THE EFFECT OF MANNITOL ON ANTIOXIDATIVE ENZYMES *IN VITRO* LONG TERM CULTURES OF *DIANTHUS TENUIFOLIUS* AND *DIANTHUS SPICULIFOLIUS*

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The decline of the number of plants species worldwide strongly imposed, besides the habitats protection and management, the use of *ex situ* conservation methods based on biotechnologies. The *in vitro* gene banking as a main direction of *ex situ* conservation relies on tissues cultures. At present, there are many papers concerning the use of in *vitro* cell and tissues cultures for propagation, preservation and introduction of the rare plants species in the recovery programs. *In vitro* active gene bank is based on living plant tissues culture maintained during medium-term period. In our laboratory, studies concerning the *in vitro* preservation of several *Dianthus* rare species involved different methods that reduced the growth of *in vitro* cultures. In this respect, the addition of mannitol was proved the most efficient for regeneration and conservation of genetic resources. We obtained *in vitro* minimized cultures by subcultivation once at 3-4 months. The mannitol induced a stimulatory effect on the regeneration capacity. The present study reports on expression of some antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) in *long term* cultures on media supplemented with different concentrations of mannitol in two rare species *Dianthus spiculifolius* and *Dianthus tenuifolius*. The results showed changing of the activity expression of SOD and CAT and significant differences in response between these *Dianthus* species to mannitol treatments. The loss of some isoforms with SOD and CAT activity of the variants maintained on medium supplemented with mannitol (0.16 M and, respectively, 0.30 M) in the cultures of *Dianthus spiculifolius* was observed. There was no difference in the enzymes expression in the case of *in vitro* cultures of *Dianthus tenuifolius*. The mannitol can act as a scavenger of hydroxyl radicals and osmoregulator which can preserve the growth and the development of *in vitro* cultures.

Key words: antioxidant enzymes, conservation, mannitol.

INTRODUCTION

The decline of the number of plants species worldwide strongly imposed, besides the habitats protection and management, the use of *ex situ* conservation methods based on biotechnologies.

The *in vitro* gene banking as a main direction of *ex situ* conservation relies on tissues cultures. At present, there are many papers concerning the use of *in vitro*

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cell and tissues cultures for propagation, preservation and introduction of the rare plants species in the recovery programs. *In vitro* active gene bank is based on living plant tissues culture maintained during medium term period. In our laboratory, studies concerning the *in vitro* preservation of several *Dianthus* rare species were made.

In this respect, there were tested different methods of slowing-down the growth of the *in vitro* material in time, work and reagents saving and in the meantime to maintain healthy and appropriate plant material for regeneration and conservation of genetic resources in the rare *Dianthus* species.

Among different methods tested (the minimal growth conditions through reduction of the light, temperature, carbon source, minerals; adding of some retardants of the growth), the use of mannitol was the most beneficial. We were being able to maintain *in vitro* miniaturized cultures in the two species studied over three years with transfers once at 3-4 months. We also observed a stimulatory effect of mannitol on the regeneration capacity.

It is already described the role of mannitol as hydroxyl radicals scavenger (Smirnoff & Cumbes, 1989; Shen *et al.*, 1997), having also the role to protect against the oxidative inactivation of the enzymes of Calvin cycle (Shen *et al.*, 1997).

The aim of our work is to study the effect of the addition of mannitol in the *long term* culture media of *D. tenuifolius* and *D. spiculifolius* on the antioxidant enzymes.

MATERIALS AND METHODS

Plant material. The *in vitro* cultures were initiated from different types of explants (uninodal stems or floral fragments). The sterilization of plant material achieved by use of a pretreatment with 70% ethanol for 30 min was followed by immersion in 0.1% $HgCl_2$ for 5-7 min. For *in vitro* culture initiation and regeneration of viable plants, there were tested more variants of culture media having in composition macro- and microelements in accordance with Murashige & Skoog (1962) formula supplemented with Gamborg (B5) vitamins. For induction of proliferate processes, dedifferentiation and redifferentiation, were added different balances of growth factors. The best results in micromultiplication were obtained on medium supplemented with 1 mg/l benzyl aminopurine (BAP), 1 mg/l kinetin (kin), 0.2 mg/l naphthylacetic acid (NAA) and 1 mg/l gibberelic acid (GA). For cultures preservation there was maintained *long term* culture on MS medium without hormones, but supplemented with different concentrations of mannitol: 0.08 M (1), 0.16 M (2), 0.32 M (3) and 0.49 M (4). The cultivation was made in culture chamber at 25°C, with a photoperiod of 16/8 h and an illumination of 4000 lux.

