Assessing the impact on virus transmission and insect vector behavior of a viral mixed infection in melon

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Summary

Mixed viral infections in plants are common, and can cause synergistic or antagonistic interactions. Except in complex diseases with severe symptoms, mixed infections frequently remain unnoticed, and their impact on insect vector transmission is largely unknown. In this work, we considered mixed infections of two unrelated viruses commonly found in melon plants, the crinivirus *Cucurbit yellow stunting disorder virus* (CYSDV) and the potyvirus *Watermelon mosaic virus* (WMV), and evaluated their vector-transmission by whiteflies and aphids, respectively. Their dynamics of accumulation were analyzed until 60 days post-inoculation (dpi) in mix-infected plants, finding reduced titers of WMV and much higher titers of CYSDV compared to single infections. At 24 dpi, corresponding to the peak of CYSDV accumulation, similar whitefly transmission rates were obtained when comparing either individual or mix-infected plants as CYSDV sources, although its secondary dissemination was slightly biased towards plants previously infected with WMV, regardless of the source plant. However, at a later time point mix-infected plants partially recovered from the initially severe symptoms, and CYSDV transmission became significantly higher. Interestingly, aphid transmission rates both at early and late time points were unaltered when WMV was acquired from mix-infected plants despite its reduced accumulation. This lack of correlation between WMV accumulation and transmission could result from compensatory effects observed in the analysis of the aphid feeding behavior by Electrical Penetration Graphs. Thus, our results showed that mix-infected plants could provide advantages for both viruses, directly favoring CYSDV dissemination while maintaining WMV transmission.

Introduction

Transmission to susceptible hosts is a key step in the cycle of pathogens that relies mainly on the activity of insect vectors, like many plant viruses (Whitfield, Falk, and Rotenberg 2015). As in other parasitic relationships, plant pathogenic viruses could affect the host-vector interaction in order to improve their transmissibility, inducing changes in the host morphology or physiology (Culver and Padmanabhan 2007), or manipulating the vector behavior (Lefèvre et al. 2009a; Mauck 2016a; Mauck, Chesnais, and Shapiro 2018). Here, we aimed to evaluate how mixed viral infections in plants could affect vector transmission traits.

Plant viruses are distributed worldwide having a significant impact on plant survival and production of crops (Alexander et al. 2014). Considering the important ecological roles played by viruses, their relationships with hosts and organisms acting as vectors are being explored (Eigenbrode et al. 2002; Mauck, De Moraes, and Mescher 2010; Mauck et al. 2015). Indeed, recent reports documented cases in which plant viruses can manipulate their hosts, and consequently modify the attractiveness for vectors and modulate transmission (Peñaflor et al. 2016; Mauck et al. 2012). Observed effects include modification of host defense responses (Petek et al. 2014; Zhang et al. 2012), changes in the nutritional quality of the plant (Alexander et al. 2014; Su et al. 2015), or alterations of visual traits and production of volatiles conditioning attraction (Ingwell, Eigenbrode, and Bosque-Pérez 2012; Liu et al. 2013). Since transmission to new hosts determines in part the fitness of the pathogen, vector performance may have a direct impact on the ecology and evolution of the viruses they transmit (Gutiérrez et al. 2013; Stafford, Walker, and Ullman 2011).

Nevertheless, our understanding of how such interactions function in complex ecological environments like natural and agricultural ecosystems is still limited. In these situations, plants frequently interact with several pathogens simultaneously, and the composition and dynamics of ecological communities can be significantly affected (Mauck, De Moraes, and Mescher 2015; Mauck et al. 2015; Stout, Thaler, and Thomma 2006). In particular, mixed infections of plant viruses are common, and current evidence suggests that mixed infections are the rule and not the exception in nature (Mascia and Gallitelli 2016; Roossinck, Martin, and Roumagnac 2015; DaPalma et al. 2010). In addition, several important viral diseases of plants are the outcome of interactions between several viral agents (Syller 2012).

In nature, viral mixed infections can be generated by the transmission of different viruses by different vectors, or by the same vector carrying the different viruses (Syller 2014). The result of a mixed infection varies according to the type of interaction between the viruses involved, from synergism to antagonism (Mascia and Gallitelli 2016; Syller 2012). In short, in a synergistic interaction, at least one of the viruses is benefited by the presence of the other(s). The benefit is often quantifiable as an increase in the viral titer, and consequently sometimes the resulting symptoms are more severe compared to a single infection. On the contrary, during an antagonistic interaction, the presence of more than one virus is detrimental for at least one of them, and this is often reflected in a decrease of the viral titer. The order of infection might influence the outcome in virulence and titers (Saldaña, Elena, and Solé 2003; Chávez-Calvillo et al. 2016).

As mentioned, few researchers have explored mixed viral infections with respect to their effect on host-pathogen-vector relationships in agricultural ecosystems (Salvaudon, De Moraes, and Mescher 2013; Syller 2012; Syller and Grupa 2016). In the present work we focused our research in a mixed viral infection to shed some light in the possible ecological outcomes regarding vector transmission. The work was carried out in melon (*Cucumis melo*, L.), an economically relevant crop with global production close to 30 million tons in 2016 (www.fao.org). As in other crops, a significant part of the potential production of melon could be lost due to diseases caused by different pathogens, including viruses (Oerke 2006). Moreover, field surveys have reported high occurrence of multiple viral infections in melon and other cucurbits (Juarez et al. 2013). To bring our research closer to current problems, we worked with two viruses that frequently infect melon: the crinivirus *Cucurbit yellow stunting disorder virus* (CYSDV), and the potyvirus *Watermelon mosaic virus* (WMV), transmitted respectively by whiteflies and aphids (Desbiez et al. 2011; Navas-Castillo, López-Moya, and Aranda 2014). Nowadays, CYSDV can be found throughout many tropical and subtropical areas where cucurbits are cultivated (Wintermantel et al. 2017). It has a bipartite single-strand plus-sense RNA genome. As other criniviruses, CYSDV is restricted to the phloem of the susceptible plants, being transmitted by whiteflies, such as *Bemisia tabaci* Gennadius, in a semipersistent manner (Célix et al. 1996; Navas-Castillo, López-Moya, and Aranda 2014). On the other hand, WMV (formerly also known as WMV2) presents a worldwide distribution, predominantly in temperate regions, and has a large host range, being able to infect more than 170 plant species (Desbiez and Lecoq 2004). As other potyviruses, WMV presents a positive-sense, single-stranded RNA genome that is translated into a

polyprotein subsequently processed by viral-encoded proteinases into functional products (Valli, García, and López-Moya 2015). The infection occurs in most plant tissues, and its transmission is mediated by different aphids, including *Myzus persicae* Sulzer, in a nonpersistent manner (Díaz-Pendón et al. 2005).

The objective of our study was to evaluate mixed infections of CYSDV and WMV in melon, in particular to know if the presence of both viruses could influence their vector transmission. To answer this, we first compared the viral load of each virus either in single or in mix-infected plants, following their accumulation along a period of 60 days, and complemented the quantification measurements with observations of symptoms. Then, transmission experiments were performed under laboratory conditions to evaluate the influence of different plants (single- or mix-infected) as virus sources. Furthermore, the crinivirus transmission was also analyzed under field conditions. Regarding the transmission of WMV, the acquisition from single or mix-infected plants was compared using Electrical Penetration Graph (EPG) to measure the number and duration of the different phases and sub-phases.

Our results reveal that mixed infections could affect the transmissibility of these two viruses. This knowledge could provide scientific-based advice to minimize virus damage, thus improving melon production and quality. More generally, our findings may help to recognize the importance and ecological impact of mixed viral infections on vector transmission and virus dissemination.

Results

Mix-infected plants accumulated more CYSDV and less WMV compared to single infections

Levels of CYSDV and WMV were compared between single and mix-infected plants during 60 days after virus inoculation. To mimic natural conditions, infected plants were obtained using viruliferous whiteflies for CYSDV and aphids for WMV. Samples were collected every 12 days to quantify viral loads. In all time-points the titer of CYSDV was higher in mix-infected compared to single infected plants, with the major peak of accumulation occurring at 24 dpi during mixed infections (Fig. 1). On the other hand, WMV titers were lower in mix-infected plants in comparison to single infections, for most of the analyzed time points. Statistical comparisons were performed first for paired values, using t-test for those cases where normality and homoscedasticity was confirmed,

and Mann-Whitney for the rest, finding significance at all time points except at 12 dpi (not shown). The dynamics of the curves of virus accumulations were also analyzed by ANOVA, finding dependency of viral accumulation both for single vs. mixed infections in the two cases (CYSDV and WMV), and of time for CYSDV.

