PEROXIDASE AND SUPEROXIDE DISMUTASE ACTIVITIES IN BEAN IN RESPONSE TO OZONE

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SUMMARY

After treatment with a pulse of ozone (120 ppb, 4 h), enzymatic activities involved in the detoxification of reactive oxygen species [superoxide dismutase (SOD), total peroxidase (PX), ascorbate peroxidase (APX), syringaldazine peroxidase (SPX) and guaiacol peroxidase (GPX)] were determined in the primary leaves of two cultivars of bean (*Phaseolus vulgaris*), which on the basis of macroscopic responses were designated ozone-sensitive (cv. Pinto) and ozone-resistant (cv. Groffy). Although slightly higher in the sensitive cultivar, all PX activities were found to be at nearly comparable levels in the untreated plants of both cultivars; hovewer for SOD, constitutive levels were about twice as high in the resistant cultivar. The response to ozone developed rather slowly, being maximal after 48-96 h. Exposure to ozone caused a large increase of SOD and PX activities in the sensitive Pinto, and smaller variations in the resistant Groffy. The largest variations (up to 7-11 fold increases) were observed for APX, SPX and SOD activities in Pinto. We conclude that the induction of antioxidant enzymes does not represent a major defence mechanism against ozone in bean, but appears to be a secondary consequence of ozone injury.

RIASSUNTO

ATTIVITÀ PEROSSIDASICA E SUPEROSSIDO DISMUTASICA IN FAGIOLO IN RISPOSTA ALL'OZONO. Attività enzimatiche ritenute implicate nella detossificazione delle specie attive di ossigeno [superossido dismutasi (SOD), perossidasi totali (PX), ascorbato perossidasi (APX), siringaldazina perossidasi (SPX) e guaiacolo perossidasi (GPX)] sono state determinate 0, 4, 24, 48 e 96 h dopo l'inizio di una fumigazione con ozono (120 ppb per 4 h) nelle foglie primarie di due cultivar di fagiolo (*Phaseolus vulgaris*), che – sulla base della risposta macroscopica – erano indicate come ozono-sensibile (cv. Pinto) e ozono-resistente (cv. Groffy). Tutte le attività PX risultavano a livelli comparabili nel materiale non trattato, anche se leggermente superiori nella cultivar sensibile; il contrario era vero per SOD. L'esposizione ad ozono causava un notevole incremento delle attività SOD e PX nelle piante di Pinto e minime variazioni nelle resistenti Groffy. Le variazioni massime (incrementi fino a 7-11 volte) erano osservate per APX, SPX e SOD in Pinto. In fagiolo, l'induzione di attività enzimatiche ad azione antiossidante non costituisce il principale meccanismo di difesa dall'ozono; essa sembra essere, piuttosto, una conseguenza del danno provocato dall'inquinante.

Key words: antioxidants, Halliwell-Asada cycle, O_3 uptake, *Phaseolus vulgaris*.

INTRODUCTION

Oxidative stress by ozone (O_3) in plants has been extensively investigated in recent years. Toxicity of O_3 derives from its high redox potential which leads to the formation of the so called 'reactive oxygen species' (ROS), such as hydrogen peroxide (H_2O_2), superoxide radicals, hydroxyl radicals and singlet molecular oxygen. These species are highly destructive: they react quickly and indiscriminately with biomolecules mainly causing lipid peroxidation and ozonolysis, and protein denaturation, which in turn cause impairment of fundamental metabolic processes (Heath, 1994). For some time now differential responses to O_3 have been described, dependent both on environmental influences and on genotype. Apart from exclusion mechanisms (based on differential stomatal absorption of the pollutant) and from repair processes (which involve resynthesis of macromolecules which have been damaged), partial insensitivity to O_3 is based on the biochemical ability to counteract oxidative stress or to prevent the formation of injurious molecules ('tolerance').

As oxidative stress is a feature of all aerobic organisms, they have evolved an impressive array of protective mechanisms designed to keep these deleterious re-

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actions to a minimum. For instance, ROS are by-products of electron transport in chloroplasts, mitochondria and plasma membranes (Salin, 1987; Winston, 1990). These protective mechanisms include induction of specific antioxidant enzymes and synthesis of scavengers such as polyamines, ascorbic acid and glutathione, which compete for the radicals and remove them from the reaction (Kangasjaervi *et al.*, 1994).

