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ORIGINAL PAPER

Fungi from the black cutworm *Agrotis ipsilon* oral secretions mediate plant–insect interactions

Xuewei Chen[1,2] · Michelle Peiffer[2] · Ching-Wen Tan[2] · Gary W. Felton[2]

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Abstract

It is well known that the interactions of insect herbivores and their host plant can be mediated by microbes. Our central hypothesis is that herbivore-associated fungi might directly or indirectly affect plant–insect interactions. In this study, we identified five orally secreted fungi from field-collected black cutworm, *Agrotis ipsilon*, including *Aspergillus parasiticus*, *Aspergillus niger*, *Geotrichum candidum*, *Fusarium subglutinans*, and *Mucor circinelloides* f. *lusitanicus*. We found that caterpillars inoculated with *F. subglutinans* or *M. circinelloides* f. *lusitanicus* induced higher defense responses in plants, but with different patterns between different plants. These herbivore-induced defense responses reduced the growth of caterpillars. However, direct application of fungi to mechanically wounded tomato did not induce JA-related defense responses. The application of regurgitant from fungi-inoculated or non-inoculated caterpillars, suggested that regurgitant might be responsible for the fungi-mediated defense response in plants against caterpillar attack. Furthermore, both *F. subglutinans* and *M. circinelloides* f. *lusitanicus* benefited caterpillar growth when they fed on detached tomato leaves, but had no influence when caterpillars fed on artificial diet. Our finding suggests that insect-associated fungi could influence plant–insect interactions by indirectly mediating plant defense responses, and directly affecting caterpillar performance on host plants.

Keywords Oral secretions · Fungi · Phytobiome · Microbiome · Induced defenses · Plant–herbivore interaction · Tomato · Maize

Introduction

During insect herbivory on plants, an array of wounding and feeding cues are detected by plants (Acevedo et al. 2015; Alborn et al. 1997; Bonaventure et al. 2011; Pinto et al. 2019). Perceiving theses damage-associated molecular patterns (DAMPs) and/or herbivore-associated molecular patterns (HAMPs), plants active a cascade of specific defenses that are regulated through a complex phytohormone system (Stahl et al. 2018). The crosstalk between jasmonic acid (JA) and salicylic acid (SA) signaling pathways plays a crucial role in plant defense response to types of attacks (Malook et al. 2019; Turner et al. 2002). Herbivores can also manipulate the phytohormone crosstalk using effectors to suppress plant defenses (Acevedo et al. 2019; Chen et al. 2019; Musser et al. 2005; Xu et al. 2019).

Mounting studies have demonstrated that insect herbivores could co-opt symbiotic microbes to modulate the interactions between herbivores and their host plants. Our colleagues have previously reported that symbiotic orally secreted bacteria in herbivores, such as Colorado potato beetle, false potato beetle, and in fall armyworm, could suppress JA-mediated plant defenses [polyphenol oxidase (PPO) activity] in tomato (Acevedo et al. 2017; Chung et al. 2013; Wang et al. 2016, 2017). In another case, chaperonin GroEL from the endosymbiont *Buchnera aphidicola* of aphids enhanced plant resistance against aphids by eliciting defense responses in transgenic *Arabidopsis* plants (Chaudhary et al. 2014). These symbionts could also exert different effects on their host and multitrophic interactions. For

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✉ Gary W. Felton
gwf10@psu.edu

[1] Department of Entomology, China Agricultural University, Beijing 100193, China

[2] Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA

example, *Wolbachia* and *Spiroplasma* could affect insect performance by alerting the growth, survival, and reproduction of their insect host (Jiggins et al. 2000; Moriyama et al. 2015). *Staphylococcus sciuri* from the pea aphid could mediate insect interactions with natural enemies by attracting and enhancing the efficiency of aphid natural enemies (Leroy et al. 2011).

Herbivorous insects can acquire microorganisms that are present on the plant, which many may originate from the soil or rain (Hannula et al. 2019). It has been reported that a fungus, *Fusarium solani* strain K (FsK), which is an endophytic fungal isolate that colonizes tomato root tissues, can reduce the damage of *Nesidiocoris tenuis*, by induction of JA defenses and the overproduction of ethylene (Garantonakis et al. 2018). *Arabidopsis* plants become significantly more susceptible to *Mamestra brassicae*, upon exposure to the volatiles of *Sclerotinia sclerotiorum* and *Rhizoctonia solani* fungi, suggesting that a prior fungal volatile exposure can negatively affect plant resistance to insects (Moisan et al. 2019). Endophytic fungi can also affect the growth of foliar feeding insects with different patterns of specialism of the herbivore (Gange et al. 2012). However, there remain many unexplored mechanisms of how insect-associated fungi mediate plant–insect interactions. Here we addressed that herbivore-associated fungi may influence the interactions with their host plants and the performance of insects.

In this study, we used culture-dependent methods to isolate several fungi from the oral secretions of the black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), a destructive pest of many crops, including nearly all vegetables and many important grains (Rings et al. 1974). In this investigation, we determine if these fungi could have direct and/or indirect effects on host plant defenses in tomato and maize.

Martials and methods

Plants and insects

Tomato (*Solanum lycopersicum*, var. Better Boy) and maize (*Zea mays*, var. B73) were grown in greenhouse with a photoperiod of 16:8 h (L:D) at The Pennsylvania State University (University Park, PA, USA). Plants were fertilized with Osmocote Plus (15-9-12) (Scotts, OH, USA) after transplant. Tomato with four to five fully expanded leaves, and maize with six to seven fully expanded leaves were used for experiment.

