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ELICITORS OF PLANT DEFENSIVE SYSTEMS REDUCE INSECT DENSITIES AND DISEASE INCIDENCE[1]

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Abstract—Some elicitors of plant defensive systems can induce biochemical changes that enable the plant to reduce disease incidence; however, little is known about the effect of these induced responses on insect herbivores. We approached this problem using exogenous field applications of several abiotic elicitors of defensive systems in tomatoes (*Lycopersicon esculentum*), and evaluated the ability of the elicitors [benzo(1,2,3)thiadiazole-7-carbothioic acid (*S*)-methyl ester (BTH, Actigard); Probenazole; chitosan; salicylic acid; KeyPlex 350; KeyPlex DP2; and KeyPlex DP3] to reduce pest densities and to provide cross-resistance against various insect herbivores and pathogens. Only BTH provided cross-resistance and significantly reduced the incidence of bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*), early blight (*Alternaria solani*), leaf mold (*Fulvia fulva*), and leafminer larval densities (*Liriomyza* spp.). The effects on leafminer larval densities were more pronounced during the early stages of plant development. A trend of reduced densities of whiteflies (*Bemisia argentifolii*) and powdery mildew (*Oidium* sp.), although not significant, was also found on the BTH-treated plants. Other elicitors had no significant effect on insect populations, but Probenazole and KeyPlex 350 significantly reduced bacterial spot and early blight incidence. The antiherbivore effects of BTH on leafminers was confirmed in a laboratory two-choice experiment. Adult leafminers preferred untreated plants to the BTH-treated tomatoes as ovipositioning host plants, generally corresponding

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with larval performance. BTH induced high levels of pathogenesis-related proteins in tomato plants including peroxidase, lysozymes, chitinase, and β-1,3-glucanases. The possible cross-resistance role of these proteins is discussed. The demonstration that exogenous induction of plant defensive systems in the field can result in lower damage caused by various pathogens and insects, supports the hypothesis that plant defensive systems may be general.

Key Words—Elicitor, induced response, leafminers, pathogenesis-related protein, plant defense, tomatoes, whitefly.

INTRODUCTION

Plant pathogenesis-related (PR) proteins have been defined as those proteins that are induced and newly expressed in pathological or related situations (van Loon et al., 1994). This definition includes proteins that are induced as a result of phytophagous insects and other herbivore attacks. PR proteins such as chitinases and β-1,3-glucanases can degrade the cell walls of some phytopathogens and consequently may play a part in the host plant's defensive system (Dixon et al., 1994; Graham and Sticklen, 1994). PR proteins are induced by abiotic substances such as ethylene, chitin, chitosan, etc., and their evaluation may also raise the resistance level to plant pests (Dixon et al., 1994; Graham and Sticklen, 1994).

Insect herbivores often induce PR proteins in their plant hosts as a result of feeding (Stout et al., 1994). Mayer et al. (1995) have reported that acidic chitinases in citrus roots are induced as a result of West Indies sugarcane rootstalk borer weevil (*Diaprepes abbreviatus* Li) larvae feeding on the roots and that induction is dependent on rootstock variety.

Various insects feeding on tomatoes induce PR proteins. This may indicate that PR proteins play a role in a plant's defense against insect attacks. Stout et al. (1994) have reported that polyphenol oxidase, peroxidase, lipoxygenase, and two proteinase inhibitors are elevated in tomato plants fed on by tomato fruitworm [*Helicoverpà zea* (Boddie)], leafminer [*Liriomyza trifolii* (Burgess)], and russet mites [*Aculops lycopersici* (Massee)]. In addition, feeding by the silverleaf whitefly (*Bemisia argentifolii* Bellows & Perring) has been shown to induce chitinases, β-1,3-glucanases, and peroxidases in tomatoes (Mayer et al., 1996).

