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ORIGINAL PAPER

In vitro manipulations in St. John's wort (*Hypericum perforatum* L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD analysis

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Abstract The present study describes the potential of in vitro grown adventitious roots of *Hypericum perforatum* L. commonly known as St. John's wort at low nutrient and auxin levels in the liquid medium for micropropagation. Roots were regenerated from shoot-derived callus on MS medium containing 4.0 mg l^{-1} Indole-3 acetic acid (IAA). IAA and Indole-3 butyric acid (IBA) were equally effective for the induction of roots from shoot cultures. Half strength MS medium containing 1.0 mg l^{-1} IAA was most found suitable for culturing roots in liquid medium. A total biomass of 4.13 ± 0.67 g comprising 226 ± 34.4 shoots and shoot buds along with roots was obtained per culture starting with 200 mg roots inoculum. Pretreatment with kinetin (2.0 mg l^{-1}) enhanced the shoot multiplication. Shoots proliferated profusely from excised roots in static liquid medium supported with glass bead matrix. Growtek™ vessel was found suitable and cost effective system for high throughput plantlet production. In vitro grown roots regardless of their source of origin were an excellent and easy to handle source of explant for aseptic production of plantlets without loosing the morphogenetic potential over the generations. The plants exhibited 84–99% similarity among themselves through RAPD. The in vitro shoots produced can either be multiplied or rooted perpetually, and alternatively they can also be explored for the in vitro production of hypericin and hyperforin.

Keywords Depression · Growtek · Glass beads · *Hypericum perforatum* · Hypericin · Hyperforin · Micropropagation · RAPD · RFLP

Abbreviations

IAA	Indole-3 acetic acid
IBA	Indole-3 butyric acid
NAA	α-Naphthelene acetic acid
2,4-D	2,4, Dichloro phenoxy acetic acid
BAP	6-Benzylaminopurine
Kn	Kinetin (6-Furfurylaminopurine)
TDZ	Thidiazuron
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism

Introduction

Hypericum perforatum L. (family—Clusiaceae) commonly known as St. John's wort is a distinguished medicinal plant naturally occurring in relatively dry temperate zones of Europe and North America. In India, it is distributed in the temperate Western Himalayan region between 3,000 and 9,000 ft altitude. St. John's wort has potential in the treatment of mild to moderate form of depression (DeSmet and Nolen 1996) due to the active principles "Hypericin and Hyperforin" and has become popular medicinal plant due to its anti (retro) viral activity, anticancer, bactericidal, anti-inflammatory and sedative properties (Vatikutti and Ciddi 2005). Formulations of St. John's wort worth of an

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estimated value of US$ 210 and 570 million in the USA and worldwide, respectively (Zobayed and Saxena 2003). In vitro culture is an attractive tool for mass multiplication and production of secondary metabolites from *H. perforatum* (Cellarova et al. 1992; Zdunek and Alfermann 1992; Murch et al. 2000; Pretto and Santrarem 2000; Zobayed et al. 2004). In vitro growth, multiplication and production of phytochemicals are governed by factors such as explant selection, its age and plant growth regulators. The roots as a source of explants for in vitro propagation have been reported only in few species (Bhat et al. 1992; Kelkar and Krishnamurthy 1998; Zobayed and Saxena 2003; Vinocur et al. 2000). Generally excised roots grow continuously in vitro. Although, loss of morphogenetic potential may sometimes occur with repetitive subculture, however, the manipulation of culture medium can salvage or alter the growth pattern. *Agrobacterium rhizogenes* mediated transformed roots have wide acceptance and potential for large-scale culture for secondary metabolite production and plant regeneration, however such roots usually produce opine like substances, having lethal effect on mammalian cells (Paek et al. 2005). Therefore attention is now being focused towards exploring the potential of adventitious roots for in vitro manipulation. Further use of bioreactors for micropropagation and culture of roots (transformed as well as adventitious) for the production of useful phytochemicals has gained considerable attention during the last few years (Ziv 2005; Hvoslef-Eide et al. 2005; Srivastava and Srivastava 2007). Large-scale culture of roots in novel bioreactors can provide the optimal conditions for biomass and secondary metabolite production comparable to (or higher than) that in native roots. Emphasis has been laid on designing appropriate bioreactors by considering several physical and chemical parameters (nutrient availability, nutrient uptake and oxygen depletion in the medium, shear sensitivity) and other factors such as support matrix suitable to culture delicate and sensitive plant roots, (Kim et al. 2002; Srivastava and Srivastava 2007). Growtek culture vessel has been successfully used for cost effective mass cloning of several economically important plants and hairy root culture (Dey 2002). The maintenance of genetic integrity of micropropagated plants with respect to the mother plant is the most vital consideration for upholding certain agronomic traits using elite genotype over natural seedlings. As the organized meristems are generally considered to be resistant to genetic changes occurring during cell division and differentiation under in vitro conditions therefore this mode is considered to give rise to genetically uniform and true to type plants (Rani and Raina 2000). The somaclonal variant frequency depends upon several factors such as the species, donor genotype, explants type, composition of the culture medium, conditions of the physical culture and the duration between successive subcultures (Ahuja 1987). In the pursuit of all these considerations, present piece of work offers a very simple technique for micropropagation of *H. perforatum*, using in vitro grown adventitious roots developed from two different origin in the liquid medium, their further scaling up in Growtek vessel and assessment of clonal fidelity of the micropropagated plants regenerated via roots from both the origin through RAPD analysis.

