Phytopathol. Mediterr. (2002) 41, 39–44

Salicylic acid enhances resistance in cowpea against *Meloidogyne incognita*

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Summary. Salicylic acid (10 mM) sprayed on cowpea leaves inoculated with *Meloidogyne incognita* reduced nematode infection and promoted plant growth. Salicylic acid did not kill nematodes in an *in vitro* test and induced expression and accumulation of pathogenesis related-1 protein in the leaves of sprayed plants. The presence of Tween-20 enhanced the effect of salicylic acid on the accumulation of pathogenesis related-1 protein.

Key words: root-knot nematode, systemic acquired resistance, *Vigna catjang*.

Introduction

Plants have a number of defense mechanisms against pathogens. In addition to constitutive resistance, plants can activate protective mechanisms upon infection with widely different pathogens. This is termed induced or acquired resistance. Resistance could be expressed locally but also systemically in tissue remotely located from the initial treatment (systemic acquired resistance, SAR) (Ross, 1961). This form of resistance protects plants from a broad spectrum of pathogens and works systemically in many cases (Klessig and Malamy, 1994; Schneider *et al*., 1996; Mauch-Mani and Metraux, 1998). In the resistant tissue there is the expression of a complete set of defense-related genes whose products, for example in tobacco leaves infected with tobacco mosaic virus (TMV), include five or more families of pathogenesis-related (PR) proteins usually 10 to 40 KDa in size (Carr and Klessig, 1989). The synthesis of these proteins have been detected in a wide variety of plants including, in addition to tobacco, tomato, cucumber, potato, cowpea, rice, soybean, sugarbeet, wheat, barley, maize, beans and carrot (Carr and Klessig, 1989; White *et al.,* 1989; Nasser *et al.,* 1990; Cordero *et al.,* 1992; Klessig and Malamy, 1994). The exogenous application of salicylic acid (SA) could induce expression of the same set of defense-related genes that are activated by TMV infection (Ward *et al*., 1991; Durner *et al.,* 1997). In recent literature SA has been reported as an endogenous signal for the activation of certain plant defense responses, including PR gene expression and enhanced resistance to pathogens (Greenberg *et al.,* 1994; Conrath *et al.,* 1995), but for some virus infections several authors demonstrated that neither

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PR protein nor exogenous SA induced resistance (Pennazio *et al*., 1983; Roggero and Pennazio, 1988, 1991). The expression of PR genes and the induction of SAR can, however, occur even without SA accumulation by means of a synthetic compound such as 2, 6-dichloroisonicotinic acid (Vernooij *et al*., 1995). In plant-nematode interactions, newly synthesized proteins including PR proteins have been found in potato plants infected with the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* (Hammond-Kosack *et al.,* 1989; Rahimi *et al.,* 1993, Rahimi *et al.,* 1996), but studies are rather limited. We decided therefore to investigate the effect of exogenous SA both on the synthesis of PR protein and on resistance to *Meloidogyne incognita* infection of cowpea (*Vigna catjang* Walp.).

Materials and methods

Nematode and plant material

Second-stage juveniles (J2) of *Meloidogyne incognita* (Kofoid and White) Chitwood were allowed to hatch from egg masses obtained from a culture maintained on cowpea in the Department of Zoology at Santiniketan, West Bengal, India. Active J2 obtained from egg masses were kept in cavity blocks containing sterile tap water, each block containing 100±15 juveniles. Cowpea plants (cv. Pusa Ruby) susceptible to *M. incognita* were used for the experiments.

