Establishment and analysis of fast-growing normal root culture of *Decalepis arayalpathra*, a rare endemic medicinal plant

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Fast-growing normal root culture of *Decalepis arayalpathra*, a rare endemic medicinal plant was established from leaf and inter nodal explants of *in vitro*-raised shoot cultures in Murashige and Skoog (MS) medium containing 2.5 mg/l 6-benzyladenine (BA), 0.5 mg/l 2-isopentenyladenine (2-ip) and 0.5 mg/l α-naphthaleneacetic acid (NAA). Shoot cultures were maintained on MS agar medium supplemented with 0.5 mg/l BA and 0.05 mg/l 2-ip or and 0.05 mg/l NAA and subcultured at 5-weeks interval. Leaf explants incubated in total darkness in half-strength MS medium supplemented with 0.5 mg/l IBA and 0.2 mg/l NAA favoured induction of roots in 89% of the cultures with highest biomass of roots (5.820 g). A 100 mg fresh root tissue cultured in 80 ml half-strength MS liquid medium supplemented with 0.2 mg/l IBA and 0.1 mg/l NAA, under continuous agitation (80 rpm), yielded 3.433 g and 0.734 g fresh and dry weight of roots, respectively. Roots grown in this optimal medium produced maximum compound, 2-hydroxy-4-methoxy benzaldehyde (0.16%) after 6 weeks of culture. The root cultures were maintained up to the 7th passage without decline of growth.

DECALEPIS arayalpathra (Joseph & Chandras) Venter., (*Janakia arayalpathra* Joseph & Chandras) (Periplocaceae) is a perennial woody laticiferous shrub[1,2] with slender, spreading branches. The plant is endemic to southern forests of the Western Ghats region of Kerala (South India)[3], distributed at an elevation of 800–1200 m and growing in the crevices of rocks. The monoliform tuberous roots of the plant are highly aromatic and the native Kani tribes use it as an effective remedy for peptic ulcer, cancer-like afflictions and as a rejuvenating tonic[4]. Recent pharmacological investigations of the root extract of the plant revealed immunomodulatory and anticancer properties[5]. The tubers are being ruthlessly collected from its natural habitat by the local Kani tribes. This has led to the acute scarcity of the plant. Consequently, it has been enlisted as endangered plant species[6]. The natural regeneration as well as conventional propagation of this plant is beset with several factors like poor fruit set, seed germination and rooting on stem cuttings. Considering the urgent need for *ex situ* conservation, an *in vitro* propagation system was recently achieved through single shoot formation from field-derived nodal explants of this plant[7].

In chemical constituents, tuberous roots of the plant are closely similar to that of *Hemidesmus indicus* R.Br., Indian Sarasaparilla and *Decalepis hamiltonii* Weight & Arn, an allied species which are widely used in the traditional system of medicine as a blood purifier and as a flavouring agent for the preparation of soft drinks and bakery products. It is interesting to note that a recent investigation carried out by George *et al.*[8] revealed the potent insecticidal activity of tuberous roots of *D. hamiltonii*. However, due to the scarce availability of *D. arayalpathra,* an extensive investigation on its wide unexposed potentialities has not been exploited as in *H. indicus* or *D. hamiltonii*. Aromatic compound 2-hydroxy-4-methoxy benzaldehyde is the main chemical constituent giving flavour to the roots of these plants.

It is important to note that in approximately 60% of the medicinal plants used in the traditional systems of medicine (Ayurveda, Siddha, Unani), roots are the principle material for drug preparation[9]. It is estimated that more than 90% of the plant species used by the industry is collected from the wild and more than 70% of the plant drugs involved destructive harvesting[10] and very few are in cultivation. Development of biotechnological methods such as micropropagation, cell/root and hairy root cultures is one of the major solutions to circumvent these problems. On this line, development of fast-growing root culture system offers unique opportunities for providing root drugs in the laboratory, without resorting to field cultivation. Moreover, development of root culture is highly advantageous, as it is an alternative method for clonal propagation and germplasm conservation[11]. The present study offers a fast-growing normal root culture and detection of a root-specific aromatic compound, 2-hydroxy-4-methoxy benzaldehyde.