Materials and methods

Plant material

Plants of *H. perforatum* L. collected from Bageshwar (Uttrakhand, India) were maintained under glass house conditions at CIMAP research farm, Lucknow.

Initial establishment of aseptic cultures

The young shoots were surface sterilized with 70% (v/v) ethyl alcohol for 30 s and 0.1% (w/v) $HgCl_2$ for 3 min followed by repeated rinsing with sterile distilled water. Initially, aseptic culture were established by culturing 2–3 cm long excised shoot explants with 1–2 node(s) containing axillary buds on MS (Murashige and Skoog 1962) medium supplemented with Kn and 2,4-D alone or in combinations (0.0, 0.25, 0.5, 1.0, 2.0 and 5.0 mg l^{-1}). For all the experiments pH of the medium was adjusted to 5.86 ± 0.02 prior to autoclaving at 15 lbs. at 121°C for 15 min. The culture conditions (temperature 25 ± 2°C, 14 h photo-period using cool white florescent light, and light intensity 20 µmol m^{-2} s^{-1}) were maintained throughout the course of study. Minimum six replicates were maintained for each treatment. Shoot multiplication and callus initiation response was examined after 8 weeks of culture.

Root induction from callus culture

The callus was transferred on IAA, IBA, or NAA (1.0, 2.0 and 4.0 mg l^{-1}) alone or in combination with Kn (0.1, 0.2 and 0.4 mg l^{-1}) in 100-ml flask containing 40 ml medium with 3% (w/v) sucrose and 0.8% (w/v) Agar (Hi-Media). Medium without any hormone served as control. Observations were recorded on six replicates per treatment and experiments were repeated three times. Frequency of root induction was examined after 8 weeks of culture.

In vitro rhizogenesis in micro-shoots

In vitro grown shoots of 3.0–4.0 cm length (bunch of 3–4) were excised and transferred on full and half strength MS

medium supplemented with different auxins viz. IAA, IBA and NAA (1.0, 2.0 and 4.0 mg l^{-1}). Half and full strength MS medium without any auxin served as control. About 15 replicates were maintained for each treatment. Root growth and root organogenesis, number of roots/explant was quantified after 6 weeks of inoculation. The number of roots per shoot bunch was compared by ANOVA. The Student *t*-test was applied to compare individual treatment.

Growth of excised roots on semi solid medium

Root segments of about 2.0 cm length without any lateral branches were excised from callus or shoot cultures and sub cultured separately in petriplates (in triplicates) on full and half MS medium containing IAA and IBA (1.0, 2.0 and 4.0 mg l^{-1}). Medium devoid of any auxin served as control.

Growth of excised roots in liquid medium

Root segments (200 mg) of about 2.0–3.0 cm length excised from shoot or callus cultures grown on semisolid medium were transferred to full and half strength MS liquid medium containing 0.5, 1.0, 2.0 and 4.0 mg l^{-1} IAA or IBA in 250-ml flask containing 30 ml medium. Medium without any auxin served as control. Culture flasks were incubated at 75 rpm on a rotary shaker in culture room. Shoot regeneration potential of in vitro roots was recorded for three subsequent generations. Effect of supplementation of different levels (0.1, 0.5, 1.0 and 2.0 mg l^{-1}) of cytokinins (TDZ, BAP and Kn) in the culture medium on morphogenetic potential of roots was also examined. Growth parameters such as total number of shoots and shoot buds, length of the shoots along with growth of roots were recorded. Similar parameters were recorded after 6 weeks for the growth of roots in 250-ml flask containing 30 g glass beads (3.0–4.0 mm) as support matrix and Growtek™ vessel (Tarsons, India) containing 250 ml of liquid medium under static conditions. In Growtek vessel, about 1.0 g root tissue was inoculated on the raft. Minimum three replicates were maintained for each treatment and experiment was repeated twice and analysed by ANOVA. The growth index was calculated by following the formula:

$$\text{Growth Index} = \frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{Initial fresh weight}}.$$

