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Full Length Research Paper

Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum* Sant. et Fernand.

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The micro-cloned plantlets of *Chlorophytum borivilianum* registered more than 95% establishment in soil following treatment with various bio-inoculants namely; *Glomus aggregatum*, *Trichoderma harazianum* and *Piriformospora indica* whereas *Azospirullum* sp. (CIM-azo) and Actinomycetes sp. (CIM-actin) showed only up to 85% plantlet establishment. The un-rooted shoots were also treated with these bio-inoculants, for *in vivo* root induction and increased survival rate/establishment frequency when transferred to soil. The un-rooted shoots also showed *in vivo* rooting (50%) when treated with mycorrhiza *Glomus aggregatum* (VAM) and *Trichoderma harazianum.* The genetic fidelity testing of micro-cloned, bio-hardened progeny based on a RAPD analysis using 40 random decamer DNA primers indicated a strong uniformity in relation to the parent genotype.

Key words: ***Chlorophytum borivilianum***, bio-inoculants, bio-hardening, genetic fidelity, RAPD analysis.

INTRODUCTION

The transfer of ***in vitro*** raised plantlets to ***ex vitro*** conditions is one of the most critical factors in the micro-propagation process and cause of higher production costs. High mortality is often observed upon transfer to ***ex vitro*** conditions as the cultured plants have non functional stomata, weak root system and poorly developed cuticle. In order to increase growth and reduce mortality in plantlets at the acclimatisation stage, research has been focused on the control of the environmental conditions (both physical and chemical). A biological approach to reduce the stress of acclimatisation and provide faster growth of plantlets is the establishment of arbuscular mycorrhizal (AM) fungi association. The VAM-***Glomus aggregatum*** reduces the osmotic potential of plantlets. This response may be useful pre-adaptation for ***in vitro*** developed plantlets during transfer to the acclimatisation stage (Elmeskaoui et al., 1995).

Chlorophytum borivilianum (Family Liliaceae), finds wide applications in more than 100 Ayurvedic preparations. The dried fasciculed storage roots of this herb, popularly known as “Musli”, have strong aphrodisiac, anti-stress and immuno-modulatory properties due to the presence of steroidal saponins and polysaccharides (Kirtikar and Basu, 1975). In the indigenous market, the dried musli roots are priced at Rs. 800 - 1800 per kg whereas it is traded at Rs 3000 per kg in the international market (Agriculture & Industry Survey, June 1999 pp. 20). The plant primarily propagates through roots due to poor seed setting and viability (<20%). The bulk of industry’s demand is met through collection from wild forests, hence threatening its extinction (Narasimham and Ravuru, 2003). To meet industry’s requirement for ***Chlorophytum*** roots, alternate propagation strategies for generating uniform, quality disease-free planting material for its comercial cultivation are urgently needed. Tissue culture-based propagation techniques in ***C. borivilianum*** have so far met with limited success because of high mortality at transplantation stage (Purohit et al., 1994a, b; Arora et al., 1999; Dave et al., 2003, 2004; Rizvi et al., 2007). These efforts, though indicated the strong possibility of micro-propagating ***Chlorophytum*** plants via vegetative buds or somatic embryogenesis, suffered from poor multiplication rate, low establishment in soil, cytological instability and early loss of regeneration potential of the ***in***

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vitro cultures. Dave et al. (2003) also tried to incorporate an ***in vitro*** hardening step using a soilrite mix and a pulse treatment of abscisic acid to improve field transplantation. To ameliorate, this problem, an attempt has been made to use different mycorrhiza and some microbial agents such as ***Glomus aggregatum***, ***Trichoderma harazianum***, ***Piriformospora indica, Azospirullum*** sp. (CIM-azo) or ***Actinomycetes*** sp. (CIM-actin) which could be inoculated to the roots of such plants to avoid the fungal attack especially those causing root rot. The earlier reports also lack the testing of genetic uniformity of the tissue culture-raised progeny of ***C. borivilianum*** based on DNA markers. The present report describes that using VAM and ***Trichoderma harazianum*** high frequency establishment of true to type micro-cloned ***C. borivilianum*** plants can be successfully obtained as evidenced by their RAPD profiling.

