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SOME HISTOLOGICAL AND BIOCHEMICAL ASPECTS OF PEA RESISTANCE AND SUSCEPTIBILITY TO *HETERODERA GOETTINGIANA*

by

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Infestation by many *Heterodera* spp. usually causes cell wall dissolution and syncytia formation in the tissues of their host-plants (Nemec, 1932; Mankau and Linford, 1960; Johnson and Fushtey, 1966; Endo, 1964; Cole and Howard, 1958).

The pea cyst nematode, *H. goettingiana* Liebscher, is an important potential plant pathogen of many *Papilionaceae* spp. on which the histological and biochemical modifications induced by the root invasion of the parasite are not known. The object of the present study was to throw some light on those aspects of the histopathology and biochemistry of its host-parasite relationships which have not been described previously.

MATERIALS AND METHODS

Two pea (*Pisum sativum* L.) varieties were used: « Verdone Fulminante » highly susceptible to *H. goettingiana,* and « Triofin » which in a previous experiment (Di Vito *et al.*, unpublished) had shown some resistance to the nematode. The plants were grown in pots filled either with soil free of *H. goettingiana* or with soil infested with the nematode at the rate of 50 cysts/100 g of soil. Biochemical analyses were made of whole root systems of three-week old plants.

Histochemical methods. Root sections of three-week old seedlings

were fixed in FAA, dehydrated in an ethanol series, embedded in paraffin wax and sectioned at 25 μm thickness. Sections were stained with safranin and fast-green or acid fuchsin and azure B.

Phenolic compounds. Whole root systems were homogenized in acetone cooled to -20° C to obtain an enzymatic acetone powder according to the method of Nanson (1955). The acetone extracts were evaporated at 40° C to give 1 ml of extract equivalent to 1 g of fresh roots. In the experiments crude acetone extracts were used.

The quantity of phenolic compounds in the extracts was determined colorimetrically by using Folin's reagent (Johnson and Schaal, 1957). Chlorogenic acid was used as a standard. Monophenols were separated from polyphenols on a neutral aluminium oxide column which adsorbed only the polyphenols (Zucker and Ahrens, 1958). Quantities of polyphenols were calculated from the differences between total phenols and monophenols.

The influence of phenolics on IAA-oxidase. The interaction of pea root phenols with IAA-oxidase was measured *in vitro* by the method of Galston and Dalberg (1954). The 10 ml of reaction mixture contained: 6 ml 1/15 M phosphate buffer pH 6.1, 1 ml 10^{-3}M $MnCl_2$ solution, 1 ml 5×10^{-3}M indoleacetic acid (IAA) solution, 1 ml of peroxidase solution as IAA-oxidase, i.e. horseradish peroxidase Koch-Light Lab. of the activity 60 U/mg and diluted with 0.1 mg/10 ml of buffer. The phenolics obtained as acetone extracts from the pea roots were added at the rate of 0.1 ml per system. Controls had no root extracts.

In plants the phenolics occur mainly as glycosides. Generally, glycosides are not capable of modifying the activity of IAA-oxidase to as great a degree as their aglycones. Therefore, nonhydrolysed extracts or extracts hydrolysed with HCl to give 1% of HCl concentration were introduced into the peroxidase systems.

Activity of IAA-decarboxylase. This activity was measured using IAA labelled in carboxyl. 20 mg portions of enzymatic acetone powders obtained from whole root system were placed in Warburg flasks with 1 ml of solution containing 0.14 μg IAA-1-C^{14}, spec. activity 10 mCi/mMole. To prevent bacterial contamination 0.5 fm of streptomycin was added to each flask. The p-hydroxybenzoic acid 10^{-5}M or acetone extract from roots of the appropriate pea variety were used as cofactor of decarboxylase at the rate corresponding to 10 mg of fresh root weight. The $^{14}CO_2$ produced during the 4-hour enzymatic

reaction at 25° C was absorbed on small paper disks soaked with a saturated water solution of barium hydroxide. At the end of the reactions the disks were disintegrated ultrasonically and suspended in 10 ml of Bray solution (8 g butyl-NPD, 60 g naphthalene, 100 ml methanol and dioxane added to 1 liter of volume). Radioactivity of the $^{14}CO_2$ as $Ba^{14}CO_3$ was determined in a SL-30 Intertechnique liquid scintillation spectrometer.

