Journal of Agricultural Technology 2012 Vol. 8(5): 1727-1744
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

In vitro micropropagation of *Hildegardia populifolia* (Roxb.) Schott & Endl., an endangered tree species from Eastern Ghats of Tamil Nadu, India

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A.R. Lavanya, S. Muthukrishnan, V. Kumaresan, J.H. Franklin Benjamin and M.V. Rao (2012) *In vitro* micropropagation of *Hildegardia populifolia* (Roxb.) Schott & Endl., an endangered tree species from Eastern Ghats of Tamil Nadu, India. Journal of Agricultural Technology 8(5): 1727-1744.

Hildegardia populifolia (Roxb.) Schott & Endl., an endangered tree species was investigated for its *in vitro* regeneration potential using axillary bud and apical bud explants obtained from 2 month old *in vitro* seedlings. Shoot induction response was best on MS medium supplemented with BA (1.0 mg/l) + KIN (0.5 mg/l) where 100% explants responded with an average shoot number (9.0) and shoot length (3.1) after 25 days of culture. However, multiplication and elongation was recorded when BA (1.0 mg/l) + KIN (0.5mg/l) treated shoot clusters were transferred to MS medium contain GA_3 (1.0 mg/l). Rhizogenesis was observed on MS medium supplemented with IBA (2mg/l), plantlets produced through micropropagation were hardened with the survival success of 100%. The efficient plantlet regeneration protocol developed would aid *ex situ* conservation of this endangered species.

Introduction

Hildegardia is a genus of 11 species of Malvaceous trees; placed in sub family Sterculioideae/tribe Sterculieae. It has a pantropical distribution. Locally known as "Malaipuvarasu" in Tamil, is one of the endemic and endangered tree species of the Eastern Ghats of peninsular India. It was earlier known to be represented by a sole surviving population comprising about 20 trees in Kalarayan Hills of Tamil Nadu (Ahmedullah, 1990). Its conservation status has been variously assessed as Critically Endangered (Sarcar and Sarcar, 2002), Endangered (Ahmedullah, 1990; Walter and Gillet, 1998), Vulnerable (Reddy *et al*., 2001). The World Conservation Monitoring Centre (1998) assessed the conservation status of this species as Critically Endangered. Rao *et al*. (1998)

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recognized five subpopulations of this endangered species in Rayalaseema District of Andhra Pradesh. It is a xerophytic tree, very resistant to drought and grows on the steep, rocky, dry, and poor stony soils. This tree has both economic and ethnic values. The fiber obtained from this tree is economically valuable. Ligno-cellulose fibers often possess good mechanical properties at low density, which makes them fit as reinforcements in the preparation of natural fiber reinforced polymer composites which is referred as "Green Composites."(Varada Rajulu *et al.,* 2001). Many reports indicate that *Hildegardia* fiber can be favorably considered as reinforcement in green composites (Varada Rajulu *et al.,* 2005; Li *et al*., 2004). The stem bark is also used for dog bite and cure malaria (Reddy *et al*., 2001). Many secondary metabolites viz. bergenin, lupin 3-one, β-sitosterol, lanosterol, n-octacosanol, stigmasterol, phytosterols, caffeic acid (hydrocarbon), amides, flavanoids, tannins, furanocoumarin derivatives namely bergapten and bergapton have been isolated from different parts of the tree (Kirana *et al*., 2009), while many more may still be unidentified. Various parts of the tree are used in various ayurvedic commercial preparations like Panchavalkal extract (http://www.padmavatiherbal. com/leucowincapsule02.htm), Lakshadi guggal (Baidyanath pharmaceuticals http://www.madanapalas.com/lakshadiguggul- p-533.html).

Conventionally, the tree is propagated by seeds, which remain viable for a few months. Also, the vegetative propagation by cutting is not efficient under varied climatic conditions. Because of its medicinal importance and potential, there is a need to carry out rapid mass propagation of the species. The *in vitro* culture can be the alternative for the continuous provisions of the woody plantlet stocks for large scale field cultivation (Ahmad and Anis, 2007; Beruto *et al*., 2004; Husain *et al*., 2008). Anuradha and Pullaiah (2001) reported callus induction from shoot explants of *Hildegardia populifolia* on MS+BA (2.0 mg/l). In the present paper, we report the accomplishment of a highly efficient micropropagation system.

