ORIGINAL PAPER

# Exogenous auxin regulates $H_2O_2$ metabolism in roots of tomato (*Lycopersicon esculentum* Mill.) seedlings affecting the expression and activity of CuZn-superoxide dismutase, catalase, and peroxidase

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**Abstract** The aim of this study was to test the effect of auxin treatment on selected parameters of the redox metabolism in roots. We found that auxin application results in a reduction in the $H_2O_2$ level in roots. The hormone stimulated CuZn-superoxide dismutase, but simultaneously increased the activities of catalase, cell wall bound ferulic acid peroxidase, and soluble peroxidase i-zoenzymes. The analysis of the expression of genes coding for the cytosolic izoform of CuZn-superoxide dismutase, catalase, and cell wall associated peroxidase (TPX 1) involved in cell wall stiffening and lignification revealed the stimulatory effect of exogenous auxin on the expression of the aforementioned genes. The enzyme activity and gene expression in the roots of control and auxin-treated plants were studied in daily intervals, during a 3-day-long growth cycle. The stimulatory effect of auxin on the enzymatic activity was transient with the highest stimulation observed on the second day of treatment. On the third day, the activities of the enzymes decreased. The maximal enzyme activities were preceded by a rise in gene expression. The increase in the level of CuZn-superoxide dismutase and catalase transcripts were detected after 1 day of auxin treatment. Then the expression of the aforementioned genes decreased. The period of auxin-dependent stimulation of the TPX 1 gene expression encompassed the first and the second day of treatment. Auxin stimulated CuZn-superoxide dismutase and catalase activities only in the

distal zone of the root while peroxidase activity was increased by auxin in the distal as well as in the proximal parts of the organ.

## Introduction

Auxin has a central role in the regulation of two processes shaping root system architecture: elongation growth and lateral root formation (Casimiro et al. 2001; Cleland 2004). However, the auxin-induced signaling pathways are involved in the complex relations with other endo and exogenous factors. Among them, the components of cellular redox systems are thought to have a prominent place in the regulation of auxin-dependent processes. Auxin-induced reactions are often enhanced by oxidative stress (Pasternak et al. 2007; Potters et al. 2007). It was found that auxin-dependent inhibition of cotyledon and root elongation in *Arabidopsis* seedlings was mimicked by exposure to radical oxygen species (ROS)-generating compounds; paraquat, and alloxan. Moreover, elevated ROS formation increased the number of adventitious roots formed in response to exogenous auxin. Mild oxidative stress stimulated the formation of cell clusters and plantlet regeneration from auxin-treated root segments. These data suggest that oxidative stress inducing agents are involved in an auxin-driven process leading to cell division and differentiation (Pasternak et al. 2005).

Redox agents are also involved in an auxin-dependent patterning in the root apical meristem. The cells of the quiescent center that accumulate high auxin levels are characterized by the oxidized status of ascorbate and

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glutathione and the overproduction of ROS. On the other hand, in rapidly dividing cells of the proximal meristem, the reduced status of ascorbate and glutathione predominates and the ROS levels are low. It was hypothesized that auxin, by regulating redox status of the discrete zones within the root apical meristem, may act as positional signal and mediate meristem patterning (Jiang et al. 2003).

The reactive oxygen species may also function as a downstream component in auxin-mediated signaling pathway during gravitropic response in maize roots. Joo et al. (2001) reported that auxin redistribution induced by gravitropic stimulus, induces ROS production which mediates gravitropic response.

Reactive oxygen species are also involved in the auxin-dependent regulation of cell elongation. It has been shown that the stimulation of coleoptile growth of maize seedlings by auxin is accompanied by the release of ROS in the cell wall (Schopfer et al. 2002). In contrast, when auxin inhibits maize root growth, there is a decrease in ROS production (Liszkay et al. 2003). In maize coleoptiles auxin was found to promote the production of superoxide radicals ($O_2^-$) in the reaction catalyzed by Fe or Cu which can reduce $H_2O_2$ forming extremely reactive hydroxyl radicals ($^.OH$) (Schopfer 2001) produced by cell wall peroxidase (Liszkay et al. 2003). It is known that besides the $H_2O_2$-scavenging reactions of the peroxidative cycle, class III peroxidases may produce extremely reactive hydroxyl radicals through the hydroxylic cycle. The $O_2^-$ released by NADPH oxidase can convert peroxidase into compound III, which catalyzes the generation of $^.OH$ from $H_2O_2$ in the cell wall. $^.OH$ in cell walls and in apoplastic spaces could cleave cell wall polysaccharides such as pectin and xyloglucan, which results in cell wall loosening and, in consequence, accelerates elongation growth (Passardi et al. 2004; Liszkay et al. 2003). It was proposed that $^.OH$ cleaves cell wall polysaccharides which results in cell wall loosening and, therefore, allowing for the elongation (Fry 1998; Schopfer 2001).

The aim of the present study was to test the response of the ROS-scavenging enzymes in tomato roots to the exogenously applied auxin. We analyzed the activities of superoxide dismutase (SOD), catalase (CAT), soluble peroxidase (POX), and cell wall bound ferulic acid peroxidase (FPOX) in control and auxin-treated roots. The levels of transcripts coding for the cytosolic CuZnSOD and CAT as well as the expression of the TPX1 gene, coding for a cell wall-targeted peroxidase, were determined. A tomato peroxidase gene, TPX1, encoding a basic izoenzyme is specifically expressed in roots. Its product is believed to be involved in lignin synthesis and cell wall cross-linking (Medina et al. 1999; Quiroga et al. 2000, 2001). We demonstrated that the auxin treatment results in transient increase in transcript levels and activities of the

aforementioned enzymes and showed that the exogenous auxin affects the $H_2O_2$ content and ROS distribution in the roots of tomato seedlings.

## Materials and methods

### Plant material and growth conditions

Seeds of tomato (*Lycopersicon esculentum* Mill, cv. Ozarowski) were surface sterilized for 10 min in 50% Clorox (about 2% $Cl_2$) and washed three times for 5 min in sterile distilled water. Then the seeds were aseptically sown on sterile filter paper soaked with autoclaved distilled water and were incubated for 4 days at 25°C in darkness. Next, the germinating seeds were transferred to petri dishes and cultured in a thin film of liquid medium composed according to Murashige and Skoog (1962). Seedlings were then grown on a basal medium (BM, control) or on the medium supplemented with 1 µM indole-3-acetic acid (IAA). The IAA solution was filter sterilized before adding to the autoclaved medium. The culture was continued for 3 days until the lateral roots appeared.

