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Review Article

Mixed viral infections in vegetable crops: biochemical and molecular aspects

Mario Sánchez-Sánchez, [1]Laboratorio de Fisiología Vegetal, Centro de Investigación en Alimentación y Desarrollo, Hermosillo, 83304, Sonora, México; **Irasema Vargas-Arispuro,** Laboratorio de Ciencia de los Alimentos, Centro de Investigación en Alimentación y Desarrollo, Hermosillo, 83304, Sonora, México; **Juan Manuel Tovar-Pedraza,** Laboratorio de Fitopatología, Coordinación Regional Culiacán, Centro de Investigación en Alimentación y Desarrollo, Culiacán, 80110, Sinaloa, México; [1]**Cristóbal Joel González-Pérez**; [1]**Emmanuel Aispuro-Hernández,** [1]**Eber Addí Quintana-Obregón,** CONAHCYT-Centro de Investigación en Alimentación y Desarrollo, A.C. Coordinación de Tecnología de Alimentos de Origen Vegetal. Hermosillo, 83304, Sonora, Mexico; [1]**Miguel Ángel Martínez-Téllez*.**

***Corresponding Author**:
Miguel Ángel Martínez-Tellez
norawa@ciad.mx

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ABSTRACT

Mixed viral infections refer to the coinfection of two or more viruses in the plant, which regularly lead to exacerbated symptoms on leaves and fruits. The dynamics of coinfections may follow either a synergistic, antagonistic, or neutral interaction that impacts the severity of the symptoms and infection. Mixed viral infections occur due to the convergence of fundamental characteristics reviewed in this manuscript. The virus–host plant interrelationship influences the establishment and spread patterns of mixed viral infections. Attention should be drawn to potential changes in the dynamics of transmission and prevalence of plant viral diseases due to the effect of anthropogenic and natural alterations to complex agroecological systems or their components, including hosts, reservoirs, vectors, ecological niches, and the emergence of new virus strains.

Keywords: antagonism, coinfection, synergism, virus, virus-host interactions

Introduction

Mixed viral infections in plants, also known as coinfections, are a complex and common phenomenon in plants. Previously, it was estimated that mixed infections were scarce and restricted to complex geographic areas; however, a recent analysis of the "virome" in horticultural crops by next-generation sequencing has provided evidence of the frequency of coinfections by multiple viral agents (Moreno and López-Moya, 2020). This has contributed to understand the viral coinfections, the

virus–plant relationships, and the mechanisms for infection establishment, disease development, and virus spread. Unlike monoinfection and coinfections have some distinctive features, such as the presence of multi-host-associated virus vectors (Syller, 2014), sudden and enhanced symptom onset, and disease progression (Jacobson *et al*., 2018). Coinfections will result in either beneficial, neutral, or counterproductive virus–virus interrelations.

Synergistic effects are frequently among viruses that belong to different species (from different genera or the same genus), (Miranda-Campaña *et al*., 2024; Kwon *et al*., 2023; Takeshita *et al*., 2012) but also, among viruses that share a vector, such as those that are transmitted by insects such as whiteflies, aphids, thrips, planthoppers, leafhoppers, and beetles. The virus species within the genera *Crinivirus*, *Potyvirus*, and *Begomovirus* are the most reported cases of synergic interactions in vegetable crops (Wintermantel *et al*., 2008; Lucía-Sanz and Manrubia, 2017; Escobedo Garcia-Medrano *et al*., 2022; Tamborindeguy *et al*., 2023).

Antagonism had been reported to occur in related virus species and implies a mixed infection where two viral elements compete to establish the infection in the plant (Mascia and Gallitelli, 2016), resulting in a virus with better fitness that the other. This trait causes the least favored virus to quickly decrease its titers. From an adaptative point of view, there is activation of host defense responses that preventing the subsequent infection by a secondary virus (Singhal *et al*., 2021). Competition for host resources is the most widely accepted hypothesis that partly explains why most cases of the virus–virus interaction results in monoinfection (Singhal *et al*., 2021), such as in begomovirus mixed infections (Silva *et al*., 2014).

Superinfection exclusion (SIE) is a type of antagonism that is specific to viruses of the same species, mild viruses, or viroid strains that protects the plant from disease resulting from a subsequent encounter with a severe strain of the same virus (Ziebell and Carr, 2010). In this phenomenon the first virus blocks infection of a second virus and causes pathogenicity and severity below the initial one, thus providing hidden protection only against related viruses or virus strains (Cwick *et al*., 2022; Tsuda *et al*., 2007). When SIE is used as an agricultural practice it is called cross-protection which is established as a widely used management strategy to reduce crop losses caused by some viral diseases (Xu *et al*., 2022; Lecoq and Katis, 2014; Zhou *et al*., 2019). Thus, cross-protection is specific to strains of the same virus, compared to antagonism, where two different viruses are considered in the infection process (Pal and Gardener, 2006).

Finally, neutral coinfection occurs when the viral species coinfects, cause damage to the plant (Vinodhini *et al*., 2021), and their titers remain intact. It is suggested that this type of interaction is under studied because the symptoms caused by a virus mask the symptoms of the other agent (Singhal *et al*., 2021). Antagonistic and neutral infections are scarcely reported and have not been analyzed in depth in

comparison with synergism. However, phenotypic diversity emerges every time, which improves our understanding of viral pathogenesis (Syller and Grupa, 2016). In this work, we have limited our analysis to vegetables of agro-industrial interest such as *Capsicum* spp., *Solanum lycopersicum,* and some other Cucurbitaceae. Although mixed viral infections began to be described in the 1970s, in this work we described current reports of mixed viral infections in vegetable crops reported in the last ten years (Table 1). genomic tools have helped to understand their nature and led to significant advances in the identification of this type of infection. This review explores the biochemical and molecular aspects of mixed viral infections in agronomic plants, focusing on three fundamental aspects that make mixed infections possible, first: plant components involved in mixed viral infections; second: RNA interference system, and finally: viral components reported in mixed infections.