Materials and methods

Establishment of aseptic intact seedlings

In this study, the dry follicles of *Hildegardia populifolia* were collected in the month of November 2004 from Kalrayan hills (300-600 m), Eastern Ghats of Tamil Nadu, India. Healthy seeds were removed from follicles by water floatation method for further treatment. To raise the seedling, three types of treatments were adopted. In the first treatment, seeds were directly sown in the pots containing sand and soil and irrigated frequently. In the second treatment,

the seeds were treated with Conc. H_2SO_4 for different time duration (5, 15, 30, 45, 60 and 90 min) and the seeds were scrubbed over rough surface to soften the hard outer coat and washed thoroughly in running tap water. Then, the seeds were sown in the soil and sand mixture followed by proper irrigation. In the third treatment, seeds were treated with Conc. H_2SO_4 to soften the outer hard seed coat for different time duration (5, 15, 30, 45, 60 and 90 min). The acid treated seeds were scrubbed over rough surface and the seeds were washed thoroughly in running tap water. The seeds were soaked in sterile distilled water and placed in a rotary shaker for the uniform imbibitions of water by the softened seeds for 2 days to improve the breakage of seed coat. On the third day, the acid treated seeds were again washed thoroughly in sterile distilled water after 1-2 drops of liquid detergent (Teepol) for 5 min. The washed seeds were disinfected by rinsing in 70% ethyl alcohol for 5 minutes and followed by aqueous solution of 0.1% (W/V) mercuric chloride ($HgCl_2$) for 10 minutes.

Then, the seeds were rinsed 4-5 times with sterile distilled water. Sterilized seeds were aseptically inoculated in both MS medium supplemented with GA_3 (1.0 mg/l) and cotton soaked with sterile water. The inoculated seeds were incubated under optimal culture condition. The *in vitro* raised seedlings were transferred to earthen pots containing soil and sand (1:1) and maintained in the garden. The explants were collected from two months old *in vitro* derived seedling.

Surface sterilization

Axillary and apical bud explants (2 cm length) from healthy plants were initially washed with few drops of liquid detergent (Teepol) for 5 min followed by rinsing in running tap water. The washed explants were disinfected by rinsing in 70% ethanol for 3 min and with aqueous solution of 0.1 % (W/V) mercuric chloride for 5 min. Finally the explants were rinsed 4-5 times with sterile distilled water and cut into pieces each 0.5-1.0 cm long. Nodal segments containing the nodal region with axillary buds were inoculated on culture medium.

Micropropagation in vitro

Various types of 1-2 cm length explants (axillary and apical bud) were taken on 2 month old *in vitro* seedlings (Fig 3 a). They were surface sterilized and transferred individually to cultures containing 20 ml of MS solid medium. Benzylamino purine (BAP) and kinetin (KIN) 0.5 - 3 mg/L combined with GA_3 (0.1 to 3mg/l) was added to the medium. For each type of explant, a total of 25 explants per medium were defined. The tubes were stored to culture at a room

temperature of 27 ± 1ºC under a photoperiod of 16 h photoperiod with a light intensity of 35μΣ $m^{-2}S^{-1}$. A measurement at 25 days and another measurement at 45 days of incubation were taken for all the treatments. The measured parameters were the number of shoots and length of the growths.

Effect of additives and media

The axillary and apical bud segments with induced shoot buds on MS medium supplemented with BA (1.0 mg/l), KIN (0.5 mg/l) and GA_3 (1.0 mg/l) (the medium yielding maximum number of healthy looking shoot buds), were taken out from this medium. These were then used as the explants to study the influence of different additives (adenine, adenine sulphate, glutamine, sodium citrate) on shoot multiplication. The MS medium supplemented with BA (1.0 mg/l), KIN (0.5 mg/l) and GA_3 (1.0 mg/l) was further modified with different concentrations and combinations of adenine, adenine sulphate, glutamine, sodium citrate (10 -50 mg/l) for evaluating the effect of these additives on shoot proliferation and multiplication (Table 3). The number of shoots per explant and the length of the shoots were recorded after 30 days of culture. Every treatment contained 25 replicates and the experiment was repeated thrice. The shoots so multiplied were separated into clumps of 8-10 shoots after 30 days and sub-cultured on medium (giving maximum number of shoots, as observed from Table 1). This process was repeated for the study of the effect of different media (MS, WPM, B_5, mMS) on shoot multiplication (Fig. 2). Every treatment contained 25 replicates and the experiment was repeated thrice.

Rooting and transplantation of the regenerated plantlet

The individual shoots regenerated from shoot explant were transferred to rooting medium consisting of MS medium + auxins [IBA and NAA (0.1 – 3.0mg/l)]. Four weeks old *in vitro* raised plantlets with well developed roots were removed from the culture medium and roots were washed thoroughly under tap water. Plantlets were transferred to plastic containers and earthen pots containing mixture of sand: soil: vermiculite mix (1:2:1) maintained inside a culture room at 25 ± 2˚C and 35μΣ $m^{-2}S^{-1}$ light intensity provided by cool white fluorescent tubes and 70-80% relative humidity was maintained by covering the plant with polythene bags. Plantlets were watered with half-strength MS basal solution devoid of sucrose and meso-inositol at 3 days intervals for a period of 3 weeks. The acclimatization plantlets were then transferred to pots containing soil and kept under shade for another 8 weeks before transferring to the field.

Culture media and conditions

Four basal media were compared: Murashige and Skoog (MS) medium (1962); Woody Plant Medium (WPM) (Lloyd and McCown. 1980); B_5 medium (Gamborg *et al*., 1968); Murashige and Skoog medium modified (MMS) (Mathur *et al*., 1995). The basal medium was added with 30 g/l sucrose. Different concentrations of phytohormones (BA, KIN, and GA_3) were used. Media were solidified with Agar (3 g/l); pH was adjusted to 5.5 – 5.7 before autoclaving (120°C for 20 min). All the cultures were maintained in sterilized culture room at 26 ±2°C, under 16/8 h light regime provided by cool white fluorescent light (60 µmol-2s-1 light intensity) and with 55 - 60% relative humidity.

