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The Effect of Nitrogen Concentrations on the Growth and Development of *Stevia rebaudiana* (Bertoni) and Production of Stevioside and Rebaudioside A.

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ABSTRACT

Stevia rebaudiana (Bertoni) is a herbaceous plant that has an increasing demand in agricultural industry worldwide. This is due to the presence of the sweet glycosides in the leaves that are hundreds times sweeter than sugar and have zero calories. The presence of sweet glycosides in *S. rebaudiana* (Bert.) leaves had cause a high demand for its function as sugar alternatives and sweetening agent in diabetic patient's food and beverage. There were many researches done on this herb in increasing its yield such as growth regulator manipulation, light, water and temperature controlling in greenhouse and genetic modification. However, there is no study had been done on the macronutrient manipulation in *S. rebaudiana* (Bert.), especially nitrogen (N). The major sources of N for plants are nitrate and ammonium and are crucial in the synthesis of proteins and other organic compounds. The aim of this study was to examine the effect of N concentrations on the growth and development of *S. rebaudiana* (Bert.) and production of stevioside and rebaudioside A by using plant tissue culture technique. From this study, it was found that normal (1x) N concentration produced the highest numbers of leaves (13.88±1.55), number of branches (1.56±0.37), number of nodes (6.84±0.56), number of shoots (1.24±0.09), fresh biomass (0.04±0.01g), dry biomass (0.004±0.001g). Whereas, media without N (0 N) had produced the highest shoot length (3.41±0.31cm). Meanwhile 8x N concentration produced the highest stevioside yield (3.46±0.01mg/ml) and 4x N concentration produced the highest rebaudioside A yield (1294.11±1.93mg/ml). Different N concentrations in the plant tissue culture media had different effects on the growth and development of *S. rebaudiana* (Bert.) and production of stevioside and rebaudioside A. Normal N concentration is best for producing the highest numbers of leaves, number of branches, number of nodes, number of shoots, fresh biomass and dry biomass. The media without N is best for producing the highest shoot length. Moreover, 8x N and 4x N concentration is best for stevioside and rebaudioside A production, respectively.

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INTRODUCTION

Stevia rebaudiana (Bertoni) or popularly called as stevia is widely used as natural sweeteners for diabetic patients where it is either used as a substitute for sucrose in food and beverages or used together with sucrose. It is a shrub native of Paraguay and Brazil and the common name of this plant in the Guaraní language of Paraguay is Caá hê-é or sweet weed, sweet leaf, sweet herb and honey leaf (Ahmed *et al*., 2007). The species leaves are estimated to be 300 times sweeter than sugar cane (Chalapathi *et al*., 1997). *S. rebaudiana* (Bert.) plant is one of 154 members of the genus Stevia and one of two species that produce sweet steviol glycosides (Madan *et al*., 2010). In a few countries, stevia has been consumed as a food and medicine (ethnobotanical) for many years, including Japan and Paraguay. *S. rebaudiana* (Bert.) leaves have a very high demand in the world market for its popular uses in medicine and as a sweetener in drinks. Stevioside, the most abundant sweet constituent of the leaves of species, and an ent-kaurene diterpene diglycoside, was first isolated in impure form in the first decade of the twentieth century and sparingly soluble in water (0.13%) (Bertoni, 1905; 1918). Rebaudiose A, the second most abundant ent-kaurene glycoside occurring in the leaves of *S. rebaudiana* (Bert.) is better suited than stevioside for use in foods and beverages, because it is not only more water soluble (0.80%) but it also exhibits a pleasanter taste (Kinghorn and Soejarto, 1991; Tanaka 1997).

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Plant macronutrients are any nutrient elements that are required in excess of 10 mmole/kg of dry weight (Hopkins and Huner, 2009). Most of the macronutrients represent 0.1 to 5%, or 100 to 5000 parts per million (ppm), of dry plant tissue (Wiedenhoeft, 2006). N serves in the same ways within the plant, as it does in other organisms as a component of amino acids and nucleic acids. N also plays a critical role in the structure of chlorophyll, the primary light harvesting compound of photosynthesis. This, along with its structural role in amino acids, explains why plants require large amounts of N, and thus why it is often the limiting nutrient for plant growth (Boroomand and Grouh, 2012).

Nitrate is readily mobile in plants and can be stored in vacuoles, but for nitrate to do its functions in plants, it must be reduced to ammonium. There are two ways for nitrate to be assimilated in plant, which are by using nitrate reductase and nitrite reductase. Whereas, there are three ways for ammonium to be assimilated in plant, which are by using glutamine synthetase, glutamate synthase, glutamic acid dehydrogenase, transamination and amidation.

