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ORIGINAL PAPER

In vitro clonal propagation of *Clerodendrum serratum* (Linn.) Moon (barangi): a rare and threatened medicinal plant

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Abstract An in vitro process for rapid clonal propagation of *Clerodendrum serratum* (Linn.) Moon, a rare and threatened medicinal shrub, has been developed. Nodal stem segments having axillary bud, taken from field-grown plant, showed bud-break within 15 days of culture on modified Murashige and Skoog (MS) (Physiol Plant 15:473–497, 1962) medium supplemented with 0.25 mg/l each of 6-benzylaminopurine and indole-3-acetic acid along with 15 mg/l adenine sulphate (AdS). Regenerated shoots could be further multiplied on the same agarified morphogenetic medium in presence of 0.5 mg/l 2-chloroethyltrimethyl ammonium chloride with increased concentration of AdS, i.e., 30 mg/l. A group of five shoots used as inoculum produced on an average 4.98 new shoots per original shoot after 4 weeks of subculture. Shoots excised from cultures of proliferating shoots were rooted in half-strength MS medium having 1 mg/l indole-3-propionic acid. In vitro rooted shoots—plantlets—grew luxuriantly under field conditions and came to flowering after 10 months of transplantation. The genetic fidelity of in vitro-raised field-grown plants and their mother plant was ascertained by random amplified polymorphic DNA markers. The protocol developed holds good for in vitro cloning of *C. serratum*.

Keywords 2-Chloroethyltrimethyl ammonium chloride · Cloning · Genetic fidelity · Nodal stem segment

Abbreviations

AdS	Adenine sulphate
BAP	6-Benzylaminopurine
CCC	2-Chloroethyltrimethyl ammonium chloride
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPA	Indole-3-propionic acid
MS medium	Murashige and Skoog (1962) medium
NAA	α-Naphthaleneacetic acid
NOA	α-Naphthoxyacetic acid
RAPD	Random amplified polymorphic DNA

Introduction

Clerodendrum serratum (L.) Moon (Verbenaceae) is a small perennial shrub growing in moist deciduous forests and occasionally in plains of peninsular India and the Western and Eastern Himalayas up to 1,400 feet above sea level. The leaf and root of this plant have great medicinal value. Root bark contains mainly sapogenins (Rangaswami and Sarangan 1969), while leaves contain flavonoids and phenolic acids (Rastogi 1999). The root has been traditionally used in Ayurveda and Siddha systems of medicine for treatment of chronic bronchial asthma and other respiratory diseases, different types of fevers and skin infections. It is one of the ingredients of the ayurvedic drug, "Kasadamana," an effective expectorant and antitussive remedy (CSIR 2001).

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It also forms an ingredient of the Siddha drug, "Siruteku," with significantly high antibacterial properties (Narayanan et al. 2004). It is an excellent antihistaminic and antiallergic agent (Gupta and Gupta 1968; Gupta et al. 1967) as well as an immunomodulator (Juvekar et al. 2006) and also has antinociceptive and anti-inflammatory properties (Narayanan et al. 1999).

Although *C. serratum* is recorded to occur in South Africa, Madagascar, South Asia and South East Asian countries, in India, this species has been regionally assessed as "vulnerable" in Northern India (WWF India and ZOO/CBSG India 1997) and "endangered" in Chhattisgarh and Madhya Pradesh region (Ved et al. 2003). Over collection and unsustainable extraction of wild plants for medicine and trade coupled with poor seed viability are the major factors for the continuous decline of its natural populations in India. Hence, development of an in vitro method for rapid clonal multiplication of *C. serratum* is warranted for providing uniform raw material for medicinal uses as well as for its conservation.

Presently, there is no published report on in vitro clonal propagation of *C. serratum.* In the present communication, an in vitro method for rapid clonal propagation of *C. serratum* employing nodal stem segments is reported for the first time.

