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SHORT COMMUNICATION

SAR induction in tomato plants is not effective against root-knot nematode infection

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Abstract Systemic Acquired Resistance (SAR) is one of the defence mechanisms plants employee against pathogen attack, resulting in resistance and protection of distal tissues. It is characterized by the accumulation of salicylic acid (SA) and the expression of a group of pathogenesis-related (PR) genes; synthetic analogues of SA, such as INA and BTH, can induce SAR. The effects of INA and BTH on tomato plant development and on the tomato–*Meloidogyne javanica* interaction were investigated, and the response of both SAR and nematode pathogenesis to changes in the expression of several SA-responsive PR genes, *PR-1b*, *PR-2* and *PR-5,* was determined. In addition, the response of calmodulin and *PR-3* transcripts – two SA-independent genes – to SAR elicitor chemicals was also studied. All elicitor-treated plants exhibited successful infestation by *M. javanica*, although a significant reduction of gall numbers was observed. INA was more efficient than BTH at inducing the SAR markers *PR-1b* and *PR-2*, but the root–knot nematode apparently suppressed the expression of these genes. This downregulation of gene expression following nematode infestation was also detected with regard to *PR-3* expression. On the other hand, expression of *PR-5* was not affected by elicitor treatment, though *Meloidogyne* infestation induced its expression.

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Anti-pathogen plant defence involves several inducible mechanisms, including some systemic responses, which can prevent secondary infection against a broad-spectrum of microbes. Salicylic acid (SA)-dependent systemic acquired resistance (SAR) and SA-independent wound response pathways are the most studied inducible responses (Ryals et al. 1996; Sticher et al. 1997). Cell death usually occurs at the local site of pathogen attack, and causes a hypersensitive response (HR); in parallel, the accumulation of SA and pathogenesis-related (PR) proteins is detected. Some of these PR gene products exhibit antimicrobial activity and restrict the growth and spread of the attacking species (Klessig and Malamy 1994). SA is an important signal molecule in the SAR transduction pathway. Exogenously-supplied SA induces resistance and the same set of genes as stimulated through pathogen-induced SAR. Inhibition of SAR development by the expression of salicylate hydroxylase increased the susceptibility of plants to infection (Durrant and Dong 2004).

In addition to pathogens, SAR is induced by exogenously applied chemicals (Conrath et al. 2006). Other well-known chemical inducers of PR

genes include 2,6-dichloroisonicotinic acid (INA), benzol (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (both with structures similar to SA) and L-β-amino butyric acid (BABA) (Görlach et al. 1996; Lawton et al. 1996; Bovie et al. 2004). The induction of SAR seems to be regulated by a complex signal transduction process (Pietersen and Van Loon 1999).

PR genes can be useful molecular markers for the expression of SAR, although the specific subset of PR genes appropriate for an interaction can vary from species to species. In *Arabidopsis*, tobacco and tomato, SAR genes include *PR-1* (unknown function), *PR-2* (β-1-3 glucanase) and *PR-5* (thaumatin-like protein). Other PR genes are induced by ethylene and jasmonic acid (JA) (e.g., *PR-3*, chitinase); some can be SA responsive, while others are not (e.g., PDF1.2, defensin). Variation in PR gene expression might be explained by the regulation of elements within their promoters (Lebel et al. 1998).

Root-knot nematodes are a group of obligatory biotrophic pathogens, which live in the soil and interact with the root systems of a great number of plant species. Some of these plants have economic relevance and nematode infection can result in significant deterioration in plant health and subsequent crop losses, such as in the interaction between tomato (*Lycopersicon esculentum*) and *Meloidogyne spp.* The juvenile nematode, at the second stage (J2), penetrates the intercellular space at the root tip and moves up the plant to the vascular cylinder, where it selects three to four cells in the protoxylem tissue on which to feed. These cells suffer physiological and molecular alterations, producing giant cells. Hypertrophy of the surrounding tissue leads to the development of galls; these are macroscopic structures visible on infected roots (Hussey et al. 2002; Williamson and Gleason 2003; Davis et al. 2004).

