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Full Length Research Paper

Physiological and molecular characterization of *in vitro* cultures of an endemic medicinal herb, *Chlorophytum borivilianum*, under abiotic stress

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This investigation was carried out to evaluate the effects of abiotic stress on the *in vitro* cultures of *Chlorophytum borivilianum*. Regenerated plantlets were re-inoculated on media containing different concentrations of sodium chloride (0, 34.2, 68.4, 136.8 and 171.0 µM) and mannitol (0, 10.6, 21.3, 42.7 and 53.4 µM), and thus subjected to *in vitro* salinity and drought stress. Both salinity and drought conditions affected all the morphological parameters and decreased growth performance at a higher concentration of sodium chloride and mannitol, respectively. The chlorophyll content decreased with time, while proline and protein content increased in the *in vitro* stress plant. The control and stress tolerant plantlets were subjected to random amplified polymorphic DNA (RAPD) analysis using 24 random decamers primers. Only 15 RAPD primers showed polymorphism and a total of 52 polymorphic loci were scored. The control plants showed the highest similarity with the drought stress plantlet, while plant under salinity stress showed least similarity. Two groups were generated from the RADP data in the dendrogram after UPGMA cluster analysis based on Jaccard's similarity estimates for the RAPD data. The 2 dimensional scaling by principal component analysis (PCA) was in agreement with the similarity index.

Key words: Abiotic stress, proline, *Chlorophytum borivilianum,* salinity, mannitol, random amplified polymorphic DNA (RAPD) markers.

INTRODUCTION

Chlorophytum borivilianum has unique pharmaceutical and neutraceutical properties. The plant has a flavonone glycoside, which is a powerful uterine stimulant. Dry roots of *C. borivilianum* contain saponin (Wagle et al., 2000) and is being widely used in herbal tonics as a restorative, curative for pre-natal and post-natal illness and a remedy for diabetes, arthritis, etc. (Ramawat et al., 1996; Debnath et al., 2006). It is also used in medicine to cure various diseases like piles, diabetes, albumin urea, leucorrhoea and menorrhea. Recent pharmacological studies on tubers of *C. borivilianum* has indicated antiviral (Siddiqui, 2005), anticancer (Jamal, 2005), antioxidant (Thakur and Dixit, 2007), antidiabetic (Mujeeb et al., 2009), antistress (Deore and Khadabadi, 2009a), aphrodisiac (Thakur et al., 2009), antimicrobial (Deore and Khadabadi, 2007), hypolipidemic (Visavadiya and Narsimhacharya, 2007), hypocholesteremic (Deore and Khadabadi, 2009b), anti-inflammatory (Deore and Khadabadi, 2008) and immunomodulatory (Govindrajan et al., 2005; Thakur et al., 2006) activities. The restricted distribution and overexploitation of the plant coupled with low seed set and viability and poor seed germination rate has made it rare in the wild (Biswas et al., 2003).

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Novel propagation techniques like tissue culture can play an important role in the rapid multiplication of elite clones and germplasm conservation of *C.borivilianum* (Dave et al., 2004; Kotari and Singh, 2003; Purohit et al., 1994 a, b, c). Geetha and Maiti (2002) reported considerable variation in morphological traits among the natural populations of *C. borivilianum*, while variability in terms of biochemical characteristics has also been reported (Bhagat and Jadeja, 2003). A great degree of variability exists in this plant and systematic study has been reported for evaluation of diversity in various accessions of *C. borivilianum* using molecular markers (Joshi et al., 2008). Mathur et al. (2008) showed that no genetic fidelity exists between the plants grown *in vivo* and the micro-cloned progeny of *C. borivilianum*.

Salinity and drought resistant mutant were produced by applying abiotic stress under *in vitro* conditions. It was assumed that they may show a different morphological and physiological response and also show genetic diversity among the tolerant plantlets. This investigation was aimed to study the effect of abiotic stress viz. salinity and drought on the morphology, physiology and biochemical constituents like proline, protein and chlorophyll content in the *in vitro* grown plantlets and at the same time analyze the molecular diversity using random amplified polymorphic DNA (RAPD) molecular marker analysis among the stress grown plantlets in comparison with the control plants. The assessment of genetic identity and purity in the tolerant plantlets of *C. borivilianum* can help us in further identification of the stress related genes in this endemic medicinal plant.

MATERIALS AND METHODS

Multiplication of explants under salinity and drought stress

The cluster of multiple shoots was established from segments of inflorescence axis with axillary floral bud (Debnath, 2006), separated and re-inoculated on MS medium supplemented with 5 mg/l BAP and different concentration of sodium chloride (0, 34.2, 68.4, 136.8 and 171.0 µM) and mannitol (0, 10.6, 21.3, 42.7 and 53.4 µM) for salinity and drought stress, respectively. These growing explants were retrieved from the modified MS medium at 5, 15 and 30 days interval for evaluation of plant development and extraction, and biochemical quantification of proline, protein and chlorophyll.

Extraction and quantification of proline, protein and chlorophyll

Proline was measured as described by Bates et al. (1973). Protein extraction was performed according to Hurkman and Tanaka (1986). Protein quantification was performed according to the protocol described by Lowry et al. (1951). Chlorophyll content was estimated by measuring the absorbance of extract at 645, 652 and 663 nm for the determination of the total chlorophyll (Borah et al., 1978).

