Studii şi Cercetări	Martie 2015	Biologie	24/1	52-55	Universitatea"Vasile Alecsandri" din Bacău

MICROPROPAGATION OF *HYPERICUM PERFORATUM* L. USING $AGNO_3$ TO ENHANCE SHOOT REGENERATION

Tina Oana Cristea, Maria Prisecaru, Silvia Ambarus, Maria Calin, Creola Brezeanu, Marian Brezeanu, George Florin Şova

Key words*: organogenesis, embryogenesis, regeneration, plants*

INTRODUCTION

In genus *Hypericum* are included plant species that are widely recognized for their therapeutic efficacy, being frequently utilized in traditional medicine. One of the most recognized species of the genus is Hypericum perforatum L., St. John's wort, which is intensively used in alternative medicine, both externally for the treatment of skin wounds, eczema and burns and, internally, for disorders of the central nervous system, the alimentary tract and other purposes (Barnes, Anderson, & Phillipson, 2001).

Now-a-days micropropagation is used routinely to generate a large number of high-quality clonal agricultural plants, including ornamental, medicinal and vegetable species. The advantage of using this method of multiplication is due to the possibility to combine rapid large-scale propagation of new genotypes, with the utilisation of small amounts of original germplasm (particularly at the early breeding and/or transformation stage, when only a few plants are available), and the generation of pathogen-free plants.

Each specie respond differently to „in vitro" conditions. There is a range of morphogenetic respons that starts from the total lack of answer („recalcitrant species") to a large number of somaclones that can be easily regenerated from small parts of the mother plants.

Ethylene is a gaseous plant hormone produced by tissues that, as shown by many *in vitro* studies, affects callus growth, shoot regeneration and somatic embryogenesis *in vitro.* Due to the fact that all the processes of plant cells and tissue culture *in vitro* are developed in a rather closed medium (culture recipients with limited or no gaseous exchange) the influence of ethylene materialized through abscission, senescence and growth retardation can negatively affect the success of the *in vitro* regeneration. The accumulation of this gas in the culture recipients inhibits the shoots and embryo initiation and growth disabling the ability of regeneration of plantlets from different plant tissues. Silver nitrate it is recognised in literature as being one of the most effective inhibitor of ethylene action. Through its involvement in regulating the production or action of ethylene, silver nitrate proved to be an important tool in modulating, to a certain extent, the growth and development of some tissue cultures *in vitro.*

The aim of the present study is to determine whether and which concentration of silver nitrate can improve the regeneration system for the establishment of a rapid and efficient micropropagation protocol.

The hormonal formula utilised was determined to be the most effective in our previous experiments.

MATERIAL AND METHODS

Plant growth conditions

The explants were collected from valuable mother plants maintained at Vegetable Research Station Bacau in controlled conditions. Young shoots of 1.5 -2 cm length were excised from actively growing plants.

Sterilization

The defoliated shoots were first washed in tap water and the sterilized in 0.1% HgCl2 for 15 minutes, and 3 rinses in sterile distilled water.

Culture techniques

The shoots were then utilized as donor source for explants. The apexes of ~ 1,5 cm were excised and inoculated on Murashige -Skoog, 1962 culture basal medium supplemented BAP – 8.9 µM and IAA – 1.1 µM supplemented with different concentration of $AgNO_3$ (5 µM, 10 µM, 15 µM, 25 µM, 30 µM*)* (table 1). To all these variants 30 g/l sucrose and 8 g/l agar were added. The pH of the medium was established at 5,8 before the autoclavation at 121^0C for 25 minutes.

Cultures were incubated at 24±1°C under 16 hr photoperiod of 3000-lux light intensity.

The cultures were transferred at a 3 weeks interval on fresh media, for a period of 90 days.

Observation of shoot multiplication and growth were recorded at weekly intervals. After three weeks, shoots of above 3 cm length were harvested and subcultured on the same medium.

Rooting and acclimatization

After 3 to 4 weeks, when regenerated shoots reached a length of more than 4.0 cm, they were separated and transferred on MS basal medium supplemented with 2.7 µM NAA for rooting. The rooted plantlets were transferred to the hydroponics conditions in bottles and hardened by maintaining a high humidity (90% RH) during first week of hardening, which was gradually decreased and it resulted in more than 95% survival of plantlets.

