IN VITRO CONSERVATION STRATEGY IN *VERONICA MULTIFIDA* SSP. *CAPSELLICARPA* (DUBOVIK) A. JELEN

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In the last decades, the biodiversity had a rapid decline, being necessary to approach the conservation using both *in situ* and *ex situ* strategies. The aim of our study was to establish a reproducible *in vitro* conservation methodology in the rare taxon *Veronica multifida ssp. capsellicarpa*. An efficient medium-term conservation strategy was developed in *Veronica multifida ssp. capsellicarpa* involving an efficient regeneration protocol, a medium-term maintaining method and plant evaluation procedures. The preservation on the media variant with mannitol 3% and 6% ensured the stop of the growth, the limitation of lateral shoots formation and an acceptable rooting, the plant material having a good vigour. The biochemical analyses proved that there are not differences between clones on the same medium variant, the prolonged maintenance on mannitol added media ensuring the limitation of the growth and reduced supplementary handling of the *in vitro* preserved material during 3 months without transfers. This medium-term preservation conditions do not affect the viability and the variability of the plants. From these medium-term cultures healthy plants can be regenerated whenever it is necessary to reintroduce the taxon in the origin habitats or in other collections or to provide material for international changes of germplasm.

Key words: *Veronica multifida ssp. capsellicarpa* (Dubovik) A. Jelen, *in vitro* conservation, medium-term preservation, mannitol.

INTRODUCTION

Since 1948, an important nature conservation support has been established through founding the *International Union for Conservation Nature and Natural Resources* – IUCN (Gland, Switzerland). The main goal of IUCN is to sustain and to assist the global biodiversity conservation.

The integrated approach of the biodiversity conservation involves the complementary use of the two different strategies: *in situ* (in the natural habitats) and *ex situ* (out of natural habitats). The selection of the best strategy depends on the species IUCN status, on the biological characteristics, on the geographical area, on the density of natural populations and on the accessibility of plant material.

In the last decades, the biodiversity had a rapid decline, 8321 plant species became endangered between 1996-2004 (www.iucn.org).

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The most common forms of *ex situ* conservation are the field collections and the botanical gardens. The plants are also preserved in seed banks, using *in vitro* cultures and the cryopreservation method.

The *in vitro* culture has proved an important role in the management and conservation of genetic resources of endangered plant species (Engelmann 1997; Lynch, 1999; Benson, 1999; Cachiță-Cosma *et al.*, 1999, Cachiță-Cosma, 2005; Sarasan *et al.*, 2006).

Several *ex situ* modern techniques for storage of vegetative propagated species have already been developed. Taking into account the duration, there are two main categories of conservation (Engelmann and Engels, 2002):

– medium-term conservation, the germplasm being preserved as aseptic plant tissues cultures using slow-growth procedures;

– long-term conservation, the plant material being stored in liquid nitrogen (cryopreservation).

Medium-term conservation involves the initiation of *in vitro* aseptic cultures using different collecting methods, explants and sterilization procedures, the establishment of regeneration protocols and the maintenance of regenerative tissues cultures in minimal conditions using different methods to diminish the growth and differentiation.

Slow-growth procedures allow the preservation of the clonal plant material with periodic sub-culture, the period of time depending on species, the interval between subcultures being extended (Cha-um S., 2006).

There are several methods for the reduction of the cultures growth rate. In most cases, there is applied the low temperature treatment (Dale, 1980; Henshaw *et al.*, 1985; Ruredzo & Hanson, 1991), often used in combination with low light intensity or even with darkness (Mullin & Schlegel, 1976). Temperatures in the range of 0-5°C are employed with cold tolerant species, but for tropical species which are generally sensitive to cold temperatures, the range varies between 15° and 20°C. It is also possible to limit the growth by modifying the culture medium composition, mainly by reducing the sugar and/or mineral elements concentration (Ng and Ng, 1991) and by reduction of the oxygen level available to the cultures by covering explants with a layer of liquid medium or mineral oil (Bridgen and Staby, 1981) or adding osmotic active substances (Henshaw *et al.*, 1980, Ng and Ng, 1991; Ruredzo and Hanson, 1991).

