Research Article

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Mycorrhiza-induced resistance in citrus against *Tetranychus urticae* is plant species dependent and inversely correlated to basal immunity

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Abstract

BACKGROUND: Mycorrhizal plants show enhanced resistance to biotic stresses, but few studies have addressed mycorrhiza-induced resistance (MIR) against biotic challenges in woody plants, particularly citrus. Here we present a comparative study of two citrus species, *Citrus aurantium*, which is resistant to *Tetranychus urticae*, and *Citrus reshni*, which is highly susceptible to *T. urticae*. Although both mycorrhizal species are protected in locally infested leaves, they show very distinct responses to MIR.

RESULTS: Previous studies have indicated that *C. aurantium* is insensitive to MIR in systemic tissues and MIR-triggered antixenosis. Conversely, *C. reshni* is highly responsive to MIR which triggers local, systemic and indirect defense, and antixenosis against the pest. Transcriptional, hormonal and inhibition assays in *C. reshni* indicated the regulation of jasmonic acid (JA)- and abscisic acid-dependent responses in MIR. The phytohormone jasmonic acid isoleucine (JA-Ile) and the JA biosynthesis gene *LOX2* are primed at early timepoints. Evidence indicates a metabolic flux from phenylpropanoids to specific flavones that are primed at 24 h post infestation (hpi). MIR also triggers the priming of naringenin in mycorrhizal *C. reshni*, which shows a strong correlation with several flavones and JA-Ile that over-accumulate in mycorrhizal plants. Treatment with an inhibitor of phenylpropanoid biosynthesis C4H enzyme impaired resistance and reduced the symbiosis, demonstrating that phenylpropanoids and derivatives mediate MIR in *C. reshni*.

CONCLUSION: MIR's effectiveness is inversely correlated to basal immunity in different citrus species, and provides multifaceted protection against *T. urticae* in susceptible *C. reshni*, activating rapid local and systemic defenses that are mainly regulated by the accumulation of specific flavones and priming of JA-dependent responses.
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Keywords: priming; mycorrhiza-induced resistance; *Citrus reshni*; *Rhizophagus irregularis*; *Tetranychus urticae*; flavonoids

1 INTRODUCTION

The two-spotted mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is a polyphagous and cosmopolitan herbivore that feeds on a wide variety of plant species and families. The spider mite is a cell content feeder with a stylet that penetrates leaf tissue and feeds on single mesophyll cells. This feeding style induces characteristic leaf chlorotic spots at the feeding sites that impact plant physiology causing global economic losses.[1–3]

The first barriers that mites encounter in their host plants are phytoanticipins located at trichomes, such as acyl sugars.[4] In addition, type VI trichomes contain several methyl ketones that are toxic for mites.[5,6] The next phase of the interaction between the mite and the plant happens when mites inject their stylets through either stomatal openings or epidermal cell apoplasts.[7,8] A recent study by Arnaiz *et al*. suggested that at this stage mite perception would be mediated by the tioredoxinh5 (TRXh5) protein located at the cell membrane that regulates the *S*-nitrosylation pattern of a THIORREDOXIN INTERACTING RECEPTOR (TIRK). This interaction activates downstream reactive nitrogen and oxygen species leading to jasmonic acid isoleucine (JA-Ile) accumulation and plant resistance against *T. urticae*.[9] Although not well understood, sensing mite herbivore-associated

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molecular patterns activates a plethora of hormonally regulated molecular defenses, leading to an accumulation of specialized metabolites and defensive proteins with antimicrobial properties.[10–12] Furthermore, as part of the mites' external digestion, breakdown products are generated by digested cells and these are perceived by surrounding cells as damage-associated molecular patterns in addition to silk or feces which are recognized as external.[13]

Although *T. urticae* is considered polyphagous,[7] it can adapt to specific hosts.[14] The adaptation of *T. urticae* to multiple hosts involves two different strategies. The first is detoxification of the host defenses, by overexpression of carboxyl/cholinesterases, cytochrome P450 monooxygenases, glutathione *S*-transferases (GST) and UDP glycosyltransferases that degrade, modify or detoxify plant toxins.[15,16] Second, through host manipulation, mites suppress host defenses regulated by the salicylic acid (SA)- and jasmonic acid (JA)-dependent pathways.[17,18] Secreted salivary proteins known as tetranins can counteract host defenses.[19] A recent study demonstrated that adapted lines of *T. urticae* attenuate the expression of plant defense genes such as *Leucine aminopeptidases 1/2*, *Arginase 1/2* and *Threonine Deaminase 2*.[20] This study suggests that keeping high levels of digestive cathepsin-L activity, and high P450, esterase and GST detoxifying activities are major mechanisms for adaptation.

Plant defenses against spider mites are mediated by JA.[7,10,21] Nevertheless, abscisic acid (ABA) or SA has also been found to increase during *T. urticae* infestation.[22-24] ABA was found to be negatively correlated with resistance against both *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) and *T. urticae* during drought stress experiments,[24] whereas SA levels do not contribute directly to resistance in *Arabidopsis* or citrus,[10,21] although SA may have a role in tomato plants.[25] Although it remains to be fully understood, ABA and SA may modulate the JA-dependent response during mite attacks, because in some cases, negative cross-talk between these hormones seems nonfunctional,[26,27] whereas in other interactions with specialist mites such as *T. evansi*, salivary effectors suppress SA and JA, resulting in increased reproduction of the specialist.[17] In fact, ectopic expression of the effector proteins Tu28 and Tu84 from *T. urticae* in *Nicotiana benthamiana* Domin (Solanaceae) leads to a downregulation of SA- and JA-dependent defenses and increased oviposition.[18] In addition to the direct activation of defense genes, JA is known to regulate glucosinolate accumulation in *Arabidopsis* in response to mite attack,[28] as well as the transcriptional activation of genes regulating terpenoids, lignin and flavonoids, leading to their accumulation in bilberry or tea.[29] Agut *et al.* found a positive correlation between JA priming in resistant citrus genotypes and the flavonoids hesperetin and naringenin.[10] In the current research, we studied two citrus species with distal basal immunity: *Citrus aurantium* Linneo (Rutaceae) with a high level of mite resistance and *Citrus reshni* hort. ex Tanaka (Rutaceae) that allows better performance by *T. urticae*.[10,30]

T. urticae not only adapts to multiple hosts, but also renders them ineffective within a few years.[31] An innovative alternative for the control of pests includes the use of microbial inoculants to enhance plant defense. The plant immune system can be potentiated by different stimuli, such as beneficial microorganisms, leading to an enhanced state in which plants display higher levels of resistance against attackers. This process is known as induced resistance (IR).[32] The association between plants and arbuscular mycorrhizal fungi (AMF) improves plant fitness,[33–35] tolerance under unfavorable environmental conditions[36–38] and defense against pests and pathogens.[39–42] Mycorrhiza-induced resistance (MIR) is mainly mediated by JA-dependent defenses. Studies on MIR against mites are rare in woody plants, although there are indications that symbiosis triggers the JA pathway, as well as genes related to the biosynthesis of flavonoids during MIR in poplar species.[43] In fact, mycorrhizal plants accumulate higher concentrations of specialized metabolites with antibiotic properties such as phenylpropanoid derivative compounds.[43] Because JA mediates both resistance against mites and MIR, it is likely that symbiosis generates a synergistic metabolic rearrangement in the host, providing enhanced resistance.

In a previous study with the mite-resistant species *C. aurantium*, we showed that symbiosis with *Rhizophagus irregularis* further boosts local resistance by triggering a concomitant increase in JA, *LOXD* and the phenylpropanoids cinnamic acid and diconiferyl alcohol, as well as several flavonoids.[44] By contrast, MIR was ineffective in triggering antixenosis or systemic resistance. In this study, we performed comparative experiments on both *C. aurantium* and *C. reshni* species to determine the differential impact of MIR related to basal immunity. Interestingly, we observed that sensitivity to MIR is dependent on plant species and is inversely correlated to basal immunity, with greater efficiency in highly susceptible species. The mite-susceptible *C. reshni* can perceive MIR in locally infested leaves similar to *C. aurantium*, but it is also protected by promoting antibiosis in systemic tissues, antixenosis and indirect resistance during symbiosis. In addition, we explore the mechanisms mediated by phytohormones and specialized metabolites during MIR in *C. reshni*. Our results indicate that MIR provides multifaceted resistance in *C. reshni* that is regulated by JA and ABA priming, as well as flavone accumulation, whereas phenylpropanoid precursors are reduced, probably to provide moieties for downstream specialized metabolites biosynthesis.

