Research Article

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Compatibility of mycorrhiza-induced resistance with viral and bacterial entomopathogens in the control of *Spodoptera exigua* in tomato

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Abstract

BACKGROUND: Arbuscular mycorrhizal fungi (AMF) are soil-borne microorganisms that establish mutualistic associations with roots of most terrestrial plants. This symbiosis results in nutritional and defensive benefits to the host plant, usually conferring protection against biotic stresses, but its indirect impact on third trophic levels is still unknown. In the present work, we explore whether the symbiosis of tomato plants with *Funneliformis mosseae* (and/or exposition to herbivory) influences the interaction of the generalist pest *Spodoptera exigua* (Lepidoptera: Noctuidae) with bacterial (*Bacillus thuringiensis*) and viral (baculovirus, SeMNPV) natural entomopathogens.

RESULTS: Symbiosis with AMF and previous herbivory reduces the relative growth of *S. exigua*, increases its susceptibility to a sublethal dose of *B. thuringiensis* and has positive or neutral impact on the lethality of SeMNPV. Reduction of the phenoloxidase activity, a marker of the insect immune response, was associated with the larval feeding on plant material previously exposed to herbivory but not to the AMF. In addition, no changes in the insect gut microbiota could be associated with the observed changes in larval growth and susceptibility to the entomopathogens.

CONCLUSION: Our findings provide the first evidence of compatibility of AMF symbiosis in tomato with the use of bacterial and viral entomopathogens, contributing to the development of novel approaches to combine the beneficial effect of AMF and entomopathogens in biological pest control.
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Keywords: arbuscular mycorrhizal fungi; *Spodoptera exigua*; microbial entomopathogens; *Solanum lycopersicum*; integrated pest management

1 INTRODUCTION

Plants must constantly cope with adverse environmental factors, consequently they have evolved several strategies to face them.[1,2] They display multiple defence mechanisms to deal with diverse stressors.[3] The plant microbiota is known to modulate such defence mechanisms and beneficial soil microorganisms can increase plant stress resistance/tolerance.[4,5] In this context, the association with arbuscular mycorrhizal fungi (AMF) deserve special attention. These obligate biotrophs belong to the phylum *Glomeromycota* and form symbiotic interactions with more than 80% of land plants, including most agricultural crops.[6] In this interaction, AMF colonize the root cortex and develop an extraradical mycelium, increasing the acquisition of water and inorganic nutrients (mainly phosphate and ammonia) of the plant. In return, the fungus receives photosynthates for the maintenance of mycorrhizal structures.[7] Besides improving plant nutritional status and growth, this symbiosis also improves the ability of the plant to overcome abiotic stresses such as salinity, drought or the presence of heavy metals.[8,9]

Mycorrhization is also involved in enhancing plant defense against a broad spectrum of pathogens and pests, a phenomenon known as mycorrhiza-induced resistance (MIR).[10–12] The

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symbiosis with AMF stimulates the immune system of the plant, leading to a primed state that implies a more efficient activation of defensive responses after exposure to biotic attack.[13,14] In the absence of stress, the defenses of mycorrhizal plants are slightly activated, hence allowing the plants to redirect resources to other biological functions (low-cost defensive strategy).[10,15] In the presence of stress, mycorrhizal plants can trigger a faster defense response, both below and above ground.[12,16] The protective role of mycorrhization has been proven at root level against soil-borne pathogens, nematodes or root-chewing insects[17–19] whereas in aboveground tissues MIR enhances resistance against necrotrophic pathogens and generalist chewing insects.[12] The mechanisms that drive MIR in aboveground tissues are still elusive, but there is emerging evidence showing transcriptional and metabolic reconfiguration in the leaves of mycorrhizal plants which led to MIR-primed responses.[20–24] Besides defense priming, some basal changes occur in leaves of mycorrhizal plants. For example, mycorrhizal plants often contain higher amounts of bioactive phenolic metabolites in their leaves than nonmycorrhizal plants. However, these metabolic responses are highly specific to each AMF–plant species combination.[22]

For orchestrating a full defense response against herbivores, plants rely on the jasmonic acid (JA) signalling pathway, which is a conserved core pathway that is activated after insect feeding and leads to the accumulation of defensive compounds such as secondary metabolites (e.g. terpenoids, phenolics and alkaloids compounds) and proteins (e.g. protease inhibitors, polyphenoloxidases).[1,25] These defensive molecules deter insect herbivory by directly impairing insect growth.[26] In addition, they interplay with the herbivore microbiome,[27] indirectly altering insect fitness, and may make target insects more susceptible to biotic stresses such as the entomopathogens.[28] These are a group of diverse microorganisms that are pathogenic to insects. They include bacteria, viruses, fungi and nematodes that are widely used in pest control in organic farming or integrated pest management strategies.[29–31] Among them, *Bacillus thuringiensis* and baculoviruses are two of the most successful organisms used in the control of lepidopteran larvae.[32,33] Both infect target insects by ingestion and lead to insect death within a few days.[34,35] Besides their direct mode of action,[36–38] research in recent decades has revealed the existence of complex interactions among these two entomopathogens, plant defenses[39,40] and the gut microbiome[41–44] that can lead to a faster death of target insects, perhaps targeting the immune system or facilitating the weakening of insect protective barriers such as the peritrophic membrane, which can in turn facilitate the start of secondary infections.[45]

