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TRANSVERSE THIN CELL LAYER CULTURE FOR HIGH FREQUENCY SHOOT GERMINATION IN *BOERHAAVIA DIFUSA* L., ITS CONSERVATION AND ASSESSMENT OF GENETIC FIDELITY

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Abstract

Boerhaavia diffusa, an important perennial member of the family Nyctaginaceae is known since centuries for its high medicinal value. Extensive uses of *B. diffusa* in traditional medicinal system have promoted its large scale collection from its wild natural habitats and this has resulted into depletion of this plant. Therefore, an efficient and high frequency plant regenerartion protocol using transverse thin cell layer (tTCL) culture system has been developed in *B. diffusa*. The percent of shoot proliferation, number of shoots per responsive explants and average shoot length were found to be greater for tTCL-derived cultures as compared to nodal segments and shoot tips-derived cultures. Among different media combination used, 1.5 mg/l BAP + 0.5 mg/l NAA was most appropriate combination for tTCL-derived cultures. Earliest and maximum rooting was achieved in half-strength MS media. After rooting tTCL-derived *in-vitro* propagated plantlets were successfully acclimatized and transferred to field condition. From tTCL-derived plantlets, nodal segments were taken and synthetic seeds were prepared which after re-growth were found to be morphologically similar to the mother plant. Genetic fidelity of tTCL-derived plants was tested by using two DNA based molecular markers *i.e.*, RAPD and ISSR markers. All the tested plants were found to be genetically identical to the mother plant. The present study provides a novel approach for high frequency shoot germination in *B. diffusa* through tTCL-method, which can be applied commercially, as well as a basis for genetic engineering experiments due to fast and efficient *in-vitro* propagation of genetically alike plants.

Key words: Transverse thin cell layer, *Boerhaavia diffusa*, RAPD, ISSR, BAP

Abbreviations

tTCL: Transverse thin cell layer
SAM: shoot apical meristem
BAP: 6-benzylaminopurine
2, 4D: 2,4-Dichlorophenoxyacetic acid
NAA: 1-Napthaleneacetic acid
RAPD: Random amplified polymorphic DNA
ISSR: Inter-simple sequence repeats

Introduction

Boerhaavia difusa, an important member of the family Nyctaginaceae, has a long history of use as medicinal herb and has been mentioned in many ancient Indian medicinal systems such as Charaka Samhita, Sushrita Samhita, Ashtaanga Hridaya and Chakradatta. In Indian pharmacopoeia, the plant as a whole has been documented in the name of the drug 'punarnava' attributed to its diuretic property (Chopra 1969). As per the Ayurvedic and Unani system of ancient Indian medicine, this plant has the property to cure around 22 different kinds of illness. Not only in Indian medicinal system, Brazilian pharmacopoeia has also documented 23 medicinal properties of this important medicinal plant (Patil and Bhalsing, 2016). *B. diffusa* is known to be effective against arthritis, joint pain, rheumatism, jaundice, hepatitis, abdominal tumors, heart troubles, asthma, seminal weakness, sprain, inflammation and gastro-entritic problems (reviewed by Patil and Bhalsing 2016).

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Recently, reports have documented the antileishmanial (Kaur *et al.* 2015) and neuroprotective (Ayyappan *et al.* 2015) effects of *B. diffusa*. Each Plant part possess some therapeutic potential owing to the presence of one or more therapeutically active compounds. It is known to possess a set of medicinally important metabolites, amongst which punarnavin (an alkaloid) is the most significant. Other important chemical constituents are boeravinones A-H, eupalitin, β-sitosterol-β-D-glucoside and β-sitosterol (Bharali *et al.* 2003; Chaudhary and Dantu 2011).

In nature, *B. diffusa* propagates majorly through seeds and less often *via* stem cutting. However, poor seed viability and low germination frequency restrict its large-scale production (Pinki and Khalkho 2012). Due to its immense medicinal property and extensive use in traditional medicinal system, its mass scale collection from its wild natural habitats has resulted in to depletion of this plant. Biotechnological approaches such as plant tissue culture provides an effective alternative for large-scale propagation of plants with low seed viability or germination frequency.

Thin cell layer (TCL) technique of *in-vitro* propagation provides advantages over conventional *in-vitro* culture methods in terms of overall plantlet production (Lakshmanan *et al.* 1995). This method was first elucidated by Tran Thanh Van in 1981. The TCL culture method includes a small sized (0.1-5 mm) explants, excised from different tissues such as shoots, stem nodes, hypocotyls etc), either transversally (tTCL) or longitudinally (lTCL). Transverse thin cell layer (tTCL) culture technique has been successfully applied in various plant species (Nhut *et al.* 2000; Leguillon *et al.* 2003; Ghnaya *et al.* 2008; Swarna and Ravindharan 2013).

Compared with the huge demand of the plant, relatively fewer attempts were made for *in-vitro* propagation of *B. diffusa* and that only through conventional tissue culture methods such as nodal or SAM (shoot apical meristem) culture using different media composition (Roy 2008; Patil and Bhalsing 2015; Bhansali *et al.* 1978, Shrivastava and Padhya 1995; Nagarajan *et al.* 2005). Ragi and Shibu (2014) had reported *in-vitro* propagation of *B. diffusa* through leaf explants.

