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Research Article

Trichoderma harzianum strain T22 enhances direct and indirect defenses in tomato plants against the stink bug Halyomorpha halys

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Authors

Tuğcan Alınç
Corresponding Author
Submitting Author

Affiliations
- Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo Viale delle Scienze, Palermo, Italy

Ezio Peri

Affiliations
- Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo Viale delle Scienze, Palermo, Italy

Livio Torta

Affiliations
- Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo Viale delle Scienze, Palermo, Italy

Salvatore Guarino

Affiliations
- Institute of Biosciences and Bioresources (IBBR), National Research Council of Italy (CNR), Palermo, Italy

Guglielmo Puccio

Affiliations

- Institute of Biosciences and Bioresources (IBBR), National Research Council of Italy (CNR), Palermo, Italy

Francesco Mercati

Affiliations

- Institute of Biosciences and Bioresources (IBBR), National Research Council of Italy (CNR), Palermo, Italy

Stefano Colazza

Affiliations

- Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo Viale delle Scienze, Palermo, Italy

Antonino Cusumano

ORCiD
https://orcid.org/0000-0001-9663-9164

Affiliations

- Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo Viale delle Scienze, Palermo, Italy

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1 **Title**

2 ***Trichoderma harzianum* strain T22 enhances direct and indirect defenses in tomato plants against the**
3 **stink bug *Halyomorpha halys***

4 **Running title:** *Trichoderma*-mediated tomato defenses against the stink bug *Halyomorpha halys*

5

6 **Authors**

7 Tuğcan Alınç[1*], Ezio Peri[1], Livio Torta[1], Salvatore Guarino[2], Guglielmo Puccio[2], Francesco Mercati[2], Stefano
8 Colazza[1], Antonino Cusumano[1]

9 [1]Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo Viale delle Scienze,
10 Palermo, Italy

11 [2]Institute of Biosciences and Bioresources (IBBR), National Research Council of Italy (CNR), Palermo, Italy

12

13 ***Corresponding author:** tugcan.alinc@unipa.it

Abstract

BACKGROUND: Plant interactions with beneficial microbes can modulate defense responses against insect herbivores, yet the mechanisms remain poorly understood for herbivorous stink bugs. This study evaluated the impact of the plant growth-promoting fungus, *Trichoderma harzianum* T22, on direct and indirect defenses of tomato plants challenged by the invasive stink bug *Halyomorpha halys*.

RESULTS: Inoculation of tomato plants with *T. harzianum* T22 reduced the relative growth rate of *H. halys* nymphs, accompanied by enhanced expression of defense-related genes involved in the Jasmonic Acid and Salicylic Acid pathways, notably *ToPIN2*, *ToLOXD* and *ToPR1*. Behavioral assays revealed that females of the egg parasitoid *Trissolcus japonicus,* one of the main natural enemies of *H. halys,* were more strongly attracted to volatiles emitted from inoculated plants induced by stink bug feeding and oviposition. However, no effect of fungal inoculation was detected in the chemical composition of plant volatiles.

CONCLUSION: These findings demonstrate that *T. harzianum* T22 enhances tomato responses against *H. halys* by strengthening defense signaling pathways and modulating indirect defenses that facilitate natural enemy recruitment. Overall, these results suggest that using plant-growth-promoting fungi to enhance plant defenses is a promising crop protection strategy for controlling stink bug pests.

Keywords: beneficial soil microbes, defense signaling pathways, oviposition-induced plant volatiles, *Trissolcus japonicus*, tomato

1 INTRODUCTION

Plants live in complex environments where they interact with a diverse community of below and aboveground organisms, such as microbes and insects.[1] These interactions play a critical role in defining plant health and productivity by mediating both antagonistic and mutualistic relationships among organisms.[2,3] Insect herbivores and plant pathogens can negatively impact plants by feeding on tissues or causing infections, whereas beneficial microbes can enhance plant growth, metabolism and defenses.[4, 5]

The interest for beneficial microbes is rapidly increasing in consideration of their capacity to enhance plant defenses against biotic stressors.[6, 7] For instance, inoculation with plant growth-promoting rhizobacteria (PGPRs), arbuscular mycorrhiza fungi (AMFs) and plant growth-promoting fungi (PGPFs) has been shown to enhance plant defenses against a broad spectrum of insect herbivores through both direct and indirect defense mechanisms.[8-12] Beneficial microbes can modulate plant defense signaling pathways, such as Jasmonic acid (JA) and Salicylic acid (SA), that reduce insect herbivore performance or induce a state of alert (called "defense priming"), leading to a more effective direct defense response against future herbivory.[13-15] Beneficial microbes can also affect indirect plant defenses by modulating the release of volatile organic compounds (VOCs), such as herbivore-induced plant volatiles (HIPVs) and oviposition-induced plant volatiles (OIPVs), which in turn attract natural enemies such as parasitoids or predators.[16-18]

Among beneficial microbes, fungi of the *Trichoderma* genus are well-studied in rhizosphere microbiome and are well known to induce systemic resistance (ISR) in plants by modulating defense signaling pathways against plant pathogens.[10, 19] In addition, recent studies have demonstrated that *Trichoderma* species can also mediate plant defenses against insect herbivores leading to reduced feeding damage or increased mortality rates.[20] For example, inoculation of *Trichoderma harzianum* strain T22 enhanced tomato defenses against the aphid *Macrosiphum euphorbiae* (Thomas) and *Bemisia tabaci* (Gennadius).[20-21] Besides, these beneficial fungi can also affect the chemical composition of plant volatile profiles to enhance the recruitment of natural enemies towards to plants attacked by insect herbivores.[22-25] However, current evidence is still limited, and further

research is needed to assess how widespread the effects of *Trichoderma*-mediated plant defenses are against herbivore pests.

Here investigated the effects of *T. harzianum* T22 on tritrophic interactions focusing on the invasive stink bug *Halyomorpha halys* (Stal) and its associated egg parasitoid, *Trissolcus japonicus* (Ashmead). *Halyomorpha halys* has become an important invasive pest in Europe and USA that can feed upon more than 300 species of annual and perennial plants, many of which are of economic importance.[26-28] Among the natural enemies, the egg parasitoid *T. japonicus* is a key biological control agent.[26, 29] To examine direct defenses, we evaluated the performance of *H. halys* nymphs on tomato plants that were either inoculated with *T. harzianum* T22 or left untreated. We also investigated the underlying molecular mechanisms by quantifying the expression of key defense-related genes in response to stink bug feeding. To assess indirect defenses, we conducted behavioral assays and VOC chemical analyses to determine whether *T. harzianum* T22 enhances the attraction of *T. japonicus* to inoculated tomato plants induced by *H. halys* oviposition.

