When viruses play team sports: mixed infections in plants

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Abstract

The pathological importance of mixed viral infections in plants might have been underestimated except for a few well-characterized synergistic combinations in certain crops. Considering that the host ranges of many viruses often overlap, and that most plant species can be infected by several unrelated viruses, it is not a surprise to find more than one virus simultaneously in the same plant. Furthermore, dispersal of the great majority of plant viruses relies in quite efficient transmission mechanisms mediated by vector organisms, mainly but not exclusively insects, that can contribute to the occurrence of multiple infections in the same plant. Recent works using different experimental approaches are showing that mixed viral infections can be remarkably frequent, up to a point that they could be considered the rule more than the exception. The purpose of the present review is to describe the impact of multiple infections, not only on the participating viruses themselves, but also on their vectors and on the common host. From this standpoint, mixed infections arise as complex events that involve several cross-interacting players, and consequently require a more general perspective than the analysis of single-virus/single-host approaches for a full understanding of their relevance.

[194 words]

Introduction

The diversity of viruses is extraordinary, with virtually all kind of cellular life forms being susceptible to be parasitized by many different viruses, and obviously, plants are not an exception (Wolf et al., 2018). Plant viruses are grouped in numerous families (about 30) and genera (more than 145), with an extraordinary diversity, what makes them one the most abundant group of pathogens (near 1500 species listed in the last report of the International Committee on Virus Taxonomy ICTV, available at https://talk.ictvonline.org/files/master-species-lists/m/msl/8266), being responsible for a large number of all known plant diseases. Nowadays, our knowledge of the virosphere is facing a major challenge to classify and accommodate many recently discovered viruses (Koonin and Dolja, 2018; Zhang et al., 2019). One of the reasons explaining why this enormous diversity was somehow overlooked until now comes from the fact that attention was mostly focused on agents of diseases, and in the case of

plants, only viruses causing economic losses in crops and ornamentally valuable species have been deeply studied for decades. A particularly interesting case of this tendency to bias our knowledge is the frequent existence of mixed infections in plants occurring when they invade the same host. This common situation was clearly underestimated in available studies, with not so many reported examples, despite the fact that many plant viruses are generalists and thus able to infect different plants, both crops and wild plants (Alexander et al., 2014; Elena et al., 2009). Furthermore, many vectors of plant viruses are polyphagous, or are organisms associated with multiple hosts, being capable to transmit more than one virus to the same plant (Syller, 2014). All these conditions argues in favor of a more frequent presence of multiple viruses in plants, and indeed metagenomics approaches using high-throughput next generation sequencing techniques have started to reveal the real extent of mixed infections between known and occasionally unknown viruses in the virome in plant hosts, either wild plant or crops (Mascia and Gallitelli, 2016; Rwahnih et al., 2009; Roossinck et al., 2010; Roossink et al., 2015). Since the field of plant virology in the coming years will be undoubtedly dominated by a much more frequent detection of mixed viral infections, we think it is timely to review what it is known about them, acknowledging that they can be quite complex, and that their establishment relies not only on the properties of each one of the actors involved (viruses, common host, and vector(s)), but also (maybe mainly) on the relationship and interactions between them. Moreover, other layers of complexity can be added to the multiple viral infections up to the ecological sphere, as the presence of natural enemies of the vectors, or the influence of non-viral pathogens in the host plant. However, those aspects are explored elsewhere and will not be addressed in the present review (Dáder et al., 2012; Jeger et al., 2011a, 2011b; Prager et al., 2015). Similarly, the importance of multi-pathogenic attacks (Abdullah et al., 2017) and even the combination of biotic and abiotic stresses in more holistic approaches (Saijo and Loo, 2019) are bringing the attention of researchers. Nevertheless, we are going to concentrate here in the combination of heterologous viruses, although it is important to keep in mind that these cases are only a subset of many other putative relationships between the numerous sources of stress that plant must face.

Some mixed viral infections known up to date are the origin of huge economic losses in important crops worldwide, as they cause a decline in plant vigor and

productivity. Logically, these cases have been studied in depth. As a first example, the corn or maize lethal necrosis (MLN) disease that affect one the main crop species in the world, causing economically devastating losses. This disease results from the combination of the Machlomovirus Maize chlorotic mottle virus (MCMV) and different members of the family *Potyviridae* (Mahuku et al., 2015; Redinbaugh and Stewart, 2018; Stewart et al., 2017; Scheets, 1998; Uyemoto et al., 1980).

The sweet potato viral disease (SPVD) is another good example of the consequences of a well-characterized mixed viral infection (Clark et al., 2012). Sweet potato is considered a crop essential for food security in extended areas, as it is a basic dietary component for large populations in China and Africa. One of the peculiarities of sweet potato compared to other crops relays in its rapid multiplication by vegetative propagation of cuttings, a rather beneficial agronomic trait that implies a high risk of accumulating pathogens, in particular viruses. Probably most marketable sweet potatoes are infected simultaneously by several viruses of the remarkable large list of over 30 different viruses that can infect this plant. The SPVD results from the synergistic interaction of isolates of the crinivirus *Sweet potato chlorotic stunt virus* (SPCSV) and the potyvirus *Sweet potato feathery mottle virus* (SPFMV), and can cause losses up to 90% of the crop yield (Karyeija et al., 2000). Another well-known viral combination in the same crop is the sweet potato severe mosaic disease (SPSMD), caused again by a synergism between SPCSV and the ipomovirus *Sweet potato mild mottle virus* (SPMMV) (Mukasa et al., 2003; Tugume et al., 2005).

Cassava is also another important staple food crop with a high incidence of multiple viral infections. Again, the vegetative propagation is often perpetuating infections by many viruses (Jacobson et al., 2018; Legg et al., 2015; Rey and Vanderschuren, 2017; Zinga et al., 2013). Recent research works are revealing some viral combinations particularly complex in this host (Abarshi et al., 2012; Carvajal-Yepes et al., 2014; Reddy et al., 2012).

A last example of a crop with important problems caused by multiple viruses is grapevine. In this case, the cultivation method relying on grafting and propagation of clonal varieties also can favor mixed infections. Currently, approximately 70 virus and virus-like agents have been documented in different grapevine cultivars worldwide (Martelli, 2014). The grapevine leafroll disease (GLD) is an extreme example of a multiple

viral infection, as it is a complex disease caused by the association of up to eleven *Grapevine leafroll-associated viruses* (GLRaVs) (Naidu et al., 2014, 2015).

Although all these examples, together with the above mentioned new knowledge that metagenomics approaches are bringing to the surface, reveal that mixed infections occur in a high frequency, a vast number of research efforts in plant virology still deal only with single infections. This might respond to the difficulties and constraints of working with several pathogens at the same time from a methodological standpoint, and also to the tendency to simplify questions, a logical first approach to any scientifically-oriented endeavor. Undoubtedly, the field of plant virology has been (and still is) particularly successful to provide novel and valuable knowledge that extends to the adoption of valid control measures against many viral diseases. But in most cases there are still complexities that remain to be elucidated, and we believe it is timely to recognize the importance of mixed viral infections. Here we try to summarize a selection of the most relevant literature available about the topic, with emphasis on the outcomes for each one of the players involved, and focusing mainly on plant viruses transmitted by insects, as they represent the major group of plant virus vectors. Nevertheless, we include a few examples of viruses disseminated through contact, or even several cases in which subviral agents participate in multiple infections. The review has been organized in three parts, referring to the impact of mixed infections to viruses, vectors and host plants. However, as a cautionary warning, this separation was adopted only to facilitate the classification of topics, and it is important to keep in mind that mixed infections often relies in the participation of the three mentioned players. Thus, the expected effects of mixed infections most probably will have consequences to each one of the players, as represented schematically in figure 1. A concluding remarks section includes a general discussion on the implications of mixed viral infections, addressing their importance for the ecology of viruses, and for the control strategies against viral pathogens.

Impact on viruses

During a mixed viral infection, the viruses involved may interact between them in a range that goes from synergism to neutralism to antagonism, having a direct impact into the plant host, and also potentially in their relationships with vector organisms. The

outcome would depend on the different aspects and parameters evaluated, such as the inside-host interactions between viruses, or the virus adaptation to the host (Mascia and Gallitelli, 2016; Syller, 2012). When there are no changes in viral accumulation (or their dynamics) during the mixed infection compared to the single infections with the individual viruses, the interaction is often called neutral, and the phenotypes (or symptoms in broad sense) can be the same as those observed in single infections, instead of the frequent occurrence of host affectations with additive symptom expression (Mascia and Gallitelli, 2016). On the other hand, if there are differences between the single and the mixed infections, the interactions can be classified as synergistic or antagonistic.

In a **synergistic interaction**, at least one of the viruses is beneficed by the presence of the other(s), what is manifested by an increase in the viral titer and/or pathogenicity (or other supra-level properties, like the capacity to disseminate efficiently by the vector organism) when compared to a single infection. The resulting symptoms are often more severe than those observed in single infections of the same viruses. There are several reasons that could explain, individually or combined, this effect (see figures 2 and 3 for schematic representations). It can be caused by an increase in replication; or by the possibility to invade new plant tissues; and even by the interference with plant defenses (particularly, but not necessarily unique, with post-transcriptional gene silencing mechanisms). Consequently, synergisms may have a high economic impact when occurring in crops because the resulting diseases are likely to become more severe. As an example we can cite the serious epidemic of cassava mosaic disease, caused by a mixed infection between isolates of *African cassava mosaic virus* (ACMV) and the *Uganda strain of East African cassava mosaic virus* (EACMV-UG), both from the genus *Begomovirus*, and leading to huge annual economic losses (Legg and Fauquet, 2004). Many other cases are mentioned in Table 1. A frequent combination of synergistic interaction involves a potyvirus with different unrelated viruses, in most cases clearly favoring the second (Pruss et al., 1997), with the involvement of the potent RNA silencing suppressor HC-Pro of potyviruses (Valli et al., 2018). Interestingly, an exception is the SPVD mentioned in the introduction, where the potyvirus partner SPFMV is the one that benefits from the presence of the crinivirus SPCSV (Karyeija et al., 2000), and in this case it was observed that the RNA silencing function of SPFMV was

shifted to a different gene product other than HC-Pro (Mingot et al., 2016; Untiveros et al., 2016).

