International Journal of Plant Developmental Biology ©2010 Global Science Books

Effect of Plant Growth Regulators on Callus Formation, Plant Regeneration and Hypericin Production in *Hypericum mysorense* Hyne

H. P. Shilpashree[1] • V. Ravishankar Rai[2*]

[1] Department of Biotechnology, University of Mysore, Mysore, Karnataka, India
[2] Department of Microbiology, University of Mysore, Mysore, Karnataka, India

Corresponding author: * **rrai33@hotmail.com**

ABSTRACT

Hypericum mysorense Hyne is a pharmaceutically important medicinal plant found in Western Ghats of India. In this study a new system was developed for *in vitro* plant regeneration through callus formation and hypericin production. Leaf explants were inoculated onto MS (Murashige and Skoog) medium supplemented with different concentrations and combinations of 6-benzyl adenine (BA), kinetin (KIN), α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D). Highest callus proliferation was observed when MS medium contained BA and NAA (1.0 mg/L each). Best shoot induction and multiplication was recorded on MS medium supplemented with 1.0 mg/L BA. A significant difference was recorded in average number and length of shoots/explant among the different concentrations of BA and KIN investigated. Roots were induced on MS media in the presence (0.5 mg/L) and absence of indole-3-acetic acid (IAA). Regenerated plantlets were successfully established in soil with a 98% survival rate. The concentration of hypericin was evaluated in different tissues of *in vitro* and *ex vitro* grown plants by using high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Hypericin levels were higher in callus (5.0 µg/g, w/v) and leaves (4.1 µg/g, w/v) of *in vitro* plantlets than those of *ex vitro* leaf samples (3.1 µg/g, w/v). Results confirmed that plant growth regulators play an important role in the production of hypericin under controlled *in vitro* conditions. This is the first ever report on a rapid plant regeneration system for *H. mysorense* and provides an avenue for conservation strategies and phytomedicine production.

Keywords: bioactive compound, HPLC, *in vitro* cultures, medicinal plant, micropropagation, plant growth regulator
Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; **BA**, 6-benzyl adenine; **HPLC**, high performance liquid chromatography; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **KIN**, kinetin; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid; **TLC**, thin layer chromatography

INTRODUCTION

Hypericum is a large genus of herbs and shrubs belonging to the family Hypericaceae, which grows widely in temperate regions. These species have been used as traditional medicine due to their wound-healing (Yazaki and Okuda 1990), bactericidal (Ishiguro *et al.* 1998), anti-inflammatory (Dias *et al.* 1998) and antidepressant (Butterweck 2003) characters. *Hypericum* spp. contain a complex mixture of bioactive substances mainly phloroglucinols, naphtodianthrone, flavonoids, phenylpropanes, essential oils and xanthones, which possess a wide array of biological properties (Greeson *et al.* 2001; Patočka 2003).

Among them, *Hypericum mysorense* is an important medicinal plant found in the southern parts of Karnataka at 900-1500 m above sea level. Extracts of *H. mysorense* have been reported for its *in vitro* cytotoxicity and anti-tumour activity tested against HEp-2, RD, Vero and DLA cell lines (Vijayan *et al.* 2003). Also extracts of *H. mysorense* exhibited significant antiviral activity against herpes simplex virus type-I (Vijayan *et al.* 2004) and antimicrobial activity against several bacterial and fungal pathogens (Pulok *et al.* 2002). Many essential fatty acids, which have been identified from leaves and flower extracts, showed antimicrobial activity against human pathogens (Shilapshree and Ravishankar 2009). A few natural compounds have also been identified with antifungal activity (Vishwakarma *et al.* 1983).

Hypericin is a natural pigment commonly present in *Hypericum* spp., has antidepressant (Butterweck *et al.* 2002) and antiviral activity against HIV (Lavie *et al.* 1989; Park *et al.* 1998), and is a potential antitumour photosensitiser for photodynamic cancer therapy (Vantieghem *et al.* 2001; Agostinis *et al.* 2002). Considering the pharmaceutical significance, commercial application and its possible use in therapeutics, it is important to screen *Hypericum* spp. for the presence of hypericin and to increase production using *in vitro* cultures. Plant cell culture is often an effective system to study the biological significance of bioactive metabolites under *in vitro* conditions as well as for producing natural products for bioprocess applications (Yanpaisan *et al.* 1999). Therefore, *in vitro* cultivation of *H. mysorense* would be an effective technique to provide plant material with a uniform phytochemical profile.

Previous research has been conducted to mass multiply *Hypericum* spp. using different plant growth regulators (PGRs) in *H. perforatum* (Santarém and Astarita 2003; Gadzovska *et al.* 2005), *H. foliosum* (Moura 1998), *H. polyanthemum* (Bernardi *et al.* 2007), *H. canariense* (Mederos 1991), *H. erectum* (Yazaki and Okuda 1990), *H. patulum* (Baruah *et al.* 2001) and *H. brasiliense* (Cardoso and Oliveira 1996). Investigations in *Hypericum* spp. have been reported for the production of hypericin in *in vitro* cell cultures by using PGRs as enhancers (Kartnig and Brantner 1990; Kartnig *et al.* 1996; Gadzovska *et al.* 2005; Liu *et al.* 2007a).

The aim of the present study was to develop an efficient protocol for rapid mass multiplication of *H. mysorense*

Received: 23 November, 2009. Accepted: 1 August, 2010.

Original Research Paper

using different PGRs and simultaneously to investigate the effect of PGRs on hypericin production in *in vitro* cultures.