Materials and methods

Establishment of aseptic shoot culture

Growing twigs from fresh flush of shoots were collected from a 3-year-old plant growing in the herbal garden of Deendayal Research Institute, Chitrakoot, Madhya Pradesh. Explants comprising single node stem segments of 2–3 cm in length were washed thoroughly in running tap water for 2 h, pretreated with 5% v/v Labolene (GlaxoSmithKline Pharmaceuticals Ltd, Mumbai, India) solution containing tween 20 (two drops per 100 ml of solution) for 10 min and surface-sterilized with 0.1% $HgCl_2$ solution for 15 min. The surface-sterilized explants were trimmed at the cut ends and cultured in the nutrient medium.

Culture media and conditions

The modified Murashige and Skoog (1962; MS) medium was used in the present investigation, which differed from the original MS medium in having the following: $MgSO_4{\cdot}7H_2O$ 450 mg/l, thiamine–HCl 0.2 mg/l, pyridoxine–HCl 0.1 mg/l, Glycine 3 mg/l, $(NH_4)_2SO_4$ 250 mg/l and ascorbic acid 10 mg/l. The pH of all the media was adjusted to 5.8 before adding 0.75% (w/v) Qualigens agar (GlaxoSmithKline Pharmaceuticals Ltd, Mumbai, India) and autoclaved at 121°C (1.08 kg cm^{-2}) for 15 min. The cultures were incubated under 25 μmol m^{-2} s^{-1} quantum flux density for 15 h/day at 27 ± 1°C temperature and 70 ± 5% relative humidity.

Shoot induction and multiplication

For bud-break nodal stem segment explants were cultured in modified MS medium supplemented with 0.25 mg/l 6-benzylaminopurine (BAP), 0.25 mg/l indole-3-acetic acid (IAA) and 15 mg/l adenine sulphate (AdS). The regenerated shoots having two nodes were excised and subcultured in the same medium for induction of multiple shoots. For augmenting the growth and rate of multiplication of regenerated shoots, 2-chloroethyltrimethyl ammonium chloride (CCC) at the concentration of 0.5 mg/l was incorporated in the same medium. In addition, the effect of different concentrations of adenine sulphate, i.e., 15, 30 and 50 mg/l, was also monitored for further improving the response. The proliferating shoots were subcultured on fresh medium as single shoot or in a group of five and ten shoots to study the effect of initial inoculum on the rate of shoot multiplication.

Rooting of shoots

For root induction, approximately 3-cm-long shoots, excised from cultures of proliferating shoots, were inoculated on half-strength MS medium supplemented with different auxins, namely, IAA, indole-3-butyric acid (IBA), indole-3-propionic acid (IPA), α-naphthaleneacetic acid (NAA) and α-naphthoxyacetic acid (NOA) at the concentration of 0.25 mg/l. In case of IAA and IPA, other concentrations (0.1, 0.5, 0.75, 1 and 1.25 mg/l) were also used besides 0.25 mg/l.

Acclimatization of in vitro-raised plantlets

The rooted shoots—plantlets—were taken out from the culture tubes, their root system washed off under running tap water to remove traces of medium and transplanted in a potting mixture comprising garden soil and leaf mold (3:1) in 10-cm earthen pots and kept for 30 days in hardening room where they were acclimatized by growing them first in high humidity (99% RH) and gradually the humidity was reduced to 70% during a period of 30 days. The hardened potted plants were kept in glasshouse for about 2 months and finally transferred to field.