Assessment of clonal fidelity of the plants regenerated from adventitious roots

Genetic fidelity between control (parent), adventitious roots from two different sources (in vitro shoots and callus) and randomly selected in-vitro raised plants maintained in glass house was assessed by PCR based RAPD analysis. DNA from roots and fresh young leaf tissue (1.0 g) of the test samples was isolated following the protocol developed by Khanuja et al. (1999). The samples were marked from 1 to 15, where sample 1 represents mother plant, samples 2 and 3 was designated to the roots obtained from shoot cultures and callus, respectively, and samples 4–15 were randomly selected in vitro raised plants grown in glass house conditions. PCR was carried out in a total volume of 25 µl with each reaction tube comprising of 25–30 ng of template DNA, 0.3 units of Taq DNA polymerase, 0.25 µl of each dNTPs, 1.5 mM $MgCl_2$ (buffer) and 5 pmol of random primers. Decanucleotide MAP1-20 primers designed by Khanuja et al. (2000) were custom synthesized from M/s Genei, Bangalore, while OPA1-20 primers were supplied by Operon Technologies Inc. Germany (Table 1). Amplification reaction was carried out using Bio-Rad i-cycler version 4.006. After initial denaturation at 94°C for 5 min, PCR was run for 45 cycles consisting of a denaturation step (1 min), a 35°C primer annealing step (1 min) and 72°C amplification (2 min) step, at the end, final amplification was appended (72°C) for 5 min. Amplified DNA was loaded on 1.2% agarose gel in TAE buffer stained with 0.5 µg/ml ethidium bromide and photographed on a Gel documentation Polaroid system. Bands were scored as present (1) or absent (0) for each sample. NTSys PC version 2.02j was used to perform cluster analysis of the complete data set. Similarity estimates were calculated by using Nei and Li coefficients (Nei and Li 1979) and cluster analysis was carried out by UPGMA method (Unweighted pair group method arithmetic mean averages) and represented as dendrogram.

Results and discussion

Establishment of aseptic cultures

Profuse shoot multiplication and callus induction was observed after 8 weeks of culture on MS medium

Table 1 The nucleotide sequences of primers used for RAPD analysis

Primer	5′–3′	Primer	5′–3′
OPA-5	AGGGGTCTTG	OPA-20	GTTGCGATCC
OPA-7	GAAACGGGTG	MAP-7	CACCCTGCGC
OPA-8	GTGACGTAGG	MAP-8	CTATCGCCGC
OPA-10	GTGATCGCAG	MAP-9	CGGGATCCGC
OPA-12	TCGGCGATAG	MAP-13	GTGCAATGAG
OPA-13	CAGCACCCAC		

containing 5.0 mg l^{-1} Kn and MS medium supplemented with 0.5 mg l^{-1} 2,4-D and 0.25 mg l^{-1} Kn, respectively. These shoot and callus cultures were used as mother source in the ensuing studies.

Root induction from callus culture

Data recorded after 8 weeks of inoculation of callus revealed that, amongst all the auxins i.e. IAA, IBA, or NAA tested only IAA supported faster root growth, whereas NAA favored only callus proliferation. First sign of root induction from callus was recorded after 3 weeks of inoculation on MS medium containing 4.0 mg l^{-1} IAA. Highest number of roots (48.0 ± 6.06/culture) with an average length of 2.25 ± 0.78 cm was recorded on IAA (4.0 mg l^{-1}) supplemented medium (Table 2, Fig. 1a). Callus cultured on hormone free MS medium did not evoke any root induction.

Table 2 Auxin induced root growth from callus cultures

IAA (mg l^{-1})	Kn	No. of roots Per culture	Root length (cm)
1.0	–	9 ± 1.55	2.1 ± 0.5
2.0	–	11 ± 1.41	2.22 ± 0.81
4.0	–	48 ± 6.06	2.25 ± 0.78
1.0	0.1	8 ± 4.33	2.0 ± 0.5
2.0	0.1	7 ± 1.78	1.96 ± 0.86
4.0	0.1	12 ± 2.89	2.15 ± 0.6
1.0	0.2	11 ± 3.46	2.0 ± 0.4
2.0	0.2	7.2 ± 1.47	1.5 ± 0.45
4.0	0.2	5 ± 2.52	1.4 ± 0.3
1.0	0.4	6.2 ± 1.16	1.6 ± 0.54
2.0	0.4	5.5 ± 2.64	1.2 ± 0.22
4.0	0.4	2.25 ± 1.26	1.8 ± 0.78

