Peroxidase isoenzymes from *Meloidogyne* spp. cultured on different hosts

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SUMMARY

Four peroxidase isoenzymes were separated by IEF analysis from extracts of adult females of *Meloidogyne* spp. propagated on tobacco. Only adult females of *Meloidogyne* and *Globodera* spp. showed peroxidase activity by IEF. Males, eggs, and free living nematodes did not show any peroxidase activity under the conditions of our tests. An increased level of peroxidase activity was observed in infected plants during the early stages of invasion by *Meloidogyne*. This increase was associated with the appearance of new multiple forms of peroxidase on tomato, cucumber, tobacco and courgettes. The cuticle of adult females of *Meloigodyne* spp. did not show any peroxidase activity. The greatest activity was associated with the gelatinous matrix and some parts of the female body. On the basis of these results the hypothesis is advanced that peroxidase increases may constitute a defence mechanism of plants against invasion or damage caused by nematodes. Peroxidase profiles of female nematodes were different in populations cultured on several different hosts.

RÉSUMÉ

Isoenzymes peroxydasiques de Meloidogyne *spp. élevés sur différents hôtes*

Quatre isoenzymes peroxydasiques ont été isolées par isofocalisation à partir d'extraits de femelles adultes de *Meloidogyne* spp. élevés sur tabac. Seules les femelles adultes de *Meloidogyne* spp. et de *Globodera* spp. montrent une activité peroxydasique par la méthode utilisée. Les mâles, les œufs et les nématodes libres ne montrent aucune activité peroxydasique dans les conditions des expériences réalisées. Une augmentation du niveau de l'activité peroxydasique est observée chez les plantes infestées lors des premiers stades de la pénétration par *Meloidogyne*. Cette augmentation est associée à l'apparition de formes multiples de peroxydases chez la tomate, le concombre, le tabac et la courgette. La cuticule des femelles adultes de *Meloidogyne* spp. ne montre aucune activité peroxydasique; la plus importante activité est associée à la gelée entourant les œufs et à quelques régions du corps de la femelle. Fondée sur ces résultats, l'hypothèse est avancée que l'accroissement des peroxydases pourrait constituer un mécanisme de défense de la plante contre l'invasion par les nématodes et les dommages qu'ils causent. Les profils peroxydasiques des femelles de nématodes présentent des variations entre populations élevées sur des hôtes différents.

Peroxidases are a group of enzymes found in a number of higher plants (Stafford, 1974) and are responsible for the oxidation of a number of hydrogen donors. Infection of some plants by parasitic nematodes appears to correlate with an increase in peroxidase activity (Giebel, Krenz & Wilski, 1971; Gregory & McClure, 1978). The significance of this phenomenon is poorly understood.

Many investigations have indicated the involvement of nematode peroxidase in giant cell formation. Linford (1937) has shown that nematodes inject saliva through the stylet into the plant cells leading to giant cell formation, and nematodes stimulated certain enzymes in the host cells during the feeding process (Veech & Endo, 1969).

Hussey and Krusberg (1971) reported that peroxidase activity was not detected in extracts of *Ditylenchus dipsaci* by polyacrylamide gel electrophoresis. However Hussey and Sasser (1973) found a peroxidase isoenzyme in the stylet exudate of *Meloidogyne incognita* and suggested that this enzyme was involved in giant cell induction and maintenance. Gregory and McClure (1978) proposed that peroxidase isolated from roots was of nematode origin and was related to the feeding process, whereas Starr (1979) concluded that the enzyme was not involved in giant cell host-parasite interactions. A similar conclusion was reached by Jones (1980), who suggested that peroxidase activity in the stylet of *M. javanica* was of host origin. Recently, Veech, Starr and Nordgren (1987) reported that no peroxidase was re-

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vealed in the stylet exudate of *M. incognita* separated electrophoretically.

However, several acrylamide gel electrophoretic studies have indicated the effect of different host plants on both general protein and certain enzyme patterns. Hussey, Sasser and Huising (1972) and Hussey and Sasser (1973) reported that the host plant has a great influence on the activity of peroxidase isoenzymes. They also reported that two peroxidase isoenzymes were detected in females of *M. incognita* reared on tomatoes, but no peroxidase activity was found when tobacco was the host.

The present study was carried out to determine the level of peroxidase activity in certain free-living and plant parasitic nematodes and to examine the changes in peroxidase activity of different varieties of plants in response to inoculation with *Meloidogyne* spp. The effect of the host plant on nematode peroxidase isoenzyme patterns was also studied.

Materials and methods

Propagation and culture of nematodes

M. incognita was propagated on tomato *(Lycopersicon esculentum)* cv. Marglobe, cucumber *(Cucumis)* cv. National pickling and tobacco *(Nicotiana tabacum)* cv. Samsun. *M. javanica* was cultured on tomato, tobacco and courgettes (*Cucurbita* spp.) the last serving as the non-specific host. Infected seedlings were grown in pots containing steam sterilized soil (John Innes No. 2 compost) for two weeks under normal greenhouse conditions (25-27 °C, relative humidity 80 % and day length of 16 hours). Six uninfected seedlings of each host were grown under the same conditions and used as controls.

