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ORIGINAL PAPER

The establishment of an *in vitro* gene bank in *Dianthus spiculifolius* Schur. And *D. glacialis* ssp. *gelidus* (Schott Nym. et Kotschy) Tutin: II. Medium-term cultures characterization in minimal growth conditions

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Abstract

The plant germplasm collections represent an important way for ex situ conservation of plant species on short, medium or long-term period of time.

In order to establish a reproducible protocol for the introduction to in vitro gene bank collection based on tissue cultures of two rare taxa ***-Dianthus*** *spiculifolius and* ***D. glacialis*** *ssp.* ***gelidus,*** *morphological, developmental and biochemical changes (antioxidant enzymes and protein spectra) after the prolonged culture in the presence of three levels of mannitol (0.16 M, 0.32 M and 0.49 M) as growth retardant were evaluated.*

Our results showed the positive effect of the mannitol for in vitro conservation in restricted growth conditions, its presence being compatible with the plant survival and the regeneration capacity. An important aspect was the induction and development of somatic embryos in the presence of mannitol as osmotic active factor. The extended culture in the minimal growth conditions, determined the increase of antioxidant enzyme activity and some small variations in enzyme spectra as peroxidase, superoxid dismutase, catalase and protein patterns.

The alterations in the proteins and enzymes pattern comparing to the control can be associated to the prolonged culture, to the desiccation stress, to the adaptation mechanisms as response to stress and developmental processes.

Keywords: *in vitro* gene bank, rare taxa, medium-term cultures, antioxidant enzymes pattern, *Dianthus spiculifolius*, *D. glacialis* ssp. *gelidus*.

Introduction

The conservation programs involve the approach of a wide variety of complementary strategies including studies of ecology, genetics, the natural habitat management, *in situ* conservation and also *ex situ* conservation in living collections (field and seed collections, botanic gardens, *in vitro* gene banks)(Sarasan & al. [19].

For the conservative purposes, the integration of different strategies is strongly imposed, *in vitro* methods having benefits and allowing the successful preservation of endangered species (Benson [2]; Linch, [14]; Bunn & al. [4]; Sarasan & al. [19].

The plant germplasm collections represent a tool for *ex situ* conservation of crops or wild plants species on short, medium or long-term, reducing the collecting pressure and ensuring the plant material. Some species are already extinct in the wild or endangered, *ex situ* conservation through modern biotechnologies offering the possibility to rescue the natural heritage of plant biodiversity. The active *in vitro* gene banks can ensure the preservation of plant germplasm for distribution, international changes and repopulation programs.

In vitro cultures allow the preservation of species especially with different problems (with recalcitrant seeds, with sterile seeds, extinct in the wild), being necessary reduced spaces for preservation of the accessions. The plant material is not exposed to different destructive factors and can be illimitably multiplied all year round. On the other hand, the *in*

vitro techniques need qualified staff and require specific reagents and facilities that involve energy consumption.

There are also necessary extensive studies for every species concerning *in vitro* protocols of regeneration and preservation and the interactions between physical medium factors and genetic stability have to be monitored. Plants should be tested if any somaclonal variation or abnormality occurred during to preservation procedures.

The use of non-cryogenic methods for the achievement of plant gene bank or collection represents a viable alternative (Goldmirzaie &Toledo [7]

Our studies are focused on the medium-term cultures in two rare taxa: *Dianthus spiculifolius* Schur. and *D. glacialis* ssp. *gelidus* (Schott Nym. et Kotschy) Tutin, as a tool for an *in vitro* gene bank establishment.

The elaboration of a complete *in vitro* conservation methodology involved many steps: the plant material collecting from the natural habitats, the use of adequate sterilization procedures and aseptic *in vitro* culture initiation, the characterization of *in vitro* reactivity, the optimization of the regeneration rate, the elaboration of medium and long-term maintaining procedures, the plant material characterization and evaluation.

Finally, this elaborated methodology is used for the introduction in a gene bank of new accessions of the target species.

A *sine qua non* condition for *in vitro* conservation is the maintenance of the viability and regenerative capacity of plant material.

In the two rare taxa studied, the medium-term cultures maintained under minimal growth condition were characterized after the prolonged culture in the presence of three concentrations of mannitol as osmoregulator factor and an evaluation at morphological and biochemical level was performed.

Material and Methods

In vitro methods

For medium-term cultures, the tissues cultures were maintained in limitative conditions in 6 cm Petri dishes during several months as samples with reduced growth at 25^0C, at 2000 lux illumination and 16/8 photoperiod.

The culture media used are based on MS formula (Murashige& Skoog [15] added with B5 Gamborg vitamins mixture[6], with sucrose in usual concentration of 30 g/l (0.087 M), solidified with 8g/l Merck agar and at 5.7-5.8 pH.

The osmolite used for growth reduction was mannitol in 3 concentrations: 0.16M, 0.32M and 0.49 M. The MS medium supplemented with sucrose in normal concentration and B5 vitamins was used as control.