DNA extraction

The plantlets were maintained for 90 days in the same media to test their tolerance. The most tolerant plantlets that grew in sodium chloride and mannitol were compared with the control. Two to three healthy shoots were collected from each plant. The shoots samples (100 mg) were ground to a fine powder with liquid nitrogen in a mortar and genomic DNA was extracted following the CTAB (cetyltrimethylethyl ammonium bromide) method as described by Li et al. (2001) with some modifications.

PCR amplification with RAPD marker

The PCR reaction was carried out in a 20 µl reaction volume containing 10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40, 1.5 mM $MgCl_2$, 2 µM of each dNTP (MBI Fermentas, Germany), 0.9 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 20 ng of template DNA and 4 µM of each RAPD primers (Table 1). The RAPD studies were carried out with 24 RAPD primer pairs. Amplification was carried out in eppendorf thermal cycler programmed at 1 cycle of 3 min at 95°C, 44 cycles of 1 min at 94°C, 36°C (depending on primer) for 1 min and 72°C for 1 min, and a final extension step of 72°C for 10 min. PCR products were resolved in 2% agarose [Cambrex Bio Science Rockland (Lonza), Inc., USA] gel. The image was captured with the help of Bio-Rad gel documentation system.

Statistical analysis

The amplified fragments of RAPD markers were scored manually. Markers producing weak bands of negligible intensity and smears were excluded from the final data analysis. Banding profile was scored with 1 indicating the presence and 0 indicating the absence of a band to construct a binary qualitative data matrix. Pair-wise comparisons of genotypes were employed to calculate the Jaccard's similarity coefficient. A dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA) and computation for multivariate analysis was done using the computer programme NTSYS-pc version 2.02e.

Resolving power (Rp) and marker index (MI) of the RAPD primers, that is, the ability of a primer or technique to distinguish between large number of genotypes was determined as described by Prevost and Wilkinson (1999). The polymorphism information content (PIC) expressing the discriminating power of the locus taking into account not only the alleles that are expressed, but also relative frequencies and frequency of alleles per locus, expressed as: $PIC = 1-\sum P_i^2$ was calculated as suggested by Lynch and Walsh (1998), where p_i is the frequency of *i*th (presence) allele. Inter-individual relationships in multidimensional space were examined by principal coordinate (PCA) analysis using Program XLstat. The PCA analysis used a similarity matrix derived from NTSYS 2.02e genetic distance. The principal coordinates were plotted in three dimensional space using Xlstat software.

RESULTS

Multiplication of explants under salinity and drought stress

Growth rate of the *in vitro* grown plantlet under salinity (NaCl) and drought (mannitol) stress was slow, reduced and stunted but was steady and sustainable as compared to the growth of the control plant. At higher concentration of mannitol (53434 µM), no morphological differentiation was observed. Non-viability and necrosis of the tissue occurred.

Table 1. Details of polymorphic RAPD markers used, indicating number of alleles amplified and number of polymorphic alleles with Rp, PIC and MI values.

S/N	Primer	Primer length (bp)	Total bands	Polymorphic locus	Rp	PIC	MI
1	OPO6	10	11	3	0.48	0.32	0.03
2	OPO7	10	9	0	0.55	0.4	0.04
3	OPO10	10	7	2	0.55	0.4	0.05
4	OPA13	10	4	0	0.55	0.4	0.1
5	OPA12	10	4	0	0.55	0.4	0.1
6	OPA16	10	7	0	0.55	0.4	0.06
7	OPA17	10	6	2	0.48	0.36	0.06
8	OPA18	10	6	1	0.52	0.38	0.06
9	OPA19	10	7	5	0.67	0.28	0.4
10	MAP1	10	8	2	0.46	0.37	0.46
11	MAP3	10	7	5	0.75	0.26	0.038
12	MAP4	10	7	4	0.4	0.3	0.037
13	MAP8	10	8	0	0.55	0.39	0.049
14	MAP9	10	15	10	0.38	0.295	0.019
15	MAP12	10	8	3	1.01	0.326	0.04
16	MAP15	10	7	3	0.84	0.35	0.05
17	MAP16	10	11	3	0.73	0.369	0.03
18	MAP18	10	4	3	0.36	0.289	0.057
19	MAP19	10	8	0	0.55	0.396	0.0566
20	OPA20	10	10	1	0.83	0.388	0.0388
21	MAP13	10	10	5	0.51	0.37	0.0376
	Total (average)		164 (34.44)	52 (10.92)	12.27 (2.58)	7.44 (1.56)	1.81 (0.38)

Tolerance to mannitol stress was achieved till 21.37 µM concentration where 2 to 3 shoot buds were recorded. Apart from shooting, roots appeared at 10.68 and 21.37 µM concentration after 9 days of culture under mannitol stress. Percentage of rooting was enhanced at lower concentration of mannitol.

It became evident that lower concentrations of NaCl promoted differentiation of shoots from the nodal region in 70 to 90% cultures, respectively after 15 days. Reduced growth in shoots development was observed at 171 µM NaCl concentration. The maximum number of shoots per culture was at 34.22 µM NaCl concentration. After 45 days of culture at 171 µM NaCl concentration, adverse effect on plant growth was evidenced by yellowing and necrosis. No root formation was found under salt stress.

