International Journal of Advanced Multidisciplinary Research (IJAMR)
ISSN: 2393-8870
www.ijarm.com
Coden:IJAMHQ(USA)

Research Article

SOI: http://s-o-i.org/1.15/ijarm-2016-3-1-9

In vitro plant regeneration of *Hydrocotyle conferta* Wight -A medicinally important of Southern India

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Keywords

Hydrocotyle conferta,
callus,
regeneration,
organogenesis.

Abstract

An efficient plant regeneration system for *Hydrocotyle conferta* Wight, an endemic herb of Western Ghats of South India, was established. The investigated factors influencing callus induction and plant regeneration included sterilizing agents, explant type, culture medium, and combinations of plant growth regulators in the medium. Five minutes treatment of explants with 0.2% $HgCl_2$ was more suitable for sterilization of leaf explants than NaOCl and H_2O_2. Leaves as explants were superior to petioles, internodes and roots, and MS medium was better than WPM and B_5 media. It was found that 9.1 µM 2, 4-D-dichlorophenoxyacetic acid (2, 4-D) and 4.4 µM 6-benzyladenine (BA) in the medium was the best hormone combination for promoting callus induction. The highest shoot regeneration frequency (99%) was achieved from callus cultured on MS medium supplemented with 2.3 µM 2, 4-D and 8.9 µM BA. The rooted shoots were successfully transplanted in soil, with over 91% survival. Establishment of an efficient plant regeneration procedure provides a basis for rapid *in vitro* propagation of *Hydrocotyle conferta*.

Introduction

Hydrocotyle conferta Wight (Apiaceae) is a herbaceous endemic species to Western Ghats of South India (Nayar, 1996) which is growing in limited areas of high altitudes of Nilgiri hills and Palni hills. *Hydrocotyle conferta* is also included in the Red Data Book of Indian plants (Nayar and Sastry, 1988). As a result of the degradation and destruction of natural habitats and due to fast depletion of rain forests, many endemic species have become threatened *in situ* and need conservation.

The use of advanced biotechnological methods of culturing plant cells and tissues offers a useful means for conserving and rapidly propagating valuable, rare and endangered species (Fay, 1992). Many important Indian endemic and endangered plants have been successfully propagated *in vitro*, either by organogenesis (Sharma & Wakhlu, 2011; Joshi et al., 2004) or by somatic embryogenesis (Ignacimuthu *et al*., 1999; Prakash *et al*., 2001; Martin, 2004; Paramageetham *et al*., 2004). Genus *Hydrocotyle* is an important group of medicinal plants which are having potential neutraceutical and pharmaceutical properties due to the presence of asiaticosides (Sakia *et al*., 2006). *In vitro* propagated plants of many species have found to be uniform, showing less variation in their content of secondary metabolites than their wild counterparts (Yamada *et al*., 1991). This paper gives an account *in vitro* propagation of an endemic plant species *Hydrocotyle conferta* by using leaf explants via callus regeneration.

Materials and Methods

Plant material for callus initiation

Plants of *Hydrocotyle conferta* were collected from Palni hills of Western Ghats and grown in earthen pots under green house condition. The young shoots with roots were collected from the garden grown plants and washed with running tap water for 15 min. The internodes, petioles, leaves and roots were cut separately and they were washed with Tween 20 (Merck, India) detergent solution (5% v/v) for 10 – 15 min. In order to solve the serious contamination problem, encountered in all the explants of *Hydrocotyle conferta* culture, three sterilizing agents for plant tissues, Sodium hypochlorite

(NaOCl), Hydrogen peroxide (H_2O_2) and Mercuric Chloride ($HgCl_2$) were tested at various concentrations in different time intervals for their sterilization efficiencies. The surface sterilization of explants was followed by rinse with sterile distilled water 3 - 4 times to remove trace of disinfectants. The whole process was carried out under the laminar air flow chamber.

Culture media

For culture media, MS (Murashige and Skoog's medium), B_5 (Gamborg's medium) and WPM (Woody plants medium) were tested for their ability to produce callus with different explants. For medium effect, the explants were cultured in each of the three media containing 4.5 µM 2, 4-D and 2.2 µM BA. For explant effect, leaves, petioles, internodes and roots were cultured on MS medium supplemented with 4.5 µM 2, 4-D and 2.2 µM BA. Fifteen explants were used in each treatment (three replicates) and the callus induction frequency was assessed after four weeks.

