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IN VITRO REGENERATION OF PLANTLETS FROM NODAL EXPLANTS OF *NYCTANTHES ARBOR-TRISTIS* LINN. AND EVALUATION OF GENETIC FIDELITY THROUGH RAPD ANALYSIS

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ABSTRACT
Plantlet regeneration in *Nyctanthes arbor-tristis* (Family: Oleaceae), a medicinal shrub, from nodal and shoot tip explants on basal MS medium was studied. Maximum number of adventitious shoots were differentiated from nodal explants on MS medium supplemented with 8μM BA (6.2 $\pm$ 0.83). The MS medium fortified with 8μM BA and 2μM NAA induced callus mediated organogenesis, and highest multiple shoot proliferation (10.4 $\pm$ 0.89) from the nodal explants. MS medium with 6μM BA and 3μM NAA showed maximum (5.54 $\pm$ 0.32cm) elongation of shoots. The elongated shoots, incubated overnight on one fourth MS medium with IBA (2.0μM), were successfully rooted on plant growth regulator free one fourth strength liquid MS medium. Regenerated plantlets were successfully established in soil where 80 $\pm$ 2% of them survived into morphologically normal and fertile plants. Nine responded RAPD primers were used to assess genetic fidelity of regenerated plantlets along with the donor plant. These nine primers generated 24 amplified products (275 to 1150bp), and the homogenous amplification pattern confirmed the genetic uniformity of the *in vitro* regenerated plantlets and substantiated the efficacy of this protocol for *in vitro* propagation of *N. arbor-tristis,* to meet the demand of herbal industry.

INTRODUCTION

Nyctanthes arbor-tristis Linn. (Family- Oleaceae), an important medicinal species, is commonly known as 'Parijatha' or 'Night Jasmine' for its fragrant flower. The plant is described in literature as Harsingar, Shephalika, Paghala and Gangasiuli. *N. arbor-tristis*, a large shrub by habit, is a native of tropical and subtropical countries including India. Its occurrence has been noted from the sub-himalayan region to southward of Godavari as wild. The plant parts including leaves, inflorescences, seeds and barks have been extensively used in traditional system of medicine for various ailments like asthma, diuretics, cancer, rheumatism, sciatica, gout, malaria, liver dysfunction, skin diseases and worm infection of intestine (Kritikar and Basu, 1993; Khatun *et al.*, 2003; Tuntiwachwuttiku *et al.*, 2003; Sasmal *et al.*, 2007; Vats *et al.*, 2009; Sandhar *et al.*, 2011; Nirmal *et al.*, 2012). Besides the plant is also known to possess anti-arthritic (Rathore *et al.*, 2007), anti-malarial (Rout *et al.*, 2007), anti-spasmodic (Das *et al.*, 2010), anti-helmintic (Das *et al.*, 2010), hepatoprotective (Hukkeri *et al.*, 2006), anti-inflammatory (Singh *et al.*, 2008), immunostimulant (Kannan *et al.*, 2007), anti-microbial (Mahida *et al.*, 2007; Vats *et al.*, 2009), anti-viral (Gupta *et al.*, 2005), anti-leishmanial (Tandon *et al.*, 1991; Khatune *et al.*, 2001; Poddar *et al.*, 2008) and decongestant properties (Sandhar *et al.*, 2011). The plant parts has been used in several ayurvedic preparations- 'Cholesterol Protection' by Maharshi Ayurveda Products International (www. mapi. com), 'Bio-nyctanthes' skin cream by Botique (www.mall.coimbatore.com), 'Geriforte' by ADI (www.ayurvedicdrugindex.com), 'Kamalahar' by Khatore Pharmaceuticals (www.khatore pharma. com) and 'AYU coldavin granules' by Sharangdhar Pharmaceuticals Pvt. Ltd. (www.spplpune.com). The present day requirement of *N. arbor-tristis* for the herbal industry is met from the natural populations, causing unrestricted large-scale exploitation from the natural habitat. Constraints like poor seed germinating ability (Rout *et al.*, 2007) poses problem for its seed based propagation and hence its garden cultivation and the species is under the threat perception for most probable extinction in future. Hence, an immediate need for accessing the natural population leading to rapid multiplication of this drug yielding plant has become imperative. Tissue culture technology can provide an alternative way to substantiate the rapid multiplication of elite clone and the germplasm conservation (Vasil, 1988). There are some reports of *in vitro* propagation *N. arbor-tristis* through direct shoot regeneration from different explants including node (Siddique *et al.*, 2006) and shoot meristem (Rout *et al.*, 2007, 2008). However, in these reports *in vitro* multiplication rate was low and the genetic fidelity of regenerated plantlets was not assessed either at cell or molecular level. The improvement of micro propagation efficiency and genetic uniformity of the plantlet is desirable to meet the need of elite clone of *N. arbor-tristis*. However genetic variability often arises as a manifestation of epigenetic influence or changes in the genome of differentiating somatic cells induced by tissue culture conditions (Larkin and Scowcroft, 1981; Muller *et al.*, 1990). Therefore, genetic stability of *in-*

