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Effects of NaCl on the response of *Mesembryanthemum crystallinum* callus to *Botrytis cinerea* infection

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Abstract

Callus of the halophyte *Mesembryanthemum crystallinum* was used to study the effect of NaCl on the response to *Botrytis cinerea* infection. The fungus easily colonized the callus surface and the intercellular spaces. However, in the NaCl-adapted tissues the incidence of penetration was 67 % lower than in the inoculated control tissue. The modification of the infection pattern found in the salt-adapted callus could be related to metabolic adaptations to salinity. This was manifested by the enhanced antioxidant potential of ascorbate, the up-regulated activities of ascorbate peroxidase, as well as guaiacol and syringaldazine peroxidases together with the increased detoxification capacity of glutathione transferase in the NaCl-adapted callus. The post-inoculation changes in NaCl-adapted and non-adapted calli were roughly similar and supported the prooxidative nature of *B. cinerea* infection.

Additional key words: ascorbate, ascorbate peroxidase, glutathione, lipid peroxidation, plant-pathogen interaction, salinity.

Introduction

In the natural environment, plants routinely experience a combination of different abiotic stresses, often accompanied by pathogen attack. There is emerging evidence that the biotic interaction might be affected by abiotic stresses, leading to changed response to pathogens (Baker *et al.* 2000, Wiese *et al.* 2004). In recent years *Mesembryanthemum crystallinum* has become a model for research on plant abiotic stress tolerance (Bohnert and Cushman 2000). The mechanism of salt-adaptation in this facultative halophyte includes transition from C_3 photosynthesis to Crassulacean acid metabolism (CAM, Cushman and Bohnert 1997). Thus, NaCl-adaptation, the CAM-related mechanisms, or both could affect the response of NaCl-adapted *M. crystallinum* plants to subsequent biotic stress. In order to reduce the complication of CAM induction or other organ-specific responses associated with the whole plant system, we used the NaCl-adapted callus of *M. crystallinum* to study the effect of NaCl on the response to infection at the cellular level. As CAM-like mode of photosynthesis may appear in *M. crystallinum* callus grown at high irradaince (Ślesak *et al.* 2003), we used callus grown at low irradance known to be insufficient for C3-CAM transition (Miszalski *et al.* 2001).

The common background of different stresses is related to changes in the prooxidant/antioxidant equilibrium in plant cells. Reactive oxygen species (ROS) and antioxidants are an interacting system that resets the cellular redox homeostasis to ensure an adequate response under most, if not all stresses (Foyer and Noctor 2005). Salt stress is often associated with increased generation of ROS such as H_2O_2 and superoxide radical

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Abbreviations: AA - reduced ascorbate; APX - ascorbate peroxidase; CAM - crassulacean acid metabolism; DHA - dehydroascorbate; dpi - days post inoculation; EC - electrical conductivity; GSH - reduced glutathione; GSH-Px - glutathione peroxidase; GSSG - oxidized glutathione; GST - glutathione-S-transferase; O_2^- - superoxide radical; PODg - peroxidase assayed with guajacol; PODs - peroxidase assayed with syringaldazine; ROS - reactive oxygen species; WC - water content; TBARS - thiobarbituric acid reactive substances.
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anion (O_2^-), and lipid peroxidation (Aghaleh *et al*. 2009) and adaptation to salinity induces changes in the antioxidant system (Bartels and Sunkar 2005). Similarly, the overproduction of ROS is one of the most important symptoms of plant-pathogen interactions (Wojtaszek 1997) and antioxidants regulate ROS concentration and their signaling potential, simultaneously protecting a plant cell from possible harmful effects of ROS (Foyer and Noctor 2005). The generation of ROS assists the colonization of plants by *Botrytis cinerea* (Von Tiedemann 1997, Muckenschnabel *et al.* 2002) and high activity of the antioxidants in the infected tissues could be a limiting factor for *B. cinerea* infection progress (Govrin and Levine 2000, Kuźniak and Skłodowska 2005). In plant cells, ascorbate and glutathione and related enzymes *i.e*. ascorbate peroxidase (APX), glutathione-S-transferase (GST) and glutathione peroxidase (GSH-Px) are important in modulating the defense response under stress *via* redox mechanisms. Moreover, class III peroxidases, which use phenolics as the preferential electron donors, play a part of defense mechanisms against pathogens at the interface cell wall/plasma membrane (Hiraga *et al.* 2001).

