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BIOCHEMICAL INVESTIGATION DURING DIFFERENT STAGES OF *IN VITRO* PROPAGATION OF *STEVIA REBAUDIANA*

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Abstract

In vitro propagation of *Stevia rebaudiana* Bert, an important non-caloric sweetening herb was carried out to explore its potential for micropropagation (both from apical and nodal meristem) and callogenesis (using leaf, node and internode as explant). MS basal medium with 1.0 mg l^{-1} of BAP was found to be the best medium for shoot formation, with 90% shoot formation response within 12 days of meristem inoculation, both from shoot apical and nodal meristem. Maximum shoot multiplication response (90%) was also obtained in MS medium having 1.0 mg l^{-1} of BAP, with average of 8.6 shoots per culture vial having an average shoot length of 6.0 cm. The best *In vitro* rooting response (96%) was recorded on MS medium containing 1.0 mg l^{-1} NAA within 7.3 days of inoculation. When well developed *In vitro* plants were shifted for hardening on a mixture of sand + soil + peat (1:1:1) 90% success was recorded. For callogenesis leaf explant proved to be the best followed by nodal and internodal explant. The highest response of callus induction from leaf explant was obtained on MS medium supplemented with 3.0 mg l^{-1} 2,4-D while nodal and internodal explants showed best results for callogenesis in MS medium supplemented with 3.0 mg l^{-1} NAA and 1.0 mg l^{-1} BAP. The highest total soluble protein contents and the peroxidases activity were estimated in the six week old callus cultures derived from leaf explant.

Abbreviations: MS= Murashige and Skoog's medium; BAP= 6-Benzylamino-purine; 2,4-D= 2,4 dichlorophenoxyacetic acid; IAA= Indole acetic acid NAA= α- Naphthaleneacetic acid

Introduction

Stevia rebaudiana Bert., the most valuable tropical medicinal plant, belongs to the family *Asteraceae*. It is one of the 154 members of genus *Stevia* which produce sweet steviol glycosides (Soejarta *et al*., 1982). It is a natural sweet herb native of northeastern Paraguay (Savita *et al*., 2004). *Stevia* is diploid plant, having 11 pairs of chromosome (Frederico *et al*., 1996) with critical day length of 13 hours (Zaidan *et al*., 1980).

An interesting feature of this plant is intense sweetness of leaves and aqueous extracts. Steviosides, sweet crystalline diterpene glycosides, which gives sweet taste to the plant are noncaloric and 200-300 times sweeter than sugar (Midmore & Rank, 2002). Typical proportions, on a dry weight basis, for the four major glycosides found in the leaves of wild *Stevia* plants is 9.1% stevioside, 3.8% rebaudioside A, 0.6% rebaudioside C and 0.3% dulcoside (Bhosle, 2004). Steviol glycosides are derived from mevalonic acid pathway. Stevioside are 110 to 270 times sweeter than sucrose, rebaudioside A are 150 to 320 times, rebaudioside C is 40 to 60 times and Dulcoside A is 30 times sweeter than sucrose (Tanaka, 1997). Diet conscious and diabetic persons with hyperglycemia can use steviosides as an alternative sweetener (Din *et al*., 2006). Stevioside can also be used as an antihyper glycaemic (Gregersen *et al*., 2004), antihypertensive (Ferri *et al*., 2006), anti-tumor (Yasukawa *et al*., 2002) drug.

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Stevia can regenerate by crown division, trampled of stem into the ground and from seeds. As the seeds of *Stevia* are very small in size and infertile, large scale mechanized production of *Stevia* through seeds is not fruitful (Savita *et al*., 2004). Plant tissue culture or micropropagation can be used for rapid propagation and conservation of such valuable and endangered plant species (Nalawade *et al*., 2002), which are difficult to propagate by conventional methods. This technique allows rapid multiplication, lack of seasonal restriction, provides sufficient number of plants in very short span of time, self incompatible inbred lines can be maintained. Micropropagation ensures the production of disease free, high yielding and premium quality planting material for automation (Chawla, 2000).

When plants are grown *In vitro* they come under stress due to accumulation of ammonia in culture vial, which sometimes leads towards somaclonal variation. To enhance tolerance under stress conditions, the levels of low molecular weight antioxidant and activity of antioxidant enzymes, such as guaiacol peroxidase, superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase, is generally increased in plants (Foyer *et al*., 1997). Therefore it is very important to note the level of these enzyme at different level of *In vitro* propagation particularly during different stages of callogenesis and somatic embryogenesis where culture are maintained for longer time under *In vitro* conditions.