How the metabolic reconfiguration undergone by herbivory and/or plant mycorrhization impacts susceptibility to entomopathogens of phytophagous larvae has not yet been thoroughly investigated. In the present work, we compared the effects of mycorrhizal and non-mycorrhizal *Solanum lycopersicum* tomato plants in the absence or the presence of herbivory on the growth of larvae from the armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) and their susceptibility to *B. thuringiensis* subsp. *aizawai* (Xentari) and the Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV). We also analysed the changes in the prophenoloxidase, a marker of the insect immune status, and midgut microbiota. Lyophilized instead of fresh leaves were used to discriminate the effect of the herbivory-induced phytochemicals from the changes associated with plant mycorrhizal status exclusively. We used these data to address four major questions: Are plant defenses enhancing susceptibility to selected entomopathogens? Is this higher susceptibility increased when mycorrhization is present? Are plant defenses altering the insect immune system and/or gut microbiota? Are these changes further modulated by the presence of AMF? Our results reveal that mycorrhization of tomato plants in combination with previous herbivory increases the susceptibility of *S. exigua* to the bacterial entomopathogen and does not interfere with the lethality of the viral entomopathogen, suggesting that the use of both methods in the field (AMF and use of *B. thuringiensis* or baculovirus) may be combined with promising results.

2 MATERIALS AND METHODS

2.1 Insects

Spodoptera exigua eggs were provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland) and maintained in our laboratory (Valencia, Spain) by continuously rearing on artificial diet supplemented with 0.05% of tetracycline[46] at 25 ± 3 °C with 70 ± 5% relative humidity and a photoperiod of 16 h light:8 h dark.

2.2 Tomato plants, mycorrhization and diet preparation

Solanum lycopersicum cv. Moneymaker (MM) plants were grown at the Zaidín Experimental Station (CSIC, Granada). Tomato seeds were surface sterilized and germinated in sterile vermiculite. Seedlings at two cotyledons stage were inoculated with the AMF *Funneliformis mosseae* (BEG 12) (Nicolson and Gerdemann) Gerdemann et Trappe (Banque Européenne des Glomales [BEG] code 12) at transplanting by mixing the AMF inocula with the growing substrate, as previously described in Rivero *et al*.[12] Plants were randomly distributed and grown in a controlled greenhouse at 24/16 °C with a 16 h:8 h diurnal photoperiod. After 6 weeks of growth to allow the establishment of the mycorrhizal symbiosis, herbivory was initiated by infesting plants with two second-instar *S. exigua* larvae per plant, which were placed on a leaflet from the third true using 30 mm Ø clip-cages to limit the feeding area and avoid their escape. Clip-cages were moved into new leaflets every 2 days to make sure they always had food available. All the plants showed significant damage to leaves. Accordingly, four groups of plants were generated depending on the presence or absence of mycorrhization and/or herbivory: control Nm− (absence of both factors) and treatments Nm+ (exposition to herbivory), Fm− (presence of mycorrhization) and Fm+ (presence of both factors) (Fig. 1). Each treatment consisted of 12 plants growing in a randomized position in the greenhouse. Plants were harvested 8 weeks after mycorrhiza inoculation (15 days after starting of herbivory). On harvesting, tomato leaves were immediately frozen with nitrogen liquid and stored at −80 °C. An aliquot of each root system was reserved for mycorrhizal assessment. Mycorrhizal colonization was evaluated by ink-staining fungal structures within the roots according to Vierheilig *et al*.,[47] and the percentage of root length colonized was quantified under a light microscope (Eclipse 50i microscope; Nikon, Japan) using the gridline intersection method.[48] Mycorrhizal colonization was confirmed in all inoculated plants (Fm), with an average of 12% of the root length colonized by the fungus. Absence of mycorrhizal colonization was confirmed in Nm plants. No significant differences in shoot or root fresh weight were found between mycorrhizal and non-mycorrhizal plants (*t*-test, $n = 10$, $P > 0.05$).

The frozen leaf material was fully ground in liquid nitrogen until leaf powder was obtained, which was lyophilized and stored until diet preparation. Plant-derived diets were prepared by mixing the lyophilized material in 1.8% agar containing 5% of the

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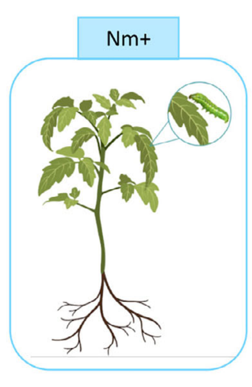

Figure 1. General diagram of the treatments applied to *S. lycopersicum* plants. Four groups of tomato plants were classified according to the presence of mycorrhization and/or herbivory. Nm−, non-mycorrhizal plants without previous herbivory, used as control; Nm+, non-mycorrhizal plants exposed to herbivory by *S. exigua* larvae for 15 days; Fm−, mycorrhizal plants associated with the arbuscular mycorrhizal fungus *F. mosseae*; Fm+, mycorrhizal plants exposed to *S. exigua* herbivory.

compounds used for the standard artificial diet[49] to obtain a suitable diet for *S. exigua* larvae.