MATERIALS AND METHODS

Collection of plant material

H. mysorense samples were collected in July from the Western Ghats of Karnataka, India. Young leaves were collected from a one-year-old plant and were cut transversally into 0.5-1.0 cm fragments. The explants were washed in running tap water for 30 min to remove surface adherents. Following this, explants were treated with an antibiotic (ampicillin, 100 mg/L) and fungicide (bavistin, 30 g/L) to prevent contamination. Later, explants were surface sterilized using an aqueous solution of 0.01% mercuric chloride (w/v) for 2 min and rinsed in sterile distilled water 2-3 times.

Culture medium, PGRs and growth conditions

1. Callus initiation

The leaf explants (1-2 cm in size) were cultured on MS medium (Murashige and Skoog 1962) containing sucrose (30 g/L) and different combinations of PGRs, including BA, KIN, NAA and 2,4-D for callus initiation (**Table 1**). The pH of all media was adjusted to 5.8 with 1N HCL or 1N NaOH before the addition of a gelling agent, phytagel (2.5 g/L). Then, medium was dispensed into 15-mL aliquots into 250 mL culture bottles and autoclaved at 121°C for 20 min All cultures were incubated at 22 ± 2°C under a 16-h photoperiod of 60 $\mu molm^{-2}s^{-2}$ irradiance provided by white fluorescent tubes (40V, Phillips, India) for 1-2 weeks. Control explants were cultured on medium devoid of PGRs. The frequency of callus formation was determined 2-3 weeks after culture initiation.

2. Shoot initiation and multiplication

To induce shoot initiation callus cultures were transferred to MS medium containing different concentrations of BA and KIN. After 2 weeks' incubation shoots were transferred to MS medium containing different concentrations of BA (0.5-2.0 mg/L) and KIN (0.5-2.0 mg/L) for multiple shoot proliferation. Cultures were serially subcultured every 4 weeks to maximize shoot multiplication. The number and length of shoots were recorded before each subculture.

3. In vitro rooting and acclimatization

Regenerated shoots that had elongated to 8-12 cm were excised and transferred to MS full- and half-strength medium with different concentrations of IAA and IBA or without PGRs for root induction. The percentage of rooting, average number and length of roots per explant was recorded after 2-3 weeks of incubation (**Table 2**). Regenerated plantlets were removed from culture tubes, washed gently under running tap water to remove adherent media and transferred to pots containing Soilrite® (Indo American Hybrid Seeds Ltd., India). *In vitro* derived plants were covered with polythene bags in order to maintain ambient humidity at 90% for 2-3 weeks. Later, acclimatized plants were gradually exposed to greenhouse (25 ± 22°C) conditions, which were maintained under partial shade for 2 weeks before subsequent transfer to the field. The plants were supplemented daily with $1/10^{th}$ of the MS medium for hardening. The survival rate was recorded once a week for 2 months.

Extraction of hypericin and HPLC analysis

Extraction of *in vitro* samples from both callus and young leaves were performed by using freeze-dried, lyophilized powdered plant material of 30 days culture, and then stored at -40°C until chemical analysis. *Ex vitro* samples (natural plants) were collected directly from a one-year-old plant, dried and powdered by using a mortar for hypericin extraction. 10 g of each sample was pulverized in 100 mL chloroform using a mortar to remove pigments (including carotenoids), lipids and resinous substances. The lipophilic fractions of all the extracts were filtered, dried and again extracted with 50 mL methanol using an ultrasonic sonicator (Ultra sonic FS-14 Sonicator, Fisher Scientific, Canada) for 16 min at 20 kHz. The extracts were evaporated to dryness under reduced pressure and purified by column chromatography using Sephadex LH-20 (Sigma, India). 100 g of Sephadex LH-20 was weighed and left in 900 ml of methanol overnight. The column material was then packed into a 3.5 cm diameter and 90 cm length glass column with a teflon top. 1.0 g of carotenoid-pigment free extract was dissolved in 5 ml of methanol and loaded into the Sephadex LH-20 column. The column was eluted with methanol at the flow rate of 1.2 mL/min. The first 250 ml of fraction was assumed as a void volume. The fractions were collected with a fraction collector; a total of 55 fractions of 5 ml per tube were collected. Later all the fractions were subjected to UV spectroscopy (Eppendorf, Germany) measurements by scanning from 200-600 nm. The initial fractions were found to contain flavonoids with maximum absorbance at 260-350 nm. Remaining fractions were measured at 590 nm since hypericin has highest absorbance at 590 nm (Dias *et al.* 1999). Hypericin was detected in the 54^{th} fraction with maximum absorbance at 590 nm. Simultaneously fractions were checked for the presence of hypericin by using HPLC (Shimadzu Corporation, Japan. Model-LC-10 ADVP Pump and SPD-10AV UP UV- Vis detector). For separation HPLC column used was C-18 column (150 × 4.6 mm, 5 µm) (Phenomenex, India). The mobile phase consisted of an isocratic mixture of methanol: phosphate buffer: ethyl acetate (370: 130: 100 w/w/w). The pH of the phosphate buffer (5.0 mM) (Sigma, India) was adjusted to 2.1 by using phosphoric acid (Merck, India). The flow rate, column temperature and detection wavelength were set at 1.0 mL/min, 30°C and 590 nm, respectively. The sample injection volume was 10 µL and the run time was 45 min. The quantity of compounds was calculated by an external standard calibration curve of hypericin in the concentration range 0.5-100 µg/mL ($r^2 = 0.999$). The HPLC chromatogram was monitored at 590 nm and the obtained data were compared with authentic standard hypericin (Sigma, India), based on retention time, UV spectrum and peak area by using Shimadzu LC Solution Software.

The presence of hypericin was also confirmed by using thin layer chromatography (TLC). TLC silica gel 60 F_{254} aluminum sheets of 20 × 20 cm in size (Merck, India) was used with toluene: ethyl acetate: formic acid (50: 40: 10 v/v/v) as the mobile phase. The fractions obtained from column chromatography were subjected to TLC along with standard hypericin. The TLC plates were sprayed with vanillin (1%) and sulphuric acid (1%) reagent (Merck, India). Later TLC plates were dried and heated to 120°C on a hotplate for 5 min for the identification of hypericin.

