



Ex vitro rescue, physiochemical evaluation, secondary metabolite production and assessment of genetic stability using DNA based molecular markers in regenerated plants of *Decalepis salicifolia* (Bedd. ex Hook.f.) Venter

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Received: 14 August 2017 / Accepted: 2 November 2017 / Published online: 17 November 2017
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Abstract

To ensure replenishment, a refined protocol for micropropagation of *Decalepis salicifolia* (Bedd. ex Hook.f.) Venter, a critically endangered and endemic medicinal plant, was developed using mature nodal explants. A high frequency shoot regeneration system was obtained on Murashige and Skoog (MS) medium comprised of 6-benzyladenine (BA) (5.0 μM) + α -naphthalene acetic acid (NAA) (0.5 μM) + adenine sulphate (ADS) (30.0 μM) corresponds to a highest mean number of 9.97 ± 0.01 shoots/explants with maximum shoot length of 6.46 ± 0.1 cm. Successful rooting in microshoots was achieved on half strength MS medium supplemented with indole-3-butyric acid (IBA) (2.5 μM). A maximum of 6.10 ± 0.07 roots/microshoot with average root length of 2.30 ± 0.06 cm was obtained. As much as 90% plantlets survived when Soilrite™ was used as planting substrate and finally established in soil without any casualty and morphological variation. Acclimatized plantlets were screened for pigment content, net photosynthetic rate (PN), stomatal conductance (Gs) and transpiration rate (E) during subsequent days of acclimatization as well as the changes in antioxidant were also evaluated. A steady rise was observed in the activity of superoxide dismutase (SOD) for initial 21 days and then after a decrease was found showing improved acclimatization efficiency of the plant. Similarly, the activities of catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) enzyme shows reliable increase as the days of acclimatization advanced which play their precautionary role against oxidative damage to the plant. The genetic fidelity of the in vitro raised plantlets with that of mother plant was further confirmed using random amplified polymorphic DNA (RAPD) and inters simple sequence repeats (ISSR) analysis. Additionally, the effect of acclimatization on the biosynthesis of 2-hydroxy-4-methoxybenzaldehyde (2H4MB) in the root system was also evaluated in relation to their biomass production. Maximum fresh weight (4.9 g/plant), dry weight (0.65 g/plant) of roots and 2H4MB content ($6.8 \mu\text{g ml}^{-1}$ of root extract) was obtained after 10 weeks of acclimatization. Accelerated multiplication rate with the stability of genetic virtue, physiological and biochemical parameters assure the efficacy of the protocol developed for the propagation of this critically endangered medicinal plant.

Keyword Acclimatization · Antioxidant · 6-Benzyladenine · ROS · 2H4MB

Introduction

Decalepis salicifolia (Bedd. ex Hook.f.) Venter (family—Apocynaceae) is a critically endangered and endemic

medicinal plant with very restricted distribution in Western Ghats of India. The present-day position of the plant comes under critically endangered categories as listed by IUCN (International Union for conservation of nature) due to over exploitation and loss in habitat. This species is endemic to Palakkad and Idukki district of Kerala and it is also found in Coimbatore and Dindigul districts in Tamil Nadu, India. The moniliform tuberous root is the most important part of the plant containing 2H4MB which gives vanilla like aroma to the root (George et al. 2011). The plant is used to treat skin diseases, tuberculosis, asthma and intestinal disorders

Communicated by Ali R. Alan.

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(Radhakrishnan et al. 1998) and shown antiulcer activities (Rao et al. 2004). The compound 2H4MB have larvicidal activities (Akenga et al. 2005), taste modifying activities (Mukonyi and Ndiege 2001), tyrosinase inhibitor activity (Kubo and Kinst-Hori 1999), antimicrobial and antioxidant (Wang et al. 2010). This compound has also been reported in *Decalepis hamiltonii* (Nagarajan et al. 2001a), *D. arayalpathra* (Verma et al. 2014) and *Hemidesmus indicus* (Nagarajan et al. 2001b). Western Ghats being as UNESCO world heritage site and is one of the eight hottest hot-spot of biological diversity in the world (Myers et al. 2000). However, a large number of plant species are being eroded due to various reasons and many of them are poorly understood because of the lack of researches conducted on them. Indiscriminate collection of roots questioned the wild stand of this lactiferous shrub in the past years in the Western Ghats, India. The unprocessed way of regeneration and propagation is obstruct due to poor fruit set and low seed germination. Such kind of orphan plant needs special attention as it represents a restricted geographical distribution.

Decalepis salicifolia is heightening towards vulnerable categories as their pharmacological wealth is known. Although its over-utilization and medicinal inference, there is no description for the reintroduction of this critically endangered plant. Plant tissue cultures techniques offer possible plan for ex vitro conservation, mass scale production and bioactive compound production to meet the industrial demands. George et al. (2011) screened the root of *D. salicifolia* and establish the presence of 2H4MB as a major constituents. However, improved mass propagation and conservation strategies have been proposed for *D. hamiltonii* (Sharma et al. 2014) and *D. arayalpathra* (Ahmad et al. 2017) using biotechnological approaches. The proposed work has been under taken to evolve an efficient and reproducible micropropagation system for *D. salicifolia* through mature nodal explants. In vitro raised clones were validated for genetic homogeneity by using molecular markers (RAPD, ISSR). Characterization of antioxidant enzymes along with physiochemical parameters during the acclimatization period was also studied. The desired compound (2H4MB) is also identified and quantified in relation to the biomass of root system of micropropagated plantlets.

Materials and method

Plant material, media and culture conditions

The healthy and mature shoots from field grown plant of *D. salicifolia* were taken from a 3-year-old growing plant in the Department net house, A.M.U. Shoots were assiduously washed for 30 min using tap water followed by treatment with 1% (w/v) Bavistin (Carbendazim Powder, BASF India

Ltd.) and 5% (v/v) labolene for 20 min respectively. The explants were washed with sterilized double distilled water (SDDW) followed by surface disinfect under horizontal laminar flow hood using 0.1% (w/v) mercuric chloride (HgCl₂) solution for 2.5 min. It was further succeeded by 3–4 times washing with SDDW before the inoculation. Nodal segment with two axillary buds were taken as explants for inoculation.

MS (Murashige and Skoog 1962) medium augmented with distinct plant growth regulators (PGRs) and 3% sucrose (Qualigens, India) was used throughout the experiments. Agar (Qualigens) at 0.8% was used as solidifying agent, pH was set as 5.8. About 15 ml of culture medium was dispersed in each culture tube (25 × 150 mm, Borosil, India) and Erlenmeyer flasks (100 ml) and were sealed with non-absorbent cotton plugs. The sterilization of medium was achieved by autoclaving at 121 °C and $1.06 \times 10^5 \text{ Nm}^{-2}$ (Pa) for 15 min. After inoculation the cultures were incubated at 16 h photoperiod under $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ of photosynthetic photon flux density (PPFD) furnished by fluorescent tubes (40 W; Philips, India) and $55 \pm 5\%$ relative humidity at 24 ± 2 °C during the experimentation.

Shoot induction and multiplication

To standardize the response of different PGRs on axillary shoot regeneration, the excised nodal segments were inoculated aseptically on full-strength MS medium augmented with cytokinins like 6-benzyladenine (BA), 2-isopentanyladenine (2iP) and kinetin (Kn) at distinct molarity (1.0, 2.5, 5.0 and 7.5 μM) with or without auxins like α -naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) at various concentrations (0.1, 0.5 and 1.0 μM). To improve the shoot regeneration, various growth additives (GAds) viz. glutamine (Glu), phlouroglucinol (PG) and adenine sulphate (ADS) at various molarity (10, 20, 30 and 40 μM) were supplied with optimal cytokinin and auxin combination.