vitro regenerated plantlets has always been assessed during standardization of an efficient *in-vitro* propagation protocol. Molecular marker based on either gene or gene products are quite stable, uniform, quick, reliable and reproducible. Random amplified polymorphic DNA (RAPD) is a kind of molecular marker (Williams *et al.*, 1990) based on DNA sequence polymorphism and it has been successfully employed in assessing the genetic uniformity of *in-vitro* regenerated plantlets in several species (Rout *et al.*, 2001; Tang, 2001; Feuser *et al.*, 2003; Mishra *et al.*, 2006; Santosh *et al.*, 2008; Maharana *et al.*, 2012). Therefore, the present study has been initiated to standardize a simple protocol for *in-vitro* propagation of this important medicinal plant *N. arbor-tristis* from the explants *in vivo*, and to access the genetic fidelity of *in vitro* regenerated plantlets using RAPD markers.

MATERIALS AND METHODS

Plant materials and explant preparation

Fresh young nodes, shoot tips and apical leaves were excised from 2 year-old *N. arbor-tristis* growing in the experimental garden of MITS, Rayagada, Odisha (Altitude-206m above sea level, 19º11′NL and 83º25′EL). The explants were washed thoroughly under running tap water for 3 min, immersed in 2% Teepol (Merck, India) for 2min and washed thoroughly with double distilled water (ddH_2O). Subsequently, the explants including nodes shoot tips and leaves were surface sterilized using 0.05% $HgCl_2$ for three, one and one minutes respectively. Surface sterilized explants were rinsed three to four times (3 min each) using sterile ddH_2O to remove the traces of $HgCl_2$.

Culture medium and conditions

The culture medium was that of MS (Murashige and Skoog, 1962) with 555μM Meso-inositol (Hi-media, India) and 3% (w/v) Sucrose (Hi-media, India). The medium was augmented with different concentrations and combinations of N^6-Benzyladenine (BA), Kinetin, α-Napthalene acetic acid (NAA) and Indole butyric acid (IBA) (Hi-media, India). The pH of the medium was adjusted to 5.7 prior to gelling it with 0.8% (w/v) Agar-agar (bacteriological grade, Hi-media, India). All the media contained in culture vessels (25×150mm culture tube and 150mL culture jar containing 12mL and 30mL media respectively) were autoclaved at 104 kpa and 121ºC for 30 min. One explant (~25mm^2) was cultured in each tube/jar and cultures were incubated at 25±1ºC, 60-70% relative humidity and 16h photoperiod of 35μ $Em^{-2}s^{-1}$ irradiance level provided by cool white fluorescent tubes (CG, India).

Effect of plant growth regulators for multiple shoot induction and shoot elongation

The experiment was designed to study the effect of different concentrations and combinations of cytokinins (BA and Kinetin) and auxins (IBA and NAA) on shoot bud regeneration, multiple shoot induction and elongation. After 10 weeks of culture, the number of shoots or shoot initials per explant was counted.

Rooting of the elongated shoots and acclimatization

Elongated shoots with 5-6 leaves (>3 cm long), excised *in vitro*, were incubated overnight in one fourth MS medium fortified with NAA (0.4, 1, 2, 3μM) and IBA (0.4, 1, 2, 3μM) individually and in combination, and then implanted on half strength semi solid and liquid MS medium, respectively. Each treatment was replicated five times using single plantlet for each treatment. Plantlets with well-developed roots were transferred to plastic cups containing autoclaved sand and soil (1:1) and maintained in same environmental condition for one week. The plantlets were watered regularly with $1/10^{th}$ strength MS liquid medium. Subsequently, plantlets were transferred to earthen pots containing coarse sand, compost and garden soil (1:1:2) and kept in shade for two weeks before transferring to the experimental garden.

Observation and statistical analysis

Visual observations were made every week and data on explant response, number of shoots per explant and number of roots per shoot was recorded at the end of 10^{th} week, 8^{th} week and 2^{nd} week, respectively. Each treatment consists of five replicated culture vessels and was repeated thrice, and the standard deviation was calculated. Data on multiple shoot induction, shoot elongation and rooting were statistically analyzed using a completely randomized block design and means were evaluated at $p = 0.05$ level of significance using Duncan's multiple range test (Harter, 1962). For this SPSS V 8.0.1 software was used with parameters - one way ANOVA and homogeneity of variance.

