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Parasitic Wasp Mediates Plant Perception of Insect Herbivores

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Abstract

Microplitis croceipes is a solitary parasitoid that specializes on noctuid larvae of *Helicoverpa zea* and *Heliothis virescens.* Both the parasitoid and its hosts are naturally distributed across a large part of North America. When parasitoids deposit their eggs into hosts, venom and polydnaviruses (PDVs) are also injected into the caterpillars, which can suppress host immune responses, thus allowing parasitoid larvae to develop. In addition, PDVs can regulate host oral cues, such as glucose oxidase (GOX). The purpose of this study was to determine if parasitized caterpillars differentially induce plant defenses compared to non-parasitized caterpillars using two different caterpillar host/plant systems. *Heliothis virescens* caterpillars parasitized by *M. croceipes* had significantly lower salivary GOX activity than non-parasitized caterpillars, resulting in lower levels of tomato defense responses, which benefited parasitoid performance by increasing the growth rate of parasitized caterpillars. In tobacco plants, parasitized *Helicoverpa zea* caterpillars had lower GOX activity but induced higher plant defense responses. The higher tobacco defense responses negatively affected parasitoid performance by reducing the growth rate of parasitized caterpillars, causing longer developmental periods, and reduced cocoon mass and survival of parasitoids. These studies demonstrate a species-specific effect in different plant-insect systems. Based on these results, plant perception of insect herbivores can be affected by parasitoids and lead to positive or negative consequences to higher trophic levels depending upon the particular host-plant system.

Keywords Parasitoid · Plant defenses · Herbivore · Oral secretions · Saliva · Glucose oxidase · Tritrophic interactions

Introduction

Caterpillar parasitism commonly occurs in nature, depending on location and host plant species. For example, larval parasitism rates of large white butterflies (*Pieris brassicae*) can range over 35% on cabbage (Razmi et al. 2011) and more than 70% of fall armyworm (*Spodoptera frugiperda*) were parasitized on corn (Ashley et al. 1983; Ashley 1986). Moreover, corn earworm (*Helicoverpa zea*) parasitism rates can range from 50% to 82% (King and Coleman 1989; Tipping et al. 2005; Young and Price 1975). The parasitoids attacking these caterpillars alter their host's physiology in many ways, some of which can have cascading effects across multiple trophic levels. For example, several recent studies showed that parasitoids can indirectly affect plant defense responses through changing host caterpillar's oral secretions and feeding behavior. Poleman et al. (2011) found that the color of oral regurgitant from *Pieris rapae* and *P. brassicae* caterpillars changed after parasitization by *Cotesia glomerata* and *Hyposoter ebeninus*. In addition, the regurgitant of parasitized caterpillars induced higher transcription of plant defense-related genes in cabbage, thus reducing diamondback moth ovipositional preference. More recently it was reported that cabbage plants expressed unique transcriptional levels (enhanced expression of glucosinolate metabolic genes) and produced different volatile compounds when being fed on by parasitized caterpillars (*Pieris spp.*) (Zhu et al. 2015). Additionally, cabbage plants produced 1.5 times more indole glucosinolates (glucobrassicin and neoglucobrassicin) when damaged by parasitized *Trichoplusia ni* due to more plant tissue consumption (Ode et al. 2015).

Oviposition by parasitoids such as braconid and ichneumonid wasps massively transforms their caterpillar host's physiology when they inject a cocktail of eggs, venom and polydnaviruses (PDVs) into caterpillar hosts (Asgari 2006; Burke and Strand 2012). PDVs function as a mutualist for the wasp larvae development because they express virulence genes to suppress host's immune response and ensure parasitoid larval develop (Asgari and Rivers 2011; Burke and

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Strand 2012; Fathpour and Dahlman 1995; Hegazi et al. 2005; Tanaka and Vinson 1991). Earlier study showed that PDV genes are expressed in multiple host caterpillar tissues including the salivary glands (Bitra et al. 2011). Labial gland proteins in caterpillar saliva play a crucial role in providing cues that trigger or suppress plant defense responses (Acevedo et al. 2015; Rivera-Vega et al. 2017, 2018). Thus, parasitoids may strongly influence the perception of insect herbivores by plants via manipulating caterpillar salivary cues.

PDVs play an important and complex role in plant-insect interactions by not only suppressing the host caterpillar's immune system, but also by indirectly downregulating plant defenses for their own benefit (Tan et al. 2018). Corn earworm (*H. zea*) parasitized by *M. croceipes* had lower elicitor activity in their saliva (i.e., glucose oxidase, GOX) and significantly downregulated tomato defense-related gene expression and defense protein activities during feeding, compared with non-parasitized caterpillars. The ultimate cause of downregulation of plant defense responses was due to the activity of the parasitoid's obligate mutualist, PDV. PDVs suppressed GOX gene expression and activity in parasitized caterpillar salivary glands, thereby downregulating plant defense responses. The lower induced plant defenses benefitted the parasitoid by promoting parasitized caterpillar growth, producing heavier cocoon masses and overall higher parasitoid survival (Tan et al. 2018). In other studies, salivary elicitors were downregulated in *P. brassicae* parasitized by *C. glomerata*; the PDV and venom of the parasitoid suppressed the expression of the elicitor gene glucose dehydrogenase (GDH) (Cusumano et al. 2018) and of the enzyme β-glucosidase in the caterpillars' salivary glands (Zhu et al. 2018).

These studies demonstrate that plants can distinguish damage by parasitized and non-parasitized caterpillars and respond accordingly. The induction or suppression of plant defenses caused by parasitoids may rely on the changes in physiology (saliva cues) or behavior (feeding amount) traits of host caterpillars. For some parasitoid species the host caterpillar as well as the host plant can vary. For example, *M. croceipes* (Hymenoptera, Braconidae) is a solitary endoparasitoid of several noctuid species, including *Helicoverpa zea*, *Heliothis virescens* and *Heliothis subflexa*, all of which are naturally distributed in North America (Hopper and King 1984; Lewis and Snow 1971; Smith et al. 1976). *Helicoverpa zea* and *H. virescens* are generalist herbivores that feed on many plant families, including Solanaceae, Fabaceae, Malvaceae, Plantaginaceae, Geraniaceae and Asteraceae (Fitt 1989; Neunzig 1963; Stadelbacher et al. 1984). Insect oral secretions may mediate plant defenses differentially depending on host plant identity (Acevedo et al. 2015). For example, GOX can cause induction/suppression in tomato/tobacco plant defenses in a species-specific manner (Musser et al. 2002; Tian et al. 2012; Zong and Wang 2004).

