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Oxidative Stress in Horseradish (*Armoracia lapathifolia* Gilib.) Tissues Grown *in vitro*

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Summary

In a previous study it was reported that transformed tissue of horseradish (*Armoracia lapathifolia* Gilib.), obtained by infection of leaf explants with *A. tumefaciens*, developed two tumour lines with different morphology. One line grew as a completely unorganized tissue (TN – tumour tissue), while the other line grew as a partially organized teratogenous tumour with malformed hyperhydric shoots (TM – teratoma tissue), but did not regenerate the whole plant of normal morphology. The factor responsible for this problem could be the increased production of reactive oxygen species (ROS). Therefore, in this study a possible involvement of activated oxygen metabolism in dedifferentiation and hyperhydricity in TM and TN tissues is investigated. Elevated values of malondialdehyde and protein carbonyl contents found in TM and TN, in comparison with plantlet leaf, confirm the presence of oxidative stress. However, lower H_2O_2 content was measured in TM and TN. Lipoxygenase (LOX) activity was more pronounced in TM and especially in TN compared to leaf, which suggests that the LOX-dependent peroxidation of fatty acids might be one of the causes of oxidative damage. Moreover, significantly higher peroxidase (PRX) and ascorbate peroxidase (APX) activity as well as the increased number of their isoforms was found in transformed TM and TN in comparison with leaf. On the other hand, significantly lower superoxide dismutase (SOD) activity was found in TM and TN, which correlates with lower H_2O_2 content. High catalase (CAT) activity measured in leaf and partially organized TM is consistent with the role of CAT in growth and differentiation. In conclusion, in horseradish transformed tissues that underwent dedifferentiation and hyperhydricity, prominent oxidative damage was found. This result suggests that oxidative stress could be associated with the inability of partially organized teratogenous TM to regenerate plantlets with normal morphology.

Key words: antioxidative enzymes, horseradish, isoenzyme pattern, oxidative stress, tissue culture, tumour tissue

Introduction

Oxidative stress is a common physiological stress. Reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are major agents causing oxidative stress (*1*). Their uncontrolled production can significantly affect plant cell growth and metabolism leading to the damage of proteins, membrane lipids, nucleic acids, and chlorophyll directly or through the formation of secondary toxic substances (*2*), which, in turn, can lead to a decrease in plant productivity. To prevent

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the harmful effects of ROS, plants activate antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), peroxidase (PRX), and ascorbate peroxidase (APX), and stimulate the production of antioxidant molecules including ascorbic acid, glutathione and α-tocopherol, which scavenge ROS (*3*). This integrated system prevents oxidative damage in general, and it is therefore a common component of the response of plants to numerous stresses. It has been shown that artificial environmental conditions of *in vitro* culture may alter oxidative metabolism and predispose tissues to the damaging effects of ROS (*1*). Namely, in plants grown in tissue culture, some of the protective systems against ROS can be disrupted, which is followed by an increased level of these highly reactive molecules (*3*). This is then reflected in alterations of antioxidative enzyme activities and antioxidants (*2*,*4*–*6*), and subsequently in altered morphology of plant tissue grown *in vitro* (*7*). Some of the *in vitro* grown plants become abnormal with a translucent aspect due to chlorophyll deficiency and high water content. This phenomenon is called hyperhydricity, which can irreversibly evolve towards the loss of the regenerative ability of the tissue and is also characterized as a juvenilization process (*4*). The excessive water accumulation in plant tissue, the most characteristic symptom of hyperhydricity, can generate aeration stress, which depletes oxygen levels in the cells (*8*) and therefore brings tissues to the near-hypoxia state (*4*). In plants, a deficiency in oxygen dramatically reduces the efficiency of cellular adenosine triphosphate (ATP) production, which has diverse ramifications for cellular metabolism and developmental processes (*9*). The presence of oxidative stress has already been reported in hyperhydric tissues of several plant species (*2*,*5*, *6*,*10*).