Agrotis ipsilon were collected from the ears of sweet corn located at Penn State University Russell E. Larson Research Farm in 2015. The field-collected black cutworms were used for fungal community analysis.

A laboratory colony of *A. ipsilon* was purchased from Benzon Research (Carlisle, PA, USA), and maintained at Penn State University. Larvae were reared on an artificial diet (Chippendale 1970) containing antibiotics (5 mg/100 mL streptomycin, 100 mg/100 mL Aureomycin, and 50 mg/100 mL FABCO-I), in a growth chamber at 25 ± 1 °C with a photoperiod of 16:8 h (L:D). Pupae were collected and kept in petri dishes till emerged, then transferred to a plastic jar to lay eggs.

Detection of *A. ipsilon* regurgitant on tomato leaves

To determine the secretion of regurgitant during *A. ipsilon* feeding, a fluorescent dye method described by Peiffer and Felton (2009) was used. Briefly, 10 µg of Alexa 488 carboxylic acid esters dye (Invitrogen, CA, USA) in 50 µL of water was placed on 0.5 g artificial diet and fed to the newly molted 6th instar black cutworm overnight to allow them to consume the entire piece of diet. Control caterpillars were fed diet supplemented with 50 µL of water in place of dye. The next morning, caterpillars were placed on leaves to feed. After they consumed about 1 cm^2 of leaf, caterpillars were removed, and the feeding side on the leaf excised and placed on a glass microscope slide, covered with a glass coverslip and secured with tape. One microlitre regurgitant was used as positive control. And a series of diluted regurgitant solutions were prepared for a standard curve. The fluorescence was observed with an Olympus FV1000 Laser Scanning Confocal Microscope (Olympus, America Inc., Melville, NY, USA) with an excitation of 488 nm. For each one, three visual fields were imaged at ×10 and relative intensity of fluorescence measured with FV10-ASW version 1.6. The standard curve was used to estimate the amount of regurgitant secreted onto leaf.

Isolation and identification of orally secreted fungi

About 15 *A. ipsilon* caterpillars were collected from the corn field each time, and the collection was performed for twice. At the same day of caterpillar collection, six of them were used to collect the regurgitant immediately, and 4 replicates were performed for each caterpillar. For regurgitant collection, the outer surface of caterpillars were sterilized with 70% ethanol and rinsed by sterile water, then the regurgitation droplet was collected using a sterile pipette tip. One microlitre of regurgitant was diluted 1:100 in sterile water, and plated on potato dextrose agar (PDA) media at 25 ± 1 °C. Three to four days later, expected spores from distinguishable fugal colonies were selected and re-plated on PDA media. Pure isolates were then used for DNA extraction. The mixture of conidia and hyphae was collected from PDA plate and ground to fine powder using Geno/Grinder® 2000 (OPS Diagnostics, USA). Total genomic DNA was obtained

using DNeasy® Plant Mini Kit by Qiagen (Valencia, CA) following the manufactures protocol. PCR was performed to identify pure isolates, and a set of primers was used to amplify specific genes (listed in Table 1). The PCR products were directly delivered to the PSU Genomics Core Facility for sequencing. The sequences obtained were BLAST at NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Fungi-mediated herbivore-induced plant defense responses

To evaluate the influence of isolated fungi on plant defenses triggered by caterpillar feeding, fungi-inoculated and/or non-inoculated black cutworms were allowed to feed on plant leaves, and the defense-related protein activity and gene expression were measured. The caterpillars were confined in a clip cage (with an approximate diameter of 27 mm) to standardize feeding damage among treatments. The fungal isolates were cultured on PDA plates for three to four days. The conidia and hyphae were removed from the medium and suspended in 20 mL sterile water and transferred to the 50 mL Falcon® tubes. The Falcon® tubes were vortexed and then centrifuged 15 min at 10,000×*g*, the supernatant discarded, and then resuspended in 5 mL sterile water to get fungal solution. 100 µL of the fungal solution or sterile water was pipetted onto detached plant leaves, and air-dried before fed to premolt 5th instar black cutworm for 48 h to allow them to consume the entire plant tissue, until the 2nd day of the 6th instar. The caterpillars used in experiment were transferred from artificial diet with antibiotics to artificial diet without antibiotics since the fourth instar to make sure the habitation of fungi successful. Fungi-inoculated or non-inoculated caterpillars were caged on the second leaf (as counted from the top of the plant) of corresponding species of plant. A set of healthy plants without cage clipped was used as untreated control. The caterpillar and cage were removed after caterpillar consumed the confined area; usually it took four to five hours for tomato, but 12 h for maize due to a lower feeding rate. 48 h later, 50 mg of leaf tissue around the wounding area was collected to measure activity of defense response-related protein. The leaf samples were treated with liquid nitrogen and stored at − 80 °C for following experiment.

To determine the defense protein responses, two JA pathway markers, polyphenol oxidase (PPO) and trypsin proteinase inhibitor (TPI) activities were evaluated, and activity of a SA pathway-related protein, peroxidase (POD), was measured. Plant samples were ground with liquid nitrogen using Geno/Grinder®. To measure PPO activity, 1.25 mL of phosphate buffer (0.1 M, pH 7.0) with 5% polyvinylpyrrolidone (PVP) (Alfa Aesar, MA, USA) was added immediately. Samples were vortexed and kept on ice for 5 min before centrifugation (4 °C, 11,000×*g* 10 min). Then 5 µL supernatant of each sample was mixed with 200 µL 3 mM caffeic acid (Sigma-Aldrich C0625, USA) in 96-well plate, and recorded change in absorbance at λ450 for 5 min using the SpectraMax® 190 plate reader (Molecular Devices, CA, USA). The protein concentration was determined using Bradford method (Bradford 1976) and enzyme activity expressed as mOD/min/mg protein.