Mix-infected plants at late time points recovered from the severe symptoms caused by WMV alone

Observation of symptoms were performed during 60 days to compare single and mix-infected plants. In our growing conditions plants with individual infections showed the expected symptoms described for each virus: severe mosaic and puckering with distortion of all leaves in WMV infected plants; and mild leaf mottling, interveinal chlorosis and yellowing, mainly visible in older leaves in CYSDV infected plants (Fig 2a). Interestingly, symptoms in mix-infected plants were very similar to those of a single infection by WMV during approximately the first half of the experiment (Fig 2b), but during the following thirty days, plants recovered and moderately regained growth and vigor, with a final aspect that was more similar to CYSDV single infected plants (Fig 2b and c). The same pattern of symptoms development was observed and reproduced in all repetitions of the experiments.

CYSDV transmission rates were similar at 24 dpi when comparing single or mixed infected source plants, and both under controlled or field conditions

To determine if the mixed infections had some impact on the dispersal of the viruses by their natural vectors we performed transmission experiments.

First, we analyzed the transmission of CYSDV under controlled laboratory conditions, comparing whiteflies that acquired the virus either in single or in mix-infected plants. Plants at 24 dpi were chosen as virus sources due to the highest CYSDV accumulation in mix-infected plants (Fig 1). Insects were allowed to acquire the virus during 48 h on infected plants, and then the viruliferous whiteflies were transferred to uninfected receptor plants for virus inoculation during 48 h. The transmission rates were determined at 18 dpi, testing CYSDV presence by RT-PCR. No significant differences were found (Chi-square) between transmission rates for each individual experiment when the whiteflies acquired the virus in mixed infected compared to single infected plants (Fig 3), although the pooled values reached a significative ($P<0.05$) higher transmission rate for

CYSDV, a result totally unexpected if we take into account the much lower viral load of CYSDV alone compared to CYSDV+WMV at the chosen time point for the acquisition. Transmission experiments were also performed in field conditions during two consecutive years (see Experimental Procedures) in order to determine if the transmission rates of CYSDV were influenced either by the source plants (single or mixed infected at 24 dpi) and/or by the receptor plants (uninfected or WMV-infected). In these field conditions, the results (Figs 4 and 5, supplemental tables S2 and S3) showed larger differences in transmission rates depending on the year, probably reflecting higher mobility and activity of the vectors caused by diverse environmental clues (most likely temperature), thus the comparisons were done separately for each year. Images of the experimental setup, and the complete set of values are provided as supporting information (Fig S1, Tables S2 and S3).

In the first experiment, transmission rates were almost identical when the viral source plants were either single or mix-infected plants, suggesting that the acquisition of CYSDV was not altered by the presence of WMV in mixed infections (Fig 4). Although transmission was slightly higher to WMV-infected than to healthy receptor plants from the two kinds of inoculum sources, the differences were not statistically significant ($t=1.06$ and 1.62 for single or mixed infected source plants).

The experimental design in the second year was modified to allow comparison of inoculation choices to either uninfected or WMV-infected plants alternated in every net-structure. The results (Fig 5, sections a and b) confirmed absence of significant differences ($t=1.13$ and 1.06 for single or mixed infected sources) between the transmission rates to WMV-infected plants compared to uninfected plants, but the slight preference of viruliferous whiteflies to transmit the crinivirus to plants previously infected with the potyvirus was observed in two of the three repetitions performed, regardless of the source of virus acquisition. In order to quantify this putative preference to pre-infected plants, a model was tested for the dependance of percentage of infection as linear function of the pre-infected state of the receptor plant, which resulted in 14% of the infection rate variability explained by the model, although without statistical significance ($p=0.23$). Therefore, further data will be required in order to confirm the potential pre-infection effect.

Reduced WMV levels in mix-infected plants does not result in less transmission of WMV by aphids at 24 dpi

To test if the mixed infection affected transmission of WMV, a different methodology was adopted, taking into consideration the differences of the non-persistent and semipersistent transmission processes. Apterous aphids were fed on a leaf of a single or mixed infected melon plant to acquire the virus, and subsequently manually transferred to test plants for virus inoculation. After insecticide treatment, the plants were observed until appearence of WMV symptoms. Despite the lower viral titers in the mix-infected plants at 24 dpi (Fig 1, and upper panel of Fig 6), the transmission rates when the insects acquired the virus in a mixed infected plant compared to a single infected one were not significantly different in two out of the three individual experiments, neither in the pooled values (Fig 6).

Aphid feeding behavior was altered in mix-infected plants

The feeding behavior of aphids was monitored using EPG to determine if it was influenced by single or mixed infections in the source plants. We fed wired aphids on i) uninfected control plants; ii) single-infected plants with CYSDV; iii) single-infected plants with WMV; and iv) mixed-infected plants with both CYSDV+WMV. The results indicated that the presence of CYSDV alone did not have any major effect on the behavior of aphid vectors compared to control uninfected plants, with the most relevant parameters showing similar values or minor changes not associated with transmission-relevant activities (Table S4). However, we observed that aphids spent more time without probing on plants infected with WMV compared to double-infected plants. Also, longer duration of the intracellular punctures (detectable as potential drops or Pds) was observed in plants infected with CYSDV+WMV, with differences after grouping the data both by insect and by event (Table 5). The statistical analysis of the duration of each of the sub-phases of the Pds confirmed the existence of significant differences in the case of the sub-phase II-3 (Fig 7 a), which was previously associated with acquisition of non-persistently transmitted viruses (Collar, Avilla, and Fereres 1997). The duration of subphase II-3 of the Pds (Fig 7 b, and complete data set in Table S5), and also the number of pulses (Fig 7 c) were significantly higher in plants infected with CYSDV+WMV.

At a late time point, mix-infected plants used as source produced similar rates of aphid transmission of WMV, but higher rates of whitefly transmission of CYSDV.

The observed recovery of plants with mixed infections of WMV + CYSDV at late time points (Fig 2) prompted us to perform additional measurements of vector

transmission rates. For these experiments, five groups of 10 test plants were considered as biological repetitions for each one of the four cases: whitefly-transmission of CYSDV and aphid-transmission of WMV, using as virus sources for acquisition either single (CYSDV or WMV, respectively) or mixed (CYSDV+WMV) infected plants at 52 dpi. Results are shown in Fig 8.

The transmission of CYSDV was much higher (98%) from mix-infected plants compared to single infected source (62%), with significant differences (Chi-square, df=1, $P < 0.0001$), strongly contrasting with the results obtained at 24 dpi (Fig 3). Comparing the source plants at these two time points, the recovered double-infected plants correlated with a better performance as virus source for whitefly transmission. On the other hand, WMV transmission rates were 74 and 67 % respectively from single and mixed infected sources, not showing significant differences (Chi-square test, df=1, P value 0.4429), reaching values in the same range as those found at 24 dpi (Fig 6), despite the clear differences in WMV accumulation at late time points of infection between single and mix-infected plants.

Discussion

Plants naturally infected by more than one virus has been reported often (Juarez et al. 2013; Peñaflor et al. 2016; Roossinck et al. 2010; Tugume, Mukasa, and Valkonen 2016; Zinga et al. 2013). However, not many studies addressed the ecological impact of such complex pathosystems (Elena, Fraile, and García-Arenal 2014). The interactions between hosts, pathogens and vectors could result in an equilibrium with significant effects on each of the three players (Mauck 2016b), as expected in any parasitic interaction (Lefèvre et al. 2009b). Antecedents pointing to the importance of these relationships during mixed infections have been described. For instance, potentially relevant ecological repercussions of complex host-pathogens interactions have been found between unrelated pathogens, like bacteria and viruses (Shapiro et al. 2012, 2013). Concerning viruses, the recently reviewed ecological implications of host manipulation (Mauck, Chesnais, and Shapiro 2018) derive mainly from reports related to single infections (Eigenbrode et al. 2002; Ingwell, Eigenbrode, and Bosque-Pérez 2012; Mauck, De Moraes, and Mescher 2010; Stafford, Walker, and Ullman 2011), although a recent work considered double infections caused by two persistently transmitted viruses (Chen et al. 2018). Repercussions on other combinations of viral infections, such as non-

persistently and semipersistently transmitted viruses (Bragard et al. 2013), are mostly unknown.

