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Generation of superoxide anion and induction of superoxide dismutase and peroxidase in bean leaves infected with pathogenic fungi*

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Generation of superoxide anion ($O_2^{\cdot-}$) and peroxidase activity were significantly increased in bean leaves infected with incompatible and compatible pathogens: *Botrytis fabae* and *Botrytis cinerea*, respectively, but the induction was greater on direct inoculation with *B. fabae*, than with *B. cinerea*. A slightly higher $O_2^{\cdot-}$ level was also detected in the parts of leaves surrounding the inoculation side. Overproduction of $O_2^{\cdot-}$ was observed earlier than the increase in peroxidase activity. Pretreatment of the leaves with methyl jasmonate enhanced both $O_2^{\cdot-}$ production and peroxidase activity following inoculation with *B. cinerea*. Induction of superoxide dismutase activity after the infection was less pronounced than changes in $O_2^{\cdot-}$ level. The differences in the rate of NADH oxidation in the extracts from control and inoculated leaves, correlated with the differences in the rate of $O_2^{\cdot-}$ production. The results indicate that $O_2^{\cdot-}$ level is one of the essential factors responsible for the difference in the interactions between bean plant and compatible and incompatible pathogens.

Plant resistance against microbial invasion is often associated with a hypersensitive reaction (HR) which leads to restricted necrotic lesions in the site of infection, clearly separated from surrounding healthy tissue. This localized death of host cells and necrosis deprives pathogens of the ability to penetrate neighbouring tissue, confines their growth to a small plant area and thus counteracts pathogen spread. The hypersensitive cell necrosis in the site of fungal or bacterial attack is characteristic of the incompatible plant-pathogen interaction (plant-resistant) (Mehdy, 1994; Baker & Orlandi, 1995). When the plant is invaded by a compatible pathogen (plant-susceptible), HR is not observed and the disease expands from the infection site due to the plant-compatible interaction. Biochemical events responsible for HR and their sequence are little known.

The increased generation of oxygen active species, as one of the earliest detectable plant defence responses, has been shown to occur in some plant cells in the incompatible interactions with pathogens (Doke, 1983; Tenhaken *et al.*, 1995; Kozłowska & Floryszak-Wieczorek, 1995). It is suggested that a high level of $O_2^{\cdot-}$ may

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Abbreviations: HR, hypersensitivity reaction; SOD, superoxide dismutase; $O_2^{\cdot-}$, superoxide anion JA, jasmonic acid; MeJA, methyl jasmonate; NBT, nitroblue tetrazolium.

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cause damage of membranes and disturb membrane potential, leading to a rapid death of cells invaded by pathogens (Adam *et al.*, 1989).

Antioxidative defence systems, both non-enzymatic and enzymatic, counteract toxic action of active oxygen species outside the necrotized area during HR. Superoxide dismutase is likely to be the first enzyme able to scavenge $O_2^{\cdot-}$ and its activity usually increases with overproduction of superoxide anions (Gupta *et al.*, 1993; Benes *et al.*, 1995; Adam *et al.*, 1995).

Peroxidase is assumed to fulfil different functions in defence mechanisms, e.g. oxidation of phenols to their derivatives toxic for pathogens, catalysis of polymerization of cinnamyl alcohol to lignin (a barrier difficult to be penetrated by microorganisms), metabolism of active oxygen forms, especially H_2O_2 (Gaspar *et al.*, 1985; Patykowski *et al.*, 1988; Baker *et al.*, 1995).

Jasmonates, natural hormonal plants regulators represent one of many biochemical processes to be directly or indirectly involved in resistance mechanism (Dixon *et al.*, 1994). It has been reported that they may enhance activation of various defence reactions (Dittrich *et al.*, 1992; Kauss *et al.*, 1992).

The purpose of this work was to compare $O_2^{\cdot-}$ level and the activities of superoxide dismutase and peroxidase in bean leaves infected with a compatible pathogen — *B. cinerea* and an incompatible pathogen — *B. fabae*. Moreover the effect of methyl jasmonate (MeJA) on $O_2^{\cdot-}$ and on the above enzyme activities were examined.