Undoubtedly, a crucial role in the defense against O_3 injury can be played by oxidative stress enzymes, particularly: superoxide dismutases (SOD) along with catalases and peroxidases (PX), that act on the end product (H_2O_2) of SOD activity. It has been reported that O_3 increases the activities of several of these enzymes and this induction is considered a protective reaction of plants against this pollutant (and others) (Lee and Bennett, 1982; Bowler *et al.*, 1992; Tanaka, 1994). However, contradictory reports exist and whether this increase is actually part of a defence response or is a secondary consequence of injury is a subject of debate. Increases in activity are sometimes greatest in plants that show highest sensitivity to O_3 (Curtis *et al.*, 1976; Tuomainen *et al.*, 1996).

Working with a single kind of plant (in terms of resistance/sensitivity) may be limiting, as the biochemical variations observed may not be attributable to a single mechanism. Differential systems, such as cultivars of plant species showing strong differences in response to standard exposures to O_3 provide a useful tool to investigate mechanisms of toxicity and defence. In the present work we aimed to study the enzymatic responses of two bean cultivars exposed to O_3 under controlled conditions. Particular attention was paid to Cu/Zn SOD (the predominant class in young bean leaves; Chanway and Runeckles, 1984), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and syringaldazine peroxidase (SPX). The first three enzymes play a role in detoxifying superoxides and H_2O_2, respectively; the last one is involved in lignin biosynthesis. In more detail, PXs catalyze two reactions: (*i*) the reduction of H_2O_2 by ascorbate to form dehydro-ascorbate (basic PX, ascorbate-linked PX, Thom and Maretzki, 1985); (*ii*) the reaction with cinnamic acid (acidic PX, involved with lignification, syringaldazine-linked PX, Heath, 1988).

MATERIALS AND METHODS

Plant material and fumigation treatment. Two cultivars of bean (*Phaseolus vulgaris* L.) were selected: Pinto, O_3-sensitive (which is used to detect the presence of O_3 - Oshima, 1974) and Groffy, O_3-resistant (Tonnejick, 1983). Seeds were germinated in a greenhouse, transferred to containers and maintained under ordinary agronomic conditions. After 15 days, the plants were selected for uniformity. Exposures to O_3 were carried out in a controlled environment fumigation apparatus (Lorenzini *et al.*, 1994) when plants had the primary (unifoliate) leaves fully expanded. The fumigation system was continuously ventilated with charcoal-filtered air maintaining two complete air changes per min. Temperature was maintained at 20 ± 1°C, R.H. 85 ± 5% (vpd 0.4 kPa), PAR 530 μmol m^{-2} s^{-1}, 14 h per day photoperiod. O_3 was generated by electric discharge with a Fischer 500 air-cooled apparatus supplied with pure oxygen, and mixed with the inlet air entering the fumigation chamber; its concentration in the chamber was continuously monitored with a photometric Monitor Labs analyzer, model 8810, connected to a PC. Standard fumigations lasted 4 h and the target O_3 constant concentration was 120 ppb (235 μg m^{-3} at standard temperature and pressure). Plants were pre-adapted to the chamber conditions for 24 h. Control plants were maintained in charcoal-filtered air. Fumigated plants were maintained in the same growth chamber. Fifteen plants of each cultivar were used in each test. The experiment was replicated twice.

Calculation of O_3 uptake. The O_3 influx (F_{O3}) was calculated according to the following flux equation (Fick's law) (Unsworth, 1982):

$$F_{O3} = \Delta[O_3] \times g_{O3} \quad (1)$$

where $\Delta[O_3]$ is the difference between O_3 concentrations in air and inside the leaf. This latter was assumed to be zero (Laisk *et al.*, 1989). The conductance for O_3 (g_{O3}) was completely ascribed to the stomatal component, which was calculated multiplying the conductance for water vapour by 0.613, the ratio of the binary diffusivities of water vapour and O_3. Stomatal conductance was determined with a diffusion porometer (LI-COR 700). Uptake through the cuticle was ignored, because the cuticle is highly impermeable to O_3 when compared to open stomata (Kerstiens and Lendzian, 1989). Boundary layer resistance was neglected as the leaves were kept fluttering slightly by air circulation during the entire fumigation time. Cumulative O_3 uptake for the entire fumigation period was estimated by integration of Equation 1.