Induction of defensive systems in plants can be achieved and enhanced during abiotic elicitors. These are compounds that act as signals that stimulate the synthesis of natural products, phytoalexins, and PR proteins that reduce pest damage (Benhamou and Theriault, 1992; Ebel and Cosio, 1994). If an elicitor induces a general defense response in the host plant, we would expect to detect effects on a variety of pests, i.e., pathogens and insects. Little is known about the effect of PR proteins on insect herbivores and whether or not exogenic elicitors can provide cross-resistance.

In this study, we examined the ability of several abiotic elicitiors to reduce disease incidence and insect herbivore densities in the field. In controlled laboratory experiments, we characterized the induction of PR proteins by the most promising elicitor and confirmed its anti-herbivore effects on the preference and performance of leaf-mining insects.

METHODS AND MATERIALS

Culture Procedure and Field Plot Experimental Design. Six-week-old Agriset tomatoes (Speedling Inc., Bushnell, Florida) were transplanted on March 26, 1996, at the University of Florida, Agricultural Research and Education Center, Ft. Pierce, Florida. Raised beds (15 cm high, 1.06 m wide) were spaced at 2.13-m centers. Plants were transplanted 0.6 m apart in the center of each bed. Beds were covered with black polyethylene mulch and watered with subsurface irrigation. Diamond R-7315 fertilizer (Diamond R Fertilizer Co., Winter Garden, Florida) was incorporated into the beds at a rate of 50-200-50 kg/acre of N-P-K, respectively. In addition, Diamond R 7314 was applied to the bed surface at rates of 199-299-498 kg/acre N-P-K. An additional single foliar fertilization of 20-20-20 N-P-K was applied three weeks after transplanting at a rate of 2.27 kg/379 liters. Two beds, each with 12 plants, were considered as plots. Plots were arranged as randomized complete block replicates with five replicates. Buffer zones (3.6 m) were maintained between plots. No pesticides were used on the plots at any time during the experiment. However, buffer zones were sprayed twice with the herbicide Gramoxone (ICI, Wilmington, Delaware), at the rate of 0.7 liters/acre.

Chemical Treatments. Tomatoes were treated with six different materials as follows: Actigard [benzo(1,2,3)thiadiazole-7-carbothioic acid(*S*)-methyl ester (BTH) (Novartis Crop Protection, Inc., Greensboro, North Carolina] was applied every three weeks (total four applications; Figure 1a). Foliar applications of BTH [1.85 g ai (active ingredient)/3.8 liter water] were made using a hand

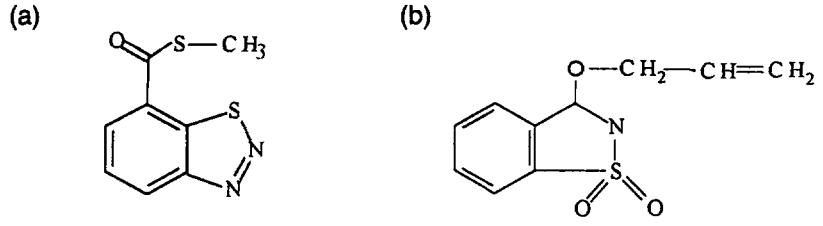

FIG. 1. Structures of (a) Actigard [benzo(1,2,3)thiadiazole-7-carbothioic acid (*S*)-methyl ester] BTH and (b) Probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide).

sprayer. The nutritional products, KeyPlex 350, KeyPlex DP2, and KeyPlex DP3 (Morse Enterprises, Miami, Florida) were applied to the soil every two weeks (six applications) at a rate of 50 ml (0.5%, v/v) per plant. A mixture of salicylic acid and chitosan was applied to the soil every two weeks (total of six applications) at rates of 50 ml (0.0015% + 0.00003% w/v respectively) per plant. Probenazole [3-allyloxy-1,2-benzisothiazole-1,1-dioxide (Meiji Seika Kaisha, Ltd., Tokyo, Japan)] was mixed with the soil only once during transplanting at rates of 6 kg/acre (Figure 1b). Control plants did not receive any chemical treatments. Except for Probenazole, the first treatment was 24 hr before transplanting. When BTH was applied, the remaining plants received water applications. Likewise, when soil applications of the other products were made, the BTH- and Probenazole-treated plants received 50 ml water. The KeyPlex 350, and the salicylic acid + chitosan mixture treatments ended with four replicates.