MATERIAL AND METHODS

Establishment of aseptic cultures

C. borivilianum material was obtained from Guna area of Madhya Pradesh, India. The stem disc explants obtained from fresh tubers were initially cleaned manually with scalpel blade to peel off the dead skin and washed thoroughly in running tap water to remove soil particles. They were then rinsed with 1% (v/v) solution of Cetavelon (cetrimide) for about 10 - 12 min, washed with 70% (v/v) ethanol for 30 s and surface sterilized with 0.1% $HgCl_2$ (w/v) solution for 3 - 5 min. The sterilized explants after washing with sterile distilled water (3 - 4 times) were then implanted onto the modified Murashige and Skoog's (1962) agar-gelled medium fortified with various concentrations/combinations of growth hormones, 3% sucrose, and 100 mg l^{-1} myo-inositol. The pH of the medium was adjusted to 5.8 ± 0.03 before autoclaving at 1.04 kg/cm^2 pressure and 121°C temperature for 15 - 20 min. The cultures were incubated at 25 ± 3°C in diffused light under 60 - 70% RH in the culture room.

Preparation of bioinoculants

The five bioinoculants used in the present study were prepared as follows. (i) ***Glomus aggregatum*** - an efficient VAM fungus was maintained and multiplied as pot culture or soil inoculum with its natural host (***Cymbopogon martini*** var. ***motia***) in sterilised soil-filled earthen pots. Shade dried soil inoculum containing 8 spores per gram of soil were spread at a depth of ca. 3 cm. in the sterilised potted soil before transplanting ***in vitro*** regenerated single non-rooted shoot or rooted plantlet of ***C. borivilianum.*** Transplantation in non-mycorrhizal soil served as the control. (ii) ***Azospirullum*** (***CIM-azo***) and (iii) ***Actinomycetes*** (***CIM-actin***) were streaked densely onto potato-dextrose agar medium and incubated at 30 ±1°C. After 48 h the cultures were scraped separately under sterile conditions. Spore suspensions of 4 x 10^6/ml density were prepared to treat the ***in vitro*** generated propagules before transplanting. (iv) ***Trichoderma harazianum*** and (v) ***Piriformospora indica*** cultures were also streaked on potato-dextrose agar medium and incubated at 25±1°C for 7 - 10 days. The spores were scrapped and suspended in sterilized distilled water and used as inoculum at a density of 2 x 10^6 spores/ml. The micro-shoots /rooted plantlets were dipped in these spore suspensions for 30 - 60 s before transplanting in soil. Non-treated propagules served as control.

Molecular characterization of micro-cloned plant

Genomic DNA was isolated from fresh and young leaves of hardened ***in vitro*** developed and ***in vivo*** growing plants of ***C. borivilianum*** after a month of their transplantation in pots using the method of Khanuja et al. (1999). PCR amplification was carried out in 25 µl volume using 40 decamer primers. The reaction mixture consisted of 0.6 units of Taq DNA Polymerase, 0.25 µl each of dNTP (10 mM), 1X polymerase buffer (2.5 µl) and 5 pmol of decanucleotide primer [20 MAP primers (custom-made by Bangalore Genie, India); 7 OPA and 13 OPO primers (procured from Operon Technologies, USA)]. The amplification was carried out using the thermal cycler [DNA Engine PTC - 2000 (M.J. Research, USA)] following the protocol of Khanuja et al. (2000). The amplification products were separated electrophoretically on 1.2% Agarose gel containing 0.5 µg/ml ethidium bromide using 50 mA current for 3 h. The bands were visualized and photographed using Gel Documentation System (Pharmacia Biotech, USA).

