In Vitro Cell. Dev. Biol.—Plant 37:232–236, March–April 2001
DOI: 10.1079/IVP2000133

1054-5476/01 $10.00+0.00

IN VITRO PLANT REGENERATION IN *HOLARRHENA ANTIDYSENTERICA* WALL., THROUGH HIGH-FREQUENCY AXILLARY SHOOT PROLIFERATION

SUMITA RAHA AND SATYESH CHANDRA ROY*

Department of Botany, Centre of Advanced Study, University of Calcutta, 35 Ballygunge Circular Road, Calcutta 700 019, India

(Received 22 May 2000; accepted 29 September 2000; editor Z. -H. Xu)

Summary

An efficient, rapid and large-scale propagation of the woody, aromatic and medicinal shrub, *Holarrhena antidysenterica*, through *in vitro* culture of nodal segments with axillary buds, is described. N^6-benzyladenine used at 15 μM was the most effective in inducing bud break and growth, and also in initiating multiple shoot proliferation at the rate of 43 microshoots per nodal explant with axillary buds, after 30 d of culture. By repeated subculturing of nodal explants with axillary buds, a high-frequency multiplication rate was established. Efficient rooting was achieved with 35 μM indole-3-butyric acid which was the most effective in inducing roots, as 80% of the microshoots produced roots. Plantlets went through a hardening phase in a controlled plant growth chamber, prior to *ex vitro* transfer. Micropropagated plants established in garden soil were uniform and identical to donor plants with respect to growth characteristics and vegetative morphology.

Key words: Holarrhena antidysenterica; nodal explants; axillary buds; micropropagation.

Introduction

Holarrhena antidysenterica Wall. (Apocynaceae) is a woody, aromatic, deciduous lactiferous medicinal shrub or sometimes a small tree, 9–10 m high. This species is distributed throughout India especially in the wet forests and tropical Himalayas, ascending to an altitude of 1200 m. The bark and roots are an excellent remedy for both acute and chronic dysentery, especially in cases where there is excessive blood with mucus and colic pain associated with stools (Ghose, 1984). Due to large-scale and unrestricted exploitation to meet increasing demands by the pharmaceutical industries, coupled with limited cultivation and insufficient attempts for its reforestation, the wild stock of this important plant species has been markedly depleted. The shrub bears seeds only during the onset of the monsoon rains and its natural rate of multiplication is limited.

In recent years, there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Arora and Bhojwani, 1989; Bera and Roy, 1993; Sharma et al., 1991; Sudha and Seeni, 1994; Sahoo and Chand, 1998; Wawrosch et al., 1999). There have been previous reports on phytochemical studies from callus cultures of *Holarrhena* (Heble et al., 1976). This communication reports a protocol for rapid clonal propagation through high-frequency axillary shoot proliferation from nodal explants, followed by successful *ex vitro* establishment of regenerated plants.

*Author to whom correspondence should be addressed: Email ari@cal2.vsnl.net.in

Materials and Methods

Plant material. Young healthy plantlets of *Holarrhena antidysenterica* Wall., each bearing three to five nodes, were collected from Jhargram areas of Medinipur, West Bengal and maintained in clay pots with soil, at 30 ± 2°C and 70–80% relative humidity. Nodes, with axillary buds and shoot tips measuring 0.5 ± 0.1 cm in length were excised from the donor plants and were washed thoroughly, first under running tap water followed by washing in double-distilled water containing 7.5% (v/v) Teepol®. They were then surface-sterilized with 70% ethanol for 40 s followed by 0.1% (v/v) $HgCl_2$ (Qualigen, India) solution for 10 min and finally rinsed six or seven times with sterile double-distilled water.

Culture medium and conditions. The culture medium used for the present work was MS medium (Murashige and Skoog, 1962) supplemented with 100 mg l^{-1} (w/v) *myo*-inositol and 3% (w/v) sucrose. The medium was further supplemented with cytokinins: 6-benzyladenine (BA), kinetin, 2-isopentenyl adenine (2iP) and adenine sulfate ($AdSO_4$) at 5, 10, 15, 20 and 40 μM, respectively. The pH of the medium was adjusted to 5.8 before the addition of 0.8% agar (BDH, India). All the chemicals used were of analytical grade (Sigma & Merck). Molten medium (20 ml) was dispensed into borosil tubes and 100 ml in Erlenmeyer flasks (Borosil). The culture vials containing media were autoclaved at 121°C and 104 kPa for 20 min. The surface-disinfected explants were implanted vertically on the culture medium (one explant per tube and 4 ± 1 per flask). All the cultures were maintained in a growth cabinet at 25 ± 1°C, under 16 h photoperiod of 35–50 $\mu mol\ m^{-2}\ s^{-1}$ irradiance provided by cool white fluorescent tubes (Phillips, India) and with 60–65% relative humidity. After 4 wk, the shoots and number of leaves were averaged and shoot lengths were measured.

