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DIFFERENCE IN ISOPEROXIDASE ACTIVITIES OF TOMATO ROOTS SUSCEPTIBLE AND RESISTANT TO ROOT-KNOT NEMATODES

by

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Summary. Peroxidase from tomato roots susceptible and resistant to root-knot nematodes was purified and its molecular weight calculated by analytical gel-filtration HPLC. Anionic and cationic peroxidases were separated and their kinetic properties measured with guaiacol, syringaldazine and *p*-phenylendiamine/pyrocathecol as substrates. A marked difference was found between affinities for syringaldazine of cationic peroxidases from susceptible and resistant roots. Isoperoxidases were solubilized from cellular particulate fractions of both root samples, and their activity characterized with the above-mentioned substrates. Reaction rates of mitochondrial guaiacol peroxidase from resistant roots were found to be about 5-fold higher than those from susceptible roots. Six isozymes were distinguished from anionic peroxidase by anionic-exchange chromatography.

Peroxidase is involved in the response of plants to stress and pathogen attacks (Castillo, 1992; Moerschbacher, 1992). Although many reports have indicated an increase of this enzyme activity in plant tissues responding to pathogens (Geiger *et al.*, 1989; Lagrimini and Rothstein, 1987; Zacheo *et al.*, 1993), it must be taken into account that peroxidase is constituted by a very high number of isoforms with marked difference in their specificity to the substrates used for the analytical tests (Gaspar *et al.*, 1986). Consequently, when an increase of plant peroxidase activity in relation to a stress event is reported, it should be clearly pointed out which isoform has been monitored. In fact, it has been reported that soluble isoperoxidase content was differently affected in susceptible tomato roots infested by the nematode *Meloidogyne incognita*, with respect to uninfested roots. Guaicol, syringaldazine (Syr) and *p*-phenylendiamine/pyrocathecol (PPD-PC) isoperoxidase activity respectively decreased, was left unaffected and increased (Molinari, 1991a). Furthermore, toma-

to roots cultured *in vitro* and resistant to nematodes had their glutathione and ascorbate peroxidase activities lowered, PPD-PC peroxidase activity unchanged and Syr-peroxidase activity greatly enhanced, after a treatment with the herbicide paraquat (Molinari, 1991b).

The physiological roles of each peroxidase isoform are still being debated, although important associations have been identified to date, as in the case of Syr-peroxidase, which has been reported to have a primary role in lignin deposition in plant cells (Gross, 1980). It is also reasonable to argue that isoperoxidases which have diverse functions are differently distributed in the various compartments of plant cells. Soluble peroxidases are usually detected as cationic or basic isoforms, whilst membrane bound peroxidases as anionic or acid isoforms (Mäder *et al.*, 1985).

In this paper, a separation of cationic and anionic peroxidases in tomato roots, susceptible and resistant to root-knot nematodes, has been carried out. The peroxidase activity of these two fractions has been characterized in its affin-

ity to three opportune substrates and a comparison between them and between the same fraction coming from the two different root samples has been made. Data show that isoperoxidases from roots, genetically differing in their response to nematode attack, present kinetic properties with significant differences.

Materials and methods

Seeds of tomato (*Lycopersicon esculentum* Mill.) cvs Roma VF and Rossol, respectively susceptible and resistant to root-knot nematodes, were germinated in sterilized quartz sand. Five days after germination, seedlings were transferred into 3 cm-diam clay pots containing quartz sand (5 seedlings per pot). After 10 days seedligns were used for the extraction of enzymatic fractions from the roots. Plants were maintained at 26 °C, illuminated for 12 h a day and watered with Hoagland's solution twice a day.

After thoroughly washing the seedlings with distilled water, roots were separated from the shoots with a scalpel, weighed (50-100 g, varying in each experiment) and kept in an ice-bath. Then roots were placed in ice-cold 0.1 M potassium phosphate buffer, pH 6.0, (w/v ratio 1:2) and rapidly cut with scissors to obtain a coarse homogenate. This homogenate was ground by a Polytron® PT-10-35 (Kinematica GmbH-Switzerland) and filtered through four layers of gauze. The filtrate was centrifuged for 10 min at 500 g. The pellet was washed twice and used as the crude cell-wall fraction. The supernatant was further centrifuged for 15 min at 12,000 g; the pellet of this centrifugation was considered as the mitochondrial fraction. Finally, the last supernatant obtained was centrifuged at 100,000 g for 90 min to obtain the microsomal fraction. The suspension resulting from the centrifugation, containing most of the cytosolic proteins, was then added with ammonium sulphate to 70% saturation. After standing overnight, the residue was collected by centrifugation, redissolved in a minimal volume of 0.1

M potassium phosphate buffer, pH 6.0, and dialyzed against 0.1 M of the same buffer, pH 7.0, containing 0.1 M KCl. The dialysate was centrifuged and the supernatant was ultrafiltrated at 4 °C through a YM ultrafiltration membrane (10,000 molecular weight cut off, Amicon Co.) in Centricon-10 micro-concentrators. This ultrafiltrate was used for further purification steps.

