BIOLOGIA PLANTARUM 55 (1): 164-168, 2011

BRIEF COMMUNICATION

In vitro cloning of *Azadirachta indica* from root explants

K. ARORA[1], M. SHARMA[1], J. SRIVASTAVA[2], S.A. RANADE[2] and A.K. SHARMA[1]*

Tissue Culture Laboratory, National Botanical Research Institute, Lucknow-226001, India[1]
Plant Molecular Biology (Genomics) Division, National Botanical Research Institute, Lucknow-226001, India[2]

Abstract

In vitro cultures of *Azadirachta indica* A. Juss. were raised by first culturing the root segments on modified Murashige and Skoog (MS) medium supplemented with 8.88 μM 6-benzylaminopurine (BAP), 9.84 μM N^6-(2-isopentenyl) adenine (2iP), 5.71 μM indole-3-acetic acid (IAA), 81.43 μM adenine hemisulphate and 2.27 μM putrescine for 2 d followed by their transfer to the same medium except containing one-tenth of the initially used concentrations of BAP, 2iP and IAA. The regenerated shoots sustained proliferation in the basal medium supplemented with 1.11 μM BAP, 1.43 μM IAA and 135.72 μM adenine hemisulphate. The isolated shoots were rooted to produce plantlets in the presence of 2.46 μM indole-3-butyric acid (IBA). The plantlets showed uniform luxuriant growth under field conditions. True-to-type nature of the field-grown root-regenerated plants was ascertained by random amplified polymorphic DNA (RAPD) analysis.

Additional key words: auxins, cytokinins, genetic stability, neem, RAPD, root-regenerated plants.

_____

Azadirachta indica A. Juss. (family *Meliaceae*), popularly known as neem, is a multipurpose tree which has acquired significance for its commercial uses in pharmaceutical and agro-industries. The main limitations in sexual propagation are the recalcitrant nature of seeds with short span of viability and high heterozygosity (Ezumah 1986, Sacande *et al*. 2001). Vegetative propagation by cuttings, however, has constraints due to problems of rooting, availability of cuttings of right maturity, particular season of the year and presence of disease causing organisms within the material (Dogra and Thapliyal 1996). Therefore, it is imperative to use plant tissue culture methods. Micropropagation of neem tree through tissue culture has been reported by employing nodal stem segments (Chaturvedi *et al*. 2004b, Arora *et al*. 2010) and leaf segments (Ramesh and Padhya 1990, Arora *et al*. 2009). Here, we report an *in vitro* method employing root explants for cloning *A. indica* leading to production of true-to-type plantlets. The fidelity of the method has been proved by carrying out random amplified polymorphic DNA (RAPD) analysis of mother tree as well as of the plants regenerated through root segments.

Plantlets of *Azadirachta indica* A. Juss. were produced employing nodal stem segments of a 40-year-old tree following the protocol reported earlier by Arora *et al*. (2010). Roots measuring about 3 cm in length, excised from *in vitro*-grown plantlets were cultured in liquid medium which is a modification of Murashige and Skoog (1962; MS) medium except containing 6.25 mM NH_4NO_3, 4.95 mM KNO_3, 1.36 mM $CaCl_2 . 2\ H_2O$, 1.10 mM KH_2PO_4, 1.83 mM $MgSO_4 . 7\ H_2O$, 0.49 μM pyridoxine-HCl, 39.96 μM glycine, 2 % sucrose and addition of 0.35 mM Na_2SO_4, 0.76 mM $(NH_4)_2SO_4$, 23.73 μM L-arginine, 68.42 μM L-glutamine, 63.45 μM cysteine-HCl, 0.2 μM D-biotin, 0.13 μM riboflavin, 56.77 μM ascorbic acid and removing *m*-inositol, supplemented with 0.05 μM indole-3-butyric acid (IBA) and

