Journal of Pest Science (2026) 99:17
https://doi.org/10.1007/s10340-025-01966-3

RESEARCH

Beneficial soil fungi induce resistance to the tomato leaf miner *Tuta absoluta* through primed accumulation of antiherbivory compounds

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Received: 11 July 2025 / Accepted: 9 November 2025

Abstract

Tuta absoluta is an invasive insect pest and major threat to global tomato production, as current management approaches fail to lower its incidence below the targeted economic threshold. While microbe-induced resistance (microbe-IR) is widely documented under controlled conditions, its implementation in the field is challenging due to context-dependency and our limited knowledge on the underlying mechanisms. We recently showed that different fungal bioinoculants reduced the natural incidence of *T. absoluta* as part of Integrated Pest Management under real production conditions. Here we focus on the underlying mechanisms studying the ability of these fungi to boost tomato direct defenses against the pest and exploring the metabolic changes involved. *Trichoderma afroharzianum*, *Funneliformis mosseae* and *Rhizophagus irregularis* consistently enhanced tomato resistance to *T. absoluta* across different experimental conditions. Untargeted metabolomics revealed a metabolic reprogramming in leaves of the inoculated plants and primed responses to the attacker associated to the microbe-IR phenotype. Upon herbivory, fungal-inoculated plants showed a limited activation of the carbohydrate and vitamin metabolism, both important for insect nutrition, and an increase of the phenylpropanoid metabolism related to defense. We identified metabolites whose concentrations negatively correlate with *T. absoluta* fitness and show a primed accumulation in resistant plants. Among them, azelaic acid and feruloylputrescine showed anti-herbivore activity, inhibiting the development of the leaf miner when exogenously applied to tomato plants. The results demonstrate that root-colonizing fungi prime the plant's ability to activate its secondary metabolism in response to herbivory, triggering microbe-IR that can effectively contribute to control important pests as *T. absoluta*.

Keywords Arbuscular mycorrhizal fungi · Defense priming · Metabolomics · Microbe-induced resistance · *Trichoderma* · *Tuta absoluta*

Introduction

The tomato leaf miner *Tuta absoluta* poses a major threat to global greenhouse and open-field tomato production (Desneux et al. 2010; Biondi et al. 2018). Tomato—*Solanum lycopersicum*—serves as the main host plant for larval instars, which feed on leaves, stems, and fruits, leading to

Communicated by Antonio Biondi.

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Published online: 05 December 2025

80–100% crop losses in the absence of appropriate control measures (Desneux et al. 2010).

Chemical pesticides are widely used for *T. absoluta* management (Biondi et al. 2018; Desneux et al. 2021), but the rapid emergence of insecticide resistance severely compromises their effectiveness in controlling the pest (Biondi et al. 2018; Guedes et al. 2019). They also have a negative impact on non-target beneficial insects, including natural enemies, causing a counterproductive reduction of natural pest control (Arnó and Gabarra 2011; Soares et al. 2019; Nozad-Bonab et al. 2021). Integrated pest management (IPM) programs have significantly reduced the reliance on chemical solutions for pest control. This is accomplished by combining various sustainable strategies alongside judicious chemical usage. IPM strategies, for example, encompass biological control using natural enemies, pest monitoring, mass trapping, mating disruption using pheromones, along with multiple agronomic and cultural practices (Biondi et al. 2018; Desneux et al. 2021). Regarding biological control by microorganisms, it primarily relies on bacterial and fungal entomopathogenic abilities. Currently commercial formulations of the bacterium *Bacillus thuringiensis,* highly effective against *T. absoluta,* are widely used in tomato production IPM programs (Biondi et al. 2018; Desneux et al. 2021).

Microbes can also aid plants in combating insect pests through mechanisms not directly related to pathogenicity. Indeed, their ability to stimulate the plant defenses, triggering induced resistance (IR) to a broad spectrum of pests and pathogens (Pieterse et al. 2014; de Kesel et al. 2021), represents a promising yet unexploited niche for agrotechnological innovation. Many soil-borne microorganisms live in association with plant roots, establishing mutualistic interactions and improving plant health (Olanrewaju et al. 2019; Harman et al. 2021). Plant-growth promoting rhizobacteria (PGPR) such as *Bacillus* spp. and *Pseudomonas* spp., fungi from the genus *Trichoderma* and arbuscular mycorrhizal fungi (AMF) are among the most studied plant-associated microorganisms able to trigger microbe-IR to diverse pests and diseases (Meena et al. 2020; Fiorilli et al. 2024; Flors et al. 2024). Indeed, recent reports suggest the efficacy of microbe-IR to control *T. absoluta*. For example, few studies show the potential of some beneficial microbes from the groups of PGPR, AMF and other endophytic fungi to negatively impact the leaf miner performance (Agbessenou et al. 2020; Aprile et al. 2022; Shafiei et al. 2022; Salazar-Mendoza et al. 2025; Qian et al. 2025). Remarkably, we recently showed the efficacy of microbe-IR triggered by some AMF and *Trichoderma* strains under commercial production conditions, with agronomic management already incorporating IPM methods (Minchev et al. 2024). Thus, experimental evidence supports that microbe-IR can be a valuable tool to complement and improve IPM programs. However, the complexity of microbe-IR functioning in the field, which may involve direct and indirect defense activation, requires a deep understanding of the operating mechanisms to fully exploit their individual contributions to the observed crop protection.

Microbe-IR is usually associated to a faster and more effective activation of plant defense responses upon challenge, known as defense priming (Mauch-Mani et al. 2017). Indeed, priming of inducible defenses is a crucial plant strategy to face insect herbivores (Wilkinson et al. 2019). Upon insect herbivory plants activate an arsenal of inducible defenses including direct and indirect defense mechanisms (Kesler and Baldwin 2002; Wilkinson et al. 2019). Indirect defenses involve plant traits such as volatile organic compounds that enhance plant attractiveness to natural enemies of herbivores (Erb and Reymond 2019; Wilkinson et al. 2019). In contrast, direct inducible defenses are the ones that include plant traits that directly antagonize the attacker (Kesler and Baldwin 2002; Mithöfer and Boland 2012). For example, direct plant defense responses to insect herbivory involve the synthesis of antifeedant proteins and the activation of the plant secondary metabolism, which play a key role for plants to resist herbivory (Mithöfer and Boland 2012; Erb and Reymond 2019). Some important families of secondary metabolites with anti-herbivore properties are alkaloids, terpenoids, and phenolic compounds including flavonoids and phenylpropanoids among others (Mithöfer and Boland 2012).

In many pathosystems, microbe-IR has been associated to primed activation of jasmonic acid dependent defenses, including a reprogramming of the plant secondary metabolism (Song et al. 2013; Pieterse et al. 2014; Kaling et al. 2018; Schoenherr et al. 2019; Gruden et al. 2020; Mhlongo et al. 2020). In fact, some studies revealed primed accumulation of alkaloids and other secondary metabolites in plants inoculated with AMF or *Trichoderma* against different pests as the generalists *Spodoptera exigua* or *Macrosiphum euphorbiae* (Coppola et al. 2019; Rivero et al. 2021; Dejana et al. 2022).

The plant response to the specialist leaf miner *T. absoluta* has been explored in the last years by transcriptomics (Chen et al. 2021; D'Esposito 2021) and metabolomic approaches, and the accumulation of organic acids and phenolamides have been proposed to play an important role (de Falco et al. 2019; Roumani et al. 2022). Yet, our knowledge on the metabolic responses to *T. absoluta* of plants associated to beneficial microbes is scarce. Only recent targeted analyses showed primed accumulation of total phenols and flavonoids in plants colonized by AMF or *Pseudomonas* in response to the herbivore (Shafiei et al. 2024; Zhao et al. 2024). However, we lack a general overview of the metabolic reprogramming undergone by plants successfully displaying microbe-IR to *T. absoluta*.

In the present study, we investigate the ability of different soil-borne beneficial microorganisms, commonly used as bioinoculants, to boost plant direct antiherbivory defenses by inducing resistance against the major pest *T. absoluta*. The protective efficacy of the inoculants against the pest was shown under agronomic management for commercial tomato production (Minchev et al. 2024). We hypothesized that this microbe-IR against *T. absoluta* is associated with boosted plant resistance through metabolic reprogramming of direct plant defenses. Through an untargeted metabolomics approach, we aimed to identify metabolic fingerprints associated with successful microbe-IR to *T. absoluta.* Our results are expected to support that these bioinoculants can successfully contribute to the sustainable management of *T. absoluta* through priming of anti-herbivore bioactive compounds.

