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EFFECT OF TEMPERATURE ON RESISTANCE AND BIOCHEMICAL CHANGES IN TOMATO INOCULATED WITH *MELOIDOGYNE INCOGNITA*

by
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Summary. Resistance of VFN8 tomato seedlings to *Meloidogyne incognita* was measured at 27°C and 34°C by recording juvenile penetration, number of host necroses and galls. At temperature of 34°C resistance was reduced; the necrotic reaction was much less and many more galls developed on the roots than at 27°C. Polyphenoloxidase and peroxidase activity increased in the infested roots at 27°C, but not in infested roots grown at 34°C. Activity of an O_2^- generating system and lipid peroxidation increased at 27°C but decreased at 34°C. SOD and catalase activity were enhanced in inoculated roots at 34°C but remained unchanged at 27°C. It is suggested that the higher temperature induced disease susceptibility in the infested tomato roots because of a decline in superoxides and hydrogen peroxide consequent on the increased activity of SOD and catalase.

The gene responsible for resistance to root-knot nematode *Meloidogyne incognita* (Kofoid *et* White) Chitw. has been designed as *Mi* (Medina-Filho and Tanksley, 1983). The nature of this gene is not yet clearly defined (Sidhu and Webster, 1981). The source of resistance to root-knot nematode in all currently available tomato cultivars was obtained from a wild species of *Lycopersicon peruvianum*. The resistance to *M. incognita* is significantly diminished at temperatures higher than 28°C (Dropkin, 1969). Heat treatment does not appear to change the cell structure but it is possible that it may prevent the interaction of membrane proteins with the nematode secretions and so prevent the hypersensitive reaction (Webster, 1975). Higher temperatures lead to an increase in the number of *M. incognita* juvenile that invade resistant tomato cultivars and a higher percentage of juveniles are able to produce galls (Dropkin, 1969; Zacheo and Bleve-Zacheo, 1984). The fact that plants become efficient hosts at high temperatures is probably due to the quantitative and/or qualitative differences in the enzymatic reaction occurring in the plant-nematode relationship.

It is known that heat treatments of plants alter the outcome of host-parasite interactions. For example, heat treatment increases the susceptibility of plants to fungal pathogens; also, preinoculation heat shock inhibits papilla formation, phytoalexin accumulation and hypersensitive death in plants inoculated with fungi (Bell, 1981). The specific action of heat shock on disease resistance is unknown, although recent work has shown that heat shock causes the temporary inhibition of normal protein synthesis while increasing the syntesis of a few «heat shock proteins». The identity, however, of the stress proteins remains obscure (Cooper and Ho, 1983).

This investigation was initiated to analyze the influence of heat treatment, which results in modification of resistance to *M. incognita*, on oxidative enzymes in resistant tomato roots. Nematode infestation stimulates production of oxygen free radicals (O_2^-) and decreases the level of superoxide dismutase (SOD) (Zacheo and Bleve-Zacheo, 1988). Free radicals induce lipid peroxidation which is considered to be an important mechanism of membrane deterioration during ageing of plant tissues (Dhindsa *et al.*, 1981). We have, therefore, examined the relationship between heat treatment and lipid peroxidation, free radical production and superoxide dismutase activity.

Materials and Methods

Seedlings of tomato cultivar VFN8, resistant to *M. incognita*, were grown at 27°C and transplanted at 3-4 cm height into 5 cm diameter clay pots containing quartz sterilized sand. The pots were placed in a growth chamber at 27°C or 34°C, 65% Rh, 5000 lux and

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watered with Hoagland's solution. After a week, the seedlings were inoculated with 60 *M. incognita* second stage juveniles and five days later the plants were removed. Ten infested root systems were stained in boiling acid fuchsin-lactophenol and necroses, galls and nematodes from each root system were counted. For histological observations infested or galled roots were fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer pH 7.2 for 6 hours at 4°C, rinsed several times in the same buffer and post-fixed in 2% osmium tetroxide for 4 hours at 4°C. The roots were dehydrated in an ethanol series and embedded in Spurr's medium. Two microns thick sections were stained with toluidine blue and examined with a light microscope.