Rooting, acclimation and field relocation

For the induction of root in the microshoots various auxins like indole-3-butyric acid (IBA), α -naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) and at various concentrations (1.0, 2.5 and 5.0 μM) were supplied with 1/2 MS medium to optimise the best rooting efficiency. MS medium lacking any auxins was served as control. The plantlets with fully developed root and shoot system were isolated from culture flask, washed gently to remove adhered agar particle followed by transferring to thermal cups (expanded polystyrene) containing autoclaved Soilrite™ (75% Irish peat moss and 25% horticulture grade expanded perlite) (Keltech Energies Ltd., India) and

watering was done periodically. To optimize the maximum moisture, the micropropagated plantlets were covered with transparent plastic bag for 14 d and maintained in the culture room at similar physiological conditions as described for multiplication in material and method section. The polythene bag was gradually opened to maximize the plant adaptation towards new environments. After 28 days of plantation, the hardened plants settled to clay pots containing garden soil and green manure (2:1) and kept in the culture room initially for 2 weeks and then they were transferred to green house followed by transfer in natural environment.

Physiochemical analysis

Estimation of chlorophyll and carotenoid content

Methodologies used by Sahai and Shahzad (2013) were used to evaluate the chlorophyll and carotenoid content. Ten micropropagated plantlets were preferred randomly, fully expanded leaves were tabbed at day 0 (control) and after 7, 14, 21 and 28 days of acclimatization and followed by extraction with 80% (v/v) acetone and filtered with whatman No. 1 filter paper. The absorbance for chl a, chl b and carotenoids were monitored at 663, 645 and 466 nm respectively by using Ultraviolet–Visible (UV–Vis) spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Kyoto, Japan).

Net photosynthetic rate and related attributes

PN, Gs and E were investigated by using Infrared gas analyzer (IRGA), a handy photosynthetic system (Li-COR 6400, LI-COR, and Lincoln, NE, USA). The estimation was carried out followed by the methodologies of Ahmad et al. (2017).

Electrolyte leakage (EL) and lipid peroxidation

The permeability of the membrane was evaluated by following the methodologies of Lutts et al. (1995). After gently washing the leaves, leaf disc (2–3 mm²) were made by a punch player. The leaf disc were dipped in 12 ml DDW and incubated for 24 h in a closed culture vial and electrical conductivity of the solution (EC₁) was calculated. After that, autoclaved at 15 psi and 121 °C for 18 min followed by cooling at 25 °C and the electrical conductivity (EC₂) was calculated. EL was calculated as: $EL (\%) = EC_1/EC_2 \times 100$. Thiobarbituric acid reactive substances (TBARS) content was evaluated following the protocol by Faisal and Anis (2009).

Antioxidant enzyme analysis

2.0 ml of extraction buffer consisting of 1% of polyvinylpyrrolidone (PVP) and Triton X-100, and 0.11 g ethylenediaminetetra acetic acid (EDTA) is used to homogenize the 0.5 g fresh and healthy leaf in mortar and pestle followed by filtration with whatman No. 1 filter paper. The filtrate was centrifuged at 15,000 rpm for 20 min using high-speed centrifuge. The process of extraction will perform in dark and assaying of enzyme was carried out with supernatant.

SOD (EC 1.15.1.1) action was evaluated by following the protocol of Dhindsa et al. (1981). The reaction mixture consisting of 13 mM methionine, 0.5 M phosphate buffer at pH 7.2, 1.2 mM riboflavin, 63.3 mM nitroblue tetrazolium (NBT) 0.1 mM EDTA and 0.1 ml enzyme extract and incubated for 15 min at 25 °C. Tube showing no illumination and equal concentration of reaction mixture served as blank and the absorbance was read at 560 nm. 50% inhibition of NBT reduction corresponds to one unit of SOD activity.

CAT (EC 1.11.1.6) action was evaluated by using the methodologies of Aebi (1984). 50 mM phosphate buffer (pH 7.0) and 100 µl enzymes extract was used to prepare the volume (3 ml) of assay mixture and H₂O₂ disappearance in the supernatant was regulated by measuring the fall in absorbance at 240 nm. The reaction began with addition of 10 mM H₂O₂. The coefficient of absorbance 0.036 mM⁻¹ cm¹ was used to calculate CAT activity.

APX (EC 1.11.1.11) action was evaluated by following the methodologies of Nakano and Asada (1981). The fall in absorbance due to disruption of ascorbate with enzyme was monitored at 290 nm The reaction mixture was consisting of 50 mM phosphate buffer (pH 7.5), 0.1 mM H₂O₂, 0.1 mM EDTA, 0.5 mM ascorbate, and 0.1 ml enzyme extract and activity of enzyme is represented as EU mg⁻¹ protein min⁻¹.

GR (EC 1.6.4.2) action evaluated by following the methodologies of Foyer and Halliwell (1976). Enzyme extract (0.1 ml) added to assay mixture contained 50 mM phosphate buffer (pH 7.5), 1.0 mM EDTA, and 0.2 mM NADPH (Nicotinamide adenine dinucleotide phosphate), and 0.5 mM glutathione disulfide (GSSG) to began the reaction. The action of the enzyme was measured by using the coefficient of absorbance of 6.2 mM⁻¹ cm⁻¹ indicated as EU mg⁻¹ protein min⁻¹.

Genomic DNA extraction

The genetic fidelity was performed by using RAPD and ISSR technique. Nine micropropagated plantlets of *D. salicifolia* were selected for DNA extraction using CTAB (cetyl-methylammonium bromide) method following the protocol of Doyle and Doyle (1990) and extracted DNA was screened for clarity (A₂₆₀/A₂₈₀ ratio) on UV–vis spectrophotometer. A set of ISSR (UBC, Vancouver, BC, Canada) and RAPD

(kit OPL) primers were used for PCR analysis and reaction was executed on a thermocycler (Biometra, T Gradient Thermoblock, Germany). The reaction mixture of 20 μl for PCR amplification was prepared with 10X buffer (2 μl), 10 mM dNTPs (0.4 μl), 25 mM MgCl_2 (1.2 μl), 3 Unit Taq polymerase (0.2 μl), 2 μM primers and 40 ng template DNA. PCR amplification cycle was set as 45 cycles consisting of a 94 °C denaturation step for 5 min without any repeat, a 55 °C annealing for 1 min and an elongation step of 72 °C for 1 min. A final extension was achieved at 72 °C for 10 min. The amplified products were separated on electrophoresis in 0.8% (w/v) agarose gels with 4 μl ethidium bromide in TAE buffer (pH 8.0) run at 50 V for 2 h and visualized on a UV trans-illuminator (BIO RAD, Hercules, CA, USA).

