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ORIGINAL ARTICLE

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Fungal pathogen-induced changes in the antioxidant systems of leaf peroxisomes from infected tomato plants

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Abstract Peroxisomes, being one of the main organelles where reactive oxygen species (ROS) are both generated and detoxified, have been suggested to be instrumental in redox-mediated plant cell defence against oxidative stress. We studied the involvement of tomato (*Lycopersicon esculentum* Mill.) leaf peroxisomes in defence response to oxidative stress generated upon *Botrytis cinerea* Pers. infection. The peroxisomal antioxidant potential expressed as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GSH-Px, EC 1.11.1.19) as well as the ascorbate-glutathione (AA-GSH) cycle activities was monitored. The initial infection-induced increase in SOD, CAT and GSH-Px indicating antioxidant defence activation was followed by a progressive inhibition concomitant with disease symptom development. Likewise, the activities of AA-GSH cycle enzymes: ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2) as well as ascorbate and glutathione concentrations and redox ratios were significantly decreased. However, the rate and timing of these events differed. Our results indicate that *B. cinerea* triggers significant changes in the peroxisomal antioxidant system leading to a collapse of the protective mechanism at advanced stage of infection. These changes appear to be partly the effect of pathogen-promoted leaf senescence.

Keywords Antioxidant defence · Ascorbate-glutathione cycle · *Botrytis* · *Lycopersicon* · Peroxisomes

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Abbreviations AA: Reduced ascorbate · APX: Ascorbate peroxidase · CAT: Catalase · DHA: Dehydroascorbate · DHAR: Dehydroascorbate reductase · GR: Glutathione reductase · GSH: Reduced glutathione · GSH-Px: Glutathione peroxidase · GSSG: Oxidised glutathione · MDHAR: Monodehydroascorbate reductase · ROS: Reactive oxygen species · SOD: Superoxide dismutase

Introduction

At the subcellular level a successful plant defence against oxidative stress induced by unfavourable environmental factors requires a cooperative interaction between the antioxidant systems localised in different compartments (Morita et al. 1999; Kingston-Smith and Foyer 2000; Mittova et al. 2000). Over the past few years a substantial progress has been made in the functional characterization of plant peroxisomes and in dissecting their potential role in plant cell defence against oxidative stress (Reumann 2000; del Río et al. 2002; Palma et al. 2002; Hayashi and Nishimura 2003).

Peroxisomes are ubiquitous organelles in plant cells with an oxidative type of metabolism fulfilling a range of metabolic functions. In addition to the role of leaf peroxisomes in the photorespiratory C_2 cycle, specialised plant peroxisomes are also involved in β-oxidation of fatty acids and the glyoxylate cycle in oilseeds as well as ureide metabolism in root nodules of leguminosae (del Río et al. 2003). Peroxisomes, containing a set of antioxidant enzymes and molecules, contribute also to the reduction of oxidative stress (Willekens et al. 1995; del Río et al. 2002). However, as an inevitable consequence of their oxidative type of metabolism, peroxisomes are simultaneously a significant source of ROS. Superoxide is generated by membrane proteins via NAD(P)H oxidation and by xanthine oxidase in the peroxisomal matrix (del Río et al. 2002). The production of H_2O_2 takes place via flavin-containing oxidases and superoxide (O_2^-)

dismutation both in matrix and in peroxisomal membrane (Corpas et al. 2001; del Río et al. 2002; Igamberdiev and Lea 2002). In leaf peroxisomes O_2^- dismutation is catalysed by MnSOD in the organelle matrix (Sandalio et al. 1987; Palma et al. 1998), and in peroxisomes from oilseed plants by CuZnSOD in the matrix and by a membrane-bound MnSOD on the cytosolic side of the peroxisomal membrane (Sandalio et al. 1997; Corpas et al. 1998). Hydrogen peroxide produced in peroxisomes is mostly decomposed by CAT isoenzymes localised in the matrix, usually in the form of organised structures called crystalline cores (Willekens et al. 1995; Igamberdiev and Lea 2002).

