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Micropropagation and Evaluation of Genetic Variability Among *in Vitro* Regenerants of *Naravelia zeylanica* (L.) DC.

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ABSTRACT

An efficient protocol was developed for the regeneration of plantlets via direct and indirect organogenesis from stem nodal segments of *Naravelia zeylanica* (L.) DC. (Ranunculaceae). The interaction of 6-benzyl aminopurine (BAP) with α-naphthalene acetic acid (NAA) in Murashige and Skoog (1962) medium induced a varied morphological response. A maximum of 17.6 ± 1.5 shoots per explant were obtained in the presence of 4.0 mg l^{-1} BAP and 0.6 mg l^{-1} NAA on MS medium. A higher concentration of BAP (5.0- 8.0 mg l^{-1}) and NAA (1.0-2.0 mg l^{-1}) favoured callogenesis while a lower range (2.0-3.0 mg l^{-1} BAP and 0.25-5.0 mg l^{-1} NAA) induced adventitious shoot buds and 11.7 ± 1.3 plantlets were obtained from stem calli. The excised microshoots rooted well on MS basal medium without plant growth regulators. Morphologically direct organogenesis regenerants were similar to *in vivo* plants while the callus regenerants exhibited abnormal development after acclimatization. The survival rate of the plantlets derived from direct organogenesis was 96% whereas in callus regenerants it declined to 72%. Random Amplified Polymorphic DNA (RAPD) markers were employed to analyze the level of genetic variation among the plantlets of direct and indirect organogenesis. Five groups of Operon primers – B 11-20, C 01-20, D 01-10, E 01-10, and F 11-20 – were screened, from which six were selected, among which OPF-17 and OPF-19 gave clear and distinguishable bands. A dendrogram was constructed using Euclidean distances by Ward's method. RAPD marker fingerprinting allowed a rapid assessment of the level of genetic variation among the regenerants.

Keywords: fingerprinting, genetic variability, organogenesis, Ranunculaceae, RAPD marker

INTRODUCTION

Plants are the valuable source of a vast array of chemical compounds; they synthesize and accumulate extractable organic substances in quantities sufficient to be economically useful raw materials for various commercial applications. Due to destruction of their habitat and over-exploitation many of the endemic Indian medicinal plants are on the verge of extinction. *Naravelia zeylanica* (L.) DC. (Ranunculaceae) is a woody climber distributed in the Western Ghats of India (Saldanha 1984). *N. zeylanica* is useful in the treatment of pitta, helminthiasis, dermatopathy, leprosy, rheumatalgia, odontalgia, colic inflammation, wounds and ulcers (Praveendhar and Ashalatha 2003). The root and stems have a strong penetrating smell (Warrier *et al*. 1995). In the Indian system of medicine, 'Ayurveda', the plant is used to relive malarial fever and headache while root and stem paste is applied externally for psoriosis, itches and skin allergy (Harsha *et al*. 2002). In Kerala, India *N. zeylanica* is used as a source of drug for intestinal worms, skin disease, leprosy, and toothache (Sivarajan and Balachandran 1958) The traditional medicine practitioners residing in the vicinity of Bhadra Wild Life Sanctury, Karnataka, India are using the leaf and stem juices for treating psoriasis and dermatitis. Many pharmaceutical industries in India (Hindustan Liver Ltd., Mumbai; Himalayan Drug House, Bangalore) are engaged in the production of skin ointments from this plant. Biosystematically, this species holds much importance because only two stove-climbing species are reported in the genus *Naravelia* (Manjunath *et al*. 2004). Destruction of the natural habitat and commercial exploittation of this species from natural resources has resulted in the dwindling of populations in the Central Western Ghats of India.

In vitro propagation is a promising tool for the rapid multiplication of threatened and endangered medicinal plants either through direct organogenesis (Rout *et al*. 2000; Vespasiano *et al*. 2003; Biondo *et al*. 2004) or indirectly through the calli (Faisal 2003; Li *et al*. 2004; Emma *et al*. 2005). A literature survey indicated that an *in vitro* protocol has not yet been standardized for this rare climber. In view of its medicinal importance and threatening status *in vitro* studies were undertaken to regenerate plantlets directly through shoot multiplication and indirectly through the callus phase.

