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METABOLIC CHANGES IN ENZYME LEVELS IN POTATO ROOTS INFESTED BY POTATO CYST-NEMATODES, *GLOBODERA PALLIDA* (Pa3) AND *GLOBODERA ROSTOCHIENSIS* (Ro1)

by
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Why some pathogens are capable of establishing infection in certain plants while the majority are not, has been one of the fundamental questions in plant pathology.

In plants attacked by nematodes selective changes occur in the metabolism either as consequence of the establishment of a susceptible host-pathogen interaction or as a result of resistance between host and parasite. Several models for resistance/susceptibility have been developed based on biochemical changes (Kaplan and Keen, 1980; Veech, 1981; Giebel, 1982; Zacheo, 1986). None, however, is sufficiently supported to be considered established. There are many reports of enhanced peroxidases (PO), polyphenoloxidase (PPO) and ascorbic acid oxidase following the interaction of nematodes with resistant plants and this has led to the hypothesis that these enzymes may be important in the defense mechanism of the host (Brenneman and Black, 1979; Lazarovits and Ward, 1982; Zacheo *et al.*, 1983).

During nematode invasion a common reaction in resistant plants is the hypersensitive response (HR), which consists of a rapid localized necrosis of a few cells directly in contact with the pathogen and which is considered to be an expression of plant resistance. Recently it has been reported that resistant tomato roots responded to infection with *Meloidogyne incognita* by generating O_2^- (Zacheo and Bleve-Zacheo, 1985, 1986). The O_2^- generation was also found to be activated by the increase in the number of juveniles that had penetrated the roots. Microscopic detection of O_2^- generation, by observing accumulation of blue formazan

derived from the reduction of nitroblue tetrazolium (NBT), showed that O_2^- may be locally generated in the cells around the feeding site of the nematode, during the initial stage of feeding, before the tissues necrotize. It has been suggested that O_2^- generation may be mediated by a NADPH oxidase (Zacheo and Bleve-Zacheo. 1987). The deleterious effects of the radicals in the root cells are prevented by the action of superoxide dismutases (SOD), which are a group of metalloenzymes which transform the O_2^- radicals to H_2O_2 which is then transformed by catalase to harmless $O_2 + H_2O$. In susceptible tomato roots infested by *M. incognita* SOD activity considerably increased in comparison with uninfested controls whereas in roots of resistant cultivars, in which there was hypersensitive reaction (necrosis), there was a slight decrease during nematode infestation (Zacheo *et al.*, 1982; Zacheo and Bleve-Zacheo, 1985).

Investigations on histological changes associated with resistance indicate that juveniles are able to penetrate roots of resistant hosts and to initiate the development of syncytia, similar to those in susceptible hosts, but these degenerate after a few days (Hoopes *et al.*, 1978).

Observations on the biochemical interaction between the potato cultivars Avanti and Agria and *Globodera rostochiensis* (Woll.) pathotype Ro1 and *Globodera pallida* (Stone) pathotype Pa3 are presented in this paper. Cv. Agria is resistant to Ro1 but not to Pa3 (Rice *et al.*, 1985); cv Avanti is susceptible to both nematode pathotypes.

Materials and Methods

Sprouted potato tuber pieces of cvs Avanti (susceptible) and Agria (resistant to Rol) were kept at 16°C until the roots had grown to about 20 mm long. They were then transferred to clay pots containing 10 ml sterilized sand. A suspension of 100 second-stage juveniles of *G. rostochiensis* pathotype Ro1 or *G. pallida* pathotype Pa3 (Dutch populations) was added to each pot. The pots were divided in two groups, one uninoculated and used as control and the other inoculated.

Five days after nematode inoculation the roots were recovered; root tissues (20 g f.wt) were homogenized in 100 mM EPPS buffer, pH 8.5, in a Potter homogenizer cooled in ice. The resultant slurry was filtered through 4 layers of cheesecloth and centrifuged at 10,000 g for 20 min. The resultant supernatant was centrifuged at 131,000 g for 1 hr to yield a pellet of microsomal membranes. The pellet was resuspended to form a membrane suspension [2 mg protein/ml in 2 mM EPPS (pH 8.5)] (Mayak

et al., 1983) and used for enzymatic assay. The supernatant was precipitated in 80% saturated ammonium sulphate and the resultant precipitate was resuspended and dialysed against 20 mM phosphate buffer pH 7.8. The dialysed material was centrifuged at 10,000 g×10 min and the supernatant was used for enzymatic assay and proteins.

