudiana A, are the most abundant chemical compounds that can be found in the Stevia leaves. These compounds are 300 times sweeter than the cane sugar (Goyal and Goyal 2010), Basically, these metabolized compounds leave the body without accumulation. Other than its value as sweeteners, Stevia and its glycosides have pharmacological effects against several diseases such as cancer, diabetes mellitus, hypertension, inflammation, cystic fibrosis, obesity, and tooth decay. Several studies have shown that steviol glycosides found in Stevia are not teratogenic, mutagenic or carcinogenic and cause no acute and subacute toxicity (Huang et al 2009). In addition, Stevia had shown antioxidant activity due its high content of phenolic compound compared to the other plant (Shukla et al 2009).

According to the US Food and Drug Administration in December 2008, FDA issued a letter declaring Stevia plant extract as generally recognized as safe (GRAS) for use in beverages, foods and sweeteners, and allowed its use in US food production. There is an increasing demand for phenolic compounds and other bioactive compounds of Stevia as important constituents for human diet and high potential therapeutic effects.

Recently, Stevia is one of the plants that is domesticated due to its high medicinal and commercial value; therefore, it is necessary to cultivate it for commercial purposes. However, the propagation of Stevia is laborious since the seed germination percentage is very low due to its self incompatibility, and its insect pollinated clear seeds are infertile (Debnath 2008, Raina et al 2013). The vegetative propagation is limited also since it has a low number of planting material that can be obtained in a single plant. As a result, it is difficult to reach the commercial quantities needed in the market. To address this problem, tissue culture technique is developed to produce large number of plant materials in a very short period of time for commercial purposes. In addition, the advanced propagation technique of Stevia to generate a higher level of steviol glycoside (Gupta et al 2010) and other valuable compounds of Stevia especially the phenolic compounds is being done. Several studies reported that Stevia plant parts such as leaves, nodes, and shoot tips can be used to raise plants in vitro (Naz and Hashmi 2008). In a similar study using a suspension culture, embryogenesis was achieved and the level of stevioside content was increased due to the addition of ammonium nitrate (Sharma et al 2015).

There are several reported methods to enhance the production of secondary metabolites. One of these methods is the UV light exposure which acts as an abiotic stress that can affect the physiological processes and morphology of the Stevia

and at the same time produce secondary metabolites as defense mechanism and adaptation to the stressful environmental condition.

The study aimed to evaluate the responses of Stevia leaves and internodes as explants to different combinations and levels of 2,4-dichlorophenoxy acetic acid (2,4-D) and 6-benzylaminopurine (BAP) on callus induction; establish the appropriate levels of 2-4 D and BAP for callus induction; and to determine the total phenolic content of Stevia callus as influenced by the length of exposure to ultraviolet (UV) light radiation.

MATERIALS AND METHODS

Collection of Plant Materials

The sources of the explants were obtained directly from a Stevia plant grown in the Ornamental Area of the Department of Horticulture, College of Agriculture and Food Science, Visayas State University, ViSCA, Baybay City, Leyte, Philippines. The mother plant was then transferred into the screen house for maintenance. The mother plant was pretreated with fungicide (Dithane) once-a-day for three (3) days prior to preparation of explants.

Figure 1. Stevia plant

Preparation of Leaf and Internode Explants

The freshly collected Stevia leaves and internodes were washed with liquid soap under running tap water and wiped dry with cotton. A 70% ethanol was used to clean the explants and to remove the trichomes where the contaminants were attached. Then, it was treated in 20% commercial bleach solution and decanted after 5mins of manual agitation. Finally, all explants were immersed in with 20% commercial bleach solution for further surface sterilization for 5mins inside the

laminar flow hood before rinsing thoroughly with sterilized distilled water three times. The leaves and internodes of Stevia were excised aseptically using a sterilized scalpel measuring 5mm for leaf explant and 10mm long for internode explants and inoculated in the sterilized culture vessels.

