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ABBREVIATIONS

APX = Ascorbate peroxidase

BTH = Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester

CAT = Catalase

CDNB = 1-chloro-2,4-dinitrobenzene

DAB = 3,3-diaminobenzidine

DCFH-DA = 2',7'-dichlorofluorescein diacetate

DHA = Dehydroascorbic acid

DHAR = Dehydroascorbate reductase

ELISA = Enzyme-linked immunosorbent assays

GR = Glutathione reductase

GSH = Reduced glutathione

GSSG = Oxidized glutathione

GST = Glutathione S-transferase

 $H_2O_2 = Hydrogen peroxide$

HR = Hypersensitive reaction

INA = 2,6-dichloroisonicotinic acid

NBT = Nitroblue tetrazolium

 $O_2^{\cdot \cdot}$ = Superoxide anion radical

 $^{1}O_{2} = \text{Singlet oxygen}$

 $OH^{\cdot} = Hydroxyl radical$

PDA = Potato dextrose agar

POX = Guaiacol peroxidase

PUFA = Polyunsaturated fatty acids

RT-PCR = Reverse transcription-polymerase chain reaction

ROS = Reactive oxygen species

SA = Salicylic acid

SAR = Systemic acquired resistance

SOD = Superoxide dismutase

TDB = Triethanolamine-diethanolamine buffers

TMV = Tobacco mosaic virus

DAI = Days after inoculation

hai = Hours after inoculation

1. INTRODUCTION

1. Reactive oxygen species (free radicals)

Recently, it turned out that there is a certain balance between the action of reactive oxygen species (ROS) and antioxidants in microorganisms, plant as well as in animal cells. As a result of stress or infection, this balance is abnormal or does not exist. Thus, the role of ROS in different forms of disease resistance or in symptom expression of susceptible plants seems to be pivotal.

The element atmospheric oxygen (chemical symbol O) which exists as a "double" molecule, two atoms being joined together to give O_2 (dioxygen). It is an essential element for all respiring organisms, however some oxygen species, in an activated status, tend to be highly toxic causing oxidative stress.

Reactive oxygen species (ROS) are involved in many important processes in plants (Elstner et al., 1994). ROS are believed to have important roles in plants in general and in plant-pathogen interactions in particular. They are involved in signal transduction, cell wall reinforcement, hypersensitive response (HR) and phytoalexin production, and have direct antimicrobial effects (Abdou et al., 1993; Galal et al., 1993; Király et al., 1993). The main toxic ROS are the superoxide anion radical (O_2), hydrogen peroxide (H₂O₂), hydroxyl radical (OH) and singlet oxygen (1O_2) in the biological systems (Elstner, 1982, 1987; Tzeng and DeVay 1993). As is seen, some ROS are free radicals and some are reactive molecules.

In a normal healthy plant or animal, the molecular oxygen is reduced to water. However, several active oxygen species are produced during the course of reduction. Free radicals are very reactive because they have unpaired shell electrons. Activation of oxygen can either occur chemically or physically. Chemical oxygen activation yields the superoxide radical (O_2) after a one-electron reduction. Two-electron reduction of dioxygen and the dismutation of superoxide yield hydrogen peroxide (H_2O_2). Hydrogen peroxide can accept electrons from metal ions such as Fe²⁺ or from certain semiquinones, forming the hydroxyl radical (OH).





2. Oxidative stress

Oxidative stress is caused by herbicides, infections (biotic stress) and abiotic stresses, such as air pollution, high light intensity, heat shock etc. Oxidative stress in the cells results in damaging the membranes, the lipids, amino acids, nucleic acids, pigments and proteins. The end result could be senescence or cell death.

Oxidative stress has a key role in the plant senescence (Leshem, 1988, Pethő, 1993). As a result of senescence, superoxide is produced in a high level in the membranes and, at the same time, some antioxidants such as SOD are reduced in the activity (Droillard et al., 1987, 1989). Interestingly, the antioxidant vitamin C and E content also decreased in the plants parallel with the senescence (Kunert and Ederer, 1985).

It is well known that antioxidants are activated in those plants, which are resistant to some ROS productions. It was found in the chloroplasts of the resistant Canada horseweed (*Conyza bonariensis*) that the activity of SOD, ascorbate peroxidase and glutathione reductase was higher than in the sensitive control plants (Gressel and Shaaltiel, 1987).

ROS may damage plant membranes causing cell and tissue necroses. Many researchers found that, as a result of bacterial infections, the lipid peroxidation and the amount of ROS increased in the diseased plants (Keppler and Novacky, 1986, 1987; Keppler and Baker, 1989; Ádám et al., 1989).

Hydroxyl radical is one of the most reactive and toxic chemical groups. It has key role in the initiation of lipid peroxidation as well as in the oxidation of amino acids and nucleic acids (Halliwell, 1974). Recently it is known that ROS may have important role in causing chemical stresses in plants induced by ozone O_3 , SO_2 , NO_x , herbicide actions and infections and the end result of this damage is cell or tissue necrosis (Duke, 1985; Shaaltiel et al., 1988; Ádám et al., 1989; Király et al., 1993; Tzeng and DeVay, 1993; Baker and Orlandi, 1995).

It was demonstrated that the polyunsaturated fatty acids (PUFA) are very sensitive to lipid peroxidation (Sundquist and Fahey, 1989). Free radicals can remove one hydrogen atom from the fatty acids chains therefore, it can initiate the lipid peroxidation (Elstner, 1982; Kappus, 1985).

Production of free radicals (first of all superoxide) in the membranes resulting in lipid peroxidation of the fatty acids which are more and more saturated, and then the level of phospholipids and galactolipids begin to decrease. These changes lead to disintegration of the membranes: the fluidity of the membranes is decreasing, the permeability is increasing and therefore, the cell and tissue necrosis (HR) develops (Ádám et al., 1989).

The first records on the role of ROS in diseased plants were published in the 1980's. It was shown that cercosporin (toxin isolated from the fungus *Cercospora beticola*) can produce O_2^{-} if it is illuminated, therefore inducing membrane damages and necrosis in infected tobacco plants and in tobacco cell suspension (Daub and Briggs, 1983). Potato tubers infected with *Phytophthora infestans*, produced large amount of O_2^{-} (Doke, 1983). It has been shown that tobacco plants infected with *Pseudomonas syringae* increases O_2^{-} production and lipid peroxidation three hours after infection and before the appearance of the HR-type necroses (Ádám et al., 1989).

3. Role of antioxidants

Antioxidants are substances that delay or inhibit oxidative damage to target molecules such as lipids, proteins, nucleic acids and carbohydrates. Antioxidants might protect a target by:

- Scavenging oxygen-derived species, either by enzymes or by direct chemical reaction (in which case the antioxidant will be used up as the reaction proceeds).
- (ii) Minimizing the formation of oxygen-derived species.
- (iii) Binding metal ions needed to convert poorly reactive species (such as O_2^{-} and H_2O_2) into nasty ones (OH).

- (iv) Repairing damage to the target.
- (v) Destroying badly damaged target molecules and replacing them with new ones.The human body uses all these mechanisms.

The nonenzymatic antioxidant scavengers: ascorbic acid (vitamin C), glutathione, albumin, ∞ -tocopherol (vitamin E) and carotenoids etc. The enzymatic antioxidant scavengers: superoxide dismutase (SOD), catalase (CAT), peroxidase, glutathione peroxidase, glutathione S-transferase, glutathione reductase, ascorbic acid oxidase, ascorbic acid peroxidase, dehydro- ascorbate reductase (DHAR) etc.

It was found in tobacco, wheat and rice that one can suppress the necrosis caused by infections by applying ascorbic acid and glutathione exogenously (Oku, 1960; Farkas et al., 1960). Recently this was strengthened by Ádám et al. (1989) by delaying the HR with the application of antioxidants. Also, in barley infected with powdery mildew, necrosis can be inhibited with some antioxidants (Hafez and Király, 2003) and HR-type necrosis in tobacco infected with TMV either at 20° C or 30° C (Hafez et al., 2003).

SOD, as an enzymatic antioxidant, eliminates the O_2^- by dismutation to H_2O_2 and O_2 (Gupta et al., 1993). Catalase is the most important enzyme to decompose H_2O_2 . Peroxidases can also play role in the decomposition. This detoxification is carried out in the glutathione cycle (Foyer and Halliwell, 1976; Schmidt and Kunert, 1987), which later termed as the ascorbate/glutathione cycle (Nakano and Asada, 1981). This system not only decomposes H_2O_2 but also the lipid hydroperoxides.

4. Induced resistance

A common response to necrogenic pathogen infection is the development of systemic acquired resistance (SAR) to a subsequent pathogen attack. This induced resistance or SAR results in broad-spectrum, non-specific immunity in non-infected parts of the plant and provides protection against several subsequent pathogens and non-pathogens (Chester, 1933; Ross, 1961; Kuć, 1982; Ryals et al., 1994, 1995).

The accumulation of salicylic acid (SA) is an important component in the signal transduction pathway leading to SAR (Métraux et al., 1990; Malamy et al., 1990). It was suggested that the role of SA in SAR signal transduction can inhibit catalase activity, leading to elevated levels of H_2O_2 which could in turn function as a second messanger of SA in SAR signal transduction (Chen and Klessig, 1991; Chen et al., 1993).

The use of chemicals to activate SAR-type reaction provides novel alternatives for disease control in agronomic systems. SA is the only plant-derived substance that has been demonstrated to be an inducer of SAR (White, 1979; Antoniw and White, 1980; Ward et al., 1991). 2,6-dichloroisonicotinic acid and its methyl ester (both referred to as INA) were the first synthetic chemical compounds shown to activate SAR-type reaction, thus providing broad spectrum disease resistance (Métraux et al., 1991; Vernooij et al., 1995).

Interestingly enough, the synthetic chemical benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) was also demonstrated to be a potent SAR activator (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996) that supplies protection in the field against some diseases in several crops. Thus, BTH and INA seem to be proper compounds for practical agronomic use (Hafez et al., 2004).

5. The aim of my research

The interaction of ROS and antioxidants has a very important role not only in several human diseases but also in plants. Therefore, I tried to focus on four unknown aspects of plant disease resistance and plant/pathogen interaction in connection with this interaction.

1. I tried to have a deeper insight into the role of hydrogen peroxide in powdery mildew infected barley on symptom expression

As is known, resistance of barley to infection of powdery mildew (*Blumeria graminis* f. sp. *hordei*) may or may not be associated with the hypersensitive response (HR). It was also shown earlier that the HR is a consequence, not the cause, of plant resistance to fungal pathogens (Király et al., 1972). It was shown later that HR and resistance are two separate phenomena also in the cases of viral and bacterial infections. Furthermore, several investigations pointed out that plant tissue necrotization, including the HR phenomenon, is associated with the generation of reactive oxygen species (ROS). Therefore, I tried to answer the question, what is indeed the role of the ROS (hydrogen peroxide) in symptom expression of powdery mildew resistant barley carrying the genes *Mla12* or *Mlg* which determine HR-type resistance and in race-non-specific resistant plants carrying the gene *mlo5* or in susceptible barley expressing the gene *Mlo*?

2. The second aspect related to an interesting phenomenon, namely immunization of plants with very low concentrations of hydrogen peroxide

Recently, it was shown that it is possible to immunize plants against abiotic stresses by spraying leaves with low concentration of H_2O_2 inducing augmented antioxidant activities in treated leaves. This resulted in suppression of abiotic stresses (Gechev et al., 2002). This is a non-specific form of plant resistance, which is effective against the appearance of normosensitive necrotic symptoms. It is associated with the activation of the antioxidant capacity of the host plant. Oxidative stress and cell necrotization caused by ROS are characteristic of the susceptible plant. Resistance means suppression of normosensitive necrotic symptoms and in some cases suppression of growth of necrotrophic pathogens. Thus, I intended to answer the following:

- Would it be possible to suppress necroses associated with infection of TMV, *Pseudomonas syringae* pv. *phaseolicola* and *Botrytis cinerea* by pre-treatment of leaves with low concentrations of H₂O₂?
- (ii) Is it also possible to suppress necroses with the application of SOD and CAT?
- (iii) What about the multiplication of viral and bacterial pathogens in the necrotized susceptible tissues and in the resistant leaf tissues, which latter have been immunized by treatment with H₂O₂?
- (iv) Is there any increase or decrease in the antioxidant level?

3. It is known that on temperatures which are above 28°C the *N*-gene encoded necrotization is not expressed in the TMV inoculated local lesion tobacco plants but the virus spreads and multiplies systemically

I wanted to answer the following questions:

- (i) Is it possible to induce HR type necroses in virus-infected local lesion tobacco even at 30°C with the application of ROS?
- (ii) Is it possible to reverse the action of ROS at 30°C with the application of antioxidant enzymes?
- (iii) What is the concentration of TMV at 30°C in necrotized and non-necrotized tissues?
- (iv) What are the level of superoxide and hydrogen peroxide at 30°C?

(v) Are there changes in the antioxidant activities as determined either by biochemical assays or gene expression using the RT-PCR technique?

4. The fourth aim of my research was to get more information about a chemically induced resistance

2,6-dichloroisonicotinic acid (INA) treatment of barley plants induced HR-type necroses in the susceptibe Ingrid barley carrying the gene *Mlo*. I tried to answer the following questions:

- (i) What is the relation between the INA treatment and the level of ROS (first of all H₂O₂) in barley plants?
- (ii) Whether or not INA-pretreatment significantly influences activities of some antioxidant enzymes or gene expression using RT-PCR technique?

2. REVIEW OF THE LITERATURE

Doke in 1983 observed the generation of ROS during the infection of potato tuber tissue with races of the potato blight fungus *Phytophthora infestans*. After his publication several papers were published in the literature (Király et al., 1991, Sutherland, 1991, Tzeng and DeVay, 1993, Mehdy, 1994, Baker and Orlandi, 1995, Fodor et al., 1997) on the role of ROS in disease resistance.

As regards resistance to ROS, it was found that natural mutants of a weed *Conyza* bonariensis which are resistant to superoxide (therefore resistant to paraquat) are also resistant to other agents producing O_2^{-} (Gressel and Shaaltiel, 1987). It was shown also that the O_2^{-} -resistant tobaccos were tolerant to several infections and environmental stresses (which usually induce production of ROS) because of the high capacity of antioxidants (Ádám et al., 1990, Barna at al., 1993).

Recently, several investigators have shown that many pathogenic and abiotic stresses are accompanied by rapid production of reactive oxygen species (ROS) which cause damage of plant tissues (Elstner and Osswald, 1994, Baker and Orlandi, 1995, Hückelhoven, et al., 1999, Piedras, et al., 1998, Hippeli, et al., 1999, Grant and Loake, 2000). Leshem (1988) found that during senescence formation of reactive oxygen species and activity of lipoxygenase enzyme is increasing and the antioxidant capacity is decreasing in plants.

1. Role of hydrogen peroxide in symptom expression of barley susceptible and resistant to powdery mildew

It has been known since a long time that resistance of barley to infection of powdery mildew (*Blumeria graminis* f. sp. *hordei*) may or may not be associated with the hypersensitive response (HR). As shown previously by several workers, host cultivars carrying the gene *Mla12* express a typical HR-associated race-specific resistance. However, cultivars carrying the gene *mlo5* exhibit race-nonspecific (general) resistance which is not associated with the HR (Jørgensen, 1988, Hückelhoven et al., 1999).

It was shown earlier that the HR is a consequence, not the cause, of plant resistance to fungal pathogens (Király et al., 1972). Recently, several laboratories (Yu et al., 1998, Bendahmane et al., 1999, Cole et al., 2001, Schoelz et al., 2003) demonstrated that HR and resistance are two separate phenomena also in the cases of viral and bacterial infections.

Furthermore, several investigations pointed out that plant tissue necrotization, including the HR phenomenon, is associated with the generation of reactive oxygen species (ROS) (Doke, 1985, Király et al., 1993., Baker and Orlandi, 1995, Barna et al., 2003, Hückelhoven and Kogel, 2003). A few publications also referred to the *in vitro* as well as *in vivo* sensitivity of plant pathogens to the action of ROS (Tzeng and DeVay, 1993, Király et al., 1993, Ouf et al., 1993, Wu et al., 1997, Király and El-Zahaby, 2000., El-Zahaby et al., 2004).

The interaction between barley (*Hordeum vulgare*) and the powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*) has been intensively studied in terms of plant defence reactions (Panstruga and Schulze-Lefert, 2002; Collinge et al., 2002). The plant response to pathogen attack includes alterations of the cell wall, production of reactive oxygen species, phenolic metabolites and a hypersensitive cell death reaction (HR) as well as accumulation of pathogenesis-related (PR) proteins.

