

Original Research Article

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Stimulation of Hypericin Induction in Calli Cultures of *Hypericum sinaicum* (L.)

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Abstract	Article Info
<p>Sufficient and promising protocol for enhancement and production of hypericin in calli cultures of <i>Hypericum sinaicum</i> was established. The highest value of calli cultures was produced from nodal segment and root explants respectively. MS-medium supplemented with 3 mg/l of 2,4-D gave the best results of calli production under dark condition. However, the lowest calli frequency (28 and 20%) and fresh weights (1.4 and 1.0 g/Jar) were estimated with nodal and root explants cultured on MS medium supplemented with 0.25 mg/l 2,4-D + 0.25 mg/l TDZ under light condition. Calli growth dynamics were investigated during five weeks of cultivation. Total hypericin produced by calli cultures derived from nodal segment and root explant were evaluated using HPLC.</p>	<p>Accepted: 11 April 2016 Available Online: 06 May 2016</p> <hr/> <p>Keywords</p> <p>Calli Growth dynamic Hypericin <i>Hypericum sinaicum</i></p>

Abbreviations: BA: N6-Benzyladenine; B5: Gamborg medium; 2, 4-D: 2,4-Dichlorophenoxyacetic acid; HPLC: High performance liquid chromatography; Kin: Kinetin; LS: Linsmaire and Skoog medium; MS: Murashige and Skoog medium; NAA: Naphthalene acetic acid; *H. sinaicum*: *Hypericum sinaicum*.

Introduction

Plants are essential to humanity for food, environmental intensification and personal fulfillment. Plants are also the foundations of healthy ecosystems ranging from the Arctic to the tropics. Propagation of plants holds tremendous potential for the production of high-quality plant-based medicines (Murch et al., 2000). The genus *Hypericum* belongs to the *Hypericeae* family which encompasses approximately 460 species accommodate in 36 sections throughout the world (Robson, 2006). The genus *Hypericum* includes both annual and perennial herbaceous plants, as well as woody, shrubs and small trees. The genus is of great economical and medicinal importance for containing of phytochemical constituents (Messina, 2006). *Hypericum* species carry secretory structures in above-ground organs (Soelberg et al., 2007),

which are sites of synthesis and accumulation of biologically active substances (Ciccarelli et al., 2001; Onelli et al., 2002), primarily hypericin, pseudohypericin and their derivatives protohypericin and protopseudohypericin (Bombardelli and Morazzoni, 1995). Hypericin and pseudohypericin, which are the basic chemicals of *Hypericum* extracts, are formed in sepals, petals, and stamens of *Hypericum* species (Zobayed et al., 2006), and these compounds have photodynamic, antiviral, antibacterial, antidepressant and antitumoral activities (Agostinis et al., 2002; Guedes and Eriksson, 2005).

H. sinaicum is one of the endemic species in Sant Katherin Protectorate South Sinai, Egypt (Khafagi and Omar, 2012). The importance of this species as a phyto-pharmaceutical, especially hypericin is a substance

isolated from *Hypericum spp.* Hypericin belongs to group of compounds known as naphodianthrones. Hypericin is a secondary plant metabolite of *Hypericum* and the amount of hypericin strongly depend upon the source of the plant material (Wagner and Bladt, 1994).

Over the last decades, plant cell and tissue cultures represent a promising and a unique source for producing plant-derived medicinal metabolites under controlled culture conditions for decades (Jacqueline et al., 1999). In addition, compounds from tissue cultures are more easily purified because of the simple extraction processes used and the absence of contaminants such as pigments, thus consequently, reducing the production and processing costs (Bourgaud et al., 2001). Callus consist of an amorphous mass of loosely arranged thin walled parenchyma cell arising from the proliferating cell of the parent tissue frequently, as a result of wounding a callus is formed at the cut end of a stem or root. As well as callus tissues are an important factor in the establishment of cell cultures.

Production of secondary metabolites *via* plant cell and tissue cultures has various advantages such as obtaining desirable secondary metabolites and large scale production (De Boer et al., 2004). In recent years, plant cell culture techniques has been commonly applied to the production of many useful secondary metabolites, including pharmaceuticals, pigments, and other bioactive compounds (Verpoorte et al., 2002; Bourgaud et al., 2001). Several valuable studies have been performed on the production of useful secondary metabolites in plant cell cultures and organ cultures of some *Hypericum* species. The relationship between biosynthesis of hypericin compounds and *in vitro* development of differentiated structures in *Hypericum* plants has also been investigated (Coste et al., 2011; Palmer and Keller, 2011).