Plant components involved in viral mixed infections

Mono and mixed infections can be difficult to detect, since damage caused in various parts of the plant such as buds, leaves, flowers, and fruits, can be confused with a lack of nutrients or damage caused by herbicides (Gergerich and Dolja, 2006). Another important aspect is the affinity of each virus involved to certain components of the plant. Besides, certain components of the host cell assist viruses in entry, move, and translocate from cell to cell or from one tissue to another. Therefore, determining plant components that are involved in mixed viral infections is a topic of great importance.

Plasmodesmata (PD) are cytoplasmic communication channels between plant cells that allow the symplastic transport of macromolecules vital for plant growth and development (Figure 2). However, plant viruses use this space for movement during the infection process. Plasmodesmata-located protein 5 (PDLP5) acts as an inhibitor of PD trafficking (Lee *et al*., 2011). To elucidate the characteristics of this protein Kutsher *et al*. (2021) investigated tomato and tobacco plants infected with viruses and through Green Fluorescent Protein assay (GFP) the location, quantification, and effect of PDLP5 on PD were determined. In tobacco leaves, *Tobacco mosaic virus* (TMV) and *Tomato brown rugose fruit virus* (TBRFV) increased PDLP5-GFP levels in PD compared to the control, whereas no significant differences were observed in tomato leaves. Conversely, infection with *Tomato yellow leaf curl virus* (TYLCV) decreased PDLP5-GFP accumulation in tomato leaf PD compared to the control, without any significant effect on tobacco leaf PD. Although, viral inoculations and effects were evaluated separately and not in mixed viral infection, the research demonstrated clear differences in the molecular mechanisms involved in the different types of infection. The infection by *Tobamovirus* species led to an increase in the expression of a specific protein

Table 1. Mixed viral infections reported for vegetable crops in the last 10 years.

Source/ Mixed infection type	Genera included	Viruses/ Symptoms	Highlight	Country and reference
Tomato/ Synergism	*Potexvirus*, *potyvirus* and *Amalgavirus*	*Potato virus Y* (PVY), *Pepino mosaic virus* (PepMV), and STV / chlorosis, deformation, and depressed spots of dark color in leaves.	There was an increased severity of symptoms in co-infected plants by comparison to plants only infected with PepMV. There was a high rate of STV transmission and association with tomato symptoms.	Italy Iacono *et al.* (2015)
Capsicum sp./ Synergism	*Orthotospovirus* and *Potyvirus*	PVY and *Tomato spotted wilt virus* (TSWV) / Stunting, leaf chlorosis, mottling, and curling	High incidence of mixed viral infections associated with *Frankliniella occidentalis* and *Myzus persicae*.	Zimbabwe Karavina *et al*. (2016)
Tomato/ Synergism	*Tobamovirus*	PaMMV and TBRFV / Necrotic local lesions were observed on the leaves.	Acceleration of symptoms and infection. Both viruses interfere with the hypersensitive response.	Israel Luria *et al*. (2018)
Tomato/ Synergism	*Begomovirus*	TYLCV, *Tomato leaf curl betasatellite* (ToLCB), and *Mungbean yellow mosaic Indian virus* (MYMIV) / yellowing, curling, and stunting symptoms.	Begomoviruses which do not commonly infect tomatoes, such as MYMIV, could be spread as a passenger of TYLCV in tomatoes.	Oman Shahid *et al*. (2019)
Tomato / Synergism	*Begomovirus* and *Crinivirus*	TYLCV and ToCV / Data not shown.	TYLCV and ToCV coinfection induced shifts in the expression of genes involved in transport and energy metabolism.	China Ding *et al*. (2019)
Tomato / Synergism	*Begomovirus*	*Tomato golden mottle virus* (TGMV) and *Tomato severe leaf curl virus* (ToSLCV) / reduced growth, mosaic, and chlorosis.	High populations of *B. tabaci* are associated with infections of different species of begomovirus, but in this study the number of whiteflies was low.	Mexico Zuñiga-Romano *et al*. (2019)
Squash and tomato / Synergism	*Begomovirus* and *Crinivirus*	*Cucurbit leaf crumple virus* (CuLCrV), *Cucurbit yellow stunting disorder virus* (CYSDV), and TYLCV / Severe crumpling and downward curling of leaves.	Effect of whitefly acquisition of TYLCV and CuLCrV on virus load, and examination of the whitefly preference and fitness.	USA Gautam *et al*. (2020)
Tomato/ Synergism	*Tobamovirus* and *Potexvirus* (see symptoms in Figure 3).	TBRFV and PepMV / Scarred or open unripe fruits, along with narrow or yellow patched leaves, shoestring-like leaves.	Mild PepMV infections preceded by TBRFV can induce characteristic symptoms of aggressive PepMV strains.	Israel Klap *et al*. (2020)
Tomato/ Synergism	*Begomovirus*	TYLCV, *Tomato yellow leaf curl Kanchanaburi virus* (TYLCKaV), and *Pepper yellow leaf curl Indonesia virus* (PepYLCIV) / Mild yellowing, curling, and stunting.	Lack of effectiveness of *Ty* resistance genes against mono and mixed infections of distinct local begomoviruses from Southeast Asia.	Japan Koeda *et al*. (2020)
Tomato/ Synergism	*Crinivirus* and *Begomovirus*	ToCV and TYLCV / Decreased height and weight of stems.	TYLCV presented tropism for upper foliage in contrast to ToCV, which was present mainly on lower leaves.	China Li *et al*. (2021)
Tomato/ Synergism	*Begomovirus*	*Tomato yellow spot virus* (ToYSV), *Tomato yellow spot alphasatellite* (ToYSA), *Euphorbia yellow mosaic virus* (EuYMV), and *Euphorbia yellow mosaic alphasatellite* (EuYMA)/Severe mosaic, leaf deformation, curling, and dwarfism.	Some begomoviruses can increase symptom severity and either promote or restrict host accumulation of other viruses.	Brazil Nogueira *et al*. (2021)

Table 1. Continue.