The aim of the present study was to optimize an *in-vitro* regeneration protocol *via* tTCL method that greatly facilitates the mass propagation of *B. diffusa*, wherein influence of growth regulators on proliferation rate, growth of shoots and rooting potential were investigated. Further the ability of the regenerated rooted plantlets to acclimate to ex-vitro conditions and their conservation through synthetic seed production was optimized. In addition, the genetic stability of the acclimated plants was verified through DNA fingerprinting using RAPD and ISSR markers.

Materials and methods

Plant material, explant preparation, and surface sterilization

Explants (nodal segments/shoot tips) of *B. diffusa* were obtained from field grown plants (maintained at Botanical Garden of Banaras Hindu University, Varanasi, India). The collected explants were thoroughly washed for 30 minutes under running tap water and then treated with 2 % (v/v) cetrimide (disinfectant; ICI India) along with 2-3 drops of Tween-20 (Hi-media, India) for 8-10 min. The detergents and disinfectants were then removed by washing under running tap water. The washed explants were taken to laminar flow hood for further sterilization by treating explants with 70 % (v/v) ethanol for 30 s and 0.05 % (w/v) freshly prepared mercuric chloride for 3-5 min. The explants were then washed (3-4 times) with sterile distilled water. The sterilized explants were transversally sliced from the nodal region into 1-4 mm thick sections which were used as tTCL explants for inoculation onto the MS medium supplied with different concentration of growth regulators. In addition to tTCL, nodes and shoot tips were also used as explants for comparative response.

Growth media and culture conditions

For shoot regeneration, the tTCL nodal segments/ nodes/shoot tips were inoculated onto the MS medium (Murashige and Skoog,1962) supplied with 3 % (w/v) sucrose and 0.8 % (w/v) agar (Merck India Ltd, Mumbai, India). The MS medium was supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP), 1-Napthaleneacetic acid (NAA) and kinetin (Table 2) (Sigma Chemical Co., St Louis, MO, USA). The resultant regenerated shoots produced on different concentrations and combinations of growth media, were further sub-cultured onto the full-strength MS medium devoid of any growth regulator, MS medium supplemented with different combinations of auxins and modified MS (half strength) for the shoot elongation and root induction. The cultures were incubated in a growth chamber preset at 24±2°C temperatures and 40 μmol m^{-2} s^{-1} light intensity by using white fluorescent tubes (Philips, India). Light was set as to provide 16 hour photoperiod.

Acclimatization of rooted plantlets

Rooted plantlets with 4-5 fully expanded leaves, retrieved from tTCL nodal segments were transferred to

plastic pots containing a mixture of sand and soil (1:1 w/w). In order to maintain high humidity, all the plantlets were covered with polyethylene bag and were regularly irrigated with water. These potted plantlets were kept at a temperature of 25 ± 1°C in artificial light (irradiance of 45 μmol m^{-2} s^{-1}) provided by white fluorescent tubes for 3-4 weeks and then the pots were gradually transferred to direct sunlight. After 2-3 months, all acclimatized plants were transferred to natural field conditions.

Encapsulation of explants for synthetic seed production

For production of synthetic seeds, solutions of 4 % sodium alginate and 100 mM $CaCl_2$ were prepared and sterilized by autoclaving. Nodal segments from plants generated through tTCL-explant were cut into appropriate size and dipped into sodium alginate solution. Drops of sodium alginate solution each containing one explant (shoot tips/nodal segment) were poured into 100 ml of $CaCl_2$ solution with the help of a 5 mm-wide sterile plastic pipette. The $CaCl_2$ solution containing explant-containing droplets was kept for 25 minutes under constant shaking in order to ensure complete crosslinking of sodium alginate and $CaCl_2$. After 25 minutes of crosslinking produced slightly hard, translucent spherical beads of Calcium Alginate which were collected, and washed with sterile distilled water. Thereafter the beads were blotted onto the autoclaved filter paper and finally transferred onto the solid MS medium (half strength basal, full strength basal, full strength basal + 1mg BAP/l) and on sterile soil directly for germination. All procedures were performed under aseptic condition in triplicates.

Assessment of genetic stability of acclimated plantlets

Leaf tissues from a mother plant which was used as a source of explants for tTCL culture, and six randomly selected 3 weeks-old acclimated plants raised through tTCL method were collected for assessment of their genetic similarity. Genomic DNA from all the seven leaf samples (1 mother plant and 6 acclimated plants) was isolated using cetyltrimethylammonium bromide (CTAB) method described by Khanuja *et al*. (1999). The quantity and quality of the isolated DNA were estimated by running aliquots on 0.8% agarose gel and by taking the absorbance at 260 nm. About 25 ng DNA was taken for both RAPD and ISSR reactions.