The behavior of medium- term tissue cultures was evaluated after 3 and 6 months of maintenance and the whole interval of medium-term preservation reached 24-36 months.

The biochemical analyses

In order to identify the effects of prolonged maintenance on media supplemented with mannitol as osmolite and retardant growth factor in *Dianthus* medium–term cultures, the antioxidant enzyme spectra of the peroxidase (POX), catalase (CAT), superoxid dismutase (SOD) and cytosolic protein pattern after the 4 months and 12 months of culture were analyzed.

The enzyme extraction was performed by grinding the plant tissue in 50 mM Tris-HCl buffer pH 8, contained 2 mM Na_2EDTA, 4% PVP at 4^0C for 2h. The extract was centrifuged at 15.000 rpm for 20 min and the supernatant was used for electrophoresis analysis.

Electrophoresis was carried out at 4ºC in 10% polyacrylamide gel (respectively 8% for peroxidase and catalase) in standard 0.05M Tris-glycine buffer pH 8.3. Samples were loaded

into each well and then electrophoresis was made at 10 mA through the stacking gel for 30 min and 15 mA through the separating gel for 2h. After electrophoresis, for locating SOD activities on gel, was used 2.45 mM NBT (nitro blue tetrazolium salt), 28mM TEMED and $2.8X10^{-5}$ M riboflavine in 36mM phosphate buffer pH 7.8, for bands with CAT activities were used 0.003% H_2O_2 in 0.01 M phosphate buffer, pH 7, and then a 2% potassium ferricyanide and 2% ferric chloride solution. To detected POX activities the gel was soaked in 0.5M acetate buffer at pH 5 containing 0.08% benzidine and H_2O_2.

Results

In the studied taxa having a very good *in vitro* reactivity and high growth rate, in the first phase of the establishment of the protocols for *in vitro* gene bank, the multiplication methods have been already elaborated. The studies concerning the possibility to maintain the tissues cultures in growth limited conditions previously made conducted to the conclusion that sugar alcohols such as mannitol is appropriate for this purpose (Holobiuc & Blindu [11].

Previous results showed that in the presence of different levels of mannitol, the growth was slowed down, the explants survived and after 1 month of culture, *in vitro* developmental processes were induced, direct shoots formation from axillary meristems occurred (Holobiuc et al, 2009 in press).

The extension of the duration of tissues cultures preservation in medium-term conditions to 12 months was performed through periodical subcultures after 2-3 months interval .The characterization of these cultures was done taking into consideration: the survival, the growth, the regeneration capacity, antioxidant enzymes and proteins spectrum.

Morphological observations concerning the tissues cultures maintained in the presence of mannitol comparing to the control were also made (table 1). Owing to high number of the regenerants even in restrictive conditions and their small height, it was difficult to make these observations.

In the case of the control (hormone-free medium), the inoculated single node shoots elongated, formed 2-4 lateral shoots, rooted and after 2 months have been in optimal state for *ex vitro* transfer. In this case, the continuing *in vitro* maintenance involved additional handling: the transfer in greater vessels, the use of more culture medium quantity or the detachment of new stem fragments recultured on fresh medium. In this respect, the medium-term procedures have an important role in a collection or gene bank to reduce these supplementary actions.

In the first phase of the initiation of medium-term cultures, starting from single nodes stem fragments, the stress was higher owing to the collection of the explants from the donor plants and the culture in artificial conditions. For the beginning, mannitol acted as an osmolite conducting to the increase of medium osmotic pressure and decreasing water osmotic potential. Later, the mannitol uptake in the cell took place, acting as an osmoregulator factor and hydroxyl radical scavenger (Smirnoff & Cumbes [21]; Shen & al [20]. Our previous studies showed that addition of mannitol induced the increase of the antioxidant enzyme activity in the initial stages of culture, in correlation to the concentration of mannitol added in the medium culture and the peculiarities of the two species studied (*D. spiculifolius* and *D. glacialis* ssp. *gelidus)*.

According to mannitol level added in the culture medium, along the prolonged duration, a recovery of the cultures occurred, conducting to regeneration from lateral meristems (fig. 1A); the viability the plant material was also maintained in condition of the reduced growth of the cultures.

The lowest level of the mannitol (0.16 M) induced a positive effect on the regeneration through direct shooting, the growth of the shoots was lower compared to the control but superior compared to the highest level of mannitol.

The extension of the culture during several months on mannitol-supplemented media at 0.32 M was associated to somatic embryogenesis induction.

In an intermediate phase (2-3 months), two developmental ways may coexist; at the base of mini-plants leaves starting from their lateral meristems or somatic embryos were induced (unpublished data). Lately, in the prolonged cultures somatic embryogenesis was a dominant process.