Production of stevioside and rebaudioside A can be optimised by using biotechnological tool. Hence, the purpose of this study was to determine the optimum concentration of N for the growth and development and the desired sweet glycosides (stevioside and rebaudioside A) yield by using plant tissue culture technique.

MATERIALS AND METHODS

Media preparation:

The manipulation of N in this study was based on the MS media (Murashige and Skoog, 1962) where the source of N in MS media were ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3). While in this study, only ammonium nitrate (Table 1) were used as it still can produced NH_4^+ and NO_3^- ions without the presence of potassium nitrate. The reason for this modification is, to reduce unwanted and redundant reaction in the study.

The media used in this study were prepared according to the modified MS media in Table 1. The manipulation of N concentration in the media can be seen in Table 2. When the concentration of N manipulated, the concentration of other macronutrients remain normal (1x concentration). The concentrations of macronutrients stock in the Table 2 were 10x more concentrated than normal modified MS media. For each litre media prepared, 1 litre distilled water (dH_2O), 30g sucrose, 3.3g agar (gelrite), micronutrient per litre as in original MS media and 10ml macronutrient as in Table 2 were used. The media and hand tools that were used in this study were sterilized (121 °C at 15psi for 20 minutes) by using autoclave machine.

Table 1: Formulation of modified MS media.

Modified MS Media		
Component	Mol. (mM)	Macronutrient
Ammonium nitrate (NH_4NO_3)	20.61	N
Potassium hydroxide (KOH)	18.79	K
Calcium chloride ($CaCl_2$)	2.99	Ca
Magnesium chloride hexahydrate ($MgCl_26H_2O$)	1.50	Mg
Sodium phosphate monobasic (NaH_2PO_4)	1.25	P
Manganese sulfate monohydrate ($MnSO_4\ H_2O$)	0.11	S

Table 2: Macronutrient stock with different N concentration (g/100ml).

	0	0.5 X	1 X	2 X	4 X	8 X
N	0	8.25	16.50	33.00	66.00	132.00
K	10.54	10.54	10.54	10.54	10.54	10.54
Ca	3.32	3.32	3.32	3.32	3.32	3.32
Mg	3.05	3.05	3.05	3.05	3.05	3.05
P	1.50	1.50	1.50	1.50	1.50	1.50
S	0.20	0.20	0.20	0.20	0.20	0.20

In vitro sample preparation:

Mature *S. rebaudiana* (Bert.) plants (4 months) were obtained. The leaves and roots of the plant were removed. The remaining stems were then cleaned and sterilized by using 5.95% sodium hypochlorite, anti-microbes and anti-fungus to remove pathogens. These twigs then were cut at the nodal section to get the node part (0.5cm each) and cultured onto the jar containing modified MS media. The growth conditions for these plantlets were 27°C with 16 hours photoperiod (2000 lux). After 1 month, the plantlets were subcultured into modified MS media with N concentration manipulated (Table 2). The study were done with 5 replicationS (1 replicate = 5 plantlets in a jar with media) for each N concentrations.

S. rebaudiana (Bert.) plantlet physical readings:

After 1 month, the plantlets were harvested, and cleaned. The physical characteristics (number of leaves, number of branches, number of nodes, number of shoots, shoot length (cm), fresh biomass (g) and dry biomass (g)) were measured and recorded.

Crude extraction of S. rebaudiana (Bert.):

Harvested plantlets were dried in the oven (40°C for 48 hours). The whole *in vitro* plantlets were ground using a blender. Ground *S. rebaudiana* (Bert.)samples were then extracted with different extraction solvents which were aqueous (dH_2O) and methanol (Fisher Scientific). Nishiyama *et al*. (1991) found that by using water as solvent for extraction of stevioside, it can increase the extraction efficiencies up to 98%. Whereas, Asrul *et al*. (2013) found that methanol is the best solvent for extracting rebaudioside A. Samples were immersed into extraction solvent with a ratio of 1g : 100ml for 24 hours. Then, the extracts were filtered using a filter funnel and filter papers (Whatman 41). The extracts were filtered again with syringe filters (PTFE membrane, pore size 0.2 μm) into sterilized glass vials.

HPLC method:

The crude extracts of *in vitro* samples of *S. rebaudiana* (Bert.)were inserted into HPLC vials (1ml each). The column used in this analysis was Agilent's Zorbax 300SB-C18 (5μm pore, 4.6mm x 150mm). The column temperature is 40^{o}C. The mobile phase was acetonitrile and aqueous solution (ratio of 80:20 respectively). The detector was UV detector at 210nm. Flow rate of the system was 1ml/min and the injection volume of 10μl for each sample with 3 times injections. The concentrations of standards injected into the HPLC instrument were shown in Table 3.

Table 3: Concentrations of standards.

