7-7-2007

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Article *in* Journal of Chemical Ecology · September 2007

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Induction of Systemic Acquired Resistance in Cotton Foliage Does Not Adversely Affect the Performance of an Entomopathogen

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Received: 22 January 2007 / Revised: 8 May 2007 / Accepted: 11 June 2007 /
Published online: 7 July 2007

Abstract Baculoviral efficacy against lepidopteran larvae is substantially impacted by the host plant. Here, we characterized how baculoviral pathogenicity to cotton-fed *Heliothis virescens* larvae is affected by induction of systemic acquired resistance (SAR). Numerous studies have shown that SAR induced by the plant elicitor benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) can protect against plant pathogens, but reports on the impacts of SAR on chewing herbivores or on natural enemies of herbivores are few. We found that BTH application significantly increased foliar peroxidase activity, condensed tannin levels, and total phenolic levels but did not alter dihydroxyphenolic levels. Consumption of BTH-treated foliage did not influence *H. virescens* pupal weight or larval mortality by the microbial control agent *Autographa californica* multiple nucleopolyhedrovirus any more than did consumption of untreated foliage. Thus, activation of SAR, although it did not protect the plant against a chewing herbivore, also did not reduce the effect of a natural enemy on a herbivore, indicating that SAR and microbial control agents may be compatible components of integrated pest management.

Keywords Baculovirus · *Heliothis virescens* · Actigard · BTH · Tritrophic interactions · Microbial control

Introduction

Systemic acquired resistance (SAR) is a long-lasting response typically induced in plants in reaction to pathogen infection (reviewed in the work of Durrant and Dong 2004). Systemic acquired resistance involves induction of multiple plant defensive compounds, including PR-1, β-1,3-glucanases, chitinases, and peroxidases (Gaffney et al. 1993; reviewed in the paper of Van Loon and Van Strien 1999). SAR activation essentially "inoculates" the plant,

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protecting against future infection by pathogens (Friedrich et al. 1996). The plant elicitor benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), also known as acibenzolar-*S*-methyl and Actigard®, is a functional analog of salicylic acid that activates SAR in both monocots and dicots (Gorlach et al. 1996; Lawton et al. 1996). Foliar application of BTH induces pathogen response genes typically associated with SAR and provides protection against an array of bacterial, fungal, and viral plant pathogens in cotton and other plants (Friedrich et al. 1996; Gorlach et al. 1996; Inbar et al. 1998; Colson-Hanks et al. 2000; Maxson-Stein et al. 2002; Bokshi et al. 2003; Zhu et al. 2003; Baysal et al. 2005). Further, foliar application of BTH to tomato foliage was shown to decrease leafminer and whitefly densities (Inbar et al. 1998; Nombela et al. 2005) and aphid population growth (Boughton et al. 2006). Because of the efficacy of BTH in protecting against phytopathogens and some types of insects in several cropping systems, this elicitor, along with other SAR activators, may be used with increasing frequency in integrated pest management (IPM) systems (reviewed in the study of Vallad and Goodman 2004).

While SAR-activated plants may be protected from phytopathogens, they may also be more vulnerable to chewing herbivores. Lepidopteran larvae feeding on naïve plants induce a variety of directly toxic and/or antinutritive compounds through the jasmonate-dependent wound response pathway (reviewed in the work of Duffey and Stout 1996; Bi et al. 1997a; Chen et al. 2005; reviewed in the paper of Felton 2005). The compounds induced in response to herbivore feeding reduce lepidopteran growth and can alter lepidopteran susceptibility to insect pathogens, depending on the system (Ali et al. 1998; Hoover et al. 1998b). SAR induction can inhibit this jasmonate-dependent response pathway (reviewed in the study of Felton and Korth 2000; Thaler et al. 2002; Cipollini et al. 2004) and may make plants previously challenged by phytopathogens more suitable hosts for lepidopteran larvae than naïve plants or plants previously damaged by chewing herbivores (Stout et al. 1999; Inbar et al. 2001; reviewed in Rostas et al. 2003). Although the apparent potential for SAR induction to influence lepidopteran susceptibility to insect pathogens and other natural enemies is great, the extent to which there is compatibility of SAR with natural enemies of lepidopterans has not been fully explored (but see the work of Rostas and Hilker 2003 for a review).

