Zoophytophagous predator-induced defences restrict accumulation of the tomato spotted wilt virus

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Running title: Zoophytophagy restricts TSWV

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Abstract

BACKGROUND: The use of zoophytophagous predators in protected crops has been widely adopted to manage pests in Southern Europe. We hypothesized plant defence responses would be induced by zoophytophagous predators and this induction could affect plant virus occurrence; the phytophagy of these predators induces plant defences similarly to that of viral infection. Therefore, we evaluated whether or not mirid predator activated plant defences limited the accumulation of *Tomato Spotted Wilt Virus* (TSWV) in mechanically infected sweet pepper.

RESULTS: Our results revealed TSWV accumulation in mirid-punctured plants to be significantly lower than in intact plants; this is most likely associated with the upregulation of the JA pathway triggered by mirid phytophagy.

CONCLUSION: Activation of induced defences by mirid predators has been demonstrated for the first time to limit the accumulation of TSWV in sweet pepper. This novel approach can offer new control strategies for the management of plant diseases.

Keywords: *Nesidiocoris tenuis*, *Macrolophus pygmaeus*, *Tomato spotted wilt virus*, plant defences, biological control

1 INTRODUCTION

In Europe, throughout the last ten years, biological control in protected crops has been widely adopted for pest management.[1–3] The case of sweet pepper and tomato in South-eastern Spain could be a paradigmatic example of how biological control based on the use of omnivorous predators has environmentally, socially and economically transformed an entire region of more than 30,000 ha of protected crops.[4,5] In this short period of time the agricultural paradigm in this zone has evolved from chemical dependency to the implementation of an integrated pest management program based on the release and conservation of natural enemies; where preventive and sustainable control methods are now prioritized.[3,6]

In sweet pepper, (*Capsicum annuum*), the release of two generalist predators native to the Mediterranean region, the predatory mite *Amblyseius swirskii* (Athias-Henriot) (Acari: Phytoseiidae) together with the minute pirate bug *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) results in highly efficient management of the two key sweet pepper pests; the western flower thrip, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) and the whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae).[7–9] Moreover, recent studies with the mirid predators, *Nesidiocoris tenuis* (Reuter) and *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae), sustained even better biological control results in this crop since these two are also able to control aphid species.[10–12] Similarly in tomatoes, the cosmopolitan predatory mirid *N. tenuis* enables effective control of *B. tabaci* and the tomato borer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae),[6,13,14] an important invasive tomato pest detected for the first time in Spain in 2007.[15]

Zoophytophagy is a special case of omnivory; predators belonging to this group use a mixture of both prey and plant resources to complete development and reproduction.[16] Zoophytophagous predators can affect herbivore populations directly by preying upon them as well as indirectly through plant-mediated effects.[17–27] Plant responses to herbivory feeding are known to result in a stunning array of structural, chemical, and protein-based defences designed to detect invading organisms and stop them before they are able to cause extensive damage.[28–31] Zoophytophagous predators have been observed to induce both direct and indirect plant defences in sweet pepper and tomato. In sweet pepper, the phytophagy of the anthocorid *O. laevigatus* and the mirids *N. tenuis* and *M. pygmaeus* activated the jasmonate acid (JA) and salicylic acid (SA) signalling pathways and triggered the release of an altered blend of volatiles (green leaf volatiles, terpenoids and methyl salicylate). Those volatiles repelled *B. tabaci* and *F. occidentalis* and at the same time attracted the whitefly parasitoid,

Encarsia formosa (Gahan) (Hymenoptera: Aphelinidae). [26,27,32] Similar results have been obtained in tomato with the mirid predators, *N. tenuis*, *M. pygmaeus* and *Dicyphus bolivari* (Lindberg) [= *D. maroccanus* (Wagner)], yet the specific responses were attributed to each predator species in these cases. Thus, while plants punctured by *N. tenuis* repel *B. tabaci* and *T. absoluta*, the phytophagy of *M. pygmaeus* and *D. bolivari* did not repel *B. tabaci* and even attracts *T. absoluta*. In contrast, the feeding activity of these three mirids results in an attraction of *E. formosa*.[18,20–24] Furthermore, the feeding behaviour of these zoophytophagous predators has been verified to induce direct defences through the activation of the JA pathway with an increase in protease inhibitor activity.[20,23,24] Plants previously induced by mirids have been found to reduce the establishment and performance of important pests such as *B. tabaci*, *F. occidentalis* and the two-spotted spider mite, *Tetranychus urticae* (Koch) (Acari: Tetranychidae) in sweet pepper,[19,26] along with *T. urticae* in tomato.[18,20,24]

