Regeneration of Genetically Uniform *Boerhaavia diffusa* by Culture of Nodal Explants and Synthetic Seeds

Avik Ray • Sabita Bhattacharya*

Department of Botany, Bose Institute, 93/1, A.P.C. Road, Kolkata – 700 009, India
Corresponding author: * sabita@bosemain.boseinst.ac.in

ABSTRACT

Multiple shoot production was achieved in *Boerhaavia diffusa* through axillary branching and differentiation of adventitious shoot buds at the base of nodal explants, cultured on Murashige and Skoog (MS) medium containing cytokinin alone or in combination with auxin. Application of kinetin and indole-3-acetic acid, both at 0.5 mg l^{-1} was most effective for shoot regeneration, resulting in an average of 3.6 shoots/explant within 30 days of culture. Re-use of nodal explants cyclically from newly regenerated microshoots increased the rate of regeneration and made the process repetitive. The shoot tips and nodal explants encapsulated with sodium alginate-polymerized MS nutrient (synthetic seeds) could be stored at 4°C for a period of 10 weeks with a 64% conversion rate. The morphologically identical plants derived from synthetic seeds and through multiple shoot formation were all genetically identical when checked by Randomly Amplified Polymorphic DNA (RAPD) analysis.

INTRODUCTION

Plants have been, and still are a rich source of various natural products. About a half of today's best selling drugs owe their origin to natural products or their derivatives (Cragg *et al*. 1997). It was estimated that there are about 121 pharmaceutical compounds used as medicine worldwide that are derived from plants, out of which 101 plant species are in general the primary sources for 119 of these compounds (Cragg *et al*. 1997). In recent years, awareness of using non-synthetic drugs has increased quite rapidly. Natural compounds obtained from herbs are still the major contributor to the traditional systems of medicines like Ayurveda, Unani, Siddha, etc. Besides commonly used medicinal plants for extraction of secondary metabolites, sometimes weeds provide important compounds and serve as source of medicines for indigenous people and have a highly significant representation in indigenous pharmacopeias in relation to other types of plants (Stepp and Moerman 2001). *Boerhaavia diffusa* (Family – Nyctaginaceae) is one such small herbaceous plant that needs no introduction to Indian people. It has been in use in different parts of India to cure several diseases like asthma, urinary disorders, jaundice, etc. (Awasthi and Verma 2006). It was revealed that this species possesses several other highly important pharmaceutical properties including anti-cancer, anti-HIV (Mehrotra *et al*. 2002), anti-hepatitis (Thyagarajan *et al*. 2002). For industrial use, tons of *B*. *diffusa* raw materials are being collected every year (Anon 2000; Bodeker and Burford 2007) from India and as a consequence of this unsustainable collection, it is feared that this plant species will be severely depleted, or become extinct, at least from some of its natural habitats within a decade (Singh 2007). *Ex situ* conservation and development of suitable agro-technology for cultivation are the two means by which the species can be made available in future (Pandey and Shukla 2001; Singh 2007). To date there are no established agro-techniques for cultivation or any suitable method of germplasm conservation available for this perennial grassland herb. Moreover, recent studies have shown that the natural populations (seed-borne) of *B*. *diffusa* are extensively variable in their genetic nature (Shukla *et al*. 2003), which may cause inconsistencies in the plant's medicinal quality (Debnath *et al*. 2006; Mathur *et al*. 2008).

Tissue culture-based technology, widely accepted for clonal propagation (Bajaj 1997) can be employed for overcoming problems in ensuring consistent regeneration of this species. Furthermore, this technology offers options to study germination, storage and viability of artificially developed germplasm, particularly for those species (e.g., grassland herbs), the seeds of which are difficult to collect in homogenous form from the mixed seed population deposited in pasture fields or in cultivated lands. Important role of biotechnological methods in germplasm conservation and propagation has been described by Kavyashree *et al*. (2006) and Ray and Bhattacharya (2008).

For *B*. *diffusa*, although a few reports are available on *in vitro* regeneration (Bhansali *et al*. 1978; Naushaba *et. al*. 2004; Roy 2008), evidence of work for producing genetically uniform population of this species along with methods for storing its germplasm is still lacking.

In this regard, our study aimed to clonally propagate *B*. *diffusa* through cyclic regeneration to increase production rate, to develop synthetic seeds by encapsulating shoot tips/ nodal segments to study their storage and subsequent conversion and finally to validate the true-to-typeness of all regenerants through genetic fingerprinting.