Protein extraction. Whole primary leaves were collected at the end of the fumigations and after 24, 48 and 96 h. The midvein was removed, and samples were homogenized with an Omnimixer Ultra Turax (10,000 rpm for 1 min) in 66 mM phosphate buffer pH 7.4 containing 100 mM NaCl (1 g leaf powder in 5 cm^3 of ice

cold buffer). After filtration through Miracloth (Calbiochem) the material was centrifuged at 10,000 *g* for 10 min. All operations were performed at 4°C. Aliquots of the supernatant were used to measure the enzymatic activities. Protein concentration of the enzyme extract was determined by the protein-dye binding method with BSA as the standard (Bradford, 1976).

Determination of enzymatic activities. SOD activity was determined by the xanthine oxidase cytochrome c method, according to McCord and Fridovich (1969). SOD activity of bean leaf extract is nearly completely inhibited by the presence of 2 mM potassium cyanide, implying that it must be predominantly ascribed to Cu/Zn SOD (Hassan and Scandalios, 1990). Total PX activity was monitored using 2,2'-azino di-[3-ethyl-benzothiazoline-(6)sulphonic acid] as a substrate, according to Werner *et al.* (1970). The reaction was carried out at pH 6.0, in the presence of 2 mM ABTS and 1mM H_2O_2 and monitored spectrophotometrically at 405 nm. The absorbance increase at this wavelength corresponds to the formation of the ABTS+ cation, which is produced by the reaction. GPX activity was determined in a reaction mixture that contained 50 mM NaH_2PO_4 (pH 6.1), 16 mM guaiacol, 2 mM H_2O_2 and 10 µl of leaf extract (Castillo and Greppin, 1986). The activity was measured by monitoring the increase in absorbance at 470 nm during the polymerization of guaiacol to tetraguaiacol. APX activity was determined in a reaction mixture (1 cm^3) which contained 50 mM NaH_2PO_4 (pH 7.0) 0.1 mM EDTA, 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 20 µl of leaf extract. The activity was measured by monitoring the rate of ascorbate oxidation at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) (Luwe *et al.*, 1993). SPX activity was assayed in 100 mM phosphate buffer (pH 6.0) containing 1.6 mM H_2O_2 and 0.1 mM syringaldazine dissolved in methanol/dioxane 1:1. The increase in absorbance was monitored at 530 nm (Pandolfini *et al.*, 1992). For all but SPX enzymatic activities, one international unit was calculated as the enzyme quantity that transforms 1 µmol of substrate in 1 min. For SPX, one arbitrary unit was calculated as the variation of absorbance (ΔA_{530} $min^{-1}mg^{-1}$ protein, Ranieri *et al.*, 1995).

RESULTS

Visible injury. As expected, only the sensitive cv. Pinto showed visible injury, in the form of small white necrotic lesions (flecks), confined to the interveinal margins of the upper leaf surface (Craker and Starbuck, 1972). These symptoms appeared about 24 h after fumigation.

Ozone uptake. O_3 uptake during the fumigation was rather similar in the two cultivars (Fig. 1). Estimated cumulative uptake was 345 ± 92.2 mmol O_3 m^{-2} in Pinto *vs* 330 ± 6.8 mmol O_3 m^{-2} in Groffy ($P > 0.05$, Student's *t*-test).

Total protein content. The total leaf extractable protein content did not change significantly in response to O_3 exposure (data not shown).