DNA marker technology is extensively used for the evaluation of relatedness among the clones of the same species. In conventional methods careful phenotypic evaluation is necessary at all stages of development of the regenerants, whereas DNA marker technology helps the genetic variants to overcome many problems faced during conventional screening. In *in vitro* studies DNA markers may be used as tools to analyze somoclonal variation among the regenerants. Many researchers have employed Random Amplified Polymorphic DNA (RAPD) fingerprinting (Williams *et al*. 1990) for the identification of genetic variability among the clones of the same species (Teixeira da Silva 2005). Using this technique, an unlimited number of polymorphic bands can be produced with relative ease from minute amounts of genomic DNA (Welsh and McClelland 1990).

The present paper has great relevance from a conservation point of view and reports on the rapid multiplication of plantlets via direct and indirect organogenesis from the stem explants of *Naravelia zeylanica*. The variations among the regenerants were evaluated using RAPD fingerprinting.

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MATERIALS AND METHODS

In vitro studies

Tender twigs 3-4 cm long of *Naravelia zeylanica* were collected from a healthy plant growing in the University Medicinal plant garden located in Bhadra Wild Life Sanctuary, Karnataka, India. The twigs were thoroughly washed under running tap water for 25-30 min and then rinsed in a solution containing the surfactant Tween-20 (two drops in 100 ml solution) Subsequently, they were surface-sterilized with 0.1% (w/v) $HgCl_2$ solution for 2-3 min, followed by three to five rinses with sterile distilled water in a clean air cabinet. The surface-sterilized explants were trimmed to 0.5-1.5 cm in length, comprising a single node each. The explants were carefully inoculated onto callogenic and caulogenic media. The culture media consisted of MS salts (Murashige and Skoog 1962) augmented with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar (Hi-Media, India). For adventitious shoot organogenesis the media was supplemented with 3.0-5.0 mg l^{-1} 6-benzyl aminopurine (BAP) and 0.4 to 0.8 mg l^{-1} α-naphthalene acetic acid (NAA). The callogenic media consisted of 5.0 to 8.0 mg l^{-1} BAP and 1.0 to 2.0 mg l^{-1} NAA. The callus-differentiating media was supplemented with 2.0 to 3.0 mg l^{-1} BAP and 0.25 to 0.5 mg l^{-1} NAA. The pH of the medium was adjusted to 6.0-6.2 and autoclaved at 105 kPa and 121°C for 20 min. Fifty ml of medium was dispensed into sterilized 50 x 110 mm culture bottles (Varsha Storage Racks, Bangalore, India) closed with ebonite caps. The cultures were incubated at 28 ± 2°C and 60 µmol m^{-2} s^{-2} light intensity under a 12 h photoperiod with cool-white fluorescent tubes (Philips, India) with 55% relative humidity (RH). For *in vitro* rooting, individual microshoots 6-8 cm long with 4 to 5 leaves were aseptically excised from the culture and transferred to MS basal medium for root initiation. The rooted plants were removed from the culture bottles, washed with sterile distilled water and transferred to plastic pots with sterile vermiculite:perlite:peat moss (1:2:3 v/v/v) (Dugar Industries, India) for hardening. The plantlets were placed in a growth chamber at 70% RH, 28 ± 2°C under a 12-h photoperiod for acclimatization. The plants were fertilized with $1/8^{th}$ MS macronutrients twice during the course of acclimatization at an interval of 4-5 weeks. Established plants were placed in 20 cm diameter pots with sand:soil mixture (1:1) and transferred to a mist chamber (RH 80%; 34 ± 2°C) for hardening. These hardened plants were field-transferred and survival rate was recorded.