Material and methods

Beneficial microorganisms

Funneliformis mosseae BEG12 (Fm) was maintained in an open pot culture of *Trifolium repens* mixed with *Sorghum vulgare* plants growing in a vermiculite-sepiolite (1:1) substrate in a greenhouse. The inoculum consisted of substrate containing infected root fragments, mycelia and spores (Rivero et al. 2021).

Rhizophagus irregularis MUCL 57021 (Ri) was grown in vitro on minimal (M) medium using *Agrobacterium rhizogenes*—transformed carrot (*Daucus carota*) roots as a host (St-Arnaud et al. 1996). For spore extraction, citrate buffer 0.01 M (pH = 6) was added to a sporulating culture in a proportion 3:1 (v/v) and placed in a rotary shaker for 1 h to dissolve the agar. The spores were recovered from the solution using sieves with different sizes (250 and 53 µm) and re-suspended in sterile tap water at final concentrations 1000 spores/ml (Minchev et al. 2021).

Trichoderma afroharzianum T22 (T22) was grown on potato dextrose agar (PDA, Difco) for 7 days at room temperature. Spores were collected from sporulating plates in sterile tap water, the concentration of the spore suspension was quantified using a Bürker-Türk counting chamber and adjusted to 1×10^7 spores/ml (Minchev et al. 2021).

Bacillus amyloliquefaciens CECT8238 (Ba) was cultured on tryptone soya agar (TSA, Oxoid) for 24 h at 28 °C. A flask containing 25 ml of DSM (Difco sporulation medium) (Nicholson and Setlow 1990) was inoculated with a single colony from the TSA culture and incubated for 48 h at 28 °C in a rotatory shaker (200 rpm). The spore concentration was quantified using a Bürker-Türk counting chamber, centrifuged at 5000 rpm for 15 min and the spores were re-suspended in sterile tap water to a final concentration of 1×10^7 spores/ml (Minchev et al. 2021).

Pseudomonas azotoformans F30A (Pa) was cultured on TSA for 24 h at 28 °C. Liquid pre-culture was prepared using tryptone soya broth (TSB, Oxoid) inoculated with a single bacterial colony and incubated overnight at 28 °C with rotary shaking at 200 rpm. TSB media (25 ml) was inoculated with 1 ml of pre-culture and placed in a rotatory shaker (200 rpm) at 28 °C. After 150 min of incubation, with bacterial growth in exponential phase, the cell concentration was calculated measuring the O.D. (620 nm). The bacterial culture was centrifuged at 5000 rpm for 15 min and the bacterial cells were re-suspended in sterile tap water to a final concentration of 1×10^7 cfu/ml (Minchev et al. 2021).

Bacterial treatments, Ba and Pa, as well as T22, were inoculated by pipetting the microbial suspensions to the roots during transplantation at a concentration 1×10^7 cfu/plant. In the case of mycorrhizal treatments, Ri was applied by pipetting the spore suspension to the roots at a concentration of 1000 spores/plant and Fm was inoculated by mixing the growing substrate with 10% (v:v) of the inoculum. A non-inoculated treatment (Ni) where only water without any microbial propagules was added to the roots was included as a control.

Plant material and growing conditions

Solanum lycopersicum cv. Money maker seeds (Vreeken's Zaden, The Netherlands) were surface sterilized by immersion in 5% sodium hypochlorite solution for 10 min followed by at least 3 washing steps in sterile water for 10 min each. The surface sterilized seeds were sown in a sterile vermiculite and incubated for 7 days in a greenhouse at 24 °C:18 °C day:night with a photoperiod 16 h:8 h light: dark and 60% of relative humidity. Tomato seedlings were transferred to 300 ml pots containing gamma-irradiated, nutrient poor sandy soil (BVB, The Netherlands) and sterile vermiculite (1:1) mixture, and inoculated with the microbial treatments described below. Inoculated plants were randomly distributed and grown for 6 weeks in a greenhouse under the same climatic conditions described above. Plants were watered once per week with water and twice per week with Long Ashton nutrient solution (Hewitt 1966) with reduced phosphorous concentration (50% of the standard concentration) to ensure mycorrhizal establishment.

Tuta absoluta rearing

Tuta absoluta colony was maintained, at 22 °C with photoperiod 16 h:8 h day:night and 60% of relative humidity, in rearing cage of 60 cm × 60 cm × 60 cm (length x width x height) with tomato (*Solanum lycopersicum* cv Money maker) plants as a host. New tomato plants were exposed

to *T. absoluta* adults for 24 h for oviposition. After egg hatching, larvae were left to reach L2 instar and used in the bioassay.

Root microbial colonization

PGPR and *Trichoderma* root colonization was confirmed through microbial DNA detection by qPCR using microbe-specific primers as previously described by Ramirez-Serrano et al. (2024).

For mycorrhizal colonization, roots were washed upon harvesting, cleared with 10% KOH, and stained with 5% ink in 2% acetic acid (García et al. 2020). The percentage of root length colonized by the AMF was quantified using the gridline intersection method (Giovannetti and Mosse 1980) under a stereo microscope Motic SMZ.

Plant and insect bioassays

Controlled conditions: Detached leaves bioassay

A total of nineteen six-week-old plants per treatment were used. The third true leaf of each plant was detached using a scalpel and placed in a Petri dish (150 mm diameter) with filter paper on the bottom, previously moistened with 3 ml of sterile water to prevent desiccation. Each leaf was infested with two second-instar *T. absoluta* larvae. All Petri dishes with the infested leaves were maintained at 22 °C until the emergence of the *T. absoluta* adults. The percentage of larvae that reached adult stage was evaluated for each treatment.

Semi-controlled conditions: whole plant bioassay

A total of twelve six-week-old plants per treatment were placed in individual rearing cages (30 cm × 30 cm × 30 cm) and infested with three second-instar *T. absoluta* larvae on the third true leaf of each plant. All cages with the infested plants were placed in a greenhouse without any control of the climatic conditions. Plants were maintained until the end of the bioassay with the same watering regime and nutrient supply as described above. Leaflets from the infested plants presenting damage by *T. absoluta* (+H) and leaflets from non-infested plants (–H) from all treatments were collected separately 48 h after infestation, immediately frozen in liquid nitrogen and stored at − 80 °C until their use for metabolomic analysis. Three weeks after infestation, when all surviving larvae reached the pupal stage, pupae from each plant were collected, placed separately in plastic cups, and incubated at 22 °C until adult emergence. The percentage of larvae reaching the pupal and adult stages was evaluated for each treatment.

Functional analysis of primed compounds

Selected compounds with primed accumulation in microbial inoculated plants in response to *T. absoluta* were purchased to test their effect on the insect development. The treatments tested were azelaic acid (AZA, Sigma-Aldrich, Germany), feruloylputrescine (FP, AKos Consulting & Solutions Deutschland GmbH, Steinen, Germany) and p-coumaric acid (CA, Sigma-Aldrich, Germany). Six weeks old plants without any microbial inoculation were used for this bioassay. One fully developed leaf from each plant was detached and the petiole dipped in 2 ml of aqueous solution containing 100 ppm of the compounds and 0.002% EtOH. A combined treatment (MIX) was included consisting of a mixture of the three compounds in a final concentration of 100 ppm for each compound as described by Rivero et al. (2021). Control treatment was mock treated with the same solution but without any of the tested compounds. Leaves were maintained until the full absorption of the aqueous solution, after that were placed in Petri dishes (150 mm diameter) with filter paper on the bottom previously moistened with 3 ml of sterile water to prevent desiccation. Ten biological replicates were used for each treatment and each replicate was infested with three second-instar *T. absoluta* larvae. All Petri dishes with the infested leaves were maintained at room temperature (≈ 22 °C) until the emergence of the *T. absoluta* adults. The percentage of larvae that reached adult stage was evaluated for each treatment.