This study examines whether NaCl adaptations affect the response of *M. crystallinum* callus to *B. cinerea* infection. It focuses on the microscopic analysis of infection and biochemical aspects related to the prooxidant-antioxidant equilibrium in the plant cells. We report *B. cinerea* infection-induced changes in lipid peroxidation, ascorbate and glutathione contents as well as APX, GSH-Px and GST activities in callus tissues grown in the absence (control) or presence of NaCl (NaCl-adapted). The activities of guaiacol (PODg) and syringaldazine (PODs) peroxidases as well as proline content were also determined.

Materials and methods

Callus of *Mesembryanthemum crystallinum* L. was grown as described by Ślesak *et al.* (2003) under irradiance of 70 - 80 μmol m^{-2} s^{-1} (16-h photoperiod) and temperature of 23 °C. NaCl-adapted cultures growing on the medium supplemented with 0.1 M NaCl were used for experiments after four subcultures. The biomass and the growth index [GI = (final biomass-initial biomass)/initial biomass] were determined over a period of 10 sub-cultures. The 2-week-old cultures in the exponential phase of growth and maintained in the presence or absence of NaCl, were inoculated with *Botrytis cinerea* isolate 1631, originally collected from strawberry and provided by Bank of Plant Pathogens (Poznań, Poland). The spore suspension containing 5×10^5 spores cm^{-3}, 5 mM glucose and 2.5 mM KH_2PO_4 was applied to the tissue surface, 0.1 cm^3 per culture tube. The respective mock-inoculated tissues were treated with 5 mM glucose and 2.5 mM KH_2PO_4.The cultures were taken for analysis one, two and three days post inoculation (dpi).

The water content (WC) and electrical conductivity (EC) in the control and NaCl-adapted callus tissues were determined at the time of inoculation. WC was evaluated according to formula: WC [%] = [(FM-DM)/FM] × 100, where FM was fresh mass and DM dry mass. The WC of the NaCl-adapted callus was given in relation to the control set at 100 %.

Salinity of the cell sap was measured in terms of electrical conductivity (EC) using a conductivity meter (*SevenEasy*, *Mettler*, Toledo, Spain).

Callus fragments taken 3 dpi were fixed for 4 h in 3 % glutaraldehyde in 0.1 M cacodylate buffer (pH 6.8), supplemented with 0.05 % $CaCl_2$ and 0.1 M glucose. After washing in the buffer and postfixation in 1 % osmium tetroxide they were prepared for electron microscopy as described earlier (Gabara *et al*. 2003). Sections were cut on *Reichert* ultratome and the semithin (1 μm thick) sections after staining with toluidine blue were photographed in *Zeiss* (Jena, Germany) light microscope while ultrathin ones (70 - 90 nm) stained with uranyl acetate followed by lead citrate were analyzed in *Jeol 1010* (Tokyo, Japan) electron microscope at 80 kV. The number of hyphae in the inoculated callus tissues was calculated in 40 micrographs per case (each comprised 18 400 μm^2 surface area of callus).

Callus was homogenized (1:5, m/v) in 0.05 M ice-cold potassium phosphate buffer (pH 7.0) containing 1 M NaCl, 1 mM ethylenediaminetetraacetic acid, 1 % (m/v) polyvinylpyrrolidone. After centrifugation (20 000 *g*, 15 min) the supernatant was used to measure the enzyme activities as well as lipid peroxidation, proline and glutathione contents. For determination of ascorbate, the tissue was homogenized in 10 % (m/v) ice-cold trichloroacetic acid.

Lipid peroxidation was estimated spectrofluorometrically (Yagi 1982), by measuring the concentration of thiobarbituric acid reactive substances (TBARS), and calculated in terms of 1,1,3,3-tetraethoxypropane used as a standard. Free proline concentration was determined according to Bates *et al.* (1973) and estimated by referring to a standard curve for L-proline.

The activities of ascorbate peroxidase (APX; EC 1.11.1.11), glutathione peroxisase (GSH-Px; EC 1.11.1.19), glutathione-S-transferase (GST; EC 2.5.1.8) as well as ascorbate and glutathione contents were determined as described earlier (Kuźniak and Skłodowska 2001). The activity of guiacol peroxidase (PODg; EC 1.11.1.7) was measured as enzymatic oxidation of guaiacol (ε = 26.6 mM^{-1} cm^{-1}) according to Maehly and Chance (1954). The activity of syringaldazine peroxidase (PODs; EC 1.11.1.7) based on

the oxidation rate of syringaldazine (ε = 27.0 mM^{-1} cm^{-1}; Quiroga *et al.* 2000) was determined as the increase in absorption at 530 nm, in the presence of 15 mM H_2O_2 in 50 mM sodium-phosphate buffer (pH 6.0) and 2.9 mM syringaldazine.