2 MATERIALS AND METHODS

2.1 Insects rearing

The colony of *H. halys* was established from field-collected individuals around Turin, Italy and reared in cages (47.5 × 47.5 × 47.5 cm; BugDorm-44545, Mega View Science Co., Ltd. Taichung, Taiwan) under controlled conditions (24 ± 2 °C; 70 ± 5% RH; 16L:8D photoperiod) at the Entomology Laboratory, University of Palermo (Palermo, Italy). The colony was fed with seasonal organic fresh vegetables and hazelnut seeds. Food was replaced every two days and water was provided with soaked cotton wool in small containers. Egg masses were collected daily and used to maintain both *H. halys* and *T. japonicus* colony.

The colony of *T. japonicus* was established from wasps emerging from *H. halys* sentinel egg masses located in fields around Turin, Italy and was reared on *H. halys* egg masses in the laboratory. Wasps were maintained in 85-ml glass tubes, fed with a honey–water solution (80:20 v/v) and kept in a controlled environmental room (24 ± 2°C; 70 ± 5% RH; 16L:8D photoperiod). *Halyomorpha halys* egg masses were singly glued onto a strip of paper and exposed to two mated female wasps for 24 h. After emergence, male and female wasps were kept together to allow mating. Before the bioassays, 3–5 days-old female wasps were individually isolated in a 2 mL vial with a drop of honey-water for 24 h and then transferred to the bioassay room for 1 hour in order to be acclimatized. The tested females were naïve with no prior exposure to host or plant materials.

2.2 Fungal culture and seed treatment

Trichoderma harzianum T22 was isolated from Trianum-P (Koppert Biological Systems, The Netherlands) in which the fungus is the active ingredient.[10,30] The isolate was routinely grown and sub-cultured at room temperature on potato dextrose agar (PDA; Oxoid). Spores were harvested from PDA plates by flooding them with sterile distilled water and adjusting the concentration to 10^7 $ml^{-1}$of conidial suspension. The tomato seeds were treated as described by Coppola *et al.*[22] Briefly, the surface of the seeds was sterilized for 5 minutes with 1% (v/v) sodium hypochlorite and then rinsed in sterile distilled water. The seeds were coated with *T. harzianum* T22 conidial suspension or with sterile distilled water as a control. Following 24 hours of air

94 desiccation, dried seeds were germinated on water-moistened filter paper in a sterile Petri dish at 25°C in the
95 dark. Germinated seeds were transferred to sterile soil-filled trays and maintained at 20 ± 2°C and 14L:10D
96 photoperiod in a growth chamber. Tomato seedlings were then transplanted into 14-cm-diameter plastic pots
97 after three weeks. Each plant in the experiments was around 20 cm height with 3–4 fully expanded leaves.

98 **2.3 Performance bioassays**

99 Insect performance on tomato plants, inoculated by *T. harzianum* T22 was investigated by exposing each plant
100 (inoculated or water control) to 3rd instar nymphs of *H. halys*. Prior to bioassays, nymphs were weighed on a
101 Kern ABS-N analytical balance (Kern & Sohn, Germany) and then allowed to feed on the plants for 1 week
102 under controlled conditions (24 ± 1 °C; 70 ± 5% RH and 14L:10D photoperiod). During bioassays, plants were
103 enclosed together with 5 insects inside a nylon mesh bag (size = 30 cm × 40 cm; mesh count = 300 mesh/cm^2).
104 After 1 week, nymphs were removed and re-weighed. To assess insect performance, nymph mortality (i.e. %
105 dead nymphs in relation to total nymphs) and nymph relative growth rate were calculated. To account for the
106 mortality effect on relative growth rate, we averaged the weight of nymphs that were initially enclosed in the
107 plant (i.e. initial average weight) as well as the weight of the nymphs that survived at the end of the experiment
108 (i.e. final average weight). Thus, nymph relative grow rate was recorded as: (final average weight - initial
109 average weight)/ initial average weight * 100. For each treatment, 10 replicates were carried out.

110 **2.4 Plant induction, isolation of RNA and RT-qPCR**

111 In order to analyze the effects of *T. harzianum* T22 on tomato molecular responses to *H. halys*, the transcript
112 levels of three key marker genes implicated in signaling defense pathways were investigated. *ToLOXD* was
113 selected as a marker for the JA-signaling pathway whereas *ToPR1* was used for the SA-signaling pathway.
114 Additionally, the expression levels of *ToPIN2*, a gene coding for a protein inhibitor, were also recorded.

115 The youngest fully expanded leaf was exposed to a 4- to 5-day-old female of *H. halys* by enclosing each insect
116 in a clip cage (4 cm in diameter and 1 cm in height) with a mesh-covered hole (3 cm in diameter). The rim of
117 the clip cage was covered with a sponge ring to prevent any damage to the leaf. The insects were allowed to

feed for 8, 24 or 72 h, then the insects were removed, and leaf disks (3.8 cm in diameter) were excised from the treated leaf to be stored at −80 °C until gene expression analyses. Tomato plants were either inoculated with *T. harzianum* T22 and then induced by stink bug feeding (treatment 'T22Hh') or were only exposed to stink bug feeding (treatment 'Hh'). As controls, we used leaf disks collected from plants with empty clip-cages to assess gene expression level in the absence of herbivory (undamaged, non-inoculate plants). Five biological replicates, each consisting of a leaf disk per plant and per treatment, were performed. Total RNA was isolated from each replicate using the ISOLATE II Plant RNA kit from Bioline according to the manufacturer's instructions. RNA quality was assessed using an Agilent Bioanalyzer RNA nanochip (Agilent, Wilmington, DE, USA). Two µg of total RNA was reverse-transcribed into cDNA using Bio-Rad's iSCRIPT cDNA synthesis kit in a 40 µL reaction volume according to the manufacturer's instructions. RT-qPCR experiments for selected genes were performed on four biological replicates for each treatment. Primers (Tables S1) were designed according to Coppola *et al,*[31] and De Palma *et al,*.[32] All reactions were run in technical triplicate on a Step OnePlus Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green detection chemistry, as previously described.[33] Expression levels were assessed using the $2^{-\Delta\Delta Cq}$ method.[34]

2.5 Y-tube bioassays

Plant treatments

The tomato plants were treated according to the following conditions: (1) *H. halys* females were allowed to feed and oviposit on the non-inoculated plants (FO); (2) *H. halys* females were allowed to feed and oviposit on the plants previously inoculated with *T. harzianum* T22 (T22-FO); (3) as controls (CTRL) non-inoculated, non-infested plants were used.

