Induction of systemic resistance and defense-related enzymes in tomato plants using *Pseudomonas fluorescens* CHAO and salicylic acid against root-knot nematode *Meloidogyne javanica*

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Abstract: Root-knot nematodes (*Meloidogyne* spp.) are the most economically important group of plant parasitic nematodes on many crops worldwide. Resistance-based management is considered as one of the most sound and effective strategies against these pathogens. Plant-mediated systemic resistance against the *M. javanica* in tomato cv. CALJN3 was triggered using salicylic acid (SA) and *Pseudomonas fluorescens* CHAO as elicitors. The effect of each elicitor was assayed by (1) the calculation of nematode indices including the number of nematode galls, egg masses and eggs/egg mass; (2) the analysis of changes in the concentration of reactive oxygen species (ROS); and (3) monitoring the activities of their scavenging enzymes *viz.* superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT). The results indicated that SA/bacterial elicitors induced the removal of high concentrations of the toxic ROS via an increase in the activity of their scavenging antioxidant enzymes, especially that of catalase. Moreover, pre- or post-treatment application of the elicitors significantly reduced the number of galls, egg masses or eggs of *M. javanica* in infected tomato plants as compared to the control. The results of the present study support the involvement of the elicitor-induced ROS and related scavenging enzymes for stimulating plant defense reactions in a moderately resistant tomato challenged with *M. javanica*.

Key words: catalase, elicitor, hydrogen peroxide, peroxidase, reactive oxygen species, superoxide dismutase

Introduction

Root-knot nematodes are an economically important polyphagous group of highly adapted obligate plant parasites. There is a worldwide distribution of these parasites (Moens *et al*. 2009). Historically, recommended control practices against this group of nematodes include preplant and/or post-plant nematicide applications. However, considering environmental and human health awareness issues, alternative strategies (e.g. host plant resistance) inevitably should be investigated and implemented (Nyczepir and Thomas 2009).

Plant resistance against pathogens (e.g. plant parasitic nematodes) could be successfully induced by the biotic inducers, as well as application of different abiotic agents (Ramamoorthy *et al*. 2001; Branch *et al*. 2004; Edreva 2004; McKenry and Anwar 2007; Jagdale *et al*. 2009; Kone *et al*. 2009). Since the rhizosphere provides the first line of defense for roots against attack bysoil-borne pathogens (e.g. plant parasitic nematodes), it is generally accepted that rhizosphere bacteria are ideal biocontrol agents (Hasky-Günther *et al*. 1998). Several studies suggest that induced systemic resistance could be one of the promising mechanisms of these bacteria for suppression of root-knot nematodes (Oostendorp and Sikora 1990; Sikora 1992; Sikora and Hoffmann-Hergarten 1992; van Loon *et al*. 1998; Anita *et al*. 2004; Siddiqui and Shaukat 2004; Bakker *et al*. 2007; Sikora *et al*. 2007). Certain strains of fluorescent pseudomonads are able to suppress plant parasitic nematodes (Sikora 1992; Santhi and Sivakumar 1995; Siddiqui *et al*. 2001; Anita *et al*. 2004; Siddiqui and Shaukat 2002, 2005), but salicylic acid does not seem to be involved in triggering the plant response against *Meloidogyne javanica* (Siddiqui and Shaukat 2004, 2005) or *Heterodera schachtii* (Siddique *et al*. 2014).

One of the most rapid defense responses following pathogen recognition is the so-called "oxidative burst", which constitutes the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($OH\cdot$) at the site of nematode invasion (Baker and Orlandi 1995). The oxidative burst is thought to be required for several defense responses as well as for direct antimicrobial action, lignin

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formation, stiffening of cell walls, and the hypersensitive response (Mehdy 1994; Baker and Orlandi 1995; Wojtaszek 1997). Organisms protect themselves against this oxidative stress by the synthesis or inducing of various enzymatic or non-enzymatic antioxidants (Saed-Moucheshi *et al*. 2014). The major ROS-scavenging enzymes of plants includes superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and some other enzymatic antioxidants that are in charge in the ascorbate-glutathione cycle (Van Peer *et al*. 1991; Williamson and Hussey 1996; Vidhyasekaran 2002; Tománková *et al*. 2006; Saed-Moucheshi *et al*. 2014).

