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BRIEF COMMUNICATION

Rapid *in vitro* propagation of *Holarrhena antidysenterica* using seedling cotyledonary nodes

K. MALLIKARJUNA* and G. RAJENDRUDU

Department of Botany, Sri Venkateswara University, Tirupati-522510, India

Abstract

A rapid *in vitro* propagation of *Holarrhena antidysenterica* has been developed. Seedling cotyledonary nodes on Murashige and Skoog medium (MS) containing 2 mg dm^{-3} N^6-benzyladenine (BA) produced highest number of multiple shoots. The shoot numbers were increased further upon subculture on MS medium supplemented with 0.5 mg dm^{-3} BA. By repeated subculture of derived shoots, a high multiplication rate was established. The excised shoots were rooted on MS basal medium without growth regulators. The *in vitro* formed shoots were also rooted *ex vitro* by dipping them in 2 mg dm^{-3} of indole-3-butyric acid (IBA) solution for 2 min before transferring them onto the hardening medium. Successful hardening and further establishment (survival 90 %) of micropropagated plants under natural conditions was observed.

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

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Holarrhena antidysenterica Wall. belonging to the family *Apocynaceae* is medicinally important tree species. The regeneration through seeds is not sufficient due to poor seed viability and dependence on season (Ravindra *et al.* 2005). Clonal propagation like rooting of cuttings and grafting has not been successful (Ahmed *et al*. 2001). Although a few attempts were made on *in vitro* propagation of *H. antidysenterica* (Ahmed *et al*. 2001, Raha and Roy 2001, 2003, Ravindra *et al.* 2005, Mallikarjuna and Rajendrudu 2007), studies on *in vitro* propagation using aseptic seedling explants are lacking. Earlier studies (Mallikarjuna and Rajendrudu 2007) have shown that mature nodal bud culture was effective for the production of genetically stable plants but the technique is rather difficult due to frequent contamination, presence of latex and highly differentiated tissue. However, aseptic seedling tissues are free from secondary metabolites and microbial contamination and hence widely used for micropropagation. In addition to this, *in vitro* culture using seedling results in the production of genetically variant plants which may contain desired traits. Nevertheless, the progeny of single (seed-derived) seedling should be genetically homogenous if multiplied *via* micropropagation *in vitro*. In this paper, based on detailed study, we report rapid *in vitro* propagation of *H. antidysenterica* using seedling explants, namely cotyledonary nodes.

Five hundred seeds of *Holarrhena antidysenterica* Wall. were collected from 15 to 20-year-old trees from Tirumala Hills, Andhra Pradesh, India and were initially washed with 5 % *Teepol* (*Chemisynth*, New Delhi, India) for 15 min followed by washing in running tap water and finally 4 - 5 times with distilled water. The seeds were surface sterilized by dipping in 0.1 % $HgCl_2$ solution for 5 min and in 70 % alcohol for 15 s. Each surface sterilization was followed by 5 - 6 rinses in sterile double distilled water. The seedlings were grown in test tubes containing 10 - 15 cm^3 of water-agar medium (0.8 % agar and 2 % sucrose), Murashige and Skoog (1962; MS) liquid medium and MS half strength liquid medium, separately.

Different explants like shoot tips, cotyledonary nodes, cotyledon and hypocotyl segments were excised from 15- to 20-d-old seedlings and these were cultured on selected media to assess their morphogenic response. Initial experiments were conducted using N^6-benzyl-

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Abbreviations: BA - N^6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; CH - kasein hydrolysate; CM - coconut milk; Kn - kinetin (6-furfuryl aminopurine); MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; TDZ - thidiazuron.

* Corresponding author's present address: Department of Botany, Acharya Nagarjuna University, Nagarjuna Nagar-522510, India; e-mail: mallikarjunaanu@gmail.com

adenine (BA; 0.5 - 5.0 mg dm^{-3}) alone or in combination with α-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) in MS medium. The effect of BA in 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 addition to this, growth adjuvants like coconut milk (CM) and casein hydrolysate (CH) 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 - 5.8 with 0.1 M HCl and 0.1 M NaOH. All cultures were incubated at 25 ± 2 ºC, a relative humidity of 50 - 60 % and 16-h photoperiod (irradiance of 15 μmol m^{-2} s^{-1}; cool white fluorescent tubes). 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 the time and cost and to increase the efficiency of micropropagation *ex vitro* rooting of microshoots was also attempted. Microshoots were initially dipped in 2 mg dm^{-3} of IBA solution for 10 min, 5 min and 2 min and then planted in plastic trays containing *Vermiculite* (Mallikarjuna and Rajendrudu 2007). For acclimatization, 1-month-old plantlets were removed from the medium, thoroughly washed with sterile water, dipped for 15 min in 0.1 % (m/v) *Bavistin* (*BASF India*, Hyderabad, India) and transplanted to plastic pots containing autoclaved *Soilrite (Keltech Energies*, Bangalore, India) for 1 month. The plantlets were irrigated with quarter strength MS solution without sucrose for one week, then with tap water, after which they were gradually transferred to *in vivo* conditions. Each treatment contained 20 replicates and each experiment was repeated thrice. Statistical analysis was carried out using Tukey´s test (Sokal and Rohlf 1998) in *SPSS* software package (*SPSS Inc*, Bangalore, India) 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.