Although *H. sinaicum* is a very valuable medicinal plant but no method has been reported about *in vitro* study such as callus induction and *in vitro* propagation of this plant. Therefore, we conducted this study to assay the effects of plant growth regulators on callus induction and hypericin accumulation.

Materials and methods

Plant materials

Seeds of *H. sinaicum* for the present study were kindly provided by Dr. Hussein Taha, Plant Biotechnology Department National Research Centre, Cairo, Egypt.

Methods

Initiation of sterilized *H. sinaicum* seedlings

H. sinaicum seeds were surface sterilized with 70% (v/v) ethanol solution for 1 min, followed by immersion in a 20 % (v/v) commercial Sodium Hypochlorite (5.25 CL) for 10 min., and rinsed three times with sterile distilled water. Sterile seeds were germinated and placed on the culture medium in jars 200 ml contained 25ml each of three types of full and half strength of nutrient basal media containing, either MS salts (Murashig and Skoog, 1962), LS salts (Linsmaier and Skoog, 1965), or B5 salts (Gamborg et al., 1986), 0.7% (w/v) agar, and 30 g/l sucrose for 4 weeks. The pH of all types of nutrient media was adjusted to 5.8 before adding gelling agents. The media were autoclaved for 20 min.

All cultures were incubated under $26 \pm 1^\circ\text{C}$ in a growth chamber with a daily 16-h photoperiod under standard cool white fluorescent tubes at light intensity $20\text{-}25 \mu\text{mol m}^{-2} \text{s}^{-1}$. After 20-30 days, jars were checked and the germination percentages were scored. This experiment was consisted of 10 jars per type of nutrient medium as replicates.

Establishment of *H. sinaicum* L. calli cultures

Two types of explant nodal segment (3mm) and root (4mm) were excised from the previously *in vitro* germinated seedlings for callus induction. Explants were cultured on different types of culture media to test the effect of different exogenous growth regulators on callus induction. The following modified MS-media were used.

- MS+ 1mg/l (NAA) + 1mg/l (BA)
- MS+ 1mg/l (NAA) + 3mg/l (BA)
- MS+ 1mg/l (NAA) + 5mg/l (BA)
- MS+ 1mg/l (2,4 D)
- MS+ 3mg/l (2,4 D)
- MS+ 5mg/l (2,4 D)
- MS+ 1mg/l (2,4 D) +1 mg/l (kin)
- MS+ 1mg/l (2,4 D) +3 mg/l (kin)
- MS+ 1mg/l (2,4 D) +5 mg/l (kin)
- MS+ 0.25 mg/l (2,4D) + 0.25mg/l (TDZ)
- MS+ 0.25 mg/l (2,4D) + 0.5mg/l (TDZ)
- MS+ 0.25 mg/l (2,4D) + 0.75mg/l (TDZ)
- MS+ 0.25 mg/l (2,4D) + 1mg/l (TDZ)

The replicates of each treatment consisted of five replicates. Cultures were incubated under $26 \pm 1^\circ\text{C}$ in a growth chamber with a daily 16-h photoperiod under standard cool white fluorescent tubes at light intensity $20\text{-}25 \mu\text{mol m}^{-2} \text{s}^{-1}$, and/ or in darkness for 4 weeks. Jars were checked regularly for callus induction. After 4 weeks the following parameters were recorded.

- 1- Percentage of explant forming callus.
- 2- Fresh weight (g/jar).
- 3- Callus figuration

The obtained calli cultures were sub-cultured on fresh modified MS media every month.

Calli growth parameters

Normal curve: According to Smith (2000) initiated calli cultures were divided to fragments ~ 750 mg/ jar (150 ml) and placed on a fresh nutrient medium modified with the best concentrations of growth regulators used for callus induction. This experiment was monitored for four weeks and incubated at $26 \pm 1^\circ\text{C}$.

Growth rate (Gr): According to Dung et al. (1981) fresh weights of calli cultures (g / day) induced from nodal and root explants, were collected weekly intervals over the 5 weeks of cultivation. Four replicates of each explant were used and the growth rate calculated according the following equation:

$$(Ge - G0) / 7$$

Where,

Ge = the weight of callus (g) at the end of 2nd, 3rd, 4th or 5th week;

G0 = the weight of callus at the end of previously week i.e., 1st, 2nd, 3rd or 4th week of cultivation.