Regeneration and successful propagation of genetic stable plants are prerequisites for any *in vitro* conservation effort.

All these methods need several researches regarding: the initiation of *in vitro* aseptic cultures and regeneration protocol, the monitoring of the interraction between different chemical and physical factors (medium compounds, retardants, temperature, light quality, photoperiod), the evaluation of the response of tissues cultures to medium-term conservation, the evaluation of physiological changes and of regenerants variability.

The aim of our study was to establish a reproducible *in vitro* conservation strategy in *Veronica multifida ssp. capsellicarpa.*

Veronica multifida ssp. capsellicarpa (Dubovik) A. Jelen is a perennial, rare taxon (Oltean *et al.*, 1994, Oprea, 2005) originated from Dobrogea, belonging to *Scrophulariaceae* family.

MATERIALS AND METHODS

The origin material was provided by PhD. Negrean Gavril, the explants were collected from a plant growth in the Botanical Gardens Bucharest.

The explants used for the aseptic tissue cultures initiation were leaves fragments, young inflorescences, single node stem fragments. The sterilization procedure was: the washing in running tap water for two hours, pre-sterilization in 70^0 alcohol for 30 seconds, sterilization with $HgCl_2$ 0.1% during 7 minutes and, finally, three washings with sterile distilled water.

For the induction of regeneration, several media variants based on MS formula (Murashige&Skoog, 1962) were tested, among them, 7 variants had good results in the shoots induction (Table 1). The standard MS medium was improved through the addition of sucrose at 30 g/l, B5 vitamins mixture (Gamborg *et al.*, 1968) and different growth factors and other compounds.

The plant material used for medium-term cultures was obtained using a previously established micropropagation protocol using a Murashige-Skoog medium (Murashige & Skoog, 1962), modified by adding 30 g/l sucrose, B5 Gamborg vitamins, supplemented with BAP (1 mg/l), Kin (1 mg/l), NAA (0.2 mg/l), and MES (0.1 g/l) (M5 variant) which ensure the regeneration of vigorously rooted shoots (Holobiuc *et al.*, 2006).

For the medium-term preservation experiments different media variants based on the reduction of macroelements concentration from the MS formula, on the reduction of carbon source level or on the presence of moderate osmotic stress induced through the addition of mannitol were tested (Table 2). Single node 0.5 cm stem fragments detached from the regenerated shoots were used as explants (5/Petri dish × 4 repetitions). After 2 months, the percent of neo-formed shoots/variant, the tissue water content, the mean number of nodes/explant, the percent of rooted regenerants were recorded.

To estimate the tissue water content the fresh weight (FW) and the dry weight (DW) were registered. The tissue water content formula was WC = (FW-DW)/FW × 100 (ISTA, 1993). The fresh weight was determined on the plant material detached from the basal rooted *in vitro* shoots. The basal segments were kept on the culture medium. The dry weight was determined after the maintenance of plant material at 60 °C, for 3 days.

Table 1

The media variants which induced good regeneration response in *V. multifida ssp. capsellicarpa*

Components		Media variants						
		I1	I2	I3	I4	I5	I6	I7
Macroelements		MS	MS	MS	MS	MS	MS	MS
Microelements		MS	MS	MS	MS	MS	MS	MS
B complex		B5	B5	B5	B5	B5	B5	B5
Growth factors (mg/l)	BAP	0.1	1	1	1	1	1	2
	Kin	–	1	1	1	1	1.5	–
	ANA	0.01	0.2	0.2	0.2	0.2	0.25	0.5
	GA_3	–	–	–	–	–	1	–
Other compounds (g/l)	AC	–	–	–	–	–	–	0.5
	MES		–	–	–	0.1	–	–
	PVP		–	10	–	–	–	–
	Glut	0.2	–	–	0.5	–	0.25	–
Agar (g/l)		10	10	10	10	10	10	10

Legend: MS-Murashige&Skoog medium; B5-Gamborg vitamins; BAP-benzyl aminopurine; Kin-kinetin; NAA-alpha-naphthyl acetic acid; GA_3-gibberellic acid; Glut-glutamine; PVP-polyvinyl pyrrolidone, CA-active charcoal.

