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BRIEF COMMUNICATION

# *In vitro* propagation and isozyme polymorphism of the medicinal plant *Hypericum brasiliense*

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## Abstract

A study of the genetic variability of a population of *Hypericum brasiliense* was made using several isozyme systems as well as an investigation of the morphogenic potential of apical buds from plants at different development stages (juvenile and adult) using *in vitro* culture. The results from nine isozymes systems showed low polymorphism in the alleles. Apical buds from juvenile plants originated plantlets with apical dominance and fast growth while those from adults led to the development of flower buds.

*Additional key words*: *in vitro* flowering, isozymes.

*Hypericum brasiliense* is a species with great pharmacological potential containing substances such as xanthone, acyl-phloroglucinols, flavonoids rutin and quercetin, and betulinic acid (Rocha *et al.* 1994, 1995). Despite of that it has not been domesticated yet. *H. brasiliense* is widely distributed in Brazil, in high altitude regions from Bahia, Minas Gerais, Rio de Janeiro, São Paulo, Paraná as far as Santa Catarina (Jimenez 1980, Robson 1987).

The *Hypericum* (*Gutiferae*) genus consists of more than 400 species grouped in 31 sections. The genus is variable in many traits and there is little information on the reproductive systems (Mártonfi and Brutovská 1996). However, it has already been ascertained that different species populations of the genus can present intra and inter genotypic populational variation (Matzk *et al.* 2001). Being a plant with medicinal potential, one of the pre-requisites for pharmaceutical exploitation is the reproduction of homogenous varieties in order to maintain the quality control in terms of the contents of the bioactive principles (Steck *et al.* 2001). Different techniques such as the morphological traits, mutant genes, isozymes, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and microsatellites have been used for the identification and characterization of the genetic variability in medicinal plants (Alfenas 1998). In addition, from the practical and pharmacological point of view, plants genetically identical produced by meristem propagation is not technologically difficult (Rout *et al.* 2000).

The most studied species in the genus *Hypericum* is *H. perforatum* and the genetic variability of different populations has been described in the literature and biotechnological techniques have been used to select genetically superior individuals to produce natural products (Mártonfi and Brutovská 1996, Matzk *et al.* 2001, Steck *et al.* 2001).

Thus, the objective of this work was to study the genetic variability of a population of *H. brasiliense* and to establish an *in vitro* propagation methodology to enable future studies to select superior lines in terms of secondary metabolites production for commercial exploitation.

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*Abbreviations:* ACPH - acid phosphatase; AK - adenylate kinase; BSA - bovine serum albumin; DIECA - diethyldithiocarbamic acid; EDTA - ethylenediaminetetraacetic acid; EST - esterase; GOT - glutamate oxalacetate transaminase; IDH - isocitrate dehydrogenase; MDH - malate dehydrogenase; MS - Murashige and Skoog; PGI - phosphogluco isomerase; PGM - phosphoglucomutase; PVP - polyvinylpyrrolidone; SKDH - shikimate dehydrogenase.

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Seeds from several *H. brasiliense* plants were collected in the city of Ibiúna, São Paulo State, Brazil and taxonomically identified by Instituto de Botânica do Estado de São Paulo (SP 337800). They were mixed and germinated in vermiculite at 20 °C in natural light.

Ten two-year-old plants were used to estimate the genetic variability of the population from Ibiúna. The existence of a asexual reproductive system was studied using isoenzymes to analyse the genetic similarity among seedlings (approximately 0.3 cm) derived from seeds of the same fruit (germinated on Petri dishes at 20 °C in light) and the mother plant. Six-month-old plants (referred here as juvenile stage) and one-year-old plants (adult stage) were used as explant sources for the tissue culture experiments.

