A root symbiont rewires tomato polyamine network to enhance herbivore resistance

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SUMMARY

- Plants rely on specialized metabolites to defend against herbivores, yet how root-associated symbiotic microbes influence their regulation, coordination, and overall contribution to defense remains widely unknown.
- We hypothesized that symbiotic microbes can reprogram specialized metabolic networks, thereby enhancing herbivore resistance, with a particular focus here on polyamine metabolic network. Using the fungal symbiotic endophyte *Trichoderma harzianum*, tomato, and the herbivore *Spodoptera exigua*, we combined greenhouse bioassays with molecular, genetic, and metabolomic analyses to investigate how *Trichoderma* symbiosis reshapes the plant polyamine metabolic network, and affects plant-herbivore interactions.
- We found that in the absence of the root symbiosis, herbivory primarily activated polyamine uptake transport and catabolism, reflecting a stress-driven turnover response. *Trichoderma* symbiosis markedly reconfigured the polyamine network. Symbiosis primed uptake transport and catabolic responses, enhanced polyamine flux through activation of the ornithine decarboxylase pathway, and redirected polyamines into conjugated metabolites with anti-herbivore activity. Genetic analyses confirmed that this metabolic rewiring contributes to *Trichoderma*-induced resistance to herbivory, linking primary metabolic routes to the accumulation of specialized defense compounds.
- Our study highlights root symbionts as key modulators of plant metabolism, showing that specialized metabolite diversity can be shaped by symbiotic interactions.

Keywords: Endophyte; herbivore resistance; polyamine metabolism; root symbiosis tomato defense; *Trichoderma* sp.

INTRODUCTION

Plants have coexisted and coevolved with insect herbivores for over 400 million years (Ehrlich & Raven, 1964; Labandeira & Currano, 2013). Over this vast evolutionary timespan, they have evolved a diverse arsenal of adaptive strategies to deter herbivory. Specialized metabolites including terpenoids, alkaloids, phenylpropanoids, fatty acid derivatives, and amino acid derivatives serve as pivotal chemical defenses in plant-herbivore interactions (Erb & Kliebenstein, 2020; Xu & Gaquerel, 2025). Their production relies on primary metabolic pathways that provide precursors, reducing power, and energy, resulting in tightly regulated biosynthesis (Li *et al*., 2024). Despite their recognized importance for plant fitness and survival, the ecological functions of many specialized metabolites in complex environments remain poorly understood (Huang & Dudareva, 2023), and the regulatory networks controlling their biosynthesis, transformation, and homeostasis are still largely unresolved (Kliebenstein, 2023).

Polyamines such as putrescine, spermidine, and spermine are involved in the regulation of critical plant processes, including growth, development and abiotic stress tolerance (Blázquez, 2024). More recently, they have further emerged as relevant regulators of plant defense against insect herbivory (Malik *et al*., 2014; Subramanyam *et al*., 2015; Zhang *et al*., 2023). These small nitrogen-containing molecules can act directly against herbivores and feed into the biosynthesis of diverse specialized defense compounds, linking primary and specialized metabolism (Alamgir *et al*., 2016; Kaur *et al*., 2010; Kerchev *et al*., 2012; Sivakumar *et al*., 2022; Zu *et al*., 2024). Polyamine levels are finely tuned through a dynamic network of biosynthesis, interconversion, transport, catabolism, and conjugation, enabling plants to adjust to developmental and environmental cues (Blázquez, 2024). In most plants, putrescine can be synthesized via the ornithine decarboxylase (ODC) or arginine decarboxylase (ADC) pathways providing metabolic flexibility (Cermanová *et al*., 2025). Downstream, spermidine and spermine are produced via aminopropyl transfer reactions, while catabolism mediated by diamine and polyamine oxidases maintains homeostasis and generates signaling molecules such as hydrogen peroxide (Salvi & Tavladoraki, 2020). Conjugation with phenylpropanoids produces phenolamides, which contribute to anti-herbivore defense (Gaquerel *et al*., 2014). Although polyamines and their derivatives are increasingly recognized as hallmarks of plant defense, the roles of individual network components and the mechanisms regulating their activity under complex ecological contexts remain unclear.

Plants also share an ancient coevolutionary history with symbiotic microbes, including mycorrhizal fungi and plant growth-promoting rhizofungi and rhizobacteria (Delaux & Schornack, 2021). These interactions, widespread across terrestrial ecosystems, are increasingly recognized as major drivers of plant metabolic diversity (Delaux & Schornack, 2021; de Vries & Feussner, 2024). Root symbionts can prime local and systemic defenses, enhancing resistance to pathogens and herbivores through microbe-induced resistance (MIR, Pieterse *et al*., 2014). MIR often reshapes the plant metabolome, boosting specialized metabolites including phenolamides, alkaloids, phenylpropanoids, glucosinolates, and terpenoids, which contribute to herbivore toxicity (Abbas

et al., 2024; Lv *et al*., 2024; Pangesti et al., 2016; Papantoniou *et al*., 2021; Rivero 2021, 2025; Van Hee *et al*., 2025). However, despite evidence of MIR-driven metabolic reprogramming, how root symbiosis influences the evolution, regulation, and coordination of specialized metabolic networks remains unresolved.

Experimental evidence indicates that plant polyamine metabolism, including biosynthesis, accumulation and conjugation can be modulated by functionally diverse root-associated mutualists, such as mycorrhizal fungi, *Trichoderma* spp., and plant growth promoting rhizobacteria (Brotman *et al*., 2012; Coppola *et al*., 2019; Minchev *et al*., 2026; Nikhil *et al*., 2024; Plett *et al*., 2020; Rivero *et al*., 2021; Salazar-Badillo *et al*., 2015). However, it remains largely unexplored how microbial symbioses influence the overall functioning of the tightly regulated polyamine metabolic network, and how modulation of specific network components contributes to the plant's adaptive responses to herbivory. We hypothesize that root symbiotic microbes adjust this coordinated network, enhancing the plant's capacity to respond to herbivore attack. Using the MIR-inducing symbiotic fungus *Trichoderma harzianum* (hereafter Trichoderma), we aimed to understand whether and how root symbiosis reshapes the polyamine network and how its key functional elements contribute to enhanced resistance against herbivory. We employed tomato (*Solanum lycopersicum*) and the herbivore *Spodoptera exigua*, combining greenhouse bioassays with molecular, genetic, and metabolomic approaches.

Our study shows that Trichoderma root symbiosis primes herbivore-induced polyamine uptake transport and catabolism, while simultaneously activating de novo biosynthesis via the ODC pathway and driving conjugation into putrescine-derived phenolamides with anti-herbivore activity. These findings reveal that root symbionts not only amplify existing defense responses but can also restructure metabolic pathways, expanding the diversity and complexity of the specialized metabolite landscape under herbivore pressure. Our work provides a mechanistic framework for understanding how symbiotic microbes can fine-tune plant specialized metabolism to enhance adaptation and resistance in complex ecological contexts.

MATERIAL AND METHODS

Plant, fungal and insect material

We used tomato (*Solanum lycopersicum*) cultivar Moneymaker. For functional analyses we used the RNA interference (RNAi) transgenic lines *SiADC, SiODC* (González-Hernández *et al*., 2022), and their corresponding parental Moneymaker line carrying the empty vector pBIN19-RNAi. Seeds were kindly provided by the Biochemistry and Biotechnology Group (Universitat Jaume I, Castellón, Spain). *Nicotiana benthamiana* plants were used for transient expression assays. The root endophytic fungus *Trichoderma harzianum* was maintained on potato dextrose agar at 4°C as part of our laboratory (MolEcoLab) collection. The *T. harzianum* inoculum was prepared according to Martinez-Medina *et al*. (2009). Batches from the same *Spodoptera exigua* (Lepidoptera: Noctuidae) laboratory colony were used for the different experiments (kindly provided by Prof. Herrero, Valencia University, Spain). Larvae were reared with artificial diet

(Hoffman *et al*., 1966) in a growth chamber maintained at 23 °C, 12 h/ 12 h diurnal photoperiod and 50 % relative humidity.

Plant growth and fungal inoculation and quantification

Tomato seeds were surface-sterilized in 5% NaClO4, rinsed thoroughly with sterile water, and germinated for 10 days in sterilized vermiculite at 25°C. Tomato seedlings were then transferred to 600 mL pots containing a sterile 1:1 mixture (v : v) of sand and vermiculite. They were assigned to one of the treatment groups: noninoculated plants, or plants inoculated with *T. harzianum*. Inoculation with *T. harzianum* was achieved by mixing the inoculum with the substrate to a final density of 1×10^6 conidia g^{-1} before transplanting (Papantoniou *et al*., 2021). The plants were then placed in a completely randomized design in a glasshouse compartment with a 24 ± 3°C, 16-h light and 8-h dark cycle, and 60% relative humidity. The plants were watered three times a week: 2 days using tap water and 1 day using Hoagland nutrient solution (Hoagland & Arnon, 1950). Five weeks after transplanting, the plants were used for experiments. Before using the plants, we confirmed that *T. harzianum* had efficiently colonized the potting substrate of the corresponding treatments by using the plate count technique and potato dextrose agar amended with 50 mg L^{-1} rose bengal, and 100 mg L^{-1} streptomycin sulphate according to Papantoniou *et al*. (2021). The number of *T. harzianum* CFU in the potting media of *T. harzianum*-inoculated plants was similar to the initial inoculation values (i.e., 1×10^6 conidia g^{-1}). *T. harzianum* endophytic colonization was confirmed in surface-sterilized roots for all the tomato lines according to Martínez-Medina *et al.* (2017), and using the specific primers Tef1-F: 5′-GGTACTGGTGAGTTCGAGGCTG-3′ and Tef1-R: 5′- GGGCTCGATGGAGTCGATAG3′, which are specific for the constitutively expressed *Trichoderma* translation elongation factor-1α gene. For *N. benthamiana* plants, seeds were germinated for 10 days in peat at 24°C. Seedlings were then transferred to 600 mL pots containing a sterile 1:1 (v : v) mixture of peat and vermiculite. Plants were randomly distributed for each treatment and maintained in a growth chamber at 24 ± 3°C, 16-h light and 8-h dark cycle, and, 60% relative humidity. The plants were watered three times a week: 2 days using tap water and 1 day using Hoagland nutrient solution (Hoagland & Arnon, 1950).

