

Plant regeneration from callus cultures of *Vitex trifolia* (Lamiales: Lamiaceae): a potential medicinal plant

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Abstract: Regeneración de plantas a partir de cultivos de callos de *Vitex trifolia* (Lamiales: Lamiaceae): una planta medicinal potencial. *Vitex trifolia* is a shrub species with popular use as a medicinal plant, for which leaves, roots and flowers have been reported to heal different distresses. The increasing exploitation of these plants has endangered its conservation, and has importantly justified the use of biotechnological tools for their propagation. Our aim was to present an efficient protocol for plant regeneration through organogenesis; and simultaneously, to analyze the genetic homogeneity of the established clonal lines by Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) markers. Plantlet regeneration was achieved in callus cultures derived from stem, leaf and petiole explants of *V. trifolia* on a differently supplemented Murashige & Skoog medium, and incubated at 25±2°C under a light intensity of 61µmol/m²s from cool white fluorescent lamps and a 16h photoperiod. The rate of shoot bud regeneration was positively correlated with the concentration of hormones in the nutrient media. Shoot buds regenerated more rapidly from stem and petiole explants as compared to leaf explants on medium containing 11.10µM BAP in combination with 0.54µMNAA. Addition of 135.74-271.50µM adenine sulphate (Ads) and 0.72-1.44µM gibberellic acid (GA₃) to the culture medium increased the growth of shoot buds. The highest rate of shoot bud regeneration responses was obtained in stem explants using 11.10µM BAP in combination with 0.54µM NAA, 271.50µM Ads and 1.44µM GA₃. *In vitro* rooting of the differentiated shoots was achieved in media containing 1.23µM indole butyric acid (IBA) with 2% (w/v) sucrose. Regenerated plantlets were successfully established in soil with 86% survival under field condition. Randomly Amplified Polymorphic DNA and Inter Simple Sequence Repeat markers analyses have confirmed the genetic uniformity of the regenerated plantlets derived from the second up to fifth subcultures. This protocol may help in mass propagation and conservation of this important medicinal plant of great therapeutic potential. Rev. Biol. Trop. 61 (3): 1083-1094. Epub 2013 September 01.

Key words: *in vitro*, inter simple sequence repeats, medicinal plant, micropropagation, randomly amplified polymorphic DNA, plant regeneration.

The growing worldwide demand of traditional medicinal plants has made large-scale commercial cultivation and genetic improvement imperative. *Vitex trifolia* is one of such plants, belonging to the family Verbenaceae, which has great therapeutic potential. The root is used in the treatment of painful inflammations, cough and fevers. Leaves are reported to be useful in conditions of loss of memory, loss of hair, leucoderma and tuberculosis.

Flowers are effective in treating fevers and fruits in treating amenorrhoea (Oommen *et al.* 2000). Besides, the fruits contain an alkaloid, vitricine which is used in treating coryza, fever, headache, photopsia, vertigo, ophthalmalgia, glaucoma, rheumatism and neuralgia (Prajapati *et al.* 2003). Pharmaceutical companies largely depend upon material procured from naturally occurring stands which are being depleted rapidly. Thus, over exploitation concerns about



possible extinction of the species, provide a significant justification for the development of *in vitro* propagation techniques for this crop. On the other hand, scarce availability of planting material, slow regeneration in nature and lack of cultivation practices might be the possible reasons for multiplication of this plant species by shoot organogenesis from callus cultures, an effective method for multiplication of medicinal plants (Grewal & Atal 1976, Khanna *et al.* 2006). Though *in vitro* multiplication of *V. trifolia* was achieved through clonal propagation through meristems culture (Hiregoudar *et al.* 2006), to date, there are no reports on plant regeneration via callus culture in this species.

Generally, organogenic differentiation is considered a useful method in achieving a high frequency of shoot regeneration within a short period of time. However, there is a chance in occurrence of somaclonal variations among the sub-clones of parental line. The frequency of these variations varies with the source of explants, media composition and cultural conditions (Damasco *et al.* 1996, Salvi *et al.* 2001). The cryptic genetic defects arising via somaclonal variation in the regenerants is a potential drawback when the propagation of an elite species is intended, due to uncontrol lable and unpredictable nature of variation which seriously limits the utility of the micropropagation system. A number of molecular markers can be used to assess the genetic fidelity of *in vitro* derived clones. However, RAPD and ISSR markers are very simple, fast, cost effective, highly discriminative and reliable, require a small quantity of DNA, and do not need any prior primer sequence information (Cassells *et al.* 1997, Lakshmanan *et al.* 2007). In this paper we report, for the first time, a rapid micropropagation protocol for *V. trifolia* through callus cultures and the genetic homogeneity of established clonal lines using RAPD and ISSR markers.