### Determination of the $H_2O_2$ concentration

The hydrogen peroxide level in roots was analyzed with the method of Veljovic-Jovanovic et al. (2002). Briefly, the samples were homogenized in liquid nitrogen and extracted with the 100 mM Tris–HCl buffer pH 7.2, 1 mM EDTA and centrifuged for 3 min at 10,000$g$ at 4°C. The supernatant was extracted with equal volume of chloroform:methanol (1:1) and centrifuged again. The upper water phase was destined for $H_2O_2$ determination. The reaction mixture was composed of 0.1 M phosphate buffer, pH 6.5, 3.3 mM 3-dimethylaminobenzoic acid (DMAB), 0.007 mM 3-methyl-2-benzothiazoline hydrazone (MBTH), 100 µl of the $H_2O_2$-containing solution and 50 µg of radish peroxidase in the volume of 3 ml. The samples were incubated for 20 min at 37°C. After that, the $A_{590}$ nm was measured. The values of absorbance were compared with a $H_2O_2$ standard curve in the range of 0.1–1.0 µM.

### Enzyme extractions and assays

The plant tissue was homogenized at 4°C in the homogenization buffer composed of 100 mM phosphate buffer, pH 7.8, 1 mM EDTA, 8 mM $MgCl_2$, 4 mM DTT and 0.1% Triton X-100. The homogenate was centrifuged at 4°C for 10 min at 10,000$g$. A supernatant containing the extracted enzyme was used for the assays of catalase, superoxide dismutase, and peroxidase activity.

The activity of catalase (CAT, EC 1.11.1.6) was assayed according to Rao et al. (1996). The measurement of CAT activity was based on the following decrease of $A_{240}$ nm in 1 ml of the reaction mixture composed of 100 mM phosphate buffer, pH 7.0, 30 μl enzymatic extract and 1.5 μl of 30% (v/v) $H_2O_2$. The decrease of $A_{240}$ was followed at 25°C against a plant extract-free blank. The activity of CAT was expressed as the number of μmol of $H_2O_2$ decomposed during 1 min by 1 mg of total protein.

The CAT activity was visualized after the electrophoretic separation of 50 μg of total proteins isolated from roots by nondenaturing, nonreducing PAGE using a separating gel containing 6% (w/v) acrylamide and a stacking gel containing 4% (w/v) acrylamide. After the electrophoresis, the gels were incubated twice in distilled water for 15 min. After that, the gels were transferred for 10 min to 0.003% (v/v) $H_2O_2$. Finally, the gels were briefly washed and placed in the staining solution containing 1% (w/v) $K_3Fe(CN)_6$ and 1% (w/v) $FeCl_3$. Both compounds of the staining solution were mixed directly before usage. After 10-min-long incubation, the catalase izoforms appeared as yellow bands on a dark-green background (Romero-Romero et al. 2005).

The activity of the izoforms of superoxide dismutase (SOD, EC 1.15.1.1) was determined after the electrophoretic separation of protein extract in the polyacrylamide gel. Samples containing 200 μg of total protein were subjected to discontinuous PAGE under non-denaturing, non-reducing conditions as described by Laemmli (1970) using a stacking gel containing 4% (w/v) acrylamide and a separating gel containing 12% (w/v) acrylamide. After the completion of electrophoresis, the gels were stained for the activities of SOD. To visualize the SOD activity the method of Rao et al. (1996) was applied. First, the gels were incubated for 25 min 100 mM phosphate buffer, pH 7.8 containing 2.5 mM nitroblue tetrazolium (NBT). Then the gels were transferred to the solution containing 28 mM tetramethyl ethylene diamine and 28 μM riboflavine in 100 mM phosphate buffer, pH 7.8 and kept for 25 min in the darkness with gentle agitation. After that, the gels were placed in the distilled water and exposed to white light for 10–15 min until the bands were visible. SOD izoenzymes appeared as colorless bands on a deep-blue background. The stained gels were scanned and the intensity of bands was determined by densitometry using Image Gauge V3.46 software. The identification of SOD izoforms was based on the inhibitory effect of KCN and $H_2O_2$ on the activity of SOD izoenzymes. After the accomplishment of the electrophoresis the gels were incubated in the NBT-containing buffer, prepared as described above but supplemented with either 5 mM KCN or 5 mM $H_2O_2$. Following the 25-min-long incubation, the procedure of the visualization of the SOD activity was performed.

In order to separate peroxidase (POX, EC 1.11.1.7) izoforms, 50 μg of total protein was subjected to native electrophoresis on polyacrylamide gel. The separating gel containing 7.5% (w/v) acrylamide and a stacking gel containing 4% (w/v) acrylamide were used. After the electrophoresis the gels were immersed in the solution containing 0.5% (w/v) benzidine and 0.03% (v/v) $H_2O_2$ in 50 mM phosphate buffer, pH 6.0 for 15 min. Peroxidase izoforms stained dark-blue.

Ferulic acid-acid dependent peroxidase (FPOX) was determined in soluble fraction prepared as described above and in the cell wall preparations. The cell wall fraction was obtained according to Lin and Kao (2001). First, the roots were homogenized in ice-cold 100 mM phosphate buffer, pH 5.8. The homogenate was then centrifuged at 1000 $g$ and washed four times with phosphate buffer. The pellet was collected and suspended in 1 M NaCl for 2 h with shaking at 30°C. After that, the samples were centrifuged and the supernatant was used for enzyme assays. Reactions were developed according to Córdoba-Pedregosa et al. (1996) with slight modifications in a final volume of 1 ml containing 50 μg of sample protein, 100 mM sodium phosphate buffer, pH 6.0, 0.1 ml of 0.3% (v/v) $H_2O_2$ and 0.1 mM ferulic acid. Reactions were followed spectrophotometrically for 1 min at 310 nm. Corrections were made by subtracting the changes in absorbance observed in the absence of enzyme extract.

Total protein was assayed according to Bradford (1976) with BSA as a standard.

Histochemical procedures

The hydrogen peroxide and superoxide radical were localized in roots according to Mellersh et al. (2002). $H_2O_2$ was visualized by incubating the seedlings for 2 h in the 5.5 mM $3',3$-diaminobenzidine hydrochloride (DAB). $H_2O_2$-rich regions stained brown. The sites of superoxide radical production in roots were localized by incubating the seedlings in 0.005% NBT dissolved in 100 mM phosphate buffer, pH 7.5 for 10 min. After that time, incubation was interrupted by replacing the NBT solution with phosphate buffer. The sites of superoxide radical accumulation stained dark blue.

Radical oxygen species localization in roots was also analyzed using the confocal microscopy after staining the tissue with the fluorescent dye, $2', 7'$-dichlorofluorescin (DCFH) diacetate. In order to load the dye into the cells the roots were incubated for 15 min in 50 mM phosphate buffer (pH. 7.5) containing 50 μM DCFH-diacetate. After that, the roots were rinsed with the phosphate buffer and imaged in an Eclipse (Nicon) confocal microscope using 488 nm excitation and 525 nm emission (Zhang et al. 2001).

Total RNA isolation

The root samples of the weight of 30–40 mg were rinsed in distilled water, frozen in liquid nitrogen and kept at −80°C until used. Total RNA was isolated from the roots of tomato seedlings using a MasterPureTM RNA Purification Kit (Epicenter, USA) according to the producer's instructions and then suspended in 30 μl of TE buffer and stored at −80°C.