The first fully developed leaf of the plants (treatments 1–2) was enclosed in a mesh bag (20 × 25 cm) and exposed to a gravid *H. halys* female for 24 h under controlled conditions (24°C; 70% RH; 16 h:8 h L:D). Plants were discarded if no oviposition was observed on the leaves after 24 hours. Stink bug feeding was visually confirmed by observing females inserting their stylets into leaf tissues. Insects were removed after 24 h and

tomato plants were used for bioassays. In control plants (treatment 3), the first fully developed leaf of was also enclosed in a mesh bag as described above.

Bioassays

The olfactory response of *T. japonicus* to volatile compounds emitted by differently treated tomato plants was investigated using a Y-tube olfactometer made from a polycarbonate body (stem 9 cm; arms 8 cm at 130" angle; ID 1.5 cm) sandwiched between two glass plates. A stream of medical-grade compressed air (approximately 80:20, N_2:O_2) coming from the cylinder, humidified by bubbling through a water jar, was regulated in each arm by a flowmeter at about 0.5 l min^{-1}. The device was illuminated from above by two 22-W cool white-fluorescent tubes (full spectrum 5900 K, 11 W; Lival, Italy) and from below by an infrared source (homogeneous emission of wavelengths at 950 nm provided by 108 LEDs). Before entering the olfactometer arms, each air stream passed through a cylindrical glass cylinder (Ø = 12 cm: h = 52 cm) containing a tomato plant (see above for treatments). Wasp females were released individually into the entrance of the Y-tube olfactometer. After evaluating 5 parasitoid females, the stimuli were switched to avoid unforeseen bias in the set-up. At the conclusion of the bioassays, the polycarbonate olfactometer and all glass components were cleaned using acetone and deionized water. The glass components were baked at 180°C overnight.

The behavior of wasps was recorded for ten minutes using a monochrome CCD video camera (Sony SSC M370 CE) with a 12.5–75 mm/F 1.8 zoom lens. To exclude visible wavelengths, the camera lens was equipped with an infrared pass filter (Kodak Wratten filter 87 A°). A video frame grabber (Studio PCTV–Pinnacle Systems, Mountain View, CA) was used to digitize analog video signals from the camera, which was then processed by XBug, a video tracking and motion analysis program.[35] Wasp response was measured in terms of residence time, i.e., the time spent by the wasps in each arm during the entire bioassay for 10 mins in the dark room under controlled conditions.

To evaluate the olfactory responses of *T. japonicus* to volatile compounds of tomato plants, the following pairwise combinations were tested: (i) feeding + oviposition *versus* control (FO *vs* CTRL); (ii) feeding + oviposition *versus* feeding + oviposition + T22 (FO *vs* T22-FO). Each choice bioassay was replicated with four 4 plant pairs and 10 female wasps per plant pairwise combination (about 40 wasps in total number).

2.6 Headspace collection and chemical analysis of VOCs

VOCs were collected from differently treated plants (see Section Plant treatments) in order to investigate whether differences in volatile profiles could explain the parasitoid behavior displayed in olfactometer bioassays. For each treatment, 5 replicates were carried out. Headspace collections were conducted using plants placed in a cylindrical glass chamber (inner Ø= 10 cm, h = 30 cm) and VOCs were collected for 24 h using adsorbent traps made by glass tubes filled with Porapak Q (Sigma Aldrich; 60 mg, 80–100 mesh). The traps were pre-cleaned with hexane and then heat-conditioned for at least 2 h in a stream of nitrogen (100 mL/min). After collection, the traps were eluted with 400 µL of hexane and the extracts were stored at −20 ◦C in glass vials with Teflon cap liners until used for gas chromatography and mass spectrometry (GC-MS) analysis.

GC-MS analysis of the VOCs was performed by injecting 1 µL of hexane extract in an Agilent 6890 GC system interfaced with an MS5973 quadruple mass spectrometer equipped with a DB5-MS column in splitless mode. Injector and detector temperatures were 260 °C and 280 °C respectively. Helium was used as the carrier gas at a flow rate of 1.6 mL/min. The GC oven temperature was set at 40°C and then increased by 10°C/min to 250°C, with initial and final hold times of 5 and 20 min, respectively. Electron impact ionization spectra were obtained at 70 eV, recording mass spectra from 40 to 550 amu. Peak integration was carried out using ChemStation software. Tentative identification of compounds was carried out using the NIST11 database and by comparison of the retention index calculated which were obtained by the injection of a 10-ppm solution of linear hydrocarbons C7-C30. In addition, synthetic standards of α-pinene, β-myrcene, γ-terpinene, limonene, and β-caryophyllene were injected for identification confirmation.

2.7 Statistical analysis

Logistic regression with binomial error distribution and log-link function was used to test whether stink bug mortality on tomato plants was affected by the *T. harzianum* T22 inoculation treatment, while ANOVA was used to test whether stink bug relative growth rate was affected by fungal inoculation treatment. ANOVA was also used to test if transcript levels of plant genes were significantly affected by the treatments (UD, Hh, and T22Hh) and by different stink bug feeding times (8, 24, and 72 h). Shapiro-Wilk normality test[36] was applied for data distribution evaluation. Post-hoc differences among treatments were tested using Tukey tests.

Olfactometer data were analyzed using linear mixed models (LMMs), with plant treatment included as a fixed factor and parasitoid identity nested within each plant pair as a random factor to account for pseudoreplication. Separate LMMs were applied for each pairwise treatment comparison. The significance of the fixed effect was assessed using Likelihood Ratio Tests (LRTs), and model fit was evaluated through inspection of residual plots.[37]

For the analysis of volatile emissions, the measured peak areas were first divided by the aboveground fresh weight of each plant. These normalized values were then analyzed using non-metric multidimensional scaling (NMDS) based on a Bray–Curtis distance matrix.[38] To assess significant differences in the chemical composition of the VOC blends, a permutational multivariate analysis of variance (perMANOVA) was conducted with 1,000 permutations. This analysis was performed using the *adonis2* function from the Vegan package in R. All statistical analyses were conducted using R software, version 3.6.2 (R Core Team https://www.R-project.org/)

208 **3 RESULTS**

209 **3.1 The effect of *T. harzianum* T22 on insect performance**

210 Inoculation of tomato plants with *T. harzianum* T22 did not affect the mortality rate of 3rd instar nymphs of *H.*
211 *halys* (GLM, $\chi 2 = 0.008$, df=1, P =0.927) (Fig. 1a). However, *H. halys* nymphs that fed on *T. harzianum* T22
212 inoculated tomato plants exhibited a lower weight compared to nymphs that fed on non-inoculated tomato
213 plants (ANOVA, $F = 5.843$, df=1 P =0.041) (Fig. 1b).

