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Rapid Method for *In vitro* Multiplication of Hypericin Rich Shoots of *Hypericum perforatum*

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Abstract: *Hypericum perforatum* is a high value medicinal herb possessing a broad range of pharmacological activities. These medicinal properties are attributed to the presence of major compound hypericin. In current study, the effect of different growth hormones and media combinations along with various growth parameters including light and temperature were analyzed for hypericin production in *in vitro* grown shoot cultures of *H. perforatum*. Rapid method for micropropagation with an average of 36.3 shoots per explant in 5-6 days was developed. Highest hypericin content of 0.119 µg/mg was detected in shoots grown on MS medium containing sucrose (3%), IBA (3mg/l), KN (1mg/l) and agar (0.9%) at 25±2 °C under light conditions after two months of incubation. In addition, sustention of *in vitro* grown shoots after hardening under greenhouse conditions was also observed showing comparable hypericin content in hardened and field grown plants after 2 years. Therefore, current study reports the rapid method for *in vitro* multiplication for hypericin rich shoots of *H. perforatum* under optimized light and temperature conditions. To the best of our knowledge, this is the first report on comparative analysis of hypericin production in hardened and field grown shoots of *H. perforatum*. Hence, the outcome is an endeavor to meet the increasing industrial demands by providing quality rich raw material using tissue culture techniques.

Keywords: Hypericin, *Hypericum perforatum*, Micropropagation, HPLC

1. Introduction

Hypericum perforatum commonly known as St. John's Wort is an important medicinal herb which belongs to Hypericaceae family. It mainly possesses antidepressant activity and used to treat various disorders like fibrosis, neuralgia, depression, anxiety, skin wounds, eczema, burns, diseases of the alimentary tract etc (Yadollah-Damavandi *et al.,* 2015; You *et al.,* 2015). These medicinal properties are attributed to the presence of various classes of biochemical compounds such as naphthodianthrones (hypericin and pseudohypericin), flavonoids (rutin, hyperoside, isoquercitrin, quercitrin and quercetin), phloroglucinols (hyperforin and adhyperforin), tannins (cathechin and epicatechin), proanthocyanidins (procyanidin B2) and bioflavonoids (biapigenin and amentoflavone) (Nahrstedt and Butterweck, 1997). But in recent years, interest has arisen in hypericin which is one of the major compounds of this plant and used as a potential photosensitizing anticancer agent (Gioti *et al.,* 2005). Several studies have also shown powerful antineoplastic activity of hypericin (Agostinis *et al.,* 2000; Hostanska *et al.,* 2003). *Hypericum* preparations are generally standardized based on defined hypericin concentrations of 0.3-0.5% (Linde *et al.,* 1996; Li and Fitzloff, 2001) and worth of an estimated value of US$ 210 and 570 million in the USA and worldwide, respectively (Zobayed and Saxena, 2003). Hypericin accumulates predominantly in foliar parts of the plant (Soelberg *et al.,* 2007). In natural habitat *H. perforatum* propagates by means of runners or from seeds but tissue culture techniques can be used as an alternative option for rapid multiplication of this medicinally important plant species for hypericin production. Various tissue culture techniques have already been employed for the micropropagation, regeneration (Pretto and Santarem, 2000; Zobayed and Saxena, 2003; Franklin and Dias, 2006; Liu *et al.,* 2007; Cui *et al.,* 2010; Palmer and Keller, 2010; Coste *et al.,* 2011) and production of valuable secondary metabolites (Bais *et al.,* 2003; Mulinacci *et al.,* 2008) from this plant species. However, reports on rapid multiplication along with hypericin production are very scarce. Therefore, current study aims in development of rapid method for micropropagation and *in vitro* production of hypericin through shoot cultures of *H. perforatum*.

