Arch. Biol. Sci., Belgrade, 62 (3), 553-558, 2010 DOI:10.2298/ABS1003553J

SUPEROXIDE DISMUTASE ACTIVITY AND ISOENZYME PROFILES IN BULBS OF SNAKE'S HEAD FRITILLARY IN RESPONSE TO COLD TREATMENT

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Abstract – The activities and isoenzyme profiles of superoxide dismutase (SOD) in *in vitro Fritillaria meleagris* bulbs in response to cold treatment (4 ^{0}C) were investigated. Differences in SOD activity and isoenzyme profiles in bulbs under standard growth conditions, six weeks chilling, as well as seven days after the completion of cold treatment are presented. SOD activity initially decreased but then rapidly increased seven days after cold treatment. Four isoforms of SOD are active under standard and chilling conditions, while three isoforms are presented 7 days after cold treatment. Native gel electrophoresis indicated the presence of mitochondrial and chloroplast localized SODs.

Keywords: *Fritillaria meleagris*, chilling, oxidative stress

UDC 577.151.64:57.08

INTRODUCTION

Fritillaria (*Liliaceae*) includes 100 species of bulbous plants found throughout the temperate parts of the Northern Hemisphere. *Fritillaria* sp. are often used as garden plants, and the bulbs of various species have been used in traditional Chinese medicine for more than 2000 years (Li et al., 1999, 2000, 2001). *Fritillaria meleagris* (snake's head fritillary, chequered daffodil, leper lily) is native to Europe, but in many places it is endangered and rarely found in the wild (Ilijanić et al., 1998). The species is protected by law in Serbia. Snake's head fritillary is a great survivor well adapted to cold conditions as it completes all its growth, flower and seed production in the early spring before the onset of summer. It is mainly propagated by bulbs and hardiness to the cold varies greatly with the bulb origin, depending on temperature and its duration. Bulbs of snake's head fritillary belong to "Hardy II type" which means that they can survive temperatures of –10 ^{0}C in nature.

In vitro propagation techniques are an important aid for the rapid multiplication of all *Fritillaria* species (Sun and Wang, 1991). Plant regeneration of snake's head fritillary by micropropagation has been reported from shoot cultures (Kukulczanka et al., 1989) as well as by somatic embryogenesis (Subotić at al., 2004).

Tissue cultures of fritillaries require low temperature for successful growth. Calli and bulblets are normally induced at standard growth room temperature but require a cold treatment of 2-15^{o}C in order to develop further into *in vitro* formed bulblets (Peak, 1996).

There is increasing evidence that environmental stress, such as chilling or freezing temperatures, can lead to dysfunction and an increased production of activated oxygen species (AOS) such as superoxide, hydrogen peroxide and hydroxyl radicals (McKersie and Lesham, 1994; Kuk et al., 2003). Cold acclimation increases tolerance to AOS in cereals and correlates with an increase in antioxidant enzymes (Anderson et al., 1995; Scebba at al., 1998, 1999). Antioxidant enzymes play a key role in the elimination of reactive oxygen. A first line of defense against oxyradical-mediated injuries are superoxide dismu-

tases (SOD), metal containing enzymes that catalase the dismutation of superoxide radicals to oxygen and hydrogen peroxide (Bowler et al., 1992). In plants, three basic forms of the enzyme exists, as classified by their active site and metal ion: mitochondrial manganase (MnSOD), cytosolic and chloroplasts copper/zinc (Cu/ZnSOD), and chloroplastic iron forms (FeSOD). These isoenzymes differ in their sensitivity to H_2O_2 and KCN and can be easily distinguished by gel electrophoresis. The number of isoenzymes of each basic type of SOD varies greatly from plant to plant, as does the relative abundance of each enzyme. Chloroplastic SOD is often the most abundant SOD present in green leaves, whereas in germinating seedlings and in etiolated material the cytoplasmic and mitochondrial SODs are prevalent (Bowler et al., 1994).