MATERIALS AND METHODS

Bean plants (*Phaseolus vulgaris* L.) cv. Gold Saxa were grown in a growth chamber under a 16 h light/8 h dark photoperiod at 22°C. *Botrytis cinerea* and *Botrytis fabae* were used as pathogens. At the age of 3 weeks the leaves were inoculated with a conidial suspension of *B. cinerea* or *B. fabae*. A part of the plants 3 days before inoculation were sprayed with a solution of MeJA. The inoculated leaves were harvested 3, 24 and 72 h after inoculation. The fragments of leaf tissue from the sites of inoculation and of the surrounding tissues were collected separately. Non-infected plants, treated and non treated with MeJA, were examined parallelly with the infected ones. Both pathogens were grown on potato dextrose agar in Petri dishes for 10 days at 26°C. To induce formation of conidia the cultures were exposed to UV light for 24 h, then they were washed with sterile water. The suspension containing 6×10^5 conidia in 1 ml was used for inoculation of plants. The inoculum was dropped on the upper surface of the leaves and then the plants were incubated in a humid chamber at relative humidity of 80–90% at 22°C.

Assay for generation of superoxide anion. The detection of $O_2^{\cdot-}$ was based on the reduction of nitroblue tetrazolium (NBT) according to Doke (1983). The leaf discs (ϕ 1 cm) were cut out with a cork borer. Five fresh leaf discs were immersed in 3 ml 0.01 M potassium phosphate buffer, pH 7.8, containing 0.05% NBT and 10 mM NaN_3 for 1 h. Then the mixture was heated at 85°C for 15 min and cooled. The ability of the discs to reduce NBT was expressed in absorbance units at 580 nm per 1 h per 5 discs.

Enzyme assays. Leaf tissue, 0.5 g, was homogenized in 5 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1% insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 15000 × *g* for 10 min and the supernatant obtained was used to assay superoxide dismutase (SOD) and peroxidase activities and determine NADH oxidation. All steps in the preparation of the enzyme extract were carried out at 0–4°C.

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT according to Beauchamp & Fridovich (1971). One unit of SOD was defined as the amount of enzyme that inhibited the rate of NBT reduction by 50%.

Peroxidase activity was assayed colorimertically with guaiacol as a substrate (Maehly & Chance, 1954). For the assay 0.5 ml of diluted enzyme extract, 0.5 ml 0.05 M acetate buffer, pH 5.6, 0.5 ml 0.06 M H_2O_2 and 0.5 ml 0.02 M guaiacol were used. The linear increase in absorbance at 480 nm was monitored for 4 min at 30°C. The amount of the enzyme producing the increase in absorbance equal 1.0 per 1 min was defined as one activity unit.

NADH-oxidation. Oxidation of NADH was followed at 30°C by measuring the decrease in absorbance at 340 nm (ε = 6.22 $mM^{-1}cm^{-1}$) (de Marco & Roubelakis-Angelakis, 1996). The reaction mixture consisted of 0.5 ml 1 mM

NADH, 1.5 ml 0.2 M potassium phosphate buffer, pH 7.0, and 0.5 ml of the enzymatic extract. Oxidation of NADH was carried out in the presence or absence of H_2O_2 (0.1 mM final concentration). The rate of NADH oxidation is expressed in μmoles of oxidized NADH per 1 g of fresh weight per 10 min.

The values presented in the diagrams are averages of 3 experiments.

