

## Perspective

## Advancing tomato crop protection: Green leaf volatile-mediated defense mechanisms against *Nesidiocoris tenuis* plant damage

Laura Depalo<sup>a</sup>, Carolina Gallego<sup>b</sup>, Raúl Ortells-Fabra<sup>b</sup>, Carlos Salas<sup>b</sup>, Rafael Montalt<sup>b</sup>, Alberto Urbaneja<sup>b</sup>, Meritxell Pérez-Hedo<sup>b,\*</sup>

<sup>a</sup> Department of Agricultural and Food Sciences, Alma Mater Studiorum-Università di Bologna, Viale G. Fanin, 42, 40127 Bologna, Italy

<sup>b</sup> Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología, (IVIA), CV-315, Km 10.7, 46113 Moncada, Valencia, Spain

## HIGHLIGHTS

- *Nesidiocoris tenuis*: highly efficient as biological control agent but damages tomato plants.
- Tomato plant defenses are activated by the GLV (Z)-3-HP.
- Increasing plant defenses reduces *N. tenuis* damage.
- Reduced callose deposition limits tissue damage.

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## ABSTRACT

Although *Nesidiocoris tenuis* is highly effective as a biological control agent, it can also damage tomato plants due to its zoophytophagous behavior. When *N. tenuis* pierces the stems and petioles of tomato plants with its stylets, it triggers callose deposition and subsequent cell death, resulting in blocked nutrient transport, floral abortions, or wilting of tender shoots. Recently, it has been shown that exposure of tomato plants to the green leaf volatile (Z)-3-hexenyl propanoate [(Z)-3-HP] activates defensive mechanisms, including the regulation of genes involved in the synthesis and degradation of callose. In this study, conducted under greenhouse conditions, we tested the hypothesis that damage caused by *N. tenuis* could be reduced by exposing tomato plants to (Z)-3-HP through polymeric dispensers. Tomato plants exposed to (Z)-3-HP and non-exposed control plants were inoculated with *N. tenuis*. *Nesidiocoris tenuis* established in both groups with no significant differences between the two treatments. However, as hypothesized, the damage caused by *N. tenuis* was significantly lower in the plants exposed to (Z)-3-HP. Gene expression analysis of salicylic, jasmonic, and abscisic acids, along with histochemical staining methods, was used to compare the defensive responses of tomato plants infested solely with *N. tenuis* versus those infested with *N. tenuis* and exposed to (Z)-3-HP. Our findings confirm the influence of (Z)-3-HP exposure on differential defensive activation between treatments and reduced callose deposition in (Z)-3-HP-exposed plants. These results pave the way for improved management of *N. tenuis* by enhancing the plant's defenses based on inter-plant communication.

## 1. Introduction

The application of zoophytophagous predatory bugs in vegetable crops is widely used around the world for biological control against whiteflies, lepidopterans, aphids, and leafminers (Urbaneja et al., 2022). *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) is one of the most effective predatory mirid bugs, although it is associated with some negative effects due to its facultative phytophagy. This insect

spontaneously colonizes agroecosystems in the presence of prey. *Nesidiocoris tenuis* is also mass-reared and commercially utilized, which makes it a valuable component of integrated pest management (IPM) programs in Southern Europe (Pérez-Hedo et al., 2021a; van Lenteren et al., 2018). Nonetheless, in the absence of prey, its presence can transition from desirable to unwanted due to the significant damage it can cause to plants (Castañé et al., 2011; Chinchilla-Ramírez et al., 2021; Hall et al., 2021; Pérez-Hedo et al., 2024; Sanchez and Lacasa, 2008).

\* Corresponding author.

E-mail address: [perez\\_merhed@gva.es](mailto:perez_merhed@gva.es) (M. Pérez-Hedo).

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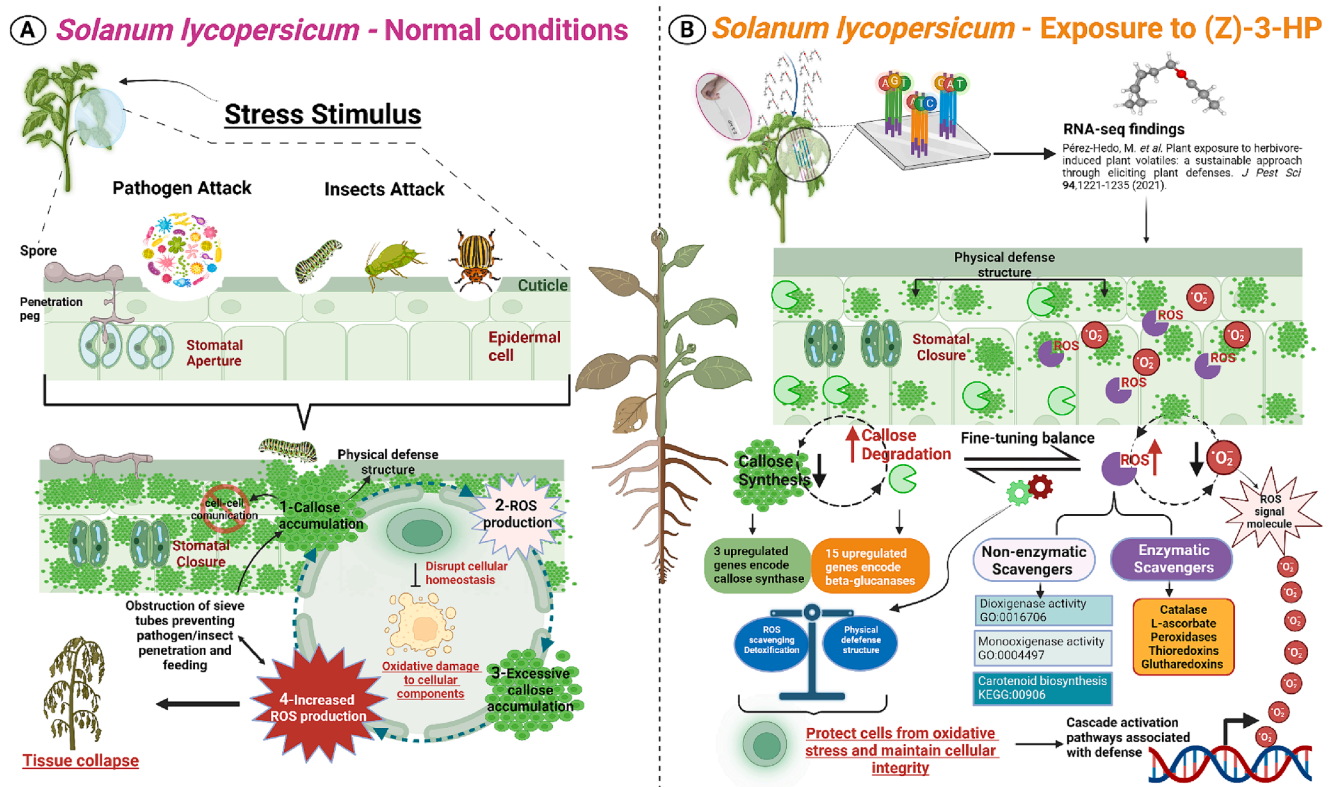
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The development of necrotic rings on stems, the abortion of small fruits and flowers, reduced vegetative growth, and blemishes on fruits are typical symptoms that occur in tomatoes as a result of *N. tenuis*'s phytophagy (Pérez-Hedo and Urbaneja, 2016). Insecticide applications are necessary in cases of high population levels (Biondi et al., 2016; Ingels et al., 2022; Moerkens et al., 2020). Therefore, the equilibrium of this tritrophic system, which is composed of plants, prey, and predators, must be carefully managed to maintain effective biological control while also minimizing damage from phytophagy (Desneux et al., 2022; Giorgini et al., 2019; Moerkens et al., 2020; Pérez-Hedo et al., 2017; Thominé et al., 2020; Yao et al., 2022).