Transient *PHT2* overexpression

The plasmid pUC57_*SlPHT2* (GeneScript Biotech Corporation), harboring the *SlPHT2* coding sequence flanked by attL recombination sites, was used as entry clone for a Gateway™ LR recombination reaction (Invitrogen) with the binary destination vector pGWB2, generating a construct for constitutive overexpression under the CaMV 35S promoter (Nakagawa *et al*., 2007). The resulting expression plasmid (Agro::PHT2), along with the empty pGWB2 vector used as control (Agro-inoculated), was introduced into *Agrobacterium tumefaciens* strain GV3101:pmp90 by electroporation. Transformed strains were incubated overnight at 28 °C with continuous shaking. Cells were then collected by centrifugation and resuspended in infiltration buffer (10 mM MES, pH 5.7; 10 mM $MgCl_2$; and 10 μM acetosiryngone). Final suspensions were adjusted to an

OD_{600} of 0.3 and used to infiltrate the fifth and sixth fully expanded leaves (counting from the base) of five-week-old *N. benthamiana* plants with a 2 mL needleless syringe. 48 h after the inoculation the plants were used for the experiments (Voinnet *et al.,* 2003).

Virus-induced *PHT1/PHT2* silencing

Tobacco rattle virus (TRV)-based silencing vectors pTRV1, pTRV2::00 (empty vector), and pTRV2::*SlPDS* were kindly provided by Dr. Rodríguez Bejarano (University of Málaga, Spain). To silence *PHT1/2* genes, a 302 bp gene-specific fragment was amplified using a GeneScript plasmid containing the full-length *PHT2* sequence (pUC57_SlPHT2) as a template (GenBank: ON248951.1; Solgenomics: Solyc11g071480). Target selection was performed using the VIGS tool available on the Sol Genomics Network (http://vigs.solgenomics.net) choosing a region with high distributions of n-mers in both genes and none off-targets in the other two *PHT* genes (*PHT3* and *PHT4*), using the specific primers for Construct 1 listed in Table S1. The amplified fragment was digested using *EcoRI* and *XhoI* restriction enzymes and ligated into the pTRV2 vector also digested with the same enzymes. To corroborate the observed phenotypes, all experiments were independently repeated using a second, redesigned silencing construct (Construct 2, Table S1). pTRV1, pTRV2::00, pTRV2::SlPDS, and pTRV2::*PHT1/2* were introduced into *Agrobacterium tumefaciens* strain GV3101 pmp90 through electroporation. Transformed strains were cultured in Luria–Bertani (LB) medium supplemented with 10 mM MES and 20 µM acetosyringone at 28°C for 24 h, with appropriate antibiotics. Bacterial cultures were harvested by centrifugation and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.7, 100 µM acetosyringone) to an optical density at 600 nm (OD_{600}) of 0.5. Suspensions were incubated in darkness at room temperature for 2–3 h prior to infiltration. Equal volumes of *Agrobacterium* cultures containing pTRV1 and pTRV2 derivatives were mixed at a 1:1 ratio before plant inoculation. Tomato seedlings at the two-true-leaf stage were infiltrated with the *Agrobacterium* suspension at the axillary region of the stem using a 1 mL syringe fitted with a needle (Pastor-Fernandez *et al.,* 2024). Fourteen days after inoculation, the plants were used for experiments.

Plant infestation and assessment of herbivore performance

Five weeks after transplanting tomato plants, the corresponding plants were infested with *S. exigua* larvae. Infestation of tomato plants was achieved by placing five second-instar larvae on the apical leaflet of the third fully expanded leaf (counted from the top). Larvae were confined using a clip cage, and empty clip cages were placed on the leaves of uninfested plants, according to Rivero *et al*. (2025). After one day of herbivory, larvae were removed from the plants and reserved. Damaged apical leaflets were individually harvested and stored at −80°C for subsequent analysis. For undamaged plants, leaf material collection was performed similarly. Following leaf sampling, all larvae corresponding to the same plant were immediately returned to the same plant, to the following leaflet of the same leaf that they were previously feeding on. The entire leaf was enclosed using a mess bag, and larvae were allowed to feed *ad libitum* on the entire leaf. Larvae were removed and their weight and mortality were recorded periodically during

the time of the bioassay. Larvae were returned to the same plant, on one leaf above the one they were previously feeding on, to ensure that larvae had enough food during the entire bioassay.

For *N. benthamiana* plants at 48 h post-infiltration, one of the two infiltrated leaves was harvested and flash-frozen in liquid nitrogen for gene expression and metabolite analyses. The other leaf was used for herbivore bioassays. Five second-instar *S. exigua* larvae were placed directly on the infiltrated leaf and confined using a fine-mesh exclusion bag to prevent larval escape. Larval weight was recorded periodically. When the leaf material was depleted, larvae were carefully transferred to a new *N. benthamiana* plant that had been infiltrated with the same construct at the same time point, ensuring uniform expression levels.

Simulated herbivory treatment

We performed two rows of punctures onto each side of the midvein of the apical leaflet of the third fully expanded leaf (counted from the top) using a fabric pattern wheel. Inmediatley, 5 µL of a 5 mM (Mes-NaOH, pH 6.0) dilution of *S. exigua* oral secretion (OS) was applied to the puncture wounds with a gloved finger. Oral secretions from third instar larvae were collected with a pipette according to (Bricchi *et al*., 2013), pooled and kept at −20°C until use. The OS quantity was adjusted after several trials.

Bioassay on artificial diet

Caffeoylputrescine (BLDpharm, Germany) and feruloylputrescine (TargetMol, United States) were purchased to test their direct effect on *S. exigua* performance. Both compounds were incorporated, separately or together, into the artificial diet (Hoffman *et al*., 1966) at a final concentration of 1 µg mL^{-1} (Rivero *et al*. 2021). Four different diet formulations were prepared: artificial diet supplemented with caffeoylputrescine (CP), artificial diet supplemented with feruloylputrescine (FP), artificial diet supplemented with both feruloylputrescine and caffeoylputrescine (FP+CP), and a control diet supplemented with acidified Milli-Q water (H2Oac, 0.01 % glacial acetic acid), corresponding to the solvent used for compound dissolution. Thirty second-instar *S. exigua* larvae were assigned to each diet treatment and reared under controlled conditions in a growth chamber maintained at 23 °C, with a 12 h light/12 h dark photoperiod and 50% relative humidity. Larval performance was assessed by monitoring larval weight over a period of 6 days.

RNA extraction and gene expression analysis

Total RNA from fresh leaves was extracted by using the RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 0.5 µg using PrimeScript RT reagent kit (Takara). Quantitative PCR was conducted using the SYBR PREMIX EX TAQ (Takara) following the manufacturer’s instructions and a thermal cycler (Bio-RAD, CFX Opus 384) with the following cycling program: 30 sec 95°C, 40 cycles of 5 sec 95°C and 60°C 34 sec, followed by a dissociation process of 95°C 15 sec, 60°C 1 min and 95°C 15 sec. The sequences of the gene-specific primers are shown in Table S2.

Before normalization, we analyzed the expression of three different tomato housekeeping genes under the different conditions of our study: actin (Solyc03g078400), β-tubulin (Solyc04g081490), and elongation factor 1-α (Solyc06g005060). We used the Normfinder software (https://moma.dk/normfinder-software) to find the optimal normalization gene. According to the results, expression values were normalized using the housekeeping gene elongation factor 1-α (*EF-1α*). The normalized data were further processed by the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001).

Measurement of arginine decarboxylase and ornithine decarboxylase enzymatic activities

Arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) enzymatic activities were determined according to Wu *et al*. (2012). Briefly, 0.2 g of fresh plant tissue was homogenized in extraction buffer (0.1 M phosphate buffer, pH 5.6, 5 mM EDTA, 1 mM pyridoxal phosphate, 0.01 mM polyvinyl pyrrolidone, 10 mM dithiothreitol (DTT), and 0.43 mM sodium thiosulfate). The homogenate was centrifuged at 12.000 *g* at 4 °C for 40 min. A volume of 0.8 mL of the supernatant was mixed with 1 mL of reaction mix (0.1 M Tris-HCl buffer, pH 7.5, 5 mM EDTA, 40 µM pyridoxal phosphate, and 5 mM DTT). To assess enzymatic activity, 0.2 mL of either 25 mM L-Arg (for ADC) or 25 mM L-Orn (for ODC) was added, and the mixture was incubated at 37°C for 60 min. Control reactions in which the substrate was substituted with perchloric acid, were included to determine background activity. Following incubation, 0.5 mL of the sample was mixed with 1 mL of 2 M NaOH and 10 µL benzoyl chloride. The mixture was stirred for 20 s, and incubated for 30 min at 37°C. Subsequently, 2 mL of saturated NaCl and 2 mL of 100% ether were added, followed by a centrifugation at 1500 *g* for 5 min at 4°C. A 1 mL aliquot of the ether phase was collected, evaporated at 50 °C in a water bath, and the remainder was dissolved in 1 mL of 100% methanol. The benzoylated polyamine derivatives were quantified at 254 nm using a GeneQuant 1300 spectrophotometer (Harvard Bioscience Inc., Holliston, MA, USA). Enzyme activity was calculated based on the increase in absorbance relative to control samples. One unit of enzymatic activity was defined as an increase of 1.0 absorbance unit at 254 nm during 1 min.