Previously, in an attempt to obtain transformed plants of horseradish (*Armoracia lapathifolia* Gilib.), leaf explants of the plantlets were transformed with a wild strain B6S3 of *Agrobacterium tumefaciens* (*11*). Subcultured on hormone-free MS medium, transformed tissues continued to grow either as a completely unorganized tissue which never expressed any morphogenic capacity (TN – tumour tissue) or as teratogenous tumour (TM – teratoma tissue) with malformed hyperhydric shoots. TM spontaneously formed unorganized tissue, which subsequently produced new shoots, but never developed roots (*12*). This inability of plant tissues to respond to culture manipulations for the desired outcome severely limits the number of transgenic plants that can be regenerated. The factor responsible for this problem could be the production of ROS, which can cause growth inhibition, cell death, or alter plant metabolic pathways leading to poor regeneration of plants (*1*). Altered metabolism and the stress-induced morphogenic response are part of a general acclimation strategy, whereby plant growth is redirected to diminish stress exposure (*13*). Biochemical markers of morphogenesis, investigated in our previous study, indicate that horseradish dedifferentiated TM and TN tissues might be exposed to oxidative stress (*14*). Therefore, the objective of the present study is to establish the presence of oxidative stress and to compare the activities of antioxidative enzymes in horseradish normal tissue (leaf) and dedifferentiated transformed tissues (TM and TN) grown *in vitro*.

Materials and Methods

Horseradish tissue culture

Horseradish plantlets and both transformed tissue lines, tumour (TN) and teratoma (TM), were propagated *in vitro* on a solid Murashige and Skoog (MS) nutrient medium (*15*) containing 8.5 g/L of agar and 30 g/L of glucose without any growth regulator. Culture conditions were: 24 °C, 16-hour light/8-hour dark period and irradiation of 33 μmol/(m^2·s). Tissues were subcultured every three weeks. Twelve days after being transferred to the fresh nutrient medium, tissue explants were used for extract preparation. The transformed character of TN and TM cells had previously been confirmed by testing octopin production and by PCR amplification of a 336-bp fragment of the *6a* gene from TL-DNA (*12*).

Lipid peroxidation, hydrogen peroxide and carbonyl content

The level of lipid peroxidation was determined according to the modified method of Heath and Packer (*16*). Tissue extracts were prepared by grinding 50 mg of lyophilized tissue in 1.5 mL of 50 mM potassium phosphate buffer, pH=7.0. The homogenates were centrifuged at 20 000×*g* and 4 °C for 15 min. A volume of 400 μL of supernatant was mixed with 600 μL of 0.3 % 2-thiobarbituric acid in 10 % trichloroacetic acid (TCA). After heating at 95 °C for 30 min, the mixture was cooled in an ice bath and centrifuged at 10 000×*g* for 10 min. The absorbance of the supernatant was measured at 532 nm and correction for unspecific turbidity was done by subtracting the absorbance at 600 nm. The content of lipid peroxides was expressed as total 2-thiobarbituric acid reactive metabolites (TBARS), mainly malonyldialdehyde (MDA) per mg of protein using a molar absorption coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide was extracted by homogenizing 40 mg of fresh tissue in 1 mL of ice-cold acetone (*17*). After centrifugation at 10 000×*g* and 4 °C for 10 min, the supernatant was mixed with titanium reagent (Fluka, Darmstadt, Germany) and ammonium solution to precipitate the titanium-hydroperoxide complex. The mixture was centrifuged at 10 000×*g* and 4 °C for 10 min and the precipitate was dissolved in 2 M H_2SO_4 and then recentrifuged. The absorbance of the supernatant was measured at 415 nm. The amount of H_2O_2 was calculated using a standard curve prepared with known concentrations of H_2O_2 and expressed per g of fresh mass.

For carbonyl quantification, the reaction with 2,4-dinitrophenylhydrazine (DNPH) was used basically as described by Levine *et al.* (*18*). Lyophilized tissue was homogenized in 50 mM potassium phosphate buffer, pH=7.0, as described for LOX activity. After centrifugation at 20 000×*g* and 4 °C for 15 min, the supernatants (200 μL) were combined with 300 μL of 10 mM DNPH in 2 M HCl. After 1-hour incubation at room temperature, the proteins were precipitated with cold 10 % TCA and the pellets were washed three times with 500 μL of ethanol/

ethylacetate (1:1 by volume) to remove excess reagent. The precipitated proteins were finally dissolved in 6 M urea prepared in 20 mM potassium phosphate buffer (pH=2.3) and the absorption at 370 nm was measured. Protein recovery was estimated for each sample by measuring the absorbance at 280 nm. Carbonyl content was calculated using a molar absorption coefficient for aliphatic hydrazones of 22 mM^{-1} cm^{-1} and expressed per mg of protein.