Experimental design and data collection

All the experiments were conducted as a randomized complete design. For each experiment, a minimum of 25 replicates were taken and repeated thrice. Observations of the culture were made every week and data related to shoot multiplication (frequency of response, number of shoots and shoot length) were collected 25 to 45 days after culture. Comparisons between treatments were made with Duncan's new multiple range test (DMRT) (Duncan, 1955).

Results and discussions

Micropropagation of woody and semi-woody trees is reported as problematic (Babaoglu *et al*., 2002) and to obtain a material without viruses is a long-term, complex process. To obtain stocks without viruses, it is necessary to use seeds without viruses as rootstocks and seedlings. For this purpose, many *in vitro* experiments have been conducted for economically and endangered important trees (Onay *et al*., 2000; Adıyaman *et al*., 2004; Tilkat *et al*., 2005; Isıkalan *et al*., 2008; Sambe *et al*., 2010; Kasumu *et al*., 2010; Girijashankar 2011). This study used axillary and apical bud of *Hildegardia populifolia*, which is an economical important and endangered woody tree, and the effects of different PGRs on *in vitro* micropropagation were investigated.

Seed germination

The test with tetrazolium chloride was 100% positive. All batches of 50 treated seeds belonged to group 1 of the scale of Moore. Therefore, these seeds were able to germinate when they were subjected to favorable conditions. They

thus seem viable and constitute seeds with very high probability of germination. For the control batch, no seed germination was recorded. As for the other treatments, there was a rate germination of 30% for the treatment of 5 min sulphuric acid and 45% for the treatments of 15 min. 70% and 85% in the respectively 30 and 45 min. The high germination (98%) was reached for the treatments of 60 min. The increase of time the germination rate was decreased (Fig. 1).

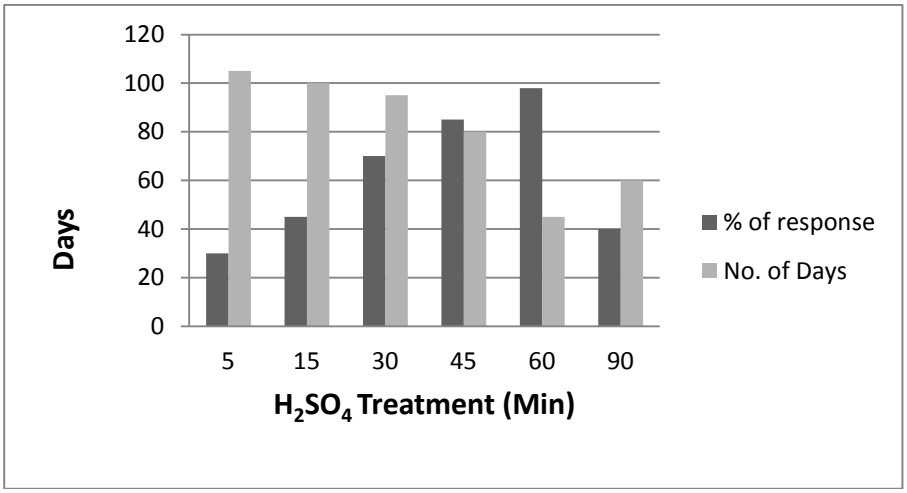

Fig. 1. Germination response of *Hildegardia populifolia* seeds treated with H_2SO_4

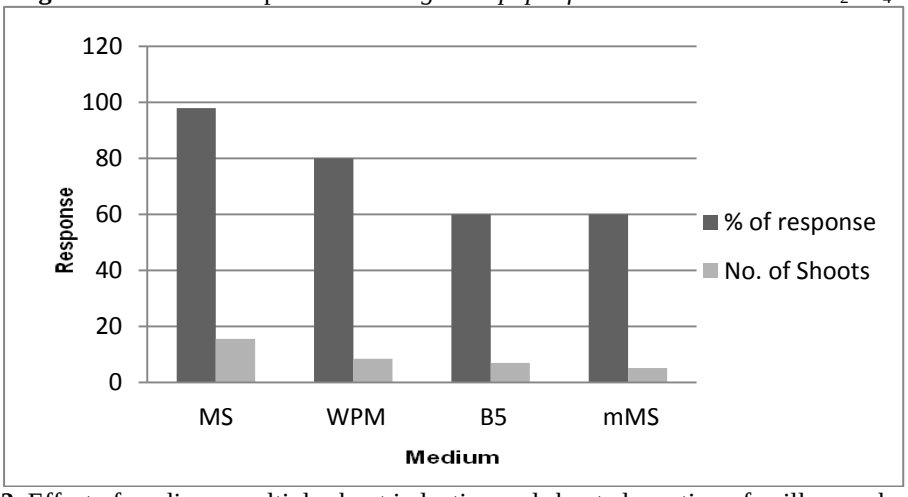

Fig. 2. Effect of media on multiple shoot induction and shoot elongation of axillary and apical bud explants of *Hildegardia populifolia* supplemented with BA (1.0mg/l) + KIN (0.5mg/l) + GA_3 (1.0mg/l) + glutamine (50 mg/l), after 45 days