The present investigation describes procedures for micropprpagation and callogenesis of *Stevia rebaudiana*. It also describes the changes in peroxidases contents during different stags of callus growth and regeneration.

Materials and Methods

In vitro grown *Stevia rebaudiana* plants were taken from the Seed Center, University of the Punjab, Lahore, Pakistan. For sterilization, explant was first washed with running tap water, and then treated with house hold detergent for five minutes. This was followed by second washing with tap water to remove all the traces of detergent. The explant was then treated with 10% Sodium hypochlorite solution for 15 minutes. After discarding Sodium hypochlorite, the explants were washed three times with sterilized distilled water to remove all the traces of Sodium hypochlorite. The sterilized explants were then inoculated by proper dissecting and sizing of the about 4-5 mm were excised from stevia plants.

Explants of shoot apical and nodal meristem were inoculated in liquid as well as solid MS media (Murashige & Skoog, 1962) supplemented with different concentrations of auxins and cytokinins either alone or in combination with each other. pH of the medium was adjusted to 5.7 with 0.1 N solution of NaOH or HCl and 0.7% agar was used for solidification of medium. The medium was autoclaved at 121ºC and 15 Ibs/Inch^2 pressure for 15 minutes. Cultures were maintained under fluorescent light having 2500 lux light intensity. The incubation temperature was 26^{o}C ± 1^{o}C with 16 hour light and 8 hour dark period in every 24 hour cycle. The data was recorded for days for shoot formation, number of shoot per culture vial, days for shoot multiplication, days for root induction and for number of roots per plant.

For hardening well developed *In vitro* plants were transferred into pots containing different media (autoclaved sand, sand+soil and sand+soil+peat in the ratio of 1:1, 1:1:1 respectively). Potted plants were brought out from green house into open sun light after three week of hardening and eventually these plants were shifted into the field for further growth.

To determine the total soluble protein contents and peroxidase activity, 2.0 grams of plant material was crushed in the ice chilled pestle and mortor containing 0.2 g of PVP (polyvinylpyrrolidine) with 8 ml of 0.1M phosphate buffer at 4°C. For the extraction of total soluble protein contents, slurry was centrifuged at 10,000 rpm for 10 mins at 4°C while for the extraction of peroxidases, the slurry was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was used for further analysis. Biuret method of Racusen & Johnstone (1961) was adopted for the estimation of total soluble protein contents. For the estimation of peroxidases, method proposed by Racusen & Foote (1965) was used.

The experimental design was completely randomized with ten replicate cultures for each hormonal treatment and each experiment was repeated thrice. Analysis of variance (ANOVA) depicting significance among means was calculated by Duncan,s new multiple range test (Steel & Torrie, 1980).

Results and Discussion

Micropropagation: For shoot formation in *Stevia rebaudiana*, MS medium containing BAP (1.0 mg l^{-1}) provided best result (90%) both from shoot apical meristem within 11.6 days of explant inoculation (Table 1). Slavova *et al*., (2003) also used only BAP for shoot formation in *Stevia rebaudiana*. In case of kinetin mediated MS medium, 0.25 mg l^{-1} of Kinetin provided good results from apical meristem. Tadhani *et al*., (2006) reported that 4.0 mg l^{-1} of kinetin showed maximum shoot formation response. Nuutila *et al*., (2002) and Ali & Afghan (2003) reported that cultivation of same species may differ drastically in their requirement for essential medium components. In the case of nodal meristem, rate of shoot formation was high but it took more time than shoot apical meristem.

When BAP was used in combination with kinetin, the maximum shoot formation response was found to be 73% at 2.0 mg l^{-1} BAP with 0.25 mg l^{-1} Kinetin. Sivaram & Mukundan, (2003) observed maximum shoot induction from shoot apex, nodal and leaf explants on Murashige & Skoog (MS) medium supplemented with 6-benzyladenine (BA; 8.87 µM) and indole-3-acetic acid (5.71µM). Smitha *et al*., (2005) reported that when 0.05 mg l^{-1} kinetin was added in MS mediun which already had 1.0 mg l^{-1} of BAP, the production of dark green healthy shoot enhanced.

Table 1. Shoot induction from shoot apical and nodal meristem of *Stevia rebaudiana* cultured on MS medium supplemented with different growth hormones alone and in combination.