In vitro rhizogenesis in micro-shoots

Data recorded after 6 weeks revealed that IAA and IBA (1.0, 2.0 or 4.0 mg l^{-1}) were equally effective for the root induction in shoot cultures (Fig. 1b). Lower levels (1.0 and 2.0 mg l^{-1}) of IAA and IBA were more effective than higher level (4.0 mg l^{-1}). IBA in the half strength MS medium at all the three levels tested showed 100% root induction response in shoot cultures. Root induction frequency was higher on half MS than full MS medium. NAA at all the levels in both half and full strength MS medium was found inhibitory for root initiation and growth and supported only the callus growth. A comparative data on auxin induced rhizogenic response in shoot cultures is presented in Table 3.

Growth of excised roots on semisolid medium

Inoculation of roots on semi solid medium showed that root growth was best supported by half MS medium containing IAA (4.0 mg l^{-1}) after 6 weeks (Fig. 1c). On an average 14.6 ± 1.82 roots with 1.82 ± 0.62 cm length were produced per explant (one cm section of root) (Table 4). Full strength MS medium was found to favor more callus formation rather than root growth. Direct shoot bud regeneration was not observed on semisolid medium. IAA or IBA (1.0–4.0 mg l^{-1}) were more or less equally effective for root growth on both half and full strength MS medium.

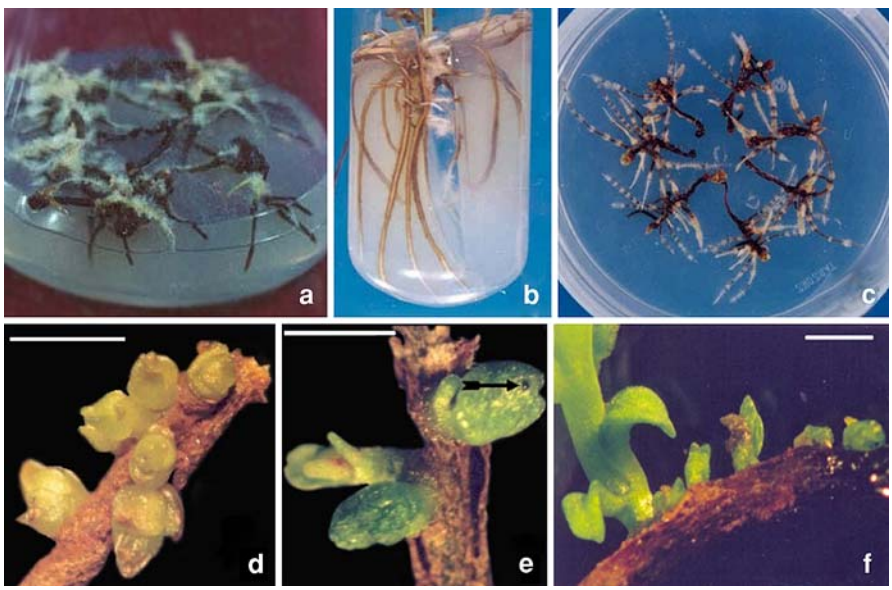

Fig. 1 Organogenesis in *H. perforatum*: root induction in callus culture on MS + 4.0 mg l^{-1} IAA (**a**); root induction in shoot culture on ½ MS + 1.0 mg l^{-1} IAA (**b**); growth of excised root on ½ MS + 4.0 mg l^{-1} IAA (**c**); shoot bud induction in excised roots in vitro, bar-5 mm (**d**); arrow denotes the presence of hypericin glands in nascent shoots, bar-5 mm (**e**) and complete shoot with all developmental stages on a single root explant, bar-2 mm (**f**)

Table 3 Auxin induced rhizogenic response in *H. perforatum* shoot cultures on half and full strength MS medium

