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BRIEF COMMUNICATION

In vitro culture of *Capparis decidua* and assessment of clonal fidelity of the regenerated plants

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Abstract

A protocol for *in vitro* multiplication of *Capparis decidua* (Forsk.) Edgew. has been developed from cultured leaves procured from multiplying axillary shoots on the cultured nodal explants. The highest efficiency of shoot formation was observed on Murashige and Skoog (MS) medium containing 2 mg dm^{-3} benzyladenine (BA) and 0.5 mg dm^{-3} 1-naphthaleneacetic acid. The regenerated shoots were transferred to MS medium containing 3 mg dm^{-3} BA for growth and proliferation. Shoots above 2 cm in length were transferred to MS medium supplemented with 1 mg dm^{-3} indole-3-butyric acid plus 0.5 mg dm^{-3} indole-3-acetic acid for root induction. No variation was detected among the micropropagated plants by randomly amplified polymorphic DNA (RAPD) markers.

Additional key words: adventitious shoot regeneration, growth regulators, leaf explant, micropropagation, RAPD, root induction.

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Capparis decidua (Forsk.) Edgew. (family *Capparaceae*) is an important medicinal and agroforestry plant endemic to Thar desert of Asia (Khan and Frost 2001). It can thrive very well under drought conditions. It propagates at a very low rate in nature *via* root suckers and seed germination. The percentage of seed germination is poor due to low seed viability. The plant is facing depletion of natural populations due to overexploitation, change in climatic condition, cutting of plants in natural habitat for urbanization and agricultural reasons (Khan *et al.* 2003).

We have earlier reported micropropagation of *Capparis decidua* through shoot multiplication using nodal or shoot tip explants (Tyagi and Kothari 1997, 2001) and also by somatic embryogenesis (Tyagi *et al*. 2005). Leaves can provide an ideal explant for genetic transformation.

The tissue culture induced somaclonal variation is quite common and can pose a problem to the genomic integrity of regenerated plants (Soniya *et al.* 2001). Several strategies have been adopted to analyze the genetic stability of the tissue culture raised progenies (Mohammadi and Prasanna 2003, Joshi and Dhawan 2007). Randomly amplified polymorphic DNA (RAPD) analysis, which uses short primers of arbitrary sequences, has been found sensitive for detecting variations among individuals between and within species (Welsh and McClelland 1990). RAPD markers have been used successfully to assess genetic stability among micropropagated plants of many plant species (Soneji *et al.* 2002).

Objective of the present study was to regenerate adventitious shoot from leaves of *in vitro* growing axillary shoots and to ascertain their clonal fidelity. This method alleviates the need for regular supply of nodal segments from field grown trees as the *in vitro* leaf culture provide large number of true-to-type clonal plants.

The selected mother plant was approximately 20 years old elite tree naturally growing at Padampura Jaipur. It is less spine genotype with 4-m straight trunk and high yield of fruits. Regeneration from nodal segments of this elite mother plant was attempted as per the protocol described by us previously (Tyagi and Kothari 2001). Briefly, the procedure involved culturing of nodal explants from soft twigs of the mother tree, *in vitro* axillary shoot multiplication, rooting and subsequent hardening. The *in vitro* leaves were obtained from growing axillary shoots. These leaves required no sterilization; they were excised in laminar airflow bench and transferred to culture media.

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Abbreviations: BA - benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid; RAPD - randomly amplified polymorphic DNA.

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Basal nutrient medium used in this study was Murashige and Skoog (1962; MS) medium with 3 % sucrose (m/v) and 0.8 % agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C temperature and 1.2 - 1.3 kg cm^{-2} pressure for 20 min. Leaves were inoculated intact in 100 cm^3 *Borosil* Erlenmayer flasks. All cultures were maintained at 28 ± 1 °C under 16-h photoperiod and irradiance of 25 μmol m^{-2} s^{-1} (white fluorescent tubes). Different auxins indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and benzyl-adenine (BA) were incorporated in the basal media singly or in combinations (Table 1). Observations were taken on a weekly basis. Data were analyzed using one way analysis of variance (*ANOVA*) and mean values comparisons among treatments were made by the least significant difference test using 25 replicates.

The primary cultures were transferred to fresh medium after 5 weeks of incubation. In the subsequent passages the regenerating masses were divided into pieces and subcultured on medium containing 3 mg dm^{-3} BA for further multiplication The shoot regenerating clusters at the time of sub culturing had at least one or two shoot buds.