Activity of tryptophan-decarboxylase. This enzyme was determined by a method similar to that described above for IAA-decarboxylase, except that 20 mg portions of enzymatic acetone powder were placed in Warburg flasks with 2 ml of solution containing 0.14 μg DL-tryptophan-1-C^{14} (spec. activity 30 m/Ci/mMole) and 68 μg of non-labelled DL-tryptophan. Also, tryptophan-decarboxylase activity was measured in two systems: (i) with both 0.2 mM pyridoxal phosphate as cofactor, and 0.2 μM $MnCl_2$ or, (ii) without pyridoxal phosphate but with acetone extract from roots of the appropriate pea variety and at the rate corresponding to 10 mg of fresh root weight.

Proline and hydroxyproline were determined in enzymatic acetone powders of root systems. The material contained both cytoplasmic and cell wall proteins, but there were no free amino acids. Powders were hydrolyzed with 6N HCl for 18 hours at 110° C.

Proline was determined in hydrolysates by using the spectrophotometric method of Bergman and Loxley (1970). This method is based on proline reaction with ninhydrin to give a red pigment. Interference from other amino acids was eliminated by a nitrozation procedure.

Hydroxyproline was determined by using the colorimetric method of Neumann and Logan (1950), with p-diethylaminobenzaldehyde as a reagent. The results were calculated in relation to the weight of acetone powder samples.

RESULTS AND DISCUSSION

Infestations of the susceptible pea variety 'Verdone Fulminante' by *H. goettingiana* caused a reduction of the root system. Invasion of the roots led to the production of typical syncytia (Fig. 2 B) or enlarged cells with thickened and lysed cell walls (Fig. 2 C). This

did not occur in the resistant variety 'Trofin' exposed to the nematode (Fig. 1) although it was observed that the walls of the cells surrounding the nematodes were lignified and eventually the cells became necrotic (Fig. 2 A). The buildup of lignin-like substances in necrotic areas was confirmed by a histochemical test using azure B which stained the affected areas green (Nemec, 1962).

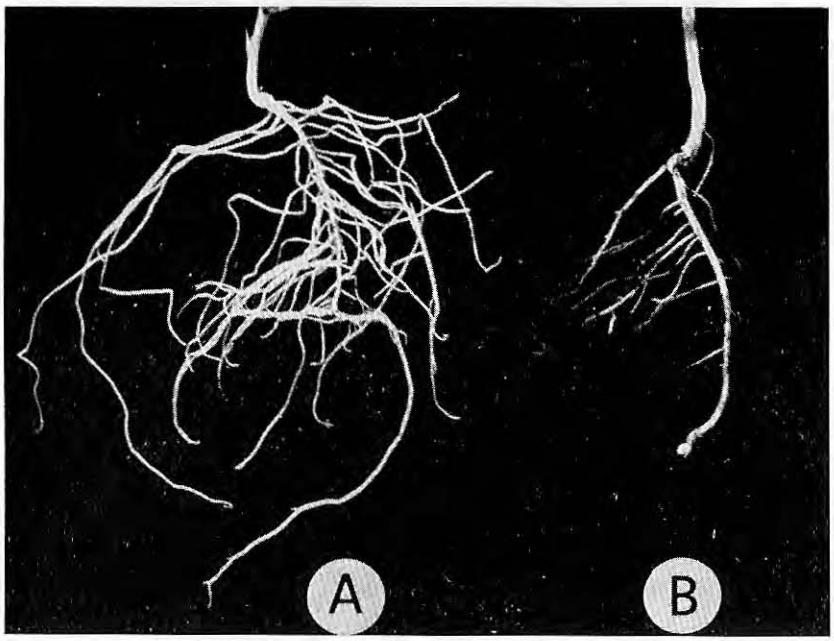

Fig. 1 - Root systems of 3-week old pea plants infested with *H. goettingiana*: A, resistant variety Triofin and B, susceptible variety Verdone Fulminante.

