Enhancing tomato crop resilience to water stress: the role of *Trichoderma afroharzianum* T22 and *Nesidiocoris tenuis* management

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With 4 figures and 1 table

Abstract: Global warming substantially threatens agricultural systems worldwide, with specific consequences for tomatoes, such as increased water demands and heightened pest pressures. Adopting sustainable farming practices is imperative, and *Trichoderma* species emerge as a valuable tool that enhances tomato growth, pest resilience, and stress tolerance. The integration of this approach into existing Integrated Pest Management (IPM) strategies requires careful consideration. A paradox arises in Southern Europe, where tomato cultivation heavily relies on predatory mirid bugs, especially *Nesidiocoris tenuis*, for pest control. While *N. tenuis* effectively controls tomato pests, it can also harm plants by inducing callose deposits, wilting, and yield losses, mainly when prey availability is scarce. This study delves into critical questions surrounding the concurrent use of *T. afroharzianum* T-22 and *N. tenuis* in tomato crops under water stress. We employed a randomized block design to examine three key factors: 1) varying levels of water stress, 2) the presence or absence of *T. afroharzianum* T-22 inoculation, and 3) the release or non-release of *N. tenuis*. Our findings demonstrate that *T. afroharzianum* T-22 enhances tomato growth under water stress and mitigates the adverse impact of *N. tenuis* on plant development. Additionally, *T. afroharzianum* T-22 inoculation did not affect *N. tenuis* performance, but it did reduce oxidative stress caused by *N. tenuis*, thus diminishing the plant damage attributed to this predatory mirid. These results hold significance for advancing pest management and promoting sustainable horticulture in a world grappling with the challenges of a warming climate.

Keywords: zoophytophagous predator; microbe; biological control; necrotic rings; callose; glucanase

1 Introduction

In the context of global climate change, global warming is a significant threat to agricultural systems (Tilman 1999). This issue affects food security and the sustainability of cropping systems worldwide, including tomato crops (Sato et al. 2006). As temperatures rise, water requirements for tomato crops increase due to higher evapotranspiration, worsening water scarcity and leading to the accumulation of salts in the soil (Flores-Saavedra et al. 2023). Under these conditions, crops become more vulnerable to pests and diseases (English-Loeb et al. 1997). The way forward includes adopting sustainable agricultural practices, innovative crop management, and climate-resilient varieties to protect crops from the impacts of global warming (Altieri & Nicholls 2017).

The beneficial fungus *Trichoderma* spp. (Hypocreales: Hypocreaceae) can enhance the growth and health of tomato crops (Harman et al. 2004). Benefits include increased plant growth and yields, better resistance to pests and diseases, improved tolerance to abiotic stress factors like drought, heat, and salinity, and enhanced nutrient availability (Woo et al. 2023). This makes *Trichoderma* spp. good candidates to counteract some negative effects of climate change in tomato cultivation (Vimal et al. 2017). However, incorporating *Trichoderma* spp. into current tomato Integrated Pest Management (IPM) protocols requires understanding how it interacts with existing methods and tools.

In Southern Europe, the main pest control strategy in tomato cultivation involves using predatory mirid bugs (Hemiptera: Miridae), which has significantly increased in recent years (Pérez-Hedo et al. 2017; 2021). In South Spain, *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) is successfully integrated into IPM programs for tomato crops (Pérez-Hedo & Urbaneja 2016). Most tomato pests, such as

Tuta absoluta and *Bemisia tabaci*, are controlled by introducing and conserving *N. tenuis* (Calvo et al. 2009; Mollá et al. 2011; Urbaneja et al. 2012). However, *N. tenuis* feeding on plant vascular tissues can cause severe damage, such as necrotic rings, phloem obstruction with callose deposits, wilting leaves, flower abortion, fruit punctures, and yield loss (Chinchilla-Ramírez et al. 2021). The extent of plant damage is inversely proportional to prey availability (Sánchez 2008). When pest pressure is low, these plant damages have led to considering *N. tenuis* as a significant pest (Moerkens et al. 2020).

Several important questions arise regarding utilizing *Trichoderma* spp. and managing pests using *N. tenuis* in tomato crops. Firstly, the phytophagous behavior of *N. tenuis* in tomatoes could increase the plant's water requirements, as its feeding significantly upregulates the abscisic acid (ABA) metabolic pathway (Pérez-Hedo et al. 2015). Therefore, it is necessary to explore the potential counteractive effects of *Trichoderma* spp. inoculation against water stress in the presence of *N. tenuis*. Secondly, it has been shown that inoculating *Trichoderma* spp. in tomatoes can affect the performance of insects that feed on the plant (Di Lelio et al. 2023). Thus, it is important to understand whether the presence of *Trichoderma* spp. influences the performance of *N. tenuis* itself. Finally, *Trichoderma* spp. triggers host systemic resistance (ISR), which involves upregulating defense mechanisms and synthesizing secondary metabolites like polyphenols and flavonoids to protect plant cells against oxidative stress (Jung et al. 2012; Martínez-Medina et al. 2013). It also increases the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), to counteract reactive oxygen species (ROS). Additionally, it stimulates enzymes like galactosidases that help degrade callose (Mastouri et al. 2012). Therefore, it is relevant to explore whether *Trichoderma* spp. inoculation can reduce the damage caused by *N. tenuis* by mitigating oxidative stress and reducing callose deposition around the necrotic rings formed by *N. tenuis*. Answering these questions is crucial for refining integrated pest management strategies and promoting sustainable horticultural practices in the face of global warming.

2   Materials and methods

A randomized block design was set up to address the previously posed questions with the combined use of *Trichoderma afroharzianum* T-22 [formerly *Trichoderma harzianum* strain T22 (Chaverri et al. 2015)] and *N. tenuis* under a water stress scenario. This design tested three factors: 1) water stress with three levels, 2) inoculation or non-inoculation of *T. afroharzianum* T-22, and 3) release or non-release of *N. tenuis*. This design yielded 12 different combinations, each of which was replicated six times (Fig. S1 A). Each replicate (plant) was individually placed in mesh boxes measuring 60 × 20 × 20 cm (BugDorm-1 Insect Tents; MegaView Science Co., Ltd., Taichung, Taiwan).