There are some particularities in the interactions leading to conceptually clear synergisms that not always are easy to identify such as that, because they affect traits beyond the measurable viral load. For instance, one kind of synergism could be the so called helper dependence, in which one of the viruses involved is defective in one or more essential functions that in turn can be supplied by the other virus, the helper, acting as a provider or facilitator (Mascia and Gallitelli, 2016). In an extreme situation of this, one virus might become a sort of parasite of the other, such as in the interaction between Groundnut rosette virus (GRV), its satellite RNA (satRNA) and Groundnut rosette assistor virus (GRAV) that together causes the Groundnut rosette disease (GRD): GRV and its satRNA depend on the coat protein of GRAV to be encapsidated and transmitted by the aphid vector *Aphis craccivora* (Murant, 1990). Another similar situation is the vector-mediated transmission by complementation in trans, also named heterologous complementation, or transcomplementation. In certain viruses, such potyviruses and caulimoviruses, there are non-structural proteins denominated helper components that are needed for vector transmission, acting as specific molecular bridges between the virus particles and the vector (Pirone and Blanc, 1996). It has been observed in mixed infections involving potyviruses and other unrelated viruses that these last ones may benefit from the action of helper components to be transmitted. For instance, Potato aucuba mosaic virus (PAMV) is the only known member of the genus *Potexvirus* that can be transmitted by aphids thanks to transcomplementation when present in a mixed infected plant together with a potyvirus (Kassanis, 1961; Manoussopoulos, 2001).

Contrarily to the cases mentioned, in the frame of an **antagonistic interaction** the presence of more than one virus results detrimental for at least one of them, for instance with a measurable decrease in the viral load. In general it has been postulated that most of the described antagonisms need the interaction, direct or indirect, between proteins from the viruses involved in the mixed infection (DaPalma et al., 2010; Díaz-Muñoz, 2019). Comparing to synergistic interactions there are less examples of antagonism, as expected because attenuation instead exacerbation is more likely to remain unnoticed. Furthermore, in a theoretical situation of synergism plus antagonism

(as in the last column of the lower panel in figure 1), it is more likely that the outcome were described as synergistic for the enhanced virus, simply because not enough attention was paid to the alleviated partner. As well documented examples, we can mention the suggested antagonism between isolates of *Potato virus X* (PVX) and *Potato virus Y* (PVY) in tobacco (Ross, 1950), and the recent work in papaya with mixed infections between isolates of *Papaya ringspot virus* (PRSV) and *Papaya mosaic virus* (PapMV) (Chávez-Calvillo et al., 2016). Also we can mention the antagonism observed in tomato between isolates of *Tomato torrado virus* (ToTV) and *Pepino mosaic virus* (PepMV), where the severity of ToTV symptoms was unaffected, but differences in virus accumulation were observed in mix-infected plants with the titer of ToTV slightly increased in the early moments of the infection, whereas that of PepMV was strongly reduced at all time-points, including a pronounced decrease at later times. In this case, effects on the epidemiology of viruses have been reported (Gómez et al., 2009, 2010). As an additional case, in *Nicotiana benthamiana* plants an antagonism was reported between two tobamoviruses, *Hibiscus latent Singapore virus* (HLSV) and *Tobacco mosaic virus* (TMV), with decreased levels of HLSV if compared with a single infection, whereas those of TMV remained almost unaltered (Chen et al., 2012).

An interesting type of antagonistic interaction is the **cross-protection**, also known as homologous interference or super-infection exclusion that takes place when the presence of one virus prevents or interferes with a subsequent infection by another virus generally, but not exclusively, homologous (Syller and Grupa, 2016). In the context of agricultural activity, cross-protection can be very useful for biological control against viral diseases, mainly in cases where no genetic resistance mechanisms are known, or they cannot be easily implemented. In such scenario, avirulent or mild protecting viruses can be inoculated in crop plants to prevent aggressive viral infections in the field, acting as a kind of "vaccine" and reducing economic losses. Successful cases of cross-protection are the control of *Citrus tristeza virus* (CTV), *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ringspot virus* (PRSV) (Ziebell and Carr, 2010).

An important factor that influences the outcome of a mixed infection is at which moment the viruses arrive and infect the host. **Co-infection** is defined when the viruses simultaneously infect the host, arriving at roughly the same time, whereas **super-infection** refers to mixed infections where the viruses arrive at different moments

(Saldaña et al., 2003). In nature, occurrence of each one depends on the viral density and the number of healthy hosts available during the epidemic episode: at the beginning, with low viral densities and high number of uninfected plants, the occurrence of co-infections can be favored; later, as the diseases spread and the numbers of healthy host plants decrease gradually whereas the viral density increases, super-infections become more frequent (Moya et al., 2001; Saldaña et al., 2003). The moment of infection might influence virus evolution, through its impact on virus fitness, that increases in co-infections but does not differs from a single infection situation in super-infections (Moya et al., 2001). Also, the outcome of super-infections may be dramatically influenced by aspects as the order of infection with the viruses involved, and the time-frame between the infections, and for both co-infections and super-infections, differences in the capacity of viruses to invade or not certain tissues have been observed. Next, a series of examples are provided to illustrate the different outcomes that can result.

In the previously mentioned work in papaya, the outcome of the mixed infection was different when the plant was infected first with PapMV and then with PRSV, causing the antagonism mentioned, whereas if the order was the inverse, or if the viruses arrived simultaneously, the result was a synergism (Chávez-Calvillo et al., 2016). Similarly, the example given above of an antagonistic interaction between HLSV and TMV occurred in the frame of a co-infection, but authors mentioned that in a super-infection the presence of HLSV protected the host against a late arrival of TMV, thus acting in practical terms as a cross-protection event (Chen et al., 2012). Another example of conditional outcome derives from one of the first synergisms described: tobacco plants infected with isolates of *Potato virus Y* (PVY) and *Potato virus X* (PVX) (Goodman and Ross, 1974; Smith, 1931; Vance, 1991). When PVX was inoculated on a tobacco previously infected with PVY, the levels of accumulation of PVX increased considerably compared with the single infected control. However, this synergism was compromised if the time between the infections with the two viruses was higher than 24 hours, and some years later, it was demonstrated that the silencing suppressor of the potyvirus PVY (HC-Pro) was one of the factors involved in the outcome of the synergism (González-Jara et al., 2005). As a final example of the different relationships occurring between viruses in mixed infections, we can cite a work in tomato plants involving mixed infections of *Tomato*

chlorosis virus (ToCV) and *Tomato spotted wilt virus* (TSWV) that resulted in a synergism that even can end with the death of susceptible plants. In those plants there was a high increase of ToCV accumulation whereas the levels of TSWV remained unaltered. Co-infection of TSWV-resistant plants with ToCV did not alter the resistance behavior of the plants, however, and most interestingly, when the resistant plants were challenged in a super-infection, with ToCV arriving before TSWV, the resistance was lost. This observation suggested that ToCV might need a certain time to affect the defense response in the resistant plants, such as requiring enough time to express some proteins acting as suppressors of RNA silencing, or to reach a certain accumulation level (García-Cano et al., 2006). However, the interference with the plant defense mechanisms by viral proteins is probably not the only component that determines if an interaction will be synergic or antagonistic: some proteins may facilitate the replication, inter-cellular movement and/or the inside-host spread of the other virus (Elena et al., 2014).

Mixed infections by the same combination of viruses but in different host plants may also lead to different outcomes, for instance in the virus accumulation levels, the affectations to the host, and the transmission by vector organisms. We will consider these aspects in different sections, but it is relevant to stress that virus-virus interactions are highly dependent of the environment (i.e, the common host) where they are present together. As an example, we can cite a mixed infection between two criniviruses, *Tomato chlorosis virus* (ToCV), and *Tomato infectious chlorosis virus* (TICV) in two different host plants: *Physalis wrightii* and *Nicotiana benthamiana* (Wintermantel et al., 2008). Compared to single infections, the titer of both viruses decreased in mixed infections of *P. wrightii,* whereas in *N. benthamiana* TICV titers increased and ToCV decreased. Vector transmission efficiencies by *Trialeurodes vaporariorum* were directly correlated to the accumulation levels of both viruses, with more transmission of TICV from mixed infected plants (this case fits in the proposed effect of the last column in figure 1). Even more interestingly, the vector *Trialeurodes abutilonea* was able to transmit TICV from mixed infected plants, even if it was described as non-vector for this virus alone (Duffus et al., 1996). This suggests that a complementation for transmission might have taken place, with ToCV providing one or more factors necessary for the *T. abutilonea*–TICV association and subsequent transmission, in a process independent of host factors as the virus was transmitted from both hosts (Wintermantel et al., 2008).