In the case of cucurbits, available surveys revealed that mixed viral infections are quite common (Gil-Salas et al. 2012; Sufrin-Ringwald and Lapidot 2011; Juarez et al. 2013). We focused our work on melon plants infected by two virus that generate important economic losses: WMV and CYSDV (Abrahamian and Abou-Jawdah 2014; Juarez et al. 2013). Mixed viral infections were established experimentally using aphids and whiteflies, and the viral load for each individual virus was determined during 60 dpi. Our results showed that CYSDV accumulated to higher titers in mixed compared to single infected plants at all time-points with a peak at 24 dpi, while the viral load of WMV was consistently lower in mix-infected plants than in single infections. These results confirmed similar kinetics reported during mixed infections involving a potyvirus and other plant viruses, including criniviruses: the most common pattern revealed that in mixed infections the accumulation of the potyvirus tend to remain similar or lower compared to single infections, while accumulation increases for the non-potyvirus partner (Murphy and Bowen 2006; Wang et al. 2009). This usual outcome might be due, at least partially, by attenuation of the host RNA silencing-related antiviral defense by the strong activity of the RNA silencing suppressor helper-component protease HC-Pro present in most potyviruses (Valli et al. 2018). This hypothesis is also indirectly supported by the atypical case of the mixed infection between the potyvirus *Sweet potato feathery mottle virus* (SPFMV) and the crinivirus *Sweet potato chlorotic stunt virus* (SPCSV) in sweet potato plants, in which the potyvirus does not generate a synergism in the usual direction, a fact that could be related with the almost complete lack of RSS activity in the SPFMV HCPro (Mingot et al. 2016). In the case of a mixed infection of two potyviruses like WMV and *Zucchini yellow mosaic virus* (ZYMV) in squash (*Cucurbita pepo*), authors found that ZYMV replicated at similar rates in single and mixed infections, whereas WMV accumulated to significantly lower levels in the presence of ZYMV, although it was still readily transmitted from mix-infected plants (Salvaudon, De Moraes, and Mescher 2013).

The virus-virus interaction during mixed infection is considered essential for the combined disease. Outcomes can be classified as antagonistic or synergistic, with synergisms being caused in many cases by combinations of unrelated viruses (Syller 2012). At the same time, the possible effects in the plant caused from mixed infections could range from beneficial, to neutral or detrimental. CYSDV and WMV belong to very

distinct viral groups, crinivirus and potyvirus, being transmitted respectively by non-related insects, semipersistently by whiteflies, and non-persistently by aphids. A superficial analysis of these differences can lead to the assumption that they might not share the same niches (such as host tissues being infected), and thus might not compete for vector transmission or host resources, which could lead to synergism rather than antagonism (DaPalma et al. 2010). In fact, this is roughly what we observed until around the midpoint of the kinetics of virus accumulation. Unexpectedly, the situation appeared to be somehow reversed after a prolonged double-infection period, revealing a more complex interaction between the two viruses. The severity of symptoms caused by CYSDV infection have been directly correlated to the viral load in the plant (Eid et al. 2006; Marco et al. 2003), and thus the higher titers of CYSDV in our mix-infected plants could be one of the factors leading to stronger disease symptoms during the initial stages of the double infection. However, we observed that mix-infected plants began to recover gradually, starting around 36-48 dpi, and by 60 dpi the symptoms were considerably less severe in the mix-infected plants compared to the ones infected only with WMV. Symptom recovery has been described in some viral infections as related to RNA silencing mechanisms (Lim et al. 2011), but further studies will be needed to determine if this is also the case for CYSDV+WMV.

When we analyzed the CYSDV transmission rates to healthy plants in laboratory conditions at 24 dpi, we found no significant differences when comparing single or mix-infected plants as virus sources, despite the higher titers in the second case. In fact, a report showed that accumulation of CYSDV associated with an increase of symptoms might negatively influence transmission, at least in non-cucurbit hosts (Wintermantel et al. 2016). However, the situation was totally reversed at 52 dpi, with mix-infected plants becoming a better source for virus transmission. It is not easy to explain these differences in transmission rates at 24 dpi and 52 dpi, especially considering that the viral load was lower in the late time point (Fig 1). We can speculate that the severely deformed leaves of the mix-infected plants at the early time point could cause steric difficulties for the whiteflies to reach the phloem and acquire the virus, while the larger and less deformed leaves of CYSDV single infected plants were more accessible for vectors. This circumstance might disappear later after the recovery of the plants with WMV+CYSDV, leading to a more comparable topology of leaves where the higher virus load would become determinant for transmission success. Such interactions have been described in certain conditions, for instance when the presence of a satellite modifies transmissibility

of the helper virus (Escriu, Perry, and García-Arenal 2000). However, we cannot rule out other explanations. For instance, in other combinations involving phloem-restricted viruses, co-infection with viruses not restricted to the vasculature can remove phloem limitations and make the virus more accessible for vectors, as in the case of *Potato leafrol virus* (PLRV) and the potyvirus *Potato virus A* (PVA) (Savenkov and Valkonen 2001). Additional work will be needed to verify these or other hypothesis.

When CYSDV transmission was tested in field conditions, similar rates were obtained when comparing single or mixed-infected plants, suggesting that the acquisition process was not affected, regardless of the presence or absence of WMV in the source plants. However, in these experiments we observed a slightly higher transmission towards WMV pre-infected plants compared to uninfected test plants (Fig 4). This observation suggested that the dissemination of CYSDV might be biased towards plants previously infected by potyviruses. Indeed, a repetition of the field experiments allowing free-choice to the viruliferous whiteflies during the inoculation period confirmed that the virus was again preferentially inoculated to WMV pre-infected plants than to uninfected controls (Fig 5 a, b). In other words, the influence of the potyvirus presence in mixed infection could be operating not only in the acquisition and transmission of the unrelated crinivirus at late time points, but also in the inoculation process to favor mixed infections. Such effects of vector attraction are more likely to be expected in semipersistently transmitted viruses, while for non-persistently transmitted viruses such as WMV, effects on acquisition will be more relevant for the transmission outcome. The explanation of this possible preference for WMV pre-infected plants during inoculation of CYSDV by viruliferous whiteflies will require further experimentation, but we can hypothesize that mechanisms like a reduced expression of JA-responsive genes, shown in potyvirus-infected plants (Westwood et al. 2014), could increase attraction to whiteflies.

The results of WMV transmission experiments showed mostly unaltered rates both at early and late time points, regardless of the observed changes in viral load with reduced virus accumulation in mix-infected plants. The transmission process was further analyzed by EPG to determine the aphid feeding behavior. This technique has been used in different studies (Garzo et al. 2016; Moreno et al. 2012; Munster et al. 2017), but to our knowledge this is the first case in which plants single or mix-infected with viruses were compared. The behavior of aphids was recorded when feeding on uninfected, CYSDV-infected, WMV-infected, or mixed (CYSDV+WMV) infected plants, and significant differences were found for transmission relevant parameters between WMV-single and

double-infected plants. Our results clearly showed that aphids, when feeding on mix-infected plants, spent more time in the subphase correlated with acquisition of non-persistent viruses (Collar, Avilla, and Fereres 1997), and also produced more pulses during intracellular stylet punctures (Pds). Interestingly, no differences in number and duration of Pd waveforms per insect were observed between uninfected plants and single infected plants with either WMV or CYSDV alone, suggesting that the observed changes in aphid behavior on mix-infected plants might be indeed attributed to the simultaneous presence of both viruses. For the future, it remains to be determined if these changes respond to differential attractiveness and/or nutritional conditions of plants, or alterations in the defense mechanisms of plants during mixed infections, and how these specific changes are caused by the simultaneous presence of the two viruses and not by each one individually. It is relevant to recall that the transmission mechanisms are different for criniviruses and potyviruses, which can have implications for the manipulative strategies of vector-borne pathogens, as suggested recently (Mauck, Chesnais, and Shapiro 2018). In fact, manipulative strategies by vectors have been reported only for a few cases of mixed infections (Rochow 1972; Froissart, Michalakis, and Blanc 2002; Bourdin and Lecoq 1991; Perry and Francki 1992), but the present work, addressing the transmissibility from co-infections of a potyvirus and a crinivirus, suggests that the possible effects of mixed infections on vector transmission might be more general than previously considered.

To summarize, we observed that titers of CYSDV remained higher in doubly infected plants, therefore increasing the chances of being transmitted, while the plants were able also to support more whitefly vectors after recovering from the initial stronger symptoms. Indeed, our experiment at 52 dpi showed a clear increase of the transmission rate for CYSDV when acquired from mix-infected plants. Regarding the other virus partner, transmission experiments indicate that WMV could also benefit from its presence in mixed infections, even if the viral titers were lower compared to single infected plants in most time points, because in this case the manipulation of aphids could somehow compensate the lower titers by extending the feeding subphase related to virus acquisition. In other words, during mixed infections CYSDV might enhance its accumulation in plants less severely affected (and thus, prone to survive longer and in better shape) and consequently its chances of dissemination, and at the same time the less abundant WMV might be maintaining the same transmission rates thanks to alterations in the feeding behavior of its vector. Thus, and from an ecological point of view, mix-

infected plants would confer adaptive advantages to both viruses for their respective dissemination. The implications of multiple infections for the evolution of pathogens (Tollenaere, Susi, and Laine 2016) are being explored in some virus pathosystems (Ali and Roossinck 2017), and our results suggest that vectors might be key elements in the outcome, along with some effects derived from the frequent presence of more than one pathogen in mixed infections. Although additional work will be needed to elucidate all the mechanisms operating during mixed infections, we believe that our results might be useful to explore how the interactions and their dynamics can conditions the available strategies to control viral diseases in crops.