Table 2

Media variants tested for medium-term conservation in *V. multifida ssp. capsellicarpa*

Media variant	Macroelements	Microelements	Sucrose content (%)	Mannitol concentration (%)
M1	MS	MS	3	–
M2	MS	MS	1	–
M3	MS	MS1/2	3	–
M4	MS	MS1/2	1	–
M5	MS	MS 1/4	3	–
M6	MS	MS 1/4	1	–
M7	MS	MS1/10	3	–
M8	MS	MS1/10	1	–
M9	MS	MS	3	3
M10	MS	MS	3	6
M11	MS	MS	3	9

THE BIOCHEMICAL ANALYSIS

The biochemical analyses were performed after 8 months of shoots medium-term maintenance on mannitol supplemented media.

The extraction of soluble cytosolic proteins was performed by grinding of the plantlets in 0.1 M phosphate buffer, pH 7 at 4°C. After centrifugation at 15 000 rpm for 10 min, the supernatant was used for electrophoretic analysis.

Polyacrylamide (PAA) gel electrophoresis. The electrophoretic analyses were carried out by the samples migration at 90/120V, 5h, in a discontinuous system using a running gel 8% PAA, a stacking gel 4% PAA and a buffer Tris-Gly 0.05M, pH 8.3. The running marker was bromphenol blue.

Detection of izoenzymes. The electrophoretic bands were developed using:

- 0.2 % α- and β-naphthyl phosphate as substrate and 0.05% Fast Blue BB in 0.1 M phosphate buffer, pH 6.5, for EST activity. The bands were stained in red.
- 0.08% benzidine in 0.5 M acetate buffer, pH 5 and hydrogen peroxide for POX activity. The band appeared very fast in blue colour that turned into brown.

RESULTS AND DISCUSSIONS

Veronica multifida ssp. *capsellicarpa* proved to have a good *in vitro* reactivity on all culture media variants tested.

Generally, different combinations of cytokinines BAP (0.1-2 mg/l) or/and kinetin (1-1.5 mg/l) and ANA (0.01-0.2 mg/l) promoted the regeneration through axillary shooting or direct morphogenesis (Fig. 1). The mean number of shoots/ explants varied between 5-30, according to the medium formula used. The addition of glutamine as supplementary source of nitrogen (0.2-0.5 g/l) and PVP had a beneficial role on the regeneration rate.

The regenerants can be easily acclimatized on perlite/ground mixture (1/3).

Owing to the good regenerability and high growth rate, for conservative purpose it is important to establish an efficient protocol to maintain as long as possible viable, regenerative cultures with limited growth and without affecting the genetic stability.

The single node stem fragments maintained *in vitro* during 2 months showed different responses concerning the vigour, the vitrification, the formation of new lateral shoots, the growth expressed as the mean number of nodes /explant, the rooting rate (% of rooted shoots) according to medium culture variant (Table 3).

In this taxon, even on hormone free medium, a lateral shoots formation occurs at the level of the nodes (of axillar meristems). For the prolonged *in vitro* maintenance for *ex situ* conservation purpose, it is necessary to limit the growth and the differentiation processes, meantime the vigour of plant material has to be good.

