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Effect of Reactive Oxygen Species on Plant Pathogens in planta and on Disease Symptoms

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It was shown that reactive oxygen species (ROS) produced by two chemical systems or applied directly can alter symptom expression and block pathogen growth *in planta.* This was demonstrated for diseases caused by four obligate and three facultative pathogens, respectively. When ROS were applied to the infected plants very early after inoculation, symptoms were fully suppressed. If application of ROS to leaves inoculated with biotrophic pathogens occurred 2–4 days after inoculation, hypersensitive type necrotic symptoms (HR) characteristic for resistant plants appeared in the leaves of susceptible cultivars instead of normal pustules containing mycelia. In the case of diseases caused by facultative pathogens only the size of the necrotic spots were diminished or in some cases no visible necroses were produced. The action of ROS were reversed in some host–pathogen combinations by the application of antioxidants, such as superoxide dismutase (SOD) or catalase and resulted in the development of normal disease symptoms. This indicated that superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) were the most important ROS involved in the inhibition of pathogen growth *in planta* and in symptom development.

Keywords: Catalase, superoxide dismutase, reactive oxygen species, hypersensitive response.

Plant disease resistance which is associated in many cases (but not always) with the hypersensitive response (HR) is the result of the incompatible interaction between the host and the pathogen (Dangl and Jones, 2001; Dinesh-Kumar et al., 2000; Feys and Parker, 2000; Heath, 2000; Király et al., 1991; Lam et al., 2001; Staskawicz et al., 1995; Stuiver and Custers, 2001). However, it is not known for certain what is responsible for the limitation or killing of the invading pathogen in an incompatible host–pathogen interaction. The sequence of events during the HR was elucidated during the past two decades: (1) a striking early host reaction is the accumulation of reactive oxygen species (ROS), this is often described as the "oxidative burst" that is similar to the reaction found in mammalian phagocytes; (2) ion fluxes and disintegration of membranes; (3) activation of genes encoding enzymes of phytoalexin and lignin synthesis as well as lytic enzymes (such as glucanases and chitinases) also called pathogenesis related (PR) proteins. It has proven difficult to evaluate whether the accumulation of phytoalexins, lignin and PR proteins would be cause or consequence of resistance.

Tissue necrosis, as a very early response, cannot be the only cause of limitation of invading pathogen, because necrotrophic fungi and bacteria prefer necrotic cells and they need necrotized tissues for colonization. It has also been shown that HR is possibly a consequence, not the cause, of disease resistance (Király et al., 1972). Something else is blocking pathogens in the resistant plant. ROS, phytoalexins and PR proteins (e.g. chitinase) are considered frequently as candidates for that function.

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The role of ROS in plant responses to infections has been treated by several papers (Alvarez et al., 1998; Apostol et al., 1989; Baker and Orlandi, 1995; Doke et al., 1991; Fodor et al., 1997, 2001; Király et al., 1991; Mehdy, 1994; Sutherland, 1991; Tzeng and DeVay, 1993) and a few publications refer to the *in vitro* sensitivity of plant pathogens to the action of ROS. Thus, Tzeng et al. (1990) and Jordan et al. (1992) pointed out that plant pathogenic microorganisms and host tissues are differentially sensitive to ROS. It is of particular interest that transgenic potato plants that express a foreign gene (glucose oxidase), and thereby generating H_2O_2 production, exhibited disease resistance for several pathogens (Wu et al., 1995). Antioxidants may profoundly influence sensitivity. *Phytophthora infestans* (Doke et al., 1991; Ivanova et al., 1991; Király et al., 1993; Merzlyak et al., 1990), *Pyricularia oryzae* (Aver'yanov and Lapikova, 1988), *Pseudomonas syringae* (Ádám et al., 1990; Király et al., 1993; Minardi and Mazzucchi, 1988), *P. syringae* pv. *phaseolicola* and pv. *pisi* (Király et al., 1993, 1997), *Erwinia amylovora* (Király et al., 1997), *Xanthomonas campestris* pv. *citri* (Doke and Shibata, 1987) and pv. *pelargonii* (Király et al., 1997), *Cercospora* spp. (Daub and Payne, 1989), *Botrytis cinerea* (Király et al., 1993), *Erysiphe graminis* (Király et al., 1993), *Puccinia graminis* (Ouf et al., 1993) and tobacco mosaic virus (Fodor et al., 1997; Király et al., 2002) have been studied mostly *in vitro* in relation to the action of prooxidants and/or antioxidants.

We investigated the possible role of ROS in suppressing or killing pathogens in planta when ROS ($O_2^{\bullet-}$ and H_2O_2) were administered directly or via chemical generating systems applied to leaves of different host plants infected by obligate and facultative pathogens.

Materials and Methods

Host–pathogen pairs

The following host–pathogen pairs were involved in this study:

I. DISEASES CAUSED BY OBLIGATE PATHOGENS

Powdery mildew disease of barley *(Hordeum vulgare/Blumeria graminis* f. sp. *hordei)*
Powdery mildew disease of cucumber *(Cucumis sativus/Sphaerotheca fuliginea)*
Leaf rust disease of wheat (*Triticum aestivum/Puccinia recondita* f. sp. *tritici*)
Stem rust disease of wheat (*Triticum aestivum/Puccinia graminis* f. sp. *tritici*)

II. DISEASES CAUSED BY FACULTATIVE PATHOGENS

Yellow leaf spot disease of wheat (*Triticum aestivum/Pyrenophora tritici-repentis,* anamorph: *Helminthosporium tritici-repentis*)
Bean anthracnose disease *(Phaseolus vulgaris/Colletotrichum lindemuthianum)*
Bean halo blight disease (*Phaseolus vulgaris/Pseudomonas syringae* pv. *phaseolicola*)
Tobacco HR-type necrosis (*Nicotiana tabacum/Pseudomonas syringae* pv. *phaseolicola*)

Host plants

The following five plant genera and species, including twelve cultivars, were used in this study: Barley *(Hordeum vulgare)* cultivars Amsel, Emir, GK-Omega, Botond and Ingrid, wheat *(Triticum aestivum)* cultivars Brevit, Marquis and a Göttingen selection, bean *(Phaseolus vulgaris)* cultivars Pinto, Processor, Red Kidney and Saxa, cucumber *(Cucumis sativus)* cultivar Budai Csemege and tobacco *(Nicotiana tabacum)* cultivar Samsun.