Biochemical analyses. The enzyme extraction was performed by freezing and grinding of plantlets in 50 mM phosphate buffer pH 7, contained 2 mM Na_2EDTA, 5 mM β-mercaptoethanol, 4% (w/v) PVP at 4^0C for 24h. The extract was centrifuged at 18 000 rpm for 20 min and the supernatant was used for electrophoretic analysis. Electrophoresis was carried out at 4^oC in 10% polyacrylamide gel (respectively 8% for catalase) in standard Tris-glycine buffer pH 8.3. Samples were loaded into each well and then migrated at 10 mA through the stacking gel for 30 min and 20 mA through the separating gel for 90 min. After electrophoresis to locate SOD activities on gel there was used 2.45 mM NBT, 28 mM TEMED and 2.8×10^{-5} M riboflavin in 36mM phosphate buffer pH 7.8, for bands with CAT activities there were used 0.003% H_2O_2 in 0.01 M phosphate buffer, pH 7, and then a 2% potassium hexacyanoferrate and 2% iron (III) chloride solution and to emphasize POX activities the gel was soaked in acetate buffer pH 5 containing 0.08% benzidine and H_2O_2. The protein bands were visualised by Coomassie Brilliant Blue stain.

RESULTS AND DISCUSSION

The preservation on short and medium term proceeds from the tissue culture which can ensure the plant regeneration when it is necessary. In medium term preservation, *in vitro* culture conditions were combined with limitative growth factors for retardation of plant development. The maintaining of an osmotic stress in moderate range can provide a prolonged period of subcultivation. The advantage of this method is represented by the possibility to restore the normal condition of *in vitro* culture for plants propagation and further acclimatization. In case of the rare and endangered plant, the *in vitro* cultures were used with success for conservation of genetic resource and enhancement of an individual number in species with reproductive problems or limited populations.

Both of the studied species, *Dianthus tenuifolius* and *Dianthus spiculifolius*, had a high reactivity for *in vitro* culture. The previous researches (Cristea *et al.*, 2002) showed that the explants cultivation on different media with NAA and BAP in proportion of 1 to 10 induced a shoot regeneration rate of 20-30 shoots/explant.

In our experiments, the culture of the node explants on medium supplemented with two cytokinins (BAP and kin) associated with NAA and GA induced shoot regeneration with a higher rate (50-100 shoots/explant). The rhizogenesis was also present on every variant of culture media which was tested. The subcultivation of these shoots led to the increase of a regenerated number by direct caulogenesis on media without hormones.

As the rate of shoots growth registered for both species was high, it was necessary to maintain *in vitro* culture on media that induced retardation of development for medium term preservation.

The addition of mannitol in a concentration of 0.16 M and respectively 0.32M in culture medium led to an optimal preservation of cultures for about 6 months. The method proved useful for maintaining the proliferate tissues collection in a minimal space without labour for frequent subcultivations.

The mannitol added to medium cultures causes a mild osmotic stress to cell cultures and, in time, this treatment may activate an adaptive mechanism that can render the cells tolerant to more severe treatments. During the culture on medium supplemented mannitol, the cells take up mannitol that can act as an osmoprotectant. For example, exposure of tobacco LT cells to mild osmotic stress appears to be sufficient to induce tolerance to vitrification, but it is conceivable that uptake of mannitol by the tobacco cells improves survival rates (Reinhoud, 1996).

