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INHIBITION OF H_2O_2-DEGRADING ENZYMES IN THE RESPONSE OF *Mi*-BEARING TOMATO TO ROOT-KNOT NEMATODES AND SALICYLIC ACID TREATMENT

by

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Summary. Near-isogenic lines of tomato, resistant and susceptible to root-knot nematodes, were inoculated with (a)virulent *Meloidogyne incognita* juveniles and treated with the defense reaction elicitor salicylic acid. Catalase activity was significantly inhibited in resistant roots by either nematode infestation or salicylic acid treatment. Such inhibition did not occur in susceptible roots and in resistant roots inoculated with a virulent isolate of *M. incognita*. The H_2O_2-degrading activities of catalase and ascorbate peroxidase were both inhibited in the upper green tissues of the resistant seedlings 24 hours after nematode inoculation, although, they were restored or enhanced compared with those of controls 48 h after inoculation. Resistant seedlings incubated overnight in salicylic acid showed a marked inhibition of catalase activity in green tissues whilst such inhibition was much less consistent with susceptible seedlings.

There is presently little doubt about the association of the hypersensitive reaction (HR) with salicylic acid (SA), at least in some specific plant-pathogen interactions (Graham and Graham, 1999). Particularly, SA seems to be generated during HR and to play a role in the cell death programme associated with the HR. Mode of action of SA is controversial but several reports have shown that SA at high concentrations (1 mM) can unspecifically bind to and inhibit heme-containing enzymes such as catalase (CAT), ascorbate peroxidase (APX), or aconitase (Ruffer *et al.*, 1995; Sticher *et al.*, 1997). The main physiological role of CAT and peroxidase is H_2O_2 degradation. Therefore, an inhibition of such activities 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). However, the role of H_2O_2 as a second messenger for SA has been questioned (Sticher *et al.*, 1997).

Resistance of tomato to root-knot nematodes (RKN) is also characterized by a rapid HR which follows the attempt by the invading juvenile (J2) to establish a feeding site (Williamson, 1998). The tomato *Mi-1.2* gene that confers such a resistance against RKN has been cloned and characterized as a member of the leucine zipper, nucleotide binding, leucine rich repeat (LRR) family of plant genes (Milligan *et al.*, 1999). At present we can only speculate on the biochemical events following recognition and leading to HR in incompatible *Meloidogyne*-tomato interaction (Molinari, 1996, 1999a).

The attempt to detect transcripts specifically related to *Mi* response of tomato roots against RKN attack was unsuccessful (Lambert *et al.*, 1999). On the other hand, PAL, which is the key

enzyme of the phenyl-propanoid pathway, seems to be readily and specifically induced after RKN attack of resistant tomato and soybean (Brueske, 1980; Edens *et al.*, 1995). Moreover, catalase activity was inhibited in the incompatible *Meloidogyne*-tomato interaction *in vivo* and *in vitro*, as early as 24 hours after inoculation (Molinari, 1999b). Conversely, an early augmentation of catalase activity was found either with compatible interaction or paraquat-treated cultured roots (Molinari, 1991; Molinari, 1999b).

The aim of this paper is to accumulate evidence on the relationship between SA and the inhibition of H_2O_2-degrading activities occurring in the events specifically associated with RKN challenge to *Mi*-bearing tomato seedlings.

Materials and methods

Seedlings of the near-isogenic lines of tomato *Lycopersicon esculentum* Mill. cv. Motelle, which carries the gene *Mi* conferring resistance to root-knot nematodes (RKN), and cv. Moneymaker which is susceptible to RKN attack, were used for inoculation with *Meloidogyne incognita* (Kofoid *et* White) Chitw. Seeds were germinated in a sterilized mixture of peat and soil at 25-27 °C and the seedlings grown to the four true-leaf stage. Seedlings were then inoculated with nematodes according to two different experimental procedures described below. J2, used for inoculation, were obtained by incubation of egg masses in distilled water at 27 °C. Concentration of J2 in small volumes of sterilized water was achieved by filtering through 1 µm filters (Whatman type) and collecting them after repeated washes. One virulent line of *M. incognita*, which had been previously selected on *Mi*-bearing tomato (cv. VFN8) in the glasshouse and named SM4, was also used to infect resistant roots. Groups of five seedlings, thoroughly washed with tap water, were transferred into two sets of 8-cm clay pots each containing: i) a suspension of active *M. incognita* J2 (150/seedling); ii) a solution of 1 mM salicylic acid (SA); untreated seedlings were used as controls. For experiments with resistant seedlings a higher density of avirulent inoculum (500 J2/seedling) and an additional set of pots containing a suspension of virulent *M. incognita* J2 (150/seedling) were tested. All the pots were filled with sterilized quartz sand, watered with tap water and kept for 24 h in a glasshouse at 24-26 °C with constant illumination.

