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In vitro Plant Regeneration in *Gymnema sylvestre* R. Br.: Influence of Season and Subculture Induced Oxidative Stress

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ABSTRACT

Nodal segments from field grown, Gymnema sylvestre plants were cultured on Murashige and Skoog's (MS) medium supplemented with benzyl adenine (BA), Kinetin and naphthalene acetic acid (NAA) during different seasons. Explanting season between November-March was found to be ideal to obtain optimal culture initiation and regeneration with less contamination. Shoot proliferation was highest on MS basal medium supplemented with 2 mgL^{-1} BA, 0.2 mgL^{-1} kinetin and 0.05 mgL^{-1} NAA. Best rooting response was obtained on half-strength MS medium supplemented with IBA (3 mgL^{-1}). Hardening of the rooted plants was successful on soil: sand: vermicomposte (2:1:1) potting mixture. Active proliferation and growth of explants and plantlets, and also subculture induced oxidative stress were confirmed determining the changes in antioxidants and antioxidant molecules. The concentrations of different antioxidants and activities of antioxidant enzymes varied with the stage of morphogenesis and subculture. Steady increase in ascorbate (ASC) content was noted throughout regeneration, while higher levels of reduced glutathione (GSH) content was observed during shoot proliferation and rooting. Increase in the activities of ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (POX) was noticed during shoot organogenesis, while peak activity of GR was noticed during rooting. Increased levels of SOD and GST activities were observed after second subculture, which might contribute in circumventing stress and their involvement in stress tolerance.

Key words: *Gymnema sylvestre; Seasonal influence; Oxidative stress; Antioxidants; Antioxidant enzymes.*

INTRODUCTION

Gymnema sylvestre R. Br. A valuable medicinal plant belonging to family Asclepiadaceae is distributed over most parts of India and Africa. In spite of its wide distribution it is considered as an endangered plant due to its overexploitation to meet the requirement of pharmacologically active metabolites[1].

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In addition, it possesses anti-microbial, anti-hypercholesteromic, hepatoprotective activities, it is also used in the treatment of asthama, eye complaints, inflammation and snake bites. Thus various reports on its multiple uses,[2-5] particularly as potent remedy for diabetes attracted attention for utilization of this plant for its active principles, collectively known as gymnemic acid. Plant organogenesis *in vitro* is a more controllable process than embryogenesis[6]. Different morphogenetic pathways, i.e. shoot or root formation, may be induced in culture by application of the appropriate hormones in the medium. Although the exact nature of these hormonal signals may vary between species, the balance of auxin to cytokinin has been found to have a consistent effect on the type of regenerated organs. A relatively high ratio between auxin and cytokinins promotes the regeneration of roots. Whereas, shoot organogenesis is a preferred type of differentiation on culture media supplemented with high cytokinin and low auxin concentration[7,8]. Aerobic organisms respond to both stress and normal physiological stimuli, such as hormones, by producing reactive oxygen species (ROS) generally in defined subcellular locations[9]. Reactive oxygen species (ROS) such as superoxide, H_2O_2 and lipid hydroperoxides are toxic cellular metabolites which are rapidly detoxified by various enzymatic and non-enzymatic scavengers. The role of ROS in plant growth and development is substantiated by the interplay of ROS with a number of phytohormones. Moreover, ROS have been implicated as second messenger in several plant hormone responses[10] and serve as signaling molecules regulating important biological processes in both animal and plant cells[11]. Production of ROS serves highly localized and specific signaling functions in stress responses and in normal physiological processes, as well as in the growth and development[12,13]. It is suggested that uncontrolled reduction of free radicals, by employing antioxidants that scavenge ROS, can lead to the disruption of metabolic pathways that are essential for tissue and organ differentiation[14]In plants, the line between normal physiological responses and stress responses is arbitrary, which continuously adapt to environmental changes such as light, temperature, humidity and salinity. The mechanism that maintain the internal reducing environment during normal physiological process includes the oxidants, these range from maintaining high levels of reducing small molecules, such as glutathione and ascorbate, to the many enzymes, such as superoxide dismutase, catalase and many other peroxidases, that further reduce ROS to water. Although ROS have become the focus of many studies due to their dual function as toxic and signaling molecules, very little has been reported regarding the requirement and involvement of ROS during plant differentiation *in vitro*[14]. In an attempt to understand the biochemical changes associated with subculture induced oxidative stress during *in vitro* plant regeneration in *Gymnema sylvestre*, and as well during explant proliferation/regeneration, the present study was undertaken to determine the changes in non-enzymatic antioxidants like ascorbate and glutathione, antioxidant enzymes like peroxidase, ascorbate peroxidase, catalase, superoxide dismutase, glutathione reductase and glutathione-S-transferase.

MATERIAL AND METHODS

Plant material

Apical growing twigs (10-20 cm) were collected randomly from two-three year old *Gymnema sylvestre* plants growing in campus garden for micropropagation studies during two consecutive years (pre-flowering and flowering season) in five seasons viz., winter (November-January), spring (February-March), summer (April-May), rainy (June-August) autumn (September-October). The cuttings were defoliated leaving the petiole stumps just after collection and processed for surface sterilization. Biochemical assays were carried out at different stages starting from explant initiation, proliferation (competence stage), shoot induction and development, in

particular emergence of first pair of leaves (20 to 40 days after inoculation onto medium). After further proliferation and growth, shoots of about 40 to 60 days old were removed from the medium and subjected for biochemical assays, this corresponds to shoot elongation period. Similarly after subjecting shoots for root induction explants (plantlets) were removed from the medium for biochemical assays (60-80 days). To study even the stress imposed on the proliferating tissue/shoots due to subculture during every subculture (for every 20 days) the biochemical assays were carried out in the regenerating shoots, on the day of shoot transfer onto fresh medium (i.e., 20, 40, 60 and 80 days), and as well after 5 days of subculture (i.e., shoot transfer onto fresh medium, 25, 45 and 65 days), for a period of 80 days.

