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MEDICINAL PLANTS

In vitro propagation and assessment of genetic uniformity along with chemical characterization in *Hildegardia populifolia* (Roxb.) Schott & Endl.: a critically endangered medicinal tree

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Abstract

Hildegardia populifolia (Roxb.) Schott & Endl. is a critically endangered medicinal tree species which also provide high-quality natural fibers. The present study was focused to develop reliable micropropagation protocol where nodal segment explants were inoculated on Murashige and Skoog medium (MS) and Woody Plant Medium (WPM) augmented with various cytokinins alone or with auxins. WPM was more effective producing up to 10.67 ± 0.88 shoots per explant with average shoot length of 4.20 ± 0.10 cm from nodal explants cultured for 6 wk on medium containing 5.0 μM 6-benzylaminopurine (BA) and 2.0 μM 1-naphthaleneacetic acid (NAA). Microshoots rooted efficiently with a 24-h pulse treatment *ex vitro* with 200 μM indole-3-butyric acid (IBA) producing 5.33 ± 0.33 roots per microshoot with mean root length of 4.77 ± 0.07 cm. The *ex vitro* rooting and simultaneous acclimatization resulted in a 98.33 ± 1.67% survival rate in soilrite. Genetic stability was confirmed by using DNA-based molecular markers. Various photosynthetic and biochemical parameter coupled with scanning electron microscopic analysis of leaves revealed the healthy adaptation of the plantlets to natural conditions. Gas chromatography and mass spectrometry analysis of leaf of mother and regenerated plantlets was performed to compare secondary metabolites.

Keywords Micropropagation · Murashige and Skoog medium · Woody Plant Medium · Plant growth regulators

Introduction

Hildegardia populifolia (Roxb.) Schott. & Endl. is a medium size deciduous tree, belonging to the family (Malvaceae) growing to a height of 15 to 20 m and easily recognizable by its smooth, pale green, fiber-rich stem bark (Lavanya *et al.* 2014; Raju *et al.* 2014). It is native to Eastern Ghats of Andhra Pradesh and Tamil Nadu in India (Ahmedullah and Nayar 1987; World Conservation Monitoring Centre 1998; Sarcar and Sarcar 2002). The distribution of the tree is very restricted in the forested eastern slopes of the Kalrayan Hills where approximately twenty trees survive (Ahmedullah 1990; Nayar and Sastry 1990; Oldfield *et al.* 1998). According to the IUCN Red List of Threatened Species (1998:e.T33656A9801072), this plant is in the critically endangered category (World Conservation Monitoring Centre 1998<www.iucnredlist.org>). This tree is utilized for its stem bark fibers, wood, and gum by local people leading to the habitat clearance and loss of this species.

H. populifolia has both economic and ethnic values. Leaves and stem bark of the plant are used for malaria and dog bite treatment in the traditional system of medicinal practice of Tamil Nadu and Andhra Pradesh (Varaprasad *et al.* 2009). Methanolic leaf extract and stem bark of this tree are reported to have pharmacologically significant compounds with antioxidant, anti-diabetic, antimalarial, anti-inflammatory, anti-cancer, antimicrobial activities and healing properties against a number of human pathogens (Lavanya *et al.* 2012; Saradha and Paulsamy 2012a and 2013; Gritto *et al.* 2015; Subbalakshmi and Pullaiah 2015). The fiber obtained from stem bark of *H. populifolia* is economically valuable because they are arranged in uniaxial direction, slightly inter-woven, and loosely bound together. It is used as a substitute for synthetic fibers, carbon, and glass for packaging and medical applications and is used as a substitute for plastic (Li *et al.*

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2004; Rajulu *et al*. 2004 and 2005; Guduri *et al*. 2006; Jeevan Prasad Reddy *et al*. 2009; Dani *et al*. 2011). Previous investigations indicate that its fiber can be used as reinforcement in green composites as they are completely biodegradable (Li *et al*. 2004; Rajulu *et al*. 2005). These fibers are used for their strength, even at low density, to make natural fiber reinforced polymer composites (Rajulu *et al*. 2003). The fiber extracted from the stem bark is also used for domestic purposes for making ropes and baskets by the local population (Raju *et al*. 2014).

It is presumed that anthropogenic interferences, habitat loss, and intrinsic and extrinsic factors have resulted in poor *H. populifolia* regeneration and low seed viability (Nayar and Sastry 1990; Anuradha and Pullaiah 2001). Fungal infections during maturation stage may damage the cotyledons and embryos thus drastically reducing the number of viable seeds and is one of the reasons for the limited survival rate and reduced population size of seedlings in their natural habitat (Anuradha and Pullaiah 2004). Raju *et al*. (2014) reported that an attempt to germinate seeds in a forest nursery sown in polythene bags contains fertile soil and watered daily showed a germination rate of only 0.42%. The seeds germinated after 18 wk and the subsequent growth of seedling was very low. However, Saradha and Paulsamy (2012b) reported that *H. populifolia* could be clonal propagation through mature stem cuttings, although Lavanya *et al*. (2012) reported that this approach is not efficient to apply the mature stem cutting method under varied climatic conditions to repopulate this species to its habitat. Furthermore, *H. populifolia* seeds are heterozygous in nature and impossible to use for pure breeding lines. Because of these limitations, *in vitro* regeneration of *H. populifolia* was considered as a means to increase the numbers of this endangered species.

Development of *H. populifolia in vitro* cultures using nodal explants from a *H. populifolia* mother plant was initiated to regenerate true-t-type plants for large-scale production and conservation. Nutrient media (MS-Murashige and Skoog Medium and WPM-Woody Plant Medium) were compared to obtain the best morphogenic response. Considering the importance of acclimatization of *in vitro* raised plantlets, parameters such as pigment content, net photosynthetic rate, and antioxidant enzymes were also screened, in addition to ultra-structural studies of leaf texture from mother and *in vitro* raised plantlets. Gas chromatography and mass spectrometry were used to analyze the important metabolites in *H. populifolia*.

Materials and Methods

Collection of explants and establishment of aseptic cultures Nodal segment (NS) explants of *H. populifolia* were excised from a 3-y-old established plant (mother plant) in the green house of the Botany Department, A.M.U., Aligarh, U.P., India. Before the inoculation, the NS were thoroughly rinsed in tap water for 30 min followed by a treatment with 1% (w/v) Bavistin solution (carbendazim powder, BASF India Ltd., Mumbai, India) for 30 min. The NS were also cleaned with 5% (v/v) Teepol (liquid detergent) for about 15 min, surface sterilized with 0.1% (w/v) freshly prepared $HgCl_2$ (Qualigens, Mumbai, India) solution for 3 min, and rinsed with sterilize DDW (double distilled water) for 4 to 5 times in a laminar air flow hood.

Murashige and Skoog Medium (Murashige and Skoog 1962) and Woody Plant Medium (McCown and Lloyd 1981) were used in all the experiments. A single NS explant was inoculated per culture tube containing 20 mL nutrient medium augmented with 0 to 10.0 μM 6-benzyladenine (BA), kinetin (Kn), or thidiazuron (TDZ) for shoot induction and multiplication. For further growth and proliferation, the optimized cytokinin treatment was tested in combination with 0 to 3.0 μM indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), or 1-naphthaleneacetic acid (NAA). Medium devoid of any plant growth regulators was the control. The media were also augmented with 3% (w/v) sucrose (Qualigens Fine Chemicals) and 0.8% (w/v) agar (Bacteriological grade, Himedia, Mumbai, India). The pH of the medium was adjusted to 5.8 using 1 N HCl or 1 N NaOH, autoclaved for 15 min at 121°C and 15 psi pressure, and dispensed in 25 × 150 mm culture tubes (Borosil, Mumbai, India) and 100 cm^3 Erlenmeyer flasks (Borosil). All the cultures were incubated under standard culture room conditions, *i.e.*, 25 ± 2°C temperature, 55% ± 5% relative humidity, and 16/8 h photoperiod having 50 μmol m^{-2} s^{-1} PPFD (photosynthetic photon flux density) supplied by cool fluorescent lights (40 W, Philips, India).

