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ACTIVATION OF DETOXIFYING ENZYMES IN TOMATO ROOTS FOLLOWING PARAQUAT TREATMENT AND NEMATODE INFECTION

by
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Summary. Highly reactive oxygen radicals are often involved in plant stress. Plants react to the environmental adversity by activating enzymes involved in oxygen detoxification. Tomato roots were analyzed after nematode infection and paraquat treatment. It was found that paraquat increased the superoxide dismutase activity more than nematode infection and this was accompanied by an increase in peroxidase activity. Cytoplasmic and cell wall peroxidases from infected and paraquat treated seedlings were examined. Pronounced differences were noticed between the paraquat-treated and untreated tissue extracts. Gel electrophoresis showed the presence of an anionic fast migrating isoperoxidase group with a high syringaldazine-oxidase activity. It appears that paraquat induced a greater increase of the two detoxifying enzymes than nematode infection. Increase of superoxide dismutase and peroxidase activity results to be an adaptive response which provides the plant with protection against biotic or abiotic stress.

In plants, environmental adversity leads to the activation of an oxidative metabolism. The by-product of oxidative stress is the generation of superoxides and enzymatic dismutation, i. e. the interaction of two superoxide radicals in such a way that one is reduced and the other oxidized to yield hydrogen peroxide (H_2O_2). Although neither superoxide nor hydrogen peroxide at physiological concentrations seem particularly harmful, their toxicity *in vivo* arises by their interaction. In the presence of iron salts, the combination of superoxide radicals and hydrogen peroxide leads to the rapid formation of the highly reactive hydroxyl radical (OH·) which is thought to be responsible for oxygen cytotoxicity *in vivo*. Living cells have evolved a wide range of enzymatic mechanism to lower the levels of such deleterious oxygen species. These mechanisms include superoxide dismutase (SOD), catalase and peroxidase. SOD scavenged superoxide radicals, and hydrogen peroxide generated as a result of this reaction is scavenged by catalase and peroxidase. The role played by the oxy-radical in plant disease (Doke *et al.*, 1987) and the defense mechanism activation by injured tissue (Zacheo and Bleve-Zacheo, 1988) has been well documented. Paraquat (1,1'-dimethyl-4,4'-dipyridinium-bis-dimethyl sulphate), a herbicide widely used, can act as an *in vivo* generator of superoxide radical. It forms relatively stable paraquat radicals that react very rapidly with dioxygen to generate superoxide radical (Hassan and Fridovich, 1978).

The increased intracellular flux of O_2^-, under appropriate conditions, causes the induction of SOD (Rabinowitch, 1983; Zacheo *et al.*, 1991). Since hydrogen peroxide is generated by the reaction catalyzed by SOD, it is reasonable to hypothesize that peroxidase within the cell may be regulated to cooperate with SOD in facilitating the detoxification. We believe that in plant stress, the determining factor is the ratio between superoxide and hydrogen peroxide within the cells. In an attempt to clarify some of the questions about plant stress, we compared the behaviour of detoxifying enzymes, i. e. SOD and peroxidase, in tomato roots infected with *Meloidogyne incognita* (Kofoid *et* White) Chitw. and/or treated with paraquat. Here we report the qualitative and quantitative differences of SOD and peroxidase isozymes in tomato roots under biotic and/or abiotic stress.

Materials and methods

Seeds of tomato cv. Roma VF were germinated in sterilized quartz sand. Uniformly germinated seedlings were transferred into 3 cm clay pots containing quartz sand, exposed to 10 µM paraquat (PQ) and divided into two groups, one uninfested and used as the control, and the other immediately inoculated with active juveniles of *Meloidogyne incognita* race 2. Measurements (time course) were made on seedlings at 0, 2, 4, 7, 10, 14 and 21 days or in other experiments 7 days after inoculation with nematodes. Root tissues were homogenized in 100 mM EPPS (N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid) buffer, pH 7.8 containing 1% of insoluble polyvinylpyrrolidone, in a Potter homogenizer cooled in ice. The resultant slurry was filtered through 4 layers of cheesecloth and centrifuged

at 10,000 g for 20 minutes. The pellet was washed 6 to 8 times with distilled water by homogenizing the cell sediments in a potter. The washed walls were used for protein and peroxidase assay (Stafstrom and Staehelin 1986).