Determination of free polyamines content

The content of free putrescine (Put), spermidine (Spd) and spermine (Spm) in leaf extracts was measured as described in Ariz *et al*. (2013) with some modifications. Fresh plant tissue was ground with liquid nitrogen and homogenized in 5% aqueous $HClO_4$ (w/w) at a ratio of 10:1 (v/w), supplemented with an internal standard solution (2 mM 1,6-hexanediamine) at 0.1:1 (v/w). Homogenates were shaken, incubated for 1 h at 4°C, and centrifuged at 15 000 *g* for 15 min at 4°C. Aliquots (0.2 mL) of the supernatant were mixed with 0.4 mL of 3 M aqueous Na_2CO_3 and 0.4 mL of 0.12 M dansyl chloride in acetone, followed by incubation at 60°C in darkness for 1 hour. To stop the reaction, 0.1 mL of 0.87 M proline were added and the mixture was further incubated for 30 min. Dansylated polyamines were extracted using 5 Ml of ethyl acetate, centrifuged at 3000 *g* for 5 min at room temperature and the organic phase was collected and evaporated under reduced pressure at 40°C. The residue was resuspended in 0.5 ml of methanol

and filtered through a 0.45 µm nylon filter. Free polyamine separation and quantification were carried out by high-performance liquid chromatography (HPLC) using a Waters system (616 pump, 717 Plus Autosampler, and 2475 Fluorescence Detector; Waters, Milford, MA, USA). A Tracer Excel 120-ODS-A column (3 µm, 4.6 × 150 mm; Teknokroma, Barcelona, Spain) maintained at 30°C was used as the stationary phase for separation. The mobile phase consisted of solvent A (water) and solvent B (methanol), with a constant flow rate of 0.5 mL min^{-1}. An increasing concentration gradient was applied, solvent B going from 58% to 100% over 44 min, held for 4 min, then returned to 58% over 3 min, and equilibrated for an additional 3 min. Fluorescence detection was performed at an λ_{ex} of 350 nm and an λ_{em} of 515 nm. Quantification of polyamines was performed using an internal standard and calibration curves prepared with authentic standards of Put, Spd, and Spm.

Determination of hydrogen peroxide content

The content of hydrogen peroxide was determined by ferric thiocyanate method after H_2O_2 extraction from 200 mg of leaf homogenized in cold with 2 mL of 0.1% TCA. The samples were centrifuged at 4^0C for 15 min at 17100 *g*. The H_2O_2 concentration was determined in the supernatant using a modification of the ferric thiocyanate method (Sagisaka *et al*., 1976). A hydrogen peroxide solution was used to setup a standard curve.

Liquid chromatography and electro-spray ionization (LC-ESI) mass spectrometry

Extraction of metabolites from freeze-dried leaves (20 mg) was performed by using MeOH:H2O (10:90) containing 0.01% of HCOOH, as described by Rivero *et al*. (2021). Extracts were injected into an Acquity ultra-performance liquid chromatography system (UPLC; Waters, Mildford, MA, USA), which was interfaced with a hybrid quadrupole time-of-flight equipment (Q-TOF-MS Premier, Waters, Mildford, MA, USA). Analytes were eluted with an aqueous methanol gradient containing 0.01% HCOOH. Solvent gradients and further chromatographic conditions were performed as previously described (Agut *et al*., 2014). LC separation was performed using an ultra-performance liquid chromatography (UPLC) Kinetex C18 analytical column with a 5 µm particle size, 2.1 × 100 mm (Phenomenex, Madrid, Spain). To accurately identify the signals detected, a second fragmentation function was introduced into the TOF analyser. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a fragmentation spectrum of each analyte (Agut *et al*., 2014; Gamir *et al*., 2014). Ornithine and arginine were identified by using commercial standards. To specifically identify phenolamides, we focused our analysis in hydroxycinnamic acid conjugates of polyamines, using the retention times and mass fragmentation patterns of reference compounds for caffeoylputrescine and feruloylputrescine, as well as the characteristic mass fragmentation of feruloyldicoumaroylspermidine.

Statistical analysis

Statistical analyses were performed using R software (R version 4.4.3) in RStudio (v1.4.1564). Data normality and homogeneity of variances were assessed using the Shapiro–Wilk and

Levene's tests, respectively. Kaplan–Meier survival curves were generated for herbivore performance assays using the *survival* and *survminer* packages. One-way ANOVA was used to analyze larval weight datasets, with pairwise comparisons conducted using Student's *t*-test ($P \leq 0.05$). Two-way ANOVA models were applied to evaluate the effects of Trichoderma treatment and genotype on larval weight data; and to evaluate the effects of herbivory (or herbivory mimic) and Trichoderma treatment on gene expression and metabolite. Tukey's test ($P \leq 0.05$) was used for overall comparisons among treatment groups, and *t*-tests were used for pairwise comparisons. All graphical outputs were generated using the *ggplot2* package. All experiments were repeated at least twice, with similar results.

RESULTS

Trichoderma symbiosis reduces the performance of *Spodoptera exigua*

We first tested whether Trichoderma symbiosis modulates tomato resistance to *S. exigua*. In greenhouse bioassays, larvae fed on Trichoderma-inoculated plants gained significantly less weight compared to those on noninoculated plants (Fig. 1A), whereas overall larval survival was not affected (Fig. 1B). Notably, during the first 6 days of herbivory larvae showed a marginally lower probability of survival on Trichoderma-inoculated plants (Cox regression, days 1-3: exp(coef) = 4.271, P=0.08). Trichoderma inoculation did not affect plant growth (Fig. S1). These results indicate that Trichoderma symbiosis reduces the performance of *S. exigua*, primarily by impairing larval weight gain.

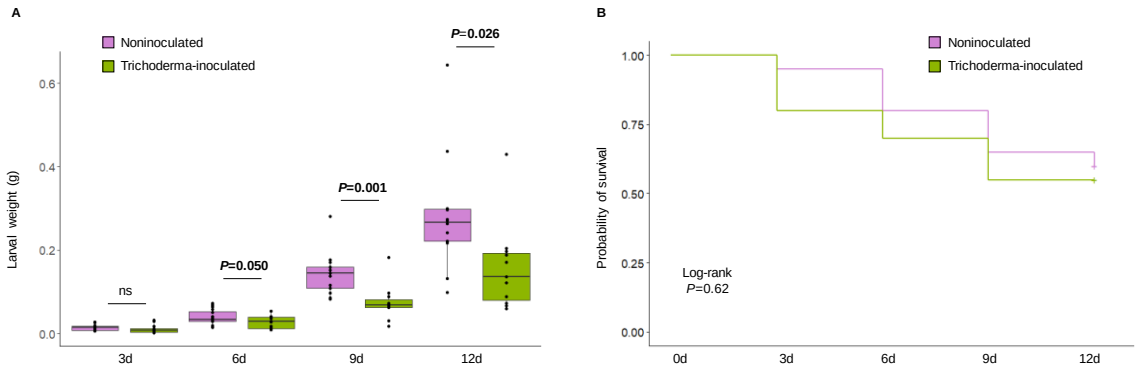

Figure 1. Impact of *Trichoderma harzianum* root colonization on *Spodoptera exigua* larval performance. (A) Larval weight and (B) survival probability were periodically assessed for *S. exigua* feeding on leaves from tomato plants root inoculated or not with *T. harzianum*. (A) Boxplots represent the interquartile range (IQR); horizontal lines indicate medians, whiskers indicate 1.5 times the IQR, and dots represent individual biological replicates (n = 15-20). *P* values (bold, $P < 0.05$) indicate results of *t*-tests between inoculated and noninoculated plants at each time point after infestation: 0d, 3d, 6d, 9d, and 12d. (B) Kaplan-Meier survival curves (n = 20 per treatment). Significant differences between treatments were determined using log-rank tests. The experiment was repeated three times, yielding similar results.

Trichoderma symbiosis activates the ODC-branch of the polyamine biosynthesis pathway following herbivory

Polyamine biosynthesis in tomato proceeds through two main branches: the ODC branch and ADC branch (Fig. S2). In the ODC branch, herbivory in noninoculated plants did not modify the transcript levels of *ORNITHINE DECARBOXYLASE 1/2* (*ODC1*, *ODC2*); and Trichoderma colonization alone, also had not effect on *ODC1*, *ODC2* expression (Fig. 2A, Table S3). By contrast, in Trichoderma-inoculated plants herbivory induced *ODC1* and *ODC2*, leading to significantly higher transcript levels compared to non-challenged plants (Fig. 2A, Table S3). Along similar lines, following herbivory, Trichoderma-inoculated plants displayed elevated ODC enzymatic activity (Fig. 2B) and higher ornithine levels (Fig. 2C), compared to noninoculated plants. Together, these results indicate that in response to herbivory, Trichoderma symbiosis activates the ODC pathway.

The ADC branch was only marginally affected. Neither herbivory nor Trichoderma inoculation significantly altered the expression of *ARGININE DECARBOXYLASE 1/2* (*ADC1*, *ADC2*) (Fig. 2D, Table S3), though a weak trend toward higher expression of *ADC1* was observed in Trichoderma plants under herbivory. Similarly, *AGMATINE IMINOHYDROLASE* (*AIH*) and (*N-CARBAMOYLPUTRESCINE AMIDOHYDROLASE* (*CPA*) transcripts (Fig. 2E, Table S3), ADC enzymatic activity (Fig. 2F), and arginine content (Fig. 2G) were unaffected. Noticeably, Trichoderma symbiosis enhanced *ARGINASE2* (*ARG2*) expression upon herbivory (Fig. 2H, Table S3), whereas *ARGINASE1* (*ARG1*) remained unchanged (Fig. S3). *ARG2* encode a jasmonate- and wound-inducible arginase that catalyzes the conversion of arginine into ornithine the substrate of the ODC branch, thereby potentially fueling ODC-mediated polyamine biosynthesis (Chen *et al*., 2024). These results indicate that following herbivory, Trichoderma activates de novo polyamine biosynthesis, a response that was no evident in noninoculated plants. Our results further demonstrate that this activation is primarily mediated through the ODC branch.

