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The establishment of an *in vitro* gene bank in *Dianthus spiculifolius* Schur and *D. glacialis* ssp. *gelidus* (Schott Nym. et Kotschy) Tutin: I. The initiation of a tissue collection and the characterization of the cultures in minimal growth conditions

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Abstract. In the last decades the plants have to cope with the warming of the climate. As a consequence of this process more than half of the plant species could become vulnerable or threatened until 2080. Romania has a high plant diversity, with endemic and endangered plant species, the measures of biodiversity conservation being necessary. The integrated approach of biodiversity conservation involves both *in situ* and *ex situ* strategies. Among *ex situ* methods of conservation, besides the traditional ones (including field and botanic collection and seed banks), *in vitro* tissues techniques offer a viable alternative. The germplasm collections can efficiently preserve the species (of economic, scientific and conservative importance), in the same time being a source of plant material for international exchanges and for reintroduction in the native habitats.The "*in vitro* gene banking" term refers to *in vitro* tissues cultures from many accessions of a target species and involves the collection of plant material from field or from native habitats, the elaboration of sterilization, micropropagation and maintaining protocols. These collections have to be maintained in optimal conditions, morphologically and genetically characterized. The aim of our work was to characterize the response of the plant material to the minimal *in vitro* growth protocol for medium-term cultures achievement as a prerequisite condition for an active gene bank establishment in two rare Caryophyllaceae taxa: *Dianthus spiculifolius* and *D. glacialis* ssp. *gelidus*. Among different factors previously tested for medium-term preservation in *Dianthus* genus, mannitol proved to be more efficient for minimal cultures achievement. *In vitro,* the cultures were evaluated concerning their growth, regenerability and enzyme activity (POX, SOD, CAT) as a response to the preservation conditions in the incipient phase of the initiation of the *in vitro* collection. The two species considered in this study showed a good *in vitro* behavior when using the mannitol as growth retardant.
Key words: *in vitro* gene bank, growth retardation, *Dianthus spiculifolius*, *D. glacialis* ssp. *gelidus*, antioxidant enzymes activity and spectrum.

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Introduction

The strong industrialization, the major land use, the overexploitation, the habitats fragmentation and destruction, and climatic changes determine European Flora to become among the most globally threatened (http://ec.europa.eu/environment/life/publications), 4,700 endemic vascular plants being in danger of extinction.

Nowadays, the stoping of the loss of plant genetic resources represents a constant concern of scientists. The classical conservation approaches require large space, lands and high labor expenses and expose the plant material to environmental damaging and pests. The integrated approach of biodiversity conservation involves both *in situ* and *ex situ* strategies.

Several authors sustained the important role of *in vitro* conservation methods in the management and conservation of genetic resources of endangered plant species (Zăpârțan 1996, 2001, Engelmann 1997, Lynch 1999, Benson 1999, Engelmann & Engels 2002, Cachiță-Cosma & Halmagyi 2005, Holobiuc 2006, Sarasan et al. 2006).

The medium-term conservation relies on the *in vitro* germplasm maintenance during months or years using slow-growth procedures made through the tissues cultures in restrictive conditions, periodical sub-culture and the duration of the transfers depends on the species (Cha-um et al. 2006).

Many methods for the reduction of the growth were reported: the low temperature treatment (Ruredzo & Hanson 1991), often used in combination with low light intensity or with darkness (Mullin & Schelegel 1976), the reduction of the sugar and/or mineral elements level (Ng & Ng 1991), the decrease of oxygen available to the cultures (Bridgen & Staby 1981) or adding osmotic active substances (Staritsky et al. 1986, Ng & Ng 1991, Goldmirzaie & Toledo 1999).

An *in vitro* collection (active gene bank) represents an *ex situ* conservation, involving the regeneration, characterization and evaluation methods, the plant material being disposable to international exchanges.

An active collection is commonly duplicated in a base collection and it is often stored under medium with long-term storage.

Romania has a high diversity and richness of plants species, but a lot of them are rare and/or threatened, preservation measures being necessary. There are 102 taxa with different IUCN categories belonging to the Caryophyllaceae family (~7%), 29% of them are *Dianthus* species and subspecies (Oltean et al. 1994), fact that justifies the *ex situ* conservation approaches.

Dianthus spiculifolius Schur is an endemic species from South-Eastern Carpathian Mountains, a perennnial plant, growing on calcareous rocks, vegetatively propagated and through seeds, belonging to plant associatiations with high conservative importance.