Culture initiation

Most investigators working with trees have found that seeds and juvenile tissues are more amenable for *in vitro* manipulations than explants taken from trees in the adult growth phase (Purohit and Dave, 1996; Sunnichan *et al*., 1998). The development of primordial shoot from the axil of axillary and apical bud segment (especially in trees) is termed as bud break stage (BB). Multiple shoot formation was observed to be highest in MS fortified with 1 mg/L BAP and 0.5 mg/L KIN in both explant types (Table 1; Fig. 3 d). This composition is named as shoot induction medium (SIM). Both BB and multiple shoot induction occurred on SIM media, so there were no separate media compositions for these stages. Shukor *et al.* (2000) reported shoot initiation occurred from nodal explants of *Acacia auriculiformis* even at low concentrations of BA (0.1 to 0.5 mg/L). Likewise Oliveira *et al*. (2012) reported that BA did not promote better multiplication compared to the control in *Pinus taeda*. Hussain *et al*. (2008) obtained shoot initiation from aseptic seed cultures of *Sterculia urens* with 5µM TDZ.With regard to the combination study of the influence of the BA and KIN, the statistical analysis showed that the average number of shoots resulting from the axillary buds and apical buds.

Table 1. Multiple shoot induction of axillary and apical bud explants of 2 month old *in vitro* seedlings of *Hildegardia populifolia* grown on MS medium supplemented with cytokinins, after 45 days

Plant growth regulators (mg/l)	**Axillary bud**			**Apical bud**		
	Shoot bud induction (%)	Shoot number	Shoot length (cm)	Shoot bud induction (%)	Shoot number	Shoot length (cm)
BA						
0.5	93.3^{bc}	5.9^{fg}	1.4^{g}	78.6^{de}	4.3^{e}	2.1^{f}
1.0	100.0^{a}	7.0^{d}	1.5^{f}	85.3^{c}	5.0^{bc}	2.4^{de}
2.0	96.0^{b}	5.2^{h}	1.5^{f}	80.0^{d}	4.1^{f}	2.3^{e}
3.0	89.3^{c}	4.7^{i}	1.3^{h}	73.3^{f}	3.8^{h}	1.9^{g}
4.0	85.3^{d}	4.0^{j}	1.0^{i}	68.0^{g}	3.5^{i}	1.7^{h}
5.0	80.0^{e}	3.8^{jk}	0.8^{k}	64.0^{gh}	3.0^{j}	1.4^{j}
KN						
0.5	85.3^{d}	4.0^{j}	2.0^{d}	70.6^{fg}	3.0^{j}	3.1^{bc}
1.0	77.3^{ef}	3.5^{k}	1.7^{e}	66.0^{gh}	2.7^{k}	2.9^{c}
2.0	70.6^{g}	3.0^{l}	1.6^{f}	61.3^{h}	2.5^{l}	2.5^{d}
3.0	65.3^{h}	2.9^{lm}	1.4^{g}	58.6^{h}	2.1^{m}	2.0^{fg}
4.0	62.6^{hi}	2.5^{n}	1.1^{h}	52.0^{i}	1.9^{n}	1.7^{h}
5.0	58.6^{ij}	2.1^{n}	1.0^{i}	49.3^{j}	1.5^{no}	1.3^{k}
TDZ						
0.5	60.0^{i}	2.0^{no}	1.2^{h}	50.6^{ij}	1.8^{no}	2.1^{f}
1.0	54.6^{k}	1.9^{o}	1.1^{i}	45.3^{k}	1.7^{no}	2.0^{fg}
2.0	52.0^{kl}	1.9^{o}	0.9^{j}	38.6^{l}	1.6^{no}	1.7^{h}
3.0	48.0^{m}	1.7^{op}	0.8^{k}	36.0^{lm}	1.3^{p}	1.6^{i}
4.0	40.0^{n}	1.4^{p}	0.6^{l}	32.0^{n}	1.1^{p}	1.2^{k}
5.0	33.3^{o}	1.1^{p}	0.5^{m}	28.0^{n}	0.9^{p}	1.0^{l}
BA + KN						

1.0 + 0.05	96.0[b]	7.6[c]	2.0[e]	86.6[bc]	4.9[bc]	2.4[de]
1.0 + 0.1	98.6[ab]	8.1[b]	2.4[c]	92.0[b]	5.1[b]	3.0[bc]
1.0 + 0.5	100.0[a]	9.0[a]	3.1[a]	95.0[a]	6.0[a]	3.8[a]
1.0 + 1.0	90.6[bc]	6.8[de]	2.9[b]	89.3[bc]	4.7[d]	3.2[b]
1.0 + 2.0	86.6[cd]	6.1[f]	2.2[d]	84.0[c]	4.0[fg]	2.9[c]

Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

The best multiple shoot initiation was obtained on the MS medium supplemented with BA 1 mg/L+ KIN 0.5mg/L with a shoot number of 9.0 and 6.0 per explant, respectively, on the 25th day of culture. Cytokinins induce bud break by activation of meristems and cause shoot proliferation. The outgrowth of axillary buds is well correlated with the cytokinins level in the bud. It had been suggested that cytokinins independently regulate the growth of axillary buds and apical buds (Shimizu-Sato and Mori, 2001). Axillary bud was the ideal explant for shoot initiation and shoot multiplication of this species when compared to the apical bud. Shoot regeneration percentage was higher in axillary bud (100%) when compared to the apical bud (95%). GA_3 enhances the shoot multiplication efficacy in axillary bud explants than compared to the apical bud explants and shoot length was increased by 3.5 cm by the addition of 1mg/L GA_3 to the medium containing BA 1 mg/L+ KIN 0.5mg/L in the apical bud explants (Table 2, Fig. 3 e). Many studies have reported the effective elongation of in vitro shoots by the GA_3 in the medium (Anwar *et al*., 2008). Hussain *et al*. (2008) reported in *Sterculia urens,* the addition of GA_3 to the medium containing BA or TDZ had no stimulatory effect on the frequency of shoot regeneration and the number of shoots per seedling but it enhanced the shoot elongation. On the other hand, some other studies find transfer of shoots to low PGR (lower to the level on which shoot multiplication achieved) medium or PGR free medium as a successful way for elongation of shoots (Sivanesan and Jeong, 2010). Likewise Isikalan *et al*. (2008) reported the effect of the explants in *Amygdalus communis* the addition of 1mg/L GA_3 to the medium containing BA 1 mg/L+ KIN 0.5mg/L had no stimulatory effect on the frequency of shoot regeneration and the number of shoots per seedlings but it enhanced the shoot elongation. Similar result has been reported in *Murraya koenigii* (Bhuyan *et al*., 1997).

Table 2. Multiple shoot induction and shoot elongation of axillary and apical bud explants of 2 month old *in vitro* seedlings of *Hildegardia populifolia* grown on MS medium supplemented with BA (1.0 mg/l), KN (0.5mg/l) and GA_3, after 45 days

GA_3 (mg/l)	Axillary bud			Apical bud		
	Shoot bud induction (%)	Shoot number	Shoot length (cm)	Shoot bud induction (%)	Shoot number	Shoot length(cm)
0.1	80.0^{d}	6.1^{cd}	3.5^{cd}	72.0^{c}	6.0^{c}	5.7^{c}
0.5	86.6^{b}	7.8^{bc}	4.0^{c}	76.6^{ab}	6.9^{b}	5.9^{bc}
1.0	90.0^{a}	10.5^{a}	5.2^{a}	80.0^{a}	8.2^{a}	6.5^{a}
2.0	84.0^{bc}	8.3^{b}	4.8^{ab}	69.3^{cd}	6.5^{bc}	6.0^{b}
3.0	78.6^{de}	7.0^{c}	3.0^{cd}	62.6^{e}	5.2^{d}	5.1^{d}

Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

Influence of different additives on shoot multiplication

In order to prevent the premature leaf fall and to further enhance the shoot multiplication, different additives viz. adenine, adenine sulphate, glutamine, sodium citrate were supplemented, in different concentration and combinations, to the MS medium (BA (1.0 mg/l) + KIN (0.5mg/l) + GA_3 (1.0 mg/l)). The inclusion of adenine to the shoot multiplication medium exhibited no significant effect on shoot multiplication; however, a significant influence on premature leaf fall was observed. Adenine in the form of ADS can stimulate cell growth and shoot multiplication probably by acting as organic nitrogen source and/or acting as pre-cursor for natural cytokinin synthesis. Adenine sulphate (ADS) was added in various concentrations (10-50 mg/l), the shoot multiplication was found to increase significantly when compared to the adenine. The addition of ADS played an effective role to enhance the conversion procedure in our study. The result demonstrated the role of auxin-cytokinin which successfully promoted the conversion in an enhanced mode, in the presence of ADS. According to Gantait and Mandal (2010), ADS acts as an elicitor or enhancer of growth in combination or synergism with endogenous or exogenously supplemented PGRs. Similar effects of ADS have been reported in *Petrocarpus marsupium* also (Husain *et al*., 2008). The promotive role of ADS for shoot multiplication in *Ficus religiosa* has also been reported by Deshpande *et al*. (1998). Sivanesan and Jeong (2007), tested the effects of Adenine sulphate on shoot bud proliferation in *Sida cordifolia*, the media contain 1.0 mg/l in shoot multiplication medium did not improve shoot induction frequency but did favour shoot proliferation. The effect of the sodium citrate had no

