American Journal of Biology and Life Sciences
2015; 3(2): 36-42
Published online April 10, 2015 (http://www.openscienceonline.com/journal/ajbls)

Open Science

Micropropagation of *Chlorophytum borivilianum*: *In vitro* Clonal Fidelity Test and Antioxidant Enzymatic Study

Sunita[1, *], Sonali Jana[1], G. S. Shekhawat[2]

[1]Department of Bioscience and Biotechnology, Banasthali University, Banasthali Rajasthan, India
[2]Department of Botany, Jai Narain Vyas University, Jodhpur Rajasthan, India

Email address

sunitabanasthali@gmail.com (Sunita)

To cite this article

Sunita, Sonali Jana, G. S. Shekhawat. Micropropagation of *Chlorophytum borivilianum*: *In vitro* Clonal Fidelity Test and Antioxidant Enzymatic Study. *American Journal of Biology and Life Sciences*. Vol. 3, No. 2, 2015, pp. 36-42.

Abstract

The regeneration of *Chlorophytum borivilianum* is generally through tuberous roots, which have become scarce in nature. Seed germination is only 14-16%, thus an *in vitro* method for conservation and multiplication of this species could be valuable. *In vitro* clonal propagation of safed musli has been achieved on MS medium augmented with auxins: 2, 4-Dicholorophenoxyacetic acid (2, 4-D), 1-Naphthaleneacetic acid (NAA) and cytokinins: Benzyladenine (BA), Kinetin (Kn). There was a 90% shoot multiplication response obtained at MS + 4.4 µM BA. Isolated shoots were transferred on ½ MS medium supplemented with 2.4 µM IBA (indole-3-acetic acid) which gave 90% rooting response, were further acclimatized and hardened to garden successfully. *In vitro* regenerated plants have been tested for clonal fidelity using RAPD markers. It is first report on biochemical changes of catalase, peroxidase, super oxide dismutase, protein and chlorophyll content during *in vitro* regeneration in *C. borivilianum*.

Keywords

Chlorophytum borivilianum, RAPD, Catalase, Genetic Fidelity, Superoxide Dismutase

1. Introduction

Chlorophytum borivilianum Santapau. et R.R. Fern., Asparagaceae, commonly known as 'safed musli', is an endangered plant (Purohit et al.1994) which also holds the position of one of the rare medicinal herbs of India (Narasimham and Ravuru 2003). *C. borivilianum* is mainly growing in thick forests in its natural form. It is an herb with sub-erect lanceolata leaves. There are about 256 varieties of Chlorophytum and 17 among them are found in India (Maiti and Geetha 2005); the species is valued for dried fasciculated storage roots. It is used in many Ayurvedic vital tonics and aphrodisiac formulations (in old literature under *C. arundinaceum*) (Purohit et al.1994). These roots possess immunomodulatory, adaptogenic properties and are used traditionally to cure impotency, sterility and boost male potency (Agrawal et al. 2013). The main active principles of roots are saponins and alkaloids which act as stimulants, metabolic enhancers and anti-tumour activity (Mimaki et al.1996; Qiu et al. 2000). Due to its therapeutic activity and diversified uses, demand of *C. borivilianum* is increasing in International and Indian markets. Hence, a highly effective micropropagation technique could be useful for the cultivation of the species and *ex situ* conservation.

There are only few reports on the multiplication of this species through explants such as young shoot bases (Purohit et al.1994) stem disc (Joshi et al. 1999; Suri et al. 1999) leaf (Gaikwad et al. 2003) and encapsulated shoot buds (Dave et al. 2003). Many reports have focused on the medicinal and agronomical aspect of *C. borivilianum*, but little attention has been drawn to the alteration in antioxidant metabolism of the plant when grown *in vitro*. Generally, micropropagated plants from the preformed structures such as shoot tips and axillary buds maintain clonal fidelity (Ostray et al. 1994). However, the possibility of generating somaclonal variations still exists (Rani and Raina 2000; Bindiya and Kanwar 2003). Since, clones of elite species are in greater demand than variants, random amplified polymorphic DNA (RAPD) is the most

efficient and the cheapest tool for the detection of genetic variability in plants (Fernando et al. 1996; Cassells et al. 1997; Hussain et al. 2008).

Plants can modify their growth, development, and physiology according to a variable environment. This ability of plants plays a key role in determining their tolerance to stress and maintains efficient growth. (Lee et al. 2007). During *in vitro* micropropagation, when explants are brought to artificial conditions, these undergo a series of physiological and metabolic changes. Hence, exposure of plants to any type of stress leads to the generation of Reactive Oxygen Species (ROS). The ROS may react with proteins, lipids and DNA, causing oxidative damage and impairing normal function of cells.

In this communication, we report for the first time an effective protocol for high frequency regeneration through nodal segment cultures of *C. borivilianum*. RAPD analysis has been performed on regenerated plants to confirm the genetic identity of the clones. Furthermore, we have also examined the changes of antioxidant enzyme activity of catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD), along with protein and chlorophyll content during *in vitro* morphogenesis and a comparison have been done amongst *in vitro* and *in vivo* plantlets.