Five days after nematode inoculation, 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 35% saturated ammonium sulphate and the resultant precipitate (fraction I) was resuspended and dialysed against 20 mM phosphate buffer pH 8.5. The supernatant was then saturated in 80% ammonium sulphate and the resultant precipitate (fraction II) was recovered as before. The dialysate was centrifuged at 10,000 g for 10 min and the supernatant was used for enzymatic assay and proteins.

The superoxide dismutase activity (SOD) was assayed according to the procedure described by Furusawa *et al.* (1984); peroxidase activity (PO) was assayed with guaiacol by the method of Evans (1970); polyphenoloxidase activity (PPO) 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; catalase activity was measured by following the disappearance of hydrogen peroxide at 240 nm (Maxwell and Bateman, 1967). Determination of lipid peroxidation was measured as reported by Dhindsa *et al.* (1981).

The level of superoxides (O_2^-) in the root tissues was measured in terms of formazan (reduced nitroblue tetrazolium) (Doke, 1983). Superoxide level generated by membranes was assayed by determining the reduction of extracellular cytochrome c (Weening *et al.*, 1975; Doke and Chai, 1985). Protein was determined according to Lowry *et al.* (1951).

All values are the means of four experiments and each experiment employed three or four replicates ± SE (standard error).

Results

Tomato seedlings of cv. VFN8 were completely resistant to *M. incognita* juveniles when held at 27°C. Roots exposed to juveniles for a period of 5 days at 27°C showed local necrotic reactions but an absence of galls (Fig. 1a). The rate of nematode penetration into the roots of VFN8 increased with increasing temperature. The number of juveniles that penetrated the root seedlings grown at 34°C was 40% more compared with those grown at 27°C. Plants held at 34°C developed galls (Fig. 1b) and 30% of the juveniles that invaded the roots continued to develop (Table I). Necroses were observed in seedlings held at 34°C, but these were 60% less than the number of necroses on the roots at 27°C.

TABLE I - *The effect of temperature on juvenile penetration and host response to infection by* M. incognita *in tomato cv. VFN8.*

Treatment	Number of juveniles	Number of necroses	Number of galls/seedling
27°C	8.03 ± 0.50	3.30 ± 0.20	none
34°C	11.56 ± 0.90	1.51 ± 0.10	3.77 ± 0.20

At both temperatures, *M. incognita* produced typical conspicuous brown lesions on the tomato roots. Sections of infested roots at 27°C and 34°C, observed by