Dianthus gelidus Schott, Nyman et Kotschy is sub-endemic taxon for Southern and Eastern Carpathians, perennial growing in alpine area, with similar reproduction to other *Dianthus* taxa, the integrating plant associations having also high conservative value. In "The Critical List of Vascular plants from Romania", *Dianthus glacialis* ssp. *gelidus* is considered a rare endemic taxon for Eastern Carpathians (Oprea 2005).

Dianthus spiculifolius has already been extensively studied concerning *in vitro* regeneration (Zăpârțan 1995, Butiuc-Keul & Deliu 2000, Cristea et al. 2002, 2004, Holobiuc et al. 2004-2005).

Dianthus glacialis Haencke ssp. *gelidus* was also *in vitro* characterized concerning the regeneration (Holobiuc & Blîndu 2006), and subsequently, the micropropagation methodology in different rare *Dianthus* taxa was improved, but the medium-term cultures in restricted growth conditions have not been yet characterized.

Our aim was to characterize the response of the plant material in the phase of the initiation of medium-term cultures in restricted growth conditions, that represent a prerequisite for an active *in vitro* gene bank establishment in two rare Caryophyllaceae taxa: *Dianthus glacialis* Haencke ssp. *gelidus* (Schott, Nyman & Kotschy) and *Dianthus spiculifolius* Schur. These taxa are considered rare (Oltean et al. 1994, Boşcaiu et al. 1994, Dihoru & Dihoru 1994, Oprea 2005).

Materials and methods

The origin of the plant material

For *Dianthus spiculifolius* Schur., mature plants and seeds were collected from Bucegi Massif: - Valea cu Brazi (1300 m),Vârful Furnica (1400 m), Vârful cu Dor (1700 m) and Piatra Craiului Massif, at Umerii Pietrei Craiului (1600 m) and Diana (1500 m). For *D. glacialis* ssp. *gelidus* (Schott, Nyman et Kotschy) Tutin, mature plants and seeds were collected from Bucegi Massif: Valea Babei (1800 m) and Babele (2000 m).

In vitro methods of culture

The elaboration of *in vitro* conservation methodology involved the collection of plant material from their habitats, the aseptic cultures establishment, the evaluation of the regenerative capacity, the testing of various limiting growth factors for medium term maintenance, the biochemical characterization of the plant material after different periods of time.

Shoots fragments from the proliferative cultures were used for medium-term maintaining cultures initiation. Among different methods previously tested (Holobiuc et al. 2004, 2005), as the use of abscisic acid, the reduction of mineral salts or of sucrose content, only mannitol addition in moderate concentrations in the culture medium has proven to be efficiently and was used for further studies.

The initiation of cultures were performed starting from single node stem fragments sterilized according an previously established procedure (Holobiuc et al. 2004-2005, 2006) using the washing in tap water during 2 hours, the short immersion in 70^0 alcohol, the use of mercuric chloride 0,1% as disinfection agent, for 5-6 minutes and finally three washing in sterile distilled water. The same methods, but with prolonged exposure to sterilizing agent at 7-8 minutes was used for seeds disinfection. The sterile seeds were cultured on M1 medium variant (Table 1) and have germinated into plants.

For medium-term cultures, we used single node stem fragments taken from *in vitro* regenerated plants or seedlings and cultured in 6 cm Petri dishes (5 inocula/dish), 5 repetitions/ variant, maintained at 25^0C, at 2000 lux illumination and 16/8 photoperiod.

The culture media used are based on MS formula (Murashige & Skoog 1962), added with Gamborg vitamins (Gamborg et al. 1968), with sucrose in usual concentration of 30 g/l (0.087 M), solidified with 8 g/l agar (Merck) and pH at 5.8. The osmolite used for growth reduction was mannitol added in 3 concentrations (Table 1). The behavior of medium term tissue cultures was for instant evaluated after 1-2 months of culture and the biochemical analysis in the incipient phase of the initiation of the cultures (after 2 and 4 weeks) were made to determine if the used protocol ensured the preservation with minimal growth and allowed the survival and adaptation of the explants in the restrictive conditions.

The biochemical analysis

The biochemical analysis are focused on the variation in antioxidant enzyme activity and expression in tissues cultures of *D. spiculifolius* and *D. glacialis* ssp. *gelidus,* on media

Table 1 The media used for medium-term cultures in *Dianthus spiculifolius* and *D. glacialis* ssp. *gelidus*

Composition		Medium variants			
		M1 (Control)	M2	M3	M4
Macroelements MS		MS	MS	MS	MS
Microelements MS		MS	MS	MS	MS
Vitamins		B5	B5	B5	B5
Others compounds	Man (M)	-	0.16	0.32	0.49
	Su (M)	0.087	0.087	0.087	0.087

Legend: MS- Murashige & Skoog- medium, B5 - Gamborg vitamins mixture, Man - mannitol, Su - sucrose

supplemented with different concentration of mannitol, after 2 weeks and 4 weeks, in the initiation phase of medium-term cultures.