Besides CAT, H_2O_2 in peroxisomes is also detoxified by the AA-GSH cycle and glutathione-dependent peroxidase (Jiménez et al. 1997; del Río et al. 1998; Churin et al. 1999). According to the proposed model based on the intraperoxisomal distribution of the AA-GSH cycle components, the membrane-bound APX, in a collaboration with MDHAR, degrade H_2O_2 that can diffuse from peroxisomes (Mullen and Trelease 1996; Reumann 2000), as well as H_2O_2 being formed by O_2^- dismutation on the cytosolic side of the peroxisomal membrane. DHAR and GR located in the peroxisomal matrix accomplish detoxification of H_2O_2 produced in the matrix in sequential ascorbate- and glutathione-dependent reactions (Jiménez et al. 1997; del Río et al. 1998; Corpas et al. 2001). The AA-GSH cycle also provides NAD^+ for peroxisomal metabolism and GSH protects the flavin-containing oxidases against photoinactivation (Schäfer and Feierabend 2000; Corpas et al. 2001). Thus, peroxisomes are one of the main cellular organelles where ROS are both generated and detoxified and they appear to be instrumental in redox-homeostasis-mediated defence against abiotic and biotic stresses. This concept has been further highlighted by recent reports of López-Huertas et al. (2000) that demonstrated H_2O_2-mediated induction of peroxisome biogenesis genes in response to wounding and pathogen infection. Moreover, peroxisomes possessing the capacity to generate and release into the cytosol signalling molecules such as O_2^-, H_2O_2 and nitric oxide (NO) (Corpas et al. 2001) could be involved in signal transduction pathways playing a key role in redox-regulated responses to pathogen attack and abiotic stresses (Grant and Loake 2000; Neill et al. 2002).

The peroxisomal antioxidant system, and especially the main ROS-scavenging enzymes—SOD and CAT have been shown to be sensitive to environmental stresses (Willekens et al. 1995; Romero-Puertas et al. 1999; Oksanen et al. 2003). These antioxidant systems have also been reported to be affected by senescence (Pastori and del Río 1997). However, although it is known that stress that generates H_2O_2 upregulates peroxisome biogenesis (López-Huertas et al. 2000), evidence that the peroxisomal AA-GSH cycle is involved in cellular response to pathogen-induced oxidative stress is still limited. In this work, the effect of *B. cinerea* infection on the antioxidant system of peroxisomes isolated from tomato leaves was studied to get a further insight into the possible involvement of these organelles in plant defence response to oxidative stress generated during pathogenesis. Profiles of changes in the activity of peroxisomal ROS-scavenging enzymes, SOD and CAT, as well as of the AA-GSH cycle and GSH-Px have been analysed.

Materials and methods

Plant material and pathogen infection

Tomato (*Lycopersicon esculentum* Mill) plants cv "Perkoz" were grown from seeds (PNOS, Ożarów Mazowiecki, Poland) in a growth chamber as previously described (Kuźniak and Skłodowska 2001). Six week-old plants were inoculated with spore solution containing *B. cinerea* Pers.:Fr. conidia (2×10^6 spores ml^{-1}), 5 mM glucose and 2.5 mM KH_2PO_4. The inoculation solution was sprayed on both leaf surfaces of all leaves. After inoculation plants were kept at 100% relative humidity to ensure spore germination and to facilitate infection. Analyses were performed 1, 2, 3 and 4 days post inoculation (dpi).

Isolation and characteristics of leaf peroxisomes

Peroxisomes were isolated from the first, second and third true leaves by differential and Percoll density-gradient centrifugation. Leaves (70 g) without the main midribs were cut into pieces, chilled in an ice bath for 20 min and homogenised in a blender for 2×15 s in an ice-cold isolation medium (1:5 w/v). The medium (pH 7.4) contained 20 mM Tris–HCl, 0.3 M mannitol, 1 mM EDTA, 20 mM sodium ascorbate, 0.1% bovine serum albumin and 2 mM $MgCl_2$. The homogenates were filtered through six layers of gauze and centrifuged at 200 *g* for 20 min to remove chloroplasts. The supernatants were centrifuged again at 15,000 *g* for 10 min to recover peroxisomes. The pellet was gently resuspended in a medium containing 0.3 M mannitol, 0.05% bovine serum albumin, 1 mM EDTA, 10 mM Tris–HCl, 1 mM sodium ascorbate and 0.3 M sucrose (pH 7.2) and separated on a continuous Percoll density-gradient containing sucrose as osmoticum (30,000 *g*, 45 min). The peroxisomal band located at the bottom was identified on the basis of activity of specific marker enzymes: hydroxypyruvate reductase (EC 1.1.1.29) and CAT (EC 1.11.1.6) assayed according to Schwitzguebel and Siegenthaler (1984) and Dhindsa et al. (1981), respectively. The collected fraction was diluted ten times with buffer containing 10 mM Tris–HCl (pH 7.2), 0.3 M mannitol, 1 mM EDTA, 1 mM sodium ascorbate and pelleted at 15,000 *g* for 10 min. In order to remove Percoll the washing procedure was repeated twice. All operations were carried out at 0–4°C. The buffers lacking sodium ascorbate were used when ascorbate content was determined.