Experimental plan

Aseptically germinated seeds of cowpea cv. Pusa Ruby were sown in 32-cm-diameter pots (one seed/pot) containing an autoclaved mixture of clay soil and composted manure (2:1, v:v). The pots were divided into 7 groups of 10 pots each. The groups were: uninoculated unsprayed (UU); inoculated unsprayed (IU); inoculated and sprayed with 0.5% Tween-20 (IT); uninoculated and sprayed with 10 mM SA (USA); uninoculated and sprayed with 10 mM SA containing 0.5% Tween-20 (USAT); inoculated and sprayed with 10 mM SA (ISA); and inoculated and sprayed with 10 mM SA containing 0.5% Tween-20 (ISAT). When the plants were at the 6-leaf stage, the IU, IT, ISA and ISAT groups were inoculated with 1,400±100 freshly hatched *M. incognita* J2 in 5 ml of water. The J2 were injected into the soil at a depth of 2 cm, halfway between the plant and the side of the pot. SA was applied as a foliar spray by an atomizer 24 h before inoculation. During spraying, the soil surface underneath each plant was covered with polyethylene sheeting. Plants in the uninoculated unsprayed and inoculated unsprayed groups received a distilled water spray. Spraying was repeated after 4 days. The plants were regularly watered and the experiment was conducted outdoors at ambient atmospheric temperature (28±3°C) and humidity (75±4%). To assess the effect of SA on *M. incognita,* the sterile tap water in the cavity blocks (see Nematode and plant material section) where active *M. incognita* J2 were kept, was pipetted out and immediately replaced by 1 ml of 10 mM SA. Two cavity blocks containing only sterile tap water served as negative controls. Mortality of the nematodes at room temperature (27±2°C) was recorded every hour for 6 hrs.

At 45 days after inoculation, shoot length, shoot weight, root length, root weight, root nodule number, root gall number and number of eggs per g of root were recorded. Nematode eggs were extracted from the roots with the sodium hypochlorite method (Hussey and Barker, 1973). Three samples of root pieces were taken at random from each plant group and the total protein fraction in each sample was determined by the Folin-phenol method (Lowry *et al.,* 1951). The experiment was repeated twice with similar results. The data from the first experiment are here presented. Data were analyzed by ANOVA and the means were separated by the least significant difference (LSD) test (P=0.05).

PR proteins extraction

PR proteins were extracted from the leaves of the various groups 2 days after the second spraying. Three samples of fresh leaves per group of plants were taken at random, mixed and chopped into pieces. Two g of these fresh leaves from each group were homogenized in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 12 mM 2-mercaptoethanol/phenylmethylsulfonyl fluoride (10 μg ml^{-1}). After clarification by centrifugation at 12,000 *g* (20 min, 4°C), the supernatant was fractionated by 12% SDS/PAGE and the separated proteins were stained with Coomassie blue following the method of Laemmli (Laemmli, 1970). For western blot analysis the homogenate was fractionated by 12% SDS-PAGE, and the separated proteins were electropho-

retically transferred to a nitrocellulose filter. Immunoblot analysis was performed using mouse monoclonal antibody MAb 2-11G5 against tobacco acidic PR-1 protein at a dilution of 1:1000 (Conrath *et al.,* 1995).

Results

No mortality of nematodes was recorded at 10 mM SA.

The sprayed plants did not show any toxic effect, in the form of wilting or yellowing of leaves, in the next 30 days after the second spraying.

The pot test indicated that: (i) SA increased growth of inoculated plants in terms of shoot length, shoot weight and root length as compared with inoculated unsprayed plants; (ii) root gall number and number of eggs in roots were significantly greater in inoculated untreated plants than in inoculated treated plants; (iii) root protein content increased significantly in all SA-treated groups compared with unsprayed inoculated plants; (iv) SA containing Tween did not differ from SA alone in its effect on the bio-mass and root gall number of the cowpea plants (Table 1). SA induced expression of PR-1 protein (16 KDa) in both uninoculated and inoculated SA-sprayed cowpea leaves (Fig. 1 and 2). The presence of Tween-20, which may increase the uptake of SA into the leaves, enhanced the effect of SA on the expression and accumulation of PR-1 protein (Fig. 1). Tween-20 alone sprayed on the leaf surface did not induce expression of PR-1 protein in cowpea leaves. No PR-1 protein accumulated in unsprayed plants, whether inoculated or uninoculated (Fig. 1 and 2).

Discussion

SA is known to be an endogenous regulator of localized and systemic acquired resistance. When some plants become infected with disease organisms, SA accumulates in them, and the exogenous application of SA in these plants without infection could induce expression of the same set of defense-related genes as when they were infected (Klessig and Malamy, 1994). Some of the genes activated during the induction of SAR by exogenous SA encode a group of PR proteins (Ryals *et al.,* 1994). The antimicrobial and enzyme activities of these gene products suggest that they may play a role in the induction and maintenance of SAR in plants (Ohashi and Ohshima, 1992). However, the function of the PR-1 protein has not yet been clearly understood. Changes in the gene expression of potato leaves after root infection with the cyst nematode *Globodera rostochiensis* induced the expres-

Table 1. Increase in cowpea growth, decrease in *Meloidogyne incognita* infestation of cowpea and root protein content following treatment by exogenous salicylic acid at a 10 mM concentration.

