UNIVERSIDAD DE MURCIA

Escuela de Doctorado

TESIS DOCTORAL

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Cucurbit-virus-aphid microbiome interaction in the development of viral diseases

AUTORA Dª. Celia de Moya Ruiz

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“Non est ad astra mollis e terris via”
“Memento vivire”

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RESUMEN

1. RESUMEN

La producción de hortalizas se enfrenta a constantes amenazas debido a la alta frecuencia de plagas y patógenos que afectan significativamente tanto la calidad como el rendimiento de los cultivos. En la producción de cucurbitáceas, *Aphis gossypii* Glover es la plaga más prevalente en la cuenca mediterránea, causando graves daños al actuar como vector de numerosas enfermedades virales. Entre los virus transmitidos destacan el *Polerovirus CABYV* (cucurbit aphid-borne yellows virus, CABYV), *Cucumovirus CMV* (cucumber mosaic virus, CMV), *Potyvirus citrulli* (watermelon mosaic virus, WMV), *Potyvirus cucurbitaflavitesselati* (zucchini yellow mosaic virus, ZYMV) y *Potyvirus papayanuli* (papaya ringspot virus, PRSV), por su amplia distribución en las zonas productoras. Además, en los últimos años, las infecciones mixtas causadas por más de un virus en la misma planta se han reconocido como un factor biótico común en el desarrollo de las epidemias. Estas infecciones mixtas combinadas con factores abióticos como el aumento de temperaturas, pueden influir en la dinámica epidemiológica de los cultivos. En este contexto, un conocimiento más profundo de las interacciones entre pulgón, virus y planta, considerando los efectos combinados de estos factores bióticos y abióticos, resulta esencial para entender la compleja epidemiología de las enfermedades virales en cultivos de cucurbitáceas y así contribuir al diseño de estrategias efectivas para su prevención y control. Además, estudios recientes destacan la relevancia del microbioma asociado al pulgón en su aptitud fisiológica y en la transmisión de virus (persistentes y no persistentes), abriendo nuevas perspectivas para el desarrollo de estrategias innovadoras en el manejo de plagas. Por ello, integrar estos conocimientos multidisciplinarios es clave para proteger la sostenibilidad de los sistemas agrícolas frente a un contexto global de cambio climático y presión fitosanitaria creciente.

El objetivo principal de esta tesis doctoral ha sido (**I**) evaluar el estado y la distribución actual de los virus trasmitidos por pulgón en las principales zonas productoras de cucurbitáceas de España (Región de Murcia, Alicante y Castilla-La Mancha) a través de la monitorización molecular de diferentes explotaciones agrícolas (cultivo de melón y de sandía). Ampliar nuestro conocimiento sobre la influencia de

factores (**II**) bióticos (infecciones virales mixtas simultáneas y secuenciales) y (**III**) abióticos (temperatura) en los patrones epidemiológicos de las principales enfermedades virales presentes en estos cultivos. Y finalmente, (**IV**) examinar el papel de los huéspedes e infecciones virales en la estructura y composición del microbioma asociado a pulgón (*Aphis gossypii* Glover).

En primer lugar, se realizó una revisión bibliográfica profunda del estado actual y distribución de los virus transmitidos por pulgón en las principales regiones productoras de cucurbitáceas en España en el periodo comprendido entre 2011 y 2022, incluyendo información relevante acerca de la sintomatología, gama de huéspedes, vectores de transmisión, distribución geográfica, prevalencia y estrategias de control para estas virosis. Tras esto se estudió **(I)** la distribución de los principales virus transmitidos por pulgón incluyendo aquellos que son transmitidos por mosca blanca en muestras sintomáticas con amarilleos y mosaicos de dos cultivos de cucurbitáceas (melón y sandía) de tres zonas productoras de España (Región de Murcia, Alicante y Castilla-La Mancha) durante los años consecutivos 2021, 2022 y 2023. Se observó que CABYV y WMV fueron los virus más ampliamente distribuidos en los tres años y cultivos, tanto en infección simple como en infección mixta. Sin embargo, una proporción importante de muestras con síntomas tipo amarilleo fueron negativas para los virus analizados. Usando una metodología de detección independiente de secuencia, identificamos un nuevo polerovirus, *Polerovirus PABYV* (pepo aphid-borne yellows virus, PABYV), que no había sido descrito anteriormente en los cultivos de cucurbitáceas de España, aunque ya estaba presente en otras regiones de Europa. Este estudio mostró que PABYV emergió en 2018 y que había permanecido oculto bajo los síntomas de amarilleo causados por CABYV, puesto que fue detectado con alta frecuencia en infecciones mixtas con CABYV. Además, con esta metodología se detectó el virus críptico *Alphaendornavirus cucumis* (cucumis melo endornavirus, CmEV) por primera en muestras de calabaza. La caracterización genética de estas poblaciones mostró que mientras que PABYV y CmEV son poblaciones genéticamente homogéneas, los aislados contemporáneos de CABYV han reemplazado a los aislados antiguos. Estos nuevos aislados parecen causar síntomas más graves en planta y tener una mayor acumulación. *En conjunto, los hallazgos obtenidos subrayan la necesidad de establecer sistemas de*

vigilancia fitosanitaria más eficaces y continuos, así como de fomentar la investigación enfocada en la detección de infecciones virales mixtas y emergentes, con el objetivo de mejorar las estrategias de diagnóstico y control. Además, se evidencia la importancia de considerar enfoques de análisis más amplios y no basados exclusivamente en los de virus conocidos, dado que nuevas amenazas pueden estar enmascaradas por síntomas previamente atribuidos a patógenos ya identificados.

En segundo lugar, **(II)** se estudió cómo el orden temporal de las infecciones mixtas podría afectar la dinámica de las poblaciones virales como un factor biótico crítico en el desarrollo de las enfermedades virales. Para ello, se hizo un ensayo experimental en el que se evaluó la carga viral de CABYV (modo de transmisión persistente) y WMV (modo de transmisión no persistente) en plantas de melón bajo diferente orden de infección (secuencial) y de forma paralela bajo infección simultánea (co-infección). La co-infección de CABYV y WMV resultó en una mayor acumulación de ambos virus, lo que sugiere un efecto doble sinérgico. En el caso de las infecciones secuenciales, se observó que el orden temporal de la infección afecta el tipo de interacción entre estos dos virus en melón, con una interacción sinérgica cuando CABYV precede a WMV y una interacción antagónica cuando WMV precede a CABYV. Con este estudio, se destaca la importancia de las interacciones bióticas en la configuración de la epidemiología viral y la dinámica de las enfermedades de las plantas en los agro-ecosistemas. Estos hallazgos tienen implicaciones importantes en la comprensión de la epidemiología viral en los cultivos, ya que sugieren que no solo la presencia de múltiples virus influye en la evolución de una enfermedad, sino también el momento en que estos virus infectan a la planta. Este factor temporal podría ser determinante en la intensidad de los síntomas, la eficiencia de transmisión por vectores y la propagación del virus en campo. *En conjunto, este estudio subraya la necesidad de incorporar el análisis de interacciones bióticas dinámicas en los modelos epidemiológicos de enfermedades virales de plantas. Comprender cómo estas interacciones dependen del orden de infección no solo mejora nuestro conocimiento de los mecanismos patogénicos, sino que también podría influir en el diseño de estrategias de manejo más precisas y sostenibles, orientadas a limitar los daños causados por infecciones virales complejas en los agro-ecosistemas.*

En tercer lugar, **(III)** se estudió cómo la combinación de factores abióticos (temperatura) y bióticos (infección viral por WMV) tienen un efecto en la respuesta de la planta y por lo tanto en la interacción virus-planta. Para ello, se examinó el efecto de la infección por WMV y el estrés por calor (20/14 °C, 26/20 °C y 32/24 °C) en la respuesta génica de dos especies de planta (melón y calabacín) con alta y baja tolerancia a estrés por temperatura mediante un estudio de transcriptómica por 3' mRNA-seq. Además, se cuantificó la carga viral de WMV bajo las diferentes condiciones mencionadas. Los resultados mostraron que la carga viral de WMV fue mucho mayor en las plantas de calabacín que de melón y que además fue dependiente de temperatura y el grado de tolerancia de las plantas a esta. En cuanto a la respuesta génica de las plantas a ambos estreses, se observó que el porcentaje de genes diferencialmente expresados (DEGs) fue mayor en las plantas termo-susceptibles para ambas especies de cucurbitáceas en la combinación de infección viral y bajas temperaturas. Por otro lado, se encontraron DEGs exclusivos en la respuesta a la combinación de ambos estreses para las variedades termo-tolerantes y en concreto, cuatro genes ortólogos ligados al estrés por temperatura y por infección viral en melón y calabacín. Tres de ellos, MELO3C023308, MELO3C024920 y Cp4.1LG05g12560 mostraron una expresión significativamente dependiente de la temperatura bajo infección por WMV y potencialmente codifican, una proteína F-box**,** un transportador de iones metálicos y un factor relacionado con la fotomorfogénesis, respectivamente**.** Este trabajo, muestra la complejidad de las interacciones virus-planta bajo la combinación de estreses abióticos y bióticos; y proporciona información sobre el desarrollo de estrategias para mejorar la resiliencia de las plantas en condiciones climáticas cambiantes. Estos hallazgos destacan que la resiliencia de las plantas frente a infecciones virales no puede entenderse sin considerar el entorno ambiental, y en particular el impacto del estrés térmico, que puede potenciar o mitigar los efectos de la enfermedad. Asimismo, la identificación de genes clave involucrados en la respuesta combinada a factores bióticos y abióticos abre nuevas posibilidades para el mejoramiento genético de cultivos más resistentes, especialmente relevantes en el contexto de un clima global cambiante. *En conjunto, este trabajo refuerza la idea de que una comprensión integrada de los estreses múltiples es esencial para diseñar estrategias agrícolas más sostenibles y eficaces, capaces de anticipar los*

efectos de las condiciones ambientales extremas sobre la salud vegetal y la incidencia de enfermedades virales en cultivos estratégicos como las cucurbitáceas.

Por último, **(IV)** se evaluó cómo la estructura y composición del microbioma de pulgón (*A. gossypii* Glover) es alterado por su alimentación en diferentes especies de plantas y cuando están infectadas con CABYV (transmisión persistente) y/o WMV (transmisión no persistente). Usando análisis de secuenciación del gen 16S rRNA, se comparó la comunidad bacteriana de pulgones alimentándose en plantas de melón, incluyendo la transición a plantas de pepino y posteriormente de regreso a plantas de melón. Además, se evaluó el microbioma de pulgones alimentándose en plantas de melón infectadas con CABYV y WMV, tanto en infecciones simples como mixtas. En este estudio, se observó que la diversidad de la comunidad de bacterias fue mayor en los pulgones alimentados en melón que en pepino por lo que el microbioma se ve fuertemente afectado por el huésped del que se alimenta. También se observó que la diversidad microbiana fue mayor en los pulgones alimentados en plantas sanas que en plantas infectadas. Los géneros más abundantes fueron *Buchnera* sp. y *Arsenophonus* sp., cuyas abundancias se vieron modificadas en función de la alimentación del pulgón, observándose una correlación negativa entre ambos géneros cuando estos se alimentaron de plantas infectadas. En general, estos resultados ofrecen nuevas perspectivas sobre la interacción entre la comunidad bacteriana en las poblaciones de *A. gossypii* Glover que se alimentan de diferentes huéspedes, incluidas plantas de melón infectadas con virus. Estos resultados ofrecen una comprensión más profunda de los mecanismos ecológicos y microbiológicos que median la interacción entre vectores y virus de plantas, con implicaciones relevantes para la epidemiología de las virosis en cultivos. El microbioma del vector, lejos de ser una comunidad estable y pasiva, actúa como un componente dinámico que responde a condiciones externas como el hospedador vegetal y su estado sanitario, pudiendo afectar, directa o indirectamente, la eficiencia de transmisión de virus, el comportamiento del insecto y su aptitud*. En conjunto, este trabajo destaca la importancia de considerar al vector como un sistema holobiótico, cuya capacidad de transmisión y adaptación está influida no solo por factores genéticos o ambientales, sino también por las complejas interacciones entre su microbiota y el entorno agroecológico. Este enfoque integrado*

podría abrir nuevas vías para el desarrollo de estrategias de control más precisas, que incluyan la manipulación del microbioma vectorial como herramienta para reducir la propagación de virus en los cultivos agrícolas.

En definitiva, los resultados obtenidos de esta tesis doctoral contribuyen a generar un conocimiento fundamental sobre los patrones epidemiológicos de las enfermedades virales en estos cultivos considerando el papel fundamental de los tipos de infecciones mixtas y la temperatura como factores bióticos y abióticos que modulan el curso de las enfermedades virales; además de caracterizar el bacterioma en las poblaciones de pulgones mediante enfoques multinivel integrados que podrían abrir un nuevo paradigma con el fin de generar información fundamental sobre los mecanismos ecológicos detrás de los efectos de las bacterias y virus en los pulgones. Esto permitirá explorar nuevas estrategias de biocontrol aplicado y, a su vez, proporcionar un avance significativo en la búsqueda a largo plazo para controlar las plagas de pulgones y las enfermedades virales en los cultivos hortícolas.

ABSTRACT

2. ABSTRACT

Vegetable production faces constant threats due to the high frequency of pests and pathogens that significantly affect both the quality and yield of crops. In cucurbit production, *Aphis gossypii* Glover is the most prevalent pest in the Mediterranean basin, causing severe damage by acting as a vector for numerous viral diseases. Among the viruses transmitted, *Polerovirus CABYV* (cucurbit aphid-borne yellows virus, CABYV), *Cucumovirus CMV* (cucumber mosaic virus, CMV), *Potyvirus citrulli* (watermelon mosaic virus, WMV), *Potyvirus cucurbitaflavitesselati* (zucchini yellow mosaic virus, ZYMV), and *Potyvirus papayanuli* (papaya ringspot virus, PRSV) stand out due to their widespread distribution in production areas. In recent years, mixed infections caused by more than one virus in the same plant have been recognized as a common biotic factor in the development of epidemics. These mixed infections, combined with abiotic factors such as rising temperatures, can influence the epidemiological dynamics of crops. In this context, a deeper understanding of the interactions between aphid, virus, and plant—taking into account the combined effects of these biotic and abiotic factors—is essential to comprehend the complex epidemiology of viral diseases in cucurbit crops and to contribute to the design of effective prevention and control strategies. Moreover, recent studies highlight the relevance of the aphid-associated microbiome in its physiological fitness and in the transmission of both persistent and non-persistent viruses, opening new perspectives for the development of innovative pest management strategies. Therefore, integrating this multidisciplinary knowledge is key to safeguarding the sustainability of agricultural systems in the face of a global context of climate change and increasing phytosanitary pressure.

The main goal of this thesis has been to (**I**) assess the current status and distribution of aphid-transmitted viruses in the main cucurbit-producing regions of Spain (Región de Murcia, Alicante y Castilla-La Mancha) through the monitoring of different agricultural fields. Additionally, the study aimed to expand our understanding of the influence of (**II**) biotic (simultaneous and sequential mixed infections) and (**III**) abiotic (temperature) factors on the epidemiological patterns of major viral diseases affecting these crops, as

well as to (**IV**) examine the role of host plants and viral infections in shaping the structure and composition of the microbiome associated with *Aphis gossypii* Glover.

First, a comprehensive literature review was conducted to examine the current state and distribution of aphid-transmitted viruses in the main cucurbit-producing regions of Spain during the 2011–2022 period. This included relevant information on symptomatology, host range, transmission vectors, geographic distribution, prevalence, and control strategies. This was followed by **(I)** a study of the distribution of the main aphid-transmitted viruses, including some whitefly-transmitted viruses, in symptomatic cucurbit crop samples (melon and watermelon) showing yellowing and mosaic symptoms, collected from three major production regions of Spain (Murcia, Alicante, and Castilla-La Mancha) between 2021, 2022, and 2023. CABYV and WMV were the most widely distributed viruses across all three years and both crops. However, a significant proportion of yellowing-type symptomatic samples tested negative for the analyzed viruses. Using a sequence-independent detection method, a novel polerovirus was identified, *Polerovirus PABYV* (pepo aphid-borne yellows virus, PABYV), not previously reported in cucurbit crops in Spain, although it was already present in other regions of Europe. This study showed that this virus emerged in 2018 and had remained undetected, masked by symptoms attributed to CABYV, as it was frequently found in mixed infections with CABYV. Additionally, *Alphaendornavirus cucumis* (cucumis melo endornavirus, CmEV), a cryptic Alphaendornavirus, was detected for the first time in pumpkin samples. Genetic characterization revealed that while PABYV and CmEV populations are genetically homogeneous, contemporary CABYV isolates have replaced older variants, appearing to cause more severe symptoms and higher viral accumulation. *Overall, the findings highlight the need to establish more effective and continuous phytosanitary surveillance systems, as well as to promote research focused on the detection of mixed and emerging viral infections, with the aim of improving diagnostic and control strategies. Moreover, the importance of considering broader analytical approaches—beyond those based exclusively on known viruses—is evident, since new threats may be masked by symptoms previously attributed to already identified pathogens.*

Second, it was studied **(II)** how the temporal order of mixed infections could influence viral population dynamics, as a critical biotic factor in disease development. An experimental assay was conducted to assess the viral load of CABYV (persistently transmitted) and WMV (non-persistently transmitted) in melon plants under different infection sequences (sequential) and in co-infection scenarios (simultaneous). Co-infection led to a greater accumulation of both viruses, indicating a dual synergistic effect. In sequential infections, the order of infection significantly influenced virus interaction: synergy occurred when CABYV preceded WMV, while antagonism was observed when WMV preceded CABYV. This study highlights the importance of biotic interactions in shaping viral epidemiology and the dynamics of plant diseases in agro-ecosystems. These findings have significant implications for understanding viral epidemiology in crops, as they suggest that not only the presence of multiple viruses influences disease development, but also the timing of virus infection in the plant. This temporal factor could be decisive in symptom severity, vector transmission efficiency, and virus spread in the field. *Together, this study underscores the need to incorporate the analysis of dynamic biotic interactions into epidemiological models of plant viral diseases. Understanding how these interactions depend on the order of infection not only enhances our knowledge of pathogenic mechanisms but could also influence the design of more precise and sustainable management strategies aimed at limiting damage caused by complex viral infections in agro-ecosystems.*