MATERIALS AND METHODS

Plant material and surface sterilization:

This study was conducted at the Directorate of Medicinal and Aromatic Plants Research (DMAPR), Anand, Gujarat, India. Shoots (5-7cm long) collected from healthy plants of *V. trifolia* grown at the gene bank of Directorate of Medicinal and Aromatic Plants Research, Boriavi, Anand, Gujarat, India. Shoots were washed in 2% (v/v) detergent solution 'Teepol' (Qualigen, India) and surface sterilized in 0.1% (w/v) aqueous mercuric chloride (HgCl₂) solution for 20 minutes after rinsing 4-5 times with sterile distilled water, leaves, petioles and stem internodes were cut in to smaller segments (60x50mm, 50x40mm and ~ 0.5cm, respectively) for use as the explants.

Culture medium: The explants were placed on semi-solid (0.8% agar) basal MS medium (Murashige & Skoog 1962) supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP: 0.0, 2.22, 4.44, 6.65, 8.88, 11.10, 13.32μM), kinetin (Kn: 0.0, 2.32, 4.64, 6.96, 9.28, 11.60, 13.92μM), adenine sulphate (Ads: 67.87, 135.75 and 271.50μM), 1-naphthalene acetic acid (NAA: 0.0, , 2.69, 8.06, 10.74, 13.43, 16.11μM), 2,4-dichlorophenoxy acetic acid (2,4-D: 0.0, 2.26, 4.52, 6.78, 9.04, 11.3, 13.56μM) and gibberellic acid (GA₃ : 0.00, 0.29, 0.72, 1.44 μM) for callusing and organogenesis. The media pH was adjusted to 5.8 using 0.1N HCl or 0.1N NaOH before autoclaving. Routinely, 25mL of the liquid medium with 0.8% (w/v) agar (Qualigen, India) was dispensed into culture tubes (25x100mm), plugged with non-absorbent cotton and sterilized at 121°C and 104kPa for 15min. The cultures were maintained by regular subcultures at 4-week intervals on similar fresh medium.

Culture condition: At the time of callus initiation, all cultures were incubated at 25±2°C

under a light intensity of $61\mu\text{mol}/\text{m}^2\text{s}$ from cool white fluorescent lamps (Philips, India) under 16h photoperiod. For organogenesis, approximately, $200\pm 10\text{mg}$ of fresh callus was placed in each culture tube containing different concentrations and combination of cytokinins (BAP, Kn and Ads) and auxins (NAA and 2,4-D). The different regeneration media were presented in table 1.

TABLE 1
Media formulated for shoot bud regeneration

MS medium +3% sucrose	BAP (μM)	NAA (μM)	Ads (μM)	GA ₃ (μM)
M1	4.44	0.054	0.00	0.00
M2	6.66	0.54	0.00	0.00
M3	11.10	0.54	0.00	0.00
M4	13.32	0.54	0.00	0.00
M5	8.88	0.54	0.00	0.00
M6	11.10	0.54	271.50	0.00
M7	13.32	0.54	271.50	1.44
M8	11.10	5.37	0.00	0.00
M9	13.32	10.74	0.00	0.00

The cultures were incubated separately under 16 and 24h photoperiods with a light intensity of $61\mu\text{mol}/\text{m}^2\text{s}$ from cool white fluorescent lamps at $25\pm 2^\circ\text{C}$.

Induction of rooting and acclimatization: Excised shoots (1-2cm) regenerated from the callus were cultured on rooting medium fortifying with basal MS semi-solid medium either alone or in combination with different concentrations of IBA (0.49, 0.73, 0.98, $1.23\mu\text{M}$) or NAA (0.54, 0.81, 1.07, $1.34\mu\text{M}$) and 2% (w/v) sucrose for root initiation. One excised shoot was cultured in each tube ($25\times 150\text{mm}$) containing 15mL of the culture media. The cultures containing different concentrations of IBA and IAA were incubated separately under 16 and 24h photoperiods with cool, white fluorescent lamps at $25\pm 2^\circ\text{C}$ temperature. Rooted plantlets were thoroughly washed to remove the adhering gel and planted in 2.5cm earthen pots containing a sterile mixture of soil, sand and well decomposed manure in the ratio of

1:1:1 (v/v/v), and were kept in the greenhouse for acclimatization.

