ORIGINAL PAPER

Light-induced production of antidepressant compounds in etiolated shoot cultures of *Hypericum hookerianum* Wight & Arn. (Hypericaceae)

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Abstract *Hypericum hookerianum* is a lesser known ethnomedicinal plant having wound healing, antitumor and anti-HSV-1 properties. Isolated nodes of in vitro shoots subcultured in the dark for 4 weeks on half strength Murashige and Skoog medium solidified with Gelzan (1.5 g l^{-1}), and supplemented with 2.325 μM kinetin produced 8.0 ± 0.40 etiolated shoots of 5.0 ± 0.62 cm length at 74 % efficiency versus 9.2 ± 0.6 healthy shoots of 4.4 ± 0.5 cm obtained from nodes in light at 96 % efficiency. Low concentrations of hypericin were found in wild plant [0.35 ± 0.09 mg g^{-1} dry weight (DW)] and control green shoot cultures (0.91 ± 0.03 mg g^{-1} DW). Etiolated shoots exposed to a 12 h photoperiod (50 μmol m^{-2} s^{-1}) through 1–25 days turned red incrementally due to synthesis and accumulation of 0.1–3.83 mg g^{-1} DW hypericin in sub-epidermal cortical cells of the stem and varied shaped cells of the distorted mesophyll. Flavonoid and anthocyanin concentrations of the etiolated shoots subjected to the 12 h photoperiod were 3–5 fold higher than the control shoot cultures while total chlorophylls [1.97 ± 0.05 mg g^{-1} fresh weight (FW)] of the light exposed shoots were significantly less compared to the control (2.86 ± 0.18 mg g^{-1} FW) and natural plant (6.82 ± 0.29 mg g^{-1} FW). HPLC analysis of shoot extracts revealed the presence of 0.14 ± 0.03, 0.16 ± 0.02 and 1.45 ± 0.16 mg g^{-1} DW hyperforin in wild plant, control shoot cultures and etiolated shoot cultures illuminated for 25 days, respectively. Despite a reasonable presence in etiolated shoots (0.61 ± 0.15 g^{-1} FW), total phenols did not increase significantly during illumination. The results indicate light induced synthesis of anti-depressant phenolic derivatives (hypericin, hyperforin and flavonoids) in etiolated shoot cultures of *H. hookerianum*.

Keywords *Hypericum hookerianum* · Shoot cultures · Hypericin · Hyperforin · Flavonoids · Light induced synthesis

Abbreviations

DW   Dry weight
FW   Fresh weight
HPLC High performance liquid chromatography
Kin   Kinetin
MS   Murashige and Skoog

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Introduction

Higher plants are significant sources of new chemicals and medicinally useful agents. Time tested traditional therapy especially followed in the rural areas of developing countries, makes use of plant extracts or their active principles for curative effects on human body and mind. In fact, instances of plants collected by local communities in the wild and used as traditional medicines in these countries becoming valuable sources of best-seller drugs worldwide are not uncommon (Bruni and Sacchetti 2009). Species of the genus *Hypericum* are known worldwide for traditional and modern medicinal uses and rural and ethnic communities of different countries have long used *Hypericum* preparations as an external anti-inflammatory and healing

remedy for the treatment of eczema, swellings, wounds, burns, anxiety and mental disorders. In the recent past, bioactivity-guided assays confirmed the anti-plasmodial activity of stem bark extracts of *H. lanceolata*, traditionally used in Cameroon to control malaria (Zofou et al. 2011). However, lack of reproducible activity for more than 40 % of plant extracts (Poulev et al. 2003) has led to limited commercial success in terms of product development. This major constraint may be overcome with biotechnological interventions to willfully induce production of secondary metabolites in tissue and cell cultures using abiotic or biotic elicitors.

Among *Hypericum* species, largely due to historical use, *H. perforatum* remains the most researched and top selling herbal antidepressant while others have been surveyed mainly as a source of novel chemicals (Lotocka and Osinska 2010). Consequently, hypericin is now reported in a number of species and also investigated for its structural, biological and pharmacological properties (Karioti and Bilia 2010). An endemic Turkish species, *H. leptophyllum*, lacks both hypericin and hyperforin (Camas et al. 2012) while *H. adenotrichum* contains hypericin and no hyperforin (Cirak et al. 2009). Other species such as *H. triquetrifolium*, *H. montanum*, *H. montbretti*, *H. sinarium*, *H. scabrum* and *H. sampsonii* are even better sources of hypericin than *H. perforatum* and are being evaluated for domestication and cultivation so as to increase their economic importance in the world market (Liu et al. 2007; Ayan and Çirak 2008; Kusari et al. 2009; Odabas and Cirak 2011). Presumably because it is unstable and not visibly pigmented as hypericin, hyperforin has been investigated only in exceptional species. The rapid growth of international markets for St. John's wort products coupled with low and varying endogenous concentrations of hypericin and hyperforin in cultivated plants has created a need for developing environmentally sustainable and economically viable bioproduction systems using in vitro cultures as part of *Hypericum* biotechnology (Bertoli et al. 2010). In fact, interest in *Hypericum* tissue cultures for biotechnological applications was triggered by discovering new activities of the marker compound, hypericin and its derivatives (Čellárová 2011). Since most of the important secondary metabolites are synthesized or accumulated in the aerial parts (stem, leaf and flowers) of the plant (Soelberg et al. 2007; Kirakosyan et al. 2008) and undifferentiated cell cultures yield notoriously low concentrations of the compounds (Kirakosyan et al. 2004), large scale shoot cultures may be a promising approach for successful bioproduction. *Hypericum* species producing more in artificial culture environment than under natural conditions and shoot cultures offering excellent sources of both hypericin and hyperforin are vouched for by many workers (Smith et al. 2002; Kirakosyan et al. 2004; Karppinen et al. 2006;