2. Materials and Methods

2.1. Explant Preparation

Young shoots approximately 3.0 cm in length of *C. borivilianum* were collected during month of January from the medicinal plant garden at Banasthali University. The explants were surface sterilized with Tween-20 and kept under running tap water for 15 minutes. Under aseptic conditions these explants were further sterilized with 0.2% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 5 min and rinsed with distilled water thrice till traces of $HgCl_2$ were removed. The pH of the media was adjusted to 5.8 using 0.1N HCl or 0.1N NaOH prior to autoclaving. The MS medium was gelled with 0.8% (w/v) agar (Qualigen, India). In every flask 30-35 ml of media were dispensed (100 ml flask, Borosil) plugged with non-absorbent cotton and sterilized at 121˚C, 1.06 kPa pressure for 15 min.

2.2. Culture Conditions

The cultures were maintained by regular subculture onto fresh medium with same composition at an interval of 4 weeks. The cultures were incubated at a temperature of 25±2˚C, relative humidity of 55±4% and photoperiod 16/8 h (light/dark) with light provided by white florescent lamps with a photosynthetic photon flux (PPF) of 40µmol m^{-2} s^{-1}.

2.3. Shoot Regeneration and Rooting

The explants were placed on semi-solid basal Murashige & Skoog's (1962) medium supplemented with different concentrations of BA (0.8-8.8 µM), NAA (1.0-10.7µM) in combination as well as singly, with 3% sucrose. Regenerated shoots were transferred to various strengths of MS in combinations of auxins IBA (0.5-2.4 µM), IAA (0.5-2.4 µM), NAA (2.6-16 µM). Individual shoots separated from clusters were trimmed from the top leaving approximately 1 cm at the base. Regenerated shoots obtained by subculturing on suitable hormonal composition, were subjected to rooting medium. Callusing was not observed on any of the tried concentration. The shoot length ranged between 3-3.5 cm were tried for rooting. Effect of shock treatment in which plant roots were subjected to ½ MS with 24.6 µM IBA for 2 days in dark conditions for rooting response was explored. Acclimatization was also done for all regenerated plantlets.

2.4. Hardening and Acclimatization of Regenerated Plantlets

Plantlets with well developed roots of length approximately 5.0 cm were removed from the culture medium. The roots were gently washed under running tap water to remove the traces of medium adhered to the roots. The plantlets were transferred to plastic cups which were covered with glass beakers to retain moisture and then to jam bottles containing soil rite. After 30 days, these plants were suitable to be transferred to green house.

2.5. Genetic Fidelity Test of Regenerated Plantlets

Genomic DNA was isolated from fresh and young leaves of 6 hardened *in vitro* developed and 2 *in vivo* grown plants of *C. borivilianum* (Doyle and Doyle 1990). DNA concentration and purity was also determined by running the samples on 0.8% agarose gel. Ten arbitrary decamers were used for Polymerase Chain Reaction (PCR) (Table 1). Amplification reactions were performed in 25µl volume containing 10× assay buffer (100 mM Tris-HCl; pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1 % gelatin), 200 µM dNTPs (dATP, dCTP, dGTP and dTTP), 10 µM primer, 0.2 unit of Taq DNA polymerase (Bangalore Genei, Bangalore, India) and 30 ng of template DNA. The amplification reaction was carried out in a DNA thermal cycler (Eppendorf AG, Hamburg, Germany)

Table 1. *Effect of BA and NAA on in vitro shoot multiplication of C. borivilianum after 30 days of culture. n = 5 [Values are Means ± SE].*

BA (µM)	NAA (µM)	Regeneration (%)	Number of shoots	Shoot height (cm)
0.8	5.3	20	1.83±0.40	1.88±0.29
2.2	5.3	30	2.70±0.41	3.7±0.21[a]
4.4	-	90	7.45±0.34	8.65±0.14
4.4	0.5	5	6.50±0.23[a]	4.0±0.34[b]
4.4	1.0	7	5.99±0.11	4.2±0.33[b]
4.4	2.6	10	4.60±0.16	3.5±0.76[a]
4.4	5.3	20	6.22±0.43	2.6±0.54
4.4	10.7	-	-	-
8.8	-	80	6.50±0.34[a]	7.5±0.22

Means having the same letter in each column do not differ significantly at P<0.05 (LSD test)

The amplification was carried out using thermal cycler following 1 cycle of denaturation at 94° C then 30 cycles of annealing (1 min at 94° C, 30 s at 48° C and 60 s at 72° C) followed by1 extension cycle (2 min at 72° C). Samples were then subjected to short spin to collect the mixture. This mixture was loaded on the 1.5% (w/v) agarose gel. These were then stained with ethidium bromide and visualized using Gel Documentation System. Banding pattern was then compared with donor plants to find out the clonal fidelity.

