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IN VITRO CONSERVATION OF THE RARE PLANT *VERONICA MULTIFIDA* L. SSP. *CAPSELLICARPA* DUBOVIK A. JELEN

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Abstract: The aim our work was to establish *in vitro* cultures in *Veronica multifida* ssp. *capsellicarpa* for *ex situ* conservation. We also characterized at the biochemical level the regenerated shoots using the peroxidases and catalases spectrum determination. The optimum regeneration way is the direct morphogenesis, which allowed the obtaining of vigorous rooted plantlets, suitable for *ex vitro* transfer. The analysis of peroxidase spectrum allowed the identification of 4 loci. The absence of some proteins with peroxidase activity is correlated with the presence of mannitol added in culture medium.

The catalases activity was identified in the samples cultured on media supplied with PVP and mannitol. It is possible that these supplements have the capacity to induce the activity of some enzymes with scavenger role for H_2O_2. The micropropagation protocol established in *Veronica multifida* ssp *capsellicarpa* can be extended in other related species.

Keywords: in vitro, conservation, morphogenesis, catalase and peroxidase spectrum

Introduction

In the last decades, as a result of plant diversity losses, the interest for conservation of endemic, rare and endangered plant species increased. The endemits represent species that originated from the ancient flora, with differentiated evolution, being maintained in particular local conditions [18]. The endemits are considered taxonomic or phyto-geographic units, with origin and spread in a limited area [22].

In Europe, from about 12.000 plant species, over 2000 have been considered rare or endangered [1].

Ciocârlan [6] considered that in Romania the endemic taxons represent ~ 5% from the whole number of species (81% being rare). In 1994, Boşcaiu [1] reported that 608 taxons (17.6% from the total) have different vulnerability grades. According to Oltean et al. [16], from 1483 taxons and infra-taxons existing in the Red List of the Vascular Plants in Romania, 3% are endangered and 12% are vulnerable. Dihoru et Dihoru [7] considered that 4.5% species (1189) are endemites from a total of 3976 species belonging to Romanian Flora.

Owing to reduction of the number of the plant species, at the international level it was established the Convention on Biological Diversity (CBD), which was sign by Romania in 1992 and ratified in 1994 (the 58th law). The Convention on Biological Diversity initiated the Global Strategy for The Plant Conservation, which was adopted at the 6th meeting of Parts Conference (COP 6) from Haga Convention (2002).

Plant biotechnology has an important role for *ex situ* conservation. Its use involves the elaboration of the methods for collection, sterilization, regeneration, germoplasm conservation, as well as the evaluation and characterization of the stability /variability of the species preserved in collection.

The use of *in vitro* culture and the preservation through different methods of the plant material represents an efficient and viable alternative for the protection of endangered species gene fund [2, 3, 8, 9, 10, 11, 23].

Material and Methods

Veronica multifida L. *ssp capsellicarpa* Dubovik. A. Jelen, perennial species is spreaded in steppe area and in the sunny meadows, in Dobrogea Central Pontic.

The plant material used for the initiation of *in vitro* cultures was originated from a specimen from the Bucharest Botanic Garden *ex situ* collection. PhD. Negrean Gavril first collected the plant from the origin habitat.

There were tested various explant types (leaves, young inflorescences, single node fragments) the induction of aseptic tissues cultures. The explants were washed in running tap water for 1 hour, then rinsed for 30 seconds in 70º ethyl alcohol and sterilized with $HgCl_2$.

For the initiation of *in vitro* cultures, severa desinfected with 0,1 % mercuric chloride for 6 minutes, followed by three washing with sterile distilled water. Different culture media were used in this experiment, being studied the effect of different variants for *in vitro* initiation, multiplication of the tissue cultures and regeneration (Tab. 1).

The cultures were maintained at 25±2^o C temperature regime, at 87 μmol/m^2/s light intensity of and 16/8 h photoperiod.

The tested medium variants consisted in MS salts [14] or N6 salts [5] supplemented with B_5 vitamins [12]. In the culture media, different combinations of growth factors and supplements were added.