RAPD Marker analysis

Genomic DNA was isolated from tender leaves (~1.2g) of 10 numbers of plantlets regenerated *in vitro* from MS medium with 8μM BA and 2μM NAA and the donor plant by using SDS method (Dellaporta *et al.*, 1983) with few modifications (Mishra *et al.*, 2006). DNA was dissolved in 10m*M* Tris and 1m*M* EDTA ($T_{10}E_1$) buffer. DNA concentration and purity was measured by using UV–Vis spectrophotometer (UV 1601, Shimadzu, Japan) with $T_{10}E_1$ buffer (pH 8.0) as blank. For further confirmation the quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gel along with diluted uncut lambda DNA as standard. DNA from 10 regenerated plantlets and donor plant were equilibrated to concentration of 10ng/μL using $T_{10}E_1$ buffer. For RAPD analysis PCR amplification of 20ng genomic DNA was carried out using thirty standard decamer oligonucleotide primers (OPA01-20 and OPB01-10) (Operon Tech., Alameda, CA, USA) as described by William *et al.* (1990). Each amplification reaction mix of 25μL contained the 20ng template DNA, 2.5μL of 10X assay buffer (100m*M* Tris. Cl, pH 8.3; 0.5*M* KCl; 0.1% Gelatin), 2m*M* $MgCl_2$, 200μM each of the dNTPs, 20ng primer, 1U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was performed in a thermal cycler (Veriti-96, Applied Biosystem, USA) programmed for initial denaturation of 5min at 94ºC; 45 cycles of 2min denaturation at 94ºC, 1min annealing at 37ºC and 2min elongation at 72ºC and final elongation step at 72ºC for 5min. The PCR products were separated on 1.4% agarose gel containing Ethidium bromide solution (@ 0.5μg/mL of gel solutions) in submarine electrophoresis apparatus (Tarson, India) using TAE (40m*M* Tris acetate; 2m*M* EDTA) buffer at constant 50V for about 3h. A gel loading buffer (20% Sucrose; 0.1 *M* EDTA, 1% SDS; 0.25% Bromophenol blue; 0.25% Xylene cyanol) was used as tracking dye. Amplified DNA fragments were visualized

and documented using Geldoc-XR system (Bio-Rad laboratories Inc.,USA). The size of the amplicons was determined using 250bp DNA ladder, (Bangalore Genei Pvt. Ltd., Bangalore, India) as standard and TOTAL LAB SOLUTIONS- V 2003.02 software. To test the reproducibility the reactions were repeated twice.

RESULTS

Effects of Plant growth regulators on shoot regeneration

The response of three different explants to various concentrations of BA and kinetin was studied. The frequency of shoot formation was influenced by the type of the explant, choice of the cytokinin and its dosage. Direct shoot bud differentiation was observed as protrusions at the leaf disc of nodal segments and shoot tip after two weeks of culture initiation (Fig.1a). By the end of 5th week, these protruded structures developed into shoot initials without intervening callus phase and most of them were flanked by green leaves (Fig. 1b). By the end of 10th week well developed multiple shoots were initiated from both the explants. But the leaf explants responded only to non-organogenic callus formation (Fig. 1c). Both the shoot tip and node explants cultured on MS medium without any cytokinin also responded very slowly giving rise to one plant each, while the leaf explant was not responded at all. The highest frequency of shoot formation (6.2 ± 0.83) was observed from node explants on MS with 8μM BA (Table 1, Fig. 1d). Similarly, three to four shoots (mean value- 2.8 ± 0.45) were developed from shoot tip on MS medium with 8μM BA and 4μM kinetin (Fig. 1e). Of the two cytokinins used, BA was more effective than kinetin in inducing

Table 1: Effect of different cytokinins (BA and Kn) on development of multiple shoots of *N. arbor-tristis* from three different explants (Node, shoot tip and leaf) after 10 weeks*

