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The oxidants and antioxidant enzymes in tomato leaves treated with o-hydroxyethylorutin and infected with *Botrytis cinerea*

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Abstract

Application of o-hydroxyethylorutin restricted the development of *Botrytis cinerea* in tomato leaves. Superoxide anion and hydrogen peroxide generation rates and changes in superoxide dismutase, peroxidase and catalase activities were studied in uninfected tomato plants, in plants infected with *B. cinerea*, and in plants treated with o-hydroxyethylorutin and infected with pathogen. About two times higher hydrogen peroxide concentration were found in plants treated with o-hydroxyethylorutin and infected with the pathogen at the early infection stages compared with untreated infected plants. *In vitro* tests showed that germination of *B. cinerea* conidia was significantly inhibited by H_2O_2. Higher H_2O_2 concentrations were needed to inhibit mycelial growth. The results indicate that o-hydroxyethylorutin triggers hydrogen peroxide production in tomato plants and suggest that enhanced levels of H_2O_2 are involved in restricted *B. cinerea* infection development.

Abbreviation: APX – ascorbate peroxidase; AOS – active oxygen species; CAT – catalase; NBT – nitro blue tetrazolium; SOD – superoxide dismutase.

Introduction

It has been reported that production of active oxygen species (AOS) such as $O_2^{\bullet -}$, $OH^{\bullet}$, H_2O_2, during the so-called oxidative burst, is one of the earliest and most effective defence reactions of plants. AOS have been suspected to play a role in many defence processes including direct antimicrobial action, lignin formation, phytoalexin production, the hypersensitive response and triggering of systemic acquired resistance (Mehdy, 1994; Baker and Orlandi, 1995; Peng and Kuć, 1992; Tenhaken et al., 1995; Chen et al., 1993). There are strong suggestions that among the generated AOS, H_2O_2 plays a central role in plant defence responses (Bestwick et al., 1998; Mehdy et al., 1996; Wu et al., 1995). AOS produced via an oxidative burst may also deleteriously affect the host cells themselves, thus in plant tissues they are under control of antioxidant defences which comprises enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases, together with low-molecular-weight antioxidants: α-tocopherol, flavonoids, ascorbate and glutathione (Alscher et al., 1997; Baker and Orlandi, 1995; Low and Merida, 1996).

Plant defence responses can be activated upon infection with pathogen as well as following treatment with elicitors. Various substances have been demonstrated to be elicitors of disease resistance in plants (Cohen, 1994; Wendehenne et al., 1998). Induced disease resistance is the phenomenon by which a plant mobilises its own defence mechanism to restrict disease development. When applied on tomato leaves, o-hydroxyethylorutin limited *B. cinerea* infection development (Małolepsza et al., 1998). o-Hydroxyethylorutin is semi-synthetic, water soluble, derivative of quercetin-3-rhamnoglucoside (rutin)

(Bruneton, 1995). Rutin represents the naturally-occurring form of quercetin, which is the predominant flavonoid found in plants, useful in the pharmaceutical, food and chemical industries (Bruneton, 1995; Miniati and Montanari, 1998; Glinka et al., 1995). Flavonoid compounds are popular secondary metabolites in vascular plants. They are known to be induced in plants by exposure to UV and other types of stresses. They often accumulate in response to wounding, pathogen infection, high light, ozone and nutrient deficiency (Dixon and Paiva, 1995). These stress conditions tend to produce AOS in cells. There are suggestions that flavonoids may contribute to the overall mechanisms for protecting cells from oxidative damage by acting as antioxidants in addition to their action as optical filters (Gould et al., 1995; Yamasaki et al., 1997). However, the function of flavonoid compounds in plant tissues is not clear. It has been pointed out that flavonoids can have pro-oxidant effects under some conditions and thus should not be simplistically classified as antioxidants (Cao et al., 1997; Rice-Evans et al., 1997).