Our primary hypothesis was that the *M. croceipes* parasitoid can manipulate host caterpillar salivary cues and thereby suppress plant defenses in tomato and tobacco. First, we investigated interactions between the caterpillar *H. virescens* and the parasitoid -*M. croceipes* in tomato, and secondly between *H. zea* and *M. croceipes* in tobacco.

Materials and Methods

Insects

Corn earworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*) eggs were purchased from Benzon Research (Carlisle, PA). Larvae of both species were fed on artificial diet (Peiffer and Felton 2005) and reared individually until pupal formation. Thirty to 40 pupae were kept in a container [19 cm (diameter) × 28 cm (height)] and a 10% sugar solution was provided as food for adults. Eggs were collected daily for the experiments. The two colonies were kept in our lab for multiple generations.

Microplitis croceipes pupae were kindly provided by Dr. Henry Fadamiro (Auburn University) and a colony was established and maintained in our lab. Briefly, 10 *H. zea* caterpillars (second and/or third instars) were exposed to one female parasitoid for 1 h in a petri dish (9 cm diameter). Twenty-40 female parasitoids were used for every group of larvae exposed to parasitism. After parasitization, caterpillars were fed on artificial diet and reared individually. Parasitoid pupal cocoons were collected, and emerged adults were kept in a container (27 cm × 15 cm × 11 cm) with a 20% honey solution. All insect colonies were reared in a growth incubator (25 ± 2 °C, 16 L:8D).

Plants

Tomato (*Solanum lycopersicum* cv. Betterboy) and tobacco (*Nicotiana tabacum* cv. Xanthi) seeds were sown in potting soil (Sunshine Mix4 Aggregate Plus, Sungrow Horticulture) in a greenhouse (16 L:8D) at Pennsylvania State University. After germination, tomato (2 week-old) and tobacco (3 week-old) seedlings were transferred to pots (10 cm × 10 cm × 9 cm), and 3 g of fertilizer (Osmocote, 15–9-12) was applied. Plants were watered daily. Tomatoes with five fully expanded leaves (4–5 week-old) and tobacco plants with six fully expanded leaves (7–8 week-old) were used in the following experiments.

Caterpillar Salivary Glucose Oxidase Enzyme Activities

To determine how parasitism affects insect GOX enzyme activity, labial glands were collected and examined from parasitized and non-parasitized caterpillars. On the last day of the

second instar (with head capsule slippage), *H. zea* and *H. virescens* larvae were parasitized by *M. croceipes* females. Caterpillars were removed immediately following a single oviposition by the female parasitoid and reared individually.

Salivary glands were collected from parasitized (P) caterpillars 6 d after parasitization (Tan et al. 2018). Non-parasitized (NP) caterpillar salivary glands were collected at the same developmental stage as P-caterpillars. GOX enzyme activity was analyzed as described previously (Eichenseer et al. 1999). Briefly, labial glands were homogenized with 30 μL of phosphate buffer (0.1 M, pH 7) using a grinder (Pellet Pestle motor, Kontes). Supernatant was collected after centrifugation (4 °C, 11,000 rpm, 10 min). Five μL of each sample was mixed with 200 μL of substrate (1.3 mg dianisidine-HCL (Sigma D-3252), 2.5 mL of phosphate buffer (0.1 M, pH 7), 0.5 ml of D-glucose (100 mg/mL, Aldrich 253,073), and 20 μL of horseradish peroxidase (1 mg/mL, Sigma P2088)) and the change in absorbance value was measured at 460 nm using a microplate reader (Spectramax 190, Molecular Devices). Protein concentration in each sample was quantified by Bradford assay using BSA (bovine serum albumin, Fraction V, Omnipur) as the protein standard (Bradford 1976).

Plant Defense Responses

To determine how parasitized caterpillars influence plant-defense responses, we used three treatments in the experiment: Parasitized-caterpillar feeding (P), non-parasitized caterpillar feeding (NP), and intact control plants (C). One P- or NP-caterpillar was placed in a clip cage on the third (counting from the bottom) terminal leaflet of each tomato plant. This method controlled for caterpillar leaf consumption (3.15 cm^2), location and duration of feeding. In the control treatment, an empty cage was placed on the plant. Leaf cages were removed when the *H. virescens* caterpillar consumed all leaf tissue inside the cage within 10 h. The same methods were used for the *H. zea*-tobacco system. Briefly, one P- or NP-caterpillar was caged on the 4th leaf of each tobacco plant and the clip cage was removed when the caterpillar had consumed the entire leaf area inside the cage within 12 h.

Twenty-four hours after placing the caterpillars on the plants, 100 mg of the treated leaflet was collected. RNA extraction, cDNA synthesis and qRT-PCR analysis were performed as described (Tan et al. 2018). Reference genes (actin and ubiquitin) were used and the relative expression of target genes was compared with intact controls (C) by using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Primers used in this assay are listed in Table 1.

Forty-eight hours after caterpillar feeding, 50 mg of plant tissues were collected from the treated leaf for polyphenol oxidase (PPO), peroxidase (POD) and trypsin inhibitor (TI) enzyme assays. PPO and POD assays were performed as described by Felton et al. (1989). Briefly, samples were powdered with a Genogrinder (Spex Sample Prep 2000) and phosphate butter (1.25 mL, 0.1 M, pH 7) with 5% PVP (Alfa Aesar 41,631) was added to each sample. Samples were set on ice for 10 min. Supernatant was collected after centrifugation (4 °C, 11,000 rpm, 10 min). For PPO activity, 5 μL of sample was added to 200 μL of caffeic acid (3 mM, Sigma C0625) and 5 μL of supernatant was mixed with 10 μL of hydrogen peroxide (3%, CareOne) and 190 μL of guaiacol (3 mM, Sigma G5502) for POD assays. The change in absorbance at 450 nm was recorded in a plate reader (Spectramax 190, Molecular Devices) for both PPO and POD assays. Protein concentration in each sample was quantified by Bradford assay using BSA as the protein standard (Bradford 1976).