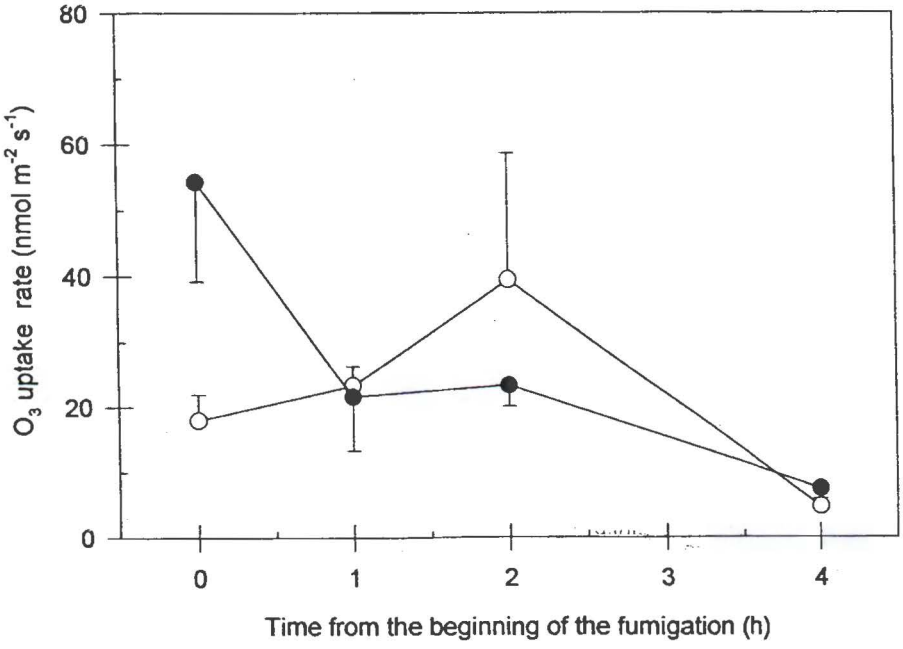

Fig. 1. Time course of ozone uptake during the fumigation (120 ppb, 4 h) in bean cv. Pinto (open circles) and cv. Groffy (closed circles); vertical bars represent SD (where they do not appear, their value is negligible).

Superoxide dismutase activity. Under non-stress conditions, Cu/Zn SOD specific activity (*i.e.* referred to the protein content) in Groffy was about twice that in Pinto (53.0 ± 3.00 *vs* 25.3 ± 6.51 U mg^{-1} protein, $P < 0.01$, Student's *t*-test). Following exposure to O_3, a dramatic increase in SOD activity was observed in Pinto (9 fold increase) within 48 h after the treatment (Fig. 2). Two days later, the activity decreased, although it remained about 7 times higher than the background. In contrast, the Cu/Zn SOD activity in cv. Groffy only showed minimal variations as a result of fumigation.

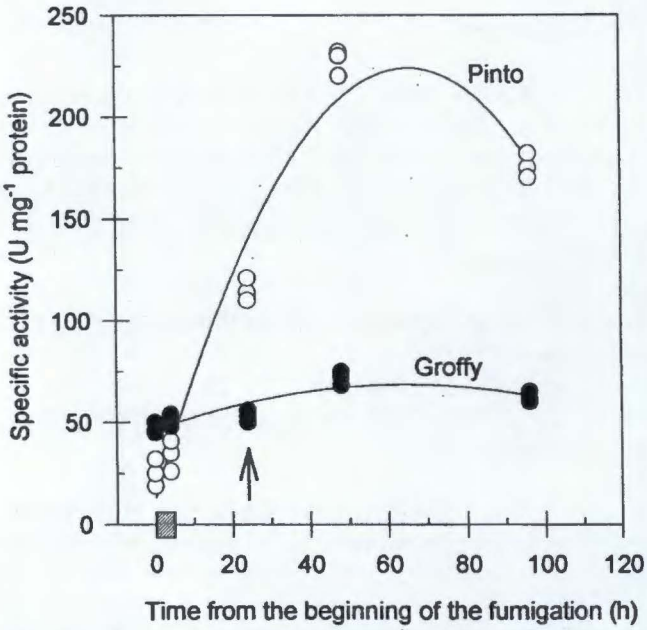

Fig. 2. Time course of superoxide dismutase activity in leaf extracts of bean, cvs Pinto (open circles) and Groffy (closed circles) exposed to 120 ppb ozone for 4 h. The dashed box on the abscissa indicates the fumigation period. The arrow indicates the appearance of visible injury on the sensitive Pinto plants. Each point represents the average of the replicates within each experiment.

