MICROPOPAGATION OF STEVIA

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ABSTRACT

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The experiment was conducted at Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, during the period of July to December, 2005. *In vitro* propagation of Stevia was attempted by multiple shoot regeneration from the explants (shoot tips and nodal segments) followed by rooting of multiplied shoots. MS media in two different strengths (full MS and half MS) were used as culture media for shoot proliferation as well as root regeneration. Cytokinins viz. BAP and Kn were used for shoot multiplication in four concentrations (0, 0.5, 1.0, and 1.5 mg/l). Auxins viz. NAA and IBA were used for root induction of microcuttings in four concentrations (0, 0.5, 1.0, and 1.5 mg/l). Shoot tips showed better response for shoot proliferation than nodal segments. MS media in half and full strength were equally effective in shoot proliferation. BAP (1.0 mg/l) was superior to all other hormonal treatments for shoot proliferation. Full MS was superior to half MS. Full MS supplemented with NAA (1.5 mg/l) was the best medium for rooting of microcuttings.

Keywords: Stevia, *in vitro* propagation, explants

INTRODUCTION

Stevia (Stevia rebaudiana Bert.) is a semi bushy herb with natural sweetening compounds that are non-calorific, and is 230 times sweeter than sucrose (Kinghorn, 1987). Stevia is helpful for hypoglycemia and diabetes because it nourishes pancreas and thereby helps to restore its normal function (Soejarto et al., 1983) and Oviedo (1971) reported a 35.2% fall in normal blood sugar levels 6-8 hours following the ingestion of Stevia leaf extract. Seed germination rate is often poor, less than 10% are common. (Miyazaki and Wantenabe, 1974). Because of instability of plantlets produced from stem cutting, micropropagation is the most reliable method of Stevia propagation (Darekar, 2004). The present investigation was undertaken to identify the suitable sources of explants for micropropagation and to find out suitable media for micropropagation.

MATERIALS AND METHODS

The experiment was conducted at Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh during the period of July-December, 2005. The materials and methods used in these investigations are described below:

***Plant materials*:** Shoot tips and nodal segments were used as explants for shoot multiplication. All the explants were collected from 12-14 months old yard grown plants at Biotecnology Laboratory, BSRI. The explants were cut into small pieces (about 1.5 cm long) and then washed with 1% savlon with constant shaking for 5-6 minutes and washed thoroughly with distilled water (7-8 times). Then the explants were taken under laminar air flow cabinet and surface sterilized with 0.1% mercuric chloride solution for 5 minutes under aseptic condition followed by washing 7-8 times with sterilized distilled water. Then explants were inoculated aseptically on culture medium.

For root induction, shoot proliferated on culture medium are taken under laminar air flow cabinet and aseptically cut (into small pieces of 1 cm) and transplanted on root induction medium.

Culture medium

Shoot proliferation medium: MS (Murashige and Skoog, 1962) media in two strengths i.e., full MS and ½ MS (all the ingredients of MS medium were reduced to half of its original concentration) supplemented with eihter 6- Benzyladenopurine (BAP) or kinetin (Kn) at varying concentrations (0. 1.0, 2.0 and 3.0 mg/l) were prepared for shoot proliferation. After mixing all stock solutions (for preparation of MS media) 3% and 1.5% sugar was added for full MS and ½ MS respectively then the pH of the media was adjusted to 5.7-5.8. The agar (.06%) was dissolved and the medium was dispensed in the test tubes and capped with cotton plugs. Test tubes were then autoclaved at 121^0C at 15 psi for 20 minutes.

Root induction medium: Full MS and ½ MS media supplemented with either 1-Napthaleneacetic acid (NAA) or Indole-3-butyric acid (IBA) at varying concentrations (0, 0.5, 1.0 and 1.5 mg/l) were prepared for rooting. Sugar was added as above. Then the pH of the medium was adjusted to 5.8. To solidify, 0.6% agar was added to the

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medium. Then the medium was dispensed in test tubes and sterilized as mentioned above. In each of the treatment 24 shoots were inoculated.