Assays of enzymatic activities

Total soluble proteins were extracted by grinding 50 mg of lyophilized tissue in 1.5 mL of 50 mM potassium phosphate buffer, pH=7.0. The buffer used for the extraction of APX was supplemented with 0.5 mM ascorbate. The insoluble polyvinylpyrrolidone (PVP) was added to tissue samples in the proportion of 1:5 (10 mg of PVP to 50 mg of lyophilized tissue) prior to grinding in the mixer mill (MM 200, Retsch, Haan, Germany) for 3 min at 30 Hz. The homogenates were centrifuged at 20 000×*g* and 4 °C for 15 min. The supernatants were transferred to the centrifugal filter devices (Centricon®, 10 MWCO, Millipore, Billerica, MA, USA) and centrifuged at 5 000×*g* and 4 °C for 60 min. The obtained filtrate was centrifuged again for 10 min at 20 000×*g* at 4 °C. The supernatant was collected and protein content was determined according to Bradford (*19*) using bovine serum albumin as a standard. The obtained supernatants were used for the following enzyme assays.

LOX (EC 1.13.11.12) activity was determined according to Axelrod *et al.* (*20*). The 1-mL reaction mixture contained 50 mM phosphate buffer (pH=7.0), 0.5 mM linoleic acid, and 20 μL of crude extract. Increase in absorbance due to the formation of conjugated diene fatty acid hydroperoxide was recorded at 234 nm (ε=25 mM^{-1} cm^{-1}). The activity was expressed as μmol of product per min per mg of protein.

PRX (EC 1.11.1.7) activity was assayed by monitoring the increase in absorbance at 430 nm due to the oxidation of pyrogallol (ε=2.6 mM^{-1} cm^{-1}), as described by Nakano and Asada (*21*). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH=7.0), 20 mM pyrogallol, 5 mM H_2O_2, and 20 μL of enzyme extract. PRX activity was expressed as μmol of purpurogallin (product of pyrogallol oxidation) per min per mg of protein.

APX (EC 1.11.1.11) activity was measured in the presence and absence of the specific inhibitor *p*-hydroxy mercury benzoic acid (*p*-HMB), which inhibits class I APX (*22*). The activity was determined by the decrease in absorbance at 290 nm (ε=2.8 mM^{-1} cm^{-1}), as described by Nakano and Asada (*21*). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH=7.0), 0.5 mM ascorbate, 10 mM H_2O_2, and 120 μL of enzyme extract. In the inhibition assay the final concentration of *p*-HMB was 0.5 mM. APX activity was expressed as μmol of oxidized ascorbate per min per mg of protein.

CAT (EC 1.11.1.6) activity was assayed by measuring the decrease in absorbance at 240 nm (ε=36 mM^{-1} cm^{-1}), according to Aebi (*23*). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH=7.0), 20 mM H_2O_2, and 100 μL of enzyme extract. CAT activity was expressed as μmol of decomposed H_2O_2 per min per mg of protein.

SOD (EC 1.15.1.1) activity was determined by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (*24*). The reaction mixture was composed of 13 mM methionine, 0.075 mM NBT, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.002 mM riboflavin, and different volumes of enzyme extract in 50 mM phosphate buffer (pH=7.8). The mixture was placed in a light box for 14 min. The increase in absorbance due to formazan formation was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50 %.

Electrophoretical separation of isoenzymes

Tissue extracts were analyzed electrophoretically under native conditions using vertical polyacrylamide 10 % (by mass per volume) slab gels with the buffer system of Laemmli (*25*). Approximately equal amounts of proteins, 100 μg per well, were loaded and electrophoresis was performed at 4 °C. For the separation of APX isoenzymes, the running buffer contained 2 mM ascorbate and the gel was prerun for 30 min.

For PRX detection, the gels were equilibrated with 50 mM potassium phosphate buffer (pH=7.0) for 30 min, then incubated in 50 mM potassium phosphate buffer (pH=7.0) containing 20 mM pyrogallol and 4 mM H_2O_2, until brown bands appeared (*26*).

APX activity was assayed according to Mittler and Zilinskas (*27*). Firstly, the gel was equilibrated with 50 mM potassium phosphate buffer (pH=7.0) and 2 mM ascorbate for a total time of 30 min; the equilibration buffer was changed every 10 min. The gel was subsequently incubated for 20 min in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2; in the inhibition assay the final concentration of 0.5 mM *p*-HMB was applied. Prior to gel incubation, H_2O_2 was added to the solution. The gel was briefly washed in 50 mM potassium phosphate buffer (pH=7.0) and incubated in the potassium phosphate buffer (pH=7.8) containing 28 mM N,N,N′,N′-tetramethylethylenediamine (TEMED) and 2.45 mM NBT for 15 min. When achromatic bands appeared, the reaction was stopped by a brief wash with distilled H_2O.