Different somatic embryogenesis phases such as globular or cotyledonary stages were identified in fresh samples analyzed in light microscopy (fig 1 B). Somatic embryos are individualized, bipolar structures, without vascular connections with tissues from which they derived and having root meristem. These somatic embryos germinated forming roots in precocious stages and evolved in mini-plants. They did not grow if were maintained on the same medium and were able to form new embryogenic structures (unpublished data). If these regenerants are transferred on hormone-free medium they are normally developed and are converted into viable plants, transferable *ex vitro* after 2-3 months.

The highest level of mannitol (0.49 M) determined in the first month of culture a severe stress conducting to the necrosis of the leaves of the explant and a slower evolution of lateral meristems.

Despite to the growth retardation effect, after 2-3 months, a regeneration from the lateral meristems also occurred (Holobiuc &Blindu [11].

Table 1. Observations concerning the behavior of the tissues cultures maintained *in vitro* collection in *D. spiculifolius* and *D. glacialis ssp. gelidus* during 3 and 6 months

Taxon	Medium-term maintaining media				
	Culture duration	**Control**	**MS+ 0.16 M mannitol**	**MS+ 0.32M mannitol**	**MS+ 0.49 M mannitol**
D. spiculifolius	3 months	Growth stagnation owing to the consumption of culture medium and reduced space in the culture vessel. The plants start to degenerate. It is necessary the transfer on fresh medium and larger recipients or *ex vitro* transfer on pots.	20-30 regenerants induced through direct morphogenesis with 1-2 nodes of 1-1,5 cm height, /initial inoculum (single node stem fragment)	50-100 regenerants of 1-2 mm height with short roots / initial explant ; Somatic embryogenesis occurred	50-70 regenerants/ initial inoculum; do not exceed -2 mm height of mini-plants; Somatic embryogenesis occurred with high rate
	6 months	The *in vitro* plants cannot be maintained in optimal conditions	Shoots of 1-2 nodes and 1,8-2 cm height with good developed roots	1-2 mm height of the regenerants with short roots; the subcultures owing high regenerative rate are necessary.	The regenerants did not evolve into plants and did not grow; The transfer of the cultures owing high regenerative rate is necessary; the embryogenic structures continued to be induced, but the basal part of the aggregate degenerated.
D. glacialis* ssp. *gelidus	3 months	Growth stagnation owing to the consumption of culture medium and reduced space in the culture vessel. The plants start to degenerate. It is necessary the transfer on fresh medium and larger recipients or *ex vitro* transfer on pots.	Single node shoots of 1 cm height with good developed roots; about 15-20 shoots/initial inoculum	Growth stagnation, about 40-50 regenerants, 1-2 mm high with short, well developed roots/initial inoculum; somatic embryogenesis occurred.	30-60 regenerants ~1 mm height with own roots developed/ initial inoculum; the growth arrest.
	6 months	The *in vitro* plants cannot be maintained in optimal conditions	Over 20 single node shoots of 1 cm height with strongly secondary hairy roots developed as a network/initial explant	Embryogenic aggregates with embryos in high number; appeared mini-plants of 1-2 mm height with short roots.	Embryogenic aggregates with embryos in high number; mini-plants of 1-2 mm height with short roots; tendency of etiolation and degeneration of the base of the aggregate; a continued production of somatic embryos took place.

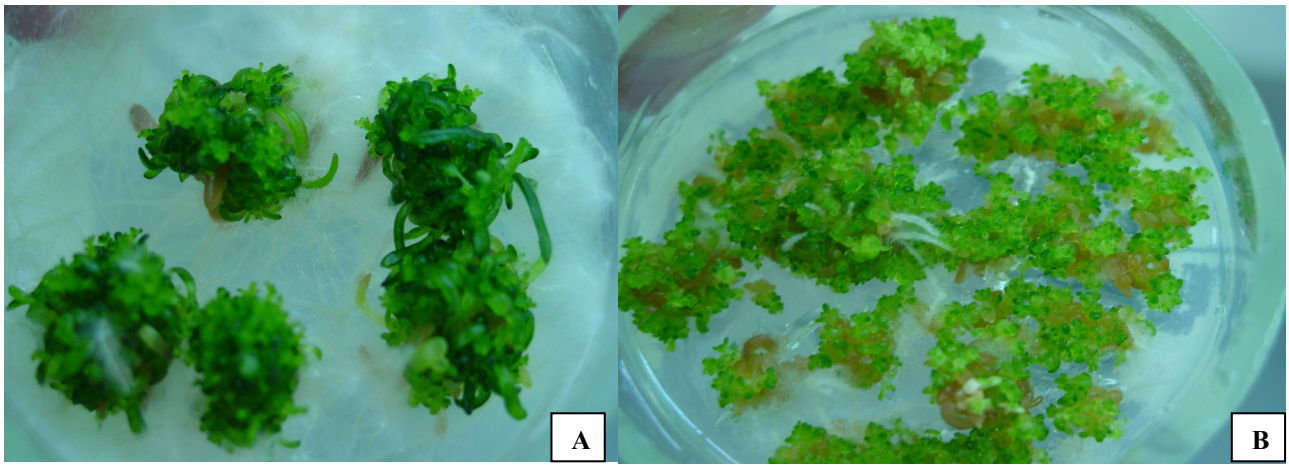

Fig. 1. **A** - Regenerative cultures in *D.glacialis* ssp. *gelidus* maintained during 6 months in the presence of 0.32 M mannitol;
B - High embryogenic cultures of *D. glacialis* ssp. *gelidus* cultured during 6 months in presence of 0.49 M mannitol.