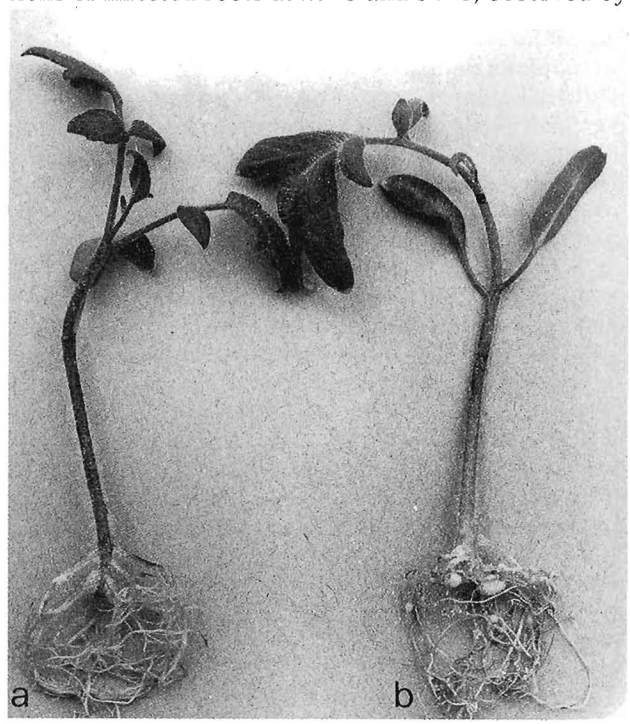
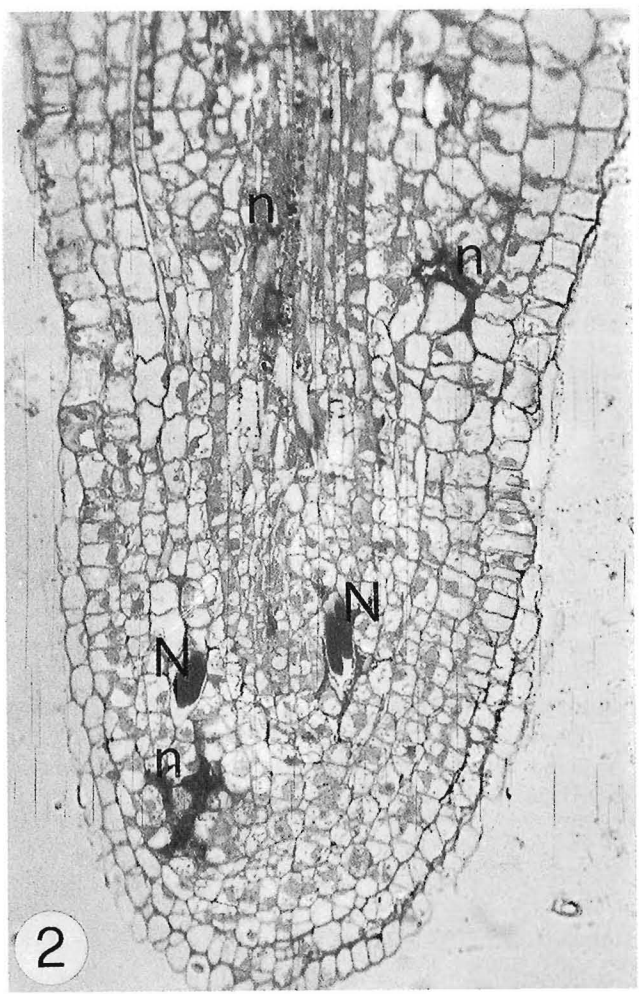


Fig. 1 - Tomato seedlings of cv. VFN8 infested with *M. incognita*. a) infested plants grown at 27°C; b) numerous and well developed galls in roots of a plant grown at 34°C.

light microscopy, showed differences in plant response. The most obvious response to infection by *M. incognita* was the presence of necrotic areas in the penetrated cells of the roots held at 27°C; these cells were clearly necrotic because they stained intensely with toluidine blue (Fig. 2). Multinucleate cells, in which the nematode was feeding, were observed in the galls on roots grown at 34°C. The gross structure of the giant cells appeared very similar to that recorded in susceptible infested roots. The tissues delimiting the giant cells were clearly stressed, with scattered necroses, which caused the collapse of some cells; however, the physiological development of giant cells can be compared to that of the susceptible roots, because the nematode development seemed to progress regularly (Fig. 3).

Activity of enzymes, measured in the extracts of tissues from healthy and infested tomato roots grown at the two temperatures, 27°C and 34°C, resulted significantly changed, 5 days after treatment. A distribution of ascorbic acid oxidase among the fractions as described in Materials and Methods is shown in Table II. Heating alone activated this enzyme in two of the three extracted fractions. Microsomal ascorbic acid oxidase was significantly lowered in infested roots grown at 27°C compared with uninfested roots grown at the same temperature; the microsomes extracted from plants at 34°C showed little change in ascorbic acid oxidase activity. The activity of this enzyme at 27°C in the fraction II was lower in infested than in healty root tissue and increased by over 24% in infested tissue grown at 34°C compared with uninfested tissue. No difference in ascorbic acid oxidase activity was observed in the heated and nonheated tissue either infested or uninfested in fraction I (Table II).

Polyphenoloxidase activity in VFN8 tissues is shown in Table III. The enzyme activity in response to inocu-



