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Carbohydrate Concentration Influences on *In Vitro* Plant Regeneration in *Stevia rebaudiana*

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Summary

In the present study effect of various carbon sources such as sucrose, glucose, fructose and maltose was investigated on *in vitro* shoot regeneration of *Stevia rebaudiana* using nodal explants. The frequency, growth and multiplication rate were highly influenced by the type and concentration of carbon sources used. The highest number of shoots (21.4±0.80), shoot length (5.36±0.55) was obtained on MS medium supplemented with 4% fructose. The least number of shoots (1.3±0.47) was obtained on 6% glucose and the least shoot length (1.22±0.25) was observed on 1% sucrose. Among the different carbon sources used in the present study, fructose at 4% proved to be better choice for multiple shoot regeneration followed by sucrose, maltose and glucose, from nodal explants of *Stevia rebaudiana*.

Key Words: *Stevia rebaudiana*, Carbohydrates, Fructose, Sucrose, Maltose, Glucose, Nodal explants, Multiple shoot regeneration

Introduction

Stevia botanically called as *Stevia rebaudiana* (also called sweet leaf, or sugar lear) is a genus of about 150 species of herbs and shrubs in the asteraceae family [1]. In Telugu, *Stevia* is called Madhu patri, in Tamil Seeni tulsi, in Sanskrit Madhu patra and Madhu parani in Marathi. *Stevia* has many favourable and exciting health benefits and it is completely non-toxic. The herb is nutrient rich, containing substantial amounts of protein, calcium, phosphorus, sodium, magnesium, zinc, rutin, vitamin A, vitamin C and other nutrients, yet has no caloric value [2]. *Stevia* contain a stevioside, a secondary metabolite responsible for the sweetness and the leaf by itself is about 20 to 30 times sweeter than sugar. Experiments proved that stevioside is 300 times sweeter than sucrose, apart from being a calorie free sugar [3]. Hence, *Stevia* has been named as calorie free "Bio-Sweetener of high quality". It is estimated that about 30 million Indians are presently suffering from diabetes and it is estimated that by 2025 India's contribution to the diabetic global population would be a whooping 80 million. Therefore the wave of 'sugar free' has become a welcome trend. Stevia shows calorie free high potency sweetener, does not contain calcium cyclamate, saccharin and aspartame and causes no side effects [4]. As a response, many no-calorie synthetic alternatives of sugar popularly known as artificial sweeteners have been discovered and replacing sugar in food and beverage industry.

In 1931, two French chemists isolated the glycosides that give *Stevia* is taste. These extracts were named as stevioside, rebaudioside A, rebaudioside C and dulcoside. Stevioside and rebaudioside induces insulin secretion [5]. Besides, stevioside acts as anti-tumour agent [6]. *Stevia* possess antifungal and antibacterial property also in addition to its other versatile uses [7]. It can be safely used in herbal medicines, tonics for diabetic patients and also in the daily usage products like mouthwashes and tooth pastes. *Stevia* has proved to give exceptional benefits when used regularly in skin care. It also has a healing effect on blemishes, wounds, cuts and scratches. *Stevia* is helpful in weight and blood pressure management [8].

In the present investigation experiments were performed to determine the effect of different carbon sources at different concentrations on shoot multiplication from axillary bud explants of *Stevia rebaudiana*.

Materials and Methods

Collection of Plant material

The plants are collected from Suveda Ayurvedic Pharmacy, P.V.Puram, Tirupati, Andhra Pradesh, India and grown in the nursery of Department of Biotechnology, S.V. University, Tirupati.

Surface sterilization

Explants were washed thoroughly under running tap water to remove the traces of dust etc. followed by treatment with 10% teepol/tween-20 for 5 minutes. Then the explants were sterilized in 70% ethanol for a minute, and finally with 0.01% $Hgcl_2$ for 1-2 minutes and washed 3-4 times with sterile double distilled water.

Culture medium

Axillary bud explants (1-2 cms) were inoculated on MS medium [9] containing 3% sucrose and gelled with 0.8% agar supplemented with different carbohydrate sources such as sucrose, fructose, glucose and maltose (1-6 %) cultured on 2 mg/L BAP. The pH of the medium was adjusted to 5.8 before

gelling with agar and autoclaved for 20 minutes at 121^{0}C for 15 lbs pressure.

Sub culturing

Subculturing was carried out at regular intervals of thirty days. Visual observations of the cultures were taken for every transfer and the effects of different treatments were quantified on the basis of percentage of cultures showing response.