_____

Received 15 April 2009, *accepted* 15 January 2010.
Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; IPA - indole-3-propionic acid; 2iP - N^6-(2-isopentenyl) adenine; MS medium - Murashige and Skoog medium; NAA - α-naphthaleneacetic acid; NOA - α-naphthoxyacetic acid; PVP - polyvinylpyrollidone; RAPD - random amplified polymorphic DNA; TDZ - thidiazuron; Z - zeatin.
Acknowledgements: The authors thank the Director, National Botanical Research Institute (NBRI), Lucknow for the facilities provided. The first author also thanks the Council of Scientific and Industrial Research (CSIR), India, for awarding her Senior Research Fellowship. Thanks are also due to Mr. R.S. Tripathi and Mr. O.P. Sharma for technical assistance.
* Corresponding author: fax: (+91) 522 2205536, e-mail: aks_nbri@rediffmail.com

adjusted to pH 5.2. These excised root cultures served as stock cultures for regeneration experiments employing root explants. Root cultures were subcultured after every 60 d.

Initially, for inducing differentiation in segments of roots, taken from 2-year-old root cultures, 6-benzylaminopurine (BAP) at different concentrations (0.55, 0.67, 0.88 and 1.11 µM) in combination with different concentrations of N^6-(2-isopentenyl) adenine (2iP) (0.62, 0.74, 0.98, 1.23 µM), with 0.57 µM indole-3-acetic acid (IAA) and 81.43 µM adenine hemisulphate was incorporated in regeneration medium. The regeneration medium is a modified MS medium with change in concentrations of NH_4NO_3 (18.74 mM), KNO_3 (14.79 mM), $Mg_2SO_4.7\,H_2O$ (1.83 mM), pyridoxine-HCl (0.49 µM) and addition of $Ca(NO_3)_2.4\,H_2O$ (0.42 mM), Na_2SO_4 (0.70 mM), K_2SO_4 (0.57 mM), L-arginine-HCl (47.45 µM), L-glutamine (171.06 µM), ascorbic acid (141.95 µM) and removing *m*-inositol. In addition, root explants were also subjected to a pulse treatment in which explants were firstly cultured on regeneration medium supplemented with a higher concentration of BAP (8.88 µM), 2iP (9.84 µM) and IAA (5.71 µM) along with 81.43 µM adenine hemisulphate for durations of 1, 2, 3, 4 or 5 d and subsequently cultured on the same medium but containing one-tenth concentrations of BAP, 2iP and IAA along with the same concentration (81.43 µM) of adenine hemisulphate.

For augmenting shoot bud differentiation as also differentiation of lesser number of aberrant regenerants, the effect of incorporation of 22.69 µM putrescine at initial step and after 2 d its one-tenth concentration (2.27 µM) at subsequent step, was studied. In addition, the effect of different lower concentrations (1.13, 2.27 and 5.67 µM) of putrescine at both the steps of pulse treatment (initial and after 2 d) was also investigated to further improve the response. Incubation period for each experiment was 60 d. Each experiment was set with 20 replicates having 3 segments of root in each and repeated twice.

For production of plantlets, about 3 cm long shoots, excised from 30-d-old cultures of proliferating shoots, were rooted in rooting medium which is another modification of MS medium, modified by reducing the concentrations of NH_4NO_3 (6.25 mM), KNO_3 (4.95 mM), $CaCl_2.2\,H_2O$ (1.36 mM), $Mg_2SO_4.7\,H_2O$ (0.61 mM) and pyridoxine-HCl (0.49 µM), adding $(NH_4)_2SO_4$ (0.76 mM), Na_2SO_4 (0.70 mM), ascorbic acid (56.77 µM), D-biotin (0.2 µM), L-arginine-HCl (47.46 µM) and removing *m*-inositol. Rooting medium is supplemented with 2.46 µM IBA. The isolated *in vitro*-rooted shoots (plantlets) were transplanted to soil in pots after being acclimatized in half-strength Knop's solution fortified with trace elements and iron of MS medium.

The pH of all media was adjusted, before adding 0.75 % (m/v) agar (*HiMedia* Laboratories, Mumbai, India) and the media were sterilized by autoclaving at 1.08 kg cm^{-2} for 15 min. Growth regulators and putrescine were filter (0.2 µm) sterilized and added to autoclaved medium after it had cooled to below 50 °C. Cultures were incubated under 25 µmol m^{-2} s^{-1} photon flux density for 15-h photoperiod except for excised root cultures which were maintained in total dark. Temperature and relative humidity of the culture room were maintained at 27 ± 1 °C and 70 ± 4 %, respectively.

