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# *IN VITRO* MICROPROPAGATION OF *HYPERICUM PERFORATUM* L. II. SAINT JOHN'S WORT CLONE SELECTION AND REGENERATION

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## INTRODUCTION

*Hypericum perforatum* L. (St. John's wort) plant is used in traditional medicine to treat various diseases. The plant contains a series of active principles relevant for their therapeutic action: the hypericin-type diantrone derivatives (hypericin and pseudo hypericin), volatile oil, which contains monoterpene and sesquiterpene, flavonoside under glycoside form and free aglycons (hyperine, rutoside, cvercetol, cvercetine, kaempferol) and biflavonoides, hyperforin, galactose, carotenes, vitamin C, vitamin PP, saponine, phenolic acids and a series of xanthones, mineral salts (Weiss, 1991; Ciulei, *et al*., 1993; Kartning, *et al*.,1996; Čellárová, 1997; Deltito and Beyer, 1998; Dias *et al*., 1998; Tămaş, 1999; Stănescu *et al*., 2004; Ayan *et al*., 2005). Currently, St John's wort is widely used as an herb remedy for the treatment of mild to moderate depression (De Smet and Nolen, 1996).

Plant cell and tissue culture were set up for a series of herbs among which St. John's wort *Hypericum perforatum* (Erzen- Vodenik and Baricevic, 1996), by using the shoot tips as explants. There were subsequent problems related to obtaining sterile explants in carrying out tissue culture of this species.

With respect to tissue cultures of species such of *Hypericum* genus information is scarce. The paper of Čellárová *et al*. (1992) on *H. perforatum* species reports on obtaining explants by sterile germination of seeds and the approach of the variability of some morphological and histological characteristics and structure of regenerants. Broderick *et al*. (1996), BEŽO and ŠTEFÚNOVÁ (2001) on *H. perforatum* species, developed a rapid technique of callus tissue propagation and of shoot multiplication and a system to produce high level hypericin in *in vitro* culture. Other authors use leaf explants for *in vitro* culture initiation (Pretto *et al*., 2003; Ayan *et al*., 2005). For *H. erectum* species - *in vitro* culture was carried out in order to trigger procianidine and to carry out quantitative analysis of specific polyphenols from procianidine (Yazaki & Okuda, 1990).

A series of papers report on the influence of some substances induced in the nutritious medium on some physiological and bioproductive parameters. Thus, supplementing medium with 0.001% (w/v) Pluronic F-68 (non-ionic surfactant), after 60 days of culture, induces a 40% growth of the mean fresh weight of regenerants and an increase of 34% of regenerated shoot number. Nonetheless, the concentration growth to 0.1% (w/v) of the same compound decreased by 15% both the number of regenerated shoots and their fresh weight (Brutovska *et al*., 1994). It was also proved that in *H. perforatum* cell suspension culture, the jasmonic-acid induced hypericin production (Travis *et al*., 2002).

As *H. perforatum* is one of the species that undergo multiple forms of reproduction in the wild, several reproductive processes were identified in wild harvested plants (Matzk *et al*., 2004). This reproductive flexibility has led to chemical variability in field grown plants. Therefore, the in *vitro* cloning has an advantage for the multiplication of valuable individuals from a bioproductive viewpoint as well.

In the experimental series we aimed at micropropagation of *H. perforatum* plant biological material selected in the Piatra-Neamt experimental field. Considering the preliminary results obtained by cultivating *H. perforatum,* caulinary explants, the aim of this study was to focus on the optimising the regeneration processes (caulogenesis, risogenesis), the adjustment and transfer of fully adjusted plants onto the experimental field.

## MATERIAL AND METHODS

Plantlets were obtained by seed germination under aseptic conditions on agar medium and the sterile segments represented the explants used. In order to initiate and cultivate callus tissues it was necessary to use a 1:2 and 1:1 ratio, respectively, of exogenous auxines and cytokinines. MR- base medium was used containing macroelements and microelements (Linsmaier and Skoog, 1965), 20g/l glucose, 100 mg/l meso-inositol, 2 mg/l glycine and 7.5% agar.