Experimental Procedures

Plants, insects and viruses

Seeds of the virus-susceptible melon cultivar "Piel de sapo" PS T111, provided by Semillas Fitó (Spain) were germinated after 5 min treatment with the fungicide Captan in aqueous solution (3g/l). Plants were grown in chambers at 22-25ºC and 16/8 h light/dark cycles.

A clonal population of the aphid *Myzus persicae,* named MP89 and originally from the ICA-CSIC (Madrid, Spain), was maintained on tobacco plants (*Nicotiana tabacum,* cv Xanthi). A colony of *Bemisia tabaci* biotype MEAM1 (formerly biotype B) was provided by Dr. R. Gabarra (IRTA-Cabrils, Barcelona, Spain), and was raised on tomato plants (*Solanum lycopersicum*, cv Micro-Tom). Plants supporting insects for laboratory experiments were kept in separated growth chambers at 23-25ºC and 16/8 h light/dark cycles. For field experiments with whiteflies, individuals from a colony of *B. tabaci* biotype MED (former Q) were used (Rodríguez-López et al. 2012).

The Spanish isolate of the crinivirus CYSDV, named CYSDV-AlLM, was kindly provided by Dr. M.L. Gómez-Guillamón (IHSM “La Mayora”, Málaga, Spain), and was maintained through periodical whitefly transmission on susceptible host plants.

The initial infection of WMV was achieved using an infectious full-length clone kindly provided by Dr. C. Desbiez (INRA–PACA, Avignon, France), following described procedures (Desbiez et al. 2011), and the virus was later propagated through aphid transmission.

CYSDV and WMV transmission experiments

Both CYSDV and WMV isolates were transmitted to melon plants using their natural vectors. Experimental conditions for CYSDV transmission were set after testing temperature and the number of viruliferous whiteflies, with acquisition and inoculation access periods of 48h each, choosing a temperature of 25 ºC and a ratio of 20 whiteflies per plant. A controlled-vacuum hand-trap and clamp-cages were used for handling insects during transmission to individual plants. In experiments with a large number of test plants, enough viruliferous whiteflies to reach the chosen rate were released inside insect-proof cages containing the plants to be inoculated.

The transmission of WMV by aphids was performed essentially as described before for other potyviruses (Ruiz-Ferrer et al. 2005). Briefly, aphids were fasted for 3h hours and then allowed to acquire and inoculate the virus during periods of 10-30 min in each case, transferring them manually from the source to the receptor plant with the help of a paintbrush, until 10 viruliferous aphids were placed on each individual test plant. Vectors were then killed with Confidor insecticide, and the plants were maintained to determine transmission rates by presence of visual symptoms for WMV, or through molecular diagnostic for CYSDV (see below).

The CYSDV field transmission experiments were performed inside net-structures (insect proof walk-in 5 × 5 × 2 m) within a tunnel net house and under natural temperature and light conditions (Fig S1) (Rodríguez-López et al. 2012). Tree plants infected with CYSDV or co-infected with CYSDV+WMV were used as inoculum source, surrounded by receptor plants (n=20), either uninfected or previously infected with WMV, distributed in a circle of about 2 m diameter. About 800 non-viruliferous whiteflies were released in the center of the source plants, leading to a vector density of 40 whiteflies/plant. After 4 days the receptor plants were treated with insecticide and transferred to a greenhouse for up to 30 days, using tissue print to determine the presence of CYSDV. Field experiments were performed in two consecutive years, using three independent net structures for each combination of source (CYSDV or CYSDV+WMV) and receptor (uninfected or WMV) plants (no choice, total 12 net structures). In free-choice experiments, 10 uninfected plants and 10 WMV-infected plants were distributed in alternated positions (3 net structures for each type of source plants).

Sampling, RNA extraction and detection of viruses

Sampling of representative tissues was standardized to account for growth differences between uninfected, single, and double virus-infected plants. Two leaf discs

(1 cm diameter) were taken from the second to the latest fully-expanded leaf at each sampling time point, regardless of size and position within the plant. Samples were immediately frozen in liquid nitrogen and ground in Tissue lyser (QIAGEN). Total RNA was extracted using TRIzol reagent (Invitrogen) according to provider instructions, including an additional ethanol precipitation to improve purity. Quality and concentration were estimated in NanoDrop® spectrophotometer (ND-8000).

The High-Capacity cDNA Reverse Transcription kit (Applied Biosystems ™) was used to generate cDNA from 1μg of total RNA, following the manufacturer protocol. The cDNAs served as templates for amplification of viral sequences by polymerase chain reaction (PCR) using TaKaRa ExTaq® commercial kit. The number of cycles and conditions for amplification (temperature and extension time) were optimized for each combination of primers. Virus specific oligos (Table S1) were used to amplify an 813 nt fragment in the CP region of WMV, and a 563 nt fragment near the 3' end of the genomic RNA2 of CYSDV (Marco et al. 2003). DNA fragments were analyzed by electrophoresis on agarose gels.

Quantification of viral load by RT-qPCR

Virus quantification was performed with cDNAs generated from 1 μg of total RNAs, that were previously treated with DNase (ThermoScientific), by RT-qPCR and comparison to standard curves representing known dilutions of *in vitro* transcripts.

To generate virus transcripts, specific oligos were designed to amplify and clone fragments of approximately 1.5 kb located near the 5' ends of viral genomic RNAs in each case (Table S1). The plasmid pGEMT-Easy (Promega) was used to clone fragments deriving from the 5' ends of RNA 1 and RNA 2 of CYSDV, generating constructs RNA1ORF1CYSDV and RNA2Hsp70hCYSDV with the viral sequences under the control of T7 promoter to allow *in vitro* transcription with genome orientation (+ sense). For WMV, the complete P1 coding region was cloned into pENTR/D-TOPO, and transferred by Gateway recombination to the vector pDEST 14 (Invitrogen) under the control of T7 promoter in genome orientation (+ sense). Constructs were verified by restriction analysis and sequencing. Transcripts were obtained with linearized plasmids as templates using the MEGAscript T7 kit (Ambion), quantified and used as standards in RT-qPCR.

Two pairs of specific oligos were designed (https://www.genscript.com/ssl-bin/app/primer) to amplify fragments ranging from 80 to 150 base pairs (bp) for each

viral RNA (S1 Table), and those leading to better linear regression with the reference were selected in each case. SYBRGreen (Roche) was used to detect PCR products in a Ligth Cycler 480 (Roche) equipment, using 50 ng of cDNAs as template in the PCR reactions

Screening of CYSDV infected plants through tissue print and molecular hybridization

The presence of CYSDV before the onset of symptoms was determined by tissue printing and hybridization with a virus-specific probe (Más and Pallás 1995). Briefly, the plasmid pLM15/24 (Marco et al. 2003) kindly provided by Dr. M. Aranda (CEBAS-CSIC, Murcia), containing a fragment of 5437 bp from the virus RNA1 under the control of the SP6 promoter, was linearized with the restriction enzyme XbaI, and *in vitro* transcription was carried out using the DIG NORTHERN kit (Roche), following manufacturer's instructions, to obtain negative sense transcripts labeled with digoxigenin. The transcripts were hydrolyzed to shorter size using a carbonate buffer treatment, ethanol precipitated, and resuspended until use (Marco et al. 2003).

Cross sections of petioles were printed by pressing on positively charged Nylon membranes (Roche), and the nucleic acids fixed by crosslinking with 120 mJ/cm^2 (UVC500 crosslinker, Amersham Biosciences). The membrane was incubated with prehybridization solution (20x SSC, 10% N-Lauryl sarcosine, 50% Formamide, 10% SDS and 2% commercial blocking agent from Roche) at 65ºC for 3h, before adding the denatured probe (heated for 10 minutes at 65 °C) and incubated for 14-16 h at 65°C. Processing included: two 10 min rinses (2x SSC, 0.1% SDS); two 15 min rinses (0.1x SSC, 0.1 % SDS); 5 min in washing solution (0.1 Maleic acid, 0.15M NaCl pH7.6, 0.3% Tween 20); 1h blocking (0.1 Maleic acid, 0.15 M NaCl pH 7.6, 1% commercial blocking agent of Roche); 1 h incubation with anti-DIG antibody (Roche) at the recommended concentration; two 15 min rinses in washing solution. The signal was finally visualized in a ChemiDoc chemiluminescence equipment (Bio-Rad), using CDP-Star substrate (Roche) in detection buffer (0.1M TrisHCl pH 9.5, 0.1M NaCl).