Seed germination and growth of seedlings was found effective on MS liquid medium (Fig. 1*A*). Among the various explants inoculated, shoot induction was found only with cotyledonary nodes while others formed callus. Shoot tips showed only elongation without shoot multiplication. Hence, further studies on shoot multiplication were carried out using cotyledonary node explants.

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

BA [mg dm^{-3}]	Regeneration frequency [%]	Number of shoots [$explant^{-1}$]	Length of shoots [cm]
0.5	85.4 ± 0.14^{c}	4.7 ± 0.33^{a}	1.3 ± 0.10^{b}
1.0	59.3 ± 0.17^{a}	25.8 ± 0.34^{d}	3.0 ± 0.28^{c}
2.0	94.3 ± 0.10^{e}	60.1 ± 0.42^{e}	4.7 ± 0.04^{d}
3.0	86.4 ± 0.11^{d}	8.2 ± 0.23^{b}	0.8 ± 0.04^{a}
4.0	86.7 ± 0.72^{d}	8.5 ± 0.24^{b}	1.5 ± 0.04^{b}
5.0	72.4 ± 1.07^{b}	13.2 ± 1.07^{c}	1.2 ± 0.08^{b}

Table 2. Effect of application of BA along with NAA on induction of multiple shoots from cotyledonary nodes. Observations recorded after 8 weeks of culture. Means ± SE, n = 20. Means having the same letter in each column do not differ significantly at $P < 0.05$.

BA [mg dm^{-3}]	NAA [mg dm^{-3}]	Regeneration frequency [%]	Number of shoots [$explant^{-1}$]	Length of shoots [cm]
0.5	0.1	80.5 ± 0.15^{d}	13.5 ± 0.15^{c}	4.4 ± 0.12^{e}
0.5	0.5	76.7 ± 0.11^{c}	5.9 ± 0.34^{a}	3.8 ± 0.03^{c}
1.0	0.1	85.5 ± 0.12^{f}	16.0 ± 0.23^{d}	3.7 ± 0.04^{b}
1.0	0.5	91.4 ± 0.13^{h}	18.5 ± 0.23^{e}	5.1 ± 0.03^{f}
2.0	0.1	91.1 ± 0.21^{h}	40.1 ± 0.54^{g}	5.8 ± 0.06^{g}
2.0	0.5	77.3 ± 0.10^{c}	19.5 ± 2.18^{f}	4.6 ± 0.04^{e}
3.0	0.1	77.3 ± 0.19^{c}	20.0 ± 2.10^{f}	2.3 ± 0.04^{a}
3.0	0.5	82.4 ± 0.10^{e}	20.0 ± 0.29^{f}	2.4 ± 0.05^{a}
4.0	0.1	56.3 ± 0.14^{a}	13.0 ± 0.12^{c}	3.7 ± 0.03^{b}
4.0	0.5	60.4 ± 0.14^{b}	9.85 ± 0.23^{b}	3.7 ± 0.06^{b}
5.0	0.1	86.4 ± 0.12^{g}	9.0 ± 0.18^{b}	4.8 ± 0.04^{e}
5.0	0.5	82.5 ± 0.09^{e}	6.4 ± 0.25^{a}	4.2 ± 0.03^{d}

Initial experiments were conducted using MS with different concentrations of BA. Cotyledonary nodes on MS medium without growth regulators formed only two shoots. Cotyledonary nodes produced highest number (60.1) of multiple shoots on MS medium with 2.0 mg dm^{-3} BA with high frequency (94.3 %) of regeneration (Fig 1*B,* Table 1). When compared with our previous report (Mallikarjuna and Rajendrudu 2007), cotyledonary nodes responded better than the mature nodal buds, but this decreased at higher concentrations of BA. 2.0 mg dm^{-3} BA on other basal media like WPM and B5 tested in the study did not show marked influence on shoot multiplication and frequency of regeneration. BA (2.0 mg dm^{-3}) along with NAA did not show marked effect on shoot number but increased shoot length (Table 2). BA in combination with NAA produced shoots with thick leaves, fleshy internodes with basal callus. High frequency (94.4 %) of regeneration was achieved on MS medium supplemented with 5 mg dm^{-3} BA along with 0.1 mg dm^{-3} IAA (Table 3). BA (2.0 mg dm^{-3}) in combination with IAA was less effective in shoot multiplication when compared to BA + NAA and BA alone (Table 3). BA (2.0 mg dm^{-3}) in combination with 3 mg dm^{-3} Kn produced shoots with highest length (6.8 cm) but showed decrease in shoot number and shoot length at higher concentrations (data not shown). Kn alone or in combination with BA was also less effective in shoot production compared to BA. Less number of shoots with low frequency of regeneration (48.3 %) was