Observations of cultures and presentation of results: Twenty cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures responding to callusing, percentage of organogenic calli/culture, percentage of shoot buds/culture, mean percentage of rooting and number of roots/shoot were statistically analyzed by Duncan's multiple range test. Between the treatments, the average figures followed by the same letter were not significantly different at $p < 0.05$ (Harter 1960).

DNA extraction and PCR amplification: DNA was extracted from fresh leaves derived from both micropropagated and field-grown donor plant by the cetyltrimethyl ammonium bromide (CTAB) method (Bousquet *et al.* 1990) with minor modifications; 1% polyvinylpyrrolidone (PVP) was added to remove polyphenols. DNA quantity was estimated spectrophotometrically (VARIAN, Cary 300, USA) by measuring the absorbance at 260nm. Sixteen plants were assessed from which one is the donor plant and other 15 are *in vitro* derived plants.

The RAPD analysis was performed according to Williams *et al.* (1990) and ISSR analysis according to Zeitkiewicz *et al.* (1994). RAPD and ISSR amplifications were performed routinely using PCR mixture ($25\mu\text{L}$) containing 25ng of genomic DNA as template, 10 X PCR buffer, $200\mu\text{M}$ dNTPs (MBI Ferment Inc. USA), 1U of Taq polymerase (Bangalore Genei, India) and 15ng of RAPD primer or 40ng of ISSR primer. The amplification was carried out in a thermal cycler (S1000; Bio-Rad, Hercules, USA). In RAPD, PCR was performed at initial temperature of 94°C for 5min for complete denaturation. The second step consisted of 42 cycles having three ranges of temperature, i.e. 92°C for 1min for denaturation of template DNA, 37°C for 1min for primer annealing, 72°C for 2min for primer extension, followed by running the samples at 72°C for

7min for complete polymerization. For ISSR the same temperature profile was followed, but the primer annealing temperature was set at 5°C lower than the melting temperature. The PCR products obtained from RAPD were analyzed in 1.5% agarose gel whereas the ISSR products were analyzed in 2% agarose gel. The size of the amplicons were estimated using O' Gene Ruler™100 bpplus DNA ladder (MBI-Fermentas, Vilnius, Lithuania) and documented in the GelDoc (Bio-Rad, Hercules, USA).

Amplified DNA markers were scored as present or absent for both the micropropagated and the mother plants. Electrophoretic DNA bands of low visual intensity that could not be readily differentiated as present or absent were considered ambiguous markers which were not scored.

RESULTS

Callus induction: Calli were initiated from stem, petioles and leaves within 18-20 days of culture on MS basal medium supplemented with different concentrations of 2,4-D or NAA in combination with either Kn or BAP. Initially, small greenish white calli developed on the cut ends within 18-20days of inoculation which subsequently covered the entire surface of the explant (Fig. 1A). There was no sign of callus initiation in the explants cultured in only MS media devoid of cytokinins and auxins. Globular and greenish calli developed on the surface of the explants 3-4 weeks after culture initiation on MS basal medium supplemented with varying concentrations of BAP with NAA. The medium containing 2,4-D (9.05-11.3µM) and BA (0.04-0.44µM) promoted rapid callus growth from stem, petiole and leaf explants which subsequently turned brown within four weeks of culture. Similarly, also rapid callus induction was obtained in the medium supplemented with TDZ (0.45-2.27µM) + Ads (271.50µM) from the leaf and stem explants which were soft or gelatinous depending on the TDZ level; the rate of callus growth was faster in the first four-weeks of culture after which it declined. Though slow callus growth took

place in media supplemented with 11.10µM BAP and 0.54- 2.70µM NAA in stem, petiole and leaf, however the best granular callus growth was observed in the medium containing 0.44µM BAP and 16.11µM NAA. However, callus morphology varied with different plant growth regulators used in the MS culture medium. The appearance of the calluses varied with all the three explant types and media. The calli derived from stem and petiole explants were invariably greener and more granular than the leaf-derived callus on the media containing 0.44 -11.10µM BAP with 0.54-16.11µM NAA. Higher concentration of NAA induced compact calli which is not desirable for plant regeneration. Media containing Kn in combination with either NAA or 2,4-D did not induce any callus growth. Of the three explant types used, early callus growth was observed in stem

