In Vitro Cell. Dev. Biol.—Plant 42:394–398, September–October 2006
DOI: 10.1079/IVP2006797

1054-5476/06 $18.00+0.00

HIGH-FREQUENCY INDUCTION OF MULTIPLE SHOOTS AND CLONAL PROPAGATION FROM RHIZOMATOUS NODAL SEGMENTS OF *HOUTTUYNIA CORDATA* THUNB. – AN ETHNOMEDICINAL HERB OF INDIA

SASWATI CHAKRABORTI, SANGRAM SINHA, AND RABINDRA K. SINHA*

Cytogenetics and Plant Tissue Culture Laboratory, Department of Life Science, Tripura University, Suryamaninagar, 799 130, Tripura, India

(Received 13 September 2005; accepted 25 April 2006; editor F. Engelmann)

Summary

This study reports an efficient and direct shoot bud differentiation and multiple shoot induction from nodal segments of underground stoloniferous rhizomes of *Houttuynia cordata* Thunb. The frequency of shoot bud regeneration was influenced by the type of cytokinin and concentrations. Among the various concentrations used, benzylaminopurine (BAP, 17.74 μ*M*) or kinetin (Kn, 18.58 μ*M*) was found to be most effective for rapid and maximum shoot bud differentiation. The number of shoots per explant was higher (20.00 ± 2.61) on Murashige and Skoog (MS) medium supplemented with Kn (18.58 μ*M*) compared to BAP and 6-γ,γ-(dimethyl-allylamino)-purine (2iP) during initial 40-d-old culture. Subsequent shoot differentiation and multiplication were achieved in MS medium containing 9.29 μ*M* Kn and 15% (v/v) coconut milk. Elongation and growth of multiple shoots were also obtained on MS medium containing either 2.32 μ*M* Kn or 2.46 μ*M* 2iP alone. The rate of shoot multiplication during subcultures declined with an increase in the size of proliferating shoot cluster. Reducing shoot cluster size to three to four shoots and subculturing together in shoot multiplication medium resulted in a better shoot multiplication and growth, which could be maintained for 2 yr. The elongated shoots (>20 mm) were successfully rooted on MS medium supplemented with 19.60 μ*M* indole-3-butyric acid. Regenerated plants were successfully established in soil and were found to be healthy and uniform. The protocol reported in this study can be used for conservation and utilization of elite clone of *H. cordata*.

Key words: ethnomedicinal herb; *Houttuynia cordata*; micropropagation.

Introduction

Houttuynia cordata Thunb. belongs to the family *Saururaceae*, is commonly known as Chinese lizard tail. It is a perennial herb with stoloniferous rhizome having two distinct chemotypes (Tutupalli et al., 1975; Brown, 1995). The Chinese chemotype of the species is growing wild and semi-wild conditions in the north-east of India (Kanjilal and Dev, 1937; Dev, 1983; Mukherjee and Roy, 1987). The ethnobotanical significance of the species having vegetable, food and age-old therapeutic uses among the different communities of this region, has been well documented (Sinha et al., 2005). A number of bioactive chemical constituents and medicinal properties of the leaves and stoloniferous rhizome of *H. cordata* have also been reported (Rastogi and Mehrotra, 1993; Kyoko et al., 1994; Park et al., 2000). The species population in the north-east of India is very restricted and known to propagate slowly by stoloniferous rhizomes. The seed viability is almost lacking and vegetative proliferation during winter is very poor. Unsustainable extraction, multiple ethnobotanical uses, and huge utilization of the plant resource from the wild have caused the species to be vulnerable in its natural habitat. Tissue culture techniques have been a useful means for rapid *ex situ* multiplication of endangered species and increasingly exploited in number of plants with reproducible success (Wochok, 1981; Edson et al., 1997; Hall, 2000). Therefore, establishment of *in vitro* culture aimed at regeneration and efficient shoot multiplication of *H. cordata* is an effective measure for rapid propagation and conservation of elite clones throughout the year. Few reports are available on plant regeneration of *H. cordata* using vegetative nodal segments and shoot tip culture (Borthakur et al., 1999; Handique and Bora, 1999; Wu et al., 2004). Regeneration of *H. cordata* using leaf callus culture was also reported by Tauzuki et al. (1995). However, in the present work, we describe an efficient and reproducible method for enhanced shoot multiplication and clonal propagation of *H. cordata* using underground stoloniferous nodal segments of the rhizome for the first time. The present work also highlights the nature and extent of shoot multiplication under varying incubation periods with different growth regulators.