RESULTS

Rhizogenesis of *in vitro* developed shoots

To induce rhizogenesis in the regenerated shoots, 4 - 6 cm long shoots growing in 4.0 mg/l BAP were excised from the multiple clusters and transferred on to a total of sixteen medium combinations involving BAP, IBA or adenine sulphate (Table 1). Best rooting response was observed on MS basal media containing 40 or 80 mg l^{-1} adenine sulphate (Figure 1). Root emergence (2 - 3 per shoot) became evident on this medium within 3 - 6 days of transfer. Higher level of adenine sulphate also favoured better root elongation. When IBA (1.5 mg l^{-1}) was also supplemented in adenine sulphate containing medium the rooting response was delayed by 10 - 12 days but number of roots per shoot was significantly enhanced. The elongation of these roots was, however, very slow in comparison to those growing in presence of adenine sulphate alone. Addition of BAP in the rooting medium was generally found inhibitory for rooting. The rooted plantlets grown on 40 - 80 mg l^{-1} adenine sulphate containing medium also exhibited the formation of typical musli fingers towards the end of the culture passage (Figure 1D). The entire duration from explant implantation to plantlet development varied from 45 - 60 days with a multiplication rate of 1: 25 per culture per ***in vitro*** cycle. The rooted plants registered an establishment rate of 25 - 40% in the soil in glass house (Figure 1E, F).

Bio-hardening and soil transplantation of micro-cloned progeny

To assess the efficacy of different bio-inoculants in increasing the ***in vivo*** establishment rate of the micro-cloned propagules 25 - 35 days old ***in vitro*** rooted plantlets (8 - 10 cm long) were taken out from the culture vessel, thoroughly washed with tap water to remove adhering agar and treated with bio-inoculants namely; ***Glomus aggregatum***, ***Trichoderma harazianum***, ***Pirifor-***

Table 1. Rooting response of micro-cloned shoots as a function of growth regulators supplementation in ***C. borivilianum***.

Medium composition	Rooting response*					
	After 15 days			After 30 days		
	% Cultures responded	No. of roots/shoot	Mean root length (cm)	% Cultures responded	No. of roots/shoot	Mean root length (cm)
MS+AD (40)**	80.0	2 - 3	1.3	100.0	3 - 5	4.0
MS+BAP(1.0)+ IBA (1.5) + AD (40)	00.0	0.0	0.0	5.0	1 - 2	0.5
MS+BAP (2.0) + IBA (1.5) +AD (40)	11.0	2 - 4	0.4	11.0	2 - 4	0.8
MS+ IBA (1.5) +AD (40)	18.0	1 - 2	1.0	100.0	10 - 16	1.7
MS + AD (80)	66.0	1 - 2	0.6	80.0	5 - 7	4.5
MS+BAP (1.0) + IBA (1.5) + AD (80)	00.0	0.0	0.0	25.0	1 - 3	0.3
MS+BAP(2.0)+ IBA (1.5) +AD (80)	25.0	1 - 2	0.2	40.0	2 - 3	0.3
MS+IBA (1.5) + AD (80)	50.0	3 - 4	0.7	70.0	7 - 8	1.2

*Data is presented as mean performance of 25 replicates.
**All concentrations are in mgl^{-1}.

mospora indica, Azospirullum (CIM-Azo) or actinomycetes (CIM-Actin), before transferring them to sterilized soil mixture (soil : sand : organic manure : 1:1:1) in pots in the glass house. Un-rooted shoots (4 – 6 cm long) from the multiple clusters were also treated in a similar manner to examine whether the bio-inoculants could also induce ***in vivo*** rooting in them. The results are presented in Table 2 and Figure 2. While un-rooted micro shoots that were not treated with any bio-inoculant failed to survive in soil, the ***in vitro*** rooted plantlets without bio-inoculant treatment registered a survival frequency of 76, 32 and 15% after 15, 30 and 45 days of transferring. In the present study, establishment frequency of 96% has been obtained when the ***in vitro*** rooted plantlets were treated with ***Glomus aggregatum, Trichoderma*** and ***Piriformosopra*** before transferring to pots***.*** One important point to mention here is that even the un-rooted shoots could form roots under ***in vivo*** conditions when treated with ***G. aggregatum*** and ***Trichoderma*** with more than 50% plantlet establishment, while about 43% when treated with ***Piriformosopra indica.*** Amongst the various bio-inoculants tested, ***G. aggregatum*** and ***T***. ***harazianum*** were most effective in terms of establishment as well as subsequent growth and elongation of the transplanted propagules. Treatments involving CIM-actin and CIM-azo though supported high establishment rates, were found to suppress plant growth. Interestingly, bio-inoculants namely ***Glomus, Trichoderma*** and ***Piriformosopra*** could also support induction of ***in vivo*** rooting in 80 - 85% of the un-rooted shoots within 10 - 15 days of transplantation. The frequency of establishment of such rooted shoots after 30 days of transfer was, however, low (40 - 55%) when compared with that of ***in vitro*** rooted plantlets.