	25%	50%	100%
Stevioside 98% purity (diluted into 1g: 10ml (w/v))	0.2551 mg/ml	0.5102 mg/ml	1.0204 mg/ml
Rebaudioside A 95% purity (diluted into 1g: 10ml (w/v))	260.43 mg/ml	520.85 mg/ml	1041.7 mg/ml

Statistical analysis:

All the readings were recorded and analysed with IBM SPSS Statistics 20. The mean differences were set to be significant at the 0.05 level. The post hoc tests after the one way ANOVA was performed were Tukey HSD and Dunnett (2-sided) t-tests. Homogeneity of variance test was also done using Tukey HSD (subset for alpha = 0.05).

Results:

A) Plant growth and biomass:

i) Number of leaves:

Fig. 1 showed the morphology readings of *S. rebaudiana* (Bert.)obtained from the modified MS media with different N concentrations. For number of leaves (Fig. 2), Tukey's HSD and Dunnett (2-sided) t-tests ($F(5,144)=4.643$, $p=0.001$, $\eta^2=0.139$) showed that 1x N concentration produced the highest number of leaves (13.880±1.552) while 8x N concentration produced the lowest number of leaves (6.400±0.879). However, these two experimental groups did not significantly differ than the control group.

ii) Number of branches:

The number of branches (Fig. 3) were highest (1.560±0.366) in normal media (1x N concentration), while 8x N concentration did not produced any branches (0.000). However, these two experimental groups did not significantly differ than the control group ($F(5,144)=3.301$, $p=0.007$, $\eta^2=0.103$).

iii) Number of nodes:

1x N concentration produced the highest number of nodes (Fig. 4) (6.840±0.556) and did not differ significantly than the control group while 8x N concentration produced the lowest number of nodes (3.160±0.394) and differ significantly than the control group ($F(5,144)=5.417$, $p=0.000$, $\eta^2=0.158$).

iv) Number of shoots:

The number of shoots (Fig. 5) was highest (1.240) in media with 0, 0.5x and 1x N concentrations, while 4x N concentration produced the lowest number of shoots (0.920±0.128). However, all of the experimental groups did not significantly differ among each other ($F(5,144)=1.438$, $p=0.214$, $\eta^2=0.048$).

v) Shoot length:

Furthermore, the shoot length (Fig. 6) of *S. rebaudiana* (Bert.) plantlets were highest (M=3.412±0.306cm) in media without N (control group, 0 N) and significantly differ than other experimental groups while 8x N concentration produced the shortest shoots (1.088±0.110cm) ($F(5, 144)=20.170$, $p=0.000$, $\eta^2=0.412$).