Proteins were extracted according to the following methods. Roots were excised from the shoots, then a series of root samples from each batch were carefully rinsed with distilled water, dried, weighed, cut with a scalpel into very small pieces and then put separately in 1,5 ml Eppendorf tubes containing 150 µl of a grinding buffer consisting of 20% sucrose, 0.1 M Trizma-Base, 0.08 M boric acid, pH 8.4, 2.5 mM EDTA, and the protease inhibitors PMSF (1 mM), pepstatin (1 µM) and leupeptin (1 µM). Samples were homogenized on ice with a small metal pestle connected to a rotor and centrifuged at 10,000 rpm for 3 min in a bench centrifuge. Supernatants were stored at -80 °C or directly used for enzyme activity assays; in a second set of experiments extracts of roots and of mixed shoots and leaves (greens) were analyzed. Roots and greens were collected from each treatment and hand-homogenized in chilled 2-ml porcelain mortars using proper volumes of the Grinding Buffer. Then, the coarse homogeneates were transferred into 2-ml glass potter and further gently ground by a glass pestle connected to a motorized drive. Homogenates were centrifuged at 10,000 rpm for 3 min in a bench centrifuge and supernatants used as enzyme extracts. Samples used for detection of isozyme electrophoresis profiles were ultrafiltered at 4 °C through YM-ultrafiltration membranes (10,000 molecular weigh cut off, Amicon Co.) in Centricon-10 micro-concentrators. Protein content of the samples was determined according to Lowry *et al.* (1951).

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-phosphate, 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 of enzyme. The spectrophotometric assay for ascorbate peroxidase activity was performed after the method of Gerbling *et al.* (1984). Reaction mixture contained 0.1 M TES, pH 7.0, 0.1 mM EDTA, 1 mM ascorbate from fresh stock solution (100 mM), 0.13 mM H_2O_2, 25 µl sample extract, in 0.5 ml final volume. Decrease in absorbance at 298 nm was followed as ascorbate was oxidized and one unit of enzyme expressed the ability to oxidize 1 µmole ascorbate min^{-1} (ε=0.8 mM^{-1} cm^{-1}).

Results

Near-isogenic tomato seedlings of cv. Motelle, carrying the *Mi*-gene, and cv. Moneymaker, susceptible to RKN, were inoculated with virulent and avirulent *M. incognita* J2 or incubated overnight in 1 mM SA. CAT activity in root extracts was determined as early as 24 h after nematode inoculation or immediately after SA treatment and compared with that of uninfested and untreated roots (Table I). Experiments with 150 and 500 J2 per seedling both showed that CAT activity of resistant roots infested with avirulent J2 was significantly inhibited with respect to uninfested roots; inoculation with 500 J2 per seedling resulted in a higher inhibition. Roots infested with a virulent line of *M. incognita* (SM4) did not show any significant change of activity, whilst CAT activity of infested susceptible roots was enhanced by nematode attack. SA treatment caused CAT inhibition in resistant roots but seemed not to have any effect on susceptible roots (Table I).

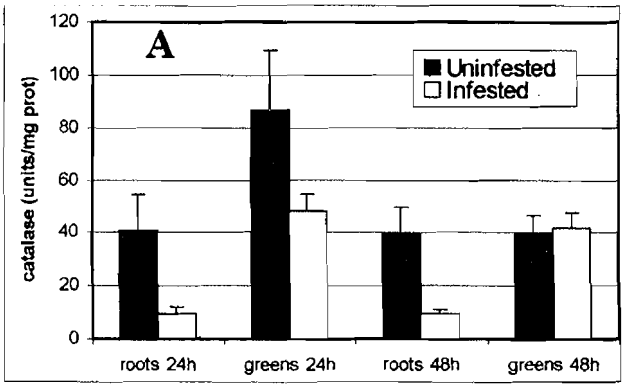
CAT and APX activities in the incompatible tomato-RKN interaction were then monitored 24 and 48 h after inoculation both in root and mixed leaf and shoot (greens) extracts (Fig. 1A-B). The levels of these two H_2O_2-degrading enzymes were lower than controls in roots 24 h and 48 h after inoculation, whilst in greens inhibition was seen at 24 h, but the activities were restored or even enhanced, compared with controls, 48 h after inoculation. Overnight SA treat-

TABLE I - *Catalase activity of near isogenic tomato cvs Motelle and Moneymaker inoculated with avirulent* Meloidogyne incognita *and incubated in 1 mM salicyic acid (SA). Catalase activity of root extracts was recorded 24 hours after treatment and compared to that of untreated seedlings (controls). Resistant seedlings were also inoculated with a virulent line of* M. incognita *(SM4).*

Tomato cv	Inoculum level J2/seedling	Activity[a]			
		Controls	*M. incognita*-inoculated		SA-treated
			avirulent	SM4	
Motelle (resistant)	150	19.2	14.1*	21.1 ns	14.5*
Motelle (resistant)	500	18.4	9.7**	–	14.5 ns
Moneymaker (susceptible)	150	11.4	22.5**	–	12.9 ns

ns = not significant; * *P = 0.05;* ** *P = 0.01 according to L.S.D. test, n = 6*

[a] expressed as units/g roots fresh weight.



