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MORPHOLOGY AND ENZYME HISTOCHEMISTRY IN GERMPLASM PEA ROOTS ATTACKED BY *HETERODERA GOETTINGIANA*

by
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Summary. The host-parasite relationships between *Heterodera goettingiana* Liebscher and pea accessions (*Pisum sativum* L.) were examined through serial sections of infested rootlets. Nematode feeding stimulated formation of syncytia in both susceptible and resistant host tissues 4 days after inoculation. The size of syncytia increased with time after inoculation. At 10 days in all the susceptible accessions the multinucleate syncytium was characterized by dense, granular cytoplasm. In all of the resistant accessions the mechanism of resistance involved degeneration of the syncytial cytoplasm and the nematode failed to develop to maturity. Localization for peroxidase and esterase activity was carried out on 4 day-inoculated root tissue. Light and ultraviolet light microscope observations of tissues revealed that both enzyme activities were increased in the susceptible and the resistant accessions following nematode invasion, but with higher levels in the resistant tissues. The results are discussed in relation to the possible role of peroxidases and esterases in the response of pea root to nematode infection.

The mechanism of penetration and establishment of various *Heterodera* spp. in their hosts and the related host response has been reported for a number of species (Riggs *et al.*, 1973; Jones, 1981; Yu and Steel, 1981). The ability of the soybean cyst nematode (*Heterodera glycines*), the potato cyst nematode (*Globodera* sp.) and the sugar beet cyst nematode (*H. schachtii*) to cause considerable stunting and/or death of the hosts has been well documented by histological, histochemical and ultrastructural studies (Endo and Veech, 1970; Endo, 1978; Krauthausen and Wyss, 1982; Rice *et al.*, 1985 and 1987; Bleve-Zacheo and Zacheo, 1987; Bleve-Zacheo *et al.*, 1990; Melillo *et al.*, 1990).

The cyst nematodes damage root tissues by forming syncytia through the dissolution of cell walls and the fusion of the cytoplasm from contiguous cells. Early development of syncytia at the feeding sites is comparable in susceptible and resistant hosts. The syncytial cells associated with nematodes in resistant hosts are disorganized and become lysed in a relatively short time (ten days), the second-stage juveniles that penetrate the root remain close to this feeding site and the majority fails to develop, although a few females may become adult in the incompatible situation and produce at least a few eggs (Acedo *et al.*, 1984). In susceptible hosts, cells are incorporated into the syncytium and their further proliferation proceeds unchecked. In this context, the susceptible host permits rapid nematode growth and reproduction.

The establishment and maintenance of the syncytium requires stimuli which one presumes to be associated with the injection of secretions from the nematodes into host cells. This suggests a co-evolution between cyst nematodes and their hosts. Stone (1979) reported that the co-evolution between potato cyst nematodes and their hosts is the selection of genes in hosts conferring resistance to nematodes. Natural sources of resistance have been found for the potato cyst nematodes (Rice *et al.*, 1985) and *H. schachtii* (Yu and Steel, 1981) but so far no source of resistance has been identified in pea cultivars against *H. goettingiana*.

Little information is available on the interaction of pea with *H. goettingiana* (Di Vito and Perrino, 1978; Zacheo *et al.*, 1981 and 1986). The present study initiated to define anatomical changes induced by *H. goettingiana* in pea germplasm lines and to establish the levels of activity of peroxidase and esterases in compatible/incompatible interactions.

Materials and methods

Juveniles of *H. goettingiana* were obtained from cysts collected from the roots of pea growing in pots containing field soil infested with the pea cyst nematode. Seeds of six accessions of germplasm pea: MG 101877 a and MG 101877 b (*Pisum arvense* L., collected in Ethiopia), MG

101956 a and MG 101956 c [*P. elatius* (Steven) Schmalh. obtained from Netherland collection], MG 101748 (*P. arvense* L., collected in South Hungary) and MG 103738 (*P. sativum* sp. *transcaucasicum* Govorov, obtained from Gatersleben collection), collected and multiplied at the Istituto of Germoplasma, Bari, were germinated under 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* was added to each pot.