Pasqua et al. 2006; Kornfeld et al. 2007; Karakas et al. 2009; Coste et al. 2011; Manero et al. 2012). Advantages of using shoot cultures for secondary metabolite production, especially for the development of uniform and high quality phytopharmaceutical products, have been reviewed (Kirakosyan et al. 2004).

Hypericum hookeranium Wight and Arnott (Hooker's wort) is a lesser known round topped shrub with weakly spreading, non-erect branches distributed in high altitude (>2,000 m) forests of Sikkim, Khasi and Jaintia hills in northern India and Nilgiri and Palni hills in southern India (The Wealth of India 1995). Mostly known for its ornamental value due to its golden yellow flowers, the shoot extracts of this plant are used by the Toda tribe of the Nilgiri hills as an antimicrobial agent to ward of skin infections and to treat wounds, burns and conditions like anxiety and inflammation (The Wealth of India 1995; Mukherjee and Suresh 2000; Vijayan et al. 2004). Because of these medicinal attributes, the species was further evaluated and found to possess antimicrobial (Mukherjee et al. 2001), antitumor (Dongre et al. 2008), antiviral (Vijayan et al. 2004) and antioxidant (Chandrashekhar et al. 2009) properties. Earlier, as part of a patented process, we raised shoots and shoot forming callus cultures of this species, the latter yielding up to 4.25 % DW hypericin (Padmesh et al. 2008). Since biomass production steadily declined in successive subcultures of the productive caulogenic calluses, and the cultures eventually ceased to grow, etiolated shoot cultures were subsequently raised as a suitable alternative to provide a ready source of shoots for induced synthesis of metabolites in light. In this paper, we report for the first time significant production of all the important antidepressant compounds (hypericin, hyperforin, flavonoids) in light induced etiolated shoot cultures of *H. hookerianum* to promote their eventual use as an antidepressive phytomedicine.

Materials and methods

Plant material

Voucher specimen of *H. hookerianum* collected from Pambar shola forest adjacent to Kodaikanal town of Palni hills in the Western Ghats was identified by Dr. A.G. Pandurangan, Systematic Botanist and deposited in the herbarium (TBGT-46936) of Tropical Botanic Garden and Research Institute, Palode, Kerala. Axillary shoot cultures were raised using isolated nodal explants (Padmesh et al. 2008). Shoots regularly sub-cultured at 6 week intervals using nodes and maintained in Murashige and Skoog (1962) Gelzan (1.5 g l^{-1}) medium supplemented with

2.325 μM Kinetin (Kin) served as the source of nodal explants used for the experiments.

Photoactivated hypericin synthesis in etiolated shoot cultures

Defoliated single node cuttings (0.8–1.4 cm) were dissected out of young shoots of a selected wild plant collected from Pambar shola forest as well as from the in vitro axillary shoot cultures of the same plant. Nodal cuttings of field origin were surface sterilized using 0.1 % HgCl$_2$ solution containing a drop of Tween-20 for 4 min and rinsing thrice in sterile distilled water as described before (Padmesh et al. 2008). Four nodal cuttings were transferred on to 50 ml half strength MS Gelzan nutrient medium in each 250 ml flask containing 2.325 μM Kin and incubated in the dark in a cabinet covered with black cloth and maintained in the culture room at 25 ± 2 °C to multiply etiolated shoots and in light (50–60 μmol m^{-2} S^{-1}) to induce formation of green shoots. After 4 weeks of incubation in dark, part of the flasks having etiolated shoot cultures were transferred to light at 25 ± 2 °C under 12 h photoperiod. Concentrations of pigments, hypericin and other bioactive metabolites were estimated using 200 mg dry weight (DW) samples after 1, 2, 3, 5, 10, 15 and 25 days. Nodal explants of the remaining etiolated shoots were used for subculture and multiplication of shoots in the dark at 6 week intervals. Shoots differentiated from the nodal explants of the wild plant were multiplied by dissecting out and sub-culturing the nodal explants (0.8–1.2 cm) under 12 h photoperiod in the culture room. Four to six week old shoot cultures were used as a control. Young shoots of the wild plant served as another control.

Extraction and quantification of hypericin

Air dried 200 mg shoot samples harvested from control green shoot cultures and etiolated shoot cultures induced to synthesize metabolites by exposing to light for different days and wild plants were extracted with 10 ml chloroform under sonication in a Sonics Vibra Cell Sonicator (USA). After filtration, each sample was re-extracted thrice with 10 ml methanol under sonication and evaporated to dryness. The solid phase containing hypericin was dissolved in 5 ml methanol and filtered through 0.22 μm Waters syringe filters.

