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QUARTERLY

Effect of high temperature treatment of *Vicia faba* roots on the oxidative stress enzymes in leaves

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The following types of superoxide dismutase (SOD) have been found in the leaves of *Vicia faba*: one isoenzyme of Mn-SOD and four isoenzymes of Cu/Zn-SOD. The treatments of roots with boiling water caused an increase of SOD activity in the leaves. The highest increase was measured after 5 s of the treatment. It was accompanied by a significant increase in catalase activity. Analysis of cell fractions' revealed an increase of SOD activity in the plastids and mitochondria isolated from the leaves of those plants whose roots were heat-treated. However, there was no distinct change of SOD activity in the cytosolic fraction. The possibility of an electric wave intervention inducing oxidative stress in the leaves is discussed.

Many environmental conditions, such as drought [1], chilling [2, 3], air pollution [4], herbicides [5] or pathogenic injury [6] may induce oxidative stress in plants. In such a case, toxic reactive species of oxygen are formed, i.e. the superoxide radical ($O_2^{\cdot-}$), and H_2O_2. High amounts of reactive oxygen species destroy all the cellular constituents.

Superoxide dismutases are metalloproteins which convert $O_2^{\cdot-}$ to O_2 and H_2O_2 as the first link in the enzymic scavenging system of reactive oxygen species. Hydrogen peroxide is also toxic for the cell. In the presence of metal cations, especially Fe^{2+}, it reacts with $O_2^{\cdot-}$, producing the strongest known oxidizer — the hydroxyl radical $^{\cdot}OH$ [7, 8]. Catalase decomposes H_2O_2 to water and oxygen, mainly in the peroxisomes [8, 9], but also in cytosol, mitochondria and chloroplasts [7, 10].

In plants three isoforms of SOD, differing in the metal present at the active site, have been identified: copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD), and evolutionarily the oldest — iron superoxide dismutase (Fe-SOD). The Fe-SOD is present mainly in the plastids of some plants. Mn-SOD is usually present in the mitochondrial matrix, but can also be found in peroxisomes and chloroplasts [11]. The Cu/Zn-SOD is present in the cytosol, chloroplasts [11, 12], mitochondria and glyoxysomes [11].

Previous research was related to the organs which were directly stress-treated [5, 13–18]. The aim of our study was to state whether high temperature treatment of roots has any

Abbreviations: CAT, catalase; PPFD, photosynthetic photon flux density; SOD, superoxide dismutase.

influence on the activity antioxidant enzymes, of such as superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) in *Vicia faba* leaves. Such influence could indicate that a communication between different organs of the plant does exist. It is assumed that the first reaction of the plant parts subjected to stress is generation of electric potentials [19]. Gaspar *et al.* [20] considered the possibility of peroxidase activity modulation as a consequence of electrical wave passage. During previous experiments, after heat treatment of *Vicia faba* roots, transmission of an electrical wave to the leaves was observed [21]. Therefore, a possible connection between this effect as well as changes in SOD and catalase activities, have been examined.

MATERIALS AND METHODS

Plant material. Germinating seeds of *Vicia faba* cv. Nadwislanski were placed individually in perforated plastic test-tubes and grown in aerated water culture (0.1 mM KCl, 0.1 mM NaCl, 0.2 mM $CaCl_2$) of an air-conditioned glasshouse for 14 days. The air temperature was 25/22°C (16/9 h day/night cycle) and the relative air humidity was 55–66%. The plants were additionally irradiated with sodium incandescent lamps Sont Argo (380 W) ensuring an irradiation intensity of 380 $\mu mol\, m^{-2}\, s^{-1}$ photosynthetic photon flux density (PPFD) at the plant level. Seedlings of about 10 cm length from the top to hypocotyl with the first fully expanded leaf (about 7 cm from hypocotyl) were used. The roots of seedlings were heated with boiling water for 5, 20, 70 or 200 s, then cut off. In order to avoid direct influence of temperature upon leaves, each plant's root system was put on a special plastic plate which enabled hypocotyl isolation from the roots. The flow of boiling water was directed to the root system. After having heated the roots, the hypocotyl was cut off above the plate. The control plants were not heat-treated but their roots were also cut off. Just after cutting off the roots, the first well formed leaf of each plant was removed and placed in distilled water for 30 min at room temperature.