For histological analysis differentiating root segments were fixed in FAA solution consisting of formalin: glacial acetic acid : alcohol (70 %) in the ratio of 4:6:90 (v/v). Standard procedure of paraffin embedding (Johansen 1940) was followed for the histological studies. Ethyl alcohol-xylol series were used for dehydration and infiltration. Sections of favourably responded and properly wax-embedded explants were cut at 10 µm thickness by a rotary microtome. They were processed and stained in Harris's haematoxylin and safranin, used as the differentiating stains. Permanent slides were made using Canadian balsam. Slides were observed under the light microscope (*Leica ATC 2000*, Wetzlar, Germany; software *Digi 3*) and photographs were taken.

Twelve field-grown root-regenerated plants were randomly selected for RAPD analysis. Total DNA was extracted from fresh, young leaf tissues of root-regenerated plants and mother tree (40-year-old tree) according to Farooqui *et al*. (1998) and based on method proposed by De Kochko and Hamon (1990) for cotyledon of seeds. RAPD analysis was carried out using 7 randomly chosen decamer primers (F and U kits) obtained from *Operon Technologies*, Alameda, USA. Polymerase chain reaction (PCR) was performed in accordance with the protocol of Williams *et al*. (1990) with modifications in annealing temperature (35 °C instead of 36 °C) and the durations of PCR steps of denaturation, annealing and extension that were reduced to 45 s, 45 s and 1 min instead of 1 min, 1 min and 2 min respectively in the original paper of Williams *et al.* (1990). DNA amplification was performed in a PTC-200, DNA engine, (*MJ Research*, USA) for 45 cycles. The amplifications were carried out in 0.025 cm^3 of final reaction volume containing 0.0025 cm^3 of 10× assay buffer, 0.002 cm^3 of 0.8 mM dNTP mix (0.2 mM each), 10 pmol of RAPD primer, 1.5 mM $MgCl_2$, 1.5 units of *Taq* DNA polymerase (*Bangalore Genei*, Bangalore, India) and 30 ng of genomic DNA. PCR was initiated by a denaturation step of 94 °C for 3 min followed by 44 cycles each, consisting of denaturation for 45 s, annealing at 35 °C for 45 s and extension at 72 °C for 1 min. The final cycle was allowed for 5 min at 72 °C extension. After completion of the PCR cycles, the amplification products were separated electrophoretically on 1.5 % agarose gel in 0.5× TBE buffer. Before loading the samples tracking dye was added to the sample. Low range DNA ruler (*Bangalore Genei*) was used as molecular size marker. After the completion of electrophoresis, the gel was stained in ethidium bromide solution and destained in sterilized single distilled water and it was photographed on UV transilluminator, using *Night Hawk*™ (Huntington

Station, USA) gel documentation system. All the reactions were repeated at least twice and only the consistently reproducible bands were considered.

Data were analyzed by analysis of variance (*ANOVA*) and a Duncan's multiple range test (DMRT). All statistical analyses were performed using the *SPSS* statistical software package.

Table 1. Effect of duration of pulse treatment on regenerant differentiation in root segments after 60 d. Means ± SE of 20 replicates, containing 3 segments each. Mean values followed by different superscript letters within a column are significantly different at $P \leq 0.05$, as determined by DMRT.

Duration of pulse [d]	Response [%]	Bud number [explant^{-1}]	Aberrant regenerants	Callusing
1	13.3	2.0 ± 0.27^{b}	6.2 ± 0.40^{d}	-
2	40.0	2.8 ± 0.19^{c}	5.7 ± 0.23^{bc}	-
3	30.0	2.3 ± 0.22^{b}	5.8 ± 0.14^{c}	+
4	6.6	2.2 ± 0.25^{b}	5.3 ± 0.09^{b}	+
5	0	0^{a}	0^{a}	-

Stock cultures of excised roots, which were maintained in root culture medium supplemented with 0.05 µM IBA showed sustained growth of roots without any decline for the past 2 years (Fig. 1*A*). In order to induce regenerant differentiation in segments of roots taken from 2-year-old excised root cultures, the root explants were cultured in different concentrations of BAP

Table 2. Effect of different concentrations of putrescine at initial and subsequent treatment on regenerant differentiation in root segments after 60 d. Means ± SE of 20 replicates, containing 3 segments each. Mean values followed by different superscript letters within a column are significantly different at $P \leq 0.05$, as determined by DMRT.