significant effect on shoot multiplication. The inclusion of glutamine at different concentrations (10-50 mg/l) to the SM, having 50 mg/l glutamine, increased the shoot multiplication as well as shoot length as compared to that on SM with 10 mg/l adenine only (Table 3). The most suitable concentration of glutamine was 50 mg/l, showing nearly 0.5 times increase in the shoot multiplication. Shoots exhibited a significant increase in length (2–3 cm) on multiplication medium after 3–4 weeks of culture. However severe leaf fall and yellowing of leaf and stem was observed on medium lacking glutamine. The early leaf fall could effectively be controlled, throughout this study, by the use of glutamine (50 mg/l). Glutamine is reported to promote the shoot proliferation in many plant species like *Vitex negundo* (Stephen *et al*., 2010), *Ficus religiosa* (Siwach and Gill, 2011). The efficacy of organic nitrogen source particularly glutamine for multiplication and maintenance of healthy *in vitro* tissue for long time periods have been reported by many workers (Sanjaya *et al*., 2005; Green *et al*., 1990; Ogita *et al*., 2001; Vasudevan *et al*., 2004). This shows the necessary requirement of this organic nitrogen source for long term maintained cultures of *Hildegardia populifolia*. This exhibited the effective role of glutamine in shoot multiplication as compared to that of adenine, ADS and sodium citrate, as reported above (Table 3). The shoots exhibited good vigour as well proper leaf expansion and shoot elongation. In the present study, glutamine promoted the shoot multiplication and was more effective compare to adenine and ADS.

Table 3. Multiple shoot induction and shoot elongation of axillary and apical bud explants of 2 month old *in vitro* seedlings of *Hildegardia populifolia* grown on MS medium supplemented with BA (1.0 mg/l), KIN (0.5mg/l) and GA_3 (1.0 mg/l) and additives, after 45 days

Additives (mg/l)	**Axillary bud**			**Apical bud**		
	Percentage of response (%)	Shoot number	Shoot length (cm)	Percentage of response (%)	Shoot number	Shoot length (cm)
Adenine						
10	70.0^{hi}	4.2^{g}	3.8^{ef}	55.0^{g}	3.4^{g}	4.0^{e}
25	65.3^{j}	3.5^{gh}	3.0^{h}	50.6^{h}	3.0^{gh}	3.8^{ef}
50	58.6^{k}	2.9^{h}	2.8^{i}	46.6^{i}	2.5^{h}	3.1^{g}
ADS						
10						
25	86.6^{d}	7.0^{e}	3.9^{ef}	72.0^{c}	6.9^{d}	4.0^{e}
50	90.0^{bc}	8.0^{d}	4.4^{c}	75.0^{b}	7.5^{cd}	4.7^{c}
Glutamine	81.3^{f}	7.3^{de}	4.0^{e}	69.3^{cd}	6.4^{de}	3.9^{ef}
10						

25	85.3^{de}	9.8^{c}	4.3^{cd}	69.3^{cd}	7.7^{c}	5.2^{b}
50	90.6^{b}	12.5^{b}	5.0^{b}	74.6^{bc}	9.8^{b}	6.0^{b}
	95.0^{a}	15.6^{a}	5.4^{a}	80.0^{a}	13.2^{a}	6.8^{a}
Sodium citrate						
10						
25	77.3^{g}	6.0^{ef}	3.5^{g}	57.3^{fg}	4.3^{f}	3.7^{ef}
50	80.0^{fg}	6.5^{ef}	3.8^{ef}	65.3^{e}	5.8^{e}	4.5^{cd}
	72.0^{h}	5.7^{f}	3.0^{h}	60.0^{f}	5.0^{ef}	4.0^{e}

Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

Rooting and acclimatization

Rooting of the *in vitro* regenerated shoots was not observed on PGRs free medium. Hence the elongated shoots were transferred to semi-solid rooting medium, supplemented with different combinations of IAA, NAA and IBA, along with 50 mg/l glutamine (to avoid leaf fall) (Table 4). Rooting was induced with different frequencies in different media. More than 85% shoots rooted well on 2.0 mg/l IBA. Maximum frequency of root induction 98.6% in axillary bud explants and 90% root induction in apical bud explants was observed on the 2.0 mg/l IBA. The same medium also resulted in significantly higher mean number of roots per shoot and the roots obtained were also sufficiently long on it, so this medium was considered as optimal rooting medium. The best rates of rooting were obtained on the microplants resulting from the axillary bud (98.6%) after an induction rhizogene of 24 h in the presence of NAA 0. 5 mg.L -1 (Fig. 3 f). It is nearly same for those formed starting from the apical bud (97.3%) after an induction rhizogene of 48 h with NAA (0.5 mg/L). The best root elongations were obtained after an induction with the IBA (3.0 mg/l). This hormone has always been a potential auxin that induces rooting in *in vitro* regenerated shoots (Iriondo *et al.*, 1995; Rajore and Batra, 2005; Rajeswari and Paliwal, 2008). Therefore, the rooting at *H.populifolia* is dependent on the hormonal treatment and the NAA seems to be better than the IBA to induce rooting. IAA did not aid rhizogenesis in *H. populifolia* the results of which are not shown here. Indeed, NAA is more favourable to the rooting of *in vitro* plants of *Acacia senegal* (Badji *et al*., 1991), *Lotus alpinus* (Laberche *et al*., 1995), *Gleditsia triacanthos* (Basbaa *et al*., 1993), *Citrus jambhiri* (Savita *et al*., 2012). On the other hand, Sané *et al.* (2001) had obtained a rate rooting of 80% on *Acacia tortilis* subsp. *raddiana* after a treatment with IBA compared to the NAA. Altaf (2006) in Kinnow tree, Gokhale and Bansal (2009) in *Oroxylum indicum* obtained the rooting of the developed shoots in half strength MS medium with addition of IBA 2 mg/l and

1 mg/l respectively. The roots, in general, were more developed in the cotyledonary explants where more side ramifications were observed.