High performance liquid chromatography (HPLC) analysis of the filtered extracts was carried out using an UV–visible detector working at 590 nm in a Shimadzu 1100 system. The hypericin elution program was done isocratically with a C$_{18}$ (125 × 4 mm) reversed phase column using a mobile phase consisting of (A) acetonitrile, (B) methanol and water (70:30) in the A:B ratio 20:80.

Aliquots (50 μl each) of the green and red samples were injected into the column at 0.25 ml min^{-1} flow rate. Elution was monitored at 590 nm and the eluted hypericin was identified on the basis of the retention time by comparison with retention time of the reference standard (Sigma-Aldrich, USA). The amount of hypericin in individual samples was calculated from the calibration curve prepared in the range of 0.5–100 μg ml^{-1} (r^2 > 0.99). Each sample was analyzed thrice and the mean value was taken for calculation.

Extraction and quantification of hyperforin

Equal quantities [500 mg fresh weight (FW)] of shoot samples from wild plant, control green shoot cultures and etiolated shoot cultures light induced to synthesize metabolites for 30 days were ground in liquid nitrogen under dim light using pestle and mortar to obtain a homogeneous powder and extracted with 96 % ethanol (50 ml) for 72 h at room temperature. The extracts were centrifuged at 10,000 RPM in a Kubota 6,200 centrifuge for 10 min. and the supernatants filtered through 0.22 μm Waters syringe filters. Reverse phase HPLC analysis of each filtered extract to determine hyperforin was carried out using a UV–visible detector working at 273 nm in a Shimadzu 1100 system. The hyperforin elution program was performed isocratically using a C$_{18}$ reversed phase column using a mobile phase consisting of acetonitrile: 0.1 % orthophosphoric acid (9:1) at 0.25 ml min^{-1} flow rate with simultaneous UV (273 nm) detection of hyperforin. Aliquots (50 μl each) were injected and eluted and hyperforin was identified on the basis of the retention time by comparing retention time of the reference standard (Sigma-Aldrich). The mean value obtained in three separate assays was converted and expressed on gram DW basis.

Estimation of total chlorophylls, phenols, anthocyanins and flavonoids

Chlorophylls, phenols, anthocyanins and flavonoids were extracted by homogenizing 500 mg green and red pigmented shoot culture samples and tender shoots (stem + leaves) of wild plant in a glass pestle and mortar using 10 ml each of 80 % cold acetone, 0.01 % acidified methanol and 80 % aqueous methanol respectively and filtering individual extracts through a 200μ nylon screen. Each residue was re-extracted once with the respective solvent and the combined extracts of each were centrifuged at 4,000 RPM in a Kubota 6,200 high speed centrifuge. Supernatants were collected and made up to 20 ml each with the respective solvent before measuring absorbance at 645 and 663 nm for chlorophylls, 506, 510 and 700 nm for

anthocyanins and 510 nm for flavonoids. Total chlorophyll concentration in each sample was calculated using the constants of Porra (2002). The Folin–Ciocalteu method was used to determine total phenolic content of the sample (0.1 ml) as described by Singleton and Rossi (1965). A calibration curve was prepared at 765 nm using gallic acid at concentrations of 0.4–1.6 mM. Anthocyanins in acidified methanol extract were estimated following the method of Wrolstad et al. (1990). Flavonoids in the methanol extracts were estimated by the aluminium chloride method described by Zhishen et al. (1999). All the values were expressed on fresh weight basis and each value represents mean ± SD of five different determinations.

Each experiment on culture initiation using young nodal explants of the selected wild plant and isolated nodes of shoot cultures consisted of at least 15 replicates with four explants per culture flask and was repeated thrice. Data presented are the mean values with standard deviations. All the values of phytochemicals and pigments were expressed on gram DW or FW basis and each value represents the mean ± SD of three to five separate determinations. The data were also subjected to a one-way analysis of variance (ANOVA) and the significance of difference between means at 5 % was determined by Scheffe's multiple comparison Test using Microsoft Excel (DSAASTAT) software (Table 1).

Results

More than 92 % of the shoot culture derived nodal explants sub-cultured in light in half strength MS medium using 2.325 μM Kin responded with the formation of callus free 9.2 ± 0.6 axillary shoots of 4.4 ± 0.5 cm free from callusing and morphological abnormalities in 4 weeks. Nodal explants of the selected wild plant cultured under identical conditions were somewhat slow to respond at low frequency (62 %) and produced less number of shoots

(5.2 ± 0.5) of similar length after 6 weeks. Both these green shoot culture types contained somewhat uniform yet higher concentration of hypericin (0.91 ± 0.03 mg g^{-1} DW) than young shoot materials of wild mother plant (0.35 ± 0.09 mg g^{-1} DW). Shoot culture derived nodes sub-cultured in the dark showed signs of initial browning for a week but resident meristems in the nodal joints actively proliferated to produce 8.0 ± 0.40 thinner and somewhat longer (6.0 ± 0.62 cm) etiolated shoots with markedly reduced number, thickness and size of leaves in 4–5 weeks (Fig. 1a). The reduced leaves were thin and papery in shoots cultured for 6 weeks and more. Somewhat greenish brown callusing observed in 20 % of the dark grown nodal explants did not interfere with the formation of the etiolated shoots. Remarkable changes occurred when the etiolated shoots acclimatized to dark conditions for 4 weeks (28–30 days) were exposed to 12 h photoperiod. A prominent and first visible change after light exposure was red pigmentation of the leaf faster than stem in 24 h (Fig. 1b) which coincided with the synthesis of 0.102 mg g^{-1} DW hypericin. Light exposed etiolated shoots turned increasingly red, indicating incremental synthesis (Fig. 2) and accumulation of hypericin more or less free from interference of chlorophylls and anthocyanins up to 72 h of illumination. A time course assay of the pigments and metabolite production corresponding to increased red pigmentation revealed linear synthesis of hypericin, flavonoids and anthocyanins to fetch 2.31 ± 0.34 mg g^{-1} DW, 2.1 ± 0.18 mg g^{-1} FW and 1.1 ± 0.18 mg g^{-1} FW respectively after 15 days of illumination at 12 h photoperiod. The stems and leaves also became thicker and broader during 1–25 days of illumination which correlated well with the visible and recorded linear increase in the concentration of hypericin and flavonoids (Figs. 1c, 2). Hypericin producing red shoots continued to grow slowly and multiply as evidenced from formation of new red pigmented buds/shoots only from the base of the existing shoots in contact with the nutrient