The interaction between viruses inside a host cell (as represented schematically in figure 2) can be highly influenced by the **multiplicity of infection (MOI**) that indicates the number of viral genomes present in the same cell (Gutiérrez et al., 2010). There are some indications that the MOI is controlled by virus-encoded functions, such as the suppression of RNA silencing mechanisms (Donaire et al., 2016). In mixed infections of *N. benthamiana* with two genotypes of TMV, it was observed that the MOI was higher at initial stages, and then changed (Gonzalez-Jara et al., 2009). Determining the values of MOI and how are they regulated can help to decipher the relationships between dynamics of virus populations and the control of genome copy numbers (Gutiérrez et al., 2012). Beyond the cellular level but closely related with this topic, is the **tissue tropism** or the specificity of each virus to infect only some cell types or tissues (Mascia and Gallitelli, 2016). During infection of a host plant, viruses could be found in different cells, from all types, to only those found in vascular tissues, or exhibit for instance restrictions to enter meristems or invade seeds. In the case of mixed viral infections, we could expect modifications and changes in host responses that might condition the pathology of the viruses involved. These responses, for instance, might alter the tissue tropism, a phenomenon observed mainly in phloem restricted viruses (Mascia and Gallitelli, 2016). In single infections of *Nicotiana benthamiana* plants, Tomato yellow spot virus (ToYSV) localizes in mesophyll cells, while Tomato rugose mosaic virus (ToRMV) is restricted to the phloem. However, when the plant is infected by both viruses, the localization of ToRMV changes, and the virus becomes able to invade mesophyll cells as well (Alves-Júnior et al., 2009). Also in *N. benthamiana*, the mixed infections between isolates of the potyvirus *Potato virus A* (PVA) and the luteovirus *Potato leaf-roll virus* (PLRV) allows the last one to infect all cell types in leaves, whereas in single infection it is restricted to the phloem. A possible explanation would be that movement proteins from PVA could complement movement deficiencies of PLRV (Savenkov and Valkonen, 2001). Similarly, in *Nicotiana clevelandii* and *N. benthamiana* plants mix-infected with PLRV and the umbravirus *Pea enation mosaic virus-2* (PEMV-2), PLRV gained the capacity to be mechanically transmitted as it could be located in mesophyll cells. Again, a modification in the cell-to-cell movement and the interference with the host RNA silencing mechanisms by the PEMV-2 may explain this observation (Ryabov et al., 2001). Additional examples of similar outcomes are: the mixed infection

by the begomovirus *Abutilon mosaic virus* (AbMV) and either the potyvirus *Cowpea aphid-borne mosaic virus* (CABMV) or the tombusvirus *Artichoke mottled crinkle virus* (AMCV), that provoked occasionally the localization of AbMV in other tissues than the phloem (Sardo et al., 2011), and the case of a movement-deficient strain of CMV in zucchini squash assisted in systemic spread by co-infection with a strain of the potyvirus *Zucchini yellow mosaic virus* (ZYMV) (Choi et al., 2002).

Besides these modifications in tissue tropism and virus distribution within the host, in some cases the mixed viral infection can even alter the host range. An example is the case of two sobemoviruses, *Southern cowpea mosaic virus* (SCPMV) and *Southern bean mosaic virus* (SBMV), that can infect *Vigna unguiculata* (cowpea) and *Phaseolus vulgaris* (common bean) respectively but not reciprocally. Even if the genomic RNA of SCPMV can replicate in bean cells, it cannot assembly viral particles. However, in co-infection with SBMV it was observed that SCPMV accumulated in an encapsidated form both in inoculated and systemic bean leaves. Nevertheless, when the experiments were performed inversely, authors observed that SCPMV did not complement the host range restriction of SBMV in cowpea. Thus, the presence of SBMV modified the host range of SCPMV allowing it to infect bean, but SCPMV did not alter the host range of SBMV, as it was unable to infect cowpea (Hacker and Fowler, 2000).

Impact on vectors

Vectors are key players in many virus life-cycles, as a majority of plant viruses rely on them for dispersion and existence along time. The virus transmission process by a vector organism involves host-finding, acquisition (often through feeding, as in the case of phytophagous vectors), transport, and finally inoculation or delivery of virus to a new host plant. As the mobile partners in a plant-virus-plant interaction leading to dispersal of infection, the vectors can show preferences for different host plants, where to acquire first, and to inoculate later, and that will condition the virus spread process. The importance of mixed viral infections in the aphid-transmission of plant viruses was already reported in a review article rich in examples (Rochow, 1972), dealing with points like the behavior of vectors, or even recombination and transcapsidation events. All these key concepts might have an impact on virus evolution.

Additionally, some recent works are showing that the vector behavior and/or physiology can be influenced by changes in the plant induced by viruses, what can in turn condition the spread and epidemiology of the viruses (Bosque-Pérez and Eigenbrode, 2011; Bak et al., 2017). Therefore, in the case of mixed infections, the possibility of manipulative strategies needs to be considered. For instance, a surprisingly example of vector manipulation by viruses was described in rice plants, and reflects the relationships of two reoviruses, *Southern rice black-streaked dwarf virus* (SRBSDV) and *Rice ragged stunt virus* (RRSV) with their insect vectors: the white-backed planthopper (WBPH, *Sogatella furcifera*) transmits SRBSDV, while the brown planthopper (BPH, *Nilaparvata lugens*) transmit RRSV (Wang et al., 2014). Preference assays using a Y-shape olfactometer-based device demonstrated that plants infected with SRBSDV modified the behavior not only of its vector WBPH, but also of the non-vector BPH. Virus-free WBPH were more attracted to infected plants than viruliferous insects, and viruliferous insects were more attracted to healthy plants than to infected ones: this result is consistent with manipulation of the vector to increase virus spread through virus-induced host modifications. Interestingly, the preference assays with BPH, a non-vector of SRBSDV, showed only preference of rice plants infected with SRBSDV over a healthy plant, when they were viruliferous for RRSV, therefore favoring the occurrence of mixed infections. Further experiments are needed to elucidate the mechanisms involved in this behavior, and explain why viruses may manipulate host selection in order to favor the existence of mixed infections.

The apparent manipulation of the vector by the virus often triggers an increase in the transmission rates, and consequently in the virus spread, what assures the existence of the virus species in the long term. In other words, virus acquisition will be increased by vector attraction to infected plants, and transmission will be improved by attraction to healthy plants where the virus can be inoculated. This concept is known as the **vector manipulation hypothesis,** and it was proposed to explain the different approaches employed by the pathogen to enhance its dissemination (Ingwell et al., 2012). The virus may influence directly the vector and modify its behavior, mainly in viruses that are transmitted in a persistent way while the virus is inside the body of the vector, as in the case of the thrips *Frankliniella occidentalis* (Pergande) feeding behavior altered when infected by the tospovirus *Tomato spotted wilt virus* (TSWV): infected

males feed more than uninfected ones, thus increasing the possibility of virus inoculation (Stafford et al., 2011). This proposed general concept is still awaiting further studies to verify how common it is, and especially it will be interesting to measure transmission efficiencies and vector behavior parameters in mixed infections. For instance, a recent research effort in our laboratory has incorporated measurements of aphid feeding behavior through Electrical Penetration Graphs (EPG), finding that a particular sub-phase related with virus acquisition was prolonged by aphids feeding on melon plants co-infected by the potyvirus *Watermelon mosaic virus* (WMV) and the crinivirus *Cucurbit yellow stunting disorder virus* (CYSDV), compared to single infected controls. Interestingly, this behavior might compensate a reduction in WMV load, thus assuring efficient transmission of the potyvirus (Domingo-Calap, Moreno, Diaz-Pendón, Moreno, Fereres and López-Moya, submitted).

Also, viruses may influence indirectly the vectors by modifications in the host infected plant that will have an impact on the vector (Dietzgen et al., 2016). The manipulations exerted by the viruses often result in a positive or neutral effect on the transmission by vectors, and are similar between different viruses depending on their mode of transmission (described in more detail in the next section) (Mauck et al., 2012). As an example we can cite the mixed infection of *Potato virus Y* (PVY) and *Potato leafroll virus* (PLRV) in potato plants that altered the performance of their most effective vectors, the aphids *Myzus persicae* and *Macrosiphum euphorbiae*. It was seen that for both vectors the fecundity increased in mixed infected potato plants, as well as their preference for those plants (Srinivasan and Alvarez, 2007). According to the authors, this behavior could be explained, at least in part, by an increase of sugars and amino acids in the phloem as a result of an inhibited phloem transport in mixed-compared to single-infected plants. Additionally, it is likely that, when together, both viruses manipulate somehow the plant to make it more attractive to the insects by visual and olfactory clues. As mixed infections of those viruses are quite frequent in potato plants, the quality of hosts and the behavior of vectors during this interaction could have important epidemiological repercussions (Chatzivassiliou et al., 2008). Another example of this kind of multiple-level interactions involving vectors is provided by the above mentioned mixed infection of WMV and CYSDV in melon: our work showed a late time recovery phenotype of the mix-infected plants after a first onset of strong synergistic damage,

resulting in a better condition and development, and thus resulting in more foliage surface available for acquisition of the viruses by vectors (Domingo-Calap, Moreno, Diaz-Pendón, Moreno, Fereres and López-Moya, submitted).

An interesting feature related with mixed infections is that some viruses gain the ability to be transmitted by vectors that are not their natural vectors when they are infecting alone a susceptible plant. As we already mentioned, the case of PAMV showed that a virus belonging to a taxonomic group, potexviruses, characterized by only contact dissemination, can acquire an efficient vector-mediated transmissibility during mix-infection with a potyvirus. The key molecular feature here was the presence of a conserved motif DAG, typical of potyviruses, in the potexvirus, as demonstrated in an elegant work through mutagenesis of the PVX CP to engineer the same aphid-transmissibility feature (Baulcombe et al., 1993).

Acquisition of new vector capacities was seen also in mixed infections by viruses from the family *Luteoviridae,* or in co-infections of unrelated viruses with a luteovirus. The general phenomenon can be named **genomic masking** or **transcapsidation**, and basically consist in a viral genome being encapsidated by the capside of other virus present at the same time in the plant, generating a sort of chimeric new entity with shared properties (represented in figure 2 by orange+blue or blue+orange virus particles). Examples of this were observed in mixed infected plants in which a virus gained transmissibility by different species of vectors. For instance, the aphid *Sitobion avenae* is a good vector of the MAV strain of the luteovirus *Barley yellow dwarf virus* (BYDV-MAV) in single infections, while the aphid *Rhopalosiphon padi* specifically transmit the strain RPV of the polerovirus *Cereal yellow dwarf virus* (CYDV-RPV). However, in plants mix-infected by both viruses, BYDV-MAV became transmissible also by *R. padi*, because the RNA of BYDV-MAV was encapsidated inside CYDV-RPV capsids, what conferred the transmissibility by *R. padi* (Creamer and Falk, 1990; Rochow, 1970). Similar results were described in oat and wheat mixed infected by two strains of BYDV (BYDV-MAV and BYDV-PAV), where besides transcapsidation, alterations in transmission specificity and synergism in symptoms where observed (Baltenberger et al., 1987; Creamer and Falk, 1990).