To characterize the genotypic diversity ten adult individuals (numbered from 1 to 10) and 0.3 cm long plantlets, derived from the seeds of a single fruit from individual number 10, were used. Small pieces of leaf tip tissue from each adult plant and whole plantlets were squashed in approximately 0.02 and 0.005 $cm^3$, respectively, of extraction buffer (0.1 M Tris-HCl, pH 7.0, containing 0.02 M sucrose, 0.6 g PVP, 0.13 mM EDTA; 0.15 % BSA, 0.13 % DIECA, 0.6 % Borax and 10 % β-mercaptoethanol (modified from Sun and Ganders 1990). The extracts were absorbed in rectangles (0.4 × 10 mm for adults plants and 0.4 × 5 mm for plantlets) of *Whatman No. 3* paper, that were applied in 1 cm (adults) and 0.5 cm (plantlets) thick starch gel (8.5 %). Three buffer systems were used: *1*) electrode - 0.04 M citric acid, pH adjusted to 6.1 with N-(3-amino-propyl)-morpholine; gel-electrode buffer in 1:20 dilution (Clayton and Tretiak 1972); *2*) electrode - histidine 0.065 M, pH adjusted to 6.5 with citric acid; gel electrode buffer at 1:4 dilution (modified by Stuber *et al.* 1977); *3*) electrode - 0.6 g lithium hydroxide; 5.9 g boric acid and 1.1 g EDTA in 100 $cm^3$ water (pH 8.0) gel electrode buffer at 1:10 dilution. For the system 1 the power supplier was set at continuous 150 V, system 2 - 150 V, and system 3 - 30 mA. The run was stopped when the tracking dye reached 9 cm from the sample application. All the process, from extraction to the end of the run, was carried out at 4 °C.

Nine enzymes were analyzed in adult leaf tissues: buffer system 1 - phosphogluco isomerase (PGI, EC 5.3.1.9), glutamate oxalacetate transaminase (GOT, EC 2.6.1.1), malate dehydrogenase (MDH, EC 1.1.1.37); buffer system 2 - isocitrate dehydrogenase (IDH, EC 1.1.1.42), shikimate dehydrogenase (SKDH, EC 1.1.1.25); buffer system 3 - phosphogluco mutase (PGM, EC 2.7.5.1), adenylate kinase (AK, EC 2.7.4.3); acid phosphatase (ACPH, EC 3.1.3.2), estarase (EST, EC 3.1.1.1). Only buffer system 3 was used for the plantlets with enzymatic system PGI. The development of the enzyme activities were carried using different recipes: ACPH, EST, SKDH (Alfenas 1998); IDH, GOT, PGI (Corrias *et al.* 1991) and AK, PGM, MHD (Soltis *et al.* 1983).

The banding pattern for each individual was interpreted from direct observation of the zymograms. Alleles were identified by their mobility relative to the most common allele in the population. The alleles of plantlets were identified by comparison with alleles of the mother plant. The genetic variation was estimated by the proportion of loci polimorphic (P; 0.95 criterion), mean number of alleles per locus (A), observed (Ho) and expected (He) mean heterozigosity, the latter calculated according to Nei's unbiased estimated (1978). All calculations were done with the *TFPGA* program.

Approximately 0.5 cm long apical buds collected from six-month-old plants (juvenile stage) and one year old plants (adult stage) were used as explant sources. The explants were sterilized in an aseptic hood as follows: 30 min washing in running tap water, immersion in 70 % ethanol for 30 s and then in commercial sodium hypochloride (0.4 % active hypochlorite) for 30 min, and finally rinsed in sterile water for six times. The sterile explants were excised and inoculated in liquid or solid media (0.7 % agar) MS medium (Murashige and Skoog 1962) supplemented with 3 % sucrose, pH 5.8, without addition of growth regulators. For liquid medium, a inoculation was also carried out in test tubes (25 × 150 mm) containing 10 $cm^3$ liquid culture medium using paper bridges to support the explants. For each media, 30 inoculated explants were kept in a growth chamber at 25 ± 1 °C and 16-h photoperiod (25 μmol $m^{-2}$ $s^{-1}$). After 20 d, the number of buds, height and presence or absence of flowering and rooting were recorded.

We obtained 12 loci presenting good electrophoresis resolution. Except for PGI (Table 1), all the other enzymes were monomorphic. Therefore, considering the parameters analysed the Ibiúna population of *H. brasiliense* showed very low genetic variability (P = 8.3 %; Ho = 0, He = 0.05; A = 1.16). In order to obtain more information on the evolutionary mechanisms responsible for such genetic pattern a large number of populations need to be studied.

Only PGI were analysed in *H. brasiliense* plantlets obtained from the seeds of a single fruit from individual 10, since it was the only polymorphic isoenzyme system and therefore the only one that could be used to estimate the genetic similarity between the mother plant and the progeny. The zymograms showed that PGI are monomorphic in the plantlets and identical to the mother plant, suggesting the existence of a asexual reproductive system.