2. Materials and Methods

2.1. Selection of Plant Material

H. perforatum plants were procured from the National Bureau of Plant Genetic Resources (NBPGR), Regional Research Station, Phagli, Shimla, H.P., India and maintained in green house of Jaypee University of Information Technology, Waknaghat, H.P., India at 25±2°C, 80–90% relative humidity under natural light conditions.

2.2. Media Preparation and Establishment of Shoot Cultures

Shoot apices were surface sterilized using 0.5% bavistin and 0.1% mercuric chloride followed by 4–5 washings in sterile water. Different media combinations having Murashige and Skoog (MS) media (Murashige and Skoog, 1962) supplemented with 3% sucrose with different concentrations of indole-3-butyric acid (IBA), 6-benzylaminopurine (BAP), kinetin (KN) were prepared. The pH adjusted to 5.7, 0.9% agar was added and 50 ml of media was dispensed in each jar prior to autoclaving at 121°C, 15 lb inch-^{2} pressure for 20 minutes. Sterilized shoot apices were cultured on above mentioned media combinations and kept at 25±2°C, with 70% relative humidity, 16 h day/8 h night photoperiod at photosynthetic photon flux density of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India) for shoot proliferation in a plant tissue culture chamber. Data was recorded for number of days required for multiple shoot formation, number of shoots and shoot length. The experiments were performed in triplicates and repeated twice.

2.3. Optimization of Tissue Culture Conditions for Hypericin Production

Effect of different growth hormones along with various growth parameters were analyzed for hypericin production in *in vitro* grown shoot cultures of *H. perforatum*. The best media combination showing highest hypericin content was used for optimizing growth parameters *viz.* temperature, light condition and solid/liquid media. Shoot cultures on optimized media were further incubated at two different temperatures (25±2°C and 15±2°C) in both solid (with agar) and liquid (without agar) media under light as well as dark conditions.

2.4. Root Induction in Shoot Cultures

Individual shoots were excised from the parent cultures and transferred to MS media supplemented with different concentrations of IBA, NAA and KN for root induction. The pH of medium was adjusted to 5.7 prior to autoclaving. The cultures were incubated under the same photoperiod conditions as mentioned above. Data was recorded for number of days required for root initiation, number of roots and average root length. The experiments were performed in triplicates and repeated twice.

2.5. Hardening of In Vitro Grown Plantlets

Fully grown plantlets were taken from the rooting media, washed under running tap water to remove traces of agar and transferred to pots containing sand: soil: vermiculite (1:1:1) in green house conditions for hardening. Initially, the plantlets were covered with glass jars for 10–15 days to provide sufficient humidity and were taken off every day for 1–2 h so as to acclimatize them to external environment. Hardened plants were maintained in green house for 2 years under the natural light conditions. Data was also recorded for percent survival of plants. Shoots were collected at different time durations and stored at -80°C for further HPLC analysis. Comparative analysis for hypericin production was done in hardened and field grown plants of *H. perforatum*.

2.6. Quantification of Hypericin

Shoot tissues were ground to a fine powder in liquid nitrogen. 100 mg of powdered material was percolated with 10ml methanol and sonicated for 1 hour in a water bath sonicator. After sonication, samples were incubated at room temperature for 3-4 hours and centrifuged at 6000 rpm for 15 minutes. The methanolic extract was filtered through 0.22 µm membrane filter. Hypericin was quantified by using the method reported by (Li and Fitzloff, 2001) with slight modifications on HPLC System equipped with Waters 515 HPLC pumps, Waters 717 autosampler, Waters 2996 photodiode array detector and Empower software. Waters Spherisorb reverse phase C18 column (4.6mm x 250mm, 5µm) was used and 20µl of sample/standard was injected into the column for analysis. The column was eluted in gradient mode with a flow rate of 1ml/min at detection wavelength of 590nm. The cycle time of analysis was 65 min at 30°C. The mobile phases consisted of water (A, containing 20% methanol and 0.5% TFA) and acetonitrile (B, containing 10% methanol and 0.5% TFA). The analyses followed a linear gradient program. Initial conditions were 90% A; 0–20 min, changed to 30% A; 20–25 min, to 10% A; 25–30 min, to 0% A kept to 60 min; 60–65 min, went back to 90% A. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standard procured from Sigma Aldrich. Standard was prepared by dissolving 1 mg of pure hypericin standard in 1ml methanol to prepare a stock solution of 1000 ppm. 50 ppm of working standard was prepared from stock solution by dilution with methanol.