Materials and Methods

Underground stoloniferous rhizomes of *H. cordata* Thunb. were collected from the wild germplasm maintained at the university's experimental garden. Stoloniferous rhizomes are spreading in nature and characterized by distinct nodes with fine roots. The nodal segments of the rhizome were used as explants.

Initiation of aseptic culture. Uprooted stoloniferous rhizomes were thoroughly washed with water and subsequently nodal segments measuring 15–20 mm were surface disinfected, washing with 5% Ezee detergent solution for 20 min. This was followed by repeated washing with sterile distilled water. Nodal segments were surface sterilized with 0.1% $HgCl_2$

*Author to whom correspondence should be addressed: Email khsinhark@yahoo.co.in

TABLE 1

MORPHOGENETIC RESPONSES OF STOLONIFEROUS NODAL EXPLANTS OF *H. CORDATA* CULTURED ON MS MEDIUM SUPPLEMENTED WITH DIFFERENT CYTOKININS

	20 d	30 d	40 d		
Cytokinin (μ*M*)	No. of shoots per explant	No. of shoots per explant	No. of shoots per explant	Shoot size (mm)	Percentage of shoots size (>10 mm in shoot cluster)
BAP					
2.22	2.20 ± 1.33[z]	6.00 ± 2.28	11.00 ± 2.28	8.68 ± 3.65	44
8.87	4.40 ± 1.36	11.00 ± 2.00	16.40 ± 2.24	7.88 ± 3.31	40
17.74	9.80 ± 2.99	13.00 ± 2.83	19.40 ± 4.63	10.44 ± 5.04	4
Kn					
2.32	2.00 ± 1.41	6.80 ± 1.33	11.20 ± 1.72	9.84 ± 3.15	56
9.29	5.00 ± 1.41	9.20 ± 0.75	13.80 ± 2.23	6.96 ± 3.01	24
18.58	7.60 ± 1.36	12.80 ± 2.04	20.00 ± 2.61	9.36 ± 4.25	28
2iP					
2.46	1.80 ± 0.75	5.20 ± 1.33	10.00 ± 1.41	8.32 ± 3.53	32
9.84	5.80 ± 2.48	8.80 ± 1.47	15.00 ± 2.28	6.44 ± 2.47	20
19.68	9.20 ± 2.31	12.20 ± 2.56	17.80 ± 2.48	6.08 ± 2.04	8

[z]Each value represents mean ± SD of five replications.

FIG. 1. *a–j. In vitro* propagation and shoot multiplication of *Houttuynia cordata* Thunb. *a*, Direct induction of multiple shoot buds and shoots from rhizomatous nodal segment. *b*, Shoot multiplication and growth in MS medium with Kn (18.58 μ*M*) of 40-d-old culture. *c*, Elongation and proliferation of shoots in MS media containing 2.32 μ*M* Kn (*bar* = 1.5 cm). *d*, Stout and stunted growth of leafy shoots in media with BAP (17.74 μ*M*). *e*, Elongation of shoots in a cluster supported by 2iP (2.46 μ*M*). *f*, Hairy roots formation (*arrow*) from the node of differentiated shoots. *g*, Elongated well-developed shoots. *h*, Rooting of shoots after 20 d of culturing on the rooting medium. *i*, Regenerated plantlet with well-developed leaves and roots in filter water for hardening. *j*, Plantlets after being successfully transferred to soil.