It was thus intriguing to study whether the increased resistance of tomato plants to infection with *B. cinerea* induced by o-hydroxyethylorutin treatment was mediated by an oxidant/antioxidant system. In this paper $O_2^{\bullet -}$ and H_2O_2 generation rates and SOD, peroxidases and CAT activities were compared in uninfected and *B. cinerea* infected tomato plants as well as in ones treated with o-hydroxyethylorutin and infected with pathogen.

Materials and methods

Plant material

Tomato plants (*Lycopersicon esculentum* Mill. cv 'Perkoz') were grown in soil in a growth chamber with a 16 h photoperiod at 350 $\mu E\ m^{-2}\ s^{-1}$ light intensity at 23 °C. At the age of one month plants were taken for experimentation. Some of the plants were sprayed with 0.5 mM o-hydroxyethylorutin solution; the other plants were sprayed with water. Two hours later, the second, fully expanded leaves were removed from untreated-control and plants treated with o-hydroxyethylorutin and placed in 10 cm diameter Petri dishes containing filter paper moistened with 5 ml of water. The excised leaves were either inoculated with 5 µl drops of *B. cinerea* conidial suspension ($1 \times 10^6\ ml^{-1}$) or left uninoculated. The Petri dishes containing the excised leaves were incubated in the growth chamber. Leaves were examined and harvested 2, 6, 24 and 48 h later.

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B. cinerea *culture*

B. cinerea in stock culture was maintained on potato dextrose agar in the dark at 24 °C. The conidial suspension was obtained by washing potato dextrose agar slant cultures with tap water; $1 \times 10^6\ ml^{-1}$ conidial suspension was used to inoculate tomato leaves.

The effect of H_2O_2 on the germination of B. cinerea *conidia* in vitro

A known quantity of H_2O_2 was added to 25 ml of conidial suspension samples in sterile tap water in 100 ml Erlenmayer flasks. The final concentrations of H_2O_2 in cultures were 0.5, 5, 10, 50, 100 mM. The samples were incubated at 24 °C in the dark with gentle rotation. Control conidial suspensions were incubated in sterile tap water. The percentage of germinated spores was determined microscopically after 24 h. Spores were considered germinated when the length of germ tubes exceeded the diameter of the spore.

Some of the conidial suspension cultures were incubated in 0.5, 5, 50 mM H_2O_2 for 2 h. Then the conidia were centrifuged out and washed three times with sterile tap water and incubated as described above. The germinated spores were counted after 24 h.

The effect of H_2O_2 on B. cinerea *mycelium growth*

A known quantity of H_2O_2 was added to potato dextrose agar medium just before its gelling. Final concentrations of H_2O_2 were 0.5, 1, 5, 10, 50, 100 mM. Medium (15 ml) including H_2O_2 was transferred to Petri dishes and inoculated with one piece of mycelium (10 mm in diameter). The dishes were incubated at 24 °C in the dark and mycelial diameters were measured after 48 h.

Nitro blue tetrazolium reducing activity

Measurement of nitro blue tetrazolium (NBT) reduction, a method used for the determination of $O_2^{\bullet -}$, was described by Doke (1983). Five leaf discs (0.5 cm diameter) were immersed in 3 ml 0.01 M potassium phosphate buffer pH 7.8 containing 0.05% NBT and 10 mM NaN_3 for 1 h. The mixture was then heated at 85 °C for 15 min and cooled. The reducing activity of NBT by the discs was expressed as increased absorbance at 580 nm $h^{-1}\ l^{-1}$ g of fresh weight.

Assay of hydrogen peroxide concentration

Hydrogen peroxide was measured by the method described by Capaldi and Taylor (1983) with slight modification. Leaves were ground in 5% TCA (2.5 ml per 0.5 g leaves tissue) with 50 mg active charcoal at 0 °C and centrifuged for 10 min at 15 000*g*. Supernatant was collected, neutralised with 4 N KOH to pH 3.6 and used for H_2O_2 assay. The reaction mixture contained 200 µl of leaf extract, 100 µl of 3.4 mM 3-methylbenzothiazoline hydrazone (MBTH). The reaction was initiated by adding 500 µl of horseradish peroxidase solution (90 U 100 ml^{-1}) in 0.2 M sodium acetate (pH 3.6). Two minutes later 1400 µl of 1 N HCl was added. The A_{630} was read after 15 min.