The SOD was assayed according to the procedure described by Furusawa *et al.* (1984); peroxidase activity was assayed with guaiacol by the method of Evans (1970); poliphenoloxidase activity was determined by measuring the absorbance at 480 nm of dopaquinone, the oxidation product of L-DOPA, supplied as substrate (Lazarovits and Ward, 1982); ascorbic acid oxidase activity was measured by following the disappearance of ascorbic acid at 265 nm (Maxwell and Bateman, 1967).

The level of superoxides (O_2^-) was measured in terms of reduced cytochrome c (Weening *et al.*, 1975) of formazan (reduced nitroblue tetrazolium) (Doke, 1983). Protein was determined according to Lowry *et al.* (1951).

Results

Effects of nematode infestation on peroxidase activity.

The microsomal and soluble peroxidase activities in the roots of the two potato cultivars after inoculation with *G. rostochiensis* pathotype Ro1 or *G. pallida* pathotype Pa3 are shown in Table I. Microsomal peroxidase levels increased significantly in the roots of cv Avanti after infestation with either of the pathotypes; there was no significant change in peroxidase levels in cv Agria. In cv. Agria (resistant to Ro1; susceptible to Pa3) soluble peroxidase increased when exposed to nematode infestation compared

Table I - *Microsomal and soluble peroxidase activities in potato roots resistant (R) and susceptible (S) infested by* G. rostochiensis *(Ro1) and* G. pallida *(Pa3). Peroxidase activity is expressed as* OD_{470} */ min × mg of proteins.*

Cultivar	Treatment	Peroxidase	
		Microsomes	Soluble Fraction
AVANTI (S)	Uninfested	16.2 ± 0.4	19.3 ± 0.4
»	Pa3	20.3 ± 0.9	19.9 ± 0.5
»	Ro1	24.1 ± 0.6	20.2 ± 0.4
AGRIA (R)	Uninfested	20.3 ± 0.7	15.5 ± 0.4
»	Pa3	19.3 ± 1.0	20.1 ± 0.5
»	Ro1	22.6 ± 8.0	25.1 ± 0.7

with the uninfested control. Nematode infestation had no effect on peroxidase activity in cv Avanti (susceptible). However, there is evidence that increased PO activity was differently located in the two cultivars. In cv Avanti nematode infestation increased PO activity in the microsomal but not in the soluble fraction; the increase was 50% higher in plants infested by the pathotype Ro1 compared with the uninfested control and less when the plants were infested by the pathotype Pa3. In cv. Agria the level of soluble peroxidase activity increased by 66% in the resistant host-pathogen combination (Ro1) and 33% in the susceptible one (Pa3).

Polyphenoloxidase activity.

Microsomal PPO activity decreased in both potato cultivars when exposed to infestation by pathotype Pa3, whereas no changes in activity were observed in the soluble fraction. In potato roots infested by pathotype Ro1 a slight increase in PPO activity occurred in both the susceptible and the resistant cultivars. This increase was greater in the microsomal than in the soluble fraction (Table II).

Ascorbic acid oxidase

The activity of ascorbic acid oxidase showed no appreciable changes in the microsomal and soluble fractions of cv. Avanti infested by pathotype Pa3 but there was a significant increase in roots infested by the pathotype Ro1. In cv. Agria infestation by pathotype Pa3 decreased the ascorbic acid oxidase activity in the microsomal fraction whereas the activity increased in roots infested by Ro1 pathotype. No changes were detected in the soluble fraction (Table III).

Superoxide (O_2^-) generation.

In uninfested roots of both cultivars NBT was reduced to formazan (a blue coloured compound) (Table IV). In cv. Avanti infested with pathotypes Pa3 or Ro1 there was a pronounced decrease in the NBT reducing activity compared with that in uninfested controls: 24% for Pa3 and 21% for Ro1. There was no significant change in O_2^- production in cv. Agria infested with Pa3 but it increased by about 30% when infested with Ro1. In the microsomal fraction isolated from the potato roots, the increases or decreases in cytochrome c reducing activity followed those of NBT reduction in the whole roots (Table IV). Microsomes isolated from roots infested by pathotype Pa3 lowered the capacity to reduce cytochrome

Table II - *Activity of microsomal and soluble polyphenoloxidase extracted from resistant and susceptible potato roots infested by* G. rostochiensis *(Ro1) and* G. pallida *(Pa3). PPO is expressed as* OD_{480} */ min × mg of proteins.*