Standards and reagents

Authentic standards, antibiotics, PGRs and reagents were purchased from Sigma, India and HPLC grade solvents were purchased from Merck, India. Standard stock solutions was prepared at 1.0 mg/mL concentration using HPLC grade methanol and stored at -20°C.

Statistical analysis

Data on the development of *in vitro* cultures was based on 15 replicates. For hypericin, quantification was based on three independent experiments. Mean values were subjected to analysis of variance (ANOVA) using SPSS software (v. 16.0). Significant differences were compared by Duncan's multiple range test at $P = 0.05$. The results are expressed as means ± standard error (SE).

RESULTS

Effect of PGRs on callus induction and shoot multiplication

Leaf explants cultured on MS medium supplemented with different PGRs were able to initiate callus within 2 weeks of incubation (**Table 1**). No response was seen in PGR-free medium (control). The highest percentage callus formation

Table 1 Effect of different growth regulators present in MS medium on callus initiation of *Hypericum mysorense* using leaf as explants.

Growth regulators in MS medium (mg/L)	Percentage of callus formed
BA (1.0)	35.1 ± 1.09 b
BA (2.0)	45.2 ± 1.02 a
KIN (1.0)	30.6 ± 0.94 c
KIN (2.0)	19.2 ± 1.71 d
BA (1.0) + NAA (0.5)	81.2 ± 0.98 b
BA (1.0) + NAA (1.0)	96.4 ± 1.02 a
BA (2.0) + NAA (0.5)	67.5 ± 1.23 c
BA (2.0) + NAA (1.0)	63.9 ± 1.34 d
BA (1.0) + 2,4-D (0.5)	31.3 ± 1.81 b
BA (1.0) + 2,4-D (1.0)	39.5 ± 1.01 a
BA (2.0) + 2,4-D (0.5)	24.3 ± 1.22 c
BA (2.0) + 2,4-D (1.0)	19.1 ± 1.91 d
KIN (1.0) + NAA (0.5)	53.8 ± 1.22 b
KIN (1.0) + NAA (1.0)	61.9 ± 1.44 a
KIN (2.0) + NAA (0.5)	33.7 ± 1.03 c
KIN (2.0) + NAA (1.0)	28.3 ± 2.01 d
KIN (1.0) + 2,4-D (0.5)	32.4 ± 1.09 a
KIN (1.0) + 2,4-D (1.0)	26.5 ± 1.21 b
KIN (2.0) + 2,4-D (0.5)	20.7 ± 1.42 c
KIN (2.0) + 2,4-D (1.0)	15.3 ± 1.53 d

Values represent mean ± standard error. Values followed by a letter are significantly different according to Duncan's multiple range test at $P = 0.05$.

Table 2 Effect of different auxins present in MS medium on root formation in *Hypericum mysorense*.

Medium + auxins (mg/L)	Percentage of rooting	Number of roots (cm)	Length of roots (cm)
MS + IAA (0.1)	25.1 ± 0.22 c	5.21 ± 1.22 b	3.15 ± 1.09 b
MS + IAA (0.5)	97.3 ± 2.34 a	11.20 ± 2.11 a	5.10 ± 0.12 a
MS + IAA (1.0)	65.6 ± 2.90 b	2.14 ± 1.34 c	1.17 ± 0.33 c
1/2MS + IAA (0.1)	34.2 ± 3.45 c	4.01 ± 1.82 b	2.80 ± 0.91 b
1/2MS + IAA (0.5)	57.0 ± 4.10 a	3.45 ± 1.28 c	3.33 ± 1.45 a
1/2MS + IAA (1.0)	49.5 ± 3.22 b	5.71 ± 2.34 a	2.01 ± 1.45 c
MS + IBA (0.1)	21.2 ± 1.09 b	3.12 ± 3.43 a	1.04 ± 1.72 c
MS + IBA (0.5)	30.1 ± 2.33 a	2.44 ± 3.91 b	1.97 ± 1.92 a
MS + IBA (1.0)	18.2 ± 1.99 c	1.22 ± 2.37 c	1.54 ± 2.09 b
1/2MS + IBA (0.1)	23.3 ± 1.57 a	2.56 ± 2.14 b	1.60 ± 1.03a
1/2MS + IBA (0.5)	20.5 ± 1.71 b	3.24 ± 2.27 a	1.01 ± 1.68c
½ MS + IBA (1.0)	16.1 ± 1.31 c	1.21 ± 1.57 c	1.23 ± 1.39b
MS free (Control)	74.1 ± 2.25 a	8.16 ± 2.62 a	4.55 ± 2.11a
1/2MS free	57.3 ± 1.78 b	6.80 ± 1.15 b	3.91 ± 2.10 b

Values represent mean ± standard error. Values followed by a letter are significantly different according to Duncan's multiple range test at $P = 0.05$.