Rooting and acclimatization For *ex vitro* rooting, the basal cut end of regenerated healthy microshoots with well-expanded leaves was removed from culture and dipped in 0, 100, 150, 200, 250, or 300 μM of IAA, IBA, or NAA for 24 h and then planted in small thermocol cups (height 8.2 cm, top diameter 7.2 cm, bottom diameter 4.2 cm, purchased from Gulmarg Chemicals & Scientific Works, Aligarh, India) containing sterilized soilrite (Keltech Energies Pvt. Ltd., Bengaluru, India) and kept in the culture room for 4 wk at 25 ± 2°C and 16/8 h photoperiod. These thermocol cups were enclosed within transparent polythene bags, having small perforations for the purpose of gaseous exchange and to stabilize relative humidity after being placed in the culture room. After 4 wk, rooted regenerated plantlets were transferred to thermocol cups containing three different planting substrates: garden soil plus manure (3:1), soilrite, and vermicompost and kept in culture room for 4 wk. These regenerated plantlets were regularly irrigated with 1/4 strength MS salt solution (without vitamins) followed by tap water for 2 wk. Polythene bags were then removed and the plantlets were placed under direct exposure to the fluorescent light in culture room for another

2 wk, moved to the green house for 2 wk, and then transplanted to the clay pots containing garden soil followed by transfer from the green house to the field.

Genetic fidelity To confirm the genetic fidelity, nine acclimatized regenerated plantlets were randomly selected and compared with the mother plant. Genomic DNA was isolated from fresh leaf tissues of *H. populifolia* using cetyltrimethyl ammonium bromide (CTAB) method according to Doyle and Doyle (1990). The purity ($A_{260/280}$ ratio) of isolated DNA was performed on a UV-vis spectrophotometer. A total set of 10 random amplified polymorphic DNA (RAPD) (OPL Kit) and 10 inter simple sequence repeats (ISSR) (UBC, Vancouver, Canada) primers were used for PCR analysis on a thermocycler (Biometra, T Gradient, Thermoblock, Germany). The reaction mixture preparation, PCR amplification, and amplicons separation were performed by following the methods of Ahmad *et al*. (2018b).

Scanning electron microscopy analysis For SEM analysis, leaf samples were taken from *in vitro* cultured plantlets and from the 4-wk-old acclimatized plants. The leaves were fixed in glutaraldehyde (Merck, Merck Specialties Pvt. Ltd., Mumbai, India) and kept at room temperature for 2 h followed by gradual dehydrated with an increasing alcohol step-wise series (30%, 50%, 70%, 90%, and 100%) with 15 min at each step. The samples were subjected to critical point drying and then abaxial surface of leaves coated with gold. Samples were mounted on aluminum stubs with double-sided adhesive tape (3M, Sumaré, Brazil) and examined by SEM (JSM-6510, LV-JEOL, Tokyo, Japan) at 15 kV where all images of leaf surface were processed digitally.

GC-MS analysis Mature and healthy leaves were used from 4-wk-old successfully acclimatized plant as well as from a mother plant for GC-MS analysis. Ten replicates were used for the GC-MS study. Harvested leaves were washed and air dried for 2 to 3 d and then crushed to a fine powder using a mortar and pestle. One gram of powder was dissolved in 50 mL of methanol and contents were extracted for 24 h. The methanolic leaf extract was centrifuged at 5000 RCF for 5 min and supernatant filtered with an aminigen syringe filter (0.22 μm, Micropor, Genetix Biotech Asia Pvt. Ltd., New Delhi, India) to remove any remaining residues. Finally, the total volume of each extraction was brought up to 10 mL with methanol solvent and used for phytochemical profiling. One microliter of methanolic leaf extract was used as sample and manually injected into RTX-5 column of GCMS (QP-2010 Ultra, Shimadzu, Kyoto, Japan) operating at 1000 eV ionization energy using helium as carrier gas at 173 kpa inlet pressure. Identification and confirmation of phytochemicals was performed using the database of the National Institute of Standards and Technology (NIST) and Wiley Library for mass spectra to determine their molecular weight (MW).

Physiological and biochemical studies *In vitro* raised plantlets showing healthy growth were selected for physiological and biochemical analysis. A set of ten micropropagated plantlets were randomly selected and maintained separately from the other plants in the culture room. Leaf samples were taken at transplantation (0 d, control) and after 7, 14, 21, and 28 d of acclimatization.

Total chlorophyll content and net photosynthetic rate estimation To estimate the total chlorophyll content of the leaves, 100 mg of fresh tissues was taken from interveinal areas of leaves and crushed in acetone (80%, 5 mL) using mortar and pestle and filtered using Whatman's No.1 filter paper. The optical density (O.D.) of filtrate was read at wavelengths 645 and 663 nm for chlorophyll evaluation using a UV-visible spectrophotometer. The estimation of total leaf chlorophyll content was performed according to Mackinney (1941).

Expanded plant leaves were selected for the estimation of net photosynthetic rate (P_N). This was evaluated by using a LI-COR 6400 infrared gas analyzer (IRGA; LI-COR Biosciences, Lincoln, NE) at 800 μmol/m^2s photosynthetically active radiation (PAR) between 11:00 a.m. and 12:00 noon under the clear sunlight using a leaf chamber. The monitoring was based on the exchange of CO_2 between leaf and atmosphere.

Biochemical enzymes evaluation To evaluate superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX) antioxidant enzyme activity, 0.5 g of fresh leaf tissues was homogenized in 2.0 mL of extraction buffer composed of 1% (w/v) of polyvinylpyrrolidone (PVP), 1% (v/v) of Triton X-100, and 0.11 g of ethylene diamine tetra acetic acid (EDTA) in water using a pre-chilled mortar and pestle. Homogenate was filtered with Whatman's No.1 filter paper and then centrifuged at 15000 RCF for 20 min using a high-speed centrifuge (Remi Instruments Ltd., Mumbai, India). The extraction was performed in darkness at 4°C and the supernatant was used as a crude extract for enzyme assays. The activity of SOD, CAT, GR, and APX was evaluated according to Dhindsa *et al*. (1981), Aebi (1984), Rao (1992), and Nakano and Asada (1981) respectively and quantified as enzyme units (EU) mg^{-1} protein.

Statistical analysis To analyze regeneration percentage, data for number of shoots per explants and shoot length were recorded after 6 wk of culture while for rooting, experiment data were recorded after 4 wk of culture. Twenty replicates per treatment with three repetitions

were taken to conduct the *in vitro* propagation experiments and the obtained data was analyzed by one-way analysis of variance (ANOVA) using SPSS Version 16 (SPSS Inc., Chicago, IL). Duncan's multiple range test (DMRT) at $P \leq 0.05$ was used to carry out the significance of difference among means and the results were denoted as mean ± SE. Graphically, the data were presented by using Sigma Plot ver. 10.0 (Systat Software, Inc., San Jose, California).

Results and Discussion

***In vitro* plant regeneration** An improved micropropagation protocol has been developed for *H. populifolia*. Types and concentration of plant growth regulators and basal media had a significant effect on shoot and root formation. Comparative nutrient media comprising of MS and WPM basal salts were used to evaluate the best morphological response of the explants. In the present study, NS explants were inoculated on MS and WPM basal media with or without (control) PGRs (Fig. 1*a*). WPM was the superior nutrient medium compared with MS. BA, Kn, and TDZ (0, 0.5, 2.5, 5.0, 7.5, and 10.0 μM) were tested with both nutrient media. Initially, the explants swelled within 1 wk and, after 12 d, green protuberance appeared followed by organized shoot buds with leaf primordial after 18 d. Among the cytokinins tested, WPM supplemented with of 5.0 μM BA was found most significant with a maximum of 9.33 ± 0.33 shoots per explant; mean shoot length (SL) of 3.57 ± 0.09 cm from over 93% of the cultures was recorded after 6 wk (Table 1, Fig. 1*c*). Lowering or increasing the concentration of BA affected shoot number with 2.67 ± 0.88 and 4.00 ± 0.58 shoots per explant using 0.5 and 10.0 μM BA, respectively (Table 1). MS medium containing 5.0 μM BA gave rise to 7.67 ± 0.33 shoots per explant with a mean SL of 3.07 ± 0.07 cm from approximately 88%the cultures after 6-wk incubation (Table 1, Fig. 1*b*). BA was effective in bud breaking and promoted formation of multiple shoots due to enhanced permeability across the plasma membrane and its high cellular uptake (Malik *et al*. 2005). Superiority of BA among cytokinins has been also established by several workers such as in *Trichosanthes dioica* (Saurabh *et al*. 2017) and *Rauwolfia serpentine* (Zafar *et al*. 2019).

