

Secondary metabolism in micropropagated *Hypericum perforatum* L. grown in non-aerated liquid medium

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Abstract *Hypericum perforatum* L. is a medicinal plant that has been extensively studied because of its bioactive properties. The objective of this study was to establish a system that could lower the cost of in vitro propagation by using liquid medium, as well as to evaluate the secondary metabolism in the systems tested. Nodal segments of *H. perforatum* were obtained from in vitro shoots and grown in three liquid culture systems: total immersion (TI), partial immersion (PI), and paper bridge support (PB). Semi-solid medium (3 g L^{-1} PhytigelTM) was used as control (SS). The organogenic responses were evaluated, and phenolic compounds, hypericin, and the activity of polyphenol oxidases (PPO) and peroxidases (POX) were quantified. After 80 days of culture, induction and proliferation of adventitious shoots were similar in the PI and SS systems (65.3 and 71.3 shoots, respectively), whereas PB resulted in the fewest shoots per explant (29.5 shoots). Longer shoots were obtained under the PI conditions. Hyperhydricity was observed in the shoots from the TI system. Browning was visible in shoots from the TI and PB systems. The highest concentrations of phenolic compounds and hypericin were observed in shoots derived from PI and PB, at 80 days of culture. POX activity was higher in shoots cultured in PI at 40 days, whereas PPO was significantly more active at 80 days of culture. Likely, POX was more related to shoot growth, whereas PPO played a later role in response to the culture environment and medium stress.

Keywords Hypericin · Liquid cultures · Micropropagation · Phenolic compounds · Polyphenol oxidases · Peroxidases

Introduction

Micropropagation allows the controlled production of genetically uniform and pathogen-free plants, and makes studies on secondary metabolites feasible. Most of the micropropagation processes are carried out on solidifying media, using gelling agents to create a substrate on which plant tissues are cultured (Robert et al. 2008). The agar system seems to be suitable for most species, although several studies have reported the successful use of liquid media in different culture systems (Ziv 2005; Cui et al. 2010; Pati et al. 2011). Liquid systems for tissue culture have several advantages over gelled media, including reduced cost of media preparation, lack of impurities from the solidifying agent, and greater efficiency in transferring plantlets to ex vitro environment. Liquid medium has also been shown to allow more rapid growth of plants (Adelberg 2006; Kämäräinen-Karppinen et al. 2010). In several liquid systems, however, plantlets or explants are completely submerged in the medium during the culture, which may result in hyperhydricity of plant tissues (Paek et al. 2005; Coste et al. 2011; Ivanova and Van Staden 2011). This physiological condition is characterized by several morphological alterations including a glossy waterlogged-tissue appearance and disordered growth in the shoot system (Ziv 2005, Jausoro et al. 2010). It has been correlated to water availability, microelements and/or hormone imbalance (Wu et al. 2009). Besides hyperhydricity, agitation or aeration applied to the liquid system may cause mechanical stress on plant tissues (Ziv 2005; Afreen 2006).

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In order to avoid these problems, other procedures have been developed, including culture supports such as paper bridges, liquid medium overlaying and temporary immersion system (Berthouly and Etienne 2005; Yan et al. 2010; Siddiqui et al. 2011). Although these systems have proved to be efficient for growing many plant species, these environments may cause oxidative stress in the cultured tissues, causing browning and ultimately cell death.

In vitro culture is likely to be indirectly affected by polyphenol oxidases (PPO, EC 1.14.18.1 or EC 1.10.3.2) and peroxidases (POX, EC 1.11.1.7), antioxidative enzymes known to be involved in organogenic processes and stress responses (Kormutak and Vookova 2001; Ozyigit 2008; Abbasi et al. 2011). Peroxidases catalyze the oxidation of organic substrates, including phenolics, in the presence of hydrogen peroxide, and have been implicated in the processes of plant growth, development, defense, and cell wall formation (Hatzilazarou et al. 2006). PPO is a nuclear-encoded copper-containing enzyme that catalyzes the oxidation of phenols to *o*-quinones (Sahoo et al. 2009), and plays a role in pigment formation, oxygen scavenging, and the defense mechanism against insects and plant pathogens (Tang and Newton 2004). Plant phenolics also function as modulators of indole acetic acid (IAA) catabolism and increase the rigidity of plant cell walls, acting as molecular bridges between cell wall components (Ozyigit 2008). Hence, adjusting the balance between these enzymes and the levels of their substrates might be a strategy of tissues to cope with the stress inherent in in vitro systems, and might determine the regeneration capability of the explant.

