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

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1 Zoophytophagous predator-induced defences restrict accumulation of 2 the tomato spotted wilt virus

3 Running title: Zoophytophagy restricts TSWV

4

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15 Abstract

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

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

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

29

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

32

331 INTRODUCTION

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

42 In sweet pepper, (*Capsicum annuum*), the release of two generalist predators native to the
 43 Mediterranean region, the predatory mite *Amblyseius swirskii* (Athias-Henriot) (Acari:
 44 Phytoseiidae) together with the minute pirate bug *Orius laevigatus* (Fieber) (Hemiptera:
 45 Anthocoridae) results in highly efficient management of the two key sweet pepper pests; the
 46 western flower thrip, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) and the
 47 whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae).⁷⁻⁹ Moreover, recent studies
 48 with the mirid predators, *Nesidiocoris tenuis* (Reuter) and *Macrolophus pygmaeus* (Rambur)
 49 (Hemiptera: Miridae), sustained even better biological control results in this crop since these
 50 two are also able to control aphid species.¹⁰⁻¹² Similarly in tomatoes, the cosmopolitan
 51 predatory mirid *N. tenuis* enables effective control of *B. tabaci* and the tomato borer *Tuta*
 52 *absoluta* (Meyrick) (Lepidoptera: Gelechiidae),^{5,13,14} an important invasive tomato pest
 53 detected for the first time in Spain in 2007.¹⁵

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

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

78 Regardless of the above mentioned studies, more investigations are needed to expand our
79 understanding of plant mediated effects on pest and disease management induced by
80 zoophytophagous predators. Interestingly, an important facet of research, previously not
81 addressed but already hypothesized, is the evaluation of plant mediated effects of
82 zoophytophagous predators on viral and microbial infection.^{18,33} Recently, beneficial microbes
83 have been observed to modulate the performance of zoophytophagous predators.^{34,35} The
84 colonization of tomato plants by the endophytic fungi *Fusarium solani* strain K reduces the
85 capability of *N. tenuis* to induce necrotic rings on tomato stems and leaves. The upregulation
86 of ethylene and JA pathways induced by *F. solani* give protection to tomato from *N. tenuis*
87 feeding.³⁴ An interaction between the pepino mosaic virus (PepMV) and the mirid *M.*
88 *pygmaeus* has been also found. The severity of crop damage caused by *M. pygmaeus* is
89 significantly enhanced when tomato plants are infected with PepMV.³⁶ This interaction was
90 attributed to the antagonistic effects of SA-mediated responses on JA-mediated responses,
91 since PepMV infection induces the SA defence pathway³⁷ meanwhile *M. pygmaeus* mainly
92 activates the JA pathway.^{20, 22} Additionally, tomato plants with high expression of methyl
93 jasmonate are less likely to be infected with the *Tomato yellow leaf curl virus* (TYLCV).³⁸
94 Therefore, we hypothesized that possible interaction can occur between induced defences by
95 zoophytophagous predator influence the incidence of plant viruses.

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

100by several thrips species; with *F. occidentalis* being its main vector. Eradication or control of
101TSWV has become even more difficult by the emergence of resistant TSWV isolates in
102pepper.⁴¹ Herein, we evaluated the effect of plant defence activation on TSWV multiplication
103by quantifying TSWV RNA accumulation. Plant defence activation was confirmed by analyzing
104gene expression of defence pathways. The implications of these results to improve TSWV
105disease management in pepper are discussed.

