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Phytopathology

Co-infection of tomato plants with *Tomato yellow leaf curl virus* and *Tomato chlorosis virus* affects the interaction with host and whiteflies

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Keywords: *Tomato yellow leaf curl virus*, *Tomato chlorosis virus*, co-infection, synergism, *Bemisia tabaci*, host choice, visual, olfaction.

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ABSTRACT

Susceptible plants infected by single or multiple viruses can differ in symptoms and other alterations influencing virus dissemination. Furthermore, behavior of viruliferous vectors may be altered in certain cases to favor acquisition and inoculation processes conductive to virus transmission. We explored single and mixed infections frequently occurring in tomato crops, caused by two viruses transmitted by the whitefly *Bemisia tabaci*: *Tomato yellow leaf curl virus* (TYLCV, *Begomovirus*, *Geminiviridae*) and *Tomato chlorosis virus* (ToCV, *Crinivirus*, *Closteroviridae*). Co-infection of both viruses in tomato plants showed more severe symptoms at late stages compared to single infections, although at earlier stages the interaction began with attenuation. This asymmetric synergism correlated with the dynamics of ToCV accumulation and expression of the salicylic acid (SA) responsive gene PR-P6. Visual and olfactory cues in whitefly preference were evaluated under controlled conditions in choice assays, testing viruliferous and non-viruliferous adult whiteflies. In experiments allowing both visual and olfactory cues, whiteflies preferred symptomatic leaflets from plants infected either with TYLCV alone or with TYLCV andToCV, over those infected with ToCV alone or non-infected leaflets, suggesting that TYLCV drove host selection. Odor cues tested in Y-tube olfactometer assays showed neutral effects on whiteflies' preference, while bioassays comparing the attractiveness of colored sticky cards confirmed preference towards sectors colored to mimic TYLCV symptomatic leaves compared with asymptomatic leaves. Our results show that the presence of co-infecting viruses affect the host and could potentially alter the behavior of insect vectors.

Keywords: *Tomato yellow leaf curl virus*, *Tomato chlorosis virus*, co-infection, synergism, *Bemisia tabaci*, host choice, visual, olfaction.

INTRODUCTION

Most plant viruses causing economically important diseases in agriculture rely on vectors such as insects for their transmission. The process of virus transmission is highly influenced by their interactions with both the host and the vector (Hogenhout et al., 2008; Ng & Falk, 2006). Virus infection frequently induces changes in host plants that are conducive to vector transmission. Such changes in the plant physiology alters visual and olfactory cues from infected plants that mediate vector attractiveness, preference and feeding behavior (Fereres & Moreno, 2009; Johnston & Martini, 2020; Mauck et al., 2016). In addition, some plant viruses can directly affect vector behavior and response to plant cues with ecological and epidemiological consequences (Blanc & Michalakis, 2016; Eigenbrode et al., 2018; Moreno-Delafuente et al., 2013).

Although in recent years there has been an increasing number of studies on the effects of plant virus infection on host plants that appear to enhance transmission by insect vectors, only a few studies have documented such effects in natural systems where mixed viral infections are very common (Moreno & López-Moya, 2020). Interactions between plant viruses in mixed infections may range from antagonism to synergism (DaPalma et al., 2010; Moreno & López-Moya, 2020; Syller, 2012). In most cases, antagonistic interactions occur when the presence of one virus is detrimental for at least one the other coinfecting viruses present, for instance with a decrease in the replication or transmission rate (Chávez-Calvillo et al., 2016; Syller & Grupa, 2016). Conversely, synergistic interactions enhance the fitness of one or more of the viruses which may result in increases of virus titers often with more severe symptoms in the host plant, although the

impact on insect vector behavior is often unknown (García-Cano et al., 2006; Mascia & Gallitelli, 2016; Syller, 2012). A third situation is neutralism, when coexisting viruses do not influence each other (Mascia & Gallitelli, 2016; Syller & Grupa, 2016). The outcome of such interactions may have important epidemiological consequences that merit being explored experimentally.

This study focuses on single and mixed virus infection by two emerging viruses that frequently co-occur in susceptible tomato (*Solanum lycopersicum* L.) crops: *Tomato yellow leaf curl virus* (TYLCV, genus *Begomovirus*, family *Geminiviridae*) and *Tomato chlorosis virus* (ToCV, genus *Crinivirus*, family *Closteroviridae*), both transmitted by the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae). TYLCV is one of the most devastating and widespread viruses of cultivated tomatoes in tropical and subtropical regions. Tomato plants affected by TYLCV exhibit characteristic symptoms of stunting, yellowing, upward curling of leaves, and suffer premature dropping of flowers and reduction of marketable fruits that can result in 100% yield loss when infections occur during early growth stages (Yan et al., 2021). In contrast, symptoms caused by ToCV are less severe and usually appear irregularly distributed in tomato-infected plants, including interveinal yellowing chlorotic areas, bronzing and red patches. The yellow leaf disorder syndrome caused by this crinivirus is frequently similar to abiotic or nutritional disorders and seems to have comparatively less economic importance than other viral diseases. Emergence of ToCV and TYLCV has been associated with the global spread of the whitefly *B. tabaci* in tropical and warm regions worldwide (Fereres, 2015; Fiallo-Olivé and Navas-Castillo, 2019; Gilbertson et al., 2015; Rojas et al., 2018). TYLCV has a single-stranded circular DNA genome and is

transmitted in persistent manner, which means the virus move through the insect vector from the midguts to the salivary glands via haemolymph, thus have a latency period within the vector before transmission and can remain viruliferous for up to several days (Díaz-Pendón et al., 2010; Hogenhout et al., 2008). Meanwhile, ToCV has a bipartite genome of positive single-stranded RNA and is transmitted in a semi-persistent manner, thus the virus is retained in the foregut of the whitefly ranging from few hours to days and can be transmitted readily after acquisition (Ng & Falk, 2006; Orfanidou et al., 2016).

The interest for studying mixed infections caused by TYLCV and ToCV is demonstrated by a recent independent publication in which a detailed description of the synergistic interaction between the two pathogens is provided (Li et al., 2021). In their paper, authors found no differences in transmission efficiencies under near-saturating conditions for either of the two viruses when comparing acquisitions from single- and mixed-infected plants (Li et al., 2021).

The goal of the present study was to evaluate the effects of single and mixed infections involving TYLCV and ToCV in tomato plants in order to understand how the virus-induced changes might impact on the whitefly vector's preference. To do so, we first analyzed the development of symptoms, their severity and relative abundance of each virus through time, mainly confirming previously published observations in the independent study mentioned above (Li et al., 2021). Then, we examined under our conditions whether the individual or simultaneous presence of these viruses might affect host plant traits that influence visual and olfactory cues for the vector, and tested them on both viruliferous and non-viruliferous whiteflies. With this knowledge we gain a better

understanding of ToCV and TYLCV mixed infections in tomato plants and evaluate their impact on vector-virus-host plant relationships that could help to design measures for disease control.

MATERIAL AND METHODS

Tomato plants and whitefly colony

The insect vector and tomato lines used in this work were the *B. tabaci* and virus susceptible tomato cv. Moneymaker. In addition, plants of the wild tomato S. *habrochaites* former *f. glabratum* (accession PI 134418) were used for the preliminary control tests in Y-tube olfactometer experiments (see below). All seeds were provided by the IHSM-CSIC germplasm collection. Plants were individually sown in plastic pots of 12 cm diameter containing vermiculite and were grown in an insect-free growth chamber with light intensity at 250 $\mu mol \cdot s^{-1} m^{-2}$ with a 16 h/d photoperiod (25 °C day / 20 °C night) and 70% relative humidity. Virus-free *B. tabaci* Mediterranean (MED) individuals were obtained from a colony originating from individuals collected during field visits in Malaga (Spain) and reared on melon (*Cucumis melo* L. cv. ANC42, IHSM seedbank collection) plants within wooden cages covered with insect-proof nets, in an insect-proof glasshouse with temperature control (22–27°C day and 17–20°C night) and light supplementation when needed.