Peroxidase activity was solubilized from the particulate fractions obtained by adding 1 mg of the detergent taurocholate per mg of protein of the particulate suspensions. After stirring for about 30 min on ice, the suspensions were centrifuged and supernatant tested for syringaldazine peroxidase activity. After the solubilization, percentages ranging from 20 to 60% of the total syringaldazine peroxidase activity of the particulate fractions were recovered in the supernatants with the specific activity improved approximately 3-fold. To compare peroxidase activity extracted from the particulate fractions with that from the cytosolic fraction, 1 mg taurocholate was added per mg of protein of the latter. All the samples were finally dialyzed against 0.1 M KPi, pH 7.0, concentrated by ultrafiltration and then used for the measurement of kinetic parameters.

Aliquots of cytosolic fractions were injected into a Sephadex G-100 column (1.6x40 cm) equilibrated in 50 mM phosphate buffer, pH 7.6, containing 0.15 M NaCl. Flow rate was set at 4 ml/h and 70 1 ml-fractions were collected. Fractions 39-40-41 contained most of the peroxidase activity assayed with syringaldazine, guaiacol and PPD-PC. These fractions were pooled and concentrated by ultrafiltration. This sample was analyzed by high performance gel permeation chromatography (HPLC) through a 7.5x600 mm analytical Ultropac TSK G3000 SW column, protected by an Ultropac precolumn TSK SWP 7.5x75 mm. The column was equilibrated with 50 mM phosphate buffer, pH 7.6, plus 0.15 M NaCl at a flow rate of 0.5 ml/min. The detector was set at 280 and 407 nm by a dual channel signal mode. Chromatograms were memorized and analyzed by SystemGold software. Mo-

lecular weights of peroxidases were determined by calibrating the column with apoferritin, alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase, cytochrome c and aprotinin (Sigma).

Peroxidase from cytosolic fractions was also purified by means of cationic-exchange chromatography through a CM-Sepharose column (1.6x18 cm). Column was equilibrated with 10 mM sodium phosphate buffer, pH 5.8, at a flow rate of 6 ml/h. Samples were injected and 1 ml-fractions were collected. After 3 hours, cationic fraction was eluted by adding 1 M NaCl to the running buffer. Proper samples of the 70 fractions collected were assayed for absorbance at 280 nm and syringaldazine peroxidase activity. The peak of anionic peroxidase was at the 5th fraction, that of the cationic peroxidase was at the 50th fraction (not shown). The three richest fractions in activity of the two peaks were pooled and defined as anionic and cationic fractions.

Anionic fraction of tomato root peroxidase from cv. Roma VF was injected into a DEAE-Sepharose column (1.6x36 cm), equilibrated with 10 mM sodium phosphate buffer, pH 6.9, at a flow rate of 6 ml/h. After 1 h run a continuous gradient between 0.25 and 1 M NaCl was started to separate the various isoforms within the anionic fraction. 131 1-ml fractions were collected and, at intervals of five, absorption at 280 nm and syringaldazine activity were detected.

Spectrophotometric assays of isoperoxidase activities were all carried out in a 1 ml final volume with protein content from the various samples not exceeding 0.2 mg. Lineweaver-Burk (double reciprocal) plots were used to calculate kinetic parameters of the various cellular and chromatography purified fractions. Assay mixtures for guaiacol and syringaldazine oxidation contained, in 1 ml of 0.05 M phosphate buffer, pH 6.0, different concentrations of guaiacol ranging 0.1-1 mM and of syringaldazine ranging 3-50 μM, respectively. Reactions were started by 2 mM H_2O_2 and oxidation rates were recorded at 470 nm for guaiacol assay and at 530 nm for syringaldazine assay. PPD oxidation was monitored at 557 nm, in TRis-HCl buffer, pH 7.6, in presence of 4.5 mM pyrocathecol. Concentration range was 14-350 μM and reaction started by 2 mM H_2O_2. Detection of peroxidase activity in the chromatographic experiments was made with 50 μM syringaldazine as substrate.