106

1072 MATERIAL AND METHODS

1082.1 Plants, insects, and virus isolate

109Sweet pepper plants [*Capsicum annuum* (Solanaceae)] cv ('Salmerón') (California rojo,
110Mascarell semillas S.L, Valencia, Spain) were used in the experiments herein described. Two
111weeks after germination the seedlings were transplanted to plastic pots (8 × 8 × 8 cm)
112containing a mixture of soil with peat moss and were maintained undisturbed at 25 ± 2°C, with
113constant relative humidity of 65% ± 5%, and a photoperiod of 14:10 h (light: dark). Plants were
114irrigated twice a week. Pesticide-free sweet pepper plants were used for the experiments at 6
115weeks of age (approximately 20 cm high). Fourth instar nymphs of *N. tenuis* and *M. pygmaeus*
116were provided directly by Koppert Biological Systems, S.L. (Águilas, Spain). *Tomato spotted wilt*
117*virus*, TSWV PVR isolate (TSWV-PVR), from the IVIA plant virus collection was used.⁴² The virus
118was maintained in *Nicotiana benthamiana* Domin (Solanales: Solanaceae). Preliminary
119research showed that the sweet pepper cultivar used in our experiments can be successfully
120infected with TSWV-PVR when mechanically inoculated.

1212.2 Biological assays

122Three treatments were assayed: i) *N. tenuis*-punctured plants, ii) *M. pygmaeus*-punctured
123plants and iii) intact plants (control plants free of arthropod contact). Mirid-punctured plants
124were obtained by individually exposing sweet pepper plants to either 20 *N. tenuis* or 20 *M.*
125*pygmaeus* fourth instar nymphs in a 30 x 30 x 30 cm plastic cage (BugDorm-1 insect tents;
126MegaView Science Co., Ltd, Taichung, Taiwan). Nymphs were selected instead of adults to
127avoid defence induction by adult oviposition.²¹ All nymphs were removed twenty-four hours
128after placing them on the plants. Ten replicates per treatment were considered. Each replicate
129consisted of a plastic cage 60 x 60 x 60 cm (BugDorm-2; MegaView Science Co., Ltd, Taichung,
130Taiwan), inside which 4 pepper plants of the corresponding treatment were introduced. A total
131of 40 plants were used per treatment. Cages were maintained in a climate chamber at the
132same environmental conditions as described above (Fig. 1).

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

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

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

154 2.3 Quantification of TSWV infection by RT-qPCR

155 Total RNAs from 0.1 g of fresh leaf tissue from TSWV-infected and non-infected sweet pepper
156 plants were extracted using TRIzol (Invitrogen, CA, USA) as described above. RNA
157 concentrations were measured in duplicate with the UV-Vis spectrophotometer nanodrop
158 1000 (Thermo Scientific, Waltham, MA, USA) and adjusted to approximately 10 ng/μl to
159 normalize the different extractions. Aliquots were stored at -80°C until use. RT-qPCR was
160 carried out using the LightCycler® 480 System (Roche Molecular Systems, Inc., Switzerland),
161 using 25 μL of a reaction mix that contained 12.5 μL LightCycler® 480 Probe Master Mix
162 (ROCHE), 4.38 μL of RNase-free water, 15 units (U) RT Multiscribe Reverse Transcriptase (Life
163 Technologies, Rockville, MD, USA), 2 U of RNase inhibitor (Applied Biosystems, Foster City, CA,
164 USA), 5 μM of primers 1M-F and 1M-R, 0.25 μM TaqMan® MGB probe and 5 μL of total RNA

165 (~10 ng μL^{-1}). The Thermo cycling conditions consisted of reverse transcription at 48°C for 30
166 min, incubation at 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min.⁴⁴