Source/ Mixed infection type	Genera included	Viruses/ Symptoms	Highlight	Country and reference
Tomato/ Synergism	*Begomovirus* (see symptoms in Figure 1).	*Tomato yellow mottle virus* (ToYMoV), *Tomato yellow mottle disease* (ToYMoD), and *Tomato leaf curl Sinaloa virus* (ToLCSiV) / Vein clearing, crumpling, yellow mosaic, and stunted foliar.	Begomoviruses synergistic interaction will lead to increased disease severity.	Costa Rica Maliano *et al*. (2022)
Tomato/ Synergism	*Begomovirus* and *Crinivirus*	TYLCV and ToCV / Severe curling and extensive yellowing in upper leaves, necrosis of newly emerging leaves.	Suggests antagonism and synergism occur at early and late stages, respectively.	Spain Ontiveros *et al*. (2022)
Peppers/ Synergism	*Begomovirus*	PepYLCIV, TYLCKaV, and ToLCNDV / Yellow mosaic, vein banding, cupping upward, leaf curling, reduced size, and stunting.	Severe monoinfection caused by PepYLCIV led to mixed infections with TYLCKaV. PepYLCIV and TYLCKaV infected resistant varieties of pepper plants without showing symptoms of infection.	Indonesia Wahyono *et al*. (2023)
Tomato/ Synergism	*Tobamovirus* and *Potexvirus*	TBRFV and PepMV / Necrosis and chlorosis	Presence of PepMV and TBRFV in imported tomatoes in Florida grocery stores.	USA Yilmaz and Batuman (2023)
Tomato/ Synergism and Antagonism	*Amalgavirus*, *Cucumovirus* and *Potexvirus*	STV, *Cucumber mosaic virus* (CMV), and PepMV / Strong leaf deformation, severe symptoms of mosaic, differences in height and weight concerning controls.	STV increased viral titer and induced symptoms by CMV at early infection stages, whereas PepMV titer did not change even though PepMV exacerbated symptoms.	Spain Elvira-González *et al*. (2021)
Tomato/ Antagonism	*Begomovirus vs Tobamovirus*	TYLCV and *Tomato mottle virus* (ToMoV) / Leaf curling, yellowing, and mosaic symptoms.	TYLCV produced an antagonistic effect on ToMoV systemic infection.	USA McLaughlin *et al*. (2022)
Tomato/ Antagonism	*Begomovirus vs Begomovirus*	ToSRV and ToRMV/ Yellow mosaic and leaf distortion	Genes involved in replication (CR) are responsible for antagonism.	Brazil Nogueira *et al*. (2023)
Capsicum sp. / Neutral	*Cucumovirus* and *Potyvirus*	CMV, *Capsicum chlorosis virus* (CaCV), and *Groundnut bud necrosis virus* (GBNV) / Concentric chlorotic ring spots, necrosis along with mosaic, and leaf malformation	Amino acid substitution of Ser129 by Pro129 of the CMV coat protein is required for mixed infection.	India Vinodhini *et al*. (2021)
Tomato and muskmelon / Neutral	*Begomovirus*	TYLCV, *Cotton leaf curl Gezira virus* (CLCuGeV), *Okra yellow crinkle Cameroon alphasatellite* (OYCrCMA), and *Okra leaf curl Oman betasatellite* (OLCuOMB) / Leaf curling, yellowing, and mosaic symptoms.	First report of mixed infection of bipartite and monopartite begomoviruses associated with tomato and melon DNA satellites.	Saudi Arabia AlHudaib *et al*. (2022)
Chili /No available	*Orthotospovirus*, *Potyvirus*, *Macluravirus*, *Cucumovirus*, *Tobamovirus* and *Begomovirus*	CaCV, *Chilli veinal mottle virus* (ChiVMV), *Large cardamom chirke virus* (LCCV), CMV, PMMoV, and *Chilli leaf curl virus* (ChiLCV) / Mottling yellow-mosaic, leaf curling, chlorotic and necrotic spots.	Widespread occurrence of mixed infections. First report of the detection of six chili viruses.	India Devi *et al*. (2022)
Tomato/ Synergism	*Tobamovirus* and *begomovirus*	ToMV, *Tomato golden mosaic virus* (ToGMoV), and *Pepper huasteco yellow vein virus* (PHYVV) / foliar necrosis, yellow mottling, green vein banding, blistering, upward and downward curling, leaf malformation, and chlorosis.	The co-infection of ToMV with begomovirus resulted in increased disease severity, reduced plant growth, and higher viral accumulation. ToMV exhibited a high mutation rate	Mexico Gonzalez-Perez *et al.* (2024)