Protein was determined by the method of Bradford (1976) using standard curves prepared for bovine serum albumin.

The data are means ± SD from 3 - 5 independent replications, each including 2 parallel measurements. The data were subjected to analysis of variance, and the means were compared by Tukey's test at 5 % probability.

Results

The NaCl-adapted callus, with EC of the cell sap of 1860 μS cm^{-1}, showed no salt-induced injury symptoms and its growth kinetics was similar to that in the cultures maintained in the absence of NaCl (the cell sap EC of 660 μS cm^{-1}). Both control and NaCl-adapted cultures demonstrated good proliferation capacity, with an average growth index of eight for 10 subcultures analyzed, and the fastest growth in the middle of the 4-week culture period, when they were taken for experiments. The NaCl-adapted callus was more friable and pale green in comparison to the control being soft in texture and deep green. It turned yellow at the end of the culture period, demonstrating the symptoms of senescence earlier than the control. The NaCl-adaptation reduced the WC by 22 % and significantly increased proline content (11-fold), but did not affect the protein content (Table 1).

Table 1. Water content (WC) and contents of proline and protein in control and NaCl-adapted callus cultures of *M. crystallinum.* Means ± SD of five independent replicates and refer to callus cultures at the moment of inoculation. Values followed by different superscript letters in each column differ significantly ($P < 0.05$).

Callus	WC [%]	Proline content [nmol g^{-1}(f.m.)]	Protein content [mg g^{-1}(f.m.)]
Control	100	15.18 ± 3.96^a	3.415 ± 0.515^a
NaCl-adapted	78	174.34 ± 3.83^b	3.675 ± 0.520^a

The first visible symptoms of colonization by *B. cinerea* on the control and NaCl-adapted callus developed 1 dpi in the form of sparse hyphal growth in the immediate vicinity of the point of inoculation. The fungus preferentially grew at the callus surface and in the intercellular spaces of its peripheral cells. The plant cells adjacent to the penetrating hyphae remained intact. In the control, the growth rate of *B. cinerea* as well as its proliferation toward the basipetal regions of the callus were intensive and 3 dpi the mycelium occupied the whole callus (Fig. 1*A*). Conversely, in the NaCl-adapted tissues (Fig. 1*B*) the fungus grew almost exclusively on the tissue surface and the number of hyphae diminished from 41.46 ± 3.30 (control) to 13.88 ± 1.26, as calculated per 18 400 μm^2 of callus surface area. In the control, hyphae characterized by numerous branching were filled with dense cytoplasm (Fig. 1*A*,*C*) while in the NaCl-adapted callus they were strongly vacuolated (Fig. 1*B*,*D*), thinner and without signs of branching (Fig. 1*B*). In the cytoplasm of the hyphae penetrating the control (Fig. 1*C*) and NaCl-adapted (Fig. 1*D*) callus numerous organelles, *e.g.* ribosomes, mitochondria, nuclei and vacuoles were present, reflecting their active metabolic state.

In spite of the above-mentioned differences, *B. cinerea* seemed to enter the cells of control and NaCl-adapted callus tissues in the same way. At first, at the apex of hyphae, near the callus cell wall, the electron-transparent vesicles embedded in electron dense material, reticulate in structure accumulated (Fig. 1*E*). Than, a pore, much smaller in diameter than the fungal hyphae, appeared in the callus cell wall (Fig. 1*F*). The penetration tube attained its smallest diameter while it entered the callus cell (Fig. 1*G*). Inside the cell, the diameter of the hyphal tube increased markedly and the reticulate material and vesicles were no longer visible in the vicinity of its apical part (Fig. 1*H*). Moreover, the inward indentation of the host cell wall at the point of penetration indicated a mechanical interference (Fig 1*I*). No alteration of the plant cell wall distant from the penetration site was recorded.

We examined also the effect of NaCl on *B. cinerea* growth *in vitro*. NaCl in the concentration range from 0.1 to 0.8 M did not inhibit the pathogen growth on plates of potato dextrose agar (data not shown).