Recent studies have demonstrated that the phytophagy of predatory mirid bugs triggers the release of herbivore-induced plant volatiles (HIPVs) (Pérez-Hedo et al., 2022). These HIPVs can repel or attract herbivores and their natural enemies and even alert neighboring plants to activate similar defense mechanisms (Pérez-Hedo et al., 2015; Pérez-Hedo et al., 2018b). The activation of these plant defenses can be instrumental in managing pests and disease infestations (Bouagga et al., 2020; Lins et al., 2014; Pappas et al., 2017, 2016, 2015; Pérez-Hedo et al., 2018a). In a further step, polymeric dispensers releasing a synthetic version of the mirid-induced plant volatile, (Z)-3-hexenyl propanoate [(Z)-3-HP from here onwards] at a constant rate, were applied in commercial tomato greenhouses for over two months to activate defenses in exposed plants. The exposition of this volatile resulted in a 60% reduction in damage from *Tuta absoluta* (Meyrick) (Lepidoptera:

Gelichiidae) without negatively impacting plant growth and yield production (Pérez-Hedo et al., 2021b). Furthermore, synthetic versions of this HIPV have been tested as inducers of plant defense in tomatoes and sweet peppers, yielding promising results (Depalo et al., 2022; Pérez-Hedo et al., 2018b; Riahi et al., 2022; Silva et al., 2021). Activating defenses in tomato plants can have additional positive effects for pest and disease management, such as increased trichome density (Riahi et al., 2023).

The exposure of tomato plants to (Z)-3-HP has been demonstrated to initiate a sophisticated molecular response within the plants (Pérez-Hedo et al., 2021b). Among other effects, exposure to (Z)-3-HP significantly influences gene expression related to both the synthesis and degradation of callose (Fig. 1). Furthermore, exposure to (Z)-3-HP initiates a significant increase in antioxidant defenses by upregulating the genes that encode enzymes such as ascorbate peroxidase and glutathione S-transferases. These enzymes are crucial in neutralizing reactive oxygen species (ROS) and managing oxidative stress effectively, as depicted in Fig. 1B. The adaptive response extends beyond the activation of antioxidant pathways to also include the regulation of processes essential for tissue repair and the suppression of pathways that could lead to cell death. This finely tuned modulation of defense pathways highlights the sophisticated adaptive mechanisms that tomato plants utilize to enhance their survival and recovery from biotic stress. These findings led to the hypothesis that activating the defenses of the tomato plant through (Z)-3-HP exposure would reduce the damage caused by



**Fig. 1.** Flowchart of comparative defense responses of *Solanum lycopersicum* under biotic stress and exposure to (Z)-3-hexenyl propanoate, adapted from Perez-Hedo et al. (2021b). (A) Flowchart depicting the defense response of *S. lycopersicum* under normal conditions and biotic stress. The stress stimulus initiates the response, inducing both callose deposition and the generation of reactive oxygen species (ROS) as integral components of the plant's defense mechanism. This process may culminate in the over-accumulation of callose, subsequently amplifying ROS production. These cascading events contribute to oxidative stress, which ultimately leads to cellular damage. (B) Flowchart illustrating the defense response of *S. lycopersicum* upon exposure to (Z)-3-HP. The stimulus triggers the activation of a significant number of genes associated with the antioxidant response, leading to the mobilization of antioxidant machinery aimed at scavenging and neutralizing ROS. Concurrently, activating genes involved in callose degradation and remodeling facilitates restoring the cell wall's structural integrity and functionality. This concerted effort culminates in the recovery of cell wall integrity, enabling the plant to mitigate the effects of stress and resume its regular growth and developmental processes.

*N. tenuis*.

In this study, we assessed the establishment of *N. tenuis* and the resulting injury to tomato plants primed for defense by exposure to (Z)-3-HP. Gene expression analysis was performed to determine the defensive response of tomato plants infested solely with *N. tenuis* versus those inoculated with *N. tenuis* and exposed to (Z)-3-HP. Additionally, histochemical staining methods were employed to identify and quantify the physiological strategies that tomato plants adopt to cope with damage.

## 2. Materials and methods

### 2.1. Plants, insects, and HIPV

Pesticide-free *Solanum lycopersicum* cv. Moneymaker plants were used in all the experiments. Two weeks after germination, the plants were individually transplanted into plastic pots measuring 8 × 8 × 8 cm and maintained in a climate-controlled chamber at the Instituto Valenciano de Investigaciones Agrarias (IVIA) at a temperature of 25 ± 2 °C, relative humidity (RH) 65 ± 10%, and a photoperiod of 14:10 h (L:D). Plants used in the bioassays were approximately four weeks old and about 20 cm in height.

Fourth-instar nymphs of *N. tenuis* were provided directly by Koppert Biological Systems, S.L. (Águilas, Spain) and fed *ad libitum* with eggs of *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae) until they reached adulthood. Adults, 0–48 h old, were used in the experiments.

The synthetic standard of the volatile compound (Z)-3-HP (purity > 97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. *Nesidiocoris tenuis* development and plant damage

The phytophagous activity of *N. tenuis* was evaluated in a greenhouse experiment involving tomato plants subjected to continuous exposure to (Z)-3-HP [Nt+(Z)-3-HP plants] and control plants not exposed (Nt plants). The trial was conducted in Moncada, Valencia, Spain (39°35'20.1"N 0°23'49.8"W), from September to October 2022, under a temperature range of 25 ± 3 °C, relative humidity of 65 ± 10%, and natural photoperiod. Each group of Nt+(Z)-3-HP plants and Nt plants was housed in separate greenhouse chambers with identical environmental characteristics and conditions to avoid interference from volatiles between the two treatments.

Six cages (60 cm × 60 cm × 60 cm) (BugDorm-2 insect tents, MegaView Science Co., Ltd., Taichung, Taiwan) each containing four tomato plants, were placed within each chamber.

Plants were continuously exposed to (Z)-3-HP using low-density polymeric dispensers (Kartell, Fisher Scientific SL, Madrid, Spain) that maintained a constant release rate of 9.6 mg/day (Pérez-Hedo et al., 2021b). Each dispenser was filled with cotton wool soaked in 1 ml of pure (Z)-3-HP. One dispenser was hung using a wire 15 cm above the plants within each cage, while an empty dispenser was hung within each control treatment cage.