HPLC analysis of 2H4MB content in the root system

Plant material, extract and standard preparation

The plant material, extract and standard were prepared according to the methodologies followed by Ahmad et al. (2017). The roots were collected from micropropagated plantlets in Soilrite™ after 0, 2, 4, 6, 8 and 10 weeks of acclimatization for qualitative and quantitative analysis of the compound. Fresh weight of roots was taken, and then dried at room temperature, dry weight calculated and squashed in mortar and pestle with liquid nitrogen. n-hexane (150 ml) was added to the dried root powder (1 g) and incubated on shaker at 120 rpm. Solvent was evaporated at 50 °C on water bath and 100 ml of methanol was used to maintain the volume following the filtration with a syringe filter (0.22 μm , Genetix, Bangalore, India). Standard compound 2H4MB (98% purity) (purchased from Sigma Aldrich, New Delhi, India) stock solution of 1000 $\mu\text{g}/\text{ml}$ was processed by dissolving 10 mg 2H4MB in 10 ml of methanol. Different concentration gradient was prepared with methanol and all working solution was stored at 4 °C.

Chromatographic conditions

Waters system (Milford, MA, USA) equipped with C18 analytical column (250 \times 4.6 mm, 5.0 μm) (Waters) was used for HPLC. 10 μl of sample was injected and quantification was carried out at 280. Data acquisition was achieved by using Empower2 software and reporting on Windows 2000™ platform. Methanol with water (70:30) in combination was preferred for mobile phase, pH adjusted at 5.0 and flow rate was adjusted to 1 ml min^{-1} . The chromatograms were adjusted at 280 nm. UV scan was measured from 210 to 400 nm. The corresponding retention time (RT) of both standard and sample was affirmation of the presence of the compound.

Statistical analysis

For statistical analysis, all the experiment were conducted with 20 replicates and repeated three times. One-way ANOVA using SPSS version 16 (SPSS Inc., Chicago, IL, USA) was used for data analysis of different parameter. The significance of differences among means was attributed using Tukey's test at 5% level of significance and data represented as mean \pm standard error (SE).

Results and discussion

In vitro regeneration system

Direct regeneration is the most relevant approach for clonal propagation in several medicinal plants (Sadeghi et al. 2015). The influence of different cytokinin concentration has been screened for direct shoot regeneration (Fig. 1a; Table 1). Only single shoot emerges on control treatment indicating the application of PGRs. MS medium fortified with different cytokinin like BA, Kn and 2ip brings changes in single nodal explants and bud development was observed within 6–8 days of inoculation. BA was proved to be the best among different cytokinin used in terms of shoot proliferation and shoot length. MS medium with BA (5.0 μM) induced 2.06 ± 0.09 shoots per explant with 2.84 ± 0.09 cm shoot length in $88.6 \pm 1.07\%$ of cultures after 6 weeks. BA beyond the optimum concentration did not bring any change in regeneration efficiency while the application of other two cytokinin i.e. Kn and 2 iP was unable to evoke any good response instead exhibited retarded growth in regenerants thereby stunted shoots were produced. Kn and 2 iP at 5.0 μM induced 1.62 ± 0.1 and 1.42 ± 0.05 shoots per explant with 2.60 ± 0.07 cm and 1.93 ± 0.05 cm shoot length in 76.6 ± 1.07 and $62.6 \pm 1.00\%$ cultures, respectively in 6 weeks of incubation. The stimulating effect of BA has also been reported in other medicinal plant species such as *Pistacia lentiscuss* (Kılınc et al. 2015) and *Crataegus arotina* (Nas et al. 2012). BA being as the first generation synthetic cytokinin elicit an efficient proliferation, bud break, multiple shoot formation and it is might be due to its more permeability across plasma membrane and high cell uptake (Malik et al. 2005).

The combination treatment of cytokinin and auxin was also evaluated to boost the regeneration efficiency of the explants. Application of optimum concentration of BA (5.0 μM) with various auxins (NAA, IAA and IBA) at definite concentration produced significant improvement in regeneration and growth in shoots. Combination of BA with NAA (0.5 μM) induced 4.04 ± 0.05 shoots per nodal segments with 4.99 ± 0.05 cm shoot length in $90.2 \pm 1.28\%$ of cultures in 6 weeks (Fig. 1b; Table 2). Jaykumar et al. (2015)



Fig. 1 Establishment of *D. salicifolia* culture. Nodal segments at MS medium—3 weeks old culture (a), shoot regeneration on MS medium+BA (5.0 μ M)+NAA (0.5 μ M)—6 weeks old culture (b), shoot multiplication on MS medium+BA (5.0 μ M)+NAA

(0.5 μ M)+ADS (30.0 μ M)—6 weeks old culture (c), In vitro rooting on 1/2 MS medium+IBA (2.5 μ M)—4 weeks old culture (d), exposed view of above culture (e), an acclimatized plantlet in Soilrite™-4 weeks old (f)

also found improved results in combination treatment of BA with NAA in *Salacia chinensis*. Other two auxin IAA and IBA also showed some improvement but lesser effective as

compare to BA. However, on the above media tested, poor leaf expansion, abscission of leaf and early leaf fall were observed in the shoots regenerated on cytokinin alone or in

Table 1 Effect of different cytokinin on in vitro shoot induction of *D. salicifolia* after 6 weeks of culture

Cytokinin (μM)	% Response	Mean no. of shoot	Mean shoot length (cm)
Control	10.0 \pm 0.70 ⁱ	1.00 \pm 0.00 ^f	1.40 \pm 0.07 ^{fg}
BA (1.0)	54.0 \pm 1.41 ^{eg}	1.30 \pm 0.07 ^{cdef}	1.52 \pm 0.06 ^{ef}
BA (2.5)	67.4 \pm 1.07 ^{cd}	1.55 \pm 0.08 ^{bcd}	1.84 \pm 0.05 ^{de}
BA (5.0)	88.6 \pm 1.07 ^a	2.06 \pm 0.09 ^a	2.84 \pm 0.09 ^a
BA (7.5)	69.6 \pm 0.92 ^c	1.74 \pm 0.09 ^b	2.52 \pm 0.05 ^b
Kn (1.0)	51.2 \pm 0.96 ^g	1.10 \pm 0.07 ^{ef}	1.36 \pm 0.05 ^{fg}
Kn (2.5)	64.2 \pm 1.15 ^{de}	1.26 \pm 0.09 ^{def}	2.06 \pm 0.09 ^{cd}
Kn (5.0)	76.6 \pm 1.07 ^b	1.62 \pm 0.10 ^{bc}	2.60 \pm 0.07 ^{ab}
Kn (7.5)	68.6 \pm 1.07 ^{cd}	1.30 \pm 0.07 ^{cdef}	2.32 \pm 0.08 ^{bc}
2iP (1.0)	42.8 \pm 1.10 ^h	1.02 \pm 0.04 ^f	1.18 \pm 0.08 ^g
2iP (2.5)	53.6 \pm 1.30 ^{fg}	1.22 \pm 0.05 ^{def}	1.60 \pm 0.07 ^{ef}
2iP (5.0)	62.6 \pm 1.00 ^e	1.42 \pm 0.05 ^{bcd}	1.93 \pm 0.05 ^d
2iP (7.5)	56.8 \pm 1.15 ^f	1.24 \pm 0.04 ^{def}	1.61 \pm 0.10 ^{ef}

(Data represent mean \pm SE of 20 replicates per treatment in 3 repeated experiments. Mean values followed by the same letter are not significantly different according to Tukey's test at 5% probability)

Table 2 Effect of different combination of auxin with BA (5.0 μM) on shoot regeneration through nodal segment of *D. salicifolia* after 6 weeks of culture