Preparation of enzyme extracts

Leaf tissue of 0.5 g was homogenised in 5 ml of 50 mM phosphate buffer pH 7.0 containing 1 N NaCl, 1% PVP MW 40 000, 1 mM ascorbate at 4 °C. After centrifugation at 15 000*g* for 15 min the supernatant was collected.

Assay of superoxide dismutase (SOD) (EC 1.15.1.1) activity

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Beauchamp and Fridovich (1971). The 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 20 µl enzyme extract. Riboflavin was added last and the reaction was initiated by placing the tubes 30 cm below two 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 10 min. Switching off the light stopped the reaction and the tubes were covered with black cloth. Non-illuminated tubes served as control. The absorbances at 560 nm were read. The volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

Assay of guaiacol peroxidase (PO) (EC 1.11.1.7) activity

Peroxidase activity was assayed colorimetrically with guaiacol as substrate (Maehly and Chance, 1954). Enzyme extract of 0.5 ml, 0.5 ml of 0.05 M acetate buffer pH 5.6, 0.5 ml 0.06 M H_2O_2 and 0.5 ml of 0.02 M guaiacol were used. The linear increases in absorbance at 480 nm were monitored for 4 min at 30 °C. The increase in absorbance equal to 1 in 1 min incubation was assumed to be one activity unit.

Assay of ascorbate peroxidase (APX) (EC 1.11.1.11) activity

APX activity was determined spectrophotometrically by a decrease in absorbance at 265 nm (ε = 13.7 mM^{-1} cm^{-1}) (Nakano and Asada, 1981). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 5 mM ascorbate, 0.5 mM H_2O_2 and enzyme extract. Addition of H_2O_2 started the reaction. The rates were corrected for the non-enzymatic oxidation of ascorbate by the inclusion of reaction mixture without enzyme extract. Enzyme activity was expressed in µmol ascorbate min^{-1}.

Assay of calalase (CAT) (EC 1.11.1.6) activity

CAT activity was determined by consumption of H_2O_2 (Dhindsa et al., 1981). The reaction mixture contained 50 mM potasium phosphate buffer pH 7.0, 15 mM H_2O_2 and plant extract. The consumption of H_2O_2 was monitored spectrophotometrically at 240 nm (ε = 45.2 $mM^{-1}cm^{-1}$). Enzyme activity was expressed in µM H_2O_2 min^{-1}.

Assay of protein content

Protein was determined by the method of Bradford (1979) with standard curves prepared using bovine serum albumin (Sigma).

Statistical analysis

The significance of differences between mean values obtained from four independent experiments with three replicates each was determined by Students' *t*-test.

Results

When tomato leaves were inoculated with *B. cinerea*, the first symptoms of infection – necrotic lesions – were noticed 48 h after inoculation. The lesions were expanding and about 80% of the leaf surface was affected after 7 days. In leaves sprayed with o-hydroxyethylorutin and inoculated with fungus, infection development was strongly inhibited (Figure 1); lesions

