Enhanced multiplication and improved *ex vitro* acclimatization of *Decalepis arayalpathra*

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Abstract

The proposed work describes a protocol for high-frequency *in vitro* regeneration through nodal segments and shoot tips in *Decalepsis arayalpathra,* a critically endangered medicinal liana of the Western Ghats. Nodal segments were more responsive than shoot tips in terms of shoot proliferation. Murashige and Skoog's (MS) basal medium supplemented with 5.0 µM 6-benzyladenine (BA) was optimum for shoot initiation through both the explants. Among different combinations of plant growth regulators and growth additive screened, MS medium added with 5.0 µM BA + 0.5 µM indole-3-acetic acid + 20.0 µM adenine sulphate effectuated the highest response: 11.8 shoots per nodal segment and 5.5 shoots per shoot tip with mean shoot length of 9.2 and 4.8 cm, respectively. Half-strength MS medium with 2.5 µM α-naphthalene acetic acid was optimum for *in vitro* root induction. The plantlets with the well developed shoot and root were acclimatized in *Soilrite*™ with 92 % survival rate in the field conditions. During acclimatization, chlorophyll content, net photosynthetic rate, stomatal conductance, and transpiration rate were gradually changed in dependence of formation of new leaves. Further, the changes in activities of antioxidant enzymes, *i.e.*, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) as well as activity of carbonic anhydrase were also observed: a continuous rise in SOD activity, but a rise and fall in the activities of CAT, APX, and GR were also noticed. Maximum fresh mass (3.1 g plant^{-1}), dry mass (0.35 g plant^{-1}) of roots and 2-hydroxy-4-methoxybenzaldehyde content of 9.22 µg cm^{-3}(root extract) were recorded after 8 weeks of acclimatization.

Additional key words: antioxidants, auxins, 6-benzyladenine, carbonic anhydrase, chlorophyll, photosynthesis, stomatal conductance, transpiration.

Introduction

Decalepis arayalpathra (Joseph & Chandras) Venter. (syn. *Jankia arayalpathra* Joseph & Chandras) is a perennial lactiferous liana of the family *Apocynaceae* (Joseph and Chandrasekharan 1978, Venter and Verhoveven 1997), endemic to a few forest parts of the Thiruvananthapuram district of Kerala, Tirunelveli and Kanyakumari district of Tamil Nadu. The tuberous roots are the most important part of the plant used for medicinal purposes (Shine *et al.* 2007). In the recent study, Verma *et al.* (2014) reported that the tuberous roots of this plant contain an isomer of vanillin 2-hydroxy-4-methoxybenzaldehyde (2H4MB). The 2H4MB has antimicrobial, antioxidant, and anti-fungal activities against seed borne fungal pathogens and it is a flavouring agent (Wang *et al.* 2010). This species comes under the high conservation concern as declared by The National Biodiversity Authority of India. Its natural regeneration is hampered by poor fruit set, low seed germination, and hard to root stem cuttings (Sudha and Seeni 2001). Therefore, there is an urgent need to conserve and propagate this species through the application of plant tissue culture.

Very few studies concern the micropropagation of this critically endangered plant (Sudha *et al.* 2001, 2005,

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Abbreviations: APX - ascorbate peroxidase; ADS - adenine sulphate; BA - 6-benzyladenine; CA - carbonic anhydrase; CAT - catalase; Chl - chlorophyll; E - transpiration rate; Glu - glutamine; GR - glutathione reductase; g_s - stomatal conductance; 2H4MB - 2-hydroxy-4-methoxybenzaldehyde; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; 2iP - 2-isopentyladenine; Kn - kinetin; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; PG - phloroglucinol; PGR - plant growth regulator; P_N - net photosynthetic rate; SOD - superoxide dismutase.

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2013, Gangaprasad *et al.* 2005). Gangaprasad *et al.* (2005) were able to regenerate only one shoot in 30 d of culture. Sudha *et al.* (2001) established the protocol for the quantitative detection of 2H4MB using thin layer chromatography in fast growing root culture. Sudha *et al.* (2013) further achieved *Agrobacterium rhizogenes* mediated transformation for enhanced production of 2H4MB in *D. arayalpathra.* However, this communication describes the effect of acclimatization on the synthesis of 2H4MB content in the root which is not reported yet for this plant. Moreover, there is no report on the antioxidant enzyme, stomatal conductance, transpiration rate, and related physiological attributes of *in vitro* raised *D. arayalpathra* plantlets during their establishment in *ex vitro* conditions.

The proposed work was taken to develop a reliable protocol for micropropagation of this critically endangered species. To achieve the aim, nodal segments and shoot tips were screened on media supplemented with different phytohormones and growth additives. The present study also reports a comprehensive description of morphogenetic events and acclimatization effects on different physiological parameters. Finally, the identification and quantification of 2H4MB in the root system of *in vitro* raised and acclimatized plantlets was carried out.

Materials and methods

Plant material, media, and culture condition: Young shoots (4.0 to 6.0 cm) of *Decalepis arayalpathra* (Joseph & Chandras) Venter. were collected from a 3-year-old plant growing in the Department of Botany, Aligarh Muslim University. The plant is taxonomically confirmed by the taxonomist Prof. M. Badruzzaman Siddiqui, Department of Botany, Aligarh Muslim University and the specimen is submitted to the department herbarium with accession No. 34173. The collected shoots were thoroughly washed under running tap water for 30 min to remove the dust particles. Thereafter, they were treated with 1 % (m/v) fungicide *Bavistin* (*BASF*, Mumbai, India) for 30 min followed by wash in 5 % (v/v) liquid detergent *Labolene* (*BASF*) for 15 min and then, 6 - 7 times in sterilized double distilled water. Subsequently surface sterilization was carried out under horizontal laminar flow hood by using 0.1 % (m/v) $HgCl_2$ solution for 3 min. It was followed by rinsing 4 - 5 times with sterilized double distilled water before the inoculation. Two type of explants were used: shoot tips (0.8 - 1.2 cm) and nodal segments (1 - 1.5 cm) having one apical and two axillary buds.