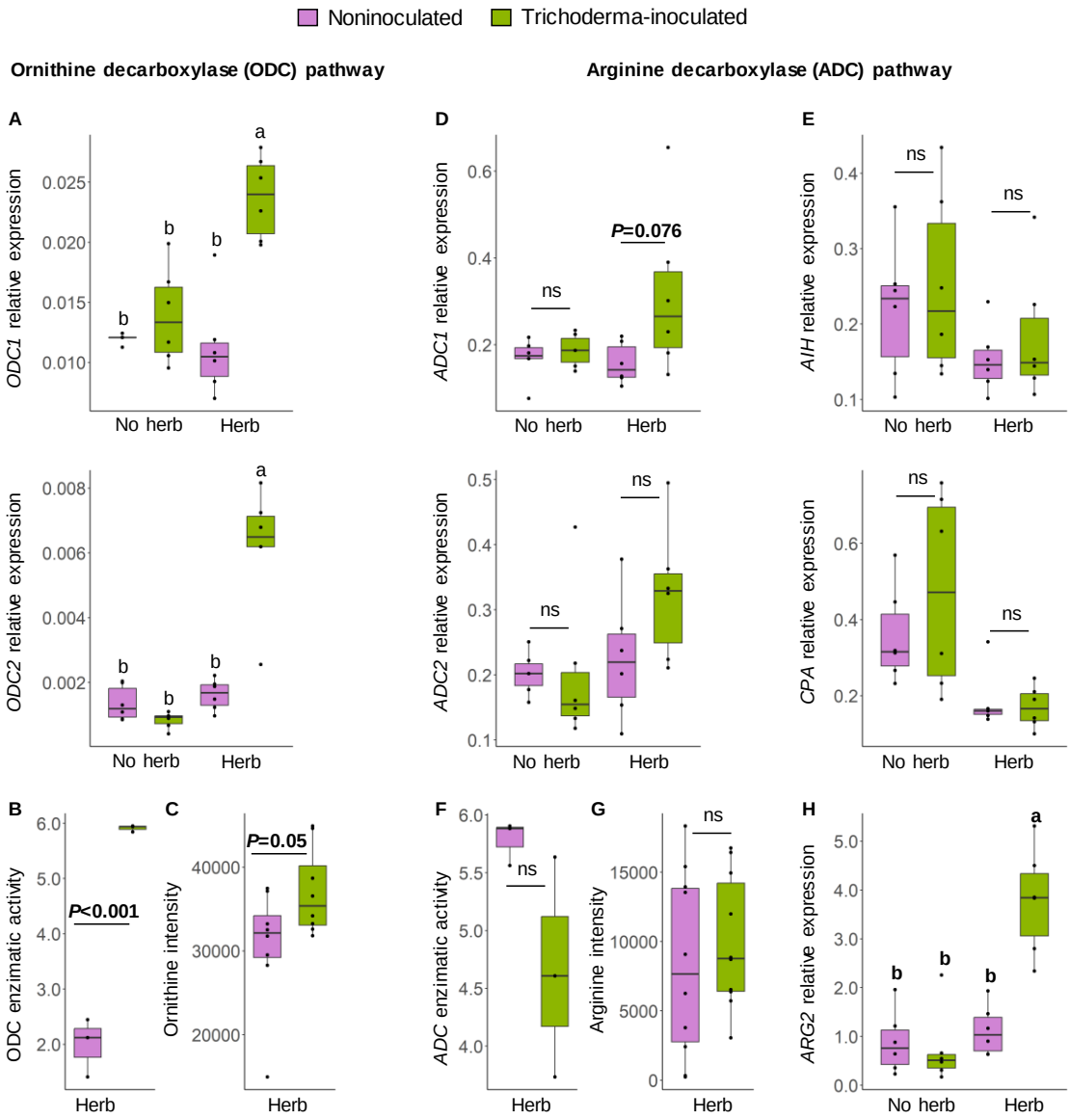

Figure 2. Effect of *Trichoderma harzianum* root colonization and *Spodoptera exigua* herbivory on ODC- and ADC-branch of polyamine biosynthesis pathway. (A, B, C) Effect of *T. harzianum* on the ornithine decarboxylase (ODC) pathway: (A) transcript levels of ORNITHINE DECARBOXYLASE 1/2 (*ODC1* and *ODC2*); (B) ornithine decarboxylase enzymatic activity; and (C) ornithine content. (D, E, F, G, H) Effect on the arginine decarboxylase (ADC) pathway: (D) transcript levels of ARGININE DECARBOXYLASE 1/2 (*ADC1* and *ADC2*); (E) transcript levels of *AGMATINE IMINOHYDROLASE* (*AIH*) and *N-CARBAMOYLPUTRESCINE AMIDOHYDROLASE* (*CPA*); (F) arginine decarboxylase enzymatic activity; (G) arginine levels; and (H) *ARGINASE 2* (*ARG2*) transcript levels. Plants were inoculated or not with *T. harzianum* and analyzed under control conditions or after 1 day of *S. exigua* feeding. Boxplots represent the IQR; horizontal lines indicate medians, whiskers 1.5 times the IQR, dots individual biological replicates (n = 6-10). Different letters indicate significant differences (Tukey's post hoc test, $P < 0.05$), and P values indicate significant t-test results ($P < 0.05$). ns, non-significant. The experiment was repeated twice, yielding similar results.

The ODC-branch is required for Trichoderma-induced resistance against herbivory

To determine the relative contribution of the ODC- and ADC-branch to Trichoderma-induced resistance, we performed bioassays using RNAi lines impaired in each branch (*SiODC* and *SiADC*; González-Hernández *et al*., 2022). In empty vector (EV) plants, Trichoderma significantly reduced larval weight gain, with effects evident at 6 and 9 days of herbivory (Fig. 3, Table S3). Trichoderma-mediated protective effect was completely abolished in the *SiODC* line, where larvae grew equally well on inoculated and noninoculated plants (Fig. 3, Table S3). By contrast, resistance was only partially lost in *SiADC* plants: Trichoderma inoculation reduced larval weight at early time points (day 3), but the effect disappeared at later stages (days 6 and 9) (Fig. 3, Table S3). Importantly, *T. harzianum* colonization was confirmed in silenced and EV plants at comparable levels. Altogether, these findings indicate that polyamine biosynthesis is required for Trichoderma-induced resistance, with the ODC branch being required under our assay conditions, and the ADC branch contributing just partially.

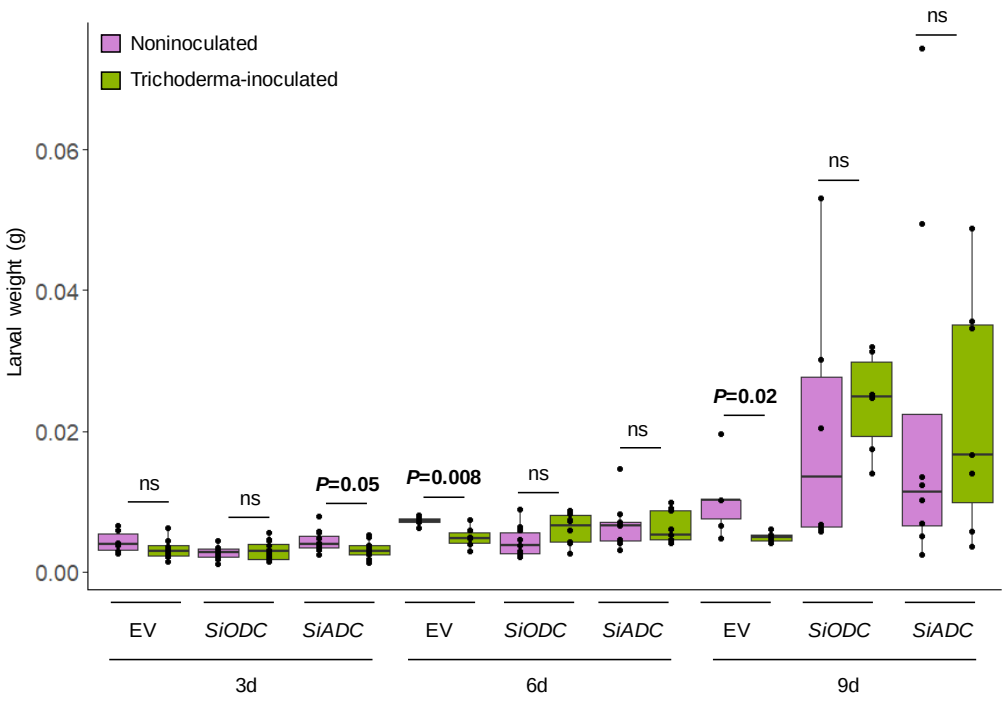

Figure 3. Role of the ODC- and ADC-branch of polyamine biosynthesis in Trichoderma-induced resistance to *Spodoptera exigua* herbivory. Larval weight was periodically measured for *S. exigua* feeding on leaves of tomato lines silenced for *ORNITHINE DECARBOXYLASE* (*SiODC*) or *ARGININE DECARBOXYLASE* (*SiADC*), and empty vector controls (EV), either inoculated or not with *T. harzianum*. Boxplots represent the IQR; horizontal lines indicate medians, whiskers 1.5 times the IQR, and dots individual biological replicates (n = 6-10). *P* values indicate significant *t*-test results ($P < 0.05$) comparing inoculated and noninoculated plants for each line and time point after infestation: 3d, 6d and 9d. ns, non-significant. The experiment was repeated twice, yielding similar results.

Trichoderma symbiosis limits herbivory-induced free polyamine accumulation without altering interconversion

We next explored the impact of Trichoderma symbiosis and herbivory on free polyamine pool, including putrescine, spermidine and spermine. In noninoculated plants, herbivory did not affect putrescine but increased spermidine and spermine (Fig. 4A, Table S3). Strikingly, in Trichoderma plants, herbivory did not trigger free polyamine accumulation (Fig. 4A, Table S3). Moreover, following herbivory, a significantly lower accumulation of putrescine and spermidine was observed in Trichoderma plants compared to noninoculated plants (Fig. 4A, Table S3). Thus, the activation of polyamine biosynthesis by Trichoderma symbiosis was not accompanied by enhanced accumulation of free polyamines, suggesting downstream mobilization.