In barley, early penetration resistance and physical reinforcement of the cell wall by appositions (syn. papillae) are typically observed in race-non-specific resistance responses, such as those governed by the *mlo* alleles. HR is the predominant race-specific plant response determined by different R genes. H_2O_2 accumulation is regularly associated with the HR (Hückelhoven and Kogel, 2003). H_2O_2 was identified also as a signal molecule that activates expression of several genes in plants (Desikan et al., 2000). Several enzymes produce hydrogen peroxide (or active intermediates) in plants including NADPH oxidase, peroxidases, oxalate oxidase and amine oxidases (Bolwell and Wojtaszek, 1997).

2. Immunization of tobacco with hydrogen peroxide against oxidative stress caused by viral, bacterial and fungal infections

It was shown (Becana et al., 1998, Noctor and Foyer, 1998) that if plants are exposed to various unfavorable environmental conditions (abiotic stresses) this leads to increased production and accumulation of reactive oxygen species (ROS), such as superoxide anion, H_2O_2 and hydroxyl radicals. This process referred to as the oxidative burst.

It was found (Pinhero et al., 1997, Zhang, et al., 1995) that stress-resistant plants often possess elevated activities of antioxidant enzymes, such as superoxide dismutase, catalase, peroxidases and glutathione reductase etc. Holmberg and Bülow (1998) found that in transgenic plants which overexpress antioxidant enzymes also express stress tolerance. Alvarez et al. (1998) and Lamb and Dixon (1997) referred to the fact that low concentration of H_2O_2 in plants may take an active part in the activation of antioxidants. H_2O_2 is the most stable ROS and it can rapidly diffuse across cell membranes. However, H_2O_2 at high concentrations is toxic and can trigger programmed cell death (HR).

Recently, Grant and Loake (2000) and Foyer et al. (1997) have shown that H_2O_2 at non-toxic concentrations can be a signalling molecule that mediates plant responses to a variety of biotic and adverse abiotic stress factors. A transient increase in the endogenous level of H_2O_2 obtained by exogenous application of salicylic acid or heat can lead to subsequent thermotolerence in mustard seedlings (Dat et al., 1998) and potato (Lopez-Delgado et al., 1998). Anderson et al. (1995) found that in maize, protection against chilling injury can be achieved by a transient increase in endogenous H_2O_2 levels during lowtemperature acclimation.

It was found by Prasad et al. (1994a, 1994b) that pre-treatment with H_2O_2 or abscisic acid (ABA) protects maize seedlings from chilling injury by induction of peroxidases and mitochondria catalase. H_2O_2 is implicated also as part of a systemic signal that sets up an acclimatory response to high light stress in *Arabidopsis* (Karpinski, et al., 1999). Bowler and Fluhr (2000) have shown that there is an intriguing possibility that moderately elevated levels of H_2O_2 can protect plants from different stress factors. A special type of resistance to normosensitive necrotic symptoms, could be associated with the activation of antioxidants (Fodor et al., 1997, Király et al., 2002). In this latter case cell necrotization and oxidative stress caused by ROS are characteristic of the susceptible plant. Thus, resistance in this case, means suppression of normosensitive necrotic symptoms, which are causing yield loss. Suppression of symptoms is the result of the activated antioxidant capacity.

Halliwell and Gutteridge (1999) point out that oxidative stresses usually up-regulate the antioxidant defense systems *in vivo*. As a result of this type of up-regulation of antioxidants may cause suppression of necrotic symptoms (resistance).

Recently, it was shown by Gechev et al. (2002) that it is possible to "immunize" tobacco plants by spraying leaves with low concentration of hydrogen peroxide (H_2O_2) against abiotic stresses. They have shown that a spray with 5 mM H_2O_2 induced augmented antioxidant activities in treated leaves which resulted in suppression of necroses caused by a catalase inhibitor or high light intensity. In further experiments Vandenabeele et al. (2003) demonstrated that this mild oxidative stress may induce expression of several genes associated with up-regulation of tolerance to abiotic stresses.

Uchida et al. (2002) found that pretreating rice seedlings with low levels (10 μ M) of H₂O₂ or NO[•] permitted survival of more green leaf tissue, and of higher quantum yield for photosystem II, than in non-treated controls, under salt and heat stresses. It was also shown that the pretreatment induces not only active oxygen scavenging enzyme activities, but also expression of transcripts for stress-related genes encoding sucrose-phosphate synthase, Δ' -pyrroline-5-carboxylate synthase, and the small heat shock protein 26.

Geetha and Shetty (2002) found that chemical induction of resistance in pearl millet against downy mildew disease (*Sclerospora graminicola*) is possible by treating seeds of highly susceptible cultivars with the resistance activator benzothiadiazole (BTH) (CGA 245704), calcium chloride (CaCl₂) and hydrogen peroxide (H₂O₂). BTH in 0.75%, 90 mM CaCl₂ and 1.0 mM H₂O₂ were effective in managing the disease by giving 78%, 66% and 59% protection, respectively. *In vivo* quantification of *Sclerospora graminicola* by an enzyme-linked immunosorbent assay confirmed the reduced fungal biomass in the treated plants.

It is also possible to "immunize" bacteria to the adverse action of ROS. Nakjarung et al. (2003) exposed cells of *Agrobacterium tumefaciens* to very low concentration of H_2O_2 which induced high catalase and peroxidase activities, helping thereby bacterial cells to survive in the host plant.

3. Role of reactive oxygen species (ROS) and antioxidants in TMV-induced necrotization associated with resistance of an N gene encoding tobacco

Doke and Ohashi (1988) and Allan and Fluhr (1997) found that in the TMV-infected N gene-expressing local lesion tobacco hosts a superoxide (O₂⁻) generating system is induced. However, this is not the case with systemic hosts lacking the N gene. ROS were associated only with the formation of necrotic symptoms and were absent when virus multiplication and spread were accompanied by non-necrotic symptoms.

It was found (Király et al., 2002) that in a transgenic local lesion host (NahG tobacco) which produces large necrotic lesions upon TMV infection, an increased level of O_2^- production was found at the edge of necrotic spots, as compared to the control nontransgenic tobacco.

Fodor et al. (1997, 2001) found that activation of ROS with infections and stresses is counteracted by up-regulation of antioxidant defense systems. This is true even in tobacco plants in which systemic acquired resistance (SAR) was induced. In TMV-infected Xanthi-nc tobacco (a local lesion host) antioxidants were activated not only in the infected leaves but also in the remote non-infected upper leaves of the same plants. However, Király et al. (2002) found that in a transgenic line of the same Xanthi-nc tobacco (NahG), in which the SAR was inhibited, the antioxidants were down-regulated.

Some investigators claimed that ROS could be responsible not only for necrotization of the host tissues but also for resistance. However, as early as 1972 it was proposed that host resistance and host cell death responses are two seperate phenomena (Király et al., 1972). This idea was recently supported by new experiments (Bendahmane et al., 1999, Cole et al., 2001, Yu et al., 1998).

It was shown as early as 1931 that HR-type necroses caused by TMV were overcome at temperatures above 28°C and the virus replicated and moved systemically in the originally

resistant *N* gene expressing plants (Samuel, 1931). However, if infected plants were moved to low temperatures (20-25°C), very intensive necrotization of tissues (HR) occurred.

4. Chemically induced resistance in barley by INA

Several chemicals have been reported to induce resistance against pathogens when applied to plants (Kessman et al., 1994). SAR is associated with accumulatin of salicylic acid (SA) both in the primarily infected leaves and later in the remote uninfected ones (Malamy et al., 1990 and Métraux et al., 1990). When salicylic acid was exogenously applied to tobacco or *Arabidopsis*, it induced resistance as a biological inducer (White 1979, Ward 1991, Uknes et al. 1992, 1993). Salicylic acid is crucial for the development of SAR, although other mobile signals seems to be resposible for the systemic action. Recently, it was suggested that a lipid transfer protein may signal the biochemical changes in the plants exhibiting SAR (Maldonado et al., 2002). In transgenic tobacco and *Arabidopsis* plants, which express a gene, whose product converts SA to catechol, SAR did not develop (Gaffiney et al., 1993).

It was shown that SA may play a primary role in up-regulating active antioxidative defense (Fodor et al., 1997). It was also determined that a systemic microoxidative burst develops in plant-fungus (Doke et al., 1996) and plant-bacterium (Alvarez et al., 1998) interactions.

Recently, it was also demonstrated by using the electron paramagnetic resonance spectroscopy technique (Fodor et al., 2001) that in remote leaves of Xanthi-nc tobacco virus-induced systemic resistance is associated with a small oxidative burst. Solymosy et al. (1959) have shown since a long time that exogenously applied antioxidants may suppress TMV-induced necrotization in tobacco leaves. Furthermore, several observations reffered to the function of plant antioxidants in resistance (Király, 2000 and Mittler, 2002).

Exogenous application of SA or its functional analogues, such as benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), and 2,6-dichloroisonicotinic acid (INA) confer increased resistance to TMV (White, 1979; Friedrich et al., 1996).

2,6-dichloroisonicotinic acid (INA) is a structural analogue of SA and an activator of acquired resistance. INA induces expression of the same set of SAR genes that are induced by either SA treatment or various infectious agents (Ward et al., 1991 and Uknes et al., 1992). It is also effective in transgenic NahG plants that are unable to accumulate SA. Therefore, INA induces the SAR signal transduction pathway by acting either at the same site or downstream of SA accumulation (Vernooij et al., 1995). In the susceptible barley, which treated with INA, the mechanism of INA-induced resistance seems to be a phenocopy of the mechanism governed by the *Mlg* locus. Acquired resistance correlates with high-level transcript accumulation of barley defense-related genes encoding pathogenesis related protein-1, peroxidase and chitinase (Kogel et al., 1994).

3. MATERIALS AND METHODS

3.1. Plant materials

Tobacco (*Nicotiana tabacum* L.) cultivar Xanthi-nc, which is a local lesion (HR) host of TMV, was used in this study.

Susceptible barley (*Hordeum vulgare* L.) near-isogenic lines of cultivar Ingrid carrying the genes *mlo5*, *Mla12* and *Mlg* for resistance against the powdery mildew (*Blumeria graminis* f. sp. *hordei* DC. (Speer) race A6) were used (obtained from Professor K-H. Kogel, Plant Pathology Department of the Justus Liebig University, Giessen, Germany).

Seeds of the above mentioned cultivars were sown in soil and grown under greenhouse conditions at 18-23°C, with 16 hours photoperiod per day using supplemental light, 75-80% relative humidity as described by Fodor et al. (1997).

3.2. Pathogens

3.2.1. Viral pathogen

Tobacco mosaic virus (TMV) U1 strain was used in this study. TMV was maintained in susceptible tobacco (*Nicotiana tabacum*) cultivar Samsun-nn in the greenhouse.

3.2.2. Fungal pathogens

The following fungal pathogens were involved in this study: barley powdery mildew, *Blumeria graminis* f. sp. *hordei* race A6. It was maintained on susceptible barley seedlings in the greenhouse. Conidia of the pathogen were powdered onto leaves of susceptible and resistant barley. *Botrytis cinerea* Pers., strain Bc-1 (isolated from tomato) kindly supplied by Prof. László Vajna, Department of Plant Pathology, Plant Protection Institute, Hungarian Academy of Sciences, Hungary. This fungus was maintained on potato dextrose agar (PDA) medium.

3.2.3. Bacterial pathogens

Pseudomonas syringae pv. *phaseolicola* (Burkholder) Young, Dye and Wilkie, GSPB 1205 kindly supplied by K. Rudolph, Göttingen, Sammlung Phytopathogener Bakterien, Germany. It was maintained or cultured King's medium (King et al., 1954). Slant cultures

3.3. Procedures of inoculation

TMV-inoculated leaves of *N. tabacum* cv. Samsun-nn showing typical disease symptoms were ground (50 mM sodium phosphate buffer, pH 7.0) in a mortar and the homogenates were used for inoculations of tobacco Xanthi-nc without an abrasive.

Barley powdery mildew was maintained on susceptible barley seedlings in the greenhouse. First leaves of the 7-day-old barley seedlings were inoculated with the fungus by shaking conidia from diseased leaves of barley plants.

Botrytis cinerea was maintained on potato dextrose agar (PDA) medium. The medium was inoculated by putting a small agar disc containing the fungus mycelium (7 day-old-culture) on the middle of the plate. A little agar disc containing the fungus mycelium put into the surface of the tobacco leaves. Inoculated leaves were held in Petri dishes at 20°C for one week.

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

3.4. Artificial production of necroses at 30°C with reactive oxygen species (ROS) and direct application of H₂O₂

Two chemical systems to produce superoxide radical and hydrogen peroxide and a direct application of hydrogen peroxide were used in this study.

3.4.1. The riboflavin/methionine photochemical system

The photogeneration of superoxide anion in chemical systems, which contain riboflavin and methionine, is well known (Koryka-Dahl and Richardson, 1978; Jordan et al., 1992). When riboflavin absorbs visible light it becomes excited and the presence of methionine initiates redox reactions. In the course of these processes riboflavin is univalently reoxidized with the formation of superoxide anion (O_2 ⁻). Several investigators have also shown that in the living systems several other reactive oxygen species (ROS) can be produced, such as H₂O₂, OH⁻, ¹O₂, which also may contribute to the toxicity of this photochemical system (Tzeng, 1989; Tzeng and Lee, 1989; Tzeng et al., 1990).

Inoculated leaves were detached three days after infection with TMV and put on the riboflavin/methionine solutions in Petri dishes. Six ml of mixture containing 266 or 532 μ M riboflavin as well as 10 or 20 mM L-methionine, respectively was poured on filter paper in each Petri dish. The Petri dishes were illuminated (100 μ E m⁻² s⁻¹) in an incubator at 30°C for three days. The same treatments were conducted with inoculated intact leaves by infiltration with a syringe. TMV-infected leaves (without riboflavin treatment) and riboflavin treated healthy leaves (without infection) were used as controls.

3.4.2. The ROS-producing glucose-glucose oxidase system and direct application of H₂O₂

In this system the enzyme acts aerobically upon glucose and generates O_2^{-} and H_2O_2 (Wu et al., 1997). Six ml of solutions containing 50, 100, 150, 200, 250 and 300 units of glucose oxidase/ml and 2 mM glucose were poured on filter paper in each Petri dish or injected into intact leaves. TMV inoculated leaves were detached three days after inoculation and put into these Petri dishes for three days. TMV-infected leaves (without glucose-glucose oxidase) and glucose–glucose oxidase treated healthy leaves (without virus infection) were used as controls.

Direct application of 10, 25, 50, 100, 150 and 200 mM H_2O_2 was carried out by injecting the intact leaves or treating the detached leaves in Petri dishes as described above.

3.5. The reversible action of antioxidant enzymes against ROS

3.5.1. In tobacco infected with TMV

The action of ROS at 30°C was reversed by treatment with 4000 U/ml of superoxide dismutase (SOD) (E.C.1.15.1.1) and 5000 U/ml of catalase (CAT) (E.C.1.11.1.6). SOD and CAT were applied to leaves treated with riboflavin/methionine three days after inoculation with TMV. CAT was applied on leaves treated with glucose-glucose oxidase or directly treated with H_2O_2 three days after inoculation with TMV.

3.5.2. In barley infected with powdery mildew

Barley leaves were injected with a water solution containing 2500 units superoxide dismutase (SOD) and 5000 units catalase (CAT)/ml and leaves were sprayed with H_2O_2 after water evaporation. Leaves were infected with the powdery mildew fungus after H_2O_2 treatment.

3.5.3. In tobacco infected with viral, bacterial and fungal pathogens

Tobacco leaves inoculated with TMV, *P. syringae* pv. *phaseolicola* and *Botrytis cinerea* were injected immediately after inoculation with a solution which contained 5000 units catalase (CAT) and 2500 units superoxide dismutase (SOD)/ml.

3.6. Determination of concentration of TMV

3.6.1. In tobacco infected with TMV at 30°C

Xanthi-nc tobacco plants infected with TMV and treated with riboflavin, glucoseglucose oxidase or H_2O_2 were kept at 30°C. TMV-infected tobacco pants held at 20°C served as controls. Four days after inoculation necroses (HR) appeared. Leaf samples were homogenized with PBS extraction buffer in ratio 1:5 (50 mM phosphate buffer, pH 7.4, containing 0.8% Tween 20) and then, diluted further for 1:10, 1:20 and 1:50. Enzyme-linked immunosorbent assays (ELISA) were performed for determining concentration of TMV according to Clark and Adams (1977) and Tobiás et al. (1982), using a TMV kit of Bioreba (Art. No. 190412 and 190422). The extinction values were measured with Multiskan ELISA Reader at 405 nm, after 10, 20 and 30 minutes of incubation with the substrate.