Growth regulators	Composition (mg l^{-1})	No. of shoot per culture	Average shoot length after 20 days (cm)	Rate of shoot multiplication (%)
MS medium	-	1.6 ± 0.274^{d}	2.0 ± 0.471^{d}	50
MS medium + BAP	0.50	3.6 ± 0.982^{bcd}	3.0 ± 0.471^{cd}	76
	1.00	**8.6 ± 0.721^{a}**	**6.0 ± 0.471^{a}**	**90**
	1.50	5.6 ± 0.721^{bc}	4.0 ± 0.471^{bc}	86
	2.00	3.3 ± 0.720^{cd}	3.3 ± 0.720^{bcd}	80
MS medium + Kinetin	0.50	3.0 ± 0.471^{d}	2.3 ± 0.272^{cd}	70
	1.00	4.0 ± 0.471^{bcd}	5.0 ± 0.471^{ab}	73
	1.50	6.0 ± 0.471^{b}	6.0 ± 0.471^{a}	76
	2.00	2.6 ± 0.274^{d}	4.0 ± 0.471^{bc}	70

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new Multiple Range Test.

The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

Shoot multiplication: The maximum number of shoots i.e., 8.6 shoots per culture vial with average shoot length of 6.0 cm was obtained after 20 days of inoculation, on MS basal medium having 1.0 mg l^{-1} of BAP (Fig. 1d, Table 1). In case of Kinetin mediated MS medium, 7.6 shoots per culture vials were obtained at 1.5 mg l^{-1} of kinetin. Tadhani *et al*., (2006) reported maximum number of shoots on MS medium supplemented with 0.6 mg l^{-1} of BA. Smitha *et al*., (2005) induced multiple shoots from shoot buds of *Stevia rebaudiana* with multiplication rate up to 1: 25 on a modified MS medium supplemented with 1.0 mg l^{-1} of benzyladenin. In this study it was also found that explant showed better shoot formation response in liquid medium as compared to the solid medium (Fig. 1a,1b; data not given). In liquid medium, the close contact of the tissue with the medium may facilitate the uptake of nutrients and phytohormones, leading to better shoot growth (Sandal *et al*., 2001).

Rooting: Root induction was observed in hormone free MS basal medium also, but best rooting response (96% with 7 roots per plant within 5 days of inoculation on rooting medium) was obtained on full strength MS medium supplemented with 1.0 mg l^{-1} of NAA (Fig. 1e; Table 3). Slavova *et al*., (2003) obtained 84% to 99% rooting on MS medium supplemented with NAA. Tadhani *et al*., (2003) reported initiation of rooting with in 6-7 days and obtained maximum number of roots on medium supplemented with 1.0 mg l^{-1} of IBA. Ferreira & Handro, (1988) reported that addition of auxin to the rooting medium (especially 0.1 mg l^{-1} IBA) favored root formation in *Stevia rebaudiana*. Smitha *et al*., (2005) recorded ≥ 90% rooting in modified MS medium supplemented with 1.5 mg l^{-1} indole-3-butyric acid.

Hardening and acclimatization: Well developed *In vitro* plants were shifted in different media for hardening acclimatization of micropropagated plants of *Stevia rebaudiana* in the glass house was achieved with 90 % survival rate in medium having autoclaved sand + soil + peat in 1:1:1 ratio (Fig. 1f; Table 4).

Callogenesis: During the *In vitro* initiation of callus, the cell differentiation and specialization that occurs in parent plant is reserved and cells of the explant become dedifferentiated (Evan *et al*., 2003).

In the present study best response for callogenesis was obtained from leaf followed by nodal explants, while internodal explants showed poor response. Din *et al*., (2006) found internodal segments as a best explant for callus induction, followed by leaf explant and poorest response by nodal explant.

Callus formation was observed, when auxin 2, 4-D or NAA were used alone or supplemented with small amount of cytokinin (BAP). There was a wide range of variation in days for callus initiation response and percentage of callus formation. The genes affecting structure and type of plant development, also influences callus formation. This depicts the involvement of inheritance in callus growth (Turhan, 2004). Among all treatments, the highest rate of callus from leaf explants (96%) within 11 days of inoculation was observed on MS basal medium containing 3.0 mg l^{-1} of 2,4-D (Table 5). When the 2,4-D was used in combination with BAP, increase in rate of callus induction was noticed in nodal and internodal explants as compared to 2,4-D alone (Table 6). The nodal and internodal explants showed maximum callus induction response i.e., 90 % (within 9 days of inoculation) and 73 % (within 7.3 days of inoculation) respectively on the MS medium supplemented with 3.0 mg l^{-1} NAA + 1.0 mg l^{-1} BAP (Table 7). Din *et al*., (2006) observed the highest amount of callus on MS medium containing 3.0 mg l^{-1} 2,4-D (Fig. 2c).