Peroxidase activity of some other nematodes was also determined. *Globodera rostochiensis* Ro 1 and *G. pallida* Pa 1 were grown on potato cv. Pentland Dell in pots and white females were extracted by hand. *Cephalobus* sp. was cultured on small pieces of peeled surface-sterilised potato kept in a crystallizing dish with some distilled water; infection was by direct transfer of nematodes using a sterile needle. *Panagrellus redivivus* was cultured at 23 °C in glass crystallizing dishes on an autoclaved mixture of whole wheat flour and distilled water. *Turbatrix aceti* was cultured on 30 % (v/v) malt or wine vinegar in tap water. *Ditylenchus dipsaci* fourth stage larvae were extracted from dried, infected field beans *(Vicia faba)* and allowed to revive in distilled water before use. Experimental procedures were the same as detailed below for *Meloidogyne;* 35 white females of *Globodera* spp. or 10 µg wet weight of each of the other species were used for each assay.

Recovery of female *Meloidogyne*

The procedure used was similar to that previously described by Hussey (1971). For each assay, 50 females were removed by hand using a pipette under a dissecting microscope and placed in cold distilled water. Subsequently the females were rinsed three times with cold 0.01 M phosphate buffer (pH 7.5) containing 0.85 g NaCl and 0.01 g $MgCl_2$ per 100 ml. The cleaned females were weighed (10 µg wet weight) in polyethylene microcentrifuge tubes (Camlab) and either used immediately for analysis or stored at - 15 °C.

To obtain cuticles, females were ruptured in polyethylene microcentrifuge tubes using a glass rod. Cuticles were removed from the tube with a pipette into distilled water in an excavated glass block. They were viewed under a binocular microscope and washed several times until they were free from tissue debris.

Extraction of nematode proteins

Twenty minutes before electrophoretic separation, the frozen females were homogenized in ice-cold 0.1 M Tris.-HCI buffer (pH 7.8) and centrifuged at 5000 r.p.m. for 10 min at 4 °C. The clarified supernatant was introduced immediately into the electrophoresis cell. The concentration of proteins in the extracts was estimated by Bradford's (1976) method, using bovine serum albumin (BSA; Sigma) as the standard.

Isoelectric focusing

Peroxidase isoenzymes were separated by flat-bed isoelectric focusing (IEF) Multiphor equipment (LKB) utilizing a self-regulating power supply, using Ampholine (pH 3.5-9.5 LKB). The cathode and anode electrode strips were soaked in 1 M NaOH and 1 M H_3PO_4 respectively, and were placed directly onto the ends of a gel and two platinum wires connected from the power supply were then laid on the top of these strips. The gel was placed on a platen which was also a cooling stage, kept at 4 °C, and prefocused for 30 min before 0.03-0.05 ml samples of nematode extracts or 0.01 ml of root extracts were applied on the well surface of the gel. Electrofocusing was achieved in 3 h with a constant power of 800 V.

Determination of the pH gradient

At the end of the running time the power supply was temporarily disconnected and readings were taken at 5 min intervals from the anode to the cathode using a surface pH electrode (Pye-Unicam). Finally the gel was refocused for a further 10 min to improve the definition of the bands.

ENZYME VISUALIZATION

Peroxidase activity was visualized by incubating the gels for 5 min at room temperature in a mixture of 0.5 % (w/v) benzidine dissolved in 10 ml of acetic acid and made up to 100 ml with distilled water. They were then placed in distilled water containing 0.3 % (v/v) hydrogen peroxide (H_2O_2).

PEROXIDASE ACTIVITY ASSAY

Peroxidase activity was assayed as previously described by Stermer and Hammerschmidt (1984).

For histochemistry, live females or females fixed with 10 % formaldehyde for 20-30 min and rinsed with water were used. The females were embedded in OCT (Miles) freezing medium in a soft plastic lid. When frozen, the complete block was removed from the lid and mounted with a drop of water onto the chuck of a cryostat freezing microtome.

Sections were cut at a thickness of 7-10 µm and placed on cooled slides which were thinly smeared with glycerine albumen. The slides were placed in warm water to remove the OCT medium and subsequently stained with the same solutions as those used for enzyme detection.

Results

Separations of multiple forms of peroxidase isozymes by PAG-isoelectrophoresis were repeated four times and were reproducible (Figs 1, 2). A total of seventeen isoperoxidase patterns with isoelectric points (Pls) between pH 5 and pH 8 were resolved from preparations of infected and uninfected roots of tomato, cucumber, tobacco and courgettes, four to five bands from female *M. incognita* cultured on these plants were detected but the fifth band was inconsistant.

Three new isozyme bands (Nos. 7, 9, 10) appeared in infected roots of tomato (Figs 1, 2, 5). Two strong bands (Nos. 2, 3) and two weak (Nos. 1, 15) were obtained from extracts of females cultured on tomato. The number of peroxidase isoenzymes increased in infected compared to uninfected roots of cucumber. One strong band (No. 7) and two weak bands (Nos. 5, 9) were present in infected roots. A strong acidic band (No. 17) was shown at the edge of the gel in both infected and uninfected roots, whereas four bands (Nos. 1, 2, 3, 4, 5) were detected from nematodes propagated on the same host. The highest concentration was always present in the giant cell.