167

1682.4 Plant gene expression

169 In a previous work, we showed how sweet pepper plants cv Lipari were activated defensively
170 when exposed to adults of both *N. tenuis* and *M. pygmaeus*.²⁶ In this work, unlike the previous
171 work, the cultivar Salmeron and fourth instar nymphs of both mirid species, were used.
172 Therefore, to confirm that sweet pepper plants used in this experiment were defensively
173 activated, plant gene expression analysis were performed. The relative expression of three
174 marker genes, commonly used as indicators of JA, SA and ABA-related defences, was
175 estimated:²⁶ (i) *PIN2* (wound-induced proteinase inhibitor II precursor) a marker gene for JA,
176 (ii) *PR1* (basic PR-1 protein precursor) a marker gene for salicylic acid (SA), and (iii) *ASR1*
177 (abscisic acid stress ripening protein 1) a marker gene for ABA signalling pathway. Total RNA
178 (1.5 μg) was extracted using TRIzol (Invitrogen, CA, USA) according to the manufacturer's
179 instructions.^{23,26} The RNA was treated with a Turbo DNA-free DNase kit (Applied Biosystems)
180 according to the manufacturer's protocol to eliminate any traces of genomic DNA. cDNA was
181 later synthesized using a prime script™ RT reagent kit (perfect real time) (TAKARA Bio, CA,
182 USA). Real-time PCR amplifications were performed with Maxima SYBR Green qPCR Master
183 Mix (Thermo Fisher Scientific, MA, USA). PCR reactions were run in duplicate, in accordance
184 with manufacturer recommendations. Quantitative PCR was carried out using the LightCycler®
185 480 System (Roche Molecular Systems, Inc., Switzerland), under standard amplification
186 conditions.²⁶ EF1 (elongation factor-1) was used as a standard control gene for normalization.

1872.5 Statistical analysis

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

193

1943 RESULTS

1953.1 Plant defence by mirids restrict TSWV infection

196TSWV titer increased with time in intact sweet pepper plants; it reached a maximum at 21 dpi.
 197However, it remained low and almost constant with time in both *N. tenuis*- and *M. pygmaeus*-
 198punctured plants (Fig. 2). No significant differences for TSWV titer were found at day 7 nor day
 19914 post inoculation ($F_{2-29} = 1.018$; $P = 0.3748$ and $F_{2-29} = 1.788$; $P = 0.1865$, respectively).
 200However, at 21 dpi TSWV titer was significantly higher in intact sweet pepper plants as
 201opposed to that in plants punctured with both mirids ($F_{2-29} = 36.25$; $P < 0.0001$). At day 21 intact
 202sweet pepper plants presented chlorotic flecking on the leaves, while these symptoms were
 203not observed in either of the two mirid phytophagy exposure treatments (Fig. 3). No virus
 204contamination was detected in the negative control plants.

2053.2 Phytophagy of mirids alters JA pathway

206Both *N. tenuis* and *M. pygmaeus* were found to influence the upregulation of JA pathways in
 207the apical part of exposed sweet pepper plants when compared to intact plants. The relative
 208expression of the corresponding defence genes, *PIN2* (JA pathway), significantly increased in
 209mirid-punctured plants ($F_{2-17} = 7.251$; $P = 0.0063$; Fig. 4a) compared to intact plants. Only *N.*
 210*tenuis* was able to upregulate the gene *PR1* (SA pathway) ($F_{2-17} = 7.440$; $P = 0.0057$; Fig. 4b). In
 211contrast, the *ASR1* gene (ABA pathway) was not significantly upregulated in mirid-punctured
 212plants when compared to intact sweet pepper plants ($F_{2-17} = 1.190$; $P = 0.3313$; Fig. 4c).

2134 DISCUSSION

214Two predators used extensively in biological control programs have been found, for the first
 215time, to limit the accumulation of one of the most important widespread plant viruses. The RT-
 216qPCR revealed that three weeks after the mechanical inoculation of TSWV, the number of RNA
 217copies in mirids-punctured plants were significantly lower in comparison to intact plants.

218The production of a number of plant hormones are directly related to the process of virus
 219infection; especially the JA and SA pathways.⁴⁵ Some components of these pathways function
 220as necessary signalling molecules that modulate responses to different stimuli.^{31,47,48} Exogenous
 221treatments with methyl jasmonate (MeJA) or JA have been shown to reduce incidence of viral
 222infection. For example, tomato plants treated with MeJA were less infected with TYLCV.³⁷ The
 223accumulation of *Cucumber mosaic virus* (CMV) in *Momordica charantia* L. (Cucurbitales:
 224Cucurbitaceae) was significantly suppressed when plants received an exogenous application of
 225JA.⁴⁹ On the other hand, the infection process of CMV in *M. charantia* was almost unaffected
 226by the exogenous application of SA, hence revealing how JA, not SA, inhibited virus infection.⁴⁹
 227The activation of the JA pathway is precisely what the phytophagy of the mirids in sweet