The optimum concentration of BA (5.0 μM) was used in combination with 1.0, 2.0, and 3.0 μM NAA, IAA, or IBA in WPM and MS media (Table 2). Between the two basal media and cytokinin-auxin combinations tested, WPM supplemented with 5.0 μM BA and 2.0 μM NAA produced the maximum number of shoots per explant (10.67 ± 0.88) and greatest average SL (4.20 ± 0.10 cm) from over 98% of the cultures after 6 wk of incubation (Table 2, Fig. 1*e*). This is supported by Nakagawa *et al*. (2005) and Samantaray *et al*. (2013) where the combined effect of cytokinin and auxin was effective in regulation of apical dominance and morphogenesis in *Petunia hybrida* and *Vitex trifolia*, respectively. In our study, MS medium augmented with 5.0 μM BA and 2.0 μM NAA showed improved shoot proliferation but less effective in comparison with WPM medium as only 8.33 ± 0.33 shoots per explant were produced with an average SL of 3.37 ± 0.03 cm from over 93% of the cultures after 6 wk of incubation (Table 2, Fig. 1*d*). There are reports demonstrating the synergism of cytokinin and auxin in promoting *in vitro* culture from medicinal plants such as *Decalepis salicifolia* (Ahmad *et al*. 2018b) and *Magnolia sirindhorniae* (Cui *et al*. 2019).

Rooting and acclimatization For *ex vitro* rooting, 4 to 6-cm-long microshoots were excised from WPM supplemented with 5.0 μM BA and 2.0 μM NAA. These microshoots were treated with 100, 150, 200, 250, and 300 μM IAA, IBA, or NAA for 24 h under sterile conditions and transferred directly to the thermocol cups containing sterilize soilrite (Fig. 2*a*). The optimal treatment was 200 μM IBA which produced 5.33 ± 0.33 roots per microshoot with an average root length of 4.77 ± 0.07 cm after 4 wk following a 24-h pulse treatment (Table 3, Fig. 2*b*, *c*). The lower concentrations of the auxins tested failed to induce rooting in the microshoots. The IBA-treated roots were healthy in nature, thick, and profusely branched, while short, fragile, fibrous roots having lesser number of secondary branching were observed on IAA and NAA at similar concentrations. Increased concentration of the auxin PGRs beyond the optimal level resulted in reduced root production after similar incubation periods (Table 3). A critical step in micropropagation is the transfer of regenerated plants from *in vitro* to greenhouse conditions. Regenerated plantlets with fully expanded leaves and well-developed root system were again hardened in thermocol cups, now containing three different planting substrates: garden soil and manure (3:1), soilrite, and vermicompost. Among these potting substrates, soilrite was the optimal substrate for acclimatization of regenerated plantlets with over 98% survival (Fig. 3). Soilrite gave optimal rooting because it is porous in nature and has good water holding capacity because of the presence of peat moss and vermiculite to support a healthy root system. After acclimatization in soilrite, the regenerated plantlets were transferred to garden soil and exhibited nearly 80% survival in greenhouse conditions. After 4 mo in soil, the plants had normal growth and morphology similar to those in nature (Fig. 2*d*, *e*).

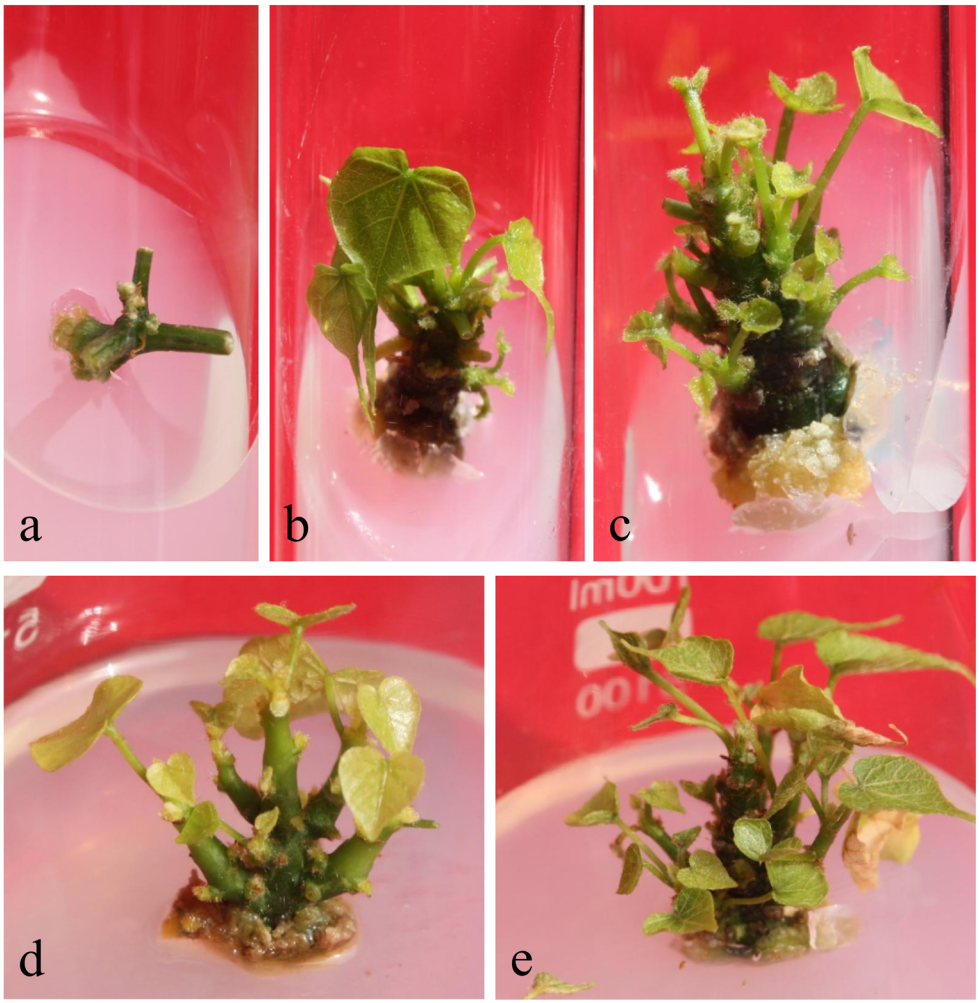

Figure 1 *In vitro* plant regeneration and establishment of *Hildegardia populifolia* (Roxb.) Schott. & Endl. (*a*) Nodal explant inoculated on Woody Plant Medium (WPM) basal medium. (*b*) Shoot induction and regeneration on Murashige and Skoog (MS) + 5.0 μM 6-benzyladenine (BA). (*c*) Shoot induction and regeneration on WPM + 5.0 μM BA. (*d*) Multiplication and proliferation of shoots on MS + 5.0 μM BA +2.0 μM 1-naphthaleneacetic acid (NAA). (*e*) Multiplication and proliferation of shoots on WPM + 5.0 μM BA +2.0 μM NAA.