Dry matter content (%): The fresh weight (g) of different calli cultures were dried at 50°C for 48 hrs and the dry matter content was estimated according the following equation:

$$\text{Dry matter content (\%)} = (\text{Dry weight}/\text{Fresh weight}) \times 100$$

Statistical analysis

The obtained results were analyzed by ANOVA. The obtained results were statistically appraised according to Snedecor and Cochran (1980).

Sample preparation for chemical analysis

Derived calli from nodal and root explants were air dried on a filter paper to wipe away the agar and excised water. Moreover, *in vitro* germinated plants of *H. sinaicum* on MS medium free growth regulators were used as control treatment. All samples were dried in oven for 3 days at 50°C and grounded with a coffee grinder. Further, all dried samples were extracted and analyzed by HPLC to detect and determination of hypericin content.

HPLC analysis

This part of study was carried out in Polish Academy of Sciences (Department of Biochemistry- Faculty of Agriculture and Biology- Warsaw University of Life Sciences- SGGW) e-mail: kbioch@sggw.pl

The HPLC system was composed of a binary pump (Model 1525, Waters Corporation, Milford, MA, USA), a fluorimetric detector (Model 474, Waters; $\lambda_{\text{ex}}=236$ nm/ $\lambda_{\text{em}}=592$ nm), an autosampler (Model 717plus, Waters) and a personal computer with Breeze data acquisition and integration software (Waters). Chromatographic separations were performed at ambient temperature on a C8 column (Symmetry 4.6 \times 150 mm, 5 μm , Waters) fitted with a C8 guard column (Symmetry 3.9 \times 20 mm, 5 μm , Waters) using gradient eluents. Eluents A consisted of 60 mM phosphate buffer pH 6.8 and eluent B consisted of 80%:20% (v/v) acetonitrile: H₂O. The mobile phase started with eluent A: eluent B at 80%:20%, gradient programs of mobile phase (concentrations and run time) are shown in Table 1. The total run time was 100 min. the flow rate of mobile phase was 1 ml/ min. the injection volumes were 20 μl .

Table 1. Gradient program of mobile phase.

Time (min.)	Eluent A (%)*	Eluent B (%)**
0	80	20
18	0	100
25	0	100
27	80	20
30	80	20

+1 additional minute of column flushing with A: B 80%: 20% before injection; Where: *Eluent A was 60 mM phosphate buffer pH 6.8; **Eluent B was 80% acetonitrile: 20% H₂O.

The relationship between the peak size and the amount of hypericin expressed as the sum of hypericin was tested. The linearity of a method was tested for the concentrations from 0.5 mg of hypericin/ ml. the equation of a straight line was $y = ax + b$, the regression coefficient $r = 0.9995$ as shown in Figs. 1 and 2.

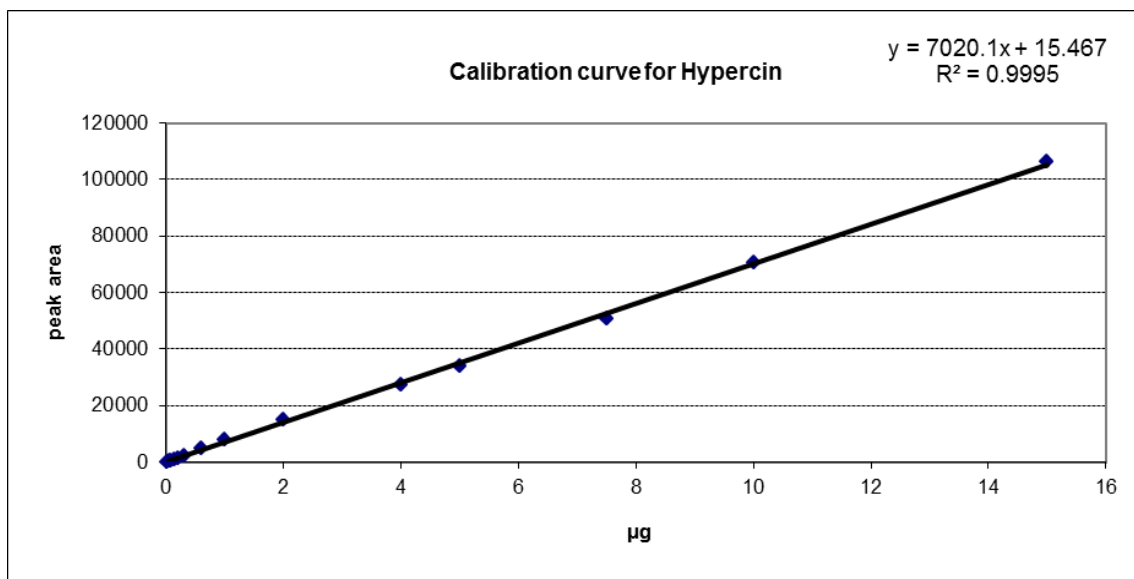


Fig. 1: Hypericin calibration curve.