Barley cultivars Amsel and Emir, wheat cultivar Göttingen selection and bean cultivar Processor were kindly supplied by the Institute for Plant Pathology and Plant Protection, University of Göttingen, Germany. All other cultivars used were obtained from the Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary. Seeds of the above mentioned plants were sown into soil in plastic pots 10 or 15 cm diameter containing a mixture of soil:peat:sand, 2:1:1, v/v/v and grown under greenhouse conditions. Temperature was 18–23 °C, with 16 hours photoperiod per day using supplemental light by mercury lamps (HQL) with a light intensity of 160 $\mu E\ m^{-2}\ s^{-1}$ and relative humidity 75–80%. Leaves of seedlings were artificially inoculated with their pathogens. The inoculated leaves were detached at different time period after establishment of the pathogen and were treated with the reactive oxygen species (ROS) producing systems and directly with H_2O_2.

Pathogens

POWDERY MILDEW PATHOGENS

Barley powdery mildew, *Blumeria graminis* f. sp. *hordei* race C17 Am (obtained from Dr. M. Niemann, Institute for Plant Pathology and Plant Protection, University of Göttingen, Germany), A6 from the University of Giessen and a Hungarian isolate were maintained on susceptible barley seedlings in the greenhouse. A Hungarian isolate of cucumber powdery mildew *(Sphaerotheca fuliginea)* was maintained on cucumber plants in the greenhouse. First leaves of 7-day-old barley seedlings or 21-day-old cucumber seedlings were inoculated with their powdery mildew fungal pathogens by shaking conidia from diseased leaves of barley or cucumber plants, respectively. The inoculated plants were held in a moist chamber for 1 day at 18–20 °C.

RUST PATHOGENS

Wheat leaf rust, *Puccinia recondita* f. sp. *tritici* race 77 and wheat stem rust, *Puccinia graminis* f. sp. *tritici* race 218 were maintained on seedlings of a susceptible wheat cultivar in the greenhouse. First leaves of 7-day-old wheat seedlings were inoculated by spraying them with a uredospore suspension of the pathogens and held in a moist chamber for 1 day at 18–20 °C.

WHEAT YELLOW LEAF SPOT PATHOGEN

Pyrenophora tritici-repentis (anamorph: *Helminthosporium tritici-repentis*) obtained from Prof. H. Fehrmann, Institute for Plant Pathology and Plant Protection, University of Göttingen, Germany, was cultivated on PDA medium. Spores were collected from 14-day-old cultures using sterile water (Lamari and Bernier, 1991). The suspension was

adjusted to 4×10^3 spores ml^{-1} using a haemacytometer. Spore suspension was sprayed onto the 3rd leaves of 21-day-old wheat plants. Inoculated plants were held in a moist chamber for 2 days at 18–20 °C.

BEAN ANTHRACNOSE PATHOGEN

Spore suspension was prepared from 3-week-old colonies of the fungal pathogen *Colletotrichum lindemuthianum.* A Göttingen isolate was cultivated on Mathur's medium (Mathur et al., 1950). Five ml of sterile water was added to each Petri dish and the surface of the culture was scraped to dislodge the spores. The suspension was adjusted to 1×10^7 spores ml^{-1} using a haemacytometer (Tu and Aylesworth, 1980). First and second trifoliate leaves of 4-week-old French bean plants were inoculated by spraying the spore suspension on leaves and then held in a moist chamber for 2 days at 18–20 °C.

BEAN HALO BLIGHT PATHOGEN

Pseudomonas syringae pv. *phaseolicola* strain S2 has been cultivated on sucrose nutrient agar (SNA) medium. Bacterial cell suspension was prepared from one-day-old cultures. The cell concentration was adjusted turbidimetrically to 10^7 cells ml^{-1}. Primary leaves of 10 to 12-day-old seedlings of French beans were inoculated by spraying the bacterial suspension onto the abaxial leaf surface with an atomizer until the exposed area became watersoaked. Any excess inoculum was carefully blotted from the leaf surface.

TOBACCO HR-TYPE NECROSIS CAUSED BY BACTERIA

The 24-hour-old cultures of the bacterial cell suspension from *Pseudomonas syringae* pv. phaseolicola strain S2 cultivated on KB medium was injected into the leaves of tobacco cultivar Samsun. The cell concentration used in this case was 10^8 cfu/ml sterile tap water.

Production of reactive oxygen species (ROS)

Two chemical systems to produce superoxide radical and other reactive oxygen species (ROS) in liquid as well as in agar media were applied in this study.

(1) THE PHOTOCHEMICAL SYSTEM OF RIBOFLAVIN-METHIONINE MIXTURE

The photogeneration of superoxide anion ($O_2^{\bullet-}$) in chemical systems which contain riboflavin and amino acids (such as methionine) was published earlier (Korycka-Dahl and Richardson, 1978). When riboflavin absorbs visible light it becomes excited and in the presence of e. g. methionine it initiates redox reactions. In the course of these processes riboflavin is univalently reoxidized with the formation of $O_2^{\bullet-}$. It is also known (Tzeng, 1989; Tzeng and Lee, 1989) that in the living systems several other ROS can be produced, such as H_2O_2, $OH^{\bullet}$, $^1O_2$, which also may contribute to the toxicity of this photochemical system. Tzeng and DeVay (1993) claim that in the riboflavin–methionine system another radical, the methyl radical ($CH_3^{\bullet}$), may also play an important role in the toxicity of this free radical-producing system.