Analysis of cell fractions was carried out on fresh leaves, while for the remaining determinations the plant material was frozen in liquid nitrogen.

Analysis of cell fractions. For cell fraction analysis, leaves (10 g) were ground in 20 ml of a solution of 0.4 M sucrose, 165 mM Tricine, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA and 10 mM dithiothreitol (DTT), pH 7.5. Macerated material was passed through four layers of cheesecloth and centrifuged at 2000 ***g*** for 5 min. The plastid-containing pellet was resuspended in 0.4 M sucrose, 10 mM Tricine, 1 mM EDTA and 10 mM DTT, pH 7.2. The supernatant was centrifuged at 12000 ***g*** for 10 min, and then retained as the cytosolic fraction. The mitochondria-containing pellet was resuspended in 0.4 M sucrose and mitochondria were further purified on a 0.6 M sucrose cushion and centrifuging at 10000 ***g*** for 20 min. The mitochondrial pellet was resuspended in the resuspending medium. Mitochondria and plastids were lysed by repeated freezing/thawing cycles and then samples were centrifuged at 10000 ***g*** to remove particles [18]. Then the cellular fractions were resuspend in the K-phosphate buffer (pH 7.8) containing 0.01 M EDTA and 0.5% bovine serum albumin (BSA). All other steps were the same as during preparation of crude extracts for electrophoresis.

Preparation of crude extracts. Leaves (5 g) were ground for 5 min in a mortar at 4°C with K-phosphate buffer (pH 7.8) containing 0.01 M EDTA and 0.5% BSA. The homogenate was centrifuged at 10 000 ***g*** for 15 min. Then, the supernatant was dialyzed against the same buffer and the amount of protein was measured. For electrophoretic separation, the buffer contained 0.1% *N,N,N',N'*-tetramethylenediamine (TEMED) and 0.15% DTT.

Enzyme assays. SOD isoenzymes were separated by native PAGE using 11.5% polyacrylamide gel at pH 7.8 and running buffer of 0.1 M Tris/glycine. Electrophoresis was performed at 4°C, at a constant voltage of 180 V for 60 min with the Bio-Rad Mini Protean device. Then, gels were stained by the method of Beauchamp & Fridovich [22] in 50 mM K-phosphate buffer, pH 7.8, containing: 2,2'-di-*p*-nitrophenyl-5,5-diphenyl-

3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride (nitrobluetetrazolium), 26.5 mM riboflavine and 26.5 μM TEMED in darkness for 20 min. After having been soaked, gels were exposed to the natural light — about 100 μmol m^{-2} s^{-1} (PPFD) — for 30 min in K-phosphate buffer, and transferred to 1% (v/v) acetic acid to stop the reaction. Isoenzyme profile was visualized using inhibitors of Cu/Zn-SOD and Fe-SOD (1 mM KCN and 5 mM H_2O_2) according to Beauchamp & Fridovich [22].

SOD activity was quantified on dialyzed crude extract by the method of McCord & Fridovich [23]. One unit was defined as the amount of enzyme necessary for 50% inhibition of cytochrome *c* in a coupled system with xanthine and xanthine oxidase.

Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm in a reaction mixture containing 50 mM phosphate buffer, pH 7.0, and 15 mM hydrogen peroxide [24].

Proteins were determined according to Bradford [25], using bovine serum albumin as a standard.

For crude extracts, all analysis were performed in 10 biological replicates, while for cell fraction's analysis in 5 replicates. All reported S.E. values were calculated using the mean square of the error term from each analysis.

