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Tissue culture of *Hypericum brasiliense* Choisy: Shoot multiplication and callus induction

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Abstract

Hypericum brasiliense, a non-domesticated plant has been shown to have useful medicinal properties. This plant has not been cultivated so a protocol for mass propagation based on selection of superior clones was developed and a protocol established for the culture of callus cells that could be used for *in vitro* metabolite production. A micropropagation method based on amplification of nodal buds was developed, by selection, from ten seedling clones that were examined for growth rate, multiplication rate and rooting. The effect of various basal media, growth regulator types and concentrations were examined for optimal callus induction. Optimal callus induction occured on either Murashige and Skoog's or Gamborg's media supplemented with 1 to 2 mg l^{-1} of 2,4-dichlorophenoxyacetic acid.

Abbreviations: B5 – Gamborg's medium; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – indolacetic acid; MS – Murashige & Skoog's medium; NAA – naphtaleneacetic acid

Introduction

Hypericum brasiliense is a wild, perennial shrub which has not been reported to be cultivated and occurs naturally in the high altitude fields of the Southeast and South regions in Brazil (Jimenez, 1980). Although the plant is not cultivated it is harvested in the wild and processed for medicinal use.

Recently it was demonstrated that species of the genus *Hypericum* contain flavonoids, bisphloroglucinols, hypericine, pseudohypericine and tannins (Kartnig & Brantner, 1990) that display cell growth-inhibitory and antimicrobial activities (Seabra & Correia Alves, 1989; Cardona, 1990; Decosterd *et al.*, 1989; Ishiguro *et al.*, 1990; Serkedjieva *et al.*, 1990; Yazaki & Okuda, 1990; Liebes *et al.*, 1991; Seabra & Correia Alves, 1991), antiallergenic (Oganesyan *et al.*, 1991; Saraf, 1991), antiinflamatory (Fernandez & Alcaraz, 1991), analgesic (Picq *et al.*, 1991) and cytotoxic activities (Kaneda *et al.*, 1991). Hypericine, a major metabolite of the genus, has potent antiviral properties (Nakanishi *et al.*, 1989; Liebes *et al.*, 1991; Gai *et al.*, 1993; Moraleda *et al.*, 1993; Stevenson & Lenard, 1993; Fehr *et al.*, 1994; Weber *et al.*, 1994 and Fehr *et al.*, 1995).

Investigations on the micropropagation of *H. perforatum* L. have been recently reported (Cellárová *et al.*, 1992a, 1992b; Büter *et al.*, 1994) and a few species within *Hypericum* have also been used to produce callus cultures (Kartizig & Brantner, 1990; Yazaki & Okuda, 1990) but no reference on the *in vitro* culture of *H. brasiliense* was found in the literature. Phytochemical and pharmacological studies with *H. brasiliense* showed that its medicinal properties are similar to related species (Rocha, 1991). Therefore the major objective of the present study was to establish an efficient, easy and reproducible method for multiplication of selected clones and to establish friable calluses that

could be used to iniate suspension cultures for studies on the biosynthesis of pharmacologically important secondary metabolites.

Materials and methods

Germination and multiplication

Seeds were collected in the field and surface sterilized with 0.5% NaOCl for 3 min, rinsed 3 times in sterile deionized water and cultured on 1/2 strength MS (Murashige & Skoog, 1962) medium without growth regulators.

From this population 10 plants, derived from seeds of different fruits were randomly selected and micropropagated using nodal segments, with attached leaves, as explants. Multiplication and rooting were performed on a basal medium comprising the salts and vitamins of MS without growth regulators. The medium was adjusted to pH 6.0 prior to autoclaving (15 min at 120°C). The explants were cultured in 250 ml flasks containing 25 ml of culture medium, at 26° C under a regime of 16 h light (50 μmol m^2 s^{-1}) and 8 h dark and relative humidity of 50%. Each clone was represented by a pool of 50 explants obtained from various nodal positions, with the exception of the apical node, of the original mother plant.

Propagation efficiency was determined by measuring the final height of the plantlets, the number of nodes and the percentage of rooting among the 10 clones (50 replicas per clone, 10 flasks with 5 explants). The data were recorded 6 weeks after node culture initiation. Results were analysed by variance analysis and hypothesis testing (Statigraphics, Statistical Graphic System version 2.6).

Callus induction

Nodal segments (1.0 to 1.5 cm in height) were excised from 3-4 week old *in vitro* grown plants and transferred to MS or B5 (Gamborg, 1968) basal media supplemented with 3% and 2% sucrose, respectively and various concentrations of 2,4-D, NAA and IAA. Six replicate flasks, with 10 explants in each flask, for each growth regulator concentration, were used to measure callus initiation. Cultures were maintained at 26°C, with a 16-h photoperiod (50 μmol m^2 s^{-1}), and subcultured every 2 weeks. The percentage of explants forming callus was scored after 30 days.

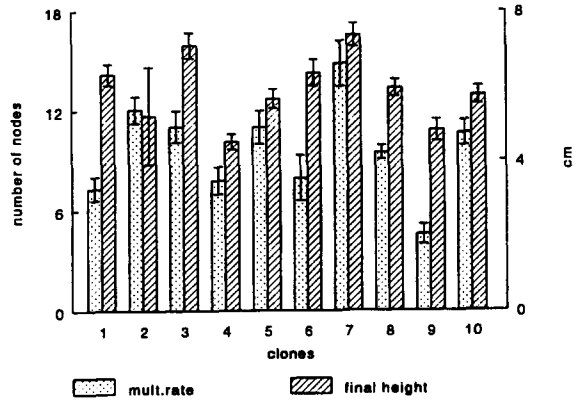

Fig. 1. Multiplication rate and mean final height of 10 clones of *H. brasiliense* grown on MS medium. Multiplication rate: averarage number of nodes produced after six weeks on micropagation medium for each node inoculated. Final height: mean final height of plantlets, in cm, after six weeks on micropropagation medium. Ten replicate flasks, containing five explants for each clone. The vertical lines indicate the standard errors.