Polyamine interconversion was not significantly altered by Trichoderma symbiosis. In noninoculated plants, herbivory upregulated *SPERMIDINE SYNTHASE* (*SPDS*, Factor Herbivory $P<0.001$, Table S3), whereas *SPERMINE SYNTHASE* (*SPMS*) remained unaffected (Fig. 4B, Table S3). Herbivory also enhanced *S-ADENOSYLMETHIONINE DECARBOXYLASE 1* (*SAMDC1*, Factor Herbivory $P< 0.005$, Table S3) whose encoded enzyme provides aminopropyl donors for spermidine and spermine synthesis. Trichoderma inoculation did not significantly affect *SPDS*, *SPMS*, or *SAMDC1* (Fig. 4B, Table S3). These results indicate that Trichoderma suppresses free polyamine accumulation during herbivory without affecting interconversion, pointing to alternative metabolic fates.

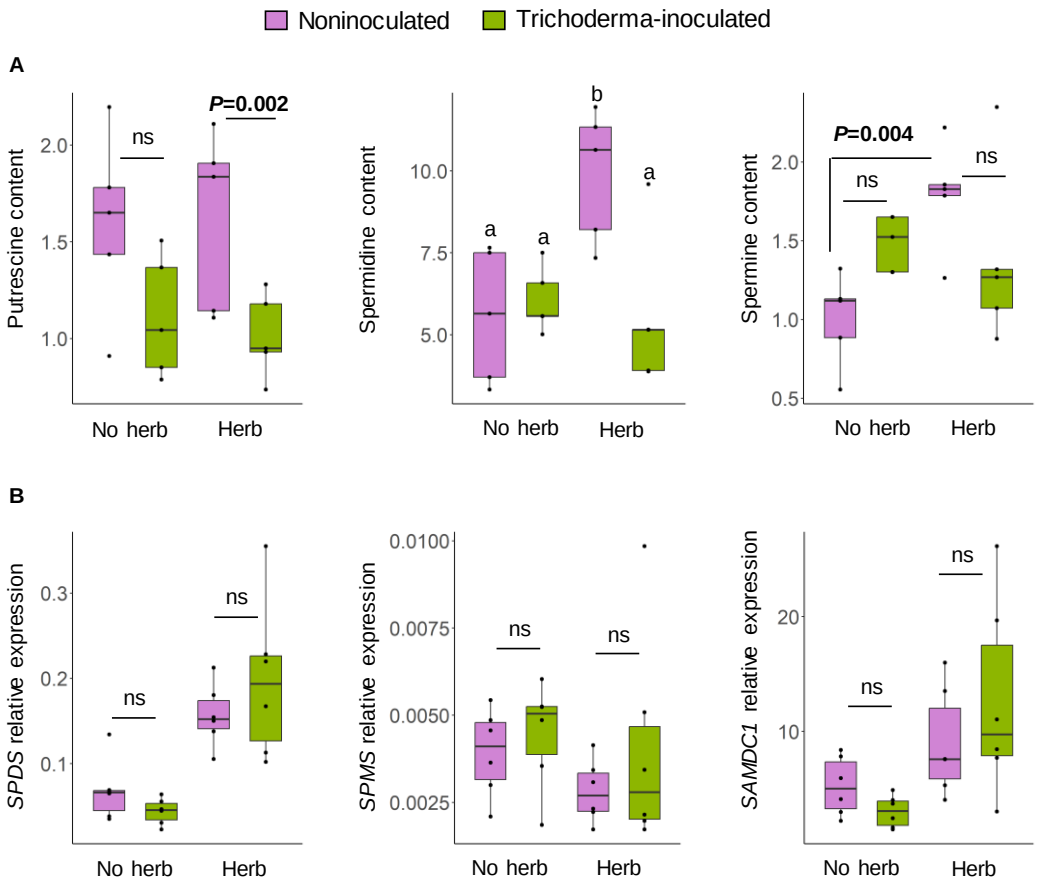

Figure 4. Impact of *Trichoderma harzianum* root colonization and *Spodoptera exigua* herbivory on free polyamine levels and interconversion. (A) Free polyamine content: putrescine, spermidine, and spermine. (B) Expression of polyamine interconversion-related genes: *SPERMIDINE SYNTHASE* (*SPDS*), *SPERMINE SYNTHASE* (*SPMS*), and *S-ADENOSYLMETHIONINE DECARBOXYLASE 1* (*SAMDC1*). Plants were inoculated or not with *T. harzianum* and analyzed under non-herbivory or 1-day herbivory conditions. Boxplots represent the IQR; horizontal lines indicate medians, whiskers 1.5 times the IQR, and dots individual biological replicates (n = 5–6). Different letters indicate significant differences (Tukey's post hoc test, $P < 0.05$). *P* values indicate significant *t*-test results ($P < 0.05$). ns, non-significant. The experiment was repeated twice, yielding similar results.

Trichoderma symbiosis enhances herbivore-induced polyamine uptake transport and catabolism

We analyzed key genes related to polyamine transport and catabolism. In noninoculated plants, herbivory induced *PUTRESCINE UPTAKE TRANSPORTER 3* (*PUT3*). This effect was significantly amplified in Trichoderma-inoculated plants (Fig. 5A, Table S3), suggesting that Trichoderma symbiosis reinforces putrescine mobilization during herbivore attack. By contrast, expression of *DIAMINE OXIDASES 1/2/3* (*DAO1-3*) involved in putrescine catabolism, was unaffected by either treatment (Fig. 5B, Table S3), indicating that this branch of putrescine turnover remained unchanged.

Herbivory increased the expression of *POLYAMINE OXIDASE 1* (*PAO1*, Factor Herbivory *P*<0.001, Table S3) but not *POLYAMINE OXIDASE 2* (*PAO2*, Fig. 5C, Table S3). Remarkably, herbivore-triggered enhancement of *PAO1* was stronger in Trichoderma plants, compared to noninoculated plants, while Trichoderma did not affect *PAO2* (Fig. 5C, Table S3). PAO1 functions as a spermidine oxidase, producing hydrogen peroxide (H_2O_2) and other reactive byproducts (Hao *et al*., 2018). Consistently, an herbivory mimic treatment (mechanical wounding plus *S. exigua* oral secretions) triggered H_2O_2 production (Factor Herbivory *P*< 0.001, Table S3), which was significantly higher in Trichoderma-inoculated plants (Fig. 5D, Table S3). Together, these findings indicate that Trichoderma symbiosis enhances herbivore-induced *PUT3* expression and PAO1-mediated catabolism, likely redirecting free polyamines toward oxidative turnover products, including H_2O_2.

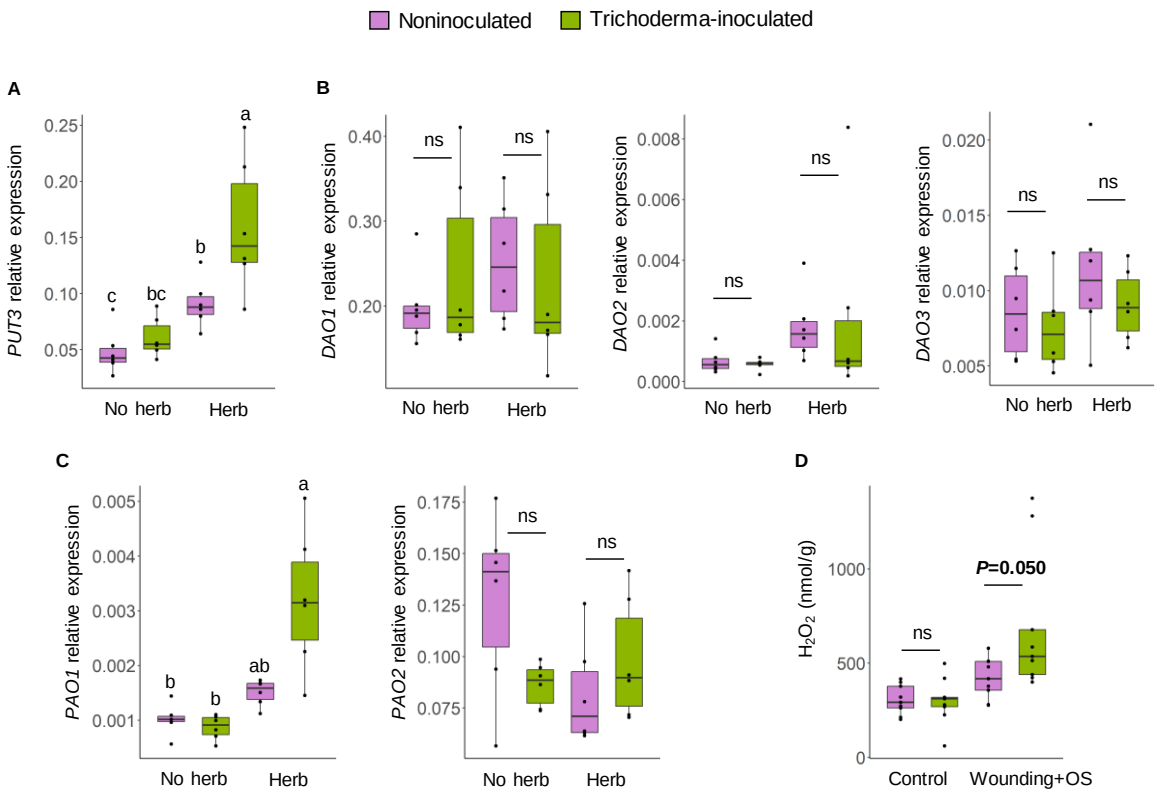

Figure 5. Effect of *Trichoderma harzianum* root colonization and *Spodoptera exigua* herbivory on polyamine uptake transport and catabolism. Transcript levels of (A) *PUTRESCINE UPTAKE TRANSPORTER 3* (*PUT3*), (B) *DIAMINE OXIDASE 1/2/3* (*DAO1*, *DAO2* and *DAO3*), and (C) *POLYAMINE OXIDASES 1/2* (*PAO1* and *PAO2*). (D) Hydrogen peroxide levels. Plants were inoculated or not with *T. harzianum* and analyzed under non-herbivory conditions or after 1 day of *S. exigua* feeding (in A-C), or 20 min after simulated herbivory (wounding + oral secretion, OS, in D). Boxplots represent the IQR; horizontal lines indicate medians, whiskers 1.5 times the IQR, dots individual biological replicates (n = 6-10). Different letters indicate significant differences (Tukey's post hoc test, *P* < 0.05). *P* values indicate significant *t*-test results (*P* < 0.05). ns, non-significant. The experiment was repeated twice, yielding similar results.