Monitoring of the feeding behavior of aphids by EPG

Feeding behavior of *M. persicae* on melon plants was analyzed by EPG (Tjallingii 1978). Activities up to 1-hour were recorded from individual insects feeding on plants: i) not inoculated, ii) infected with CYSDV, iii) infected with WMV, or iv)

infected with CYSDV+WMV. Adult apterous aphids were connected to a gold wire (3 cm long, 20 μm of diameter) with a drop of silver conductive paint (Pelco® Colloidal Silver no. 16034, Ted Pella Inc., Redding, CA, USA) on the dorsum. The wire was glued to a copper nail and connected to an EPG probe following standard procedures (Carmo-Sousa et al. 2014). Each wired insect was placed on the leaf of a plant, with the circuit closed through the plant substrate. Four plants were tested in parallel using a 4-channel device (Giga-4; EPG Systems, Wageningen, The Netherlands), and 20 to 30 repetitions were recorded. EPG data acquisition was carried out using the Stylet+ computer program (EPG-Systems, Wageningen, The Netherlands), to identify EPG waveforms described for aphids (Tjallingii 1988) corresponding to non-probing (np), intercellular apoplastic stylet pathway (C), and intracellular stylet puncture (Pd). No other EPG waveforms were observed during the 1h recording time. Behavioural variables were processed using a previously described MS Excel Workbook (Sarria et al. 2009).

EPG variables (mean ± SE) were calculated (Backus et al. 2007) to determine: proportion of individuals that produced a specific waveform type (PPW); number of wave-form events per insect (NWEI); total waveform duration (min) per insect (WDI); and total waveform duration (min) per event (WDE). Data were analyzed after transformation using ln(x+1). Because of the non-Gaussian distribution of the data, a Mann-Whitney U test was conducted in GraphPad prism 6.0.

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Table Caption

Table 1. Selected EPG variables describing the feeding behavior of *M. persicae* on single and mixed virus infected melon plant

Figure Legends

Figure 1. Kinetics of viral loads in single and mix-infected plants. Quantification of CYSDV (left) and WMV (right), showing viral loads in single (blue) and mixed (red) infected plants. Each point represents 2 independent biological replicates of individually infected plants (dataset n=15), collected at 12, 24, 36, 48 and 60 d.p.i. The mean values are indicated by the horizontal lines, connected through an adjusted curve, and surrounded by gray areas representing 95% confidence intervals.

Figure 2. Symptomatology of infected plants. A) Details of leaves with symptoms caused by single infection with WMV (upper panel) or CYSDV (lower panel). B) Representative single and mix-infected plants photographed at the indicated time-points. C) Side-by-side representative plants of the four categories (from left to right: non-infected, CYSDV, WMV, CYSDV+WMV) photographed at 24 and 48 dpi. corresponding with the development used as virus sources during vector-transmission experiments

Figure 3. CYSDV transmission in controlled conditions at 24 dpi. Pooled values of virus accumulation are plotted on the upper panel (compared by U Mann-Whitney test) above the tabulated results of the three repetitions of transmission, with number of infected plants/number of plants tested for each independent experiment, followed by the values of two-tailed Chi-square tests (df=1). Transmission rates are showed graphically using pink for CYSDV infected vs. green for uninfected test plants.

Figure 4. Transmission of CYSDV under field conditions. Average of transmission rates in three individual net-structures, with 20 test plants each, either uninfected or previously infected by WMV, and surrounding single infected CYSDV plants (left panel),

or mixed infected CYSDV+WMV plants (right panel) as sources of inoculum. No significative differences were found (t-test).

Figure 5. Free-choice transmission of CYSDV under field conditions. A) Graphical representation of comparative transmission of CYSDV to alternated plants, 10 uninfected and 10 previously infected with WMV, using single infected CYSDV plants as source of inoculum; results for each individual net-structure are represented individually. B) Similar experiment as in a) with mixed infected CYSDV+WMV plants as inoculum source. Analysis of data was performed with an association test.

Figure 6. WMV transmission in controlled conditions at 24 dpi. Pooled values of virus accumulation are plotted on the upper panel (compared using t-test analysis) above the tabulated results for the three repetitions of transmission, with number of infected plants/number of plants tested for each independent experiment, followed by the values of two-tailed Chi-square tests (df=1). Transmission rates are showed graphically using blue for WMV infected vs. green for uninfected test plants.

Figure 7. EPG monitoring of aphid feeding behavior. A) Example of a representative EPG record with a potential drop (Pd) in which the different sub-phases (II-1, II-2 and II-3) can be identified. B) Comparison of the duration of the sub-phases corresponding to Pds between aphids fed on single or mix-infected plants c) Comparison of number of pulses of subphase II-3 between aphids fed on single or mix-infected plants. The error bars represent ±1 SEM. ** $P<0.005$.

Figure 8. CYSDV and WMV transmission in controlled conditions at 52 dpi. The transmission rate of CYSDV by whiteflies (upper panel) and WMV by aphids (lower panel) is shown for five independent experiments using 10 plants each, with the number of infected plants/number of plants tested for each independent experiment, and the pooled total followed by the value of a two-tailed Chi-square test (df=1). Statistical significant differences are indicated with P-value in bold. Transmission rates are depicted graphically using the same color code as in previous figures: pink for CYSDV-infected, blue for WMV-infected, and green for uninfected test plants.

Supporting Information Legends

Table S1. List of primer sequences and their uses

Table S2. CYSDV transmission in field conditions from single and mixed-infected plants to uninfected or WMV-infected in different net-houses.

Table S3. CYSDV transmission in field conditions from single and mixed-infected plants to uninfected or WMV-infected plants in the same net-structures, in alternate positions.

Table S4. Comparative analysis of EPG parameters in aphids feeding on uninfected, CYSDV, WMV, and WMV+CYSDV infected plants

Table S5. Monitoring of intracellular subphases during potential drops (Pd) detected in aphids feeding behavior by EPG

Figure S1. Secondary CYSDV transmission in field conditions.

Table 1. Selected EPG variables describing the feeding behavior of *M. persicae* on single and mixed virus infected melon plant

Variables[1]	Treatment	PPW	NWEI	P[2]	WDI	P[2]	WDE	P[2]
Np	WMV	30/30	0.80±0.17 (0.48-1.04)	0.73	3.07±0.27 (2.28-3.32)	**0.04**	2.10±0.53 (0.65-3.18)	**0.03**
	CYSDV + WMV	28/28	0.82±0.23 (0.30-1.18)		2.96±0.28 (2.19-3.39)		2.00±0.46 (0.84-2.98)	
C	WMV	30/30	0.77±0.18 (0.30-1.00)	0.48	2.75±0.31 (2.08-3.25)	0.06	1.69±0.55 (0.94-3.05)	0.74
	CYSDV + WMV	28/28	0.80±0.22 (0.30-1.15)		2.91±0.27 (2.36-3.26)		1.68±0.59 (0.92-3.26)	
Pd	WMV	30/30	1.14±0.26 (0.60-1.59)	0.12	1.75±0.25 (1.11-2.13)	**0.02**	0.70±0.08 (0.52-1.12)	**<0.0001**
	CYSDV + WMV	28/28	1.24±0.23 (0.70-1.62)		1.90±0.22 (1.29-2.20)		0.74±0.11 (0.54-1.18)	

[1] For each treatment, the following parameters were measured: Np, non-probe activity; C, intercellular stylet pathway; Pd, short intracellular punctures. PPW means proportion of individuals that produced the waveform type, with NWEI being the number of waveform events per insect, WDI the waveform duration per insect, and WDE the waveform duration per event. Durations are expressed in minutes for Np and C, and in seconds for Pd. Values are indicated as mean ± 1 SEM (range given in parentheses).

[2] Statistical comparisons between the two treatments were performed for each parameter with non-parametric U-Mann Whitney test, considering non Gaussian distribution variables. Bold-type indicates significant differences ($p<0.05$)