Initial conc. [µM]	Subsequent conc. [µM]	Response [%]	Bud number [explant^{-1}]	Aberrant regenerants
22.7	2.27	40	3.3 ± 0.18^{b}	1.8 ± 0.17^{b}
1.13	1.13	50	4.5 ± 0.51^{c}	1.4 ± 0.12^{b}
2.27	2.27	60	8.0 ± 0.14^{d}	1.1 ± 0.79^{ab}
5.67	5.67	20	1.2 ± 0.11^{a}	0.3 ± 0.04^{a}

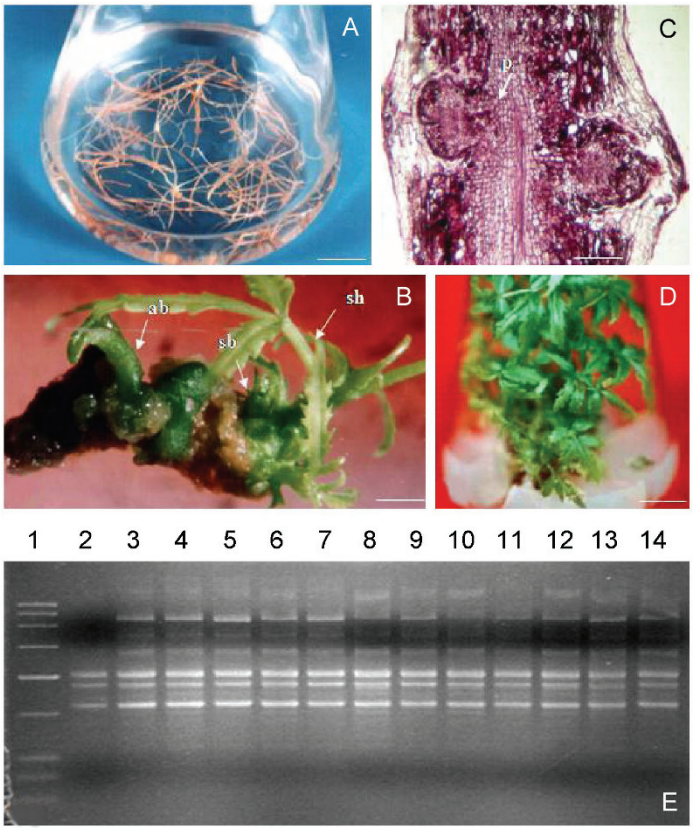

Fig. 1. *A* - Sustained growth of excised roots in prolonged culture (2 years) in root culture medium supplemented with 0.05 µM IBA (*bar* = 12.5 mm). *B* - Direct differentiation of shoot buds (sb), shoots (sh) and aberrant regenerants (ab) in root segment (*bar* = 0.69 mm). *C* - A longitudinal section of responded root explant showing endogenous differentiation of meristemoids from the pericycle (p) juxtaposed to the vascular tissue of explant beneath the cortical tissue (*bar* = 0.05 mm). *D* - Proliferation of shoots regenerated from segments of roots in regeneration medium supplemented with 1.11 µM BAP, 1.43 µM IAA and 135.72 µM adenine hemisulphate (*bar* = 15.3 mm). *E* - RAPD profile of field-grown plants of neem regenerated from root segments obtained with primer OPF-04. *Lanes*: 1 - low range DNA ruler, 2 - the mother plant, 3 to 14 - root-regenerated plants.