3. Results

3.1. Establishment of Multiple Shoots and Root Induction

The surface sterilized shoot apices of *H. perforatum* were cultured on MS media containing BAP (0-3 mg/L), IBA (0-3 mg/L) and KN (0-4 mg/L) in different combinations at 25±2°C. Out of tested different media combinations for multiple shoot formation, MS medium containing KN (3mg/L) + sucrose 3% (w/v) + agar 0.9% (w/v) was found to

be best medium for multiple shoot formation. Total 36.3 shoots were induced from single explant within 5-6 days of culturing (Table 1; Fig 1). This media combination was found to very rapid with higher number of shoot formation.

Table 1. *Effect of different concentrations and combinations of growth hormones on shoot multiplication.*

MS + growth hormones (mg/L)			Parameters of shoot multiplication		
BAP	IBA	KN	Days to multiple shoot formation	Shoots per explant (Mean ± S.E.)	Shoot length (cm) (Mean ± S.E.)
0	0	0	18-20	10.3 ± 0.5	6.6 ± 0.5
1	0.5	0	9-10	15.3 ± 0.5	8.9 ± 0.5
2	1	0	9-10	17.6 ± 0.3	8.8 ± 0.5
0	0	1	8-9	22.2 ± 0.6	10.4 ± 0.5
0	0	3	5-6	36.3 ± 0.3	12.2 ± 0.5
1	2	3	9-10	21.3 ± 0.3	9.7 ± 0.3
1	3	4	6-8	30.6 ± 0.3	8.5 ± 0.6
0	2	4	5-6	35.3 ± 0.3	10.6 ± 0.5
3	3	2	8-9	21.6 ± 0.3	11.1 ± 0.3

Fig 1. *Multiple shoot formation in H. perforatum shoot explant grown on MS medium supplemented with KN (3mg/L) + sucrose 3% (w/v) + agar-agar 0.9% (w/v).*

Table 2. *Effect of different concentrations and combinations of growth hormones on root multiplication.*

MS + growth hormones (mg/L)			Parameters of root formation		
NAA	IBA	KN	Days to root initiation	Number of roots per shoot explant (Mean ± S.E.)	Root length (cm) (Mean ± S.E.)
0	0	0	20-22	3.3 ± 0.3	1.8 ± 0.5
1	0.5	0	10-12	3.5 ± 0.6	2.6 ± 0.5
1	2	1	10-12	4.8 ± 0.6	3.5 ± 0.5
0	3	1	7-8	5.8 ± 0.3	3.8 ± 0.5
3	0	1	9-10	4.8 ± 0.5	2.2± 0.5
1	3	1	9-10	5.0 ± 0.5	2.5± 0.3
2	4	2	10-12	5.2 ± 0.5	2.8 ± 0.6
0	4	2	7-8	4.7 ± 0.6	3.1 ± 0.5
1	2	0	10-12	4.9 ± 0.6	2.9 ± 0.3

Fig 2. *Fully developed H. perforatum plantlet on root induction medium (MS medium supplemented with IBA (3mg/l) + KN (1mg/l) + sucrose 3% (w/v) + agar-agar 0.9% (w/v)).*

In vitro grown shoots were transferred into MS media supplemented with different concentrations and combinations of NAA, IBA and KN for root induction. Highest root induction with 5.8 roots per shoot explants was observed within 7-8 days of culturing in MS medium containing IBA (3 mg/l) and KN (1 mg/l) (Table 2; Fig 2).