Trichoderma symbiosis triggers the biosynthesis and accumulation of putrescine-derived phenolamides

Polyamine conjugation with hydroxycinnamic acids represents a key metabolic branch regulating polyamine homeostasis. In noninoculated plants, herbivory specifically induced the expression of *PUTRESCINE HYDROXYCINNAMOYL TRANSFERASE 2* (*PHT2*) but not *PUTRESCINE HYDROXYCINNAMOYL TRANSFERASE 1/3* (*PHT1*, *PHT3*), or SPERMIDINE HYDROXYCINNAMOYL TRANSFERASE (*SHT*) (Fig. 6A, Table S3). In tomato, an additional member of this family, *PUTRESCINE HYDROXYCINNAMOYL TRANSFERASE 4* (*PHT4*), is present, but it appears transcriptionally inactive in foliar tissue under the conditions tested. Strikingly, in Trichoderma-inoculated plants, herbivory elicited a stronger induction of both *PHT1* and *PHT2* compared to noninoculated plants. Moreover, Trichoderma symbiosis enhanced *PHT1* expression even in the absence of herbivory, whereas *PHT3* and *SHT* remained unaffected (Fig. 6A, Table S3).

Metabolite profiling identified two main polyamine conjugates: caffeoylputrescine (CP) and feruloylputrescine (FP), that were confirmed by standards (Fig. 6B, Fig. S4). We further identified feruloyldicoumaroylspermidine, putatively annotated based on MS fragmentation patterns (Fig. S4). In noninoculated plants, herbivory did not significantly alter CP levels, but decreased FP accumulation (Factor Herbivory, $P < 0.001$, Table S3) (Fig. 6B, C; Table S3). Remarkably, upon herbivore challenge, Trichoderma symbiosis led to enhanced accumulation of CP and FP compared with noninoculated plants. Furthermore, Trichoderma increased FP levels even in the absence of herbivory (Fig. 6B). Together, these results demonstrate that Trichoderma symbiosis activates the conjugation branch of the polyamine metabolic network, promoting both constitutive and herbivore-induced accumulation of putrescine-derived phenolamides, particularly CP and FP.

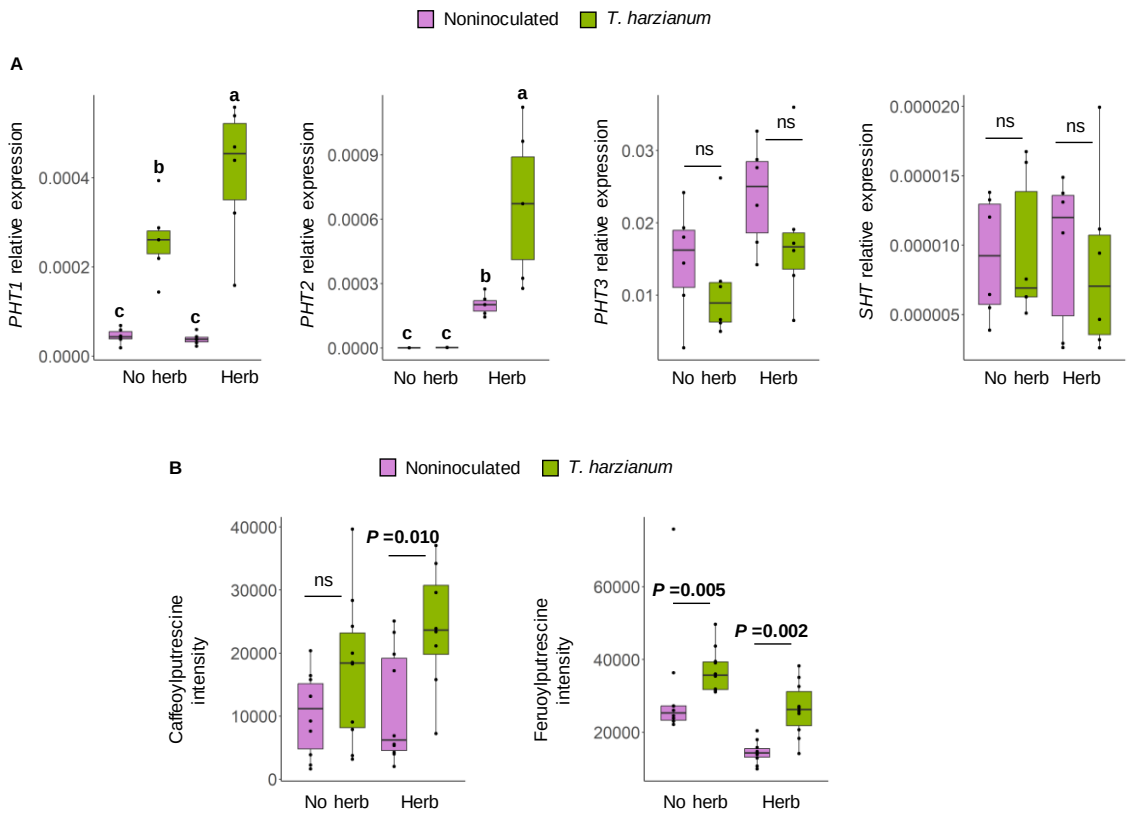

Figure 6. Impact of *Trichoderma harzianum* root colonization and *Spodoptera exigua* herbivory on phenolamide biosynthesis. (A) Transcript levels of polyamine conjugation-related genes *PUTRESCINE HYDROXYCINNAMOYLTRANSFERASE 1/2/3* (*PH1*, *PHT2*, *PHT3*), and *SPERMIDINE HYDROXYCINNAMOYL TRANSFERASE* (*SHT*). (B) Intensity level of the polyamine conjugates caffeoylputrescine and feruloylputrescine from leaf samples analyzed by liquid chromatography-mass spectrometry (LC-MS). Plants were inoculated or not with *T. harzianum* and analyzed under non-herbivory conditions or after 1 day (in A) and 2 days (in B) of *S. exigua* feeding. Boxplots represent the interquartile range (IQR); horizontal lines indicate medians, whiskers 1.5 times the IQR, and dots individual biological replicates (n = 6-10). Different letters indicate significant differences according to Tukey's post hoc test (P < 0.05). (*P* < 0.05). *P* values (bold, *P* < 0.05) indicate *t*-test results between inoculated and control plants. ns, non-significant. The experiment was repeated twice, yielding similar results.

The biosynthesis and accumulation of putrescine-derived phenolamides contributes to *S. exigua* performance

We next transiently overexpressed *PHT2* (Fig. S5). This gene was selected because it displayed the strongest induction in response to both herbivory and Trichoderma symbiosis. In greenhouse bioassays, *PHT2* overexpression led to a significant increase in CP accumulation, whereas FP levels remained unchanged (Fig. 7A). Larvae feeding on *PHT2*-overexpressing *Nicotiana benthamiana* plants gained less weight than those feeding on agroinoculated controls, with the effect being significant at both 3 and 9 days after herbivory (Fig. 7B). These results indicate that elevated *PHT2* expression promotes CP accumulation and reduce *S. exigua* growth.

We used virus-induced gene silencing (VIGS) to suppress *PHT2* expression. Due to the high sequence similarity between *PHT1* and *PHT2*, both genes were silenced simultaneously (Fig. S5). TRV::PHT1/2 plants accumulated significantly less FP after herbivory compared with TRV::00 controls (Fig. 7C), while CP was undetectable in these samples. Larvae feeding on TRV::PHT1/2 plants showed greater weight gain, particularly at 6 days after feeding, and marginally higher at 9 days (Fig. 7D). Noticeably, these results were independently confirmed using an additional, different silencing construct: TRV::PHT1/2 plants accumulated significantly less FP, while CP was undetectable; and larvae feeding on TRV::PHT1/2 plants showed greater weight gain (Fig. S6).