The supernatant was made up to 1.37 M by adding solid ammonium sulphate slowly over 1 h and stirred for an additional hour. The suspension was centrifuged and the supernatant adjusted to 3.71 M $(NH_4)_2SO_4$ as described above. The suspension was then centrifuged and the precipitate dissolved in 50 mM potassium phosphate (Pi), pH 7.0, and dialyzed against the same buffer. The dialyzed protein solution was centrifuged and concentrated on Amicon membrane Centricon 10 (Renaldo *et al.*, 1981). This fraction was used as a crude extract for enzymatic assays and proteins. The samples were partially purified by mixing for 90 minutes 1:1 v/v of pre-swollen resin of DEAE-Sepharose CL-6B (Pharmacia), equilibrated in 50 mM phosphate buffer pH 7.0 and then filtered through a Millipore apparatus equipped with a 0.22 μm filter. The recovered resin was mixed for 90 min with Pi buffer containing 0.5 M NaCl and then filtered as described before. Part of the pigments present in the protein extracts remained attached to the resin. The filtrates of the first and second steps were mixed and dialyzed against Pi buffer. This procedure was repeated twice. The resultant solutions were concentrated by a Centricon 10 membrane and used to assay SOD activity or to separate the SOD isoenzymes. Protein concentration was measured by the protein-dye binding assay (Bradford, 1976). The SOD activity was assayed according to the procedure described by Furusawa *et al.* (1984). Samples (6 μg of proteins) were analyzed by non denaturating sodium dodecyl sulphate (SDS)-PAGE carried out according to Laemmli (1970) by using 10-13% linear gradient polyacrylamide gels. Gels were electrophoresed for 1 h at a current of 40 mA and 900 Volts at 7 °C. The SOD was visualized on the gels by the staining technique of Beauchamp and Fridovich (1971). Inhibitor studies were performed on the gels to distinguish between Cu/Zn-SOD isozymes (Sandalio *et al.*, 1987) in presence of 2 mM KCN or 5 mM H_2O_2.

Anionic peroxidases were purified from the crude extracts by HPLC ion exchange chromatography through DEAE-Sepharose CL-6B (Pharmacia) column (2.5 x 20 cm) equilibrated with 50 mM Na-K phosphate buffer, pH 7.2. The cationic proteins that did not adsorbe to the DEAE column were recovered in the flow-through fraction. The anionic proteins were eluted with a linear gradient from 0.25 to 0.5 M NaCl in 50 mM Pi pH 7.0. Flow rate was 0.5 ml min^{-1} and 0.5 ml fractions were collected. The detector was set at 280 and 407 nm. Fractions with Reinheitzahl (RZ) value (A_{407}/A_{280}) exceding 1 were pooled and concentrated (Centricon 10) for enzyme assay.

Cell wall bound peroxidase and soluble peroxidase activity was assayed spectrophotometrically with two different electron donors. Syringaldalzine-oxidase was assayed in 50 mM Pi pH 7.0 containing 25 μM syringaldalzine and 250 μM H_2O_2 and p-phenilenediamine-pyrocatechol (PPD-PC)-oxidase in 50 mM Pi pH 7.0 containing 0.17 mM PPD, 4.5 mM PC and 250 μM H_2O_2. The increase in assorbance was recorded at 530 and 575 respectively (Imberty *et al.*, 1985). The isoperoxidases were separated by SDS-PAGE and stained by using three different chromogens, benzidine, syringaldazine and PPD-PC. Polypeptides were analyzed on discontinuos SDS-PAGE (see before), after denaturation by boiling the samples for 3 min in presence of 1% SDS and 4% mercaptoethanol. Gels were stained with 0.2% Coomassie blue and destained in a solution of acetic acid (7%) – methanol 5%. The molecular weights of the isoenzymes were determined on SDS-PAGE by using as standard: apoferritin (443,000), alcohol dehydrogenase (150,000), bovine serum albumine (66,000), ovoalbumin (45,000), carbonic anhydrase (29,000), cytochrome c (12,400) and aprotinin (6,500).