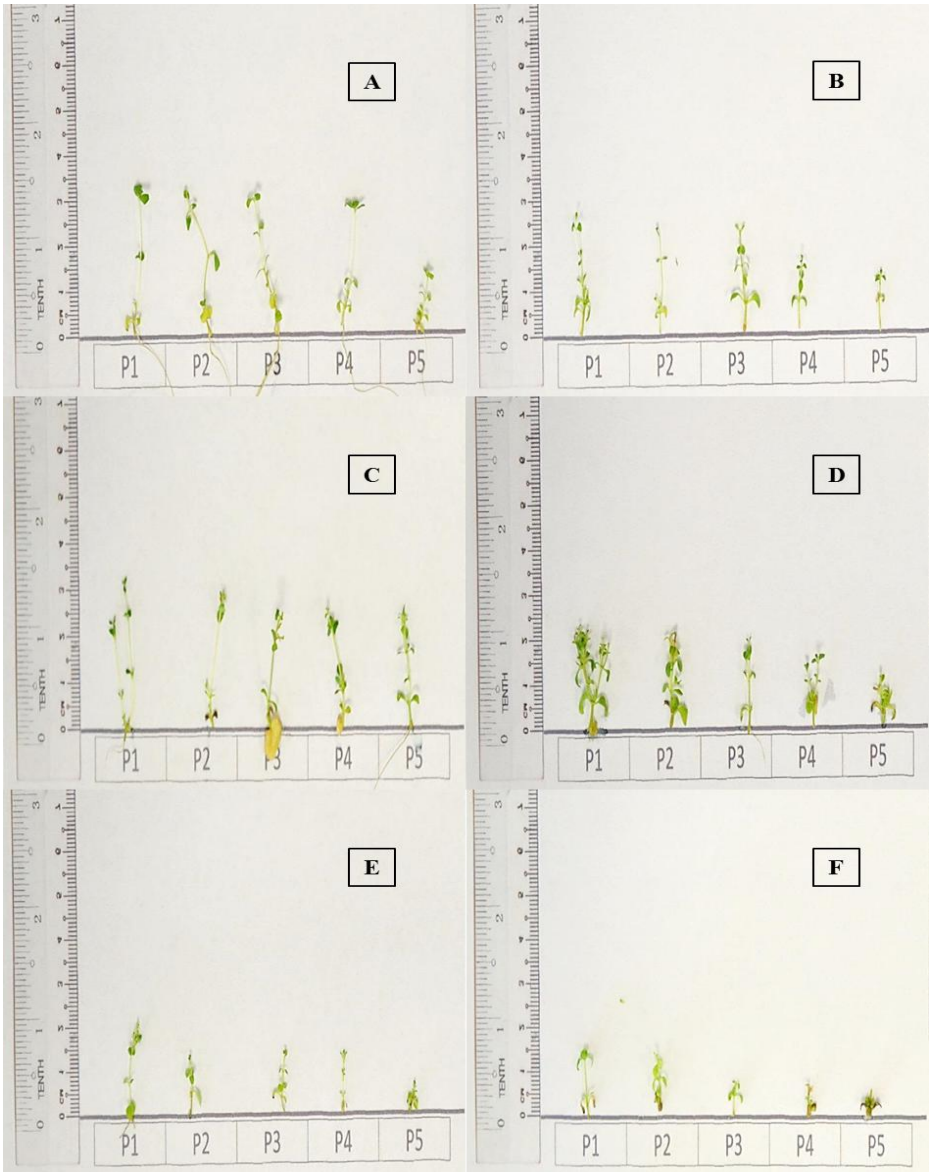

Fig. 1: *S. rebaudiana* (Bert.) plantlets from media with different N concentrations: A) 0 N; B) 0.5x N; C) 1x N; D) 2x N; E) 4x N and F) 8x N.

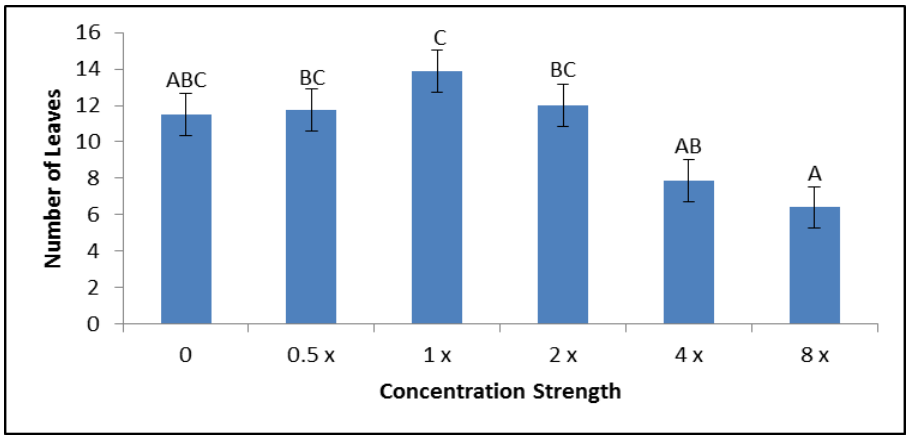

Fig. 2: Effect of N concentrations toward number of leaves of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

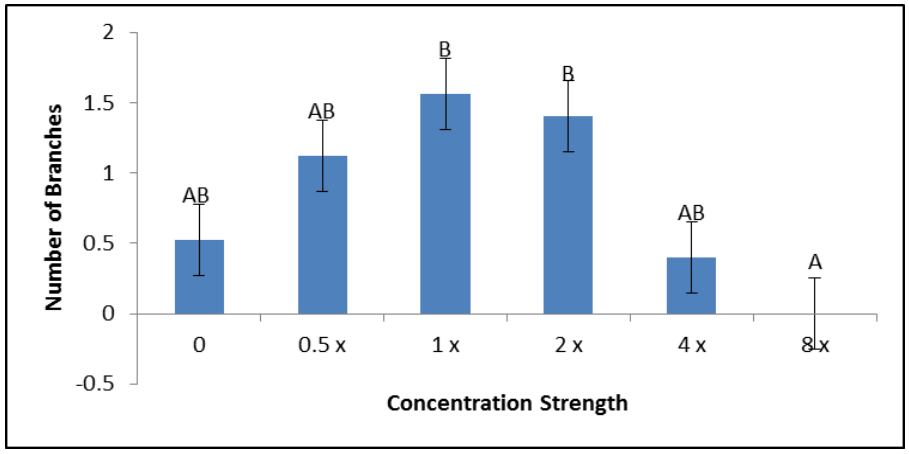

Fig. 3: Effect of N concentrations toward number of branches of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

