Studii şi Cercetări	Martie 2016	Biologie	25/1	104-109	Universitatea"Vasile Alecsandri" din Bacău

USE OF *IN VITRO* CULTURES TO OBTAIN PLANT MATERIAL AT THE SPECIES *VERONICA OFFICINALIS* L., NEEDED TO MAKE BIOPRODUCTS

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***Key words**: Veronica officinalis, in vitro culture; caulogenesis, micropropagation, bioproducts*

INTRODUCTION

Veronica officinalis L., native to Europe and Western Asia, commonly known as speedwell, gypsyweed or Pauls' betony etc., is used as a medicinal plant because of its content of tannins, bitter substances, volatile oil, saponins, flavones and glycosides (Bojor O., Alexan M., 1998; Crişan G., 2007; Mocan A., 2015). Because of these bioactive compounds, speedwell, also called "the basic cure of all evil" is recommended for its actions and effects both internally – stomachic, cholagogue, digestive tonic, gentle expectorant, anti-catarrhal, diuretic, astringent, hemostatic, cleansing, anti-inflammatory –, and externally – healing skin wounds and scars, astringent, hemostatic (Bojor O., Alexan M., 1998; Robu T, Milică C, 2004; Riley D. S., 2012). Because of its beneficial effects, the plant has also been called *Ehrenpreis* (*Ehre* = honour, esteem; *Preis* = price, prize), which emphasizes the place of honour held by the plant at ancient Germans before the arrival of the Romans.

In our country, the plant was long used empirically and only recently for its therapeutic virtues. With the development of Phytochemistry research and access to literature from abroad, speedwell drew the attention of Romanian researchers (Danila D. et al., 2011; Crişan G. C., 2015). Currently, there are few herbal pharmaceuticals containing *Veronica officinalis* extract and identifying an unconventional technology of multiplication would provide the raw material required to produce the extracts needed to make bioproducts.

Among the many advantages provided by the *in vitro* culture technology at plants, there are: the possibility to *preserve the valuable qualities of a genotype* by cloning it, as well as the *induction of genetic variability* in order to obtain genotypes (somaclones) that are more valuable in terms of the content of bioactive compounds. In the first case, there is used the meristem culture, and in the second case, indirect organogenesis - *via callus*, the culture of organs, tissues and cells, or protoplasts etc. (Ghiorghiță G, Nicuță Petrescu D., 2005).

The idea of inducing callusogenesis in the *in vitro* culture at medicinal plants (including at *Veronica officinalis*) could result in improving the content of active ingredients, both qualitatively and quantitatively. The plants regenerated *via callus* or the callus itself obtained for different nutritious versions may contain different amounts of active ingredients specific to the plant or even some new active principles, which are not typically found in plants of the spontaneous flora (Ghiorghiță G., 1992). These determinations require specific biochemical analyses of the callus or the regenerants obtained *in vitro* for the different nutritional versions.

MATERIAL AND METHODS

To initiate *in vitro* cultures of *Veronica officinalis* L. there were used young shoots harvested in May 2015 from a population at the edge of a beech forest in the village of Luncani, the commune of Mărgineni, Bacău County. The shoots brought into the laboratory were the source for explants, represented by apices, nodes, internodes and leaf fragments.

The biological material taken from the wild flora was washed with tap water and then subjected to sterilization in the laminar air flow cabinet. The sterilization procedure covered several steps: immersion of the fragments of shoots into a 1 ‰ mercuric chloride solution for 5 minutes, immersion into a 5% chloramine T solution for 15 minutes. To remove the sterilizing agents, the explants were rinsed 3 times with sterile distilled water. For some of the culture vessel, the sterilization procedure was repeated, due to massive infection occurring 3-4 days after inoculation. Thus, in the case of the culture vessels where the infection process did not affect all the explants, they were immersed again in 5% chloramine T solution for 20 minutes. In this way, about 10% of the explants were saved and evolved with no further problems.

As a basic nutrient medium there was used Murashige-Skoog (1962), with sucrose (30 mg/l) as the carbon source, and agar - agar (8.5 g/l) for solidification.

To obtain sterile explants, fragments of plant material (shoot apices and nodes) were inoculated initially on several nutritive options: MS without phytohormons, and MS supplemented with 1 and 2

mg/l BAP. The neoplantlets obtained on the initial media represented the source of explants for highlighting the *in vitro* behaviour on different nutritious versions, supplemented with different combinations and concentrations of phytohormones (Table 1).

To resume the growth processes, the inoculated vials were placed in a growth chamber – SANYO, under controlled temperature and light.