Regardless of the above mentioned studies, more investigations are needed to expand our understanding of plant mediated effects on pest and disease management induced by zoophytophagous predators. Interestingly, an important facet of research, previously not addressed but already hypothesized, is the evaluation of plant mediated effects of zoophytophagous predators on viral and microbial infection.[18,33] Recently, beneficial microbes have been observed to modulate the performance of zoophytophagous predators.[34,35] The colonization of tomato plants by the endophytic fungi *Fusarium solani* strain K reduces the capability of *N. tenuis* to induce necrotic rings on tomato stems and leaves. The upregulation of ethylene and JA pathways induced by *F. solani* give protection to tomato from *N. tenuis* feeding.[34] An interaction between the pepino mosaic virus (PepMV) and the mirid *M. pygmaeus* has been also found. The severity of crop damage caused by *M. pygmaeus* is significantly enhanced when tomato plants are infected with PepMV.[36] This interaction was attributed to the antagonistic effects of SA-mediated responses on JA-mediated responses, since PepMV infection induces the SA defence pathway[37] meanwhile *M. pygmaeus* mainly activates the JA pathway.[20, 22] Additionally, tomato plants with high expression of methyl jasmonate are less likely to be infected with the *Tomato yellow leaf curl virus* (TYLCV).[38] Therefore, we hypothesized that possible interaction can occur between induced defences by zoophytophagous predator influence the incidence of plant viruses.

In this research, we focused on evaluating whether plant defences triggered separately by *N. tenuis* or *M. pygmaeus* affect the multiplication of the *Tomato Spotted Wilt Virus* (TSWV) in sweet pepper. TSWV is one of the most harmful plant viral pathogens, ranking second in the list of the most important plant viruses worldwide.[39,40] It is transmitted in a persistent manner

by several thrips species; with *F. occidentalis* being its main vector. Eradication or control of TSWV has become even more difficult by the emergence of resistant TSWV isolates in pepper.[41] Herein, we evaluated the effect of plant defence activation on TSWV multiplication by quantifying TSWV RNA accumulation. Plant defence activation was confirmed by analyzing gene expression of defence pathways. The implications of these results to improve TSWV disease management in pepper are discussed.

2 MATERIAL AND METHODS

2.1 Plants, insects, and virus isolate

Sweet pepper plants [*Capsicum annuum* (Solanaceae)] *cv* ('Salmerón') (California rojo, Mascarell semillas S.L, Valencia, Spain) were used in the experiments herein described. Two weeks after germination the seedlings were transplanted to plastic pots (8 × 8 × 8 cm) containing a mixture of soil with peat moss and were maintained undisturbed at 25 ± 2°C, with constant relative humidity of 65% ± 5%, and a photoperiod of 14:10 h (light: dark). Plants were irrigated twice a week. Pesticide-free sweet pepper plants were used for the experiments at 6 weeks of age (approximately 20 cm high). Fourth instar nymphs of *N. tenuis* and *M. pygmaeus* were provided directly by Koppert Biological Systems, S.L. (Águilas, Spain). *Tomato spotted wilt virus*, TSWV PVR isolate (TSWV-PVR), from the IVIA plant virus collection was used.[42] The virus was maintained in *Nicotiana benthamiana* Domin (Solanales: Solanaceae). Preliminary research showed that the sweet pepper cultivar used in our experiments can be successfully infected with TSWV-PVR when mechanically inoculated.