Culture conditions

All the media were fortified with 20 g/l sucrose (Qualigens, India) and gelled with 0.8% agar (Sd-fine chemicals, India), and the pH was adjusted to 5.8 after adding the growth regulators. The media were steam sterilized in an autoclave under 1.5 kg/cm^2 and 121^0 C under a 16 h photoperiod supplied by cool white fluorescent tubes. Atleast fifteen cultures were raised for each treatment and all the experiments were performed three times. The callus induction frequency was calculated as the total number of explants developing callus divided by the total number of explants inoculated, and expressed as a percentage.

Effect of plant growth regulators on callus induction, shoot regeneration and rooting

MS basal media containing different concentrations and combinations of plant growth regulators (NAA, KN, 2, 4-D and BA) were investigated for their effects on callus induction from leaf explants. Subculture was carried out at 3 weeks intervals, and callus induction frequency was measured after 5 weeks.

After 5-6 weeks of subculture, calli were transformed on MS-based regeneration media containing different concentrations of cytokinins (BA, KN) and auxins (NAA, 2, 4-D). The effects of different plant growth regulators on shoot regeneration were examined after 40 days.

Shoots with 3 cm in height were excised and transferred to rooting medium: half strength MS medium containing different concentrations of IAA (Indole -3- Acetic acid), NAA or IBA (Indole -3- Butyric acid). The cultures were incubated under 16 h photoperiod for 20 days until the explants developed the roots. Then the rooting frequency was measured.

Acclimatization and transplantation of plantlets

The rooted plantlets were removed from the culture tubes and washed with tap water to remove trace of agar. Then the plantlets were planted onto polycups containing a mixture of finely chopped peatmass and sterilized garden manure in 1:1 ratio. The polycups were covered with transparent polythene cover to maintain humidity until the development of new leaves for 10 days. Then the polycups were transferred to green house and polythene covers were removed. Quarter strength MS major salts solution poured with 5 days intervals up to 40 days of hardening and followed by pouring of tap water. After 60 days the frequency of survival was calculated.

Statistical analysis

Fifteen replicates were maintained for each experiment and each experiment was repeated thrice to ensure the reliability of data. The effect of plant growth regulators on callus induction, shoot regeneration and rooting was analyzed by ANOVA. Tukey comparison test was used to compare the means and standard deviations (SD).

Results and Discussion

In initial experiments, we failed to establish aseptic *in vitro* cultures of *Hydrocotyle conferta* from the explants (leaves, petioles, internodes and roots) collected from plants growing in the natural habitat due to endogenous bacterial and fungal contaminations. To overcome this problem, plants collected from the natural habitat were transplanted to pots, and grown under controlled, greenhouse conditions described earlier (Franatale *et al*., 2002). New shoots, sprouted after one month of transplanting were used in the present investigation. All the explants of *Hydrocotyle conferta* were treated with three types of disinfectants in the present study. Of the three disinfectants, $HgCl_2$ was better than NaOCl and H_2O_2 for sterilizing all the four kinds of explants (Leaves, petioles, internodes and roots) to achieve different degrees of callus induction frequency. In a preliminary experiment, surface sterilized explants of leaves, petioles, internodes and roots were cultured on MS medium containing 4.5 µM 2, 4-D and 2.2 µM BA. Callus efficiency was observed in all the explants treated with three kinds of sterilizing agents. However, callus formation in internodes, roots and petioles was lesser than the leaf explants (data not shown). Hence further studies were carried out using only the leaf explants. Leaf explants treated with 0.2 % $HgCl_2$ for 5 min were found best for sterilization and callus induction, and the callus induction frequency was 86% (Table 1).