The synthesis of the first strand of cDNA and RT PCR

The reverse transcription reaction was performed using the superscript II reverse transcriptase (Invitrogen, USA) and random hexamers following the producer's instructions. After the accomplishment of the synthesis the reaction mixture was stored at −20°C until it was used as a template in the PCR. The gene specific primer pairs for coding sequences of the genes of tomato cytosolic CuZnSOD, catalase, cell wall associated peroxidase (TPX1), and 18S rRNA were designed based on the sequences available in the NCBI database. The following primer pairs were used in the experiments (from 5′ to 3′), CuZnSOD forward: TGTGGTTCATGAGCTTGAGG; CuZnSOD reverse: TTGGAGTCAAACCAACCACA, NCBI accession number: X14040, expected product size: 153 bp, CAT forward: GATGAGCACACTTTGGAGCA, CAT reverse: TGCCCTTCTATTGTGGTTCC, NCBI accession number: AF112368, expected product size: 145 bp, TPX1 forward: TGCAGCATTGACAACACGTA, TPX1 reverse: TCTTCCCATTTTCTCCATCG, NCBI accession number: L13654, expected product size: 112 bp, 18S rRNA forward: AAACGGCTACCACATCCAAG, 18S rRNA reverse: CCTCCAATGGATCCTCGTTA, NCBI accession number: AY552528, expected product size: 110 bp. The specificity of primers was checked with BLAST software.

The fragments of transcripts were amplified by PCR in the reaction mixture composed of 80 μM dNTP mix, 250 μM forward and reverse starters, 0.6 U of DyNAzyme II polymerase (Finnzymes, Finland), 1 μl cDNA and appropriate polymerase buffer in the volume of 20 μl. PCR was performed in the MasterCycler Gradient (Eppendorf, Germany) at 59°C annealing temperature for all genes. The products of PCR amplification resulted in a single band of the predicted molecular mass. These were analyzed on 2% agarose gels against a 1-kb DNA ladder (Fermentas, Germany) containing the following fragments: 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 bp.

Real-time PCR

The samples were composed of 1 μl cDNA, 200 μM dNTP, 250 μM forward and reverse primer, 0.005 μg

SYBR Green, 0.6 U polymerase DyNAzyme II (Finnzymes, Finland), working concentration of DyNAzyme II buffer, and water to the final volume of 20 μl. Real-time PCR reactions were run in triplicates on a Quantica device (Techne, Great Britain). After a 1 min denaturation step at 95°C, samples were run for 40 cycles of 15 s at 95°C, 20 s at 59°C and 30 s at 72°C. SYBR Green fluorescence was recorded after the elongation step of each cycle. PCR crossing points (Cp) were determined with the second derivative maximum method (Luu-The et al. 2005). After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 70 to 95°C with simultaneous fluorescence monitoring. Serial dilutions of cDNA were used to trace the calibration curves which were used to determine the values of amplification efficiency ($E$). Real-time PCR efficiencies were calculated from the slopes of the calibration curves according to $E = 10^{[-1/\text{slope}]}$. The SOD, CAT, and TPX 1 transcripts (target genes) were normalized to 18s rRNA (reference gene) and their relative expression levels were calculated according to Pfaffl's formula:

$$R = \frac{\left(E_{\text{target gene}}\right)^{\Delta C\text{ptarget(control-sample)}}}{\left(E_{\text{reference gene}}\right)^{\Delta C\text{preference(control-sample)}}}$$

where $R$ is a relative expression ratio of the target gene expressed in a sample versus a control in comparison to a reference gene (18s rRNA). $E_{\text{target gene}}$ is the real-time efficiency of the target gene transcript; $E_{\text{reference gene}}$ is the real-time PCR efficiency of the reference gene transcript (18s rRNA); $\Delta C p_{\text{target}}$ is the $C$p deviation of control−sample of the target gene transcript, $\Delta C p_{\text{reference}}$ is the $C$p deviation of control-sample of the reference gene (Pfaffl 2001). In our studies, the samples isolated from roots after 4 days of germination (before the start of the culture on basal or auxin-containing media) were considered as the control.

Statistics

Statistical differences between mean values were determined with the Student's $t$-test. Differences at the level of $P < 0.05$ were considered as significant. Each experiment was repeated three times. Each time the roots excised from at least 50 seedlings were used to obtain the total RNA sample or the extract for the single measurement of the enzyme activity or $H_2O_2$ concentration. Mean and standard error were calculated. Error bars shown in all figures represent standard errors calculated from all repetitions of each experiment.

## Results

### Superoxide dismutase, catalase, soluble peroxidase, and ferulic acid peroxidase in the roots of control and auxin-treated seedlings of tomato

The root growth rate of auxin-treated seedlings decreased throughout the entire culture period (Table 1). Simultaneously, exogenous auxin induced numerous lateral roots (data not shown). In order to discriminate between the effects of auxin on the activities of superoxide dismutase, catalase, and peroxidase in the elongating (distal) and in the lateral root-forming (proximal) part of the root, the enzymes were assayed separately in the protein extracts obtained from segments isolated from proximal or distal parts of the roots of tomato seedlings.

Distal halves of the roots of tomato seedlings were equipped with two CuZnSOD izoenzymes (Fig. 1). It has been observed that the activities of both CuZnSOD enzymes were stimulated in distal halves of roots of seedlings grown on the medium supplemented with auxin when compared to the control. The highest stimulatory effect of auxin was observed on the second day of culture (Fig. 1).

Four distinct SOD izoforms were detected in total protein extracts isolated from the proximal halves of tomato seedling roots; two MnSOD izoforms (MnSOD I and MnSOD II), and two CuZnSOD izoforms (CuZnSOD I and CuZnSOD II (Fig. 2). It was observed that CuZnSOD izoenzymes did not significantly change the activity during the culture period. Their activities were not affected by auxin treatment. The activities of the MnSOD izoforms decreased after the onset of culture and remained similar in plants grown on both control- and auxin-supplemented medium (Fig. 2).

The catalase activity in the roots of tomato seedlings was attributed to one izoform of this enzyme (Fig. 3a, c). In the distal halves of roots grown on the basal medium, catalase activity gradually decreased during subsequent days of culture. In the presence of auxin, the decrease in the enzyme's activity was transiently interrupted on the second day of culture when the activity significantly increased (Fig. 3d). On the other hand, CAT activity in proximal parts of the roots gradually decreased both in control plants as well as in those treated with auxin (Fig. 3b).

When protein preparations were subjected to native electrophoresis and monitored for POX activity, higher intensity of the bands was observed if extracts were obtained from the roots of auxin-treated seedlings when compared to the control. In the proximal halves of the roots, auxin application resulted in the enhancement in the intensities of most of the existing POX izoforms and caused the synthesis of a new isoform. In distal part of the root, treatment with auxin resulted in the synthesis of three new izoforms, in addition to enhancing the intensities of the existing ones (Fig. 4).