2 MATERIALS AND METHODS

2.1 Plant material and mycorrhizal inoculation

Both citrus species were grown under exactly the same conditions for comparative experiments. Two-month-old *C. reshni* and *C. aurantium* seeds were germinated and grown in vermiculite in a climate chamber with a 16:8 h light/dark photoperiod, 24 °C/18 °C day/night temperature and 50–70% relative humidity (RH). *Rhizophagus irregularis* (BEG121 isolate) (former *Glomus intraradices*) was maintained in a soil-based inoculum and inoculated as described by Manresa-Grao *et al.*[44] Control plants were watered once with a filtrate of the mycorrhizal (AM) inoculum, without the AMF spores, to replicate the microbiome. All plants were watered with modified Hoagland's solution; the modification involved the use of a commercial microelements mix (Nutrishell®, Agrimor-Zenagro, Spain),[45] and no pesticide was applied. To ensure that symbiosis was established 1 month after AMF inoculation, four mycorrhized plants were collected at random, and staining and quantification were performed as described by Vierheilig *et al.* and Giovannetti and Mosse, respectively to determine the levels of colonization.[46,47]

2.2 Spider mite and phytoseiids stock colonies

The *T. urticae* colony was obtained from local orchards (Castelló, Spain) and maintained on pesticide-free lemon fruits [*C. limon* (L)] at 50–70% RH, 25 °C and under the natural photoperiod. Cohorts were prepared for assays that required synchronized gravid females, which were transferred from the lemon colony

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to detached *Citrus clementina* Tanaka (Rutaceae) leaf units as described in Agut *et al*.[48] Two days later, the females were removed and eggs were maintained on the leaves until the hatching larvae reached the adult stage 13 days later. For the adaptation experiments, a line from festuca was collected from orchards containing artificial covers of *Festuca arundinacea* and maintained in the laboratory on the same host.

Colonies of *Phytoseiulus persimilis* Athias-Henriot and the omnivore *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) were obtained from Koppert (Spical® and Spidex®, respectively). Populations were maintained on bean leaflets and supplied twice a week with pollen and a mixture of different *T. urticae* stages, as described by Cabedo-López *et al*.[49] Two-day-old adult mites including *T. urticae* and phytoseiids were starved for an additional day before the assays.

2.3 Direct responses in mycorrhizal citrus plants

To determine the direct effects of MIR in citrus plants, we assessed the relative foliar damage and the number of eggs laid by *T. urticae*. Mites were confined to the infested leaves (two mites per plant, fifth and sixth true leaves) using wet cotton to prevent mite dispersal. Isolated leaves were then infested with five adult females from the cohort described above. Three days later, leaves were detached, and the numbers of adults and eggs were counted. Leaves were cleaned and scanned to determine the relative foliar damage using GIMP software (GNU Image Manipulation Program, version 2.10.20) by normalizing the damaged area to the total area of each leaf. Treatment groups used for gene expression, non-targeted analysis and hormone quantification were as follows: non-mycorrhizal (NM); non-mycorrhizal infested (NM inf); mycorrhizal (AM) and mycorrhizal infested (AM inf).

2.4 RNA extraction and quantitative polymerase chain reaction quantification

RNA extraction from leaves was performed as described by Kiefer *et al*.,[50] with some modifications indicated in Manresa-Grao *et al*.[44] The RNA concentration was adjusted to 1 μg/μL using a NanoDrop--2000 (Thermo Scientific). Following DNase (Thermo Fisher Scientific) treatment, complementary DNA was obtained using the PrimeScript™ RT Reagent Kit (Takara, Valencia, Spain). Primers for *PR-3*, *LOX2*, *PR5*, *NPR1*, *ABA-4* and *C4H* were used for quantitative real-time polymerase chain reactions (qPCR; Supporting Information, Table S1) using AceQ Universal SYBR Green Qpcr Master Mix (Vazyme, Madrid, Spain).[10,51] *NPR1* was obtained from the citrus EST database (https://harvest.ucr.edu/). *UPL7* and *Actin* were used as housekeeping genes to normalize the results, as described by Manresa-Grao *et al*.[44,52]

2.5 Liquid chromatography, electrospray ionization mass spectrometry and full-scan data

For hormone quantification, 1 mL of MeOH/H_2O (10:90) supplemented with 0.01% HCOOH containing a mixture of internal standards—abscisic acid-d6 (ABA-d6), jasmonic-2,4,4d3-(acetyl-2,2-d2) acid (JA-d_5) (Sigma-Aldrich) and jasmonic acid isoleucine-$^{13}C_6$ (JA-Ile-$^{13}C_6$) —was added to 30 mg of freeze-dried leaf tissue to a final concentration of 5 ppb. Samples were centrifuged, the supernatant was collected in a new tube and filtered, and 5 μL of a 1:3 dilution was injected into an Acquity UPLC system (Waters, Milford, MA, USA) coupled with a triple quadrupole mass spectrometer using a UPLC Kinetex 2.6 μm EVO C18 100 A, 2.1 × 50 mm (Phenomenex) column in negative ionization mode (ESI−). External calibration curves were prepared with pure standards. The transitions used were selected as follows: ABA (269 > 159); ABA-d6 (269 > 159); 12-oxo-Phytodienoic Acid (OPDA) (291 > 165); JA-d5 (214 > 61); JA-Ile (322 > 130); JA-Ile-c6 (328 > 136). Further extraction and chromatographic details are given in Sánchez-Bel *et al*.[53]

For untargeted metabolomics, six biological replicates of powdered freeze-dried tissue were mixed with 1 mL of MeOH/H_2O (30:70) with 0.01% HCOOH. Samples were centrifuged and filtered, and the supernatant was collected in a new tube. Five microliters of a 1:3 dilution were injected into an Acquity UPLC system coupled to a hybrid quadrupole time-of-flight mass spectrometer (QTOF), SYNAPT G2-S high-definition tandem mass spectrometry (MS/MS) detector (Waters) with positive and negative ionization modes (ESI+ and ESI−, respectively), using a reverse Kinetex C18_EVO analytical column (2.6 mm particle size, 50 mm × 2.1 m; Phenomenex). Further extraction and chromatographic details are given in Sanmartín *et al*. and Manresa-Grao *et al*.[44,54]

Raw data transformed into a.cdf format (Databridge; MassLynx) were processed as described in Manresa-Grao *et al*.[44] Data in the. cdf format were processed using R and the Centwave algorithm from XCMS. Isotope and adduct corrections (MarVis Filter), heatmaps (MarVis Cluster) and pathways (MarVis Pathway) were obtained from the MarVis Suit 2.0 software.[55] A Kruskal–Wallis test ($P < 0.05$) was performed to compare signals from different treatments. Supervised sparse partial least squares discriminant analysis (sPLS-DA) was obtained by applying normalization by median followed by cube root transformation and Pareto scaling (Metaboanalyst 5.0). Heatmaps were obtained by applying the same statistical data analysis as mentioned for the sPLS-DA, using hierarchical clustering, Euclidean distance measure and Ward clustering method. The criteria for identification were the exact mass, retention time and spectrum fragmentation as described in Gamir *et al*. and Schymanski *et al*.[56,57] The relative amount of each metabolite was calculated by normalizing the peak intensity to the sample weight. Compounds that did not appear in the libraries were identified using different online databases (PubChem, Massbank) and an internal library of real standards using CromaLynx 4.1 (Waters, Micromass, Manchester, UK).

2.6 Antixenosis and indirect defense bioassays

Olfactometer assays were performed as described by Bruin *et al*.[58] A Y-tube comprised a 4-cm diameter glass tube with two arms, each 13.5 cm long, containing a Y-shaped metal wire that was 1 mm in diameter and the same length as the Y-tube in the core of the olfactometer. Two 5-L glass vessels were used to contain the plant samples with a pump-out system (charcoal-filtered air flow of 1.5 L/min). Plants were conditioned to the vessels for 30 min before the experiments. To determine the choice, an adult female was placed at the start of the metal wire. A positive choice was noted when mites reached the end of one of the arms within 5 min; otherwise, 'no-choice' was recorded. Plants were switched every 12 mites to avoid any possible artifacts; mites were used only once. Three replicate experiments with a minimum of 45 choosing mites per replicate and four plants per combination were tested. Antixenosis was tested using symbiosis and infestation as variables in *C. reshni*, but also in comparative assays with *C. aurantium*, as indicated in the Results section.

C. reshni plants and two natural enemies of the spider mite, the specialist predator *P. persimilis* and the omnivore *N. californicus*, were tested to evaluate indirect defense in mycorrhizal plants. Phytoseiids were given a choice between NM and AM plants that

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were either naive or contained prey. Three replicate experiments with a minimum of 45 choosing phytoseiids per replicate and four plants per combination were tested. Treatment groups used for olfactometer assays were as follows: NM, NM inf, AM and AM inf.

2.7 Exogenous application of inhibitors to disrupt ABA and the phenylpropanoid pathway

To block the phenylpropanoid pathway, we used the cinnamate-4-hydroxylase (C4H) inhibitor piperonylic acid (PIP). A 300 μM solution[59] containing 0.1% dimethylsulfoxide and 0.01% Tween-20 was prepared, and mock controls were treated with the same solution except PIP. In the first set of experiments, to determine the importance of flavonoids for the establishment of symbiosis, 24 h before mycorrhization, half of the non-mycorrhizal plants were sprayed with PIP, and the other half were sprayed with the mock solution. After 24 h, all plants were mycorrhized, and the treatment was repeated 2 and 4 weeks after mycorrhization. Root samples were harvested 1, 3 and 5 weeks after mycorrhization to quantify colonization levels. In a second set of experiments, once symbiosis was established, half of the mycorrhizal and non-mycorrhizal plants were sprayed with the PIP solution, and the other half were sprayed with the mock solution. Plants were infested with five females of *T. urticae* per leaf (two leaves per plant) 24 h after treatment. Leaves were harvested and scanned to estimate relative foliar damage at 5 days post infestation (dpi). Treatment groups used for olfactometer assays were as follows: NM inf, non-mycorrhizal infested treated with PIP (NM PIP), AM inf and mycorrhizal infested treated with PIP (AM inf PIP).