Monitoring of Insect Populations. The total number of mines of *Liriomyza* spp. (Diptera: Agromyzidae) on the adaxial surface of the three terminal leaflets of the seventh leaf from the top of the plant were counted, as suggested by Schuster and Beck (1992). Ten leaves per plot (randomly selected) were counted every week. Whitefly eggs and nymphs were counted on the terminal trifoliate using a stereomicroscope. Because whitefly densities were low, we examined the entire abaxial surface of these leaflets. Trifoliate area was measured after counting, using a leaf area meter (LI 3000, Lambda Instruments Corp., Lincoln, Nebraska).

Yellow sticky traps (7.6 × 12.7 cm; Olson Products Inc., Medina, Ohio) were placed in the center of each plot between beds at 50.8 cm height. Adult *B. argentifolii* were counted weekly. Insect populations (adults and immatures separately) for each sampling date were subjected to two-way analysis of variance (Sokal and Rohlf, 1981) with date and treatments as main effects. Data analyses followed Student-Newman-Keuls test (S-N-K) at the 0.05 level of significance. A Pearson correlation analysis was used to examine the relationships between densities of immature whiteflies and adults that were counted on the yellow traps. Mean numbers of insects were subjected to square root transformation before analysis.

Monitoring Disease Incidence. We monitored the incidence of several diseases including *Xanthomonas campestris* pv. *vesicatoria* (Doidge) dye (bacterial spot), *Fulvia fulva* (Cooke), Cif. (leaf mold of tomato), *Alternaria solani* Sorauer (early blight), and *Oidium* sp. (powdery mildew of tomato). Readings of each disease were taken from all the plants in the field on June 17, 1996. The plants were manipulated so that stem and leaf surfaces could be viewed; thus, the disease ratings are on a whole-plant basis. Disease rankings were 0 = no symptoms observed; 1 = a few lesions present on less than 10% of foliage; 2 =

scattered lesions on up to 50% of the foliage; 3 = lesions present on most of the foliage; 4 = very high incidence of lesions on most foliage.

Laboratory Experiments with BTH. Because of the encouraging results of the BTH effect on leafminers (*Liriomyza* sp.) in the field trials (see results), we tested BTH efficacy in controlled laboratory experiments. One-month-old Agriset tomatoes (Speedling Inc.) were maintained in a greenhouse in 5.7-cm-diameter pots with Metro Mix 500 growing medium (Grace Sierra, California). Half the plants ($N = 14$) were treated with three foliar applications (every two weeks) of BTH (1.85 g ai/3.8 liters water), as in the field experiments. Control plants were sprayed with water. Four days after the last application, 14 pairs of control and BTH-treated plants were used for choice and performance experiments with leafminers. Each pair of plants was placed in a 15.2-liter bucket sealed with cellophane. Six unsexed, newly emerged, adult leafminers [*Liriomyza trifolii* (Burgess)] were introduced into each bucket. Adults were allowed to feed and oviposit for 24 hr and then removed. Plants were maintained in a controlled atmosphere room (50% relative humidity, 28°C) throughout the experiment. One week later, whole plants were placed horizontally in a plastic dish, and puparia were collected. The experiment was terminated 13 days after the removal of the adults. Adult leafminer host preference was measured by counting the number of mines, oviposition, and feeding punctures per plant and per leaf area. Survival (based on collected puparia) was calculated as an indication of larval performance. Leafminers were from a culture initiated in 1983 and maintained on cowpea, *Vigna sinensis* (*Stickm.*). The data were analyzed using the paired comparison t test (Sokal and Rohlf, 1981). The number of mines, oviposition, and feeding punctures, as well as the number of infested leaves and leaflets, were subjected to square root transformation before analysis.