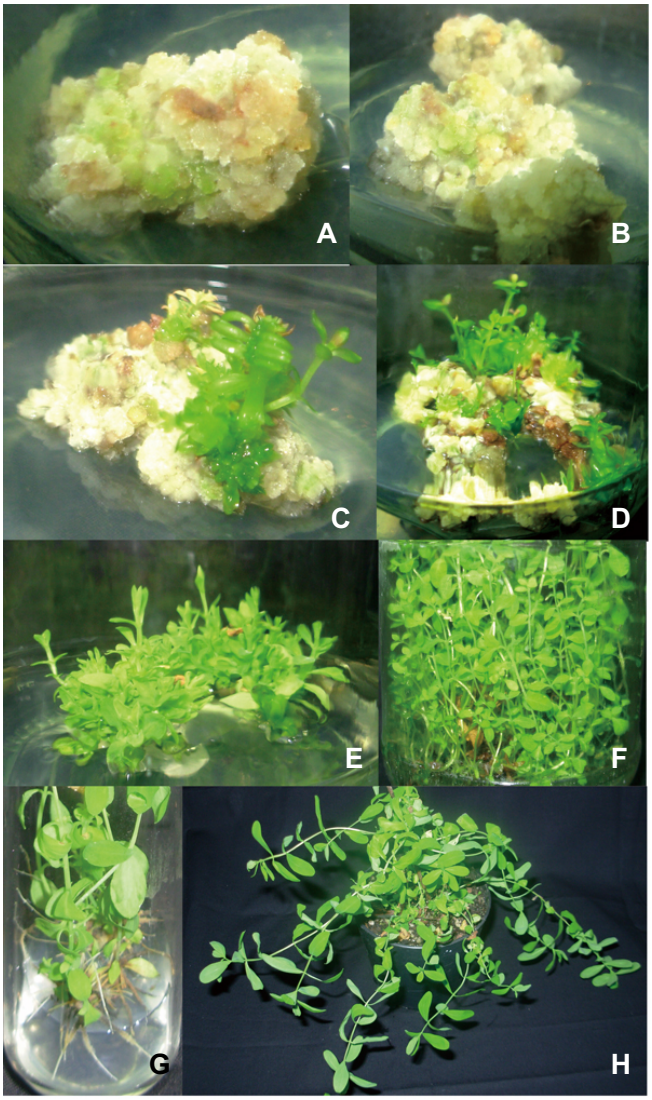

Fig. 1 ***In vitro*** **plant regeneration of** ***Hypericum mysorense.*** (**A-B**) Callus formed on MS medium with BA + NAA at (1.0 mg/L) after 3 weeks of incubation. (**C-D**) Axilary shoots developed on the surface of callus on MS medium containing BA (1.0 mg/L). (**E-F**) Multiple shoot proliferation and elongation on MS medium containing BA (1.0 mg/L). (**G**) Roots formation after one week of incubation on MS medium containing IAA (0.5 mg/L). (**H**) Acclimatized *in vitro* plant developed in greenhouse conditions.

occurred on MS medium supplemented with BA + NAA at 1.0 mg/L (96.4 ± 1.02%) (**Figs. 1A, 1B**). However, 1.0 mg/L KIN +1.0 mg/L NAA initiated little callus (61.9 ± 1.44%) compared to 1.0 mg/L BA + 1.0 mg/L NAA. 2,4-D, along with BA and KIN initiated callus within 1 week but necrotic callus was gradually observed. Therefore, the BA + NAA combination at 1.0 mg/L was the best PGR combination to obtain maximum fresh weight of healthy callus.

The maximum shoot initiation and multiplication through callus was observed on MS medium with 1.0 mg/L BA within 2 weeks (**Fig. 1C-F**). Shoots also could be proliferated in MS medium containing 2.0 mg/L BA, but shoot multiplication was reduced after 2-3 weeks of incubation, leading to shoot hyperhydricity, decreased vigor then death. Shoots induced on KIN (1.0 mg/L) medium were stunted and had a slow multiplication rate (61.2% shoots produced/explant). MS medium supplemented with only 1.0 mg/L BA could efficiently and constantly initiate and multiply shoots (98.8%) within 18 days of incubation. The number and length of shoots regenerated per explants is shown in **Fig. 2**.

Effect of auxins on root induction

Rooting was observed in the presence or absence of PGRs. Roots of 2–5 cm in length were produced within a period of 2 weeks; these branched profusely (**Fig. 1G**) in MS medium containing 0.5 mg/L IAA and in PGR-free medium with a high survival percentage (97.0 ± 2.34 and 74 ± 2.25, respectively). At higher concentration of IBA (1.0 mg/L) roots became necrotic and were not considered for further investigations. The percentage of rooting, average number and length of roots formed are shown in **Table 2**.

Acclimatization

A crucial aspect of *in vitro* propagation is to acquire regenerated plants that are capable of surviving in a natural environment. The acclimatization stage was successfully performed by transplanting the plantlets to pots containing Soilrite® in greenhouse conditions. The regenerated plantlets were successfully acclimatized with a 98% survival rate (**Fig. 1H**). The plants did not show detectable variation with respect to morphological or growth characteristics when compared with the respective parent plant.

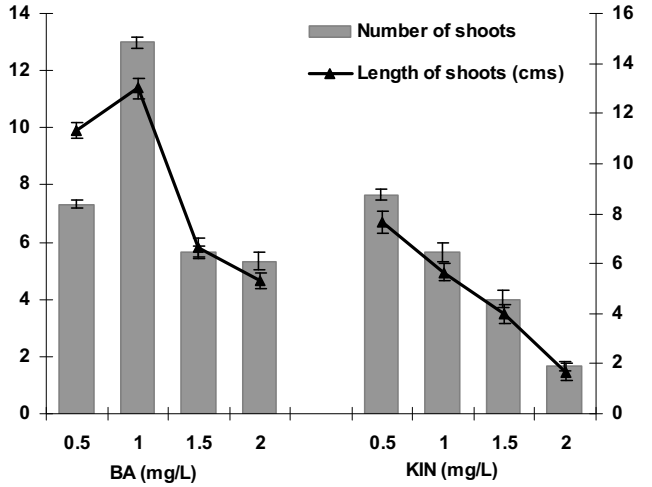

Fig. 2 Effect of cytokinins (BA and KIN) on shoot multiplication of *Hypericum mysorense.* Data represented as mean values. The significant differences were compared by using Duncan's multiple range test at P = 0.05 using SPSS software (v. 16.0). Error bar indicates standard deviation of fifteen replicates.