To measure peroxidase (POD) activity, samples were prepared in phosphate buffer (0.1 M, pH 7.0) with 5% PVP. Then 5 µL supernatant of each sample was mixed with 10 µL 3% (v/v) hydrogen peroxide (H_2O_2), and 190 µL 3 mM guaiacol (Sigma-Aldrich, USA) in 96-well plate, and absorbance at λ450 was recorded for 5 min. POD activity was expressed as mOD/min/mg protein.

For TPI activity assay, 1.25 mL of extraction buffer (0.046 M Tris-base and 0.0115 $CaCl_2$, pH 8.1) with 5% PVP was added, and kept on ice for 10 min before centrifugation (4 °C, 11,000×*g* 10 min) to get sample supernatant. Then 10 µL of sample was mixed with 10 µL 20 µg/mL trypsin (Sigma-Aldrich T1426, USA) and 80 µL Tris buffer

Table 1 Primers used in this study

Locus	Lineage	Primer name	Sequence (5′–3′)	References
Internal transcribed spacer (ITS) region of the rRNA	All fungi	ITS1F	CTTGGTCATTTAGAGGAAGTAA	Anderson et al. (2003)
		ITS4	TCCTCCGCTTATTGATATGC	
Calmodulin	*Aspergillus*	CMD5	CCGAGTACAAGGAGGCCTTC	Hong et al. (2005)
		CMD6	CCGATAGAGGTCATAACGTGG	
Translation elongation factor 1-alpha	*Fusarium*	ef1	ATGGGTAAGGAAGACAAGAC	Maciá-Vicente et al. (2008)
		ef2	GGAAGTACCAGTGATCATGTT	
Calmodulin	*Fusarium*	CL1	GARTWCAAGGAGGCCTTCTC	O'Donnell et al. (2000)
		CL2A	TTTTGCATCATGAGTTGGAC	
18S ribosomal RNA	*Mucor*	MucL1	TGATCTACGTGACATATTTCT	Machouart et al. (2006)
		MR1	AGTAGTTTGTCTTCGGTCAA	

(0.046 M, pH 8.1), and wait for 10 min. Then added 100 µL of *p*-toluene-sulfonyl-L-arginine methyl ester (TAME) (0.002 M; Sigma T4626, USA), and absorbance values were read at 247 nm for 5 min. Percentage inhibition was calculated by comparing the activity of sample with standard trypsin activity.

We have performed our experiment with five fungal species in tomato, only caterpillars inoculated with *F. subglutinans* or *M. circinelloides* f. *lusitanicus* altered defense responses in tomato, so we performed the other experiments with only *F. subglutinans* and *M. circinelloides* f. *lusitanicus*.

Application of fungi onto wounded tomato leaves

To evaluate the direct influence of fungi on plant defense responses, the fungal solution was directly applied to mechanically wounded tomato leaf, then PPO and TPI, as well as POD activities were measured. Briefly, the second leaf from the top of plant was mechanically wounded using a wounding tool (approximately with a 1.0 cm diameter) and 20 µL of fungal solution described as above was immediately applied to the wounded area. Wounded plants applied with water were used as control, and a set of untreated controls was included. 48 h post-treatment, 50 mg plant tissue was collected for protein activity assay. The protein activities of PPO, POD, and TPI were conducted as described above.

Feeding bioassay with defense-induced plant tissues

To assess the effect of induced defenses on the black cutworm, the relative larval growth rate was determined on previously induced plant leaves. Plant defense responses were triggered by fungi-inoculated or non-inoculated caterpillars feeding as described above. 48 h after treatment, the damaged leaves were excised and fed to the 1st day 4th instar caterpillars. After 48 h feeding, caterpillars were weighed, and the relative growth rate was calculated as (final weight − initial weight)/(initial weight × no. of days).

Bioassay by directly feeding with fungi

To evaluate the direct influence of fungi on caterpillar growth, the relative growth rate of caterpillars fed with fungi on artificial diet or tomato leaves were monitored. In this experiment, 100 µL of fungal solution or sterile water was pipetted onto artificial diet or detached tomato leaves (the tomato leaves were kept on 2% agar embedded cups to maintain humidity and hydration of the leaves). After air-drying the solution, first day fourth instar caterpillar were weighed and placed in individual diet cups. The fungal diet was replaced every two days and larvae were weighed every two days. The relative growth rate was calculated as (final weight − initial weight)/(initial weight × no. of days).

Plant responses to application of regurgitant and saliva from fungi-inoculated *A. ipsilon*

To determine if regurgitant is responsible for the differences that we observed in plant defense responded to fungi-inoculated or non-inoculated caterpillar feeding, regurgitant were applied to mechanically wounded plant leaves. Regurgitant from at least 10 fungi-inoculated or non-inoculated caterpillars was collected and diluted 1:20 in 20 µL phosphate buffer (0.1 M, pH 7.0), and applied onto the mechanically wounded area.

To determine if saliva is responsible for the differences that we observed in plant defense responded to fungi-inoculated or non-inoculated caterpillar feeding, labial glands homogenization were applied to mechanically wounded plant leaves. Briefly, labial glands from at least 10 fungi-inoculated or non-inoculated caterpillars were collected and homogenized with 20 µL phosphate buffer (0.1 M, pH 7.0) per pair of glands. The protein concentration was determined using Bradford method, and 20 µg protein in 20 µL phosphate buffer were applied onto the mechanically wounded area.