DNA extraction

Leaves from the *in vivo* mother plant and the regenerants derived from direct and indirect organogenesis were collected and used for DNA extraction. To test for the optimal isolation protocol, the methods of Dellaporta *et al*. (1983), Cao and Oard (1997), were subjected to DNA isolation, and that of Porebski *et al*. (1997) with some modifications was found to be the most suitable to get more and better DNA for our research purpose. The choice of extraction protocol depended on the plant sample and in order to remove phenolics or mucilaginous substances we had to modify the protocol, as described next. For DNA extraction 500 mg of leaf tissue in 20 ml of extraction buffer contained 3% w/v CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2% PVP and 1% β-mercaptoethanol, 100 mM Tris-HCl, pH 8.0. The phenolic and mucilagenous substances were removed by the addition of 2% polyvinylpyrrolidone (PVP) in the extraction buffer. The contents were preheated to 65°C for 1 h. The centrifuge tube was brought to room temperature and 6 ml of chloroform:isoamyl alcohol (24:1) were added. The contents were mixed well by inverting the tube gently 25-30 times, then spun at 7,000 rpm for 15 min. The supernatant was transferred to a fresh tube and this clean-up step was repeated until a clear supernatant was obtained. The supernatant was kept overnight at 4°C to precipitate DNA by adding half of the volume of 5.0 M NaCl and one volume of isopropanol. The content was centrifuged at 10,000 rpm for 20 min to obtain a DNA pellet, which was washed with 70% ethanol. The dried DNA pellet was resuspended in 500 µl of TE (Tris EDTA) buffer. Contaminating RNA was removed by digestion with 10 µg of RNase (Genei, Bangalore, India) for 60 min at 37°C. Proteins were removed by digestion with 25 µg of Proteinase-K (Genei, Bangalore, India). The DNA was further purified by extracting twice with an equal volume of distilled phenol followed by an equal volume of phenol:chloroform (1:1) and finally with an equal volume of chloroform. The DNA was precipitated by the addition of one volume of isopropanol and spun at 5,000 rpm for 5 min. The final pellet was dissolved in 300 µl TE. The quality was verified by electrophoresis on a 1% agarose gel stained with 0.15 µg/ml ethidium bromide (EtBr) (Sambrook *et al*. 1989).

DNA amplification

The basic protocol reported by Williams *et al*. (1990) for PCR was followed with slight modifications. A single decamer of arbitrary sequence was used in each PCR reaction and reproducible fingerprint profiles were carried out in 25 µl reaction mixture containing template DNA (25 ng), 10 pmol decamer primer (Operon technologies, USA), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.05% Triton X-100, 1.0 unit of Taq DNA polymerase and 200 µM of each dNTP (Genei, Bangalore, India). The mixture was overlaid with one drop of mineral oil to prevent evaporation of the reaction mixture. Amplification was performed in a PTC 150 thermal cycler (MJ Research Inc, USA) for 38 cycles after an initial denaturation at 94°C for 6 min. In each cycle, denaturation for 1 min at 94°C, annealing for 1 min at 33°C and extension for 2 min at 72°C was programmed with a final extension step at 72°C for 6 min. After the 38 cycles the amplified product was subjected to electrophoresis.

DNA electrophoresis

The PCR products were electrophoresed in 1% agarose gel using 1X TAE buffer stained with 0.15 µg/ml EtBr. Wells were loaded with 25 µl reaction mixture and 5.0 µl of loading dye (40% sucrose (w/v), bromophenol blue and xylene cyanol, 0.25% each) and spun briefly in a micro centrifuge before loading (Sambrook *et al*. 1989). Electrophoresis was conducted at 45 V for 3 h and the gel was documented using a gel doc system with EASY WIN 32 type RH-2.1 (Herolab, Germany).

Primer screening

The DNA samples isolated from the clones of direct and indirect organogenesis were subjected to RAPD analysis using five groups of Operon primers (B 11-20, C 01-20, D 01-10, E 01-10 and F 11-20). Six primers produced recognizable polymorphic bands from which only two primers that showed clear bands were selected. The size of the bands was determined and compared with a 1000 base pair (bp) DNA ladder (Genei, Bangalore, India). Fragments of identical size of the amplified DNA of the same primer were considered to be the same corresponding to one locus.

Data analysis

For each hormonal concentration the explants/callus were inoculated in ten replicates. The mean number of shoots organized directly from the explants or differentiated through the calli was evaluated at the end of the sixth week of incubation. The analysis of variance was carried out using Duncan's Multiple Range Test (Duncan 1955). Statistical significance was determined at the 5% level, using statistical software SPSS Inc. (Chicago, USA). A dendrogram of amplified band was constructed using Euclidean distances by Ward's method (Hemanthkumar *et al*. 2001; Prakash *et al*. 2005).