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

The peroxidase (POX) activity was detected by incubation of the cellular extract in a guaiacol solution 30 mM in the presence of 3% H_2O_2. The enzyme activity was determined as the variation of O.D. at 470 nm per minute. The superoxid dismutase (SOD) activity detection was based on the enzyme capacity to stop the reduction of NBT (nitro blue tetrazolium salt) with super oxide radicals (Beauchamp & Fridovich 1971). One SOD activity unit represents the enzyme quantity, which inhibits the reduction of the substrate with 50%, at 560 nm. The catalase activity (CAT) was estimated by the consumption of H_2O_2, from the reaction mixture, at 240 nm. One enzyme unit neutralizes one H_2O_2 µmol in 1 minute, at 25°C and pH 7.

The protein content was assayed by the Bradford`s (1976) method, using BSA as a standard. For each variant, three different samples were analyzed.

Electrophoresis was carried out at 4°C in 10% polyacrylamide gel (respectively 8% for peroxidase and catalase), in standard 0.05M Tris-glycine buffer pH 8.3. Samples were loaded into wells. The electrophoresis was carried out at 10 mA, through the stacking gel for 30 min and 15 mA, through the separating gel for 2 hours. After electrophoresis, for locating SOD activities on gel 2.45 mM NBT, 28 mM TEMED and $2.8X10^{-5}$ M riboflavine in 36 mM phosphate buffer pH 7.8 was used, 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 and for detecting POX activities the gel was soaked in 0.5M acetate buffer pH 5 containing 0.08% benzidine and H_2O_2.

Results

An important condition of the medium-term preservation besides the reduction of the growth rate is the maintenance of the viability of the *in vitro* plant material and a satisfactory regeneration rate.

Generally, the regeneration pathway in *Dianthus* species is the direct morphogenesis, the multiple axillary shooting, respectively, which undergoes with high rates. In the case of *D. spiculifolius*, after 2 months, the number of regenerants can reach 83 regenerants/ inoculum, and in *D. glacialis* ssp. *gelidus*, the recorded mean number of regenerants was 43.3 (Holobiuc & Blîndu 2006), on the media supplemented both cytokinine and auxin. The regenerated plants grow with high rate, and the *ex vitro* acclimatization can be made without problems. Owing these aspects, the *in vitro* prolonged maintenance is difficult, because are necessary supplementary handling for transfers and, also, large culture vessels and space, the acquirement of minimal growth procedures being strongly imposed.

Compared to other methods of growth reduction tested before by us (the use of abscisic acid for the indirect osmotic stress induction through the reduction of intra-cellular level of water, the reduction of sucrose or/ and salts concentration from culture media and the low temperature), had not significant effects in the growth retardation (Holobiuc & Blîndu 2006). Only the mannitol addition at moderate concentrations in the culture media as osmolite conducted to positive results concerning the reduction of the growth and the induction and maintenance of the regeneration.

After one month of culture, on control medium variant without mannitol, explants belonging to both taxa showed a normal elongation of the shoots (Fig. 2c, 2d) with good rhizogenesis process.

The comparative analysis of the cultures maintained during one month in the presence of mannitol, showed that in the presence of mannitol as osmolite, a limitation of the growth of the shoots fragments in *D. spiculifolius* (Fig. 2 c, e, g, i) and *D. glacialis* ssp. *gelidus* cultures (Fig. 2 d, f, h, j) was determined. Despite to the growth reduction effect

on all mannitol added media variants, from the lateral meristems of the explants buds were induced (Fig. 1). Similar development did not occur on mannitol-free medium in the absence of growth factors. These propagules grown into 2 nodes shoots on 0.16 M mannitol added medium, but remained at single node on 0.32 M added variant and arrested as small buds on 0.49 M mannitol supplemented medium

In the case of 0.49 M mannitol level, the inhibition of the growth was stronger between 2 weeks and 1 month of culture (Fig. 2i, j). The leaves of the explants etiolated and underwent necrosis owing to osmotic stress and the induction of buds occurred slower. Although after 2 months also a good regeneration was registered, other buds were developed, probably owing to plant cells capacity to overcome the stress after the intra-cellular accumulation of mannitol.