2.1   Plant, *T. afroharzianum* T-22 and *N. tenuis*

A total of 72 tomato plants (*Solanum lycopersicum* L.), specifically of the MoneyMaker cultivar, were utilized in the study. The germination process involved these plants in a substrate mixture composed of professional peat (Pro5050, Projar, Quart de Poblet, Valencia, SP) and vermiculite (Vermiculata nº2, Projar, Quart de Poblet, Valencia, SP), adhering to a 3:1 ratio. After approximately two weeks, each plant was transplanted into pots measuring 8 × 8 × 8 cm, utilizing the same soil and vermiculite blend. The plants were then individually placed in their respective boxes and distributed according to the treatment and experimental design. The plants were not fertilized during the entire trial.

The individuals of *N. tenuis* were provided by Bioline Agrosciences, S.L. (Almeria, Spain) (Nesiline©), while *T. afroharzianum* T-22 (Trianum-P©) was supplied by Koppert Biological Systems S.L. (Almeria, Spain). Upon the development of three true leaves on the tomato plants (3 weeks after sowing), they were subjected to inoculation with *T. afroharzianum* T-22. The inoculation quantity corresponded to the manufacturer's recommended field dose for potted plant cultivation, involving 30 grams of water-soluble granules for every 1000 plants (Koppert 2023). The exact dose per plant was applied through the irrigation water based on the field capacity of each pot (the volume of water that does not lead to percolation).

The seedbed and plant growth were carried out in one growth room, while the development of the experiment took place in another independent growth room. The environmental conditions in both growth rooms were maintained at 25 ± 2 °C, 50% ± 10% relative humidity, and a photoperiod cycle of 14L:10D hours. The light was supplied by Gro fluorescent tubes (GRO-LUX 36W, Sylvania, London, UK).

2.2   Water stress level

One week after the inoculation of *T. afroharzianum* T-22, water stress was initiated in each plant (Fig. S1 B). Plants were watered twice weekly, with each watering delivering the corresponding volume of water (Table 1). The watering requirements were established considering the plants' physiological demands and the crop coefficient (Kc), with calculations performed using the field water balance method outlined in Allen et al. (1998). This approach was applied across three treatments, each involving 24 plants. The trial began with an irrigation volume per watering of 81 mL for the standard water volume treatment (100%), gradually increasing to 160 mL. In the other two treatments, the irrigation volume was reduced to 75% of the standard water requirement for the moderate water stress treatment and to 50% of the standard water requirement for the higher water stress treatment. The irrigation volume applied during the trial can be found in Table 1.

Table 1. Water volume in milliliters applied per plant during each irrigation to achieve the three distinct levels of water fulfillment.

Water requirements	100%	75%	50%
From 1 to 10 days	81	61	41
From 11 to 20 days	90	68	45
From 21 to 30 days	118	88	59
From 31 days	160	120	80

2.3   *N. tenuis* release

One week after the inoculation of *T. afroharzianum* T-22, when the plants had about 3 to 4 true leaves (around 12–13 cm in height, measured from the base at soil level to the highest apical tip), a pair of *N. tenuis* (one male and one presumably mated female, approximately 4–5 days old) was released per plant in the treatments involving the mirid release. The *N. tenuis* population was fed with eggs of *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) throughout the entire duration of the experiment. Eggs were offered on the adhesive part of a Post-it sticker (3 cm²) (Post-it® 3M. Madrid, Spain), where they had been placed under a binocular stereoscope.

2.4   Evaluation

For eight weeks, with a weekly interval, plant development was assessed by measuring plant height (cm) and the number of leaflets per plant. Similarly, every week, the number of *N. tenuis* per plant (adults and nymphs) was counted with the naked eye, and the damage caused by *N. tenuis* was measured, including the number of necrotic rings and the number of wilted leaflets on each plant. A wilted leaflet is a leaflet that exhibits signs of wilting and is characterized by a dried-out appearance. For more details about gradient damage caused by *N. tenuis*, see Depalo et al. (2024).

2.5   Callose and glucanase quantification

The concentration of callose was determined from plant tissue following the method described by Köhle et al. (1985). For quantification, the apical part of the plant was selected because it is the area inhabited by *N. tenuis*, and it is in this plant stratum where damage mainly occurs. Therefore, it is in the apical part where the callose mobilized by the plant's defense system is expected to repair the damage. The apical part was considered the tender, growing area where the young leaves with their small leaflets, the small stems connecting them, and the shoot tips were located. The leaves were immediately frozen in liquid nitrogen, crushed in a mortar and pestle, and the resulting powder was introduced into 2 mL Eppendorf tubes, which were then stored at −80 °C. Thirty milligrams of crushed tissue were homogenized in 800 µl of 1M NaOH in new 2 mL Eppendorf tubes. The mixture was incubated for 30 minutes at 80 °C in a water bath, vortexing every 10 minutes, and then brought to room temperature in cold water. Afterward, it was centrifuged

at 12,000 rpm for 30 minutes at 4 °C. Two hundred µl of the supernatant were transferred to fresh 1.5 mL Eppendorf tubes containing 1.25 mL of a mixture of methyl blue (in a 3:1 ratio of 0.1% methyl blue to 1M glycine, pH 9.5) of dark blue color. It was incubated for 20 minutes in a water bath at 50 °C, during which a discoloration of the dark blue was observed. Afterward, an additional 30-minute incubation was allowed until room temperature was reached. To quantify callose, fluorescence spectrophotometry equipment (Multiskan SkyHigh Reader, Thermo Scientific, Waltham, MA, USA) was used with an excitation wavelength of 400 nm and an emission wavelength of 510 nm. Three blanks were prepared using 200 µl of 1M NaOH as a sample. A calibration curve was used to quantify callose. The calibration curve was prepared using callose standards at known concentrations, thus allowing the correlation of fluorescence intensity with the amount of callose present in the samples. The amount of callose was expressed in mg/mL. This was achieved by interpolating the fluorescence values obtained from the samples into the previously established calibration curve, which provides the concentration of callose corresponding to the measured fluorescence intensity.

