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Herbivore-Induced Defenses in Tomato Plants Enhance the Lethality of the Entomopathogenic Bacterium, *Bacillus thuringiensis* var. *kurstaki*

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Abstract

Plants can influence the effectiveness of microbial insecticides through numerous mechanisms. One of these mechanisms is the oxidation of plant phenolics by plant enzymes, such as polyphenol oxidases (PPO) and peroxidases (POD). These reactions generate a variety of products and intermediates that play important roles in resistance against herbivores. Oxidation of the catecholic phenolic compound chlorogenic acid by PPO enhances the lethality of the insect-killing bacterial pathogen, *Bacillus thuringiensis* var. *kurstaki* (*Bt*) to the polyphagous caterpillar, *Helicoverpa zea*. Since herbivore feeding damage often triggers the induction of higher activities of oxidative enzymes in plant tissues, here we hypothesized that the induction of plant defenses would enhance the lethality of *Bt* on those plants. We found that the lethality of a commercial formulation of *Bt* (Dipel® PRO DF) on tomato plants was higher if it was applied to plants that were induced by *H. zea* feeding or induced by the phytohormone jasmonic acid. Higher proportions of *H. zea* larvae killed by *Bt* were strongly correlated with higher levels of PPO activity in the leaflet tissue. Higher POD activity was only weakly associated with higher levels of *Bt*-induced mortality. While plant-mediated variation in entomopathogen lethality is well known, our findings demonstrate that plants can induce defensive responses that work in concert with a microbial insecticide/entomopathogen to protect against insect herbivores.

Keywords Biological control · *Helicoverpa zea* · Microbial insecticide · Peroxidase · Polyphenol oxidase · Tritrophic interactions

Introduction

Plants strongly influence the degrees of protection they obtain from microbial insecticides through numerous mechanisms (Cory and Hoover 2006; Shikano 2017). Plant structures, such as leaves, can protect entomopathogenic microbes on the plants by shading the microbes from harmful UV irradiation and being washed away by rain (Ment et al. 2017), while the surface chemistry of plants, such as high pH and allelochemicals, can inactivate some entomopathogens (McLeod et al. 1977; Stevenson et al. 2010; Young et al. 1977). Moreover, plants indirectly influence the effectiveness of microbial insecticides by altering the physiology of pest insects (Lampert 2012; Shikano 2017). These can include plant nutritional and allelochemical effects on insect immune functioning (Lee et al. 2006; Povey et al. 2014;Shikano et al. 2010, 2015b; Smilanich et al. 2011, 2017) and physical barriers to entomopathogens, such as the cuticle and peritrophic matrix (Lee et al. 2008; Plymale et al. 2008).

Microbial insecticides based on food-borne entomopathogens require target insects to ingest the entomopathogens as they feed on plants. Thus, entomopathogens come in contact with an array of phytochemicals that are released as the insect chews on plant tissues. Interactions between entomopathogens and phytochemicals in the insect's digestive tract can significantly influence the successful establishment of infections (Cory and Hoover 2006; Shikano 2017). For example, in numerous caterpillar–baculovirus systems, the potency of baculoviruses can vary widely depending on the species and genotypes of plants ingested with the infectious virus particles (Ali et al. 1998; Hoover et al. 1998a, b; Keating et al. 1988; Shikano et al. 2017a, b; Wan et al. 2016). Even variation within an individual plant, such as vegetative and reproductive parts ingested with the virus, can influence virus lethality (Ali et al. 1998). Most notable

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is the inhibition of virus-induced mortality when virus particles were ingested on plants that were previously damaged by caterpillar feeding (i.e., induced plants) (Hoover et al. 1998a; Shikano et al. 2017b).