214 **3.2 The effect of *T. harzianum* T22 on gene transcript levels**

215 A significant upregulation of *ToLOXD* in tomato plants inoculated with *T. harzianum* T22 and infested with
216 *H. halys* (T22Hh) was found for 8h compared to non-inoculated but infested plants (Hh) ($p < 0.001$) (Fig. 2a;
217 Table 1). However, for the same time point, no significant difference was observed between controls (UD)
218 and infested - but non-inoculated – plants (Hh). At the 72h time point, no significant differences were recorded
219 among treatments (Fig. 2a; Table 1). Feeding damage by *H. halys* resulted in activation of *PIN2* at all three
220 timepoints ($p < 0.001$) (Fig. 2b; Table 1). In fact, *T. harzianum* T22 significantly upregulated the expression
221 of *PIN2* in T22Hh compared to Hh and control samples along the three time points investigated. The effects
222 of *T. harzianum* T22 were also observed in the transcript levels of the SA-marker gene *PR1* among treatments,
223 as significant differences were recorded at 8h and 72h of *H. halys* feeding time ($p < 0.05$) (Fig. 2c; Table 1),
224 with higher *PR1* expression level in T22Hh than in Hh. No differences in terms of transcript levels of *PR1*
225 were observed among treatments at the 24h timepoint.

226 **3.3 The effect of *T. harzianum* T22 on *T. japonicus* behavior**

227 *Trissolcus japonicus* females were significantly attracted to the volatile compounds emitted by tomato plants
228 induced by *H. halys* feeding and oviposition compared to control tomato plants (F_O vs CTRL: $\chi^2=9.250$,
229 df=1, P=0.002) (Fig. 3). Furthermore, inoculation with *T. harzianum* T22 resulted in significantly higher
230 attractiveness for *T. japonicus* to volatiles emitted by tomato plants induced by feeding + oviposition compared

231 to non-inoculated plants induced by *H. halys* feeding + oviposition (F_O_T22 vs F_O: χ^2=5.989, df=1, P=
232 0.014).

233 **3.4 The effect of *T. harzianum* T22 on chemical composition of VOCs**

234 In total, 24 different volatile organic compounds were detected in the headspace of the differently treated
235 tomato plants (Table 2). A variety of monoterpenes and sesquiterpenes were detected across all treatments.
236 When the total VOC blend was considered, no significant differences due to root colonization by *T. harzianum*
237 T22 were found between FO and T22-FO plants (perMANOVA, F = 0.403, df = 1, P = 0.953). The major
238 compounds consistently emitted included α-pinene, (+)-2-carene, *β*-phellandrene, 1,3,5-cycloheptatriene
239 (3,7,7-trimethyl-) and *α*-phellandrene. Plants subjected to *H. halys* activity, regardless of the *T. harzianum* T22
240 inoculation, generally exhibited higher VOC emission levels compared to control plants. Although these
241 differences in emission quantities were apparent—such as increased emissions of α-pinene, (+)-2-carene, *β*-
242 phellandrene, and caryophyllene in *H. halys* -exposed plants—no statistically significant differences were
243 found.

244

4 DISCUSSION

This present study provides the first comprehensive evidence that inoculation of tomato plants with the plant growth-promoting fungus *T. harzianum* T22 enhances both direct and indirect defenses against the stink bug pest *H. halys*. In particular, we found multiple lines of evidence of this effect: 1) *T. harzianum* T22 mediates direct defense of tomato plants by reducing the performance of *H. halys* nymphs; 2) *T. harzianum* T22 enhances expression of marker genes involved in defense signaling pathways in response to *H. halys* feeding; 3) *T. harzianum* T22 enhances indirect defense of tomato plants by increasing the recruitment of the egg parasitoid *T. japonicus*.

In terms of direct defenses, we found that root inoculation with the beneficial microorganism did not affect the mortality of *H. halys* nymphs. Instead, the effect was observed on the relative growth rate of the nymphs, which was reduced when they fed tomato plants previously inoculated with *T. harzianum* T22. Little is known on *Trichoderma*-mediated plant defenses against stink bugs, with the exception of *Nezara viridula* (L.). In this species, a similar effect was observed on both tomato[39] and sweet pepper,[40] suggesting a pattern in which *T. harzianum* T22 protects solanaceous plants against stink bugs by negatively affecting their feeding behavior rather than their survival. Furthermore, Van Hee *et al,*[41] observed a reduction in leaf damage in plants inoculated with *T. harzianum* T22 compared to non-inoculated plants, as the number of stink bug feeding punctures was reduced in the former. Our results are in accordance with previous studies that showed that *Trichoderma* species can promote direct plant defenses against a wide range of herbivores.[22,42] For example, the survival of the aphid *M. euphorbiae* was significantly reduced by tomato root colonization by *Trichoderma atroviride* strain P1.[43] Negative effects on insect performance were also observed in *S. littoralis* caterpillars feeding on tomato, where inoculation with *T. harzianum* T22 caused gut dysbiosis.[44]

As beneficial soil microbes can affect plant defenses by altering signaling pathways, the effect of *T. harzianum* T22 inoculation on tomato defenses may be mediated by changes in plant gene expression and metabolism, ultimately inhibiting the development of the stink bug *H. halys*. Previous works have shown that the beneficial effect of *Trichoderma* species against herbivores is dependent on both JA and SA defense - signaling pathways

in tomato plants.[20,24,45] Our results support these findings, as an effect of root inoculation with *T. harzianum* T22 was found for *ToLOXD* at 8h, with significantly higher gene expression level in tomato plants induced by *H. halys* feeding compared to non-inoculated but feeding induced plants. Also, an increase in expression levels in another JA-related gene, *ToPIN2* was found between inoculated versus non-inoculated tomato plants across all timepoints (8h, 24h, and 72h). This gene was also upregulated in studies investigating the effect of *T. harzianum* T22 on *N. viridula* feeding on tomato.[39] In response to herbivore damage, *PIN2* proteins are activated both locally and systemically in leaves and contribute to resistance against herbivores, which subsequently exhibit reduced growth and development.[46, 47] In addition, a significant upregulation of *ToPIN2* was found in tomato plants induced with salivary extracts of *H. halys*.[48] Thus, it is possible to argue that the upregulation of *ToPIN2* observed in our study may contribute to the reduced relative growth rate of *H. halys* nymphs feeding on inoculated tomato plants. However, we cannot exclude the involvement of the SA-signaling pathway, as we also observed an upregulation of the *ToPR1* gene which occurred already 8 hours after *H. halys* feeding on *T. harzianum* T22- inoculated plants compared to non-inoculated plants. Taken together, these results suggest that both JA- and SA-defense signaling pathways are involved in fungal-mediated defenses against *H. halys*.