The β-1,3-glucanase activity was also measured in the apical part of the plant collected above by determining the release of reducing sugars from laminarin, as Denault et al. (1981) described. This allowed us to calculate the enzyme activity based on the generated rates of reducing sugar. From frozen leaf material, 0.1 g of tissue was weighed and homogenized with 1 mL of sodium acetate buffer on ice. The mixture was then centrifuged at 12,000g at 4 °C for 10 minutes, and the resulting supernatant was used as the enzymatic extract. Next, 50 µL of the extracts were transferred into 0.25 mL Eppendorf tubes, and 50 µL of a 0.25% laminarin solution was added. For control replicates, 50 µL of distilled water was mixed with 50 µL of the 0.25% Laminarin solution. Three control replicates were prepared. The extract and substrate were thoroughly mixed and then heated in a thermocycler at 37 °C for 10 minutes. After incubation, the tubes were brought to room temperature. To prepare the glucose standards, 100 µL of glucose standards were transferred into 0.25 mL Eppendorf tubes. Then, 100 µL of DNS reagent was added to each control and blank sample. The mixtures were thoroughly mixed with a pipette. The thermocycler was set to heat the tubes at 90 °C for 10 minutes and then at 25 °C for 2 minutes. Subsequently, 150 µL of the reaction mixture was transferred to a 96-well flat-bottomed polystyrene microplate. Using fluorescence spectrophotometry equipment (Multiskan SkyHigh Reader, Thermo Scientific, Waltham, MA, USA), the absorbance at 540 nm was measured. The units used to express β-1,3-glucanase activity are U/g of glucanase activity. Here, "U" represents enzyme units, and the enzymatic activity is measured in terms of the amount of enzyme that releases 1 micromole of glucose per minute under the assay conditions. Therefore, β-1,3-glucanase activity is expressed in units per gram of plant material (U/g).

2.6 Callose deposition

A parallel experiment was conducted to study the histochemistry of the callose deposits following the same methodology and schedule described previously. Four treatments were tested: control plants, plants inoculated with *T. afroharzianum* T-22, plants inoculated with *N. tenuis*, and plants inoculated with both *T. afroharzianum* T-22 and *N. tenuis*. Five replicates (plant) per treatment were performed. In this case, the plants were watered at the normal frequency corresponding to the previously described 100% treatment. Seven days after the release of *N. tenuis*, necrotic rings were observed in treated plants compared to control plants without mirids. At this point, plant material was collected from both control and *T. afroharzianum* T-22-inoculated plants and plants exhibiting necrotic rings due to *N. tenuis* damage in the corresponding treatments. Sections from intact and *T. afroharzianum* T-22-inoculated plants were collected from the same stratum where the necrotic rings were found in the plants with *N. tenuis*. The plant material used included petiole sections taken immediately below the formation of the necrotic ring, ranging from 2 to 5 mm in diameter. These sections were then collected and fixed in FAA solution, following the protocol (formalin, glacial acetic acid, 70% ethanol, 1:1:18, v/v) as described by Johansen (1940). The plant samples were completely submerged in this solution for two weeks. Subsequently, they were washed with distilled water to remove any traces of the fixative solution. The plant sections were introduced for the staining process in 2 mL Eppendorf tubes with a stock solution of dark blue methyl blue (in a 1:1 ratio of 0.1% methyl blue to potassium phosphate buffer 1M, pH 6.5). After being submerged for 24 hours, the fluorescence of the samples was observed under a Nikon SMZ800N microscope (Nikon Corporation, Kanagawa, Japan) equipped with a CoolLED pE-300Lite integrated epifluorescence system with a GFP-L filter. At least 10 sections (2 per replicate/plant) were examined for each treatment, and photographs were taken with an XM full HD995 Nikon digital microscopy camera. Callose deposits produce a bright green fluorescence color under UV light. The amount of callose deposited was quantified by measuring the fluorescence area in each petiole section according to the method described by Scalschi et al. (2015). The analysis of fluorescent deposits corresponding to stained callose was performed by counting the number of pixels using GIMP (GNU Image Manipulation Program).

2.7 Data analysis

During the trial, the accumulated values days (over the eight-week sampling period for plant height, leaflets, *N. tenuis*, necrotic rings, and wiltings were calculated, following the procedures outlined in Stansly et al. (2005a, b). Value days accumulated (= area under the weekly incidence curve) were calculated by summing the trapezoidal areas formed by the measurement values at different points in time using the formula:

$$AVD = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) \times 7$$

where AVD is accumulative values days, y_i is the measurement value on day t_i, n is the number of observations, and 7 (days) is the interval of time between samplings.

Subsequently, the resulting estimates of insect and damage accumulation over the experiment, as indicated by the area under the weekly incidence curve, underwent statistical analysis. Plant height, leaflets and the amounts of callose and glucanase were analyzed using a Generalized Linear Mixed Model (GLMM) incorporating three factors and their interactions, utilizing a normal distribution and an identity link function, appropriate for continuous data. Conversely, the analyses of *N. tenuis* counts, necrotic rings, and wilting symptoms were conducted using a GLMM with two factors and their interaction, employing a negative binomial distribution with a log link function to accommodate the count nature of the response variables. Mean separation for the water stress factor was performed using the Least Significant Difference (LSD) method with a significance level of $P < 0.05$. Callose deposition was analyzed using one-way ANOVA, followed by a comparison of means using Tukey's test at a significance level of $P < 0.05$. All statistical analyses were carried out using IBM SPSS Statistics 22 for Windows (IBM Corp., Armonk, NY, United States).

3 Results

3.1 Plant growth

The height of the tomato plants was affected by the three treatments under study in this work (Fig. 1A). The plants inoculated with *T. afroharzianum* T-22 had approximately 24% greater height than the non-inoculated ones ($F_{1, 60} = 95.18$; $P < 0.001$). In contrast, the presence of *N. tenuis* significantly reduced the height of the tomato plants by approximately 11% compared to the plants where the mirid predator had not been released ($F_{1, 60} = 24.49,45$; $P < 0.001$). The reduction in the water requirements of the tomato plants significantly affected the plant height ($F_{2, 60} = 19.04$; $P < 0.001$), with differences observed among the three different levels of water stress. The interaction between *T. afroharzianum* T-22 and *N. tenuis* was significant ($F_{1, 60} = 8.234$; $P = 0.006$), showing that the height reduction due to the presence of *N. tenuis* was lower in the plants inoculated with *T. afroharzianum* T-22 (−13%) compared to the non-inoculated ones (−25.%) (Fig. 1A). The rest of the interactions between factors did not prove significant for the plants' height.