Caulogenesis was performed by placing the callus on MS and MR variant agar media, where by inducing cytokinins (kinetin, 6 benzyl-amino purin =

BA) and auxines (IAA, NAA) separated or in different combinations, 10 media variants were obtained (B02, B05, B10, K0l, K02, K05, K10, KB, RM, MR1, MR2 (Table 1). Risogenesis was performed on simple agar MS medium, simple MR and MRI (supplemented with 0.5 mg/l acid-indolilbutiric).

The cultures were maintained in culture chamber under stable semi climatic conditions, at 20oC, 16-hour light and 8-hour darkness.

After having formed roots, the new plantlets were taken out from the Erlenmeyer flasks; small agar medium was cleaned off their roots. After successive washing, they were transferred in sterile-soil pots or in hydroponic cultures. In both cases, they were covered with glasses. Throughout this adjusting period, water equilibrium was stimulated by temporary taking off the glasses and the adjusted plants were transplanted onto the experimental field.

## RESULTS AND DISCUSSIONS

Callus was obtained on agar Murashige Skoog (MS) culture media and Linsmaier Skoog revised medium (RM), supplemented with auxines and cytokinins in different concentrations (Amariei *et al*., 2000). Despite the good semi-climatic conditions secured, the growth of the set culture cells, especially during the first stage, was slow. From a morphological viewpoint, the tissue cultures obtained displayed a wide variety. Thus, considering the phonotype aspect (colour and consistence), we obtained dark green compact callus and green callus with many growing buds, and slightly friable green-yellowish callus.

With a view to *inducing caulogenesis*, a series of studies with concern to the interaction of cytokinins concentration in the medium and the St. John's wort callus morphogenetic response (or the sterile explants fragment). To this aim, MS-base medium was supplemented by variously concentrated BA in different concentrations (de la 0.1 mg/l up to 1 mg/l), a medium on which the inoculated callus recorded a multiple shoot differentiation (Table 1). In another series of experiments - when a different but similarly concentrated cytokinine was used, i.e. kinetin, (0.1-1mg/l)-it was noticed that while kinetin concentrations ranging between 0.1-0.5 mg/l, induced shoot differentiation, the 1 mg/l concentration produced necrosis. Hence, in the case of tissue culture and *H. perforatum* plant regeneration experiments, the use of BA plant hormone is more advantageous than kinetin.

Table 1. Morphogenetic response of H. *perforatum* L. explants on different culture media variants

| No. | Var. | Growth regulators (mg / l) | | | | | | Morphogenetic response induced |
|---|---|---|---|---|---|---|---|---|
| | | BAP | Kin | NAA | 2,4- D | IAA | IBA | |
| 1. | B02 | 0.2 | - | - | - | - | - | Multiple shoot formation |
| 2. | B05 | 0.5 | - | - | - | - | - | Multiple shoot formation |
| 3. | B10 | 1.0 | - | - | - | - | - | Multiple shoot formation |
| | | | | | | | | Multiple shoot formation |
| 4. | K01 | - | 0.1 | - | - | - | - | Shoot regeneration |
| 5. | K02 | - | 0.2 | - | - | - | - | Multiple shoot formation |
| 6. | K05 | - | 0.5 | - | - | - | - | Shoot regeneration |
| 7. | K10 | - | 1.0 | - | - | - | - | Significant necrosis |
| 8. | KB | 0.5 | 1.0 | 0.5 | - | - | - | Multiple shoot formation |
| 9. | RM | 1.0 | - | 0.5 | - | - | - | Multiple shoot formation |
| 10. | RM | 1.0 | - | - | - | 0.5 | - | Abundant shooting |
| 11. | MS | - | - | - | - | - | - | Good risogenesis |
| 12. | MR | - | - | - | - | - | - | Good risogenesis |
| 13. | MRI | - | - | - | - | - | 0.5 | Very good risogenesis |

Our results are confirmed by studies conducted by Čellárová *et al.,* (1995) who, still with concern to St. John's worth, obtained similar data using RM-base medium. The authors previously cited also used 2iP, in of 0.1 – 0.5 mg/l concentrations beside BA and K – as plant hormone supplement; in this case shoot differentiation was mainly obtained as explant response.