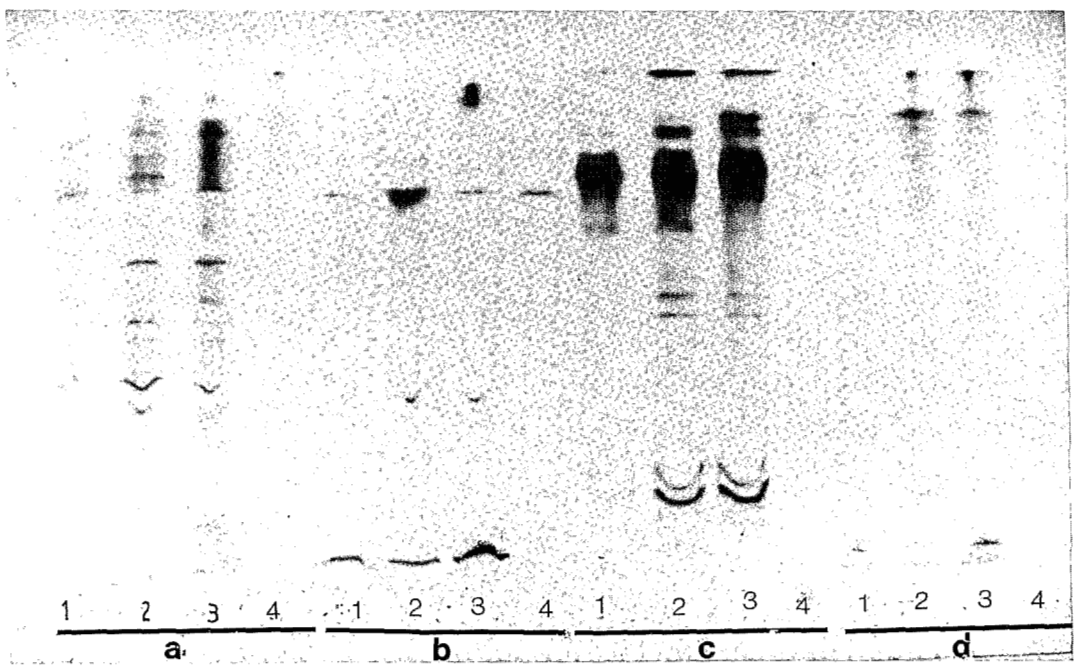

Fig. 1. Peroxidase isoenzyme pattern obtained by isoelectrofocusing from infected root tissues of different hosts within 1-6 days after infection with *Meloidogyne incognita*. 1 : Uninfected roots (control); 2 : Infected roots; 3 : Infected roots (giant cells); 4 : Adult females (A : tomato; B : cucumber; C : tobacco; D : Courgette).

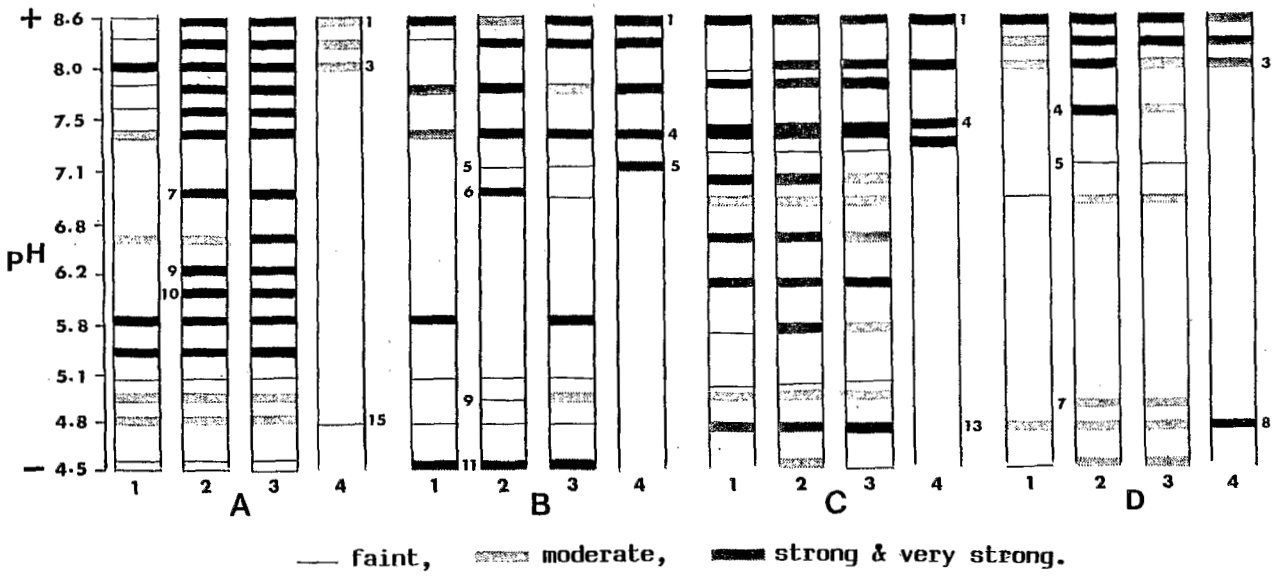

Fig. 2. Schematic peroxidase isoenzyme patterns obtained by isoelectrofocusing from infected root tissues of different hosts 1-6 days after infection with *Meloidogyne incognita*. Numbers on tracks for each host refer to the loction of important bands (see text); 1 : Uninfected roots (control); 2 : Infected roots; 3 : Infected roots (giant cells); 4 : Adult females (A : tomato; B : cucumber; C : tobacco; D : courgette).