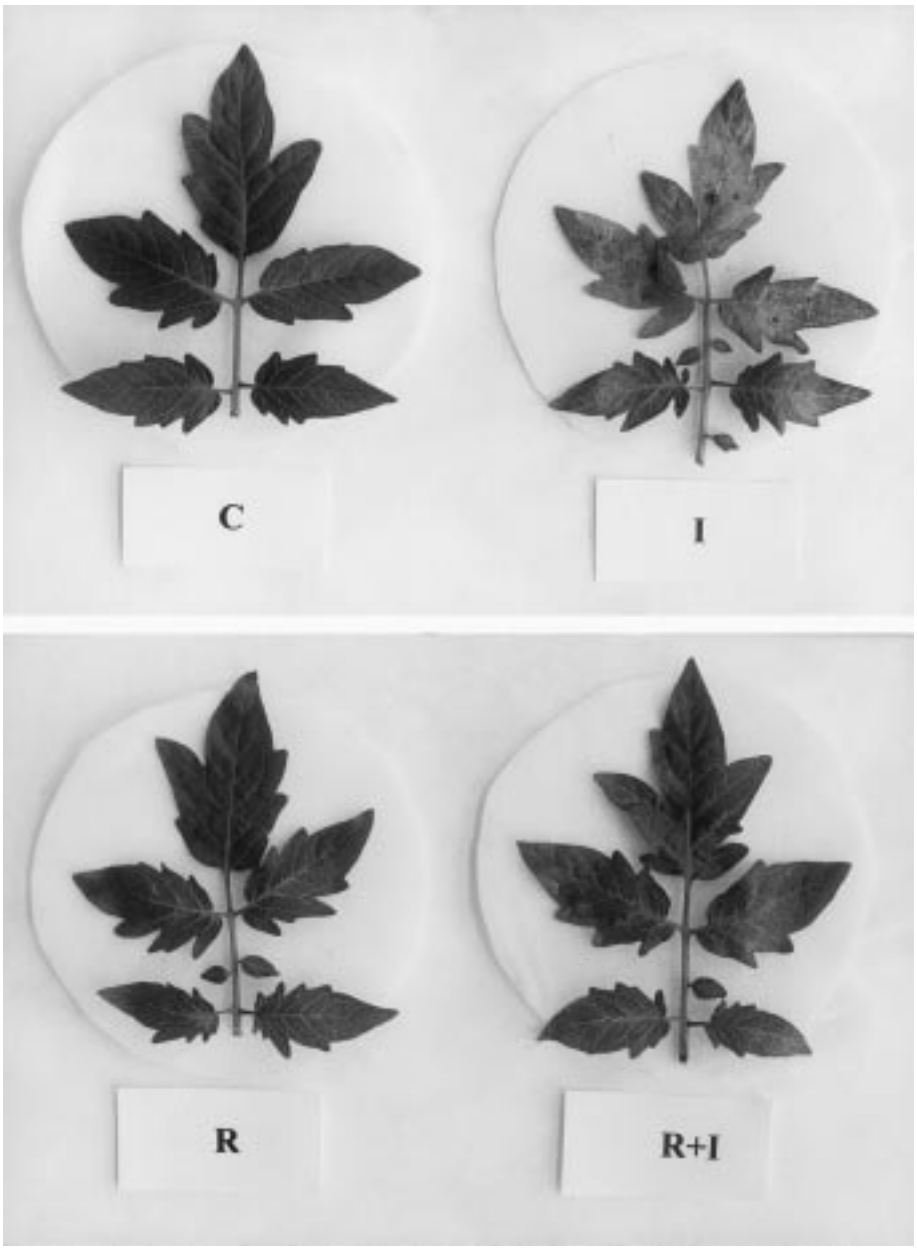

Figure 1. *B. cinerea* infection development in tomato leaves: control (C), infected with pathogen (I), treated with o-hydroxyethylorutin (R), pretreated with o-hydroxyethylorutin and infected with pathogen (R + I).

were visible on not more than 20% of these leaves after 7 days.

No significant changes in $O_2^{\bullet-}$ generation were noticed in any of the leaves. $O_2^{\bullet-}$ generation in leaves inoculated with *B. cinerea* was only slightly higher than in control ones 2 and 6 h after infection. Small increases in $O_2^{\bullet-}$ generation in leaves treated with only o-hydroxyethylorutin persisted during the whole experiment (Figure 2).