MATERIALS AND METHODS

Micropropagation

1. Collection of plant materials and disinfection of explants

Healthy and actively growing *B. diffusa* plants were collected from Bose Institute campus before flowering and identified properly at species level. Nodal explants (0.8-1.0 cm with a single node in

Original Research Paper

each), after excised from younger vegetative parts of the plant, were surface sterilized initially by cleaning thoroughly with running tap water and then treated stepwise with aqueous solution of a mixture of 0.1% Bavistin (Carbendazim, BASF, Mumbai, India) and 0.2% Pantomycin (Streptomycin sulphate, Tetracyclin tetrachloride, ARIES, Mumbai, India) for 1 h. Finally, under aseptic conditions, explants were dipped in 0.1% mercuric chloride for 2 min with mild stirring. Each treatment followed 3-4 rinses in sterile double distilled water. Before inoculation, the axillary regions were made clear by excising leaf lamina, leaving only petiolar parts at the nodes.

2. In vitro culture condition and shoot regeneration and rooting

Sterilized explants were inoculated in 100 ml Erlenmayer flasks, each containing 30 ml semi-solid (gelled with 0.8% agar powder Type I, Himedia Laboratories Pvt. Ltd., Mumbai, India) MS medium (Murashige and Skoog 1962) at pH 5.6-5.8, fortified with 3% sucrose. Sterilization was done under 103kPa pressure and 121°C for 15 mins in an autoclave. For shoot regeneration, different concentrations of plant growth hormones (PGRs; 0.1, 0.5, 1.0 and 2.0 mg l^{-1}) like kinetin, 6-benzyl aminopurine (BAP), and thiadiazuron (TDZ) were applied singly or in combination with indole-3-acetic acid (IAA) or naphthalene acetic acid (NAA). Cultures were incubated under a 18 h photoperiod provided by cool white fluorescent lamps (Philips, India; intensity 2500 lux) and maintained at 22 ± 2°C. Routine subcultures were done at every 30 days' intervals. Data on shoot response (%) and total number of shoots regenerated were recorded after 60 days culture. Results on the effect of BAP and TDZ on shoot regeneration were not presented because the data generated from the experiment were insufficient to do so.

Some of the regenerated leafy shoots were used routinely as the source of nodal explants. Cyclic supply of *in vitro* multiplied shoots in initiating new cultures was referred to as 'cyclic regeneration' after the term used earlier by Keller *et al*. (1997).

For rooting, regenerated shoots (1.0-1.5 cm), harvested from medium containing kinetin or kinetin-IAA were cultured in MS with IAA/NAA at 0.5, 1.0 and 2.0 mg l^{-1} or without PGRs. Data on root initiation time, total number of roots and their length (cm) were recorded after 30 days.

Storage and conversion of synthetic seeds

1. Preparation and storage conditions

Shoot tips and nodal explants (0.4-0.6 cm), excised from axenic regenerated shoots were suspended in sterile gelled MS, containing 3% sucrose at pH 5.7 (gelling agent = sodium alginate, Sigma, 3% w/v). The suspension was then loaded in a sterile Pasteur pipette (0.5-0.8 cm diameter) and ejected drop-wise (each drop containing single explant) into 150 ml solution of MS nutrient components and CaCl$_2$ (100 mM) in a beaker. The droplets, forming semi-transparent beads were kept in the same solution with continuous stirring for 30 min to complete polymerization of sodium alginate. The solution was decanted and the alginate beads (approximately 0.5-0.6 cm diameter) were washed repeatedly (at least twice) with sterile distilled water to remove excess CaCl$_2$, and finally the adhering water was soaked with sterile filter paper. The resultant beads – the 'synthetic seed' or in short 'synseeds' ('syn-s' – shoot tip inside or 'syn-n – nodal explant inside) were placed on semi-solid MS medium (3% sucrose, w/v) without growth hormone in petriplates or conical flasks.

Encapsulated propagules were incubated at different temperatures (20, 12 and 4°C) under continuous light (1500 lux) and conversion was recorded at intervals of two weeks. Storage in total darkness, diffused light and 18 h photoperiod were abandoned after trial tests because these conditions were unsuitable for viability and storage of synthetic seeds.