Elongated shoots (> 2.0 cm) derived from actively multiplying cultures were separated and cultured on MS medium supplemented with IBA or IAA (0.1 - 3 mg dm^{-3}) used alone or in different combinations (Table 2). Different conditions were screened for obtaining best root formation. Plantlets with fully developed root system were transferred to pots containing peat moss and garden soil.

For histological studies the leaf with adventitious buds was fixed in FAA (formaldehyde/glacial acetic acid/ 70 % ethyl alcohol 5:5:90; v/v). Dehydration was done through graded tertiary butyl alcohol (TBA) series and embedding was done in a saturated paraffin wax. Embedded material was sectioned at 10 μm thickness on a rotary microtome. Paraffin wax was removed by xylene and 1.0 % (m/v) safranine was used for staining. Tissues were thoroughly washed to remove excess stain and then dehydrated in a graded ethanol series.

DNA was extracted from fresh leaves by the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) derived from forty randomly selected *in vitro* raised plantlets from the first progeny of leaf derived plants and the stock plant raised by nodal explant which was source of leaf explant and also from the field grown mother plant. Another *C. decidua* plant which was shrub in appearance was selected as control. Approximately, 1 g of fresh leaves was ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to a 25 cm^3 tube with 5 cm^3 of CTAB buffer: 10 % (m/v) CTAB, 5 M NaCl, 50 mM EDTA, 1 M Tris-HCl pH 8.0, and 0.2 % (v/v) β-mercaptoethanol. The homogenate was incubated at 60 °C for 2 h, extracted with an equal volume of chloroform:isoamyl alcohol (24:1).

Twenty arbitrary 10-base primers (*Operon Technologies*, Alameda, USA) were used for polymerase chain reaction (PCR). Amplification reactions were performed in 1.25 mM dNTP, 20 ng of the primer, 1× Taq polymerase buffer, 0.5 U of Taq DNA polymerase (*Genei*, Bengaluru, India) and 40 ng of genomic DNA. DNA amplification was performed in a DNA thermocycler (*Corbett Research*, Sydney, Australia) programmed for 40 cycles: 1st cycle of 5 min at 95 °C, 1 min at 37 °C and 2 min at 72 °C; then 39 cycles each of 1 min at 95 °C, 1 min at 37 °C, 2 min at 72 °C followed by one final extension cycle of 7 min at 72 °C. Amplified products were electrophoresed in a 1.2 % (m/v) agarose (*Sigma*, St. Louis, USA) gels with 1× TBE buffer, stained with ethidium bromide, and photographed under UV radiation. The sizes of the amplification products were estimated according to a 100-bp ladder (*MBI Fermentas*, USA). All the reactions were repeated at least thrice. Amplified DNA markers were scored as band present or absent both in the regenerated and the mother plants. Electrophoretic DNA bands of low visual intensity were considered ambiguous markers and not scored.

The present investigation was carried out to explore the morphogenic potential of leaves of *C. decidua* by

Table 1. Adventitious shoot formation from cultured leaf explant of *Capparis decidua* after 5 weeks. Means ± SE, $n = 25$. Means followed by different letters are significantly different at $P = 0.05$ according to the least significant difference test.

Growth regulators [mg dm^{-3}]	Shoot number
BA 0.5	3.2 ± 1.3^{a}
BA 2.0	4.6 ± 2.8^{b}
BA 3.0	5.5 ± 3.0^{b}
BA 2.0 + NAA 0.1	12.8 ± 5.6^{c}
BA 2.0 + NAA 0.5	20.0 ± 2.0^{d}
BA 2.0 + IAA 0.1	9.1 ± 1.3^{e}
BA 2.0 + IAA 0.5	6.6 ± 2.7^{f}

Table 2. Rooting response of regenerated shoots on different hormonal combination in MS medium. Means ± SE, $n = 25$. Means followed by different letters are significantly different at $P = 0.05$ according to the least significant difference test.