The reduction of the sucrose content at 1% limited the lateral shoots formation and rooting. The variants of media with macroelements reduced at 1/2 also determined a reduction of the two parameters, but in all these media (M2-M4), the growth of the shoots was not limited. On the variants supplemented with reduced carbon source (sucrose) at 1 %, the vigour of the shoots is low owing to

their heterotrophic nutrition being necessary an exogenous source of carbon (the photosynthesis process cannot ensure alone the polysaccharides production).

Fig. 1. Direct morphogenesis and rooting in *V. multifida ssp. capsellicarpa.*

Table 3

Different effects of media variants on *in vitro* maintained explants

Media variant	Effects				
	Vigour	Vitrification process	% of explants with axillar shooting	Mean number of nodes/explant/variant	% of rooted shoots/variant
M1-control	medium	absent	85	2	85
M2	low	present	55	2.5	65
M3	medium	present	65	2.5	60
M4	low	present	55	2.5	55
M5	medium	absent	75	1.5	60
M6	low	present	45	1.5	60
M7	medium	absent	30	1	75
M8	low	absent	35	1	75
M9	Medium to high	absent	15	2.5	50
M10	Medium to high	absent	15	1.5	60
M11	high	absent	5	1	15

On MS media with 1/4 and 1/10 macronutrients content and 3% sucrose level, the lateral shoots formation and growth is limited and the rooting is satisfactory. In the case of 1% sucrose content, the lateral shooting is more reduced and vitrification present.

On the maintaining media supplemented with mannitol in different concentrations (M9-M10), the growth and lateral shoots formation is strongly reduced, but at the highest mannitol level (9%) the rooting (% of rooting explants/ variant) is affected. Although, the explants had a good vigour.

Some of the quantified parameters (vigour, % of explants with lateral shooting, the mean number of nodes/explant/variant, the tissue water content) were reduced on the media variants supplemented with mannitol (Fig. 2). Only tissue water content was higher in shoots cultured on M9 media variant (Fig. 3).

The media variants with nutrient deficiency and 3% sucrose (M3, M5, and M7) induced a decrease of dry weight (Fig. 3) which is in concordance with other papers (Tewari R.K. *et al.*, 2003).

Taking into account the dry weight of the samples, the reduction of sucrose content at 1% influenced negatively this parameter. Also, in the case of M6, M7, M8 variants characterized with reduction of MS macroelements at ¼ and 1/10 level, the dry weight decreased, fact correlated with limited growth and low development of lateral shoots. In the case of mannitol supplemented media, in M9 and M10 variants with moderate concentrations, the dry weight increased correlated with the good vigour despite the growth reduction (Fig. 4).

Shoots maintained on M9, M10 and M11 media variants were used for biochemical analysis in order to verify if the prolonged culture in osmotic stress conditions induced some undesired variability.