2. Test for conversion

To study the effect of storage duration (2, 4, 6, 8, 10 weeks) and temperature (20, 12 and 4°C) on conversion, synthetic seeds, maintained under continuous light were exposed (each time 10 seeds from each set) to normal culture conditions (25°C; 2500 lux; 24h photoperiod) and allowed to resume growth. Emergence of shoot tips by rupturing the bead matrix was considered as conversion. The conversion frequency (percentage) was recorded from each experimental set at 2 week interval starting from the first day of storage. Plantlets raised from synseeds were treated with IAA at 1.0 mg l^{-1} for better rooting.

Acclimatization and hardening

The well rooted plantlets obtained through micropropagation and synseeds were transferred from culture medium stepwise to sterile soilrite and soilrite-garden soil (1:1) mixture in plastic pots (both covered with polythene sheets) and maintained at 25°C, 2500 lux for the first 7 days, then transferred to the glasshouse. Finally, the hardened plants were transplanted to earthen pots with soil and were maintained in natural environment after removing the covers. The percentage survival was recorded in all sets eight weeks after transfer to soil.

Genetic fidelity test

For testing genetic fidelity of the micropropagated *B. diffusa* plants RAPD analysis was conducted. DNA was extracted from the donor plant (maintained in a separate pot) and from 14 randomly selected hardened plants (7 each from micropropagated and synseed-derived) after 8 weeks of their transferring to soil under greenhouse condition.

1. DNA extraction

For each sample, DNA was extracted from 400 mg of fresh leaves according to the method of Doyle and Doyle (1987). It was purified with RNAaseA (Sigma) and concentration was measured by UV spectrophotometer (Shimadzu Corp. Japan) at 260 nm. Purified DNA was stored at 4°C and used as template for RAPD study.

2. RAPD analysis

Polymerase chain reactions were carried out in 25 μl volumes with 50 ng of template DNA, 1 unit of Taq polymerase (Integrated DNA Technologies, USA), 1X reaction buffer, 250 μM of dNTP (Integrated DNA Technologies, USA), 2.5 mM MgCl$_2$ and 50 ng RAPD primer (Operon Tech, USA and Bangalore Genei, India). The amplification was carried out in a DNA thermal cycler (Gene Amp PCR System 2400, Perkin Elmer Corporation, USA) in the following way: initial 3 mins at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. An additional cycle of 10 mins at 72°C was used for final extension. The amplified products were electrophoresed in 1.5% agarose gels in 1X TBE (Tris-Borate-EDTA) buffer for at least 1 h at 65-70V. The gels were stained with 0.5 μg/ml ethidium bromide, checked under UV light, scanned and photographed in gel documentation system (Gel-doc 1000, Bio-Rad Laboratories, USA).

All the reactions were repeated at least in triplicate, and only consistently reproducible bands were considered. For each primer, RAPD bands were scored on the basis of visual inspection of the gel photograph and molecular weights were estimated by the Software Quantity One 1-D Analysis Software (version 4.6.2, Bio-Rad). The 30 10-mer primers were used for RAPD study, of which 20 were from Operon technologies (OPB11-20, OPC 1, OPC 2, OPC4, OPC 6, OPC 9, OPC 11, OPC 15, OPC 18, OPC 20) and 10 self-designed primers were obtained from Bangalore Genei (BG1-10).

Statistical analysis

Each treatment consisted of 10 replicates and each experiment was repeated three times. All data were subjected to analysis of variance (ANOVA) and comparisons of means were made with least significant difference test at the 5% level of probability (excluding the data generated from experiments with BAP and TDZ).

RESULTS AND DISCUSSION

Shoot multiplication

Nodal explants were cultured in MS containing cytokinins like kinetin, BAP and TDZ, each of which was applied singly or in combination with IAA at 0.1-2.0 mg l^{-1}. It was observed that kinetin, alone or jointly with IAA (both at 0.5 mg^{-1}) was most effective in inducing shoot regeneration. In this hormone combination, the highest number of shoots per explant (on average 3.6) was obtained (**Table 1**). During culture, callus was formed at nodal region and at the base of the explant in the presence of all hormones tested, regardless of its kind, though callus growth was quite restricted in medium containing kinetin. Unlike the other two cytokinins, BAP and TDZ, kinetin-induced shoots were normal in appearance and growth. TDZ was least effective for shoot regeneration at any applied concentration. Shoots obtained on treatment with BAP were observed to be abnormally shaped, stunted, bearing small leaves and without regeneration power. Superiority of kinetin over other PGRs in inducing shoot regeneration of *B. diffusa* was observed in callus-mediated regeneration (Bhansali *et. al*. 1978) and that directly from explants (Baig *et al*. 2004).