One class of insect natural enemies are the baculoviruses, a group of orally infectious, dsDNA viruses of arthropods that commonly infect lepidopteran larvae (reviewed in the paper of Bonning 2005). The family Baculoviridae is divided into the genera Granulovirus and Nucleopolyhedrovirus (NPV) (Theilmann et al. 2005). NPVs are found in nature as environmentally resistant proteinaceous occlusion bodies (OB) on host plant foliage, and susceptible larvae become infected when they consume at least one OB (reviewed in the study of Bonning 2005). Upon ingestion, the OB is degraded in the alkaline midgut of the host (Pritchett et al. 1982), releasing occlusion-derived virions, which pass through the peritrophic matrix and infect midgut epithelial cells (Granados and Lawler 1981). NPVs replicate in the nucleus of infected cells; progeny virions bud from infected midgut cells and enter associated tracheolar cells and hemocytes, spreading the infection to other tissues, eventually killing the host.

Most baculoviruses are highly host specific, infecting, at the extremes, from 1 to 40 lepidopteran species (reviewed in the paper of Cory and Myers 2003). Because of their high degree of host specificity, baculoviruses have been considered candidate pest control agents (reviewed in the work of Szewczyk et al. 2006). The potential of baculoviruses as bioinsecticides has not been fully realized due in part to the high cost of production, inactivation by ultraviolet light, and a slow speed of kill. As these issues are addressed, baculoviruses could be implemented more frequently as a part of IPM strategies. Thus, it is important to assess how baculoviral efficacy is influenced by other components of IPM. In

this study, we analyzed whether cotton foliar peroxidase activity and quantities of phenolic compounds change after SAR induction by BTH. Furthermore, we evaluated the influence of SAR induction on pathogenicity of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) to *Heliothis virescens* larvae.

Methods and Materials

Insect Rearing Heliothis virescens eggs were acquired from the North Carolina State University Insectary, from a colony that started in 1997 (Raleigh, NC, USA). Larvae were grown in groups through the third stadium on semisynthetic diet (Southland Products, Lake Village, AR, USA) in wax-lined paper cups (Xpedx, Harrisburg, PA, USA) at 25°C and a 16L:8D photoperiod.

Plant Growing Conditions Cotton, *Gossypium hirsutum* cv. Maxxa, seeds were donated by the California Planting Cotton Seed Distributors (Bakersfield, CA, USA). Seeds were sown individually in sterile soil in plastic pots, fertilized at planting with slow-release 14–14–14 fertilizer, and grown in an Conviron® environmental chamber under a day:night temperature regime of 29:24°C and a 16L:8D photoperiod.

Plant Treatments BTH (Actigard®) was provided by Syngenta/Novartis (Greensboro, NC, USA). A 0.056-g/l solution of BTH (Actigard® 50WG) in ultrapure water was sprayed to run off on upper leaf surfaces of six-node cotton plants. Control plants were left untreated or sprayed with ultrapure water. Plants to be treated were removed from the chamber for application of solutions to prevent drift to control plants. The concentration of 0.056 g/l used is similar to concentrations of 0.035 g/l (Colson-Hanks and Deverall 2000) and 0.05 g/l (Jabaji-Hare and Neate 2005) applied to cotton foliage in previous studies, yet is much lower than the 0.8 g/l applied by Inbar et al. (2001). We found higher concentrations than those used here phytotoxic to this cultivar of cotton.

Four days after treatment, the top two fully expanded leaves of all control and BTH-treated plants were removed. Foliage removed for use included leaves 5 and 6 (the cotyledons were not counted) from eight-node plants. A subsample of intact leaves from the water and BTH treatment groups was frozen in liquid nitrogen and stored at −80°C for phytochemical assays. Foliage from untreated and BTH-treated plants was fed to larvae to determine the influence of SAR activation on pupal weight and viral mortality.