Isoenzyme patterns from tobacco plants exhibited the same banding with infected and uninfected roots. Control bands were less intense. Four bands (Nos. 1, 2, 3, 4) and one acidic band (No. 13) were obtained from females reared on tobacco.

Isoenzyme patterns from courgettes showed slight differences in intensity between infected and uninfected root-tissues with two weak bands (Nos. 5, 7). Female extracts from the same host exhibited four bands (Nos. 1, 2, 4, 5).

Data in Table 1 indicate that only adult females show peroxidase activity. Four to five bands were obtained from *Meloidogyne* sp. cultured on different hosts, whereas a total of two bands were detected from *Globodera* spp. cultured on potato plants. No peroxidase activity was detected either in *Ditylenchus dipsaci* or in the free-living nematodes *Turbatrix aceti*, *Panagrellus redivivus*, and *Cephalobus* sp.

Cuticles isolated from females of *Meloidogyne* spp. and analyzed by isoelectric focusing showed no peroxidase activity. Histochemical staining revealed peroxidase activity in some parts of the female body (Fig. 3 E). The greatest activity was associated with the gelatinous matrix (Fig. 3 B). Eggs did not show any peroxidase (Fig. 3 D).

The results shown in Fig. 4 provide clear evidence of the increase in peroxidase in root-tips removed within six days after inoculation in tomato, and after two days in cucumber and tobacco. Greatest peroxidase activity was always detected in the plant tissues adjacent to the nematodes. Female extracts showed very low peroxidase activity.

Quantitative relations between isoperoxidase extracts from uninfected roots and roots infected by *Meloidogyne* spp. for six days is represented by densitometric tracings (Fig. 5). Three extra bands (Fig. 5; arrows) were revealed in infected roots. The peroxidase profiles obtained from different populations of *Meloidogyne* spp. cultured on four different hosts after isoelectrofocusing were also studied (Fig. 6). Two bands (Pl. 7.91, 7.93) and one weak band (Pl. 7.25) were detected from extracts of females of *M. Incognita* (Bangladesh population) and *M. javanica*, while *M. incognita* (Newcastle population) had four bands (Pl. 8.21, 7.91, 7.83, 6.05). Four bands (Pl. 8.70, 8.20, 7.91, 7.93) and one acidic band (Pl. 4.83) were detected from *M. incognita* (Leeds population) from tomato. A very strong band (Pl. 6.05) and three less strong bands (Pl. 8.21, 7.25, 6.02) were observed from females reared on cucumber. Females reared on tobacco had four distinct bands (Pl. 8.20, 7.92, 7.22, .05). Three bands (Pl. 8.20, 7.95, 7.92) and one acidic weak band (Pl. 4.82) were obtained from females reared on courgettes. A common band (Pl. 8.21) was detected among the four populations of *Meloidogyne* spp. Isoperoxidase patterns of *M. incognita* and *M. javanica* cultured on the same host were not significantly different, except for one acidic weak band (Pl. 4.82) which appeared from females extracted from tomato plants. Using a narrow range ampholine (pH 5-8), similar results were obtained in isoperoxidase patterns for *M. incognita* populations cultured on different hosts (Fig. 7).