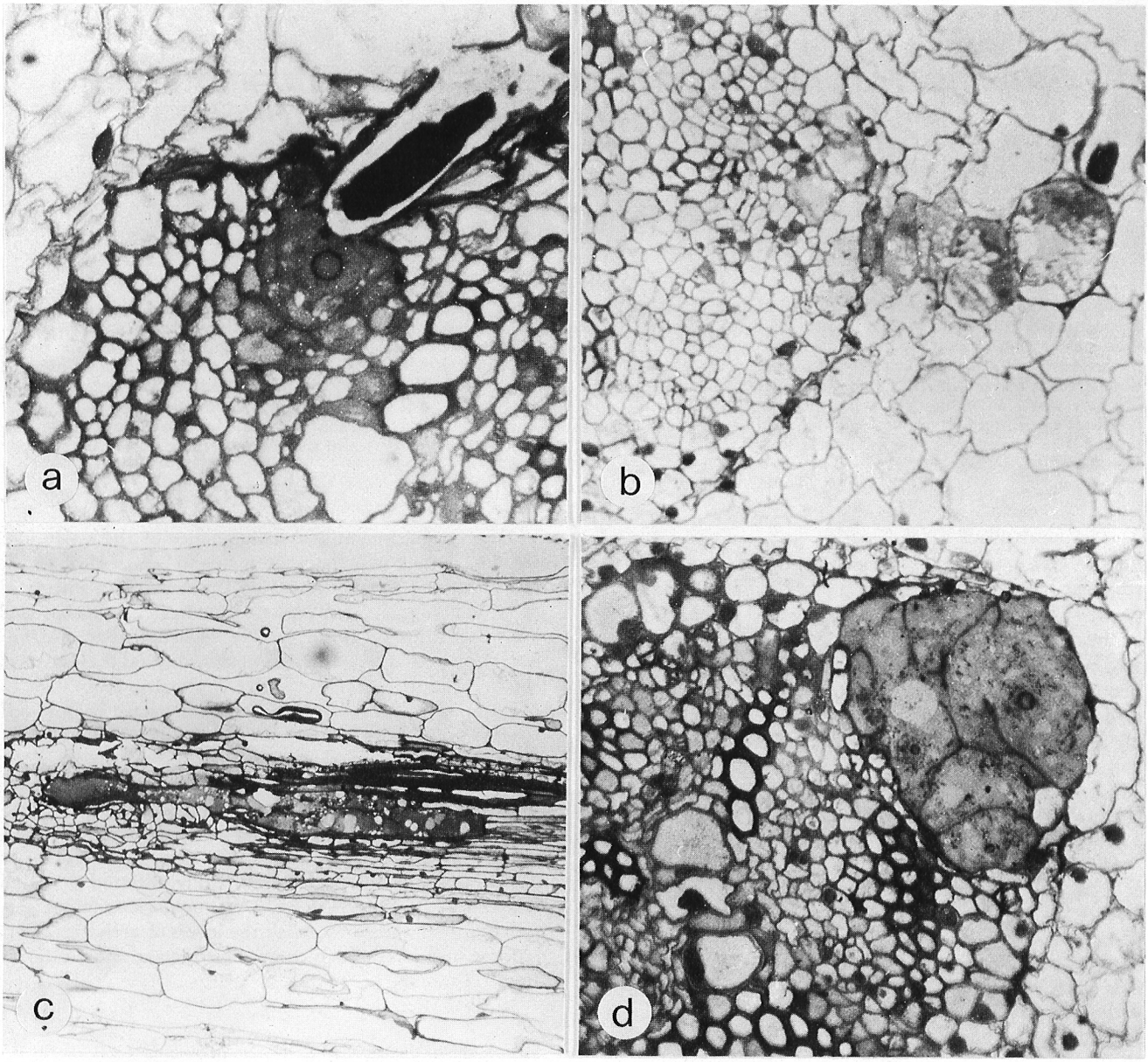

Fig. 1 - Micrograph of pea roots and feeding sites of *Heterodera goettingiana* in susceptible hosts. (a, b) Cross section of MG 101877a root with syncytia associated with a female (a) and a male (b) 4 days after nematode inoculation. Note, the former is within the stele and the latter is in the cortex. Both structures initiate from the endodermal layer, which appears to be more necrotized in (a), (a × 560, b × 370). (c) Longitudinal section of well developed syncytium in MG 101877 a, 10 days after nematode inoculation. The cytoplasm content is deeply stained and dense. The syncytial components are widely extended along the root axis and the necrotic area is very reduced (× 200). (d) Cross section of a syncytium in MG 101956c 10 days after nematode inoculation. The structure is strictly related to endodermal cells, phloem and xylem elements. Cell wall stubs and hypertrophied nuclei are clearly visible. Note the absence of vacuoles and dense cytoplasm (× 370).

The inoculated plants were maintained at 17°C in growth chambers.