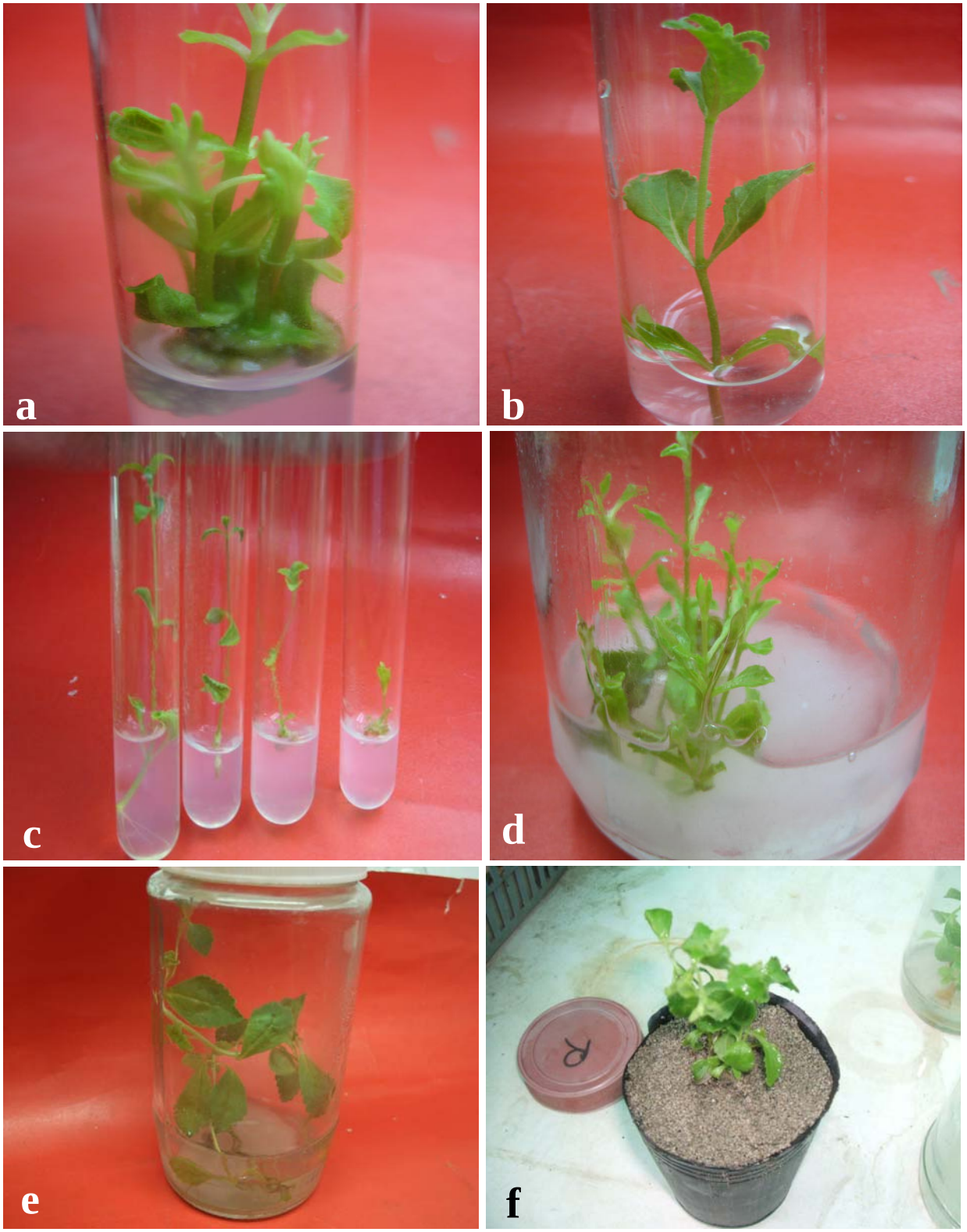

Fig. 1. Micropropagation of *Stevia*. (a),(b) Shoot formation from apical shoot meristemin solid and liquid MS medium supplemented with 1.0 mg l^{-1} of BAP; (c) Different stages of shoot formation from apical shoot meristem (after 7, 14, 20, and 25 days) inoculated in MS medium with 1.0 mg l^{-1} of BAP; (d) Shoot multiplication in liquid MS basal medium supplem- ented with 1.0 mg l^{-1} of BAP; (e) *In vitro* rooting in MS basal medium supplemented with 1.5 mg l^{-1} IAA; (f) Hardening of *In vitro grown Stevia plant* in sand.