Results

Guaiacol and syringaldazine isoperoxidase activities were distributed in the various cellular

TABLE I - *Total activities and percentages of guaiacol (ΔA_{470} min^{-1} x total volume of the fraction) and syringaldazine (Δ_{530} min^{-1} x total volume of the fraction) isoperoxidases of cellular fractions isolated from tomato roots susceptible (Roma VF) and resistant (Rossol) to root-knot nematodes.*

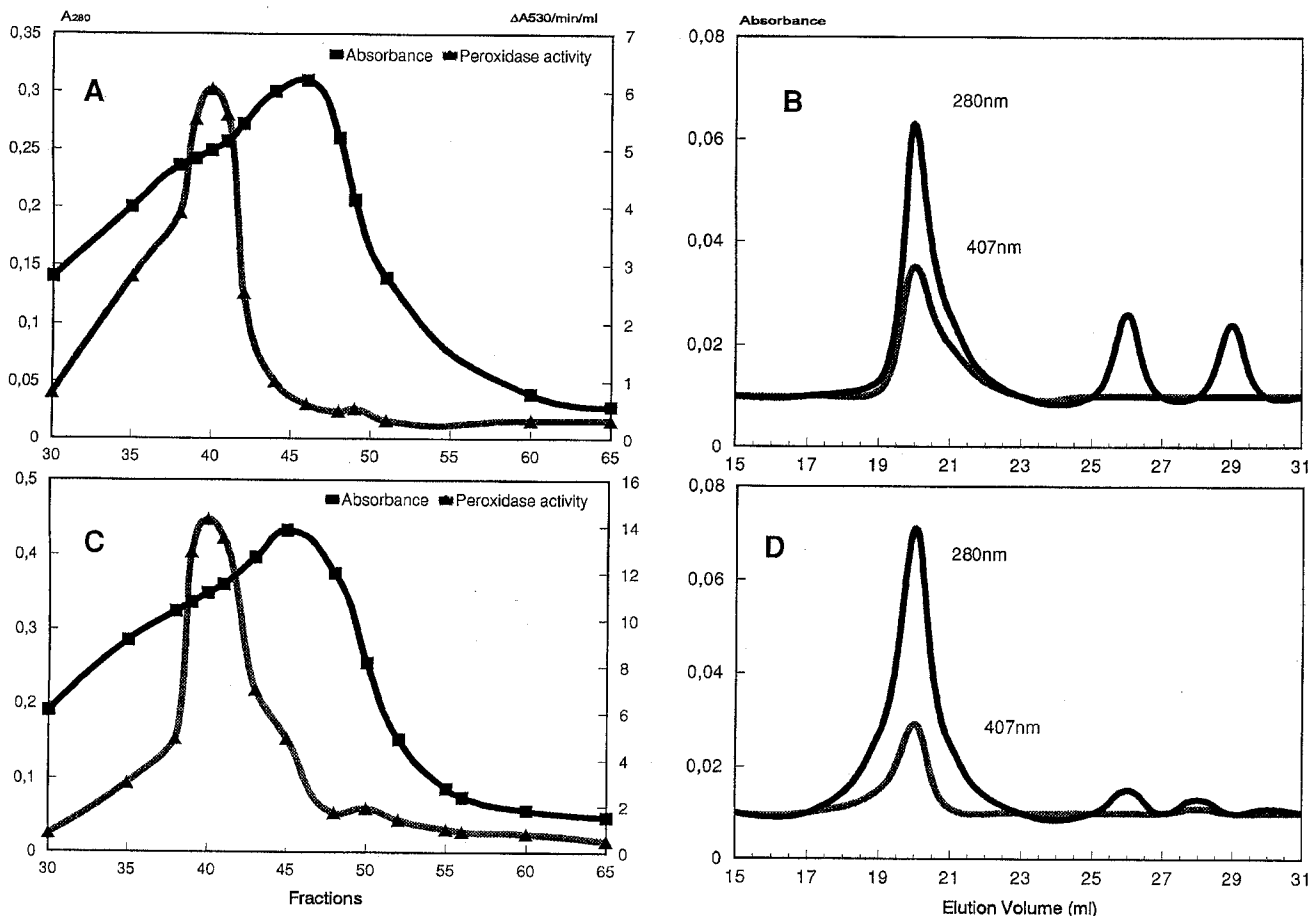| Cellular Fractions	Roma VF				Rossol			
	Guaiacol isoperoxidase		Syringaldazine isoperoxidase		Guaiacol isoperoxidase		Syringaldazine isoperoxidase	
	Total activity	%	Total activity	%	Total activity	%	Total activity	%
Cell-walls	25	2	110	3	54	5	758	7
Mitochondria	290	36	1500	39	283	26	4263	40
Microsomes	111	14	496	13	240	22	1250	12
Cytosol	389	48	1738	45	506	47	4444	41

