Multiple shoot formation in *Hypericum perforatum* L. and hypericin production

Eliane Romanato Santarém and Leandro Vieira Astarita*

*Laboratório de Biotecnologia Vegetal, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6681, Prédio 12C, sala 253, CEP 90619-900, Porto Alegre, RS, Brasil; *Corresponding author: astarita@pucrs.br*
Received: 06/01/2003, Accepted: 12/03/2003

Hypericum perforatum is a traditional medicinal plant with wound healing and antidepressive properties. Among the secondary compounds of interest is hypericin, a naphtodianthrone that seems to participate in the medicinal effects of this species. The aim of this work was to obtain an efficient micropropagation system of *H. perforatum* and to compare the hypericin content between *in vitro* and field-grown plants. Cultures were initiated from nodal segments of mature plants inoculated onto MS medium supplemented with 4.5 µM BA, kinetin, thidiazuron, individually or in combination with 0.05 µM NAA. Organogenic explants were observed on medium with either BA or kinetin alone or in combination of these with NAA. Subculture of organogenic explants onto the proliferation medium containing 4.5 µM BA promoted the organogenic response. The highest average of shoot production (52.6 shoots) was obtained on those explants induced in the presence of BA and NAA. Rooted plantlets were successfully acclimated. Analysis of hypericin contents showed that levels found in callus represented only 0.11 % of what was detected in adult plants, while shoots and leaves from *in vitro* plants showed similar hypericin levels to those found in the leaves of the field-grown plants, suggesting that the accumulation of this compound is related to leaf differentiation.

Key words: medicinal plant, micropropagation, organogenesis, St. John´s Wort.

Formação de múltiplas gemas em *Hypericum perforatum* e produção de hipericina: *Hypericum perforatum* é uma planta medicinal que apresenta propriedades cicatrizante e antidepressiva. Entre os compostos secundários de interesse está a hipericina, uma naftodiantrona que parece participar dos efeitos medicinais dessa espécie. Os objetivos deste trabalho foram propor um sistema otimizado de micropropagação de *H. perforatum* e comparar a quantidade de hipericina nas plantas produzidas *in vitro* e no campo. As culturas *in vitro* foram iniciadas a partir de segmentos nodais de plantas adultas inoculados em meio MS, suplementado com 4,5 µM de BA, cinetina ou tidiazuron, individualmente ou combinados com 0,05 µM de ANA. Explantes organogênicos foram obtidos em meio com 4,5 µM de BA ou cinetina, ou combinações destes com ANA. A subcultura dos explantes no meio de proliferação contendo 4,5 µM de BA promoveu a organogênese. A maior média de brotos obtida na fase de proliferação foi de 52,6 brotos, naqueles explantes induzidos na presença de BA e ANA. As plântulas enraizadas foram aclimatadas com sucesso. A análise da quantidade de hipericina demonstrou que os calos acumularam somente 0,11 % da concentração de hipericina encontrada nas plantas adultas, enquanto nos brotos e nas plântulas regeneradas *in vitro*, os níveis de hipericina foram similares àqueles das plantas cultivadas em campo, sugerindo que o acúmulo de hipericina está relacionado com a diferenciação do tecido foliar.

Palavras-chave: erva de São João, micropropagação, organogênese, planta medicina.

Hypericum perforatum (St. John's Wort) is an herbaceous perennial species well known as a medicinal plant, which has been used since ancient times for its antiviral, wound-healing and antimicrobial properties, and recently, as an antidepressant (Vitello, 1999). The naphtodianthrones, hypericin and pseudohypericin, appear

to contribute to the antidepressant action of this species, and most of *Hypericum* phytomedicines are currently standardized according to their hypericin content (Briskin, 2000). Hypericin is found in dark glandular structures located on the margins of leaves and petals (Fornasiero et al., 1998).

In vitro systems have been reported as an effective tool for obtaining genetically uniform plants, which can be the source for less variable pharmaceutical preparations. Plant regeneration of *Hypericum* species has been achieved using as explants whole seedlings or their excised parts (Cellárová et al., 1992), hypocotyl sections (Murch et al., 2000) and leaves (Pretto and Santarém, 2000), using various types and concentrations of cytokinins and auxins. Due to the climatic conditions in Southern Brazil, blooming of *H. perforatum* is not promoted and seeds are difficult to obtain. Therefore, procedures that optimize the micropropagation from vegetative parts are desirable. Moreover, tissue culture plays an important role in the studies of plant secondary metabolism. The available data suggest that secondary metabolite synthesis by *in vitro* cultured cells can differ from those of whole plants, which many times limit the application of this technology for pharmaceutical applications. Studies on *in vitro* cultured cells and tissues have demonstrated that hypericin contents are variable and often low, thus, the use of elicitors has been suggested as an alternative for the stimulation of the production of this biologically active compound (Kirakosyan et al., 2000; Walker et al., 2002). Therefore, the aim of this report was to optimize the micropropagation of *Hypericum* plants from vegetative parts and evaluate the content of hypericin in different stages of the organogenic process, comparing to its content in naturally growing plants.