CIM-azo treatment was not found effective in eliciting an ***in vivo*** rooting response, whereas CIM-Actin was found effective to a limited extent (25 - 30% establishment). These bio-inoculants have not been used in any earlier micro-propagation study in the genus ***Chlorophytum.*** In previous reports only up to 66.6% plantlet survival was observed with an additional ***in vitro*** hardening step (Dave et al., 2003) whereas in another species of ***Chlorophytum*** i.e. ***C. arundinaceum***, Lattoo et al. (2006) observed up to 90% success when transferred in earthen pots.

Genetic fidelity testing of micro-cloned progeny using RAPD analysis

Optimum conditions for DNA extraction from ***C. borivilianum*** leaves for reproducible PCR amplification were identified. Genetic fidelity of randomly selected thirteen micro-cloned plants out of a total progeny of 100 ***in vivo*** established plants was tested through RAPD analysis. Out of 40 different decamers tested, 24 produced amplification products that were monomorphic across all micro-cloned plants (Table 3). The 16 other primers did not yield good amplified fragments. A total of 79 amplified reproducible monomorphic bands were scored with three different sets of 24 decamer primers (14 MAP, 3 OPO, 7OPA primers; Table 3). The number of bands per primer ranged from 1 band in MAP 9 and MAP10 to 6 bands in OPO 6. All the primers were found to be monomorphic with a very low percentage of polymorphism (>1%). The size of the monomorphic DNA fragments produced by these 24 primers ranged from 0.125 to 2.027 bp (Table 3). Primer OPA 18 produced 5 DNA fragments common to all 13 micro-cloned and the parent plants, but in primer