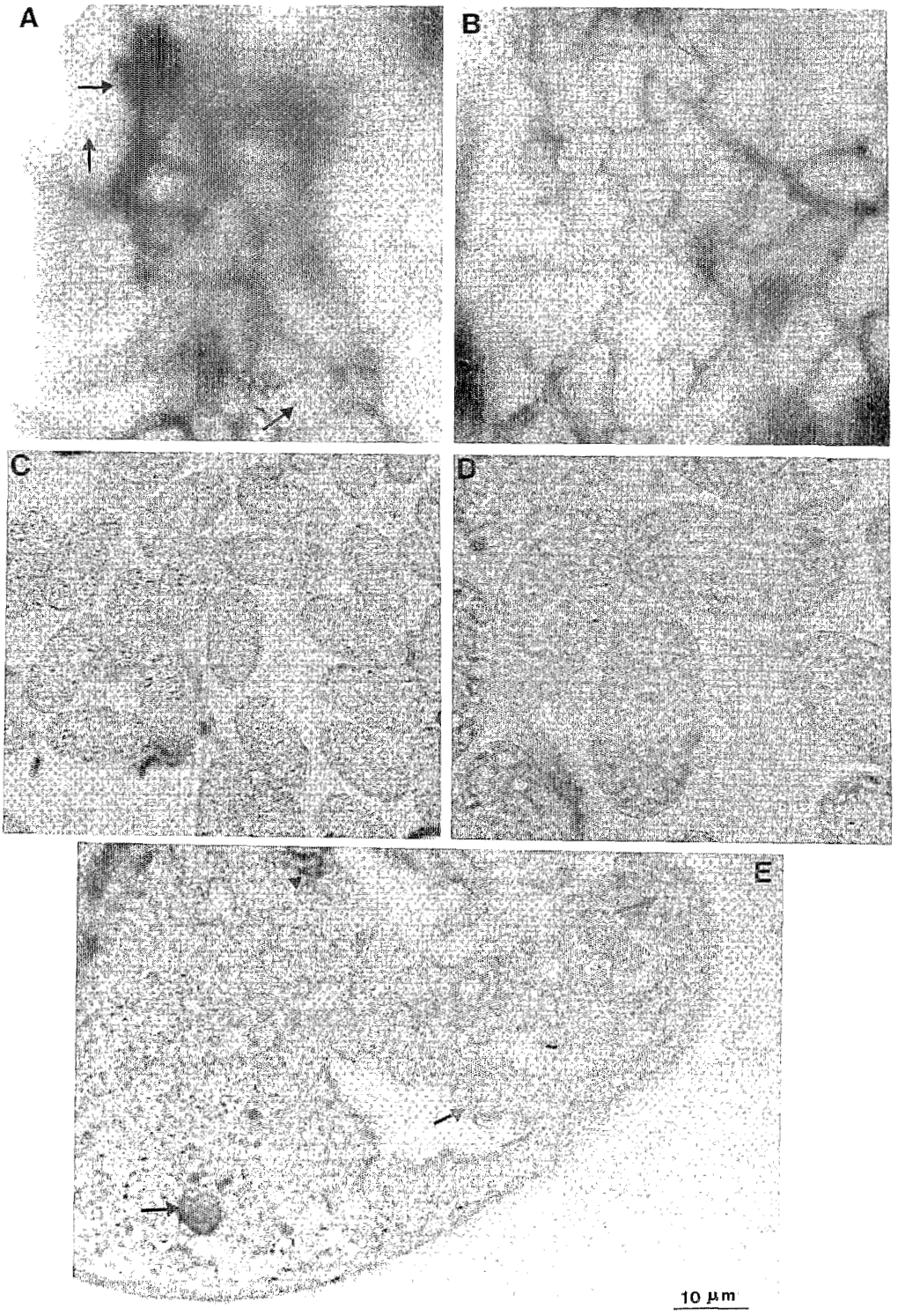

Fig. 3. Peroxidase activity of *Meloidogyne incognita*. A : Gelatinous matrix (stained for peroxidase activity); B : Gelatinous matrix (unstained); C : Mature eggs (stained for peroxidase activity); D : Mature eggs (unstained; control); E : Cross section of female; arrows indicate the position of peroxidase activity.