Values were obtained from densitometric scanning of the gels stained for peroxidase activities. All reported values are the means of three different experiments and triplicate analyses were performed on each extract.

Results

Effects of paraquat and nematode infection on SOD content.

In preliminary experiments, tomato seedlings were exposed to different concentrations of PQ (O - 200 μM). Concentration of 10 μM PQ inhibited the growth of the seedlings by about 50%. At 10 μM PQ, no effect was detected on nematode growth and reproduction. The addition of 10 μM paraquat in the watering solution diminished the growth rate of the plants, but increased the total SOD content of the roots. The changes of SOD activity is clearly illustrated by the data obtained from ammonium sulphate (Fig. 1) and DEAE purified fractions (Table I). The time course indicat-

Table I - *Activity of SOD isoenzymes in tomato roots following treatment with paraquat and nematode infection. Enzymes were extracted 7 days after treatments. The CN-insensitive Mn-SOD was differentiated from the CuZn-SOD by performing the reaction in presence or absence of 2 mM cyanide. Values are means of three different experiments. Activity is expressed as U mg^{-1} of proteins.*

	Uninfected	Infected	Paraquat	Paraquat + infected
SOD	62	96	115	116
CuZn-SOD	20	36	42	43
Mn-SOD	42	60	73	73

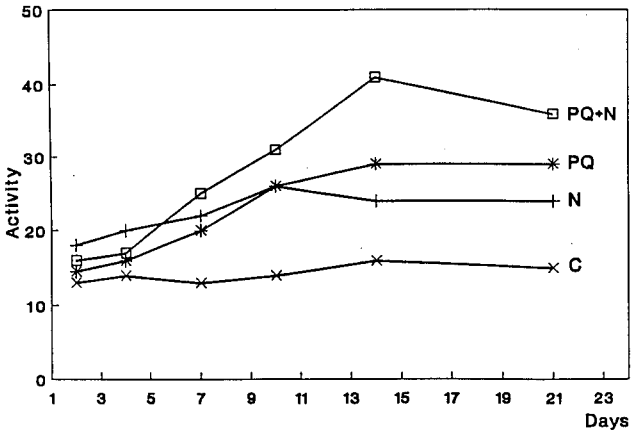

Fig. 1 - Time course for induction of superoxide dismutase activity in roots of tomato plant (Roma) uninfected (C) and infected with *M. incognita* (N), or treated with 10 µM paraquat (PQ) and both paraquat treated and nematode infected (N + PQ). Activity is expressed as U mg^{-1} of proteins. Values are mean of activity from three representative experiments.

ed that little changes in SOD activity were detected in the ammonium sulphate fractions of paraquat-treated roots during the first two days of incubation. Thereafter there was a rapid increase in SOD activity, reaching a maximum level of about two times that of the control value at the 14th day. Root infection by nematodes leads to about a doubling in the total SOD activity 10 days after infection. As a consequence of the concomitant root infection and paraquat treatment, the activity of SOD increased almost linearly and reached a value three times more than in the untreated control. Because of interferring substances present in ammonium sulphate extracts, it has been difficult to distinguish the different isoforms of SOD. In order to avoid this interference, the ammonium sulphate fractions of proteins were partially purified from total protein extract. A substantial enrichment in SOD was obtained after the proteins were purified on DEAE - Sepharose. On the basis of specific activity the partially purified SOD was estimated to be five times higher than that found in crude extracts (Table I). Spectrophotometric analysis of SOD activity showed that tomato roots contained about 30% of cyanide and H_2O_2 sensitive superoxide dismutase activity and 70% insensitive to both inhibitors. KCN and H_2O_2 are commonly used to distinguish between MnSOD (resistant to both H_2O_2 and KCN) and Cu, Zn-SOD (sensitive). Under the procedure described here the activity of Mn-SOD was predominant in tomato roots (Table I). Analysis of SOD activity in infected or/and paraquat treated tissue revealed that both Cu/Zn-SOD and Mn-SOD increased under stressed conditions. These results were confirmed by subsequent analysis of the purified proteins on non-denaturating polyacrylamide gel electrophoresis (Fig. 4). Two main bands (c, d) were identified, a slower-moving Mn-SOD (resistant to both H_2O_2 and KCN) and a quickly migrating SOD, that are most likely cytosolic Cu/Zn-SOD. Two more bands (arrows) slower-migrating than Mn-SOD were also identified as KCN and H_2O_2 resistant. These two last could be a dimeric or multimeric subunit composition of Mn-SOD.