A set of untreated control plants was included. Plant tissues were collected 48 h after treatment for PPO, POD, and TPI activities assay.

Results

Regurgitant detection and identification of orally secreted fungi

After *A. ipsilon* had fed overnight on artificial diet spiked with fluorescent dye, we detected their regurgitant on tomato leaves. In this experiment, the regurgitant collected after the caterpillars ate spiked diet was highly fluorescent and fluorescence was detected on all feeding leaves. Based on the standard curve, the estimated average amount of regurgitant on tomato leaves was 0.23 µL/cm during feeding bout (Supplemental Fig. 1).

Through culture-dependent isolation, we isolated five different fungi from oral secretions of *A. ipsilon*. Based on sequence-dependent identification, we identified two *Aspergillus* fungi, one *Geotrichum* fungus, one *Fusarium* fungus, and one *Mucor* fungus, using the primers located in internal transcribed spacer region of rRNA. Furthermore, we used *Aspergillus* genus-specific primers for calmodulin, *Fusarium* genus-specific primers for translation elongation factor 1-alpha and calmodulin, and *Mucor*-specific primers located in 18S rRNA to analyze the sequences by comparison with

NCBI GenBank database. We identified these fungi as *A. parasiticus*, *A. niger*, *G. candidum*, *F. subglutinans*, and *M. circinelloides* f. *lusitanicus*.

Fungi-mediated herbivore-induced plant defense responses

To determine whether the fungal strains established successfully, the regurgitant were collected from fungi-inoculated or non-inoculated caterpillars, and applied to PDA plate. Additionally, plant tissues damaged by fungi-inoculated or non-inoculated caterpillars were cultured on PDA plate to assay the presence/absence of the fungi in the damaged leaves (Supplemental Fig. 2).

To determine the effect of fungi on induced plant defenses, caterpillars were pre-fed with/or without fungi and allowed to feed on plant leaves. Caterpillars inoculated with both *F. subglutinans* and *M. circinelloides* f. *lusitanicus* induced significantly higher PPO and TPI levels in tomato compared with the non-inoculated treatment group (Fig. 1a). There was a markedly different defense response in maize, where plants fed by caterpillars exhibited even lower PPO and TPI activities than untreated plants, and only caterpillars inoculated with *M. circinelloides* f. *lusitanicus* induced significantly higher levels of TPI compared to plants damaged by non-inoculated caterpillars (Fig. 1b). However, no difference was observed in POD activities between the treatment damaged by fungi-inoculated or non-inoculated caterpillars in both tomato and maize.

Caterpillars inoculated with *A. parasiticus*, *A. niger*, or *G. candidum* had no significant alteration of plant defense, and the results were presented in Supplemental Fig. 3.

Plant responses to direct application of fungi

We observed no differences when we applied fungal solution directly on mechanically wounded tomato plants in both PPO and TPI activities, compared with the set of plants treated with sterile water after mechanical wounding. However, POD was induced significantly by application of both *F. subglutinans* and *M. circinelloides* f. *lusitanicus* compared with the sterile water control (Fig. 2). These results indicate that the effects of the fungi are likely indirect in that they are