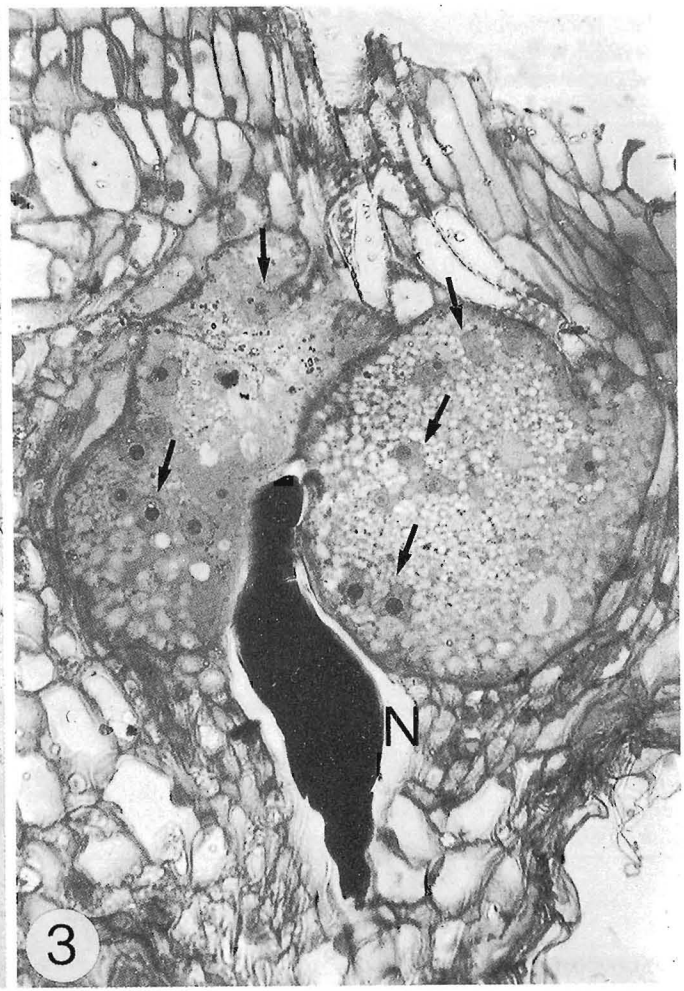


Fig. 2 - Longitudinal section through a root tip infested with *M. incognita* at 27°C. Note necroses (n) in the cells penetrated by nematode juveniles (N). × 80.
Fig. 3 - Longitudinal section through the galled tissue of a root held at 34°C. Two giant cells with numerous nuclei (arrows) and third stage juvenile (N) feeding on them are clearly visible. Necrosis and collapsed cells are evident all around the giant cells. × 80.

lation markedly increased in all the fractions extracted from the tissues grown at 27°C. The activity of PPO in the roots tended to be lower at 34°C than at 27°C. In infested plants at 34°C a slight increase in the PPO level was recorded only in the microsomal fraction but this was lower than that in the extracts of infested plants at 27°C (Table III).

Extracts of heated (34°C) noninoculated plants showed a significant increase in peroxidase activity over the control at 27°C (Table IV). More importantly, there was a decrease in the level of peroxidase activity in all the fractions obtained from infested roots at 34°C; there were 40%, 60% and 10% reduction in peroxidase activity in microsomes, fraction I and fraction II, respectively, of the infested tissue compared with the uninfested grown at the same temperature. In plants grown at 27°C, peroxidase activity was enhanced by nematode infestation and was particularly high in the microsomes and fraction I.

TABLE II - *Effect of heat treatment on the ascorbic acid oxidase activity in VFN8 tomato roots infested or uninfested by* M. incognita. *The enzyme activity is expressed as μg of oxidized ascorbic acid × 10 mg of proteins.*

Treatment	Ascorbic acid oxidase		
	Microsomes	Fraction I	Fraction II
Uninoculated 27°C	0.57 ± 0.05	0.19 ± 0.03	1.65 ± 0.08
Inoculated 27°C	0.44 ± 0.02	0.19 ± 0.02	1.41 ± 0.08
Uninoculated 34°C	0.79 ± 0.07	0.20 ± 0.08	1.78 ± 0.07
Inoculated 34°C	0.84 ± 0.03	0.18 ± 0.07	2.22 ± 0.17

TABLE III - *Polyphenoloxidase activity in resistant tomato roots grown at 27°C and 34°C infested or uninfested by* M. incognita. *PPO is expressed as* $OD_{480} \times min \times mg$ *of proteins.*