Virus isolates and Virus Inoculation

The viruses used in this study were the ToCV isolate Pl-1-2 (García-Cano et al., 2010) maintained at IHSM-CSIC "La Mayora" in tomato cv. Moneymaker by periodic transmission with *B. tabaci* and the Israel strain of TYLCV (Genbank AC: AJ489258). To obtain TYLCV-viruliferous whiteflies, non-viruliferous whiteflies were given a 24-h acquisition access period (AAP) on tomato plants four weeks after their agroinoculation with TYLCV, as previously described in Pereira-Carvalho et al., (2015) . Similarly, viruliferous whiteflies with ToCV were obtained by 24 h AAP onto ToCV-infected tomato plants maintained by whitefly-mediated inoculations. After the AAP, whiteflies were transferred to healthy plants at the three-leaf growth stage for a 48-h inoculation access period (IAP). The same procedure was used for obtaining healthy (mock-inoculated) tomato plants, except that non-viruliferous whiteflies were placed on non-infected plants. Transmission of each virus for the establishment of single infection was performed using 20 viruliferous and 20 non- viruliferous whiteflies per test plant in clip-on cages. Co-infections were established by using 40 viruliferous whiteflies; 20 containing ToCV and 20 TYLCV. Following IAP, the clip cages were removed and the infested leaf was excised from the plant at 7 days to avoid eclosion of eggs putatively laid by adults during the IAP. The plants were maintained in an insect-free growth chamber (see above). Virus-inoculated plants were checked by tissue blot hybridization 3 weeks after inoculation to verify infection condition as described in Navas-Castillo et al. (1999) and Fortes et al. (2012). Similarly, viruliferous whiteflies were also regularly checked by PCR to verify their viruliferous condition (Macedo et al., 2015).

Evaluation of symptoms and virus quantification

Symptom severity of infected and control plants were recorded at 7, 14, 21 and 28 days post-inoculation (dpi) using a 0–5 scale, where 0 was assigned to asymptomatic plants and 5 was assigned to the most severe symptoms observed as described in Ferrero et al. (2020). The second most recently expanded leaf from the apex was harvested at 7, 14 and 21 days post-infection (dpi). For each infection condition, time point and replica, the leaf tissue from 6 infected plants was pooled and used in downstream analysis. A total of three biological replicates were processed per condition and time point. Total DNA and RNA were isolated with the DNeasy Plant Mini Kit (Qiagen) and Trizol reagent (Ambion) and then treated with RNase-Free DNase (Qiagen) and DNase-Free RNase (Roche), respectively. The cDNAs were generated from 500 ng of total RNAs using BioRAD iScript™ cDNA Synthesis Kit in a reaction volume of 20 µl. Quantitative PCR (qPCR) were done using as template 1 µl of cDNA or 500 ng of total DNA using TAKARA SYBR Green PCR kit on a CFX96 Real-time PCR detection system (Bio-Rad, USA). The following cycling conditions were used: 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification of specific RNAs and TYLCV DNA were normalized to the elongation factor 1-α (SlEF1α) gene and tomato 25S ribosomal RNA genes, respectively (López-Ráez et al., 2010; Rodríguez-Negrete et al., 2014). Primers for tomato PR-P6 and Pin-II amplification were reported by Sarmento et al. (2011) and Uppalapati et al. (2005). Gene-specific primer pairs for virus quantification were designed using the Primer Blast tool available online (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The set primers MA1178 (5´ ACCGGGCGCAGTTCATACAA 3´)/MA1179

(5´ CCGACAAGAAACAGCGCTCC 3´) and MA1461 (5´ CCTGGATTGCAGAGGAAGATAGT 3´)/MA1462 (5´ TGGTACAACGTCATTGATGACGT 3´) were used to amplify 173-nucleotides of the ToCV coat protein gene and 152-nucleotides of the Replication enhancer of TYLCV, respectively. Each primer pair was evaluated by a standard curve with six points and three replicates to obtain efficiency rates (E) of 102.56% (for ToCV) and 106.05% (for TYCLV) (E=10(1/slope) −1, expressed as percentages) with R^2=0.99 correlation values for the curves. Relative quantifications were measured by using the comparative Ct method, as described by Livak and Schmittgen (2001), using co-infected samples at 7 dpi as a calibrator.

Whitefly dual and olfactometer choice bioassays

Dual-choice assays were performed by pairwise comparisons of tomato leaflets (10-leaf-growth stage) of mock-inoculated and 21dpi single and co-infected with ToCV and TYLCV. For each comparison, a total of four leaflets were placed in a plastic cage (25x25 cm) equidistant to a flight release platform disposed in the middle of the cage. The procedure was similar as described by Rodríguez-López et al. (2011) and the experimental design of the cages is illustrated in Fig. S1. Each whitefly was briefly placed on ice before being disposed in the release platform and only the first choice was recorded. Longer than 15 min choosing periods were excluded, and each whitefly was only tested once. A total of 60 adults of *B.tabaci* (replicates) were individually released per pairwise comparison. Both viruliferous and non-viruliferous *B.tabaci* were used for each test (without

sex distinction), and they were individually collected with a controlled-vacuum hand trap one hour before the beginning of the experiments.

A Y-tube olfactometer (Analytical Research Systems, Gainesville, FL, USA) was used to test whether volatile cues are involved in whitefly choice responses in the absence of visual stimuli (Fig. S2). The olfactometer consisted of a glass Y-tube (28 cm long stem and 20 cm long arms) connected to two separate glass chambers (40 cm height and 20 cm inner diameter) through silicone tubes. Each glass jar contained a single test plant and was connected to an air delivery system (ARS, Gainesville, FL, USA) to ensure a purified and humidified airflow. After a short period on ice, a single whitefly was placed in the intersection of both arms of the Y-tube and later an input flow was pulled across the silicone tubing from the glass chambers to the Y-tube. Thus, the whitefly was able to make a choice based on the reception of the volatiles emitted by the plants by moving through one of the arms. Crossing a limit line at 6 cm into either arm for more than 3 minutes was considered as a choice. The insect was excluded from the sampling and replaced after 15 min if the established choice was not completed. An airflow of 0.3 liters per minute (LPM) was chosen for the assays and generated with a vacuum pump connected to the olfactometer. A preliminary control test was used to validate experimental parameters, verifying repellences to *S.habrochaites* accession PI 134418 (Bleeker et al., 2009, 2011; Momotaz et al., 2010), compared to tomato cv. Moneymaker plants. A total of 35 whiteflies out of 40 preferred Moneymaker plants.

Whitefly adults were starved for one hour before the experiments began, and a total of 60 adults of each non-viruliferous, ToCV- and TYLCV-viruliferous

whiteflies per comparison were individually used as replicates. Both glass chambers were covered to avoid whitefly detection of visual cues during the experiments. Dual-choice and olfactometer tests for all comparisons were run in parallel, using new plants for each of the replications and systematically alternating the spatial orientations of infected and mock-inoculated plants. The bioassays were conducted in a room chamber with light intensity at 250 $\mu mol \cdot s^{-1} m^{-2}$, maintained at 27 °C and 70% relative humidity.