However, accumulation of mannitol may have adverse effects, even though mannitol is a compatible solute, the target plant may not tolerate high levels of mannitol. The appropriateness of the term compatible solute for osmolytes is questionable because marginal accumulation can induce pleiotropic effects (Hare *et al.*, 1998). For instance, plants that accumulated higher levels of mannitol had severe abnormalities including sterility, stunted growth, twisted heads and curled leaves (Abebe *et al.*, 2003). Exogenous application of Gly to nonaccumulating plants was found to osmotic-induced accumulation of Pro (Gibon *et al.*, 1997) and stress-induced accumulation of Pro also results in a reduced growth (Hare and Cress, 1998).

Our results demonstrate that the medium supplemented with 0.16 M allowed development of plantlet which can be ever transferred on proliferate medium. Also addition of mannitol to culture medium promotes the secondary somatic embryogenesis to lateral bud level. The higher concentration of mannitol established maintain the embryonic culture in early stages of development (Fig.1d and 2d).

Mannitol is a polyol normally synthesized in numerous species of vascular plants, which serves as an alternate metabolic reserve as well as an osmoprotectant. Mannitol accumulation increases when plants are exposed to a low water potential and the most obvious function of this compound is in osmotic adjustment. Therefore, mannitol has been proposed to enhance tolerance to water deficit stress primarily through osmotic adjustment (Loester *et al.*, 1992). However, further studies concluded that the amount of mannitol accumulated was inadequate to account for osmotic effects (Tarczynski *et al.*, 1993, Thomas *et al.*, 1995). The amount of mannitol accumulated and its effect on osmotic adjustment was less than that of other carbohydrates, suggesting that the beneficial effect of mannitol resulted from other protective mechanisms than osmotic adjustment (Abebe *et al.*, 2003).

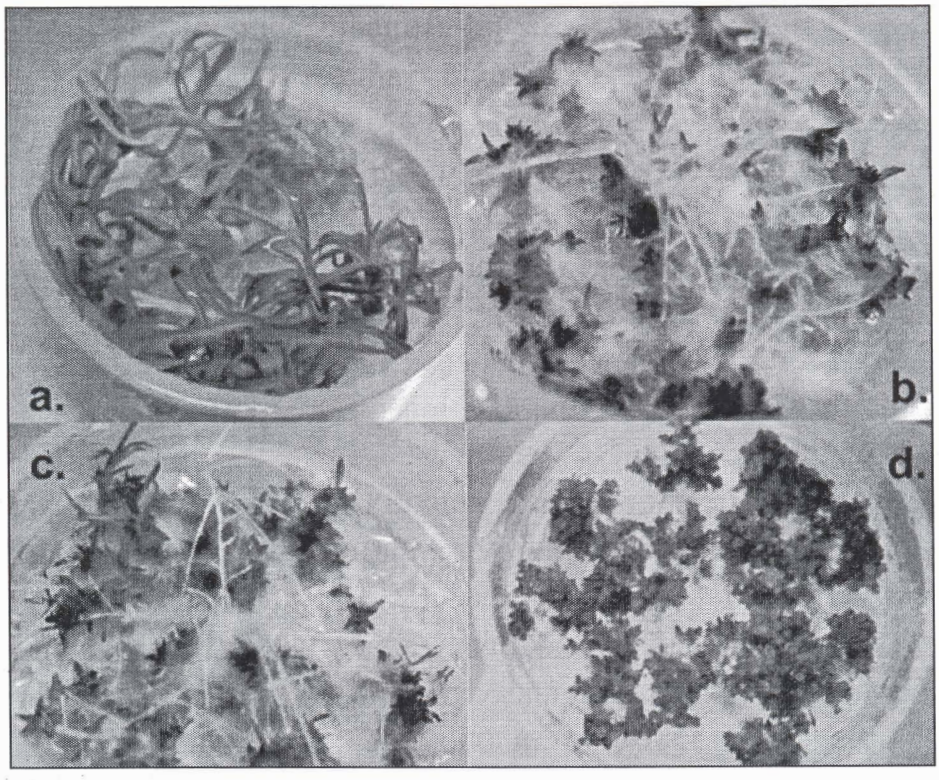

Fig. 1. Regenerative culture in *Dianthus spiculifolius* (a) and long term cultures on media supplemented with a different concentration of mannitol 0.16 M (b), 0.32 M (c) respectively 0.49 M (d) after 6 months of preservation.