Media		Rooting response (%)	No. of roots/ shoot bunch	Average root length (cm)
Full MS				
mg l^{-1}	0	33.3	2.3 ± 0.6[a]	1.25 ± 0.2
IAA	1	100	18.7 ± 1.6	3.8 ± 0.3
	2	80	14.1 ± 5.7	4.0 ± 0.7
	4	85	8.1 ± 2.4	3.5 ± 0.2
IBA	1	100	17.6 ± 2.5	4.0 ± 0.5
	2	85	13.14 ± 2.4	3.8 ± 0.4
	4	64.3	9.9 ± 2.1	2.5 ± 0.4
NAA	1	7	2 ± 0.0	1.0 ± 0.1
	2	0	–	–
	4	0	–	–
Half MS				
mg l^{-1}	0	37.5	4.33 ± 0.6	2.0 ± 0.1
IAA	1	100	17.7 ± 2.3	4.5 ± 0.8
	2	100	16.3 ± 2.2	4.0 ± 0.6
	4	92.3	13 ± 2.7	3.7 ± 0.3
IBA	1	100	17.8 ± 3.2	2.9 ± 0.2
	2	100	17.3 ± 1.8	2.2 ± 0.2
	4	100	11 ± 2.2	3.1 ± 0.3
NAA	1	9	2 ± 0.0	1.4 ± 0.1
	2	7	3 ± 0.0	1.0 ± 0.2
	4	8	3 ± 0.0	0.9 ± 0.1

[a] Mean ± SD

Table 4 Effect of IAA and IBA on growth of excised roots on semi-solid medium

Auxin (mg l^{-1})		Number of secondary roots/root explant	
		MS	½MS
IAA	0.0	3.33 ± 1.8	4.43 ± 1.72
	1.0	5.3 ± 1.78	7.0 ± 2.08
	2.0	3.0 ± 0.82	7.3 ± 1.11
	4.0	2.86 ± 0.69	14.6 ± 1.82
IBA	1.0	6.0 ± 1.01	7.3 ± 1.96
	2.0	4.6 ± 1.38	10.9 ± 2.32
	4.0	4.0 ± 1.18	13.3 ± 2.08

Growth of excised roots and de novo shoot regeneration in liquid medium

It was observed that varying levels of IAA (0.5, 1.0, 2.0 or 4.0 mg l^{-1}) supplemented to half strength MS medium supported both root proliferation as well as shoot regeneration from cultured root explants, whereas same levels of IAA with full strength MS medium supported only root proliferation. Shoot bud initiation from in vitro roots started after 2 weeks of inoculation and small nodule like structures appeared predominantly on the proximal end (Fig. 1d) after 4 weeks and rarely on the distal end. Hypericin glands were quite prominent (Fig. 1e). Older roots were comparatively thicker, dark brown in color and woody (corky) in appearance, produced shoot buds (Fig. 1f), whereas the younger roots were slim, thread like and off white, did not produce shoots. Such a polarization of morphogenetic response in root explants has also been observed earlier (Kelkar and Krishnamurthy 1998; Vinocur et al. 2000). Data recorded after 6 weeks of bud initiation showed that roots were more copiously branched, longer and thicker in half MS than full strength MS medium. Root growth and shoot regeneration response in half strength MS medium containing 1.0 mg l^{-1} IAA was highest amongst all other level tested (Table 5).

Using 200 mg roots as inoculum, a total biomass of about 4.13 ± 0.67 g comprising 226 ± 34.4 shoots and shoot buds per culture along with roots was obtained (Table 5, Fig. 2a). On an average 9.4 shoot buds/cm developed from a single root in liquid culture medium. Average length (5.61 ± 1.0 cm) of roots was higher than that (1.82 ± 0.62) obtained on semisolid medium. When the roots after shoot bud regeneration were cultured on half MS semisolid medium containing IAA (1.0 mg l^{-1}), shoot development started within 2 weeks of culture, however, length of shoots was much shorter than that obtained in liquid medium. A higher growth index with enhanced shoot number was recorded in liquid medium supported with

Table 5 Response of adventitious roots in vitro: effect of media strength and IAA concentration

Medium	IAA (mg l^{-1})	No. of shoots/culture	Biomass (g)		
			Shoot	Root	Total
MS	0.0	–	–	0.408 ± 0.09	0.408 ± 0.09
	0.5	–	–	0.731 ± 0.79	0.731 ± 0.79
	1.0	–	–	0.977 ± 0.12	0.977 ± 0.12
	2.0	–	–	1.706 ± 0.12	1.706 ± 0.12
	4.0	–	–	2.141 ± 0.67	2.141 ± 0.67
½MS	0.0	–	–	2.728 ± 0.25	2.728 ± 0.25
	0.5	149 ± 41[a]	1.03 ± 0.13	2.338 ± 0.37	3.408 ± 0.45
	1.0	226 ± 34.4	1.533 ± 0.44	2.592 ± 0.43	4.125 ± 0.67
	2.0	216 ± 32.4	1.204 ± 0.12	2.566 ± 0.6	3.77 ± 0.63
	4.0	98 ± 11.5	0.898 ± 0.12	2.2 ± 0.21	3.098 ± 0.14

[a] Mean ± SD

glass beads and Growtek under static conditions. A comparative account of the morphogenic response in different type of physical conditions of the culture is presented in the Table 6.