2.6. Biochemical Study of in vitro and *in vivo* Plantlets

2.6.1. Enzyme Extraction

Tissue (0.5 g) was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.05% Triton X-100, 1 mM polyvinylpyrrolidone, and 1 mM ascorbate. After centrifugation of homogenate at 5,000×g for 20 min at 4°C, the supernatant was used to measure the activities of antioxidative enzymes. The sample was obtained by homogenizing the *in vitro* shoot, regenerated plant with roots and *in vivo* plants. Protein estimation was carried out by method of Lowry et al. (1951).

Catalase activity was measured by the method of Aebi (1974). The assay system comprised of 50 mM phosphate buffer (pH 7.0), 20 mM H_2O_2 and a suitable aliquot of enzyme in the final volume of 3 ml. Decrease in the absorbance was recorded at 240 nm. The molar extinction coefficient of H_2O_2 at 240 nm is 0.04 μM^{-1} cm^{-1}.

Super oxide dismutase activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971) with 3 ml reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 mM Riboflavin, 0.1 mM EDTA and a suitable aliquot of enzyme extract. This reaction mixture was incubated for 30 min under fluorescent lamp. A tube containing enzyme was kept in dark served as blank while the control tube without enzyme was kept in dark served as blank and the control tube without enzyme kept in light as control. The absorbance was taken at 560 nm. Calculations were made by using an extinction coefficient: 100 $mM^{-1}cm^{-1}$.

Peroxidase (POD) assay was done by homogenising the samples in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. POD activity was determined by monitoring the formation of tetraguaiacol (extinction coefficient, 6.39 mM^{-1} cm^{-1}) at 436 nm during enzymatic reaction from initial substrate guaiacol in presence of H_2O_2 following the method of Pütter (1974). The reaction was initiated by adding 0.1 mM H_2O_2.

Protein was estimated by protocol of Lowry et al. (1951). For this estimation homogenization of leaf was done, to which copper sulphate ($CuSO_4$) prepared in 1% potassium sodium tartrate was added and incubation was done at room temperature for 10 min. After that 0.5 ml folin's reagent was added and mixed well. All the tubes were incubated for 30 min in dark. Blue colour developed and absorbance was recorded at 660 nm.

For estimation of photosynthetic pigment chlorophyll 'a', 'b' & total chlorophyll, tissue (0.25 gm) was homogenized in chilled 80% acetone in dark. After centrifugation of homogenate at 10,000g for 10min at 4ºC, absorbance of supernatant was taken at 645, 663, and 665 nm (Arnon, 1949).

2.6.2. Experimental Design and Statistical Analysis

All data were recorded after 3 weeks of initiation of multiple shoots as well as root initiation. All experiments were conducted with minimum of five replicates per treatment. The data were analyzed statistically using SPSS version 16. The significance of differences among mean was carried out using one way ANOVA range test at $P<0.05$. The results are expressed as the mean ± standard error of five replicates per treatment.

3. Results

3.1. Shoot Regeneration

After seven days of culture bud break was recorded in cultured nodal explants with pre-existing axillary single bud. No callusing was observed and the explants remained green throughout that duration. Optimized response for shoot multiplication was obtained at BA 4.4µM. Number of shoots per explants was 7.45±0.34 with shoot length of 8.65±0.14 cm after 4 weeks of culture (Table 2 and Fig.1 (A), (B) & (C)). There is no significant difference among the treatments applied ($P<0.05$).

Table 2. *Effect of IBA and NAA on root induction in C. borivilianum. Data recorded on third week of transfer to the rooting medium. n = 5 [values are mean±SE].*

Basal medium	PGR (µM)	Rooting (%)	No. of roots	Length of roots (cm)
MS	0.5 IBA	75%	8.2±0.43	3.02±0.66[a]
MS	2.4 IBA	80%	7.6±0.66[a]	4.78±0.58[b]
MS	22 IBA	85%	7.3 ±0.55	2.35±0.24
½ MS	2.4 IBA	90%	10.0±0.25	5.76±0.26
MS	2.6 NAA	80%	6.5±0.66	3.02±0.44[a]
MS	5.3 NAA	90%	9.2±0.75	4.82±0.05[b]
½ MS	8.0 NAA	75%	7.7±0.33[a]	2.45±0.24
½ MS	16 NAA	60%	7.6±0.21[a]	3.00±0.44[a]
¾ MS	0.5 IAA	-	-	-
¾ MS	2.4IAA	-	-	-

Means having the same letter in each column do not differ significantly at $P<0.05$ (LSD test)

3.2. Rooting of Regenerated Shoots

On ½ MS supplemented with 2.4µM IBA 90% of the shoots were successfully rooted (Fig.1 (D), (E) & (G)). The fibrous adventitious roots originating from the basal shoot part were 10±0.25 in number with an average length of 4.5 cm (Table 3). There is no significant difference among the treatments applied ($P<0.05$). These results are in consonance with Samantaray and Maiti (2010), where rooting was

achieved upon transferring the shoots onto half strength MS medium 233 supplemented with IBA and 2% sucrose. In the present study a novel approach for rooting was tried in which, the rooted plantlets were dipped in ½ MS (without agar) + 24.6 µM IBA for 48 hours in dark (Fig. 1(F)). This much of high concentration of IBA was like a shock to the newly emerged roots. An interesting observation was recorded, roots tried in shock treatment were thicker and elongated, comparatively to roots on agar medium were thin and less elongated (Table 4).

