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The Effect of Potassium 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) 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. 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 potassium (K). Potassium ion (K^+) is the most abundant cellular cation and so is required in large amounts by most plants. The aim of this study was to examine the effect of K 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 4x K concentration produced the highest numbers of leaves (22.00±2.21), number of branches (2.96±0.48), number of nodes (9.40±0.85), number of shoots (1.80±0.14), highest shoot length (2.95±0.12cm), fresh biomass (0.07±0.01g) and dry biomass (0.006±0.001g). Meanwhile control group (0 K concentration) produced highest stevioside concentration (4.26±0.00mg/ml) and 2x K concentration produced the highest rebaudioside A yield (1293.06±0.58mg/ml). Different K 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. 4x K concentration is best for producing all of the studied physical characteristics while 0 K and 2x K concentration is best for stevioside and rebaudioside A production, respectively.

Keywords: Stevia rebaudiana (Bertoni), potassium (K), potassium ion (K+), stevioside, rebaudioside A.

INTRODUCTION

Stevia rebaudiana (Bertoni) is an herbaceous plant that has an increasing demand in agricultural industry worldwide. It is a shrub native of Paraguay and Brazil and commercially known as sweet weed, sweet leaf, sweet herb and honey leaf. 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. The species leaves are estimated to be 300 times sweeter than sugar cane [5]. *S. rebaudiana* (Bert.) plant is one of 154 members of the genus Stevia and one of two species that produce sweet steviol glycosides [14]. Stevioside is the most abundant sweet constituent of the leaves of species and was first isolated in impure form in the first decade of the twentieth century and sparingly soluble in water (0.13%) [3,4]. On the other hand, rebaudiose A is the second most abundant sweet constituent in the leaves of *S. rebaudiana* (Bert.) and it is better suited than stevioside for use in foods and beverages. This is because it is not only more water soluble (0.80%), but it also exhibits a pleasanter taste [11,20].

The available form of potassium (K) in environment that can be readily absorbed by plant roots is potassium ion (K^+). It is a highly mobile

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macronutrient. Deficiency symptoms first appear in older leaves. Plant requires 250 mmol/kg of K^+ for normal growth in environment [7]. In Murashige and Skoog plant tissue culture media [16], 18.79 mmol/l (normal concentration) of K^+ is adequate to sustain the cultured plant's growth. 18.79 mmol/l of K^+ was the basis of K concentration manipulation in the plant tissue culture media in this study.

K^+ is an activator for a number of enzymes, most notably those involved in photosynthesis and respiration. Starch and protein synthesis are also mediated by K^+ [18]. Other than that, K^+ serves an important function in regulating the osmotic potential of cells. As an osmoregulator, K^+ is a principal factor in plant movements, such as the opening and closure of stomatal guard cells and the sleep movements, or daily changes in the orientation of leaves [18]. Because it is highly mobile, K^+ also serves to balance the charge of both diffusible and non-diffusible anions.

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

Materials and Methods

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 16h photoperiod (2000 lux). After 1 month, the plantlets were subcultured into modified MS media with K concentration manipulated (Table 1). The study were done with 5 replication (1 replicate = 5 plantlets in a jar with media) for each K concentrations.

Table 1: Macronutrient stock with different K concentration (g/100ml).

	0	0.5x	1x	2x	4x	8x
N	16.50	16.50	16.50	16.50	16.50	16.50
K	0	5.27	10.54	21.08	42.16	84.32
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

Media preparation:

The manipulation of K^+ in this study was based on the MS media [16] where the source of K^+ in MS media are potassium phosphate monobasic (KH_2PO_4), potassium iodide (KI) potassium nitrate (KNO_3). While in this study, potassium hydroxide (KOH) (Table 2) was used as it still can produced K^+ ions without the presence of potassium phosphate monobasic, potassium iodide and 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 2. The manipulation of K^+ concentration in the media can be seen in Table 1. When the concentration of K^+ manipulated, the concentration of other macronutrients remain normal (1x concentration). The concentrations of macronutrients stock in the Table 1 were 10x more concentrated than normal modified MS media. For each litre media prepared, 1l distilled water (dH_2O), 30g sucrose, 3.3g agar (gelrite), micronutrient per litre as in original MS media and 10ml macronutrient as in Table 1 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 2: 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

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.):

Then, the 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 [17] found that by using water as solvent for extraction of stevioside, it can increase the extraction efficiencies up to 98%. Whereas, Asrul *et al*. [1] 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 sterelized 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 machine 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 used Tukey HSD (subset for alpha = 0.05).