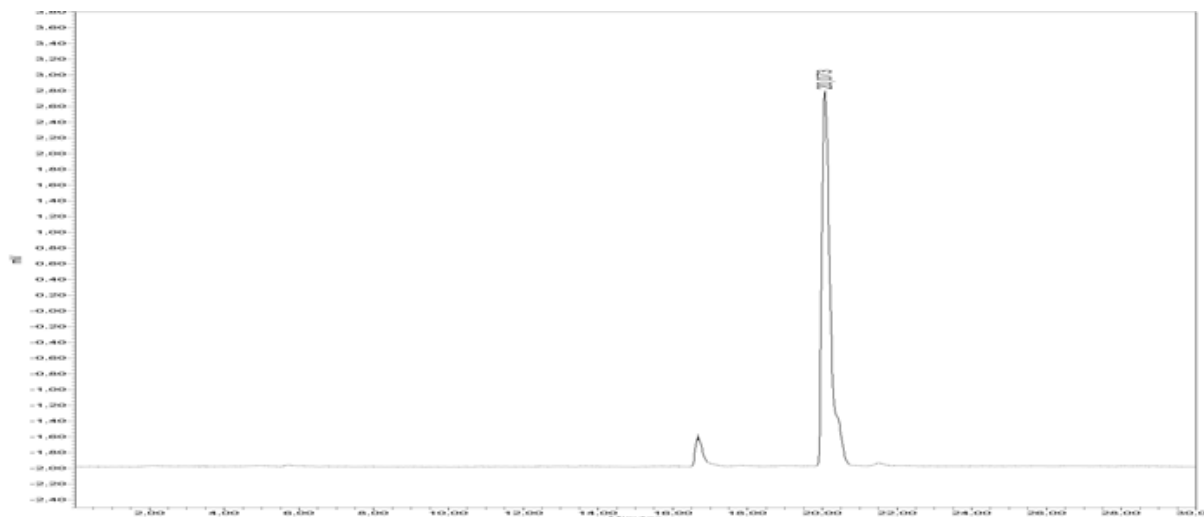


Fig. 2: Hypericin standard curve.

Results

In-vitro seeds germination

Full and half salt strengths of basal MS, LS and B₅ nutrient media were investigated for *H. sinaicum* seeds germination under aseptic conditions. Cultures were incubated for 28 days at 26±1°C under light illumination (16/8hrs). Data tabulated in Table 2 showed that the highest percentages of seeds germination (84%) was recorded with full salt strength of MS media compared other used nutrient media as shown in Fig. 3. However, the lowest percentage of seed germination (20%) was obtained with half salt strength of B₅ nutrient media.

Table 2. Effect of different nutrient media on percentage of *in vitro* *H. sinaicum* seeds germination.

Nutrient media	Germination (%)
MS	84 ± 0.33 ^A
½ MS	72 ± 0.38 ^{AB}
LS	60 ± 0.57 ^B
½ LS	40 ± 0.64 ^C
B ₅	64 ± 0.45 ^B
½ B ₅	20 ± 0.55 ^D
LSD _{0.05}	1.435

Each value is the average of 10 replicates ± Standard error; Average values within the column followed by the same letters are not significantly differed at 5% level.



Fig. 3: *H. sinaicum* L. seeds germination on full salt strength of MS media.