Aseptic shoot cultures of *D. arayalpathra* were raised through single shoot formation from nodal explants derived from field-grown plants on agar-based Murashige and Skoog (MS)[12] medium with 2.5 mg/l 6-benzyladenine (BA), 0.5 mg/l 2-isopentenyladenine (2-ip) and 0.5 mg/l α-naphthaleneacetic acid (NAA)[7]. Shoot cultures were regularly subcultured in the medium supplemented with 0.5 mg/l BA and 0.05 mg/l 2-ip or with 0.05 mg/l NAA at 5-weeks interval. Transversely-split leaf segments of well-expanded leaf from second to fifth nodes below the shoot tip, and 1.5–2.0 cm long internodal segments below the terminal end of the long shoots maintained in subculture medium, were used as explants for the induction of roots. They were placed horizontally (dorsal surface down) on 50 ml agar gelled (0.6% w/v; Hi-Media Pvt Ltd, India) full- and half-strength MS medium supplemented with varied concentrations of different auxins, indole-3-

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acetic acid (IAA), indole-3-butyric acid (IBA) and NAA in 250 ml Erlenmeyer flasks. The pH of the medium was adjusted to 5.8 before adding agar and autoclaved at 121°C and 108 kPa for 20 min. The shoot cultures were incubated at 24 ± 2°C under 16 h photoperiod at a photon flux density of 20–50 μmol m^{-2} s^{-1} from daylight fluorescent tubes (Philips India Ltd, Mumbai). The explants for root induction were incubated under the conditions similar to those for shoot cultures or under total darkness.

After six weeks of initiation, roots were separated from the explants aseptically and the nutrient medium sticking on the roots was removed. Thereafter, roots were cut into 1.0–1.5 cm long segments and fresh root tissue (100 mg fw) was subcultured into 80 ml aliquots of half-strength MS liquid medium supplemented with IAA, IBA and NAA (0.1–0.5 mg/l) in 250 ml Erlenmeyer flasks. The cultures were kept under continuous agitation at 80–90 rpm in a gyrotory shaker (Brunswick, USA) and incubated under light (16 h photoperiod) or total dark condition. The root growth in different auxin regimes was assessed in terms of fresh and dry weight after six weeks of culture. Regular subculture was done by inoculating growing root tip (0.8–1.0 cm) in the optimal medium (half-strength MS liquid medium with 0.2 mg/l IBA and 0.05 mg/l NAA) at 4-weeks interval. The root biomass was determined up to a period of eight weeks and the production of 2-hydroxy-4-methoxybenzaldehyde was assessed. Fresh weight of the root was determined by blotting the harvested roots on filter paper after a gentle wash in distilled water. Dry weight was obtained after drying them at room temperature for 48 h. Biomass was considered as fw/dw per 80 ml medium.

Phytochemical analysis was done by extracting 5 g of the dried root powder (obtained at different stages of growth time) with petroleum ether (60–80°C) for 48 h in a Soxhlet apparatus. The extract so obtained was chromatographed on thin layer chromatographic plates (TLC) with reference compound 2-hydroxy-4-methoxybenzaldehyde (Fluka chemicals and Co, Switzerland) and analysed in a solvent system, petroleum ether (60–80°C) : ethyl acetate (9:1). The quantitative determination was done by HPLC (Shimadzu CR 7A e plus, with detector SPD-10A UV-VIS, column – CLC-ODS, λ-max 278, methanol : water : 36% acetonitrile :: 45 : 55 : 2) with a flow rate of 1.5 ml/min and with a known quantity of the reference compound.

Explants cultured on full-strength MS medium always induced profuse callusing which subsequently turned brownish and hindered initiation of roots. Therefore, explants were cultured on half-strength MS medium (Table 1). The influence of full ionic concentration, especially of nitrates in MS medium, may be responsible for the profuse induction of callus. Roots initiated within 2–3 weeks on leaf explants followed by internodal explants (3–4 weeks) in most of the treatments, under total darkness. Whereas, under light condition (16 h photoperiod), rooting was delayed (5–6 weeks) in both explant types. A minimum of 4–5 roots on leaf explants and 2–3 roots on internodal explants were observed. Even though rooting was preceded by callusing at various degrees in few auxin treatments on either explants, efficiency of rooting was more with less degree of callusing in leaf explants (Figure 1 *a*) than internodes (Figure 1 *b*). Of the various auxin supplements, the response of the explants was higher in a combination of low concentration of NAA with high concentra-

Table 1. Effect of different auxins in half-strength MS medium on root induction of *in vitro*-derived leaf and internodal segments of *D. arayalpathra*, under total darkness