Table 1 Anti-depressant compounds present in tender shoots of wild plant, green shoot cultures multiplied in MS medium supplemented with 2.325 μM KIN and etiolated shoot cultures multiplied using 2.325 μM KIN and exposed to light (50–60 μmol m^{-2} S^{-1}) for 25 days

Chemical constituent (mg g^{-1} DW or FW)	Wild plant	Control green shoot culture	Photoactivated dark grown shoot culture
Hypericin	1.01[b] ± 0.015	0.08[c] ± 0.002	3.83[a] ± 0.12
Hyperforin	0.14[b] ± 0.050	0.16[b] ± 0.005	1.45[a] ± 0.23
Flavonoids	8.26[a] ± 0.080	2.82[c] ± 0.10	7.51[b] ± 0.11
Chlorophyll	6.82[a] ± 0.076	2.86[b] ± 0.23	1.97[c] ± 0.12
Anthocyanins	1.85[a] ± 0.050	0.19[c] ± 0.10	1.01[b] ± 0.02
Total phenols	1.92[a] ± 0.030	0.28[c] ± 0.03	0.61[b] ± 0.10

Results are the mean values of three separate determinations. Mean values in the same row sharing different letters are statistically significant according to Scheffe's test ($p \leq 0.05$). Hypericin, hyperforin and total phenol values are expressed in mg g^{-1} DW and flavonoids, chlorophylls, anthocyanins and total phenols in mg g^{-1} FW

medium. Nodal explants of the etiolated shoots were also sub-cultured in the same medium to produce 8.05 ± 0.25 shoots of 5.75 ± 0.71 cm length without decline but with further reduction in thickness and size of the leaves at least through 6 subculture cycles.

Chlorophyll synthesis in the light exposed etiolated shoot cultures followed a different pattern. Detected only 3 days after transfer to light, chlorophyll synthesis was slow and a concentration of 0.05 ± 0.008 mg g^{-1} FW was recorded after 5 days. A steady increase in concentration followed thereafter resulted in the accumulation of 1.6 ± 0.14 mg g^{-1} FW total chlorophyll in 15 days (Fig. 2). A time course assay of the related pigments (flavonoids, anthocyanins) indicated their absence in the etiolated shoot cultures; starting from day 2, a gradual increase in their concentrations was recorded (1.37 ± 0.30 and 1.08 ± 0.10 mg g^{-1} FW respectively on day 10) after which the former steadily increased to reach 2.1 ± 0.18 mg g^{-1} FW after 15 days. A significant presence (0.41 ± 0.02 mg g^{-1} DW) of total phenols recorded in

the etiolated shoots persisted up to 10 days before showing a marginal increase after 15 days. Free hand sections of the red pigmented shoots exposed to light for 15 days showed the distinct presence of red pigmented hypericin rich cells in the sub-epidermal cortical region of the stem (Fig. 1d) and such cells of varied and uneven shape and size occupying entire mesophyll of the red pigmented leaves of the shoot (Fig. 1e). The hypericin over producing leaves invariably had distorted anatomy as they were devoid of typical palisade and spongy parenchyma of the normal green leaves. The mesophyll anatomy of the upper greener and red leaves of the shoots, however, was somewhat normal with palisade and spongy parenchyma interspersed with red pigmented hypericin producing cells.