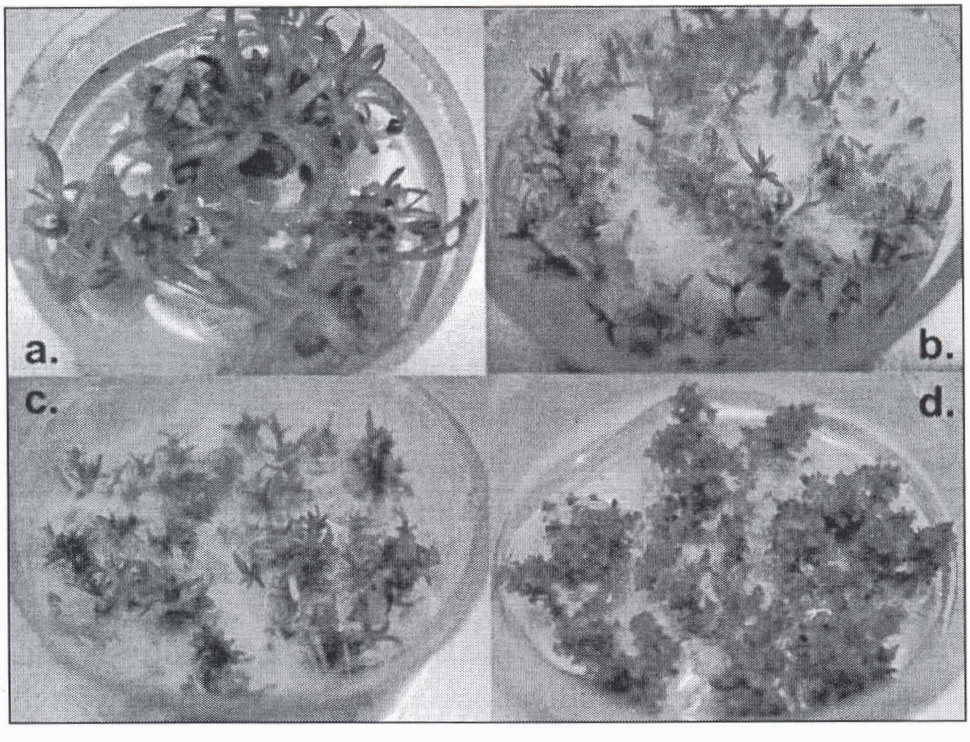

Fig. 2. Regenerative culture in *Dianthus tenuifolius* (a) and long term cultures on media supplemented with a different concentration of mannitol 0.16 M (b), 0.32 M (c) respectively 0.49 M (d) after 6 months of preservation.

Besides its function in osmotic adjustment, mannitol improves tolerance to stress through scavenging of hydroxyl radicals ($OH^{\bullet}$) and stabilization of macromolecular structures (Crowe *et al.*, 1992, Shen *et al.*, 1997). The importance of mannitol as a scavenger of the hydroxyl radical has been demonstrated *in vitro* (Smirnoff and Cumbes, 1989) and *in vivo* using transgenic tobacco (Shen *et al.*, 1997). The mechanism, by which mannitol decreases damages due to hydroxyl radicals, is still unknown.

In order to find a correlation between production of ROS, we studied the effect induced by the exogenous application of mannitol to antioxidant enzymes expression in long term cultures.

Oxidative stress is common in plants during water stress. Reactive oxygen species (ROS) including superoxide, peroxide and hydroxyl radicals, in general react aggressively with biological molecules and can cause lipid peroxidation, breakdown of macromolecules and damage to nucleic acids (Smirnoff, 1998). However, production of ROS is an unavoidable process in illuminated chloroplasts. Superoxide is mainly produced from photoreduction of oxygen *via* PS I. Most peroxide is then produced through disproportionation of superoxide by superoxide dismutases (SOD). Additionally, hydroxyl radicals are produced in Haber-Weiss or Fenton reactions through the interaction of H_2O_2 and superoxide or directly from H_2O_2 in the presence of transitions metals, such as Fe^{+2} and Cu^{+2} (Halliwell and Gutteridge, 1990). The catalases (CAT) can consume H_2O_2 preventing the amino acid oxidation or eliminated H_2O_2 before it can be converted to hydroxyl radical. In contrast to the detoxification system for H_2O_2 and superoxide, an enzyme system that could scavenge the short-lived hydroxyl radicals has not been identified. Because of their reactivity and short lifetime, protection from damage *in vivo* is then best achieved by mechanisms that prevent hydroxyl formation.