Untargeted metabolomics

LC-ESI full scan mass spectrometry

Thirty milligrams of freeze-dried leaf material (six biological replicates per treatment) were homogenized at 4 °C in 1 ml of MeOH: H2O (30:70) containing 0.01% of HCOOH. After that, the homogenate was centrifuged at 15 000 g for 15 min at 4 °C, the supernatant was recovered and filtered through 0.2 µm cellulose filters (Regenerated Cellulose Filter, 0.20 µm, 13 mm D. pk/100; Teknokroma). Twenty microliters of the filtered supernatant were injected into an Acquity ultra performance liquid chromatography system (UPLC, Waters, Mildford, MA) interfaced with a hybrid quadrupole time-of-flight instrument (QTOF-MS Premier, Waters, Mildford, MA). Six independent biological replicates per treatment were randomly injected. To elute analytes, a gradient of methanol and water containing 0.01% HCOOH was used. The LC separation was performed using an UPLC Kinetex 2.6 µm particle size EVO C18 100 A, 50 × 2.1 mm (Phenomenex). Subsequently, a second fragmentation function was introduced into the TOF analyser to identify the signals detected. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a

fragmentation spectrum of each analyte (Gamir et al. 2014). Chromatographic conditions and solvent gradients were established as described by Gamir et al. (2014).

Full scan data analysis

Positive and negative electrospray ionization (ESI) signals were analyzed independently to obtain a global view of the data conduct. For ESI positive, the instrument detected 3387 signals and, for ESI negative, 1878 signals (Table S1). The raw data files acquired with the Masslynx 4.1 software (Masslynx 4.1, Waters) were transformed into.cdf files with Databridge tool. The files were then processed with the software R using the XCMS algorithm (Smith et al. 2006) to obtain the peak peaking, grouping and signal corrections. Metabolite amounts were analyzed based on the normalized peak area units relative to the dry weight. Adduct and isotope correction, Kruskal–Wallis test ($p < 0.05$), filtering, clustering and pathway categorization were carried out with the packagesMarVis filter, MarVis cluster and MarVis pathway, which are integrated in the Marvis suit 2.0 (Kaever et al. 2015). To obtain the overall behavior, data obtained in positive and negative ESI were combined using the same software. When several signals corresponded to the same metabolite, we kept the signal displaying the highest intensity range across treatments. Metabolite identification was carried out based on exact mass accuracy and fragmentation spectra matching with different online database. The database KEGG (https://www.genome.jp/kegg/) was used for exact mass identity, and the Massbank and the Metlin databases (www.massbank.jp; www.masspec.scripps.edu) were used for fragmentation spectrum analysis.

Statistical analysis

Data were analyzed using R statistical language, version 4.0.5 (R Development Core Team 2021) and figures were produced using the package ggplot2 (Wickham 2009). The effect of microbial treatments on the percentage of larvae reaching adult stage in the detached leaves bioassay and the comparisons between the microbial treatments and the control was assessed using a Pearson's Chi-squared test with Yates' continuity correction. In the whole plant bioassay, to test the effect of the microbial treatments on *T. absoluta* development we used generalized linear models with binomial distribution and logit link function. Differences in the percentage of root length colonized by AMF, and the effect of microbial treatments and infestation on metabolite accumulation were assessed using a linear modeling. Post-hoc comparisons among microbial treatments were based on a Fisher's LSD. Model validation was performed graphically by inspecting the residuals and fitted values (Zuur and Leno, 2016).

Spearman's rank correlation analyses between insect performance and plant metabolism were performed using the *cor.test* function of *stats* R package. Then, the *MetaboAnalystR* package (Pang et al. 2020) was used to filter and transform the data prior hierarchical clustering of chromatographic signals and treatments. Variance filtering was based on the interquartile range (IQR), with 10% signals filtered out. Data was cube root-transformed and range scaled (mean-centered and divided by the range of each variable). The R package *ComplexHeatmap* (Gu 2022) was used to generate a heatmap of the general overview of the tomato plants metabolome depending on microbial inoculations and herbivory status was generated based on the Euclidian distances. A second heatmap focused on a group of signals correlated with insect performance based on Pearson's distances was performed using the same package.

The effect of the exogenous leaf application of the pure compounds on the percentage of larvae reaching adults was assessed with exact binomial test.

Results

Microbe-induced resistance remains robust across different setups

To explore the mechanisms underlying microbe-IR against *T. absoluta*, we first set different bioassays under controlled or semi-controlled conditions. The tested microbes (*F. mosseae, R. irregularis, T. afroharzianum, B. amyloliquefaciens and P. azotoformans*) were selected because of their confirmed ability to protect tomato plants against different pathogens (Minchev et al. 2021), and their performance under agronomic conditions reducing natural incidence of *T. absoluta* infestation (Minchev et al. 2024).

Different mechanisms could account for the reduced incidence of *T. absoluta* infestation in the inoculated plants observed in the field, including potentiation of direct defenses, changes in pest attraction, or synergies with the different IPM methods, for example potentially improving natural enemies attraction (Minchev et al. 2024). To specifically address the contribution of plant direct defenses, we established different non-choice bioassays to test larval performance in whole plant and detached leaves bioassays. First, we confirmed efficient root colonization by the inoculated microbes in all treatments. PGPR and *Trichoderma* colonization was confirmed by qPCR in the inoculated plants. For mycorrhizal treatments, symbiosis establishment was confirmed with root colonization levels of 83% and 40% for *R. irregularis and F. mosseae* inoculated plants, respectively, in the experiment under controlled conditions, and 66% and 56%, respectively, in the semi-controlled conditions experiment (Table S2).