observed on MS medium supplemented with TDZ (data not shown). Addition of CH or CM caused the development of shoots with profuse callus. The explants showed a decrease in regeneration potential when subcultured continuously in the same regenerating medium. The lower concentration of BA than that used in the initial culture was effective in shoot multiplication. BA at 0.5 mg dm^{-3} was found optimal for shoot induction during subculture (Fig. 1*D*).

Roots were readily initiated from shoots within two weeks after their transplantation onto auxin free MS basal medium (Fig. 1*E*). Auxins (NAA, IAA and IBA) showed the induction of profuse callus rather than roots from base of shoots. *Ex vitro* rooting of microshoots was achieved by dip treatment in 2 mg dm^{-3} of IBA for 2 min just before transferring them onto hardening medium (Mallikarjuna and Rajendrudu 2007). The rooted microshoots were hardened (Fig. 1*F*) and 80 - 90 % of them survived when transferred to the field.

Most of the studies carried out on tree species have utilized seeds and juvenile tissue that are more amenable to *in vitro* manipulations and are more responsive than tissue from mature plants (*e.g.* Salahuddin *et al*. 2005, Pranati *et al*. 2007). In addition to this, seedling tissues are free of endophytic microbes and phenolic compounds. Hence, in the present study juvenile explants namely cotyledonary nodes were selected.

Tissues from different plant organs may need different nutritional requirements for normal growth (Murashige and Skoog 1962). Therefore, it is essential to standardize the medium that can fulfill the specific requirements of selected tissue (Bhojwani and Razdan 1983). In the case of *H. antidysenterica*, optimum response was found on MS medium with 3 % sucrose and 0.8 % agar. This may be attributed to a greater demand of nitrogen and potassium containing compounds, which induce a great amount of new proteins (Guru *et al*. 1999). These components are lower in B5 and WPM as