The activity of ferulic acid peroxidase was assayed in soluble and cell wall-bound fraction isolated from the roots of tomato seedlings. The FPOX activity associated with the cell wall was approximately four times higher than in the soluble fraction (Fig. 5). The activity of the soluble FPOX did not differ between extracts isolated from roots of control or auxin-treated plants. In contrast to the soluble fraction, the FPOX activity of the cell wall bound enzyme was strongly stimulated in the roots exposed to auxin on the second and third day of culture when compared to the untreated control (Fig. 5).

### The expression of CuZnSOD, CAT, and POX genes in the roots of control and auxin-treated seedlings of tomato

The relative level of the cytosolic CuZnSOD transcript decreased in the roots of control plants during the first 2 days of culture. On the other hand, in the presence of auxin, on the first day of culture, the SOD transcript level remained stable or even slightly increased; however, it dropped down on the following day (Fig. 6a). The analysis of the expression of the catalase gene revealed that the CAT transcript level transiently increased in roots grown on the auxin-supplemented medium on the first day of culture. On the second day, the transcript level sharply decreased. In contrast to auxin-treated plants, in control roots, a gradual decrease in the level of catalase mRNA was observed during the 3 days of culture (Fig. 6b). The expression of TPX1 gene coding for the tomato cell wall peroxidase (Botella et al. 1993; Medina et al. 1999) was increased in the roots grown in the presence of auxin when

**Table 1** Effect of auxin on the primary root elongation

| Day 0 | Day 1 | | Day 2 | | Day 3 | |
|---|---|---|---|---|---|---|
| | Control | IAA | Control | IAA | Control | IAA |
| 9.4 ± 1.7 | 23.6 ± 1.3 | 12.7 ± 2.0 | 36.7 ± 2.8 | 17.5 ± 2.3 | 49.7 ± 2.8 | 22.3 ± 2.3 |

Roots were measured in daily intervals during the 3-day-long culture in the medium with or without 1 µM IAA. Data represent mean length of the primary root (mm) ± SD

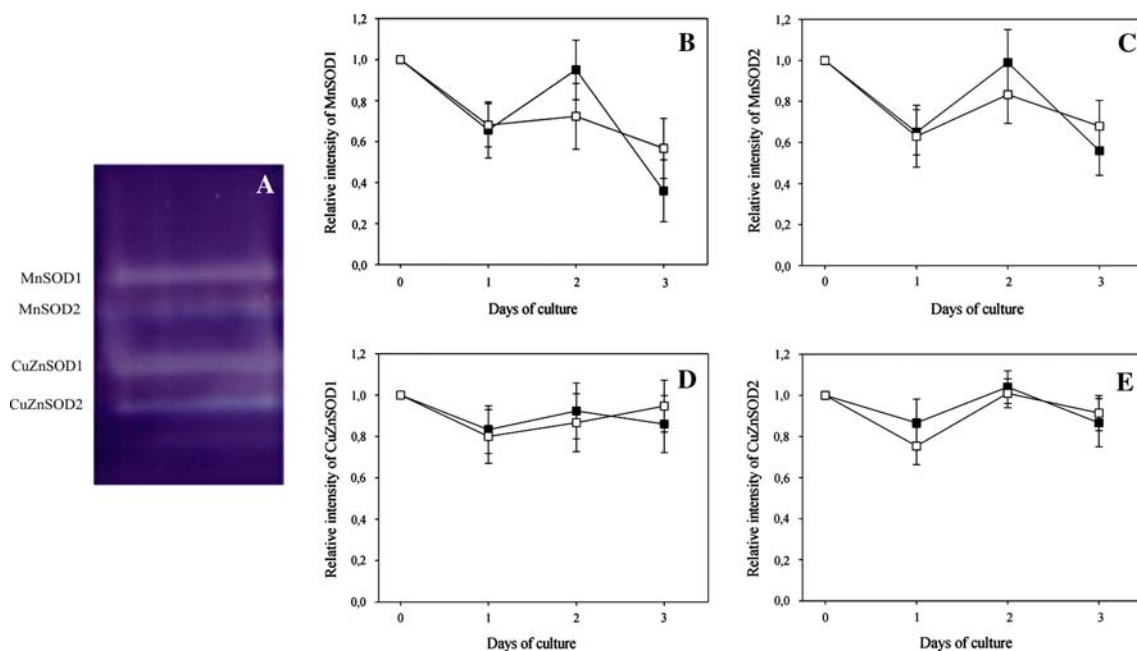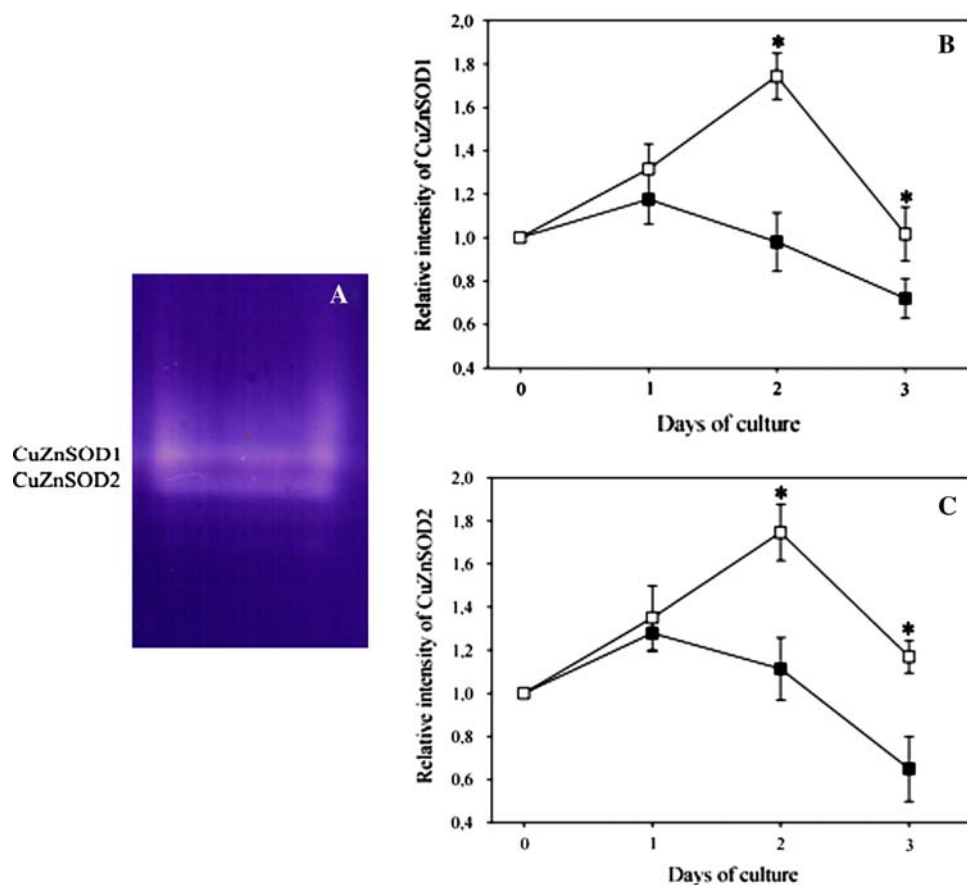Fig. 1 Superoxide dismutase activity in the distal parts of the roots of tomato seedling cuttings. Native gel stained for SOD activity revealed two CuZnSOD izoenzymes (a). Changes in the staining intensities of CuZnSOD1 (b) and CuZnSOD2 (c) in the distal parts of the roots of seedlings cultured on BM (*filled symbols*) or in the presence of 1 µM IAA (*empty symbols*) were followed at 1-day intervals during culture period. *Asterisk* denotes significant differences from control with $P < 0.05$