1

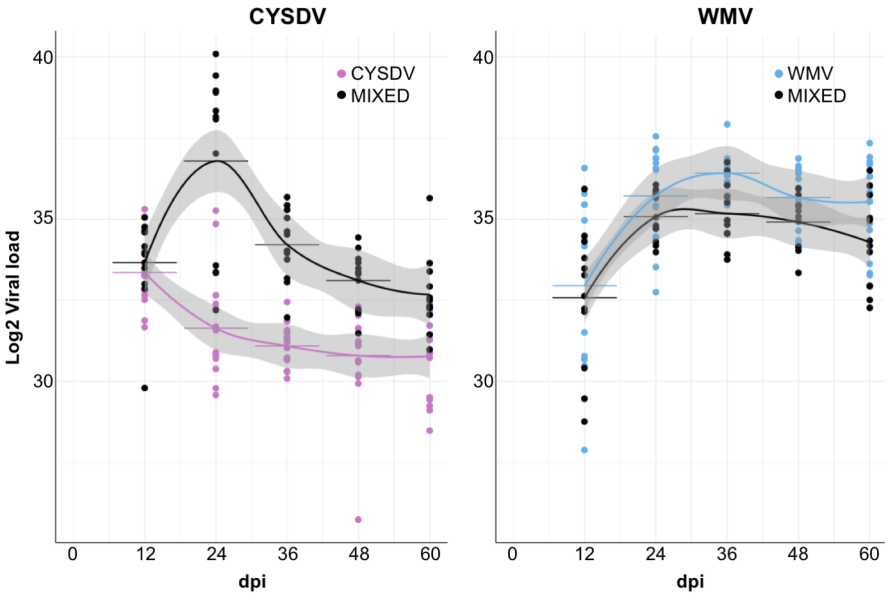
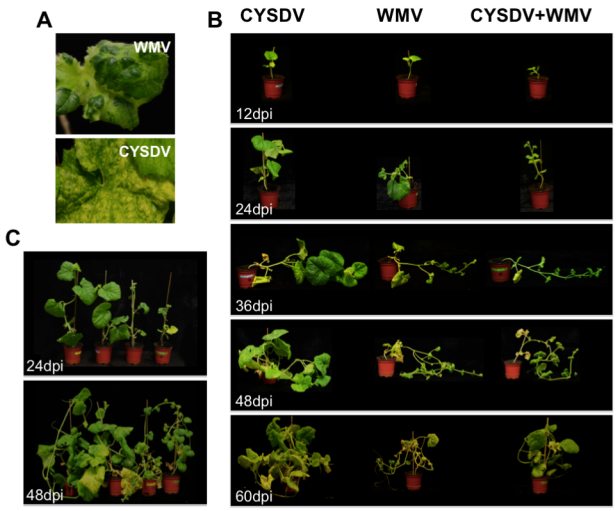

CYSDV
WMV
CYSDV
MIXED
WMV
MIXED
Log2 Viral load
40
35
30
0
12
24
36
48
60
dpi

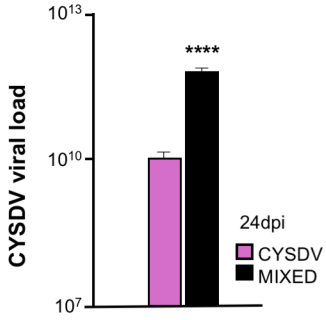
Fig.1


A
WMV
CYSDV
B
CYSDV
WMV
CYSDV+WMV
12dpi
24dpi
36dpi
48dpi
60dpi
C
24dpi
48dpi

1

2 Fig.2

3



Inoculum source		CYSDV	CYSDV+WMV	Chi-square , df	P value
Transmission rates	Exp.1	27/40	19/40	3.27 , 1	0.07
	Exp. 2	15/39	14/40	0.10 , 1	0.75
	Exp. 3	30/40	22/40	3.52 , 1	0.06
Total		72/119	55/120	5.16 , 1	**0.02**
Average		61%	46%		

CYSDV transmission

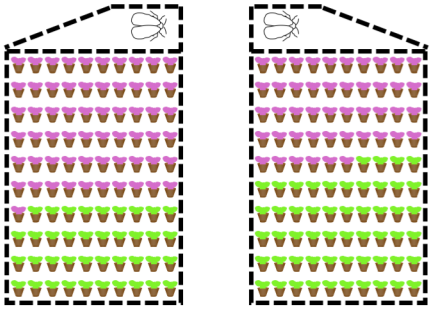
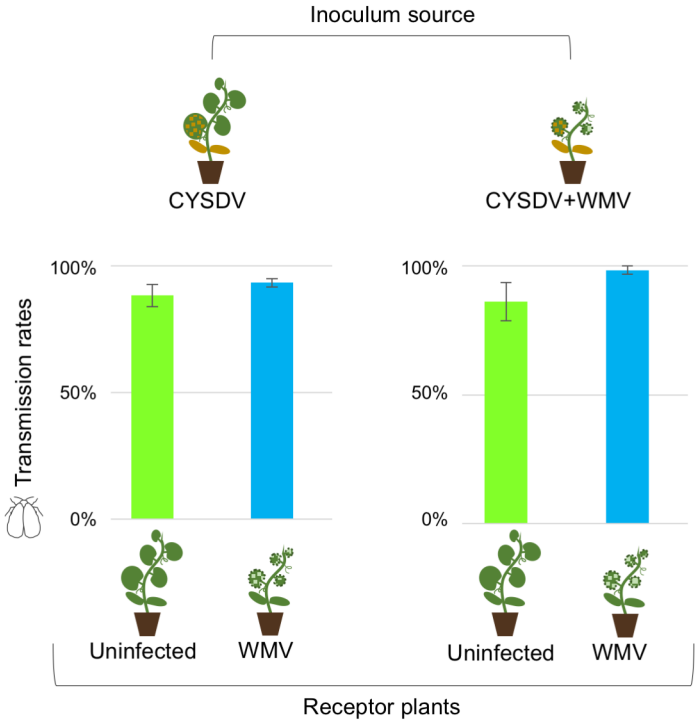


Fig.3


Inoculum source
CYSDV
CYSDV+WMV
100%
50%
0%
Transmission rates
Uninfected
WMV
Uninfected
WMV
Receptor plants

1

2 Fig.4

3

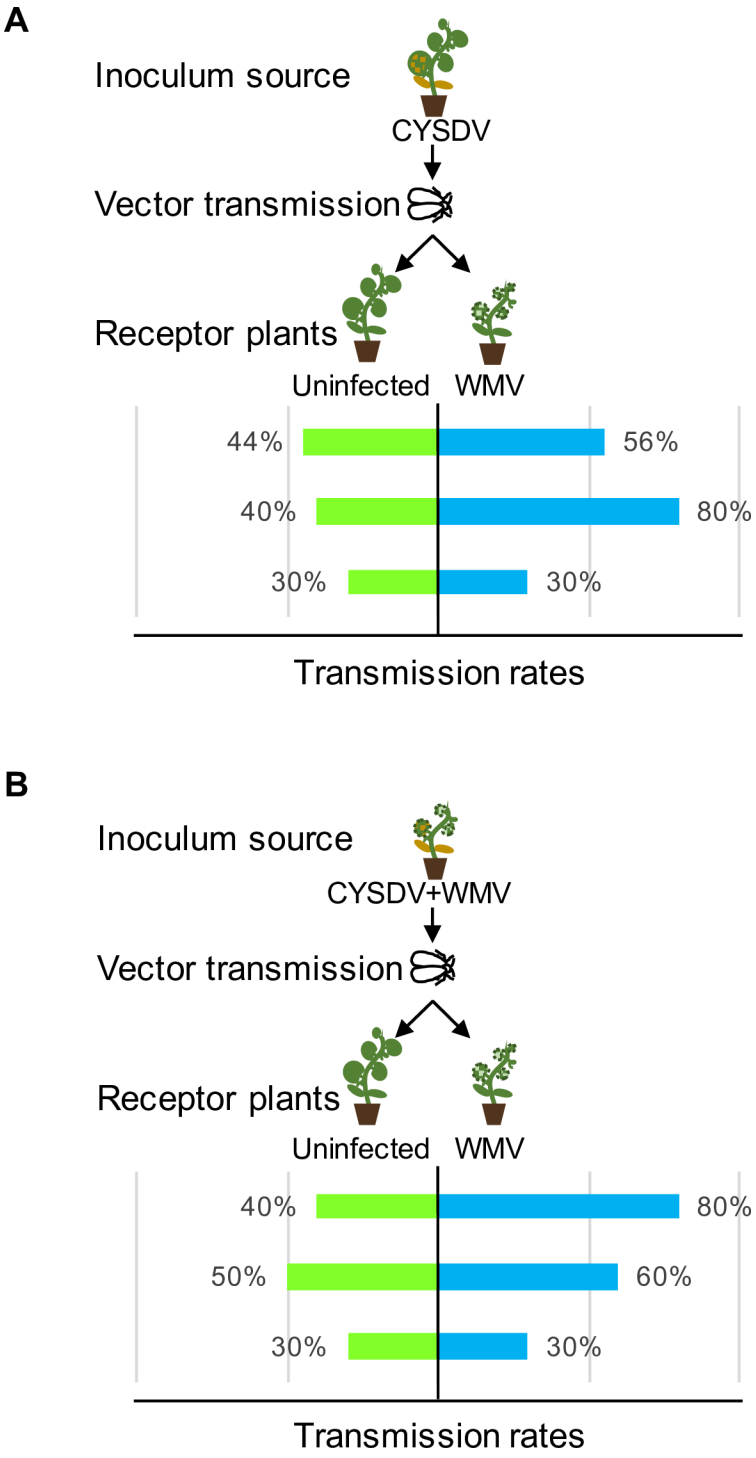
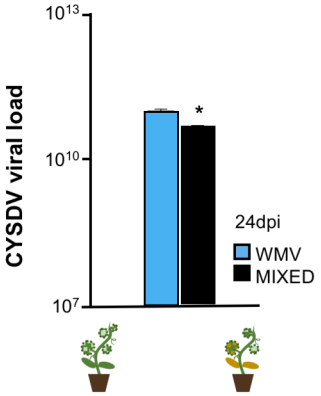