Table 4. Rooting response of *in vitro* raised shoots of *Hildegardia populifolia* grown on MS medium supplemented with auxins, after 45 days

Plant growth regulators (mg/l)	Axillary bud				Apical bud			
	Percentage of response (%)	Number of roots	Root length (cm)	Shoot with basal callus (%)	Percentage of response (%)	Number of roots	Root length (cm)	Shoot with basal callus (%)
IBA								
0.1	78.6^{e}	5.0^{d}	7.0^{cd}	86.6^{h}	72.0^{d}	4.3^{e}	6.3^{d}	81.3^{g}
0.5	85.3^{d}	5.8^{c}	7.5^{c}	88.0^{g}	78.6^{c}	5.0^{d}	7.0^{bc}	85.3^{f}
1.0	92.0^{b}	6.5^{b}	8.3^{b}	93.3^{d}	85.3^{b}	5.5^{bc}	7.9^{ab}	89.3^{de}
2.0	98.6^{a}	7.0^{a}	9.0^{a}	95.0^{bc}	90.0^{a}	6.2^{a}	8.1^{a}	90.0^{d}
3.0	90.0^{bc}	6.2^{bc}	8.5^{ab}	92.0^{e}	86.6^{ab}	5.8^{b}	7.5^{b}	88.0^{e}
NAA								
0.1	69.3^{g}	3.5^{f}	4.0^{de}	97.3^{b}	61.3^{ef}	3.0^{g}	3.0^{e}	96.0^{ab}
0.5	75.0^{ef}	4.0^{e}	4.2^{d}	98.6^{a}	65.0^{e}	3.4^{f}	3.5^{de}	97.3^{a}
1.0	66.6^{gh}	3.8^{ef}	3.7^{e}	96.0^{b}	58.6^{f}	3.1^{fg}	2.9^{ef}	94.6^{b}
2.0	61.3^{i}	2.9^{g}	3.3^{ef}	90.6^{f}	52.0^{g}	2.7^{gh}	2.5^{f}	92.0^{c}
3.0	57.3^{ij}	2.6^{gh}	2.9^{ef}	88.0^{g}	48.0^{gh}	2.2^{i}	2.1^{f}	89.3^{de}

Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

According to Khatun *et al*. (2003) and Srivatanakul *et al.* (2000) the easy rooting aptitude was found to be related to its high endogenous auxin content. Thus, the addition of auxin leading to an increase in the effect of exogenous auxin in medium may disrupt the mobilization of total auxin (endogenous + exogenous) resulting consequently in morphogenesis disequilibrium for the profit of callogenesis. On the other hand, there might be a relationship between root production and fiber producing plants. In fact, the induction of fiber formation is controlled by auxin and gibberellin produced in leaves, and by cytokinin produced in roots (Aloni *et al*., 2007). Thus, plantlets of *Corchorus olitorius*, a high fiber content species, rapidly produced roots on hormone free MS medium (Khatun, 2002; Huda *et al*., 2007).

Acclimatization

In vitro formed plantlets were transferred to soil, sand and vermiculite mix (2:1:1) and were maintained in culture room at 26 ± 2°C, under plastic sheets for 25–30 days. Periodical removal of plastic sheets, so as to lower down the high atmospheric humidity gradually, was observed as effective way for the

acclimatization process as more than 85 % of the plantlets survived through this. Plantlets were watered with 1/4 strength MS basal solution devoid of sucrose and meso-inositol at 3 days intervals for a period of 3 weeks. The acclimatized plantlets were then transferred to pots containing soil and kept under shade for another 8 weeks before transferring to the field (Fig. 3 g). After it, the plantlets were transplanted to soil conditions where more than 95 % plantlets showed the successful establishment. According to Mapes *et al.* (2004), the plants resulting from *in vitro* culture had a finer cuticle in general than that of the mother plants, which caused their fast desiccation when the relative humidity was lowered by passage in greenhouse. Because of these characteristics, the control of the process of adjustment of the *in vitro* produced plants was necessary to limit the water loss and to have a good level of survival after recovery (Carreto, 1992). The substrate is also important for plant acclimatization. Rajeswari and Paliwal (2008) obtained the best rate (75%) of survival of the plant *Albizia odoratissima* after acclimatization on vermiculite substrate, while Ndiaye *et al*. (2006) reported 100% of survived plantlets of *Bambusa vulgaris* after acclimatization in pots containing sterile perlite-peat. In total, 95% of potted *H. populifolia* plantlets survived acclimatization and were kept in a greenhouse for 8 weeks before transplanting to an experimental field plot. After one year, *H. populifolia* plantlets ranged from 100 – 150 cm in height and had a 100% survival rate in the experimental field plot (Fig. 3 h).

