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INFLUENCE OF EXOGENOUS CARBOHYDRATES ON SUPEROXIDE DISMUTASE ACTIVITY IN *TRIFOLIUM REPENS* L. EXPLANTS CULTURED IN VITRO

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The effect of four sugars (sucrose, glucose, fructose, maltose) used as carbon source in liquid medium on superoxide dismutase (SOD) activity was studied. Little is known about how different soluble sugars used in plant tissue culture can affect SOD, an antioxidant enzyme involved in regulating the level of reactive oxygen species (ROS) in plant cells. In extracts of 15-day-old cotyledons and hypocotyls of *Trifolium repens*, four SOD forms were detected by native PAGE and activity staining: MnSOD, FeSOD, Cu/ZnSOD I and Cu/ZnSOD II. Activity of FeSOD and Cu/ZnSOD II was significantly lower in cotyledons cultured on media supplied with different carbohydrates than in control cotyledons cultured on medium without sugars, or was absent altogether. FeSOD was absent in hypocotyls obtained from seedlings grown on sterile filter paper (SFP); those cultured on sucrose-supplemented medium showed weak FeSOD activity. FeSOD appeared to be the most labile SOD form in *T. repens* cotyledons. MnSOD activity was highest in cotyledons cultured on sucrose-, fructose- and maltose-based media. Cu/ZnSOD I activity was highest in cotyledons cultured on medium with glucose and fructose. Sucrose strongly inhibited both FeSOD and Cu/ZnSOD II activity. The results indicated that various carbohydrates affect the activity of SOD forms in *T. repens* cotyledons. The decrease in activity of FeSOD and Cu/ZnSOD II, putatively located in chloroplasts, might suggest an inhibitory effect of some carbohydrates on photosynthesis in cotyledons of *T. repens*. On the other hand, supply of sucrose, fructose and maltose increased the activity of the putative mitochondrial form of MnSOD. This result might indicate sugar-induced production of ROS during respiration in mitochondria.

Key words: *Trifolium repens*, plant tissue culture, SOD pattern.

INTRODUCTION

Plants are very often exposed to unfavorable environmental conditions. The physiological and biochemical changes appearing in response at all levels of the plant organization are generally called stress reactions (Lichtenthaler, 1996). In cultures in vitro as well, plants are exposed to conditions deviating from those of the natural environment (Gaspar et al., 2002). One of the most important differences lies in the composition of nutritive media. Carbohydrates are essential biomolecules needed for the growth and development of plants in vitro (Gamborg et al., 1976; Bogunia and Przywara, 2000; Ślesak and Przywara, 2003). The role of soluble sugars, not only as sources of energy and carbon skeletons but also as signalling molecules during development and gene expression, has been intensively studied (e.g., Rolland et al., 2002; Gibson, 2003).

Stress reactions are very often associated with increased production of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$), which lead to oxidative stress in plant cells. The term 'oxidative stress' refers to a serious imbalance between the production and removal of ROS (Halliwell, 1997). ROS are generated during the life

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Abbreviations: DTT – dithiothreitol; EDTA – ethylenediamine tetraacetic acid; EGTA – ethylene glycol-bis (β-aminoethyl ether) N,N,N',N-tetraacetic acid; NBT – nitroblue tetrazolium salt; PAGE – polyacrylamide gel electrophoresis; ROS – reactive oxygen species; SFP – sterile filter paper; TEMED – N,N,N',N-tetramethylethylenediamine; Tricine – N-[tris-(hydroxymethyl)methyl]glycine; Tris – tris(hydroxymethyl) aminomethane.

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of a plant as a by-product of aerobic metabolism (for review see: Bartosz, 1997; Mittler, 2002; Vranová et al., 2002). In plant cells, chloroplasts, mitochondria and peroxisomes are important endogenous generators of ROS (Dat et al., 2000; Foyer and Noctor, 2000; Vranová et al., 2002). ROS levels can increase due to various environmental factors such as light, drought, temperature stress, salinity, heavy metals, pathogens and air pollutants (Bartosz, 1997; Mittler, 2002). An excess of ROS causing oxidative stress is harmful to cells, as these molecules can damage nucleic acids, proteins and lipids. Plants have evolved several defense systems to avoid oxidative stress. This system consists of low-molecular compounds and antioxidant enzymes (Dat et al., 2000). The most stable ROS, hydrogen peroxide, is involved in different morphogenic and embryogenic processes in plant tissue culture (Cui et al., 1999; Papadakis et al., 2001; Libik et al., 2005).

