

Micro-propagation of *Hypericum gaitii* Haines, an endangered medicinal plants : assessment of genetic fidelity

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Abstract An efficient in vitro protocol was established for mass production of *Hypericum gaitii* Haines, an important medicinal plant. Multiple shoots were induced from apical and axillary meristems on Murashige and Skoog medium supplemented with 1.0–3.0 mg/l 6- benzylaminopurine (BAP) or Kinetin and 3 % (w/v) sucrose. Inclusion of 0.5 mg/l 1-naphthaleneacetic acid (NAA) in the culture medium to enhance the rate of shoot elongation and multiplication. The rate of multiplication was higher when the cultures were incubated under 16 h photoperiod rather than the continuous light. Rooting was readily achieved upon transferring the microshoots onto full strength MS basal semi-solid medium supplemented with 0.5–1.0 mg/l IBA after 15 days of culture. Medium having NAA along with BAP did not show the induction of rooting. Micro-propagated plantlets were acclimatized and successfully grown in soil mixture. About 50 % of micropropagated plantlets were hardened in the polyhouse and successfully established in the soil. Inter Simple Sequence Repeat DNA marker was used to detect the variability among the micropropagated plants developed through in vitro. The results showed that there was no polymorphism among the micropropagated plants. Further study is necessary to enhance the survivality rate of in vitro raised plantlets of *Hypericum gaitii* for commercialization.

Keywords In vitro · Medicinal plants · Growth regulators · ISSR marker · Genetic stability

Introduction

Hypericum is one of the nine genera that belongs to the clusoid clade of the Malpighiales [7, 25]. It is a large genus of herbs and shrubs that belongs to family Hypericaceae, widely grown in temperate regions. The genus comprises of over 484 species widely found in Europe, Asia, Northern Africa and America, that are placed in 36 taxonomic sections on the basis of morphology and biogeography [2]. In cold temperate regions, *Hypericum* is native mainly to lowland and upland areas, while in the tropics and warm temperate areas it is almost always confined to high elevation mountain habitats. *Hypericum* karyology is quite diverse, as basic haploid chromosome numbers of $x = 6–10$ and 12 have been reported [13, 17]. Robson [17] reported that $n = 12$ is the ancestral chromosome number within *Hypericum*. Ruhfel *et al.* [21] proposed the inclusion of another 10 taxa based on phylogenetic analysis for a total of 494 species. Out of these species, about 29 species occur in India [24]. Among 29 species, 13 were native to the Himalayan region and remaining 16 species were non-natives. Three species namely *Hypericum assamicum*, *H.gaitii* and *H.gracilipes* were endemic and 6 species, viz., *H.cordifolium*, *H.dyeri*, *H.oblongifolium*, *H.podocarpoides*, *H. tenuicaule* and *H.williamsii* extended their distribution to the Himalayan region of Pakistan, Afghanistan, Bhutan and Nepal, hence have been identified as near endemic. The information based on diversity pattern, habitats, nativity, climate and diversity of soil [22]. These species have been used as traditional medicine for wound healing, bactericidal and anti-inflammatory properties [3, 11, 26]. Extracts of the crude drug are widely used in

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the treatment of mild and moderate depression. *Hypericum* species has potential for curing many diseases like cancer, AIDS, tumour etc. and is very popular today in different countries of the world especially as an antidepressant [5]. The literature indicates that many species of the genus are also economically important which are used as medicinal, fodder, fuel, edible, dye, etc. In spite of such high economic value, there is scanty information available, which contains comprehensive information on the diversity, distribution, habitat preference, nativity, endemism, status and indigenous uses of the species of genus *Hypericum*. *Hypericum gaitii* is a tall shrub, bushy, with branches erect to ascending which is now endangered plant species to the Eastern Ghat region of India. The major threat to *H. gaitii* is habitat loss because of high anthropogenic pressures. Both in-situ and ex-situ conservation strategy should be adopted to save this remarkable species from the verge of extinction. Development of protocol for in vitro propagation of *H. gaitii* would be an alternative for conservation. There is urgent need to develop efficient protocol on rapid propagation and to establish genetical uniformity for this plant species. Gadzovska et al. [6] developed an efficient protocol for micropropagation and also improve the production of hypericin and pseudohypericin from calli, microshoots and in vitro raised plantlets of *H. perforatum*. Further, Pavlik et al. [16] achieved the higher production of hypericin and hyperforin from cell culture of *Hypericum perforatum* in the presence of polyethylene glycol and saccharose containing medium. There is no report on in vitro plant regeneration of *H. gaitii*. The present investigation deals with the standardization of in vitro protocol by using plant growth regulators, modified culture condition and by periodic evaluation of the genetic stability of in vitro raised plants for commercial utilization as well as ex vitro conservation.