Plant growth regulator conc. (μm)		Explant response in term of number of shoots and shoot initials per explant (Mean ± SD)		
BA	Kn	Node	Shoot tip	Leaf
Control		1.2 ± 0.45^{ab}	0.0 ± 0.0^{a}	
2.0	-	1.2 ± 0.45^{ab}	0.0 ± 0.0^{a}	
4.0	-	1.8 ± 0.45^{abcd}	0.4 ± 0.55^{ab}	
6.0	-	3.2 ± 0.83^{ghi}	1.2 ± 0.45^{cde}	
8.0	-	6.2 ± 0.83^{k}	1.2 ± 0.45^{cde}	
10.0	-	3.6 ± 0.55^{hi}	1.8 ± 0.45^{ef}	
-	2.0	1.0 ± 0.0^{a}	0.4 ± 0.55^{ab}	Only Callus
-	4.0	1.6 ± 0.55^{abc}	0.4 ± 0.55^{ab}	developed
-	6.0	1.6 ± 0.55^{abc}	0.6 ± 0.55^{abc}	
-	8.0	2.4 ± 055^{cdefg}	1.4 ± 0.55^{def}	
-	10.0	2.8 ± 0.45^{efgh}	1.8 ± 0.45^{ef}	
2.0	2.0	1.8 ± 0.45^{abcd}	1.0 ± 0.0^{bcd}	
2.0	4.0	2.2 ± 0.83^{cdef}	1.4 ± 0.55^{def}	
2.0	6.0	2.4 ± 0.55^{cdefg}	1.6 ± 0.55^{def}	
4.0	2.0	1.6 ± 0.55^{abc}	1.4 ± 0.55^{def}	
4.0	4.0	2.8 ± 0.45^{efgh}	1.6 ± 0.55^{def}	
4.0	6.0	2.6 ± 0.55^{defg}	1.6 ± 0.55^{def}	
6.0	2.0	4.0 ± 0.71^{i}	1.8 ± 0.45^{ef}	
6.0	4.0	5.0 ± 0.71^{j}	2.0 ± 0.71^{fg}	
6.0	6.0	2.0 ± 0.71^{bcde}	0.6 ± 0.55^{abc}	
8.0	2.0	3.6 ± 1.14^{hi}	1.4 ± 0.55^{def}	
8.0	4.0	3.0 ± 0.71^{fgh}	2.8 ± 0.45^{h}	
8.0	6.0	2.8 ± 0.45^{efgh}	2.6 ± 0.55^{gh}	

*Means within a column having the same letter are not statistically significant ($p = 0.05$) according to Duncan's multiple range test (SPSS V 8.0.1)

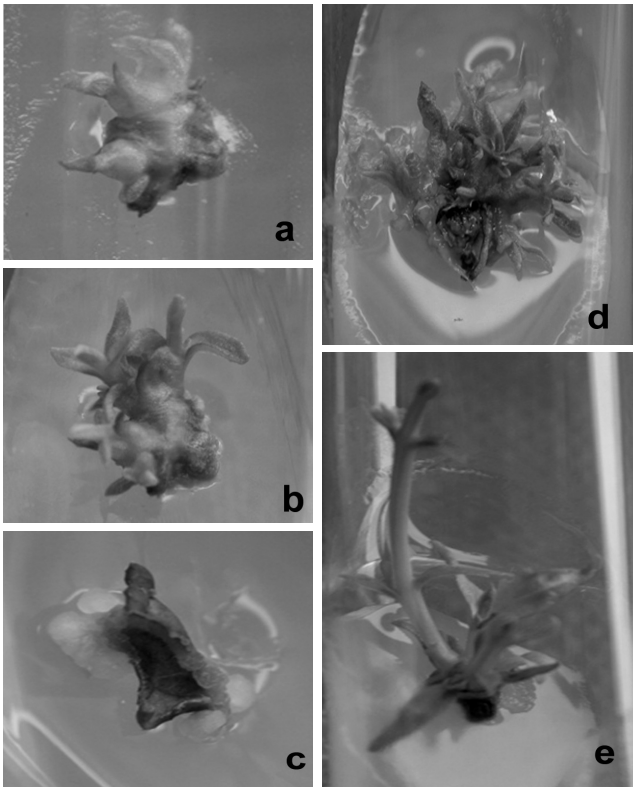

Figure 1(a-e): Plant regeneration in *N. arbor-tristis* through direct organogenesis. Protuberances from the node explant (a), Multiple shoot initials after 5th week on MS + 8.0μM BA (b), response of leaf explant showing callus formation (c), well developed multiple shoots after 10th week from node explants on MS + 8.0μM BA (d) and multiple shoot regeneration from shoot tip after 10th week on MS + 8.0μM BA + 4.0μM Kn (e)

multiple shoots from node explants, but the shoot length varied randomly irrespective of explant type. The regeneration frequency increased with increment in the concentration of cytokinins and 8μM BA was found to be optimal for maximum frequency of shoot bud formation from node explant. With BA concentrations above 8μM, the frequency of shoot bud regeneration decreased drastically (Table 1). To assess the combined effect of cytokinin and auxins on differentiation and organogenesis, the MS medium was supplemented with various combinations of BA, NAA and IBA and its effect on shoot regeneration frequency and elongation of shoots was noted. The highest frequency of shoot regeneration was obtained from node explants in comparison to shoot tip (Table 2). The cultured explants gave rise to greenish compact callus, covering the entire surface of the explant, by the end of 3rd week (Fig. 2a, b). By the end of 10th week, maximum number of healthy shoots including shoot initials (10.4 ± 0.89) were obtained in MS medium with 8μM BA and 2μM NAA from node explants (Fig. 2c). Similar to the response of node explant, maximum 3 to 4 number shoots (2.4 ± 0.45) regenerated in MS medium with 10 μM BA and 2μM NAA from the shoot tip.