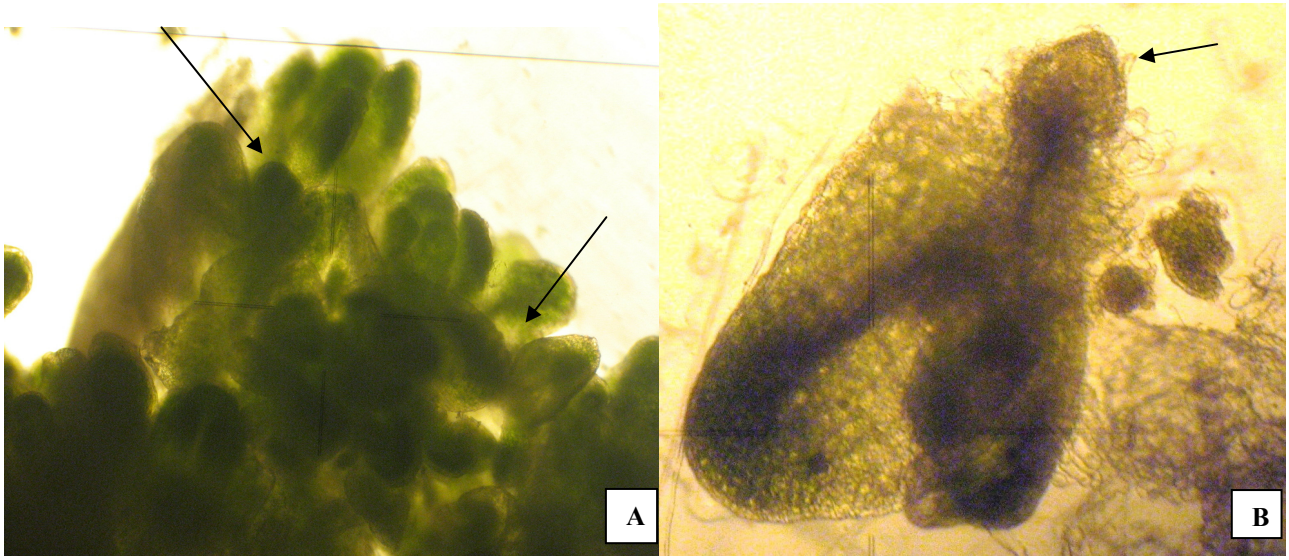

Fig. 2. **A -** The induction of the development of the lateral meristems (arrows) into axillary shoots in regenerative cultures of *Dianthus spiculifolius* in minimal growth conditions in presence of 0.32 M after 2 months of culture (X100).
B - Early somatic embryogenesis in *D. glacialis* ssp. *gelidus* (X 800) – globular and cotyledonar embryos with root meristem (arrow).

Concerning the biochemical studies, in the case of SOD spectra, previous results showed appearance of new isoform with SOD activity at 0.32 M and 0.49 M mannitol level in the first month of culture (Holobiuc et al., 2009, in press).

This pattern was maintained in *D. spiculifolius,* after 4 months of culture on media supplemented with mannitol (Fig. 3 A- see arrows), but after 12 months the disappearance of this supplementary band and the decrease of SOD activity were observed.

Similarly, a high activity of SOD isoenzymes on media added with mannitol after 4 months from initiation of the medium-term cultures were detected in *D. glacialis* ssp. *gelidus*, while a decrease in enzyme activity occurred after 12 months of culture (fig.3B).

In the cultures preserved in the presence of high level of mannitol (0.49M) the appearance of a highly expressed band with a different molecular weight or electric charge was detected (Fig. 3B- see arrow), fact that could indicate the induction of some alterations at genetic or epigenetic level.