Results:

A) Plant growth and biomass:

Fig. 1 showed the morphology readings of *S. rebaudiana* (Bert.) obtained from the modified MS media with different K concentrations. Tukey's HSD and Dunnett (2-sided) t-tests showed that 4x K concentration produced highest number of leaves (22.00±2.21) (Fig. 2), highest number of branches (2.96±0.48) (Fig. 3), highest number of nodes (9.40±0.85) (Fig. 4), highest number of shoots (1.80±0.14) (Fig. 5), highest shoot length (2.95±0.12cm) (Fig. 6), highest fresh biomass (0.07±0.01g) (Fig. 7) and highest dry biomass (0.006±0.001g) (Fig. 8). On the other hand, control group (0 K concentration) produced lowest number of leaves (7.16±0.56), lowest number of branches (0.08±0.08), lowest number of nodes (3.88±0.27), lowest number of shoots (1.00±0.00), lowest shoot length (1.89±0.17cm), lowest fresh biomass (0.02±0.00g) and lowest dry biomass (0.002±0.000g). These two experimental groups were statistically different.

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 control group (0 K concentration) produced highest stevioside concentration (4.26±0.00mg/ml) while 0.5x K concentration produced lowest stevioside concentration (2.92±0.00mg/ml). These two experimental groups differ significantly. Moreover, 2x K concentration produced highest rebaudioside A concentration (1293.06±0.58mg/ml) (Fig. 11) while control group (0 K concentration) produced lowest rebaudioside A concentration (746.16±1.23mg/ml). These two experimental groups were also differ significantly.

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	1.72	y = 2.1795x + 9.1627	0.9999
*y = peak area, x = concentration			