At 4 and 10 days after inoculation segments of infested roots were excised and fixed for 4 h in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer pH 7.2 post-fixed in 2% osmium tetroxide in the same buffer for 2 h, dehydrated in a graded ethanol series and embedded in Spurr's medium (1969). Thick, transverse and longitudinal sections (2 μm) were cut using a LKB ultratome IV, transferred onto glass slides, dried and stained with 1% toluidine blue in 1% borax. Slides were examined and photographed using a light microscope. Enzyme localization was carried out as described by Gahan (1984). Unfixed, frozen sections of root segments of infested and uninfested germplasm pea, 4 days after nematode inoculation, were prepared with a 2800 Frigocut REICHERT-JUNG automatic cryostat at cutting speed of 5 and section thickness setting 17 μm.

To test for peroxidases, sections were incubated in a medium containing lead nitrate, homovanillic acid and rhodamine B as substrate in 0.2 M sodium acetate buffer at pH 6. Freshly prepared 1% H_2O_2 was added. The sections were observed under UV using a Leitz Dialux microscope equipped with a mercury vapor lamp and a violet filter assembly transmitting wavelengths from 361-435 nm.

To test for esterases, the sections were reacted at 37°C using naphthol AS-D acetate as substrate and fast blue BB as the diazonium salt in Tris-HCl buffer at pH 6.5 for 60 min and observed under a bright field microscope. The esterase reaction employs a diazonium salt which can couple readily with polyphenolics present in some root cells, expecially those involved in nematode infection, at the pH employed. However, there is no difficulty in distinguishing between the esterase enzyme product, which is an intense blue colour, and the polyphenolics which are yellow-brown stained.

Results

Syncytia were induced by *H. goettingiana* in all six accessions of pea roots. The structural features of syncytia under the light microscope were similar four days after nematode inoculation, in terms of cell wall dissolution, dense cytoplasm and absence of vacuoles (Figs. 1a, 2a, 2b, 3b). The feeding nematode was located close to endodermal cells, which became necrotic, expecially those subjected to mechanical damage by the invading parasites. The syncytium extended longitudinally by incorporating cells of the pericycle and vascular parenchyma by gradual dissolution of cell walls and merged as one continuous multinucleate cytoplasmatic unit (Figs. 1a, 2a, 2b, 3b). A characteristic type of syncytium was associated with those nematodes that developed to males in both compatible and incompatible combinations. Figures 1a and b compare syncytia associated with female and male nematodes in compatible hosts. Male feeding sites were usually found in the cortex, syncytium expansion starting from the pericycle to the cortical layers. Figures 3a and b compare those induced by male and female nematodes in incompatible combination. A large number of cortical cells were involved in the syncytial unit, which also appeared to incorporate endodermis and pericycle (Fig. 3a). The syncytia in susceptible roots fixed on day 10 reached a large size and developed along the root axis bordering on xylem vessels. Their cytoplasm stained deeply, hypertrophied nuclei were irregularly shaped and central vacuoles were absent-all the typical features of functional feeding sites (Figs. 1c, 1d). In transverse section syncytial components were observed to maintain their contact with endodermis and pericycle, but these were then displaced by the syncytium enlargement (Fig. 1d). At 10 days after nematode inoculation, the accessions of 101956a, 101748, 103738 and 101877b developed an incompatible reaction. The juveniles failed to develop beyond the third stage (Figs. 2d, 3d) and their associated syncytia were almost degenerate (Figs. 2c, d, 3c, d) indicating that the nematodes had not obtained adequate nutrients. Despite this host response, the size of the syncytia, considering the number of cells that were incorporated, had greatly increased (Figs. 2c, d, 3c, d). In these syncytia the cytoplasm was reduced to a very thin layer around the outer walls and very few organelles were present, but there were differences between the four accessions tested. The reduction in cytoplasmic density was more evident in the combination MG 101748 and MG 103738 — *H. goettingiana* (Figs. 2c, 3c). Figures 2d and 3d show that the wall thickenings of syncytia induced in MG 101956a and 101877b were less pronounced than in the other two combinations (Figs. 2c, 3c) and cellular content was continuous through the spaces between cell wall fragments. Fig. 3c (MG 103738) and Fig. 3d (MG 101877b) clearly show the differences between the two syncytia. In MG 103738 thickened cell walls and cytoplasm reduced to deposits of amorphous dark material indicate that it was a non-functioning system; the syncytial volume in MG 101877b root was extremely vacuolated but still physiologically active.

Histochemistry

Localization of enzyme activities was identified by fluorescent reaction product for peroxidases and coloured reaction product for esterase activity in thick sections focused on encounter sites.