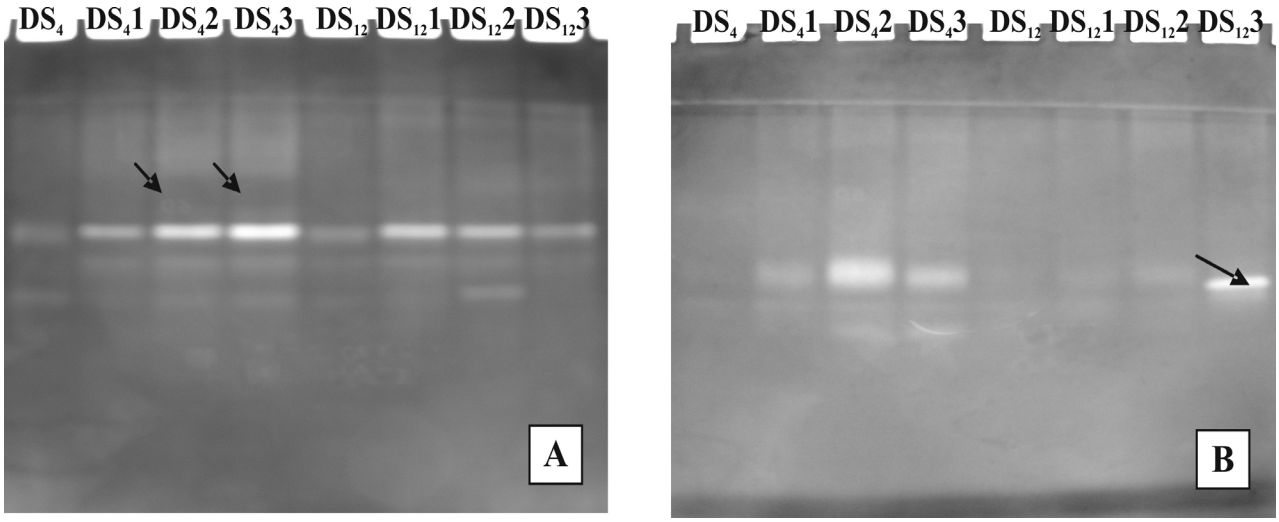

Fig. 3. The SOD electrophoretic spectra in *D. spiculifolius* (A) and *D. glacialis ssp. gelidus* (B) after the 4 months (DS_4 -DG_4), and 12 months (DS_{12} -DG_{12}) of the culture on media with different concentrations of mannitol- 0.16 M (1), 0,32 M (2) and 0.49 M (3).

Corelated to the pattern of the proteins migration (fig.4 A and 4 B), can be considered that the prolonged exposure to mannitol at high concentration determined the alteration of protein expression pattern, thus some bands dissapeared and others are expressed according to the level of the mannitol in both studied plant taxa.

In the case of POX, the prolonged maintenance in the presence of the mannitol determined an increase of enzyme activity, but compared to the control (mannitol free variant) some bands dissapeared (fig.5A, 5B). At high level of mannitol, after 12 months of culture, although the activity was high, the bands separation was low in the both taxa studied (fig.5).

However the CAT expression pattern (fig.6) did not showed a high variability, though in *D. spiculifolius* a new single isoform appeared in the fast migration zone at 12 months of culture in presence of 0.32 M mannitol (fig.6 A). It could be possible that this single expression to be an artifact, but our repeated analyses confirmed the presence of this isoenzyme with CAT activity.

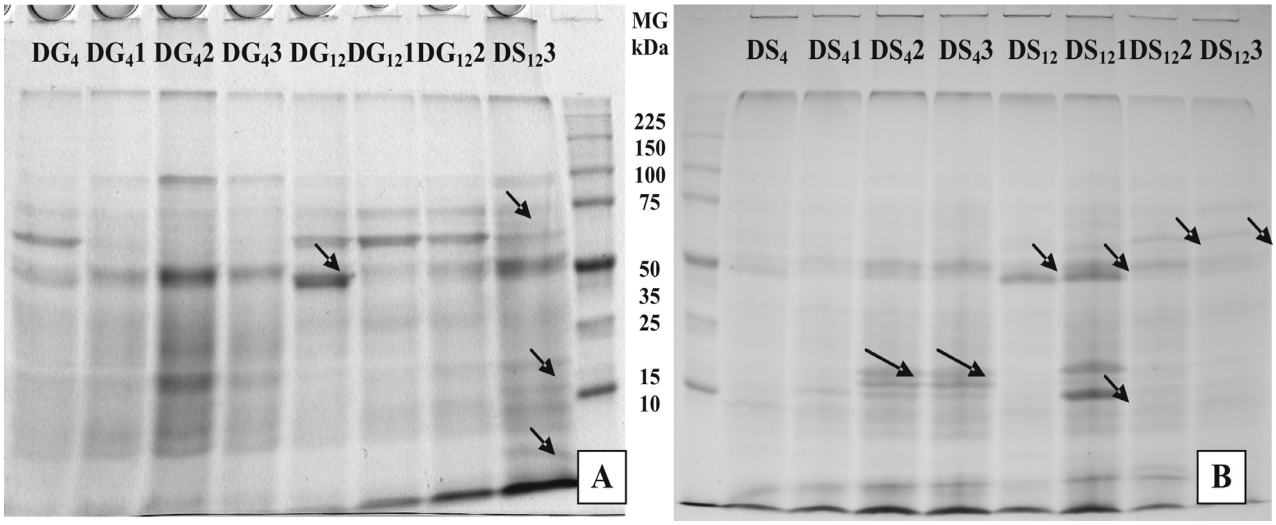

Fig. 4. The cytosolic proteins pattern in *D. glacialis ssp. gelidus* (A) and *D. spiculifolius* (B) after 4 months (DS_4-DG_4), and 12 months (DS_{12}-DG_{12}) of the culture on media with different levels of mannitol- 0,16 M (1), 0,32 M (2) and 0,49 M (3).