Stem segments of *H. perforatum* were excised from one-year-old plants and were surface disinfected (Pretto and Santarém, 2000). Under aseptic conditions, nodal segments (ca. 2 mm) were excised from the stems, being the leaves and petioles discarded. The basal medium for shoot induction contained MS salts and vitamins (Murashige and Skoog, 1962), 30 $g.L^{-1}$ sucrose and 3 $g.L^{-1}$ Phytagel™. The pH was adjusted to 5.8. In order to test the effect of different growth regulators on shoot induction, 4.5 $\mu mol.L^{-1}$ of benzyladenine (BA), kinetin or thidiazuron (TDZ) were incorporated into the basal medium individually or in combination with 0.05 $\mu mol.L^{-1}$ 1-naphtaleneacetic acid (NAA). Hormone-free medium was used as control. Subcultures to fresh medium were carried out every 20 d. Proliferation of the induced shoots was evaluated by transferring the organogenic explants to a MS-based medium supplemented with 4.5 $\mu mol.L^{-1}$ BA.

Each treatment consisted of six flasks with five explants per flask. The experiment was repeated twice. All the cultures were maintained at 25 ± 2 ºC, under 16 h photoperiod at 30 $\mu mol.m^{-2}.s^{-1}$ irradiance. The organogenic efficiency was recorded from the number of organogenic explants per total number of explants 60 d after culture initiation. The number of shoots induced per explant was also evaluated after 60 d. The shoot multiplication was recorded after 30 d of cultivation on proliferation medium.

Elongated shoots from proliferation medium were rooted on half-strength MS medium (1/2 MS) containing 20 $g.L^{-1}$ sucrose, 5.4 $\mu mol.L^{-1}$ indolebutyric acid (IBA) and 3 $g.L^{-1}$ Phytagel™ (Pretto and Santarém, 2000). *In vitro* plantlets with well-developed roots were acclimated as previously reported (Pretto and Santarém, 2000) and the survival frequency was recorded after 10 d.

Callus, *in vitro* shoots, aerial parts of *in vitro* plantlets and aerial parts of field plants (1 g fresh weight) were analyzed for hypericin content. Samples were homogenized at room temperature with 10 mL methanol and 5 mL of the crude extract were filtered through 0.45 µm filter and Sep-Pack™ C18 cartridges. Hypericin was eluted with 10 mL methanol. Samples were maintained at –20 ºC until chromatographic analyses were carried out. All solvents were purchased from Mallinkrodt (USA). The HPLC analyses were carried out in a Gilson System (Gilson Medical Eletronics, France) equipped with a 321 model pump, operated at room temperature (25 ± 2 ºC). Separations were performed on a MetaSil ODS column (5 µm; 150 x 4.6 mm) and detection was achieved with a UV/Vis detector set at 590 nm. The chromatographic data were recorded and processed on Unipoint® System software. Gradient was formed between two mobile phases: phase A consisted of 0.01 % of phosphoric acid in water and phase B was a mixture of methanol: acetonitrile (70:30, v/v). The analysis followed a linear gradient programmed as 60 % to 100 % B from 0 to10 min and kept at 100 % B from 10 to 15 min. The flow rate was kept constant at 1 $ml.min^{-1}$ and injection volume was 20 µL. HPLC analysis was performed by using a six point calibration curve generated with authentic hypericin (Calbiochem, USA). All parameters were analyzed by analysis of variance

(ANOVA). Variance homogeneity was verified through a graphical representation of residuals. Data from organogenic frequency were transformed using $x'=\text{arcsen}\sqrt{x/100}$ (Fowler and Cohen, 1990). HPLC results from four replicates for each sample were compared using ANOVA. Means were separated by Tukey Test, $p \leq 0.05$. The statistical analysis was performed using Statistica 4.3 for Windows™.

De novo shoot organogenesis was induced on nodal segments of *H. perforatum* cultivated on medium containing cytokinins alone or in combination with NAA. The organogenic frequency, as well as the number of shoots induced, varied according to the growth regulator used. The highest organogenic frequency was obtained from explants cultivated on medium supplemented with cytokinins and auxins (table 1). No organogenic response was obtained in explants grown on medium supplement with NAA as the only growth regulator. Besides the organogenic response, callus formation was observed on the explants cultivated in presence of TDZ, NAA or combination of both, at frequencies of 65.4 %, 60.7 % and 57.7 %, respectively (data not shown). No callus formation was induced on the other media tested. In *H. perforatum* callus formation has been obtained from leaf explants (Pretto and Santarém, 2000) and from stamens (Kirakosyan et al., 2000) and calli were the intermediate step to regenerate shoots. In our study, calli were maintained under a continuous growth cycle and samples were taken for hypericin analysis.

