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Effects of Acibenzolar-S-Methyl Application to *Rotylenchulus reniformis* and *Meloidogyne javanica*[1]

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Abstract: Effects of acibenzolar-s-methyl, an inducer of systemic acquired resistance in plants, on *Rotylenchulus reniformis* and *Meloidogyne javanica* in vitro and in vivo were determined. A single foliar application of acibenzolar at 50 mg/liter (5 ml of solution per plant) to 7-day-old cowpea or soybean seedlings decreased *R. reniformis* and *M. javanica* egg production by 50% 30 days after inoculation. The mechanism of acibenzolar on plant-parasitic nematodes was then investigated. Acibenzolar at 50 to 200 mg/liter did not affect movement of *R. reniformis* and *M. javanica* or penetration of second-stage juveniles (J2) of *M. javanica* on cowpea. However, *M. javanica* development was slowed and fecundity was reduced in plants treated with acibenzolar. On average, 50% of J2 that penetrated acibenzolar-treated cowpeas developed into mature females with eggs, whereas the other 50% exhibited arrested development. The number of eggs per egg mass was 450 in water-treated cowpeas, whereas the number declined to 250 in acibenzolar-treated plants. Acibenzolar may be responsible for stimulating the plants to express some resistance to the nematodes.

Key words: acibenzolar-s-methyl, cowpea, *Meloidogyne javanica,* reniform nematode, root-knot nematode, *Rotylenchulus reniformis,* soybean, systemic acquired resistance.

Systemic acquired resistance (SAR) is a natural inducible plant defense response that is expressed throughout the plant in response to pathogen infection (Ryals et al., 1996). The effectiveness of SAR is long lasting and broad spectrum. It is mediated by salicylic acid (SA) and associated with the activation of pathogenesis-related (PR) genes (Van Loon and Van Strien, 1999). In tobacco, SAR induction reduced disease symptoms of fungal, viral, and bacterial diseases (Vernooij et al., 1995). In *Arabidopsis,* SAR induction following infection with *Turnip crinkle virus* protected the plant from *Pseudomonas syringae* pv. *tomato* DC 3000 and *Turnip crinkle virus* (Uknes et al., 1993).

SAR also can be induced artificially by chemicals. Salicylic acid (Ward et al., 1991; White, 1979), 2,6-dichloroisonicotinic acid (INA) (Métraux et al., 1991; Vernooij et al., 1995), and acibenzolar-s-methyl (formerly called benzothiadiazole or BTH) are three such compounds. Acibenzolar activates resistance against diseases in wheat (Görlach et al., 1996), tobacco (Friedrich et al., 1996), *Arabidopsis* (Lawton et al., 1996), maize (Morris et al., 1998), tomato (Benhamou and Bélanger, 1998a), and cucumber (Benhamou and Bélanger, 1998b). Acibenzolar is currently registered as a control of downy mildew of leafy vegetables, bacterial leaf spots of tomatoes, and blue mold of tobacco (Anonymous, 2001).

Whereas no product is currently labeled for activity against nematodes, SAR has been induced during plant-parasitic nematode infection. Kempster et al. (2001) reported induction of resistance in *Trifolium repens* to *Heterodera trifolii* by acibenzolar and, when applied as a 50-µM root drench, reduced *H. trifolii* fecundity and increased the number of distorted females. Owen et al. (2002) demonstrated that single or multiple applications of acibenzolar (Bion 50WG, Novartis, Greensboro, NC) at 50 mg a.i./liter decreased egg production by 40% to 80% in a mixed population of *Meloidogyne javanica* and *M. arenaria* in grapevines 10 weeks after inoculation. In a second series of experiments, they also showed that acibenzolar neither inhibited penetration of the nematodes into grapevine roots nor affected egg viability or hatch after 24-hour exposure. In this series of experiments, our objective was to determine the effect of acibenzolar on mobility, penetration, development, and reproduction of *R. reniformis* and *M. javanica* in cowpea and soybean.

Materials and Methods

Nematodes: Nematode cultures were maintained on plants in a greenhouse. *Rotylenchulus reniformis* were propagated on 'California Black Eye' cowpea. Mixed vermiform stages of the nematodes obtained from soil extraction by the combined screening-funnel technique (Ayoub, 1980) were used within 1 day of extraction. Egg masses of *M. javanica* cultured on 'Orange Pixie' tomato were handpicked and placed in a Syracuse dish containing distilled sterile water. Second-stage juveniles (J2) that emerged within 24 hours were used as inoculum for experiments.