Results and discussion

Germination and multiplication

Approximately 50% sterilized seeds produced normal plants on the germination medium. After 8 weeks *in vitro* grown seedlings produced 10-12 nodes and were 8-9.5 cm high. Nodal segments isolated from these plants developed into normal shoots and subsequently rooted on MS medium devoid of growth regulators, a similar result was found with *H. perforatum* when grown either on RM (Cellárová *et al.*, 1992a) or MS medium (Büter *et al.*, 1994). The growth of preexistent buds and root formation occurred within two weeks of inoculation.

Although shoot apices presented higher morphogenetic potential than nodal segments, they were not used as explants in order to prevent differences in plantlet development due to the differential balance of auxins and cytokinins.

Fifty percent of the clones produced 10 or more nodes within 6 weeks, clones 2 and 7 were significantly (p=0.05) the most efficient node producers (Fig.1). The differences in final height were smaller than those for node number, with clone 3 and 7 showing the greatest increase in height over the test period (Fig. 1).

No significant differences were observed among the 10 clones for adventious root formation.

The linear amplification of pre-existing buds was considered a very convenient micropropagation system for the rapid production of plants in *H. brasiliense*,

Table 1. Effect of different growth regulators, in combination with MS or B5, on callus formation using nodal explants.

MS basal medium		%*	B5 basal media	%*
2,4D	(2)	58,8a	(2)	82,2a
	(1)	62,2a	(1)	68,8a
	(0,5)	35,5b	(0,5)	56,6b
NAA	(10)	90,0a	(10)	88,8a
	(5)	83,3a	(5)	53,3b
	(1)	44,4b	(1)	33,3b
IAA	(20)	33,3a	(20)	42,2a
	(10)	17,7b	(10)	21,1a
	(5)	14,4b	(5)	17,7b

*Percentage of nodal explants forming callus
Within a combination, the percentage followed by the same letter do not differ significantly (p=0.05).
Numbers between parenthesis represents concentration (mg l^{-1}).

because it is easily manipulated as multiplication and rooting occured on the same medium.

Callus induction

Preliminary experiments showed that White's medium (White, 1943) and various cytokinins in combination with various auxins did not support callus growth of explants. B5 and MS with similar combinations of auxins and cytokinins or cytokinins alone gave little or poor growth of callus specially when grown in the dark. Explants inoculated on media with both classes of growth regulators tended to produce roots with little or no callus growth, unlike *H. perforatum* that when grown in the presence of an auxin and a cytokinin (2,4-D/kin) promoted callus formation (Cellarova *et al.*, 1992a).

Nodal explants, excised from clones 3 and 4 cultured on MS and B5 media supplemented with 2,4-D, NAA and IAA were evaluated for appearance of callus and percentage of roots producing callus. Callus initiation was more efficient on B5 basal medium with any of the growth regulators used, and was first observed five days after culture initiation. Callus initiation only occured after 10 days when explants were cultured on MS medium.

Calluses produced on both MS and B5 media supplemented with 2,4-D(0.5, 1.0 and 2.0 mg l^{-1}) were friable and brownish. The medium containing 0.5 mg.l^{-1} 2,4-D produced the least callus. Callus induction was equally efficient at 2 or 1 mg l^{-1} 2,4-D with MS or B5. Friable calluses were used to initiate cell suspension cultures in liquid MS supplemented with 1 mg l^{-1} of 2,4-D, which will be the subject of further investigations. Calluses obtained with NAA, in combination with MS or B5 were green and heterogeneous, containing mostly dense and compact areas. Although 10 mg l^{-1} NAA with B5 produced a significantly (p=0.05) higher percentage of explants forming callus than 5 mg l^{-1}, the calluses looked very similar in appearance. A lower concentration of NAA (1.0 mg l^{-1}) induced regeneration of shoots, as observed in *H. perforatum* (Büter *et al.*, 1994) and adventitious root formation on both basal media. Callus induction on IAA supplemented medium (5, 10 or 20 mg l^{-1}) was proportional to concentrations used but the percentage of callus initiation was inferior to the other two auxins (Table 1). Calluses produced on IAA were compact and green during the first 4 weeks. After this period, organogenesis ocurred, specially root proliferation (20 mg l^{-1}), though *de novo* bud formation was also observed on the surface of leaves and stem segments of the explants on 10 mg l^{-1} IAA.

Callus formation was stimulated by several auxins. MS or B5 with 2,4-D and NAA gave better results than with IAA, a weaker auxin which is degraded more rapidly.

The selection of the medium for callus induction, however, will depend on the objective of the work. If the main goal is regeneration, IAA and low concentrations of NAA are indicated; when the objective is suspension culture, 2,4-D is the most appropiate growth regulator.

H. brasiliense can be easily established and grown in culture and shares with *H. perforatum* the same ability of high regeneration capacity *in vitro* (Cellarova *et al.*, 1992a). Propagation of selected clones can be readily obtained via node multiplication on growth regulator-free medium, which should result in minimal somaclonal variation in recovered plants. Studies on meristems show that, even though some variation might be found in plants derived from the development of pre-existing buds, this is not a very common event (Illg, 1990). Concerning genetic stability, the accuracy of this method is considered as very high, when compared to other micropropagation systems (Gratapaglia & Machado, 1990).

Callus can be produced in the presence of only one auxin, being either 2,4-D or NAA. 2,4-D provided optimal callus initiation and optimal friability for the

establishment of suspension cultures which will be the subject of further studies.

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