After 25 days of light exposure, analysis of solvent extracts from the red pigmented shoots versus control green shoots showed far less chlorophyll in both shoot culture types than in the leaves of wild plant (Table 1). Significantly elevated levels of most of the antidepressant
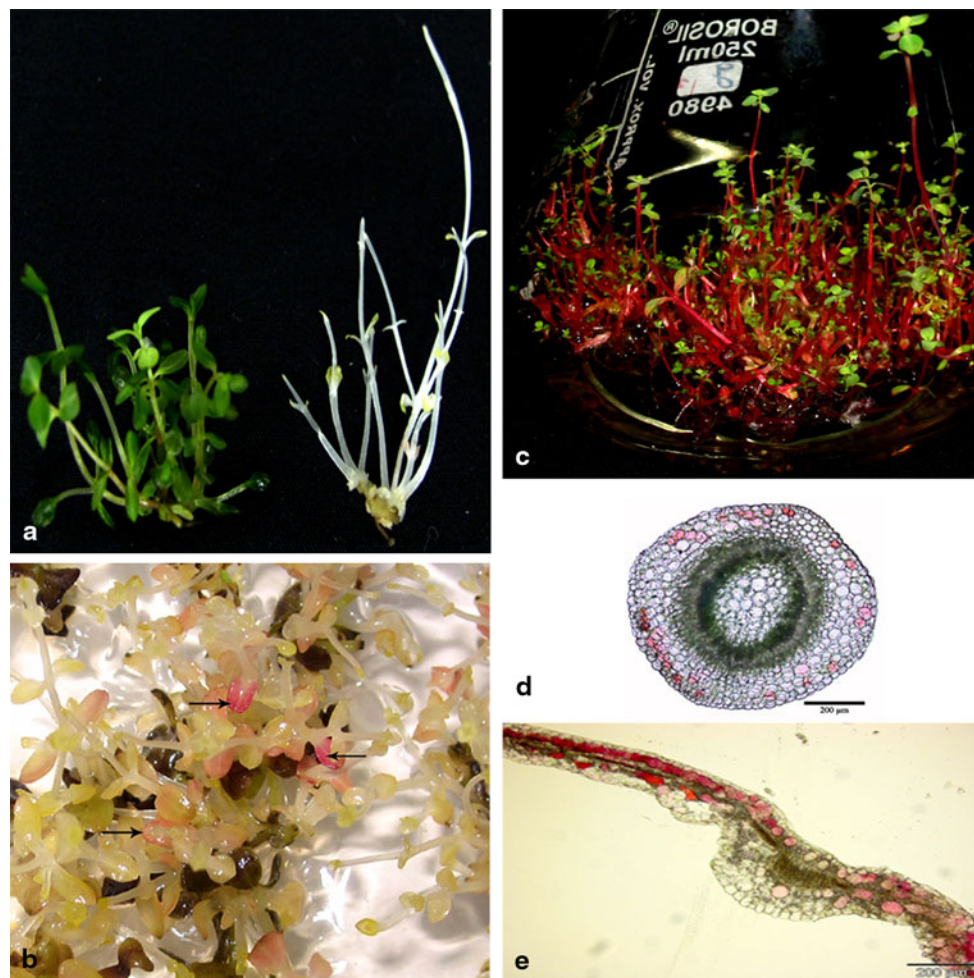

Fig. 1 a *Green* and etiolated shoots differentiated upon in vitro derived node cultures after 4 weeks of culture in *light* and *dark* respectively. **b** Visible signs of hypericin synthesis (indicated by *arrows*) in etiolated shoot cultures after 24 h of illumination **c** Multiplying hypericin rich shoots after 25 days of illumination. **d, e** Transverse sections of stem and basal leaf of 15 day light induced hypericin rich shoot showing distribution of hypericin rich cells. (Color figure online)
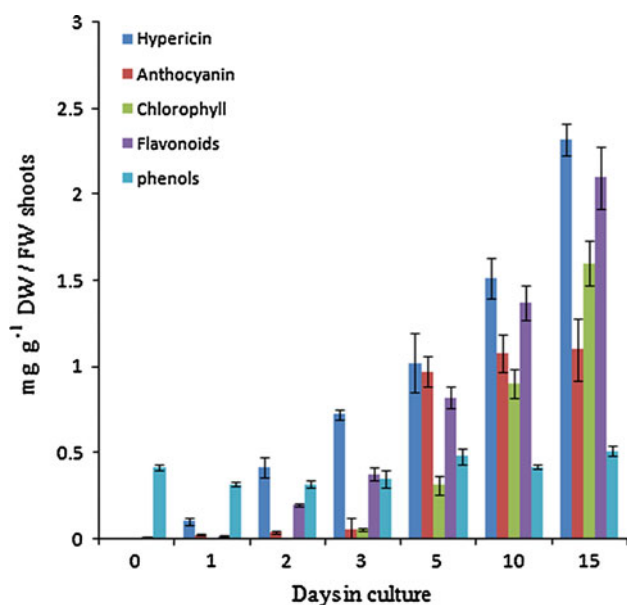
Fig. 2 Metabolite synthesis as a function of period of light exposure in etiolated shoot cultures. Data are mean values obtained from three independent determinations. *Bars* represent standard errors of the mean

compounds (hyperforin, hypericin, flavonoids) were found in the red shoots which were less chlorophyllous (1.97 ± 0.12 mg g^{-1} FW) than the normal green shoot cultures (2.86 ± 0.18 mg g^{-1} FW); both these shoot culture types were 2.5–3 fold less chlorophyllous than the young leaves of the wild plant (6.82 ± 0.076 mg g^{-1} FW). The red shoots in particular contained nearly 2 (0.61 ± 0.13 mg g^{-1} FW), 3 (7.51 ± 0.11 mg g^{-1} FW), 5 (1.01 ± 0.02 mg g^{-1} FW) and 4 (3.83 ± 0.12 mg g^{-1} DW) fold higher concentrations of total phenols, flavonoids, anthocyanins and hypericin than the control green shoot cultures (0.28 ± 0.03 mg g^{-1} FW, 2.82 ± 0.10 mg g^{-1} FW, 0.19 ± 0.10 mg g^{-1} FW, 0.91 ± 0.03 mg g^{-1} DW) respectively. While total phenol content was strikingly low both in the control green and red shoot cultures, concentrations of total flavonoids, and to some extent anthocyanins, recorded in red shoot cultures were high and comparable to those of wild plants. HPLC analysis of hyperforin in 25 day light induced red shoot culture, control green shoot cultures and wild plant samples showed that chromatographic analysis time per sample was 15.0 min with hyperforin eluting at retention times of about 8.595 (Fig. 3a, b) min for the red, wild and green samples with peak areas of 64,228, 76,429 and 72,210 respectively. Among the three samples, the light induced red shoots had reproducibly higher content of hyperforin (125 µg ml^{-1}) than the control green shoot culture and wild plant samples (3.2–5.8 µg ml^{-1}). These values were extrapolated to arrive at 1.45 ± 0.23, 0.14 ± 0.050 and 0.16 ± 0.005 mg g^{-1} dry mass respectively for the three samples.