Molecular analysis

Total genomic DNA was extracted from young leaves of in vitro-raised field-grown plants of *C. serratum* and mother

plant by cytyl trimethyl ammonium bromide (CTAB) procedure (Saghai-Maroof et al. 1984). Ten arbitrary decamer primers (Bangalore Genei, India) were used for polymerase chain reaction (PCR). PCR was performed in 20-ml reaction mixture containing 5 ng template DNA, 1 unit of Taq DNA polymerase, 100 μM dNTPs, 1 μM primer, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9), 50 mM KCl and 0.01% gelatin. PCR amplification was performed using a PTC-100 Peltier Thermal Cycler (MJ Research, USA) under the following conditions: preheating for 4 min at 94°C; 45 cycles of 15 s at 94°C, 45 s at 36°C and 1.5 min at 72°C and elongation was completed by a final extension of 4 min at 72°C. After amplification, the PCR product was resolved by electrophoresis in 1% agarose gel with 1× Tris–borate–EDTA (TBE) buffer. Bands were visualized by staining with ethidium bromide (0.5 μg/ml) under UV light and photographed.

Statistical analysis

The data were collected after 4 weeks of culture for shoot regeneration as well as rooting in isolated shoots. There were 10 replicates per treatment and the experiments were repeated thrice. The results are expressed as a mean ± SE of three experiments. The data were analyzed by Student's unpaired *t* test and treatment mean values were compared at $P \leq 0.05$–0.001.

Results and discussion

Nodal stem segment explants cultured on modified MS medium supplemented with 0.25 mg/l each of BAP and IAA along with 15 mg/l AdS showed 25% bud-break within 15 days of culture and the regenerated shoots attained an average height of approximately 4 cm in an incubation period of 30 days (Fig. 1a). Regenerated shoots after excision from mother explants grew only in height and showed no proliferation in the same morphogenetic medium. Proliferation of shoots could be induced by incorporating 0.5 mg/l CCC in the morphogenetic medium in the presence of high concentration of AdS (30 mg/l). Further increase in the concentration of AdS from 30 to 50 mg/l was detrimental for growth of regenerated shoots. For induction and multiplication of shoots from various explants of woody species, superiority of BAP over other cytokinins has already been established (Murashige 1974; Chaturvedi et al. 2004). The usefulness of AdS for improved multiplication of shoots is well documented by many workers (Rao and Bapat 1978; Reuveni et al. 1990).

To achieve optimum shoot proliferation, different initial inoculums comprising single shoot as well as groups of five and ten shoots were cultured on the same morphogenetic medium supplemented with 0.25 mg/l each of BAP and IAA along with 30 mg/l AdS and 0.5 mg/l CCC. It was observed that a group of five shoots, when taken as initial inoculum, produced maximum number of shoots, i.e., 24.9 shoots that comes to 4.98 shoots per shoot (Fig. 1b), whereas a decline in the rate of proliferation of shoots was observed with initial inoculum of 10 shoots where a maximum of 30.4 shoots, i.e., only 3.4 shoots per shoot were formed (Table 1). More or less similar results have been reported in *Clerodendrum colebrookianum* by Mao et al. (1995). Furthermore, shoots produced from inoculum comprising a group of 10 shoots during successive subcultures exhibited the problem of vitrification probably due to crowding of shoots. However, no or very little proliferation was obtained when single shoot was cultured in the same treatment. Hence, the inoculum comprising a group of five shoots was found ideal for achieving optimum rate of multiplication of shoots during prolonged culture.

For root induction in excised shoots, amongst different auxins used at the concentration of 0.25 mg/l, IAA and IPA were found more effective, as 60% rooting was obtained in both the cases in comparison to IBA, where only 40% rooting was achieved, while in NAA and NOA callus was formed at the base of shoots with poor, i.e., 20% and no rooting, respectively. Hence, for improving the rooting percentage, different concentrations (0.1, 0.25, 0.5, 0.75, 1 and 1.25 mg/l) of only IAA and IPA were used (Table 2). Amongst IAA and IPA, the latter was found to be more effective as at 1 mg/l concentration of IPA 100% rooting was obtained (Fig. 1c). Furthermore, the number of roots formed per shoot was also maximum, i.e., 14.3 in this treatment, which resulted in more than 90% ex vitro survival of plantlets. Although IPA is a mild auxin, its effectiveness in rooting has been reported by Shanker Rai and Jagadish Chandra (1987) and Cheetam et al. (1992). Contrary to this, in case of other members of the family Verbenaceae, like *Vitex negundo*—a woody shrub (Sahoo and Chand, 1998)—and *Tectona grandis* (a large tree), poor rooting was observed in medium supplemented with IPA (Devi et al. 1994; Shirin et al. 2005). The in vitro-raised plants in field registered uniform luxuriant growth with 100% survival and came to flowering after approximately 10 months of transplantation (Fig. 1d). In the present study, no differences were observed between mother plant and plantlets regenerated from nodal stem segment explants by RAPD analysis. Out of 10 primers screened, six primers produced clear and scorable amplification products. Each primer produced a unique set of amplification products ranging in size from 300 to 2,500 bp (Fig. 1e with primer P8: AGGGCCGTCT). The number of bands for each primer varied from four in P2 to seven in P8. However,