Foliar Assays Peroxidase activity was measured in untreated and BTH-treated cotton foliage using a guaiacol/hydrogen peroxide substrate by measuring the change in absorbance at OD_{470}, as described previously (Bi et al. 1997a), except that the assay was modified for use with a microplate reader. Peroxidase activity is reported as change in absorbance, with one unit of activity=0.001 ΔOD_{470} per milligram of foliage per minute. Three untreated and three BTH-treated plants were used for the peroxidase assay, with three measurements conducted per plant. Leaves from positions 5 and 6 were combined and used from each plant.

Phenolic compounds were extracted from water-treated and BTH-treated cotton foliage in 50:50 acetone to water at 60°C for 20–24 hr. This extract was used for the following assays. Total phenolics were measured with the Folin-Ciocalteau reagent with caffeic acid as the standard at OD_{720} (Singleton and Rossi 1965). Dihydroxyphenolics were measured by using the 0.5% diphenylborinic acid–ethanolamine complex with chlorogenic acid as the

standard at OD_{365} (Broadway et al. 1986). Condensed tannins were measured with the vanillin–HCl assay with catechin as the standard at OD_{500} (Price et al. 1978; Bi et al. 1997a). A SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA) was used for all of the above spectrophotometric measurements. For the dihydroxy- and total phenolic assays, five water-treated and three BTH-treated plants were used, with three measurements made per plant. For the condensed tannin assay, three water-treated and two BTH-treated plants were used, with three measurements made per plant. Leaves from positions 5 and 6 were combined and used from each plant. The same foliar samples were used for all of the above phenolic assays.

Influence of SAR Induction on Time to Pupation and Pupal Weight Newly molted second instars of *H. virescens* were transferred individually to 30 ml plastic cups containing artificial diet or foliage from BTH-treated or untreated cotton plants and were allowed to feed *ad libitum*. Larvae were maintained at 28°C until pupation, and fresh foliage was provided as needed. Weight was recorded at pupation, with 31–38 pupae weighed per treatment group. Foliage was taken from 50 BTH-treated and 50 control plants to feed larvae; within each treatment group, foliage was randomly distributed among larvae. Foliage was harvested from treated plants 4–7 d after BTH application from nonsquaring plants. We have found that foliar peroxidase activity remains significantly elevated in BTH-treated plants relative to controls for at least 7 d after treatment. Foliage was concurrently harvested from control plants.

Virus Preparation AcMNPV-hsp70/*lacZ*, provided by Suzanne Thiem (Michigan State University, East Lansing, MI, USA), was amplified in *H. virescens* larvae. Occlusion bodies (OB) were harvested from virus-killed cadavers and partially purified by using ultrapure water as described previously (Hoover et al. 1995), quantified with a hemacytometer, and stored at 4°C in neutrally buoyant 60% glycerol with 0.002% sodium azide (Engelhard et al. 1994). We used a genetically modified construct of AcMNPV because, although this experiment did not require lacZ expression, concurrent experiments did, and this gene does not affect viral performance.

Influence of SAR Induction on Viral Mortality of H. virescens Newly molted fourth instars of *H. virescens* were transferred individually to a 30-ml plastic cup containing artificial diet or foliage from BTH-treated or untreated cotton plants and were allowed to feed for 8 hr. Eight hours postmolt was used because it was the earliest time point at which the midgut was full of plant material or artificial diet for all larvae based on the time at which frass was first produced. The susceptibility of *H. virescens* larvae to fatal viral infection decreases as larvae age within an instar, so controlling for age postmolt is essential (Washburn et al. 1995). Foliage was taken from 35 BTH-treated and 35 control plants to feed larvae. Within each treatment group, harvested foliage was randomly distributed among larvae.

After feeding for 8 hr, larvae were microinoculated *per os* with 95 OBs of AcMNPV-hsp70/*lacZ* in a 1-ml aliquot by using a PAX 100 microapplicator (Burkard Scientific, Middlesex, UK) holding a 1-ml tuberculin syringe with 32-gauge blunt needle (Popper Precision Instruments, Lincoln, RI, USA). After inoculation, the larvae were returned to their respective preinoculation food of artificial diet or foliage from BTH-treated or untreated cotton plants. Insects were maintained at 28°C and were checked daily for mortality or pupation. All cotton-fed larvae were transferred to artificial diet after molting to the fifth instar for ease of handling. This transfer does not influence mortality since larvae clear midgut infections during the first molt after viral challenge (Washburn et al. 1995). Two trials were performed with larvae fed with BTH-treated and untreated cotton foliage,

and three trials were performed with larvae fed with artificial diet. The first trial included all three treatment groups; subsequent trials included artificial diet and either BTH-treated or untreated cotton foliage. Some 25 to 30 artificial diet-fed larvae and 40–50 cotton-fed larvae were inoculated for each trial.