used in combination with different concentrations of 2iP along with 0.57 µM IAA and 81.43 µM adenine hemisulphate. Maximum regenerant differentiation was obtained in 0.88 µM BAP and 0.98 µM 2iP along with 0.57 µM IAA and 81.43 µM adenine hemisulphate but it was associated with callusing. The combinations of cytokinins effective for neem differentiation were reported by Ramesh and Padhya (1990) and Eeswara *et al*. (1998). Addition of adenine hemisulphate has also been reported to stimulate shoot bud formation (Ramesh and Padhya 1990, Vila *et al*. 2003). Intervening callusing during regenerant differentiation could be reduced to a good extent and the number of regenerants increased by giving pulse treatment in consonance with the requirement of explants to be exposed to a growth regulator for a minimum length of time for a given morphogenetic event to be initiated (George 1993). Sequential culture of explants from higher to lower concentration of morphogenetic stimuli has been found useful also in other plant species (Singh *et al*. 2002, Pati *et al*. 2004). The pulse treatment comprised culturing of explants initially in high concentrations of BAP, 2iP and IAA, *i.e.*, 8.88 µM, 9.84 µM and 5.71 µM, respectively, together with 81.43 µM adenine hemisulphate for different duration of time (1, 2, 3, 4 and 5 d) followed by their transfer to one-tenth concentrations of BAP, 2iP and IAA, *i.e.*, 0.88 µM, 0.98 µM and 0.57 µM, respectively, along with 81.43 µM adenine hemisulphate (Table 1). Considering different duration of time, the aforesaid pulse treatment applied for 2 d was found optimum for inducing direct regenerant differentiation from root explants without intervening callusing. However, in the differentiated regenerants, there was more number of aberrant regenerants (meristematic nodules and embryo-like structures) as compared to shoot buds. There are reports of simultaneous regeneration of more than one type of morphogenetic structures (D'Onofrio and Morini 2003/2004). Whilst shoot buds gave rise to shoots, the meristematic nodules underwent the process of organogenesis and consequently either budded into similar nodular structures or differentiated into roots rarely into shoot buds as well as bipolar structures having shoot and root on opposite ends giving rise to plantlets and the embryo-like structures showing arrested growth. Furthermore, it was observed that by incorporating 22.7 µM putrescine to the optimum pulse treatment at the initial step followed by its one-tenth concentration at the subsequent step, the process of regenerant differentiation was augmented in respect of formation of more number of shoot buds as compared to aberrant regenerants (Table 2). The promotive effect of exogenous polyamines on shoot regeneration has also been reported in *Brassica campestris* (Chi *et al*. 1994) and *Corylus avellana* (Nas 2004). The number of differentiated shoot buds could be increased further from 3.3 to 8.0 coupled with differentiation of lesser number of aberrant regenerants when the same lower concentration (2.27 µM) of putrescine was used at both the steps of optimum pulse treatment (Table 2; Fig. 1*B*). This is in agreement with the results obtained by Purohit *et al*. (2007) in *Achras sapota* in which putrescine in combination with BAP was found to be most effective in shoot multiplication. It has been reported that exogenous application of polyamine (putrescine) may reduce the production of ethylene and enhance morphogenesis (Bais *et al*. 2000). There are other reports also where root segments have been demonstrated to produce regenerants *in vitro*, as in case of *Populus tremula* (Vinocur *et al*. 2000), *Eleutherococcus koreanum* (Park *et al*. 2005) and *Lycium barbarum* (Hu *et al*. 2008).

Histological studies of differentiating root segments revealed the origin of regenerants from the pericycle tissue (Fig. 1*C*). The significance of shoot meristem differentiation from pericycle tissue lies in the fact that the direct regenerant differentiation from this tissue will result into the formation of true-to-type plants, since the pericycle tissue is genetically stable and represents true-to-mother type genetic make-up (Yeoman and Street 1977). The origin of regenerants from pericycle was also demonstrated in other woody plant species such as *Populus deltoides* (Chaturvedi *et al*. 2004a) and *Melia azedarach* (Vila *et al*. 2005).

Differentiated shoot buds developed into shoots which proliferated further in regeneration medium in the presence of 1.11 µM BAP, 1.43 µM IAA and 135.72 µM adenine hemisulphate (Fig. 1*D*). The isolated shoots rooted 100 % in rooting medium supplemented with 2.46 µM IBA. The *in vitro*-rooted shoots (plantlets) were hardened first in an inorganic salt solution under culture room conditions for about 30 d and then grown in potted soil under glasshouse conditions and after about 6 months of growth they were transplanted in the field. The root-regenerated plants in field showed uniform luxuriant growth.