The effect of abiotic stress (salinity and drought) on the morphology, physiology and biochemical constituents

Effect on shoot bud proliferation

With the increase in time, there was an increase in shoot proliferation in almost all the cultures (stress and without stress). The cultures under high NaCl and mannitol concentration did not show any increase in the number of shoot buds. However, the cultures at lower concentration of stress showed the shoot proliferation but stunted growth (Figure 1A).

Effect on shoot length

There was an increase in shoot length in all the cultures. The cultures under drought stress in the mannitol supplemented MS medium showed a different type of growth pattern in contrast to shoot length. Under low concentration of mannitol (34.22 µM) there was an increase in shoot length but at high concentration (171 µM), there was negligible increase in the shoot length. The plants underwent necrosis and blackening and there was no growth. Similar results were obtained in NaCl stress plant (Figure 1C).

Relationship of fresh weight to dry weight

The results of the ratio of fresh weight to dry weight showed interesting future indications. Cultures without stress showed a general tendency of an increasing fresh weight to dry weight ratio whereas cultures under NaCl and mannitol stress showed a decreasing tendency. It was also noted that a steep increase in the ratio among the cultures under stress was highest after 5 days at

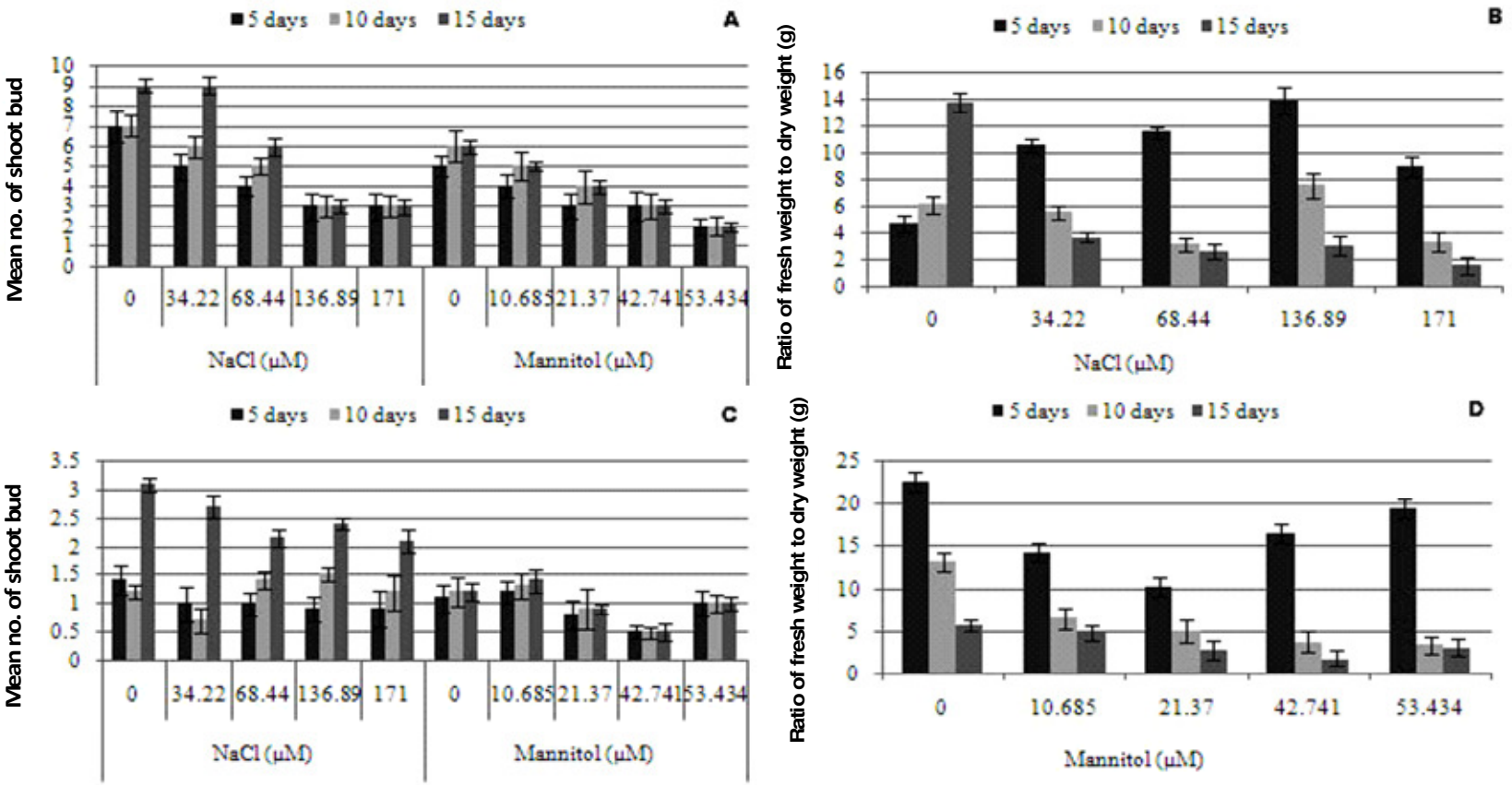

Figure 1. The effect of abiotic stress on the morphogenesis of *C. borivilianum.* (A) Effect of salinity and drought stress on mean number of shoot bud; (B) relationship of fresh weight to dry weight under salinity stress; (C) effect of salinity and drought stress on shoot length.; (D) relationship of fresh weight to dry weight under drought stress.wet, weight