Materials and methods

Plant material and explant source

Actively growing young shoots of *Hypericum gaitii* were collected from greenhouse grown plants and washed with 2 % (v/v) detergent 'Teepol' (Qualigen, India) for 5 min and washed with running tap water for 10 min. Further, the explants were surface sterilized with 0.1 % (w/v) mercuric chloride aqueous solution for 5 mins followed by wash in sterile double distilled water at least four times. The apical (~ 0.5–1.0 cm) and nodal explants (~ 2–3 cm) in length were used as explant source and cultured aseptically.

Culture medium and condition

Both apical and nodal explants were implanted into semi-solid basal Murashige and Skoog (MS) medium [15] containing

30 g/l sucrose and supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP: 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l), kinetin (Kn: 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) and 1-naphthalene acetic acid (NAA : 0.0, 0.25 and 0.5 mg/l) or IAA for shoot proliferation and multiplication. The pH of the media was adjusted between 5.6–5.8 using 0.1 N NaOH or 0.1 N HCl before autoclaving. Routinely, 25 ml of the molten medium was dispensed into culture tubes (25 × 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheesecloth and sterilized at 121 °C and 1.06 Kg/cm² pressure for 15 min. The cultures were maintained at 25 ± 2 °C under 16 h photoperiod from cool, white fluorescent lamps. The cultures were maintained by regular subcultures at 4-week intervals on fresh medium with the same compositions.

Induction of rooting and acclimatization

For root induction, excised micro-shoots were transferred to full and half strength of basal MS medium supplemented with different concentrations of IBA or NAA (0.0, 0.5, 1.0, 1.50 and 2.0 mg/l) and 2 % (w/v) sucrose. One excised shoot was placed in each tube (25 × 150 mm) having 15 ml of the culture media. All the culture tubes were incubated at 25 ± 2 °C under 16 h photoperiod with cool, white fluorescent lamps. Rooted micro-propagules were thoroughly washed to remove the adhering gel and planted in 5 cm plastic cups containing a sterile mixture of sand, soil and cow-dung manure in the ratio of 1:1:1 (v/v) and kept in the greenhouse for acclimatization.

Observation of cultures and presentation of results

Twenty cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and mean percentage of rooting were statistically analyzed [8].

DNA isolation and quantification

Genomic DNA was extracted by the CTAB method of Doyle and Doyle [4] with modifications. Leaf tissue (1.0 g) derived from in vitro-raised plantlets and mother plant of *Hypericum gaitii* was grounded in liquid nitrogen to a fine powder with a chilled mortar and pestle and added to preheated, modified CTAB buffer [2 % (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2 % (v/v) 2-mercaptoethanol]. Extraction with chloroform: isoamyl alcohol (24:1) was performed twice. RNase A (final concentration 10 mg/1000 µl) was added, followed by incubation at 37 °C for 60 min. Final centrifugation at 5000 g for 5 min was performed to remove any impurities and suspended in TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA pH 8.0). DNA was examined by

gel electrophoresis in 0.8 % (w/v) agarose in Tris-Acetate-EDTA (TAE) buffer prestained with ethidium bromide (5 mg/10 ml). Electrophoresis of DNA was performed at 50 V for 45 min and DNA profile was visualized with a UV trans-illuminator. The concentration of DNA was estimated by comparing it with an uncut lambda DNA marker (EMerck Bioscience, India).

ISSR analysis

Initial optimization of polymerase chain reaction (PCR) was done for Inter Simple Sequence Repeats (ISSR) assay, including concentration of Template DNA, primer, MgCl₂, number of PCR cycle and annealing temperature. Twenty-five microliter reaction mixture for ISSR assay contained 25 ng template DNA, 100 mM each dNTPs, 15 ng of decanucleotide primers (M/S EMERCK Bioscience, India), 1 µl Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01 % gelatin) and 0.5 U Taq DNA polymerase (M/S EMerck Bioscience, India). A total of 1.5 mM MgCl₂ was used in the case

of ISSR. DNA amplification was performed in a thermal cycler (PeQlab, Germany) programed as follows: preliminary activation at 94 °C for 5 min followed by 45 cycles of 1 min at 94 °C, 1 min at annealing temperature (varied from primer to primer) and 1 min at 72 °C, with a final extension at 72 °C for 10 min. Amplification products were visualized in 1 % (w/v) agarose gel for electrophoresis in 1 % TAE pre-stained with ethidium bromide. The 3.0-kb plus ladder (M/S EMerck Bioscience, India) was run in each gel as size marker. Gel photographs were scanned through Gel Documentation System (UVITEC, Cambridge, UK) and the amplification products size were evaluated using the software Quantity One (Bio-Rad, USA).