Because of the therapeutic importance of St. John's wort (*Hypericum perforatum* L.; Hypericaceae) as an antidepressant, antiviral, antineoplastic (Karioti and Bilia 2010) and antioxidant (Silva et al. 2005), the use of this plant has increased considerably during the past decade. Among the secondary metabolites present in St. John's wort, hyperforin, hypericin and pseudohypericin seem to be responsible for most of the medicinal properties found in this species. Moreover, ethanolic extracts contain abundant amounts of phenolic compounds and phenolic acids (Silva et al. 2005; Diniz et al. 2007; Figueiró et al. 2010), which have antioxidant properties.

Many studies have been carried out with *H. perforatum*, aiming to scale up the production of secondary metabolites. However, in plants grown in natural environments, the production and quality of these compounds may be affected by genotype, different environmental conditions, and biotic stresses (Filippini et al. 2010). Clearly, in vitro culture has been an option for plant multiplication and production of valuable compounds from this species (Pretto and Santrarám 2000; Karppinen et al. 2006; Santarém and Astarita 2003; Franklin and Dias 2006; Don Palmer and Keller 2010). While the efficiency of gelled medium for

propagation of *Hypericum* is undoubted, some investigators have described the use of liquid cultures for micropropagation (Zobayed et al. 2004; Goel et al. 2008; Cui et al. 2010; Coste et al. 2011). Nevertheless, reports on the combination of an efficient protocol for micropropagation and high production of bioactive molecules from secondary metabolism are very uncommon. Hence, the objective of this study was to establish a low-cost liquid system for propagation of adventitious shoots of *H. perforatum*, and to determine how the systems evaluated modulate the shoot metabolism during micropropagation.

Materials and methods

Plant material

Explants of *Hypericum perforatum* consisted of nodal segments (approximately 0.5 cm) obtained from in vitro shoots maintained on MS medium (Murashige and Skoog 1962) supplemented with 1 mg L⁻¹ 6-benzyladenine (BA), 30 g L⁻¹ sucrose, and 3 g L⁻¹ PhytigelTM. The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Explants were excised from elongated shoots that had been cultured on hormone-free medium for 20 days. Proliferative cultures were maintained at 25 ± 2°C under a 16 h photoperiod at a photosynthetic flux of 32.6 μmol m⁻² s⁻¹, provided by cool daylight fluorescent lamps.

Experimental systems

Explants were inoculated into the following liquid-culture systems: (1) Total immersion (TI), where explants were completely submerged in the medium; (2) partial immersion (PI), using a thin layer of medium in the bottom of a flask, so that the plant material was partially in contact with the medium; and (3) paper bridge support (PB), in which a strip of filter paper (4 × 7 cm) was used to support the explant and establish contact with the medium. Semi-solid medium (3 g L⁻¹ PhytigelTM) was used as control (SS) (Fig. 1a–d). Vessels with a volume of 150 mL were used for all treatments. No agitation was applied.

