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Effect of Bavistin and Silver Thiosulphate on *In Vitro* Plant Regeneration of *Stevia rebaudiana*

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Summary

Effect of Bavastin (fungicide), Silver thiosulphate (Ehtylene inhibitor) on shoot regeneration using axillary bud explants of *Stevia rebaudiana* was studied. In Bavastin supplemented medium multiple shoots were induced from axillary bud explants. Bavistin in combination with BA induced maximum number of shoots (6.4±0.2). Ethylene inhibitor silver thiosulphate also favoured the shoot morphogenesis. At lower concentration of silver thiosulphate (10-40 μM/L) maximum number of shoots (2.1-3.2) were obtained. All the *in vitro* raised shoots with a length of 3-5 cm were transferred to rooting medium. The best rooting response was observed on 2.0mg/L IBA. The well rooted plantlets were transferred to polybags containing soil + vermiculite in 1: 1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions for maximum survivability.

Key Words: *Stevia rebaudiana*, Bavistin, Silver thiosulphste, BA, Shoot regeneration

Introduction

In recent years there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and aromatic medicinal plants [1].

Stevia is an outstanding herb bearing leaves of very refreshing sweet taste and remarkable health promoting activities. *Stevia* is botanically called as *Stevia rebaudiana* and is belongs to asteraceae family [2]. In Telugu, *Stevia* is called Madhu patri, in Tamil Seeni tulsi, in Sanskrit Madhu patra and Madhu parani in Marathi. 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 [3]. *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 [4]. 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 [5]. 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.

Stevia possess antifungal and antibacterial property also in addition to its other versatile uses [6]. 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* is helpful in weight and blood pressure management [7].

The present study was aimed to understand the influence of bavistin (a fungicide), silver thiosulphate (ethylene inhibitor) on *in vitro* shoot regeneration 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, Tirupathi, A.P., India.

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 [8] containing 3% sucrose and gelled with 0.8% agar supplemented with bavistin (50-300 mg/L) alone or in combination with BA, Kn (2.0 mg/L) and STS (silver thiosulphate) (10-120 μM/L). The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 minutes at 121^0C 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

Effect of bavistin on the in vitro plant regeneration from axillary bud explants of field grown S. rebaudiana plants

The axillary bud explants cultured on MS medium supplemented with 200 mg/L bavistin is the most effective in terms of regeneration frequency (96%), number of shoot bud regeneration (3.6 ± 0.1). The maximum shoot length (8.1 ± 0.1) was obtained in 150 mg/L of bavistin (Table-1; Figure-1). Bavistin (100 mg/L) in combination with BA (2.0 mg/L) showed the increased shoot number (6.4 ± 0.20), decreased frequency of regeneration (90) and the shoot length (7.0 ± 0.1 cm) when compared with the cultures having only bavistin. Bavistin with kinetin also showed less regeneration frequency (86), shoot length (6.8 ± 0.3) and an increase in shoot number (4.0 ± 0.1). MS basal medium with 3% sucrose and devoid of growth regulators and bavistin does not show any regeneration.

Table-1: Effect of bavistin singly and in combination with plant growth regulators on *in vitro* shoot organogenesis from axillary bud explants of field grown plants of *S. rebaudiana* plants; Observation: after 4 weeks; values are mean ± SE of 20 independent determinations

Bavistin (mg/L)	BA (mg/L)	Kn (mg/L)	Frequency of shooting response (%)	Number of shoots / explant	Length of shoot (cm)
50	-	-	78	2.0 ± 0.12	6.6 ± 0.4
100	-	-	82	2.4 ± 0.4	7.5 ± 0.1
150	-	-	94	3.1 ± 0.2	8.1 ± 0.1
200	-	-	96	3.6 ± 0.1	6.3 ± 0.6
300	-	-	80	2.1 ± 0.1	6.0 ± 0.5
100	2.0	-	90	6.4 ± 0.2	7.0 ± 0.1
100	-	2.0	86	4.0 ± 0.1	6.8 ± 0.3
-	2.0	-	88	8.2 ± 0.3	5.0 ± 0.9
-	-	2.0	75	3.5 ± 0.1	7.1 ± 0.1