136.8 µM NaCl concentration, whereas the highest in terms of mannitol concentration (53.43 µM) was observed after 5 days (Figure 1B and D). Hence, it could be concluded that 136.8 µM NaCl and 53.43 µM mannitol concentration may be the highest concentration tolerable by the *in vitro* cultures. These plantlets were allowed to grow in the conditioned medium and also subcultured in the same medium. After 3 subcultures, the plantlets at higher concentrations of mannitol showed negligible growth. Only plantlets under 10.685 µM mannitol concentration showed growth and were later used for RAPD analysis. The plantlets in the NaCl containing media grew well in comparison to the mannitol containing media. The cultures were green and proliferating even at 68.44 µM NaCl concentration and were later used for RAPD analysis.

Effect on shoot length to root length

The roots originated after 10 days of stress under drought conditions. Although under the salinity stress, there was no root development, there was

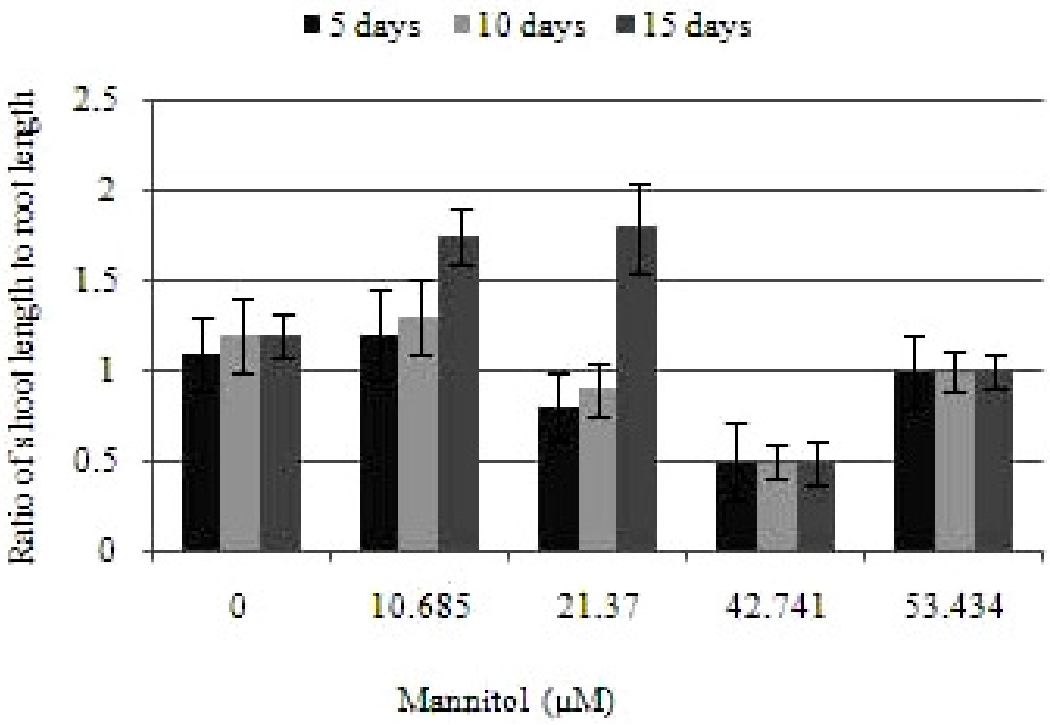

Figure 2. Ratio of shoot length to root length under drought stress.

no root development, there was a declining relationship between the number of shoots and the shoot length in the *in vitro* cultures under salinity stress. There was a significant difference in the roots developed at low concentration (10.68 µM) of mannitol as compared to the high concentration (21.37 µM). A relationship of shoot to root length under drought was also studied. There was a significant change in the ratio after 15 days in comparison with the results after 5 and 10 days. It was also noted that at 21.37 µM mannitol concentration, the shoot to root ratio was the most significant, whereas the optimum was noted at 10.69 µM (Figure 2). Hence, it can be concluded that for future experimentation, 10.68 µM mannitol in MS medium can be the most suitable concentration for further studies on stress regulation.

Effect on proline content

In this study, an increase in free proline content was observed in all the *in vitro* plants with increasing concentration of NaCl and mannitol in the medium. Surprisingly, there was a decreasing proline concentration in the cultures without stress. Proline is considered to be the stress marker. The maximum amount of proline was noted at 34.22 µM NaCl concentration and the optimum at 68.44 µM NaCl concentration. Under drought conditions, the highest proline content was found at 21.37 µM mannitol concentration and the optimum was at 10.68 µM mannitol concentration (Figure 3A and B).

Effect on protein content

In the study, it was observed that with the progression of time, the protein content increased in all the cultures with and without stress, although in cultures under mannitol stress, the behavior of protein content was not always uniform (Figure 3C and D).

Chlorophyll content

The chlorophyll content in the cultures without stress showed an increasing tendency, whereas the cultures under stress showed a decreasing content with time (Figure 3E and F). The cultures under stress showed reduced metabolism and this attribute was prominent in the cultures under stress.

