# Growth Conditions in In Vitro Culture Can Induce Oxidative Stress in *Mammillaria gracilis* Tissues

**Biljana Balen · Mirta Tkalec · Dubravko Pavoković · Branka Pevalek-Kozlina · Marijana Krsnik-Rasol**

**Abstract** In vitro propagated plants of *Mammillaria gracilis* Pfeiff. (*Cactaceae*) develop calli without any exogenous growth regulators. This habituated tissue spontaneously regenerates morphologically normal as well as hyperhydric shoots. In this study, a possible involvement of activated oxygen metabolism in habituation and hyperhydricity in in vitro propagated plants of *Mammillaria gracilis* Pfeiff. (*Cactaceae*) was investigated. Significantly higher malondialdehyde (MDA) and carbonyl contents as well as hydrogen peroxide ($H_2O_2$) production were observed in habituated callus (HC), hyperhydric regenerated shoots (HS), and tumors (TT) in comparison to normal regenerated shoots (NS). Lipoxygenase (LOX) activity showed a similar trend, with a clear increase in activity in HC and HS. The activities of antioxidative enzymes, namely, peroxidase (POX), ascorbate peroxidase (APX), and catalase (CAT), were also higher in HC, HS, and TT, whereas an increase in superoxide dismutase (SOD) activity was observed in HC and HS. The majority of antioxidative isoenzymes were common to all cactus tissues, although a few tissue-specific bands were noticed. Significant decreases in phenylalanine ammonia lyase (PAL) activity, total phenolic content, and lignification were found in HS, HC, and TT in comparison to NS. Our results showed the appearance of a prominent oxidative stress in HC, HS, and TT as well as a strong induction of the antioxidant system indicating that activated oxygen metabolism could be involved in habituation and hyperhydricity and linked to the loss of tissue organization in *M. gracilis*.

**Keywords** Antioxidative enzymes · Habituation · Hyperhydricity · *Mammillaria gracilis* Pfeiff. · Oxidative stress · Tumor tissue

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## Introduction

Native cacti occur exclusively in the New World, which is characterized by long dry periods with short rains. As a result of living in these conditions, cacti developed certain adaptations, including the special physiologic adaptation Crassulacean Acid Metabolism (CAM) photosynthesis. CAM plants are largely dependent upon nocturnal accumulation of $CO_2$ for their photosynthesis because their stomata are closed during the day, retarding water loss. As CAM plants, cacti are highly affected by artificial environmental conditions in the tissue culture (Malda and others 1999). In a water-rich environment their stomata stay open during the day; consequently, photosynthesis is stimulated and photorespiration is more intensive. In vitro propagated plants of *Mammillaria gracilis* Pfeiff. (*Cactaceae*) develop calli without any exogenous growth regulators. In this hormone-independent (habituated) callus, a spontaneous regeneration of morphologically normal and hyperhydric shoots occurs (Krsnik-Rasol and Balen 2001). Hyperhydricity is a physiologic disorder frequently affecting shoots cultured in vitro and is associated with reduced transpiration and excessive water uptake that could

reduce the level of oxygen within tissues to near-hypoxia (Gaspar and others 2000; Franck and others 2004). It can arise as the result of different stressing conditions in in vitro culture such as high humidity, high levels of growth regulators, gas accumulation in the atmosphere of the jars, and light intensity (George 1996). Hyperhydric shoots of *M. gracilis* are translucent, more round in shape, partially covered with an irregular pattern of softer spines, and exhibit high water content and chlorophyll deficiency. The anatomy of these hyperhydric shoots is also altered by a poorly developed cell wall and chloroplast degeneration (Poljuha and others 2003). Habituation and hyperhydricity are both considered to be part of a neoplastic progression leading, in the short term, to a loss of cell differentiation or regenerative ability and, in the long term, to cancer or death of the plant (Gaspar and others 2000). Therefore, cactus cells were transformed using *Agrobacterium tumefaciens* to obtain dedifferentiated tumor tissue which could be compared with habituated and hyperhydric tissues (Krsnik-Rasol and Balen 2001).

In plants growing in in vitro culture some of the protective systems against reactive oxygen species (ROS) can be disrupted, which leads to increased levels of these highly reactive molecules (Olmos and others 1997; Dewir and others 2006). Toxic levels of ROS can generate oxidative stress leading to damage of proteins, membrane lipids, nucleic acids, and chlorophyll (Saher and others 2005). To prevent the harmful effects of ROS, plants activate antioxidative enzyme systems, including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), peroxidase (POX), and ascorbate peroxidase (APX), and stimulate the production of antioxidant molecules such as ascorbic acid and glutathione (Cassells and Curry 2001). Artificial environmental conditions in the tissue culture can also influence and modify tissue growth and induce spontaneous changes from the characteristic organization pattern to unorganized callus that can be reflected in alterations of antioxidative enzyme activities as well as of antioxidants (Cassells and Curry 2001; Franck and others 2004; Saher and others 2004, 2005; Dewir and others 2006). It has been suggested that aberrations in plant tissue culture, such as habituation and hyperhydricity, are induced by oxidative stress (Kevers and others 2004). However, literature about oxidative stress in hyperhydric tissues is scarce and restricted mainly to carnation, *Prunus*, *Narcissus*, and *Euphorbia* (Olmos and others 1997; Franck and others 1998; Chen and Ziv 2001; Dewir and others 2006).