2.3 Growth bioassay and larval development

With the aim of studying the effects of mycorrhization and previous herbivory on larval growth, newly molted fourth-instar *S. exigua* larvae were reared on the different leaf-based diets for 48 h. For that, disposable polypropylene boxes (11 cm × 8 cm × 5 cm) with the top cover replaced by paper (airflow window) were used. Each box contained four *S. exigua* larvae, previously weighed (in grams) in a precision balance (Sartorius MC-1 Analytic AC 120S; Göttingen, Germany) with an accuracy of 0.1 mg. The boxes were maintained in an insect chamber at 25 ± 1° C with a photoperiod of 16 h light:8 h dark. To prevent the impact on the weight gain of larval moulting, larvae were pooled per treatment, and replicated and weighed at 48 h. Weight increase was recorded and relative growth (RG) was estimated as grams of biomass acquired per gram of initial body weight.[50] Three independent biological replicates were performed. Statistical differences in relative growth between treatments were identified using two-way ANOVA with mycorrhization and herbivory as factors, followed by a Tukey HDS multiple comparison test using R Statistical Software (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). Levene and Shapiro–Wilk tests were applied to determine homoscedasticity and normality of data, respectively.

2.4 Interaction with natural entomopathogens

The effect of mycorrhization and herbivory on the susceptibility of *S. exigua* larvae to *B. thuringiensis* and baculovirus (SeMNPV) was tested using the droplet feeding method. Specifically, newly molted second-instar larvae were placed in groups during the infection process using independent Petri dishes for each treatment, where previously 4-μL droplets were arranged in circle. Each droplet contained 10% sucrose, phosphate-buffered saline (PBS; pH 7.4), 0.05% tracking dye phenol red and a sublethal concentration of one of the two pathogens. For Bt infections, we used *Bacillus thuringiensis* subsp. *aizawai* (Xentari; Kenogard S.A, Barcelona, Spain), previously dissolved in water, at two concentrations (1 and 3 mg/mL). For BV infections, we used a viral suspension containing 2×10^4 occlusion bodies (OBs) per milliliter from SeMNPV (SP2 strain).[51] We conducted previous assays to estimate the sublethal and lethal concentrations of the pathogens under our experimental conditions. After 20 min, larvae with red-coloured bodies were selected for the next step, thus ensuring only larvae that had ingested the entomopathogen were selected. Selected larvae were placed individually in a single well (2 cm × 2 cm × 2 cm) of a bioassay tray and fed with a piece of the different plant-based diets for 48 h at 25 ± 1 °C. Each well was sealed with microperforated adhesive tape (product no. 9074-L; Frontier Agricultural Sciences).

The plant-based diet was then replaced by artificial diet (prepared without antibiotic addition) and mortality was recorded every 24 h for 7 and 8 days (Bt and SeMNPV, respectively) from the beginning of the bioassay. In Bt assays, four independent replicates were performed with the sublethal dose (1 mg/mL), whereas two were carried out with the lethal dose (3 mg/mL). In SeMNPV assays, three independent replicates were conducted. A total of 16 larvae were used per treatment and replicate. Survival curves were assessed using the Kaplan–Meier method and compared using the log-rank analysis (Mentel–Cox test) (GraphPad software Inc., San Diego, CA, USA).

2.5 Phenoloxidase enzymatic assays

To evaluate the effect of mycorrhization and herbivory on the *S. exigua* immune system, the hemolymph from the larvae used in the growth assay was extracted immediately after weighing to measure the phenoloxidase (PO) enzymatic activity, a marker of the insect immunity. In brief, the posterior proleg of each larva was cut, and the hemolymph was collected with a micropipette and pooled by treatment (four larvae each). Then, hemolymph was centrifuged at 500 × *g* for 2 min at 4 °C to remove the hemocytes and kept on ice. Four microliters of cell-free hemolymph, 46 μL of PBS and 50 μL of the substrate L-dopamine (100 μg/mL in PBS) were added to each well in a 96-well microtiter plate placed on ice. PO activity was determined by monitoring the increase of absorbance at 492 nm for 30 min using an Infinite 200 PRO multimode plate reader (TECAN Group Ltd, Switzerland). The activity of the enzyme was obtained as the initial velocity (Vo) of the reaction, measuring the change in absorbance per time.

Changes in PO activity in the larval hemolymph could be due to the effect of the different treatments on insect immune status, but also direct interaction of plant metabolites on the PO enzymes. To test that, PO inhibition assay to assess the direct effect of the plant extract on these enzymes was also performed. For this purpose, leaf powder from the different treatments was mixed vigorously

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with methanol (50 mg/mL), incubated for 10 min and centrifuged at maximum speed for 10 min at 4 °C. The supernatant was collected and used as plant metabolites source. A solution containing 2 μL of plant extract, 100 μL of L-dopamine (100 μg/mL in PBS) and 100 μL of larval hemolymph derived from L5 larvae feeding on an artificial diet (20 mg of lyophilized hemolymph in 1 mL of PBS) was added to each well in a 96-well microtiter plate. After incubating for 15 min at room temperature, PO activity was monitored and calculated as described above.

