Effects of Inducers of Systemic Acquired Resistance on Reproduction of Meloidogyne javanica and Rotylenchulus reniformis in Pineapple

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Abstract: The potency of the inducers of systemic acquired resistance (SAR), acibenzolar-s-methyl, DL-α-amino-n-butyric acid (AABA), DL-β-amino-n-butyric acid (BABA), γ -amino-n-butyric acid (GABA), p-aminobenzoic acid (PABA), riboflavin, and salicylic acid (SA), in reducing reproduction of Meloidogyne javanica and Rotylenchulus reniformis in pineapple was investigated. All inducers were applied as foliar sprays to 1-mon-old pineapple plants (20 ml/plant) grown in 22-cm-diam. pots in the greenhouse. Two days after application, 10,000 eggs of M. javanica or R. reniformis were inoculated onto the plants. Six months after inoculation, nematode reproduction was measured. Acibenzolar decreased R. reniformis egg production by 58% compared to the nontreated control ($P \le 0.05$). Acibenzolar, BABA, and riboflavin reduced M. javanica egg production by 60% to 64% compared to the nontreated control ($P \le 0.05$). The point in the pineapple SAR pathway that each compound activates may explain the differing results between M. javanica and its giant cells and R. reniformis and its syncytia. Foliar application of acibenzolar at 100 and 200 mg/liter decreased by 30% and 60%, respectively, the number of M. javanica eggs as compared to the nontreated control. Fresh shoot weight of pineapple treated with 50, 100, 200, and 400 mg/liter acibenzolar was reduced by 1.2%, 3.3%, 9.9%, and 33% compared to the nontreated pineapple, respectively ($P \le 0.05$). Foliar application of acibenzolar may activate intrinsic resistance of pineapple to M. javanica and R. reniformis and may have a role in the sustainable management of nematodes in pineapple.

Key words: acibenzolar-s-methyl, Ananas comosus, DL-α-amino-n-butyric acid, DL- β -amino-n-butyric acid, γ -amino-n-butyric acid, Meloidogyne javanica, p-aminobenzoic acid, pineapple, reniform nematode, riboflavin, root-knot nematode, Rotylenchulus reniformis, salicylic acid, systemic acquired resistance.

Systemic acquired resistance (SAR) is the ability of plants to become resistant after prior infection by pathogens, exposure to stress, or application of chemical inducers (Sticher et al., 1997). An initial recognition event leads to the production of signals translocated endogenously to plant parts that are remote from the initial site of infection. SAR has been described against fungal, bacterial, and viral pathogens in over 30 plant species belonging to both dicotyledonous and monocotyledonous plants (Sticher et al., 1997).

Evidence of a phenomenon similar to SAR also has been reported for plant-parasitic nematodes. Ibrahim and Lewis (1986) found that 'Centennial' soybean, which is normally susceptible to Meloidogyne arenaria, showed increased resistance to this nematode when the plant was pre-inoculated with M. incognita. Ogallo and McClure (1995) found that the prior inoculation of nematode species, M. incognita or M. javanica, onto tomato cultivar Celebrity and pyrethrum clone 223, that were naturally incompatible with these nematodes, induced resistance to M. hapla that was naturally compatible with these plants. Reproduction of M. hapla decreased 84% on potted tomato, 72% on potted pyrethrum, and 55% on field-grown pyrethrum. Induction of resistance was observed 10 d after the incompatible reaction was initiated. To exclude the competition of space and infection site of nematode in a single root system, Ogallo and McClure (1996) employed a splitroot assay and demonstrated that the reproduction ratios of the challenge nematode, *M. hapla*, were reduced on tomato plants that had been infected with *M. incognita* compared to those that had no prior infection. The authors postulated that the mechanism may have been the result of biochemical substances that were elicited in one side of the root system where incompatible reaction occurred and then expressed systemically.

Various natural or synthetic chemicals can induce SAR. Salicylic acid (SA) and jasmomic acid (JA) are natural compounds, and their potency in inducing SAR is documented (Schneider et al., 1996). Phosphate salts, powdered SiO₂ preparations, Si, or polyunsaturated and oxygenated fatty acids such as arachidonic, linolenic, and oleic acids were effective in inducing SAR in some solanaceous plants (Sticher et al., 1997). The product of the hrp gene (HrpZpss) from Pseudomonas syringae also induced SAR in cucumber (Smith et al., 1991). The synthetic compounds propenazole (Oryzemate) and 2,2-dichloro-3,3-dimethylcyclopropane carboxylic acid (WL 28325) were described as inducers of resistance against rice blast (Araki and Kurahashi, 1999; Yoshioka et al., 2001). DL-3-aminobutytric acid (BABA) induced SAR in tomato, potato, and tobacco to fungal diseases (Cohen, 1994; Cohen and Gisi, 1994). INA (2,6-dichloroisonicotinic acid) is also an inducer of SAR in a number of dicotyledonous and monocotyledonous plants (Métraux et al., 1991).