Third, it was investigated **(III)** how the combination of abiotic (temperature) and biotic (viral infection) factors affects plant responses and, consequently, virus–plant interactions. It was examined the effects of WMV infection and heat stress (20/14 °C, 26/20 °C, and 32/24 °C) on the gene expression profiles of two plant species (melon and zucchini) with high and low heat stress tolerance using 3’ mRNA-seq transcriptomic analysis. WMV viral load was also quantified under the different conditions. Results showed that WMV accumulation was significantly higher in zucchini than in melon and was dependent on both temperature and the plant’s tolerance level. Regarding gene expression responses, a higher percentage of differentially expressed genes (DEGs) was found in heat-susceptible plants of both species under combined virus and low-temperature stress. Moreover, specific DEGs were identified in thermotolerant varieties

exposed to both stresses, including four orthologous genes linked to temperature and viral stress responses in melon and zucchini. Three of them, MELO3C023308, MELO3C024920, and Cp4.1LG05g12560, showed significantly temperature-dependent expression under WMV infection and potentially encode an F-box protein, a metal ion transporter, and a photomorphogenesis-related factor, respectively. This work highlights the complexity of virus-plant interactions under combined abiotic and biotic stresses and provides insights for developing strategies to enhance plant resilience under changing climatic conditions. These findings emphasize that plant resilience to viral infections cannot be understood without considering the environmental context, particularly the impact of heat stress, which can either amplify or mitigate disease effects. Furthermore, identifying key genes involved in the combined response to biotic and abiotic factors opens new possibilities for breeding more resistant crops, especially relevant in the context of global climate change. *Overall, this study reinforces the idea that an integrated understanding of multiple stresses is essential for designing more sustainable and effective agricultural strategies, capable of anticipating the effects of extreme environmental conditions on plant health and the incidence of viral diseases in key crops such as cucurbits.*

Finally, it was evaluated **(IV)** how the structure and composition of the aphid (*A. gossypii* Glover) microbiome is altered by feeding on different plant species and in the presence of CABYV (persistent transmission) and/or WMV (no-persistent transmission) infections. Using 16S rRNA gene sequencing, it was compared the bacterial communities of aphids feeding on melon, followed by transitions to cucumber and back to melon plants. It was assessed the microbiome of aphids feeding on melon plants infected with CABYV and WMV, in both single and mixed infections. This study revealed that bacterial diversity was higher in aphids feeding on melon compared to cucumber, indicating that the host plant strongly influences the aphid microbiome. Additionally, microbial diversity was greater in aphids feeding on healthy plants than on infected ones. The most abundant bacterial genera were *Buchnera* sp. and *Arsenophonus* sp., with their relative abundances affected by feeding conditions, and a negative correlation between the two was observed in aphids feeding on infected plants. In general, these results provide new insights into the interaction between the bacterial community in

populations of A. gossypii Glover feeding on different hosts, including virus-infected melon plants. They offer a deeper understanding of the ecological and microbiological mechanisms that mediate the interaction between vectors and plant viruses, with important implications for the epidemiology of viral diseases in crops. The vector's microbiome, far from being a stable and passive community, acts as a dynamic component that responds to external conditions such as the plant host and its health status, potentially affecting—directly or indirectly—the efficiency of virus transmission, insect behavior, and fitness. *Overall, this work highlights the importance of considering the vector as a holobiont system, whose transmission and adaptation capacities are influenced not only by genetic or environmental factors but also by the complex interactions between its microbiota and the agroecological environment. This integrated approach could open new avenues for developing more precise control strategies, including manipulation of the vector microbiome as a tool to reduce virus spread in agricultural crops.*

The results of this doctoral thesis contribute to a fundamental understanding of the epidemiological patterns of viral diseases in cucurbit crops, highlighting the pivotal role of mixed infections and temperature as biotic and abiotic modulators of disease progression. Furthermore, by characterizing the aphid bacteriome through integrated multi-level approaches, this work opens new avenues for exploring the ecological mechanisms underlying virus and bacterial interactions in aphids. These findings may facilitate the development of novel biocontrol strategies and represent a significant advance in the long-term efforts to control aphid pests and viral diseases in horticultural crops.

LIST OF ABBREVIATIONS

3. LIST OF ABBREVIATIONS

Virus abbreviations (in alphabetic order)

ACMV *Begomovirus manihotis* (African cassava mosaic virus)

AWMV *Potyvirus algeriaense* (Algerian watermelon mosaic virus)

BChV *Polerovirus BCHV* (beet chlorosis virus)

BICMV blackeye cowpea mosaic virus

BPMV *Comovirus siliquae* (bean pod mottle virus)

BPYV *Crinivirus pseudobetae* (beet pseudoyellows virus)

BTMV *Potyvirus betaceum* (beet mosaic virus)

BWYV *Polerovirus BWYV* (beet western yellow virus)

BYDV barley yellow dwarf virus

BYMV *Potyvirus phaseoluteum* (bean yellow mosaic virus)

BYV *Closterovirus flavibetae* (beet yellows virus)

CABYV *Polerovirus CABYV* (cucurbit aphid-borne yellow virus)

CaMV *Caulimovirus tessellobrassicae* (cauliflower mosaic virus)

CCYV cucurbit chlorotic yellows virus

CFMMV *Tobamovirus maculafructi* (cucumber fruit mottle mosaic virus)

CGMMV *Tobamovirus viridimaculae* (cucumber green mottle mosaic virus)

CHIKV *Alphavirus chikungunya* (chikungunya virus)

CLSV *Aureusvirus cucumis* (cucumber leaf spot virus)

ClYVV *Potyvirus trifolii* (clover yellow vein virus)

CmEV *Alphaendornavirus cucumis* (cucumis melo endornavirus)

CMoV *Umbravirus maculacarotae* (carrot mottle virus)

CMV *Cucumovirus CMV* (cucumber mosaic virus)

CpCDV *Mastrevirus cicerparvi* (chickpea chlorotic dwarf virus)

CPMV *Comovirus vignae* (cowpea mosaic virus)

CuLCrV *Begomovirus cucurbitae* (cucurbit leaf crumple virus)

CuSBV Cucumber soil-borne virus

CymMV *Potexvirus cymbidii* (cymbidium mosaic virus)

CVYV *Ipomovirus cucumisvenaflavi* (cucurbit vein yellows virus)

CYSDV *Crinivirus cucurbitae* (cucurbit yellow stunting disorder virus)

DENV *Orthoflavivirus dengue* (dengue virus)

EMDV *Alphanucleorhabdovirus melongenae* (eggplant mottled dwarf virus)

HIV-1 *Lentivirus humimdef1* (human immunodeficiency virus 1)

MNSV *Gammacarmovirus melonis* (melon necrotic spot carmovirus)

MWMV *Potyvirus citrullimoroccense* (Moroccan watermelon mosaic virus)

OrMV Ornithogalum mosaic virus

ORSV *Tobamovirus odontoglossi* (odontoglossum ringspot virus)

PABYV *Polerovirus PABYV* (pepo aphid-borne yellows virus)

PapMV *Potexvirus papayae* (papaya mosaic virus)

PEMV *Enamovirus PEMV* (pea enation mosaic virus)

PepGMV *Begomovirus capsicummusivi* (pepper golden mosaic virus)

PepMV *Potexvirus pepini* (pepino mosaic virus)

PepMoV *Potyvirus capsimaculae* (pepper mottle virus)

PeVYV-2 *Polerovirus PEVYV2* (pepper vein yellows virus-2)

PeWBVYV *Polerovirus PEWBVYV* (pepper whitefly borne vein yellows virus)

PHYVV *Begomovirus capsicumhuastecoense* (peper huasteco yellow vein virus)

PILV *Capulavirus plantagonis* (plantago lanceolata latent virus)

PLRV *Polerovirus PLRV* (potato leaf roll virus)

PPV *Potyvirus plumpoxi* (plum pox virus)

PRSV *Potyvirus papayanuli* (papaya ring spot virus)

PSV *Cucumovirus PSV* (peanut stunt virus)

PVA *Potyvirus atuberosi* (potato virus A)

PVX *Potexvirus ecspotati* (potato virus X)

PVY *Potyvirus yituberosi* (potato virus Y)

SARS-CoV-2 *Betacoronavirus pandemicum* (severe acute respiratory syndrome coronavirus 2)

SLCV *Begomovirus cucurbitapeponis* (squash leaf curl virus)

SMV *Potyvirus glycitessellati* (soybean mosaic virus)

SqMV *Comovirus cucurbitae* (squash mosaic virus)

SRBSDV *Fijivirus boryzae* (southern rice black-streaked dwarf virus)

TeMV *Potyvirus telfairiae* (telfairia mosaic virus)

TEV *Potyvirus nicotianainsculpentis* (tobacco etch virus)

TGMV *Tobamovirus mititessellati* (tobacco mild green mosaic virus)

TMV *Tobamovirus tabaci* (tobacco mosaic virus)

TNV tobacco necrosis virus

ToCV *Crinivirus tomatichlorosis* (tomato chlorosis virus)

ToLCNDV *Begomovirus solanumdelhiense* (tomato leaf curl New Delhi virus)

ToMoV *Begomovirus solanumvariati* (tomato mottle virus)

TSWV *Orthotospovirus tomatomaculae* (tomato spotted wilt virus)

TuMV *Potyvirus rapae* (turnip mosaic virus)

TYLCV *Begomovirus coheni* (tomato yellow leaf curl virus)

WLMV *Potyvirus citrullufolimaculae* (watermelon leaf mottle virus)

WMV *Potyvirus citrulli* (watermelon mosaic virus)

WmCSV *Begomovirus citrulli* (watermelon chlorotic stunt virus)

ZIKV *Orthoflavivirus zikaense* (zika virus)

ZYFV *Potyvirus pepo* (zucchini yellow fleck virus)

ZYMV *Potyvirus cucurbitaflavitesselati* (zucchini yellow mosaic virus)

Other abbreviations (in alphabetical order)

3' UTR 3' untranslated region

5' UTR 5' untranslated region

ABA Abscisic acid

AMOVA Analysis of molecular variance

ANOVA Analysis of variance

ASVs Amplicon sequence variants

BIC Bayesian information criterion

CP Coat protein

CRISPR Clustered regularly interspaced short palindromic repeats

CT Threshold cycle

CuGenDB Cucurbit genomics database

D Tajima's D

DEG Differentially expressed genes

dN Number of non-synonymous substitutions per non-synonymous site

DNA Deoxyribonucleic acid

DPI Days post-inoculation

dS	Number of synonymous substitutions per synonymous site
dsDNA	Double-stranded DNA
EMBL-EBI	European molecular biology laboratory - European bioinformatics institute
EPPO	European plant protection organization
ETI	Effector-triggered immunity
eIF4E	Eukaryotic translation initiation factor 4E
FDR	False discovery rate
GO	Gene ontology
gRNA	Genomic RNA
Hd	Haplotype diversity
HTS	High-throughput sequencing
KST	Nucleotide sequence-based statistic
log2FC	Log2 fold change
NB-LRR	Nucleotide-binding Leucine-rich repeat proteins
NCBI	National center for biotechnology information
NGS	Next generation sequencing
Nt	Nucleotide
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PCA	Principal component analysis
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance

PI	Genetic diversity
RawPE	Raw paired-end reads
RdRp	Viral RNA dependent RNA polymerase
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RT	Reverse transcription
RT-qPCR	Quantitative reverse transcription PCR
SA	Salicylic acid
SCI	Science citation index
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SRA	Sequence Read Archive
TS	Thermo-susceptible
TT	Thermo-tolerant
UPGMA	Unweighted pair-group method with arithmetic mean

INTRODUCTION

4. INTRODUCTION

Plant diseases have significant social, economic, and political impacts by affecting essential aspects of food security, including the availability, access, utilization, and stability of crops (Tronsmo et al., 2020; Ristaino et al., 2021). A plant disease is defined as any deviation from the plant's normal growth and development caused by a pathogen. While a pathogen is the causal agent of a disease and is typically a microorganism, virus, viroid, fungus or fungus-like organism, or nematode. In some cases, abiotic stress factors such as drought, extreme temperatures, and nutrient deficiencies, can also lead to disease-like symptoms (Tronsmo et al., 2020). Plant pathologists have commonly defined diseases as the interaction between a susceptible host plant, a virulent pathogen, and favorable environmental conditions, which can be simply illustrated by the disease triangle, as the essential conditions for disease development (Jeger, 2020). However, the development of a disease in plants can be more complex, which is why other models have been proposed that include pathogen transmission vectors (Jones and Naidu, 2019), considering the environment as a factor capable of interacting and influencing the three fundamental pillars of disease development (**Figure 1**).

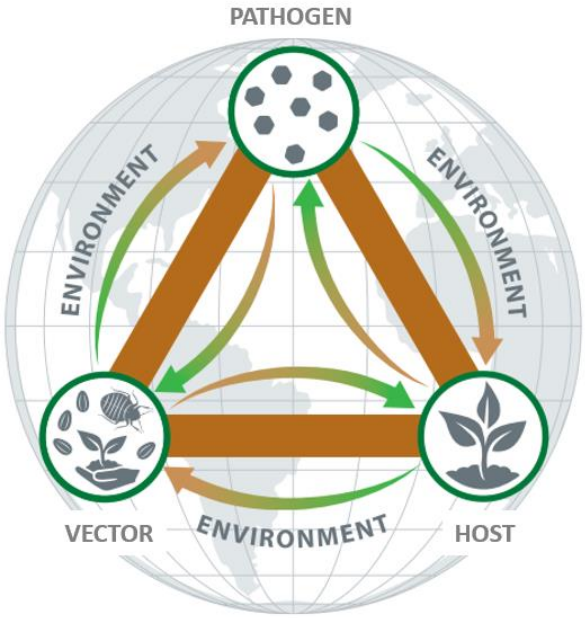

Figure 1. Disease triangle for a pathosystem including transmission vectors. The arrows indicate the type of interaction with the environment, pathogen, host, and vector. Modified image from Jones & Naidu, 2019.

The environment is defined as the set of abiotic and biotic factors that can have a positive, negative, or neutral effect on the development of a disease (Trebicki, 2020).

Some of the most studied abiotic factors in disease development have been alterations in CO_2 concentration, temperature changes, and water availability (Montes and Pagán, 2022; Roussin-Léveillée *et al.*, 2024). Biotic factors include insect pests, weeds, or species interactions, with mixed infections being recognized as a key factor in the development of viral diseases (Alcaide et al., 2020; Moreno and López-Moya, 2020).

In this context, since the beginning of plant domestication, diseases have been present in wild plant communities (Jones, 2006). However, the genetic homogeneity of cultivated populations in monocultures, the high nutritional status of plants, climate change, globalization, and the movement of materials from one region to another have contributed to the spread of pathogens and vectors, exposing agro-ecosystems to the emergence of diseases, leading to epidemics and even pandemics (Lebeda and Burdon, 2023; Trebicki, 2020). Indeed, the traditional disease triangle can be extended into a disease tetrahedron by incorporating a fourth component, human activity, which plays a significant role in plant-pathogen interaction (Tronsmo et al., 2020). Thus, an emerging pathogen can be defined as "the causative agents of infectious diseases whose incidence is increasing following its appearance in a new host population or whose incidence is increasing in an existing host population as a result of long-term changes in its underlying epidemiology" (Elena et al., 2014). Viruses are among the pathogens with the greatest potential to cause emerging diseases and, therefore, epidemics and pandemics (Anderson et al., 2004; Vurro et al., 2010; Ristaino et al., 2021; Jones, 2021). One of the reasons why viruses are responsible for nearly half of global epidemics (Anderson et al., 2004) is their ability to be transmitted primarily by insect vectors (Reynolds et al., 2006). These insect vectors can travel long distances, thereby contributing to the spread of diseases. This is why most efforts are focused on managing and controlling these pests as a preventive measure for viral diseases in plants (Damicone et al., 2007; Eigenbrode et al., 2018; Hooks and Fereres, 2006; Jones, 2006; Rekika et al., 2008). Due to insecticide limitations and rising insect resistance, interest is growing in the insect microbiome as an eco-friendly alternative for pest control highlighting the need for integrated management of these diseases, taking into account the new challenges faced by humanity.

The aim of this thesis was to assess the epidemiological status of the most important cucurbit crops (melon and watermelon) in the main production regions of Spain

(Alicante, Region of Murcia, and Castilla-La Mancha) through monitoring the major virus diseases transmitted by aphids and whiteflies during the period from 2021 to 2023. After understanding the status of these crops, the study delved into the understanding of these viral diseases by examining the effects of abiotic factors (temperature) and biotic factors (mixed infections) on the host and the behavior of the viruses, which are key in the development and evolution of these diseases. Finally, the microbiome of the primary transmission vector of these viral diseases was studied as a potential tool to combat these viral diseases.

4.1. Cucurbit crops

Cucurbitaceae and *Solanaceae* families are among the most important crops in Mediterranean horticultural systems due to their economic value and intensive cultivation in both greenhouses and open fields (FAO, 2025). The *Cucurbitaceae* family consists of 1,000 species, with 10 of them being the most important due to their widespread cultivation around the world, and another 23 species with less significance, cultivated on a local scale (Chomicki et al., 2020). Among the most widely cultivated species globally are watermelon, with an annual production of approximately 104,932,071 tons, followed by cucumber with 97,814,093 tons, and melon and pumpkin with 29,541,294 and 23,681,846 tons, respectively, according to the latest 2023 annual production data (FAO, 2025). In the Mediterranean region, the most cultivated species include cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* Thunb.), pumpkin (*Cucurbita maxima* Duch. and *Cucurbita moschata* Duch.), and zucchini (*Cucurbita pepo* L.), with Spain being one of the leading producers (Eurostat, 2025). Specifically, according to data from the "*Encuesta sobre Superficies y Rendimientos de Cultivos*" (ESYRCE) from the "*Ministerio de Agricultura, Pesca y Alimentación*" (MAPA, 2025), the largest cultivated area of cucurbits in Spain is dedicated to melon (22,400 hectares), followed by watermelon (11,221 hectares), pumpkin (5,988 hectares), and zucchini (3,493 hectares), with cucumber being grown on a smaller scale (1,580 hectares) according to available 2024 data. For the two major crops, the Region of Murcia is the primary melon producer (yield in kg/ha), followed by Castilla-La Mancha, Andalucía, and the Comunidad Valenciana (**Figure 2**). In contrast, for watermelon cultivation, the Region of Murcia is the second-largest producer after

Castilla-La Mancha, followed by Andalucía and the Comunidad Valenciana (MAPA, 2025) (**Figure 2**).

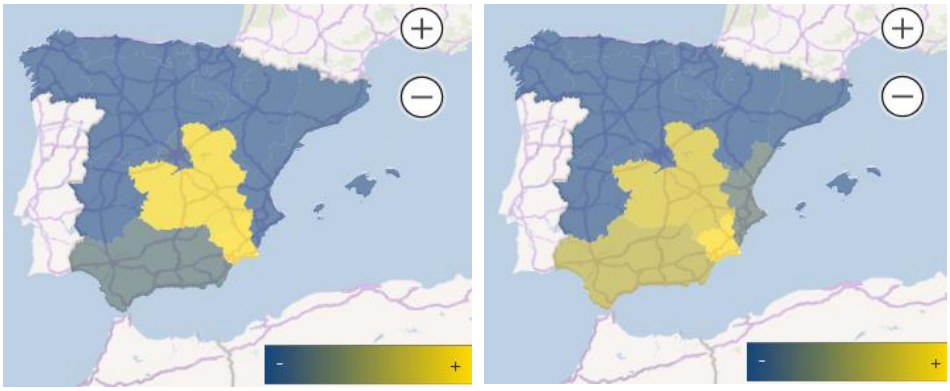

Figure 2. Map of the autonomous communities with the largest cultivated areas for melon (left) and watermelon (right) production in Spain in 2024. Source modified from ESYRCE (MAPA, 2025).