Fluorescent microscope examination revealed that in homovanillic acid-treated root tissues of MG 101956c with nematodes the reaction product in terms of brillant yellow spots were strongly located only in those cells affected by the presence of the parasite (Fig. 4a). In MG 103738 the reaction product was distributed not only in the cells directly affected by nematode injury but also in the outer cells, indicating the dispersion of intense enzyme

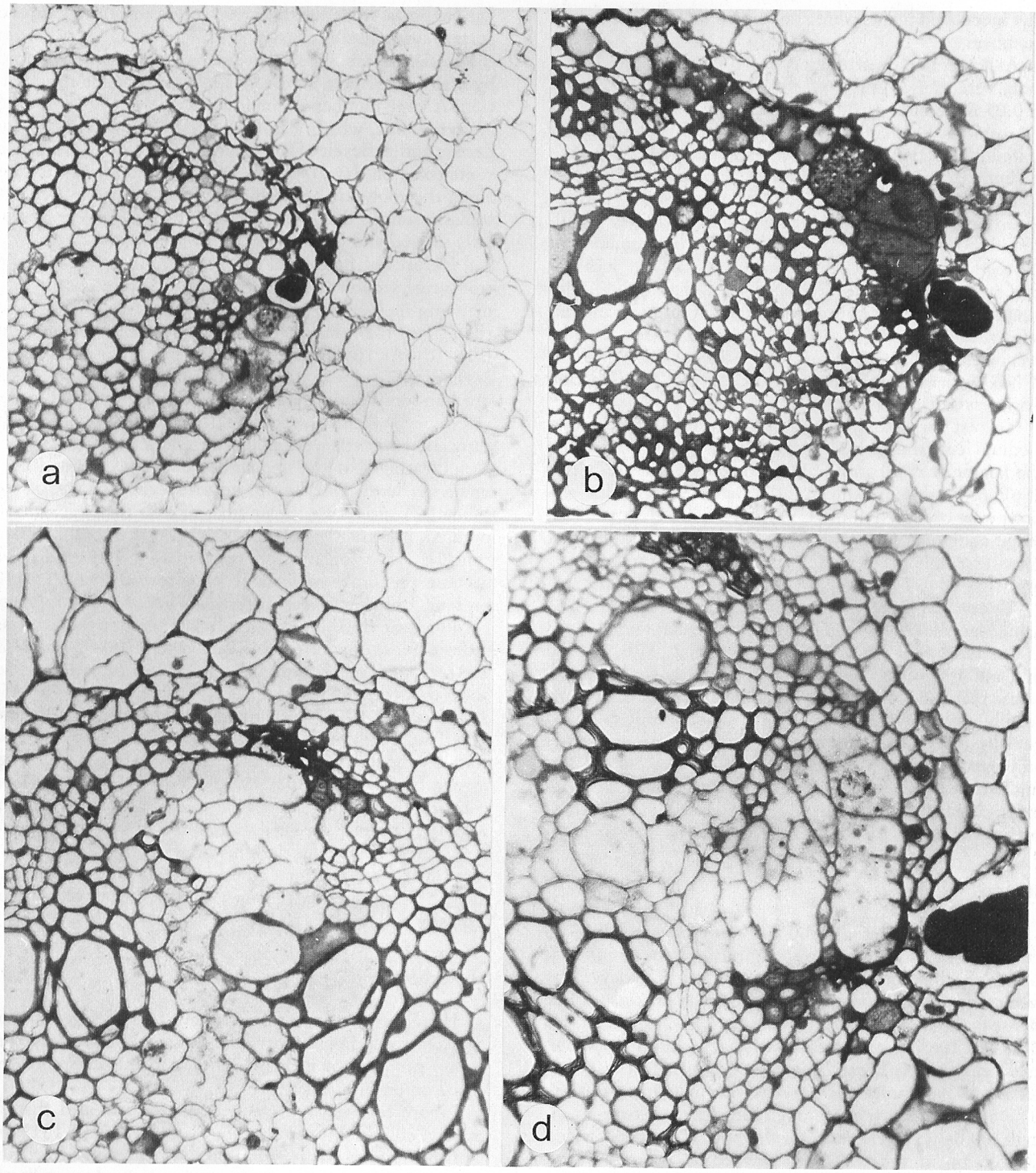

Fig. 2 - Micrograph of cross sections of resistant pea roots. Juveniles have stimulated syncytial formation, 4 days after inoculation in MG 101748 (a) and MG 101957a (b) respectively. In affected cells wall breakdown is evident and cell contents are still dense. Necrotic cells are present around the periphery of each syncytium (a × 380, b × 400). Syncytia, 10 days after nematode inoculation, greatly enlarged by incorporation of additional cells (c, d). They have the same shape and are related with vessels as syncytia in susceptible hosts but their cytoplasm is almost completely degenerated, particularly in MG 101748 (c). Some third stage juveniles are surrounded by dead cells (d), (c × 380, d × 380).