2.2 Biological assays

Three treatments were assayed: i) *N. tenuis*-punctured plants, ii) *M. pygmaeus*-punctured plants and iii) intact plants (control plants free of arthropod contact). Mirid-punctured plants were obtained by individually exposing sweet pepper plants to either 20 *N. tenuis* or 20 *M. pygmaeus* fourth instar nymphs in a 30 x 30 x 30 cm plastic cage (BugDorm-1 insect tents; MegaView Science Co., Ltd, Taichung, Taiwan). Nymphs were selected instead of adults to avoid defence induction by adult oviposition.[21] All nymphs were removed twenty-four hours after placing them on the plants. Ten replicates per treatment were considered. Each replicate consisted of a plastic cage 60 x 60 x 60 cm (BugDorm-2; MegaView Science Co., Ltd, Taichung, Taiwan), inside which 4 pepper plants of the corresponding treatment were introduced. A total of 40 plants were used per treatment. Cages were maintained in a climate chamber at the same environmental conditions as described above (Fig. 1).

Once the experimental design was assembled, six pepper plants per treatment were removed to quantify the transcriptional response of the genes involved in defence responses. The apical region of the sweet pepper plants (the first 5 cm of the plant formed by the apical stem and young leaves) were cut and then ground in liquid nitrogen for RNA extraction. Next, the leaves of all remaining pepper plants for all three treatments (34 plants in each treatment) were mechanically inoculated with TSWV-PVR (Fig. 1). Inoculation was performed by rubbing a dilution of the following leaf extract inoculation solution (1:20, w:v) onto pepper leaves with a cotton bud and celite (diatomaceous earth).[43] The inoculation solution was obtained by grinding 250 mg of TSWV infected *N. benthamiana* leaves in a mortar in a mixture containing 5 ml 0.05 M phosphate buffer, pH 7.2; 0.2% 2-mercaptoethanol; 1% polyvinylpyrrolidone (average molecular weight 10.000).[43]

One plant per replicate and treatment was removed at 7, 14 and 21 days after inoculation (dpi), respectively, to quantify virus accumulation (n=10). As above, the apical region of each plant was excised and immediately immersed in liquid nitrogen for subsequent RNA extraction (Fig. 1). The remaining four plants per treatment were used to visually detect the virus symptoms.

In addition, a negative control treatment for the virus inoculation was also performed (mock inoculation). For this, ten plastic cages were also arranged with the same conditions as described above. Four intact pepper plants were placed inside each cage. Samples were collected at 7, 14, and 21 days post inoculation (dpi) to check and verify the absence of any contamination.

2.3 Quantification of TSWV infection by RT-qPCR

Total RNAs from 0.1 g of fresh leaf tissue from TSWV-infected and non-infected sweet pepper plants were extracted using TRIzol (Invitrogen, CA, USA) as described above. RNA concentrations were measured in duplicate with the UV-Vis spectrophotometer nanodrop 1000 (Thermo Scientific, Waltham, MA, USA) and adjusted to approximately 10 ng/µl to normalize the different extractions. Aliquots were stored at -80°C until use. RT-qPCR was carried out using the LightCycler® 480 System (Roche Molecular Systems, Inc., Switzerland), using 25 µL of a reaction mix that contained 12.5 µL LightCycler®480 Probe Master Mix (ROCHE), 4.38 µL of RNase-free water, 15 units (U) RT Multiscribe Reverse Transcriptase (Life Technologies, Rockville, MD, USA), 2 U of RNase inhibitor (Applied Biosystems, Foster City, CA, USA), 5 µM of primers 1M-F and 1M-R, 0.25 µM TaqMan®MGB probe and 5 µL of total RNA

(~10 ng µL−1). The Thermo cycling conditions consisted of reverse transcription at 48°C for 30 min, incubation at 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min.[44]

2.4 Plant gene expression

In a previous work, we showed how sweet pepper plants cv Lipari were activated defensively when exposed to adults of both *N. tenuis* and *M. pygmaeus*.[26] In this work, unlike the previous work, the cultivar Salmeron and fourth instar nymphs of both mirid species, were used. Therefore, to confirm that sweet pepper plants used in this experiment were defensively activated, plant gene expression analysis were performed. The relative expression of three marker genes, commonly used as indicators of JA, SA and ABA-related defences, was estimated:[26] (i) *PIN2* (wound-induced proteinase inhibitor II precursor) a marker gene for JA, (ii) *PR1* (basic PR-1 protein precursor) a marker gene for salicylic acid (SA), and (iii) *ASR1* (abscisic acid stress ripening protein 1) a marker gene for ABA signalling pathway. Total RNA (1.5 µg) was extracted using TRIzol (Invitrogen, CA, USA) according to the manufacturer's instructions.[23,26] The RNA was treated with a Turbo DNA-free DNase kit (Applied Biosystems) according to the manufacturer's protocol to eliminate any traces of genomic DNA. cDNA was later synthesized using a prime script™ RT reagent kit (perfect real time) (TAKARA Bio, CA, USA). Real-time PCR amplifications were performed with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, MA, USA). PCR reactions were run in duplicate, in accordance with manufacturer recommendations. Quantitative PCR was carried out using the LightCycler® 480 System (Roche Molecular Systems, Inc., Switzerland), under standard amplification conditions.[26] EF1 (elongation factor-1) was used as a standard control gene for normalization.