Another batch of 28 tomato plants (half treated with BTH), maintained and treated similarly to those used for the leafminer experiment, was used to determine the biochemical effects of BTH on tomatoes. Leaves were randomly harvested when the leafminer experiment was terminated and kept in the freezer (−20°C) until analysis. Foliar protein was measured by the Bradford (1976) procedure. The induction of several PR proteins (chitinase, peroxidase, β-1,3-glucanase, and lysozyme) was measured with enzymatic and immunological methods described previously (Mayer et al., 1995).

RESULTS

Leafminer Populations in Field Experiments. Leafminer populations increased relatively fast on the young tomato plants. Leafminer larval population densities increased rapidly and reached a peak four weeks after transplanting, followed by a gradual but consistent reduction throughout the experiment (Figure

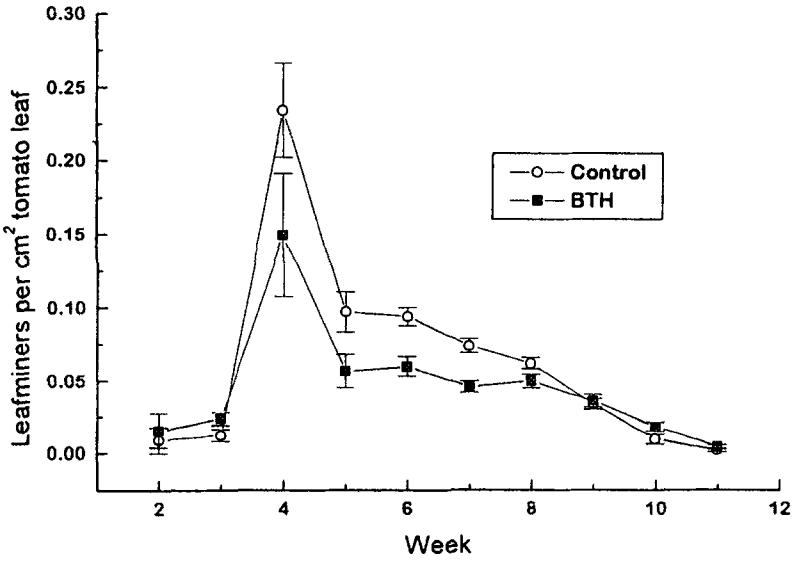

FIG. 2. Effects of Actigard (BTH) applications on larval leafminer populations in tomatoes. BTH significantly reduced leafminer density in weeks 4, 5, and 6 after transplanting ($F_{6,26} = 2.2$, $P < 0.05$). Other elicitors had no effect on leafminers at any time (S-N-K, $P > 0.05$) and therefore are not shown. Error bars indicate 1 SE.

2). Only in BTH-treated plants and only during the peak of the population density did we find a significant reduction in the number of leafminers per trifoliate ($F_{6,26} = 2.2$, $P < 0.05$). Compared with the control plants, BTH-treated plants had 34.7% fewer leafminers per square centimeter leaf area four weeks after transplanting, 40% fewer leafminers at five weeks, and 33% fewer leafminers at six weeks (Figure 2). Other treatments did not significantly reduce leafminer larval populations at any time (S-N-K, $P > 0.05$).

Whitefly Population in Field Experiments. In contrast to leafminers, whitefly populations took more than two months to build up. None of the treatments was found to be statistically significant in reducing whitefly egg (Figure 3), nymph (Figure 4), or adult (Figure 5) densities. However, two trends were observed. First, control plants supported more whiteflies than did any of the treatment groups. Second, BTH-treated plants had consistently lower whitefly adult, nymph, and egg densities compared with other treatments and the control. We found a strong correlation between adult whiteflies caught on traps and whitefly nymphs ($r = 0.69$, $P < 0.01$) and eggs ($r = 0.61$, $P < 0.01$) counted on the leaves.