3.6.2. In tobacco infected with TMV and pre-treated with low concentration of H₂O₂

Xanthi-nc tobacco plants pre-treated with low concentration of H_2O_2 were inoculated with TMV one day after the treatment. Plants infected with TMV were kept in the greenhouse for three days. TMV-infected untreated plants served as controls. Enzyme-linked immunosorbent assays (ELISA) were performed for determining concentration of TMV as mentioned above.

3.7. Determination of concentration of bacteria

Infection with *P. syringae* pv. *phaseolicola* was carried out one day after treatment with low concentration of H_2O_2 and kept in the greenhouse for one day. Plants infected with the bacterium served as controls. To determine the bacterial concentration, we used the plate-count technique according to Klement et al., (1990) 24, 48 and 72 hours after inoculation.

3. 8. Application of H₂O₂ to intact and detached barley leaves

Leaves of 8-10-day-old barley seedlings were inoculated with *Blumeria graminis* f. sp. *hordei*. Some intact inoculated leaves were detached at different time periods after

inoculation. Either intact or detached leaves were sprayed with 10-500 mM H_2O_2 one, two and three days after inoculation. The water solution of H_2O_2 contained 0.5% Tween. Leaves, which were treated with H_2O_2 only or infected only with the pathogen, served as controls.

3.9. Application of low concentration of H₂O₂ to tobacco leaves

Tobacco (*Nicotiana tabacum*) cultivar Xanthi-nc, which is a local lesion (HR) host of TMV, was used and planted as mentioned.

The fourth and fifth true leaves of 8-10-week-old plants were treated by spraying the plant leaves with an aqueous solution of H_2O_2 . Plants were sprayed with 5, 7, 10, and 12.5 mM H_2O_2 solution. The control plants were treated with water alone. After one day of H_2O_2 treatments a suspension of the U1 strain of TMV were prepared for the infection. Numbers and diameters of necroses were counted.

Infection with *Pseudomonas syringae* pv. *phaseolicola* was conducted one day after the treatment with 5, 7 and 10 mM H_2O_2 . The age of bacterial culture was 24 hours and its concentration was 10^8 /ml.

Infection with *Botrytis cinerea* was conducted one day after the treatment with 5, 7 and 10 mM H_2O_2 and the age of culture was 7 days.

3.10. Action of H₂O₂ on the mycelial growth of Botrytis cinerea

Petri dishes which contained potato dextrose agar (PDA) medium were inoculated by putting a small agar disc containing the fungal mycelium (7 day-old-culture) in the middle of the plate. Several holes were made with a corkborer in the agar plates around the inoculum. Different concentrations of H_2O_2 (2.5, 5, and 10 mM) were poured into the holes. H_2O_2 solution were supplied every day to fill the holes. The mycelial growth of the fungus in Petri dishes was checked after 3-5 days.

3.11. Chemical induction of resistance by 2,6-dichloroisonicotinic acid (INA)

Susceptible barley seedlings (*Hordeum vulgare* cultivar Ingrid) were treated with 2,6dichloroisonicotinic acid (INA) applied as a soil-drench (6 mg/litre soil) 3-4 days after sowing. Inoculation with the powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) was carried out 4 days after INA treatment (7-8 days after sowing). Leaf samples were harvested 12, 18, 24, 36, 48, 60, 72, 84 and 96 hours after inoculation with the powdery mildew fungus. Level of hydrogen peroxide (H_2O_2) was determined using the 3,3-diaminobenzidine technique (Hückelhoven et al., 1999). Activities of antioxidant enzymes, such as superoxide dismutase (SOD), dehydroascorbate reductase (DHAR) were also determined by biochemical and gene expression studies spectrophotometrically and applying the RT-PCR techniques, respectively.

3.12. Histochemical analysis of ROS

3.12.1. Detection of superoxide

Histochemical staining for superoxide production in leaf tissue was based on the ability of O_2^- to reduce nitro blue tetrazolium (NBT). Superoxide was visualised as a purple discoloration of NBT. Leaf discs (2 cm in diameter) were vacuum infiltrated or injected (Hagborg, 1970) with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 w/v % NBT (Sigma-Aldrich, Steinheim, Germany) according to the procedure of Ádám et al. (1989). NBT-treated samples were incubated under daylight for 20 min and subsequently cleared in 0.15 % trichloroacetic acid (w/v) in ethanol: chloroform 4:1 (v/v). The solution was changed once during the next 48 h of incubation (Hückelhoven et al., 1999). Subsequently, leaves were stored in 50% glycerol prior to microscopic evaluation. Discoloration of leaf discs was quantified using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA).

3.12.2. Detection of hydrogen peroxide

3.12.2.1. Application of DAB staining

 H_2O_2 was visualised as a reddish-brown coloration of 3,3-diaminobenzidine (DAB). Detection of H_2O_2 was performed using 0.1% DAB-uptake method as described by (Thordal-Christensen et al., 1997, Hückelhoven et al., 1999). Leaf discs (2 cm in diameter) were vacuum infiltrated with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 w/v % DAB (Fluka, Buchs, Switzerland). DAB-treated samples were incubated under daylight for 2 hours, and subsequently, cleared in 0.15 w/v % trichloroacetic acid in ethanol:chloroform 4:1 v/v for 1 day. Cleared samples were washed with water and placed in 50% glycerol prior to evaluation. Discoloration of leaf discs was quantified using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA).

3.12.2.2. Using xylenol orange dye

To detect H_2O_2 spectrophotometrically with a peroxidase independent reaction, a xylenol orange based method was used according to the method of Gay et al., (1999). Leaf

tissue (0.25 g) was ground in a mortar in boric acid/borax buffer (pH 8.4) mixed up 0.61 g boric acid in 150 ml water and 0.95 g borax in 50 ml water. The homogenate was centrifuged (12000 rpm, 15 min, 4°C). 200 μ l supernatant was combined with 1 ml xylenol orange solution. The xylenol orange solution was prepared just before usage mixing 100 μ l solution A (25 mM FeSO₄, 25 mM (NH₄)₂SO₄, 25 mM H₂SO₄) and 10 ml solution B (125 mM xylenol orange from Sigma and 100 mM sorbitol). After 30-min incubation at 25°C the absorption in A₅₆₀ was evaluated with a spectrophotometer. For quantification of the measured xylenol orange values, a standard curve was made applying dilution series of H₂O₂.

3.12.2.3. Using 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye

2',7'-dichlorofluorescein diacetate (DCFH-DA) reacts with H_2O_2 in the presence of peroxidase yielding the fluorescent dichlorofluorescein (DCF). We used the method described by Lu and Higgins (1998) with little modification. To measure fluorescence by spectrofluorometer the DCFH-DA stock solution was diluted to 0.04 mM in phosphate buffer and injected into leaf tissues (Hagborg, 1970). After 8 minutes 0.1 g leaf tissues were taken and frozen in liquid nitrogen. The samples were ground in liquid nitrogen and the powder was dissolved in 400 μ l 2 mM NaCN. The samples were centrifuged at 15000 g for 15 min at 4 °C and diluted thousandfold in distilled water. Fluorescence was measured by a spectrofluorometer (FluoroMax-3, Yobine Yvon, France) with excitation at 488 nm and emission at 525 nm. Samples without DCFH-DA injection were used as absolute control.

The mode of action of this dye is as follows: injected DCFH-DA diffuses through the cell membrane and it is hydrolysed by intracellular esterases into DCFH, which is believed to remain trapped within the cell. DCFH, a non-fluorescent compound, is able to react with reactive oxygen species (ROS), particularly with H_2O_2 in the presence of peroxidases, generating the fluorescence compound 2',7'-dichlorofluorescein (DCF).

3.13. Biochemical assays of antioxidant enzymes

For enzyme assays in tobacco and barley plants, 0.5 g leaf material was homogenized at 0-4°C in 3 ml of 50 mM TRIS buffer (pH 7.8), containing 1 mM EDTA-Na₂ and 7.5% polyvinylpyrrolidone. The homogenates were centrifuged (12,000 rpm, 20 min, 4°C), and the total soluble enzyme activities were measured spectrophotometrically in the supernatant. All measurements were carried out at 25°C, using the model UV-160A spectrophotometer (Shimadzu, Japan).

3.13.1. Activity of ascorbate peroxidase (APX)

Activity of APX (E.C. 1.11.1.11) was determined spectrophotometrically according to Nakano and Asada (1981) and Asada (1992). The reaction mixture contained in a final volume of 2.25 ml: 2 ml of 0.2 M TRIS/HCl buffer (pH 7.8), 100 μ l of 0.25 mM ascorbic acid solution, 100 μ l of 0.5 mM hydrogen peroxide solution and 50 μ l leaf extract supernatant (0.5 g plant material/3 ml homogenizing buffer containing 2 mM ascorbic acid). The control reaction was carried out without H₂O₂. The components (without H₂O₂) were mixed and the absorption change was registered in quartz cuvette for 3 min at 290 nm. Immediately after this period 100 μ l of H₂O₂ was given into the cuvette, the solution was homogenized, and the absorbance change was recorded again for 3 min. The result was determined from the decrease in absorbance at 290 nm as ascorbate was oxidized with the extinction coefficient 2.8 mM⁻¹ cm⁻¹ (Klapheck et al., 1990).

3.13.2. Activity of catalase (CAT)

Activity of CAT (E.C. 1.11.1.6) was determined spectrophotometrically according to Aebi (1984). Decomposition of H_2O_2 by catalase results in the decrease of the ultraviolet absorption of hydrogen peroxide at 240 nm. Enzyme activity can be calculated from this decrease. The reaction mixture contained, in a final volume of 2.15 ml, 2 ml 0.1 M Naphosphate buffer (pH 6.5), 100 µl hydrogen peroxide and 50 µl leaf extract supernatant. The solution is mixed, then the absorption change is registered for 3 min at 240 nm using a quartz cuvette.

3.13.3. Activity of dehydroascorbate reductase (DHAR)

Activity of DHAR (E.C. 1.8.5.1) was determined spectrophotometrically according to Asada (1984). The reaction mixture contained, in a final volume of 2.3 ml, 50 mM sodium phosphate buffer (pH 6.5), 0.5 mM dehydroascorbate (DHA), 1.0 mM reduced glutathione (GSH), 0.1 mM EDTA and 0.1 ml supernatant. The assay was carried out in quartz cuvettes following the increase in absorbance at 265 nm due to the formation of ascorbate with extinction coefficient 14 mM⁻¹ cm⁻¹ (Klapheck et al., 1990). The reaction rate was corrected for the non-enzymatic reduction of dehydroascorbate by GSH.

3.13.4. Activity of glutathione reductase (GR)

Activity of GR (E.C. 1.6.4.2) was determined spectrophotometrically according to the method of Halliwell and Foyer (1978) which was modified by (Klapheck et al., 1990). The reaction mixture contained, in a final volume of 2.5 ml: 0.1 mM EDTA, 1 mM oxidized glutathione (GSSG), 0.1 mM NADPH, 0.1 M Tris-HCl buffer (pH 7.8), and 0.1 ml supernatant. The assay was determined in plastic cuvettes following the decrease in absorbance at 340 nm due to the oxidation of NADPH with extinction coefficient 6.2 mM⁻¹ cm⁻¹ (Smith et al., 1989, Klapheck et al., 1990). The reaction rate was corrected for any NADPH oxidation carried out without GSSG for each sample.

3.13.5. Activity of glutathione S-transferase (GST)

Activity of GST (E.C. 2.5.1.18) was determined spectrophotometrically at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate with the extinction coefficient 9.6 mM^{-1} cm⁻¹ according to Habig et al. (1974) with certain modifications (Mauch and Dudler, 1993, Marrs, 1996).

The reaction mixture contained, in a final volume of 2.75 ml, 2 ml of 0.1 M Naphosphate buffer (pH 6.5) containing 0.1 mM EDTA, 150 μ l of 1 mM CDNB, 500 μ l of 3.6 mM GSH and 0.1 ml leaf extract supernatant (0.5 g plant material/3 ml homogenizing buffer). The control reaction occurred without leaf extract supernatant. The components were mixed and the absorption change was registered in plastic cuvettes for 3 min at 340 nm. Immediately after this period, 0.1 ml of leaf extract supernatant was given into the cuvette, the solution was homogenized, and the absorbance change was recorded again for 3 min. Substrate (CDNB) was prepared in ethanol and the final ethanol concentration was always between 3-4%.

3.13.6. Activity of guaiacol peroxidase (POX)

Activity of POX (E.C. 1.11.1.7) was determined spectrophotometrically at 480 nm based on the formation of tetraguaiacol. The reaction mixture contained, in a final volume of 2.21 ml, 2 ml of 0.1 M Na-phosphate buffer (pH 6.5) containing 1 mM EDTA-Na, 100 μ l of 1 mM guaiacol solution, 100 μ l of 1 mM H₂O₂ and 10 μ l leaf extract supernatant (0.5 g plant material/3 ml homogenizing buffer). The control reaction was carried out without H₂O₂. The components (without H₂O₂) were mixed and the absorption change was registered in plastic cuvettes for 3 min at 480 nm. Immediately after this period 100 μ l of H₂O₂ solution was given

into the cuvettes, the solution was mixed, and the absorbance change was recorded again for 3 min.

3.13.7. Activity of NADPH oxidase

For assay of enzyme cell-free homogenate were prepared as described above. Leaf tissue was homogenized with different extraction buffer: Na-phosphate (50 mM) buffer pH 7.0, Na₂S₂O₅ 0.1% PVPP (insoluble, added directly to sample just before grinding, 0.1 g/g). The homogenate was centrifuged (14,000 g, 15 min., 4°C), and the total soluble NADPH oxidase activity was determined in the supernatant spectrophotometrically as before at 530 nm. Assay buffer: NADPH 0.15 mM, NBT 0.3 mM, HEPES (pH 7.5) 50 mM and SOD from horseradish (optional, 100 U/ml. The reduction of O₂⁻⁻ by NBT is an indicator of the activity of NADPH oxidase. The negative test which include SOD to determine the inhibition of O₂⁻⁻ reduction is the real indication of NADPH oxidase activity as described by Ott et. al., (2000).

3.13.8. Activity of superoxide dismutase (SOD)

The activity of SOD (E.C.1.15.1.1) enzyme was determined spectrophotometrically according to the method of Paoletti and Mocali (1990).

Reagents and solutions: Triethanolamine-diethanolamine (TDB) buffers (100 mM each). The pH (7.4) was adjusted by cc. HCl; NAD(P)H (7.5 mM); EDTA-MnCl₂ (100 mM EDTA and 50 mM MnCl₂); Mercaptoethanol (10 mM).

Conditions of the assay: Each set of assay must include its own control. The control consists of 0.8-ml TDB buffer, 4 μ l NADPH, 25 μ l EDTA-MnCl₂ and 0.1 ml of sample. The absorbance was recorded by a spectrophotometer (Shimadzu UV-160A, Japan) at 340 nm for 5 min and then we added 0.1-ml mercaptoethanol solution into the cuvettes. We monitored the decrease of absorbance for another 20 min. The calculation was carried out using a calibration curve prepared by SOD purchased from Sigma Co. (Germany).

3.14. Gene expression of antioxidants

3.14.1. Total RNA isolation from tobacco and barley plants

Young upper leaves of TMV-inoculated and mock-inoculated tobacco plants kept at 20 and 30°C in growth chambers were used 0, 6, 12 and 24 hours after inoculation. Untreated

tobacco plants kept in growth chambers at 20 and 30°C were used 0 and 6 hours after exposure to the indicated temperatures.

Young leaves of barley plants were used 0, 12, 24, 48, 72 and 96 hours after treatment with dichloroisonicotinic acid (INA), untreated plants served as controls.

For each individual sample, at least 200 mg of fresh leaves were homogenized in a pre-cooled mortar (-20°C) in liquid nitrogen until a fine powder was obtained. 100 mg of homogenized tissue was transferred into a 2 ml eppendorf tube (100 mg = about 0,5 ml leaf tissue), the rest of the tissue was put in another 2 ml Eppendorf tube to be used as stock.

For plant total RNA isolation, 100 mg homogenized plant material was used by applying a total RNA isolation minicolumn kit (Viogene). Briefly, samples were mixed with 450 μ l of homogenization buffer and applied to a shearing minicolumn. The flow-through fraction was collected, mixed with about half volume of 96-100% ethanol and applied to an RNA minicolumn. Subsequently, the minicolumns were washed three times with washing buffer in order to remove contaminating materials. The RNA fraction remaining on the minicolumns was then eluted with 40-50 μ l of sterile distilled water and stored at -70°C.

3.14.2. Determination of RNA concentration spectrophotometrically

The absorption maximum of RNA is at a wavelength of 260 nm, while that of proteins at 280 nm in the UV range. Assays of RNA concentration were done at 260 and 280 nm (the latter for indication of protein contamination). The ratio of absorbance values measured at 260 and 280 nm (A_{260}/A_{280}) indicates purity of the RNA sample, values between 1.6 and 2.0 are acceptable. RNA samples were diluted 100× in sterile distilled water to 500 µl total volume. Assays were carried out in a 1ml quartz cuvette, sterile distilled water was used as a reference.