Inoculation technique

General aseptic techniques concerning *in vitro* culture of the explants were followed in the present experiment. After surface sterilization, the explants were cut into very small pieces (about 1cm) and inoculated onto shoot induction and proliferation media. When the shoot was fully grown (for 1 month), the shoots were taken out from the test tube in a laminar airflow hood and were cut (about 1cm). Then the micro-cuttings were subcultured on root induction media (Figure 1). All inoculations and aseptic manipulations were carried out in a laminar airflow cabinet. Before use, the working surface of the cabinet was cleaned by swabbing with 90% ethyl alcohol and UV light (for 20 min) to reduce the chances of contamination. The instruments like scalpels, forceps, needles, etc. were sterilized by an alcoholic dip followed by flaming inside the laminar airflow cabinet. Other requirements like Petri dish, bottles, conical flasks, cotton, distilled water etc. were sterilized by steam sterilization method. Before the onset of inoculation, hands were washed thoroughly by soap and then swabbing with 70% ethyl alcohol. Cutting and transfer of the explants were carried out taking all possible care to ensure contamination free inoculation.

Culture environment

All cultures were grown in an air-conditioned culture room illuminated by 40 W white fluorescent tubes with an intensity varied from 2000-3000 lux. The photoperiod was maintained as 16 hours light and 8 hours dark. The temperature of the culture room was maintained at 25 ± 1^0C.

Data collection

Visual observation of culture was made every day. Data were recorded for days of shoot initiation, number of shoots and number of micro-cuttings, shoot length, days of root initiation, and number of roots and length of roots. Except days of shoot and root initiation all data were collected 25 days after the inoculation.

Data analysis

All data collected were assessed by analysis of variance for factorial complete randomized design (CRD) using computer software MSTAT-C (MSTAT Development Team, 1988). Duncan's Multiple Range Test (DMRT) was applied for means separation.

Hardening of Plantlets

Rooted plantlets were transplanted to soil in a polyethylene packs or small pot. The plantlets were transplanted to soil mixture containing 50% soil and 50% sand and then hardened in relatively cooler place (at $\leq 30\ ^0$C) than the normal hardening site. Plantlets were hardened for 2-3 weeks in hardening shade.

Plantation of Plantlets to the Soil

After 2-3 weeks of hardening of plant-lets they become 7-12 inches in height and strong enough for plantation to soil under normal condition. Therefore, they are then transplanted to fields or to big pots where they were grown. Care was taken of so that roots did not get any damage during plantation.

RESULTS AND DISCUSSION

Shoot induction and proliferation

Both shoot tip and nodal segment explants produced multiple shoots. Shoot tip and nodal segments responded differently in terms of days of shoot initiation, number of shoots per culture, and number of micro-cuttings per culture (Table 1). Initiation of shoots in shoot tip explants was earlier and significantly different from that in nodal segments (3.23 and 5.26 days respectively). Remarkably higher number of shoots per culture was obtained using shoot tip as explant than the nodal segment (1.98 and 1.76 respectively). However, both the explants were statistically similar in terms of length of shoots. Shoot tip produced significantly higher number of micro-cuttings per culture than the nodal segments (6.38 and 5.83 respectively). Shoot tip, nodal segment were also used by the followings researchers. Sivaram and Mukundan (2003) reported shoot formation using shoot apex, nodal and leaf segment as explants. Primary leaf and shoot tip were used for shoot multiplication by Sritongkum in 1995. Acuna *et al.* (1997) used only nodal segment for multiple shoot production in Stevia.

The effect of MS media strengths (full MS and ½ MS) was significantly different in terms of number of shoot per culture and length of shoot (Table 2). The number of shoots per culture in full MS was significantly higher than the number of shoots per culture in ½MS media (1.96 and 1.78 respectively). Similarly, the length of shoot in full MS was significantly higher than ½ MS media (6.64 cm and 5.56 cm respectively). However, the effects of MS strengths (MS and ½ MS) were statistically similar for days of shoot initiation and number of micro-

cuttings. Therefore, ½ MS medium instead of MS full strength may be used for shoot regeneration of stevia. Acuna *et al.* (1997) used MS medium with 50% macroelements for *in vitro* culture of nodal segments.

The effect of hormones on shoot proliferation is shown in Table 3. BAP shows the significantly higher number of shoots (1.96) and micro-cuttings (5.63) per culture than the number of shoots (1.77) and micro-cuttings (4.76) per culture of Kn. However, BAP and Kn were statistically similar in terms of days of shoot initiation and length of shoots. Interaction effects of explant, MS strength, Hormones and their concentrations on days of shoot initiation, number of shoots per culture, length of shoots and number of micro-cuttings per culture is shown in Table 4. Shoot tip cultured on full MS media supplemented with BAP (0 mg/l) and Kn (0 mg/l) shows the minimum days (2.90 days) of shoot initiation. While, shoot tip cultured on full MS media supplemented with BAP (1mg/l) showed remarkably the highest number of number of shoots (3.10) and micro-cuttings (12.30) per culture. However, shoot tip cultured on ½ MS media supplemented with BAP (0 mg/l) and Kn (0 mg/l) showed the highest length of shoots (8.10 cm).