RESULTS

A significant increase in NBT reduction indicating O_2^- generation was evident as early as 3 h after inoculation with either pathogen (Fig. 1). In the sites of direct inoculation with *B. cinerea* and *B. fabae* the O_2^- level was doubled or reached a level nearly 3.5 times as high, respectively, as in the non-inoculated leaves. Twenty four hours after inoculation the difference in O_2^- generation between the leaves inoculated with *B. fabae* and the control persisted at the same level but the difference between the sites of inoculation with *B. cinerea* and the control leaves evidently diminished. After 72 h the O_2^- generation in the sites inoculated with either pathogen decreased to the level only slightly higher than that of the control. Some increase in O_2^- generation was also observed in parts of the leaves surrounding the inoculation sites. Up to 24 h after inoculation this increase was similar for both pathogens and was higher by about 30% above the control. After 72 h the O_2^- level in the surrounding tissues was nearly doubled in the leaves inoculated with *B. cinerea* but dropped down to the level of the control in the leaves inoculated with *B. fabae*.

After inoculation with *B. cinerea* the increase in O_2^- level was markedly higher in the leaves previously sprayed with MeJA than in the non-pretreated leaves (Fig. 2). MeJA was the most effective at 2 mM concentration as at higher concentration it necrotized the tissue and its effect on O_2^- level diminished.

In the leaves infected with either *B. fabae* or *B. cinerea* no changes in SOD activity were noticed 3 h after inoculation (Fig. 3). After 24 h the activity was increased in the case of *B. cinerea* infection by 40% and then decreased, while in the leaves infected with *B. fabae* the increase in SOD activity was evident only as late as 72 h after inoculation. In the non-inoculated parts of the leaves the enzyme activity increased by about 20% of the control value 24 h after inoculation in the case of both pathogens and persisted to the end of the experiment. Treatment with MeJA did not influence SOD activity (not shown).

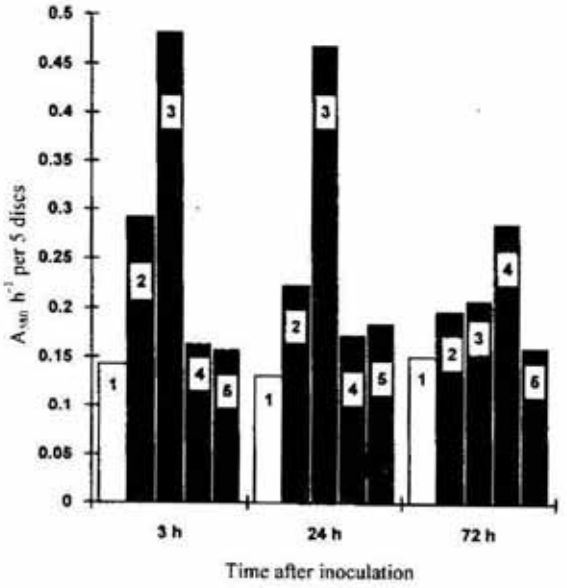

Fig. 1. Changes in NBT reducing activity in bean leaves after inoculation with B. cinerea and B. fabae.
Leaves: 1, control; 2, area directly inoculated with *B. cinerea*; and 3, with *B. fabae*; 4, area surrounding sites inoculated with *B. cinerea*; and 5, *B. fabae*.

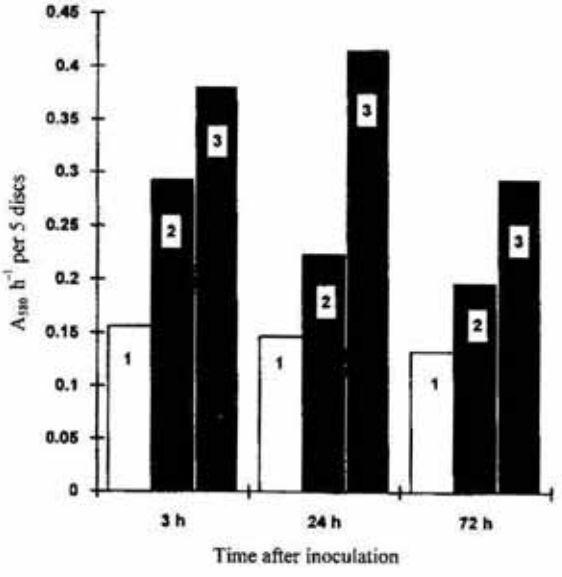

Fig. 2. Effect of pretreatment with methyl jasmonate on NBT reducing activity in bean leaves after inoculation with B. cinerea.
Leaves: 1, treated with MeJA; 2, inoculated with *B. cinerea*; and 3, pretreated with MeJA and inoculated with *B. cinerea*.