228pepper plants stimulates, which could be the explanation for the minor infection by TSWV
229shown in our experiments. Nevertheless, SA also plays an important role in plant defence
230against certain plant viruses. SA exogenous treatments have been reported to reduce the coat
231protein levels of *Tobacco Mosaic Virus* (TMV) and *Potato Virus X* (PVX) during their interactions
232with *N. benthamiana* plants.⁴⁶ Both MeJA and Methyl salicylate (MeSA) are required for the
233systemic resistance response of *N. benthamiana* plants against TMV.⁵⁰ The foliar application of
234MeJA at early stages of TMV infection followed by a later application of SA activated the
235strongest systemic defence response and upregulated the expression of defence related genes
236against TMV.⁵⁰ This is also consistent with another study which showed plant resistance to a
237broad spectrum of RNA viruses could be improved with the application of JA and SA.⁴⁷ Future
238identification of the roles of hormones in plant-virus interactions, how these hormones may
239interact with other biotic stressors, and cross talk among hormone pathways is still needed to
240fully understand the mechanisms by which plants resist infection.

241Sweet pepper plants defensively activated by mirids became less attractive to *F. occidentalis*;²⁶
242the TSWV vector. Interestingly, TSWV infected plants are more attractive to the vector, *F.*
243*occidentalis*, than healthy plants; indeed thrips themselves develop faster on TSWV infected
244plants.⁴⁸ How mirid induced plant responses influence these TSWV-thrips interactions is not
245known, hence further research is needed to evaluate how mirid plant puncturing can limit viral
246infection of TSWV transmitted by thrips. However, not only the mutualistic interactions
247occurring between mirids and plants but also the interactions between vectors and viruses can
248affect the final response of the plant.⁴⁸ Additionally, environmental conditions, the presence of
249alternative food on the plant (pollen and nectar) and the presence of prey are crucial factors to
250be considered for further evaluation of plant mediated effects by mirids and its impact on the
251accumulation of TSWV in sweet pepper plants.

252Current control strategies for TSWV include elimination of infected plants, use of clean stock
253material, exclusion of thrips with greenhouse screens or air locks, and introduction of natural
254enemies.⁴⁹⁻⁵¹ As these control strategies are only partially successful, additional measures are
255needed to limit virus spread. Until recently, resistance to TSWV was obtained through the
256introgression of the two main resistance genes, *Sw5* and *Tsw*, in tomato and pepper,
257respectively. However, the emergence of resistant TSWV isolates (as the one used in our
258experiment)⁴³ has limited the durability of this strategy.^{52,53} Therefore, breeding for durable
259TSWV resistance in plants is still a challenge upon which our results could provide new insight
260into plant viruses resistance. Probably, the activation of JA signalling pathway through genetic
261and chemical manipulation might improve plant defence against plant viruses.

262The possible implementation of strategies based on the above mentioned hypothesis has been
263verified in young plants; the size which is similar to those habitually transplanted from the
264nursery. Previously, nursery inoculation with mirids was proposed since the activation of
265defence responses reduces the infestation of important pests such as the whitefly, *B. tabaci* in
266sweet pepper and tomato plants^{23,26} along with the two-spotted spider mite, *T. urticae* in
267tomato plants.¹⁸ Our results support this strategy since the plants would also be protected
268from diseases such as the TSWV. In this sense, sweet pepper plants can be kept defensively
269activated (upregulated JA pathway) up to 14 days after a single 24 h exposure to mirids.²⁶ The
270same time period of defence activation was obtained also in *M. pygmaeus*-infested tomato
271plants.²⁰ In zones where transplanting occurs at the end of summer there is great insect vector
272pressure, thus protecting young plants from viral infection is crucial. Therefore, these results
273promote the use of biological control which could limit viral incidence at the beginning of the
274cultivation period. Further research must clarify the duration of defence activation under field
275conditions when a part of high vector pressure, the plant is subjected to multiple infestations
276which could work synergistically or antagonistically with each other to activate or block the
277metabolic pathways responsible for defences.⁵⁴