Table 1. Effects of different explants on days of shoot initiation, number of shoots per culture, length of shoots and number of micro-cuttings per culture

Explants	Days of shoot initiation	Number of shoots per culture	Length of shoots (cm)	Number of micro-cuttings per culture
Shoot tip	3.23b	1.98a*	6.38	6.08a
Nodal segment	5.26a	1.76b	5.83	4.31b

**figures within columns bearing different letters are significantly different by DMRT at p=0.05*

Table 2. Effects of strengths of MS media on days of shoot initiation, number of shoots, length of shoots and number of micro-cuttings per culture

MS Strength	Days of shoot initiation	Number of shoots per culture	Length of shoots (cm)	Number of micro-cuttings per culture
MS	4.26	1.96a*	6.64a	5.42
1/2 MS	4.23	1.78b	5.56b	4.96

*figures within columns bearing different letters are significantly different by DMRT at p=0.05

Table 3. Effect of different hormones on days of shoot initiation, number of shoots per culture, length of shoots and number of micro-cuttings per culture

Hormones	Days of shoot initiation	Number of shoots per culture	Length of shoots (cm)	Number of micro-cuttings per culture
BAP	4.27	1.96a*	5.87	5.63a
Kn	4.21	1.77b	6.33	4.76b

**figures within columns bearing different letters are significantly different by DMRT at p=0.05*

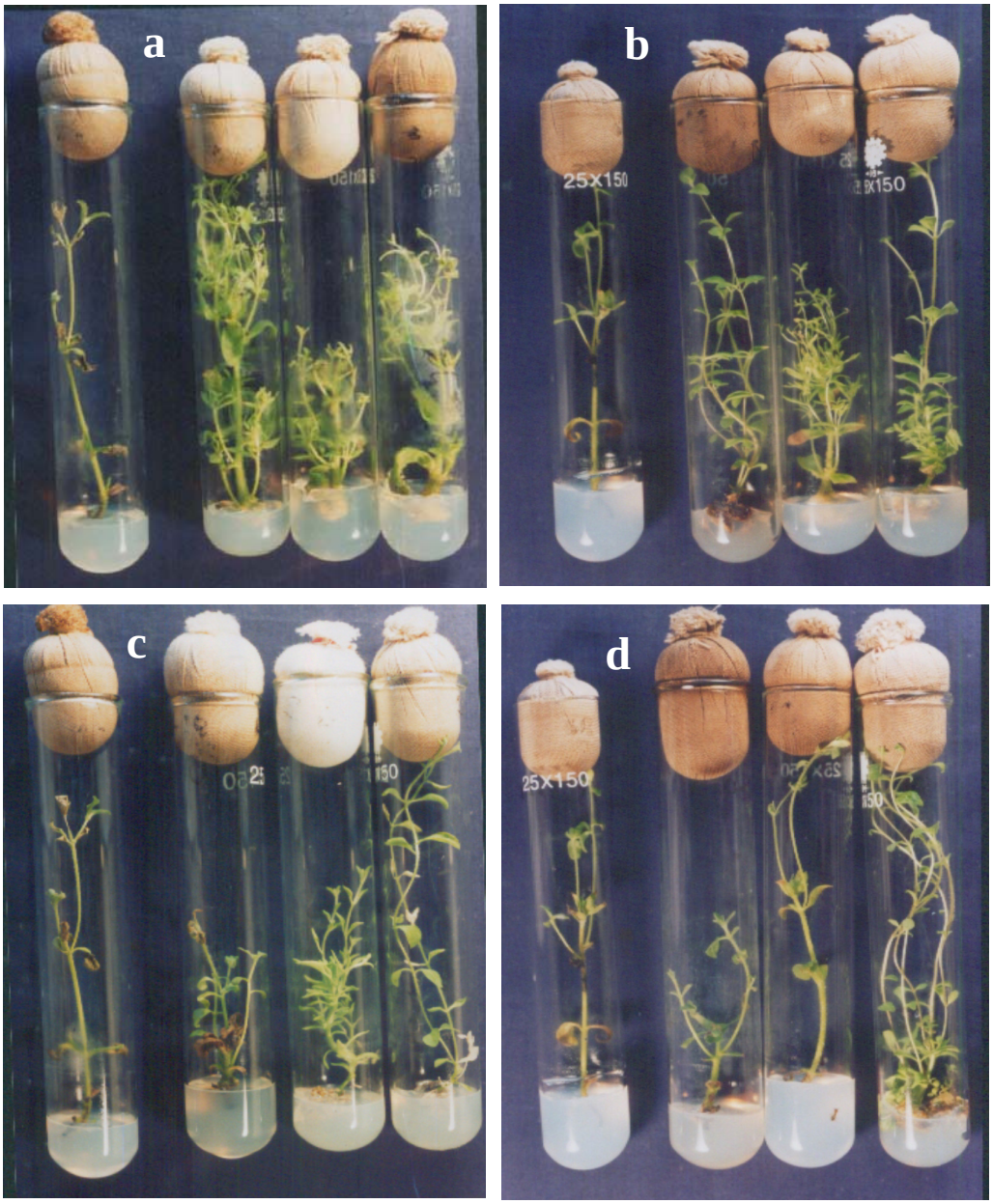

Figure 1. Effect of different media on shoot induction from shoot tips-full MS media with increasing concentrations of BAP (a), ½ MS media with increasing concentrations of BAP (b), full MS media with increasing concentrations of Kn (c), and ½ MS media with increasing concentrations of Kn (d). Tubes arranged from left to right represent 0, 1, 2, and 3 mg/l respectively.

Table 4. Interaction effects of explant, MS strength, hormones and their concentration on days of shoot initiation, number of shoots per culture, length of shoots and number of micro-cuttings per culture