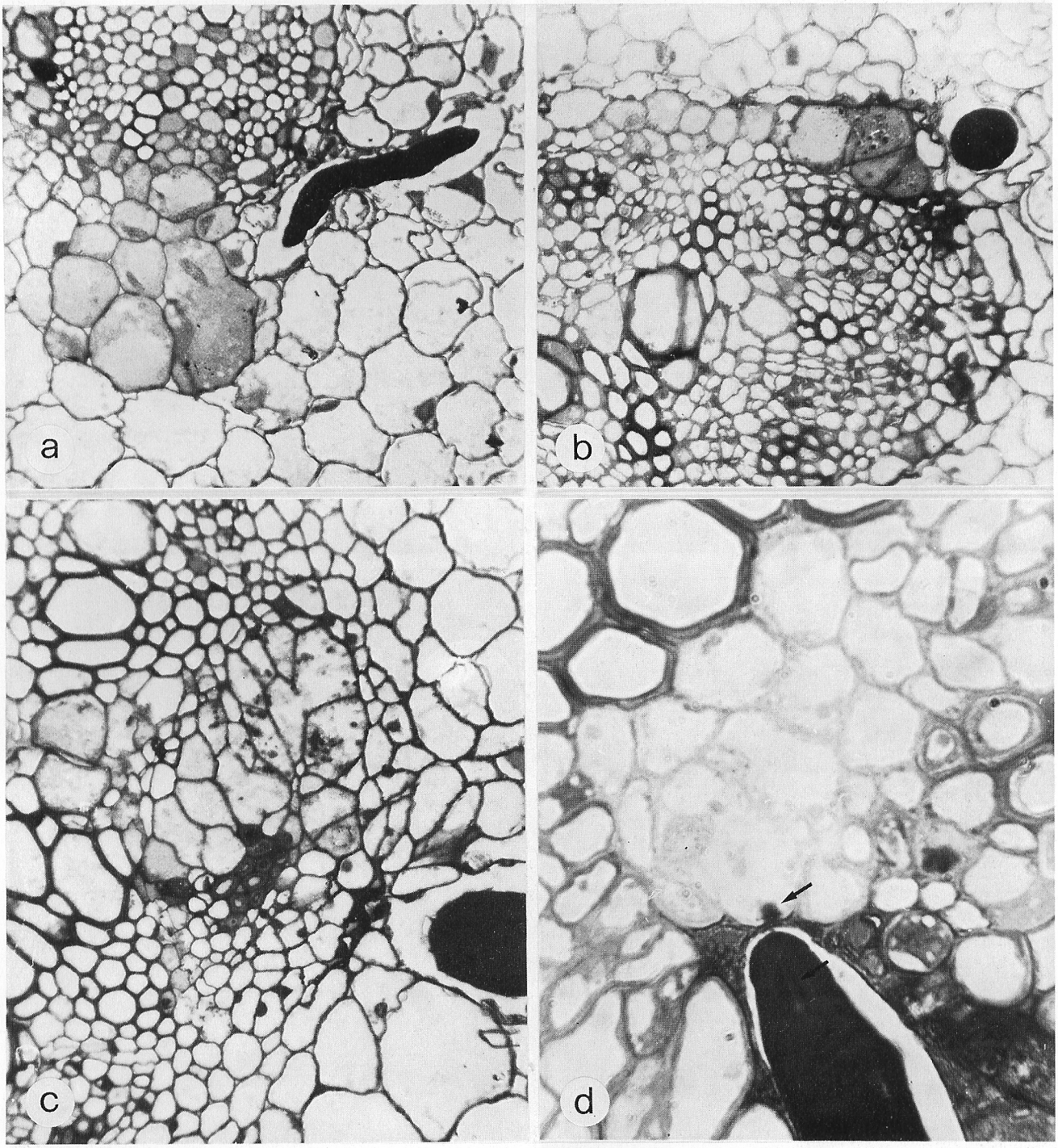

Fig. 3 - Micrograph of cross sections of MG 103738 (a, c) and MG 101877b (b, d) infested roots. The syncytium in a is located in the cortex and pericycle and is associated with a male, 4 days after inoculation. Note the great number of incorporated cells and hypertrophied nuclei. Syncytium in b is similar to those in Fig. 2a, b and delimited by necrotic cells (a × 380, b × 380). Very large syncytia in c and d 10 days after nematode inoculation. Cytoplasmic contents are transformed into amorphous dark material in c and reduced to cell wall layer but still synthetizing in d. Note the stylet of the juvenile and the plug (arrows) into the syncytial cells (d) (c × 360, d × 770).