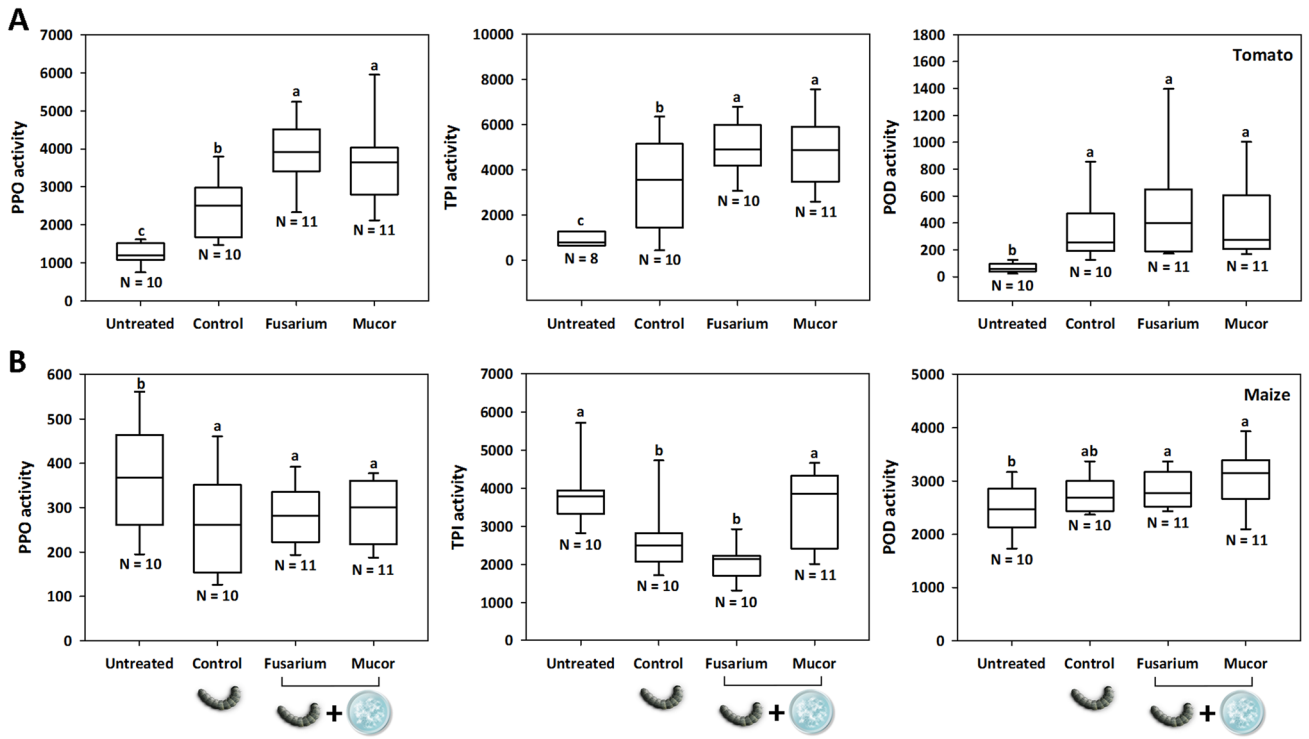

Fig. 1 Polyphenol oxidase (PPO), trypsin proteinase inhibitor (TPI), and peroxidase (POD) activities of plants fed on by *Agrotis ipsilon*. Plants were damaged by caterpillars which were pretreated with fungal solution of *Fusarium subglutinans* (Fusarium), or *Mucor circinelloides* f. *lusitanicus* (Mucor), or without fungi (control), and a set of undamaged plants was included as negative control (untreated). Mean values and replicate numbers are indicated in the figures, different letters represent significant differences obtained with one-way ANOVA followed by LSD test ($P<0.05$). **a** PPO ($F_{3,38}=20.854$, $P<0.001$), TPI ($F_{3,35}=14.746$, $P<0.001$), and POD ($F_{3,38}=4.103$, $P=0.013$) activities in tomato. **b** PPO ($F_{3,38}=2.504$, $P=0.074$), TPI ($F_{3,37}=9.812$, $P<0.001$), and POD ($F_{3,38}=3.435$, $P=0.026$) activities in maize

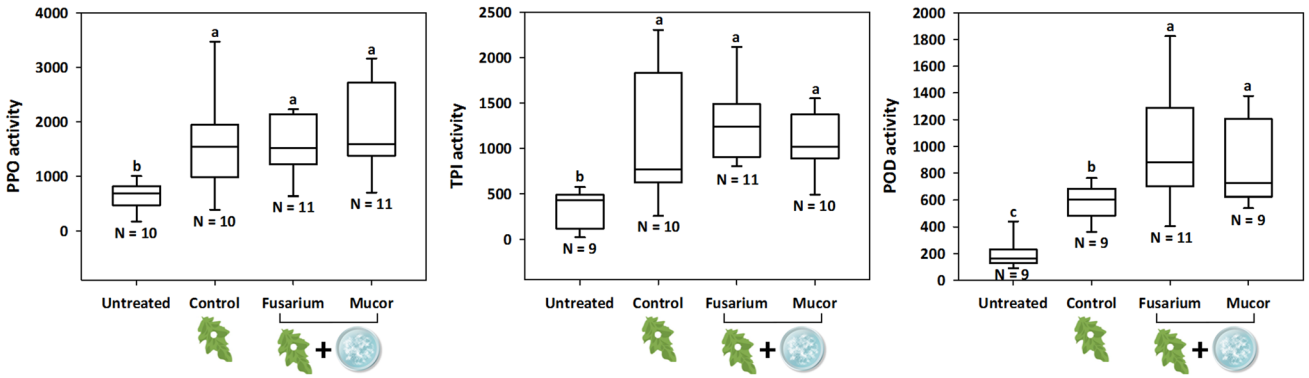

Fig. 2 Activities of defense proteins in tomato. Plants were applied with *Fusarium subglutinans* (Fusarium), or *Mucor circinelloides* f. *lusitanicus* (Mucor), or without fungi (Control) after mechanically wounded, and a set of undamaged plants was included as negative control (Untreated). Mean values and replicate numbers are indicated in the figures, different letters represent significant differences obtained with one-way ANOVA followed by LSD test ($P<0.05$). Activity of polyphenol oxidase (PPO) ($F_{3,38}=6.153$, $P=0.002$), accumulation of trypsin proteinase inhibitor (TPI) ($F_{3,36}=7.390$, $P=0.001$), and activity of peroxidase (POD) ($F_{3,34}=14.101$, $P<0.001$)

potentially mediating differences in salivary or regurgitant components.

Herbivore-induced plant responses affect caterpillar growth

Plants were damaged by either fungi-inoculated or non-inoculated caterpillars, and then the leaves were fed to non-inoculated fourth instar caterpillars (results shown in Fig. 3). In tomato, the growth of caterpillars on treated leaves was lower than on untreated control leaves. The relative growth rate of larvae fed on the tomato leaves damaged by caterpillars inoculated with either *F. subglutinans* or *M. circinelloides* f. *lusitanicus* were significantly lower than those fed on leaves damaged by non-inoculated caterpillars.