Fig. 1 - Gel permeation classic chromatography of cytosolic fractions from susceptible (A) and resistant (C) tomato roots through a Sephadex G-100 (1.6 x 40 cm) column. Purified peroxidase from susceptible (B) and resistant (D) roots were analysed for their degree of purity and for calculation of their molecular weight by high performance gel permeation chromatography on an Ultropac TSK G3000 SW (7.5x600 mm) column. Elution was achieved with 50 mM phosphate buffer (pH 7.6) plus 0.15 M NaCl.

fractions, isolated from tomato roots susceptible (cv. Roma VF) and resistant (cv. Rossol) to root-knot nematodes (Table I). Distribution of these isoperoxidases was similar in both cultivars, with most of the activity retained in the mitochondrial and cytosolic fractions.

A purification of peroxidase activity was attempted starting from cytosolic fractions. Samples were injected into a gel filtration column by which cytosolic proteins were separated according to their molecular weights; 1-ml frac-

tions of the eluate were collected and their absorption at 280 nm and peroxidase activity, with syringaldazine as substrate, detected. One peak of peroxidase activity was found at the 40th fraction, whilst a much smaller one was found at the 49-50th fraction (Fig. 1 A-C). The three fractions with the highest activity (39-40-41) of each sample were pooled and peroxidase activity of the pools was measured with syringaldazine, guaiacol and PPD/PC as substrates. The improvement of the specific activity with all the

substrates tested ranged between 2 and 3-folds (not shown). These purified fractions were analyzed for their molecular weight by means of a passage through a HPLC gel filtration analytical column, which revealed peaks with the Reinheitzahl (RZ) value (A_{407}/A_{280}) of about 0.5 (Fig. 1 B-D). The molecular weight of peroxidase from both samples was calculated to be 40,000 according to the calibration set with standard proteins of known molecular weight (Fig. 2).

Cation-exchange chromatography carried out on cytosolic samples of the tomato roots tested revealed the existence of two different fractions rich in peroxidase activity: an anionic fraction which did not bind to the column and passed with the equlibration buffer and a cationic fraction which strongly bound to the column and was then eluted by 1 M NaCl. Also if in the cationic fractions the yields were about 31-36% of the Syr-peroxidase activity injected, the im-provement in specific activity was about 5-fold with cv. Roma VF and 12-fold with cv. Rossol; no improvement of the specific activity was observed with the anionic fractions (Table II).

Substrate affinities (K_m) for guaiacol, syringaldazine and p-phenylendiamine of the anionic and cationic peroxidases were calculated by means of Lineweaver-Burk plots (Fig. 3). A marked difference was found between K_m values for syringaldazine of cationic peroxidases from susceptible (300 µM) and resistant (60 µM) tomato roots. Moreover, anionic and cationic isoperoxidases from susceptible roots have, apparently, higher affinity for guaiacol than those purified from resistant roots. On the other hand, cationic isoforms seem to be generally more specific for p-phenylendiamine (K_m = 100 µM), whilst anionic isoforms more specific for syringaldazine (K_m = 20-30 µM) as substrates, in both cultivars.
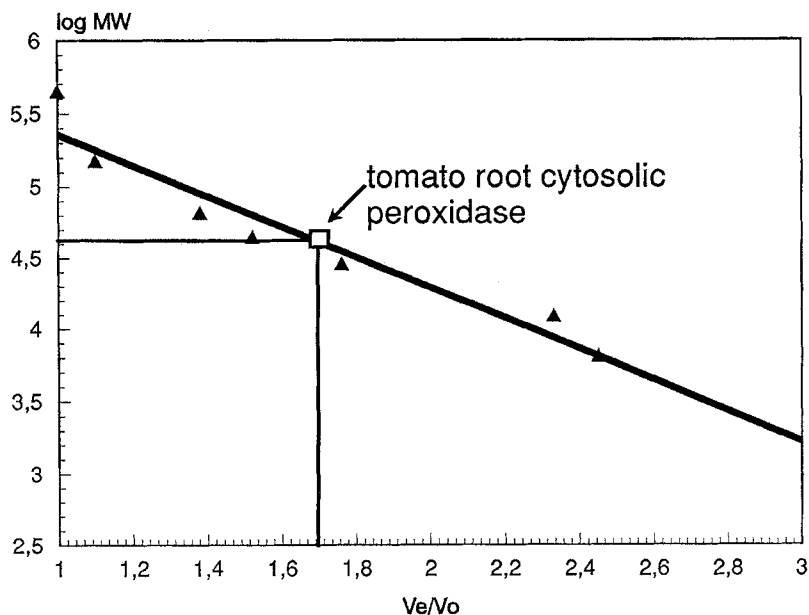

Fig. 2 - Estimation of the molecular weight of tomato root cytosolic peroxidase by gel filtration (HPLC). An Ultropac TSK-G3000 SW (7.5x600 mm) column was calibrated with known protein standards, including apoferritin (443,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), cytochrome c (12,400) and aprotinin (6,500).