To inhibit ABA production, different aliquots of plants were sprayed with 100 μM of the inhibitor of the phytoene desaturase enzyme (PDS) fluoridone [FLU; 1-methyl-3-phenyl-5-[3-(trifluoromethyl) phenyl]-4-(IH)-pyridinone; in 0.1% ethanol], with 100 μM ABA (in 0.1% ethanol) or with 0.1% ethanol. All the treatments were supplemented with 0.01% Tween-20 as a wetting agent. After 24 h, plants were infested with five *T. urticae* per leaf (two leaves per plant), and 7 days later, leaf damage was estimated. Treatment groups used for olfactometer assays were as follows: non-mycorrhizal infested (NM), non-mycorrhizal infested treated with ABA (NM inf ABA); mycorrhizal infested (AM inf), non-mycorrhizal infested treated with FLU (NM inf FLU) and mycorrhizal infested treated with FLU (AM inf FLU).

2.8 Systemic responses in *C. reshni*

Before infestation, the lower and medium parts of the plants were covered with wet cotton to separate local and distal parts and prevent mite dispersal. The lower part of half of the mycorrhizal and non-mycorrhizal plants was randomly infested with ten mites from the cohort described above. Three days after the first infestation, two leaves from the upper part of each plant were isolated and infested with five 2-day-old adult females per leaf. Five days later, leaves from the upper part were detached, and the number of eggs was determined. The groups were: non-mycorrhizal locally infested (NM), non-mycorrhizal infested in systemic leaves (NM SR), mycorrhizal locally infested (AM) and mycorrhizal infested in systemic leaves (AM SR).

2.9 Statistical analyses

Shapiro–Wilk test and Levene's test were used to check normality and homoscedasticity of the data, respectively. The Mann–Whitney *U*-test was performed to analyze the local number of eggs and relative damage because the data were not normally distributed. Chemical inhibition and systemic number of eggs were compared using *t*-tests. A generalized linear model (GLIM) was applied in the case of relative gene expression and hormone quantification because the data did not fulfill the normality assumption, comparing each treatment to the control (NM). Olfactometer assays were analyzed using a binomial test from a 50:50 distribution. Statistical analyses (*t*-test, binomial test, GLIM) were performed with SPSS 25.0 (SPSS, Inc., Chicago, IL, USA).

3 RESULTS

3.1 Arbuscular mycorrhizal symbiosis triggers resistance in *C. reshni* plants against *T. urticae* by reducing leaf damage and egg oviposition

Mite adaptation to different plant species is linked to the performance and damage inflicted on the host plant.[14,28] To assess the relevance of adaptation in our pathosystem, we used *T. urticae* adapted to *F. arundinacea*, which showed reduced and equal levels of mite reproduction in both citrus species (Supporting Information, Fig. S1). By contrast, in experiments using mites adapted to the clementine citrus host for more than 50 generations, increased mite populations were found in *C. reshni* compared with *C. aurantium*, which shows elevated levels of basal immunity to the mite (Supporting Information, Fig. S1). Hence, differences in basal immunity are relevant only when the pest becomes a serious threat to the host. Furthermore, these observations confirmed previous studies in these citrus species infested with citrus-adapted lines.[10,30] Subsequently, we used citrus-adapted *T. urticae* lines for the remaining experiments.

Previously, we demonstrated that symbiosis in *C. aurantium* enhances resistance in locally infested leaves.[44] In the current work, we performed a comparative study of MIR between both citrus species. Three-month-old *R. irregularis*-colonized and non-mycorrhizal plants were infested. Colonization of roots by *R. irregularis* reached 36% after 35 days of inoculation in both rootstocks. Mite performance was examined at 3 dpi in locally infested leaves (Fig. 1). Symbiosis significantly reduced the number of eggs laid in mycorrhizal plants in both citrus species (Fig. 1(A), (B)). None of the treatments affected mite survival during the assay. In addition, MIR reduced the area of leaf damage compared with non-mycorrhizal *C. reshni* plants, which exhibited a 40% increase in damage at 72 hpi (Fig. 1(C)).

3.2 MIR triggers priming of specific components of the JA- and ABA-dependent pathways in *C. reshni* against *T. urticae*

We have previously shown that *C. reshni* is unable to mount an efficient response against *T. urticae*[10]; remarkably, MIR was fully functional in protecting this species against the mite. Hence, we studied mechanisms activated during the immune responses in mycorrhizal plants. Despite its high susceptibility to mites, the enhanced resistance displayed by mycorrhizal *C. reshni* correlated with priming of the biosynthesis gene *LOX2* at 24 hpi and the marker gene *PR3* at 48 hpi (Fig. 2(A)). Participation of the JA pathway in MIR was further confirmed by OPDA levels, which were significantly higher in mycorrhizal plants compared with control levels at 24 and 48 hpi. This may be indicative of a greater availability of jasmonate precursors to generate the active hormone. In fact, the levels of JA-Ile were significantly higher in AM-infested plants than in NM and NM-infested plants at 48 hpi (Fig. 2(B)). Analysis of the *NPR1* marker gene and the levels of SA in non-mycorrhizal *C. reshni* showed that both were induced following

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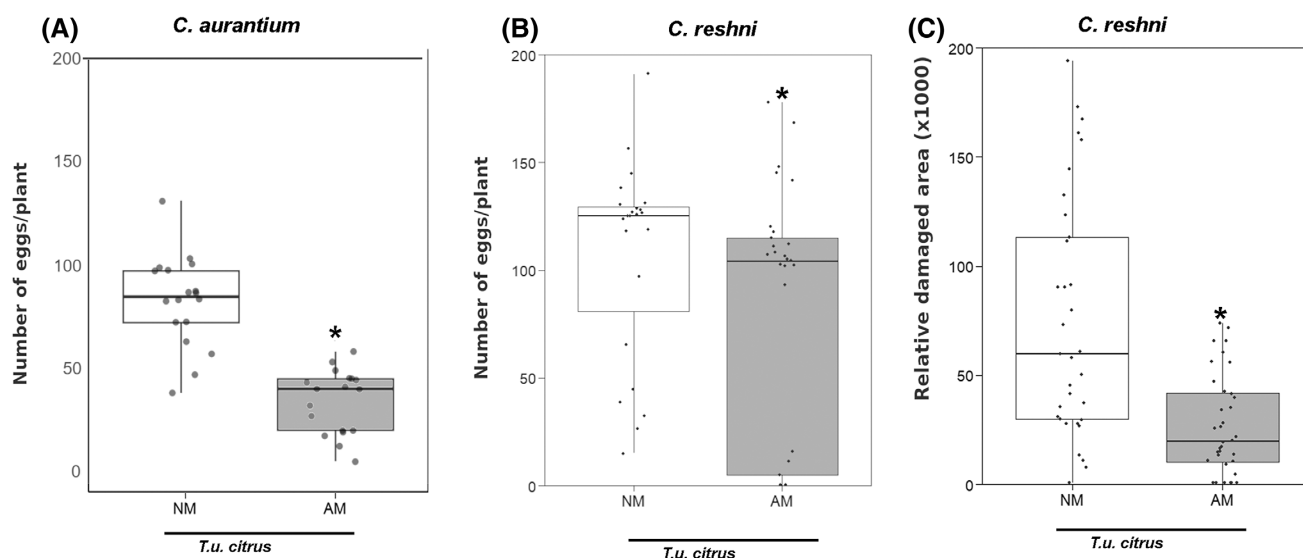

Figure 1. Comparison of mycorrhiza-induced resistance impact on spider mite performance in two citrus species, *Citrus aurantium* and *Citrus reshni*. (A, B) Number of laid eggs per plant in mycorrhizal (AM) and non-mycorrhizal (NM) plants. Three-month-old plants were infested with five synchronized females (two leaves per plant). Asterisks indicate significant differences between treatments ($P < 0.05$; Mann–Whitney U-test; $n = 18–24$). (C) Area of relative damage in *C. reshni* at 72 hpi. Three independent assays were carried out with similar results. Box plots show the results of all the experiments. Asterisks indicate significant differences between treatments ($P < 0.05$; Mann–Whitney U-test; $n = 36$). See Supporting Information, Table S4 for statistical details.

infestation at 24 hpi (Supporting Information, Fig. S2). This increase probably attenuates JA-related defenses in non-mycorrhizal *C. reshni* upon mite infestation (Fig. 2). Notably, this attenuation is not observed in mycorrhizal plants, which still show strong accumulation of JA-Ile and *LOX2* and *PR3* transcripts.