Elongation of shoots

The shoots, elongated on various concentrations of BA and/or kinetin and the time required for elongation was more in comparison with development of shoot initials. However, the

shoots showed differential rate of elongation with respect to its origin from either node or shoot tip. The shoots originated from the node explant showed highest elongation (5.54 ± 0.32cm) in MS medium fortified with 6 μM BA and 3μM NAA (Fig. 2d). However, the shoots originated from the shoot tip showed maximum elongation (4.76 ± 0.17cm) in MS medium fortified with 8μM BA and 3μM NAA.

Rooting of shoots

Elongated and well developed shoots (>3 cm long) were excised from the shoot clumps, implanted in half strength MS medium with different sucrose concentration, and combination of IBA and NAA did not induce rhizogenesis. In the second phase, the shoots were pretreated in one fourth MS medium supplemented with various concentration and combination of NAA and IBA (Table 3) for overnight in floating condition and then transferred to half-strength semi-solid MS medium free of plant growth regulator which responded for rooting. But the roots were developed from the stem region (1 to 2cm above the cut end of the shoot and the penetrated into the medium (Fig. 2e). If the separated shoots were placed in half strength MS medium in floating condition, the roots were developed from the cut end (Fig. 2f) and the growth was faster. Among the pretreatment combinations, seven different treatments responded for rooting whereas in others only callus formed. The frequency of rooting varied from 66.66% to 93.24 % (Table 3) with auxin type, auxin concentration and auxin combination. Maximum number of roots (6.8 ± 0.55) and highest rooting frequency was observed in case of overnight incubation in half strength MS medium fortified with 2μM IBA (Fig.2f).

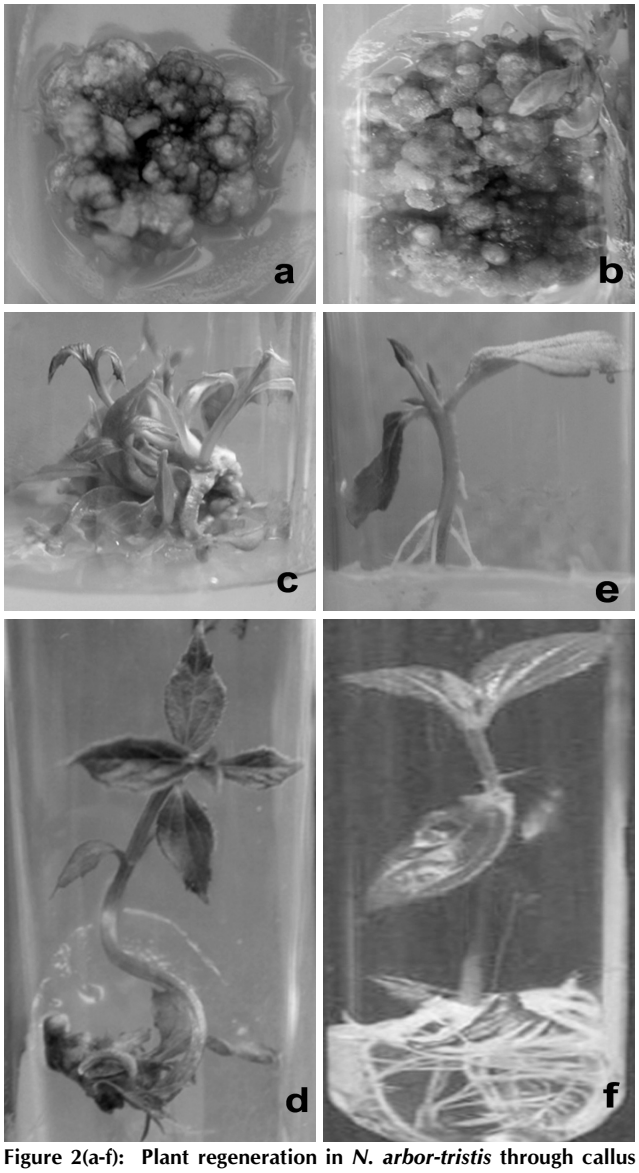

Figure 2(a-f): Plant regeneration in *N. arbor-tristis* through callus mediated organogenesis. Hard, compact greenish calli developed from node explants after 3rd week (a), Greenish, hard, compact calli developed from shoot tip after 3rd week on MS + 8μM BA + 2μM NAA (b), Multiple shoot regeneration from the nodal callus after 10th week (c), Elongation of shoots originated from nodal explants on MS + 6μM BA + 3μM NAA (d), Shoot showing rooting from the stem region on PGR free half strength semi solid MS medium(e), Shoot showing profused rooting on PGR free half strength liquid MS medium (f)