Surface sterilization and culture establishment

In order to control contaminants from *ex vitro* explants, different sterilizing agents $HgCl_2$, NaOCl, bavistin and cetrimide at various concentrations and for different time durations were tried during November-March of a calendar year. Ten explants were tested per sterilization method. *Ex vitro* explants were washed under running tap water for 5 min, and then in neutral detergent Tween-20 (2 to 3% in distilled water (v/v)) along with bavistin and cetrimide at different concentration for 5min. Washed explants were rinsed in double distilled water and brought under laminar airflow hood for further surface sterilization treatments. Explants were subsequently exposed to $HgCl_2$/NaOCl at various concentrations for different durations. After exposure to surface sterilants, the explants were washed in sterile distilled water (3-4 times) and immersed in antioxidant solution (0.01% PVP and citric acid) for 5 min. Explants were trimmed and dried on pre sterilized filter paper sheets. Data on asepsis was recorded after a period of 3 weeks.

Shoot proliferation

Nodal explants were aseptically inoculated to MS medium[15] supplemented with (Murashige and Skoog 1962), 30 g l^{-1}sucrose and 7.5 g l^{-1} agar. Media pH were adjusted to 5.8 prior to autoclaving at 121^{o}C for 20 min. For shoot induction and proliferation medium was supplemented with different concentrations of cytokinin and auxin. Cytokinins BA and kinetin at 0.0, 0.2, 0.5, 1, 2 and 5 mg l^{-1}were tested either alone or in combination. Auxins NAA, IAA and IBA at 0.05, 0.1, 0.2, 0.5 and 1 mg l^{-1}were tested in combination with cytokinins. All the cultures were maintained at 25 ± 2 ^{o}C under a 16 h photoperiod (supplied by two Philips TL 40 W fluorescent tubes). Newly developed shoots were separated and subcultured for further multiplication. Explant multiplication frequency was assessed by determining the percentage of explants producing shoots and average number of shoots per single explant and also average length of the individual shoot.

Rooting and acclimatization

For rooting shoots longer than 3-4 cm were transferred to either half and full strength MS medium supplemented with auxins IBA, IAA and NAA at various concentrations. Regenerated plantlets with well established roots were removed from culture vessels and washed gently with sterile double distilled water to remove the traces of agar-agar and successfully transferred to plastic pots (7.5 cm in diameter) containing autoclaved mixture of garden soil : sand : vermicomposte (2:1:1). Humidity was maintained by covering with polythene covers for an initial period of 20 days and watered daily with half-strength MS inorganic nutrients before subsequent transfer to the field.

Protein and enzyme extraction

Leaf material (0.5g) was homogenized in 4 ml 100 mM potassium buffer (pH 7.5) containing 5 mM DTT (w/v), 5 mM EDTA (w/v), 0.2 mM phenyl methyl sulphonyl fluoride (PMSF), 2.5% PVP at 4^{o}C. The homogenate was filtered through four layers of muslin cloth and centrifuged at 10,000 rpm for 20 min at 4 ^{o}C, using cooling centrifuge and the supernatants were used for protein estimation and enzyme assays. For assay of ascorbate peroxidase (APX) activity leaf material was homogenized in 3 ml of 100 mM sodium

phosphate buffer (pH 7.0) containing 5mM sodium ascorbate and 1mM EDTA. For SOD assay leaf material was homogenized in 4 ml extraction buffer containing 100 mM potassium phosphate buffer (pH 7.0) and 1% PVP in a pre-chilled mortar and pestle. Protein content was determined using bovine serum albumin as a standard[16].

Assays for antioxidants and antioxidant enzymes

Total ascorbate (ASC) and reduced glutathione (GSH) estimation:

Ascorbate was determined according to the modified procedure[17]. Briefly, leaf material (500 mg) was homogenized into 2.5 ml of 5% metaphosphoric acid and centrifuged at 22,000×*g* for 15 min at 25 ^{o}C. The supernatant was initially treated with reducing agent dithiothreitol (for reducing dehydroascorbate to ASC). 0.2 ml supernatant was added to 0.5 ml of 150 mM phosphate buffer (pH 7.4) containing 5mM EDTA and 0.1 ml of 0.5% (w/v) *N*-ethylmaleimide. After adding 0.4 ml of 10% (w/v) trichloroacetic acid, 0.4 ml of 44% (v/v) orthophosphoric acid, 0.4 ml of 4% (w/v) 2,2'-bipyridyl in 70% (v/v) ethanol and 0.2 ml of 3% (w/v) ferric chloride, the mixture was incubated at 40 ^{o}C for 40 min. Absorbance was measured at 525 nm. Total ascorbate was calculated using a standard curve for pure ascorbate.Extraction and estimation of reduced glutathione was carried out using 5, 5'-dithiobis-2-nitro benzoic acid (DTNB) reagent[18]. Leaf material (500 mg) was homogenized into 2.0 ml of 5% sulfosalycylic acid and centrifuged at 12,000×*g* for 15 min at 25 ^{o}C. The supernatant (1.0 ml) was neutralized with 1.5 ml of 0.5 M potassium phosphate buffer containing 5 mM EDTA (pH 7.5), 0.2 ml of 6 mM 5, 5'-dithiobis 2 nitro benzoic acid (DTNB) prepared in methanol. Absorbance was measured at 412 nm at 25 ^{o}C. Concentration of the glutathione was determined using a standard calibration curve of glutathione.Catalase (CAT; EC 1.11.1.6) activity was assayed from the rate of H_2O_2 decomposition as measured by the decrease in absorbance at 240 nm (E = 39.4 mM^{-1} cm^{-1})[19]. The reaction mixture contained 100 mM phosphate buffer (pH 7.0), 6.6 mM H_2O_2 and 50 µl plant extract.Peroxidase (POX; EC 1.11.1.7) activity was measured by monitoring the increase in absorbance at 420 nm (E = 26.6 $mM^{-1}cm^{-1}$)[20]. The reaction mixture contained 100 mM phosphate buffer (pH 7.0), 0.1 µM EDTA, 5.0 µM guaiacol, 15.0 µM H_2O_2 and 50 µl enzyme extract.Superoxide dismutase (SOD; EC 1.15.1.1) activity was estimated by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm[21]. The assay mixture in 3 ml contained 50 mM phosphate buffer, pH 7.8, 9.9 mM L-methionine, 57 mM NBT, 0.025% (w/v) Triton X-100, and 0.0044% (w/v) riboflavin.Ascorbate peroxidase activity (APX; EC 1.11.1.11) was determined, measuring the decrease in absorbance at 290 nm due to ascorbate oxidation (E = 2.8 mM^{-1} cm^{-1})[22]. The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate and 1.2 mM H_2O_2.Glutathione reductase (GR; EC 1.6.4.2) activity was assayed by monitoring NADPH oxidation at 340 nm (E = 6.2 mM^{-1} cm^{-1})[23]. The assay mixture (1 ml) contained 100 mM K-phosphate buffer (pH 7.8), 2 mM EDTA, 0.5 mM oxidized glutathione, 0.2 mM NADPH and enzyme extract. Glutathione-S-transferase (GST; EC 2.5.1.18**)** activity was determined by measuring the increase in absorbance at 340 nm (E = 9.6 mM^{-1} cm^{-1}), incubating reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) as substrates[24]. The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 6.5), 5mM GSH, 0.4 mM CDNB and enzyme extract.

Statistical analysis

All the experiments were repeated thrice with at least 10 explants per treatment and data recorded as mean and standard error using Windows Microsoft excel.

RESULTS AND DISCUSSION

Surface sterilization

Explant surface sterilization individually with either $HgCl_2$ (mercuric chloride) or NaOCl (sodium hypochlorite) treatments were found

to be ineffective in controlling the contamination. Even though $HgCl_2$ treatment was found to be effective at higher concentration, but caused severe damage to explants. While, NaOCl was found to be ineffective that poorly inhibited the microbial load, besides causing severe damage to explants. $HgCl_2$ treatment (0.05 % for 5 min or 0.1% for 3 min) yielded only 40.33±1.02 % contamination free explants (data recorded after 15-20 days of inoculation). Inclusion of antimicrobials like bavistin (0.12%) and cetrimide (0.08%) along with 3 drops Tween-20 in the pre-treatment solution before $HgCl_2$ wash reduced the contamination rate convincingly to 18±1.02 %.

Shoot proliferation

Incorporation of various concentrations of BA and kinetin in the MS basal medium increased shoot proliferation significantly. BA at 2 mg l^{-1} increased bud sprouting frequency to 72.86±1.85 % and induced a mean of 2.0±0.24 shoots per explants (Table1). Kinetin alone was found to be not effective as that of BA, 0.2 mg l^{-1} kinetin had shown 46.21±1.02 % of bud sprouting frequency with 1.30±0.33 shoots per explant. At 2 mg l^{-1} BA with or without 0.2 mg l^{-1} kinetin with various combinations of auxins treatment resulted in varying degree of responses (Table 2). BA in combination with kinetin increased the degree of response in terms of explant bud sprouting frequency, mean shoot number per explant and shoot length. 2 mg l^{-1} BA in combination with 0.2 mg l^{-1} kinetin and 0.05 mg l^{-1} NAA induced multiple shoots, at an average of four shoots per explant (Figure 1). Once culture conditions were optimized for shoot induction, the *in vitro* regenerated shoots were further multiplied by sub-culturing on optimized medium containing 2 mg l^{-1} BA, 0.2 mg l^{-1} kinetin and 0.05 mg l^{-1} NAA.

Rooting

In the present work, rooting using three auxins IBA, NAA, IAA achieved comparable rates *in vitro* (Table 3). Other factors which influenced rooting were strength of MS-salts, charcoal and the length of regenerated shoots. Reducing the MS-salts to half of its initial concentration increased the frequency of rooting to more than two folds. Incorporation of charcoal in the rooting media (0.05-0.08%) reduced the basal callusing and also increased growth of the roots. Successful root establishment was achieved in individual shoots of length 4-5cm on MS medium (half-strength) supplemented with IBA (3 mgl^{-1}) and presence of 0.05% activated charcoal, after 10-15 days in root induction medium. Of the three auxins tested IBA was found to be more effective in root induction in terms of root length and number followed by NAA and IAA. Roots induced with IBA were thick and long and also the survival percent of regenerated plants was better (60.11±0.95 %) compared to NAA (33.88±0.88%) and IAA (22.56±1.45%) (Figure 1).