ABA- and JA-related defenses were shown to participate in the response to wounding and herbivory.[60] Barczak-Brzyzek *et al*. suggested that *ABI-4* is essential for *Arabidopsis* protection against spider mites.[23] Analysis of ABA-signaling in MIR showed that *ABA-4* transcripts accumulated at 48 hpi, as well as ABA at 48 hpi in infested mycorrhizal plants (Fig. 3(A), (B)). This suggests the participation of both JA and ABA-signaling in mediating MIR against the mite. The relevance of ABA was further tested by treating plants with ABA and the biosynthesis inhibitor fluoridone. Fluoridone blocks the activity of the PDS, which participates in the biosynthesis of phytofluene, a precursor of carotenes and ABA.[61] Plants treated with the hormone showed no reduction in leaf damage but more importantly, treatment with the inhibitor completely abolished MIR against the mite (Fig. 3(C)).

3.3 Intact flavonoid metabolism is essential for functional MIR against mites in *C. reshni*

Mycorrhization induces strong metabolic changes in primary and secondary metabolism of different plant species, such as *Vitis vinifera*,[62] *Solanum lycopersicum*[23,41-48,50-53,55-63] and *C. aurantium*.[44] To elucidate whether there is a metabolomic rearrangement that may contribute to resistance, we harvested leaves at two different timepoints following infestation: 24 hpi, with no visible symptoms, and 48 hpi, with the occurrence of first visual damage. We performed an extensive metabolomic analysis using non-targeted Liguid Chromatography coupled to a Tim-e-of-Flight mass spectrometer (LC-QTOF). Before statistical filtering we obtained 9804 features. The obtained features were analyzed with Metaboanalyst 5.0 and MarVis Suit 2.0. sPLS-DA (Supporting Information, Fig. S3(A)) using all signals from ESI+ and ESI− ionization modes showed that AM plants separated from NM plants irrespective of the infestation at both time points; however, at 24 hpi infested mycorrhizal plants (AM inf) showed stronger separation, suggesting a higher impact of the combined treatments (symbiosis and infestation). Following analysis of variance, we performed a heatmap analysis with the significant features using combined ESI+ and ESI− modes at 24 h and 48 hpi (1600 and 1017 respectively; Supporting Information, Fig. S3(B)). At 24 hpi, a cluster of features was over-accumulated exclusively in AM-infested plants, thus showing a priming profile; these signals accumulated more in AM plants after infestation. Neither mock nor infested non-mycorrhizal plants showed differences in the accumulation of signals. Once compounds with priming profiles were selected, a pathway ontology classification was obtained using MarVis software based on exact mass identification (Supporting Information, Table S2 and Fig. S4). At 24 hpi, the biosynthesis of specialized metabolites, mainly flavonoids, terpenoids and polyketides, covered most signals (Supporting Information, Fig. S4(A)). Nevertheless, at 48 hpi, there was a reduction in the number of signals from these pathways, but an increase in oxocarboxylic acid and carbohydrate metabolism together with cofactors and vitamins and, to a lesser extent, compounds from lipid metabolism (Supporting Information, Fig. S4(B)).

Because the major changes supporting a primed regulation related to MIR are visible at 24 hpi, we used this timepoint for the precise identification of the candidate features by using an internal library containing more than 250 compounds from secondary and primary plant metabolism. Using the ChromaLynx tool from MassLynx 4.2, we identified 57 compounds (Supporting Information, Table S3). An sPLS-DA containing only identified compounds showed a priming profile of AM-infested plants that separated clearly from the remaining treatments (Fig. 4(A)).

These changes were mainly observed in Component 1 in the sPLS-DA, which explained 35.1% of the differences, indicating a strong impact of symbiosis after infestation. Furthermore, NM plants had similar behavior despite infestation, because they overlapped with the other treatments. These results are indicative of a faster

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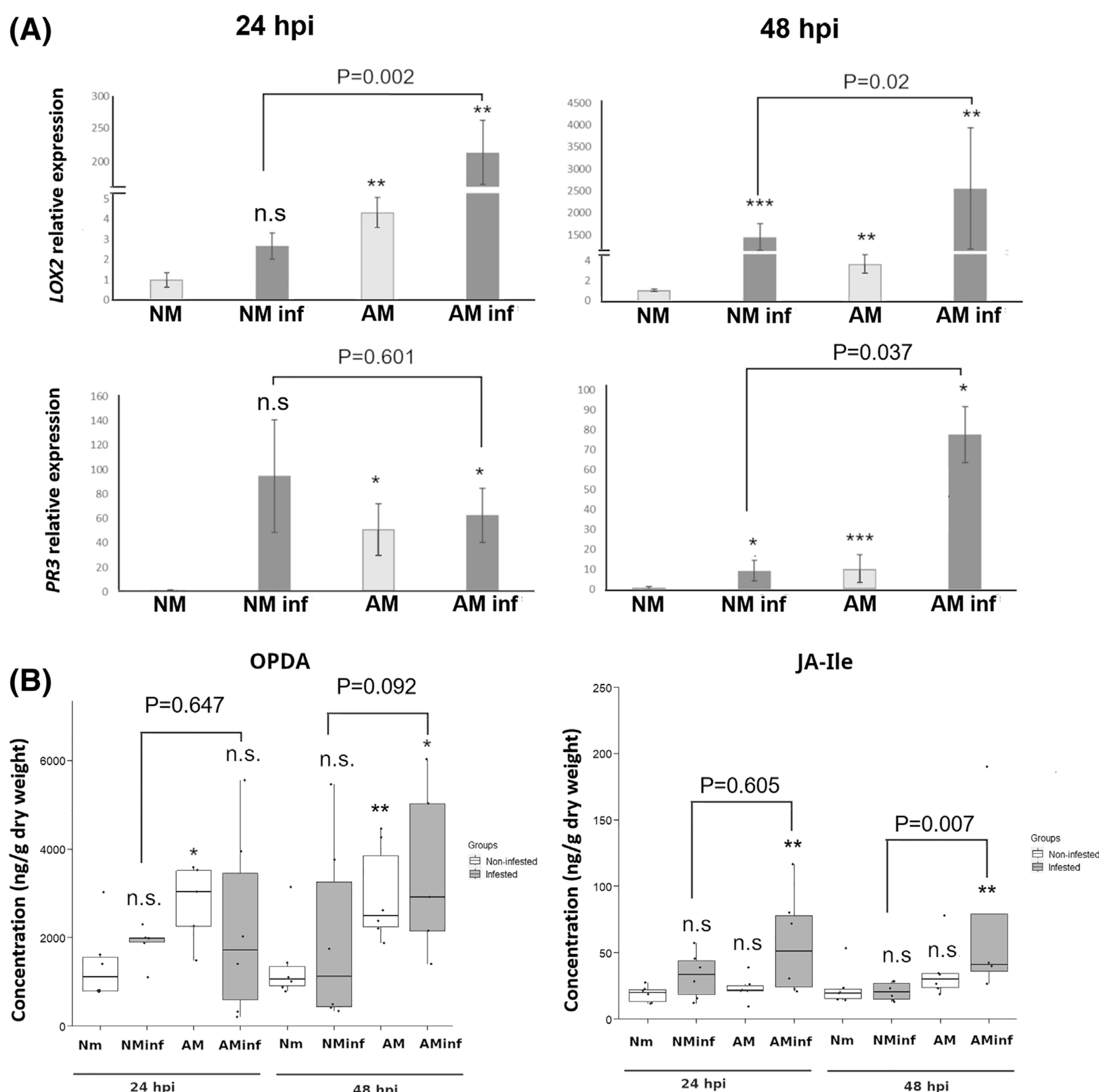

Figure 2. Effects of mycorrhization on the jasmonic acid (JA)-dependent defense pathway in locally infested leaves of *Citrus reshni*. (A) Relative gene expression of the JA biosynthetic gene *LOX2* and the signaling marker gene *PR3* at 24 and 48 hpi. Three independent replicates were carried out per analysis, and six plants were used per condition. Bars show the average of all the experiments. Different treatments: NM inf, non-mycorrhizal infested plants; AM, mycorrhizal non-infested plants; AM inf, mycorrhizal infested plants were compared with the control (NM, non-mycorrhizal non-infested plants). Asterisks indicate significant differences compared with NM [***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; generalized linear model (GLIM), $n = 18$]. NM inf and AM inf were also compared using GLIM, and the P value is presented in the figure. (B) Levels of 12-oxo-Phytodienoic Acid (OPDA) and jasmonic acid isoleucine (JA-Ile) at 24 and 48 hpi. There were three independent replicates per analysis, and six plants per condition. Asterisks indicate significant differences compared with NM (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; GLIM, $n = 6$). NM inf and AM inf were also compared using GLIM, and the P value is presented in the figure. See Supporting Information, Table S4 for statistical details.

preparation and response in mycorrhizal plants. These compounds are likely candidates to mediate MIR against mites because they accumulate in mycorrhizal plants upon infestation, showing a priming profile. Pearson correlation analysis according to the accumulation of metabolites showed high correlation between levels of flavanones, lignans and Trp metabolism (Supporting Information, Fig. 4(B)). Notably, most phenylpropanoids accumulated less in AM inf plants than in NM inf plants, except for two identified caffeoyl acid derivatives (Supporting Information, Fig. S5(A)). By contrast, most flavones, such as baicalein, apigenin and its glucoside, and 5-hydroxy-7,8-dimethoxyflavone, spinosyn, eupatilin and eriocitrin displayed priming profiles in mycorrhizal plants upon infection, whereas other flavones did not (Supporting Information, Fig. S5(B)). Interestingly, the flavonols 5-deoxykaempferol and kaempferitrin (Supporting Information, Fig. S5(C)) and the flavanone naringenin were the only identified phenylpropanoids of the flavone family that showed a priming profile, suggesting a role in MIR against the mite (Supporting Information, Fig. S5(C)).