In plant tissue culture, the method of explant preparation, the medium composition, limited gas exchange and high relative humidity may induce oxidative stress in plant cells (Benson et al., 1997; Cassells and Curry, 2001). Changes in the activity of several antioxidant enzymes in plant tissues cultured in vitro have been also described (for review see: Benson et al., 1997; Gaspar et al., 2002). The primary scavenger of ROS is the enzyme superoxide dismutase (SOD; EC 1.15.1.1), which converts superoxide to hydrogen peroxide and oxygen. Based on the metals present at the active site, three classes of SOD have been identified in plants: copper/zinc SOD (Cu/ZnSOD), iron SOD (FeSOD) and manganese SOD (MnSOD) (Alscher et al., 2002).

There are few data on the influence of the carbohydrate composition of the culture medium on SOD activity in explants. Recently, the relationships between soluble sugars and ROS production or between soluble sugars and ROS responses have been studied intensively (for review see: Couée et al., 2006). Little is known about how different sugars commonly used in plant tissue culture can affect SOD activity. Changes in SOD activity can be an indicator of the level of ROS production and detoxification during in vitro culture. The objective of this study was to test our hypothesis that sucrose, glucose, fructose and maltose can influence the activity of SOD forms identified in cotyledons and hypocotyls of *Trifolium repens* cultured in vitro.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of white clover (*Trifolium repens* L.) were surface-sterilized by soaking in 70% (v/v) ethanol for 30 sec and then in commercial bleach diluted 1:1 with distilled water for 15 min, followed by three rinses with sterile distilled water. Surface-sterilized seeds were germinated in Petri dishes on a double layer of moistened sterile filter paper and incubated at 25 ± 3°C under a 16 h photoperiod (cool-white fluorescent tubes, 70–100 μmol photons m^{-2} s^{-1}). Explants of cotyledons (with petioles) and hypocotyls (both ~5 mm long) obtained from 7-day-old seedlings were cultured on liquid MS (Murashige and Skoog, 1962) medium supplied with either sucrose, maltose, glucose or fructose. The molar concentrations of all carbohydrates added were 87 mM (equivalent to 3% solution of sucrose). The control MS medium, not supplemented with sugars, contained only mannitol (87 mM). All media used had almost the same osmolarity (data not shown).

Explants were placed in 150 ml Erlenmeyer flasks containing 25 ml medium, and cultured using a laboratory shaker (KS 250 basic IKA LABORTECHNIK) at 100 rpm. Twenty explants were placed in each flask; there were 6 independent replicates of each treatment variant. For identification of SOD forms, some seedlings were cultured in Petri dishes on moistened sterile filter paper (SFP explants). After 15 days of culture, SFP explants, explants cultured on MS without carbohydrates (control), and explants cultured on MS supplemented with different sugars were sampled for SOD assays.

PREPARATION OF SOLUBLE PROTEINS (CRUDE EXTRACT) FROM EXPLANTS

Explants (0.2–1 g f.w.; 60 from 3 replicates: 3 × 20 explants) were mixed in 1:1 or 1:2 ratios (g fresh weight : ml homogenization buffer) and then homogenized in a cooled mortar. Homogenization buffer contained 100 mM Tricine adjusted with 1 M Tris to pH 8.0, 3 mM $MgSO_4$, 1 mM DTT and 3 mM EGTA. Insoluble material was removed by centrifugation (~2 min at 12000 g). Protein fractions were stored at –40°C until further use.

NATIVE PAGE, STAINING OF SOD FORMS AND ASSESSMENT OF SOD ACTIVITY

Native polyacrylamide gel electrophoresis (PAGE) was performed as described previously by Miszalski et al. (1998). Protein extract was applied to each lane. SOD activity in the gels was visualized by activity staining according to Beauchamp and Fridovich (1971). After 20–25 min of dark incubation at room temperature in standard staining buffer (50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA, 2.8 mM TEMED, 22 μM riboflavin and 245 μM NBT), gels were exposed to