The number of leaflets was greater by approximately 45% in the plants inoculated with *T. afroharzianum* T-22 than in those that were not ($F_{1, 60} = 450.83$; $P < 0.001$), while the number of leaflets was not affected by the presence or absence of *N. tenuis* ($F_{1, 60} = 0.005$; $P = 0.943$)
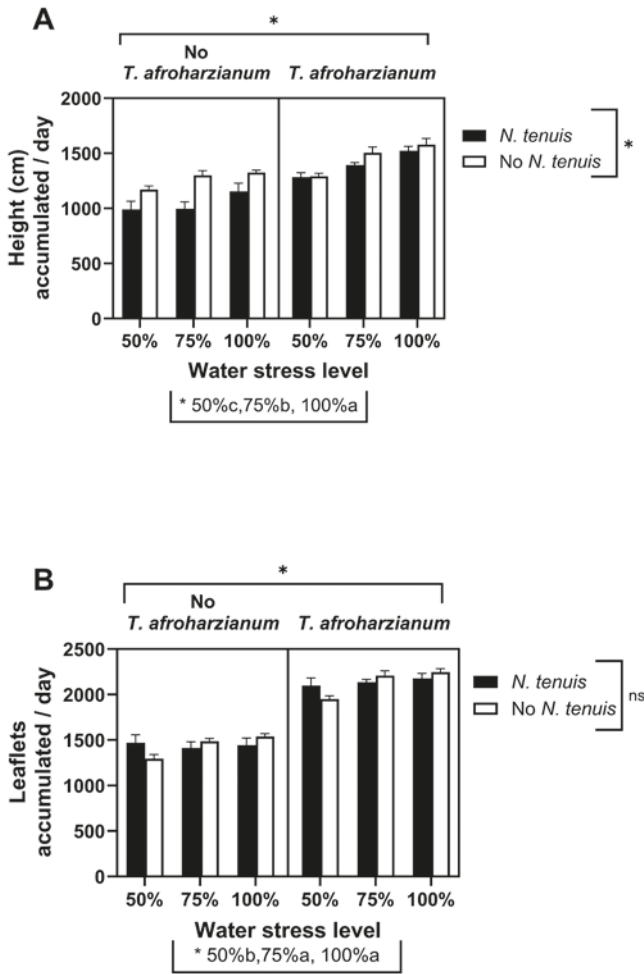
Fig. 1. (A) Average height (cm) (±SE) and (B) average number of leaflets (±SE) accumulated per day for the three treatments considered in the experiment: 1) varying levels of water stress, 2) *T. afroharzianum* T-22 inoculation or non-inoculation, and 3) *N. tenuis* release or non-release. The asterisk indicates statistical differences within each factor (ns = not significant), and in the case of the water stress factor, different letters following the asterisk denote significant differences among the three levels of the factor (three-way ANOVA; Tukey test, $P < 0.05$).

(Fig. 1B). The reduction in the water supplied to the tomato plants decreased the number of leaflets ($F_{1, 60} = 7.245.46$; $P = 0.002$), but this decrease was only significant in the 50% treatment with a reduction of −6% compared to the 75% and 100% treatments. None of the interactions between the factors studied proved significant for the number of leaflets.

3.2 *N. tenuis* performance and plant damage

The inoculation of *T. afroharzianum* T-22 ($F_{1, 30} = 0.006$; $P = 0.939$) and the different watering levels ($F_{1, 30} = 0.691$; $P = 0.509$) did not affect the number of *N. tenuis* per plant among treatments (Fig. 2A). However, the number of necrotic rings ($F_{1, 30} = 15.37$; $P < 0.001$) and the number of wilted leaflets ($F_{1, 30} = 5.089$; $P = 0.032$) were significantly
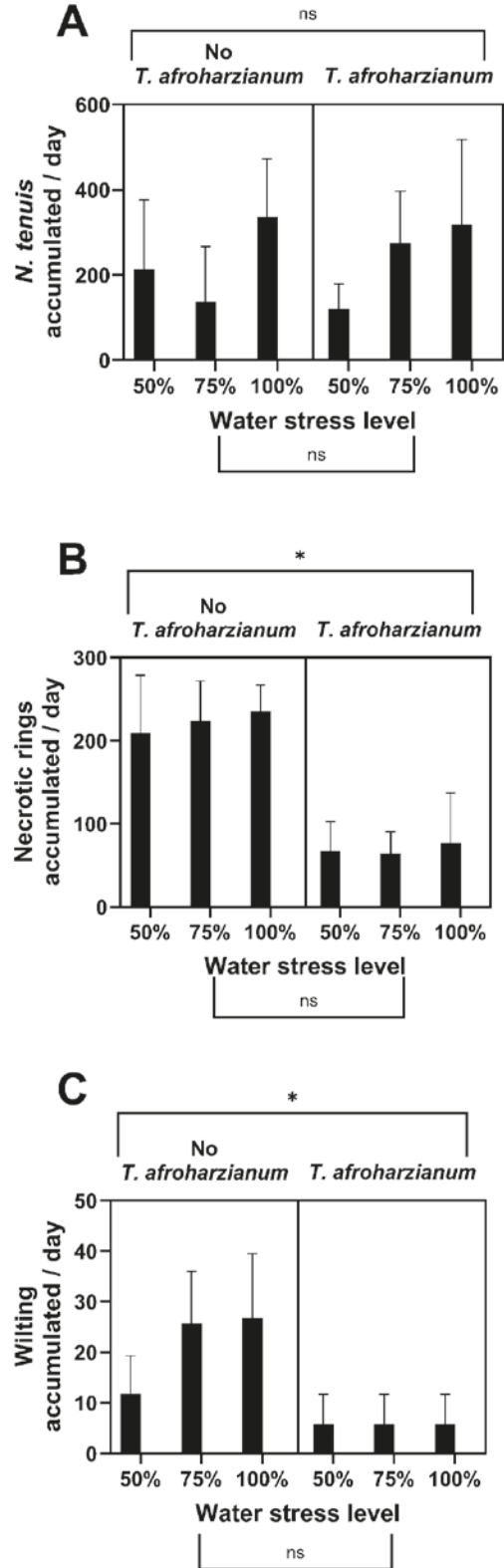

Fig. 2. (A) Number of *N. tenuis* (±SE), (B) number of necrotic rings (±SE) and number of wiltings, (C) accumulated per day for the three treatments considered in the experiment: 1) varying levels of water stress, 2) *T. afroharzianum* T-22 inoculation or non-inoculation, and 3) *N. tenuis* release or non-release (Three-way ANOVA; Tukey test, $P < 0.05$).

lower in the plants that had been inoculated with *T. afroharzianum* T-22 compared to those that had not (−69% and −72%, respectively) (Fig. 2B, C). Water stress did not affect the damage caused by *N. tenuis* in the tomato plants (necrotic rings: $F_{2, 30} = 0.074$; $P = 0.929$; Wilting: $F_{2, 30} = 0.499$; $P = 0.612$). None of the interactions between the studied factors were significant for the *N. tenuis*, necrotic rings, and wilting variables.