In leaves treated with o-hydroxyethylorutin and inoculated with *B. cinerea*, the H_2O_2 concentration increased significantly, about twice above untreated,

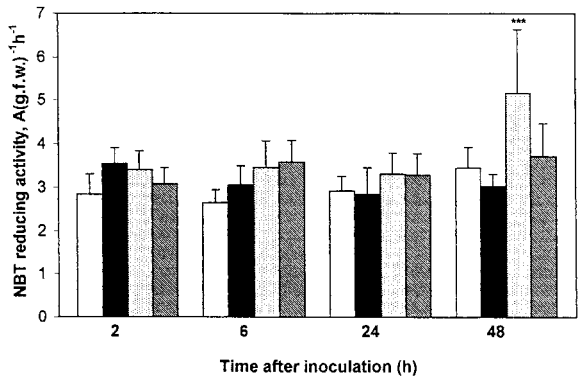

Figure 2. Time course of NBT reducing activity in tomato leaves treated with o-hydroxyethylorutin and infected with *B. cinerea.* Values represent the mean and SE from four independent experiments with three replicates each, $n = 12$; $*$, $**$ and $***$ indicate values that differ significantly from the control at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; □ – untreated uninfected plants, ■ – plants infected with *B. cinerea*, ■ – plants treated with o-hydroxyethylorutin, ■ – plants treated with o-hydroxyethylorutin and infected with *B. cinerea*.

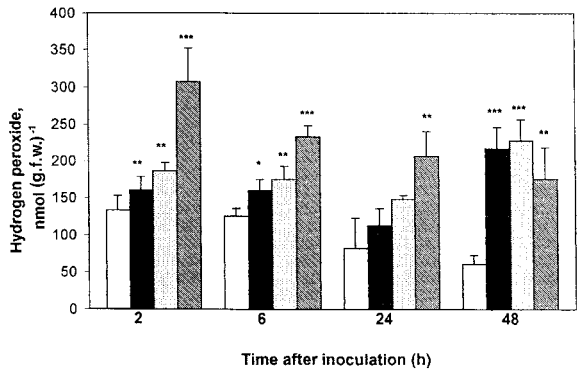

Figure 3. Time course of changes in H_2O_2 concentration in tomato leaves treated with o-hydroxyethylorutin and infected with *B. cinerea*. For explanation see Figure 2.

uninoculated leaves, as early as 2 h after inoculation, and the high level of H_2O_2 concentration in these leaves was observed during the whole studied time (Figure 3). The H_2O_2 concentration in leaves inoculated with *B. cinerea*, but not treated with o-hydroxyethylorutin, as well as in those only treated with o-hydroxyethylorutin increased less visibly after 2, 6 and 24 h; in these leaves H_2O_2 concentration was greatest after 48 h.

The influence of H_2O_2 on conidial germination and mycelium growth of *B. cinerea* was estimated *in vitro* to elucidate whether H_2O_2 was able to inhibit the infection start and development. Significant inhibition of *B. cinerea* conidial germination was evident at 0.5 mM and was almost complete at 5 mM H_2O_2 (Figure 4). Even 2 h incubation of conidia in 5 mM H_2O_2 solution reduced the conidial germination by 40% and in 50 mM about 80% (Figure 5). Higher concentrations of H_2O_2 were needed to inhibit mycelial growth. Mycelial growth was significantly inhibited at 50 mM H_2O_2 and completely inhibited at 100 mM (Figure 6).

There were no significant changes in SOD activities in tomato plants (Figure 7). Peroxidase activity increased in tomato leaves inoculated with *B. cinerea* after 24 h. In leaves treated with o-hydroxyethylorutin prior to inoculation, increased levels of peroxidase were evident only after 48 h (Figure 8). APX and CAT activities were similar or a little lower in tomato leaves treated with o-hydroxyethylorutin – uninoculated and

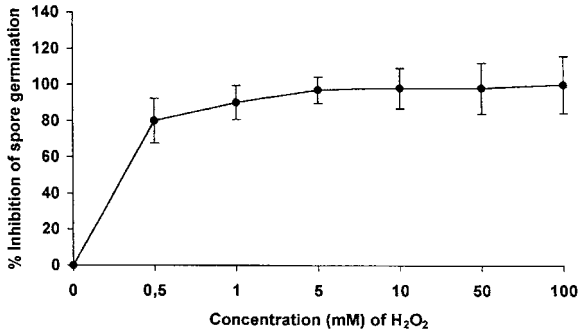

Figure 4. Effect of H_2O_2 on germination of conidia of *B. cinerea*. Values represent the mean and SE from three independent experiments with five replicates each.