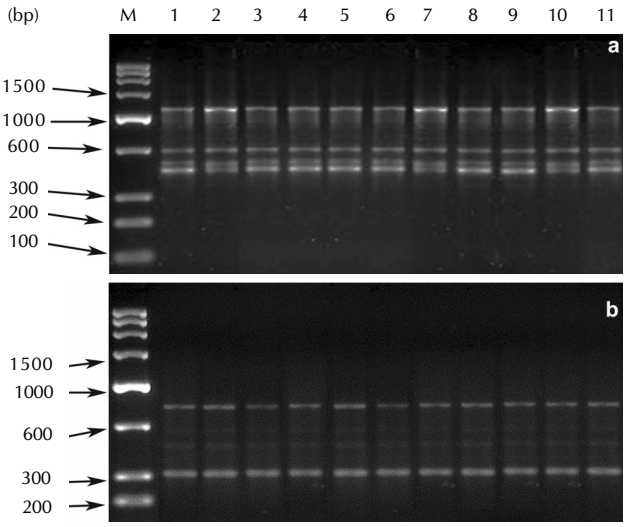

Figure 3(a-e): Electrophoretic pattern of PCR amplified DNA fragments of the donor plant (1) and ten *in vitro* regenerants (2 to 11) using primer OPA 02 (a) and OPA 4 (b). The lane 'M' represents molecular weight marker containing 250 bp ladder

Acclimatization and field transfer

After four weeks, the rooted plantlets were transferred into plastic pots containing autoclaved sand and soil mixture (1:1) and were maintained in the culture room for two weeks, transferred to shade and finally to field conditions. The survival rate was 80.0 ± 2.0%. The regenerated plantlets did not show any variation in morphology or growth characteristics when compared with the respective donor plant.

RAPD analysis to test genetic fidelity

The genetic fidelity of *in vitro* regenerated plantlets was analysed using RAPD marker assay. Among the RAPD primers (OPA 01-20 and OPB 01-10 series) used, nine primers responded for amplification (Table 4) and produced 24 scoreable RAPD bands with size ranging from 275bp to 1150bp. The analysis revealed quite homogenous banding pattern (Fig. 3a-b). The bands were uniformly present in all 11

Table 2: Effect of different plant growth regulators in combination on multiple shoot bud regeneration and elongation shoots derived from different explants of *N. arbor-tristis* after 8th weeks*