For CAT detection, the gels were incubated in distilled water for 45 min and then in 0.003 % H_2O_2 solution for 10 min. The gels were then washed in distilled water and stained in a mixture of 2 % $FeCl_3$ and 2 % $K_3Fe(CN)_6$ for 10 min (*28*).

After electrophoresis, a modified photochemical method of Beauchamp and Fridovich (*24*) was used to detect SOD activities on the gels. The gel was soaked in 50 mM potassium phosphate buffer (pH=7.8) containing 2.45 mM NBT for 25 min, and then in the dark in the same buffer containing 28 mM TEMED and 0.028 mM riboflavin for 15 min. The gel was illuminated in the same solution for 10–20 min with gentle agitation to initiate the photochemical reaction. The enzymes appeared as colourless bands on a purple background. Assignment of SOD isoforms was performed by selective inhibition of gels in 2 mM potassium cyanide (inhibition of Cu/Zn-SODs) or 5 mM H_2O_2 (inhibition of Fe-SODs

and Cu/Zn-SODs) for 30 min before staining for SOD activity.

Gels were scanned using an HP ScanJet 3400C scanner (Hewlett-Packard Company, Palo Alto, CA, USA). The data are representative of two independent experiments.

Statistics

All results were expressed as means of at least six replicates from three experiments followed by corresponding standard errors. Data were statistically compared by analysis of variance (ANOVA) using the STATISTICA v. 8.1 software package (StatSoft, Inc, Tulsa, OK, USA), and differences between the means were considered statistically significant at $p<0.05$ by the least significant difference (LSD) test.

Results

Lipid peroxidation, hydrogen peroxide and carbonyl content

The measurement of MDA, H_2O_2, and protein carbonyl contents as parameters of oxidative stress showed significant differences among different horseradish tissues. MDA and carbonyl content were significantly higher in TM and TN in comparison with the leaf (Table 1). Interestingly, the H_2O_2 content was the highest in the leaf, while the lowest value was obtained in the TM tissue (Table 1). In parallel with the high content of MDA and carbonyl, LOX activity was significantly elevated in TM and particularly in TN, whereas the leaf had almost three times lower activity than TN (Table 1).

Assays of enzymatic activities and isoenzyme pattern

Analyses of antioxidative enzymes (PRX, APX, SOD and CAT) also demonstrated significant differences in their activities among the analyzed horseradish tissues. In comparison with leaf, TM and TN exhibited significantly higher PRX activity, which was the highest in TN tumour (Fig. 1a). In total, five isoperoxidases were resolved on the gel and marked as P1–P4 according to their rising mobility towards the anode (Fig. 1b). In-gel PRX activity was in accordance with the spectrophotometrically obtained results; while leaf exhibited only 3 faint PRX isoforms (P1, P2 and P4), increased number of isoenzymes was noticed in TM (five; P1–P5) and TN (four; P1, P3–P5) with strong staining intensity (Fig. 1b).

In the absence of *p*-HMB inhibitor, TM and TN exhibited significantly higher APX activity than the leaf

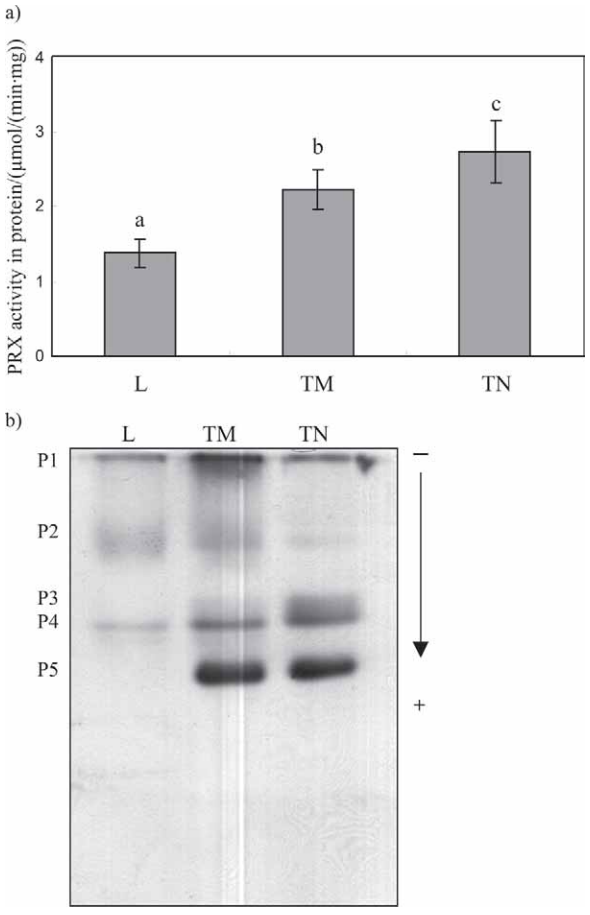

Fig. 1. PRX activity in horseradish tissues: a) values are mean± S.E. based on six replicates. Bars with different letters are significantly different according to LSD test at $p<0.05$; b) isoenzyme pattern of PRX in horseradish tissues. Equal amounts of proteins (50 μg) were loaded on the gel. L – leaf, TM – teratoma tissue, TN – tumour tissue