Fig. 2 Superoxide dismutase activity in the proximal parts of the roots of tomato seedling cuttings. Native gel stained for SOD activity revealed two CuZnSOD and two Mn SOD izoenzymes (a). Changes in the staining intensities of MnSOD1 (b), MnSOD2 (c), CuZnSOD1 (d), and CuZnSOD2 (e) in the proximal parts of the roots of seedlings cultured on BM (*filled symbols*) or in the presence of 1 µM IAA (*empty symbols*) were followed. *Asterisk* denotes significant differences from control with $P < 0.05$

**Fig. 3** Catalase activity in the roots of tomato seedlings. Native gel stained for CAT activity revealed one catalase izoenzyme in proximal (**a**) and distal (**c**) part of the root. Changes in the CAT activity in the proximal (**b**) and distal (**d**) parts of the roots of seedlings cultured on BM (*filled symbols*) or in the presence of 1 μM IAA (*empty symbols*) were followed at 1-day intervals during culture period. 1 unit = 1 μmol $H_2O_2$ reduced $min^{-1}$ (mg of total protein)$^{-1}$. *Asterisk* denotes significant differences from control with $p < 0.05$
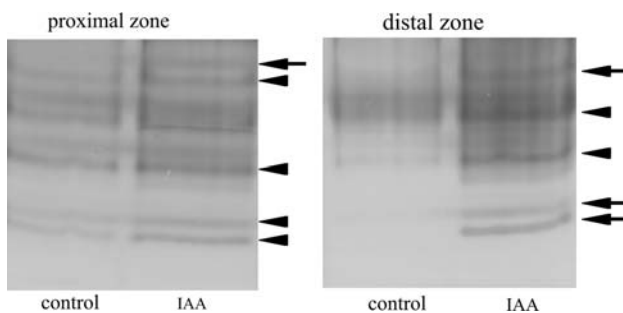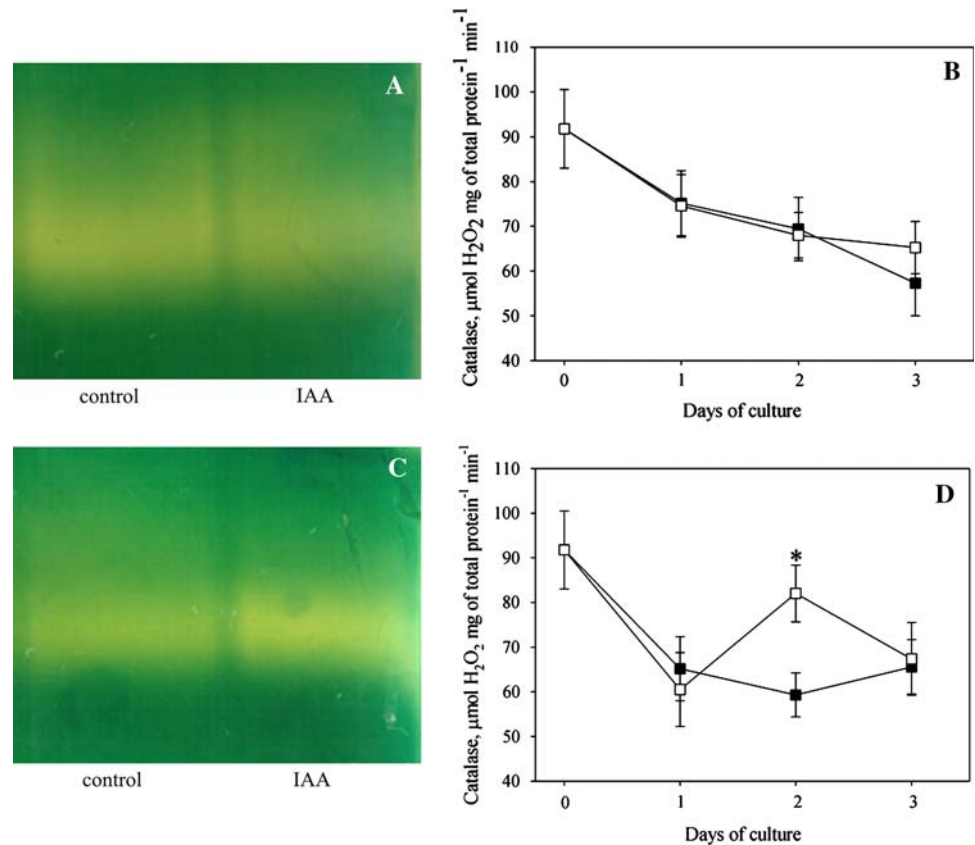


**Fig. 4** The patterns of POX izoenzymes in the proximal and distal zones of the roots of tomato seedlings grown on BM (control) or in the presence of 1 μM IAA (IAA). POX izoenzymes were visualized after native electrophoresis of protein extracts isolated on the third day of culture. Equal amounts of protein (50 μg) from proximal or distal zone of roots were loaded on the gel. *Arrows* indicate the izoforms which were present in the roots of auxin-treated plants and absent in control ones. *Arrowheads* indicate the izoforms whose staining intensities were preferentially enhanced by auxin treatment

compared to the control. In the roots of control seedlings a slight increase in the expression level was observed during the culture period. In the roots of the auxin-treated plants, the expression level was significantly higher after 1 day of auxin treatment; however, the strongest increase in the TPX 1 gene expression was detected after 2 days of culture on the auxin-supplemented medium. On the third day of culture the level of TPX 1 transcript decreased in the roots of the auxin-treated seedlings (Fig. 6c).

### $H_2O_2$ content in roots of tomato seedlings

It has been observed that the endogenous level of $H_2O_2$ in roots of tomato seedlings grown on auxin-supplied medium, measured during the 3-day-long culture period, was oscillating around the values determined in the starting material. The roots of seedlings grown on basal medium were characterized by a significant increase in $H_2O_2$ concentration observed on the second day of culture. Hydrogen peroxide content slightly decreased on the following day; nevertheless it remained three times higher than in roots of seedlings grown in the presence of IAA (Fig. 7).