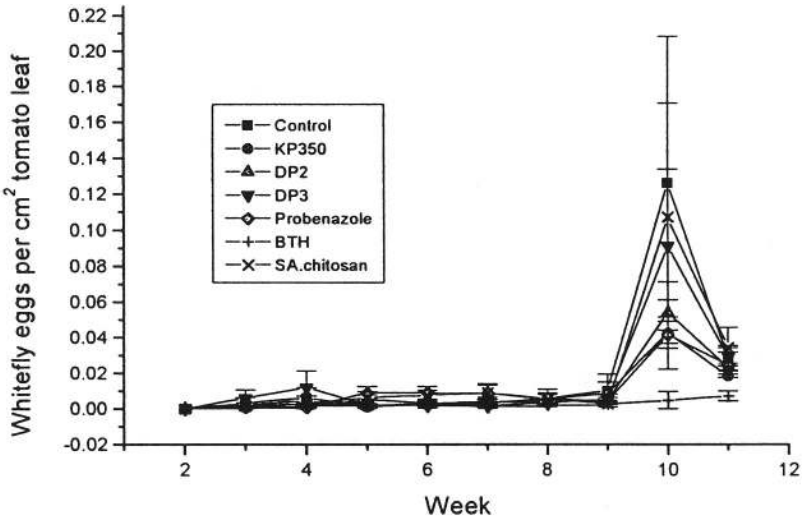

FIG. 3. Effects of elicitor applications on whitefly egg densities in tomatoes. None of the treatments had significant effect at any time ($F_{6,26}$ = 1.58, NS). Error bars indicate 1 SE.

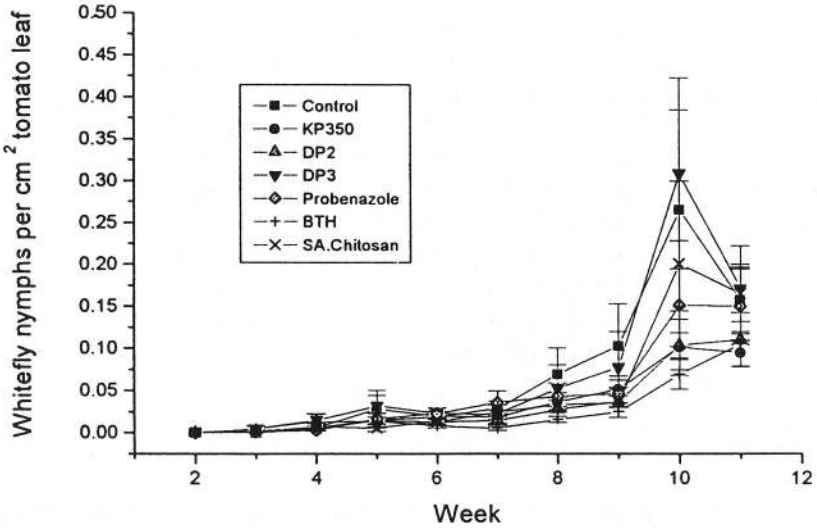

FIG. 4. Effects of elicitor applications on whitefly nymph densities in tomatoes. None of the treatments had significant effect at any time ($F_{6,26}$ = 1.57, NS). Error bars indicate 1 SE.