Peroxidase induction

Soluble peroxidases (1.37-3.7 M ammonium sulphate saturation) from control, paraquat-treated and nematode-infected tomato roots were examined spectrophotometrically by two different chromogens syringaldazine and PPD-PC. Important differences were obtained with the two substrates: oxidation of syringaldazine and PPD-PC might then be catalyzed by different isoenzymes. As shown in Fig. 2, syringaldazine oxidase activity increased in the cytoplasmatic fraction almost linearly starting 48 h after paraquat

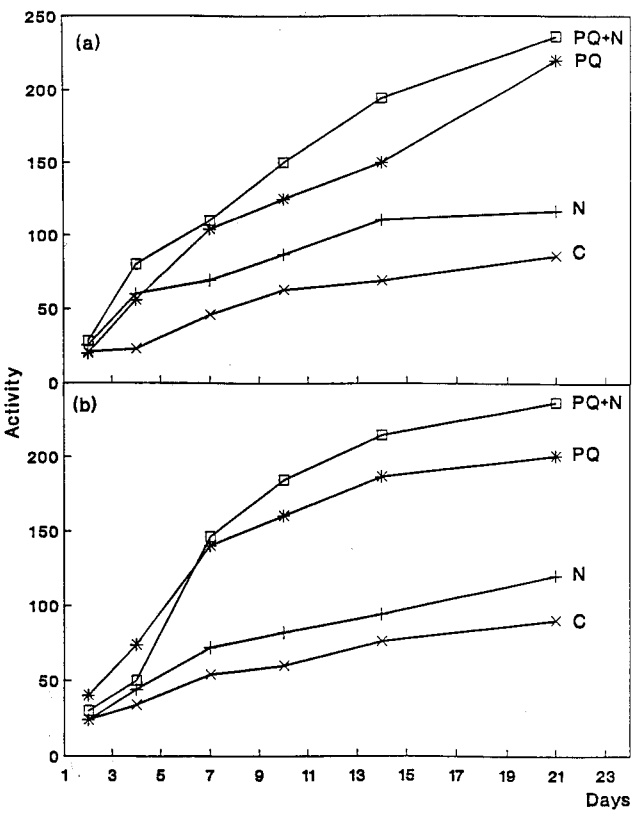

Fig. 2 - Time course of nematode infection and paraquat treatment on syringaldazine-oxidase in cytosol (a) and cell wall (b). Peroxidase activity is expressed as O.D. min^{-1} mg^{-1} of proteins. (Legend: see Fig. 1).