Some studies have been carried out to characterize the reproduction pathway of some *Hypericum* species (Mártonfi and Brutovská 1996, Matzk *et al.* 2000, 2001). *H. perforatum* reproduction was described by Noak in 1939 (quoted by Brutovská *et al.* 1998) who ascertained that about 97 % of the seeds developed apomictically

and that only 3 % were formed from sexual fusion. Later chromosomal studies reported that the chromosome number is variable (2n=16, 32 and 48) probably in consequence of the occurrence of the facultative apomixis in the species (Robson and Adams 1968, Robson 1981). Using the cytometric flow techniques, Matzk *et al.* (2000) verified the possible pathways of reproduction in *H. perforatum* and *H. patulum*, concluding that the seed formation in the first species would occur sexually by pseudogamy (apomixis) and parthenocarpy, and in the second species would form from sexual fusion and apomixis. Later, Matzk *et al.* (2001) analysed seeds from apomictic *H. perforatum* species and observed the existence of genetic control for such an event.

The observed genetic similarity between the mother plant and its progeny, and existence of facultative apomixy in species genetically close to *H. brasiliense* suggest the possible occurrence of this type of reproduction system also in this species.

Table 1. Number of individuals and allelic frequencies in PGI locus.

| Alleles | Number of individuals | Allelic frequency |
|---|---|---|
| $PGI^1$ | 10 | 0.5 |
| $PGI^2$ | 10 | 0.2 |
| $PGI^3$ | 10 | 0.3 |

Regarding secondary metabolite production for pharmacological application, the low genetic variability of this population of *H. brasiliense* and the probable occurrence of apomixy are positive aspects in terms of stable production of these substances. On the other hand, for a breeding program the selection based on genotypes is not recommended. In such case, studies on the variation of these compounds in response to environmental conditions might be an alternative.

Apical bud culture in MS medium without growth regulators supplementation resulted in plantlet growth whose development occurred with apical dominance. The development in solid culture medium was similar to that obtained in nodal culture of *H. brasiliense* without growth regulators (Cardoso and Oliveira 1996) and *H. perforatum* (Čellárová *et al.* 1992, Murch *et al.* 2000).

The plantlets derived from juvenile explants (six-month-old plants) presented better growth in liquid culture medium compared to solid medium, being taller and with more buds. However, this was not observed in plantlets derived from explants from adult plants (Table 2). The plantlets from juvenile explants generally showed, in culture media, longer internodes when compared with those plantlets formed from the nodal culture in solid culture medium (Cardoso and Oliveira 1996). These authors reported that for 7 cm high plants there was a mean of 11.5 buds, whereas in the present study 7 buds were found on 8 cm tall in solid culture medium and 10.5 buds on 12.5 cm long plants in liquid medium. Longer internodes ease handing during the replication phases to obtain large quantities of plantlets, as the performance of an explant, for plantlet regeneration under controlled conditions, does not depend only on age, but also on size (Grattapaglia and Machado 1998).

Table 2. Number of buds, height, flowering and rooting of *H. brasiliense* plantlets grown *in vitro* in solid and liquid culture medium for 20 d, derived from apical buds of juvenile and adult plants.

| | Solid medium | | Liquid medium | |
|---|---|---|---|---|
| | juvenile | adult | juvenile | adult |
| Number of buds | 7 ± 1.5 | 5 ± 0.9 | 10.5 ± 1.1 | 7 (± 0.7) |
| Height [cm] | 8 ± 0.9 | 6 ± 0.8 | 12.5 ± 1.3 | 5 (± 0.7) |
| Flowering [%] | 0 | 0 | 0 | 100 |
| Rooting [%] | 100 | 100 | 100 | 100 |

Plantlet rooting was observed in liquid and solid media and in all developmental stages (Table 2). Eventually it was observed the presence of buds in the roots, which in liquid medium developed in new plantlets (data not shown), indicating the possibility to use roots as explants.

Explants from adult plants grown in liquid medium had their buds differentiated into floral buds, resulting in flowering. This was not observed in explants obtained from juvenile plants in liquid and solid media (Table 2). I*n vitro* flowering has already been detected in other species (Margara 1969, Sehgal *et al.* 1993, Jumin and Nito 1995, Franklin *et al.* 2000) and it seems to be related with the explant age in *Aratemisia annua* (Gulati *et al.* 1996) and water stress in *Polypleurum stylosum* (Sehgal *et al.* 1993).

Compared with plants derived from seeds and growing in a greenhouse (which takes approximately six months to reach 15 cm), *in vitro* propagation seems to be a better way to propagate *H. brasiliense*, since plantlets approximately 12 cm long were obtained in 20 d and can already be used as explant sources for large scale plant production. Being genetically homogeneous, these plants could be exploited for pharmacological purposes.

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