Table 1. Callus induction frequency (%) of leaf explants of *Hydrocotyle conferta* under different sterilization conditions

Sterilizing Agent	Concentration (%)	1 min	5 min	10 min	15 min
$HgCl_2$	0.1	0	68.57 ± 5.06^{b}	26.18 ± 0.12^{d}	0
	0.2	0	86.23 ± 5.77^{a}	46.67 ± 6.15^{c}	0
	0.5	15.21 ± 5.03^{e}	38.21 ± 4.03^{cd}	0	0
NaOCl	0.1	0	0	12.46 ± 2.12^{e}	3.42 ± 0.10^{f}
	0.2	0	12.52 ± 0.56^{e}	38.61 ± 4.09^{cd}	0
	0.5	4.34 ± 0.15^{f}	36.84 ± 3.61^{cd}	30.29 ± 5.01^{cd}	0
H_2O_2	10	0	26.67 ± 2.51^{d}	38.42 ± 3.46^{cd}	26.12 ± 2.12^{d}
	20	0	18.00 ± 1.73^{e}	11.21 ± 1.40^{e}	0
	35	15.21 ± 1.62^{e}	12.2 ± 1.76^{e}	2.32 ± 0.10^{f}	0

Data represent means ± SD based on 15 replicates. Different letters after means indicate significant differences at $p<0.05$.

Culture medium and explant type were found to significantly influence callus induction and regeneration. Three basal media (MS, B_5, WPM) containing the same hormones (4.5 µM 2, 4-D and 2.2 µM BA) were used in this study. MS medium was the most favorable for callus proliferation, while callus could not be induced on B_5 medium (Fig.1a). Many earlier studies have reported that MS medium is a superior medium to propagate a few Apiaceae members *in vitro* (Tripathi and Tripathi, 2003).

In the present study, we found that leaf explants were better for callus induction than other explants such as petiole, internodes and roots (Figure 1b). In early studies with *Cannabis sativa* (Slusarkiewicz-Jarzia *et al*., 2005) reported that the callus proliferation potential varies with developmental stages of the various explants. The advantages of leaf as an explant, such as its easy acquisition mean that the procedure can be carried out in all seasons.

Callus was induced from the leaf explant using MS basal medium supplied with both auxins and cytokinins (Table 2). When cultured on media containing no plant growth regulators, no callus was induced regardless of explant type. Application of 2, 4-D alone showed no effect on callus induction, but the combined use of auxin and cytokinin significantly improved callus induction and growth. The use of 2, 4-D and BA together were superior to the use of other auxin and cytokinin combinations tested for callus induction (Fig. 1b). The highest callus induction frequency (100%) was achieved from leaf segments cultured on medium containing 9.1 µM 2, 4-D and 4.4 µM BA (Table 2). The calli were greenish white or greenish yellow. The calli were maintained by subculture at 3-week intervals. Petiole explants could induce less callus proliferation but produced adventitious shoots directly.

Table 2. Effect of plant growth regulators (PGR) on callus induction and shoot regeneration of leaf explants of *Hydrocotyle conferta*

Callus induction		Shoot regeneration	
PGR (µM)	Frequency (%)	PGR (µM)	Frequency (%)
2, 4-D 2.3	0	KN 4.7 + 2, 4-D 2.3	39.0 ± 4.3^{b}
2, 4-D 4.5	0	KN 9.3 + 2, 4-D 2.3	40.2 ± 2.4^{b}
2, 4-D 9.1	0	KN 9.3 + 2, 4-D 4.5	52.3 ± 5.2^{b}
2, 4-D 4.5 + KN 2.3	33.3 ± 2.3^{c}	BA 4.4 + 2, 4-D 2.3	80.3 ± 4.1^{a}
2, 4-D 4.5 + KN 4.7	18.0 ± 1.7^{d}	BA 8.9 + 2, 4-D 2.3	99.0 ± 1.7^{a}
2, 4-D 9.1 + KN 4.7	42.3 ± 5.0^{c}	BA 8.9 + 2, 4-D 4.5	$94.7 + 3.0^{a}$
2, 4-D 4.5 + BA 2.2	79.0 ± 8.4^{b}	KN 4.7 + NAA 2.7	0
2, 4-D 4.5 + BA 4.4	66.7 ± 5.0^{b}	KN 9.3 + NAA 2.7	12.2 ± 2.3^{d}
2, 4-D 9.1 + BA 4.4	86.9 ± 2.0^{a}	KN 9.3 + NAA 5.4	11.6 ± 1.8^{d}
NAA 5.4 + KN 2.3	9.0 ± 1.6^{d}	BA 4.4 + NAA 2.7	11.0 ± 4.1^{d}
NAA 5.4 + KN 4.7	2.4 ± 1.2^{e}	BA 8.9 + NAA 2.7	25.2 ± 5.1^{c}
NAA 10.7 + KN 4.7	10.2 ± 2.0^{d}	BA 8.9 + NAA 1.0	18.1 ± 4.0^{cd}
NAA 5.4 + BA 2.2	12.6 ± 1.8^{d}		
NAA 5.4 + BA 4.4	4.2 ± 2.0^{e}		
NAA 1 0.7 + BA 4.4	13.7 ± 2.1^{d}		