In our experiment the modified method of Tzeng and DeVay (1989) was used. The mixture contained 266 μM riboflavin and 10 mM L-methionine (in the case of bean leaves 133 μM riboflavin and 5 mM methionine). Water agar medium was prepared with or without riboflavin and methionine and then autoclaved at 121 °C for 20 min. Each Petri dish (diameter 90 mm) contained 10 ml medium.

To investigate the effect of ROS on pathogens and symptoms, one part of Petri dishes were immediately illuminated and after different illumination periods they were transferred back to the incubator. The other part of Petri dishes were incubated without illumination. The illumination process was made in an incubator at 25 °C. The light intensity was approximately 100 μE m^{-2} S^{-1}, using six Tungsram 15 WF33 cool white fluorescent tubes. The distance between the Petri dishes and the light source was 30 cm.

As regards the action of our photochemical system on pathogens *in planta,* we determined the biocidal activity of the superoxide-producing mixture in infected leaves on the basis of suppression of symptoms and the multiplication of bacterial cells. After inoculation leaves were detached and put on the riboflavin-methionine mixture in Petri dishes. Ten ml mixture containing 266 μM riboflavin and 10 mM L-methionine was poured on filter paper in each Petri dish. All Petri dishes were illuminated for 2 hours in an incubator at 25 °C (100 μE m^{-2} S^{-1}). To determine bacterial number in the leaves, four discs were punched out from the infected area with a sterile cork borer (12 mm diameter) from four different leaves. Samples were homogenized in 5 ml cold sterile saline in phosphate buffer (0.05 M phosphate buffer pH 7.0, in 0.15 M NaCl). Tenfold dilutions were made from the homogenates and 0.1 ml from each sample was plated on the surface of SNA medium. Petri dishes were held in a dark incubator for 48 hours at 27 °C. The number of colony forming units (cfu) were counted and calculated for 1 cm^2 leaf area. The biocidal activity of the photochemical mixture was determined by comparing the number of cfu in the water treated control leaves to that in the leaves treated with the riboflavin–methionine mixture.

(2) THE ROS- PRODUCING XANTHINE–XANTHINE OXIDASE SYSTEM

In this system the enzyme acts aerobically upon xanthine and generates superoxide ($O_2^{\bullet-}$) and H_2O_2. It has been shown that superoxide alone is not enough to cause cell death in animal cells (cf. Halliwell and Gutteridge, 1999; Link and Riley, 1988), however it contributes to the generation of H_2O_2 and probably to the formation of $OH^\bullet$ which are more toxic reactive oxygen species.

In our experiments the method of Doke and Shibata (1987) was used. The xanthine–xanthine oxidase mixture was prepared in 10 ml 0.1 M phosphate buffer (pH 7.8). The concentration of xanthine was 1 mM and each Petri dish contained 3.3 units xanthine oxidase. Inoculated leaves were put in these Petri dishes and other Petri dishes containing only buffer or buffer and xanthine without xanthine oxidase were used as control. The samples were incubated at 25 °C for 2,5 hours (in the case of bean leaves we used 2 h incubation and the concentration of xanthine oxidase was 2.5 units).

Fresh test solution from either riboflavin–methionine or xanthine–xanthine oxidase system was prepared before each experiment.

DIRECT APPLICATION OF H_2O_2

We treated attached barley leaves with H_2O_2 solution (H_2O_2 in tap water containing 0.1% Tween 20) by spraying barley leaves with 10, 100, 200, 400, 500 and 700 mM H_2O_2 one day before or one, two and three days after inoculation with powdery mildew.

Antioxidant enzymes

To demonstrate the role of $O_2^{\bullet -}$ anion and H_2O_2 in the killing action of the ROS-producing chemical systems, horseradish superoxide dismutase (E.C.1.15.1.1) and bovine liver catalase (E.C.1.11.1.6) enzymes were used. In the case of SOD 2000 or 2500 U and from catalase 4000 or 5000 U were applied in each Petri dish mixed with the ROS-producing systems.

Application

The time period between inoculation of host plants and the treatment with the ROS producing chemical systems were different depending on the particular host–pathogen relationship.

Results

Powdery mildew of barley (Blumeria graminis *f. sp.* hordei)

Four compatible and three incompatible plant cultivar/pathogenic race combinations were used in this experiment. The compatible combinations were as follows: cultivar Amsel/mildew race C17Am, cultivars Emir and GK Omega/Hungarian mildew isolate and cultivar Ingrid/mildew race A6. The incompatible combinations were: cultivar Amsel/Hungarian isolate, cultivar Emir/race C17Am and Ingrid expressing the gene *Mla*/race A6. Inoculated leaves were put on a solution or filter paper or on agar containing the ROS-producing riboflavin–methionine mixture (266 μM riboflavin and 10 mM methionine). We regularly used 2 or 3 hours for illumination (100 μE m^{-2} s^{-1}) and the time period between inoculation and illumination was different. As regards the ROS-producing xanthine–xanthine oxidase system, the inoculated leaves were put on a solution containing 1 mM xanthine and 3.3 units of xanthine oxidase for 2 or 3 hours. After treatments, leaves were transferred to Petri dishes on wet filter paper.