(Fig. 2a). The inhibition assay revealed significantly higher activity of *p*-HMB-insensitive class III APX in TM in comparison with leaf, while the highest value was obtained in TN. The higher activity of *p*-HMB-sensitive class I APX was again obtained in TM and TN; the ratios between different tissues were similar to those obtained without an inhibitor (Fig. 2a). Native polyacrylamide gel electrophoresis separated ten ascorbate isoperoxidases. They were marked as APX1–APX10 (Fig. 2b). Isoform APX6 was present in all investigated tissues. APX7 band was present only in the leaf extract, while APX5 isoform

Table 1. Differences in H_2O_2, MDA and protein carbonyl content, and LOX activity in horseradish leaf (L), teratoma tissue (TM) and tumour tissue (TN)

		In protein		
	H_2O_2 content in fm	MDA content	Carbonyl content	LOX activity
	μmol/g	nmol/mg	nmol/mg	μmol/(min·mg)
L	$(78.96\pm2.78)^a$	$(4.94\pm0.25)^b$	$(399.19\pm20.23)^c$	$(0.024\pm0.002)^c$
TM	$(0.061\pm0.02)^c$	$(7.24\pm0.21)^a$	$(587.98\pm44.20)^b$	$(0.042\pm0.003)^b$
TN	$(10.74\pm1.28)^b$	$(7.50\pm0.54)^a$	$(693.56\pm30.14)^a$	$(0.067\pm0.004)^a$

Means±S.E., $N=6$; values marked with different letters are significantly different according to LSD test at $p<0.05$; fm=fresh mass

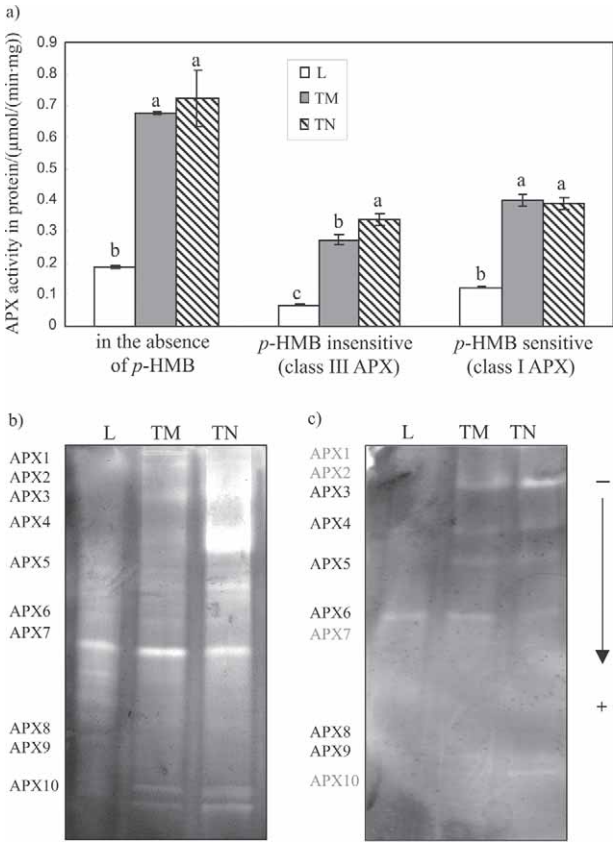

Fig. 2. APX activity in horseradish tissues: a) values are mean± S.E. based on six replicates. Bars with different letters are significantly different according to LSD test at $p<0.05$; b) isoenzyme pattern of APX in horseradish tissues; c) isoenzyme pattern of APX in horseradish tissues after inhibition assay with 0.5 mM *p*-HMB. Present APX isoforms are marked with black letters (APX) and missing isoforms are marked with grey letters (APX). Equal amounts of proteins (50 μg) were loaded on the gel. L – leaf, TM – teratoma tissue, TN – tumour tissue

was characteristic for TN. TM and TN tissues exhibited common isoforms of APX3, APX4 and APX8–10, although the staining of APX3 and APX4 was stronger in TN tissue. In the *p*-HMB inhibition assay, APX1, APX2, APX7 and APX10 (class I APX) isoforms were missing, whereas the staining intensity of APX3 and APX4 bands was weaker (Fig. 2c) in comparison with the intensity without inhibitor in TN tissue (Fig. 2b).

