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Micropropagation of *Hypericum maculatum* Cranz an important medicinal plant

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Abstract

The purpose of this work was to establish an effective in vitro propagation protocol for Hypericum maculatum Cranz using nodal segments explants taken from the aseptically germinated seedlings. Explant browning, a major problem for regeneration, was overcome by adding ascorbic and citric acid (each 50 mg/L) to all prepared media containing growth regulator combinations The nodal pieces inoculated individually in MS basal medium supplemented with 0.5 mg/L 2iP + 0.2 mg/L BA + 0.1 mg/L K + 0.05 mg/L NAA produced multiple small shoots (30 ± 0.6) with an average height of 3.1 ± 0.2 cm. For shoot elongation and rooting, these shoots were transferred on half-strength MS basal medium containing 0.5 mg/L GA_3 and two different auxins, IAA and IBA, at three concentrations (0, 0.5 and 1.0 mg/L). The most effective culture medium for root number, root length, and shoot height was the half-strength MS basal medium with 1.0 mg/L IAA. Rooted plantlets were transferred to pots containing perlite for acclimatization, for a period of three weeks, and further on soil. An average of 97 – 100% acclimatized plantlets survived after two months of transferring into the soil.

Keywords: *Hypericum maculatum* Cranz, medicinal plants, micropropagation, rooting, acclimatization

Abbreviations: N^6-benzyladenine (BA), γ,γ-(dimethylallyl) aminopurine (2iP), kinetin (6-furfurylaminopurine) (KIN); thidiazuron [N-phenyl-N'-(1,2,3-thidiazol-yl)urea] (TDZ), indole-3-acetic acid (IAA); indole-3 butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and giberellic acid (GA_3).

Introduction

The species of the genus *Hypericum* have been used as a remedy since ancient times particularly to treat ulcers, burns, wounds, abdominal pains and bacterial diseases. Recently it has received attention in clinical trials for the treatment of depression and viral diseases [1; 2]. The antidepressant activity is attributed to hypericins, hyperphorins, and flavonoids [2]. Although the probable parent species, diploid *H. maculatum* Crantz is given in several pharmacopoeias together with *H. perforatum* as a component of the drug *Herba hyperici*, rather little attention was paid to its secondary metabolites and their dynamics [3]. In spite of this, many authors discovered that *H. maculatum* plants synthesize and accumulate hypericin and falvonoids, in amounts close to those detected for *H. perforatum* [3; 4; 5].
MURCH et al. [6] underlined the fact that remarkable differences were found regarding the hypericin content, or other compounds, of different comercial extracts of *Hypericum*.
This variability is presumably the result of several factors, including genotype, weather, and the method used for plant production [6; 7]. Moreover, the plants are usually grown in the field, and are therefore susceptible to contamination by pollutants and to infestation of pathogens and insects that can compromise the quality of the products. One solution to the problems faced by the phytopharmaceutical industry is the development of *in vitro* systems for the production of medicinal plants.

In vitro culture has been demonstrated as a viable option for multiplying *Hypericum* species, the attention of scientists being mainly directed towards the multiplication of *Hypericum perforatum* species [8; 9; 10].
Analyzing the existing literature we found out that other 15 *Hypericum* species were micropropagated through in vitro culture techniques for both multiplication purposes as well as for the production of secondary metabolites. Plant regeneration of *Hypericum* species has been achieved using as explants whole seedlings or their excised parts [11], hypocotyl sections [6], nodal segment [12] and leaves [13], using various types and concentrations of cytokinins and auxins.
As far as *Hypericum maculatum* is concerned, we found only two research articles in the specialty literature, regarding the in vitro cultivation of this species only as plant cell suspensions for hypericin, pseudohypericin and flavonoids production [14; 15]. Therefore, the objective of our current research was to develop an *in vitro* protocol for the micropropagation of *H. maculatum* to facilitate production of consistent plant material for industrial and research applications.