Multiplication of shoot cultures. Primary shoots formed *in vitro* were sectioned into one-node pieces after removing the leaves. The nodal segments containing the axillary buds were cultured in MS fortified with BA 15 μM for further multiplication. Subsequent subcultures were at 4 wk intervals.

Rooting of shoots. For root induction, excised shoots (3–5 cm) with four or more leaves were harvested from each subculture and transferred to: (1) MS medium without growth regulators; (2) MS medium with auxins, indole-3-butyric acid (IBA), indoleacetic acid (IAA) and α-napthaleneacetic acid (NAA) (5, 10, 15, 25 and 35 μM). After 4 wk, the lengths of the roots initiated were measured and the total number of roots were counted.

Acclimatization and transfer of plantlets to soil. Plantlets with well-developed roots were removed from culture, washed gently under running tap water, and transferred to plastic pots (6 cm diameter) containing 'soilrite' (Indo American Hybrid Seeds Ltd., Bangalore, India). These were watered with 1/8 MS basal salt solution, devoid of sucrose and inositol every 5 d for 2 wk. The plantlets were maintained in a glasshouse set at 25 ± 1°C, 80–85% relative humidity and irradiance provided with cool white fluorescent tubes. After 2 wk, these plantlets were transferred to clay pots containing a mixture of 'soilrite' and autoclaved garden soil and were exposed to natural light by covering them with transparent polythene bags punched with four holes to allow gaseous exchange. These were kept under shade in a net house for another 2 wk before transferring outside under full sun.

Statistical analysis. Data presented in the tables are treatments consisting of 10 replicates (culture tubes) and the experimental unit was a single explant per tube. Each experiment was replicated 10 times. Data were analyzed using analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) was used to compare the means.

Results and Discussion

Establishment of cultures. Shoot apices and nodes with axillary buds from 3-wk-old plants were used as primary explants. The nodal explants and shoot apices failed to respond morphogenetically to a growth regulator-free MS medium. Using cytokinins, the initial multiplication rate varied between 6 and 14 shoots per explant depending on the type of explants, concentration and type of growth regulator used. Shoot proliferation in cultures of nodes with axillary buds as well as shoot apices was a function of cytokinin activity (Table 1). Of all the four cytokinins tested, BA was the most effective and 15 μ*M* was found to be optimum for inducing maximum shoot multiplication, shoot length and leaf numbers, respectively (Table 1). There was a linear correlation between the increase in BA concentration up to the optimal level (15 μ*M*) and number of shoots per explant. In contrast, higher concentrations of BA (20 μ*M* and above) suppressed shoot formation during the same 4 wk culture period.

MS containing 15 μ*M* BA induced shoot bud break in 90% of the nodal explants. The explants cultured on this medium showed their first response by initial enlargement of the nodes with axillary bud following bud break within 10–12 d. An average of 43 shoot buds developed within 15–20 d (Table 1; Fig. 1b) and each developing shoot bud elongated within another 10 d. These microshoots attained a maximum average length of 4.5 cm and had 16 leaves per shoot (Table 1). However, only about 40% of the shoot apices responded in MS with 15 μ*M* BA within 15 d and multiplied to produce an average of 13 shoots in 30 d. Because of the superior *in vitro* response of the nodes with axillary buds, compared to the apical shoot buds, only the former was used in subsequent experiments. Data obtained on using shoot tips as explants are shown in Table 1.

The stimulating effect of BA on bud break and multiple shoot formation has been reported earlier for several medicinal and aromatic plant species including *Chlorophytum borivilianum* (Purohit et al., 1994), *Ocimum* sp. (Patnaik and Chand, 1996), *Piper* spp. (Bhat et al., 1995), *Pogostemon cablin* (Kukreja et al., 1990), *Withania somnifera* (Sen and Sharma, 1991), *Litchi chinensis* (Das et al., 1999) and *Anacardium occidentale* (Bogetti et al., 1999). Reduction in the number of shoots generated from each node at BA concentrations higher than the optimum level was also reported for several medicinal plants (Kukreja et al., 1990; Sen and Sharma, 1991).