To gain additional insight into the relevance of flavonoids in MIR, we studied the expression profile of *C4H*, which catalyzes the hydroxylation of cinnamic acid to generate *p*-coumaric acid.[59,64] This is an early step in the biosynthesis of phenylpropanoids that are precursors of flavonoids. *C4H* expression was over-accumulated in AM plants at 24 hpi, whereas it showed a priming profile at 48 hpi, suggesting its role in MIR (Fig. 5(B)). To gain mechanistic insight into the relevance of

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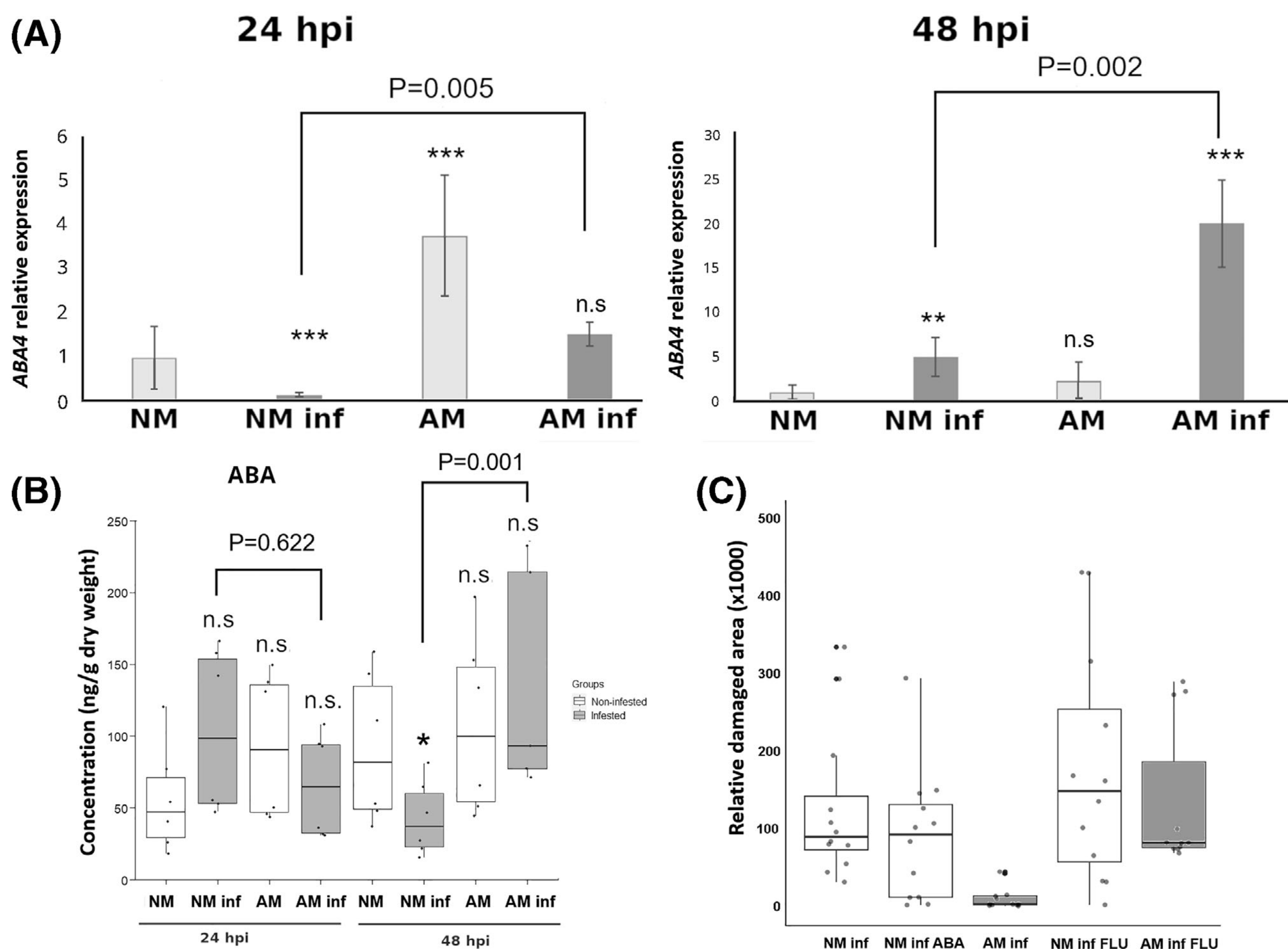

Figure 3. Effects of mycorrhization on the abscisic acid (ABA)-dependent pathway in locally infested leaves of *Citrus reshni*. (A) Relative gene expression of the signaling (ABA-4) ABA-related gene at 24 and 48 hpi. There were three independent replicates per analysis, and six plants per condition. Bars show the average of all the experiments. Non-mycorrhizal infested plants (NM inf), mycorrhizal non-infested plants (AM) and mycorrhizal infested plants (AM inf) were compared with non-mycorrhizal non-infested plants (NM). Asterisks indicate significant differences (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$) for the generalized linear model (GLIM, $n = 18$). NM inf and AM inf were also compared using GLIM, and the P value is presented in the figure. (B) Levels of ABA at 24 and 48 hpi. There were three independent replicates per analysis, and six plants per condition. Asterisks indicate significant differences compared with NM (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$) for the GLIM, $n = 6$). NM inf and AM inf were also compared using GLIM, and the P value is presented in the figure. (C) Mycorrhizal and non-mycorrhizal plants were treated either with mock [0.1% ethanol (EtOH)], ABA or fluoridone (FLU). One day after treatments, four plants per condition were infested with five *Tetranychus urticae* per leaf (two leaves per plant). The experiment was repeated three times. Box plots show the results of all the experiments. Asterisks indicate significant differences at $P < 0.05$ (t-test, $n = 12$). See Supporting Information, Table S4 for statistical details.

phenylpropanoids, and more specifically C4H activity, we used the specific inhibitor PIP. Continuous treatments by spraying PIP reduced the number of roots showing mycorrhizal structures by 50% (Fig. 5(A)). To avoid a reduction in symbiosis interfering with MIR, in the next experiment, we treated mycorrhizal and non-mycorrhizal plants with PIP only 24 h before infestation and determined the leaf damage at 24 hpi. The C4H inhibitor did not alter basal resistance to the mite, although it fully abolished MIR (Fig. 5(C)), giving support to the relevance of phenylpropanoids and their derivatives for a functional MIR.

3.4 Systemic resistance in *C. reshni* against *T. urticae* is boosted in mycorrhizal plants

Despite its high susceptibility to mites, *C. reshni* retains the ability to display systemic resistance to secondary infestations.[48] In addition, AM symbiosis was shown to enhance systemic resistance in several plant species,[65,66] but conversely *C. aurantium* was impaired in systemic resistance triggered by MIR.[44] To test the impact of MIR in systemic tissues of *C. reshni* to secondary infestations a bioassay was performed. Lower leaves were infested with ten mites that were confined to central leaves using wet cotton, which was considered the systemic resistance treatment (SR). At 72 hpi, we performed a secondary infestation in the upper leaves, without removing the mites from the first infestation, using synchronized females to test mite oviposition (Fig. 6). Seven days later, NM SR plants showed a 25% reduction in egg-laying in systemic tissues. Interestingly, the number of eggs in AM SR plants was further reduced by 30% compared with AM control plants and by 44% compared with NM SR plants (Fig. 6).

