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CHANGES OF CATALASE AND SOD ACTIVITIES IN THE EARLY RESPONSE OF TOMATO TO *MELOIDOGYNE* ATTACK

by

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Summary. Excised roots of *Mi*-bearing resistant tomato were inoculated with *Meloidogyne* spp. in agar. Variations of antioxidant enzyme activity were detected as early as 24 hours after the inoculation. Catalase activity was inhibited by the infestation. Catalase inhibition was also observed in roots 24 hours after seedlings were inoculated in pots with second-stage juveniles. The catalase inhibition was specific for resistant cultivars whilst susceptible cultivars had their catalase content enhanced by root-knot nematode infestation. Superoxide dismutase activity did not show reproducible changes due to nematode infestation. Catalase inhibition is indicated as a mechanism by which hydrogen peroxide level is increased in resistant roots thus triggering a hypersensitive response and producing a chemical toxic to nematodes.

Tomato resistance to root-knot nematodes (RKN) of the genus *Meloidogyne* has been recently and extensively reviewed (Liharska and Williamson, 1997). The best characterized and most used resistant gene is the dominant *Mi*-gene, which induces a hypersensitive response (HR) in root cells infested by the nematodes. It is well known that *Mi*-gene is ineffective with *M. hapla* (Hadisoeganda and Sasser, 1982) and infestation of *Mi*-carrying tomatoes by *M. hapla* normally produces fully susceptible reactions. Conversely, it has been reported that infestation *in vitro* resulted in an incompatible reaction characterized by inhibited growth and extensive necrosis (Molinari and Miacola, 1997b)

Mi-mediated HR occurs as early as 12 hours after inoculation of roots with nematode juveniles (Paulson and Webster, 1972). Studies on the early *Meloidogyne*-tomato interaction are scarce and the first biochemical events triggering HR are far from clear (Molinari, 1996). The lack of simple isolated elicitors extracted from RKN prevents experiments being undertaken in which root cells in cultures may be used to monitor the early events following recognition of the parasites, as has been done for different plant-pathogen interactions (Apostol and Heinstein, 1989; Baker and Orlandi, 1995). Therefore, infestation of whole plants by living nematodes is normally used to detect the root response, which prolongs and complicates the collection, and interpretation of data.

One of the earliest events which is considered most important for the realization of HR in plants is the generation of active oxygen species (AOS) which produces H_2O_2 (Levine *et al.*, 1994). H_2O_2 acts as a signal for gene activation, a trigger for hypersensitive cell death as well as a powerful antimicrobial agent. Catalase is a very active enzyme which specifically destroys H_2O_2 in cells. The high level of H_2O_2 required in HR may be obtained by a strong inhibition of catalase which must be specific for the tissues involved and temporarily limited; it is currently accepted that such an inhibition is probably due to the generation of salicylic acid, which is

a strong inhibitor of the enzyme (Chen *et al.*, 1993). Since spread of H_2O_2 in cells leads ultimately to cell death, cells normally maintain high catalase activity in order to neutralize this toxic chemical.

Catalase inhibition is a highly specific process. An unspecific oxidative stress, such as paraquat treatment, was experimentally imposed on resistant tomato roots cultured *in vitro*, and was found to cause a marked increase in catalase activity, as a defense reaction (Molinari, 1991). Conversely, in the incompatible combination between bean leaves and *Pseudomonas syringae* pv. *phaseolicola* catalase activity dropped markedly in the inoculated leaf area (Milosevic and Slusarenko, 1996).

Unfortunately, direct evidence of early production of AOS in incompatible nematode-plant interactions is not available although it has been reported with different pathogens attacking resistant plants (Kauss, 1990). However, RKN have been found to contain high levels of antioxidant enzymes, such as catalase, superoxide dismutase and ascorbate peroxidase, although they lacked glutathione peroxidase (Molinari and Miacola, 1997a). It is reasonable that endoparasitic nematodes have developed such an active AOS-detoxifying enzyme system to counteract AOS production which is, presumably, one of the earliest defense reactions of the resistant plants that are penetrated. In this regard, virulence on *Mi*-bearing tomato cvs may be seen as the ultimate and successful adaptation of nematode populations to AOS production associated with plant resistance. This is suggested by the finding that *M. hapla* and virulent populations of *M. incognita* selected in a glasshouse showed higher and additional isoforms of antioxidant enzyme activities compared to avirulent counterparts (Molinari and Miacola, 1997a; Molinari, 1999).