Culture Medium Preparation

All explants were cultured in a Murashige and Skoog (MS 1962) medium supplemented with 30g L^{-1} of sucrose and 5g L^{-1} of agar with the addition of different concentrations and combinations of plant growth regulators such as 2,4-dichlorophenoxy acetic acid (2,4-D) and benzylaminopurine (BAP). The pH was adjusted to 5.8 using 1.0N HCl or 1.0N NaOH prior to dispensing. The culture vessels containing 10mL of medium treatment were sterilized by autoclaving at 121°C at a pressure of 15psi for 20min. Cultures were incubated for 4 weeks at 25±2°C, under cool white fluorescent light having 2500 lux light intensity in an 8h light and 16h dark light cycle.

UV Light Exposure

After four weeks of culture incubation, the Stevia calli were exposed to UV radiation inside the laboratory using the laminar flow hood UV (UV-B) with exposure time of 1, 3, and 5h, for 4 days (Table 1). Each culture was placed diagonally for complete exposure of the callus to UV rays.

Table 1. Length of UV Light Exposure

Factor 1 (Treatment Code)	Length of Exposure to UV light (Hour per day)
E_0	Control (no exposure)
E_1	1
E_2	3
E_3	5

Preparation of the Callus Extract

After the Stevia calli were exposed to UV radiation, the control and the irradiated calli were then collected from the medium using forceps and weighed using analytical balance. One gram callus for each treatment was macerated and transferred to a tube with cover and added with 3mL pure methanol and incubated at room temperature for 24h free from light for complete extraction. The extract was filtered using the filter paper and 1mL of the filtrate was obtained and placed in the tube with cover and stored at 4°C for further analysis.

Determination of Total Phenols

Folin-Ciocalteu assay method was used for the determination of the total phenolic content (Kaur and Kapoor 2002). Gallic acid was used as standard. The reaction mixture consisted of 1mL of plant extract added with 5mL distilled

water, then mixed thoroughly with 0.5mL of Folin-Ciocalteu (1:10) reagent (Scharlau, Scharlab S.L.). After 5min, 1.5mL of 20% sodium carbonate solution was added and was allowed to stand for 60mins. The sample was measured at 650nm using UV-Vis Spectrophotometer (Shimadzu UV-1800) and the total phenolic content was calculated from the calibration curve and expressed as mg gallic acid per g of callus weight.

Treatments, Experimental Design and Statistical Analysis

The experiment was laid out in a 2-Factor Factorial Experiment in Completely Randomized Design (CRD) with three replicates per treatment and ten sample cultures per replicate (Table 2). The obtained data on total phenolic content was subjected to analysis of variance (ANOVA) to determine the significant differences among treatment combinations of UV light exposure and medium composition. Multiple comparison procedure was done with the use of the Tukey's Honest Significant Difference (HSD) Test for differences between means at 5% level of significance.

Table 2. Media Treatments for callus induction using leaf and internode explants of Stevia rebaudiana Bertoni

Factor 2 (Treatment Code)	Medium Composition
T_0	MS alone
T_1	MS + 1.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP
T_2	MS + 1.0 mg L^{-1} 2,4-D + 3.0 mg L^{-1} BAP
T_3	MS + 1.0 mg L^{-1} 2,4-D + 4.0 mg L^{-1} BAP
T_4	MS + 1.5 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP
T_5	MS + 1.5 mg L^{-1} 2,4-D + 3.0 mg L^{-1} BAP
T_6	MS + 1.5 mg L^{-1} 2,4-D + 4.0 mg L^{-1} BAP

*MS, Murashige and Skoog (1962) ; 2,4-D, 2,4-dichlorophenoxy acetic acid); BAP, 6-benzylaminopurine

RESULTS AND DISCUSSION

Effects of Different Combination and Levels of BAP and 2, 4-D on Callus Induction of Stevia Leaf and Internode Explants

An intermediation ratio of auxin and cytokinin have been widely used to generate callus (Ikeuchi et al 2013), and several studies reported that different explants have the ability to produce unorganized cells or to form into a new regenerated plant using different combinations of phytohormones (Pawar et al 2015). In the present study, it was found out that stevia leaf and internode explants have different responses to different combination and concentration of 2,4-D and BAP after 4 weeks of incubation (Figure 2).