Total peroxidase activity. Background levels of specific PX activity were slightly different in the two cultivars (2.5 ± 0.25 U mg^{-1} protein in Pinto *vs* 1.8 ± 0.24 in Groffy, $P < 0.05$, Student's *t*-test). Exposure to O_3 caused in Pinto a sharp but temporary increase in PX activity, which reached a three fold increase 48 h after the beginning of fumigation (Fig. 3). PX activity was twice that of the background 96 h after the beginning of fumigation. The behaviour of Groffy was different: a progressive increase was observed from the end of fumigation, with a maximum 2.4-fold increase above background at 96 h.

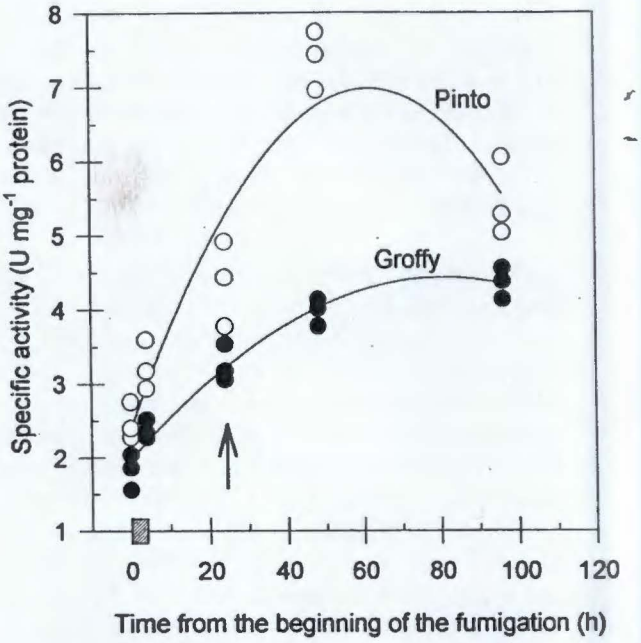

Fig. 3. Time course of total peroxidase activity in leaf extracts of bean, cvs Pinto (open circles) and Groffy (closed circles) exposed to 120 ppb ozone for 4 h. The dashed box on the abscissa indicates the fumigation period. The arrow indicates the appearance of visible injury on the sensitive Pinto plants. Each point represents the average of the replicates within each experiment.

Electron-donor-specific peroxidase activities. Minimal differences in the specific activities monitored were detected in the background values of the two cultivars. While no apparent effect was induced by fumigation in Groffy, APX activity in ozonated leaves of Pinto, at the end of the experimental period, was 11.4 times higher than the background (Fig. 4, A). In the same way, GPX activity was stimulated by O_3 treatment especially in Pinto, with an increase over background which reached a factor of about 4. Only a slight increase was observed in Groffy (Fig. 4, B). Fumigation also stimulated SPX activity in Pinto much more than in Groffy (Fig. 4, C).

DISCUSSION

As O_3 uptake in the two cultivars was very similar, their differential response is not related to an exclusion mechanism, in agreement with the report of Hucl *et al.* (1982). Biochemical mechanism(s) should therefore be involved. Physiological and genetic evidence indicates that ROS scavenging systems should play an important role in stress protection (Pell and Reddy, 1991).