RAPD analysis

For RAPD analysis, polymerase chain reaction (PCR) amplification was performed in a reaction volume of 25 μl containing 25 ng genomic DNA, 1.7 M oligonucleotide primer, 200 μM dNTPs (50 μM of each), 2.5 μl 10x PCR buffer with 15 mM $MgCl_2$, 10 pmol of primer, and 1 U Taq DNA polymerase (Bangalore Genei, India). Amplification was performed in an *i*-Cycler (Bio-Rad, USA) with the following program: 5 min at 94 ºC for initial denaturation, followed by 44 cycles of denaturing at 94ºC (45 s), annealing at 36ºC (45 s) and extension at 72ºC (60 s) followed by a final extension of 8 min at 72ºC. Out of 8 arbitrary primers tested, only 4 produced readable and reproducible bands. Primer details are listed in Table 1.

ISSR analysis

PCR using 10 randomly selected microsatellite primers (FermentasInc, MD, USA) were performed and 4 of them produced readable and reproducible bands and therefore were taken forward for analysis. For ISSR amplification, the concentration and reaction volume were same as for the RAPD analysis. The PCR program consisted of 5 min at 94ºC for initial denaturation, followed by 44 cycles of denaturing at 94ºC (45 s), annealing at 36-45ºC (45 s) and extension at 72ºC (60 s) and a final extension of 8 min at 72ºC. The reactions were performed in an *i*-Cycler (Bio-Rad, USA).

Statistical Analysis

Each experimental set consisted of 10 replicates and each experiment was repeated thrice. Data were expressed as mean±S.E. of the number of replicates. Through one way ANOVA statistical significance among different experimental sets was analyzed by Duncan's multiple range test (DMRT) ($P < 0.05$) through SPSS software (SPSS Inc.,Version 16.0).

Results

Influence of plant growth regulators on shoot induction

Three different kinds of explants (SAM, nodes and tTCL segments) were inoculated on to the basal MS medium and three other groups of MS medium supplemented with different combinations of plant growth regulators. First group was comprised of four concentrations of BAP (0.75, 1, 1.5 and 2 ml/L). Second group was comprised of four concentrations of BAP (0.75, 1, 1.5 and 2 ml/L) each with a constant supplementation of 0.5 mg/L NAA and similarly third group was comprised of four concentrations of BAP (0.75, 1, 1.5 and 2 ml/L) each with a constant supplementation of 0.5 mg/L kinetin.

The effect of different plant growth regulator on shoot induction efficiency of all the three explants is presented in Table 2. Among all three kinds of explants (shoot tips, nodal segment and tTCL segment), tTCL segment

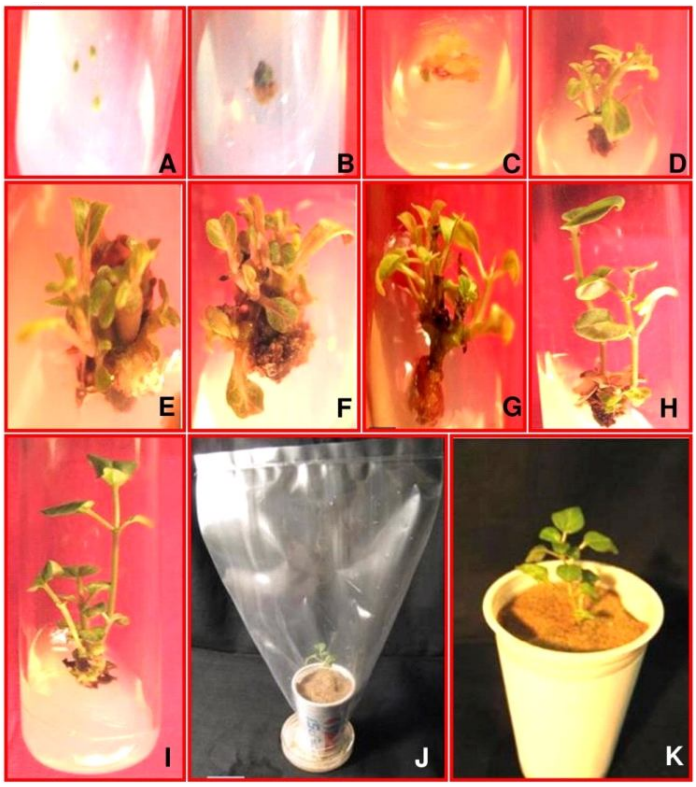

Fig.1: Shoot regeneration from transverse thin cell layer (tTCL) derived cultures of *Boerhaavia diffusa*. (A) Transverse thin cell layer inoculated on to the media. (B) to (I) different stages of *in-vitro* proparagtion on MS media supplemented with BAP (1.5 mg/l) and NAA (0.5 mg/l). (I) and (J) Acclimatization of the *in-vitro* propagated plantlets.