The amount of liquid medium added to the culture vessels varied according to the system used: 10 mL for TI and PB, 3 mL for PI, and 20 mL for SS. The induction phase consisted of MS medium supplemented with 1 mg L⁻¹ BA, 0.01 mg L⁻¹ naphthaleneacetic acid (NAA), and 30 g L⁻¹ sucrose in order to obtain multiple shoots. After 20 days, organogenic explants were transferred to MS liquid medium with 1 mg L⁻¹ BA and 30 g L⁻¹ sucrose for shoot multiplication, under the same systems used in the induction phase. The amounts of medium were maintained as mentioned above for each

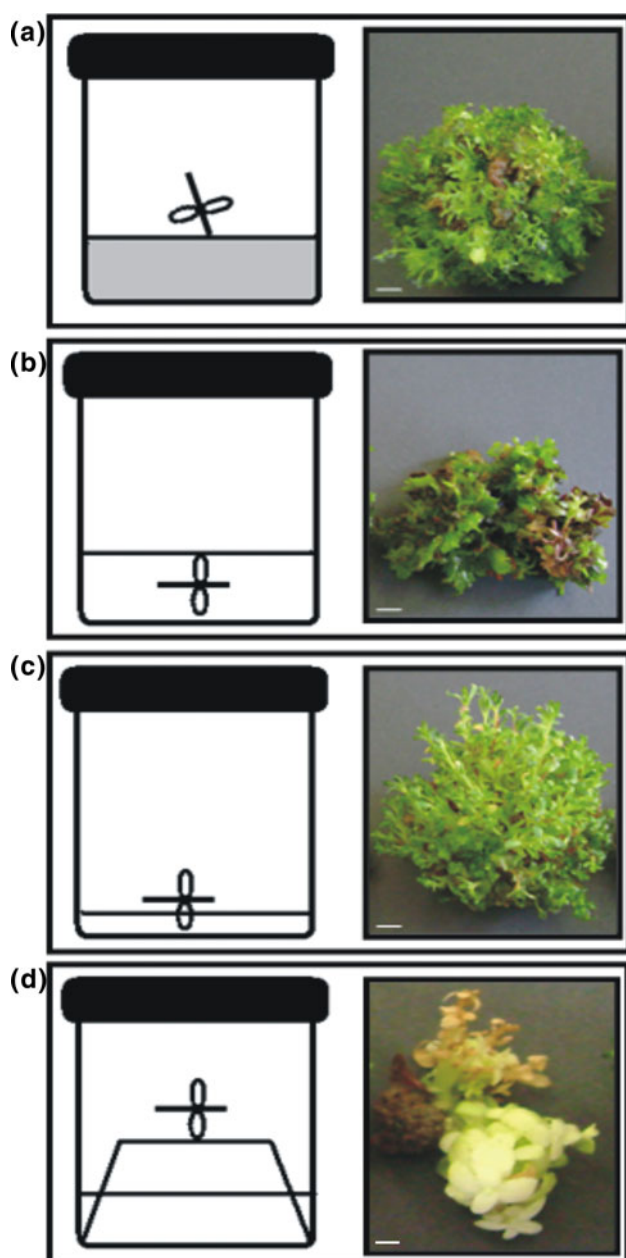


Fig. 1 Adventitious shoots of *H. perforatum* cultivated in different medium systems. **a** semi-solid, **c** partial immersion, and **d** paper bridge, after 80 days of cultivation; **b** total immersion after 40 days. Note in **b** the hyperhydric shoots induced on explants completely submerged in liquid medium (TI system). Bars = 0.5 cm

system, with the exception of the PI system, in which the amount of medium was doubled every 20 days for keeping the plant material under partial immersion. At day 60, the volume for TI system was 20 mL of medium for total immersion of the tissues. At this time point volumes of liquid medium were similar in PI, TI and SS systems. Subcultures into fresh medium were carried out every

20 days. Cultures were maintained in the same conditions described above. The organogenic responses were expressed in terms of percentage of responding explants, number of adventitious shoots formed per explant, and mean length of the longest shoot at 20-day intervals, during 80 days of culture.

Quantification of secondary metabolites and enzymes

Adventitious shoots (1 g of fresh mass) were randomly taken within each treatment, blot dried on sterile filter paper and ground in 10 mL of 80% (v/v) methanol at room temperature. Extracts were filtered and centrifuged at $1,250\times g$ for 15 min. Total phenolic compounds were analyzed in the supernatant by a colorimetric method as described previously (Poiatti et al. 2009). Briefly, 100 μ L of extract was mixed with 2.5 mL Folin-Ciocalteu reagent (ImprintSul Ltda, Brazil) and 0.7 M Na_2CO_3 . Samples were incubated at 25°C in the dark for 30 min and absorbance was measured at 765 nm. Gallic acid was used as the standard. The contents of total phenolic compounds were expressed as mg g^{-1} of fresh mass (FM).