278Herein included is a new perspective which had not been previously considered in the use of
279biological control programs with zoophytophagous predators; the ability of *N. tenuis* and *M.*
280*pygmaeus* to influence the reduction of TSWV infection incidence. New research lines should
281explore defence response activation against other diseases such as those caused by fungi and
282bacteria along with how pathogenic microbes may modulate mirid performance.^{34,35} In
283conclusion, our results provide insights for future studies that can further strengthen pest and
284disease management programs based on these plant-predator-virus interactions.

285

286

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- 449

450 Figure captions**451 Figure 1.**

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

456 Figure 2.

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

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464 Figure 3.

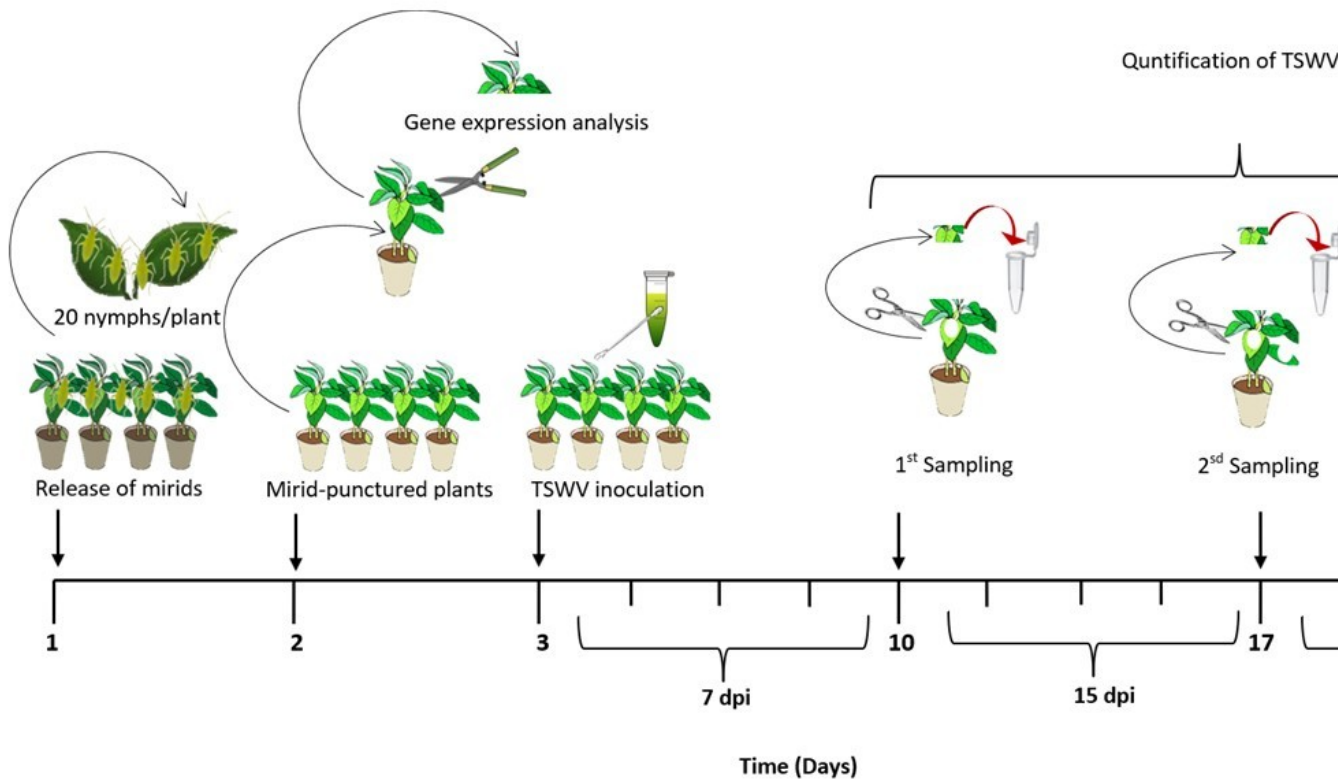
465 Symptoms of TSWV in sweet pepper leaves at 21 days post inoculation (dpi), (a) intact plants,
466 (b) *N. tenuis*-punctured plants and (c) *M. pygmaeus*-punctured plants.

