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ROLE OF PEROXIDASE AND ESTERASE ISOENZYMES IN PEA ROOTS INFECTED WITH *HETERODERA GOETTINGIANA*

by
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Summary. A cytochemical and biochemical study of peroxidase and esterase activity of pea roots susceptible or resistant to *Heterodera goettingiana* has shown that the levels of activity of the two enzymes appeared to be related to infection and differed with regard to pea resistance. Infected tissues of resistant roots showed a marked increase in peroxidase activity, which became perceptible within 24h of infection and continued for several days; cycloheximide treatment prevented this induction. The high level of peroxidase is to be considered part of a general activation of the cell metabolism, which takes the form of *de novo* synthesis of enzymatic proteins with peroxidase activity. The peroxidases and carboxylesterases localized near the infected tissues appear to catalyze the formation of suberin. The activity of these enzymes seems to precede the suberization of cell walls. Presumably, the deposition of suberin aids in the defense of the plant by forming a barrier that blocks the pathogen. We therefore propose a correlation of the two enzymes as a rapid and sensitive response in the incompatible pea-cyst nematode interaction.

Invasion of plants by pathogens, including nematodes, affects the whole metabolism of the host and consequently, unspecific alterations of enzyme activities may occur (Endo and Veech, 1970; Giebel *et al.*, 1971; Goodman *et al.*, 1986). Peroxidase activity is particularly prone to increase in plants during pathogenesis (Gaspar *et al.*, 1982) and the increase is suggested to be associated with resistance (Reuveni and Ferreira, 1985). Resistance reactions that involve tissue necrosis are accompanied by stronger and earlier increases than susceptible reactions and it has been suggested that the rapid increases in peroxidase activity may be part of the plant defense against parasites (Goodman *et al.*, 1986). In general the stimulation of peroxidase activity in response to infection, constitutes a good indication of a general increase in oxidative processes (Gaspar *et al.*, 1982; Zacheo *et al.*, 1990).