Culture conditions

The growth room conditions maintained for *in vitro* cultures were 26 ± 2^{0}C and 60-70% relative humidity, light intensity was 3000 lux with a photoperiod of 18 hrs day light and 6 hrs dark. Each experiment was conducted at least thrice with 20 replicates per treatment

Results

Influence of different carbohydrates on multiple shoot regeneration

Among the different carbohydrate sources used, fructose performed well followed by sucrose, maltose and glucose in keeping shoot number constant. The maximum shoot number (21±0.6) was recorded at MS medium supplemented with 4% of fructose with a maximum mean shoot length of (5.36±0.55) (Table-1 and Figure-1). Least mean number of shoots 1.3±0.47 was observed in MS medium supplemented with glucose 6% with a mean shoot length of 1.5±0.35. Highest frequency of shoot regeneration was observed both at 4% fructose and 3% of sucrose. In maltose the highest mean number of shoots 9.0±0.56 was observed at 4% whereas 3% of maltose gave 8.8±0.84 as mean number of shoots. Less number of shoots 2.44±0.51 were observed at 1% of maltose. In all concentrations tested the second highest mean number of shoots 17.7±1.08 was observed at 3% of sucrose with a shoot length of 3.89±0.60 and 1.33±0.50 was the mean number of shoots observed at 1% sucrose. The regeneration frequency, number of shoots and shoot length was increased in carbohydrate concentration upto (1-4%). Further increasing carbohydrate concentration (5-6%) resulted in reduced shoot frequency, shoot number and shoot length was observed. Well developed shoots with a length of (3-5cm) were excised and transferred to MS medium supplemented with different concentrations of auxins such as IBA, IAA and NAA (0.1–2.0 mg/l). In all the concentrations tried, exogenous supply of auxins favoured the root formation and root primordial appeared between 7-10 days of inoculation. High rooting frequency (98%) with highest number of roots (30.3±1.85) were obtained in IBA (0.5 mg/L). The maximum root length (3.8±0.10) was observed at IAA (0.1 mg/l) (Table-2 and Figure-1). Higher concentrations of IBA, IAA and NAA showed the organogenic callus formation. From the results it is evident that lower concentration of carbohydrates favours the shoot organogenesis where as at higher concentration it diminishes the shoot regeneration.

Table.1: Effect of different carbohydrate sources on multiple shoot regeneration from nodal explants of *Stevia rebaudiana*

Carbohydrate source	Concentration (%)	Regeneration frequency (%)	Mean no. of shoots	Mean length of shoots (cm)
Glucose	1	59	2.0±0.42	2.15±0.05
	2	61	2.2±0.53	2.68±0.07
	3	66	3.6±0.55	2.96±0.0
	4	82	4.75±0.4	4.68±0.39
	5	70	2.16±0.45	2.23±0.18
	6	64	1.3±0.47	1.5±0.35
Fructose	1	73	3.27±0.49	2.2±0.18
	2	79	3.36±0.47	3.71±0.16
	3	86	8.55±0.03	3.62±0.06
	4	95	21.4±0.8	5.36±0.55
	5	90	11.22±1.0	4.55±0.36
	6	74	7.87±0.35	5.01±0.47
Maltose	1	62	2.44±0.55	2.54±0.27
	2	65	2.78±0.0	2.26±0.11
	3	81	8.8±1.08	1.97±0.66
	4	76	9.0±0.3	2.34±0.23
	5	72	3.8±0.05	2.81±0.21
	6	64	4.0±0.39	1.76±0.12
Sucrose	1	68	1.33±0.51	1.22±0.25
	2	82	10.92±0.55	2.33±0.17
	3	98	17.7±0.84	3.89±0.24
	4	86	4.5±0.56	2.01±0.12
	5	79	4.33±0.25	2.10±0.28
	6	77	1.75±0.0	2.48±0.29

Observation: after 4 weeks, values are mean ± SE of 20 independent determinations.

Table.2: Root organogenesis of *in vitro* derived shoot lets in MS medium supplemented with various concentrations of auxins such as IBA, IAA and NAA

Plant growth regulator (mg/L)			Frequency of root formation (%)	Mean number of roots/ shoot	Mean length of root (cm)	Callus
IBA	IAA	NAA				
0.1	-	-	88	21.7±0.85	3.17±0.08	-
0.5	-	-	96	27.0±0.57	3.76±0.14	-
1.0	-	-	98	30.3±1.85	2.73±0.49	-
2.0	-	-	80	22.5±0.75	2.12±0.80	C^{++}
-	0.1	-	83	17.3±0.88	3.8±0.10	-
-	0.5	-	91	22.0±0.57	3.73±0.21	-
-	1.0	-	88	20.2±0.94	2.17±0.33	C^{+}
-	2.0	-	72	11.4±0.60	1.90±1.20	C^{+}
-	-	0.1	85	12.2±0.94	3.4±0.12	-
-	-	0.5	91	16.6±1.20	2.53±0.08	C^{+}
-	-	1.0	94	15.6±0.88	1.53±0.26	C^{+}
-	-	2.0	81	7.5±0.62	1.20±0.15	C^{++}