When the time period between inoculation and the ROS-producing treatments (using the two chemical systems) was less than 2 days, practically no symptoms appeared *(Fig. 1)* whether the combination was a compatible or an incompatible one. One can suppose that the pathogen was killed before the establishment of infection (formation of haustoria) and this was the cause of the lack of symptoms *(Table 1).*

When inhibition exerted by ROS produced in the two chemical systems was applied 2 or more days after inoculation, different symptoms appeared on compatible combinations. When ROS acted 2 days after inoculation, the compatible combination produced necrotic symptoms on the susceptible cultivar. This necrotization is similar to the HR that

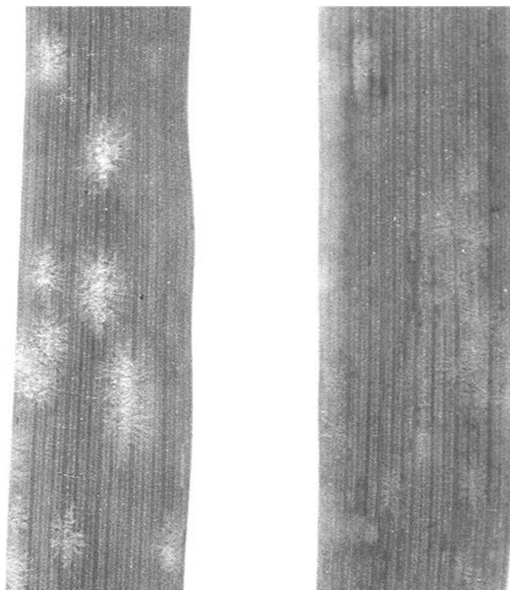

Fig. 1. Effect of ROS produced in a riboflavin-methionine mixture on symptom expression of powdery mildew on a susceptible barley cultivar (Amsel). Left: control (not treated). Right: leaf treated with a ROS-producing mixture 1.5 days after inoculation with *Blumeria graminis* f. sp. *hordei* race C17 Am

Table 1

The sensitivity of compatible and incompatible host-pathogen combinations of powdery mildew infected barley cultivars to ROS produced in two chemical systems

Host-pathogen combination	Symptoms			
	The time period between inoculation and ROS treatment			
	0–40 hours	2 days	3 days	4 days
Compatible H/P relationships				
Emir/Hungarian isolate	none	necroses	few d. b. mycelium	d. b. mycelium
GK-Omega/Hungarian isolate	none	necroses	few d. b. mycelium	d. b. mycelium
Incompatible H/P relationships				
Amsel/Hungarian isolate	none	none	small necroses	necroses
Emir/Cl7 Am isolate	none	none	small necroses	necroses

ROS = reactive oxygen species
H/P = host-pathogen
d.b. = dead/brown

usually appears in the incompatible combinations when the host is resistant. When ROS acted only 3 or more days after inoculation, some brown and dead mycelium and necrotic spots were seen on the compatible leaves *(Fig. 2)*.

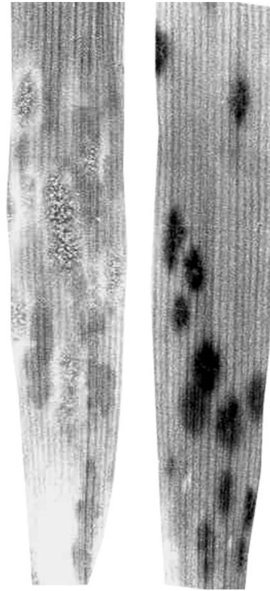

Fig. 2. Necrotic spots in a susceptible barley cultivar (Amsel) as a result of treatment with a riboflavin-methionine mixture and inoculated with *Blumeria graminis* f. sp. *hordei* race C17 Am. Left: control (not treated). Right: leaf treated with the ROS-producing mixture 3 days after inoculation

As regards the incompatible host–pathogen combinations, cultivars Emir and Amsel were used in combination with races C17 Am and Hungarian isolate, respectively. By applying the two chemical systems, no necrotic symptoms appeared on the leaves whether ROS acted 1 or 2 days after inoculation *(Fig. 3).* However, when the application of ROS occurred 3 days after inoculation, very small necrotic symptoms were formed on the incompatible (resistant) leaves. This means that in the latter experiment the fungus was only partially inhibited by ROS or it acted late and the pathogen was able to cause normal HR (necrotic spots).

To know more about the concentration of ROS which can inhibit the pathogen *in planta,* different concentrations of H_2O_2 solutions were sprayed onto attached barley leaves one day before inoculation or one, two and three days after inoculation with powdery mildew. It was shown that 10, 100 and 200 mM concentrations of H_2O_2 applied one day after inoculation suppressed or killed the pathogen even in an incompatible host–pathogen combination (Ingrid expressing the gene *Mla* inoculated with race A6 of powdery mildew). In this case there was no symptom expression in leaves whether the fungus was a virulent or an avirulent one. This corresponds to the action of riboflavin–methionine and xanthine–xanthine oxidase complexes applied 0–40 hours after inoculation. It was also shown that 100, 200 and 400 mM concentrations of H_2O_2 applied two or three days after inoculation suppressed the pathogen and green islands were appeared however, 500 mM H_2O_2 applied two or three days after inoculation produced HR-type necroses in

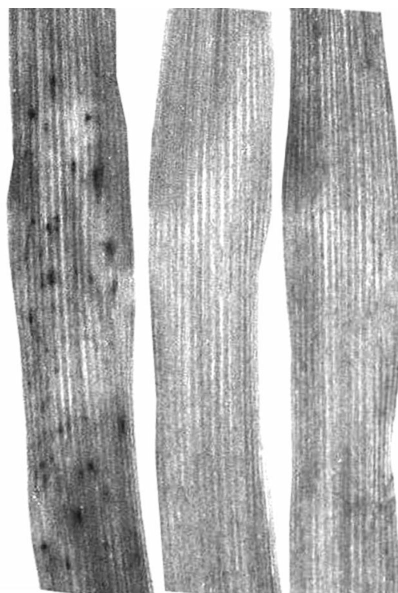

Fig. 3. Effect of ROS produced in two chemical systems in a resistant barley cultivar (Emir) inoculated with *B. graminis* f. sp. *hordei* race C17 Am. Left: control, not treated but inoculated. Middle: leaf treated with the xanthine–xanthine oxidase system 1.5 days after inoculation. Right: leaf treated with the riboflavin-methionine mixture 1.5 days after inoculation

the comptible host–pathogen combination (Ingrid inoculated with race A6). This corresponds to the action of riboflavin–methionine and xanthine–xanthine oxidase complexes applied two days after inoculation.