3.3. Hardening and Acclimatization

For acclimatization plants were transferred to plastic cups containing soil: soilrite (1:3), which were covered with glass beakers to retain moisture (Fig. 1 (H) & (I)). After 30 days these plants were transferred to green house and then successfully hardened in the field (Fig. 1 (J)). Rooted plantlets acclimatized in the greenhouse for 2 weeks and 75% of transferred plants to the field survived. At this point, the plants grew normally and no gross morphological variation was noticed.

Table 3. *Comparative analysis of plants grown on Agar medium and plants given a shock treatment n = 5 [values are mean±SE].*

S.No.	Basal medium	Average root Number		Average root Length (cm)	
	MS+PGR(µM)	Before shock treatment	After shock treatment	Before shock treatment	After shock treatment
1.	MS+0.5 IBA	-	8.2±0.43	-	3.02±0.66[a]
2.	MS+2.4 IBA	3.3±0.45	7.6±0.66[a]	3.00±0.16[a]	4.78±0.58[b]
3.	MS+22 IBA	5.6±0.36[a]	7.3 ±0.55	1.90±0.08	2.36±0.24
4.	½MS+2.4 IBA	5.9±0.5[a]	10.0±0.25	4.89±0.48	5.76±0.26
5.	MS+2.6 NAA	-	6.5±0.66	-	3.02±0.44[a]
6.	MS+5.3 NAA	2.3±0.43	9.2±0.75	3.65±0.6[a]	4.82±0.05[b]
7.	½MS+8.0NAA	-	7.7±0.33[a]	-	2.45±0.24
8.	½MS+16 NAA	-	7.6±0.21[a]	-	3.00±0.44[a]
9.	¾MS+0.5 IAA	-	-	-	-
10.	¾ MS+2.4IAA	-	-	-	-

Means having the same letter in each column do not differ significantly at P<0.05 (LSD test)