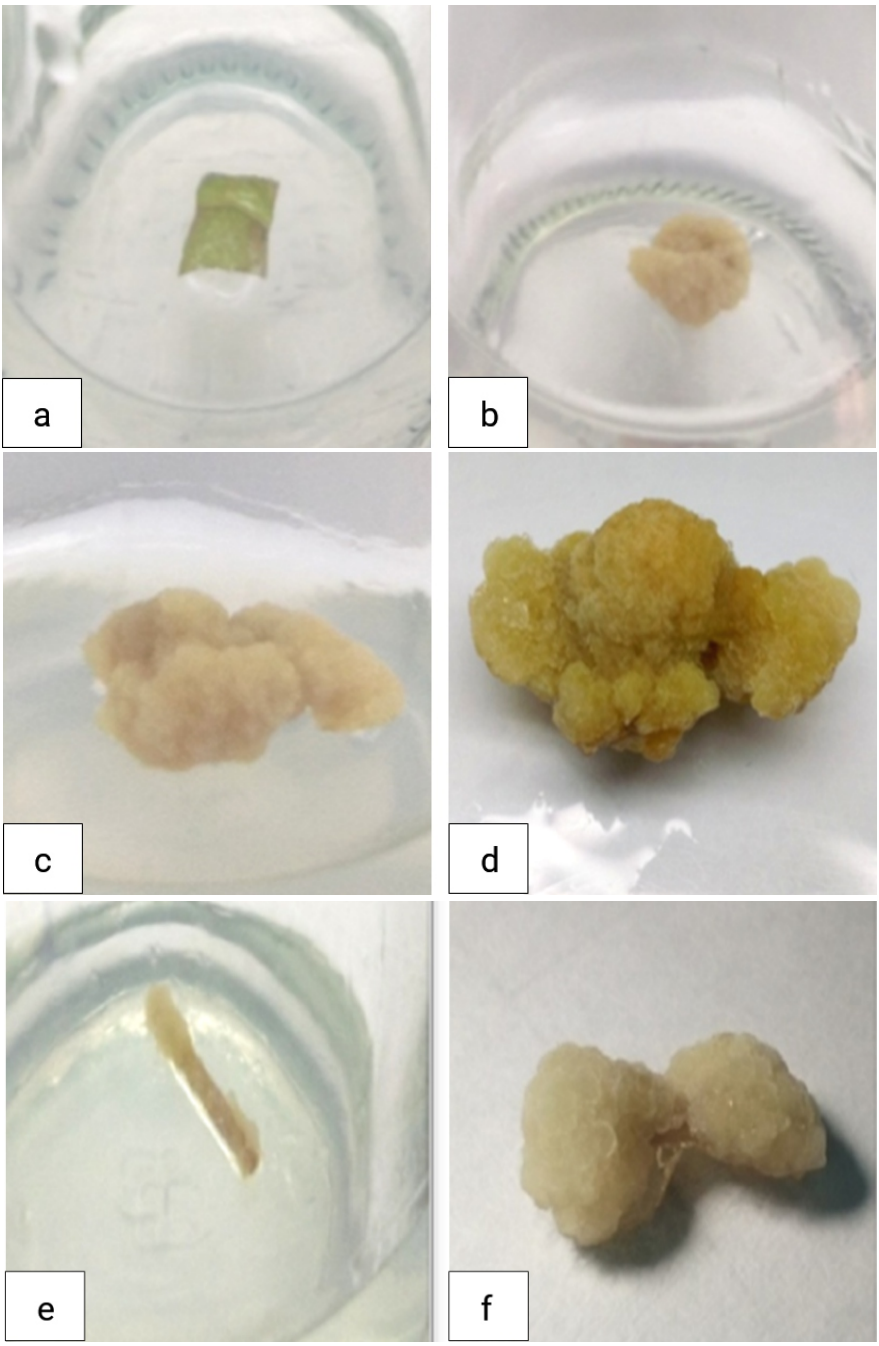

Figure 2. Responses of leaf and internode explants inoculated to MS medium supplemented with different combination and level of BAP and 2,4-D after 4 week incubation: (a-d) leaf explants at (a) 5 days, (b) 2 weeks, (c) 3 weeks and (d) 4 weeks of incubation, (e-g) internode explants at (e) 2 weeks, (f) 3 weeks and (g) 4 weeks of incubation.