Table 1. Effect of various cytokinins in different basal media on shoot regeneration from *in vivo* nodal explants of *Hildegardia populifolia* (Roxb.) Schott. & Endl. after 6-wk culture

Plant growth regulators (μM)			Murashige and Skoog medium			Woody Plant Medium		
BA	Kn	TDZ	% response	Mean number of shoots per explant	Mean shoot length (cm)	% response	Mean number of shoots per explant	Mean shoot length (cm)
0.0	0.0	0.0	0.0 ± 0.0^{i}	0.0 ± 0.0^{h}	0.0 ± 0.0^{k}	0.0 ± 0.0^{k}	0.0 ± 0.0^{h}	0.0 ± 0.0^{l}
0.5			55.00 ± 2.89^{gh}	1.33 ± 0.33^{fg}	1.87 ± 0.03^{ef}	63.33 ± 1.67^{gh}	2.67 ± 0.88^{def}	3.17 ± 0.12^{cd}
2.5			63.33 ± 1.67^{def}	5.33 ± 0.67^{bc}	2.23 ± 0.07^{c}	85.00 ± 2.89^{bc}	6.00 ± 0.58^{b}	3.20 ± 0.06^{bcd}
5.0			88.33 ± 1.67^{a}	7.67 ± 0.33^{a}	3.07 ± 0.07^{a}	93.33 ± 1.67^{a}	9.33 ± 0.33^{a}	3.57 ± 0.09^{a}
7.5			70.00 ± 2.89^{cd}	6.33 ± 0.33^{b}	2.73 ± 0.12^{b}	78.33 ± 1.67^{cd}	8.67 ± 0.33^{a}	3.43 ± 0.12^{ab}
10.0			53.33 ± 1.67^{gh}	3.67 ± 0.88^{de}	2.17 ± 0.13^{cd}	75.00 ± 2.89^{d}	4.00 ± 0.58^{cd}	3.33 ± 0.03^{abc}
	0.5		51.67 ± 1.67^{gh}	1.00 ± 0.00^{g}	1.13 ± 0.09^{j}	58.33 ± 1.67^{h}	1.00 ± 0.00^{g}	1.67 ± 0.03^{jk}
	2.5		65.00 ± 2.89^{def}	1.00 ± 0.00^{g}	1.40 ± 0.11^{hij}	66.67 ± 3.33^{efg}	1.33 ± 0.33^{fg}	2.13 ± 0.09^{i}
	5.0		68.33 ± 1.67^{de}	1.67 ± 0.33^{fg}	1.63 ± 0.09^{fghi}	71.67 ± 1.67^{def}	2.67 ± 0.88^{def}	2.40 ± 0.11^{h}
	7.5		78.33 ± 1.67^{b}	4.33 ± 0.67^{cd}	2.37 ± 0.12^{c}	86.67 ± 1.67^{ab}	5.00 ± 0.58^{bc}	2.97 ± 0.03^{de}
	10.0		75.00 ± 2.89^{bc}	2.67 ± 0.33^{ef}	1.73 ± 0.09^{efg}	76.67 ± 1.67^{d}	3.33 ± 0.33^{de}	2.67 ± 0.07^{fg}
		0.5	48.33 ± 1.67^{h}	1.00 ± 0.00^{g}	1.50 ± 0.05^{ghi}	50.00 ± 2.89^{i}	1.67 ± 0.33^{fg}	2.03 ± 0.09^{i}
		2.5	61.67 ± 1.67^{ef}	2.00 ± 0.58^{fg}	2.27 ± 0.09^{c}	73.33 ± 1.67^{de}	3.67 ± 0.33^{cde}	2.77 ± 0.07^{ef}
		5.0	58.33 ± 1.67^{fg}	1.33 ± 0.33^{fg}	1.93 ± 0.03^{de}	65.00 ± 2.89^{fgh}	2.33 ± 0.67^{efg}	2.50 ± 0.11^{gh}
		7.5	41.67 ± 1.67^{i}	1.00 ± 0.00^{g}	1.67 ± 0.09^{efgh}	46.67 ± 3.33^{i}	1.00 ± 0.00^{g}	1.77 ± 0.03^{j}
		10.0	33.33 ± 3.33^{j}	1.00 ± 0.00^{g}	1.37 ± 0.07^{ij}	35.00 ± 2.89^{j}	1.00 ± 0.00^{g}	1.43 ± 0.09^{k}

Values of mean ± SE represent data of three repeated experiments with 20 replicates. Same *letter* within *columns* not significantly different at $P \leq 0.05$, using Duncan's multiple range test (DMRT). *BA*, 6-benzyladenine; *Kn*, kinetin; *TDZ*, thidiazuron

Table 2. Effect of different combination of auxins with BA (5.0 μM) in different basal media on shoot multiplication through nodal segments of *Hildegardia populifolia* (Roxb.) Schott. & Endl. after 6-wk culture

Plant growth regulators (μM)			Murashige and Skoog medium			Woody Plant Medium		
NAA	IAA	IBA	% response	Mean number of shoots per explant	Mean shoot length (cm)	% response	Mean number of shoots per explant	Mean shoot length (cm)
0.0	0.0	0.0	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	0.0 ± 0.0^{f}	0.0 ± 0.0^{g}	0.0 ± 0.0^{e}	0.0 ± 0.0^{h}
1.0			78.33 ± 1.67^{b}	6.67 ± 1.67^{abc}	2.83 ± 0.07^{b}	85.00 ± 2.89^{bc}	7.33 ± 1.20^{bc}	3.73 ± 0.07^{b}
2.0			93.33 ± 1.67^{a}	8.33 ± 0.33^{a}	3.37 ± 0.03^{a}	98.33 ± 1.67^{a}	10.67 ± 0.88^{a}	4.20 ± 0.10^{a}
3.0			80.00 ± 2.89^{b}	7.67 ± 0.88^{ab}	3.10 ± 0.06^{ab}	91.67 ± 1.67^{ab}	8.67 ± 0.33^{ab}	3.97 ± 0.03^{ab}
	1.0		36.67 ± 3.33^{e}	3.00 ± 0.58^{d}	1.10 ± 0.15^{e}	45.00 ± 2.89^{f}	3.33 ± 1.33^{d}	1.57 ± 0.09^{g}
	2.0		41.67 ± 1.67^{e}	3.67 ± 1.45^{d}	2.27 ± 0.17^{c}	53.33 ± 1.67^{e}	4.33 ± 0.33^{cd}	3.03 ± 0.09^{c}
	3.0		38.33 ± 1.67^{e}	3.33 ± 0.33^{d}	1.43 ± 0.09^{de}	46.67 ± 3.33^{ef}	4.00 ± 0.58^{d}	2.30 ± 0.10^{e}
		1.0	60.00 ± 2.89^{d}	4.67 ± 0.67^{cd}	1.37 ± 0.12^{de}	61.67 ± 1.67^{d}	5.33 ± 1.33^{cd}	1.93 ± 0.13^{f}
		2.0	73.33 ± 1.67^{b}	5.67 ± 0.67^{abcd}	2.43 ± 0.09^{c}	78.33 ± 1.67^{c}	6.33 ± 0.33^{bcd}	3.20 ± 0.06^{c}
		3.0	66.67 ± 1.67^{c}	5.00 ± 0.58^{bcd}	1.70 ± 0.11^{d}	65.00 ± 2.89^{d}	6.00 ± 1.15^{bcd}	2.67 ± 0.12^{d}

Values of mean ± SE represent data of three repeated experiments with 20 replicates. Same *letter* within *columns* not significantly different at $P \leq 0.05$, using Duncan's multiple range test (DMRT). *IBA*, indole-3-butyric acid; *IAA*, indole-3-acetic acid, *NAA*, 1-naphthaleneacetic acid

Figure 2. *Ex vitro* rooting in *Hildegardia populifolia* (Roxb.) Schott. & Endl. (*a*) Microshoot treated with 200 μM indole-3-butyric acid transplanted in soilrite potting substrate. (*b*) *Ex vitro* rooted microshoot after 4 wk of culture. (*c*) Exposed view of above culture. (*d*) Regenerated plantlet hardened in earthen pots containing garden soil. (*e*) Successfully established an acclimatized plant.