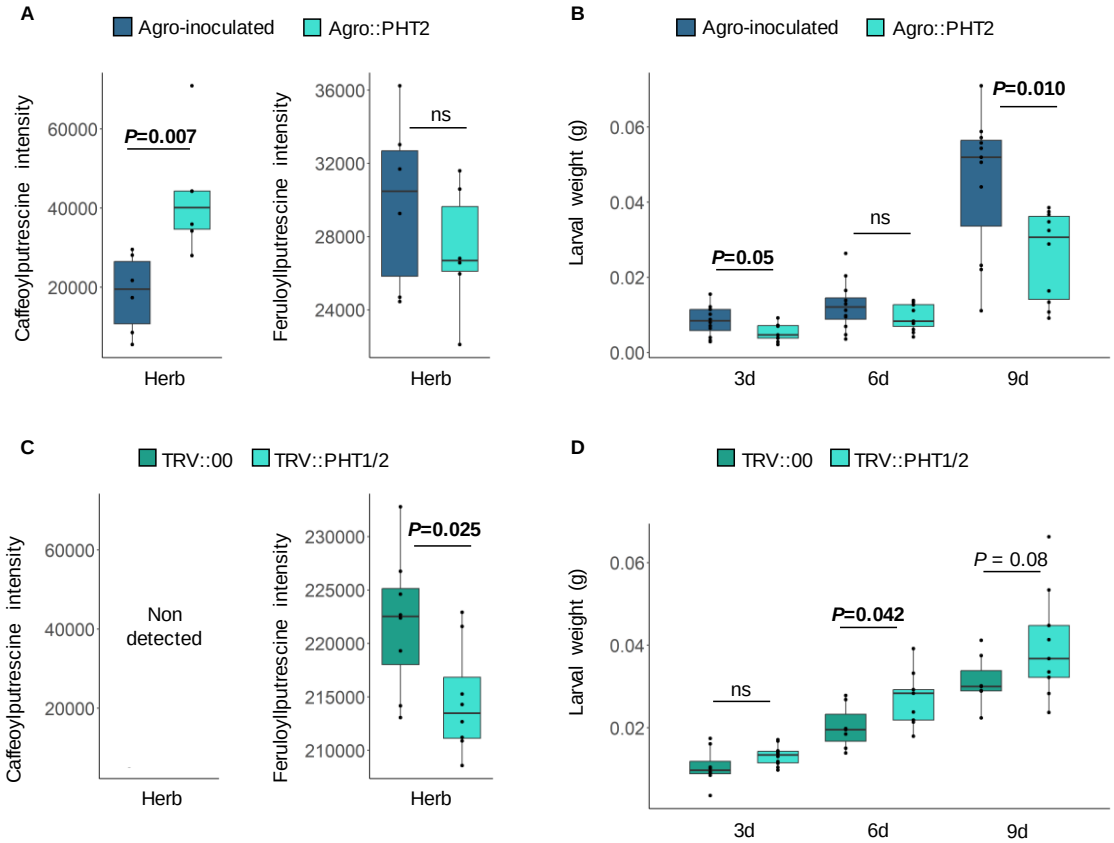

Figure 7. Impact of putrescine-derived phenolamides biosynthesis in plant resistance to herbivory. (A) Intensity of caffeoylputrescine and feruloylputrescine in *Nicotiana benthamiana* plants overexpressing *PUTRESCINE HYDROXICINNAMIC ACID TRANFERASE 2* (Agro::PHT2) and agroinoculated controls (Agro-inoculated). (B) Larval weight of *S. exigua* feeding on leaves of Agro::PHT2 or Agro-inoculated *N. bethamiana* plants. (C) Intensity of feruloylputrescine in tomato plants silenced in *PUTRESCINE HYDROXICINNAMIC ACID TRANFERASE 1/2* (TRV::PHT1/2) and in TRV controls (TRV::00). (D) Larval weight of *S. exigua* feeding on leaves of TRV::PHT1/2 or TRV::00 tomato plants. Boxplots represent the interquartile range (IQR); horizontal lines indicate medians, whiskers 1.5 times the IQR, and dots correspond to individual biological replicates (n = 6-12). *P* values (bold, $P < 0.05$) indicate *t*-test results between treatments. ns, non-significant.

To further corroborate the direct impact of CP and FP on *S. exigua* performance, we conducted a bioassay using artificial diet supplemented with the pure compounds. Application of either CP or FP alone resulted in a transient reduction in larval weight (Fig. 8, Table S3). Specifically, CP supplementation reduced larval weight after 2 days of feeding, whereas this effect was no longer evident at later time points (days 4 to 6; Fig. 8, Table S3). In the case of FP, larval weight reduction was observed at 3 days, with no significant effects detected at the remaining days of the assay. Remarkably, the combined application of CP and FP led to a sustained and significant reduction in larval weight throughout the entire bioassay (Fig. 8, Table S3). These results indicate that, while CP and FP individually exert mild (still significant) effects on larval performance, their combined presence produces a consistent negative impact on herbivore growth. Together, these findings demonstrate that enhanced biosynthesis and accumulation of putrescine-derived phenolamides, mediated primarily by PHT1/2 activity, reduces *S. exigua* performance. Moreover, they support the view that Trichoderma-induced activation of this conjugation pathway constitutes a mechanistic component of microbe-induced resistance to herbivory.

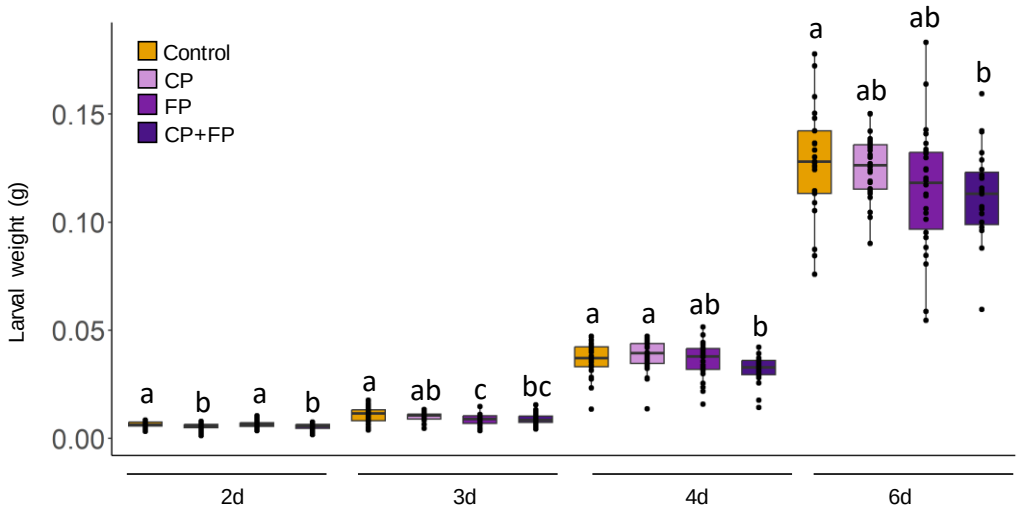

Figure 8. Direct effects of caffeoylputrescine and feruloylputrescine in *S. exigua* larval growth. Larval weight of *S. exigua* was monitored over time in individuals fed on an artificial diet supplemented with caffeoylputrescine (CP), feruloylputrescine (FP), or a mixture of both compounds (CP+FP). Control larvae were fed an artificial diet supplemented with acidified Milli-Q water, corresponding to the solvent used for compound dissolution. Box plots represent the interquartile range (IQR), the bisecting line represents the median, the whiskers represent 1.5 times the IQR, and the dots represent data points (n= 25 -30 biological replicates). Different letters at each time point indicate statistically significant differences among treatments according to Tukey's post hoc test ($P < 0.05$).

DISCUSSION

Root symbionts are increasingly recognized as modulators of polyamine metabolism in their host plants, influencing biosynthesis, accumulation, and conjugation (Brotman *et al*., 2012; Coppola *et al*., 2019; Minchev *et al*., 2026; Nikhil *et al*., 2024; Plett *et al*., 2020; Rivero *et al*., 2021; Salazar-Badillo *et al*., 2015). However, despite these advances, how symbiotic associations rewire the integrated functioning of the polyamine metabolic network, and how such network-level adjustments shape plant defensive phenotypes, remains unresolved. Polyamine metabolism operates as a highly dynamic and interconnected system, coupling biosynthesis, transport, catabolism, and conjugation to generate a wide array of specialized metabolites. Through this organization, the polyamine network functions as a central regulatory hub linking primary metabolism with chemical defense and broader stress-adaptation processes (Blazquez, 2024). Consistent with our hypothesis, our findings show that the root symbiont *Trichoderma harzianum* reconfigures the tomato polyamine metabolic network, at multiple levels, enhancing resistance to herbivory through coordinated regulation of its major functional branches. Trichoderma symbiosis boosts herbivore-induced uptake transport-catabolism module, activates de novo polyamine biosynthesis via ODC-dependent pathway, and stimulates conjugation with hydroxycinnamic acids to produce phenolamides with anti-herbivore activity. Together, these responses show that the symbiotic fungus not only primes herbivore-triggered polyamine responses, but also engages additional metabolic branches, thereby increasing the complexity and diversity of the polyamine network, expanding thus the metabolic landscape of the host under herbivore pressure.

In the absence of the root symbiont, herbivory primarily activated the polyamine network branch associated with uptake transport (*PUT3*), spermidine synthesis (*SPDS*), and catabolism (*PAO*), leading to H_2O_2 accumulation and reflecting a general stress-induced turnover response. Polyamine oxidation and the resulting reactive oxygen species have been implicated in plant resistance to a variety of stresses, including herbivory (Yang *et al*., 2024; Yoda *et al*., 2006; Zu *et al*., 2024). In non-colonized plants, these polyamine-mediated responses likely represent a broad metabolic adjustment to oxidative stress rather than a targeted defense program. Remarkably, Trichoderma symbiosis strongly amplified this herbivore-induced module, enhancing *PUT3* expression and promoting *PAO1* induction, accompanied by increased H_2O_2 accumulation. These findings indicate that the symbiont primes oxidative polyamine turnover, likely reinforcing herbivory-induced resistance response. Consistent with this interpretation, Trichoderma-mediated resistance has been linked to enhanced redox signaling and controlled production of reactive oxygen species (ROS), which function both as antimicrobial and anti-herbivore agents and as central signals in defense regulation (Contreras-Cornejo *et al*., 2011; Kerchev *et al*., 2012; Kumar *et al*., 2023; Mahanta *et al*., 2025). While symbiont-triggered ROS can originate from multiple enzymatic sources, our results suggest that the primed polyamine uptake-catabolism module may contribute to H_2O_2 dynamics during herbivory, although dedicated functional validation is still required. Notably, recent work shows that PUT3 and several polyamine-modifying enzymes form a coordinated regulatory module within the same genomic cluster in tomato,

supporting the emerging view that polyamine turnover and H_2O_2 homeostasis constitute an integrated stress-response node (Yang et al., 2024).