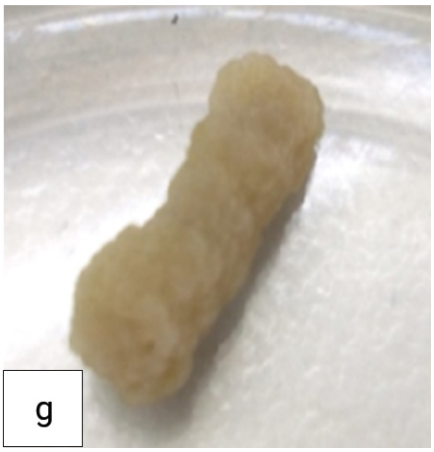

Figure 2. continued

Stevia Callus Formation

Callus formation is observed in response to stresses such as wounding or pathogen infection as well as the different concentration and combination of plant growth regulators. In addition, different explants have also different responses to the formation of callus (Ikeuchi et al 2013). The callus initiation was observed in both explants but was first observed in the cut margin of the leaf explants in culture medium supplemented with BAP and 2,4-D. Earliest period (11 days) of callus formation for leaf explants was observed in MS medium supplemented with 1.5mg L^{-1} 2,4-D + 3.0mg L^{-1} BAP (T_5), while the longest callus induction (15 days) was observed in 1.0mg L^{-1} 2,4-D + 3.0mg L^{-1} BAP (T_2) (Figure 4). Meanwhile in internode explants, early callus formation (16 days) was observed in MS culture medium added with 1.0mg L^{-1} 2,4-D + 4.0mg L^{-1} BAP (T_3) while the longest period of response (20 days) was observed in 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4) (Figure 3).

A week after inoculation, MS + 1.5mg L^{-1} 2,4-D + 3.0mg L^{-1} BAP (T_5) had the highest percentage of callus formation (34.78%) in leaf explants (Table 3). Mean percentage of callus formation after four weeks showed no significant difference among treatments with different levels of 2,4D and BAP. The result suggests that any of the treatment levels can be used for callus induction of stevia. Likewise, among all culture medium that was used, MS + 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (95.24%) in leaf explants and MS + 1.0mg L^{-1} 2,4-D + 4.0 BAP mg L^{-1} (90%) in internode explants showed the highest percentage of callus formation while MS alone did not formcallus.

In the present study, both leaf and internode explants exhibited callus formation cultured on MS medium supplemented with 2,4-D and BAP. However, leaf explants formed callus earlier than the internode, but after 2 weeks of incubation callus formation was exhibited in all treatments except control and in culture medium with MS + 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4). This result signifies that leaf explants have more meristematic region which is composed of active cells that has the potential to respond immediately to suitable medium for callus induction compared to the internode explants (Ikeuchi et al 2013).