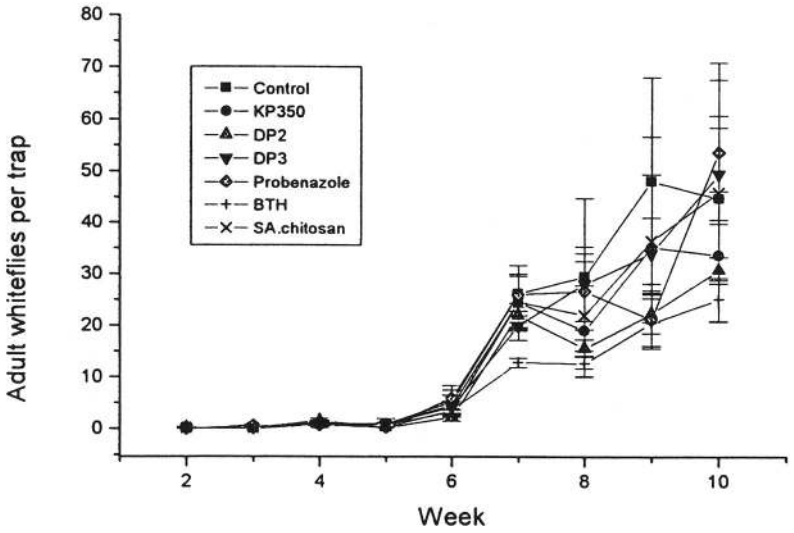

FIG. 5. Effects of elicitor applications on whitefly adult populations in tomatoes. None of the treatments had significant effect at any time ($F_{6,26} = 1.08$, NS). Error bars indicate 1 SE.

Disease Incidence in Field Experiments. Disease incidence for each of the treatments is given in Table 1. There were no significant differences for any of the treatments on powdery mildew incidence ($F_{6,26} = 1.08$, NS). The incidence of bacterial spot ($F_{6,26} = 11.1$, $P < 0.01$) and early blight ($F_{6,26} = 6.9$, $P < 0.01$) was significantly different from controls for Probenazole, KeyPlex 350, and BTH treatments. Only BTH-treated plants had a significantly lower incidence of leaf mold ($F_{6,26} = 4.3$, $P < 0.01$).

Induction of PR Proteins by BTH. Applications of BTH to tomato plants significantly induced PR proteins (Table 2). Foliar levels of chitinase, β-1,3-glucanase, and lysozyme were about two fold higher in the BTH-treated plants compared to the controls. Induction of peroxidase was less pronounced but highly significant. Significantly different levels of total proteins were not found in the BTH-treated plants. Unlike the field experiments, some phytotoxic effects were observed in the laboratory experiments where BTH-treated plants appeared to be smaller.

Effect of BTH on Leafminers in Laboratory Experiments. The effect of BTH on leafminer preference and performance is summarized in Table 3. Adult female leafminers recognized BTH-treated plants as less preferable hosts for oviposition. The number of eggs laid and mine densities were lower on the

TABLE 1. DISEASE INCIDENCE IN TOMATOES TREATED WITH DIFFERENT ELICITORS

Treatment	Disease rating[a]			
	Powdery mildew (*Oidium* sp.)	Bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*)	Early blight (*Alternaria solani*)	Leaf mold (*Fulvia fulva*)
Control	0.33a	2.47a	2.24a	2.08a
Salicylic acid + chitosan	0.69a	2.54a	2.23a	1.59a
KeyPlex 350	0.80a	1.70c	1.61b	1.77a
KeyPlex DP2	0.60a	2.12ab	2.62a	1.99a
KeyPlex DP3	0.45a	2.42a	2.16a	2.06a
Probenazole	0.20a	1.78b	1.53b	1.99a
BTH	0.28a	1.31c	1.50b	0.99b

[a]Numbers followed by like letters within columns indicate no significant differences.

TABLE 2. EFFECT OF BTH ON INDUCTION OF DEFENSIVE PROTEINS IN TOMATOES[a]

Treatment	Protein (mg/g tissue)	Peroxidase (ΔA_{510}/min/g tissue)	Lysozyme (ΔA_{510}/min/g tissue)	Chitinase (ΔA_{510}/min/g tissue)	β-1,3-Glucanase (mmol Glc/min/g tissue)
Control	0.5 ± 0.02	29.4 ± 1.4	124.5 ± 12.5	1.7 ± 0.1	0.39 ± 0.02
BTH	0.65 ± 0.06	36.9 ± 2	317.9 ± 32.2	3.3 ± 0.2	0.84 ± 0.07
t	1.7 NS	3.02*	5.5*	5.7*	5.7*

[a]Values are mean ± SE; NS, nonsignificant; *$P < 0.01$; each treatment with 14 replicates.

BTH-treated plants. The incidence of adult feeding on BTH-treated plants was not significant. BTH-treatments reduced the number of leaves and leaflets mined by approximately half. Survival of leafminer larvae was not lower on the BTH-treated plants.