There was a different scenario in maize where caterpillars fed on plant leaves damaged by *F. subglutinans*-inoculated or non-inoculated caterpillars exhibited higher growth rates compared to the treatments fed on untreated corn leaves or *M. circinelloides* f. *lusitanicus*-inoculated caterpillars

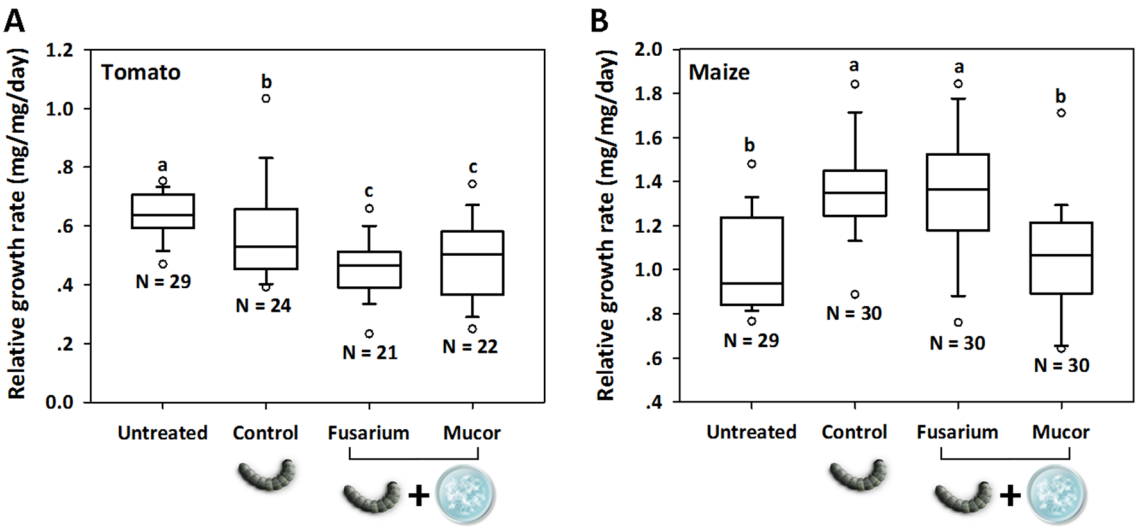

Fig. 3 Relative growth rate of *Agrotis ipsilon* fed on defense responses induced plant leaves. Defense responses were triggered by caterpillars feeding which were pretreated with fungal solution of *Fusarium subglutinans* (Fusarium), or *Mucor circinelloides* f. *lusitanicus* (Mucor), or without fungi (Control), and a set of undamaged plants was included as negative control (Untreated). Mean values and replicate numbers are indicated in the figures, different letters represent significant differences obtained with one-way ANOVA followed by LSD test ($P<0.05$). **a** Relative growth of *A. ipsilon* fed on defense responses triggered tomato leaves ($F_{3,92}=10.817$, $P<0.001$). **b** Relative growth of *A. ipsilon* fed on defense responses triggered maize leaves ($F_{3,115}=13.968$, $P<0.001$)

damaged corn leaves. These results were consistent with the defense responses to these treatments (Fig. 1).

The direct influence of fungi on individual fitness of caterpillars

When caterpillars were fed the respective fungi, their fitness varied among diets (Fig. 4). The result showed that larvae pretreated with either *F. subglutinans* or *M. circinelloides* f. *lusitanicus* showed faster growth on tomato leaves compared to non-inoculated larvae (Fig. 4a). However, either *F. subglutinans* or *M. circinelloides* f. *lusitanicus* had no effect on the growth of black cutworm when they fed on artificial diet (Fig. 4b).

Effect of regurgitant and saliva on plant defense responses

To estimate if regurgitant or saliva is responsible for the defense response mediated by fungi, we applied the regurgitant or homogenization of labial glands from fungi-inoculated or control caterpillars onto mechanically wounded tomato leaves, and assayed PPO and TPI activities (Fig. 5). The results showed that application of regurgitant from fungi-inoculated caterpillars had a trend towards higher defense responses compared to fungi-free control, but the regurgitant from *M. circinelloides* f. *lusitanicus*-inoculated was the only treatment that was statistically higher than the PBS-wounded control (Fig. 5a). Application of homogenization of labial glands from fungi-inoculated caterpillars did not affect plant defense responses compared with the controls (Fig. 5b).

Discussion

Microbes are ubiquitous, including symbionts associated with herbivores and plants that mediate plant–herbivores interactions (Casteel and Hansen 2014; Douglas 2016; Mason et al. 2019a). Insect symbionts can directly affect these interactions by facilitating nutrition, digestion, and detoxification, or by interfering with plant defenses (Brownlie et al. 2009; Brune and Dietrich 2015; Chung et al. 2013; Hansen and Moran 2014; Hosokawa et al. 2010; Sabree et al. 2009). However, in some cases herbivore-associated microbes betray their hosts. In one instance, herbivore-associated bacterial exacerbate defenses in plants by triggering salivary elicitors such as glucose oxidase (GOX) that elicit defenses in tomato and maize (Wang et al. 2017, 2018). In another example, the toxic effects of plant defenses known to disrupt the insect gut lining (e.g., chitinases, protease, trichomes) are exacerbated by gut bacteria that leak into the hemolymph of the insect (Mason et al. 2019b). In this study, we isolated symbionts in oral secretions from field-collected black cutworm and tested how these symbionts influence interactions with plant defenses.