SOD in the plasma membrane of pea and maize roots, would implicate their specific role in the antioxidative protection of membrane constituents, as well as in the redox communication between apoplast and symplast, which is part of the signaling processes (Kukavica at al., 2007).

The objectives of this research were to measure the activity and compare the isoenzyme profiles of SOD in *F. meleagris* bulbs, formed in culture *in vitro*, in response to 6 weeks of cold treatment.

MATERIALS AND METHODS

Tissue culture

In vitro cultures of *F. meleagris* were established as earlier reported by Subotić et al. (2004) and Nikolić at al. (2006). Bulbs were formed indirectly from calli derived on MS medium (Murashige and Skoog, 1962) containing 3% sucrose, 0.7% agar, casein hydrolysate 250 mgL^{-1}, L-proline 250 mgL^{-1}, adenine sulfate 80 mgL^{-1}, thidiazuron (TDZ, 1.0 mgL^{-1}). Isolated bulbs were cultured on MS hormone free medium at 25°C with 16 h light / 8 h night, intensity of 40 μmol $m^{-2}s^{-1}$. Similar light regimes were used for the six week 4°C cold treatment. Each treatment consisted of 80 to 100 bulbs with three replications. All media were adjusted to pH 5.8 with 1N NaOH and autoclaved at 121°C for 25 min.

Enzyme extraction

Frozen (-70°C) bulbs (0.5 g) were homogenized in 4 ml of 0.1 M potassium phosphate extraction buffer (pH 6.8, containing 200 mg insoluble PVP and PMSF). The homogenate was centrifuged for 5 min at 10000 ×g at 4°C. The supernatant was separated and stored at -70°C.

SOD activity

SOD activity was determined according to a modified method of Beyer and Fridovich (1987). The supernatant was added to a reaction mixture containing potassium phosphate buffer pH 7.8, 0.1 mM EDTA, 12 mM L-methionine, 75 μM nitroblue tetrazolium (NBT) and 2 μM riboflavin to a total volume of 3 ml. The reaction mixture was kept under a fluorescent light for 15 min at 24°C. One SOD unit was described as the amount of enzyme needed to reduce the NBT ratio to 50%. NBT reduction ratios were measured with a spectrophotometer at 550 nm.

SOD electrophoresis

Proteins were separated by native polyacrylamide gel electrophoresis (PAGE) using 5% stacking and 10% running gels with a buffer consisting of 0.025M Tris and 0.192 M glycine (pH 8.3) at 100 V for 3.5 h. The total amount of protein applied per lane was 20 μg. After electrophoresis, the gels were incubated with 1 mM KCN and 30% H_2O_2 followed by incubation with a reaction mixture (0.1 M EDTA, 0.098 mM NBT, 0.030 mM riboflavin and 2 mM N,N,N,N-tetramethylethylendiamine in K-phosphate buffer, pH 7.8) for 30 min in the dark. The gels were washed in distilled water and visualized with regular light.

RESULTS AND DISSCUSION

The procedure for *in vitro* propagation of snake's head fritillary used in this study is a simple and

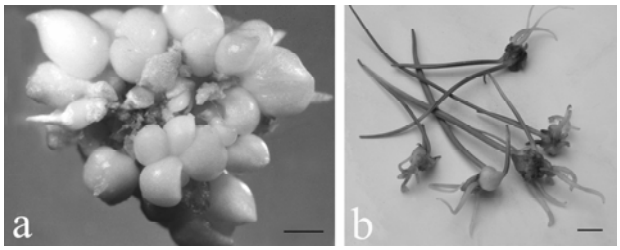

Fig. 1. Effect of cold treatment on bulbs of snake's head fritillary. **a** Bulbs formed on MS medium with TDZ (1mgL^{-1}), **b** Bulblets one month after cold treatment at +4ºC before planting in greenhouse; the bar is 10 mm.