Reproduction: One 5-day-old 'California Black Eye' or 'Lee' soybean seedling was transplanted into an 11-cm-diam. clay pot filled with autoclaved sandy loam soil. Acibenzolar-s-methyl (1,2,3-benzothiadiazole-7-carbothioic acid S-methyl ester), provided under the commercial name Actigard 50 WC by Syngenta, was prepared as an aqueous solution. The cowpea or soybean seedlings were treated with a foliar spray of 0, 50, 100, or 200 mg acibenzolar/liter 2 days after transplanting when the seedlings had two true leaves. To prevent

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runoff of acibenzolar into the soil, soil surfaces were covered with plastic sheets while the foliar spray was applied. Each plant received 5 ml of solution. Two days after acibenzolar application, 2,000 mixed vermiform stages of *R. reniformis* were inoculated onto each cowpea seedling and the equivalent amount of *M. javanica* J2 applied to soybean seedlings. Inoculum was pipeted near the roots in 1-ml aliquots. Plants were placed on a greenhouse bench in a randomized complete block design of five replicates per treatment. Plants were assessed for acibenzolar phytotoxicity, indicated by discoloration and deformation of leaves, throughout the experiment. Plants were watered daily and fertilized weekly with 5 ml of full-strength N-P-K (Plant Growth)/3.78 liters of water. Thirty days after inoculation, the extent of *R. reniformis* infestation in cowpea was determined by shaking the roots in NaOCl (Byrd et al., 1972) and extracting vermiform stages from 250 cm^3 soil (Byrd et al., 1976; Jenkins, 1964). In soybean, *M. javanica* eggs were collected using a NaOCl extraction technique (Byrd et al., 1972). Fresh shoot and root weights were determined from cowpea and soybean. Dry shoot and root weights were recorded after 72 hours at 50 °C. The experiment was repeated once.

Mobility: Twenty vermiform stages of *R. reniformis* or 20 24-hour-old J2 of *M. javanica* were placed in 2-cm-diam. watch glasses filled with 240 µl of 0, 50, 100, or 200 mg acibenzolar/liter. Seven replicate watch glasses of each concentration were placed in a 90-cm-diam. petri dish, covered to limit solution evaporation, and maintained at 25 °C. Nematode mobility was determined under a stereomicroscope at 24, 72, and 120 hours after exposure. Mobility was determined by noting nematode response after prodding with a dental pick. The experiment was arranged in a randomized design of four treatments, each replicated seven times. The experiment was repeated once.

Penetration: One 5-day-old cowpea seedling was transplanted into a 5-cm-diam. plastic cup containing sandy soil. Two days later, 5-ml foliar applications of 0, 50, 100, or 200 mg acibenzolar/liter were made to the transplants. Two hundred *M. javanica* J2 in 1 ml of distilled water were inoculated onto the roots of the cowpea plant 2 days after the acibenzolar application. Cowpea seedlings were maintained in a greenhouse for 2 days. Seedlings were uprooted and washed carefully in water. Roots were cleared in 1.5% NaOCl, stained with acid fuchsin (Byrd et al., 1983), and the number of J2 in the roots counted. The experiment was arranged in a randomized design with five replications and repeated once.

Development: The method was followed as in the penetration experiments but after allowing 2 days for nematode penetration. Cowpea seedlings were gently uprooted, rinsed in water, and replanted into 11-cm-diam. clay pots filled with sterilized sandy loam soil. All plants were maintained in a greenhouse. Thirty days after J2 inoculation, the number of life stages in the roots was determined by staining roots with acid fuchsin in lactoglycerol (Hooper, 1986) and observing nematodes under a stereomicroscope. Life stages of *M. javanica* were separated into two groups: immature stages (vermiform, sausage-shaped juveniles and females without egg masses) and mature females with egg mass attached. The experiment was arranged in a randomized complete block design with five replications and repeated once.

Fecundity: The procedure was similar to the development study. At 30 days post inoculation, the number of eggs per egg mass was determined by randomly picking 10 egg masses per root system into 2-cm-diam. watch glasses filled with 1% NaOCl. The number of eggs in each egg mass was counted using a stereomicroscope. The experiment was arranged in a randomized complete block design with five replicates and repeated once.