Results

Shoot initiation and multiplication

Both apical and nodal explants of *Hypericum gaitii* are cultured on MS medium supplemented with different concentrations

Table 1 Effect of cytokinins and auxins on shoot multiplication of *Hypericum gaitii* after 4 weeks of culture

MS + Growth Regulators (mg/l)				
Kn (mg/l)	BAP (mg/l)	IAA (mg/l)	NAA (mg/l)	Av. No. of multiple shoots per explant ± SE* (Av. length of the shoot (cm))
0	0	0	0	0
0.50	0	0	0	0
1.0	0	0	0	1.2 ± 0.3 a (1.4 ± 0.5)
1.5	0	0	0	1.5 ± 0.4 a (1.5 ± 0.4)
2.0	0	0	0	2.2 ± 0.5 c (1.6 ± 0.6)
3.0	0	0	0	2.6 ± 0.8 c,d (1.7 ± 0.7)
0	0.5	0	0	1.2 ± 0.6 a (1.6 ± 0.5)
0	1.0	0	0	2.6 ± 0.6 c,d (1.8 ± 0.7)
0	1.5	0	0	3.2 ± 0.8 e (2.1 ± 0.8)
0	2.0	0	0	3.5 ± 0.6 e,f (2.2 ± 0.7)
1.0	0	0.25	0	1.2 ± 0.4 a (0.75 ± 0.02)
2.0	0	0.25	0	1.2 ± 0.2 a (1.0 ± 0.5)
2.0	0	0.50	0	1.5 ± 0.5 a (1.0 ± 0.4)
1.0	0	0	0.25	1.8 ± 0.8 b (1.5 ± 0.6)
2.0	0	0	0.50	2.2 ± 0.4 c (1.6 ± 0.7)
3.0	0	0	0.50	2.8 ± 0.6 c,d (1.6 ± 0.8)
0	1.0	0.25		1.2 ± 0.7 a (1.2 ± 0.6)
0	1.5	0.25		2.5 ± 0.8 b (1.4 ± 0.7)
0	2.0	0.50		2.6 ± 0.6 c (1.3 ± 0.6)
0	1.0	0	0.25	3.6 ± 0.5 e,f (2.5 ± 0.4)
0	2.0	0	0.50	4.3 ± 0.7 g (2.8 ± 0.6)
0	3.0	0	0.50	4.5 ± 0.8 g (2.6 ± 0.7)

*Mean of 20 cultures per treatment; repeated thrice

a-g Means having the same letter in a column were not significantly different by Post-Hoc Multiple Comparison test

$P < 0.05$ level

and combination of cytokinin and auxins to develop shoot proliferation and multiplication. The micro shoots were initiated from the axillary explants within 2 weeks of inoculation supplemented with BAP or Kn alone and in combination with either NAA or IAA. Of the different growth hormones tested, BAP was the most effective for shoot proliferation and multiplication. The medium having kinetin helped shoot elongation but did not enhance shoot multiplication. The maximum number of shoot proliferation and multiplication was observed in axillary meristems cultured on MS medium supplemented with 0.5–2.0 mg/l BAP and 0.5–3.0 mg/l Kn within 4 weeks of culture under 16 h photoperiod (Table 1). Both apical and axillary shoots were proliferated and elongated to 1.5–2.8 cm within 4 weeks of culture (Fig. 1a and b). There was no sign of shoot proliferation when explants were cultured in media devoid of cytokinin or auxins. At higher concentrations of BAP, the rate of shoot proliferation declined. The maximum rate of shoot elongation and proliferation was achieved on MS medium supplemented with 2.0 mg/l BAP. Prolonged culture on the proliferation and multiplication media resulted in the blackening of the basal ends of the developing shoots. There were differences among the treatments for both the percentage of cultures with multiple shoots and the mean number of shoots/culture. Both apical and axillary meristems were grown early in liquid medium with similar composition. The number of multiple shoots per explant ranged from 1.2 to 4.5 depending on the culture medium within 4 weeks of culture (Fig. 1c). The highest percentage of cultures with multiple shoots (62.6) was observed on media containing 3.0 mg/l BAP and 0.5 mg/l NAA when the cultures were incubated for 4 weeks (Table 1).