Materials and methods

Plant materials *(explant sources)*

The seeds of *Hypericum maculatum* Crantz (collected from Baisoara, jud. Cluj, Roumania) were sterilized with 70% (v/v) ethanol for 1 min immersion and subsequently in a 0.1% (w/v) $HgCl_2$ solution for 5 min, followed by three washes of sterilized distilled water. For germination seeds were placed on the culture medium in conical flasks contained approximately 25ml of half-strength MS basal medium (Murashige and Skoog, 1962) [16] solidified with 0.7 % (w/v) agar, for 4 weeks. The pH of media was adjusted to 5.7 before adding gelling agents. The media were autoclaved at 1.1 kg cm^{-2} ($121^{o}C$) for 20 min. The seeds were germinated at 25 ± 1°C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}).

Shoot induction and multiplication

Under aseptic conditions, the shoot nodal segments (ca. 1 cm) having leaf discarded were excised from the three-weeks-old seedlings, being leaves discarded. Five excised nodal segments were cultured in 100 ml conical flask on full-strength MS basal medium solidified with 0.75 % (w/v) agar. For eradication of browning exudates arisising from the cut tissue which lead to the death of the explants, this medium was supplemented with antioxidant (citric and ascorbic acids). In order to test the effect of different growth regulators on shoot induction and multiplication, 0.5 mg/L BA, K, 2iP or TDZ were incorporated into the basal medium, individually or in combination with 0.05 mg/L NAA. Hormone-free medium was used as control. Subcultures to fresh medium were carried out every 30 d. The organogenic efficiency was recorded from the number of organogenic explants per total number of explants 40 d after culture initiation. The number of shoots induced per explant was also evaluated after 40 d.

Shoot elongation and rooting

For rooting and elongation, the micro-shoots (around 3 cm height) placed on half-strength MS medium (1/2 MS) containing 20 g/L sucrose, 0.5 mg/L GA_3 and two different auxins, IAA and IBA, at three concentrations (0, 0.5 and 1.0 mg/L). The medium was solidified with 7.3 g/L agar and dispersed at 30 ml per flasks. Four shoots were cultured in each flask and were incubated at $25\pm1^{o}C$ in a growth chamber with a 16-h photoperiod, under

standard cool white fluorescent tubes (25 μmol s^{-1} m-2) for 20 days. Number of roots formed, root length and shoot height were evaluated at the end of this period root. After 20 days, the rooted plantlets washed to remove agar from their roots, were transferred to the pots containing autoclaved perlite, covered with polythene bags to maintain high humidity and kept at 25°C in a growth chamber for 1 week. After 1 week the bags were removed gradually. These plants were then transferred to the green house.

Results and discussion

Shoot induction and multiplication

Although *H. maculatum* is a valuable medicinal plant, there is no study about *in vitro* propagation of this plant. We, therefore, aimed to develop an *in vitro* culture protocol for the high-efficiency regeneration of plants by shoot organogenesis.

De novo shoot organogenesis was induced on nodal segments of *H. maculatum* cultivated on MS 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. No organogenic response was obtained in explants grown on medium supplemented with NAA as the only growth regulator (Table 1).

Table 1. The effect of different plant growth regulators on shoot induction and proliferation from nodal segmets of *Hypericum maculatum* Cranz after 6 weeks of culture on MS medium

Growth regulators (mg/L)	Organogenic explants (%)	No of shoots/explant	Shoot height (cm)	Number of roots/explant
Control	12.7±0.9	1.2±0.1	3.1±0.1	3.6±0.1
0.5 BA	48.2±4.2	45.3±2.2	0.3±0.0	0.0
0.5 KIN	30.6±2.9	8.6±0.3	1.0±0.0	0.0
0.5 2iP	25.4±1.8	4.5±0.2	1.8±0.0	0.0
0.5 TDZ	19.2±1.1	27.2±1.5	0.1±0.0	0.0
0.05 ANA	0	0	0	0
0.5 BA + 0.05 NAA	78.2±4.8	58.6±3.6	0.5±0.0	0.2±0.0
0.5 KIN + 0.05 NAA	46.7±3.2	14.4±0.5	1.8±0.1	0.3±0.0
0.5 2iP + 0.05 NAA	39.6±1.7	8.3±0.4	2.4±0.2	0.6±0.0
0.5 TDZ + 0.05 NAA	22.3±2.1	43.1±3.2	0.3±0.0	0.3±0.0
0.5 2iP + 0.2 BA + 0.1 KIN + 0.05 NAA	89.7±4.2	30.0±0.6	3.1±0.2	0.8±0.0

Values represent the mean ± SD

In the first days of culture shoots will arise from the axilary buds of nodal explants, but at the end of culture period they will cover explants entirely (Fig. 1A, 1B and 1C). The number of shoots induced on the nodal segments varied with the type of cytokinin. Thus, the highest average shoot number was obtained in the organogenic explants induced on the presence of BA combined with NAA (58.6 shoot per explant) (Table 1and Fig. 1B).