Treatment[a]	Cowpea plants growth				*Meloidogyne incognita* infestation			Root protein content
	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Root nodule No.	Root gall No.	Eggs g^{-1} root	(mg g^{-1})
UU	264.6±6.5 a[b]	229.3±5.6 a	43 ±3.9 a	15 ±1.4 f	313 ±12.2 a	_	_	7.5±0.67 a
IU	182.6±5 de	165 ±4.2 cd	28.6±2.4 c	31 ±5 a	166 ±9.8 g	745 ±18.7 a	8400±348.2 a	4.2±0.65 bc
IT	175.4±4.9 e	157.5±5.3 d	31 ±1.8 bc	29.3±3.1 a	167.3±7.5 fg	738 ±29.4 a	7900±141.4 a	4 ±0.50 c
USA	255 ±5.3 a	225 ±6.8 ab	37 ±3.2 ab	22.3±2.2 bcdef	251 ±7.7 e	_	_	6.2±0.57 a
USAT	261.5±6 a	218 ±5.3 ab	41.6±3.1 a	20 ±2.8 def	274.6±8.8 cd	_	_	6.6±0.68 a
ISA	242 ±3.7 bc	215 ±3.7 ab	36 ±5.3 abc	21.6±3.5 cdef	265 ±6.9 d	423.2±17.7 bc	3800±277.8 bc	6.4±0.43 a
ISAT	235.6±7.4 c	209 ±6 b	40 ±6.1 a	17 ±3.5 ef	285 ±6.4 bc	410 ±28.8 c	3500±179.4 c	7.1±0.56 a

[a] Cowpea seedlings with six compound leaves were sprayed with salicylic acid at a 10 mM concentration. Each plant was inoculated with 1400±100 second-stage juveniles of *M. incognita* 24 h after the first treatment. Data are means of ten replicates. UU, uninoculated untreated; IU, inoculated untreated; IT, inoculated and treated with Tween; USA, uninoculated and treated with salicylic acid; USAT, uninoculated and treated with salicylic acid containing Tween; ISA, inoculated and treated with salicylic acid; ISAT, inoculated and treated with salicylic acid containing Tween.

[b] Different small letters in a column indicate significant differences according to the least significant difference test (P=0.05).

-, no root galls or eggs in this group.

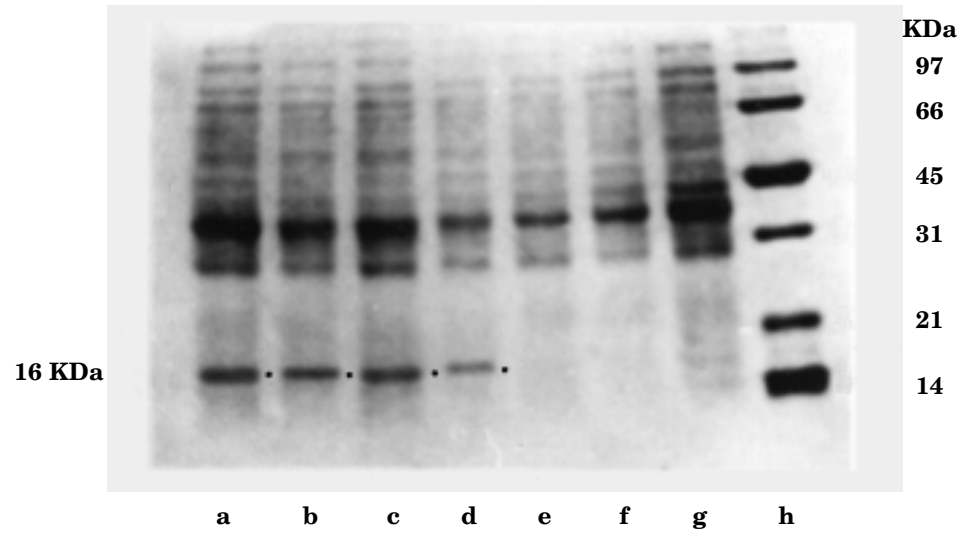

Fig. 1. SDS-PAGE of proteins extracted from cowpea plants. Acidic PR proteins were extracted from leaves on day 2 after the 2nd treatment. Separated proteins were stained with Coomassie Blue. Lane a, inoculated and treated with salicylic acid containing Tween; lane b, inoculated and treated with salicylic acid; lane c, uninoculated and treated with salicylic acid containing Tween; lane d, uninoculated and treated with salicylic acid; lane e, inoculated and treated with Tween; lane f, inoculated untreated; lane g, uninoculated untreated; lane h, molecular weight marker. The induced band is indicated with a dot to the right of the respective lanes.