In general, the nematode infection elicits important changes in plant gene expression (Gheysen and Fenoll 2002), but the majority of upregulated genes seems to be induced through the establishment of the feeding site (Puthoff et al. 2003). These are related to metabolic pathways, cell-cycle progression, water transport and cell-wall expansion (Potenza et al. 2001; Vercauteren et al. 2002; Gal et al. 2006). Some of them are also induced during the host-cyst nematode interaction and the host-*Rhizobium* symbiosis (Koltai et al. 2001; Favery et al. 2002; Bird 2004). On the other hand, several host defence genes are downregulated during attack, suggesting that the nematode actively suppresses the host defence response (Gheysen and Fenoll 2002; Jammes et al. 2005).

In the tomato–*Meloidogyne* incompatible interaction the Mi-1 gene confers resistance against three species of *Meloidogyne* (Williamson and Gleason 2003). In addition, SA is part of the Mi-mediated resistance (Branch et al. 2004), though the participation on susceptible tomato plants responses is not well known. On the other hand, JA treatment enhances Mi-mediated resistance and reduces the *Meloidogyne javanica* reproduction on susceptible tomato plants (Cooper et al. 2005).

The objective of the present work was to study the induction of resistance to root-knot nematodes in tomato plants by application of SAR inducers. Treatments involved application of INA and BTH, SA analogues, to susceptible tomato plants either via leaves or roots. Plant development and *M. javanica* pathogenesis were monitored. In addition, effects on the expression of some plant defence responses were assessed by analysis of the expression of SA-independent genes such as calmodulin and *PR-3* (chitinase), as well as several SA-dependent PR genes (*PR-1a*, *PR-1b*, *PR-2* and *PR-5*).

Experiments were initiated with 100 tomato plants per experiment; the protocol is represented in Fig. 1. Experiments were repeated three times, to collect different plant samples and to conduct separated analyses, in order to determine significant variations by the average value and the standard deviation (SD) in the parameters studied.

Seeds of a nematode-susceptible tomato variety (*L. esculentum* cv. Marmande) were sterilized for 10 min in 10% hypochlorite, then washed several times with sterile water. Seeds were sown in pots (15 cm diam 4 seeds per pot) containing sterile, sandy loam. Pots were watered every other day with 100 ml of Gamborg B5 Medium in mineral nutrient solution (Duchefa Biochemie, Haarlem, The Netherlands). Seeds were germinated in a growth chamber with a previous vernalization treatment for 4 days at 4 °C. Plants were grown at 25 °C, with a 16-h light:8-h dark photoperiod, until they reached the third leaf stage, at which time they were treated with the SAR inducers (Fig. 1). Suspensions of wettable powder formulations were prepared with a concentration of 1 mM BTH (BION, Syngenta Agro, Madrid, Spain) and 1.25 mM