Table 1. Variants of nutritive medium with different hormonal factors utilized for "in vitro" regeneration

Variant	V0	V1	V2	V3	V4	V5
$AgNO_3$	-	5 µM	10 µM	15 µM	25 µM	30 µM

After acclimatization, the regenerants were planted in a potting mixture of sterilized sand + vermiculite (1:1 ratio) in plastic cups, hardened in a mist chamber (80% relative humidity) for 2 weeks before transfer to a green house.

RESULTS AND DISCUSSIONS

The results obtained suggest that the poor regeneration response found on control variant (without $AgNO_3$, variant V1) may be associated with ethylene production by the *in vitro* cultured cells or tissues.

The initiation of regenerative processes were accomplished at 6 days after the explant`s inoculation on the aseptic nutritive medium. First, 1 or 2 small buds were observed, and then the evolution was positive - in almost two weeks, 9 shoots were developed on the initial explant of only 1.5 – 2.0 cm long.

As it is shown in graph 1 the addition of silver nitrate improved the ability of *Hypericum* shoots to regenerate on MS medium supplemented with BAP-8.9 µM and NAA 2.7 µM. On all variants the results are higher when compared with the control (variant V1) were no silver nitrate was added. The results are presented as percentage from the total reactive explants.

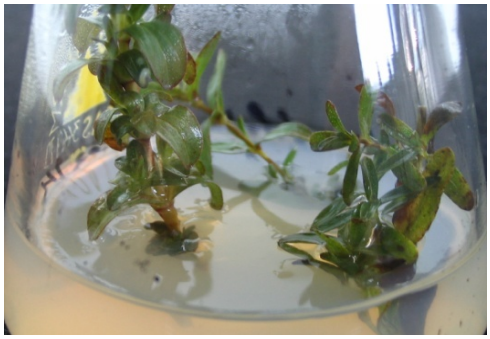
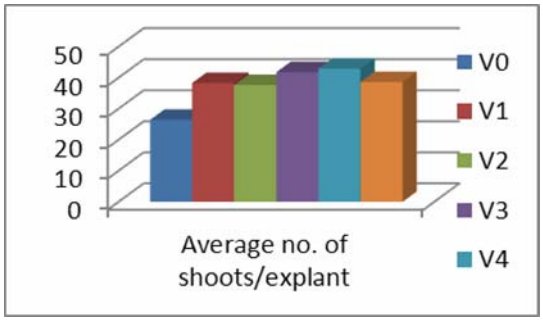


Fig. 1. Tips at with small outgrowth at the base of the explant

Not all the inoculated explants had the same morphogenetic reaction, due to the fact that there are functional differences between the similar morphologic explants. In this stage of ontogenetic development there are different particularities in the organogenesis reaction of the explant, particularities that lies on the specific totipotence of each explant.



Graph 1. The "in vitro" morphogenetic reaction of *Hypericum perforatum* L. explants on the cultivation medium

On the other hand, a part of the tips were eliminated due to their gradual degeneration (reaching to necrosis), or to the secondary contamination of the recipients. The morphogenetic reaction of the *Hypericum perforatum* explants was favorable, the initiation and development of the regenerative structures were followed by the rapid development of the neopropagules.

Fig. 2. General aspects during regeneration process

Adventitious shoots developed directly from the base of the explant and initially appeared as small outgrowths that developed gradually in shoots. At the base of the initial shoot started to appear new shoots with one or two branches, which allowed the continuation of the regeneration process.

Regeneration percentage was affected by silver nitrate concentration. Of the various levels of $AgNO_3$ tested, the variant with 25 µM proved to be

most effective, as on this medium not only the number of shoots per explant was maximal but also the number of explants producing multiple shoots was the highest. On lower concentration of silver nitrate from 5 µM to 10 µM the number of shoots per culture was reduced. Similarly on higher concentrations of $AgNO_3$ (30 $AgNO_3$) the number as well as the length of shoots was similarly reduced.

Gradually the shoots that were at the best stage of development were inoculated on rooting medium, which should allow the initiation and development of roots.

Figure 3. Shoots on rooting medium

10 - 12 days after the inoculation of the shoots on the rooting mediums, the plants were transferred on hydroponics medium and kept about four days covered with a plastic foil, in the culture room. Subsequently, they were day by day acclimatized to room atmosphere.

Surviving plants, nearly 90-92%, were transferred to the greenhouse and grown to maturation.

CONCLUSIONS

The results of the present study demonstrated the fact that *Hypericum perforatum* is a specie suitable for cultivation in „in vitro" conditions, the morphogenetic reaction of the explants being positive – with the initiation and development of the regenerative structures were followed by the rapid development of the neopropagules.