Effects of plant infection on leaf coloration and evaluation of colored sticky traps

Optical methods based on image analysis were used to estimate differences in color between leaves of healthy (mock-inoculated), ToCV- and TYLCV-infected tomato plants. Three random measures per leaf were recorded using a colorimeter model CR-400 (Konica Minolta, Inc., Tokyo, Japan) with a 1cm-diameter aperture. The CIE Lab three-dimensional color space was used to evaluate color attributes of the samples. In this color space, the L*value indicates lightness, varying from black (L = 0) to white (L = 100), the a* value characterizes the color of the region from green (−a*) to red (+a*), and the b* value indicates the color in the range from blue (−b*) to yellow (+b*). Numerical values of a* and b* were converted into hue angle (H) using the Konica Minolta CR-400 Utility software. The H value is expressed in degrees: 0° (+a axis) represents red; 90° (+b axis) represents yellow; 180° (-a axis) is green and 270° (-b axis) represent blue color (Barrantes et al., 2016; Sacks & Francis, 2001). The colorimeter was

previously calibrated following the manufacturer's instructions and each L*, and H value was calculated by the formulas described by Sacks and Francis, (2001).

Colored sticky cards were designed to conduct choice assays to evaluate the influence of visual stimuli on *B. tabaci* in absence of olfactory cues. A total of 24 cards (8x10 cm) colored to mimic TYLCV symptomatic leaves at 21 dpi and asymptomatic leaves were disposed alternately on both sides of a cardboard surface (21x29.7 cm) and used for each replicate. Three glass boxes were placed inside a greenhouse under natural light and controlled temperature (25 ± 2°C) to test the visual responses of whiteflies, either non-viruliferous or viruliferous for ToCV or TYLCV. Two sticky cardboards were suspended from the top of each glass box (65 x 77 x 56 cm) and groups of 100 whiteflies were released per comparison. The cards were collected after 8h to record the number of whiteflies trapped on each sector. The assays were replicated three times for the different categories of viruliferous or non-viruliferous whiteflies.

Data analysis

Statistical analysis was performed using the program IBM SPSS v.26.0 (IBM, Armonk, NY, USA) and the statistical software R for Windows (R Foundation for Statistical Computing, Vienna, Austria). Means of severity of symptoms and the relative quantification of the responsive genes PR-P6 and Pin-II were analyzed by a one-way ANOVA. An independent t-test was performed to estimate statistical differences of TYLCV and ToCV accumulation between single- and double-infected plants. For the dual-choice assays, the host-settling preferences of viruliferous and non-viruliferous *B.tabaci* were analyzed by applying a chi-

square test to the data of each pairwise comparison. The chi-square test was also used to evaluate host preference behavior of whiteflies in the olfactometer assays. Colorimeter data were submitted to the Shapiro-Wilk normality test and then compared by a one-way ANOVA to correlate color components of mock-inoculated and single-infected leaves with ToCV and TYLCV. Numbers of whiteflies collected from sticky cards were analyzed by a Wald test ($p<0.05$) using a general linear model (GLM) following a Poisson distribution.

RESULTS

Mixed infections of ToCV and TYLCV induce more severe symptoms in tomato plants than single infections at late time points

Under our growing conditions tomato plants infected by TYLCV and ToCV in single or co-infection started displaying symptoms around 7 dpi (Fig. 1). ToCV-infected plants only exhibited slight symptoms of interveinal chlorosis and mild yellowing with no evidence of stunting, always with a rating ≤2. As expected, tomato plants infected with TYLCV exhibited significantly more severe symptoms than ToCV-infected plants (Table S1 and Fig. S3) with scores around 4 at 28 dpi, and included leaf curling, yellowing and plant stunting. However, we observed that ToCV and TYLCV produce different symptomatic outcomes during mixed infection over time. Symptoms ranged from moderate (values 1-2) to severe (values 4 -5), suggesting that antagonism and synergism occurred respectively at early (weeks 1 and 2) and late stages (from week 3 onwards) of mixed infection with the two viruses. The development of symptoms resembled those of single-

infected with ToCV during the first observations, but were followed by a dramatic increment of severity (severe curling and extensive yellowing in upper leaves) that led to necrosis of the newly emerging leaves and sometimes even to the death of the plant in the final stages (Fig. 1), a situation which was not observed in any of the plants inoculated solely with one virus.

Time-dependent antagonistic and synergistic interactions correlate with significant changes in ToCV accumulation

In order to determine whether symptom severity could be correlated with viral accumulation in infected tissues, we estimated the relative abundance of TYLCV and ToCV in single and mixed-infected tomato plants by qPCR and at 7, 14 and 21 dpi (Figs. 2A and 2B). Samples at 28 dpi were not considered because severe necrosis in many apical leaves of co-infected plants precluded sampling of enough material for representative analysis.

The results showed the pattern of TYLCV DNA accumulation was similar between single infection and co-infection with ToCV (Table S2) where the total amount of viral DNA increased drastically from 7 to 14 dpi and slightly decreased at 21 dpi (Fig. 2A). The same trend was observed for ToCV RNA accumulation in single infection, reaching a peak at 14 dpi with a slight decrease at 21 dpi. However, a pattern of reduced ToCV RNA in co-infection with TYLCV was observed at 7 and 14 dpi compared with plants infected by ToCV alone, while the opposite trend was observed at 21 dpi: ToCV reached a higher accumulation in co-infection compared to single infection (Fig. 2B).

Taken together, these results suggest that changes in symptoms of mixed-infected plants between antagonism (14 dpi) and synergism (21 dpi) seemed to correlate with the dynamics of ToCV accumulation: the relative abundance of ToCV significantly increased in mixed-infected plants at 21 dpi (Fig. 2B) when the increment of the severity of symptoms suggests a synergistic interaction between both viruses (Fig. 1).

Dynamics of expression corresponding to defense-related genes in single and mixed infections

To investigate the putative involvement of known pathways corresponding to plant immune responses, we monitored PR-P6 and Pin-II gene expressions. PR-P6 and Pin-II are respectively considered molecular markers for two major pathways: systemic acquired resistance (SAR) related to responses mediated by salicylic acid (SA), and induced systemic resistance (ISR) related to responses mediated by jasmonic acid (JA) (Ament et al., 2004; Wasternack et al., 2006). To verify whether the expression of these genes could be triggered by the different viral infections alone or combined, we analyzed their relative expression by RT-qPCR in systemic leaves at 7, 14 and 21 dpi.

We observed no significant differences in PR-P6 expression patterns at 7 dpi between single infected and co-infected plants. Furthermore, the PR-P6 RNA levels peaked at 14 dpi in infected plants with TYLCV alone, with significantly higher values than those detected in plants either co-infected or infected only with ToCV (Table S3), except for one ToCV sample that showed a similar value to those shown by TYLCV. This variability might reflect local differences related to

the sampling process and the irregular distribution of criniviruses. After these peaks in single infections, PR-P6 expression significantly decreased at 21 dpi for both plants infected with TYLCV or with ToCV alone (Table S3), but in contrast, the values continued to increase in co-infected plants, resulting in higher levels compared to single infections (Fig. 3A). This delay in the pattern of PR-P6 expression for mixed-infected plants roughly followed symptom severity, suggesting that its expression might be correlated with the severity of symptoms. No significant differences in Pin-II RNA expression were observed at 7 dpi between singly infected and co-infected plants, while at 14 dpi a significant lower expression was observed in infected plant with ToCV than those in TYLCV singly or co-infected tomato plants (Fig. 3B, Table S3). The Pin-II RNA accumulation reached its maximum at 21 dpi for all treatments, with the highest induction levels detected in plants infected with TYLCV (Fig. 3B, Table S3).