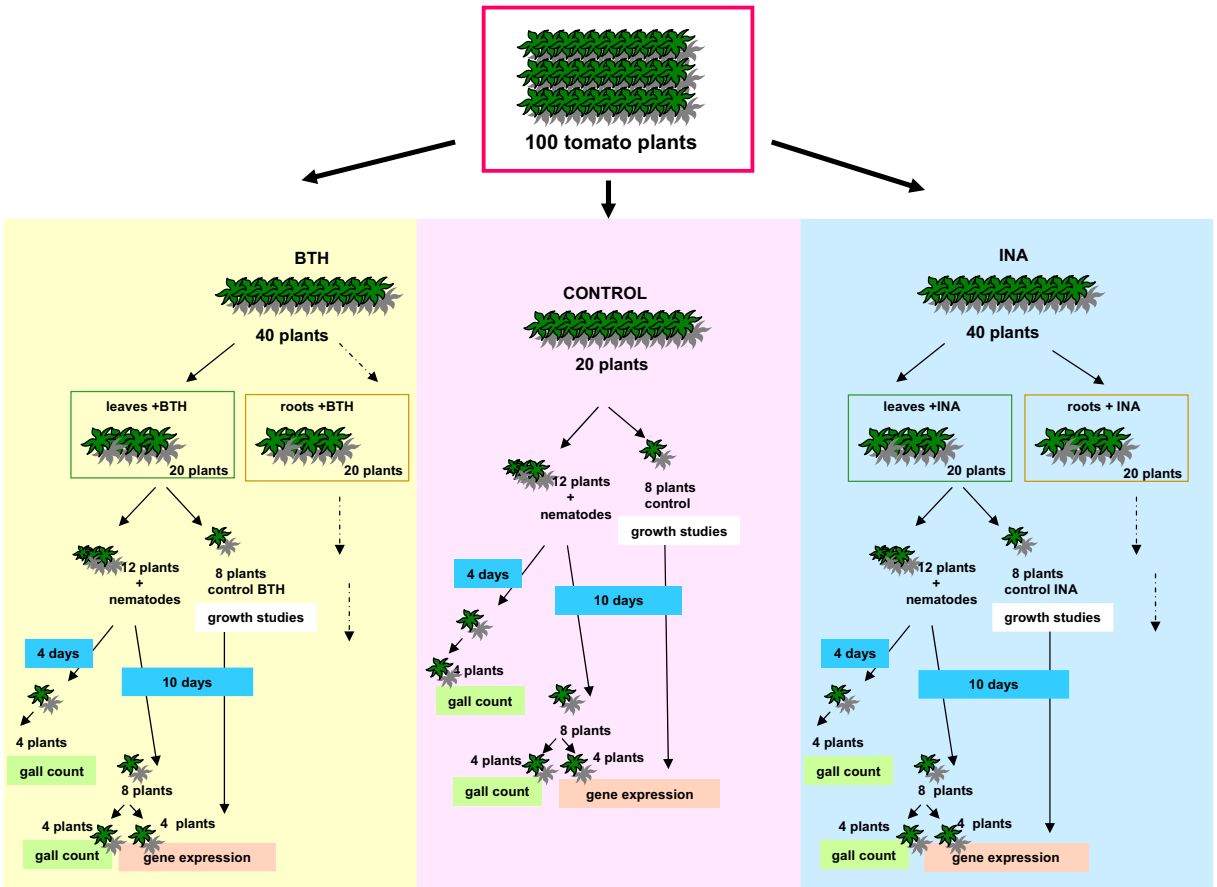

Fig. 1 Representation of the experimental design. One hundred tomato plants were exposed to the various treatments and sequentially analysed

INA (Aldrich, Milwaukee, Wisconsin, USA). These inducer chemical preparations were added to the Gamborg solution and applied independently by spraying approximately 50 ml on the three leaves, or to the roots as a soil drench (100 ml). Control plants were treated with water only. Two days after the application of elicitors, tomato plants (12 per treatment) were inoculated with 200 J2 nematodes per pot; remaining plants (four per treatment) were not inoculated (Fig. 1). The length of stems and roots (maximum length) were measured 30 days after application of the elicitors (Fig. 2).

The root-knot nematode, *M. javanica*, obtained from Dr. F.M.W Grundler (Institute of Plant Protection, Vienna, Austria), was maintained in our laboratory, growing *in vitro* on cucumber roots. Cucumber seeds were germinated in Petri dishes containing Gamborg medium and 1% Daishing Agar, at 28 °C in the dark. The developed cotyledons were removed and the roots were inoculated with four to five egg masses per plate. The infected roots were allowed to produce new egg masses until maturity at approximately 45 days, at which time the egg masses were collected and incubated at 28 °C in sterilized water for 48 to 72 h. After they hatched, the second stage juveniles (J2) were used to inoculate tomato plants by applying (using a pipette) an aqueous suspension of nematodes close to the root system at a rate of approximately 100 J2 animals per plant.

Four and 10 days after inoculation, roots from half the plants were stained to estimate the level of nematode infestation. Root samples were cleared in 0.5% sodium hypochlorite for 1 min and washed to eliminate traces of the bleach. Nematodes were then stained by boiling the roots in acid fuchsin (0.05%) in lactophenol. After the staining solution was cooled to room temperature (approximately 4 min), plant tissues were destained in an acetic acid, glycerol and water