Auxin (mg/l)			Frequency of response (%)		Mean fresh weight (g)	
IAA	IBA	NAA	LF	IN	LF	IN
0.2	–	–	30.3	12.2	1.273 ± 0.075	0.360 ± 0.032
0.5	–	–	39.5	15.1	1.427 ± 0.034	0.425 ± 0.005
0.5	–	0.2	78.2	36.4	4.538 ± 0. 43	3.106 ± 0.010
0.5	0.2	–	42.3	18.2	2.525 ± 0.009	0.868 ± 0.007
1.0	–	0.2	62.1	25.9	3.560 ± 0.010	1.565 ± 0.009
–	0.2	–	48.2	16.0	3.000 ± 0.030	1.120 ± 0.013
–	0.5	–	52.0	20.3	3.775 ± 0.012	2.065 ± 0.002
–	0.5	0.2	89.1	43.2	5.820 ± 0.024	4.106 ± 0.011
0.2	0.5	–	56.7	22.7	2.890 ± 0.009	0.952 ± 0.005
–	1.0	0.2	71.5	34.0	4.010 ± 0.016	1.860 ± 0.023
–	–	0.2	38.2	19.1	2.386 ± 0.022	0.725 ± 0.015
–	–	0.5	45.0	21.3	3.225 ± 0.060	0.737 ± 0.042
–	0.2	0.5	57.6	31.7	4.108 ± 0.047	2.869 ± 0.012
–	0.2	1.0	52.4	28.2	2.620 ± 0.038	1.110 ± 0.020

Observations were made after 6 weeks; mean (n = 15); LF, leaf; IN, internode.

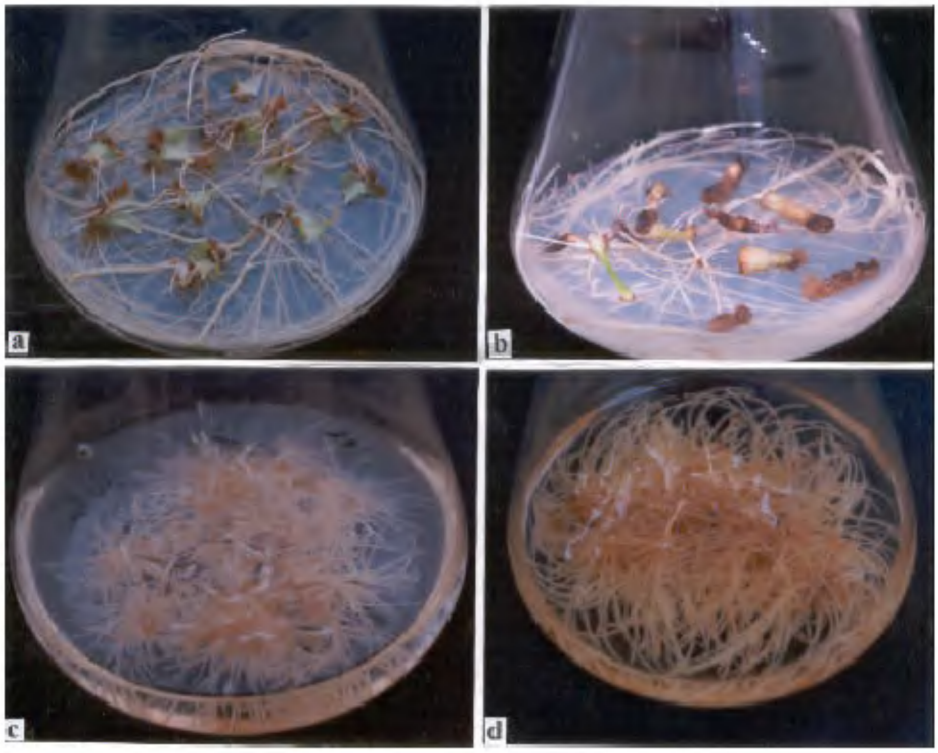

Figure 1. Initiation and establishment of normal root culture from *in vitro* leaf and internodal segments of *D. arayalpathra*; Root initiation on ***a***, leaf segments; and ***b***, on internodal segments cultured in half-strength MS medium with 0.5 mg/l IBA and 0.2 mg/l NAA after 6 weeks incubation in total darkness; root growth after ***a***, 4-weeks and ***d***, after 6 weeks incubation in darkness in half-strength MS liquid medium with 0.2 mg/l IBA and 0.1 mg/l NAA.

tions of IBA or IAA (Table 1). Leaf explants (89%) incubated in total darkness in half-strength MS medium supplemented with an optimum combination of IBA (0.5 mg/l) and NAA (0.2 mg/l) showed rapid induction of roots and higher growth (5.820 g fw) after a period of 6 weeks (Table 1). IAA–NAA combinations suppressed the lateral root formation compared to IBA–NAA combinations. However, in both combinations of auxins, significant length of roots (15–20 cm) was noticed. The influence of individual auxins on root growth was negligible. Unlike IAA and IBA, the roots initiated in the medium containing NAA alone at high concentrations were thick, short and occasionally callused.