The presence of TYLCV conditions the host plant preference responses of *B. tabaci*

To investigate whether viral infections might interfere with vector attraction, we tested whiteflies' preferences comparing infected versus mock-inoculated plants at 21 dpi. This time point was chosen based on the differential expression of Pin-II and PR-P6 in the conditions analyzed, which suggested that the plants were clearly responding to the infections (Fig. 4). The experiments were repeated independently with naive whiteflies (non-viruliferous) and with whiteflies previously fed on virus-infected plants, therefore becoming viruliferous for either one of the individual viruses considered. Significantly, more non-viruliferous and

ToCV-viruliferous whiteflies preferred to land on infected leaves than on control leaves both for TYLCV and for mixed infections. A similar pattern was found for TYLCV-viruliferous whiteflies with a preferential attraction to the leaves of plants with TYLCV, whereas plants harboring mixed infections were not significantly more attractive than uninfected controls, although the number of whiteflies was consistently higher on infected plants. In contrast, whiteflies did not discriminate between leaves from plants infected with ToCV alone and mock leaves or TYLCV from mixed infection, regardless of the non-viruliferous or viruliferous conditionof the insects (Fig. 4). Overall, enhanced whitefly attraction was observed towards TYLCV single or mixed infection plants compared with plants infected by ToCV, suggesting that the presence of TYLCV might drive host attractiveness, either alone or in mixed infection with ToCV, and regardless of their viruliferous condition as either naive or ToCV-viruliferous. Interestingly, although the trends are similar, the TYLCV-viruliferous whiteflies only discriminated significantly between leaflets of mock and TYLCV-infected plants, and it seems that the preferences shared by non-viruliferous and ToCV-viruliferous whiteflies were less marked in this case, suggesting a possible effect of the virus presence in the behavior of the vector.

Visual cues rather than olfactory stimuli are responsible for whiteflies' attraction towards TYLCV-infected tomato plants

We performed a series of assays to determine if the TYLCV infection induces attraction based on odor or visual cues among whitefly vectors in tomato plants. Since the infection with TYLCV was shown to be determinant in the arena studies

reported above, we concentrated our efforts on comparing mock and TYLCV-infected plants.

In the Y-tube olfactometer bioassays, volatiles emitted from either plants systemically infected with TYLCV or mock-inoculated plants flowed through both arms of the Y-tube. Individual non-viruliferous and viruliferous whiteflies were released from the base of Y-tube stem and allowed to make the choice to fly toward either arm of the Y-tube avoiding visual detection of the plants. Whiteflies showed no preference for either mock or TYLCV-infected plants, suggesting that olfactory cues have neutral effects in the initial choice of *B. tabaci* (Fig. 5A). Furthermore, we tested the whiteflies' preference in dual assays for mock-inoculated versus TYLCV-infected leaflets at 4 dpi, when the symptoms were not yet developed. The results showed no significant differences in the preferences of either viruliferous or non-viruliferous whiteflies (Fig. S4) suggesting that visual cues are responsible for the attraction of whiteflies to TYLCV-infected plants.

We then investigated the response of whiteflies to colored sticky traps in absence of olfactory stimuli (Fig. S5). Comparing TYLCV symptomatic plants with mock-inoculated or asymptomatic infected plants (before the onset of symptoms) we observed that TYLCV infection had a significant effect on plant coloration. There was no significant difference in the mean values of parameters describing the color between mock-inoculated and asymptomatic TYLCV-infected leaves, while TYLCV symptomatic plants revealed significant differences in terms of $a*$, $b*$, L* and H (Table 1). This reflects an increase in yellow coloration in TYLCV-infected plants. When traps displaying the different colors were used in choice experiments, significantly more whiteflies were caught on the card sectors

mimicking TYLCV symptomatic leaves than on sectors representing the color of asymptomatic plants, and the differences were maintained for both viruliferous and non-viruliferous whiteflies (Fig. 5B). Overall, these results suggest that visual signals associated to the color yellow seem to play an important role in whiteflies' preference responses.

DISCUSSION

In this study, we found a synergistic interaction between TYLCV and ToCV, characterized by an increase in the severity of symptoms in the late stages of the observation, with a significant increase in ToCV RNA accumulation in plants co-infected with TYLCV. Previous reports studying mixed infections by TYLCV and ToCV (Li et al., 2021; Seo et al., 2018) showed a reasonable level of coincidence with our results. However, these studies were performed in other experimental conditions, in particular using different tomato cultivars, virus isolates, and even inoculation procedures (grafting vs whitefly). All these differences may cause discrepancies in outcomes, making difficult their direct comparison. In the first of these two previous studies, the authors focused on differential gene expression associated with the development of distinct symptoms induced by ToCV and TYLCV in tomato plants (cv. Tenten), testing only samples in upper leaves at eight weeks after grafting infections and giving no information about earlier time points (Seo et al., 2018). The second paper reported clear synergistic effects in a different cultivar of tomato (cv. Zhong Za 9) at late time points in the co-infection (Li et al., 2021). This coincides with our data in a different cultivar of tomato (cv. Moneymaker), but under our conditions we also noticed that the synergism was

preceded by antagonism in the early stages (7 and 14 dpi) not reported in the different periods of observation during the experiments described in the aforementioned papers.

These differences could be attributed to additional divergences beyond the plant materials, such as the virus isolates used (local Qingdao field isolates were used for the studies in China, while we used viruses previously characterized in Spain), and also to the growing conditions, in particular the temperature, which was higher (27 ± 2 ºC) in the greenhouse experiments conducted in China (Li et al., 2021) than in our growth chambers. Indeed, temperature has previously been shown to affect synergistic interactions (Aguilar et al., 2015), and is a key factor for virus-virus interactions (Alcaide et al., 2021). In the case of TYLCV + ToCV, the differences might only affect the dynamics to reach the final stages when the outcomes are mostly coincidental, although this hypothesis will require further experiments and appears to be irrelevant for the clear synergism at later time points.

Among the numerous examples of viral synergistic interactions in mixed infections, the crinivirus ToCV appeared to cause interesting interactions with unrelated viruses. Indeed, mixed infections of ToCV and *Tomato spotted wilt virus* (TSWV) of susceptible tomato plants resulted in an exacerbation of ToCV-associated symptoms with a remarkable increase of ToCV accumulation, whereas TSWV accumulation was not altered but the resistance in tomato cultivars carrying the Sw-5 gene was compromised by pre-infection with ToCV (García-Cano et al., 2006). Also, Wintermantel et al. (2008) reported that co-infection of ToCV with the crinivirus *Tomato infectious chlorosis virus* (TICV)

altered the accumulation of each virus in a host-specific manner: while in *Nicotiana benthamiana* TICV and ToCV titers increased and decreased respectively, in *Physalis wrightii* the titers of both TICV and ToCV decreased in comparison with single infections (Wintermantel et al., 2008). However, a different scenario was found in the early stage of infection in which mixed infections repressed the symptoms induced by TYLCV and ToCV accumulation compared with the corresponding single-inoculated plants, showing an asymmetric synergistic effect between both viruses. A recent study demonstrated that interactions between synergistically interacting viruses can be asymmetric (Tatineni et al., 2019). Moreover, both antagonistic and synergistic interactions were observed between unrelated *Papaya ringspot virus* (PRSV) and *Papaya mosaic virus* (PapMV), depending on their order of infection. Synergism occurred in plants inoculated first with PRSV and later with PapMV, but antagonism occurred if PapMV was first inoculated, a situation that could derive from the prior activation of the salicylic acid-associated defense response against PRSV (Chávez-Calvillo et al., 2016). Interestingly, in our case we found that the asymmetric synergistic effect between both viruses correlated with a higher accumulation of ToCV and expression of PR-P6, although it is difficult to ascertain if the altered expression of PR-P6 is a direct cause or a consequence of the exacerbated disease symptoms and/or virus accumulation.