3.14.3. Formaldehyde-agarose gel electrophoresis of plant RNA

RNA integrity and concentration was checked by formaldehyde-agarose gel electrophoresis (Rapley and Manning, 1998). The integrity and concentration of ribosomal RNA (rRNA) bands visible in the gel is an indication of the quality and amount of mRNA present in the samples. Electrophoresis running buffer was prepared from a 10× stock (0.2 M 3-(N-Morpholino)propane sulfonic acid, MOPS, pH 7.0 adjusted with HCl; 0.1 M sodium acetate; 10 mM EDTA, pH 8.0). A formaldehyde-agarose gel was used for electrophoresis (1 % w/v agarose and 5% v/v formaldehyde in gel). Samples were prepared by denaturing 1-5

 μ g of plant RNA at 95°C for 5 min then placing the RNA samples on ice and adding an equal volume of 2× RNA loading buffer (Fermentas). Electrophoresis was done at 50-60 V until rRNA bands have moved ca. two thirds of the way along the gel (4-6 V/cm length of gel). rRNA bands were visualized under a UV transilluminator at 254 nm.

3.14.4. Gene expression analysis on the mRNA level

For gene expression analysis on the mRNA level, a two step reverse transcriptionpolymerase chain reaction (RT-PCR) procedure was applied (Fermentas) following the manufacturers instructions. Briefly, in the reverse transcription step, 1.5-3 μ g of total RNA was used for synthesis of first strand cDNA with the aid of an oligo (dT)₁₈ primer (0.5 μ g/ μ l). The cDNA strands developed are copies of mRNA-s representing all genes expressed in the given plant tissue at the time of sample preparation. Subsequently, in the polymerase chain reaction step, specific primer pairs were used to assay expression of genes of interest. PCR reactions were done with relatively low cycle numbers (22-28 cycles) in order to maintain initial differences in target transcript amounts as much as possible. Expression of a tobacco actin and a barley ubiquitin gene served as a constitutive control.

Primers used in the **RT-PCR** the following: 5'assays were CGGAATCCACGAGACTACATAC-3' (5' 5'primer [forward]) and GGGAAGCCAAGATAGAGC-3' (3' primer [reverse]) for a 230-bp tobacco actin (NtAct) cDNA fragment (Genbank accession X69885); 5'-GAAACAGTGGCTGCAGTGCC-3' (5'primer) and 5'-GTGATACCCAATTGGTGC-3' (3'primer) for a 520-bp tobacco alternative oxidase (NtAOX) cDNA fragment (Genbank accessions S71335 and X79768); 5'-AACCACAGGGCTACAAAT-3' (5'primer) and 5'-GAGCAGAACGAGCATCAC-3' (3'primer) for a 681-bp tobacco NADPH oxidase (NtRBOH) cDNA fragment (Genbank accessions AJ309006 and AF506374); 5'-GCCGTCCTTAGCAGCAGT-3' (5'primer) and 5'-ACAAGCAACCCTTCCACC-3' (3'primer) for a 420-bp tobacco cytosolic Cu/Zn superoxide dismutase 5'-(NpSOD1) cDNA fragment (Genbank accession X55974); TCCGCTTGATGTGACTAAA-3' (5'primer) and 5'-TCCACCCACCGACGAATA -3' (3'primer) for a 501-bp tobacco catalase (NgCAT1) cDNA fragment (Genbank accession 5'-GCGACACCTCTGTATGAA-3' 5'-AF006067); (5'primer) and ATGAAAGAAGACCTCCAA-3' (3'primer) for a 223-bp tobacco BAX inhibitor (NtBI1) cDNA fragment (Genbank accession AF390556); 5'-CAAGGCTCACGGACCATA-3' (5'primer) and 5'-ACTTCCTGCGAAACAACG-3' (3'primer) for a 277-bp tobacco

cytoplasmic dehydroascorbate reductase (*NtDHAR*) cDNA fragment (Genbank accession AY074787); 5'-ACCCTCGCCGACTACAACAT-3' (5'primer) and 5'-CAGTAGTGGCGGTCGAAGTG-3' (3'primer) for a 270-bp barley ubiquitin (*HvUbi2*) cDNA fragment (Genbank accession M60175), and 5'-GACCGAGGTCTGCGTCAAG-3' (5'primer) and 5'-TCAGCAATCCATTTGCCATC-3' (3'primer) for a 240-bp barley cytoplasmic dehydroascorbate reductase (*HvDHAR*) cDNA fragment (Genbank accession).

3.15. Statistical analysis

At least three or four independent parallel experiments were carried out in each case. All experiments were conducted in a completely randomized design with 3-5 replicates for each treatment by using the standard method of completely randomized design, illustrated by Cochran and Cox (1957). The significance of the differences between mean values was evaluated by Student's *t*-test according to Clark (1980). The estimation of the standard error for the percentage of values was carried out as illustrated by Strickberger (1968).

3.16. Origin of chemicals and enzymes

All reagents used in the experiments described above were standard laboratory grade. CAT, SOD, NBT, INA were purchased from Sigma-Aldrich Chemical Co., St.Louis, Missouri, USA. DAB dye was obtained from Fluka Co., Buchs, Switzerland. Glucose oxidase, DHA, GSH, GSSG and DCFH-DA were purchased from Sigma-Aldrich Chemical Co., Steinhein, Germany. Riboflavin, L-methionine and H₂O₂ were purchased from Reanal Co., Budapest, Hungary. ELISA was performed by using a TMV kit of Bioreba AG, Germany. For plant total RNA isolation a minicolumn kit (Viogene Biotek Corp., Taiwan) was used. For gene expression analysis, on the mRNA level, a two-step reverse transcriptionpolymerase chain reaction (RT-PCR) procedure was applied by using a kit provided by Fermentas Life Sciences (Lithuania).

4. RESULTS

4.1. Role of hydrogen peroxide in symptom expression of barley susceptible and resistant to powdery mildew

4.1.1. Compatible (susceptible) host/ pathogen combination

Infection of barley cultivar Ingrid with race A6 of powdery mildew resulted in a susceptible reaction of the host, e.g. production of fungal mycelia and conidia. We have shown that by treating barley leaves with chemicals which produce ROS, symptom expression of susceptible cultivars profoundly changed and the host exhibited HR-type necrotic spots, similar to the resistant reaction (Király and El-Zahaby, 2000; El-Zahaby et al., 2004).

In this study I applied H_2O_2 as a spray, 1, 2 and 3 days after inoculation on intact as well as on detached leaves. 50 mM solution of H_2O_2 was effective in the case of intact attached leaves and 25 mM H_2O_2 was inhibitory in the case of detached leaves. Leaf detachment was carried out immediately after inoculation with the pathogen. When I sprayed barley leaves with a H_2O_2 solution one day after inoculation, no symptoms of susceptibility or resistance were expressed at all, because the pathogen was supposedly killed or inhibited before establishment of infection. However, if I treated intact leaves with 50 mM H_2O_2 or detached leaves with 25 mM H_2O_2 2 or 3 days after inoculation, HR-type tissue necrotization characteristic of resistant cultivar/avirulent pathogenic race combinations was induced (Fig. 1). Spraying leaves only with H_2O_2 did not cause any symptoms.

The HR-type necrotic symptoms caused by leaf treatments with H_2O_2 were fully reversed (inhibited) when I injected a mixture of SOD and CAT into infected leaves before spraying leaves with H_2O_2 (Fig. 1) (as regards the concentrations see also Materials and Methods). This experiment demonstrated that the resistant reaction with HR-type symptoms was indeed induced by the combined action of infection and a certain amount of H_2O_2 .



Fig. 1. Induction of HR in a susceptible cultivar Ingrid. **(A):** Leaves infected with powdery mildew. **(B):** Leaves treated only with 25 mM H_2O_2 . **(C):** Induced HR with the stimulated necrotization in infected and H_2O_2 -treated leaves. Treatment was applied after establishment of infection (2 days after inoculation). **(D):** The stimulated HR was reversed in infected and H_2O_2 -treated leaves which were injected with SOD + CAT.

4.1.2. Resistant cultivar carrying the gene *Mla12* (incompatible combination)

This type of host/pathogen combination expresses HR-type resistance to some races of the fungus (e.g. race A6). When I sprayed intact leaves of cultivar Ingrid *Mla12* with H_2O_2 as early as one day after inoculation, the concentration of 50 mM H_2O_2 inhibited the development of hypersensitive necrosis (HR) and the resistant plant remained symptomless because the powdery mildew pathogen was supposedly killed before establishment of infection. Similar suppression of HR-type symptoms was experienced with detached leaves when I applied 25 mM H_2O_2 (Fig. 2C). Leaf detachment was carried out immediately after inoculation (Fig. 2).

On the other hand, when I sprayed intact barley leaves with 50 mM H_2O_2 2-3 days after inoculation, tissue necrotization was stimulated: the number of leaf necroses increased, as compared to the control infected leaves (Fig. 3). Treatment of detached leaves with 25 mM H_2O_2 2 days after inoculation also stimulated the development of HR. The number of necroses also increased (Fig. 3C).



Fig. 2. Inhibition of HR in a resistant combination of barley cultivar Ingrid (*Mla12*) infected with race A6 of powdery mildew. (A): Infected leaves exhibiting HR. (B): Uninfected leaves treated with 25 mM H_2O_2 . (C): Symptomless infected leaves treated with H_2O_2 one day after inoculation. (D): Infected and H_2O_2 -treated leaves injected with SOD and CAT. The original necrotic symptoms of HR were restored.



Fig. 3. Stimulation of HR in the resistant cultivar Ingrid (*Mla12*) infected with race A6 of powdery mildew. (A): Infected leaves exhibiting HR. (B): Uninfected leaves treated with 25 mM H₂O₂. (C): Stimulated necrotization in infected and H₂O₂-treated leaves. Treatment was applied 2 days after inoculation (after establishment of infection). (D): Stimulation of HR was reversed in infected and H₂O₂-treated leaves which were injected with SOD + CAT.

Stimulation of HR caused by H_2O_2 -treatments was reversed by injection of a mixture of SOD and CAT into infected leaves before the H_2O_2 -treatments (Fig. 3D). Injection of antioxidants into leaves which were treated with H_2O_2 one day after inoculation resulted in reversal of the action of H_2O_2 , namely infected leaves produced regular HR, similar to the control infected leaves (Fig. 2D) or typical symptoms of susceptibility (Fig. 1D). It was concluded that the antioxidant capacity of these compounds protected leaves from the effects of H_2O_2 on symptom expression.

It seems reasonable to suppose that the action of H_2O_2 is required for the stimulated HR-producing ability of infection when I applied H_2O_2 -treatment 2-3 days after inoculation, however when H_2O_2 -treatment was applied early (one day) after inoculation it inhibited the pathogen and the production of HR.

4.1.3. Resistant cultivar carrying the gene *mlo5* (race non-specific resistance)

It is known (Jørgensen, 1988) that barley cultivars carrying the gene *mlo5* exert resistance to most of the pathogenic races of *Blumeria graminis* f. sp. *hordei*. In other words, these plants express a general, race non-specific resistance, which is not associated with the HR.

If I sprayed intact barley leaves with 50 mM H_2O_2 one day after inoculation, no symptoms were detected in leaves. However, treatment with H_2O_2 2 or 3 days after inoculation induced HR-type necroses in infected leaves (Fig. 4C). Necroses were induced with 50 or 25 mM H_2O_2 in case of attached and detached leaves, respectively. Detachment of leaves was carried out one day after inoculation. Treatments only with H_2O_2 did not induce any symptoms in barley leaves (Fig. 4).

It was also demonstrated in these experiments that injection of leaves with antioxidants (SOD plus CAT) reversed the HR-inducing ability of H_2O_2 -treatments in both attached and detached inoculated leaves (Fig. 4D), indicating that a certain amount of H_2O_2 is required for the production of HR-type necroses. This experiment supports the hypothesis that the capacity to develop HR is suppressed in susceptible as well as in the *mlo5* resistant cultivars (Büschges et al., 1997; Shirasu and Schulze-Lefert, 2000; Hückelhoven et al., 2003).



Fig. 4. Induction of HR in barley cultivar Ingrid *mlo*) with race A6 of powdery mildew. (A): Infected leaves with powdery mildew. (B): Uninfected leaves treated with 25 mM H_2O_2 . (C): Infected and H_2O_2 -treated leaves with symptoms of HR. Treatment was applied after establishment of infection (2 days after inoculation). (D): Infected and H_2O_2 -treated leaves injected with a mixture of SOD and CAT. Symptoms of HR were reversed to the original non-HR-type resistance.

4.1.4. Resistant cultivar carrying the gene Mlg

This type of host/pathogen combination expresses invisible HR-type resistance to some races of the fungus *Blumeria graminis* f. sp. *hordei*. (e.g. race A6). When I sprayed attached barley leaves with 50 mM H_2O_2 2 days after inoculation, tissue necrotization was stimulated and visible HR-type leaf necroses appeared. Treatment of detached leaves with 25 mM H_2O_2 2 days after inoculation also stimulated the development of HR (Fig. 5C).

Stimulation of HR caused by H_2O_2 -treatments was also reversed in this case by injection of a mixture of SOD and CAT into infected leaves before the H_2O_2 -treatments (Fig. 5D). It was concluded that the antioxidant capacity of these compounds protected leaves from the effects of H_2O_2 on symptom expression. It would seem that the action of H_2O_2 is required for the development of visible HR-producing ability of infection when I applied H_2O_2 -treatment 2-3 days after inoculation.


Fig. 5. Stimulation of HR in the resistant cultivar Ingrid (*Mlg*) infected with race A6 of powdery mildew. (A): Infected leaves (B): Uninfected leaves treated with 25mM H_2O_2 . (C): Stimulated necrotization in infected and H_2O_2 -treated leaves. Treatment was applied 2 days after inoculation (after establishment of infection). (D): Stimulation of HR was reversed in infected and H_2O_2 -treated leaves which were injected with SOD + CAT.

- 4.2. Immunization of tobacco with low concentration of hydrogen peroxide against oxidative stress caused by viral, bacterial and fungal infections
- 4.2.1. Effect of pre-treatment of leaves with H₂O₂ on necrotic symptoms caused by viral, bacterial and fungal infections

Xanthi-nc tobacco leaves located in the middle of a 12-leaf-plant were pre-treated with H_2O_2 one day before inoculation of leaves with a virus (TMV), a bacterium (*P. syringae* pv. *phaseolicola*) and a fungal pathogen (*Botrytis cinerea*). Pre-treatment consisted of a single spray applied to both sides of leaves with a solution of H_2O_2 . Concentration of the solution of H_2O_2 was 7, 5, 5 and 5 mM in the case of viral, bacterial and fungal infections, respectively. All of these infections caused necrotic symptoms on the plants: the viral infection induced a hypersensitive response (HR) on Xanthi-nc tobacco, the bacterial infection also caused HR-type necrosis on its non-host plant and the fungal infection induced non-HR type necroses on its susceptible host plant.

In all these three cases necrotic symptoms were suppressed significantly (Table 1 and Figs. 6, 7, 8).

	Treatment	Number of lesions (cm	²) Lesion size (mm)
Control (TMV only	7)	8.53 ± 1.85	1.57 ± 0.29
$7 \text{ mM H}_2\text{O}_2 + \text{TMV}_2$	V	5.80 ± 1.30 **	0.70 ± 0.11 *
$10 \text{ mM H}_2\text{O}_2 + \text{TM}_2$	IV	5.90 ±1.25 **	0.77 ± 0.20 *
12.5 mM H ₂ O ₂ + T	MV	6.07 ± 1.7 **	0.84 ± 0.15 *
SOD + CAT + TM	V	5.90 ± 1.05 **	0.79 ± 0.14 *

Table 1. Number and size of TMV lesions on tobacco Xanthi-nc that pre-treated with H₂O₂ and superoxide dismutase (SOD) plus catalase (CAT)

Number and size of TMV lesions were inspected visually on the third and fourth leaves of 8-weeks old tobacco Xanthi-nc. Treatment with low concentration of H_2O_2 was carried out one day before TMV infection. SOD and CAT were injected into tobacco leaves 30 min before TMV infection. TMV lesion size (mm) and lesion number were detected 3 days after infection. Values are means \pm SD based on three independent assays. I measured the lesions from three plants in each experiment. * Significant differences between control and immunized plants with H_2O_2 or SOD and CAT injected leaves (p < 0.05). ** Significant differences (p < 0.01).

If I applied a mixture of SOD and CAT (3000 and 5000 units/leaf, respectively) to the leaves by injecting tissues with a syringe immediately after inoculation, we determined very effective suppression of tissue necrotization in all cases (Figs. 6, 7, 9).