Forty-eight hours after the plants were setup, 22 couples of *N. tenuis* were released into each cage. *Nesidiocoris tenuis* was used at a dose exceeding the recommended amount for augmentation into greenhouses [0.5–1 individual per plant (Pérez-Hedo et al., 2021b)], to ensure plant damage. Individuals of *Nesidiocoris tenuis* were fed *ad libitum* throughout

the experiment with *E. kuehniella* eggs sprinkled on the plants.

After releasing the predators, each plant in each cage was sampled at seven-day intervals over five weeks to count the number of nymphs produced by the released adults, the number of adults that survived, the number of necrotic rings (distinguishing between mild and severe damage), the number of stems/petioles bent as result of necrotic rings, and the number of aborted stems/petioles due to necrotic rings. A ring was considered mild if it did not completely encircle the stem, while a ring was considered severe if it developed over the entire circumference of the stem. This distinction also coincided with the width of the ring, which was less developed in mild cases than in severe ones, where numerous necrosed cells were already observed. Criteria for differentiating the damage gradient caused by *N. tenuis* on tomato plants are illustrated in Fig. 3.

### 2.3. Defensive gene expression

Gene expression analysis of three marker genes was conducted to compare the defensive responses of three experimental treatments under the same greenhouse conditions detailed above. The two treatments mentioned earlier, consisting of Nt+(Z)-3-HP plants and Nt plants, were compared to control plants (without *N. tenuis* inoculation or exposure to (Z)-3-HP).

Nt+(Z)-3-HP plants and Nt plants were placed in the same chamber described in the previous trial. Control plants were maintained in a separate chamber to prevent potential contamination from volatiles, ensuring identical conditions. The marker genes used as indicators for the activation of abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) signaling pathways were: *ASR1* (abscisic acid stress ripening protein 1), *PIN2* (wound-induced proteinase inhibitor II precursor), and *PR1* (pathogenesis-related protein precursor), respectively.

Each treatment consisted of four cages (60 × 60 × 60 cm; BugDorm-2 insect tent), each containing seven tomato plants, with one dispenser hung as previously described. Forty-eight hours after plant set up, one *N. tenuis* couple per plant was released into each cage of both Nt+(Z)-3-HP plants and Nt plants treatments. Eggs of *E. kuehniella* were also added *ad libitum* in the control treatment.

Samples of the apical part of each plant were randomly collected at 2, 7, 14, and 21 days after the release of *N. tenuis*. The samples were ground in liquid nitrogen for RNA extraction using NZYol (NZYTech, Lisbon, Portugal). One microgram (µg) of each RNA sample was treated with the TURBO DNA-free™ Kit (Ambion®, Life Technologies, CA, USA) to remove any contaminating DNA. Reverse transcription (RT) was performed, and cDNA was synthesized using the PrimeScript™ RT Reagent Kit (TAKARA Bio, CA, USA). Real-time PCR amplification was conducted on the LightCycler® 480 System (Roche Molecular Systems, Inc., Switzerland), using NZYSpeedy qPCR Green Master Mix (2x) (NZYTech, Lisbon, Portugal), following the protocol described by Bouagga et al. (2018). The relative quantification of gene expression was performed using the experimental design and analysis methodology for the Relative Standard Curve Method (Applied Biosystems). The primer sequences for the defensive genes *ASR1*, *PIN2*, *PR1*, and the housekeeping gene *EF1* (Elongator factor 1), used as a standard control gene for normalization, are provided in Table 1 (Pérez-Hedo et al., 2015).

**Table 1**

Primers employed for the quantification of *EF1* (elongation factor-1), *PR1* (pathogenesis-related protein precursor), *ASR1* (abscisic acid stress ripening protein), and *PIN2* (JA-regulated defense protein) genes.

Gene	Primer forward (5' → 3')	Primer reverse (5' → 3')
<i>EF1</i>	5-GATTGGTGGTATTGGAACGTGC-3	5-AGCTTCGTGGTGCATCTC-3
<i>PR1</i>	5-CTCATATGAGACGTGCGAGAAG-3	5-GGAAACAAGAAGATGCAGTACTTAA-3
<i>ASR1</i>	5-ACACCACCACCACCACCTGT-3	5-GTGTGGTGTGCATGTTGTGGA-3
<i>PIN2</i>	5-GAAAATCGTTAATTTATGCCAC-3	5-ACATACAAACTTCCATCTTTA-3

## 2.4. Measuring callose deposition intensity

Callose deposition was examined in plants 21 days after treatment. A total of 8 leaf petiole samples, ranging from 2 to 5 mm in diameter and 10 mm in length, were cut with a scalpel from both Nt+(Z)-3-HP plants and Nt plants, which exhibited severe necrotic rings due to *N. tenuis* damage. These samples were collected and fixed for two weeks in FAA solution composed of 37% formaldehyde, 100% glacial acetic acid, 95% ethanol, and distilled water in a volume ratio of 50:5:10:35 ml (Johansen, 1940). Fixed samples were stored at 4 °C until the histological observations were made. Subsequently, they were rinsed with distilled water to eliminate any residual fixative solution and then sectioned into thin discs approximately 0.5 mm thick using a histological scalpel. Petiole sections were stained in 2 ml Eppendorf tubes containing a stock solution of dark blue methyl blue [(1:1) 0.1% methyl blue:potassium phosphate buffer 1 M, pH 6.5]. After 24 h immersion, the fluorescence of the petiole sections was observed using a Nikon SMZ800N microscope (Nikon Corporation, Kanagawa, Japan) equipped with a CoolLED pE-300Lite integrated epifluorescence system with a GFP-L filter. The images were captured using an XM full HD995 Nikon digital microscopy camera. Callose deposits generate a bright green fluorescence color under UV light. The amount of callose deposited was quantified by measuring the fluorescence area in each petiole section following the method outlined by Scalschi et al. (2015). Analysis of fluorescent deposits corresponding to stained callose was conducted by analyzing the number of pixels using GIMP (GNU Image Manipulation Program).

## 2.5. Statistical analyses

All statistical analyses were conducted in GraphPad Prism 9.3.1.471 for Windows (GraphPad Software, San Diego, California, United States). A two-way ANOVA with repeated measures was run to compare the number of nymphs and adults of *N. tenuis*, and the occurrence of different necrotic rings caused by *N. tenuis*, between Nt+(Z)-3-HP plants and Nt plants. The significance level was set at  $P < 0.05$ , with comparisons of means performed using Tukey's test. The "time" factor was considered fixed, together with the "treatment" factor, to assess the impact of different dates and treatments on the measurements. Additionally, the interaction between date and treatment was investigated. For each time interval, data on the relative expression of defense genes and callose deposition were analyzed using one-way ANOVA, followed by a comparison of means using Tukey's test at a significance level of  $P < 0.05$ .

## 3. Results

### 3.1. *Nesidiocoris tenuis* development and plant damage

The numbers of adults and nymphs of *N. tenuis* were not different between Nt+(Z)-3-HP plants and Nt plants (adults:  $F_{1,40} = 0.6075$ ,  $P = 0.459$ ; nymphs:  $F_{1,40} = 2.985$ ,  $P = 0.115$ ) (Fig. 2). In both treatments, the number of *N. tenuis* adults significantly decreased over time ( $F_{1,40} = 80.08$ ,  $P < 0.001$ ) (Fig. 2A), while the number of nymphs of *N. tenuis* significantly increased ( $F_{4,40} = 145.5$ ,  $P < 0.001$ ) (Fig. 2B). No interaction between the two factors was found for either the adults ( $F_{4,40} = 0.798$ ,  $P = 0.538$ ) or the nymphs ( $F_{4,40} = 1.738$ ,  $P = 0.161$ ).