The molecular details of the mechanism underlying the interaction between these two viruses have not yet been clarified; however, we might speculate that blockage of the RNA silencing antiviral response by viral suppressors of RNA silencing (VSR) could be part of the mechanisms involved in synergism and

antagonism in plants (Carrington et al., 2001). Similarly to other plant viruses, ToCV and TYLCV encode multiple VSRs (Cañizares et al., 2008; Luna et al., 2012) which may act at different stages of infection (Díaz-Pendón and Ding, 2008), although these interactions may occur for more complex reasons than the action of a few viral proteins (Latham and Wilson, 2008). In one of the previous works involving mixed infection of TYLCV and ToCV, the differential gene expression associated with the development of distinct symptoms induced by ToCV and TYLCV in tomato plants was explored (Seo et al., 2018). Unfortunately, as we discussed earlier, the analyzed samples corresponded to late time points in the infection process, and consequently any possible asymmetric synergistic effect between both viruses was not observed.

Recent studies have uncovered novel strategies that insect-borne plant viruses might adopt to manipulate the host defenses, like the emissions of plant volatile organic compounds, or enhance the plant's nutritional quality to improve the suitability of the vector and its attraction to the infected host plant, thereby impacting plant–insect interactions (Pan et al., 2021; Pieterse et al., 2012; Ponzio et al., 2013). For example, infection of tobacco plants with *Tomato yellow leaf curl China virus* and *Arabidopsis* with *Cucumber mosaic virus* could repress the JA-mediated defenses against the whitefly and aphid, respectively. Jasmonate is commonly considered in studies of the effects of virus infection on plant–insect interactions, mainly as a result of its direct involvement in plant defense against insect herbivores and production of a volatile blend (Lewsey et al., 2010; Luan et al., 2013; Zhang et al., 2012). Similarly, the manipulation of the SA-signaling pathway by viruses may affect host attractiveness to insect herbivores (Chisholm

et al., 2018; Tomitaka et al., 2015). Our data showed that whiteflies did not discriminate between plants infected with TYLCV alone or mixed-infected plants, regardless of the non-viruliferous or viruliferous condition of the insects (Fig. 4), despite the fact that the mixed infection causes more severe symptoms than in a single virus infection with up-and-down regulation of expression of SA-related gene PR-P6 and JA-related gene Pin-II, respectively. It seems reasonable to postulate that in our pathosystem the vector attraction may not depend on the induction of the signaling pathway of the host hormone JA or SA. Additional studies are needed to draw more definitive conclusions on the impact of host hormones on whitefly preference.

In our dual-choice experiment, a preferential whitefly attraction to the leaves of plants with TYLCV or mixed infections was observed, whereas plants with single ToCV infections were not significantly more attractive than uninfected controls (Fig.4), regardless of whitefly viruliferous condition, suggesting that TYLCV drives host attractiveness in mixed infection with ToCV. For example, the fact that plants with mixed infections were more attractive than plants with ToCV infections or uninfected controls suggests that ToCV present in co-infected plants might benefit from enhanced whitefly transmission opportunities created by TYLCV-driven changes in host traits, thus providing more opportunities for ToCV to interact with insects, which might favor their spread.

We hypothesized these whitefly preferences were likely mediated by plant-derived olfactory and visual cues, as whiteflies use both to locate, select and accept their host plants. Our data obtained in the Y-tube experiment showed that volatiles emitted by tomato plants infected with TYLCV had neutral effects in

viruliferous and non-viruliferous whiteflies compared with mock-inoculated plants. Our findings, however, differed from Johnston and Martini, (2020) who also work on the TYLCV-tomato pathosystem, where *B. tabaci* attraction to olfactory cues from TYLCV symptomatic tomato plants was reported. They also differed from those that suggest that begomovirus can manipulate whitefly olfactory response by switching their preference to non-infected plants once the virus is acquired (Fang et al., 2013; Legarrea et al., 2015). However, this type of manipulation induced by begomovirus was not observed for ToCV, where non-viruliferous *B. tabaci* MEAM1 preferred volatiles from mock-inoculated tomato plants, while viruliferous whiteflies showed no preference (Fereres et al., 2016). Conversely, Shi et al. (2018) found that non-viruliferous MEAM1 were attracted to ToCV-infected plants and viruliferous MED preferred to settle on non-infected plants rather than on ToCV-infected plants. This inconsistency indicates that virus effects on host–vector communication are complex and may include effects on the host phenotype and vector physiology.

Our subsequent data showed that the leaf-color profiles were altered to a much greater extent by TYLCV than by ToCV or Mock plants, suggesting that attraction based on visual cues drives selection of *B. tabaci*. Moreover, the significant differences in H and b* values found in measurements of the external color of TYLCV-infected plants reveal a higher expression of a yellow color than either healthy, ToCV-infected or asymptomatic TYLCV-infected leaflets, which may enhance the attractiveness of *B. tabaci*. In fact, this visual influence is consistent with the work developed by Mound, (1962) and Johnston and Martini (2020), which documented that whiteflies are strongly attracted to the color yellow,

suggesting that it may influence the host selection mechanism. Recently these results have been supported by their application in many management strategies to control whitefly populations as seen, for example, in the use of yellow sticky traps or the development of optical barriers to manipulate the sunlight spectrum (Antignus, 2010; Gerling & Horowitz, 1984; Moreau & Isman, 2011).

Our study constitutes a further effort to investigate the consequences of co-infection of ToCV and TYLCV on whitefly-related plant traits. TYLCV and ToCV are two emerging viruses with very similar ecological niches; both are transmitted by the same vector *B. tabaci* and have overlapping host ranges. Therefore, mixed infections of TYLCV and ToCV are expected to be common in crops in areas where these two viruses are present, with important epidemiological consequences that may be unpredictable. In fact, superinfection of TSWV-resistant plants carrying the Sw5 with ToCV resulted in a breakdown of the resistance to TSWV (García-Cano et al., 2006). Moreover, recent studies have reported the emergence of different aggressive TYLCV (TYLCV-IS76 and TYLCV-IL[IT:Sic23:16]) outbreaks in resistant tomatoes carrying the TY-1 gene in Spain, Morocco and Italy (Belabess et al., 2015; Panno et al., 2018; Torre et al., 2018). Since ToCV is widely distributed in these areas, mixed infections of resistant-breaking variants of TYLCV and ToCV could compromise the resistance conferred by TY-1, and should be seen as a warning for tomato breeders and growers in Mediterranean countries. Interestingly, Ty-1 resistance was shown to be compromised upon a mixed infection of TYLCV with CMV (Butterbach et al., 2014). The complexities of these kinds of mixed viral infections go beyond the interactions between the viruses involved and the host plant suffering them, and

also can influence the behavior of the insect vectors. We believe these studies yield relevant information regarding the impact of mixed infections in the spread of both viruses, which could be important for achieving a better understanding of the epidemiological consequences of ToCV and TYLCV mixed infections.

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Table caption

Table 1. Mean and standard deviation values of color differences corresponding to mock-inoculated, asymptomatic and symptomatic TYLCV-infected tomato leaves. Values of L*, a*, b* and H parameters are listed. Significant differences are indicated by different letters according to one-way ANOVA with $P < 0.05$.

Figure legends:

Fig. 1. Evolution of symptoms in single- and mixed-infected plants. Mean severity of symptoms (from 0 = absence to 5 = maximum severity) observed on different days post-inoculation (dpi) in tomato cv. Moneymaker plants singly infected with *Tomato yellow leaf curl virus* (TYLCV) or *Tomato chlorosis virus* (ToCV), co-infected with both viruses (ToCV+TYLCV), or mock-inoculated. Mean ± standard error values corresponding to 6 plants per treatment are indicated.