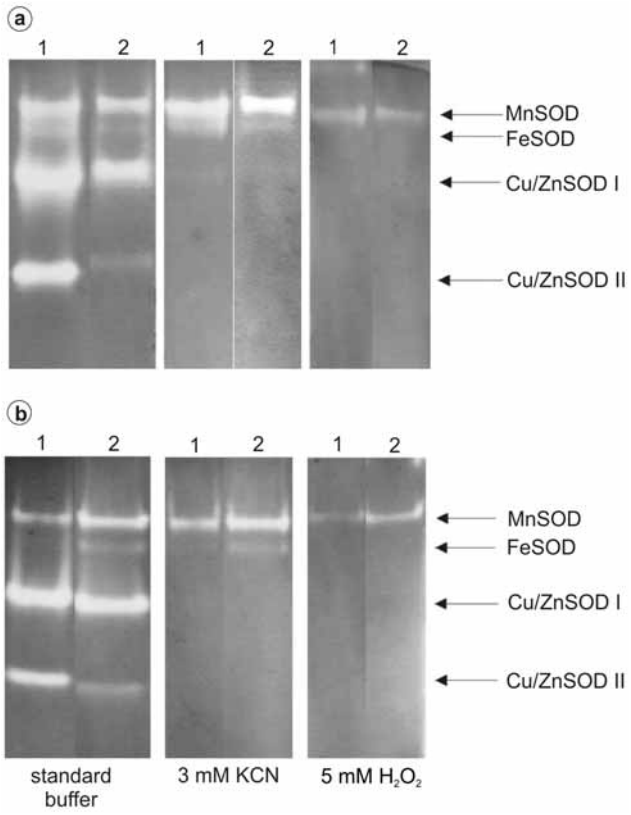

Fig. 1. Native polyacrylamide gel electrophoresis (PAGE) of different SOD forms in *T. repens* cotyledons (**a**) and hypocotyls (**b**). Inhibitors were KCN and H_2O_2: 40 μg protein was loaded on the gels. Lane 1 – SFP cotyledons and SFP hypocotyls; Lane 2 – cotyledons and hypocotyls cultured on MS supplied with 3% sucrose.

artificial white light until the SOD activity bands became visible. To inhibit Cu/ZnSOD and FeSOD activity, H_2O_2 was added to a final concentration of 5 mM to the staining buffer. To inhibit Cu/ZnSOD activity, gels were incubated in buffer containing 3 mM KCN. Gels were scanned using the Bio-Print® system (Vilber-Lourmat, France). The activity of different forms was expressed in arbitrary units per 30 μg protein.

PROTEIN DETERMINATION

Protein concentrations were determined according to Bradford (1976) using the BioRad protein assay (U.S.A.) following the manufacturer's instructions. Bovine serum albumin (BSA) SIGMA-ALDRICH was used as a standard.

STATISTICAL ANALYSIS

One-way ANOVA and Bonferroni's multiple comparison post-test were used for statistical analyses.

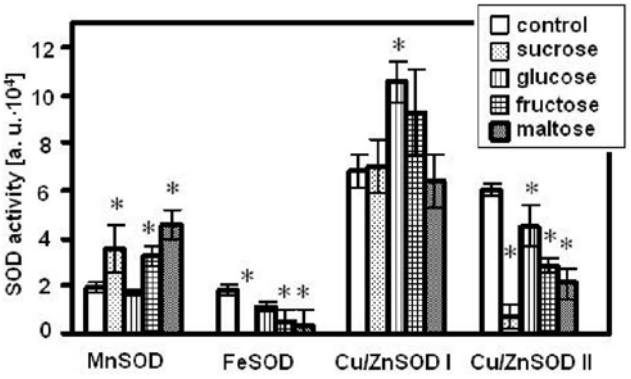

Fig. 2. Activity of SOD forms in *T. repens* cotyledons (a.u., arbitrary units) after 15 days of culture on control media and media supplemented with different carbohydrates; 30 μg protein was loaded on gels; values are means ± SD (n=4). Asterisks above bars indicate significant differences from the control at $p < 0.05$.

RESULTS

SOD IDENTIFICATION

To identify SOD forms, extracts from *Trifolium repens*, explants cultured on sterile filter paper (SFP) and extracts from explants cultured on MS medium supplied with 3% sucrose were analyzed. Two SOD forms, MnSOD and FeSOD, were detected by native PAGE; two Cu/ZnSODs were detected by activity staining, denoted Cu/ZnSOD I and Cu/ZnSOD II (Fig. 1a,b). There were no differences in the occurrence of these forms between SFP cotyledons and cotyledons cultured on MS supplied with sucrose (Fig. 1a). In the presence of sucrose, the activity of FeSOD and Cu/ZnSOD II was distinctly lower than in SFP cotyledons (Fig. 1a). The four mentioned forms were also identified in the control cotyledons (Fig. 2).