TABLE II - *Purification of peroxidase obtained by means of cation-exchange chromatography of cytosolic fractions from both susceptible (Roma VF) and resistant (Rossol) tomato roots to root-knot nematodes. Enzyme activity was detected with syringaldazine as substrate.*

	total proteins (mg)	Specific Activity ΔA_{530} min^{-1} mg^{-1}	Total activity	Yield (%)	Improvement
Roma VF					
Sample injected	4.2	65.5	275.0		
Anionic Fraction	2.4	30.7	73.6	27	–
Cationic Fraction	0.25	344.0	86.0	31	5.2
Rossol					
Sample injected	6.8	40.0	270.0		
Anionic Fraction	1.0	40.2	40.2	15	–
Cationic Fraction	0.2	490.0	98.0	36	12.2

Peroxidase was solibilized from cell walls, mitochondria and microsomes, isolated from both susceptible and resistant roots. Isoperoxidases of the particulate fractions were analyzed for their affinity towards the same substrates used with purified ionic isoforms (Fig. 4). There was an evident difference with mitochondrial peroxidase between susceptible and resistant roots. Guaiacol peroxidase activity from resistant roots was about 5-fold higher than that from susceptible roots. On the contrary, Syr-peroxidase activity was about 3-fold lower. Generally, Syr-peroxidase activity was higher in all particulate fractions isolated from susceptible roots than in those isolated from resistant roots, and, affinities to guaiacol by peroxidases from resistant roots were much lower than those from susceptible roots.

Syr-peroxidase activity extracted from the particulate fractions of both cultivars showed a high affinity for the substrate, comparable with that of cytosolic ionic isoforms, whilst guaiacol and PPD-PC isoperoxidases had a much lower affinity for their respective substrates than that of the cytosolic ionic isoforms.

Finally, the anionic fraction from roots of cv. Roma VF was subjected to an anionic-exchange chromatography for the detection of isozymes that could be separated by this technique. Proteins bound to the DEAE-Sepharose column and were eluted with a continuous gradient of 0.25-1 M NaCl. Six isozymes were clearly distinguished by this method but major peaks (isozyme 2-3) did not show any improvement in the affinity to syringaldazine compared with the starting sample (Fig. 5).

Discussion

This paper presents novel data on the purification and kinetic characterization of peroxidase from tomato roots, susceptible and resistant to root-knot nematodes. Peroxidase from the cytosolic fraction had a molecular weight of 40,000, which is in good agreement with peroxidase isolated from other plant sources (Van den Berg and Van Huystee, 1984; Castillo and Greppin, 1986).