When roots were cultured in liquid medium 0.1, 0.5, 1.0 or 2.0 mg l^{-1} of TDZ and BAP, the roots turned tuberose and shoot bud regeneration was not observed. However, culturing of roots on 2.0 mg l^{-1} Kn for 2 weeks followed by their transfer to half MS medium containing 1.0 mg l^{-1} IAA enhanced the shoot regeneration frequency (247 ± 41) (Fig. 2c). Root borne shoots were rooted and converted into plantlets and the plants exhibited over 85% hardening in the glass house (Fig. 2g). All the regenerants were morphologically similar.

Assessment of clonal fidelity of the plants regenerated from adventitious roots

About 48 amplified reproducible bands were produced by RAPD analysis of 12 in vitro raised plants, control parent plant and root cultures from two sources using 11 random decanucleotide primers. The number of bands produced per primer ranged from 2 to 6 (Fig. 3).

All the primers were polymorphic and produced different amount of polymorphism, out of 48 bands 21 (43.75%) were monomorphic and remaining 27 (54.25%) were polymorphic. Cluster analysis represented as dendrogram on the basis of similarity co-efficient generated from RAPD data of 48 bands exhibited a similarity range from 0.84 to 0.99. All the 20 samples were grouped into one major cluster at 84% level (Fig. 4). In vitro roots derived from shoots and callus cultures shared 99–95% similarity with mother plant.

From the present study, it is clear that variability is present in the micropropagated plants of *H. perforatum*. The polymorphism in amplified bands might result from changes in either the sequence of the primer-binding site (e.g. point mutation) or changes, which alter the size or prevent successful amplification of target DNA (e.g. insertions, deletions, inversions) (Williams et al. 1990). Using the RAPD technique, the presence of genetic variations in micropropagated plants arose from axillary bud explants have been reported (Hashmi et al. 1997; Watanabe et al. 1998; Bindiya and Kanwar 2003). On the other hand, several reports have been published, where no somaclonal variation could be detected using molecular markers (Rani and Raina 2000). Whether this is due to the lack of variation or detection method that is sensitive enough still is a debatable issue. However, such analysis examine only a fraction of the total genome, therefore, DNA fragment polymorphic profiles may not have detected genetic variability but more probably stability in particularly selected sequences (Harding 2004). This variability may be due to accumulating mutations during the process of indirect organogenesis and long-term clonal growth. However, in the current report this genetic variability coupled with secondary metabolite studies could be of great economic importance. Somaclonal variations studies in micropropagated plants in *H. perforatum* have been carried out using RFLP (Haluskova and Cellarova 1997) and cytogenetic analysis (Brutovska et al. 1998), however such studies based on the RAPD analysis are not available. Moreover technical complexities of RFLP, relatively high cost and wide spread use of radioisotopes in the detection method are some of the disadvantages for its routine application in micropropagation system. Therefore RAPD can circumvent some of the problems associated with RFLP analysis. To best of our knowledge this is claimed to be the first report of analysis of clonal fidelity of plants regenerated from adventitious roots. From the presented result it is demonstrated that genetic integrity of micropropagated plants should be ascertained before transferring hardened plants to field.