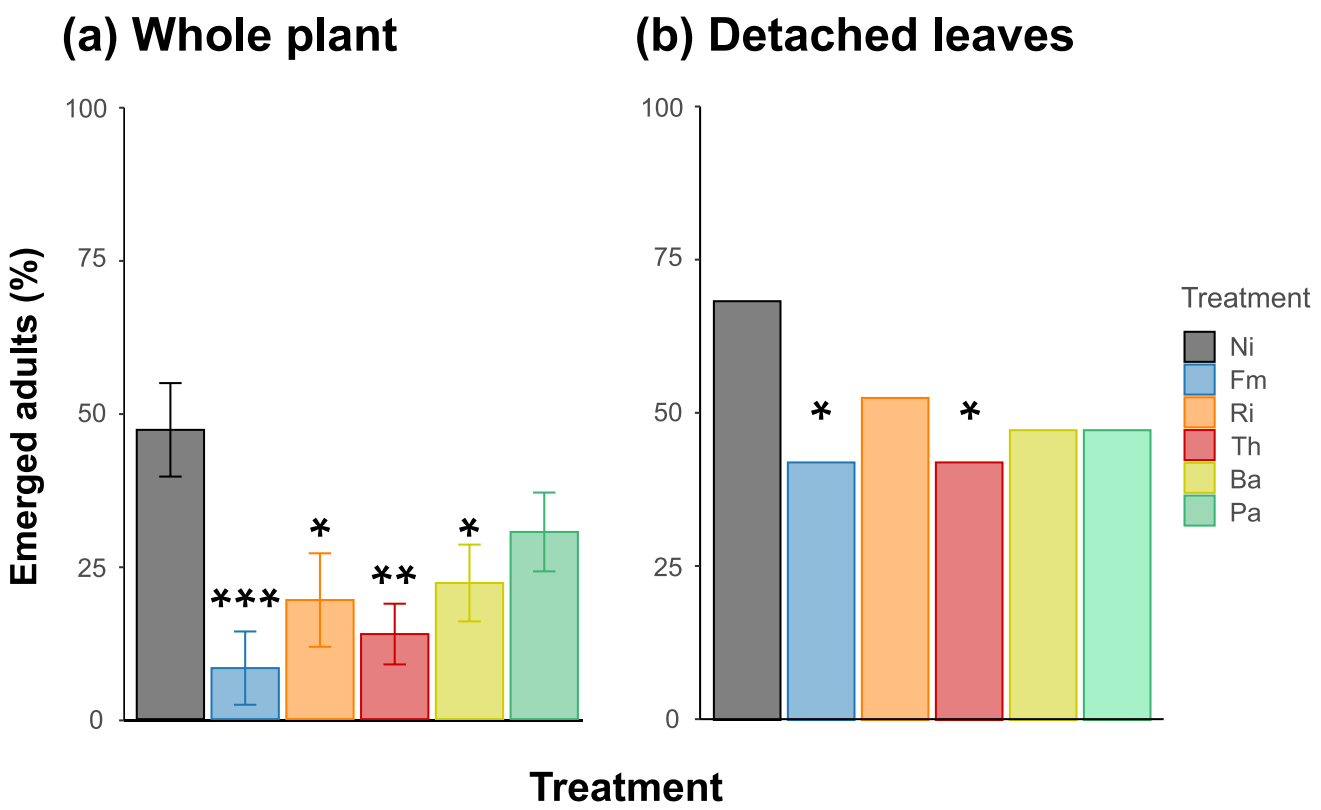

Fig. 1 Microbe-Induced Resistance against *Tuta absoluta* in different setups varying the degree of experimental control. Plants were inoculated with *Funneliformis mosseae* (Fm), *Rhizophagus irregularis* (Ri), *Trichoderma afroharzianum* T22 (T22), *Bacillus amyloliquefaciens* (Ba) and *Pseudomonas azotoformans* (Pa). Non- inoculated (Ni) plants were included as control. **a** Plants were grown in a greenhouse with controlled climatic conditions, and the insect bioassay was performed on whole plants in insect cages in a greenhouse without any climatic control. Bars represent the mean percentage of *T. absoluta* larvae reaching adults for each treatment and error bars represent standard errors of the means. Asterisks indicate statistically significant differences compared to the control based on generalized linear model with binomial distribution and logit link function followed by LSD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n = 12). **b** Plants were grown in a greenhouse with controlled climatic conditions; the herbivory bioassay was performed on detached leaves. Bars represent the percentage of *T. absoluta* larvae reaching adults for each treatment. Asterisks indicate statistically significant differences compared to the control based on Pearson's Chi-squared test with Yates' continuity correction (*$p < 0.05$, n = 38)

We assessed the induced resistance capacity in plants growing in pots inside insect cages placed in a greenhouse without artificial light nor climatic control. All microbial treatments reduced the percentage of larvae that reached adult stage, but in *P. azotoformans*-inoculated plants the reduction was not significant (Fig. 1a). In contrast, the three fungi *F. mosseae, R. irregularis* and *T. afroharzianum*, and the bacteria *B. amyloliquefaciens* significantly reduced the percentage of larvae reaching the adult stage with 83%, 60%, 70% and 53%, respectively as compared to the non-inoculated control (Fig. 1a), with the strongest effect found in *F. mosseae* and *T. afroharzianum*. The results are consistent with those obtained under production conditions including IPM (Minchev et al. 2024), confirming the higher efficacy of these fungal inoculants to enhance plant resistance against *T. absoluta*.

This reduction in the number of adults results from the cumulative negative effect of the microbial inoculations on the insect life cycle, with a non-significant reduction on the proportion of individuals reaching the pupal stage (44%, 38%, 38% and 22% reduction in *F. mosseae, R. irregularis, T. afroharzianum* and *B. amyloliquefaciens*, respectively, (Fig. S1a), and a significant reduction of the percentage of pupae reaching adult stage by 65%, 47% and 32% in *F. mosseae, T. afroharzianum* and *B. amyloliquefaciens* respectively, compared to the non-inoculated control (Fig. S1b).

The ability of the root colonizing microbes to protect leaves against the pest was further confirmed in experiments using detached leaf bioassays. All microbial treatments reduced the percentage of *T. absoluta* larvae that

reached the adult stage, but the effect was only significant in *T. afroharzianum* and *F. mosseae* inoculated plants, reducing the percentage of larvae reaching the adult stage by 38% compared to the non-inoculated control (Fig. 1b). Thus, the bioassays consistently show that microbial inoculation leads to changes in the leaves that impact the performance of the larvae feeding on them.

Microbe-IR is associated with differential metabolic reprograming in response to *Tuta absoluta*

To explore the changes in the plant metabolic profile that may lead to the negative impact on pest performance, we conducted an untargeted metabolomics analysis on leaf samples 48 h post infestation from the experiment in semi-controlled conditions. We focused on the fungal treatments, as they showed more consistent protection across the different experimental scales. Thus, we compared leaf samples from non-inoculated control plants and plants inoculated with *F. mosseae, R. irregularis* and *T. afroharzianum,* infested or not with the herbivore.

In total 496 signals displaying statistically significant changes among treatments were detected (Table S1). While a heatmap visualization of these signals clearly revealed metabolic rearrangement in leaves infested with *T. absoluta* (Fig. 2), hierarchical clustering separated first the samples into two main groups, non-mycorrhizal and mycorrhizal treatments. This separation was driven by the presence of distinct clusters containing metabolites exclusively over accumulated in mycorrhizal plants regardless of the presence of the leaf-miner. Whitin the non-mycorrhizal group, plants were further subdivided based on their inoculation status with or without *T. afroharzianum*, revealing a pronounced effect of *T. afroharzianum* inoculation on the metabolome of tomato leaves, even in the absence of herbivory. Each of these groups (Nm, T22) was finally split depending on the presence or absence of herbivore attack (Fig. 2). In contrast, within the mycorrhizal group, plants clustered primarily according to the herbivory condition rather than the AMF species, supporting a consistent common metabolic impact of the different mycorrhizal inoculants first, but then herbivory has a dominant effect over symbiotic species-specificity at the metabolic level (Fig. 2).

Differentially accumulated metabolites correlate with *T. absoluta* performance

After this general overview, we searched for specific metabolites contributing to the enhanced resistance observed in the fungal-inoculated plants by focusing on a set of 85 signals significantly correlating with insect performance. Among them, 56 signals exhibited a negative correlation with *T. absoluta* adult emergence, while 29 showed positive correlation (Fig. 3, Table S1). Remarkably, clustering analysis on this set of signals relocated herbivory challenged *T. afroharzianum* inoculated plants near attacked mycorrhizal plants, grouping in a cluster of treatments displaying IR phenotype against *T. absoluta* (Fig. 3).

In response to *T. absoluta*, *F. mosseae* and *T. afroharzianum* grouped more closely with each other than the two AMF treatments did. Notably, these results are consistent with the different levels of microbe-IR displayed, where *F. mosseae* and *T. afroharzianum* caused a greater reduction in the percentage of emerged adults than *R. irregularis*.

Feature clustering allowed us to discriminate between three main profiles of chromatographic signals potentially with distinct impacts in insect performance.

The first cluster (C1, Fig. 3a) encompasses signals corresponding to metabolites whose accumulation might benefit *T. absoluta,* as they positively correlated with the insect fitness and were less abundant in resistant plants. This pattern could reflect a defense strategy by microbe-inoculated (resistant) plants that limits the ability of the insect to optimally exploit the plant resources, unlike in non-inoculated controls.

The other two clusters (C2 and C3, Fig. 3a) corresponded to metabolites likely detrimental to *T. absoluta*, as they negatively correlated with insect performance. The second cluster included a group of signals whose accumulation was strongly reduced in non-inoculated plants upon leaf-miner attack. These metabolites remained at higher levels in mycorrhizal infested plants, likely due to a greater basal accumulation compared to non-inoculated ones (C2, Fig. 3a). The third cluster consisted of a group of metabolites showing a priming profile, over-accumulated in microbe-inoculated plants in response to the herbivore (C3, Fig. 3a). These primed metabolites could represent potentially bioactive compounds with anti-herbivore properties.