Biochemical analyses of pea root tissues, directly affected by the nematodes, were made with whole root systems only. In any case, changes in the metabolism of infected plants were probably proportional to the number of larvae entering the roots. We did not ascertain the degree of root infestation and probably the number of larvae in the roots of each variety was different. Hence, metabolic changes measured after root invasion by *H. goettingiana* indicate only a trend, i.e. an increase or a decrease in the level of particular metabolites or enzyme activities.

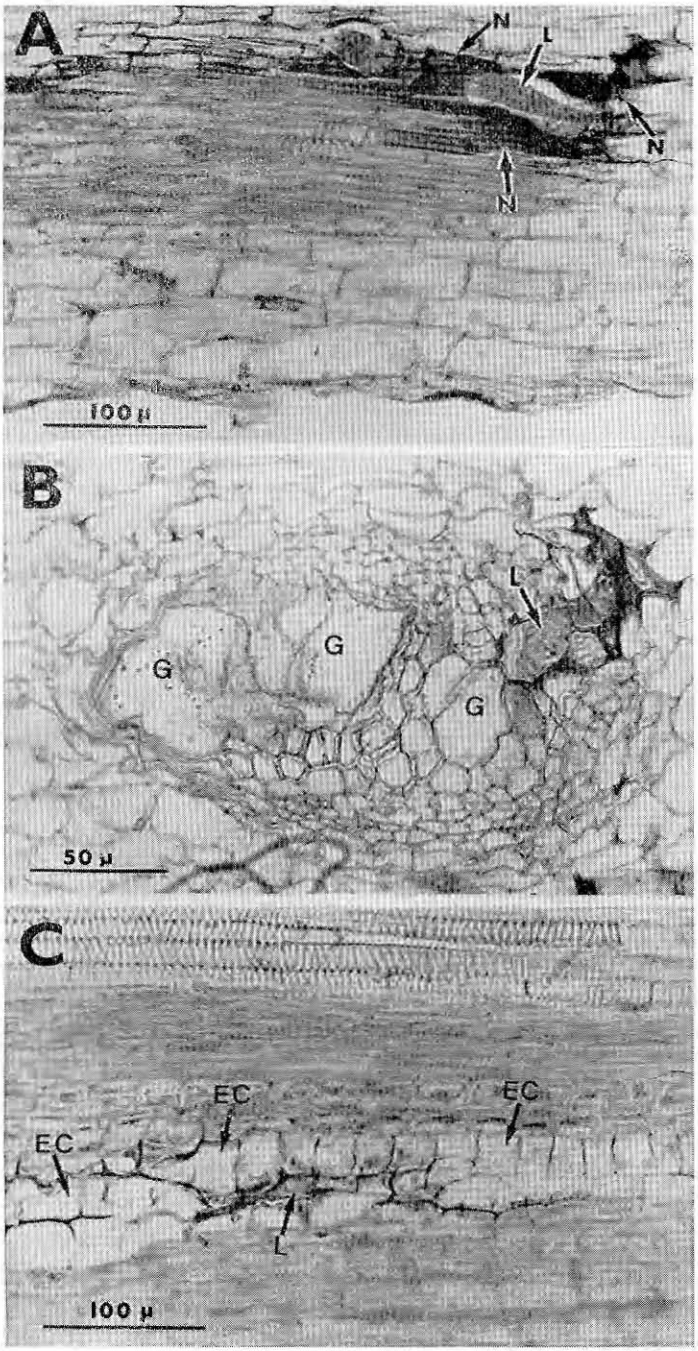

Fig. 2 - Root tissue response to *H. goettingiana*: A, resistant variey Triofin, longitudinal section of root; B, susceptible variety Verdone Fulminante, a transverse section of adventitious root; C, Verdone Fulminante, longitudinal section; visible are: larva (L), necroses (N), syncytia (G) and enlarged cells (EC).

The phenolic content in the roots of the susceptible pea variety was similar to that in the resistant one. After infestation with nematodes, the level of total phenols increased in both varieties, but to a greater degree in the susceptible variety. It is interesting to note that after nematode infestation of the susceptible variety, the ratio of mono- to polyphenols markedly decreased (Table I).