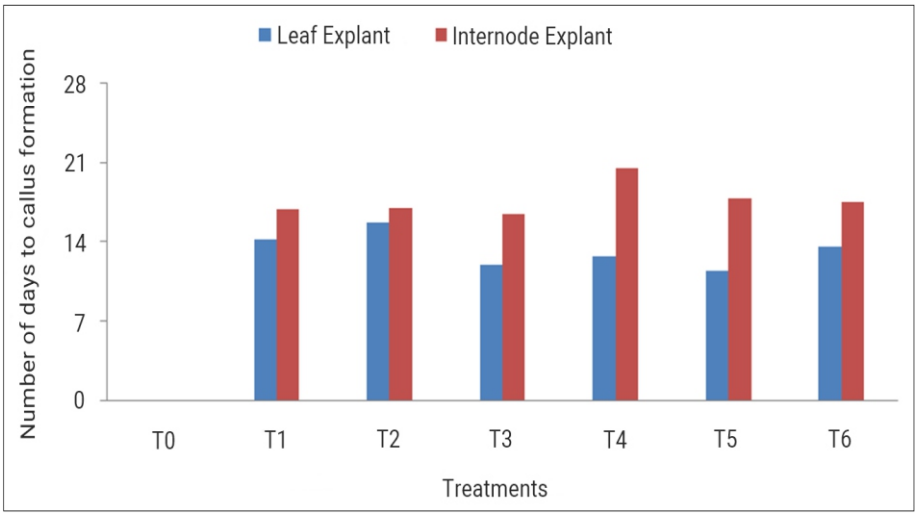

Figure 3. Number of days to callus formation in leaf and internode explants inoculated in MS medium supplemented with different combination and level of 2,4-D and BAP.

Treatments:

T_0.MS alone

T_1.MS + 1.0mg L^{-1} 2,4-D + 2mg L^{-1} BAP

T_2. MS + 1.0mg L^{-1} 2,4-D + 3mg L^{-1} BAP

T_3.MS + 1.0mg L^{-1} 2,4-D + 4mg L^{-1} BAP

T_4.MS + 1.5mg L^{-1} 2,4-D + 2mg L^{-1} BAP

T_5.MS + 1.5mg L^{-1} 2,4-D + 3mg L^{-1} BAP

T_6.MS + 1.5mg L^{-1} 2,4-D + 4mg L^{-1} BAP

Table 3. Percentage of callus formation in leaf and internode explants of *Stevia rebaudiana* Bertoni inoculated to MS medium supplemented with 2,4-D and BAP after 4 weeks from inoculation

Treatment code	Medium Composition	Percentage Callus Formation				Mean ± SE
		1st week	2nd week	3rd week	4th week	
Leaf Explant						
T_0	MS alone	0	0	0	0	0.00 [a] ±0
T_1	MS + 1.0 mg L^{-1} 2,4-D + 2.0 BAP mg L^{-1}	16.00	52.00	68.00	92.00	92.59 [b] ±3.70
T_2	MS + 1.0 mg L^{-1} 2,4-D + 3.0 BAP mg L^{-1}	10.71	42.86	64.29	89.29	89.63 [b] ±5.79
T_3	MS + 1.0 mg L^{-1} 2,4-D + 4.0 BAP mg L^{-1}	9.09	59.09	81.82	81.82	83.33 [b] ±16.67
T_4	MS + 1.5 mg L^{-1} 2,4-D + 2.0 BAP mg L^{-1}	23.81	61.9	80.95	95.24	95.24 [b] ±4.76
T_5	MS + 1.5 mg L^{-1} 2,4-D + 3.0 BAP mg L^{-1}	34.78	65.22	91.3	86.96	89.81 [b] ±4.76
T_6	MS + 1.5 mg L^{-1} 2,4-D + 4.0 BAP mg L^{-1}	12.00	52.00	72.00	88.00	89.76 [b] ±12.35
Inter node Explant						
T_0	MS alone	0	0	0	0	0.00 [a] ±0
T_1	MS + 1.0 mg L^{-1} 2,4-D + 2.0 BAP mg L^{-1}	0	17. 24	62.07	82.76	83.33 [b] ±8.82
T_2	MS + 1.0 mg L^{-1} 2,4-D + 3.0 BAP mg L^{-1}	0	10.00	76.67	83.33	83.33 [b] ±3.33
T_3	MS + 1.0 mg L^{-1} 2,4-D + 4.0 BAP m g L^{-1}	0	26.67	83.33	90.00	90.00 [b] ±5.77
T_4	MS + 1.5 mg L^{-1} 2,4-D + 2.0 BAP mg L^{-1}	0	0	40.00	86.67	86.67 [b] ±3.33
T_5	MS + 1.5mg L^{-1} 2,4-D + 3.0 BAP mg L^{-1}	0	20.00	70.00	86.67	86.67 [b] ±8.82
T_6	MS + 1.5 mg L^{-1} 2,4-D + 4.0 BAP mg L^{-1}	0	3.33	60.00	80.00	80.00 [b] ±5.77

*Results presented as mean ± standard deviation

*Means sharing a letter in the group label are not significantly different at the 5% level of significance.