We wanted to see whether one could reverse the action of ROS on symptom expression by applying enzymatic antioxidants, such as superoxide dismutase (SOD) or catalase. Both enzymes were applied at the time when ROS were developed in the chemical systems. Cultivar GK Omega was infected by the Hungarian mildew isolate (compatible combination). Two days after inoculation the riboflavin–methionine mixture was applied in a solution that also contained 2500 units of SOD. As a result of the influence of this antioxidant enzyme, the action of ROS was reversed. Instead of necrotic symptoms normal mycelium developed on the surface of inoculated susceptible leaves. In another experiment (cultivar GK Omega/Hungarian mildew isolate) infected leaves were floated on the xanthine–xanthine oxidase solution that contained 2500 units of SOD or 5000 units of catalase. Weak mycelial development was experienced in both cases instead of necrosis. In an incompatible combination (cultivar Amsel/Hungarian mildew isolate) the solution containing the xanthine–xanthine oxidase mixture plus SOD or catalase or both antioxidants together was applied one day after inoculation. The concentration of SOD was 2500 units and that of the catalase was 5000 units. Separately, as well as in combination, the two antioxidants reversed the action of ROS, i. e. regular necroses (HR-type symptoms) appeared on leaves of the resistant cultivar as a result of the action of antioxidants, however

no hypersensitive necroses were seen on leaves that were treated only with ROS one day after inoculation. All of these experiments supported the hypothesis that ROS are able to inhibit powdery mildew *in planta* and this inhibition is SOD or catalase sensitive reaction. In other words, superoxide and H_2O_2 could be the ROS that are indeed active in inhibition of the pathogen *in planta.*

Powdery mildew of cucumber (Sphaerotheca fuliginea)

One compatible plant–pathogen combination was used in this experiment (cultivar Budai csemege/Hungarian mildew isolate). Both chemical systems were applied to inhibit the fungus *in planta* by producing ROS. In both cases the time period of treatment was 2,5 hours. Not more than 3 or 4 days time period between inoculation and the application of ROS was necessary to inhibit the fungus *in planta* and produce necrotic symptoms instead of the normal mildew development *(Fig. 4).* When this time period was 5 or 7 days, small amount of brown mycelium or abundant brown mycelium, respectively was seen on the surface of the leaves, indicating that the action of ROS was late to inhibit or kill the fungus in due time.

Fig. 4. Inhibition of symptom expression in cucumber cultivar Budai Csemege caused by *Sphaerotheca fuliginea* under the influence of ROS produced in a riboflavin-methionine mixture. Left: control, not treated but inoculated. Right: leaf treated with the ROS-producing system 4 days after inoculation

Leaf rust of wheat (Puccinia recondita *f. sp.* tritici)

The combination of wheat cultivar Brevit/leaf rust race 77 resulted in a compatible host–pathogen pair. For the production of ROS both chemical systems were used in this

experiment. Treatment lasted for 2,5 hours. Not more than 3 or 4 days time period between inoculation and the application of ROS was necessary to inhibit the rust *in planta* and produce necrotic (HR-like) symptoms instead of the normal rust pustules *(Fig. 5).* When this time period was only 2 days, usually no symptom expression was seen on infected wheat leaves. When this time period increased to 5 or 7 days, small pustules or normal rust pustules were formed, respectively and it was seemingly late to inhibit or kill the rust fungus. It was possible to reverse the inhibitory action of ROS on symptom expression also in this host–pathogen combination by applying a combination of SOD and catalase in 2500 units and 5000 units, respectively. Both enzymes were applied to leaves of cultivar Brevit when ROS were developed in the xanthine–xanthine oxidase system (data not shown).

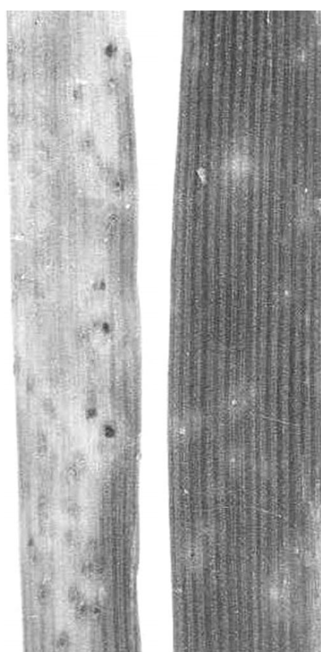

Fig. 5. Suppression of wheat rust pustules on cultivar Brevit inoculated with *Puccinia recondita* f. sp. *tritici* as a result of treatment of leaves with the xanthine–xanthine oxidase system. Left: control, not treated but inoculated. Right: leaf treated with the ROS-producing system 3 days after inoculation

Wheat stem rust (Puccinia graminis *f. sp.* tritici)

Wheat cultivar Marquis/stem rust race 218 was a compatible host/pathogen combination. The method of this experiment was the same as in the case of leaf rust. Three days time period between inoculation and the application of ROS resulted in complete inhibition of the rust fungus. In this case no symptoms were seen on the leaves. When this time period increased to 4 days, HR-like necroses appeared on the leaves, indicating that the fungus was able to establish itself and cause necrotic symptoms in a susceptible host which

is characteristic for the incompatible host/pathogen combinations (for the resistant cultivars). Five days time period was late to inhibit the pathogen, and accordingly, small and few rust pustules appeared on the inoculated leaves *(Fig. 6).*