2.5 Statistical analysis

The relative expression of defence genes was analysed using one-way analysis of variance (ANOVA), followed by a comparison of means (Tukey's test) at $\alpha < 0.05$. Data from RNA quantification of TSWV isolates were log (concentration +1) transformed prior to analysis using ANOVA to differentiate between treatments for each of the three post inoculation days (7, 14 and 21 dpi), followed by comparison of means (Tukey's test) at $\alpha < 0.05$.

3 RESULTS

3.1 Plant defence by mirids restrict TSWV infection

TSWV titer increased with time in intact sweet pepper plants; it reached a maximum at 21 dpi. However, it remained low and almost constant with time in both *N. tenuis*- and *M. pygmaeus*-punctured plants (Fig. 2). No significant differences for TSWV titer were found at day 7 nor day 14 post inoculation ($F_{2\text{-}29}$ = 1.018; P = 0.3748 and $F_{2\text{-}29}$ = 1.788; P = 0.1865, respectively). However, at 21 dpi TSWV titer was significantly higher in intact sweet pepper plants as opposed to that in plants punctured with both mirids ($F_{2\text{-}29}$ =36.25; P < 0.0001). At day 21 intact sweet pepper plants presented chlorotic flecking on the leaves, while these symptoms were not observed in either of the two mirid phytophagy exposure treatments (Fig. 3). No virus contamination was detected in the negative control plants.

3.2 Phytophagy of mirids alters JA pathway

Both *N. tenuis* and *M. pygmaeus* were found to influence the upregulation of JA pathways in the apical part of exposed sweet pepper plants when compared to intact plants. The relative expression of the corresponding defence genes, *PIN2* (JA pathway), significantly increased in mirid-punctured plants ($F_{2\text{-}17}$ = 7.251; P = 0.0063; Fig. 4a) compared to intact plants. Only *N. tenuis* was able to upregulate the gene *PR1* (SA pathway) ($F_{2\text{-}17}$ = 7.440; P = 0.0057; Fig. 4b). In contrast, the *ASR1* gene (ABA pathway) was not significantly upregulated in mirid-punctured plants when compared to intact sweet pepper plants ($F_{2\text{-}17}$ = 1.190; P = 0.3313; Fig. 4c).

4 DISCUSSION

Two predators used extensively in biological control programs have been found, for the first time, to limit the accumulation of one of the most important widespread plant viruses. The RT-qPCR revealed that three weeks after the mechanical inoculation of TSWV, the number of RNA copies in mirids-punctured plants were significantly lower in comparison to intact plants.

The production of a number of plant hormones are directly related to the process of virus infection; especially the JA and SA pathways.[45] Some components of these pathways function as necessary signalling molecules that modulate responses to different stimuli.[31,47,48] Exogenous treatments with methyl jasmonate (MeJA) or JA have been shown to reduce incidence of viral infection. For example, tomato plants treated with MeJA were less infected with TYLCV.[37] The accumulation of *Cucumber mosaic virus* (CMV) in *Momordica charantia* L. (Cucurbitales: Cucurbitacea) was significantly suppressed when plants received an exogenous application of JA.[49] On the other hand, the infection process of CMV in *M. charantia* was almost unaffected by the exogenous application of SA, hence revealing how JA, not SA, inhibited virus infection.[49] The activation of the JA pathway is precisely what the phytophagy of the mirids in sweet