Changes in phenylalanine ammonia lyase (PAL) activity and the accumulation of various phenolic compounds were often observed in plants subjected to various stresses (Ye and others 2006), and it is believed that PAL could be involved in the resistance to a variety of biotic and abiotic

stresses. Hyperhydric tissues are associated with low phenolics and high water content (Perry and others 1999) and are also known to show changes, mostly a decrease, in PAL activity and failure of lignin biosynthesis. It is even suggested that these deficiencies might be responsible for the hyperhydric phenotype (Olmos and others 1997; Saher and others 2004; Dewir and others 2006).

In this study we investigated the possible involvement of activated oxygen metabolism in habituation and hyperhydricity in *M. gracilis* tissue culture. The majority of previous studies were oriented toward establishing the changes in the antioxidative system response in hyperhydric shoots in comparison to normal plants. In addition to comparison between callus-regenerated normal shoots (NS) and hyperhydric shoots (HS), we extended our research to habituated callus (HC) as well as to tumor tissue (TT), for which it was not possible to find any extensive study.

## Materials and Methods

### Plant Material and Water Content

*M. gracilis* plants were propagated in vitro under a 16-h/8-h light/night photoperiod (light intensity = 90 $\mu$E s$^{-1}$ m$^{-2}$) at 24°C on solid Murashige and Skoog (MS) nutrient medium (0.9% agar, 3% sucrose) (Murashige and Skoog 1962) without any growth regulators. In the culture conditions, the majority of cactus plants produced abundant calli. The spontaneously formed calli were detached from the plants and subcultivated on the same nutrient medium every 4 weeks as a hormone-independent habituated callus (HC) tissue (Krsnik-Rasol and Balen 2001). The callus had a snowy surface and a rather compact yellowish or light green inner portion. In the callus culture, which expressed high morphogenic capacity, regeneration of normal shoots (NS) and hyperhydric shoots (HS) occurred simultaneously. NS were green, covered with spines, and had normal growth. HS were translucent, light green, spheral shaped, and partially covered with softer spines (Poljuha and others 2003). The water content in HC and HS was 2.2 and 2.5 times, respectively, higher than in NS. On the surface of the disc-like explants, which were cut from *Mammillaria* plants grown in vitro, suspension of *Agrobacterium tumefaciens* wild-type B6S3 was applied and the primary crown gall tumor was obtained (Krsnik-Rasol and Balen 2001). This primary tumor was subcultivated every 4 weeks and a stable line of tumor tissue (TT) was established. TT was yellowish to orange-brown and never expressed any organogenic potential. It was considered hyperhydric tissue because the content of the water in this tissue was 2.8 times higher than in NS.

Three weeks after subcultivation to the fresh nutrient medium, explants of all cactus tissues were used for extract preparation.

## Lipid Peroxidation

The level of lipid peroxidation was determined according to the modified method of Heath and Packer (1968). Lyophilized tissues (60 mg) were homogenized in 0.25% (w/v) 2-thiobarbituric acid in 10% (w/v) trichloracetic acid. 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 g of dry weight using an extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$.

## Hydrogen Peroxide and Carbonyl Content

$H_2O_2$ was extracted by homogenizing 60 mg of lyophilized tissues in 1 ml of ice-cold acetone (Mukherjee and Choudhari 1983). After centrifugation at 10,000 $g$ and 4°C for 10 min, the supernatant was mixed with titanium reagent (Fluka) 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 dry weight.

For carbonyl quantification, the reaction with 2,4-dinitrophenylhydrazine (DNPH) was used basically as described by Levine and others (1994). 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 h incubation at room temperature, the proteins were precipitated with cold 10% (w/v) trichloroacetic acid and the pellets were washed three times with 500 µl of ethanol/ethylacetate (1/1 v/v) to remove excess reagent. The precipitated proteins were finally dissolved in 6 M urea 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 absorption at 280 nm. Carbonyl contents were calculated using a molar absorption coefficient for aliphatic hydrazones of 22 mM$^{-1}$ cm$^{-1}$ and expressed per g of dry weight.

## 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 extraction of APX was supplemented with 0.5 mM ascorbate. The insoluble PVP (10 mg) was added to tissue samples prior to grinding in the mixer mill (MM200, Retsch, Germany) for 3 min at 30 Hz. The homogenates were centrifuged at 20,000 $g$ and 4°C for 15 min. 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 (1976) using bovine serum albumin as a standard. Obtained supernatants were used for the following enzyme assays.

Lipoxygenase (LOX; EC 1.13.11.12) activity was determined according to Axerold and others (1981). The 1.0-ml reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.5 mM linoleic acid, and 20 µl crude extract. Increase in absorbance due to the formation of conjugated diene fatty acid hydroperoxide was recorded at 234 nm ($\varepsilon = 25$ mM$^{-1}$ cm$^{-1}$). The activity was expressed as µmol of product per min per mg of proteins.

POX (EC 1.11.1.7) activity was assayed by monitoring the increase in absorbance at 430 nm due to the oxidation of pyrogallol ($\varepsilon = 2.6$ mM$^{-1}$ cm$^{-1}$), as described by Nakano and Asada (1981). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 20 mM pyrogallol, 5 mM $H_2O_2$, and 20 µl enzyme extract. POX activity was expressed as µmol of purpurogallin (product of pyrogallol oxidation) per min per mg of proteins.