Data Analysis Data were analyzed with SAS (SAS Institute 2003). Measurements of foliar peroxidase activity and quantities of phenolic compounds were compared between control and BTH-treated plants using the general linear model with Tukey post hoc analysis (proc GLM). The effects of food and BTH treatment on *H. virescens* time to pupation and pupal weights were also determined using the general linear model with Tukey post hoc analysis (proc GLM). The influence of food, BTH application, and their interaction on the proportion of *H. virescens* larvae killed by virus was assessed using the analysis of variance for categorical data (proc CATMOD) (Zar 1999).

Results

Foliar BTH treatment increased cotton peroxidase activity 2.5-fold over that observed in untreated plants (F=6.49, df=1, P=0.029; Fig. 1), indicative of induction of systemic acquired resistance (SAR) (Inbar et al. 2001). Benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester application also resulted in large increases in certain phenolic classes. For instance, we observed a nearly twofold increase in total phenolics (F=31.62, df=1, P=0.011; Fig. 2) and a 2.4-fold increase in condensed tannins in foliage of BTH-treated plants (F=12.58, df=1, P=0.038; Fig. 3) compared with water-treated controls. However, application of BTH did not significantly affect the foliar concentration of dihydroxyphenolics (F=1.31, df=1, P=0.335; Fig. 3).

Consumption of control foliage by *H. virescens* larvae increased time to pupation, relative to artificial diet and BTH-treated foliage (F=24.82, df=2, P<0.001) (Fig. 4). Interestingly, the developmental rate of larvae fed with artificial diet and those fed with BTH-treated foliage was similar (Fig. 4). In contrast, consumption of induced foliage did not impact pupal weight (Fig. 5) nor did we observe a marked increased in consumption of BTH-treated cotton foliage relative to untreated foliage (data not shown). The pupal weights of larvae fed with untreated or induced foliage were similar (F=0.66, df=1, P=0.421).

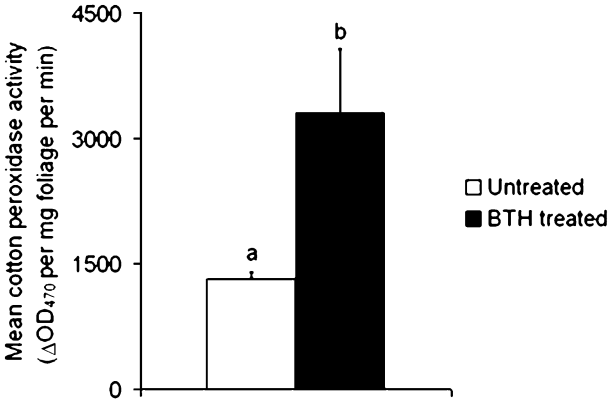

Fig. 1 Mean peroxidase activity measured at 96 hr post-spray, shown as change in activity per milligram of cotton foliage per minute. *Error bars* represent one standard error of the mean; N=3 plants per treatment. The general linear model with Tukey post hoc analysis was used to compare peroxidase activities; *small letters* indicate significant differences between treatments (F=6.49, df=1, P=0.029)

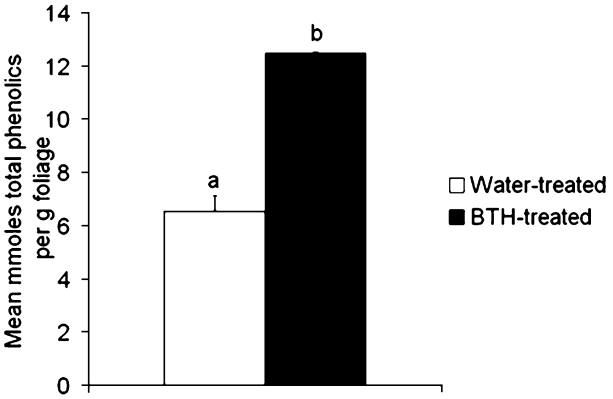

Fig. 2 Mean concentrations of total phenolics measured at 90 and 96 hr post-spray with water or BTH. *Error bars* represent one standard error of the mean. N=5 plants for water treatment and 3 for BTH treatment. The general linear model with Tukey post hoc analysis was used to compare levels of phenolics; *small letters* indicate significant differences between treatments (F=31.62, df=1, P=0.011)