B. cinerea induced TBARS accumulation, both in the callus maintained in the absence and presence of NaCl. However, only in the infected control tissue 3 dpi a significant increase in TBARS content was found (Table 2). The basal activities of APX, PODg and PODs were remarkably higher in the NaCl-adapted tissues than in the control (Table 2). The most pronounced effect was observed for PODs activity which in the NaCl-adapted callus increased over 9-fold, on average. No significant difference in APX activity between the inoculated and non-inoculated control tissues was noticed whereas in the NaCl-adapted callus it decreased 2 and 3 dpi by 37 and 44 %, respectively. *B. cinerea* induced a notable increase in PODg activity 2 dpi and 3 dpi in the control cultures and 2 dpi in the salt-adapted ones. Although PODg activity was considerably higher in the NaCl-adapted tissues, the inoculation-induced activity increase was much stronger in the control cultures. The activity of

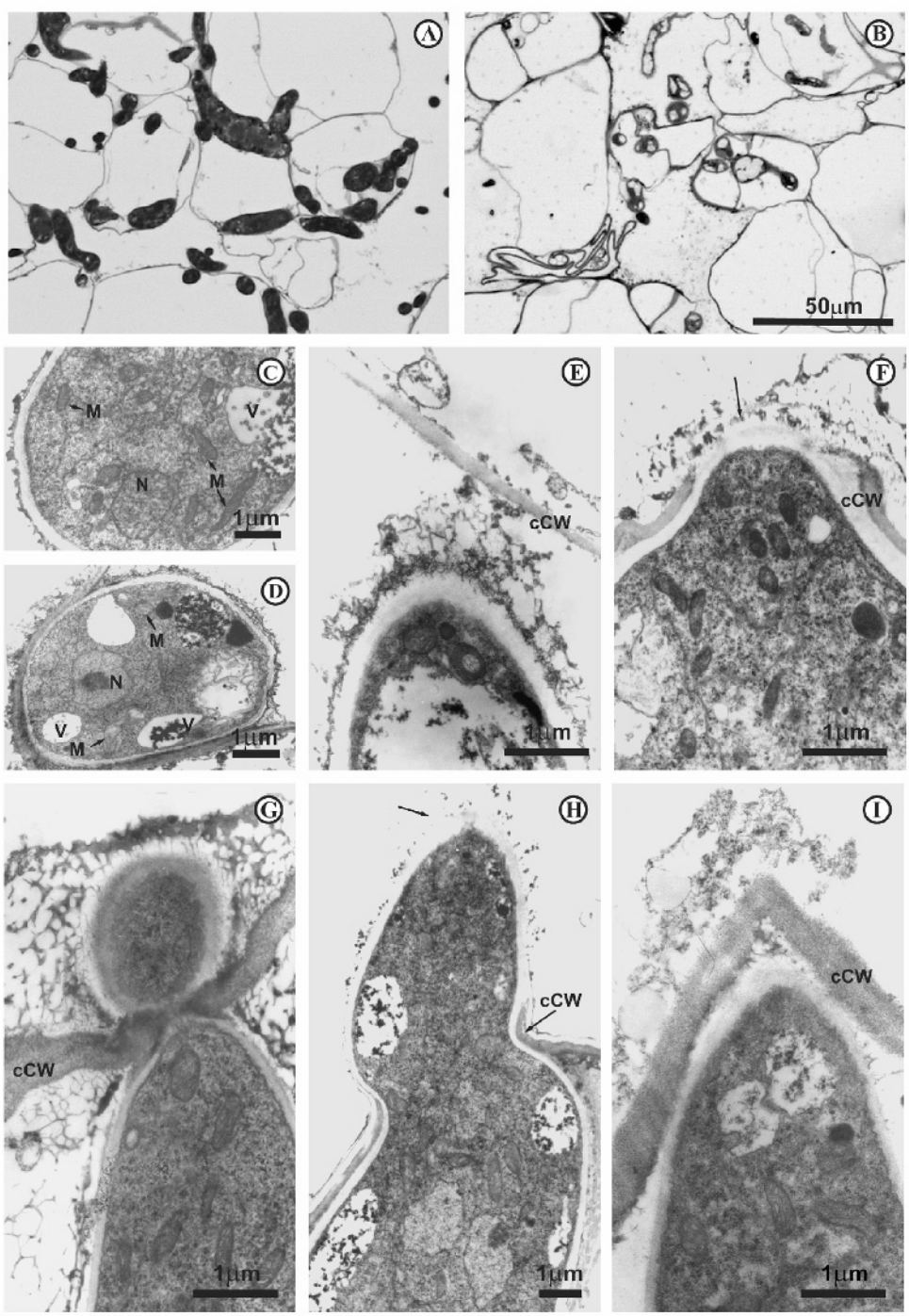

Fig. 1. *Mesembryanthemum crystallinum* infected with *Botrytis cinerea, A,B* - semithin sections stained with toluidine blue; *C* to *I* - ultrathin cross sections; *A,C,G,H* - control; *B,D,F,I* - NaCl-adapted callus; *A* - numerous hyphae, sometimes branching, filled with cytoplasm are present in the callus cells and in the intercellular spaces; *B* - a few hyphae, strongly vacuolated, without branching, are visible in the callus cells and in the intercellular spaces; *C* - dense cytoplasm, nuclei (N), ribosomes, mitochondria (M) and vacuoles (V) are seen.; *D* - hyphae in the NaCl-adapted callus are thinner and strongly vacuolated; *E* - apex of hyphae covered with many vesicles and reticulate material; part of the latter is in the vicinity of callus cell wall (cCW); *F* - hyphae entering a callus cell; the plant cell wall in a close contact with the hyphae apex (*arrow*) and the remnants of the material visible at the apex of hyphae in Fig. 1*E* are present; G - *B. cinerea* penetrating the plant cell through a pore in the cell wall; material reticulate in structure, probably representing the