### $H_2O_2$ and superoxide radical localization in tomato roots

DAB staining revealed that in both distal and proximal parts of roots $H_2O_2$ accumulation started from the second day of culture. The most intensive staining in the distal parts of roots was seen in the apical part of the root including the root cap, apical meristem, and the root elongation zone. Approximately the same areas of $H_2O_2$ accumulation were observed in the distal parts of the control and auxin-treated roots (Fig. 8). DAB staining was seen on the second day of culture in the lateral root
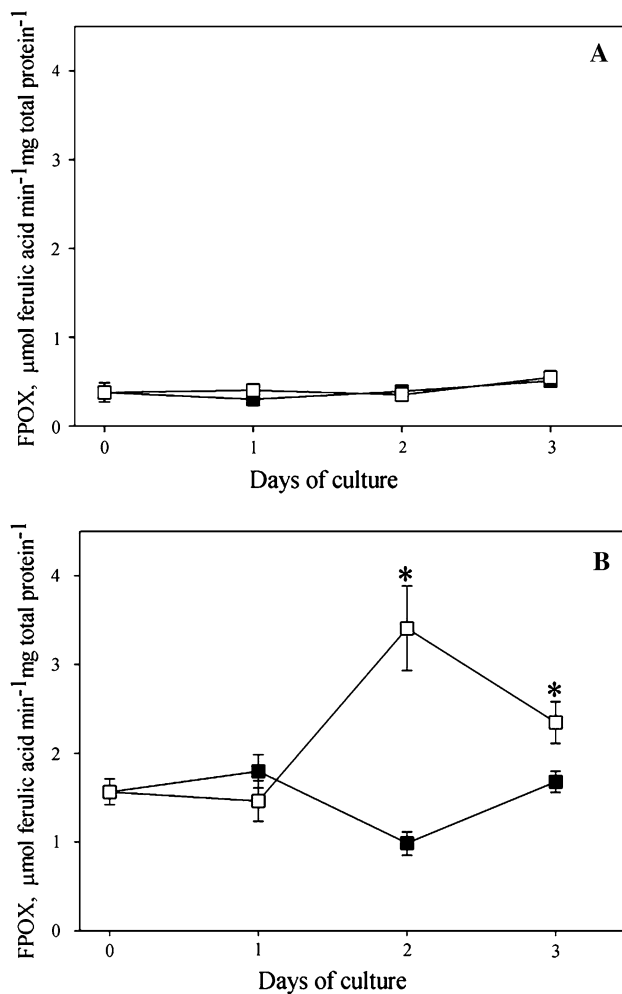
**Fig. 5** The activity of ferulic acid peroxidase (FPOX) in the roots of tomato seedlings cultured on BM (*filled symbols*) or in the presence of 1 µM IAA (*empty symbols*) during the 3-day-long culture period. The enzyme's activity was assayed in the soluble fraction (**a**) and in a fraction ionically bound to the cell wall (**b**). 1 unit = 1 µmol ferulic acid oxidized min$^{-1}$ (mg of total protein)$^{-1}$. *Asterisk* denotes significant differences from control with $P < 0.05$

primordia and on the third day of culture in the apices of lateral roots. In the proximal regions of the lateral roots DAB staining was less intense (Fig. 8).

NBT staining for $O_2^-$ was seen throughout the culture period in the root cap of the primary root. Moreover, a zone of NBT staining was seen in the root elongation zone. There was less NBT staining within this zone if the roots were grown in the presence of auxin. On the third day of culture, NBT staining in the elongation zone of the roots of the auxin-treated plants was not observed (Fig. 8H"). In the proximal part of the root, on the second day of culture NBT selectively stained lateral root primordia, and the tips of the lateral roots (Fig. 8).
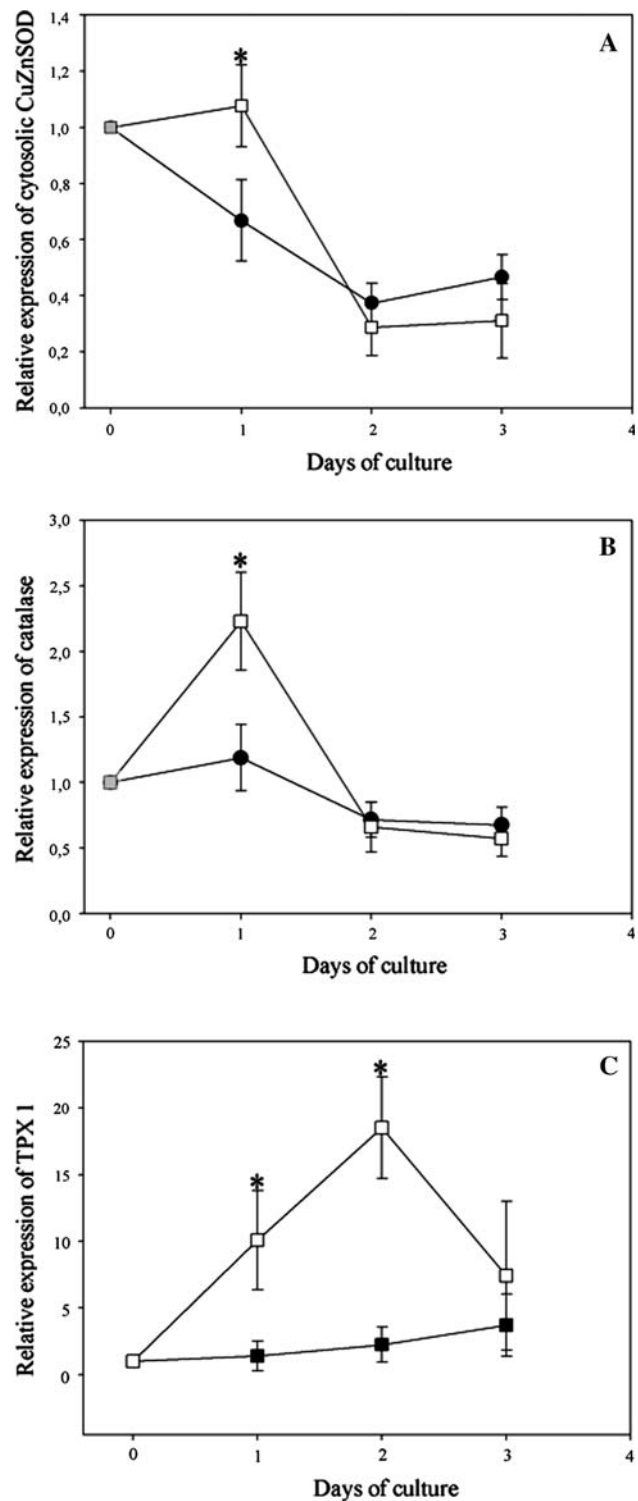
**Fig. 6** Relative expression of cytosolic CuZnSOD (**a**), catalase (**b**), and TPX1 gene coding for cell wall peroxidase (**c**) in the roots of tomato seedlings grown on BM (*filled symbols*) or in the presence of 1 µM IAA (*empty symbols*). *Asterisk* denotes significant differences from control with $P < 0.05$
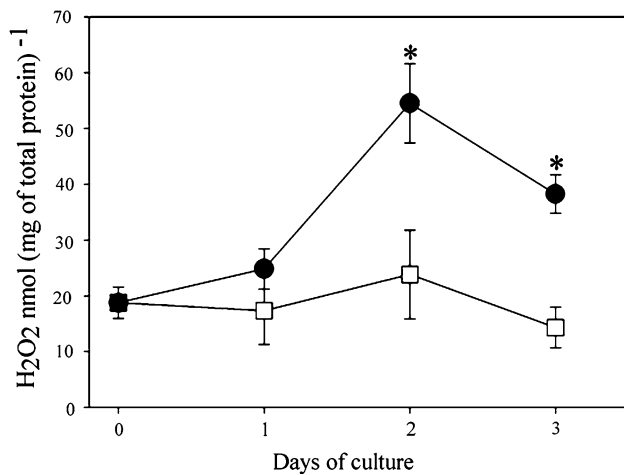
**Fig. 7** Changes in the $H_2O_2$ level in the roots of tomato seedlings during the culture on BM (*filled symbols*) or in the presence of 1 μM IAA (*empty symbols*). *Asterisk* denotes significant differences from control with $P < 0.05$