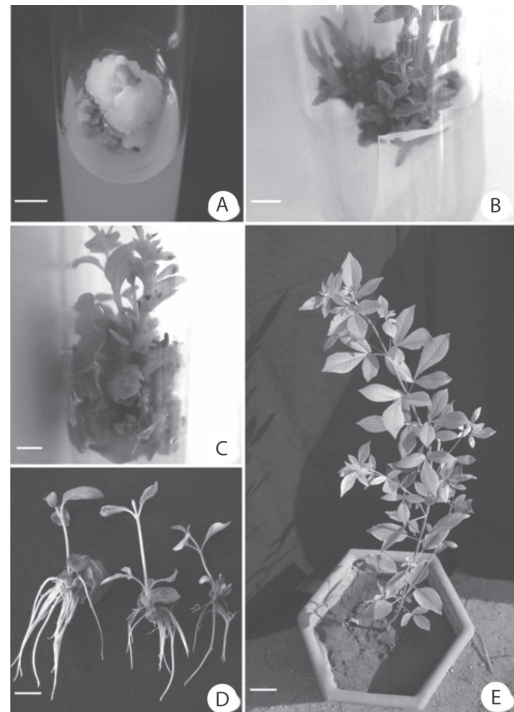


Fig. 1. Callus initiation and plant regeneration in *Vitex trifolia*. (A) - Callus initiation from stem explants (bar=10mm). (B) & (C) - Regeneration of shoot buds from stem derived callus (bar=10mm). (D) - Root induction in micro shoots (bar=50mm). (E) - Plantlets established in soil (bar=0.5cm).

explants followed by petiole and leaf explants. Leaf explants produced callus at a relatively slow rate. Young leaf and mature stem explants responded better than mature leaf and young stem explants respectively (data not shown).

Differentiation of shoots from callus:

After eight weeks on callus induction medium (MS+0.44 μ M BAP+16.11 μ M NAA) the calli were subcultured into media containing different concentrations of BAP, Kn and NAA for shoot bud regeneration. The calli differentiated into green nodular structures which developed into dark green shoot buds in the media supplemented with 10.0 -13.32 μ M BAP and 0.54 μ M NAA. Kinetin or BAP alone did not induce any morphogenic response, and also the combination of BAP or Kn with 2,4-D did not help in regeneration of shoot buds. The addition of adenine sulphate (271.50 μ M) and GA₃ (1.44 μ M) in the culture medium, however, resulted in quick growth of shoot buds within four weeks of culture (Fig. 1 B and C). A very high percentage of regeneration was observed in mature stem (86.67%) followed by petiole (76.67%) and leaf (58.33%) explants derived calli cultured in media supplemented with 13.32 μ M BAP+0.54 μ M NAA (Table 2). Regeneration of shoot buds also took place at low concentration BAP, but less frequently.

With the increase of NAA concentration (5.37-10.74 μ M) in the medium, the rate of regeneration slowed down. The percentage of shoot bud regeneration and the frequency of regenerated shoot/culture varied from 7.25 to 16.0, 3.45 to 10.85, and 2.60 to 9.80 in stem, petiole and leaf explants, respectively (Table 1).

Among the five subcultures at four week intervals, the higher regeneration potential and shoot buds per culture were observed in the 2nd subculture. After five to six subcultures of callus, loss of organogenic potential was observed even when cultured on similar medium or modified medium (data not shown). The regeneration frequency in cultures from stem, petiole and leaf explants was found to be best under 16h photoperiod as compared to the cultures under continuous light, which was not conducive to shoot bud regeneration and multiplication.

