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BRIEF COMMUNICATION

High frequency *in vitro* propagation of *Holarrhena antidysenterica* from nodal buds of mature tree

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Abstract

An *in vitro* method for propagation of *Holarrhena antidysenterica* Wall. has been developed using nodal explants from mature trees growing in the field. Irrespective of concentrations and combinations of growth regulators used, the axillary and terminal buds sprouted and elongated when inoculated on Murashige and Skoog (MS) medium. The highest numbers of shoots were formed when sprouted shoots were subcultured from MS basal medium onto MS medium containing 2 mg dm^{-3} N^6-benzyladenine (BA) and 0.5 mg dm^{-3} α-naphthalene acetic acid (NAA). The shoot number further increased upon subculture on MS medium containing 0.5 mg dm^{-3} BA. By repeated sub-culturing of shoots derived from nodal axillary buds, a high frequency multiplication rate was established. The elongated shoots were excised and rooted in auxin free MS basal medium. *Ex vitro* rooting of *in vitro* formed shoots was achieved upon dipping the microshoots for 2 min in 2 mg dm^{-3} of indole-3-butyric acid solution. Successful field establishment and high (80 - 90 %) survival of plants was observed.

Additional key words: benzyladenine, *ex vitro* rooting, indole-3-butyric acid, micropropagation, naphthalene acetic acid.

Holarrhena antidysenterica Wall. (*H. pubescence*) is a typical Indian medicinal plant widely used as a cure for dysentery and diarrhea. This medicinal plant is propagated by seeds but its seed viability and germinability are very poor. Clonal propagation through conventional methods of propagation like rooting of cuttings and grafting has not been successful (Ahmed *et al*. 2001). Although some attempts were on tissue cultures of *H. antidysenterica* (Panda *et al.* 1991, 1992a,b, Ahmed *et al.* 2001, Raha and Roy 2001, 2003, Agrawal *et al*. 2005), rapid *in vitro* propagation has not been achieved. In this paper, based on a detailed study, we report rapid *in vitro* production of *H. antidysenterica* using nodal buds of mature tree. We report the production of more number of shoots with high frequency of regeneration than the earlier reports (Ahmed *et al.* 2001, Raha and Roy 2001, Agrawal *et al*. 2005).

Nodal buds were collected from 15 - 20 year old trees from Tirumala forest of Andhra Pradesh, India, and were initially washed with running tap water for 15 min to remove the latex, and then with 5 % *Labolene* for 15 min followed by washing in running tap water and then 4 - 5 times with distilled water. Surface sterilization was done by treating explants with 0.1 % mercuric chloride for 2 min and with 70 % alcohol for 15 s and each surface sterilization was followed by 5 - 6 rinses in sterile distilled water. The explants were then inoculated on agar gelled medium in 25 × 150 mm culture tubes or 150 cm^3 conical flasks. Initial experiments were tried by incorporating N^6-benzyladenine (BA; 0.5 - 5 mg dm^{-3}) alone and in combination with α-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) (0.1 - 0.5 mg dm^{-3}) in Murashige and Skoog (MS) medium. After assessing the effect of BA on shoot multiplication on MS medium, its effects on the other basal media like Woody Plant medium (WPM; Lloyd and McCown 1980) and B5 (Gamborg *et al.* 1968) were tried along with different cytokinins like kinetin (Kn) and thidiazuron (TDZ). In

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Abbreviations: BA - N^6-benzyladenine; IBA - indole-3-butyric acid; IAA - indole-3-acetic acid; Kn - kinetin (6-furfuryl aminopurine); NAA - α-naphthalene acetic acid; MS - Murashige and Skoog (1962) medium; TDZ - thidiazuron.
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addition to this, growth adjuvants like coconut milk, casein hydrolysate, and antioxidants like ascorbic acid, polyvinylpyrrolidone (PVP) and activated charcoal were added to the media to improve the shoot initiation and multiplication efficiency. The medium was fortified with 3 % (m/v) sucrose and pH was adjusted to 5.6 to 5.8 with 0.1 M HCl and 0.1 M NaOH. The microshoots of 4 - 5 cm length were dissected from proliferating shoot cultures and inoculated on agar gelled medium containing various strengths of MS salts with or without auxins for *in vitro* rooting. To reduce time and cost and to increase the efficiency of micropropagation the *ex vitro* rooting of microshoots were also tried. Microshoots were initially dipped in 2 mg dm^{-3} indole-3-butyric acid (IBA) solution for 10 min, 5 min, and 2 min and then planted in plastic trays containing vermiculate. Microshoots dipped in IBA solution for 2 min developed roots and found useful for micropropagation. After inoculation all the cultures were incubated in a culture room at 25 ± 2 °C with a relative humidity of 50 - 60 % and 16-h photoperiod at photon flux density of 15 µmol m^{-2} s^{-1} from white cool fluorescent tubes. Each treatment contained 20 replicates and each experiment was repeated thrice. Statistical analysis was carried out using Tukeys test (Sokal and Rohlf 1998) in *SPSS* software package to test which groups are significantly different from each other and whether means can be divided into groups that are significantly different from each other.