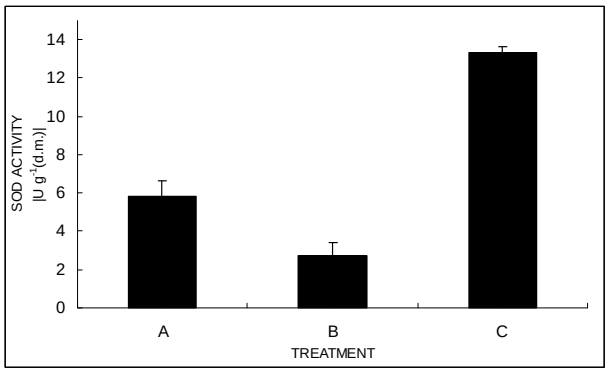

Fig. 2. SOD activity of snake's head fritillary bulbs after exposure to lower temperature. SOD in bulbs grown at 25ºC (A), 4C (B) and seven days after 4ºC treatment (C). SOD activity unit (U) as described in Materials and Methods.

practical method for the rapid mass production of bulblets. Numerous white bulbs are constantly produced for more than four years (Fig. 1a). Dormancy occurs during the annual life cycle of the bulb and it is essential for normal development. Bulbs of *F. meleagris* formed *in vitro* also require a dormant period. Cold treatment (4ºC) has a positive effect on breaking dormancy, resulting in the increased rooting and sprouting of *in vitro* bulbs. Rooting after six weeks of chilling is two times higher (-60 %) than in control bulbs grown under standard conditions. Shoot and root length is also greater in the chilled bulbs. Accumulation of soluble sugars, mainly monosaccharides as well as polyols, occurs in bulbs and have been found to increase in response to cold treatment (Nikolić et al., 2008). After six months treatment of bulbs at low temperature, with one additional month under standard conditions, whole bulblets are formed and ready for planting (Fig. 1b).

SOD activity in *F. meleagris* bulbs before, during and after cold treatment is presented in Table 1. In standard growth conditions (25ºC) the SOD activity is 5.83 units and decreases two-fold during the cold treatment. High SOD activity has been shown to be associated with stress tolerance in plants due to the overproduction of the reactive O^{2-} which is produced under stress (Bowler et al., 1992). In maize there are many reports suggesting that scavenging enzyme activity has an essential role in chilling tolerance (Seppänen and Fagerstedt, 2000). The SOD system in bulbs of snake's head fritillary is very efficient and SOD activity increases more than three-fold seven days after cold treatment, compared to standard growth conditions (Fig. 2). Plant species that exhibit lower inactivation of antioxidant enzymes during chilling as well as rapid restoration of enzymatic activities after exposure to lower temperatures are more cold-resistant to oxidative stress (Bowler et al., 1992).

Investigation of the snake's head SODs isoenzyme involved and active during this process of growth initiation indicated that four isoforms are present in bulbs of snake's head fritillary cultured at 25ºC and 4ºC (Fig. 3 A, B). Two of the isoforms are MnSOD while two lower weight forms are FeSODs. Seven days after cold treatment the two FeSODs are still active but only one mitochondrial MnSOD isoform retains activity (Fig. 3 C). Oxygen radicals are by-products of many biological oxidation reactions occurring in different subcellular locations (Bowler et al., 1994). In rice, the induction of SOD activity in response to chilling is localized in the roots where MnSOD is active and FeSOD or Cu/ZnSOD are not observed in any treatment (Kuk et al., 2003). McKersie et al., (1993) have shown that transgenic alfalfa overexpressing MnSOD had better recovery after freezing stress and better winter survival in field trails. It is suggested that overexpression of MnSOD minimizes the accumulation of oxygen free radicals after freezing stress and increased mitochondrial respiration and sugar metabolism has been observed to enhance MnSOD