For trypsin inhibitor (TI) activity assays, samples were powdered with a Genogrinder and 1.25 mL of assay buffer (0.046 M Tris and 0.0115 M $CaCl_2$; pH 8.1; 5% PVP) was added. Supernatant (4 °C, 11,000 rpm, 10 min) was collected for TI activity measurements. Ten microliters of each sample was mixed with 10 μL of Trypsin (20 μg/mL, Sigma T1426) and assay buffer (80 μL). Ten minutes later, TAME (p-toluene-sulfonyl-l-arginine methyl ester, 100 μL, 0.002 M, Sigma T4626) was added and absorbance values were recorded at 247 nm (Chung et al. 2013). Percent inhibition of each sample was calculated by comparing to the activity of trypsin and assay buffer alone. Protein concentration in each sample was quantified by the Bradford assay (Bradford 1976) using BSA as the standard.

Insect Saliva and Plant Defense Responses

To verify if saliva is responsible for the different levels of plant defense responses that we observed from P- and NP-caterpillar feeding, plant defense responses were evaluated after applying insect saliva to wounded leaves. Caterpillars were parasitized as described above (plant defense responses). Salivary glands were collected from P-caterpillars 6 d after parasitization (Tan et al. 2018). NP-caterpillar salivary glands were collected at the same developmental stage as P-caterpillars. Glands were homogenized with phosphate buffer (0.1 M, pH 7) and supernatant was collected after centrifugation (4 °C, 7500 rpm, 10 min). Protein concentration in the supernatant was quantified by Bradford assay (Bradford 1976) using BSA as the standard. A serrated wounding tool (Bosak 2011) was used to wound the third terminal leaflet of tomato plants and the fourth leaf of tobacco plants (counting from the bottom); then 15 μL (1 μg/μL protein) of saliva homogenate was applied immediately from P- or NP-caterpillars. The control group consisted of intact control plants without treatment. For gene expression experiments, samples of the wounded leaf were collected 24 h after treatment. PPO, POD and TI activities were analyzed 48 h after treatment as described above. For all subsequent experiments, treatment of leaves

Table 1 Primer pairs used for tomato gene expression

Gene name	Description	Species	Forward	Reverse	Accession No.
CysPI	Cysteine proteinase inhibitor	Tomato	GGTGAAGGAATGGGAGGACT TCAA	GGAGGTTTGGGAATGGAACA TTGG	AF198390
PPOB	Polyphenol oxidase B	Tomato	TTCGCGAGTGGGAATACCTC GTTT	AGTCAGGGACTGTTTGGACA CGAA	Z12834
UBI	Ubiquitin	Tomato	GCCAAGATCCAGGACAAGGA	GCTGCTTTCCGGCGAAA	X58253
ACT	Actin-7	Tomato	AGGTGTTATGGTCGGAATGG	TCATCCCAATTGCTGACTATACC	AB199316

with saliva from caterpillars was conducted on wounded leaves or leaflets.

Caterpillar Host Performance

To evaluate the effect of plant-defense responses on P-caterpillar performance, larval relative growth rate experiments were performed. There were three groups in the experiment: caterpillars feeding on plants treated with P-caterpillar saliva or NP-caterpillars saliva, and intact control plants (C). Plants were treated with labial salivary gland homogenate as described above. Forty-eight hours after treatment, the treated leaf was collected for bioassay. Third instar *H. zea* and *H. virescens* of similar body size were selected and parasitized by *M. croceipes*. Caterpillars were weighed and then fed on the treated tomato/tobacco leaves in plastic cups lined with 2% agar to keep leaves moist. Forty-eight hours later, caterpillars were reweighed and relative growth rate was calculated as follows: (final weight − initial weight)/(average weight × no. of days) (Mohan et al. 2008).

Parasitoid Performance

To determine if different levels of induced plant defense responses caused by P- and NP-caterpillars influence parasitoid development, we conducted parasitoid performance experiments. Tobacco plants were treated with labial salivary gland homogenate from *H. zea* caterpillars as described above. Forty-eight hours after treatment, the treated leaf was collected and placed in a plastic cup lined with 2% agar to keep leaves moist. Third instar *H. zea* of similar body size were parasitized by *M. croceipes* and fed on one of three treatments: P-caterpillar-treated plants, NP-caterpillar-treated plants or intact control plants (*H. zea*: total $n = 35$; five replicates of seven individuals per treatment). Leaves were replaced every other day to keep food fresh until parasitoid larvae pupated. Larval duration, cocoon weight, pupal duration, total development time, larval mortality, cocoon formation failure rate, adult emergence rate, and survival rate were recorded. Cocoon weight was measured 2 days after cocoon formation, and adult emergence rate was calculated 30 days after cocoon formation. For the percentage of larval mortality, percentage of cocoon formation failure rate, percentage of adult emergence, and percentage of total survival, data were calculated from five replicates with $n = 7$ individuals per treatment.

Statistical Analyses

Data were transformed as needed to obtain a normal distribution and to address residuals with heterogeneity of variance; SAS 9.4 (SAS Institute Inc) was used for data analyses. Insect labial gland GOX enzyme activities were compared between treatments using Student's t test. Plant-defense responses (gene expression and TI and PPO activities), caterpillar performance bioassays (RGR) and parasitoid performance (larval duration, cocoon weight, pupal duration, total development period, percentage of larval mortality, cocoon formation, adult emergence, and total survival) were analyzed using one-way ANOVA (Proc GLM), followed by means comparisons using Fisher's Least Significant Difference (LSD) test (significance level, $P < 0.05$).