The number of necrotic rings caused by *N. tenuis* was lower in (Z)-3-HP-exposed plants compared to Nt plants ( $F_{1,40} = 11.27$ ,  $P = 0.007$ ) (Fig. 3A). In both treatments, the number of necrotic rings significantly increased over time ( $F_{4,40} = 111.7$ ,  $P < 0.001$ ). However, in the control treatment, the weekly increase was higher, as indicated by the interaction between factors ( $F_{4,40} = 19.73$ ,  $P < 0.001$ ). When analyzing the total number of necrotic rings based on their severity, the number of mild necrotic rings was similar in both treatments ( $F_{1,40} = 0.117$ ,  $P = 0.739$ ) (Fig. 3B). The number of severe rings, bent stems, and aborted

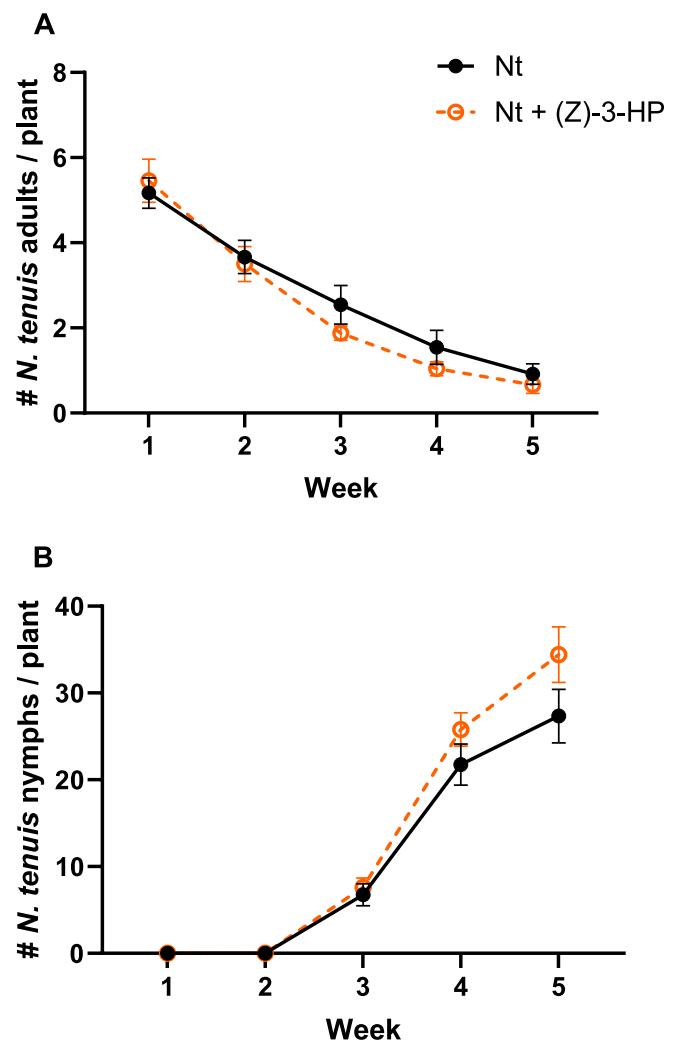
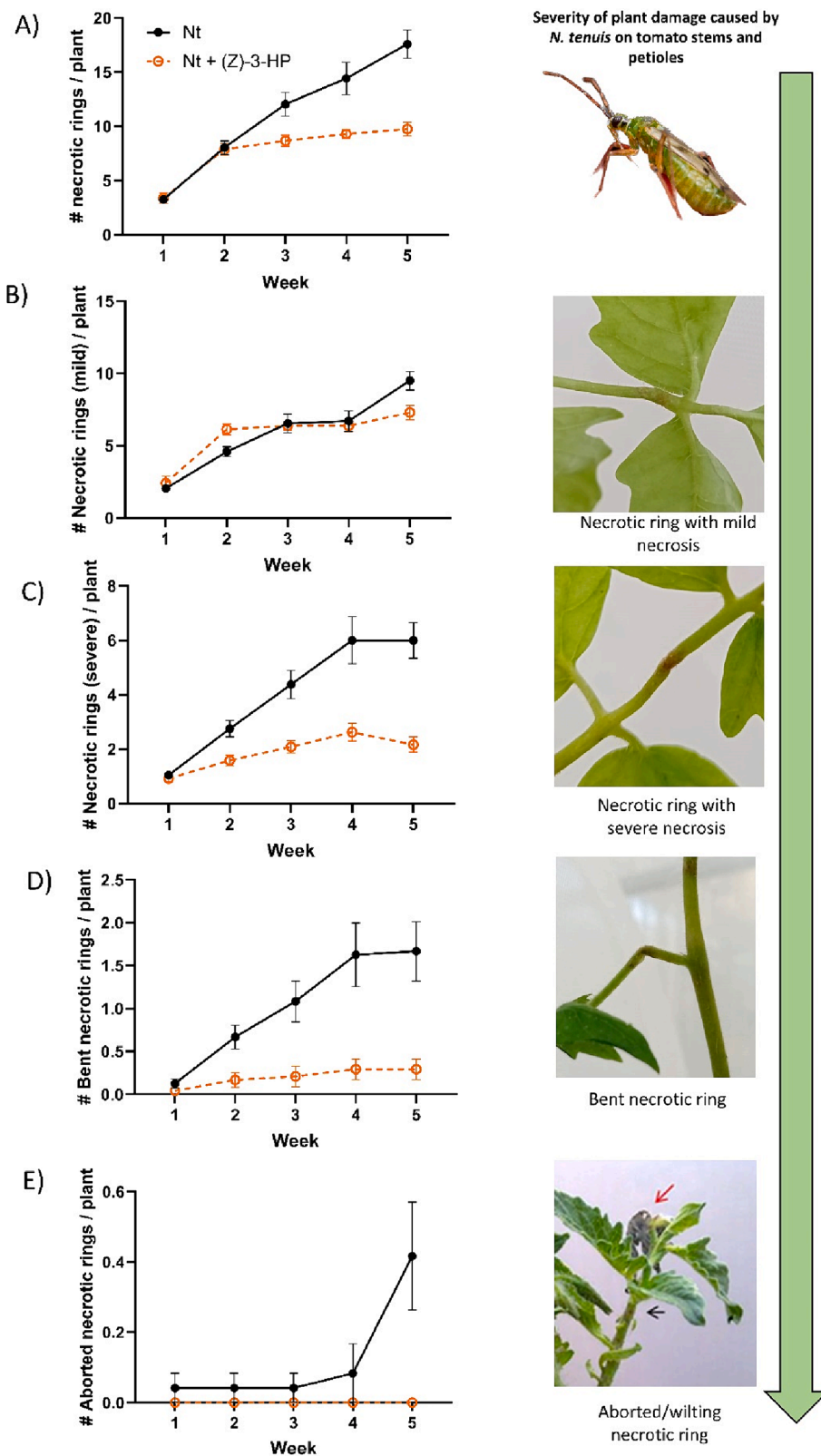


Fig. 2. Number (mean  $\pm$  SE) of nymphs and adults of *Nesidiocoris tenuis* per tomato plant (cv. Moneymaker) when comparing the predatory mirid development in tomato plants exposed to (Z)-3-hexenyl propanoate [(Z)-3-HP] compared to untreated tomato plants (Control).