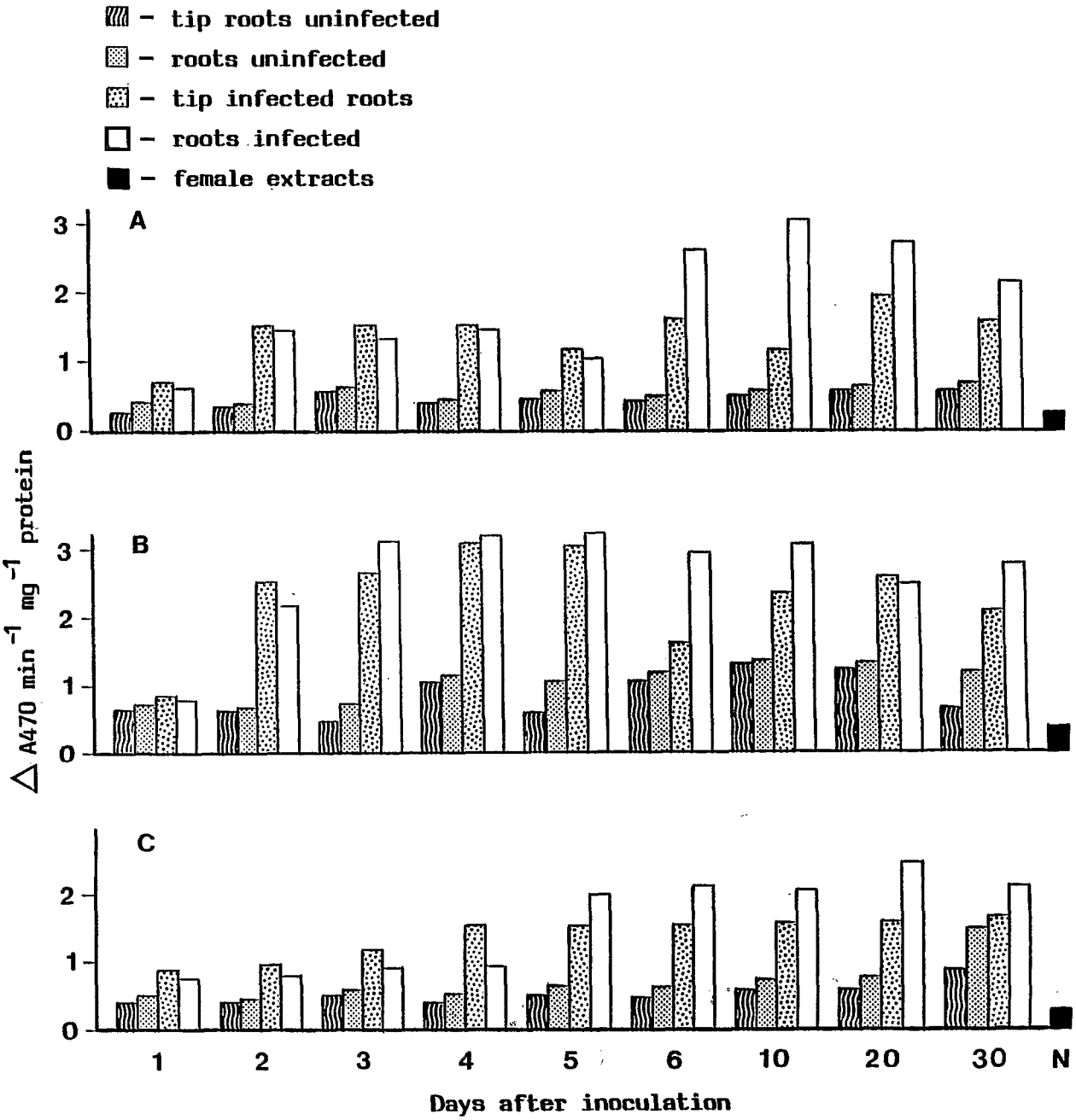

Fig. 4. Isoperoxidase activity from root tissues inculated with *Meloidogyne incognita*. Increase in peroxidase activity is represented as increase in absorbance (min^{-1} mg^{-1} protein) using guaiacol as the hydrogen donor. A : Tomato; B : Cucumber; C : Tobacco.

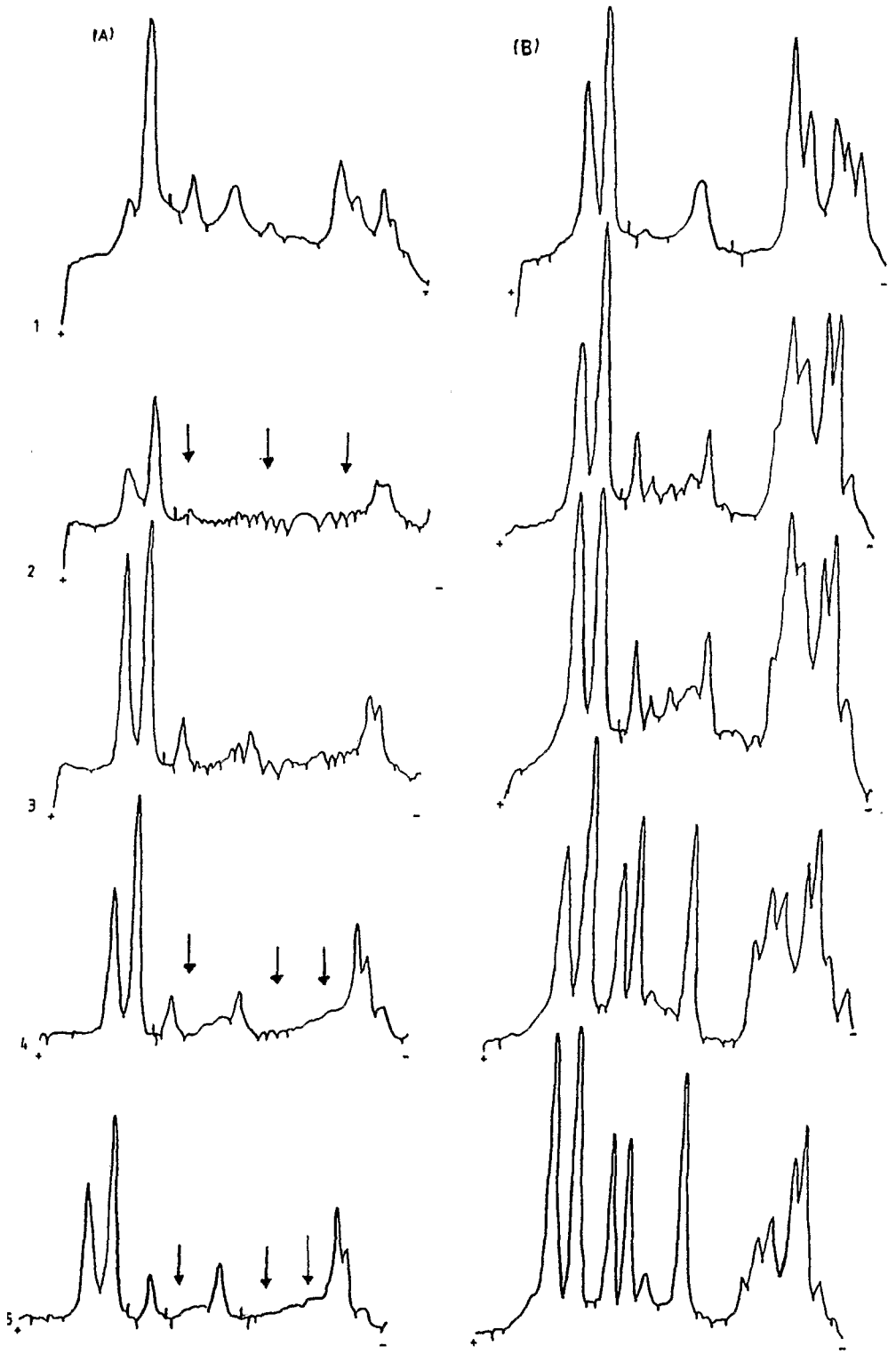

Fig. 5. Densitometric scans of tissues 1-6 days after infection with *Meloidogyne incognita* on tomato. Arrows indicate the missing bands (see text) (A : control; B : infected).