Table 1: Effect of cytokinins on morphogenesis in nodal explants

Cytokinin	Concentration (mg l^{-1})	% of response	Days to bud sprout	No. of Shoots per explant
BA	0.2	38.67±0.88	20.75±1.57	1.0±0.23
	0.5	55.33±2.20	20.33±0.88	1.0±0.32
	1	70.86±1.85	18.86±1.02	1.67±0.33
	2	72.86±1.85	15.67±1.45	2.0±0.24
	5	64.24±1.0	15.56±1.20	1.33±0.33
	10	60.0±2.08	12.00±1.52	1.33±0.67
Kinetin	0.2	46.21±1.02	21.56±0.97	1.30±0.33
	0.5	45.33±1.75	21.33±1.25	1.0±0.28
	1	36.55±1.33	20.55±0.88	1.0±0.24
	2	35.86±1.62	18.45±1.33	1.0±0.22
	5	35.67±0.89	18.16±1.95	1.0±0.05
	10	30.33±0.66	17.28±1.45	1.0 ±0.03

Table 2: Effect of cytokinin and auxin combinations on morphogenesis

BA (mgl^{-1})	Kinetin ($mg\ l^{-1}$)	NAA ($mg\ l^{-1}$)	% of Explant response	Average No. of shoots per explant	Average shoot length (cm)	Basal callus
2	0.5	0.05	84.66±1.02	4.01±0.67	3.76±0.75	-
2	0.5	0.1	82.21±1.56	3.67±0.66	3.56±0.97	-
2	0.5	0.2	81.37±0.56	3.33±0.33	3.18±1.11	-
2	0.5	0.5	78.48±1.21	2.67±0.66	3.18±1.54	+
2	0.5	1	77.00±1.45	2.67±0.67	2.9±0.09	+
2	0.5	0.05	84.66±1.02	4.01±0.67	3.76±0.75	-
---	---	**IBA ($mg\ l^{-1}$)**	---	---	---	---
2	0.5	0.05	74.48±1.33	3.06±0.67	3.04±0.88	-
2	0.5	0.1	74.89±0.76	2.67±0.67	3.29±0.77	+
2	0.5	0.2	73.34±0.88	2.67±0.33	2.92±1.22	+
2	0.5	0.5	73.15±1.56	2.67±0.33	2.57±1.76	++
2	0.5	1	73.00±1.19	2.33±0.67	2.55±1.02	++
---	---	**IAA ($mg\ l^{-1}$)**	---	---	---	---
2	0.5	0.05	73.45±0.88	2.67±0.33	2.89±0.88	+
2	0.5	0.1	72.55±0.76	2.33±0.33	2.75±0.96	+
2	0.5	0.2	72.12±1.56	2.33±0.67	2.71±1.02	+
2	0.5	0.5	72.16±0.69	2.07±0.66	2.53±0.75	++
2	0.5	1	72.06±1.74	2.34±0.33	2.4±1.22	+++

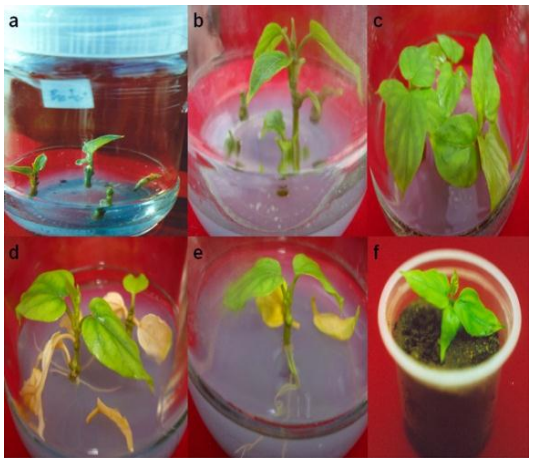

Figure 1: *In vitro* plant regeneration in nodal explants, a, b & c. Shoot induction and regeneration in nodal explants,d & e. Rooting; f. Hardening

Influence of explanting season on culture initiation

Establishment of cultures from *ex vitro* explants was found to vary depending on the explanting season. Nodal explants collected in pre-flowering season during the months November-January showed low contamination rate followed by February to March, while contamination rate was more severe during April-May followed by June-August, and September-October. In the flowering season, contamination rate was high during September-October, followed by previous period June-August while low contamination rate was documented during February-March (Tables 4 & 5). Explants collected during months November-January and subsequent culture initiation showed the best response not only in terms of axillary bud sprouting but also in shoot vigor in terms of shoot length and shoot number during pre-flowering season, while in flowering season best response was documented during February-March.

Table 3: Effect of different auxins on root induction in *in vitro* shoots

Auxin	Concentration (mg l^{-1})	Rooted Shoots (%)	Mean no. of roots/shoot	Mean root length (cm)	Plant survival (%)
NAA	1	25.61±1.22	2.55±0.55	0.81±0.63	9.77±1.56
	2	31.55±1.02	4.13±0.78	1.21±0.49	20.11±1.41
	3	60.23±0.98	5.75±1.11	2.35±1.06	30.14±1.71
	4	65.71±0.88	7.21±1.63	6.62±1.22	33.88±0.88
	5	57.42±1.02	5.96±1.45	4.52±1.09	32.79±1.06
IBA	1	34.71±0.86	4.66±1.21	2.94±0.95	35.31±1.22
	2	40.32±1.02	9.74±0.99	2.94±0.95	35.31±1.22
	3	71.95±0.67	15.79±0.56	8.26±0.56	60.11±0.95
	4	74.33±1.11	15.12±1.65	7.78±1.02	52.46±1.78
	5	75.51±1.26	14.45±1.21	7.31±1.29	50.12±1.56
IAA	1	22.62±0.95	1.43±0.88	0.52±1.55	07.12±0.55
	2	28.66±1.22	3.66±0.95	3.54±1.11	22.56±1.45
	3	45.23±1.05	5.24±1.09	3.54±1.11	22.56±1.45
	4	50.51±1.45	5.54±1.02	2.72±1.02	30.15±0.89
	5	52.92±0.79	5.10±1.56	1.43±0.55	30.95±1.22

Table 4: Effect of explant harvest period (season) on shoot induction efficiency during pre-flowering season