Table 1: Sequences of RAPD and ISSRs primers used in the present study

Primer	Sequence (5' to 3')
RAPD Primer	
OPO-16	TCGGCGGTTC
OPO-11	GACAGGAGGT
OPB-07	GTGACGCAG
OPB-05	TGCGCCCTTC
ISSR Primer	
UBC-807	AGAGAGAGAGAGAGAGT
UBC-841	GAGAGAGAGAGAGAGACTC
UBC-811	GAGAGAGAGAGAGAGAC
UBC-899	CATGGTGTTGGTCATTGTTCCA

showed maximum response in all sets of media combination. The overall response of explants was analyzed by measuring three parameters: percent shoot proliferation, number of shoots per responsive explants and average shoot length. In MS medium devoid of any growth regulator, percent shoot proliferation was 47, 36 and 54% for SAM, nodal segments and tTCL segments respectively. Maximum percent shoot proliferation (88.8 %) for tTCL was obtained after inoculation on to 1.5 mg/l BAP and 0.5 mg/L NAA. Number of shoots per responsive explants was higher for tTCL segments as compared to SAM and nodal explants at every concentration of BAP, NAA and kinetin. Among different concentration of plant growth regulator, 1.5 mg/l BAP and 0.5 mg/L NAA-supplemented MS media produced maximum number of shoots *i.e.*, 7 per responsive tTCL explants. Further the average shoot length for all three kinds of explants was found to be highest in MS media supplied with 2 mg/L BAP and 0.5 mg/L NAA. For tTLC, average shoot length was found to be 1.54, 1.13, 1.4, 1.61, 1.58, 1.18, 1.92, 3.01, 3.72, 1.14, 1.96, 2.14 and 2.44 in MS basal, MS+0.75 mg/l BAP, MS+1.0 mg/l BAP, MS+1.5 mg/l BAP, MS+2.0 mg/l BAP, MS+0.75 mg/l BAP+0.5mg/l NAA, MS+1.0 mg/l BAP+0.5mg/l NAA, MS+1.5 mg/l BAP+0.5mg/l NAA, MS+2.0 mg/l BAP+0.5mg/l NAA, MS+0.75 mg/l BAP+0.5mg/l KIN, MS+1.0 mg/l BAP+0.5mg/l KIN, MS+1.5 mg/l BAP+0.5mg/l KIN and MS+2.0 mg/l BAP+0.5mg/l KIN respectively.

As evidenced from the data presented in table 2, the most efficient shoot induction was obtained for tTCL explants; they were taken further for rooting under different media combination, acclimatization and genetic stability check. Fig. 1 describes the pictorial view of tTCL-mediated *in-vitro* regeneration of *B. diffusa*.

Effect of different medium and auxin (NAA/2,4D) combination on rooting of tTCL-derived microshoots

The effect of MS salt strength and different auxin supplementation on rooting of microshoots generated through tTCL segments is presented in Table 3. Half-strength MS medium was found to be most efficient in inducing rooting response when compared to full strength or quarter strength MS medium. Number of roots and root length was found to be highest in case of half-strength MS medium. Of the two auxins tested (2, 4D and NAA) NAA alone was found to induce rooting earlier (8-9 days) as compared to 2, 4D alone, where rooting was induced after 14-15 days after inoculation. However, when NAA and 2, 4D was used in combination, rooting was induced within 9-10 days *i.e.*, earlier than 2, 4D alone and later than NAA alone. The percent root response was also found to be highest in media containing both NAA and 2, 4D however the value was insignificantly higher than half-strength MS medium. The most prompt induction was recorded in half-strength MS medium where rooting was induced in 7-8 days. Full-strength MS media devoid of auxins, produced 4-5 roots per plantlets with an average root length of 3.2 cm. The