DISCUSSION

Mayer et al. (1995, 1996) have suggested that plant defensive proteins might be used to control some insect pests. Chitosan and salicylic acid are known

TABLE 3. EFFECT OF BTH ON ADULT LEAFMINER HOST SELECTION AND LARVAL SURVIVAL (PERFORMANCE)[a]

Treatment	Mines/plant	Mines/cm^2	Eggs/cm^2	Feeding/cm^2	Leaves infested	Leaflet infested	Larval survival (P)
Control	3.2 ± 0.6	0.15 ± 0.03	0.17 ± 0.04	0.7 ± 0.2	2.4 ± 0.1	5.5 ± 1.2	0.22 ± 0.05
BTH	1.6 ± 0.5	0.08 ± 0.02	0.09 ± 0.02	0.53 ± 0.19	1.1 ± 0.34	2.5 ± 0.8	0.16 ± 0.05
Paired t	3.3*	2.2*	2.3*	0.6 NS	2.9*	3.2**	1.4 NS

Survival rate is the ratio between the number of eggs laid and puparia collected at the end of the experiment. The experiment was designed and analyzed as paired comparison ($n = 14$).

[a] Values presented as mean and SE; *$P < 0.05$; **$P < 0.01$; NS, nonsignificant.

elicitors of PR proteins in many different types of plants (Benhamou and Theriault, 1992; Ebel and Cosio, 1994; Klessig and Malamy, 1994), and salicylic acid has been associated with insect resistance in rice plants (Ishii et al., 1962). Probenazole is an elicitor that is used to reduce fungal diseases in rice (Midoh and Iwata, 1996), and BTH is marketed as an activator of plant defensive systems (Ciba-Geigy, 1995). KeyPlex 350 is a micronutrient preparation that is believed to be an elicitor (G. Butler, Morse Enterprises, personal communication) and the KeyPlex DP series are test products that use KeyPlex 350-based materials.

We have demonstrated that applications of BTH to tomato plants induced several known PR proteins and that applications effectively reduced the incidence of diseases and populations of insect herbivores. Tomato resistance is based on a complex of defensive systems that include PR proteins, proteinase inhibitors, polyphenol oxidases, and phytoalexins (Duffey and Stout, 1996). The induction of some of the proteins measured is thought to have multiple negative effects on pathogens and insect pests. Chitinase degrades chitin, a major component of pathogen cell walls (Graham and Sticklen, 1994). Chitinase may severely affect insects by damaging chitin-based structures such as the peritrophic membrane that provides a physical barrier to ingested pathogens and other substances that pose a hazard to the insect. Chitinases can also act as α-amylase inhibitors and interfere with digestion of plant parts (Ary et al., 1989). Induction of chitinase activity may interfere with insect development, feeding and growth, facilitate microbial infection, and finally cause death (Shapiro et al., 1987; Wang et al., 1996). Recently, Wang et al. (1996) reported 100% larval mortality of the grain beetle, *Oryzaephilis mercator*, six days after feeding on 2% chitinase obtained from transgenic tobacco. Lysozymes are defensive enzymes that protect plants against bacterial pathogens (Boller et al., 1983). These hydrolases act on murein, a peptide glycan cell wall component of bacteria. Their exact impact on insects is unknown; however, they may affect intestinal flora during ingestion and digestion and subsequently affect development.

Peroxidases are induced in tomato plants following pathogen and insect damage. Peroxidases are involved in production and polymerization of phenolics, lignification, and hypersensitive responses, limiting the possibility of disease spread (Bowles, 1990). Peroxidases also have negative effects on food digestibility and protein availability to herbivorous insects (Duffey and Felton, 1991; Duffey and Stout, 1996). β-1,3-Glucanase hydrolyzes β-1,3-glucans, which are major components of the surface structure and cell walls of many microbial and fungal pathogens (Bowles, 1990) but have no apparent effect on insects.