The highest SOD activity was obtained in the leaf extract, while both TM and TN had significantly lower values (Fig. 3a). Five SOD isoenzymes were separated electrophoretically and marked as SOD1–SOD5 (Fig. 3b). Isoforms SOD2 and SOD3 were detected in all tissues. SOD4 and SOD5 isoenzymes were revealed only in the leaf while SOD1 isoform was characteristic for transformed tissues (TM and TN). SOD inhibitors were applied to allow the identification of Mn-SOD, Fe-SOD, and Cu/Zn-SOD isoenzymes. Incubation of gels in 3 mM potassium cyanide or 5 mM H_2O_2 before staining for SOD activity identified isoenzymes SOD2 and SOD3 as Mn-SOD (no inhibition by KCN and H_2O_2), isoenzymes SOD4 and SOD5 as Cu/Zn-SOD (inhibition by both KCN and H_2O_2), and SOD1 as Fe-SOD (no inhibition by KCN) (Fig. 3c).

The lowest CAT activity was measured in the TN. Significantly higher value was obtained in TM and especially in leaf, in which CAT activity was five times higher than in the TN (Fig. 4a). Electrophoretic analysis revealed two CAT isoforms whose staining intensities were in accordance with the data obtained by spectrophotometric measurements; CAT1 isoenzyme was common to all horseradish tissues with the highest staining intensity in the leaf extract, while the CAT2 band was detected only in the TM (Fig. 4b).

Discussion

ROS have an important role in the metabolism and development of aerobic organisms and, although they can induce considerable cellular damage, these molecules are also important signalling compounds (*13*). Studies of a diverse range of *in vitro* plant systems have shown that they produce ROS which then may affect the morphogenic responses of cells grown *in vitro* (*7*). Previously, biochemical markers of morphogenesis analyzed by means of peroxidase and phenylalanine-ammonia lyase activity suggested the possible presence of oxidative stress in horseradish partially organized teratoma with hyperhydric shoots (TM) and completely unorganized tumour (TN) tissues (*14*). In this study, the measurements of MDA and protein carbonyl contents showed significantly elevated values in TM and TN, hence confirming the presence of oxidative stress in these tissues. Similar results were obtained in tumour tissue of cactus *M. gracilis* (*6*). Moreover, hyperhydric shoots of *Euphorbia milii* (*5*), *Prunus* (*29*), carnation (*2*) and *M. gracilis* (*6*) also exhibited higher contents of MDA and protein carbonyl, which is in correlation with hyperhydric morphology observed in the shoots of TM. These results suggest that oxidative stress occurs in tissues undergoing dedifferentiation and hyperhydricity, which consequently causes cellular damages in *in vitro* grown tissues. Oxidative damage of membranes leads to a loss of function, and, eventually, to a breakdown in structural integrity, which can subsequently lead to changes in tissue morphology (*1*). Changes in the membrane composition occur by peroxidation of lipids, which results from the enzymatic activity of LOX or from autocatalytic oxidation by activated oxygen species (*4*). Studies of lipid peroxidation in plant tissue cultures demonstrated that toxic aldehydes are produced during culture initiation and throughout routine subculturing (*1*). Moreover, lipid peroxidation has already been associated with early dedifferentiation processes in *Vitis vinifera* (*7*). In our study, LOX activity was significantly more pronounced in partially organized TM, and especially in completely unorganized TN, in comparison with leaf. This result suggests possible involvement of the lipoxygenase-dependent peroxidation of fatty acids in the observed damages and altered morphology.