Nutrient medium comprising of Murashige and Skoog (1962; MS) salts and vitamins added with required plant growth regulators (PGRs), 3 % (m/v) sucrose (*Qualigens*, Mumbai, India) and 0.8 % (m/v) agar (*Qualigens*). Before autoclaving at 121 °C and 1.06×10^5 N m^{-2} for 15 min, the pH of the medium was adjusted to 5.8 by 1 M NaOH and 1 M HCl. The MS medium was allocated to the culture tubes (25 × 150 mm, *Borosil*, Mumbai, India) or Erlenmeyer flasks (100 cm^3). All the cultures were kept in culture room at a 16-h photoperiod, a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (40 W; *Philips*, Kolkata, India), a relative humidity of 55 ± 5 %, and a temperature of 24 ± 2 °C.

For shoot regeneration, nodal segments and shoot tips were inoculated aseptically in culture tubes containing 20 cm^3 of full-strength MS medium supplemented with different concentrations (1.0, 2.5, 5.0, and 7.5 µM) of cytokinins like 6-benzyladenine (BA), kinetin (Kn), 2-isopentanyladenine (2iP) with or without auxins like indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) at different concentrations (0.1, 0.5, and 1.0 µM). The plant growth regulator (PGR) free MS basal medium was served as control.

To refine shoot regeneration, different growth additives like glutamine (Glu), adenine sulphate (ADS) and phloroglucinol (PG) at different concentrations (10, 20, 30 and 40 µM) were used to optimize the combination of cytokinin and auxin. The PGRs and additives were obtained from *Duchefa* (Haarlem, The Netherlands). After proper regeneration of shoots, the explants were transferred to Erlenmeyer flasks containing 50 cm^3 of optimized combination of PGRs and additives. The non-absorbent cotton wrapped properly in muslin cloths was used for plugging the culture tubes and flasks.

For *in vitro* rooting, healthy microshoots were excised from proliferating cultures and transferred to full- and half-strength MS medium with or without auxin like NAA, IAA, and IBA at different concentrations (0.1, 0.5 and 1.0 µM). MS medium devoid of any auxins was used as control.

Acclimatization: The rooted plantlets were gently washed under running tap water up to the removal of adhering medium. After that they were transferred to *Thermocol* (expanded polystyrene) cups having autoclaved *Soilrite*™ (75 % Irish peat moss and 25 % horticulture grade expanded *Perlite*) (*Keltech Energies*, Garansur, India) and soaked with water. To assure the maximum air humidity, the plantlets were closed with thin polyethene membrane, for initial 2 weeks and kept in culture room at the same conditions as described for multiplication. After 2 weeks, the polythene bag was repeatedly opened to gradually adapt plants to environmental conditions. Successfully acclimatized plantlets were transferred to clay pots with garden soil and green manure (2:1) and initially (4 weeks) kept in the culture room. Then they were transferred to the greenhouse for further 4 weeks and finally they were transferred to field and grown under full sun.

Physiological analyses: For the estimation of chlorophyll (Chl) *a*, Chl *b*, and total Chl content ten micropropagated plantlets were selected. The green leaves at 3 - 4 nodes from the base were taken at transplantation day (day 0, control) and after 7, 14, 21, and 28 d of acclimatization and extracted with 80 % (v/v) acetone. The estimation was carried out spectrophotometrically at 663 and 645 nm according to Sahai and Shahzad (2013) using UV-visible spectrophotometer (*UV-1700 Pharma Spec, Shimadzu,* Kyoto, Japan).

Net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) were evaluated by using a portable photosynthetic system (*Li-COR 6400,* Lincoln, NE, USA). The third fully expanded leaves were taken between 11:00 and 12:00 and measured in a a single leaf chamber at PPFD of 800 μmol $m^{-2}s^{-1}$, CO_2 concentration of 600 μmol mol^{-1}, a relative humidity of 85 %, and a temperature of 25 °C

For assessment of the activity of antioxidant enzymes, 0.5 g of fresh leaves was homogenized in pre-chilled mortar and pestle in extraction buffer (2.0 cm^3) consisting of 1 % (m/v) polyvinylpyrrolidone (PVP) and *Triton X-100*, and 0.11 g of ethylenediaminetetraacetic acid (EDTA). The homogenate was filtered through four layers of cheese cloth and then it was centrifuged at 27 600 *g* for 20 min in high-speed centrifuge (*Remi Instruments*, Mumbai, India). Extraction was processed in the dark and at 4 °C and a supernatant was used for enzyme assay (the above mentioned spectrophotometer was used).

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by the method of Dhindsa *et al.* (1981). A reaction mixture consisted of 13 mM methionine, 0.5 M phosphate buffer (pH 7.5), 1.3 mM riboflavin, 63 mM nitroblue tetrazolium (NBT), 0.1 mM EDTA, and 0.1 cm^3 of enzyme extract. It was incubated under 15 W fluorescent lamp (*Philips*) at 25 °C for 15 min, and the absorbance was monitored at 560 nm. The SOD inhibits photochemical reduction of NBT and one unit of its activity corresponds to 50 % inhibition of NBT reduction.