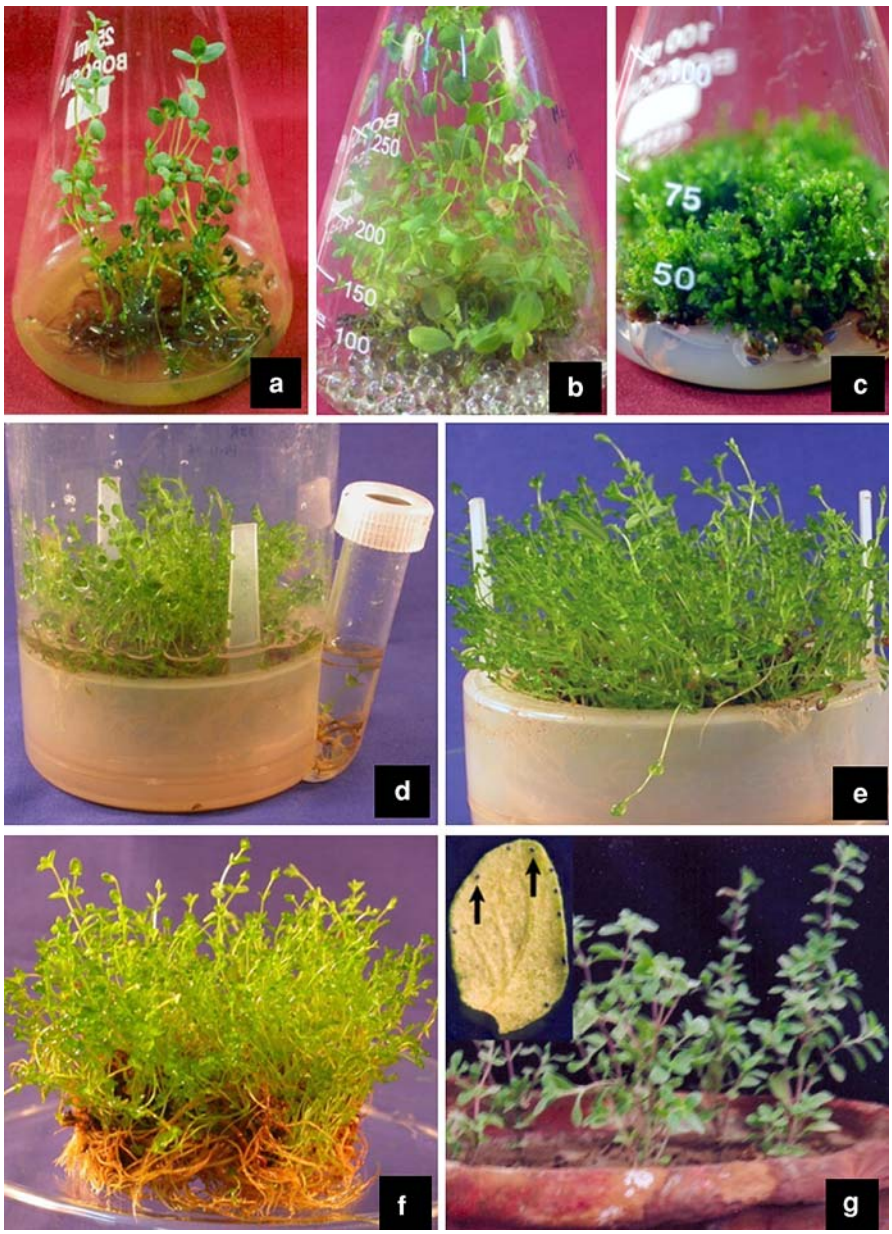

Fig. 2 In vitro shoot multiplication in *H. perforatum*: in liquid ½ MS + 1.0 mg l^{-1} IAA (**a**); glass bead supported static liquid ½ MS + 1.0 mg l^{-1} IAA (**b**); MS + 2.0 mg l^{-1} Kn (**c**); Growtek bioreactor (½ MS + 1.0 mg l^{-1} IAA) (**d–e**); shoot biomass (**f**) and plants in glass house, arrow shows the presence of hypericin glands (inset) (**g**)

Table 6 comparative account of auxin induced caulogenic response of in vitro roots of *H. perforatum* cultured in different physical states of the medium

Growth parameters	Semi-solid	Shake flask	Static with glass beads	Growtek
Biomass (g)	1.78 ± 0.36[a]	4.13 ± 0.78	4.86 ± 0.65	56 ± 10.8
Shoot biomass (g)	0.53 ± 0.12	1.53 ± 0.32	1.67 ± 0.26	21.3 ± 1.6
Growth index (GI)	7.9 ± 1.46	19.65 ± 3.23	23.3 ± 2.81	55 ± 9.6
No. of shoots	141 ± 21.4	226 ± 34.4	244 ± 12.3	b
Shoot length (cm)	0.9 ± 0.4	4.5 ± 2.7	4.9 ± 2.8	4.8 ± 3.1

[a] Each value represents mean ± SD of three replicates

[b] Due to their large number, shoots in Growtek could not be quantified therefore shoot biomass was measured

Present investigations demonstrated that in vitro grown adventitious roots irrespective of its source of origin serve as alternative source of explant for single step in vitro production of plantlets. Further, liquid medium was optimal for faster root and shoot growth. Present study also substantiated earlier findings where seedling root