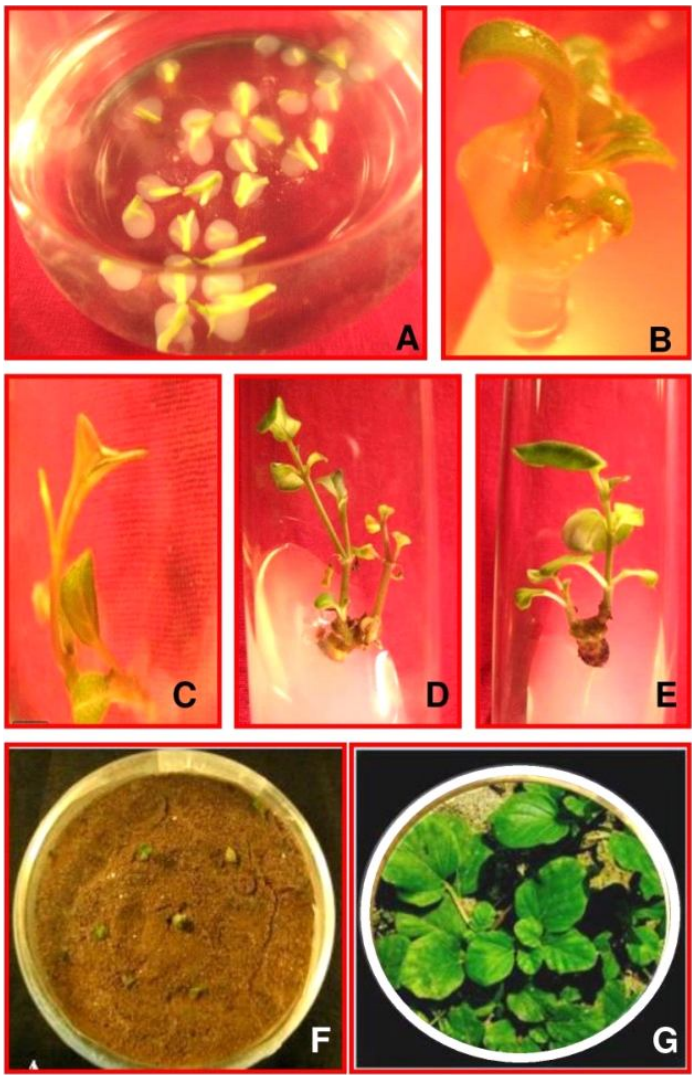

Fig. 2: Storage and regrowth of *Boerhaavia diffusa* through synthetic seed production. (A) Synthetic seeds in MS liquid medium. (B) to (D) regrowth of synthetic seeds inoculated onto MS medium. (E) and (F) Regrowth of synthetic seeds in sterile soil.

half-strength MS medium gave the best rooting response where 13-14 roots per plantlets was produced with an average root length of 5.8 cm.

Acclimatization of tTCL-derived rooted plantlet to ex-vitro conditions

All the tTCL-generated rooted plantlets were successfully acclimatized to ex-vitro conditions. With the survival frequency of 98 %, all the rooted plantlets were well established in soil. These plantlets grew well and possessed more number of branches within a month without showing any detectable morphological variation or abnormality.

Storage and re-growth of tTCL-derived plantlets through synthetic seeds

Synthetic seeds were successfully produced by using nodal explants from *in-vitro* raised *B. diffusa* through tTCL

Table 2: Effect of different concentrations of growth regulators on shoot multiplication and its response on the inoculated explant

			Percent response of shoots			Number of shoots per explant			Shoot length		
BAP	NAA	KIN	SAM	NODE	tTCL	SAM	NODE	tTCL	SAM	NODE	tTCL
0	0	0	47.22 ± 1.38^{bc}	$36.11 \pm .77^{bc}$	54.17 ± 2.40^{de}	2.86 ± 0.18^{g}	3.06 ± 0.14^{e}	3.95 ± 0.07^{e}	1.41 ± 0.02^{ef}	1.09 ± 0.09^{f}	1.54 ± 0.12^{f}
0.75	0	0	50 ± 2.40^{abc}	38.88 ± 3.67^{abc}	55.55 ± 1.38^{de}	3.63 ± 0.12^{ef}	3.96 ± 0.16^{cd}	4.01 ± 0.14^{e}	0.85 ± 0.07^{hi}	1.09 ± 0.09^{f}	1.13 ± 0.03^{g}
1	0	0	51.38 ± 1.38^{ab}	43.05 ± 1.38^{abc}	59.72 ± 2.77^{cd}	3.93 ± 0.08^{de}	4.76 ± 0.03^{ab}	4.95 ± 0.07^{d}	1.07 ± 0.04^{gh}	1.1 ± 0.1^{f}	1.4 ± 0.03^{fg}
1.5	0	0	52.77 ± 1.38^{ab}	47.22 ± 1.38^{abc}	68.05 ± 1.38^{bc}	4.26 ± 0.08^{bcd}	4.86 ± 0.06^{ab}	5.18 ± 0.05^{bcd}	1.31 ± 0.10^{fg}	1.09 ± 0.09^{f}	1.61 ± 0.08^{efg}
2	0	0	61.11 ± 3.67^{a}	48.61 ± 1.38^{ab}	79.17 ± 2.40^{ab}	4.56 ± 0.14^{abc}	4.9 ± 0.17^{ab}	5.57 ± 0.13^{bc}	1.38 ± 0.04^{efg}	1.29 ± 0.09^{ef}	1.58 ± 0.09^{fg}
0.75	0.5	0	$44.44 \pm .38^{bc}$	34.72 ± 3.67^{c}	45.83 ± 2.40^{cde}	2.75 ± 0.02^{g}	2.93 ± 0.08^{e}	3.9 ± 0.05^{e}	0.63 ± 0.08^{i}	1.19 ± 0.01^{ef}	1.18 ± 0.04^{g}
1	0.5	0	51.38 ± 1.38^{ab}	45.83 ± 2.40^{abc}	56.94 ± 3.67^{ef}	3.79 ± 0.05^{def}	4.1 ± 0.11^{cd}	4.68 ± 0.19^{d}	1.74 ± 0.08^{cd}	1.51 ± 0.08^{de}	1.92 ± 0.05^{def}
1.5	0.5	0	55.55 ± 3.67^{ab}	50 ± 2.40^{a}	88.88 ± 1.38^{a}	5.03 ± 0.17^{a}	4.9 ± 0.1^{ab}	7.01 ± 0.09^{a}	2.14 ± 0.07^{ab}	2.25 ± 0.06^{b}	3.01 ± 0.09^{b}
2	0.5	0	54.17 ± 2.40^{ab}	48.61 ± 1.38^{ab}	81.94 ± 2.77^{a}	4.83 ± 0.12^{ab}	5.06 ± 0.08^{a}	5.71 ± 0.10^{b}	2.40 ± 0.02^{a}	2.65 ± 0.04^{a}	3.72 ± 0.03^{a}
0.75	0	0.5	38.88 ± 3.67^{c}	34.72 ± 2.77^{c}	41.67 ± 2.40^{f}	3.29 ± 0.10^{fg}	3.72 ± 0.03^{d}	3.83 ± 0.12^{e}	0.61 ± 0.05^{i}	1.23 ± 0.03^{ef}	1.14 ± 0.06^{g}
1	0	0.5	47.22 ± 1.38^{bc}	40.27 ± 2.77^{abc}	52.78 ± 1.38^{def}	4.1 ± 0.11^{cde}	4.8 ± 0.05^{ab}	5.1 ± 0.09^{cd}	1.68 ± 0.01^{cde}	1.48 ± 0.05^{de}	1.96 ± 0.02^{de}
1.5	0	0.5	52.77 ± 1.38^{ab}	50 ± 2.40^{a}	56.94 ± 2.77^{cde}	4.09 ± 0.04^{cde}	4.38 ± 0.06^{bc}	5.58 ± 0.15^{bc}	1.97 ± 0.04^{bc}	2 ± 0.05^{bc}	2.14 ± 0.08^{cd}
2	0	0.5	54.16 ± 0^{ab}	48.61 ± 2.77^{ab}	56.94 ± 1.38^{cde}	4.60 ± 0.11^{abc}	4.66 ± 0.12^{ab}	5.1 ± 0.11^{cd}	1.61 ± 0.08^{def}	1.68 ± 0.05^{cd}	2.44 ± 0.03^{c}