Fig. 2. Kinetics of viral loads in single- and mixed-infected plants. Relative quantification of *Tomato yellow leaf curl virus* (TYLCV) (A) and *Tomato chlorosis virus* (ToCV) (B), showing viral loads in single-infected (blue for TYLCV and yellow for ToCV) and mixed-infected (green) plants at 7, 14, and 21 days post-inoculation (dpi). Each point represents the relative quantification of viral accumulation in the independent biological replicates with pooled samples of six infected plants per treatment. Mean values are indicated by the represented lines. The relative quantifications were calculated using the comparative cycle threshold method (Livak and Schmittgen ,2001). The co-infected samples at 7 dpi were chosen as calibrators for comparisons. Relative quantification of specific RNAs and TYLCV DNA were normalized to the expression of the elongation factor 1-α (SlEF1α) and the tomato 25S ribosomal RNA genes, respectively.

Fig. 3. Relative accumulation of the salicylic acid (SA) responsive PR-P6 gene (A) and the wound-induced proteinase inhibitor II (Pin-II) (B) in single- and mixed-infected tomato plants at 7, 14 and 21 dpi. Each point represents the relative quantification of the levels of transcript corresponding to independent biological replicates after pooling samples of six infected plants per treatment. Mean values

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Fig. 4. Viruliferous and non-viruliferous *Bemisia tabaci* preference responses in a dual-choice bioassay. Number of whiteflies that landed on TYLCV-infected (blue), ToCV-infected (yellow), mixed-infected (green) or mock-inoculated (grey) leaflets were recorded in each pairwise comparison. Whitefly icons represent the infectious condition of the whiteflies tested: white for non-viruliferous and blue and yellow for TYLCV- and ToCV-viruliferous whiteflies, respectively. Asterisks indicate significant differences between pairs of treatments ($p<0.05$) based on chi-square analysis.

Fig. 5. Behavioral responses of *Bemisia tabaci* to olfactory and visual stimuli. (A) Percentage of whiteflies responding to volatiles coming from mock-inoculated and TYLCV-infected plants connected to a Y-tube olfactometer with a continuous active air flow. Individual adult whiteflies were released in the principal arm and their responses were recorded individually during a 15 min time interval. A positive response was considered when whiteflies remained for 3 min across the border line of the lateral tubes. A total of 60 replicates (whiteflies) per test were used. (B) Visual attractiveness of *B. tabaci* towards colored sticky cards. Bars represent the mean ± SE of whiteflies collected on cards colored to mimic TYLCV symptomatic leaves compared with mock-inoculated leaves. Whitefly icons represent the infectious condition of the whiteflies tested: white for non-

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e-Xtras

Legends for Supplemental Materials

Figure S1. Experimental design of dual-choice arena cages. A total of four leaflets per pairwise comparison are disposed surrounding the flight release platform. Leaflets belonged to the two treatments compared (represented by yellow and green for ToCV- and mixed-infected, respectively) were placed alternately for each test.

Figure S2. Set-up of the Y-tube olfactometer used to test *B. tabaci* preferences to volatiles emitted by mock or TYLCV-infected plants. Blue arrows represent the airflow direction.

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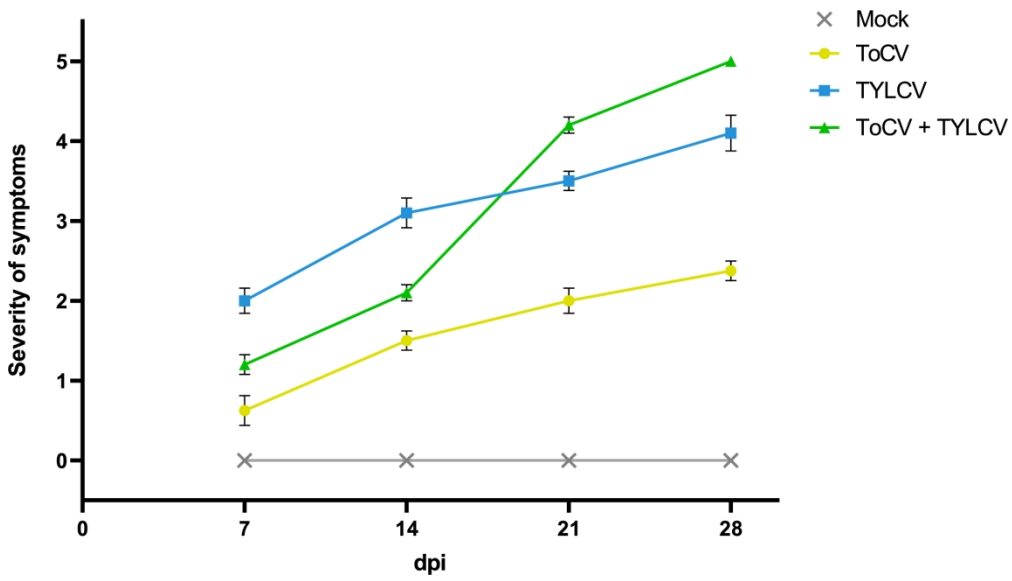

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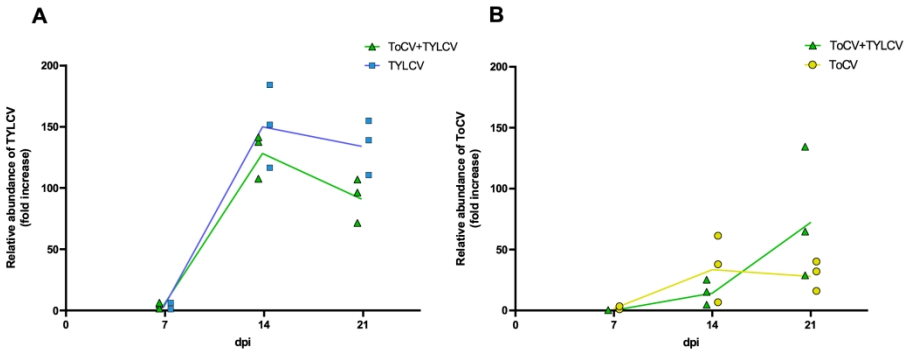

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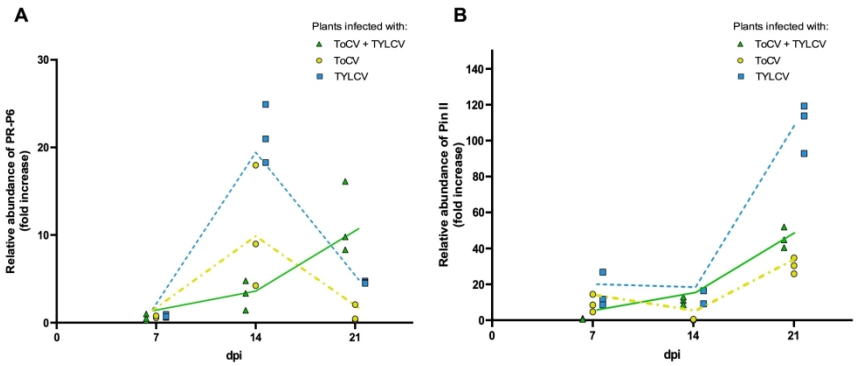

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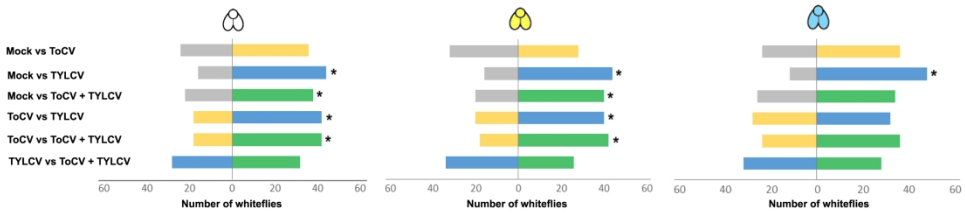
218x95mm (300 x 300 DPI)