The most widely used bacteria for the control of numerous insect pests are the *Bacillus thuringiensis* (*Bt*) sub-species, comprising about 98% of formulated, sprayable bacterial insecticides (Bravo et al. 2011; Lacey et al. 2015). *Bt* is a gram-positive bacterium that, during sporulation, produces crystalline inclusions containing insecticidal proteins called δ-endotoxins. The inclusions solubilize in insect midguts, releasing the δ-endotoxins, which upon proteolytic activation by endogenous *Bt* and exogenous larval midgut proteases, interact with specific binding sites on the midgut brush border membrane. After binding, pores form in the midgut cells that result in cell lysis, and eventually septicemia as the bacteria enter and replicate in the hemolymph (Bravo et al. 2007; Raymond et al. 2010; Vachon et al. 2012). Spores can also germinate in the gut prior to invasion of the haemocoel. These vegetative bacterial cells can produce other virulence factors involved in the destruction of the midgut tissues, which include other insecticidal toxins (Vip proteins) and an array of phospholipases and proteases (Brar et al. 2007; Estruch et al. 1996; Raymond et al. 2010). Numerous studies have demonstrated varied levels of mortality when *Bt* is used against a particular pest on different plants (Ali et al. 2004; Appel and Schultz 1994; Bauce et al. 2002; Carisey et al. 2004; Hwang et al. 1995; Jafary et al. 2016; Janmaat and Myers 2007; Janmaat et al. 2007; Kouassi et al. 2001; Meade and Hare 1993, 1994; Paramasiva et al. 2014). Tannins in plants have been repeatedly implicated in decreased effectiveness of *Bt* formulations against a variety of insects (Appel and Schultz 1994; Lord and Undeen 1990; Lüthy et al. 1985; Navon et al. 1993). Increasing levels of nicotine, an alkaloid in tobacco plants, had bactericidal effects on *Bt* var. *kurstaki,* decreasing mortality of *Manduca sexta* larvae (Krischik et al. 1988).

In contrast, some allelochemicals have been shown to increase *Bt* lethality. MacIntosh et al. (1990) demonstrated that the seed extracts from 21 agricultural plants significantly enhanced the toxicity of *Bt* var. *kurstaki* and var. *tenebrionis* toxins against lepidopteran and coleopteran insects, respectively. They also showed that a variety of plant and animal protease inhibitors enhanced the activities of *Bt* var. *kurstaki* toxins (MacIntosh et al. 1990). Other allelochemicals, including the non-protein amino acid, L-canavanine, increased the lethality of *Bt* commercial formulations to *Manduca sexta* (Felton and Dahlman 1984), and the simple phenolic compounds, resorcinol and gallic acid, increased the toxicity of *Bt* var. *galleriae* toxin to *Helicoverpa armigera* (Sivamani et al. 1992).

The plant oxidative enzymes polyphenol oxidases (PPO) and peroxidases (POD) play important roles in resistance against herbivores. These enzymes activate certain *o*-dihydroxyphenolics (e.g., chlorogenic acid) to *o*-quinones (e.g., chlorogenoquinone), which in turn alkylate dietary proteins and reduce their nutritive qualities for herbivores (Felton et al. 1989, 1992). Interestingly, Ludlum et al. (1991) found that the addition of chlorogenic acid and PPO to artificial diet enhanced the lethality of *Bt* var. *kurstaki* to *Helicoverpa zea.* They demonstrated that the formation of *o*-quinones and subsequent alkylation of the *Bt* protoxins enhanced the solubilization and/or proteolysis of the protoxins *in vivo*.

In tomato, feeding damage caused by common caterpillar pest species, such as *Helicoverpa zea*, *Manduca sexta* and *Spodoptera frugiperda*, strongly induce both PPO and POD activities (Acevedo et al. 2017; Stout et al. 1994, 1996, 1998; Thaler et al. 1996). Therefore, we hypothesized that the induction of higher plant PPO and POD activities in response to caterpillar feeding would enhance the ability of a foliar application of *Bt* to infect and kill the caterpillars. Similar to our hypothesis, Olsen et al. (2005) found that endotoxin (Cry1Ac)-producing *Bt* transgenic cotton plants that were induced by *H. armigera* feeding were dramatically more toxic to *H. armigera* that subsequently fed on those plants. They suggested that interactions between plant induced defensive chemicals and the Cry1Ac toxins may have enhanced the toxicity of the plants, since they detected no changes in the level of Cry1Ac expression or in the concentration of the *Bt* toxin (Olsen et al. 2005).

Materials and Methods

Insects

H. zea lab colonies were established from eggs purchased from Benzon Research (Carlisle, PA, USA). Lab colonies were reared on a wheat germ and casein-based artificial diet (including antibiotics) (Chippendale 1970) in 30 ml plastic cups, and maintained in a growth chamber at 27°C and 16:8 h (L:D) photoperiod. Larvae used in the experiments were reared in 128-well insect rearing trays (Frontier Agricultural Sciences, Newark, DE, USA) for 3 d on antibiotic-free artificial diet at 25°C and 16:8 h (L:D) photoperiod. Three-day old larvae were used in all *Bt* mortality assays.

Plants

Tomato (*Solanum lycopersicum* L. cv. Better Boy) plants were maintained in a temperature-controlled greenhouse at 25°C and 16:8 h (L:D) photoperiod, supplemented with high-pressure sodium lights. Plants were grown in 10 cm plastic pots in professional growing mix (Sunshine Mix 4 Aggregate Plus) with 3 g of Osmocote Plus slow release fertilizer (15-9-12; Scotts). Plants for the four trials were grown July – September 2017.