The quality of the peroxisomal fraction was monitored by measurements of organellar marker enzyme activities and chlorophyll content. The activitiy of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) as a specific marker for cytosol, and fumarase (EC 4.2.1.2) and NADH:cytochrome c oxidoreductase (EC 1.6.99.3) activities as specific mitochondrial markers were assayed using a Sigma diagnostic kit and according to Hatch (1978) and Douce et al. (1972), respectively. Chlorophyll was extracted with 80% acetone and measured according to Porra et al. (1989). The activities of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) as a specific marker enzyme for chloroplast stroma, and acid phosphatase (EC 3.1.3.2) being a marker for vacuoles were measured according to Bradbeer (1969) and assayed using a Sigma diagnostic kit, respectively. The intactness of peroxisomes isolated from control and inoculated leaves was determined on the basis of enzyme latency calculated for enzyme activities in peroxisomes in the isotonic medium and in the peroxisomal extract using the formula of Burgess et al. (1985):

$$\left[100 - \left(\frac{\text{Activity intact}}{\text{Activity burst}} \times 100\right)\right] = \%\text{latency}$$

Taking into account the permeability of the peroxisomal membrane for H_2O_2 being the substrate for CAT and the intraperoxisomal enzyme distribution, latency was calculated for hydroxypyruvate reductase, as a specific peroxisomal marker enzyme and APX and GR representing the AA-GSH cycle.

Antioxidant enzyme assays and determination of ascorbate and glutathione

The peroxisomal pellet was homogenised in 0.05 M ice-cold potassium phosphate buffer (pH 7.0) containing 1 M NaCl, 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone and the supernatant was used for the determination of SOD, CAT, APX, MDHAR, DHAR and GSH-Px activities as well as the glutathione content. For determination of ascorbate, the pellet was homogenised in 6% (w/v) ice-cold trichloroacetic acid. SOD activity assay was run according to Minami and Yoshikawa (1979). The activity unit (50% inhibition) was defined according to McCord and Fridovich (1969). APX activity was assayed following the oxidation of ascorbate at 265 nm ($\epsilon = 13.7\ \text{mM}^{-1}\ \text{cm}^{-1}$) by a modified method of Nakano and Asada (1981) and DHAR was determined by following the formation of ascorbate at 265 nm ($\epsilon = 14.6\ \text{mM}^{-1}\ \text{cm}^{-1}$) according to Hossain and Asada (1984). The activity of MDHAR was determined following the oxidation rate of NADH at 340 nm ($\epsilon = 6.22\ \text{mM}^{-1}\ \text{cm}^{-1}$) as described by Kuźniak and Skłodowska (2004). The activity of GR was assayed by determination of GSSG-dependent oxidation of NADPH at 340 nm ($\epsilon = 6.22\ \text{mM}^{-1}\ \text{cm}^{-1}$) as described by Beutler (1975). GSH-Px (EC 1.11.1.19) activity was determined according to the method of Hopkins and Tudhope (1973) with *t*-butyl hydroperoxide as a substrate. The activities of APX, MDHAR, DHAR, GR and GSH-Px were expressed in nmol min^{-1} mg^{-1} protein. Ascorbate was determined according to the colorimetric bipyridyl method as described by Knörzer et al. (1996). Total ascorbate was assayed by adding dithiothreitol to the samples for reducing DHA to AA. DHA concentration was calculated by subtracting the AA value from the total ascorbate. For determination of glutathione the sample obtained as described for enzyme assays was deproteinized immediately using 30% $HClO_4$. The levels of non-protein sulfhydryl groups (–SH) were determined colorimetrically with 5,5′-dithiobis-2-nitrobenzoic acid according to Brehe and Burch (1976). For specific assay of GSSG the GSH could be masked by derivatisation with 2-vinylpiridine in the presence of triethanolamine for 60 min at 20°C. The concentrations of non-enzymatic antioxidants were expressed in nmol mg^{-1} protein. All assays were performed spectrophotometrically (UNICAM UV300 UV-visible spectrometer) at 25°C.

Other determinations

Proteins were measured according to Bradford (1976) with bovine serum albumin as a standard.

Statistical analysis

The significance of differences between mean values was determined by a non-parametric Mann–Whithey Rank Sum Test. Differences at $P < 0.05$ were considered significant. All mean values ($n = 4$–6) are presented ± SD.

Results

The purity of the peroxisomal fraction was monitored by determination of chlorophyll content and specific marker enzyme activities (Table 1). Peroxisomes were adequately separated from cytosol as judged on the basis of glucose-6-phosphate dehydrogenase activity. The cross-contamination of organelles was in the range routinely found for subcellular fractionation of leaves reflecting relatively clean separation of peroxisomes from chloroplasts and mitochondria (Table 1). The fumarase specific activity in the peroxisomal band accounted for 3.5% of the total enzyme activity confirming a minor contamination of the fraction with mitochondria. The intactness of peroxisomes was determined on the basis of enzyme latency. The latency values calculated before inoculation for hydroxypyruvate reductase, APX and GR were 80, 77 and 95%, respectively. The comparative analysis of enzyme latency 4 dpi revealed that *B. cinerea* infection did not significantly modify the integrity of isolated organelles (data not shown).