RAPD analysis

All the tolerant samples were scored for the presence and absence of the RAPD bands (1 for presence and 0 for absence of the character) (Figures 4 and 5) and the data were entered into a binary matrix as discrete variables, and this data matrix was subjected to further analysis. 21 out of 24 RAPD primers were used for the molecular marker analysis of the three samples of *C.* conditions from the same eco-geographical regions of India. A total of 52 polymorphic locus were scored. The

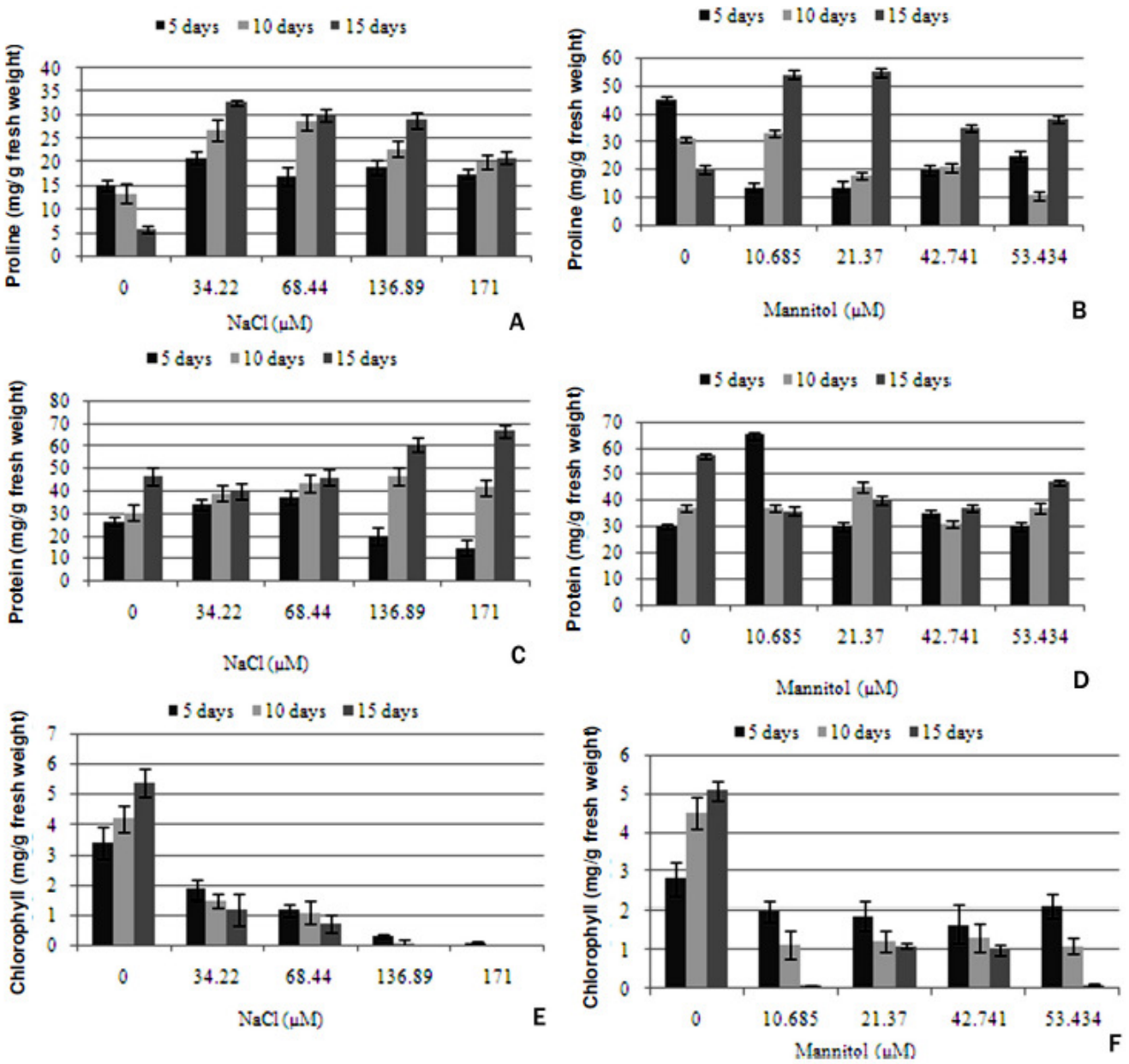

Figure 3. Effect of abiotic stress on the physiology and biochemistry of *C. borivilianum*. (A) Proline content in cultures under salinity stress; (B) Proline content under drought stress; (C) protein content under salinity stress; (D) protein content under drought stress; (E) effect of salinity on chlorophyll content; (F) effect of drought on chlorophyll content.

study revealed that the number of bands per primer ranged from 1 to 15 with an average of 10.92 bands per primer. The resolving power (Rp) of the 21 RAPD markers ranged from 0.4 to 1.01 with an average of 2.58 per primer and MI of the 21 RAPD markers ranged from 0.02 to 0.46 with an average of 0.38 per primer. The markers with the high Rp values were more informative as they were able to distinguish more number of *C. borivilianum* treated by stressed conditions. Polymorphism information content for the RAPD ranged from 0.26 to 0.4 with an average value of 1.56 per primer (Table 1).