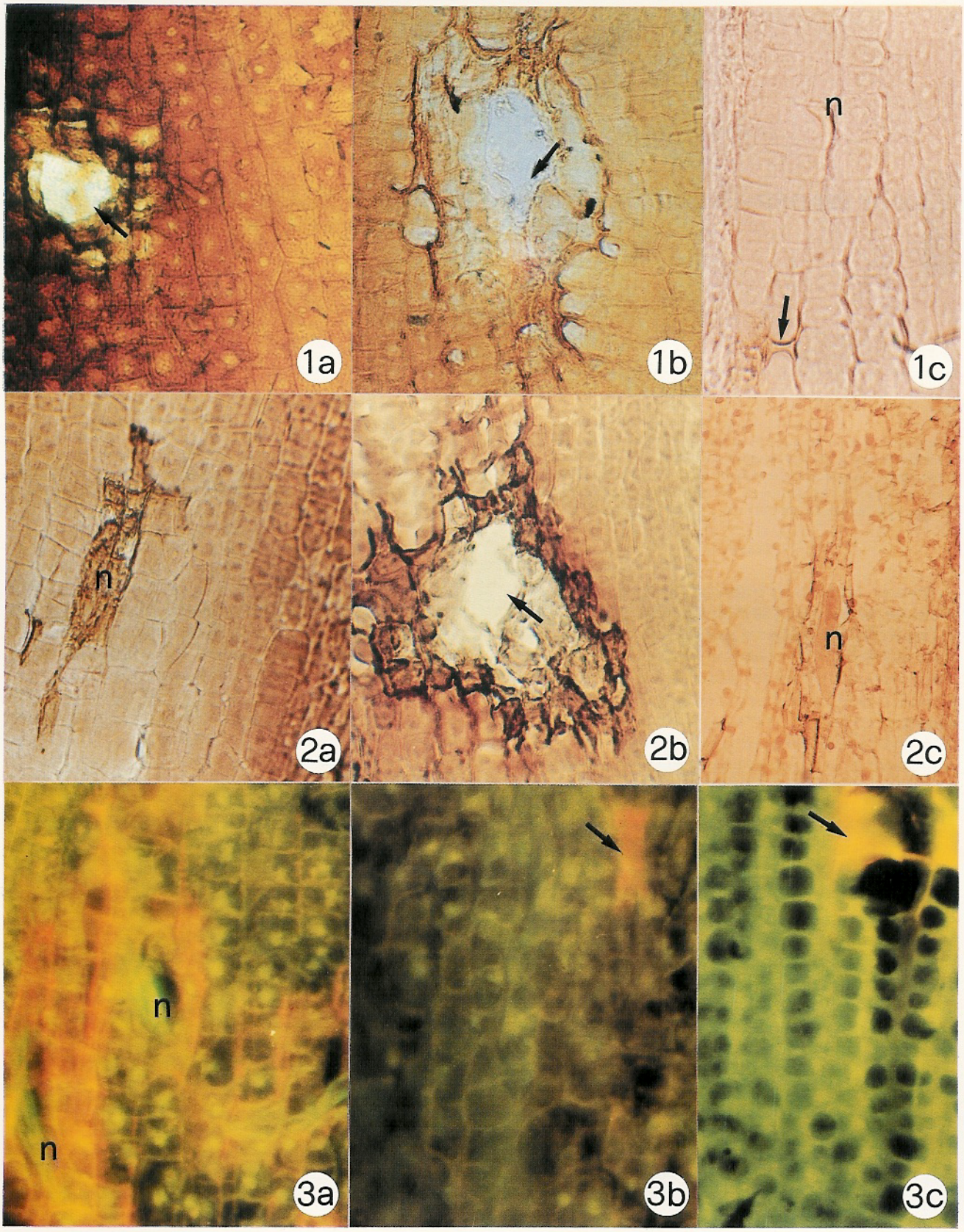
Peroxidases have been shown to catalyze the polymerization of phenolic compounds into lignin and recently Espelie *et al.*, (1986) have demonstrated that the polymerization of the aromatic monomers of suberin are biosynthesized similarly. The induction of new cell wall biosynthesis has been proposed as an important defense mechanism in response to pathogen attack. Plants contain a number of peroxidase isoenzymes that differ in substrate specifity and molecular form. Plant peroxidases also have a broad subcellular distribution (Gaspar, 1986).

The present study concerns the localization of peroxidase activities related to infection of *Heterodera goettingiana* Liebscher in resistant and susceptible pea root tissues by means of cytochemical and biochemical studies with different substrates. The distribution of esterases has also been examined. Esterase activity is known to be concerned with secondary metabolism and, in particularly, is thought to be involved, at least in part, with cell wall changes during differentiation (Gahan, 1981), stress or infection (Onyia and Gahan, 1984, Melillo *et al.*, 1989). We examine the possible involvement of both enzymes, peroxidases and esterases, in the resistant host-nematode relationship.

Materials and methods

Plant material and inoculation

Seeds of an accession of germplasm pea MG 103738 (*Pisum sativum* sp. *transcaucasicum* Govorov, obtained from Gatersleben collection) and Progress 9 (commercial variety), respectively resistant and susceptible to *H. goettingiana*, were sterilized with 1% sodium hypochlorite and germinated in sterile conditions. When root initials appeared, the seedlings were transplanted into clay pots containing 10 ml of sterilized sand and simultaneously a suspension of 50 second-stage juveniles of *H. goettingiana*, sterilized with 0.1% of streptomycin sulphate (15 minutes) and 0.05% hibitane (15 minutes), was added to each pot. In a parallel experiment pea seedlings were incubated in 10 µg/ml cycloheximide, a protein synthesis inhibitor, for 4 h before nematode inoculation. The plants were then watered with the same solution during all the experiment. Infected plants were placed in growth chambers at 17°C. Root apices and root segments were excised 24 h and 4 days after nematode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inoculation, respectively, for histochemical analysis. Whole roots, for biochemical extraction, were recovered 24 h, 4 and 20 days after nematode inoculation.

Histochemical tests

Enzyme localization was carried out on unfixed, longitudinal, frozen sections from excised material prepared with a 2800 Frigocut Reichert-Jung automatic cryostat at cutting speed of 5 and section thickness setting 17 µm.