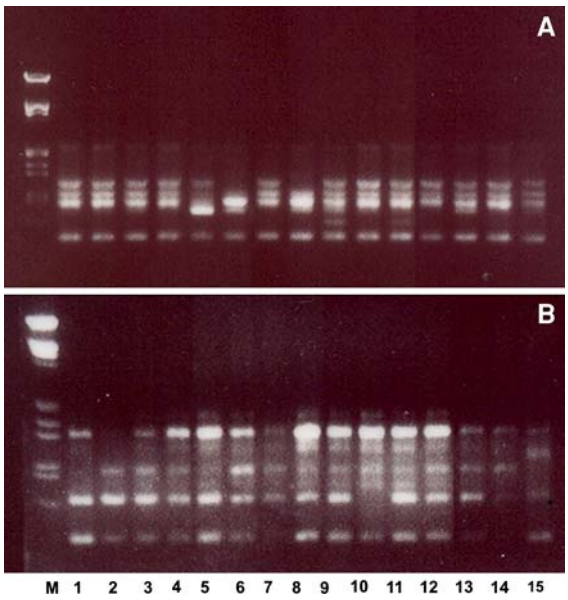

Fig. 3 RAPD profile of *H. perforatum* root cultures and tissue culture raised plants as shown by random primers MAP-8 (**a**) and OPA-5 (**b**), respectively, where M = λ marker DNA *EcoRI*/*Hind*III, 1 = mother plants, 2 and 3 = root derived from shoot and callus cultures and 4–15 = micro propagated plants growing in glass house

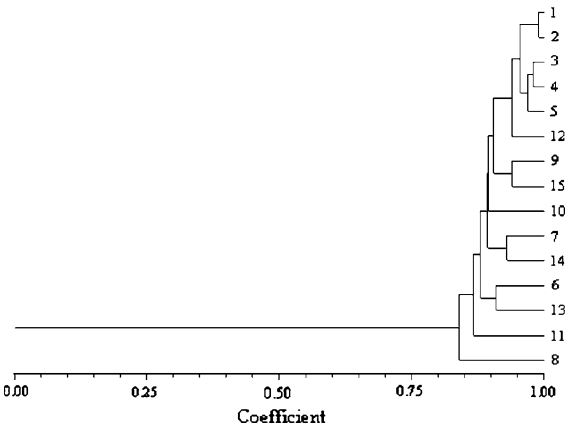

Fig. 4 UPGMA based dendrogram showing similarity among in vitro raised plants of *H. perforatum* derived from adventitious roots

segments were used as explant source (Zobayed and Saxena 2003), however the current work distinctively elaborates the use of adventitious roots developed from in vitro shoots and callus tissue for plantlet production. Moreover, the present investigation describes a low nutrient, low auxin requiring and cytokinin independent single step system for production of multiple shoots through de novo shoot regeneration, which could probably be triggered by the endogenous cytokinin levels. Addition of cytokinin (Kn) also enhanced the shoot multiplication efficiency. This may be helpful in studying the basic aspects of organogenesis as well as applied aspects such as cost effectiveness and ease of handling. In our study, TDZ could not incite the shoot morphogenesis as reported in earlier finding (Zobayed and Saxena 2003). The proximal sections produced large number of buds per section, and the total number of buds from sectioned roots was higher than in entire non-sectioned roots. It is likely that the proximal zone, being closer to the shoot, contained higher levels of endogenous hormones or growth factors required for bud regeneration. It is also possible that the actively elongating distal and middle zones have a limited number of competent responding cells. This could also be attributed to the cut surfaces which are better exposed to the medium or to the effect of wounding, which is known to promote re-differentiation in isolated explants in vitro (Vinocur et al. 2000). Inclusion of support matrix like glass beads in the static liquid cultures enhanced the shoot multiplication and elongation (Fig. 2b). Glass beads have also successfully been used as a support matrix for cost efficient propagation of *Rauwolfia serpentina* (Goel et al. 2007). In the current study also, glass beads rendered the root tissue to remain in partially submerged condition and static nature of glass bead supported cultures and Growtek vessel further proved as a cost efficient means in terms of energy input. Therefore, the chosen culture vessel system (Growtek) is efficient, simple, time saving and cost effective system for high through put plantlet production (Fig. 2d, e). In addition, it is easier to harvest the biomass. Although Growtek^{TM} is a laboratory scale bioreactor, further scale-up is possible however; more studies may still be required to adopt the current protocol at large scale. Dey (2002), successfully used Growtek culture vessel for cost effective mass cloning of several economically important plants. Improved nutrient and oxygen uptake apparently enhanced biomass growth because tissue is supported on a floating raft that keeps the tissue in constant contact with the medium. There was an incessant production of roots along with shoots in the same medium making this a single step. The in vitro roots retained morphogenetic potential over the generations. The micro-shoots produced can further be multiplied or rooted, or alternatively they can also be explored for their potential for aseptic biomass production for secondary metabolites. In vitro roots are a highly potential source for root or shoot biomass production and the use of large-scale liquid cultures using Growtek vessel can further augment proliferation and growth in large size bioreactors.

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