The transcapsidation phenomenon is not restricted to luteovirus, and was also observed in viruses of the genus *Umbravirus* and some luteovirus-associated RNAs

(specific sub-viral RNA replicons) that do not encode for a capsid protein. Their aphid-transmission capacity is absolutely dependent on the transcapsidation with other co-infecting luteovirus (Syller, 2000; Casteel and Falk, 2016).

It is worth mentioning here a related situation known as **heteroencapsidation**, where a virus genome can acquire partially the properties of the particles of another one through heterologous encapsidation. For instance, electron microscopy was used to demonstrate that a non-aphid-transmissible isolate of the potyvirus *Zucchini yellow mosaic virus* (ZYMV) can produce heteroencapsidated particles in mixed infection with the potyvirus *Papaya ring spot virus* (PRSV), and thus being efficiently transmitted by aphids (Bourdin and Lecoq, 1991). Other well documented examples involves whitefly-transmitted begomovirus from the family *Geminiviridae*, where the frequent recombinations that occurs during mixed infections are one of the main sources of variability in this genus. To cite one case of heteroencapsidation, in a mixed infection between *Tomato leaf curl Palampur virus* (ToLCPalV) with *Tomato leaf curl New Delhi virus* (ToLCNDV) in tomato and cucurbits, it was demonstrate the whitefly transmission of recombinants (Kanakala et al., 2013). From an epidemiological point of view, these transcapsidation events occurring in the frame of a mixed viral infection can have a huge influence on the spread of a given virus, allowing it to be putatively transmitted by new vectors and arriving to new hosts, and thus contributing to speed up speciation events. In fact, recent taxonomical proposals based in the similarities of viral polymerases (Wolf et al., 2018) argues in favor of evolution processes involving capture and switching of capsid genes, with mixed infections representing a key step to explain how these kind of events could happen.

As mentioned, viruses are able to manipulate the host plant using different strategies that modify the vector biology, fitness and behavior, and consequently might have an impact on virus transmission and disease dynamics (Fereres and Moreno, 2009). This is known as **indirect mutualism** and confer advantages to the vector that would result in an increase of virus transmission and, consequently spread (Li et al., 2014). Such modifications in the host mainly rely on nutritional changes and on a manipulation of the defense responses, making the plant more attractive and suitable for the vector (Bak et al., 2017; Casteel and Falk, 2016; Casteel et al., 2014; Casteel et al., 2015; Luan et al., 2014; Li et al., 2014). This process is not random and the host alterations are strongly

related with the **transmission mode** of each virus (Mauck et al., 2012; Mauck, 2016). Briefly, plant viruses transmitted by insects are classified in two major groups, each one sub-divided in two more groups, according to some parameters needed for acquisition, retention and inoculation. The time duration of those periods are related with the tissue tropism and the localization inside the host, and on the other side with the insect. In the first mode of transmission, called “noncirculative” the virus does not need to enter the insect body and the virion particles are retained in the stylet or foregut region of the vector. If the retention of the virus in the vector is brief (seconds to minutes), viruses are classified in the sub-group following a “nonpersistent” transmission mode, and if it takes minutes to hours they are classified as “semipersistent”. On the other hand, the viruses that cross cellular barriers and reach the body cavity of the insect to end up in the salivary glands are named “circulative” or “persistent”. If they only circulate inside the vector are classified as “nonpropagative”, and if there is multiplication inside the insect they are called “propagative” (Kaur et al., 2016; Casteel and Falk, 2016; Mauck et al., 2018). Thus, a host plant that is modified to be attractive (by visual and olfactory clues) even if its quality as feed for a phytophagous insect is reduced, will be well adapted for a more efficient transmission of a nonpersistent virus: the vector is first attracted to the infected plant, but when it feeds on it (and thus acquire the virus in a brief lapsus of time), the insect does not receive enough gratification to colonize and remain in the plant, and then goes to another plant carrying the virus. On the other hand, viruses that are transmitted in a semipersistent or persistent manner will get benefit if they manipulate the host to promote long-term feeding, thus favoring acquisition of the virus (Mauck et al., 2012). These attraction-repulsion processes can be quite dynamic, and some works have described that feeding preferences and behavior of vectors could change after virus acquisition (Ingwell et al., 2012; Moreno-Delafuente et al., 2013; Rajabaskar et al., 2014). Collectively, all these studies suggest that viruses might have been favored by natural selection to adopt strategies to enhance its transmissibility/dispersion in the context of mixed infections, using for that purpose many different leverages, from altering vector behavior via controlling vector interactions with their host plants, to other mechanisms controlling transmissibility. The last column in the upper panel of figure 1 tries to illustrate that vectors can be essential parts of the pay-off mechanisms achieved and favored during mixed infections.

Impact on plants

The host plant is the organism to be preserved in agricultural production, and consequently the attention about the outcomes of mixed viral infections, considered as pathogenic attacks, has been mainly focused on the synergistic or antagonistic responses. We have already mentioned those along the previous sections, and an extensive catalogue of known cases is provided in Tables 1 and 2, with comments primarily dedicated to provide information on how the different host plants are impacted by the simultaneous presence of the viruses mentioned. In this section we will add some additional comments about a few cases that illustrate the wide variety of effects caused by the presence of multiple viruses in a host plant, in particular effects that go beyond the expected additive response to the individual viruses.

An example of host modification induced by a mixed infection can be found in grapes of plants infected with isolates of *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine virus A* (GVA), and *Rupestris stem pitting-associated virus* (RSPaV) (Giribaldi et al., 2011). A proteomic analysis of samples from such mixed infected plants revealed several changes in the host, with an expected major influence in the oxidative stress responses (in the fruit skin), and in the cell structure (in the pulp). The oxidative stress responses can be induced by biotic stresses, including several pathogens (Demidchik, 2015; Muthamilarasan and Prasad, 2013). On the other hand, the alterations on cell structure might be linked to the cytoskeleton and the movement of viruses (Lucas, 2006; Henry et al., 2006).

Another key element in the responses of plants towards viruses is the RNA silencing or post-transcriptional gene silencing pathways. The RNA silencing is a mechanism present in all eukaryotes that regulates many essential processes, such as chromatin modification, DNA methylation, and transposon activity among many others, and relies in the activity of several types of the so called “small RNAs”. In plants, it is also one of the most important antiviral defense systems induced in response to both RNA and DNA viruses (Baulcombe, 2004; Csorba et al., 2009; Wang et al., 2012; Vaucheret, 2006). We have already anticipated in previous sections the importance of the mechanisms of RNA silencing for the outcome of mixed infections. In fact, all plant viruses encode at least one RNA silencing suppressor (Csorba et al., 2015), and therefore

during mixed infections we should consider that at least two of them might be acting simultaneously. As indicated, the numerous cases of potyviruses contributing to synergistic responses during multiple infections have been associated with the strong activity of HC-Pro as RNA silencing suppressor (Pruss et al., 1997). Also within the same family *Potyviridae*, a deep-sequencing study carried out in wheat showed that the synergistic mixed infection caused by the combination of the tritimovirus *Wheat streak mosaic virus* (WSMV) and the poacevirus *Triticum mosaic virus* (TriMV) caused major changes in the endogenous small RNA profile of the host plant, with an expected impact in the defense responses that differs between single and mixed infections (Tatineni et al., 2014).

The influence of the host plant in the outcome of viral mixed infections has been mentioned already, and there are some additional examples in table 1. It has also been reported that in some synergistic viral combinations, the outcome varies depending on the host plant cultivar. For instance, the previously cited synergistic interaction between WSMV and TriMV induced different disease outcomes depending on the wheat cultivar (Tatineni et al., 2010). Other examples illustrate the influence of the cultivar in the outcome of the mixed infection, as the co-infections between (i) *Tomato chlorosis virus* (ToCV) and *Tomato infectious chlorosis virus* (TICV); (ii) *Potato virus X* (PVX) and *Potato virus Y* (PVY) or *Tobacco etch virus* (TEV); and (iii) *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV) (Wintermantel et al., 2008; Gonzalez-Jara et al., 2004; Mendez-Lozano et al., 2003). All these cases probably reflect differences in the RNA silencing and antiviral responses of the host in a way that might play important roles not only in the establishment but also in the development of the disease. As a consequence, the management of viral diseases through control measures would need to consider the effects of mixed infections. Particularly worrisome would be the risk of compromising genetic resistance, as in the case of ToCV and TSWV already discussed (García-Cano et al., 2006). Further investigations will be useful to determine if other resistances can be as well compromised by the combined actions of viruses during mixed infections.

Concluding remarks

In this review we have pointed out several aspects related to mixed viral infections that support their great relevance not only for pathology but also for plant

virus ecology and epidemiology. These two later disciplines are partially related but deserve specific attention. When talking about ecology, we refer to the interactions of the virus populations with the environment and its components, whereas the term epidemiology is more linked to the disease caused, and how it is spread in the frame of complex associations between the viruses and the host plants, passing through the decisive participation of vectors (Jones, 2014). Understanding why and how pathogenic viruses can spread in a given ecosystem is the main challenge of the epidemiology, but in a mixed infection scenario, the factors controlling the spread become more complex, and it is absolutely essential to understand them to build effective strategies to control the frequent diseases caused by multiple viral infections.