Table-2: Effect of different concentrations of silver thiosulphate (STS) supplemented in MS medium, on regeneration of plantlets from axillary bud explants of *S. rebaudiana*; Observation: after 4 weeks; values are mean ± SE of 20 independent determinations

STS (µM/L)	Frequency of regeneration (%)	Number of shoots / explant	Length of shoot (cm)
10	83	3.2 ± 0.1	4.3 ± 0.1
20	95	2.5 ± 0.3	4.9 ± 0.1
30	90	1.9 ± 0.1	5.5 ± 0.3
40	74	2.1 ± 0.2	5.8 ± 0.2
50	79	1.4 ± 0.2	3.1 ± 0.4
80	54	1.2 ± 0.3	4.5 ± 0.2
100	42	1.0 ± 0.1	3.7 ± 0.1
120	40	1.0 ± 0.1	3.4 ± 0.2

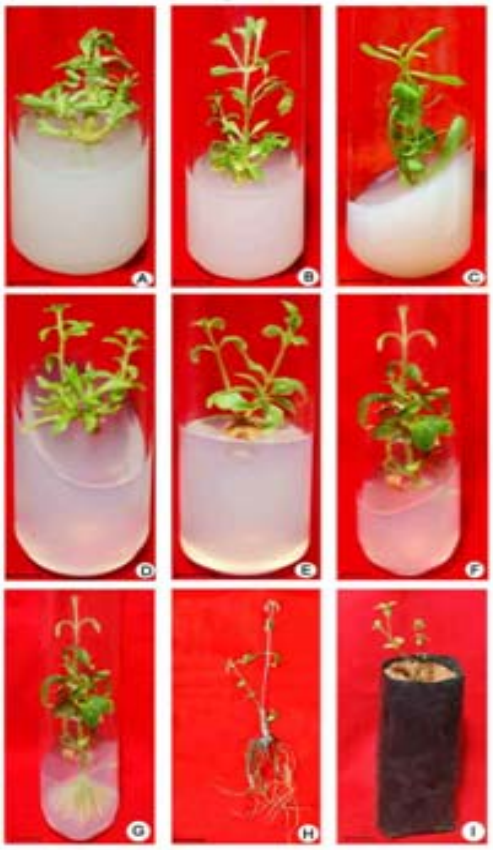
Table-3: Root organogenesis of *in vitro* derived shoot lets in MS medium supplemented with various concentrations of auxins such as IBA and NAA

Plant growth regulator (mg/L)		Frequency of root formation (%)	Mean number of roots / Shoot	Mean length of root (cm)	Callus
IBA	NAA				
0.1	-	74	7.0 ± 1.22	4.52 ± 0.93	-
0.5	-	83	17.3 ± 0.88	3.8 ± 0.10	-
1.0	-	91	22.0 ± 0.57	3.73 ± 0.21	-
2.0	-	88	20.2 ± 0.94	2.17 ± 0.33	C$^+$
3.0	-	72	11.4 ± 0.60	1.90 ± 1.20	C$^+$
-	0.1	82	8.3 ± 0.88	4.9 ± 0.62	-
-	0.5	85	12.2 ± 0.94	3.4 ± 0.12	-

-	1.0	91	16.6 ± 1.20	2.53 ± 0.08	C^{+}
-	2.0	94	15.6 ± 0.88	1.53 ± 0.26	C^{+}
-	3.0	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