Induction of Plant Defenses

In total, four trials were conducted. We altered the ages of the plants and methods of plant induction among trials in an effort to produce different levels of constitutive and induced PPO and POD activities. Five-leaf stage plants were used in trials 1 and 2, and four-leaf stage plants were used in trials 3 and 4. In trials 1 and 2, two fifth instar *H. zea* larvae were placed on the youngest fully expanded leaf in individual clip-cages. One cage was placed on the terminal leaflet and the other cage was placed on the adjacent leaflet. Larvae fed for up to 3 h until the entire leaflet area inside the cage had been consumed (3.14 cm^2). In trial 3, individual larvae were caged on three leaflets. No insects or cages were placed on the non-induced treatment plants. In trial 4, we exogenously applied jasmonic acid (JA), a phytohormone that upregulates defenses against chewing herbivores in plants, including tomato (Thaler et al. 1996). Entire plants were sprayed with a 2 mM solution of JA until runoff (approx. 5 ml per plant), and then air-dried. JA was dissolved in 1 ml of 95% ethanol and dispersed in ultrapure water to the desired concentration. Non-induced plants were sprayed with the same concentration of ethanol in ultrapure water. Since induction of PPO and POD activities in tomato foliage reaches maximal levels at approximately 48 h after *H. zea* feeding damage (Stout et al. 1994) and JA application (Thaler et al. 1996), plants were used in bioassays 48 h after treatment. Only the *H. zea*-damaged leaflets and leaflets from the youngest fully expanded leaf of JA-treated plants were used for experiments. Leaflets in corresponding positions were used from non-induced plants. There were 12, 11, 10 and 10 induced plants and 11, 10, 10 and 10 non-induced plants used for plant enzyme activity assays and *Bt* mortality assays in trials 1, 2, 3 and 4, respectively. Enzyme activity assays and *Bt* mortality assays were conducted with leaflets collected from the same induced and non-induced plants.

Plant Enzyme Activity and Protein Concentration Assays

A sample of leaf tissue (approx. 50 mg) was collected from the herbivore-damaged and JA-treated terminal leaflet from each induced plant and from the terminal leaflet of each non-induced plant. Samples were weighed to the nearest 0.1 mg, placed individually in a 2 ml microcentrifuge tube, frozen immediately in liquid nitrogen and stored at –80°C. Frozen leaf samples were homogenized to a fine powder using a GenoGrinder 2000 (OPS Diagnostics, Bridgewater, NJ, USA). A modification of the technique of Bi and Felton (1995) was used to measure PPO and POD activities. Briefly, the powdered samples were suspended in 1.25 ml of ice-cold extraction buffer, which consisted of 5% of cross-linked polyvinylpyrrolidone (Alfa Aesar, Ward Hill, MA, U.S.A.) in 0.1 M potassium phosphate buffer (pH 7.0). Samples were vortexed for 10 s, kept on ice for 5 min, then centrifuged at 11,000 × g for 10 min at 4 °C. The resulting supernatant was used to measure PPO and POD activities.

For PPO activity, 5 μl of supernatant was combined with 200 μl of 3 mM caffeic acid in 0.1 M potassium phosphate buffer. The change in absorbance was measured in a 96-well microplate at 450 nm for 10 min using a Spectra Max 190 microplate reader (Molecular Devices, Silicon Valley, CA, USA). For POD activity, 5 μl of supernatant were combined with 10 μl of 0.3% hydrogen peroxide and 190 μl of 3 mM guaiacol in 0.1 M potassium phosphate buffer. The change in absorbance was measured at 450 nm for 10 min.

The concentration of protein in each sample was measured using the Bradford protein assay (Bradford 1976). This measurement was used to calculate the concentration of protein per unit leaflet tissue and the activities of PPO and POD relative to the total protein content in the leaflet (mOD/min/mg protein).