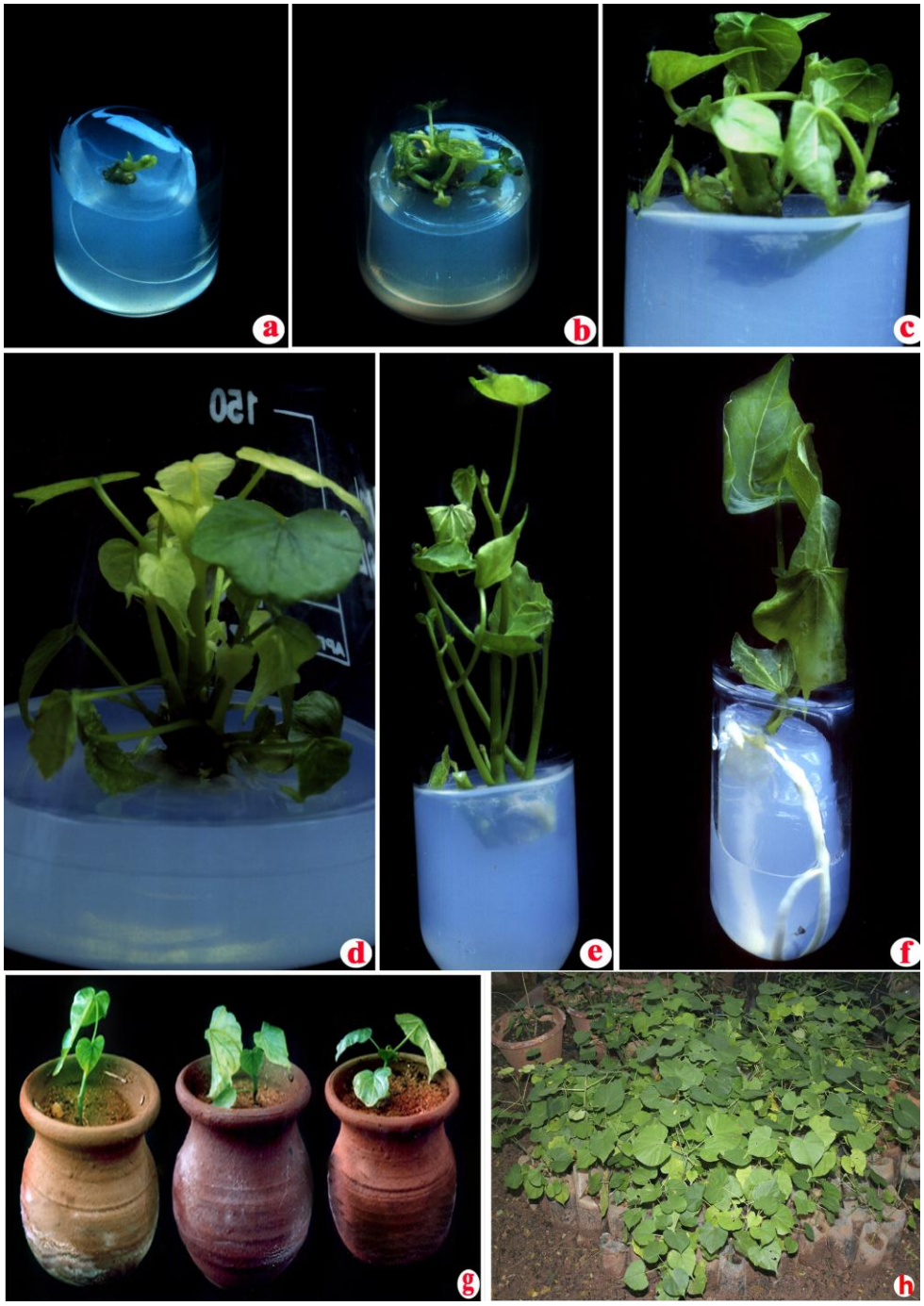

Fig. 3. Micropropagation of *Hildegardia populifolia*. (a) Shoot initiation; (b, c & d) Multiple shoot formation; (e) Shoot elongation. (f) Rooting; (g) Hardening; (h) Experimental field plant.

Conclusion

This study provides an efficient *in vitro* propagation method for the economically important and endangered tree species *H. populifolia*, using a simple protocol for producing uniform plants in a relative short time and with high multiplication rate. The highest percentage of elongated and rooted plantlets was on MS + BA (1.0mg/l) + KN (0.5mg/l) + GA_3 (1.0mg/l) + glutamine (50 mg/l), after 45 days. Rhizogenesis was observed on MS medium supplemented with IBA (2 mg/l) and plantlets produced through micropropagation were hardened with survival rate of 100%. The protocol developed would aid *ex situ* conservation of this endangered species.

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(Published in September 2012)