In hypocotyls cultured on MS supplemented with sucrose, four SOD forms were detected, whereas in SFP hypocotyls only three SOD forms were identified: MnSOD, Cu/ZnSOD I and Cu/ZnSOD II (Fig. 1b). In SFP hypocotyls, FeSOD was absent but Cu/ZnSOD II activity was much higher than in hypocotyls cultured with sucrose (Fig. 1b). Three forms of SOD were also observed in control hypocotyls, as in the SFP hypocotyls (data not shown).

SOD ACTIVITY

There were evident differences among SOD patterns in the control and plant material cultured in the presence of different carbohydrates. The level of SOD activity was dependent on the medium composition and explant type.

For hypocotyls cultured on media supplied with different carbohydrates, the activity of SOD forms was almost similar for all tested culture media. Only Cu/ZnSOD I and Cu/ZnSOD II activity was lower in explants from sucrose-supplemented medium (data not shown).

In extracts from cotyledons cultured on control medium (without carbohydrates) and on media supplied with different carbohydrates, SOD activity differed between forms. MnSOD activity was significantly higher in cotyledons cultured on sucrose-, fructose- and maltose-supplemented medium than in control cotyledons (Fig. 2). Cu/ZnSOD I activity was higher in cotyledons cultured on glucose than in the control (Fig. 2). Cu/ZnSOD II activity was significantly lower in cotyledons cultured on medium supplied with sucrose, glucose, fructose and maltose, than in the control (Fig. 2). The presence of sucrose in the medium inhibited FeSOD activity in cotyledons; on glucose-supplemented medium, FeSOD activity decreased but not significantly (Fig. 2). This result differs from that in the assays of SOD forms, in which the band of FeSOD activity was weak (Fig. 1a). FeSOD is probably the most labile SOD form of all the classes of SOD detected in *T. repens* (see Discussion). In cotyledons cultured on sucrose-supplemented medium, however, FeSOD activity was weaker than in other growth conditions, or even absent (Figs. 1, 2). Generally, it should be noted that FeSOD activity was the SOD form most strongly inhibited in all the carbohydrate-supplementation treatments (Fig. 2). Cotyledons cultured on media with different carbohydrates had lower FeSOD and Cu/ZnSOD II activity than the control (Fig. 2).

DISCUSSION

The three classes of SOD identified in higher plants are located in different compartments of the plant cell. FeSODs are present in chloroplasts, MnSODs in mitochondria and peroxisomes, and Cu/ZnSODs in chloroplasts, cytosol, and possibly extracellular spaces (for review see: Alscher et al., 2002). In this study, native PAGE and activity staining disclosed four SOD forms in extracts from *Trifolium repens* explants. In *T. repens* and *T. pratense*, previously only three SOD forms were identified in roots, shoots and leaf tissues: MnSOD and two Cu/ZnSOD isoforms (Palma et al., 1993; Tang et al., 1999; Mascher, 2002). In *T. pratense* shoots the fast-moving Cu/ZnSOD isoform (Cu/ZnSOD II) has been described as the chloroplastic isoform of this enzyme (Mascher et al., 2002). In other Leguminosae species such as *Pisum sativum* and *Medicago sativa*, apart from the three SOD forms mentioned above, FeSOD has also been detected (Hernández et al., 1999; McKersie et al., 1999; Samis et al., 2002). In our experiments the activity level of SOD forms was dependent on the medium composition (Figs. 1, 2). The patterns of SOD forms clearly differed between SFP cotyledons and plant material cultured on medium with sucrose (Fig. 1). In cotyledons cultured on medium with different carbohydrates, FeSOD and Cu/ZnSOD II activity was lower than in control cotyledons. In SFP hypocotyls, FeSOD was absent. These results strongly suggest that FeSOD is the chloroplastic form, as in other plants (Alscher et al., 2002). We did not identify the localization of Cu/ZnSOD I and Cu/ZnSOD II, but data obtained in other plants of the Leguminosae family strongly indicate that Cu/ZnSOD I is a cytosolic enzyme and that Cu/ZnSOD II is a chloroplastic SOD form in addition to FeSOD (Hernández et al., 1999; McKersie et al., 1999; Tang et al., 1999; Mascher et al., 2002; Samis et al., 2002). In cotyledons of *T. repens*, FeSOD was present in SFP explants and explants cultured on medium without carbohydrates. In hypocotyls and cotyledons cultured on medium with sucrose, FeSOD activity was weak or absent. This indicates that sucrose might be at least partly responsible for inhibition of FeSOD in photosynthesizing tissues such as cotyledons. Another possible explanation may be the effect of oxygen deprivation in cotyledons in liquid culture; hypoxia in liquid culture medium could decrease FeSOD and Cu/ZnSOD II activity as compared to the levels in explants growing on moistened filter paper. Blokhina et al. (2003) discussed the effect of oxygen deprivation stress on SOD activity. The higher activity of SOD forms observed in SFP cotyledons as compared to that in cotyledons cultured on media supplemented with various sugars might be due to sugar starvation stress. Increased SOD activity has been observed in sugar-starved embryos of *Lupinus angustifolius* (Morkunas et al., 2003). It should be noted that the FeSOD activity bands in our experiments were quite labile: for example, FeSOD activity band was weak in cotyledons cultured on medium supplemented with sucrose, and absent in cotyledons from sucrose-supplemented medium in another set of experiments (Figs. 1a, 2). Similar findings on FeSOD activity have been reported in alfalfa (McKersie et al., 1999). Probably this is why other authors could not detect FeSOD in *Trifolium* sp. (Tang et al., 1999; Mascher et al., 2002).