Table 1 Marker enzyme activities and chlorophyll concentration in peroxisomes isolated from tomato leaves

Marker	Activity/ concentration
Glucose-6-phosphate dehydrogenase (nmol mg^{-1} protein)	7.30
$NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (μmol mg^{-1} protein)	ND
Chlorophyll (μg mg^{-1} protein)	2.46
Acid phosphatase (μmol mg^{-1} protein)	ND
Fumarase (nmol mg^{-1} protein)	24.10
NADH:cytochrome c oxidoreductase (nmol mg^{-1} protein)	8.10
Catalase (mmol mg^{-1} protein)	1.51
Hydroxypyruvate reductase (μmol mg^{-1} protein)	10.07

ND not detected

In peroxisomes isolated from *B. cinerea*-infected tomato leaves an induction of the total SOD activity was found 1 dpi with a maximum activity increase of 153% of control ($P < 0.05$) whereas there was an activity decrease of 35% ($P < 0.05$) 4 dpi (Fig. 1). *B. cinerea* infection markedly affected the activity of CAT and the changes were concomitant with those observed for SOD. A strong CAT activity increase of 188 and 165% of control ($P < 0.05$) was found on the first and second days post-inoculation, respectively (Fig. 2). The initial induction of CAT activity was followed by a decline to 41% of control ($P < 0.05$) 4 days after inoculation (Fig. 2). Moreover, in peroxisomes isolated from control leaves we observed similar SOD and CAT activity fluctuations. They were evident on the second day when the enzyme activities reached the highest levels that then remained stable until the end of the examined period (Figs. 1, 2).

The peroxisomal ascorbate pool was notably diminished in response to *B. cinerea* infection (Table 2). A progressive significant ($P < 0.05$) reduction in both reduced ascorbate (AA) and total ascorbate pools was found, starting from the second dpi. After 4 dpi, AA and total ascorbate concentrations were reduced to 24 and 32% ($P < 0.05$) of those in control, respectively (Table 2). With the exception of the first day, the ascorbate content decrease did not coincide with dehydroascorbate (DHA) accumulation, and the absolute DHA concentration in peroxisomes from inoculated leaves was from 34% to 59% ($P < 0.05$) lower than controls. However, as a consequence of the simultaneous progressive decrease in AA content, the ascorbate redox ratio in peroxisomes from inoculated tissues was lower than in controls. After 4 days, the DHA in peroxisomes isolated from diseased leaves accounted for up to 61% of the total ascorbate pool whereas in control leaves the DHA concentration did not exceed 48% of the total ascorbate content (Table 2).

Similar to *B. cinerea*-induced changes reported for the ascorbate pool, the reduced glutathione (GSH) and total glutathione concentrations decreased from day 1 onwards (Table 3). The GSH concentration was from 56% to 73% ($P < 0.05$) lower than in controls and the reduction in total glutathione content was within the range of 39–57% ($P < 0.05$). In peroxisomes isolated from control leaves a gradual decrease in GSH as well as in the total glutathione concentration was observed starting from the third day (Table 3). In peroxisomes from infected leaves the concentration of oxidized glutathione (GSSG) was increased when compared with control peroxisomes, except for the second day. The maximum GSSG accumulation, up to twice as high than controls ($P < 0.05$), occurred on the first and fourth dpi. Following *B. cinerea* infection a decrease in the peroxisomal GSH/GSSG ratio, being the consequence of both

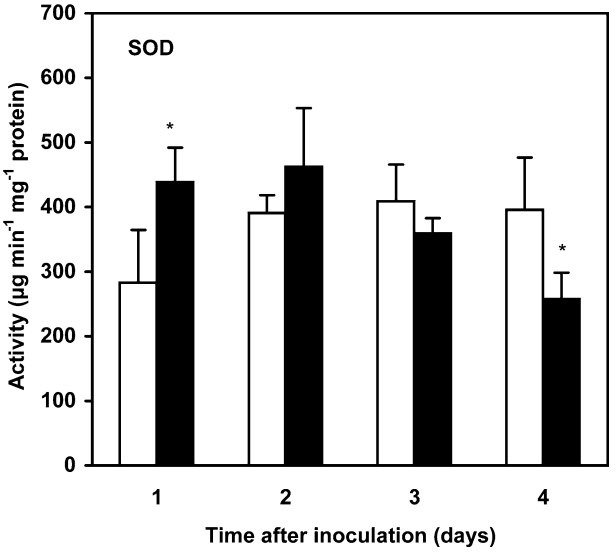


Fig. 1 SOD activity in peroxisomes from *B. cinerea*-inoculated tomato leaves. *Open bars*, *closed bars B. cinerea* infected