	Days of shoot initiation	Number of shoots per culture	Length of shoots (cm)	Number of micro-cuttings per culture
Shoot tip: MS : BAP : 0 mg/l	2.90g	1.40ef*	8.10abc	4.70e-i
: MS : BAP : 1 mg/l	3.20fg	3.10a	8.35ab	12.30a
: MS : BAP : 2 mg/l	3.30fg	2.75abc	4.30f-j	6.40d-g
: MS : BAP : 3 mg/l	3.50efg	2.50a-d	4.80ef	6.60d-g
Shoot tip: MS : Kn: 0 mg/l	2.90g	1.40ef	8.10abc	4.70e-i
: MS : Kn: 1 mg/l	3.40efg	2.10b-e	7.30f-i	5.80d-h
: MS : Kn: 2 mg/l	3.50efg	1.90de	4.50f-j	4.30ghi
: MS : Kn: 3 mg/l	3.30 fg	2.80ab	7.80a-d	6.60d-g
Shoot tip: 1/2 MS: BAP : 0 mg/l	3.10 g	1.00f	9.40a	2.10i
: 1/2 MS: BAP : 1 mg/l	3.20 fg	3.00a	6.20b-i	7.60b-f
: 1/2 MS: BAP : 2 mg/l	3.20 fg	1.90de	3.55hij	7.90d-e
: 1/2 MS: BAP : 3 mg/l	3.40 efg	1.70ef	3.45ij	6.40d-g
Shoot tip: 1/2 MS: Kn: 0 mg/l	3.10 g	1.00f	9.40a	2.10i
: 1/2 MS: Kn: 1 mg/l	3.30 fg	1.50ef	3.48ij	2.20i
: 1/2 MS: Kn: 2 mg/l	3.20 fg	1.60 ef	6.95a-g	7.00c-g
: 1/2 MS: Kn: 3 mg/l	3.10 g	2.00 cde	6.35b-h	10.50ab
Nodal segment: MS : BAP : 0 mg/l	5.50 bc	1.50 ef	7.85 a-d	2.90 hi
: MS : BAP : 1 mg/l	4.30b-g	1.80def	7.25a-e	6.90c-g
: MS : BAP : 2 mg/l	7.50a	2.10b-e	6.30b-h	4.50f-i
: MS : BAP : 3 mg/l	3.90d-g	1.80def	5.05d-j	3.90ghi
Nodal segment: MS : Kn: 0mg/l	5.50bc	1.50ef	7.85a-d	2.90hi
: MS : Kn: 1 mg/l	4.10c-g	1.50ef	6.15b-i	3.20hi
: MS : Kn: 2 mg/l	5.80b	1.40ef	5.50b-j	2.90hi
: MS : Kn: 3 mg/l	5.50bc	1.80def	7.10a-f	8.10bcd
Nodal segment: 1/2 MS: BAP : 0 mg/l	5.40bcd	1.60ef	6.80a-g	2.10i
: 1/2 MS: BAP : 1 mg/l	5.40bcd	2.00cde	6.90a-g	9.90abc
: 1/2 MS: BAP : 2 mg/l	4.90b-e	1.60ef	2.80j	2.80hi
: 1/2 MS: BAP : 3 mg/l	5.60bc	1.70ef	2.80j	3.00hi
Nodal segment: 1/2 MS: Kn: 0 mg/l	5.40bcd	1.60ef	6.80a-g	2.10i
: 1/2 MS: Kn: 1 mg/l	5.20bcd	1.70ef	4.15g-j	2.80hi
: 1/2 MS: Kn: 2 mg/l	5.40bcd	2.00cde	5.25c-j	4.20ghi
: 1/2 MS: Kn: 3 mg/l	4.70b-f	2.50a-d	4.65c-j	6.70d-g