Cotyledons cultured on media supplemented with carbohydrates (sucrose, glucose, fructose or maltose) showed much lower activity of SOD forms than control cotyledons, or even no activity. Probably sugar in the medium inhibited the activity of the putative chloroplastic forms of SOD: FeSOD and Cu/ZnSOD II. MnSOD activity was highest on medium supplemented with sucrose, fructose and maltose (Fig. 2). This result indicates that sucrose, fructose and maltose are utilized by explants, and

that the products of disaccharide hydrolysis (glucose and fructose) stimulate respiration in mitochondria, which would be reflected in higher MnSOD activity. The effective uptake of sucrose has been observed in *Brassica napus* explants (Ślesak et al., 2004). Bowler et al. (1989) concluded that sugars did not induce the mRNA for MnSOD in *Nicotiana plumbaginifolia* cell suspension cultures through an osmotic effect, and that induction of *MnSOD* mRNA by carbohydrates may rather reflect their role as carbon source, because the cytochrome *c* oxidase activity necessary for respiration was clearly stimulated by sucrose. *MnSOD* mRNA was also induced by sucrose in *Hevea brasiliensis* (Miao and Gaynor, 1993).

Cu/ZnSOD I activity was highest in cotyledons cultured on medium supplemented with glucose. Most likely this sugar caused the strongest oxidative stress in them. Cu/ZnSOD II activity was inhibited on media supplemented with sucrose, glucose, fructose and maltose. Together with the finding of very weak FeSOD activity, these results point to inhibition of photosynthetic activity, mainly by sucrose and maltose, in cotyledons of *T. repens*. A relationship between photosynthesis and the activity of SOD forms located in chloroplasts seems likely. Sugar-induced inhibition of photosynthesis-related genes has been described in many species; it overrides regulation by light, tissue type and developmental stage (Smeekens, 2000; Pego et al., 2000; Rolland et al., 2002). Apart from photosynthesis, another important source of ROS in plant cells is respiration (Foyer and Noctor, 2000). The increase in the activity of putatively mitochondrial MnSOD in the presence of sucrose, fructose and maltose might indicate stimulation of $O_2^{-\cdot}$ production, a substrate for SOD, on the mitochondrial electron transport chain. It should be emphasized that sugars apparently assume a dual role with respect to ROS. Soluble sugars can be involved in ROS-producing metabolic pathways such as respiration. Sugars may also feed NADPH-producing pathways, such as the oxidative pentose-phosphate pathway, which can contribute to the ROS scavenging system (for review see: Couée et al., 2006). The results are interesting in the context of the role of soluble sugars as signalling molecules activating/deactivating specific genes on both major metabolic pathways, such as photosynthesis and respiration. High sugar levels may correspond to activation of some ROS-producing pathways (e.g., respiration), and reduction of other ROS-producing processes (e.g., photosynthesis). SOD probably is one of the major antioxidant enzymes involved in regulation of sugar-dependent ROS production, because different SOD forms are present in all cellular compartments linked with sugar metabolism.

The results of our experiments confirmed that various sugars affect the expression pattern and the activity of SOD forms in cotyledons of *T. repens* in liquid culture media. The activity of SOD forms putatively associated with chloroplasts is down-regulated in response to the presence of soluble sugars in culture media. These results might suggest an inhibitory effect of some carbohydrates on photosynthesis. On the other hand, some soluble sugars can enhance respiration via increased activity of putatively mitochondrial MnSOD. Regulation of ROS level by SOD activity, together with metabolism of soluble sugars, most likely plays an important role in the development and morphogenetic response of cultured explants.

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