The hypericin levels were determined from extracts obtained from adventitious shoots (1 g) ground in 10 mL of 100% (v/v) methanol. The extracts were filtered in SepPackTM cartridges (Walters, USA), and quantitative estimation of hypericin was performed by HPLC (Agilent Technologies, USA), at 590 nm as reported by Santarém and Astarita (2003). Hypericin amounts were expressed as $\mu\text{g g}^{-1}$ FM.

The activities of the enzyme polyphenol oxidase (PPO) and peroxidases (POX) were quantified in extracts obtained from shoots (1 g) ground in 5 mL of 50 mM sodium phosphate buffer (pH 7.0), supplemented with 2% (v/v) Triton X-100 and 1% (w/v) polyvinylpolypyrrolidone (PVPP). Extracts were filtered and centrifuged at $2,500\times g$ for 15 min at 5°C, and the supernatant was collected for determination of protein content and enzyme assay. PPO activity was determined using chlorogenic acid as the substrate at 400 nm in a spectrophotometer, according to Poiatti et al. (2009). Specific enzyme activity was defined as the change in absorbance $\text{min}^{-1} \text{mg}^{-1}$ protein. The activity of peroxidases was determined in a spectrophotometer by the oxidation of guaiacol at 420 nm, using the extract described above. The reaction mixture contained 50 mM sodium phosphate buffer (pH 6.0), 0.1 M guaiacol as substrate and 10 mM hydrogen peroxide. Specific enzyme activity was expressed as μ katal mg^{-1} protein. The protein content in the enzyme extracts was measured by the method of Bradford (1976), using bovine serum albumin as a standard.

Statistical analysis

The number and length of shoots and the percentage of organogenic explants were analyzed from 15 replications every 20 days over a period of 80 days. Two time points (40 and 80 days) were used to evaluate the levels of the secondary metabolites and the activity of POX and PPO, using four replications for each treatment. Experiments were repeated twice. Data were analyzed by one-way analysis of variance (ANOVA) for each time point to determine the differences among treatments. Means were separated using the Tukey test ($\alpha = 0.05$). The results are presented as the mean $\pm$ standard error (SE).

Results

Effects of liquid medium on induction and growth of adventitious shoots

Organogenic response was observed in all treatments and ranged from 67.1 to 88.5% according to the system tested, although no significant difference was observed among the systems ($P = 0.295$, data not shown). Callus formation was observed on the explants, except in the SS system. The highest intensity of callus formation was recorded on the explants cultured in the TI system.

Differences in the number of shoots were evident among the systems. Shoot induction and proliferation were more rapid in the partial-immersion (PI) and semi-solid (SS) systems, showing significant differences at each time point analyzed. The treatment consisting of submerged explants (TI) resulted in a significantly lower mean of shoots per explant until day 40 (Table 1) and symptoms of hyperhydricity were observed in some shoot clusters. Hyperhydric shoots showed morphological alterations such as highly translucent, curled and thickened leaves as well as disordered growth (Fig. 1b). However, it seems that once shoots from TI system grew partially out of the medium in the period of 60–80 days, multiplication of shoots was recovered and reached 46.6 shoots per explant (Table 1), and hyperhydricity was then reduced. The use of a thin layer of medium in the PI system prevented the symptoms of hyperhydricity, and growth was similar to that observed in the control-induced shoots (SS; Fig. 1a, c). In spite of the high induction of organogenic explants observed in all treatments, the paper bridge condition was the least efficient for shoot multiplication, resulting in significantly fewer shoots (Table 1; Fig. 1d). Overall, explants cultured in PI and SS systems produced more shoots than those in all other systems, reaching means of 65.3 and 71.3 shoots per explant at day 80, respectively (Table 1).