Further, larvae fed with artificial diet displayed dramatically higher pupal weights than larvae fed with either treated or untreated cotton foliage (F=95.24, df=2, P<0.001; Fig. 5).

Although some classes of phenolics and peroxidase activities were induced by BTH treatment, consumption of induced foliage did not significantly influence *H. virescens* larval susceptibility to a well-known natural enemy of this host, the baculovirus AcMNPV (Fig. 6). Larvae fed with BTH-treated cotton foliage experienced a similar level of viral mortality as larvae fed with untreated foliage (χ^2=1.11, df=1, P=0.292). However, when larvae consumed induced or uninduced foliage, they were 1.3-fold more resistant to mortal infection than larvae fed with artificial diet (χ^2=21.64, df=1, P<0.001; Fig. 6).

Discussion

Application of BTH to foliage of a number of plant species is known to induce SAR, conferring resistance to phytopathogens (Friedrich et al. 1996; Inbar et al. 1998; Maxson-

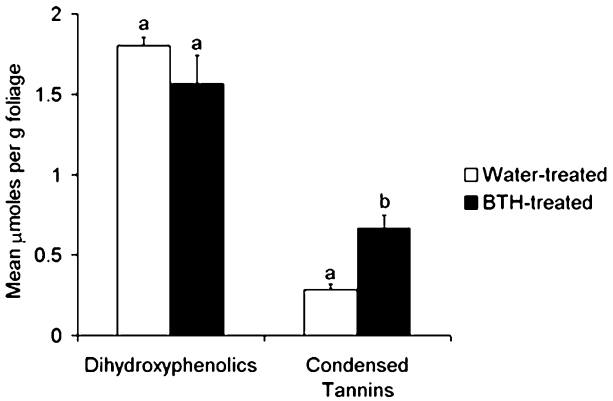

Fig. 3 Mean concentrations of dihydroxyphenolics and condensed tannins measured at 90 and 96 hr post-spray with water or BTH. *Error bars* represent one standard error of the mean. For the dihydroxyphenolics assay, N=5 plants for water treatment and 3 for BTH treatment. For the condensed tannin assay, N=3 plants for water treatment and 2 for BTH treatment. The general linear model with Tukey post hoc analysis was used to compare levels of phenolics; *small letters* indicate significant differences between treatments (dihydroxyphenolics: F=1.31, df=1, P=0.335; condensed tannins: F=12.58, df=1, P=0.038)

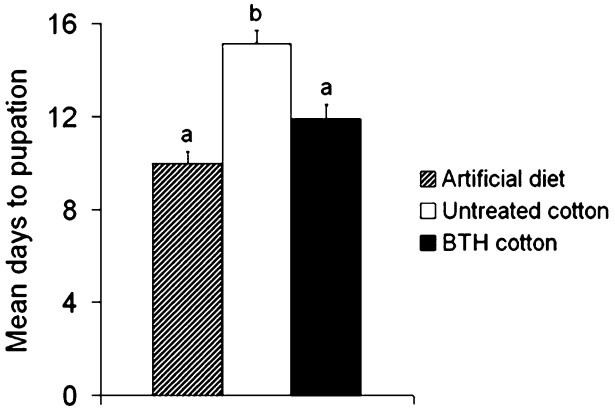

Fig. 4 Mean time to pupation of *H. virescens* fed with artificial diet or foliage from untreated or BTH-treated cotton plants from second instar to pupation. *Error bars* represent one standard error of the mean; N=35 larvae fed with artificial diet, 37 fed with untreated foliage, and 22 fed with BTH-treated foliage. The general linear model with Tukey post hoc analysis was used to compare times to pupation; *small letters* indicate significant differences between treatments (F=24.82, df=2, P<0.001)