Catalase (CAT) (EC 1.11.1.6) activity was measured according to Aebi (1984). The assay mixture (3 cm^3) contained 50 mM phosphate buffer (pH 7.0) and 0.1 cm^3 of enzyme extract. The reaction began with addition of 10 mM H_2O_2. The H_2O_2 disappearance was monitored at 240 nm. The activity was calculated by using the coefficient of absorbance 0.036 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). The enzymatic breakdown of ascorbate and so decrease in absorbance was monitored at 290 nm. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.5), 0.1 mM H_2O_2, 0.1 mM EDTA, 0.5 mM ascorbate, and 0.1 cm^3 of enzyme extract. One unit of enzyme activity indicates the amount of enzyme necessary to decompose 1 μmol of ascorbate per min.

Glutathione reductase (GR) (EC 1.6.4.2) activity was determined according Foyer and Halliwell (1976). The assay mixture contained 50 mM phosphate buffer (pH 7.5), 1.0 mM EDTA, 0.2 mM NADPH, and 0.5 mM glutathione disulfide (GSSG). The reaction was started by adding enzyme extract (0.1 cm^3). The activity of the enzyme was evaluated by using the coefficient of absorbance of 6.2 $mM^{-1}cm^{-1}$.

Carbonic anhydrase (CA) activity was assayed according to Dwivedi and Randhawa (1974). Leaf samples (200 mg) chopped into small pieces (1 - 2 mm) were inserted in 10 cm^3 of 0.2 M cystein in a Petri dish at 0 - 4 °C. Before transfer to the test tube containing 4 cm^3 of phosphate buffer (pH 6.8) they were dried by blotting paper. Then 3.4 cm^3 of 0.2 M sodium bicarbonate in 0.02 M sodium hydroxide solution and 0.2 cm^3 of 0.002 % (m/v) bromothymol blue were added. After shaking, the tube was kept at 0 - 4 °C for 20 min and the rection mixture was titrated against 0.05 M HCl after the addition of 0.2 cm^3 of methyl red indicator.

2-hydroxy-4-methoxybenzaldehyde (2H4MB) content in the roots: For high performance liquid chromate-graphy (HPLC) analysis, standard 2H4MB (98 % purity) was obtained from *Sigma-Aldrich* (New Delhi, India). Methanol and deionised water were purchased from *SDH* (Mumbai, India). A stock solution of 2H4MB was prepared by dissolving 10 mg 2H4MB in methanol (10 cm^3) and different concentrations (20, 30, 40, 50, 100, and 200 μg cm^{-3}) of standard solution were prepared by dilution with methanol and used to obtain calibration curve.

The roots were collected from plants grown in *Soilrite*™ after 0, 2, 4, 6 and 8 weeks of acclimatization. Fresh mass of roots were taken, then roots were dried in shade at room temperature (25 ± 2 °C) and crushed in a pre-chilled mortar and pestle. Root powder (1 g) was dissolved in *n*-hexane (150 cm^3) and shaked at 120 rpm and 25 °C for 2 h. The evaporation of solvent was carried out on water bath at 50 °C. Final sample was prepared by adding 100 cm^3 of methanol and filtered through a 0.22 μm syringe filter (*Genetix*, New Delhi, India).

An HPLC assay was performed on *Waters* system (Milford, MA, USA) armed with *Waters 2707* auto sampler, *Waters Delta 600* pump and *996 Photodiode Array* detectors. The injected amount of the sample was about 10 mm^3. Quantification of the compound was carried out at 280 nm on C18 analytical column (250 × 4.6 mm, 5.0 μm) (*Waters*). *Empower2* software was used for the data acquisition and reporting on *Windows 2000*™ platform. The combination of methanol and water (70:30) was used as mobile phase at pH 5. The flow rate was about 1 cm^3 min^{-1}. The solvent was filtered through a nylon filter. The chromatograms were recorded at 280 nm. The presence of the compound was confirmed by comparison of retention time (RT) of standard with that of the sample. Ultra violet (UV) scan was monitored from 210 to 400 nm.

Statistical analyses: All the experiments were conducted with 20 replicates and repeated thrice. The data were analyzed by one-way *ANOVA* using *SPSS v. 16* (*SPSS*

Inc., Chicago, IL, USA). The data for shoot multiplication and *in vitro* rooting were recorded after 6 and 4 weeks of culture, respectively. The data for physiological and biochemical activities were taken after 0, 7, 14, 21, and 28 d of acclimatization. The HPLC analysis was based on 3 separate measurements of single extract and the data were collected after 0, 2, 4, 6, and 8 weeks of acclimatization. The significance of differences among means was evaluated by Tukey's test at 5 % level of significance.

Results and discussion

On medium without PRGs, a shoot tip elongated into single shoot and a nodal segment induced only two shoots but no multiplication was noticed (Fig. 1*A*).

Fig. 1. Shoot regeneration from nodal segments of *D. arayalpathra*. *A* - Nodal segments cultured on MS basal medium for 3 weeks. B - *S*hoot regeneration after 6 weeks on MS medium + 5.0 µM BA + 0.5 µM NAA. C - Shoot proliferation after 6 weeks on MS medium + 5.0 µM BA + 5.0 µM NAA + 20.0 µM ADS. *D,E* - *In vitro* rooting after 4 weeks on ½ MS medium + 2.5 µM NAA. *F* - An acclimatized plantlet in *Soilrite*™ (after 4 weeks).