Acibenzolar-s-metyl (benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl), formally called benzothiadiazole (BTH), is a novel disease-control compound that induces SAR (Anonymous, 2001). Exogenous application of acibenzolar to tobacco and *Arabidopsis* leaves activated a number of SAR-related genes, leading to enhanced pathogen defense (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996). In addition, acibenzolar induced resistance in cucumber against *Pythium ultimum* and in tomato against *Fusarium oxyspo-*

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rum f. sp. radicis-lycopersici (Benhamou and Bélanger, 1998a, 1998b).

Meloidogyne javanica and Rotylenchulus reniformis are the major nematode pathogens associated with yield suppression of pineapple (Ananas comosus) in Hawaii (Rohrbach and Apt, 1986). With either nematode, overall pineapple yields are decreased because the non-regenerative pineapple root system is damaged (Rohrbach and Apt, 1986). The yield of the plant crop may be reduced but the ratoon crop may be devastated or even fail since the number, vigor, and size of pineapple suckers producing the ratoon crop depend heavily on the health of roots developed during the first crop (Rohrbach and Apt, 1986).

If the nematode numbers are not reduced to low levels, the damage they cause can result in the death of pineapple, undersized pineapple fruits, or precocious flowering (Apt and Caswell, 1988; Sipes et al., 2005). With the phase-out of methyl bromide and regulation of 1,3-dichloropropene (Schmitt and Sipes, 1998), a nontoxic, environmentally friendly alternative is required for controlling nematodes in pineapple cultivation. The objectives of this series of experiments were to: 1) evaluate several compounds, suspected of inducing SAR in pineapple, for effectiveness in controlling M. javanica and R. reniformis, 2) determine the effectiveness of different concentrations of acibenzolar in reducing reproduction of M. javanica and R. reniformis, and 3) determine the phytotoxicity of acibenzolar to pineapple.

MATERIALS AND METHODS

Nematodes: Meloidogyne javanica and R. reniformis were reared on Solanum lycopersicum 'Orange Pixie,' and Vigna unguiculata 'California Black Eye,' respectively. After 6 wk, nematode eggs were extracted using 1% NaOCl (Byrd et al., 1972).

Activity of SAR inducers: Crowns of A. comosus 'Smooth Cayenne,' weighing between 65 and 86 g, were planted in 22-cm-diam. clay pots filled with steam-sterilized sandy loam soil. When pineapple plants were 1-monold, foliar spray applications of 20 ml aqueous suspension of 2.38 mM acibenzolar-s-methyl (50% a.i.) (Actigard 50 WG: Syngenta, Greensboro, NC), 20 mM DL-α-amino-n-butyric acid (AABA) (99% a.i.), 20 mM DL-β-amino-n-butyric acid (BABA) (99% a.i.), 20 mM y-amino-n-butyric acid (GABA) (99% a.i.), 20 mM paminobenzoic acid (PABA) (99% a.i.), 10 mM salicylic acid (SA) (99% a.i.), or 0.5 mM riboflavin (99% a.i.) were made. The soil surface was covered with plastic sheet to prevent run-off. Two days after foliar application, approximately 10,000 eggs of M. javanica or R. reniformis in 10 ml of water were introduced onto the pineapple roots.

The experiment consisted of nine treatments (one nematode-noninoculated control, one nematode-

inoculated control, and seven combinations of chemical and nematode-inoculated treatments). Each treatment was replicated five times. Treatments were arranged in randomized complete blocks on a bench in the greenhouse. Each nematode constituted a separate experiment. Pineapple plants were watered daily and fertilized as needed. Each experiment was conducted twice.

Six months after inoculation, nematode reproduction and plant growth were assessed. Pineapple shoots were separated from the roots by cutting off the base of each plant and weighing. Roots were washed gently with water, cut into small pieces, and shaken individually in 1% NaOCl (Byrd et al., 1972) to collect *M. javanica* eggs. Then roots were placed in a 50°C oven for 72 hr before recording dry root weights. For *R. reniformis*-inoculated pineapple, vermiform stages of the nematode were extracted from 250 cm³ soil by elutriation and centrifugation (Jenkins, 1964; Byrd et al., 1976).