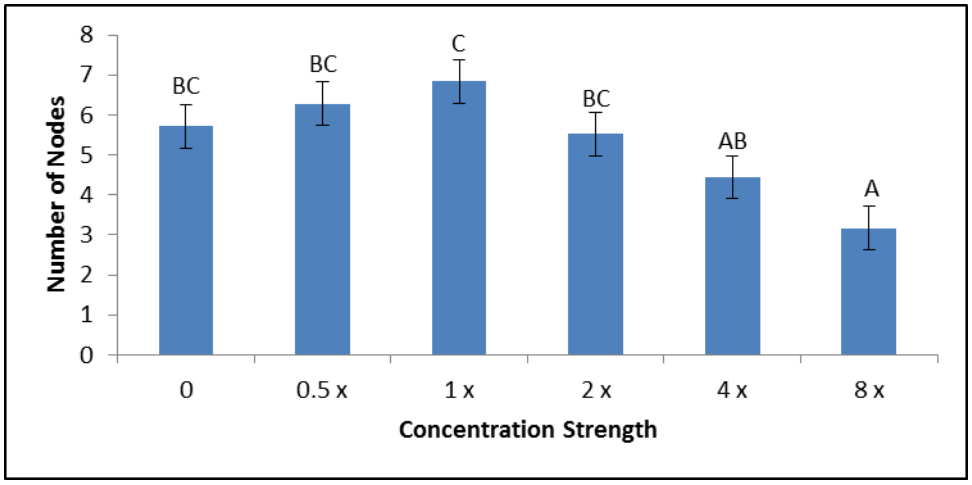

Fig. 4: Effect of N concentrations toward number of nodes of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

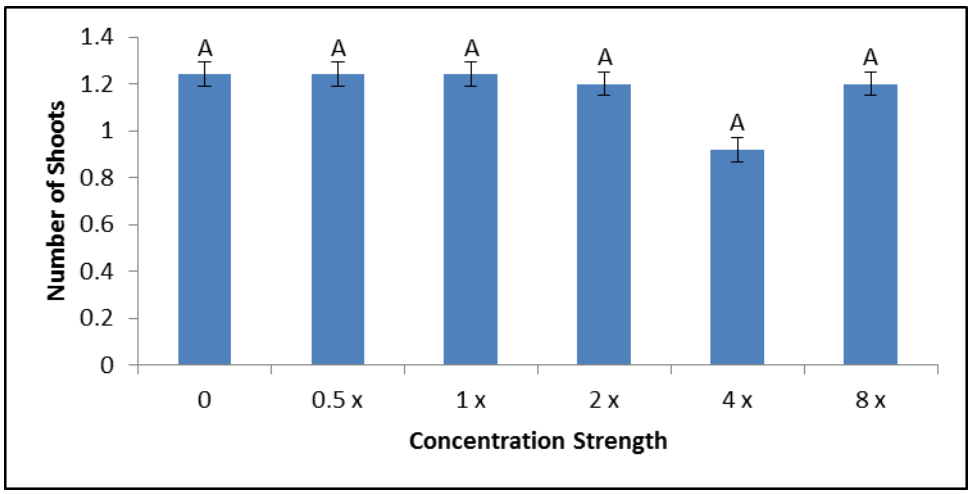

Fig. 5: Effect of N concentrations toward number of shoots of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

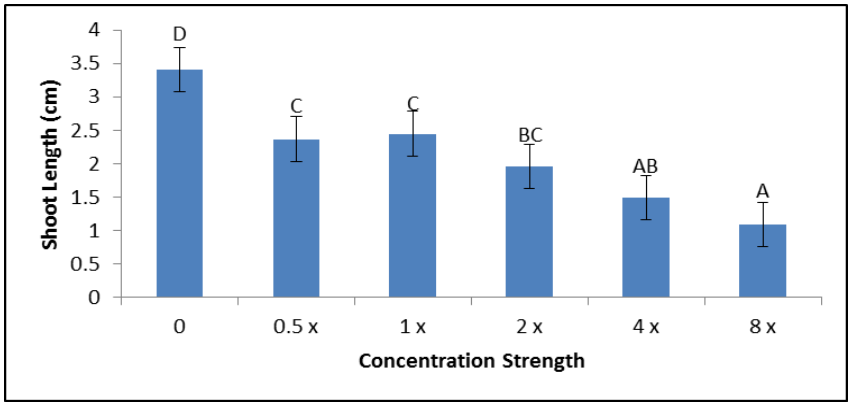

Fig. 6: Effect of N concentrations toward shoot length (cm) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

vi) Fresh and dry biomass:

The fresh biomass (Fig. 7) were highest (0.0428±0.0087g) in normal media (1x N concentration) while 8x N concentration produced the lowest fresh biomass (0.0131±0.0015g). These two experimental groups did not significantly differ than the control group ($F(5,144)=3.776$, $p=0.003$, $\eta^2=0.116$). On the other hand, dry biomass (Fig. 8) were also highest (0.0039±0.0010g) in normal media (1x N concentration) while 4x N concentration produced the lowest dry biomass (0.0017±0.0004g). These two experimental groups were also did not significantly differ than the control group ($F(5,144)=2.349$, $p=0.044$, $\eta^2=0.075$).