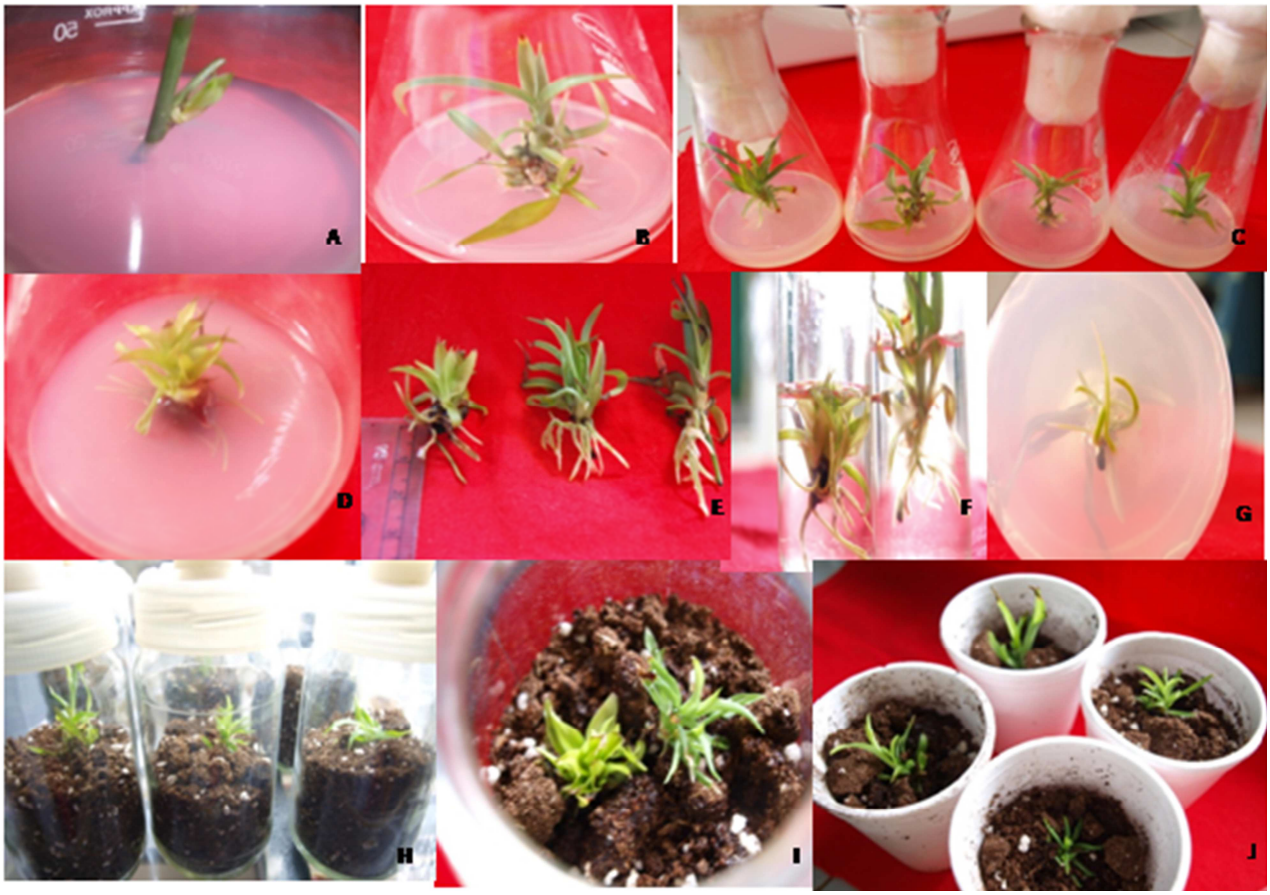

Fig. 1. *Different stages of micropropagation of C. borivilianum. A. shoots bud initiation on MS + BA (4.4µM). B. Shoots after 15 Days of culture shows multiplication. C. Multiplying shoots after 30 of days of culture. D. Rooted plant on ½ MS + 2.4 µM IBA. E. Root initiation and elongation in regenerated shoots 15, 20 and 25 days old plantlets. F. Plantlets immersed in suspension culture with ½ MS +5mg L^{-1} IBA in dark for 2 days, roots were more prominent and sturdy. G. Plant with roots in agar medium, H- I. Plants in soil: soilrite (1:3) before acclimatization, J- Acclimitized plants after 10 days in culture room.*

3.4. Genetic Fidelity Check of Regenerated Plants

Ten random primers were tested as shown in Table 4, two (S2 & S3) were selected on the basis of their clarity in the electrophoresis banding patterns (Fig. 2). In the present study, the bands amplified through RAPD showed monomorphism with the mother plant and were also similar among the *in vitro* raised plants. There are similar reports on *C. borivilianum* using RAPD markers; where genetic stability has been proved (Samantaray and Maiti, 2010; Samantaray et al. 2011). All RAPD profile analysis from micropropagated plants was genetically similar to mother plants. Therefore these regenerated plants are true copies of donor plants and this mode of regeneration (direct) has not resulted in any kind of variations. So, regeneration using nodal explants can be a boon for producing elite and endangered plants, highly in demand for their medicinal properties.

3.5. Biochemical Estimation of Enzymes

In biochemical estimation of catalase enzyme, it was found that capacity of *in vitro* grown regenerated plants to reduce hydrogen peroxide is higher than the *in vivo* plants. The highest activity shown by regenerated plants was 5.3 $mM\mu g^{-1}min^{-1}$. Increase in CAT activity during *in vitro* growth is in agreement with the result of Gupta and Datta (2003) as reported in *Gladiolus hybridus* Hort. Similar pattern of difference in enzymatic activity of *in vitro* shoots and regenerated plants is also revealed by Misra et al. (2010) while studying the *in vitro* propagation of Asiatic Hybrid Lily. The high activity of CAT in regenerated plant could be due to higher content of H_2O_2 in regenerated shoot (Meratan et al. 2009). The results of superoxide dismutase and peroxidase activity are in accordance with that of CAT, the enzyme activity of SOD and POD was found higher in regenerated plants i.e. $9.7mM\mu g^{-1}min^{-1}$ and 10.0 $mM\mu g^{-1}min^{-1}$ respectively (Fig. 3). CAT and POD are known to play a role in growth and differentiation (Gaspar 1995; Molassiotis et al. 2004) and their high activity could be correlated to the process of differentiation that occurred during shoot and root induction. SOD activity directly modulates the amount of ROS and higher SOD activity contributes to detoxification of superoxide. (Gupta and Datta 2003) The changes in antioxidant enzymatic activities appeared to be related to the mode of plant regeneration. The total protein content has a tendency to decrease with the developmental stage of shoot. The highest protein content was observed in *in vitro* shoots while the lowest protein content was found in *in vitro* rooted plant. The concentration of protein *in vitro* shoots, *in vitro* regenerated plants and *in vivo* plants is found to be 0.181, 0.140 and 0.146 (mgg^{-1}) respectively (Fig.4). Similar pattern of changes in protein content was also obtained by Sharifi and Ebrahimzadeh (2010). This might be due to synthesis of certain amino acid or polypeptides required to initiate shoot bud initiation and their depletion led to rhizogensis (Panigrahi et al. 2007).

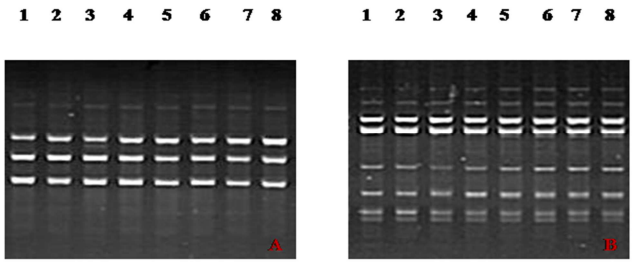

Fig. 2. *RAPD profile of field grown mother plant and micropropagated plants of C. brovilianum generated by primer S2 and S3 (Fig A and B) Lane 1,2 shows banding profile of mother plants and lane 3 to 8 represents banding pattern of 6 different micropropagated plants*