3.3 Quantification of callose and glucanase activity and callose deposition histochemistry

The phytophagy of *N. tenuis* increased the amount of callose in the apical part of the tomato plant compared to control plants without *N. tenuis* ($F_{1, 60} = 76.56$; $P < 0.001$) (Fig. 3A). In contrast, plants inoculated with *T. afroharzianum* T-22 had lower callose levels than those not inoculated ($F_{1, 60} = 7.702$; $P = 0.007$). The different levels of water stress did not affect the amount of callose ($F_{2, 60} = 0.042$; $P = 0.959$). The interaction between *T. afroharzianum* T-22 and *N. tenuis* was significant ($F_{1, 60} = 7.413$; $P = 0.008$), as can be observed in Fig. 3A. The inoculation of *T. afroharzianum* T-22 led to a smaller increase in callose in the *N. tenuis* treatment compared to the condition without *T. afroharzianum* T-22 inoculated. The rest of the interactions were not significant.

On the contrary, glucanase activity was higher in the plants inoculated with *T. afroharzianum* T-22 than in the non-inoculated ones ($F_{1, 60} = 37.09$; $P < 0.001$) (Fig. 3B). Neither the presence of *N. tenuis* ($F_{1, 60} = 0.117$; $P = 0.733$) nor the different levels of water stress ($F_{2, 60} = 0.626$; $P = 0.538$) affected glucanase activity. None of the interactions were significant.

3.4 Callose deposition

The callose deposits in tomato petiole sections at 7 days after the release of *N. tenuis* revealed significant differences among the four treatments studied ($F_{3, 37} = 243.0$; $P < 0.001$) (Fig. 4). Plants without *N. tenuis* inoculation showed low callose deposition in both the *T. afroharzianum* T-22-inoculated plants and control plants compared to those where *N. tenuis* had been released. The plants with *N. tenuis* inoculation displayed strong fluorescence due to callose deposition predominantly accumulating in the vascular tissue. However, there was less callose deposition in the plants with *N. tenuis* that had previously been inoculated with *T. afroharzianum* T-22.

4 Discussion

Our results demonstrated that *T. afroharzianum* T-22 significantly enhanced tomato growth under water stress conditions, consistent with previous research indicating its role in improving plant tolerance to abiotic stress (Woo et al. 2023). Furthermore, the study found that inoculating *T. afroharzianum* T-22 did not negatively impact the establishment of *N.*
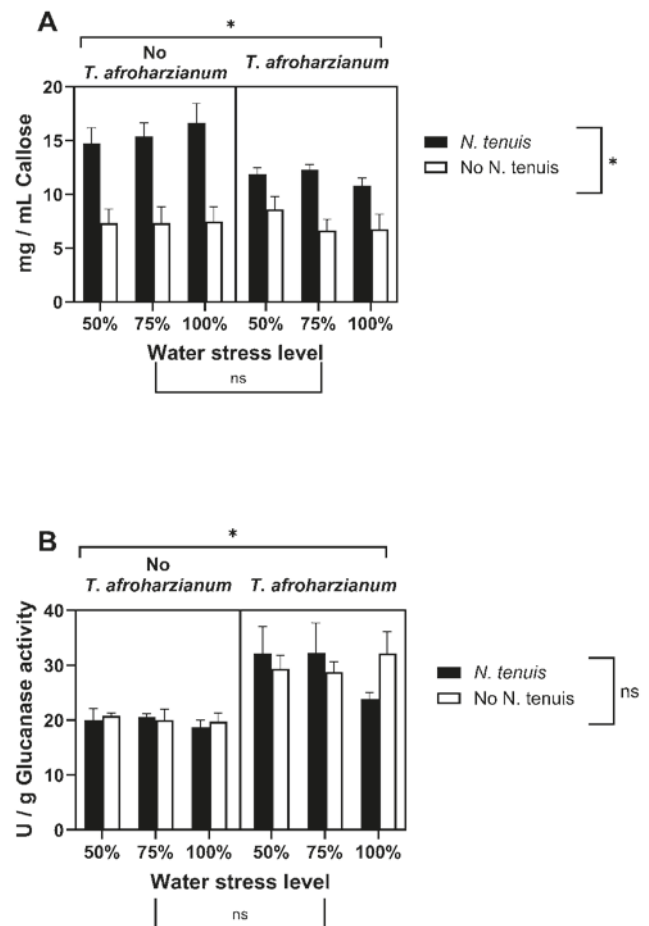
Fig. 3. (A) Amount of callose (mg/mL) (±SE) and glucanase activity (U/g) for the three treatments considered in the experiment: 1) varying levels of water stress, 2) *T. afroharzianum* T-22 inoculation or non-inoculation, and 3) *N. tenuis* release or non-release. The asterisk indicates statistical differences within each factor (ns = not significant) (Three-way ANOVA; Tukey test, $P < 0.05$).

tenuis. Interestingly, *T. afroharzianum* T-22 inoculation also mitigated the negative effects of *N. tenuis* on plant growth and reduced the callose deposition induced by *N. tenuis* in tomato plants. Consequently, this beneficial fungi alleviated damage caused by this predatory mirid, evidenced by decreased necrotic rings and wilting. These results underscore the potential of *T. afroharzianum* T-22 as a valuable tool for enhancing tomato crop growth, with significant implications for agricultural productivity.