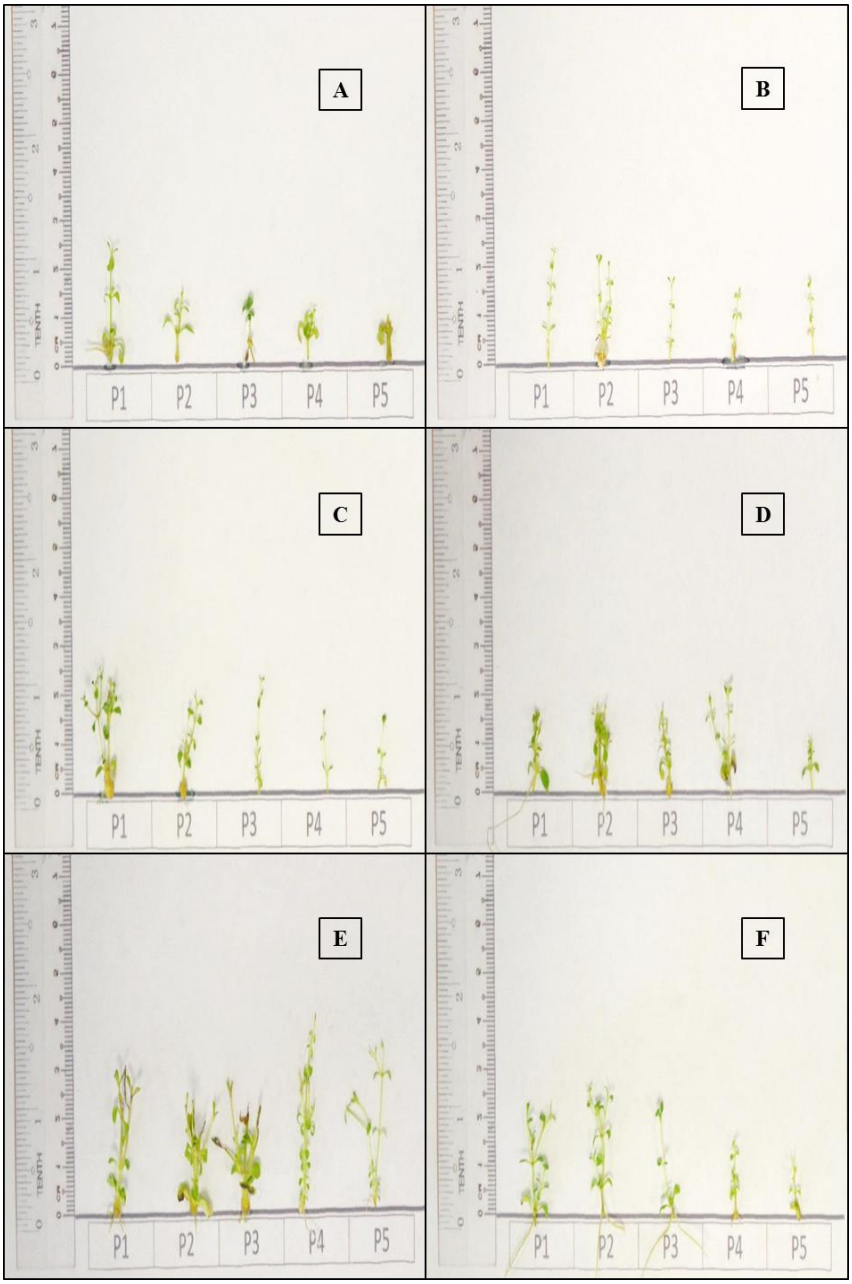

Fig. 1: *S. rebaudiana* (Bert.) plantlets from media with different K concentrations: A) 0 K; B) 0.5x K; C) 1x K; D) 2x K; E) 4x K and F) 8x K.

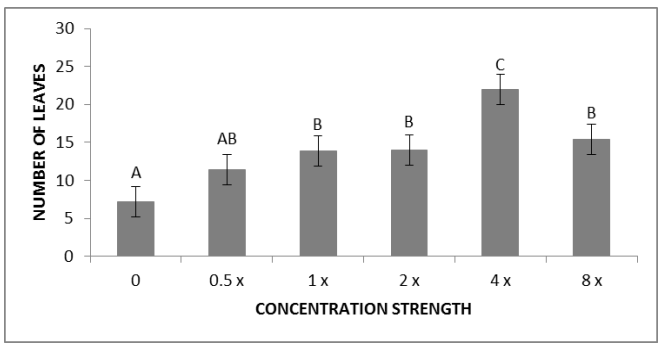

Fig. 2: Effect of K concentrations toward number of leaves of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

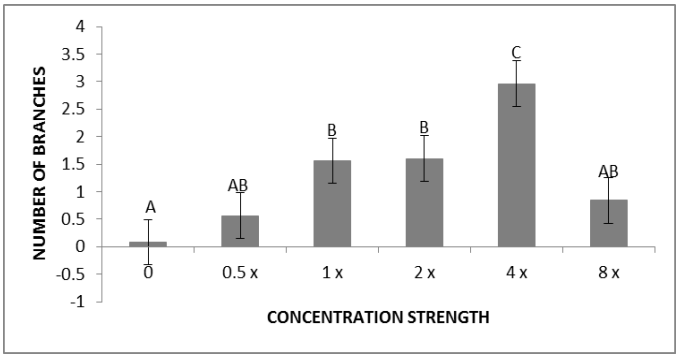

Fig. 3: Effect of K concentrations toward number of branches of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