D. glacialis ssp. *gelidus* was more susceptible in the phase of initiation of medium-term cultures at 0.49 M mannitol concentration comparing to *D. spiculifolius*.

The maintenance of the single node stem fragments on the media, supplemented with 0.16 M, 0.32 M and 0.49 M mannitol during 1 month, determined the regeneration with rates ranging between 3-7 lateral buds/inoculum in *D. spiculifolius* and 2-5 in *D. glacialis* ssp *gelidus*, in absence of any growth factor (Table 2). This aspect is important because the minimal growth procedure used by us is compatible with the explants survival and regeneration induction and maintenance.

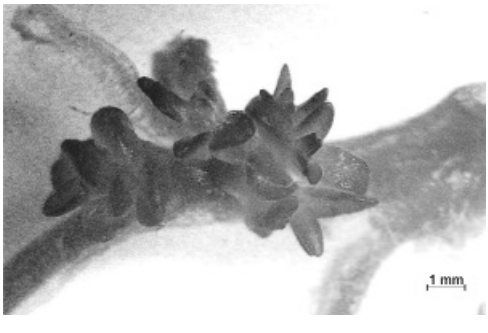

Figure 1 The induction of the development of buds from lateral meristems from single node stem fragments cultured on mannitol (0.32M) added medium in absence of growth factors.

After 2 months, the rate of regeneration increased in both species at 10-15 regenerants/ initial explant. Generally, the rooting process was good, the levels of 0.16 M and 0.32 M, mannitol even stimulated the formation of normal and "hairy roots".

Taking into account that the levels of 0.32 and 0.49 M of mannitol determined a reduction of the growth higher than at 0.16 M concentration, with the maintenance of viability and regeneration, these levels are recommended for medium-term maintenance of these plant material in a tissue collection.

It is already known that mannitol can induce moderate osmotic stress, reducing the osmotic potential in culture medium (Thompson et al. 1986), meantime being a compatible solute, which does not disturb proteins structure, protecting SH-enzymes from oxidative inactivation (Shen et al. 1997a).

Mannitol can be absorbed from culture media at the cell level. The intracellular accumulation contributed to the osmotic adjustment potential and improved tolerance to desiccation. To counteract reactive oxygen species (ROS) resulted from oxidative stress generated by desiccation, and for the restoration of the cells homeostasis, the plants have antioxidant equipments, which inactivated ROS by transformation in stable compounds, to avoid the cellular damages.

The antioxidant enzyme as superoxide dismutases, cytosolic catalases or peroxisomal glutathione peroxidases, can act together with other endogenous reducing agents, as ROS scavengers in the plant cell.

Taking into account the behavior of mannitol in supplemented media and its particular function as hydroxyl radicals scavenger (Smirnoff & Cumbes 1989) besides its osmolite function, a correlation between the concentration of mannitol in culture media and different antioxidant enzyme was studied.

After 2 weeks of culture on medium supplemented with mannitol, it was observed an increase of the CAT activity correlated with administration of mannitol and reactivity of species (Fig 3).

In *D. spiculifolius*, a high activity was determined on variant added with 0.32 M mannitol and in *D. glacialis* ssp. *gelidus* the increase