All the *C. borivilianum* treated by the stress conditions could be distinguished from each other at the level of 1 to 52 polymorphic locus between individuals in pairwise comparison over all the amplified 21 RAPD markers. Similarity index of pair-wise comparisons estimated on the basis of all the 21 primers ranged from 0.7613 to 0.8182. The untreated plant showed the highest similarity with mannitol stress treated plant (0.8182), while NaCl treated plant showed least similarity with the untreated plant (0.7613). Four primers were selected from this study with high resolving power. Future studies with

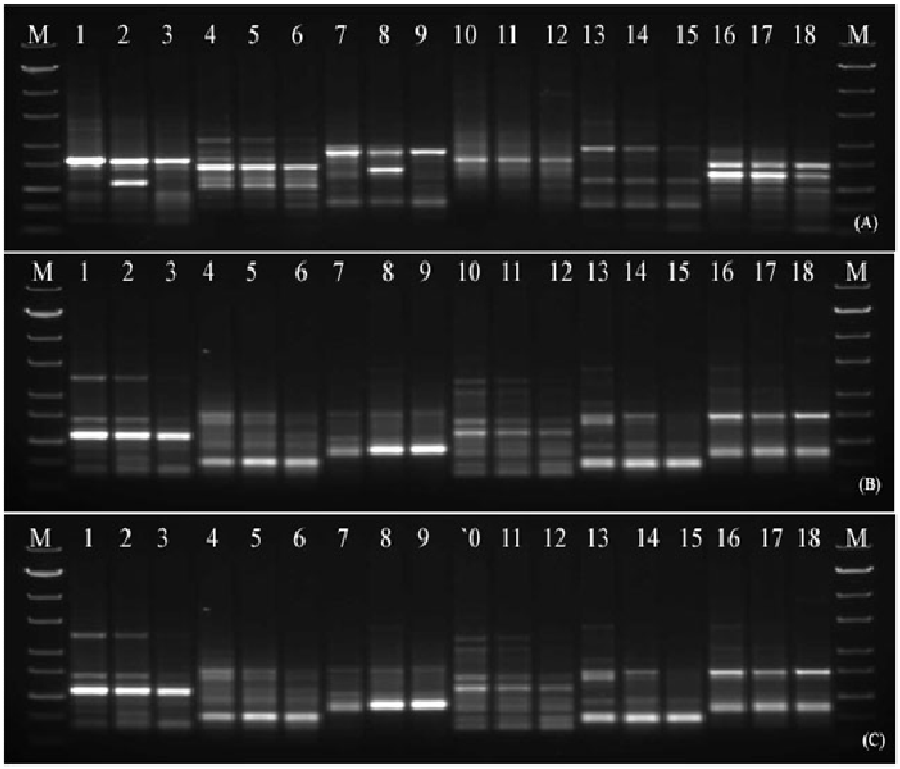

Figure 4. RAPD marker profiles of three samples of *Chlorophytum borivilianum* treated by stressed conditions generated with RAPD primers *(a)* M-Marker, Lane 1-3 OPO6 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 4-6 OPO7 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 7-9 OPO10 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 10-12 OPA13 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 13-15 OPA12 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 16-18 OPA16 (CB-Normal, CB-Mannitol, CB-NaCl) *(b)* M-Marker, Lane 1-3 OPA17 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 4-6 OPA18 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 7-9 OPA19 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 10-12 MAP1 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 13-15 MAP3 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 16-18 MAP 4 (CB-Normal, CB-Mannitol, CB-NaCl) *(c))* M-Marker, Lane 1-3 MAP8 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 4-6 MAP12 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 7-9 MAP9 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 10-12 MAP15 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 13-15 MAP16 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 16-18 MAP18 (CB-Normal, CB-Mannitol, CB-NaCl).

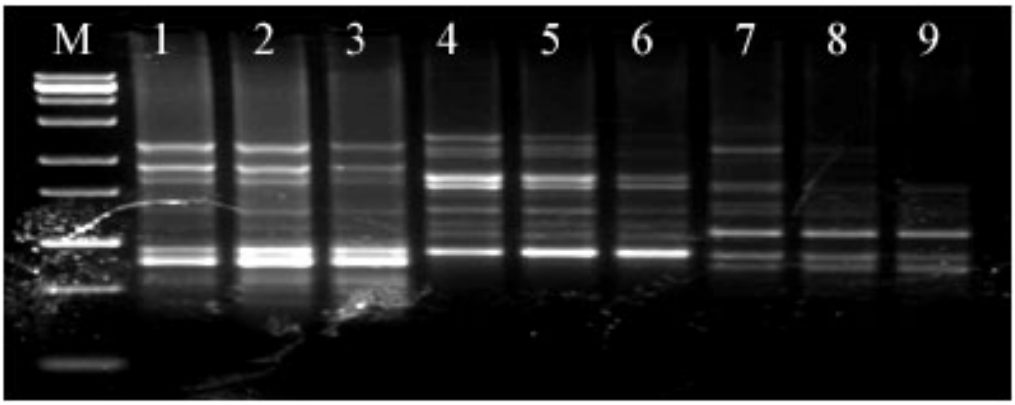

Figure 5. RAPD marker profile of normal and stress plantlet of C. borivilianum using RAPD universal decameric markers) M-Marker, Lane 1-3 MAP19 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 4-6 OPA20 (CB-Normal, CB-Mannitol, CB-NaCl), Lane 7-9 MAP13 (CB-Normal, CB-Mannitol, CB-NaCl).