Fig. 6. Inhibition of leaf necroses with the application of low concentration of H_2O_2 in TMV-infected resistant Xanthi-nc tobacco leaves. **(A):** Leaf inoculated only with TMV. **(B):** Leaf sprayed with 5 mM H_2O_2 one day before inoculation with TMV. **(C):** Leaf inoculated with TMV and tissues injected immediately after inoculation with a mixture of superoxide dismutase and catalase (3000 and 5000 units/leaf, respectively) by a syringe.



Fig. 7. Inhibition of leaf necroses with the application of low concentration of H_2O_2 in resistant Xanthi-nc tobacco leaves infected with *Pseudomonas syringae* pv. *phaseolicola.* (A): Leaf inoculated only with *Pseudomonas syringae* pv. *phaseolicola.* (B): Leaf sprayed with 7 mM H_2O_2 one day before inoculation with the bacterium. (C): Leaf inoculated with the bacterium and immediately after inoculation tissues injected with a mixture of superoxide dismutase and catalase (3000 and 5000 units/leaf, respectively) by a syringe.



Fig. 8. Inhibition of leaf necroses with the application of low concentration of H_2O_2 in *Botrytis cinerea*-infected Xanthi-nc tobacco leaves. **Left:** Leaf inoculated only with *Botrytis cinerea*. **Right:** Leaf sprayed with 5 mM H_2O_2 one day before inoculation with *Botrytis cinerea*.



Fig. 9. Inhibition of leaf necroses with the application of low concentration of H_2O_2 in *Botrytis cinerea*-infected Xanthi-nc tobacco leaves. **Left:** Leaf inoculated only with *Botrytis cinerea*. **Right:** Leaf inoculated with *Botrytis cinerea* and tissues injected immediately after inoculation with a mixture of superoxide dismutase and catalase (3000 and 5000 units/leaf, respectively) by a syringe.

4.2.2. Antioxidant enzymes

On the basis of the previous results of Gechev et al. (2002) obtained with abiotic stresses and our above-mentioned results with the action of SOD and CAT on suppression of necrotization, we investigated changes of several antioxidant enzymes in infected leaves, which were pre-treated, with low concentration of H_2O_2 . Pre-treated leaves were inoculated with TMV and *P. syringae* pv. *phaseolicola* one day after pre-treatment. Activities of antioxidant enzymes were determined 2, 3 and 4 days after inoculations. Catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POX), glutathione S-transferase (GST), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) were investigated. As is seen in Figs. 10 and 11 the activities of CAT, APX and POX increased significantly in immunized plants.



Fig. 10. Activities of CAT, POX and APX in Xanthi-nc tobacco leaves (8-10 weeks old) 2, 3 and 4 days after TMV-inoculated (DAI) and pre-treated with low concentration of H_2O_2 . Healthy: Leaves treated with water (mock-inoculated). TMV: Leaves infected only with TMV. 7 mM H_2O_2 : Leaves pre-treated only with 7 mM H_2O_2 . H₂O₂+TMV: Leaves pre-treated with 7 mM H_2O_2 and inoculated with TMV.



Fig. 11. Activities of CAT, POX and APX in Xanthi-nc tobacco leaves (8-10 weeks old) 2, 3 and 4 days after inoculated (DAI) with *Pseudomonas syringae* pv. *phaseolicola* and pre-treated with low concentration of H_2O_2 . **Healthy:** Leaves treated with water (mock-inoculated). **P.phas.:** Leaves inoculated only with *Pseudomonas syringae* pv. *phaseolicola*. **5 mM H₂O₂:** Leaves pre-treated only with 5 mM H₂O₂. **H**₂**O**₂+**P.phas.:** Leaves pre-treated with 5 mM H₂O₂ and inoculated with the bacterium.

4.2.3. Multiplication of viral and bacterial pathogens in immunized leaf tissues

It is known since a long time that pathogens are arrested in plants exhibiting the hypersensitive response (HR). This response was suppressed in our experiments in the immunized (pre-treated with low concentration of H_2O_2) leaves, thus we wanted to see whether or not pathogens will grow and multiply more intensively in the immunized than in the non-immunized tissues. It turned out that the concentration of TMV remained unchanged (indeed, insignificantly increased) in the pre-treated (immunized) leaves (Fig. 12). The same is true for the multiplication of *P. syringae* pv. *phaseolicola* in leaves pre-treated with low concentration of H_2O_2 (Fig. 13). These results refer to the action of pre-treatment with H_2O_2 to tissue necrotization but not to the multiplication of pathogens. In other words, immunization results in resistance to symptoms but not necessarily to the pathogens.

This hypothesis was supported by another experiment in which necrotic symptoms were suppressed by a combined application of two antioxidants, such as SOD and CAT. Multiplication of pathogens did not change in leaves in which necrotic spots (HR response) were suppressed, as compared to control leaves in which the necrotic spots (HR response) were developed.



Fig. 12. Concentration of TMV in tobacco leaf extracts (10, 20 and 50 times dilutions), as determined with the ELISA test at different time points. TMV only: Leaf inoculated only with TMV. TMV+Hydrogen peroxide: Leaf treated with 7 mM H_2O_2 and inoculated with TMV one day after the treatment. TMV+SOD+CAT: Leaf inoculated with TMV and immediately infiltrated with a mixture of superoxide dismutase and catalase (3000 and 5000 units/leaf, respectively) by a syringe.



Fig. 13. Concentration of *Pseudomonas syringae* pv. *phaseolicola* in Xanthi-nc tobacco leaves. P.phas.only: Leaf inoculated only with *Pseudomonas syringae* pv. *phaseolicola*. P.phas.+ Hydrogen peroxide: Leaf sprayed with 5 mM H_2O_2 one day before inoculation with the bacterium. P.phas +SOD+CAT: Leaves inoculated with the bacterium, and immediately after inoculation, tissues injected with a mixture of superoxide dismutase and catalase (3000 and 5000 units/leaf, respectively) by a syringe.

Interestingly, 5 mM H_2O_2 also did not influence the growth of *Botrytis cinerea* in an artificial medium (Fig. 14). However, pre-treatment of leaves with this low concentration of H_2O_2 suppressed the non HR-type necrotic spots caused by infection of this fungus.



Fig.14. The action of H_2O_2 on the mycelial growth of *Botrytis cinerea*. Left: Petri dish which contained potato dextrose agar (PDA) medium was inoculated by putting a small agar disc containing the fungal mycelium (7 day-old-culture) in the middle of the plate and distilled water were poured into the wells served as control. **Right:** Petri dish which contained PDA medium was similarly inoculated and 5 mM H_2O_2 was poured into the wells.

- 4.3. Role of reactive oxygen species (ROS) and antioxidants in TMV-induced necrotization associated with resistance of an N gene encoding tobacco
- 4.3.1. Action of reactive oxygen species on tissue necrotization caused by TMV at high temperature (30°C)

As is known, TMV infection induces necrosis in Xanthi-nc tobacco leaves under less than 28°C, but there is no necrotization above 28°C. However, if we applied ROS-producing chemical systems or applied H_2O_2 directly to the leaves, it was possible to induce HR-type necroses even at 30°C (Figs.15-17).

In one experiment we poured a riboflavin/methionine mixture (266 μ M riboflavin and 10 mM methionine) on filter paper in each Petri dish and put the detached infected tobacco leaves in the dishes for three days and illuminated (Fig.15). In another experiment we injected the intact leaves with the ROS-producing chemical mixture as was mentioned in the Materials and Methods. In this case the concentration of the riboflavin was 532 μ M and that of the methionine was 20 mM.



Fig. 15. Induction of leaf necroses at 30°C with the application of the ROS-producing riboflavin/methionine mixture (266 μ M riboflavin and 10 mM methionine) and illuminated the tobacco Xanthi-nc leaves. (A): Leaf infected only with TMV. (B): Leaf infected with TMV and treated with the ROS-producing mixture. (C): Leaf treated with the ROS-producing riboflavin/methionine mixture only.

In a separate experiment we applied a glucose-glucose oxidase system. As is seen in Fig. 16, only the solution containing 200 units/ml glucose oxidase and 2 mM glucose was able to induce necroses in Petri dishes even at 30°C. Necroses also appeared if we injected the intact tobacco leaves with a solution which contained 250 units/ml of the enzyme and 2 mM glucose.



Fig. 16. Induction of leaf necroses at 30°C with the application of the ROS-producing glucose-glucose oxidase system. **Upper row left:** Leaf inoculated only with TMV. **Upper row right:** Leaf inoculated with TMV and treated with the glucose-glucose oxidase system. **Lower row left:** Leaf treated only with water. **Lower row right:** Leaf treated only with the glucose-glucose oxidase system.

If we put 4 ml of 25 mM H_2O_2 on filter paper in each Petri dish or infiltrated the leaves with a solution containing 50 mM H_2O_2 , tissue necroses were produced in the infected leaves even at 30°C (Fig. 17).



Fig. 17. Induction of leaf necroses at 30°C with the direct application of hydrogen peroxide (H₂O₂). **(A):** Leaf infected only with TMV **(B):** Leaf infected with TMV and treated with H₂O₂ **(C):** Leaf treated only with H₂O₂.

It is important to point out that in the controls (TMV inoculated leaves or uninfected leaves in which only the ROS-producing chemical systems or H_2O_2 were applied) tissue necroses did not appear at 30°C.

4.3.2. Action of antioxidants against reactive oxygen species at high temperature (30°C)

To demonstrate the roles of O_2 ⁻ anion and H_2O_2 in the killing action of ROSproducing chemical systems, horseradish SOD was used (4000 U/Petri dish) and bovine catalase (5000 U/Petri dish) at the same time when we poured a riboflavin/methionine mixture (266 μ M riboflavin and 10 mM methionine) on filter paper in each Petri dish. Then we put the detached infected tobacco leaves in the dishes for three days and illuminated. The necrosisinducing ability of riboflavin/methionine mixture was reversed, and, accordingly, necroses did not appear at 30°C, in spite of the action of ROS. In the control the same treatment was applied without SOD (Fig. 18).



Fig. 18. Reversible action of leaf necroses at 30°C with the application of the antioxidant enzymes superoxide dismutase (SOD) and catalae (CAT) in Xanthi-nc tobacco leaves. **Left:** Leaf infected with TMV and treated with the ROS-producing mixture (riboflavin/methionine). **Right:** Leaf infected with TMV and treated with the ROS-producing mixture plus SOD (4000 U/Petri dish) and CAT (5000 U/Petri dish).

CAT (5000 U/Petri dish) was also used in two separate experiments together with the glucose-glucose oxidase system (200 units of the enzyme/ml plus 2 mM glucose) or the direct application with H_2O_2 , as described above in Materials and Methods. CAT reversed the necrosis-inducing action of the glucose-glucose oxidase system or H_2O_2 . In the case of controls the same treatments were applied without CAT (Figs 19-20).



Fig. 19. Reversible action of leaf necroses at 30°C with the application of the antioxidant enzyme catalase (CAT) in Xanthi-nc tobacco leaves. **Left:** Leaf infected with TMV and treated with the ROS-producing glucose-glucose oxidase system. **Right:** Leaf infected with TMV and treated with the ROS-producing system plus CAT (5000 U/Petri dish).



Fig. 20. Reversible action of leaf necroses at 30° C with the application of the antioxidant enzyme catalase (CAT) in Xanthi-nc tobacco leaves. **Left:** Leaf infected with TMV and treated with H₂O₂. **Right:** Leaf infected with TMV and treated with H₂O₂ plus CAT (5000 U/Petri dish).

4.3.3. Concentration of TMV in leaves in relation to tissue necrotization

It is known from previous results (Samuel, 1931; Holmes, 1938; Kassanis, 1952; da Graca, et al. 1976) that the virus does not spread in a local lesion host which produces HR-type necroses in leaves. Consequently, the virus content is also reduced, as compared to a systemic host. However, in our experiments it was shown that the concentration of TMV determined with the ELISA-test, did not depend on necrotization of tissues, however, it depended on temperature (Fig. 21).

In this case tissue necroses were produced at 30°C because the virus-inoculated leaves were treated with the ROS-producing chemical systems or with H_2O_2 . Thus, necrosis itself cannot cause limitation of virus multiplication. Concentration of TMV in necrotized leaves of Xanthi-nc tobacco held at 20°C was significantly lower than in necrotized leaves at 30°C where necroses were induced by H_2O_2 or the ROS-producing chemicals (Fig. 21). If I compared TMV concentration in infected leaves held at 20°C (with necrosis) with 30°C (without necrosis), there was a substantial increase in the concentration of TMV in plants held at 30°C (Fig. 21). It would seem that the high temperature, not the lack of HR (necrotization), permits increase in virus concentration at 30°C.



Fig. 21. Concentration of TMV in Xanthi-nc tobacco leaf extracts (50 times dilution), as determined with the ELISA test. TMV/30: Leaf inoculated with TMV and held at 30°C. TMV/20: Leaf inoculated with TMV and held at 20°C. TMV+G.OX./30: Leaf inoculated with TMV and treated with the glucose-glucose oxidase system at 30°C. TMV+RF./30: Leaf inoculated with TMV and treated with the riboflavin/methionine system at 30°C. TMV+H₂O₂/30: Leaf inoculated with TMV and treated with H₂O₂ at 30°C.

4.3.4. Levels of superoxide (O₂^{•-}) and hydrogen peroxide (H₂O₂) in leaves of Xanthi-nc tobacco at low and high temperatures

From the previous experiments one can conclude that the HR-type necroses caused by TMV is in association with the presence or accumulation of reactive oxygen species (ROS). I determined the level of superoxide by infiltrating leaves with nitroblue tetrazolium (NBT) at 20°C and 30°C. The amount of superoxide was substantially reduced at 30°C, as compared to 20°C. This occurred in healthy as well as in virus infected leaves (Fig. 22).

The level of hydrogen peroxide was also determined by infiltrating the leaves with 3,3-diaminobenzidine (DAB) at 20°C and 30°C. The amount of H_2O_2 at 30°C increased slightly but not significantly in both healthy and TMV-infected leaves, as compared to leaves held at 20°C (Fig. 22).



Fig. 22. Levels of superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2) in TMVinoculated and healthy Xanthi-nc leaves at low (20°C) and high temperatures (30°C) 24, 36, 48 and 60 hours after inoculation (hai). The fifth and sixth true leave were inoculated with TMV and immediately held at 20°C and 30°C. Mock inoculated leave held also at 20°C and 30°C. Healthy 20°C: Leaf treated with water and held at 20°C. Healthy 30°C: Leaf treated with water and held at 30°C. TMV 20°C: Leaf inoculated with TMV and held at 20°C. TMV 30°C: Leaf inoculated with TMV and held at 30°C. Means of three independent experiments are shown. At least three independent samples were analyzed in each experiment. Error bars present \pm SD.

4.3.5. Levels and activities of antioxidant enzymes

Activities of catalase (CAT), superoxide dismutase (SOD), dehydroascorbate reductase (DHAR) and NADPH oxidase were determined spectrophotometrically at 20°C and 30°C. The level of NADPH oxidase at 20°C and 30°C was determined by the reduction of O_2^- by NBT (an indicator of the activity of NADPH oxidase). The negative test, which includes SOD to determine the inhibition of O_2^- reduction, is the real indication of NADPH oxidase activity. We found that activity of NADPH oxidase was reduced significantly in healthy as well as in TMV-infected leaves at 30°C, as compared to that at 20°C (Fig. 23). SOD activity did not change significantly. Activity of CAT was slightly lower at 30°C in the healthy and infected leaves, as compared to that at 20°C (Fig. 23). However, DHAR activity was significantly higher at 30°C in the healthy as well as in infected leaves, as compared to that at 20°C (Fig. 23).



Fig. 23. Activities of CAT, DHAR and NADPH oxidase in TMV-inoculated and healthy leaves of Xanthi-nc tobacco at low (20°C) and high (30°C) temperatures 2, 2.5, 3, 3.5, 4 and 5 days after inoculation (DAI). The fifth and sixth true leaves were inoculated with TMV and immediately put under low and high temperatures, uninoculated plants were also tested under low and high temperatures. **Healthy 20°C:** Leaf treated with water and held at 20°C. **Healthy 30°C:** Leaf treated with water and held at 30°C. TMV 20°C: Leaf inoculated with TMV and held at 30°C. See legend to Fig. 22 for details.

4.3.6. Gene expression of the antioxidant enzymes on the mRNA level

We found that in the TMV-infected Xanthi-nc tobacco expression (transcript levels) of an alternative oxidase (*NtAOX*) and the NADPH oxidase (*NtRBOH*) gene was suppressed at high temperature (30°C), as compared to ambient temperatures (20°C) when assayed by RT-PCR (Figs. 24 and 25).