To stimulate the cito-differention process, glutamine was added in the culture medium. MES (morfolin-etansulfonic acid), PVP (polyvinyl pyrrolidone), active charcoal were used to counteract the effects of the oxidative stress and for the retention of the phenolic compounds released in the culture media by the tissues.

Mannitol was used as osmolite, being known its role as carbon source and as scavenger of hydroxyl radicals.

For the evaluation of the regeneration process efficiency, it was registered the mean number of regenerants /explant. For biochemical characterization of *in vitro* regenerated shoots, there were analysed the isoperoxidases and catalases spectrum.

Protein Extraction

The plant material, represented by shoots, was grinded with a mortar pestle. The enzyme extract was made using Tris-HCl buffer 20 mM, pH 8 (10 mM $NaHCO_3$, 10 mM $MgCl_2$, 0,1 mM Na_2EDTA, 10 mM ß-mercapto-ethanol, 10% sucrose, 0.1% Triton X-100, PVP 4% at 4° C for 24h). After centrifugation at 18000 rpm for 20 minutes, the supernatant was used for electrophoresis analysis.

The electrophoresis analysis

It was prepared a running gel consisted in 8% polyacrilamide and a stacking gel 5% polyacrilamide. It was used Tris-glycine 0.05M, pH 8.3, as migration buffer. The protein separation was performed at 4°, 10 mA for staking gel and at 15 mA for the running gel, during 2 hours. The running marker was blue bromphenol.

Enzyme staining.

For peroxidases used a mixture made from the substrate, H_2O_2 and benzidine in acetate buffer. The bands appeared brown.

For catalases determination, the gel was first incubated in 0.003% hydrogen peroxide, in 0.01M phosphate buffer, pH 7, for 10 minutes and than in potassium ferricyanide and ferric chloride solutions.

The Blue Berlin complex results from the reaction of hydrogen peroxide which reduces the ferricyanide to ferrocyanide and reacts with ferric chloride. In this way, the catalases bands appear yellow colored on the blue-green background.

Results and Discussions

The successful of *in vitro* culture and conservation depends on the optimal choose of the explants and on the efficiency of the sterilization method.

Generally, to ensure the stability of micropropagated and preserved material is preferred to use the non-adventive propagation system. In the recalcitrant species, all the possible ways of multiplication are welcome. The most important step for the establishment of a *ex situ* conservation protocol is the initiation of aseptic proliferative cultures. After this step, the multiplied material could be *in vitro* maintained, cryo-preserved or used in restoration programs.

Veronica multifida ssp *capsellicarpa* species (*Scrophulariaceae* family) had a good *in vitro* behaviour on almost all the tested media. Different developmental processes were induced. The registered mean number of regenerants /explant varied between 6.3 (M2) and 22.5 (M6) (Fig. 1) The ideal explants were the uninodal shoots and young inflorescences.

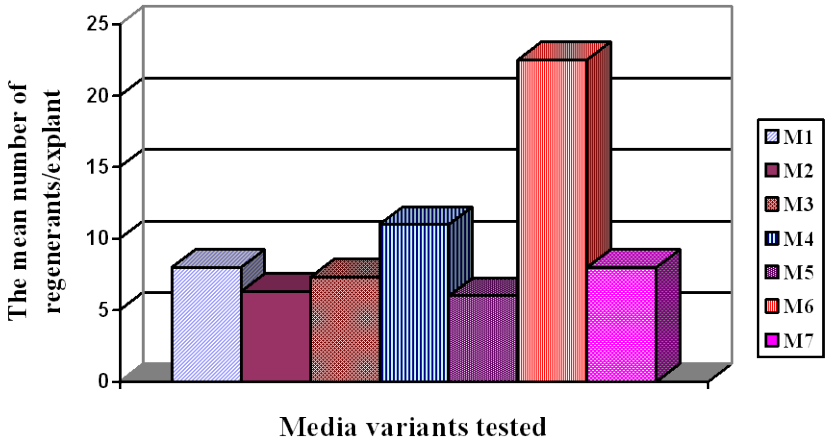

Fig. 1: The mean number of regenerants / explant / different media variants.

On the M1 medium variant was induced a multiple axillary shooting, starting from lateral meristem of the uninodal shoots. The M2, M3, M4, M5, M7 variants allowed direct morphogenesis from foliar explants, uninodal fragments and young inflorescences.