Results

Tomato-Heliothis Virescens-Microplitis Croceipes System

To determine if *M. croceipes* can influence *H. virescens* salivary enzyme activity with subsequent effects on induced plant defenses from parasitized caterpillar feeding, insect labial gland GOX activity and plant defense responses were measured. Parasitized *H. virescens* caterpillars had significantly lower GOX activity in their labial glands compared with non-parasitized caterpillars (Fig. 1). Tomato plants showed lower defense protein activities (POD and PPO) when fed on by P-caterpillars compared with NP-caterpillars (Fig. 2).

To determine if insect saliva was responsible for the observed differences in induced plant defenses, tomato defense-related gene expression and defense protein activities were measured after insect saliva application to wounded leaflets. Tomato plants showed lower transcript (*PPOB* and *CysPI*) levels and enzymatic activities (POD, PPO and TI) when saliva was applied from P-caterpillars compared with NP-

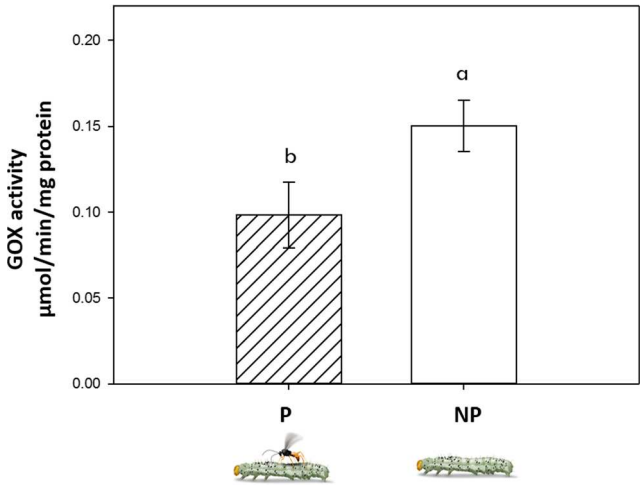

Fig. 1 ***Heliothis virescens*** **GOX enzyme activity at six days after being parasitized.** Values are untransformed means ± SEM. Different letters indicate significant differences between treatments. Treatments include parasitized (P) and non-parasitized (NP) caterpillars. Student's t test; $n = 13$, $F_{(1, 24)} = 4.58$, $P = 0.044$

caterpillars (Fig. 3). These results indicate that saliva was the factor responsible for the lower induction of tomato defense responses after parasitization.

To determine if difference in induced plant defenses influenced parasitoid performance, larval relative growth rate (RGR) were measured. Parasitized *H. virescens* had lower RGR when fed NP-caterpillar treated tomato plants (higher plant defenses) than the P-caterpillar treatment (Fig. 4). These results indicate that parasitized *Heliothis virescens* caterpillars had lower GOX activity in their labial glands after being parasitized and induced lower defense responses in tomato plants. Lower plant defenses induced by parasitized *H. virescens* caterpillars *benefitted* parasitoid performance by promoting parasitized caterpillar host growth.

Tobacco-*Helicoverpa zea*-Microplitis Croceipes System

To determine if *M. croceipes* parasitized *H. zea* could affect plant defense responses in a different plant system, tobacco plants were used in this study. In tobacco, GOX suppresses plant induced defense responses (Musser et al. 2002; Zong and Wang 2004). Therefore, we expected results from this experiment to be the opposite of what we saw in tomato, given that in tomato plant defenses are induced by GOX (Tian et al. 2012). *Microplitis croceipes* parasitized *H. zea* had significantly lower GOX gene expression at two days post parasitism (Tan et al. 2018) and showed lower GOX activity in their labial glands six days after being parasitized (Fig. 5), which in turn triggered higher plant defense protein activities (PPO and TI) compared with the NP-caterpillar treatment in tobacco (Fig. 6).