3.5 Mycorrhizal symbiosis enhances the antixenosis of *T. urticae* and attraction to the natural enemy *P. persimilis*

Non-mycorrhizal *C. reshni* infested with *T. urticae* is highly attractive to conspecifics.[48] In our study, among the pathways induced in mycorrhizal plants, flavonoids and terpenoids represented more than 50% of the over-accumulated compounds. Among

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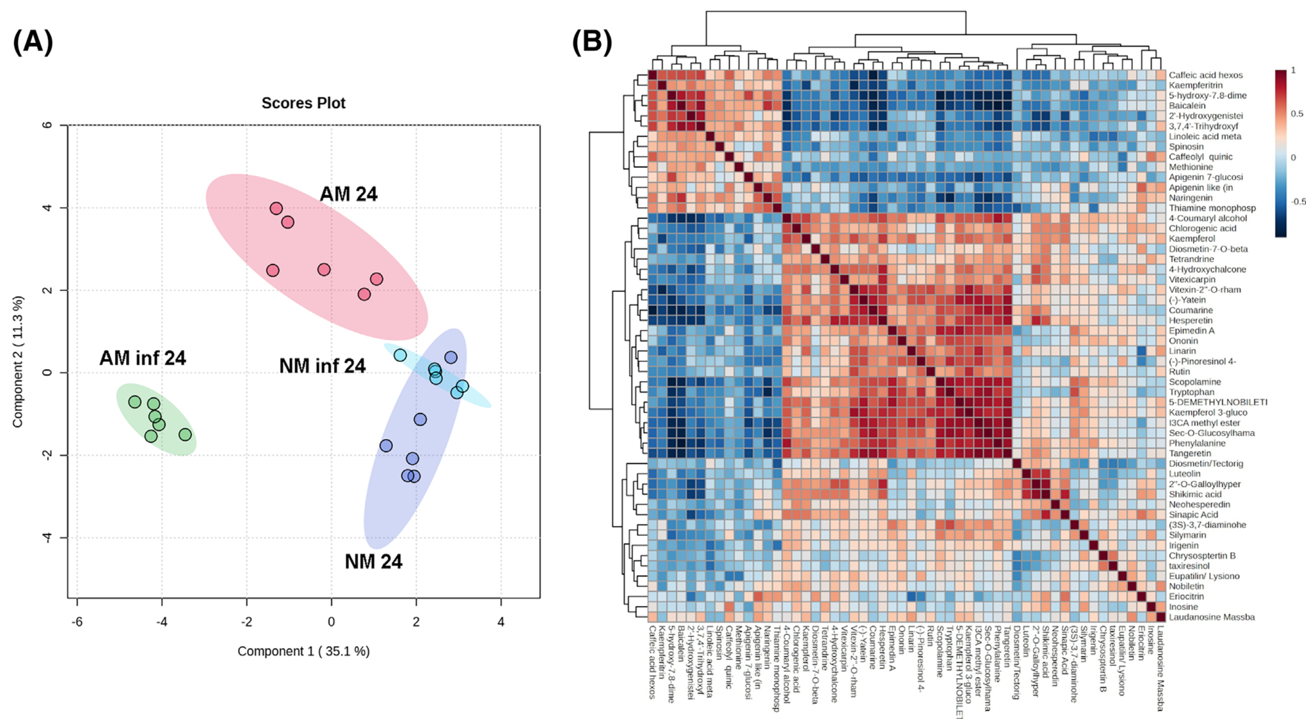

Figure 4. Impact of mycorrhization on the metabolomic profile of locally infested leaves of *Citrus reshni* plants at 24 hpi. (A) Sparse PLS discriminant analysis (sPLS-DA) obtained from a non-targeted analysis using Liquid Chromatography coupled to Time-of-Flight mass spectrometer (UPCL-QTOF). (B) Correlation plot of identified markers from the non-targeted metabolomic analysis of locally infested leaves at 24 hpi. Positive correlations are presented in red, and negative correlations are presented in blue.

them, we tentatively identified geranyl diphosphate, a precursor of volatile terpenoids (Fig. 7(A)). Note that mycorrhization impacts the synthesis of volatile compounds in response to herbivory.[67,68] This prompted us to explore whether symbiosis changes the behavior of the mite pests and their natural enemies. Y-tube assays showed that in the absence of herbivory, the mites preferred NM over AM plants, confirming that AM plants are less attractive to *T. urticae* (Fig. 7(B)). The fact that mites prefer previously infested *C. reshni* hosts over non-infested plants[48] suggests that mites can perceive self-volatiles, choosing plants that already contained conspecifics. This behavior changed when comparing AM and AM-infested hosts, because mites did not show a difference in the selection. Notably, when a choice between NM-infested and AM-infested hosts was given, the mycorrhizal plants were strongly repellent. Importantly, Manresa-Grao *et al.* showed that symbiosis does not affect choice in *C. aurantium*. We also performed two-choice bioassays offering combinations of the susceptible *C. reshni* and the resistant *C. aurantium* in the presence of conspecifics and symbiosis (Fig. 7(C)).[44] Cruz-Miralles *et al.* showed that naive *C. reshni* plants are more attractive to *T. urticae* than *C. aurantium*.[69] Surprisingly, symbiosis changed the behavior of the mite, which selected both genotypes equally, hence abolishing the attraction of the susceptible *C. reshni*. Furthermore, in the presence of a previous infestation, the strong attractiveness of *C. reshni* was also abolished in mycorrhizal plants, and mites did not choose between *C. aur*-AM-infested or *C. resh*-AM-infested plants.

P. persimilis is a specialized predator that feeds on Tetranychids, whereas *N. californicus* also feeds on pollen. Two-choice trials showed that, in the presence of prey, *P. persimilis* chooses infested plants irrespective of mycorrhization (Fig. 8(A)). Interestingly, in the absence of the prey and hence prey odors, *P. persimilis* preferred AM plants over NM plants. When we performed assays with *N. californicus*, the phytoseiid preferred NM inf plants rather than NM plants, as described previously by Cabedo-López *et al.*[49] However, this selection did not occur in mycorrhizal plants. (Fig. 8(B)). Note that *N. californicus* did not have a clear selection between AM and NM plants either in the presence or absence of mycorrhization; hence, it seems that symbiosis has no influence on the choices of *N. californicus* (Fig. 8(B)).

4 DISCUSSION

In the current study, we present a comparative analysis of MIR functionality in two different citrus species, one resistant to *T. urticae* and another highly susceptible. Along with the investigations, we confirmed that MIR is context-dependent, and is related to basal immunity because it is more effective in citrus species displaying higher susceptibility. Mycorrhizal symbiosis in *C. reshni* provides multifaceted resistance that reduces oviposition on local and systemic tissues, triggers antixenosis and improves natural enemy attraction.

SR provides boosted protection in the plant that has perceived primary infestation by an insect.[70] Both citrus species, *C. aurantium* and *C. reshni*, show enhanced SR in response to a secondary infestation by *T. urticae*.[70] Conversely, both species behave differently when mycorrhized, because *C. aurantium* is impaired in displaying MIR-triggered SR,[44] whereas mycorrhizal *C. reshni* strongly reduces egg-laying following the perception of a primary infestation. This indicates that *C. reshni* rootstock is highly sensitive to MIR, and this interaction has a strong impact at both the local and systemic level.

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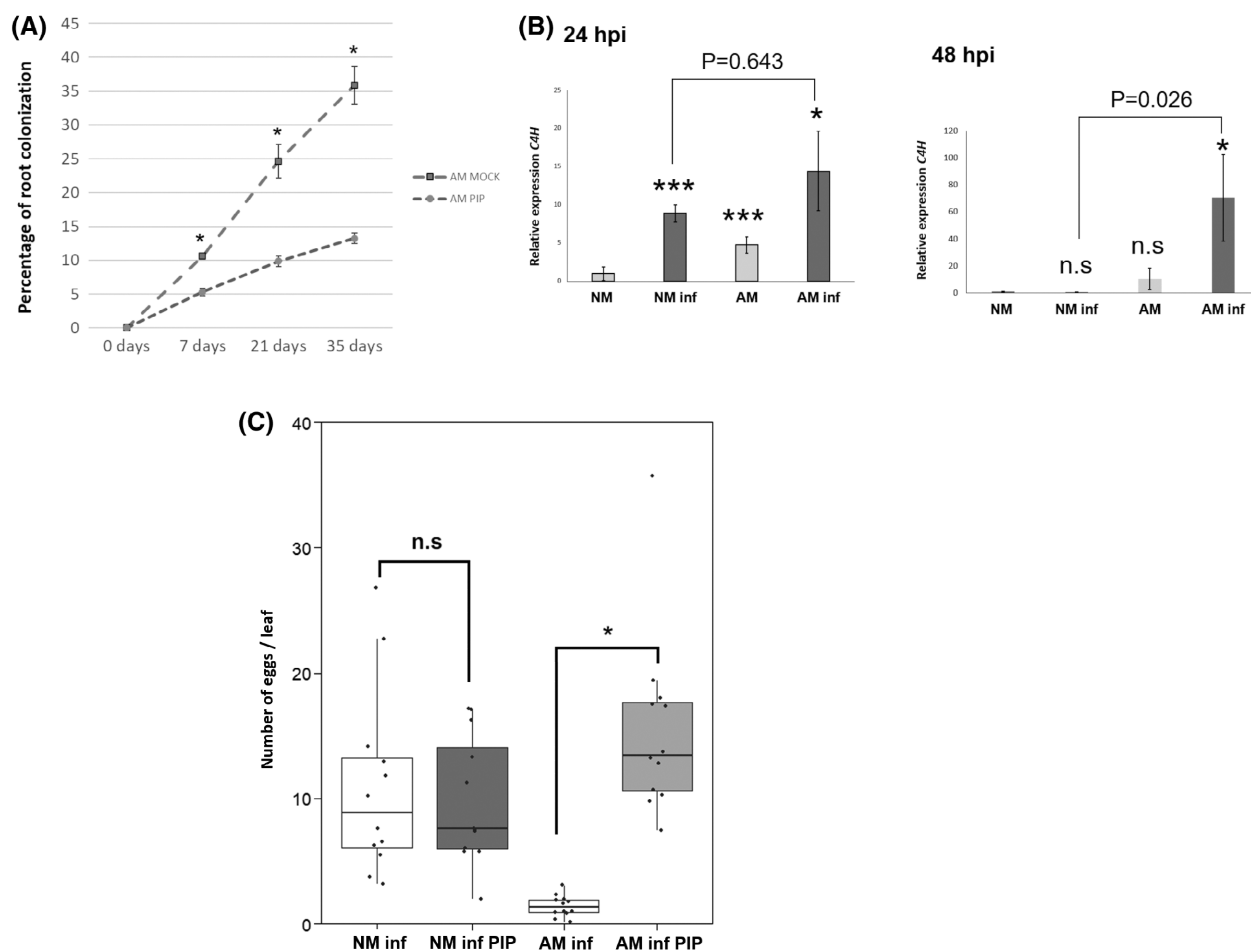