Treatment	Polyphenoloxidase activity		
	Microsomes	Fraction I	Fraction II
Uninoculated 27°C	13.20 ± 0.71	3.28 ± 0.19	7.35 ± 0.78
Inoculated 27°C	16.26 ± 0.65	4.65 ± 0.20	10.20 ± 1.00
Uninoculated 34°C	10.50 ± 0.64	3.01 ± 0.22	6.88 ± 0.25
Inoculated 34°C	12.40 ± 0.85	3.98 ± 0.22	6.31 ± 0.30

TABLE IV - *Peroxidase activity in resistant tomato roots infested and uninfested by* M. incognita *at two different temperatures, 27°C and 34°C. Enzyme activity is expressed as* $OD_{470} \times min \times mg$ *of proteins*

Treatment	Peroxidase activity		
	Microsomes	Fraction I	Fraction II
Uninoculated 27°C	12.67 ± 1.10	1.15 ± 0.08	5.98 ± 0.78
Inoculated 27°C	19.47 ± 1.85	1.36 ± 0.06	7.40 ± 0.29
Uninoculated 34°C	13.65 ± 0.92	1.62 ± 0.06	7.20 ± 0.32
Inoculated 34°C	7.84 ± 0.65	1.04 ± 0.04	6.59 ± 0.31

The data presented in Table V provide evidence that production of free radicals in root tissues was increased by nematode infestation at 27°C; heat treatment, on the contrary, induced a considerable decline in both infested and uninfested tissues. With nematode infestation at 27°C, microsomal membranes isolated from root tissues showed an increase of cytochrome c reduction, reflecting an increase of O_2^- production; at 34°C there was a decrease in O_2^- production in both inoculated and uninoculated tissues. Nematode infestation at 27°C increased the level of lipid peroxidation but this decreased with heat treatment particularly in infested tissues. Treatment at 34°C increased SOD activity in extracts of root tissues (Table VI) and this reached the maximum in fraction I of the inoculated tissues.

Catalase activity increased in healthy tissues at 34°C and reached a maximum value (4-fold) in the microsome fraction. Nematode infestation had no apparent effect on catalase activity at 27°C but caused a decline in activity at 34°C compared with the uninfested control at the same temperature (Table VII).

TABLE V - *Superoxide generation in roots and membranes and lipid peroxidation in membranes of tomato resistant to* M. incognita. *Plants infested or uninfested by nematodes were grown at 27°C and 34°C. Superoxides in root tissue were expressed as (a)* $OD_{580} \times h \times g$ *dry weight of reduced NBT and in membranes as (b) μg of reduced cytochrome c × min × 100 mg of proteins (c) lipid peroxidation was expressed as concentration of nmoles MDA × mg of proteins.*

Treatment	Superoxide generation		Lipid peroxidation
	Root tissue (a)	Membranes (b)	Membranes (c)
Uninoculated 27°C	343 ± 13	3.60 ± 0.19	1.12 ± 0.06
Inoculated 27°C	440 ± 18	4.46 ± 0.16	1.52 ± 0.08
Uninoculated 34°C	220 ± 10	2.73 ± 0.12	1.03 ± 0.04
Inoculated 34°C	215 ± 9	2.54 ± 0.11	0.79 ± 0.06

TABLE VI - *Superoxide dismutase activity in heated and not heated VFN8 tomato roots, expressed as units × mg proteins. The enzymes were extracted from infested and uninfested roots after* M. incognita *invasion.*

Treatment	Superoxide dismutase activity		
	Microsomes	Fraction I	Fraction II
Uninoculated 27°C	2.55 ± 0.21	2.26 ± 0.15	7.28 ± 0.37
Inoculated 27°C	2.60 ± 0.10	2.50 ± 0.17	7.19 ± 0.18
Uninoculated 34°C	3.01 ± 0.16	3.40 ± 0.16	8.62 ± 0.64
Inoculated 34°C	3.19 ± 0.18	3.43 ± 0.34	8.67 ± 0.63

TABLE VII - *Catalase activity in the extracts from VFN8 tomato roots held at 27°C or 34°C and infested or uninfested by* M. incognita. *Enzyme activity is expressed as* $OD_{240} \times min \times mg$ *proteins.*