In order to determine shoot regeneration output, three culture media were chosen, e.g. MS (KB variant) and RM, and RMI on which the best caulogeneses were previously recorded. Selecting callus according to phenotypical traits manifested proved that the most rapid caulogenesis was obtained in the green callus, covered with a large number of growing buds. With a view to caulogenesis, at this stage, the callus generated by each inoculated segment was sectioned and transferred into Erlenmeyer flasks with culture media afore mentioned. Shoots formation needed 12 to 24 days. Over this period the number of shoots on the surface of inoculated callus grew as detailed in Table 2 below.

Table 2. *Hypericum perforatum* L. shoot inducing and differentiating

| Culture Medium | Explants | Morphological response after 4 weeks | No. of shoots formed after 8 weeks |
|---|---|---|---|
| KB | callus | Multiple shoot formation | 168 |
| | callus | Moderate caulogenesis | 79 |
| | callus | Reduced caulogenesis | 57 |
| | callus | Shoot formation | 147 |
| | callus | Multiple shoot formation | 158 |
| | callus | Multiple shoot formation | 172 |
| | callus | Moderate caulogenesis | 82 |
| | callus | Satisfactory caulogenesis\ | 104 |
| | callus | Satisfactory caulogenesis | 99 |
| | callus | Multiple shoot formation | 158 |
| | callus | Multiple shoot formation | 137 |
| Mean | | | 136.10 |
| RM | callus | ± Good shoot differentiation | 145 |
| | callus | Good shoot differentiation | 168 |
| | callus | Good shoot differentiation | 287 |
| | callus | Very good shoot differentiation | 452 |
| | callus | Very good shoot differentiation | 351 |
| | callus | ± Good shoot differentiation | 147 |
| | callus | Very good shoot differentiation | 581 |
| | callus | Very good shoot differentiation | 430 |
| | callus | Very good shoot differentiation | 342 |
| | callus | Good shoot differentiation | 195 |
| | callus | Very good shoot differentiation | 509 |
| Mean | | | 330.70 |

In order to pass over the winter during shoot stage the newly formed *H. perforatum* shoots reaching 3 to 4 cm in length were separated and further transferred on identical multiplication media. At this stage, new vigorous shoots – either singular or four to six-branched ones were noticed (Photo 1 and 2).


1


2

3

*H. perforatum* L. shoots – stage preliminary to rooting (Photo 1 and 2); rooting stage (Photo 3)

*Regenerated shoot rooting* is a very important step in all cases, especially when initiation of an experimental lot in field is pursued, for analysing regenerants from the point of view of their biology, genetic characteristics and bioproductivity. To this aim, well formed shoots were excised and transferred into Erlenmeyer flasks with agar simple MS, simple MR and MRI (supplemented with 0.5 mg/l acid-indolilbutiric). Using the three medium types in the risogenesis inducing stage (Table 1) allowed for a comparative evaluation of the process in its interaction with nutritious medium depending on mineral substance content, i.e., the lack or presence of plant hormone with a well-known stimulating role.

As a result of this experiment, single or branched *stem H. perforatum* shoots proved a good risogenesis on all media used. This process needed a longer time (6 to 7 weeks) on MR medium, a shorter one on MS (5 - 6 weeks), and the shortest period recorded (3 - 4 weeks) on MRI medium.

Moreover, culture phenotype recorded modifications during risogenesis although shoots similar in length and vigour were inoculated. Regenerated shoots recorded a significantly different development on risogene media used with a view to root generations. The most vigorous shoots were noticed on MS medium which is richer in organic substances than MR and MRI. Therefore, the media for risogenesis must be carefully chosen according to

different criteria: the time factor – in which case MRI medium is more advantageous (shorter time), or the need for more vigorous plants then MS (hormone-free) medium would required a longer time (Photo 3).

The rate of St. John's wort roots inducing and formation - in *in vitro* culture ranges between 87 - 98%. The best values (98%) were recorded on MRI culture medium.