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 (Ros-Barceló and others 2006). The activity was determined by the decrease in absorbance at 290 nm ($\varepsilon = 2.8$ mM$^{-1}$ cm$^{-1}$), as described by Nakano and Asada (1981). 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 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 proteins.

CAT (EC 1.11.1.6) activity was assayed by measuring the decrease in absorbance at 240 nm ($\varepsilon = 36$ mM$^{-1}$ cm$^{-1}$), according to Aebi (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 20 mM $H_2O_2$, and 100 µl enzyme extract. CAT activity was expressed as µmol of decomposed $H_2O_2$ per min per mg of proteins.

SOD (EC 1.15.1.1) activity was determined by measuring inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). 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%.

For PAL (EC 4.3.1.5) activity, lyophilized cacti tissue (20 mg) was homogenized by grinding in the mixer mill in 2.5 ml of 0.2 M borate buffer (pH 8.8) containing 5 mM 2-mercaptoethanol and 100 mg of PVP. The homogenate was centrifuged at 20,000 $g$ for 20 min at 4°C and the supernatant was applied to a Sephadex G-25 column (2 cm × 10 cm) equilibrated with 0.2 M borate buffer (pH 8.8). The PAL assay was performed according to Saunders and McClure (1975), at 40°C in an assay mixture consisting of 0.5 ml enzyme extract and 0.5 ml of 2% L-Phe in 0.2 M borate buffer (pH 8.8). The activity was assayed by monitoring the increase in 290 nm against a control without L-Phe over a period of 3 h at 20-min intervals. The rate of appearance of trans-cinnamic acid was taken as a measure of enzyme activity using an increase of 0.01 $A_{290}$ equal to 3.09 nmol of trans-cinnamic acid formed. The PAL activity is expressed in pkat trans-cinnamic acid per g of dry weight.

Electrophoretical Separation of Isoenzymes

Tissue extracts were analyzed electrophoretically in native conditions using vertical polyacrylamide 8% (w/v) slab gels with the buffer system of Laemmli (1970). Approximately equal amounts of proteins, 100 μg per well, were loaded and electrophoresis was performed at 4°C. For separation of APX isoenzymes, the running buffer contained 2 mM ascorbate and the gel was prerun for 30 min.

For POX 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 (Chance and Maehly 1955).

APX activity was assayed according to Mittler and Zilinskas (1993). Following electrophoretic separation, 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 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. The $H_2O_2$ was added to the solution

just prior to gel incubation. The gel was briefly washed in 50 mM potassium phosphate buffer (pH 7.0) and incubated in 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 $H_2O_2$ solution (0.003% v/v) for 10 min. The gels were then washed in distilled water and stained in a 1:1 mixture of 2% (w/v) $FeCl_3$ and 2% (w/v) $K_3Fe(CN)_6$ for 10 min (Woodbury and others 1971).

After electrophoresis, a modified photochemical method of Beauchamp and Fridovich (1971) was used to locate SOD activities on gels. The gel was first soaked in 50 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 2.45 mM NBT for 25 min, briefly washed, then soaked in the dark in 100 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 0.028 mM riboflavin for another 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 colorless bands in 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 and the staining intensity was quantified using the image-processing software ImageTool 3.0 (University of Texas, Austin, TX). The data are representative of two independent experiments.

Total Phenolics Content

Total phenolics were extracted from 20 mg lyophilized tissue in the volume of 1 ml of 80% MeOH. Samples were sonicated twice for 10 min at 40°C and centrifuged at 20,000 $g$ for 10 min. Extract solution (0.1 ml) was diluted with 1.5 ml distilled water, 0.1 ml Folin–Ciocalteu reagent was added, and the tube was shaken thoroughly. After 3 min, 0.3 ml of 2% $Na_2CO_3$ solution was added and the mixture was allowed to stand for 30 min at 40°C with intermittent shaking. Absorbance was measured at 765 nm after the mixture was cooled down to room temperature (Slinkard and Singleton 1997). Phenolic content was expressed as μg of gallic acid equivalents per g of dry weight.

Lignin Determination

Lignin and lignin-like polymers were semiquantitatively estimated in the plant material essentially as described by Sancho and others (1996). Cacti tissue (1 g) was rinsed in

boiling water and the insoluble material was pelleted by centrifugation and rinsed again in 100% ethanol. The dry residue was dissolved in 2.5 ml of 18% hydrochloric acid in ethanol for 2.5 h. After this period, 20 μl of 10% phloroglucinol-HCl was added to 1 ml of the previously used solution and the absorbance was recorded at 540 nm after 6 h. The results were expressed as $A_{540}$ per g of dry weight.

## 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 7.1 software package (StatSoft, Inc., Tulsa, OK), and differences between means were considered statistically significant at $p \leq 0.05$ by the least significant difference (LSD) test.

## Results

The measurement of MDA, $H_2O_2$, and carbonyl contents as parameters of oxidative stress showed significant differences between different cactus tissues. MDA, $H_2O_2$, and carbonyl contents were significantly higher ($p \leq 0.05$) in HC, HS, and TT than in NS (Table 1). Moreover, among all cactus tissues the callus revealed significantly the highest MDA and carbonyl contents, which were 3 and 1.5 times higher than in the NS, respectively. In parallel with the high amounts of MDA and carbonyl content, LOX activity was significantly more pronounced in HC and HS, whereas the NS had the lowest LOX activity (Table 1). Again, the HC revealed significantly the highest value and its activity was four times higher than in the NS.

