The zoophytophagous predator *Pilophorus clavatus* (Hemiptera: Miridae) induces plant defences in citrus

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Short title: Induced defences by *P. clavatus*

Abstract

The zoophytophagous mirid *Pilophorus clavatus* has been recently identified inhabiting citrus orchards in the Mediterranean region, where it feeds on several important citrus pests. In this work, we investigated whether the plant feeding by *P. clavatus* could induce defensive responses in citrus plants. Here, we show for the first time that the *P. clavatus* herbivory triggers the accumulation of the stress-related hormones salicylic acid (SA) and jasmonic acid (JA) in citrus plants. Moreover, the SA and JA pathways enhanced plant defence mechanisms as the expression of genes encoding enzymes from both biosynthetic and responsive pathways were upregulated in *P. clavatus* punctured plants. We also investigated whether the induced defences could affect the plant host selection of *Tetranychus urticae* and the predatory mites *Phytoseilus persimilis* and *Neouseiulus californicus*. Neither *T. urticae* nor *N. californicus* preferred the odour source emitted by intact or *P. clavatus*-punctured plants in a Y-tube olfactometer assay. However, *P. persimilis* were significantly attracted to *P. clavatus*-induced plants. The performance of *T. urticae* was also compared when mites were released on control or previously *P. clavatus*-induced plants. Compared to the control, the infestation of *T. urticae* was significantly reduced up to 70% on those citrus plants previously activated *by P. clavatus*. Our results show for the first time that feeding of *P. clavatus* on citrus plants can have a dual beneficial effect due to its known predatory action and, at the same time, by inducing the plant's immune system.

Keywords: biological control, plant host selection, *Tetraychus urticae, Phytoseilus* persimilis, *Neouseiulus californicus*, HIPVs

Authors' contributions: MP-H and AU conceived the idea. MP-H, MD, and AU designed the research methodology. MD, OR-R, MA-V, and MP-H performed the experiments. MP-H, MD, and AU analysed the data. All the authors discussed the drafts, took part in writing the manuscript, and gave final approval for publication.

Key Message

- *Pilophorus clavatus* is a zoophytophagous mirid recently identified in citrus crops preying on key pests.
- We demonstrate that the plant-feeding behaviour of *Pilophorus clavatus* induces defences in citrus plants.
- The feeding activity of *Pilophorus clavatus* upregulates SA and JA pathways.
- *Pilophorus clavatus*-induced plants become more resistant to the attack of *Tetranychus urticae*.
- Our results are a starting point in studying zoophytophagous predators as inducers of the citrus plant immune system.

Introduction

Citrus crops are characterised by having a large number of both phytophagous arthropods and natural enemies (Urbaneja et al. 2020a). In an ideal scenario, only a few phytophagous arthropods escape the action of natural enemies and can become citrus pests. The major part of the arthropods inhabiting citrus orchards is usually under control thanks to the action of a large and complex group of natural enemies. Nevertheless, this biocontrol balance has been broken in most citrus-producing areas due to the presence of serious diseases that limit citrus production, such as huanglongbing (HLB), citrus Tristeza virus (CTV), citrus variegated chlorosis and citrus leprosies, which are transmitted by psyllids, aphids, certain Cicadellidae and *Brevipalpus* spp. (Acari: Tenuipalpidae), respectively (Urbaneja et al. 2020a). These diseases have compromised conservation biological control due to the multiple applications of broad-spectrum insecticides to control their insect vectors. Such is the case of the Asian and African citrus psyllids *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) and *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), the vectors of HLB incurable disease (Ferrarezi et al. 2020). One of the few citrus-producing areas in the World that is absent from these severe diseases, or in the case of CTV, citrus are cultivated on tolerant rootstocks (Bruessow et al. 2010), is the Spanish citrus orchards. That is why the number of pesticides used in this citrus area is relatively low compared to other citrus areas. Most currently permitted pesticides used are also highly selective against natural enemies. (MAGRAMA 2014; Urbaneja et al. 2020b, 2022). In addition, an abrupt reduction in the number of pesticides available has also occurred (UE 2009; European Commission 2017). This soft pesticide management has led to the appearance in the Spanish citriculture of some natural enemies that had gone unnoticed earlier or had been undervalued (Bouvet et al. 2019). One of these natural enemies is the predatory mirid bug *Pilophorus clavatus* (L.) (Hemiptera: Miridae).

Pilophorus clavatus was reported for the first time in the citrus growing areas in Valencia (Spain) by Bouvet et al. (2019) (cited as *Pilophorus* cf *gallicus*), actively preying on the California red scale *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae). In that work, gut content analysis confirmed the predatory activity of *P. clavatus* on *A. aurantii* from spring to autumn, higher in spring. More recently,

in citrus trees from the same geographical area, Mansour et al. (2021) observed that population levels of *P. clavatus* were positively correlated with the citrus leaf miner predation *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae). Although these two studies revealed the predatory potential of *P. clavatus* in citrus orchards, the possible interaction of this predatory mirid bug with citrus plants was unknown. It is precisely the last aspect that is the focus of our present work.

Predatory mirid bugs have zoophytophagous behaviour. It means they can feed on arthropod prey or the plant they inhabit (Wheeler 2001). When feeding on the plant, the plant can identify this feeding activity as a herbivore attack and launch defence mechanisms (Pérez-Hedo et al. 2021). It has been demonstrated that several species of predatory mirid bugs, such as *Nesidiocoris tenuis* Reuter, *Macrolophus pygmaeus* Rambur, *Dicyphus bolivari* Lindberg, and *Macrolophus praeclarus* (Distant), can activate the immune system in tomato (Pérez-Hedo et al. 2015b, a, 2021; Pappas et al. 2015), and sweet pepper plants (Zhang et al. 2018, 2019; Bouagga et al. 2018a, 2020). Mirid herbivory triggers direct defensive plant responses, reducing pests' performance by producing toxic and anti-nutritive compounds. Mirid herbivory also induces indirect responses mediated by Herbivore Induced Plant Volatiles (HIPVs). The emission of HIPVs can make the induced plants more attractive to natural enemies or even repel certain pests (Zhang et al. 2021; Pérez-Hedo et al. 2022). However, whether *P. clavatus* can induce defences in citrus plants is unknown.