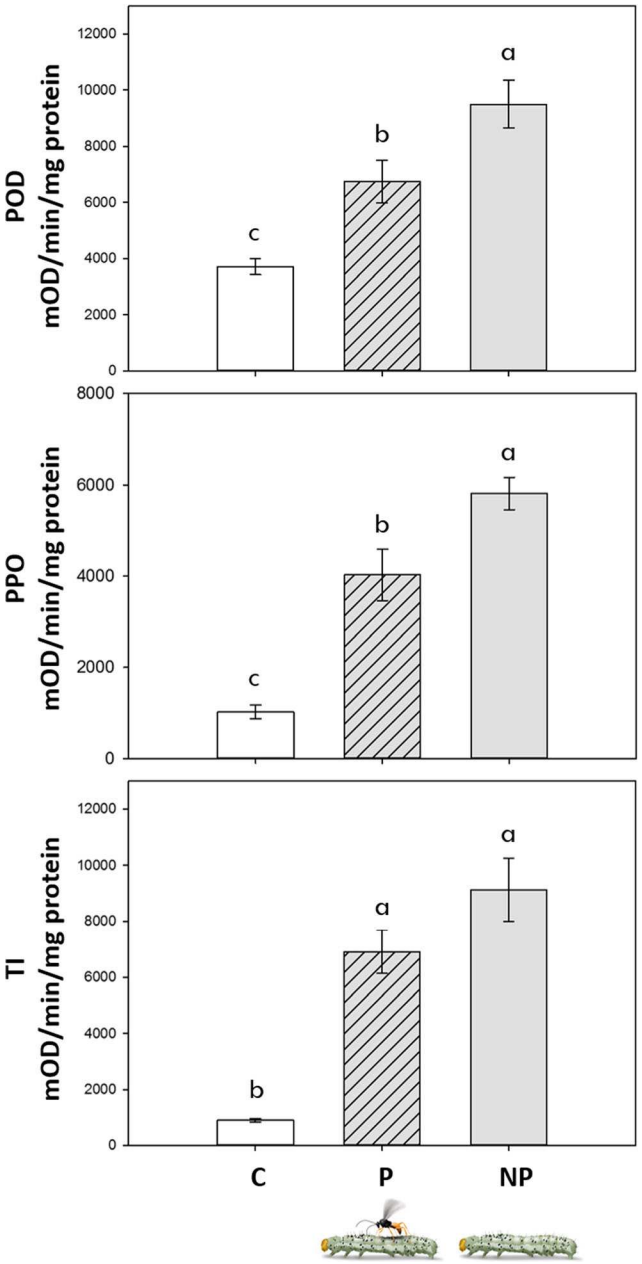

Fig. 2 Effect of ***Heliothis virescens*** **caterpillar parasitism on induction of tomato defensive proteins.** Values are untransformed means ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, $\alpha = 0.05$; POD, $n = 6–9$, $F_{(2, 21)} = 23.59$, $P < 0.0001$; PPO, $n = 7–9$, $F_{(2, 21)} = 61.50$, $P < 0.0001$; TI, n = 6–9, $F_{(2, 23)} = 139.92$, $P < 0.0001$. C, intact control plant; NP, plant fed on by non-parasitized caterpillar; P, plant fed on by parasitized caterpillar; POD, peroxidase PPO, polyphenol oxidase; TI, trypsin inhibitor

To determine if insect saliva was responsible for the observed difference in induced plant defenses, tobacco defense protein activities were measured after application of insect saliva to wounded leaflets. Saliva from P-caterpillars induced higher plant defense protein activities (PPO and TI) in tobacco plants than in plants treated with saliva from NP-caterpillars (Fig. 7). These results indicate that saliva is the factor

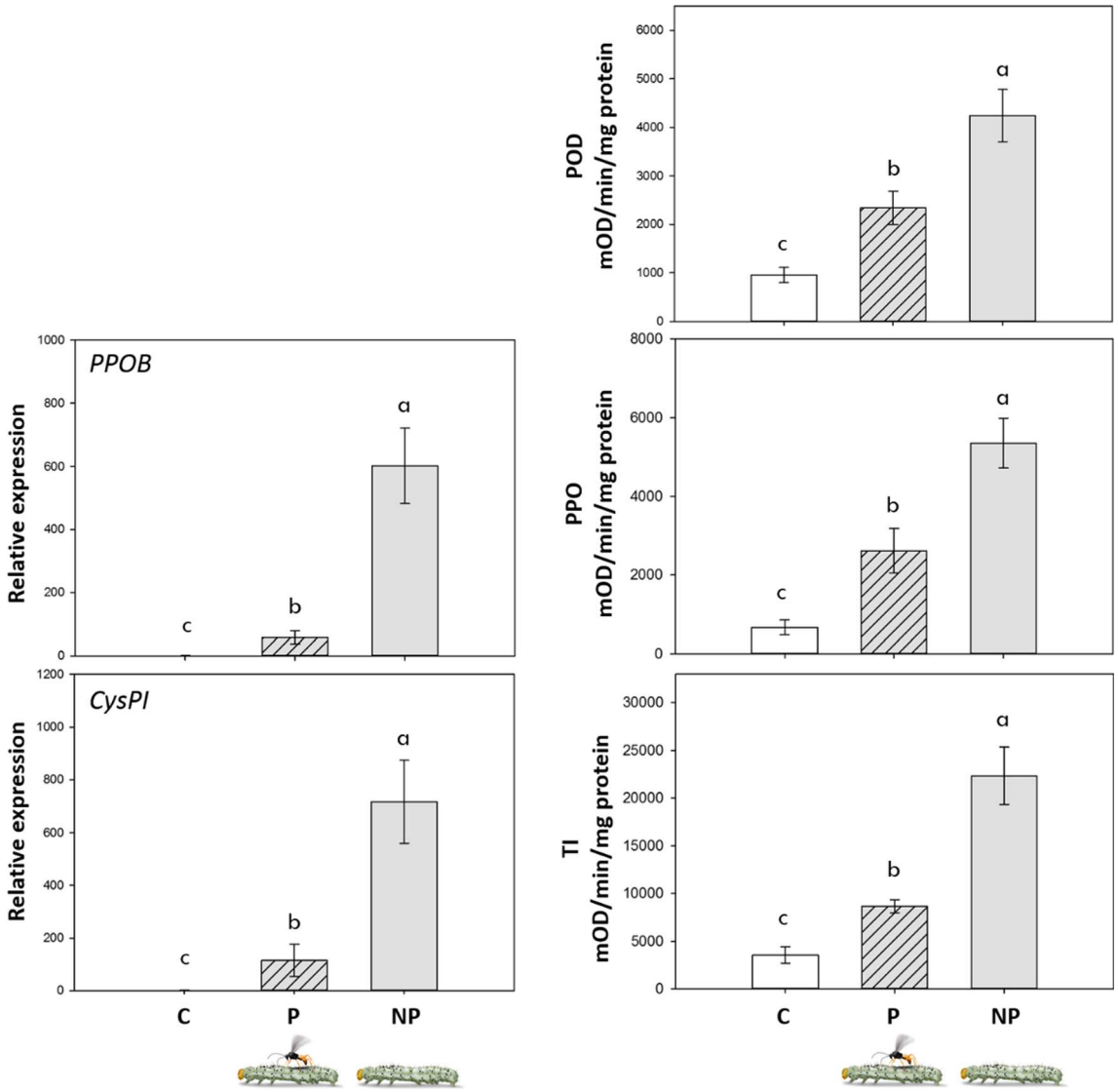

Fig. 3 Plant defense gene expression levels and protein activities in tomato plants treated with saliva of non-parasitized caterpillars (NP), parasitized caterpillars (P) and unwounded control (C). Gene expression levels and protein activities were measured 24 h and 48 h after saliva application respectively. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, $\alpha = 0.05$: *PPOB*, $F_{(2,\ 7)} = 76.38$, $P < 0.0001$; *CysPI*, $F_{(2,\ 24)} = 40.10$, $P < 0.0001$; POD, $F_{(2,\ 41)} = 27.54$, $P < 0.0001$; PPO, $F_{(2,\ 41)} = 28.19$, $P < 0.0001$; TI, $F_{(2,\ 41)} = 43.19$, $P < 0.0001$). C, intact control plant; P, plant treated with parasitized *Heliothis virescens* caterpillar saliva (15 μl (1 μg/μl protein)); NP, plant treated with non-parasitized caterpillar saliva (15 μl (1 μg/μl protein))

responsible for the higher induction of tobacco defense responses after caterpillars were parasitized.