In terms of indirect defenses, several studies have shown that plant colonization by beneficial soil microbes can affect the foraging behavior of larval parasitoids.[15,22,49,50] On the contrary, to the best of our knowledge, only a handful of studies have investigated how beneficial soil microbes can affect plant defenses by enhancing the recruitment of parasitoids that kill herbivore eggs. Our olfactometer results showed that inoculating tomato roots with *T. harzianum* T22 significantly enhanced the attraction of the egg parasitoid *T. japonicus* towards plants induced by *H. halys* feeding and oviposition. Similarly, *T. harzianum* T22 also enhanced the attraction of the egg parasitoid *T. basalis* towards tomato and sweet pepper plants induced with *N. viridula* eggs.[25, 51] Taken together, these studies show that plant defenses against insect egg deposition can be further modulated by the colonization of beneficial soil microbes, adding an extra layer of complexity to plant–insect–microbe interactions. Although chemical analysis of plant volatiles showed apparent differences in emission quantities

among treatments, no significant effect of fungal inoculation was detected on the chemical composition of the VOC profiles. Beneficial microbes have been shown to alter plant VOC profiles by enhancing or suppressing specific volatile emissions.[52] Yet, the lack of changes detected in the blend of plant volatiles, which contrasts with the behavioral responses of the egg parasitoid *T. japonicus*, suggests that the parasitoid's olfactory repertoire is more sensitive than the detection limits of current analytical techniques. In fact, olfactory responses of foraging parasitoids and predators are not always fully explained by chemical analyses.[25,53,54,55] Furthermore, insect parasitoids can detect changes in the ratios of specific volatiles within the blends adopting the so called "ratio-specific odor recognition".[56] To better understand which chemical compounds are especially important in driving parasitoid olfactory responses, further investigations should be carried out with the aid of gas chromatography coupled with electroantennographic detection (GC-EAD), as this technique has proven effective in detecting biologically active compounds.[57]

This study provides evidence that inoculation of tomato plants with *T. harzianum T22* boosts multiple lines of defense when plants are attacked by the invasive stink bug *H. halys*. This plant-growth-promoting fungus indeed modulates both direct defenses, by reducing the performance of the stink bug, and indirect defenses, by enhancing the recruitment of egg parasitoids. Overall, these results suggest that the use of plant-growth promoting fungi to enhance plant defenses in crop protection appears a promising strategy to control an important group of pests such as herbivorous stink bugs in order to reduce pesticide application.

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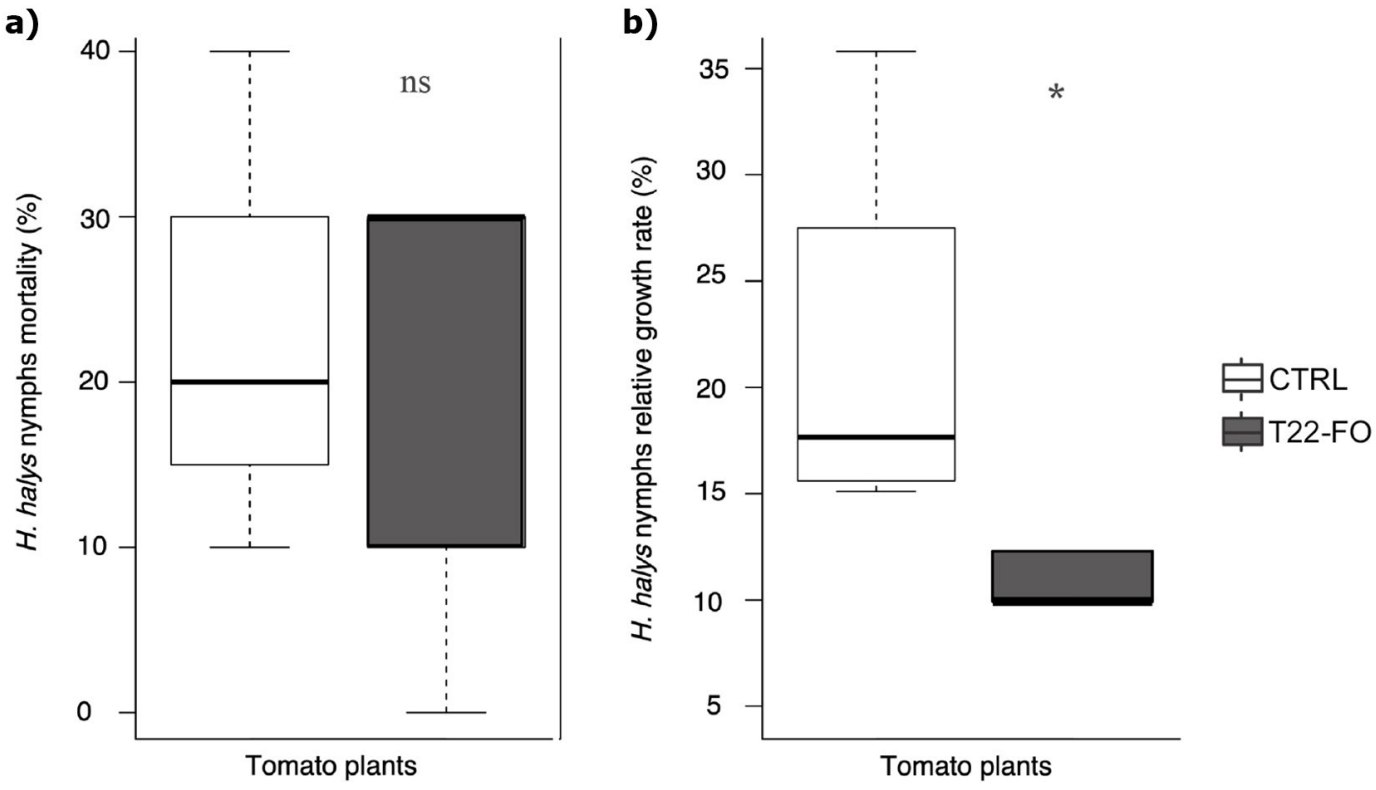
452 **Figures and legends**