Cultivar	Treatment	Polyphenoloxidase	
		Microsomes	Soluble Fraction
AVANTI (S)	Uninfested	2.3 ± 0.1	3.3 ± 0.1
»	Pa3	2.0 ± 0.1	3.5 ± 0.1
»	Ro1	2.8 ± 0.2	3.5 ± 0.1
AGRIA (R)	Uninfested	3.5 ± 0.1	3.0 ± 0.1
»	Pa3	2.9 ± 0.1	3.1 ± 0.1
»	Ro1	3.9 ± 0.1	3.2 ± 0.1

Table III - *Activity of ascorbic acid oxidase in potato cultivars infested and uninfested by* G. rostochiensis *(Ro1) and* G. pallida *(Pa3). The enzyme activity is expressed as µg of ascorbic acid × 10 mg of proteins.*

Cultivar	Treatment	Ascorbic Acid Oxidase	
		Microsomes	Soluble Fraction
AVANTI (S)	Uninfested	1.23 ± 0.06	1.44 ± 0.04
»	Pa3	1.27 ± 0.07	1.49 ± 0.08
»	Ro1	1.39 ± 0.07	1.60 ± 0.06
AGRIA (R)	Uninfested	1.06 ± 0.10	0.61 ± 0.03
»	Pa3	0.69 ± 0.03	0.68 ± 0.07
»	Ro1	1.34 ± 0.05	0.67 ± 0.07

Table IV - *Reduction of nitroblue tetrazolium (NBT) and cytochrome c by whole roots or by isolated membranes, respectively, following infestation with* G. rostochiensis *(Ro1) and* G. pallida *(Pa3). NBT reduction is expressed as* OD_{580} */ h × g dry weight. Cytochrome c is expressed as µg of cytochrome × min × 100 mg of proteins.*

Cultivar	Treatment	NBT Reduction	Cytochrome c reduction
		Roots	Microsomes
AVANTI (S)	Uninfested	193 ± 5.3	3.20 ± 0.08
»	Pa3	148 ± 8.4	2.03 ± 0.06
»	Ro1	153 ± 9.4	2.97 ± 0.10
AGRIA (R)	Uninfested	165 ± 9.4	3.46 ± 0.10
»	Pa3	175 ± 8.9	3.67 ± 0.13
»	Ro1	216 ± 10.6	4.36 ± 0.10

c by about 37% in cv. Avanti and did not cause any changes in cv. Agria. The cytochrome c reducing activity increased in cv. Agria but decreased in cv Avanti after infestation with pathotype Ro1.

SOD changes in infested and uninfested potato roots.

Table V shows the SOD activity in infested and uninfested potato roots. The changes can be viewed as occurring in two opposite ways, the first being a significant increase, the second a reduction of SOD activity. In cv. Avanti, susceptible to both pathotypes, SOD activity increased as a consequence of nematode infestation. The enzyme activity was twice that measured in the uninfested control in both the microsomal and soluble fractions. In the cv. Agria infestation with pathotype Pa3 (susceptible combination) caused a slight decrease in SOD activity. Inoculation of cv. Agria with pathotype Ro1, which induces an incompatible reaction, caused a drastic reduction of SOD activity of about 50% in microsomes and 60% in the soluble fraction.

Table V - *Superoxide dismutase (SOD) activity expressed as units × mg of proteins. The enzymes were extracted from infested and uninfested potato roots after* G. rostochiensis *(Ro1) and* G. pallida *(Pa3) inoculation.*

Cultivar	Treatment	Superoxide Dismutase	
		Microsomes	Soluble Fraction
AVANTI (S)	Uninfested	2.74 ± 0.1	8.65 ± 0.5
»	Pa3	4.76 ± 0.2	17.85 ± 0.4
»	Ro1	5.01 ± 0.1	19.91 ± 0.5
AGRIA (R)	Uninfested	4.04 ± 0.2	18.54 ± 0.6
»	Pa3	3.66 ± 0.1	17.23 ± 0.9
»	Ro1	2.36 ± 0.1	7.09 ± 0.5

Discussion

The results obtained from the present study showed that nematode infestation induced i) increase of the peroxidase activities in both susceptible and resistant cultivars, although the elicited increase was differently located; ii) no significant increase in polyphenoloxidase and ascorbic acid oxidase in both cultivars examined; iii) increase of O_2^- production and decrease of SOD activity in resistant host-pathogen

combination; iv) decrease in O_2^- generation and a concomitant increase in SOD activity in the susceptible host-pathogen combination.