In recent years, *N. tenuis* has proven to be a valuable resource for biological pest control in tomato cultivation under Mediterranean conditions in Southern Europe (Pérez-Hedo et al. 2021; van Lenteren et al. 2018). However, its implementation is not without significant challenges. While *N. tenuis* plays an essential role in pest management, its presence can adversely affect tomato crops (Pérez-Hedo & Urbaneja 2016). In this work, the release of *N. tenuis* was associated with reducing the height of tomato plants, a critical indicator
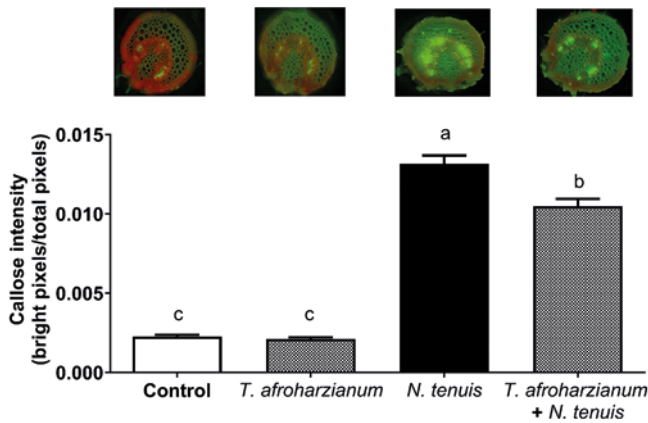
Fig. 4. Stained cross-sections of tomato petioles showing callose deposits under the epifluorescence microscope with a UV filter and quantification of fluorescent deposits corresponding to callose intensity, measured as the average number of bright pixels/total number of pixels using GIMP (GNU Image Manipulation Program) for each of the four treatments studied: control plants, plants inoculated with *T. afroharzianum* T-22, plants inoculated with *N. tenuis*, and plants inoculated with both *T. afroharzianum* T-22 and *N. tenuis*. Significant differences based on ANOVA and Tukey's multiple comparison tests are indicated by different letters ($P < 0.05$).

of plant performance and health. These findings underscore the need to address this dilemma (Pérez-Hedo et al. 2024), as the effectiveness of *N. tenuis* is undeniable in a Mediterranean context, where pests can pose a considerable threat to tomato production. Here, the study proposes that the simultaneous application of *T. afroharzianum* T-22 can offer a practical and balanced solution. The interaction between *T. afroharzianum* T-22 and *N. tenuis* reduces the damage *N. tenuis* causes. The damage caused by *N. tenuis*, such as necrotic rings and wilting, did not increase with reduced water availability in tomato plants. This contrasts with our initial hypothesis, which postulated that decreased water availability would exacerbate the damage caused by *N. tenuis*. This discrepancy suggests that other factors might be influencing the relationship between water stress and the damage caused by *N. tenuis*.

Nesidiocoris tenuis is a cell-rupture feeder (Chinchilla-Ramírez et al. 2021). In this feeding strategy, common among mirids (Wheeler 2000), the insect lacerates plant tissues with its stylet movements and injects watery saliva into surrounding cells, creating pockets of diluted cell contents that are eventually ingested. These punctures, coupled with an increase in callose levels as a response to the damage, trigger an oxidative response in the area where tomato plants are affected by the punctures, leading to the phenotypic appearance of necrotic rings (accumulation of browned cells) (Hori et al. 2000). Callose deposition is a common plant defense response to wounding and pathogen attack, acting as a physical barrier to limit the spread of damage and infection. However, excessive callose accumulation can impede cellular repair and recovery. In this study, we observed that *T.*

afroharzianum T-22-inoculated plants exhibit an increase in β-1,3-glucanase activity. β-1,3-glucanase plays a crucial role in degrading callose deposits, facilitating cell wall remodeling and repair processes. This enzymatic activity helps to alleviate the negative effects of excessive callose accumulation, promoting faster healing of damaged tissues. Our results demonstrated that the interaction between *T. afroharzianum* T-22 and *N. tenuis* was significant. Specifically, the inoculation of *T. afroharzianum* T-22 led to a greater reduction in callose levels in the *N. tenuis* treatment compared to the absence of *T. afroharzianum* T-22. This indicates that *T. afroharzianum* T-22-inoculated plants showed less damage and lower callose contents. The increased β-1,3-glucanase activity observed in *T. afroharzianum* T-22-inoculated plants likely facilitated the degradation of callose deposits, aiding in the repair of cell damage caused by *N. tenuis* feeding. By reducing callose accumulation, *T. afroharzianum* T-22 helps to prevent the formation of necrotic rings and promotes faster healing of the punctures inflicted by *N. tenuis*.

Research on *Trichoderma* species, such as *T. afroharzianum* T-22 and *Trichoderma asperellum*, has revealed their capacity to produce several cell wall-degrading enzymes, including β-1,3 glucanase (Almeida et al. 2007; Noronha et al. 2000; Ramada et al. 2010). The evaluation of production and activity of β-1,3-glucanase represents important parameters to be analyzed during the screening of efficient candidates of *Trichoderma* spp. for use as a biocontrol agent (Almeida et al. 2007).

Trichoderma afroharzianum T-22 triggers various defense mechanisms in host plants (Di Lelio et al. 2023; Martínez-Medina et al. 2013). These mechanisms include the induction of inducible defense systems, the production of secondary metabolites, increased levels of defensive enzymes, the initiation of systemic acquired resistance (SAR), and the modulation of hormonal signaling pathways, such as salicylic acid (SA) and jasmonic acid (JA) (Woo et al. 2023). On the other hand, *N. tenuis* activates the abscisic acid and jasmonic acid (JA) signaling pathways, leading to a series of remarkable plant responses (Pérez-Hedo et al. 2015). Therefore, exploring the potential synergistic effects when both *T. afroharzianum* T-22 and *N. tenuis* are used together to induce plant defenses is particularly interesting. The impact of both *T. afroharzianum* T-22 and *N. tenuis* varies depending on the tomato cultivar, highlighting the need to investigate this synergistic effect across different tomato cultivars. Previous studies have indicated that while *Trichoderma* spp. inoculation can reduce pest (or predator, as in this study) damage to tomato plants and may also positively influence their population growth. For instance, *Trichoderma* spp. has been shown to increase insect pest population growth and attractiveness to natural enemies such as predatory mirids, leading to faster development of the predator (Caccavo et al. 2022; Coppola et al. 2017). This dual effect could have unpredictable implications for integrated pest management. This underscores the complexity of *Trichoderma* spp. interactions and the

need for careful consideration when integrating it into pest management strategies.

While the results of this study are encouraging and suggest a promising path for using *T. afroharzianum* T-22 in tomato cultivation alongside a pest management strategy based on the use of *N. tenuis*, it is essential to acknowledge that laboratory research provides only a partial view of agricultural reality. For these findings to become standard practices and translate into tangible benefits for farmers and the environment, conducting further research under field conditions is crucial. We have already initiated this validation in commercial crop fields. Although the results of these field investigations have not yet been published, initial indications are promising, and the results obtained in this study have already been confirmed (Unpublished results). We have also observed how *N. tenuis* has effectively maintained key pests, such as *T. absoluta* and *B. tabaci*, under control.