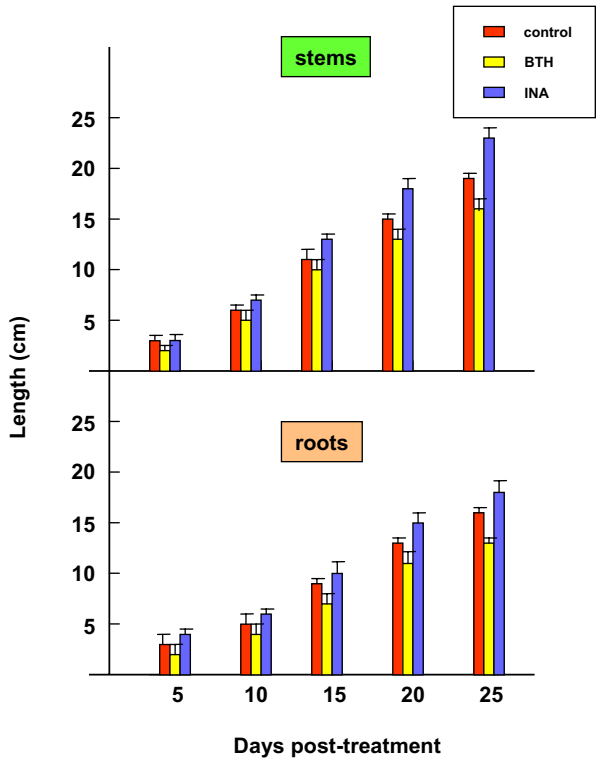

Fig. 2 Growth of plants after SAR inducer treatments. Error bars represent the SD obtained from 12 different plants. Experiments were conducted in triplicate (four plants were measured per experiment)

(1:1:1) solution. The destaining solution was changed several times until tissue colour was relatively clear and stable (approximately 24 h). The number of galls was counted in stained roots by stereo microscopy (Fig. 3).

Samples from leaves and roots were collected from half of the infected and non-infected plants (Fig. 1), frozen immediately in liquid nitrogen and stored at −80 °C until used. Total RNA was obtained with the SV Total RNA Isolation System (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions, after tissue extraction. Expression of the studied defence genes was established by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from each sample and random primers were used in the cDNA synthesis using the RETROScript Kit (Ambion, Austin, Texas, USA). Ten microliters of each cDNA were used for amplification in the presence of Super Taq DNA polymerase (Ambion). Specific primers were designed from their gene sequences deposited in GenBank, using the PRIME software, with a selected annealing temperature of 60°C (Table 1). The quantification was accomplished using the Quantum*RNA* 18S Internal Standards Kit (Ambion), according to the manufacturer's recommendations. This kit allows a multiplex RT-PCR reaction to be run with 18S primers (as an endogenous control), 18S competimer primers and the specific primers. The

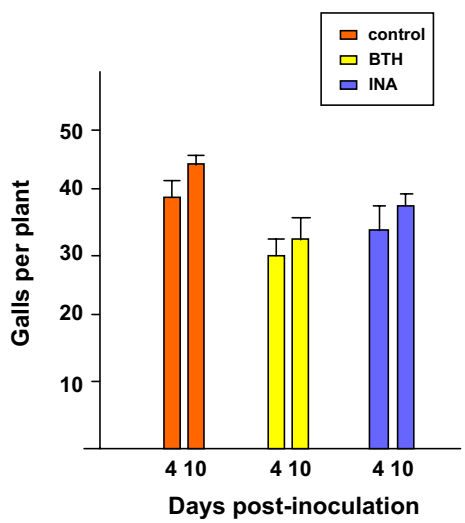

Fig. 3 *Meloidogyne javanica* infection rate in tomato roots under different treatments. The number of galls per plant was estimated by microscopic examination of stained roots. Bars represent the SD obtained from 12 separated plants. Experiments were conducted in triplicate (four plants were analyzed per experiment)

Table 1 Specific primers employed in RT-PCR reactions

Genes	Sequences of oligonucleotides
PR-1b	Forward: 5′-CCCAAAATTCACCCCAAGAC-3′ Reverse: 5′-CTACACTTTTTTCCACCAACAC-3′
PR-1a	Forward: 5′-TGATGCTCAAAATTCACCCC-3′ Reverse: 5′-TTCCGACACCCACAATTCC-3′
PR-5	Forward: 5′-GCAACAACTGTCCATACACC-3′ Reverse: 5′-AGACTCCACCACAATCACC-3′
PR-2	Forward: 5′-AAGTATATAGCTGTTGGTAATGAA-3′ Reverse: 5′-ATTCTCATCAAACATGGCGAA-3′
PR-3	Forward: 5′-AAAACAGCAACTACAACT-3′ Reverse: 5′-TACCTCCTGTAAAATCCA-3′
Calmodulin	Forward: 5′-GCACGGAAGATGAAGGACAC-3′ Reverse: 5′-GCAAGCATCATACGGACAAAC-3′

level of product from the gene of interest was expressed as a percentage of the corresponding product amount from the control. Several conditions were determined empirically, including the cycle number (25 cycles for *PR-1a* and *PR-1b* and 20 cycles for the remaining genes *PR-2, PR-3, PR-5* and calmodulin) and the optimum ratio of 18S primers:competimers (1:9 for *PR-1a and PR-1b* genes and 2:8 for the remaining genes).