pepper plants stimulates, which could be the explanation for the minor infection by TSWV shown in our experiments. Nevertheless, SA also plays an important role in plant defence against certain plant viruses. SA exogenous treatments have been reported to reduce the coat protein levels of *Tobacco Mosaic Virus* (TMV) and *Potato Virus X* (PVX) during their interactions with *N. benthamiana* plants.[46] Both MeJA and Methyl salicylate (MeSA) are required for the systemic resistance response of *N. benthamiana* plants against TMV.[50] The foliar application of MeJA at early stages of TMV infection followed by a later application of SA activated the strongest systemic defence response and upregulated the expression of defence related genes against TMV.[50] This is also consistent with another study which showed plant resistance to a broad spectrum of RNA viruses could be improved with the application of JA and SA.[47] Future identification of the roles of hormones in plant-virus interactions, how these hormones may interact with other biotic stressors, and cross talk among hormone pathways is still needed to fully understand the mechanisms by which plants resist infection.

Sweet pepper plants defensively activated by mirids became less attractive to *F. occidentalis*;[26] the TSWV vector. Interestingly, TSWV infected plants are more attractive to the vector, *F. occidentalis*, than healthy plants; indeed thrips themselves develop faster on TSWV infected plants.[48] How mirid induced plant responses influence these TSWV-thrips interactions is not known, hence further research is needed to evaluate how mirid plant puncturing can limit viral infection of TSWV transmitted by thrips. However, not only the mutualistic interactions occurring between mirids and plants but also the interactions between vectors and viruses can affect the final response of the plant.[48] Additionally, environmental conditions, the presence of alternative food on the plant (pollen and nectar) and the presence of prey are crucial factors to be considered for further evaluation of plant mediated effects by mirids and its impact on the accumulation of TSWV in sweet pepper plants.

Current control strategies for TSWV include elimination of infected plants, use of clean stock material, exclusion of thrips with greenhouse screens or air locks, and introduction of natural enemies.[49–51] As these control strategies are only partially successful, additional measures are needed to limit virus spread. Until recently, resistance to TSWV was obtained through the introgression of the two main resistance genes, *Sw5* and *Tsw*, in tomato and pepper, respectively. However, the emergence of resistant TSWV isolates (as the one used in our experiment)[43] has limited the durability of this strategy.[52,53] Therefore, breeding for durable TSWV resistance in plants is still a challenge upon which our results could provide new insight into plant viruses resistance. Probably, the activation of JA signalling pathway through genetic and chemical manipulation might improve plant defence against plant viruses.

The possible implementation of strategies based on the above mentioned hypothesis has been verified in young plants; the size which is similar to those habitually transplanted from the nursery. Previously, nursery inoculation with mirids was proposed since the activation of defence responses reduces the infestation of important pests such as the whitefly, *B. tabaci* in sweet pepper and tomato plants[23,26] along with the two-spotted spider mite, *T. urticae* in tomato plants.[18] Our results support this strategy since the plants would also be protected from diseases such as the TSWV. In this sense, sweet pepper plants can be kept defensively activated (upregulated JA pathway) up to 14 days after a single 24 h exposure to mirids.[26] The same time period of defence activation was obtained also in *M. pygmaeus*-infested tomato plants.[20] In zones where transplanting occurs at the end of summer there is great insect vector pressure, thus protecting young plants from viral infection is crucial. Therefore, these results promote the use of biological control which could limit viral incidence at the beginning of the cultivation period. Further research must clarify the duration of defence activation under field conditions when a part of high vector pressure, the plant is subjected to multiple infestations which could work synergistically or antagonistically with each other to activate or block the metabolic pathways responsible for defences.[54]

Herein included is a new perspective which had not been previously considered in the use of biological control programs with zoophytophagous predators; the ability of *N. tenuis* and *M. pygmaeus* to influence the reduction of TSWV infection incidence. New research lines should explore defence response activation against other diseases such as those caused by fungi and bacteria along with how pathogenic microbes may modulate mirid performance.[34,35] In conclusion, our results provide insights for future studies that can further strengthen pest and disease management programs based on these plant-predator-virus interactions.

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Figure captions

Figure 1.

Time line presenting pepper defence activation by either *N. tenuis* or *M. pygmaeus* fourth instar nymphs, gene expression analysis, TSWV inoculation on mirid-punctured plants and intact plants, and TSWV quantification using RT-qPCR at 7, 14 and 21 days post inoculation (dpi).