Our results showed changing of the activity expression of SOD and CAT and significant differences in response between these *Dianthus* species to mannitol treatments. The loss of some isoforms with SOD and CAT activity of the variants maintained on medium supplemented with low concentrations of mannitol (0.16 M and, respectively, 0.32 M) in the cultures of *Dianthus tenuifolius* was observed. There was no difference in the enzymes expression in the case of *in vitro* cultures of *Dianthus spiculifolius*.

The expression of SOD was modified when the mannitol was added in long term culture after successive subcultivation and at the same time with the increase of mannitol concentration. If the treatments with low concentrations of mannitol suppressed the activity of some SOD and CAT isoforms, the higher concentration of mannitol induced expression of new isoforms with SOD and POX activity.

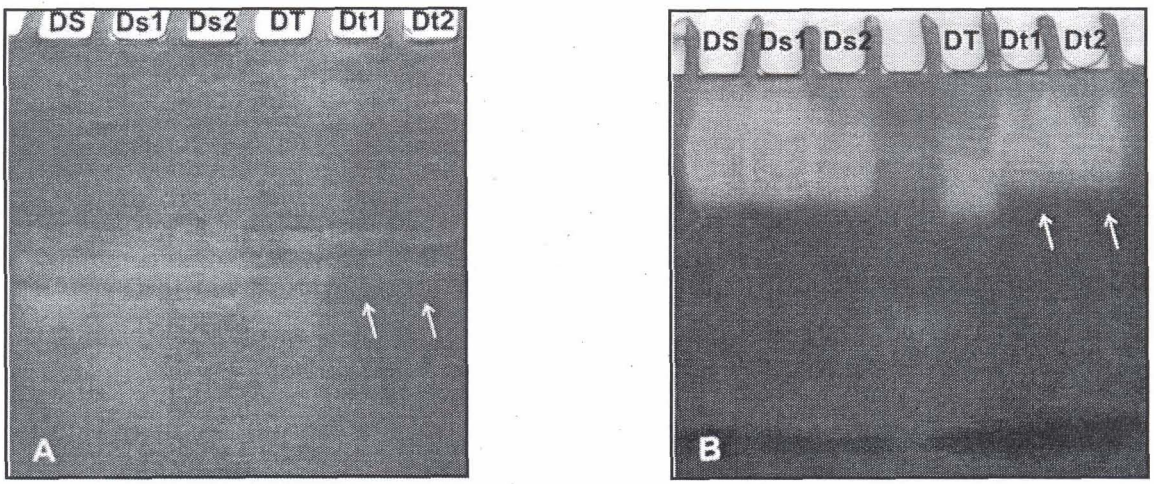

Fig. 3. The electrophoretic spectra of CAT (A) and SOD (B) in long term cultures of *Dianthus spiculifolius* (DS) and *Dianthus tenuifolius* (DT) on media supplemented with 0.08 M (1) and 0.16 M (2) of mannitol.