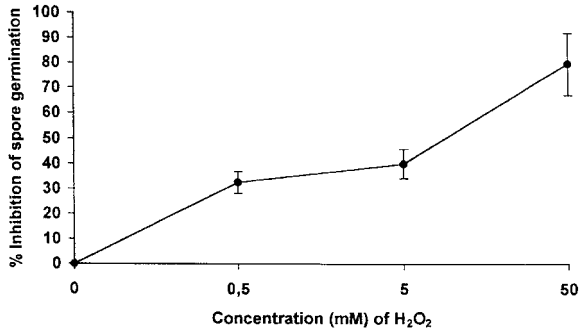

Figure 5. Effect of H_2O_2 pretreatment on germination of conidia of *B. cinerea*. Values represent the means and SE from three independent experiments with five replicates each.

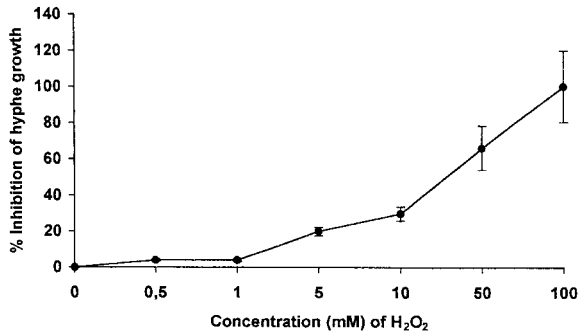

Figure 6. Effect of H_2O_2 on hyphal growth of *B. cinerea*. Values represent the mean and SE from three independent experiments with five replicates each.

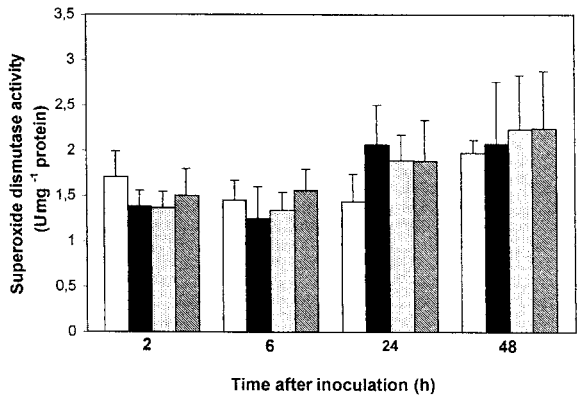

Figure 7. Time course of changes in SOD activity in tomato leaves treated with o-hydroxyethylorutin and infected with *B. cinerea*. For explanation see Figure 2.

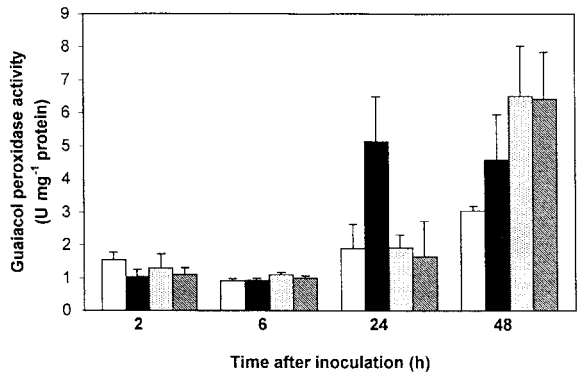

Figure 8. Time course of changes in guaiacol PO activity in tomato leaves treated with o-hydroxyethylorutin and infected with *B. cinerea*. For explanation see Figure 2.