Statistical differences in PO activity were identified using two-way ANOVA with mycorrhization and herbivory as factors, followed by a Tukey HDS multiple comparison test using R Statistical Software (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). The normality of the data sets was assessed by a Shapiro–Wilk test and homoscedasticity by Levene's test.

2.6 Gut microbiota composition and diversity

To test if feeding on the different plant-based diets could influence the gut microbiota of *S. exigua*, we carried out metagenomic sequencing of the guts extracted from the larvae used in the growth bioassay and PO quantification. An additional treatment (AD) represented by larvae of the same instar raised side-by-side during 48 h in 100% artificial diet without antibiotic was added. Each sequenced sample (AD, Nm−, Nm+, Fm−, Fm+) was composed of a pool of four larvae and three independent biological replicates per treatment were analysed. In brief, after 48 h of feeding, the whole gut of each larva was dissected with forceps, homogenized in Luria–Bertani medium supplemented with 10% glycerol and kept at −80 °C until DNA extraction. Total DNA extraction from the homogenized guts was carried out using a MasterPure DNA purification kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions, followed by PCR amplification and sequencing of the 16S rRNA (V3-V4 region). Sequencing was performed as previously described in Martínez-Solís *et al*.[44] using a 2 × 300-pb paired-end run on a MiSeq sequencing platform (Illumina) at the Foundation for the Promotion of Health and Biomedical Research (FISABIO, Valencia). The PRINSEQ-lite program[52] was used to evaluate the quality of the obtained reads, setting the following parameters: min_length, 50; trim_qual_right, 20; trim_qual_type, mean; trim_qual_window, 20. Paired reads were joined using *fastq-join* from the ea-tools suite.[53] Then, filtered and demultiplexed sequences were processed with software QIIME v.1.9.[54] using default parameters. The sequences, from a total of 15 samples, were clustered in operational taxonomic units (OTUs) of 97% sequence identity using *de novo* OTU picking. After filtering the unassigned *Chloroflexi* and *Cyanobacteria* taxa, bacterial composition was determined for the 20 most abundant genera and represented in Excel software. In addition, the OTU table data was transformed and normalized (CSS (cumulative sum scaling) + log with total sum normalization) using Calypso software (version 8.2) to generate a canonical correspondence analysis (CCA) plot showing the relationship among mycorrhization/herbivory (exposure factors) and larval gut microbial communities at genus level. Determination of alpha diversity (Shannon index) and linear discriminant analysis (LDA) effect size (LEfSE) was undertaken at genus level using mycorrhization and herbivory as factors.

To calculate the bacterial load in each sample, total DNA was amplified using universal primers for the 16S rRNA gene by performing a specific quantitative PCR (qPCR) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction was carried out using 5× HOT FIREpol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) in a total reaction volume of 20 μL. To estimate the bacterial concentration, the threshold cycle (Ct) values of our samples were compared with those from a standard curve of known *Escherichia coli* DNA concentrations. Statistical analysis was performed using one-way ANOVA with a Tukey's multiple comparison test (GraphPad software Inc., San Diego, CA, USA).

3 RESULTS

3.1 Effect of mycorrhization and herbivory on larval development

To understand how mycorrhization (Fm−), herbivory (Nm+) or a combination of both (Fm+) impact on the development of fourth-instar *S. exigua* larvae, we measured the relative growth of caterpillars after feeding on the different plant diets for 48 h (Fig. 2(A)). A significant decrease of about 2-fold in relative growth was observed after herbivory (Nm+ and Fm+) but not after mycorrhization and both factors interacted in a significant way (mycorrhiza: $F_{1,8} = 3.727$, $P = 0.090$; herbivory: $F_{1,8} = 21.939$, $P = 0.002$; interaction: $F_{1,8} = 5.435$, $P = 0.048$). No larval mortality was observed in either treatment.

3.2 Influence of mycorrhization and herbivory on larval susceptibility to entomopathogens

The effect of mycorrhization and herbivory on the susceptibility of *S. exigua* to two natural bacterial (*B. thuringiensis*) and viral (SeMNPV) pathogens was evaluated. Susceptibility of larvae to *B. thuringiensis* was analyzed at sublethal and lethal concentrations. At the sublethal concentration (1 mg/mL), no mortality was observed for the insects feeding on diet based on control plants (Nm−) whereas about 20% decrease in survival was observed when infected larvae were reared on Nm+, Fm− or Fm+ diets ($\chi^2 = 12.31$, df = 3, $P = 0.0064$; Fig. 2(B)). There were no significant differences among these three treatments. In contrast, when larvae were exposed to a higher concentration of *B. thuringiensis* (3 mg/mL), no differences in survival were observed between the larvae feeding on the different treated plant-based diets and the control Nm− ($\chi^2 = 2.114$, df = 3, $P = 0.5492$; Fig. 2(C)).