The combination of cytokinins and auxin promoted shoot formation. The number of shoots induced on the nodal segments varied with type of cytokinin. Explants cultivated on medium containing BA and NAA resulted in 40.6 shoots per explant after 60 d in culture (figure 1A), while the other cytokinins used for shoot induction were not as effective even in combination with NAA (table 1). The effects of auxins and cytokinins on shoot multiplication have been reported for *Hypericum* species. In *Hypericum foliosum*, the highest number of shoots was obtained on media supplemented with BA and NAA, simultaneously (Moura, 1998). Similar results were also reported for *H. canariensis* (Mederos, 1991). Among the cytokinins, TDZ was reported to be more efficient than BA on promoting adventitious shoots in *H. perforatum*. In hypocotyl sections, the presence of 5 µmol.L^{-1} TDZ produced 54 shoots per explant while BA was not effective for inducing shoots (Murch et al., 2000). In our experiments, the addition of TDZ to the culture medium resulted in the lowest organogenic frequency and average shoot number per explant (table 1), indicating that the organogenic response might be related to the initial explant used for micropropagation.

Table 1. Shoot induction and proliferation from nodal segments of *Hypericum perforatum* cultured on different concentrations and types of cytokinins. Data of organogenic explants and shoot induction were obtained after 60 days in culture, and proliferated shoots were recorded after 30 d on 4.5 µmol.L^{-1} BA.

Growth regulators used for shoot induction (µmol.L^{-1})	Organogenic explants (%)[a]	Average number of shoots	
		Induction	Proliferation
Control	0 d	0 c	-
4.5 BA	31.5 bc	16.9 b	31.3 b
4.5 KIN	20.6 cd	20.7 ab	32.5 b
4.5 TDZ	19.9 c	6.0 c	14.3 bc
0.05 NAA	0 d	0 d	21.2 bc
4.5 BA + 0.05 NAA	55.6 a	40.6 a	52.6 a
4.5 KIN + 0.05 NAA	39.6 ab	5.5 c	33.8 b
4.5 TDZ + 0.05 NAA	44.4 ab	3.7 c	7.4 c

[a] Data from organogenic frequency were transformed using $x'=\text{arcsen}\sqrt{x/100}$. Means followed by different letters in each column differ significantly according to Tukey's Test at the probability level p £ 0.05. - : the non-organogenic explants from the control treatment were not transferred to proliferation medium.

The subculture of the shoot-forming explants onto the proliferation medium (4.5 µmol.L^{-1} BA) promoted the organogenic response, regardless the treatment used for shoot induction. However, the highest average shoot number was obtained in the organogenic explants induced on the presence of BA combined with NAA. The inhibitory effect of TDZ on shoot proliferation was overcome when the explants were transferred to the medium containing BA.

Roots were successfully induced on shoots of *H. perforatum* when transferred to 1/2 MS medium supplemented with 4.5 µmol.L^{-1} IBA after 14 d (figure 1B). Plantlets with well-developed roots were successfully hardened off in the non-sterile conditions and transferred to greenhouse. The survival frequency was 98 %.

Production of hypericin was evaluated at different stages of the organogenic process. Callus, shoots and plantlets were analyzed for identification and quantification of this compound and the data was compared to the hypericin content found in the field-grown plants. Preliminary analysis has not shown significant differences in the hypericin levels among the *in vitro* shoots from the different induction treatments (data not shown). Therefore, shoots from the 4.5 µmol.L^{-1} BA + 0.05 µmol.L^{-1} NAA were used for further chromatographic analyses. Hypericin contents are showed in the figure 1C. The level of hypericin in callus was very low, representing only 0.11 % of the amount found in the field-grown plants. It has been reported that callus initiated from stamens of *H. perforatum* showed only traces of hypericin or pseudohypericin (Kirakosian et al., 2000).