These crops are grown in a wide range of agro-ecosystems, from soil-less greenhouse production to dryland farming conditions (Lecoq & Katis, 2014). Like other crops, during their domestication, traits such as increased fruit size and sweetness, reduced acidity, seed dormancy, and better adaptation to specific environmental conditions have been selected (Chomicki et al., 2020; Wan Shafiin et al., 2021). However, this artificial selection has led to a reduction in genetic variability, making these crops more vulnerable to both abiotic and biotic stresses (Grumet et al., 2021). Given the economic importance of these crops, there is an increasing effort to develop varieties that are tolerant or resistant to both abiotic and biotic stresses (Gaba et al., 2004; Mondal et al., 2020; Parvathi et al., 2022).

4.2. Plant Viruses

4.2.1. Survival strategies and transmission modes of plant viruses

The first virus ever described in history was *Tobamovirus tabaci* (tobacco mosaic virus, TMV), identified in the 1890s by M.W. Beijerinck as the causal agent of leaf spots in tobacco plant (Beijerinck, 1898). Since then, hundreds of viruses have been described as pathogens responsible for numerous plant diseases (Crawford, 2002). In fact, viral diseases cause annual yield losses estimated at 30 billion dollars in agricultural crops worldwide (Tatineni & Hein, 2022). However, not all viruses cause diseases, as four different survival strategies and co-evolution have been described for plant viruses: acute, chronic, persistent or cryptic, and endogenous (Roossinck, 2010). Viruses that do

not cause diseases in plants, called persistent or cryptic viruses, are characterized by vertical transmission and typically cause asymptomatic infections, as is the case with members of the family *Partitiviridae*. Endogenous viruses, on the other hand, are integrated into the plant's genome as remnants of ancient viral infections, capable of being activated under certain conditions. Additionally, there are those with an "acute" survival strategy, characterized by causing symptomatic infections that can be horizontally transmitted. When these infections persist over time, they may be referred to as chronic viruses (the fourth survival strategy), sometimes without clearly observable symptoms (Roossinck, 2010).

Clearly, the most studied viruses are those that cause diseases in plants due to the severe consequences they have for crops. These viruses, as mentioned earlier, have the ability to be transmitted vertically by seed (Pagán, 2022), and more commonly horizontally through the soil, by contact, or via arthropod, nematode and fungal vectors (Nault, 1997; Lecoq & Katis, 2014), with insects being the main transmission vectors (Hogenhout et al., 2008; Peters et al., 2024). Historically, insect-vectored viruses have been classified according to the mode of transmission into *circulative* viruses, if they enter the insect's cells and are transported by the hemolymph; and *non-circulative* viruses, if the virus-insect interaction occurs superficially in the mouthparts and foregut. Additionally, circulative viruses can be further classified into *propagative* (if they replicate within the insect) or *non-propagative* (Ng and Perry, 2004). On the other hand, according to the duration of virus retention, they have been classified as *persistent*, *semi-persistent*, or *non-persistent* (Fereres and Raccah, 2015). Non-circulative viruses include those classified as *non-persistent* and *semi-persistent*, where the insect remains viruliferous for a short period of time. *Non-persistent* viruses are those with a short acquisition and inoculation period, typically ranging from seconds to minutes (Gadhave et al., 2020). This group includes, among others, the genera *Potyvirus* and *Cucumovirus*, both transmitted by aphids. *Semi-persistent* viruses are those with a retention period of minutes to hours, with notable genera such as *Caulimovirus* transmitted by aphids and *Crinivirus* transmitted by whiteflies (Whitfield et al., 2015). Circulative viruses include those classified as *persistent*, which have an acquisition and retention period ranging from hours to days, with insects potentially remaining viruliferous for weeks (non-propagative) or throughout their life (propagative) (Ng and Perry, 2004). Within the

propagative circulative viruses, prominent genera include *Tospovirus* transmitted by thrips and *Nucleorhabdovirus* transmitted by planthoppers. For non-propagative circulative viruses, notable genera include *Luteovirus* and *Polerovirus*, both transmitted by aphids, and *Begomovirus* transmitted by whiteflies (Whitfield et al., 2015).

4.2.2. Current status of cucurbit crops in the Mediterranean basin

Although there is no data on the economic losses caused by viral diseases in cucurbit crops, globally, around 70 virus species have been identified affecting cucurbit crops (Lecoq & Desbiez, 2012; Lecoq & Katis, 2014). In the Mediterranean Basin, in 1977, 11 viruses were described affecting these crops (Lovisolo, 1977). Since then, additional viruses have been identified, and currently, about 28 viruses are known to threaten cucurbit crops (**Figure 3**) (Lecoq and Desbiez, 2012; Radouane et al., 2021). Among these viruses, some of the most important due to the significant losses they cause are primarily those transmitted by whiteflies and aphids. Among the viruses transmitted by whiteflies, those belonging to the *Geminiviridae* family stand out, such as *Begomovirus solanumdelhiense* (tomato leaf curl New Delhi virus, ToLCNDV), *Begomovirus cucurbitapeponis* (squash leaf curl virus, SLCV), *Mastrevirus cicerparvi* (chickpea chlorotic dwarf virus, CpCDV), and *Begomovirus citrulli* (watermelon chlorotic stunt virus, WmCSV); and those belonging to the *Closteroviridae* family, such as *Crinivirus pseudobetae* (beet pseudoyellows virus, BPYV), and *Crinivirus cucurbitae* (cucurbit yellow stunting disorder virus, CYSDV) (Lecoq and Desbiez, 2012; Radouane et al., 2021). Among the aphid-transmitted viruses, those belonging to the *Bromoviridae* family are prominent, such as *Cucumovirus CMV* (cucumber mosaic virus, CMV); *Potyviridae* including *Ipomovirus cucumisvenaflavi* (cucumber vein yellowing virus, CVYV), *Potyvirus citrulli* (watermelon mosaic virus, WMV), *Potyvirus citrullimoroccense (*Moroccan watermelon mosaic virus, MWMV), *Potyvirus cucurbitaflavitesselati* (zucchini yellow mosaic virus, ZYMV), and *Potyvirus papayanuli* (papaya ring spot virus, PRSV); Lastly, those from the *Luteoviridae* family, where *Polerovirus CABYV* (cucumber aphid-borne yellows virus, CABYV) stands out as the most significant (Lecoq and Desbiez, 2012; Radouane et al., 2021). There are also other viruses, although in smaller proportion, transmitted by seeds or by contact, fungi, beetles, and leafhoppers such as *Tobamovirus viridimaculae* (cucumber green mottle mosaic virus, CGMMV), *Gammacarmovirus*

melonis (melon necrotic spot virus, MNSV), *Comovirus cucurbitae* (squash mosaic virus, SqMV), and *Alphanucleorhabdovirus melongenae* (eggplant mottled dwarf virus, EMDV), respectively (Lecoq and Desbiez, 2012). The epidemiological situation of cucurbit crops in Spain will be discussed later as part of the first chapter of this thesis.

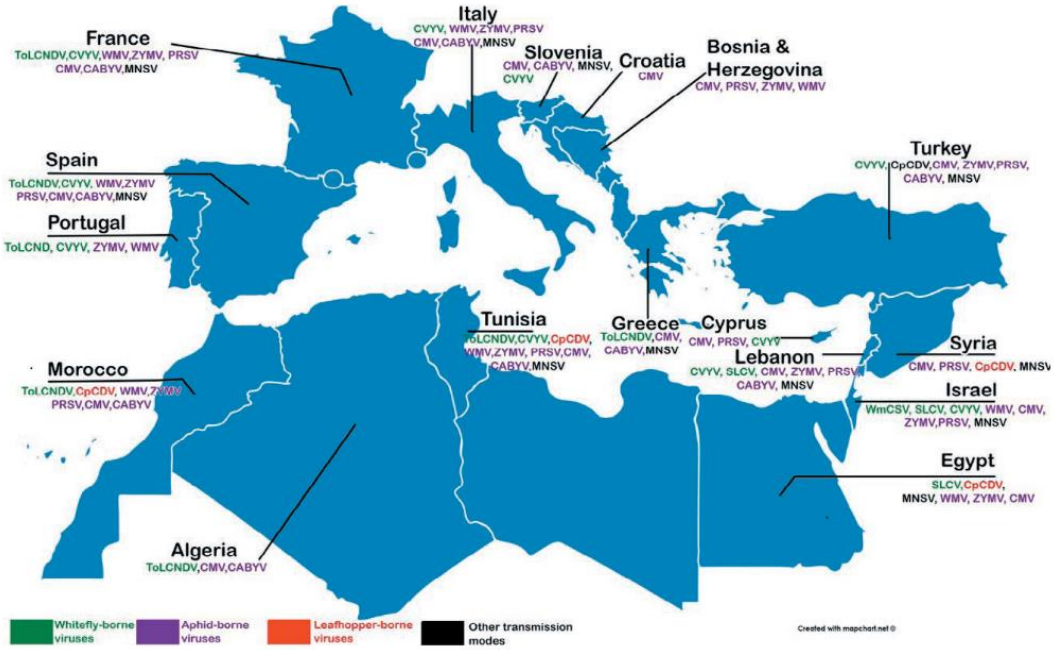

Figure 3. Geographical distribution of viruses affecting cucurbit crops in the Mediterranean Basin (Radouane et al., 2021)

The most common symptoms caused by these viruses in cucurbit crops include mosaic patterns and leaf curling, yellowing and necrosis of leaves, as well as fruit deformity, reduced size, and mottling, which affect the aesthetic value and yield of the fruits (Radouane et al., 2021).

Although the epidemiological pattern observed in cucurbit crops appears to be consistent over time (Rabadán et al., 2023), it is important to highlight that the incidence of these diseases and their geographical distribution are constantly changing and expanding, leading to emerging diseases (Roossinck and García-Arenal, 2015; McLeish et al., 2019). This is directly due to factors such as the simplification of ecosystems, climate change, the movement of plant material, and the Mediterranean climate's favorable conditions for vector insects, among others (Ertunc, 2020; Radouane et al., 2021; Rojas and Gilbertson, 2008). For example, in recent years, the emergence of viral diseases in cucurbit crops in Spain has been identified, such as cucurbit chlorotic yellows

virus (CCYV), transmitted by whiteflies, which has been detected in watermelon and zucchini crops (Alfaro-Fernández et al., 2022). Additionally, possibly linked to climate change, among other causes, there is also the emergence of previously unreported viral diseases in other regions of Europe, such as CABYV in Germany, Slovenia, the Netherlands, and Bulgaria (Mehle et al., 2020; Menzel et al., 2020; Minicka et al., 2020; Radeva-Ivanova et al., 2022), or two related viruses, such as in the case of *Coguvirus citrulli* (watermelon crinkle leaf-associated virus 1, WCLaV-1) and *Coguvirus henanense* (watermelon crinkle leaf-associated virus 2, WCLaV-2) in Slovenia and Italy, affecting watermelon crops (Parrella, 2025; Vučurović *et al*., 2025). Thus, one of the major challenges during the monitoring and surveillance of these crops is the difficulty in distinguishing between plants affected by one or more viruses that can cause similar symptoms, in addition to those caused by abiotic or nutritional stress (Mascia and Gallitelli, 2016; Syller, 2012). Although significant progress has been made in viral disease diagnostic methods, from the use of techniques such as serology or molecular methods to more advanced ones like next-generation sequencing and remote sensing as non-invasive tools (Boonham et al., 2014; Gaborjanyi et al., 2003; Hasiów-Jaroszewska et al., 2021), addressing these complexities is crucial for effective viral diagnosis and long-term disease management, thus improving viral disease control measures in crops.

4.3. Biotic and abiotic factors

Plants, like animals, have their own mechanisms of adaptation to both abiotic and biotic factors, mediated by complex molecular responses that trigger increased resistance or tolerance to deficiencies and/or diseases (Roussin-Léveillée et al., 2024; Walling, 2009). While the plant response to nutritional deficiencies has been widely studied (Pessarakli, 2015), some of the most important abiotic factors today are those directly related to climate change and global warming, such as drought, salinity, and rising temperatures (Parrotta et al., 2023). The increase in global average temperature directly affects numerous physiological processes in plants, including seed germination, development, and photosynthetic and reproductive processes (Ahanger et al., 2017). The study of the genetic and molecular changes that occur in response to temperature stress is crucial to understanding the adaptation and thermo-tolerance processes of

crops, with some of the most widely studied mechanisms being those based on heat shock proteins (HSPs) and enzymes that detoxify reactive oxygen species (ROS), such as ascorbate peroxidase (APX) (Ahanger et al., 2017). However, the increase in temperature not only affects the host but also has a direct effect on plant pathogens, potentially triggering or worsening diseases (Singh et al., 2023). In fact, many pathogens give rise to emerging diseases when environmental conditions are favorable for them and vulnerable for the host (Singh et al., 2023). Most plants are capable of resisting pathogens through specific interactions between the pathogen's avr (avirulence) gene loci and the corresponding plant disease resistance (R) allele loci (Jeffery and Jonathan, 2001). It is known that an innate response is triggered, involving molecules called "pathogen-associated molecular patterns (PAMPs)," which ultimately lead to the synthesis of defense hormones such as salicylic acid, jasmonic acid, and ethylene (Walling, 2009). The response to viruses is similar to that of other pathogens (**Figure 4**) (Mandadi and Scholthof, 2013). There is scientific evidence that temperature changes (an abiotic factor) have an effect on the development of viral diseases (a biotic factor), modifying their epidemiology (Trebicki, 2020). In this regard, while the host's response to abiotic and biotic factors has been well studied independently, little is known about

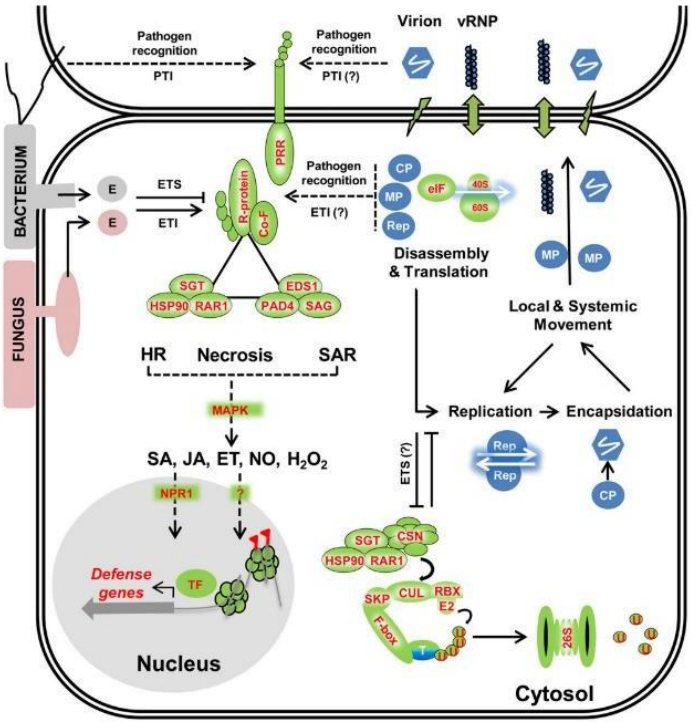

Figure 4. Antiviral immune response analogous to bacterial and fungal immune responses (Mandadi & Scholthof, 2013).

the joint response of the host to the combination of abiotic and biotic stresses as a key aspect in the host-pathogen interaction and, therefore, in the development of a disease.

4.3.1. Biotic factors: Impact of mixed infections on the ecology and evolution of diseases

Biotic stress is defined as that caused by pathogenic fungi, bacteria, nematodes, herbivores, and viruses, which trigger a plant response through the activation of genes related to stress and resistance (Schulze et al., 2019). Among all pathogens, viruses are the leading cause of plant diseases (Anderson et al., 2004; Jones and Naidu, 2019). Most plant viruses are generalist species whose ability to be transmitted by polyphagous insects allows them to infect a wide variety of hosts, including both crops and wild plants, which contributes to the high frequency of mixed infections in nature (Moreno and López-Moya, 2020; Rabadán et al., 2023). In fact, a high proportion of plants, both cultivated and wild, have been observed to be co-infected by multiple viruses or strains (Roossinck et al., 2010; Tugume et al., 2016). Thus, mixed infections are recognized as another form of biotic stress and are defined as the simultaneous presence of multiple viruses or an infection by one virus followed by a subsequent infection by another, which may or may not be related to the first (McLeish et al., 2019; Saldaña et al., 2003). These mixed infections have direct consequences on the development and dynamics of diseases, as they can lead to changes in virulence, transmission rate, symptom expression, and viral fitness compared to single infections (Moreno and López-Moya, 2020; Tollenaere et al., 2016). Traditionally, three types of interactions have been defined: a *neutral interaction* occurs when there are no changes in viral accumulation or symptom expression for either of the viruses involved compared to their respective single infections on the other hand, a *synergistic* or *antagonistic interaction* is defined when at least one of the viruses benefits or is harmed by the presence of the other, respectively, affecting viral accumulation and/or pathogenicity (Moreno and López-Moya, 2020; Syller, 2012). However, alternative classifications have been proposed that consider the reciprocal response of each virus's fitness in a mixed infection, with fitness understood as a virus's ability to replicate and increase its viral accumulation in a host (Alcaide et al., 2020). Therefore, six types of interactions are established: in a *neutral interaction*, both viruses respond similarly in single and mixed infections without

changes in viral fitness; in a *neutral-antagonistic interaction*, one virus's fitness is reduced while the other remains unaffected; in a *neutral-synergistic interaction*, one virus benefits while the other remains unaffected; in an *inverse interaction*, one virus's fitness increases while the other's decreases; and lastly, in *a double-synergistic* or *double-antagonistic interaction*, both viruses either increase or decrease their fitness, respectively (Alcaide et al., 2020) (**Figure 5**).

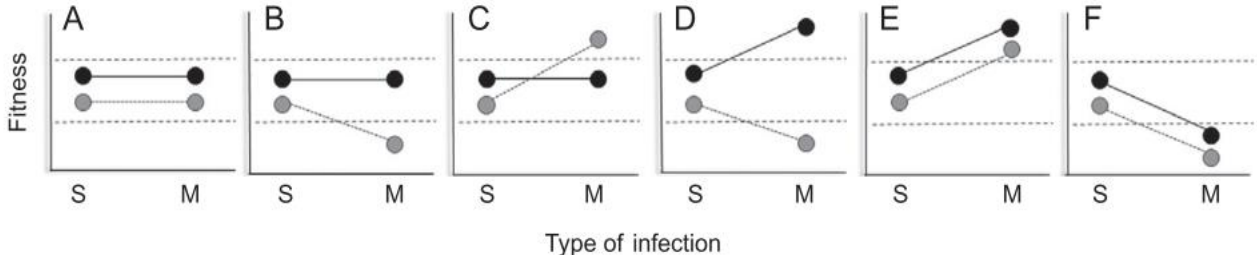

Figure 5. Classification of virus interactions within the host into simple (S) and mixed (M) infections, where black and grey circles represent both related and unrelated viruses (Alcaide et al., 2020).