Although the number of shoots, for this variant, is very high, their length and height is very low (ca 0.5 cm) (Table 1, Fig.1B). The same aspects may be observed in the case of explants grown on the variant supplemented with TDZ combined with NAA, but the number and especially the length of shoots/explant was much lower (Table 1). As far as the influence of other cytokinins on nodal segment explants of *H. maculatum* is concerned, we observed that KIN and 2iP enhanced especially the elongation of newly formed shoots (Table 1). In this context, the hormonal variant comprising 2iP, KIN, BAP and NAA had a positive effect on both number and length of shoots induced on the nodal segments of *H. maculatum,* (Table 1 and Fig. 1C).

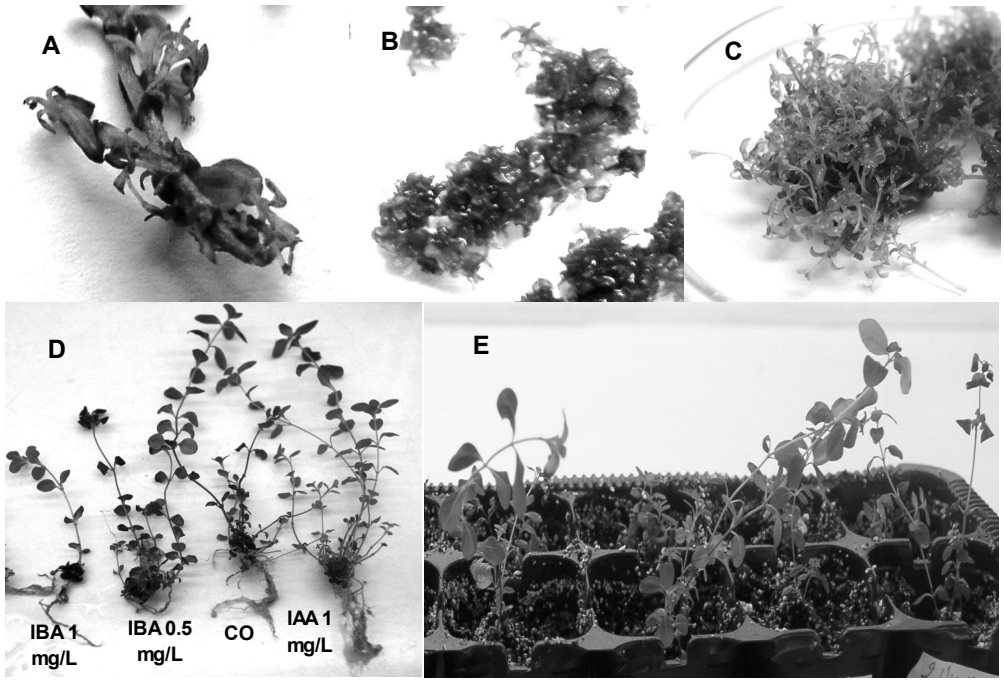

Fig.1. Regeneration of *Hypericum maculatum* from the nodal explants

A) Shoot regeneration from the nodal explants 20 days after cultivation on MS solid media supplemented with 0.5 mg /L BA and 0.05 mg/L NAA;
B) Shoot regeneration from the nodal explants 40 days after cultivation on MS solid media supplemented with 0.5 mg /L BA and 0.05 mg/L NAA;
C) Shoot regeneration from the nodal explants 40 days after cultivation on MS solid media supplemented with 0.5 mg /L 2iP, 0.2 mg/L BA, 0.1 mg/L KIN and 0.05 mg/L NAA;
D) Rooting of regenerated shoots on half-strength MS basal medium containing 0.5 mg/L GA_3 and two different auxins, IAA and IBA, at three concentrations (0, 0.5 and 1.0 mg/L). CO = control;
E) Regenerated plants in the pot one month after the cultivation in the growth chamber