Discussion

Selection of plants on the basis of ethno-medicinal and ethno-pharmacological considerations has given a higher hit rate than research based on synthesis and has contributed to successful development of therapeutically important molecules such as vincristin, vinblastine, reserpine, curcumin, azadirachtin, artimisinin, piperin and campto-thecin. Selection of *H. hookerianum* for the present investigation was based on use of its leaf and stem extracts by the traditional healers of the Toda tribe of the Nilgiris to find remedies for skin infections, wounds, inflammation and anxiety (Vijayan et al. 2004; The Wealth of India 1995) and validation of these and other properties by pharmacological assays (Mukherjee and Suresh 2000; Mukherjee et al. 2001; Vijayan et al. 2004; Dongre et al. 2008; Chandrashekhar et al. 2009). Loss of this species in the depleted forests of Nilgiris in the Western Ghats region forced earlier investigators to use horticultural collections of the Ooty Botanic Garden for conducting pharmacological investigations (Mukherjee and Suresh 2000). South of Nilgiris, this species is naturally distributed in isolated pockets on the banks of Pambar river in the Palni hills but subjected to natural and anthropogenic hazards including habitat degradation and loss of seeds in the flowing waters of Pambar river. Consequently, self-perpetuating populations of the species do not occur anywhere in the Western Ghats of India. Perusal of the literature revealed no tissue culture work on *H. hookerianum* and no chemical basis established for the published papers on pharmacological aspects. In fact, Vijayan et al. (2004) felt a need for phytochemical characterization of the species to develop new and effective antiviral agents. The present investigation has brought to light for the first time that young shoots of this species are a source of both hypericin and hyperforin and the chlorophyllous shoot cultures of the species and photoinduced red shoot cultures in particular are even better sources of these compounds. Since availability of the species is scarce and the remaining plants in their native habitats in Palni hills face anthropogenic threat, successful initiation of 5.2 ± 0.5 shoots at 62 % rate and multiplication of 9.2 ± 0.6 shoots at 92 % rate in nodal explant cultures would ensure availability of shoots for future recovery and product development programs.

Plants have the ability to synthesize many phenolic substances and their oxygen-substituted derivatives. In St. John's wort plants grown in natural environments, the production and quality of phenolic constituents may vary due to genotype of the plants, different environmental conditions, and biotic stresses (Filippini et al. 2010). Controlled production of medicinal plants is essential for uniform preparations to meet the increasingly stringent safety requirements of regulatory agencies (Bruni and