Susceptibility to baculovirus was evaluated using a sublethal concentration of SeMNPV suspension (2×10^4 OBs/ml). Only feeding on mycorrhizal plant (Fm−)-based diet caused a significant increase in mortality when compared to the control (Nm−) ($\chi^2 = 3.944$, df = 1, $P = 0.0470$; Fig. 2(D)).

3.3 Impact of treatments on larval immunity

Changes in the insect metabolism may weaken its immune status and in turn make insects more susceptible to pathogens. Hence, we asked whether feeding on plants that had been mycorrhized or previously exposed to herbivory may alter insect immune defences. To test this, we focused on the analysis of one of the key components of the insect immune system, PO, whose activity in the hemolymph is a widely used marker of the immune status. Two-way ANOVA showed that only previous herbivory had a significant effect on the activity of PO (mycorrhiza: $F_{1,8} = 1.361$, $P = 0.277$; herbivory: $F_{1,8} = 6.665$, $P = 0.033$; interaction: $F_{1,8} = 0.010$, $P = 0.924$). (Fig. 3(A)). To verify that reduction in PO activity was not driven by the presence of PO inhibitors in the lyophilized leaves, we performed an inhibition assay that confirmed that none of the different treatments inhibited PO activity in insect hemolymph (mycorrhiza: $F_{1,8} = 2.061$, $P = 0.189$;

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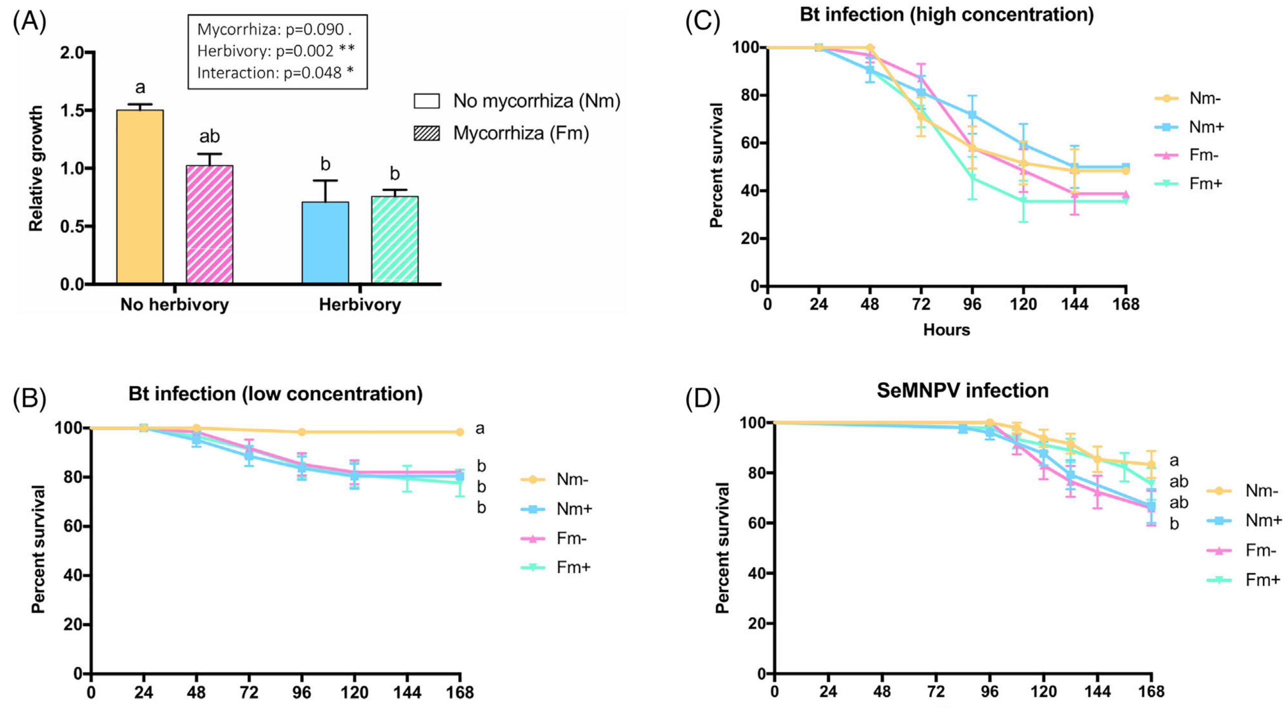

Figure 2. Effect of mycorrhization and herbivory on the development and susceptibility of *S. exigua* larvae to bacterial and viral pathogens. (A) Larval development, represented as relative growth, after feeding on plant-based diets for 48 h. Mean values were analyzed with two-way ANOVA with mycorrhization and herbivory as factors followed by a Tukey HSD *post hoc* test. Error bars represent standard error of the mean (SEM). (B) Percentage of survival of larvae infected with Xentari (*Bacillus thuringiensis* subsp. *aizawai*) at 1 mg/mL (sublethal) and (C) 3 mg/mL (lethal). (D) Percentage of survival of larvae infected with SeMNPV virus at a sublethal concentration (2×10^4 OBs/ml). Mortality curves were plotted using the Kaplan–Meier method and statistical analyses were performed using the log-rank (Mantel–Cox) test. Error bars depict standard errors (SE). Different letters denote significant differences among the treatments. Nm−, non-mycorrhizal plants without previous herbivory; Nm+, non-mycorrhizal plants exposed to herbivory; Fm−, mycorrhizal plants; Fm+, mycorrhizal plants exposed to herbivory.

herbivory: $F_{1,8}$ = 0.010, P = 0.924; interaction: $F_{1,8}$ = 0.157, P = 0.702) (Fig. 3(B)).