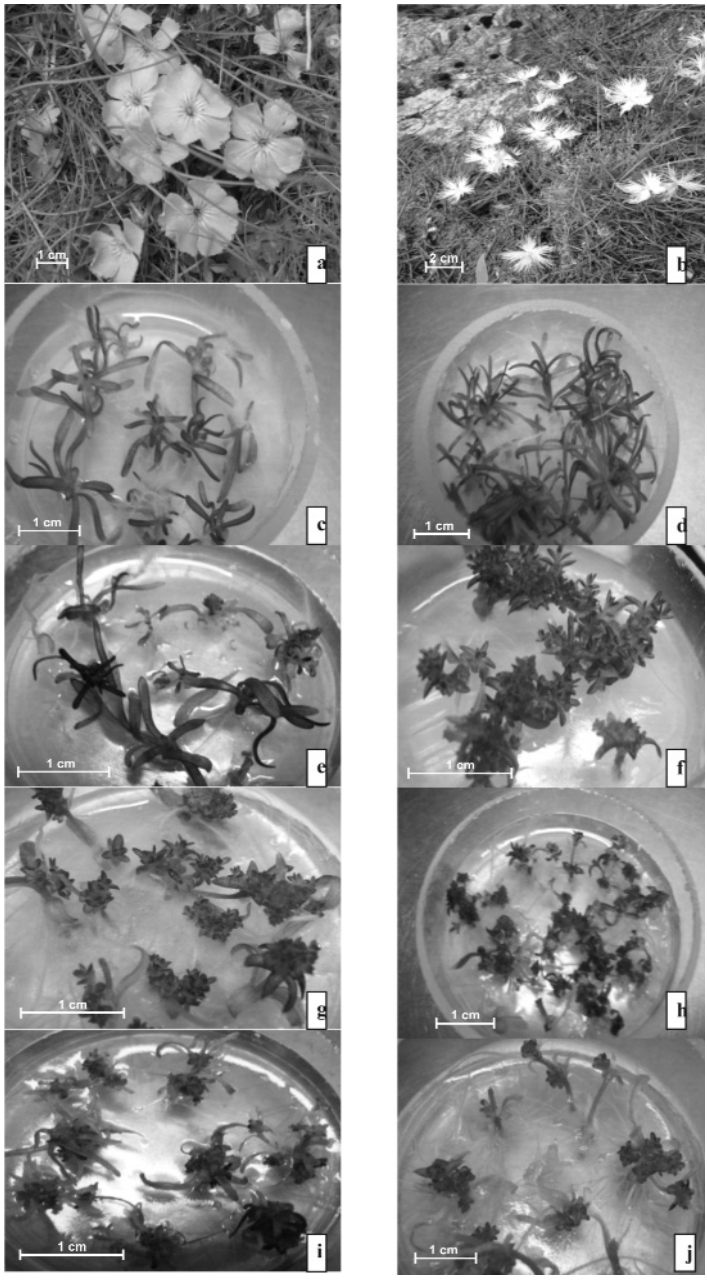

Figure 2 a - *In situ Dianthus spiculifolius* Schur, Valea cu Brazi, Sinaia; b - *In situ Dianthus glacialis* ssp. *gelidus*, Valea Babei; c - *D. spiculifolius* single node stem fragments cultured on control medium after 1 month; d. - *D. glacialis* ssp. *gelidus* on control medium after 1 month; e - *D. spiculifolius* - on 0.16M mannitol supplemented medium, after 1 month ; f - *D. glacialis* ssp. *gelidus* in presence of 0.16 M mannitol after 1 month; g. - *D. spiculifolius* on 0.32M mannitol supplemented medium, after 1 month; h - *D. glacialis* ssp. *gelidus* cultured in presence of 0.32 M mannitol after 1 month; i - *D. spiculifolius* -on 0.49 M mannitol supplemented medium, after 1 month; j - *D. glacialis* ssp.*gelidus* cultured in the presence of 0.49 M mannitol after 1 month.

Table 2 Behavior of medium - term tissue cultures in the two *Dianthus* taxa after 1 and 2 months in limitative growth conditions in the presence of mannitol

Variants	*D. spiculifolius*							
	1 month				2 months			
	growth (cm)	rooting	regeneration /inocullum	Obs.	growth (cm)	rooting	regeneration /inocullum	Obs.
Control	2- 3	+++	1-2	Lateral shoots	> 10	+++	-	Suitable for *ex vitro* transfer
M1 (+0.16 M mannitol)	>0.5	++	3-7	Induction of buds from axilar meristem	0.5-1	+++	10-20	Growth at 1-2 nodes, with short and hairy roots
M2 (+0.32 M mannitol)	0.5	++	3-5	Induction of buds from axilar meristem	0.5-1	+++	5-10	Growth at 1-2 nodes, with short and hairy roots
M3 (+0.49 M mannitol)	<0.5	++	1-2	Initial leaves etiolated; Induction of buds from axilar meristem	< 0.5	++	7-10	several small buds/ explant; growth stagnation

Variants	*D. glacialis* ssp. *gelidus*							
	1 month				2 months			
	growth (cm)	rooting	Regeneration / inocullum	Obs.	growth (cm)	rooting	regeneration / inocullum	Obs.
Control	1.5-2	+++	-	No lateral shoots	2-3	+++	-	Suitable for *ex vitro* transfer
M1 (+0.16 M mannitol)	0.5	+	1-3	Induction of buds from axilar meristem reduced roots	0.5	+	10-15	single node shoots formation
M2 (+0.32 M mannitol)	0.5	+++	2-5	Hairy roots	0.5	+	5-10	single node shoots formation
M3 (+0.49 M mannitol)	< 0.5	++	2-4	Leaves necrosis, slow induction of buds on lateral meristem,	<0.5	+	10-15	several small buds/ explant; short roots