Our results regarding *H. maculatum* are consistent with those reported for the other *Hypericum* species. Thus, in *Hypericum foliosum*, the highest number of shoots was obtained on media supplemented with BA and NAA, simultaneously [17]. Similar results were also reported for *H. canariensis* [18] and *H. perforatum* [8, 11, 12]. Among the cytokinins, TDZ was reported to be more efficient than BA on promoting adventitious shoots in *H. perforatum* in hypocotyl sections [6]. On the other hand studies performed with nodal explants of *H. erectum*, showed that TDZ had a stimulating effect on shoot multiplication [19]. Nevertheless, in our experiments with *H. maculatum*, even if similar with those performed on *H. perforatum* [12] the addition of TDZ to the culture medium resulted in the lowest organogenic frequency and average shoot number per explant, indicating that the organogenic response might be related to the initial explant and *Hypericum* species used for micropropagation.

Shoot elongation, rooting, and hardening

One physiological process with negative impact on the duration of the in vitro cultures of *Hypericum* species is the partial or total absence of roots. This is not the case for other species, where these roots are constantly regenerating from the explants used for shoot regeneration and multiplication. Many authors found out that shoots regenerated from *Hypericum* explants are very small, their length varying between 0.1 si 2.0 cm. For these reasons it is necessary to introduce another suplimentary step to the in vitro culture, for the regeneration and rooting of shoots.. Thus, for shoot elongation and rooting in *H. maculatum*, individual shoots (around 3 cm height) were transferred on half-strength MS basal medium containing 0.5 mg/L GA_3 and two different auxins, IAA and IBA, at three concentrations (0, 0.5 and 1.0 mg/L). These two types of auxines had different effects on both the process of rhyzogenesis and caulogenesis. Thus, roots regenerated from shoots cultivated on MS basal medium with IBA are longer and thicker, but les numerous, comparatively with roots formed from shoots cultivated on MS basal medium with IAA or on basal medium lacking growth hormones. These roots proved to be shorter and thiner. Also, shoots cultivated on culture media with IAA grew longer than those cultivated on culture media with IBA, simultaneously a multiplication being observed (Table 2 and Fig. 1D).

Table 2. Effect of different concentrations of IBA and IAA added to half-strength MS bazal medium on elongation and root proliferation of *H. maculatum* shoot cultures, after 20 days.

IAA (mg/L)	IBA (mg/L)	% of shoots rooted	No of roots per shoot	Length of roots (cm)	Height of shoots (cm)
0	0	55.2±2.7	4.68±0.8	2.62±0.2	5.71±0.1
0.5	0	78.4±3.3	5.74±0.2	3.01±0.1	6.31±0.2
1.0	0	91.8±4.5	6.69±0.6	3.87±0.2	6.42±0.4
0	0.5	75.6±2.4	4.11±0.2	5.02±0.4	4.15±0.5
0	1.0	71.3±1.1	3.02±0.1	4.12±0.3	3.11±0.1

Values represent the mean ± SD

The most effective culture medium for root number, root length, and shoot height was the half-strength MS basal medium with 1.0 mg/L IAA (Table 2, Fig..). Similar results were also reported for *H. perforatum* [12, 20, 21, 22], *H. canariensis* [18] and *H. heterophyllum* [23].

Plantlets with well-developed roots were successfully hardened off in the non-sterile conditions and transferred to greenhouse and soil (Fig. 1E). The survival frequency was 97 - 100%.

Conclusions

An efficient protocol for the micropropagation of *H. maculatum* was established by using four plant growth regulator mixtures. The results of these investigation cleary show that nodal segments are capable of producing multiple shoots *in vitro*, which can then be rooted to form complete plantlets. This system will be utilized for the useful compounds of *H. maculatum* and will also allow tge possibility of genetic engineering for improving the content in medicinal compounds.

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