In this paper it is shown that catalase inhibition occurs early and also specifically in *Mi*-bearing tomato roots attacked by avirulent *Meloidogyne* populations, thus suggesting that, at least, H_2O_2 cellular level increases in incompatible RKN-tomato interaction leading to the hypersensitive cell death which stops nematode reproduction.

Materials and methods

Seeds of the resistant tomato (*Lycopersicon esculentum*, Mill.) cvs VFN8 (Petoseed Co., Inc., California) and Rossol (Peto Italiana s.r.l., Parma), and the susceptible cv. Roma VF were used. The populations of *M. incognita* (Kofoid *et* White) Chitw. and *M. hapla* Chitw. were from suceptible tomato cultures grown in a glasshouse. Seeds were germinated in a sterilized mixture of peat and soil at 27-29 °C and seedlings allowed to grow to the four true-leaf stage. Seedlings were then inoculated with nematodes according to two different experimental procedures as described below. Second-stage juveniles used for inoculation were obtained by incubation of egg masses in distilled water at 27 °C; concentration of juveniles in small volumes of sterilized water was achieved by filtering through 1 µm filters (Whatman type) and collecting them after repeated washes.

In the first experimental procedure seedlings were thoroughly washed with tap water and then transferred into 8-cm clay pots containing sterilized quartz sand. Half of the pots of seedlings were inoculated with 150-200 active J2 per seedling, the other half was left uninfested as a control. Pots were put in a growth chamber at 27 °C and illuminated throughout the experiment. After rising, roots were excised from their shoots and tested for enzyme activity as early as 24 h after inoculation.

In the second experimental procedure roots were excised from the shoots and put on 2% agar Petri dishes, which were divided into two sections by removing a central strip of the agar. Sterilized water (250 µl) containing approx. 2000 active J2 was uniformly poured on one section of each dish and the same amount of the water without nematodes was added to

each control section. The Petri dishes were then transferred into a growth chamber at 27 °C in the dark. Roots were tested for enzyme activity as early as 24 h after inoculation.

Each batch of roots coming from experiments involving inoculation in pots and in Petri dishes were divided into three small samples of comparable consistence which were separately washed, drained and weighed. Each sample was put in a 1.5 ml Eppendorf tube containing 150 µl of 0.1 M Na, K-phosphate buffer, pH 6.0. Homogenization was carried out in ice using a steel pestle fitted to the tubes and connected to a motorized drive. Three cycles of 1 min at maximum speed were used to ensure uniform conditions of homogenization. The homogen-ates were centrifuged at 3000 rpm for 10 min in a bench centrifuge and supernatants collected and promptly used as samples for enzyme activity and isozyme electrophoresis profile detection or kept at -80 °C until required.

Protein content was determined by the Lowry procedure with bovine serum albumin as the standard (Lowry *et al.*, 1951).

All measurements were carried out spectroscopically by means of a 557 Perkin-Elmer double-beam spectrophotometer.

Superoxide dismutase. SOD activity was assayed as the percentage of inhibition on the reduction of cytochrome *c* (80 µM) by the xanthine[1] oxidase (20 mU) system in 1 ml assay medium of 0.1 M Na-K Pi, pH 7.8, 20 mM NaN_3 and 1 mM EDTA. Standard reactions were carried out with 25-50 µl of extraction buffer which was substituded with identical amount of sample suspension in the enzyme activity tests. A high concentration of NaN_3 was added to completely inhibit the cytochrome oxidase present in the crude extracts. Reactions were started by adding xanthine oxidase and monitored at 550/540 nm. One unit of SOD activity represents the amount of enzyme able to produce a 50% inhibition on standard reaction (Furusava *et al.*, 1984).

Catalase. CAT activity was measured as the initial rate of disappearance of hydrogen peroxide (Chance and Mahley, 1955), using 20 mM H_2O_2 and 25 µl sample extract in 0.1 M Na-Pi, pH 7.0 (0.5 ml final volume). The rate of H_2O_2 disappearance was followed as decrease in the absorbance at 240 nm and oxidation of 1 µmole H_2O_2 min^{-1} (ε=0.038 mM^{-1} cm^{-1}) represented one unit fo enzyme.