The combination of *T. afroharzianum* T-22 and *N. tenuis*, with their benefits for tomato growth and pest control, is a promising example of how a holistic approach can contribute to sustainable agriculture. This balanced strategy promises greater crop resilience in the face of climate change and provides a roadmap for ensuring food security in a warming world.

References

Allen, R. G., Pereira, L. S., Raes, D., & Smith, M. (1998). Crop evapotranspiration-Guidelines for computing crop water requirements-FAO Irrigation and drainage paper 56. *Fao, Rome, 300*(9), D05109.

Almeida, F. B. dos R., Cerqueira, F. M., Silva, R., Ulhoa, C. J., & Lima, A. L. (2007). Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: Evaluation of coiling and hydrolytic enzyme production. *Biotechnology Letters, 29*(8), 1189–1193. https://doi.org/10.1007/s10529-007-9372-z

Altieri, M. A., & Nicholls, C. I. (2017). The adaptation and mitigation potential of traditional agriculture in a changing climate. *Climatic Change, 140*(1), 33–45. https://doi.org/10.1007/s10584-013-0909-y

Caccavo, V., Forlano, P., Mang, S., Fanti, P., Nuzzaci, M., Battaglia, D., & Trotta, V. (2022). Effects of *Trichoderma harzianum* strain T22 on the arthropod community associated with tomato plants and on the crop performance in an experimental field. *Insects, 13*(5), 418. https://doi.org/10.3390/insects13050418

Calvo, J., Bolckmans, K., Stansly, P. A., & Urbaneja, A. (2009). Predation by *Nesidiocoris tenuis* on *Bemisia tabaci* and injury to tomato. *BioControl, 54*(2), 237–246. https://doi.org/10.1007/s10526-008-9164-y

Chaverri, P., Branco-Rocha, F., Jaklitsch, W., Gazis, R., Degenkolb, T., & Samuels, G. J. (2015). Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. *Mycologia, 107*(3), 558–590. https://doi.org/10.3852/14-147

Chinchilla-Ramírez, M., Garzo, E., Fereres, A., Gavara-Vidal, J., ten Broeke, C. J. M., van Loon, J. J. A., … Pérez-Hedo, M. (2021). Plant feeding by *Nesidiocoris tenuis*: Quantifying its behavioral and mechanical components. *Biological Control, 152*, 104402. https://doi.org/10.1016/j.biocontrol.2020.104402

Coppola, M., Cascone, P., Chiusano, M. L., Colantuono, C., Lorito, M., Pennacchio, F., … Digilio, M. C. (2017). *Trichoderma harzianum* enhances tomato indirect defense against aphids. *Insect Science, 24*(6), 1025–1033. https://doi.org/10.1111/1744-7917.12475

Denault, L. J., Glenister, P. R., & Chau, S. (1981). Enzymology of the mashing step during beer production. *Journal of the American Society of Brewing Chemists, 39*(2), 46–52. https://doi.org/10.1094/ASBCJ-39-0046

Depalo, L., Gallego, C., Ortells-Fabra, R., Salas, C., Montalt, R., Urbaneja, A., & Pérez-Hedo, M. (2024). Advancing tomato crop protection: Green leaf volatile-mediated defense mechanisms against *Nesidiocoris tenuis* plant damage. *Biological Control, 192*, 105517. https://doi.org/10.1016/j.biocontrol.2024.105517

Di Lelio, I., Forni, G., Magoga, G., Brunetti, M., Bruno, D., Becchimanzi, A., … Pennacchio, F. (2023). A soil fungus confers plant resistance against a phytophagous insect by disrupting the symbiotic role of its gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America, 120*(10), e2216922120. https://doi.org/10.1073/pnas.2216922120

English-Loeb, G., Stout, M. J., & Duffey, S. S. (1997). Drought stress in tomatoes: Changes in plant chemistry and potential nonlinear consequences for insect herbivores. *Oikos, 79*(3), 456–468. https://doi.org/10.2307/3546888

Flores-Saavedra, M., Plazas, M., Vilanova, S., Prohens, J., & Gramazio, P. (2023). Induction of water stress in major *Solanum* crops: A review on methodologies and their application for identifying drought tolerant materials. *Scientia Horticulturae, 318*, 112105. https://doi.org/10.1016/j.scienta.2023.112105

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004). *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nature Reviews. Microbiology, 2*(1), 43–56. https://doi.org/10.1038/nrmicro797

Hori, K., Schaefer, C. W., & Panizzi, A. R. (2000). Possible causes of disease symptoms resulting from the feeding of phytophagous Heteroptera. *Heteroptera of Economic Importance*, 11–35.

Johansen, D. A. (1940). *Plant Microtechnique*. New York, NY, USA: McGraw-Hill Book Company, Inc.

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., & Pozo, M. J. (2012). Mycorrhiza-Induced Resistance and Priming of Plant Defenses. *Journal of Chemical Ecology, 38*(6), 651–664. https://doi.org/10.1007/s10886-012-0134-6

Köhle, H., Jeblick, W., Poten, F., Blaschek, W., & Kauss, H. (1985). Chitosan-elicited callose synthesis in soybean cells as a Ca²⁺-dependent process. *Plant Physiology, 77*(3), 544–551. https://doi.org/10.1104/pp.77.3.544

Koppert, B. S. (2023). *Trianum-P*. https://www.Koppert.Es/Trianum-p.