Figure 5. Effects of the chemical inhibition of the phenylpropanoid pathway in *Citrus reshni* mycorrhizal plants using piperonylic acid (PIP). (A) Percentage of mycorrhizal colonization in *C. reshni* plants treated with PIP. Plants were treated either with mock (0.1% dimethyl sulfoxide) or PIP at 0, 14 and 28 days after *Rhizophagus irregularis* inoculation. Roots were harvested at the indicated time points to determine the level of colonization. Asterisks indicate significant differences within time points (*t*-test, $n = 9$). (B) Relative gene expression of the phenylpropanoid biosynthesis gene *C4H* at 24 and 48 hpi. There were three independent replicates per analysis, and six plants per condition. Asterisks indicate significant differences compared with non-mycorrhizal non-infested plants (NM) (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$) for the generalized linear model (GLIM, $n = 18$). Non-mycorrhizal infested plants (NM inf) and mycorrhizal infested plants (AM inf) were also compared using GLIM, and the *P* value is presented in the fig. (C) Chemical treatments of PIP. Non-mycorrhizal and mycorrhizal plants were treated 24 h before infestation either with mock (0.1% dimethyl sulfoxide) or PIP. Four plants per condition were infested with five synchronized females per leaf (two leaves per plant). The number of eggs was determined 5 days after infestation. The experiment was repeated three times. Asterisks indicate significant differences compared with the untreated controls ($P < 0.05$; *t*-test, $n = 12$). See Supporting Information, Table S4 for statistical details.

We previously demonstrated that *C. aurantium* is insensitive to antixenosis triggered by symbiosis.[44] Furthermore, infested *C. reshni* is highly attractive to conspecific mites.[69] Notably, the ontology classification of signals based on exact mass identification showed that some signals in the terpenoid pathway were primed by MIR. Symbiosis completely reversed the attraction, because *C. reshni* plants, irrespective of the infestation, were less attractive to the mite. In addition, symbiosis reverses the preference of *C. reshni* over *C. aurantium* because both were equally chosen when mycorrhized. From these results, we inferred a change in Herbivor-e-Induced Plant Volatiles (HIPVs) during MIR, hence, we performed bioassays of indirect resistance which demonstrated that mycorrhizal plants can alter *P. persimilis* preference because the predator preferred mycorrhizal *C. reshni* in more than 60% of the choices in the absence of infestation. This was unexpected because previous studies have shown that the specialist *P. persimilis* prefers its prey independently of the host.[49] Despite both *N. californicus* and *P. persimilis* being described as antagonistic, previous studies mentioned no interference in egg cross-laying or a presence in plants where the other species is established.[71]

MIR has a different impact on both citrus species despite both responding similarly at the local level. Therefore, we delve into mechanistic studies that may explain MIR in *C. reshni*. Previous studies on basal immunity against spider mites in tomato,[11] pepper,[53] and citrus[31] have demonstrated that *T. urticae* infestation triggers JA-related defenses and plant resistance relies on more efficient JA-dependent responses.[10,53] Furthermore, MIR was also found to trigger JA-dependent responses against fungal pathogens and upon herbivory by *Spodoptera exigua*.[72,73] Notably, our transcriptional studies of the mycorrhizal *C. reshni* revealed priming of *LOX2* at 24 hpi, which resembles the fast *LOX2* induction described in the basal response of the resistant genotype *C. aurantium*.[10] The activation of biosynthesis genes in

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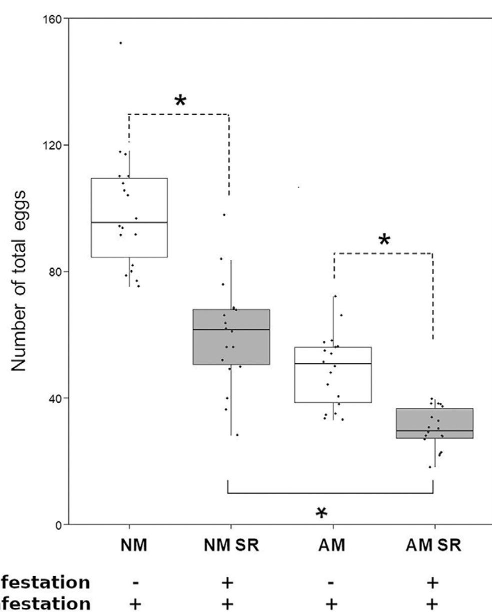

Figure 6. Systemic resistance of mycorrhizal *Citrus reshni* plants. Plants were locally infested either with mock [non-mycorrhizal non-infested (NM) and mycorrhizal non-infested (AM)] or with ten mites per plant (NM SR and AM SR; SR systemic treatment); the mites were restricted to the bottom leaves using humid cotton wool. Seventy-two hours after the first infestation, all the plants were infested with five mites (two systemic leaves per plant). Seven days later, the number of total eggs per plant was determined. Six plants per condition were used. The assay was repeated three times. Box plots show the results of all the experiments. Asterisks indicate significant differences ($P < 0.05$; *t*-test, $n = 18$) between treatments. See Supporting Information, Table S4 for statistical details.

mycorrhizal plants correlates with priming of JA-Ile. The signaling gene *PR3* was also primed 1 day later (at 48 hpi).

Treatments with Methyl Jasmonate (MeJA) to mimic herbivory revealed a greater number of metabolic changes compared with *T. urticae*-infested plants, indicating that mites interfere with host defenses.[53,74] Host manipulation was recently shown to be one of the mechanisms of *T. urticae* adaptation in addition to detoxification.[20] The susceptible citrus genotype *C. reshni* infested with adapted *T. urticae* showed weak JA-dependent responses, whereas the resistant *C. aurantium*, despite concomitant SA induction, did not show any repression of jasmonates.[10] In our study, we observed that irrespective of symbiosis, the mite triggers SA accumulation and *NPR1*; however, only the mycorrhizal plants showed induction of OPDA and priming of JA-Ile, indicating that AMF-colonized hosts hamper the negative cross-talk, allowing more efficient defenses. It is worth mentioning that *COI1*-independent pathways regulated by OPDA also participate in plant defenses.[73,75] The elevated levels of OPDA in mycorrhizal plants may be indicative of an alternative independent role of OPDA during MIR, although this requires further specific study.

In response to wounding and chewing arthropod herbivory, plants activate JA and ABA-signaling pathways, both showing a synergistic function in response to chewing insects.[60,76] The infestation in mycorrhizal *C. reshni* triggers the *ABA-4* gene involved in the biosynthesis of *t*-neoxanthin,[77] accordingly ABA levels were primed by MIR at 48 hpi. External ABA treatments did not show any impact on damage levels in the absence of symbiosis. Conversely, treatments with fluoridone, the ABA synthesis inhibitor, completely abolished MIR, restoring non-mycorrhizal levels of leaf