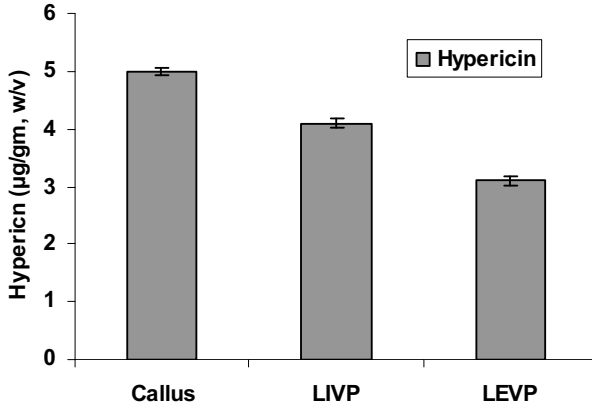

Fig. 3 Comparison of hypericin levels in callus, LIVP (leaves of *in vitro* plants) and LEVP (leaves of *ex vitro* plants) of *Hypericum mysorense*. Data represented as mean values. The significant differences were compared by using Duncan's multiple range test at *P* = 0.05 using SPSS software (v. 16.0). Error bar indicates standard deviation of three replicates.

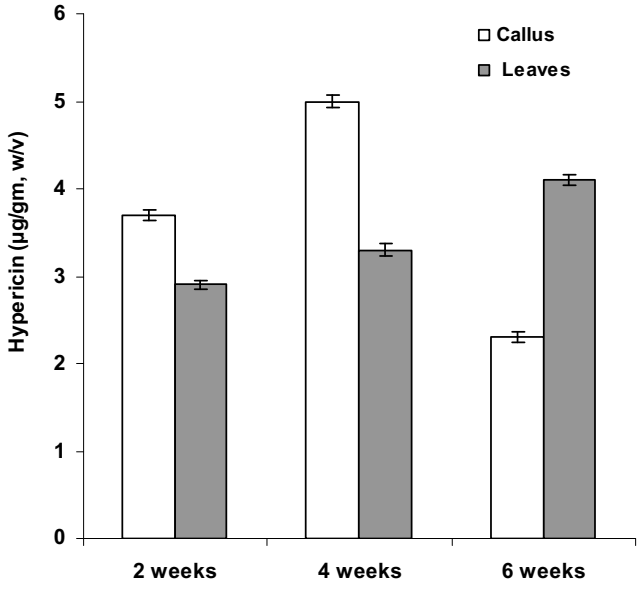

Fig. 4 Comparison of hypericin levels produced during different stages of *in vitro* plant development of *Hypericum mysorense*. Data represented as mean values. The significant differences were compared by using Duncan's multiple range test at *P* = 0.05 using SPSS software (v. 16.0). Error bar indicates standard deviation of three replicates.

Identification and quantification of hypericin

HPLC analysis of the methanolic extracts of *in vitro* (callus and leaves) and *ex vitro* (leaves) samples allowed hypericin to be identified and quantified by comparing with an authentic standard sample on the basis of retention time, UV spectrum and peak area of the HPLC chromatogram. Under optimized condition the retention time of standard hypericin was recorded at 15.85 min which was the same for all the samples tested. The presence of hypericin was also confirmed by using TLC. Hypericin spot was visualizated as red after spraying with vanillin (1%) and sulphuric acid (1%) reagent.

The highest amount of hypericin was present in callus (5.0 µg/g, w/v) compared to leaves (4.1 µg/g, w/v) of *in vitro* cultures (**Fig. 3**). Hypericin levels were also evaluated after every 2 weeks of incubation during *in vitro* developmental stages of 2-, 4- and 6-week cultures. Most hypericin was detected in the 4th week of callus culture (5.0 µg/g, w/v) and leaves (4.1 µg/g, w/v) collected at the 6th week of culture (**Fig. 4**). Leaf extracts of *ex vitro* plantlets contained 3.1 µg/g (w/v) of hypericin.

Effect of PGRs on hypericin production

To study the effect of PGRs on hypericin production, *in vitro* cultures grown in the presence and absence (control) of PGRs were used. Leaf extracts of *in vitro* cultures grown in the presence of PGRs accumulated significantly large amounts of hypericin while a trace amount was detected in the control. Among all the *in vitro* cultures examined, most hypericin was noted in callus (2.3-5.0 µg/g, w/v) followed by leaf extracts (2.9-4.1 µg/g, w/v). The HPLC profile of isolated and standard hypericin from callus cultures is shown in **Fig. 5**. Callus did not form in PGR-free medium (control) for hypericin identification. Therefore, BA and NAA at 1.0 mg/L present in MS medium act as complementary inducers for the production of hypericin in callus cultures. Similarly, BA (1.0 mg/L), when present alone in MS medium, influenced multiple shoot proliferation and stimulated hypericin levels in leaves. The HPLC analysis of leaf extracts of *ex vitro* cultures showed less hypericin (3.1

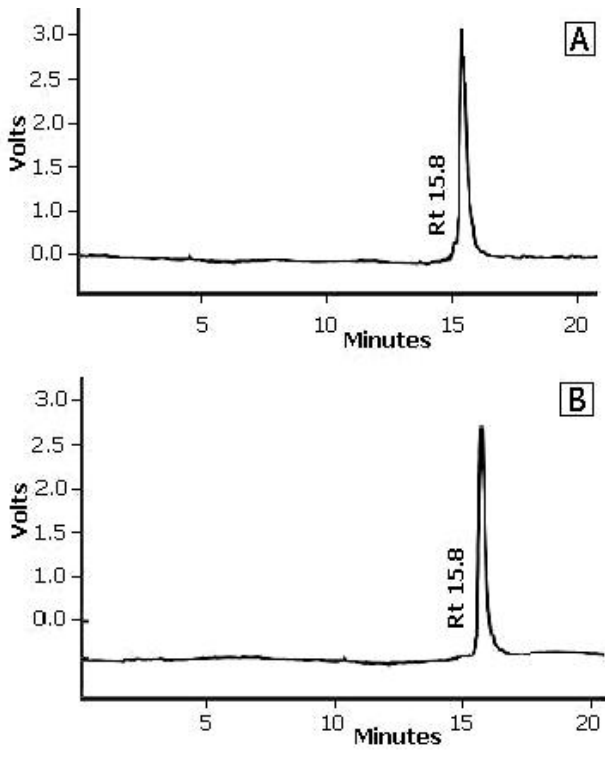

Fig. 5 HPLC profile of isolated hypericin from callus culture of *Hypericum mysorense*. (**A**) Isolated hypericin. (**B**) Standard hypericin.