Breaking of two axillary buds (within seven days) was the first sign of growth shown by nodal explants (**Fig. 1A, 1B**). Thereafter, the sprouting buds grew rapidly to form axillary branches with distinct nodes, at each of which foliated oppositely arranged leaves (**Fig. 1C**). Subsequently, the basal part of the explant in contact with the medium gradually thickened, and new shoots emerged from it (**Fig. 1D, 1E**). This pattern of regeneration tallied with that observed in *B. diffusa* growing in pasture land (Pandey and Shukla 2001). One important observation made in the present experiment was that the adventitious shoots, irrespective of their origin, had strong apical dominance and that by removing tips, axillary buds at lower nodes could be stimulated to develop new branches. This phenomenon led to the utilization of young shoots in cyclic micropropagation i.e., making the process recurrent. In this way, it was possible to harvest approximately 1000 *B. diffusa* microshoots within one year period starting from a single explant. A high frequency regeneration protocol following cyclic production of shoots were developed in *Alstroemeria* (Lin *et al*. 2000), *Allium sativum* (Keller 1997), and *Crataeva nurvala* (Walia *et al*. 2007).

As a result of excising tips from growing shoots, the number of regenerants gradually increased in individual cultures giving a bushy appearance. Interestingly, in quite older cultures, the leaf lamina, petioles and sometimes stems of the shoots turned red (**Fig. 1F**) possibly due to the synthesis of betanin (Stintzing *et al*. 2004). The *in vitro* production of the natural red pigment from *B. diffusa* (Stintzing *et al*. 2004) may be exploited in natural pigments industries. The growing interest in plant-based natural pigments and betanin, in particular as a food colorant has recently been reviewed (Moreno *et al*. 2008).

Table 1 Effect of kinetin alone and in combination with IAA on shoot regeneration from nodal explants of *Boerhaavia diffusa* (data collected after 30 days culture).

Growth hormones (mg^{-1})		Shoot response (%)	Number of shoots regenerated/explant (mean ± SD)[*]	Rooting response (%)
Kinetin	Kn + IAA			
0.1	-	100 a	1.68 ± 0.23 c	87.1 ± 2.9 a
0.5	-	100 a	3.20 ± 0.56 ab	79.5 ± 3.0 ab
1.0	-	100 a	1.86 ± 0.29 c	45.4 ± 3.5 c
2.0	-	89 ab	1.08 ± 0.16 c	11.7 ± 2.0 d
-	0.1 + 0.1	100 a	2.10 ± 0.18 bc	74.8 ± 4.1 b
-	0.5 + 0.5	100 a	3.60 ± 0.68 a	81.4 ± 3.8 ab
-	1.0 + 1.0	87 b	2.28 ± 0.54 abc	78.3 ± 1.0 ab
-	2.0 + 2.0	57 c	1.06 ± 0.25 c	82.9 ± 5.0 ab

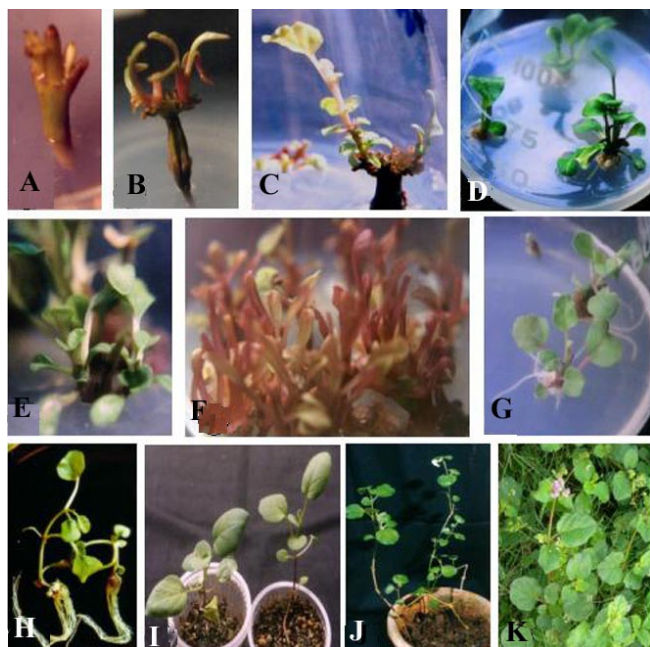Means followed by same letter in a column are not significantly different at 5% level.