To get insights into the biological meaning of those changes, we used the MarVis-pathway tool (Kaever et al. 2015), through exact mass match, to predict the pathway ontology of the differentially accumulated compounds. This was achieved for 31% of the signals correlating with insect performance. The pathway characterization revealed differential accumulation of several metabolic pathways among the different clusters C1, C2 or C3 (Fig. 3b). For instance, the metabolites comprised in C1 (positively correlating with *T. absoluta* performance) were mostly involved in the metabolism of cofactors and vitamins, amino acid and carbohydrate metabolism, and degradation of aromatic compounds. The most representative pathway in C2 was carbohydrate metabolism, while 2-oxocarboxilic acid metabolism was exclusively present in this cluster. Phenylpropanoid and amino acid metabolism also appeared as major pathways in C2. In C3, the higher proportion was for signals involved in phenylpropanoid and amino acid (in particular

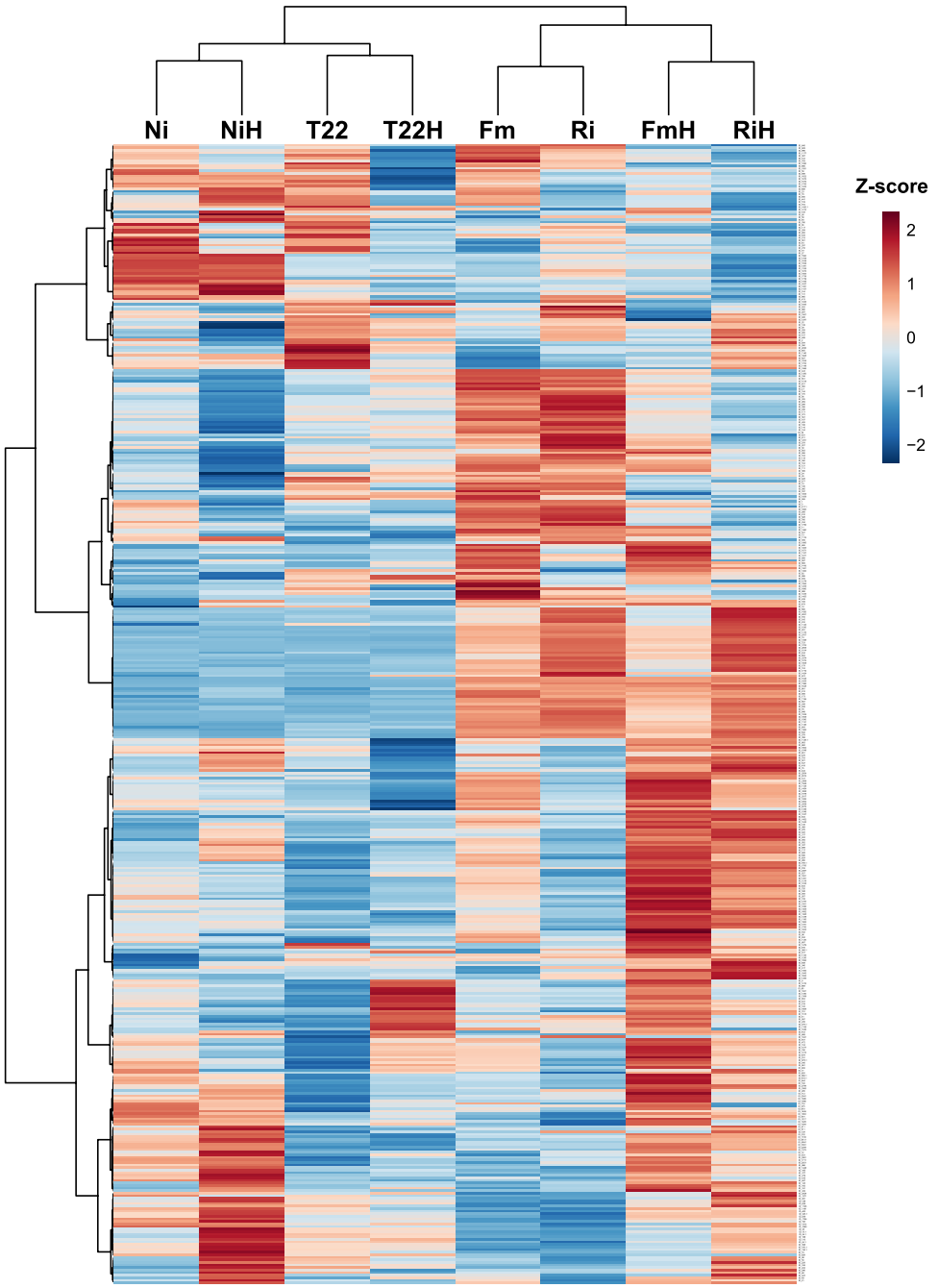

Fig. 2 Metabolic rearrangement in tomato plant leaves in response to *Tuta absoluta* attack. Overview 48 h post infestation in the treatments exhibiting efficient protection under semi-controlled conditions**.** Hierarchical clustering and heatmap analysis representing the signals from positive and negative ESI with statistically significant differences among treatments based on Kruskal–Wallis test ($p < 0.05$, n = 6). Non-inoculated control plants (Ni), plants inoculated with *Funneliformis mosseae* (Fm), *Rhizophagus irregularis* (Ri) or *Trichoderma afroharzianum* T22 (T22). Treatments infested with *T. absoluta* larvae are indicated with "H"

phenylalanine) metabolism, and aromatic compound degradation (Fig. 3b).

Remarkably, most signals in C1, positively correlated with *T. absoluta* adults (the higher signal intensity, the higher adult emergence, Fig. 4a), and in C3, correlating negatively with adult emergence (the higher signal intensity, the lower adult emergence, Fig. 4b), showed consistent accumulation patterns in resistant plants regardless of the fungal species inoculated. Specifically, we found 16 and 24 signals common to the 3 fungal treatments for C1 and C3 respectively (Fig. 4c, d). However, *F. mosseae* and *T. afroharzianum* displayed a higher

resemblance in their response to the attack as evidenced by the additional signals shared by these two fungal treatments but not by the *R. irregularis* treatment. Again, although all three microbe-inoculated treatments displayed very similar pattern of response to the herbivory, the magnitude of the response was higher in *F. mosseae* and *T. afroharzianum* plants, the plants displaying higher degree of resistance in our different experiments.

Identification of metabolites with potential antiherbivory properties

We then attempted the identification of the metabolites corresponding to the signals with relevant profiles through analysis of the exact mass and fragmentation spectra of the signals. While none of the signals within the C1 cluster were identifiable (Fig. 4E), the analyses allowed the identification of some known defense-related compounds in C3. Among them, we confirmed that azelaic acid (AZA) and coumaric acid (CA) were significantly more accumulated in *F. mosseae* and *T. afroharzianum* inoculated plants in response to *T. absoluta* (Fig. 4F). Furthermore, we found chlorogenoquinone (ChQ) to be over-accumulated in both mycorrhizal treatments in response to the challenge, while in *T. afroharzianum* it was highly accumulated in absence of herbivory (Fig. 4F).

In addition, we performed a targeted search of feruloylputrescine (FP), a metabolite previously found to be primed in tomato plants inoculated with *F. mosseae* attacked by the leaf-chewing caterpillar *S. exigua* (Rivero et al. 2021). Search by exact mass and fragmentation spectra allowed us to accurately identify and quantify the levels of FP in our samples, confirming a primed accumulation by both AMF in response to *T. absoluta* (Fig. 5).

Primed metabolites display anti-herbivore activity against *Tuta absoluta*

Functional analysis of the commercially available identified metabolites showing primed profiles (AZA, FP and CA), and a combination of them (MIX), was performed to evaluate their effect on the development of *T. absoluta*. For that, the pure chemicals were applied using the herbivory bioassay on detached leaves as in Fig. 1, as we confirmed the bioassay is suitable to test IR. While 70% of the larvae reached the adult state when feeding in leaves from the control treatment, the percentage when feeding on leaves treated with AZA and FP was significantly reduced to 44 and 50%, respectively, (37 and 29% of reduction respectively) (Fig. 6). The treatment with CA did not have any impact on *T. absoluta* development. The combined treatment with AZA, FP and CA also reduced the percentage of emerging adults to 53% (24% of reduction compared to the control), in a similar range to the application of individual compounds, so the bioassay does not support a synergistic or additive effect among these compounds (Fig. 6). Overall, the negative effect of AZA and FP on *T. absoluta* development confirmed the antiherbivory effect of these compounds showing primed accumulation in plants displaying microbe-IR.

Discussion

Our research demonstrates that the fungal bioinoculants *F. mosseae*, *R. irregularis* and *T. afroharzianum* T22, consistently trigger microbe-IR, efficiently protecting tomato plants against the devastating insect pest *T. absoluta*. The untargeted metabolomics analysis performed reveals that the higher resistance against the leaf miner in the microbe elicited plants involves metabolic reprograming of direct plant defenses, fully supporting our hypothesis. We identified metabolic fingerprints and primed accumulation of antiherbivore bioactive compounds associated to the efficient microbe-IR in tomato against *T. absoluta*.