µg/g, w/v) than *in vitro* cultures (**Fig. 3**). The hypericin in the parent plant accumulated to 3.0 µg/g, (w/v) in leaf samples (pers. obs.), which is similar to *ex vitro* (3.1 µg/g, w/v) samples. Therefore, callus culture is the best source for the large-scale production of hypericin and PGRs are necessary for the biosynthesis of this bioactive compound during *in vitro* plant regeneration of *H. mysorense*.

DISCUSSION

Effect of PGRs on callus formation

The PGRs used for callus formation varied at different PGR combinations. MS medium supplemented with 1.0 mg/L BA and 1.0 mg/L NAA was most effective to initiate and multiply callus, which was intact and yellowish. In contrast, in the other treatments MS medium containing 2,4-D (0.1 mg/L) + BA (0.5 mg/L) callus was dark brownish and less intact and became necrotic after 2 weeks' incubation. A similar observation was recorded in *H. perforatum* cultures (Pretto and Santarém 2000). According to previous reports, 2,4-D (0.2 mg/L) + KIN (0.02 mg/L) were effective PGRs for callus induction in *H. perforatum* cell suspension cultures (Bais *et al*. 2002; Walker *et al.* 2002). Similarly Ayan *et al*. (2005) reported optimum callus formation in the presence of 2,4-D + KIN at 0.5 mg/L. Also, callus cultures have been developed in *H. perforatum* by culturing leaf explants in the presence of 2,4-D + KIN at 0.1 mg/L (Wójcik and Podstolski 2007). In *H. brasiliense*, nodal explants could only form callus in the presence of 2,4-D (2.0 mg/L) or NAA (10 mg/L) using either MS or B_5 medium (Cardoso and Oliveira 1996). In the present study BA (1.0 mg/L) + NAA (0.5 mg/L) resulted in good callus formation (Table 1), but callus was dark brownish, possibly indicating the presence of phenolic acids. Similar observation was repoted in *H. perforatum in vitro* cultures (Gadzovska *et al.* 2003). In this study MS medium with BA and NAA at 1.0 mg/L were essential to establish *H. mysorense* callus cultures.

Effect PGRs on shoot initiation and multiplication

The highest frequency of shoot initiation and multiplication through callus was recorded in the presence of 1.0 mg/L BA alone (**Fig. 2**). Similar results were observed in previous studies in *H. perforatum* by using BA at 1.0 mg/L (Čellárová *et al.* 1992; Pretto and Santarém 2000; Ayan *et al.* 2005) and BA at 0.4 mg/L in *H. polyanthemum* (Bernardi *et al.* 2007). However, cytokinins were frequently used in combination with auxins for shoot regeneration of different *Hypericum* species: BA (0.1-1.0 mg/L) + NAA (0.5 mg/L) in *H. canariense* (Mederos 1991) and *H. foliosum* (Moura 1998); BA (1.0 mg/L) + IAA (0.5 mg/L) in *H. perforatum* (Wójcik and Podstolski 2007). In the present study high concentrations of BA (2.0 mg/L) reduced the shoot length and multiplication after 2-3 sub cultures (**Fig. 2**). A similar observation was noted in *H. perforatum* (Gadzovska *et al.* 2003). Therefore, standardization of *in vitro* plant regeneration of a particular species is important for further investigation of pharmaceutically active compounds.

Effect of PGRs on root formation

IBA, IAA and NAA have commonly been used to improve rooting in different *Hypericum* species. The differential response of auxins has been reported in previous studies. In *H. canariense* roots were induced either in the presence of IBA (1.0 mg/L) or NAA (0.5 mg/L) (Mederos 1991). In *H. perforatum* IAA and IBA at 0.05-1.0 mg/L were the most effective for root formation (Gadzovska *et al.* 2005). However, these PGRs did not efficiently induce roots in *H. foliosum* (Moura 1998). Half-strength MS medium with or without IBA (1.0-1.5 mg/L) were also successful in inducing roots in *H. perforatum* (Pretto and Santarém 2000; Santarém and Astarita 2003). In the present investigation MS medium with 0.5 mg/L IAA and PGR-free medium were more effective in initiation and elongation of roots, respectively (**Table 2**). Roots induced on IAA-containing medium were thick and adventitious while those induced in the presence of IBA were thin, fragile and short.

The benefit of any micropropagation system can be fully realized by transferring the plantlets from tissue culture to *ex vitro* conditions (Hazarika 2003). Regenerated plantlets grew well in greenhouse conditions without any phenotypic changes with 98% survival in the field. Therefore, callus culture could be used for the large-scale production of hypericin and PGRs are necessary for the biosynthesis of the bioactive compound, hypericin.