Cation-exchange chromatography revealed the presence of two distinct peroxidase fractions: an anionic fraction, which did not bind to the CM-Sepharose column, and a cationic fraction, which was retained by the column and
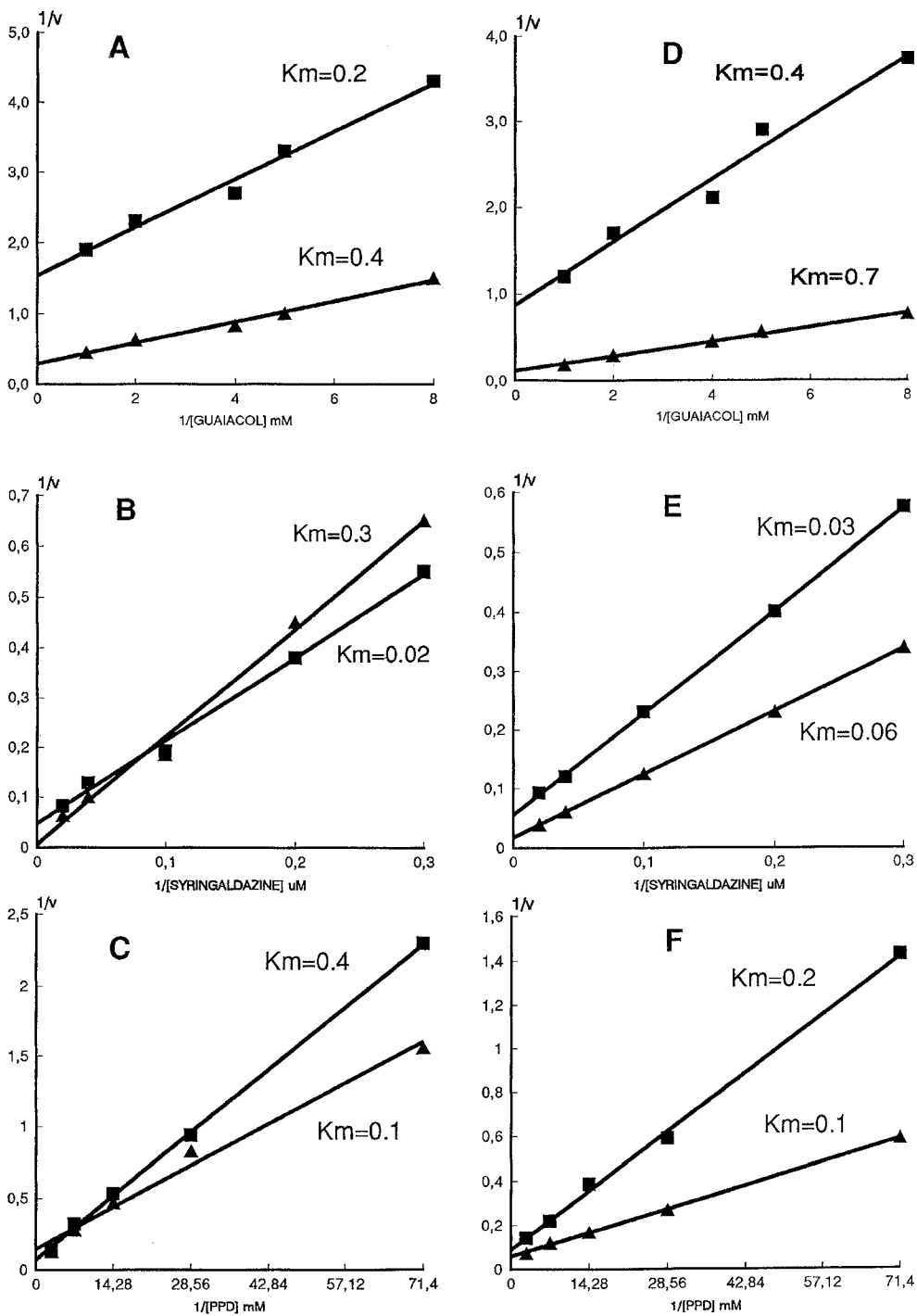
Fig. 3 - Double reciprocal plots of the initial rate (v) of substrate oxidation by anionic (■) and cationic (▲) peroxidases, from susceptible (Roma VF, A,B,C) and resistant (Rossol D,E,F) tomato roots, purified by cation-exchange chromatography carried out on cytosolic fractions. Assays