It is well described that one of the main challenges for bioinoculants application in crop protection is the reproducibility of the results under field conditions, as environmental variability can compromise microbe-IR efficacy (di Lelio et al. 2021; Lee Díaz et al. 2021). However, we previously showed these microbes to efficiently reduce natural incidence of *T. absoluta* under agronomic conditions of intensive agriculture, with efficient IPM methods already in place (Minchev et al. 2024).

As plant defenses combine both direct and indirect ones, isolating the specific contribution of direct plant defenses is difficult under the multitrophic context of agronomic management. Indeed, indirect defenses such as the impact of microbial inoculation on the performance of natural enemies released as part of IPM may also contribute to the observed effect in the field (Schausberger et al. 2012; Papantoniou et al. 2022). By using non-choice bioassays, in which natural enemies and potential effects on attraction/repellence are excluded, we were able to address the particular contribution of the impact on plant direct defenses to the IR phenotype. Upon confirmation of the efficacy of microbe-IR under semi-controlled conditions (a non-choice bioassay but maintaining the characteristics of production greenhouses) we explored the leaf metabolic changes associated with microbe-IR to *T. absoluta*. In this way, we could address the specific contribution of the microbe-IR on plant direct defenses, independently of the contribution of the other IPM methods. While bacterial and fungal inoculantes were tested, for the biochemical analysis, we focused on the mechanisms underlying the IR success of the fungal inoculants *F. mosseae*, *R.*

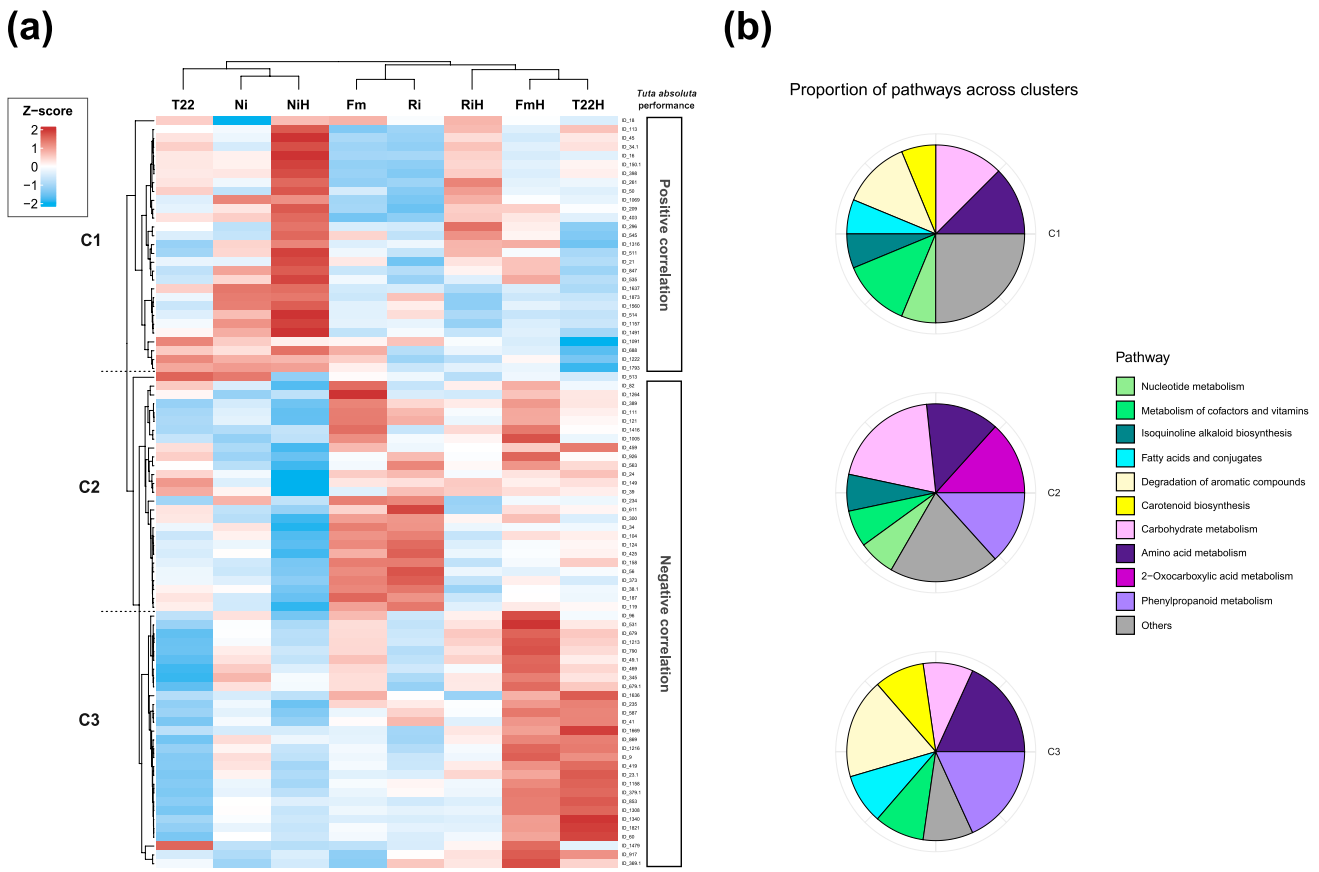

Fig. 3 Metabolic features and pathways likely contributing to microbe-induced resistance to *Tuta absoluta.* **A** Hierarchical clustering and heatmap analysis representing 85 signals exhibiting either positive or negative correlation with *T. absoluta* performance. **B** Pathways predicted for tentative metabolites correlated with *T. absoluta* emergence. Non-inoculated control plants (Ni), plants inoculated with *Funneliformis mosseae* (Fm), *Rhizophagus irregularis* (Ri) or *Trichoderma afroharzianum* T22 (T22). Treatments infested with *T. absoluta* larvae are indicated with "H". Three main clusters of features are represented as C1, C2 and C3. The former includes chromatographic signals positively correlated with *T. absoluta* adult emergence, while C2 and C3 grouped the signals displaying negative correlations with insect performance

irregularis and T. afroharzianum T22 because of the consistency and context-stability of the IR conferred across the different settings tested. Indeed, it is described that fungi are usually more resilient to environmental challenges (de Vries et al. 2018) so they are expected to perform better under the changing conditions of crop production—usually a challenge for microbials performing well under optimized lab conditions. Our mechanistic analyses revealed that root inoculation with these beneficial fungi leads to a significant metabolic rearrangement in leaves, especially upon herbivory, resulting in the primed accumulation of defensive compounds that would likely be a strong contribution to the effect observed in the field, likely complementing the other control strategies in place under IPM.

Previous studies on microbe-IR against phytophagous insects revealed that microbial inoculations trigger metabolic changes in the plant upon herbivory, likely related to the enhanced plant resistance. For example, *F. mosseae* primed the accumulation of defensive compounds like alkaloids, fatty acid derivatives and phenylpropanoid-polyamine conjugates in response to *S. exigua* (Rivero et al. 2021). *Trichoderma afroharzianum* T22 inoculation increased the accumulation of defense-related secondary metabolites such as alkaloids, phenolic acids and flavonoids in response to *Macrosiphum euphorbiae* (Coppola et al. 2019). In addition, inoculation of tomato roots with *T. harzianum* T78 was shown to negatively impact *Manduca sexta* development through alteration of the plant and insect metabolomes (Papantoniou et al. 2021). Hence, we hypothesized that the protection achieved by fungal inoculation of roots is associated to differential reprogramming of plant secondary metabolism and primed accumulation of bioactive compounds associated to defense. We also hypothesized that there may be a common core of compounds related to resistance by different microbes, so a microbe-IR fingerprint. To test our hypothesis, we performed an untargeted metabolomic