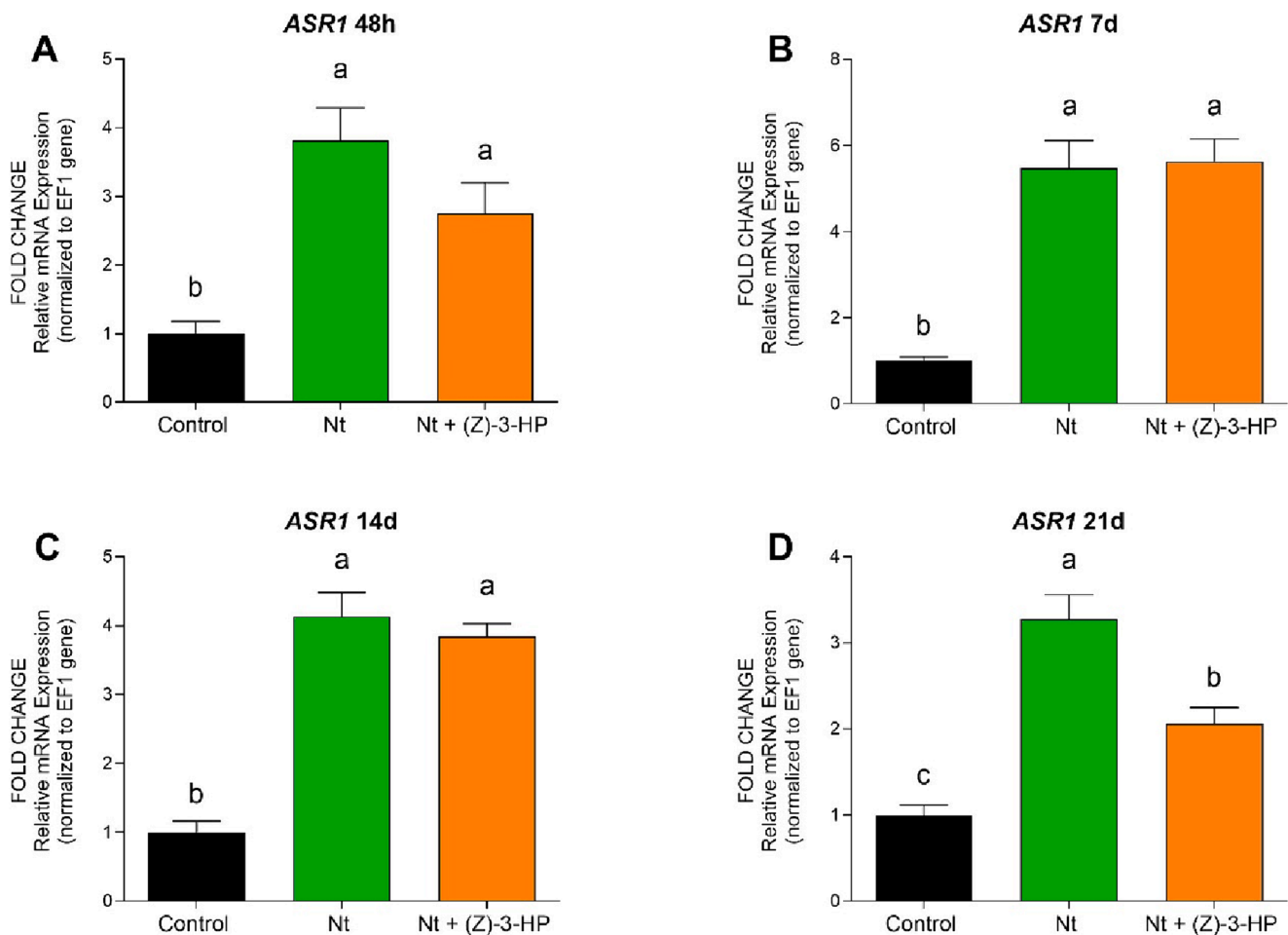
stems, resulting from necrotic rings, were significantly higher in the control treatment ( $F_{1,40} = 20.43$ ,  $P = 0.001$ ;  $F_{1,40} = 12.83$ ;  $P = 0.005$ ; and  $F_{1,40} = 5.161$ ;  $P = 0.046$ , respectively) (Fig. 3C-E). All categories of damage (including mild ring, severe ring, bent stem, and aborted stem) experienced significant increases over time ( $F_{4,40} = 61.14$ ,  $P < 0.001$ ;  $F_{4,40} = 44.92$ ,  $P < 0.001$ ;  $F_{4,40} = 19.73$ ;  $P < 0.001$ ; and  $F_{4,40} = 10.00$ ;  $P < 0.001$ , respectively). This increase was different between treatments, as demonstrated by the interaction between the two factors ( $F_{4,40} = 5.478$ ,  $P = 0.001$ ;  $F_{4,40} = 13.71$ ,  $P < 0.001$ ;  $F_{4,40} = 10.42$ ;  $P < 0.001$ ; and  $F_{4,40} = 10.00$ ;  $P < 0.001$ , respectively).

### 3.2. Defensive gene expression

The analysis of the relative expression levels of genes *ASR1*, *PRI1*, and *PIN2* in response to treatments revealed significant transcriptional differences. The *ASR1* gene exhibited significant upregulation in all three treatments throughout the entire 21-day duration of the experiment (Fig. 4), resulting in expression levels approximately four times higher in Nt plants and in Nt+(Z)-3-HP plants compared to those observed in the control plants at 2 days ( $F_{2,18} = 13.65$ ;  $P = 0.001$ ) (Fig. 4A), 7 days ( $F_{2,17} = 39.24$ ;  $P < 0.001$ ) (Fig. 4B), and 14 days ( $F_{2,20} = 50.01$ ;  $P < 0.001$ )



**Fig. 3.** Damage caused by *N. tenuis* in tomato plants exposed to (Z)-3-hexenyl propanoate [(Z)-3-HP] compared to untreated tomato plants (Control) (cv. Money-maker). Damage was assessed in terms of necrotic rings (A), ranging from a mild presence of necrotic ring (B), clear and evident necrotic damage (severe necrotic ring, C), bending of the stem or petiole due to the necrotic ring (bent necrotic ring, D), and ultimately, the abortion or drying of the stem or petiole (aborted/wilting necrotic ring, E).



**Fig. 4.** Transcriptional response of the defense-related gene *ASR1* in tomato plants with the release of *N. tenuis* (Nt), tomato plants with the release of *N. tenuis* and exposed to (Z)-3-HP [Nt + (Z)-3-HP], and control plants. Data are presented as the mean independent transcript expression analyses relative to the constitutive *EF1* gene  $\pm$  SE (n = 7). Significant differences based on ANOVA and Tukey's multiple comparison tests are indicated by different letters ( $P < 0.05$ ).