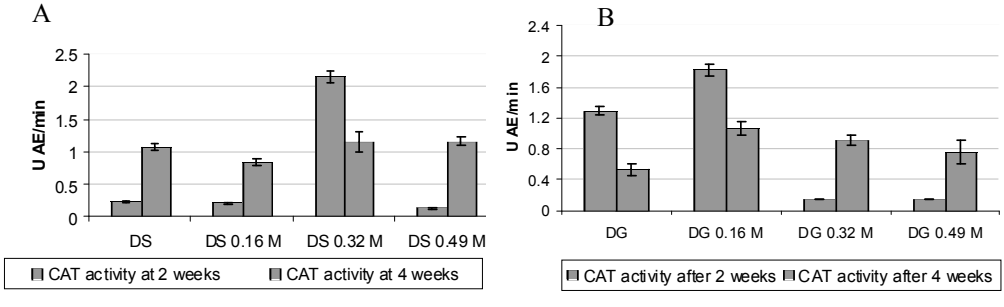

Figure 3 The CAT activity after 2 and 4 weeks of cultivation on medium supplemented with different concentration of mannitol (0.16 M, 0.32 M and 0.49 M) in *D. spiculifolius* (DS) and *D. glacialis* sp. *gelidus* (DG).

occurred at 0,16 M mannitol (Fig. 3 A and B), while at higher concentrations of mannitol the activity was diminished.

After 4 weeks of culture, in *D. spiculifolius*, the CAT activities detected in the presence of mannitol were closed to the control, but increased, compared to the levels registered at 2 weeks interval (Fig. 3A).

In *D. glacialis* ssp. *gelidus*, the CAT activity detected in the samples cultured on media supplemented with mannitol was higher, compared to the control, and increased than those determined after 2 weeks of culture in the same conditions (Fig. 3B).

The electrophoresis spectra after 2 weeks of culture showed the induction of an isoform of CAT in *D. spiculifolius* related to high reactivity and adaptation of this species of restrictive conditions, after 4 weeks this isoform disappeared, the spectra being similarly for the two species (Fig. 4).

In *Dianthus spiculifolius* cultures, SOD activity had significant increase on the media variant with high concentration of mannitol (0.49M), in early stage of adaptation to stress (Fig. 5 A). After 4 weeks, the SOD activity decreased, correlated with the increase of concentration mannitol from culture medium, but slight increased than control variant (Fig. 5 A). The SOD activity in *D. glacialis* ssp. *gelidus* compared to *D. spiculifolius* did not present a peak of activity for any treatment. In the first 2 weeks, the SOD activity decreased with the mannitol concentration and was higher than in control. After 4 weeks, the SOD activity increased with the mannitol level (Fig. 5 B).

The reactivity of the both species was different taking into account the SOD activity, although the patterns of SOD expression were similarly after 2 weeks of culture. A new SOD isoform appeared at 0.32 M mannitol after 4 weeks both in *D. spiculifolius* and *D. glacialis* ssp. *gelidus* cultures (Fig. 6), correlated with the induction of direct morhogenesis and adaptation at particular conditions.

Initially, in *D. glacialis* ssp. *gelidus*, the

Figure 4 The electrophoretic spectra of CAT after 2 weeks (A) and 4 weeks (B) of culture on media supplemented with mannitol in concentration of 0.16 M (1), 0.32 M (2), 0.49 M (3); DS - *D. spiculifolius*, DG - *D. glacialis* ssp. *gelidus*

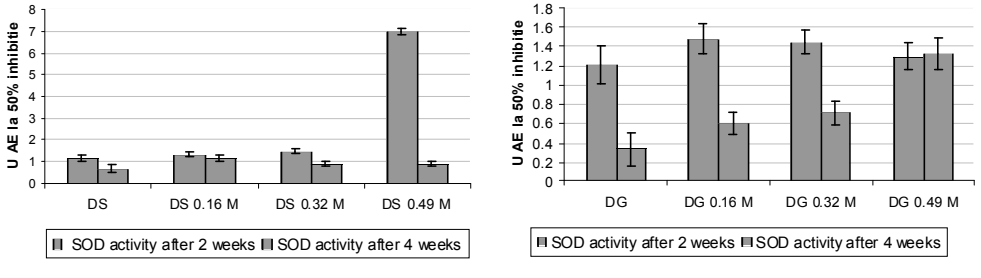

Figure 5 The SOD activity after 2 and 4 weeks of culture on medium supplemented with different concentration of mannitol (0.16M, 0.32M and 0.49M) in *D. spiculifolius* (DS) and *D. glacialis* ssp. *gelidus* (DG)