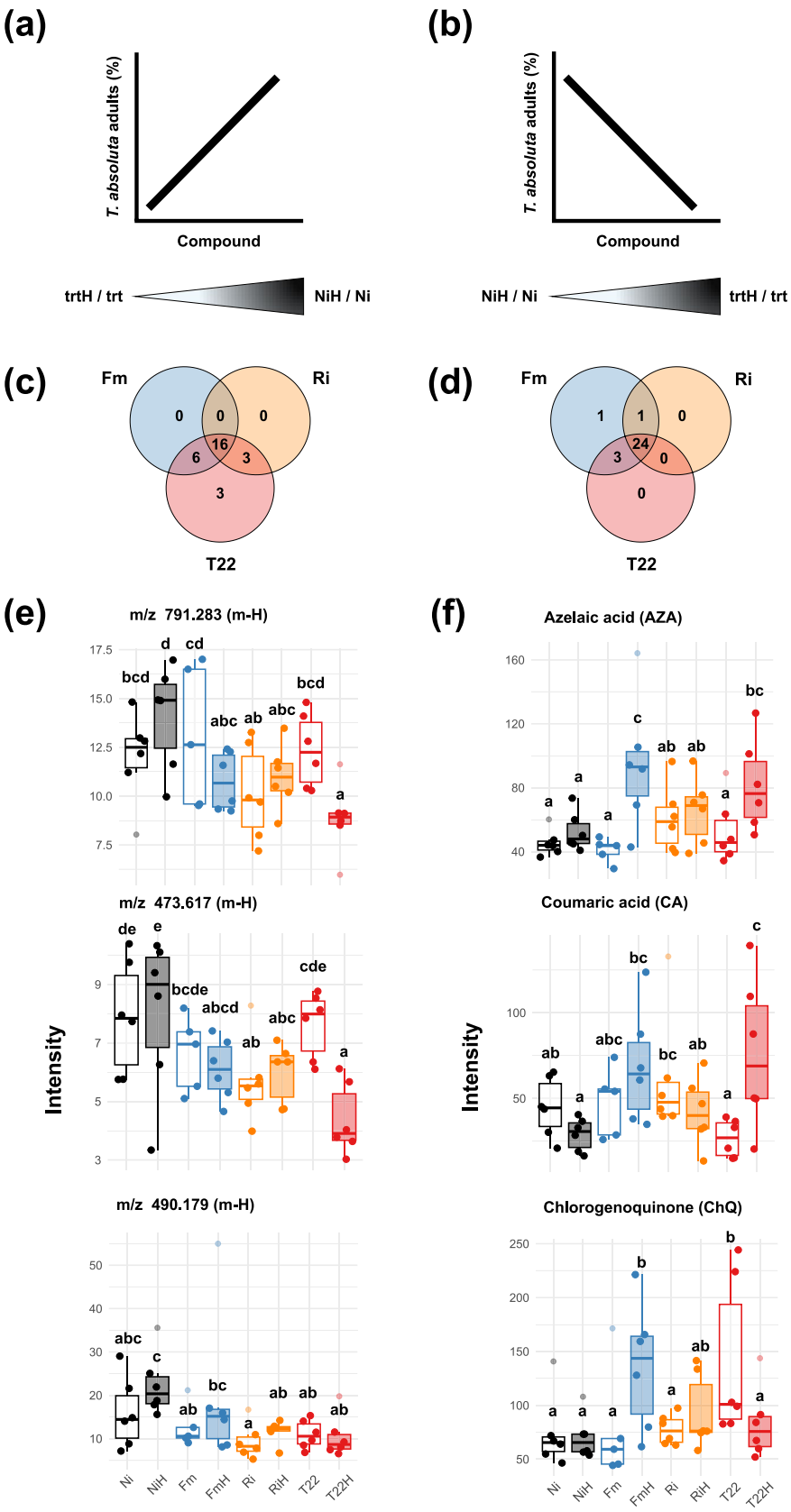

Fig. 4 Metabolic profiles of microbe-inoculated plants related to the microbe-IR phenotype. Left panels correspond to chromatographic signals positively correlated with *Tuta absoluta* adult emergence. Right panels represent a set of signals negatively correlated with *T. absoluta* performance that exhibited priming profiles. **a** Schematic representation of metabolites potentially beneficial to *T. absoluta* and less accumulated in microbe-inoculated plants in response to the leafminer than in control plants. **b** Schematic representation of inducible metabolites likely detrimental to *T. absoluta* and over accumulated in microbe-inoculated plants compared to non-inoculated ones in response to the insect attack (priming profile). **c** Venn diagram for signals corresponding to C1 profile. **d** Venn diagram for signals corresponding to C3 profile. **e** Examples of metabolites located in intersections of the venn diagram for C1; this is shared by all microbe-inoculated plants or only by couples of them **f** Examples of metabolites located in intersections of C3 venn diagram. Fungal treatments (trt) include plants inoculated with the arbuscular mycorrhizal fungi *Funneliformis mosseae* (Fm) or *Rhizophagus irregularis* (Ri) or plants inoculated with *Trichoderma afroharzianum* T22 (T22). Non-inoculated (Ni) plants were included as control. Infestation with *T. absoluta* larvae is labeled as "H". In boxplots, dots represent raw data. Boxes represent the interquartile range, inner lines in bold represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range and empty dots represent outliers. Treatments not sharing a letter are statistically different based on one-way ANOVA followed by Fisher's least significant difference (LSD) post hoc test ($p < 0.05$, n = 6)

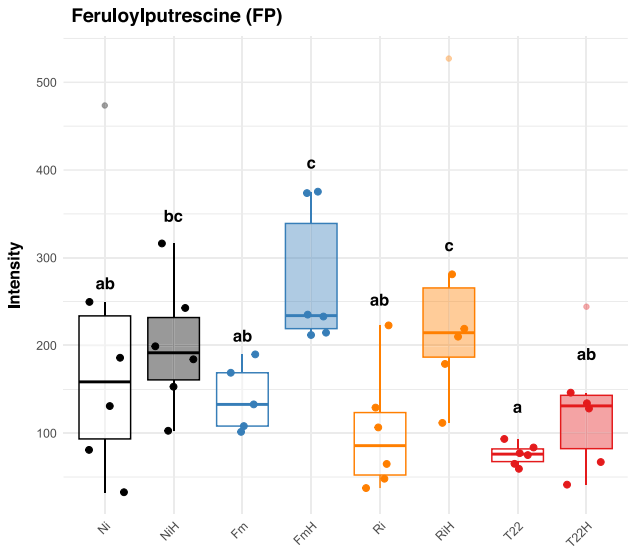

Fig. 5 Primed accumulation of feruloylputrescine in mycorrhizal treatments. Accumulation of feruloylputrescine in non-infested tomato plants and plants infested with *Tuta absoluta*. Non-inoculated plants (Ni), plants inoculated with the arbuscular mycorrhizal fungi *Funneliformis mosseae* (Fm) or *Rhizophagus irregularis* (Ri) or plants inoculated with *Trichoderma afroharzianum* T22 (T22). Plant infestation with *T. absoluta* larvae is labeled as "H". Dots represent raw data. Boxes represent the interquartile range, inner lines in bold represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range and empty dots represent outliers. Treatments not sharing a letter are statistically different based on one-way ANOVA followed by Fisher's least significant difference (LSD) post hoc test ($p < 0.05$, n = 6)

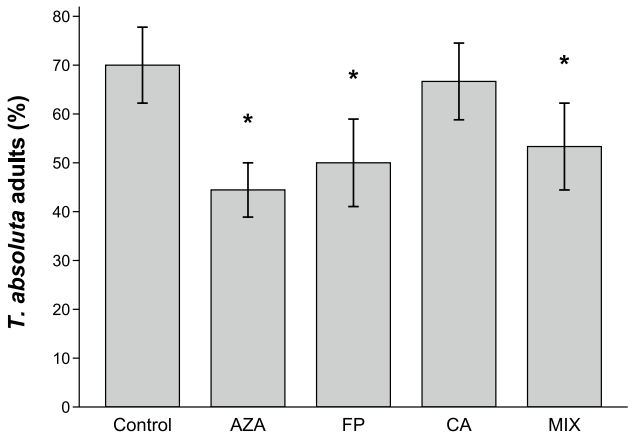

Fig. 6 Functional analysis of the identified primed metabolites in microbe-inoculated plants. Effect of exogenous application of the pure compounds (100 ppm) on *Tuta absoluta* development evaluated as percentage of larvae reaching adult stage. Mock treated (Control), azelaic acid (AZA), feruloylputrescine (FP), p-coumaric acid (CA), combined treatment (MIX) consisting in a mixture of the three compounds. Asterisks indicate statistically significant differences compared to the control based on exact binomial test ($p < 0.05$, n = 30)

analysis to explore overall metabolic rearrangement occurring in leaves when attacked by *T. absoluta* larvae. Indeed, we detected groups of metabolites whose accumulation in plant shoot has likely influenced pest performance either positively or negatively. Moreover, we found similarities in the patterns triggered by the different IR eliciting microbes, including enhanced accumulation of bioactive compounds able to inhibit *T. absoluta* performance.