Concentrations of acibenzolar-s-methyl: Smooth Cayenne' pineapple crowns, weighing between 75 and 85 g, were sprayed with 20 ml aqueous suspension of 0, 50, 100, or 200 mg acibenzolar/liter water and planted in 22-cm-diam. clay pots containing sterilized loamy soil. Ten thousand eggs of *M. javanica* or *R. reniformis* in 10 ml aliquots were inoculated around the base of each crown. Pots were arranged in randomized blocks with five replications of each treatment. Each nematode represented a separate experiment. Pineapples were sprayed again 1 and 3 mon later with the same concentration of acibenzolar. To prevent runoff of acibenzolar into the soil, the soil surface was covered with plastic sheet while the foliar spray was applied.

Fresh shoots and dry roots of pineapple were weighed as previously described 12 mon after planting. The reproduction of *R. reniformis* was determined by shaking the roots in NaOCl (Byrd et al., 1972) and extracting vermiform stages from 250 cm³ soil (Jenkins, 1964; Byrd et al., 1976). Reproduction of *M. javanica* was measured by shaking roots in NaOCl to extract eggs (Byrd et al., 1972). Nematode eggs were counted. The experiment was conducted twice.

Phytotoxicity of acibenzolar-s-methyl: The procedure was the same as used in the previous experiment (concentrations of acibenzolar) except that the plants were not inoculated. Concentrations of acibenzolar tested were 0, 50, 100, 200, and 400 mg/liter, and the experiment was conducted twice.

Data Analysis: Data from both tests of an experiment were analyzed for homogeneity of variance. The repeat tests were found to be homogenous for each experiment, and their data were combined for further analysis. When appropriate, means were separated using Duncan's multiple-range test (DMRT) and orthogonal comparison (SAS Institute, Cary, NC). Regression

analysis was performed by Excel 2002 SP3 (Microsoft, Redmond, WA).

RESULTS

Activity of SAR inducers: In pineapple infected with *M*. javanica, application of SAR inducers lowered nematode reproduction ($P \le 0.05$) (Fig. 1A). The SAR compounds differed ($P \le 0.05$) in their effects on nematode egg production. Acibenzolar, BABA, and riboflavin decreased reproduction the most. These SAR inducers reduced M. javanica reproduction by 59%, 62%, and 69%, respectively, compared to the watertreated control.

Shoot weight of pineapple was lower ($P \le 0.05$) in M. javanica-infected than in the noninoculated plants (Fig. 1B). The application of SAR inducers to M. javanicainfected pineapple did not increase the shoot weight compared to pineapple infected with M. javanica but without SAR inducer application. Shoot weight of pineapple in the M. javanica-inoculated regime differed among SAR compounds ($P \le 0.05$) (Fig. 1B). Salicylic acid (SA) stunted pineapple.

A similar trend was shown in pineapple root weight (Fig. 1C). Meloidogyne javanica caused a reduction in pineapple root mass compared to the noninoculated plants ($P \le 0.05$). The application of SAR inducers to M. javanica-infected pineapple did not improve the root weight as compared to those infected with M. javanica but without SAR inducer application. The root morphology and structure of pineapple treated with SAR inducers was not different from that of pineapple without SAR inducer treatments based on visual observation. Among SAR inducers, root weight was lower in plants treated with SA (Fig. 1C). However, the reduction was less than that observed for shoot weight.

In pineapple inoculated with R. reniformis, nematode reproduction was not reduced by most SAR inducers (Fig. 2A). However, the SAR inducer acibenzolar reduced reproduction of R. reniformis by 51% ($P \le 0.01$) compared to water-treated pineapple. Shoot weight was

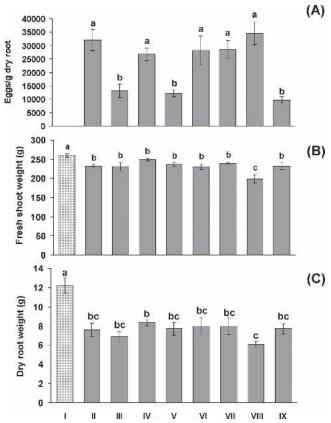


Fig. 1. Effects of different SAR inducers on Meloidogyne javanica in pineapple. (A) The number of M. javanica eggs per g dry root extracted from pineapple treated with water or different SAR compounds. Shoot (B) and root (C) weight of pineapple noninoculated (I) or inoculated with M. javanica (II-IX) and treated with different SAR compounds. Data were obtained 6 mon after a single application of SAR compounds and are means of two pooled tests. Treatments with the same letter are not different $(P \ge 0.05)$ according to Duncan's multiple range test. (I) noninoculated, (II) water, (III) acibenzolar-s-methyl, (IV) AABA, (V) BABA, (VI) GABA, (VII) PABA, (VIII) SA, and (IX) riboflavin. Bars = \pm SE.