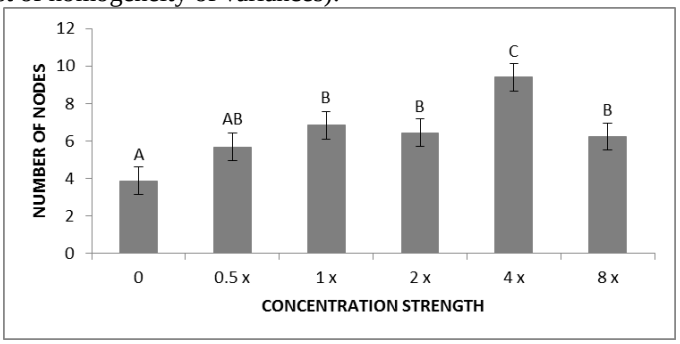

Fig. 4: Effect of K concentrations toward number of nodes of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

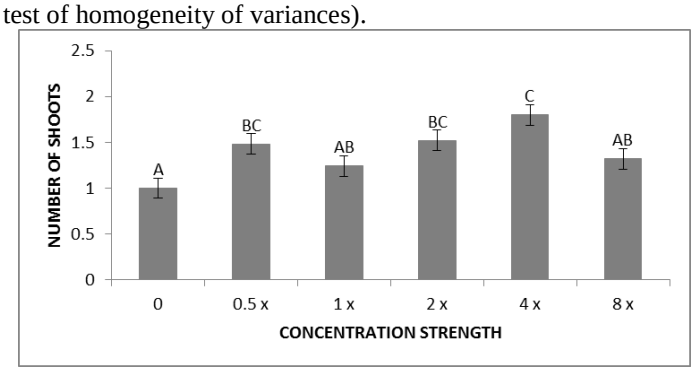

Fig. 5: Effect of K concentrations toward number of shoots of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

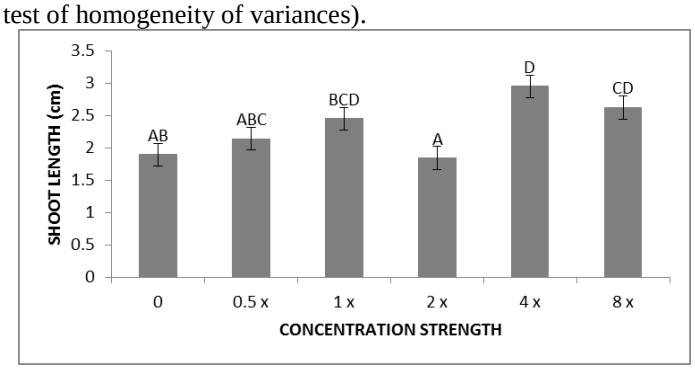

Fig. 6: Effect of K concentrations toward shoot length (cm) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

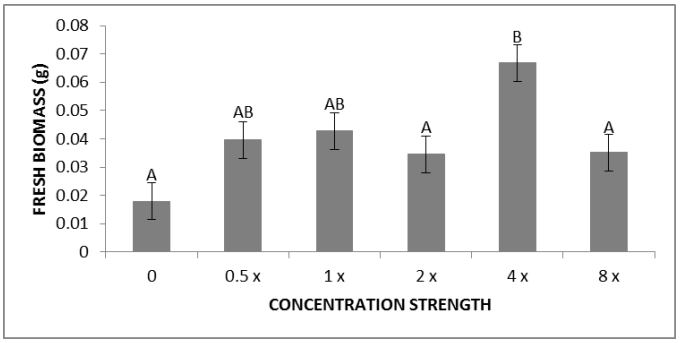

Fig. 7: Effect of K concentrations toward fresh biomass (g) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

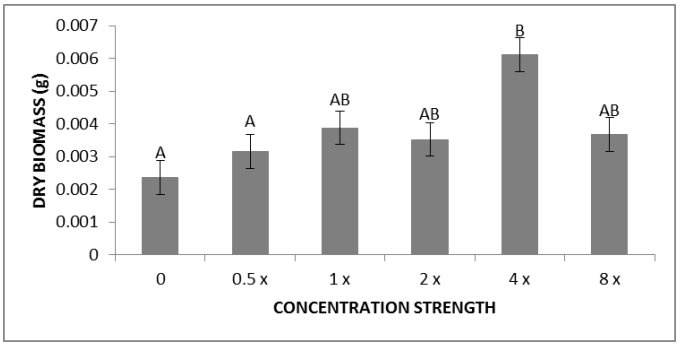

Fig. 8: Effect of K concentrations toward dry biomass (g) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

The value of F ratio, significance level and eta square of all parameters that had been observed can be seen in Table 5.