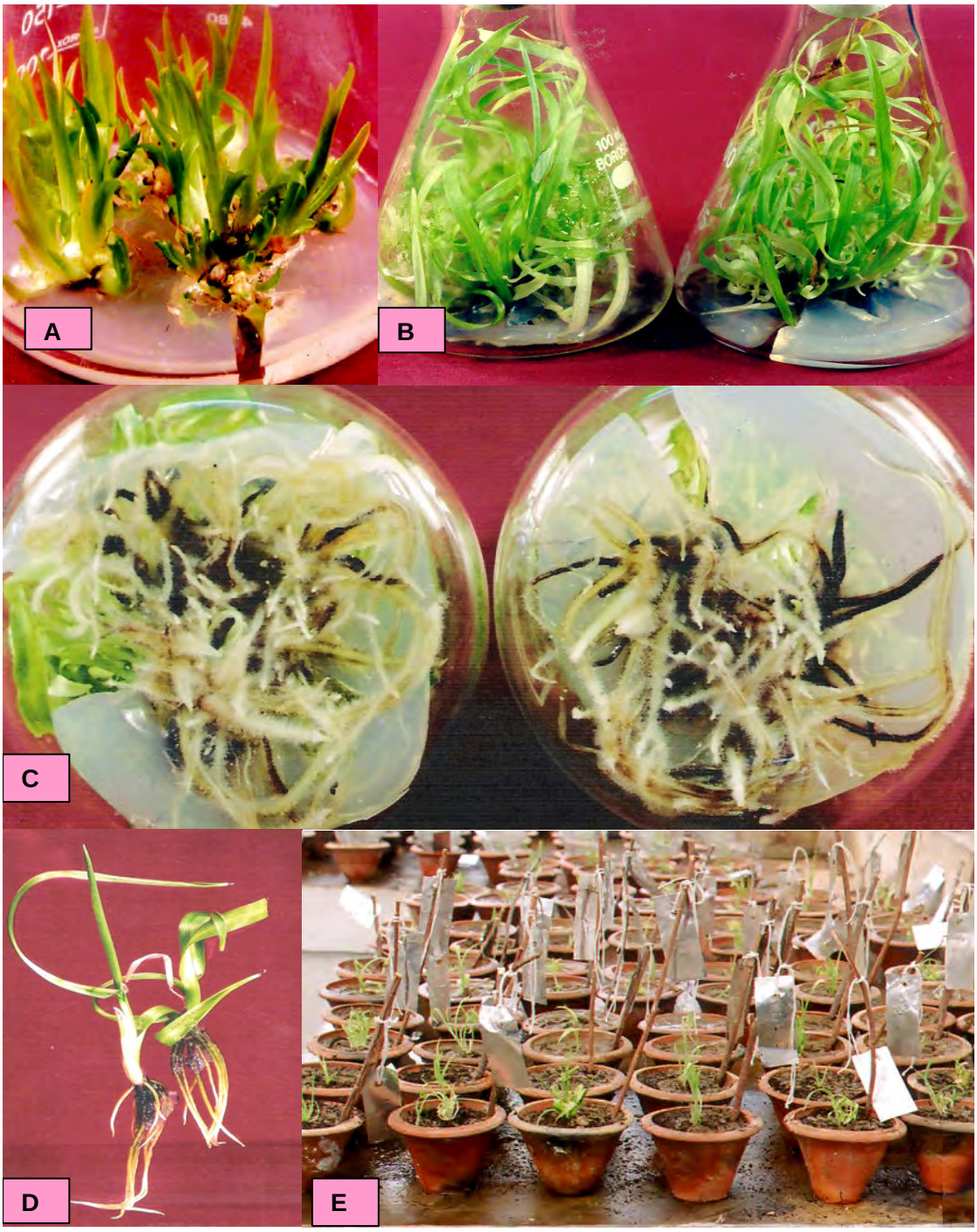

Figure 1. ***In vitro*** micro-cloning of ***C. borivilianum***. **A.** Multiple shoots formed on MS medium with 4.0 mg/l BAP; **B.** Rooted plants on MS basal medium fortified with 40 mg/l adenine sulphate; **C.** Profuse rooting and finger formation in six week old culture on rooting medium; **D.** Rooted plantlet before transfer to pots ; **E.** Hardening of tissue culture-raised plants in the glasshouse.

OPA 19 and MAP 13 only three monomorphic fragments were obtained whereas MAP 16 showed four monomorphic bands common to all 13 plants (Figure 3 A-D). Cluster analysis, done on the basis of similarity coefficient generated from RAPD profiles, indicated that all the 13 micro-cloned plants along with the parent were genetically alike and could be grouped into one major cluster at nearly 99% similarity level (Figure 3 E). These plants were successfully transferred to the CIMAP Resarch Farm with 100% survival. Field performance of

Table 2. Comparative efficacy of various bio-Inoculants on ***in vivo*** acclimatization of ***C. borivilianum*** shoots/plantlets developed ***in vitro.***

Bio-inoculants used	*In vitro* plantlets transferred	% Survival after		
		15 days	30 days	60 days
Glomus aggregatum	With root	100	86	86
	Without root	85	57	55
Piriformospora indica	With root	100	86	86
	Without root	100	43	43
Actinomycetes	With root	100	85	80
	Without root	43	29	29
Azospirillum sp.	With root	83	83	80
	Without root	43	Nil	Nil
Trichoderma harazianum	With root	100	86	86
	Without root	100	55	55
Control	With root	76	32	15
	Without root	Nil	Nil	Nil