**figures within columns bearing different letters are significantly different by DMRT at p=0.05*

Root induction in micro-cuttings

Roots induction in micro-cuttings cultured on full MS and ½ MS media was significantly different for days of root initiation, number of roots per plant and length of roots is presented in the Table 5. Rooting was observed significantly earlier in full MS than in ½ MS (7.57 and 10.56 days respectively). Statistically significant and the higher number of roots per plantlet was observed in full MS than roots per plantlet in ½ MS (8.16 and 6.06 respectively). The higher length of roots was observed in full MS than length of roots in ½ MS (3.11 and 2.19cm respectively).

The effects of hormones and their concentrations on days of root initiation, number of roots per plantlet and length of roots are shown in Table 6. The days of root initiation was least in treatments without any hormone (7.25). Significantly, the highest number of roots per plant (10.25) was recorded in IBA (1.5 mg/l). The highest length of root (3.47) was recorded in treatment without any hormone. The roots found in treatments without any hormone are much weaker (Figure 2) and their number is much lower than the other hormonal treatments.

Sritongkum (1995) found optimal condition for root induction in micro-cuttings (derived from culture of shoot tip) was MS medium supplemented with 0.01 mg/l NAA and MS medium without plant growth regulator.

Interaction effects of MS strengths, hormones and their concentrations on days of root initiation, number of roots per plantlet and length of roots is shown in Table 7. The minimum days (6.5) of root initiation was observed in full MS media supplemented with NAA (1.0 mg/l). The remarkably highest number of roots per plantlet (13.80) was observed in full MS media supplemented with NAA (1.5 mg/l). However, the highest length of roots (3.80 cm) was observed in ½ MS media without any hormone. The effect of different MS strengths, hormones and their concentrations used for rooting is shown in Figure 2. It must be mentioned that the rooted plantlets die sooner in rooting medium. It has been recoded that above 80% of the plantlets die after 30 days of inoculation.

Table 5. Effects of MS strengths on days of root initiation, number of roots per plant and length of roots

	Days of root initiation	Number of roots per plantlet	Length of roots (cm)
MS	7.57b	8.61a*	3.11a
1/2 MS	10.56a	6.06b	2.19b