The changes in peroxidase activities indicated both similarities and differences in the responses of potato cultivars to infestation with pathotype Pa3 and Ro1. It is believed that different isozymes, which may be related to their differential distribution within the cells, were activated. The increase of peroxidases which accompanied the infestation of both pathotypes on cv. Avanti was localized in the microsomal but not in the soluble fraction. By contrast in cv. Agria, resistant to Ro1, the increase in peroxidase activity was associated with soluble fraction.

In general, increase in peroxidase activity has been reported following interaction of plants with potential pathogens. This led to the hypothesis that the peroxidases may be important in the defense mechanism of plants, but their precise role has remained obscure. The differences in localization of potato peroxidases may represent differences in their function. The increase in microsomal PO activity of susceptible cultivars suggests that peroxidases are implicated in syncytia formation (Huang *et al.*, 1971) and could be one of the groups of hydroxyproline rich proteins in cell walls, the levels of which determine wall extensibility and cell growth (Ridge and Osborne, 1971). The increase of soluble PO activity in resistant cultivar following nematode infestation could be interpreted as a response of infested tissue by the production of diffusible stimulus. The significance of this stimulus could be to activate with other enzymes such as polyphenoloxidase, ascorbic acid oxidase etc., a mechanism of resistance leading to the hypersensitive reaction. Peroxidase could constitute a first line of defense against plant pathogens, but the exact nature of the putative role of peroxidase still needs to be defined. It has been suggested that proteolytic or polysaccharide-degrading enzymes (Jones, 1981) are injected into the root tip by the invading nematode; thus the cellular pH could be altered. Because of an interrelationship among peroxidase isozymes and pH has been demonstrated (Srivastava and van Huystee, 1973), the changes in pH induced by nematode in infested roots may explain the physiological changes associated with the activity of this diversifunctional enzyme.

Some authors (Yokota and Yamazaki, 1965; Elstner and Heupel, 1976) have demonstrated superoxides (O_2^-) production during the enzymatic oxidation of NAD (P)H by peroxidases. In the present experiments an O_2^- generating NADPH oxidase in the membrane rich fraction of resistant potato root tissues was found to be enhanced by nematode invasion. The O_2^- production was also found to be enhanced in whole root tissue of the resistant cultivar. From this result it is postulated that a novel O_2^-

generating NADPH oxidase may be activated in infected, hypersensitively reacting, but not in susceptible tissues of potato roots. The results confirm the previous finding (Zacheo and Bleve-Zacheo, 1987) that necrotizing tissue of tomato resistant cultivar, infested by *M. incognita* was more active than the surrounding tissue. Thus O_2^- activation may be one of the earliest biochemical reactions in the hypersensitive response which occur in the host cells. In this sense, the enzymatic activation is interpreted as a key reaction triggering the hypersensitive reaction, following penetration of juveniles in the host (Doke, 1985). The detail of the role of enzymes that generate O_2^- in plants remains to be elucidated. The propensity of infested tissue to produce superoxides is significantly controlled by superoxide dismutase.

In resistant cultivars nematode invasion is accompained by an increase of O_2^-, but by a decreased activity of SOD. The evidence of this correlation lies mainly in the finding that a decline in SOD activity could result in a greater availability of free radicals. The decline in SOD in response of resistant potato roots to nematode invasion may be a preparatory action for such a defence system, allowing the formation of superoxide radicals. On the contrary in the susceptible host-pathogen combination the increased level of endogenous SOD would suppress the enzymatic production of superoxides by scavenging action.

SUMMARY

Changes in peroxidase, polyphenoloxidase, ascorbic acid oxidase, superoxide dismutase and production of superoxides were investigated in potato roots resistant and susceptible to *Globodera rostochiensis* pathotype Ro1 and *G. pallida* pathotype Pa3. Peroxidase activities increased in the soluble fraction of the resistant cv and in the microsomal fraction of the susceptible cv. The activities of polyphenoloxidase and ascorbic acid oxidase were less affected by nematode infestation. The susceptible cv showed a decrease in NBT and cytochrome c reduction and an increase of superoxide dismutase activity. In the resistant cv the nematode infestation elicited the generation of O_2^- and a decrease in SOD activity. It is postulated that a novel O_2^- generating system may be activated in infected resistant cv because of the decreased activity of SOD.

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