Irrespective of concentrations and combinations of BA with other plant growth regulators the apical and axillary buds sprouted and elongated to 1.5 - 2.5 cm length on MS medium (Fig. 1*A*). Other cytokinins namely kinetin and TDZ along with BA, in combination with auxins (IAA and NAA) also showed similar effect. Hence the sprouted shoots from MS medium were excised and transferred on to MS shoot multiplication medium containing cytokinins and auxins for shoot culture. High frequency of regeneration (93.5 %) with maximum

Fig. 1. *A* - Single shoot initiation from axillary and terminal buds of mature explant grown on MS medium supplemented with 0.5 mg dm^{-3} BA; observations recorded after two weeks (*bar* = 3.8 mm). *B* - Induction of multiple shoots when grown on MS medium containing 2 mg dm^{-3} BA and 0.5 mg dm^{-3} NAA; observations recorded after four weeks (*bar* = 5.5 mm). *C* - Elongation of multiple shoots during sub-culture on MS medium containing 0.5 mg dm^{-3} BA; observation recorded after four weeks (*bar* = 6.2 mm). *D* - Healthy root growth on micro-shoots when culture on MS full strength basal medium; observations were recorded after six weeks (*bar* = 6.2 mm). *E* - *Ex vitro* rooting of *in vitro* formed shoots by dipping in 2 mg dm^{-3} IBA for two min before transferring them on vermiculite for acclimatization; Observations recorded after 20 d. *F* - Hardening of *in vitro* grown plants in root trainers containing *Vermiculite* and sand (1: 1); 10 d after transplantation.

Table 1. Effect of different concentrations of BA on induction of multiple shoots from mature nodal buds. Observations recorded after 8 weeks of culture, means ± SE, $n = 20$. Mean values having the same letter in each column do not differ significantly at $P < 0.05$ (Tukey test).

BA [mg dm^{-3}]	Frequency of regeneration [%]	Number of shoots [explants^{-1}]	Length of shoots [cm]
0.5	87.3 ± 0.20^{e}	16.7 ± 0.98^{c}	2.4 ± 0.13^{d}
1.0	82.2 ± 0.20^{c}	11.7 ± 0.39^{b}	1.5 ± 0.07^{b}
2.0	93.5 ± 0.10^{f}	53.2 ± 0.18^{e}	4.0 ± 0.02^{f}
3.0	85.3 ± 0.13^{d}	26.8 ± 0.35^{d}	1.9 ± 0.09^{c}
4.0	76.4 ± 0.08^{b}	11.0 ± 0.39^{b}	3.0 ± 0.11^{e}
5.0	70.4 ± 0.08^{a}	3.5 ± 0.29^{a}	0.8 ± 0.05^{a}

Table 2. Effect of combined application of BA along with NAA on induction of multiple shoots from mature nodal buds. Observations recorded after 8 weeks of culture, means ± SE, $n = 20$. Mean values having the same letter in each column do not differ significantly at $P < 0.05$ (Tukey test).

BA + NAA [mg dm^{-3}]	Frequency of regeneration [%]	Number of shoots [explants^{-1}]	Length of shoots [cm]
0.5 + 0.1	75.5 ± 0.16^{c}	16.0 ± 0.54^{f}	2.9 ± 0.17^{a}
0.5 + 0.5	82.4 ± 0.08^{e}	6.3 ± 0.33^{b}	4.2 ± 0.04^{d}
1.0 + 0.1	75.4 ± 0.08^{c}	10.4 ± 0.66^{c}	3.4 ± 0.11^{b}
1.0 + 0.5	82.5 ± 0.05^{e}	13.2 ± 0.48^{d}	5.7 ± 0.06^{g}
2.0 + 0.1	90.4 ± 0.52^{g}	41.0 ± 0.53^{g}	3.2 ± 0.06^{d}
2.0 + 0.5	90.5 ± 0.11^{g}	66.3 ± 0.32^{h}	4.4 ± 0.03^{e}
3.0 + 0.1	76.5 ± 0.13^{c}	13.3 ± 0.30^{d}	3.5 ± 0.03^{b}
3.0 + 0.5	80.5 ± 0.11^{d}	15.5 ± 0.47^{e}	3.6 ± 0.03^{b}
4.0 + 0.1	85.4 ± 0.19^{f}	6.1 ± 0.21^{b}	3.5 ± 0.03^{b}
4.0 + 0.5	50.5 ± 0.10^{b}	4.2 ± 0.02^{a}	4.1 ± 0.03^{c}
5.0 + 0.1	85.4 ± 0.14^{f}	4.8 ± 0.02^{a}	4.8 ± 0.02^{f}
5.0 + 0.5	30.5 ± 0.25^{a}	4.3 ± 0.024^{a}	4.2 ± 0.24^{d}