Although numerous studies have been conducted on mixed viral infections in different pathosystems (Moreno and López-Moya, 2020), most of these studies experimentally carry out simultaneous infections over time, with only a few considering sequential infections (the order of infection) as a key factor in disease development (Moya-Ruiz et al., 2024). Since most plant viruses are transmitted by insect vectors (Ng and Falk, 2006), mixed viral infections in crops result from multiple transmission events within the same plant, either as simultaneous (co-infections) or sequential infections (one after another). In fact, it has been observed that simultaneous infection of sugar beet plants with *Potyvirus betaceum* (beet mosaic virus, BTMV), and *Closterovirus flavibetae* (beet yellows virus, BYV) resulted in a synergistic interaction, while sequential infection with BTMV followed by BYV days later led to a neutral interaction (Borgolte et al., 2024; Wintermantel, 2005). In another study, it was observed that simultaneous or sequential infection with PRSV followed by *Potexvirus papayae* (papaya mosaic virus, PapMV) resulted in greater symptom expression in papaya plants than sequential infection with PapMV followed by PRSV (Chávez-Calvillo et al., 2016). In contrast, another study found a synergistic interaction between *Potyvirus glycitessellati* (soybean mosaic virus, SMV) and *Comovirus siliquae* (bean pod mottle virus, BPMV) and *Comovirus vignae* (cowpea mosaic virus, CPMV), regardless of whether the infection was simultaneous or sequential (Anjos et al., 1992). Therefore, the temporal order of different viruses arriving at the same plant can inevitably affect the ecology and

evolution of viral diseases. These studies highlight the importance of the order of infection as a key biotic factor in the development and dynamics of viral diseases in plants. So, further studies considering the order of infection are necessary to gain a better understanding of the ecological mechanisms driving viral epidemiology.

4.3.2. Abiotic factors: Effect of temperature on plant-virus interactions

Climate change, particularly the rise in temperature, alters the dynamics of plant pathogens and their interaction with hosts, increasing the risk of disease outbreaks in crops (Elad and Pertot, 2014; Lahlali et al., 2024; Raza and Bebber, 2022). Temperature changes, whether mild or moderate, whether low or high, have a variable and direct effect on plant responses, potentially leading to greater tolerance or susceptibility to diseases depending on the pathosystem in question (Bostock et al., 2014). Moreover, it has been observed that temperature-induced responses also affect plants' increased resistance or susceptibility to herbivorous insect pests (Nechols et al., 2020) as well as to pathogens (Kassanis, 1952; Laine, 2007). Although the response is variable depending on the pathosystem, some studies have shown that *Potyvirus rapae* (turnip mosaic virus, TuMV) and *Potyvirus plumpoxi* (plum pox virus, PPV) exhibit lower accumulation at high temperatures, which is associated with milder symptoms (Aguilar et al., 2015; Chung et al., 2016; Szittya et al., 2003). In contrast, in Chinese cabbage, it has been observed that TuMV causes more severe symptoms at 28°C, which coincides with higher viral accumulation. Another study evaluated the impact of thermal stress on the mutation rate, such as in the case of *Potexvirus pepini* (pepino mosaic virus, PepMV) in tomato, where the mutation rate increased at 30°C compared to 20°C (Alcaide et al., 2021). On the other hand, at low temperatures, plants may experience a slowdown in metabolic processes, which could affect the efficiency of their defense mechanisms and, in turn, influence replication, viral accumulation, and thus the development of the disease (Garcia-Ruiz, 2018).

Most studies focused on plant responses to abiotic and biotic factors have been conducted independently, and while these factors have been extensively studied (Hirt and Shinozaki, 2003; Thakur and Sohal, 2013), few have considered the plant's response to the combination of both types of stress (Ben Rejeb et al., 2014). In the few studies that do exist, a highly varied response is observed, with positive or negative effects on

the host depending on the type of combined stresses (Suzuki et al., 2014). In the case of studies based on the combination of abiotic stresses, these show that the plant's response is different from what would occur in the presence of a single stress, potentially leading to either antagonistic or synergistic effects for the plant (Mittler and Blumwald, 2010). All of this suggests that the physiological, molecular, and genetic responses of plants to combined stresses can be complex, making it impossible to predict the effects by studying these stresses individually (Atkinson and Urwin, 2012). In fact, some studies have examined the connections between metabolic pathways in response to the combination of stresses and how prior exposure to a biotic stress can improve the plant's tolerance or resistance to an abiotic stress, and vice versa, a phenomenon known as "cross-tolerance" (Ben Rejeb et al., 2014; Tippmann et al., 2006). For example, it has been observed that viral infection can enhance tolerance to water stress due to changes in the host's metabolic profile (Xu et al., 2008). On the other hand, other studies show that an increase in temperature favors a higher accumulation of *Potyvirus yituberosi* (potato virus Y, PVY) in thermo-sensitive plants compared to thermo-tolerant ones, possibly due to a reduction in pathogen-related proteins mediated by salicylic acid (SA) in plant defense, which may increase the plant's susceptibility to viral infection (Fesenko et al., 2021; Makarova et al., 2018; Spechenkova et al., 2021). In this context, given current climate trends and the importance of viral diseases, it is necessary to conduct studies on the host's response to the combination of temperature stress and viral infection in order to identify varieties with greater tolerance to these stresses.

4.4. The aphid insect vector

4.4.1. Control and management of aphid-transmitted viruses in cucurbit crops in Spain

The fact that plants are sessile organisms with an impermeable cuticle has led to most plant viruses being transmitted by vectors, which facilitates their entry into cells and allows their spread among different hosts (Fereres and Raccah, 2015). Among the main transmission vectors are insects, particularly those belonging to the order Hemiptera, as they are capable of transmitting up to 55% of most vector-borne viruses (Hogenhout et al., 2008; Peters et al., 2024). Within the Hemiptera order, some of the most important insects for transmitting plant viruses are: cicadellids, fulgoromorphs,

whiteflies, and aphids, with aphids being the most significant due to their ability to transmit the highest number of viruses (Hogenhout et al., 2008). This is likely because their piercing-sucking mouthparts make them efficient vectors without causing severe damage to the plant, and they are the only ones capable of transmitting viruses after a brief exploration of a leaf (Bragard et al., 2013). Most aphid vectors belong to the subfamily Aphidinae in the suborder Sternorrhyncha, and they are characterized by very high reproductive rates, which promote the spread of viruses over both short and long distances (Ng and Perry, 2004). On the other hand, aphids are considered a pest since, at high population densities, they deplete plant nutrients, leading to their weakening. Additionally, their saliva and honeydew excretions can cause leaf deformities, growth retardation, and the appearance of fungal diseases. However, the greatest risk associated with these insects is their role as virus vectors, as infected plants tend to become more attractive to them due to yellowing of their leaves and the increase in free amino acids, thus promoting viral spread (Sorensen, 2009). Aphids are globally distributed, primarily in temperate climates, and around 450 species have been identified, though 14 are recognized as pests, causing serious problems to crops: *Aphis craccivora*, *Aphis fabae*, *Aphis gossypii*, *Aphis spiraecola*, *Rhopalosiphum maidis*, *Rhopalosiphum padi*, *Schizaphis graminum*, *Acyrthosiphon pisum*, *Diuraphis noxia*, *Lipaphis pseudobrassicae*, *Macrosiphum euphorbiae*, *Myzus persicae*, *Sitobion avenae*, and *Therioaphis trifolii* (Sorensen, 2009), among these, *Aphis gossypii* and *Myzus persicae* are the dominant species in Spain (Hooks and Fereres, 2006; Kassem et al., 2013). The life cycle of aphids can vary depending on the species. In some species, the cycles include periods of alternated parthenogenetic reproduction and sexual reproduction generations, as is the case for *Myzus persicae*, while other species reproduce continuously by parthenogenesis, such as *Aphis gossypii* in temperate regions, where males are rare (Blackman and Eastop, 2017; Singh and Singh, 2016). The parthenogenetic periods coincide with long days (summer), while sexual reproduction occurs during the short days (autumn), leading to the egg stage that can withstand winter (Singh and Singh, 2016). Furthermore, aphids exhibit different types of polymorphism, including wingless and winged females, with the latter enabling the dispersion of their populations over long distances (Dixon, 1977). Climate change and increasingly mild winters are causing some aphid species to remain active throughout

the year with parthenogenetic reproduction cycles, leading to earlier aphid outbreaks (Simon and Peccoud, 2018).

Traditionally, aphid pests and, consequently, viral diseases have been controlled through preventive measures such as the use of pesticides and/or cultural practices. These may include crop protection with covers, biological control through the use of predators, or intercropping and trap crops (Ben-Issa et al., 2017; Blackman and Eastop, 2017; Hull, 2002; Painkra et al., 2019). For instance, in open-field cucurbit crops, it is recommended to protect plants from vector insects in the first few weeks after sowing by using perforated plastic tunnels and agrotextile covers (**Figure 6**). In greenhouses, it is important to ensure the tight sealing of the structure, and an integrated management approach with insecticide treatments is advisable to prevent initial outbreaks and their spread to secondary foci (de Moya-Ruiz et al., 2024). However, the indiscriminate use of pesticides, restrictions on their use, and the rapid adaptation of aphids have led to the emergence of insecticide resistance in major transmission vectors, including *A. gossypii* and *M. persicae* (Collins and Schlipalius, 2018; Simon and Peccoud, 2018). Therefore, the most desirable measure currently for controlling viral diseases is the use of resistant

Figure 6. Agrotextile covers (left) and perforated plastic tunnels (right) as methods of protection and prevention against insect vectors of viral diseases (de Moya-Ruiz et al., 2024).

varieties (Gómez et al., 2009). Specifically, for cucurbit crops, some lines with genetic resistance and tolerance to ZYMV, WMV, CMV, and PRSV viruses have been described, as well as melon varieties resistant to *A. gossypii* or to MWMV, and cucumber varieties resistant to CVYV, ZYMV, PRSV, CMV, and CGMMV using CRISPR/Cas9 (Boissot et al., 2016; Chandrasekaran et al., 2016; Díaz-Pendón et al., 2004; Diaz et al., 2003; Fuchs and

Gonsalves, 2008; Gómez et al., 2009; Pechar et al., 2022). Although the use of resistant varieties is the most desirable approach, these resistances are often partial and depend on environmental conditions, vector pressure, virus, and plant status (de Moya-Ruiz et al., 2024). For this reason, the primary strategy used for managing pests and viral diseases in cucurbit crops is the control of aphid populations. However, with the European Union legislation on pesticide use and the lack of effective methods to protect crops from aphids, it is necessary to implement integrated pest management and continue researching to find innovative and sustainable strategies to protect crops from pests and diseases.

4.4.2. Bacterial community composition of insect vectors as a future strategy for pest and disease control

It is estimated that insect pests are responsible for approximately 40% of crop losses worldwide (Rupawate et al., 2023). Due to limitations in the use of insecticides, as well as the increasing resistance of insects to these chemicals, there is growing interest in exploring approaches that emphasize efficiency and the long-term sustainability of agroecosystems as eco-friendly alternative for pest management, aiming to keep pest populations below economically damaging levels (Qadri et al., 2020; Omkar, 2021; Sugio et al., 2015). Integrated Pest Management (IPM) combines strategies such as the cultivation of resistant plant, monitoring and identification of pests, preventive and control measures, biotechnology, and cultural and biological control, among others (Omkar, 2021; Rupawate et al., 2023). Biological control has often served as a fundamental pillar in the development of IPM, highlighting the use of natural enemies as predators, parasitoids, entomopathogenic fungi and bacteria (Omkar, 2021). Recently has incorporated insect symbionts as a novel and promising tool to effectively reduce pest-related economic losses since dysbiosis of gut microbiota is detrimental to insect survival (Rupawate et al., 2023; Zhang et al., 2023). Although insect-associated microbial diversity has been described to be very low (10-15 operational taxonomic units (OTUs)) (Sugio et al., 2015), insect endosymbionts are generally classified into three categories: *obligate, facultative*, and *phytopathogenic symbionts*. *Obligate symbionts* (also called primary) are typically localized in specialized cells called bacteriocytes, which are situated near the insect's digestive system. These symbionts are transmitted

vertically and are essential for the host, often providing nutritional and fitness-related benefits (Oliver et al., 2010). *Facultative symbionts* (also called secondary), while also mainly transmitted vertically, can occasionally be acquired from the environment. Unlike obligate symbionts, they are not restricted to bacteriocytes and may inhabit various tissues. These symbionts have been associated with diverse functions, such as pesticide detoxification, for instance, *Sphingomonas* has been shown to mediate resistance to imidacloprid in the cotton aphid (Lv et al., 2023). They may also influence insect phenotype, such as *Rickettisella* in the pea aphid, which enhances pigment synthesis, altering body color and affecting susceptibility to predation or parasitism (Qadri et al., 2020). Additionally, facultative symbionts may modulate plant defense systems and offer protection against biotic stressors, and under certain conditions, they can become pathogenic (Oliver et al., 2010). Finally, *phytopathogenic symbionts* depend on insects for transmission and are typically involved in insect-plant interactions. A notable example is *Xylella fastidiosa*, which is transmitted by leafhoppers and spittlebugs, and is pathogenic in over 100 plant species causing disease (Rapicavoli et al., 2018; Rupawate et al., 2023). Moreover, recent studies have shown a correlation between the microbiomes of the soil, plant, and insect vectors, indicating that most of the insect vector's microbiome originates from the plant stems, with a smaller contribution from the soil (Li et al., 2023; Malacrino et al., 2021). The reciprocal influence among these microbial communities across the soil–plant–vector may be leveraged to develop innovative microbiome-based strategies for pest management (Wolfgang et al., 2023).

Aphids frequently establish mutualistic associations with endosymbionts. Among these, *Buchnera aphidicola* stands out as an obligate symbiont, a Gram-negative bacterium that is essential for aphid survival, as it provides 10 essential amino acids that the aphid is unable to synthesize on its own (Baumann et al., 1995; Shigenobu and Wilson, 2011). In addition to *Buchnera*, nine facultative symbionts have traditionally been described: *Serratia symbiotica*, *Hamiltonella defensa*, *Regiella insecticola*, *Rickettsia*, *Rickettsiella*, *Spiroplasma*, *Wolbachia*, *Arsenophonus*, and the Pea Aphid X-type Symbiont (PAXS) (Guo et al., 2017). These facultative symbionts have been implicated in various biological processes, including host specialization, resistance to both abiotic and biotic stresses, and the reproduction and development of the aphid

(Guo et al., 2017). Although the aphid microbiome has traditionally been described as limited in terms of the number of associated symbiont, typically no more than three to seven OTUs per sample (Sugio et al., 2015), an increasing number of studies on the microbiome of *Aphis gossypii* have identified additional symbionts whose functions remain unknown. For instance, field populations of *A. gossypii* have revealed the presence of genera not previously reported, such as *Acinetobacter*, *Brevundimonas*, *Pseudoxanthomonas*, *Kosakonia*, and *Exiguobacterium*, among the most abundant facultative symbionts, alongside the primary symbiont *Buchnera* and other traditionally described as facultative symbionts (Zhang et al., 2021). Notably, *Arsenophonus* has emerged as the most abundant facultative symbiont and the second most prevalent bacterial taxon after *Buchnera* in the *A. gossypii* microbiome (Gallo-Franco et al., 2019; Xu et al., 2020; Zhang et al., 2021; Zhao et al., 2016). Additionally, variations in the microbial community of *Aphis gossypii* have been observed depending on the host plant it feeds on. For example, in a study analyzing field-collected aphid populations, facultative symbionts such as *Flavobacterium* and *Pantoea* were detected, with clear differences in microbial diversity linked to the host plant (Ma et al., 2021). Similar host-dependent differences have been documented in other studies, where aphids feeding on cucurbits showed greater microbial diversity compared to those feeding on other plant species (Ma et al., 2021; Xu et al., 2020, 2023). These studies also reported shifts in the abundance of facultative symbionts such as *Acinetobacter*, *Stenotrophomonas*, *Pseudomonas*, and *Novosphingobium* (Xu et al., 2023). However, no study has yet examined how the *A. gossypii* microbiome changes after transfer to an alternative host and return to the original, leaving unclear the role of microbiota in aphid adaptation to different host plants. Nevertheless, it is also important to consider that a significant portion of the herbivorous insect microbiome originates from the host plant and the surrounding soil. Studies have shown that bacterial genera commonly found in soil can also be present in both plants and insects, likely due to surface or internal interactions between these microbial communities (Li et al., 2023; Malacrino et al., 2021). For example, the genus *Flavobacterium* constitutes a substantial component of the microbial communities associated with plant roots and leaves across a wide variety of plant species (Kolton et al., 2016). So, the roles of many of these genera in aphid survival, development, or even in the transmission of viral diseases remain largely unknown.

Although little is currently known about the relationship between the microbiome of *A. gossypii* and the transmission of plant viruses, a Bayesian modeling study involving luteoviruses, *Buchnera*, and aphids revealed that the relative abundance of *Buchnera* depends on both the aphid species and virus acquisition (Enders and Hefley, 2023). Another study found that *Buchnera* may facilitate the transmission of CMV by altering the host plant's volatile profile (Shi et al., 2021). In fact, *Buchnera* is also known to produce symbionin proteins similar to GroEL, which have been implicated in the transmission of circulative plant viruses as treatments with antibiotics that eliminated *Buchnera* in aphids led to a 70% reduction in the transmission of luteovirids (Pinheiro et al., 2015; Van den Heuvel et al., 1994). Moreover, other facultative symbionts are also known to produce GroEL-like proteins, suggesting they may also play a role in virus transmission. Furthermore, it has also been observed that secondary symbionts can function as co-obligate symbionts, compensating for the essential role of *Buchnera* in certain aphid species (Chong and Moran, 2018; Koga et al., 2003; Manzano-Marín et al., 2023; Monnin et al., 2020). Further research is needed to understand the effects and functions of these microbial communities in aphids, particularly considering the interactions between symbionts. Gaining insight into these dynamics could contribute to the development of new strategies for controlling these pests.

The investigation of pest insect microbiomes holds significant potential for the development of novel pest control strategies, exerting both direct and indirect effects comparable to those of conventional insecticides (**Figure 7**) (Zhang et al., 2023). For example, the use of antibiotics or antimicrobial peptides (AMPs) to eliminate insect symbionts has been explored on different insect pest as fruit fly or chinch bug, as well as, the introducing or replacement of a symbiont on stink bugs and aphids in order to reduce survival rates or fecundity (Qadri et al., 2020; Rupawate et al., 2023). Also, the use of microbial insecticides applied directly to the field using pathogenic or toxic microbes as the active ingredient (Qadri et al., 2020). Another technique is the Sterile Insect Technique (SIT) or Incompatible Insect Technique (IIT), which are used to interfere or modulate the host's innate microbiota, through which males are made incompatible for reproduction (Rupawate et al., 2023). This method can impact the insect microbiome and has proven effective in controlling other pests, such as the mosquito (*Aedes aegypti)*,

by reducing the incidence of Dengue (De Castro Poncio et al., 2021). On the other hand, as indirect effects, gut bacteria can produce volatile organic compounds (VOCs) that attract other pests or natural enemies, or engage in synergistic interactions with other bacterial species that may ultimately be lethal to the insect pest (Zhang et al., 2023). The use of symbiont-mediated RNAi to target specific genes in host insects, which has been demonstrated in various pest species, or CRISPR/Cas9 gene editing tool for genomic manipulation of insect symbionts offering a promising avenue for the development of innovative pest control strategies (Rupawate et al., 2023). Another emerging pest control methods is paratransgenesis which consists of using symbiotic bacteria as gene expression vectors to introduce foreign genes into insects that interferes with pathogen development or insect fitness (Qadri et al., 2020; Zhang et al., 2023). Given the potential of studying associated symbionts as a tool for pest control, further research is needed to better understand the interactions between symbionts, the potential adaptation of microbiota-mediated aphids to the transition between different host plant species, and how they may influence the transmission of both persistent and non-persistent viruses.