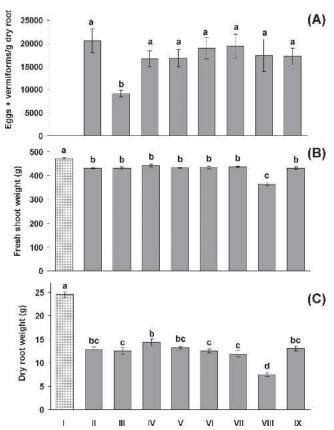


Fig. 2. Effects of different SAR inducers on Rotylenchulus reniformis in pineapple. (A) The number of eggs and vermiform stages per g dry root extracted from pineapple inoculated with R. reniformis and treated with water or different SAR compounds. Shoot (B) and root (C) weight of pineapple noninoculated (I) or inoculated with R. reniformis (II-IX) and treated with water or different SAR compounds. Data were obtained 6 mon after treatment and are means of two pooled tests. Treatments with the same letter are not different $(P \ge$ 0.05) according to Duncan's multiple range test (I) uninoculated, (II) water, (III) acibenzolar-s-methyl, (IV) AABA, (V) BABA, (VI) GABA, (VII) PABA, (VIII) SA, and (IX) riboflavin. Bars = ± SE.

lower in *R. reniformis*-inoculated pineapple than in noninoculated plants ($P \le 0.01$) (Fig. 2B). SAR inducer application did not result in increased plant growth. As in the *M. javanica* experiments, SA reduced growth of pineapple ($P \le 0.05$) (Fig. 2B).

Rotylenchulus reniformis also caused a reduction in root mass of pineapple when the noninoculated and inoculated plants were compared ($P \le 0.01$) (Fig. 2C). In addition, application of SAR inducers did not increase root weight of pineapple. Application of SAR inducer did not affect pineapple root morphology and architecture. SA reduced pineapple root mass more than any other compound ($P \le 0.05$).

Concentrations of acibenzolar-s-methyl: Reproduction of M. javanica on pineapple decreased with the increase of acibenzolar concentrations (Fig. 3A). The relationship between the number of M. javanica eggs per g of dry root and the concentration of acibenzolar was linear (Y = 16798 – 53X, r^2 = 0.89, where Y = the number of nematode eggs and X = mg/liter of acibenzolar). At

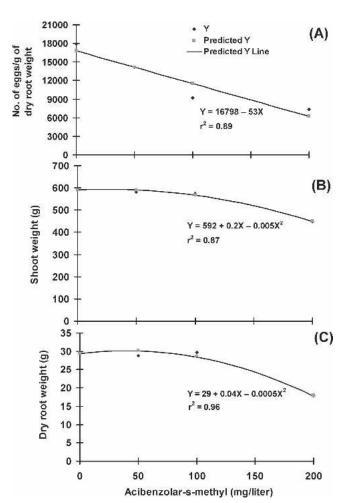


FIG. 3. Effect of foliar spray of acibenzolar-s-methyl on reproduction of *Meloidogyne javanica* and growth of pineapple. (A) Number of nematode eggs per g dry root. Shoot (B) and root (B) weight of pineapple inoculated with *M. javanica* and treated with different concentrations of acibenzolar-s-methyl. Data were collected 1 yr after inoculation and are means of two pooled tests.

100 mg acibenzolar/liter, nematode reproduction was reduced by 31.5% compared to the nontreated control.