Previous works have shown that citrus plants activate defence mechanisms in response to herbivory by the two-spotted spider mite *Tetranychus urticae* Kock (Acari: Tetranychidae) (Agut et al. 2015), as well as to the phytophagy of the zoophytophagous predatory mite *Euseius stipulatus* Athias-Henriot (Acari: Phytoaeiidae) (Agut et al. 2014; Cruz-Miralles et al. 2019). Both species upregulated the salicylic acid (SA), jasmonic acid (JA), and flavonoid defensive pathways in sour orange, but only the JA pathway was upregulated on Cleopatra mandarin. Interestingly, citrus plants are even capable of activating defensive responses to the presence of *Neoseiulus californicus* (McGregor), a predatory mite that does not feed on the plant. In this case, the JA and flavonoid defensive pathways were downregulated in sour orange, whereas the JA was upregulated in Cleopatra mandarin. It has been hypothesised that

citrus plants can recognise specific signals emitted by this *N. californicus* (trail) or its footprints (Cruz-Miralles et al. 2019; Cruz-Miralles et al. 2021). Therefore, in this work, we study whether herbivory by the predatory mirid bug *P. clavatus* triggers defensive responses in citrus plants. To do this, we first quantified the expression of marker genes for the salicylic acid (SA) and jasmonic acid (JA) pathways in plants previously exposed to *P. clavatus*. Second, plant host selection was studied in a Y-tube olfactometer assay when control and *P. clavatus*-induced plants were offered to *T. urticae* and the two predatory mites *N. californicus* and *P. persimilis*. Finally, the performance of *T. urticae* on previously exposed plants to *T. urticae* was studied and compared to a control treatment.

Materials and Methods

Plants material and insects

Four months old *Citrus sinensis* L. Osbeck *cv.* Pineapple plants (approximately 20 cm in height) were used in all experiments. Citrus seeds were sown on a sterile substrate composed of peat moss, coconut fibre and silica sand (50:25:25) in seedling trays. Three weeks after germination, seedlings were individually transplanted into plastic pots (8 × 8 × 8 cm). Citrus plants were maintained at 25 ± 2 °C, with a constant relative humidity of 65% ± 5% and a photoperiod of 14:10 h (light: dark). All citrus plants were pesticide-free and watered twice a week. One of the waterings was fertigated at 2% with a citrus-specific fertiliser composed of $NH_4H_2PO_4$ at 0.115 g/L, KNO_3 at 0.065 g/L, $Ca(NO_3)_2$ at 1.25 g/L and synthetic chelating at 0.018 g/L [Sequestrene® (Syngenta NK 138Fe, Basel, Switzerland)].

Females of *T. urticae* were obtained from a colony available at the Instituto Valenciano de Investigaciones Agrarias (IVIA) since 2011. The colony was initially collected and renewed annually from clementine orchards in the region of La Plana (Castelló, Spain). The colony was maintained on lemon fruits in a climatic chamber at 25 ± 2 ºC, 65 ± 10 % RH and a photoperiod of 14:10 h (light: dark) (Argolo et al. 2014). The phytoseiids *P. persimilis* and *N. californicus* were provided directly by Koppert Biological Systems S.L. (Murcia, Spain). The predatory mirid *P. clavatus* used to induce citrus plants was

also obtained from a colony established at the IVIA. The initial individuals that started the colony were collected in experimental citrus orchards at the IVIA during the spring of 2019. The *P. clavatus* colony was maintained on green bean pods (*Phaseolus vulgaris* L. Fabales: Fabaceae) with *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) frozen eggs as supplementary food inside plastic cages of 30 × 30 × 30 cm (BugDorm-1 insect tents; MegaView Science Co., Ltd., Taichung, Taiwan), under controlled environment conditions (25 ± 2 ºC, 65 ± 10 % RH and a photoperiod of 14:10 h [light: dark]). Mite, predatory mite females, and *P. clavatus* females, less than five days old, were isolated and starved in a Petri dish (9 cm in diameter) for 24 h before their use in the Y-tube experiments under the same environmental conditions mentioned above.

Pilophorus clavatus-punctured plants were obtained by exposing citrus plants to seven couples (male and female) of *P. clavatus* for 48 h in a 30 × 30 × 30 cm plastic cage (BugDorm-1 insect tents). Plants and insects were left undisturbed under the same environmental conditions mentioned above. All individuals were removed from plants before the beginning of each trial.

Plant hormone determination

Jasmonic Acid (JA) and Salicylic acid (SA) were quantified in leaves of untreated control (not been in contact with any arthropod since germination), and *P. clavatus* punctured plants, using the extraction protocol adapted from Seo et al (2011). Briefly, collected leaves were grounded in liquid nitrogen and stored at -80 °C. About 150 mg of leave tissue was freeze-dried and suspended in 80 % methanol and 1 % acetic acid solution, which included the respective internal standards (OlChemim Ltd., Olomouc, Czech Republic). The mixture was shaken for 1 h at 4 °C, and the extract was maintained at -20 °C overnight. After that, the extract was centrifuged at 14,000 g to obtain the supernatant, in which methanol was evaporated. Then the extracts were dissolved in 1 % acetic acid and passed through an Oasis HLB reverse phase cartridge. A reverse-phase 2.6 µm diameter Accucore UHPLC column (Thermo Fisher Scientific, San Diego – CA, USA) was used for the extraction, washed with a gradient of acetonitrile (2 % - 55 %) and 0.05 % acetic acid at a constant speed for 22 min. SA and JA were detected

in a mass spectrometer (ESI–MS/MS; Thermo Fisher Scientific, San Diego – CA, USA), and Targeted Selected Ion Monitoring and Electrospray Ionization in the negative mode was used for the detection of both plant hormones. The extracts' concentrations of SA and JA were determined using embedded calibration curves always with the internal standard [2H4]SA and dhJA (OlChemim Ltd., Olomouc, Czech Republic) using the Xcalibur 4.0 and TraceFinder 4.1 SP1 programs. Hormone concentration was expressed as ng of SA or JA per ng fresh weight (ng/g FW).