An important work front to fight against the negative effects of the mixed infections is to interfere with the transmission by vectors. As mentioned before, the dispersion of the majority of plant viruses rely on the action of vectors, on one hand as an efficient way to overcome the sessile nature of the plants, and on the other hand to provide effective means to cross the strong cell wall and deliver viruses into the susceptible parts of the cell. Usually, a viral infection begins with the specific interaction of the vector and the virus, mediated by proteins from both of them (Gutiérrez et al., 2013). As discussed in this review, in the frame of a mixed infection, affectations and changes both in vectors and in viruses may occur and have an influence on the transmission. Thus, understanding in detail those modifications may be essential to interfere with key steps in the interaction vector-virus and interrupt the viral cycle, limiting the spread of the infection (Whitfield and Rotenberg, 2015).

The knowledge generated by the study of mixed viral infections could become a rich source of useful information, for instance to design effective control measures, or even to engineer resistant plants against viruses. As mentioned in the **"impact on viruses" section**, one example of this later strategy is represented by cross-protection, based in the antagonism between viruses, and consisting in the activation of plant defenses (such as the mentioned RNA silencing mechanisms) by the first virus, thus preventing or alleviating the damaging infection by the second. This strategy has been successfully applied to protect plants with a high economical interest like citrus, cucurbits and papaya, through inoculation of plants with a mild strain of a virus, preventing the infection by a more virulent strain in the field (Ziebell and Carr, 2010).

However, we must recognize that the complexities of the interactions require more investigations. Indeed, when designing strategies to engineer resistant plants against viruses, it is worth taking into account the impact that possible mixed infections in nature can have. One relevant example reports the expression of an artificial microRNA (amiRNA) targeting the viral RNA-silencing suppressor HC-Pro of *Turnip mosaic virus* (TuMV) in *Arabidopsis thaliana* transgenic plants. High levels of resistance against the infection by the homologous virus were observed, however, if the plant was previously infected by *Tobacco rattle virus* (TRV), *Cauliflower mosaic virus* (CaMV) or *Cucumber mosaic virus* (CMV), the resistance was lost and TuMV has able to infect the plant in the frame of a mixed infection with one of the other mentioned viruses (Martínez et al., 2013).

The relationship between viruses in the frame of a mixed infection can have a clear impact on the evolution of each virus involved, as the competition for the host resources influences directly the fitness of viruses. This competition can be specified at many levels, some of them depicted schematically in figure 2, and others still waiting to be analyzed in depth. Among new aspects that might require attention are for instance those occurring due to the different mechanisms of translation, or the conflicting situation prompted by the different codon usages of the viral partners and the host (Adams and Antoniw, 2004; Belalov and Lukashev, 2013; Miras et al., 2017). As a result of all these competitions, mixed infections must be considered as another key factor that influences virus evolution, and indeed recent studies have proposed models to predict the long-term evolution of viruses in the frame of mixed infections (Alizon et al., 2013; Escriu et al., 2003; Elena et al., 2014; Tollenaere et al., 2016). Virulence increases can be predicted due to the competitive advantage of virulent parasites, as the mixed infections are prone to alter the immune defense and cause phenotypic changes in the host. Thus, a higher virulence can be consequence of the competition for resources during a mixed infection (Choisy and de Roode, 2010).

To confirm these predictions and incorporate the derived knowledge into strategies of virus control, more research will be highly desirable. The presence of more than one virus in the plant also makes possible genetic exchanges between them, and even the formation of new hybrid viruses containing parts from the genomes of the distinct viruses, through reassortments or recombinations (DaPalma et al., 2010).

Eventually, speciation events might take place, therefore contributing to the expansion of the global virome. Moreover, it seems that the choice of a given host by a given virus is not random, and it is driven by some still unknown mechanisms, what reinforces the importance of multiple infections from an evolutionary and epidemiologic point of view (Malpica et al., 2006).

Finally, recognizing the importance of mixed infections could be also instrumental for a safer biotechnological exploitation of plant viruses (Psin et al. 2019). For instance, we must take into account that when using a viral vector to express a protein or a valuable product in a susceptible plant that can be infected with another virus, we are dealing with a particular scenario of mixed infection, and thus we must be aware of the possible consequences this can have to the pathosystem under study. On the other hand, the examples existing in nature where plants are capable to tolerate, survive and even thrive while supporting a multiple and highly variable virome, as in the case of sweet potatoes, deserve attention from researchers, since understanding how this particular plants can stand the multiple infections might provide clues for dealing with damage caused by viral pathogens in other crops. Genomic studies might give clues on key traits that could eventually provide wide range tolerances to multiple stresses.

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Table 1. Examples of mixed viral infections in plants caused by pairs of viruses

Virus 1 [a]	Virus 2 [b]	Host plants [c]	Comments	References
Abutilon mosaic virus (AbMV) (Begomovirus)	Cucumber mosaic virus (CMV) (Cucumovirus)	*Nicotiana benthamiana* and Tomato (*Solanum lycopersicum*)	Synergism between RNA and ssDNA viruses. Change in the tissue tropism of AbMV (no phloem-limitation)	Wege and Siegmund, 2007
Abutilon mosaic virus (AbMV) (Begomovirus)	Tomato mosaic virus (ToMV) (Tobamovirus)	*Nicotiana benthamiana* and Tomato (*Solanum lycopersicum*)	Negative effect of ToMV on AbMV accumulation and a synergistic enhancement in pathogenicity	Pohl and Wege, 2007
African cassava mosaic virus (ACMV) (isolate ACMV-CM) (Geminivirus)	East African cassava mosaic Cameroon virus (EACMCV) (Geminivirus)	*Nicotiana benthamiana* and *Nicotiana tabacum* (protoplasts)	Synergism with increase in symptom severity and viral accumulation of both viruses. PTGS involved in the interaction	Vanitharani et al., 2004
African cassava mosaic virus (ACMV) (Geminivirus)	Uganda strain of East African cassava mosaic virus (EACMV-UG) (Geminivirus)	Cassava (*Manihot esculenta*)	Synergism and cause of the Cassava mosaic disease (CMD). Field survey in Central African Republic	Zinga et al., 2013
Bean pod mottle virus (BPMV) (Comoviridae)	Soybean mosaic virus (SMV) (Potyviridae)	Soybean (*Glycine max*)	Synergism. Effects on biochemical traits of the plant, and on behaviour and performance of vectors	Peñaflor et al., 2016; Lee and Ross, 1972
Bean pod mottle virus (BPMV) (Comovirus)	Bean yellow mosaic virus (BYMV) or Peanut mottle virus (PMV) (Potyvirus)	Soybean (*Glycine max*)	Neutralism	Anjos et al., 1992
Bean yellow mosaic virus (BYMV) (Potyvirus)	Pea mosaic virus (PMV) (Potyvirus)	Pea (*Pisum sativum*)	A non-aphid-transmissible isolate of BYMV depends on mixed infection with PMV to be transmitted	Hobbs and McLaughlin, 1990
Beet mild yellowing virus (BMYV) (Polerovirus)	Groundnut rosette virus (GRV) (Umbravirus)	*Nicotiana clevelandii; Nicotiana benthamiana*	BMYV became mechanically transmissible	Mayo et al., 2000
Cassava brown streak virus (CBSV) (Ipomovirus)	Ugandan cassava brown streak virus (UCBSV) (Ipomovirus)	Cassava (*Manihot esculenta*)	Synergism and cause of the cassava brown streak disease (CBSD)	Jacobson et al., 2018; Rey and Vanderschuren, 2017
Cowpea chlorotic mottle virus (CCMV) (Bromovirus)	Soybean mosaic virus (SMV) or Peanut mottle virus (PMV) (Potyvirus) or Tobacco ringspot virus (TRSV) (Secoviridae)	Soybean (*Glycine max*)	Effect of mix infections on plant characteristics and chemical composition. No synergism observed	Demski and Jellu, 1975

Cucumber mosaic cucumovirus (CMV) (Cucumovirus)	Blackeye cowpea mosaic potyvirus (BICMV) (Potyvirus)	Cowpea (*Vigna unguiculata*), *Cassia obtusifolia, Cucumis sativus, Cucurbita pepo, Glycine max, Nicotiana tabacum, Phaseolus lunatus, Phaseolus vulgaris*	Synergism and cause of the Cowpea stunt disease. In greenhouse can reduce yield of cowpea. Aphids can transmit both viruses from mixed infected plants	Pio-Ribeiro et al., 1978
Cucumber mosaic cucumovirus (CMV) (Cucumovirus)	Blackeye cowpea mosaic potyvirus (BICMV) (Potyvirus)	Cowpea (*Vigna unguiculata*)	Synergism. Severe symptoms might not be only due to an increase on CMV levels	Anderson et al., 1996
Cucumber mosaic virus (CMV) (Cucumovirus)	Turnip mosaic virus (TuMV) (Potyvirus)	*Nicotiana benthamiana*	Synergism. Analysis of the CMV 2b protein function in the mixed infection	Takeshita et al., 2012
Cucumber mosaic virus (CMV) (Cucumovirus)	Pepper mottle virus (PepMoV) (Potyvirus)	Pepper (*Capsicum annuum*)	Synergism. Detailed evaluation of symptom severity and statistical proof of synergy	Murphy and Bowen, 2006
Cucumber mosaic virus (CMV) (Fny-CMV and LS-CMV) (Cucumovirus)	Watermelon mosaic virus (WMV) (Potyvirus)	Zucchini squash (*Cucurbita pepo*)	Synergism with a high increase of CMV levels	Wang et al., 2002
Cucumber mosaic virus (CMV) (Fny-CMV and LS-CMV) (Cucumovirus)	Zucchini yellow mosaic virus (ZYMV) (Potyvirus)	Zucchini squash (*Cucurbita pepo*) and melon (*Cucumis melo*)	Strong synergistic pathological responses	Wang et al., 2002
Cucumber mosaic virus (CMV) (Fny-CMV) (Cucumovirus)	Zucchini yellow mosaic virus (ZYMV) (Potyvirus)	Cucumber (*Cucumis sativus*) and bottle gourd (*Lagenaria siceraria*)	Quantification of synergism by real-time RT-PCR	Zeng et al., 2007
Cucumber mosaic virus (CMV) (impaired in long-distance, strain M-CMV) (Cucumovirus)	Zucchini yellow mosaic virus (ZYMV) (strains ZYMV-A and -AG) (Potyvirus)	Zucchini squash (*Cucurbita pepo*)	Synergism in pathology and gain of long-distance movement for M-CMV	Choi et al., 2002
Lettuce infectious yellows virus (LIYV) (Crinivirus)	Turnip mosaic virus (TuMV) (Potyvirus)	*Nicotiana benthamiana*	Synergistic interaction with enhanced accumulation of LIYV, mediated by the entire TuMV or only the TuMV P1/HC-Pro sequence	Wang et al., 2009b