Table I - *Phenolic content in roots of 3-week old pea plants.*

Variety	mg of phenols in 1 g of fresh roots			ratio m/p
	total t	mono m	poly p	
Verdone Fulminante				
healthy	0.21	0.13	0.08	1.6
infested	0.52	0.23	0.29	0.8
Triofin				
healthy	0.22	0.13	0.09	1.3
infested	0.38	0.20	0.18	1.1

IAA-oxidase activity can be modified by phenolic compounds. It is known that polyphenols usually synergize IAA action, while monophenols often antagonize it (Henderson and Nitsch, 1962; Tomaszewski and Thimann, 1966). Phenols present in acetone extracts of healthy roots did not influence the breakdown of IAA by peroxidase but the extract obtained from infested roots inhibited this destruction. The extract of the susceptible variety was about 3-times more active than that of the resistant plant. The greatest effect occurred with the hydrolysed extracts (Fig. 3). It is therefore suggested that this inhibitor (or inhibitors) of IAA-oxidase is a phenolic compound, and is a component of root extracts appearing in plant tissues after nematode infestation.

In healthy roots, the IAA-decarboxylase activity at pH 4.5 was at a similar level in both varieties. But at pH 6.0 this activity in the susceptible variety was twice that in the resistant one. After

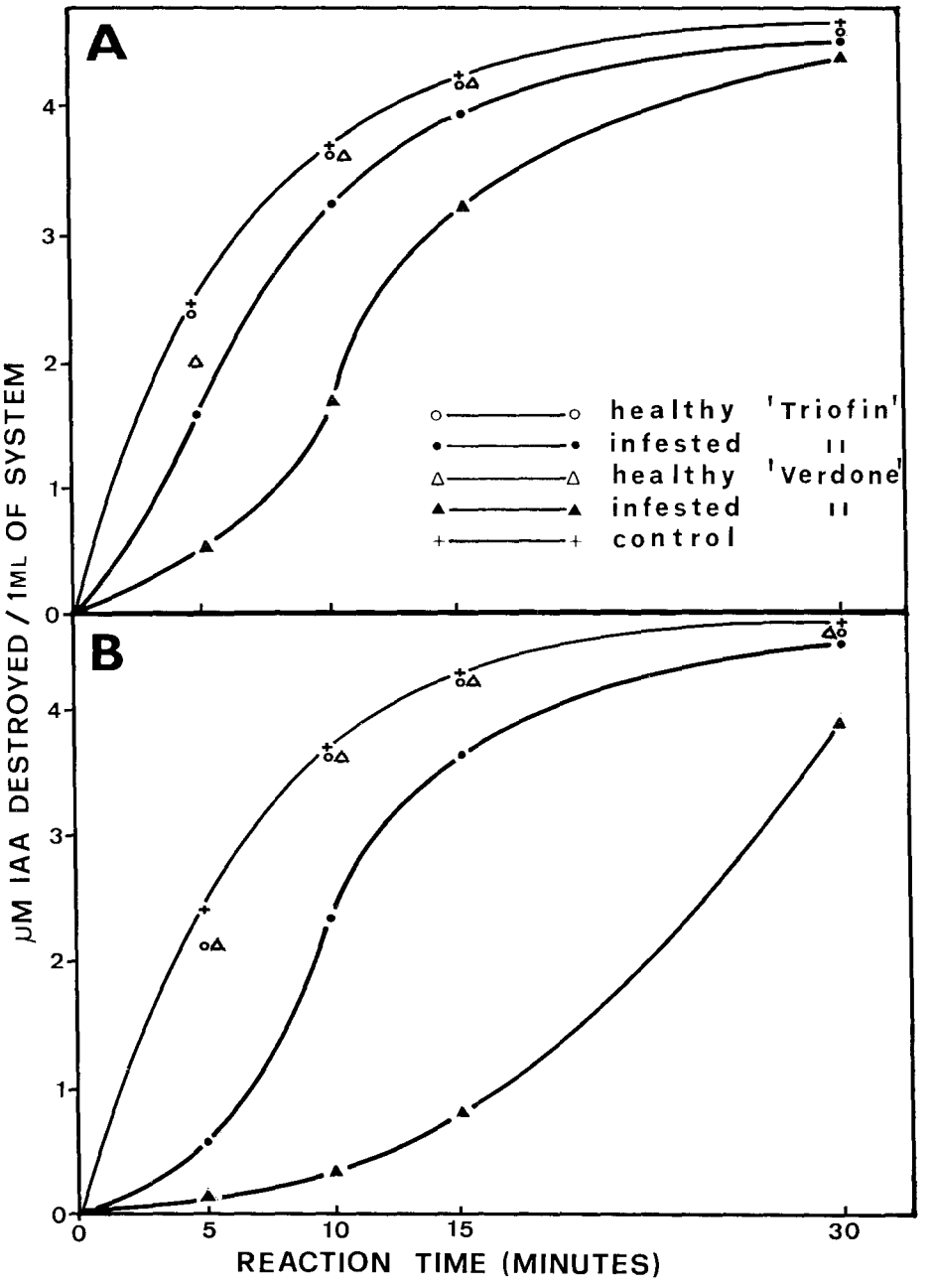

Fig. 3 - Destruction of IAA in peroxidase system with extracts of healthy or infested roots of 'Triofin' and 'Verdone Fulminante' pea: A, systems with non-hydrolysed root extracts; B, systems with root extracts hydrolysed with 0.1 N HCl.

infestation the enzyme activity generally decreased, but more so in the susceptible plant and at pH 6 (Table II).

The tryptophan-decarboxylase activity of healthy roots at pH 7.4 was about 10-times higher than at pH 4.5. After root infestation this activity generally decreased. It seems that in infested roots the tryptophan decarboxylase inhibitor is more active in the resistant variety (Table III). The pH of the tissue is probably an important factor limiting enzyme activity. Healthy roots of potato varieties both resistant and susceptible to *H. rostochiensis* Woll. have approximate pH. In giant cells formed in susceptible roots as consequence of nematode invasion the pH increased to about 6.6 whereas in resistant roots at sites of nematode feeding it decreased to about 4, and even more in the necrotic area (Piegat *et al.