plant cell wall material, visible mostly around the hyphae apex; *H* - a further step of hyphae penetration, note the absence of the material at the apex of hyphae (*arrow*); *I* - deformation of the callus cell wall by the apex of growing hyphae. The *E* - *I* stages were similar in the control and NaCl-adapted cells.

Table 2. The effects of *Botrytis cinerea* infection on lipid peroxidation, the activities of peroxidases and glutathione transferase in *Mesembryanthemum crystallinum* callus tissues grown in the absence (-NaCl) and presence of NaCl (+NaCl). Means ± SD of five independent replicates. Values with different letters indicate significant differences between treatments on individual days at $P < 0.05$.

Parameter	dpi	-NaCl control	*Botrytis cinerea*	+NaCl control	*Botrytis cinerea*
TBARS [nmol mg^{-1}(protein)]	1	2.94±0.64 ^{a}	3.55±0.78 ^{a}	2.07±0.72 ^{a}	2.64±0.57 ^{a}
	2	2.95±0.78 ^{ab}	3.51±0.36 ^{bc}	2.38±0.28 ^{a}	2.46±0.29 ^{a}
	3	2.98±0.91 ^{a}	4.91±0.27 ^{b}	2.18±0.57 ^{a}	2.96±0.41 ^{a}
APX [U mg^{-1}(protein)]	1	0.49±0.08 ^{a}	0.44±0.04 ^{a}	1.27±0.25 ^{b}	1.10±0.19 ^{b}
	2	0.70±0.12 ^{ac}	0.62±0.10 ^{ac}	1.19±0.04 ^{b}	0.75±0.13 ^{c}
	3	0.45±0.08 ^{a}	0.43±0.05 ^{a}	1.15±0.07 ^{b}	0.64±0.10 ^{c}
PODg [U mg^{-1}(protein)]	1	0.76±0.26 ^{a}	0.72±0.12 ^{a}	3.82±0.79 ^{b}	3.56±0.60 ^{b}
	2	0.70±0.13 ^{a}	1.66±0.25 ^{b}	3.19±0.71 ^{c}	4.25±0.57 ^{d}
	3	0.58±0.08 ^{a}	1.06±0.13 ^{b}	4.64±0.15 ^{c}	3.99±0.87 ^{c}
PODs [U mg^{-1}(protein)]	1	0.16±0.03 ^{a}	0.39±0.08 ^{b}	2.28±0.28 ^{c}	2.02±0.38 ^{c}
	2	0.26±0.03 ^{a}	0.54±0.05 ^{b}	1.54±0.07 ^{c}	1.84±0.26 ^{c}
	3	0.26±0.04 ^{a}	0.53±0.03 ^{b}	2.43±0.15 ^{c}	3.99±0.87 ^{d}
GSH-Px [U mg^{-1}(protein)]	1	35.76±9.76 ^{a}	41.86±10.5 ^{a}	31.62±6.99 ^{a}	46.61±14.7 ^{a}
	2	45.45±0.16 ^{a}	69.20±10.9 ^{b}	34.49±6.86 ^{c}	32.38±1.51 ^{c}
	3	36.18±7.10 ^{ab}	25.16±1.57 ^{bc}	36.47±2.00 ^{a}	20.42±3.91 ^{c}
GST [U mg^{-1}(protein)]	1	78.00±9.43 ^{a}	93.00±6.41 ^{b}	111.00±3.41 ^{c}	145.90±16.9 ^{d}
	2	79.30±0.89 ^{a}	141.90±20.3 ^{bd}	123.40±25.1 ^{bc}	184.00±33.0 ^{dc}
	3	100.00±31.0 ^{a}	160.97±43.2 ^{ab}	151.30±5.53 ^{bc}	210.48±27.3 ^{bd}