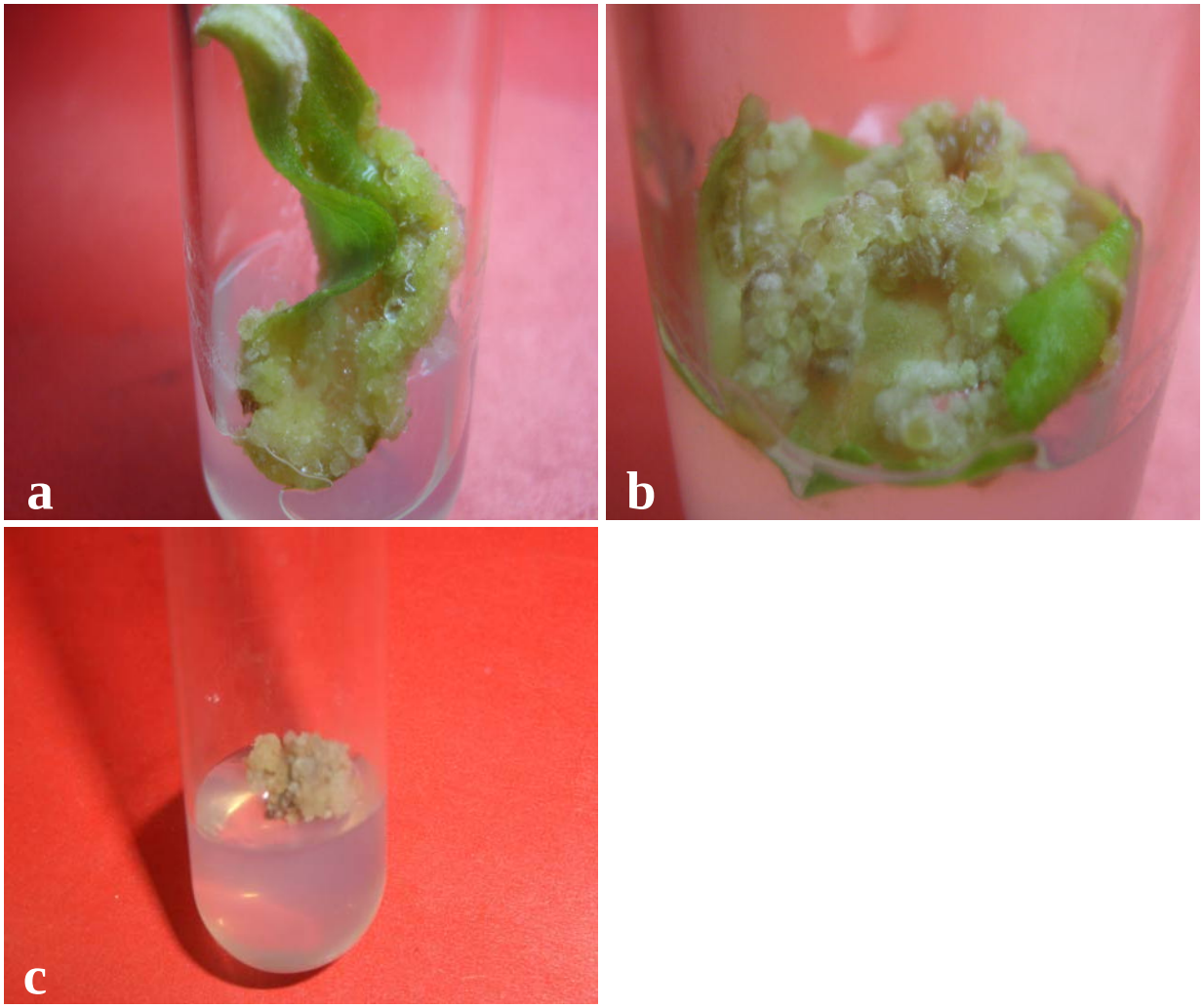

Fig. 2. Callogenesis in *Stevia*. (a), (b) Callus induction in leaf explant in light and dark respectively inoculation in MS medium supplemented with 3.0 mg l^{-1} of 2, 4-D; (c) Callus induction in nodal explant inoculated in MS medium supplemented with 3.0 mg l^{-1} of NAA with 1.0 mg l^{-1} of BAP.

Table 2. *In vitro* shoot multiplication of *Stevia rebaudiana* cultured on MS medium supplemented with different concentrations of BAP and Kinetin.