RESULTS AND DISCUSSIONS

In the opening stage of the *in vitro* culture of *Veronica officinalis* L., we encountered some problems in that, after the sterilization of the plant material harvested from the spontaneous flora, we found a high percentage of infected plantlets. Therefore, the percentage of explants that survived was quite low (10%).

On most nutrient media supplemented with growth regulators, the node and apex explants had a good reaction, generating new shoots and roots. The aspect and number of shoots and the rizogenetic intensity varied depending on the type and concentration of the growth regulators present in the nutrient medium.

In general, to induce callus there is used a hormonal balance in favour of auxins since this type of phytohormone is responsible for cell dedifferentiation and callus occurrence. Although we respected the conditions recommended by the literature, we were surprised to find that in the media that contained only auxins (in various concentrations), the inoculated node explants formed shoots. The emergence of the callus was visible only at explants inoculated on the versions BD_1 (apices, nodes, leaves, internodes) and on N_2 and A_2 (leaves and internodes).

The apices and nodes of the shoots grown on the MS medium supplemented with 2 ml/l BAP had a poor reaction. There developed small shoots, which generated sporadic roots in the nutrient medium. Growing these explants on the MS medium supplemented with BAP (1 ml/l) and IAA (0.1 ml/l) resulted in the generation of new vigorous shoots from the basal nodes (2 shoots/node) and few roots. The shoots had long internodes and large leaves. On the new shoots formed, at the level of each pair of nodes, after about 1-1.5 months of *in vitro* culture there occurred new shoots (about 2-3/node) representing the 2nd generation. Thus, in each vessel, after about 2 months of cultivation, there could be seen clumps of shoots. The roots, formed at the basis of the initial explant, increased in length, were thin, white and without secondary ramifications. Some shoots had short adventitious roots at their upper nodes.

By supplementing the nutrient medium with BAP and IBA, the nodes and apices generated 1-2 shoots/explant. They were small, "atomy", compared with the shoots generated on other nutritional formulas, but had large leaves, well-developed and heavily coloured in green. On the BB1 medium there was no rizogenesis.

The presence of BAP in the nutrient medium together with NAA also induced the formation of shoots from the nodes and apices inoculated on this version (BN_1). There were formed 2 shoots/node. These were vigorous, with large leaves and stems thicker than on BA_1. From the upper nodes there were formed adventitious, long roots of approximately 2 cm that presented secondary ramifications. On the medium, the explants formed sporadic long roots (about 3-4 cm in length). In some vials, where the bedding of the plant material was not made before 2.5 to 3 months, at the basis of nodal explants there was formed a compact green callus with low proliferation, which turned brown in time.

Inoculating the apex and node explants on the version BD_1 (1 ml/l BAP + 0.5 ml/l 2,4-D) resulted in the generation of a coarse lime-green callus which covered the basal stems and leaves that were in direct contact with the medium; it showed average proliferation and had no regenerative capacity after transfer to other nutritive alternatives. After about 1-1.5 months of cultivation it degenerated.

Table 1. Hormonal versions used to highlight the morphogenetic reaction of some explants of *Veronica officinalis* L.

No	Hormonal formula	GROWTH REGULATORS (ml/l)					
		IAA	IBA	NAA	2,4-D	BAP	KIN
1.	MS	-	-	-	-	-	-
2.	A2	2	-	-	-	-	-
3.	N2	-	-	2	-	-	-
4.	D2	-	-	-	2	-	-
5.	B2	-	-	-	-	2	-
6.	BA1	0.1	-	-	-	1	-
7.	BB1	-	0.5	-	-	1	-
8.	BD1	-	-	-	0.5	1	-
9.	KN	-	-	0.5	-	-	1
10.	BN1	-	-	0.5	-	1	-
11.	ND0.5	-	-	0.3	0.5	-	-

The leaves and internodes explants also generated a coarse, lime-green, white-green callus, with reduced proliferation on the version BD_1, the callus being non-regenerative.

A good morphogenetic reaction was manifested by the apices of shoots on the version KN (1 ml/l Kinetin + 1ml/l NAA). The reaction consisted of intensive shoots; the shoots were vigorous, with large thick leaves. At the basis of the explants there formed long, fibrous, white roots without secondary ramifications.

The presence of auxins in the nutrient media, in the absence of cytokinins, induced a different morphogenetic reaction to the shoots apices and nodes. Thus, the inoculation of these explants on the version N_2 (2ml/l NAA) resulted in the generation of new small shoots, some of them anomalous, with poor growth, presenting leaves with chlorophyll deficiencies; rizogenesis was also highlighted on this hormonal formula, the roots being reduced in number/explant, but thicker, with secondary branches and green in colour.