Data analysis: Data from the two repeats of each test were tested for homogeneity of variance; if there was no difference, the data were pooled. The combined or individual data from each test were analyzed for variance. When appropriate, means were separated using Duncan's multiple-range test (DMRT).

Results

Because variance between repeats of each study was homogeneous, data were combined and analyzed together.

Reproduction: Fresh and dry weights of roots and shoots of cowpeas did not differ among treatments, and the reproduction of *R. reniformis* was lower on cowpeas treated with 50, 100, or 200 mg acibenzolar/liter compared with the plants receiving no acibenzolar (Table 1). Acibenzolar treatment at 50 and 100 mg/liter decreased the number of eggs per root system and the number of vermiform stages by 44% and 60%, respectively, as compared to 0 mg/liter acibenzolar. An increase of acibenzolar concentration to 200 mg/liter, however, did not enhance the effectiveness of the chemical. In the *M. javanica* and soybean system, fresh

Table 1. Effect of acibenzolar-s-methyl on cowpea plants in the greenhouse and on the fecundity of *Rotylenchulus reniformis* 30 days after inoculation.

Acibenzolar treatment (mg/liter)	Root weight (g)		Shoot weight (g)		*R. reniformis*[a]
	Fresh	Dry	Fresh	Dry	
0	8.5 ± 0.89	0.4 ± 0.04	26.1 ± 2.84	6.5 ± 0.83	34,840 a
50	8.4 ± 0.71	0.5 ± 0.03	24.7 ± 5.36	5.7 ± 0.71	19,440 b
100	7.5 ± 1.19	0.5 ± 0.06	25.7 ± 2.56	5.8 ± 0.92	14,100 c
200	7.7 ± 0.25	0.5 ± 0.04	26.0 ± 0.74	6.0 ± 0.12	14,280 c

Data are means of two tests pooled. Means within a column followed by the same letter are not different ($P \geq 0.05$) according to Duncan's multiple-range test.

[a] No. of eggs/root system + No. of juveniles in 250 cm^3 soil.

and dry weights of roots and shoots of soybean also were not different among treatments, and acibenzolar treatment at 50, 100, and 200 mg/liter decreased egg production of *M. javanica* as compared to no treatment (Table 2). However, in contrast to *R. reniformis,* application of acibenzolar at 50 mg/liter lowered the number of eggs per root system of *M. javanica.* In addition, acibenzolar did not show greater effectiveness at concentrations greater than 50 mg/liter. In both the cowpea and soybean tests, acibenzolar as high as 200 mg/liter did not cause phytotoxicity to the plants. Dry roots and shoot weights of cowpea plants were 0.4 to 0.5 and 5.7 to 6.5 g, respectively, and did not differ among treatments ($P \geq 0.05$). Similarly, dry root and shoot weights of soybean plants did not differ between 0 mg/liter and 200 mg/liter and ranged between 0.4 to 0.5 and 1.4 to 1.7 g, respectively.

Mobility: Acibenzolar as high as 200 mg/liter did not adversely affect vermiform stages of *R. reniformis* or J2 of *M. javanica* in vitro (Table 3). The number of immobile *R. reniformis* and J2 of *M. javanica* did not differ between the control and any concentration of acibenzolar. The mobility of *R. reniformis* was reduced 20% within 1 day in the water and acibenzolar solutions. In 5 days, 60% of *R. reniformis* vermiforms were immobile. *Meloidogyne javanica* was much more active, with only 8% of the nematodes immobile after 5 days in all treatments.

Penetration: Acibenzolar did not affect *M. javanica* penetration. The percent penetration averaged 10% over all treatments (Table 3). The number of J2 in the roots treated with 50 to 200 mg acibenzolar/liter ranged from 18 to 24 J2/plant, as compared with 20 J2/plant with 0 mg acibenzolar/liter.