453 **Fig. 1.**



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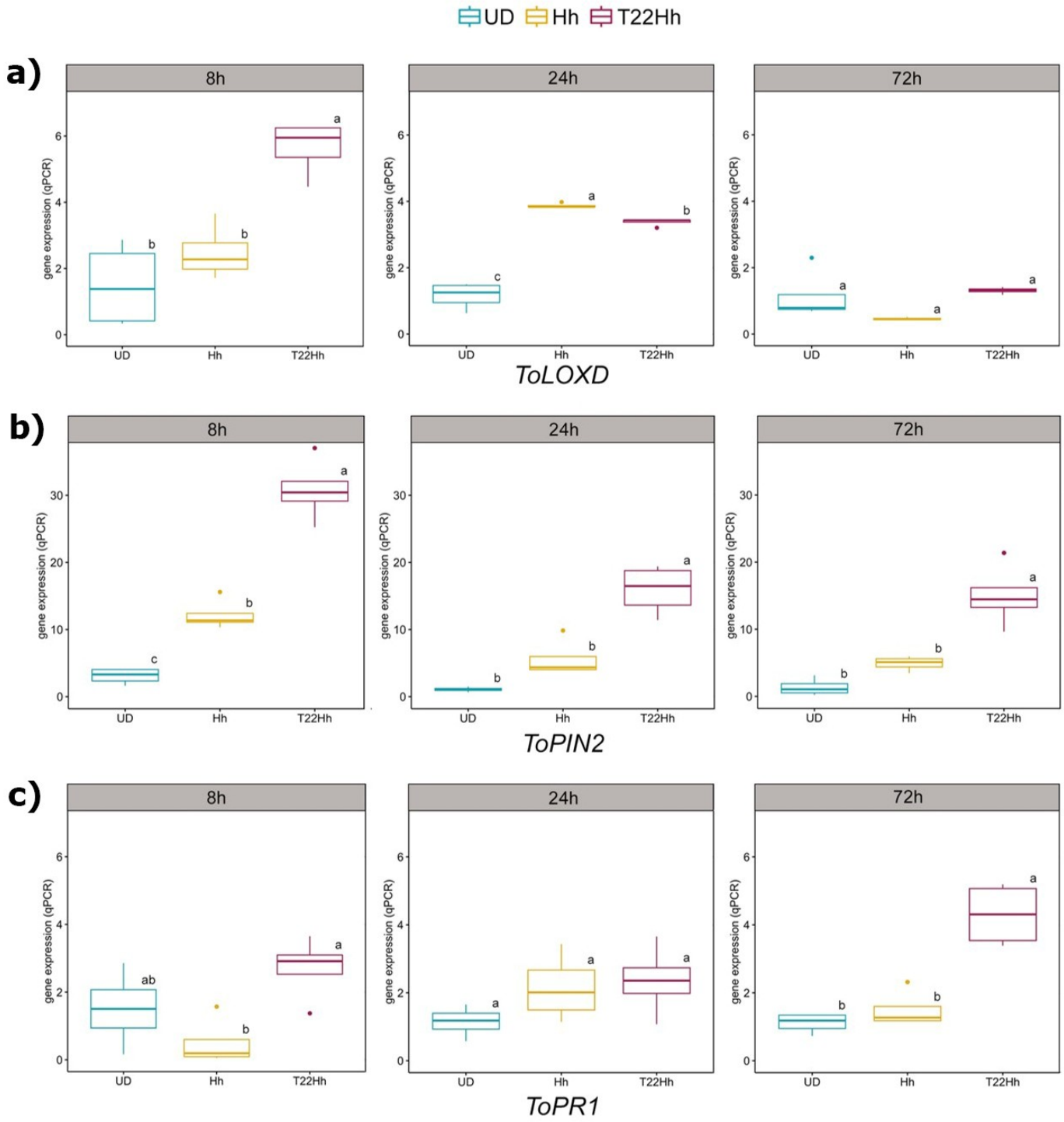
456

457 **Fig. 1.** Percentage of mortality (a) and relative growth rate (b) of *Halyomorpha halys* nymphs feeding on
458 *Trichoderma harzianum* T22 inoculated (T22-FO) versus control non-inoculated (CTRL) tomato plants. Bold
459 horizontal lines show medians, boxes contain the 25th–50th percentiles, whiskers show the upper and lower
460 quartiles and points show outliers (ANOVA, $P < 0.05$, ns = no significant differences).

461

462 **Fig. 2**



463

464 **Fig. 2**. Effects of tomato root colonization by *Trichoderma harzianum* T22 on relative expression of defense
465 marker genes. Expression levels of the JA-marker gene *ToLOX D* (a), protein inhibitor gene *ToPIN2* (b) and
466 SA-marker gene *ToPR1* (c) were analyzed in the leaves of tomato plants at 8h, 24h and 72h. UD = non-
467 inoculated undamaged plants; Hh = non-inoculated plants damaged by *H. halys* feeding; T22Hh = plants

468 inoculated with *T. harzianum* T22 and subsequently damaged by *H. halys* feeding. Bold horizontal lines show
469 medians, boxes contain the 25th–50th percentiles, whiskers show the upper and lower quartiles and points
470 show outliers. Different letters indicate statistically significant differences (ANOVA followed by Tukey post
471 hoc test, $P < 0.05$, ns = no significant differences).

472

473 **Fig. 3**

474

475

476 **Fig. 3.** Residence time (mean percentage ± SE) of *Trissolcus japonicus* females in each arm of Y-tube
477 connected to differently treated tomato plants. Treatments were non-inoculated and uninfested plants (CTRL),
478 non-inoculated – *Halyomorpha halys* oviposited plants (FO) and Trichoderma inoculated – *H. halys* oviposited
479 plants (T22-FO). Data were analyzed by means of GLMs (* asterisk indicates statistical significance; ns: not
480 significant, n = number of replicates)