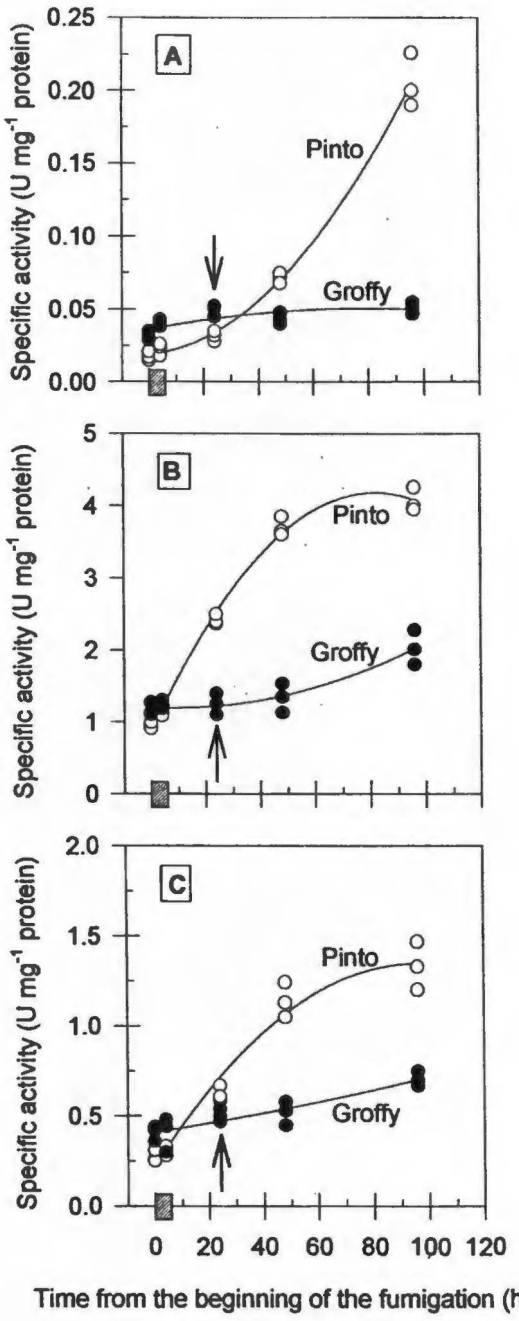

Fig. 4. Time course of ascorbate- (A), guaiacol- (B) and syringaldazine- (C) peroxidase activities in leaf extracts of bean, cvs Pinto (open circles) and Groffy (closed circles) exposed to 120 ppb ozone for 4 h. The dashed box on the abscissa indicates the fumigation period. The arrow indicates the appearance of visible injury on the sensitive Pinto plants. Each point represents the average of the replicates within each experiment.

Previous investigations on bean have shown PX increases as a consequence of exposure to O_3 (*e.g.* Astorino *et al.*, 1995; Brunschoen-Harti *et al.* 1995). In our work, significant increases of the enzymatic activities investigated (*i.e.* Cu/Zn SOD, total PX, APX, GPX, SPX) were clearly observed only in the O_3-sensitive cultivar. The increase of APX activity in Pinto occurred 48 h after fumigation, clearly after the appearance of symptoms. The increases of GPX and SPX were concomitant or occurred soon after the symptoms. Rather similar results have been obtained for APX in tobacco and GPX in *Arabidopsis thaliana* by Willekens *et al.* (1995) and Kubo *et al.* (1995), respectively, but not for APX in *A. thaliana*, whose activity was enhanced before the appearance of symptoms (Kubo *et al.*, 1995).

The fact that PX activities were induced more rapidly and intensively in the O_3-sensitive than in the resistant plants, only in concomitance or after the appearance of symptoms, strongly suggests, in agreement with the results of previous work (Dass and Weaver, 1972; Endress *et al.*, 1980; Bender *et al.*, 1990) that increased PX is a secondary consequence of O_3-induced injury. Unfortunately the usefulness of PX as a quantitative marker of O_3 injury seems to be minimal, due to the interference of other environmental factors (Petolino *et al.*, 1983).

Constitutive levels of SOD activity in the untreated resistant plants were about twice those found in the sensitive ones. The significance of this finding should be established with an higher number of bean cultivars. No correlation was found between O_3 sensitivity and constitutive levels of SOD in ten bean cultivars (McKersie *et al.*, 1982). The huge differences in O_3 sensitivity in tobacco between cv. Bel-W3 (supersensitive) and Bel-B (resistant) are not related to basic differences in SOD and APX levels (Tanaka *et al.*, 1990). Tanaka *et al.* (1985) found no remarkable differences in constitutive SOD, GPX and APX activities among six spinach cultivars differing in response to O_3.

SOD activity has been similarly enhanced by O_3 in a resistant and in a sensitive cultivar (McKersie *et al.*, 1982) and increases in SOD have been related to O_3 tolerance in snapbean (Lee and Bennett, 1982). Our results confirm the strong association between injury induced by O_3 and SOD activity found by Pitcher *et al.* (1992). SOD activities in response to O_3 in other plants may vary in a conflicting fashion and there is no clear evidence for SOD involvement in O_3 resistance (Bowler *et al.*, 1992). The view that induction of antioxidant enzymes does not represent a major defence system against O_3 in bean is thus reinforced by the present work.

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