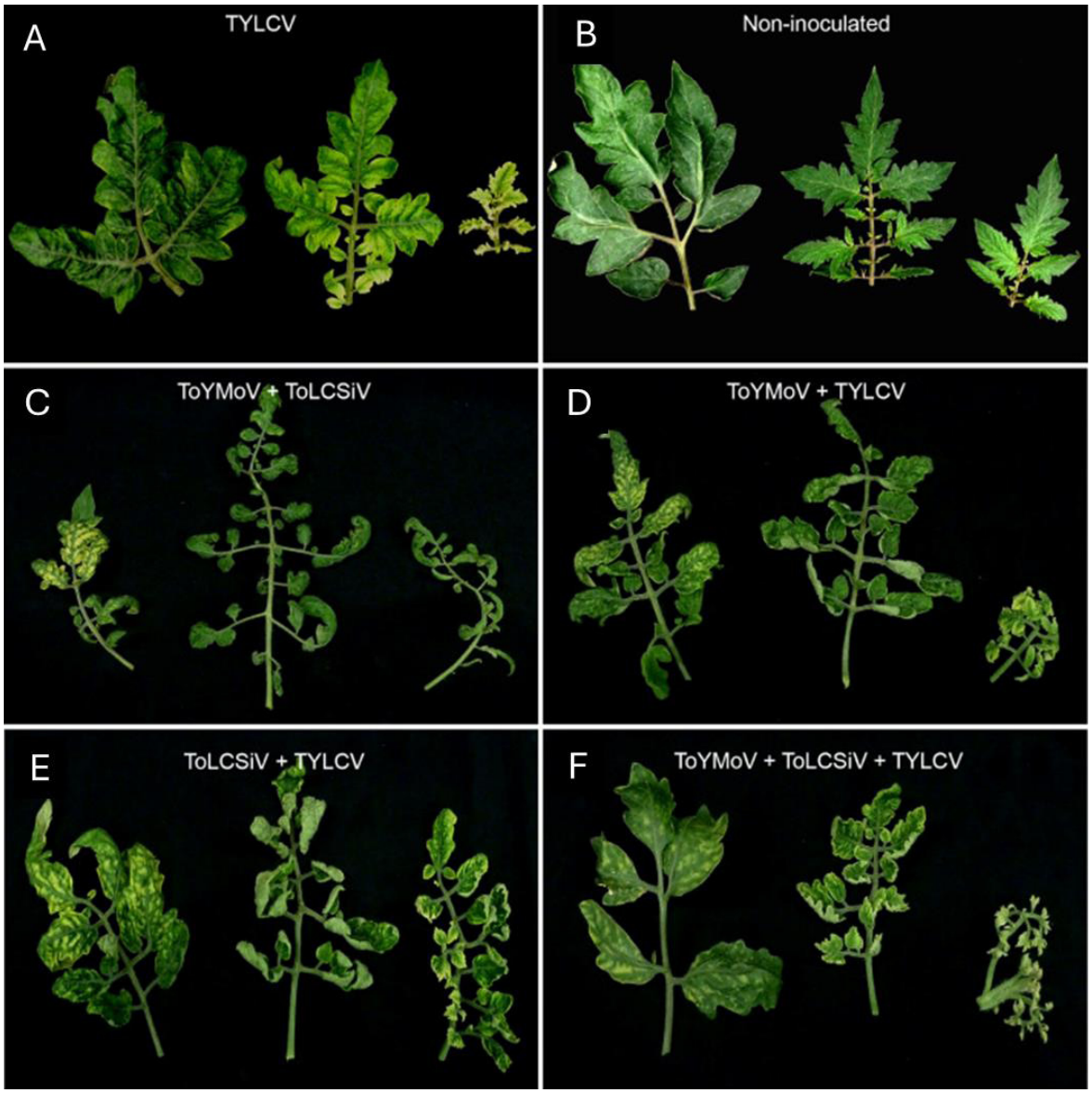

Figure 1. Symptom transition in the lower, middle, and upper leaves of tomato plants agroinoculated with the infectious cloned DNA-A and DNA-B components of isolates of TYLCV from Costa Rica (ToYMoV-[CR:Gre:GR1:90]) and *Tomato leaf curl Sinaloa virus* (ToLCSiV-[CR:Lib:L1:02]) from Costa Rica and the infectious clone of the genomic DNA of TYLCV from the Dominican Republic (TYLCV-[DO]) individually or in all combinations. (A) TYLCV; (B) non-inoculated tomato plant; (C) ToYMoV and ToLCSiV; (D) ToYMoV and TYLCV; (E) ToLCSiV and TYLCV; (F) ToYMoV, ToLCSiV and TYLCV. Plants were photographed 21 d after agroinoculation (with permission of Maliano *et al*., 2022).

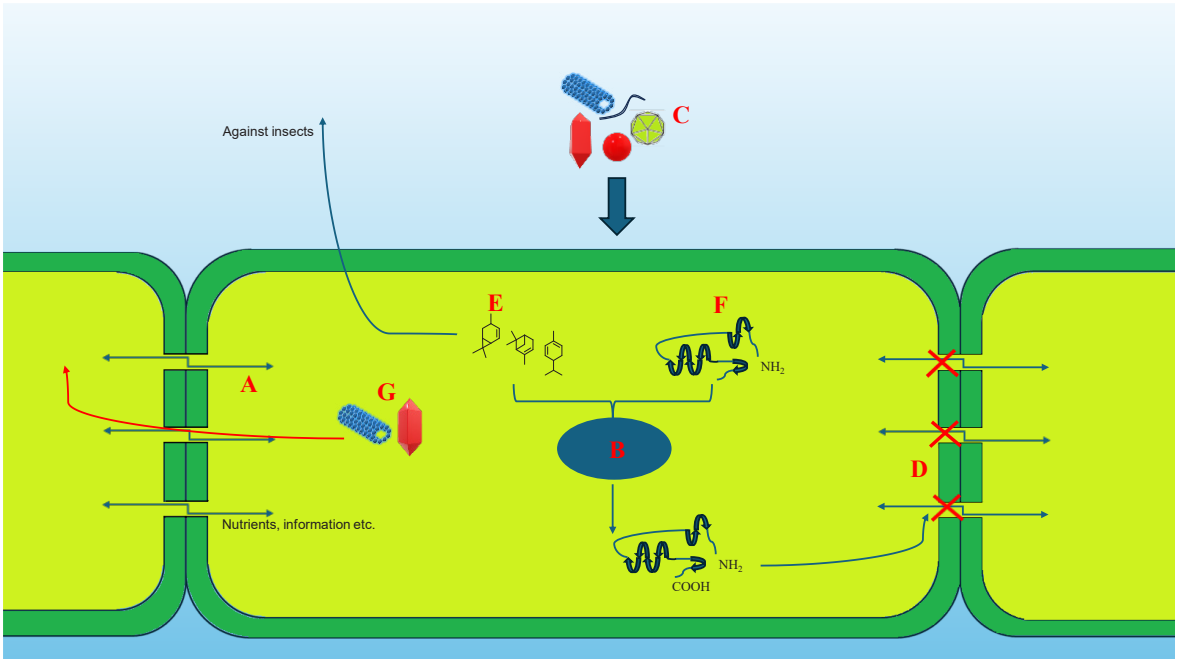

Figure 2. Plant components involved in viral mixed infections. Normal communication processes between plant cells involve the interrelation of structures such as plasmodesmata (A) and nucleus (B), the former are conducted in the cell walls that allow the passage of molecules between cytoplasm. In the presence of a viral infection (C), the cell counteracts the action through well-defined biochemical pathways such as inhibition of plasmodesmata function and intracellular trafficking (D), production of specific response metabolites (They can occur before and during the development of the viral infection), (E) or increase in the production of proteins involved in detoxification processes (F). In other hand, after a successful internalization by two viruses occurs synergistic effect of mixed viral infection (G).