During assays for *NtAOX* two bands of similar size were visible but, according to Chivasa et al. (1997), the lower band (520 base pairs long) corresponds to *NtAOX*. In fact, the 520 bp band reflects expression of two tobacco alternative oxidase genes of the same size which are both induced during an HR in response to TMV infection (Chivasa et al., 1997; Chivasa and Carr, 1998).

In untreated plants kept in a greenhouse environment (G0, see Fig. 24) expression of *NtAOX* was not significantly different from that found in plants kept in growth chambers at 20°C for 6 hours. However, in uninfected plants kept at 30°C, *NtAOX* expression was highly suppressed, as compared to that in plants kept at 20°C for 6 hours. In mock inoculated plants, *NtAOX* expression was also suppressed at 30°C, 12 hours after treatment. Interestingly, suppression of *NtAOX* was quite strong in TMV-infected plants kept at 30°C, 6 and 12 hours after virus inoculation (Fig. 24).



Fig. 24. Changes in expression of an alternative oxidase gene (*NtAOX 1-2*) in Xanthinc tobacco at 30°C in comparison to 20°C in response to mock inoculation (mechanical stress) and inoculation with tobacco mosaic virus (TMV) at different time points after treatments. Untreated plants were either in a winter greenhouse environment (G0) or exposed to 20 and 30°C in growth chambers. RT 0 = negative control of reverse transcription (no RNA template present); PCR 0 = negative control of polymerase chain reaction (no cDNA template present). Gene expression assays were done by a two step reverse transcription-polymerase chain reaction (RT-PCR) procedure. A tobacco actin gene (*NtAct*) served as a reference of gene expression.

NtRBOH expression in untreated plants kept in the greenhouse (G0, see Fig. 25) was somewhat lower than that in plants kept in growth chambers at 20°C for 6 hours. However, *NtRBOH* expression was highly suppressed in untreated plants kept at 30°C for 6 hours, as compared to that in plants kept at 20°C. In mock inoculated plants, suppression of *NtRBOH* transcripts was also visible at 30°C, 12 and 24 hours after treatment. As with *NtAOX*, suppression of *NtRBOH* was quite strong in TMV-infected plants kept at 30°C, 6 and 12 hours after virus inoculation (Fig. 25).



Fig. 25. Changes in expression of an NADPH oxidase gene (*NtRBOH*) in Xanthi-nc tobacco at 30°C in comparison to 20°C in response to mock inoculation (mechanical stress) and inoculation with tobacco mosaic virus (TMV) at different time points after treatments. Untreated plants were either in a winter greenhouse environment (G0) or exposed to 20 and 30°C in growth chambers. RT 0 = negative control of reverse transcription (no RNA template present); PCR 0 = negative control of polymerase chain reaction (no cDNA template present). Gene expression assays were done by a two step reverse transcription-polymerase chain reaction (RT-PCR) procedure. A tobacco actin gene (*NtAct*) served as a reference of gene expression.

Expression of the gene catalase (*NtCAT1*) in untreated tobacco plants kept in the greenhouse (G0, see Fig. 26) was not different from that found in plants kept in growth chambers at 20°C for 6 hours. In mock inoculated plants *NtCAT1* did not change at 20°C and 30°C for 6, 12 and 24 hours after treatment. In TMV-infected plants, slight induction of *NtCAT1* was visible at 30°C, 24 hours after infection (Fig. 26).



Fig. 26. Changes in expression of a catalase gene (*NtCAT1*) in Xanthi-nc tobacco at 30°C in comparison to 20°C in response to mock inoculation (mechanical stress) and inoculation with tobacco mosaic virus (TMV) at different time points after treatments. Untreated plants were either in a winter greenhouse environment (G0) or exposed to 20 and 30°C in growth chambers. RT 0 = negative control of reverse transcription (no RNA template present); PCR 0 = negative control of polymerase chain reaction (no cDNA template present). Gene expression assays were done by a two step reverse transcription-polymerase chain reaction (RT-PCR) procedure. A tobacco actin gene (*NtAct*) served as a reference of gene expression.

Expression of the gene superoxide dismutase (*NtSOD*) in untreated tobacco plants kept in the greenhouse (G0, see Fig. 27) was strongly suppressed as compared to that found in plants kept in growth chambers at 20°C for 6 hours. However, *NtSOD* induced in untreated plant at 20°C, as compared to that at 30°C for 6 hours. In mock inoculated plants or TMV-inoculated plant *NtSOD* did not change at 20°C and 30°C for 6, 12 and 24 hours after treatment (Fig. 27).



Fig. 27. Changes in expression of a superoxide dismutase gene (*NtSOD1*) in Xanthinc tobacco at 30°C in comparison to 20°C in response to mock inoculation (mechanical stress) and inoculation with tobacco mosaic virus (TMV) at different time points after treatments. Untreated plants were either in a winter greenhouse environment (G0) or exposed to 20 and 30°C in growth chambers. RT 0 = negative control of reverse transcription (no RNA template present); PCR 0 = negative control of polymerase chain reaction (no cDNA template present). Gene expression assays were done by a two step reverse transcription-polymerase chain reaction (RT-PCR) procedure. A tobacco actin gene (*NtAct*) served as a reference of gene expression.

Expression of the gene DHAR (*NtDHAR*) in untreated tobacco plants kept in the greenhouse (G0, see Fig. 28) was induced strongly, as compared to that plants kept in growth chambers at 20°C for 6 hours. However, *NtDHAR* was suppressed in untreated plants kept at 30°C for 6 hours, as compared to plants kept at 20°C. In mock inoculated plants, *NtDHAR* was induced at 30°C, as compared to plants kept at 20°C for 12 hours after treatment. In TMV-inoculated plant *NtDHAR* expression did not change in the all time points. However, very slight induction of *NtDHAR* was visible at 30°C, as compared to that plants kept at 20°C for 24 hours after virus inoculation (Fig. 28).



Fig. 28. Changes in expression of a dehydroascorbate reductase gene (*NtDHAR*) in Xanthi-nc tobacco at 30°C in comparison to 20°C in response to mock inoculation (mechanical stress) and inoculation with tobacco mosaic virus (TMV) at different time points after treatments. Untreated plants were either in a winter greenhouse environment (G0) or exposed to 20 and 30°C in growth chambers. RT 0 = negative control of reverse transcription (no RNA template present); PCR 0 = negative control of polymerase chain reaction (no cDNA template present). Gene expression assays were done by a two step reverse transcription-polymerase chain reaction (RT-PCR) procedure. A tobacco actin gene (*NtAct*) served as a reference of gene expression.

Expression of the Bax inhibitor gene (*NtBI-1*), which inhibits tissue necrotization in infected plants, was suppressed in untreated plants kept in the greenhouse (G0, see Fig. 29), as compared to that in plants kept in growth chambers at 20°C for 6 hours. However, in untreated plants *NtBI-1* expression was suppressed at 30°C for 6 hours, as compared to that in plants kept at 20°C. In mock inoculated plants, *NtBI-1* expression did not change at 20°C and 30°C for 6, 12 and 24 hours after treatment. Interestingly, *NtBI-1* expression was induced in TMV-infected plants kept at 30°C for 6 and 24 hours after virus inoculation (Fig. 29).



Fig. 29. Changes in expression of a Bax inhibitor-1 gene (*NtBI-1*) in Xanthi-nc tobacco at 30°C in comparison to 20°C in response to mock inoculation (mechanical stress) and inoculation with tobacco mosaic virus (TMV) at different time points after treatments. Untreated plants were either in a winter greenhouse environment (G0) or exposed to 20 and 30°C in growth chambers. RT 0 = negative control of reverse transcription (no RNA template present); PCR 0 = negative control of polymerase chain reaction (no cDNA template present). Gene expression assays were done by a two step reverse transcription-polymerase chain reaction (RT-PCR) procedure. A tobacco actin gene (*NtAct*) served as a reference of gene expression.

These results provide evidence that several antioxidant genes/enzymes and possibly programmed cell death-inhibitors such as BI-1 are responsible under high temperature (30°C) for suppression of virus-induced necrotization during HR which is caused by ROS.

4.4. Changes in prooxidants (ROS) and antioxidants in barley leaves in which resistance was induced chemically by INA

We treated barley leaves as a soil drench with an analogue of salicylic acid, 2,6dichloroisonicotinic acid (INA), which is an inducer of disease resistance, four days before inoculation with the barley powdery mildew (*Blumeria graminis* f.sp. *hordei*). HR-type necroses were induced in cultivar Ingrid, carrying the gene *Mlo* which determines susceptibility to the race A6 of the powdery mildew pathogen (Fig. 30 A, B).



Fig. 30. Stimulation of HR in the susceptible barley cultivar Ingrid infected with race A6 of powdery mildew. Left: Control infected leaves. Right: Pre-treated leaves with INA and infected with the fungus. (**A**): Detached leaves. (**B**): Intact leaves.

Interestingly, at the time of induction of HR-type necroses, accumulation of reactive oxygen species (ROS), namely hydrogen peroxide (H_2O_2), was detected by a technique using a fluorescent dye, 2',7'-dichlorofluorescein diacetate. The high level of H_2O_2 of INA-pre-treated plants was maintained even after inoculation with the pathogen (Fig. 31).



Fig. 31. Levels of reactive oxygeen species (ROS) in Ingrid barley leaves. A fluorescent dye, 2',7'-dichlorofluorescein diacetate, was injected into the leaves and fluorescence intensity was tested by a FluoroMax-3 spectrofluorometer following extraction of 2',7'-dichlorofluorescein from tissues. Levels of ROS were detected 4 days after treatment with INA and 2 days after inoculation with powdery mildew. **Untreated:** Plants treated only with water. **INA-treated:** Plants treated only with INA. **Untreated + infected:** Plants only infected with barley powdery mildew. **INA + infected:** Plants treated with INA and infected with the fungus.

INA-pretreatment significantly inhibited activities of superoxide dismutase (SOD) and dehydroascorbate reductase (DHAR) in both uninfected and infected leaves (Figs. 32, 33, 34).



Fig. 32. Activity of dehydroascorbate reductase (DHAR) which is involved in detoxification of ROS was measured with a Shimadzu UV-160A spectrophotometer. Ingrid barley leaves were harvested 4 days after INA treatment. Pre-treated plants were inoculated with powdery mildew and tested for enzyme activity 2 days after inoculation. **Untreated:** Plants treated only with water. **INA-treated:** Plants treated only with INA. **Untreated + infected:** Plants infected only with barley powdery mildew. **INA + infected:** Plants treated with INA and infected with the fungus.



Fig. 33. Activity of superoxide dismutase (as determined spectrophotometrically) in Ingrid barley leaves. Leaves were harvested 4 days after INA treatment. Pretreated plants were inoculated with powdery mildew 4 days after treatment and tested for enzyme activity 2 days after inoculation. Untreated: Plants treated only with water. INA-treated: Plants treated only with INA. Untreated + infected: Plants infected only with barley powdery mildew. INA + infected: Plants treated with INA and infected with the fungus.



Fig. 34. Inhibition of dehydroascorbate reductase (**DHAR**) and superoxide dismutase (**SOD**) in Ingrid barley leaves after treatment with INA. Activity of dehydroascorbate reductase involved in detoxification of ROS was tested with Shimadzu UV-160A spectrophotometer. Barley leaves were harvested 4 days after INA treatment. Pretreated plants were inoculated with powdery mildew and tested for enzyme activity 2 days after inoculation.

We also measured the expression of DHAR gene using the RT-PCR technique. DHAR expression did not change in plants, which were treated with INA, as compared to leaves treated with water for 12 hours after treatment (Fig. 35). However, 48 hours after treatment DHAR expression was suppressed in plants treated with INA, as compared to the control plants. Interestingly enough, DHAR was strongly suppressed in the INA-treated leaves 96 hours after treatment, as compared to the control plants (Fig. 35).



Fig. 35. Changes in expression of a cytoplasmic dehydroascorbate reductase (HvDHAR) gene in Ingrid barley in response to dichloro-isonicotinic acid (INA) soil drench treatment at different time points. Control plants were drenched with tap water (W). RT 0 = negative control of reverse transcription (no RNA template present); PCR 0 = negative control of polymerase chain reaction (no cDNA template present). Gene expression assays were done by a two step reverse transcription-polymerase chain reaction (RT-PCR) procedure. A barley ubiquitin gene (*HvUbi2*) served as a reference of gene expression.

It is suggested that down-regulation of antioxidants could be in a cause-and-effect relationship with high accumulation of hydrogen peroxide (H_2O_2) upon pre-treatment with the resistance inducer INA. Suppression of antioxidant enzymes in the inducer-treated barley during the hypersensitive response (HR) may play a role in causing cell death in the host and in the killing or restricting the pathogenic fungus.

NEW SCIENTIFIC RESULTS

My new scientific results are as follows:

- It was demonstrated that spraying barley leaves with hydrogen peroxide (H₂O₂) could alter the type of symptoms and induces HR-type necrosis in the *Mlo*-susceptible genotype. This effect could be reversed with the application of antioxidant enzymes (SOD and CAT).
- As a result of H₂O₂-treatment, the *Mla12*-resistant genotype produced HR earlier and the number of necrotic lesions increased, as compared to untreated but infected control leaves. Leaves of the resistant non-necrotic *mlo5* or *Mlg* plants exhibited HR-type symptoms. It was also possible to reverse this action of H₂O₂ with the antioxidants.
- Pre-treatment of tobacco plants with low concentration of H₂O₂ suppressed tissue necrotization caused by viral, bacterial and fungal infections. Suppression of tissue necrotization is in correlation with the high activities of several antioxidants (CAT, APX and POX). H₂O₂-treatment did not alter the multiplication (replication) of the pathogens.
- HR-type necrosis was induced in Xanthi-nc tobacco infected with TMV even at 30°C by chemical compounds, which produce ROS, or with direct application of H₂O₂. This action can be reversed with antioxidants. At 30°C the level of superoxide was significantly reduced and activity of enzymes associated with the production of superoxide as well as their gene expression were also suppressed.
- The expression of the gene BAX inhibitor was stimulated at 30°C, which corresponds to the suppression of necrotization.
- Resistance can be induced chemically with 2,6-dichloroisonicotinic acid (INA) in barley susceptible to powdery mildew. As a result of INA-treatment, level of ROS increased and the activity as well as gene expressions of antioxidants were reduced. Correspondingly, HR-type necrosis developed in the originally susceptible barley.

5. DISCUSSIONS

It is evident that tissue necrotization may or may not be associated with resistance of plants to infections and the hypersensitive reaction (HR) could be a consequence, not the cause, of disease resistance (Király et al., 1972).

Baulcombe and co-workers (Bendahmane et al., 1999) claim that "extreme resistance" to viral infections, which is not associated with HR, is the consequence of an early resistance mechanism in which arrest of viral replication is very effective. In other words, the time is too short for the development of HR (tissue necrotization), thus, resistance to the virus overrides the induction of HR. However, if the development of resistance needs a longer time period, infection is able to induce HR in the resistant host. Furthermore, if resistance develops very late, systemic spread of the virus occurs first and only subsequently can the "resistant" host develop the systemic HR. Whether or not reactive oxygen species (ROS) have role in virus resistance, remains an unresolved question. Another example of HR-independent resistance exists in tomato, which has a *Cf-9*-encoded resistance to *Cladosporium fulvum*. However, if a sustained production of the elicitor of this pathogen is carried out in the *Cf-9* resistant plant, HR-independent resistance is changed to HR-associated-type of resistance (Hammond-Kosack et al., 1995). As a rule, necrotic symptoms are caused by the production of ROS.

The HR-type resistance of plants to bacterial infections is also associated with the production of ROS. This was first demonstrated by Ádám et al. (1989). The importance of the role of ROS and antioxidants in the development of normosensetive necrotic symptoms produced by the susceptible plants, was only rarely mentioned in the literature (El-Zahaby et al., 2004).

5.1. Role of hydrogen peroxide in symptom expression of barley susceptible and resistant to powdery mildew

It was demonstrated in several laboratories that the presence of ROS in mildew resistant barley plants may be associated with the hypersensitive response and papillae formation (Thordal-Christensen et al., 1997; Hückelhoven and Kogel, 2003; Hückelhoven et al., 1999; Király and El-Zahaby, 2000; Hafez et al., 2003; El-Zahaby et al., 2004). However, regulatory compounds may modify the action of ROS. Several publications (Levine et al., 1994; El-Zahaby et al., 1995; Foyer et al., 1997) referred to the possible role of antioxidants

in balancing the action of ROS in powdery mildew-infected barley and other host/pathogen combinations. In susceptible barley leaves antioxidant reactions are induced that inhibit tissue necrotization and suppress lipid peroxidation. However, these antioxidative processes are less efficiently activated in resistant leaves which may result in necrotization (HR).