Unlike in root induction, influence of dark and light conditions was not an important factor for increased biomass production during subculture, as was noticed in *Hemidesmus indicus*[13]. Dark incubation favouring root induction may be due to the slow metabolism of endogenously or exogenously applied auxin in the dark than in the light, as stated by Norton and Boe[14]. The stimulatory effect of darkness and inhibitory effect of light condition on root induction have been well-documented in other systems[15,16]. During serial subculture, reduced concentrations of auxin favoured rapid and viable growth of roots (Table 2). NAA in subculture medium was found to be inevitable for profuse and rapid lateral root formation. The medium supplemented with 0.2 mg/l IBA and 0.1 mg/l NAA was best to produce maximum biomass (Table 2). Decrease in biomass was noticed in cultures due to low number of lateral roots because of the callusing of roots. It is important to note that in any system, the production of lateral roots is a key factor for the rapid growth and is responsible for higher biomass. Lateral root initiation on root segments in the present system was noticed on the root axis after 10th day of culture. The rapid growth started after 16–17 days by the elongation of lateral roots. Lateral roots elongated (2.0–3.5 cm) and formed a mat of roots at the end of 4 weeks (Figure 1 *c*). Maximum growth of roots was noticed at the 6th week (Figure 1 *d*), and beyond that growth slowly declined (Figure 2). During the 8th week of growth, roots turned brown, flaccid and brittle. For regular maintenance of root culture, it was found that root tips of 4-week-old culture were the best inoculum. The decline of root growth might be due to the accumulated endogenous auxin during each subculture. Most likely, the loss of root differentiation ability is one of the drawbacks of maintaining normal root culture for a long term as observed in *Duboisia* species[17].

Qualitative detection of 2-hydroxy-4-methoxy benzaldehyde in root culture using TLC indicated the same Rf value (4.2) with that of the reference standard, which

Table 2. Effect of different combinations of auxin in half-strength MS liquid medium on root biomass (fw/dw)

Auxin (mg/l)				
IAA	IBA	NAA	Mean fw (g)	Mean dw (g)
–	0.5	0.2	2.808 ± 0.035	0.650 ± 0.005
–	0.2	0.1	3.433 ± 0.024	0.734 ± 0.012
0.5	–	0.2	1.980 ± 0.036	0.429 ± 0.008
0.2	–	0.1	2.481 ± 0.021	0.600 ± 0.015
0.2	0.2	–	1.125 ± 0.019	0.290 ± 0.007

Initial root inoculum (100 mg). Observations were made after 6 weeks; mean value = 4 flasks.

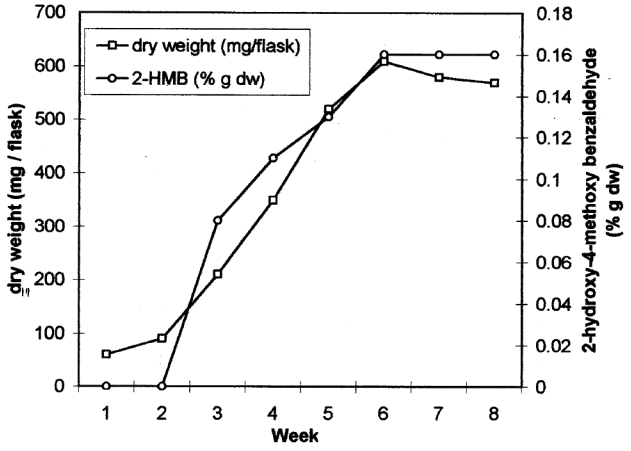

Figure 2. Time course of root growth and production of 2-hydroxy-4-methoxy benzaldehyde (2-HMB) in half-strength MS medium with 0.2 mg/l IBA and 0.1 mg/l NAA. Initial weight of inoculum was 60 mg (dry weight) in 80 ml medium.

was later confirmed by co-TLC. The quantitative estimation of the compound in the optimum medium carried out by HPLC indicated that it was not detected in the early stages of growth (Figure 2). The accumulation of the compound was closely related to the biomass production. Root culture harvested from 6th week yielded maximum concentration (0.16%) of the compound and did not increase further during continued incubation (Figure 2).

Hairy root culture systems are more efficient than normal root cultures, because of their genetic and biochemical stability over long periods and are ideal for introducing genes to elevate growth and secondary metabolism. However, normal root culture system is an alternative method for those species, which are recalcitrant to *Agrobacterium rhizogenes* infection. Before the establishment of first hairy root culture[18], normal root culture had been established for a number of species and demonstrated the ability to accumulate secondary products similar to those found in the roots of the parent plant[19,20]. But in most of these studies, callus-derived root culture systems were established.

In conclusion, the present system of normal root culture would be beneficial for the sustainable utilization of this rare endemic medicinal plant for its bioactive ingredients, thereby providing an alternative method rather than destroying whole plants that are not under cultivation.

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