Peroxidase activity was localized by using four different substrates.

a) Sections were incubated in 10 ml of 0.05 M Tris-HCl buffer, pH 7.6, containing 5 mg diaminobenzidine (DAB) and 0.2 ml of freshly diluted 0.5% H_2O_2 (from 33% stock solution, prepared just before use). After 5 minutes of incubation at 25°C the substrate was washed from the sections with distilled water (Gahan, 1984). Then the sections were mounted in Farrants' medium and observed under a light microscope.

b) Sections were incubated at 25°C in 20 ml of 0.1 M Tris-HCl buffer, pH 7.6, containing 10 mg *p*-phenylenediamine (PPD), 20 mg pyrocatechol (PC) and 0.2 ml of freshly diluted 0.5% H_2O_2, for 5 minutes and then washed with distilled water. After mounting in Farrants' medium the sections were observed under a light microscope (Gahan, 1984).

c) Sections were incubated at 25°C in 0.1% alcoholic syringaldazine, pH 5.0, and 0.03% H_2O_2 for two minutes and directly examined under a light microscope (Catesson, 1980).

d) 10 ml of 0.2 M sodium acetate buffer, pH 6.0, were supplemented with 51 mg homovanillic acid and 1 mg rhodamine B for fluorescence analysis. In another 10 ml of the same buffer, 132 mg of lead nitrate were dissolved and the solution was then added slowly to the homovanillic acid solution. Finally, 0.2 ml of freshly prepared 0.05 H_2O_2 was added. The sections were incubated at 25°C in the full reaction mixture for up to 10 minutes and then washed in ethanol to remove uncoupled rhodamine B. Sections were dehydrated, mounted in Euparal and examined with a Leitz Dialux epifluorescence microscope equipped with a mercury vapour lamp and violet filter assembly transmitting wavelenghts from 361-435 mm (Papadimitriou *et al.*, 1976). Controls for the absorption of the substrates were made by incubation of the sections in the test solution without H_2O_2 or in H_2O_2 without any other substrate.

To test for esterases, unfixed sections were reacted for 1 h at 37°C in a full medium containing naphthol AS-D acetate as substrate and fast blue BB as the diazonium salt in 0.2 M Tris-HCl buffer, pH 6.5. Control sections were pre-incubated for 1 h at 25° C in 0.2 M Tris-HCl buffer, pH 6.5, containing either 0.1 mM diisopropylfluorophosphate (DFP) or 10 mM eserine, and then transferred to the full reaction medium containing either DFP or eserine for 1 h at 37°C. All sections were rinsed in distilled water and mounted in Farrants' medium for observation in a light microscope (Gahan, 1984).

Enzyme extraction and assay

Extraction procedure and protein assay have been previously described (Zacheo *et al.*, 1990). The soluble fraction was obtained by saturation with 80% ammonium sulphate. Cell walls were obtained by centrifugation at low speed (500 g) of root homogenate. After 5 washings with the grinding medium, pellet was used as cell wall fraction

Fig. 1a - Unfixed, frozen, longitudinal section of an infection site (arrow) in a resistant pea root tip, 24 h after *H. goettingiana* infection, incubated with DAB + H_2O_2: note the strong reaction in the cells adjacent to the injured area, indicative of high peroxidase activity. x1180.

Fig. 1b - Infection site in a susceptible pea root tip incubated with DAB + H_2O_2. The reaction is diffused but less intense than in a resistant root. x1140.

Fig. 1c - Meristematic cells of a resistant 24 h infected root treated with DAB + H_2O_2. In the absence of H_2O_2 a very faint reaction in the cells and cell walls is observed. n = nematode. x1170.

Fig. 2a - Micrograph of a 24 h infected resistant root tip, incubated with PPD-PC + H_2O_2. Different intensities of brown deposits in the cytoplasm and nuclei of cells might refer to initiation of hypersensitive reaction at different times. x1100.