My results show that H_2O_2 plays a pivotal role in inducing HR as well as pathogen arrest in different barley/powdery mildew combinations.

In a compatible host/pathogen combination (cultivar Ingrid/race A6 of *Blumeria graminis* f. sp. *hordei*) H_2O_2 applied to the infected leaves 2-3 days after inoculation caused HR-type necroses characteristic of resistant cultivars (Fig. 1). Earlier it was shown in our laboratory that ROS-producing chemicals, such as the riboflavin-methionine mixture with illumination and the xanthine-xanthine oxidase mixture can cause HR-type symptoms in infected susceptible barley (Király and El-Zahaby, 2000, El-Zahaby et al., 2004). In the present study we demonstrate that H_2O_2 is responsible for host tissue necrotization and 50 mM or 25 mM H_2O_2 , applied as a spray, is necessary for induction of HR in attached and detached susceptible (*Mlo*) barley leaves, respectively. Hydrogen peroxide was also responsible for pathogen arrests because an early (one day after inoculation) application of H_2O_2 inhibited appearance of any symptoms in leaves. The role of H_2O_2 in inducing HR-type resistance in a susceptible barley cultivar was substantiated by another result: we were able to reverse the action of H_2O_2 when we injected a combination of SOD and CAT solution into infected leaves before treatment of leaves with H_2O_2 . As a result, host susceptibility was restored and the fungus produced mycelia and conidia in those leaves (Fig.1D).

In a resistant host which produces typical HR (cultivar Ingrid carrying the gene *Mla12* and infected with race A6), treatment of leaves with H_2O_2 resulted in a stimulated development of HR-type necroses and HR appeared earlier than in the untreated and detached infected leaves (Fig. 3). As in the case of the susceptible host, an early application of the H_2O_2 -spray (one day after inoculation) induced arrest of the pathogen, and consequently, no symptoms appeared (Fig. 2). The antioxidant action of SOD and CAT protected leaves from the action of H_2O_2 on symptom expression (Figs. 2D and 3D).

The race non-specific resistance of cultivar Ingrid, carrying the gene *mlo5*, against race A6 resulted in a symptomless resistance, which was not associated with the HR. However, HR was produced as a consequence of spraying leaves with H_2O_2 2-3 days after inoculation (Fig. 4). Leaf treatments only with H_2O_2 did not cause any symptoms, as was

experienced in all of our experiments. Antioxidants (SOD and CAT) prevented development of HR, (Fig. 4D), indicating that a certain amount of H_2O_2 is necessary for the induction of necrotization, which is associated with HR.

The cultivar Ingrid, carrying the gene Mlg, expresses invisible HR-type resistance to some races of the fungus. Interestingly, tissue necrotization was stimulated and visible HRtype leaf necroses appeared as a consequence of spraying leaves with H₂O₂ 2 days after inoculation. (Fig. 5). SOD and CAT prevented development of HR, indicating also that a certain amount of H₂O₂ is necessary for the induction of necrotization, which is associated with HR (Fig. 5D).

It would seem that H_2O_2 , which is produced after infection as a result of the oxidative burst, exerts a dual role in symptom expression and resistance: it may arrest pathogen growth (resistance) and induces HR-associated host symptoms. In other words, increased amounts and/or sustained production of H_2O_2 can convert susceptibility to resistance or the HRindependent resistance to HR-associated one. When we applied H_2O_2 to leaves before establishment of infection (one day after inoculation), pathogen arrest occurred early and neither susceptible nor HR-type symptoms were expressed. However, appearance of HR (necrotization) or stimulation of the HR symptoms occurred if H_2O_2 was applied to infected leaves after establishment of infection (2-3 days after inoculation).

It is important to note that in relation to other host/pathogen interactions several invesigators called the attention to the action of high doses of H_2O_2 in resistance and the role of low doses in signalling stimulated antioxidant actions (Doke, 1985, Levine et al., 1994, Gechev et al., 2002, Vandenabeele et al., 2003). It remains to be seen whether in our powdery mildew-infected barley plants an artificial supply of H_2O_2 influences the natural antioxidant capacity of the host? This would balance the harmful effects of ROS and expression of symptoms.

Several research groups hypothesized that the ability for resistance, which is associated with HR, is suppressed in barley cultivars susceptible to powdery mildew infection. These plants express the gene *Mlo* that may function as a negative regulator of plant resistance and tissue necrotization. It was also claimed that the *mlo5* resistance, which is not associated with HR, could be the consequence of another type of negative regulation of HR

where the pathogen arrest (resistance) is uncoupled from HR (Büschges et al., 1997, Shirasu and Schulze-Lefert, 2000, Hüchelhoven et al., 2003).

One can suppose that the negative regulation of host resistance and tissue necrotization in both susceptible and the *mlo5*-resistant barley plants means a limited production of H_2O_2 . Artificial supply of this compound, as a spray to infected leaves, can induce HR-associated resistance in both susceptible and *mlo5*-resistant cultivars. Release of this negative control by H_2O_2 can be reversed by injecting tissues with antioxidants, such as SOD and CAT. It is not known at present whether H_2O_2 can influence the natural antioxidant capacity of leaves, as was shown earlier in naturally infected barley plants (El-Zahaby et al., 1995).

5.2. Immunization of tobacco with low concentration of hydrogen peroxide against oxidative stress caused by viral, bacterial and fungal infections

As a result of infection of plants with pathogens, different tissue necrotizations may develop in the hosts. We inoculated the Xanthi-nc tobacco variety with a viral, a bacterial and a fungal pathogen. Tobacco mosaic virus (TMV) and *P. syringae* pv. *phaseolicola* induced HR-type necroses in tobacco because this plant variety was an incompatible host for the virus and a non-host for the bacterium. In both cases, HR was associated with plant resistance. However, *Botrytis cinerea* was a compatible fungal pathogen for Xanthi-nc tobacco. In this case, necrotic symptoms developed as a result of host susceptibility, not resistance. Thus, the necrotic spots could be regarded as normosensetive, not hypersensitive, responses.

It is important to point out that in the development of both the HR and the normosensitive necrotic response reactive oxygen species (ROS), such as H_2O_2 , may have role. As is known, the harmful effects of ROS (oxidative stress) can be counteracted by the enzymatic and non-enzymatic antioxidants. Halliwell and Gutteridge (1999) have the opinion that often the only evidence that oxidative stress has occurred *in vivo* may be the upregulation of antioxidant enzyme systems. Gechev et al. (2002) have shown that pre-treatment of tobacco plants with low concentration of H_2O_2 (a mild oxidative stress), stimulates a few antioxidant enzymes and makes plant tolerant to abiotic stresses. Furthermore, Vandenabeele et al. (2003) demonstrated that this mild oxidative stress might induce expression of several genes associated with up-regulation of several signal transduction components and tolerance against abiotic stresses.
I have shown that pre-treatment of Xanthi-nc tobacco with low concentration of H_2O_2 one day before inoculation with the pathogens, suppressed the HR-type necrotic symptoms caused by either TMV or the *Pseudomonas* bacterium and the normosensitive necrotic symptoms caused by the fungal pathogen, *Botrytis cinerea*. This pre-treatment enhanced activities of three antioxidant enzymes. Artificial application of two antioxidant enzymes (SOD and CAT) also suppressed the development of necroses casued by viral, bacterial or fungal pathogens. On the basis of this experiments one can suppose that the immunization of tobacco against pathogenic stresses was determined by up-regulation of the antioxidant enzymes, such as catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POX).

As regards suppression of the spread and multiplication of the virus associated with HR, the generally accepted hypothesis was that the cause of plant virus resistance was the appearance of tissue necroses upon infection. However, this hypothesis was recently criticized on the basis of new experiments (Bendahmane et al. 1999 and Cole et al. 2001) showing that resistance is independent of tissue necrosis. This idea is supported by our results, according to which the lack of necrotization in a local lesion host, as a consequence of immunization with low concentration of H_2O_2 or a direct application of SOD and CAT, did not increase viral multiplication, in comparison to control plants which were only infected with the virus. Similarly, bacterial multiplication did not increase in the immunized or SOD plus CAT-treated leaves. Earlier it was also shown that suppression of necrotic symptoms by albumintreatment did not influence bacterial number in tobacco (Király et al., 1977). It is also worth of mentioning that the low concentration of H_2O_2 (5 mM) which suppressed necroses caused by *Botrytis cinerea* in tobacco leaves did not suppress growth of this fungus in an artificial medium.

5.3. Role of reactive oxygen species (ROS) and antioxidants in TMV-induced necrotization associated with resistance of an N gene encoding tobacco

According to our results, the HR-type leaf necrotization caused by TMV infection in a local lesion host depends on the presence of ROS, such as O_2 . (superoxide), H_2O_2 (hydrogen peroxide) etc. We were able to induce HR-type necroses in virus inoculated Xanthi-nc tobacco leaves even at high temperature (30°C), where the necrotic spots developed because the leaves were treated with ROS, in addition to inoculation with TMV. ROS-treatments alone or virus-inoculation alone were not effective in inducing HR-type leaf necroses at this temperature. It

would seem that in our local lesion host the hypersensitive response is associated with the presence of a certain amount of reactive oxygen species.

As regards suppression of the spread and multiplication of the virus associated with HR, the generally accepted hypothesis was that the cause of virus resistance was the appearance of tissue necroses upon infection. However, this hypothesis was recently criticised on the basis of new experiments (Bendahmane, et al., 1999; Cole et al., 2001), showing that resistance is independent of tissue necrotization. This idea is supported by our results, according to which at 30°C the lack of necrotization in a resistant local lesion host cannot increase virus multiplication in comparison to plants in which necrotization was induced by ROS. However, high temperature itself is in a cause-and-effect relationship with increased virus content in leaves.

When we applied SOD and CAT to the infected tobacco leaves treated with ROS at 30°C, necrotization was reversed as a result of application of antioxidants. Our results support the hypothesis concerning the possible role of antioxidants in balancing the action of ROS in several host/pathogen combinations (Levine et al., 1994; El-Zahaby et al., 1995; Foyer et al., 1997; Hafez et al., 2003).

Interestingly enough, when we measured the level of ROS (O_2^- and H_2O_2) in tobacco leaves, we found that the level of O_2^- is significantly decreased at 30°C, as compared to 20°C either in the control or TMV-infected leaves. However, H_2O_2 did not change significantly. This result supports the hypothesis of Samuel (1931) that at 30°C the necroses are overcome but the virus is able to multiply and spread systemically. Furthermore, this result strongly points to the role of ROS, namely O_2^- , in the production of HR-type necrosis.

NADPH oxidase is an enzyme known to be responsible for O₂⁻ production in infected plants (Lamb and Dixon, 1997; Grant and Loake, 2000). We have shown that the activity of NADPH oxidase, as measured spectrophotometrically, was suppressed at 30°C, as compared to 20°C in uninfected control or TMV-inoculated leaves. Similarly, the expression of the gene NADPH oxidase (*NtRBOH*) was suppressed at 30°C. Therefore, it is likely that the decrease in superoxide levels detected at 30°C is a consequence of decreased gene expression and NADPH oxidase activity. Several studies demonstrated that the oxidative burst in plants could be caused by activation of an NADPH oxidase closely resembling the enzyme operating in activated neutrophils (Lamb and Dixon, 1997; Grant and Loake, 2000). Furthermore, there is genetic evidence that plant NADPH oxidases involved in HR-type cell death are activated by

MAPK kinases (Yoshioko at al., 2003). It is possible that in TMV-infected plants the decrease in NADPH oxidase activity at 30°C is a consequence of a lack of MAPK kinase activity.

Superoxide dismutase (SOD) and catalase (CAT) are very important enzymes that convert O_2 to H_2O_2 and then to water. These two enzymes are ubiquitous in aerobic organisms where they play a major role in defense against oxygen radical-mediated toxicity (Tsang et al., 1991; Mittler, 2002). We have found that expression of an SOD gene (NtSOD) was considerably lower in untreated plants kept at 30°C, as compared to 20°C. In mock or TMVinoculated plants, however, the expression of NtSOD was the same at 30°C or 20°C. It is likely that NtSOD expression did not change or was slightly lower at 30°C because the level of O₂⁻ in these plants was also lower at 30°C. Our data also demonstrate that CAT avtivity, as measured spectrophotometrically, was slightly increased at 30°C both in healthy (untreated) and virus-infected leaves, as compared to that at 20°C. On the other hand, expression of a catalase gene (NtCAT1) did not differ significantly at 20°C or 30°C, as detected by RT-PCR. It is possible that CAT level and activity did not change or increased only slightly at 30°C becuase the level of H₂O₂ was also only slightly higher at 30°C. The balance between the activity of SOD and H₂O₂-degrading antioxidant enzymes, like CAT or ascorbate peroxidase (APX), is crucial for controlling the steady-state level of the ROS (O_2^{-1} and H_2O_2) and the development of plant cell death (Mittler et al., 2002). For example, transgenic antisense tobacco plants with reduced CAT or APX expression display increased sensitivity "hypersusceptibility" to cell death during bacteria-induced HR (Mittler et al., 1999). Furthermore, increases in SOD expression are not sufficient to counteract oxidative damage and necrosis: transgenic SOD-overexpressing tobacco which is tolerant to necrosis, caused by high light intensity and low temperature, also display elevated levels of endogenous APX (Gupta et al., 1993).

Alternative oxidase (AOX) lowers the generation of ROS in the mitochondria by preventing overreduction of the cytochrome respiratory electron transport chain during stresses like aging and pathogen attack (Maxwell et al., 1999; Chivasa and Carr, 1998). Thereby it contributes to restriction of the size of necrotized viral lesions, during HR-type cell death (Ordog et al., 2002). In our study, an alternative oxidase gene (*NtAOX*) was suppressed at 30°C, as compared to 20°C both in untreated control, mock- or TMV-inoculated tobacco leaves as measured by RT-PCR techniques. The reduced expression of *NtAOX* at 30°C could be, in part at least, a consequence of the absence of necrosis caused by TMV. AOX transcript and protein

levels increase in tobacco that displays an HR during TMV-infection, but do not change during a compatible interaction, where virus localization and HR-necrosis do not occur (Chivasa and Carr, 1998; Lennon et al., 1997).

Dehydroascorbate reductase (DHAR) activity and gene expression was considerably higher at 30°C, as compared to 20°C. In mock- and TMV-inoculated plants, the gene *NtDHAR* was induced at 30°C, as compared to 20°C at 12 and 24 hours after treatments, respectively. Our results suggest that DHAR seems to play, at least a partial role, in inhibiting the formation of HR-type necroses at 30°C. This conclusion is supported by research showing that DHAR-overexpressing tobacco plants exhibit elevated levels of reduced ascorbate and glutathione, the reductant used by DHAR to recycle ascorbate (Chen et al., 2003). Such a simultaneous increase in levels of ascorbate and glutathione is a likely consequence of DHAR-induction at 30°C and could be directly resposible for the elimination of necrosis during viral HR as suggested by earlier studies (Farkas et al., 1960; Gullner et al., 1999).

We have found that expression of a Bax inhibitor-1 (*NtBI-1*) gene which inhibits necrotization in plant and animal cells (Danon et al., 2000; Bolduc et al., 2002, 2003) was induced in TMV-infected tobacco kept at 30°C 6 and 24 hours after virus inoculation. These data could explain why necrotization caused by TMV is overcome at 30°C and no visible symptoms appear. Bax inhibitor genes encode proteins proposed to be suppressors of programmed cell death (PCD). For example, *NtBI-1* can suppress PCD in human kidney cells (Bolduc et al., 2003). On the other hand, transgenic tobacco cells that express an antisense copy of the *NtBI-1* gene display down-regulation of *NtBI-1* expression and enhanced cell death during sucrose starvation and osmotic stress (Bolduc et al., 2002).

The above results indeed support the hypothesis that in virus-infected tobacco (Xanthinc) plants kept at 30°C necrotization is overcome but the virus is able to multiply and spread systemically. Furthermore, these results provide evidenceat that at high temperature (30°C) several antioxidant genes/enzymes and possibly programmed cell death-inhibitors, such as BI-1, are responsible for suppression of virus-induced necrotization during HR caused by ROS.

5.4. Changes in prooxidants (ROS) and antioxidants in barley leaves in which resistance was induced chemically by INA

2,6-dichloroisonicotinic acid (INA) has been described as a resistance-inducing chemical effective in many plant species (Kessmann et al., 1994). When I treated the

INA induced HR-type necroses in the susceptible leaves of Ingrid with accumulation of hydrogen peroxide (H_2O_2). The high level of H_2O_2 was maintained even 10 days after inoculation.