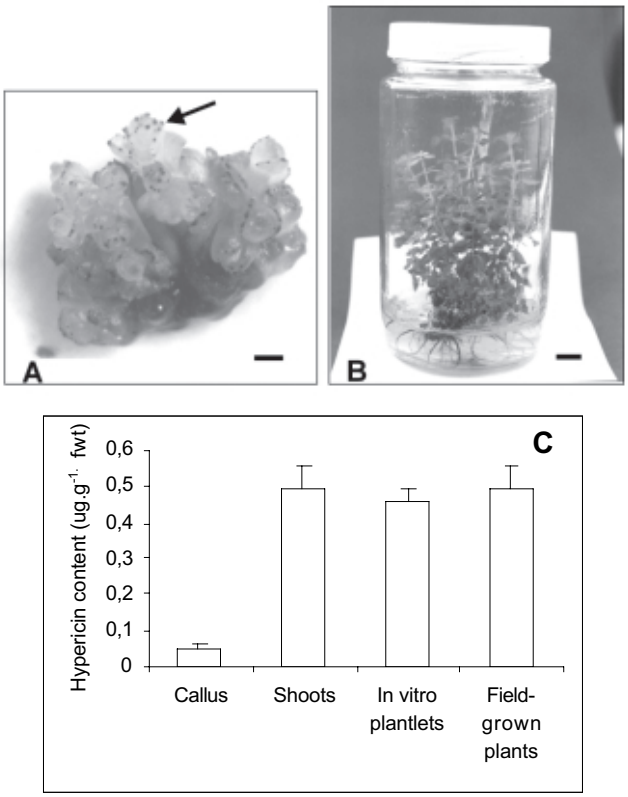

Figure 1. Organogenesis on nodal segments of *H. perforatum*. (A) Organogenic explant cultivated on 4.5 µmol.L^{-1} BA + 0.05 µmol.L^{-1} NAA for 60 d. Arrow indicates the dark glands on newly developing leaves. (B) Regenerated plantlets obtained *in vitro* on 1/2 MS medium supplemented with 4.5µmol.L^{-1} IBA after 14 d. Bars represent 15 mm. (C) Hypericin content analyzed at various stages of micropropagation of *H. perforatum*. Bars represent the standard deviation.

The biosynthesis of hypericin seems to be correlated with the morphogenesis and development of dark red oil glands in leaves of intact plants (Zdunek and Alfermann, 1992). Similar dark glands on newly developed leaves from *in vitro* plants were reported by Walker et al. (2002). Our results showed that shoots and aerial parts of the plantlets regenerated *in vitro* exhibited translucent and dark glands characteristic of *H. perforatum* (figure 1A). Unlike callus culture, higher contents of hypericin were detected in *in vitro* shoots and regenerated plantlets, reaching 0.49 and 0.46 µg.g^{-1} fresh weight, respectively. These levels are similar to those found in the naturally growing plants (figure 1C). Such observations lead to the conclusion that hypericin biosynthesis is related to leaf differentiation degree and to the formation of dark glandular structures.

The protocol here reported allows the production of up to 52 shoots per explant in 3 months of culture using nodal segments of mature plants. The chromatographic analysis of the regenerated plants of *H. perforatum* showed hypericin contents similar to those found in field-grown plants, suggesting that this micropropagation system could be used for selection of plants with high levels of hypericin.

Acknowledgments: This work was supported by Fundação de Amparo à Pesquisa do Rio Grande do Sul – FAPERGS and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq.

REFERENCES

Briskin DP (2000) Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Plant Physiol. 124:507-514.

Cellárová E, Kimákova K, Brutóvska R (1992) Multiple shoot formation in *Hypericum perforatum* L. and variability of R_0. Biol. Plant. 34 (suppl): 536.

Fornasiero RB, Bianchi A, Pinetti A (1998) Anatomical and ultraestructure observations in *Hypericum perforatum* L. leaves. J. Herbs Spices Med. Plants 5:21-33.

Fowler J, Cohen L (1990) Practical statistics for field biology. Open University Press, Buckingham.

Kirakosyan A, Vardapetyan RR, Charchoglyan AG (2000) The content of hypericin and pseudohypericin in cell cultures of *Hypericum perforatum*. Russ. J. Plant Physiol. 47:270-273.

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

Moura M. (1998) Conservation of *Hypericum foliosum*, an endemic azorean species, by micropropagation. In Vitro Cell. Dev. Biol. - Plant 34:244-248.

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

Murch SJ, Choffe KL, Victor JMR, Slimmon TY, Krishna Raj S, Saxena PK (2000) Thidiazuron-induced plant regeneration from hypocotyl cultures of St. John's wort (*Hypericum perforatum* cv. "Anthos"). Plant Cell Rep. 19:576-581.

Pretto FR, Santarém ER (2000) Callus formation and plant regeneration from *Hypericum perforatum* leaves. Plant Cell Tis. Org. Cult. 62:107-113.

Vitello B (1999) *Hypericum perforatum* extracts as potential antidepressants. J. Pharm. Pharmacol. 51:513-517.

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

Zdunek R, Alfermann W (1992) Initiation of shoot organ cultures of *Hypericum perforatum* and formation of hypericin derivatives. Planta Med. 58:621-622.