Most of the reported herbivore gut microorganisms are bacteria, but herbivores can also harbor fungi as symbionts (Hannula et al. 2019). We identified five fungi from the regurgitant of field-collected *A. ipsilon*: they were *A. parasiticus*, *A. niger*, *G. candidum*, *F. subglutinans*, and *M. circinelloides* f. *lusitanicus*. All of these fungi are ubiquitous in the soil; three of which have been reported as plant pathogens in certain instances, including *A. parasiticus*, *A. niger*, and *F. subglutinans* (Desjardins and Hohn 1997; Schuster et al. 2002). We found that larvae inoculated with

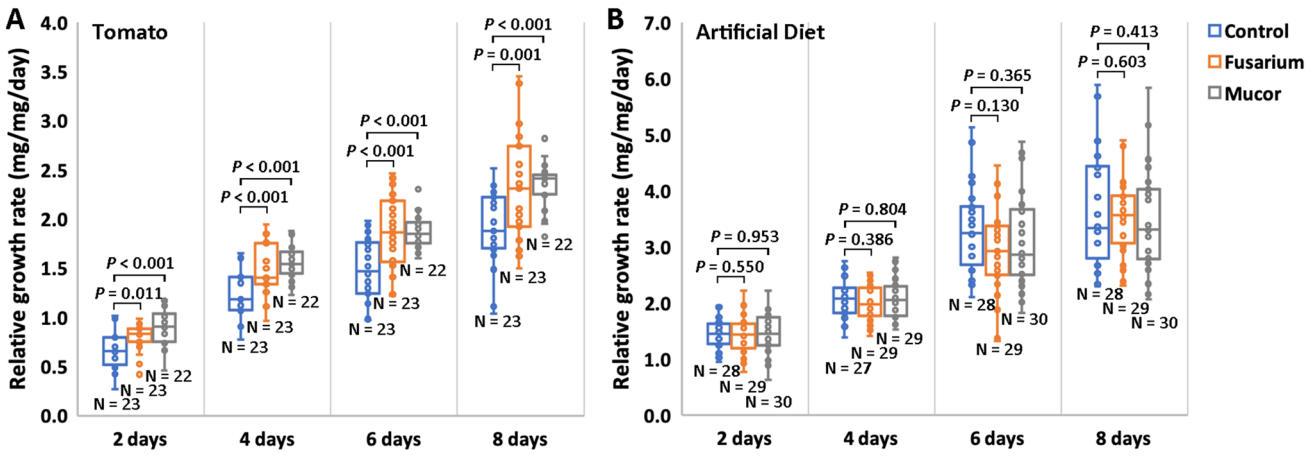

Fig. 4 Relative growth rate of *Agrotis ipsilon*. **a** Caterpillars fed with *Fusarium subglutinans* (Fusarium), or *Mucor circinelloides* f. *lusitanicus* (Mucor), or without fungi (Control) on excised tomato leaves; **b** caterpillars fed with *Fusarium subglutinans* (Fusarium), or *Mucor circinelloides* f. *lusitanicus* (Mucor), or without fungi (Control) on artificial diet. ANOVA analysis was performed followed by LSD test. *P* values compared to control, mean values, and replicates are indicated in the figures