Fig. 3 HPLC analysis showing significant presence of hyperforin in *green* (**a**) and *red* (**b**) samples with retention time 8.595. (Color figure online)
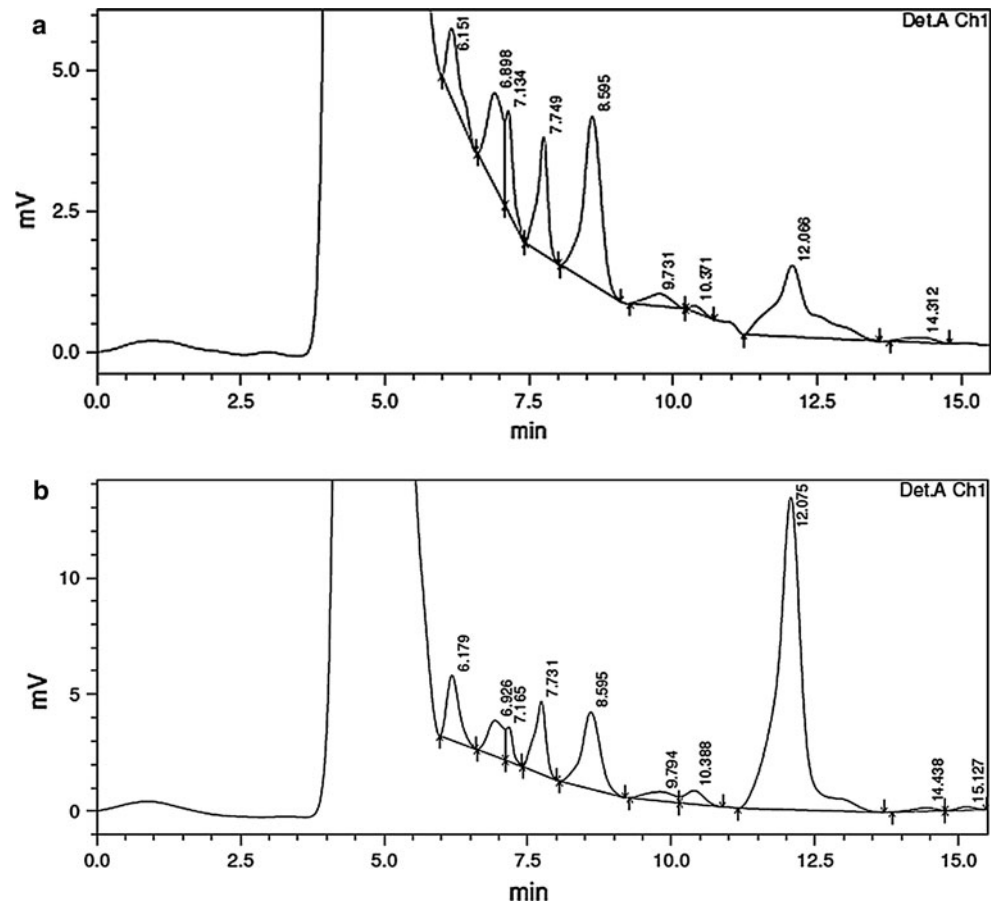
Sacchetti 2009). This could also be better achieved using in vitro raised shoot cultures of *Hypericum* species (Kirakosyan et al. 2004). The data obtained in the present study indicate that concentrations of phenolic substances vary significantly between wild plants, green shoot cultures and photoinduced etiolated shoot cultures of *H. hookerianum* and the red shoots derived from etiolated shoots contained highest concentrations of the phenolic derivatives, hypericin and hyperforin (Table 1; Figs. 2, 3). Although regulation of hypericin and hyperforin synthesis is not yet fully understood, there is substantial progress in the elucidation of biotechnological aspects especially for their enhanced production using in vitro cultures (Ramawat and Merillon 2008). The results are in consonance with the observations of others in *H. perforatum* (Pasqua et al. 2006; Karppinen 2010) and *H. hirsutum* and *H. maculatum* (Coste et al. 2011) that differentiated and stable shoot cultures are a superior alternative to cultivation and accumulation of such molecules as hypericin from secondary metabolism requires differentiated tissues or even whole plants (Santarem and Astarita 2003; Cui et al. 2010; Savio et al. 2012) to extract uniformly high quantities of hypericin. Hypericin production in differentiated shoot cultures was favored by inclusion of 0.4 m gl^{-1} 6-benzyladenine (BA) or Kin (Coste et al. 2011) and replacement of MS vitamins by

Gamborg's B5 vitamins (Danova et al. 2012) in the nutrient medium. Earlier, as part of an effort to achieve sustainable production of hypericin, we established ∞-naphthalene-acetic acid (NAA) induced shoot and shoot forming callus cultures yielding up to 4.21 mg g^{-1} DW hypericin (Padmesh et al. 2008). Since these cultures lost the ability to produce sufficient biomass in long term subcultures, 2.325 μM Kin was subsequently incorporated into half strength MS medium to induce stable production of both shoot biomass and bioactive compounds. This study demonstrates that shoot cultures form a valuable source of the marker compounds (hypericin, hyperforin) and other phenolic derivatives (flavonoids, anthocyanins) of *Hypericum* (Table 1; Figs. 2, 3) in alternating dark (30 days) and light (25 days) cycles in culture.

The near equal number (8.0 ± 0.4) of thin and long (6.0 ± 0.62 cm) etiolated shoots differentiated upon dark grown nodes and their ability to multiply without decline through subcultures in the dark indicated sufficiency of heterotrophic nutrition contributing to significant growth, regeneration and multiplication of the shoots. Besides, visible red pigmentation of the etiolated shoots first observed in 24 h of transfer to light (Figs. 1b, 2) confirmed rapid onset of photoinduced hypericin biosynthetic process and availability of sufficient precursors and cofactors

required for product synthesis in the dark grown shoots. The continued red pigmentation as a mark of linear synthesis of hypericin in light (Fig. 2) occurred free of any secretory structure otherwise present in *H. perforatum* (Pasqua et al. 2003; Kazemiani and Motallebi-Azar 2011) and *H. adenotrichum* (Yamaner et al. 2013) shoot cultures. Since low content of hypericin in *H. hirsutum* is related to feeble development of secretory glands (Gitea et al. 2010), synthesis of up to 3.83 mg g^{-1} DW hypericin in shoot cultures free of secretary glands is a significant observation. Synthesis and accumulation of both hypericin and hyperforin in light was, in a way, related to differentiation and formation of fully expanded leaves in the etiolated shoots during 1–25 day period (Fig. 1c). Differentiation and further development of leaf is known to stimulate production of hypericin (Santarem and Astarita 2003), hyperforin and flavonoids (Pasqua et al. 2003) in *H. perforatum*.