RESULTS AND DISCUSSION

The frequency of shoot organogenesis and rate of multiplication depends upon the type and concentration of exogenously supplemented growth regulators either alone or in combinations. Among the different cytokinins and auxins tested, the caulogenic efficiency of BAP and NAA has been exploited in the micropropagation of many medicinally important plant species such as *Chrysoplenium americanum* (Brission *et al*. 1988), *Andrographis alata* (Nagaraja *et al*. 2003), *Thapsia garganica* (Nokwanda *et al*. 2005) and

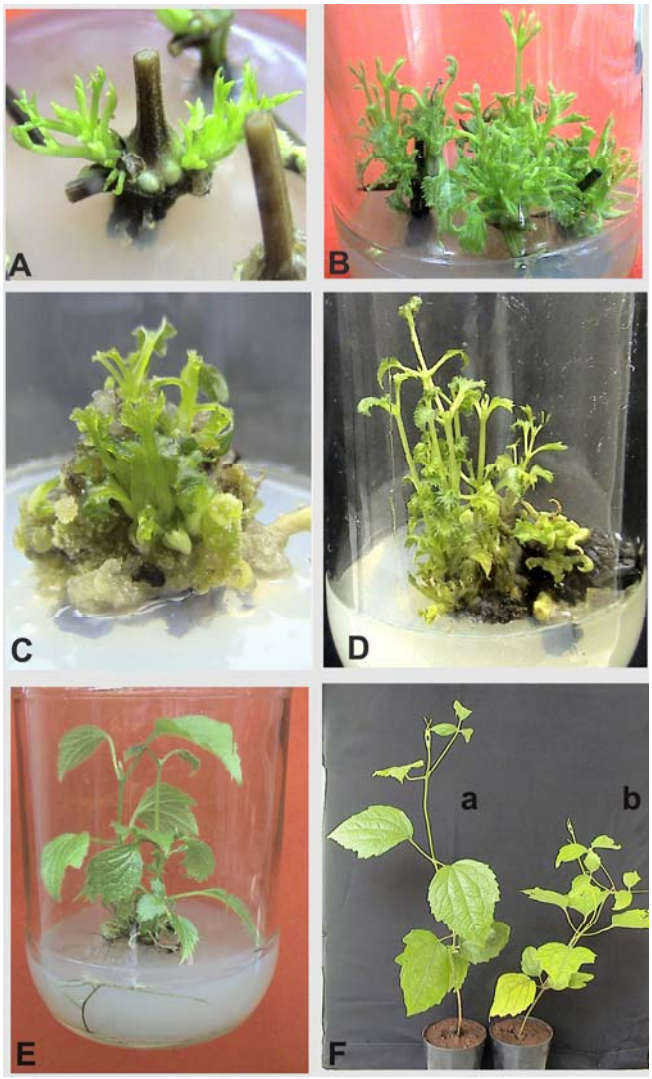

Fig. 1 (**A**) Adventitious shoot buds organised from the stem node on MS + 4.0 mg l^{-1} BAP and 0.6 mg l^{-1} NAA. (**B**) Five weeks old culture showing shoot multiplication with 17.6 ± 1.5 shoots per explant. (**C**) Shoot bud differentiation from the stem callus on MS + 2.0 mg l^{-1} BAP and 0.3 mg l^{-1} NAA. (**D**) Five weeks-old culture showing differentiation of a mean of 11.70 ± 1.34 shoots per callus. (**E**) Rhizogenesis from the shoots on MS basal medium. (**F**) a, direct organogenesis regenerant with normal growth and b, callus regenerant with abnormal growth.

Dioscorea zingiberensis (Yuan *et al*. 2005).

In our experiments on *N. zeylanica* the treatment containing 4.0 mg l^{-1} BAP and 0.6 mg l^{-1} NAA showed the best multiplication and adventitious multiple shoot induction from the nodal region of the stem explants (**Fig. 1A**). At this optimal concentration 17.6 ± 1.5 shoots per explant differentiated (**Fig. 1B**). The increase in NAA concentration with a decrease in BAP level induced calli induction and diminished the average number of shoots per explant (**Table 1**). A similar type interaction of BAP with NAA was reported on multiple shoot induction from the nodal segments of *Ochreinauclea missionis* (Naomita and Ravishankar Rai 2001), *Mecardonia tenella* (Liliana *et al*. 2006), *Psoralea corylifolia* (Anis and Faisal 2005) and *Pseudoxytenanthera stocksii* (Sanjaya *et al*. 2005).