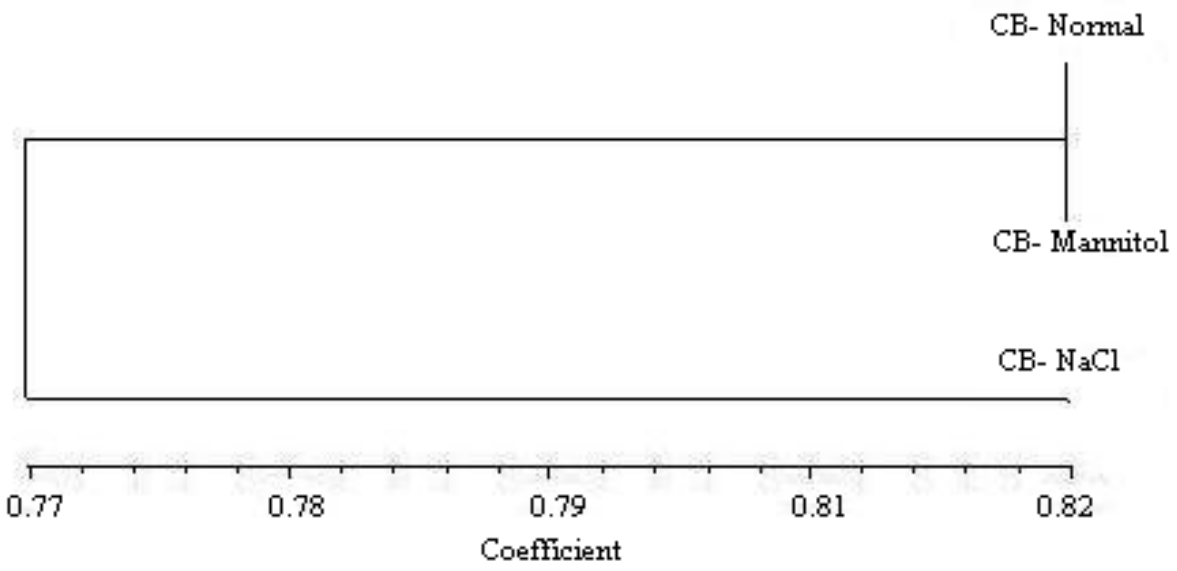

Figure 6. Dendrogram generated for the three samples of *C. borivilianum* treated with stress conditions using UPGMA cluster analysis based on Jaccard's similarity estimates for RAPD data. SM-Nor, Control (normal sample without treatment); SM-MD, sample treated with mannitol stress; SM-NaCl, sample treated with NaCl stress.

Table 2. Principal component analysis.

Parameter	F1	F2	F3
Eigen value	1.412	1.099	0.489
Variability (%)	47.071	36.626	16.303
Cumulative %	47.071	83.697	100.000

these four primers are visualized to help in predicting the polymorphism more clearly.

Cluster analysis was used to group the samples and to construct a dendrogram. The UPGMA based dendrogram obtained from the binary data deduced from the DNA profiles of the samples analyzed adds a new dimension to the genetic similarity prospective generated. Cluster analysis of the three samples of *C. borivilianum* treated by the stress conditions from *in vitro* conditions was performed using NTSYS-pc statistical package. A dendrogram was generated by UPGMA cluster analysis based on Jaccard's similarity estimates for RAPD data and it revealed two main groups (Figure 6). The two main distinct groups (I and II) had closely corresponded with their genome composition. Group I contained two samples belonging to untreated plant and mannitol stress treated plant. Group II contained only 1 sample of NaCl treated plant. This group will be studied in the future for the presence of stress related genes. The PCA analysis (Table 2) supported the UPGMA results and clarified the relationships among the stressed samples. PCA axis 1 explained 47.07% of the variance, while axis 2 explained 36.63% variance, respectively. The PCA plot (Figure 7) showed that individuals from the same parent generally clustered together. However, the control and mannitol tolerant plants were also grouped as group I and were separated from the NaCl tolerant plant (group II). Thus, it is concluded that the group II needs further study for identification of genes.

DISCUSSION

Salinity and drought are the most important stresses that adversely affect plant growth and productivity. They result in dehydration and osmotic imbalance of the cell. As the soil salinity increases, water is osmotically held in the soil and it becomes less accessible to the plant. Drought stress can occur for a variety of reasons, such as limited water availability or intense evaporation. One of the several approaches to solve problems of salinity and drought is to identify and grow stress tolerant plants. With only a few