Description of Stevia Callus

After 4 weeks of incubation, the leaf and internode callus appeared as compact, non-embryogenic, and yellowish green calli in all treatments (Figure 4, Table 4). Swelling was first observed in all treatments after one week from inoculation of all explants. However, in leaf explants, callus formation continued in the following weeks in all treatments unlike the internode explants. The cultures inoculated in MS alone turned brown in the succeeding weeks and eventually failed to initiate callus until termination

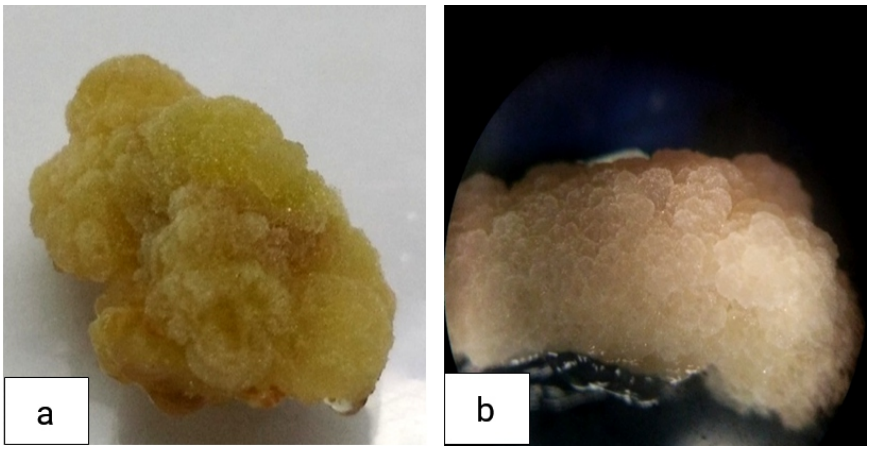

Figure 4. Stevia Callus from leaf explants (a) was compact, non-embryogenic, yellowish green and (b) light yellowish white callus in internode explants.

Stevia leaf and internode callus formed was rated following the scoring system used by Gurel et al (2001). The leaf callus obtained an average rate of 4, which indicated a measurement greater than 10mm callus form all the cultures in each treatment. However, in internode callus, a range of 5-10mm or greater than 10mm callus formation was recorded in all culture of each treatment. No rating was indicated in the control since no callus formation was observed after the explants bulged or swelled. Furthermore, the statistical analysis for callus weight both in leaf and internode explants was significantly different. Callus weight was highest at MS + 1.5mg L^{-1} 2,4-D + 3.0mg L^{-1} BAP (T_5) in both type of explants. The leaf explants had the highest callus weight average of 0.54g compared to internode explants which has 0.10g. This result signifies that, the culture medium supplemented with higher level of 2,4-D and BAP obtained a greater callus weight both in leaf and internode explant (Table 4).

The same study of Gupta et al 2010, reported that leaf segments can be the best explant for callus production of Stevia. A similar study also reported that stevia leaf and nodes explants cultured MS supplemented with 2,4-D or NAA in combination with 2.22µM BAP were effective. However, 2,4-D generated a higher frequencies of callus formation than NAA with 80%(leaf) and 60%(nodes) successful callus formation (Janarthanam et al 2009). In addition, indirect organogenesis of *Cucumis anguria* L. using the leaf and nodal explants,

it was found out that leaf explants showed maximum response in culture medium supplemented with 4.0mg L^{-1}NAA and 4.0mg L^{-1} 2,4-D compared to auxin and cytokinin alone (Jeyakumar, Vivekanandan 2015).

Table 4. Type and color of callus formed from leaf and internode explants grown in MS medium supplemented with 2,4-D and BAP after 4 weeks of incubation.