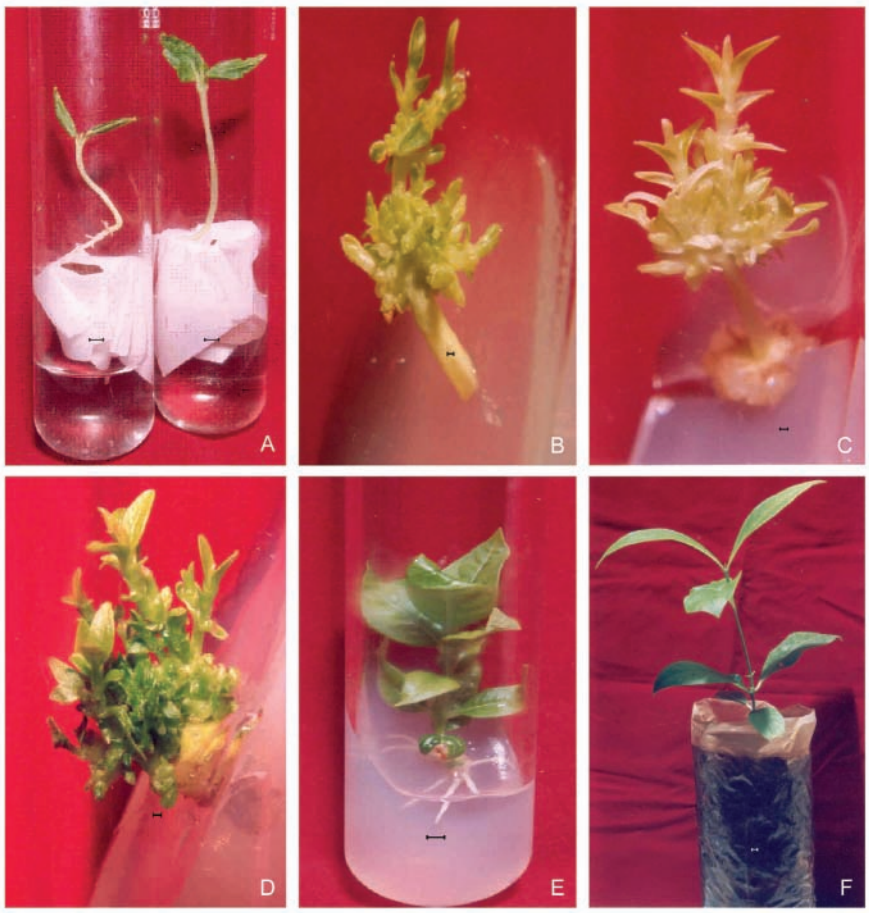

Fig. 1. *A* - Aseptic seed germination and seedling growth on MS full strength liquid medium. Observations were recorded after 20 d. (*bar* = 8.3 mm); *B* - Initiation of multiple shoots from cotyledonary node on MS medium supplemented with 2 mg dm^{-3} BA. Observations were recorded after 4 weeks (*bar* = 5.5 mm); *C* - Clump of multiple shoots developed from cotyledonary node when grown on MS medium containing 2 mg dm^{-3} BA. Observations were recorded after 6 weeks (*bar* = 4.5 mm); *D* - Development of multiple shoots from the stump of cotyledonary node when grown on MS medium supplemented with 0.5 mg dm^{-3} BA. Observations were recorded after 4 weeks (*bar* = 4.5 mm); *E* - *In vitro* rooting of microshoots on MS full strength basal medium. Observations were recorded after 3 weeks (*bar* = 8.3 mm); *F* - Micropropagated plant growing in plastic bag containing soil, organic manure, and sand (2:1:1) after 2 months of transfer (*bar* = 4.5 mm).

Table 3. Effect of different concentrations of BA along with IAA on induction of multiple shoots from cotyledonary nodes. Observations recorded after 8 weeks of culture. Means ± SE, n = 20. Means having the same letter in each column do not differ significantly at $P < 0.05$.

BA [mg dm^{-3}]	IAA [mg dm^{-3}]	Regeneration frequency [%]	Number of shoots [explant^{-1}]	Length of shoots [cm]
0.5	0.1	85.3 ± 0.08^{g}	3.5 ± 0.15^{a}	2.4 ± 0.10^{a}
0.5	0.5	80.4 ± 0.07^{f}	14.2 ± 0.26^{f}	3.2 ± 0.05^{b}
1.0	0.1	72.4 ± 0.07^{b}	13.1 ± 0.26^{e}	3.6 ± 0.03^{b}
1.0	0.5	79.6 ± 0.75^{e}	11.8 ± 0.26^{d}	4.5 ± 0.06^{c}
2.0	0.1	93.4 ± 0.07^{h}	30.1 ± 0.19^{i}	4.4 ± 0.08^{c}
2.0	0.5	86.4 ± 0.08^{g}	32.2 ± 0.40^{j}	5.0 ± 0.07^{d}
3.0	0.1	92.5 ± 0.07^{h}	15.5 ± 0.33^{g}	3.2 ± 0.04^{b}
3.0	0.5	76.5 ± 0.20^{d}	17.8 ± 0.31^{h}	2.8 ± 0.03^{a}
4.0	0.1	70.4 ± 0.09^{a}	15.8 ± 0.25^{g}	3.2 ± 0.02^{b}
4.0	0.5	75.4 ± 0.09^{c}	7.3 ± 0.21^{c}	2.9 ± 0.08^{a}
5.0	0.1	94.4 ± 0.09^{h}	11.5 ± 0.23^{d}	3.3 ± 0.04^{b}
5.0	0.5	77.2 ± 0.05^{e}	6.5 ± 0.25^{b}	4.4 ± 0.03^{c}

compared to MS. The superiority of MS medium over others found in the present study is similar to those demonstrated for many plant species such as *Hybanthus enneaspermum* (Prakash *et al*. 1999) and *Aegle marmelos* (Pranati *et al*. 2007). Formation of basal callus is a common observation in tissue culture and this may be due to the action of accumulated auxins at the basal cut ends in auxin rich plants (Bhattacharya and Bhattacharya 2001). Similarly as in the present study, cotyledonary nodes on MS medium with BA produced maximum number of shoots in *Whritia tinctoria* (Purohit *et al*. 2004).

In vitro rooting was achieved on full strength MS liquid medium without growth regulators in contrast to previous reports which showed rooting on MS medium supplemented with IBA (Ravindra *et al.* 2005, Raha and Roy 2001, 2003). Rooting of micropropagated shoots of *H. antidysenterica* on basal medium may be due to high endogenous levels of auxins. Occurrence of profuse callus from shoot bases on auxin supplemented medium supports this hypothesis. The method of *ex vitro* rooting achieved by dip treatment in 2 mg dm^{-3} of IBA of *in vitro* raised shootlets adopted in the present study reduces labor, costs and overheads as rooting and acclimatization are effectively combined into single stage of micro-propagation (Tiwari *et al.* 2002). *Ex vitro* rooted plants produced fully functional roots thus facilitating the better survival than *in vitro* rooted plants.

In the present paper, for the first time high frequency regeneration using cotyledonary nodes of aseptic seedlings of medicinally important plant species *H. antidysenterica* has been reported. The protocol described here will be useful in propagation, conservation and improvement of this threatened medicinal plant.

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