Supplementation of different cytokinins triggered the response within 4 d of inoculation and multiple shoot bud formation was noticed after 6 - 7 d of inoculation. Among the cytokinins (BA, Kn and 2ip) tested, BA was found to be the most effective for shoot proliferation. After 6 weeks, MS medium with 5.0 µM BA induced 4.0 and 2.3 shoots per explant with length of 4.8 and 2.9 cm in 85.8 and 60.0 % of cultures using nodal segments and shoot tips as explants, respectively (Table 1). Explants cultivated on medium with Kn or 2ip showed poorer response: 5.0 µM Kn induced 3.3 and 2.4 shoots per explant with length of 4.7 and 2.7 cm in 75.0 and 55.0 % cultures when using nodal segments and shoot tips as explants, respectively. The 2ip at the same concentration induced only 3.0 and 2.0 shoots per explants with length of 4.6 and 2.0 cm in 64.2 and 50.6 % cultures using nodal segments and shoot tips, respectively. Supremacy of BA among the cytokinins for multiple shoot induction is also reported in other species such as *D. hamiltonii* (Anitha and Pullaiah 2002, Sharma *et al*. 2014a) and *Gymnema sylvesters* (Thiyagarajan and Venkatachalam 2013) and might be due to its higher transport across the plasma membrane and higher ability of the cell to metabolize it compared to the other cytokinins (Malik *et al.* 2005).

To improve the regeneration efficiency of both the explants, cytokinin-auxin combinations were also tested. Among different combination, nodal segments and shoot tips cultured on MS medium augmented with 5.0 µM BA and 0.5 µM NAA induced 6.2 shoots per nodal segments with 5.8 cm shoot length in 93.6 % of cultures and 4.0 shoots per shoot tip with 2.3 cm shoot length in 72.8 % response for shoot tip after 6 weeks of culture (Fig. 1*B*, Table 2). When increasing and decreasing the concentration of NAA beyond the optimum, regeneration efficiency of both the explants reduced due the intense callusing.

However, shoots induced either on cytokinin alone or in combination with auxins revealed the poor leaf development and early leaf fall. Therefore, the addition of growth additives such as ADS, Glu, and PG to the medium with 5.0 µM BA and 0.5 µM NAA was tried. This combination was proved to be superior in terms of mean number of shoots and mean shoot length induced in both the explants (Table 3). Among the all growth additives, 20.0 µM ADS proved to be optimal as it induced 11.8 shoots per nodal segment and 5.5 shoots per shoot tip with 9.2 and 4.8 cm shoot length, respectively, within 6 weeks of culture (Fig. 1*C*, Table 3). The addition of other two growth additives Glu and PG was less efficient. The addition of ADS also supported thicker stem and better overall growth and development of regenerants. Murashige (1974) has demonstrated that the addition of ADS has a stimulatory effect on the shoot bud proliferation. Further, the study in *Pterocarpus marsupium* by Husain *et al.* (2007) suggests that ADS acts as a rich source of nitrogen and its cellular uptake is very high in comparison to the other N form. The similar effect of ADS on shoot bud proliferation and shoot development was also observed in woody plant species such as *Acacia catechu* (Kaur and Kanta 2000), *Jatropha curcas* (Datta *et al.* 2007), and *Decalepis hamiltonii* (Sharma *et al.* 2014a).

Table 1. Effect of different cytokinins on shoot regeneration from nodal segments and shoot tips after 6 weeks of culture. Means ± SEs, *n* = 20. Means followed by the same letter are not significantly different at 5 % probability according to Tukey's test.

Cytokinins [µM]	Nodal segment response [%]	shoot number [explant^{-1}]	shoot length [cm]	Shoot tip response [%]	shoot number [explant^{-1}]	shoot length [cm]
Control	12.0 ± 0.0^{i}	1.6 ± 0.2^{fg}	2.7 ± 0.2^{e}	10.0 ± 0.0^{e}	1.0 ± 0.1^{d}	1.4 ± 0.1^{c}
BA (1.0)	56.8 ± 2.1^{efg}	2.2 ± 0.3^{cdefg}	3.7 ± 0.1^{bcde}	38.6 ± 2.7^{d}	1.4 ± 0.2^{cd}	1.9 ± 0.2^{bc}
BA (2.5)	66.8 ± 2.2^{cd}	3.0 ± 0.3^{bcd}	4.6 ± 0.1^{b}	54.0 ± 2.2^{ab}	1.6 ± 0.2^{bc}	2.3 ± 0.1^{ab}
BA (5.0)	85.8 ± 1.8^{a}	4.0 ± 0.4^{a}	4.8 ± 0.4^{a}	60.0 ± 2.9^{a}	2.3 ± 0.1^{ab}	2.9 ± 0.2^{a}
BA (7.5)	73.6 ± 1.6^{bc}	3.6 ± 0.4^{ab}	3.8 ± 0.2^{abcd}	56.8 ± 2.0^{a}	2.4 ± 0.2^{ab}	2.2 ± 0.1^{abc}
Kn (1.0)	54.8 ± 1.8^{fg}	1.7 ± 0.2^{efg}	3.4 ± 0.2^{cde}	42.0 ± 2.2^{cd}	1.2 ± 0.1^{cd}	2.3 ± 0.1^{abc}
Kn (2.5)	61.0 ± 1.0^{def}	2.0 ± 0.2^{defg}	4.3 ± 0.2^{bcd}	49.8 ± 2.7^{abc}	1.6 ± 0.1^{bc}	2.4 ± 0.1^{ab}
Kn (5.0)	75.0 ± 1.8^{b}	3.3 ± 0.1^{bcd}	4.4 ± 0.3^{abc}	55.0 ± 1.8^{ab}	2.4 ± 0.2^{ab}	2.7 ± 0.2^{ab}
Kn (7.5)	63.4 ± 1.1^{de}	3.5 ± 0.1^{abc}	4.2 ± 0.1^{bcd}	44.2 ± 2.2^{bcd}	2.6 ± 0.1^{a}	2.3 ± 0.1^{abc}
2iP (1.0)	45.6 ± 1.5^{h}	1.3 ± 0.2^{g}	3.4 ± 0.1^{cde}	35.0 ± 3.5^{d}	0.7 ± 0.1^{d}	2.0 ± 0.1^{abc}
2iP (2.5)	52.0 ± 1.4^{gh}	2.4 ± 0.1^{cdefg}	4.4 ± 0.2^{bc}	37.4 ± 1.7^{d}	1.4 ± 0.1^{cd}	2.5 ± 0.1^{ab}
2iP (5.0)	64.2 ± 1.4^{de}	3.0 ± 0.2^{bcdef}	4.6 ± 0.1^{bcd}	50.6 ± 1.6^{abc}	1.8 ± 0.1^{abc}	2.4 ± 0.0^{a}
2iP (7.5)	59.6 ± 1.5^{defg}	2.4 ± 0.5^{cdefg}	4.0 ± 0.3^{bcd}	41.4 ± 1.2^{cd}	2.5 ± 0.1^{a}	2.7 ± 0.2^{ab}