Expression analysis of SA and JA marker genes

The expression of genes encoding proteins from the SA and JA biosynthesis and responsive pathways was analysed in the same leaf tissue collected from control and *P. clavatus* punctured plants (eight plants per treatment) used for hormone quantification. The expression of two biosynthetic genes [*CsICS2* (encoding an isochorismate synthase from the secondary biosynthetic pathway of SA –the ICS pathway) and *CsPAL* (encoding a phenylalanine ammonia-lyase) (reviewed by Lefevere et al. (2020)] and two defensive-response genes [CsNPR1 (encoding a transcriptional activator that is translocated to the nucleus where it interacts with bZIP TFs that bind to the promoters of SA-responsive genes; Fan and Dong (2002) and CsPR5 (encoding an osmotin-like protein reviewed by de Jesús-Pires et al. (2020)] was analysed for SA.

In the case of JA, the expression of two biosynthetic genes [*CsLOX2* (encoding a lipoxygenase that catalyses the addition of molecular oxygen to the α-linolenic acid released from glycolipids of chloroplast membrane to yield the 13-hydroperoxide) and *CsAOS1-2* (encoding a CYP450 that transform the 13-hydroperoxide substrate in an unstable allene oxide intermediate)(reviewed by Li et al. (2021)] and two defensive-response genes [*CsPR3* (encoding a chitinase 4-like protein that catalyses the hydrolysis of the beta-1,4-N-acetyl-D-glucosamine linkages in chitin polymers; Thomma et al. 1998)) and *CsDTX19* (encoding a multidrug and toxin extrusion-like [MATE] transporter involved in extracellular accumulation of protective secondary metabolites, such the hydroxycinnamic acid amides [HCAAs], at the plant surface; Dobritzsch et al. (2016)] was assessed.

Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN. Mississauga – ON, Canada). RNA was treated with TURBO DNA-free™ Kit to remove contaminating genomic DNA, following the manufacturer's procedure (Thermo Fisher Scientific. VNO, Lithuania). First-strand cDNA was synthesised using Prime Script RT Reagent Kit as described by the manufacturer's procedure (TAKARA Bio. CA, USA). Quantitative Real-time PCR (qRT-PCR) was performed in a LightCycler® 480 System (Roche Molecular Systems. Inc., Switzerland), using NZYSupreme qPCR Green Master Mix (2x) (NZYTech. LIS, Portugal) as described by Bouagga et al. (2018a). *CsGAPC1* (Glyceraldehyde-3-Phosphate Dehydrogenase; formerly *GAPDH*) was the reference gene used as a standard control for normalisation. Primers sequence of the reference gene, SA and JA biosynthetic genes, and those related to the plant defence mechanisms induced by both hormones are given in Table 1. Primers for JA-related genes were designed using the Oligo 7 informatics software (Molecular Biology Insights Inc., West Cascade – CO, USA). The relative gene expression analysis was performed by the ΔCt against the reference gene (Livak and Schmittgen 2001). The occurrence of non-specific amplification products was ruled out by melting curve analysis.

Y-tube olfactometer bioassays

A Y-tube olfactometer experiment was conducted to test the olfactory responses of *T. urticae*, *N. californicus,* and *P. persimilis* to *P. clavatus*-punctured citrus plants relative to intact control plants. The Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) consisted of a Y-shaped glass tube (2.4 cm in diameter, 13.5 cm length base, and two arms 5.75 cm length each) which was connected via high-density polyethylene (HDPE) tubes to two 5 L glass jars. Each jar containing an odour source (*P. clavatus*-exposed plant or unexposed plant) was connected to a unidirectional air pump with a 150 mL/min airflow rate. A Y-shaped metallic wire with a diameter of 1 mm was inserted into the Y-tube to facilitate the movement of the mites. The experiment's environmental conditions were 23 ± 2 ºC, RH 60% ± 10, and light intensity of 2516 lux (Pérez-Hedo and Urbaneja 2015). Each individual was introduced to the entry base arm and observed for a maximum of 15 min. Once the mite walked at

least 3 cm up to one of the arms, the time was recorded, and the individual was considered a "responder". If, after 15 min, the mite had not chosen between either arm, it was excluded from the data analyses. Each individual was used only once, and the total number of replicates per species was 40 responder individuals. After five females were tested, the right and left arms were switched to avoid any spatial effect. After testing ten females, the Y-tube glass material (jars and Y-arm) was rinsed with soap, water, and acetone and was dried for 5 min, and plants (induced and intact) were replaced with new ones.

Performance of *Tetranychus urticae*

The performance of *T. urticae* when released on intact or *P. clavatus*-induced plants was tested in a bioassay under controlled conditions of 25 ± 2 ºC, 65 ± 10 % RH, and a photoperiod of 14:10 h (light: dark). For each experimental treatment (induced or intact), one citrus plant was isolated in a plastic cage (60 × 60 × 60 cm) (BugDorm-2 insect tents), and five replicates per treatment were prepared as mentioned above. To avoid volatile interferences between both treatments, one climatic chamber was assigned to the treatment with *P. clavatus*-induced plants and a second to the control treatment. Plants were artificially infested with 12 female *T. urticae* from the above-mentioned colony. Mite females were carefully placed with a soft brush on two leaves equally. Females of *T. urticae* per plant were counted visually every seven days for five consecutive weeks. Two successive replicates following the methodology described above were conducted.

Data analyses

Results are presented as mean ± standard error (SE). Data obtained from the Y-tube olfactometer experiment were statistically analysed by the Chi-square goodness of fit test based on a null model in which the two odour sources are selected with equal frequency. Data of gene expression quantification were analysed using a one-tailed Student's *t*-test (P =<0.005). Data from both *T. urticae* performance bioassays were analysed using a generalised linear mixed model (GLMM) with repeated measures.