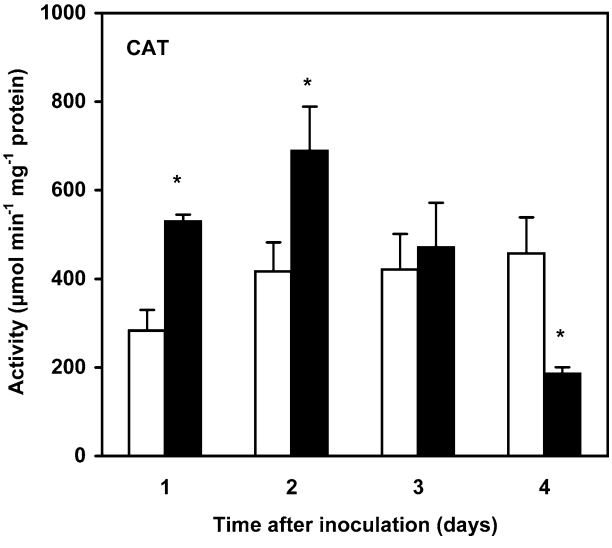


Fig. 2 CAT activity in peroxisomes from *B. cinerea*-infected tomato leaves. *Open bars* control, *closed bars B. cinerea*-infected

Table 2 Reduced ascorbate (AA) and dehydroascorbate (DHA) contents in peroxisomes from control and *B.cinerea*-infected tomato leaves

Time (days)	Metabolite content (nmol mg^{-1} protein)							
	Control				*Botrytis cinerea*			
	AA	DHA	Total ascorbate	AA/DHA	AA	DHA	Total ascorbate	AA/DHA
1	9.57 ± 0.50	6.09 ± 0.47	15.66 ± 0.98	1.57	10.50 ± 2.31	6.75 ± 1.14	17.25 ± 4.48	1.55
2	13.10 ± 2.22	7.47 ± 1.49	20.57 ± 4.17	1.75	5.04* ± 0.32	4.94 ± 1.29	9.98* ± 1.68	1.02*
3	12.08 ± 3.50	9.11 ± 1.64	21.19 ± 5.29	1.33	3.90* ± 0.26	5.09 ± 1.71	8.99* ± 1.76	0.77*
4	10.41 ± 2.70	9.74 ± 2.13	20.15 ± 5.80	1.07	2.53* ± 0.38	3.98* ± 0.30	6.51* ± 0.68	0.63*

*Indicates values that differ significantly from the control at $P < 0.05$

GSH content decrease and GSSG concentration increase, was found over the 4-day time-course of the experiment (Table 3). In contrast to the control AA/DHA ratio ranging from 1.75 to 1.07 (Table 2) the glutathione redox state in peroxisomes from non-inoculated leaves was maintained at a significantly higher level of 16.11 to 4.17 (Table 3). However, the rate and timing of decreases in the ascorbate and glutathione redox ratios in response to *B. cinerea* infection were very similar, indicating that both antioxidant pools underwent prooxidative changes (Tables 2, 3).

The activity of peroxisomal APX was markedly decreased following inoculation (Fig. 3). The strongest inhibition effect was visible on the first and fourth days when the enzyme activity was 45 and 69% ($P < 0.05$) lower than the controls, respectively. The activities of MDHAR and DHAR in general showed similar *B. cinerea*-induced changes (Fig. 3). They were slightly ($P > 0.05$) higher than in the controls only 1 day after inoculation. Thereafter, the activities of both enzymes started to decrease reaching the lowest values at the advanced stage of infection development i.e. on the fourth dpi.

The activity levels of the two enzymes related to glutathione metabolism in peroxisomes isolated from control leaves, GR and GSH-Px, were found to fluctuate over the examined period (Fig. 4). In peroxisomes from diseased leaves GR activity exhibited a slight activity increase that was restricted to the first day post-inoculation. Afterwards, it declined to a constant level being on average 25% lower than that in the control (Fig. 4). As to the GSH-Px, its activity was enhanced on the first ($P < 0.05$) and second ($P > 0.05$) days after inoculation, whereas from day 3 onwards it was significantly ($P < 0.05$) reduced down to 66% of control (Fig. 4).