3.4 Gut microbiota changes after feeding on the different diets

Gut microorganisms are critical to the nutrition, physiology and immune responses of many insect species, and have a complex interplay with both plant metabolites and entomopathogens. Thus, we studied the influence of the different plant-based diets to understand the impact of mycorrhization and herbivory on the gut bacterial communities of *S. exigua*.

Bacterial microbiota composition was determined in larval guts after 48 h of feeding on artificial or plant-based diets (Fig. 4(A)). Most abundant bacteria belonged to the *Delftia* genus, which was highly present in all the groups (with a relative abundance ranging from 10% to 50%), followed by unclassified *Oxalobacteraceae, Comamonadaceae* and *Enterobacteriaceae*. CCA showed significant differences (P = 0.038; Fig. 4(B), upper panel) in larval gut microbiota composition at the genus level among the artificial and the plant-based diets. However, these differences disappeared when only plant diets were compared (P = 0.053; Fig. 4 (B), bottom panel). Great variability and heterogeneity were observed among different diet groups but also among samples from the same group. No differences in the bacterial load (P = 0.407; Fig. 4(C)) nor in the diversity (P = 0.37; Fig. 4(D)) were observed among the different diets. LEfSE analysis revealed the existence of three genera with differential abundance according to the type of ingested diet. High abundance of unclassified *Caldilineaceae* and *Anaerobaculaceae* were detected in larvae fed on the artificial diet when compared with the plant-based diet. Unclassified *Xanthomonadaceae* were found to be more abundant in the insects fed on the Fm+ plant diet when compared to the other diets.

4 DISCUSSION

Our results show that mycorrhizal colonization of tomato roots by *F. mosseae* coupled with previous herbivory decreases *S. exigua* growth, enhances its susceptibility to *B. thuringiensis* and has positive or neutral impact on the lethality of SeMNPV. These bacterial and viral entomopathogens are widely used for the control of folivorous *S. exigua* larvae,[33,55,56] and our results support the compatibility of AMF application in tomato with the use of entomopathogens in pest management strategies.

AMF colonization is known to imply changes in plant physiology and metabolism that may enhance its resistance to foliar-feeding herbivores.[15,57–60] Our larval growth assays have revealed that *F. mossae* inoculation in combination with herbivory had a negative impact on the growth of *S. exigua*. This confirms previous observations with the same mycorrhiza–plant–insect combination,[12,59] with a related foliar-feeder, the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae),[61] with *S. exigua* feeding on *Plantago lanceolata* inoculated with *F. mossae*,[62] and also with other *Spodoptera* spp.–plant–AMF combinations.[63–66] Our approach differs from these previous studies because we used a plant-based artificial diet composed of lyophilized leaves from

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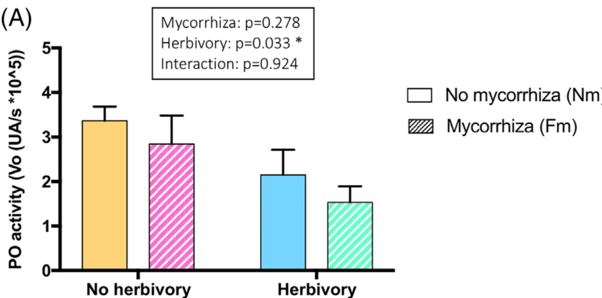

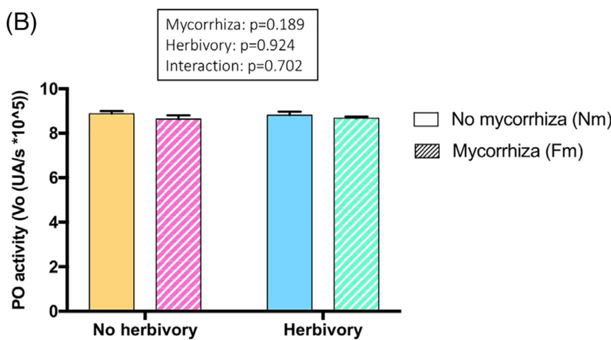

Figure 3. Effect of mycorrhization and herbivory on *S. exigua* prophenoloxidase (PO) activity. (A) Enzymatic activity of PO, represented as the initial velocity (Vo) of the reaction. (B) PO enzymatic inhibition assay using leaf extracts from the different treatments. Means were analyzed with two-way ANOVA using mycorrhization and herbivory as factors. Error bars represent standard error of the mean (SEM).