PDLP5, while viruses belonging to the genus *Begomovirus* inhibited its expression, establishing that the activity in plasmodesmata (PD) largely depends on the type of virus. Thus, it would be interesting to know what the role of PDLP5 is would be concerning mixed infection with different viral genera.

Plant metabolites encompass a vast number of organic compounds of different chemical natures, importance, and functions. They participate in the development of cellular processes, reproduction, protection, maturation, and senescence of plants (figure 1). There are scarce reports about the synthesis and functionality of plant metabolites when coinfections occur, changes in the type and accumulation patterns of phytochemicals have been observed (Zaynab *et al*., 2018).

Fereres *et al*. (2016) investigated the alterations in the production pattern of volatile compounds in tomatoes infected by the circulative *Tomato severe rugose virus* (ToSRV) and the non-circulative *Tomato chlorosis virus* (ToCV). Analysis of individual compounds showed that tomato control plants emitted significantly higher amounts of the terpenes α-pinene, 4-carene, α-phellandrene, terpinene, and

β-phellandrene than ToSRV-infected tomato plants, which indicated that ToSRV infection promotes suppression of some volatile terpenes. Additionally, the whiteflies showed a clear preference for uninfected plants, favoring the disease dispersion. These results suggested the ability of certain viruses to coevolve with their host, increasing their propagation through the modification of the synthesis patterns of volatile compounds in tomato plants. It would be interesting to discern if there is any connection in the synthesis of terpenes as a defense mechanism against insect vectors, especially for *Bemisia tabaci* (*B. tabaci*). However, the reduction in the concentration of terpenes was not associated with a greater attraction of the vector to ToSRV-infected plants.

Subsequently, De *et al*. (2018) described that the synergistic infection of *N. benthamiana* by *Potato virus X* (PVX) and *Potato virus A* (PVA) increased the expression of the helper component proteinase (HCPro), a protein that causes disturbances to the methionine cycle in the host cell. In this experiment, through gene silencing of key plant enzymes, such as *S*-adenosyl-l-methionine synthetase (SAMS) and *S*-adenosyl-l-homocysteine hydrolase (SAHH), a significant reduction in glutathione reductase (GSH) was found. GSH is an important antioxidant in plant cells, and therefore, GSH deficiency may explain the symptoms observed during mixed PVX-PVA infection.

Recently, a study published by Pollari *et al.* (2022) found that when *N. benthamiana* is infected by poty- and potexviruses, the host's metabolic response differs significantly depending on whether it is a mixed or monoinfection. In mixed infections, an increase in defense-related metabolites was found, and a low methylation potential of genetic material was suggested. Finally, it is concluded that infection-associated oxidative stress is successfully controlled in individual infections but not in synergistic infections.

Das *et al.* (2019) reported a set of secondary metabolites (terpenoids, and isoprenoids such as chlorophyll, carotenoids, phylloquinones, gibberellins, and abscisic acid), produced by *N. benthamiana* after the infection with TMV during cross-infection. Using the isobaric tags for relative and absolute quantification (iTRAQ) technique, it was possible to demonstrate during a mixed infection that a TMV-43A (a mutant where 43-nt of poly(A) were replaced), could cross-protect *N. benthamiana* plants against a wild-type strain of TMV. In cross-protected plants, amino acid biosynthesis and photosynthetic activities were increased, supported by increased accumulation of crucial 1-deoxy-*D*-xylulose-5-phosphate reductoisomerase (DXR) and geranylgeranyl diphosphate synthase (GGPS) enzymes for the biosynthesis of chlorophyll. An increase in the abundance of enzymes related to the management of reactive oxygen species (ROS) and redox homeostasis, such as thioredoxins and *L*-ascorbate peroxidase, was also found in cross-protected plants.

PD and metabolites synthesis are modified by the presence of viruses, although plant components related to mixed infections are scarce, other important sites in growth such as the apical system, or the ubiquitination mechanism could be viable for study and establish the role of these components in mixed viral infections.

RNA interference system

RNA-based silencing is a system whose main function is the maintenance of genome integrity, but plants have adapted this system as a protective mechanism, particularly against viruses. RNA-based silencing defense allows suppression of gene expression via sequence-specific interactions that are mediated by 21- to 24-nucleotide-long RNA molecules (Figure 3), (Moissiard and Voinnet, 2004; Cisneros and Carbonell, 2020). Under the premise that RNA sequences are directed to specific viral targets, it would be useful to know whether viruses with highly similar sequences can be targeted by the same microRNAs (Kwon *et al*., 2020); and