Stein et al. 2002; Bokshi et al. 2003; Baysal et al. 2005) and, in some cases, to phloem-feeding insects such as aphids and whiteflies (Nombela et al. 2005; Boughton et al. 2006). However, SAR activation by BTH or salicylic acid in tomato or cotton foliage did not influence growth rate or survival of larval *Helicoverpa zea* (Bi et al. 1997b; Stout et al. 1999; Inbar et al. 2001). We found that although SAR activation by application of BTH to cotton plants increased foliar peroxidase activity, condensed tannins, and total phenolics, consumption of induced foliage did not affect *H. virescens* pupal weight compared with water-treated or untreated foliage. Likewise, Inbar et al. (2001) reported that while BTH applied to cotton foliage activated SAR—as evidenced by increased foliar peroxidase, chitinase, and β-1,3-glucanase—consumption of foliage from BTH-treated plants did not alter growth or survival of larval *Helicoverpa armigera*. Similarly, *H. zea* larvae fed with foliage from cotton plants treated with either salicylic acid or methyl salicylate grew as well as those that fed on untreated foliage (Bi et al. 1997b).

Increases in foliar phenolic levels after activation of the SAR pathway by BTH have been recently reported in strawberries (Hukkanen et al. 2007). Here, we found a similar

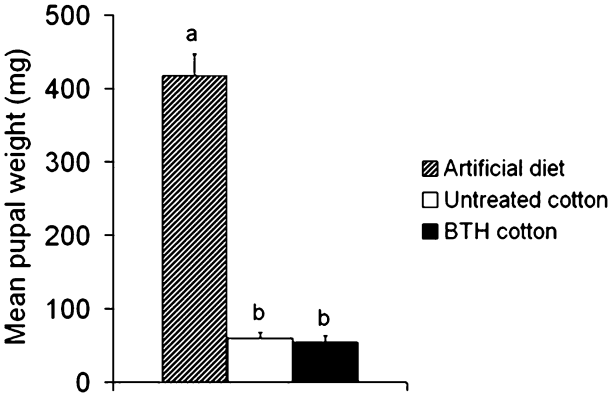

Fig. 5 Mean pupal weight of *H. virescens* fed with artificial diet or foliage from untreated or BTH-treated cotton plants from second instar to pupation. *Error bars* represent one standard error of the mean; N=35 larvae fed with artificial diet, 37 fed with untreated foliage, and 22 fed with BTH-treated foliage. The general linear model with Tukey post hoc analysis was used to compare pupal weights; *small letters* indicate significant differences between treatments (artificial diet versus cotton foliage: F=95.24, df=2, P<0.001; untreated versus BTH-treated cotton: F=0.66, df=1, P=0.421)

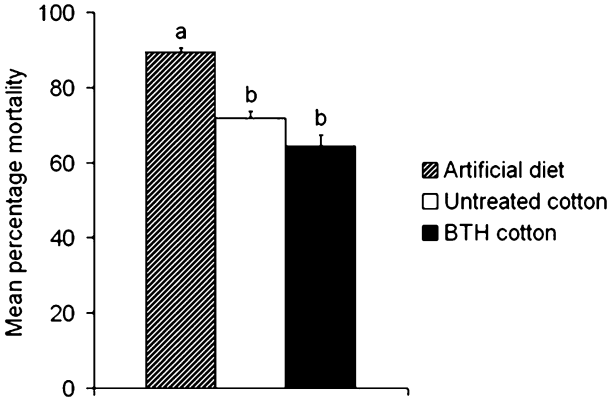

Fig. 6 Mean percentage mortality of *H. virescens* larvae fed with artificial diet or foliage from untreated or BTH-treated cotton plants before AcMNPV inoculation. The influence of food and BTH treatment on larval mortality was determined using the analysis of variance for categorical data (artificial diet versus cotton foliage: χ^2=21.64, *df*=1, *P*<0.001; untreated versus BTH-treated cotton: χ^2=1.11, *df*=1, *P*=0.292). *Error bars* represent one standard error of the mean; *N*=23–33 larvae fed with artificial diet, 38–53 larvae fed with untreated foliage, and 45–49 larvae fed with BTH-treated foliage. Two trials were performed with larvae fed with BTH-treated and untreated cotton foliage, and three trials were performed with larvae fed with artificial diet