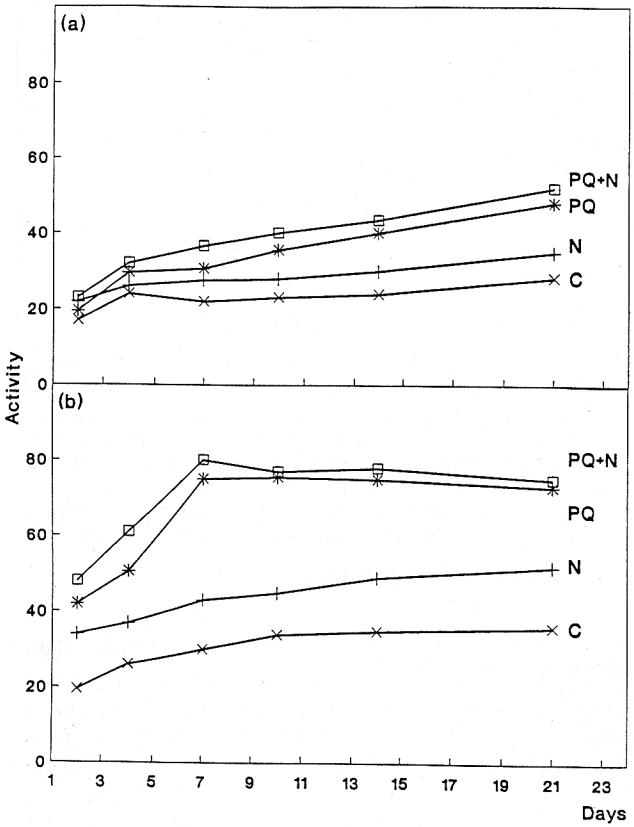

Fig. 3 - Time course of nematode infection and paraquat treatment on PPD-PC-oxidase in cytosol (a) and cell wall (b). Peroxidase activity is expressed as O.D. min^{-1} mg^{-1} of proteins. (Legend: see Fig. 1).

treatment and reached during the time course of these experiments. There was an increase of more than two fold in syringaldazine-oxidases in the extracts from plants both paraquat treated and paraquat treated and infected, while peroxidase increased slightly in extracts from infected roots (Fig. 2). Similar results were also obtained by examining peroxidases on cell wall fractions.

Extracts from tomato roots showed a slight increase in the activity of PPD-PC oxidases. These isozymes revealed a moderate increase of cell wall activities during the period from 2 to 7 days in roots treated only with paraquat and in infected roots also treated with paraquat (Fig. 3). These experiments clearly indicate that the increase of peroxidase activity on stressed plants can be attributed to the increased activities of syringaldazine oxidases. These activities were reported to be strongly bounded to the lignifying cell walls (Imberty *et al.*, 1985). As anionic peroxidases are thought to exhibit a high affinity for syringaldazine, we separated and analyzed the anionic peroxidases from crude extracts obtained from roots treated for 7 days. The anionic peroxidases accounted for nearly 80% of peroxidase activity extracted from roots and exhibited a high affinity for syringaldazine. The infected plants showed only a slight increase of anionic peroxidases when compared with paraquat treated plants. The results indicate that paraquat treatment leads to about a six fold increase in the activity of anionic syringaldazine oxidases compared with the untreated control (Table II).

All the anionic isoperoxidases present in the extracts and separated on non-denaturating SDS gel acrylamide showed a high affinity for the electron donor diaminoben-