3.2. Hardening

Well rooted *in vitro* grown plantlets of *H. perforatum* were transferred to pots containing mixture of sand:soil:vermiculite (1:1:1) for hardening and 80% of survival rate was observed after hardening under greenhouse conditions (Fig 3). The hardened plants were propagated further for two years under greenhouse conditions for the production of hypericin rich shoots.

Fig 3. *Hardened plant of H. perforatum under greenhouse conditions.*

3.3. Hypericin Production

Different media combinations along with different growth parameters were studied for optimization of hypericin production in shoot tissues of *H. perforatum*. Effect of IBA, BAP and KN was evaluated for hypericin production in shoot cultures of *H. perforatum*. MS media supplemented with IBA (3 mg/L) and KN (1 mg/L) showed the highest hypericin production (0.119 µg/mg) (Table 3, Fig 4).

Table 3. *Effect of different growth hormones on hypericin production in shoot cultures of H. perforatum.*

MS + growth hormones (mg/L)			Hypericin content
BAP	IBA	KN	(µg/mg)
0	0	0	0.000 ± 0.000
0	0	1	0.062 ± 0.004
0	0	3	0.108 ± 0.003
1	2	3	0.063 ± 0.003
1	3	4	0.085 ± 0.001
0	2	4	0.096 ± 0.001
0	3	1	0.119 ± 0.001
1	3	1	0.091 ± 0.004
2	4	2	0.054 ± 0.002
1	2	0	0.037 ± 0.002

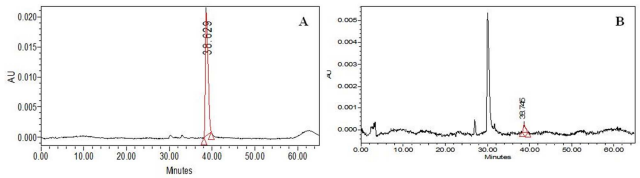

Fig. 4. *HPLC chromatograms of hypericin in (A) standard and (B) shoots of H. perforatum grown on MS media supplemented with IBA (3 mg/L) + KN (1 mg/L) + sucrose 3% (w/v) + agar-agar 0.9% (w/v) under light conditions at 25±2 °C.*

Shoots growing in this media combination were further incubated in light and dark condition on solid and liquid media at 25±2°C and 15±2°C for hypericin production. Browning of shoots was observed under dark condition. Shoots growing on solid media were found to produce higher hypericin content as compared to shoots grown on liquid media (Table 4). Higher hypericin content was observed in shoots grown on 25±2°C as compared to 15±2°C (Table 4). In addition, comparative analysis of 2 year old hardened and field grown plants was done for hypericin production. Comparable amount of hypericin was estimated in hardened and field grown plants (Table 5).

Table 4. *Effect of temperature, light condition on hypericin production in shoots growing on MS media supplemented with sucrose (3%), IBA (3 mg/L) and KN (1 mg/L) with and without agar.*

MS + sucrose + IBA + KN + agar			MS + sucrose + IBA + KN	
	25 ^{0}C	15 ^{0}C	25 ^{0}C	15 ^{0}C
Light	0.119 ± 0.001	0.009 ± 0.001	0.008 ± 0.002	0.006 ± 0.003
Dark	0.003 ± 0.002	0.001 ± 0.001	0.002 ± 0.002	0.001 ± 0.001

Table 5. *Hypericin production in hardened and field grown plants of H. perforatum.*

S. No.	Tissue Type	Hypericin (µg/mg)
1	*In vitro* grown (2 month)	0.11
2	Hardened (1st year)	0.32
3	Hardened (2nd year)	0.37
4	Field grown (1st year)	0.41
5	Field grown (2nd year)	0.44