Growth regulators	Composition (mg l^{-1})	Shoot apical meristem		Nodal meristem	
		Days for shoot formation	Rate of shoot formation (%)	Days for shoot formation	Rate of shoot formation (%)
MS medium	-	21 ± 0.942a	50	22.2 ± 0.544^{a}	53
BAP	0.25	15 ± 0.471^{bc}	56	18.3 ± 0.981^{b}	60
	0.50	14.3 ± 0.72^{bc}	63	15.3 ± 0.720^{bcde}	66
	1.00	**11.6 ± 0.721^{c}**	**90**	**12.6 ± 0.982^{e}**	**90**
	1.50	15.6 ± 0.721^{b}	60	17.6 ± 0.721^{bc}	63
Kinetin	0.25	13.3 ± 0.981^{bc}	86	15.6 ± 0.721^{bcde}	90
	0.50	15.0 ± 1.247^{bc}	70	17.3 ± 0.720^{bcd}	73
	1.50	13.0 ± 0.942^{bc}	66	14.3 ± 0.720^{de}	66
	2.00	18.6 ± 0.721^{bc}	50	15.0 ± 0.471^{cde}	53
BAP + Kinetin	0.5+0.25	12.6 ± 0.721^{bc}	46	13.6 ± 0.721^{e}	56
	1.0+0.25	13.0 ± 0.942^{bc}	50	14.0 ± 0.471^{e}	60
	1.5+0.25	13.6 ± 0.982^{bc}	56	14.6 ± 1.186^{cde}	66
	2.0+0.25	12.3 ± 0.720^{bc}	73	13.0 ± 0.471^{e}	76

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new Multiple Range Test.
The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

Table 3. Effect of different concentration of NAA and IAA on rooting of *In vitro* developed shoots of *Stevia rebaudiana*.

Growth regulators	Composition (mg l^{-1})	Days for root induction	No. of roots per plant	Rate of rooting (%)
Basal MS medium	-	14.3 ± 0.981^{bc}	2.0 ± 0.471^{d}	73
MS medium + NAA	0.5	11.3 ± 0.981^{cd}	2.6 ± 0.274^{cd}	80
	1.0	**7.3 ± 0.720^{e}**	**5.0 ± 0.471^{ab}**	**96**
	1.5	9.3 ± 0.720^{de}	4.0 ± 0.471^{abcd}	76
	2.0	10.0 ± 0.471^{de}	3.0 ± 0.471^{bcd}	73
MS medium + IAA	0.5	15.3 ± 0.720^{b}	3.0 ± 0.471^{bcd}	73
	1.0	16.3 ± 1.186^{b}	3.6 ± 0.982^{abcd}	83
	1.5	13.3 ± 0.981^{bc}	5.3 ± 0.720^{a}	93
	2.0	19.6 ± 0.721^{a}	4.3 ± 0.272^{abc}	86

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new Multiple Range Test.
The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

Table 4. Hardening of well developed *in vitro* plants.

Medium composition	Days for hardening	Rate of plant survival (%)
Autoclaved sand	40 ± 0.632^{a}	70
Sand + Soil	40 ± 0.456^{a}	70
Sand + Soil + Peat	31.8 ± 0.334^{b}	90

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range test.
The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

Table 5. Effect of different concentrations of 2, 4-D on callus induction

MS medium + 2,4-D (mg l^{-1})	Days for callus induction			Rate of callus induction (%)		
	Leaf	Node	Internode	Leaf	Node	Internode
0.0	0.0 ± 0^{c}	0.0 ± 0^{c}	0.0 ± 0^{c}	0.0	0.0	0.0
1.0	14.33 ± 0.274^{a}	15.66 ± 0.720^{a}	20.66 ± 1.274^{a}	73	46	30
2.0	11.66 ± 0.982^{b}	13.33 ± 0.272^{b}	19.33 ± 0.272^{ab}	80	50	30
3.0	**11 ± 0.471^{b}**	**12.66 ± 0.274^{b}**	**17 ± 0.471^{b}**	**96**	**73**	**50**
4.0	12 ± 0.274^{ab}	13 ± 0.471^{b}	19.33 ± 0.272^{ab}	70	60	43

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new Multiple Range Test.
The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

Table 6. Effect of different concentrations of 2, 4-D with BAP on callus induction.

MS medium + 2,4-D (mg l^{-1})	Days for callus induction			Rate of callus induction (%)		
	Leaf	Node	Internode	Leaf	Node	Internode
2.0 + 0.5	12 ± 0.471^{a}	15.66 ± 0.981^{ab}	20 ± 0.471^{ab}	**90**	63	43
2.0 + 1.0	12.33 ± 0.720^{a}	19 ± 0.942^{a}	20.33 ± 0.720^{a}	80	50	40
3.0 + 0.5	11.33 ± 0.720^{a}	15.66 ± 0.720^{ab}	$18b \pm 0.471^{bc}$	83	53	43
3.0 + 1.0	10.33 ± 0.272^{a}	14.33 ± 0.720^{b}	16.66 ± 0.272^{c}	83	**73**	**60**

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new Multiple Range Test.
The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

Table 7. Effect of different concentrations of NAA with BAP on callus induction.