Analyses of antioxidative enzymes (POX, APX, SOD, and CAT) demonstrated significant differences in their activities between different cactus tissues, showing mainly
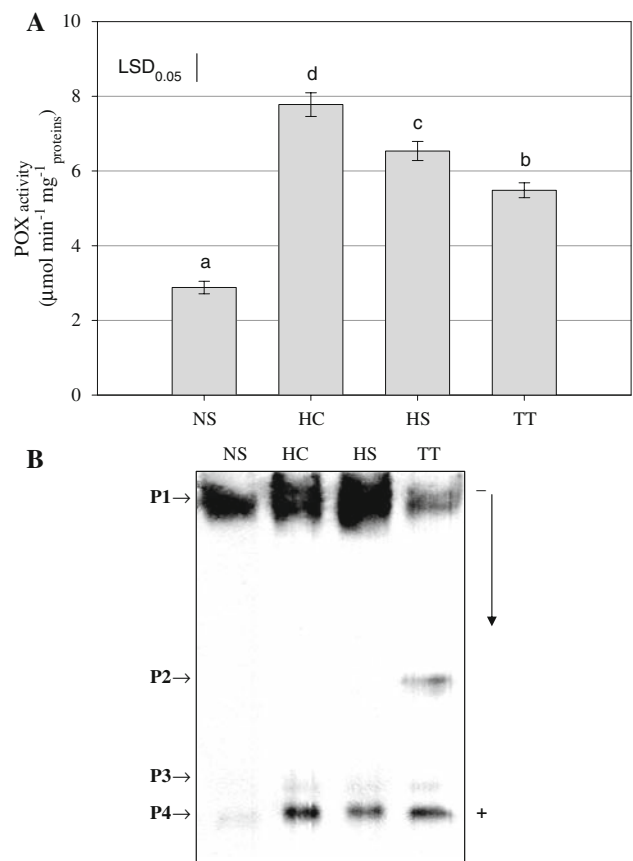


**Fig. 1** **A** POX activity in *M. gracilis* tissues. Values are mean ± SE based on six replicates. Columns marked with different letters are significantly different at $p \leq 0.05$ and the vertical line indicates the LSD value. **B** Isoenzyme pattern of POX in *M. gracilis* tissues. Equal amounts of proteins (100 μg) were loaded on the gel. NS, normal regenerated shoot; HC, habituated callus; HS, hyperhydric regenerated shoot; TT, tumor tissue

increased activities in unorganized and tumor tissues. The highest POX activity was measured in HC, in which POX activity was 2.5 times higher than in the NS, which revealed the lowest values (Fig. 1A). HS had significantly higher activity than TT (Fig. 1A). In total, four

**Table 1** Differences in MDA, $H_2O_2$, and protein carbonyl contents and LOX activity in *M. gracilis* tissues

|  | $H_2O_2$ content (μmol $g^{-1}$ DW) | MDA content (nmol $g^{-1}$ DW) | Carbonyl content (nmol $mg^{-1}$ protein) | LOX activity (μmol $min^{-1}$ $mg^{-1}$ protein) |
|---|---|---|---|---|
| Normal regenerated shoot | 2.36 ± 0.28 a | 69.94 ± 3.38 a | 34.57 ± 0.85 a | 0.07 ± 0,01 a |
| Habituated callus | 4.57 ± 0.13 b | 218.19 ± 7.69 b | 54.54 ± 1.61 b | 0.32 ± 0.05 b |
| Hyperhydric regenerated shoot | 4.11 ± 0.16 b | 163.35 ± 19.10 c | 44.59 ± 1.35 c | 0.22 ± 0.007 c |
| Tumor tissue | 4.19 ± 0.29 b | 144.83 ± 3.53 c | 46.26 ± 2.22 c | 0.11 ± 0.02 a |
| LSD (0.05) | 0.65 | 29.92 | 4.43 | 0.07 |

Values are means ± SE based on six replicates. Different letters in each column indicate significant differences between means at $p \leq 0.05$ according to LSD

DW = dry weight

isoperoxidases were resolved on the gel and marked as P1-P4 according to their rising mobility toward the anode (Fig. 1B). Isoform P1 was present in all cactus tissues as the strongest band, although densitometric analysis showed that the band-staining intensity was stronger in HC and HS than in NS and TT. All tissues were also characterized by the P4 isoperoxidase; however, in NS its intensity was very weak. Isoform P3 was detected as a faint band in HC, HS, and TT, whereas the P2 band appeared only in TT.

In the absence of *p*-HMB inhibitor, HC, HS, and TT exhibited significantly higher APX activity than NS (Fig. 2A). Moreover, the highest value was measured in HC, in which APX activity was two times higher than in the NS. The inhibition assay revealed significantly higher activity of *p*-HMB-insensitive class III APX in TT in comparison to NS. Significantly the highest *p*-HMB-sensitive class I APX activity was again obtained in HC; the ratios between different tissues were similar to those

obtained without inhibitor (Fig. 2A). Native polyacrylamide gel electrophoresis separated six ascorbate isoperoxidases. They were marked as APX1-APX6 (Fig. 2B). The bands APX2-APX6 were common to all cactus tissues, whereas the APX1 band was characteristic for TT only. Isoform APX4 was present in all cactus tissues, but according to densitometric analysis the band-staining intensity was the strongest in HC. In the *p*-HMB inhibition assay, APX2 and APX3 isoforms (class I APX) were missing in all cactus tissues, whereas the staining intensity of APX4 isoform was two to seven times weaker (Fig. 2C) in comparison to the intensity without inhibitor (Fig. 2B).