Table 5: The value of F ratio, significance level and eta square.

Parameters	F ratio (F)	Significance level (p)	Eta square value
Number of leaves	F(5,144)=12.384	p<0.000	η^2=0.301
Number of branches	F(5,144)=9.910	p<0.000	η^2=0.256
Number of nodes	F(5,144)=10.744	p<0.000	η^2=0.272
Number of shoots	F(5,144)=6.314	p<0.000	η^2=0.180
Shoot length	F(5,144)=9.119	p<0.000	η^2=0.240
Fresh biomass	F(5,144)=4.993	p<0.000	η^2=0.148
Dry biomas	F(5,144)=3.513	p=0.005	η^2=0.109
Stevioside concentration	F(5,12)=637.390	p<0.000	η^2=0.996
Rebaudioside A	F(5,12)=12171.283	p<0.000	η^2=1.000

Discussion:

The results showed that when the concentration of K was increased until 4x, the height and other physical characteristics of the plantlets were increased. The concentration of stevioside was highest in the media without K while the concentration of rebaudioside A was highest when the concentration of K was 2x. The height and other physical characteristics of *S. rebaudiana* (Bert.) plantlets were directly proportional with the increment of K concentration in the media and 4x was the concentration of K that can produce the highest of these physical characteristics. Further K concentration increment beyond 4x leads to decrement of the observed physical characteristics.

Furthermore, the results from this study showed that 0, 0.5x, 1x and 2x of K concentration in the media were deficient for *S. rebaudiana* (Bert.) to produce the highest physiological characteristics. This can be explained with the studies by other researchers. Gardia and Benlloch [9] showed that the beginning of K^+ deficiency in plants is growth retardation and the growth rate of internodes is affected. At an advanced stage of K^+ deficiency, chloroplasts [19] and mitochondria collapse [13]. K^+ deficient plants have a low-energy status [8] because K^+ is essential for efficient energy transfer in chloroplasts and mitochondria.