The interaction of root-knot nematodes with their host plants has been intensively studied and a broad range of responses from susceptibility through to resistance was established (Williamson and Hussey 1996). Several studies demonstrated the biochemical changes which resulted from these interactions (reviewed in Gheysen and Mitchum 2009; Tomczak *et al*. 2009), however, there is comparably little information regarding ROS and the related activity of enzymes in the infested plants with plant-parasitic nematodes. A gene encoding a catalase, which is induced after infection with rot bacteria, is induced both locally and systematically in potato infected with *Meloidogyne incognita* as well as *Globodera pallida* (Niebel *et al*. 1995). In tomato roots infected with root-knot nematodes, genes with homology to several known plant defense genes (including peroxidase, chitinase, lipoxygenase, and proteinase inhibitors) are induced locally within 12 h of inoculation (Lambert 1995). In two separate studies, SOD decreased during the initial days after inoculation, but increased dramatically in the formed galls of *M. incognita* on tomato roots (Zacheo and Bleve-Zacheo 1988; Vanderspool *et al*. 1994). An accumulation of peroxidase, phenylalanine ammonia lyase and catalase was shown in tomato root tissue treated with the *Pseudomonas fluorescens* isolate Pf1 in response to invasion by *M. incognita* (Anita *et al*. 2004). In a more recent study by Siddique *et al*. (2014), it was concluded that infection of *Arabidopsis thaliana* by *H. schachtii* activated the NADPH oxidases RbohD and RbohF to produce ROS, which was necessary to restrict infected plant cell death and promote nurse cell formation.

The present study was carried out to assess the induction of ROS and defense related enzymes by the *P. fluorescens* strain CHAO and salicylic acid (SA) against *M. javanica* in tomato as a result of induced systemic resistance.

Materials and Methods

Nematode material

Seedlings of tomato (*Solanum lycopersicum* L. cv. Rutgers) were selected and considered as a susceptible host for purification and for obtaining the required inoculum for the experiment. For this, the seeds were surface sterilised with 0.5% sodium hypochlorite (NaOCl), washed three times with magnesium sulfate ($MgSO_4$) 0.1 M and air-dried under the laminar flow. The seeds were sown in 1000-cm^3 pots containing steam-sterilised soil (a mix of field soil, sand, and compost at 1 : 1 : 2 ratio) or perlite. Initially, the nematode was purified on the 4–6 leaf-stage transplanted tomato seedlings, by using a single egg mass (obtained from a naturally infected tomato field in Tehran). The egg masses were subsequently reproduced on tomato plants. Using an available identification key (Karssen and van Hoenselaar 1998), and taking into consideration the morphological characters of the females and juveniles, the populations were identified as *M. javanica*.

The nematode suspension was inoculated into clay pots containing transplanted tomato seedlings by making three 2.5 cm deep holes around the seedlings (Hartman and Sasser 1985; Eisenback and Triantaphyllou 1991). When large egg masses were present on the roots, the eggs were extracted by uprooting the tomato roots and rinsing the soil off the roots with tap water. Roots were dipped in 0.5% NaOCl for 2 min (Hussey and Barker 1973). Then, the nematode suspension was poured onto a 74-µm sieve nested on a 25-µm sieve and washed with tap water to eliminate excess residues of NaOCl. Subsequently, the eggs and second stage juveniles (J_2) were put into a beaker and their numbers estimated before using them for inoculation.

Preparing the elicitors (SA and *P. fluorescens* CHAO)

For preparing each liter of 10 mM SA (Merck Co., Germany) suspension, 1.3812 g of SA was poured in 100 ml of ethanol 96% and then the solution volume was diluted to 1,000 ml by adding distilled water. Plants were inoculated with the obtained suspension by the soil-drenching method (Oka *et al*. 1999).