The RT-PCR products were separated by electrophoresis on 2% agarose gels with incorporated ethidium bromide. Amplified bands were visualized under UV light; the particular intensities were quantified and standardized to the 18S fragment intensity by the application of Kodak 1D software. All the RT-PCR reactions were completed in triplicate to verify the amplification. Resulting data are represented as bar charts (Fig. 4a–f).

The elicitor treatments were applied at both the leaf (Métraux et al. 1991; Lawton et al. 1995; Görlach et al. 1996) and root levels; experiments were conducted in triplicate. During each experiment, leaf and root results were very similar, with no significant differences in growth, nematode infection studies or gene expression analyses. Therefore, only the leaf treatment results are presented here.

Root and stem length were measured as developmental factors (Fig. 2). Although tomato plants treated with BTH were shorter than control plants, and INA-treated plants were taller (and had longer roots) than the controls, these differences were not significant.

The pathogenesis rates achieved by *M. javanica* were significantly, albeit only slightly, affected by the SAR inducers. Four days after inoculation, there was a 20% reduction in gall numbers in BTH-treated plants (compared to control plants), and a 10% reduction in INA-treated plants. After 10 days the reduction in galls increased to 28% in BTH plants and 20% in INA plants. These data indicate less of an effect than has been reported from other studies where the application of SAR inducers was found to strongly diminish the level of infection (Görlach et al. 1996; Kohler et al. 2002; Bovie et al. 2004). However, the ability of nematodes to circumvent induced resistance has been mentioned elsewhere (Gheysen and Fenoll 2002; Williamson and Gleason 2003). Our results indicated that total resistance is not imparted through the application of SA-derived elicitors, and only a partial reduction in infection rates was detected.

Analyses of SAR-gene expression, on the samples collected after different treatments, were carried out to provide information about the progression of the nematode interaction. The gene expression patterns obtained in non-infected plants indicated that INA was able to increase the expression of *PR-1b* and *PR-2* genes only in roots, while BTH activated the transcript accumulation of *PR-2* in leaves. These genes have been described as SAR markers (Dong 2001; Durrant and Dong 2004) and these results confirm that SAR was induced, at least by INA treatment. In our current investigation, a 1 mM concentration of BTH was selected as optimal. In other systems, concentrations between 0.1 mM and 10 mM were efficient at inducing PR genes and diminishing pathogen development (Görlach et al. 1996). In this study, infected plants exhibited suppressed expression of *PR-1b* and *PR-2*, in both leaf and root samples, from treated and control plants. The nematode appears to be able to overcome SAR induction and silence the activated genes. This phenomenon supports data recently presented by the group of Pierre Abad at INRA (France), where the suppression of plant defence genes was detected by microarray expression profiles in *Arabidopsis*, infected either by *M. incognita* (Jammes et al. 2005) or *Heterodera schachtii* (Puthoff et al. 2003). An

exception to this trend was an increase in the *PR-5* transcript following *M. javanica* inoculation. This same *PR-5* response has been observed during the analysis of several *Arabidopsis* mutants. The function of the *PR-5* protein might be related to water stress, which is induced during establishment of the nematode infestation (Van der Eycken et al. 1996; Escobar et al. 1999).