Table 3. The effects of *Botrytis cinerea* infection on ascorbate and glutathione contents in callus tissues grown in the absence (-NaCl) and presence of NaCl (+NaCl). For explanation see Table 2.

Parameter	dpi	-NaCl control	*Botrytis cinerea*	+NaCl control	*Botrytis cinerea*
GSH [nmol mg^{-1}(protein)]	1	2.33±0.64 ^{a}	1.64±0.48 ^{ab}	2.22±0.11 ^{a}	0.98±0.29 ^{b}
	2	2.34±0.49 ^{a}	1.49±0.50 ^{ab}	1.62±0.13 ^{a}	1.31±0.13 ^{b}
	3	2.17±0.24 ^{a}	1.47±0.50 ^{b}	1.67±0.33 ^{bc}	0.69±0.13 ^{d}
GSSG[nmol mg^{-1}(protein)]	1	0.70±0.14 ^{a}	0.87±0.25 ^{ac}	0.96±0.22 ^{ab}	0.46±0.07 ^{c}
	2	1.20±0.25 ^{a}	0.91±0.25 ^{a}	1.39±0.32 ^{ab}	0.21±0.01 ^{c}
	3	1.06±0.05 ^{a}	0.70±0.05 ^{b}	0.74±0.17 ^{b}	0.52±0.07 ^{b}
GSH/GSSG	1	3.83	1.90	2.36	2.11
	2	1.94	1.63	1.16	6.11
	3	2.04	2.10	2.24	1.31
AA [μmol mg^{-1}(f.m.)]	1	0.27±0.07 ^{ad}	0.27±0.02 ^{ac}	0.43±0.06 ^{bc}	0.49±0.06 ^{cd}
	2	0.28±0.04 ^{ad}	0.18±0.04 ^{b}	0.34±0.03 ^{c}	0.26±0.04 ^{d}
	3	0.25±0.03 ^{a}	0.10±0.02 ^{b}	0.40±0.09 ^{c}	0.43±0.12 ^{c}
DHA [μmol mg^{-1}(f.m.)]	1	0.08±0.01 ^{a}	0.08±0.01 ^{ab}	0.11±0.01 ^{b}	0.10±0.02 ^{b}
	2	0.09±0.02 ^{ad}	0.08±0.01 ^{ac}	0.12±0.01 ^{bc}	0.15±0.02 ^{bd}
	3	0.12±0.00 ^{ad}	0.06±0.02 ^{b}	0.202±0.054 ^{c}	0.11±0.03 ^{d}
AA/DHA	1	3.38	3.41	3.81	4.65
	2	3.21	2.31	2.90	1.67
	3	2.07	1.64	1.96	3.71

PODs showed a constant infection-dependent increase in the cultures grown in the absence of NaCl, being over 2-fold higher than in the control. A similar although less marked effect, starting from 2 dpi only, was found in the NaCl-adapted tissues. The activity of GSH-Px in the inoculated tissues, after initial increase in the control callus 1 and 2 dpi and in the NaCl-adapted tissue only 1 dpi, decreased by 30 and 44 % in the control and NaCl-adapted cells, respectively. GST showed a significantly higher basal activity in the NaCl-adapted cells. *B. cinerea*