The bacterium *P. fluorescens* strain CHAO was cultured on suitable media such as Nutrient Agar (NA), transferred to Nutrient Broth-medium and shaken for 48 h. The resulting suspension was centrifuged for 20 min at 4,500 rpm and subsequently, bacterial cell sediment was rinsed with distilled water. The bacterial concentration of the obtained suspension was justified at 10^{-9} (CFU/ml) by absorbance estimation at 600 nm (OD = 1) using a spectrophotometer (Thompson 1996).

Inoculation of plants with the elicitor and nematode

Initially, the 4–6 leaf-stage transplanted tomato seedlings (cv. CALJN3) in each clay pot were inoculated with 75 ml of SA or the bacterium suspension using the soil-drenching method. After 24 h, nematodes were added as a 5 ml suspension containing 2,000 eggs+J2, into each 230-cm^3 clay pot by the method discussed earlier. The pots were maintained in a greenhouse in which the greenhouse temperatures were adjusted to 27±2°C. For defense compounds and the enzyme activity assay, the plants were laid out according to a completely randomised design with three replicates for each of the four following treatments: inoculated with either the elicitor (SA/*P. fluorescens* CHAO) or nematode (*pne*); with elicitor alone (*pe*); with nematode alone (*pn*); and with distilled water as the control (*p*). For evaluatiing the nematode indices, the plants were arranged in a completely randomised design with three replicates for each of the five following treatments: plants inoculated with nematode alone (N); plants pretreated with SA/*P. fluorescens* CHAO and after 24 h with

nematode (as SN and PN, respectively), and finally, plants challenged with nematode and after 24 h, treated with SA/*P. fluorescens* CHAO (as NS and NP, respectively).

The number of galls and egg masses per g of root was determined by cutting the roots into one cm long pieces from which one g was randomly selected. The eggs of an egg mass were extracted by agitating the egg mass in a 0.5% NaOCl solution for 2 min (Hussey and Barker 1973), sieved as explained earlier, and counted. The nematode indices were evaluated 50 days after inoculation.

Assay of ROS levels in roots

Hydroxyl radical ($OH^{\cdot}$) was measured according to the method described by Van Tiedemann (1997) but with slight changes. Root tissue was immersed in 1 ml of the 1 mM 2-deoxyribose (DOR) (sigma) as scavenger/molecular probe for $OH^{\cdot}$. The assay was incubated at the dark for 45 min. Subsequently, a 0.5 ml aliquot of the solution was added to a preheated mixture containing 0.5 ml thiobarbituric acid (TBA) (Sigma) (1% w/v) in 0.05 M NaOH and 0.5 ml trichloroacetic acid (TCA) (Sigma) (2.8% w/v). The resulting solution was immediately boiled for 10 min. Finally, samples were cooled on ice for a further 10 min. Absorbance was measured at 540 nm and the results were given as absorbance units per g of fresh weight (Malolepsza and Rozalska 2005).

Hydrogen peroxide (H_2O_2) was measured using slight modifications to the method developed by Velikova *et al*. (2000). Root tissues (500 mg) were homogenised in an ice bath with 5 ml TCA (0.1% w/v). The homogenate was centrifuged at 12,000 g for 20 min and subsequently, a 0.5 ml aliquot of the supernatant was added to 0.5 ml potassium phosphate buffer 10 mM (pH 7.0) and 1 ml 1 M potassium iodide (KI). The absorbance capability of supernatant was read at λ max = 390 nm. The measured H_2O_2 content was expressed based on μm per g of root tissue (Velikova *et al*. 2000). The concentration of ROS was assessed at 24 h intervals during a one week period.

Assay of enzyme activity

The SOD quantity was measured using the method (with slight modifications) developed by Patykowski and Urbanek (2003). The three ml reaction mixture contained 1.5 ml 0.05 M sodium phosphate buffer (pH = 7.8), 0.3 ml 130 mM methionin, 0.3 ml 750 μM nitro blue tetrazolium (NBT), 0.3 ml 0.1 mM EDTA-Na_2, 0.3 ml 20 μM riboflavin, 0.01 ml enzyme extract, 0.01 ml polyvinyl polypyrrolidone (PVPP) 4% (w/v), and 0.28 ml deionized water. The reaction was started by exposing the tubes with a 30 cm distance under a 20 W fluorescent lamp for 10 min. After covering the samples with a black cloth for 10 min, the reduction activity of NBT was expressed as increased absorbance at 560 nm/h g of fresh weight (Patykowski and Urbanek 2003).