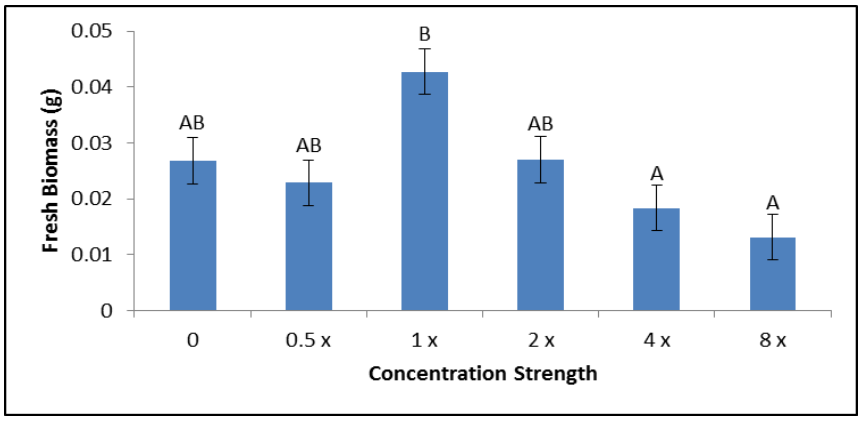

Fig. 7: Effect of N concentrations toward fresh biomass (g) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

B) Stevioside and rebaudioside A productions:

The concentration of desired sweet glycosides (stevioside and rebaudioside A) from *S. rebaudiana* (Bert.) plantlets were calculated from the sample's HPLC chromatogram and compared with the known concentration standards of both glycosides. Some of the chromatograms can be seen in Fig. 9. Table 4 shows the calibration curves for standards that were used to calculate the concentration of both sweet glycosides from the samples. The data from HPLC analyses (Fig. 10) showed the *S. rebaudiana* (Bert.) plantlets in 8x N concentration produced the highest stevioside concentration (3.464±0.006mg/ml) while 4x N concentration produced the lowest stevioside concentration (1.425±0.006mg/ml). Both of these experimental groups were significantly differ than the control group ($F(5,12)=2579.339$, $p=0.000$, $\eta^2=0.999$). Moreover, control group (0 N) produced the highest rebaudioside A (Fig. 11) concentration (1295.830±4.950mg/ml) and slightly higher than 4x N concentration (1294.108±1.925mg/ml) with both of the groups did not differ significantly. In contrast, 0.5x N concentration produced the lowest rebaudioside A concentration (527.633±5.326mg/ml) and significantly differ than the control group ($F(5,12)=7996.259$, $p=0.000$, $\eta^2=1.000$). Although without of N and 4x N concentration yielded significantly the same ($P < 0.05$) rebaudioside A concentration, only 4x N concentration were taken into account for further discussions.

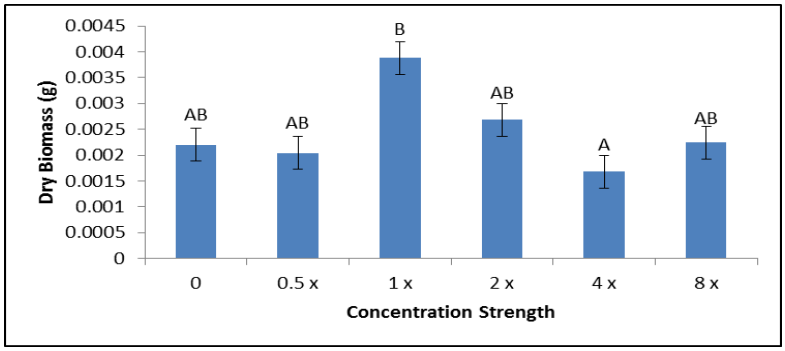

Fig. 8: Effect of N concentrations toward dry biomass (g) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