Development: Treatment of cowpea with acibenzolar delayed the development of *M. javanica.* The percentage of immature stages of *M. javanica* in roots treated with 50 to 200 mg acibenzolar/liter 30 days after inoculation was higher than plants receiving 0 mg acibenzolar/liter (Table 4). On average, 50% of J2 that penetrated 50 mg or greater acibenzolar/liter-treated plants developed into mature females with eggs, whereas the remaining nematodes exhibited arrested development. In contrast, 78% of the nematodes in 0 mg acibenzolar/liter treatment were mature females with egg masses. There was no additional delay in development at acibenzolar concentrations greater than 50 mg/liter.

Fecundity: Acibenzolar treatment reduced fecundity of *M. javanica* by an average of 40% (Table 4). In the 0 mg acibenzolar/liter treatment, females deposited an average of 450 eggs/egg mass. However, at 50 mg/liter acibenzolar, the number of eggs per egg mass was 330—a 26% decrease. At 100 mg/liter, females on acibenzolar-treated plants produced 250 eggs/egg mass—a 46% reduction. The 200-mg acibenzolar treatment had a similar 46% decrease in eggs per egg mass. Females exposed to acibenzolar typically produced greater quantities of gelatinous matrix than did unexposed females.

TABLE 2. Effect of acibenzolar-s-methyl on soybean plants in the greenhouse and on the fecundity of *Meloidogyne javanica* 30 days after inoculation.

Acibenzolar treatment (mg/liter)	Root weight (g)		Shoot weight (g)		*M. javanica*[a]
	Fresh	Dry	Fresh	Dry	
0	11.3 ± 0.83	0.5 ± 0.05	8.2 ± 0.64	1.6 ± 0.15	40,160 a
50	10.4 ± 0.26	0.5 ± 0.04	7.8 ± 0.40	1.7 ± 0.10	19,660 b
100	9.9 ± 0.52	0.4 ± 0.02	7.5 ± 0.41	1.4 ± 0.07	20,400 b
200	11.5 ± 0.36	0.5 ± 0.07	7.7 ± 0.21	1.6 ± 0.08	18,640 b

Data are means of two tests pooled. Means within a column followed by the same letter are not different ($P \geq 0.05$) according to Duncan's multiple-range test.

[a] No. of eggs/root system.

DISCUSSION

A single foliar application of acibenzolar to cowpea or soybean plants reduced reproduction of *R. reniformis* and *M. javanica.* Acibenzolar treatments delayed the development and reduced fecundity of *M. javanica,* which would account for reduced reproduction. The results also indicated that acibenzolar was not directly toxic to either nematode species. The effect of the application was successfully translocated to the roots using a foliar spray, suggesting an SAR effect. The results of this experiment were consistent with previous acibenzolar studies with nematodes (Kempster et al., 2001; Owen et al., 2002).

SAR activity against plant-parasitic nematodes is suggested by other studies. Ibrahim and Lewis (1986) demonstrated that an advance inoculation of *M. incognita* onto soybean enhanced resistance to *M. arenaria,* as demonstrated by decreased root gall and egg mass production. Eisenback and Griffin (1987) showed that resistance of tobacco NC95 to *M. incognita* was altered following the prior infection of the plant with *M. arenaria* or *M. hapla.* However, this phenomenon was not observed when *M. javanica* or *M. incognita* race 4 was used as the advance inoculum. Ogallo and McClure (1995) found that the prior inoculation of naturally incompatible nematode species, *M. incognita* or *M. javanica,* to tomato 'Celebrity' and pyrethrum clone 223 induced resistance to a compatible nematode species, *M. hapla,* in pot and field trials. The ratio of final populations to initial populations (Pf/Pi) of *M. hapla* decreased 84% on potted tomato, 72% on potted pyrethrum, and 55% on field-grown pyrethrum. With results from a split-root assay, Ogallo and McClure (1996) postulated that the mechanism may be the result of biochemical substances that were elicited in roots infected by incompatible nematodes and then expressed in uninfected roots, suggestive of SAR.

The application of other synthetic chemicals to induce SAR to plant-parasitic nematodes also has been

TABLE 3. Percentage immobility of vermiform stages of *Rotylenchulus reniformis* and second-stage juveniles (J2) of *Meloidogyne javanica* in various concentrations of acibenzolar-s-methyl. The number of J2 of *M. javanica* penetrating cowpea seedlings treated with the acibenzolar.