Plant growth regulator conc. (μm)			No. of shoots and shoot initials per explant (Mean ± SD)		Length of shoots originated from node and shoot tip (Mean ± SD)	
BA	NAA	IBA	Node	Shoot tip	Node	Shoot tip
Control			1.4±0.55 ab	0.8±0.45 bcd	2.98±0.23 ef	2.4±0.23 bc
4.0	1.0	-	3.6±0.89 defg	1.0±0.0 bcde	3.3±0.36 hi	2.9±0.2 fgh
6.0	1.0	-	4.4±0.55ghi	1.2±0.45cdef	3.4±0.16 i	3.26±0.17 jkl
8.0	1.0	-	5.2±0.45ij	1.4±0.54def	4.16±0.15 l	3.2±0.14 ijkl
10.0	1.0	-	6.8±0.84k	1.6±0.54 ef	3.82±0.18 jk	3.1±0.1 hijk
4.0	2.0	-	4.6±0.55hij	0.8±0.45 bcd	3.28±0.26 ghi	2.84±0.19 efg
6.0	2.0	-	4.6±0.89hij	0.8±0.45 bcd	3.94±0.17 kl	3.4±0.32 l
8.0	2.0	-	10.4±0.89l	1.8±0.45 f	4.4±0.29 m	3.36±0.32 kl
10.0	2.0	-	7.2±0.84 k	2.4±0.54 g	4.12±0.11l	3.26±0.09 jkl
4.0	3.0	-	2.4±0.55 c	0.6±0.54 abc	3.32±0.24 hi	3.04±0.15 ghij
6.0	3.0	-	3.8±0.84efgh	1.0±0.0 bcde	5.54±0.32 0	3.68±0.23 m
8.0	3.0	-	3.2±0.45de	1.4±0.54 def	4.9±0.1n	4.76±0.17 n
10.0	3.0	-	5.4±0.55j	1.8±0.45 f	3.8±0.28 jk	3.64±0.17 m
4.0	4.0	-	2.8±0.84 cd	0.4±0.54 ab	3.4±0.14 i	2.96±0.11fghi
6.0	4.0	-	3.4±0.55de	0.4±0.54 ab	3.04±0.09 fg	2.72±0.19 def
8.0	4.0	-	4.2±0.45fgh	1.4±0.54 def	3.08±0.08 fgh	3.06±0.17ghij
10.0	4.0	-	7.2±0.89 k	1.4±0.54 def	3.34±0.24 i	3.06±0.08ghij
4.0	-	1.0	1.0±0.0 a	0.0±0.0 a	2.46±0.21 a	-
6.0	-	1.0	1.0±0.0 a	0.0±0.0 a	2.44±0.11 a	-
8.0	-	1.0	2.0±0.7 bc	1.0±0.0 bcde	3.36±0.15 i	3.18±0.15ijkl
10.0	-	1.0	3.8±0.45 efgh	1.4±0.54 def	3.66±0.18 j	3.24±0.17ijkl
4.0	-	2.0	1.0±0.0 a	0.0±0.0 a	2.58±0.09 ab	-
6.0	-	2.0	1.0±0.0 a	0.4±0.54 ab	2.46±0.21 a	2.56±0.17 cd
8.0	-	2.0	4.2±0.84 fgh	1.0±0.0 bcde	2.86±0.21 def	2.38±0.18 bc
10.0	-	2.0	5.2±0.84 ij	1.0±0.0 bcde	2.76±0.11 cde	2.58±0.13 cde
4.0	-	4.0	2.0±0.0 bc	0.0±0.0 a	2.58±0.09 ab	-
6.0	-	4.0	2.0±0.0 bc	0.0±0.0 a	2.46±0.21 a	-
8.0	-	4.0	2.0±0.0 bc	1.0±0.0 bcde	2.72±0.11 bcd	2.06±0.17 a
10.0	-	4.0	2.0±0.0 bc	1.0±0.0 bcde	2.44±0.11 a	1.98±0.11 a

*Means within a column having the same letter are not statistically significant (p = 0.05) according to Duncan's multiple range test (SPSS V 8.0.1)

Table 3: Influence of overnight incubation of shoots in one fourth strength MS medium with two auxins on rooting of *in-vitro* derived shoots of *N. arbortristis* after two weeks*

Pretreatment with Plant growth regulator conc. (μm)		Rooting Frequency (%age)	No. of roots developed per shoots regenerated from nodal explant (Mean ± SD)
IBA	NAA		
Control		73.26	1.8±0.45 cd
0.4	-	73.26	1.0±0.45 b
1.0	-	79.92	1.4±0.55 bc
2.0	-	93.24	6.8±0.55 f
3.0	-	86.58	3.0±0.71 e
-	0.4	-	Callus; 0.0±0.0 a
-	1.0	-	Callus; 0.0±0.0 a
-	2.0	-	Callus; 0.0±0.0 a
-	3.0	-	Callus; 0.0±0.0 a
0.4	0.4	66.66	2.4±0.55de
0.4	1.0	73.26	2.4±0.45 de
0.4	2.0	-	Callus; 0.0±0.0 a
1.0	0.4	-	Callus; 0.0±0.0 a
1.0	1.0	-	Callus; 0.0±0.0 a
1.0	2.0	-	Callus; 0.0±0.0 a
2.0	0.4	-	Callus; 0.0±0.0 a
2.0	1.0	-	Callus; 0.0±0.0 a
2.0	2.0	-	Callus; 0.0±0.0 a

*Means within a column having the same letter are not statistically significant (p = 0.05) according to Duncan's Multiple range test (SPSS V 8.0.1)

samples including the donor and ten *in vitro* regenerants. Representative electrophoretic pattern of PCR amplified DNA

Table 4: Details of RAPD analysis of *in vitro* regenerated plant lets using decamer primers responded for amplification

Primer	Sequence (5′-3′)	No. of bands amplified	Range(in bp)
OPA-02	5′-TGCCGAGCTG-3′	4	485-1150
OPA-03	5′-AGTCAGCCAC-3′	4	408-615
OPA-04	5′-AATCGGGCTG-3′	4	325-810
OPA-05	5′-AGGGGTCTTG-3′	1	570
OPA-07	5′-GGTCCCTGAC-3′	2	348-460
OPA-08	5′-GTGACGTAGG-3′	1	575
OPA-09	5′-GGGTAACGCC-3′	3	275-880
OPA-13	5′-CAGCACGCAC-3′	3	500-970
OPB-01	5′-GTTTCGCTCC-3′	2	430-600

fragments of the donor plant and *in vitro* regenerated plantlets using primers OPA-02 and OPA-04, has been illustrated in Fig. 3.