Season	Contamination rate	Survival rate	% Of response	Average Shoot length	Average Shoot number
November-January	20.21±0.72	63.77±1.00	71.33±0.35	3.5±0.11	3.95±0.11
February-March	34.3±0.36	41.77±1.6	69.33±1.8	3.06±0.12	3.42±0.04
April-May	49.46±0.3	30.06±0.20	44.66±1.20	2.6±0.14	1.96±0.08
June-August	41.3±1.40	44.56±0.38	41.66±1.20	2.5±0.11	1.83±1.8
September-October	40.26±1.56	40.66±1.02	39.88±0.88	2.31±0.09	1.80±0.2

Table 5: Effect of explant harvest period (season) on shoot induction efficiency during flowering season

Season	Contamination rate	Survival rate	% Of response	Average Shoot length	Average Shoot number
November-January	38.33±0.88	41.33±0.88	57.33±1.20	2.88±0.04	2.64±0.03
February-March	27.3±1.45	57.33±0.88	65.88±0.58	3.81±0.07	3.88±0.01
April-May	34.67±0.67	53.67±1.45	48.67±0.88	3.32±0.07	3.53±0.06
June-August	47.67±0.88	33.67±0.33	37.33±1.20	2.40±0.04	1.87±0.04
September-October	68.45±0.87	30.06±1.21	34.56±0.75	2.05±0.05	1.65±0.03

Antioxidants and antioxidant enzymes

Changes in ASC and GSH content:

There was a gradual increase in ASC content during all the stages of explant growth and also after every subculture regime (25, 45, 65 days) till the root induction and growth (between 65 to 80 days), indicating the existence of non-enzymatic ROS scavenging system during the entire morphogenesis (Figure 2).

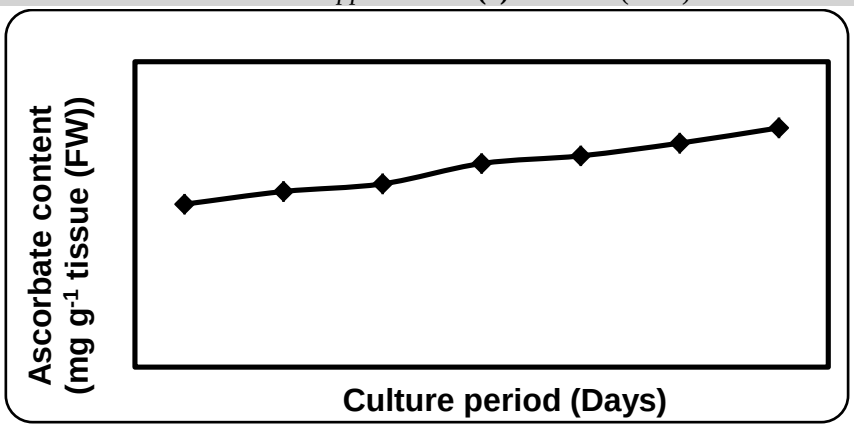

Figure 2: Changes in ascorbate (ASC) content during *in vitro* organogenesis

The GSH content was increased gradually between day 20 to day 40 (two subcultures) in culture probably indicating the active phase of cell growth, this correlated with the multiple shoot induction period during shoot regeneration. There was gradual decrease between day 40 to day 60 (after 3rd subculture) in the GSH content. The GSH content reached the maximum during root induction and growth. Even after every subculture regime also there was an increase in a GSH content indicating the prevalence of oxidative stress (Figure 3).

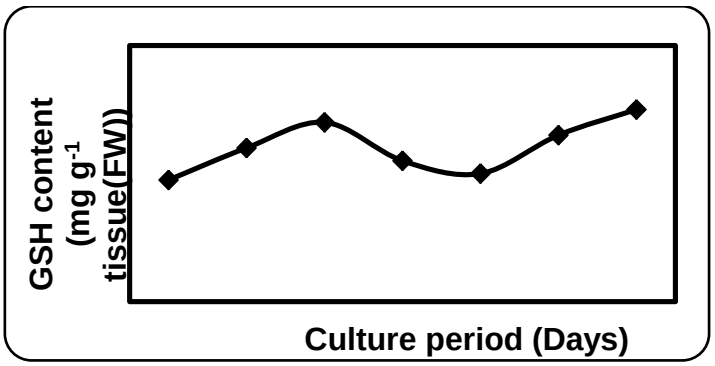

Figure 3: Changes in Glutathione (GSH) content during *in vitro* organogenesis

Changes in anti-oxidant enzymes:

Superoxide dismutase (SOD) (EC 1.15.1.1) activity increased markedly after subculture (for every 25 days) indicating the oxidative stress induced during each subculture regime. There was a rapid burst in the SOD activity after the second subculture (45 days) regime, after day 45 till day 65 there was a declining trend observed in the SOD activity, and thereafter increased during rooting stage that is in between day 65 to day 80 (Figure 4).

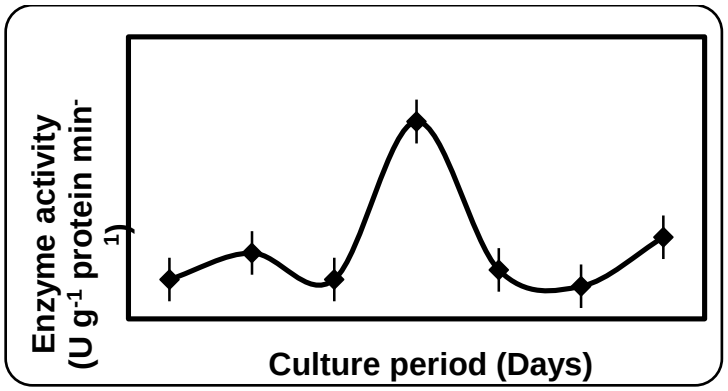

Figure 4: Changes in Superoxide dismutase (SOD) activity during *in vitro* organogenesis

Peroxidase (POX) (1.11.1.7) activity was found to be increased during the all stages of morphogenesis (shoot regeneration) and even after every subculture too till the root induction. The magnitude of elevation was more after second subculture (between 40 to 60 days of culture) and gradually declined during rooting stage (Figure 5).