In callogenic media an increase in the concentrations of BAP and NAA above 5.0 to 8.0 mg l^{-1} and 1.0 to 2.0 mg l^{-1}, respectively favoured only callus initiation from whole explants. Callus was first initiated from the node then it expanded all over the surface of the explant. Maximum proliferation of callus was noticed at 6.5 mg l^{-1} BAP and 1.5 mg l^{-1} NAA. Conversely the combination of these hormones at a lower range, i.e. 2.0-3.0 mg l^{-1} BAP and 0.25-0.5 mg l^{-1} NAA promoted shoot differentiation from the callus (**Fig. 1C**). At the optimal concentration (2.0 mg l^{-1} BAP and 0.3 mg l^{-1} NAA) a mean of 11.70 ± 1.34 shoots organised per callus mass (**Fig. 1D**). The number of shoot buds differentiated at different concentrations of BAP is depicted in **Table 2**. The frequency of shoot differentiation remained the same for more than eight subcultures (data not shown). The interaction of BAP with NAA at different concentrations induced varied morphological responses. Higher concentrations of BAP and NAA above 5.0 to 8.0 mg l^{-1} and 1.0 to 2.0 mg l^{-1}, respectively favoured callogenesis from the excised nodes; lower levels of these hormones induced shoot bud differentiation while moderate levels provoked adventitious shoot organogenesis from the nodes of the explants. A similar type of caulogenic effect of BAP and NAA was reported in the culture of stem callus of another threatened climber, *Embelia ribes* (Shankarmurthy *et al*. 2005). The effect of pH also had a profound influence on shoot multiplication and callogenesis. At normal pH (5.8) exudation of phenolics from the organogenic explants or the calli continued until the medium and the explants turned black,

Table 1 Effect of BAP and NAA on adventitious shoot bud induction and regeneration of plantlets from the stem explant of *Naravelia zeylanica*.

Growth regulators (mgl^{-1})		Number of shoot buds per explant Mean ± SD	Number of rooted plantlets per explant Mean ± SD
BAP	**NAA**		
3.0	0.4	1.60 ± 0.52 I	2.70 ± 0.95 j
3.0	0.6	2.50 ± 0.85 hi	3.70 ± 0.95 hij
3.0	0.8	3.50 ± 1.08 g	4.40 ± 1.73 gh
3.5	0.4	5.20 ± 1.32 f	7.30 ± 1.34 f
3.5	0.6	8.00 ± 1.05 e	9.20 ± 0.63 e
3.5	0.8	11.10 ± 0.88 c	12.70 ± 2.21 c
4.0	0.4	13.50 ± 0.85 b	15.10 ± 1.85 b
4.0	0.6	15.10 ± 1.45 a*	17.60 ± 1.51 a*
4.0	0.8	13.70 ± 1.34 b	15.30 ± 1.57 b
4.5	0.4	9.80 ± 1.03 d	11.30 ± 1.06 d
4.5	0.6	6.10 ± 1.20 f	8.90 ± 0.74 e
4.5	0.8	5.30 ± 1.34 f	7.80 ± 1.23 f
5.0	0.4	3.60 ± 0.97 g	5.30 ± 0.95 g
5.0	0.6	3.10 ± 0.88 gh	3.90 ± 0.74 hi
5.0	0.8	1.70 ± 0.95 i	2.90 ± 0.74 ij
F-Value		187. 25	153. 02

In each column the mean value with different alphabetical letters are signify-cantly different ($P < 0.05$).
Uses harmonic mean sample = 10.

Table 2 Effect of BAP and NAA on shoot bud differentiation from stem calli of *Naravelia zeylanica*.

Growth regulators (mgl^{-1})		% of calli involved in shoot bud differentiation	Number of shoot buds per calli Mean ± SD
BAP	**NAA**		
1.0	0.2	40	1.90 ± 0.88 g
1.0	0.3	45	4.00 ± 0.94 e
1.0	0.4	50	4.50 ± 1.65 e
1.5	0.2	60	6.10 ± 1.66 d
1.5	0.3	60	6.20 ± 1.23 d
1.5	0.4	80	7.60 ± 1.35 c
2.0	0.2	90	10.10 ± 1.52 b
2.0	0.3	90	11.70 ± 1.34 a*
2.0	0.4	90	10.50 ± 1.78 b
2.5	0.2	70	6.70 ± 1.89 cd
2.5	0.3	60	4.80 ± 0.92 e
2.5	0.4	55	3.60 ± 1.35 ef
3.0	0.2	45	2.50 ± 0.84 fg
3.0	0.3	40	2.10 ± 0.99 g
3.0	0.4	40	1.70 ± 0.82 g
F-Value			59. 49

In each column the mean value with different alphabetical letters are significantly different ($P < 0.05$).
Uses harmonic mean sample = 10.