The effect of acibenzolar on *R. reniformis* was similar to its effect on *M. javanica* (Fig. 4). The number of eggs plus vermiform stages per g of dry root was reduced by an average of 260 with each increase of 100 mg/liter of acibenzolar, represented by the regression equation Y =

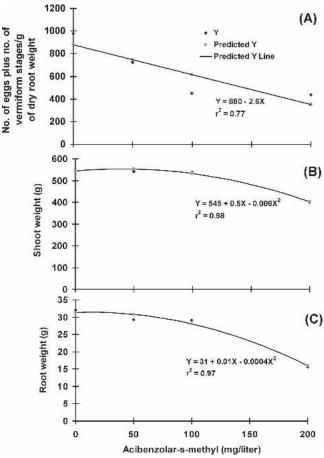


FIG. 4. Effect of foliar spray of acibenzolar-s-methyl on reproduction of *Rotylenchulus reniformis* and growth of pineapple. (A) Number of nematode eggs plus vermiform stages per g dry root. Shoot (B) and root (C) weight of pineapple inoculated with *R. reniformis* and treated with different concentrations of acibenzolar-s-methyl. Data were collected 1 yr after inoculation and are means of two pooled tests.

880 - 2.6X, $r^2 = 0.77$, where Y = the number of eggs plus vermiform stages per g of dry root and X = mg per liter of acibenzolar. Acibenzolar at 100 mg/liter reduced reproduction of R. reniformis by 30% (Fig. 4A). However, at higher concentrations acibenzolar also stunted pineapple growth (Fig. 4B,C).

Phytotoxicity of acibenzolar-s-methyl: Foliar application of acibenzolar at concentrations from 50 to 400 mg/liter caused a reduction in pineapple shoot and root growth (Fig. 5). The reduction in shoot weight was greater as the acibenzolar concentrations increased (Fig. 5A). The relationship was represented by the equation Y = $606 - 0.1X - 0.001X^2$, $r^2 = 0.99$, where Y = shoot weight (g) and X = mg per liter of acibenzolar. At 50 and 100 mg/liter, shoot weight of plants was 598.5 and 586 g (1.24% and 3.3% reduction), respectively, compared to 606 g in the nontreated control. Pineapple treated with 400 mg/liter acibenzolar grew to only 67% of that of the nontreated plants.

The relationship between root weight and acibenzolar concentration (Fig. 5B) was similar to that for shoot weight $(Y = 46 - 0.04X - 0.00005X^2, r^2 = 0.97, where$ Y = root weight (g) and X = mg per liter acibenzolar. Acibenzolar at 50 and 100 mg/liter reduced root weight by 4.6% and 8.8%, respectively, compared to the nontreated control. At 200 and 400 mg/liter, aciben-

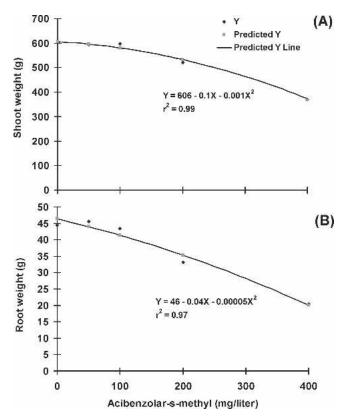


Fig. 5. Effects of different concentrations of acibenzolar-s-methyl on shoot (A) and root (B) weight of pineapple. Foliar spray of acibenzolar on pineapple was applied three times (at planting, 1 mon, and 3 mon after planting). Data were collected 1 yr after planting and are means of two pooled tests.

zolar decreased root weight 21.7% and 52.2%, respectively, relative to the nontreated control. However, acibenzolar did not alter pineapple root morphology and architecture.

DISCUSSION

SAR inducers differed in their ability to reduce nematode reproduction on pineapple. Acibenzolar, BABA, and riboflavin are among the most potent SAR inducers. Differential potency among SAR inducers and between nematode species may be due to different activation points along the signal transduction pathway of SAR. It is likely that acibenzolar induces components upstream of those triggered by BABA and riboflavin in the signal transduction pathway for resistance to rootknot and reniform nematodes. Therefore, resistance induced by acibenzolar showed a broader spectrum of control than that activated by BABA and riboflavin.

In our experiments, acibenzolar, BABA, and riboflavin did not have direct contact with nematodes in pineapple roots, thereby excluding the possibility that these chemicals have detrimental effects to the nematodes by direct contact. Since the activation of pathogenesisrelated genes (PR genes) in pineapple following the application of these inducers was not determined with SAR markers, it was not confirmed that the effect on pineapple was due to SAR. However, acibenzolar was shown to activate PR genes in tobacco, Arabidopsis, and wheat (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996). In grapevine, Owen et al. (2002) found that PR-2 protein increased in grapevine roots 5 and 28 d after foliar application of acibenzolar. BABA induced PR protein accumulation in tomato (Cohen, 1994). Dong and Beer (2000) showed that riboflavin induced expression of PR genes in tobacco and Arabidopsis and described the possible mechanisms by which riboflavin elicits SAR in plants.