RESULTS AND DISCUSSION

Figure 1 shows the electrophoretic separation of SOD in *Vicia faba* leaf extracts, derivated from the plants whose roots were heat-treated for 200 s (T) and from control plants (C). Light bands visualize individual SOD isoforms. The following types of superoxide dismutase were found: one isoenzyme of Mn-SOD and four isoenzymes of Cu/Zn-SOD. Isoenzyme Fe-SOD was not detected. Isoforms of Cu/Zn-SOD were not visible in the presence of inhibitors: KCN and H_2O_2, whereas the Mn-SOD band was resistant to both inhibitors (not shown). Identical locations of SOD band's activity and similarity in their inhibition points to lack of differences in the isoenzyme pattern of the extracts taken from the control and the stressed plants. However, the bands of enzymes were more distinctly visible in the leaves of heat-treated plants indicating increased SOD activity. Moreover, analysis of cell fractions also showed changes in SOD activity in plastid and mitochondrial fractions of leaves from heat-treated plants (Figs. 2 and 3). On the other hand, there was no distinct change of SOD activity in cytosolic fraction (Fig. 4). In each fraction the presence of Cu-SOD was found, while the manganese isoform was observed only in mitochondria and plastid fractions. These results correspond to the literature data [11, 26, 27].

The increase of SOD activity in leaves caused by high temperature can determine the extent of the oxidative stress response [28]. That is why quantitative measurements of enzyme activity were done taking into account the stress treatment time. The highest increase of SOD activity in the detached and incubated leaves occurred after 5 s of the root treatment (Fig. 5). Longer periods of heating (20–200 s) caused some decrease in SOD activity, although even after 200 s it

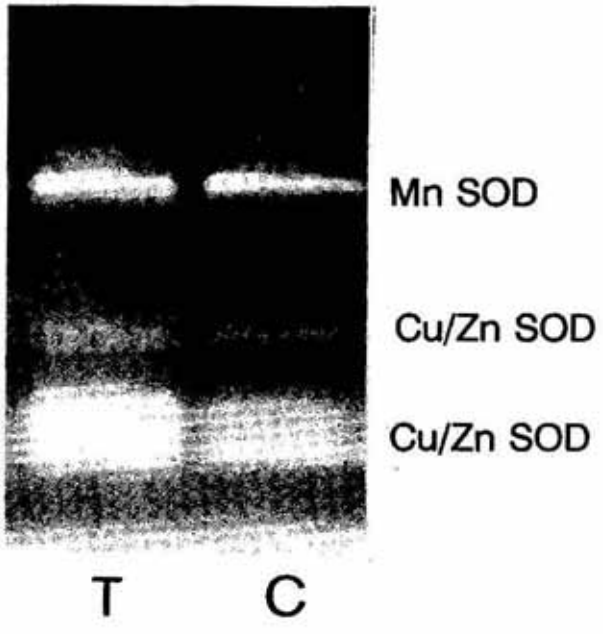

Figure 1. Superoxide dismutase isoenzyme pattern in extracts from *Vicia faba* leaves.

C, control plants; T, after 200 s of heat (100°C) treatment of the roots. Crude extracts were resolved by native PAGE, then the gel was stained for superoxide dismutase activity.

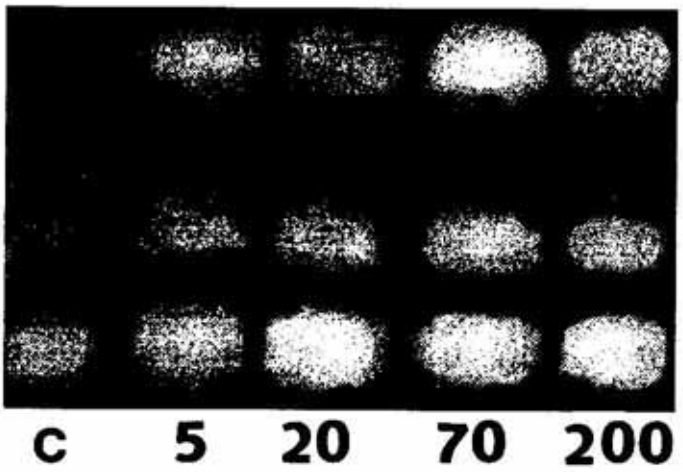

Figure 2. Superoxide dismutase isoenzyme pattern in plastid fraction of *Vicia faba* leaves, after high temperature treatment of the roots.