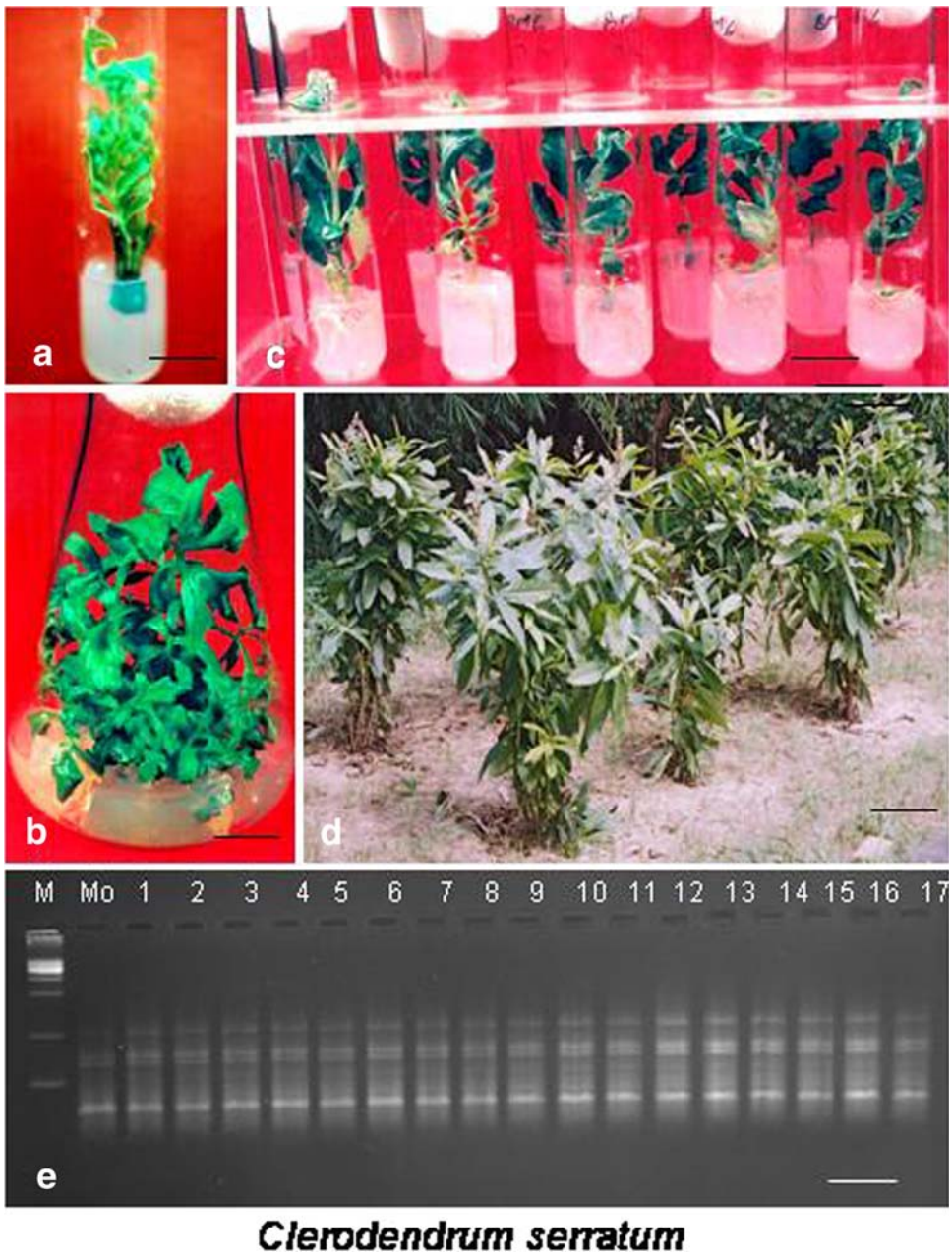

Fig. 1 In vitro cloning of *Clerodendrum serratum*. **a** Bud-break resulting into shoot formation in nodal stem segment, taken from field-grown plant, cultured on nutrient agar medium supplemented with 0.25 mg/l each of BAP and IAA and 15 mg/l AdS (*bar* 2.1 cm). **b** Proliferation of shoots in a medium containing 0.25 mg/l each of BAP and IAA, 0.5 mg/l CCC and 30 mg/l AdS (*bar* 1.85 cm). **c** Rooting in isolated shoots in half-strength MS medium supplemented with 1 mg/l IPA (*bar* 3.1 cm). **d** In vitro-raised plants in flowering after 10 months of transplantation in field (*bar* 25 cm). **e** RAPD marker analysis of in vitro-raised field-grown plants and mother plant; *lane M* 500-bp DNA size markers, *lane Mo* DNA from mother plant, *lanes 1–17* DNA from randomly selected regenerated plantlets

Table 1 Effect of initial number of shoots (inoculum) of *C. serratum* on multiplication after 4 weeks of culture

Number of shoots (inoculum)	Explants producing new shoots (%)	Average total number of shoots formed[a]
1	60	2.5 ± 0.68
5	100	24.9 ± 3.1**
10	100	30.4 ± 5.14**

[a] Values represent mean ± standard error of 10 replicates per treatment in three repeated experiments

** Significantly different at $P \leq 0.001$, according to Student's unpaired *t* test

no differences were observed between mother plant and plantlets regenerated from nodal stem segments by any six primers tested in present RAPD study. RAPD fingerprints have been shown to be useful to confirm the genetic fidelity of plantlets regenerated through in vitro culture (Rani et al. 1995; Chakrabarty et al. 2003; Dewir et al. 2005).