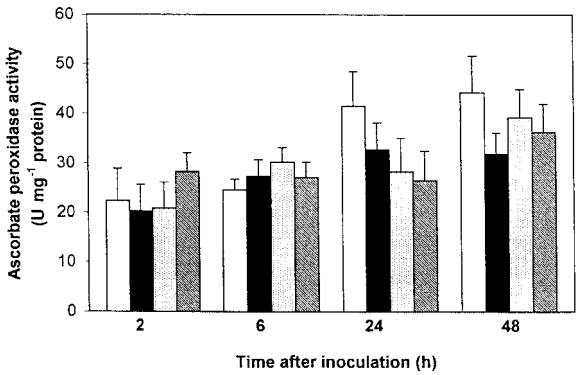

Figure 9. Time course of changes in APX activity in tomato leaves treated with o-hydroxyethylorutin and infected with *B. cinerea*. For explanation see Figure 2.

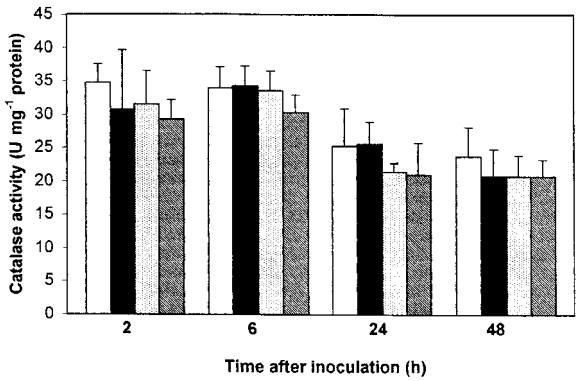

Figure 10. Time course of changes in CAT activity changes in tomato leaves treated with o-hydroxyethylorutin and infected with *B. cinerea*. For explanation see Figure 2.

inoculated with *B. cinerea* as compared with untreated and uninoculated ones (Figures 9 and 10). The changes in H_2O_2 concentrations, APX and CAT activities in untreated and uninoculated leaves observed during the experimental period may be associated with concomitant senescence processes which took part in those detached leaves.

Discussion

When tomato leaves were treated with o-hydroxyethylorutin, development of *B. cinerea* infection was limited. In the present study, the generation of AOS and antioxidant enzymes activities were examined in attempt to determine their role(s) in resistance reactions

of tomato plants to infection with *B. cinerea* induced by o-hydroxyethylorutin treatment.

About two-fold increase in H_2O_2 concentration was observed in tomato leaves treated with o-hydroxyethylorutin and inoculated with pathogen at the early infection stages. The H_2O_2 concentration reached 300 nmo1/g.f.w. and thus may play a part in restricting *B. cinerea* conidia germination, and hence prevent infection development. The values concerning the H_2O_2 level in plants might be even higher, but were probably reduced by losses during the extraction procedures. High H_2O_2 concentrations, from 800 nmol/g.f.w. to 5 μmo1/g.f.w. and even up to 1 M have been reported in plants (Bolwell and Wojtaszek, 1997, Okuda et al., 1991, Velikova et al., 2000). The results obtained from *in vitro* tests showed that *B. cinerea* conidia are sensitive to H_2O_2 and they seem to confirm the role of hydrogen peroxide in inhibition of fungal expansion in plant tissue. Even 2 h preincubation of conidia in H_2O_2 solutions strongly retarded their germination. Hydrogen peroxide is known to be involved in various plant defence responses, direct reduction of pathogen viability is one of them. Spore germination for a number of fungal pathogens has been shown to be inhibited by micromolar concentration of H_2O_2 (Peng and Kuć, 1992). H_2O_2 in concentration of 1 mM completely inhibited the growth of *Erwinia carotovora* ssp. *carotovora* and caused greater than 95% inhibition of *Phytophthora infestans* (Wu et al., 1995). Higher levels of hydrogen peroxide observed in tomato leaves are likely to be more harmful to the fungus than to the plant tissues and they restrict pathogen development. Lu and Higgins (1999) showed that green tissues of tomato plants are tolerant to high concentrations of H_2O_2; 100 mM H_2O_2 caused only insignificant necrosis when applied to tomato leaves and a concentration as high as 1 M H_2O_2 was required to cause complete leaf necrosis, whereas conidial germination of *Cladosporium fulvum* was retarded by 4–5 mM H_2O_2. As our results show, the growth of *B. cinerea* mycelium is less influenced by H_2O_2. When the process of conidia germination was not stopped at the early infection stages, mycelial growth can develop although the H_2O_2 concentrations were high. This may explain the infection development in infected leaves non-treated with o-hydroxyethylorutin in spite of high H_2O_2 concentration in them at the later experimental stages.