Surprisingly, lower H_2O_2 content was measured in TM and TN in comparison with leaf, although it is commonly considered that stress conditions promote enhanced production of H_2O_2. It is well known that H_2O_2 may also serve as a signal for a major developmental process in plants; either inhibition of H_2O_2 production or scavenging it from the system can prevent differentiation events (*30*). In the view of this knowledge, the low

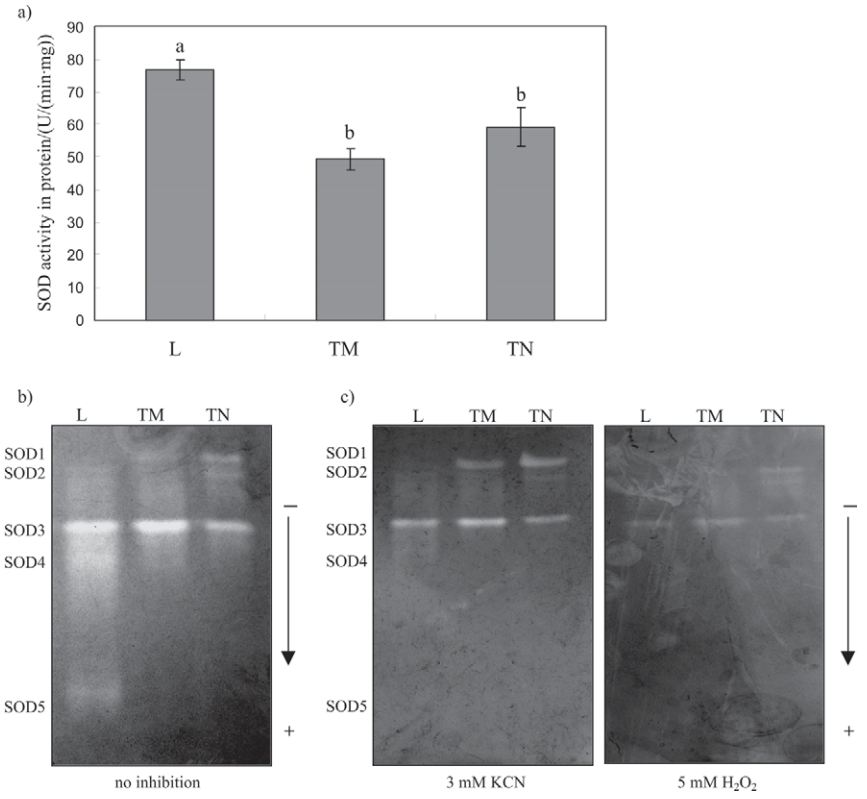

Fig. 3. SOD activity in horseradish tissues: a) values are mean±S.E. based on six replicates. Bars with different letters are significantly different according to LSD test at $p<0.05$; b) isoenzyme pattern of SOD in horseradish tissues. Equal amounts of proteins (50 μg) were loaded on the gel; c) isoenzyme pattern of SOD in horseradish tissues after incubation of gels in 3 mM potassium cyanide or 5 mM H_2O_2 before staining for SOD activity. L – leaf, TM – teratoma tissue, TN – tumour tissue

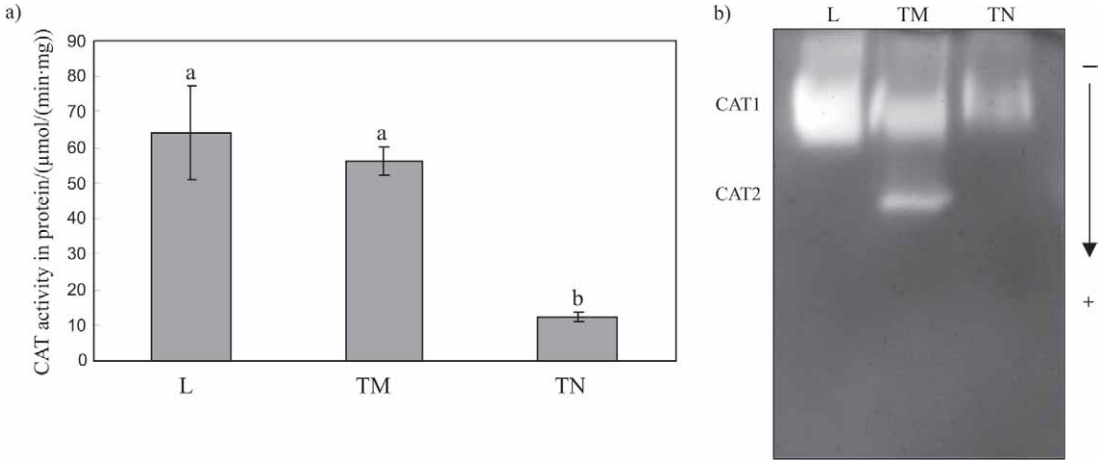

Fig. 4. CAT activity in horseradish tissues: a) values are mean±S.E. based on six replicates. Bars with different letters are significantly different according to LSD test $p<0.05$; b) isoenzyme pattern of CAT in horseradish tissues. Equal amounts of proteins (50 μg) were loaded on the gel. L – leaf, TM – teratoma tissue, TN – tumour tissue

H_2O_2 content found in transformed horseradish tissues could be correlated with their low level of morphological organization. However, these results should be additionally confirmed by other methods, like chemiluminescence, which has greater sensitivity than absorbance measurements (*31*).