Auxin (μM)	% Response	Mean no. of shoot	Mean shoot length (cm)
IAA (0.1)	66.0 \pm 1.31 ^{de}	2.36 \pm 0.09 ^c	2.56 \pm 0.08 ^c
IAA (0.5)	71.0 \pm 1.00 ^{cd}	2.81 \pm 0.06 ^{bc}	3.62 \pm 0.13 ^c
IAA (1.0)	64.2 \pm 1.24 ^a	2.42 \pm 0.13 ^c	2.50 \pm 0.16 ^e
IBA (0.1)	62.6 \pm 1.07 ^e	2.32 \pm 0.11 ^c	3.14 \pm 0.09 ^{cd}
IBA (0.5)	72.0 \pm 1.41 ^c	2.66 \pm 0.12 ^{bc}	3.38 \pm 0.14 ^{cd}
IBA (1.0)	61.6 \pm 0.93 ^e	2.42 \pm 0.13 ^c	2.96 \pm 0.10 ^{de}
NAA (0.1)	81.4 \pm 1.07 ^b	3.74 \pm 0.16 ^a	4.46 \pm 0.10 ^{ab}
NAA (0.5)	90.2 \pm 1.28 ^a	4.04 \pm 0.05 ^a	4.99 \pm 0.05 ^a
NAA (1.0)	74.4 \pm 1.28 ^c	3.12 \pm 0.08 ^b	4.30 \pm 0.14 ^b

Data represent mean \pm SE of 20 replicates per treatment in 3 repeated experiments. Mean values followed by the same letter are not significantly different according to Tukey's test at 5% probability

combination with auxin. Therefore, to improve the healthy shoot system, another set of experiment was carried out with the treatment of growth additive like ADS, Glu and PG to the MS medium. Significant improvement was obtained when different additives were tried. MS medium augmented with BA (5.0 μM) + NAA (0.5 μM) + ADS (30 μM) was found to be optimal to induce 9.97 \pm 0.01 shoots per nodal segment with 6.46 \pm 0.1 cm shoot length, after 6 weeks of incubation (Fig. 1c; Table 3). ADS being a rich source of nitrogen might have improved the multiplication rate along with the healthy leaf development and also prevention of early leaf fall. Murashige (1974) has established that the addition of adenine in the form of ADS act as a catalyst on the organization of enhanced shoot bud proliferation. The

Table 3 Effect of different growth additives on shoot regeneration from nodal segments of *D. salicifolia* after 6 weeks of culture. Means \pm SE, n = 20

Additives (μM)	Mean no. of shoot	Mean shoot length (cm)
Glu (10)	7.20 \pm 0.15 ^{de}	5.40 \pm 0.07 ^{cd}
Glu (20)	7.34 \pm 0.14 ^{de}	5.30 \pm 0.10 ^{cd}
Glu (30)	7.84 \pm 0.09 ^e	5.70 \pm 0.13 ^{bc}
Glu (40)	7.34 \pm 0.13 ^{cd}	5.40 \pm 0.2 ^{bcd}
ADS (10)	9.16 \pm 0.09 ^b	6.00 \pm 0.07 ^{ab}
ADS (20)	9.26 \pm 0.10 ^b	6.14 \pm 0.09 ^{ab}
ADS (30)	9.97 \pm 0.01 ^a	6.46 \pm 0.1 ^a
ADS (40)	9.34 \pm 0.15 ^b	6.12 \pm 0.1 ^{ab}
PG (10)	6.66 \pm 0.10 ^{ef}	4.54 \pm 0.09 ^{ef}
PG (20)	6.80 \pm 0.09 ^{ef}	4.62 \pm 0.12 ^{ef}
PG (30)	7.00 \pm 0.07 ^{def}	4.96 \pm 0.1 ^{ef}
PG (40)	6.52 \pm 0.08 ^f	4.30 \pm 0.1 ^f

Means followed by the same letter are not significantly different at 5% probability according to Tukey's test

supremacy of ADS was also observed in other medicinal plant such as *Ficus religiosa* (Siwach and Gill 2011) and *Decalepis arayalpathra* (Ahmad et al. 2017).

In vitro rooting and acclimatization

Excised shoot were transfer on full-strength MS medium (control) devoid of auxin declined to promote root initiation in microshoot. 1/2 MS medium fortified with NAA, IAA and IBA was also screened to improve the rooting. Among all the auxins screened, half MS + IBA (2.5 μM) proved to be most effective in comparison to other auxin and corresponds maximum of 6.10 \pm 0.07 roots per shoot with 2.30 \pm 0.06 cm root length in 91.0 \pm 1.0% cultures after 4

Table 4 Effect of different auxins on in vitro root induction in *D. salicifolia* after 4 weeks of culture. Means \pm SE, n=20

Auxin (μ M)	% Response	Mean no. of root	Mean root length (cm)
MS (control)	00.0 \pm 0.00 ^g	0.00 \pm 0.00 ^h	0.00 \pm 0.00 ^h
½ MS	49.4 \pm 1.07 ^e	1.58 \pm 0.16 ^{efg}	0.70 \pm 0.07 ^g
½ MS + NAA (1.0)	60.4 \pm 0.92 ^d	2.16 \pm 0.09 ^{de}	1.00 \pm 0.07 ^{efg}
½ MS + NAA (2.5)	64.0 \pm 1.41 ^d	2.66 \pm 0.10 ^d	1.52 \pm 0.13 ^{bcd}
½ MS + NAA (5.0)	72.0 \pm 1.41 ^c	3.40 \pm 0.14 ^c	1.30 \pm 0.11 ^{def}
½ MS + IBA (1.0)	83.6 \pm 1.72 ^b	4.36 \pm 0.17 ^b	1.82 \pm 0.08 ^b
½ MS + IBA (2.5)	91.0 \pm 1.00 ^a	6.10 \pm 0.07 ^a	2.30 \pm 0.06 ^a
½ MS + IBA (5.0)	72.6 \pm 1.07 ^c	4.50 \pm 0.18 ^b	1.80 \pm 0.07 ^{bc}
½ MS + IAA (1.0)	40.6 \pm 1.07 ^f	1.30 \pm 0.07 ^{fg}	1.04 \pm 0.07 ^{fg}
½ MS + IAA (2.5)	42.8 \pm 1.15 ^f	1.02 \pm 0.04 ^g	1.00 \pm 0.07 ^{efg}
½ MS + IAA (5.0)	41.6 \pm 0.92 ^f	1.62 \pm 0.13 ^{ef}	1.40 \pm 0.07 ^{cde}

Means followed by the same letter are not significantly different at 5% probability according to Tukey's test

weeks of transfer (Fig. 1d, e; Table 4). The root generated on NAA and IAA was fragile, fibrous and devoid of any branching. The demands of half-strength MS medium augmented with IBA have also been found more suitable for induction of root in microshoot of *Rhinacanthus nasutus* (Cheruvathur and Thomas 2014), *Salacia chinensis* (Jaykumar et al. 2015) and *Pittosporum eriocarpum* (Thakur et al. 2016). In contrast to the present finding, optimum rooting was observed in *D. arayalpathra* (Ahmad et al. 2017) when MS medium was fortified with NAA. Healthy plantlets were well hardened as represented in “Materials and Method” section. The hardened plant in Soilrite™ when transferred to the natural conditions showed healthy growth with 89.3% survival rate (Fig. 1f). In the field, in vitro raised plantlets showed lushy growth and exhibit similar morphological character as that of mother plant, which indicated the stability of plant free from visible abnormalities.