Induction of rooting from microshoots

The elongated in vitro grown shoots were transferred to full and half-strength MS basal medium supplemented with various concentrations of IBA or NAA with reduction of sucrose concentration from 3 % to 2 % (w/v). Elongated shoots (1–2 cm long) were rooted on full strength MS basal medium supplemented with various concentrations of IBA and NAA (Table 2). The induction of roots from microshoots were inhibited in medium without growth regulators. Root initiation took place in MS basal medium supplemented with 1.0–2.0 mg/l IBA or NAA after 3 weeks of transfer. There was also increase in shoot length after 4 weeks of culture. However, optimal rooting (86.2 %) and growth of microshoots were observed on medium containing 1.0 mg/l IBA with 2 % (w/v) sucrose (Fig. 1d). The rooting ability was reduced and lead to root necrosis with the increase in the concentration of NAA and IBA in the culture medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of IAA or IBA (Table 2).

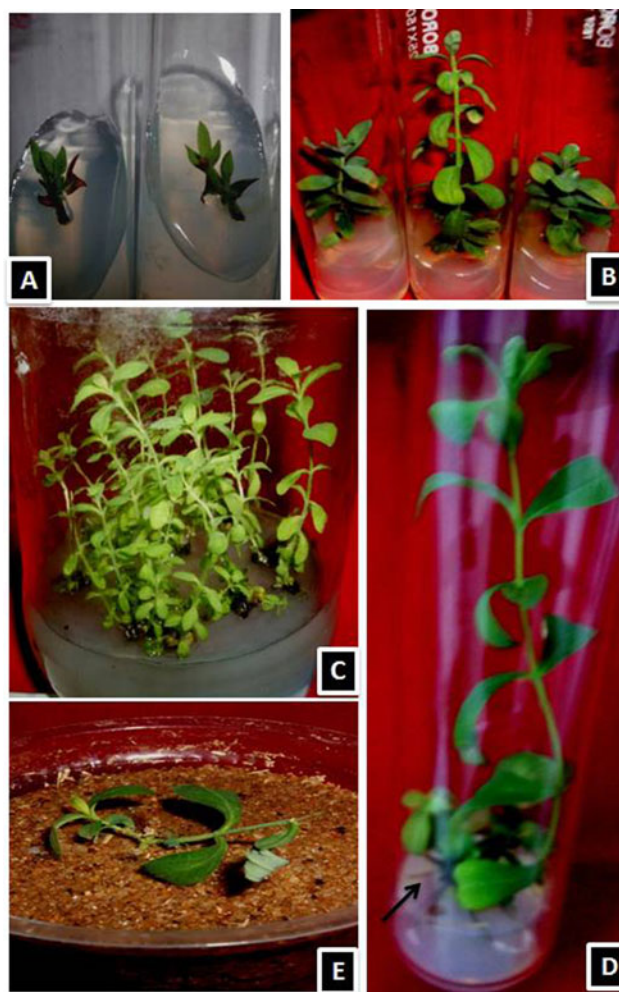


Fig. 1 In vitro propagation of *Hypericum gaitti*. **a** Proliferation of axillary shoot from nodal explants on MS medium supplemented with 3.0 mg/l BAP after 3 weeks of culture. **b** Development of shoots from nodal explants on MS medium supplemented with 3.0 mg/l BAP, and 3 % sucrose after 4 weeks of subculture. **c** Development of multiple shoots from axillary meristems on MS liquid medium supplemented with 3.0 mg/l BAP + 0.5 mg/l NAA and 3 % sucrose after 4 weeks of subculture. **d** Induction of roots from microshoots on half strength MS medium supplemented with 0.5 mg/l IBA and 2 % sucrose after 2 weeks of culture. **e** In vitro raised plantlets grown in the soil-mixture