TABLE 2

ANALYSIS OF VARIANCE SHOWING SIGNIFICANT DIFFERENCE IN THE NUMBER OF SHOOTS OF *H. CORDATA* AMONG THE GROWTH REGULATOR TREATMENTS AND DIFFERENT PERIODS OF INCUBATION IN A CONTINUOUS CULTURE

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	*F*-ratio
Between incubation periods	2	421.42	210.71	274.31***
Among treatments	8	244.64	30.58	39.81***
Residual	16	12.29	0.768	

***Highly significant at 5% level.

solution for 7–8 min, rinsed thoroughly in sterile distilled water, and cut transversely into 5–6 mm long sections. The culture medium used for adventitious shoot induction and multiplication consisted of MS basal medium (Murashige and Skoog, 1962) containing 3% sucrose, 0.8% agar, and supplemented singly with 6-benzylaminopurine (BAP), kinetin (Kn) and 6-γ,γ-(dimethyl-allylamino)-purine (2iP) at three different concentrations. All experimental media were adjusted to pH 5.7 before autoclaving at 121°C for 20 min. Cultures were incubated for 6 wk at 25 ± 2°C under a 16-h photoperiod, with the light intensity of 40 μmol m^{-2} s^{-1} provided by cool fluorescent lamps. The morphogenetic potential of the nodal segments was recorded periodically after incubation in the same culture medium for 20, 30 and 40 d, respectively. For each treatment, five replicates were used and the experiment was repeated twice. Data for shoot induction and multiplication were recorded and analyzed statistically using analysis of variance (ANOVA).

Shoot multiplication. *In vitro*-raised primary shoot clusters were used as a secondary source of explant and sectioned into small clusters of three to four shoots. Each cluster, having a mean of 3.6 ± 0.49 microshoots, was subcultured in MS basal medium supplemented with 9.29 μ*M* Kn and 15% (v/v) coconut milk (CM) and incubated for 4 wk. All the clusters were maintained under similar conditions as stated above up to the third passage of subculture. Increased shoot bud induction and multiplication were recorded in terms of number of shoots per proliferating shoot cluster. The nature of proliferating shoot clusters after each subculture were analyzed according to their rate of shoot multiplication (RSM), size and growth. The RSM was calculated as follows:

$$\text{RSM} = \frac{\text{Final shoot no. in the cluster} - \text{Initial shoot no. in the cluster}}{\text{Final shoot number}} \times 100$$

Rooting of shoots. For induction of adventitious roots, clumps of three to four microshoots (20–30 mm in height) were aseptically isolated from the proliferating shoot clusters and subcultured in MS basal medium supplemented with indole-3-butyric acid (IBA), 2,3,5-tri-iodobenzoic acid (TIBA) and 2iP at different concentrations and combinations. All the cultures were maintained by subculturing on the same medium at 3-wk intervals under the same cultural conditions. Data on rooting of regenerated shoots in different types and concentrations of growth regulators were analyzed by least standard deviation.

Results and Discussion

Initiation of aseptic culture. Adventitious shoot bud induction and differentiation of shoots from the nodal region of stoloniferous rhizomes of *H. cordata* under the influence of BAP, Kn, and 2iP at three different concentrations were carried out. Concentration-dependent shoot bud induction and proliferation in different ages of cultures were clearly recorded (Table 1). All the experimental treatments supported a varying degree of shoot bud induction and differentiation within 20 d of incubation in cultures, while new shoot bud formation and differentiation continued in subsequent 30 and 40 d of culture in the same medium (Fig. 1*a*). Among the three cytokinins, BAP (17.74 μ*M*) produced more shoots per explant during 20 d of culture. Although BAP supported early induction of shoot buds and greater increase in shoot proliferation, Kn at 18.58 μ*M* sustained the highest number of shoots (20.00 ± 2.61) during 40 d of culture (Fig. 1*b*). Using BAP (17.74 μ*M*) in place of Kn in the long-term culture, the shootlets produced were found to be much stouter and leafier (Fig. 1*d*). Higher concentrations of BAP causing stunted shoot growth were also reported by Aitken-Christie and Connell (1992). Thus, the greater effectiveness of Kn than BAP in shoot multiplication and growth has been recorded in this species. A two-way ANOVA indicated a highly significant ($P = 0.01$) influence of growth regulators on the number of proliferating shoots at three different periods of incubation in a continuous culture (Table 2).