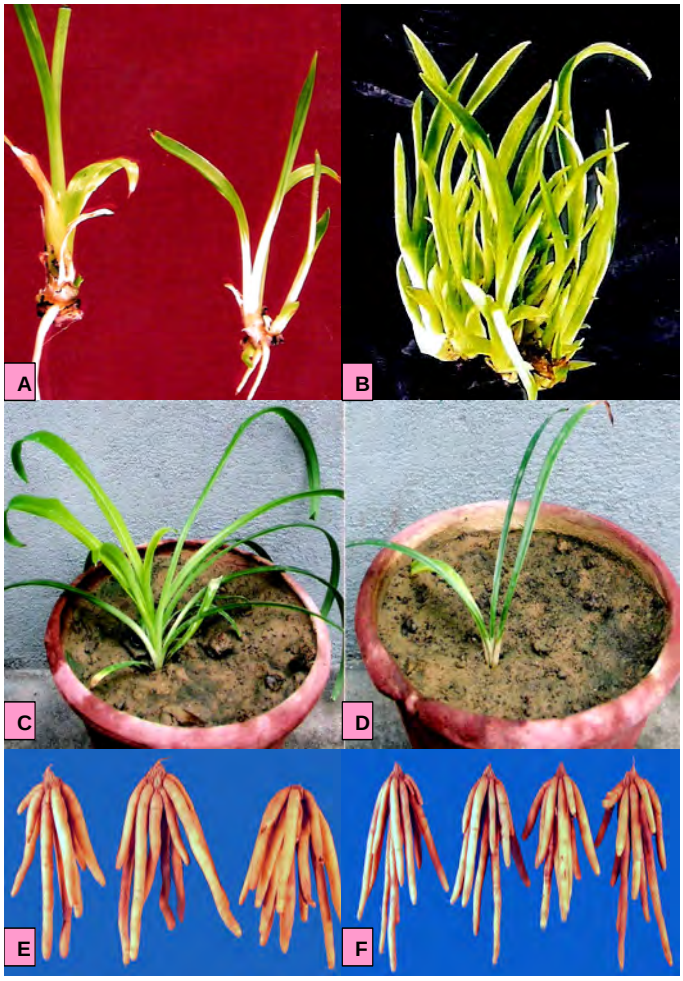

Figure 2. Bio-hardening of ***C. borivilianum*** using ***Glomus aggregatum.*** **A and B.** Rooted plantlets **(A)** and non-rooted shoots **(B)** at the time of treatment with bio-inoculants; **C and D.** Established plants in soils; **E and F.** Fingers development in field grown plants after seven months of growth.

the micro-cloned progeny was also very uniform. The first harvest data obtained so far on these plants after seven months of growth in field showed that 8 - 12 uniformly filled fasciculed root fingers with average length of 4.11 cm and mean fresh weight of 10.97 gm could be produced per plant (Figure 2) Presently they are growing in the field and will be evaluated for comparative tuber yield and chemical profile.

DISCUSSION

Rapid ***in vitro*** multiplication rate, successful acclimatization/establishment of tissue culture-raised propagules in soil, genetic uniformity and stability of the resultant progeny and cost effectiveness are key parameters of a micro-propagation protocol (Mathur and Mathur 2003; Debnath et al., 2006). The ultimate success of such efforts on a commercial scale depends upon the ease with which micro-cloned plants can be carried to the field transplantation stage at high frequency. The highly protective environment of the tissue culture-raised plants coupled with the lack of exposure and interaction with microorganisms normally found in nature, make it difficult to sustain growth and survive. It is imperative, therefore, to take into consideration the existence of mutualistic symbiosis of mycorrhiza and other associate rhizobacteria, normally present in the natural soil and also hazards of external environmental conditions. In the last few years, attempts have been made to expose the young ***in vitro***-raised plantlets to useful microorganisms (PGPRs) that promote growth and encourage mutual association that the plantlets may confront upon transplantation to natural conditions (Varma and Schuepp, 1996; Hernandez-Sebastia et al., 1999; Sahay and Varma 1999; Pandey et al., 2000). This biological hardening envisages physical, chemical and environmental conditioning of the micro-propagated plantlets.

Table 3. Description of decamer primers used for fingerprint analysis of micropropagated plants of ***C. borivilianum***.