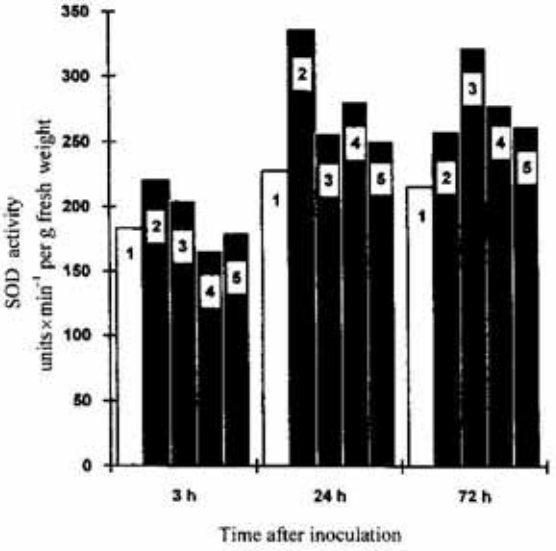

Fig. 3. Changes in superoxide dismutase activity in bean leaves after inoculation with B. cinerea and B. fabae.
Leaves: 1, control; 2, area directly inoculated with *B. cinerea*; and 3, with *B. fabae*; 4, area surrounding sites inoculated with *B. cinerea*; and 5, *B. fabae*.

Some increase in peroxidase activity measured with guaiacol was noticed as early as 3 h after inoculation with *B. fabae* (Fig. 4). In the sites inoculated with this fungus the enzyme

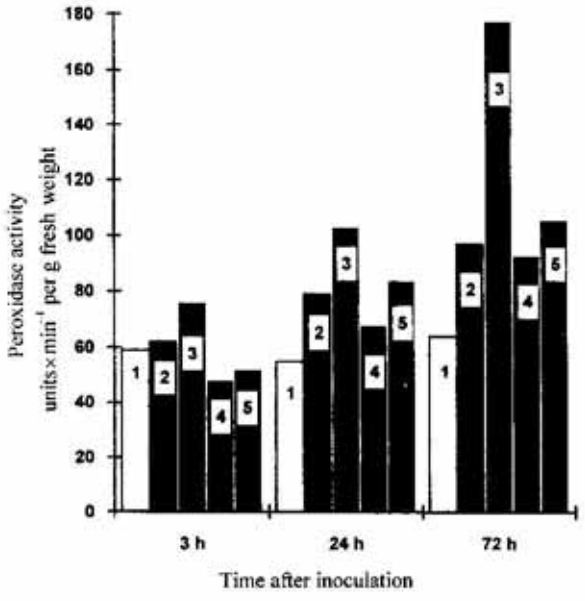

Fig. 4. Changes in peroxidase activity in bean leaves after inoculation with B. cinerea and B. fabae.
Leaves: 1, control;, 2, area directly inoculated with *B. cinerea*; and 3, with *B. fabae*; 4, area surrounding sites inoculated with *B. cinerea*; and 5, *B. fabae*.