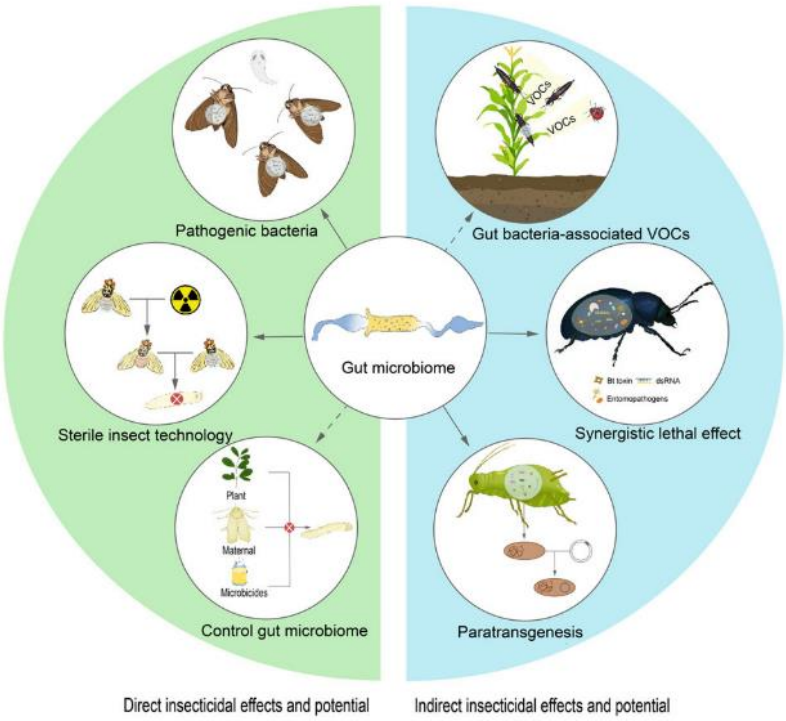

Figure 7. The significant role of gut microbiota in pest control strategies (Zhang et al., 2023).

OBJECTIVES

5. OBJECTIVES

The aim of this thesis is to understand the ecological and molecular interactions between aphid-transmitted viruses, host plants, and vectors in cucurbit crops, with a particular focus on virus distribution in Spain, mixed infection dynamics, host response under varying temperatures, and aphid vector microbiome.

More specifically, this aim was addressed through the following objectives:

1. To validate and expand studies on the distribution of aphid-transmitted viruses in melon and watermelon crops for 3 consecutive years (2021-2023) in the production areas of the Region of Murcia, Alicante, and Castilla-La Mancha, including the genetic characterization of viruses affecting cucurbit crops.
2. To examine how the temporal order of mixed infections, co- and sequential infections, could affect the dynamics of viral populations of two main virus species, CABYV and WMV, that infect melon plants.
3. To examine the individual and combined effects of viral infection and temperature variations on the gene response of susceptible melon and zucchini plants with differing levels of temperature tolerance.
4. To evaluate how the structure and composition of the aphid microbiome (*A. gossypii*) are altered by feeding on different plant species and with various types of viral infections.

RESULTS

6.1. Chapter I: Distribution and frequency of aphid-transmitted viruses in cucurbit crops

6.1.1. Sub-Chapter I.I: "*Occurrence, distribution, and management of aphid-transmitted viruses in cucurbits in Spain*"

pathogens

MDPI

Review

Occurrence, Distribution, and Management of Aphid-Transmitted Viruses in Cucurbits in Spain

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Abstract: The effectiveness of pest and disease management in crops relies on knowledge about their presence and distribution in crop-producing areas. Aphids and whiteflies are among the main threats to vegetable crops since these hemipterans feed on plants, causing severe damage, and are also able to transmit a large number of devastating plant viral diseases. In particular, the widespread occurrence of aphid-transmitted viruses in cucurbit crops, along with the lack of effective control measures, makes surveillance programs and virus epidemiology necessary for providing sound advice and further integration into the management strategies that can ensure sustainable food production. This review describes the current presence and distribution of aphid-transmitted viruses in cucurbits in Spain, providing valuable epidemiological information, including symptom expressions of virus-infected plants for further surveillance and viral detection. We also provide an overview of the current measures for virus infection prevention and control strategies in cucurbits and indicate the need for further research and innovative strategies against aphid pests and their associated viral diseases.

Keywords: aphids; cucurbits; CABYV; crop management; WMV; viral symptoms

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1. An Introduction to Viral Diseases Affecting Cucurbit Crops

Cucurbits are among the most important horticultural vegetables worldwide. The production of these vegetables (melon, watermelon, zucchini, cucumber, and pumpkin, among others) is often seriously affected by several pests and diseases [1,2]. Among them, viral diseases stand out not only for their negative impact on food quality and yield [3–7] but also for the complexity of controlling these diseases in an efficient manner and the rise in major epidemic events when control is deficient [8]. Although cucurbit crops may be grown under protected production systems (tunnels and greenhouses), they are mostly grown in open fields from spring to early autumn in temperate and subtropical regions. Herein, viral diseases that are transmitted by insect vectors are highly relevant, negatively affecting yield and quality of crop production. It has been estimated that plant viruses are responsible for around USD 30 billion in annual losses in crops [9]. Without a reliable estimation of the economic impact of viral diseases on cucurbit crops, there are 28 viral species that have been recently described to be relevant to cucurbit crops in the Mediterranean basin [3,10,11] (Table 1). It is remarkable that most of these viruses are vectored by aphids and whiteflies (order Hemiptera: *Aphidoidea* and *Aleyrodoidea*, respectively) [11,12]. Among them, cucurbit aphid-borne yellows virus (CABYV), cucumber mosaic virus (CMV), watermelon mosaic virus (WMV), and zucchini yellow mosaic virus (ZYMV) have been reported in more than half of the 26 countries of the Mediterranean basin [11–15], whereas the remaining 24 viruses have also been found to cause damages in cucurbit crops, but occasionally either at the local or regional levels. These include whitefly-borne viruses, such as cucurbit yellow stunting disorder virus (CYSDV), and tomato leaf curl New Delhi virus (ToLCNDV), with significant and negative effects on cucurbit production [16–18], or viruses that can be transmitted

either by leafhoppers, such as the rhabdovirus eggplant mottled dwarf virus (EMDV), or soil fungi, such as the carmovirus melon necrotic spot virus (MNSV). In addition, we also find seed-borne viruses, such as the tobamovirus cucumber green mottle mosaic virus (CGMMV), which is severely affecting cucurbit crops in the Mediterranean basin [3,11]. Among these, cucurbit chlorotic yellows virus (CCYV) is also worth mentioning, as this crinivirus was first described in 2004 in melon plants in Japan, and since then, it has been reported in countries such as Greece, Lebanon, Korea, India, Iran, Sudan, Taiwan, China, and the United States, where it affects different cucurbit crops [10,19–28], including the recent identification in cucumber, watermelon and zucchini crops in Spain [29,30].

Table 1. Relevant viral species known to affect cucurbit crops in the Mediterranean basin [3,11]. There are 28 virus species, which have been classified according to the vector transmission and taxonomic genus. The significant number of aphid-transmitted viruses affecting cucurbit crops is remarkable. The viruses described in this review are indicated in bold.

TRANSMISSION	GENUS	VIRUS
APHID-BORNE VIRUSES	POTYVIRUS	Algerian watermelon mosaic virus (AWMV)
		Clover yellow vein virus (ClYVV)
		Melon vein-banding mosaic virus (MVBMV)
		Telfairia mosaic virus (TeMV)
		Papaya ringspot virus (PRSV)
		Turnip mosaic virus (TuMV)
		Moroccan watermelon mosaic virus (MWMV)
		Watermelon leaf mottle virus (WLMV)
		Watermelon mosaic virus (WMV)
		Zucchini yellow fleck virus (ZYFV)
		Zucchini yellow mosaic virus (ZYMV)
	CUCUMOVIRUS	**Cucumber mosaic virus (CMV)**
	POLEROVIRUS	**Cucurbit aphid-borne yellows virus (CABYV)**
	MASTREVIRUS	Chickpea chlorotic dwarf virus (CpCDV)
WHITEFLY-BORNE VIRUSES	CRINIVIRUS	Beet pseudoyellows virus (BPYV)
		Cucurbit yellow stunting disorder virus (CYSDV)
	IPOMOVIRUS	Cucumber vein yellowing virus (CVYV)
	BEGOMOVIRUS	Squash leaf curl virus (SLCV)
		Tomato leaf curl New Delhi virus (ToLCNDV)
		Watermelon chlorotic stunt virus (WmCSV)
FUNGUS-BORNE VIRUSES	CARMOVIRUS	Cucumber soil-borne virus (CuSBV)
		Melon necrotic spot virus (MNSV)
	AUREUSVIRUS	Cucumber leaf spot virus (CLSV)
	NECROVIRUS	Tobacco necrosis virus (TNV)
SEED-BORNE VIRUSES	TOBAMOVIRUS	Cucumber fruit mottle mosaic virus (CFMMV)
		Cucumber green mottle mosaic virus (CGMMV)
BEETLE-BORNE VIRUSES	COMOVIRUS	Squash mosaic virus (SqMV)
LEAFHOPPER-BORNE VIRUSES	RHABDOVIRUS	Eggplant mottled dwarf virus (EMDV)

Within the Mediterranean basin, Spain is among the largest producer of cucurbits, with a total of 67,500 ha cultivated in the central and southeastern areas and over 3 million tonnes

harvested a year [31]. Our current observations in these crops suggest that the incidence of aphid-transmitted viruses has increased in the last few seasons [32–34], concurrently with an increase in the aphid population in the early stages of crop cultivation. Thus, a better understanding of the intricate epidemiology of cucurbit viral diseases will allow the improvement of the management strategies utilized for these pests and the aphid-transmitted viruses in crops [35,36]. Here, we review the occurrence of CABYV, CMV, papaya ringspot virus (PRSV), WMV, Moroccan watermelon mosaic virus (MWMV), and ZYMV in cucurbit crops, providing a brief description of their biology and symptom expression in the major cultivated plant species, and considering their geographical distribution and epidemiological status from 2011 to 2020 in Spain. In addition, current strategies for the prevention and control of these viral diseases are discussed, drawing attention to the need to understand the plant-aphid-virus interplay as a crucial prerequisite for improving and developing innovative and sustainable control strategies for the management of aphid pests and their associated viral diseases in agriculture. This review will contribute to the knowledge of the aphid-borne viruses in cucurbits crops in Spain, as well as to the knowledge of the epidemiological state and management of these viral diseases.

2. Description of the Main Aphid-Transmitted Virus in Cucurbits

2.1. Cucurbit Aphid-Borne Yellows Virus

CABYV is a member of the genus *Polerovirus* (family *Luteoviridae*). CABYV particles are icosahedrical with an approximate diameter of 25 nm, and its genome consists of a single-stranded positive-sense RNA molecule of about 5.7 kb [3]. It is limited to phloem tissues in infected plants and is transmitted in a persistent (circulative and non-propagative) manner by the aphid species *Aphis gossypii* Glover, *Myzus persicae* Sulzer, and *Macrosiphum euphorbiae* Thomas [37]. Based on phylogenetic analyses of full-length genome sequences, CABYV has been divided into four groups: Asian, Taiwan, Mediterranean, and recombinants [38–40]. CABYV mainly infects cucurbit species such as cucumbers, melons, watermelons, courgettes, pumpkins, and bitter cucumbers, in addition to other cultivated species such as fodder beet *(Beta vulgaris*) and lettuce (*Lactuca sativa*), and a variety of wild plant species: *Papaver rhoeas*, *Ecballium elaterium*, and *Capsella bursa-pastoris*, which could serve as reservoirs for vectors and/or sources of inoculum [11,41,42]. CABYV causes symptoms entirely in cucurbit plant species, causing significant flower abortion. Depending on the plant species, virus isolate, the age of the plant at the time of infection, and environmental conditions, yellowing is the most common symptom induced by CABYV infection, which causes systemic discoloration of the plant (Figure 1). This CABYV yellowing may vary from slight to mild thickening and brittleness, with vein banding leading to green veins (Figure 1A–D). In addition, necrotic rings are often observed on basal leaves due to starch accumulation and dehydration, with subsequent collapse (Figure 1B–D). However, new emerging CABYV variants and mixed infections may ameliorate or exacerbate plant symptoms, frustrating the phytosanitary inspections of cucurbit crops. In fact, a novel CABYV variant has recently been reported in watermelon crops, causing more severe symptoms of yellowing and interveinal chlorotic mottling not only in watermelon plants but also in zucchini and melon plants, as compared to the classical CABYV isolate [34].

2.2. Cucumber Mosaic Virus

CMV belongs to the *Cucumovirus* genus (family *Bromoviridae).* Its particles are roughly spherical and about 30 nm in diameter, and its genome has three-partite, single-stranded RNA molecules, which code for five genes [43]. CMV is transmitted in a stylet-borne non-persistent manner by 70 aphid species, including *M. persicae*, *A. gossypii*, *A. craccivora* Koch, and *A. fabae* Scopeli [44–46]. Although, it has been experimentally shown that *A. gossypii*, *A. glycines* Matsumura, *Acyrthosiphon pisum* Harris, and *Therioaphis trifolii* Monell are the most efficient vectors of CMV [47]. In addition, seed transmission of CMV takes place in some species such as spinach, zucchini, bean, and some weedy plants such as wild cucumber (*Echinocystis lobata)* and *Stellaria media* (L.) Vill [43,44,46]. In spite of this, it

has been reported that transmission rates are quite low, although higher rates have been reported. For example, transmission rates of up to 21% have been observed in cowpea seeds, which is enough to cause an epidemic [47]. Based on serology, symptomology, host range data, and nucleotide sequence of either CP or the 3′ or 5′ UTR of RNA3, CMV strains have been classified into three subgroups: IA, IB, and II [48]. While Subgroup IA and II have a worldwide distribution, Subgroup IB is mainly present in Asia, although it has also been reported in Spain, Italy, and Greece, with a few isolates in the USA and Brazil [47]. CMV has a broad host range, including monocotyledons and dicotyledons plant species, with more than 1200 known hosts [11,49]. For example, CMV infects plant species belonging to different botanical families, such as *Brassicaceae*, *Solanaceae*, *Papilionaceae*, and *Cucurbitaceae*, including weeds and ornamental plants such as *Chenopodium amaranticolor*, *Datura innoxia*, *Hibiscus rosa* or *Salvia splendens* [49–51]. Recently, it has also been reported to infect *Ocimum gratissimum* L. in Nigeria [52] and *Tylophora indica* in India [53]. In Spain, CMV has been found to be widely distributed in cucumber, melon, pumpkin, zucchini, watermelon, tomato, pepper, aubergine, tobacco, beans, celery, and borage horticultural crops, among others [42,47,50,54]. The most common symptom of CMV infection is the presence of mosaics on the leaves (Figure 2), although symptom expression varies according to the plant species, virus isolate, the age of the plant at the time of infection, and environmental conditions [55]. Moreover, CMV symptoms can be modified by the presence of RNA satellites [56]. For example, a satellite RNA, CARNA-5, has been associated with CMV, and in the case of tomato infection, it becomes lethal to the plant [47]. In our observations of CMV infection in cucurbits, mosaic patterns were commonly observed on leaves and fruits (Figure 2A–H). For instance, mild mosaics may appear in cucumber, melon, watermelon, and zucchini leaves (Figure 2A–D, respectively), including chlorotic spots and curling on melon leaves (Figure 2B), as well as yellow mottling on watermelon leaves (Figure 2C). CMV symptoms on fruits generally consist of deformation, mosaic, mottling, and stunting (Figure 2E–H), with yellow or green mosaics and enation (Figure 2E,H), and even bubbling (Figure 2G,H).

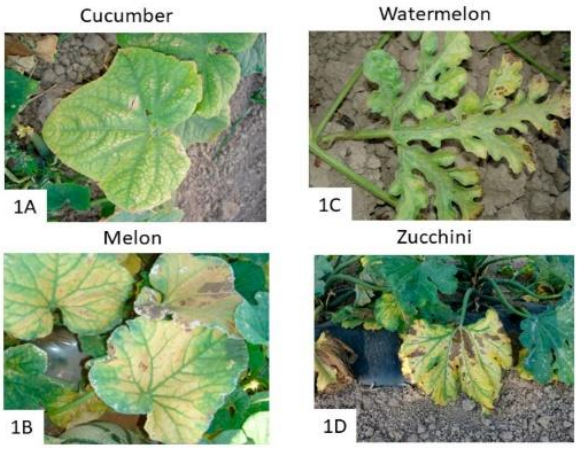

Figure 1. Symptoms of CABYV infection on cucurbit leaves. CABYV-affected plants show yellowing and vein banding symptoms on cucumber leaves (**A**), as well as on melon (**B**), watermelon (**C**), and zucchini (**D**). Additionally, necrotic rings can also be observed on basal leaves of melon (**B**), watermelon (**C**), and zucchini (**D**).

2.3. Papaya Ringspot Virus

PRSV belongs to the genus *Potyvirus* (family *Potyviridae*), and it is composed of flexuous particles of about 780 nm. Its genome consists of a unipartite linear single-stranded positive-sense RNA of 10 kb [3,57]. PRSV is transmitted by more than 20 aphid species in a non-persistent manner, including *M. persicae*, *Aulacorthum solani* Kaltenbach, *A. craccivora*, *A. gossypii*, *M. euphorbiae*, *Toxoptera aurantii* Boyer de Fonscolombe, and *Rhopalosiphum maidis* Fitch [58]. There is experimental evidence that suggests that PRSV could be transmit-

ted mechanically during the harvesting process or mechanically by contact between plant leaves [57]. In fact, there is one study in which the authors performed mechanical inoculations of PRSV in different cucurbit plants, and they observed that zucchini squash was the most susceptible species, followed by watermelon and cucumber, while the pumpkin was not infected by mechanical inoculations of PRSV [59]. Serologically, PRSV has been divided into two biotypes; the papaya-infecting biotype P (PRSV-P) and the cucurbit-infecting biotype W (PRSV-W). PRSV-P was first described from papaya in Hawaii, and PRSV-W was formerly known as watermelon mosaic virus 1 (WMV-1), which might have appeared in Australia 20 years before PRSV-P, suggesting that PRSV-P may have originated from PRSV-W [60]. PRSV has a very restricted host range, mainly infecting members of the *Cucurbitaceae* family, with the exception of the papaya from which it takes its name, and some non-cucurbit species such as *Chenopodium* [11]. In this sense, while PRSV-W isolates infect plants from *Cucurbitaceae* and *Chenopodiaceae* families, PRSV-P isolates also infect papaya plants [57]. Symptoms associated with PRSV may vary depending on the plant species. In papaya, PRSV-infected plants show typical symptoms of vein-clearing, yellow mottling, rough surface, and bristly leaf margins, stunting, and partial or complete cessation of fruiting [61]. In cucurbits, PRSV symptoms are similar to those found on papaya, consisting of chlorosis and mosaics on leaves, with curling and malformations (Figure 3A–C). In fruits, deformation, discoloration, and flower abortion are the major PRSV symptoms in melon, watermelon, and zucchini (Figure 3D–F), with dark green blisters, distortion banding, and ringspots as well. In the case of some members of the family *Chenopodiaceae*, symptoms are milder than in papaya or cucurbits crops [57].