(Fig. 4C) after the release of *N. tenuis*. The relative expression of *ASR1* significantly decreased on day 21 for the Nt+(Z)-3-HP treatment compared to Nt treatment, but it remained overexpressed compared to the control plants ( $F_{2,19} = 31.30$ ;  $P < 0.001$ ) (Fig. 4D).

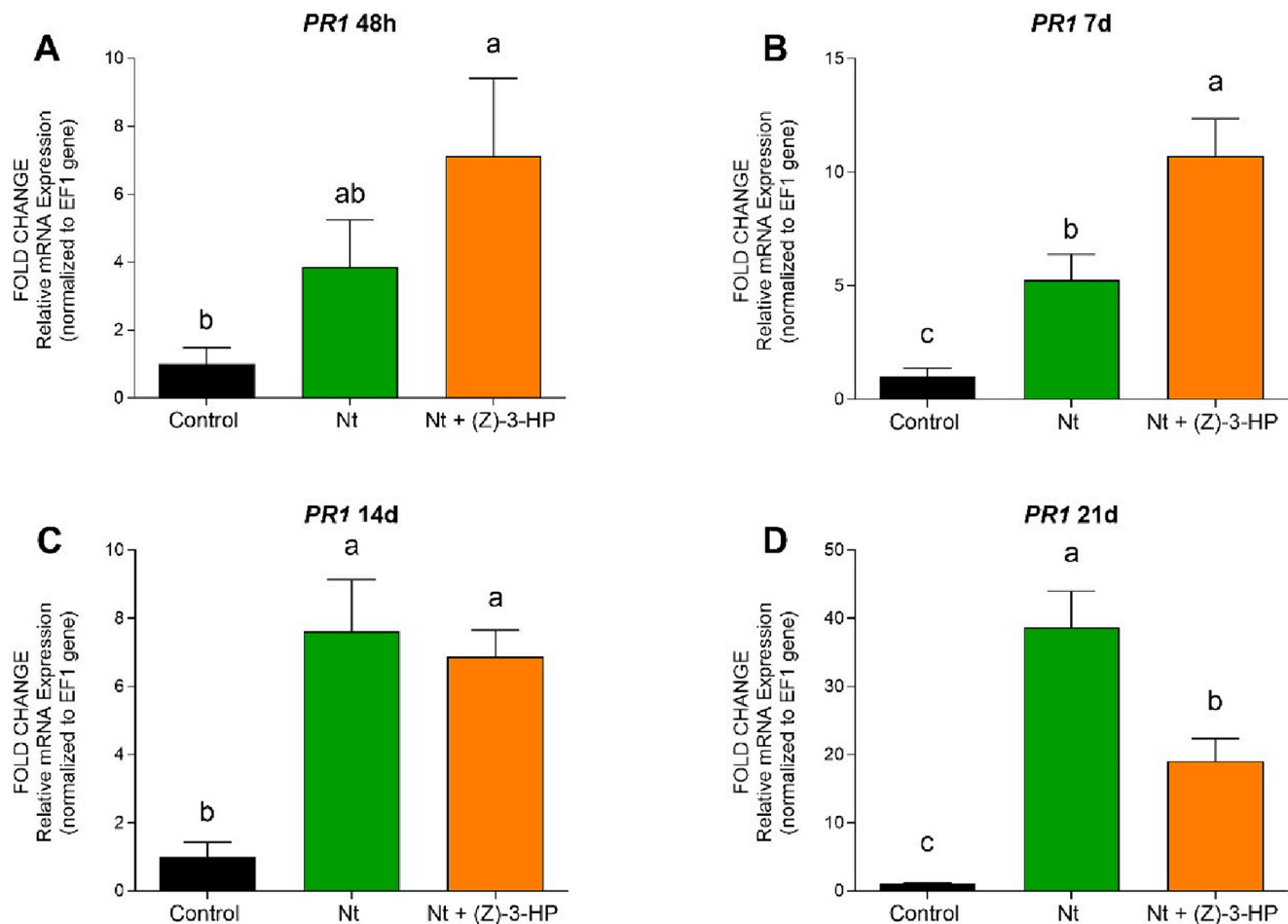
The relative expression of the *PR1* gene at 2 days was significantly different only in the Nt+(Z)-3-HP treatment, showing a fold change of nearly 7 compared to the control treatment ( $F_{2,15} = 5.693$ ;  $P = 0.017$ ) (Fig. 5A). At 7 days, *PR1* expression in the Nt+(Z)-3-HP treatment increased slightly, reaching a fold change of approximately 10 compared to the control plant. The Nt treatment during this period also showed significantly higher expression compared to the control plant, although it was lower than that of the Nt+(Z)-3-HP treatment ( $F_{2,15} = 24.65$ ;  $P < 0.001$ ) (Fig. 5B). By 14 days, the relative expression of *PR1* in Nt treatment continued to increase, matching the expression levels of the Nt+(Z)-3-HP, with a fold change approximately 7 times higher than in the control plants ( $F_{2,15} = 16.52$ ;  $P = 0.001$ ) (Fig. 5C). This increasing trend in Nt treatment persisted. By day 21, the relative expression was approximately 40-fold higher than the expression in the Nt+(Z)-3-HP treatment and, consequently, higher than the control ( $F_{2,15} = 42.53$ ;  $P < 0.001$ ) (Fig. 5D).

The significant overexpression of the *PIN2* gene at 48 h indicates a rapid response in tomato plants to mechanical wounding, with expression change values exceeding 1000-fold in Nt plants. This was significantly higher than the 300-fold increase observed in the Nt+(Z)-3-HP treatment ( $F_{2,13} = 94.59$ ;  $P < 0.001$ ) (Fig. 6A). It is noteworthy that

there was a significant declining trend in *PIN2* expression starting from 7 days. At that time, downward changes in gene expression were observed in all experimental conditions compared to the 2-day interval. The average values of up-regulation were approximately 200-fold in Nt and 110-fold in Nt+(Z)-3-HP treatments. However, although the expression levels were considerably lower than those observed at 2 days, the expression of the *PIN2* gene remained significantly up-regulated at 7 days ( $F_{2,14} = 47.31$ ;  $P < 0.001$ ) (Fig. 6B). At 14 days, *PIN2* gene expression continued this downward trend while maintaining significant gene induction in Nt and Nt+(Z)-3-HP treatments until the end of the experiment ( $F_{2,17} = 19.36$ ;  $P < 0.001$ ) (Fig. 6C). By the end of the experiment, no significant *PIN2* expression was detected in the two tested conditions, and the fold change values decreased to a level similar to that of the control plants ( $F_{2,17} = 1.086$ ;  $P = 0.363$ ) (Fig. 6D).

### 3.3. Callose deposition

The damage caused by *N. tenuis* led to a significant increase in callose deposition in tomato plants compared to control plants that did not receive *N. tenuis* ( $F_{2,21} = 14.70$ ;  $P < 0.0001$ ) (Fig. 7). Specifically, *N. tenuis* increased callose deposits throughout the vascular bundle, as depicted in Fig. 6. Conversely, plants with *N. tenuis* that were exposed to Z-(3)-HP showed a notable reduction in callose intensity within the vascular bundle ( $F_{2,21} = 14.70$ ;  $P = 0.0397$ ) (Fig. 7).