Optimization of *in vitro* culture conditions affecting on callus induction

As shown in Table 3 the effect of MS medium supplemented with various growth regulators on callus induction from nodal and root explants were investigated. The obtained results clearly showed that, the highest frequencies of calli induction (96 and 72%) were recorded

with nodal and root explants (Figs. 4B and D) cultured on MS supplemented with 3 mg/l 2,4-D, and incubated under dark condition, respectively. However, those recorded 92 and 68% under light condition (Figs. 4A and C). On same respect, the maximum values of calli fresh weight (4.8 and 3.6 g/jar) were recorded with nodal and root explants, respectively. MS medium fortified with 3 mg/l 2,4-D gave the best results of calli fresh weight compared other supplementations. In contrast, the lowest calli frequency formation (28 and 20%) and fresh weights (1.4 and 1.0 g/Jar) were estimated with nodal and root explants cultured on MS medium supplemented with 0.25 mg/l 2,4-D + 0.25 mg/l TDZ and incubated under light condition. However, those recorded 32 and 24 (%) for calli frequency, and 1.6, 1.2 g/Jar as fresh weights for nodal and root explants cultured on the same previously mentioned type of nutrient MS medium and incubated under dark condition, respectively. Regarding obtained calli phenotype it was recorded that, supplementation of MS medium with combinations of NAA and BA gave yellowish, green, friable and sometimes compact calli. Moreover, composition of MS medium with 2,4-D in combination with either Kin or TDZ produced yellow, friable and granular calli.

Table 3. Effects of MS medium supplemented with various PGR_s at different concentrations on frequencies of calli induction and calli fresh weight of nodal and root explants of *H. sinaicum* incubated under light or dark condition at 26 ± 1°C.

MS media supplemented with growth regulators (mg/l)		Under light condition at 26 ± 1°C				Under darkness conditions at 26 ± 1°C			
		Frequency of callus induction (%)		Callus fresh weight (g/jar)		Frequency of callus induction (%)		Callus fresh weight (g/jar)	
Auxin	Cytokinin	Nodal explant	Root Explant	Nodal explant	Root explant	Nodal explant	Root Explant	Nodal explant	Root Explant
1 mg/l NAA	1 mg/l BA	88	68	4.4±0.31	3.4±0.31	92	72	4.6±0.31	3.6±0.31
1 mg/l NAA	3 mg/l BA	80	64	4±0.57	3.2±0.48	88	68	4.4±0.51	3.4±0.51
1 mg/l NAA	5 mg/l BA	84	64	4.2±0.48	3.2±0.48	88	68	4.4±0.31	3.4±0.51
1 mg/l (2,4D)	0	68	60	3.4±0.31	3±0.40	72	64	3.6±0.31	3.2±0.25
3 mg/l (2,4D)	0	92	68	4.6±0.31	3.4±0.31	96	72	4.8±0.25	3.6±0.31
5 mg/l (2,4D)	0	80	60	4±0.40	3±0.40	84	64	4.2±0.25	3.2±0.25
1 mg/l (2,4D)	1 mg/l Kin	28	24	1.4±0.77	1.2±0.48	32	28	1.6±0.65	1.4±0.31
1 mg/l (2,4D)	3 mg/l Kin	36	28	1.8±0.75	1.4±0.65	40	32	2±0.57	1.6±0.65
1 mg/l (2,4D)	5 mg/l Kin	48	36	2.4±0.65	1.8±0.63	52	40	2.6±0.51	2±0.40
0.25mg/l (2,4D)	0.25 mg/l TDZ	28	20	1.4±0.65	1±0.40	32	24	1.6±0.51	1.2±0.48
0.25mg/l (2,4D)	0.50mg/l TDZ	36	32	1.8±0.25	1.6±0.31	40	32	2±0.0	1.6±0.31
0.25mg/l (2,4D)	0.75 mg/l TDZ	48	32	2.4±0.77	1.6±0.77	52	36	2.6±0.65	1.8±0.75
0.25 mg/l (2,4D)	1 mg/l TDZ	72	52	3.6±0.51	2.6±0.31	76	52	3.8±0.48	2.6±0.31

Each value is the average of 5 replicates ± standard error.