Physiochemical study

Pigments, photosynthesis and related attributes

Micropropagated plantlets when transferred to the ex vitro condition, face high light irradiance and situation demands increased in pigment content to overcome the effect. The reduction in chlorophyll content was found during the initial days of acclimatization for which Pospisilova et al. (1999) has found the reason i.e. disordered grana might be responsible for initial fall. During the first week, reduction in chlorophyll a (0.64–0.52 mg g⁻¹), chlorophyll b (0.19–0.1 mg g⁻¹) and carotenoid content (0.24–0.14 mg g⁻¹) was found (Fig. 2a, b, c). After that steady rise was found in chl a (1.14 mg g⁻¹), chl b (0.28 mg g⁻¹), and carotenoid content (0.82 mg g⁻¹) during 8 weeks of acclimatization which corresponds to increase in photosynthetic efficiency. The increased in photosynthetic

pigment content was also reported in *D. hamiltonii* (Sharma et al. 2014), *Plantago algarbiensis* Samp. and *P. almogravensis* Franco (Goncalves et al. 2017).

The very first challenge that micropropagated plantlets faces when relocated to the ex vitro condition is related to water loss due to poor stomatal functioning and lack of thick cuticular deposition (Pospisilova et al. 1999). After the initial fall in P_N, a gradual rise was found in P_N which might be due to the formation of new leaf and photosynthetic pigment. P_N becomes 2.31 μ mol CO₂m⁻²s⁻¹ after 8 week of acclimatization (Fig. 2d). An incremental practice in photosynthetic rate is not surprising as it has been also reported in several medicinal plants during ex vitro transplantation (Pospisilova et al. 1988; El-Mahrouk et al. 2016).

The plant performance in new environment is greatly influenced by the stomatal activity as its control on gaseous exchange. However, the process of acclimatization is genetically controlled. During in vitro phase the stomata remain open due to high humidity and the initial days of acclimatization (Xiao et al. 2011). Transpiration rate and stomatal conductance are directly related to the water balance in the plant body. The rate of transpiration (Tr) decreased from 0.7 to 0.4 mmol m⁻²s⁻¹ at initial days of acclimatization followed by a gradual increase was found, as the days of acclimatization increase (Fig. 2e). Similarly, stomatal conductance decreased from 0.037 to 0.016 mol m⁻² s⁻¹ during the initial days and then a gradual increase was noticed (Fig. 2f). The rise and fall in stomatal activities reflects strong regulation under ex vitro environment. The results were in accordance with the findings of Galmés et al. (2007) and Perez-Jimnez et al. (2015).

Electrolyte leakage (EL), lipid peroxidation and antioxidant enzyme activities

The light being as preliminary energy source for the growth of the plant however, excess of light intensities

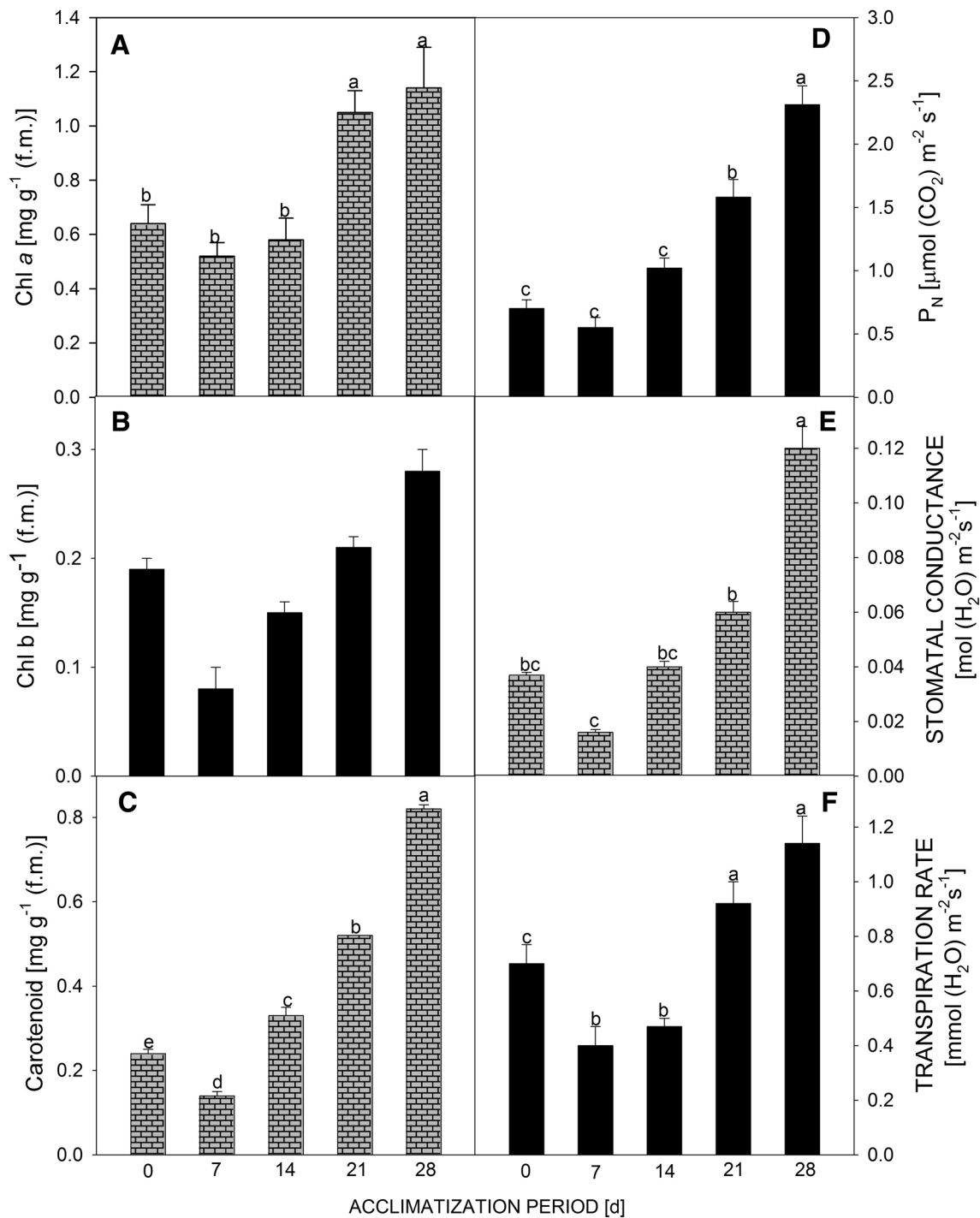


Fig. 2 Pigment content and related characteristics in *in vitro* raised plantlets of *D. salicifolia*. Chlorophyll a (**a**), Chlorophyll b (**b**), carotenoid (**c**), net photosynthetic rate (PN) (**d**), stomatal conductance (Gs)

(**e**), transpiration rate (**f**). Bars represent mean \pm SE. Bars represented by same letter within response variable are not significantly different ($P=0.05$) according to Tukey's test at 5% probability

can be harmful for the plant and leads to photoinhibition of photosynthetic light reaction and the incomplete reduction of oxygen responsible for the successive production of ROS (reactive oxygen species) i.e., hydrogen peroxide,

superoxide radical and singlet oxygen (Batková et al. 2008). The polyunsaturated fatty acid present in the plasma membrane is more prone to damage by ROS and leads damage to the plasma membrane and production of MDA as

consequence of lipid peroxidation. The leakage in electrolytes directly corresponds to the membrane permeability (Gill and Tuteja 2010). During the transfer of in vitro to

ex vitro acclimatization, the plantlets feel light stress and a maximum increase was noticed in both EL (51.8%) and TBARS [$2.06 \mu\text{mol g}^{-1}$ (f.w.)] at 7 days of acclimatization