Treatment Code	Callus Weight Mean	Rate of Callus	Callus Description
Leaf Explant			
T_0	0.00 ^{b}±0	0	Brown Explants/ Dead
T_1	0.44 ^{a}±0.01	4	compact, non-embryogenic, light yellowish green callus
T_2	0.47 ^{a}±0.07	4	compact, non-embryogenic, yellowish green callus
T_3	0.38 ^{a}±0.03	4	compact, non-embryogenic, yellowish white callus
T_4	0.40 ^{a}±0.03	4	compact, non-embryogenic, yellowish green callus
T_5	0.54 ^{a}±0.09	4	compact, non-embryogenic, yellowish white callus
T_6	0.26 ^{ab}±0.03	4	compact, non-embryogenic, yellowish white callus
Internode explants			
T_0	0.00 ^{c}±0	0	Brown Explants/ Dead
T_1	0.04 ^{b}±0.01	4	compact, non-embryogenic, light yellowish white callus
T_2	0.05 ^{b}±0.00	3	compact, non-embryogenic, yellowish white callus
T_3	0.06 ^{b}±0.01	4	compact, non-embryogenic, yellowish white callus
T_4	0.06 ^{b}±0.00	3	compact, non-embryogenic, yellowish white callus
T_5	0.10 ^{a}±0.00	3	compact, non-embryogenic, yellowish white callus
T_6	0.05 ^{b}±0.01	3	compact, non-embryogenic, yellowish white callus

*Results presented as mean ± standard deviation
*Means sharing a letter in the group label are not significantly different at the 5% level of significance
*Rate of callus: A '0-4 scale' scoring system developed for rating of callus, wherein 0 (No visible callus); 1 (Small proliferation at cut ends only); 2 (5mm callus at cut ends); 3 (5-10mm callus from all over the explants); 4 (>10mm callus from all over the explants)

Total Phenolic Content of Stevia Calli

When cultures were subjected to UV radiaton, Stevia leaf callus was observed to have affected color of calli. Irradiated calli produced brownish color all over the callus tissue (Figure 5), which may indicate that the irradiated calli have experienced abiotic stress through the UV radiation exposure and resulted to the production of secondary metabolites, specifically the phenolic compounds.

Phytochemical compounds that are present in different plant material can be obtained by milling, grinding, homogenization, and solvent extraction. Among all these steps, solvent extraction method is the best method that has always been used in recovering phytochemical compounds. The effectiveness of the extraction method depends on the solvent used, composition of phytochemical compounds, and also the presence of other interfering substances in the plant materials. The production yield of compounds in solvent extraction will depend also on the condition, pH, temperature, extraction time and the polarity of the solvent used (Do et al 2013).

Figure 5. UV Irradiated calli derived Stevia leaf explants grown in MS + 1.5mg L^{-1} 2,4-D + 2mg L^{-1} BAP (T_4) after 3h of exposure, showing brown callus tissues.

In this study, a quantitative analysis was conducted to determine the total phenolic content of UV irradiated stevia leaf callus with different time of exposure. The stevia leaf callus extract was obtained with the use of pure methanol as solvent. Methanol is colorless and volatile that is used in organic synthesis, fuel, antifreeze and as solvent. Methanol as an extraction solvent is very popular due to its high polarity that can dissociate the hydroxyl group and reported as more efficient in extracting lower molecular weight polyphenols (Dai and Mumper 2010).

The total phenolic content of stevia leaf callus extract was estimated by Folin Ciocalteu's method using gallic acid as standard. The gallic acid standard solution was obtained by plotting the concentration versus the absorbance at 650nm with a regression co-efficient (R^2)=0.997, with a linear equation standard of y=0.007x+0.049. The milligram gallic acid equivalent (GAE) per gram of callus weight was derived from the regression line of y=0.007x+0.049.