*, 1966). Possibly a similar phenomenon exists in the relation between the pea plant and *H. goettingiana.* Hence, after nematode infestation the IAA-decarboxylase activity in the roots of the resistant varieties will be higher than in susceptible ones. Conversely, tryptophan-decarboxylase activity will be higher in susceptible roots. It can be expected that the metabolic system in invaded root tissues of susceptible plants will favour the biosynthesis of auxin, e.g. from tryptophan via tryptamine, although IAA can accumulate. On the contrary, the active system of IAA-decarboxylase in roots of resistant varieties can lead to a decrease of auxin.

Viglierchio (1971) and Giebel (1974) suppose that the pathogenic changes which occur in susceptible plant tissues after nematode invasion are due to auxin secreted by the nematode or biosynthesized in diseased cells. It seems the biochemical mechanism of pathogenesis in the relationship between the pea plant and *H. goettingiana* may be similar.

Following *H. rostochiensis* invasion of resistant potato plants, the ratio of protein proline to hydroxyproline decreased, while it increased in susceptible plants (Giebel and Stobiecka, 1974). The hydroxyproline, which occurs mainly in cell wall glycoproteins, controls cell wall extensibility, cell elongation and tissue maturity (Cleland and Karlsnes, 1967; Ridge and Osborne, 1971). Hydroxyproline inhibition of cell elongation induced by IAA may be reversed by L-proline (Norris, 1967). It is hypothesised that hydroxyproline-rich protein inhibiting cell extensibility can suppress the cell hypertrophy caused by *H. rostochiensis.*

This hypothesis cannot be applied to our experiment since the

Table II - *IAA-decarboxylase activity in susceptible and resistant pea roots measured in systems with p-hydroxybenzoic acid of with correspondent root extract. The activity is expressed as dpm of $^{14}CO_2$ produced during 4-hr reaction, per 20 mg of acetone enzymatic powder.*