Table 2. Effect of different auxins on shoot regeneration from nodal segments and shoot tips after 6 weeks of culture. Means ± SEs, *n* = 20. Means followed by the same letter are not significantly different at 5 % probability according to Tukey's test.

Auxins [µM]	Nodal segment response [%]	shoot number [explant^{-1}]	shoot length [cm]	Shoot tip response [%]	shoot number [explant^{-1}]	shoot length [cm]
NAA (0.1)	75.0 ± 1.6^{b}	3.1 ± 0.1^{bc}	4.2 ± 0.2^{bc}	59.6 ± 1.2^{bc}	2.2 ± 0.1^{bc}	1.9 ± 0.2^{bc}
NAA (0.5)	$93.6 \pm .3^{a}$	6.2 ± 0.2^{a}	5.8 ± 0.3^{a}	72.8 ± 2.0^{a}	4.0 ± 0.1^{a}	2.3 ± 0.1^{abc}
NAA (1.0)	72.8 ± 2.1^{ab}	2.5 ± 0.2^{bcd}	3.9 ± 0.1^{cd}	55.2 ± 2.6^{cd}	1.2 ± 0.1^{ef}	2.9 ± 0.2^{a}
1AA (0.1)	86.8 ± 1.9^{a}	2.7 ± 0.1^{bc}	5.3 ± 0.3^{a}	63.0 ± 2.2^{bc}	1.5 ± 0.2^{def}	2.2 ± 0.1^{abc}
IAA (0.5)	86.6 ± 1.9^{a}	3.8 ± 0.1^{a}	4.7 ± 0.1^{bc}	65.2 ± 1.1^{ab}	2.5 ± 0.1^{b}	2.3 ± 0.1^{a}
IAA (1.0)	73.5 ± 1.2^{bc}	2.4 ± 0.1^{cd}	4.7 ± 0.1^{bc}	55.2 ± 1.2^{cd}	1.7 ± 0.1^{bcd}	2.4 ± 0.1^{ab}
IBA (0.1)	57.5 ± 1.4^{d}	1.8 ± 0.1^{de}	4.0 ± 0.1^{cd}	39.8 ± 2.2^{e}	1.2 ± 0.1^{ef}	2.7 ± 0.2^{ab}
IBA (0.5)	65.6 ± 1.7^{c}	2.9 ± 0.2^{bc}	4.6 ± 0.1^{bcd}	50.6 ± 1.2^{d}	2.0 ± 0.2^{bcd}	2.3 ± 0.1^{abc}
IBA (1.0)	46.4 ± 1.5^{e}	1.5 ± 0.1^{e}	3.7 ± 0.1^{d}	35.2 ± 2.0^{e}	1.0 ± 0.2^{f}	2.0 ± 0.1^{abc}

Table 3. Effect of different growth additives on shoot regeneration from nodal segments and shoot tips after 6 weeks of culture. Means ± SEs, *n* = 20. Means followed by the same letter are not significantly different at 5 % probability according to Tukey's test.

Additives [µM]	Nodal segment shoot number [explant^{-1}]	shoot length [cm]	Shoot tip shoot number [explant^{-1}]	shoot length [cm]
Glu(10)	4.3 ± 0.2^{h}	5.1 ± 0.1^{g}	$2.5 \pm\pm 0.1^{h}$	2.8 ± 0.1^{g}
Glu (20)	4.7 ± 0.1^{fg}	5.3 ± 0.2^{ef}	2.8 ± 0.1^{j}	3.0 ± 0.1^{f}
Glu (30)	4.5 ± 0.1^{gh}	5.2 ± 0.1^{fg}	2.6 ± 0.1^{i}	3.6 ± 0.1^{e}
Glu (40)	4.3 ± 0.1^{h}	4.9 ± 0.1^{h}	2.4 ± 0.1^{k}	2.6 ± 0.1^{h}
ADS (10)	6.3 ± 0.1^{b}	5.8 ± 0.1^{c}	4.2 ± 0.1^{b}	4.1 ± 0.1^{a}
ADS (20)	11.8 ± 0.1^{a}	9.2 ± 0.1^{a}	5.5 ± 0.2^{a}	4.8 ± 0.1^{a}
ADS (30)	5.3 ± 0.2^{c}	6.4 ± 0.1^{b}	4.1 ± 0.1^{c}	4.3 ± 0.1^{ab}
ADS (40)	5.0 ± 0.1^{de}	5.8 ± 0.1^{c}	4.1 ± 0.1^{c}	4.2 ± 0.1^{bc}
PG (10)	4.8 ± 0.1^{ef}	5.1 ± 0.1^{g}	3.4 ± 0.2^{e}	3.6 ± 0.1^{cd}
PG (20)	5.1 ± 0.2^{cd}	5.5 ± 0.2^{d}	3.6 ± 0.1^{d}	3.9 ± 0.1^{d}
PG (30)	5.0 ± 0.1^{de}	5.5 ± 0.2^{bd}	3.2 ± 0.1^{f}	3.5 ± 0.1^{e}
PG (40)	4.8 ± 0.1^{ef}	$5.4 \pm\pm 0.1^{de}$	3.0 ± 0.1^{g}	3.5 ± 0.1^{e}

Table 4. Effect of different auxins on *in vitro* root induction after 4 weeks of culture. Means ± SEs, n = 20. Means followed by the same letter are not significantly different at 5 % probability according to Tukey's test.