Table 3. Effect of different auxins on *ex vitro* root induction in *Hildegardia populifolia* (Roxb.) Schott & Endl. after 4-wk culture

Plant growth regulators (μM)			% response	Mean number of roots per explant	Mean root length (cm)
IAA	IBA	NAA			
0.0	0.0	0.0	00.00 ± 0.00^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
100			00.00 ± 0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{h}
150			00.00 ± 0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{h}
200			00.00 ± 0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{h}
250			36.67 ± 1.67^{e}	2.00 ± 0.58^{def}	1.50 ± 0.17^{f}
300			28.33 ± 1.67^{f}	1.33 ± 0.33^{f}	1.10 ± 0.15^{g}
	100		00.00 ± 0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{h}
	150		53.33 ± 1.67^{c}	2.67 ± 0.67^{bcde}	3.20 ± 0.15^{d}
	200		91.67 ± 1.67^{a}	5.33 ± 0.33^{a}	4.77 ± 0.07^{a}
	250		70.00 ± 2.89^{b}	3.67 ± 0.33^{b}	4.33 ± 0.12^{b}
	300		66.67 ± 1.67^{b}	3.33 ± 0.67^{bc}	3.57 ± 0.09^{c}
		100	00.00 ± 0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{h}
		150	00.00 ± 0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{h}
		200	43.33 ± 3.33^{d}	1.67 ± 0.33^{ef}	2.63 ± 0.14^{e}
		250	65.00 ± 2.89^{b}	3.00 ± 0.58^{bcd}	3.67 ± 0.09^{c}
		300	35.00 ± 2.89^{e}	2.33 ± 0.67^{cdef}	2.70 ± 0.06^{e}

The values of mean ± SE represent data of three repeated experiments with 20 replicates each. The same *letter* within *columns* is not significantly different at $P \leq 0.05$, using Duncan's multiple range test (DMRT). *IBA*, indole-3-butyric acid; *IAA*, indole-3-acetic acid; *NAA*, 1-naphthaleneacetic acid

Genetic fidelity Uniformity in the genetic makeup of the regenerants as compared with the *ex vitro* grown mother plant is of immense practical importance in clonal propagation. Genetic fidelity of regenerants has been used to identify and remove somaclonal variants to maintain the genetic uniformity of the plants. Genetic fidelity of the regenerated plantlets was assessed using RAPD and ISSR DNA molecular markers. Mother plant and nine *in vitro* raised plantlets were randomly selected from the population of healthy growing plantlets and were subjected to molecular analysis. Among the set of ten RAPD primers screened from Kit OPL, nine primers produced clear, distinct, and reproducible bands (Table 4). Out of these, OPL − 5 produced a maximum of three monomorphic bands. All the amplified bands with respect to the molecular markers were scored between 100 and 1000 bp (Fig. 4*a*).

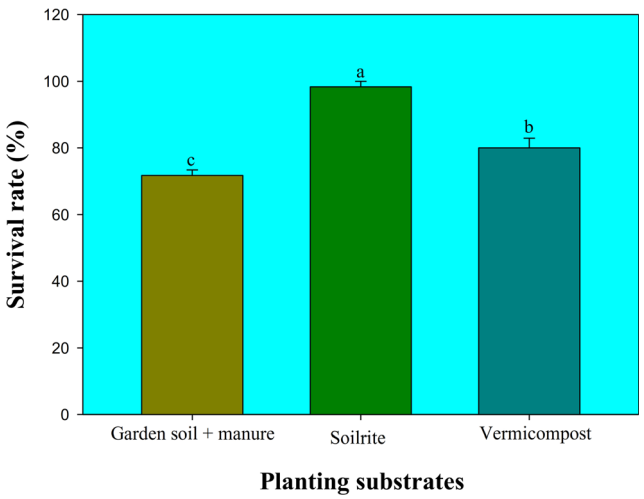

Figure 3. Effect of different planting substrates on survival rate (%) of regenerated plantlets of *Hildegardia populifolia* (Roxb.) Schott. & Endl. during acclimatization.

Table 4. Amplification products generated with randomly amplified polymorphic DNA (RAPD) markers among mother plant and *in vitro* raised plantlets of *Hildegardia populifolia* (Roxb.) Schott & Endl.

S. no.	Name of primers	Primer sequence (5′-3′)	No. of bands
1.	OPL - 01	GGCATGACCT	1
2.	OPL - 02	TGGGCGTCAA	2
3.	OPL - 03	CCAGCAGCTT	2
4.	OPL −04	GACTGCACAC	1
5.	OPL - 05	ACGCAGGCAC	3
6.	OPL - 06	GAGGGAAGAG	2
7.	OPL - 07	AGGCGGGAAC	0
8.	OPL - 08	AGCAGGTGGA	2
9.	OPL - 09	TGCGAGAGTC	1
10.	OPL - 10	TGGGAGATGG	1

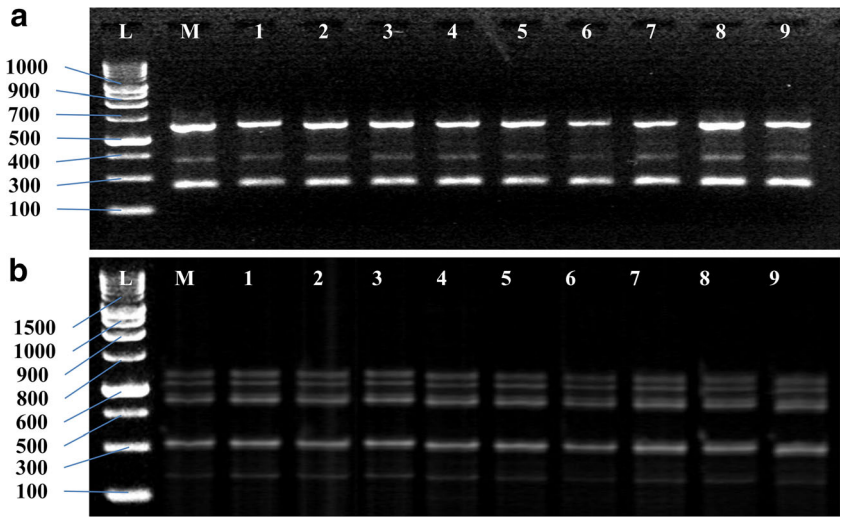

Figure 4. DNA fingerprinting of mother plant and micropropagated plants of *Hildegardia populifolia* (Roxb.) Schott. & Endl. Obtained through (*a*) random amplified polymorphic DNA (RAPD) primer (OPL - 5) and (*b*) inter simple sequence repeats (ISSR) primer (UBC - 848) showing monomorphic banding pattern. 1 to 9 *lanes* show the DNA sample from regenerated plants and *M* shows the DNA from mother plant while L represent DNA ladder.

Molecular profiling of regenerated plantlets were screened using ten UBC primers for ISSR markers, out of which nine primers produced clear and distinct bands (Table 5). UBC − 848 scored the maximum number of five monomorphic bands. In this case, more bands were depicted as compared with RAPD primers which were scored between 100 and 1500 bp of the molecular markers (Fig. 4*b*). The monomorphic banding pattern obtained from both molecular markers clearly depicted uniformity in the genetic makeup of the regenerated plants and the *H. populifolia* mother plant. Comparable results were obtained from *Inula royleana* (Amin *et al.* 2018), *Decalepis salicifolia* (Ahmad *et al.* 2018b), *Prunus cerasifera* (Nasri *et al.* 2019), and *Hemidesmus indicus* (Yadav *et al.* 2019) using RAPD and ISSR markers to confirm the genetic homogeneity.

Table 5. Amplification products generated with inter simple sequence repeats (ISSR) markers among mother plant and *in vitro* raised plantlets of *Hildegardia populifolia* (Roxb.) Schott & Endl.