Also, bud sprouting was influenced by 2iP, and this cytokinin showed second best results at concentrations of 15 μ*M*, using nodes

TABLE 1

INFLUENCE OF DIFFERENT CYTOKININS ON SHOOT BUD MULTIPLICATION OF *HOLARRHENA ANTIDYSENTERICA*, USING NODES WITH AXILLARY BUDS AND SHOOT TIPS AS EXPLANTS AND CULTURED IN MS MEDIUM

Plant growth regulator	Concentration (μ*M*)	Shoots per explant		Shoot length[a,b] (cm) (average)	Number of leaves[a,b] (average)
		Nodes with axillary buds[a]	Shoot tip[a]		
BA	5	14.667 FG	4.633 F	1.10 HIJ	4.333 JK
	10	27.767 D	9.533 CD	2.90 DE	12.667 CDE
	15	42.833 A	13.700 AB	4.50 AB	16.333 AB
	20	34.533 BC	12.400 B	3.80 C	17.667 A
	40	12.700 GHI	3.733 FGH	1.80 G	3.333 JK
Kinetin	5	8.767 I	1.167 J	0.50 K	2.333 K
	10	19.833 E	14.333 A	3.00 D	5.667 IJ
	15	29.700 D	13.300 AB	4.80 A	12.333 CDEF
	20	22.067 E	8.367 DE	2.40 EF	10.667 EFGH
	40	27.100 D	7.533 E	2.90 DE	9.667 FGH
$AdSO_4$	5	2.667 J	1.233 J	0.60 JK	2.667 K
	10	4.333 J	1.967 IJ	1.20 HI	10.000 EFGH
	15	17.933 EF	7.933 E	1.50 GH	11.000 DEFG
	20	10.067 HI	4.533 FG	1.80 G	9.333 GH
	40	2.233 J	1.733 IJ	1.60 GH	5.667 IJ
2iP	5	14.233 FGH	2.600 HIJ	0.80 IJK	3.667 JK
	10	29.333 D	10.500 C	1.90 FG	8.000 HI
	15	36.967 B	13.567 AB	4.20 BC	14.333 BC
	20	31.333 CD	12.300 B	3.90 C	13.667 BCD
	40	8.867 I	3.067 GHI	2.60 DE	9.333 GH

Data were collected after 30 d from three independent experiments, each with 10 replications, and the experimental unit was a single explant per culture vessel.

[a] Means followed by the same letter are not significantly different ($P < 0.05$) according to ANOVA and LSD multiple-range test.

[b] Scoring of shoot length and number of leaves correspond to the axillary bud explant (data on shoot length and number of leaves from shoot tip explant not shown).

FIG. 1. *In vitro* morphogenesis of *Holarrhena antidysenterica* from nodes with axillary buds. a, Multiple shoot development from nodes with axillary buds explant cultured on MS + BA (15 μ*M*) after 30 d of culture (×3). b, Profuse leaves and well-developed roots derived from subculturing four nodes with axillary buds explants in MS medium containing 15 μ*M* BA, dispensed into 250-ml flask (×0.65). c, Regenerated plantlet with well-developed leaves and roots in liquid MS medium for hardening (×0.65). d, Single plantlet prior to transfer to soil (×1.3). e, Plantlets after transfer to soil (×0.326).

with axillary buds as explants. At this concentration, an average of 36 microshoots were produced (Table 1), which were weak, hyperhydrated and leaves very much reduced in size. The maximum shoot length was 4.2 cm and the number of leaves corresponded to 13 per shoot (Table 1). 2iP was the best cytokinin for shoot multiplication in blueberry (Cohen, 1980) and garlic (Bhojwani, 1980a). However, 2iP was completely ineffective in induction of shoot multiplication in hybrid willow (Bhojwani, 1980b) and white clover (Bhojwani, 1981).