The highest SOD activity was obtained in HC, whose values were three times higher than in the NS (Fig. 3A). HS had lower SOD activity than did HC but significantly higher than NS and TT. Nine SOD isoenzymes were separated electrophoretically and marked as SOD1-SOD9



**Fig. 2 A** APX activity in *M. gracilis* tissues. Values are mean ± SE based on six replicates. Columns marked with different letters are significantly different at $p \leq 0.05$ and the vertical line indicates the LSD value. **B** Isoenzyme pattern of APX in *M. gracilis* tissues. **C** Isoenzyme pattern of APX in *M. gracilis* 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 (100 μg) were loaded on the gel. NS, normal regenerated shoot; HC, habituated callus; HS, hyperhydric regenerated shoot; TT, tumor tissue
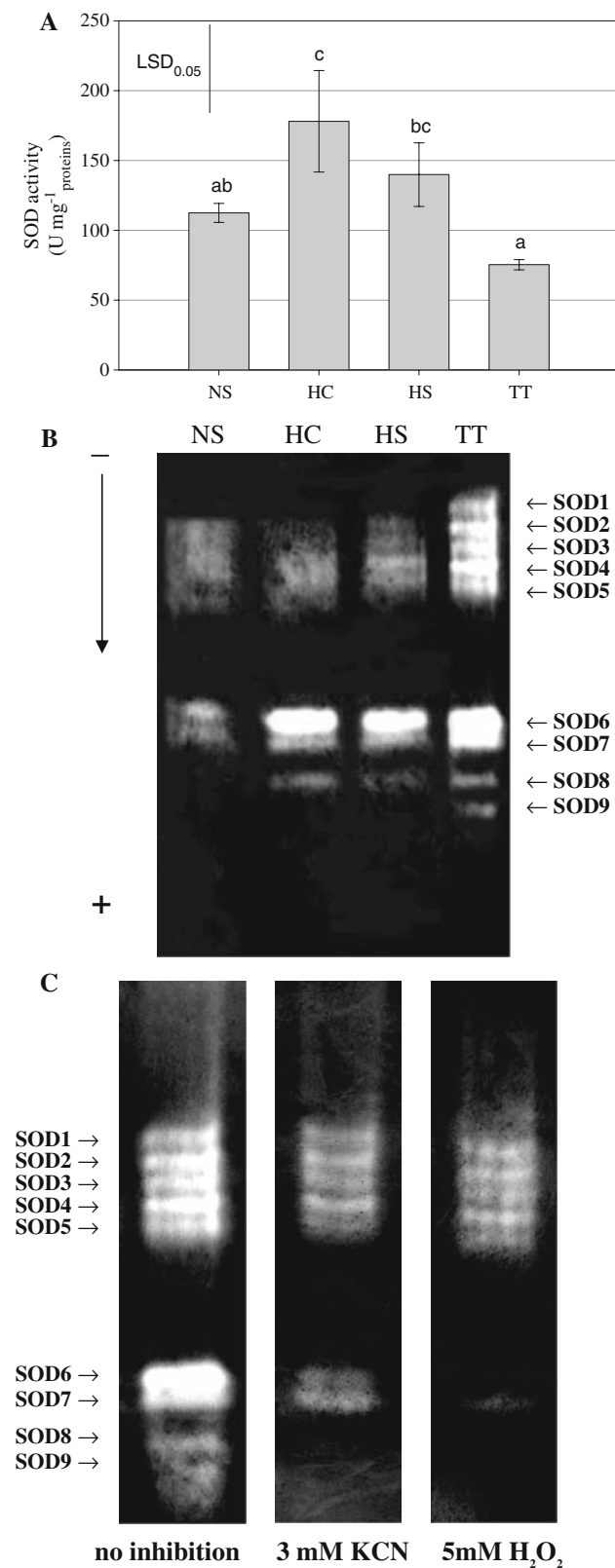
**A**