Table 1 Effect of different systems of culture on induction, proliferation and growth of adventitious shoots of *H. perforatum*

Culture systems	Days of cultivation	Number of shoots/explant	Shoot length (mm)
SS	20	3.3 $\pm$ 0.6 ^a	5.3 $\pm$ 1.0 ^a
TI		3.2 $\pm$ 0.6 ^b	6.2 $\pm$ 1.2 ^a
PI		5.5 $\pm$ 0.3 ^{ab}	6.1 $\pm$ 0.8 ^a
PB		3.9 $\pm$ 0.4 ^{ab}	4.0 $\pm$ 0.8 ^a
SS	40	20.7 $\pm$ 4.0 ^{ab}	7.0 $\pm$ 1.0 ^{ab}
TI		9.1 $\pm$ 2.1 ^c	9.2 $\pm$ 1.9 ^{ab}
PI		25.2 $\pm$ 3.7 ^a	10.5 $\pm$ 1.6 ^a
PB		14.0 $\pm$ 0.6 ^{bc}	5.4 $\pm$ 0.8 ^b
SS	60	46.8 $\pm$ 6.2 ^a	9.0 $\pm$ 1.2 ^b
TI		29.1 $\pm$ 4.7 ^b	13.1 $\pm$ 2.4 ^{ab}
PI		48.0 $\pm$ 5.6 ^a	15.9 $\pm$ 1.7 ^a
PB		21.0 $\pm$ 3.1 ^c	8.3 $\pm$ 0.7 ^b
SS	80	71.3 $\pm$ 5.7 ^a	16.2 $\pm$ 1.5 ^{ab}
TI		46.6 $\pm$ 7.7 ^b	15.5 $\pm$ 2.1 ^{ab}
PI		65.3 $\pm$ 5.5 ^{ab}	18.5 $\pm$ 0.7 ^a
PB		29.5 $\pm$ 4.8 ^c	13.5 $\pm$ 1.1 ^{ab}

Data are expressed as mean $\pm$ SE of 15 replicates. Means within columns for each time point with different letters are significantly different at $P \leq 0.05$

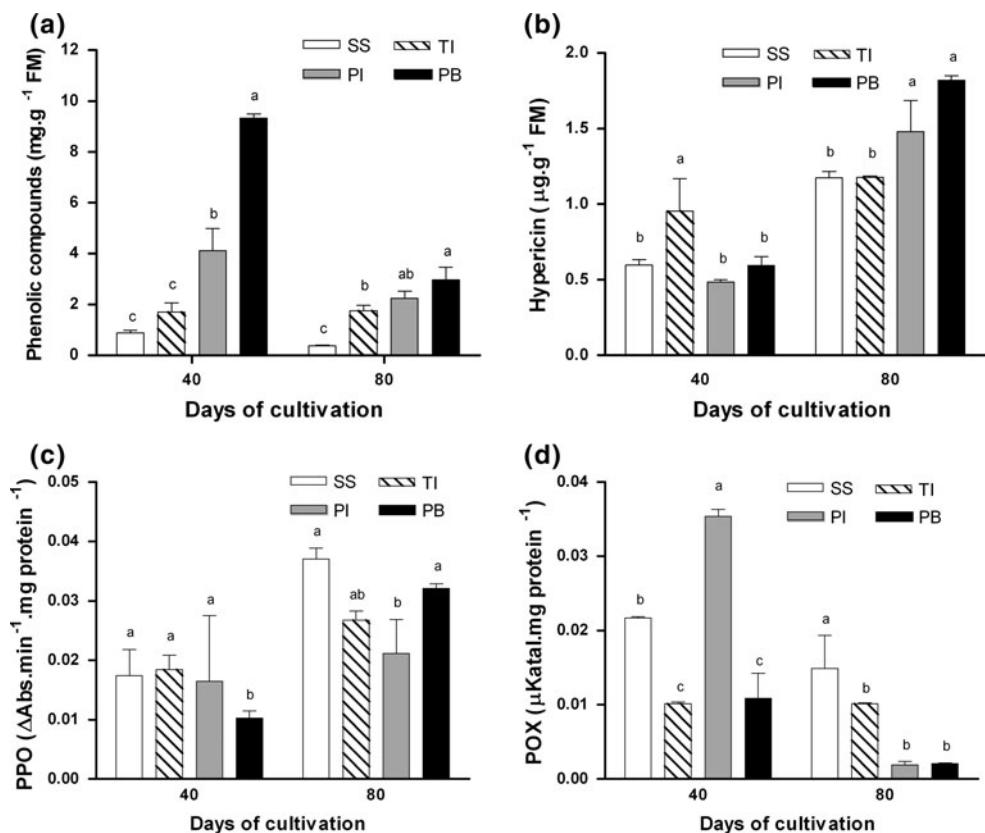
SS semi-solid, TI total immersion, PI partial immersion, and PB paper bridge