Variety	dpm of produced $^{14}CO_2$			
	in system with p-hydroxybenzoic acid		in system with pea root extract	
	pH 4.5	pH 6.0	pH 4.5	pH 6.0
Verdone Fulminante				
healthy	7570	15060	4980	13550
infested	3740	2060	2640	2160
Triofin				
healthy	7400	8520	5140	7090
infested	4740	2310	3380	1980

Table III - *Tryptophan-decarboxylase activity in susceptible and resistant pea roots measured in systems with pyridoxal phosphate or with correspondent root extract. The activity is expressed as dpm of $^{14}CO_2$ produced during 4-hr reaction, per 20 mg of acetone enzymatic powder.*

Variety	dpm of produced $^{14}CO_2$			
	in system with pyridoxal phosphate		in system with pea root extract	
	pH 4.5	pH 7.4	pH 4.5	pH 7.4
Verdone Fulminante				
healthy	230	2670	140	2530
infested	250	1830	220	600
Triofin				
healthy	220	2340	140	1720
infested	240	1990	170	330

data presented in Table IV show that there are no changes either in the proline/hydroxyproline ratio nor in the level of these amino acids in pea roots infested with *H. goettingiana*. It cannot be excluded, however, that these amino acids are involved in the mechanism of resistance or susceptibility of pea plants to the nematode, especially

Table IV - *Protein-proline and hydroxyproline levels in roots of susceptible and resistant pea varieties.*

Variety	mg of amino acid per 100 mg of sample		Ratio of PRO/HPRO
	proline	hydroxyproline	
Verdone Fulminante			
healthy	2.37	1.61	1.5
infested	2.92	1.62	1.8
Triofin			
healthy	1.90	1.51	1.3
infested	2.67	1.52	1.8

if we consider that the variety Triofin was shown to be only moderately resistant to it (very much decreased but did not prevent reproduction of the nematode). However, it can be concluded that the histological response of pea roots to *H. goettingiana* seems to be similar to the reaction observed in host-plants invaded by *H. rostochiensis, H. schachtii, H. trifolii* or *H. glycines.*

SUMMARY

Some histological and biochemical aspects of the relationship between *Heterodera goettingiana* Liebscher and pea varieties Verdone Fulminante and Triofin, respectively highly susceptible and partially resistant to the nematode, were studied. The histological reaction of pea roots following nematode invasion is similar to that induced by other *Heterodera* spp. on their hosts. The phenolic content was higher in the susceptible variety. Activity of the IAA - decarboxylase at pH6 in the susceptible variety was twice that in the resistant one and decreased more in the susceptible plants after infestation. Conversely tryptophan-decarboxylase activity, after nematode invasion, was higher in the susceptible roots. No changes were observed either in the proline/hydroxyproline ratio or in the content of these amino acids in pea roots infested by *H. goettingiana.*

RIASSUNTO

Aspetti istologici e biochimici della resistenza e suscettibilità di piante di Pisello ad Heterodera goettingiana.

Sono stati studiati alcuni aspetti istologici e biochimici delle relazioni ospite-parassita tra *Heterodera goettingiana* Liebscher e le varietà di Pisello (*Pisum sativum* L.) Verdone Fulminante e Triofin, rispettivamente molto suscettibile e moderatamente resistente al nematode. Le alterazioni istologiche osservate in

radici dopo l'invasione del nematode non differiscono da quelle causate da altre specie di *Heterodera* nei rispettivi ospiti. Il contenuto in fenoli è risultato più elevato nella varietà suscettibile e l'attività dell'acidoindolacetico-decarbossilasi, sempre nella varietà suscettibile, era, a pH 6, doppia di quella nella varietà resistente e diminuiva maggiormente nelle piante suscettibili, dopo l'invasione del nematode. Al contrario, l'attività della triptofano-decarbossilasi, dopo l'attacco del nematode, era più intensa nelle radici di piante suscettibili. Nelle radici infestate da *H. goettingiana* non è stata osservata alcuna variazione nel rapporto prolina/idrossiprolina o nel contenuto di questi amminoacidi.

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