**B**



**C**
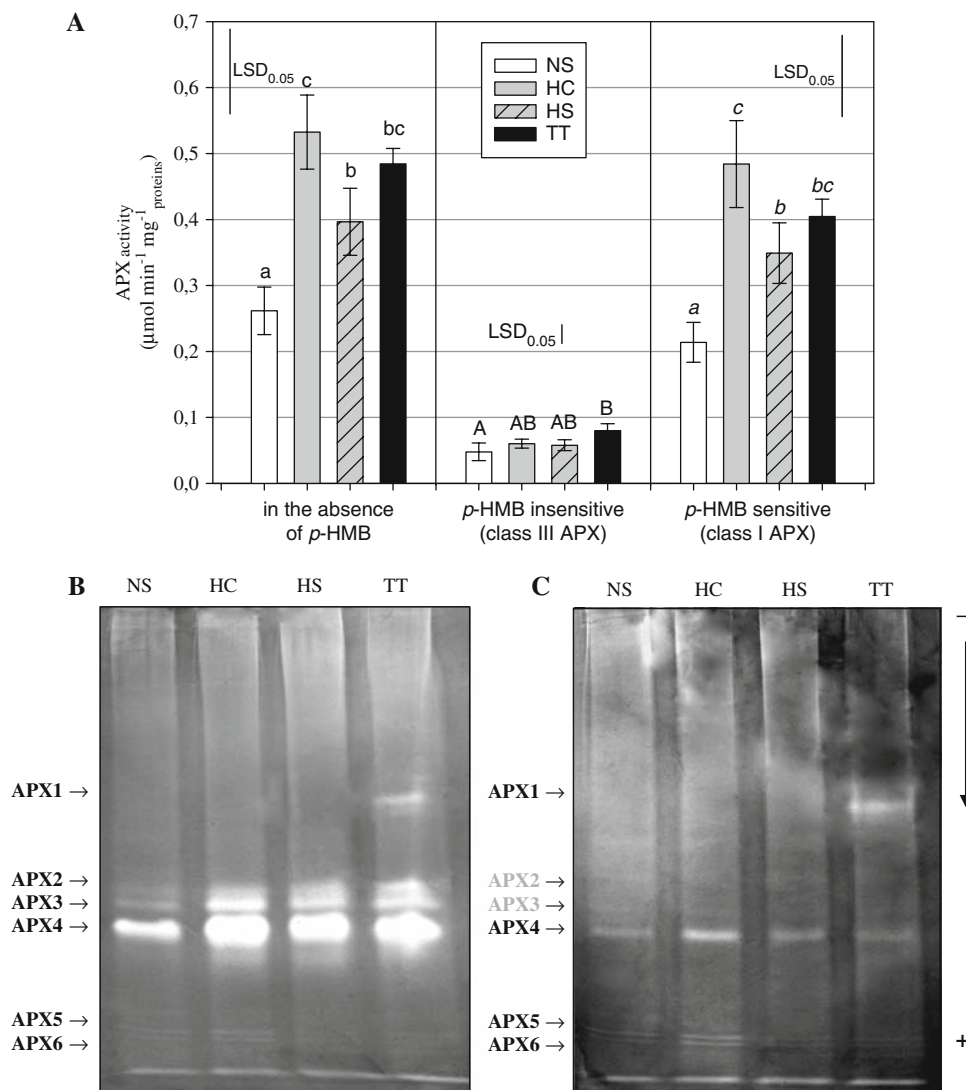


**Fig. 3** **A** SOD activity in *M. gracilis* tissues. Values are mean ± SE based on six replicates. Columns marked with different letters are significantly different at $p \leq 0.05$ and the vertical line indicates the LSD value. **B** Isoenzyme pattern of SOD in *M. gracilis* tissues. Equal amounts of proteins (100 µg) were loaded on the gel. **C** Isoenzyme pattern of SOD in tumor tissue after incubation of gels in 2 mM potassium cyanide or 5 mM $H_2O_2$ before staining for SOD activity. NS, normal regenerated shoot; HC, habituated callus; HS, hyperhydric regenerated shoot; TT, tumor tissue

inhibitors were used to allow the identification of Mn-SOD, Fe-SOD, and Cu/Zn-SOD isoenzymes. Because all the detected bands were present in TT, the inhibition test was performed in this tissue extract. Incubation of gels in 2 mM potassium cyanide or 5 mM $H_2O_2$ before staining for SOD activity identified five isoenzymes SOD1-SOD5 as Mn-SOD (no inhibition by KCN and $H_2O_2$), isoenzymes SOD8 and SOD9 as Cu/Zn-SOD (inhibition by both KCN and $H_2O_2$), and SOD6 and SOD7 as Fe-SOD (no inhibition by KCN) (Fig. 3C).

The lowest CAT activity was measured in the NS. Significantly higher values were obtained in other cactus tissues, especially in HC and HS, in which CAT activity was seven times higher than in the NS (Fig. 4A). Electrophoretic analysis revealed only one CAT isoenzyme common to all cactus tissues, but the intensity of staining was in accordance with the data obtained by spectrophotometric measurements (Fig. 4B).

Differences in phenol and lignin contents as well as in PAL activity between different tissues of *M. gracilis* were also analyzed. The highest amount of phenolic compounds was found in NS. Other cactus tissues (HC, HS, and TT) had similar phenol content, which was almost three times lower than in the NS. PAL activity showed great variability between cactus tissues; significantly the highest value was in NS and was 2.5 times higher than that measured in TT, which had the lowest PAL activity. HC revealed a significantly higher PAL activity than HS (Table 2). The estimation of lignin content showed that lignification was significantly higher in NS compared to other cactus tissues; it was 13 times higher compared to HC, 10 times higher compared to HS, and 27 times higher compared to TT (Table 2).