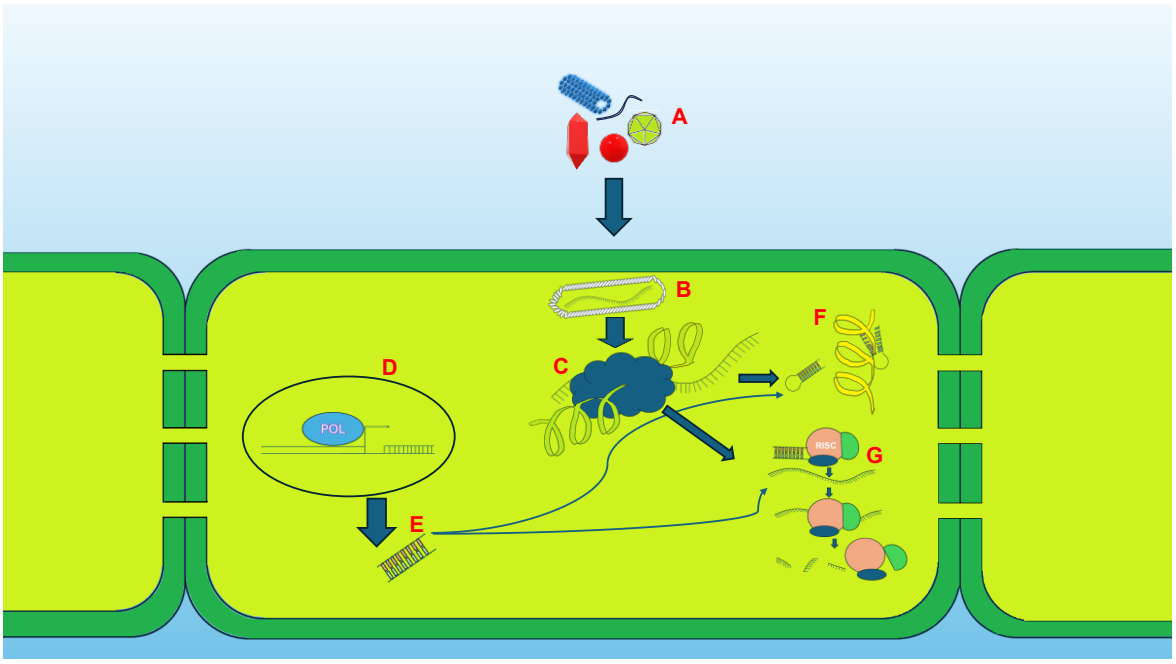

Figure 3. Gene silencing through small interfering RNA in vegetable cells. Internalization of the virus occurs (A), the viral RNA is protected by a viral capsid (B), the virus uses the cellular transcription machinery for the synthesis of viral proteins (C). DNA replication takes place in the nucleus (D), and a small interfering RNA is synthesized, which is subsequently matured and exported to the cytosol where after modifications short hairpin RNA is produced (E). Small RNA can act in several ways such as: by interfering with specific viral proteins (F), or by binding to the viral RNA and through the RNA-induced silencing complex (RISC) who interferes with the expression of viral genes (G).

which is the impact of gene silencing strategies against mixed viral infections and their effect on cross-protection (Mohamed *et al*., 2022; Leonetti *et al*., 2021). Turco *et al*. (2018) characterized the antiviral defense of cultivated solanaceous plants that carry mixed infections. In this experiment (Turco *et al*., 2018), tomato plants with resistance against the Chilean strain of PepMV (CH2) indirectly exerted a resistance effect to infection by the Peruvian genotype (LP strain) of the same virus. LP strain exposure to tomato plants preinfected with PepMV CH2 strain promoted alterations of the consensus genomic sequences in the viral species, indicating a broad potential for cross-protection Turco *et al*. (2018). Additionally, they found a broad association of small interfering RNA (siRNAs) with catalytic components of gene silencing, such as AGO 1, AGO 2, and AGO 3, involved with RNA interference (RNAi) in the cleavage of viral sequences (Figure 2). The authors remarked that the LP quasispecies (genotypic variants) undergo rapid evolution within an infected plant through the appearance and fixation of point mutations.

Additionally, CH2 quasispecies were found to evolve rapidly, which can eventually create aggressive genetic variants with severe disease symptoms, and genetic stability is considered important for cross-protection against particular viral strains (Ziebell and Carr, 2010). The effectiveness of gene sequences mediating resistance in plants against viral coinfections has been addressed. Wen *et al*. (2013) highlighted the pivotal role of the *TOM*1 gene in the replication process of *Hibiscus latent Singapore virus* (HLSV) and TMV. In a study conducted on *N. benthamiana*, the plant exhibited mild symptoms rather than severe systemic necrosis, and cross-protection against mixed infections was demonstrated. While the mechanism behind protection during double viral infection remains unknown, it is established that the *TOM* genes play a crucial role in *Tobamovirus* multiplication.

Subsequently, Moreno-Pérez *et al*. (2016) reported that *Pepper mild mottle virus* (PMMoV) with the next amino acid mutations: M138N, T43K + D50G, L13F + G66V, M138N, T43K D50G and A87G in coat protein (CP) manages to overcome mediated resistance in plants, producing pleiotropic effects and influencing the expansion of the host's range. In this experiment, phenotypes sensitive and resistant to the *L* gene of pepper were evaluated in a proficiency test, finding that the effects that break the resistance of the plant depend on the specific mutation, the genotype of the susceptible host, and the type of infection (monoinfection or mixed). Consequently, pleiotropic effects can be negative, positive, or absent based on the nature of the virus.

Wang *et al*. (2021) reported advances in the understanding of RNAi activation during viral infection. The experiments conducted in *N. benthamiana* demonstrated that wounding activated the calmodulin-3 binding transcription factor (CAMTA3) and BN2 bifunctional nuclease via the Ca^{++} ion-associated signaling cascade (Toyota *et al*., 2018). This set of proteins is responsible for assembling and stabilizing

mRNA coding for key components of the RNAi machinery. The authors mention that ARGONAUTE1 and DICER-LIKE1 play a leading role in the degradation of microRNAs. CAMTA3 or BN2 knockout plants showed increased susceptibility to *Begomovirus* and *Potyvirus* infections, and particularly, the V2 protein present in geminiviruses disrupts the calmodulin-CAMTA3 interaction to counteract RNAi defense. These findings associate Ca^{++} signaling with the initiation of RNAi-mediated defense and reveal the versatility of antiviral defense. Elvira-González *et al*. (2021) reported the profile of siRNAs obtained from *S. lycopersicum* subjected to mixed infection with unrelated viruses. In the experiment, tomato plants were inoculated with CMV, PepMV, and *Southern tomato virus* (STV), and it was found that up to 47 miRNAs were differentially expressed in plants that were infected with these viruses compared to control plants inoculated. Finally, the siRNAs with differential expression found in this work were mainly involved in fundamental plant processes, such as development, metabolism, and abiotic and biotic stress, and the frequencies of siRNAs were not uniform, nor did they show influence by the type of virus. This is one of the first reports establishing the characteristics of plant defense mediated by RNAi silencing when a plant is faced with a mixed infection.

In plants, RNAi-mediated antiviral defense is mediated by complementarity with foreign sequences (viral sequences). The synergistic characteristics due to mixed infections raise broad and robust questions about the specificity and coverage of the action of RNAi sequences against viruses involved in coinfections. Actually, few indicators evidence differences as clear as the RNAi produced by the plant to deal with monoinfection *vs.* mixed infections.