**figures within columns bearing different letters are significantly different by DMRT at p=0.05.*

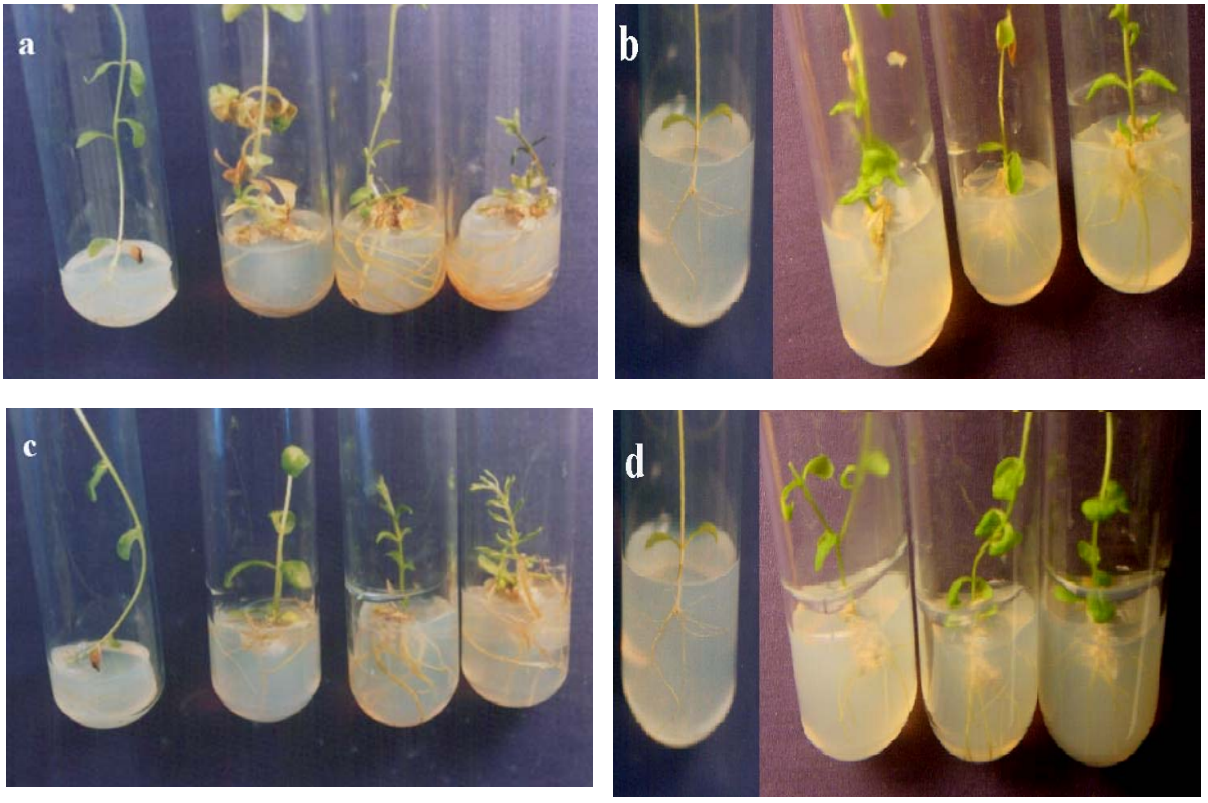

Figure 2: Effect of different media on root induction from micro-cuttings -(a) MS full strength media with increasing concentrations of NAA, (b) 1/2 MS media with increasing concentrations of NAA, (c) Full MS media with increasing concentrations of IBA, (d) 1/2 MS media with increasing concentrations of IBA. The concentrations starting from left are 0, 1, 2 and 3 mg/l respectively.

Table 6. Interaction effects of hormones and their concentration on days of root initiation, number of roots per plant and length of roots

		Days of root initiation	Number of roots per plantlet	Length of roots (cm)
NAA	: 0 mg/l	7.25b	3.30c*	3.47a
	: 0.5 mg/l	9.65a	5.70bc	2.92ab
	: 1.0 mg/l	9.35a	9.05a	2.69bc
	: 1.5 mg/l	9.55a	10.10a	2.69bc
IBA	: 0 mg/l	7.15b	3.30c	3.47a
	: 0.5 mg/l	9.50a	8.55ab	2.10cd
	: 1.0 mg/l	9.85a	8.45ab	1.76c
	: 1.5 mg/l	10.25a	10.25a	2.10cd

**figures within columns bearing different letters are significantly different by DMRT at p=0.05*

Table 7. Interaction effect of MS strengths, hormones and their concentrations on days of root initiation, number of roots per plant and length of roots

	Days of root initiation	Number of roots per plantlet	Length of roots (cm)
MS: NAA: 0 mg/l	7.50c	2.30f*	3.15abc
: NAA: 0.5 mg/l	7.10c	7.60b-e	3.65ab
: NAA: 1.0 mg/l	6.50c	11.90ab	3.65ab
: NAA: 1.5 mg/l	6.70c	13.80a	3.60ab
MS: IBA: 0 mg/l	7.30c	2.30f	3.15abc
: IBA: 0.5 mg/l	8.50bc	9.10bcd	2.58bcd
: IBA: 1.0 mg/l	8.20bc	10.80abc	2.20cde
: IBA: 1.5 mg/l	8.80bc	11.10ab	2.90abc
1/2MS: NAA: 0 mg/l	7.00c	4.30ef	3.80a
: NAA: 0.5 mg/l	12.20a	3.80ef	2.20cde
: NAA: 1.0 mg/l	12.20a	6.20def	1.73de
: NAA: 1.5 mg/l	12.40a	6.40c-f	1.77de
1/2MS: IBA: 0 mg/l	7.00c	4.30ef	3.80a
: IBA: 0.5 mg/l	10.50ab	8.00b-e	1.63de
: IBA: 1.0 mg/l	11.50a	6.10def	1.33e
: IBA: 1.5 mg/l	11.70a	9.40a-d	1.30e