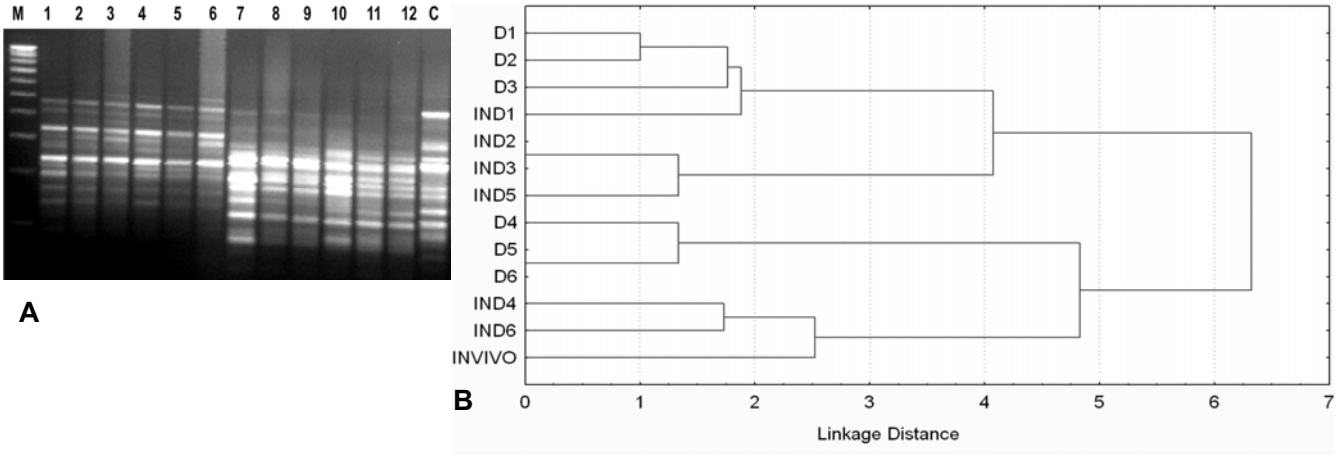

Fig. 2 (**A**) Gel profile of *Naravelia zeylanica*. M, Marker; 1-6, regenerants of direct organogenesis; 7-12, regenerants of indirect organogenesis; C, *in vivo* plant amplified using selected primer OPF-17. (**B**) Dendrogram of 12 cloned regenerants and an *in vivo* plant by using selected primer OPF-17.

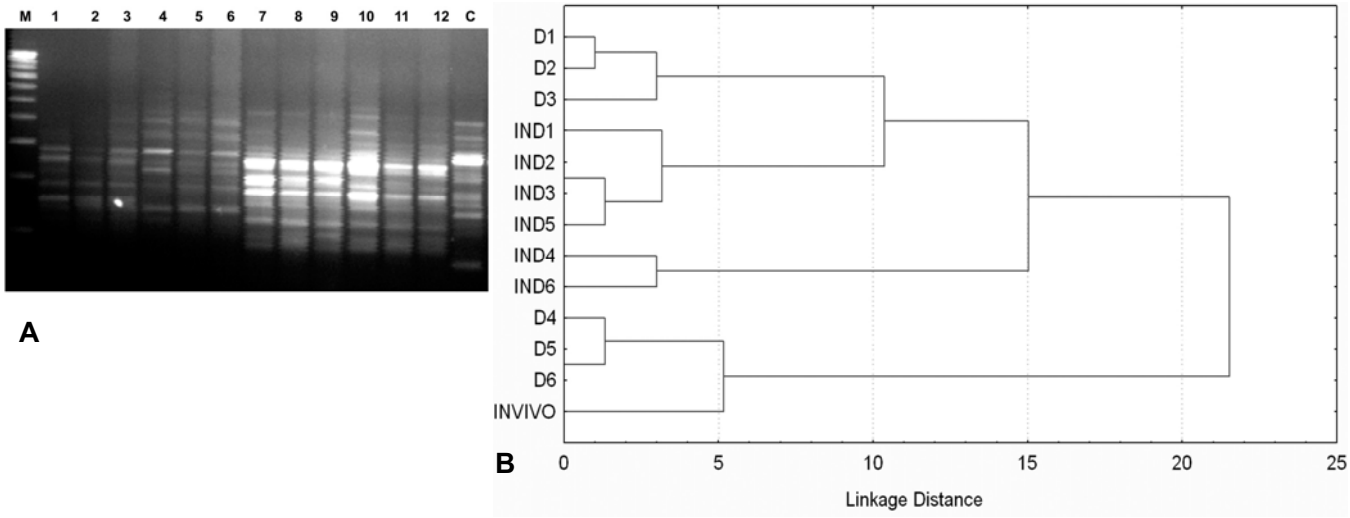

Fig. 3 (**A**) Gel profile of *Naravelia zeylanica*, M- Marker, 1-6 regenerants of direct organogenesis and 7-12 regenerants of indirect organogenesis and C - *in vivo* plant amplified by using selected primer OPF-19. (**B**) Dendrogram of 12 clones regenerants with *in vivo* plant of *Naravelia zeylanica* by using selected primer OPF-19.