CAT isozymes were separated by isoelectric focusing using PhastSystem equipment (Pharmacia Biotech, USA), which permits pre-programming of the chosen separation method. The exact replication of the method in each run is thus ensured by microprocessor control and by the use of precast 0.45 mm thick gels whose separation zone is only 3.8x3.3 cm. Features of the equipment and the separation method applied have been described by Molinari *et al.* (1996). Gels were calibrated by using a broad pI calibration kit (Pharmacia Biotech, USA) containing amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.88), human carbonic anhydrase B (6.55), myoglobin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65), and trypsinogen (9.30). CAT isozymes were stained in isoelectrofocusing gels after the method of Cardy and Beversdorf (1984). The focused gel was soaked in 0.6% H_2O_2 for 60 sec then rinsed with distilled water and immersed in 0.2% (w/v) potassium iodide and 0.1% (v/v) glacial acetic acid until transparent zones of CAT activity appeared against a dark yellow background. Gels were then immediately drained and scanned by means of a ScanJet II cx (Hewlett Packard), arranged by computer into negative images and printed.

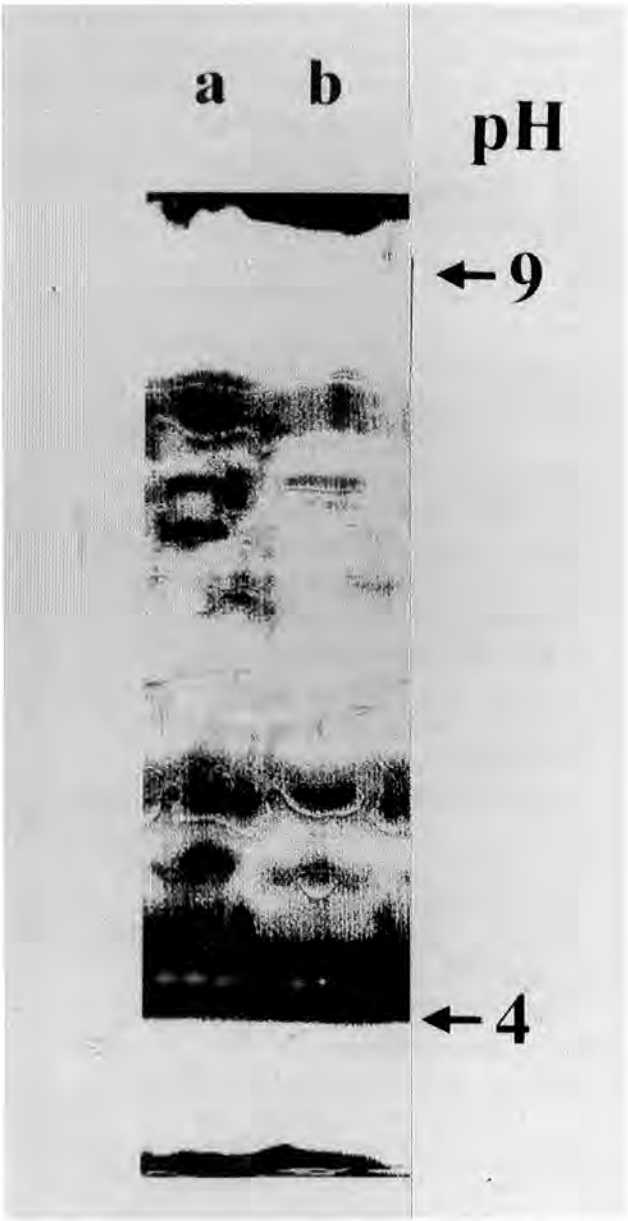
[1] (1 mM) - xanthine.

Results and discussion

In the incompatible interaction between *Meloidogyne incognita* and the resistant tomato cv. Rossol, CAT activity of infested roots, 24 h after inoculation, was about 40% lower than that of uninfested counterparts (Table I). Isoelectrofocusing of CAT isozymes of resistant control roots (a) compared with roots of seedlings 24 h after inoculation with *M. incognita* (b) is shown in Fig. 1. Basically, eight zones of CAT activity at different pI were observed in control roots, ranging from pH 8.2 to pH 4.0. The sample from infested roots completely lacked isozymes at pI 7.4 and generally showed less intense bands compared with infested roots. Conversely, compatible interactions resulted in a general augmentation of CAT activity in the infested compared with uninfested roots (Table I). No conclusions could be obtained from the results comparing variations of SOD.

To obtain data on possible early variations of antioxidant enzyme activities in root-nematode interaction *in vitro*, roots were excised, placed on agar and heavily inoculated with an excess of active J2. Again, after 24 h incubation in the dark, CAT acitvity was severely inhibited in infested roots with respect to controls only when an incompatible interaction occurred (Table II). This novel experimental approach, developed to study the early plant-nematode interaction *in vitro*, confirmed that nematode infestation specifically inhibited CAT activity of *Mi*-carrying resistant tomato cultivars. On the other hand, SOD was generally inhibited after inoculation of the roots, with no significant difference between compatible and incompatible interactions.