Bt Assays

Half of the *H. zea*-damaged and JA-treated leaflets and leaflets from non-induced plants were dipped for 10 s in a 9600 Cabbage Looper Units (CLU) ml^{-1} aqueous suspension of a commercial formulation of *Bacillus thuringiensis* var. *kurstaki* (strain ABTS-351; DiPel® PRO DF, Valent Biosciences, Libertyville, IL, USA), which contains fermentation solids, spores, and insecticidal protoxins. The suspension was constantly stirred to keep the *Bt* suspended in solution. The remaining half of the leaflets was dipped for 10 s in just ultrapure water to serve as untreated controls. All leaflets were gently agitated to remove excess water droplets and then placed on drying racks until all water droplets on the leaf surface had evaporated. The leaflets were then cut with a razor blade into equal sized pieces and placed in individual wells of a 128-well insect-rearing tray, which was lined with 1% agar to keep leaf pieces from desiccating. *Bt*-treated leaf tissues from each plant in all 4 trials were cut into 12 equal pieces. Untreated (*Bt*-free) leaf tissues from each plant in trials 1 and 2 were cut into 12 pieces. *Bt*-free leaflets in trials 3 and 4 were cut into only 8 pieces, because the leaflets were smaller than in the previous trials and more of the leaf tissues were needed to produce 12 *Bt*-treated leaflet pieces. There were 10–12 plants (i.e. replicates) per induction treatment (induced and non-induced) in each trial. One 3-d old *H. zea* larva was placed into each well and mortality was recorded 72 h later. A total of 144, 120, 120 and 120 larvae were exposed to *Bt* on induced leaflets and 132,120, 118 and 116 larvae were exposed to *Bt* on non-induced leaflets in trials 1, 2, 3 and 4, respectively. A total of 144, 132, 80 and 80 larvae were exposed to induced *Bt*-free leaflets and 132, 120, 80 and 80 larvae were exposed to non-induced *Bt*-free leaflets in trials 1, 2, 3 and 4, respectively.

Plant Resistance to *H. zea*

In the third and fourth *Bt* assay trials, four larvae feeding on the *Bt*-free leaflets from each plant (4 out of 8 control larvae) in

each plant induction treatment were haphazardly selected and weighed to the nearest 0.1 mg to assess plant-mediated effects on *H. zea* growth (total of 40 larvae per plant induction treatment in each trial). Larvae were not weighed in trials 1 and 2.

Statistical Analyses

PPO and POD activities were analyzed by two-way analysis of variance (ANOVA), with trial, plant induction treatment (induced vs. non-induced) and their interaction included as factors. PPO activity was square root transformed and POD activity was $\log_{10}$ transformed to meet the assumption of normality. *H. zea* larval weight after 72 h of feeding in trials 3 and 4 were also analyzed by two-way ANOVA, but since 4 larvae were weighed for each individual leaflet, the leaflet ID (i.e., plant ID) was included as a random effect.

Mortality in the *Bt*-treatment was corrected for control mortality in trials 3 and 4 using Abbott's formula (Abbott 1925). There was no control mortality in trials 1 and 2. Corrected *Bt*-induced mortality and control mortality for each plant were analyzed by generalized linear model, using a binomial distribution and logit link. Trial, plant induction treatment and their interaction were included as factors. Linear regression was used to determine if PPO activity, POD activity and/or tissue protein concentration were predictive of mortality from *Bt*.

Results

Plant PPO and POD Activities

Plants treated with JA or damaged by caterpillar feeding, regardless of the number of caterpillars per plant, had significantly higher PPO activity compared to control plants (Fig. 1a; Induction treatment: $F_{1,79} = 9.93$, $P = 0.002$), though the levels of PPO activity varied depending on when the plants were grown during the summer (i.e., trial; $F_{3,79} = 4.36$, $P = 0.006$). There was no interaction between induction treatment and trial ($F_{3,76} = 0.87$, $P = 0.46$).

POD activity was also significantly induced to higher levels (Fig. 1b; $F_{1,79} = 16.69$, $P = 0.0001$) and the levels of POD activity varied among trials ($F_{3,79} = 3.51$, $P = 0.02$). Although the interaction between induction treatment and trial was not significant ($F_{3,76} = 2.31$, $P = 0.08$), means contrasts of POD activity between induced and non-induced plants in each trial indicated that caterpillar feeding damage (trials 1, 2 and 3) induced significantly higher POD activity ($P < 0.05$ in each trial) whereas JA application did not ($P = 0.90$).