Maize chlorotic mottle virus (MCMV) (Machlomovirus)	Maize dwarf mosaic virus A (MDMV-A)	Corn (*Zea mays*), sorghum(*Sorghum vulgare*) and wheat (*Triticum aestivum*)	Synergism and cause of the corn lethal necrosis disease (CLND). Field survey in Kansas, US	Uyemoto et al., 1980
Maize chlorotic mottle virus (MCMV) (Machlomovirus)	Wheat streak mosaic (WSMV) (Rymovirus)	Corn (*Zea mays*), sorghum(*Sorghum vulgare*) and wheat (*Triticum aestivum*)	Synergism and cause of the corn lethal necrosis disease (CLND). Field survey in Kansas, US	Uyemoto et al., 1980
Maize chlorotic mottle virus (MCMV) (Machlomovirus)	Sugar cane mosaic Sugar cane mosaic virus-MD-B (SCMV-MD-B (formerly known as Maize dwarf mosaic virus MDMV-B) (Potyvirus)	Maize (*Zea mays*)	Synergism with high increase of MCMV levels. Decrease in chlorophyll levels and in the ratio of chloroplast to cytoplasmic rRNA	Goldberg and Brakke, 1987
Maize chlorotic mottle virus (MCMV) (Machlomovirus)	Wheat streak mosaic (WSMV) (Rymovirus)	Maize (*Zea mays*)	Synergism and cause of corn lethal necrosis (CLN, nowadays called maize lethal necrosis). WSMV infections were enhanced by MCMV and more pronounced at higher temperatures	Scheets, 1998
Maize chlorotic mottle virus (MCMV) (Machlomovirus)	Sugarcane mosaic virus (SCMV), or Maize dwarfmosaic virus (MDMV), or Johnsongrass mosaic virus (JGMV) (all Potyvirus)	Maize (*Zea mays*)	Synergism caused by co-infection of MCMV with anyone of the indicated Potyvirus. The resulting disease is known as maize lethal necrosis (MLN) and causes high economic losses.	Stewart et al., 2017
Papaya ringspot virus (PRSV) (Potyvirus)	Papaya mosaic virus (PapMV) (Potexvirus)	Papaya (*Carica papaya*)	Super-infection (first PapMV) causes antagonism, but co-infection or super-infection (first PRSV) causes synergism	Chávez-Calvillo et al., 2016
Parsnip yellow fleck virus (PYFV) (Secoviridae)	Anthriscus yellow virus (AYV) (Secoviridae)	Parsnip (*Pastinaca sativa*)	PYFV depends on mixed infection with AYV to be aphid-transmitted. Electron microscopy to detect viral particles in aphids	Murant and Goold, 1968; Murant et al., 1976
Peanut mottle virus (PMV) (Potyvirus)	Tobacco ringspot virus (TRSV) (Secoviridae)	Soybean (*Glycine max*)	Effect of mix-infection on plant characteristics and chemical composition. No synergism observed	Demski and Jellu, 1975

Pepper huasteco yellow vein virus (PHYVV) (Begomivirus)	Pepper golden mosaic virus (PepGMV) (Begomivirus)	Pepper (*Capsicum annuum*)	Synergism. Analysis of symptom severity, viral DNA concentration and tissue localization	Rentería-Canett et al., 2011
Plum pox virus (PPV) (Potyvirus)	Tobacco etch virus (TEV) (Potyvirus), or Potato virus X (PVX) (Potexvirus), or Cucumber mosaic virus (CMV) (Cucumovirus)	*Nicotiana tabacum; Nicotiana benthamiana; Nicotiana clevelandii*	Function of HC-Pro in the long-distance movement and in the control of the host range of potyviruses	Sáenz et al., 2002
Potato leaf roll virus (PLRV) (Luteovirus)	Tobacco mosaic virus (TMV) (Tobamovirus) or Potato spindle tuber viroid (PSTVd) (Pospiviroid) or Alfafa mosaic virus (AMV) (Bromoviridae) or Potato virus M (PVM) (Carlavirus) or Potato virus S (PVS) (Carlavirus) or Potato virus T (PVT) (Tepovirus) or Potato virus X (PVX) (Potexvirus)	*Solanum brevidens*	Solanum brevidens is tolerant to PLRV and accumulation of this virus did not vary in mixed infections with any of the viruses or viroids tested	Valkonen, 1992
Potato leafroll virus (PLRV) (Polerovirus)	Pea enation mosaic virus-2 (PEMV-2) (Umbravirus) or Cucumber mosaic virus (CMV) (Cucumovirus) or Potato virus X (PVX) (Potexvirus) or Groundnut rosette virus (GRV) (Umbravirus)	*Nicotiana clevelandii; Nicotiana benthamiana*	PLRV gain mechanical transmissibility when in mix infections with PEMV-2 or GRV	Mayo et al., 2000

Potato leafroll virus (PLRV) (Polerovirus)	Pea enation mosaic virus-2 (PEMV-2) (Umbravirus) or Cucumber mosaic virus (CMV) (Cucumovirus) or Potato virus X (PVX) (Potexvirus) or Tobacco mosaic virus (TMV) (Tobamovirus) or Potato virus Y (Potyvirus)	*Nicotiana clevelandii; Nicotiana benthamiana*	PEMV-2 complement PLRV mechanical transmission and facilitate its full systemic infection (cell-to-cell and inside-phloem movement)	Ryabov et al., 2001
Potato virus X (PVX) (Potexvirus)	Potato virus Y (PVY) (Potyvirus)	*Nicotiana tabacum*	Synergistic interaction with an increase of infectious PVX particles, probably related to an alteration in the relative levels of (-) and (+) strands of PVX RNA	Vance, 1991
Potato virus X (PVX) (Potexvirus)	Tobacco vein mottling virus (TVMV) or Tobacco etch virus (TEV) or Pepper mottle virus (PMV) or Potato virus Y (PVY) (Potyvirus)	*Nicotiana tabacum*	Synergism. Alteration in the replication of PVX RNA due to the expression of 5'-proximal sequences of either TVMV or TEV	Vance et al., 1995
Potato virus X (PVX) (Potexvirus)	Potato virus S (PVS) (Carlavirus)	Potato (*Solanum tuberosum*)	Increase of PVS titer and disease symptoms. More than 30 potato cultivars tested	Nyalugwe et al., 2012
Potato virus X (PVX) (Potexvirus)	Potato virus Y (PVY) (N–Wi strain) (Potyvirus)	*Nicotiana benthamiana and Nicotiana tabacum*	Synergism. Quantification of symptom expression and levels of coat proteins and suppressors of silencing under different temperature conditions and during co- or super-infection	Senanayake and Mandal, 2014
Potato virus X (PVX) (Potexvirus)	Potato virus A (PVA) (Potyvirus)	*Nicotiana benthamiana*	HCPro from PVA mediates reductions on methionine and glutathione and has a crucial role in the synergism observed	De et al., 2018
Potato virus Y (Potyvirus)	Cucumber mosaic virus (CMV) (Cucumovirus)	Tomato (*Solanum lycopersicum*)	Increase of both PVY and CMV levels. Differences with the same mix-infection in tobacco and cucumber	Mascia et al., 2010

Potato virus Y (PVY) (Potyvirus)	Tobacco etch virus (TEV) (Potyvirus)	Pepper (*Capsicum annuum*)	A non-aphid-transmissible strain of TEV depends on mixed infection with PVY to be aphid-transmitted	Simons, 1976
Potato virus Y (PVY) (Potyvirus)	Potato spindle tuber viroid (PSTVd) (Pospiviroid)	*Solanum tuberosum*	Synergism only in co-infection or super-infection with PSTVd infecting prior to PVY. PVY levels significantly higher in mixed infected plants	Singh and Somerville, 1987
Potato virus Y (PVY) (Potyvirus)	Tobacco mosaic virus (TMV) (Tobamovirus) or Potato spindle tuber viroid (PSTVd) (Pospiviroid) or Alfafa mosaic virus (AMV) (Bromoviridae) or Potato virus M (PVM) (Carlavirus) or Potato virus S (PVS) (Carlavirus) or Potato virus T (PVT) (Tepovirus) or Potato virus X (PVX) (Potexvirus)	*Solanum brevidens*	Accumulation of PVY in mixed infected plants with TMV and PSTVd increased up to 1000 fold, whereas no changes were observed in co-infections with the other viruses tested. *Solanum brevidens* is tolerant to PVY and PVX, what explains differences with other cases between PVX and PVY (usually synergistic)	Valkonen, 1992
Potato Virus Y (PVY) (Potyvirus)	Potato leaf roll virus (PLRV) (Luteovirus)	*Solanum tuberosum*	Synergism with more severe symptoms. Effect of the mixed infection on the fecundity and preference of two aphid vectors of PVY and PLRV	Srinivasan and Alvarez, 2007
Southern cowpea mosaic virus (SCPMV) (Sobemovirus)	Southern bean mosaic virus (SBMV) (Sobemovirus)	Cowpea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*)	In co-infection, the host range of SCPMV is modified, but not the one of SBMV	Hacker and Fowler, 2000
Southern rice black-streaked dwarf virus (SRBSDV) (Reoviridae)	Rice ragged stunt virus (RRSV) (Reoviridae)	Rice (*Oryza sativa*)	Synergism. Increase in replication and movement of viruses and inhibition of host immunity. Alteration on vector selection preferences tending to enhance mix infection	Li et al., 2017; Wang et al., 2014
Soybean mosaic virus (SMV) (Potyvirus)	Tobacco ringspot virus (TRSV) (Secoviridae) or Peanut mottle virus (PMV) (Potyvirus)	Soybean (*Glycine max*)	Effect of mix infections on plant characteristics and chemical composition. No synergism observed	Demski and Jellu, 1975