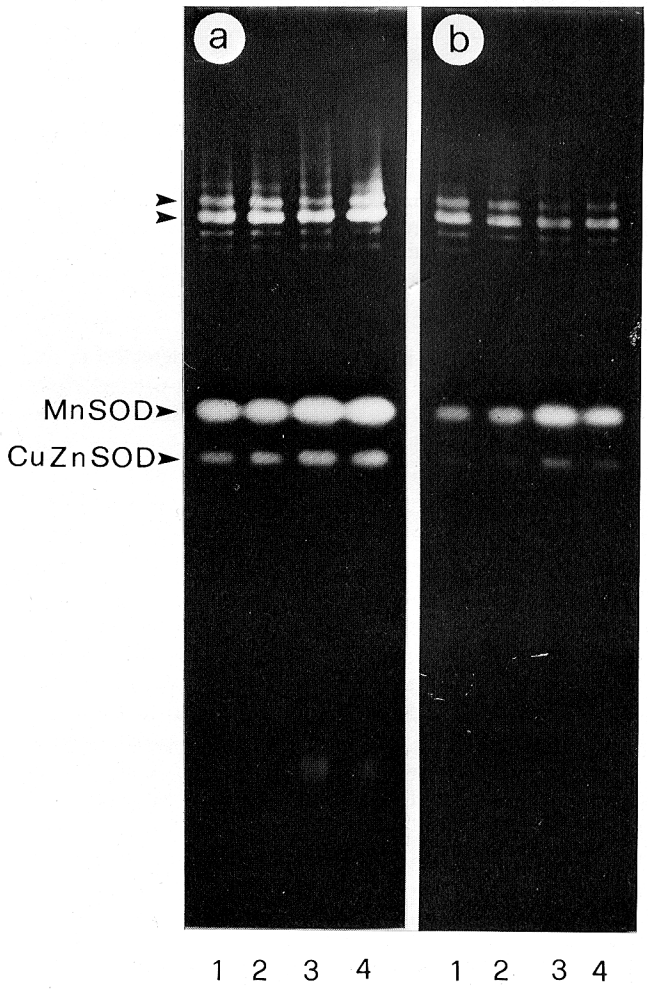

Fig. 4 - SOD activity profiles on native polyacrylamide gels of tomato plants after 7 days of nematode infection and paraquat treatment. Line 1 uninfected, line 2 infected, line 3 paraquat treated, line 4 both paraquat treated and nematode infected. Each sample loaded on the gels contains 6 µg partially purified protein. Gels were stained for SOD activity by photochemical method using NBT (a), and NBT + 2mM KCN (b). Lines 1 and 2 untreated, lines 4 and 4 paraquat-treated. Lines 1 and 3 uninfected, lines 2 and 4 infected.

Table II - *Anionic peroxidase activities in tomato roots after paraquat treatment or nematode infection. Values are means of three different experiments. Enzymes were extracted 7 days after treatments. The activity is expressed as O.D. min^{-1} mg^{-1} of proteins.*

	Uninfected	Infected	Paraquat	Paraquat + infected
Syringaldazine oxidase	90	120	550	540
PPD-PC-oxidase	26	40	105	100

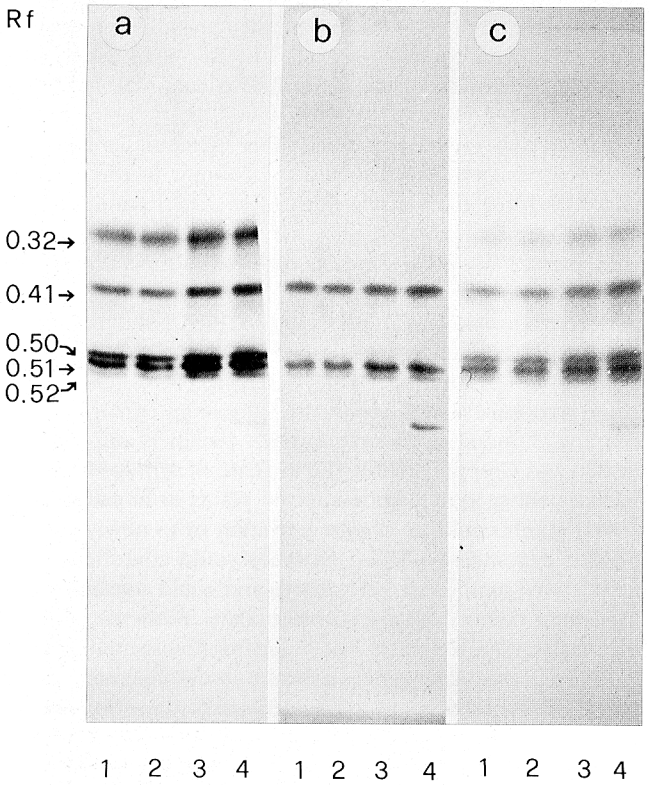

Fig. 5 - Anionic peroxidase induced by nematode infection and paraquat treatment after 7 days. Isoenzymes were separated by non denaturating SDS-PAGE and stained by benzidine (a), syringaldazine (b) and PPD-PC (c). Lines 1 and 2 untreated lines 3 and 4 paraquat-treated. Lines 1 and 3 uninfected, lines 3 and 4 infected.

zidine (Fig. 5a). The benzidine stained patterns revealed that the bands of uninfected (lane 1) and infected (lane 2) root extracts were qualitatively and quantitatively very similar, with the exception of the band with Rf 0.51 which increased in intensity in infected plant extract. Following treatment of plants with paraquat (lanes 3 and 4) four