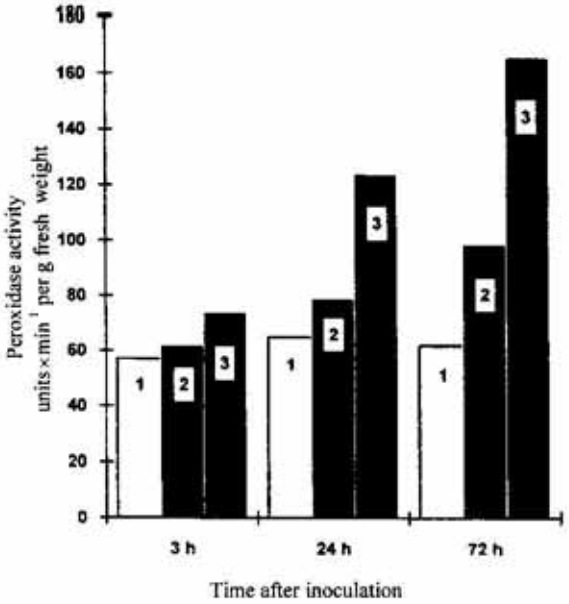

Fig. 5. Effect of pretreatment with methyl jasmonate on changes in peroxidase activity in bean leaves after inoculation with B. cinerea.
Leaves: 1, treated with MeJA; 2, inoculated with *B. cinerea*; and 3, pretreated with MeJA and inoculated with *B. cinerea*.

activity markedly increased later, and 24 h and 72 h after inoculation reached a level twice and almost 3 times as high, respectively, as compared with the control. In parts of the leaves surrounding the sites of inoculation with *B. fabae* peroxidase activity was at least by 50% higher than in the control both 24 h and 72 h after inoculation. In the case of *B. cinerea* peroxidase activity increased to a lesser extent, but in the plants treated with MeJA prior to inoculation the increase in peroxidase activity was distinctly higher (Fig. 5).

In plants NADPH or NADH oxidation is considered to constitute one of the active oxygen species generating processes (Vera-Estrella *et al.*, 1994). As shown in Fig. 6A the decrease in NADH content was faster in the reaction mixtures with the extracts from the inoculated parts of the leaves than with the control extracts. This difference was more noticeable in the leaves infected with *B. fabae* than in those infected with *B. cinerea* (Fig. 6A). Addition of H_2O_2 to the incubation mixture although resulted in a severalfold higher NADH oxidation the rate of NADH decrease with the extracts from the infected parts of the leaves was not essentially different from that with control extracts (Fig. 6B).

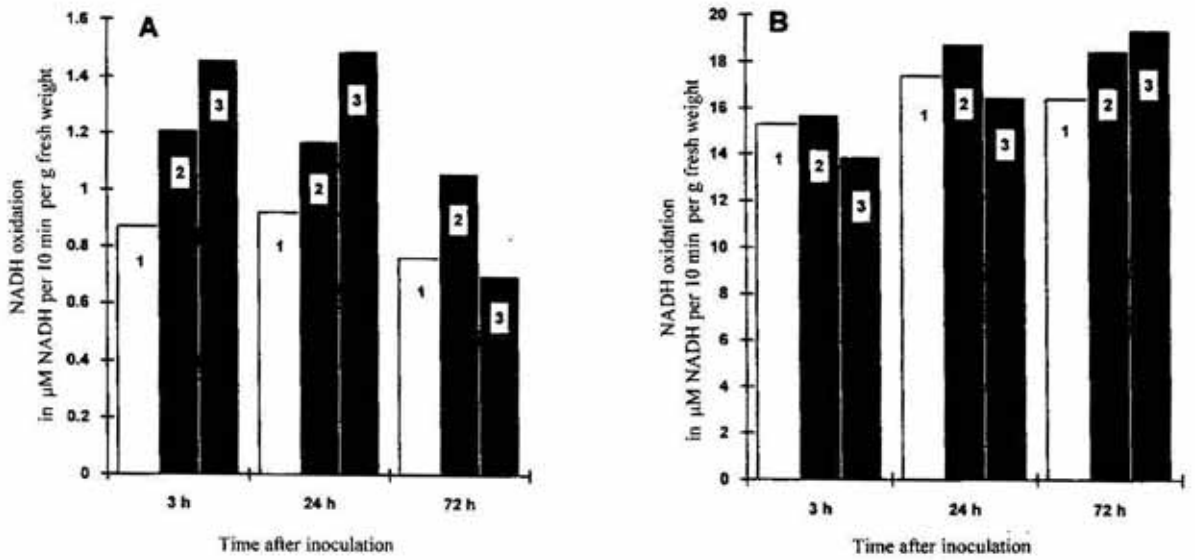

Fig. 6. Changes in the NADH-oxidation in bean leaves after inoculation with B. cinerea and B. fabae: A, in the absence of H_2O_2, and B, in the presence of H_2O_2.
Leaves: 1, control; 2, inoculated with *B. cinerea*; and 3, with *B. fabae*.