## Discussion

The possible involvement of activated oxygen metabolism in habituation and hyperhydricity has been studied in several tissue culture plants (Franck and others 1998; Saher and others 2004, 2005; Dewir and others 2006) but not in CAM plants such as *M. gracilis*. High relative humidity has been considered one of the most important environmental factors responsible for hyperhydricity in plants cultured *in vitro*. Hyperhydricity is associated with reduced

(Fig. 3B). All nine bands were present in the TT. The SOD8 band was missing in NS, whereas the SOD1 and SOD9 isoenzymes were revealed only in TT. SOD
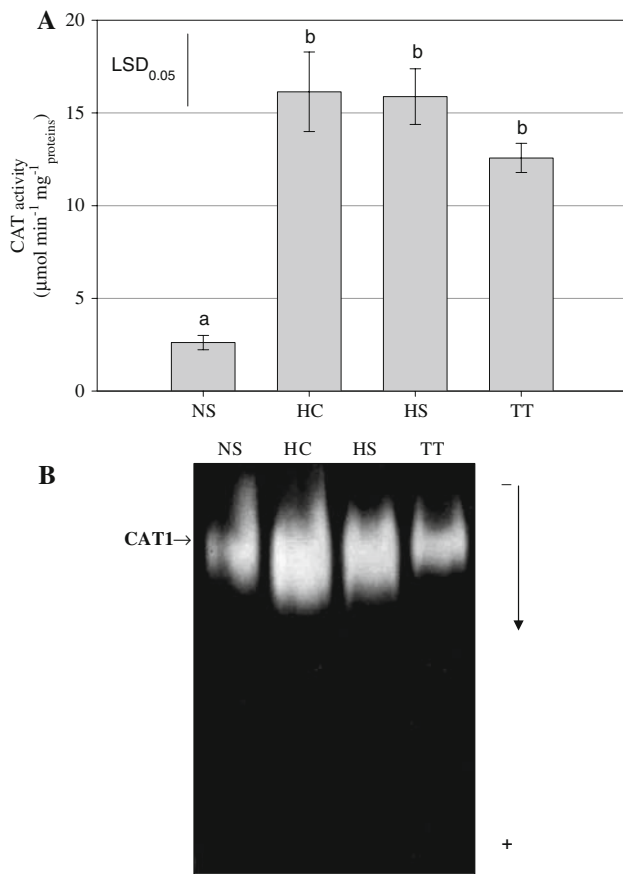
**Fig. 4** **A** CAT activity in *M. gracilis* tissues. Values are mean ± SE based on six replicates. Columns marked with different letters are significantly different at $p \leq 0.05$ and the vertical line indicates the LSD value. **B** Isoenzyme pattern of CAT in *M. gracilis* tissues. Equal amounts of proteins (100 μg) were loaded on the gel. NS, normal regenerated shoot; HC, habituated callus; HS, hyperhydric regenerated shoot; TT, tumor tissue

others 2005). Increased levels of $H_2O_2$ and lipid peroxidation, well-known biomarkers of oxidative stress, have been previously observed in hyperhydric shoots of carnation (Olmos and others 1997; Saher and others 2004) suggesting involvement of oxidative stress in hyperhydricity. On the other hand, MDA content (marker of lipid peroxidation) was lower in hyperhydric shoots of *Prunus avium* (Franck and others 1998), although abnormal morphology, less chlorophyll content, and chloroplast degradation observed in these shoots implicated oxidative stress. Results obtained from different cactus tissues showed that higher MDA content observed in HC, HS, and TT compared to NS was associated with high $H_2O_2$ content, thus indicating that oxidative stress could be responsible for the abnormal morphology in cactus hyperhydric tissues. In a previous ultrastructural study of *Mammillaria* tissues, thylakoid degradation and significantly lower concentrations of total chlorophyll, chlorophyll *a*, and chlorophyll *b* were observed in hyperhydric tissues compared to NS (Poljuha and others 2003). In plants, lipid peroxidation and membrane damage results from the enzymatic activity of LOX or from autocatalytic oxidation by ROS (Axerold and others 1981). Therefore, elevated activity of LOX measured in cactus hyperhydric tissues is in agreement with observed peroxidative damage. Similar results were obtained in *Euphorbia milli* (Dewir and others 2006) and *Prunus* hyperhydric shoots (Franck and others 1998), whereas lower LOX activity was measured in hyperhydric carnation shoots (Saher and others 2004). Carbonyl content, another reliable biomarker of oxidative stress (Dalle-Donne and others 2003), was also increased in HC, HR, and TT compared to NS, thus confirming severe oxidative stress in these tissues, especially in HC. In cacti plants grown in vitro, photosynthesis is stimulated and photorespiration is more intensive, probably due to alteration of the normal CAM pattern (Malda and others 1999). Therefore, it is possible that apart from the already mentioned conditions causing hyperhydricity, *M. gracilis* plants grown in tissue culture are exposed to higher oxidative stress.

transpiration and excessive water uptake that could reduce the level of oxygen within tissues to near-hypoxia (Franck and others 2004). Under hypoxia conditions, some of the metabolic activities that can generate ROS in plants could be disrupted, and there could be production of toxic levels of $H_2O_2$ and generation of oxidative stress (Saher and

**Table 2** Differences in total phenolics, lignin contents, and PAL activity in *M. gracilis* tissues

|  | Total phenolics (μg g$^{-1}$ DW) | Lignin (A$_{540}$ × 10$^{-3}$ g$^{-1}$ DW) | PAL activity (μmol min$^{-1}$ mg$^{-1}$ protein) |
|---|---|---|---|
| Normal regenerated shoot | 1.07 ± 0.08 a | 122.65 ± 12.35 a | 145.94 ± 5.06 a |
| Habituated callus | 0.39 ± 0.01 b | 9.52 ± 0.72 b | 124.18 ± 4.71 b |
| Hyperhydric regenerated shoot | 0.38 ± 0.02 b | 12.05 ± 1.02 b | 100.78 ± 5.45 c |
| Tumor tissue | 0.39 ± 0.01 b | 4.46 ± 0.22 b | 58.56 ± 6.31 d |
| LSD (0.05) | 0.05 | 0.018 | 14.90 |