Martínez-Medina, A., Fernández, I., Sánchez-Guzmán, M. J., Jung, S. C., Pascual, J. A., & Pozo, M. J. (2013). Deciphering the hormonal signalling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. *Frontiers in Plant Science, 4*(206). https://doi.org/10.3389/fpls.2013.00206

Mastouri, F., Björkman, T., & Harman, G. E. (2012). *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. *Molecular Plant-Microbe Interactions, 25*(9), 1264–1271. https://doi.org/10.1094/MPMI-09-11-0240

Moerkens, R., Pekas, A., Bellinkx, S., Hanssen, I., Huysmans, M., Bosmans, L., & Wäckers, F. (2020). *Nesidiocoris tenuis* as a pest in Northwest Europe: Intervention threshold and influence of Pepino mosaic virus. *Journal of Applied Entomology, 144*(7), 566–577. https://doi.org/10.1111/jen.12789

Mollá, O., González-Cabrera, J., & Urbaneja, A. (2011). The combined use of *Bacillus thuringiensis* and *Nesidiocoris tenuis* against the tomato borer *Tuta absoluta. BioControl, 56*(6), 883–891. https://doi.org/10.1007/s10526-011-9353-y

Noronha, E. F., Kipnis, A., Junqueira-Kipnis, A. P., & Ulhoa, C. J. (2000). Regulation of 36-kDa β-1, 3-glucanase synthesis in *Trichoderma harzianum. FEMS Microbiology Letters, 188*(1), 19–22. https://doi.org/10.1016/S0378-1097(00)00205-6

Pérez-Hedo, M., Riahi, C., & Urbaneja, A. (2021). Use of zoophytophagous mirid bugs in horticultural crops: Current challenges and future perspectives. *Pest Management Science, 77*(1), 33–42. https://doi.org/10.1002/ps.6043

Pérez-Hedo, M., & Urbaneja, A. (2016). The zoophytophagous predator *Nesidiocoris tenuis*: a successful but controversial biocontrol agent in tomato crops. In A. R. Horowitz & I. Ishaaya (Eds.), *Advances in Insect Control and Resistance Management* (pp. 121–138). Springer International Publishing. https://doi.org/10.1007/978-3-319-31800-4_7

Pérez-Hedo, M., Bouagga, S., Jaques, J. A., Flors, V., & Urbaneja, A., & the Pérez-Hedo. (2015). Tomato plant responses to feeding behavior of three zoophytophagous predators (Hemiptera: Miridae). *Biological Control, 86*, 46–51. https://doi.org/10.1016/j.biocontrol.2015.04.006

Pérez-Hedo, M., Ortells-Fabra, R., Alonso-Valiente, M., Ruíz-Rivero, O., & Urbaneja, A., & the Pérez-Hedo. (2024). Enhancing the biocontrol potential of the predator *Nesidiocoris tenuis* through genetic selection. *Biological Control, 188*, 105413. https://doi.org/10.1016/j.biocontrol.2023.105413

Pérez-Hedo, M., Suay, R., Alonso, M., Ruocco, M., Giorgini, M., Poncet, C., & Urbaneja, A., & the Pérez-Hedo. (2017). Resilience and robustness of IPM in protected horticulture in the face of potential invasive pests. *Crop Protection (Guildford, Surrey), 97*, 119–127. https://doi.org/10.1016/j.cropro.2016.11.001

Pérez-Hedo, M., Urbaneja-Bernat, P., Jaques, J. A., Flors, V., & Urbaneja, A., & the Pérez-Hedo. (2015). Defensive plant responses induced by *Nesidiocoris tenuis* (Hemiptera: Miridae) on tomato plants. *Journal of Pest Science, 88*(3), 543–554. https://doi.org/10.1007/s10340-014-0640-0

Ramada, M. H. S., Lopes, F. Á. C., Ulhoa, C. J., & do Nascimento Silva, R. (2010). Optimized microplate β-1, 3-glucanase assay system for *Trichoderma* spp. screening. *Journal of Microbiological Methods, 81*(1), 6–10. https://doi.org/10.1016/j.mimet.2010.01.010

Sánchez, J. A. (2008). Zoophytophagy in the plantbug *Nesidiocoris tenuis. Agricultural and Forest Entomology, 10*(2), 75–80. https://doi.org/10.1111/j.1461-9563.2007.00357.x

Sato, S., Kamiyama, M., Iwata, T., Makita, N., Furukawa, H., & Ikeda, H. (2006). Moderate increase of mean daily temperature adversely affects fruit set of *Lycopersicon esculentum* by disrupting specific physiological processes in male reproductive development. *Annals of Botany, 97*(5), 731–738. https://doi.org/10.1093/aob/mcl037

Scalschi, L., Llorens, E., Camañés, G., Pastor, V., Fernández-Crespo, E., Flors, V., … Vicedo, B. (2015). Quantification of callose deposition in plant leaves. *Bio-Protocol, 5*(19). https://doi.org/10.21769/BioProtoc.1610

Stansly, P. A., Calvo, F. J., & Urbaneja, A. (2005a). Augmentative biological control of *Bemisia tabaci* biotype "Q" in Spanish greenhouse pepper production using *Eretmocerus* spp. *Crop Protection (Guildford, Surrey), 24*(9), 829–835. https://doi.org/10.1016/j.cropro.2005.01.010

Stansly, P. A., Calvo, J., & Urbaneja, A. (2005b). Release rates for control of *Bemisia tabaci* (Homoptera: Aleyrodidae) biotype "Q" with *Eretmocerus mundus* (Hymenoptera: Aphelinidae) in greenhouse tomato and pepper. *Biological Control, 35*(2), 124–133. https://doi.org/10.1016/j.biocontrol.2005.07.004

Tilman, D. (1999). Global environmental impacts of agricultural expansion: The need for sustainable and efficient practices. *Proceedings of the National Academy of Sciences of the United States of America, 96*(11), 5995–6000. https://doi.org/10.1073/pnas.96.11.5995

Urbaneja, A., González-Cabrera, J., Arnó, J., & Gabarra, R. (2012). Prospects for the biological control of *Tuta absoluta* in tomatoes of the Mediterranean basin. *Pest Management Science, 68*(9), 1215–1222. https://doi.org/10.1002/ps.3344

van Lenteren, J. C., Bolckmans, K., Köhl, J., Ravensberg, W. J., & Urbaneja, A. (2018). Biological control using invertebrates and microorganisms: Plenty of new opportunities. *BioControl, 63*(1), 39–59. https://doi.org/10.1007/s10526-017-9801-4

Vimal, S. R., Singh, J. S., Arora, N. K., & Singh, S. (2017). Soil-plant-microbe interactions in stressed agriculture management: A review. *Pedosphere, 27*(2), 177–192. https://doi.org/10.1016/S1002-0160(17)60309-6

Wheeler, A. (2000). Plant bugs (Miridae) as pests. In C. W. Schaefer & A. R. Panizzi (Eds.), *Heteroptera of economic importance* (pp. 37–84). CRC Press.

Woo, S. L., Hermosa, R., Lorito, M., & Monte, E. (2023). *Trichoderma*: A multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. *Nature Reviews. Microbiology, 21*(5), 312–326. https://doi.org/10.1038/s41579-022-00819-5

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Fig. S1