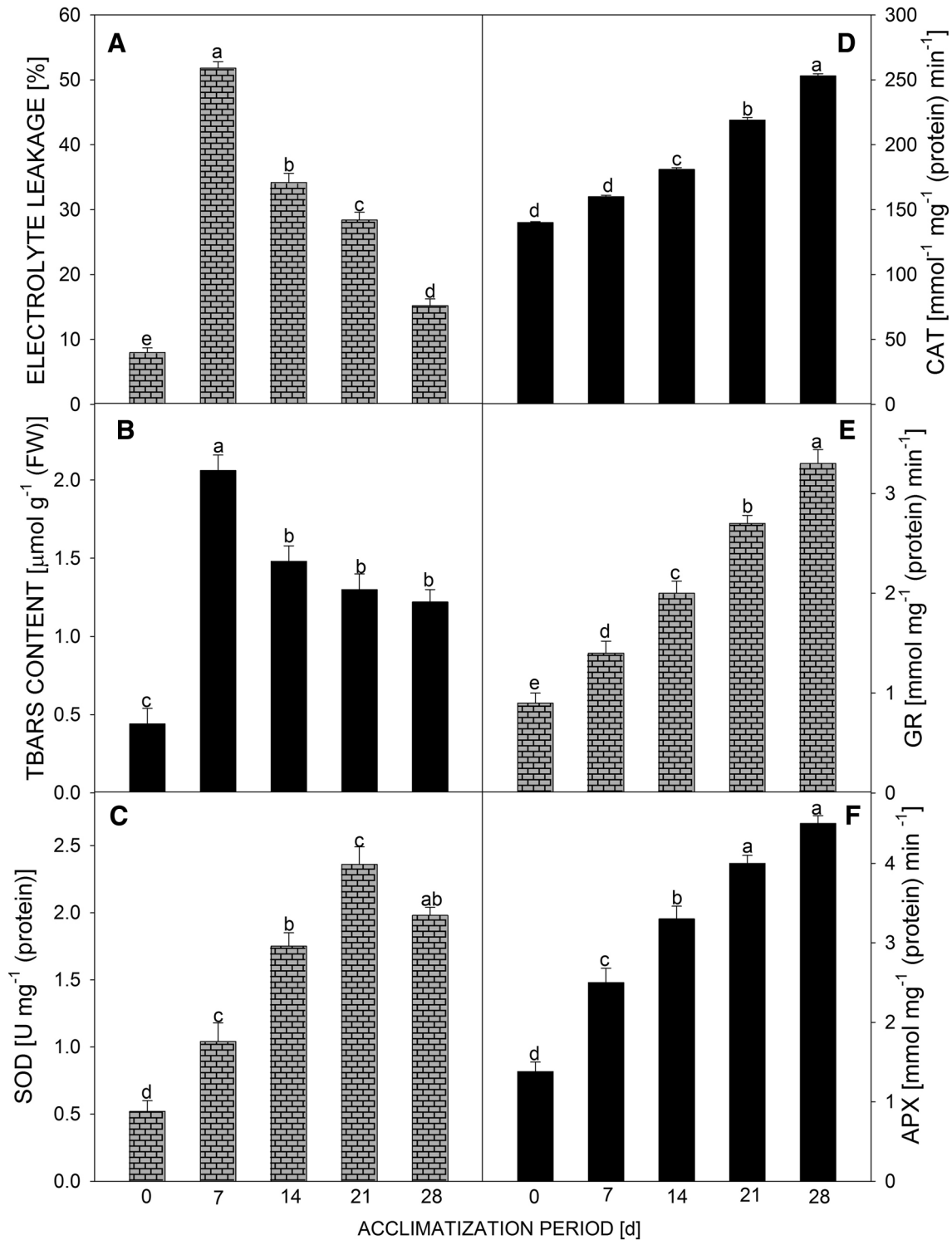


Fig. 3 Antioxidant enzymes activity in in vitro raised plantlets of *D. salicifolia*. EL (a), TBARS (b), SOD (c), CAT (d), GR (e), APX (f) Bars represent mean \pm SE. Bars represented by same letter within

response variable are not significantly different ($P=0.05$) according to Tukey’s test at 5% probability

(Fig. 3a, b). After that a gradual decrease was found due to improved acclimatization. Our finding was according to the findings of Faisal and Anis (2009) and Perez-Jimnez et al. (2015) conducted in *Rauvolfia tetraphylla* and *Cynara scolymus* respectively. The harmful effects of ROS are generally overcome by the antioxidant enzymes such as SOD, CAT, GR and APX. In this study a steady rise was found for SOD as the days of acclimatization increases, it reaches maximum value of 2.36 U μ g⁻¹ (protein) after 21 days of acclimatization and decreases to 1.98 U μ g⁻¹ (protein) after 28 days of acclimatization (Fig. 3c). Increase in H₂O₂ content corresponds the catalytic activity of SOD as it converts superoxide to H₂O₂ and oxygen (O₂) to prevent membrane oxidation and damage to the biological molecules. Moreover, increase in CAT activities (Fig. 3d) and SOD both represents photorespiratory detoxification of H₂O₂ into O₂ and H₂O in mitochondrial electron system (Scandalios 1990). CAT scavenges H₂O₂ by converting it into O₂ and H₂O and hence reduced level of H₂O₂ suggest successful acclimatization. The similar kind of behavior of these enzymes was also found in *Plantago algarbiensis* Samp. and *P. almogravensis* Franco (Gonçalves et al. 2017), *D. arayalpathra* (Ahmad et al. 2017).

Both APX and GR carried out chloroplast based detoxification of ROS via Mehler pathway (Foyer and Mullineaux

1998) and the activity of both the enzyme reached highest value of 4.5 mmol mg⁻¹ (protein) min⁻¹ and 3.3 mmol mg⁻¹ (protein) min⁻¹ respectively after 28 days of acclimatization (Fig. 3e, f). Both performed essential function by scavenging H₂O₂ in chloroplast, cytosol, vacuoles and apoplastic space (Asada 1999). If the action of SOD, CAT, APX and GR are in coordination, the rate of oxidative damage to the plant with ROS could be controlled and plant can perform better towards acclimatization process in a new environment (Yan 2009). The elevation of APX and GR is not astonished as it is previously reported in *D. arayalpathra* (Ahmad et al. 2017).