However, at shoot induction and proliferation stages both BAP and Kn were suitable at 17.74 and 18.58 μ*M*, respectively, while 2iP was less effective at any concentration. On the contrary, shoot elongation was better promoted by Kn at lower concentrations (2.32 μ*M*) with 56% shoots measuring > 10 mm in 40-d-old culture (Fig. 1*c*). MS basal medium containing 2iP (2.46 μ*M*) was also found to support shoot elongation, with thin shoots and leaves (Fig. 1*e*). In contrast, inhibition of shoot growth and elongation were recorded in all the media containing higher levels of cytokinin. This observation also corroborates findings in other species by different workers (Wareing and Phillips, 1981; Sinha and Mallick, 1991).

Shoot multiplication. The RSM in a proliferating shoot cluster was also tested by subculturing fresh in MS basal medium supplemented with Kn (9.29 μ*M*) and 15% CM at 4-wk intervals (Table 3). Clusters of microshoots with an average number of

TABLE 3

EFFECT OF SUBCULTURES ON MICROSHOOT MULTIPLICATION AND GROWTH OF *H. CORDATA*

No. of subcultures	No. of microshoots	No. of shoots in each shoot size category				RSM[z]
		< 5 mm	5– < 10 mm	10– < 20 mm	20– < 40 mm	
First	45.2 ± 4.5[y]	9.6 ± 1.9	12.6 ± 3.1	15.4 ± 3.2	7.6 ± 2.4	91.9 ± 1.4
Second	110.2 ± 7.8	36.6 ± 5.7	32.6 ± 8.6	23.4 ± 4.3	17.6 ± 3.0	58.7 ± 5.4
Third	173.4 ± 11.6	46.4 ± 6.7	50.8 ± 8.3	36.0 ± 5.4	40.2 ± 10.8	36.0 ± 6.9

Explants were grown on MS medium supplemented with 9.29 μ*M* kinetin and 15% coconut milk for 4 wk of culture.

[z]Rate of shoot multiplication.

[y]Each value represents the means ± SD of five replications.

TABLE 4

EFFECT OF DIFFERENT CONCENTRATIONS AND COMBINATIONS OF GROWTH REGULATORS ON ROOT INDUCTION AND GROWTH OF *H. CORDATA* CULTURED AFTER 20 AND 40 D OF CULTURE

		20 d after culture		40 d after culture			
Growth regulator (μ*M*)	Agar (%)	No. of roots per shoot	Root length (mm)	No. of roots per shoot	Root length (mm)	No. of roots per node	Size of nodal roots (mm)
IBA (9.80)	8	4.00 ± 1.61[y]	5.50 ± 2.16	5.70 ± 2.15	9.80 ± 2.27	–	–[z]
IBA (19.60)	8	7.00 ± 1.73	8.20 ± 1.66	7.80 ± 2.64	12.50 ± 3.85	–	–
TIBA (4.00)	8	3.60 ± 1.20	7.30 ± 3.55	5.60 ± 2.00	10.20 ± 2.95	–	–
TIBA (8.00)	8	5.60 ± 2.25	4.40 ± 1.43	7.40 ± 2.25	8.70 ± 2.49	–	–
IBA (9.80) + 2iP (2.46)	8	5.60 ± 1.80	6.60 ± 1.86	9.90 ± 2.45	10.30 ± 2.00	4.00 ± 1.26	10.40 ± 1.96
IBA (9.80) + 2iP (2.46)	9	4.30 ± 1.79	7.20 ± 1.56	8.00 ± 2.05	11.80 ± 2.68	3.70 ± 1.10	16.50 ± 5.26
LSD		1.61	2.04	2.09	2.61		

[z]– Indicates no nodal rooting.
[y]Each value represents mean ± SD of five replications.