MS medium + 2,4-D (mg l^{-1})	Days for callus induction			Rate of callus induction (%)		
	Leaf	Node	Internode	Leaf	Node	Internode
1.0 + 0.5	12.6 ± 0.721[ab]	13 ± 0.942[a]	14 ± 0.471[a]	60	70	56
2.0 + 0.5	14 ± 0.471[a]	13.6 ± 0.982[a]	14.3 ± 0.981[a]	53	60	50
3.0 + 0.5	10.6 ± 0.721[bc]	11 ± 0.471[ab]	13.6 ± 0.721[a]	56	73	60
3.0 + 1.0	**10 ± 0.471[c]**	**9 ± 0.471[b]**	**12 ± 0.471[a]**	**70**	**90**	**73**

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new Multiple Range Tests.

The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

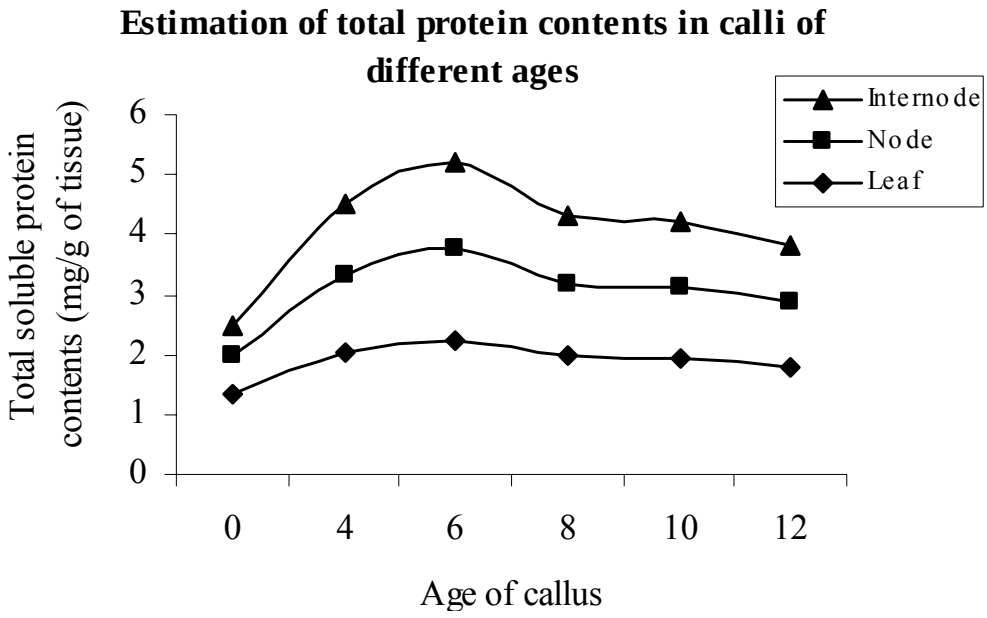

Fig. 3. Estimation of total protein contents (mg g^{-1} of tissue) in callus cultures of different ages.