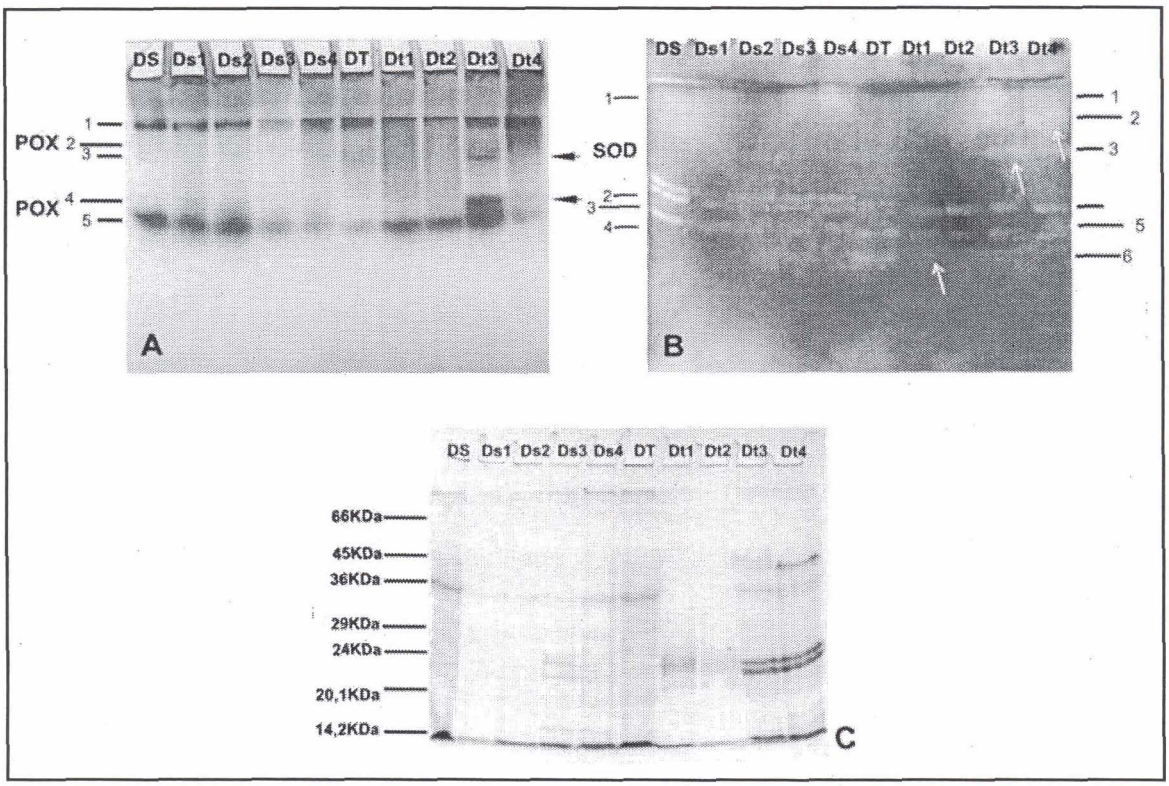

Fig. 4. The electrophoretic spectra of POX (A), SOD (B) and cytosolic proteins (C) in long term cultures of *Dianthus spiculifolius* (DS) and *Dianthus tenuifolius* (DT) on media supplemented with 0.08 M (1), 0.16M (2), 0.3 M (3) respectively 0.5 M (4) of mannitol.

Also, these findings can be related to increase of protein synthesis and appearance of new protein isoforms probably with functions in the acquisition of desiccation tolerance. For example, it seems that addition of mannitol to tobacco cells

rapidly induced gene expression of zinc finger transcription factor and a LEA 5 protein with role in the development of tolerance to vitrification (Reinhoud *et al.*, 1995).

CONCLUSIONS

In initial treatment with mannitol, this can act as a suppressor for SOD and CAT activity in *Dianthus tenuifolius*. It is possible that the mannitols be a potent ROS quencher as in the mechanism by which fungi evade ROS-mediated plant defenses (Jennings *et al.*, 1998). Also, the mannitol accumulation in the cells can affect the scavenger system of ROS, by capacity to react and readily eliminate the hydroxyl radicals changing the ROS conversion towards hydroxyl formation from superoxide and H_2O_2.

When the mannitol was applied in higher concentrations in *long term* culture, such a treatment may activate the adaptive mechanisms by which the expression of antioxidant enzymes was correlated with enhancement of water stress tolerance.

The response of antioxidant enzymes system of these two *Dianthus* species was distinct because of difference in drought resistance. The stress imposed by the exogenous mannitol applied in mentioned concentrations did not affect expression of these enzymes in *Dianthus spiculifolius*; probably this species reacts and tolerates higher concentrations of mannitol.

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