Treatment was considered a fixed factor, sampling dates were repeated measures, and replicate (block) was a random factor. The data were fitted by maximum likelihood (Laplace Approximation) to a negative binomial generalised linear mixed model (GLMM) with a log link function. Results are expressed as the mean ± standard error (SE).

Results

SA and JA are highly accumulated in citrus plants infested by *P. clavatus*

To investigate whether the phytophagy behaviour of the mirid *P. clavatus* can induce the synthesis of stress-related phytohormones, we quantify the endogenous level of SA and JA in citrus plants infested for 48 hours under controlled environmental conditions. Comparison of the SA and JA content in leaves of infested plants with those of control plants revealed that the biosynthesis of both phytohormones was strongly induced by the feeding of *P. clavatus* (t = -3.304; df = 1, 18; P = 0.004 and t = -3.422; df = 1, 18; P = 0.003, respectively) (Fig. 1). Notably, the phytophagy behaviour of *P. clavatus* increases more than three times the content of SA and nearly five times the content of JA in the leaves of infested plants compared to those of control untreated plants.

P. clavatus phytophagy enhances the expression of SA and JA pathways marker genes in citrus plants

The quantification of transcripts levels of the two marker genes encoding enzymes from the SA biosynthesis pathway (*CsICS2* and *CsPAL*) and the two SA-responsive pathway marker genes (CsNPR1 and *CsPR5*) was upregulated in citrus plants punctured by *P. clavatus* (t = -9.041, df =1, 13; P = <0.001; t = -9.224, df =1, 16; P = <0.001; t = -4.539, df =1, 14; P = <0.001; t = -6.925, df =1, 16; P = <0.001, respectively) (Fig. 2A). The expression of the two genes encoding enzymes from the JA biosynthetic pathway (*CsLOX2-1* and *CsAOS1-2*) and the two JA-responsive genes (*CsPR3* and *CsDTX19*) was also upregulated in citrus plants previously exposed to *P. clavatus* (t = -14.970; df =1, 12; P = <0.001; t = -7.

573; df =1, 14; P = <0.001; t = -6. 443; df =1, 11; P <0.001; t = -8.778; df =1, 14; P <0.001, respectively) (Fig. 2B).

P. clavatus-punctured plants do not alter plant selection by *T. urticae* or *N. californicus* but by *P. persimilis*

The two-spotted spider mite *T. urticae* and the predatory mite *N. californicus* showed no preference for the odour source emitted by intact citrus plants when compared to *P. clavatus*-punctured plants (X^2 = 0.1000; P = 0.7518 and X^2 = 3.600; P = 0.0578; respectively) (Fig. 3). However, the predatory mite *P. persimilis* chose *P. clavatus*-punctured plants when was given to choose between these and the intact control plants (X^2 = 6.140; P = 0.0144) (Fig. 3). Only 6 *T. urticae*, 2 *N. californicus* and 4 *P. persimilis* did not show any response in the T-tube experiment (Fig. 3).

P. clavatus-punctured plants reduce *T. urticae* performance

Two consecutive replicates were conducted to evaluate the performance of *T. urticae* in *P. clavatus*-punctured orange seedlings compared to untreated control. In the first experiment, the number of *T. urticae* females per citrus plant was lower in *P. clavatus*-induced plants compared to intact control plants (F = 30.559; df = 1, 58; P < 0.0001) (Fig. 4A). On day 35, the accumulated number of *T. urticae* females on plants previously exposed to *P. clavatus* was reduced by 70.1 ± 7.2 % compared to the control treatment. The experiment ended on day 35 after the release of *T. urticae* because citrus plants from the control treatment collapsed due to the extensive damage produced by the mites on the next sampling day (day 42). This same trend was observed in the *P. clavatus*-induced plants, where on day 42, similar levels of *T. urticae* were reached to those obtained seven days earlier in the control treatment (223.7 ± 31.4). In the second experiment, the number of mites per plant was also lower in *P. clavatus*-induced plants compared to the control treatment (F = 27.183; df = 1, 46; P < 0.0001) (Fig. 4B). Statistical analysis was only possible up to day 28 since from that day the leaves from control

plants began to drop and plants collapse due to the high infestation level reached by *T. urticae*. On day 28, the accumulated number of *T. urticae* females on plants previously exposed to *P. clavatus* was reduced by 54.1 ± 5.2 % compared to the control treatment. On day 35, the *T. urticae* population in *P. clavatus*-induced plants continued increasing. The leaves began to drop due to the heavy damage produced by the high population of mites on these plants.

Discussion

It is widely known that, due to their phytophagous behaviour, predatory mirid bugs induce plant responses in various plant species (see reviews Perez-Hedo et al. [2022] and Zhang et al. [2021]). This research field has increased in recent years, but as far as we know, our work describes for the first time how the phytophagy of a zoophytophagous mirid induces defences in citrus plants. Furthermore, we have described how this activation modulates plant resistance against one of the most important citrus pests, the two-spotted spider mite *T. urticae*. We hypothesise that the citrus plants have responded mainly to *P. clavatus* feeding behaviour, as we have observed with other species of zoophytophagous predators (Naselli et al. 2016; Bouagga et al. 2018a). However, because *P. clavatus* females may oviposit upon tender stems on citrus plants, we cannot rule out that some females could have oviposited on plants, resulting in plant responses. This point will need to be clarified in future works.