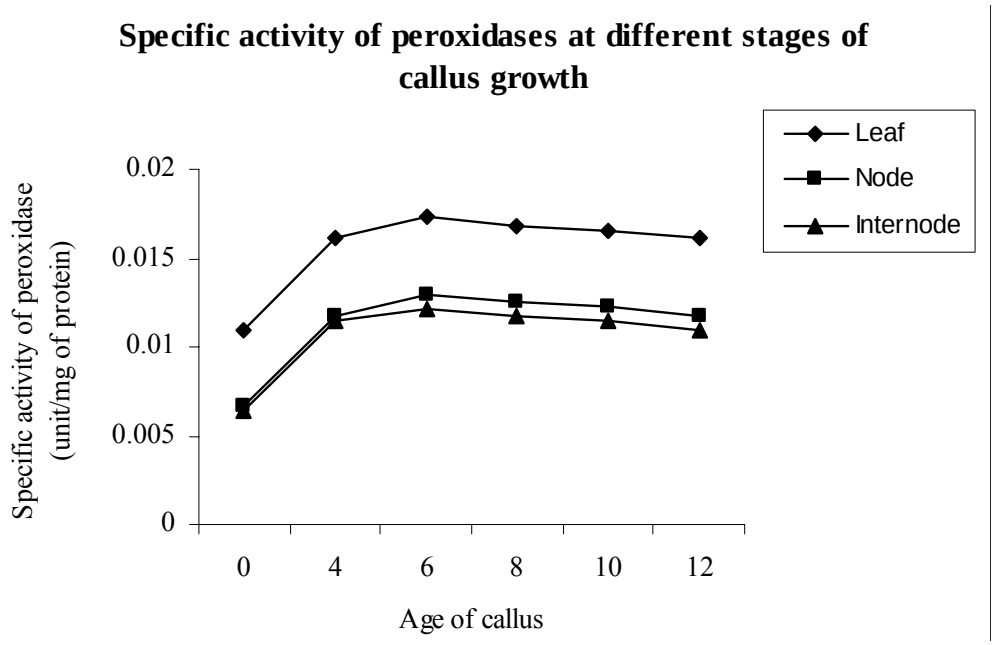

Fig. 4. Specific activity of peroxidases at different stages of callus growth.

Tadhani, *et al*., (2006) used leaves of *Stevia* for the production of callus on MS medium supplemented with 2.0 mg l^{-1} NAA and 0.3 mg l^{-1} of BA (6-benzyladenine). Sivaram & Mukundan, (2003) derived callus from on different combination of auxin and cytokinins like BA with IAA, IBA or 2,4-D and kinetin with NAA, IAA, IBA or 2,4-D. Fitho & Haltori, (1997) observed maximum callus formation response of *Stevia* on MS basal medium supplemented with 0.05 M 2,4-D. Swanson *et al*., (1992) observed the friable callus cultures from leaf explants of *Stevia rebaudiana* Bertoni cultured on MS medium supplemented with naphthalene acetic acid (NAA, 0.5 mg l^{-1}) and benzylaminopurin (BAP, 0.5 mg l^{-1}).

Many factors determine the ability of a specific tissue to form callus. Among these chemical factors, include mineral nutrition and plant growth regulators, environmental factors, such as light, temperature and humidity. At 3.0 mg l^{-1} of 2,4-D callus from different explants i.e., leaf, node and internode initiated earlier in darkens than in light, but the callus was compact and whitish brown in darkness while smooth and yellowish green in light (Fig. 2a,2b; data not given).

The results of our investigation reflect that the explants obtained from different parts of the same plant behave differently in the same culture media. The experimental result regarding callus induction and growth had shown that callus generating capacity varies with explants and media composition.

Biochemical investigations: Among different explants, the highest amount of total soluble protein contents were found in leaf explant (1.35 mg g^{-1} of tissue) while node and internode have 0.625 mg g^{-1} and 0.525 mg g^{-1} of tissue respectively. In comparison, callus of any age has more total soluble protein contents than any part of plant. The highest amount of total soluble protein contents (2.25mg g^{-1} of callus) was present in 6.0 week old callus cultures of leaf explant (Fig. 3).

In the present study, specific activity of peroxidases in different explants and in the calli of different ages was also estimated. Among different explants, leaf (callus at 0 week) had the highest specific activity (0.011 units mg^{-1} of protein) of peroxdases, and in callus cultures, the highest specific activity (0.0173 units mg^{-1} of protein) of peroxidases was estimated in 6.0 week old callus derived from leaf, after that there was a gradual decrease in the activity of peroxidases (Fig. 4). Kairong *et al*., (1999) reported that peroxidase activity is high in callus and rapidly decreases in the early days of culture differentiation. When plants are grown under *In vitro* conditions and exogenous growth regulators (auxins and cytokinins) are also present in growth medium, calli exhibit high ethylene production (Csiszar *et al*., 2003). In the result of ethylene production, defense mechanisms at a transcriptional level and generation of active oxygen species including H_2O_2 are activated, which result in increased peroxidase activities (Levins *et al*., 1995). In the calli of tea plant cultured on MS medium containing 2, 4-D (10 mg l^{-1}), 2.4 fold increase in the activity of peroxidase was observed relative to the calli grown in the absence of any phytohormone (Aoshima & Takemoto, 2006).

Peroxidases participates in lignin biosynthesis (Quiroga *et al*., 2000), in cross-linking of cell wall components in plants (Hatfield *et al*., 1999) and restrict the expansion of the cells (Andrews *et al*., 2002).

Conclusion

In present investigation protocols for *In vitro* propagation of *Stevia rebaudiana* have successfully been standardized and changes in total soluble protein contents and peroxidase activity during different stages of callus growth have been estimated. As this

sweet herb, is an important medicinal plant, it is becoming an endangered species due to its infertile and small sized seed. The methods of vegetative propagation are not efficient to save this rare plant. Therefore protocols developed in present investigation are not only useful for its mass scale propagation but also conservation of germplasm.

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