By using the cytokinins kinetin and $AdSO_4$ at 15 μ*M*, it was possible to produce an average of 30 and 17 shoots, respectively, using nodes, with axillary buds as explant. The maximum shoot length using kinetin (15 μ*M*) was 4.8 cm, with a maximum of four leaves per shoot. Similarly, $AdSO_4$ at 15 μ*M* had shoot length of

TABLE 2

INFLUENCE OF DIFFERENT AUXINS ON ROOT LENGTH AND NUMBER OF ROOTS PRODUCED PER SHOOT OF *HOLARRHENA ANTIDYSENTERICA* CULTURED IN MS MEDIUM (OBSERVATIONS WERE MADE 4 WK AFTER CULTURE)

Plant growth regulator	Concentration (μM)	Root length[a]	Root number[a]
IBA	C[b]	3.310 DE	4.80 GH
	5	3.880 D	6.40 EF
	10	4.940 BC	8.30 D
	15	5.460 B	10.20 C
	25	6.520 A	15.90 B
	35	3.540 D	17.70 A
NAA	C[b]	3.310 DE	4.80 GH
	5	0.00 H	0.00 J
	10	0.00 H	2.80 I
	15	2.70 F	8.10 D
	25	2.880 EF	6.00 EFG
	35	1.660 G	5.10 FGH
IAA	C[b]	3.310 DE	4.80 GH
	5	0.0000 H	0.00 J
	10	0.0000 H	4.40 H
	15	4.580 C	6.90 DE
	25	5.120 BC	6.60 E
	35	2.820 EF	6.10 EFG

Values are means of 10 replicates and the experimental unit was single shoot per culture vessel.

[a] Means followed by same letter are not significantly different ($P < 0.05$) according to ANOVA and LSD multiple-range test.

[b] MS medium without any growth regulator.

4.2 cm with an average of 13 leaves (Table 1). Kinetin led to the production of weak shoots with a large reduction in the number of leaves. $AdSO_4$, however, reduced shoot elongation significantly in sprouted buds and also led to the formation of stunted shoots.

Multiplication of shoot cultures. The shoot cultures were multiplied by repeatedly subculturing the *in vitro*-generated nodes with axillary buds as explants. MS with BA at 15 μM was used for shoot multiplication (Fig. 1a, b). These nodal microshoots were subcultured once every 30 d and formed new axenic shoots. The number of shoots per node retained the highest values (43 per node) (Table 1), during the first five culture passages, beyond which there was a gradual decline in multiplication rate. Shoot apices were not used for multiplication because of their comparatively poor response.

Rooting of shoots. Of the three auxins tested, IBA was the most effective (Fig. 1b). IBA at a concentration of 35 μM produced an average number of 18 roots. These roots were short, with basal callusing and had a length of 3.5 cm (Table 2) whereas the number of roots was less at a concentration of 25 μM, but the root elongation was rapid with root length being almost double (6.5 cm) (Table 2). High concentrations of NAA and IAA (25 μM) produced an average of six roots with maximum shoot length being 2.8 cm in the case of NAA and 5.1 cm in the case of IAA (Table 2). Besides, IAA also led to the formation of stout roots.

Optimum rooting response using IBA has been reported for several plants including *Syzygium alternifolium* (Wight) *walp* (Sha Valli Khan et al., 1997), *Azadirachta indica* (Eeswara et al., 1998), *Melia azedarach* (Thakur et al., 1998), *Anacardium occidentale* (Bogetti et al., 1999) and *Dendrocalamus asper* (Arya et al., 1999).

Acclimatization and field culture. Plantlets with fully expanded leaflets and well-developed roots were successfully transferred to 'soilrite' and hardened off (Fig. 1c, d) in a growth chamber for 4 wk. All the micropropagated plants survived and grew normally following transfer to soil during the monsoon rains (Fig. 1e). Transfer during the summer seasons with temperatures up to 38–42°C led to the drying and death of 70% of the plantlets. There was no detectable variation among the potted plants with respect to morphological and growth characteristics (Fig. 1e).

The protocol reported here could be used for conservation and large-scale propagation of this important woody aromatic medicinal shrub, which is restricted to tropical climates.

Acknowledgments

The authors are grateful to the UGC for financial assistance. Photographic assistance of Dr. T. K. Bera and statistical analysis by Ms. Manomita Patra are also gratefully acknowledged.

References

Arora, R.; Bhojwani, S. S. *In vitro* propagation and low temperature storage of *Sausurea lappa* C.B. Clarke—an endangered medicinal plant. Plant Cell Rep. 8:44–47; 1989.