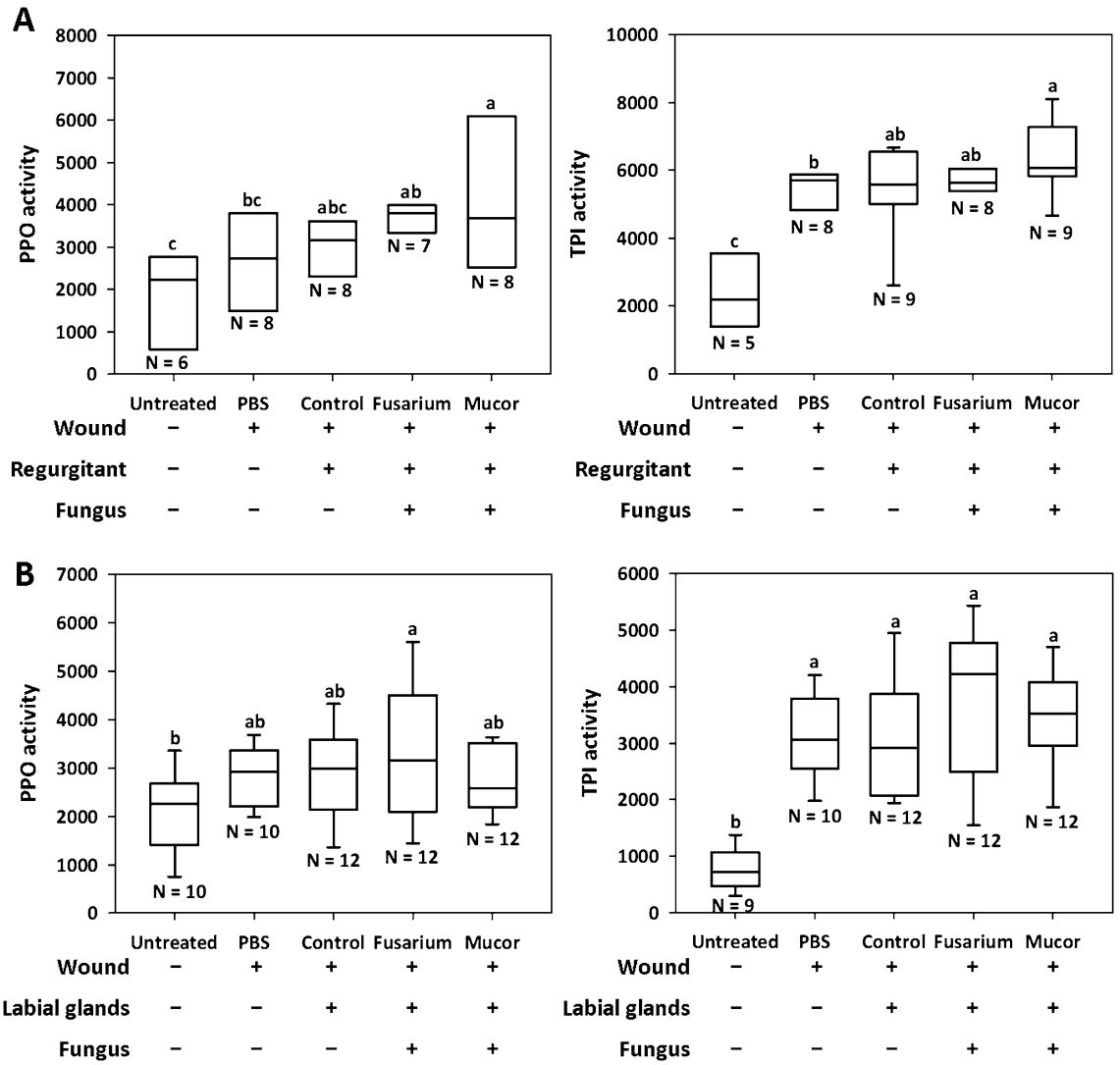

Fig. 5 Effect of regurgitant and saliva on polyphenol oxidase (PPO) and trypsin proteinase inhibitor (TPI) activities of tomato. Regurgitant or labial glands were collected from caterpillars pretreated with fungal solution of *Fusarium subglutinans* (Fusarium), or *Mucor circinelloides* f. *lusitanicus* (Mucor), or without fungi (Control). A set of undamaged plants was included as negative control (Untreated), and a set of plants was applied with PBS buffer after mechanically wound was included as treated control (PBS). Mean values and replicate numbers are indicated in the figures, different letters represent significant differences obtained with one-way ANOVA followed by LSD test ($P<0.05$). **a** PPO ($F_{4,32}=3.719$, $P=0.014$), and TPI ($F_{4,34}=11.471$, $P<0.001$) activities triggered by applications of regurgitant after mechanically wounded. **b** PPO ($F_{4,51}=2.246$, $P=0.077$), and TPI ($F_{4,50}=14.276$, $P<0.001$) activities triggered by applications of homogenization of labial glands after mechanically wounded

F. subglutinans and *M. circinelloides* f. *lusitanicus* induced higher JA-related defense responses in their host plants. In some cases, insect herbivores can deposit microbes such as bacteria on the leaf surface, which suppresses plant defenses (Acevedo et al. 2017; Chung et al. 2013). In these cases, the suppression occurs due to crosstalk between jasmonic acid (JA) and salicylic acid (SA) phytohormone signaling pathways (Chung et al. 2013; Thaler et al. 2012). However, in our study, a change of SA-responsive protein activity (POD) was not observed by feeding of caterpillars. Moreover, application of fungi with mechanical wounding did not translate to any change on PPO or TPI activities. These results suggest that insect-associated fungi more likely indirectly mediate plant defenses through other mechanisms.

The plant defense to herbivores is partially determined by the presence of herbivore-derived elicitors found in their saliva, regurgitant, oviposition secretion, and feces (Peiffer and Felton 2014; Ray et al. 2016; Reymond 2013; Wang et al. 2017). In the case of the tomato fruitworm *Helicoverpa zea*, gut bacteria triggered higher levels of the salivary elicitor glucose oxidase, which caused higher levels of defenses to be induced when they fed on tomato (Wang et al. 2017)

or maize (Wang et al. 2018). However, application of saliva from fungus-inoculated caterpillars was not significantly different from the PBS control in terms of inducing PPO or TPI.

Alternatively we hypothesized that the fungi could alter the ability of cutworm regurgitant to induce defenses. Although not all caterpillars regurgitate while feeding on plants (Peiffer and Felton 2009), we found that the black cutworm shows considerable regurgitation during feeding. Our data showing that regurgitant from *M. circinelloides* f. *lusitanicus*-inoculated caterpillars triggered significantly higher levels of TPI and PPO compared to the PBS-wounded control. These data suggest that the fungi might alter components in regurgitant that could induce defenses but further experiments are necessary. Our results were based on a single application of regurgitant to mechanically wounded plants and in the case of caterpillar feeding which is continuous and accompanied with regurgitation, the results may be more pronounced if we were better able to simulate natural feeding behaviors. The component(s) in regurgitant responsible for these effects on plant defenses remain to be identified.

Because of the induction of plant defenses, the association of the caterpillars with these two fungi would appear to be detrimental. Indeed, we observed the reduced growth of caterpillars feeding on leaves that had been previously fed on by caterpillars inoculated with the fungi. However, the effects on induction of defenses could be offset if the fungi aid in detoxification or nutrition (Hansen and Moran 2014). We found inoculation of caterpillars with either *F. subglutinans* or *M. circinelloides* f. *lusitanicus* improved their growth on tomato leaflets, but did not influence their growth on artificial diet. These fungi have been classified as pathogens of plants or mammals, particularly *F. subglutinans* which can produce toxins (Lee et al. 2014; Leslie 1995; Logrieco et al. 1996). However, our results suggest that these fungi facilitate the fitness of caterpillar to host plants, which might result from increased digestion, and/or detoxification of plant secondary metabolites.

Our study also revealed that the interactions of the microbes are host plant specific. The effects in maize were unexpected as caterpillar feeding caused a significant suppression in defenses in this system. Caterpillars inoculated with *M. circinelloides* f. *lusitanicus* triggered a slightly elevated response in TPI levels, but this was still lower than the unwounded control. Further studies are required to identify the factors in oral secretions responsible for mediating the plant defenses and the particular role(s) that the gut fungi play in each host plant system.

In summary, this study provides mounting evidence that herbivore-associated microbes are integral mediators of plant–herbivore interactions, particularly in terms of affecting the expression of plant defenses. Theories on insect–plant interactions need to account for the impact of the herbivore gut microbiomes on insect and plant fitness.

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