number of shoots (53.2 with mean shoot length of 4.0 cm) was formed on MS medium with 2 mg dm^{-3} BA and the shoot number decreased with further increase in the concentration of BA (Table 1). The shoot production further increased on MS medium containing BA (2 mg dm^{-3}) along with NAA (0.5 mg dm^{-3}) (Table 2, Fig. 1*B*). However, higher concentrations of BA along with NAA showed decrease in shoot number and increase in shoot length (Table 2). BA along with IAA was found effective in increasing shoot length considerably (data not shown). High frequency regeneration was achieved on MS medium supplemented with 2.0 mg dm^{-3} BA and 0.1 mg dm^{-3} IAA or 3.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IAA. Application of BA (2 mg dm^{-3}) along with 0.5 mg dm^{-3} NAA produced higher number of shoots than with 2 mg dm^{-3} BA plus 0.5 mg dm^{-3} IAA or BA alone. Initial study showed that MS medium was more effective in inducing multiple shoots than WPM and B5. Studies using kinetin (Kn) alone on MS medium did not evoke good shoot multiplication and had low frequency of regeneration (data not shown). However, application of BA (2 mg dm^{-3}) along with kinetin showed negative effect on frequency of regeneration and multiple shoot production. Studies using TDZ at concentrations equal to other cytokinins (Kn and BA) tested in the study did not show any response on multiple shoot production. Trials were also conducted by reducing the concentrations of TDZ in the medium. TDZ at 0.01 mg dm^{-3} produced 6.5 shoots but, high frequency of regeneration (60.4 %) was recorded on MS medium supplemented with 0.03 mg dm^{-3} TDZ (data not shown). Increasing concentration of TDZ in the MS medium decreased the shoot number, frequency of regeneration and shoot length. The highest mean shoot length (7.45 cm) was obtained on MS medium supplemented with 2 mg dm^{-3} BA along with 0.01 mg dm^{-3} TDZ. Studies on application of gibberellic acid, GA_3 (0.5 %) in combination with 2.0 mg dm^{-3} BA on shoot multiplication produced 9.8 axillary shoots per explant, and each shoot was 2.94 cm long. However, incorporation of GA_3 in the BA containing medium did not increase shoot length. Addition of 0.025 % casein hydrolysate or 10 % tender coconut milk (TCM) showed the formation of basal callus. Among all adjuvants tested in the study, casein hydrolysate was effective in inducing multiple shoots. Addition of ascorbic acid increased frequency of regeneration (96.5 %) with the lowest number of shoots per explant (data not shown). Rooting was observed on auxin free MS basal medium (Fig. 1*D*). Auxins (NAA, IAA and IBA) induced profuse callus rather than roots from the base of the shoots (data not shown). *Ex vitro* rooting of micro-shoots was achieved by dip treatment in IBA for 2 min just before transferring them onto hardening medium. Long, thick, brown, healthy roots produced during *ex vitro* rooting were better than those of *in vitro* rooting for establishment and survival of plants produced through micropropgation (Fig. 1*E*). The rooted micro-shoots were hardened (Fig. 1*F*) and had high (80 - 90 %) survival when transferred to the field.

Use of media like B5 and WPM in the present study did not favour shoot morphogenesis, instead they favoured the formation of profuse callus. The observations supported the view that certain species require ammonium or other source of reduced nitrogen for cell growth and differentiation. Formation of basal callus is a common observation in tissue culture (Bhattacharya and Bhattacharya 2001), perhaps due to the action of accumulated auxin at the basal cut end as on the cell proliferation especially in the presence of cytokinins (Marks and Simpson 1994). Basal callus formation is often observed in plants showing strong apical dominance (Preece and Sutter 1991). In the present study, formation of basal callus was also observed. In some plants it has not been possible to release axillary buds from apical dominance by manipulating the composition of the medium and the buds present on the initial explant grow to an unbranched shoot (Bhojwani and Razdan 1983). Unbranched solitary shoot formation was reported in *Ulmus punila* (Corchete *et al.* 1993). In such cases more