To localize ROS in the roots more precisely optical sections were obtained using a confocal microscope. The roots were stained with DCFH which is oxidized by ROS to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Because DCFH does not penetrate into the plasma membrane; it was applied in the form of DCFH-diacetate, which can cross the plasma membrane and after being deacetylated by endogenous esterase, liberates DCFH in the cytoplasm (Hempel et al. 1999). DCFH exhibits selectivity for $H_2O_2$; nevertheless the assay provides an integral assay for several ROS because it is likely that in vivo, other radical species are quickly converted to the more stable $H_2O_2$ (Rodriguez et al. 2002). The apices of the primary roots of the control plants showed the highest fluorescence in the root cap, distal part of the apical meristem, and the epidermis. ROS production was also observed in the elongation zone of the roots but was restricted to peripheral tissues (Fig. 9a). In contrast to the roots of the control
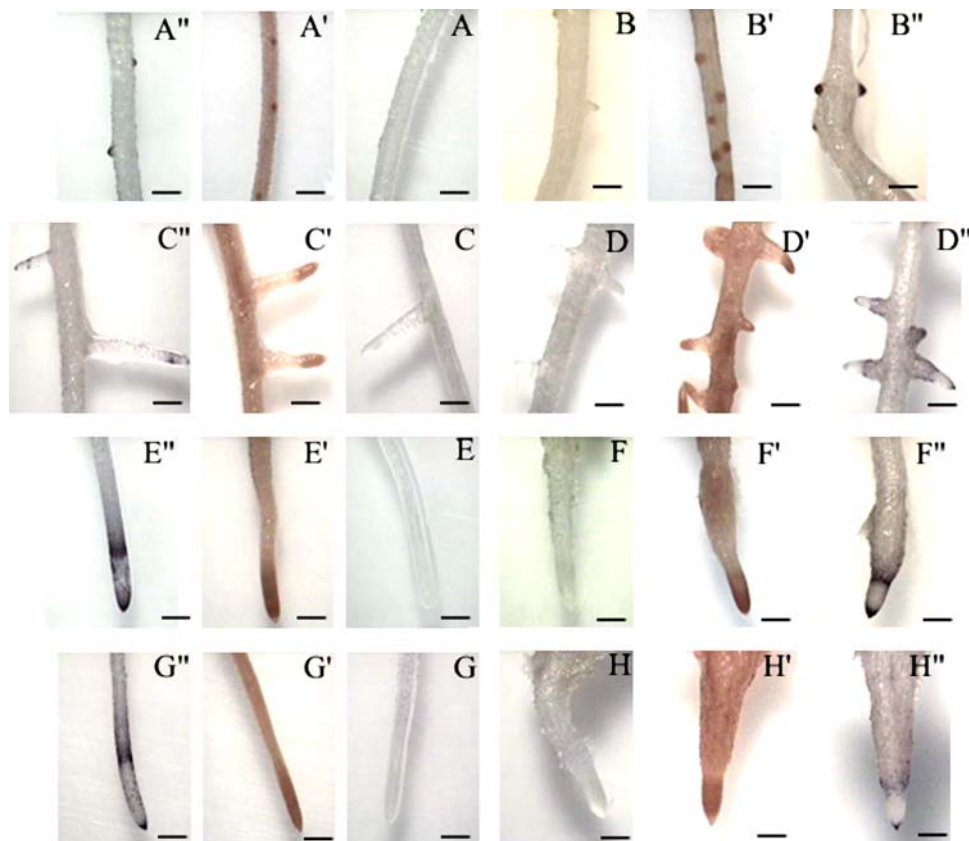


**Fig. 8** Light micrographs showing NBT (indicative of $O_2^-$) and DAB (indicative of $H_2O_2$) staining in the proximal and distal parts of the roots of tomato seedlings cultured on BM or on the medium supplemented with 1 μM IAA. (**a, a′, a″**) No stained control, DAB staining and NBT staining in the proximal parts of roots cultured 2 days on BM. (**b, b′, b″**) No stained control, DAB staining and NBT staining in the proximal parts of roots cultured 2 days on 1 μM IAA. (**c, c′, c″**) No stained control, DAB staining and NBT staining in the proximal parts of roots cultured 3 days on BM. (**d, d′, d″**) No stained control, DAB staining and NBT staining in the proximal parts of roots cultured 3 days on 1 μM IAA. (**e, e′, e″**) No stained control, DAB staining and NBT staining in the distal parts of roots cultured 2 days on BM. (**f, f′, f″**) No stained control, DAB staining and NBT staining in the distal parts of roots cultured 2 days on 1 μM IAA. (**g, g′, g″**) No stained control, DAB staining and NBT staining in the distal parts of roots cultured 3 days on BM. (**h, h′, h″**) No stained control, DAB staining and NBT staining in the distal parts of roots cultured 3 days on 1 μM IAA. *Scale bars* 1 mm
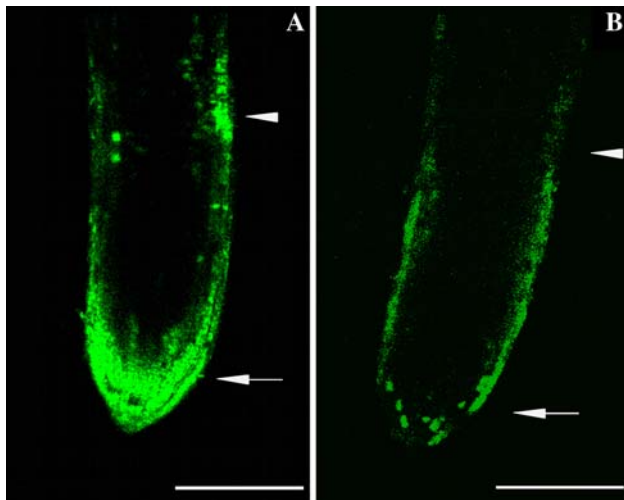
**Fig. 9** DCF fluorescence in the primary root apex of tomato seedling cultured 3 days on BM (**a**) or in the presence of 1 μM IAA (**b**). The *arrows* indicate apical root meristem, the *arrowheads* indicate the primary root elongation zone. *Scale bars* 300 μm

plants, in the roots of the auxin-treated seedlings, ROS did not accumulated in the meristem and root cap. DCF fluorescence was still present in the epidermis but was absent in the elongation zone (Fig. 9b).