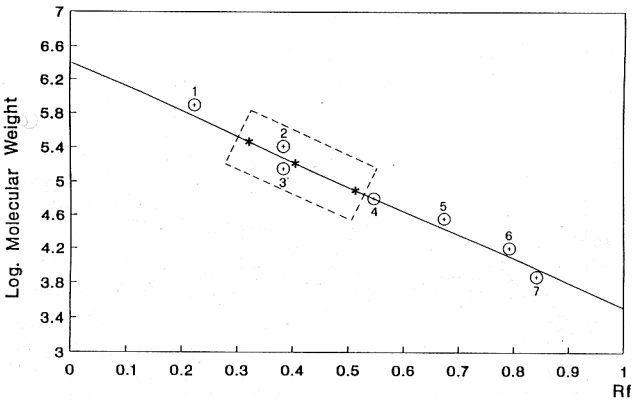

Fig. 6 - Estimation of the molecular weights of anionic peroxidases (*) by SDS-PAGE electrophoresis. Gels were calibrated with known protein standards, including (1) apoferritin (443,000), (2) alcohol dehydrogenase (150,000), (3) bovine serum albumine (66,000), (4) ovoalbumin (45,000), (5) carbonic anhydrase (29,000), (6) cytochrome *c* (12,400) and (7) aprotinin (6,500).

prominent bands (Rf 0.32, 0.41, 0.50, 0.51) and one slight (0.52) of isoenzymes were localized. When peroxidase isoenzymes were stained by syringaldazine (Fig. 5b) two main bands (Rf 0.41, 0.51) were visualized. However, the effects of paraquat on tomato roots were more evident in the band with Rf 0.51. As gels were incubated in the presence of PPD-PC, four isoperoxidases were detected. The PPD-PC isoperoxidases were more active in the paraquat treated plants (Fig. 5c, lanes 3 and 4) in bands with Rf 0.41 and 0.51. Anionic peroxidase exhibited a molecular weight of 150 kD for the isozymes showing an Rf of 0.32, of 100 kD for the isozymes with Rf 0.41 and about 54, 50 and 47 kD for the isozymes with Rf 0.50, 0.51 and 0.52 respectively (Fig. 6).

Discussion

Our interest in the SOD is primarily concerned with the role that this class of enzymes plays in biotic stress in plants and on the physiological relationship between these enzymes and the peroxidases. When tomato roots were exposed to paraquat, the SOD content increased in the tissues and this could be considered as a protective action against the herbicide. The toxicity of paraquat appears to be due to its ability to exacerbate the intracellular production of the superoxide radical (Moody and Hassan, 1984). Moreover, the presence of O_2 accentuates the sensitivity of the plants and animals (Hassan and Fridovich, 1978). SOD is a protective enzyme responsible for maintaining lower levels of superoxide radicals within the cells. It acts by catalyzing the

dismutation of two superoxide radicals to yield hydrogen peroxide and oxygen. Based on the knowledge that hydrogen peroxide is produced by the dismutation of superoxides, it could be thought that the higher SOD activity increases the production of H_2O_2. Further reduction of hydrogen peroxide inside the cell, produces reactive hydroxyl radicals which are more cytotoxic and the consequence of their increased production results from the damage caused to cellular membranes, organelles and their associated enzymes. It is imperative that hydrogen peroxide be quickly removed from the tissue. In tomato roots treated with paraquat or infected with nematodes, peroxidase activity is significantly increased together with that of SOD. This is believed to be due to an adaptative level of hydrogen peroxide produced by the augmented SOD activity. In view of the known generation of hydroxyl radical by the interaction between H_2O_2 and O_2^- (Kellogg and Fridovich, 1975) it is likely that the amount of hydrogen peroxide is important in regulating the toxicity in plant cells.