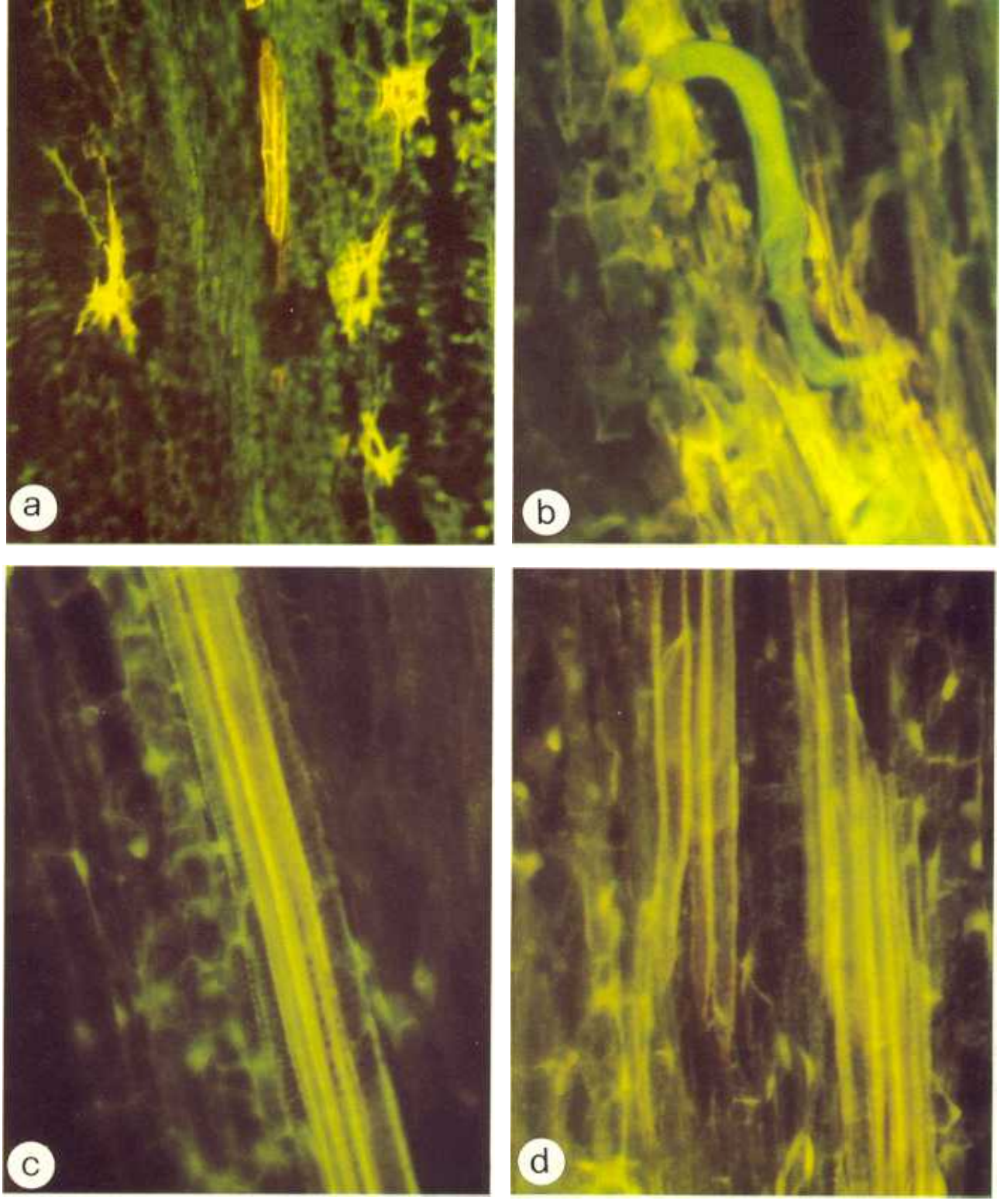

Fig. 4 - Unfixed frozen sections of pea roots reacted for peroxidase activity using homovanillic acid as substrate. (a) MG 101956c - *H. goettingiana*, 4 days after inoculation. The root cells show bright fluorescent spots at the site of nematode penetration (× 450). (b) MG 103738 - *H. goettingiana* interaction, 4 days after inoculation. A longitudinal view of the reaction involving a large number of cells either directly or indirectly injured by the nematode. Note the nematode positioned at its feeding site (× 1,000). (c) and (d). Healthy roots of MG 101956c and MG 103738 respectively. No (c) and very little (d) activity is present in the cortical cells; xylem walls reacted positively (× 1,000).