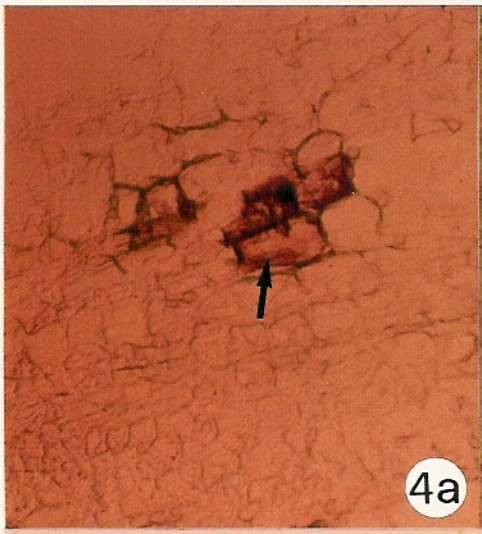
Fig. 2b - Infection site on susceptible pea 24 h infected root tip incubated with PPD-PC + H_2O_2. Apart from brownish products localized on cells penetrated by nematode (arrow) the enzyme distribution is shown on the host cell cytoplasm and nuclei but the dye intensity is weaker than in the resistant root cells. x1100.

Fig. 2c - Same sample as in (2a) after the treatment with PPD-PC +H_2O_2. Note the nematode (arrow) and dying penetrated cells, but not peroxidase activity. x1150.

Fig. 3a - Longitudinal, frozen section of MG 103738 tip 24 h after nematode infection treated with homovanillic acid + H_2O_2 showing detail of the connection between nematode presence and brilliant orange fluorescence on the cell walls. The fluorescent area is wide and involves a large number of cells which nuclei strongly reacted. x1200.

Fig. 3b - Progress 9 root tip 24 h after nematode infection treated with homovanillic acid + H_2O_2. Injured cells only fluoresce orange under violet light, but the cell walls next the wound remain unstained. x1180.