Induction of rooting in regenerated plants:

Microshoots regenerated from stem and petiole derived calli were excised and transferred to the medium with or without growth regulators. Half strength MS medium without growth regulators did not promote root induction. Roots were formed on excised shoots grown in 1/2 MS media containing either IBA (0.49-1.23 μ M) or NAA (0.54-1.34 μ M)

TABLE 2
Effect of various media on shoot bud regeneration in calli derived from stem, leaf and petiole explants of *Vitex trifolia* after four weeks of subculture

MS medium + 3% sucrose + additives	Source of callus					
	Stem		Petiole		Leaf	
	A*	B*	A*	B*	A*	B*
M1	60.00±2.23	10.90±0.32c	66.69±2.34	9.700.30b	33.33±1.7	6.50±0.26c
M2	50.00±2.23	10.65±0.36c	58.33±2.32	8.35±0.29c	48.33±2.3	4.75±0.27d
M3	48.34±2.82	9.35±0.26d	45.00±2.23	6.80±0.32d	38.35±1.7	6.75±0.25c
M4	80.00±2.23	13.10±0.31b	70.00±2.23	10.30±0.32ab	50.00±2.2	7.90±0.32b
M5	63.33±1.71	8.60±0.21d	40.00±2.24	5.80±0.24e	41.67±1.7	6.10±0.24c
M6	70.00±1.12	11.30±0.27c	45.00±2.23	6.90±0.19d	50.00±2.2	7.65±0.29b
M7	86.67±0.64	16.00±0.34a	76.67±2.33	10.85±0.51a	58.33±2.8	9.80±0.27a
M8	43.33±2.81	7.55±0.30e	35.00±1.12	3.45±0.24f	30.00±2.2	4.55±0.23d
M9	38.33±1.71	7.25±0.33e	43.33±2.42	4.00±0.25f	20.00±1.1	2.60±0.28e

A- Mean percentage of cultures regenerated; B- Average number of shoot bud/culture.

* Mean having the same letter in a column were not significantly different by Duncan's Multiple Range test p<0.05 level.

with 2% (w/v) sucrose. A high percentage of shoots (86%) rooted in the medium containing half strength MS basal salts with 1.23 μ M IBA+2% (w/v) sucrose after 11-12days of culture (Table 3). Root initials formed within 7-9 days which developed a good root system in 11-12 days (Fig. 1 D). Root initiation was achieved on medium containing either NAA (1.23 μ M) or IAA (1.43 μ M) but with intervening callus at the cut end of the shoot. The rooting of microshoots was observed to be best under 16h irradiance which was found to be inhibited in continuous light.

Acclimatization of rooted plants: Rooted plantlets were transferred into pots containing soil: sand: well decomposed cow-dung manure, in the ratio of 1:1:1 and kept in the green house for acclimatization. After one month of transfer to soil, about 90% of the plants survived in the green house (Fig. 1 E) and were subsequently planted in the field. No morphological variation was noticed.

Clonal fidelity: DNA samples from the hardened plantlets of *in vitro*-grown shoots derived from second up to fifth cycle of shoot multiplication grown in the polyhouse and the donor plants were subjected to RAPD and ISSR analysis. A total of 40 plantlets were

analyzed taking a minimum 15 plants from each culture period along with the donor plant. For RAPD analysis, a total of 60 random primers were used out of which 20 random decamer primers (Operon Technologies, Alameda, USA) were selected. In the case of ISSR, 12 out of 27 primers were selected. A total of 20 selected RAPD primers gave rise to a total of 125 scorable bands ranging from 180 to 3 000bp from which two markers (OPA & OPN 04) showing monomorphic bands are represented in figure 2 (A and B). The number of bands for each primer varied from 4-9 with an average of 6.25 bands per primer. The highest number of bands (9) was obtained with primer OPA10 and the lowest number of bands (4) amplified in OPA 05, OPA 19 and OPP 10 (Table 4). In ISSR analysis, 12 selected primers (Bangalore Genei, India) produced a total of 49 scorable bands ranging from 400 to 2 000bp (Table 5). For each primer, the number of amplified bands varied from 1-7, and a total of 49 bands were generated all of which were found to be monomorphic in nature; a common fragment size (400-1 220bp) amplified with two primers such as (AG)₈C and (AC)₈C are represented in figure 3 (A and B). The highest number of bands (7) and the lowest number of bands (1) were generated with the primer (CA)₆GG and (CT)₈T, respectively.

TABLE 3

Effect of various media on rooting of excised shoots of *Vitex trifolia* cultured on ½ MS basal salts with 2% sucrose

½ MS+Growth regulators (μ M)			% of shoots rooted	Average number roots/ shoot \pm s.e	Days to rooting
IBA	NAA	IAA			
0	0	0	0	0	0
0.05	0	0	13	1.82 \pm 0.15	12
0.49	0	0	40	3.60 \pm 0.17	11-12
1.23	0	0	86	5.68 \pm 0.13	11
0	0.05	0	20	1.18 \pm 0.12	16
0	0.53	0	30	2.11 \pm 0.12	14a
0	1.34	0	80	5.09 \pm 0.16	14a
0	0	0.06	20	0.98 \pm 0.12	18
0	0	0.57	73	3.04 \pm 0.11	18a
0	0	1.43	13	1.09 \pm 0.12	16a

* Data represent mean of 20 replicates/treatment in three repeated experiments.

a- basal callusing at the cut end.