Fig. 1 Different stages of plant regeneration from *Boerhaavia diffusa* nodal explant. (**A**) Initial response of axillary bud to nutrient medium showing increase in size; (**B**)emergence of young leaves from sprouted axillary buds; (**C**) axillary shoots with growing leaves at nodes; (**D**) emergence of adventitious shoots at the base of explant; (**E**) regenerated shoots showing strong apical dominance; (**F**) Culture of *B. diffusa* exihibiting red pigmentation after prolonged culture; (**G**) Rooting of shoots in auxin-free medium; (**H**) rooting of auxin-treated shoots; (**I**) young plants in soilrite; (**J**) hardened *B. diffusa* in earthen pots; (**K**) *in vitro* grown plants in field.

Rooting of shoots and hardening of *in vitro*-raised *B. diffusa*

Root production was infrequent from the shoots that were still attached to the explants. On transferring to MS, with or without auxin (IAA, IBA or NAA), 100% root induction was observed (**Fig. 1G**). The presence of auxin in the medium improved rooting (number and length) in comparison to what was observed in auxin-free medium. Profusely growing roots (18.4/culture) reached an average length of 9.5 cm in the presence of 2.0 mg l^{-1} IAA (**Table 2**). Over and above the normal root system, adventitious roots emerged from leaf margins and mid-veins – a phenomenon similar to that observed earlier by Srivastava and Padhya (1995).

Well-rooted plantlets of *B. diffusa* (**Fig. 1H**) were transplanted in the first phase to sterile soilrite (**Fig. 1I**) and step-wise to soilrite-soil mixture and finally to the soil in small plastic cups. For the first 7 days these were kept under culture room conditions followed by transfer to the glasshouse and finally to the field (open area). The small growing plants (**Fig. 1H**) did not need any plastic bag covering if exposed during the monsoon season, but this was required in other seasons, particularly in summer, for the first few days. The hardened plants were transferred to earthen pots (**Fig. 1J**) and after completing one flowering cycle there, the plants were sown in the field amongst the naturally grown *B. diffusa*, where they continued to grow intermingled with others (**Fig. 1K**). In summer, the percentage survival was 78% and it increased to 85% during winter and almost 100% was obtained in the rainy season.

All the tissue culture-derived plants were morphologically alike and resembled the donor (mother) plant.

Storage and regrowth of synthetic seeds

To study storage and conversion of *B. diffusa* synthetic seeds (syn-s and syn-n), these were stored at 20, 12 and 4°C under continuous light for different durations.

Table 2 Effect of different concentrations of auxins on rooting of *Boerhaavia diffusa* shoots (data collected after 30 days culture).

Auxins (mg^{-1})		Root initiation time (day)	Number of roots	Length of roots (cm)	Respondent explants (%)
IAA	0.5	6-9 days	10.6 ± 1.4 ac	4.8 ± 0.4 a	100 a
	1.0		14.7 ± 2.0 ab	7.2 ± 0.2 b	100 a
	2.0		18.4 ± 2.6 b	9.5 ± 0.05 c	100 a
IBA	0.5	8-10 days	8.1 ± 0.8 c	2.4 ± 0.2 d	95.4 ± 3.6 a
	1.0		12.5 ± 1.0 ac	5.4 ± 0.15 ae	92.3 ± 5.4 a
	2.0		13.8 ± 1.15 ad	5.8 ± 0.2 ef	100 a
NAA	0.5	8-10 days	9.4 ± 0.5 cd	3.1 ± 0.1 d	100 a
	1.0		13.2 ± 1.2 ad	6.5 ± 0.25 bf	93.2 ± 6.9 a
	2.0		12.5 ± 1.05 ac	4.8 ± 0.34 a	90.3 ± 4.4 a

Means ± SD followed by same letter in a column are not significantly different at 5% level.

Table 3 Effect of storage temperature (°C) and storage period (weeks) on conversion frequency (%) of *B. diffusa* synthetic seeds (syn-s & syn-n).