result in BTH-treated cotton foliage. However, despite the induction of condensed tannins in treated foliage, we did not observe any influence of BTH treatment on *H. virescens* pupal weight. There is a large body of literature on cotton and other plants, suggesting that condensed tannins reduce growth in lepidopteran larvae (Feeny 1968; Chan et al. 1978; Kopper et al. 2002; Nomura and Itioka 2002; but see the study of Ayers et al. 1997 for a review). Also, while consumption of condensed tannins extracted from cotton has been shown to reduce larval mass in artificial diet experiments (Chan et al. 1978; Klocke and Chan 1982; Reese et al. 1982; Navon et al. 1983), the opposite is true *in planta*, where greater levels of foliar condensed tannins have either been positively correlated with or shown not to influence larval weight (Hanny 1980; Hedin et al. 1983; Mulrooney et al. 1985; McColl and Noble 1992; Smith et al. 1992). Although our results agree with previous experiments that used plant foliage, the tannin levels observed in the plants used in our experiments were fairly low, possibly resulting from suboptimal lighting (Dudt and Shure 1994). Thus, we cannot rule out the possibility that tannin levels in our plants were simply too low to have an effect on *H. virescens* pupal weight. At the same time, inconsistencies in experimental outcomes between artificial diet and *in planta* are common and likely reflect the numerous differences in chemical composition between artificial diet and plant foliage. The activity of specific phenolic compounds is known to depend in large part on chemical mixture and the environment in which reactions occur (Bernays et al. 1989; Appel 1993; Duffey and Stout 1996; Hoover et al. 1998b, c).

Just as we did not observe an influence of SAR activation on growth of *H. virescens* larvae, we also saw no influence of induced foliage on *H. virescens* larval susceptibility to baculoviral infection despite significant increases in peroxidase activity and condensed tannins. Peroxidase, condensed tannins, and dihydroxyphenolics have been implicated in altering baculoviral pathogenicity to lepidopteran larvae (Felton et al. 1987; Keating et al. 1988; Felton and Duffey 1990; Young et al. 1995; Hoover et al. 1998a, b; Ali et al. 1999). In particular, dihydroxyphenolics and tannins decrease mortal baculovirus infection in artificial diet experiments (Keating et al. 1989; Felton and Duffey 1990; Young et al. 1995).

While these compounds were demonstrated to influence baculoviral efficacy individually, they may act differently in mixture. Hoover et al. (1998b) found that, when considered individually, both peroxidase and condensed tannins reduced mortality of *H. virescens* larvae fed with cotton foliage. However, when considered in a mixed model, peroxidase reduced larval mortality, whereas condensed tannins increased mortality (Hoover et al. 1998c). Because we measured increased levels of both of these phytochemicals in induced foliage, it is possible that the failure of induced plants to affect *H. virescens* larval mortality may be explained by offsetting influences of peroxidase and condensed tannins, as suggested by the model of Hoover et al. (1998b).

The effect of SAR may have been different if the larvae had been fed through their entire life cycle on foliage. The underlying impetus for our experimental design was to specifically address a hypothesis suggested by Hoover et al. (1998c) that states that foliar peroxidase is negatively related to larval mortality by virus during the process of initial infection. Exposure to induced foliage during the instar in which viral challenge occurs was sufficient to test the proposed hypothesis but may not reflect what would happen in the field with long-term exposure, particularly given that feeding on plants throughout an insect's life cycle can have profound effects on insect–pathogen interactions (see review by Cory and Hoover 2006).

In summary, despite significant changes in several phytochemical components in BTH-treated plants, activators of SAR are likely to be compatible with microbial control agents such as baculoviruses in an IPM system.

Acknowledgments We thank Tony Pomicter and Mike Grove for their assistance with experiments and phenolic assays and Diana Cox-Foster, Kelly Johnson, and A. Daniel Jones for their valuable discussions. We thank Syngenta for supplying the BTH. This work was a part of the dissertation of R.P. and was funded by the National Science Foundation Integrated Organismal Biology Program, Grant No. IBN-0077710.

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