IBA+ IAA [mg dm^{-3}]	Root number
0.5 + 0.1	3.5 ± 3.2^{a}
0.5 + 0.5	2.1 ± 0.8^{b}
0.5 + 1.0	7.0 ± 6.9^{c}
1.0 + 0.1	2.2 ± 1.2^{b}
1.0 + 0.5	11.8 ± 0.9^{b}
1.0 + 1.0	4.4 ± 7.0^{d}
2.0 + 0	6.2 ± 2.0^{e}
3.0 + 0	$3.6 \pm 3.0^{a\,g}$
1.0 + 0.1	5.5 ± 3.0^{f}
1.0 + 0.5	$4.1 \pm 3.4^{d\,g}$

using different combinations of growth regulators. The natural supply of leaves from the field plants is limited due to caducous nature of leaves. In the present experiment leaves were obtained from regenerated plants, there by maintaining the regular availability of explant material. Out of the various cytokinins tested, kinetin alone or in combination did not show any positive response of shoot bud formation. BA alone in concentration of 0.5 - 2.5 mg dm^{-3} initiated shoot buds (2 to 8) with or without green callus at the cut ends of the leaves. Combination of BA (0.5 - 2 mg dm^{-3}) with IAA or NAA (0.1 - 2 mg dm^{-3}) also induced shoot bud formation and this combination also did not favor callusing. Maximum direct shoot bud formation was observed on MS medium supplemented with 2.0 mg dm^{-3} BA and 0.5 mg dm^{-3} NAA (Fig. 1*A*,*B*) and developed shoots elongated up to 3 cm. All the shoots were transferred on medium with BA (3 mg dm^{-3}) only for further multiplication, and 25 to 30 shoots were induced per culture (Fig. 1*C*). Similar results have been reported in other plants (Gyves *et al.* 2001, Espinosa *et al.* 2006). The interaction of cytokinins with low concentration of auxin enhancing shoot multiplication has been reported by many workers (Sharma *et al.* 2003, Joshi and Kothari 2007, Santombi and Sharma 2008). The inclusion of higher concentration of auxin (either IAA or NAA) into the cytokinin rich medium inhibited not only shoot multiplication but also produced some compact callus at the base of the explants.

Elongated shoots derived from actively multiplying cultures were transferred for rooting on MS medium supplemented with IAA and IBA (Table 2). IAA alone (1 or 2 mg dm^{-3}) or in combination with IBA (0.1 to 0.5 mg dm^{-3}) induced roots along with callus. IBA (0.5 to 3 mg dm^{-3}) was more effective in root induction and developed 4 to 20 adventitious roots. IBA (2 mg dm^{-3}) was considered best in earlier studies (Tyagi and Kothari 1997, 2001). However, in this study when 1.0. mg dm^{-3} IBA was combined with 0.5 mg dm^{-3} IAA induction of thick healthy roots was effective in post transfer acclimatization similarly as shown in other tree species (Nanda *et al*. 2004).

This protocol required two steps: firstly initiation and multiplication of axillary shoots in nodal explant and later culture of *in vitro* grown leaves and formation of adventitious shoots. In this case it was essential to ascertain the genetic status of resultant progeny. RAPD markers were employed to characterize genetic similarity of mother plant and its progeny along with one intermediary plantlet which was initiated from nodal explant through axillary shoot multiplication. 8 out of 20 primers used in the study gave RAPD reproducible patterns, which generated bands in the size range of 0.2 to 2.8 kb for mother plant and its progenies (Table 3). Primer OPR-05 generated monomorphic bands. Seven primers were polymorphic in showing dissimilarity of the control plant with group of mother plant and its progenies. The primers OPP-12, OPE-12, OPA-3 (Fig. 1*D*,*E*) and OPS-13 were found highly reproducible. The number of bands per primer ranged from 3 in OPR-05 to 7 bands in OPA-03. The average number of bands per primer