S. no.	Name of primers	Primer sequence (5′-3′)	No. of bands
1.	UBC - 812	$(GA)_8A$	3
2.	UBC - 814	$(CT)_8A$	4
3.	UBC - 818	$(CA)_8G$	2
4.	UBC - 825	$(AC)_8T$	1
5.	UBC - 827	$(AC)_8G$	4
6.	UBC - 836	$(AG)_8YA$	3
7.	UBC - 848	$(CA)_8RG$	5
8.	UBC - 855	$(AC)_8YT$	1
9.	UBC - 868	$(GAA)_6$	2
10.	UBC - 880	$(GGGGT)_3G$	0

Ultra-structural study of *in vitro* and acclimated leaves The anatomy of *in vitro* and acclimated leaves of *H. populifolia* were compared using SEM. The control conditions during *in vitro* culture resulted in abnormal anatomy associated with low light irradiance, gaseous exchange, and nutrients availability in culture vessels. A remarkable change was observed regarding the plant's anatomy when transferred to greenhouse conditions. Electron micrographs of the abaxial surface of *in vitro* derived leaves showed highly constricted surface bearing few stomata which were deep seated and mostly closed (Fig. 5.*1a*), having unhealthy guard cells and uneven stomatal aperture (Fig. 5.*1b*). During acclimatization, regenerated plantlets slowly stabilized and achieved normal growth. Leaf characterization at this stage is a good indicator of plant health. SEM analysis of abaxial leaf surface of *ex vitro* acclimatized plantlets showed relaxed surface with numerous well-defined stomata (Fig. 5.*2a*), having clear aperture and functional guard cells as they possess both closed and open type of stomata (Fig. 5.*2b*). Related findings were also reported for *Ceratonia siliqua* (Shahzad *et al.* 2017) and *Leucospermum cultivars* (Suarez *et al.* 2019).

GC-MS analysis Previous work regarding the phytochemical analysis using different chromatographic techniques of *H. populifolia* has been reported by Saradha and Paulsamy (2013) and Gritto *et al.* (2015). However, our study is the first report with comparative analysis of mother and *in vitro* raised plantlet to examine the chemical uniformity and exploring the pharmacologically important metabolites by using GC-MS. Several compounds having pharmacological value in major and minor concentrations have been reported (Huang *et al.* 2009; Reddy and Couvreur 2009; Güneş 2013; Zalkhani and Moazedi

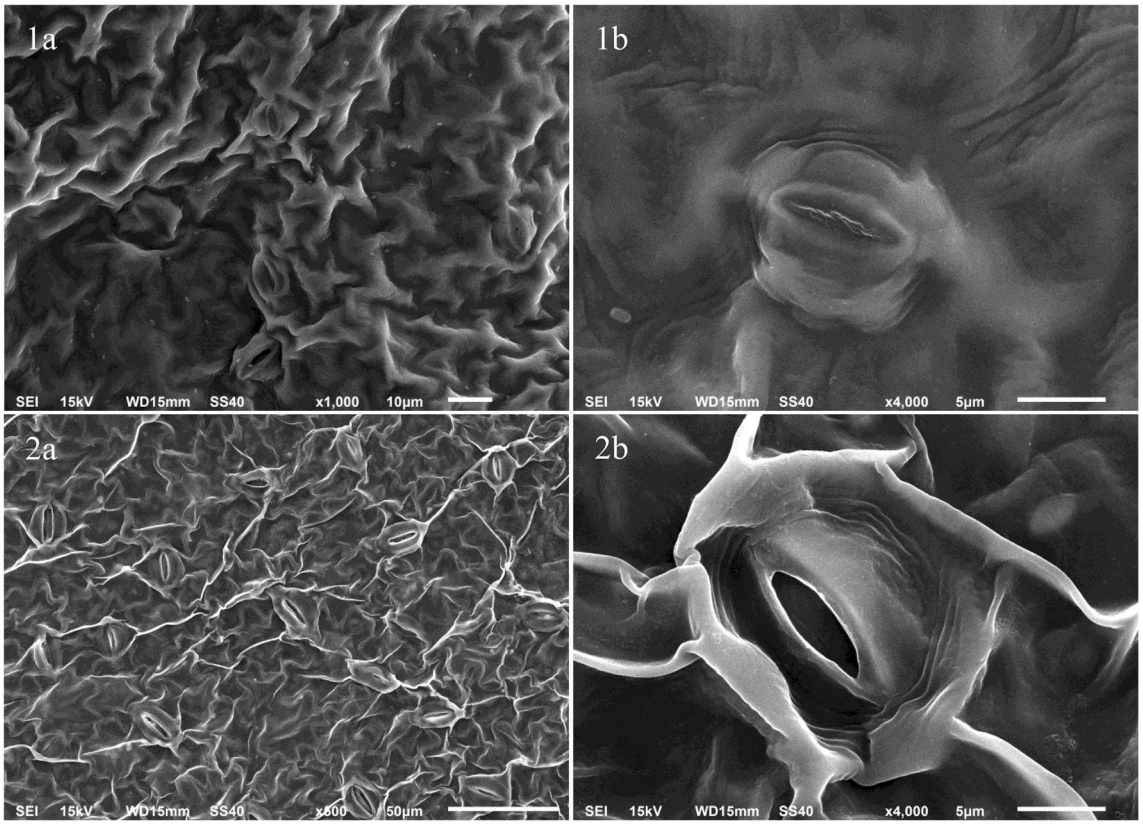

Figure 5. Scanning electron microscope (SEM) examination of *in vitro* derived leaves of *Hildegardia populifolia* (Roxb.) Schott. & Endl. (*1a*) abaxial surface of leaf showing deep seated closed stomata and (*1b*) unhealthy guard cells and uneven stomatal aperture of deformed stomata. SEM examination of leaf taken from acclimatized regenerated *H. populifolia* plantlet (*2a*) abaxial surface of leaf showing numerous well-developed stomata and (*2b*) open stomata having clear aperture.

2020). More than forty compounds have been identified when GC-MS was performed for both mother and *in vitro* regenerated plants (Supp. Table 1 & 2, Figs. 6 and 7). Methanol was found to be the optimal solvent for extraction. The names of specific compounds, their retention time (RT), concentration (area and area %), molecular formula, and molecular weight (MW) from mother and *in vitro* regenerated plant are shown in Tables 6 and 7 respectively. Compounds such as squalene, methyl commate D, vitamin E, and 9-octadecenamidewere found in higher percentage from regenerated plants in comparison with the mother plant. The higher quantity of compounds from regenerated plants might be due to stress associated with *in vitro* culture. The combination of chromatography and mass spectrometry has been widely used for the screening of metabolites in several medicinal plants. In *Cassia angustifolia* leaves, Parveen *et al*. (2016) reported GC-MS data which showed the presence of 45 different phytocomponents on the basis of comparison of the mass spectrum of each constituent with NIST and Wiley libraries. Ahmad *et al*. (2017) also reported that methanolic leaf extract of *Decalepis arayalpathra* was rich in phytochemicals in comparison with the other polar solvents. Additional reports described GC-MS results from medicinal plant species including *Zhumeria majdae* (Fallah *et al*. 2019) and *Hemidesmus indicus* (Yadav *et al*. 2019).

Physiological and biochemical studies - Total chlorophyll and photosynthetic rate evaluation During the first week, acclimatization of regenerated plants, reduction in total chlorophyll content (1.03 ± 0.14 mg g^{-1} to 0.57 ± 0.09 mg g^{-1}) was observed (Fig. 8*A*). A gradual increase occurred during 4 wk of acclimatization and reached 1.80 ± 0.06 mg g^{-1} which correspond to increased photosynthetic efficiency (Fig. 8*A*). The phenomenon of reduction in chlorophyll content during the initial d of acclimatization was attributed to the poorly developed chloroplast with disordered grana (Pospóšilová *et al.* 1999). When the days of acclimatization increased, the total chlorophyll content also increased in micropropagated plants. A similar observation was also reported in *Decalepis hamiltonii* (Sharma *et al*. 2014) and *D. arayalpathra* (Ahmad *et al*. 2018a).