4. Discussion

Plant tissue culture techniques have been emerging as a tool for *in vitro* production of highly expensive and industrially important secondary metabolites. In current study, we have optimized various tissue culture conditions for rapid hypericin production in *in vitro* grown shoots of *H. perforatum.* Out of tested different combinations of growth hormones *viz.* BAP (0-3 mg/L), IBA (0-3 mg/L) and KN (0-4 mg/L), MS medium supplemented with 3mg/L KN was found to be best medium for multiple shoot formation in this species. Results revealed that BAP did not increase the multiplication of shoots. Higher concentration of two cytokinins together (BAP and KN) proved to be inhibitory for shoot multiplication. KN alone at higher concentrations increased shoot multiplication indicating the direct relationship between growth hormones requirement and multiplication of shoots. MS media supplemented with KN have already been reported to induce multiple shoots in *Picrorhiza kurroa* (Patial *et al.,* 2012). Pretto and Santarem (2000) and Zobayed *et al.,* (2004) have already reported the mass propagation and secondary metabolites production from *H. perforatum.* In this study, rapid method of shoot multiplication was developed using shoot apex as an explant with higher number of shoot induction (36.3 shoots) within 5-6 days of subculturing. As the concentration of IBA increased and KN decreased in the medium, root formation initiated in *H. perforatum* shoots. Early root induction (7-8 days) was observed in MS medium containing IBA (3 mg/l) and KN (1mg/l).

Previous reports have shown that different concentrations and combinations of growth hormones influence the biosynthesis and accumulation of secondary metabolites in different plant species such as menthone, menthol, pulegone, and menthofuran in *Mentha piperita*, *β*-sitosterol and caffeic acid in *Sericostoma pauciflorum,* scopoletin in Angelica archangelica and berberine in *Coscinium fenestratum* (Narasimhan and Nair, 2004; Siatka and Kasparová, 2008; Jain *et al.,* 2012; Santoro *et al.,* 2013). Our results showed that MS medium supplemented with sucrose (3%), IBA (3mg/l), KN (1mg/l) and agar (0.9%) was suitable for better growth and multiplication of shoots as well as maximum accumulation of hypericin content. Similarly, Baskaran *et al.,* (2012) showed that growth and development of plants are influenced by growth hormones through their effects on physiological and biochemical processes. No hypericin content was detected in shoots grown on MS basal media, which further suggests the importance of IBA and KN for hypericin production. This media combination was also tested on two different incubation temperatures and revealed that incubating *H. perforatum* shoots at lower temperature did not show any enhancement in hypericin production. Our results are in agreement with Zobayed *et al.,* (2005) where high temperature treatment of *H. perforatum* resulted in increased hypericin levels in shoot tissues.

Further, *in vitro* grown plants were hardened and maintained in green house for 2 years and compared with field grown plants for hypericin production. Mixture of sand:soil:vermiculite (1:1:1) gave good shoot and root growth as it provided enough moisture, aeration, and micronutrients for the profuse growth of plants. Comparable amount of hypericin was estimated in hardened and field

grown plants. Sustention of *in vitro* grown plants under green house condition showing comparable hypericin content with field grown plants would be of great practical use in tapping other species of *Hypericum* genus having higher secondary metabolite content for their commercialization.

5. Conclusion

In current study, we developed a rapid protocol for *in vitro* multiplication and hypericin production in *H. perforatum* which could not be achieved through conventional methods. Media and different growth parameters have been optimized for *in vitro* production of hypericin rich shoots which would be useful for large scale production of herbal raw material. In addition, hardened *Hypericum* plants showed promising hypericin content comparable to field grown plants. Therefore, this study will open various avenues in setting up industrial scale plant tissue culture units for micropropagation and secondary metabolite production to meet industrial demands for expensive herbal formulations from *H. perforatum.*

Abbreviations

HPLC: High performance liquid chromatography
KN: Kinetin
IBA: indole-3-butyric acid
BAP: 6-benzylaminopurine
MS media: Murashige and Skoog media

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