In the proximal part of the root, both in the control plants and the auxin-treated ones, the highest DCF fluorescence was observed in the cells adjoining the basal part of the lateral root primordia. ROS also accumulated in the
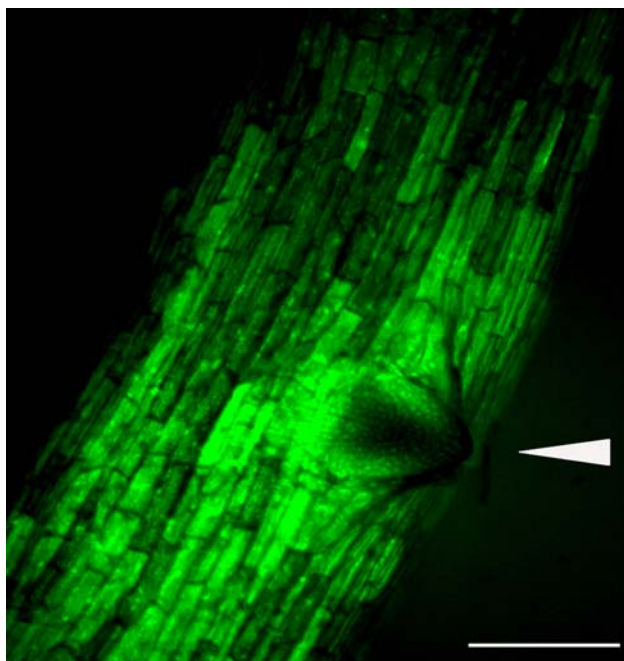


**Fig. 10** DCF fluorescence in the proximal zone of the tomato seedling root cultured 3 days on BM. *Arrowhead* indicates lateral root primordium. *Scale bars* 300 μm

lateral parts of the root primordia, while the central and apical part of the primordium was ROS-free (Fig. 10).

## Discussion

In this study we present the evidence that exogenous auxin in a synchronized manner affects the activities of the important enzymes of the redox metabolism in the roots of tomato seedlings. It was shown that auxin transiently stimulated the activities of CuZnSOD, CAT, FPOX, and soluble POX (Figs. 1, 2, 3, 4, 5). The rise in the enzyme's activity was preceded by an increase in the levels of transcript coding for the aforementioned enzymes (Fig. 6). However, it should be kept in mind that IAA is rapidly degraded in the presence of peroxidase-containing material and it is questionable that 1 μM IAA survives 3 days of culture. IAA decomposition in the culture medium is a possible reason of the transient character of the effect of hormone treatment on gene expression and enzyme activity.

The rise in the SOD activity may result in the increased production of $H_2O_2$ (Mittler 2002). However, we found that the $H_2O_2$ level in roots was lower if plants were grown in the presence of auxin when compared to the control ones (Fig. 7). The decrease in the $H_2O_2$ level in the roots exposed to exogenous auxin (Fig. 7) may be explained by the increase in the activities of the $H_2O_2$ consuming enzymes, especially CAT and POX. The stimulatory effect of auxin on SOD and CAT activity was restricted to the distal part of the root (Fig. 3). In our previous study we reported on the fact that auxin stimulates the activity of ascorbate peroxidase (APX). In contrast to CAT, auxin induced an increase in the activity of this enzyme in the proximal part of the root (Tyburski et al. 2008).

The auxin-triggered increase in the peroxidase activity may result in the cell wall rigidification as a result of the cross-linking of several cell wall compounds as extensins and polysaccharide-linked ferulates (Brett and Waldron 1996, Sanchez et al. 1996). It was shown that auxin stimulated the activity of the cell wall associated FPOX (Fig. 5) and the expression of the TPX1 gene (Fig. 6c) which was reported to encode the cell wall-targeted peroxidase supposed to be involved in cell wall cross-linking (Medina et al. 1999). These findings raise the idea that root growth inhibition by exogenous auxin may be related to the formation of the diferulic linkages between cell wall polysaccharides. However, the reduction in the root elongation rate usually occurs within 1 h of auxin treatment (Liszkay et al. 2004). In our studies, after 1 day of culture, roots of the auxin-treated plants were significantly shorter than the control ones (Table 1), whereas the auxin-dependent increase in the FPOX, as well as CuZnSOD and CAT, activity was detected after 2 days of culture. This finding

suggests, that the observed auxin effects on enzyme activities are not directly related to root growth inhibition.

NBT staining revealed $O_2^-$ production in the root elongation zone and in the root cap (Fig. 8). A similar pattern of NBT staining was observed by Liszkay et al. (2004) in the root tips of maize. We observed that NBT staining in the growing zone of the root and DCF fluorescence in the root apical meristem were reduced in the auxin-treated plants when compared to the relevant zone of the control plant roots (Figs. 8g'', h'', 9). The reduction in NBT staining is accompanied by swelling of the growth zone in auxin-treated roots. This finding is explained as a result of the auxin-induced ethylene formation, which occurs in roots exposed to supraoptimal IAA concentration (Chadwick and Burg 1970). The decrease in the $O_2^-$ production may be explained by the auxin-induced rise in SOD activity in the apical parts of roots (Fig. 1). It was already shown that the activity of apoplastic SOD may affect the concentrations of ROS in the cell wall (Ogawa et al. 1997; Schopfer et al. 2001; Karlsson et al. 2005).

The decrease in the $H_2O_2$ content resulting from auxin treatment is accompanied by a radical change in the pattern of ROS distribution in the root apex. In control plants, ROS localization with DCFH revealed the presence of ROS in the root apical meristem, whereas in the roots grown in the presence of auxin, ROS were localized only within a few cells of the meristem (Fig. 10). This finding is consistent with data reporting the antagonistic effects of auxin and ROS on cell cycle progression (Hirt 2000; Kovtun et al. 2000).

In conclusion, the data reported in the present study demonstrates that the exposition of the roots of tomato seedlings to exogenous auxin is followed by the changes in the pattern of ROS distribution in the root apex and the intensified $H_2O_2$ consumption in the roots. The effect of auxin is mediated by the ROS-scavenging enzymes, namely: CAT, CuZnSOD, and POX which respond to the presence of the exogenous hormone with the increase in the gene expression and activity levels.

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