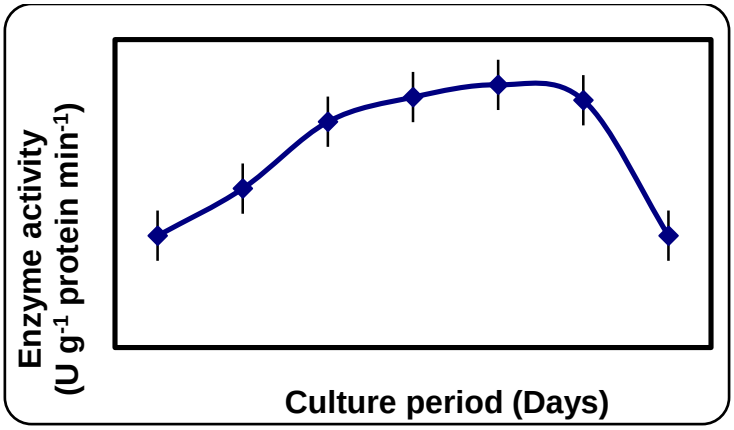

Figure 5: Changes in peroxidase activity (POX) during *in vitro* organogenesis

Catalase activity (CAT) (EC 1.11.1.6) increased during all the stages of morphogenesis. There was a steep increase in activity before second subculture (between day 20 to 40), followed by decrease in the activity after every subculture during initial five days of after subculture (between day 40 to 45th day and day 60 to 65th day), thereafter increase in the activity till the next subculture (between day 45th to 60th day) during shoot regeneration and also during root induction (between day 65th to 80th day) (Figure 6).

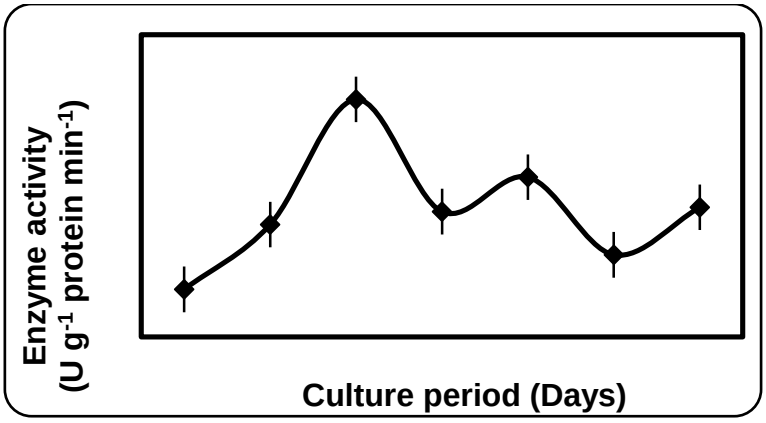

Figure 6: Changes in catalase activity (CAT) during *in vitro* organogenesis

The activity of ascorbate peroxidase (APX) (EC 1.11.1.11) was slightly increased between day 20 and day 25, followed by a steep increase till day 40 and gradually progressing to till day 60 during shoot regeneration. The increase in the activity was more pronounced between, first five days of every subculture to the day of subsequent subculture (between day 25 to day 40 and day 45 to day 60) during shoot regeneration. However, during root induction phase (between day 60 to day 80) the APX activity was found to be decreased gradually (Figure 7).

Glutathione reductase (GR) (EC 1.6.4.2) activity increased gradually during all the stages of morphogenesis (both shoot and root organogenesis) except after five days of second subculture (day 45) a slight decrease in activity was observed (between day 45 to day 60). The increase was significant during root induction period (between day 60 to day 80) (Figure 8).

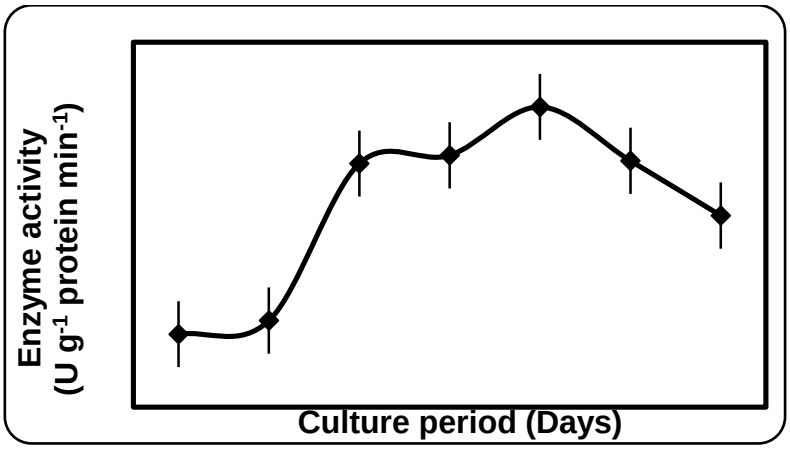

Figure 7: Changes in Ascorbate peroxidase activity (APX) during *in vitro* organogenesis