One of the groups (C1) included metabolites potentially beneficial for the leaf miner, as their accumulation correlated with an increased emergence of *T. absoluta* adults. These metabolites were more accumulated in non-inoculated controls upon herbivory. Thus, by limiting the availability of these compounds, microbe-inoculated plants could be impeding the optimal performance of the insect. Interestingly, some herbivorous insects, and particularly galling and leaf mining insects, are described to manipulate the host plant responses for their own benefit (Giron et al. 2016; Favery et al. 2020). In our study, a pathway ontology prediction points that the metabolites from this group are mainly involved in the metabolism of carbohydrates, amino acids, and vitamins. Indeed, all these metabolites are important for phytophagous insect dietary requirements. For example, some insects are not able to synthesize some vitamins and amino acids, and carbohydrates are often limiting nutrients for them (Behmer 2009). In addition, an important pathway related to plant defense against insects such as phenylpropanoid metabolisms was not present in this cluster. Therefore, it is tempting to speculate that the accumulation of the compounds in this group could result of *T. absoluta* manipulation of the plant metabolism for its own benefit, that might be less efficient in the fungal-inoculated plants. This hypothesis requires further research.

Further, we focused on a group of metabolites likely to be detrimental to *T. absoluta* negatively correlating with *T. absoluta* performance. We identified a cluster of compounds (C3) with primed accumulation in plants displaying microbe-IR—more accumulated in the inoculated plants than in the non-inoculated controls in response to the herbivore. Indeed, this group of metabolites are mainly involved in amino acid metabolism and phenylpropanoid among others, metabolic pathways described to play important role in plant defense responses to insect herbivores (Mithöfer et al. 2012; Zhou et al. 2015; Li et al. 2020; Chen et al. 2021). On one hand, amino acids can function as precursors of important plant defensive metabolites such as phenylpropanoids, but on the other hand can play an important role for herbivore growth as source of nitrogen (Zhou et al. 2015). In this line, while we found metabolites in C1 to be involved in amino acids metabolism but not in the metabolism of phenylpropanoids, compounds in C3 were involved in both pathways in agreement with the dual role of amino acids in plant–herbivore interactions.

Interestingly, upon herbivory, the metabolite accumulation pattern of *F. mosseae* inoculated plants was more similar to *T. afroharzianum* T22 than to *R. irregularis* ones. This observation matches with the higher level of resistance achieved by these two bioinoculants. A detailed analysis allowed the identification of some metabolites associated to plant defenses, specifically AZA and CA, over-accumulated in *T. afroharzianum* and *F. mosseae* in response to the leafminer. AZA, a fatty acid derivative, has been shown to operate in plant systemic immunity (Jung et al. 2009). This compound has been shown also to have direct biocidal activity, as it was found as major component among the carboxylic acids of *Zanthoxylum armatum* extract, with high larvicidal and ovicidal activity against *Spodoptera frugiperda* and *T. absoluta* (Firake et al. 2023). CA is a hydroxycinnamic acid that serves as a precursor in the biosynthesis of phenylpropanoids, secondary metabolites with wide range of functions in plant defenses against pathogen infection and insect herbivores (Dixon et al. 2002; Schott et al. 2022). For example, ChQ which results from the oxidation of chlorogenic acid by the action of polyphenol oxidases, has been demonstrated to have anti-herbivore properties (Felton et al. 1989; Kundu and Vadassery 2019). Remarkably, we found ChQ to be over-accumulated upon herbivory in mycorrhizal plants, and with higher basal levels in *Trichoderma* plants. CA and other hydroxycinnamic acids can also act as precursors of phenolamides through the conjugation to amines (Zeiss et al. 2021). Interestingly, the phenolamide FP showed primed accumulation in *R. irregularis* and *F. mosseae* inoculated plants challenged with *T. absoluta*. FP and other phenolamides have been reported to be inducible in response to different pathogens (Morimoto et al. 2018), in response to simulated herbivory (Gaquerel et al. 2014), and most importantly in response to *T. absoluta* (Roumani et al. 2022). Remarkably, primed accumulation of AZA and FP was also found in leaves of *F. mosseae* inoculated tomato plants after attack by the generalist chewing insect *S. exigua* (Rivero et al. 2021). Thus, these compounds appear as good candidates to mediate microbe-IR in tomato, as their primed accumulation appears to be a conserved trait in very different pathosystems including chewing and leaf-mining herbivorous insects.

Functional tests on the bioactivity of the identified compounds that were commercially available demonstrated the capacity of the primed metabolites AZA and FP to impair *T. absoluta* development. Thus, the functional bioassay supports the likely contribution of their high levels in inoculated plants to the microbe-IR here reported. Remarkably, AZA was also demonstrated to effectively reduce *S. exigua* performance (Rivero et al. 2021). Thus, microbe-IR is associated with plant metabolic reprogramming upon herbivory, leading to the primed accumulation of bioactive secondary metabolites. Besides helping to understand the mechanisms behind microbe-IR, this approach contributes to the discovery of new plant derived compounds with anti-herbivore activity. Moreover, it can lead to the identification of biomarkers of microbe-mediated defense priming against pests. In this regard, our findings and those previously reported by Rivero et al. (2021) are, to the best of our knowledge, are the first two studies relating AZA and FP with microbe-IR in tomato. These studies covered two different herbivores, the leaf chewer *S. exigua* and the leaf miner *T. absoluta*, and three different eliciting microbes, the AMF *F. mosseae* and *R. irregularis,* and the biocontrol fungus *T. afroharzianum*. Accordingly, we propose AZA and FP as potential metabolic biomarkers of microbe-IR against herbivorous insects in tomato. However, whether these markers are part of a common fingerprint of microbe-IR requires further research including other efficient IR-eliciting microbes. Future studies should address also the potential conservation across different plant species to evaluate the suitability of these biomarkers for IR screening strategies in different plant systems.

Regarding the consistency and efficiency of the protection of tomato plants against *T. absoluta* achieved, we previously demonstrated that these fungal inoculants consistently trigger IR protecting tomato plants in a commercial production greenhouse (Minchev et al. 2024). Yet, as the same cultivar was used, whether the microbe-IR is functional across different tomato plant genotypes requires further research. Moreover, considering the well-known context dependency of microbe-IR (Lee Díaz et al. 2021), further validation in different tomato genotypes, including commercial varieties, as well as in different environmental contexts and agronomic managements are currently underway to explore the full potential of the reported bioprotection strategy.

Overall, this study reveals that beneficial fungi like *Trichoderma* and AMF can efficiently and consistently activate IR in tomato, protecting the plants against the devastating insect pest *Tuta absoluta* under very diverse setups. We show that the inoculation boosts plant direct defenses leading to metabolic reprogramming and primed accumulation of bioactive compounds with a confirmed deleterious effect on *T. absoluta* development. These compounds, identified as part of the microbe-IR metabolic fingerprint, may serve as potential biomarkers of primed defenses, and future research may explore their potential application in screening systems for IR-triggering microbes. In summary, microbe-IR should be considered as an efficient tool for the control of severe pests, such as *T. absoluta.* By boosting plant defenses microbe-IR can be a key complement to IPM programs for optimizing sustainable crop protection in future agriculture.

Author contributions

All authors have contributed to the manuscript substantially and have agreed to the final submitted version. Conceptualization, BRS, MJP and ZMI; Investigation, BRS and ZMI; Formal analyses, BRS, VF and ZMI; Writing—original draft, BRS, MJP and ZMI; Writing—review and editing, BRS, DG, MJP, RS, VF and ZMI; Funding acquisition, DG, MJP and RS; Supervision, MJP.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10340-025-01966-3.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This study was funded by the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 765290 in the project "MiRA – Microbe-induced Resistance to Agricultural Pests", project Intramural CSIC 202440E065 (PRIMOMICS), project PID2021-124813OB-C3 funded by MICIU/AEI /10.13039/501100011033 and by ERDF, EU, "ERDF A way of making Europe", project PDC2022-133600-C2 funded by MICIU/AEI /10.13039/501100011033 and by European Union Next Generation EU/ PRTR.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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