Fig. 3c - Autofluorescence and phenol localization on penetrated cells of a resistant pea root tip when H_2O_2 was omitted from the medium containing homovanillic acid. x1150.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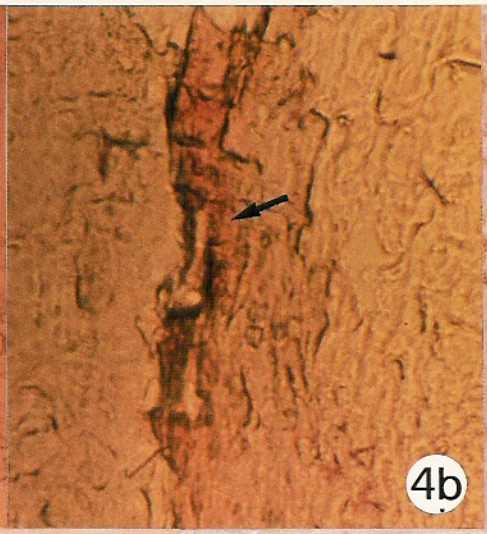
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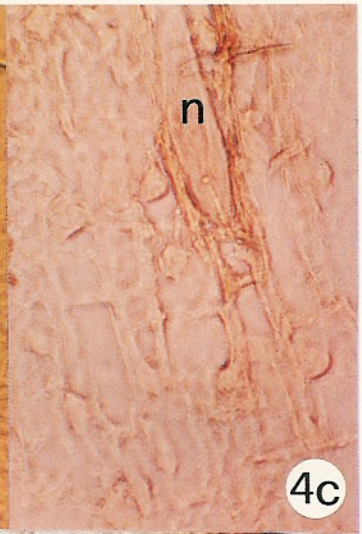
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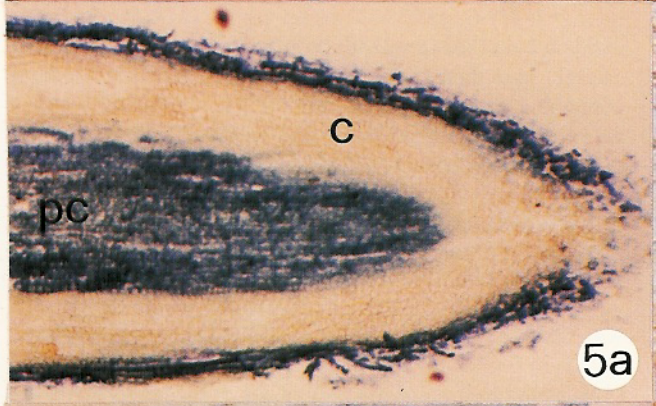
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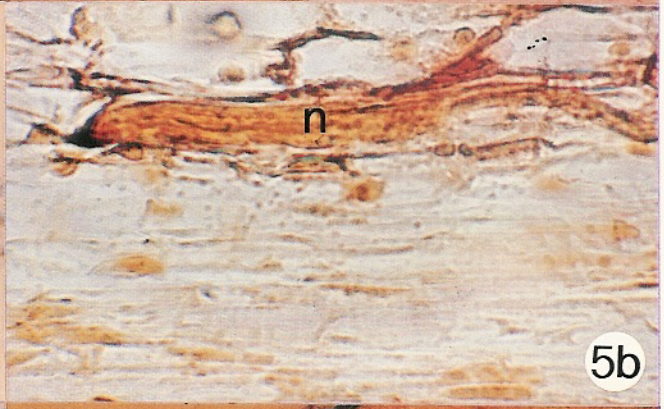
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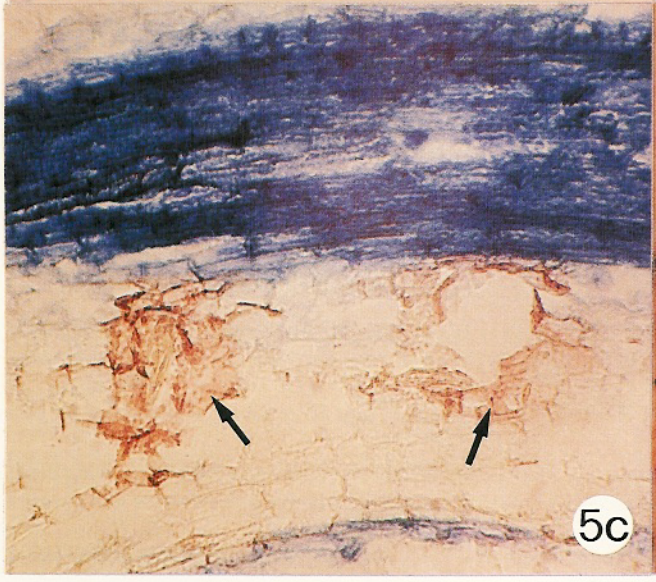
5c


5d

(Mader *et al.*, 1986). Peroxidase activity was assayed in 0.35 mM PPD, in presence of 4.5 mM PC or 50 µM of syringaldazine, 20 mM sodium phosphate buffer (pH 6.1) and 250 µm H_2O_2. The absorbance was monitored spectrophotometrically at 575 nm for PPD oxidase and 530 nm for syringaldazine oxidase.

Results

Histochemical analysis

Longitudinal sections of germplasm MG 103738 and Progress 9 pea apices, infected with *H. goettingiana* and incubated with DAB as substrate and H_2O_2 as cofactor, showed a reaction in all cells involved in nematode penetration and this was localized in cell walls, nuclei and cytoplasm (Figs. 1a, 1b). Twenty four hours after nematode inoculation brown-black granules of the reaction product also were observed in the cytoplasm of the resistant pea cells at some distance from the cells wounded by the nematode (Fig. 1a). In sections of the susceptible pea there was a weaker brown coloration either in the injured cells or in neighbouring tissues (Fig. 1b). By day 4 no more staining intensity was observed in either sample, suggesting that the major DAB-peroxidase activity developed very quickly after nematode injury. DAB oxidation *in situ* occurred in the resistant tissues incubated without addition of H_2O_2, thus indicating that hydrogen peroxide might be produced by the nematode infection, but only a faint reaction was observable (Fig. 1c). There was no reaction when H_2O_2 was used alone (Table I).