It is known since a long time that HR-type necrotic symptoms are associated with resistance to infections. HR occurs in genetically incompatible plant-pathogen interactions in the presence of functionally active barley *Mlg* and *Mla12* resistance genes (Kita et al., 1981; Koga et al., 1990; Aist and Bushnell, 1991). These genes govern resistance in a typical race-specific manner. According to Kogel et al. (1994) INA arrested the fungal growth before the formation of haustorium. The establishment of the haustorium is the important step for fungal development in the epidermal cells, since it serves to feed the pathogen. Therefore, to prevent the formation of haustoria is a very effective mechanism to induce resistance against the pathogens.

My results demonstrated that INA-treatment significantly inhibited the activity of superoxide dismutase (SOD) and dehydroascorbate reductase (DHAR) in both uninfected and infected leaves. Therefore, this could be the cause of an increase in the level of H_2O_2 which seems to play a very important role in resistance against the fungal pathogen *Blumeria graminis* f.sp. *hordei*. The expression of the gene *DHAR* was also strongly suppressed in the INA-treated leaves, as compared to the control untreated plants.

It can be concluded that, down-regulation of antioxidants could be in a cause-andeffect relationship with high accumulation of H_2O_2 upon treatment of barley leaves with INA. Suppression of antioxidant enzymes in the inducer-treated barley during the hypersensitive response (HR) can play a role in causing cell death in the host and in killing or restricting the pathogenic fungus. Thus, the induced (acquired) resistance caused by treatment of leaves with INA, which is induces a broad-spectrum resistance, resembles the mechanism which is characteristic for the so-called race specific resistance.

CONCLUSIONS

Role of hydrogen peroxide in symptom expression of barley susceptible and resistant to powdery mildew

- 1. Spraying four genotypes of barley (cultivar Ingrid) expressing the genes *Mlo*, *mlo5*, *Mla12* and *Mlg* with H₂O₂ and infected with *Blumeria graminis* f. sp. *hordei* resulted in HR-type necrosis and produced necroses earlier in the *Mla12* barley than in the control.
- 2. Treatment of barley genotypes with hydrogen peroxide (H₂O₂) before establishment of infection (one day after inoculation) resulted in inhibition or killing the pathogen and symptomless response in all of the four genotypes.
- 3. It was possible to reverse these actions of H_2O_2 with injection of barley leaves with a combination of superoxide dismutase (SOD) and catalase (CAT) before treatment with H_2O_2 .

Imunization of tobacco with low concentration of hydrogen peroxide

- 1. In tobacco leaves pre-treated with low concentration of H₂O₂ the number and size of necroses caused by tobacco mosaic virus (TMV) infection was suppressed and the antioxidant capacity augmented, as compared to the untreated but infected control. Particularly the activity of CAT, ascorbate peroxidase (APX) and guaiacol peroxidase (POX) was stimulated. Suppression of necroses and increased activity of these antioxidant enzymes were also demonstrated in pre-treated tobacco leaves infected with *Pseudomonas syringae* pv. *phaseolicola*. Pre-treatment of tobacco with H₂O₂ similarly suppressed necrotization caused by the fungal pathogen *Botrytis cinerea*.
- 2. Injection of a mixture of SOD and CAT into leaves infected with TMV, *P. syringae* pv. *phaseolicola* and *B. cinerea* significantly diminished tissue necrotization caused by these pathogens, showing that the increased activities of antioxidants could be responsible for immunization.
- **3.** Concentration of TMV and the number of bacteria did not change significantly in infected leaves, which were pre-treated with H_2O_2 . Also, viral and bacterial concentrations were not diminished when exogenously applied SOD and CAT suppressed tissue necrotization. Low concentration of H_2O_2 exerted no action on the growth of *B. cinerea* in an artificial medium.

Role of reactive oxygen species (ROS) and antioxidants in TMV-induced necrotization at high temperature

- 1. Chemical compounds which generate reactive oxygen species (ROS), such as riboflavin/methionine and glucose/glucose oxidase systems, or the directly applied H_2O_2 are able to induce HR-type necroses in Xanthi-nc tobacco infected with TMV even at $30^{\circ}C$.
- Chemically induced HR-type necrotization at 30°C was suppressed by the application of antioxidants such as SOD and CAT. In this case TMV content, as determined by the ELISA-test, did not change.
- **3**. The amount of superoxide (O₂⁻) decreased and that of the H₂O₂ slightly increased in leaves of infected and healthy Xanthi-nc tobacco at 30°C, as compared to 20°C.
- **4.** Activity of NADPH-oxidase and the mRNA levels of NADPH-oxidase as well as an alternative oxidase were also significantly lower at 30°C, as compared to 20°C. Activity of a dehydroacorbate reductase (DHAR) significantly increased at 30°C, as compared to 20°C. However, gene expressions of SOD and CAT on the mRNA level did not change. Interestingly, expression of the gene Bax inhibitor (*NtBI-1*) was stimulated in TMV-infected tobacco kept at 30°C.

Induced resistance caused by 2,6-dichloroisonicotinic acid (INA) in barley

Treatment of barley leaves with 2,6-dichloroisonicotinic acid (INA) four days before inoculation induces resistance against barley powdery mildew (*Blumeria graminis* f.sp. *hordei*). The frequency of epidermal cell death (HR) significantly increased in INA-treated barley leaves upon powdery mildew infection and resistance was accompanied by elevated level of ROS (H_2O_2) and reduced activities of SOD and DHAR

SUMMARY

- Under natural conditions barley leaves, carrying the gene *Mlo*, exhibited susceptible response to infection, the *mlo* and *Mlg* barley leaves were resistant but did not develop HR necrotic symptoms. The *Mla12* barley was resistant and developed HR-type symptoms. Under the influence of treatment with H₂O₂ (25-50 mM), leaves of the susceptible *Mlo* and the resistant *mlo5* or *Mlg* plants exhibited HR-type symptoms with tissue necroses. The *Mla12*-resistant genotype produced HR earlier and the number of necrotic lesions increased, as compared to untreated but infected control leaves. H₂O₂ alone, without infection, did not induce any visible symptoms. These experiments show that ROS may have role in symptom expression and disease resistance.
- Treatment of barley genotypes with H₂O₂ before establishment of infection (one day after inoculation) resulted in inhibition of the pathogen and symptomless response in all of the four genotypes, because the pathogen was probably killed before the establishment of infection. It was possible to reverse the inhibitory effect as well as the HR-producing actions of H₂O₂ by injection of barley leaves with a combination of superoxide dismutase (SOD) and catalase (CAT) before treatment with H₂O₂.
- In tobacco leaves pre-treated with low concentration (5-7 mM) of hydrogen peroxide (H₂O₂) the number and size of necroses caused by tobacco mosaic virus (TMV) infection was suppressed and the antioxidant capacity augmented, as compared to the untreated but infected control. Particularly the activity of CAT, ascorbate peroxidase (APX) and guaiacol peroxidase (POX) was stimulated.
- Suppression of necroses and increased activity of these antioxidant enzymes were also demonstrated in pre-treated tobacco leaves infected with *Pseudomonas syringae* pv. *phaseolicola*. Pre-treatment of tobacco with H₂O₂ similarly suppressed necrotization caused by the fungal pathogen *Botrytis cinerea*. Injection of a mixture of SOD and CAT into leaves infected with TMV, *P. syringae* pv. *phaseolicola* and *B. cinerea* significantly diminished tissue necrotization caused by these pathogens.
- Concentration of TMV and the number of bacteria did not change significantly in infected leaves which were pre-treated with H₂O₂. Viral and bacterial concentrations were also not diminished when exogenously applied SOD and CAT suppressed tissue necrotization. Low concentration of H₂O₂ exerted no action on the growth of *B. cinerea* in an artificial medium. It is concluded that spraying leaves with low concentration of H₂O₂ can

immunize tobacco leaves to necrotic symptoms caused by viral, bacterial and fungal pathogens by increasing activity of several antioxidant enzymes, but multiplication of pathogens is not enhanced.

- It is known that local lesion hosts of TMV cannot develop HR-type necroses at high temperature, such as 30°C and the concentration of TMV increases at this high temperature. Chemical compounds which generate reactive oxygen species (ROS), such as riboflavin/methionine and glucose/glucose oxidase systems, or the directly applied H₂O₂ are able to induce HR-type necroses in Xanthi-nc tobacco infected with TMV even at 30°C. It was possible to suppress the chemically induced HR-type necrotization at 30°C by the application of antioxidants, such as SOD and CAT. In this case TMV content, as determined by the ELISA-test, did not change.
- The amount of superoxide (O₂⁻) decreased and that of the H₂O₂ slightly increased in leaves of infected and healthy Xanthi-nc tobacco at 30°C, as compared to 20°C. Activity of NADPH-oxidase and the mRNA levels of the NADPH-oxidase and an alternative oxidase were also significantly lower at 30°C, as compared to 20°C. Activity of a dehydroacorbate reductase (DHAR) significantly increased at 30°C, as compared to 20°C. However, the mRNA level of SOD and CAT did not change. Interestingly, expression of the gene Bax inhibitor (*NtBI-1*) was stimulated in TMV-infected tobacco kept at 30°C. The development of HR-type necroses caused by TMV infection depends on a certain level of superoxide and other ROS. Accordingly, suppression of virus multiplication in resistant tobacco is independent of the appearance of necroses but depends on high temperature.
- 2,6-dichloroisonicotinic acid (INA) is an analogue of salicylic acid (SA) which acts as a chemical inducer of resistance. Treatment of barley leaves with INA four days before inoculation induces resistance against barley powdery mildew (*Blumeria graminis* f.sp. *hordei*). The frequency of epidermal cell death (HR) significantly increased in INA-treated barley leaves upon powdery mildew infection and accompanied by elevated levels of ROS, including H₂O₂. Activities and expression of the genes SOD and DHAR were significantly inhibited as a result of INA treatment.

ÖSSZEFOGLALÓ

- Természetes körülmények között az *Mlo* gént kifejező árpavonal fogékonyságot mutatott a lisztharmat A6 rasszával szemben. Az *mlo* és *Mlg* géneket kifejező árpavonalak ellenállóak voltak, de nem fejlesztették ki a hiperszenzitív reakciót (HR). Az *Mla12* gént expresszáló vonal HR kifejlődése mellett mutatott rezisztenciát. Ha a fiatal növények leveleit 25-50 mM H₂O₂-vel kezeltük, a fogékony *Mlo* növény HR-típusú rezisztenciát mutatott. Ugyancsak HR típusú rezisztenciát mutattak az *mlo* és *Mlg* géneket kifejező vonalak, ha H₂O₂-vel permeteztük a fertőzött leveleket. A HR-t kifejlesztő, eredetileg is ellenálló *Mla12* gént kifejező vonal a hidrogén-peroxid kezelés hatására fokozta a nekrotikus léziók számát, és az elhalások (nekrózisok) hamarabb jelentek meg a fertőzött leveleken. A H₂O₂ önmagában, fertőzés nélkül, nem okozott látható tüneteket a leveleken. Ezek a kísérletek azt mutatják, hogy a reaktív oxigén fajtáknak (ROS) meghatározó szerepük van a nekrotikus tünetek kialakításában és a rezisztenciában.
- Ha az említett négy árpagenotípust a fertőzés után igen korán (1 nappal) kezeltük H₂O₂-vel, a növények minden genotípus esetében tünetmentességet mutattak, minden bizonnyal azért, mert a kórokozót az említett ROS-kezelés elölte még a megtelepedés előtt. Ezt a kórokozó-gátlást, valamint a HR kifejlődésének gátlását meg lehetett akadályozni, ha az árpaleveleket még a H₂O₂-vel való kezelés előtt antioxidánsok (SOD és CAT) kombinációjával injektáltuk. Ebben az esetben az árpavonalak eredeti reakciója fejlődött ki.
- Dohányleveleket (Xanthi-nc dohány) immunizálni lehet a *Dohány mozaik virus* (TMV) által okozott nekrotikus tünetekkel szemben, ha a leveleket igen kis töménységű (5-7 mM) H₂O₂-vel egyszer megpermetezzük, és így egy enyhe stresszt idézünk elő a szövetekben. A tünetek visszaszorulásával együtt az antioxidáns kapacitás növekedett a levelekben. Különösen a CAT, az aszkorbát peroxidáz (APX) és a guajakol peroxidáz (POX) aktivitása fokozódott a kezelt (stresszelt) levelekben.
- Fokozott antioxidáns kapacitást, illetve a nekrózisok visszaszorulását lehetett tapasztalni akkor is, amikor az előkezelt (stresszelt) dohányleveleket a *Pseudomonas syringae* pv. *phaseolicola*-val inokuláltuk. A H₂O₂-vel való előkezelés a *Botrytis cinerea* gombával történt fertőzés által okozott, nem HR típusú nekrózisos tüneteket is visszaszorította. A

vírus, gomba illetve baktériumos fertőzések okozta nekrotikus tünetek akkor is szignifikánsan csökkentek, ha a leveleket kombinált SOD és CAT oldatokkal injektáltuk. Ebben az esetben tehát mesterségesen biztosítottuk a nagy antioxidáns kapacitást.

- A vírus illetve baktériumkoncentráció nem csökkent szignifikánsan a kis töménységű H₂O₂-vel történt immunizálás következtében. Akkor sem volt változás a kórokozók koncentrációjában, amikor a külsőleg adagolt SOD és CAT keverékével csökkentettük a nekrotikus tüneteket, tehát csak a tünetek mérséklődtek az immunizált levelekben, a kórokozók koncentrációja nem. A *B. cinerea* gomba növekedésére (mesterséges táptalajon) nem hatott az immunizálásra alkalmazott kis töménységű H₂O₂. Ezekből az eredményekből az következtethető, hogy az immunizálás oka bizonyos antioxidáns enzimek serkentett aktivitása, amely együtt jár a ROS káros hatásainak ellensúlyozásával, és ennek következtében a tüneti rezisztenciával.
- Már előbbről ismert az a tény, hogy a TMV lokálléziós gazdanövényei 30°C hőmérsékleten nem képesek HR-típusú, nekrózisos tüneteket kifejleszteni, és ezekben a növényekben a vírus koncentrációja növekszik. Ha azonban a Xanthi-nc dohányleveleket nagyobb töménységű H₂O₂-vel kezeljük, továbbá, ha a leveleket olyan vegyületekkel permetezzük, amelyek hidrogén-peroxidot és egyéb ROS-t képeznek, 30°C hőmérsékleten is kialakulnak a HR-típusú nekrózisok a TMV-vel fertőzött levelekben. A ROS-t generáló vegyület-keverékek a következők voltak: riboflavin/metionin, permetezés után a levelek meg voltak világítva, a másik ROS-generáló komplexum pedig a glukóz-glukózoxidáz volt. A mesterségesen adott antioxidánsok (SOD és CAT) ebben az esetben is képesek voltak ellensúlyozni a kémiailag indukált HR-nekrotizálódást. Itt is csak a tünetek változtak, az ELISA-teszttel meghatározott TMV-tartalom nem változott a levelekben.
- A magas hőfokon (30°C) tartott növényekben a 20°C-on tartott növényekhez képest a szuperoxid (O2⁻) szintje jelentősen csökkent. A szuperoxid bioszintézisében kulcsszerepet játszó NADPH-oxidáz aktivitása és a mRNS-szinten meghatározott génexpressziója, valamint egy alternatív oxidáz génexpressziója is csökkent. Az egyik legfontosabb antioxidáns, a dehidroaszkorbát-reduktáz (DHAR) aktivitása viszont 30°C-on fokozódott, és ez is hozzájárulhatott a nekrózisok visszaszorításához. A SOD és CAT génexpressziója nem változott. Érdekes, hogy a sejtelhalást (nekrózist) segítő *Bax* gént ellensúlyozó B*ax*-inhibitor gén (*NtBI-1*) expressziója is fokozódott 30°C-on. Ezekből a kísérletekből

kiderült, hogy a vírus által okozott HR típusú nekrózisok kifejlődése egy bizonyos mennyiségű szuperoxidtól és más ROS-tól függ, valamint az is világossá vált, hogy a magas hőmérsékleten nőtt dohánylevelekben a vírus fokozott szaporodása nem a nekrózisok hiánya miatt következett be, hanem amegemelkedett hőmérséklet miatt.

A 2,6-diklór-izonikotinsav (INA) a szalicilsav (SA) analógjának fogható fel, amely kémiai rezisztencia-indukáló vegyületként is ismert. Ha árpa növényeket 4 nappal a lisztharmattal (*Blumeria graminis* f.sp. *hordei*) történő fertőzés előtt INA-val kezeltünk, az eredetileg fogékony levelek lisztharmat-rezisztensekké váltak. A fertőzött, de INA-kezelt leveleken a HR nekrózisok kialakultak, és a ROS-szint, beleértve a H₂O₂-t is, emelkedett. Két antioxidáns enzim (SOD és DHAR) aktivitása és génexpressziója is szignifikánsan gátlódott, amely hozzájárulhatott a nekrózisok (rezisztencia) kialakulásához.

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