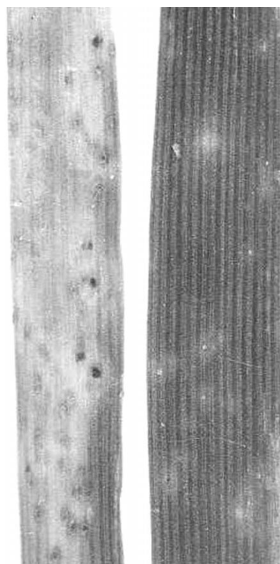

Fig. 6. Suppression of wheat stem rust pustules on cultivar Marquis inoculated with *Puccinia graminis* f. sp. *tritici* as a result of treatment of leaves with a xanthine–xanthine oxidase system. Left: control, not treated but inoculated. Right: inoculated leaf treated with the ROS-producing system 5 days after inoculation

Wheat yellow leaf spot (Pyrenophora tritici-repentis, *anamorph:* Helminthosporium tritici-repentis)

The 3rd leaves of a Göttingen wheat selection were inoculated with the spores of the fungus which is a necrotrophic pathogen, causing yellow-necrotic spots on the susceptible wheat leaves. For the production of ROS the riboflavin–methionine system was used with a 3 hours illumination. One day after inoculation leaves were put on the ROS-producing solution and illuminated. Symptom expression was fully inhibited by this treatment indicating that the fungus was also inhibited by the reactive oxygen species *(Fig. 7).* In the control (not treated) leaves typical yellow-necrotic spots appeared.

Bean anthracnose (Colletotrichum lindemuthianum)

Four bean cultivars (Red Kidney, Processor, Pinto and Saxa) were inoculated by a Göttingen isolate of *C. lindemuthianum.* For the production of ROS both chemical systems were used. Treatment with the riboflavin–methionine system was somewhat different from the other cases: the concentration of riboflavin was 133 μM and that of the methionine was 5 mM. The concentration of xanthine oxidase was also lower (2.5 units per Petri dish in 10

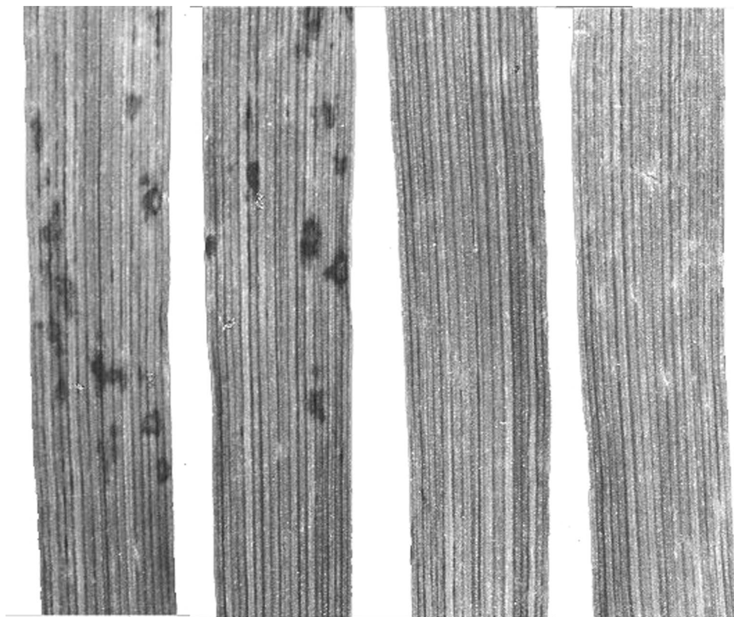

Fig. 7. Suppression of symptoms on wheat (a Göttingen selection) caused by *Pyrenophora tritici-repentis* as a result of treatment with the ROS-producing riboflavin-methionine mixture. Left two leaves: control, not treated but inoculated. Right two leaves: treated with the ROS-producing mixture 1 day after inoculation

ml reaction mixture). Treatment periods were 2 hours in each case. The time period between inoculation and the application of ROS was different: at 18 and 24 hours the inoculated leaves remained symptomless, however when this time period increased to 2 days a few very little necroses appeared on the leaves. When this period increased to 3 days or more, regular necroses were formed on the treated leaves just as many as in the control. All these results were experienced with the four bean cultivars used.

We tried to reverse the inhibitory action of ROS on symptom expression by applying SOD and catalase at the time when the ROS-producing two chemical mixtures were applied to the leaves. This experiment was carried out with cultivar Red Kidney. In the presence of 2000 units of SOD or 4000 units of catalase the ROS did not exert its inhibitory action on the necrotic symptoms. In other words, inhibition was reversed by these antioxidants. In some experiments we combined the SOD and catalase. Inhibition of the action of ROS in this case was somewhat higher than in the cases when SOD and catalase were applied separately *(Figs 8, 9).*

Halo blight of bean (Pseudomonas syringae *pv.* phaseolicola)

A compatible host–pathogen combination of French bean cultivar Red Kidney/halo blight bacterial strain S2 was used in these experiments. Both chemical systems producing ROS were applied using the same concentrations, which were used in the case of bean

Fig. 8. Inhibition of symptom expression on bean cultivar Red Kidney inoculated with *Colletotrichum lindemuthianum* as a result of treatment with the xanthine–xanthine oxidase system. Middle leaf: control, not treated but inoculated. Right leaf: leaf treated with the ROS-producing system 1 day after inoculation. Left: leaf treated with ROS-producing system plus SOD (2000 units) and catalase (4000 units) per Petri dish (10 ml reaction mixture) 1 day after inoculation