Viral protein components involved in mixed infections

The continuity of the viral lineage when infecting a plant can be considered the common purpose of plant viruses; under this premise, a virus has a series of proteins responsible for conducting three fundamental situations. First, adhesion to the host cell, followed by internalization mediated by various means (masking, protein mimicry, etc.), and finally genome replication. The probability of success in conducting all the stages will depend on factors that are not typical of viruses (defense system mediated by proteins and plant genetic material) and on their own characteristics. Then, we analyze the most recognized viral proteins and discern the intricate processes they overcome to achieve their goal. We emphasize that the structures described in this section are based on characteristics observed in monoinfection because studies evaluating the characteristics of these proteins in mixed infections are scarce.

The CP plays a fundamental role in the assembly of viral particles and has

been established as a target for the development of molecules against plant viruses (Wu *et al*., 2022). Basu *et al*. (2021) reported that in the next begomoviruses: *Tomato leaf curl New Delhi virus* (ToLCNDV) and *Tomato leaf curl Gujarat virus* (ToLCGuV) the proteins AV-2 (pre-CP) and AC-2 (transcriptional activator protein) have functioned as modulators of the viral coinfection process in tomato and *N. benthamiana*. Both viruses presented synergism in an *in vitro* assay in tomato and *N. benthamiana* plants and promoted the appearance of extreme symptoms of infection. The authors suggest that AC2 and AV2 of ToLCGuV asymmetrically increase ToLCNDV accumulation and that the presence of this type of viral protein favors suppression of gene silencing in *N. benthamiana* and tomato plants. Through a reversal of the GFP silencing assay, (briefly, transgenic plants that expressed AC2 and AV2 were validated through GFP assay; subsequently, plants were infiltrated with *A. tumefaciens* containing desired constructs) suppressor activity of the viral encoded proteins was obtained with the siRNAs isolations after 6 days post-infection. The authors suggested that AV2 suppressor activity is attributed to blocking the propagation of RNA silencing signals in the leaves and concluded that the proteins encoded by ToLCGuV promote the efficient replication of ToLCNDV via the antiviral silencing pathway.In some genera of plant viruses, CP is essential in the infection process by recruiting and interacting with various cellular structures (Bennett and Agbandje-McKenna, 2020). Mochizuki *et al*. (2014) showed that the CP mutation (P129) reduced the expression of genes related to chloroplast function and photosynthesis in CMV-infected tobacco plants. Other proteins of the replication process, such as virulence complement 2b, were also associated with asymptomatic phenotypes and low viral titers. The authors concluded that the effect of the mutation increased the capacity for pathogenicity due to CMV acquiring virulence through CP amino acid mutations, leading to structural abnormalities in chloroplasts. Subsequently, Zhang *et al*. (2017) reported that the E96 mutation of CP from *Cucumber green mottle mosaic virus* (CGMMV) is important to the developmental pathogenesis process. *In vitro* assays with *N. benthamiana* E96 CGMMV mutants produced a successful infection, and this study indicated that modifications in the CP assembly site in cells can be affected by mutations in the crucial amino acid E96. For this reason, other mutants conducted in this same study, such as E96A and E96K, and three double mutants, V94A-E96K, V97-E96K, and T104A-E96K, showed only late systemic traits of infection in *N. benthamiana*, highlighting the role of the E96 residue in the infection process.

Shi *et al*. (2023) reported that in the *Tobamovirus* genus the CP and MP display the ability to interact to facilitate viral movement through PD. It was determined *in vitro* that the MP protein region between residues 78 and 128 was important for viral movement. Specifically, the threonine residue at position 107 in MP ($T107^{MP}$) is crucial for CP-MP interaction and the T107A substitution delays systemic

CGMMV infection in *N. benthamiana*. Mutations in other evaluated residues, such as $F17A^{MP}$ and $D97A^{MP}$ inhibited CGMMV infection, suggesting that the latter have no role in the CP-MP interaction but regulate CP-independent MP function. Additionally, it was found that the coexpression of MP and CP from CGMMV in *N. benthamiana* increases the accumulation of CP and that the interaction of both proteins inhibits the defense response mediated by salicylic acid in the early stages of infection. Finally, the authors concluded that the suppression of the host's antiviral defense triggered by CP-MP interaction facilitates systemic infection.

Understanding the role of CP in the infection process has allowed notable advances that may be applied in the future for the control of viral infections in plants. Liu *et al*. (2020) reported that CGMMV mutant strains presented useful phenotypic characteristics for virus-specific control by cross-protection. Through site-directed mutagenesis, it was possible to obtain CP^{CGMMV} mutants that were subsequently inoculated in *N. benthamiana* until symptoms were observed. Specifically, the CP mutation of residue D89 (D89A) reduced symptoms of CGMMV infection in leaves and veins. The obtained CP mutant clones showed an important feature for the regulation of CGMMV virulence and can be developed as a successful model for the containment of virulent strains of this specific type of virus. Sharma *et al*. (2020) also suggested the great utility of CP^{TMV} as a model for generating mutants to confer viral resistance to future infections and develop cross-protection. Specifically, the CP^{TMV} T42W and E50Q mutants showed the presence of highly stable long nonhelical rods that interfere with the accumulation of MP and promote the molecular disassembly of TMV in the host cell. Because cross-protection is considered within mixed viral infections, the results indicate that this type of methodology could provide adequate protection against TMV infection.

We have previously described the characteristics of reported proteins during the viral infection process. Although the functions are established, new research is needed to understand what happens when orthologous proteins from different virus species interact in a mixed infection, and why not estimating to determine the extent to which a protein-protein interaction favors viral synergism or crossed protection, or even if such an interaction could trigger the development of viral antagonism.