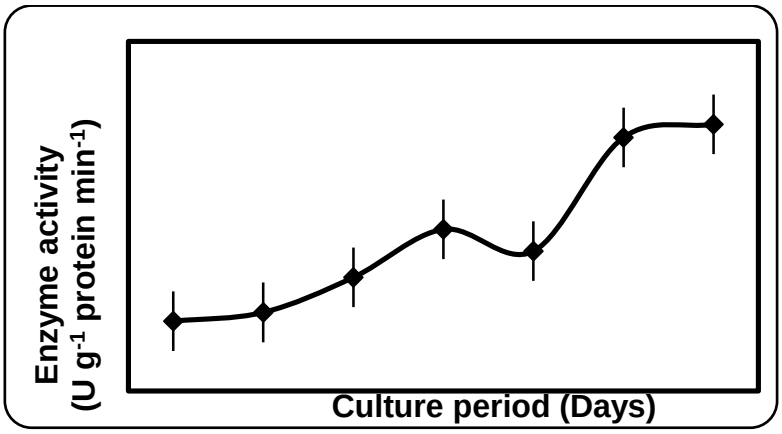

Figure 8: Changes in glutathione reductase activity (GR) during *in vitro* organogenesis

Glutathione-S-transferase (GST) (EC 2.5.1.18) activity was found to be decreased during initially after first sub-culture (between day 20 to day 25), thereafter a steep increase was observed till day 45 (between day 25 to day 45).After five days of second subculture (on day 40) the activity was decreased till the end of shoot organogenesis period (between day 45 to day 60). However a gradual increase in the GST activity was observed during rooting stage (between day 60 to day 80) **(**Figure 9).

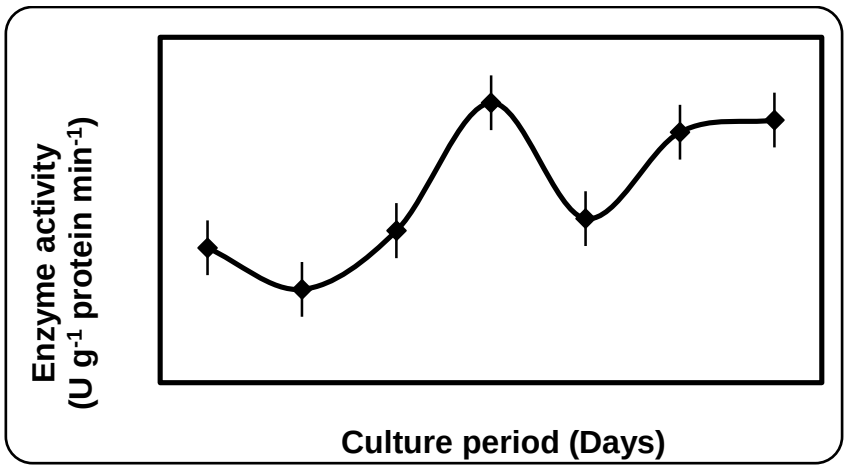

Figure 9: Changes in Glutathione S transferase activity (GST) during *in vitro* organogenesis

DISCUSSION

The customary step in the micropropagation process is to obtain aseptic culture of the selected plant material. Different types of disinfectants like bavistin, cetrimide, mercuric chloride ($HgCl_2$), sodium hypochlorite (NaOCl) were used for sterilizing the nodal explants of *Gymnema sylvestre*. Inclusion of bavistin (0.12%) and cetrimide (0.08%) along with neutral detergent in the pretreatment solution following $HgCl_2$ 0.1% for 3 min or 0.05% for 5 min exposure was found to be effective in obtaining healthy shoot proliferation with very low contamination. It has been observed that, if concentrations and duration were respectively too high or too long, the plant tissue are damaged, but too mild exposure do not eliminate the contamination by microorganisms. Hence it is important to determine the best concentration and the duration of exposure to sterilant. In the present study *in vitro* plant regeneration was achieved using different concentrations of Kn and BA alone or in combination with NAA. Our observations indicate that BA and NAA play crucial roles in multiple shoot induction from nodal explants. The addition of NAA into the medium considerably enhanced multiple shoot induction. The essentiality of a cytokinin-BA for multiple shoot induction from nodal cuttings has been reported in *Terminalia bellirica*[25], *Ceropegia intermedia*[26] and *Wattakaka volubilis*[27]. The superiority of BA over Kn in multiple shoot induction were made in the present study. However, the individual use of cytokinins was not found to give optimum results in most of the systems; the best result was obtained when Kn or BA was employed in combination with an auxin. Combination of cytokinins and auxins stimulated *in vitro* multiplication has been reported in several plant species *Aegle marmelos*[28], *Rotula aquatica*[29], *Ocimum basilicum*[30]. The synergistic effect of BA in combination with an auxin has been demonstrated in many medicinal plants of Asclepiadaceae family, such as *Holostemma annulare*[31], *Holostemma ada-kodien*[29], *Sarcostemma brevistigma*[32], *Ceropegia spiralis*[33].Explanting season was observed to influence the culture initiation and regeneration frequency during pre-flowering and flowering seasons in a year. It could be concluded that, for explantation ideal period is between November-March to obtain optimal culture initiation and regeneration with minimal contamination. The seasonal influence of explant collection on culture establishment has been reported for other plant species including *Ceratonia siliqua*[34], *Carissa carandas*[35], *Tylophora indica*[36].In the present study IBA at 3 mg l^{-1} was found to be more effective in root induction in terms of root length and number followed by NAA and IAA. The superiority of IBA in inducing rooting over the other auxins was also reported in *Decalepis arayalpathra*[37] and *Ricinus communis* L.[38] The role of IBA in root induction has been reported in medicinal plants of *Asclepiadaceae* family viz., *Ceropegia jainii*[39], *Hemidesmus indicus*[40], *Holostemma ada-kodien*[29], *Ceropegia candelabrum*[41] and *Sarcostemma brevistigma*[42]. In the present study shoot regeneration was induced by a combination of high cytokinin BA (2 mg l^{-1}) and low auxin NAA (0.05 mg l^{-1}), and rooting was achieved in the regenerated shoots by auxin alone IBA (3 mg l^{-1}) supplemented in MS medium. Generally, the *in vitro* artificial conditions generate an oxidative stress on plant cells and the association with additional factors determines specific adaptation reactions. 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[41]. In higher plants redox state plays an important role during growth and development of plant, and a reduced culture environment is required during organogenetic events characterized by active cell proliferation, and also during initial embryogenic events and somatic embryo development[43]. There is a growing body of evidence indicating that the role of ascorbate and glutathione in plants extends beyond their intensively explored antioxidant function[44]. It has been demonstrated that high ascorbate and glutathione levels are required for normal progression of the cell cycle in meristematic tissues[45]. Ascorbate is directly involved in the regulation of two processes that mediate morphogenic responses in plants: cell division and elongation. The reduced ascorbate (ASC) as well as oxidised form of this compound (dehydroascorbate, DHA) play an important