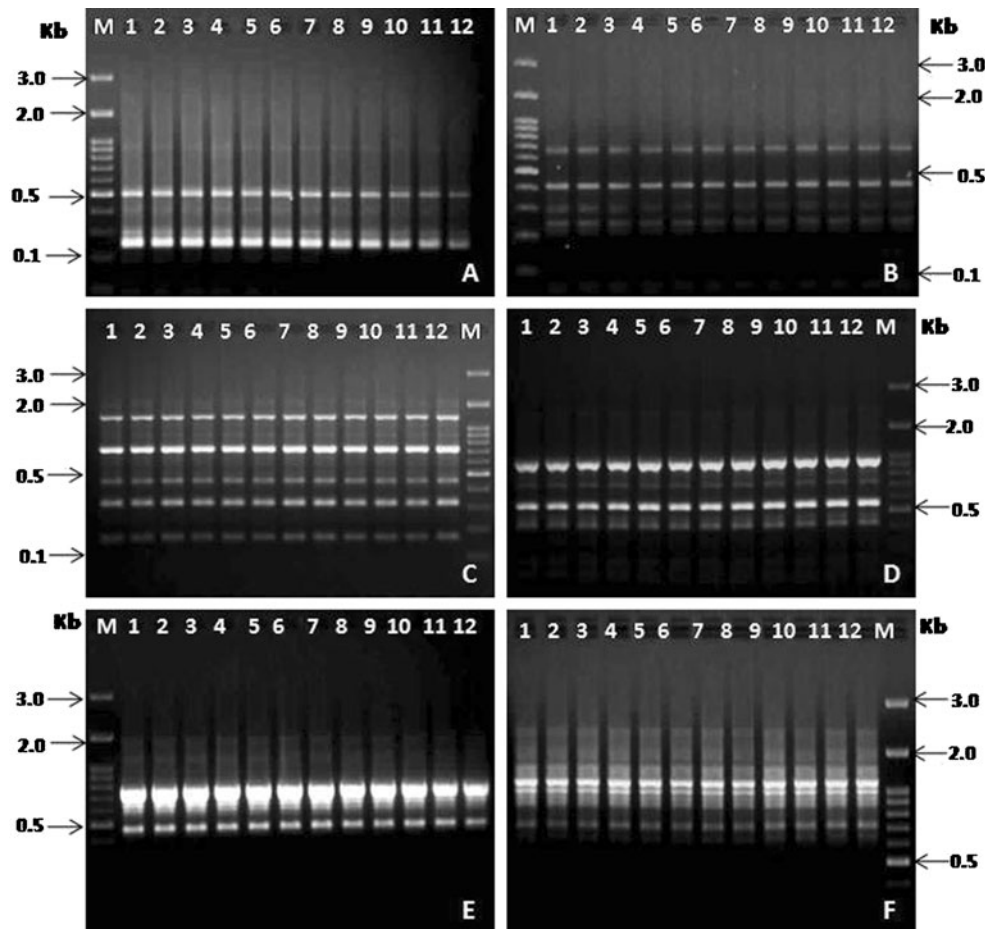
Acclimatization and field establishment

A critical aspect of in vitro micropropagation is to acquire regenerated plants that are capable of surviving in natural environment. Rooted plantlets grown in vitro were washed thoroughly in running water to remove the adhering gel, transplanted to 5 cm plastic cups containing sand for 2 weeks and subsequently transferred to garden soil, sand and cow-dung at the ratio of 2: 1: 1 (v/v). About 50 % of the rooted plantlets established in the greenhouse within 4 weeks of transfer (Fig. 1e). The plants were grew well and attained 3–4 cm height within 12 weeks of transfer. The acclimatized plants were established in the field condition and grew normally without morphological variation.

Table 2 Effect of NAA and IBA on rooting from excised shoots on ½ strength MS basal medium supplemented with 2 % (w/v) sucrose

MS + growth regulator (mg/l)		Av. percentage of rooting (Mean ± SE)*	Av. No. of roots/explant (Mean ± SE)*
IBA	NAA		
0	0	0	0
0.25	0	0	0
0.50	0	0	0
1.00	0	42.6 ± 2.2 b	4.6 ± 1.2 b
1.50	0	76.2 ± 1.5 e	5.8 ± 1.2 c
2.00	0	72.5 ± 1.2 e	6.4 ± 1.0 d
0.00	0.25	0	0
0.00	0.50	0	0
0.00	1.00	35.6 ± 2.5 a	2.6 ± 0.8 a
0.00	1.50	58.4 ± 2.6 c	4.3 ± 1.3 b
0.00	2.00	62.8 ± 2.2 d	5.1 ± 1.2 c