The activity of the peroxidase during purification, was monitored using spectrophotometric measurements of the oxidation products by guaiacol at 475 nm (Reuveni 1995). The reaction was performed in a 2 ml reaction mixture containing 20 μl 200 mM guaiacol, extract of 40 μg protein, and buffer citrate-phosphate (pH = 5.4). Ten μl of H_2O_2 (30% v/v) was added, and finally, measurements (475 nm) were made at six time points with 10 sec intervals. One unit was defined as the amount of enzyme which catalysed oxidation of 1 μmol of guaiacol per min per mg protein at 475 nm.

For assessing catalase activity, the reaction mixture contained 3 ml 50 mM sodium phosphate buffer (pH = = 7), apoplectic extract containing 30 μg protein, and 30 ml H_2O_2. The consumption of H_2O_2 was monitored by a spectrophotometer at 240 nm (Sahebani 2003). The activity of enzymes was determined at 24 h intervals during a one week period.

Statistical analysis

Variables of the experiment were subjected to analysis of variance (ANOVA) and means were compared with Duncan's multiple range tests using SAS software. Differences at $p \leq 0.05$ were considered significant.

Results

In plants treated with salicylic acid, induction of hydroxyl radical occurred more rapidly in pn treatment when compared with the *pne* or *pe* treatments. However, during the third and fourth days after inoculation, the concentration of the hydroxyl radical was significantly higher in *pne* as compared to the *pn* or *pe* treatments (Fig. 1A). Out of all the treatments, hydrogen peroxide increased more rapidly in plants treated with the pathogen and elicitor (*pne* treatment) during the first three days, but the level of hydrogen peroxide showed no significant differences during subsequent days (Fig. 1B). Salicylic acid likely has no significant effects on the induction of SOD and POX activity (Fig. 2A, B). On the other hand, SA is an effective inducer of CAT activity against invading *M. javanica*, so this enzyme activity was significantly increased in the plants treated with SA and the nematode (*pne* treatment), as compared with all other treatments (Fig. 2C).

In plants treated with *P. fluorescens* CHAO, the concentration of hydroxyl radical was significantly higher in the *pne* treatment, as compared with the *pn* treatment, during the first four-day period from inoculation, but showed non-significant differences during the subsequent days (Fig. 1C). The concentration of hydrogen peroxide was higher in the *pn* treatment, the first day after inoculation, but this concentration was higher in the *pne* treatment during the three subsequent days (Fig. 1D). There was significantly more SOD activity in the *pne* than *pn* treatment only on the third day after inoculation (Fig. 2D), but POX was significantly higher in the *pn* treatment compared to the *pne* or *pe* treatments, during the whole experiment (Fig. 2E). Plants challenged with the bacteria and nematodes (*pne* treatment) showed higher levels of CAT activity when compared with the *pe*, *pn* or *p* treatments (Fig. 2F).

Both pre- or post-treatment of infected tomato seedlings with SA or *P. fluorescens* significantly reduced the number of galls, egg masses and eggs/egg mass of *M. javanica* as compared to the control plants (Table 1). These