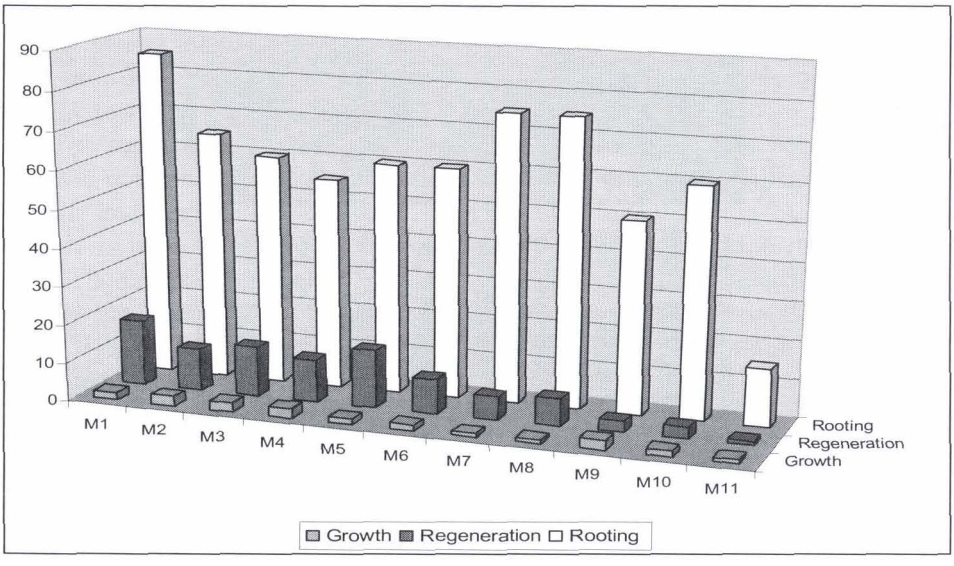

Fig. 2. The responses of *V. multifida ssp. capsellicarpa* plant material to different medium-term preservation variants.

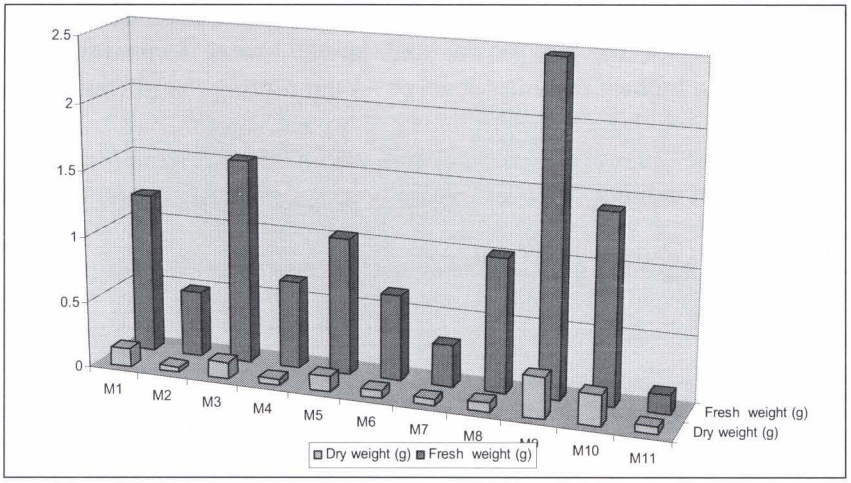

Fig. 3. Fresh and dry weight in *V. multifida ssp. capsellicarpa in vitro* explants cultured on different media variants.

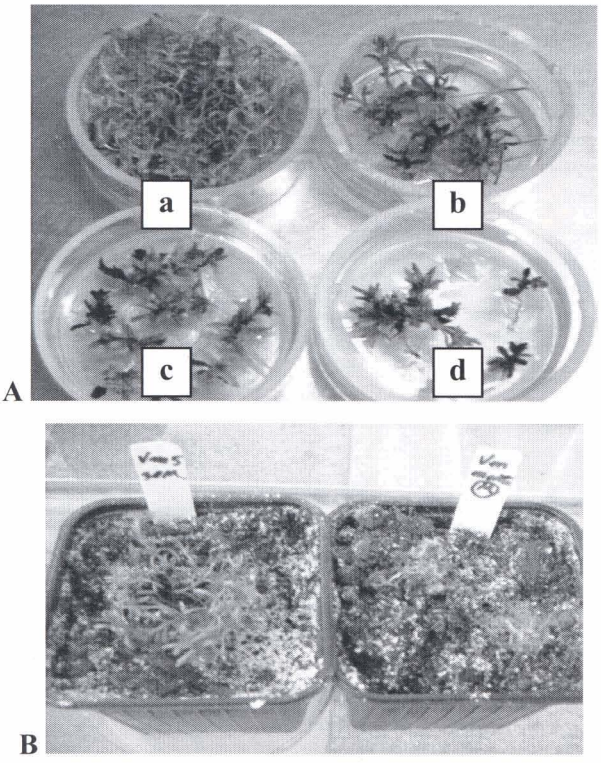

Fig. 4.A. Experimental variants used for medium term preservation: a) control, b) 3% mannitol, c) 6% mannitol, d) 9% mannitol; **B.** Acclimatised plants after medium-term preservation during 8 months.