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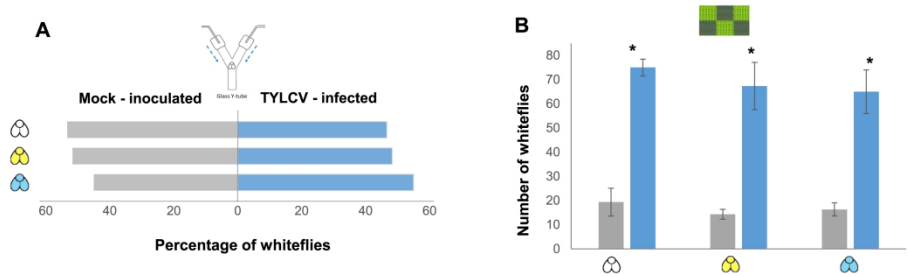

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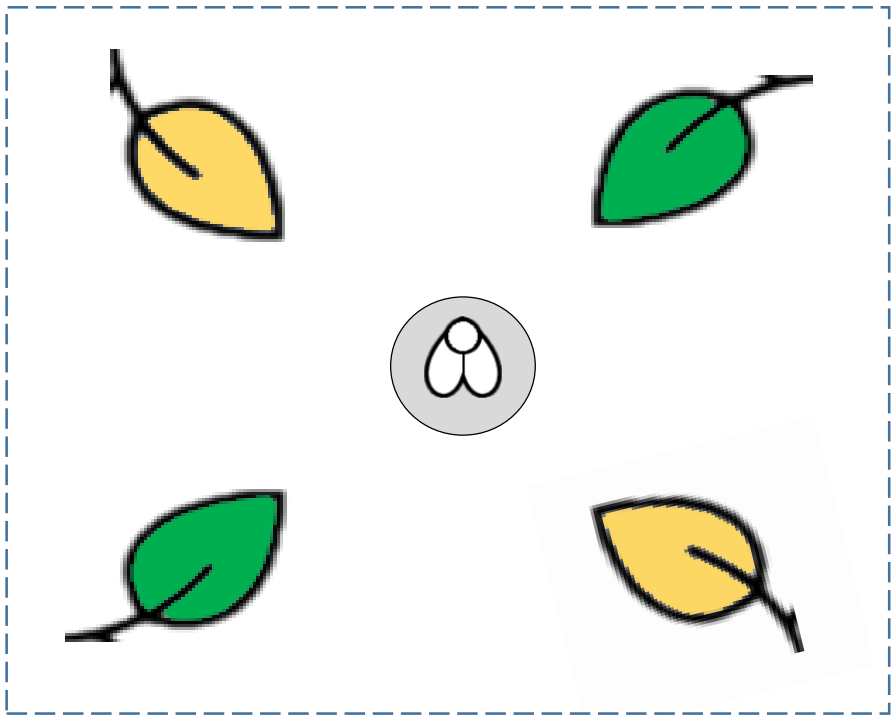
	Mock	Asymptomatic TYLCV-infected	TYLCV symptomatic
L*	35.83 ± 1.03 m	38.49 ± 0.46 m	44.78 ± 1.08 n
a*	-16.27 ± 0.84 m	-17.99 ± 0.39 m	-13.71 ± 0.72 n
b*	23.92 ± 1.37 m	27.19 ± 0.61 m	32.805 ± 0.72 n
H	124.27 ± 0.48 m	123.5 ± 0.17 m	112.68 ± 0.29 n



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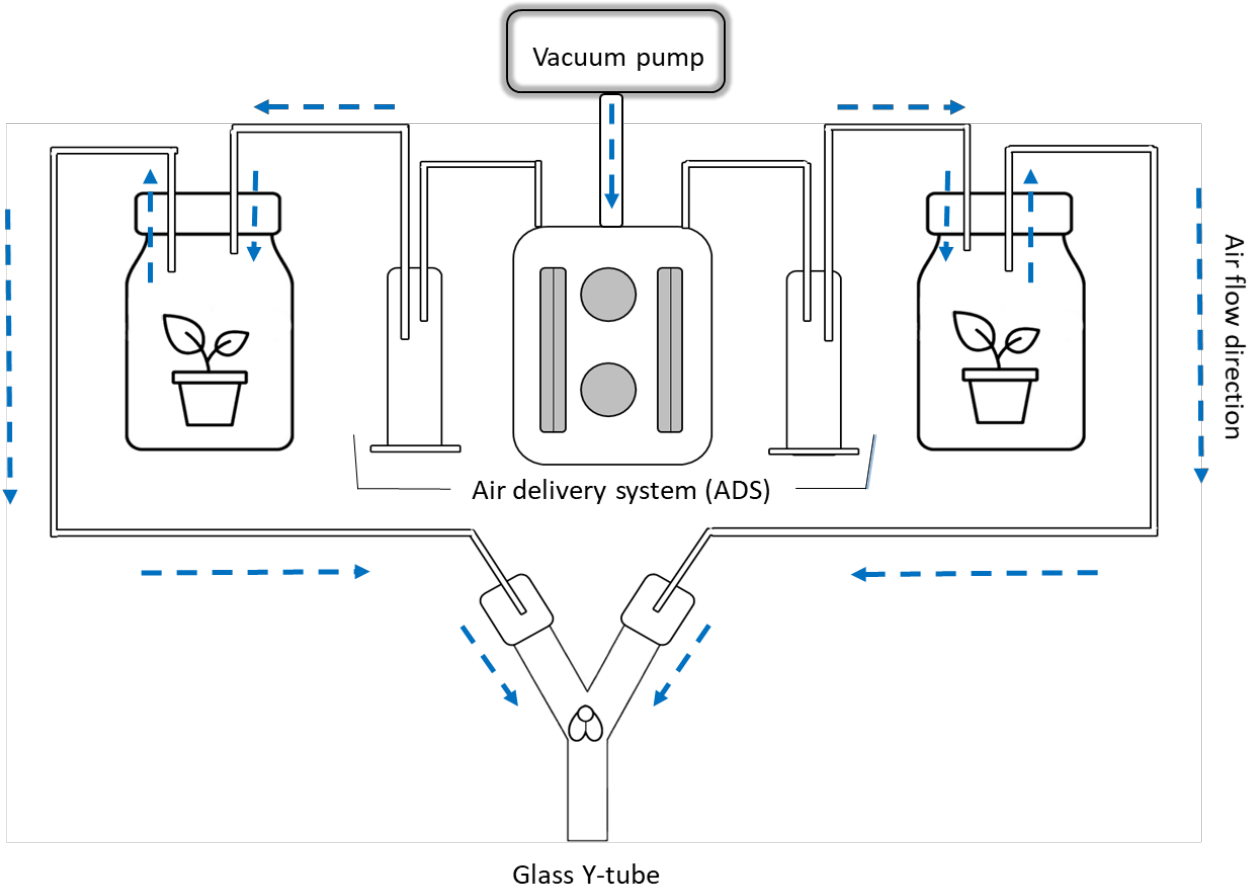