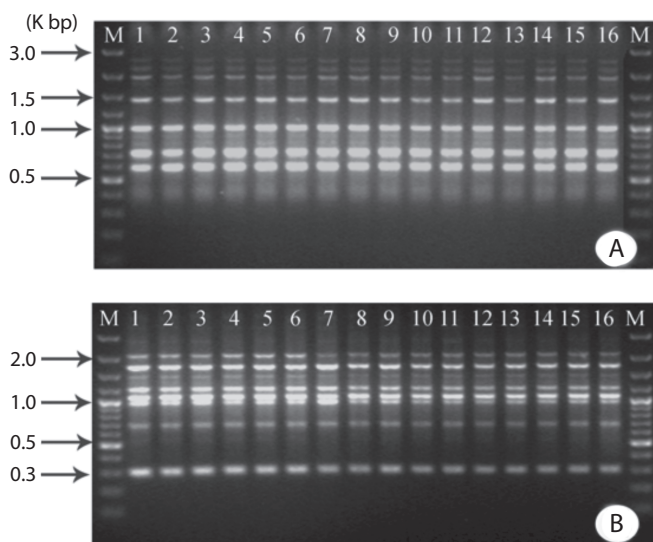


Fig. 2. RAPD banding pattern of *Vitex trifolia* with primer (A) - OPA 10 and (B) - OPN 04. (M - 100bp plus DNA ladder, Lane 1 - Donor plant, Lane 2 to 16 - callus regenerants).

TABLE 4
RAPD banding patterns of micropropagated and donor plants of *V. trifolia*

S. No.	Primer	Sequence (5'-3')	No of bands	Approx. fragment size range (bp)
1	OPA 01	CAGGCCCTTC	6	700-2 500
2	OPA 05	AGGGGTCTTG	4	1 250-2 000
3	OPA 07	GAAACGGGTG	7	450-2 000
4	OPA 09	GGGTAACGCC	6	180-2 000
5	OPA 10	GTGATCGCAG	9	600-2 700
6	OPA 11	CAATCGCCGT	5	400-1 400
7	OPA 18	AGGTGACCGT	5	300-2 000
8	OPA 19	CAAACGTCGG	4	700-3 000
9	OPN 01	CTCACGTTGG	6	650-2 900
10	OPN 02	ACCAGGGGCA	8	420-2 000
11	OPN 04	GACCGACCCA	8	300-2 100
12	OPN 05	ACTGAACGCC	8	720-2 000
13	OPN 06	GAGACGCACA	7	350-2 100
14	OPN 07	CAGCCCAGAG	6	500-1 250
15	OPN 09	TGCCGGCTTG	7	430-2 000
16	OPN 10	ACAACGTTGG	6	320-3 000
17	OPN 12	CACAGACACC	7	500-1 500
18	OPN 14	TCGTGCGGGT	7	360-1 500
19	OPP 01	GTAGCACTCC	5	700-2 300
20	OPP 10	TCCCGCTTAC	4	550-1 080
Total number of bands amplified			125	

TABLE 5
ISSR banding patterns in micropropagated and donor plants of *V. trifolia*

S. No.	Primer	Primer Sequence (5'-3')	Annealing temp(°C)	No. of bands	Approx. fragment size range (bp)
1	(CA) ₆ AC	CACACACACACAAC	40.0	3	850-1 500
2	(CA) ₆ AG	CACACACACACAAG	37.5	4	800-2 000
3	(CA) ₆ GG	CACACACACACAGG	39.0	7	400-1 200
4	(CA) ₈ AT	CACACACACACACAAT	47.0	3	950-1 350
5	(CT) ₈ TG	CTCTCTCTCTCTCTTG	56.0	4	950-1 400
6	(CT) ₈ RC	CTCTCTCTCTCTCTRC	52.5	3	900-1 400
7	(CT) ₈ T	CTCTCTCTCTCTCTT	50.5	1	600
8	(CT) ₈ G	CTCTCTCTCTCTCTG	52.0	3	520-1 200
9	(AG) ₈ C	AGAGAGAGAGAGAGAGC	51.8	6	400-1 220
10	(CT) ₈ GT	CTCTCTCTCTCTCTGT	51.5	4	550-1 200
11	(AC) ₈ C	ACACACACACACACC	52.4	5	400-1 220
12	25CC16C6	AGGGCTGGAGGAGGGC	56.0	6	400-1 650
Total number of bands amplified				49	