Effect of PGRs on hypericin production

Establishment of protocols for cultivation of medicinal plants using different PGRs to enhance the production of bioactive compounds is required for commercial and research applications. Studies have been reported for the production of hypericin and flavonoids in cell cultures of *H. perforatum* (Kartnig and Brantner 1990; Yazaki and Okuda 1990). Hypericin found in cell cultures are of the same concentration of those usually found in the whole plant (Kartnig *et al.* 1996). In *H. perforatum* callus cultures, BA (4.0-5.0 mg/L) did not changed hypericin contents (15-20 µg/g) but improved the production of hypericin (25–50 µg/g) at BA concentration range from 0.1 to 2.0 mg/L (Gadzovska *et al.* 2005). However, hypericin was identified only in *in vitro* plants (0.3 mg/g) and *in vitro* shoots (1.5 mg/g) but not in callus cultures of *H. perforatum* (Dias *et al.* 1999). In another study high level of hypericin was detected in shoots (0.5 mg/g) than in callus cultures (0.11 mg/g) (Santarém and Astarita 2003). In another study hypericin was identified in trace amounts or was absent in *H. perforatum* callus cultures compared to differentiated tissues (Kirakosyan *et al.* 2000a). In our study reports that callus (5.0 µg/g, w/v) accumulated hypericin in higher levels than leaves (4.1 µg/g, w/v). This divergent result might be due to different PGRs, culture medium and growth condition used. Verma *et al.* (2008) reported synthesis of hypericin is strongly correlated with genetic and environment factors. Hypericin levels varied from 0.26-18.0 mg/g of plant material in different *H. perforatum* collected from different locations in India, which were analyzed by using DNA markers. Natural products accumulate in cultured cells of medicinal plants at higher levels than those in native plants through optimized culture conditions (Mulabagal and Tsay 2004). Therefore, results indicated that callus cultures grown with hormonal supplementation (BA and NAA at 1.0 mg/L) were able to acquire certain degree of differentiation and capability for production of secondary metabolites like hypericin in *H. mysorense*.

CONCLUDING REMARKS

To the best of our knowledge this is the first ever report for the micropropagation of *H. mysorense* and simultaneous production of hypericin under the influence of specific PGRs. The procedure reported here allowed for micropropagation within a period of 3 months. Furthermore, standardized HPLC and TLC protocols could be used for further characterization of phytochemicals of pharmaceutical interest.

REFERENCES

Agostinis P, Vantieghem A, Merlevede W, de Witte P (2002) Hypericin in cancer treatment, more light on the way. *The International Journal of Biochemistry and Cell Biology* **34**, 221-241

Ayan AK, Çirak C, Kevseroğlu K, Sökmen A (2005) Effects of explant types and different concentrations of sucrose and phytohormones on plant regeneration and hypericin content in *Hypericum perforatum* L. *Turkish Journal of Agriculture and Forestry* **29**, 197-204

Bais HP, Walker TS, McGrew JJ, Vivanco JM (2002) Factors affecting growth of suspension culture of *Hypericum perforatum* L. and production of hypericin. *In vitro Cellular and Developmental Biology – Plant* **38**, 58-65

Baruah A, Sarma D, Saud J, Singh RS (2001) *In vitro* regeneration of *Hypericum patulum* – A medicinal plant. *Indian Journal of Experimental Biology* **39**, 947-949

Bernardi APM, Maurmann N, Rech SB, Poser GV (2007) Benzopyrans in *Hypericum polyanthemum* Klotzsch ex Reichardt cultured *in vitro*. *Acta Physiologiae Plantarum* **29**, 165-170

Briskin DP, Gawienowski MC (2001) Differential effects of light and nitrogen on production of hypericins and leaf glands in *Hypericum perforatum*. *Plant Physiology and Biochemistry* **39**, 1075-1081

Butterweck V (2003) Mechanism of action of St. John's wort in depression. What is known? *CNS Drugs* **17**, 539-562

Butterweck V, Bockers T, Korte B, Wittkowski W, Winterhoff H (2002) Long-term effects of St. John's wort and hypericin on monoamine levels in rat hypothalamus and hippocampus. *Brain Research* **930**, 21-29

Cardoso MA, Oliveira DE (1996) Tissue culture of *Hypericum brasiliense* Choisy, shoot multiplication and callus induction. *Plant Cell, Tissue and Organ Culture* **44**, 91-94

Čellárová E, Kimákova K, Brutóvska R (1992) Multiple shoot formation and phenotypic changes of R_0 regenerants in *Hypericum perforatum* L. *Acta Biotechnologica* **12**, 445-452

Dias ACP, Francisco A, Tomás-Barberán FA, Fernandes-Ferreira M, Ferreres F (1998) Unusual flavonoids produced by callus of *Hypericum perforatum*. *Phytochemistry* **48**, 1165-1168

Dias ACP, Seabra RM, Andrade PB, Fernandes-Ferreira F (1999) The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from *in vivo* and *in vitro* biomass of *Hypericum* species. *Journal of Chromatography and Relative Technology* **22**, 215-227

Gadzovska S, Maury S, Ounnar S, Righezza M, Kascakova S, Refregiers M, Spasenoski M, Joseph C, Hagège D (2005) Identification and quantification of hypericin and pseudohypericin in different *Hypericum perforatum* L. *in vitro* cultures. *Plant Physiology and Biochemistry* **43**, 591-601

Greeson JM, Sanford B, Monti DA (2001) St. Jonh's wort (*Hypericum perforatum* L.), a review of the current pharmacological, toxicological and clinical literature. *Psychopharmacology* **153**, 402-414

Hazarika BN (2003) Acclimatization of tissue cultured plants. *Current Science* **85**, 1704-1712

Ishiguro K, Nagareya N, Fukomoto H (1998) A phloroglucinol derivative from cell suspension cultures of *Hypericum perforatum*. *Phytochemistry* **47**, 1041-1043

Karppinen K, György Z, Kauppinen M, Tolonen A, Jalonen J, Neubauer P, Hohtolo A, Häggman H (2006) *In vitro* propagation of *Hypericum perforatum* L and accumulation of hypericins, pseudohypericins and phloroglucinols. *Propagation of Ornamental Plants* **6**, 170-179