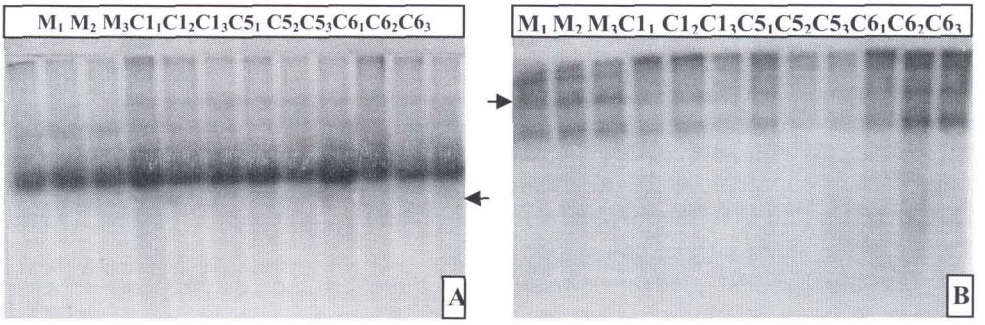

Fig. 5. Electrophoretic spectra of **POX (A), EST (B)** in cellular extracts from the plants (control-**M)** maintained on M1 media variant and clones **(C1, C5, C6)** regenerated on M 9 media variant.

The variability of the plants material cultured in medium-term preservation conditions was determined using isoenzyme analysis. It has been known that *in vitro* culture induced genetic and epigenetic changes, generic named somaclonal variation (Larkin & Scowcroft, 1981) the regenerants plants being considered as somaclones. Although, the developmental way for the micropropagation in *Veronica multifida ssp. capsellicarpa* is the direct morphogenesis which ensures a good stability of the regenerants. On the other side, the prolonged maintenance on slow growth conditions and the use of different limitant factors could determine some variations that affected the viability, the vigour, biological and biochemical characters of the preserved plant material.

The study of isoenzymatic spectra showed that there are some differences between shoots maintained for 8 months on mannitol and control (MS added with 30 g/l sucrose) media variants. There are no differences between clones 1, 5, 6 on the same media variant (Fig. 5).

The peroxidases catalyse the conversion of reactive oxygen species in stable, non-toxic compounds, having an anti-oxidant role (superoxide dismutase – SOD, cytosolic and peroxisomal catalases – CAT, glutathione peroxidase). The hydrogen peroxide is used as electron acceptor in oxido-reduction reaction (Obinger *et al.*, 1996). The peroxidases had several isoforms with multigenic determination. These enzymes have particular expression in some developmental processes (Ostergaard *et al.*, 1996), being associated with growth zones and rapid divisions and influenced by the composition of the culture medium.

In a previous study (Holobiuc *et al.*, 2006), a reduction of peroxidase activity was detected in the samples cultured on 3% mannitol supplemented medium compared to variants without this osmolite.

In the case of the clones maintained on the media variants added with mannitol, the absence of an esterase band can be observed. The mannitol induces growth plant reduction, which can be associated with differences in isoenzyme spectra.

CONCLUSIONS

A medium-term conservation strategy was developed in *Veronica multifida ssp. capsellicarpa* involving an efficient regeneration protocol, a medium-term maintaining method and plant evaluation procedures. The preservation on the media variant supplemented with mannitol 3% and 6% ensured the stop of the growth, the limitation of lateral shoots formation and an acceptable rooting. On the other side, the plant material has a good vigour.

The biochemical analyses proved that there are not differences between clones on the same medium variant, the prolonged maintenance on mannitol added media ensure the limitation of the growth and reduced supplementary handling of *in vitro* preserved material during 3 months, without transfers. These medium-term preservation conditions do not affect the viability and variability of the plants.

From these cultures, a lot of healthy plants can anytime be regenerated and acclimatized.

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