The regeneration rate depends on the supplements added in culture medium (Tab. 2), varying between 5 and 30 shoots /cultured explants.

The development of the neoplantles was achieved with a high rate on M1 variant (axillary shooting – Fig. 4) and on M5 variant (direct morphogenesis - Fig. 2). The presence of glutamine had a beneficial role.

The presence of kinetin and BAP, associated to NAA and GA_3 (M6 variant) had a stimulatory effect for shoots induction.

It was also noticed the calli formation (indirect morphogenesis – Fig.3). On M6 variant, although the number of shoots were increased, it was observed a tendency of elongation and vitrification. On M7 variant, an incresead number of vigorous and rooted shoots were induced.

Taking into account that *V. multifida* ssp. *capsellicarpa* had a good *in vitro* reactivity, it is preferable the induction of direct regeneration, trying to avoid the calli formation as a source of somaclonal variability.

The regeneration rate (10-15 shoots /explant) registered in the first step of the induction it was later amplified though subculture on the adequate culture medium, which sustains the morphogenesis process.

The new plantlets regenerated on all tested medium, are easily rooted (Fig. 5) and they can be *ex vitro* acclimatized.

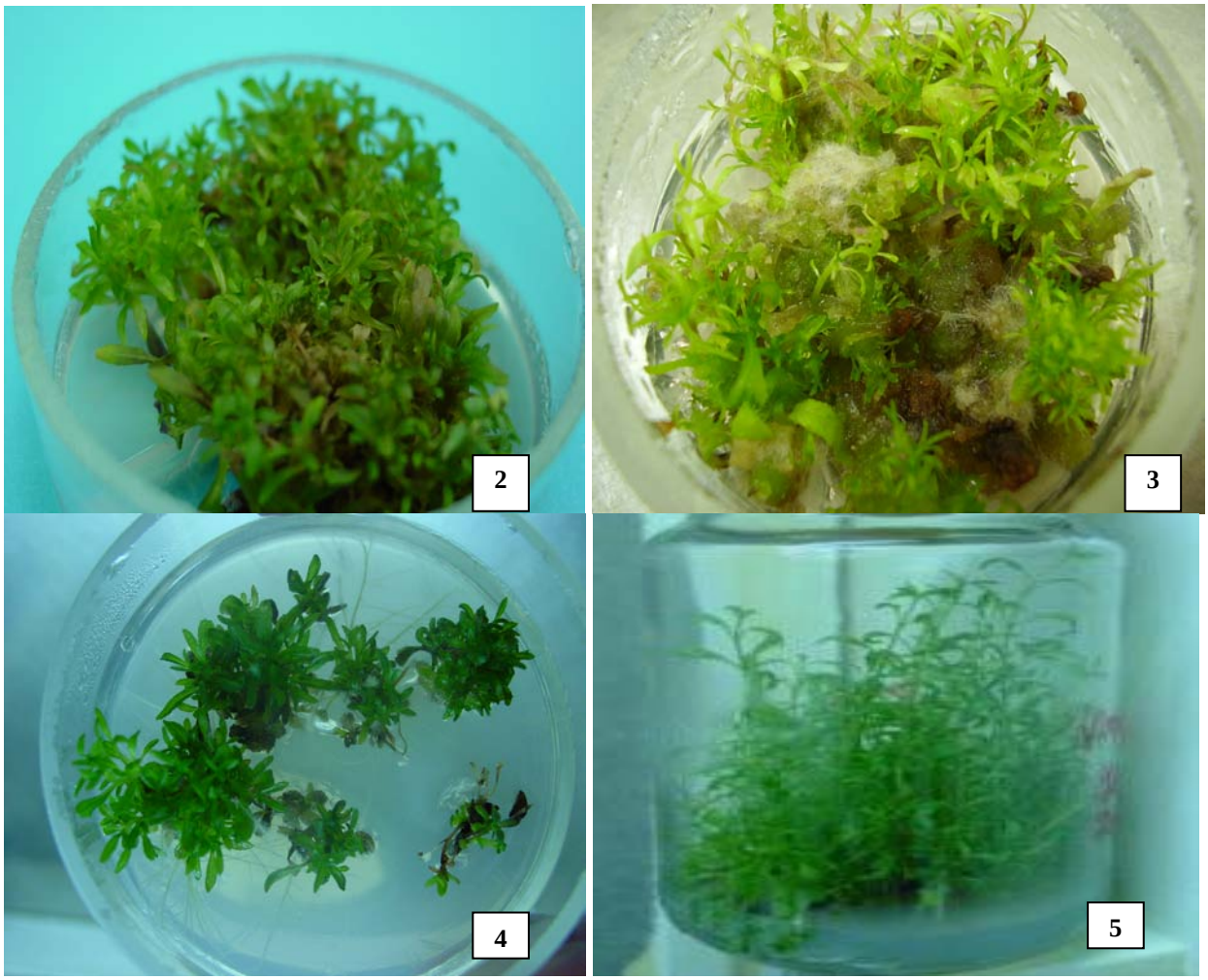

Fig. 2: Direct morphogenesis. **Fig 3:** Indirect morphogenesis. **Fig. 4:** Axillary shooting. **Fig. 5:** *In vitro* regenerated plantlet.

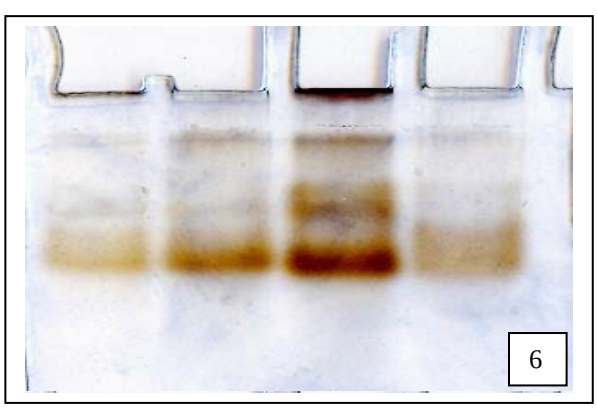

Fig. 6: Electrophoretic spectrum of peroxidases