3.6 ± 0.49 microshoots were aseptically excised from the proliferating cultures and subcultured for further shoot multiplication and growth. The nature of shoot multiplication and growth were analyzed after each subculture until the third passage. Use of CM as growth adjuvant in the shoot multiplication medium was found to support enhanced multiplication and growth of shoots. The increase in number of shoots formed was also recorded with the passages of subcultures. In contrast, the RSM gradually declined to 36.0 ± 6.93 at the end of the third subculture. Decline in RSM was also associated with better shoot growth and differentiation as observed in their categorical shoot size variation in proliferating shoot cluster (Table 3). To optimize enhanced and continued shoot multiplication, clusters of shoots obtained from the second subculture were excised into several pieces of microshoot cluster and subcultured in the same shoot multiplication medium. These practices of *in vitro* culture can be carried out several times without much decline in the RSM and maintained for a period of 2 yr in the laboratory. On the contrary, shoot elongation and growth to the optimum size >20 mm were achieved by subculturing three to four shoots together in MS medium containing low levels of cytokinin, either 2iP (2.46 μ*M*) or Kn (2.32 μ*M*). The presence of low cytokinin concentrations not only promoted shoot growth and elongation, but also produced new shoot buds at the base of shoots. This suggests suitability of the cytokinin used in the present investigation for shoot bud induction and multiplication from the rhizomatous nodal segment of *H. cordata*.

Rooting of microshoots. *In vitro* raised shoots with three to four nodes were excised from the proliferating shoot cluster and placed on MS medium (Fig. 1*g*) supplemented with different concentrations of IBA, TIBA alone, or in combination with 2iP for root induction and growth (Table 4). Among the treatments, IBA alone produced better root induction than TIBA or in combination with 2iP within 20 d of culture. Optimal root induction was observed on 19.60 μ*M* IBA alone (Fig. 1*h*). Such an efficiency of IBA on rooting was also reported in other species (Sugiura et al., 1986; Winnaar, 1988; Rani et al., 2003; Saha et al., 2003). However, prolonged incubation in root inducing medium did not support further rooting and growth. This observation, clearly suggesting the inhibitory role of auxin in subsequent cultures, is also reported in other cases (Torrey, 1976). On the other hand, addition of 2iP (2.46 μ*M*) in rooting medium containing IBA (9.80 μ*M*) not only supported rooting of microshoots at the base and hairy adventitious rooting from the nodes of the differentiated microshoots (Fig. 1*f*), but also resulted in better shoot elongation. A similar observation has also been reported in other species (Preece and Imel, 1991). Increased level of agar (0.9%) in the same rooting medium also supported better root growth, both from the base and nodal region of the microshoots during 40-d-old culture (Table 4). Hairy root formation from the nodal region is a characteristic feature of the stoloniferous rhizome of *H. cordata*.

Hardening and acclimatization. *In vitro* rooted plantlets aged 3–4 wk, after removing the agar under running tap water, were individually transferred to filter paper supported in culture tubes containing filtered water and kept as such for another 3–4 wk in shaded conditions at 30 ± 2°C (Fig. 1*i*). Finally, plants were successfully transferred to potted soil containing sand, farmyard compost, and soil (1:1:2) under 70% shade and moist conditions. Plants grown in the potted soil did not show any morphological abnormality and were found to grow uniformly healthily (Fig. 1*j*).

The present *in vitro* experiments demonstrate for the first time a procedure for the regeneration and propagation of an ethnomedicinally important herb, *H. cordata* using nodal segments of stoloniferous rhizome. In addition, the present work also outlines different cytokinin requirements to obtain a high number of shoots with a better rate of growth for *H. cordata*, and the culture as such was maintained for 2 yr without morphological variation. These advantages of the procedure have direct implications for clonal propagation and improvement of this species.

Acknowledgment

The authors are grateful to the Department of Biotechnology, Government of India, for financial assistance.

References

Aitken-Christie, J.; Connell, M. Clonal propagation;gymnosperms. In: Vasil, I. K., ed. Cell culture and somatic cell genetics of plants. New York: Academic Press; 1992:82–95.

Borthakur, M.; Singh, R. S.; Bora, P. *In vitro* regeneration of *Houttuynia cordata*: a medicinal herb. Planta Med. 65:677; 1999.

Brown, D. Encyclopedia of herbs and their uses. London: Dorling Kindersley; 1995.

Dev, D.B. The flora of Tripura, vol. II. New Delhi: Today and Tomorrow's Publisher; 1983:139–140.

Edson, J. L.; Wenny, D. L.; Leege-Brusven, A. D.; Everell, R. L. Using micropropagation to conserve threatened rare species in sustainable forests. J. Sust. For. 5:279–291; 1997.