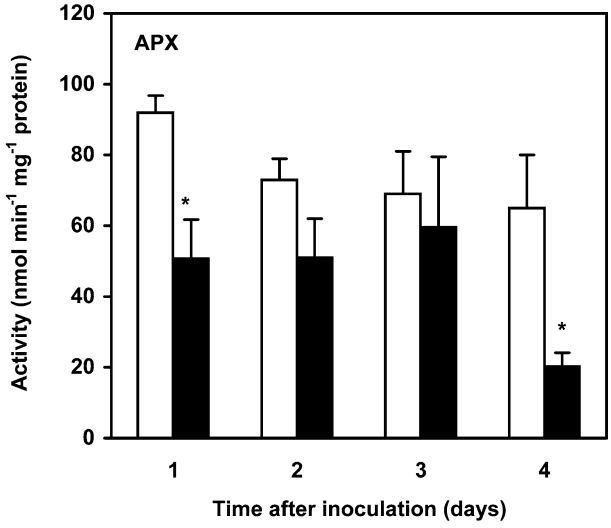
Discussion

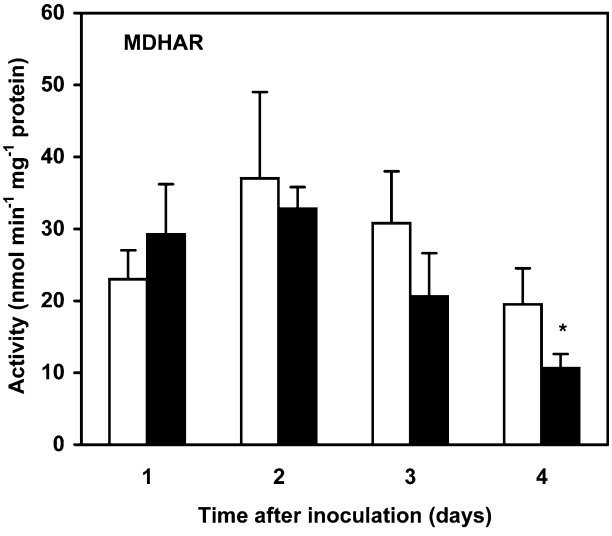
Under stress conditions peroxisomes seem to play a role that is not confined just to ROS detoxification (Foyer and Noctor 2003). We found that SOD and CAT, forming the first lane of defence against ROS, were strongly affected by *B. cinerea* infection. The total SOD activity was induced at the initial phase of tomato–*B. cinerea* interaction, before the appearance of visible infection symptoms. This could be interpreted as a defence response against the pathogen infection-imposed oxidative stress. However, the development of necrotic lesions, starting from the third day post-inoculation, was concomitant with a progressive peroxisomal SOD activity decrease. The decreasing SOD activity could favour the prooxidant shift in the peroxisomal metabolism correlating with the development of disease symptoms. CAT showed complementary pathogen-induced activity changes implying that the CAT-mediated antioxidant protection also ceased as disease started to develop. Although CAT has been reported to be induced in response to infection at both mRNA and activity levels (Niebel et al. 1995; Patykowski and Urbanek 2003), its role in this process appears to be more complex than in abiotic stress, where it is generally considered to be positively correlated with plant tolerance (Willekens et al. 1995; Miyagawa et al. 2000; Oksanen et al. 2003).

Table 3 Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents in peroxisomes from control and infected tomato leaves

Time (days)	Metabolite content (nmol mg^{-1} protein)							
	Control				*Botrytis cinerea*			
	GSH	GSSG	Total glutathione	GSH/GSSG	GSH	GSSG	Total glutathione	GSH/GSSG
1	3.25 ± 0.27	0.33 ± 0.14	3.58 ± 0.26	9.85	1.01* ± 0.03	0.65* ± 0.09	1.66* ± 0.11	1.55*
2	3.27 ± 0.36	0.27 ± 0.14	3.54 ± 0.23	12.11	1.46* ± 0.52	0.24 ± 0.04	1.70* ± 0.60	6.08
3	2.76 ± 0.19	0.43 ± 0.09	3.19 ± 0.21	6.42	0.77* ± 0.16	0.62 ± 0.14	1.39* ± 0.31	1.24*
4	1.67 ± 0.32	0.40 ± 0.15	2.07 ± 0.45	4.17	0.45* ± 0.12	0.83* ± 0.07	1.28* ± 0.16	0.54*

*Indicates values that differ significantly from the control at $P < 0.05$.