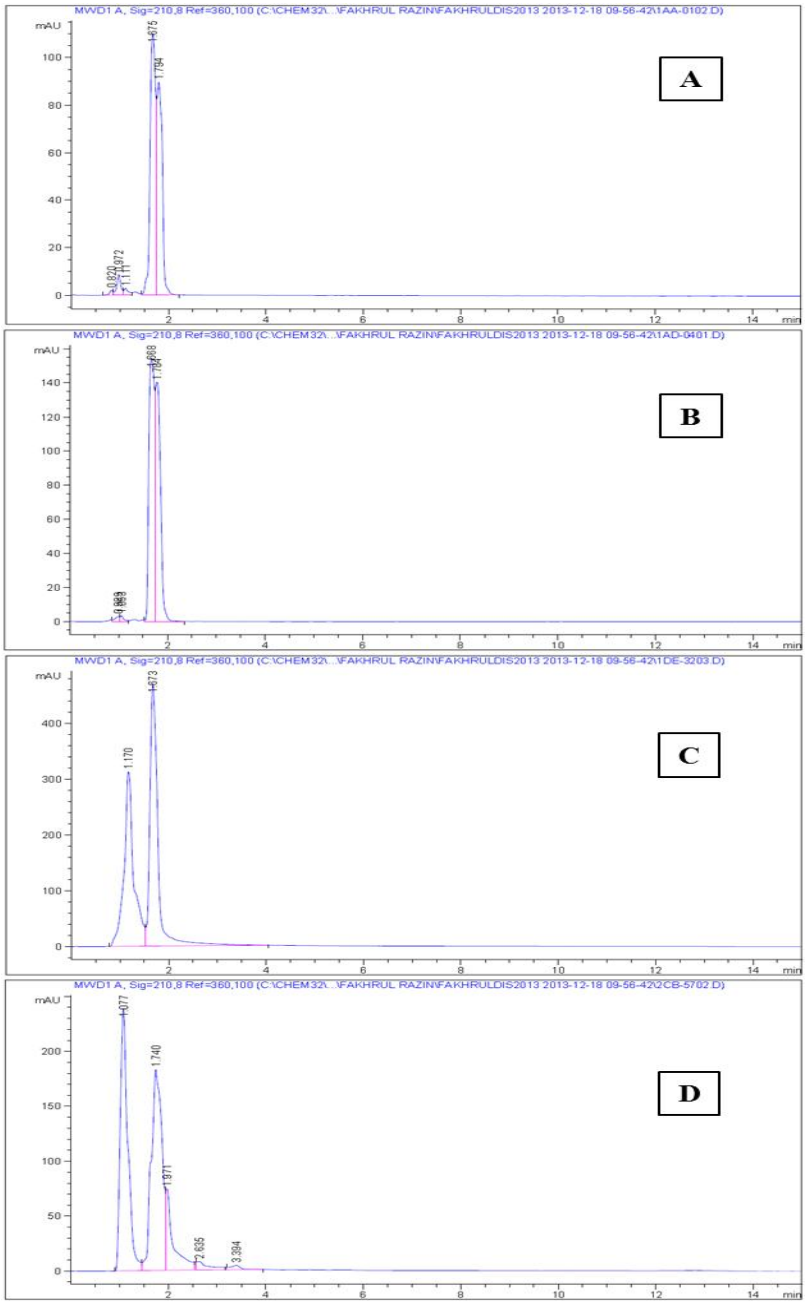

Fig. 9: HPLC chromatograms of: A) Stevioside standard; B) Rebaudioside A standard; C) 4 x N and D) 8 x N.

Table 4: Retention time, linear ranges and correlation coefficients of calibration curves for standards from HPLC.

Steviol glycosides	Retention time (min)	y = mx + c linear model *	r^2
Stevioside	1.67	y = 1538.9x + 15.699	0.9993
Rebaudioside A	1.78	y = 2.1795x + 9.1627	0.9999
*y = peak area, x = concentration			

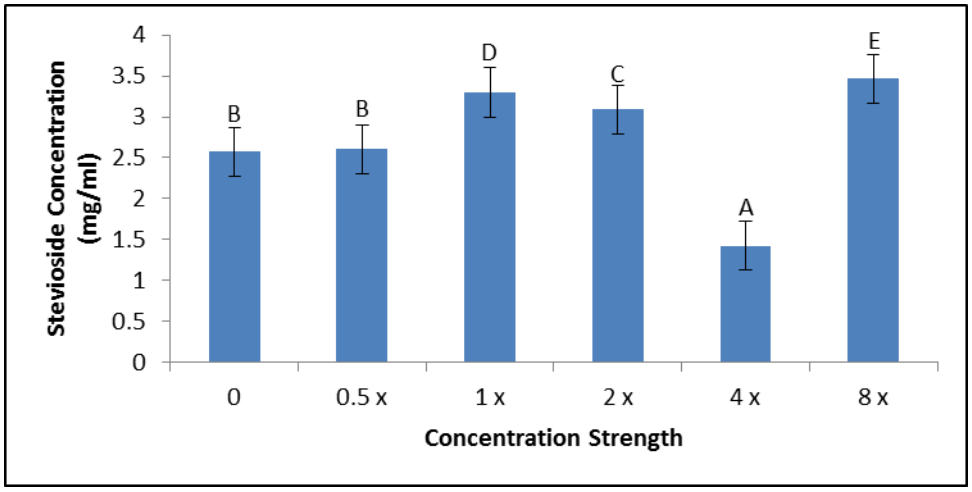

Fig. 10: Effect of N concentrations toward stevioside concentration (mg/ml) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