When PPD was used as a substrate in the presence of H_2O_2 as the hydrogen donor, enzyme activity was visualized in the tissues of both MG 103738 and Progress 9 peas (Figs. 2a, 2b). The reactant sites were localized in the cytoplasm of meristematic and differentiating cells as brown precipitate. In the nuclei, heavy deposits of reaction product were present on the nuclear membranes and nucleolus. Staining was more intense in resistant than in susceptible tissues at 24 h and 4 days, whereas there was only a very weak reaction in both control sections incubated without H_2O_2 (Fig. 2c). Sections were completely unstained when reacted only in presence of H_2O_2 (Table I).

Use of homovanillic acid in the presence of hydrogen peroxide as the hydrogen donor showed that most peroxidase activity in 24 h infected roots occurred in cell walls, which exhibited a brilliant orange deposit, and in nuclei which brightly fluoresced. Fluorescence was more diffuse in resistant (Fig. 3a) than in susceptible tissues (Fig. 3b), in the latter the reaction product being restricted to the site of nematode feeding. There was a marked increase of cell wall fluorescence after 4 day infection, indicating an increase in peroxidase activity, and many cells were involved in the reaction in both resistant and susceptible roots. Without exogenous H_2O_2 the homovanillic acid treatment did not give any reaction. Green fluorescence (autofluorescence) was observed in the tissues of both resistant and susceptible apices; injured cells were distinguishable because of the presence of a yellow fluorescent product (Fig. 3c). Reaction was nil in sections treated only with H_2O_2 (Table I).

The presence of peroxidase was also tested by using syringaldazine as a substrate. As well as a red reaction product in the cell wall and cytoplasm at the site of nematode penetration, a faint staining was observed in the neighbouring cells 24 h after nematode injury. By day 4 positive syringaldazine staining occurred in the cell walls of the tissues surrounding the injured cells, the reaction being more intense in sections of MG 103738 than in Progress 9 (Figs. 4a, 4b). When H_2O_2 was omitted in the incubation medium, the cells forming the feeding site and cells near the wound were only weakly reactive (Fig. 4c). Negative reaction was detected on sections when syringaldazine was omitted (Table I).

Localization of esterase enzyme activity during the sequence of events after penetration by *H. goettingiana* and establishment of feeding in healthy pea root tissues is illustrated in Figs. 5a-d. In both MG 103738 and Progress 9 este-

Fig. 4a - Visualization of peroxidatic activities in cells of a resistant root 4 days infected and treated with syringaldazine + H_2O_2 as substrate. The red dye, indicating syringaldazine reaction is localized only on the walls of cells directly injured by nematode. x1140.

Fig. 4b - Infection site on cells of a susceptible root 4 days after nematode penetration and treated as in (4a). Note the same localization than in cells of resistant root but with diminished dye intensity. x1120.

Fig. 4c - Nematode stylet inserting into a cell wall of a resistant root treated with syringaldazine without hydrogen peroxide. No dye is detectable on the cell walls. Light yellow colour around the nematode indicates presence of phenols. x1100.

Fig. 5 - Light micrographs of the distribution of naphthol AS-D esterase activities in resistant and susceptible pea root tip.
(a) Unfixed, frozen, longitudinal section of resistant pea root utilized as control. The uninfected root shows high esterase activity in the procambium (pc) but not in the cortex (c). x600.
(b) This control for naphthol AS-D esterase localization was treated with the inhibitor DFP. No visible blue reaction is present. x1120.
(c) The 24 h infected resistant pea root shows further increase in the level of the reaction in the procambium. In the cortical cells only phenolic compounds (arrow), yellow brown stained, are detectable. x1200.
(d) Susceptible 24 h infected pea root showing brown stain of polyphenolics (arrow) in cells penetrated by nematode and esterase end product localized in the procambium associated with cell walls. The reaction is less intense than in resistant root cells. x1160.