Observation: After 4 weeks; values are mean ± SE of 20 independent determinations; C^{+} - Poor callus; C^{++} - Moderate callus, C^{+++} - High callus

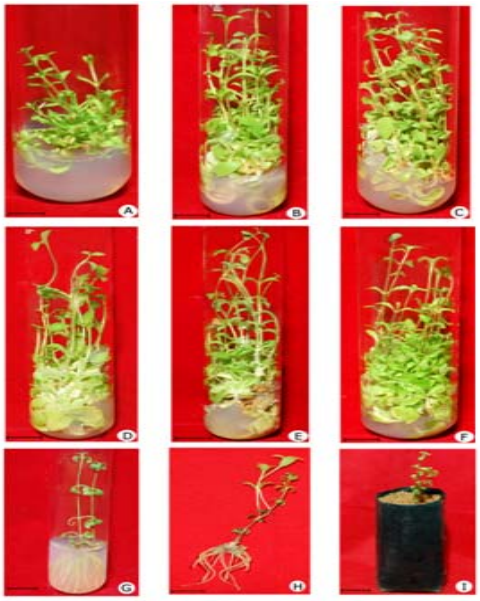

Figure-1: Effect of different carbon sources at different concentrations on shoot morphogenesis from axillary bud explants cultured on MS + 2 mg/L BAP. Shoot bud initiation from axillary bud explant
A) Sucrose (2 %) (bar 1 cm = 1.3 cm); B) Sucrose (3 %) (bar 1 cm = 1.4 cm);
C) Fructose (3 %) (bar 1 cm = 1.1 cm); D) Fructose (4%) (bar 1 cm = 1.2 cm)
E) Maltose (4%) (bar 1 cm = 1.1 cm); F) Glucose (4%) (bar 1 cm = 1.1 cm)
G) Rooting of *in vitro* regenerated shoot on half-strength MS medium supplemented with IBA 0.5 mg/L (bar 1.0 cm = 0.85) ; H) Rooted plantlet showing profuse and elongated roots culture on half-strength MS medium supplemented with NAA 0.1 mg/L (bar 1.0 cm = 1.2) ; I) Hardened plant transplanted to polybag containing garden soil and sand (bar 1.0 cm = 2.5)

Acclimatization and hardening

Well rooted plantlets were separated from the culture tubes, washed and transferred to polybags containing soil + vermiculite in 1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions. Rooted shoots showed the maximum percentage of survival.

Discussion

The growth and multiplication of shoots *in vitro* are affected by many factors [10], one of which was the concentration and type of exogenous carbon sources added to medium to serve as energy and also to maintain the osmotic potential [11]. Plant cells and tissues under *in vitro* conditions are mixotrophic in nature and needs supply of external carbon source for its metabolic activities. The solution of sucrose as the most suitable energy source for culture follows many comparisons between possible alternatives. Several carbohydrates, in addition to sucrose, are translocated and metabolized in plants [12], but little information is available regarding the effect of carbohydrates on *in vitro* embryogenesis and plant regeneration.

Sucrose have been proved to be better for shoot proliferation than other carbon sources in micropropagation of several plant species such as patchouli *Pogostemon cablin* [13] and *Centella asiatica* [14]. But in the present study high

frequency, maximum number of shoots was induced on fructose supplemented medium. The results obtained are in line with the earlier observations in *Mulbury* [15], where addition of fructose instead of sucrose in the multiplication medium increased the shoot number and also growth of the shoots. The beneficial effect of glucose on direct shoot formation was emphasized in *Prenus mume* [16]. Many authors have reported that various sources of carbon such as glucose, fructose, mannitol and sorbitol play an important role in tissue culture of *Asparagus* [17] and *Cucumber* [18]. Although sucrose has been the carbohydrate of choice in the vast majority of work on *in vitro* shoot induction and shoot development in woody species, it is not always the most effective carbon source for these purposes [19]. Even though carbohydrates are of prime importance for cell growth, maintenance and differentiation *in vitro*, the fundamental aspects of carbon utilization and metabolism in cell and tissue cultures have to be fully under stood [20]. Thus, the carbohydrate requirements have yet to be defined and optimized in micropropagation systems.

Among the different carbohydrates used fructose can be considered as best carbohydrate followed by sucrose, maltose and finally glucose in producing maximum number of shoots per explant.

Conclusion

In the present study growth of *Stevia rebaudiana* is greatly influenced by different carbon sources supplemented in the media. The present study leads to the conclusion that *stevia* cultures have quite selective carbohydrate requirements. These results could contribute to the improvement in the micropropagation of this economically important medicinal plant on commercial scale to meet the present day demand.

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