Protein Content of Leaflet Tissues

The mean protein concentration in the leaflets of induced plants (13.51 ± 0.54 μg mg^{-1}) was significantly lower than the leaflets of non-induced plants (15.61 ± 0.63 μg mg^{-1}) ($F_{1,79} = 6.65$, $P = 0.01$). Protein concentration did not significantly differ among trials ($F_{3,79} = 2.37$, $P = 0.08$) and there was no interaction between induction treatment and trial ($F_{3,76} = 2.12$, $P = 0.10$)

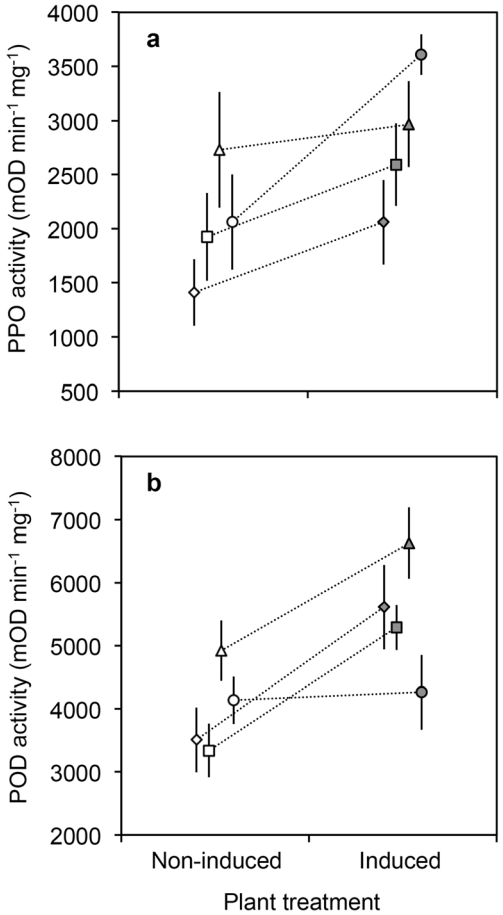

Fig. 1 Mean (**a**) PPO and (**b**) POD activities were significantly higher in induced than non-induced leaflets. Plants were induced by feeding damage sustained from two fifth instar *H. zea* larvae (trials 1 and 2), three fifth instar larvae (trial 3) and JA application (trial 4). Different symbols indicate mean (± SE) values from each trial (trial 1, diamond; trial 2, square; trial 3, triangle; trial 4, circle). Dotted lines connect treatment means in each trial for clearer visualization of induction effects

Plant Resistance to *H. zea* Larvae

Larvae fed on the *Bt*-free leaflets from plants that were induced by previous *H. zea* feeding damage (Trial 3) or JA application (Trial 4) weighed significantly less after 72 h compared to larvae fed on *Bt*-free leaflets from non-induced plants (Fig. 2; induced vs. non-induced: $F_{1,37} = 20.63$, $P < 0.0001$). This confirmed that the induction treatments increased resistance of the plants to *H. zea* by elevating the plant's antiherbivore defenses and/or decreasing nutritional (protein) quality. Larvae weighed less in trial 3 than in trial 4 ($F_{3,79} = 3.51$, $P = 0.02$), but this was not due to the use of caterpillar feeding vs. JA application for plant induction (i.e., no interaction between trial and induction; $F_{1,36} = 1.88$, $P = 0.18$).

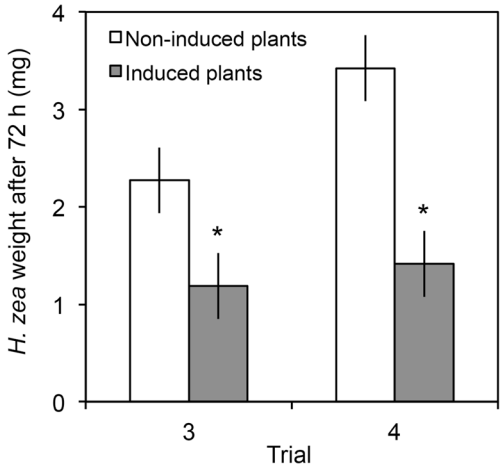

Fig. 2 Mean (± SE) weights of *H. zea* larvae were significantly lower after feeding on induced than non-induced leaflets for 72 h. These measurements were taken from the control leaflets (i.e., not treated with *Bt*) from the *Bt* assays in trials 3 and 4. Plants were induced by the feeding damage sustained from three fifth instar larvae (trial 3) and JA application (trial 4). Asterisk above bars indicates a significant difference in mean *H. zea* weight between induced and non-induced leaflets in each trial ($\alpha < 0.05$)

Bt-Induced Mortality

A significantly higher proportion of *H. zea* larvae died if they ingested *Bt* on induced leaflets than on non-induced leaflets (Fig. 3; $X^2_1 = 36.58$, $P < 0.0001$). *Bt* lethality and the degree to which it was enhanced on induced leaflets varied by trial (induction treatment by trial: $X^2_3 = 13.34$, $P = 0.004$). *Bt* lethality increased the most when plants were induced by JA application (Trial 4) compared to no induction (55% increase; means contrast, $X^2_1 = 30.80$, $P < 0.0001$). Plants induced by caterpillar feeding damage also elicited higher *Bt* lethality than undamaged plants (Trial 1: 15% increase, $X^2_1 = 2.37$, $P = 0.12$; Trial 2: 22% increase, $X^2_1 = 4.07$, $P = 0.04$; Trial 3: 14% increase, $X^2_1 = 8.62$, $P = 0.003$).