hindering the morphogenic potential of organogenisis from node and subsequent callus formation (data not shown). In contrast, at pH 6.0-6.2 normal growth and caulogenic response was observed (data not shown).

The shoot buds organised either directly from the explants or indirectly through the stem calli were harvested from the clump when they attained a length of more than 5 cm with 5-6 leaf primordia. They were transferred to basal medium without growth regulators for root induction. Irrespective of their organogenic origin 98% of the shoots produced root initials from their base. In a five-week-old culture the regenerants rooted without intervening callus and morphologically leaves were similar to *in vivo* plants with a dentate margin and trifoliate nature (**Fig. 1E**). However, the survival rate of the plantlets derived from direct organogenesis was 96% whereas in callus regenerants it declined to 72%. The morphology of the two month-old regenerants derived from direct organogenesis was similar with the *in vivo* plants. The callus regenerants showed some variations like abnormal growth, reduced height, a slender stem and curled leaves (**Fig. 1F**).

RAPD analysis reported by Welsh and McClelland (1991) and Williams *et al*. (1990) is one of the most popular DNA marker systems owing to its simple and straightforward protocol. It is a very fast and cost efficient technique and needs only nanogram amounts of template DNA and minimum laboratory equipment. RAPD analysis of the plantlets derived from direct and indirect organogenesis provides genetic support for the morphological variability observed among the *in vitro* regenerants. In this study, five groups of Operon random primers – B 11-20, C 01-20, D 01-10, E 01-10, and F 11-20 – were used for amplification, six of which, OPD-07, OPD-10, OPE-02, OPE-17, OPF, 17 and OPF-19 showed recognizable bands; from these only two (OPF 17 and OPF 19) showed a clear banding pattern. A representative of the PCR amplification product of six clones from direct and indirect organogenesis was analyzed by comparing with one from *in vivo* plants. The results showed sufficient polymorphisms to distinguish between direct and indirect organogenesis clones. Among the six selectable primers more amplification bands were exhibited with the primers OPF-17 (**Fig. 2A**) and OPF-19 (**Fig. 3A**) and each one exhibited four polymorphic bands (**Table 3**). On testing with both the markers eleven prominent bands were observed in the *in vivo* plant and also in the clones derived from direct organogenesis. In the clones of callus regenerants these bands (i.e. between 400 and 500 kb) were absent. However, organogenesis-derived regenerants exhibited prominent bands between 800 kb to 900 kb. In particular, DNA samples taken from clones 1 and 4 were very prominent.

Table 3 Amplification of DNA of the regenerants with the selected primers.

Selected Primers	Sequence	Number of fragments amplified	Number of polymorphic bands
OPD-07	TTGGCACGGG	11	4
OPD-10	GGTCTACACC	9	3
OPE-02	GGTGCGGGAA	10	3
OPE-17	CTACTGCCGT	7	2
OPF-17	AACCCGGGAA	12	4
OPF-19	CCTCTAGACC	13	4