Whether induced plant responses to pathogen and insect pests are specific or general and whether or not cross-immunization actually occurs is currently

under debate (Karban and Myers, 1989; Apriyanto and Potter, 1990; Baldwin, 1994; Hatcher, 1995). However, as pointed out by Ebel and Cosio (1994), some biotic elicitors are race-specific, providing limited defense against pathogens. Other elicitors are more general, inducing a wide range of defenses against herbivores and pathogens. Our results support this view, suggesting that the degree of specificity of the defensive response will be dependent on the nature of elicitors used. Probenazole and the Keyplex-based elicitors that were tested in the field successfully reduced disease incidence but had no effect on the insects. Induced responses resulting from BTH applications were useful in providing general protection against pests belonging to different phyla. There is a growing body of evidence that induced defenses are general and, therefore, can provide resistance across phyla. For example, Karban et al. (1987) found that induced responses in cotton following fungi and spider mite infestations had reciprocal negative effects on the two pests. Infection of cotton with *Bacillus megaterium* and *B. cereus* induced systemic production of phytochemicals that affected boll weevils (Benedict and Chang, 1991). McIntyre et al. (1981) demonstrated that TMV infection of tobacco induced general resistance against a variety of pathogens and insect herbivores. Inoculation of soybeans with extracts of *Phytophthora megasperma* induced phytoalexin production that deterred feeding by the Mexican bean beetle (Kogan and Fischer, 1991).

Leafminer density and larval performance may be affected by the level of foliar proteins (Minkenberg and Ottenheim, 1990). We found a nonsignificant trend of increased total protein content in the BTH-treated plants. Thus, total protein (i.e., nitrogen) levels in this experimental system could not explain leafminer preference and performance. Regardless of the mechanism(s) involved, our results indicate that female leafminers have the ability to discriminate between favorable and less favorable host plants for larval development, based on the level of induced defensive substances, in addition to their response to total foliar protein levels (Minkenberg and Ottenheim, 1990).

Several factors may explain the lack of effect of BTH on whiteflies compared with its effect on the leafminers. First, throughout the experiment whitefly populations remained low despite gradual increases in populations towards the end of the growing season, perhaps preventing sensitive statistical detection of treatment effects. Secondly, unlike whiteflies, leafminer populations increased sharply soon after transplanting. Indeed, at this stage, we found significant reductions in mine densities. Although induced resistance in tomatoes may be detected in all plant stages, it is most pronounced in young seedlings (Alarcon and Malone, 1995). Thaler et al. (1996) showed that field applications of jasmonic acid induced several defensive compounds in tomato seedlings. Our field experiments provide additional evidence that induced defense responses in tomatoes are more effective in young plants to a level that can reduce insect herbivore densities. In addition, whiteflies are phloem-feeders (Cohen et al., 1996), while

L. trifolii larvae feed in the palisade mesophyll (Parrella, 1987). It has long been suggested that phloem-feeders are less exposed to plant chemical defense systems. Most plant toxins and other defensive compounds including chitinase (Wang et al., 1996) and proteinase inhibitors (Walker-Simmons and Ryan, 1977) are thought to be stored in intracytoplasmic vacuoles in parenchyma and epidermal cells (e.g., Rosenheim et al., 1996, and references therein). Vascular tissue should not be considered as defenseless, but it is possible that BTH-induced substances are less effective against phloem-feeders such as whiteflies. Finally, one cannot rule out the possibility that whiteflies may be less susceptible to the effects of PR proteins.

It appears that plant defensive systems can be used to enhance insect resistance in plants. Elicitors can be used to activate plant defensive systems at desired times; however, generally they should be applied prior to having a pest problem so that the plant will have the best opportunity for resisting pests (Ciba-Ceigy, 1995). Elicitors will probably not be effective in all plants since defensive systems vary with plant variety. Neither will they be effective against all insects since insects vary widely in their abilities in overcoming plant defensive systems. Considerable research will have to conducted to determine how best to use plant defense responses in insect control strategies.

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