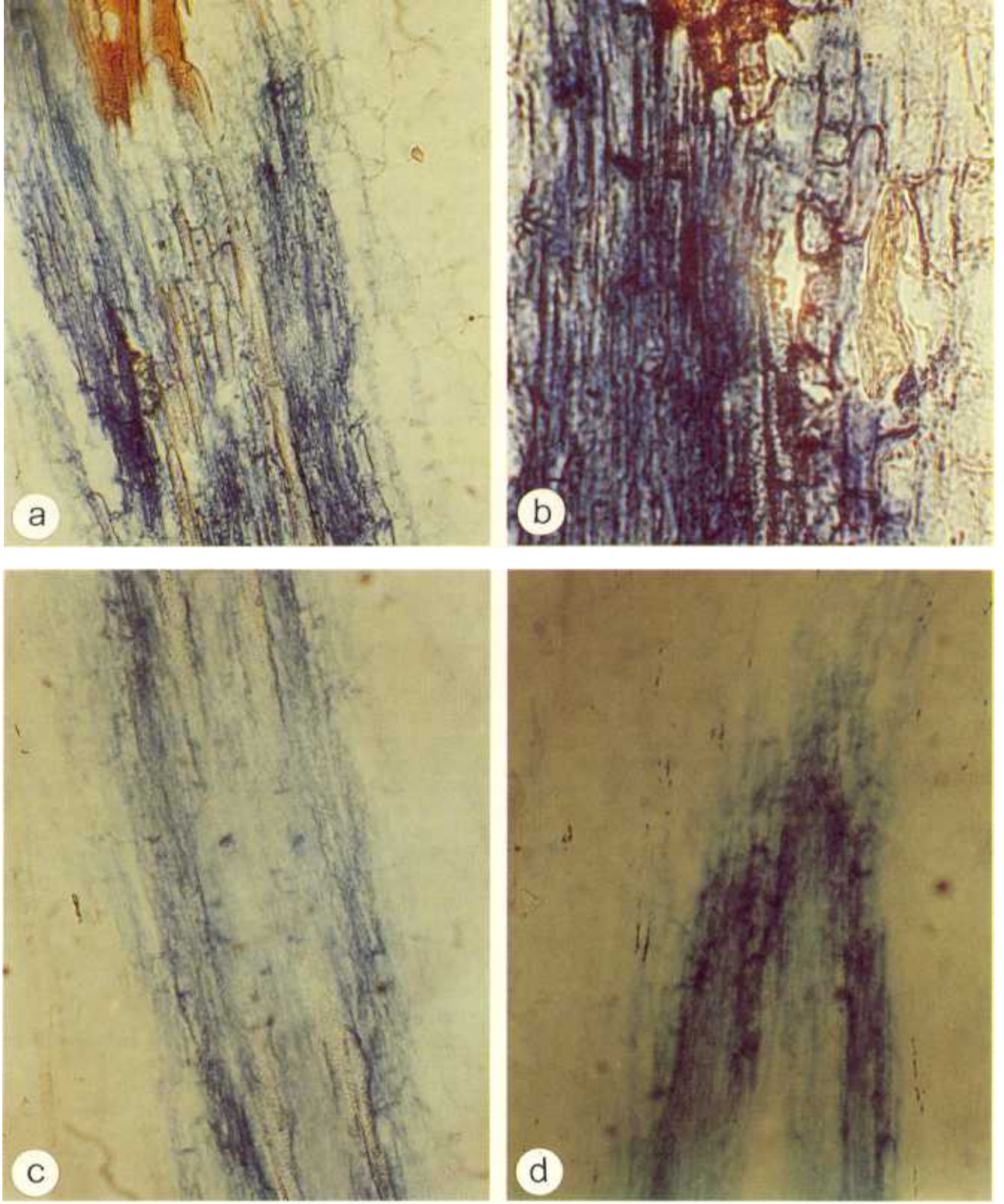

Fig. 5 - Unfixed frozen section of pea roots reacted for esterase activity using naphthol acetate AS-D as substrate and fast blue BB as the diazonium salt. (a) Longitudinal section of MG 101956c 4 days after nematode infection. The stelar region reacted for esterase activity more strongly than the healthy root (c). Esterase activity associated with nematode feeding was more pronounced in MG 103738 (b) than in healthy root (d). Note the reaction for phenolic compounds at the sites of nematode penetration (a, b) (a × 1,000, b × 1,600, c × 1,000, d × 1.000).

activity (Fig. 4b). Unaffected cells were green and vessels yellow (Figs. 4c, d). The results obtained for the unaffected root tissues used as control were almost similar. Deposits in the cells were absent, but some localization on cell walls was evident and the stelar region strongly reacted (Figs. 4c, d). The cortical cells of MG 103738 root tissues revealed the presence of peroxidase activity on the outer surface of the cell walls, more than in MG 101956c root tissues (Figs. 4c, d).