Roots were heated with boiling water for 5, 20, 70, and 200 s. The control plants were not heat-treated. Immediately, roots of each plant were cut off and leaves were incubated in distilled water at room temperature.

was significantly higher than in control plants.

It has been found that the heat shock response may be a result of changes in the oxidative-reductive environment of the cell stressed organs (i.e. leaves) to a damage occurring in a distant part of the plant can prove the passing of electrical wave, carrying information about the heat shock in roots [19, 31].

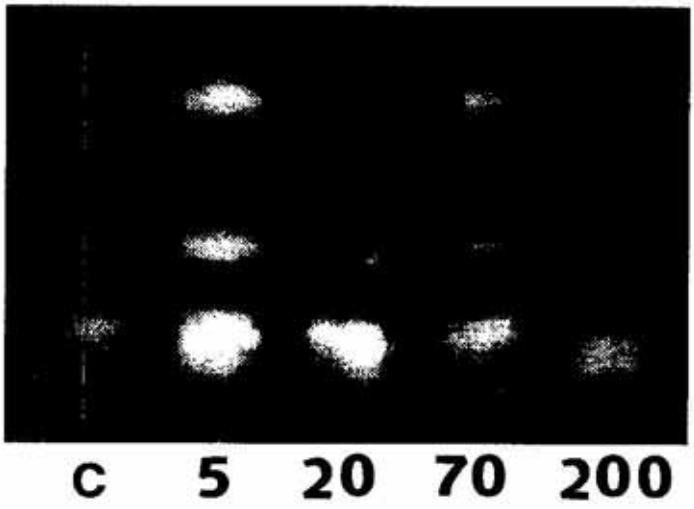

Figure 3. SOD isoenzyme pattern in mitochondrial fraction of *Vicia faba* leaves after high temperature treatment of the roots for the time indicated (in s). C, control.

or changes in the concentrations of some ions [29, 30]. These changes can be related to the electrical potential changes in the cell membranes. A very fast response (5 s) of non-

Changes in the activity of catalase which decomposes hydrogen peroxide, the product of SOD reaction, were investigated after different periods of heat-treatment. Increased

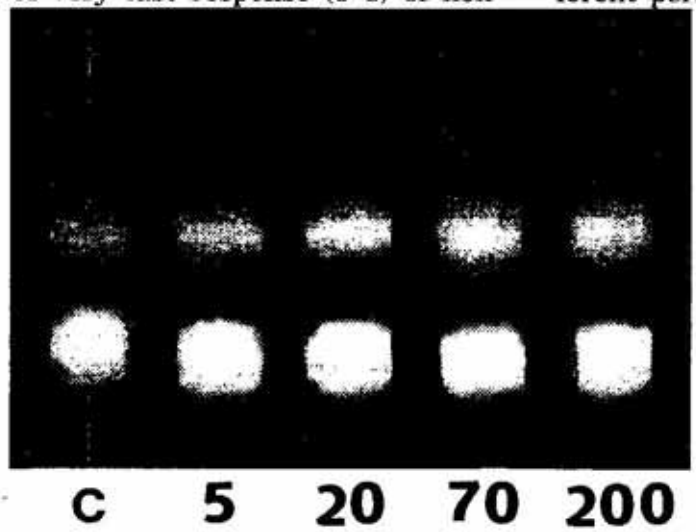

Figure 4. SOD isoenzyme pattern in cytosolic fraction of *Vicia faba* leaves after high temperature treatment of the roots for the time indicated (in s). C, control.

activity of catalase similarly as SOD activity was observed after 5 s of heating (Fig. 6). The increase in activity of the two antioxidant enzymes in the *Vicia faba* leaves indicates the transfer of information between distantly placed parts of plants as a result of the stress factor. This information can pass very rapidly, as shown by the activity of SOD as well as of catalase being the highest after 5 s of heat-treatment.