Insect herbivore attack causes an extensive rearrangement of the plant transcriptome that involves the up- and/or down-regulation of many genes (Garcia et al. 2021). The action and crosstalk coordinate this transcriptomic rearrangement among different stress-related plant hormones (Heidel and Baldwin 2004; Erb et al. 2012). This work shows that the SA and JA pathways were upregulated in *P. clavatus*-induced citrus plants. SA and JA are central in regulating defence responses to herbivores that inflict various types of tissue damage. Different approaches have shown that the SA and JA pathway has a dominant role in regulating global changes in gene expression in response to herbivory (War et al. 2011; Beyer et al. 2021) and has a key role in the acquisition of resistance against pests and

diseases in citrus (Vacas et al. 2009; Agut et al. 2014; Huang et al. 2021; Long et al. 2021; Ibanez et al. 2022). Exogenous SA treatment attenuates the occurrence of citrus canker [*Xanthomonas axonopodis* pv. *citri* (Xac)] in susceptible navel oranges (Wang and Liu 2012). Treatment of susceptible Cleopatra mandarin with MeJA –a methyl ester of JA– has been shown to reduce the oviposition of *T. urticae* (Agut et al. 2014). Zou et al. (2019) compared the susceptibility of two different citrus species against the HLB, the most devastating citrus disease worldwide. They observed that methyl salicylate (MeSA), involved in the SA signalling pathway, is a critical mobile signal for plant systematic acquired resistance (SAR), playing an essential role in citrus tolerance to (HLB). The relationship between the SA pathway and HLB was also demonstrated in transgenic plants that over-express the gene *NPR1*, an essential regulator gene from the SA signal transduction pathway that leads to SAR (Dutt et al. 2015). Over-expression of *NPR1* on transgenic citrus trees exhibited enhanced resistance to HLB and reduced disease severity. Furthermore, it has been shown that SA mediates the immune response of *Citrus sinensis* to the psyllid *D. citri* and *Candidatus* Liberibacter asiaticus (*Ca*Las: the bacterium that causes HLB) infection (Ibanez et al. 2022). Therefore, conserving *P. clavatus* in citrus plants could increase their tolerance against this threatening disease by activating the SA pathway.

Activation of the SA- and JA- mediated pathways also activates indirect defences, which trigger the emission of different volatiles blends that attract natural enemies (van Poecke and Dicke 2002; Asai et al. 2016). Some previous work has also shown that predatory mites respond to volatiles emitted by plants that had been previously exposed to zoophytophagous predators (Pérez-Hedo et al. 2018; Cruz-Miralles et al. 2021; Zhang et al. 2022). In our work, *P. clavatus*-induced plants turned out to be attractive to *P. persimilis* but not to *N. californicus*, although a trend toward *P. clavatus*-induced plants was perceived. Cabedo-López et al. (2019) discussed that the responses of predatory mites changed according to plant genotype, the presence of the prey, and the specialisation of the predator's diet. Nevertheless, our results support the idea that the two-spotted spider mite *T. urticae* did not show a preference for either odour source tested. A further step of our present work would be to study the composition of the volatiles emitted by *P. clavatus*-induced plants to discern and confirm which

344 volatiles or blend of volatiles are responsible for the attraction. In the same way, it would also be
345 interesting to study the effect of the plant-feeding by *P. clavatus* on the behavioural response of other
346 natural enemies inhabiting citrus crops.

347 We have confirmed the activation of the SA and JA pathways by the feeding activity of *P. clavatus*.
348 However, other phytohormones such the abscisic acid (ABA) or brassinosteroids (BRs) with a
349 demonstrated role in citrus defence against pests (Agut et al. 2014, 2015, 2016; Alférez et al. 2019;
350 Cruz-Miralles et al. 2019), escape to the scope of this work. Therefore, the potential response of other
351 phytohormones after *P. clavatus* feeding should be considered in future works to completely unravel
352 the metabolic interaction of this zoophytophagous predator with citrus plants.

353 When females of *T. urticae* were released on *P. clavatus*-induced plants and their number of progeny
354 was compared to control plants, the population levels of *T. urticae* were significantly lower in plants
355 previously induced by *P. clavatus*. It has been reported that plants respond directly to herbivore attacks
356 by negatively affecting the survival and reproductive success of herbivores (Soler et al. 2012), and this
357 is the case for the *P. clavatus* phytophagy observed in our study. The reduced performance of *T. urticae*
358 has been previously related to the induction of SA- and JA-related defences and the accumulation of
359 secondary metabolites such as glucosinolates in citrus and tomato plants (Kant et al. 2008; Agut et al.
360 2014, 2016). The activation of the SA signal transduction pathway has been described as a herbivore
361 repellent (Erb et al. 2012; Liu et al. 2016), although this was not observed in this work where *T. urticae*
362 did not turn out to be repelled. The activation of the JA pathway has previously demonstrated a
363 reduction in the performance of *T. urticae* (Arimura et al. 2000; Li et al. 2002; Kant et al. 2004; Ament
364 et al. 2004; Pappas et al. 2015). Ament et al (2004) suggested that direct JA-related defences reduced
365 egg viability and lengthened the embryonic period. The JA pathway upregulates proteinase inhibitors,
366 which have already been suggested to decrease the performance of *T. urticae* (Pappas et al. 2015;
367 Pérez-Hedo et al. 2018).

Here we have also shown that the activation of plant defence responses by *P. clavatus* temporarily affects the performance of *T. urticae*, returning to its basal values after a few days. This phenomenon may explain why the *T. urticae* populations had a lag of approximately one week between the *P. clavatus*-induced plants and the control treatment and why the plants induced by *P. clavatus* also collapsed by *T. urticae* infestation, although seven days later.

Given the results obtained in this work were carried out on seedlings, the next step would be to study whether this effect can be extrapolated to mature trees where *P. clavatus* populations are continuously present (Bouvet et al. 2019; Mansour et al. 2021) but where many other biotic and abiotic factors can interfere with the induction of defences. In this sense, to know if the presence of *P. clavatus* can activate defences in mature citrus plants would be of extreme interest. If this hypothesis is confirmed, the conservation of *P. clavatus* populations in adult citrus trees will make them more resilient against pests and diseases by activating their defence mechanisms, in a similar way to that reported in horticultural crops where predatory mirids are maintained (Pérez-Hedo et al. 2017, 2022; Pérez-Hedo et al. 2021).