as described in the text. K_m is expressed in mM and indicates the affinity of peroxidase towards the substrate tested.
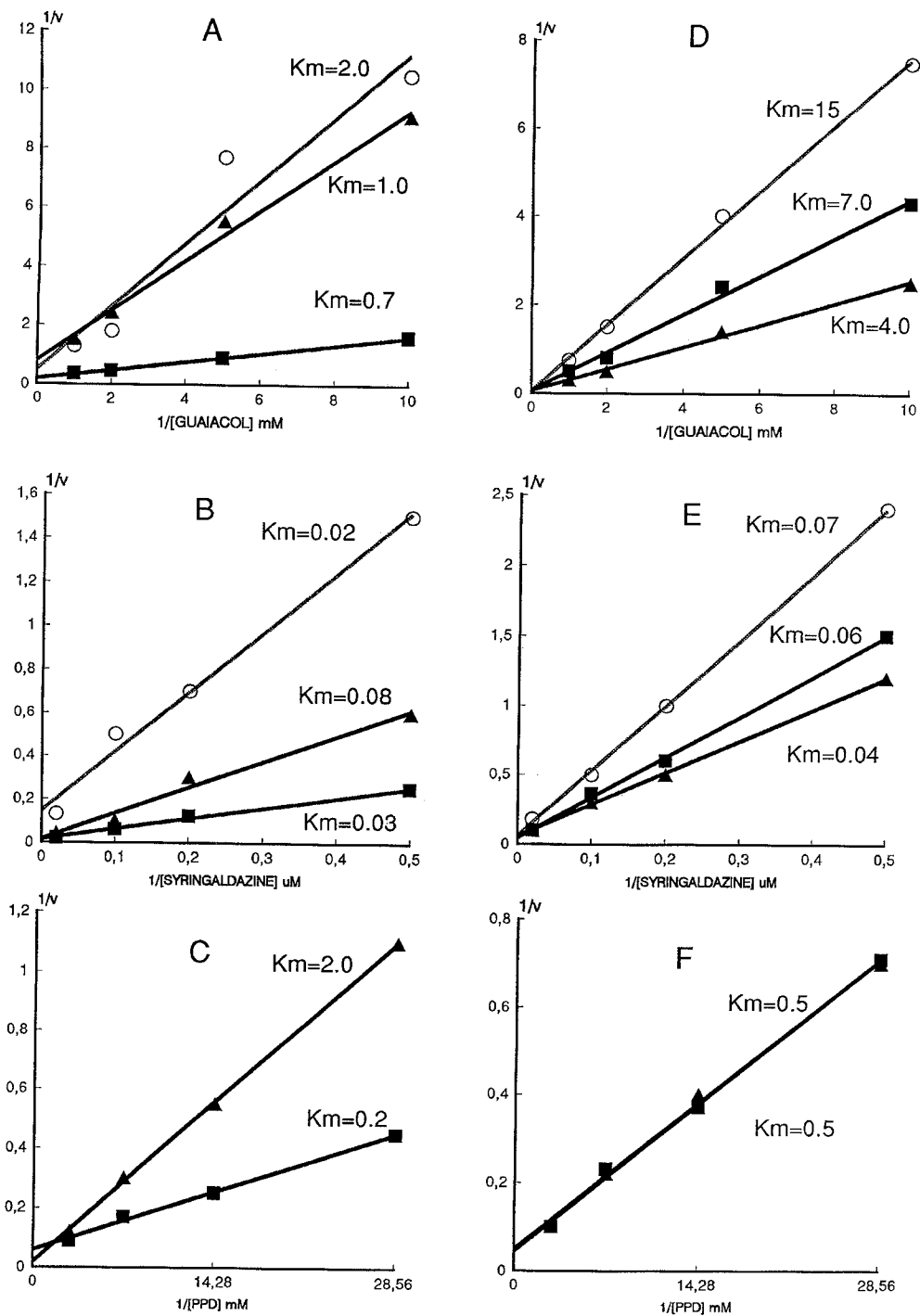
Fig. 4 - Double reciprocal plots of the initial rate (v) of substrate oxidation by peroxidase extracted from cell walls (o), mitochondria (▲) and microsomes (■), isolated from susceptible (Roma VF, A,B,C) and resistant (Rossol D,E,F) tomato roots. Assays as described in the text. K_m is expressed in mM and indicates the affinity of peroxidase towards the substrate tested.