the inoculated and/or herbivory-exposed plants. This allows the dissociation between the effects of mycorrhization and herbivory, and for their dietary outcomes on larval growth and susceptibility to entomopathogens to be tested separately. We observed that previous herbivory but not AMF colonization reduced larval growth, and that both factors interacted in a significant way. The weight decrease of *S. exigua* larvae feeding on a diet based on herbivory-exposed plants, either with or without AMF inoculation, may be related to the JA-dependent defensive response of the tomato plants, which accumulate secondary metabolites toxic to deter the herbivore.[1,67] In fact, on *S. exigua* herbivory, tomato plants undergo a dramatic metabolic reconfiguration, which has been shown by previous studies to be primed by *F. mossae* mycorrhization.[12] In contrast, in the absence of herbivory, AMF colonization has very low impact on the foliar metabolic profile,[9] although some transcriptional shifts in transcription in the levels of genes related to JA biosynthesis and response, even in the absence of any aggressor, have been reported.[68,69] Thus, the significant interaction between herbivory and AMF colonization on *S. exigua* larval growth may be explained by the effect of AMF priming on the subsequent response to herbivory. However, we should be aware that in lack of metabolic data supporting the induction of plant defences and their modulation by AMF priming, we cannot exclude other hypothesis explaining the reduced larval growth. For example, a drop in the nutritional quality of the attacked leaves might reduce the conversion efficiency of the ingested food by the larvae.[62] Previous studies have shown that in tomato, *F. mossae* inoculation increased leaf photosynthesis, nutrient absorption and altered foliar hormone homeostasis.[59] Thus,

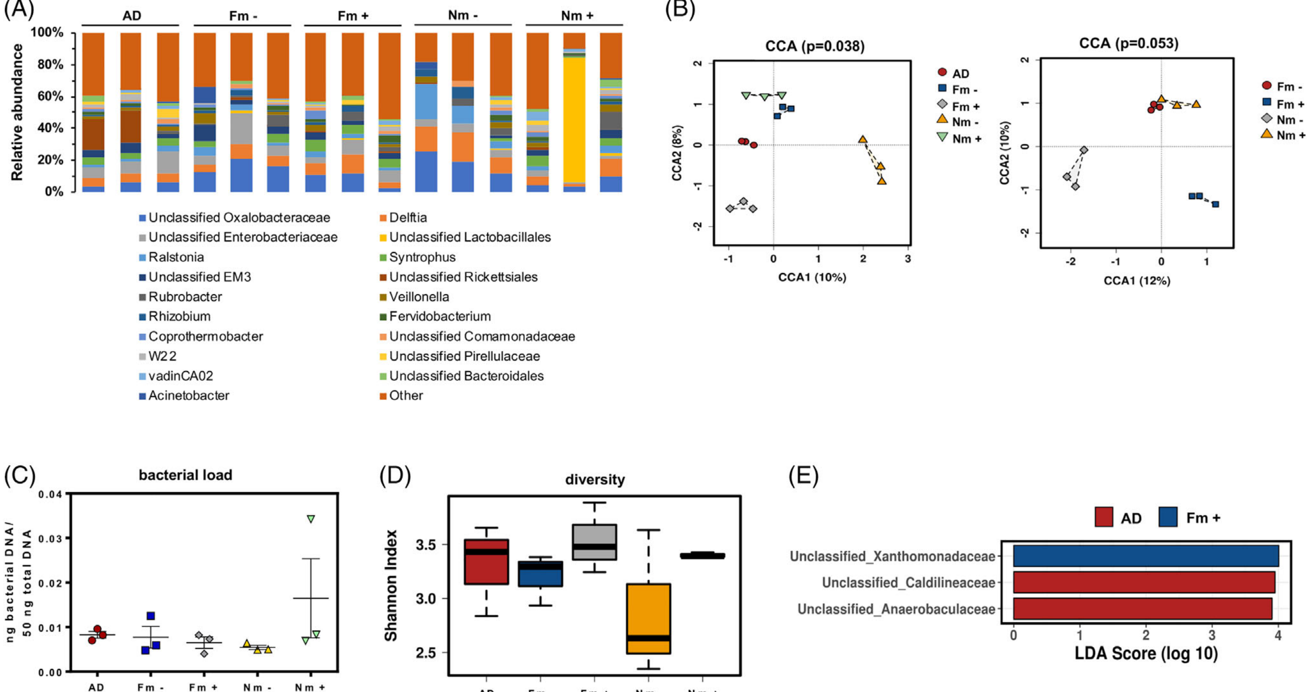

Figure 4. Effect of mycorrhization and herbivory on the gut microbiota of *S. exigua* larvae. (A) Relative abundance (percentage) of the different genera of bacteria of guts from larvae fed on the different diets for 48 h. Each bar represents a pool of four larvae. (B) Canonical correspondence analysis (CCA) showing the relationship between the composition of *S. larvae* gut microbiome at genus level and the diets tested: artificial diet (AD) and plant-based diets. (C) Bacterial load of larval guts calculated as nanogram of bacterial DNA per 50 ng of total DNA (means ± SEM). (D) Microbial diversity of larval guts represented using Shannon index. Nm−, non-mycorrhizal plants without previous herbivory; Nm+, non-mycorrhizal plants exposed to herbivory; Fm−, mycorrhizal plants; Fm+, mycorrhizal plants exposed to herbivory.