With respect to the culture period, the PI and SS systems showed the largest increases in shoot proliferation, ranging from 4.5- to 6.2-fold increases respectively, between 20 and 40 days. However, this increase in shoot proliferation decreased in most of the treatments, from 40 to 60 days and then to 80 days. Overall, at the latest time point evaluated, the proliferation rate varied from 1.2 to 1.6-fold. As shown in Table 1, during the first 20 days of culture, no differences were observed in the length of shoots among the systems tested. Differences became visible at day 40, when shoots induced in the PI system were significantly longer (10.5 mm) than in all other treatments (Table 1; Fig. 1c). Culturing in the PB system resulted in significantly smaller and chlorotic shoots (Table 1; Fig. 1d). Browning was clearly observed in the TI derived-shoots, and to a lesser extent in the SS system (Fig. 1a, b). The observed differences in growth among the systems were maintained up to the end of the culture period.

Effect of liquid medium on secondary metabolism

The secondary metabolism of *H. perforatum* was evaluated at 40 and 80 days of culture. The accumulation of total phenolic compounds was significantly higher in shoots cultured in the PB system, followed by PI, at both time points assayed (Fig. 2a). The lowest level of phenolic

Fig. 2 Secondary metabolism in adventitious shoots of *H. perforatum* cultivated in different systems. **a** Phenolic compounds, **b** Hypericin, **c** Polyphenol oxidase, and **d** Peroxidase activity. *SS* semi-solid, *TI* total immersion, *PI* partial immersion, and *PB* Paper bridge. Bars represent the standard error. Different letters represent significant differences ($P \leq 0.05$) within each time point assayed



compounds was detected in shoots from the semi-solid medium. Regardless of the treatment, levels of phenolics decreased significantly from 40 to 80 days ($P = 0.018$, data not shown).

A different response was observed in the levels of hypericin accumulated in the shoots. At day 40, the levels of this metabolite did not differ among treatments, with the exception of the TI system, which resulted in the highest concentration of hypericin ($0.95 \mu\text{g g}^{-1}$ FM; Fig. 2b). After 80 days of culture, the levels of hypericin were significantly higher in the shoots from the PB ($1.8 \mu\text{g g}^{-1}$ FM) and PI ($1.4 \mu\text{g g}^{-1}$ FM) systems, as were the levels of the phenolic compounds, which reached 3.61 and 2.14 mg g^{-1} FM, respectively (Fig. 2a, b).

The activity of PPO varied during the period of culture. The lowest activity was observed in the PB system at day 40 ($0.0102 \Delta \text{Abs. min}^{-1} \text{ mg protein}^{-1}$). On the other hand, at day 80, the highest PPO activity was found in the shoots from the PB ($0.032 \Delta \text{Abs. min}^{-1} \text{ mg protein}^{-1}$) and SS ($0.037 \Delta \text{Abs. min}^{-1} \text{ mg protein}^{-1}$) systems (Fig. 2c).

In contrast to PPO, more significant variation of POX activity was found at day 40 (Fig. 2d). The highest activity was observed in shoots from the PI system ($0.035 \mu\text{Katal mg protein}^{-1}$) followed by SS ($0.022 \mu\text{Katal mg protein}^{-1}$). At day 80, POX activity in SS-cultured shoots remained similar to the activity at day 40,

although it decreased markedly in the shoots from the PI ($0.016 \mu\text{Katal mg protein}^{-1}$) and PB ($0.010 \mu\text{Katal mg protein}^{-1}$) systems.