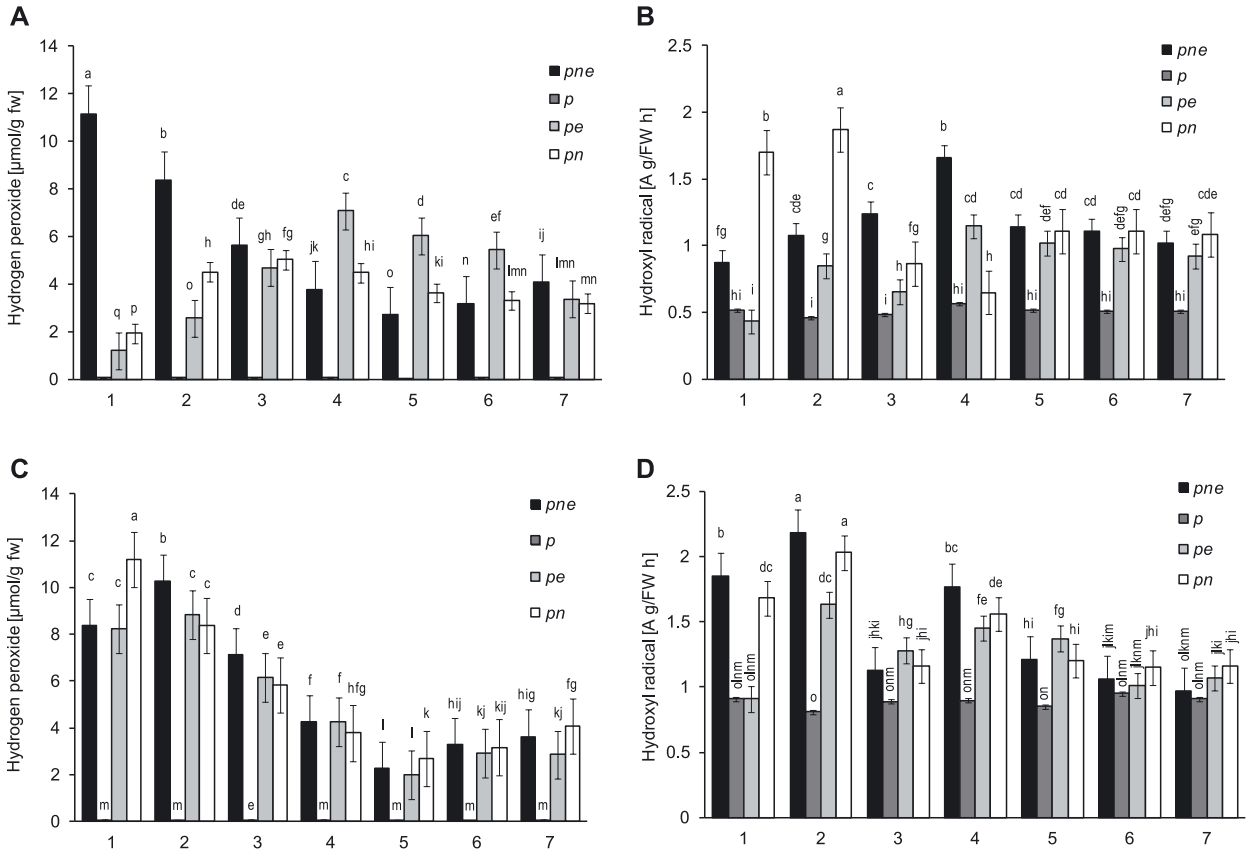

Fig. 1. Concentration of hydrogen peroxide and hydroxyl radical in the roots of tomato seedlings inoculated with either elicitor (A and B – salicylic acid; C and D – *P. fluorescens* CHAO) and *M. javanica* (*pne*); with distilled water, as the control treatment (*p*); with elicitor alone (*pe*); and with *M. javanica* alone (*pn*). Data in the horizontal lines are days after inoculation

Table 1. The number of *M. javanica* galls, egg masses and eggs/egg mass per each g root of tomato seedlings (cv. CALJN3), pre- or post-treated with salicylic acid (SN and NS, respectively) and *P. fluorescens* CHAO (PN and NP, respectively) as elicitors, 50 days after inoculation; N – plants inoculated with nematode alone

Treatment/character		Gall*	Egg mass	Eggs/egg mass
The control	N	208.3 a	262.3 a	362.3 a
Pre-treatment	SN	65.7 c	30.7 c	87.0 d
	PN	47.3 cd	45.0 c	161.7 c
Post-treatment	NS	22.0 d	20.0 c	261.7 b
	NP	105.0 b	95.7 b	280.0 b

*within a column, averages sharing a letter are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test

controlling effects were more evident in the pre-treatment than in the post-treatment application of *P. fluorescens* (for all three indices) or SA (only for eggs/egg mass).