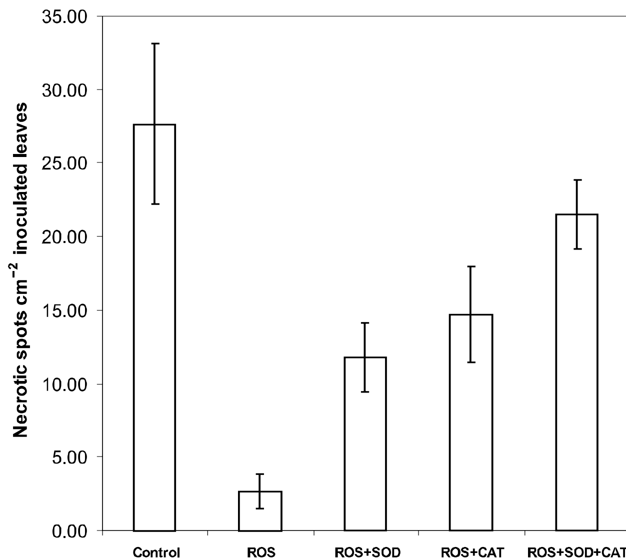

Fig. 9. Effect of ROS produced in the riboflavin-methionine mixture on the expression of necrotic spots in the bean cultivar Red Kidney caused by Colletotrichum lindemuthianum and reversing the action of ROS by SOD and catalase and their combination

anthracnose. In both cases the time period of treatment were 2 hours. We regularly waited for 30 minutes to 1 hour as a time period between inoculation and the application of ROS to evaporate the water from the intercellular space. Immediately after application of ROS, samples were taken from the leaves by a cork borer and homogenized in saline phosphate buffer. An aliquot (0.1 ml) of this suspension was plated on each Petri dish containing sucrose nutrient agar (SNA) medium for counting the number of colonies after 48 hours. As shown in *Table 2* the application of the riboflavin–methionine system significantly and importantly reduced the number of bacteria in the intercellular space. Plating was carried out less than 3 hours after inoculation.

Table 2

The killing action of reactive oxygen species on cells of *Pseudomonas syringae* pv. *phaseolicola* in the intercellular spaces of bean leaves

Treatment after bacterial inoculation	cfu per cm^2 leaf area
Control	$3478 \pm 566^{\tau}$
Reactive oxygen species-producing riboflavin-methionine system	348 ± 151

$^{\tau}$Standard deviation.

As regards the necrotized area caused by bacterial inoculation, symptom expression was fully inhibited by application of ROS. In the control (not treated) leaves typical necrotized area appeared after the watersoaking symptoms *(Fig. 10).* Using the riboflavin–methionine mixture containing 2000 units SOD and 4000 units catalase reversed the action of ROS. In other words, these antioxidants can reverse the inhibitory action of ROS in tissue necrotization.

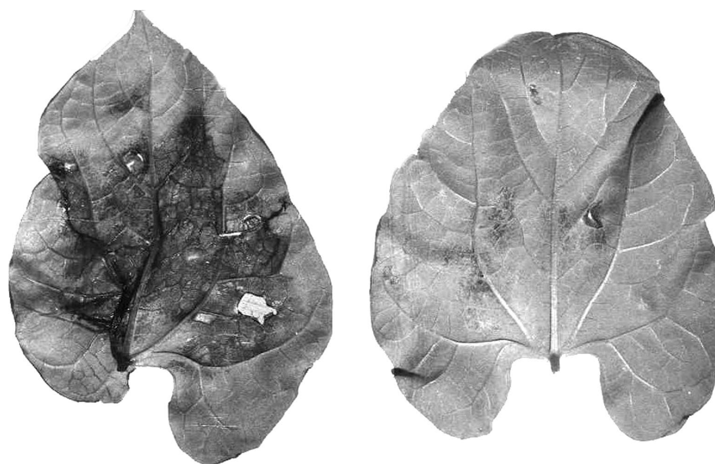

Fig. 10. Suppression of symptoms on Red Kidney bean cultivar inoculated with *Pseudomonas syringae* pv. *phaseolicola* as a result of treatment of leaves with the xanthine–xanthine oxidase system 1 hour after inoculation. Left: control, not treated but inoculated. Right: leaf inoculated and treated with the ROS-producing system

HR-type necrosis of tobacco caused by Pseudomonas syringae *pv.* phaseolicola

The non-host–pathogen combination, tobacco cultivar Samsun/*P. syringae* pv. *phaseolicola,* resulted in an incompatible host–pathogen combination. A riboflavin–methionine ROS-producing system was applied to tobacco leaves one hour after injection with the bacterial cell suspension. During the one hour time period between inoculation and application of ROS, water was evaporated from the inoculated leaf area. The time period of application of ROS was 2 hours. Leaves injected with bacterial suspension were kept in the dark on a riboflavin–methionine mixture or on water (control) and others were illuminated. After illumination samples were transferred to wet filter paper in a humid chamber and incubated at 20 °C for 24 hours under continuos light. HR-type necroses appeared in the control treatments, however ROS treatments completely inhibited the HR-type necroses in the injected area.

Discussion

Basically three plant products are considered to be responsible for inhibition or killing plant pathogens in resistant plants: reactive oxygen species (ROS), phytoalexins and pathogenesis related (PR) proteins. There is no convincing evidence supporting the *in planta* action of phytoalexins against pathogens under natural conditions although a few publications refer to slight increases in resistance in transgenic plants which express resveratrol, a foreign phytoalexin from grapewine (Hain et al., 1993; Hipskind and Paiva, 2000). Similarly, there are a few reports (cf. Zhu et al., 1994) on enhanced resistance against fungal attack by expression of PR proteins, which are indeed stress proteins. However, the role of those “defence” proteins remains questionable if one consider an important result according to which chitinase and ß-1,3-glucanase of tomato can not inhibit its pathogen, *Cladosporium fulvum* (Joosten et al., 1995). Furthermore it seems hard to accept the claim that chitinase could exert inhibition against fungi and bacteria which have no chitin in their cell walls. The possible role of ROS, first of all superoxide and hydrogen peroxide, in inhibiting pathogens in resistant plant, was recently stressed by several investigators (Baker and Orlandi, 1995; Doke et al., 1991; Grant and Loake, 2000; Joosten et al., 1995; Király et al., 1991; Levine et al., 1994; Mehdy, 1994; Staskawicz et al., 1995; Tzeng and DeVay, 1993).