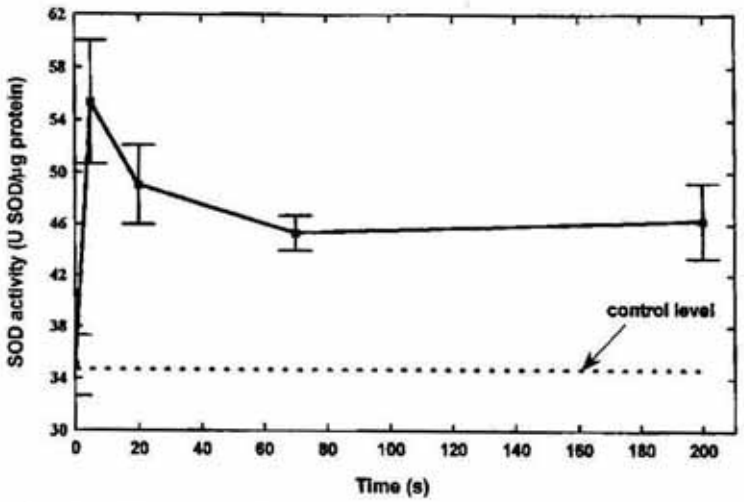

Figure 5. SOD activity in extracts from leaves of *Vicia faba* plants after high temperature treatment of the roots as described in the legend to Fig. 2.

The values are the averages of 10 series of experiments. The bars indicate the standard error.

It is probable that such a strong factor as high temperature causes a systemic reaction in plants, aimed at releasing defensive reactions and — in this particular case — antioxidant enzymes. As we have mentioned above, the short time of information transfer from roots to the leaves (5 s) suggests the electrical nature of the signal. The evidence for the electric wave passing through the *Vicia faba* plants, after the heat treatment of the roots, was previously presented [21]. Heating for 5 s of the roots induced electrical potential changes which, in the form of a depolarization wave, were transferred along the plant and reached non-stressed leaves. Gaspar *et al.* [20] suggested that stress-induced membrane depolarization leads to generation of free radicals and peroxides which cause the breakdown of membrane lipids, thereby permitting K^+ efflux and Ca^{2+} influx. Membrane depolarization, by causing Ca^{2+} passage to cytosol, may influence synthesis (or activation) of other chemical transmitters, for example: activate the genome and thus produce increase of amount of the examined enzymes. The increase in SOD and catalase activity measured after the heat treatment (5–200 s) and incubation of the leaves (30 min) observed in the presented experiments, may indicate that this electrical signal (directly or indirectly) could cause O_2^- generation. These results suggest involvement of reactive oxygen species in the transfer of information about the stress fac-

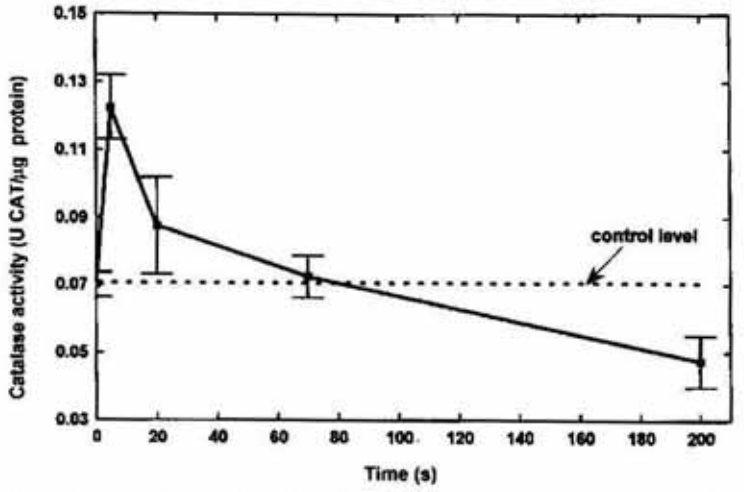

Figure 6. Catalase activity in extracts from *Vicia faba* leaves after high temperature treatment of the roots as described in the legend to Fig. 2.

The values are the averages of 10 series of experiments. The bars indicate the standard error. CAT, catalase.

tor between a plant's organs. The information reaches successive cells probably *via* symplast. Changes in activity of the antioxidative enzymes in the three studied organelles may lead to the conclusion that this signal is transmitted not only between the cells but also within the cell.

A hydraulic signal could be another potential factor, playing the role of information transmitter [32]. However, it is not known in what way this signal would increase the activity of the investigated enzymes.

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