It has been reported that auxins can induce a drastic increase in ROS generation in the cell (*32*). Moreover, auxin and oxidative stress complement each other in common stress responses. It has recently been reported that ROS, in concert with auxin, have a role in cell cycle activation of differentiated leaf cells (*33*) and that moderate oxidative stress may also accelerate cell division entry and affect the activity of the cell cycle machinery in cultured plant cells (*34*). Both horseradish transformed tissues, TM and TN, are hormone autonomous and their rapid cell proliferation is promoted by high concentrations of cytokinin and auxin (*11,12*) due to the expres-

sion of the genes present on T-DNA, which direct synthesis of these plant hormones. Among their numerous activities in cells, plant PRXs can act as IAA oxygenases; they catalyze IAA-dependent generation of superoxide radical ($O_2^{\cdot-}$) in the absence of H_2O_2 and thus are involved in H_2O_2-independent mechanism of ROS generation (*35*). Such PRXs have been shown to be an active source of $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$, which could cause oxidative damage (*36*). *In vitro* cultivated callus, hairy roots, tumour and teratoma tissues of horseradish have already been reported to exhibit high peroxidase activity in comparison with normal tissue (*14*,*37*,*38*). In the present paper, significantly higher PRX and APX activity as well as the increased number of their isoforms were found in transformed TM and TN in comparison with horseradish leaf. Therefore, high activity of PRX could be associated with increased ROS generation and appearance of oxidative damage in these tissues. Moreover, the inhibition assay revealed that in the total APX activity the major part belongs to class I APXs, involved in the removal of H_2O_2 in a reaction strictly dependent on ascorbate (*22*), which is in agreement with low H_2O_2 content in these tissues.

The lowest CAT activity was measured in TN tumour, while both leaf and partially organized TM exhibited similar significantly higher values. Horseradish TN tumour grows as an unorganized tissue which never expresses any morphogenic capacity and it lacks typical cell organization (Peharec *et al.*, unpublished data). CAT is known to play a role in growth and differentiation (*39*) and its high activity has been correlated with the process of differentiation that occurred during shoot or root induction (*40*). Similar results were reported in tissue culture of sugarbeet (*41*), gladiolus (*42*) and *Acanthophyllum sordidum* (*43*), where lower CAT activity was found in non-organogenic callus tissues in comparison with the organized shoots.

The level of SOD activity in our investigation was significantly lower in both transformed tissues (TM and TN) in comparison with the horseradish leaf. Since SOD catalyzes dismutation of $O_2^{\cdot-}$ to molecular oxygen and H_2O_2, it is possible that excess superoxide is present in these tissues, although the formation of very destructive hydroxyl (OH^{-}) and hydroperoxyl radicals ($HO_2^{\cdot}$) could not be excluded. Moreover, the results of the SOD activity measurements in different horseradish tissues could be correlated with the obtained H_2O_2 content; the leaf tissue exhibited the highest SOD activity as well as the highest H_2O_2 content, while the lowest values of both SOD and H_2O_2 were obtained in TM tissue.

There is some evidence which indicates that $O_2^{\cdot-}$ and $OH^{\cdot}$, both highly toxic oxygen species, are produced by dedifferentiated plant cultures (*1*,*7*). In our study, a prominent oxidative damage was found in horseradish transformed tissues that are undergoing dedifferentiation and hyperhydricity. Possible cause could be due to the increased PRX activity, which can produce $O_2^{\cdot-}$ and/or $OH^{\cdot}$ (*35*). However, since the LOX-mediated formation of $^1O_2$ or $O_2^{\cdot-}$ has also been reported to appear during plant tissue dedifferentiation (*1*,*44*), the other possible cause could be ascribed to the increased LOX activity. Several lines of correlative evidence suggest that LOXs function in the regulation of plant growth and development (*45*,*46*).

Conclusions

Results obtained in this study confirmed the presence of the prominent oxidative stress in horseradish TM and TN. Increased LOX activity as well as high activity of PRX, found in both transformed tissues, suggest the possible involvement of these enzymes in ROS generation and oxidative damage, which could be linked to hyperhydricity and the loss of tissue organization. These findings indicate that the production of ROS is associated with the inability of partially organized teratogenous TM to regenerate plantlets with normal morphology.

Acknowledgements

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