DISCUSSION

In vitro regeneration is a complex process and it depends upon the application of exogenous plant growth regulators in the media to make the explant organogenic competent for differentiation and this varies from species to species. In the present study, complete plantlets with well developed shoots and roots were produced from nodes of *N. arbor-tristis*, through direct adventitious shoot bud regeneration as well as through callus mediated organogenesis. Results obtained in the present study showed that the highest numbers of

adventitious shoot (6.2 ± 0.83) were developed directly from the nodal explants cultured on MS medium fortified with 8 μM BA. Increasing or decreasing this concentration reduced the number of shoots. Hence, during present study BA emerged as the preferred cytokinin indicating cytokinin specificity for multiple shoot induction in *N. arbortristis*, and thi is in confirmation with the of Maharana *et al.* (2012) for several other species. Relative thermostability and slow degradability of BA in the culture during *in vitro* regeneration (Mishra *et al.*, 2006) can be the explanation for such observation. This might be due to the thermo stability and slow degradation of BA during culture. Siddique *et al.* (2006) reported that MS medium fortified with 1μM Thidiazuron (TDZ) or 2.5μM BA was more effective for multiple shoot induction from cotyledonary node and addition NAA to MS medium with BA (2.5μM) enhanced the rate of multiplication. It was also reported that MS medium enriched with 1.5mg dm^{-3} BA, 50 mg dm^{-3} adenine sulfate and 0.1mg dm^{-3} IAA was suitable for adventitive multiple shoot induction from axillary meristem, and its elongation (Rout *et al.,* 2008). More over, the preconditioning of axillary bud in liquid MS medium with TDZ (75μM) for 16 days showed improved explant response (94%), multiple shoot induction and elongation (Jahan *et al.,* 2011). However, in the present study addition of NAA and/or IBA along with BA promoted callus mediated organogenesis and MS medium with 8 μM BA and 2μM NAA generated significant number of shoots per explant. Our results were contrasting with the results obtained for cotyledonary node explants (Siddique *et al.,* 2006) with regard to the pattern of organogenesis. However, this is the first report of callus mediated organogenesis in *N. arbor-tristis*. This differential response to organogenesis might be attributed to explant age and its composition, growth regulator combination and genotype tested. This led to the conclusion that *in-vitro* organogenesis in *N. arbor-tristis* requires a critical amount of cytokinin and auxin in addition to macro/micro-nutrient composition of media. However, the MS medium with dual cytokinin has not responded well in the present study. But Rout *et al.* (2008) reported BA with adenine sulfate showed better shoot multiplication, which might be due to the synergistic effect of adenine sulfate with BA on cell growth and shoot multiplication.

In present study, incubation in one fourth strength MS medium (2% sucrose) supplemented with 2μM IBA for over night followed by culturing on PGR free half strength MS medium culminated with better rhizogenesis both in term of rooting frequency as well as roots per shoots. IBA was also reported as most effective auxin for *in vitro* rhizogenesis in *N. arbortristis* (Rout *et al.,* 2008; Jahan *et al.,* 2011). These results again indicated the stimulatory effect of auxin for rhizogenesis in *N. arbor-tristis*. Genetic variation during *in vitro* organogenesis could influence the genetic stability of regenerated plantlets. The usefulness of RAPD as a means of molecular analysis of *in vitro* regenerated plants has been well documented in several species (Nayak *et al.,* 2003; Mishra *et al.,* 2006; Tyagi *et al.,* 2007; Maharana *et al.* 2012). In the present study, the genetic stability of the regenerated plants was screened by RAPD markers that could detect DNA sequence modifications at the primer annealing site of the genome. Thirty primers were tested to analyze 10 regenerated plants as well as the donor parent. Of these nine primers responded for genomic DNA amplification and produced 24monomorphic bands, similar to the donor plant, with a molecular weight between 275 bp to 1150bp. The observed homogeneous banding pattern might be due to lack of genetic variation in the plantlets, in concordance with their morphological appearance. The experimental evidences, as observed in the present study, substantiates the utility of RAPD markers for the test of genetic stability of *in vitro* regenerated plantlets. The efficient and the reliable plant regeneration protocol optimized during the present study can be potentially utilized for the *ex-situ* conservation as well as mass propagation of *N. arbor-tristis* clones for the herbal industry for different therapeutics purposes.

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