467

468 Figure 4.

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

475**Figure 1.**

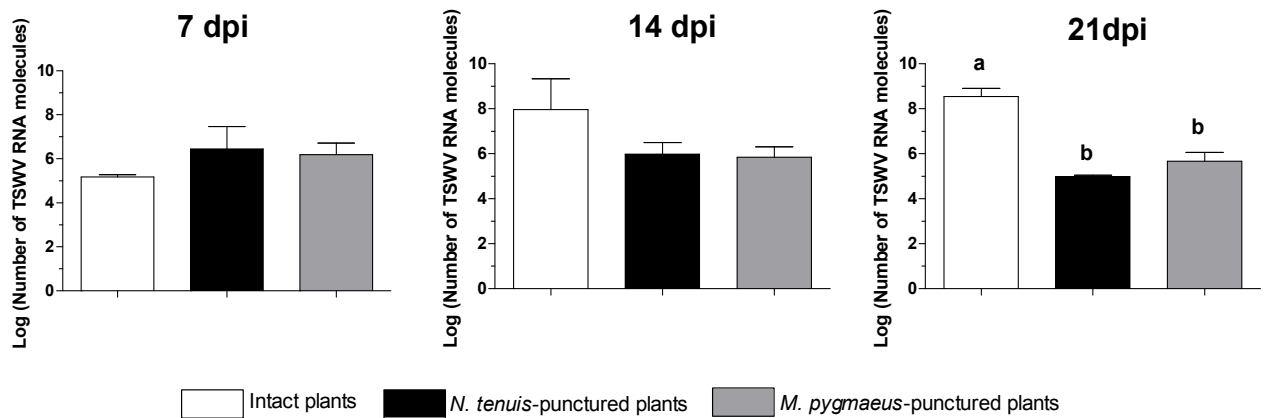


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477 Figure 2.