Auxins [µM]	Response [%]	Number of roots [shoot^{-1}]	Root length [cm]	Callogenesis
MS (control)	0.0 ± 0.0^{g}	0.0 ± 0.0^{e}	0.0 ± 0.0^{g}	+++
½ MS	46.8 ± 1.4^{f}	0.8 ± 0.0^{d}	2.3 ± 0.2^{e}	+
½ MS + NAA (1.0)	78.0 ± 0.3^{b}	1.8 ± 0.1^{c}	6.4 ± 0.1^{a}	+
½ MS + NAA (2.5)	91.6 ± 0.5^{a}	5.1 ± 0.1^{a}	4.9 ± 0.1^{b}	-
½ MS + NAA (5.0)	69.8 ± 0.3^{c}	1.2 ± 0.1^{d}	3.9 ± 0.2^{c}	++
½ MS + IAA (1.0)	78.8 ± 0.2^{b}	1.2 ± 0.1^{d}	3.5 ± 0.1^{d}	+
½ MS + IAA (2.5)	65.5 ± 0.3^{d}	3.2 ± 0.1^{b}	4.6 ± 0.1^{b}	+
½ MS + IAA (5.0)	52.2 ± 0.3^{e}	1.1 ± 0.1^{d}	2.8 ± 0.1^{e}	+++
½ MS + IBA (1.0)	0.0 ± 0.0^{g}	0.0 ± 0.0^{e}	0.0 ± 0.0^{g}	++
½ MS + IBA (2.5)	40.0 ± 0.0^{f}	0.9 ± 0.1^{d}	1.7 ± 0.0^{f}	+
½ MS + IBA (5.0)	0.0 ± 0.0^{g}	0.0 ± 0.0^{e}	0.0 ± 0.0^{g}	+++

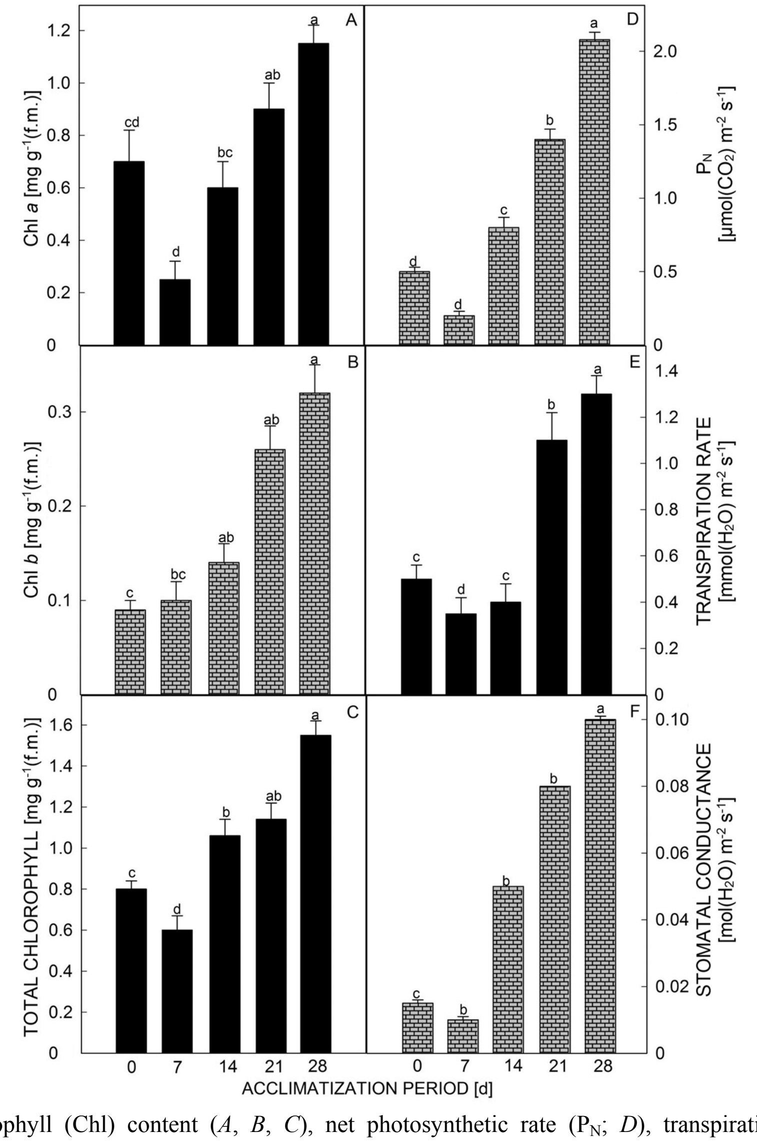

Fig. 2. Changes in chlorophyll (Chl) content (*A*, *B*, *C*), net photosynthetic rate (P_N; *D*), transpiration rate (*E*), and stomatal conductance (*F*) in micropropagated plantlets of *D. arayalpathra* during acclimatization. Means ± SEs, n = 20. Means followed by the same letter are not significantly different at 5 % probability according to Tukey's test.