POX activity registered a slow decrease at 0.32 M mannitol, while at the higher concentration of mannitol, enzyme activity was increased (Fig. 7 B). After 4 weeks of culture, the POX activities were significantly higher than control. The same pattern of activities was detected in *D. spiculifolius,* but with a maximum of activity in 0.32 M mannitol added variant (Fig. 7 A). It is possible as in *D. spiculifolius,* POX activity to be induced till 0.32 M mannitol level. The electrophoretic spectra of POX did not show modifications of expression pattern (Fig. 8), but an increase of activity on media supplemented with mannitol was observed.

Figure 6 The electrophoresis spectra of SOD after 2 weeks (A) and 4 weeks (B) of culture on media supplemented with mannitol in concentration of 0.16 M (1), 0.32 M (2) and 0.49 M (3); DS - *D. spiculifolius*, DG - *D. glacialis* ssp. *gelidus*

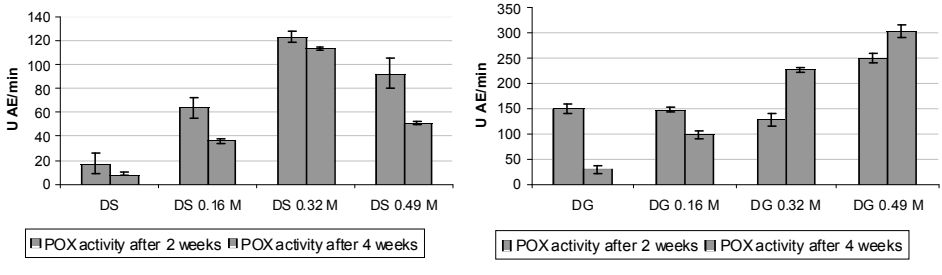

Figure 7 The POX activity after 2 and 4 weeks of culture on media supplemented with different concentration of mannitol (0.16M, 0.32M and 0.49M) in *D. spiculifolius* (DS) and *D. glacialis* ssp. *gelidus* (DG).

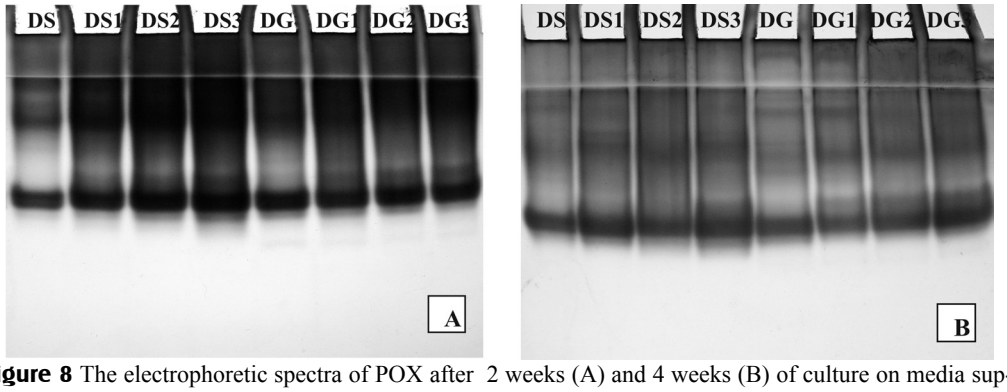

Figure 8 The electrophoretic spectra of POX after 2 weeks (A) and 4 weeks (B) of culture on media supplemented with mannitol in concentration of 0.16 M (1), 0.32 M (2), respectively 0.49 M (3); DS - *D. spiculifolius*, DG - *D. glacialis* ssp. *gelidus*

Discussion

Similar results considering the effect of mannitol on growth limitation, were reported in potato (Sarkar & Naik 1998), in medicinal plant leadwort (*Plumbago indica*)(Charoesub & Salak 2004), in *Ensete ventricosum* (Negash et al. 2001), in the rare taxon *Veronica multifida* ssp. *capsellicarpa* (Holobiuc et al. 2006, Holobiuc et al. 2008).

The addition of a sugar in the medium decreases the osmotic potential, reducing the uptake of the minerals and leading to growth retardation. The mannitol can also been absorbed in the plant cells and acts as osmolite (Thompson et al. 1986). In our case, mannitol presence in culture media induced a moderate osmotic stress which can activate the adaptation mechanisms that confer tolerance to more severe dehydration. Although, in some cases accumulation of high mannitol concentrations may have unfavorable consequences to plant development (Abebe et al. 2003), in our experiment work, besides of growth retardations, undesirable effects were not observed.