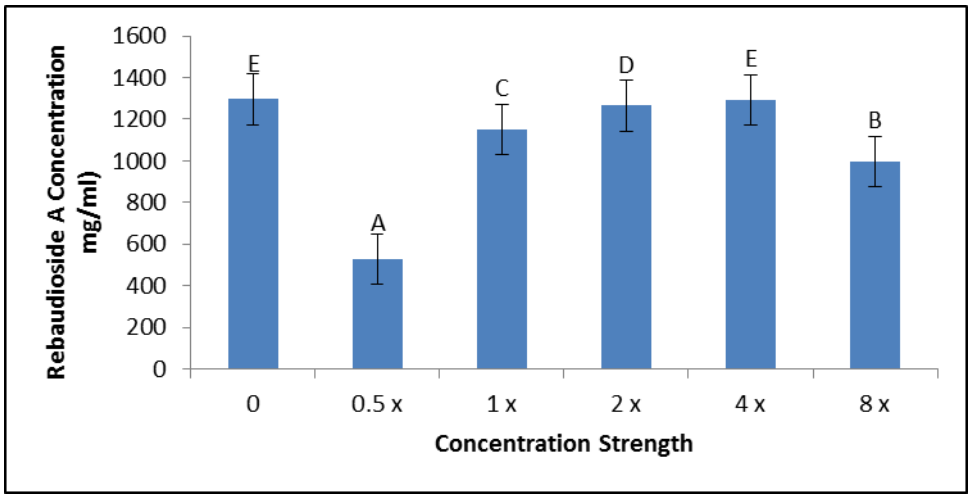

Fig. 11: Effect of N concentrations toward rebaudioside A concentration (mg/ml) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

Discussion:

The results showed that when the concentration of N was increased, the height and other physical characteristics of the plantlets were decreased while the concentrations of both sweet glycosides produced were increased. The height and other physical characteristics of *S. rebaudiana* (Bert.) plantlets were inversely proportional with the increment of N concentration in the media.

It is well known that nutrient availability controls plant development. Moreover, plant development is sensitive towards numerous of hormonal signals. Thus, there is direct relationship between nutritional and hormonal signalling. N nutrient affecting gene expression, molecular process and pathways (transport, signalling and phytohormones biosynthesis) and hence, affecting growth and development programs in roots and shoots (Gabriel *et al*., 2011). Some of the phytohormones biosynthesis involved were cytokinin and auxin biosynthesis.

Auxin is one of the major phytohormones and it has vital functions in plant growth and development. Higher amount of auxin causing longer shoot length (Edwin *et al*., 2008) and it function antagonistically with cytokinin in plants. From the study by Krouk, *et al*. (2010), the absence of N can be transduced as an increase in

auxin flux into the cell and tissue. This suggests that high nitrate supply might be inhibiting auxin biosynthesis. Similarly, cytokinin content is also under the control of N supply (Kiba *et al*., 2011 and Rahayu *et al*., 2005). Nitrate supplementation to plant roots results in a rapid increase in cytokinin levels and its translocation into the xylem vessels (Takei *et al*., 2002). Thus, when the concentration of N was increased, the amount of auxin decreased (the amount of cytokinin increased) and hence causing the height and other physical characteristics of the *S. rebaudiana* (Bert.) plantlets decreased.

Furthermore, the study by Brandle and Telmer (2007) showed that steviol glycosides were biosynthesized via the methylerythritol 4-phosphate (MEP) pathway and these processes occured in the chloroplast, endoplasmic reticulum, cytoplasm and vacuole of *S. rebaudiana* (Bert.). All 16 reactions in steviol glycosides biosynthesis involved various genes. 2 of 19 molecules in the biosynthesis have N element, which are 4-diphosphocytidyl-2-C-methyl-D-erythritol and 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate. It is known that N nutrient can affect gene expression. Therefore, as the concentration of N was increased, the concentrations of both sweet glycosides produced by *S. rebaudiana* (Bert.)were increased in this study.

Conclusion:

Different N concentrations in the plant tissue culture media had different effects on the growth and development of *S. rebaudiana* (Bert.) and production of stevioside and rebaudioside A. Normal N concentration is best for producing the highest numbers of leaves, number of branches, number of nodes, number of shoots, fresh biomass and dry biomass. The media without N is best for producing the highest shoot length. Moreover, 8x N and 4x N concentration is best for stevioside and rebaudioside A production, respectively.

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