The inclusion of 2ml/l IAA (version A_2) in the nutrient medium resulted in the elongation of apices, without generating the emergence of new shoots. The nodes issued two shoots/node, which have also grown in length, such that after about 2 months of culture, these measured about 8.6 cm in length. Shoots leaves were large, well developed and normally coloured. In some vials, however, on the A_2 medium, some explants developed slow-growing shoots with etiolated leaves, while other explants degenerated, the reaction being similar to that highlighted for the solution N_2. In the nutrient medium there also grew a strong root system from the basis of each apex or node explant, the roots being long, thick, green and without secondary branches.

Inoculating explants of leaves and internodes on the nutrient versions used in the experiment led to callus formation on the media supplemented only with auxins (N_2, A_2) or the version BD_1. The callus showed low proliferation being coarse, of a pale whitish-green, non-regenerative, ultimately turning brown.

On the MS medium with no phytohormones, there were highlighted both caulogenesis and rizogenesis at the level of apices and nodes, but the shoots were weaker, with small leaves and normally coloured. In the nutrient medium there were generated roots, in small number/explant, thin, whitish, without secondary ramifications. In some vials, on the MS medium, there developed adventitious, short, thin and white roots at the level of the upper nodes.

On the hormonal version supplemented with NAA and 2.4D, at the level of node explants, there was observed the development of a green and cream callus, with low proliferation and semi-compact consistency. The callus was initially formed at the ends of the explants and after a period of cultivation it covered the entire explant. Although transferred to other nutritious alternatives, the callus showed no caulogenetic capacity and eventually degenerated. On the same nutritional version ($ND_{0,5}$), at the end of internode explants – generating the aspect of halters – there occurred the phenomenon of caulogenesis. The callus was crisp, cream-coloured and characterized by low proliferation.

The accommodation to the septic environment of the *Veronica officinalis* neoplantlets was carried out in hydroponic system, an action that was performed without difficulty, in about 12 days. The survival rate of the in vitro plants after transfer to pots with soil was high, allowing us to appreciate that an unconventional technology of multiplication can be developed for this species.

CONCLUSIONS

- To initiate the *in vitro* culture of *Veronica officinalis* in order to obtain plant material by clonal micropropagation of valuable genotypes it is recommended to use shoots apices and nodes as explants.
- The main morphogenetic reaction highlighted at the shoots nodes and apices was direct caulogenesis, suggesting that this system of cultivation is advantageous for the production of raw materials required in the pharmaceutical industry or seedlings for establishing a culture of speedwell.
- The most efficient medium formulas for obtaining shoots are, in descending order: BA, KN, BN and MS.
- Except the version BB_1, on all the nutrient media there was highlighted rizogenesis.
- The presence in the nutrient media only of the plant hormones IAA or NAA resulted in the emergence of shoots with chlorophyll deficiencies.
- The calusogenetic reaction at the inoculated explants was highlighted sporadically, with low yields on some hormonal formulas, which allows us to appreciate that the nutrient media we tested are not profitable if the goal is to achieve a high yield of callus, or for speculation of the somaclonal variation phenomenon.
- A very good performance was also recorded when accommodating the in vitro regenerated plants to the septic environment, as well as in terms of the rate of their survival after acclimatization.

ABSTRACT

Veronica officinalis L., native to Europe and Western Asia, commonly known as speedwell, gypsyweed or Pauls' betony etc., is used as a medicinal plant because of its content of tannins,

bitter substances, volatile oil, saponins, flavones and glycosides.

Because of these bioactive compounds is recommended for its actions and effects both internally – stomachic, cholagogue, digestive tonic, gentle expectorant, anti-catarrhal, diuretic, astringent, hemostatic, cleansing, anti-inflammatory –, and externally – healing skin wounds and scars, astringent, hemostatic.

Among the many advantages provided by the *in vitro* culture technology at plants, there are: the possibility to *preserve the valuable qualities of a genotype* by cloning it, as well as the *induction of genetic variability* in order to obtain genotypes (somaclones) that are more valuable in terms of the content of bioactive compounds.

To initiate *in vitro* cultures of *Veronica officinalis* L. there were used young shoots harvested from a population at wild flora. As a basic nutrient medium there was used Murashige-Skoog (1962), enriched with several combinations and amounts of growth regulators.

Our observations led to the following conclusions: on most nutrient media supplemented with growth regulators, the node and apex explants had a good reaction, generating new shoots and roots. The aspect and number of shoots and the rizogenetic intensity varied depending on the type and concentration of the growth regulators present in the nutrient medium.

A proper morphogenetic response was noticed for the explants inoculated on the medium variants of MS enriched with 1mg/l BAP (benzylaminopurine) + 0.1 mg/l IAA (indole-3-acetic acid), 1mg/l BAP (benzylaminopurine) + 0.5 mg/l ANA (naphtyl-acetic acid) and 1mg/l Kin (kinetine) + 0.5 mg/l ANA (naphtyl-acetic acid).