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Statements & Declarations

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

M.P.H. and A.U. conceived the idea. M.P.H., M.D., O.R.R and A.U. designed the research methodology. M.D., O.R.R., M.A.V., and M.PH. performed the experiments. M.P.H., M.D., O.R.R and A.U. analysed the data. All the authors discussed the drafts, took part in writing the manuscript, and gave final approval for publication.

586 **Table 1.** List of genes and primers used in qPCR assays.

Gene[a]	Gene name	Former Name	Locus	GenBank ID	Gene ID[b]	Primer Sequence (5'→3')	PCR PRODUCT (bp)	Reference
CsGAPC1	*Glyceraldehyde-3-phosphate dehydrogenase,*	*CsGAPDH*	LOC102624117	XM_006476919	Cs5g06870	**FW:** GGAAGGTCAAGATCGGAATCAA **RV:** CGTCCCTCTGCAAGATGACTCT	75	Cruz-Miralles et al (2018)
CsPAL	*Phenylalanine ammonia-lyase-like*	*CsPAL*	LOC102620464	XM_006481431	Cs6g11940	**FW:** CACATTCTTGGTAGCGCTTTG **RV:** AGCTACTTGGCTGACAGTATTC	94	Alferez et al. (2018)
CsICS2	*Isochorismate synthase 2, Chloroplastic*	*CsICS*	LOC102630235	XM_006476588	Cs5g04210	**FW:** GGAGGAGGAGAGAGTGAATTTG **RV:** GGGTTGCTTCCTTCTACTATCC	107	
CsNPR1	*BTB/POZ domain and ankyrin repeat-containing protein*	*CsNPR1*	LOC102617188	XM_006475416	Cs4g14600	**FW:** GTACCTTGAAAACAGAGTTGGACTGG **RV:** TGCTCCTCTTGCATTTTGAAAGGTG	189	
CsPR5	*Osmotin-like protein*	*CsPR5*	LOC102607779	XM_006488230	Cs8g18200	**FW:** CATCAAGCTTCACAGTGCTTAG **RV:** CCACAACGTACAGACTGATGAC	152	Cruz-Miralles et al (2018)
CsLOX2	*Linoleate 13S-lipoxygenase 2-1, chloroplastic-like*	*CsLOX2*	LOC102629656	XR_001506736	orange1.1t03770	**FW:** GAACCATATTGCCACTTTCG **RV:** CGTCATCAATGACTTGACCA	231	Cruz-Miralles et al (2018)
CsAOS1-2	*Allene oxide synthase*	-	-	NM_001288906	Cs3g24230	**FW:** AGATCTTATTCCCGAACATGGT **RV:** CGGACTTCATCAACGGCAT	150	This work
CsPR3	*Chitinase 4-like*	-	LOC112495548	XM_025099484.1	Cs5g21850.1	**FW:** CCCCGTTGTGTCATTTAAGACTG **RV:** TCCTTATAATATCCGTTGCGAGCTTG	162	
CsDTX19	*MATE (Multidrug And Toxin Extrusion) transporter*	-	LOC102608545	XM_006464716.3	Cs1g07540.1	**FW:** TTAGTAACAGCCCTGAAATCATAAAGG **RV:** AATAGAATGTTGCCAAATTAGCCCA	159	

587 [a]Former gene names were updated based on their functional annotation according to the RefSeq Genome Sequencing Project PRJNA86123 published for *Citrus sinensis* cv Valencia at NCBI
588 (http://www.ncbi.nlm.nih.gov/refseq/). [b]Citrus Genome Database (https://www.citrusgenomedb.org/). **FW:** Forward; **RV:** Reverse.

589 **Figure captions**

590 **Figure 1.** SA and JA content in leaves of control and *P. clavatus* punctured orange Pineapple plants.
591 Hormone content was determined by ultra-high-performance liquid chromatography-mass
592 spectrometry (UHPLC–MS) using a Q-Exactive spectrometer (Orbitrap detector; Thermo Fisher
593 Scientific). Asterisks indicate significant differences (*t*-test; P<005).

594 **Figure 2**. Relative expression of genes from the SA and JA pathways in the leaves of control and *P.*
595 *clavatus*-punctured orange plants. Data represent the mean of eight plants independently analysed.
596 (**A**) Genes from the SA biosynthesis and responsive pathways: ***CsPAL*** (*Phenylalanine ammonia-lyase*),
597 ***CsICS2*** (*Isochorismate synthase-2*), ***CsNPR1*** (encoding a BTB/POZ domain and ankyrin repeat-
598 containing protein), and ***CsPR5*** (Pathogenesis-Related Gene 5, encoding an *Osmotin-like protein*). (**B**)
599 Genes from the JA biosynthetic and responsive pathways: ***CsLOX2*** (Liposygenase 2), ***CsAOS1-2*** (Allene
600 oxide synthase), ***CsPR3*** (Chitinase 4-like), and ***CsDTX19*** (MATE transporter). Asterisks indicate
601 significant differences (*t*-test; P<005).

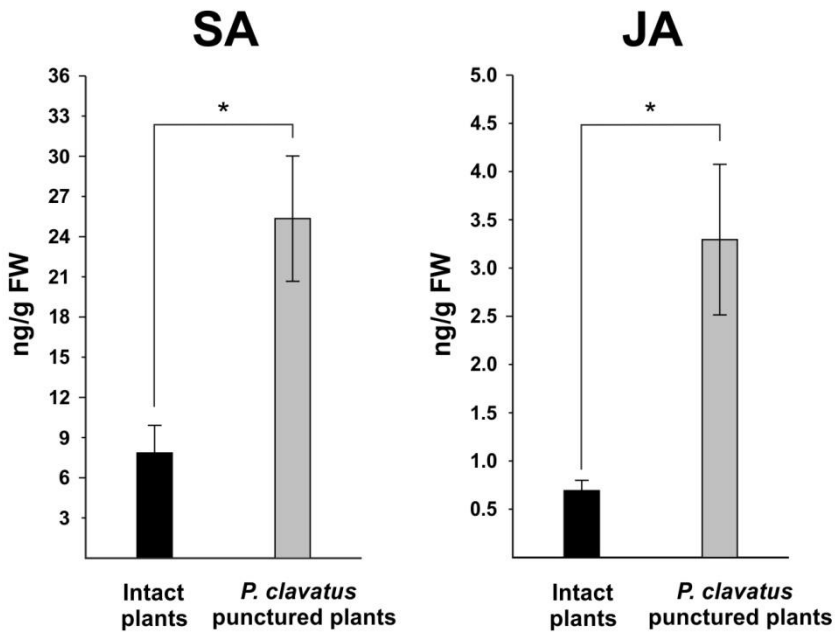
602 .**Figure 3**. Response (%) of females of *Tetranychus urticae* (*T.u.*), *Neouseiulus californicus* (*T.u.*) and
603 *Phytoseiulus persimilis* (*P.p.*) in a Y-tube olfactometer when exposed simultaneously to control and *P.*
604 *clavatus*-punctured citrus plants. “nc” indicates the number of tested females that did not make a
605 choice. Asterisks indicate significant differences in the distribution of side-arm choices (χ2 tests; P =<
606 0.05).