Salicylic acid has been found to inhibit CAT activity in many plants and thus involved in plant systemic acquired resistance (SAR) (Chen *et al.*, 1993). CAT inhibition enhances the cellular level of H_2O_2, which is presently recognized as a diffusible signal for gene activation in HR, as a trigger for hypersensitive cell death as well as a strong antimicrobial molecule (Levine *et al.*, 1994). It is worth considering that in *Meloidogyne*-tomato incompatible interaction endog-



Fg. 1 - Isoelectrofocusing of catalase isozymes of resistant tomato plants (cv. VFN8) uninfested or infested in glasshouse with *Meloidogyne incognita* 24 h after inoculation: a) control roots; b) infested roots. Electrophoresis runs loaded with approximately the same amount of proteins for both samples. Gels (4x4 cm) immediately scanned after staining, rearranged as negative images and printed. Dark bands indicate catalase activity over a white background.

TABLE I - *Variation of SOD and catalase activities (expressed as units g^{-1} root fresh wt± standard deviation) in* Meloidogyne*-tomato interactions: analyses of roots 24 hours after inoculation of seedlings with active J2 in greenhouse.*

Meloidogyne-tomato interactions *in pots*	SOD activity		CAT activity	
	Uninfested	Infested	Uninfested	Infested
incompatible				
M. incognita-Rossol	34.9±3.7	35.8±6.3	19.8±8.6	11.6±2.4
compatible				
M. incognita-Roma VF	15.6±2.5	22.2±3.8	34.0±6.3	39.8±25.3
M. hapla-Roma VF	8.9±2.6	10.0±5.1	30.3±12.9	37.8±13.2
M. hapla-VFN8	27.5±4.8	20.4±7.2	42.5±18.2	45.4±25.5
M. hapla-Rossol	29.4±8.4	12.6±4.7	17.3±10.6	51.7±12.5

TABLE II - *Variation of SOD and catalase activities (expressed as units g^{-1} root fresh wt± standard deviation) in* Meloidogyne*-tomato interactions occurring* in vitro: *analyses of excised roots 24 hours after inoculation with active J2 in agar.*

Meloidogyne-tomato interactions *in vitro*	SOD activity		CAT activity	
	Uninfested	Infested	Uninfested	Infested
incompatible				
M. incognita-Rossol	38.4±12.0	29.2±5.2	36.6±14.2	25.1±6.0
M. incognita-VFN8	52.4±9.4	56.3±7.9	53.5±15.2	34.7±16.0
M. hapla-Rossol	40.2±10.3	22.7±7.3	27.1±13.7	19.8±3.8
M. hapla-VFN8	56.2±11.2	19.0±5.2	49.7±29.9	33.7±26.2
compatible				
M. incognita-Roma VF	33.3±12.5	35.5±11.0	18.0±8.6	21.8±15.8
M. hapla-Roma VF	38.7±15.0	33.4±1.9	17.1±6.0	25.7±6.3

enous salicylic acid levels of roots may increase as has been reported for other plant-pathogen interactions (Malamy *et al.*, 1990).

The role of SOD in tomato resistance to RKN could not be ascertained by the available data since there was no reproducible variation associated with incompatible early interactions. SOD activity was found to be higher or unchanged in resistant soybean roots attacked by *M. incognita* compared with uninfested roots (Vanderspool *et al.*, 1994). However, in most, but not all, of the resistant *Meloidogyne*-tomato interactions analyzed in this paper SOD activity was lower. Such inconclusive data may be explained by the fact that SOD is probably unspecifically induced by the oxidative stress of plant response but, at the same time, it is a producer of H_2O_2, which if allowed to accumulate by catalase inhibition, irreversibly inhibits the enzyme (Hodgson and Fridovich, 1975). Indirectly, SOD inhibition may be indicative of the level of H_2O_2 accumulation in a determined plant-nematode

interaction which, as suggested above, may or may not be characterized by CAT inhibition.

In summary, these results suggest that in *Mi*-mediated *Meloidogyne*-tomato interaction CAT activity is specifically inhibited in order to impede the removal of the H_2O_2 being produced, which would act as a HR signal as well as a nematocide molecule; salicylic acid generation is tentatively indicated as responsible for this inhibition.

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