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additional experiments are needed to unravel the causes of decreased *S. exigua* growth.

All the plant diet based on either mycorrhizal (Fm−) and/or plants exposed to herbivory (Nm+ and Fm+) displayed an increased susceptibility to a sublethal concentration of *B. thuringiensis*, with no additional effects when AMF and previous herbivory were combined. Equally, a general increase in mortality of SeMNPV-infected *S. exigua* larvae was observed when they were fed on the treated plant-based diets, although only the Fm− diet led to a statistically significant difference. These observations suggest that mycorrhization and previous herbivory, individually or in combination, could enhance the susceptibility of *S. exigua* larvae to entomopathogens. This contrasts with the only previous study so far that evaluated the combination of AMF and an entomopathogenic baculovirus. García-Gómez *et al*. reported that inoculation of maize roots with a natural community of AMF (including *Glomus* spp., *Acaulospora* spp., *Gigaspora* spp. and *Intraspora* spp) increased growth of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) under certain nitrogen fertilization conditions, and reduced larval mortality by its baculovirus (SfMNPV) by about 25%.[70]

The increased lethality of *B. thuringiensis* after previous herbivory or the combination of herbivory and AMF may be explained by the defense metabolites produced by the plant after attack, which have been previously shown to influence the outcome of insect–baculovirus interactions[39,71,72] as well as increase or decrease the lethality of *B. thuringiensis* at various extents. For instance, tannins and nicotine decreased mortality of *B. thuringiensis* against a variety of insects,[73–77] whereas protease inhibitors, L-canavanine, resorcinol and gallic acid increased *B. thuringiensis* lethality.[78–80] The induction of herbivory defenses in tomato increased susceptibility to *B. thuringiensis* in the lepidopteran *Helicoverpa zea* (Lepidoptera: Noctuidae),[40] in a similar fashion to what we observed for *S. exigua*. In the tomato-*Helicoverpa* system, the increased susceptibility to *B. thuringiensis* correlated with increased plant polyphenoloxidase (PPO) and peroxidase (POD) activity, which are part of the inducible defensive armoury of tomato.[40] Interestingly, in our system we reported an increased susceptibility to *B. thuringiensis* also in mycorrhizal plants which had not been induced by previous herbivory and thus, no major changes in these defense related enzyme activities are expected.[12,59] Therefore, the enhanced lethality of *B. thuringiensis* but also of SeMNPV in larvae reared on Fm− diets may be led by other causes. The effects of plant nutrients dictate herbivore physiology, which in turn could influence their susceptibility to the third trophic levels such as the entomopathogens.[81,82]

Susceptibility to pathogens is strongly correlated with the immune status of an organism.[83,84] Thus, we sought to evaluate the effects of the plant-based diets on the larval immune system, specifically focusing on the activity of the insect's PO due to its relevant function in cellular and humoral response in insects.[85] PO enzyme is involved in the formation of melanin, which is deposited around the damaged tissue or foreign object, leading to the production of intermediate products with toxic effects against bacterial, fungal and viral agents.[86] Enzymatic assays revealed that PO activity was significantly reduced by herbivory but not by AMF or the interaction of the two factors. Additionally, inhibition assays showed that the leaf extracts from the tested plants did not suppress PO activity, ruling out the possibility of direct interference with the enzymatic activity. Thus, the results suggest that compounds induced during herbivory negatively influence the immune status of *S. exigua* larvae. The reduction in PO activity may be the outcome of direct toxic effects of leaf allelochemicals that debilitate the larval immune system or indirect consequences of the altered herbivore performance and development of the insect.[28,87]

Finally, we asked whether the different plant-based diets had an impact on the gut microbiota, which is known to influence insect physiology by the modulation of caterpillar nutrition and metabolism, development and immune responses.[88,89] Although we did not observe a major influence of the diet on the bacterial load or alpha diversity of the gut microbiota, CCA analyses revealed significant differences in bacterial composition between larvae fed on artificial diet and tomato plant-based diets. Similar changes in the microbiota composition associated with the source of the ingested food (artificial diet, pepper and tomato) were previously observed in *S. exigua* larvae by Martínez-Solís *et al*.[44] Nevertheless, when only plant-based diets were included in the multivariate canonical analysis, the differences disappeared. This may indicate that changes in the leaf nutritional quality or phytochemicals induced by mycorrhization and/or exposition to herbivory were not/or just slightly influencing the microbiota gut composition of the caterpillar.

In conclusion, AMF colonization in tomato plants increased the susceptibility of the generalist pest *S. exigua* to the bacterial entomopathogen *B. thuringiensis* and did not negatively affect the action of the viral entomopathogen baculovirus. These findings support the compatibility of AMF inoculation with the use of bacterial and viral entomopathogens, and support the design of pest control strategies combining the effects of both treatments. Nevertheless, further studies using different combinations of plants, AMF, herbivores and entomopathogens are needed to extend these results to other crops and pest systems.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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