**Fig. 5.** Transcriptional response of the defense-related gene *PR1* in tomato plants with the release of *N. tenuis* (Nt), tomato plants with the release of *N. tenuis* and exposed to (Z)-3-HP [Nt + (Z)-3-HP], and control plants. Data are presented as the mean independent transcript expression analyses relative to the constitutive *EF1* gene  $\pm$  SE ( $n = 7$ ). Significant differences based on ANOVA and Tukey's multiple comparison tests are indicated by different letters ( $P < 0.05$ ).

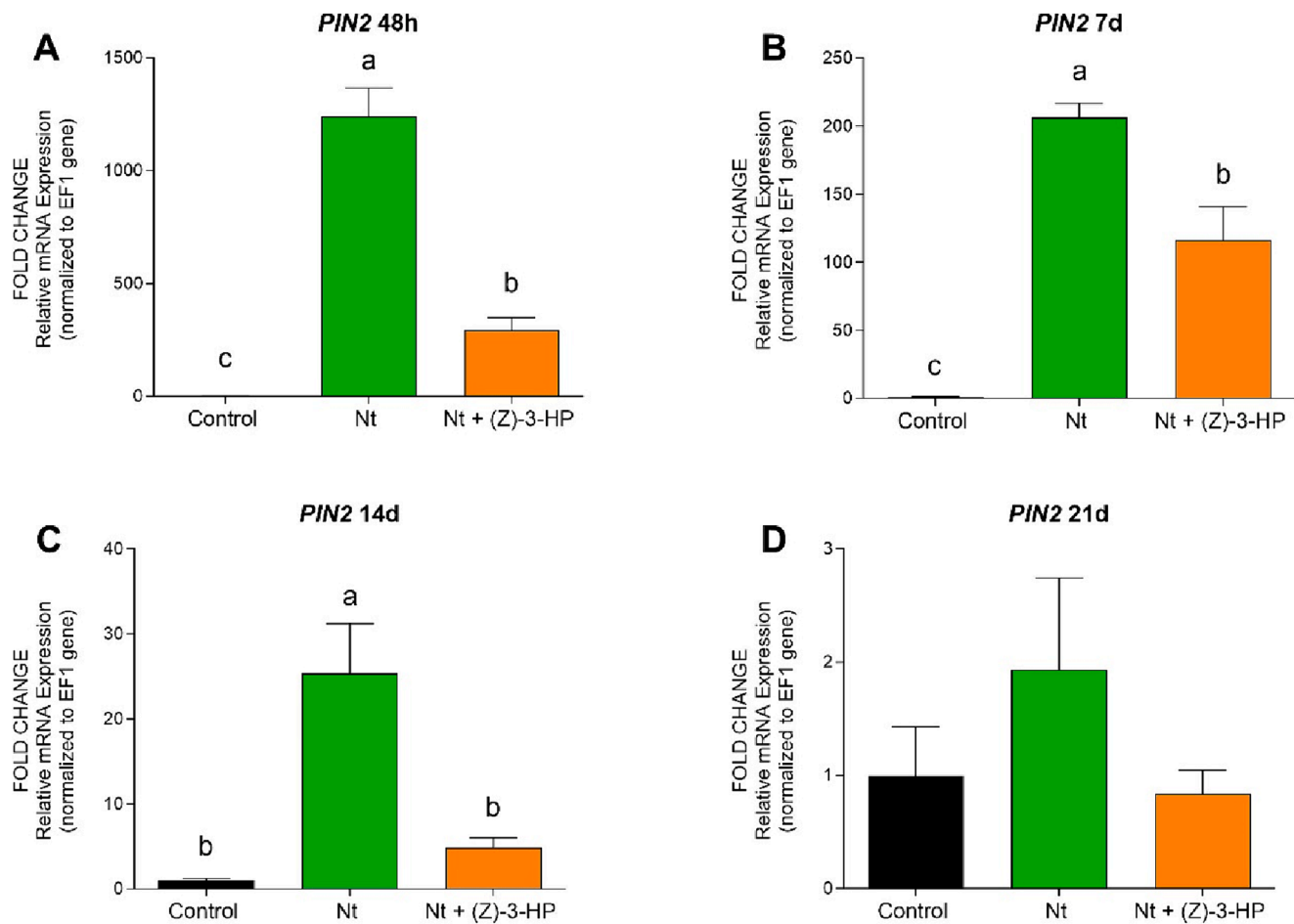
#### 4. Discussion

Our results demonstrate a reduction in damage caused by *N. tenuis* in tomato plants exposed to the volatile (Z)-3-HP; indeed, fewer necrotic rings classified as severe, and fewer bent and aborted stems were observed in this treatment compared to those in Nt plants. Our study demonstrated that eliciting tomato plants with the synthetic HIPV, (Z)-3-HP, did not impact the establishment and reproduction of the zoophytophagous predator *N. tenuis*. The number of nymphs produced on plants continuously exposed to the volatile was comparable to those produced on Nt plants. Previous studies using this volatile compound under tomato greenhouse conditions had already demonstrated that the establishment of *N. tenuis* was not affected in a (Z)-3-HP-saturated environment (Pérez-Hedo et al., 2021b), and that this volatile might even act as an attractant not only for this mirid species but also for *Macrolophus pygmaeus* (Rambur) and *Dicyphus bolivari* Lindberg (Hemiptera: Miridae) (Silva et al., 2021). Therefore, our results confirm that eliciting plants with synthetic volatiles may be compatible with the rational use of *N. tenuis* as a highly effective biocontrol agent.

Previously, Garantonakis et al. (2018) demonstrated that inoculating tomato plants with the endophytic strain *Fusarium solani* K reduced the damage caused by *N. tenuis* feeding through the activation of ethylene and jasmonic acid signaling pathways, both essential for plant resistance. Similarly, both pathways were elicited when tomato plants are exposed to (Z)-3-HP (Pérez-Hedo et al., 2021b). However, the molecular

mechanisms underlying the reduction of *N. tenuis* plant damage in tomato plants induced by (Z)-3-HP or *Fusarium solani* strain K have not been fully elucidated.

Several molecular and physiological events can contribute to a more effective adaptive response in plants exposed to (Z)-3-HP when they face stress caused by phytophagy. In plants inoculated with *N. tenuis*, the induction of defensive genes such as *PIN2*, which is mediated by jasmonic acid, exhibited a significantly higher, faster, and more sustained response compared to those inoculated with *N. tenuis* and exposed to (Z)-3-HP. In the latter condition, *PIN2* gene expression consistently remained lower over time. Indeed, the decreased activation of *PIN2* in this scenario might be due to reduced wounding pressure compared to plants exposed only to *N. tenuis*. Consequently, this results in a more regulated systemic response to wound induction, in plants exposed to the volatile compound. Nevertheless, we hypothesize that if the number of mild necrotic rings remains similar in both treatments, the exposure to (Z)-3-HP could trigger biochemical responses in tomato plants, preventing a significant portion of mild rings from advancing to severe damage and promoting their persistence in healthy tissue as the plant grows further. In this study, we observed the upregulation of the transcription factor *ASR1* in both treatments and at all time intervals. *ASR1* is crucial in various stress response pathways and enhances cellular antioxidant activity (Dominguez et al., 2021). This effect is clearly observed in tobacco plants, where the overexpression of *ASR1* leads to increased expression of ROS-related genes (reactive oxygen species) and



**Fig. 6.** Transcriptional response of the defense-related gene *PIN2* in tomato plants with the release of *N. tenuis* (Nt), tomato plants with the release of *N. tenuis* and exposed to (Z)-3-HP [Nt + (Z)-3-HP], and control plants. Data are presented as the mean independent transcript expression analyses relative to the constitutive *EF1* gene  $\pm$  SE ( $n = 7$ ). Significant differences based on ANOVA and Tukey's multiple comparison tests are indicated by different letters ( $P < 0.05$ ).