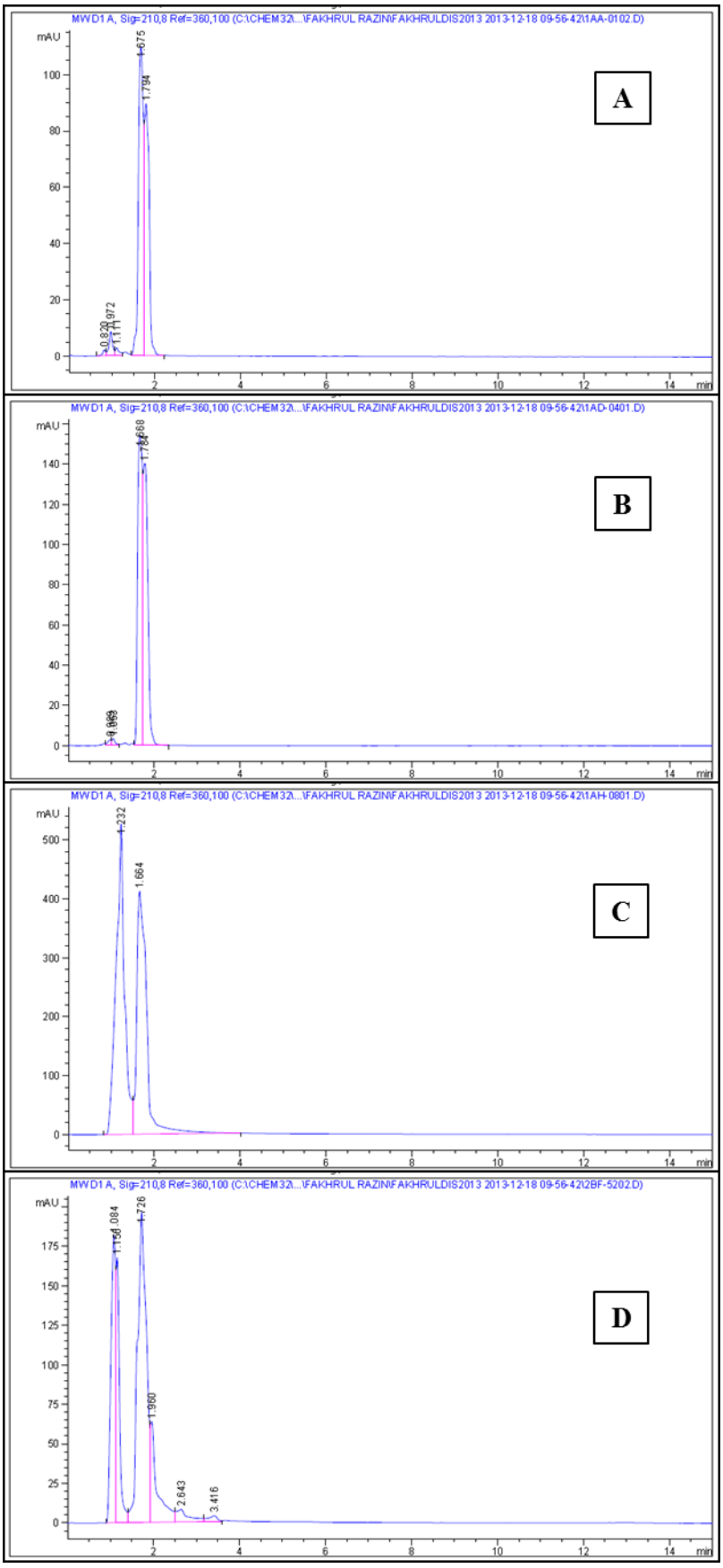

Fig. 9: HPLC chromatograms of: A) Stevioside standard; B) Rebaudioside A standard; C) 0 K and D) 2x K.

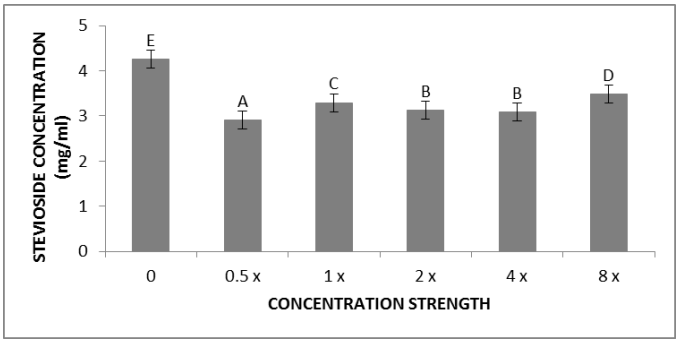

Fig. 10: Effect of K concentrations toward stevioside concentration (mg/ml) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

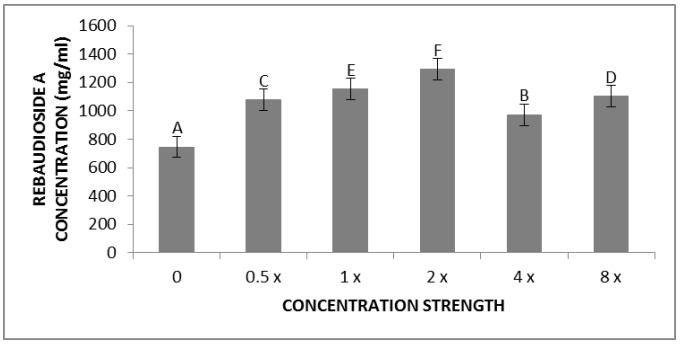

Fig. 11: Effect of K concentrations toward rebaudioside A concentration (mg/ml) of *S. rebaudiana* (Bert.) plant tissue culture (different letters in the figure indicate that they are significantly different at $p< 0.05$ according to Tukey's HSD test of homogeneity of variances).