provoked GST activity to increase in the callus grown in the absence and presence of NaCl and the strongest effect was detected 2 dpi (Table 2). The total glutathione and GSH pools significantly decreased in response to infection (Table 3). The most pronounced changes in the total glutathione content appeared in the NaCl-adapted cells. In the inoculated NaCl-adapted tissues, the total glutathione concentration was lower than in the inoculated control, reaching the minimum value of 1.21 nmol mg^{-1}(protein) 3 dpi. The changes in the GSH pool were of similar pattern but less dynamic. The GSH content decline in the inoculated control callus ranged from 29 to 36 % over the whole period. However, in the inoculated NaCl-adapted tissues a significant (56 %) decrease in GSH content 1 dpi, followed by a minor decrease of 19 % 2 dpi, was seen. Thereafter, GSH content dropped markedly to 0.686 nmol mg^{-1}(protein), being over 2-fold lower than in the inoculated control. There was no significant effect of *B. cinerea* on GSSG content in the control cultures 1 and 2 dpi whereas 3 dpi a decrease (34 %) was found (Table 3). In contrast, GSSG content in the inoculated NaCl-adapted tissues was significantly reduced 1 dpi (51 %) and 2 dpi (84 %). The glutathione redox ratio (GSH/GSSG) tended to decrease, except the significant ratio increase to 6.11 observed in the NaCl-adapted cells 2 dpi. The minimum glutathione redox ratios in the inoculated control and NaCl-adapted cultures were detected 2 and 3 dpi, respectively (Table 3).

In the NaCl-adapted callus the absolute contents of total ascorbate and AA were significantly higher than in the tissue grown in the absence of salt (Table 3). Pathogen induced a significant and progressive reduction in total ascorbate and AA contents 2 and 3 dpi in the control tissues. However, there was no significant response of the salt-adapted tissues, except for the reduction in AA content 2 dpi. In response to inoculation, the concentration of DHA decreased significantly only 3 dpi, in both salt-adapted and control tissues. The changes in ascorbate redox ratio followed those in AA and its increase in the inoculated NaCl-adapted callus found 2 dpi was the consequence of lowered DHA concentration (Table 3).

Discussion

We found that both callus types were intensively surface colonized by *B. cinerea*. However, the pathogen penetrated the underlying cells in the control and salt-adapted tissues with different incidence. The NaCl-adapted callus was penetration resistant, as the incidence of penetration was 67 % lower than in the inoculated control tissue. However, the way in which *B. cinerea* penetrated the control and NaCl-adapted cells was similar. It is tempting to speculate that it was probably the result of: *1*) structural disintegration of the plant cell wall, as indicated by the presence of the vesicles and the material reticulate in structure only in the vicinity of the apical part of hyphae successfully penetrating the plant cell wall (Fig. 1*E-G*) and *2*) mechanical interference as suggested by the inward indentation of the host cell wall at the point of penetration (Fig. 1*I*). The reason for the impeded penetration of the cells adapted to salt remains further to be elucidated. Although 0.1 M NaCl did not inhibit *B. cinerea* growth *in vitro* (data not shown), it cannot be excluded that the changed water potential in the NaCl-adapted callus negatively affected the proliferation rate of the mycelium, as water relations in the plant tissue have been ascribed a role in the interaction between *B. cinerea* and its host (Elad and Evensen 1995). Thus, the strong vacuolization of the hyphae growing in the salt-adapted callus (Fig. 1*D*) could be related to the osmotic adjustment of the fungus and the osmoregulatory function of the vacuoles.

The significance of the osmoprotective function of osmotic stress-induced accumulation of proline has been reported for many plants and for some cultured cells, including *M. crystallinum* (Yen *et al.* 1995). Proline also serves as a storage sink for carbon and nitrogen, stabilizes subcellular structures and buffers cellular redox potential under stress (Chinnusamy *et al.* 2005). Moreover, its role in scavenging ROS and protecting from ROS-induced damaging effects has been suggested (Zhu 2001). Taking into account the well-documented accumulation of ROS during *B. cinerea* infection culminating in lipid peroxidation and depletion of antioxidants (Von Tiedemann 1997, Govrin and Levine 2000, Williamson *et al.* 2007), the antioxidant potential of proline accumulated in the NaCl-adapted callus could restrict the proliferation of the fungus. Simultaneously, proline could fulfill other protective functions mentioned above rendering the NaCl-adapted cells hardly accessible to the pathogen.