Soybean mosaic virus (SMV) (Potyvirus)	Bean pod mottle virus (BPMV) (Comovirus)	Soybean (*Glycine max*)	Synergism with increase of BPMV. Localization by electron microscopy. Reduction in yields both in field and greenhouse	Anjos et al., 1992; Calvert and Ghabrial, 1983
Soybean mosaic virus (SMV) (Potyvirus)	Cowpea mosaic virus (CPMV) (Comovirus)	Soybean (*Glycine max*)	Synergism with increase of CPMV. Localization by electron microscopy	Anjos et al., 1992
Strawberry pallidosis-associated virus (SPaV) (Crinivirus)	Beet pseudo-yellows virus (BPYV) (Crinivirus)	*Fragaria x ananassa*	Causing agents of the strawberry pallidosis disease. Detection in field surveys in North America and Peru	Tzanetakis et al., 2006; Wintermantel et al., 2006
Sweet potato chlorotic stunt virus (SPCSV) (Crinivirus)	Sweet potato feathery mottle virus (SPFMV) (Potyvirus)	Sweet potato (*Ipomoea batatas*)	Together they cause the sweet potato virus disease (SPVD), a synergistic interaction with a 600 fold increase of SPFMV and severe symptoms	Karyeija et al., 2000
Tobacco etch virus (TEV)	Tobacco mosaic virus (TMV) (Tobamovirus) or Cucumber mosaic virus (CMV) (Cucumovirus) or Potato virus X (PVX) (Potexvirus)	*Nicotiana benthamiana* and *Nicotiana tabacum*	Synergism. TEV P1/ HC-Pro protein enhances the pathogenicity and accumulation of both TMV and CMV. Transactivation of PVX replication	Pruss et al., 1997
Tobacco mosaic virus (TMV) (Tobamovirus)	Hibiscus latent Singapore virus (HLSV) (Tobamovirus)	*Nicotiana benthamiana*	Evolutionary game theory to model the co-infection between TMV and HLSV and validation by qRT-PCR	Chen et al., 2012
Tomato chlorosis virus (ToCV) (Crinivirus)	Tomato spotted wilt virus (TSWV) (Tospovirus)	Tomato (*Solanum lycopersicum*)	Synergism increasing ToCV while TSWV not change. Breakdown of resistance against TSWV in plants pre-infected with ToCV, contrarily to co-infection	García-Cano et al., 2006
Tomato chlorosis virus (ToCV) (Crinivirus)	Tomato infectious chlorosis virus (TICV) (Crinivirus)	*Physalis wrightii* and *Nicotiana benthamiana*	Effects of co-infection in viruses accumulation and transmission efficiency are host specific	Wintermantel et al., 2008
Tomato chlorosis virus (ToCV) (Crinivirus)	Tomato severe rugose virus (ToSRV) (Begomovirus)	Tomato (*Solanum lycopersicum*)	Near 50% of mixed infections in field surveys, with symptoms of ToSRV enhanced in the top part of plants, and crini-like symptoms at the bottom	Macedo et al., 2014
Tomato leaf curl New Delhi virus (ToLCNDV) (Begomivirus)	Tomato leaf curl Palampur virus (ToLCPalV) (Begomivirus)	Tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*)	Genetic reassortment and heteroencapsidation. Whitefly transmission of pseudo-recombinants	Kanakala et al., 2013

Tomato rugose mosaic virus (ToRMV) (Begomovirus)	Tomato yellow spot virus (ToYSV) (Begomovirus)	*Nicotiana benthamiana* and Tomato (*Solanum lycopersicum*)	ToYSV induces a change in tissue tropism of ToRMV (exits phloem). Both positive (synergism) and negative (interference) interactions are observed depending on the infection stage	Alves-Júnior et al., 2009
Turnip mosaic virus (TuMV) (Potyvirus)	Cauliflower mosaic virus (CaMV) (Caulimovirus)	*Arabidopsis thaliana*	Effect of mixed infection on viruses infectivity, pathogenicity and accumulation. No symptom synergism	Martín and Elena, 2009
Wheat streak mosaic virus (WSMV) (Tritimovirus)	Triticum mosaic virus (TriMV) (Poacevirus)	Wheat (*Triticum aestivum*)	Cultivar-specific synergism and Influence of different temperatures. Determination of different physiological parameters. Impact on endogenous and viral small RNA profiles	Tatineni et al., 2010, 2014; Byamukama et al., 2012
Zucchini yellow mosaic virus (ZYMV) (Potyvirus)	Cucumber mosaic virus (CMV) (Cucumovirus)	Zucchini squash (*Cucurbita pepo*)	Synergism with increase of CMV levels	Fattouh, 2003
Zuchini yellow mosaic virus (ZYMV) (non-aphid-transmissible isolate ZYMV-NAT) (Potyvirus)	Papaya Ring Spot Virus (PRSV) (Potyvirus)	Melon (*Cucumis melon* L. Vedrantais)	Heteroencapsidation and gain of aphid-transmissibility	Bourdin, D and Lecoq, 1991

[a] Viruses listed in alphabetical order, indicating the acronym and their taxonomic adscription (Genus) in brackets

[b] When the bibliographical references include data for more than a pair of viruses, the second partner of every combination are mentioned but separated by "or".

[c] Common names and/or scientific names are used to identify hosts. When different plant species were tested in the same work, their names are listed

Table 2. Mixed infections involving more than two viruses in the same plant

Viruses [a]	Host [b]	Comments	References
African cassava mosaic virus (ACMV); Cassava mosaic Madagascar virus (CMMGV); East African cassava mosaic virus (EACMV); East African cassava mosaic Kenya virus (EACMKV); East African cassava mosaic Malawi virus (EACMMV); East African cassava mosaic Zanzibar virus (EACMZV); South African cassava mosaic virus (SACMV) (all Begomovirus)	Cassava (*Manihot esculenta*)	Causing agents of the Cassava Mosaic Disease (CMD). Synergism and cause of severe pandemics. Source of high recombination rates between DNAs A and B of the different virues involved in the disease.	Jacobson et al., 2018; Rey and Vanderschuren, 2017
Australian grapevine viroid (AGV) (Pospiviroidae); Grapevine leafroll-associated virus-9 (GLRaVs) (Closteroviridae); Grapevine rupestris stem pitting-associated virus (GRSPaV) (Foveavirus); Grapevine rupestris vein-feathering virus (GRVFV) (Marafivirus); Grapevine Syrah virus-1 (GSyV-1) (Marafivirus); Grapevine yellow speckle viroid (GYSVd) (Pospiviroidae); Hop stunt viroid (HSVd) (Pospiviroidae); Potato spindle tuber viroid (PSTVd) (Pospiviroid)	Common grapevine (*Vitis vinifera*)	Deep sequencing analysis of RNAs from a grapevine showing decline symptoms revealed the presence of seven different viruses and viroids	Al Rwahnih et al., 2009
C-6 virus (Carlavirus); Cucumber mosaic virus (CMV) (Cucumovirus); Sweet potato chlorotic fleck virus (SPCFV) (Carlavirus); Sweet potato chlorotic stunt virus (SPCSV) (Crinivirus); Sweet potato feathery motle virus (SPFMV); Sweet potato latent virus (SPLV) (Potyvirus); Sweet potato mild mottle virus (SPMMV) (Ipomovirus); Sweet potato mild speckling virus (SPMSV) (Potyvirus)	Sweet potato (*Ipomoea batatas*)	Synergism. The presence of a third different virus in plants affected with SPVD (SPCSV + SPFMV) increased the severity of symptoms of SPVD alone	Untiveros et al., 2007
Ipomoea vein mosaic virus (IVMV) (Portyvirus); Sweet potato chlorotic stunt virus (SPCSV) (Crinivirus); Sweet potato feathery mottle virus (SPFMV) (Potyvirus); Sweet potato virus G (SPVG)	Sweet potato (*Ipomoea batatas*)	Mix infections with 2 or 3 viruses. Synergism with SPCSV increasing the titers of potyviruses. No synergism observed between potyviruses	Kokkinos and Clark, 2006
Potato leaf roll virus (PLRV) (Polerovirus); Potato virus A (PVA) (Potyvirus); Potato virus M (PVM) (Carlavirus); Potato virus S (PVS); Potato virus X (PVX) (Potexvirus); Potato virus Y (PVY)	Potato (*Solanum tuberosum*)	Field survey in Pakistan showing synergism for almost all the mixed infections tested, combining 2 or 3 viruses	Hameed et al., 2014
Sweet potato chlorotic stunt virus (SPCSV) (Crinivirus); Sweet potato feathery mottle virus (SPFMV) (Potyvirus); Sweet potato mild mottle virus (SPMMV) (Ipomovirus)	Sweet potato (*Ipomoea batatas*)	Synergism between SPCSV + SPFMV or SPMMV, and SPCSV + SPFMV + SPMMV. Neutralism between SPMMV and SPFMV	Mukasa et al., 2006

[a] Viruses listed in alphabetical order, indicating the acronym and their taxonomic adscription (Genus) in brackets