2H4MB quantification during acclimatization

Now a great interest are being developed for separation, identification and quantification of 2H4MB from *D. salicifolia* root system due to its pharmacological value and it could be possible through HPLC. The chromatogram obtained for the extract was compared with that of standard 2H4MB peak showed similar retention time (RT) for both. Figure 4a, b clearly depict the similarity in RT of 2H4MB (3.686 min) and RT of extract (3.689 min) at same chromatographic condition. Figure 5 represent the relationship between fresh weigh, dry weight and 2H4MB content in root. Maximum

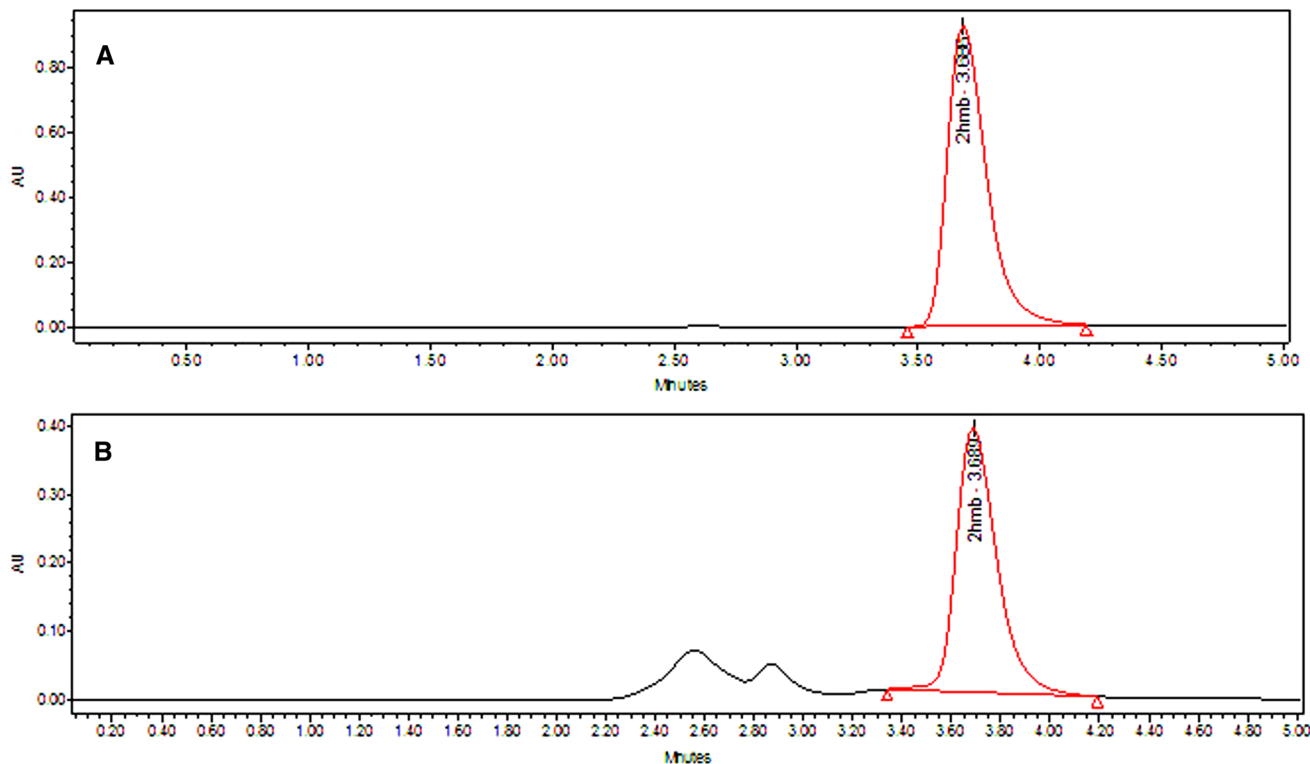
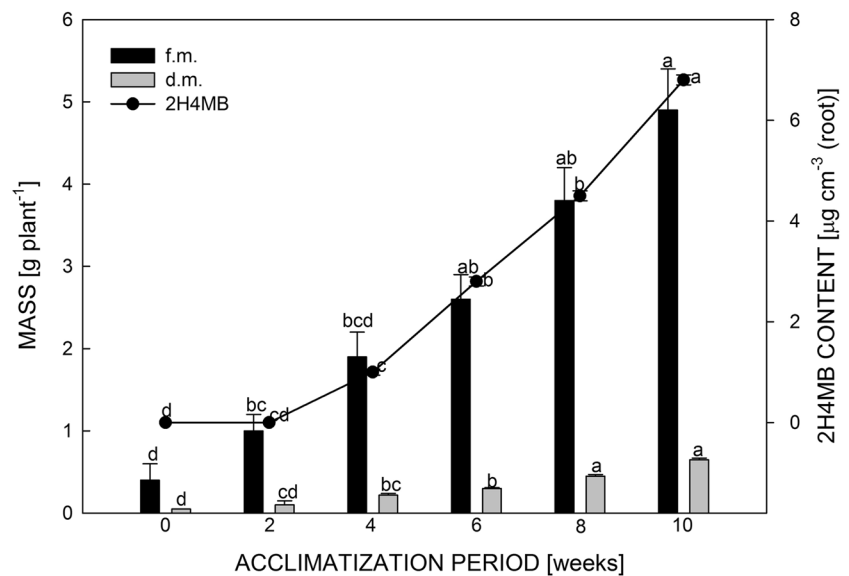


Fig. 4 HPLC chromatogram of the standard compound, i.e., 2-hydroxy-4-methoxy benzaldehyde (2H4MB) eluted at 280 nm (a), HPLC chromatogram of the root extract after 10 weeks of acclimatization at 280 nm (b)

Fig. 5 Quantification of 2H4MB in relation to the biomass of the root system of *D. salicifolia*. Bars and lines represent the data as mean \pm SE. Bars and lines represented by same letter within response variable are not significantly different ($P=0.05$) according to Tukey's test at 5% probability



fresh weigh and dry weight of root were found to be 4.6 and 0.65 g per plant and the highest accumulation of 2H4MB was recorded $6.8 \mu\text{g ml}^{-1}$ after 10 weeks of acclimatization. The results support the hypothesis that accumulation of 2H4MB start only after 2 weeks of acclimatization and increased gradually. Our results are according to the reports of Sharma et al. (2014) and Ahmad et al. (2017) they found similar kind of growth accumulation pattern in *D. hamiltoni* and *D. aryalpathra* respectively.

Genetic homogeneity assessment

Nine in vitro raised plants randomly selected including mother plant for the screening of genetic homogeneity by RAPD and ISSR markers. Clear and distinct banding with no reproducible alien bands were observed and manually scored from the gel profiles and included for final analysis (Fig. 6a, b; Tables 5, 6). Both RAPD and ISSR marker are unsophisticated and profitable in screening of genetic uniformness. Out of 10 RAPD primers screened from Kit L, 9 primers produced comprehensible and reproducible bands. Total of 42 scorable bands and highest number of bands were obtained with the primer 3 i.e., 8 and the lowest number of bands i.e., one. In case of ISSR primers, out of 10 primers used, nine primers produced clear band and a total of 50 bands were produced. Maximum number of bands were obtained in the primer UBC-825 i.e., 9 and the lowest number of bands i.e., 3. No polymorphism was detected during the analysis of tissue culture raised plant, confirming the genetic uniformity of the plant. The proposed dendrogram from the analysis also proved over

99% similarity with mother and nine randomly selected in vitro raised plantlets showing high level of monomorphism (Fig. 7). The present findings also suggest that micropropagation of *D. salicifolia* using nodal explants maintains genetic uniformity even after prolonged period under in vitro conditions. Our results are in accordance to the earlier report on genetic uniformity of in vitro derived plantlets of *Curcuma longa* (Nayak et al. 2011), *Helicteres isora* (Mariappan et al. 2016) and *Salacia chinensis* (Jaykumar et al. 2015).