The total phenolic content of Stevia calli is presented in Table 5. Treatment combination of 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4) exposed for 3h yielded the highest phenolic content of 87.71mg g^{-1} followed by 84.58mg g^{-1} in 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4) exposed for 5h, 80.23mg g^{-1}, 79.78mg g^{-1}, 77.16mg g^{-1}, 67.06mg g^{-1}, 57.52mg g^{-1} and 54.83mg g^{-1} respectively. However, it is also presented in Table 5 that during the 1h exposure of cultures inoculated in MS+1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4) and 5h exposure of cultures inoculated in MS+1.5mg L^{-1} 2,4-D + 3.0mg L^{-1} BAP (T_5) had sudden decrease in the phenolic content. It is interesting to note that the non-exposure of callus in MS+1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP medium had slight increase of total phenolic content compared to the 1h exposure. According to Nantitanon et al (2010) varying results of phenolic content could be due to several factors such as the UV exposure, environment and during the extraction done during the total phenolic determination assay.

The results obtained generally showed that both medium composition and time of exposure affected the production of phenolic content of Stevia leaf callus due to the in vitro stress condition of UV radiation experienced by leaf callus tissue. However, it was also observed that when stevia leaf callus tissue was exposed at longer time, it resulted to lesser total phenolic content compared to 3h exposure. Similarly, Manaf et al (2016) study reported also that *Echinacea purpurea* callus culture exposed

to UV-B light showed a significant increment in total phenols after 2h exposure time followed by 6h. Similarly, the stevia cultures in this study were exposed to UV-B light with less than an hour of exposure time for comparison. This study supported the research of Abou-Arab and Abu-Salem (2010), on the comparison of total phenolic contents and total flavonoid content of stevia callus and leaf, and it was found out that there were higher phenolic compound and flavonoid yield from stevia callus (33.99 and 30.03mg g^{-1} dry weight) than leaf (24.01 and 18.93mg g^{-1} dry weight).

Table 5. Total phenolic content (mg GAE per g sample) of Stevia calli as influenced by different exposure time to UV radiation.

Medium Combination	Length of Exposure	Total Phenolic Content of Ste via (mg GAE per g callus weight)
MS + 1.5mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP	0h	77.16 ^{e} ± 0.0 5
	1h	54.83 ^{h} ± 0.0 2
	3h	87.7 2^{a} ± 0.0 1
	5h	84.5 8^{b} ± 0.02 1
MS + 1.5 mg L -1 2,4 -D + 3.0 mg L^{-1} BAP	0h	57.5 2^{g} ± 0.0 1
	1h	79.7 8^{d} ± 0.0 4
	3h	80.2 3^{c} ± 0.04
	5h	67.06 ^{f} ± 0.0 1

*Results presented as mean ± standard deviation
*Means sharing a letter in the group label are not significantly different at the 5% level of significance.

Abiotic stress such as UV radiation exposure was reported to have a beneficial effect in production of secondary metabolites. Phenolic compounds are secondary metabolites that have been widely distributed in plants and are important constituents of human diet and also serve as defense mechanism against biotic and abiotic stresses (Bhattacharya et al 2010). Phenolic compounds as antioxidant have the ability to destroy free radicals due to its hydroxyl group (Pourreza 2013).

CONCLUSION

Stevia leaf explant was the suitable explants used for callus induction in stevia since it showed the earliest callus formation after 11 days of incubation. All treatment combination used successfully induced callus formation on both explants but MS + 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4) induced early callus induction in leaf explants of 95% and; MS + 1.0mg L^{-1} 2,4-D + 4.0mg L^{-1} BAP (T_3) for internode explants with 90% callus formation. From the result of total phenolic determination by Folin-Ciocalteu method, treatment combination of MS + 1.5mg L^{-1} 2,4-D + 2.0mg L^{-1} BAP (T_4) with 3h of exposure had the highest total phenolic content of stevia callus with 87.71mg g^{-1} of callus weight. Hence, both the culture medium combination and the length of exposure to UV affect the production of phenolic content of stevia calli.

RECOMMENDATION

Further studies may be conducted on different levels of BAP and 2,4D at longer exposure to UV radiation.

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