The H_2O_2 concentrations in plant tissues depend on the intensity and longevity of its production as well as on processes of its degradation. SOD, CAT and peroxidases are involved in the regulation of the level of H_2O_2 in plant tissues (Low and Merida, 1996; Lamb and Dixon, 1997; Wojtaszek, 1997). The disproportionation of the superoxide anion to hydrogen peroxide is one of the ways of H_2O_2 production in plants. We observed only slight correlation between H_2O_2 concentration, $O_2^{\bullet -}$ generation and increases in SOD activity although it is assumed that $O_2^{\bullet -}$ dismutation by SOD may be the important source of H_2O_2 in plants (Ogawa et al. 1997). Perhaps spontaneous disproportionation of superoxide radicals to H_2O_2 as well as other alternative pathways of H_2O_2 generation without going via $O_2^{\bullet -}$ might be considered in studied plants. The high concentrations of H_2O_2 in tomato plants treated with o-hydroxyethylorutin may be partially connected with, lower than in control, the activity of CAT. This enzyme is thought to play an important role in removing H_2O_2 from plant tissues. A decline in CAT activity in bean leaves following inoculation with *Pseudomonas syringe* pv. *phaseolicola* (Ádám et al., 1995; Milosević and Slusarenko, 1996) and lower level of H_2O_2-scavenging activity correlated with increase in H_2O_2 production during interaction between *Pseudomonas syringae* pv. *glycine* and soyabean suspension cells (Baker et al., 1995) were described. The increase in intracellular levels of AOS caused by inhibition of CAT and APX activities was reported in plants as a reaction to treatment with resistance inducing substances such as salicylic acid, 2,6-dichloroisonicotinic acid, benzothiadiazol (Conrath et al., 1995, Wendehenne et al., 1998).

Peroxidase activity, which is suggested to be an important component of plant stress responses, may also regulate the level of H_2O_2 in plant tissues (Bestwick et al., 1998; Takahama and Oniki, 1997). There are no essential correlations between guaiacol peroxidase activity, H_2O_2 concentration and enhanced resistance of tomato leaves to *B. cinerea* infection by o-hydioxyethylorutin treatment. The increase in peroxidase activity in leaves pretreated with o-hydroxyethylorutin and infected with *B. cinerea* at the end of experiment may be correlated with participation of this enzyme in cross-linking of cell wall proteins, polymerisation of lignin precursors and other resistance reactions. However, the earlier increase in peroxidase activity in leaves only infected with *B. cinerea* may be caused by the reaction to the invaiding pathogen as well as by the senescence processes accelerated by the infection. The increase in peroxidase activity as a consequence of senescence processes

has been reported in other plants (Abeles et al., 1988; Bartoli et al., 1995; Panava and Rubinstein, 1998). So, the high peroxidase activity in o-hydroxyethylorutin treated leaves could be also partly caused by senescence processes and may be not significantly connected with resistance.

The present study demonstrates that o-hydroxyethylorutin triggers resistance of tomato plants to *B. cinerea* infection. It seems possible that higher production of H_2O_2 in tomato plants as a result of treatment with o-hydroxyethylorutin could explain their resistance to *B. cinerea*. Our results are in accordance with other authors who suggest that hydrogen peroxide is one of the elements of plant resistance mechanism to pathogen infection.

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