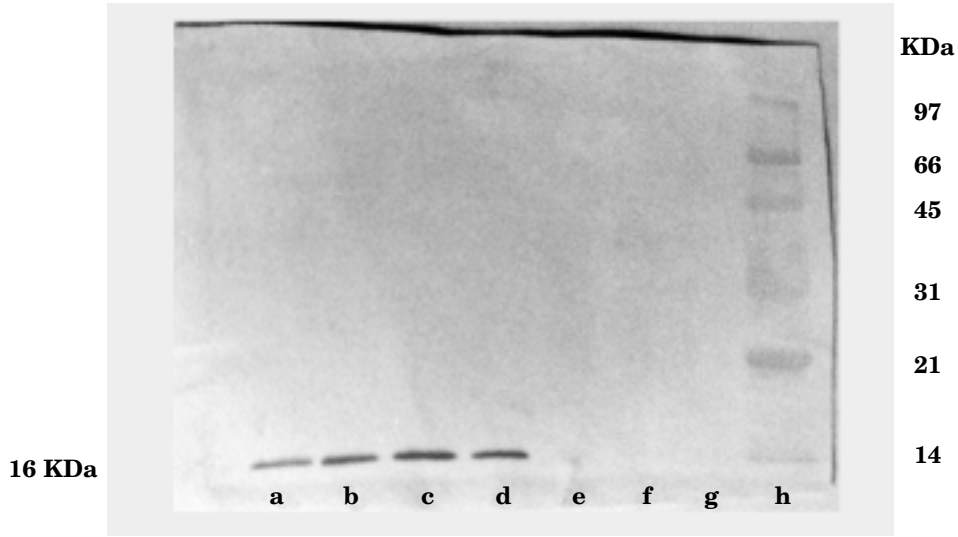

Fig. 2. Immunoblot analysis of proteins extracted from cowpea plants. Acidic PR proteins were extracted from leaves on day 2 after the 2nd treatment. Proteins were fractionated by SDS-PAGE, and immunoblotted with mouse monoclonal antibody MAb 2-11G5 against tobacco acidic PR-1 protein. lane a, uninoculated and treated with salicylic acid; lane b, uninoculated and treated with salicylic acid containing Tween; lane c, inoculated and treated with salicylic acid containing Tween; lane d, inoculated and treated with salicylic acid; lane e, inoculated and treated with Tween; lane f, inoculated untreated; lane g, uninoculated untreated; lane h, molecular weight marker.

sion of PR proteins (Hammond-Kosack *et al.,* 1989), while infection of potato roots with *Globodera pallida* induced the expression of proteins with a molecular weight of 70–82 KDa, larger than that of common PR proteins (Rahimi *et al.,* 1993). In tomato root infected with root-knot nematodes, several homologous plant-defense genes (including peroxidases, chitinase, lipoxygenase and proteinase inhibitor protein) are expressed locally within 24 h of inoculation (Lambert, 1995; Williamson and Hussey, 1996). In our experiments, spraying with SA, which induced the accumulation of PR-1 protein in the sprayed leaves, significantly increased resistance to root-knot nematode infection, reflected in a lack of nematode reproduction. It appeared that SA-spraying did not have any direct influence on plant growth but improved growth in the sprayed plants, perhaps as a result of the reduction in root-knot disease it broughtabout. The mechanisms governing SA-induced resistance to *M. incognita* in cowpea are under further investigation.

Acknowledgements

The work described here is part of a project funded by the Council of Scientific and Industrial Research, New Delhi to SPSB. We thank Prof. Daniel F. Klessig, Waksman Institute and Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, USA, for providing us with mouse monoclonal antibody MAb 2-11G5.

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Accepted for publication: February 4, 2002