Conclusions

Proposed findings account a holistic and reliable protocol for micropropagation of *D. salicifolia*. This is the first productive efforts to set up a micropropagation protocol for the plant using nodal explants, which imparts reintroduction, large-scale propagation, commercialization and ex situ conservation. DNA based markers proved to evaluate genetic fidelity and true-to-type nature of micropropagated plant. Considering the importance of the plant adjustment during acclimatization, different physiochemical parameters and biochemical characterization were also studied for the first time in this species. The results obtained with high rate of plantlets survivability could be utilized in making micropropagation and conservation of this endangered species more advantageous and profitable. The identification and quantification of 2H4MB in relation to biomass accumulation during acclimatization was also encouraging and reported for the first time. The developed protocol can be used for the

Fig. 6 DNA fingerprinting of micropropagated plants of *D. salicifolia* **a** RAPD Primer (OPL04), **b** ISSR Primer (UBC 818)

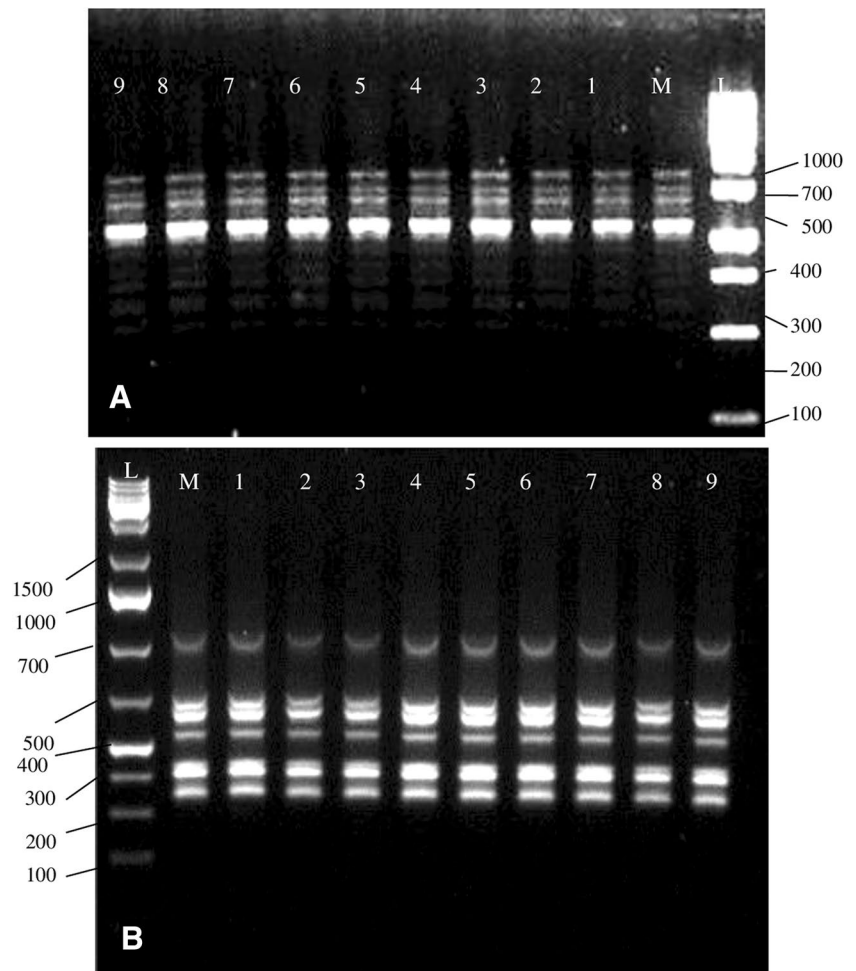


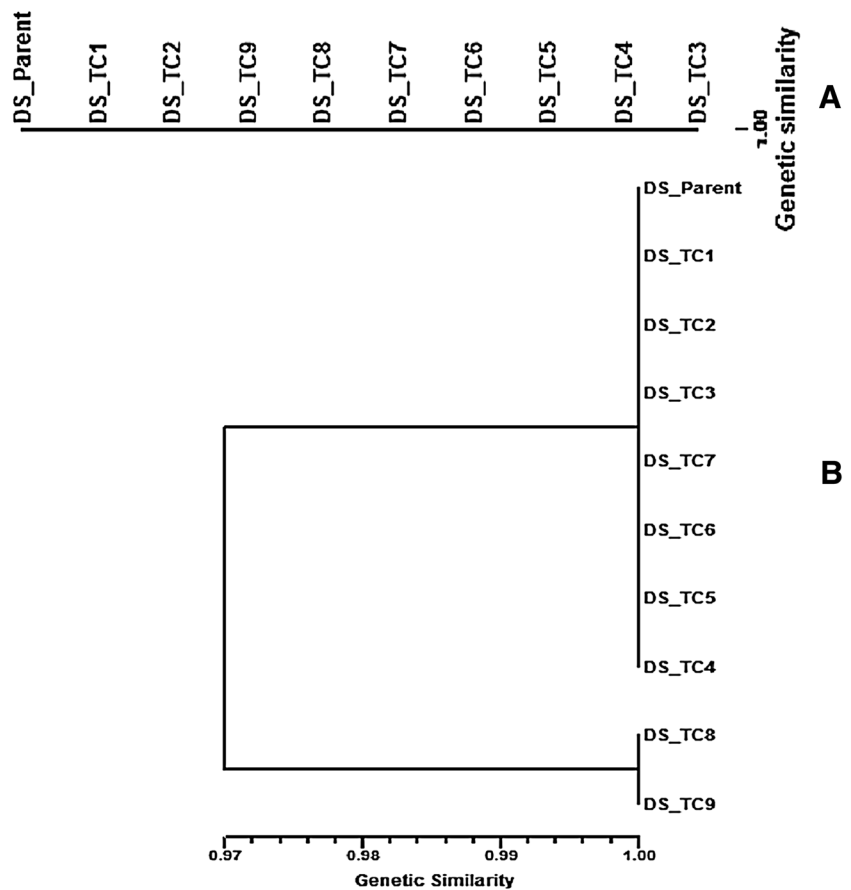
Table 5 Amplification products generated with RAPD markers among mother plant and in vitro raised plants of *D. salicifolia*

Name of primers	Primer sequence (5'–3')	Total no. of bands
OPL-01	5'GGCATGACCT3'	2
OPL-02	5'TGGGCGTCAA3'	3
OPL-03	5'CCAGCAGCTT3'	8
OPL-04	5'GACTGCACAC3	6
OPL-05	5'ACGCAGGCAC3'	5
OPL-6	5'GAGGGAAGAG3'	0
OPL-7	5'AGGCGGGAAC3'	1
OPL-8	5'AGCAGGTGGA3'	7
OPL-9	5'TGCGAGAGTC3'	6
OPL-10	5'TGGGAGATGG3'	4

Table 6 Amplification products generated with inter sequences repeats (ISSR) marker among mother plant and in vitro raised plantlets of *D. salicifolia*

Name of primers	Primer sequence (5'–3')	Total no. of bands
UBC-812	(GA)8A	3
UBC-814	(CT)8A	4
UBC-818	(CA)8G	7
UBC-825	(AC)8T	9
UBC-827	(AC)8G	5
UBC-836	(AG)8YA	6
UBC-848	(CA)8RG	7
UBC-855	(AC)8YT	0
UBC-868	(GAA)6	4
UBC-880	(GGGGT)3G	5

Fig. 7 Dendrogram obtained by molecular analysis of regenerants of *D. salicifolia* **a** RAPD, **b** ISSR



conservation of *D. salicifolia* by providing valuable resource material and might play a significant role in the delisting of species from Red Data List.

Acknowledgements Financial support by the Department of Science and Technology (DST), India, is gratefully acknowledged.

Author contributions This work was carried out in collaboration among four authors. Author Dr. AS conceived and designed the experiments. Author Dr. SS and Dr SP helped for the quantitative estimation of the chemical compound and writing of the manuscript. Author ZA performed all the experimental work, analyzed the data and wrote the manuscript. All the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors proclaimed no conflict of interest.

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