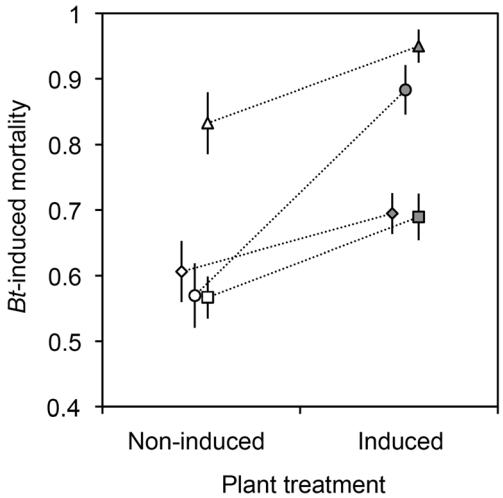

Fig. 3 Mean proportional mortality of *H. zea* larvae was significantly higher on induced leaflets treated with *Bt* than non-induced treated with *Bt.* Proportional *Bt*-induced mortality was corrected for control mortality. Twelve larvae were challenged with *Bt* on each plant, and 10–12 plants were used per plant induction treatment in each trial. Plants were induced by the feeding damage sustained from two fifth instar *H. zea* larvae (trials 1 and 2), three fifth instar larvae (trial 3) and JA application (trial 4). Different symbols indicate mean (± SE) mortality values from each trial (trial 1, diamond; trial 2, square; trial 3, triangle; trial 4, circle). Dotted lines connect treatment means in each trial for clearer visualization of induction effects

Induced plant defenses had no significant effect on the mortality of *H. zea* larvae over a 72 h period in the absence of *Bt* (i.e., control mortality) (induction treatment: $X^2_1 = 0.86$, $P = 0.35$; induction treatment by trial: $X^2_3 = 4.94$, $P = 0.18$). Mortality on *Bt*-free leaflets varied among trials ($X^2_3 = 22.72$, $P < 0.0001$), but was overall very low. Control mortality on non-induced and induced leaflets were 5% (4/80 larvae) and 3.75% (3/80 larvae), respectively in trial 3, and 0% and 5% (4/80 larvae), respectively in trial 4. No larvae died on induced or non-induced control leaflet pieces in trials 1 and 2.

Relationships Between *Bt*-Induced Mortality and Plant Enzyme Activities and Protein Content

Higher mean PPO activities from each induction treatment in each trial was tightly correlated with higher mean *Bt*-induced mortalities from each induction treatment in each trial (Fig. 4a; $F_{1,6} = 14.68$, $P = 0.009$; $R^2 = 0.71$). Higher mean POD activity was weakly associated with higher mean *Bt*-induced mortality (Fig. 4b; $F_{1,6} = 5.13$, $P = 0.06$; $R^2 = 0.46$). There was no correlation between mean tissue protein concentration and mean *Bt*-induced mortality ($F_{1,6} = 1.47$, $P = 0.27$).

When we assessed the same relationships using values from individual plants, higher PPO and POD activities were weakly associated with higher *Bt*-induced mortality on those plants (PPO: $F_{1,82} = 16.44$, $P < 0.0001$; $R^2 = 0.17$; POD: $F_{1,82} = 5.34$, $P = 0.02$; $R^2 = 0.06$). The strength of the correlations using values from individual plants were dramatically weaker compared to using the mean values, likely due to the low resolution of *Bt*-induced mortality on individual plants (only 12 larvae were challenged per plant with *Bt*). Tissue protein concentration was not significantly associated with *Bt*-induced mortality ($F_{1,82} = 2.44$, $P = 0.12$).