By the other hand, mannitol acts as a trigger in plant morphogenesis. Similarly, in other studies were reported that *in vitro* application of moderate stress factors levels showed an inductive or stimulant role on plant regeneration (Xu et al. 1990, Kong & Yeung 1995), especially in gymnosperms.

Supplementary, the mannitol can be used as carbon source and was described as hydroxyl radicals scavenger, protecting some enzymes from Calvin Cycle against oxidative inactivation (Shen et al. 1997a). In transgenic tobacco, increase of mannitol biosynthesis and catalase activity in chloroplasts determined an increase radical scavenging capacity and resistance to oxidative stress (Shen et al. 1997b).

In our study, the presence of mannitol in culture medium induced in the early stages of culture (the first two weeks) an osmotic stress (also generating an oxidative stress) which determined an adaptation of cell to dehydration conditions. The oxidative stress at cellular level conducted to the increase of the antioxidant enzymes activity. It was also reported that a high osmoticum level as polyethylene glycol or mannitol can change plants metabolism, the antioxidant balance of gluthatione in *Picea* embryos being changed (Belmonte et al. 2005).

The increase of CAT, SOD and POX activities depended on the concentration of mannitol, respectively on the osmotic potential in medium culture, correlated with a characteristic species tolerance.

D. glacialis ssp. *gelidus* was more susceptible to mannitol in the initiation phase of the culture (after 2-4 weeks), antioxidant enzymes activity increased starting from the lowest concentration tested, being maintained to high levels even after 4 weeks. On short-term, mannitol, probably acted as osmotic factor, determined the decrease of the extracellular water osmotic potential and a higher osmotic pressure and the increase of antioxidant enzyme activity.

D. spiculifolius was more tolerant to mannitol, having an increased antioxidant activity, starting from 0.32 M mannitol level at 2 weeks, while after 4 weeks the CAT activities were closed to the control.

D. spiculifolius and *D. glacialis* ssp. *gelidus* have different responses to the exposure to moderate osmotic stress, so that a reduction of antioxidant activity was registered above the threshold of sensitivity. For example, in case of CAT, the threshold of sensitivity was the level of 0.32 M in the case of *D. spiculifolius* and 0.16 M mannitol in *D. glacialis* ssp. *gelidus*. It is possible that accumulation of mannitol at intracellular level, above a certain limit, to affect ROS scavenger system, changes in the ROS conversion having as result the decrease in antioxidant enzyme activity. This idea was supported by the results obtained after 4 weeks of culture, when all antioxidant enzyme activities were diminished, but were higher than the control.

The changes of spectra and enzymes activity in restrictive growth conditions, in the phase of the initiation of the medium-term cultures, are rather connected to the adaptations to stress of the specific culture conditions, to the presence of mannitol and to species reactivity.

The established protocol, based on the culture of single node shoots on media, added with mannitol in appropriate concentrations, for each species can be used for *in vitro* medi-

um-term preservation of the two studied *Dianthus* taxa.

Conclusions

The medium-term conservation for an *in vitro* gene bank establishment can be efficiently made using a growth retardation protocol, based on mannitol as limiting factor.

After 1 month of culture, an *in vitro* development of buds at the level of the lateral meristems of the stem fragments was induced. In the case of the highest level of mannitol (0.49 M), the growth retardation effect was stronger, but also allowed the lateral meristems development into small buds.

The osmolite mannitol, at all concentrations tested, did not affect the viability of the whole explants, but in the phase of initiation of the medium-term cultures induced changes of the anti-oxidant enzyme activity. In the first 2 weeks of the initiation of in vitro medium-term cultures, mannitol acted as osmotic factor, determined the decrease of the extracellular water osmotic potential and a higher osmotic pressure, toghether with the increase of anti-oxidant enzyme activity.

After a prolonged exposure, the accumulation of mannitol at intracellular level above certain limits, probably influenced ROS scavenger system, decreasing the antioxidant enzymes activity, owing its protective role against ROS *in vitro* generated.

The method of growth retardation tested for the two *Dianthus* taxa is compatible with the survival of plant material in the condition of medium-term cultures, ensuring, besides the reduction of the growth of *in vitro* plant material, the induction of the regeneration of small propagules in absence of plant hormones. These advantages allow the maintenance of the *in vitro* collection in a reduced space, having many regenerants in every replicate, ready to use for different purposes (conservative, for exchanges of plant material or for basic and applied researches).

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