One of the main prerequisite conditions for the development of process of cloning is the maintenance of genetic stability in tissue culture-raised plants. True-to-type nature of the plants produced in the present study was ascertained by RAPD analysis. Five primers, (OPF-01, OPF-03, OPF-04, OPU-19 and OPU-20) resulted in amplification of monomorphic profiles across all the root-regenerated plants and one such profile (obtained using primer OPF-04) shown in Fig. 1*E*. RAPD has shown to be an efficient molecular marker for determining the genetic diversity among accessions of *Curcuma* spp. (Hussain *et al*. 2008). Genetic stability of *in vitro*-grown plants has also been assessed in *Dictyospermum ovalifolium* (Chandrika *et al*. 2008) and *Platanus acerifolia* (Huang *et al*. 2009) by inter simple sequence repeat (ISSR) markers. RAPD method was chosen for its rapidity, sensitivity and easy-to-use characteristics. Our results with RAPD profiles of root-regenerated plants of neem show their true-to-type nature. Thus the results provide a strong support to the use of the proposed method for *in vitro* cloning of neem, because there do not exist any genetic variations in the regenerated plantlets during culture process.

References

Arora, K., Sharma, M., Sharma, A.K.: Control of pattern of regenerant differentiation and plantlet production from leaflet segments of *Azadirachta indica* A. Juss. (neem). - Acta Physiol. Plant. **31**: 371-378, 2009.

Arora, K., Sharma, M., Srivastava, J., Ranade, S.A., Sharma A.K.: Rapid *in vitro* cloning of a 40-year-old tree of *Azadirachta indica* A. Juss. (Neem) employing nodal stem segments. - Agroforest. Syst. **78**: 53-63, 2010.

Bais, H.P., Sudha, G.S., Ravishankar, G.A.: Putrescine and silver nitrate influences shoot multiplication, *in vitro* flowering and endogenous titers of polyamines in *Cichorium intybus* L. cv. Lucknow local. - J. Plant Growth Regul. **19**: 238-248, 2000.

Chandrika, M., Thoyajaksha, Ravishankar Rai, V., Ramachandra Kini, K.: Assessment of genetic stability of *in vitro* grown *Dictyospermum ovalifolium*. - Biol. Plant. **52**: 735-739, 2008.

Chaturvedi, H.C., Sharma, M., Sharma, A.K., Jain, M., Agha, B.Q., Gupta, P.: *In vitro* germplasm preservation through regenerative excised root culture for conservation of phytodiversity. - Indian J. Biotechnol. **3**: 305-315, 2004a.

Chaturvedi, R., Razdan, M.K., Bhojwani, S.S.: *In vitro* clonal propagation of an adult tree of neem (*Azadirachta indica* A. Juss.) by forced axillary branching. - Plant Sci. **166**: 501-506, 2004b.

Chi, G.L., Lin, W.S., Lee, JEE., Pua, E.C.: Role of polyamines on *de novo* shoot morphogenesis from cotyledons of *Brassica campestris* spp. Pekinensis (Lour) Olsson *in vitro*. - Plant Cell Rep. **13**: 323-329, 1994.

De Kochko, A., Hamon, S.: A rapid and efficient method for the isolation of restrictable total DNA from plants of genus *Abelmoschus*. - Plant mol. Biol. Rep. **8**: 3-7, 1990.

D'Onofrio, C., Morini, S.: Simultaneous regeneration of different morphogenic structures from quince leaves as affected by growth regulator combination and treatment length. - Biol. Plant. **47**: 321-325, 2003/2004.

Dogra, P.D., Thapliyal, R.C.: Gene resources and breeding potential. - In: Randhawa, N.S., Parmar, B.S. (ed.): Neem. Pp. 27-32. New Age International, New Delhi 1996.

Eeswara, J.P., Stuchbury, T., Allan, E.J., Mordue (Luntz), A.J.: A standard procedure for the micropropagation of the neem tree (*Azadirachta indica* A. Juss.). - Plant Cell Rep.**17**: 215-219, 1998.