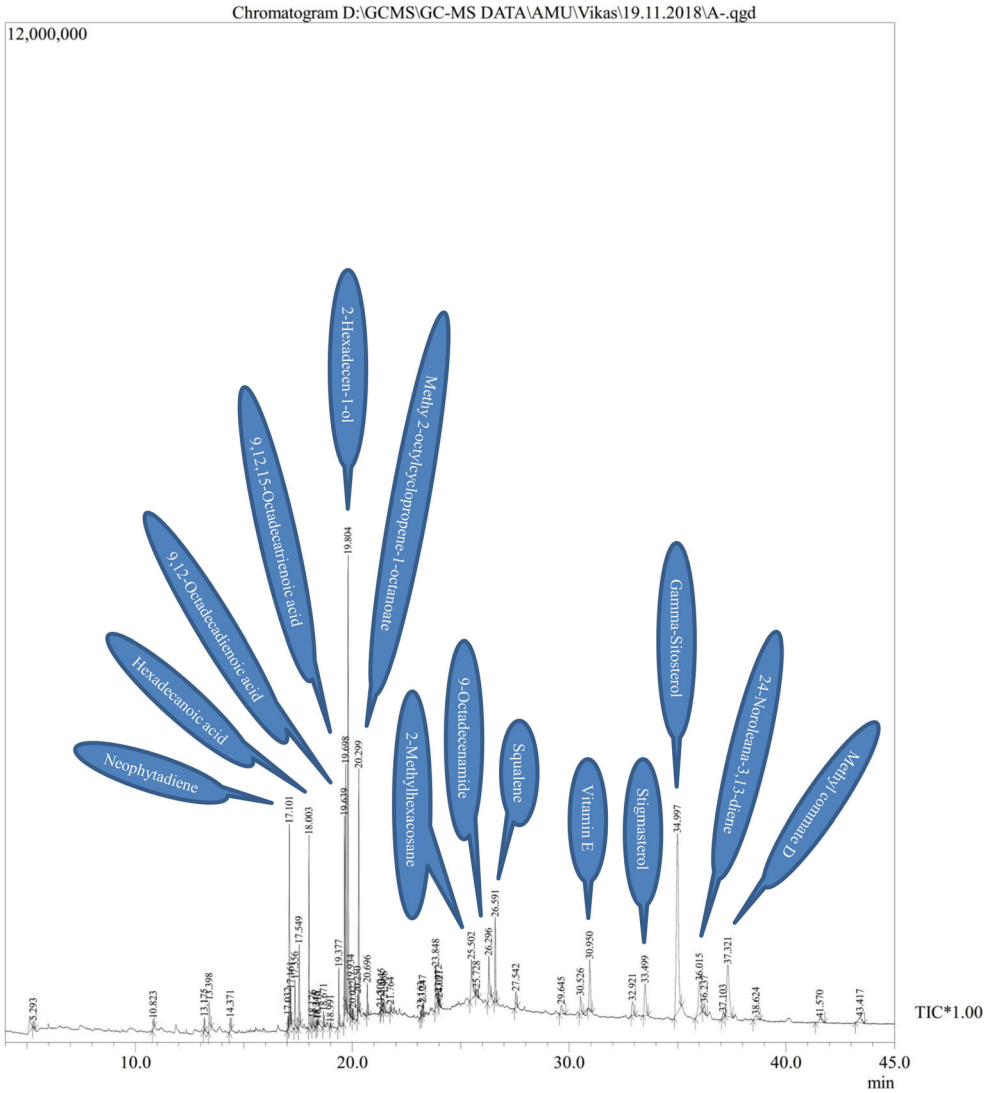

Figure 6. GC-MS chromatogram of methanolic leaf extract of mother plant of *Hildegardia populifolia* (Roxb.) Schott. & Endl.

In vitro grown plants experience low CO_2 and PPFD (photosynthetic photon flux density) with high air humidity (Van Huylenbroeck *et al.* 1998). A sudden change in the environmental conditions during regenerated plant transfer to *ex vitro* conditions results in environmental stress during plant acclimatization (Van Huylenbroeck *et al.* 1998). During the initial days of acclimatization, plants' primary challenges are water loss due to poor stomatal functioning and absence of a thick cuticle. Therefore, a decrease in PN was recorded during the first wk of acclimatization (Fig. 8*B*). However, as the days of acclimatization increased, PN increased to its maximum value of 6.57 ± 0.07 μmol CO_2 m^{-2} s^{-1} after 4 wk of acclimatization. Similar variation in PN was observed in other medicinal plants (Pospóšilová *et al.* 1988; El-Mahrouk *et al.* 2016; Yadav *et al.* 2019).

Antioxidant enzymes analysis When plantlets are transferred from *in vitro* to *ex vitro* conditions, various stresses occur which promote the production of reactive oxygen species (ROS; Batková *et al.* 2008). Plant cells induce the production of various antioxidant enzymes such as SOD, CAT, GR, and APX to overcome the harmful effects of ROS (Mitrović and Bogdanović 2008; Kayihan *et al.* 2012; Xu *et al.* 2012). Antioxidant enzymes could reduce the oxidative damage to the plant by ROS when acclimating to a new environment (Yan 2009).

H. populifolia SOD activity increased from 1.70 ± 0.11 to a maximum of 4.17 ± 0.03 unit mg^{-1} protein after 21 d and then decreased to 3.63 ± 0.09 unit mg^{-1} protein after 28 d of acclimatization (Fig. 8*C*). Superoxide is converted to H_2O_2 and O_2 by the activity of SOD, which played a significant role in the prevention of membrane oxidation and damage to biological molecules. The process of mitigating ROS is carried out by a series of affected membrane and stomatal enzymes, including SOD and APX, at the acceptor site of photosystem I (Scalet *et al.* 1995). Ahmad *et al.* (2018a) reported similar results in *Decalepis arayalpathra* where SOD activity increased