481

Fig. 4

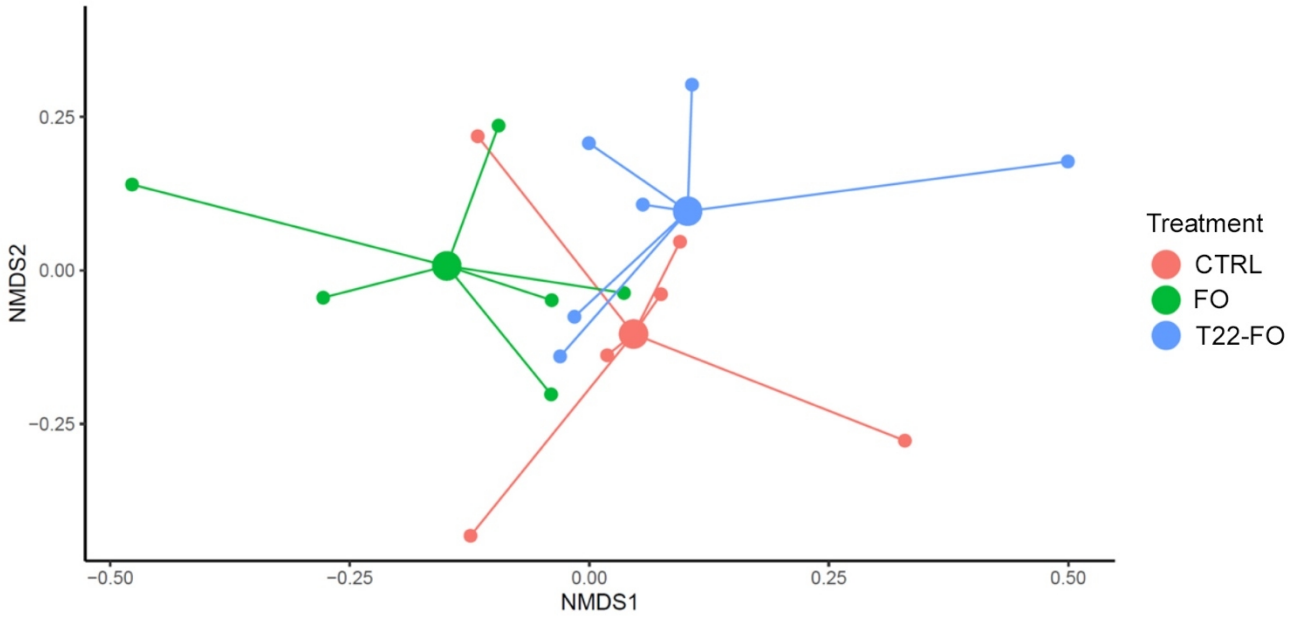


Fig. 4 Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities of relative amounts of chemical compounds found in differently treated tomato plants: *H. halys* feeding and oviposition (FO); *H. halys* feeding and oviposition + inoculation with *Trichoderma harzianum* T22 (T22-FO); non-inoculated and uninfested plants were used as control (CTRL). Small dots represent data points for individual samples (bigger dots represent the centroids) colored according to the treatments.

Table 1. Shapiro-Wilk normality and ANOVA for the marker genes (*ToLOXD, ToPR1, ToPIN2*) investigated at each feeding time (8h, 24h,72h).

Genes	**Feeding time**	**Shapiro-Wilk**		**ANOVA**				
		W	***p-value***	**df**	**Sum Sq**	**Mean Sq**	**F**	***p-value***
ToLOXD	8h	0.9246	0.3272	2	37.84	18.91	18.45	<0.0001
	24h	0.9117	0.2249	2	16.65	8.33	137.70	<0.0001
	72h	0.8346	0.0587	2	1.625	0.81	4.02	0.0565
ToPR1	8h	0.9246	0.3272	2	9.79	4.89	5.43	0.0284
	24h	0.9117	0.2249	2	3.36	16.80	2.17	0.17
	72h	0.8346	0.0593	2	24.13	12.06	28.46	<0.0001
ToPIN2	8h	0.9132	0.2347	2	1596.70	798.40	79.43	<0.0001
	24h	0.9420	0.5248	2	463.90	231.97	31.69	<0.0001
	72h	0.8749	0.0754	2	399.60	199.79	22.92	<0.0001

493 **Table 2**. Volatile organic compound (VOC) composition of tomato plants heathy, (CTRL), exposed to feeding
494 and oviposition by *H. halys* (FO) and tomato inoculated plant with *T. harzianum* exposed to feeding and
495 oviposition by *H. halys* (T22-FO). Values in the table are means of absolute peak areas ($\times 10^4$) ± SE (standard
496 error) of five biological replicates (n =5). Volatile organic compound(s) are ordered by their increasing
497 retention time (RT) and retention index (RI). VOCs were tentatively identified based on spectra, Kovats
498 retention index and database NIST 2011.