Figure 2.

Quantification of *Tomato spotted wilt virus* by real time quantitative RT-PCR at 7, 14 and 21 days post inoculation (dpi) in sweet pepper plants with three treatments: I) intact plants, II) punctured by *N. tenuis*, and III) punctured by *M. pygmaeus*. Bars correspond to the mean TSWV RNA titer (Log of the number of TSWV RNA molecules) from ten plants (n = 10). Standard errors are represented by vertical segments. Bars with different letters are significantly different (ANOVA with Tukey's multiple comparison test; $P < 0.05$).

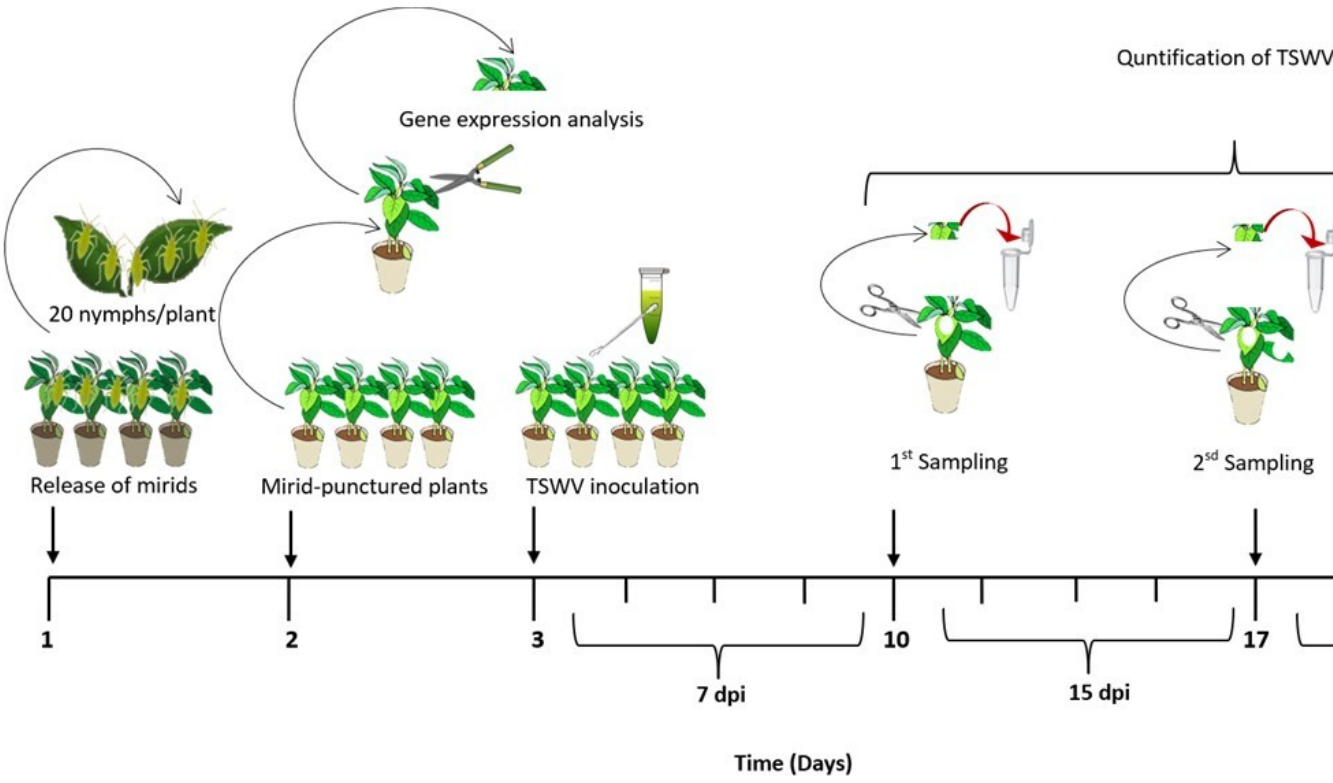
Figure 3.

Symptoms of TSWV in sweet pepper leaves at 21 days post inoculation (dpi), (a) intact plants, (b) *N. tenuis*-punctured plants and (c) *M. pygmaeus*-punctured plants.