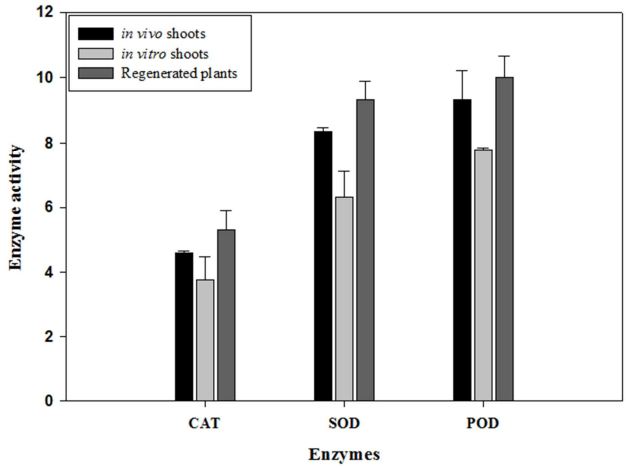

Fig. 3. *Antioxidant enzymatic activity (CAT, SOD and POD) in different stages of morphogenesis in C. borivilianum*

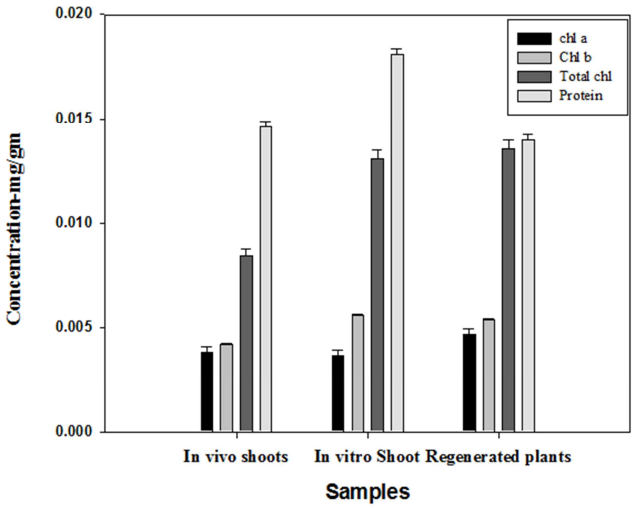

Fig. 4. *Chlorophyll content and protein concentration of different stages of morphogenesis in C. borivilianum*

The photosynthetic pigments such as chlorophyll 'a', chlorophyll 'b' and total chlorophyll content was evaluated in *C. borivilianum*. The highest level of chlorophyll 'a' was found in regenerated plants i.e. 0.00466 (mg g^{-1}fresh weight) and in *in vivo* grown plants i.e. 0.0038 (mg g^{-1} fresh weight).

Similarly level of chlorophyll 'b' was maximum in *in vitro* grown shoots i.e. 0.0053 (mg g^{-1}fresh weight) and was 0.0041(mg g^{-1} fresh weight) found in *in vivo* plants. Total chlorophyll content was highest in regenerated plants i.e. 0.01355 (mg g^{-1}fresh weight) and least content in *in vivo* grown plants. (Fig. 4)

Table 4. *List of decamer primer used for RAPD*

S.No.	Primer	Sequence 5'-3'
1.	S1	CAAACGTCGC
2.	S2	GTCGCCGTCA
3.	S3	GTCGCCGTCA
4.	S4	TCTGGTCAGG
5.	S5	TGAGCGGACA
6.	S6	ACCTGAACGG
7.	S7	TTGGCACGGG
8.	S8	GTGTGCCCCA
9.	S9	CTCTGGAGAC
10.	S10	GGTCTACACC

4. Discussion

The enhancement in number of shoots per explants has been recorded in many plant tissue culture systems containing cytokinins, particularly BAP, as a cell division growth hormone. Similar morphogenetic response has also been recorded in internodal and nodal ring regions of *Piper* species (Bhat et al.1995). The results obtained here, are akin with Kemat et al. (2010) where young shoot buds of safed musli grown on MS medium supplemented with BA and Kn were reported for shoot induction. Similarly, more than 80% callus free rooting was obtained on ¾ MS supplemented with 2 mg L^{-1} IBA (Joshi and Purohit 2012). A variety of gene expressions and protein syntheses are involved in the shoot organogenesis, which are biologically complex developmental and differentiation processes (Shi et al. 2006). There are various reports on antioxidant enzyme changes during *in vitro* studies, which have gained interest in past few years. Plant growth and productivity is adversely affected by nature's wrath in the form of various abiotic and biotic stresses. The ROS in plants can be removed by a series of enzymatic and non enzymatic detoxification systems. ROS induce defence system of plant at low concentration; on the other hand it triggers a genetically controlled death at higher concentrations (Van Breusegem et al. 2001). SOD activity directly modulates the amount of ROS and higher SOD activity contributes to detoxification of superoxide (Gupta and Datta 2003). The changes in antioxidant enzymatic activities appeared to be related to the mode of plant regeneration. The decreased chlorophyll content was obviously due to retarded growth of plant. Another reason could be due to excess salt supply through MS medium resulting in interference with the synthesis of chlorophyll. These results indicate that the organogenesis is a very complicated process involving up and down regulation of a number of antioxidant enzymes, which seem to play an important role in root/shoot organogenesis (Lee et al. 2007).