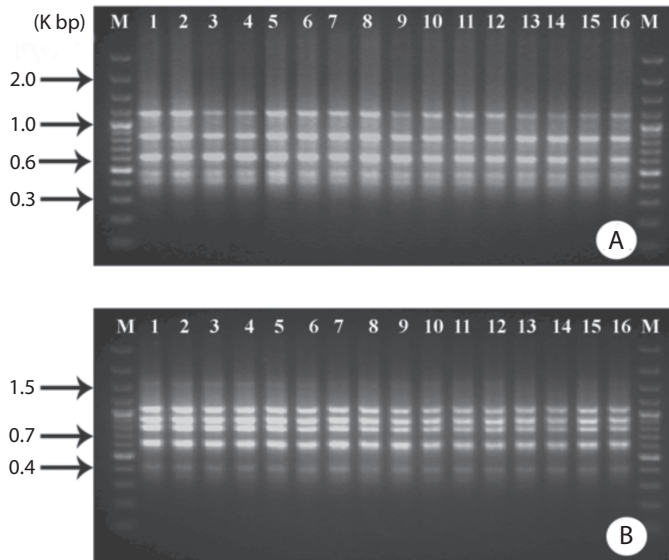


Fig. 3. ISSR banding pattern of *Vitex trifolia* with primer (A) - (AC) 8C and (B) (AG) 8C. (M - 100bp plus DNA ladder, Lane 1 - Donor plant, Lane 2 to 16 - callus regenerants).

DISCUSSION

The present study demonstrated the possibility of exploring the morphogenetic potential in callus derived from stem, petioles and leaves of *V. trifolia* with the application of various growth regulators. The importance of auxin and cytokinin balance has been reported in

regulating the apical dominance as well as morphogenetic events such as shoot formation and multiplication (Li & Bangerth 2003, Werner *et al.* 2003, Nakagawa *et al.* 2005). Also, another factor that may influence the morphogenetic events during *in vitro* culture is a possible interaction between the endogenous concentrations of plant growth regulators and those present in

the culture media (Mercier *et al.* 2003). Our results showed that BAP was more effective for shoot bud proliferation than kinetin also corroborated in Samantaray & Maiti (2008). Moreover, the concentrations 11.10-13.32 μ M of BAP in the medium favored the shoot bud proliferation and multiplication, which could be the optimum BAP concentration for cytokinin and endogenous auxin balance resulting in shoot bud regeneration. However, in the present study, combination of high concentrations of BAP with low concentrations of NAA enhanced the shoot bud multiplication as reported earlier (Prakash & Staden 2008, Amoos *et al.* 2009). The usefulness of different combinations of auxin and cytokinin for the production of organogenic and subsequent plant regeneration was well documented (Irvani *et al.* 2010, Bantawa 2011). Besides, the combination of cytokinins and auxins sometimes triggered the rate of shoot bud regeneration in various medicinal plants (Sivanesan & Jeong 2007, Samantaray & Maiti 2008, Samantaray *et al.* 2009).

Though BAP and NAA played a significant role in shoot bud regeneration, however, addition of adenine sulphate and GA₃ in the culture medium resulted in quick growth of shoot buds within four weeks of culture which is corroborated with the findings of earlier reports (Mohan & Krishnamurthy 1998, Samantaray & Maiti 2010). The percentage of shoot bud regeneration and the frequency of regenerated shoot/culture varied significantly in stem, petiole and leaf explants. The use of various explants for the production of callus and subsequent regeneration of shoot buds was well documented (Arora & Bhojwani 1989, Cacho *et al.* 1991). The differential response could be due to the growth regulators in the medium and explant types (Cacho *et al.* 1991, Pellegrineschi & Tepfer 1981). Differentiation of callus into green nodular structures followed by dark shoot buds were achieved in the media supplemented with high concentration of BAP and low concentration of NAA as reported earlier (Saxena *et al.* 1997, Samantaray & Maiti 2011). The regeneration frequency in

cultures from stem, petiole and leaf explants was found to be best under 16h photoperiod as compared to the cultures under continuous light which was not conducive to shoot bud regeneration and multiplication. Similar effects of photoperiod on shoot bud regeneration were observed in *Sesbania rostrata* (Perez-Bermudez *et al.* 1984), *Actinidia deliciosa* (Muleo & Morini 1990), *Lavandula latifolia* (Calvo & Segura 1989) and *Trema orientalis* (Samantaray *et al.* 1995).