Discussion

In the present study, the induction of anti-herbivore defenses in tomato plants significantly increased the proportion of *H. zea* larvae killed by a commercial insecticidal formulation of the entomopathogen *Bt* var. *kurstaki* (Dipel® PRO DF). The levels of *Bt*-induced mortality were correlated with the levels of PPO and POD activities in the *Bt*-treated leaflets. The positive correlations between the activities of oxidative enzymes and *Bt* lethality is consistent with the mechanism described by Ludlum et al. (1991), suggesting that the alkylation of *Bt* protoxins *in vivo* by orthoquinones formed from the catalysis

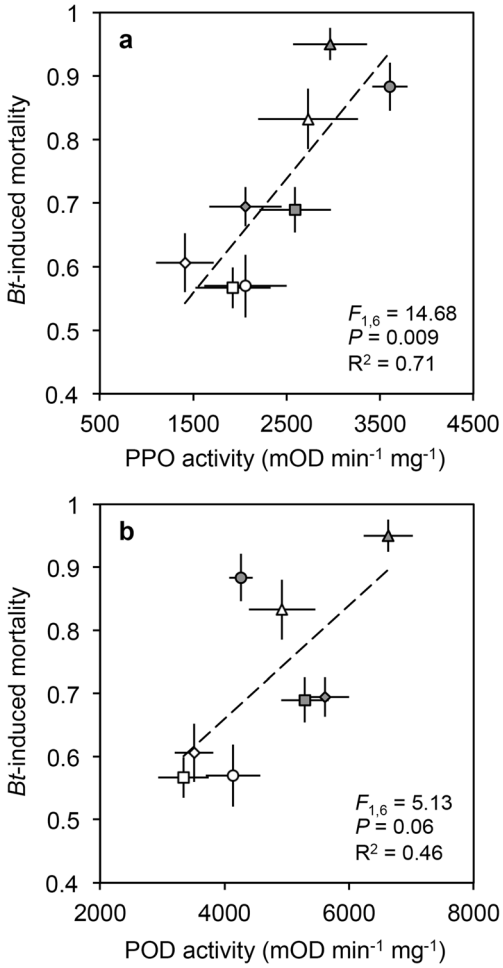

Fig. 4 Mean *Bt*-induced mortality was (**a**) strongly positively correlated with mean foliar PPO activity and (**b**) moderately positively correlated with mean foliar POD activity. Plants were induced by the feeding damage sustained from two fifth instar *H. zea* larvae (trials 1 and 2), three fifth instar larvae (trial 3) and JA application (trial 4). Different colors (white, non-induced; grey, induced) and symbols indicate mean (± SE) values from each trial (trial 1, diamond; trial 2, square; trial 3, triangle; trial 4, circle)

of chlorogenic acid by PPO subsequently increases the solubilization and/or proteolysis of the toxins (Ludlum et al. 1991). Though we did not measure it in the present study, chlorogenic acid, the major *o*-dihydroxyphenolic compound in tomato tissues, can also be induced to higher levels by herbivory (Broadway et al. 1986). Our findings are also consistent with a study by Olsen et al. (2005), which found that *Bt* transgenic cotton plants that had been induced by *H. armigera* feeding were more toxic to *H. armigera* that subsequently fed on those plants.

Interestingly, the levels of PPO activity were considerably more strongly correlated than POD activity with *Bt* lethality. While both PPO and POD catalyze phenolic oxidation, the types and mechanisms of end-product formation during catalysis by these two enzymes differ. PPO in most plants readily oxidize *o*-dihydroxyphenolics (e.g. chlorogenic acid, caffeic acid, and catechin) but has no activity on the mono-hydroxyphenolics (e.g. ferulic, p-coumaric, or p-hydroxybenzoic acids) (Constabel and Barbehenn 2008). In contrast, many PODs from plants have much broader substrate specificity with potent abilities to oxidize mono-hydroxyphenolics (Robinson and Eskin 1991), although PODs from cotton and tomato are reportedly more active on *o*-dihydroxyphenolics than on mono-hydroxyphenolics (Hoover et al. 1998b). Catalysis of phenolics by PPO directly produces quinones using O_2 as a co-substrate (Constabel and Barbehenn 2008), but because POD catalyzes oxidation of a wider range of substrates, free radical intermediates are produced in the process, including semiquinones, active oxygen species and other organic radicals (Robinson and Eskin 1991). We propose that the direct production of quinones by PPO acts to enhance *Bt* lethality on induced plants, and that the catalysis of phenolics by POD has less of an effect. This is supported by our data showing that low PPO induction in trial 1, 2 and 3 weakly increased *Bt* lethality even though there was high POD induction. Also, strong induction of PPO by JA application in trial 4 strongly increased *Bt* lethality even though JA did not induce POD. Hoover et al. (1998a) found a similar differential effect of plant PPO and POD activities on baculovirus lethality, such that PPO had no effect while POD inhibited viral disease, which was shown to occur by production of free radicals by POD activity. Similarly, on *Bt* transgenic cotton plants, the toxicity of *Bt* toxins to caterpillars were enhanced by the induction of cotton defenses against caterpillar feeding but not aphid feeding (Olsen et al. 2005); this is not surprising since these two insects use different modes of feeding and consequently induce different plant defenses (Howe and Jander 2008).