Table 1

Isoperoxidase bands of some plant-parasitic nematode species propagated on different hosts and some free-living nematodes, detected by isoelectrofocusing

Nematodes	Host or culture medium						
	Tomato	Cucumber	Tobacco	Courgettes	Potato	Wine	Flour
M. incognita	5	5	5	—	—	—	—
M. incognita	5	5	5	—	—	—	—
M. incognita	5	5	5	—	—	—	—
M. javanica	5	—	5	4	—	—	—
G. rostochiensis	—	—	—	—	1	—	—
G. pallida	—	—	—	—	1	—	—
D. dipsaci	—	—	—	n	—	—	—
P. redivivus	—	—	—	—	—	—	n
T. aceti	—	—	—	—	—	n	—
Cephalobus sp.	—	—	—	—	n	—	—

5, 1 = Number of bands; n = No reaction with IEF; — = Not tested.

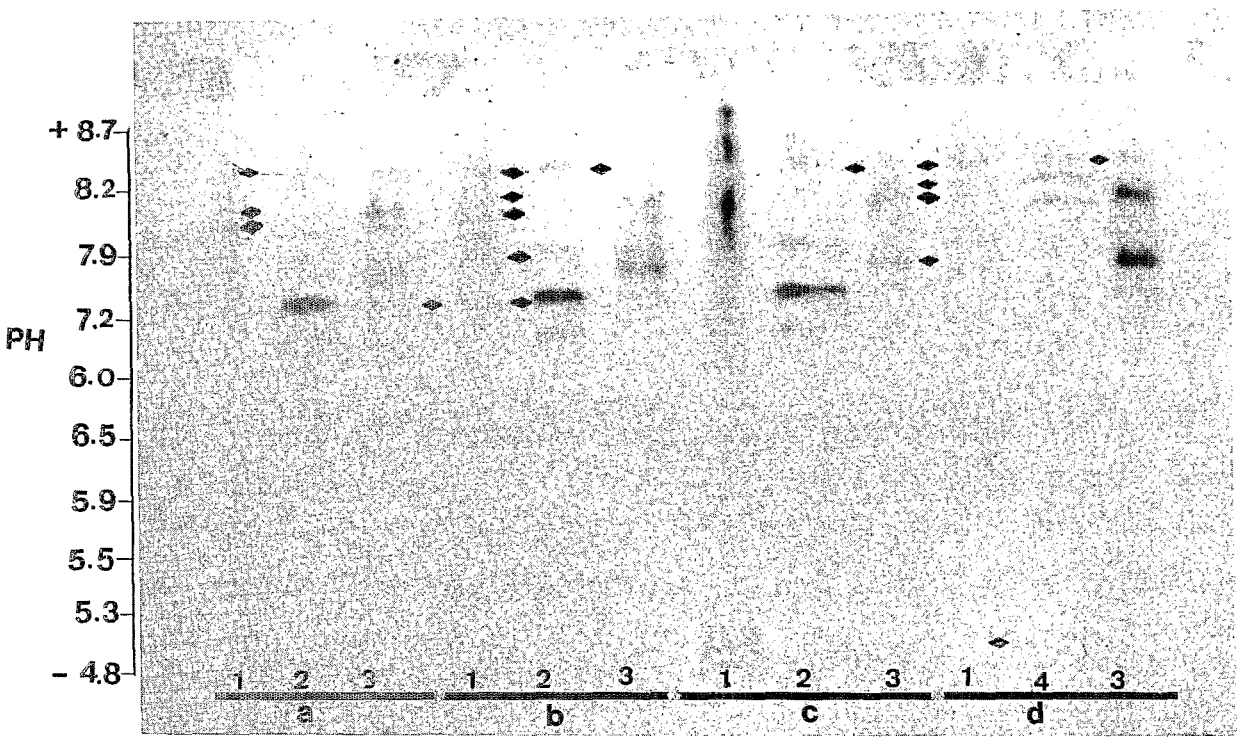

Fig. 6. Isoperoxidase profiles obtained by isoelectrofocusing of four populations of *Meloidogyne* spp. cultured on different hosts separated in the pH range 3.5-9.5. a, b, c : *Meloidogyne incognita* obtained from different parts of the world. d : *M. javanica.* (1 : tomato; 2 : cucumber; 3 : tobacco; 4 : courgette).