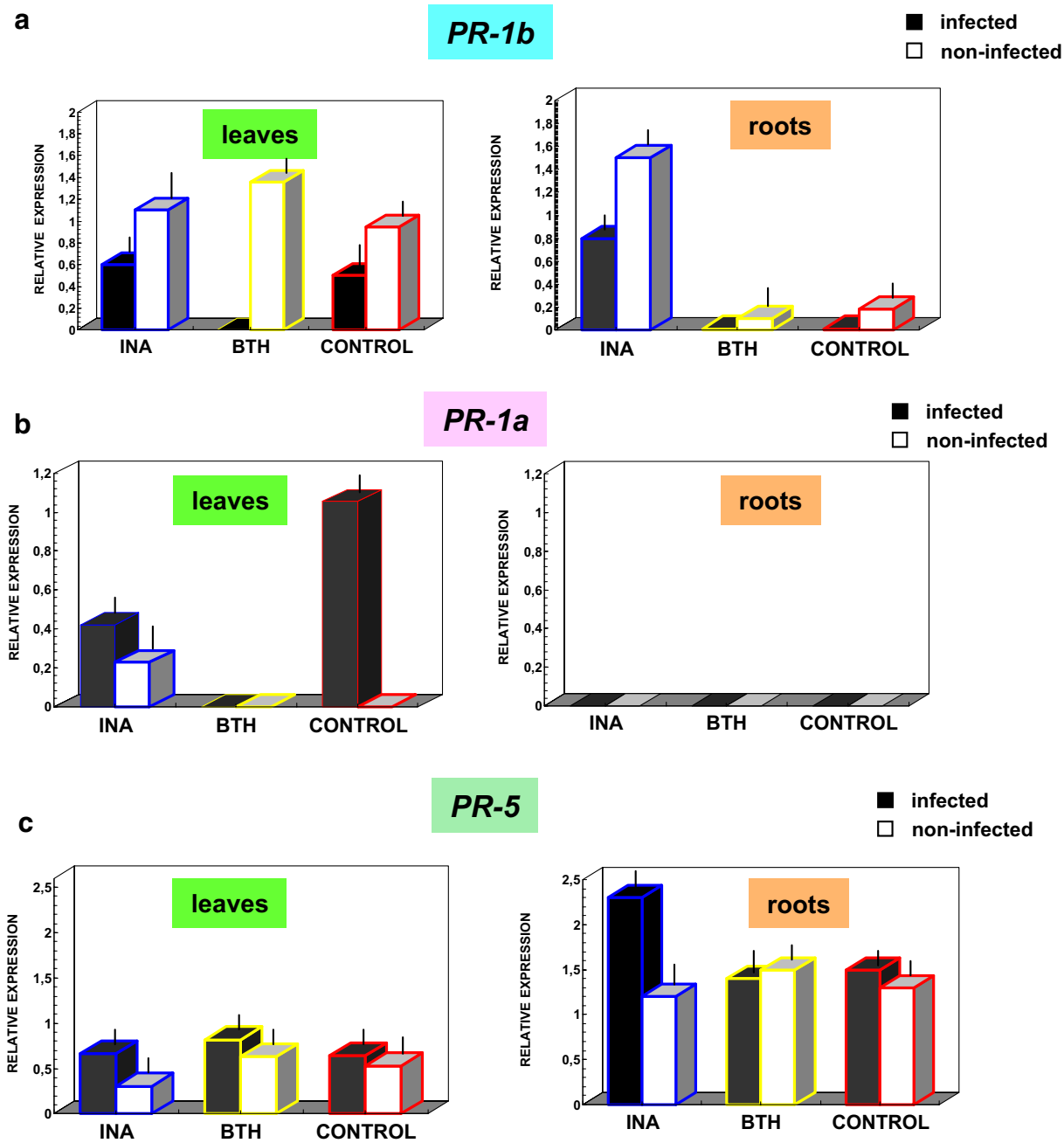

Fig. 4 Plant defence gene expression in *Lycopersicon esculentum* plants infected with *Meloidogyne javanica.* Leaves and roots (following foliar application of INA and BTH) were analyzed to determine the gene expression patterns: **a**: *PR-1b* gene; **b**: *PR-1a* gene; **c**: *PR-5* gene; **d**: *PR-2* gene; **e**: *PR-3* gene and **f**: *Calmodulin* gene. Semi-quantitative expression was obtained by RT-PCR reactions, total RNAs were extracted from three different plants per experiment. Experiments were conducted in triplicate. Bars represent the SD obtained from nine different reactions