*Mean of 15 cultures per treatment; repeated thrice

a-e Means having the same letter in a column were not significantly different by Post-Hoc Multiple Comparison test $P < 0.05$ level**Fig. 2** ISSR profiles of micropropagated plants of *Hypericum gaitti* using ISSR primers UBC-818 : 5'-CACACACACACACAG-3' (a), UBC-855 : 5'-ACACACACACACACCTT- 3' (b), USB-843 : 5'-GAGAGAGAGAGAGAGACT-3 (c), AM-6 : 5'-AGCAGCAGCAG CAGCGG-3' (d), UBC-889 : AGTCGTAGTACACACACACAC-3'

(e) and USB-810 (5'-GAGAGAGAGAGAGAT-3') (f). "M" shows the molecular marker. ISSR banding profile of mother plants "No. 1" and "No. 2 to 12" are in vitro raised plants. Arrow indicates the size of the marker

Genetic fidelity analysis

In vitro grown plantlets including mother plant were selected and used for genetic fidelity analysis. The quality of in vitro-derived plantlets was screened with ISSR primers that have showed monomorphic banding pattern among the plantlets. The banding pattern of PCR amplified product from micropropagated plantlets was found to be monomorphic with most of the primer tested. The identical ISSR banding pattern of in vitro-raised plantlets and their control mother plants are shown in Fig. 2. The size of the monomorphic banding pattern of DNA fragments produced by UBC-818, UBC-855, USB-843, AM-6, UBC-889 and USB-810 primers ranged from 200 to 1500 bp (Fig. 2). The number of monomorphic banding pattern of DNA fragments was found to be 05 in case of UBC-818, UBC-855, USB-843, 04 in case of primer AM-6, 03 in case of UBC-889 and 07 in case of primer USB-810. Most of the primers used showed identical banding patterns for DNA profiles in the plantlets when compared with those found in the original mother plant.

Discussion

The present study showed that it was possible to explore the morphogenetic potential of *Hypericum gaitii* by application of growth regulators and by modification of culture condition. Cytokinin helps for induction and multiplication of shoots derived from axillary as well as apical meristems. The regulatory action of cytokinins and apical dominance helped the in vitro shoot induction and multiplication [9, 10]. The maximum shoot induction and multiplication was observed both in apical and axillary meristems cultured on MS medium supplemented with 2.0 mg/l BAP and within 4 weeks of culture. These results are supported by the observations from previous studies in *H. perforatum* [3] and *Aloe vera* [9]. At higher concentrations of BAP, the rate of shoot proliferation declined. Similar results were reported in *Nyctanthes arbortristis* [19], *Plumbago zeylanica* [20]. Mao et al. [14] reported that BAP proved superior to other cytokinins for multiple shoot induction of *Clerodendrum colebrookianum*. Banerjee et al. [1] achieved shoot bud regeneration from callus derived from hypocotyl explants of *Hypericum perforatum* on MS medium supplemented with 1.0 mg/l thidiazuron. Our results demonstrated that the inclusion of NAA in the culture medium help higher rate of shoot multiplication. The results are consistent with earlier reports indicating cytokinins and auxins affect shoot multiplication in other plants using shoot tip or axillary bud explants [10, 19]. The interaction of photoperiod and plant growth regulators have significant effect on shoot morphogenesis as reported earlier [26]. The results also imply that there were differences among the treatments for both the percentage of culture developing multiple shoots and the mean

numbers of shoots per culture. This might be due to the balancing of the endogenous and exogenous growth regulators and the ionic concentration of nutrient salts as reported earlier in other plants [23]. The elongated shoots were rooted maximum in full strength MS basal salts supplemented with 1.5 mg/l IBA with 2 % sucrose. The rooting ability was reduced with the increase in the concentration of IBA or NAA in the medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of NAA or IBA. Similar observations were made in *Plumbago rosea* [20], *Aloe barbadensis* [12] and *Psoralea corylifolia* [18]. However, Banerjee et al. [1] achieved root initiation from young shoots of *Hypericum perforatum* on MS medium supplemented with 2.0 mg/l IAA. The rooted plantlets were established in the field and grew normally. There are many factors like length of culture periods, genotype and nature of explant, which have significant influence on the stability of the tissue cultured plants. So, assessment of genetic stability of in vitro regenerated plantlets is highly significant for further studies.

In conclusion, an attempt was made to develop an in vitro protocol for mass multiplication of *Hypericum gaitii* by manipulating the nutrient salts, growth regulators and by modification of culture conditions. The pattern of morphogenesis on various phytohormonal regimes largely confirm to those reported in other plant species [9, 26]. This investigation may be useful for conservation of rare, endangered plant species.

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