TABLE I - *Peroxidase activity. Relative intensity of the staining obtained on sections of uninfected or infected pea roots with different substrates: diaminobenzidine* (DAB), *p-phenylenediamine-pyrocatechol* (PPD-PC), *homovanillic acid* (HA), *syringaldazine* (SY), *either in the presence or the absence of* H_2O_2. *The number of* + *indicates relative staining intensity as appreciated through the light microscope.*

Pea	Treatment	Substrates								
		DAB + H_2O_2	DAB	PPD PC + H_2O_2	PPD PC	HA + H_2O_2	HA	SY + H_2O_2	SY	H_2O_2
MG 103738	Uninfected	+	0	+	0	+	0	0	0	0
	Infected	+++	0	+++	0	+++	0	++	0	0
Progress 9	Uninfected	+	0	+	0	+	0	0	0	0
	Infected	++	0	++	0	++	0	+	0	0

rase activity was associated with the rhizodermis and the stele (Fig. 5a) involving not only the cambial initials but the whole cambial zone. During the early stage of nematode infection (24h) the esterase activity appeared to increase in resistant but not in susceptible pea tissues (Figs. 5c, 5d). Cells severely damaged by nematode penetration stained yellow-brown for polyphenolics (Figs. 5c, 5d). Complete inhibition of pea esterase was observed when DFP was used as an inhibitor (Fig. 5b), but no effect was detectable with eserine. These results indicate that the esterases present in peas were carboxylesterases.

Biochemical assay

Preliminary studies performed at 24 h, 4 and 20 days after nematode infection showed that the increase in peroxidase activity reached the maximum, in the resistant roots, 4 days after infection. During susceptible interaction, not involving necrosis, lower stimulation of enzymes occurred. Very few differences between resistant and susceptible tissues were observed after 20 days. This observation indicates that enzyme stimulation occurs at very early stage of infection. Thus we performed all the experiments at fourth day after nematode inoculation.

The effect of nematode infection on the induction of peroxidase isoenzymes, both in soluble fraction and cell walls was determined. The resistant inoculated pea roots showed a 50% increase of their initial PPD-PC oxidase activity in the soluble fraction (Table II). Peroxidase activity remained constant in the same fraction of susceptible infected pea roots (Table III). In cell wall fraction PPD-PC oxidase activity was also higher in the resistant (50%) while in the susceptible there was only a slight increase (Table II, III). The increase of affinity for syringaldazine was 30% higher in resistant and nil in susceptible soluble fraction (Tables II, III). The cell wall fraction of resistant roots showed 5 fold more affinity towards syringaldazine than the susceptible roots. After nematode infection syringaldazine oxidase exhibited an increase of about 100% in resistant and only 20% in susceptible cell walls.

In a parallel experiment extracts from roots treated with a protein synthesis inhibitor (cycloheximide) showed no induction of peroxidase activity (Tables II, III). These results suggest that the increase in peroxidase activity appears to require *de novo* synthesis of protein rather than enzyme activation.

Discussion

The results extend our previous studies on peroxidase activity in pea roots infected by *H. goettingiana* (Zacheo *et al.*, 1990) and confirm that higher levels of peroxidase occurred earlier in resistant than in susceptible infected roots. As the products of oxidation by peroxidases are toxic to cells and rapid killing of the tissue can confine the nematode to the site of infection, clearly not only the timing but also the localization of the increased peroxidase activity may be decisive in the host-nematode interaction. The presence of extracellular isoenzymes (Bireka *et al.*, 1975) and their affinity for substrates for lignification (Mader and Fussl, 1982) suggest that they may be involved in the formation of a barrier substance against the pathogen. In our experiments the increase in peroxidase activity quantita-

TABLE II - *Distribution of peroxidase activity between uninfected and infected roots of MG 103738 pea resistant to* H. goettingiana; *effect of cycloheximide on peroxidase synthesis. Values are the average of three experiments ± standard deviation.*