DISCUSSION

The differences in the early O_2^- overproduction in bean leaves inoculated with the compatible *B. cinerea* and the incompatible *B. fabae* may be one of the reasons of the differences observed in the plant interactions with those pathogens. This supports the important role of O_2^- in HR involved in the resistance against incompatible pathogens (Adam *et al.*, 1989). Comparatively low O_2^- production in the leaves inoculated with *B. cinerea* could be insufficient to trigger the series of events leading to HR and to stopping of the compatible pathogen expansion. The moderately higher SOD activity observed 24 h after inoculation with *B. cinerea* might have partially counteracted the effect of the increased O_2^- level. The increase in O_2^- production in parts of the leaves surrounding the sites inoculated with *B. cinerea* could constitute an inefficient response of the tissue to penetrating hyphae. The O_2^- production, in addition to being involved in HR, may play a role in other defence responses including O_2^- functioning as an intercellular stress signal molecule triggering changes in gene expression (Tenhaken *et al.*, 1995). An increased SOD activity in plant tissue surrounding the sites which are in direct contact with pathogens may protect plants against toxic action of O_2^-.

It has been reported that JA and its methyl ester, applied as foliar sprays to potato and tomato plants induced local and systemic protection against infection with *Phytophthora infestans* (Cohen *et al.*, 1993). Stimulation of O_2^- generation in bean leaves infected with *B. cinerea* by pretreatment with MeJA had no perceptible effect on HR and spreading of the disease. This suggests that although the increase in active oxygen species is an important factor contributing to disease resistance but they coact with other defence reactions and that MeJA alone is insufficient to elicit resistance against pathogens. Dittrich *et al.* (1992) and Xu *et al.* (1994) reported on the involvement of jasmonates in elicitation of such defence reactions as biosynthesis of the pathogenesis-related proteins and phytoalexins. Experimental data indicate that pretreatment with JA can enhance the ability of plant cells to respond to pathogen attack and elicitors (Kauss *et al.*, 1992; Kauss *et al.*, 1994). Also it has been suggested that JA may activate the expression of defence genes, being thus an integral part of signal transduction systems regulating gene induction in plants (Gundlach *et al.*, 1992).

In plants, several systems have been proposed to generate active oxygen species. One of them is NADPH or NADH oxidation by oxidase or peroxidase (Doke & Chai, 1985; Mehdy, 1994; Vera-Estrella *et al.*, 1994). It has been shown that peroxidase may also be able to oxidize NADPH

or NADH in the presence of molecular oxygen, generating $O_2^{\cdot-}$, which following dismutation produces H_2O_2 (Peng & Kuć, 1992; Baker & Orlandi, 1995).

NADH content decreased during incubation of extracts from leaves inoculated with either pathogen, but this process was significantly more pronounced following infection with *B. fabae*, which is in accordance with $O_2^{\cdot-}$ generation and is consistent with the opinion that NADH is one of $O_2^{\cdot-}$ sources. The lack of such a relation in the experiment in which NADH oxidation was strongly stimulated by the presence of H_2O_2 in the reaction mixture (a typical reaction of peroxidase) is at variance with participation of peroxidase in generation of $O_2^{\cdot-}$ in the infected bean leaves. A marked increase in peroxidase activity observed in the infected leaves following $O_2^{\cdot-}$ overproduction suggests that this increase does not represent early events responsible for HR. It may be rather related, as it has been hypothesized by some authors (Moerschbacher *et al.*, 1990; Brownleader *et al.*, 1995), to utilization of H_2O_2 in strengthening of the plant cell wall *via* oxidative polymerization of the cell wall phenol constituents and protein cross-linking.

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