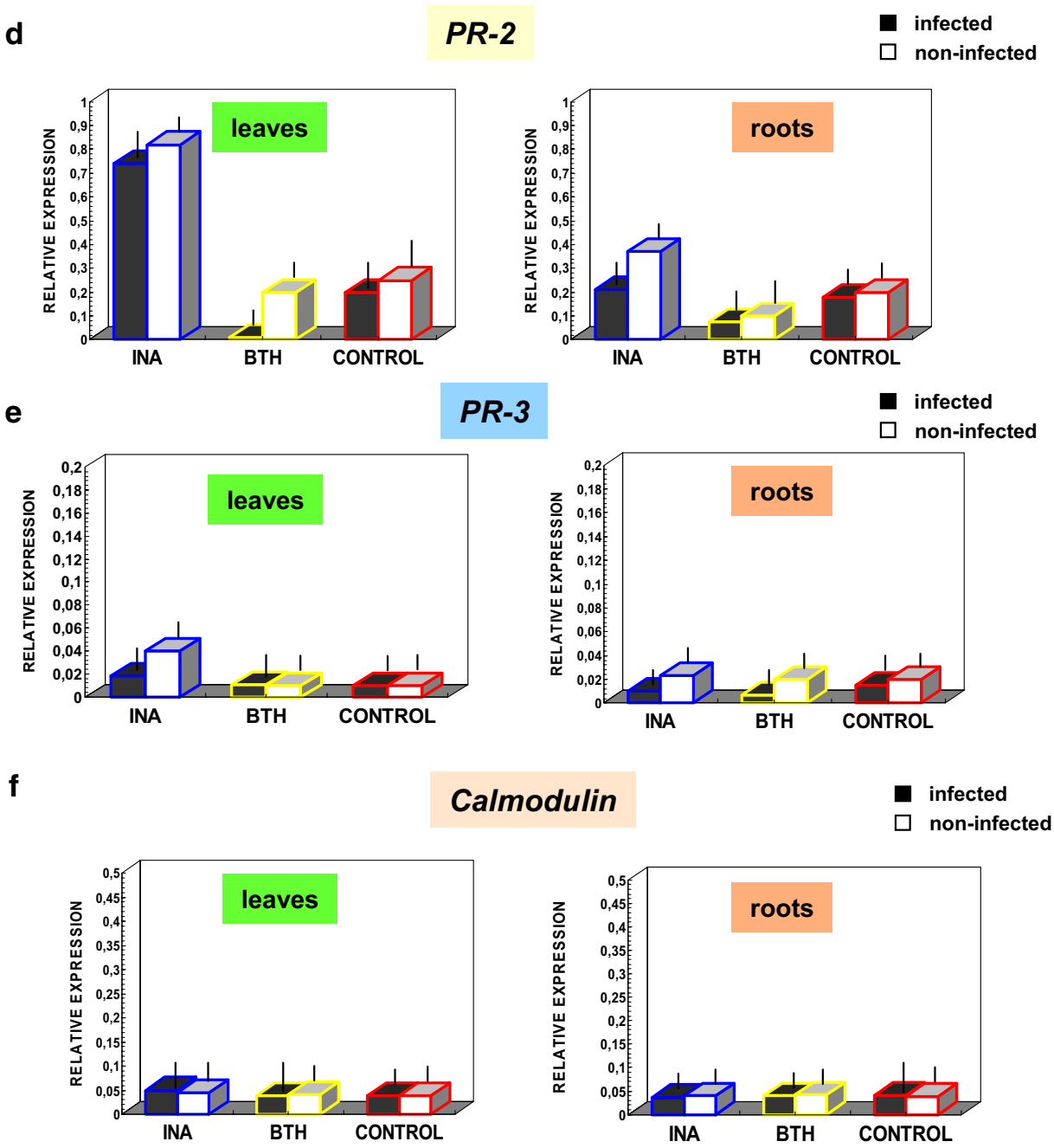

Fig. 4 (continued)

Expression of the other genes studied (calmodulin, *PR1-a* and *PR-3)* was not modified by the elicitors. The lack of induction of the *PR-1a* gene could be explained by its greater association with developmental processes than pathogen defence (Tornero et al. 1997). Similarly, calmodulin proteins have basic functions in several pathways, including those related to protein kinases and Ca^{++} binding. However, calmodulin mRNAs have been described as not being affected by the SA pathway, though the gene does contribute to the perception of pathogens and, at some point, to the signal transduction pathway (Van der

Luit et al. 1999; Yang and Poovaiah 2002). The *PR-3* gene has been described as JA-responsive, and therefore SA-independent. It might be possible that the nematode infection modified only the SA pathway, although some cross-talk between JA and SA pathways could be expected, and has been described in other systems (Spoel et al. 2003; Katagiri 2004).

In conclusion, we have observed that SA-derived elicitors were able to activate the expression of *PR-1* and *PR-2*, as SAR markers. However, this treatment only reduced the production of galls by 25% following *M. javanica* infection. This relatively high rate of pathogenesis was correlated with the silencing of PR genes induced by INA and BTH. Taken together, these data suggest that the application of SAR inducers would not be very effective at controlling root-knot nematode infestations in tomato crops.

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