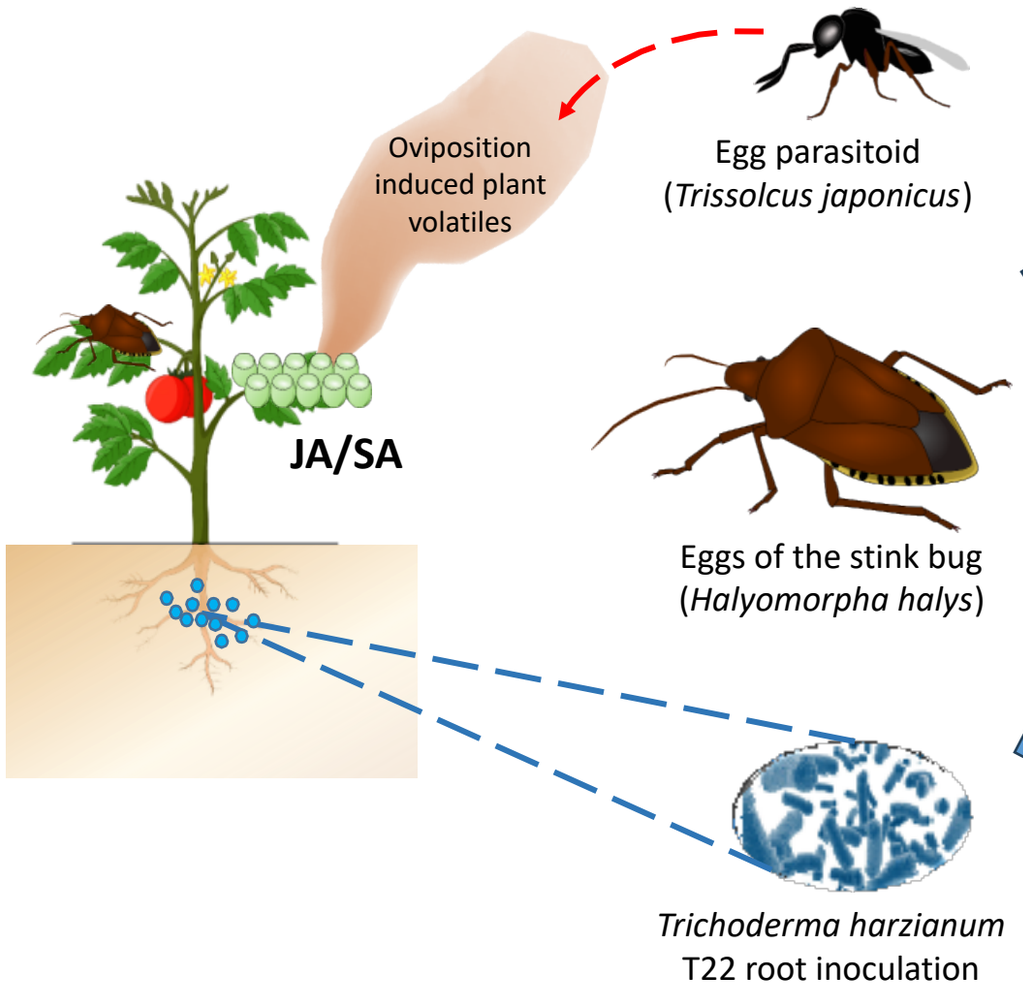
499

peak	rt	ri	chemical	FO	T22-FO	CTRL
1	11.006	925	α-tujene	32.45 ± 15.15	23.15 ± 19.97	5.15 ± 3.36
2	11.252	932	α -pinene	1421.61 ± 860.36	1394.55 ± 1157.08	246.28 ± 128.65
3	12.631	969	1,3,5-cycloheptatriene, 3,7,7-trimethyl-	1357.55 ± 921.25	1126.70 ± 922.42	149.50 ± 77.51
4	12.864	976	β-pinene	61.53 ± 37.18	77.98 ± 62.98	9.55 ± 4.85
5	13.069	981	isolimonene	167.01 ± 101.26	162.98 ± 141.85	18.09 ± 10.08
6	13.355	989	β -myrcene	68.38 ± 32.81	59.25 ± 44.63	10.48 ± 7.04
7	13.656	997	(+)-2-carene	3141.77 ± 1869.33	2915.81 ± 819.87	901.57 ± 461.19
9	13.916	1004	α-phellandrene	1185.17± 716.73	1012.53 ± 807.76	218.86 ± 120.68
10	14.284	1015	α-terpinene	663.27± 414.42	556.81 ± 486.59	76.02 ± 44.15
11	14.558	1023	p-cymene	174.19 ± 88.77	785.20 ± 737.15	21.61 ± 10.78
12	14.722	1028	limonene	220.80 ± 120.18	197.74 ± 170.51	158.96 ± 126.07
13	14.804	1031	β-phellandrene	5230.67 ± 3091.32	4282.63 ± 3097.90	1516.68 ± 695.12
14	15.719	1058	γ-terpinene	90.03 ± 47.74	74.84 ± 61.81	11.50 ± 5.99
15	16.593	1084	terpinolene	156.45 ± 93.05	98.06 ± 73.44	18.25 ± 10.57
16	17.604	1115	α-terpinene	34.40 ± 18.99	26.04 ± 22.60	3.57 ± 2.47
17	22.233	1303	unknown 1	15.49 ± 8.20	12.54 ± 7.75	1.17 ± 0.75
18	25.513	1389	(-)-β-elemene	3.95 ± 1.39	3.44 ± 1.66	0.24 ± 0.24
19	26.333	1421	caryophyllene	144.62 ± 107.24	90.17 ± 71.87	21.66 ± 13.02
20	26.557	1427	γ-elemene	3.19 ± +1.61	2.10 ± 1.04	0.56 ± 0.25
21	26.863	1441	unknown 2	8.72 ± 4.97	4.32 ± 2.69	0.46 ± 0.27
22	27.249	1456	humulene	27.52 ± 14.73	10.70 ± 8.06	1.41 ± 0.78
23	27.870	1481	germacrene d	2.27 ± 1.08	2.31 ± 1.21	0.29 ± 0.19
24	30.320	1583	caryophyllene oxide	3.43 ± 1.75	0.85 ± 0.66	0.28 ± 0.17

500

Table S1. Specific primers for quantitative PCR of plant-defense related genes

Oligonome	Name/Gene symbol	Sequence	Primer from
LoxD Fw LoxD Rv	lipoxigenase D (LOX D)	TTCATGGCCGTGGTTGACA AACAATCTCTGCATCTCCGG	Coppola et al. 2015
PIN II Fw PIN II Rv	Proteinase inhibitor II (PIN II)	CCAAAAAGGCCAAATGCTTG TGTGCAACACGTGGTACATCC	Coppola et al. 2015
PR1 Fw PR1 Rv	PR1	ATGCAACACTCTGGTGGACCTT CCATTGCTTCTCATCAACCCA	Coppola et al. 2015
Actin-fw Actin-rev	Actin	CACCACTGCTGAACGGGAA GGAGCTGCTCCTGGCAGTTT	De Palma et al. 2016

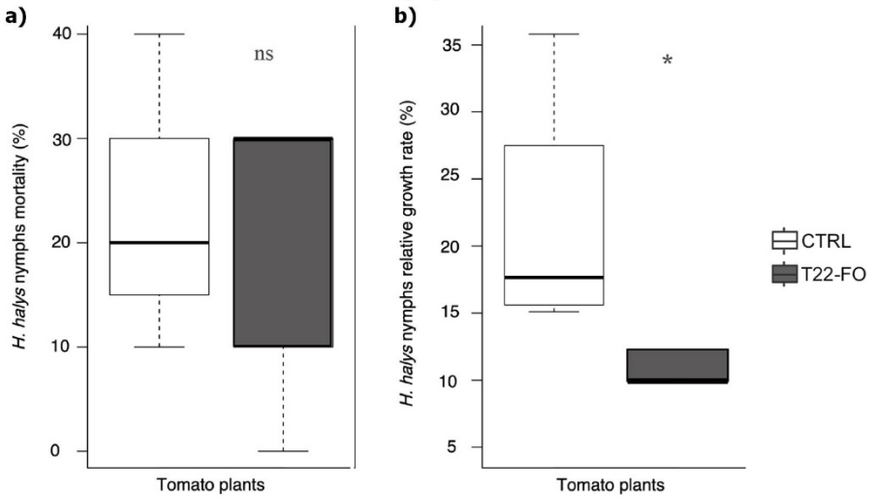


Egg parasitoid
(*Trissolcus japonicus*)

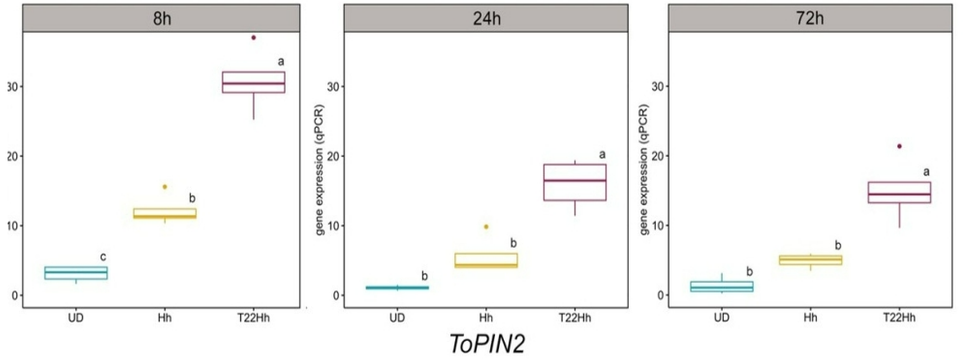
Eggs of the stink bug
(*Halyomorpha halys*)

Trichoderma harzianum
T22 root inoculation

Stink bug performance



qPCR of tomato defense-related genes



Egg parasitoid foraging behavior