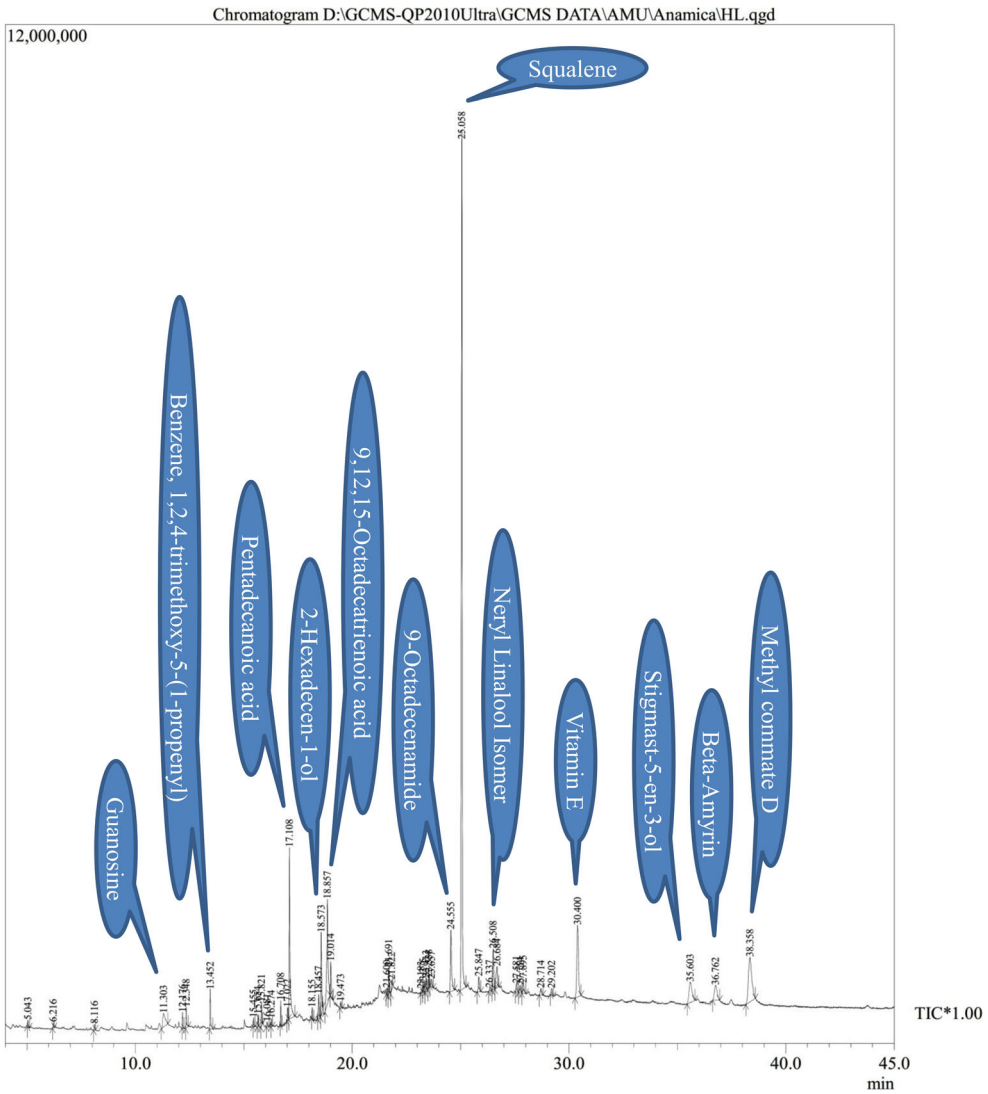

Figure 7. GC-MS chromatogram of methanolic leaf extract of 4-wk-old *in vitro* derived plantlet of *Hildegardia populifolia* (Roxb.) Schott. & Endl.

after 7 d of acclimatization and then decreased after 28 d of acclimatization.

The decreased H_2O_2 levels suggest successful acclimatization. CAT reduced the effect of H_2O_2 in peroxisomes by its conversion into H_2O and O_2. A steady and gradual increase in CAT activity was observed which peaked at 321.67 ± 1.67 unit mg^{-1} protein after acclimatizing for 28 d (Fig. 8*D*). *H. populifolia* CAT activity increased in accordance with the findings in *Decalepis arayalpathra* and *D. salicifolia* (Ahmad *et al.* 2018a, 2018b). The photorespiratory detoxification of H_2O_2 into O_2 and H_2O in mitochondrial electron system is represented by increase in both CAT and SOD activities (Scandalios 1990).

GR and APX are two important enzymes of the ascorbate glutathione cycle involved with chloroplast-based detoxification of ROS *via* the Mehler pathway (Foyer and Mullineaux 1998). *H. populifolia* GR and APX enzyme activity increased gradually with the increase in days of acclimatization and peaked after 28 d at 9.40 ± 0.06 and 6.60 ± 0.06 unit mg^{-1} protein, respectively, (Fig. 8*E*, *F*) functioning in the cytosol, chloroplast, vacuoles, and apoplast (Asada 1999). The elevation of GR and APX during plant acclimatization was previously reported in *Cynara scolymus* (Pérez-Jiménez *et al.* 2015) and *Hemidesmus indicus* (Yadav *et al.* 2019).

Conclusions

A comprehensive protocol for large-scale micropropagation of *H. populifolia* uses a comparative nutrient media study and *ex vitro* physio-chemical analysis of regenerants. The true-to-type nature of the micropropagated plant was verified using DNA-based molecular markers. An active rise and fall in photosynthetic pigments and biochemical enzymes revealed the significance of plant adjustment during acclimatization to soil and hence can be correlated with the maximum survivability of the regenerated plants.

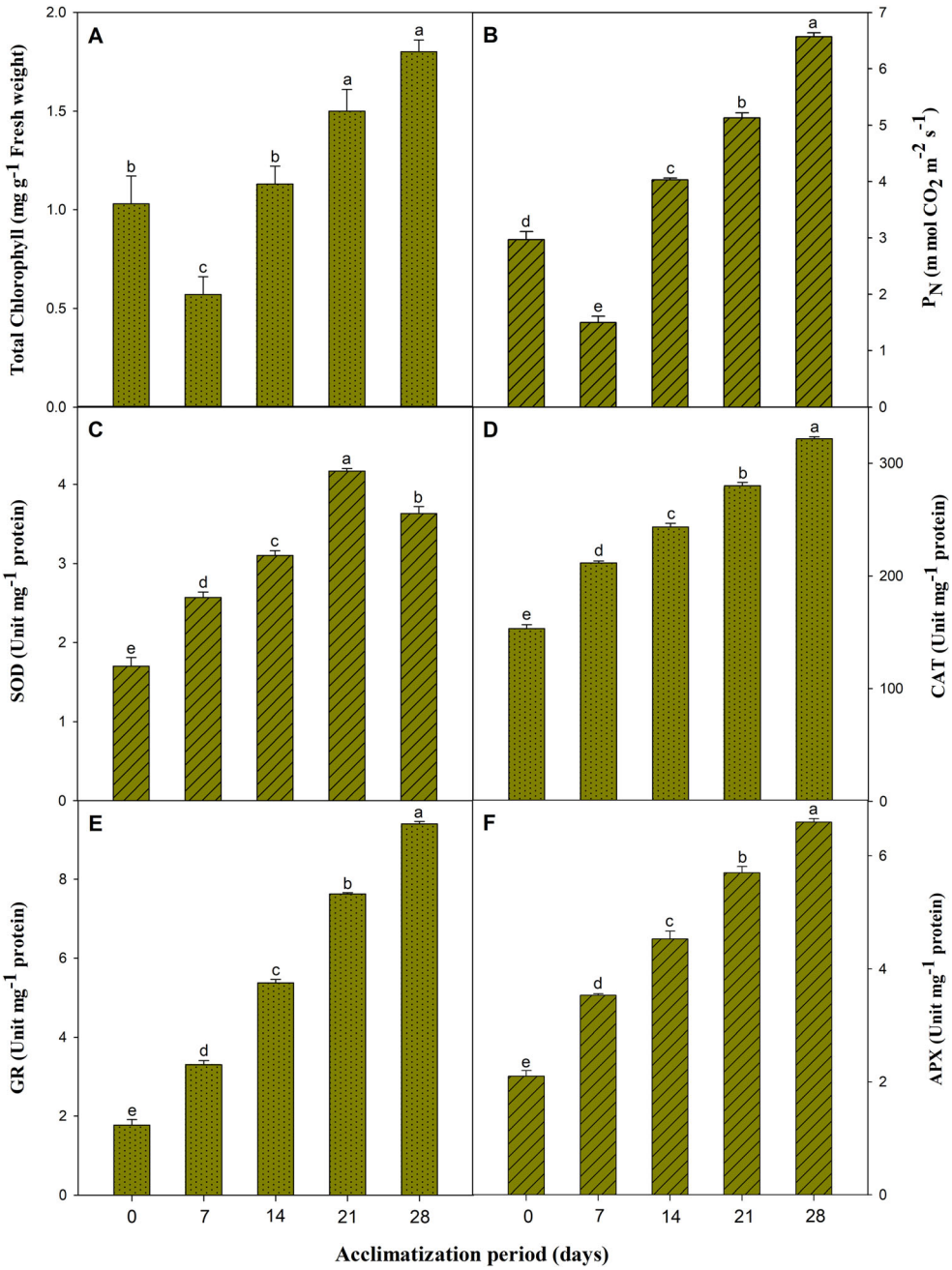

Figure 8. Changes in total chlorophyll content (*A*) net photosynthetic rate; P_N (*B*) and antioxidant enzymes activity (C-SOD, D-CAT, E-GR, F-APX) in *in vitro* raised plantlets of *Hildegardia populifolia* (Roxb.) Schott. & Endl. during acclimatization. The value of *bars* represents mean ± SE. The same *letter* above the *bars* are not significantly different at $P \leq 0.05$, using Duncan's multiple range test (DMRT).

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