buds were induced to develop by removal of existing shoots and reculture of entire plant. In the present study, mature nodal buds sprouted and elongated without multiplication hence these were sub-cultured on fresh medium at multiplication stage. Highest number of shoots were developed adventitiously from the base of mature nodal bud derived shoots. In general, high concentration of cytokinin and low concentration of auxin are required in a medium to promote the induction of shoot morphogenesis (Kohlenbach 1997), and this was found true in the present study for shoot multiplication using mature nodal bud explants. BA was found superior to other cytokinins (kinetin and TDZ) used for shoot multiplication of *Holarrhena* as it has been documented for many other plant species (Maria *et al.* 1998). Although TDZ was beneficial shoot multiplication in other plants like *Rauvolfia tetraphylla* (Mohammad *et al*. 2005), it showed no effect on shoot multiplication of *Holarrhena.* Rapid and active induction of multiple shoots was observed on the MS medium fortified with BA along with NAA. The beneficial effect of BA-NAA combination for bud induction and multiplication has been reported for other plant species like *Pterocarpus* (Tiwari *et al*. 2004). Efficient rooting of micropropagated shoots and their field establishment are the critical stages for growing plants using tissue culture technology. In the present study, root induction was achieved on auxin free basal medium as in other studies made by Reddy *et al*. (2001) and Pyati *et al*. (2002). Rooting of micropropagated shoots on basal medium may be due to high endogenous levels of auxins in *in vitro* raised shootlets. Occurrence of profuse shoot basal callus from shoot bases on auxin supplemented medium supports the above hypothesis. The method of *ex vitro* rooting of *in vitro* raised shoots eliminated one set of labour costs and overheads, as rooting and acclimatization are effectively combined into single stage of micro-propagation process and the expensive growth room space is released for other purposes. *Ex vitro* rooting technique is, therefore, highly suitable for species which exhibit rooting easily and is advantageous for woody plants in which secondary thickening is important for proper root growth. In the present investigation, high frequency regeneration of roots with high mean number and length of roots were maintained by dipping micro-shoots in IBA solution for 2 min before planting them on *Vermiculite*. Similar *ex vitro* rooting response of *in vitro* propagated shoots were also reported for other plant species such as cherry species (Kris *et al*. 2005) and *Lagerstroemia parviflora* (Tiwari *et al*. 2002).

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Khan, J.A., Dijkstra, J. (ed.): **Handbook of Plant Virology. -** Food Products Press, An Imprint of the Haworth Press, New York - London - Oxford 2006. xviii + 452 pp., USD 69.95. ISBN 13: 978-1-56022-978-0.

The “Handbook of Plant Virology” represents a valuable source of information about selected topics in plant virology; it is a comprehensive guide to the terms and expressions of plant virology complete with descriptions of plant virus families down to the generic level.The book consists of two sections.

The first section (251 pp.) contains 19 chapters dealing with plant virus taxonomy, internal and external symptoms (with color illustrations), isolation and purification of plant viruses, architecture of plant viruses, replication and gene expression of RNA and DNA viruses and viroids, various methods of virus transmission (by arthropods, fungi, nematodes, seeds and mechanical), detection and identification of plant viruses and disease diagnosis, serology, ecology and epidemiology, recombination in plant viruses, virus variability and evolution, recombinant DNA technology, and control of virus diseases and economic impact.

The second section (190 pp.) is devoted to concise descriptions of the 81 genera and 18 families of plant viruses. It is divided into 5 appendixes: 1. Description of positive-sense, single-stranded RNA viruses (families *Potyviridae*, *Sequiviridae, Comoviridae, Luteoviridae*, *Tymoviridae, Tombusviridae, Bromoviridae, Closteroviridae, Flexiviridae,* and unassigned genera). 2. Description of double-stranded RNA viruses (families *Reoviridae, Partitiviridae,* and unassigned genus). 3. Description of negative-sense, single-stranded RNA viruses (families *Rhabdoviridae*, *Bunyaviridae,* and unassigned genera). 4. Description of single-stranded DNA viruses (families *Geminiviridae* and *Nanoviridae*). 5. Description of reverse-transcribing viruses (families *Caulimoviridae, Pseudoviridae* and *Metaviridae*)

The “Handbook of Plant Virology” provides comprehensive approach to a range of topics in plant virology, it is documented with photographs, illustrations, figures, diagrams, and brief, but detailed, bibliographies. This book is very clear, informative and invaluable for all people with an interest in plant virology, as well as for research workers, educators, and students working in plant pathology, crop protection, molecular biology, and plant breeding.

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