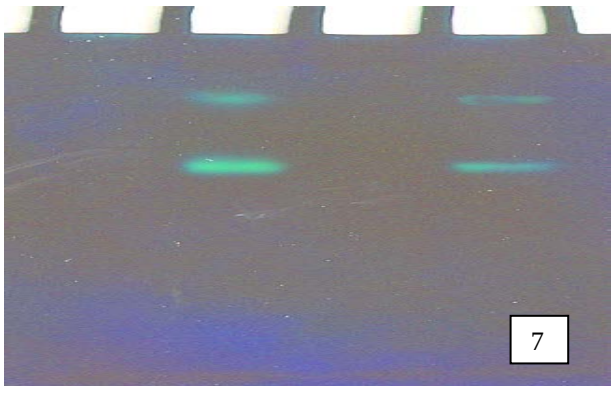

Fig. 7: Electrophoretic spectrum of catalases.

M – MS without hormones; 1. Regeneration medium with Kin, NAA, BAP, PVP; 2. Regeneration medium with Kin, ANA, BAP, GA_3 without PVP; 3. Medium without hormones supplemented with mannitol.

The M9-M13 variants media, characterized by auxine dominance determined the callusogenesis and rooting formation. In all the cases the calli were non-regenerative.

PVP, MES and mannitol were used as supplements added into the culture media. The plantlets can be maintained on mannitol-added medium during 2-3 months without subculture. PVP allows the retention of the phenolic compounds released by the explants, on the other hand, MES had no effect.

The M14 variant supplied with mannitol (30g/l) determined the lateral shoots formation and a vigorous rooting, but the plants not exceeded 2-3 nodes.

In a previous experiment [13], the mannitol was also used for the reduction of the growth and proliferation of the tissue cultures. Mannitol was described as *in vitro* [21] and *in vivo* [19] hydroxyl radicals' scavenger, having the role to protect against oxidative inactivation of some enzymes from Calvin Cycle [20].

The determination of the isoenzymatic spectrum was used to characterize the shoots regenerated according to different supplements present in the culture media.

It is already known that different types of stress generate reactive oxygen species (ROS). *In vitro* culture *per se* represents a stress factors for the cells.