607 **Figure 4**. Comparison of mite performance on intact control vs *P. clavatus*-punctured orange plants.
608 (**A**) Trail 1 and (**B**) Trial 2. Data correspond to the number (mean ± SE) of *Tetranychus urticae* females
609 per citrus plant. Asterisks indicate significant differences as detected by the generalised linear mixed
610 model (GLMM, repeated measures; P < 0.05)

611

612

Figure 1


SA
ng/g FW
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Intact plants
P. clavatus punctured plants
JA
ng/g FW
5.0
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4.0
3.5
3.0
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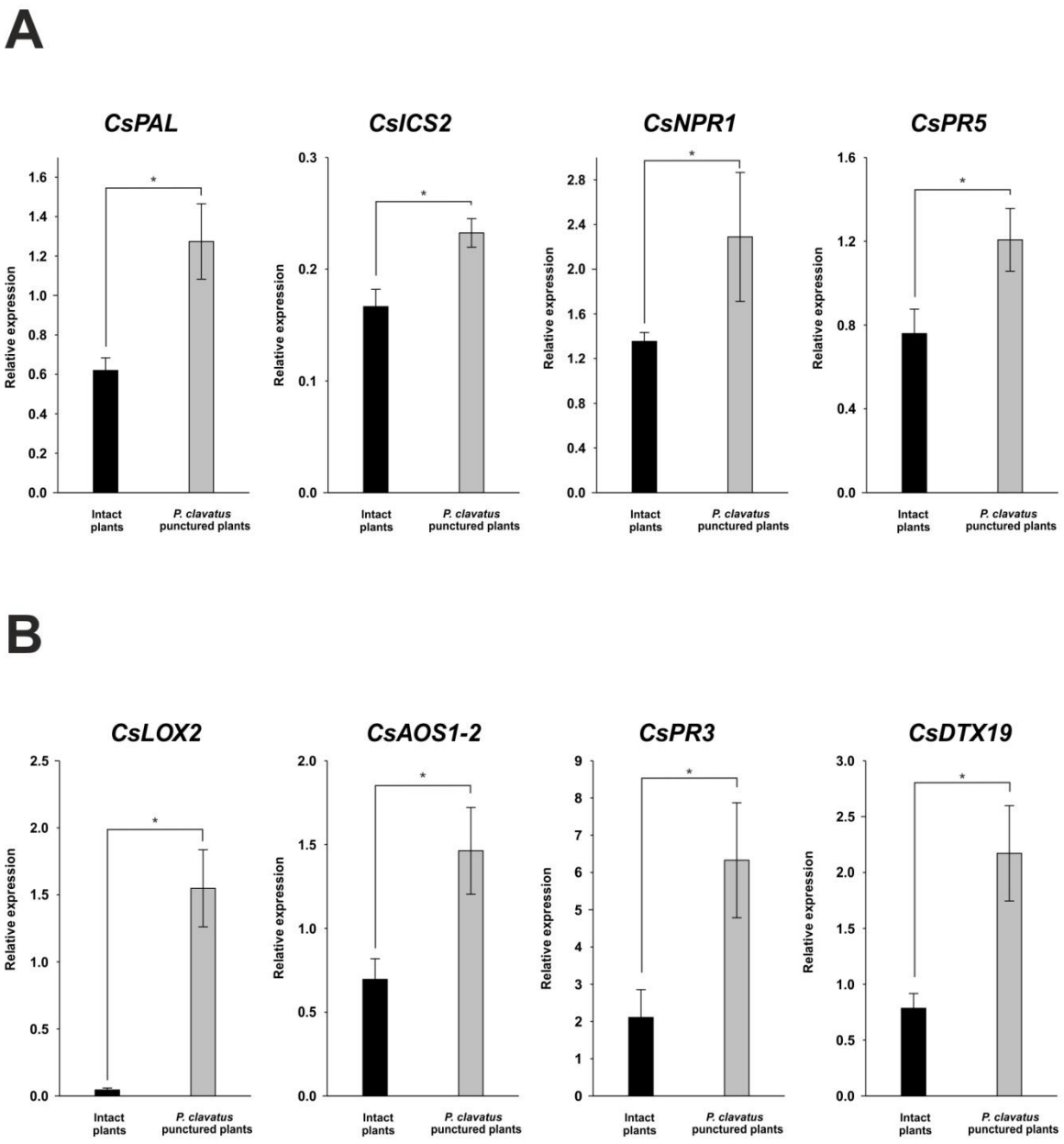
2.0
1.5
1.0
0.5
*
Intact plants
P. clavatus punctured plants