A
Inoculum source
CYSDV
Vector transmission
Receptor plants
Uninfected
WMV
44%
56%
40%
80%
30%
30%
Transmission rates
B
Inoculum source
CYSDV+WMV
Vector transmission
Receptor plants
Uninfected
WMV
40%
80%
50%
60%
30%
30%
Transmission rates

Fig. 5



Inoculum source		WMV	CYSDV+WMV	Chi-square , df	P value
Transmission rates	Exp.1	23/40	28/40	1.35 , 1	0.25
	Exp. 2	15/40	23/38	4.14 , 1	**0.04**
	Exp. 3	21/34	13/32	2.95 , 1	0.09
Total		59/114	64/110	0.93 , 1	0.33
Average		52%	58%		

WMV transmission

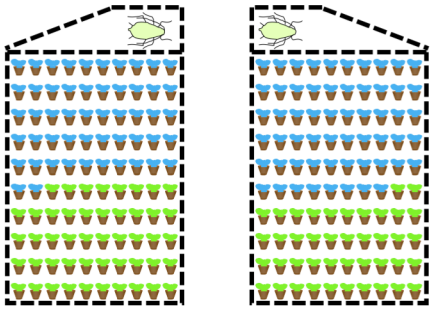


Fig. 6

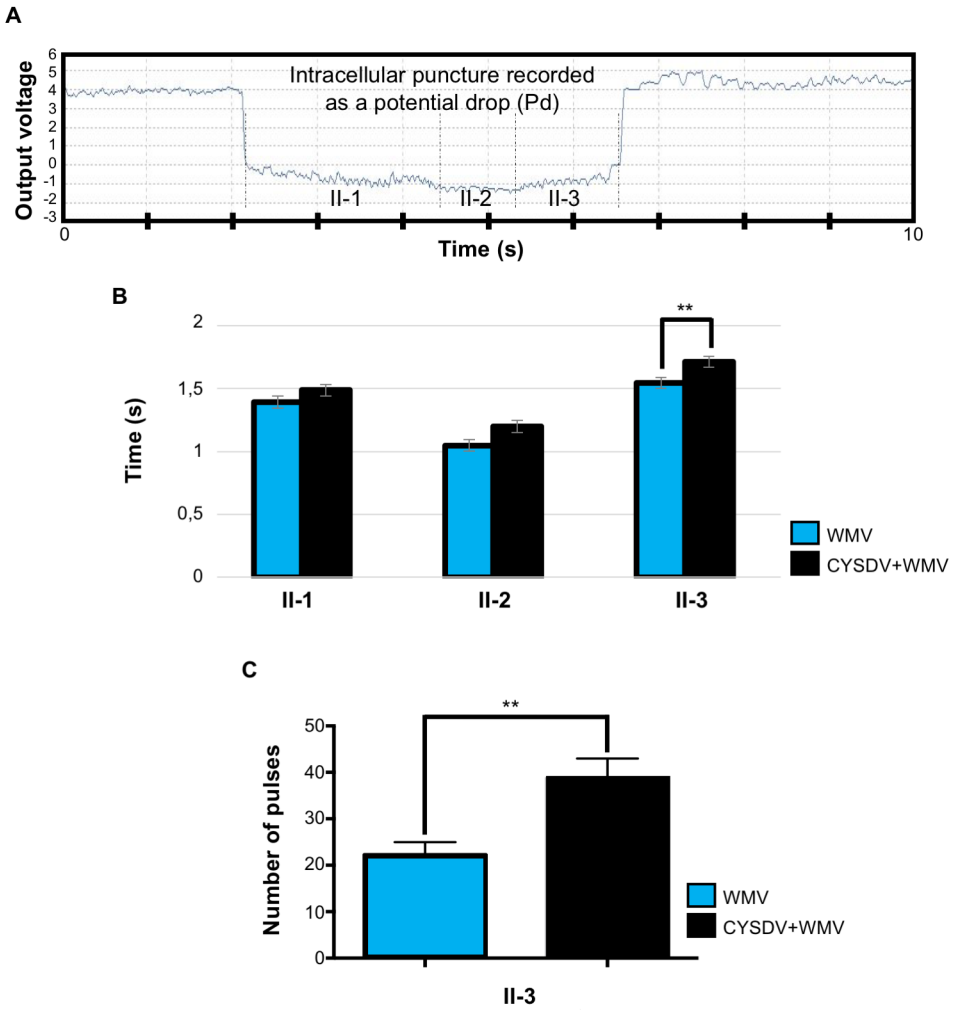
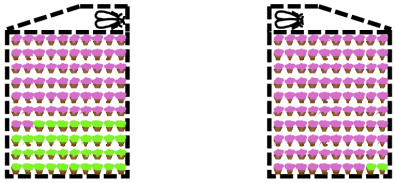

A
Output voltage
Intracellular puncture recorded
as a potential drop (Pd)
II-1
II-2
II-3
Time (s)
B
Time (s)
**
WMV
CYSDV+WMV
II-1
II-2
II-3
C
Number of pulses
**
WMV
CYSDV+WMV
II-3

Fig.7

A

Inoculum source	CYSDV	CYSDV+WMV		
Transmission rates	5/10	10/10		
	8/10	9/10		
	8/10	10/10		
	4/10	10/10		
	6/10	10/10	Chi-square , df	P value
Total	31/50	49/50	20.25 , 1	**< 0.0001**
Average %	62%	98%		
CYSDV transmission				

B

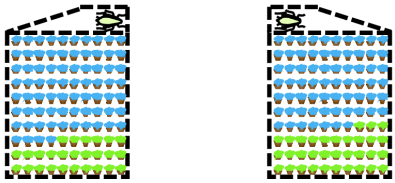
Inoculum source	WMV	CYSDV+WMV		
Transmission rates	8/10	5/9		
	7/8	8/10		
	6/9	7/10		
	5/10	7/10		
	9/10	6/10	Chi-square , df	P value
Total	35/47	33/49	0.59 , 1	0.44
Average %	74%	67%		
WMV transmission				

Fig. 7

Supplemental Table 1. List of primer sequences and their uses

Name	Sequence	Use
RNA1 CYSDV Fw	5'ATGTCGTCGTCACTAGTTGC 3'	Template for *in vitro* transcription [1]
RNA1 CYSDV Rv	5'GTCGAGACCACTCCAGTACA 3'	
RNA2 CYSDV Fw	5'AGGTGGGTAGGTGTTGACAG 3'	Template for *in vitro* transcription [1]
RNA2 CYSDV Rv	5'TGGGAAGTACCGTCTCCTTACC 3'	
P1 WMV Fw	5'**CACC**ATGGCAACAATCATGTTTGGAG 3'	Template for *in vitro* transcription [1]
P1 WMV Rv	5'TCAATAATGTTGAATATCTTCTATCTCC 3	
qPCR RNA1 CYSDV Fw	5'GACTCAAACAAGCGCGATGA 3'	Analysis of viral load (CYSDV RNA1)
qPCR RNA1 CYSDV Rv	5'GCGTTGCCGTTACTGAGGAT 3'	
qPCR RNA2 CYSDV Fw	5'TTTGTCCGCTGACGTACTGG 3'	Analysis of viral load (CYSDV RNA2)
qPCR RNA2 CYSDV Rv	5'GGTTCAATGCAACGACTCAGA 3'	
qPCR P1 WMV Fw	5'TGCAACTATTGTGGCCTTCG 3'	Analysis of viral load (WMV)
qPCR P1 WMV Rv	5'CATGGCACCTCCACCTCATA 3'	
WMV Fw	5'CTTATGGTTGTCATTGCTATG 3'	Detection of WMV
WMV Rv	5' CCCACCAACTGTTGGAAG 3'	
MA156 Fw	5' GAAGAATTCCAGGCAAGG 3'	Detection of CYSDV [2]
MA129 Rv	5' TCACATCATCAATCCAAAAG 3'	

[1] Primers were used for cloning partial virus sequences to generate *in vitro* transcripts as templates for quantitative comparisons in RT-qPCR. Non-viral sequences used for cloning purposes are highlighted in bold.

[2] Primers described by Marco et al. 2003

Supplemental Table 2. CYSDV transmission in field conditions from single and mixed-infected plants to uninfected or WMV-infected in different net-houses.

Source	Receptors	Repetition[1]	Infected plants[2]	Total	Average[3]
CYSDV	Uninfected	1	19/20	53/60	88.33%
		2	18/20		
		3	16/20		
	WMV	1	19/20	56/60	93.33%
		2	19/20		
		3	18/20		
CYSDV+WMV	Uninfected	1	15/20	50/58	86.2%
		2	20/20		
		3	15/18		
	WMV	1	20/20	59/60	98.33%
		2	19/20		
		3	20/20		

[1] Three independent net-structures, each with 20 receptor plants, all of the same type, are shown
[2] Results obtained in each independent net-house are indicated for each treatment, and pooled values and percentages are shown in the last columns.
[3] Statistical analysis were performed by two-tail t-Test comparisons of every pair of treatments (after verifying inequality of variances by F-Test). None of the paired comparisons indicated significant differences between the samples means.