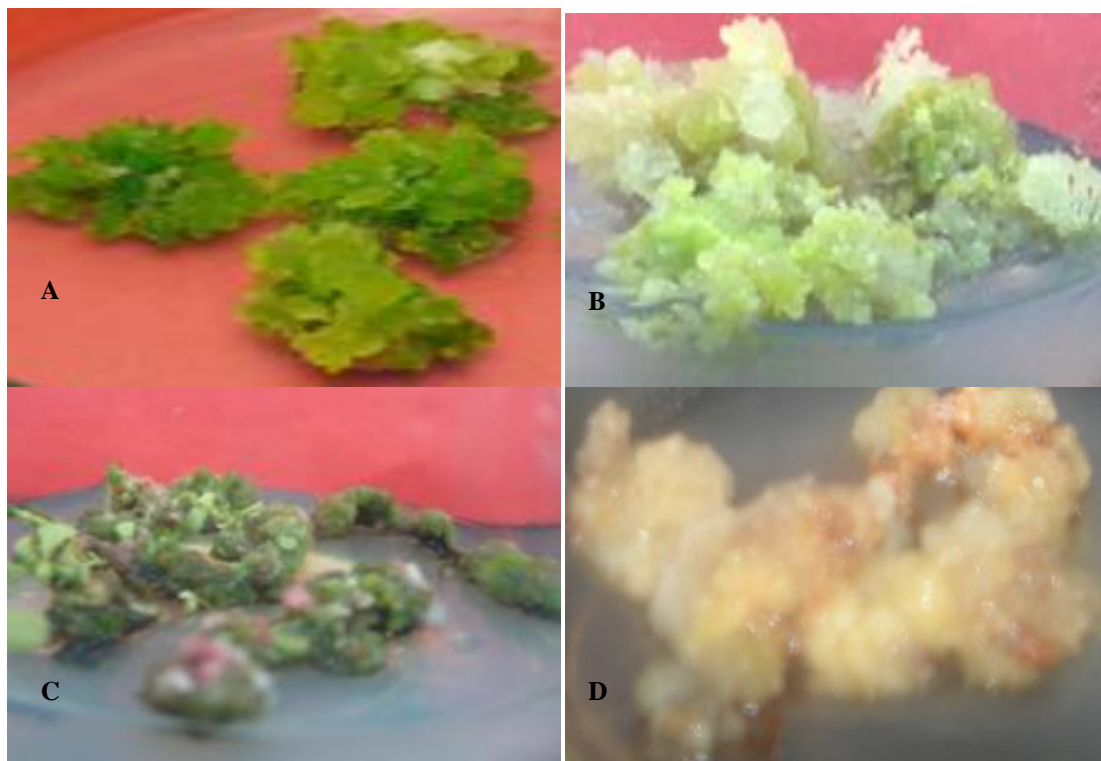


Fig. 4: Effect of MS media supplemented with 3mg/l 2,4D on calli induction from nodal explant (A and B), root explant (C and D) of *H. sinaicum* incubated under light and dark conditions.

Calli growth parameters

The growth characteristics and pattern of calli derived from nodal and root explants of *H. sinaicum* growing in MS media containing 3mg/l of 2,4D was investigated. Calli growth patterns were expressed as fresh weight (g), dry weight (g) and dry matter content (%). The growth dynamics of the obtained different calli cultures were determined weekly for five weeks of cultivation. The obtained data clearly showed that the highest values of calli fresh weight 5.4 and 4.83 (g/Jar) were recorded at the end of the 5th week of cultivation for nodal and root explants, respectively.

Regarding those dry weight and dry matter content values of the callus derived from different explants were calculated. The results clearly showed that the highest dry weights were obtained at the end of the 5th week of cultivation as shown in Fig. 5A. Also, the maximum dry matter content (82.2 and 82%) was recorded with nodal and root explant, respectively at the end of the 5th week of cultivation as shown in Fig. 5B. In addition, daily growth rates (0.33 and 0.18 g/day) were recorded at the end of the 3rd week of cultivation for nodal and root explants, respectively.

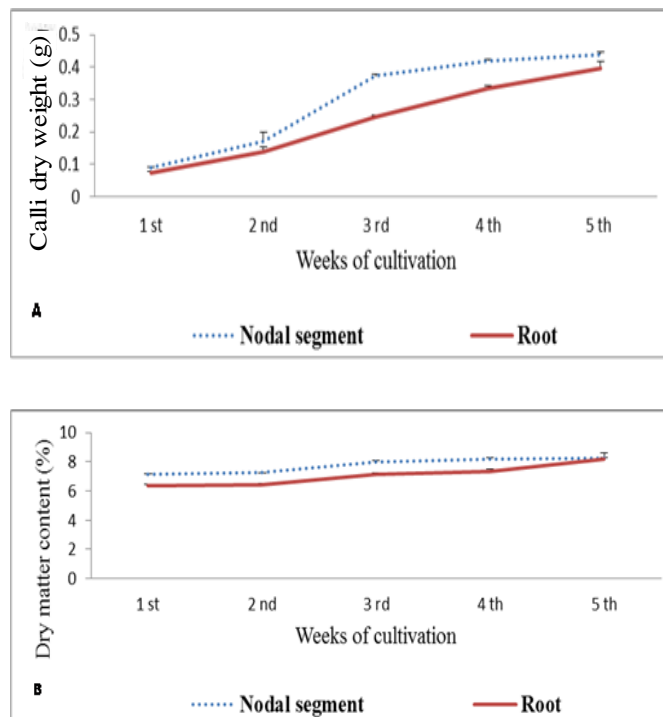


Fig. 5: Dry weight (A) and dry matter content% (B) of calli derived from nodal and root explants of *H. sinaicum* during five weeks of cultivation.