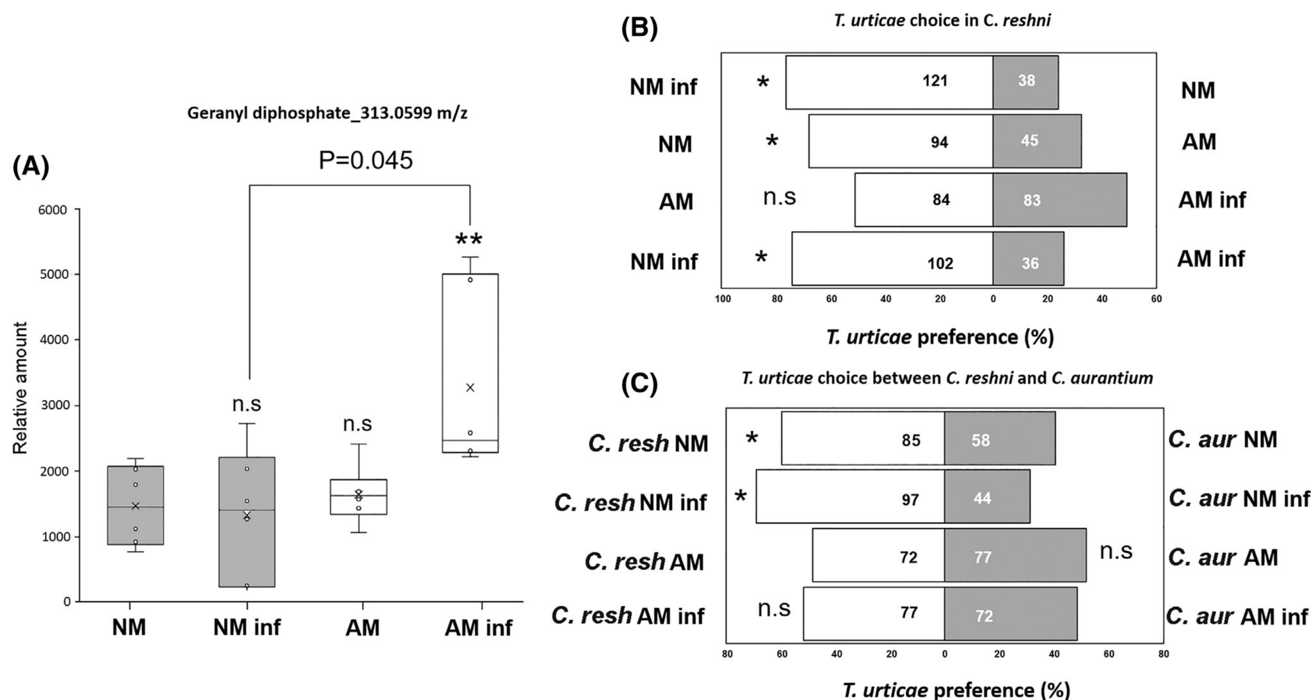

Figure 7. Antixenotic effects of mycorrhizal *Citrus reshni* plants against *Tetranychus urticae*. (A) Relative amounts of geranyl diphosphate. Asterisks indicate significant differences compared with non-mycorrhizal non-infested plants (NM) [***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; generalized linear model (GLIM), $n = 6$]. Non-mycorrhizal infested plants (NM inf) and mycorrhizal infested plants (AM inf) were also compared using GLIM, and the *P* value is presented in the figure. (B) *T. urticae* choices when exposed to odors from naive or infested (inf), mycorrhizal (AM) or non-mycorrhizal (NM) *C. reshni* plants. Different pair combinations were tested: NM *versus* NM inf; NM *versus* AM; AM *versus* AM inf; NM *versus* AM inf. Asterisks indicate significant differences between treatments for a 50:50 distribution (binomial test; $P < 0.05$). Numbers indicated the total amount of mites tested. (C) *T. urticae* choices when exposed to odors of *Citrus aurantium* and *C. reshni*. Different combinations were tested: *C. resh* NM *versus C. aur* NM; *C. resh* AM *versus C. aur* AM; *C. resh* NM inf *versus C. aur* NM inf; *C. resh* AM inf *versus C. aur* AM inf. Asterisks indicate significant differences between treatments for a 50:50 distribution (binomial test; $P < 0.05$). A minimum of four plants per treatment were used. See Supporting Information, Table S4 for statistical details.

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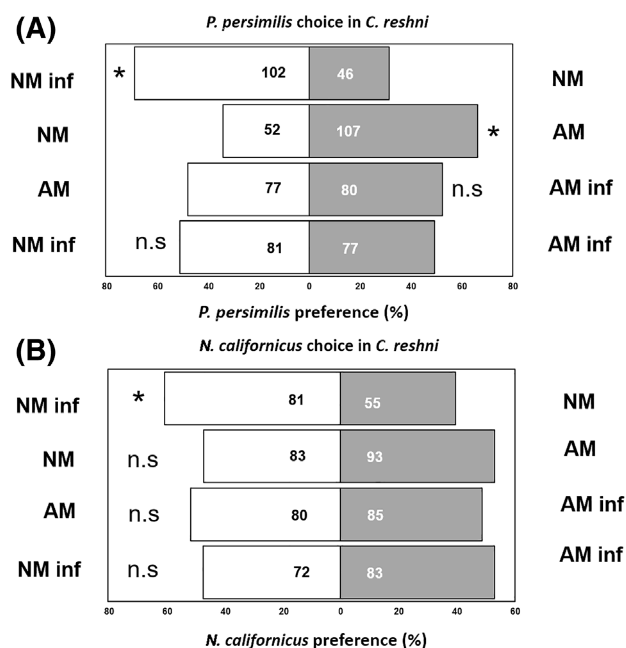

Figure 8. Indirect defense of mycorrhizal *Citrus reshni* plants. (A) Changes in the attraction of the phytoseiid *Phytoseiulus persimilis* when exposed to odors from naive or infested (inf), mycorrhizal (AM) and non-mycorrhizal (NM) *C. reshni* plants. The combinations tested were NM *versus* AM, NM inf *versus* AM inf, AM *versus* AM inf, and NM *versus* NM inf. (B) Changes in *Neoseiulus californicus* behavior when exposed to odors from naive or infested (inf) mycorrhizal (AM) and non-mycorrhizal (NM) *C. reshni* plants. The combinations tested were NM *versus* AM, NM inf *versus* AM inf, AM *versus* AM inf, and NM *versus* NM inf. All mites used in the two-choice assays, including phytoseiids, were starved 24 h before the assays. Asterisks indicate significant differences between treatments for a 50:50 distribution (binomial test; $P < 0.05$). See Supporting Information, Table S4 for statistical details.

damage. This indicates that intact ABA-signaling is required for MIR to be functional.

Jasmonates are also involved in regulating alternative defensive pathways such as terpenoids, flavonoids and phenylpropanoids in other plant species.[29] Additional research in citrus,[10] bilberry[78] and pepper[53] confirmed that jasmonates regulate the phenylpropanoid pathway during herbivory. A positive correlation between JA and flavonoid accumulation was also found in MIR against aphids[79] and mites, which trigger coumarate–coenzyme A ligase involved in phenylpropanoid biosynthesis.[43,53,80] In our study, we observed a positive correlation between the induction of JA-Ile and *LOX2* and priming of flavones and lignans at 24 hpi during MIR. In addition, the flavanone naringenin and the flavones baicalein, apigenin and its glucoside, spinosyn, eupatilin, eriocitrin and 5-hydroxy-7,8-dimethoxyflavone, which have been shown to be related to defense against herbivory,[81] are primed in mycorrhizal *C. reshni*. Previous investigations revealed that *T. urticae* exclusively induces phenylpropanoids but not flavonoid biosynthesis, potentially leading to elevated levels of SA.[53] Consequently, this can lead to a diminished build up of inhibitory compounds for the mite, such as rutin, kaempferol or quercetin.[82] Notably, the phenylpropanoids we identified (shikimic acid, 4-hydroxychalcone, ferulic acid and chlorogenic acid), except those derivatives of caffeic acid, were less accumulated in infested mycorrhizal plants, suggesting a metabolic flux for the synthesis of more complex flavonoids. Among flavonols that accumulated in infested mycorrhizal *C. reshni*, we also identified two kaempferol derivatives related to defense.[83] Collectively, flavones were consistently over-accumulated, whereas phenylpropanoids were less accumulated in AM inf plants, the balance observed between phenylpropanoids and flavonoids in basal defense responses and in MIR suggests that symbiosis prevents host manipulation.

Flavonols exuded to the rhizosphere were also suggested to participate in the presymbotic stages, promoting spore germination and root colonization.[84,85] Note that citrus is a fully mycotrophic host even when fertilized with 100% of its phosphorous needs,[44] likely because citrus produces large amounts of flavonoids.[29,86] Conversely, other crops, such as tomato, promote symbiosis only upon P depletion.[54] In this study, repeated treatments with the C4H inhibitor PIP over a period of a month reduced root colonization by *R. irregularis* by 50%, although it did not fully abolish symbiosis. Hence, phenylpropanoids and flavonoid derivatives are relevant for symbiosis in citrus, as previously shown in other crops.[85,87] Furthermore, we confirmed that C4H activity is essential for MIR in citrus, because a single treatment with PIP 1 day before infestation abolished MIR. Although the specificity of PIP inhibition of C4H has been recently questioned,[88] in our bioassays, we observed no changes in basal resistance in PIP-treated non-mycorrhizal plants.

Collectively, MIR against *T. urticae* is citrus species dependent and inversely correlated with basal immunity. MIR is fully functional in *C. reshni* and confers a multifaceted protection, reducing damage and egg-laying in locally infested leaves as well as in systemic tissues. Furthermore, despite *C. reshni* being highly attractive to the pest, when colonized by *R. irregularis* it becomes repellent and more attractive to the specialist predator *P. persimilis*. In addition, the mechanisms regulating MIR in *C. reshni* are mediated by priming some elements of the JA-dependent pathway and intact C4H activity, as well as priming of a set of specific flavones, flavonols and flavanones.

It should be noted that there is a general scarcity of the use of AMF in fruit agricultural systems like citrus. Following on from the results obtained here, we suggest the use of AMF in integrated pest management programs. Although MIR was shown to be functional in both species, more studies are needed because the level of protection is highly context-dependent.

AUTHOR CONTRIBUTIONS

MMG performed most bioassays of IR and treatments. MC performed qPCR analysis. VP and VF developed the LC–MS methods and analysis, and contributed to the writing of results and methods. PSB contributed to writing and pharmacological bioassays. AC and JJ performed choice assays with mites and natural enemies. VF and VP contributed to writing, supervised the research, designed experiments and performed hormonal analysis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Metabolights at https://www.ebi.ac.uk/metabolights, reference number MTBLS8319.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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