The present investigation provides a complete in vitro process, which is simple, reproducible and efficient for rapid clonal multiplication of an important rare and threatened medicinal shrub, *Clerodendrum serratum.*

Table 2 Effect of different concentrations of IPA and IAA as well as 0.25 mg/l of IBA, NAA and NOA on rooting of in vitro-regenerated shoots of *C. serratum* using half-strength MS medium after 4 weeks of culture

Auxin (concentration mg/l)					Rooting (%)	Number of roots/shoot[a]
IAA	IBA	IPA	NAA	NOA		
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.10					40	3.3 ± 2.10*
0.25					60	3.4 ± 2.62*
0.50					60	3.8 ± 2.65*
0.75					60	3.9 ± 1.17*
1.00					80	4.7 ± 2.3**
1.25					40	3.5 ± 2.13*
	0.25				40	2.5 ± 1.25*
		0.10			40	5.3 ± 2.80**
		0.25			60	7.1 ± 3.52**
		0.50			80	8.9 ± 4.20**
		0.75			80	9.4 ± 4.40**
		1.00			100	14.3 ± 3.49***
		1.25			40	1.5 ± 1.10*
			0.25		20	3.2 ± 1.12*
				0.25	0	0 ± 0.00

[a] Values represent mean ± standard error of 10 replicates per treatment in three repeated experiments

Significantly different at $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, respectively, according to Student's unpaired *t* test

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