Discussion

The SA/bacterial elicitor-mediated generation of ROS and the subsequent activities of the related antioxidant enzymes were examined in an attempt to determine their roles in the resistance reactions of tomato seedlings to infection with *M. javanica*.

Exogenous application of SA and *P. fluorescens* CHAO significantly reduced the root-knot nematode population in tomato roots. Root galling and egg mass/egg production in tomato due to infestation by *M. javanica* was also less in plants treated with both elicitors. This is in agreement with the works using *P. fluorescens* as elicitor against *M. incognita* (Santhi and Sivakumar 1995; Anita *et al*. 2004). The present study revealed a significant accumulation of CAT, but that of SOD or POX did not followed similar trends. The accumulation of CAT began on the first day after inoculation with the nematode and the accumulation gradually increased for up to three

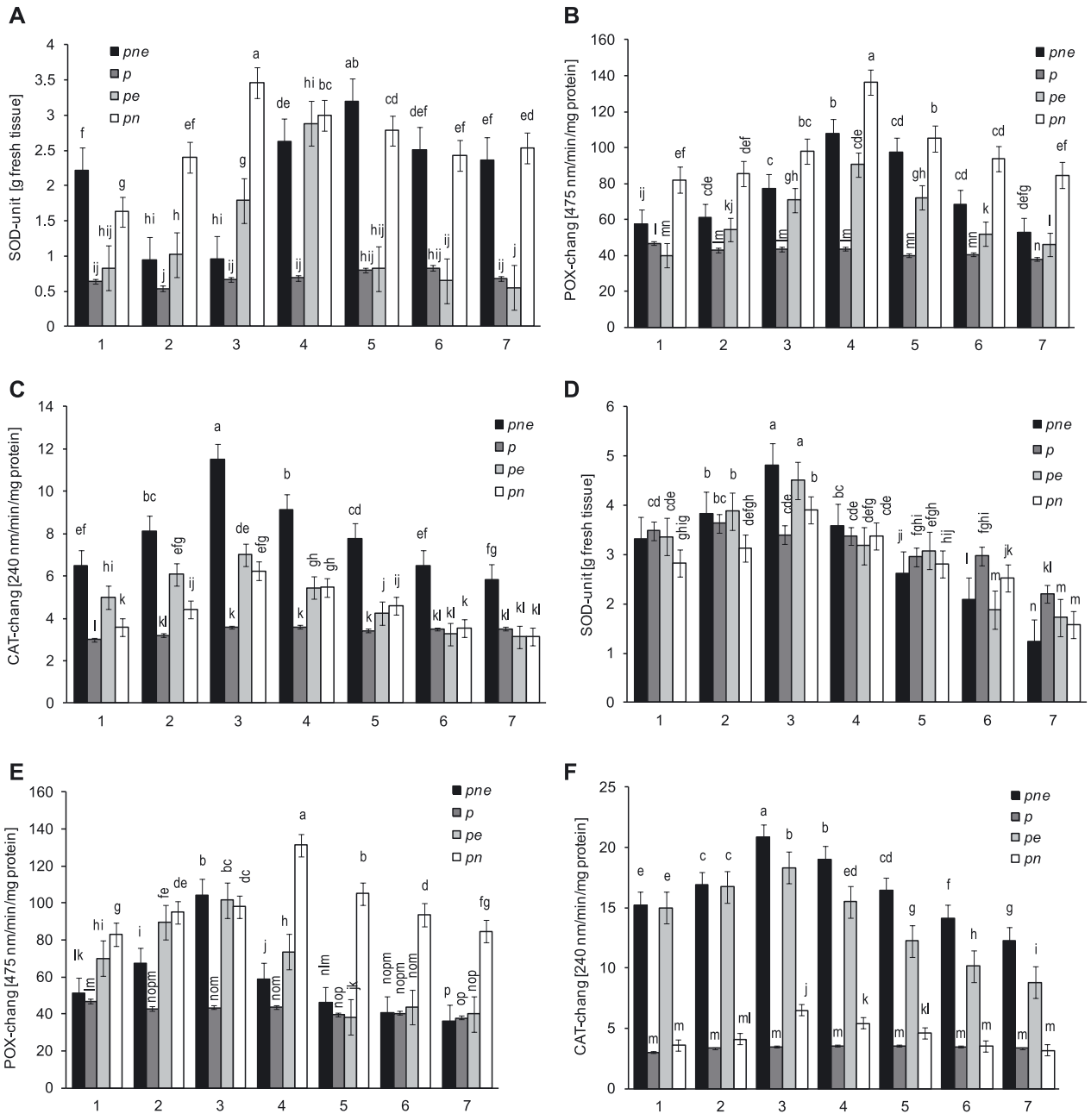

Fig. 2. Activity of superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) in the roots of tomato seedlings inoculated with either elicitor (A–C – salicylic acid; D–F – *P. fluorescens* CHAO) and *M. javanica* (*pne*); with distilled water, as the control treatment (*p*); with elicitor alone (*pe*); and with *M. javanica* alone (*pn*). Data in the horizontal lines are days after inoculation

days and then subsequently decreased till seven days after inoculation. Plants inoculated with the elicitor alone (*pe* treatment) or nematode alone (*pn* treatment) also showed an increased CAT level, but the increases were less than in the *pne* treatment. It may be concluded, that high levels of H_2O_2 in the root tissue and an increase activity of CAT at the beginning of infection may be needed to inhibit infection, but the activity of SOD or POX may not play very important roles in elicitor-mediated resistance in tomato cv. CALJN3 to *M. javanica*.

While investigating the resistance of tomato plant cultivars toward infection with *M. incognita*, Zacheo and Bleve-Zacheo (1988) reported a fall of SOD activity in hypersensitively responding tomato plants. Similar results were obtained by Vanderspool *et al*. (1994) who confirmed a decline in the activity of SOD in resistant soybean, 96 h after nematode infection. In contrast, SOD increased dramatically in galls (Zacheo and Bleve-Zacheo 1988) or as nematodes matured and enlarged (Vanderspool *et al*. 