Fractions	Treatments	PO activity as O.D./mg of proteins			
		as PPD-PC oxidase		as Syringaldazine oxidase	
		Uninfected	Infected	Uninfected	Infected
Soluble	H_2O	12.27±0.89	18.86±1.00	10.04±0.66	15.20±1.10
	Cycloheximide 10 µg/ml	11.10±0.23	11.92±0.61	10.01±0.73	10.73±0.61
Cell walls	H_2O	42.01±3.10	75.23±3.15	51.71±3.67	99.71±4.61
	Cycloheximide 10 µg/ml	22.24±2.00	23.95±1.00	36.14±2.31	37.54±1.31

TABLE III - *Distribution of peroxidase activity and effect of cycloheximide on uninfected and infected root of Progress 9 pea susceptible to* H. goettingiana. *Values are the average of three experiments ± standard deviation.*

Fractions	Treatments	PO activity as O.D./mg of proteins			
		as PPD-PC oxidase		as Syringaldazine oxidase	
		Uninfected	Infected	Uninfected	Infected
Soluble	H_2O	12.70±1.00	11.48±0.60	6.96±0.32	7.36±0.85
	Cycloheximide 10 µg/ml	9.71±0.64	10.24±0.96	4.46±0.60	5.40±0.52
Cell walls	H_2O	10.43±0.74	14.81±0.49	9.89±1.02	14.20±0.82
	Cycloheximide 10 µg/ml	8.23±0.52	8.91±0.64	10.68±0.61	10.85±1.06

tively and qualitatively determined, appeared to be considerably higher in resistant than in susceptible tissues and involved either cell walls or cytoplasmatic compartments. The results gave clear evidence for the exclusive localization of isoenzymes in a zone very close to the injured cells and in neighbouring cell layers. The increased activity of peroxidase isoenzymes detected in infected pea roots resulted cycloheximide sensitive. Synthesis of the peroxidase protein moiety is classically cycloheximide dependent (Gaspar, 1986), but the inhibitor did not affect host response associated with resistance (Cohen *et al.*, 1990). One possible explanation for these observations is that, upon nematode infection, there is a rapid accumulation of newly synthesized protein which will constitute the new cell wall. The mode of action of peroxidases in the cell wall modification is not yet characterized, although they may play a role in the resistance of pea to *H. goettingiana.* Lignin-specific peroxidases, with high affinity for syringaldazine, are reported to be involved in the control of lignification processes (Catesson *et al.*, 1986). Thus the high syringaldazine oxidase present in cell wall fraction of resistant pea roots may indicate an increase in lignin deposition.

Peroxidase isoenzymes with high affinity to PPD-PC might therefore be involved in suberization, a new hypothesis of peroxidase function, which has recently been claimed by several authors (Mader *et al.* 1986; Kolattukudy,

1981; Mohan and Kolattukudy, 1990). Suberin is a secondary metabolite, usually produced by some cells and tissues, when injured by pathogens. The physiological significance of suberin, which in contrast to lignin synthesis, is confined to only few cell layers, is to limit water loss or to provide a barrier against pathogen penetration. Mader *et al.* (1986) and Mohan and Kolattukudy (1990) reported that there are isoenzymes that always appear after wounding or infection by fungi and demonstrated their high affinity to PPD-PC. These isoenzymes are thought to be anionic isoperoxidases and to be involved in suberization but not in lignification. Recent histochemical studies demonstrated suberin deposition on cells of infected resistant pea roots (-Bleve-Zacheo *et al.*, in preparation). Because also carboxylesterases are reported to have a critical role in suberization upon wounding (Rana and Gahan, 1983) and in host-parasite interaction (Nicholson *et al.*, 1972; Takahashi *et al.*, 1985; Melillo *et al.*, 1989) we could hypothesize that there is a correlation between PPD-PC oxidases and esterases, detected in resistant pea roots, with wall suberization. Reports of lignin and suberin deposition as host response indicated that lignification occurred first, followed by suberization (Rittinger *et al.*, 1987). These findings are supported by those of Espelie *et al.* (1986), who reported that accumulation of phenolic compounds appeared to be a necessary step in the biosynthesis of suberin.

However, it is not clear how the two enzyme activities are integrated in the secondary thickening of cell walls. Nevertheless both enzymes are certainly involved in the reinforcement of the cell walls, and is not unreasonable to assume that they have a role in the containment of the nematode.

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