To determine if difference in induced plant defenses influence parasitoid performance, the relative growth rate (RGR) of parasitized caterpillars and parasitoid performance were measured. Parasitized *H. zea* caterpillars had lower RGR when fed on P-caterpillar treated tobacco plants (higher plant defense responses) (Fig. 8). Moreover, parasitoids performed worse when their host caterpillars fed on P-caterpillar treated plants compared to NP-caterpillar treatments. In fact, they had longer development times, lower cocoon weights, and lower total survival compared with those whose host caterpillars fed on NP-caterpillar treated tobacco plants (Table 2). These results indicated that parasitized *H. zea* caterpillars had lower GOX activity in their labial glands, thereby inducing higher defense responses in tobacco plants resulting in lower parasitoid performance.

Discussion

Glucose oxidase is a multifunctional enzyme that expresses widely in larvae of lepidopteran species and it tends to have higher expression in generalist/polyphagous species (Eichenseer et al. 2010). Salivary GOX has been shown to suppress defenses in several host plant species (Bede et al.

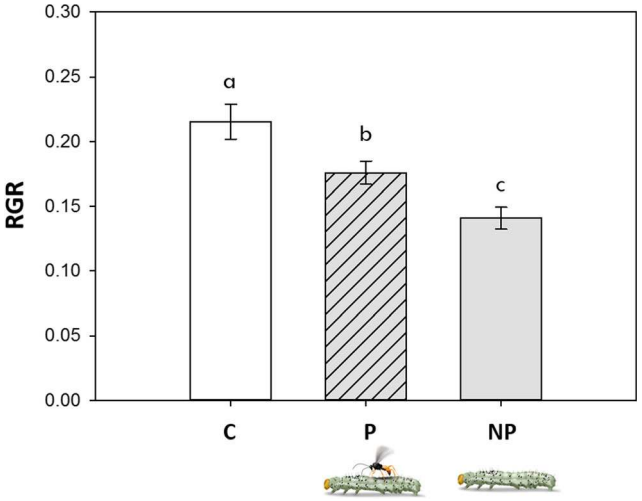

Fig. 4 Relative growth rate (RGR) of parasitized *Heliothis virescens* caterpillars feeding on tomato plants previously treated with saliva from parasitized caterpillars (P), saliva from non-parasitized caterpillars (NP) or untreated control plants (C) for 48 h. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, $\alpha = 0.05$: $n = 49–53$, $F_{(2,\ 148)} = 11.68$, $P < 0.0001$. C, intact control plant; NP, plant treated with non-parasitized caterpillar saliva; P, plant treated with parasitized caterpillar saliva

2006; Diezel et al. 2009; Musser et al. 2002). Salivary GOX is also present in folivore hymenopteran sawflies (Eichenseer et al. 2010) and in other Hymenoptera such as the honeybee, *Apis mellifera*, where it plays a crucial role in social immunity by sterilizing larval food and contributing to the antiseptic properties of honey (López-Uribe et al. 2017). Thus, GOX plays a dual role in mediating insect and plant immunity.

However, the effects of salivary GOX on plant immunity/defense are species specific: in tobacco GOX of several caterpillar species, including *H. zea, Helicoverpa armigera* and

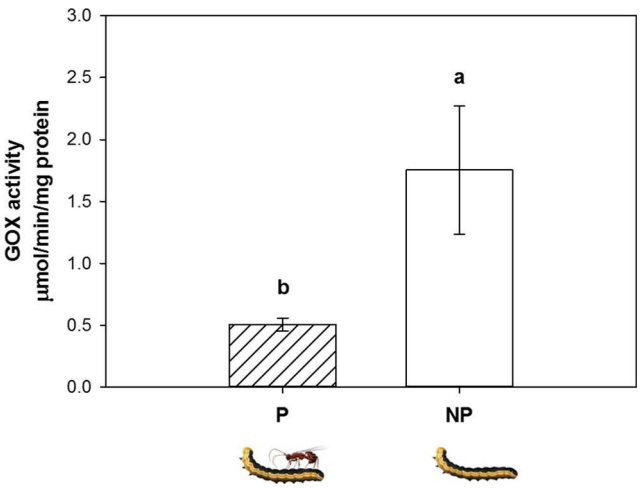

Fig. 5 *Helicoverpa zea* GOX enzyme activity at six days after being parasitized. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments. Treatments include parasitized (P) and non-parasitized (NP) caterpillars. Student's t test; $n = 8–9$, $F_{(1,\ 15)} = 7.51$, $P = 0.017$