PERSPECTIVES AND CONCLUSION

Viral control strategies depend to a large extent on the understanding of natural phenomena including viral pathogenesis and its aspects, such as mono- and coinfections. Preliminary investigations in this field may in the future be the foundation for the implementation of programs for the control of mixed viral infections in vegetables of agronomic interest. One of the most accepted theories

about mixed infections from related viruses is that since they have similar entry mechanisms into the host cell and share the arsenal of proteins involved in the infection process, the chances of developing coinfection increase (Ng and Falk, 2006; Tamborindeguy *et al*., 2023). Although this phenomenon is possible and demonstrable in multipartite viruses of the *Closteroviridae, Geminiviridae, Nanoviridae, Potyviridae*, and *Rhabdoviridae* families (Lucía-Sanz and Manrubia, 2017), it does not work the same way for other viruses. Figure 4 shows a mixed infection of *tobamovirus* with *potexvirus,* with a synergistic effect on the phenotype. However, for the *tobamovirus* genus, where some species present genetic similarity (example: *Tomato mottle mosaic virus* (ToMMV) and *Tomato mosaic virus* (ToMV)) there are no reports on mixed infections (Tettey *et al*., 2022).

Syller and Grupa (2016) highlight that related viral infections generally produce antagonism; in this context, studies conducted on *Turnip crinkle virus* (TCV) establish that positive sense (+) RNA viruses, through a viral function that is still unknown, "denies" the offspring viruses the possibility of replicating their genomes in cells from their "parents", leading to highly homologous superinfecting viruses that are indistinguishable from the progeny (Tatineni and French, 2016). Currently, SIE explains how viruses are evolutionarily selected by maintaining an optimal error frequency in the progeny genomes. Although primarily based on observations made in TCV, this new model could be applied to other viruses to understand how SIE influences evolutionarily conserved virus-encoded function that minimizes the

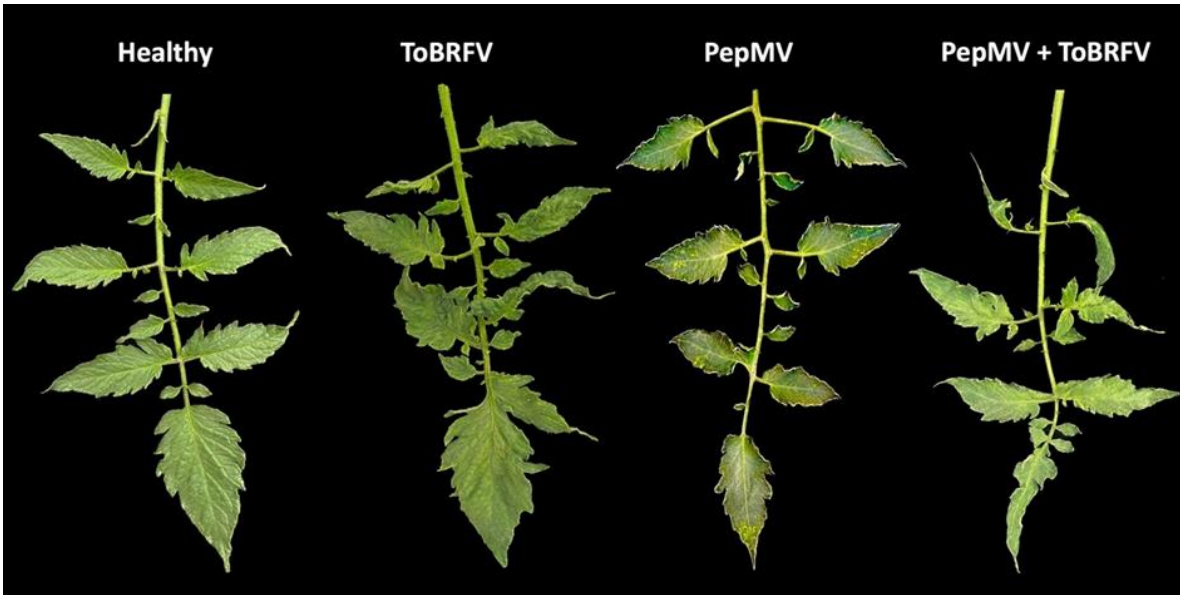

Figure 4. Symptom development on tomato leaves (cv. Macizo) infected with TBRFV and PepMV in a mono and mixed infections (synergism) at 21 days post-inoculation, (photography donated kindly by the Dr. Tovar-Pedraza group, CIAD Culiacan, México).

proliferation of replication errors. In this way, SIE is established as a molecular mechanism that promotes antagonism and must be strengthened in solanaceous and cucurbitaceous model plants (Yin *et al*., 2022; Zhou *et al*., 2019).

High-throughput sequencing (HTS) technologies make possible to discern the origins of mixed infections more accurately and quickly. Specifically, technologies such as Oxford Nanopore Technologies (ONT) focused on long reads, present sufficient sensitivity to detect related viral agents, as reported by previous authors (Abou Kubaa *et al*., 2023; Lee *et al*., 2022; van Rengs *et al*., 2022). Additionally, RNAi is becoming a versatile and ecological alternative for the protection of crops of economic interest (Bharathi *et al*., 2023). Host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS) are trends in RNAi delivery technologies. These methodologies make use of encapsulation to improve the effectiveness of gene silencing by RNAi; also, it confers protection and stability to RNA, preventing it from undergoing enzymatic or pH modifications (Mitter *et al*., 2017). However, the greatest challenge of these procedures lies in achieving stability and avoiding premature degradation of RNAi in the environment. Most of the current research on encapsulated RNAi is mainly focused on oral administration to control insects by silencing essential genes (Hernández-Soto and Chacón-Cerdas, 2021). There is incipient research on the application of RNAi for the improvement of the characteristics of crops of interest as reported by Termolino, (2021) but are no reports of this technology being used to counteract mixed viral infections. However, the first evidence of its use and effectiveness against viruses that infect crops of commercial interest has already been established in previous reports (Worrall *et al*., 2019).

Thanks to the application of new sequencing technologies, the virus-host relationship has been elucidated. Viral synergism is the most studied coinfection, and its objective is to enhance dual resources to achieve infection. By comparison, antagonism or neutral coinfection is a way for viruses to proliferate while competing with other organisms, decreasing the establishment chances of the latter. New ecological niches, hosts, vectors, and modes of transmission of viruses that infect plants are taking place in nature.

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