5. Conclusion

In recent years many research groups have been involved in establishing reliable regeneration procedure for medicinally important plants because such protocols are essential for plant improvement as well as for production of medicines. In the present work we demonstrate for the first time the regeneration of shoot and root from nodal explant and involvement of oxidative protection system during organogenesis. The plantlets regenerated by this method are true to type. This procedure can be used to conserve this endangered plant species.

References

[1] Aebi, H. 1974. "Catalases." In *Methods of Enzymatic Analysis*. Edited by H.U. Bergmeyer, pp. 673-684. New York: Verlag Chemie, Weinheim, Academic Press.

[2] Agrawal, R., Upadhyay, A., & Nayak, P. S. 2013. Drying characteristics of *Safed Musli* (*Chlorophytum borivilianum*) and its effect on colour and saponin content. Journal of Pharmacognosy and Phytotherapy 5(8):142-147.

[3] Arnon, D.I. 1949. Copper enzymes in isolated chloroplast: polyphenol oxidases *in Beta vulgaris. Plant Physiology 24:1-15.*

[4] Beuchamp, C., & Fridovich, I. 1971. Superoxide dismutase: improved assays and an acrylamide gels. Analls of Biochemistry 44: 276-287.

[5] Bhat, S.R., Chandel, K.P.S., & Malik. S.K. 1995. Plant regeneration from various explants of cultivated *Piper* species. Plant Cell Reports 14: 398-402.

[6] Bindiya, K., & Kanwar K. 2003. Random amplified polymorphic DNA (RAPDs) markers for genetic analysis in micropropagated plants of *Robinia pseudoacacia* L. Euphytica 132: 41-47.

[7] Cassells, A.C., Croke, J.T., & Doyle, B.M. 1997. Evaluation of image analysis, flow cytometry, and RAPD analysis for the assessment of somaclonal variation and induced mutation in tissue culture-derived *Pelargonium* plants. Angewandte Botanik 71(3-4): 125-130.

[8] Dave, A., Bilochi, G., & Purohit, S.D. 2003. Scale–up production and field performance of micropropagated medicinal herb 'safed musli' (*Chlorophytum borivilianum*). InVitro Cellular and Developmental. Biology- Plant 39: 419.

[9] Doyle, J.J., & Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.

[10] Fernando, A., Barney, V.E., Tohme, J., & Roca, W.M., 1996. Stability of cassava plants at the DNA level after retrieval from 10 years of *in vitro* storage. Euphytica 90: 307-313.

[11] Gaikwad, P.D., John, S.C., & Billore, M. 2003. Callus induction and regeneration studies in safed musli (*Chlorophytum borivilianum* Sant & Fern). Research on Crops 4: 249–253.

[12] Gaspar, T. 1995.The concept of cancer in *in vitro* plant cultures and the implication of habituation to hormones and hyperhydricity. Plant Tissue Culture and Biotechnology 1: 126-136.

[13] Gupta, S. D., & Datta. S. 2003. Antioxidant enzyme activities during *in vitro* morphogenesis of gladiolus and the effect of application of antioxidants on plant regeneration. Biologia Plantarum 47: 179-183.

[14] Hussain, Z., Tyagi, R.K., Sharma, R., & Agrawal, A. 2008. Genetic diversity in *in vitro*-conserved germplasm of *Curcuma* L. as revealed by RAPD markers. Biologia Plantarum 52: 627-633.

[15] Jana, S., & Shekhawat, G.S. 2010. Plant growth regulators, adenine sulfate and carbohydrates regulate organogenesis and *in vitro* flowering of *Anethum graveolens*. Acta Physiologiae Plantarum 33: 305-311.

[16] Jana, S., & Shekhawat, G.S. 2011. *In vitro* regeneration of *Anethum graveolens*, antioxidative enzymes during organogenesis and RAPD analysis for clonal fidelity. Biologia Plantarum DOI: 10.1007/s10535-011-0223-3.

[17] Joshi, A., Ganesh, R., Sharma, A., & Sharma, G. S. 1999. Plant regeneration from stem-disc derived callus cultures of *Chlorophytum borivilianum*. Annual Review of Plant Physiology 13:79–83.

[18] Joshi, N., & Purohit, S.D. 2012. Optimization of factors influencing shoot multiplication during micropropagation of *chlorophytum borivilianum* Sant. et Fernand. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences* 82: 421-429.

[19] Kemat, N., Kadir, M.A., Abdullah, N.A.P., & Ashraf, F. 2010. Rapid multiplication of safed musli (*Chlorophytum borivilianum*) through shoot proliferation. African Journal of Biotechnology 9: 4595-4600.