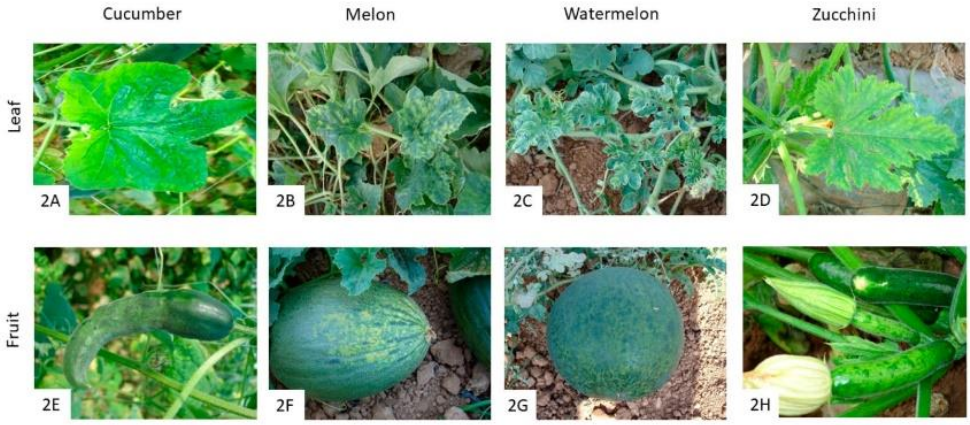

Figure 2. Symptoms of CMV infection on cucurbit leaves and fruits. CMV-affected plants show symptoms of mild mosaics on cucumber leaves (**A**), and yellow or green mosaics, deformation, and enation on cucumber fruits (**E**). In melon, mosaics, chlorotic spots, and curling on leaves (**B**), and mosaic, mottling, and stunting on fruits (**F**) are observed. In watermelon, leaves may also show mottling (**C**), and mosaic, mottling, and bubbling on fruits (**G**). Zucchini leaves show mosaic (**D**), with deformation, mosaic, mottling, and stunting on zucchini fruits (**H**).

2.4. Watermelon Mosaic Virus

WMV is another potyvirus with flexuous and filiform particles of about 760 nm in length [3]. Its genome consists of a single-stranded positive-sense RNA genome, comprising a unique open reading frame of about 10 kb that encodes for a polyprotein [62]. WMV can be transmitted in a non-persistent manner by at least 35 species of aphids in 19 genera, with the most important being *A. citricola* V. der G., *A. craccivora*, *A. gossypii*, *A. solani*, *M. euphorbiae*, *M. persicae*, and *T. citricidus* Kirkaldy. WMV has not been reported to be seed transmitted in cucumber, watermelon, melon, or zucchini [11], although it can be transmitted mechanically in laboratory conditions and through vegetative propagation in vanilla [63,64], and other species such as *C. quinoa*, *C. amaranticolor*, *Nicotiana benthamiana*, *N. glutinosa*, and *N. tabacum*, as well as cucurbits species and some legumes such as *Trifolium*

incarnatum, *T. subterraneum*, *Pisum sativum* and *Vicia faba* [65,66]. As stated before, WMV-1 is considered the W strain of PRSV, and WMV-2 is referred to as WMV [64]. WMV isolates have been classified into three molecular groups based on the amino acid sequences of the coat protein N-terminal region: Group 1 isolates, which are also known as classical (CL) isolates, Group 2 isolates, and Group 3 emerging isolates (EM), with its isolates divided into four genetic subgroups (EM1, 2, 3, and 4). These subgroups were associated with a different geographic distribution in southern France, where it was observed that wherever EM1 and EM2 were present, EM3 and EM4 were absent [11,67,68]. It has been shown that the accumulation and transmission efficiency of EM isolates is higher than CL and Group two isolates, which could explain the displacement of both groups by the EM group in the Mediterranean basin [63,69]. WMV has a wide host range, infecting over 170 species of monocotyledonous and dicotyledonous plant species, including cultivated cucurbits and peas, beans, spinach, and vanilla crops, as well as weeds (senecio, shepherd's purse, nettle or fumaria, among others) [63,64]. WMV causes typical potyvirus symptoms, although it depends on the plant species. In cucurbit leaves, mosaic patterns are commonly observed (Figure 4A–D), with vein clearing and banding in cucumber (Figure 4A), including mottling mosaics and leaf deformation in melon, watermelon, and zucchini (Figure 4B–D). In fruits, yellow and green mosaics are observed (Figure 4E–H), with enation (Figure 4E), colorless (Figure 4F,G), and blisters (Figure 4H).

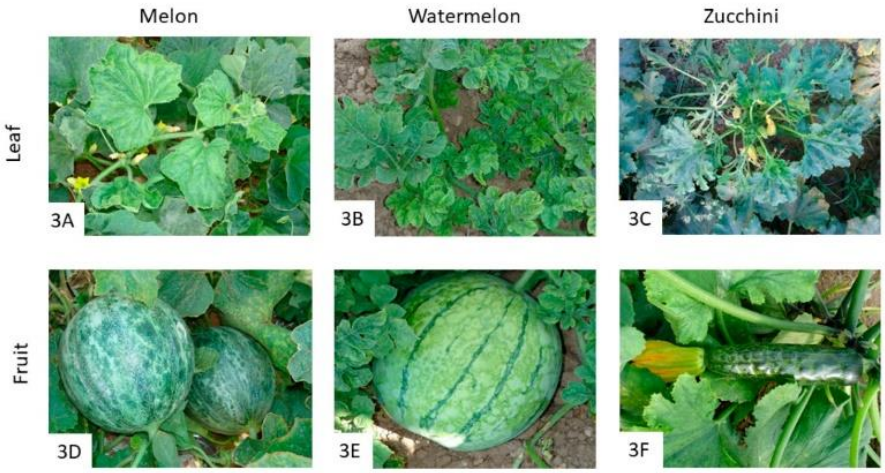

Figure 3. Symptoms of PRSV infection on cucurbit leaves and fruits. PRSV-affected plants show symptoms of chlorosis and mosaics, including curling and malformations on melon (**A**), watermelon (**B**), and zucchini (**C**) leaves. Affected fruits show deformation and discoloration, along with dark green blistering, distortion banding, and ringspots in melon (**D**), watermelon (**E**), and zucchini (**F**).

2.5. Moroccan Watermelon Mosaic Virus

MWMV is a potyvirus that results from PRSV at the genetic level, with a single-stranded RNA genome of about 10 kb [70]. Similar to WMV, this virus can be transmitted mechanically, but it is naturally transmitted by aphids in a non-persistent manner [71]. *M. persicae*, *A. gossypii*, and *A. spiraecola* Patch can efficiently transmit MWMV, with *M. persicae* and the subspecies *M. persicae nicotianae* being the most competent vectors in cucurbit crops [71]. Phylogenetic analyses have shown three genetic groups (A-C): Clade A comprises isolates from Tunisia and Europe; clade B, isolates from South Africa; and clade C, isolates from Central Africa [72]. Cucurbits are the main hosts of MWMV [64], in addition to other crops, such as papaya (*Carica papaya*) [73], and wild species, such as *Chenopodium amaranticolor*, *C. quinoa*, and *Ranunculus sardous* [72]. MWMV symptoms are similar to those of WMV, resulting a severe vein clearing and banding with dark green mosaics, including sawn edges on leaves (Figure 4A–D). Fruit symptoms consist of deformations (Figure 4E), discoloration (Figure 4F), mosaics, and blistering (Figure 4G,H, respectively).

Figure 4. Symptoms of WMV infection on cucurbit leaves and fruits. Plants affected by WMV show mosaic symptoms, including sawn edges, vein clearing, and banding in cucumber leaves (**A**). In melon, mosaic, mottling, and leaf deformation are observed on leaves (**B**), as well as on watermelon (**C**) and zucchini (**D**) leaves. Affected fruits show yellow and green mosaics, with enation and deformations in cucumber (**E**), colorlessness on melon (**F**), and blistering on watermelon (**G**) and zucchini (**H**) fruits. Similar symptoms are observed in plants affected by MWMV.

2.6. Zucchini Yellow Mosaic Virus

ZYMV also belongs to the genus *Potyvirus*, with flexuous particles of about 750 nm in length. Its genome consists of a single-stranded RNA of about 9.6 kb [74]. ZYMV is transmitted in a non-persistent manner by at least 26 aphid species. However, only a few aphid species have been tested for their transmission efficiency, apart from *A. gossypii*, *M. persicae*, *A. craccivora*, and *M. euphorbiae* [11,64]. Three genetic groups have been described in the ZYMV population: Group A, which is in turn divided into six clusters, and Group B and C. While isolates from group A have only been detected in the Mediterranean basin, isolates from groups B and C have been detected in Vietnam and China, as well as isolates from group C in Poland [11]. ZYMV has a restricted host range, primarily infecting cultivated or wild cucurbit plant species, in addition to a few weed species of the *Delphinium*, *Begonia*, and *Altheae* genera [64]. Symptom expressions of ZYMV in *Chenopodium amaranticolor* and *C. quinoa* range from local lesions or latent infections [74]. In cucurbits, ZYMV symptoms may include vein clearing, deformation, and mosaics, with bubbling, blistering, sawn edges, and enation (Figure 5A–D). In fruits, enation, mosaic, or necrotic cracks appear to be common (Figure 5F–H), with severe deformations (Figure 5E) and bubbling (Figure 5G,H).

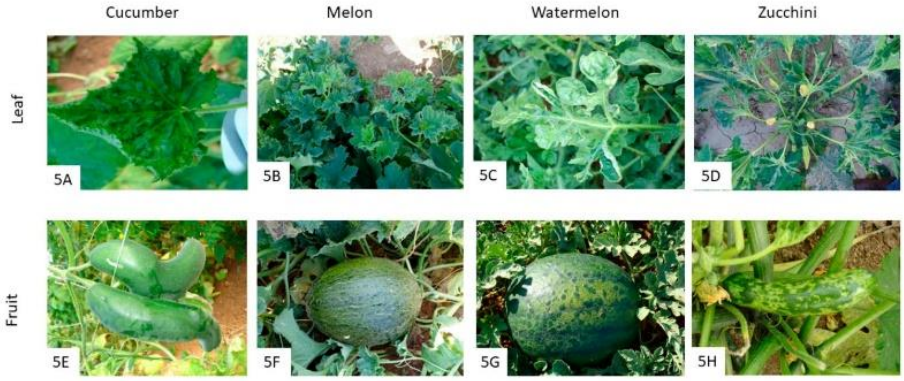

Figure 5. Symptoms of ZYMV infection on cucurbit leaves and fruits. ZYMV-affected plants show symptoms of vein clearing, deformation, mosaics with bubbling, blistering, sawn edges, and enation observed in cucumber (**A**), melon (**B**), watermelon (**C**), and zucchini (**D**) leaves. Affected fruits show enation, mosaic, or necrotic cracks, with severe deformations on cucumber (**E**) and melon (**F**), including bubbling in watermelon (**G**) and zucchini (**H**) fruits.

3. Geographical Distribution and Occurrence of Aphid-Transmitted Viruses

Since aphid-borne viruses are widely distributed in cucurbit-producing areas, it is crucial to determine which virus species are present and which factors lead to their prevalence in order to address successful crop management. On the one hand, the lack of reliable viral detection during phytosanitary inspections is a risk factor. Monitoring and surveillance programs must be accompanied by appropriate serological or molecular diagnostic methods [75], in addition to the characterization of the genetic diversity and population structure since novel variants can emerge. The monitoring based on symptom expression is often technically challenging, whereas different viruses induce similar symptoms, some plant species may be asymptomatic, or symptoms can even be mistaken for physiological disorders or nutrient deficiencies [76]. Serological methods, such as enzyme-linked immunosorbent assay (ELISA) and its variants, have been used for decades, allowing the identification and detection of viruses in a fast and easy manner [77]. Molecular methods, such as polymerase chain reaction (PCR) and all its variants, DNA microarray, as well as tissue-print and dot-blot hybridization, have high sensitivities and reasonable costs [75,77,78]. Nevertheless, current and innovative techniques, such as metagenomics high-throughput sequencing (HTS), CRISPR/Cas12, or machine learning (ML) approaches, have created a revolution in the detection of multiple viruses that are either known or unknown [79–81].

On the other hand, the combination of multiple abiotic and biotic factors can be responsible for the prevalence of viral diseases [82–87]. In cucurbit crops, the commercial exchanges of seeds, plants, or fruits are associated with the geographical distribution of these viral diseases. However, the aphid vector abundance and transmission efficiency, along with the presence of alternative host reservoirs, including other cultivated crops and wild plant host species that may overlap at spatio-temporal scales, can underlie the plant viral distribution [88,89]. Additionally, agricultural ecosystems composed of highly dense and genetically uniform host populations can favor the emergence and prevalence of viral diseases [88], where socio-cultural changes and alterations of the growing agricultural production can create fragmentations in the distribution of vectors, generating changes over the viral populations. Furthermore, several studies have reported how host preference and vector behavior influence the transmission and spread of plant viruses [35,90,91], and in turn, how environmental conditions can affect the virus prevalence and vector transmission, as climate change may have a significant influence on virus pathogenicity, ecology, and evolution [92,93]. Attention should be paid to the fact that the aforementioned factors can also be influenced by the co-occurrence of related and/or unrelated viruses in the same plant, which may consequently alter virus accumulation and transmission rates [94–96]. In this case, mixed infections of aphid-borne viruses in the same plant can be found, and some examples are gathered in Supplementary Table S1. Therefore, the lack of early and appropriate virus detection, along with the variation between several abiotic and biotic factors, have an influence on the occurrence of viral diseases, underlie their prevalence, and shape the evolutionary dynamics of each viral population.

3.1. Cucurbit Aphid-Borne Yellows Virus

CABYV is widespread in Europe, especially in the Mediterranean region, although it also occurs in Asia, parts of Africa, and North America. CABYV was first described in cucurbit production areas of France in 1992 [97]. Since then, it has spread to Cyprus, the Czech Republic, Greece, Italy (including Sicily), Spain, and Tunisia [3,5,98–100], with current detections in Poland, Slovenia, Germany, and Bulgaria [101–104]. In Spain, CABYV was first identified in 2004 in the Murcia region in melon and zucchini plants [14], and since then, it has become one of the most prevalent viruses affecting cucurbit crops reaching incidences of 83% and 66% for melon and squash samples, respectively [14,32,34]. Indeed, after combining the epidemiological data from our long-term monitoring studies (2011 to 2020) for aphid-borne viral diseases [32–34], including the last two growing seasons (2021 and 2022), CABYV was found to be the most prevalent virus in cucurbit crops. It

was detected in a high proportion of symptomatic samples of zucchini (54%), melon (40%), pumpkin (34%), and watermelon (26%) in single infections (Figure 6). Furthermore, there was also a high proportion of samples with CABYV and WMV in mixed infections in melon (24%), watermelon (32%), pumpkin (16%), and zucchini (14%) (Figure 6). The combination of CABYV with other viruses, such as ZYMV, PRSV, and MWMV, was present in low occurrence (<3%) and only detected in pumpkin and zucchini (Figure 6). In addition, CABYV is often found in the presence of other viruses in the same plant (Supplementary Table S1).

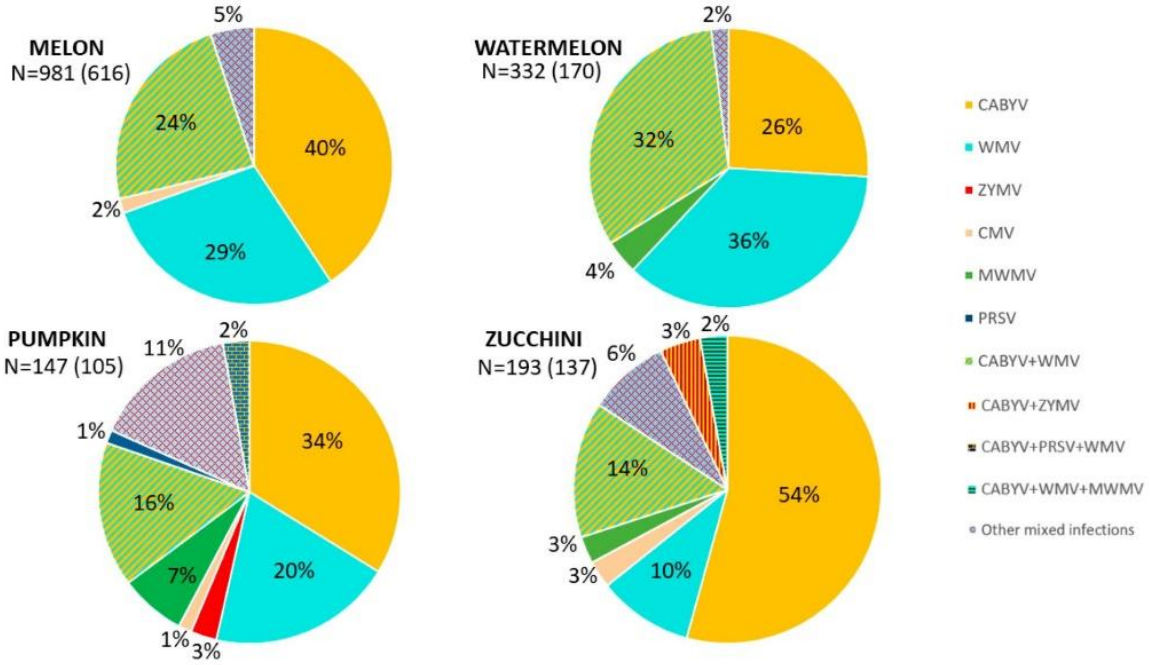

Figure 6. Occurrence and distribution of cucurbit aphid-transmitted virus in Spain. The proportion of aphid-transmitted cucurbit viruses from symptomatic cucurbit cultivated plants over 12 cucurbit growing seasons (2011 to 2022). These epidemiological data came from a long-term monitoring study (2011 to 2020) of aphid-borne viral diseases [32–34], including the unpublished data from the last two growing seasons (2021 and 2022). The proportion of each virus is represented in each slice, with a color assigned for each virus and indicated in the legend. Single infection is represented by a solid color and mixed infection by a stripped color, combining the color of each virus in a single infection. N = total samples (total positive samples detected). CABYV and WMV are the most prevalent aphid-transmitted viruses affecting cucurbit crops in Spain.