eluted with salts. Anionic and cationic peroxidases greatly differed in their kinetic properties, thus confirming possible different physiological roles (Gaspar *et al.*, 1985). These different roles might be the basis of the diverse induction of isoperoxidases observed when susceptible or resistant tomato roots are attacked by root-knot nematodes. PPD-PC isoperoxidase has been reported to increase in both the compatible and the incompatible reaction of tomato roots to *Meloidogyne incognita*, whilst syringaldazine and guaiacol isoperoxidases increased only in the incompatible reaction (Molinari, 1991a).

Generally, cationic isoforms had a high affinity for *p*-phenylendiamine and anionic isoforms high affinity for syringaldazine as substrate. To-

mato root anionic peroxidase showed K_m values for syringaldazine in the range of 20-30 μM, which reveals a very high affinity towards this substrate, higher, for instance, than that reported for peroxidase in maize roots (Grison and Pilet, 1984) and comparable to that found in leaves of *Sedum album* (Castillo and Greppin, 1986).

A consistent difference was found between syringaldazine oxidation by cationic peroxidases of susceptible and resistant roots. The oxidation proceeded more actively at low concentration of substrate in the resistant roots, whilst it became very fast towards saturating conditions of substrate in the susceptible roots. It cannot be ruled out that this difference might have a
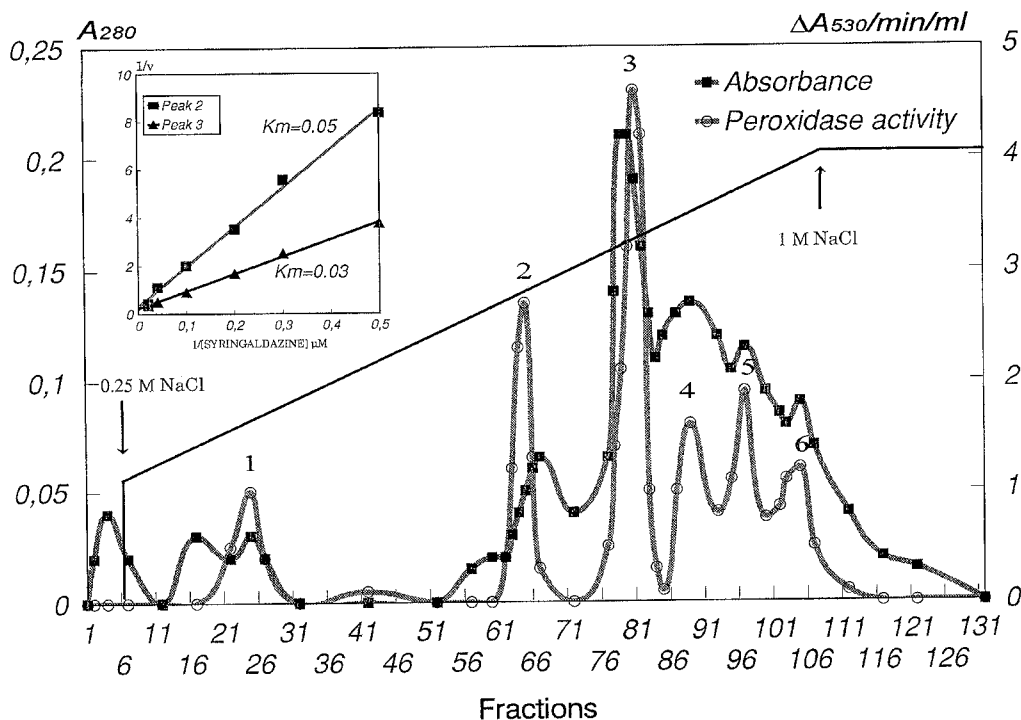

Fig. 5 - Anion-exchange classic chromatography of the anionic peroxidase of susceptible (Roma VF) tomato roots through a DEAE-Sepharose (1.6x36 cm) column. Elution of the anionic isozymes was achieved by applying a continuous gradient between 0.25 and 1 M NaCl. The insert shows double reciprocal plots of the initial rate (v) of syringaldazine oxidation by isozyme 2 and 3 isolated by means of the chromatography. K_m is expressed in mM and indicates the affinity of the isozymes towards the substrate tested.

physiological implication, although this substrate should not be specific for this peroxidase isoform. Usually, kinetics similar to that of cationic Syr-peroxidase of susceptible roots indicate a major possibility of regulation on the enzyme activity, but at this state of knowledge this is only hypothetical.

It is worth considering that specificity of each isozyme class for the proper substrate cannot be total, also because the substrates used in our assays are not physiological. In this regard, distinguishing one isoform with distinct physiological role from another is a very complex task. However, strong evidence is emerging about the existence of isoperoxidases with different physiological roles and differently located in cells. This might imply that the expression of the relative genes can be differently and independently regulated in cells.

As far as is known, peroxidases detected with different substrates can be differently induced by the same stress event. When tomato roots grown *in vitro*, susceptible or resistant to nematodes, were treated with paraquat, which promotes high levels of superoxide radicals in cells, ascorbate and glutathione isoperoxidases were differently affected with respect to syringaldazine isoperoxidase and this with respect to PPD-PC isoperoxidase. Moreover, superoxide dismutase (SOD) showed the same type of variation of ascorbate and glutathione peroxidases, whilst catalase, for example, always increased (Molinari, 1991b). Ascorbate and glutathione peroxidases, as well as SOD, belong to an enzymatic system which destroys the highly reactive oxygen radicals and their derivatives in cells (Shaaltiel and Gressel, 1986). Therefore, gene expression of these isoforms might have regulation factors more similar to a completely different enzyme than to other peroxidase isoforms having completely different cellular functions. This would mean that different genes exist for peroxidase isozyme classes having analogous physiological role.

In this paper, data are shown which confirm the existence of these two distinct ionic fractions of peroxidases in tomato roots suceptible and resistant to root-knot nematodes and their different kinetic properties with proper substrates. It is suggested that they have different physiological roles which, ultimately, would justify their different induction with a stress event such as a nematode attack. Additional information is the existence of peroxidases with different kinetic properties in the various cell compartments, but the respective roles in cells are unknown.

Further investigation is needed on the regulation of gene expression of peroxidase to clarify whether different cistrons involving the expression of functionally similar isoperoxidases exist and the substances affecting their activation or repression. The hypothesis that susceptibility and resistance to nematodes can be due also to this differential potential in induction or repression of isoperoxidases will form the framework of future research.

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