Discussion

Reports on the successful micropropagation of *H. perforatum* have demonstrated that 25–50 adventitious shoots can be produced from one explant in a culture cycle, using a variety of systems (Santarém and Astarita 2003; Franklin and Dias 2006; Don Palmer and Keller 2010). Most of the reports refer to the use of gelled media, using agar or PhytigelTM. Nevertheless, some approaches using liquid medium in bioreactors or alternative systems are also effective in increasing the production of plants from this species (Zobayed et al. 2004; Goel et al. 2008; Cui et al. 2010; Coste et al. 2011). In the current study, comparison among different systems showed that the induction and multiplication of adventitious shoots of *H. perforatum* in liquid medium using partial immersion (PI) of the explant was as efficient as culturing in semisolid medium. However, the PI system has the advantages of reduced costs because of the lack of a solidifying agent or the need for agitation. In addition, common symptoms observed in liquid cultures such as hyperhydricity and browning were

not observed in the shoot clusters derived from the PI system, in contrast to the shoots induced on completely submerged explants. Recently, faster root and shoot growth was obtained from excised roots of *H. perforatum* cultured in liquid medium using Growtek™ vessels (Goel et al. 2008), although little difference was observed between the other liquid systems (shaker or glass beads as support) and the semisolid medium. Successes in increasing the induction and proliferation of adventitious shoots on stationary liquid medium under partial immersion were also reported for other species (Paek et al. 2005).

Partial immersion of the *H. perforatum* explant also resulted in longer and more vigorous shoots when compared to all other treatments at the 40-day time point and thereafter. Heterotrophic plant growth depends on the uptake of sugar, water, and nutrients from the medium. The use of gelling agents may limit the hydraulic conductance and consequently the availability of solutes to the tissue (Adelberg 2006). It is likely that the higher rate of plant growth in liquid medium may be related to the wider contact of the tissue with the medium and the higher efficiency in the uptake of nutrients and hormones by the plant or explant. In the current study, the low rate of multiplication of shoots on the completely submerged explants may be explained by the limitation of gas exchange between tissue and environment, as well as by the occurrence of hyperhydric shoots until day 40. Likewise, shoots induced on paper bridges (PB) showed poor development and multiplication. Browning and tissue necrosis were also observed. In the PB system, a small part of the cluster was in contact with the support, which likely limited the uptake of nutrients and availability of water. The use of a thin film of medium (PI) is an alternative to promote a better culturing environment, maximizing the gas exchange between the interior and exterior of the tissue (Jackson 2003), which may have contributed to the better results obtained in our study.

The most important aspect of in vitro production of medicinal plants is that in the system, biomass accumulation must be efficiently coupled with the higher production of bioactive molecules. Indeed, in vitro systems are artificial ways of cultivating plants and may be themselves responsible for alteration in the pathways of secondary metabolite production. This is the reason for the interest in tissue culture, because pathways can be manipulated to scale up the production of valuable molecules. Cell and callus cultures have been reported as alternatives for improving the production of secondary metabolites in *H. perforatum* (Pasqua et al. 2003). However, accumulation of some molecules derived from secondary metabolism, particularly hypericin, requires differentiated tissues or even whole plants (Pasqua et al. 2003; Santarém and Astarita 2003; Cui et al. 2010). Therefore, undifferentiated