From the present study, since nematode infection into pineapple roots was not inhibited, it is most likely that plant response to M. javanica and R. reniformis occurred following nematode penetration of the roots. This could be due to the production of toxic compounds by plants that interfered with nematode development and growth or the malformation of feeding sites that deprived the nematodes of nutrition and water. Chinnasri et al. (2003) demonstrated that acibenzolar does not have nematicidal properties and does not inhibit M. javanica penetration into the roots of cowpea. Rather, the mechanisms induced by acibenzolar were a delay in nematode development and reduction of reproduction. Similar mechanisms of resistance were evident in the study of acibenzolar against Meloidogyne spp. in grapevine and Heterodera trifolii in white cover (Kempster et al., 2001; Owen et al., 2002).

To associate the expression of PR proteins with nematode reduction, the property of each PR protein family was considered. There are at least 14 families of pathogenesis-related genes (PR-1 to PR-14) associated with the development and maintenance of SAR (Van Loon and Van Strien, 1999). The proteins encoded by the PR genes have a wide range of activities and some, such as β-1,3-glucanase and chitinase, have in vitro antimicrobial activity (Ryals et al., 1996). Of all the PRprotein families, proteinase inhibitors or PR-6 are likely candidates for nematode control because they can inhibit proteolytic activity of nematode proteinases. Proteinases are enzymes localized to the intestine of plantparasitic nematodes (Lilley et al., 1996) and are capable of digesting the dietary proteins consumed by nematodes. Proteinase inhibitors are present ubiquitously in many plants, including pineapple, potato, maize, rice, cowpea, mungbean, tomato, wheat, barley, rye, and millet, and they play important roles in plant defense mechanisms (Ryan, 1990). Radovich et al. (2005) found that the activity of proteinase inhibitors was higher in R. reniformis-infected pineapple than in noninfected plants. In addition, this activity was increased at the basal portion of pineapple roots where nematode densities were high. Urwin et al. (1997) found that expression of a modified rice cysteine proteinase inhibitor in A. thaliana caused reduction of size and fecundity of females of *Heterodera schachtii* and *M. incognita*. In addition, the gene product was ingested by both nematodes, and this correlated with loss of cysteine proteinase activity in the nematode intestine.

Phytotoxicity caused by the application of SAR inducers has been increasingly documented. For example, application of acibenzolar at 300 mg/liter lowered wheat biomass and caused the plants to develop fewer shoots and ears and to produce fewer seeds (Heil et al., 2000). The effects were more pronounced when nitrogen was a limiting factor and wheat plants were in the early stage of development. The allocation cost related to metabolic competition between the processes involved with plant growth and the production of defense-related compounds could be responsible for this.

The mechanism responsible for the reduced plant fitness associated with SAR induction is not known (Cipollini et al., 2003), although resource allocation tradeoff has been widely supported as a key mechanism. Baldwin et al. (1998) found that induced responses caused an increase in nicotine content, which is a putative defense compound, and which accounted for 6% of the plant's total nitrogen. In addition, Baldwin and Callahan (1993) found that high levels of nicotine also lead to autotoxicity to plants. Recent experiments utilizing differential display or microarrays to analyze gene expression have shown that induced plant responses are associated with the coordinate up-regulation of many defense-related transcripts and the downregulation of transcripts involved in primary metabolism (Reymond et al., 2000; Hermsmeier et al., 2001). These findings support the assumption that upon induction, resources are allocated toward defense and away from primary metabolism, leading to fitness costs in the plant.

Nematode management in pineapple, which is a perennial plant, is dependent on the initial number of nematodes in the soil at planting and the increase in nematode population throughout the crop life. Both stages of nematode control are important to ensure healthy growth of the plants. Presently, pre-plant nematicides are applied to lower number of nematodes in the soil, and post-plant nematicides provide effective control of nematode populations. However, as social and environmental concerns about the residues and adverse effects of synthetic chemicals are on the rise, those efficacious nematicides will be removed from the market eventually. Therefore, effective control measures that are environmentally sound are needed. Acibenzolar at 100 mg/liter of water maintained nematode population densities at low levels for 1 yr with only small adverse effects on pineapple plant growth. Therefore, this compound may have potential as an alternative to post-plant nematicides or could be used in combination with post-plant nematicides or other nonchemical control measures in an integrated management program to achieve more effective nematode control in pineapple.

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