To conclude, *H. perforatum* is species endowed with a high *in vitro* regenerating capacity. A very efficient regenerating method was obtained when RM culture medium was supplemented only by 6-benzyl-amino. This regenerant system, having the same genetic origin, allowed for the study of somaclonal variation at various levels (morphological, cytogenetic and biochemical) followed by the individual selection of plants with the desired characteristics. *Adjustment of in vitro regenerated St. John's wort plants* - Completely regenerated *H. perforatum L.* plants, after having undergone risogenesis, were transferred and subject to adjustment with a view to their being transplanted in the experimental field.

Two experiments were initiated with a view to plant adjustment. The former began in the second half of August while the latter at the beginning of spring the following year. As to the experiment begun in August, the new plantlets were very carefully taken out of the Erlenmeyer flasks and their *in vitro* regenerated roots were cleared of the small pieces of agar medium. After successive washing, the new plantlets were transferred into fertile soil pots. In order to avoid a massive loss of water, the plants were covered with glasses, hampering thus heavy perspiration and eventually the loss of the newly formed plants. Plant adjustment required 12 to 16 days. Throughout this adjustment period, glasses were taken out so as to stimulate water equilibrium of each new plantlet transplanted. At the end of this period, the plants adjusted their water equilibrium and resisted throughout the day under outdoors climatic conditions. In this adjusting experiment (plants transferred into sterile soil), the output reached 80.05%. During this stage, the new plantlets were transplanted into the "Stejarul" Research Centre Piatra Neamț experimental field.

During the latter experiment carried out during winter and the beginning of spring, new plantlets were taken from the agar medium similarly to the previous experiment. But this time, the new plantlets were transferred into a hydroponic system instead of sterile soil pots. Plants were also covered so as to avoid heavy loss of water via leaves, in-appropriately adjusted to the new conditions (Photo 4). In this system, restoring water equilibrium of St. John's wort plants took 10 to 14 days and then they were further kept in the hydroponic system (Photo 5 -

6). Some 57 fully adjusted plants were transplanted onto the experimental field. Hydroponic culture output reached 89.4%.

4

5

6

Aspects of *ex vitro* adjustment of new plantlets obtained

## SUMMARY

Micropropagation of H. perforatum, selected in experimental field in Piatra-Neamt with a view to optimising the regeneration processes (i.e caulogenesis, risogenesis), adjustment and transfer of fully adjusted plants in the experimental field. An average of 330.70 shoots were obtained at 35 flasks 14-18 days after the inoculation of callus on RM agar culture medium, supplemented with 1.0mg/l BAP and 0.5mg/l IAA or NAA, whereas on 0.5mg/l BAP, 1.0mg/l Kin and 0.5mg/l IAA supplemented medium only 136.10 shoots were obtained. In vitro risogenesis rate is of 87 - 98%, the best values (i.e., 98%) were obtained with MRI culture medium though the most vigorous shoots were obtained on MS hormone-free medium. Plant adjustment was observed in parallel, in sterile soil pots and under hydroponic system. In both cases they are covered. Under hydroponic system, restoring plant water equilibrium took 10 to 14 days whereas the adjustment rate obtained was of 89.4%. Some 57 adjusted plants were transplanted in the experimental field.


## CONCLUSIONS

*Hypericum perforatum* shoot regeneration is a more advantageous process when using MS or BA-supplemented RM, as compared to kinetin; when shoot length reached 3 to 4 cm, the most vigorous ones – either singular or 4 to 6 -branched ones were separated and transferred on rooting media.

A good risogenesis was obtained on all three media: 6 to 7 weeks on MR medium, 5 to 6 weeks on MS medium, and 3 to 4 weeks on MRI medium. The rate of St. John's wort *in vitro* inducing and root formation ranged between 87 and 98%. The highest values (i.e. 98%) were reached on MRI culture medium.

Survival rate of new plantlets over the ex *vitro* adjustment period ranged between 80.5 and 89.4% depending on the system used – sterile soil pots or hydroponic cultures; in hydroponic cultures, water equilibrium restoring time shortened by 2 to 3 days; some 50% of the plantlets transplanted in the field (in September) survived the winter; plants grew, flowered and bore fruits and they displayed a carrying characteristic to the species.

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