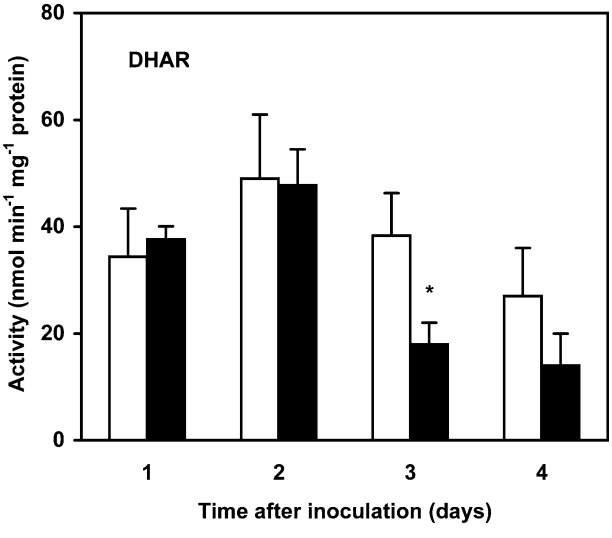
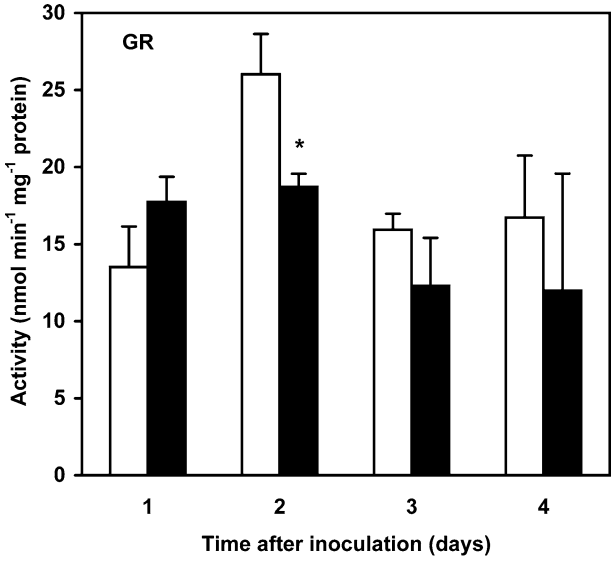


Fig. 3 The effect of *B. cinerea* infection on APX, MDHAR and DHAR activities in peroxisomes from tomato leaves. *Open bars* control, *closed bars B. cinerea*-infected

Since during plant–pathogen interaction ROS are generated mainly extracellularly (Wojtaszek 1997), the protective effect of CAT against ROS toxicity would require H_2O_2 diffusion to peroxisomes. In accordance, peroxisomal CAT has been shown to be a sink for H_2O_2 generated in other subcellular compartments under stress conditions (Willekens et al. 1997). On the other hand studies on transgenic plants revealed that the elevated level of H_2O_2 rendered them disease-resistant (Chamnongpol et al. 1998; Wu et al. 1995). Moreover, CAT can interfere with signalling functions of H_2O_2 in pathogenesis (Conrath et al. 1995). We found that peroxisomal GSH-Px followed infection-induced changes similar to those observed for CAT and SOD. Regarding that GSH-Px is considered to reduce organic hydroperoxides as well as H_2O_2 produced under stress (Eshdat



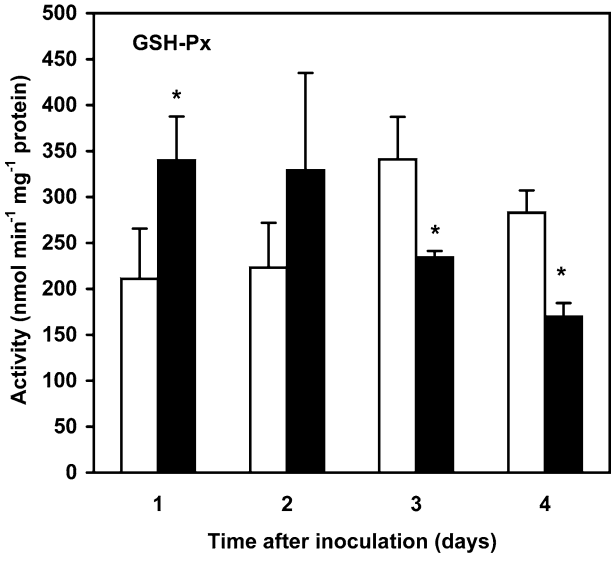


Fig. 4 The effect of *B. cinerea* infection on GR and GSH-Px activities in peroxisomes from tomato leaves. *Open bars* control, *closed bars B. cinerea*-infected

et al. 1997) its induction at the initial stage of the interaction could reflect the early necessity to detoxify H_2O_2 and/or membrane lipid hydroperoxides produced in peroxisomes after *B. cinerea* inoculation. Our results suggested that the peroxisomal antioxidant mechanism related to SOD, CAT and GSH-Px activities was mobilised before infection progress was visible and ceased when disease started to develop. Recently, NO, postulated to be generated by the peroxisomal NADPH-dependent NO synthase (Barroso et al. 1999) has been also reported to inhibit CAT and APX in tobacco peroxisomes (Clark et al. 2000).