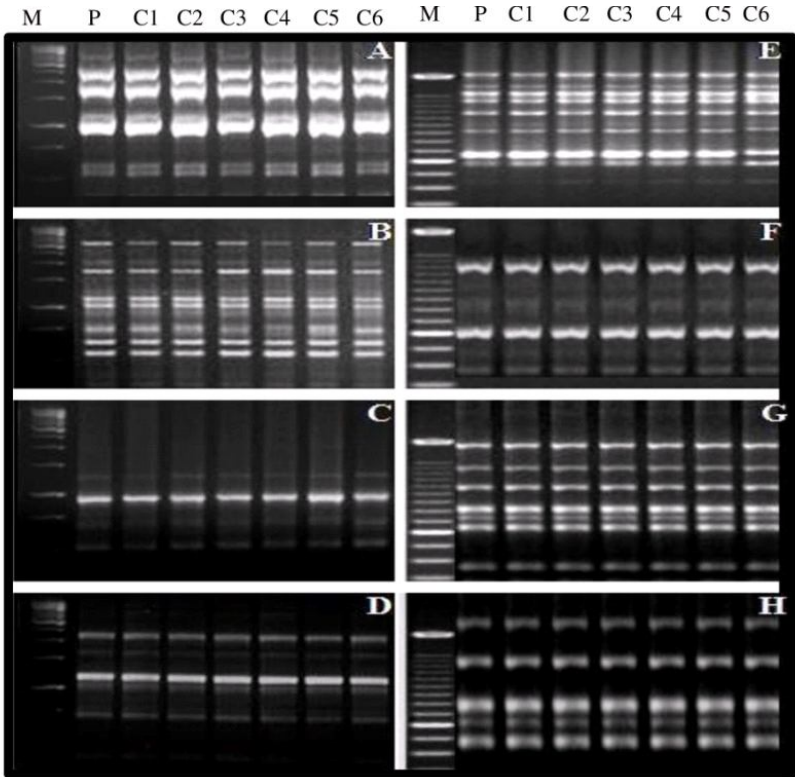

Fig. 3: RAPD (A, B, C, D) and ISSR (E, F, G, H) profiles of mother plant (P) and six randomly selected tTCL-derived plants (C1 to C6) of *Boerhaavia diffusa*. Lane 1 (M) is showing the marker.

Table 3: Effect of different concentrations of growth regulators on root induction.