As *B. cinerea* strongly enhanced peroxidase activities in both callus types, the difference in the infection pattern observed between the control and salt-adapted tissues could be attributed to the significantly higher steady state levels of activities detected in the cells adapted to NaCl. The markedly increased constitutive activities of class III peroxidases in these tissues could be considered as signs of diverse H_2O_2-mediated peroxidative processes involved in salt adaptation that modulated the infection development as well. An increased activity of a cell wall peroxidase encoded by a gene specifically up regulated by NaCl treatment was reported in tomato roots (Botella *et al.* 1994). Moreover, ROS produced under stress have been attributed the ability to elicit the peroxidase-catalysed cell wall strengthening (Quiroga *et al.* 2000, Ribeiro *et al.* 2006). The oxidation of syringaldazine, a lignin monomer analog, has been suggested to be

indicative of the synthesis of lignin and suberin (Pang *et al.* 1989). As biotic stress is perceived firstly by the apoplast, these peroxidase-mediated processes could be important in the plant cell attempt to stop pathogen spread.

Many studies have demonstrated metabolic cell reprogramming in the area of redox regulations during adaptation to stress (Foyer and Noctor 2005, Niewiadomska and Borland 2008, Liu *et al.* 2010). In line with these reports, the adaptation of *M. crystallinum* callus to NaCl was linked to the capacity to maintain significantly higher ascorbate concentration as well as GST, APX, PODg and PODs activities whereas the oxidative stress profile, in terms of lipid peroxidation, was unaffected. Thus, in the tissue adapted to salinity the prooxidant/antioxidant mechanisms operated at the basal levels completely different from those in the control callus. The changes in the control and salt-adapted cells after inoculation were, in general, qualitatively similar and supported the earlier data on the pro-oxidative nature of *B. cinerea* infection (Kuźniak and Skłodowska 2005, Muckenschnabel *et al.* 2002). The most prominent oxidative effects of the infection were expressed as a remarkable decrease in glutathione concentration together with GST activity increase, being in accordance with the suggestion that the pro-oxidative state resulting from the reduced GSH content triggers GST gene expression (Vranova *et al.* 2002). In this context, it should be mentioned that the constitutive and post-infection whole-cell contents of H_2O_2 and catalase activity did not differ significantly in the control and salt-adapted tissues (data not shown). Furthermore, the present results confirmed our previous reports on tomato that *B. cinerea* infection drastically affected the glutathione pool (Kuźniak and Skłodowska 2005). The marked increase in GST activity observed in this study could indicate higher need for detoxification of toxic endogenous compounds, potentially the products of *B. cinerea*-induced lipid peroxidation (Muckenschnabel *et al.* 2002).

We found that after infection the antioxidant defense in terms of metabolites and enzymes was roughly similar. Thus, the modification in the infection pattern could be attributed to the quantitative differences, resulting mainly from the changed equilibrium of the pro- and antioxidants in the salt-adapted callus. Many studies referred to the important role of plant tissue antioxidants in the enhanced disease resistance (Barna *et al.* 2003, Kuźniak and Skłodowska 2005), and changes in the ascorbate and glutathione contents and their redox ratios appeared to be intrinsic responses of plants to biotic stress (Kuźniak and Skłodowska 2005). In our study, the enhanced antioxidant potential of ascorbate (Table 3) and the increased activities of APX, PODg and PODs, together with the reinforced GST detoxification capacity in the salt-adapted callus (Table 2) could successfully counteract the oxidative impact of *B. cinerea* ingress, as supported by the significantly lowered level of lipid peroxidation compared to the infected control tissue (Table 2). Moreover, changed pro- and anti-oxidant equilibrium, considered an adaptation process aimed at balancing the redox-controlled regulation mechanism, could take part in creating an intracellular micro-environment that strengthened the penetration resistance of the plant tissue and modulated the infection pattern.

In conclusion, we found that the control and NaCl-adapted callus tissues of *M. crystallinum* showed differences in the development of *B. cinerea* infection. The control callus provided a more favorable environment for the fungus, enabling stronger colonization potential. The salt adaptation modulated the response to the subsequent biotic stress, as *B. cinerea* growth in the inoculated NaCl-adapted tissue was restricted, at least with respect to the penetration accessibility of the callus. Although the morphological and physiological backgrounds of this phenomenon, and the possible role of the antioxidant system need to be further elucidated, the changed pro- and antioxidant equilibrium, resulting from metabolic adaptation to salinity, is likely to be involved in the enhanced capacity to withstand the pathogen ingress observed in the NaCl-adapted callus.

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