Hypericin content ($\mu\text{g/g}$ dry weight)

Total hypericin content extracted from *in vitro* derived plantlets on free MS medium; calli cultures derived from either nodal or root explants cultured on MS medium supplement with 3mg/l 2,4D was determined and compared to hypericin standard curve (Fig. 2).

The data clearly revealed that the highest accumulation of hypericin contents 32.99 $\mu\text{g/g}$ dry weights was recorded in *in vitro* plantlets grown on free full salt strength of MS medium (Fig. 6A), while calli cultures derived from nodal and root explants were recorded 21.09, 7.23 $\mu\text{g/g}$ dry weight, respectively (Fig. 6B and C).

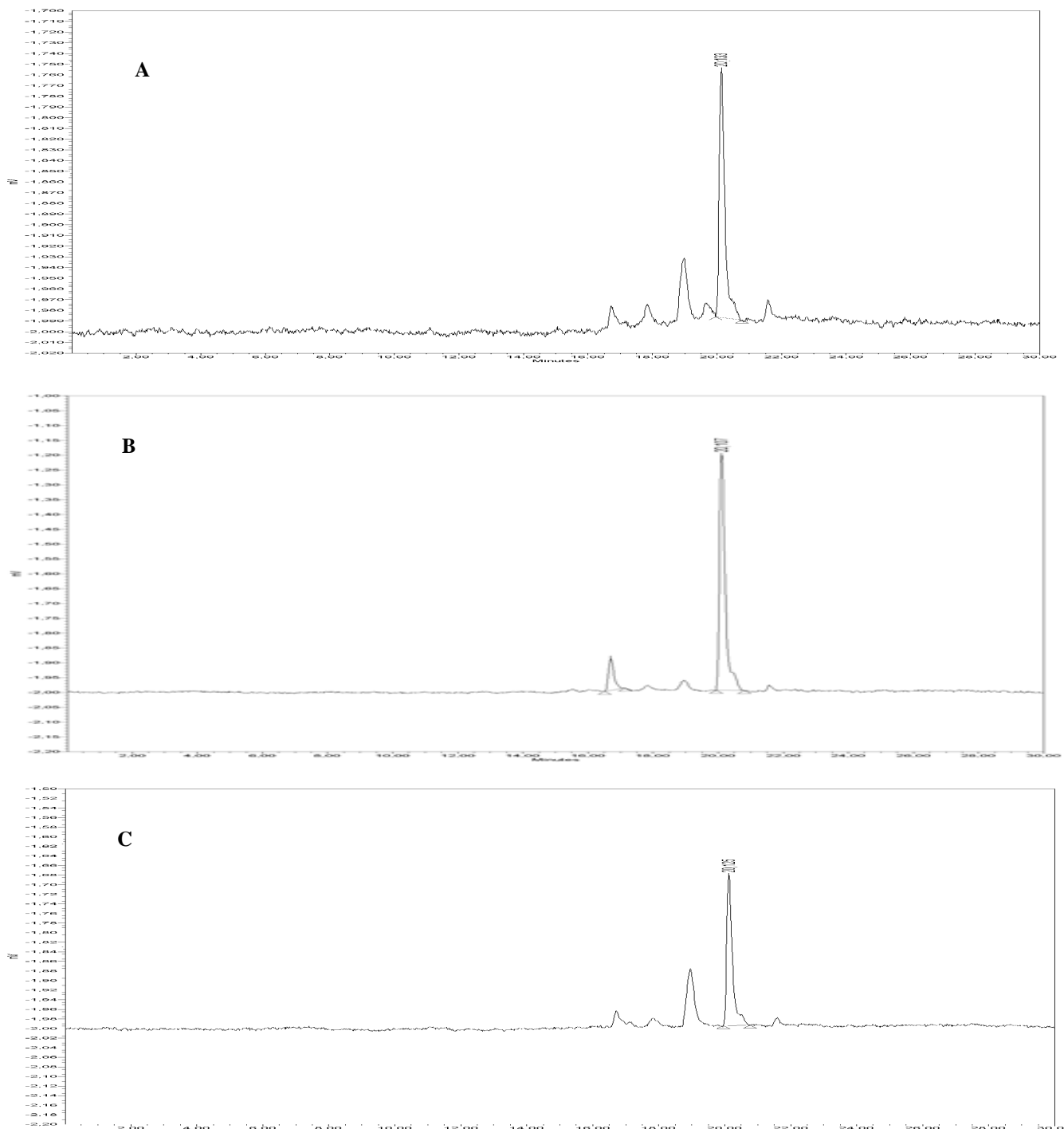


Fig. 6: HPLC peaks of hypericin extracted from; *in vitro* plantlets (control) (A); calli derived from nodal segment (B) and root explant (C) of *H. sinaicum*.

Discussion

Plant tissue culture, or the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro*, is an important tool in both basic and applied studies as well as in commercial application (Thorpe, 1990). Different approaches have been taken to enhance yields of secondary metabolites. These include cell cloning and the repeated selection of high-yielding strains from heterogeneous cell populations (Dougall, 1987). Generalizations about plant growth regulators and their use in plant cell culture media summarized by George et al. (2008) who reported that, auxins promote both cell division and cell growth. The most important naturally occurring auxin is IAA (indole-3-acetic acid), but it is limited used because it is unstable to both heat and light. 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used auxin and is extremely effective in most circumstances.

The obtained results showed that, the highest frequencies of calli induction (96%) were recorded with nodal explants grown in MS containing 3 mg/l 2,4-D, and incubated under dark condition. Pretto and Santarém (2000) reported that MS basal media containing BA in combination with 2,4-D showed the highest calli formation, while Walker et al. (2002) reported that BA in combination with 2,4-D was efficient for calli formation. Ayan et al. (2005) obtained that the highest frequency of callus induction from the leaf discs cultivated on the medium supplemented with of 2,4-D and kinetin.

According to previous reports, in combinations with 2,4-D and kinetin or BA proved to be effective plant growth regulators for callus induction and cell suspension cultures from *H. perforatum*. Regarding the stimulating effect of darkness on callus initiation and callus production, our results may support those of Yazaki and Okuda (1990) they found that highest callus initiation and mass callus production of *H. perforatum* was achieved when cultures were incubated under dark condition.