Figures 5a, b illustrate the localization of esterase activity during *H. goettingiana* infection in MG 101956c and MG 103738 root tissues respectively. In both accessions the enzyme activity was localized in the stele whose cells showed intense deep blue colour. The blue stain, however, was most intense in MG 103738 root tissues (Fig. 5b). The cells wounded by nematode penetration stained yellow-brown, indicating that they were necrotic (Figs. 5a, b). The localized deposition in the stele was also evident in the unaffected root tissues, used as control, of both accessions. The pattern of deposition was similar in both samples but the blue stain was less intense than in affected root tissues. (Figs. 5c, d).

Discussion

The pea cyst nematode, *H. goettingiana*, induced syncytial formation in all the pea accessions tested. The syncytia occurred in the same regions of the root tissues in both resistant and susceptible plants. The amount of tissue incorporated in the syncytia was similar among in all of the susceptible accessions. This is in contrast to the situation with potato-*Globodera* pathotypes and the suceptible host — *H. glycines* interaction (Hoopes *et al.*, 1978; Kim *et al.*, 1986; Melillo *et al.*, 1990). In the latter, the site and development of syncytia differed between the hosts and particularly in susceptible hosts. In pea roots the syncytium was always located in the stele in both susceptible and resistant accessions and our observations confirm the findings of other workers with respect to the formation and maintenance of the syncytium in susceptible plants. In all of the resistant accessions, however, differences were observed in the sequence of changes occurring in component cells of the syncytia. In later stages the collapse of the cytoplasm was more severe in MG 101748 and MG 103738, indicating some degree of resistance. Structural observations are insufficient, however, to establish the level of resistance and further investigations are needed to confirm these relationships. According to Hoopes *et al.* (1978), it can be concluded from these results that even the highly resistant plants have a certain degree of susceptibility in that they all respond to some extent to the stimulus to form a syncytium on which a nematode can feed and to some extent develop.

Preliminary studies on the biochemical changes induced by *H. goettingiana* demonstrated differences between pea accessions arbitrarily defined as resistant and susceptible, based on nematode numbers in the roots. Increases of ascorbic acid and peroxidase activity in resistant accessions attacked by pea cyst nematode have been reported (Zacheo *et al.*, 1981; Zacheo *et al.*, 1986).

Cytochemical studies of pea root apices demonstrated that peroxidase activity is present in virtually all cell-types and is involved with xylogenesis and in particular with lignification (Catesson *et al.*, 1978; Fielding and Hall, 1978). When the pea cyst nematodes infect the roots they induce an increase in peroxidase activity in the host tissues by forming reaction zone tissues. These reaction zone tissues exhibit much higher peroxidase activity than do healthy tissues and are more extensive in the resistant tissues. Increase in peroxidase activity has been widely reported in pathogenic infections where the stimulation of peroxidase activity is associated with resistance to infection (Coffey and Cassidy, 1984; Zacheo *et al.* 1982 and 1988). In this context, the peroxidases are assumed to operate the final step of lignin biosynthesis and this lignification may constitute an active defence mechanism against intra-tissue progression of the pathogens (Vance *et al.*, 1980).

Hydrolytic enzymes are reported to play important roles in the normal metabolism of plant cells during plant development (Chan *et al.*, 1970). In intact roots of *Pisum sativum* esterase activity is reported to be low in the cortical parenchyma and high in the rhizodermis and stele. Esterases constitute a marker of commitment to differentiation and the formation of xylem elements (Gahan, 1981).

Esterases are a complex and heterogenous group of enzymes which hydrolyse the ester link of different metabolites. However, the role of hydrolytic enzymes in plant pathological processes has not yet been well delineated. Stimulation of esterase has been detected at the encounter sites between host and parasite in fungal and nematode infection (Hwang *et al.*, 1982; Takahashi *et al.*, 1985; Melillo *et al.*, 1989). The activity of these enzymes seems to precede suberization of the cell walls or the appearance of polyphenolics. Hwang *et al.* (1982) have used the multiple form of esterases as a marker of various levels of resistance of barley to powdery mildew (*Erysiphe graminis* f. sp. *hordei*). They relate the differences in multiple form of esterases between cultivars with the different levels of resistance in barley genotypes.

In our study, the pattern of esterases was markedly different in susceptible and resistant infested pea accessions. The increased activity in resistant roots, during nematode invasion, can be related to the hypersensitive reaction. Further studies on enzyme patterns could be useful in establishing more specific and reliable criteria for the characterization of resistance of pea accessions to pea cyst nematode.

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