Importantly, our findings are only correlations and could have also resulted from a number of plant-mediated effects on *Bt* and/or insect physiology. Plants have a vast array of anti-herbivore defenses, many of which are inducible (Agrawal 2011; Chen 2008; Schaller 2008; Zhu-Salzman et al. 2008). Several allelochemicals have been linked to increased *Bt* lethality, including protease inhibitors (MacIntosh et al. 1990), simple phenolic compounds (Sivamani et al. 1992), and a non-protein amino acid (Felton and Dahlman 1984). Induced plant defenses, such as a cysteine protease in maize, can disrupt the peritrophic matrix of caterpillars (Mohan et al. 2006; Pechan et al. 2002). The peritrophic matrix is an important physical barrier against food-borne pathogens. Thus, a thinner and/or structurally damaged peritrophic matrix can increase insect susceptibility to *Bt* (Granados et al. 2001), and other pathogens (Plymale et al. 2008; Vijendravarma et al. 2015).

Allelochemicals can also influence the functioning of insect immune responses (Bukovinszky et al. 2009; Laurentz et al. 2012; Ojala et al. 2005; Smilanich et al. 2011, 2017). Plant-mediated variation in insect immune functioning is likely to influence the outcome of *Bt*-challenge because the germination of *Bt* spores and replication of *Bt* cells in the hemolymph can play an important role in insect mortality (Cornforth et al.

2015; Raymond et al. 2010). Correspondingly, humoral and cellular immune responses in the hemolymph and gut lumen, especially the levels of melanization and antibacterial activity in the hemolymph, have been associated with heightened insect resistance to *Bt* (Grizanova et al. 2014; Inagaki et al. 1992; Ma et al. 2005; Rahman et al. 2004; Shikano et al. 2015a; Tamez-Guerra et al. 2008). Additionally, plants possess their own bacteriostatic and bacteriocidal compounds that can inhibit the growth of *Bt* cells (Smirnoff and Hutchison 1965). When ingested by insects, these compounds may persist or accumulate in the insect gut and body tissues, thereby influencing *Bt* growth within the insect. For example, increasing concentrations of nicotine in *Bt*-treated diet decreased *Bt*-induced mortality in *Manduca sexta* larvae (Krischik et al. 1988). This was associated with decreasing colony growth of *Bt* on nutrient agar containing increasing concentrations of nicotine (Krischik et al. 1988). Plants also have ecological effects on entomopathogen fitness, yield and transmission (Raymond and Hails 2007; Raymond et al. 2002; Shikano et al. 2017a), which is important when pathogen cycling is a key component of control, such as for baculoviruses and fungi. Whether the insect's food plants influence *Bt* spore production and natural cycling requires further investigation.

Lastly, the macronutrient content of the food ingested before or with *Bt* spores and/or toxins can influence insect susceptibility (Deans et al. 2017; Orpet et al. 2015; Shikano and Cory 2014). Deans et al. (2017) found that *H. zea* larvae were more susceptible to *Bt* toxins if they ingested the toxins on a lower protein-to-carbohydrate ratio artificial diet, though the mechanism is unknown. Generalist lepidopteran larvae are known to increase their consumption rate to compensate for low dietary protein content (Deans et al. 2015; Lee et al. 2003; Shikano and Cory 2014) and for the presence of protease inhibitors that reduce the digestibility of proteins (De Leo et al. 1998). In our study, induced tomato leaflets had a lower concentration of protein and higher activities of oxidative enzymes that reduce the nutritive qualities of proteins, compared to non-induced leaflets. Thus, it is possible that *H. zea* larvae in our study ate more induced foliage to compensate and consequently ingested more *Bt*. Nevertheless we think that this is unlikely since there was no correlation between foliar protein concentration and levels of *Bt*-induced mortality.

To conclude, we have demonstrated a novel multitrophic interaction whereby a plant's induced anti-herbivore defenses work in concert with an insect pathogen to increase caterpillar mortality. We propose that the most likely mechanism is through the induction of PPO activity. Since PPOs are widespread in plants and are well known to be inducible, not just by herbivory but also through wounding and JA application (Zhu-Salzman et al. 2008), the enhancement of *Bt* lethality through PPO induction is likely to be relevant for other plant species. Understanding how a plant's natural defenses influence the lethality of insect pathogens is critical for predicting the effectiveness of microbial insecticide applications. Moreover, particularly for *Bt*, identifying intrinsic plant factors that enhance *Bt* toxicity could inform the development of improved insect-resistant transgenic crops.

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