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483 **Figure 3.**

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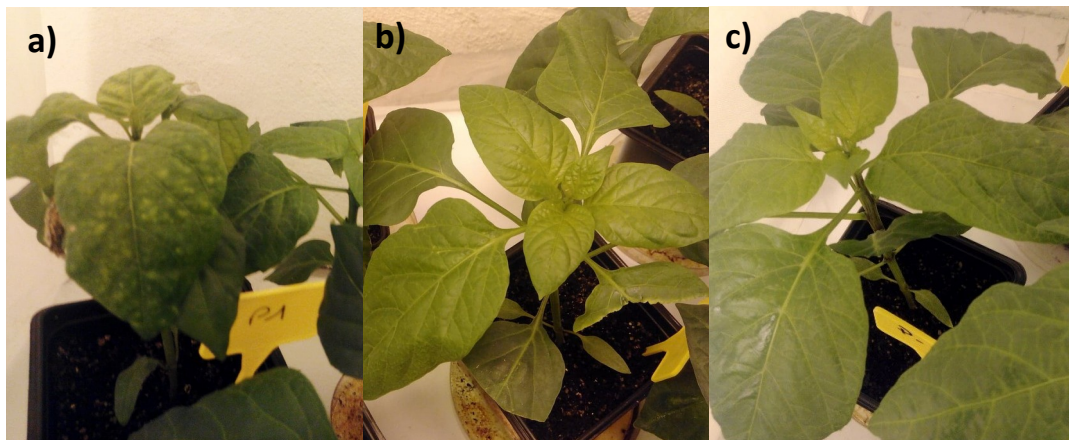
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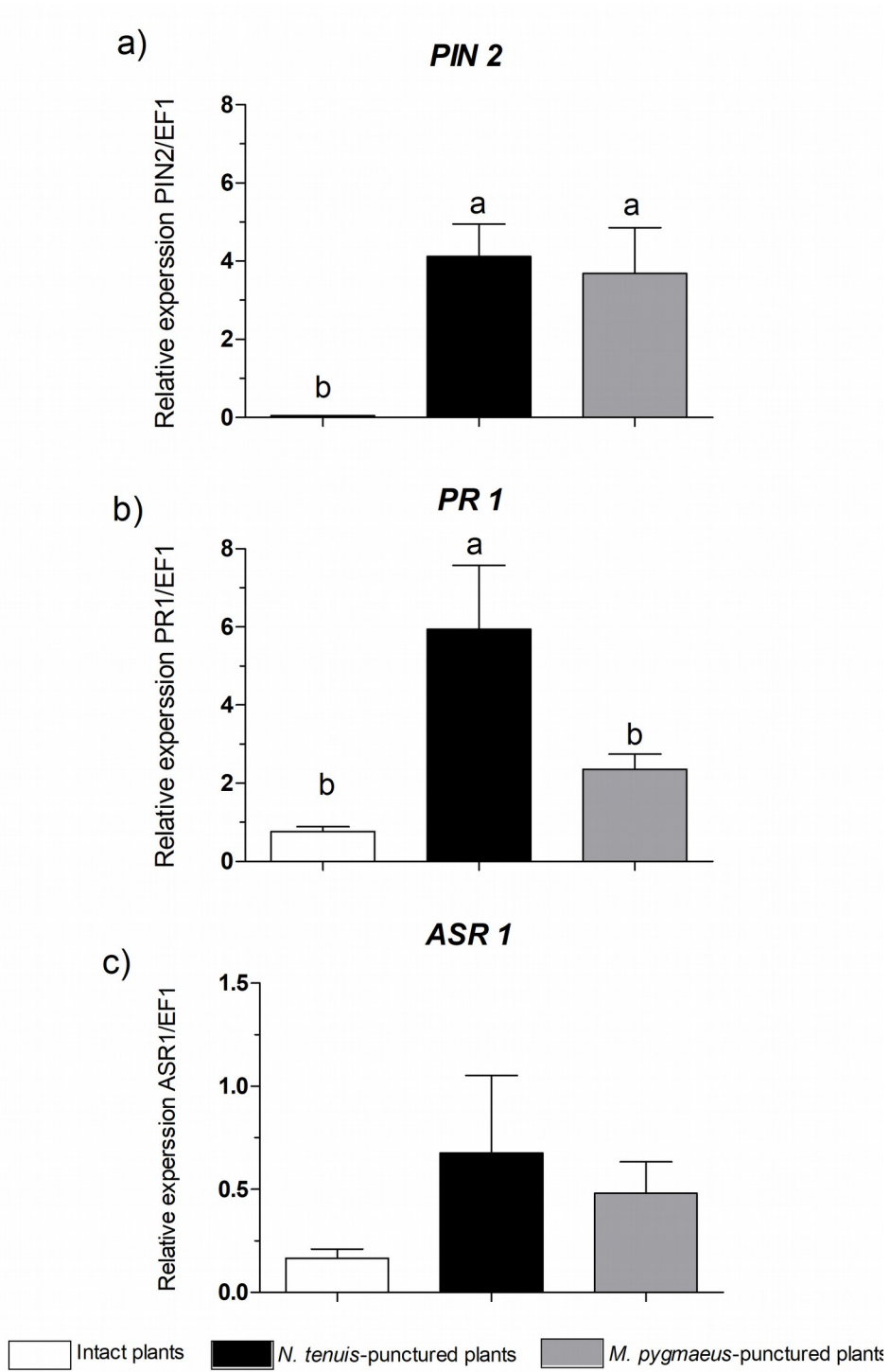
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493 **Figure 4.**



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