It was of interest to note that the patterns of both the synthetic events viz. chlorophyll synthesis and hypericin synthesis in the light exposed etiolated shoot cultures were different (Fig. 2). Active synthesis of metabolites (hypericin, flavonoids) starting at 24 h and its linearity maintained up to 15–25 days of illumination in contrast to 3 day lag followed by slow and steady synthesis of chlorophyll resulted in synthesis of 1.97 ± 0.12, versus 2.86 ± 0.23 and 6.82 ± 0.076 mg chl. in control green shoot cultures and wild plant, respectively. Accumulation of 3, 5 and 48 fold higher concentrations of flavonoids, anthocyanins and hypericin recorded in light-induced shoot cultures compared to control green shoot cultures at 25 days (Table 1) was a manifestation of enhanced productivity of the former cultures. Besides the reduced potential of the leaf cells to synthesize chlorophylls, their enhanced potential to synthesize and store hypericin was evident from microscopic observations of certain cortical cells of the stem (Fig. 1d) and all the cells of the basal red leaves (Fig. 1e) of the shoots becoming competent to produce hypericin. In the absence of specialized secretary glands, the observed structural and functional alterations of the mesophyll cells to aid enhanced production is not described in any of the shoot cultures (Pasqua et al. 2006; Karppinen 2010). Since such physiologically altered red cells were few in the stem and top leaves, overall internal structure of these organs was not as much disturbed as the basal leaves. Enrichment of secondary metabolites unfavorable to primary metabolism in the cells of the red shoots resulted in slow growth of these shoots; consequently, new shoots emerged from bases of shoots in contact with the nutrient medium. An inverse relationship between tissue growth and product synthesis is long known in plant tissue cultures.

Multiplication of the exceptionally thin and long etiolated shoots having severely reduced leaves in the dark and

the time course assay of bioactive metabolites and chlorophylls in the etiolated shoots subjected to 12 h photoperiod cycles (Fig. 2) indicated the indispensability of light for product synthesis in the increasingly red shoot cultures. Although maintained through regular subcultures at 12 h photoperiod, the control green shoot cultures showed only nominal presence of the bioactive molecules. Secondary metabolism in plants in general is inducible due to its role in plant communication and defense (Manero et al. 2012) and in vivo secondary metabolites including hypericin are components of inducible plant defense responses as inferred from their elevated synthesis in response to biotic factors (Sirvent and Gibson 2002; Yamaner et al. 2013). Many abiotic elicitors also stimulate production of important metabolites including hypericin in in vitro cultures of *Hypericum* species (Gadzovska et al. 2012) of which light as an inducible external factor for hypericin (Fig. 2) and hyperforin (Fig. 3) has not been previously described. The observed linear increase in synthesis of metabolites including hypericin (2.32 ± 0.34 mg g^{-1} DW) and flavonoids (2.10 ± 0.10 mg g^{-1} FW) with relatively less pronounced chlorophyll synthesis up to 15 days of illumination suggest that light induced etiolated shoot culture is a simple yet bioactive system for product synthesis. The unexpected presence of 0.51 ± 0.01 mg g^{-1} FW phenols in dark grown shoots may represent free phenols which on illumination declined due to possible conversion into useful phenolic derivatives as evidenced from the nil increase until 10 days and simultaneous linear increase recorded in the concentrations of hypericin, flavonoids and anthocyanins (Fig. 2). Insignificant though, concentrations of phenols higher than the values recorded in the dark grown shoots were evident from 5 days onwards. Overall, natural plants and green shoot cultures seem to be unproductive and the better option would be to use photoinduced etiolated shoots cultivated using 2.325 μM Kin for extracting significant quantities of hypericin and flavonoids after 25 days of culture (Table 1). The etiolated shoot culture system is attractive since chemical synthesis of hypericin is not economical and that of hyperforin is not practical (Nicolaou et al. 2005).

This study reveals that axillary shoot cultures raised from individual nodes and multiplied through subculture at 6 week intervals in the dark form an useful model system for light induced synthesis and extraction of significant concentrations of antidepressant hypericin (3.83 ± 0.12 mg g^{-1} DW), hyperforin (1.45 ± 0.23 mg g^{-1} DW) and flavonoids (7.51 ± 0.11 mg g^{-1} FW). Simultaneously, in order to cope up with the biosynthetic activities, the thin and long etiolated shoots with reduced and papery leaves progressively became robust and red stemmed with broad and thick leaves during the 25 day period. The results suggest dispensability of the auxin, α-napthalene acetic

acid (NAA) used earlier by our group to stimulate hypericin production in *H. hookerianum* shoot and callus cultures (Padmesh et al. 2008) and desirability of using Kin (2.325 μM) alone under dark and light regimes to initiate, multiply and extract plant specific high value chemicals from sub-culturable shoots. Hypericin production is considered to be a stress induced response as various physical, chemical, biotic and nutritional elicitors influence the same in tissue cultures (Walker et al. 2002; Pavlik et al. 2007; Cui et al. 2010). Since dark and light conditions individually failed to induce significant synthesis, it is likely that the combined dark and light treatments are stressful enough to induce significant synthesis of hypericin and other metabolites. The pronounced difference between 9 and 48 fold increases in hyperforin and hypericin concentrations, respectively, observed in the etiolated shoot cultures photoinduced for 25 days may suggest differential regulation of the synthesis of the two metabolites. Little is known about the biosynthesis of hypericin other than it lies on the polyketide pathway (Zobayed et al. 2006) and Hyp-1 (phenolic oxidative coupling protein) gene with presumed role in the biosynthesis of hypericin is stress induced (Košuth et al. 2013). Since detectable synthesis of hypericin in response to light was recorded within 24 h (Fig. 2), the etiolated shoot culture system may be useful for deciphering the biosynthetic pathway of the molecule.

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