S/N	Sequence(5'-3')	Primer	Monomorphic bands	Fragment size of scored bands
1.	5′AAATCGGAGC3'	MAP1	3	2.027 - 0.831bp
2.	5′GTCCTTAGCG3′	MAP3	2	2.027 - 1.375bp
3.	5′TGCGCGATCG3′	MAP4	5	1.904 - 0.564bp
4.	5′GCACGCCGGA3'	MAP6	3	1.375 - 0.831bp
5.	5′CACCCTGCGC3′	MAP7	2	1.300 - 0.564bp
6.	5′CTATCGCCGC3'	MAP8	4	1.584 - 0.831bp
7.	5′CGGGATCCGC3′	MAP9	1	0.947bp
8.	5′CACCCTGCGC3′	MAP10	1	0.564bp
9.	5'GTGCAATGAG3',	MAP12	3	0.947 - 0.564bp
10.	5′ AGGATACGTC 3′	MAP13	3	1.324 - 0.125bp
11.	5′GGATCTGAAC 3′	MAP15	4	1.584 - 0.831bp
12.	5′ TTGTCTCAGG 3'	MAP16	4	1.584 - 0.125bp
13.	5′CATCCCGAAC 3′	MAP18	2	1.584 - 0.831bp
14.	5′GGACTCCACG3′	MAP19	3	1.584 - 0.125bp
15.	5′CCACGGGAAG3′	OPO6	6	2.027 - 0.125bp
16.	5′CAGCACTGAC3′	OPO7	2	1.584 - 0.831bp
17.	5′TCAGAGCGCC3'	OPO10	4	2.027 - 0.564bp
18.	5′ TCGGCGATAG3'	OPA12	5	1.375 - 0.125bp
19.	5′ CAGCACCCAC3'	OPA13	5	0.947 – 0.125bp
20.	5′ AGCCAGCGAA3'	OPA16	4	1.375 - 0.125bp
21.	5′ GACCGCTTGT3′	OPA17	3	0.947 - 0.564bp
22.	5′AGGTGACCGT3'	OPA18	5	1.584 - 0.125bp
23.	5′CAAACGTCGG3'	OPA19	3	1.375 - 0.564bp
24.	5′ GTTGCGATCC3'	OPA20	2	1.584 - 0.947bp

The protocol for ***C. borivilianum*** described in this study fulfils these considerations and provides significant advancement over the previously reported methods in this medicinal herb (Dave et al., 2003, 2004; Purohit et al., 1994a). One of the unique features of this study was the incorporation of a bio-hardening step at the time of soil transplantation. Bio-inoculants such as ***Glomus aggregatum, Trichoderma harazianum*** and ***Piriformospora indica*** were found very effective in improving the establishment frequency. Bio-hardening is an emerging dimension of micro-propagation techniques (Lovato et al., 1996; Nowak, 1998; Gange and Ayres, 1999; Sahay and Verma, 1999; Pandey et al., 2000; Rai, 2001; Srivastava et al., 2002). Mycrorrhization of tissue cultured plants is believed to provide advantage to the transplanted propagules in terms of nutrient availability, soil pH, aeration and protection from water stress. Biotization with friendly associates like arbuscular mycorrhizal fungi, symbiotic endophytes or pathogen antagonists also protect the juvenile axenic plants from infestation of the harmful saprophyes (Lovato et al., 1996; Pandey et al., 2000). The colonization of ***Glomus*** and ***Trichoderma*** species is also known to reduce the osmotic potential of plants that can be critical in saving 99% similarity indices that matched well with that of the parent plant. Our findings are also in agreement with them from initial dehydration shocks (Elmeskaoui et al., 1995; Varma and Schuepp, 1996; Hernandez-Sebastia et al., 1999; Sahay and Varma, 1999). Another special observation of the present study was the ability of ***Piriformospora***, ***Trichoderma*** and ***Glomus*** to induce ***in vivo*** rooting in transplanted un-rooted shoots of ***C. borivilianum*** giving rise to more than 50% establishment.