The HR of plants that is associated with resistance is preceded by the “oxidative burst” that produces $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$ and perhaps $^1O_2$. There are plenty of experiences in relation to the important role of H_2O_2 in the inhibition of pathogens in resistant plants (cf. Király et al., 1997; Levine et al., 1994; Wu et al., 1995). However, a balance between ROS and antioxidants seems to be decisive from the point of view of resistance (El-Zahaby et al., 1995; Levine et al., 1994). As early as 1960, we have shown (Farkas et al., 1960) that non-enzymatic antioxidants, such as ascorbic acid, glutathione and cysteine, were able to suppress host tissue necrotization caused by TMV. Probably the action of ROS on symptoms was counteracted by these antioxidants. Recently, it has been demonstrated in many instances that antioxidants are able to eliminate or counteract the adverse action of ROS on symptoms as well as on pathogens. This was also a decisive aspect in

our studies demonstrating that ROS can indeed inhibit or kill pathogens *in planta* and suppress disease symptoms. Measurement of pathogen's multiplication was made only with *Pseudomonas syringae* pv. *phaseolicola* because counting the number of bacteria in infected leaf tissue is relatively easy and accurate. In the cases of fungal diseases symptom expression was evaluated.

In experiments with the powdery mildew of barley it was shown that ROS produced by the two chemical systems as well as the directly applied H_2O_2 were able to kill or inhibit the fungus *in planta* and this inhibition was a SOD or catalase sensitive reaction. Similarly, the killing action of ROS on leaf rust and on bean anthracnose fungi as well as on bacteria causing halo blight was reversed by the application of SOD or catalase. This indicates that superoxide anion and/or hydrogen peroxide are the ROS that are involved in inhibiting or killing pathogenic fungi and bacteria.

When the time period between inoculation of the host plant and the treatment with the ROS-producing chemical systems was relatively short, symptoms of the disease did not appear, indicating that the establishment and/or early multiplication of pathogens were inhibited. As regards mildew of barley, one-day time period resulted in full suppression of symptoms both in the compatible and incompatible H/P combinations. In other words, the pathogen was not able to produce even the HR characteristic for the resistant host. This shows that the fungus was killed before establishment in the host. ROS, not the HR (necrosis), was responsible for resistance in this incompatible H/P combination. This symptomless resistance seems to be identical with the immune-type resistance of cereals to rust or the extreme resistance of plants to viral infections. When this time period increased to 2 days, the compatible barley produced necrotic symptoms similar to the hypersensitive necrosis characteristic for the incompatible combinations (for the resistant host). The 3–4 days time period resulted in mycelial growth, however the mycelium in the pustules turned brown and dead.

In the case of powdery mildew of cucumber, a similar action occurred: 3–4 days time period between inoculation of host and the application of ROS resulted in necrotic symptom expression, however 5–7 days time period seemed to be late because the mycelium was able to develop. However, later it became dead (brownish).

The two rust diseases of wheat exhibited similar events. In the case of leaf rust 2 days time period and in the case of stem rust 3 days resulted in total suppression of symptoms. When this time period was 3–4 days, hypersensitive-like necroses appeared in the susceptible cultivars, indicating that the fungus was able to establish itself, however it was inhibited or killed by the ROS. Five to seven days time period resulted in the appearance of small rust pustules. In these cases the action of ROS was too late to fully inhibit the pathogens.

In this context it is worth to mention that we have shown earlier (Király et al., 1972) that selective inhibition or killing of pathogens in susceptible hosts may result in the formation of HR-type necrotic symptoms that is characteristic for the resistant host plants.

The picture is somewhat different with diseases caused by facultative pathogens. The susceptible or resistant plant exhibit equally necrotic symptoms, however, necroses are significantly smaller and appear regularly early in the resistant plant. Thus, if the pathogen is suppressed by treatment with ROS, very few or no necroses appear on the inoculated leaves (see *Table 3*).

Table 3

Alteration of symptoms of diseases caused by obligate and facultative pathogens under the influence of ROS

	Diseases symptoms caused by			
	Obligate pathogens		Facultative pathogens	
	Compatible H/P	Incompatible H/P	Compatible H/P	Incompatible H/P
Under normal condition	disease symptoms	early necrotic spots	late necrotic disease symptoms	nothing or early and small necrotic spots
Under the effect of ROS	necrotic spots	nothing	nothing	nothing

ROS = reactive oxygen species
H/P = host-pathogen relationship

The necrotic spots caused by *Pyrenophora tritici-repentis* on wheat were fully suppressed when the time period between inoculation and the application of ROS was 1 day. A similar time period (18–24 hrs) was suitable also in the case of bean anthracnose *(C. lindemuthianum)* to fully inhibit the appearance of necrotic spots. Two days time period resulted in the appearance of little necroses and 3 days permitted to produce regular necroses on bean leaves. The bean halo blight caused by a pseudomonad was even more sensitive: 30–60 minutes period was suitable to a substantial reduction of the number of bacteria in the leaf. In this case symptoms were fully suppressed.

In conclusion, reactive oxygen species, first of all superoxide and hydrogen peroxide, were able to inhibit or kill the pathogens *in planta* whether they were obligate or facultative pathogens. This inhibition basically altered the symptoms of diseases caused by both types of pathogens. The action of ROS was reversed by the application of antioxidants such as SOD and catalase, indicating that in the killing action $O2^{\bullet-}$ and H_2O_2 were the most important or at least the initial reactive oxygen species.

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