cells may not accumulate the desired metabolites in quantities sufficient for production in large scale. In our study, the hypericin content varied with the culture, and the highest levels of hypericin were first found in shoots derived from the TI system after 40 days of culture, followed by PI and PB at 80 days of culture. Specifically, in TI system, where events of hyperhydricity were observed, concentration of hypericin was higher than in the other treatments at day 40. Contrarily, in liquid-cultured shoots of *H. maculatum* high levels of hypericin have been related to the absence of hyperhydricity (Coste et al. 2011). Nevertheless, our results showed that concentration of hypericin in the shoots cultured in liquid systems was similar or higher than what was observed for SS system at any time point analyzed, regardless the symptoms of hyperhydricity observed in some shoot clusters. On the other hand, higher concentration of hypericin was observed in plantlets of *H. perforatum* cultured on semi-solid culture medium when compared to the liquid systems, although hyperhydricity has not been reported in the study (Zobayed et al. 2004). The authors suggested that the leaf glands that accumulate hypericin may rupture in contact with the medium. The lack of agitation in our liquid systems may have preserved the glands. Notwithstanding, the higher levels of hypericin found in the shoots grown on PB may also be a result of the stress caused by the system.

We also demonstrated that phenolic compounds are affected by the system used for propagation, and overall, the liquid systems resulted in higher phenolic concentration than the semi-solid system (SS). Initially, the highest level of total phenolics was found in the shoots cultured on paper bridges over stationary liquid. However, the growth rate of the shoots in this system was also slower. The significant difference observed between the PB system and the other systems tested may be explained as a result of the stress caused by the limitation of nutrients and variation of the water potential (Chen and Ziv 2003). Indeed, nutrients could have been used in response to stress rather than in growth. In spite of that, at 80 days of culture the levels of phenolics were similar between the PI and PB systems and higher than in the other treatments, although only the PB-derived shoots showed chlorosis.

The variation in the phenolic levels seemed to be related to the activity of the enzymes polyphenol oxidase and peroxidases, and may have indirect effects on organogenesis. At day 40, the high concentration of phenolics observed in the shoots from the PB system might be a consequence of the lower activity of both PPO and POX in the shoots induced and propagated in this system. PPO activity increased at the 80-day time point in the shoots from the SS, TI and PB systems, leading to a decrease in the levels of phenolic compounds. Since polyphenol

oxidases are enzymes involved in oxidation of phenolic compounds, usually in response to several types of biotic and abiotic stresses (Tang and Newton 2004; Veljovic-Jovanovic et al. 2008), the differences observed among the treatments suggest that the stress caused by nutrient diffusion or low aeration was similar for the SS, TI, and PB systems. Cultures under either hydric or nutritional stress could favor an alternative oxidase system, leading to a decrease in the phenol levels.

The higher POX activity at day 40 in comparison to day 80 may be related to the proliferation of adventitious shoots, since peroxidases are involved in cell wall formation and organogenesis (Laukkanen et al. 1999; Abbasi et al. 2011). POX activity was significantly higher in the shoots from the PI system, which may be a consequence of the growth and proliferation of shoots observed at this time point. The lowest phenolic level observed in the SS system at 80 days (Fig. 2a) may be related to a larger increase in shoot proliferation (1.46-fold) as well as to POX activity (Fig. 2d), resulting in the reduction of hypericin accumulation (Fig. 2b). On the other hand, the PI system accumulated hypericin at 80 days, coinciding with the reduction of proliferation and lower activity of POX (Fig. 2c, d). A decrease in the phenolic compounds in *H. perforatum* after 60–80 days of culture has been reported previously (Figueiró et al. 2010).

The balance between phenolic compounds and the enzymes PPO and POX may indicate an adjustment of the shoots to the different environments of culture. The increase in PPO activity coincided with the decrease in POX at each time point evaluated. The results indicated that peroxidases play a role in the first steps of organogenesis, whereas PPO is more related to the later stress response.

Our results showed that adventitious shoots of *H. perforatum* can be induced and proliferated in liquid medium without agitation, with the same efficiency as the multiplication obtained with semi-solid medium. The system of partial submersion of either explants or adventitious shoots in a thin layer of stationary liquid medium yielded both the highest accumulation of hypericin and greater proliferation of non-hyperhydric shoots. The reduction of growth and multiplication of adventitious shoots was marked by reductions of POX activity and hypericin production. Furthermore, this system reduced the costs of multiplication without the need for complex equipment, and may therefore be useful for basic and applied research.

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