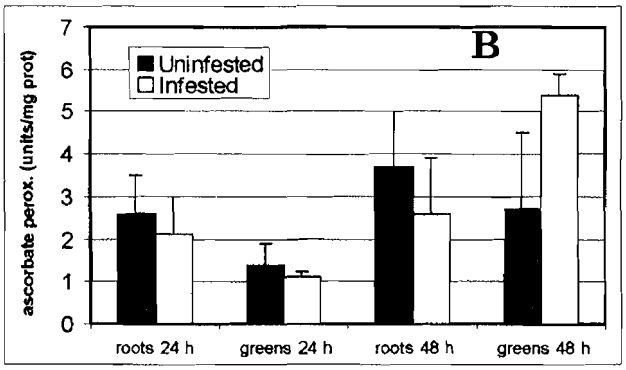


Fig 1 - Histograms of catalase (A) and ascorbate peroxidase (B) activity of resistant tomato roots and mixed green tissues (shoots + leaves) 24 and 48 h after inoculation of the seedlings with active *Meloidogyne incognita* J2 compared with that of uninoculated controls.

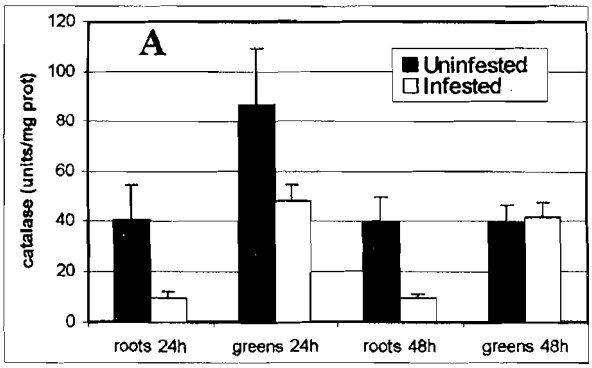


Fig. 2 - Histograms of catalase activity of near-isogenic resistant (A) and susceptible (B) tomato seedlings incubated overnight in 1 mM salicylic acid compared with that of untreated controls.

ment did inhibit CAT of resistant greens (Fig. 2A), but CAT inhibition was negligible in susceptible greens (Fig. 2B).

Discussion

Data of this paper unambiguously show that one of the earliest events occurring in the *Mi*-mediated response of tomato to incompatible RKN is the inhibition of the H_2O_2-degrading enzymes, CAT and APX. Moreover, it has been found that such inhibition is not limited to root tissue but is temporarily extended to the upper regions of shoots and leaves, far from the site of inoculation. It is likely that a diffusible signal is produced at the site of infestation and translocated upwards. Evidence is emerging to implicate SA, which is able to directly inhibit heme-containing enzymes, such as CAT and APX, as this signal (Ruffer *et al.*, 1995). SA is also generally recognized as the principal mediator of SAR (Sticher *et al.*, 1997).

High CAT activity appears to be very important in mediating compatible plant response to nematodes; one of the genes systematically induced by *M. incognita* in potato encodes a catalase (Niebel *et al.*, 1995). The early compatible reaction *in vivo* and *in vitro* of tomato to *M. incognita* is characterized by an augmentation of CAT (Molinari, 1999b). CAT is also highly active in mature tomato galls whilst APX is the most relevant antioxidant enzyme found in J2 (Molinari and Miacola, 1997a, 1997b). In contrast, CAT (and APX) inhibition seems to specifically characterize the incompatible RKN-tomato interaction. Also, SA might be produced in such an interaction and act as the inhibitor of these enzymes.

SA production would favor the impairing of H_2O_2-degrading enzyme system resulting in a rise of H_2O_2 level in cell which ultimately leads to cell death and tissue necrosis (Levine *et al.*, 1994).

Most interesting is the finding of a different sensitivity to SA between resistant and susceptible tomato plants. When resistant tomato roots

are immersed in SA solutions, this chemical is rapidly translocated upwards and inhibits CAT of the green tissue. If concentrated solutions are used (4 mM) leaf damage and necrosis are clearly visible just a few hours after immersion. Interestingly, this does not occur if near-isogenic susceptible tomato are immersed in the same solutions. In this case, seedlings appeared healthy long after immersion (Molinari, unpublished).

Future research will investigate the different sensitivity to SA between resistant and susceptible tomato isolines.

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