Arya, S.; Sharma, S.; Kaur, R.; Dev Arya, I. Micropropagation of *Dendrocalamus asper* by shoot proliferation using seeds. Plant Cell Rep. 18:879–882; 1999.

Bera, T. K.; Roy, S. C. Micropropagation of *Tylophora indica* (Burmn f.) Merr. by multiple bud formation from mature leaf explants without callus intervention. Bot. Bull. Acad. Sin. 34:83–87; 1993.

Bhat, S. R.; Chandal, K. P. S.; Malik, S. R. Plant regeneration from various explants of cultivated *Piper species*. Plant Cell Rep. 14:395–402; 1995.

Bhojwani, S. S. *In vitro* propagation of garlic by shoot proliferation. Sci. Hortic. 13:47–52; 1980a.

Bhojwani, S. S. Micropropagation method for a hybrid willow (*Salix matsudana xalba* NZ-1002). N.Z. J. Bot. 18:209–214; 1980b.

Bhojwani, S. S. A tissue culture method for a propagation and low temperature storage of *Trifolium repens* genotypes. Physiol. Plant. 52:187–190; 1981.

Bogetti, B.; Jasik, J.; Mantell, S. *In vitro* multiplication of cashew (*Anacardium occidentale* L.) using shoot node explants of glasshouse raised plants. Plant Cell Rep. 18:456–461; 1999.

Cohen, D. Application of micropropagation methods for blueberries and tamarillos. Comb. Proc. Int. Plant Prop. Soc. 30:144–146; 1980.

Das, D. K.; Shiva Prakash, N.; Bhalla-Sarin, N. Multiple shoot induction and plant regeneration in litchi (*Litchi chinensis* Sonn.). Plant Cell Rep. 18:691–695; 1999.

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

Ghose, S. C. *Holarrhena antidysenterica* and *Wrightia tinctoria*. In: Drugs of Hindustan. Calcutta: Hahnemann Publishing Co. Pvt. Ltd.; 1984:309–320.

Heble, M. R.; Narayanswamy, S.; Chadha, M. S. Metabolism of cholesterol by callus culture of *Holarrhena antidysenterica*. Phytochemistry 15:1911–1912; 1976.

Kukreja, A. K.; Mathur, A. K.; Zaim, M. Mass production of virus free patchauli plants [*Pogostemon cablin* (Blanco) Benth] by *in vitro* culture. Trop. Agric. 67:101–104; 1990.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15:473–497; 1962.

Patnaik, S. K.; Chand, P. K. *In vitro* propagation of the medicinal herbs *Ocimum americanum* L. Syn, *O. canum* Sims (hoary basil) and *Ocimum sanctum* L. (holy basil). Plant Cell Rep. 15:846–850; 1996.

Purohit, S. D.; Dave, A.; Kukda, G. Micropropagation of safed musli (*Chlorophytum borivilianum*), a rare medicinal herb. Plant Cell Tiss. Organ Cult. 39:93–96; 1994.

Sahoo, Y.; Chand, P. K. Micropropagation of *Vitex negundo* L., a woody aromatic medicinal shrub, through high frequency axillary shoot proliferation. Plant Cell Rep. 18:301–307; 1998.

Sen, J.; Sharma, A. K. Micropropagation of *Withania somnifera* from germinating seeds and shoot tips. Plant Cell Tiss. Organ Cult. 26:71–73; 1991.

Sha Valli Khan, P. S.; Prakash, E.; Rao, K. R. *In vitro* microprogation of an endemic fruit tree *Syzygium alternifolium* (Wight) walp. Plant Cell Rep. 16:325–328; 1997.

Sharma, N.; Chandel, K. P. S.; Srivastava, V. K. *In vitro* propagation of *Coleus forskohlii* Briq., a threatened medicinal plant. Plant Cell Rep. 10:67–70; 1991.

Sudha, G. C.; Seeni, S. *In vitro* multiplication and field establishment of *Adhatoda beddomei* CB Clarke, a rare medicinal plant. Plant Cell Rep. 13:203–207; 1994.

Thakur, R.; Rao, P. S.; Bapat, V. A. *In vitro* plant regeneration in *Melia azedarach* L. Plant Cell Rep. 18:127–131; 1998.

Wawrosch, C.; Maskay, N.; Kopp, B. Micropropagation of the threatened Nepalese medicinal plant *Swertia chirata* Buch.-Ham ex. Wall. Plant Cell Rep. 18:997–1001; 1999.