B. cinerea infection markedly affected the activity of the peroxisomal AA-GSH cycle. Severe perturbations in the peroxisomal non-enzymatic antioxidant pools were manifested by a significant decline in ascorbate and glutathione concentrations, GSSG accumulation and ascorbate and glutathione redox ratio decreases. We also found that in peroxisomes isolated from control leaves the AA/DHA ratio was relatively low and did not match that reported earlier for cytosol, chloroplasts and mitochondria (Kuźniak and Skłodowska 1999, 2001, 2004). Our data coincided with those of Jiménez et al. (1997) for pea leaf peroxisomes indicating that low ascorbate redox state could be a unique feature related to the oxidative type of peroxisomal metabolism.

In response to infection the peroxisomal APX activity was markedly decreased throughout the examined period. This may be partly explained by the concomitant AA concentration decline, as found for *Arabidopsis vtc*1 mutant and senescing spinach leaves (Conklin et al. 1997; Hodges and Forney 2000). Given that this membrane-bound enzyme is responsible for detoxification of H_2O_2 that escaped the action of CAT in the matrix (Del Río et al. 2002; Lisenbee et al. 2003) its activity inhibition, favouring the increase in cytosolic H_2O_2 content, could contribute to pathogen-induced perturbations of the cellular redox state. However, all the enzymes responsible for ascorbate and glutathione recycling showed a characteristic biphasic pattern of activity changes, similar to that described earlier for SOD, CAT and GSH-Px. The activities of MDHAR and DHAR as well as GR were induced only early during the plant–pathogen interaction whereas the appearance of disease symptoms was preceded or accompanied by a notable suppression of these enzymes. The decrease in activity of MDHAR, which has been shown to be regulated primarily by monodehydroascorbate radical (Arrigoni et al. 1997), could be the result of the observed APX suppression. Assuming that the transmembrane MDHAR participates in regenerating the peroxisomal and not the cytosolic ascorbate pool, its activity decline appeared to be also related to the significant ascorbate concentration decrease in peroxisomes from inoculated leaves. The fall of enzymes keeping ascorbate reduced contrasts with DHA concentration that remained lower than in the control. This indicated that either the decreasing activities of MDHAR and DHAR were still sufficient for AA regeneration or other mechanisms, e.g. a putative peroxisomal peroxiredoxin (Verdoucq et al. 1999), have been involved. With regard to GR, its declining activity appeared to be insufficient to prevent the oxidation of the glutathione pool to judge by the GSSG accumulated in peroxisomes from inoculated leaves. This pattern contrasts with that described for DHA.

Adversely to chloroplasts and mitochondria, where the H_2O_2-detoxifying role of AA-GSH cycle is highlighted, in peroxisomes enriched in CAT that detoxifies bulk amounts of H_2O_2 this mechanism could be also implicated in reoxidising NADH and NADPH indispensable for their metabolism (del Río et al. 2002). This could be of special interest regarding that *B. cinerea* infection favours leaf senescence and peroxisomes have been proposed to play a special ROS-mediated role during senescence (Pastori and del Río 1997; Distefano et al. 1999). It has been found that during senescence CAT activity was largely decreased, the constitutive MnSOD was depressed, APX as well as MDHAR activities decreased, and lipid peroxidation was enhanced (del Río et al. 1998). The changes in CAT activity could be also the effect of enhanced proteolysis catalysed by peroxisomal endopeptidases which are induced by oxidative stress in senescent leaves (Palma et al. 2002). In our study, changes in enzymes involved in the metabolism of ROS, namely CAT, SOD, GSH-Px, APX and MDHAR that were concomitant to those observed in senescent leaves could favour, at the peroxisomal level, the pathogen-induced promotion of senescence.

To conclude, our results indicated that the peroxisomal antioxidant system was significantly affected by *B. cinerea* infection. A defensive response manifested by SOD, CAT and GSH-Px activity increases was reflected at the peroxisomal level before the appearance of infection symptoms. However, as the disease advanced the peroxisomal antioxidant potential, expressed both as SOD, CAT and GSH-Px activities as well as ascorbate and glutathione contents, decreased. The same was true for the effectiveness of ascorbate and glutathione regeneration in the AA-GSH cycle. Whether changes in the peroxisomal antioxidant system solely reflect the progressive suppression in the organellar antioxidant defence as a consequence of successful pathogenesis or they play a signalling role at the cellular level remains to be elucidated. The collapse of the peroxisomal antioxidant enzymes involved in the metabolism of ROS parallel to the development of disease symptoms appears to contribute to the pathogen-induced leaf senescence.

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