Data represent means ± SD based on 15 replicates. Different letters after means indicate significant differences at $p<0.05$.

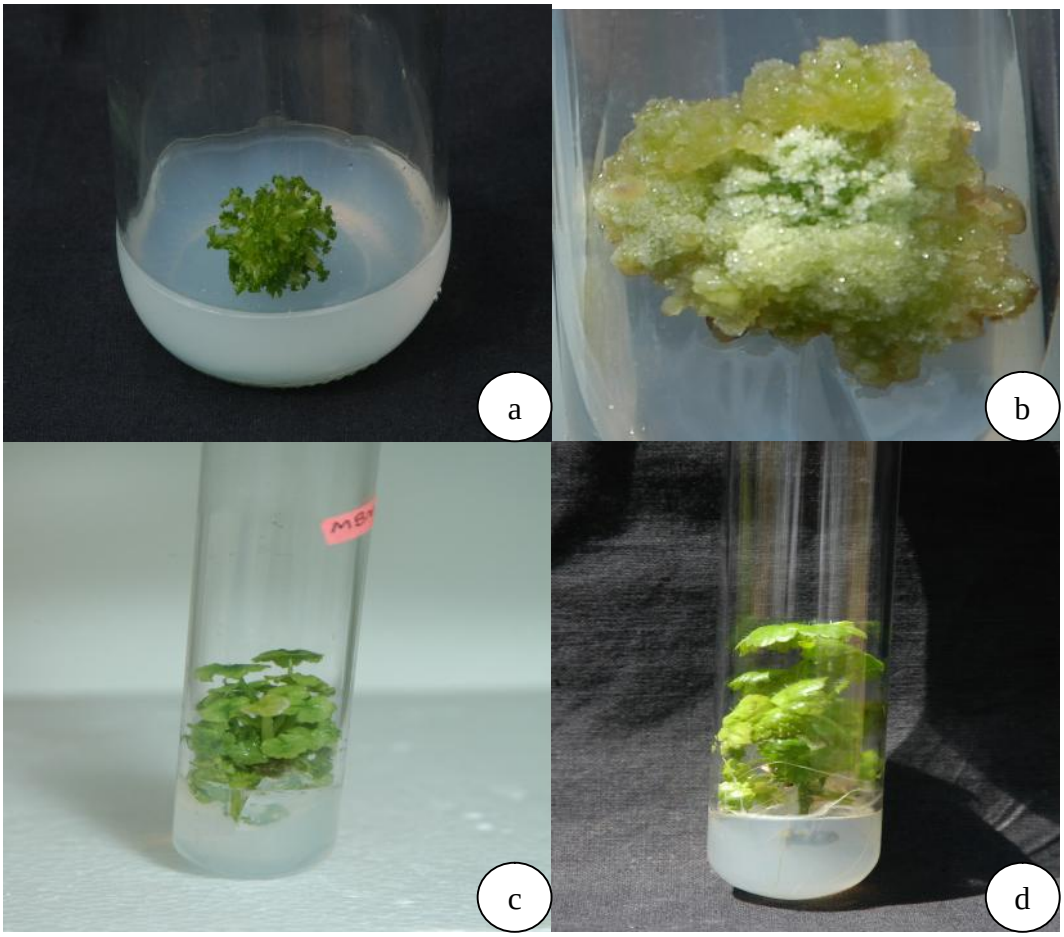

Figure 1. *In vitro* propagation of *Hydrocotyle conferta*. ***a***. Multiple shoot induction on the BAP supplemented MS medium. ***b***. Leaf callus on 2,4-D + BA supplemented medium. ***c***. Callus derived in vitro shoot. ***d***. In vitro rooting on NAA supplemented medium.