Hall, R. D. Plant cell culture initiation: practical tips. Mol. Biotechnol. 16:161–173; 2000.

Handique, P. J.; Bora, P. *In vitro* regeneration of a medicinal plant – *Houttuynia cordata* Thunb. from nodal explants. Curr. Sci. 76:1245–1247; 1999.

Kanjilal, P.C.; Dev, R. N. Flora of Assam, vol. III. New Delhi: Omsons Publishers; 1937:113.

Kyoko, H.; Kamiya, M.; Hayashi, T. Viricidal effects of the steam distillate from *Houttuynia cordata* Thunb. and its components on HSV-1, influenza virus and HIV. Planta Med. 61:237–241; 1994.

Mukherjee, A.; Roy, S. D. An account of Piperaceae and Saururaceae in the hills of Darjeeling district, West Bengal (India). J. Econ. Tax. Bot. 9:367–372; 1987.

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

Park, J. C.; Hur, J. M.; Park, S. J.; Lee, J. H.; Sung, N. J.; Choi, M. R.; Song, S. H.; Kim, M. S.; Choi, J. W. The effects of *Houttuynia cordata* on the hepatic bromobenzene metabolizing enzyme system in rats and isolation of phenolic compounds. Kor. J. Pharmacol. 31:228–234; 2000.

Preece, J. E.; Imel, M. R. Plant regeneration from leaf explants of rhododendron 'P. J. M. hybrids'. Sci. Hort. 48:159–170; 1991.

Rani, G.; Virk, G. S.; Nagpal, A. Callus induction and plantlet regeneration in *Withania somnifera* (L.) Dunal. In vitro Cell. Dev. Biol.—Plant 39:468–474; 2003.

Rastogi, R.P.; Mehrotra, B. N. Compendum of Indian medicinal plants, vol. 2. New Delhi: Lucknow and Information Directorate; 1993:376.

Saha, S.; Mukhopadhyay, M.; Mukhopadhyay, S. *In vitro* clonal propagation through bud culture of *Hemidesmus indicus* (L) R Br: an important medicinal herb. J. Plant Biochem. Biotechnol. 12:61–64; 2003.

Sinha, R.K.; Chakraborti, S.; Roy, K.; Sinha, S. Traditional knowledge on some ethnobotanical species of Tripura – its utility and future prospects In: International Conference on 'promotion and development of botanicals with international co-ordination: exploring quality, safety, efficacy and regulations', Kolkata, India; 2005:63.

Sinha, R. K.; Mallick, R. Plantlets from somatic callus tissue of the woody legume *Sesbania bispinosa* (Jacq.) W. F. Wight. Plant Cell Rep. 10:247–250; 1991.

Sugiura, A.; Tao, R.; Murayama, H.; Tomana, T. *In vitro* propagation of Japanese persimmon. HortSci. 21:1205–1207; 1986.

Tauzuki, K.; Miyasita, S.; Kono, N. Report on the plant regeneration from the callus made of the leaf segments of *Houttuynia cordata* Thunb. Bull. Nip. Vet. Anim. Sci. Univ. 44:65–68; 1995.

Torrey, J. G. Root hormones and plant growth. Annu. Rev. Plant Physiol. 27:435–459; 1976.

Tutupalli, L. V.; Chaubal, M. G. Saururaceae. V. Composition of essential oil from foliage of *Houttuynia cordata* and chemosystematics of Saururaceae. Lloydia 38:92–96; 1975.

Wareing, R.F.; Phillips, I. D. J. Growth and differentiation in plants. Oxford: Pergamon Press; 1981.

Winnaar, W. De. Clonal propagation of papaya *in vitro*. Plant Cell Tiss. Organ Cult. 12:305–310; 1988.

Wochok, Z. S. The role of tissue culture in preserving threatened and endangered plant species. Biol. Conserv. 20:83–89; 1981.

Wu, W.; Zheng, Y.; Liu, F.; Tan, G.; Ren, H.; Zhang, W. Construction of fast propagation system of *Houttuynia cordata* new line. Zhongguo Zhongyao Zazhi 29:27–28; 2004.