Treatment	Catalase activity		
	Microsomes	Fraction I	Fraction II
Uninoculated 27°C	6.00 ± 0.25	0.51 ± 0.10	0.76 ± 0.11
Inoculated 27°C	6.09 ± 0.29	0.52 ± 0.10	0.86 ± 0.09
Uninoculated 34°C	26.45 ± 1.01	0.68 ± 0.10	2.10 ± 0.06
Inoculated 34°C	17.15 ± 0.60	0.62 ± 0.10	1.86 ± 0.18

Discussion

Temperature affected the interaction between *M. incognita* and the resistant tomato cv. VFN8. When exposed to high temperature (34°C) the phenotypic expression of resistance to the nematode was modified; the necrotic reaction was much less intense, but numerous galls developed, giant cells were established and the nematode completed its life cycle. It seems reasonable to suppose that in resistant plants certain biochemical messages are activated during nematode invasion. These pre-existing messages are physically altered in some way at high temperature and block their activation. However, the biochemical nature of these messages remains obscure.

An interesting aspect of the heat treatment was the marked change in enzyme activities in tomato roots. Our investigation demonstrated a decline in PO and PPO activity in nematode inoculated plants at 34°C; in contrast, in inoculated plants at 27°C the two enzymes increased. Because of the importance of PO and PPO activity in the resistance response (Rautela and Payne, 1969; Bell, 1981; Lazarovits and Ward, 1982) it can be hypothesised that the diminished activity of these enzymes could be one cause of the induced susceptibility. It is known that the cellular events associated with necrosis closely resemble those associated with the host-specific necrogenic resistance to various pathogens (Bell, 1981). Browning in the hypersensitive reaction is mainly caused by the enzyme polyphenol oxidase which catalyses the oxidation of various o-dihydroxyphenols to quinones or free radicals, and which can react with several biological molecules, to create an environment that may be unfavourable for growth of potential pathogens (Butt, 1980). Thus the diminished activity of PPO could be a factor limiting the necrotic reaction in heat-treated plants. The specific action of peroxidase on disease resistance is unknown. The PO activity has been associated primarily with the most cathodic isozymes which appear to be responsible for lignin synthesis (Bell, 1981).

Our investigations confirm earlier reports (Doke 1985; Zacheo and Bleve-Zacheo, 1988) that an O_2^- generating system, NADPH dependent, is generated enzymatically, presumably by a membrane-associated oxidase, in infested tomato plants grown at 27°C. The increased propensity of microsomal membranes to produce superoxides, which induced lipid peroxidation (Fridovich, 1975), may reflect deterioration in the molecular organization of membranes and senescence of tissues (Mayak *et al.*, 1983). It is interesting that the superoxide generation is activated in animal as well as plant cells in relation to defence reactions. The activation of this enzymatic system could be considered as one of the earliest biochemical reactions in the hypersensitive response to occur on the host plasmamembrane. In this respect, the enzymatic activation is interpreted as a key reaction which results in the eventual death of the cells. Because of the chemical nature of O_2^- and the derived superoxides to reduce or to oxidize, it seems that they may be involved in the metabolism of polyphenols or lignification, all processes generally associated with resistance reactions in plants (Doke *et al.*, 1987). The present results show inhibition of O_2^- generating NADPH oxidase and lipid peroxidation in infested roots grown at 34°C. The lack of activation of a superoxide generating system in heat-treated plants is related to the decreased necroses and the induction of susceptibility. The cause of the induced susceptibility to *M. incognita* in plants exposed to 34°C is not clear. The heat-treated plant tissues presumably avoid the toxicity of superoxides because they are scavenged by the activation of endogenous SOD and catalase. The superoxides are converted by SOD to hydrogen peroxide which can then be removed by catalase (Fridovich, 1975).

From the foregoing it is clear that the activities of SOD and catalase could determine the abundance of superoxides, hydrogen peroxide and derived species in the root tissues. Our results suggest that heat treatment, by increasing the SOD and catalase activity, could contribute to the change from resistance to disease susceptibility. The way in which temperature stress functions in physiological terms is unknown. Nevertheless, the results from our experiments presented agree with those of others (Cooper and Ho, 1983; Ashburner and Bonner, 1979), which indicate that heat treatment of tissues represses the transcription of *Mi* genes; and consequently there is a marked shift in enzymes or substrates which results in modification of resistance to nematode.

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