Temperature (°C)	2 weeks		4 weeks		6 weeks		8 weeks		10 weeks	
	syn − s	syn − n	syn − s	syn − n	syn − s	syn − n	syn − s	syn − n	syn − s	syn − n
20°C	100 a	100a	95.1 ± 2.1 a	91.3 ± 3.9 a	-*	-*	-*	-*	-*	-*
12°C	100 a	100a	89.3 ± 3 a	86.4 ± 3.2 a	81 ± 3.6 a	79.3 ± 2.8 a	-*	-*	-*	-*
4°C	92.3 ± 3 b	90.4 ± 3.3 b	85.1 ± 2.7 a	82.5 ± 3 a	80.2 ± 3.8 a	77.1 ± 2.8 a	75 ± 2.4 a	72.1 ± 4.3 a	64.2 ± 2.7 a	61.5 ± 3.5 a

*Storage of synseed not possible due to random conversion

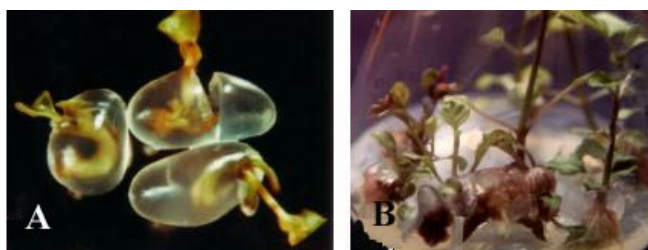Means ± SD followed by same letter in a row are not significantly different at 5% level.

Fig. 2 Plant conversion of *B. diffusa* from synthetic seeds. (A) sprouting of synthetic seeds, **(B)** normal growth and rooting of synseed derived plantlets.

Light, temperature and period of storage (weeks) affected viability and regrowth potential of the meristematic tissues of the explants embedded in the gel beads. The results (data not presented) showed that under total darkness, green explants turned gradually colourless and lost ability to perform normal physiological functions, whereas photoperiods (12 h: 12 h/18 h: 6 h) stimulated growth leading to rapid conversion, an undesirable phenomenon for storage. Preservation in continuous light showed dormancy and conversion of synseeds at variable frequencies depending on temperature (**Table 3**). Synseeds could not be stored beyond four weeks at 20 and 12°C due to random shoot formation while in storage. On the other hand, 4°C promoted extended storage up to 10 weeks with moderately high conversion (61.1% in syn-n and 64.2% in syn-s). The difference in conversion percentage between the two types of synseeds under similar storage conditions was insignificant (P=0.05). During conversion, shoot tips or axillary buds of nodal explants became activated and emerged from the bead by rupturing the gel matrix (**Fig. 2A**). At this stage, if the sprouted beads were cultured in MS containing IAA (0.5-2.0 mg l^{-1}), roots started to differentiate at the shoot base simultaneously with differentiation of new leaf primordium at the growing apex and the resultant plantlets continued growth in normal way (**Fig. 2B**). Evidences in support of suitability of low incubation temperature (4°C or around it) for successful storage and conversion of synthetic seeds for species of different taxonomic status are available, for example, in conifers (Attree and Fowke 1993), in trees – *Paulownia elongata* (Ipekci and Gozukirmizi 2003), in shrubs – *Punica granatum* (Naik and Chand 2006).

Synseed-derived hardened *B. diffusa* showed no difference with that originated from nodal explants with respect to morphological appearance.

Encapsulation of regenerable plant parts has been extensively employed nowadays to conserve, propagate, distri-bute as well as to exchange plant germplasm, particularly to solve problems arising from storage and propagation of plants. The other advantages of using synthetic seeds are ease of handling, higher scale-up potential and low-cost of production (Rao *et al*. 1998). This technique is particularly useful in grassland herbs like *B. diffusa* whose seeds are difficult to collect from a mixed seed population deposited in pasture fields or in cultivated lands. Encapsulated germplasm not only offer short-term storage, but high conversion at post storage condition can also provide readily available germplasm for rapid mass propagation of this species.

Checking genetic uniformity in tissue culture-derived *B. diffusa*

DNA extracted from leaf tissues of 14 randomly selected plants (7 each from micropropagated and synseed-derived accessions) and the donor were used in RAPD analysis. The
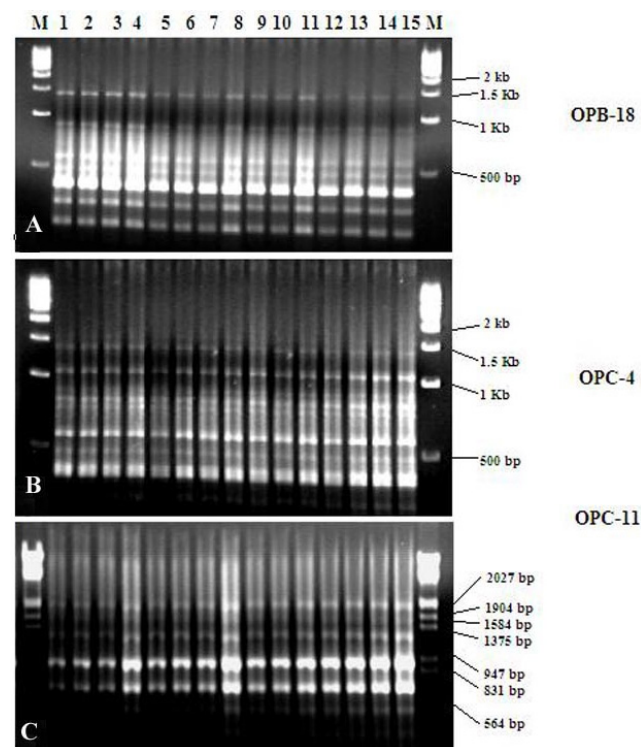