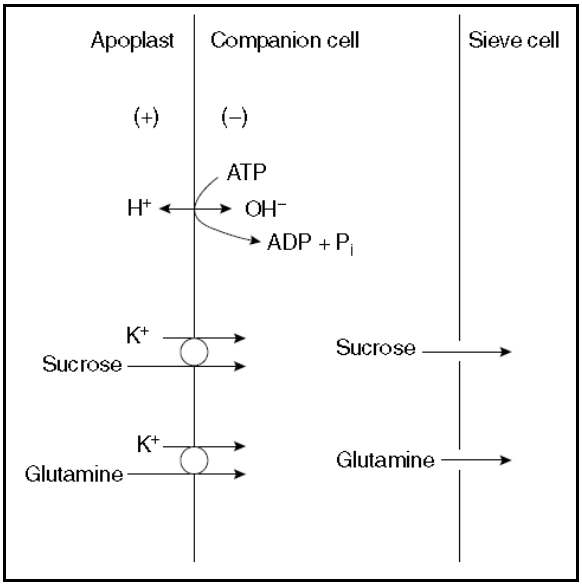

Fig. 12: Cotransport of K^+/sucrose and K^+/glutamine from the apoplast into the companion cell, and from there into the sieve cell, driven by the plasmalemma ATPase (adapted from Bel and Erven, 1979).

From this study, 8x K concentration was excess for *S. rebaudiana* (Bert.) physiological activities. The oversupply of K depresses plant growth and yield of *S. rebaudiana* (Bert.).

Study by Troufflard *et al*. [21] showed that K deficiency induces the biosynthesis of secondary metabolites in *Arabidopsis thaliana*. The similar happened to *S. rebaudiana* (Bert.) when the productions of stevioside was highest in media without K and productions of stevioside was highest in media with 2x K concentrations (K concentrations

below 4x were considered deficient, based on the results of this study).

Inorganic compounds comprise approximately 13% of the total extractables from *S. rebaudiana* (Bertoni), with K being the major inorganic constituent of the *S. rebaudiana* (Bert.) extract [6]. K plays several roles on plant physiological activity such as enzyme activation, protein synthesis, ion absorption and transport, osmotic function, long-distance transport, photosynthesis and respiration.

K^+ particularly can promote the uptake of sucrose and glutamine (amino compounds) into the sieve cells. These metabolites presumably are taken up into the sieve vessels via a K^+ cotransport (Fig. 12) [2]. Optimum amount of sucrose in the *S. rebaudiana* (Bert.) plantlets were then ensured the plantlet's optimum growth and development.

K^+ activates various enzymes. One of the enzymes is plasmalemma H^+-ATPase. This enzyme is responsible for excreting H^+ from the cell. It is assumed that K^+ binds to the enzyme surface, changing the enzymic conformation and thus leading to enzyme activation. Other function of K is in polypeptide synthesis in the ribosomes, since that process requires a high K^+ concentration [10]. There is indirect evidence that protein synthesis requires K^+ [12]. K^+ also functions as a crucial osmoticum. In most cells, the volume of the vacuole is relatively large, and its turgor is essential for the tissue turgor. Study by Mengel and Arneke [15], showed that the turgor were significantly higher in the *Phaseolus vulgaris* L treated with sufficient K^+ compared with insufficient K^+ supply. This higher turgor promoted cell expansion.

These functions were performed the best by *S. rebaudiana* (Bert.) plantlets in media with 4x K concentrations. 4x K concentrations were the optimum concentration for enzyme activation, protein synthesis, ion absorption and transport, osmotic function, long-distance transport, photosynthesis and respiration in *S. rebaudiana* (Bert.) where these were represented in the productions of the highest numbers of leaves, number of branches, number of nodes, number of shoots, highest shoot length, fresh biomass, dry biomass.

Conclusion:

Different K 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. 4x K concentration is best for producing all of the studied physical characteristics while 0 K and 2x K concentration is best for stevioside and rebaudioside A production, respectively.

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