Values are means ± SE based on six replicates. Different letters in each column indicate significant differences between means at $p \leq 0.05$ according to LSD test

DW = dry weight

A clear relationship between hyperhydricity and antioxidant enzymatic activities has been observed in micropropagated shoots of *Prunus avium* (Franck and others 1998, 2004), carnation (Olmos and others 1997; Saher and others 2004), and *Euphorbia milli* (Dewir and others 2006). The increased activity of SOD, a key enzyme involved in protecting the cells from oxidative stress by catalyzing the disproportion of $O_2^{\bullet-}$ radical to $H_2O_2$ and $O_2$, was observed in *M. gracilis* hyperhydric tissues. Together with the appearance of new Cu/Zn-SOD and Fe-SOD isoforms, which have also been induced in chloroplasts of *Mesembryanthemum crystallinum* under oxidative stress (Surowka and others 2007), these findings suggest induction of a defense mechanism against oxidative damage in chloroplasts of hyperhydric cactus tissues. However, in TT we noticed low SOD activity when measured spectrophotometrically in contrast to the increased staining intensity of all SOD isoforms. The observed discrepancy could be explained by shortcomings of SOD measurements because spectrophotometric quantification of SOD activity in crude plant extracts could be problematic due to the presence of compounds that interfere with the assay (Banowetz and others 2004). Related to the increase in SOD activity, significantly higher activities of APX and CAT were detected in HC, HR, and TT, indicating activation of enzymes involved in scavenging $H_2O_2$. The inhibition assay revealed the presence of both *p*-HMB-sensitive (class I APX) and *p*-HMB-insensitive (class III APX) ascorbate peroxidase in all cactus tissues. In the total APX activity the major part belongs to the class I APXs, which are involved in the removal of $H_2O_2$ in a reaction strictly dependent on ascorbate (Ros-Barceló and others 2006). According to the inhibition assay in gel, APX2 and APX3 could be considered as class I APX, whereas APX1, APX4, APX5, and APX6 probably belong to class III APX, although the intensity staining of APX4 was weaker in the presence of inhibitor. In the present work, we have also found a significantly higher POX activity in HC, HR, and TT than in NS; this could be connected with their role in scavenging increased $H_2O_2$, produced in hyperhydric tissues (Franck and others 2004). However, Fernandez-García and others (2008) recently proposed that the concomitant presence of higher amounts of peroxidase, $H_2O_2$, and Fe (Saher and others 2004), observed in apoplasts of hyperhydric carnation shoots, could induce a Fenton reaction generating extremely reactive hydroxyl radicals that can damage cell molecules, membranes, and other structures.

The increased activities of the antioxidative enzymes SOD, APX, CAT, and POX in HC, HS, and TT suggest an activation of a defense mechanism against the increased production of ROS in these tissues. However, the increase in lipid peroxidation and carbonylation of proteins and oxidative damage to chloroplasts (Poljuha and others 2003)

indicate that activation of these enzymes was not effective enough to prevent oxidative stress. These findings are in agreement with results on oxidative stress in hyperhydric shoots of carnation and *Euphorbia millii* (Saher and others 2004; Dewir and others 2006) and partly in agreement with results on hyperhydric shoots of *Prunus* (Franck and others 2004) and on sugarbeet callus tissue (Causevic and others 2006).

It has been suggested that deficiencies in phenolic content (Perry and others 1999) and in PAL activity and lignin content (Olmos and others 1997; Saher and others 2004) might be responsible for the hyperhydric phenotype. In our study, concomitant with the decrease in PAL activity, a decrease of the content of phenolic compounds and lignification was observed in cactus HC, HS, and TT, thus confirming this hypothesis. Hypolignification in vascular tissues, as a consequence of a reduction in cellulose and lignin biosynthesis, can alter the mechanical properties of the cell wall, which could lead to reduced cell turgor pressure, changes in the water potential, increased water uptake, and, finally, hyperhydration of tissues (Olmos and others 1997).

According to our results, TT is more similar to HC and HS than to NS. Because habituation and hyperhydricity are both considered to be a part of a neoplastic progression (Gaspar and others 2000), observed similarities could be related to a loss of cell differentiation. However, oxidative stress in TT was not as severe as in HC and HS, probably because of lower LOX activity. These results were also confirmed by lower activities of some antioxidative enzymes in TT compared with those in HC and HS. TT revealed some specific isoforms of POX, APX, and SOD enzymes which could be the consequence of *Agrobacterium* transformation (Krsnik-Rasol and Jelaska 1991; Krsnik-Rasol and Muraja-Fras 1993). Differences in the stress response between TT and both HC and HR suggest that the HR and HC phenotypes are probably due to a physiologic response leading to a precarious equilibrium between oxidative stress and antioxidant response, whereas the tumor state is probably a more stable state implicating genetic change that allows the transition to another physiologic state and a more efficient response to stress (Kevers and others 2004).

In conclusion, prominent oxidative stress and strong induction of the antioxidative system was observed in HC, HS, and TT, indicating that activated oxygen metabolism is involved in habituation and hyperhydricity as well as in loss of tissue organization in *M. gracilis* tissue culture.

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