role in the regulation of mitotic activity in the meristems[45]. In the present study it was observed that, ascorbate content was increased in a steady manner throughout all the stages of both shoot and root organogenesis (Figure 2), indicating the presence of active non-enzymatic ROS scavenging system. Previous reports suggest that ASC may enhance shoot organogenesis in tobacco callus culture[46]. The antioxidant enzyme assay can offer information concerning the reaction of the plant tissue to different kind of stresses but also to the adaptation to particular conditions. In order to determine the possible involvement of oxidative events in the induction and during the course of regeneration processes in *Gymnema sylvestre*, changes in certain antioxidant enzymes like SOD, POX, CAT, APX, GR and GST were measured. Results of the present study suggest that, peak activities of different antioxidant enzymes varied with the stage of morphogenesis and subculture (Figures 4-9). It has been reported that cytokinins stimulate the enzymes that are associated with H_2O_2 detoxification like POX, APX and CAT[47]. In the present study the shoot organogenesis has been induced by a combination of high cytokinin BA (2 mg l^{-1}) and low auxin NAA (0.05 mg l^{-1}). The cytokinin BA (2 mg l^{-1}) in the medium might have stimulated the activities of H_2O_2 detoxiying enzymes like, APX, CAT and POX activities during shoot organogenesis (till day 60), as thereafter it has been observed that, the activities of these enzymes gradually decreased from day 60 to day 80 (rooting phase), might be due to the transfer of shoots to rooting medium that lacks cytokinins.APX activity progressively increased with the reaching maximum by day 60 (during shoot organogenesis), which might be involved in the detoxification of H_2O_2 produced during dismutation of superoxide radical, that might have improved stress tolerance. Increase of peroxidase activity and isozymes have been correlated with adventitious shoot formation, root induction and somatic embryogenesis[48]. Peroxidase (POX) activity also increased progressively from day 20 to day 60 during shoot organogenesis (Figure 5). A pronounced increase in catalase activity was observed during shoot growth (between 40 to 60 days of culture). Catalase, besides its protective function against stress may also have developmental role, organized development in cultured tissue, including somatic embryogenesis is often promoted by the imposition of stress treatments[49].Low levels of SOD activity was observed during all growth stages except during second subculture period had shown a peak activity by day 40 (Figure 4). It might be implied that plant does not majorly dependent on SOD activity for detoxification of $O_2^{\bullet-}$, rather they do possess alternative, non-enzymatic routes for conversion of $O_2^{\bullet-}$ to H_2O_2 using antioxidants like glutathione and ascorbate. In support of this view, there is an active elevation in the levels of non-enzymatic antioxidants throughout the culture regime (Figures 2 & 3). Decrease in SOD activity during shoot organogenesis, were also reported in *Gladiolus*[50] and *Crocus sativus*[10].GR activity progressively increased along the developmental stages with a peak increase during rooting. GST activity also followed same pattern but peak activity was observed after second subculture indicating its involvement in stress tolerance. Lower levels of GR activity was observed during shoot organogenesis (from day 20 to day 60), thereafter the activity rose to attain maximum levels during rooting stage (between day 60 to day 80). The increase in GR levels might be attributed to provide the GSH so as to replenish the oxidized ascorbic acid levels to reduced state by the reduction of dehydroascorbate to ascorbic acid (Figure 8). This finding is consistent with an overall mode of auxin action, which triggers auxin-specific signalling pathways, oxidizing the cellular environment[51,52]. Thus GR activity could be considered as the key factor that keeps the cellular antioxidant status (reduced environment) maintained during rooting phase by providing both GSH and ascorbic acid so as to cope the oxidative state promoted by auxin. It has been reported that, artificial environmental conditions of *in vitro* culture may alter oxidative metabolism and predispose tissues to the damaging effects of ROS[53]. Namely, in plants grown in tissue culture, some of the protective systems against ROS can be disrupted, which is followed by an increased level of these highly reactive molecules[54]. This is then reflected in alterations of antioxidative enzyme activities and antioxidants[55] and subsequently in altered

morphology of plant tissue grown *in vitro*[56]. In conclusion, for culture establishment from *ex vitro* nodal explants of *Gymnema sylvestre* ideal period is between November-March months that provides optimal culture initiation and regeneration with minimal contamination. The integrated system that comprises the non-enzymatic soluble antioxidant molecules and antioxidant enzymes like SOD, POX, CAT, APX, GR and GST successfully prevented the stress induced during explant proliferation and regeneration, and also subculture. Thus all these molecules might endure the active growth of tissue/shoots/roots, which could be confirmed by observing active growth of shoots and further plantlets.

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