Full-strength MS medium without any auxin failed to induce rooting in microshoots; only callusing was noticed at the basal cut end of the microshoots. Among the treatments tested, half-strength MS with 2.5 µM NAA induced maximum of 5.1 roots per shoot with 4.9 cm root length in 91.6 % cultures after 4 weeks of transfer (Fig. 1*D*,*E*, Table 4). However, roots were very weak, fibrous, and devoid of secondary branching on medium with IAA and IBA. The need of half-strength MS medium for the growth of root has also been proclaimed in other members of *Apocynaceae* such as *Tylophora indica* (Sahai *et al.* 2010), *Gymnema sylvestre* (Thiyagarajan and Venkatachalam 2013), and *D. hamiltonii* (Sharma *et al.* 2014a). In contrast, optimum rooting was reported in *Salvia splendens* using IAA (Sharma *et al*. 2014b).

Plantlets with developed shoot and root were hardened on *Soilrite*™ as mentioned in Materials and method. The hardened plant showed 92.3 % continuity in the growth in the field conditions (Fig. 1*F*). In the field, the micropropagated plantlets showed similar morphological characters related as the mother plant without visible abnormalities.

During the first week of acclimatization, reduction in amount of Chl *a* (0.70 - 0.25 mg g^{-1}), Chl *b* (0.09 - 0.1 mg g^{-1}) and total Chl (0.8 - 0.6 mg g^{-1}) were observed. Thereafter, a gradual increase was found and Chl *a*, Chl *b,* and total Chl reached values of 1.15, 0,32, and 1.55 mg g^{-1}(f.m.) after 8 weeks of acclimatization (Fig. 2*A*,*B*,*C*). The poorly developed chloroplast containing disorganized granna might be the reason of initial reduction in chlorophyll content (Pospíšilová *et al.* 1999). As the days of acclimatization increased, formation of new leaves were taken place and chlorophyll content increased gradually that showing significance of acclimatization on chlorophyll content in *in vitro* raised plantlets. The similar trend was also noticed for chlorophyll content in *Coleus forskohlii* (Sahai and Shahzad 2013) and *Cassia occsidentalis* (Naaz *et al.* 2015).

During the transfer of *in vitro* raised plantlets to *ex vitro* condition, the environmental stresses appeared during the initial days of acclimatization. Therefore, P_N decreased from 0.5 to 0.2 µmol(CO_2) m^{-2} s^{-1} during first week of transfer. The further enhancement in P_N was due to the formation of new leaves and P_N reached 2.08 µmol(CO_2) m^{-2} s^{-1} after 4 weeks of acclimatization (Fig. 2*D*). Similar results were found in *C. forskohlii* (Sahai and Shahzad 2013) and *D. hamiltonii* (Sharma *et al.* 2014a). As CA is known to catalyze the reversible hydration of CO_2 to give the bicarbonate ion, the increased P_N was in agreement with CA activity during acclimatization which increased during acclimatization and reached its maximum after 28 d (Fig. 4).

Stomatal conductance is critical during acclimatization, as directly affects the performance of

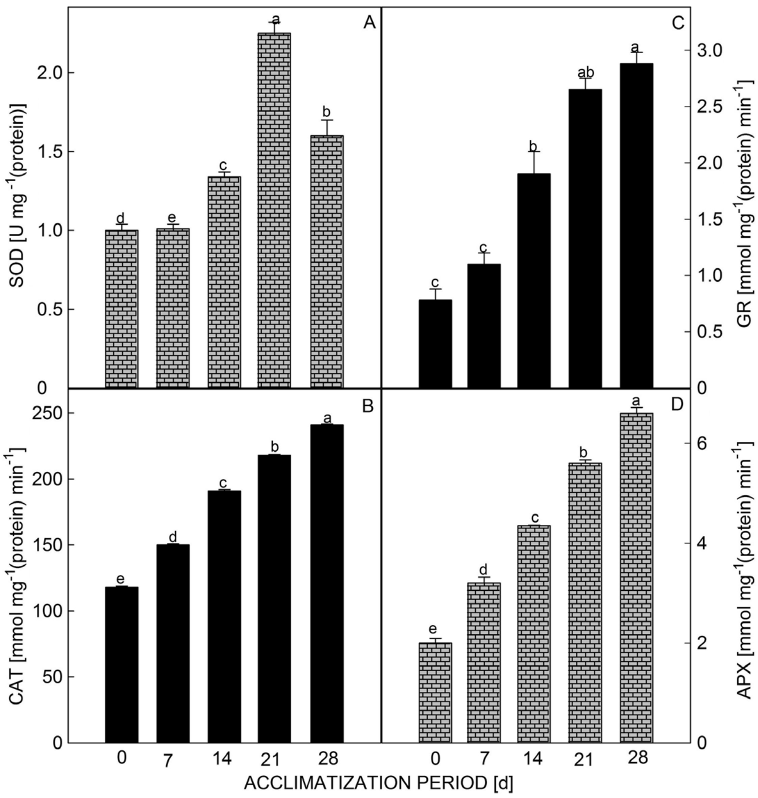

Fig. 3. Changes in antioxidant enzymes activity in micropropagated plantlets of *D. arayalpathra* during acclimatization. Superoxide dismutase (SOD; *A*), catalase (CAT; *B*), glutathione reductase (GR; *C*), and ascorbate peroxidase (APX; *D*). Means ± SEs, *n* = 20. Means followed by the same letter are not significantly different at 5 % probability according to Tukey's test.