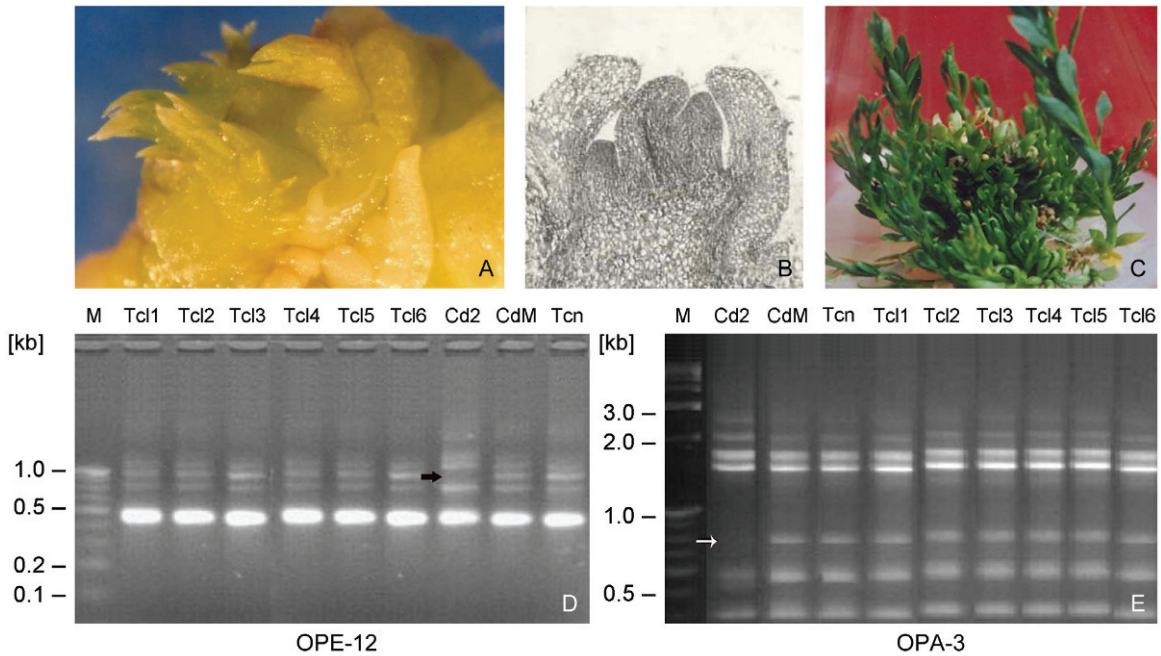

Fig. 1. *A* - Proliferation of adventitious buds from *C. decidua* leaves cultured on MS medium supplemented with 2 mg dm^{-3} BA and 0.5 mg dm^{-3} NAA after 6 week of culture. *B* - Histological section showing the initiation of the adventitious buds. *C* - Formation of multiple shoots on MS medium supplemented with 3 mg dm^{-3} BA. *D,E* - RAPD profiles of micropropagated plants from leaves and nodal segments using primers OPE-12 (*D*) and OPA-3 (*E*). *Lane* CdM shows RAPD bands from the mother plant. *Lanes* Tcl1 - Tcl6 and Tcn show RAPD products from micropropagated plants. *Arrows* indicate the DNA fragment absent in control (Cd2).

Table 3. RAPD analysis of the micropropagated and field grown mother plant.

Primer	Sequence 5' to 3'	Number of amplified bands	Fragments size [bp]
OPB-04	GGACTGGAGT	5	400-2700
OPP-12	AAGGGCGAGT	4	350-4000
OPE-12	TTATCGCCCC	4	400-3700
OPS-13	GTCGTTCCTG	5	270-1500
OPS-8	TTCAGGGTGG	6	400-2000
OPK-19	CACAGGCGGA	5	300-3500
OPR-5	CACAGCTGCC	3	270-3200
OPA-3	AATCGGGCTG	7	280-1500

was 5.6. The primers indicated absence of polymorphism in a total of 1617 loci derived from a combined analysis of RAPD markers of mother plant and its progenies. The control had different bands in respect to the *in vitro* grown plants (regenerated or mother *in vitro* plant). It was confirmed that micropropagated *C. decidua* plants obtained through leaves via adventitious shoot multiplication were genetically stable. Rady (2006), Feyissa *et al*. (2007) and Gagliardi *et al*. (2007) also confirmed complete similarity of adventitious shoot progeny with mother plant. Corresponding results were also observed in neem tree propagated *in vitro* (Singh *et al*. 2002), chestnut (Carvalho *et al*. 2004) and silver birch (Ryynanen and Aronen 2005). There are studies indicating that genetic variation is associated predominantly with plant regeneration from unorganized callus (Piola *et al*. 1999) but variations have been also reported in plantlets derived from axillary buds (Soneji *et al*. 2002) and adventitious shoots (Virscek-Marn *et al*. 1999).

Considering the importance of ensuring the genetic stability of *in vitro* plants in conservation programs (Ashmore 1997, Gagliardi *et al*. 2007), it is important to choose a regeneration procedure that does not allow or hinders induction of variation. Our experiments have shown that direct shoot formation can be induced in *C. decidua* leaf explants using MS medium containing BA in combination with NAA and demonstrated that *in vitro* leaves obtained from or near the apical meristem produced similar response. In this experiment from one leaf explant 2200 regenerated shoots were obtained in 13 weeks. The system appears to be highly effective and can be used for micropropagation and genetic transformation of *C. decidua*. Here the nodes from field grown tree are not required in large numbers as the culture of *in vitro* leaves provide large number of true to type clonal plants.

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