1994). There might be an involvement of SOD in processes related to the development and maintenance of the nematode feeding sites and this involvement might provide protection against superoxide-mediated damage in a compatible response. It appears, that generation of superoxide radicals is an important feature of the local events that occur in an incompatible interaction, while an increased scavenging activity of superoxide onions by SOD, with concurrent production of H_2O_2 occurs in a compatible interaction. Clearance of H_2O_2 may be a sub-

sequent defensive step that could be induced by the action of POX or CAT (Zacheo *et al*. 1997).

In our experiment, the concentration of H_2O_2 was increased rapidly the first and second days after inoculation, and subsequently, CAT activity increased three days after inoculation. It is supposed that the scavenging activity of CAT corresponds to the rapidity and intensity of H_2O_2 production. It was well established that CAT might be responsible for the removal of the excess of ROS during stress (Mittler 2002). However, the significant increase in CAT activity in the present study differs from the hardly distinguishable increase of this enzyme in tomato plants challenged with *M. incognita* (Rajasekhar *et al*. 1997; Anita *et al*. 2004). These differences in enzyme activity may be attributed to the resistance level of the challenged host plants (Zacheo *et al*. 1995; Rajasekhar *et al*. 1997; Tománková *et al*. 2006). Tománková *et al*. (2006) found that CAT activity differs among susceptible, moderately resistant, and highly resistant varieties of tomato plants against *Oidium neolycopersici* (the cause of powdery mildew), so activity of this enzyme showed higher accumulation in moderately resistant tomato. Zacheo *et al*. (1995) pointed out that there is a relationship between the activity of antioxidant enzymes and the resistance level of the host plants. It already has been shown that tomato cv. CALJN3 could be considered as a moderately resistant host for *M. javanica* (Maleki Ziarati 2006).

The present study revealed the involvement of elicitor-mediated induction of ROS and the scavenging enzymes in a resistant host plant (tomato cv. CALJN3) challenged with *M. javanica*. Further studies dealing other nematode species or different levels of resistance during a longer time period, are suggested. Such studies can provide for a better understanding of the potential contribution of ROS and antioxidant enzymes in the defense mechanisms of plants during compatible or incompatible interactions against plant-parasitic nematodes.

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