plant in terms of P_N and E and E determine the water status in the plant body. It was clearly shown that the E decreased from 0.5 to 0.3 mmol(H_2O) m^{-2} s^{-1} during the first week of acclimatization but thereafter a linear increase was found (Fig. 2*E*). A similar pattern was also found in the case of g_s: a decrease from 0.015 to 0.01 mol m^{-2} s^{-1} during the first week of acclimatization and thereafter a gradual increase (Fig. 2*F*). The results are in accordance with the findings of Wang *et al.* (2006) in *Camptotheca accuminata.*

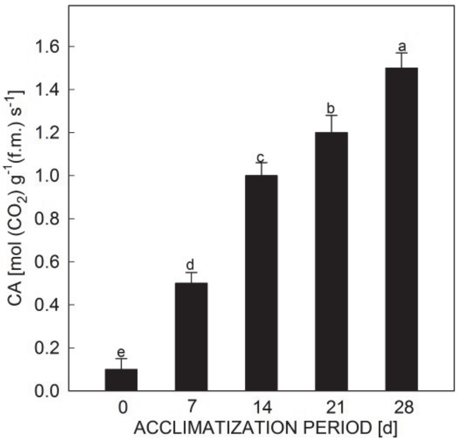

Fig. 4. Changes in carbonic anhydrase (CA) activity in micropropagated plantlets of *D. arayalpathra* during acclimatization. Means ± SEs, *n* = 20. Means followed by the same letter are not significantly different at 5 % probability according to Tukey's test.

During the transfer of *in vitro* raised plantlets to *ex vitro* condition, plantlets have to face various stresses. The availability of low CO_2 concentration, low PPFD, and high air humidity in culture tubes might be the main reasons for problem occurring in the following *ex vitro* acclimatization (Van Huylenbroeck *et al.* 1998). The stresses might induce formation of ROS. However, plant cells develop a strong defense mechanisms including SOD, CAT, APX, and GR (Mitrovic and Bogdanovic 2008, Kayihan *et al.* 2012, Xu *et al.* 2012). In the present study, SOD activity increased after 7 d of acclimatization and it decreased in day 28 of acclimatization (Fig. 3*A*). The SOD converts superoxide to H_2O_2 and O_2. This is the primary defense against the ROS. The process of detoxification is carried out by a series of membrane associated and stromal enzymes, including SOD and APX at the accepter side of photosystem I (Scalet *et al.* 1995). CAT scavenges H_2O_2 by converting it into O_2 and H_2O in peroxisomes. In the present study, gradual increase in CAT activity was noticed and reached its maximum at 28 d (Fig. 3*B*). The CAT increase is accordance with the findings of Varshney and Anis (2012) in *Tecomella undulata.* APX and GR are two important enzymes of ascorbate glutathione cycle. They play a significant role by scavenging H_2O_2 in chloroplast, cytosol, vacuoles, and apoplast (Asada 1999). The activities of both APX and GR were gradually elevated as the days of acclimatization increases (0 - 28 d) (Fig. 3*C*,*D*). The elevation of APX and GR during the acclimatization period was previously reported in *Rauvolfia tetraphylla* and *Tylophora indica* (Faisal and Anis 2009, 2010).

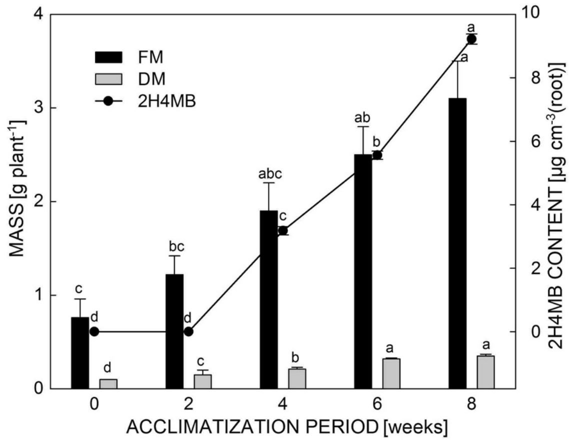

Fig. 5. Fresh mass (FM), dry mass (DM), and 2H4MB content in the roots of micropropagated plantlets *D. arayalpathra* during acclimatization. Means ± SEs, *n* = 20. Means followed by the same letter are not significantly different at 5 % probability according to Tukey's test..

The presence of the 2H4MB in the extract was determined on the basis of similarity in RT of standard compound and compound in extract (Fig. 1 Suppl.). The RT of root sample corresponds with that of the standard compound which proved the presence of 2H4MB in root extract of *D. arayalpathra*. Moreover, the peak purity was further screened and both compounds showed maximum absorption at 278.5 nm. The quantification was based on the calibration curve. The relationship between biomass production and accumulation of the compound (Fig. 5) clearly depicts that the synthesis was started after 2 weeks of acclimatization. A 2H4MB synthesis was also reported by Sharma *et al.* (2014a) in *D. hamiltonii*. Sudha and Seeni (2001) and Giridhar *et al.* (2005) also reported similar results in the normal root culture of *D. hamiltonii* and *D. arayalpathra* by using GC-MS and HPLC analysis, respectively.

Conclusions

The present protocol gives a comparative analysis of regeneration of *D. arayalpathra* through nodal segments and shoots tips. In comparison to the previous report of Gangaprasad *et al.* (2005), shoot multiplication was 11.8 times higher from nodal segments and 4.6 times higher from shoot tips. Considering the importance of the plant adjustment during acclimatization, different physiological parameters (chlorophyll content, P_N, g_s, and E) and biochemical parameters (activities of enzymes like SOD, CAT, APX, GR, and CA) were also studied which have been never reported for this plant in previous studies. The results reported in this study could be used to successful micropropagation and acclimatization of this species which can be important for 2H4MB production and the conservation of this endangered species.

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