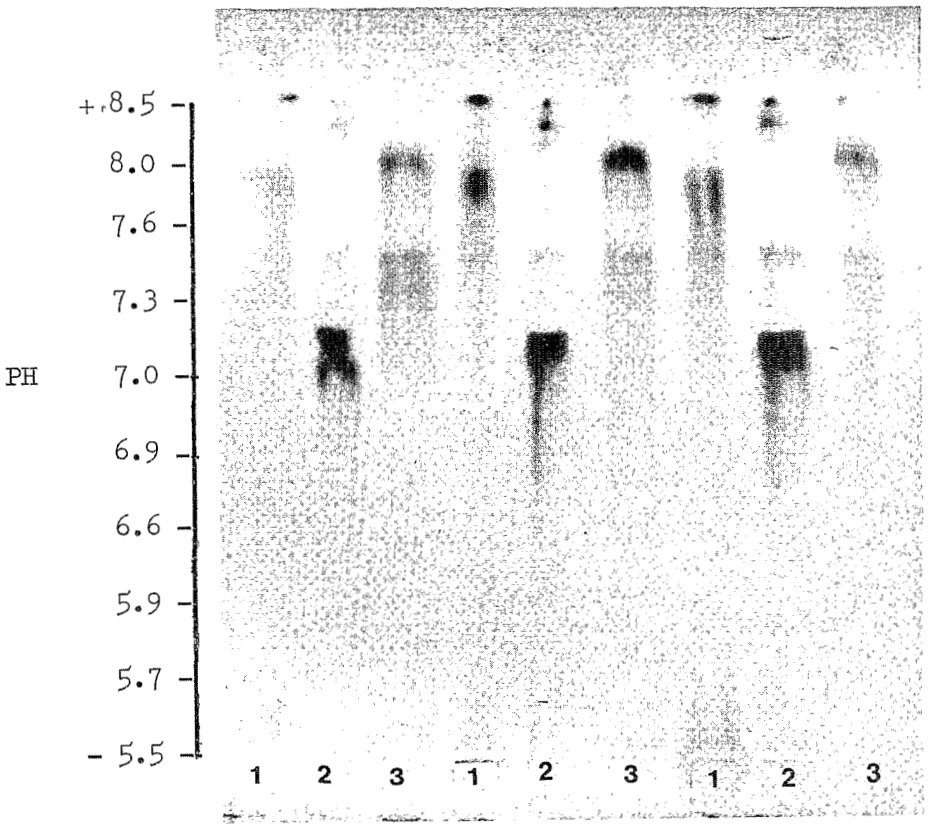

Fig. 7. Isoperoxidase profiles obtained by isoelectrofocusing of three populations of *Meloidogyne incognita* cultured on different hosts separated in the pH range 5-8. 1 : Tomato; 2 : Cucumber; 3 : Tobacco.

Discussion

This study demonstrated the presence of peroxidase activity in extracts of females of *Meloidogyne* spp. reared on tobacco. Previously, Hussey, Sasser and Huisingh (1972), Hussey and Sasser (1973), and Starr (1979) have failed to detect any peroxidase activity in extracts of females propagated on tobacco after separation by PAGE. These workers also reported that only two isoperoxidase activity bands could be detected in extracts of females propagated on tomato plants. In the present investigation two additional isoperoxidase bands were obtained from females removed from the same host. It is possible that these variable results were due to the different techniques employed. Furthermore, the majority of peroxidase isoenzymes of females extracted from tobacco are cationic proteins (Hussey & Sasser, 1973), which could not be detected in the slab-gel electrophoresis system.

The origin of the peroxidase activity in the roots in response to nematode infection is not yet understood. This study demonstrated that only plant-parasitic nematodes, in direct contact with plant-cells, showed peroxidase activity. No peroxidase activity was detected in three free-living nematodes (Table 1). The same conclusion has been reached by Hussey and Krusberg (1971). It has been suggested that the presence of peroxidase activity in the saliva exudate from the stylet of adult females might have a positive role in giant cell formation (Hussey & Sasser, 1973). In contrast, Veech, Starr and Nordgren (1987) did not find any peroxidase activity in stylet exudates separated by electrophoresis. It is unlikely that nematodes inject peroxidase into the host cells. This conclusion is supported by the fact that the enzyme activity is only present in adult females; eggs, L 2 and males showed no peroxidase activity. The invasion of nematodes into plant roots appears to lead to an increase of peroxidase in infected roots of hosts during the early stages (Gregory & McClure, 1978; Zacheo *et al.*, 1980; Raja & Dasgupta, 1986).

Huang, Lin and Huang (1971) reported that peroxidase isoenzymes of uninfected root- tissues and roots infected by *Meloidogyne* spp. were different, whereas Starr (1979) did not find any difference between galled and ungalled root- tissues. In this study some variation occurred soon after infection (Fig. 1). The appearance of

these new peroxidase bands suggests a reaction by the defence mechanism system of the host to the continuous damage by nematodes, or a direct response only by the root cells. Convincing evidence was provided that peroxidase is bound to the plant cells walls (Veech & Endo, 1969; Giebel, Krenz & Wilski, 1971) and is associated with peroxidation of phenolic substances during the formation of lignin or suberin in the cell wall (Goldberg & Catesson, 1985). Kevers and Gaspar (1985), and Asada, Oguchi and Matsumoto (1975) reported that lignin forms as a direct response to infection or wounding in plants. Giebel, Krenz and Wilski (1971) observed the " barrier " in cells with high peroxidase activity at the beginning of giant cell formation which later disappears. This observation suggests that peroxidases are produced by plants as a first reaction against nematode invasion.

ACKNOWLEDGEMENT

This paper forms part of a study for a Ph. D. degree. The author gratefully acknowledges the help of his supervisor, L. Smith, the assistance of Dr. R. N. Perry in the preparation of this manuscript and the financial support of the Hariri Foundation.

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Accepté pour publication le 14 mai 1990.

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