**figures within columns bearing different letters are significantly different by DMRT at p=0.05*

Hardening of plantlets

Rooted plantlets were transplanted to soil in a polyethylene packs or small pot (Figure 3). At normal hardening condition and on normal soil the plantlets fail to survive. The plantlets were transplanted to soil containing 50% soil and 50% sand mixture and then hardened in relatively cooler place (at about 30 ^{0}C) than the normal hardening site (Figure 3). About 69% plantlets survived this way. Plantlets were hardened for 2-3 weeks in hardening shade.

Figure 3: Hardening of Plantlets- (a) Plantlets transplanted into polypacks, (b) Plantlets under hardening shade

Plantation of hardened plantlets to the soil

After 2-3 weeks of hardening, the plantlets they became 4-7 inches in height and strong enough for plantation to soil under normal condition. Therefore, they were then transplanted to fields or to big pots where they were grown. Care was taken of so that roots do not get any damage during plantation. About 85% plantlets were survived in pots and 75% plantlets were established in field.

SUMMARY AND CONCLUSION

Shoot tip and nodal segments respond differently in terms of days of shoot initiation, number of shoots per culture, and number of micro-cuttings per culture. Initiation of shoots in shoot tip explant was earlier than in nodal segments. Remarkably higher number of shoots per culture was obtained using shoot tip as explants than using the nodal segments (1.98 and 1.76 respectively). Shoot tip produced higher number of micro-cuttings per culture (6.38) than the nodal segments (5.83).

The number of shoots per culture in full MS was significantly higher than the number of shoots per culture in ½MS (1.96 and 1.78 respectively). The length of shoot in full MS was higher than ½ MS media (6.64 cm and 5.56 cm respectively). The effects of both MS strengths were similar for days of shoot initiation and number of micro-cuttings.

BAP showed the significantly higher number of shoots (1.96) and micro-cuttings (5.63) per culture than the number of shoots (1.77) and micro-cuttings (4.76) per culture of Kn. The effects of hormones and their concentrations were significantly different in terms of number of shoots per culture, length of shoots and number of micro-cuttings per culture. BAP (1mg/l) shows the highest number of shoots (2.48) and micro-

cuttings (9.18) per culture. While treatments without any hormone shows the highest length of shoots (8.04 cm).Shoot tip cultured on full MS media supplemented with BAP (1mg/l) showed the highest number of number of shoots (3.10) and micro-cuttings (12.30) per culture. Shoot tip cultured on full MS media supplemented with BAP (0mg/l) and Kn (0mg/l) showed the minimum days (2.90 days) of shoot initiation.

Rooting was observed earlier in full MS media than in ½ MS media (7.57 and 10.56 days respectively). The higher number of roots per plantlet was observed in full MS media than roots per plantlet in ½ MS media (8.16 and 6.06 respectively). The higher length of roots was observed in full MS media than length of roots in ½ MS media (3.11 and 2.19cm respectively).

The highest number of roots per plant (10.25) was recorded in IBA (1.5 mg/l). The highest length of root (3.47) was recorded in treatment without any hormone.

The minimum days (6.5 days) of root initiation was observed in full MS media supplemented with NAA (1.0 mg/l). The remarkably highest number of roots per plantlet (13.80) was observed in full MS supplemented with NAA (1.5 mg/l). The highest length of roots (3.80 cm) was observed in ½ MS without any hormone. After hardening about 6P% plantlets survived which are then planted to the soil. About 85 plantlet survived in pots and 75 % plantlets survived in fields.

The following conclusions could be drawn from the present investigation:

1. Shoot tip was the superior explants for *in vitro* propagation of stevia.
2. MS in half strength could be used instead of full MS for shoot induction.
3. Full MS supplemented with BAP (1.0 mg/l) was superior for shoot multiplication.

MS media supplemented with NAA (1.5 mg/l) was better for root induction

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