3.2. Cucumber Mosaic Virus

CMV was first described in 1916, resulting in one of the viruses that most frequently affects cucurbit crops worldwide [43]. Since CMV also affects major annual crops, it is widely distributed in Argentina, eastern China, Croatia, France, Egypt, Greece, Israel, Italy, Japan, Poland, Portugal, Sweden, and the northeastern US [105–108]. According to molecular analyses of the genomic RNAs, CMV has been classified into two extremely heterogeneous subgroups [108]. Subgroup I has been reported in both temperature and tropical regions and is divided into two subgroups (IA and IB) based on analysis of RNA 3 open reading frames, whereas Subgroup II has been reported in cooler areas and seasons of temperate regions being Subgroup I more heterogeneous than Subgroup II [108]. In the Mediterranean basin, CMV is present in all countries [11]. In Spain, it was reported for the first time in 1995 in melon crops [109], with a high incidence in some cases [110,111]. Since then, the genetic structure of CMV in different locations in Spain has been studied, showing that most CMV isolates belong to Subgroup I, which has a wide distribution in cucurbit crops and others, such as tomato crops in simple and mixed infections [112].

Moreover, studies in other hosts, such as weeds, have shown that from 1999 to 2002, CMV was detected with a maximum incidence of 30% in weed hosts and melon in Central Spain [50]. Systematic surveys in 2003 and 2004 in open field melon and squash in the Murcia Province showed that the incidence of CMV was less than 6% and 1.5% in melon and squash samples, respectively [105]. In the Valencian Community, from 2005 to 2006, it was shown that the CMV incidence was less than 10% of the total plants from different cucurbits (melon, watermelon, pumpkin, cucumber, and squash) [69]. In a cucurbit survey study in 2018, from open fields in the Murcia Province, Castilla-La Mancha, and the Valencian Community, it was reported that CMV had a low incidence and that the CMV sequences showed a high identity between themselves and with European isolates [15]. This is in accordance with our long-term monitoring program from 2011 to 2022, which showed that CMV was only detected in symptomatic samples of melon (2%), pumpkin (1%), and zucchini (3%) [32–34] (Figure 6).

3.3. Papaya Ringspot Virus

PRSV is the cause of one of the most serious diseases affecting cucurbit crops in the Southern United States and the Antilles, although it also occurs in several Mediterranean countries (Bulgaria, Cyprus, France, Israel, Italy, Lebanon, Spain, Syria, Tunisia and Turkey) [11,57], and has even been detected in pumpkin in Sudan, zucchini in Poland and Morocco, and papaya in Argentina for the first time [113–116]. In Spain, PRSV was detected for the first time in zucchini in Malaga in 1985 [117]. In 1995, PRSV was present in melon crops in Spain with a lower incidence [109], and since then, PRSV has been present in different cucurbit crops with variable incidences between regions, in both simple and mixed infection with other viruses year by year [14,33,34,69]. Although with a low frequency in Spanish crops, PRSV has been detected in pumpkin samples (1%), with the last detection in 2019 under mixed infections with CABYV and WMV [32–34] (Figure 6).

3.4. Watermelon Mosaic Virus

WMV is widely distributed in cucurbit crops worldwide [63]. It was reported in most countries in the Mediterranean region, the first time being in 1963 in Israel. Since then, it has also been found in Italy, Tunisia, and France, among others [11], with the most recent detections in Bosnia, Herzegovina, and Poland [118,119]. In Spain, WMV is among the most widely distributed cucurbit viruses [110,117,120] and has become highly prevalent in melon and zucchini crops [14,69,109,111]. In melon crops, WMV was the most frequently found virus in surveys carried out in 1995 and 1996, although it is known that WMV has been present in field-grown melons in Spain since 1991 [109]. Cucurbit crop surveys carried out in eastern Spain in 2005 and 2006 showed that WMV was present in 27% of samples and was also detected in 36% of samples under mixed infections [69]. However, during the 2003 and 2005 seasons in Murcia, WMV incidence was 21% and 6% in melon and squash crops, respectively, where mixed infections of CABYV + WMV were present with a high frequency (18%) in melon crops [14]. Thus, and similar to the CABYV assessment from 2011 to 2022, WMV is well-recognized as a prevalent virus in melon, watermelon, pumpkin, and zucchini crops, with a higher occurrence in watermelon (36%), as compared to melon (29%), pumpkin (20%), and zucchini (10%) plants, where it is lower in single infections [32–34] (Figure 6). There was also a high frequency of mixed infections between WMV and CABYV, as mentioned above (Figure 6). The combination of WMV with others, such as CABYV and PRSV, or CABYV and MWMV in a triple infection, was found in a low occurrence (<2%) in pumpkin and zucchini (Figure 6).

3.5. Moroccan Watermelon Mosaic Virus

The initial identification and characterization of a novel WMV isolate from Morocco in 1974 resulted in the consideration of this MWMV as an emergent threat to cucurbit crops in the Mediterranean basin [11,121]. It has been described as causing damage to cucurbits crops in all producing regions, such as South, Central, and North Africa, and also European

countries such as Italy, Greece, France, Portugal, and Spain [72,121–124]. Additionally, it has been reported in the Democratic Republic of Congo in papaya, in Turkey in squash, and in Iran and Greece, in zucchini [73,124–126]. In Spain, it was described for the first time in zucchini crops [121], and since then, its occurrence has been low, although there was a resurgence in 2018 in watermelon and pumpkin crops in the cucurbit-producing areas of Murcia, Alicante, and Castilla-La Mancha [33]. MWMV was present in 4%, 7%, and 3% of symptomatic samples of watermelon, pumpkin, and zucchini, respectively, with no detection in melon [32–34] (Figure 6).

3.6. Zucchini Yellow Mosaic Virus

ZYMV was reported for the first time in 1973 in zucchini plants in Italy, and since then, it has been reported in 18 Mediterranean countries, including France, Israel, Egypt, and Turkey [11,64], as well as in watermelon crops in Serbia, and Bosnia and Herzegovina [127,128]. It has also been reported in other cucurbit species, such as bitter melon in South Korea [129] and gherkin in India [130]. In Spain, it is known that ZYMV has been present in melon crops since 1991 [109]. Since then, surveys carried out in different cucurbit crops over the years showed that ZYMV had a low incidence of less than 10% [14,69,117], as well as in mixed infection with other viruses [15]. From our long-term epidemiological data, ZYMV was found in a low occurrence in pumpkin crops (3%) from 2011 to 2022 and also in mixed infections combined with other aphid-transmitted viruses in melon, watermelon, and zucchini in a low occurrence (<1%) [32–34] (Figure 6). It is worth mentioning that the combination of ZYMV and CABYV was present in 3% of zucchini samples (Figure 6).

4. Overview of the Management of Aphid-Transmitted Viral Diseases

Despite advances in plant virus control and efforts to reduce or delay the spread and dispersal of the aphid-transmitted viruses described above, they are widely distributed, causing severe losses in yield and quality on cucurbit crops. Current methods for controlling these aphid-viral diseases result from preventive measures [131]. These include the use of plant material that is resistant and/or tolerant to these viruses, in addition to the use of virus-free material combined with cultural practices, such as weed control and crop rotation, as well as controlling vector-mediated transmission. These control options have been comprehensively described by other reviews [3,13,36,132–137], and even novel techniques based on nanotechnology have been recently reported for the management of plant virus diseases [138–140]. In cucurbit crops, it is important to consider that these crops grow in open fields and temperate growing areas, where the aphid-vector populations are also major pests that are difficult to control. This underscores a complex scenario where a profound understanding of the interplay between plant-virus-vector is crucial for the prevention and control of aphid pests and their associated viral diseases. In the following sections, we provide an overview of the general and local control measures for these aphid-transmitted viruses.

First, the most effective management option for virus control on plants is the use of resistant cultivars [141,142]. In this sense, genetic resistance and tolerance have been reported in a few melon, watermelon, squash, and cucumber lines through organogenesis or embryogenesis using agrobacterium-mediated transformation, mitigating symptom expression against ZYMV, WMV, CMV, and PRSV-W [141–143]. Potential sources of resistance to PRSV-W, WMV, and ZYMV have also been reported from melon accessions (C-189, C-105, C-885, C-769) and from wild relatives [144]. There are commercially-grown hybrid cultivars of melon with tolerance to aphids, in addition to cultivars carrying the *vat* gene, which confers resistance to both *A. gossypii* and the viruses it transmits [145]. With the means to edit cucurbit genomes directly through the revolutionary CRISPR/Cas9 approach [146], cucumber lines with mutations that disrupt the eIF4E interaction with viral proteins, which is associated with recessive resistance [147], have been reported and tested against CVYV, ZYMV, PRSV-W, CMV and CGMMV [148], and melon lines against MWMV [149]. This technology is reducing the timeframe needed to obtain cultivars carry-

ing traits of interest and is further underway to incorporate viral disease control into plant crops. Additionally, despite the fact that these viruses are mainly aphid-transmitted, in some cases seed transmission may occur, and this is why seed certification programs are mandatory to control the primary sources of viral inoculum and contribute greatly to success in controlling viral diseases [150]. Additionally, cultural practices by the use of physical barriers can readily diminish the presence of insects in the growing area. For example, in open-field production, the use of floating row covers and colored plastic mulches affects the abundance and presence of aphid species in the field production of watermelon [151]. This covering could prevent the crop, with moderate effectivity, especially against viruses that are transmitted in a non-persistent manner. In greenhouse production, the use of sticky insect traps and dense netting must be considered [134,152]. Another cultural practice is the use of border plant cultivation, known as trap crops, which can limit the spread of insect-vector-protecting cucurbit crops [13,153]. For example, in the USA, intercropping of pumpkin and sorghum can reduce the incidence of PRSV and WMV from 96% to 43%, while intercropping with other crops, such as soybean and peanut, was less effective than sorghum [154]. Additionally, tilling and the elimination of symptomatic plants or crop residues from previous seasons, including the alternative wild plants or others that can act as reservoirs and/or sources of viral inoculum, must be considered [155]. For example, it has been shown that viral incidence rates in wild plants could be one of the most important factors in explaining the epidemiological patterns of ZYMV and WMV [13,47]. Furthermore, modifications in the growing crop system may also lead to alterations in the viral population dynamics, increasing the risks of the virus emergence or outbreaks in new geographical regions [8,87]. For example, the intensification of vegetable production, increased connectivity between plant populations, along with global warming are considered to increase the risk of virus outbreaks [8,93]. In this sense, the speeding up replacement from traditional to organic/ecological crop production is thought to temporally affect the aphid pests and viral disease situation and requires a better understanding of the aphid-virus-plant interaction to understand the intricated epidemiology of the cucurbit viral diseases and to develop innovative strategies that replace the use of chemical pesticides for managing aphid-transmitted viruses in crops.

Beyond the preventive measures and cultural practices, it is worth considering that particular treatments with mineral oil sprays in the early stages of cultivation have been shown to interfere with the transmission of certain non-persistent viruses [11]. For example, there is evidence that the use of silver nanoparticles or nanomaterials by direct application to soil, seeds, or plant foliage, can have an impact on the bean yellow mosaic virus (BYMV) due to their antiviral activity [140]. In addition, in the early stages of plant cultivation, the implementation of a mild natural virus into the plant by mechanical inoculation to protect commercial crops from the same virulent virus species, known as cross-protection, has also been applied in cucurbits to minimize the risks from the ZYMV [156] and PRSV [157] diseases. However, the feasibility of the use of nanoparticles and cross-protection against insect-vectored viruses remains unknown. Finally, aphid population control is the major strategy utilized to manage these pests and to avoid the spread of viral diseases in cucurbit crops. It is thought that the efficiency of pesticide-based control may vary depending on the aphid-vector acquisition and transmission. While it could be relatively effective against persistently transmitted viruses, such as CABYV, which needs long-time acquisition and retention periods in the aphid-vector, it may have a limited effect against the non-persistently transmitted viruses (CMV, WMV, MWMV, ZYMV, and PRSV). Additionally, phytosanitary measures must be considered not only to prevent the primary plant inoculations but also to constrain the development of aphid colonies in the crop, which may help with the secondary infections and viral spread to the rest of the plot. Nevertheless, it should be noted that aphid species may become resistant to chemical compounds [153]. In fact, in accordance with the EU pesticide legislation 1107/2009 on Plant Protection Products, the negative effects caused by chemical pesticide applications, the increasing production of organic/ecological vegetables and the lack of effective methods to protect crops from

aphids, makes it necessary to integrate all available measures and to continue research to find innovative and sustainable strategies that protect crops from pests and diseases.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens12030422/s1, Table S1: Viral species combination that has been reported in mixed infections, including those particular aphid-borne viruses that have been described in this review. Refs. [158–185] are cited in Supplementary Materials.

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6.1.2. Sub-Chapter I.II: “*Revealing hidden viruses inducing similar yellowing symptoms or remaining asymptomatic in cucurbit crops*”

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ORIGINAL ARTICLE

Revealing hidden viruses inducing similar yellowing symptoms or remaining asymptomatic in cucurbit crops

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Abstract

Mixed viral infections occur frequently in plants, leading to diseases that can be difficult to diagnose, especially when an unknown virus is hidden behind expression of symptoms common to other viruses. We monitored aphid- and whitefly-transmitted viruses in two cucurbit crops (melon and watermelon) for three consecutive seasons (2021–2023) across three production regions in Spain, focusing on the common yellowing and mosaic symptoms. A total of 984 symptomatic leaf samples from 246 field plots were tested for 10 frequently found cucurbit viruses. Cucurbit aphid-borne yellows virus (CABYV) was the most prevalent virus infecting both cucurbit crops over the three seasons. Additionally, occurrence of watermelon mosaic virus (WMV) in samples with mosaic symptoms was significant. However, an important proportion of yellowing symptomatic samples tested negative for known viruses. Using a sequence-independent approach, we identified a novel polerovirus, Pepo aphid-borne yellows virus (PABYV), which was widespread, infecting both crops. Analysis of our long-term cucurbit frozen-sample collection revealed that PABYV had emerged in Spain in 2018, possibly unnoticed as its yellowing symptoms were similar to CABYV, with which it was frequently associated. Additionally, we found the cryptic Cucumis melo endornavirus (CmEV) in all tested melon samples from 2011 and, for the first time, in pumpkin. Genetic characterization of CABYV, PABYV and CmEV populations revealed a replacement of ancient CABYV isolates by contemporary ones, while PABYV and CmEV isolates were genetically homogenous among their populations. This study underlines the need for continuous surveillance and further investigation into common symptoms of mixed viral infections.

KEYWORDS
aphid-transmitted plant virus, CABYV, CmEV, mixed infections, PABYV, virus detection

1 | INTRODUCTION

Plant viral diseases lead to substantial economic losses to agriculture and cause food security concerns (Tatineni & Hein, 2022). Systematic monitoring of plant viral diseases, including symptom visualization and molecular diagnosis, has become crucial for the early and accurate detection of emerging viruses in crops (García-Arenal et al., 2000; Jeger et al., 2006; Jones, 2014; Madden et al., 2017;

Parnell et al., 2017). Additionally, comprehensive molecular genetic analyses of viral populations provide insights into the epidemiology and evolutionary dynamics of these emerging and circulating viruses (Elena et al., 2014; García-Arenal et al., 2001, 2003; Lefeuvre et al., 2019). Recent epidemiological studies have suggested the need for multiple approaches to understanding the aetiology and emergence of viral diseases. For example, with the new advent of metagenomic approaches, virome studies using high-throughput sequencing offer comprehensive characterizations of viral communities in plants (Hasiów-Jaroszewska et al., 2021; Maclot et al., 2020; Minicka et al., 2020), although these studies often leave the pathogenicity of detected viruses unclear. Also, novel methodological advances from remote sensing have provided non-invasive tools for detecting and monitoring plant diseases (Gáborjányi et al., 2003; John et al., 2023; Terentev et al., 2022), supplying valuable data for plant disease management (Oerke, 2020). However, distinguishing between plants affected by one or multiple viruses and those experiencing abiotic or nutritional stress remains challenging during surveillance of crops. In addition, related and unrelated virus species can infect the same host (i.e., mixed infections) and are indeed common in crops, being recognized as an integrated biotic factor in crop epidemics that can affect the ecology and evolution of the disease (Alcaide et al., 2020; Mascia & Gallitelli, 2016; Moreno & López-Moya, 2020; Syller, 2011). In fact, unknown viruses may remain hidden in mixed infections, leading to the misattribution of disease symptoms to known or frequently detected viruses. Addressing these complexities is essential for effective viral diagnosis and the management of long-term diseases, improving sustainable control measures for viral diseases in crops.

Cucurbit crops comprise a variety of vegetables, such as melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita maxima*), cucumber (*Cucumis sativus*) and zucchini (*Cucurbita pepo*), among others, with considerable economic importance in agriculture. These crops are persistently threatened by several plant viruses (Keinath et al., 2017; Lecoq & Desbiez, 2012;Lecoq & Katis, 2014; Radouane et al., 2021). Twenty-eight viruses have been identified as causing significant economic losses in the Mediterranean basin (Lecoq & Katis, 2014; Radouane et al., 2021). Most are mainly transmitted by insect vectors and belong to families such as *Geminiviridae*, *Closteroviridae*, *Potyviridae*, *Bromoviridae* and *Solemoviridae*; they can induce either yellowing or mosaics in plant leaves, regardless of the cultivar, environmental condition or strain (De Moya-Ruiz, Gómez, & Juárez, 2023; Lecoq & Desbiez, 2012). Among them, *Potyvirus citrulli* (watermelon mosaic virus, WMV), *Potyvirus citrullimoroccense* (Moroccan watermelon mosaic virus, MWMV), *Potyvirus papayanuli* (papaya ringspot virus, PRSV), *Potyvirus cucurbitaflavitesselati* (zucchini yellow mosaic virus, ZYMV) and *Cucumovirus CMV* (cucumber mosaic virus, CMV) are all aphid-transmitted viruses causing mosaics that are widely distributed, affecting cucurbit crops worldwide (Bertin et al., 2020; De Moya-Ruiz, Gómez, & Juárez, 2023; Desbiez et al., 2007; Juárez et al., 2013). The spread of cucurbit aphid-borne yellows virus (CABYV) is also a current concern, causing yellowing diseases in cucurbit crops, with recent outbreaks in various European countries (Desbiez et al., 2020; Mehle et al., 2019; Menzel et al., 2020; Minicka et al., 2020; Rabadán et al., 2021; Radeva-Ivanova et al., 2022). In particular, CABYV disease causes systemic discolouration of the plant, with yellowing and slight to mild thickening and brittleness depending on plant species, along with vein banding leading to green veins, accompanied by necrosis in basal leaves and fruit abortion (De Moya-Ruiz, Gómez, & Juárez, 2023). CABYV and WMV have been reported to infect major cultivated cucurbit species in Spain (Alonso-Prados et al., 2003; De Moya-Ruiz et al., 2021; Juárez et al., 2013; Kassem et al., 2007, 2013; López-Martín et al., 2024; Maachi et al., 2022; Moreno et al., 2004; Rabadán et al., 2021, 2023), with a significant association with and prevalence in mixed infections (Rabadán et al., 2023). Furthermore, our phylogenetic studies on CABYV and WMV populations in Spain revealed that CABYV populations exhibited genetic variation that may be attributed to the variation found in time and type of infection (i.e., specifically whether contemporary CABYV isolates were coming from single or mixed infection), whereas WMV populations remained genetically homogeneous (Rabadán et al., 2023; Rabadán & Gómez, 2023). This observation aligns with the identification of a novel CABYV isolate showing severe symptoms and a higher viral load compared to older isolates in cucurbit species (Rabadán et al., 2021), suggesting temporal differentiation of CABYV isolates within the Mediterranean cluster (Rabadán et al., 2023; Rabadán & Gómez, 2023), and raising the question about the current distribution of old or contemporary CABYV variants.