[20] Lee, S.H., Tewari, R.K., Hahn, E.J., & Paek, K.Y. 2007. Photon flux density and light quality induce changes in growth, stomatal development, photosynthesis and the transpiration of *W. somnifera* (L.) Dunal. Plantlets. Plant Cell, Tissue and Organ Culture 90:141-151.

[21] Lowry, O.H., Rosenberg, N.J., Farr, A.L., & Randall, R.J. 1951. Protein measurement with folin phenol reagent. The Journal of Biological Chemistry 393 193: 265-275.

[22] Maiti, S., & Geetha, K.A. 2005. Plant introduction in medicinal and aromatic plants: achievements and opportunities. Indian Journal of Plant Genetic Resources 18: 22-23.

[23] Meratan, A.A., Ghaffari, S.M. & Nikam, V. 2009. *In vitro* organogensis and antioxidant enzymes activity in *Acanthophullum sordidum*. Biologia Plantarum 53: 5-10.

[24] Mimaki, Y., Kanmoto, T., Sashida, Y., Nishino, A., Satomi, Y., & Nishino, H. 1996.Steroidal saponins from the underground parts of *Chlorophytum comosum* and their inhibitory activity on tumour promoter-induced phospholipid metabolism of HeLa cells. Phytochemistry 41:1405-1410.

[25] Misra, P., Pandey, V., & Kochhar, S. 2010. Study of antioxidant enzymes activity during organogenesis and *in vitro* propagation of *Asiatic hybrid lily*. Journal of Plant Biochemistry and Biotechnology 19: 119-122.

[26] Molassiotis, A.N., Dimassi, K., Diantidis, G., & Therios, I. 2004. Changes in peroxidase and catalase activity during *in vitro* rooting. Biologia Plantarum 48:1-5.

[27] Murashige, T., & Skoog, F. 1962. A revised medium for rapid growth and bioassays tobacco tissue cultures. Acta Physiologiae Plantarum 15: 473-497.

[28] Narasimham, K.R.S.L., & Ravuru, B.K. 2003. A note on some endangered medicinal plants as NTFPs of Eastern Ghats, Andhra Pradesh. EPTRI-ENVIS Newsletter, EPTRI, Hyderabad 9(2):11-12.

[29] Ostray, M.E., Hackett, W., Michler, C., Serres, R., & McCown, B. 1994. Influence of regeneration method and tissue culture on the frequency of somatic variation in *Populus* to infection by *Septoria musiva*. Plant Science 97: 209-215.

[30] Panigrahi, J., Behera, M., Maharana, S., & Mishra, R.R. 2007. Biomolecular changes during *in vitro* organogensis of *Asteracantha longifolia*. Indian Journal of Experimental Biology 45: 911-919.

[31] Putter, J. 1974. "Peroxidases." In *Methods of enzymatic analysis,* Edited by H.U. Bergmeyer, 85–690, 2nd ed. Elzievir, *New York*.

[32] Purohit, S.D., Dave, A., & Kukda, G. 1994. Micropropagation of safed musli (*Chlorophytum borivilianum*), a rare Indian medicinal herb. Plant Cell, Tissue and Organ Culture 39: 93-96.

[33] Qiu, S.X., Li, X.C., Xiong, Y.S., Dong, Y.M., Chai, H.B., Fransworth, N.R., Pezzuto, J.M., & Fong, H.H.S. 2000. Isolation and characterization of cytotoxic saponin chloromaloside - A from *Chlorophytum malayense*. Planta Medica 66: 587-590.

[34] Rani, V., & Raina, S.N., 2000. Genetic fidelity of organized meristem derived micropropagated plants: a critical reappraisal. In Vitro Cellular and Developmental Biology-plant 36: 319-330.

[35] Samantaray, S., & Maiti, S. 2010. An assessment of genetic fidelity of micropropagated plants of *Chlorophytum borivilianum* using RAPD markers. Biologia Plantarum 54: 334-338.

[36] Samantaray, S., Patel, T.K., Kumar, T., Geetha, K.A., & Maiti, S. 2011. Identification and assessment of genetic relationships in three *Chlorophytum* species and two high yielding genotypes of *C. borivilianum* through RAPD markers. Biologia 66(2): 244-250.

[37] Sharifi, G., & Ebrahimzadeh, H. 2010. Changes of antioxidant enzyme activities and isoenzyme profiles during *in vitro* shoot formation in saffron (*Crocus sativus* L.). Acta Biologica Hungarica 61: 73-89.

[38] Shi, X.L., Han, H.P., Shi, W.L., & Li, Y.X. 2006. NaCl and TDZ are two key factors for the improvement of *in vitro* regeneration rate of *Salicornia europaea* L. Journal of Integrative Plant Biology 48: 1185-1189.

[39] Suri, S.S., Jain, S.K., Arora, D.K., & Ramawat, K.G. 1999. *In vitro* high frequency regenaration of plantlets and tuberous root formation in *Chlorophytum borivilianum*. Gartenbauwissenschaft 64:106–110.

[40] Van Breusegem, F.M., Vernova, E., Dat, J.F., & Inze, D. 2001. The role of oxygen in plant signal transduction. *Plant Science* 161: 405-414.