Figure 4.

Relative expression of defensive genes *PIN1* (Jasmonic acid pathway) (a), *PR1* (Salicylic acid pathway) (b) and *ASR1* (Abscisic acid pathway) (c), in the apical part of sweet pepper plants previously punctured by either *N. tenuis* or *M. pygmaeus* fourth instar nymphs, and in intact plants. Data are presented as the mean of six independent analyses of transcript expression relative to a housekeeping gene ± SE ($n = 6$). Bars with different letters are significantly different (ANOVA with Tukey's multiple comparison test; $P < 0.05$).

Figure 1.



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477**Figure 2.**

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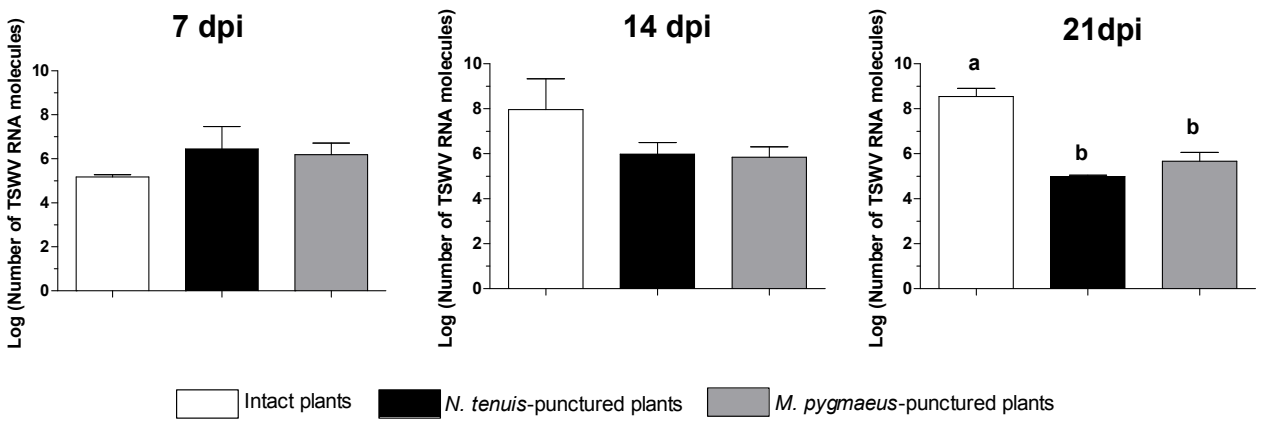

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Figure 3.