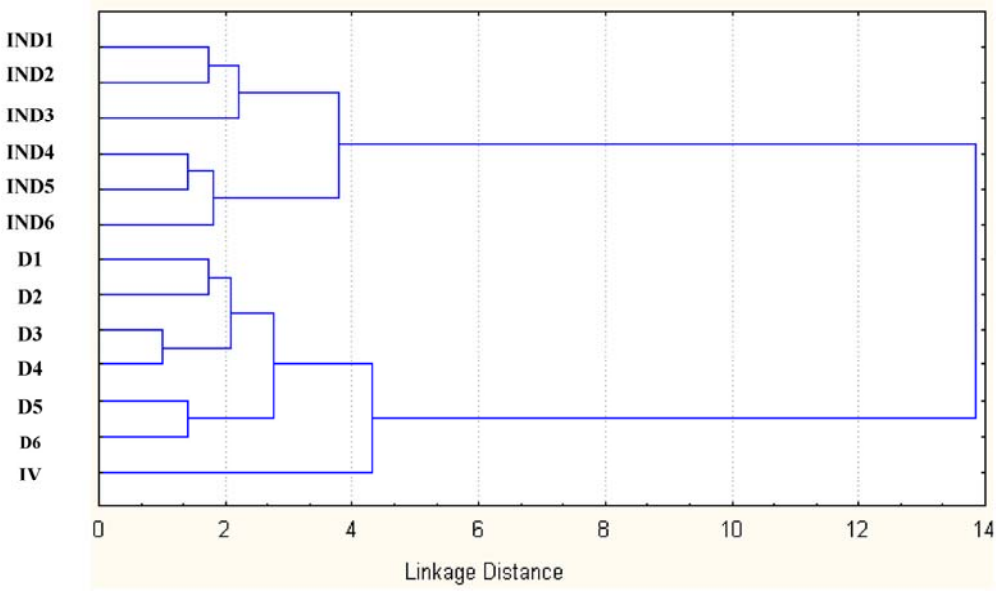

Fig. 4 Dendrogram of 13 clones belongs to direct and indirect regenerants were amplified with the primers OPF-17 and OPF-19.

These bands were absent in the *in vivo* plant.

The data from the two primers OPF-17 and OPF-19 were combined and a new cluster analysis was carried out in which the plantlets derived from direct organogenesis merged under the same cluster whereas those from indirect organogenesis showed differences in the cluster analysis. The regenerants derived from direct organogenesis were related to *in vivo* plants which indicates that they had retained their parental characteristics. The variation differentiating the callus regenerants from *in vivo* plants may be due to cytodifferentiation influenced by exogenously supplemented growth regulators. The Dendrogram (**Fig. 4**) generated by combining the data scored using two gel profiles of OPF-17 and OPF- 19 identifies clear-cut differences between direct and indirect organogenesis compared with *in vivo* plants.

The efficiency of the clone in marker analysis depends upon the functional heritability triat and environmental factors. The analysis of six clones regenerated through calli showed more polymorphic bands than the regenerants of direct organogenesis and *in vivo* plants. The dendrogram based on Ward's method revealed maximum similarity among the direct organogenesis regenerants with *in vivo* plants amplified with OPF 17 and OPF 19 (**Figs. 2B, 3B**). The transfer of the same characters from parents to clones appeared in the form of monomorphic bands. Similar types of monomorphic bands were reported among the clones derived through adeventitious shoot organogenesis of some medicinal plants (Larkin *et al*. 1981; Camlin 2001; Hosokawa *et al*. 2000). The callus regenerants showed more polymorphic bands than the *in vivo* plants. This may be due to the origin of plantlets from the cytodifferentiated cells of the callus which was induced due to the effect of exogeneously supplemented growth regulators.

In conclusion, we report on a prime protocol for the mass propagation of *N. zeylanica* both from adventitious shoot organogenesis and through differentiation from callus. With these methods of propagation, over 7,500 plantlets were obtained from direct organogenesis and over 5625 plantlets were obtained from indirect organogenesis in one year. *In vitro* mass propagation through direct organogenesis reduces the cost and completes the life cycle within a reasonable period of time and reduces the increasing demand by the pharmaceutical industry as well as for conservation of this important Indian medicinal plant. The regenerants derived through the calli showed varied phenotypic characters. This may be due to the origin of plants from hormone-induced cytodifferentiated cells. The results of this investigation indicated that the abnormal phenotypic characters of callus-derived regenerants are due to the presence of genetic variability and genetic stability of the clones of direct organogenesis is proved by a similar banding pattern. RAPD fingerprint analysis was a suitable technique to discover the identity or non-identity in *Hypericum perforatum* L. populations (Arnholdt-Schmitt 2006). It has also been used to assess the levels and patterns of genetic diversity in *Digitalis obscura* L. (Nebauer *et al*. 1999), the genetic stability of *Dioscorea bulbifera* plants regenerated from embryogenic tissues (Dixit *et al*. 2003) and the degree of genetic diversity in seven species of *Asparagus racemosus* (Shasany *et al*. 2003) and nine species of *Lippia* (Verbenaceae) (Viccini *et al*.2004). Further work is in progress on the comparative phytochemical evaluation of the clones derived from direct and indirect organogenesis by comparing with the *in vivo* plants.

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