Also in our study, the obtained results showed that *in vitro* cultivation of nodal and root explants of *H. sinaicum* during 3 weeks was more economically and significantly for calli production. These results in close with Smith (2000) who reported that, the rate of growth of calli tissue parallels in many ways the sigmoid curve seen in population of single. There are

usually five stages for callus growth rate; a lag phase in which cells prepare to divide, a period of exponential growth in which cell division is maximal, a period of linear growth in which division slows down and cells enlarge, a period of decelerating growth and stationary or no-growth period in which the number and size of cells is constant.

Hypericin content of calli derived from nodal segment cultured on MS medium containing 2,4-D was 21.09 µg/g dry weight, while this rate was 7.23 µg/g dry weight in calli derived from root explant. Hypericin content of *in vitro* plantlets derived free MS medium was 32.99 µg/g dry. A similar study on hypericin content in calli cultures of *H. triquetrifolium* Turra was conducted by Oluk et al. (2010) they detected 0.048 mg g⁻¹ amount of hypericin in calli of *H. triquetrifolium*. In another study, Santarem and Astarita (2003) reported that the level of hypericin in calli of *H. perforatum* was very low, representing only 0.11% of the amount found in the field-grown plants. Moreover, it has been reported that callus initiated from stamens of *H. perforatum* showed only traces of hypericin or pseudohypericin (Kirakosian et al., 2000). In addition, Pasqua et al. (2003) reported that although undifferentiated calli and suspension cultures in MS medium containing 1.105 mg/l 2,4-D, 0.215 mg/l Kin and 0.186 mg/l NAA was not produce hypericin or pseudohypericin but calli with vegetative buds produced hypericin (0.015 %) and pseudohypericin (0.050 %).

Conclusion

In this study, we reported for the first time a protocol for the successful callus induction, in *H. sinaicum* which would provide more homogenous source of medicine. The frequency of callus induction from nodal explants described here was high enough to encourage us to carry out, regeneration culture, genetic transformation and cell suspension culture for improvement of secondary metabolites quality and quantity.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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