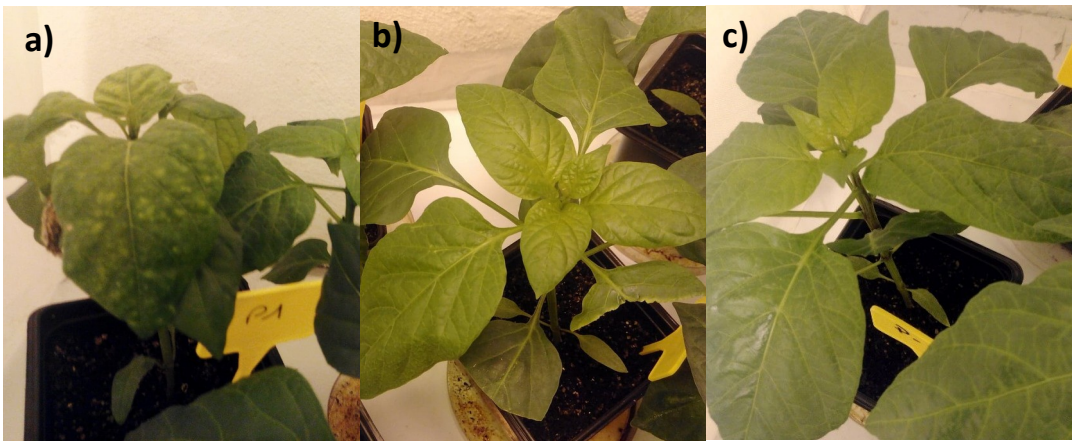
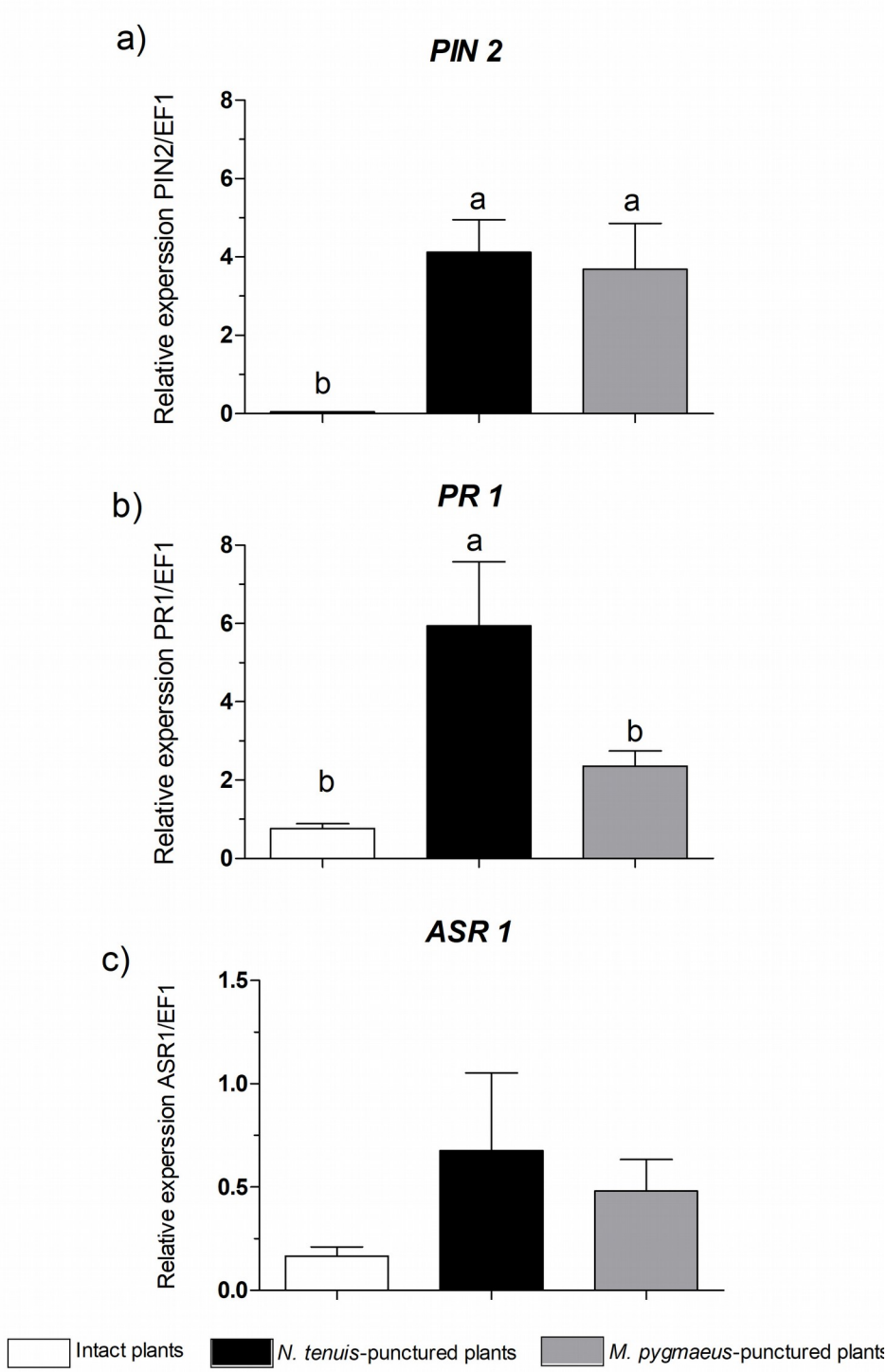


Figure 4.


a)
PIN 2
Relative experssion PIN2/EF1
8
6
4
2
0
a
a
b
b)
PR 1
Relative experssion PR1/EF1
8
6
4
2
0
a
b
b
c)
ASR 1
Relative experssion ASR1/EF1
1.5
1.0
0.5
0.0
Intact plants
N. tenuis-punctured plants
M. pygmaeus-punctured plants