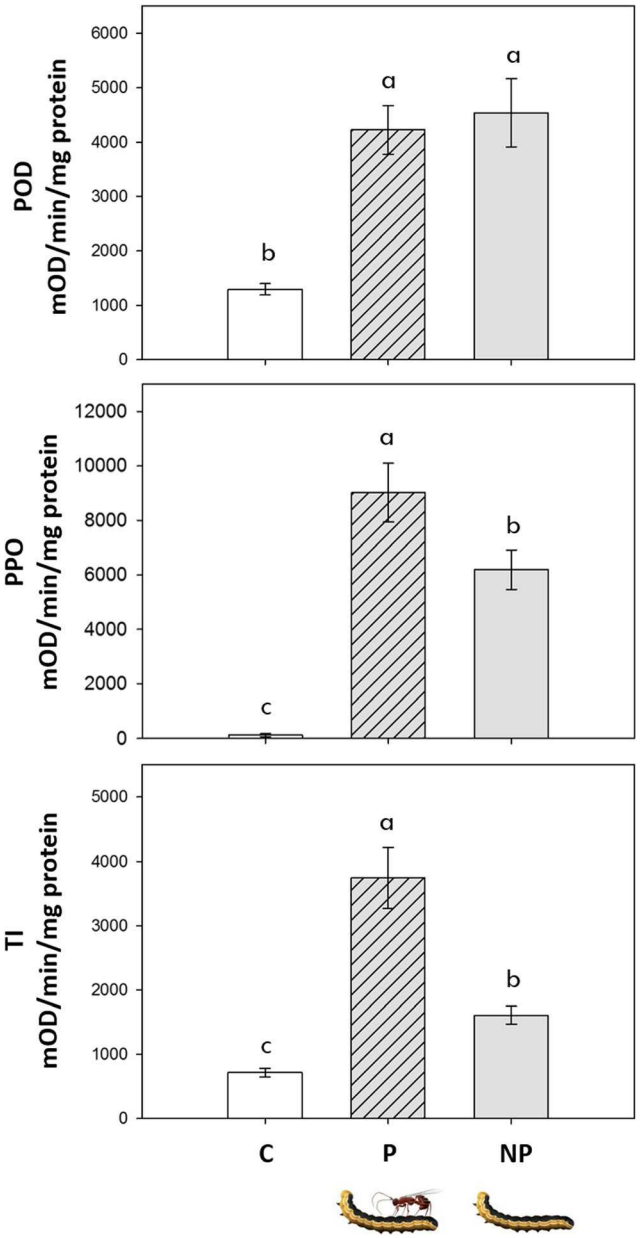

Fig. 6 Effect of *Helicoverpa zea* caterpillar parasitism on induction of plant defensive proteins. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, $\alpha = 0.05$; POD, $n = 11–15$, $F_{(2,\ 37)} = 43.43$, $P < 0.0001$; PPO, $n = 11–14$, $F_{(2,\ 35)} = 89.75$, $P < 0.0001$; TI, $n = 7–15$, $F_{(2,\ 30)} = 54.61$, $P < 0.0001$. C, intact control plant; NP, plant fed on by non-parasitized caterpillar; P, plant fed on by parasitized caterpillar; POD, peroxidase; PPO, polyphenol oxidase; TI, trypsin inhibitor

Helicoverpa assulta (Musser et al. 2002; Zong and Wang 2004) suppresses jasmonate-regulated plant defenses (defense protein activities and nicotine levels). The suppression of JA-defenses is likely due to hormonal cross-talk with salicylic acid signaling. Application of *H. zea* GOX triggered significantly higher levels of the SA-mediated PR-1a protein in *N. tabacum* (Musser et al. 2005), while salivary GOX (but not β-glucosidase activity) levels of *Spodoptera exigua* were

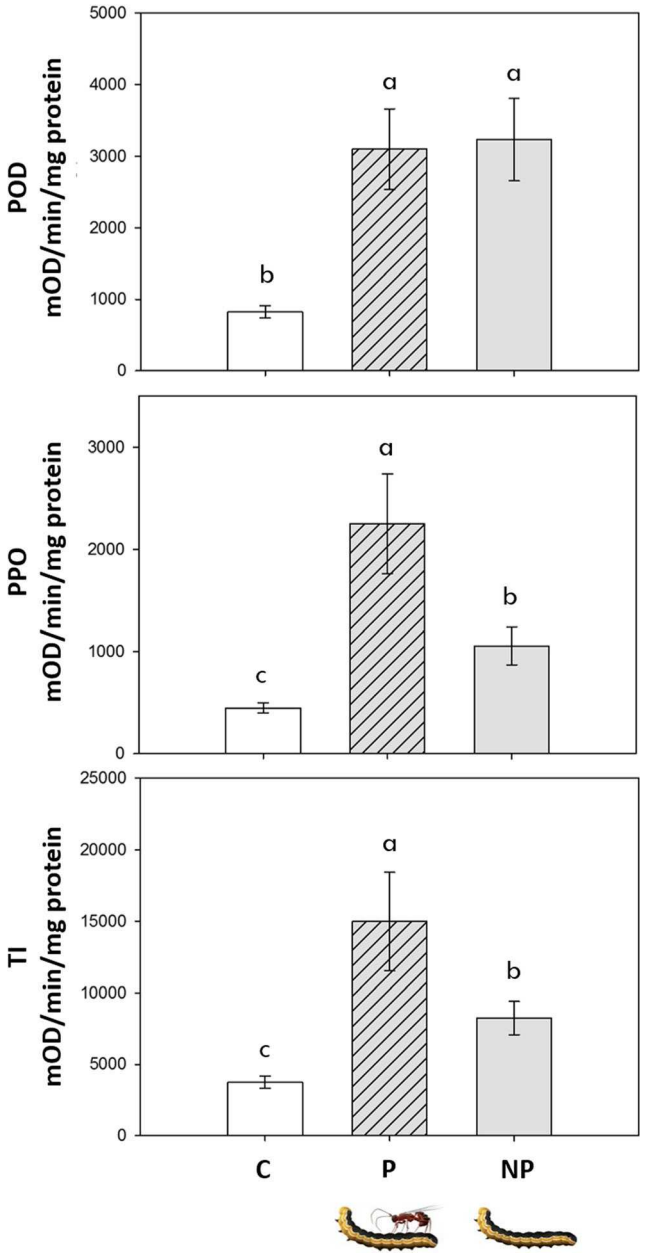

Fig. 7 Plant defense protein activities in tobacco plants treated with saliva of non-parasitized caterpillars (NP), parasitized caterpillars (P) and unwounded control (C). Defense protein activities were measured 48 h after saliva application. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, $\alpha = 0.05$: POD, $n = 10–13$, $F_{(2, 35)} = 15.60$, $P < 0.0001$; PPO, n = 10–13, $F_{(2, 35)} = 25.60$, $P < 0.0001$; TI, $n = 14$, $F_{(2, 41)} = 14.05$, $P < 0.0001$. C, intact control plant; P, plant treated with parasitized *Helicoverpa zea* caterpillar saliva (15 µl (1 µg/µl protein)); NP, plant treated with non-parasitized caterpillar saliva (15 µl (1 µg/µl protein)). POD, peroxidase; PPO, polyphenol oxidase; TI, trypsin inhibitor