This can further reduce the time required for cloning the elite selections of this medicinal herb. The present study also provides for the first time the molecular evidence for

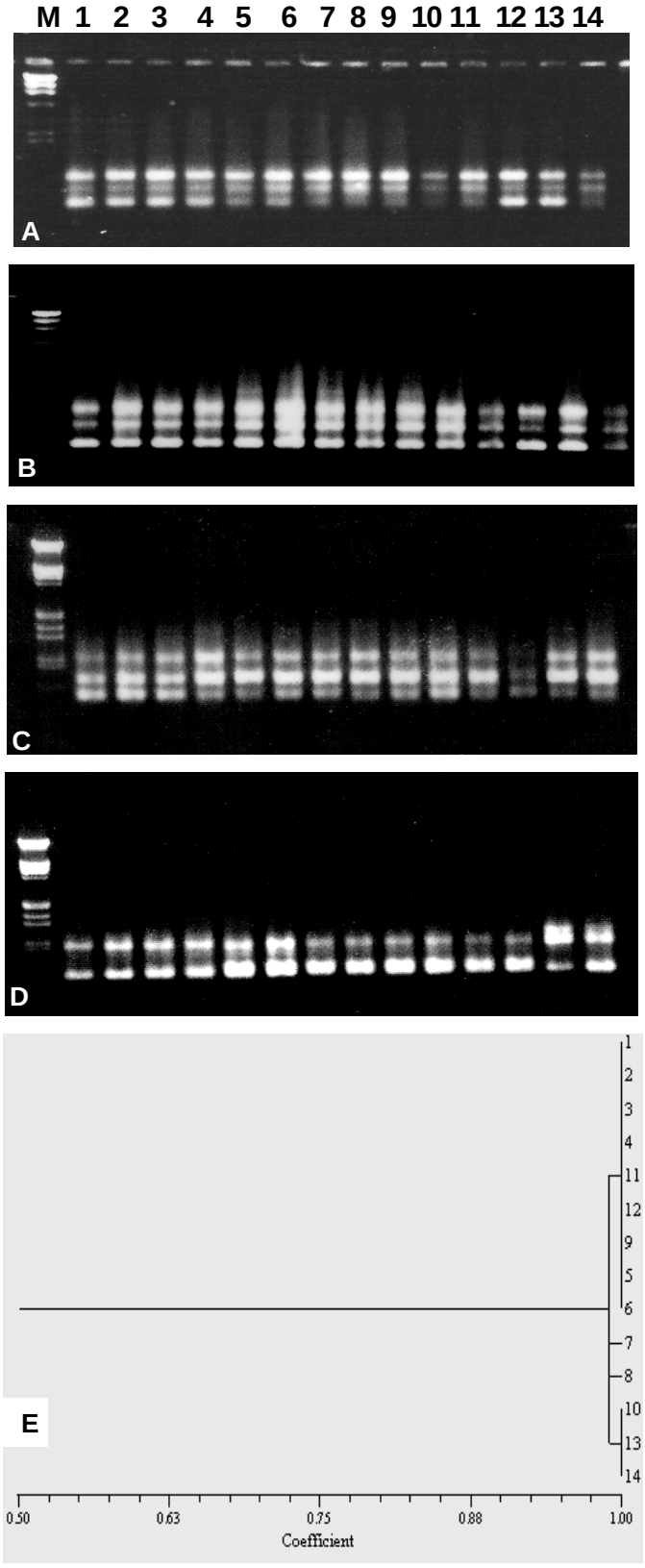

Figure 3. RAPD profiles of micro-cloned plants **(Lanes 1-13)** of ***C. borivilianum*** in relation to parent clone (Lane14) using decamer primer MAP13 **(A)**, MAP16 **(B)**, OPA18 **(C)**, OPA19 **(D)**, UPGAMA based phenogenetic relationship between regenerated progeny (**E)**

the genetic uniformity of the micro-propagated, bio-hardened plants of ***C. borivilianum***. RAPD analysis of the DNA amplified products of the micro-cloned plants exhibited high degree of monomorphism with more than 99% similarity indices that matched well with that of the parent plant. Our findings are also in agreement those of Angel et al. (1996), Lattoo et al. (2006), Rani et al. (1995) and Rout et al. (1998) who also found no RAPD fingerprint variation in ***Mannihot***, ***C. arundinaceum***, ***Populus***, ***Zingiber*** plants derived from ***in vitro*** meristem cultures, respectively.

The observed genetic uniformity might be ascribed to the employment of stem discs with pre-formed buds as starting explant for micro-cloning as proposed for axillary and apical bud explants in many plant systems (Shenoy and Vasil, 1992; Herman, 2006).

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