Fig. 3 DNA amplification profile of *B. diffusa*. (A) primer OPB - 18, **(B)** OPC – 4, **(C)** OPC - 11 (Lane - M : Marker DNA, Lane – 2-8 : DNA from micropropagated plants. Lane – 9-15 : DNA fron synseed-derived plants.

profile of the PCR-amplified product of genomic DNA of total 15 samples is shown in **Fig. 3**. Out of total 30 different decamer primers used in PCR reactions, only 3 produced definite reproducible bands. There were 19 visible bands in total and 6.3 bands per primer on average, the molecular weight of which ranged between 230 bp and 1.9 kb. The amplification profile raised with all 3 primers was found to be similar without showing any aberrations in RAPD banding pattern among the tested materials.

Fig. 3A-C demonstrates an amplification pattern generated by primers OPB18, OPC4 and OPC11 which show 7, 8 and 4 distinct bands respectively in each of the lanes of the 3 sets of the amplified products. This indicates genetic uniformity between the individual regenerants (irrespective of the source) as well as between the regenerants and their donor. This result also indicates that the present propagation system is able to produce genetically stable *B. diffusa* plants. In a number of earlier studies, RAPD was applied to examine genetic variation/stability of *in vitro* propagated medicinal plants (reviewed by Teixeira da Silva *et al.* 2005). Reliability of RAPD in detecting changes at molecular level including mutation, large rearrangements, DNA damage, etc. has been reviewed by Atienzar and Jha (2006).

Checking genetic uniformity of *in vitro* raised plants is critical and important (Mathur *et al.* 2008). This aspect has special significance in the case of *B. diffusa* because this species occurring in different geographical regions of India shows a wide range of genetic diversity (Shukla *et al.* 2003).

ACKNOWLEDGEMENTS

REFERENCES

Anon (2000) Requirements of major crude drugs (Tons/Annum) by some Indian Pharmacies. In Government of India Planning Commission (Ed) *Report of the Task Force on Conservation and Sustainable Use of Medicinal Plants*, pp 151-153

Atienzar FA, Jha AN (2006) The randon amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenic studies: a review. *Mutation Research* **613**, 76-102

Attree SM, Fowke LC (1993) Embryogeny of gymnosperm: advances in synthetic seed technology of conifers. *Plant Cell, Tissue and Organ Culture* **35**, 1-35

Awasthi LP, Verma HN (2006) *Boerhaavia diffusa* – A wild herb with potent biological and antimicrobial properties. *Asian Agrihistory* **10**, 55-68

Baig N, Shahzad A, Siddiqi SA (2004) *In vitro* clonal propagation of *Boerhaavia diffusa* L. – a potential medicinal herb. *Advances in Plant Sciences* **17**, 5-10

Bajaj YPS (1997) *Biotechnology in Agriculture and Forestry* (Vol 39) *High-Tech and Micropropagation V*, Springer-Verlag, Berlin, 395 pp

Bhansali, RR, Kumar A, Arya HC (1978) *In vitro* induction of adventitious shoots on stem explants of *Boerhaavia diffusa* L. *Current Science* **47**, 551-552

Bodeker G, Burford G (2007) Medicinal plant biodiversity and local healthcare: sustainable use and livelihood development. In Boder G, Burford G (Eds) *Traditional Complementary and Alternative Medicine*, Imperial College Press, London, pp 145-166

Cragg GM, Newman DJ, Snader KM (1997) Natural products in drug discovery and development. *Journal of Natural Products* **60**, 52-60

Debnath M, Malik CP, Bison PS (2006) Micropropagation: a tool for production of high quality plant based medicine. *Current Pharmaceutical Biotechnology* **7**, 33-34