Supplemental Table 3. CYSDV transmission in field conditions from single and mixed-infected plants to uninfected or WMV-infected plants in the same net-structures, in alternate positions.

Source	Repetition[1]	Receptors	Infected plants	Average
CYSDV	1	Uninfected	3/10	30%
		WMV	3/10	30%
	2	Uninfected	4/10	40%
		WMV	8/10	80%
	3	Uninfected	4/9	44,44%
		WMV	5/9	55,55%
	Total	Uninfected	11/29	37,93%
		WMV	16/29	55,17%
CYSDV+WMV	1	Uninfected	3/10	30%
		WMV	3/10	30%
	2	Uninfected	5/10	50%
		WMV	6/10	60%
	3	Uninfected	4/10	40%
		WMV	8/10	80%
	Total	Uninfected	12/30	40%
		WMV	17/30	56,66%

[1] The results of three independent net-structures (with 10 uninfected and 10 previously WMV-infected receptor plants in alternated positions) are shown for each treatment, with the pooled values for each category in the "Total" rows.

[2] Statistical analysis were performed by two-tail t-Test comparisons of every pair of treatments (after verifying inequality of variances by F-Test). None of the paired comparisons indicated significant differences between the samples means.

1

Supplemental Table 4. Comparative analysis of EPG parameters in aphids feeding on uninfected, CYSDV, WMV, and WMV+CYSDV infected plants

Parameter[1]	Treatment	PPW	NWEI	WDI	WDE
NP	Uninfected	20/20	0.73±0.05 (0.30-1.04) a	2.88±0.06 (2.24-3.16) a	2.04±0.04 (0.84-2.87) a
	CYSDV	23/23	0.88±0.03 (0.60-1.20) a	2.91±0.06 (2.34-3.33) a	1.90±0.04 (0.86-2.95) b
	WMV	30/30	0.80±0.03 (0.48-1.04) a	3.07±0.05 (2.28-3.32) b	2.10±0.04 (0.65-3.18) ac
	CYSDV +WMV	28/28	0.82±0.04 (0.30-1.18) a	2.96±0.05 (2.19-3.39) a	2.00±0.03 (0.84-2.98) abd
C	Uninfected	20/20	0.71±0.05 (0.30-1.04) a	2.96±0.04 (2.49-3.26) a	1.91±0.06 (1.04-3.26) a
	CYSDV	23/23	0.87±0.03 (0.60-1.18) b	2.90±0.07 (1.96-3.21) ab	1.75±0.05 (0.84-3.14) b
	WMV	30/30	0.77±0.03 (0.30-1.00) ab	2.75±0.06 (2.08-3.25) b	1.69±0.04 (0.94-3.05) b
	CYSDV +WMV	28/28	0.80±0.04 (0.30-1.15) ab	2.91±0.05 (2.36-3.26) ab	1.68±0.05 (0.92-3.26) b
Pd	Uninfected	20/20	1.28±0.04 (0.85-1.53) a	1.86±0.04 (1.38-2.09) ab	0.69±0.004 (0.51-1.14) ab
	CYSDV	23/23	1.26±0.04 (0.85-1.59) a	1.85±0.04 (1.45-2.21) ab	0.69±0.004 (0.50-1.04) a
	WMV	30/30	1.14±0.05 (0.60-1.59) a	1.75±0.05 (1.11-2.13) a	0.70±0.005 (0.52-1.12) b
	CYSDV +WMV	28/28	1.24±0.04 (0.70-1.62) a	1.90±0.04 (1.29-2.20) b	0.74±0.005 (0.54-1.18) c

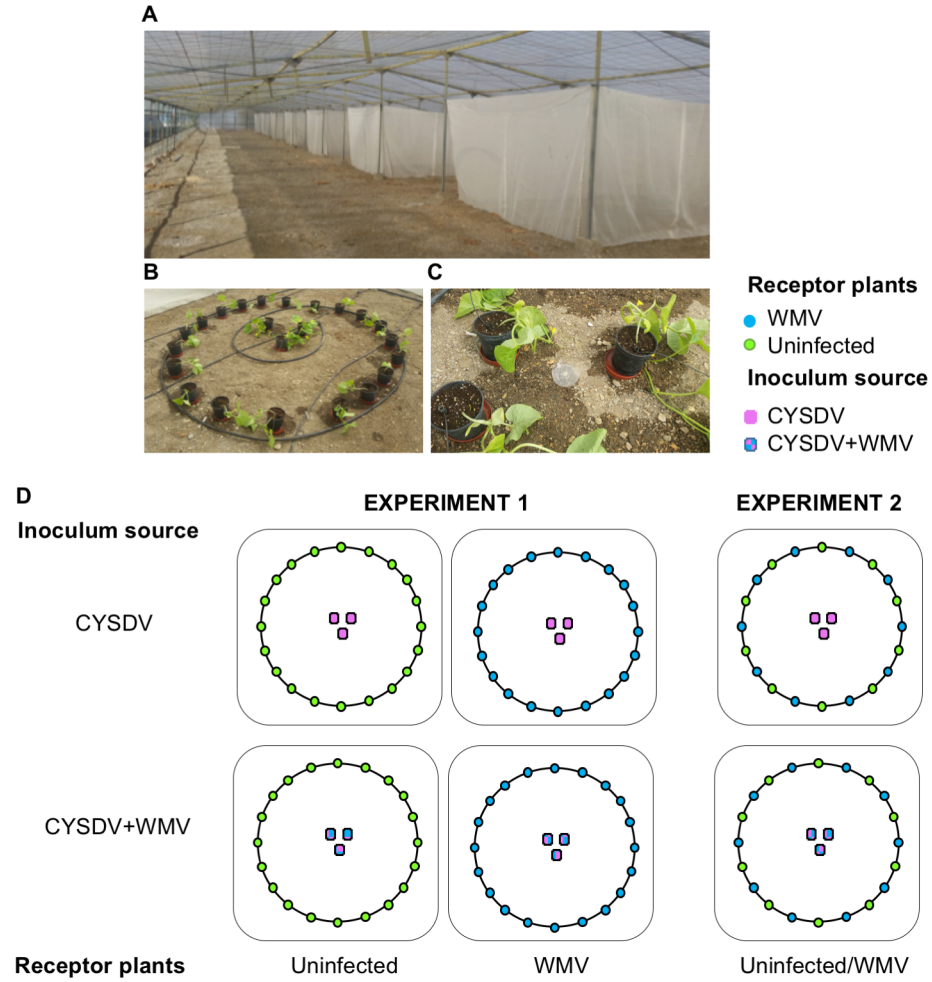
[1] Parameters measured include: NP, non-probe activity; C, intercellular stylet pathway; Pd, short intracellular punctures. PPW means proportion of individuals that produced the waveform type, with NWEI being the number of waveform events per insect, WDI the waveform duration per insect, and WDE the waveform duration per event. Durations are expressed in minutes for NP and C, and in seconds for Pd. Values are indicated as mean ± SE with extremes given in parentheses. The statistical comparisons were performed in pairs for each parameter with non-parametric U-Mann Whitney tests, considering non Gaussian distribution variables. Only values followed by different letters were found significantly different ($p<0.05$).

Supplemental Table 5. Monitoring of intracellular subphases during potential drops (Pd) detected in aphids feeding behavior by EPG

Sub-phase[1]	Treatment	PPW	WDI	P[2]
II-1	WMV	30/30	1,36±0,05(0,75-1,82)	0,09
	CYSDV+WMV	28/28	1,49±0,05(0,81-1,90)	
II-2	WMV	30/30	1,03±0,05(0,52-1,46)	0,08
	CYSDV+WMV	28/28	1,15±0,05(0,53-1,53)	
II-3	WMV	30/30	1,34±0,04(0,77-1,69)	**0,002**
	CYSDV+WMV	28/28	1,53±0,04(0,91-1,84)	

[1] The sub-phases of pd, associated with uptake (acquisition, II-3) and release (inoculation, II-1) of non-persistently transmitted viruses are identified following Martin et al., 1997. PPW means proportion of individuals that produced the waveform type, and WDI is the waveform duration per insect in seconds. Values are indicated as mean ± SE with extremes given in parentheses.

[2] Statistical comparisons between the two treatments was performed for each parameter with non-parametric U-Mann Whitney test, considering non Gaussian distribution variables. Bold-type indicates significant differences ($p<0.05$).


A
B
C
Receptor plants
WMV
Uninfected
Inoculum source
CYSDV
CYSDV+WMV
D
EXPERIMENT 1
EXPERIMENT 2
Inoculum source
CYSDV
CYSDV+WMV
Receptor plants
Uninfected
WMV
Uninfected/WMV

S1 Figure