Axillary bud explants cultured on MS basal medium supplemented with bavistin
A. Shoot bud initiation from axillary bud explant MS + bavistin (100 mg/L) (bar 1cm = 0.8 cm); B. Multiple shoot induction from axillary bud explant MS + bavistin (150 mg/L) (bar 1cm = 1.1 cm); C. Multiple shoots regenerated from axillary bud explant MS + bavistin (200 mg/L) (bar 1cm = 1 cm);
Shoot regeneration from axillary bud explants cultured on MS media supplemented with silver thiosulphate; Shoot bud initiation from axillary bud explant
D. MS + silver thiosulphate (20 µM/L) (bar 1 cm = 1.0 cm); E. MS + silver thiosulphate (40 µM/L) (bar 1 cm = 1.1 cm); F. MS + silver thiosulphate (80 µM/L) (bar 1 cm = 1.0 cm)
***In vitro* root organogenesis from regenerated shoots**
G. MS + IBA (0.1 mg/L) (bar 1cm = 1.2 cm); H. MS + IBA (0.1 mg/L) (bar 1cm = 1.0 cm)
H. Tissue cultured plantlet in polybag after hardening (bar 1cm = 2 cm)

Effect of ethylene inhibitor silver thiosulphate on the regeneration of axillary bud explants of S. rebaudiana

Bud break from nodal explants was observed *in vitro* one week after inoculation on MS basal media supplemented with different concentrations of silver thiosulphate. Silver thiosulphate, at any concentration tested, had a positive effect and showed shoot formation. Highest frequency of regeneration (95%) was observed at 20 µM/L STS and maximum number of shoots (3.2 ± 0.1) was achieved with 10 µM/L STS, added to MS medium. Maximum shoot length (5.8 ± 0.1 cm) was recorded at 40 µM/L concentration of STS (Table-2; Figure-1). Lower concentrations of STS favoured the high frequency of regeneration and highest number of shoots. The optimum range of STS concentrations recorded was between 10 – 40 µM/L.

In vitro derived shoots with a length of (3-5cm) were excised and transferred to MS medium supplemented with different concentrations of auxins such as IBA and NAA (0.1–2.0 mg/l). In all the concentrations tried, exogenous supply of auxins favoured the root formation. High rooting frequency (91%) with highest number of roots (3.73 ± 0.21) were obtained in IBA (1.0 mg/L) (Table-3 and Figure-1).

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

Bavistin appeared to have much stronger cytokinin-like activities. This is evident from a promotory effect of bavistin on shoot bud regeneration. Bavistin is a systemic fungicide that belongs to benzimidazole family [9]. It has been reported that the molecular structure of methyl benzimidazole carbamate or carbendazim (bavistin) has some resemblance to kinetin, adenine and to many other cytokinins based on adenine [10]. Earlier reports have shown that bavistin induces differentiation of roots and shoots in calli derived from carrot segment [10]. The shoot regeneration promoting activities of bavistin are results of increased biosynthesis of endogenous cytokinins with in the cultures. Bavistin was found to be the least toxic to plant cells and has a broad-spectrum fungicidal activity [11]. It has also been demonstrated that these compounds can have beneficial effects on the physiology of the plant [12]. From the present study it has been known that the usage of bavistin to control the fungal contamination does not show any negative effect on *S. rebaudiana* cultures and further promotes the shoot regeneration.

Ethylene inhibits the shoot morphogenesis and also affects the root formation. Ag^+ ions inhibit ethylene action in a wide variety of ethylene induced responses in plants by reducing the receptor capacity to bind ethylene [13, 14]. These findings are in agreement with the reports in *Decalepis hamiltonii*, where silver nitrate favoured the shoot morphagenesis [15]. The addition of STS to the regeneration media increased regeneration in both cultivars, Canino and Helena [16]. These results are in line with the present investigation, silver thiosulphate at lower concentrations (10-40 μM/L) favors the *in vitro* shoot regeneration of *Stevia rebaudiana*.

Conclusion

From the results it is evident that fungicide bavistin, ethylene inhibitor silver thiosulphate does not shows any negative effect on shoot regeneration. Thus these promissory compounds may be useful as a media supplement to develop efficient protocols for *in vitro* propagation of *Stevia rebaudiana* as it favors the shoot formation.

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