The calusogenetic reaction at the inoculated explants was highlighted sporadically, with low yields on some hormonal formulas. The acclimatisation of the vitroplants to the *ex vitro* environment encountered no problems, and took place in a hydroponic system.

REFERENCES

1. BOJOR O., ALEXAN M., 1998 – Plante medicinale de la A la Z. Ed. Ulpia Traiana, Bucureşti;
2. CRIŞAN G.C., 2015 - Genul Veronica şi Ruscus abordare fitochimică. Teză de abilitare, Universitatea de medicină şi farmacie „Iuliu Ha□ieganu" Cluj-Napoca;
3. DANILA D., STEFANACHE C., DRUTU C., TROTUS E., 2011 - Conventional and unconventional techniques for in situ conservation and capitalization in agro-systems of *Veronica officinalis*. ISHS Acta Horticulturae Vol. 955, p.137 – 142;
4. GHIORGHIȚĂ G., NICUȚĂ PETRESCU D., 2005 – Biotehnologiile azi. Ed. Junimea, Iaşi;
5. GHIORGHIȚĂ G., 1992 - Perspectivele folosirii culturilor in vitro în inducerea variabilității plantelor, Lucr. St. Stejarul, Biol. veget. exp şi genetică, 12, 97-109;
6. ROBU T, MILICĂ C, 2004 – Plante medicinale autohtone. Colecția Utilis, Institutul European;
7. MOCAN A., VODNAR D. C., VLASE L., CRIŞAN O., GHELDIU A.M., CRIŞAN G., 2015 – Phytochemical Characterization of Veronica officinalis L., V. teucrium L. and V. orchidea Crantz from Romania and Their Antioxidant and Antimicrobial Properties. Int. J. Mol. Sci. 2015, 16, 21109-21127;
8. RILEY D. S., 2012 – Veronica officinalis. Materia Medica of New and Old Homeopathic Medicines. Springer, 262 – 271;
9. CRIŞAN G., TĂMAŞ M., MICLĂUŞ V., KRAUSY T., ŞANDOR V., 2007 - A comparative study of some Veronica Species, Rev. Med. Chir. Soc. Med. Nat. Iaşi, III(1).

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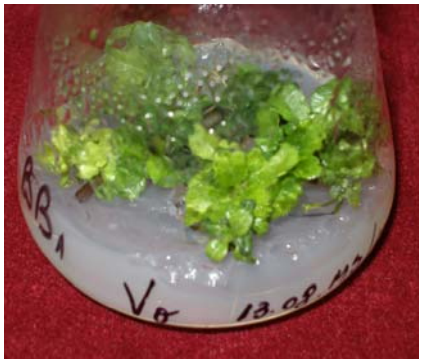
Fragments of leaves callused on BD_1 variant

Compact callus at the base of light green explants on BD_1 Variant



The evolution of shoots on BN_1

Caulogenesis on BN_1 variant

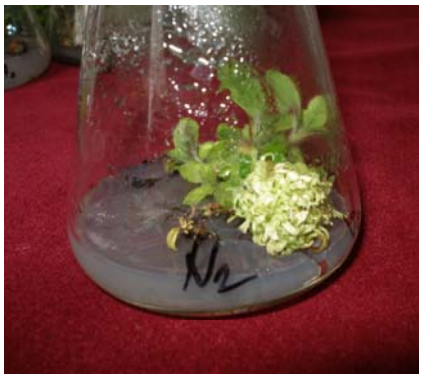
Rhizogenesis on BA_1 variant

Caulogenesis on BA_1 variant

Caulogenesis on KN_1

Rhizogenesis on KN_1

Dwarf shoots with chlorophyll deficiencies on N_2



Shoots with chlorophyll deficiencies (N_2)

Rhizogenesis on N_2

Caulogenesis on A_2

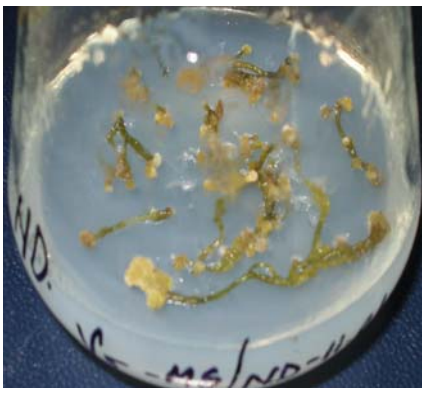
Shoots with chlorophyll deficiencies (A_2)

Rhizogenesis on A_2

Node shoots with adventitious roots (MS)

Shoots with roots (MS)

Callus on ND medium



Internodes callused on ND_{05}