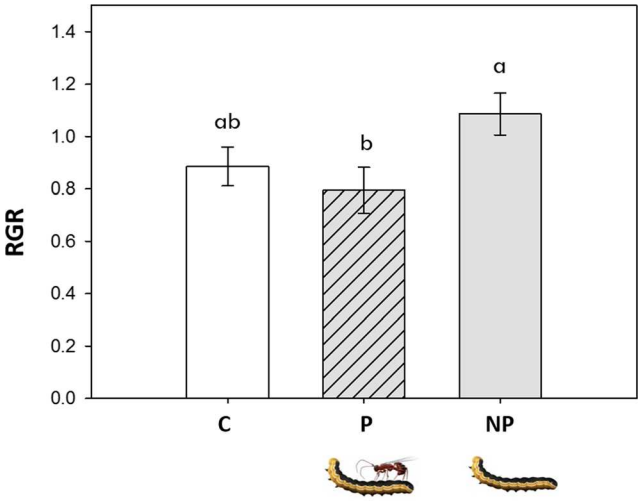

Fig. 8 Relative growth rate (RGR) of parasitized *Helicoverpa zea* caterpillars feeding on tobacco plants previously treated with saliva from parasitized caterpillars (P), saliva from non-parasitized caterpillars (NP) or untreated control plants (C) for 48 h. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, $\alpha = 0.05$: $n = 29–33$, $F_{(2, 93)} = 3.40$, $P = 0.0377$. C, intact control plant; NP, plant treated with non-parasitized caterpillar saliva; P, plant treated with parasitized caterpillar saliva

sufficient to trigger a SA burst and attenuate JA and ethylene levels in *Nicotiana atteunata* (Diezel et al. 2009). In contrast, GOX *triggers* JA-regulated defenses such as proteinase inhibitors in tomato (Tian et al. 2012) but does not elicit a significant SA burst (unpublished data; Tian et al. 2012). In other plant species the effects of GOX have been shown to be dose dependent: basal levels of salivary GOX in the European corn borer *Ostrinia nubilalis* (Louis et al. 2013) or *H. zea* (Wang et al. 2018) were not sufficient to trigger defenses in maize. However, because GOX is involved in insect immunity, it is expected that microbes could influence its expression (Wang et al. 2017). Bacteria present in the digestive tract of *H. zea* caused caterpillars to secrete more than twice as much GOX during feeding on maize leaves, which was sufficient to trigger defenses in maize (Wang et al. 2018).

Polydnaviruses associated with parasitoids play a critical role in suppressing the immune systems of their caterpillar hosts (Burke and Strand 2012). We previously showed that the polydnavirus associated with *M. croceipes* (*Mc*BV) suppressed immune related gene *gox* expression in *H. zea* (Tan et al. 2018). The downregulation of GOX by *Mc*BV had cascading effects across trophic levels. The lower expression of GOX resulted in an attenuation of plant defenses during caterpillar feeding, which in turn improved the survival and fitness of the parasitoid (and *Mc*BV). Here we report a similar phenomenon in another host of *M. croceipes*, *H. virescens*, where parasitized caterpillars had lower GOX activity and triggered lower levels of plant defenses. We hypothesized that the parasitoid would down regulate plant defenses regardless of the host plant of the caterpillar. However, when tobacco was the host plant for *H. zea*, the downregulation of salivary GOX by parasitoids had detrimental effects on the developing parasitoid by reducing their overall survival. This effect was caused by an enhanced induction of plant defenses.

Table 2 Parasitoid performance with *Helicoverpa zea* host feeding on different tobacco plant treatments

	Larval duration (day)	% Larval mortality	% Cocoon formation failure	Cocoon weight (mg)	Pupal duration (day)	Total development (day)	% Adult emergence	% Total survival
C	15.11 ± 0.28 ^{ab}	20.00 ± 5.71 ^{b}	38.33 ± 5.00 ^{a}	12.98 ± 0.33 ^{ab}	10.80 ± 0.20	25.50 ± 0.58 ^{ab}	60.00 ± 8.90	28.57 ± 4.52 ^{b}
P	15.96 ± 0.47 ^{a}	37.14 ± 3.50 ^{a}	17.00 ± 7.68 ^{b}	12.32 ± 0.51 ^{b}	11.42 ± 0.36	27.83 ± 0.96 ^{a}	66.67 ± 4.56	34.29 ± 3.50 ^{b}
NP	14.59 ± 0.23 ^{b}	17.14 ± 5.35 ^{b}	20.86 ± 3.30 ^{b}	14.00 ± 0.34 ^{a}	11.13 ± 0.24	25.19 ± 0.44 ^{b}	72.00 ± 8.46	45.71 ± 2.86 ^{a}
	$p = 0.035$ F = 3.50	$p = 0.030$ F = 4.78	$p = 0.044$ F = 4.09	$p = 0.011$ F = 4.85	$p = 0.357$ F = 1.06	$p = 0.045$ F = 3.41	$p = 0.525$ F = 0.68	$p = 0.019$ F = 5.60

C plant without any treatment, *P* saliva from parasitized *Helicoverpa zea* treated plant, *NP* saliva from non-parasitized *Helicoverpa zea* treated plant. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (one-way ANOVA)

There is an emerging body of evidence suggesting that insect herbivore-associated microbes not only mediate insect immunity but could directly (Chung et al. 2013) or indirectly mediate plant immunity by altering the expression of salivary components such as GOX (Tan et al. 2018; Wang et al. 2017, 2018). Whereas the bottom up effects of plant defense traits on parasitoids and microbes are generally well appreciated (Peterson et al. 2016; Shikano et al. 2017), the top down effects of parasitoids and herbivore-associated microbes on plant defense traits have only recently become recognized (Cusumano et al. 2018; Poleman et al. 2011; Tan et al. 2018; Zhu et al. 2018). Due to the large number of lepidopteran species (>180,000) and their associated parasitic braconid (upwards to 50,000) and ichneuomonid wasp species (60,000 to 100,000) which carry PDVs, we predict that the top down regulation of plant defense traits by parasitoids and PDVs may be a widely occurring phenomenon impacting multiple trophic levels.

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