Ezumah, B.S.: Germination and storage of neem (*Azadirachta indica* A. Juss.) seed. - Seed Sci. Technol. **14**: 593-600, 1986.

Farooqui, N., Ranade, S.A., Sane, P.V.: RAPD profile variation amongst provenances of neem. - Biochem. mol. Biol. Int. **45**: 931-939, 1998.

George, E.F.: Plant Propagation by Tissue Culture. Part I The Technology. - Exegetics, Edington 1993.

Hu, Z., Hu, Y., Gao, H.H., Guan, X.Q., Zhuang, D.H.: Callus production, somatic embryogenesis and plant regeneration of *Lycium barbarum* root explants. - Biol. Plant. **52**: 93-96, 2008.

Huang, W.J., Ning, G.G., Liu, G.F., Bao, M.Z.: Determination of genetic stability of long-term micropropagated plantlets of *Platanus acerifolia* using ISSR markers. - Biol. Plant. **53**: 159-163, 2009.

Hussain, Z., Tyagi, R.K., Sharma, R., Agrawal, A.: Genetic diversity in *in vitro*-conserved germplasm of *Curcuma* L. as revealed by RAPD markers. - Biol. Plant. **52**: 627-633, 2008.

Johansen, D.A.: Plant Microtechnique. McGraw-Hill Book Co., New York 1940.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nas, M.N.: Inclusion of polyamines in the medium improves shoot elongation in hazelnut (*Corylus avellana* L.) micropropagation. - Turk. J. Agr. Forest. **28**: 189-194, 2004.

Park, S.-Y., Ahn, J.-K., Lee, W.-Y., Murthy, H.N., Paek, K.-Y.: Mass production of *Eleutherococcus koreanum* plantlets *via* somatic embryogenesis from root cultures and accumulation of eleutherosides in regenerants. - Plant Sci. **168**: 1221-1225, 2005.

Pati, P.K., Sharma, M., Sood, A., Ahuja, P.S.: Direct shoot regeneration from leaf explants of *Rosa damascena* Mill. - *In Vitro* cell. dev. Biol. Plant **40**:192-195, 2004.

Purohit, S.D., Singhvi, A., Nagori, R., Vyas, S.: Polyamines stimulates shoot bud proliferation and multiplication in *Achras sapota* grown in culture. - Indian J. Biotechnol. **6**: 85-90, 2007.

Ramesh, K., Padhya, M.A.: *In vitro* propagation of neem, *Azadirachta indica* (A. Juss.), from leaf discs. - Indian J. exp. Biol. **28**: 932-935, 1990.

Sacande, M., Golovina, E.A., Van Aelst, A.C., Hoekstra, F.A.: Viability loss of neem (*Azadirachta indica*) seeds associated with memberane phase behavior. - J. exp. Bot. **52**: 919-931, 2001.

Singh, A.K., Chand, P.K., Patnaik, S., Chand, S.: Adventitious shoot organogenesis and plant regeneration from cotyledons of *Dalbergia sisoo* Roxb., a timber yielding tree legume. - Plant Cell Tissue Organ Cult. **68**: 203-209, 2002.

Vila, S.K., Gonzalez, A.M., Rey, H.Y., Mroginski, L.A.: *In vitro* plant regeneration of *Melia azedarach* L.: shoot organogenesis from leaf explants. - Biol. Plant. **47**: 13-19, 2003.

Vila, S.K., Gonzalez, A.M., Rey, H.Y., Mroginski, L.A.: Plant regeneration, origin, and development of shoot buds from root segments of *Melia azedarach* L. (*Meliaceae*) seedlings. - *In Vitro* cell. dev. Biol. Plant **41**: 746-751, 2005.

Vinocur, B., Carmi, T., Altman, A., Ziv, M.: Enhanced bud regeneration in aspen (*Populus tremula* L.) roots cultured in liquid media. - Plant Cell Rep. **19**: 1146-1154, 2000.

Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V.: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. – Nucl. Acids Res. **18**: 6531-6535, 1990.

Yeoman, M.M., Street, H.E.: General cytology of cultured cells. - In: Street, H.E. (ed.): Plant Tissue and Cell Culture Vol. 2. Pp. 137-176. Blackwell Scientific Publications, Oxford 1977.