In the present work the increased enzymatic activities of peroxidase together with the SOD may readily enable the cells to deal with the superoxide and hydrogen peroxide formed after paraquat treatment and nematode infection. In plants, under normal conditions a delicate balance exists between the rate of hydrogen peroxide formation, via dismutation of superoxides, and its removal by peroxidases. The increased SOD activity induced by biotic or/and abiotic stress could lead to an increase in the steady-state levels of hydrogen peroxides which may enhance the peroxidase activity. Our previous results (Zacheo *et al.*, 1991) demonstrated that simultaneous exposure of tomato roots, susceptible to *Meloidogyne incognita*, to paraquat and nematode infection induced at the nematode feeding site, not only multinucleate cells but also some small necroses in the neighbouring tissues. It is possible that in plants, the initial degree of protection is not sufficient to counteract the combined action of paraquat and nematode, and thus radicals induce damage at the membrane level. The overproduction of radicals could produce a cellular signal to increase the SOD activity and other detoxifying enzymes.

Localized necroses induced by the hypersensitive reaction (HR) in infected tissue are reported to be due to the generation of superoxide radicals when the plants are infected by incompatible pathogens. The HR is characterized by the rapid development around the infected area of necroses which prevent further spread of the pathogens. The biological source of the O_2^- is only partially defined. Some authors (Doke *et al.*, 1987; Zacheo and Bleve-Zacheo, 1988) found that large amounts of superoxides, produced in an incompatible host-pathogen interaction are generated in the membranes by NADPH-oxidase. It is interesting that increase of this oxygen species enhances lipid peroxidation (Keppler and Baker, 1989) and is correlated with decrease of SOD (Zacheo *et al.*, 1991; Dhindsa *et al.*, 1982). The physiological consequences of increased lipid peroxidation (Adam *et al.*, 1989) result from the damage caused to cellular membranes (Mayak *et al.*, 1983), organelles and associated enzymes. In a compatible situation, nematode infection provides sufficient protection by increasing SOD activity. Consequently the superoxides generated in susceptible tissue can be scavenged, the hydrogen peroxide produced eliminated by O_2-dependent enzymes, and a hypersensitive reaction does not occur.

The detoxification of H_2O_2 depends not only on the ability of endogenous enzyme scavenging the H_2O_2 (peroxidase, catalase) but also by the stimulation of such enzymes activated by the accumulation of this reactive product. The large induction of peroxidase activity, due to paraquat treatment, could be activated by the plant tissue to limit the production of the powerfull oxidant OH· by destroying H_2O_2.

Our results concerning the anionic peroxidases are in agreement with those observed by Imberty *et al.* (1985) who found that the anionic peroxidase had a particularly high affinity for syringaldazine. This electron donor has been used as a specific substrate for lignifying peroxidases allowing for active participation of syringaldazine oxidases in lignification (Goldberg and Catesson, 1985). Anionic peroxidases are actively released into the cellular free spaces of plants, in response to pollutants (Castillo *et al.*, 1984) or ozone exposure which induce the formation of superoxides and different hydroxylic radicals (Smith *et al.*, 1982; Castillo and Greppin, 1986). The increase of syringaldazine oxidases either in nematode-infected plants or in paraquat treated plants could be due to activation or to newly synthesized peroxidases. These enzymes could contribute to the effective removal of peroxidases and could constitute a first line of defence against stress in plants. From the information obtained from SDS-PAGE electrophoresis, the SOD and peroxidases isoenzymes activated in the nematode-infected tomato roots seem to be identical to those in paraquat treated roots. However, the isoenzymes isolated from the paraquat-treated roots were more active than the infected ones. With regard to the biological significance of the data reported in this paper it appears that an increase in the cell content of SOD and peroxidase is an adaptative response which provides protection against paraquat and pathogens.

Genetically engineered plants which over produce Mn-SOD significantly reduce the amount of cellular superoxide-mediate damage which normally occurs (Bowler *et al.*, 1991). We believe that the induction of SOD and peroxidase is a determining factor in the general response, via superoxide radicals, of the plants to biotic or abiotic stress. This should facilitate the determination of the molecular basis of tissue specific expression, the response to wounding, temperature, chemicals and infection, and the eventual identification of a function for each different enzyme or iso-

enzymes. With such knowledge it will be possible to engineer new plants which should allow further elucidation on the regulatory mechanism of detoxifying enzymes such as SOD and peroxidase.

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