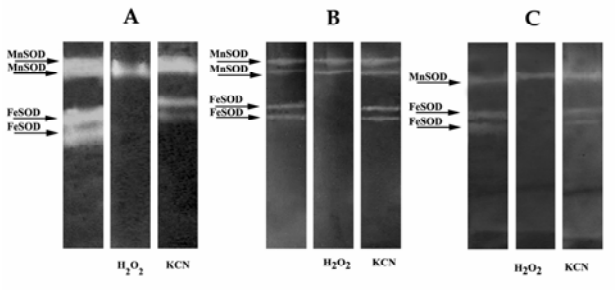

Fig. 3. Native PAGE, stained for SOD isoenzyme activities in bulbs of snake's head fritillary grown under standard growth conditions (A), after six weeks and (B) seven days of cold exposure (C). Arrows indicate SOD isoenzymes that were identified by active staining of gels after pre-incubation with KCN or H_2O_2 (5 mM each).

expression. On the other side, there is no correlation between SOD activity and freezing tolerance in potato hybrids, but the relationship between antioxidative capacity and low temperature tolerance is not always clear in other species either (Lukatkin, 2002). The observed variations in enzyme abundance in snake's head fritillary appear to be a consequence of strict regulation at the level of gene expression by environmental, but also physiological, constraints.

The distribution of SOD isoforms presumably reflects changes occurring in the subcellular sites of oxyradical formation, such as during the greening process, when photosynthetic reactions become more dominant in cell metabolism, necessitating the need for chloroplastic FeSOD. In etiolated seedlings of chickpea, the cytoplasmic and mitochondrial SODs are prevalent suggesting that metabolism at this age is mainly respiratory. As epicotyls enlarge Cu/ZnSOD appears not to be required and the sites of oxyradical formation changes as metabolism becomes photosynthetic (Hernández-Nistal et al., 2002). The constantly active forms of chloroplast FeSOD compared with MnSOD in snake's head fritillary bulbs can also be explained as a consequence of breaking dormancy and increasing photosynthesis. It is well known that photosynthesis at low temperatures may cause the production of reactive oxygen species and hence chloroplast SOD activity is required (Bowler at al., 1992).

The SOD enzyme system seems to be strictly regulated in every plant species at the gene expression level which is affected by both environmental and developmental stimuli (Bowler et al., 1994). Overproduction of the reactive O^{2-} which is produced after cold treatment of bulbs of snake's head fritillary can be explained as a consequence of both low temperature treatment and developmental changes connected with breaking the dormancy of bulbs and sprouting of shoots.

Relative changes of the particular enzyme activities should not be correlated with complete cold-resistance of the plant since cold-resistance is not determined by the activity of a single enzyme. The results presented in this work are a good start for further investigation of the oxidative system of species that are well-adapted to cold.

Acknowledgment - This research was sponsored by Ministry of Science and Technological Development of Serbia, project N° 143026B.

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АКТИВНОСТ И ИЗОЕНЗИМСКИ ПРОФИЛ СУПЕОКСИД ДИЗМУТАЗЕ У ЛУКОВИЦАМА КОЦКАВИЦЕ КАО ОДГОВОР НА ТРЕТМАН ХЛАЂЕЊЕМ

СЛАЂАНА ЈЕВРЕМОВИЋ, МАРИЈА ПЕТРИЋ, СУЗАНА ЖИВКОВИЋ, МИЛАНА ТРИФУНОВИЋ, АНГЕЛИНА СУБОТИЋ

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Проучавана је активност и изоензимски профил супероксид дизмутазе (СОД) у луковицама *Fritillaria meleagris* гајеним у условима *in vitro* као одговор на третман хлађења. Приказане су разлике у активности СОД као и изоензимским профилима код луковица на стандардним условима гајења, после шест недеља хлађења и седам дана после завршетка третмана хлађења. Активност СОД се смањује током хлађења док се значајно повећава седам дана после хлађења. Четири СОД изоформе су активне током гајења у стандардним условима и током хлађења док су три изоформе детектоване седам дана после хладног третмана. Нативна гел електрофореза је показала присуство активности митохондријалне и хлоропластне СОД.