Kartnig T, Brantner A (1990) Secondary constituents in cell cultures of *Hypericum perforatum* and *Hypericum maculatum*. *Planta Medica* **56**, 634-637

Kartnig T, Gobel I, Heydel B (1996) Production of hypericin, pseudohypericin and flavonoids in cell culture of various *Hypericum* species and their chemotypes. *Planta Medica* **62**, 51-53

Kirakosyan A, Vardapetyan RR, Charchoglyan AG (2000a) The content of hypericin and psuedohypericin in cell cultures of *Hypericum perforatum*. *Russian Journal of Plant Physiology* **47**, 270-273

Kirakosyan A, Hayashi HI, Inoue K, Charchoglyan A, Vardapetyan H (2000b) Stimulation of the production of hypericins by mannan in *Hypericum perforatum* shoot cultures. *Phytochemistry* **53**, 345-348

Lavie G, Valentine F, Levin B, Mazur Y, Gallo G, Lavie D, Weiner D, Meruelo D (1989) Studies of the mechanisms of action of the antiretroviral agents hypericin and pseudohypericin. *Proceedings of the National Academy of Sciences USA* **86**, 5963-5967

Liu XN, Zhang XQ, Sun JS (2007a) Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in *Hypericum sampsonii* and *Hypericum perforatum*. *Plant Growth Regulation* **53**, 207-214

Liu XN, Zhang XQ, Zhang SX, Sun JS (2007b) Regulation of metabolite production by precursors and elicitors in liquid cultures of *Hypericum perforatum*. *Plant Cell, Tissue and Organ Culture* **91**, 1-7

Mederos MS (1991) *In vitro* growth and multiplication of *Hypericum canariense* L. *Acta Horticulturae* **289**, 133-135

Moura M (1998) Conservation of *Hypericum foliosum* Aiton, an endemic azorean species by micropropagation. *In Vitro Cell and Developmental Biology – Plant* **34**, 244-248

Mulabagal V, Tsay HS (2004) Plant cell cultures-an alternative and efficient source for the production of the biologically important secondary metabolites. *International Journal of Applied Science, Engineering and Technology* **2**, 29-48

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-497

Park J, English DS, Wannemuehler Y, Carpenter S, Petrich JW (1998) The role of oxygen in the antiviral activity of hypericin and hypocrellin. *Photochemistry and Photobiology* **68**, 593-597

Patočka J (2003) The chemistry, pharmacology and toxicology of the biologically active constituents of the herb *Hypericum perforatum* L. *Journal of Applied Biomedicine* **1**, 61-73

Pretto FR, Santarém ER (2000) Callus formation and plant regeneration from *Hypericum perforatum* L. leaves. *Plant Cell, Tissue and Organ Culture* **67**, 107-113

Pulok MK, Saritha GS, Suresh B (2002) Antimicrobial potential of two different *Hypericum* species available in India. *Phytotherapy Research* **16**, 692-695

Santarém ER, Astarita LV (2003) Multiple shoot formation in *Hypericum perforatum* L. and hypericin production. *Brazilian Journal of Plant Physiology* **15**, 43-47

Shilpashree HP, Ravishankar RV (2009) Fatty acid composition and antimicrobial activity of leaf and flower extracts of *Hypericum mysorense*. *Medicinal and Aromatic Plant Science and Biotechnology* **3**, 117-119

Vantieghem A, Xu Y, Vandenabeele P, Denecker G, Vandenheede JR, Merlevede W, Witte PA De, Agostinis P (2001) Different pathways mediate cytochrome c release after photodynamic therapy with hypericin. *Photochemistry and Photobiology* **74**, 133-142

Verma V, Smelcerovic A, Zuehlke S, Hussain MA, Ahmad SM, Ziebach T, Qazi GN, Spiteller M (2008) Phenolic constituents and genetic profile of *Hypericum perforatum* L. from India. *Biochemical Systematics and Ecology* **36**, 201-206

Vijayan P, Vinod Kumar S, Dhanaraj SA, Mukherjee PK, Suresh B (2003) *In vitro* cytotoxicity and antitumour properties of *Hypericum mysorense* and *Hypericum patulum*. *Phytotherapy Research* **17**, 952-956

Vijayan P, Raghu C, Ashok G, Dhanaraj SA, Suresh B (2004) Antiviral activity of medicinal plants of Nilgiris. *Indian Journal of Medical Research* **120**, 24-29

Vishwakarma RA, Kapil RS, Popli SP (1983) Studies in medicinal plants, Chemical constituents of *Hypericum mysorense*. *Indian Journal of Chemistry* **22**, 612-613

Walker TS, Bais HP, Vivanco JM (2002) Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L. (St. John's wort). *Phytochemistry* **60**, 289-293

Wójcik A, Podstolski A (2007) Leaf explant response in *in vitro* culture of St. John's wort (*Hypericum perforatum* L.). *Acta Physiologiae Plantarum* **29**, 151-156

Yanpaisan, King W, NJC, Doran PM (1999) Flow cytometry of plant cells with applications in large-scale bioprocessing. *Biotechnology Advances* **17**, 3-27

Yazaki K, Okuda T (1990) Procyaninins in callus and multiple shoots of *Hypericum erectum*. *Planta Medica* **56**, 490-491

Zobayed SMA, Afreen F, Kozai T (2007) Phytochemical and physiological changes in the leaves of St. John's wort plants under a water stress condition. *Environment Experimental Botany* **59**, 109-116

Zobayed SMA, Afreen F, Goto E, Kozai T (2006) Plant environment interactions: accumulation of hypericin in dark glands of *Hypericum perforatum*. *Annals of Botany* **98**, 793-804