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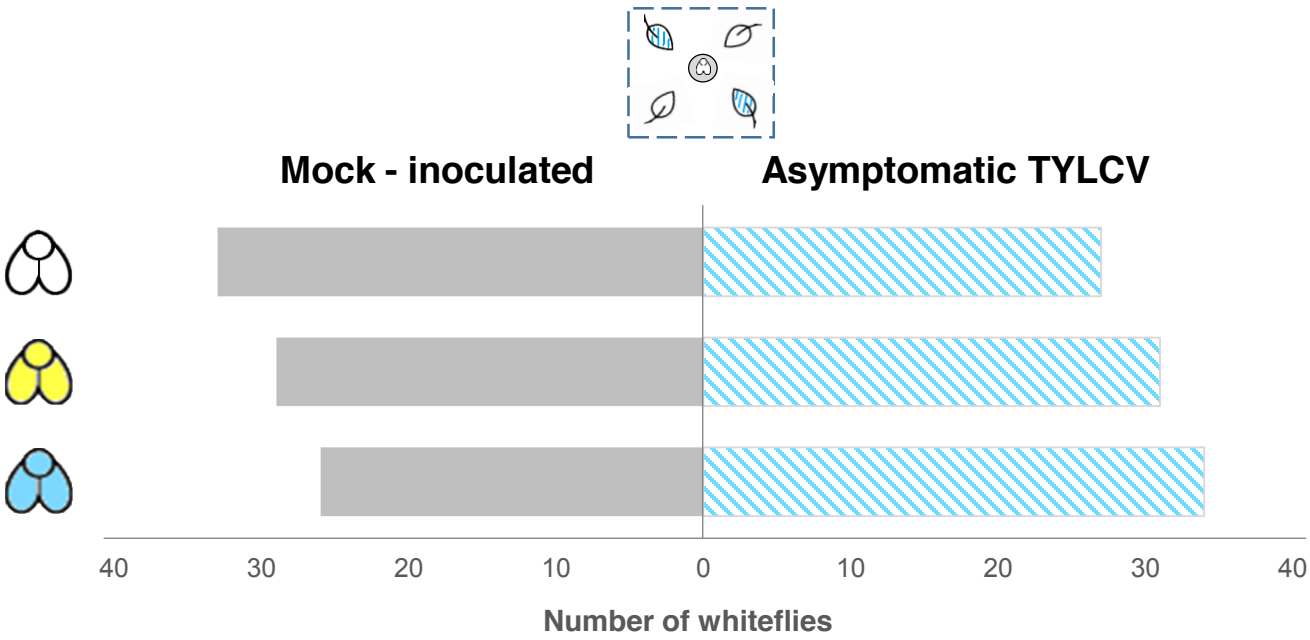

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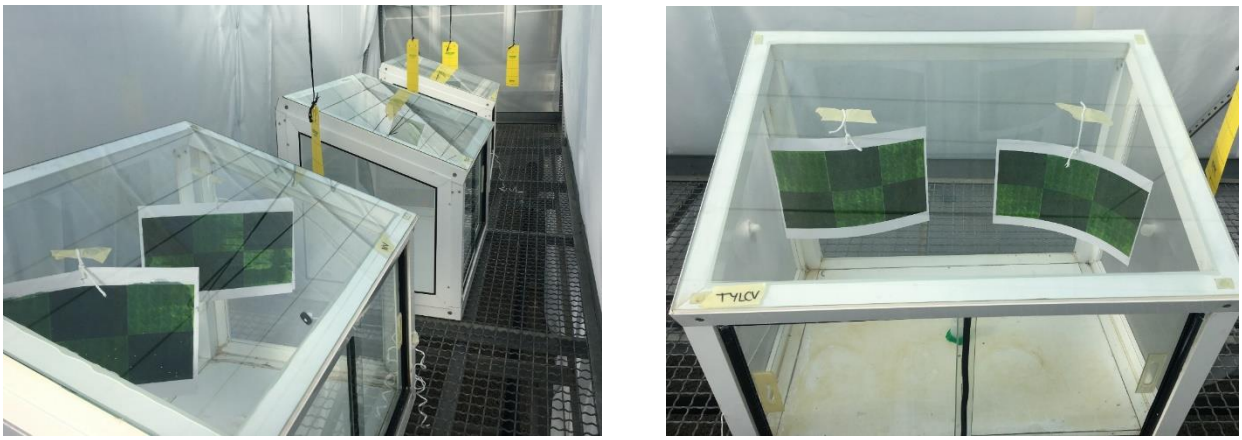

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Symptoms in plants infected with:	7 dpi			14 dpi			21 dpi			28 dpi		
	Mean ± SE	ANOVA		Mean ± SE	ANOVA		Mean ± SE	ANOVA		Mean ± SE	ANOVA	
		F	P value		F	P value		F	P value		F	P value
ToCV	0.625 ± 0.19 a			1.50 ± 0.12 a			2.00 ± 0.16 a			2.38 ± 0.12 a		
TYLCV	2.00 ± 0.16 b			3.10 ± 0.19 b			3.50 ± 0.12 b			4.10 ± 0.22 b		
ToCV + TYLCV	1.20 ± 0.12 c	26	< 0.001	2.10 ± 0.10 c	27.38	<0.001	4.20 ± 0.10 c	68.6	<0.001	5.00 ± 0.00 c	226.8	<0.001

Table S2. Numerical values and statistical analysis of the relative accumulation of TYLCV and ToCV in single- and mixed-infected plants at the indicated different days post-inoculation (dpi), as summarized graphically in Figure 2. Means of fold change ± SD and the results of the Student's t-test with $P < 0.05$ are indicated.

	7 dpi			14 dpi			21 dpi		
TYLCV accumulation in plants infected with:	**Mean fold change ± SD**	**t**	**P value**	**Mean fold change ± SD**	**t**	**P value**	**Mean fold change ± SD**	**t**	**P value**
TYLCV	2.6 ± 3.11			150.8 ± 49.73			134.88 ± 22.41		
ToCV + TYLCV	4.28 ± 2.43	-0.74	0.503	128.94 ± 18.56	0.98	0.382	91.58 ± 28.72	2.60	0.060
	7 dpi			**14 dpi**			**21 dpi**		
ToCV accumulation in plants infected with:	**Mean fold change ± SD**	**t**	**P value**	**Mean fold change ± SD**	**t**	**P value**	**Mean fold change ± SD**	**t**	**P value**
ToCV	1.8 ± 1.41			35.34 ± 10.06			29.43 ± 12.26		
ToCV + TYLCV	0.29 ± 0.14	1.84	0.139	15.08 ± 8.26	1.20	0.296	76.06 ± 53.69	-1.47	0.216

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Accumulation of PR-P6 in infected plants with:	**7 dpi**			**14 dpi**			**21 dpi**		
	Mean ± SD	**ANOVA**		**Mean ± SD**	**ANOVA**		**Mean ± SD**	**ANOVA**	
		F	**P value**		**F**	**P value**		**F**	**P value**
ToCV	0.63 ± 0.12 a			10.40 ± 6.98 a			0.95 ± 0.97 a		
TYLCV	0.75 ± 0.17 a			21.39 ± 3.34 b			4.62 ± 0.13 a		
ToCV + TYLCV	0.59 ± 0.37 a	0.08	0.922	7.02 ± 4.86 a	**12.05**	**0.008**	11.41 ± 4.14 b	**14.01**	**0.005**
Accumulation of Pin II in infected plants with:	**7 dpi**			**14 dpi**			**21 dpi**		
	Mean ± SD	**ANOVA**		**Mean ± SD**	**ANOVA**		**Mean ± SD**	**ANOVA**	
		F	**P value**		**F**	**P value**		**F**	**P value**
ToCV	9.19 ± 4.91 a			0.25 ± 0.26 a			30.28 ± 4.42 a		
TYLCV	15.66 ± 9.73 a			13.97 ± 4.25 b			108.61 ± 13.94 b		
ToCV + TYLCV	0.66 ± 0.30 a	4.28	0.07	10.85 ± 2.11 b	**20.68**	**0.002**	45.71 ± 5.84 a	**62.42**	**<0.001**