25

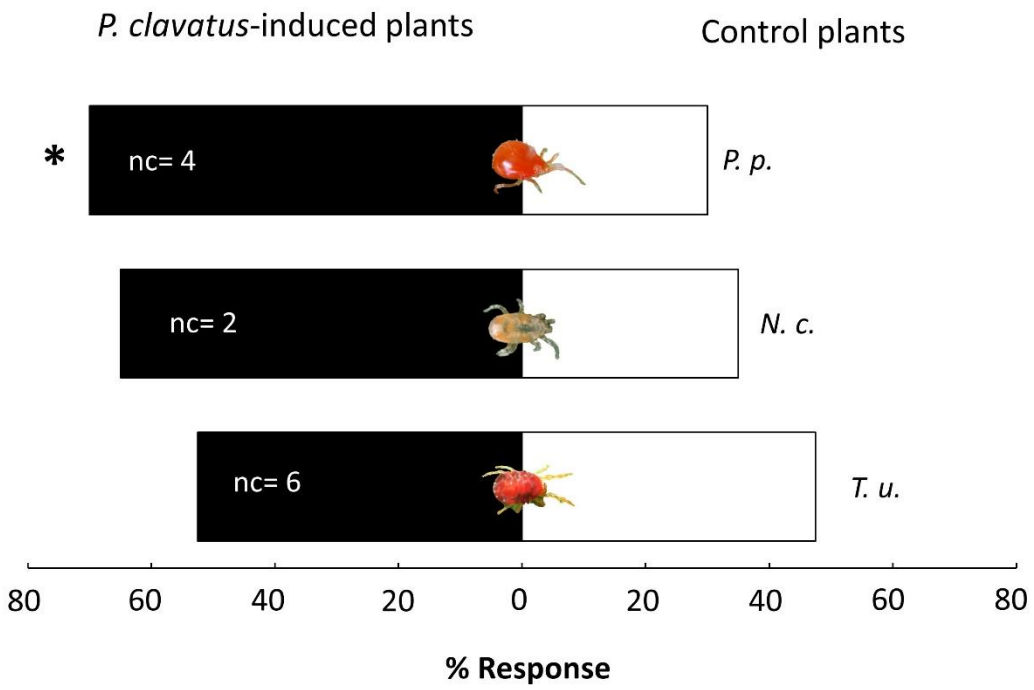
630 **Figure 2**

631



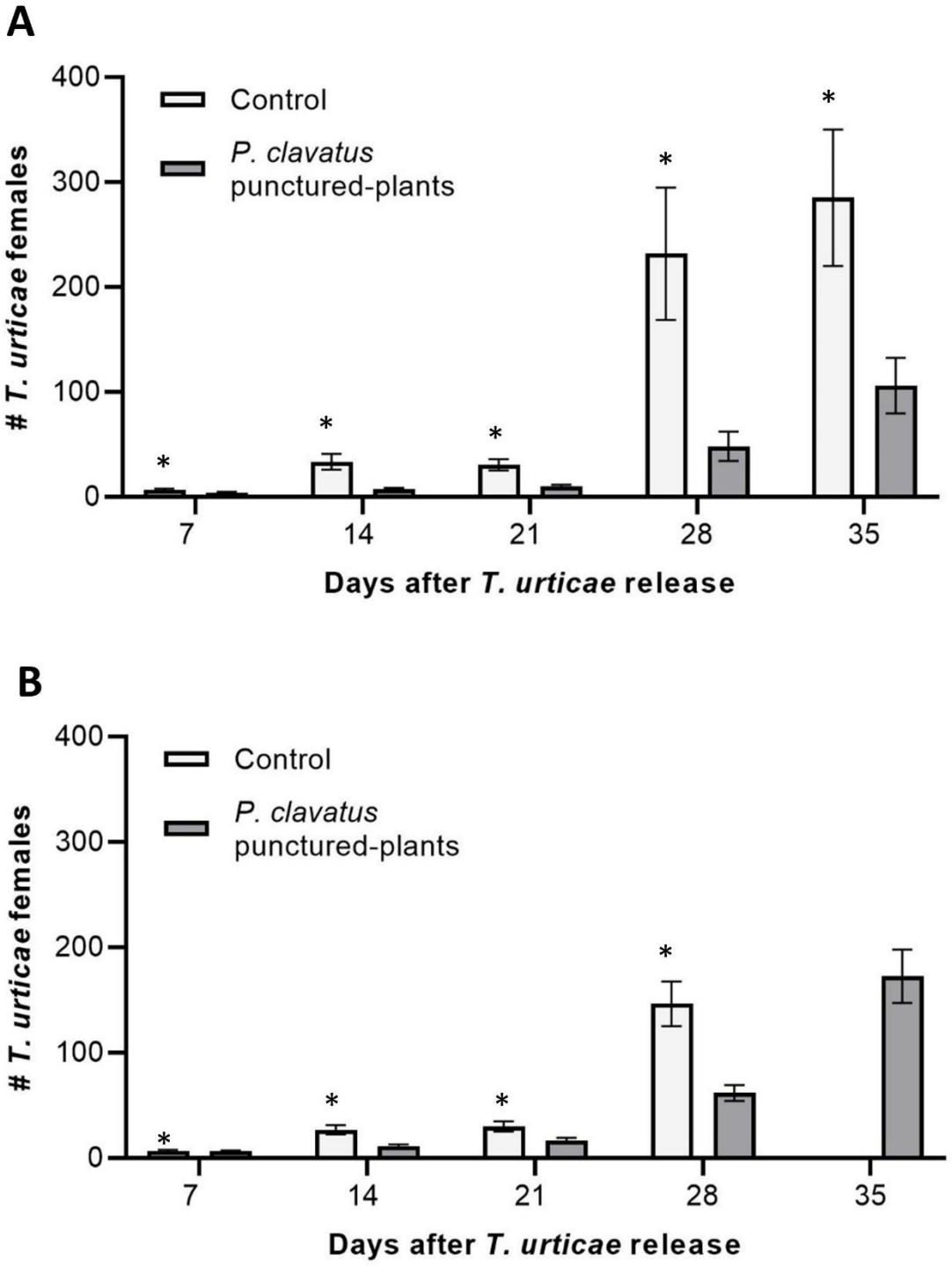
632

633 **Figure 3**



634

635 **Figure 4.**



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