	NAA	2,4 D	IBA	% root response	No. of root	root length
MS	0	0	0	55.55 ± 3.67^{cd}	4.33 ± 0.33^{ef}	3.25 ± 0.25^{b}
Half MS	0	0	0	76.38 ± 2.77^{ab}	13.33 ± 0.88^{a}	5.89 ± 0.13^{a}
Quarter MS	0	0	0	34.72 ± 1.38^{e}	3.33 ± 0.88^{f}	1.67 ± 0.06^{defg}
1/2 MS	0.5	0	0	63.88 ± 3.67^{abcd}	7.66 ± 0.88^{cde}	1.89 ± 0.15^{cdef}
1/2 MS	1	0	0	66.66 ± 2.40^{abc}	8.33 ± 0.66^{cd}	2.26 ± 0.04^{c}
1/2 MS	0	0.5	0	47.22 ± 3.67^{de}	4.66 ± 0.33^{def}	1.35 ± 0.05^{fg}
1/2 MS	0	1	0	48.61 ± 2.77^{de}	5.33 ± 0.33^{cdef}	1.66 ± 0.12^{defg}
1/2 MS	0	0	0.5	52.77 ± 2.77^{cd}	5.33 ± 0.33^{cdef}	1.96 ± 0.07^{cde}
1/2 MS	0	0	1	54.16 ± 4.16^{cd}	6.00 ± 0.57^{cdef}	2.10 ± 0.01^{cd}
1/2 MS	0.5	0.5	0	77.77 ± 3.67^{a}	12.33 ± 0.88^{ab}	3.62 ± 0.09^{b}
1/2 MS	0.5	0	0.5	56.94 ± 2.77^{cd}	6.66 ± 1.45^{cdef}	1.26 ± 0.04^{g}
1/2 MS	0	0.5	0.5	59.72 ± 5.00^{bcd}	8.66 ± 0.33^{bc}	1.52 ± 0.07^{efg}

method (Fig. 2). In order to optimize the appropriate storage parameters, these synthetic seeds were stored at three different temperatures (4, 15 and 25ºC) for different durations under continuous light. Random shoot induction after 4 weeks was observed in synthetic seeds stored at 15 and 25 ºC. Whereas no such observations were recorded for synthetic seeds stored at 4 ºC up to 10 weeks, suggesting 4ºC to be appropriate temperature for long-term (12 weeks) storage with few shoot emergence. When these synthetic seeds (stored at 4ºC) were inoculated onto the MS media, shoot were successfully emerged from both one week and four weeks old synthetic seeds. The viability of one and four weeks old synthetic seeds were tested by measuring three parameters viz. percentage of synthetic seed germination, number of shoots emerged and percent root formation on three different media combinations (full strength MS medium, half strength MS medium and full strength MS medium supplied with 1 mg L^{-1} BAP (Table 4). Highest germination rate was recorded for four weeks old synthetic seeds inoculated on to full strength MS medium supplied with 1 mg L^{-1} BAP.

Assessment of genetic stability of tTCL-derived acclimated plants

Two different molecular markers *i.e.*, RAPD and ISSR were used to assess genetic stability of tTCL-derived acclimated plants which have been shown in Fig 3. A total of 51 monomorphic bands were observed after RADP analysis while, ISSR marker produced a total of 60 monomorphic bands. None of the acclimatized plants showed

Table 4: Effect of different media strength on synthetic seeds germination

Storage duration (Week)	MS media	Percentage of syntheticseed germination (%)	Number of shoots (mean ± SE)	Root formation (%)
1	Full strength MS basal media	69.6 ± 1.85^{bc}	2.9 ± 0.18^{b}	91.0 ± 1.52^{b}
	Half strength MS basal media	47.0 ± 2.51^{d}	1.4 ± 0.20^{c}	87.6 ± 1.85^{b}
	Full strength MS basal media with 1 mg L^{-1} BAP	77.6 ± 1.20^{b}	3.1 ± 0.08^{b}	93.0 ± 1.00^{b}
4	Full strength MS basal media	77.3 ± 1.85^{b}	2.6 ± 0.12^{b}	98.6 ± 0.66^{a}
	Half strength MS basal media	64.6 ± 1.45^{c}	2.7 ± 0.08^{b}	93.0 ± 0.57^{b}
	Full strength MS basal media with 1 mg L^{-1} BAP	92.3 ± 1.20^{a}	3.8 ± 0.12^{a}	99.6 ± 0.33^{a}s

polymorphic bands. An ISSR marker displayed on an average 16 monomorphic bands while RAPD marker showed an average of 12.7 monomorphic bands. Analysis was carried out using one mother clone and 6 clones (C1 to C6) generated through tTCL culture and acclimatized to ex-vitro conditions. Based on the results obtained after RAPD and ISSR marker-assisted genetic stability analysis, no polymorphism between mother plants and acclimated clones were found.

Discussion

Direct shoot regeneration using tTCL explants provides an efficient method for rapid mass propagation of *B. diffusa*. Only conventional tissue culture methods have been implicated for *in-vitro* propagation of *B. diffusa* till date (Nagarajan *et al.* 2005; Sudarshana *et al.* 2008; Roy 2008; Saini *et al.* 2011; Patil and Bhalsing 2015). Ragi and Shibu (2014) had reported *in-vitro* clonal propagation of *B. diffusa* through leaf explants. In the present study, first we compared the shooting response among three different explants: shoot tips, nodal segments and tTCL segment grown in different media combination, by analyzing three parameters as if shoot proliferation percentage, number of shoots emerged from each responsive explants and average shoot length. The tTCL segments were found to be most responsive in terms of all the three parameters studied in all media combination used. Among different media combinations, MS medium supplied with BAP (1.5 mg/l) and NAA (0.5 mg/l) was found to be most suitable for tTCL segments and resulted in an average of 7.5 shoots per responsive tTCL explants with an average shoot length of 1.5 cm (Table 2). Such higher frequencies have also been reported in a number of other species propagated through tTCL explants (Van Le *et al.* 1998, 2002).