Acibenzolar (mg/liter)	*R. reniformis* Immobility			*M. javanica* Immobility			*M. javanica*
	1 day	3 days	5 days	1 day	3 days	5 days	Penetration[a]
0	23.7 ± 6.4	51.7 ± 4.6	57.6 ± 3.9	2.9 ± 1.0	3.6 ± 0.9	7.9 ± 1.0	20 ± 2
50	18.1 ± 6.4	52.9 ± 5.3	59.6 ± 6.7	2.1 ± 1.0	2.9 ± 1.0	8.6 ± 0.9	18 ± 1
100	23.6 ± 3.6	56.5 ± 1.4	58.9 ± 4.4	3.6 ± 1.4	4.3 ± 1.3	8.6 ± 0.9	22 ± 1
200	20.1 ± 4.2	55.1 ± 5.6	56.5 ± 3.8	3.6 ± 0.9	3.6 ± 1.4	6.4 ± 1.4	24 ± 2

Data are means of two tests pooled. Means in all columns are not different among treatments ($P \geq 0.05$).
[a] Penetration data are from a separate experiment.

demonstrated. Oka et al. (1999) demonstrated that foliar spray and soil drench of tomato with DL-β-amino-*n*-butyric acid (BABA) reduced the number of *M. javanica* eggs 30 days after inoculation. Fewer J2 and lower gall indices were found on tomato treated with 20 or 40 mM BABA. The protection of tomato by single application of BABA lasted at least 5 days after application, and postinfection spray also delayed nematode development.

The key element involving SAR induction against plant-parasitic nematodes may be the activation of plant genes that encode proteins or enzymes capable of interfering with nematode development and growth. Currently, 14 families of PR proteins have been described in different plants, some of which—such as protease inhibitors (PR-6)—may exert their inhibitory effects on nematodes (Van Loon and Van Strien, 1999). Protease inhibitors are important elements of natural plant defense and are induced as a result of damage, pathogen infection, and abiotic stress (Ryan, 1990). Protease inhibitors may reduce the proteolytic activity of the digestive protease of nematodes, thereby limiting the availability of amino acids essential to nematode growth and development. In a study with pineapple, protease inhibitor concentration was higher in pineapple inoculated with *R. reniformis* as compared to pineapple not infected with the nematode (Kelly et al., 2002). Whether or not acibenzolar exerts its effect by such a mechanism remains to be determined.

TABLE 4. Percentage of *Meloidogyne javanica* immature stages and mature females and number of eggs per egg mass in cowpea roots treated with different concentrations of acibenzolar-s-methyl 30 days after inoculation. Data of nematode development and fecundity are from two separate experiments.

Acibenzolar (mg/liter)	Immature stages[a]	Mature females with egg masses	Eggs/ egg mass
0	22 a	78 a	450 a
50	54 b	46 b	330 b
100	46 b	54 b	250 c
200	57 b	43 b	260 c

Data are means of two tests pooled. Means within a column followed by the same letter are not different ($P \geq 0.01$) according to Duncan's multiple-range test.
[a] Immature stages are vermiform and sausage-shaped juveniles and females without egg masses.

Chemical induction of SAR by acibenzolar against plant-parasitic nematodes in perennial crops may be a viable control, but in annual crops acibenzolar application may not be effective due to the inability of this compound to hinder nematode invasion or to cease nematode development in the plants. In addition, 50% reduction in the nematode reproduction by acibenzolar is not sufficient because most annual crops have about a 3-month growing season. This timing would allow nematodes to complete multiple life cycles. Nematode infection in the beginning and the continual feeding of nematodes during multiple life cycles make severe plant damage likely. However, SAR activation may be applicable for use with perennial crops that are exposed to persistent nematode invasion. In perennial crops, yield losses are dependent on long-term control of the nematode population. If nematode reproduction in a perennial crop treated with acibenzolar is reduced by 50% in the first generation, the initial nematode population of the second generation will be 50% lower than that of untreated plants. Thus, subsequent nematode generations and final population densities in perennial crops treated with acibenzolar are expected to be lower, theoretically reducing damage caused by the nematodes.

For acibenzolar to be an effective management tool against plant-parasitic nematodes, further investigation is needed. Questions of importance include if a single acibenzolar application sufficiently protects perennial crops or if multiple treatments are required. In addition, studies are needed to determine the fate of immature stages of nematodes in acibenzolar-treated plants and whether nematodes simply die or their development is delayed.

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