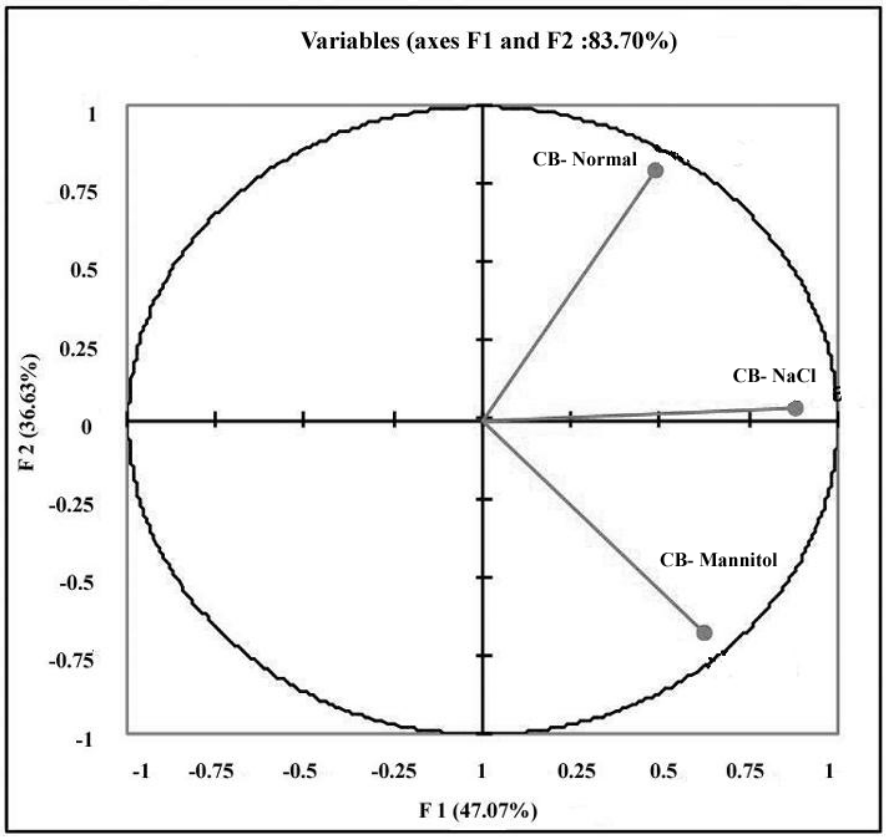

Figure 7. PCA plot generated for the three samples of *C. borivilianum* treated with stress conditions.

few exceptions, many widely used medicinal plants have not received the extensive plant physiological characterization (Elfeky et al., 2007).

The multiple shoot proliferation was in conformity with the earlier finding on monocots (Palai et al., 2000; Sachdev et al., 2002; Debnath et al., 2007). Dave and Purohit (2002) also reported that addition of BAP to the regeneration medium resulted in a significant increase in the growth and induction of shoot buds from young stem disc with shoot meristems of *C. borivilianum* as explant. Reduced growth of tissues in stressful media is a usual phenomenon and it has been interpreted that a metabolism is channeled to resist the stress. Growth response of adapted plantlets in a stressful environment imposed due to NaCl or mannitol was not identical. Mannitol is a non-ionic osmoticum, whereas NaCl is ionic in nature. The results revealed that tissue growth was more impaired due to the effect of an ionic osmoticum than a non ionic one. More tissue injury due to NaCl-shock than mannitol indicated that NaCl stress had both ionic and osmotic components (Gomes and Sodek, 1988). In comparison with the control conditions, there was a pronounced reduction of growth due to stress in the study. The chlorophyll content under salinity and drought stress also showed reduced content. One of the mechanisms of salt and drought stress in plants is the increase of root length and root branches (Ehsanpour and Amini, 2003). In this study, there was also an increase in root length but only in increasing concentrations of mannitol.

Normally, drought and salinity affects the physiology and biochemistry of plant cells under *in vivo* and *in vitro* conditions. These stresses have been reported to increase proline. The detection, stabilization and repair of stress induced damage are essential for cellular integrity.

The role of proline accumulation and its metabolism viz. tolerant to salinity and drought therefore need to be critically examined (Tani and Sasakawat, 2006). Higher plants accumulate free proline in response to external salt and drought stress. There are reports suggesting that proline at higher concentration acts as an osmoticum, a protective agent of enzyme and cellular structure and a storage compound of reducing nitrogen for rapid growth after stress (Armengaud et al., 2004; Lin and Kao, 2001; Abdel et al., 2003; Tani and Sasakawat, 2006).

The effect of salt-stress on protein content depended on the concentration of NaCl. At lower levels of mannitol and NaCl, there was an increase in protein content, but

higher concentrations caused it to decline as also reported by Abdel et al. (2003). But our results suggest that there is a continuous decrease in protein content on increasing the concentration of NaCl. It would be predicted that plants under stress would have a powerful protein turnover machinery to degrade stress-damaged and environmentally-regulated proteins.

RAPD studies showed the polymorphism among the tolerant species when compared to the control plantlets. The mannitol tolerant plants were grouped with the control plants, while the sodium chloride tolerant plants were in a separate group. This was a clear indication of the polymorphism present in the salt tolerant plants. On the basis of molecular characterization using RAPD markers in the study, we could emphasis the locus that showed banding pattern in the stress conditions. This locus if sequenced in future, can give vast data and knowledge to understand the behavior of gene which is triggered in stress conditions. This finding is in complete agreement with that of Yildirim and Akkaya (2006). Work in this aspect is in progress to identify the genes related to the stress by transcriptomic analysis. It is concluded that abiotic stresses are the major environmental challenges for medicinal plants like *C. borivilianum.*

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