Optimal rooting and growth of microshoots were observed on medium containing low concentration of IBA without intervening callus. Similar observations were made in various medicinal plants such as *Vitex agnus-castus*, *Filipendula ulmaria*, *Chlorophytum arundiacum* (Balaraju *et al.* 2008, Yildirim & Turker 2009, Samantaray & Maiti 2011). On the other hand, the irradiance showed significant effects on rooting induction (Sivanesan & Jeong 2007). The rooting in microshoots performed better under 16h photoperiods compared to continuous light. The rate of rooting dependent on growth regulators and photoperiod was also well documented (Murashige 1974, Rout *et al.* 1995).

The occurrence of genetic defects arising from variation in the regenerants seriously affects the true-to-type nature of the plants, which in turn hampers the production of a specific secondary metabolite of commercial value. Hence, the production of genetic uniform and stable plants is a prerequisite for commercial purposes (Shu *et al.* 2003). Though many PCR-based techniques such as SSR, RAPD and AFLP have been used to determine somaclonal variation of regenerants, RAPD and ISSR markers were successfully employed for the detection of variations at the genome level among tissue cultured regenerants (Taylor *et al.* 1995, Choudhuri *et al.* 2009). The variations can be due to gene amplification, chromosomal irregularities, point mutation and alteration in DNA methylation during *in vitro* culture (Saker *et al.* 2000). In the present study, amplification of genomic DNA of the arbitrarily selected *in vitro* derived progenies and

that of the donor plant of *V. trifolia* was similar which indicates the true-to-type nature of the progenies (Samantaray & Maiti 2008, 2010).

In conclusion, we established a suitable regeneration system protocol for *V. trifolia*, a potential medicinal plant, via organogenesis using three different explants viz. stem, leaf and petiole which may be very much useful for mass propagation and genetic transformation. Our results indicate the organogenic potential of *V. trifolia* is greatly influenced by NAA and BAP. Besides, the efficient usefulness of RAPD and ISSR techniques in assessing the true-to-type nature of the regenerated clones of *V. trifolia* was confirmed.

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RESUMEN

Vitex trifolia es una especie arbustiva de uso popular como planta medicinal, sus hojas, raíces y flores se han reportado para la cura de diferentes afecciones. El aumento de la explotación de estas plantas ha puesto en peligro su conservación y ha justificado el uso de herramientas biotecnológicas para su propagación. El objetivo de esta investigación fue presentar un protocolo eficiente para la regeneración de estas plantas a través de la organogénesis, y analizar la homogeneidad genética de las líneas clonales establecidas por ADN polimórfico amplificado aleatoriamente (RAPD) mediante la repetición de marcadores de inter secuencia simple (ISSR). La regeneración de plántulas se logró en cultivos de callos derivados de explantes de tallo, hoja y pecíolo de *V. trifolia* en un medio diferenciado Murashige & Skoog, que se incubaron a 25±2°C bajo una intensidad de luz de 61µmol/m²s con lámparas fluorescentes blancas y un fotoperíodo de 16h. La tasa de regeneración de brotes se correlacionó positivamente con la concentración de las hormonas en el medio nutritivo. Los brotes se regeneraron más rápidamente a partir de explantes de tallo y pecíolos en comparación con explantes de hoja. La mayor tasa de regeneración de brotes se obtuvo en los explantes de tallo utilizando 11.10µM BAP en combinación con 0.54µM NAA, 271.50µM AdS y 1.44µM GA3. Este protocolo puede ayudar a la propagación masiva y conservación de esta importante planta medicinal de gran potencial terapéutico.

Palabras clave: *in vitro*, repetición inter secuencia simple, plantas medicinales, micropropagación, ADN polimórfico amplificado aleatoriamente, regeneración plantas.

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