The plants have enzymes, which catalyse reactions of ROS transformation in stable compounds, without nocive effect on the plant cells. These enzymes with antioxidant role (superoxide dismutases, citosolic catalases and peroxisomal, glutathion peroxidases) have the role of ROS scavangers.

The peroxidases are enzymes which catalyses the oxido-reduction reactions, using H_2O_2 as electron acceptor.

The high number of peroxidases isoforms has a multigenic determination. The proteins with peroxidase activity are the result of the duplication and genetic variation and post-transcriptional and of post-translational alteration.

In most cases, there is a particular expression in a specific tissue in certain developmental stages [17]. Obinger et al., [15] showed their role in the differentiation processes.

POX play a role in the phyto-hormones metabolism, in the phenolic oxidation in quinones and condensed tannins and an important role in the wounding and the defence stresses [4]. The increased peroxydase activity is correlated to the growth zones with rapid divisions.

The isoenzymatic spectrum is used for the intra-and inter-populational diversity determination or for preserved material characterization, the methods being reproducible.

In the case of the plants of *Veronica multifida* ssp *capsellicarpa* cultured on the medium added with giberelic acid, there were identified 4 different bands for peroxidases, respectively 4 possible genetic loci which can be correlated with epigenetic variation induced by gibberelic acid, and its involvement in natural auxine –indolyl acetic acid metabolism pathway.

The absence of one band was identified in the shoots cultured on PVP supplemented medium (Fig. 6).

The reduction of peroxidase activity was also observed in the samples collected from the medium supplemented with mannitol, characterized by reduced growth. This aspect can be correlated with the antioxidant activity of the mannitol, as scavenger of hydroxyl radicals in the *in vitro* culture.

The electrophoresis spectrum of catalases (Fig. 7), shown only 2 bands (2 loci) in the case of the shoots cultured on mannitol and PVP- supplemented media. It is possible that these supplements could be involved in the induction or mediation of these enzyme activities with role in H_2O_2 neutralization produced *in vitro*.

Conclusions

- The rare species *Veronica multifida ssp.capsellicarpa* was successfully *in vitro* introduced and multiplied.
- These micropropagation methods may be extended in other rare species from the same genus or family. The optimum micropropagation way is the direct morphogenesis, which allows the vigorous rooting plantlets regeneration, suitable for *ex vitro* transfer.
- The use of medium supplied with mannitol showed a favourable effect.
- The analysis of peroxidase spectrum allowed the identification of possible 4 genetic loci correlated with the 4 bands detected. The absence of some proteins with peroxidase activity is associated to the presence of mannitol and PVP added in culture medium.
- The catalases activity was identified in the samples collected from the culture medium supplied with PVP and mannitol. It is possible that these supplements had the capacity to induce some enzymes with scavenger role for H_2O_2.

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CONSERVAREA *IN VITRO* A PLANTEI RARE *VERONICA MULTIFIDA* L. SSP. *CAPSELLICARPA* DUBOVIK. A. JELEN

(Rezumat)

A fost vizată introducerea în cultura *in vitro* în vederea conservării *ex situ* a plantei rare *Veronica multifida* L. ssp. *capsellicarpa* Dubovik. A. Jelen. Au fost caracterizaţi din punct de vedere biochimic lăstarii regenerati pe baza utilizării spectrului peroxidazelor şi catalazelor. Calea de regenerare optimă este morfogeneza directă care permite obţinerea de plantule viguroase apte pentru transfer *ex vitro*. Analiza spectrului electroforetic al peroxidazelor la nivelul lăstarilor cutivaţi pe diferite variante de medii a permis identificarea a 4 loci genetici, respectiv a 4 proteine cu activitate peroxidazică. Reducerea activităţii peroxidazice a fost corelată cu prezenţa manitolului în mediu. Activitatea catalazelor a fost identificată în probele recoltate de pe medii suplimentate cu PVP şi manitol, care au capacitatea de a induce anumite enzime cu rol de scavanger pt H_2O_2.

Protocolul de micropropagare elaborat pentru specia *Veronica multifida* ssp *capsellicarpa*, poate fi extins şi la alte specii inrudite.