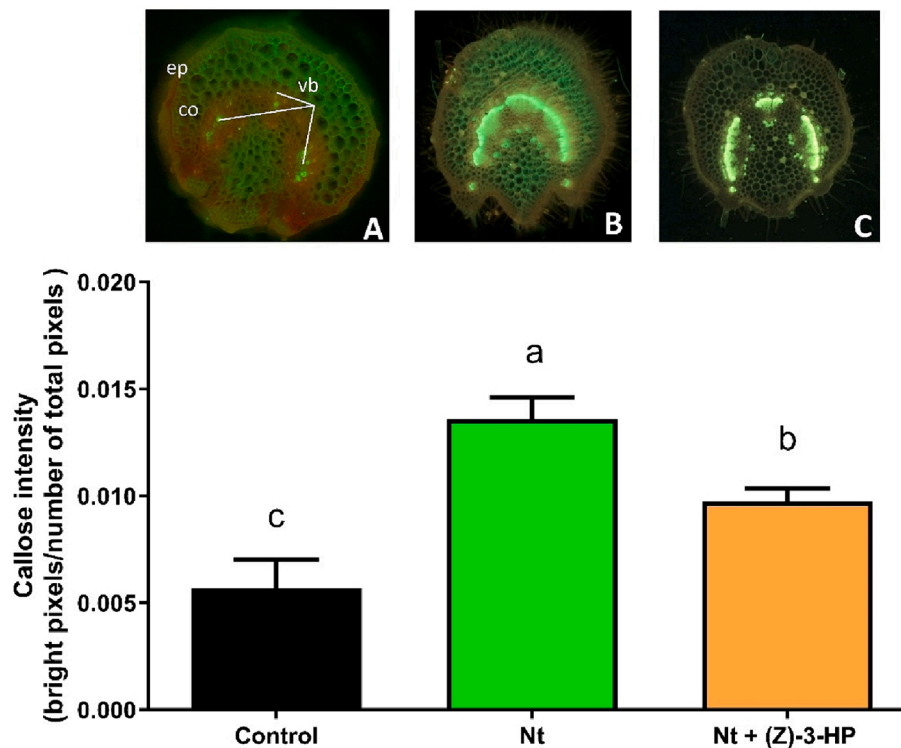
an intensified stress response (Dominguez and Carrari, 2015). Consistent with this mechanism, a stable expression of the *PR1* gene was observed in Nt+(Z)-3-HP plants, whereas in Nt plants there was a progressive increase in *PR1* gene. This pattern aligns with the oxidative stress events, such as cellular death around necrotic rings, observed in Nt plants.

The physiological disruption, caused by stress events in Nt plants, was evidenced by more severe damage from *N. tenuis* compared to plants exposed to both *N. tenuis* and (Z)-3-HP, resulting in more pronounced necrotic rings and the formation of callose deposits in vascular bundles and structural tissues. Callose deposits likely act as barriers that hinder the plant's ability to transport nutrients, water, and signaling molecules. Consequently, the tissues at the feeding sites collapse, as demonstrated by the intense callose fluorescence in the collenchyma and vascular tissues of plants attacked by mirids. In contrast, plants exposed to (Z)-3-HP displayed fewer necrotic rings and less damage overall. These plants also showed a weaker callose fluorescence signal, with callose deposits asymmetrically distributed in the vascular bundle, thus preserving areas without obstruction. Callose accumulation is a rapid defense mechanism that can enhance plant resistance against viruses and other pests. Yang et al. (2022) reported that salicylic acid (SA) positively regulates callose accumulation. Their research highlighted an enhanced defense in plant against Tomato Yellow Leaf Curl Virus (TYLCV), transmitted by

whiteflies, through the activation of the SA-related defense pathway and callose priming via SA. Our results align with these findings, demonstrating a similar pattern of callose accumulation and activation of the SA-related defense gene *PR1*. In Nt plants, *PR1* gene expression peaked at 21 days, coinciding with detection of intense fluorescence, indicating dense and compact callose deposition in the tissue. Conversely, during the same period, the callose signal was weaker in Nt+(Z)3-HP plants, and *PR1* gene expression was slightly reduced. Exposing tomato plants to (Z)-3-HP activates pathways essential for tissue regeneration while suppressing those associated with cell death (Pérez-Hedo et al., 2021b). Therefore, exposure to (Z)-3-HP may facilitate a defensive mechanism by modulating callose deposition to enhance the plant's capacity for tissue regeneration in response to damage, as observed in plants exposed to *N. tenuis* and (Z)-3-HP.

In summary, this study demonstrates that the continuous release of (Z)-3-HP via a polymer dispenser serves as a plant elicitor, priming tomato plants for enhanced defense responses and increased tolerance to stress from *N. tenuis* phytophagy. This approach effectively reduces damage caused by *N. tenuis*, limiting tissue damage while preserving its establishment, thus ensuring the maintenance of biological control of important tomato pests such as whiteflies, *Bemisia tabaci* and *Trialeurodes vaporariorum*, or the pinworm *Tuta absoluta*.





**Fig. 7.** Stained cross sections of tomato petioles showing callose deposits under the epifluorescence microscope with UV filter following conditions and quantification of callose intensity as an average number of bright pixels/number of total pixels using A. GIMP (GNU Image Manipulation Program): A) control plants, B) tissue sections with necrotic ring observations made in tomato plants with the release of *N. tenuis*, and C) tissue sections with necrotic ring observations made in tomato plants with the release of *N. tenuis* and exposure to (Z)-3-HP. The abbreviations used in the figure are as follows: ep (epidermis), co (collenchyma), and vb (vascular bundle). Significant differences based on ANOVA and Tukey's multiple comparison tests are indicated by different letters ( $P < 0.05$ ).

Future research should investigate these findings under field conditions, where various biotic and abiotic factors may influence plant defenses.

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#### CRedit authorship contribution statement

**Laura Depalo:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Carolina Gallego:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Raúl Ortells-Fabra:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Carlos Salas:** Writing – review & editing, Methodology, Investigation. **Rafael Montalt:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Alberto Urbaneja:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Meritxell Pérez-Hedo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

#### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A. U. and M.P.-H. are inventors of the Spanish Patent No. P202030330 entitled "Use of (Z)-3-hexenyl esters and method for protecting plants against pests". The other authors declare no conflict of interest.

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