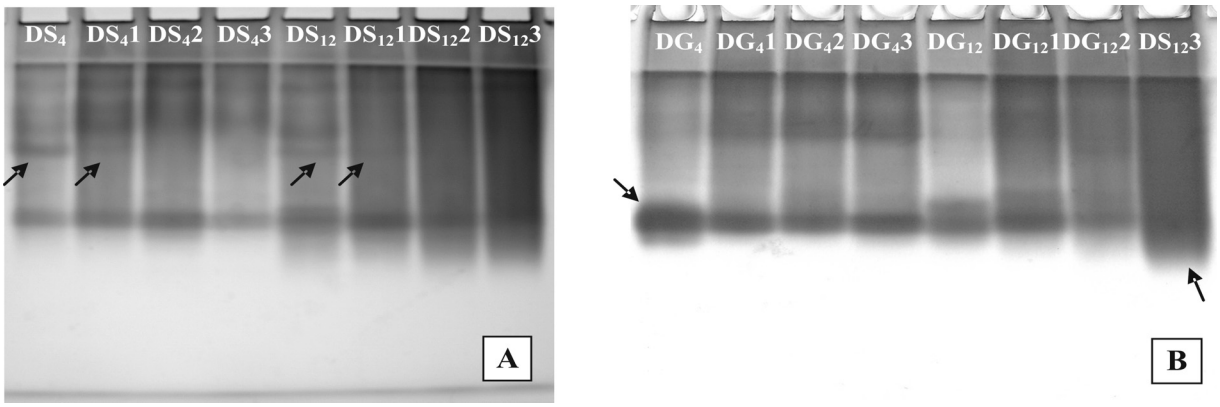

Fig. 5. The POX electrophoretic spectra in *D. spiculifolius* (A) and *D. glacialis* ssp. *gelidus* (B) after 4 months (DS_4-DG_4), and 12 months (DS_{12}-DG_{12}) of the culture on media with different concentrations of mannitol- 0,16 M (1), 0,32 M (2) and 0,49 M (3).

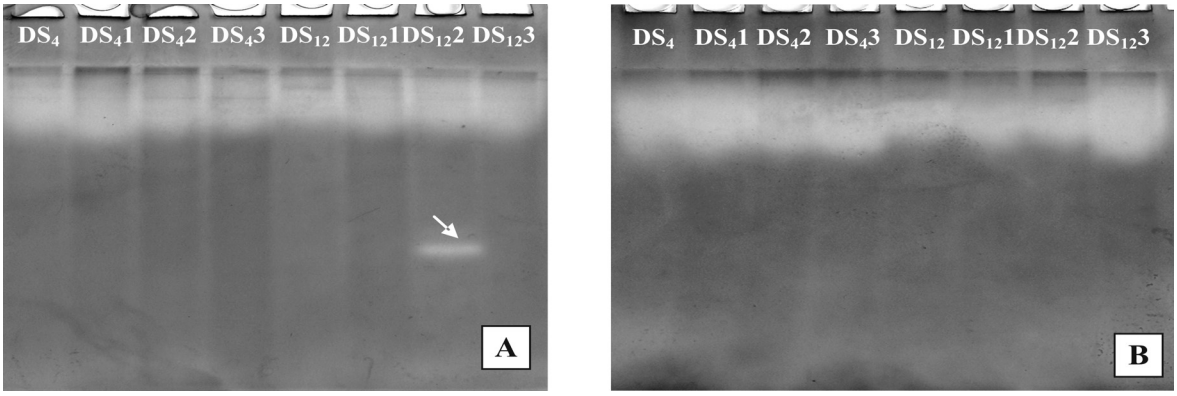

Fig. 6. The CAT electrophoretic spectra in *D. spiculifolius* (A) and *D. glacialis ssp. gelidus* (B) after 4 months (DS_4-DG_4) and 12 months (DS_{12}-DG_{12}) of the culture on media with different concentration of mannitol 0,16 M (1), 0,32 M (2) and 0,49 M (3).

Discussion

In our previous study, in the first 2 weeks after the initiation of *in vitro* medium-term cultures in the same taxa, mannitol acted as osmotic factor and determined the decrease of the extracellular water osmotic potential, a higher osmotic pressure and the increase of antioxidant enzyme activity; after a prolonged exposure, the accumulation of mannitol at intracellular level above certain limits, determined decreasing of the antioxidant enzymes activity due to its protective role against ROS (Holobiuc et al., 2009, in press).

Sugar alcohols as mannitol and sorbitol were used as osmotic factors in the plant tissue cultures for medium-term preservation (Harding et al. [9}; Goldmirzaie & Toledo [7]; Gopal & al. [8]; Borges & al. [3]. These compounds added into culture medium decreased the water osmotic potential, reduced the uptake of nutrients in the cells, thus limiting the plant growth *in vitro* culture for the preservation of plant material on medium-term.