A suitable combination of auxin and cytokinin in the regeneration medium was important for regeneration of *Hydrocotyle conferta* (Table 2). The use of BA and 2, 4-D together was superior to other combinations tested for regeneration (Fig. 1c). The highest shoot regeneration frequency (99%) was achieved with medium containing 2.3 µM 2, 4-D and 8.9 µM BA. The maximum number of shoots per callus (18.4) and the longest shoots (4.5cm) were achieved when calli were cultured on medium containing 2.3 µM 2, 4-D and 4.4 µM BA (data not shown). The time of shoot emergence, varying from 15 days to 25 days, was also affected by growth regulator treatments.

In most cases calli cultured on regeneration medium became hard and turned green, and the whole callus developed into a protuberant structure in medium containing suitable combination of growth regulators. In the present study, *Hydrocotyle conferta* leaf callus placed on medium containing 2.3 µM 2, 4-D and 4.4 µM BA led to differentiation of shoot initials 10 to 15 days later, and tiny shoot protuberances developed soon after. It differentiated subsequently into shoots 10 days later from the initiation, resulting in multiple shoots (18.4) derived from single callus. The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced. There are many reports on the regeneration of several plants via callus culture. Satheesh Kumar and Bhavanandan (1988) have reported the regeneration of shoots from callus of *Plumbago reosea* using appropriate concentrations of auxins and cytokinins.

On regeneration medium, shoots developed roots slowly. Rooting medium promoted more rapid root development. Vanegas *et al*. (2002) reported that shoots of marigold developed roots well on hormone free medium. In the present study, on hormone free medium a few roots developed from shoots of *Hydrocotyle conferta* after 18 days, and the rooting frequency was 12.2%. Rooting efficiency was improved by the addition of auxin to the medium (Table 3). The highest rooting rate (100%) was achieved from shoots cultured on half strength MS medium containing low concentrations of 0.5 or 1.1 µM NAA (Fig. 1d). IBA 1.0 µM containing medium also gave best results on rooting of *Hydrocotyle conferta* shoots (95%). Rooting

time was also shortened to 4-5 days with addition of low concentrations of NAA or IBA to the medium, and there were more roots per shoot on auxin containing medium (average 8.2) than on auxin free medium (average 3.8). After a month of acclimation, well rooted plantlets were transplanted into pots with over 91% survival under greenhouse condition.

Table 3. Effect of different auxins on rooting of *Hydrocotyle conferta* on MS half strength medium

Auxins (µM)	Rooting Rooting frequency (%)
IAA 0.6	52. 2 ± 4.0[c]
IAA 1.1	75.0 ± 4.2[b]
IAA 2.9	32.2 ± 3.4[d]
NAA 0.5	100.0 ± 0.0[a]
NAA 1.1	100.0 ± 0.0[a]
NAA 2.7	73.0 ± 3.0[b]
IBA 0.5	78.6 ± 5.2[b]
IBA 1.0	95.2 ± 1.0[a]
IBA 2.5	52.6 ± 5.0[c]
Without auxin	12.2 ± 0.5[e]

Data represent means ± SD based on 15 replicates. Different letters after means indicate significant differences at $p< 0.05$.

In this study, we established a stable and efficient system for plant regeneration of *Hydrocotyle conferta*. Only 92 days were needed from callus induction to plantlet transplantation. No significant morphological variation was observed between the 64 plants regenerated by this procedure and non-cultured donor plants. Tissue culture technology could help prevent further depletion of the natural population of this important endemic plant species in South India.

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How to cite this article:

S. Karuppusamy and **M. Jayakumar. (2016). *In vitro* plant regeneration of *Hydrocotyle conferta* Wight - A medicinally important of Southern India. International Journal of Advanced Multidisciplinary Research 3(1): 68–73.**