Silver nitrate can be successfully utilized for improving the morphogenetic reaction of *Hypericum perforatum* shoots.

In the experimental conditions tested in the present study, the tips, utilized as initial explant, allowed the regeneration of uniform and stable plants.

The shoots regeneration was accomplished through the neoformation of adventive shoots (at the basis of the inoculated tips) as well as through the multiple axillary sprouting (through the development of pre-existent meristematic centers).

The obtained experimental results encourage the continuation of the researches for the determination of all the factors that can influence the regeneration process (genotype, explant, etc). This should allow the establishment of a rapid and efficient propagation technology that permit the regeneration of a large number of plants, in short term, plants that have the same genetic background as the parental plants.

ABSTRACT

The present study was undertaken in the Laboratory of Plant Cell ad Tissue Culture from Vegetable Research and Development Station Bacau, Romania. The purpose of the study was the enhancement of plant regeneration efficiency at *Hypericum perforatum* L. cultivated in vitro. As well known silver ions, in the form of nitrate play an important role in promoting the somatic embryogenesis and organogenesis, which led its wide spread use, is in different plant tissue culture in vitro. Thus, the investigations were focused toward the establishment of its role in sustaining the plant regeneration in vitro and the achievement of a rapid multiplication procedure at *Hypericum perforatum* (a valuable herbaceous medicinal plant). The explants excised from healthy mother plants were cultured on Murashige-Skoog, 1962 medium with BAP – 8.9 µM and IAA – 1.1 µM supplemented with different concentration of $AgNO_3$ (5 µM, 10 µM, 15 µM, 25 µM, 30 µM*)*. In general, NAA alone promoted root induction at all the regenerated shoots. Rooted shoots were successfully re-established in soil under controlled conditions.

REFERENCES

1. CHEN J., ZIV M., 2003 - Carbohydrate, metabolic, and osmotic changes in scaled-up liquid cultures of Narcissus leaves. In Vitro Cell Dev Biol Plant 39:645–650;
2. COSTE A., VLASE L., HALMAGYI A., DELIU C., COLDEA G., 2011 - Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. Plant Cell Tiss Organ Cult 106:279–288;
3. DON PALMER C., KELLER WA., 2010 - Plant regeneration from petal explants of *Hypericum perforatum* L. Plant Cell Tiss Organ Cult 105:129–134;
4. GOEL MK, KUKREJA AK, BISHT NS., 2008 - In vitro manipulations in St. John's wort (*Hypericum perforatum* L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity

using RAPD analysis. Plant Cell Tissue Organ Cult 96:1–9;
5. KARPPINEN K., GYORGY Z., KAUPPINEN M., JOLONEN A., JALONEN J., NUEBAUER P., HOHTOLA A., HAGGMAN H., 2006 - In vitro propagation of *Hypericum perforatum* L. and accumulation of hypericins, pseudohypericins and phloroglucinols. Prop Ornam Plants 6: 170–179;
6. MURASHIGE T. & SKOOG F., 1962 - A revised medium for rapid growth andbioassays with tobacco tissue cultures. *Physiologia Plantarum,* 15:473-497;
7. PRETTO FR., SANTAREM ER., 2000 - Callus formation and plant regeneration from *Hypericum perforatum* leaves. Plant Cell Tiss Organ Cult 62:107–113;
8. SUDHA G.C., KRISHNAN P.N., SEENI S. and PUSHPAGANDAN P., 2000 - Regeneration of plants from *in vitro* root segments of *Holostemma annulare* (Roxb.) K. Schum., a rare medicinal plant. *Curr. Sci*. 78: 503-506;
9. ZIV M., 2005 - Simple bioreactors for mass propagation of plants. Plant Cell Tiss Organ Cult 81:277–285;
10. ZOBAYED SMA, MURCH SJ., RUPASINGHE HPV., SAXENA PK., 2004 - In vitro production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'). Plant Sci 166:333–340.

AUTHORS` ADDRESS

TINA OANA CRISTEA, MARIA CALIN, SILVIA AMBARUS, CREOLA BREZEANU, BREZEANU MARIAN, SOVA FLORIN - Vegetable Research and Development Station Bacau, Calea Barladului, No. 220, Bacau, code: 600388, e-mail: tinaoana@yahoo.com

MARIA PRISECARU - „Vasile Alecsandri", University of Bacau, Faculty of Science, Department of Biology, Ecology and Environmental Protection, Marasesti Street, no. 157, Bacau, Romania, e-mail: prisecaru_maria@yahoo.com