There are some reports according to our results in others species (Staritsky & al. [22]; Vysotskaya [23]; Negash & al. [16]; Charoensub &Phansiri, [5]; Yang & al. [24].

The level of mannitol tolerated by a certain species is different: for example in *Plumbago indica*, 20 g/l mannitol concentration favored the reduction of the plantlet growth and long- term subculture during 8 months (Charoensub & Phansiri, [5]. Although, these authors associated mannitol with a plant growth regulator to improve the response of the explants to medium-term condition preservation, shoots multiplication occurred only in the hormone presence.

Despite the induction of an osmotic stress and its role as plasmolysing agent, the mannitol was well tolerated in the studied taxa. The mannitol was chosen by us among the several previously tested

factors for the growth retardation such as the temperature decrease, the abscisic acid administration or the nutrients levels reduction (Holobiuc& Blindu, [11], because in its presence the explants and the regenerants survived in better conditions, while the growth was limited.

Generally, *in vitro* regeneration in *Dianthus* genus occurs through lateral shoots formation (direct morphogenesis).

Somatic embryogenesis was reported first time by us in *Dianthus* genus in several rare taxa to be induced in presence of moderate osmotic stress in absence of any growth regulator.

Somatic embryogenesis (SE) represents the ideal way for *in vitro* plant regeneration and the somatic embryos can be used for conservative purposes on medium or long-term period. They are more stable compared to other structures generated *in vitro* (Ozias Akins & Vasil [17].

It is possible that osmolite mannitol to influence the endogenous level of abscisic acid (ABA), similar to other osmotic factors determining the induction of SE (Robinson and Barritt [18].

In soybean, mannitol was also found to sustain the somatic embryos formation when was added in the cultured medium (which includes 2, 4-Dichlor phenoxy acetic acid as growth factor and asparagine as nitrogen source) alone or in combination with abscisic acid as desiccation factor (Yang & al [24]. In *Arabidopsis thaliana,* Ikeda-Iwai& al. [12] reported that short stress induced by mannitol, but in presence of an auxin, promotes somatic embryogenesis.

A low osmotic water potential was already proved to induce somatic embryogenesis: for example, in *Vigna aconitifolia*. Kumar et al, [13] found that sorbitol in concentration of 10-30% added in the usual culture medium induced and sustained SE on long-term.

Generally, the *in vitro* artificial conditions generate an oxidative stress on plant cells and the association with additional factors determines specific adaptation reactions.

The antioxidant enzyme assay can offer information concerning the reaction of the plant tissue to different kind of stresses but also to the adaptation to particular conditions.

Mannitol added into culture medium in moderate concentrations can be transferred into cells and can activate adaptation mechanisms which confer tolerance to severe dehydration and ensure the cell survival (Hare & al. [10].

The single modifications in the isoenzymes and/or proteins spectra could be the result of the stress induced by the high osmolality of the culture medium which also determined the growth inhibition and some necrosis of tissue cultured in contact with medium.

On the other hand, the differences in the expression of antioxidant enzymes in cultures exposed to mannitol can be due to the activation of morphogenetic processes, initially as direct morphogenesis (shoots induction) and subsequently, in presence of 0.32 M and 0.49 M mannitol as somatic embryogenesis.

Conclusions

- Despite of some variations of the biochemical parameters tested by us during the prolonged culture in the presence of the mannitol, this compound proved to be well tolerated by the plant material during several months, being compatible with the survival in the restrictive conditions and with the maintenance of the regeneration capacity in the condition of growth retardation. An original aspect of our methodology consists in the induction and development of somatic embryogenesis in *Dianthus* genus, in absence of phytohormones, although in this species usually regenerated *in vitro* by direct shooting. Using the mannitol, the regenerative cultures with growth reduction were maintained in the collection for several years and morphologically normal plants could be obtained whenever it is necessary through the transfer on hormone- free medium.

- An increase of antioxidant enzyme activity was detected in the presence of mannitol as growth retardant in the first 4 months; after 12 months of cultures, the antioxidant enzyme activity decreased, probably due to the adaptation process.

- The appearance of some new distinct bands as in case of SOD respectively CAT patterns after 12 months of culture both in *Dianthus glacialis* ssp. *gelidus* at a concentration of 0.49 M mannitol and *D. spiculifolius* at 0.32 M mannitol could be determined by genetic mutations or epigenetic changes.