Plant growth regulators like auxins and cytokinins are essentially required for rapid *in-vitro* morphogenesis such as shoot induction and development. Auxin-cytokinin action and interaction are the key determinants of *in-vitro* organogenesis. In the present study, low NAA/BAP ratio promoted shoot induction which is in agreement with the pioneer reports by Skoog and Miller (1957) suggesting low auxin/cytokinin ratio as a pre-requisite for shoot induction. The 6-BAP has previously been shown to be effective in regeneration of a number of plant species (Nayak *et al.* 1997, 2002) including *B. diffusa* (Ragi and Shibu 2014). However, a combination of 6-BAP with an auxin such as NAA was found to be more effective in regeneration of *Dendrobium firmbriatum* as compared to the regeneration frequency of 6-BAP alone (Roy and Banerjee 2003). Similar effects of high BAP and low NAA combination on maximum shoot regeneration was also observed in *Simmondsia chinensis* (Bala *et al.* 2015), *Cyamopsis tetragonaloba* (Prem *et al.* 2005) and *Echinacea purpurea* (Koroch *et al.* 2002), which gives strength to the present results showing maximum shoot regeneration on MS medium supplemented with higher BAP (1.5 mg/l) and relatively lower NAA (0.5 mg/l). After successful regeneration of *B. diffusa* shoots from tTCL segments, each tTCL-derived *in-vitro* shoots (5 – 6 cm long) were excised and transferred onto five different kinds of rooting medium *i.e.*, basal MS, MS with NAA, MS with 2,4-D, MS with NAA & 2,4-D both and modified MS (half strength MS medium) for induction of roots. Shoots inoculated onto all five different kinds of rooting media produced *in-vitro* roots with varying degree of time needed for root induction, number of roots and root length. The data shown in table 3, clearly indicated the best rooting response with lowest root induction time and highest number of roots and maximum root length in shoots grown in half-strength MS medium. When the *in-vitro* regenerated plants need to be transferred to the natural conditions, the preferred rooting medium is the half-strength MS (Ncube *et al.* 2015). The probable reason behind it is the relatively lower osmotic strength of half-strength MS as compared to full-strength MS medium, making the osmotic strength of previous one more close to the natural soils. If the *in-vitro* generated plants will be transferred from high osmotic strength environment (*i.e.*, full-strength MS) to the low osmotic-strength environment (*i.e.*, natural soils), the survival rate becomes very low and plants will collapse. Another probable reason for best rooting response in half-strength MS may be some sort of nutrient deficiency in half-strength as compared to full-strength MS, that in-turn stimulate rooting. The tTCL-derived *in-vitro* rooted plantlets (after approximately 25 days of subculture) were successfully acclimatized to ex-vitro conditions and established in natural conditions with 98 % of survivability.

The prime purpose of micropropagation is to produce genetically alike plants and therefore it becomes necessary to check the maintenance of complete genetic fidelity, failing which micropropagation cannot be considered successful. There are quite a few reports of induced somaclonal variations among micropropagated plants. Raina and Rani (2000) have stated various molecular processes governing genetic instability during *in-vitro* regeneration. We have previously reported that the plant growth regulators used in high concentration may induce genetic variability in *Artemisia annua* (Rai 2014). In the present study, RAPD and ISSR markers provided a very useful tool to study genetic stability among *in-vitro*

regenerated plants through tTCL method. RAPD marker has variously been applied for genetic fidelity test in micropropagated *B. diffusa* (Shukla *et al*. 2003; Patil and Bhalsing 2015). Here, for the first time we showed the usefulness of ISSR markers for genetic stability assessment in *in-vitro* propagated *B. diffusa*. The cumulative results obtained by genetic stability analysis using both RAPD and ISSR marker suggest that no genetic mutations have taken place during *in-vitro* regeneration (both shooting and rooting) and acclimatization process in tTCL-derived *B. diffusa*. These results strengthen the suitability of tTCL-mediated rapid regeneration of *B. diffusa* plantlets for their probable use in various genetic engineering experiments.

Conclusion

Plant regeneration protocol through tTCL method has been established for the first time in *B. diffusa*. The present study describes an efficient and reliable method for shoot regeneration from tTCL segments. A combination of BAP (1.5 mg/l) and NAA (0.5 mg/L) was most effective in shoot regeneration from tTCL-derived cultures. Further, for root induction, half-strength MS media was found to be most appropriate and the rooted plants were successfully transferred to field conditions. Nodal segments from tTCL-derived plantlets were used to produce synthetic seeds for conservation and regrowth and 4 ºC temperature was found to be most appropriate for storage of tTCL-derived synthetic seeds up to 12 weeks. All the tTCL-derived plants were morphologically and genetically identical with the mother plant as tested through RAPD and ISSR marker assisted analysis. Conclusively, tTCL-method described here provides a novel approach for high frequency shoot germination in *B. diffusa* which can be applied commercially as well as as a basis for genetic engineering experiments.

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Conflict of Interest

The authors declare no conflict of Interest.

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