Even more strikingly, Trichoderma symbiosis reconfigured the polyamine network beyond the uptake transport and catabolism modules, activating both the biosynthetic and conjugation branches, which seemed to remain largely quiescent upon herbivory in plants lacking the symbiosis. This qualitative reprogramming guided our subsequent genetic analyses toward these specific pathway components. Upon herbivory, Trichoderma specifically induced the ODC pathway, as evidenced by elevated *ODC* transcript levels, increased ornithine accumulation, and higher ODC enzymatic activity. Notably, ornithine levels rose despite enhanced ODC activity, indicating that the increase was fueled by greater precursor availability rather than reduced consumption. Consistent with this, the symbiont upregulated *ARG2*, a jasmonic acid- and wound-responsive arginase that converts arginine into ornithine, thereby sustaining the ODC-driven biosynthetic flux (Chen *et al*., 2004). Functional assays confirmed the central role of this pathway in symbiont-mediated resistance: silencing *ODC* completely abolished Trichoderma-induced protection, whereas disruption of the ADC branch caused only a partial reduction in resistance. Notably, silencing either pathway in noninoculated plants overall did not affect basal resistance to *S. exigua*, suggesting that polyamine biosynthesis contributes little to constitutive defense under these conditions (Gonzalez-Hernandez *et al*., 2022). Although direct metabolic flux measurements were not performed, our data indicate that Trichoderma symbiosis recruits the ODC pathway as a metabolic switch, channeling primary amino acid precursors into defense-oriented polyamine biosynthesis during herbivore attack. Moreover, while the ADC branch appears less central than ODC in this response, it may still contribute to the overall induction of resistance, underscoring a potential complementary role of both biosynthetic routes in shaping polyamine-mediated defense.

Concomitant with the activation of the biosynthetic module, Trichoderma symbiosis also elicited the conjugation module of the polyamine network, enhancing the formation of putrescine-derived phenolamides. These specialized metabolites are generated through the conjugation of hydroxycinnamic acids with polyamines via acyltransferases, a process described in several plant species (Bernard *et al*., 2022; Luo *et al*., 2009; Roumani *et al*., 2021). In tomato, four putative hydroxycinnamoyltransferases (PHTs) have recently been identified (Roumani *et al*., 2025), yet their role in antiherbivore defense remained unexplored, and our understanding of phenolamide metabolism and regulation is still incomplete. Here, we found that Trichoderma enhances *PHT1* and *PHT2* expression and promoted the accumulation of CP and FP. Bioassays with pure compounds revealed that both phenolamides were associated with reduced larval growth, with the strongest and most consistent effects observed when CP and FP were applied together. Consistent with this, putrescine-derived phenolamides have previously been linked to antiherbivore activity in other systems (Kaur *et al*., 2010; Minchev et al., 2026). Genetic manipulation further supported the defensive role of this conjugation pathway: transient overexpression of *PHT2* in *N. benthamiana* increased CP accumulation and impaired larval performance, whereas simultaneous silencing of *PHT1* and *PHT2* in tomato reduced FP levels

and promoted larval growth. Although the inducibility and defensive role of phenolamides vary across plant-insect systems (Gaquerel *et al*., 2014; Kim *et al*., 2011; Kundu, 2023; Li *et al*., 2020), our findings demonstrate that symbiosis-driven activation of the conjugation branch of the polyamine network constitutes a functionally relevant mechanism contributing to enhanced resistance against herbivory.

Remarkably, in non-colonized plants, herbivory did not trigger phenolamide accumulation, suggesting that activation of this branch is symbiosis-dependent. Phenolamide accumulation can be triggered by herbivory in several plants species (Alamgir *et al*., 2016; Kaur *et al*., 2010; Pearce *et al*., 1998). Yet, in certain plant-insect systems, this response is weak or absent and does not necessarily confer greater resistance emphasizing its context-dependent nature (Minchev *et al*., 2026; Roumani *et al*., 2021). Consistent with our observations, Minchev *et al*. (2026) found that *Tuta absoluta* induced FP accumulation only in mycorrhizal plants, but not in non-colonized plants. The reasons and mechanistic basis for the lack of phenolamide induction by *S. exigua* in tomato are currently unknown, but may involve *Spodoptera*-mediated suppression of host immunity (Chen *et al*., 2019) and/or nitrogen allocation trade-offs that occurs during herbivory (Matsuno *et al*., 2009; Ullmann-Zeunert *et al*., 2013). In this context, Trichoderma-induced increase in ornithine may support both enhanced ODC-mediated polyamine biosynthesis and phenolamide formation, alleviating nitrogen constraints that might otherwise limit these defenses.

Overall, our study demonstrates that a root symbiont orchestrates a coordinated reprogramming of the tomato polyamine network, linking primary metabolism to specialized metabolite production and antiherbivore defense (Fig. 9). The symbiont not only primes the uptake transport-catabolism module but also activates ODC-driven biosynthesis and phenolamide conjugation, thereby increasing the complexity, diversity, and functional contribution of the polyamine metabolomic network. Remarkably, these responses reveal that under stress, microbial symbionts can induce metabolic branches that seems to remain quiescent in the absence of the symbiont. Exploring this idea is particularly important because it challenges the prevailing view that diversification of specialized metabolites occurs predominantly as a direct response to biotic threats (Speed *et al*., 2015). By showing that symbiotic interactions can expand and reshape metabolic networks, our findings provide a mechanistic framework for understanding how root symbiosis dynamically fine-tune specialized metabolism to enhance plant adaptation and resistance under herbivore pressure.

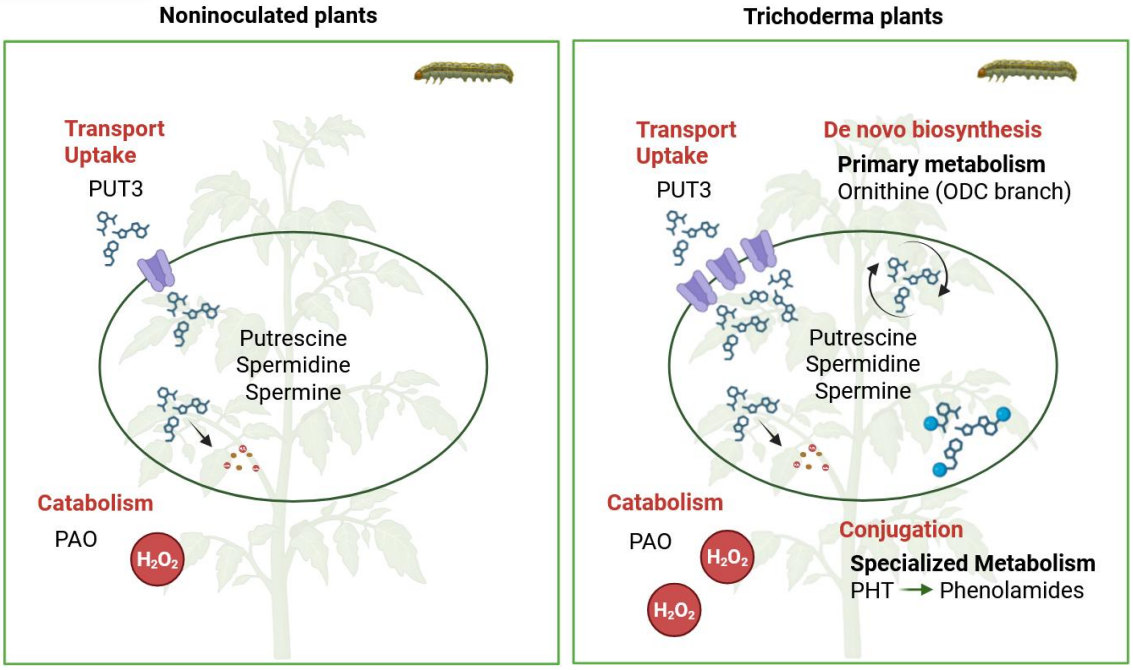

Figure 9. Trichoderma root symbiosis reconfigures the tomato polyamine network to enhance herbivore resistance. (A) In plants lacking endophytic symbiosis, *Spodoptera exigua* herbivory primarily activates the polyamine network branch associated with uptake (via *PUT3*) and catabolism (via *PAO*), leading to hydrogen peroxide (H_2O_2) accumulation. This oxidative burst can act as both a toxic defense against herbivores and a signaling cue for defense activation. (B) In *Trichoderma*-colonized roots, symbiosis primes polyamine uptake and catabolic nodes, resulting in a controlled increase in H_2O_2 production that contributes to enhanced antiherbivore resistance. Following herbivory, *Trichoderma* symbiosis further activates de novo polyamine biosynthesis through the ornithine decarboxylase (ODC) pathway and redirected free polyamines, via *PHT* activation, toward the production of putrescine-derived phenolamides with antiherbivore activity. Overall, *Trichoderma* symbiosis reorganizes the polyamine metabolic network, functionally linking primary metabolism to specialized defense pathways, thereby increasing the complexity, diversity, and functional output of the polyamine-derived metabolome.

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AUTHOR CONTRIBUTIONS

IF and SCT contributed equally. Conceptualization and Supervision: AMM. Project Administration: AMM. Funding Acquisition: MJP, VF and AMM. Methodology and Investigation: IF, SCT, BRR and PLG. Data Analysis: IF, SCT, BRR, PLG, AIGH, AC, MJP,

VF and AMM. Visualization: IF, SCT and AMM. Writing Original Draft: IF and SCT. Review & Editing: AMM

COMPETING INTERESTS

None declared.

DATA AVAILABILITY

All data are available within the article. Sequence data used in this article can be found in the Solgenomics platform (https://solgenomics.net/)

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SUPPORTING INFORMATION

Figure S1: Effect of *Trichoderma harzianum* root colonization on tomato growth.

Figure S2: Schematic illustration of polyamine metabolism in plants.

Figure S3: Effect of *Trichoderma harzianum* root colonization and *Spodoptera exigua* herbivory on the expression of *ARGINASE 1*.

Figure S4. Identification and quantification of putrescine- and spermidine-derived phenolamides

Figure S5. Expression level of *PHT1* and *PHT2* genes in *PHT2* overexpressing *N. benthamiana* plants and *PHT1/PHT2* silenced tomato plants.

Figure S6. Impact of virus-induced *PHT1/PHT2* silencing on feruloylputrescine accumulation and *Spodotera exigua* larval performance using silencing construct 2.

Table S1: *SlPHT2* gene target region and primer sequence for Virus Induced Gene Silencing (VIGS)

Table S2. Specific primers used for RT qPCR

Table S3. Results (*F* and *P* values) from 2-Way ANOVAs for the different variables presented in Figures 2-6, and 8 in main text.