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11-15

Ipekçi Z, Gözükirmizi (2003) Direct somatic embryogenesis and synthetic seed production from *Paulownia elongata*. *Plant Cell Reports* **22**, 16-24

Kavyashree R, Gayatri MC, Revanasiddaiah HM (2006) Propagation of mulberry variety – S$_{54}$ by synseeds of axillary bud. *Plant Cell, Tissue and Organ Culture* **84**, 245-249

Keller ERJ, Lesemann DE, Lux H, Maas HI, Schubert I (1997) Application of *in vitro* culture to onion and garlic for management and use of genetic resources at Gatersleben. *Acta Horticulturae* **433**, 141-150

Lin HS, De Jen MJ, Jacobson E (2000) Application of leafy explant micropropagation protocol in enhancing the micropropagation efficiency in *Alstroemeria*. *Scientia Horticulturae* **85**, 307-318

Mathur A, Mathur AK, Verma P, Yadav S, Gupta ML, Darokar MP (2008) Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum* Sant. et Fernand. *African Journal of Biotechnology* **7**, 1046-1053

Mehrotra S, Mishra KP, Maurya R, Srimal RC, Sing VK (2002) Immunomodulation by ethanolic extract of *Boerhaavia Diffusa* roots. *International Immunopharmacology* **2**, 987-996

Moreno DA, García-Viguera C, Gil JI, Gil-Izquierdo A (2008) B*etal*anins in the era of global agri-food science, technology and nutritional health. *Phytochemical Reviews* **7**, 261-280

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-479

Naik SK, Chand PK (2006) Nutrient-alginate encapsulation of *in vitro* nodal segments of pomegranate (*Punica granatum* L.). *Scientia Horticulturae* **108**, 247-252

Pandey SK, Shukla RP (2001) The regeneration pattern and population structure of *Boerhaavia diffusa* L. in relation to disturbance in grasslands of northeastern U.P. *Tropical Ecology* **42**, 137-140

Rao PS, Suprasanna P, Ganapathi TR, Vapat VA (1998) Synthetic seeds: concepts, methods and application. In: Srivastava PV (Ed) *Plant Tissue Culture and Molecular Biology*, Narosa Publishing House, New Delhi, pp 607-619

Ray A, Bhattacharya S (2008) Storage and plant regeneration from encapsulated shoot tips of *Rauvolfia serpentina* – an effective way of conservation and mass propagation. *South African Journal of Botany* **74**, 776-779

Roy PK (2008) Rapid multiplication of *Boerhaavia diffusa* L.through *in vitro* culture of shoot tip and nodal explants. *Plant Tissue Culture and Biotechnology* **18**, 49-56

Singh A (2007) *Boerhaavia diffusa*: An over-exploited plant of medicinal importance in eastern Utterpradesh. *Current Science* **93**, 446

Shukla N, Sangwan NS, Misra HO, Sangwan RS (2003) Genetic diversity analysis in *Boerhaavia diffusa* L. of different geographic locations in India using RAPD markers. *Genetic Resources and Crop Evolution* **50**, 587-601

Srivastava N, Padhya MA (1995) Punarnavin profile in the regenerated roots of *Boerhaavia diffusa* L. from leaf segments. *Current Science* **68**, 653-656

Stepp JR, Moerman D (2001) The importance of weeds in ethnopharmacology. *Journal of Ethnopharmacology* **75**, 19-23

Stintzing FC, Kammerer D, Schieber A, Adama H, Nacoulma OG, Carle R (2004) Betacyanin and phenolic compounds from *Amaranthus spinosus* L. and *Boerhaavia erecta* L. *Zeitschrift für Naturforschung* **59c**, 1-8

Teixeira da Silva JA, Giang DTT, Rashid SZ, Nhut DT (2005) Molecular marker-assisted phylogeny and breeding in ornamental, physiological model, medicinal and aromatic plants. In: Thangadurai D, Pullaiah T, Tripathy L (Eds) *Genetic Resources and Biotechnology* (Vol III), Regency Publications, New Delhi, India, pp 309-326

Thyagarajan SP, Jayaram S, Gopalakrishnan V, Hari R, Jevakumar P, Sripathi MS (2002) Herbal medicines for liver diseases in India. *Journal of Gasteroenterology and Hepatology* **17**, 370-376

Walia N, Kaur A, Babbar SB (2007) An efficient *in vitro* cyclic production of shoots from adult trees of *Crataeva nurvala* Buch. Ham. *Plant Cell Reports* **26**, 277-284