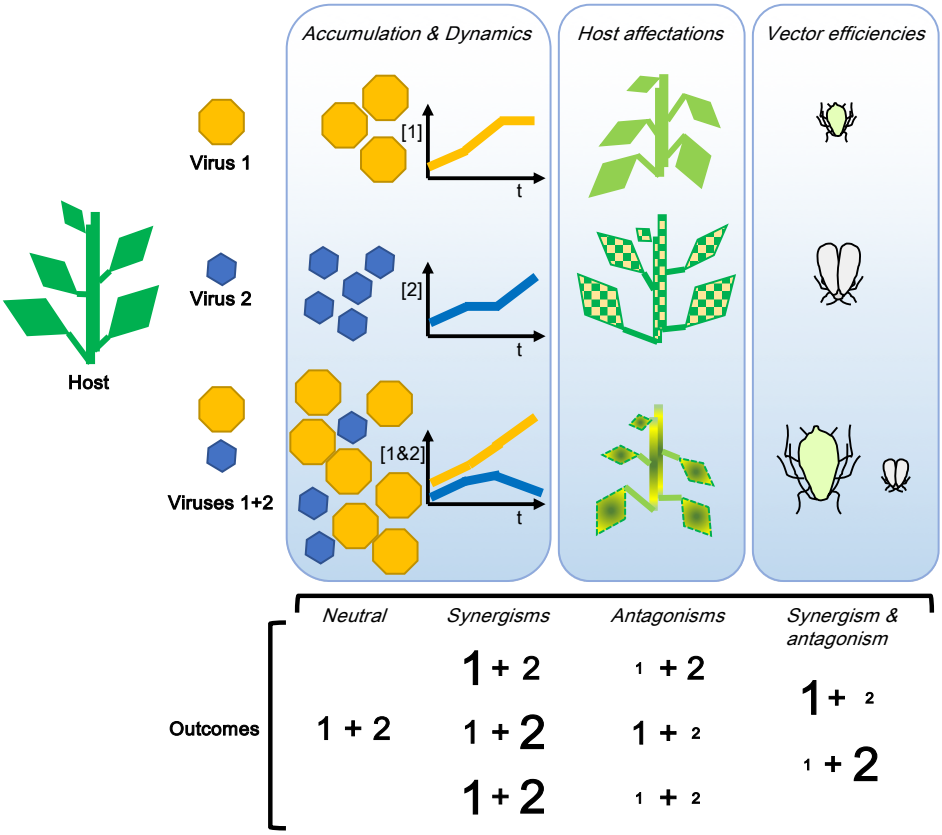
[b] Common names and scientific names are used to identify hosts.

Figure legends

Fig. 1. Different scenarios of interaction during mixed viral infections of plants. Responses in terms of virus accumulation and temporal dynamics (left column), degree of symptoms in the susceptible host (central column) and efficiency of vector transmission (right column) are schematically represented respectively for the individual infections by virus 1 or virus 2 in the first and second rows, while the third row proposes one of the possible outcomes of a mixed infection of 1+2, with increased and faster accumulation of one of the viruses (represented by number of virions and inclination of curves), causing more damage to the plant (less growth and severe symptoms), and altering their vector dissemination (efficiencies proporcional to vector sizes). For each of the parameters, combined outcomes might differ as shown in the lower part, where the size of the font represents neutral, synergistic, antagonistic or both types of responses for each virus. See text for further details.

Fig. 2. Schematic representation of interactions occurring at cellular level during co-infection by two unrelated viruses (1 and 2) of a susceptible host plant. Cell entry and exit processes, either through the action of vector organisms (inoculation and acquisition), or through movement from and to adjacent cells (cell-to-cell transport through plasmodesmata, or invasion of distant tissues during long-distance transport), as well as essential intracellular viral processes such desencapsidation, gene expression, genome replication and encapsidation of progeny, are represented by arrows of colors matching the individual viruses. The involvement of host factors and other processes are omitted for simplicity, as well as presumed differences in localization and timing of events. Outcomes of the positive and facilitative interactions are represented with arrows, while negative interactions are indicated by blockage symbols (T-shaped connectors), and neutral effects are omitted for simplicity. Representative hypothetical examples of cross-assistance/complementation of movement functions, or transcapsidations during vector transmission, are represented by question marks. See text for further details.

Fig. 3. Additional levels of interaction during viral mixed infections of plants. A schematic cross section of a leaf (epidermis, parenquima and vasculature) is shown, together with the routes of hypothetical spread of infections by a virus capable to invade all tissues (virus 1, orange arrows), and another restricted to vascular tissues (virus 2, blue arrows). Mix-infection might lead to interactions like those indicated by question marks: both facilitative and blocking outcomes of intracellular interactions (see figure 2) could influence dispersal of the viruses in the plant, and thus condition its affectation (see figure 1). Furthermore, behavior and performance of vectors responsible of transmission (here represented as different insects for each virus) might also respond positively or negatively to manipulative strategies (see figure 1). Arrows and T-shaped connectors represent positive and negative cross-interactions that can take place during mixed infections inside the plant and in the relationship with vectors.


Accumulation & Dynamics
Host affectations
Vector efficiencies
Virus 1
Virus 2
Viruses 1+2
Host
[1]
[2]
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t
Outcomes
Neutral
Synergisms
Antagonisms
Synergism & antagonism
1 + 2

Figure 1

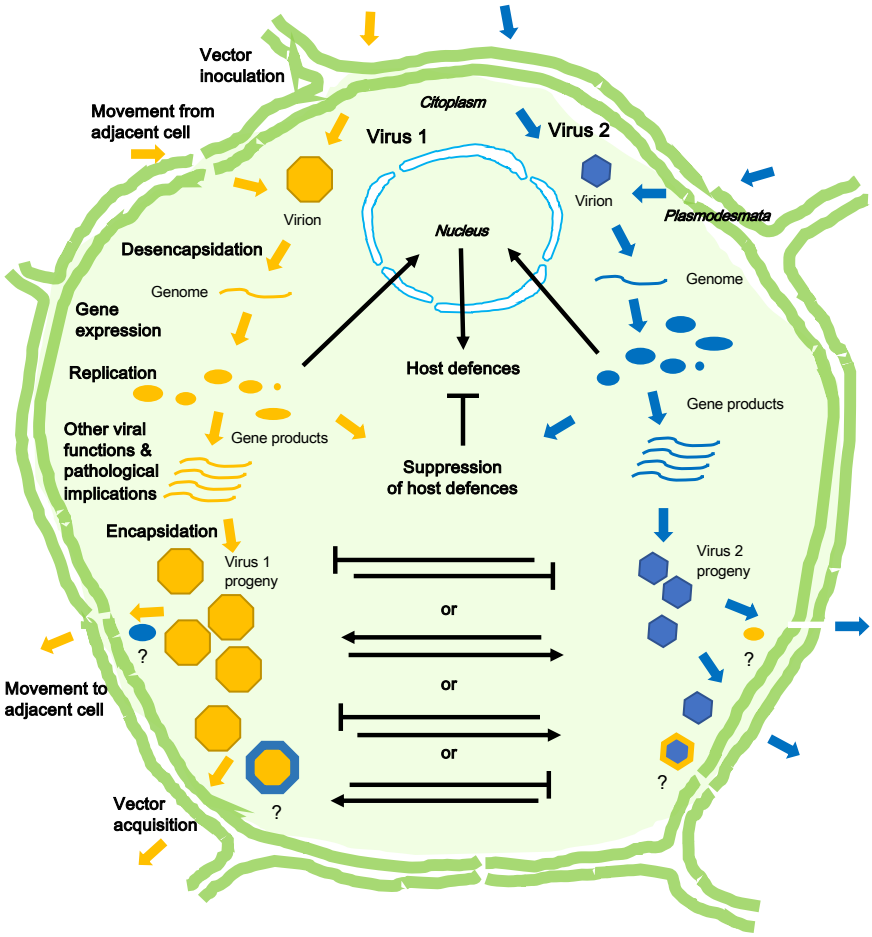
Vector inoculation
Movement from adjacent cell
Citoplasm
Virus 1
Virus 2
Virion
Virion
Plasmodesmata
Nucleus
Desencapsidation
Genome
Genome
Gene expression
Replication
Host defences
Gene products
Other viral functions & pathological implications
Gene products
Suppression of host defences
Encapsidation
Virus 1 progeny
Virus 2 progeny
or
or
or
?
?
?
?
Movement to adjacent cell
Vector acquisition

Figure 2

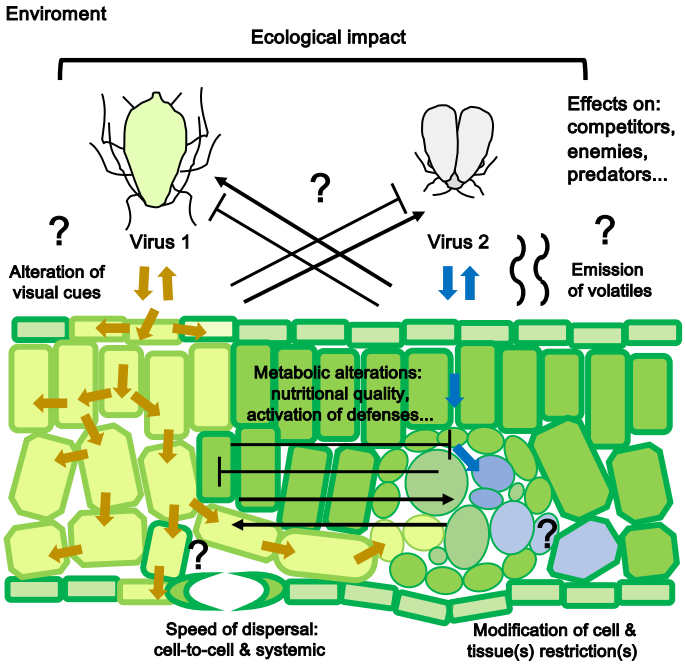
Enviroment
Ecological impact
Effects on:
competitors,
enemies,
predators...
?
?
?
Virus 1
Virus 2
Alteration of
visual cues
Emission
of volatiles
Metabolic alterations:
nutritional quality,
activation of defenses...
?
?
Speed of dispersal:
cell-to-cell & systemic
Modification of cell &
tissue(s) restriction(s)

Figure 3