References

1. P.V. AMMIRATO, *Organizational events during somatic embryogenesis* In; C.E. Somers D.SA., Hacket, W.P., Biesboer D.D. (eds.). Plant Tiss. Org. Cult. New ork, Alan R. Liss, pp. 57-81 (1987).
2. E. BENSON, *An introduction to Plant Conservation Biotechnology*. In: Benson, E. E., ed. Plant conservation biotechnology. London: Taylor& Francis, pp. 3–10 (1999).
3. M. BORGES, W. CEIRO, S. MENSES, N. AGUILERA, J. VASQUEZ, Z. INFANTE, M. FONSECA, Regeneration and multiplication of *Dioscorea alata* germplasm maintained *in vitro*. Pl. Cell. Org. Cult. **76**, 87-90 (2004).
4. E. BUNN, S. TURNER, M. PANAIA, K.W. DIXON, The contribution of *in vitro t*echnology and cryogenic storage to conservation of indigenous plants. Austr. J. Bot.**55,** 345-355 (2002).
5. R. CHAROENSUB, P. SALAK, *In vitro* conservation of Rose coloured leadwort: Effect of mannitol on growth of plantlets. Kasetsart J.(Nat.Sci.) **38,** 97-102 (2004).
6. O.L. GAMBORG, R.A. MILLER, K. OJIMA, Nutrient requirements of suspension cultures of soybean root cells. *Physiol*. Plant **15,** 473-497 (1968).
7. A. GOLDMIRZAIE, J. TOLEDO, *Noncryogenic long-term germplasm storage*. Methods in molecular Biology. Plant Cell Culture protocols. 111. R.D. Hall.ed.Humana Press Inc. Totowa (1999).
8. J. GOPAL, A. CHAMAIL, D. SARKAR, Slow-growth *in vitro* conservation of potato germplasm at normal propagation temperature. Popato Res. **45**(2-4), 203-213 (2002).
9. K. HARDING, E. BENSON, K. CLARCHER, Plant Conservation Biotechnology; An. Overview. Agro-Food Industry Hi-Tech, pp. 24-29 (1997).
10. P.D. HARE, W.A. CRESS, J. VAN STADEN, Dissecting the roles of osmolyte accumulation during stress.Plant, Cell & Environment **21** (6), 535-553 (1998).
11. I. HOLOBIUC, R. BLÎNDU, Improvement of the micropropagation and *in vitro* medium-term preservation of some rare D*ianthus* species, Contributii Botanice, **41** (2), 143-151 (2006).
12. M. IKEDA–IWAI, M. UMEHARA, S. SATOH, H. KAMADA, Stress-induced somatic embryogenesis in vegetative tissues of *Arabidopsis thaliana*. Plant J. **34** (1), 107-114, (2003).
13. A.S. KUMAR, L.O. GAMBORG, M.W. NABORS, Plant regeneration from cell suspension cultures of *Vigna aconitifoloa*. Plant. Cell Rep**.7,** 138-141(1988).
14. P.T. LYNCH, Tissue Culture Techniques *in vitro* Plant Conservation. Plant Conservation Biotechnology, ed. E. Benson, Univ. Abertay Dundee UK, pp. 4-62 (1999).
15. T. MURASHIGE, F. SKOOG, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol. Plant*., **15,** 473-477 (1962).
16. A. NEGASH, F. KRENS, J. SCHAART, B. VISSER, *In vitro* conservation of the enset under slow-growth conditions. Pl. Cell. Tiss. Org. Cult. **66**, 107-111 (2001).
17. P. OZIAS-AKINS, I.K. VASIL, *In vitro* regeneration and genetic manipulation of grasses. Physiol Plant, **73,** 565-569 (1988).
18. T.L. ROBINSON, B.H. BARRITT, Endogeous abscisic acid concentration, vegetative growth and water relations of apple seedlings following PEG induced water stress. J. Am. Soc. Hortic. Sci. **115**, 991-999 (1990).
19. V. SARASAN, R. CRIPPS, M.M. RAMSAY, C. ATHERTON, M. MCMICHEN, G. PRENDERGAST, J.K. ROWNTREE, Conservation *In Vitro* Of Threatened Plants – Progress In The Past Decade. In Vitro Cell. Dev. Biol.Plant **42**, 206–214 (2006).
20. B. SHEN, R.G. JENSEN, H.J. BOHNERT, Mannitol protects against oxidation by hydroxyl radicals. Plant. Physiol.**115**, 527-532 (1997).
21. N. SMIRNOFF, Q.J.CUMBES, Hydroxyl radical scavenging activity of compatible solutes. Phytochemistr*y* **28**: 1057-1060 (1989).
22. G. STARITSKY, A.J. DEKKERS, N.P. LOUWAARS, E.A. ZANDVORT, *In vitro conservation at reduced temperature and under osmotic stress*. In: Withers L.A. Anderson P.G. (eds). Butherwords, London Plant Tissues Culture and its Agricultural Applications, pp. 277-284 (1986).
23. O.N. VYSOTSKAYA, Long-term maintenance of strawberry plants *in vitro*. Russian J. Plant Physiol. **41**, 827-833 (19940.
24. C. YANG, T. ZHAO, D. YU, J. GAI, Somatic embryogenesis and plant regeneration in Chinese soybean (*Glycine max* (L.) Merr. - impacts of mannitol, abscisic acid, and explant age. In Vitro Dev. Biol. Plant **45**: 180-188 (2009).