In addition to these viruses, whitefly-transmitted viruses, such as *Crinivirus pseudobetae* (beet pseudo-yellows virus, BPYV), *Crinivirus cucurbitae* (cucurbit yellow stunting disorder virus, CYSDV), *Ipomovirus cucumisvenaflavi* (cucumber vein yellowing virus, CVYV), and *Begomovirus solanumdelhiense* (tomato leaf curl New Delhi virus, ToLCNDV) have also been reported to affect major cucurbit species in Spain, causing yellowing symptoms (Juárez et al., 2013, 2019; Kassem et al., 2007; López-Martín et al., 2024; Maachi et al., 2022). Recently, a new crinivirus; cucurbit chlorotic yellows virus (CCYV) has also been identified in Spain (Alfaro-Fernández et al., 2022; Chynoweth et al., 2021), which could potentially be associated with the increasing incidence of yellowing observed in crops in Spain. However, to date, with the exception of ToLCNDV, there is only limited information about the causative viruses that induce yellowing and the distribution of these aphid- and whitefly-transmitted viruses in cucurbit crops in Spain. For instance, it is unclear whether unknown viruses causing similar symptoms can remain unnoticed within mixed infections, potentially affecting the accuracy of viral diagnostics. In this study, we addressed these gaps by examining the distribution of six aphid- and four whitefly-transmitted RNA viruses in melon and watermelon crops, developed a molecular diagnostic method to enable identification of a hidden polerovirus, Pepo aphid-borne yellows virus (PABYV), and determined its emergence. The presence of the cryptic virus *Alphaendornavirus cucumis* (Cucumis melo endornavirus, CmEV) was also detected, and a comprehensive genetic characterization of CABYV, PABYV and CmEV isolates was conducted.

2 | MATERIALS AND METHODS

2.1 | Sample collection

The phytosanitary inspection of melon (*C. melo*) and watermelon (*C. lanatus*) crops was carried out seasonally from April to September, with peak surveys conducted in July and August in several field-plots located in the three main producing areas of Spain: Murcia (37°45′50.9″ N, 01°02′55.4″ W), Alicante (38°07′06.9″ N, 00°47′58.2″ W) and Castilla-La Mancha (39°11′37.8″ N, 03°12′56.7″ W). The coordinates given for each area represent the central point around which most of the plots were located within a radius of approximately 35 km. A total of 984 samples were collected from both crops, covering the growing seasons from 2021 to 2023 (Table S1). These comprised 392 melon samples from 98 field plots in Murcia, 132 from 33 plots in Alicante, and 120 from 30 plots in Castilla-La Mancha. For watermelon crops, 152 samples were taken from 38 field plots in Murcia, 92 from 23 plots in Alicante, and 96 from 24 plots in Castilla-La Mancha. During each field-plot survey, 10 apical leaf samples were collected, specifically targeting those displaying either yellowing or mosaic virus-like symptoms. Considering the large number of surveyed field plots, which were assumed to be representative of each production area and crop, four samples per plot were processed in the laboratory for total RNA extraction. Note that several plots exhibited yellowing symptoms at earlier stages (April–July) without any mosaic symptoms, whereas plots at later stages showed plants with both symptoms simultaneously (July–September). All RNA extraction and remaining duplicated plant samples were stored frozen at −80°C.

2.2 | Cucurbit virus detection

Total RNA from plant samples was extracted using TRI reagent (Sigma-Aldrich) and used for RNA virus detection by dot-blot hybridization, as previously described (De Moya-Ruiz et al., 2021; Rabadán et al., 2021, 2023). Briefly, RNA from each sample was placed on positively charged nylon membranes and fixed with an ultraviolet-light crosslinker. A dot-blot molecular hybridization was carried out using specific RNA probes for the detection of six aphid-transmitted viruses: CABYV, WMV, CMV, MWMV, PRSV and ZYMV, and four whitefly-transmitted viruses: CYSDV, CVYV, CCYV and BPYV. Most of these RNA probes (CABYV, WMV, CMV, MWMV, PRSV, ZYMV, CYSDV, CVYV and BPYV) were synthesized as described in Kassem et al. (2007) and De Moya-Ruiz et al. (2021). Additionally, RNA probes for CCYV, PABYV and CmEV were synthesized following similar approaches. Specifically, for CCYV, the coat protein (CP) gene was amplified using the primers CE-3233 Fw 5′-ATGGAGAAGACTGACAATAAACAA-3′ and CE-3234 Rv 5′-TTTACTACAACCTCCCGGTG-3′, with the Supreme NZYTaq II 2× Green Master Mix (NZYTech). Briefly, PCR was carried out using 2 μL of cDNA in a 50 μL final volume reaction mixture containing 2 μL of each primer (10 μM) and 25 μL NZYTaq II 2× Green Master Mix. The thermal cycling consisted of initial denaturation at 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 54°C for 30 s and 72°C for 1.5 min; and a final extension step at 72°C for 7 min. The CCYV isolate was kindly provided by R. Chynoweth (BASF, Murcia, Spain) (Chynoweth et al., 2021). For PABYV, the partial P1 gene was amplified using the primers CE-3511 Fw 5′-GCCCTCGCCGAAGAATATACGCG-3′ and CE-3512 Rv 5′-GGGGGCACATACGTCCCACTTG-3′, and for CmEV, the partial RNA-dependent RNA polymerase (RdRp) gene was amplified by using the primers CE-3440 Fw 5′-CACCGAAGTTGGGAGGAGAT-3′ and CE-3427 Rv 5′-CCAGTCAACCGCATACCTTC-3′, using Expand High Fidelity PCR System (Roche). Briefly, PCR was carried out using 2 μL of cDNA in a volume of 50 μL and contained 1.5 μL of each primer (10 μM), 1 μL of dNTPs (10 μM), 5 μL of Expand High Fidelity buffer (10× with 15 mM $MgCl_2$) and 1 μL of Expand High Fidelity polymerase mix. The thermal cycling consisted of initial denaturation at 94°C for 2 min; followed by 30 cycles of 94°C for 15 s, 50°C (PABYV)/56°C (CmEV) for 30 s, 72°C for 1.5 min; and a final extension step at 72°C for 7 min. Subsequently, the resultant fragments for each virus were purified and inserted into the pGEM-T Easy vector (Promega), according to the manufacturer's instructions, followed by the synthesis of specific probes through in vitro RNA transcription incorporating digoxigenin.

For detection of each virus, the membranes containing the RNA samples were incubated overnight at 68°C with specific digoxigenin-labelled probes, followed by subsequent incubation with anti-digoxigenin antibody linked to alkaline phosphatase (Anti-Digoxigenin-AP; Roche Diagnostics), and the chemiluminescent substrate CDP-Star (GE Healthcare) as described in Gómez-Aix et al. (2019). Membranes were visualized by a chemiluminescent detector (Amersham Imager 600, GE Healthcare Bio-Sciences). Additionally, reverse transcription (RT)-PCR was performed on a subset of samples to confirm dot-blot hybridization results, as previously described (Rabadán et al., 2021, 2023).

2.3 | Extraction and amplification of double-stranded RNAs

To reveal unknown viruses, we used a sequence-independent approach to amplify and clone double-stranded RNAs (dsRNAs), aiming to explore the potential presence of additional viral entities contributing to the virus-like symptoms observed (see schematic protocol in Figure 1). From duplicate samples of melon and watermelon that tested negative for any diagnosed virus, three pooled samples of each were subjected to dsRNA isolation using CF-11 fibrous cellulose chromatography (Whatman) following the protocol described by Marais et al. (2018) and Valverde et al. (1990) with some modifications. After homogenization of 100 mg leaf tissue in liquid nitrogen, the ground powder was suspended in 0.4 mL of extraction buffer (0.4 vol. of 10× STE, 0.1 vol. of 10× SDS, 0.02 vol. of bentonite (40 mg/mL), 0.02 vol of 2-mercaptoethanol and 0.5 vol of RNase-free water), together with 0.2 mL of UltraPure

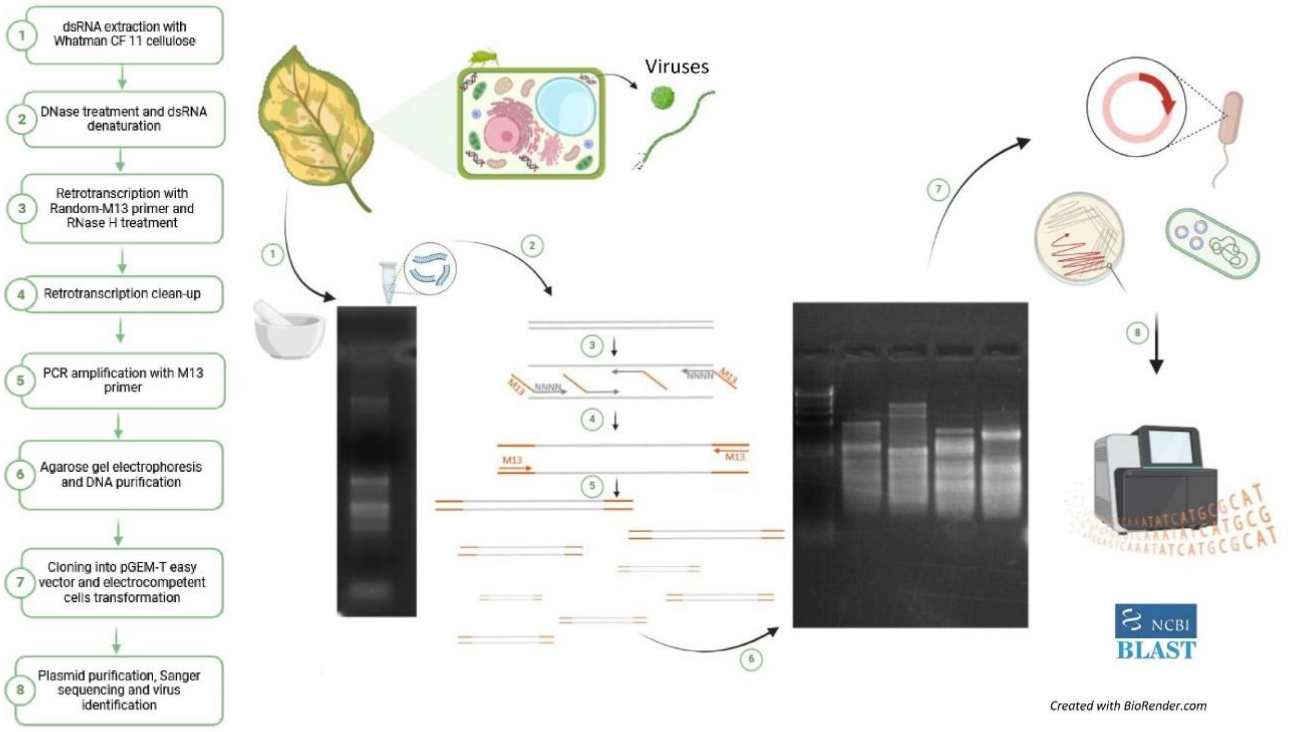

FIGURE 1 Schematic representation of the double-stranded RNAs (dsRNAs) protocol for amplification, cloning and sequencing of potential unknown viral entities.

buffer-saturated phenol (Invitrogen). The mixture was shaken for 15 min and centrifuged at 8000*g* for 20 min at room temperature. The resulting supernatant was combined with 100 mg of CF-11 cellulose, ethanol was added to a final concentration of 16.6% (vol/vol), and the mixture was centrifuged at 8000*g* for 5 min. The cellulose was washed with 0.5 mL of STE buffer containing 16.6% ethanol (vol/vol) and centrifuged at 5000*g* for 5 min; this was repeated two times. The bound dsRNA was eluted with 0.2 mL of STE buffer and centrifuged at 5000*g* for 1 min. This was repeated two times and the supernatants from each were pooled, then precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and 0.8 volume of isopropanol. After centrifugation, the pellet was washed with 1 mL of 75% (vol/vol) ethanol and resuspended in 0.05 mL of DEPC-treated water. The resuspended dsRNA was treated with DNase I following the manufacturer's protocol (Sigma-Aldrich), and subsequently, the dsRNAs were separated on a 1% agarose gel (Figure 1).

For the synthesis and amplification of cDNAs from the dsRNA templates, reverse transcription and random PCR (rPCR) were performed according to the method described by Darissa et al. (2010). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche), following the manufacturer's recommendations, with the M13-Random primer CE-2715 5′-GTAAAAC GACGGCCAGNNNNNNNNNN-3′. The cDNA was then amplified with Supreme NZYTaq II 2× Green Master Mix (NZYTech) using the universal M13 primer CE-1844 5′-GTAAAACGACGGCCAG-3′. The PCR products were cloned into pGEM-T Easy Vector following the manufacturer's recommendations (Promega) and sequenced using the Sanger method by an external sequencing service (STAB VIDA, Caparica, Portugal).

2.4 | Amplification and cloning of CABYV, PABYV and CmEV genomic fragments

Given the temporal differentiation of CABYV isolates within the Mediterranean cluster (Rabadán et al., 2023; Rabadán & Gómez, 2023), we sought to characterize the genetic diversity of CABYV, along with the new PABYV and CmEV populations identified in this study. For CABYV, the ORF2 sequences from 15 isolates (five per sampling year) were analysed. Note that ORF2 was sequenced because our previous studies (Rabadán et al., 2021, 2023) identified mutations in this region that differentiate a more pathogenic contemporary CABYV isolate (CABYV-LP63) from an older one (CABYV-MEC12.1), and we aimed to examine the prevalence of these contemporary versus ancient strains in current cucurbit crops. The ORF2 fragments were amplified by RT-PCR using the primers CE-3641 Fw 5′-ATGGCACCAACCTACTTCG-3′ and CE-2832 Rv 5′-AAACCGAGCGATTTGTAACG-3′. RT-PCR was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and the Expand High Fidelity PCR System (Roche) following the manufacturer's instructions. For PABYV, the CP sequences from 14 isolates were selected, including isolates from its first detection in 2018 and

those from different hosts (cucumber, melon, watermelon, pumpkin and zucchini) and locations (Murcia, Alicante and Castilla-La Mancha). Note that we used our long-term cucurbit sample collection for PABYV detection from 2011 (Rabadán et al., 2023). Reverse transcription of the PABYV CP sequences was carried out by the specific primer CE-3507 Rv 5′-GTTCTGGACCTGGCACTGGATG G-3′ using TransScript II Reverse Transcriptase (M-MLV, RNaseH; Transgen Biotech), and the PCR amplification of CP sequences from PABYV isolates was carried out with the specific primers CE-3506 Fw 5′-GAATACGGTCGCGGTTAGATCTAGC-3′ and CE-3507 Rv 5′-GTTCTGGACCTGGCACTGGATGG-3′, as described by Masika et al. (2022). Similarly, for CmEV, the partial RdRp sequences of 17 isolates, including samples from 2011, cucurbit hosts, and locations (as previously mentioned), were analysed. Reverse transcription of CmEV genomic fragments was carried out by the specific primer CE-3427 Rv 5′-CCAGTCAACCGCATACCTTC-3′, as described previously, and the partial RdRp sequences were amplified according to the set of primers described previously for the synthesis of the CmEV RNA probe.

The resulting PCR products for these three viral populations were purified using the GelPure kit (NZYTech), cloned into the pGEM-T Easy vector, and transformed into *Escherichia coli* Top10 competent cells to obtain two purified plasmids from each isolate for each virus. These plasmids were then sequenced by the Sanger method (STAB VIDA, Caparica, Portugal). Based on their sequence identity, one sequence from each isolate was considered for phylogenetic analysis.

2.5 | Phylogenetic analysis of CABYV, PABYV and CmEV isolates

Phylogenetic relationships among CABYV, PABYV and CmEV isolates were inferred from 15, 14 and 17 isolates, respectively. This analysis incorporated an additional 30, 17, and 5 sequences of CABYV, PABYV and CmEV, respectively, from the GenBank database, including an outgroup rooting sequence of PABYV (NC 030225.1), CABYV (JF939764) and CuEV (MT586998), respectively. Multiple sequence alignment was carried out in MEGA 11 software using MUSCLE (Kumar et al., 2016), and subsequently, we performed evolutionary analysis in MEGA 11 to find the best model to show the evolutionary and phylogenetic history. The general time reversible model was selected due to the lowest Bayesian information criterion (BIC) scores to describe the substitution pattern. The evolutionary history and phylogenetic trees were inferred using the maximum-likelihood method, with 1000 bootstrap replicates.

2.6 | Genetic diversity analysis of the CABYV, PABYV and CmEV populations

Genetic diversity (π), number of haplotypes (h), Tajima's D and nucleotide sequence-based statistic (K_{ST}) were estimated among the CABYV, PABYV and CmEV subpopulations with DnaSP 6 software (Rozas et al., 2017). Additionally, to assess the direction and strength of selection operating on the PABYV and CmEV populations, the ratio of the number of nonsynonymous substitutions per nonsynonymous site (dN), and the number of synonymous substitutions per synonymous site (dS) were calculated using the Pamilo–Banchi–Li method in MEGA 11 (Pamilo & Bianchi, 1993). The dN/dS ratio (<1 negative or purifying selection) was calculated with MEGA 11. The extent of genetic differentiation between yearly seasonal CABYV subpopulations was determined with an analysis of molecular variance (AMOVA) using R with the Poppr package (Dray & Dufour, 2007). The AMOVA significance levels were determined using the Monte Carlo test and 1000 replicates.

2.7 | Statistical analysis

The percentage of infected samples was estimated as the number of positive samples for each aphid- and whitefly-transmitted virus (either alone or in combination) relative to the total number of samples analysed across different years (2021–2023), hosts (melon and watermelon), and locations (Murcia, Alicante and Castilla-La Mancha). Additionally, the proportion for each virus was calculated based on the total number of infected/positive samples for each year, host and symptom type (yellowing and mosaics). The analysis to assess the potential association between the percentage of detected/undetected viruses and symptoms (yellowing and mosaics) was conducted using a χ^2 distribution using a binary (2×2) contingency table approach (Rabadán et al., 2023). This involved the comparison of observed and expected proportions of detected and symptomatic samples ($df = 1$, $p < 0.05$), including the number of negative samples. To compare the occurrence of virus species, the frequency of each virus was averaged through location and year and analysed using a general linear model with the response variable outcome (presence/absence) in a binomial regression and logit function. Viral species, plant hosts and locations were fitted as treatments, with their interactions as appropriate. All analyses were carried out using the package 'stats' in Rstudio.

3 | RESULTS

3.1 | Assessment of viral infections in melon and watermelon crops

A total of 644 melon and 340 watermelon samples exhibiting yellowing and mosaic symptoms were collected from the three main cucurbit-producing areas (Region de Murcia, Alicante and Castilla-La Mancha) of Spain across consecutive growing seasons (2021–2023) (Table S1). Sample collection was based on symptom differentiation (yellowing and mosaics) for each plot of melon and watermelon plants. Yellowing symptoms included vein banding and necrotic rings on basal leaves of both plant species, often accompanied by a significant fruit abortion

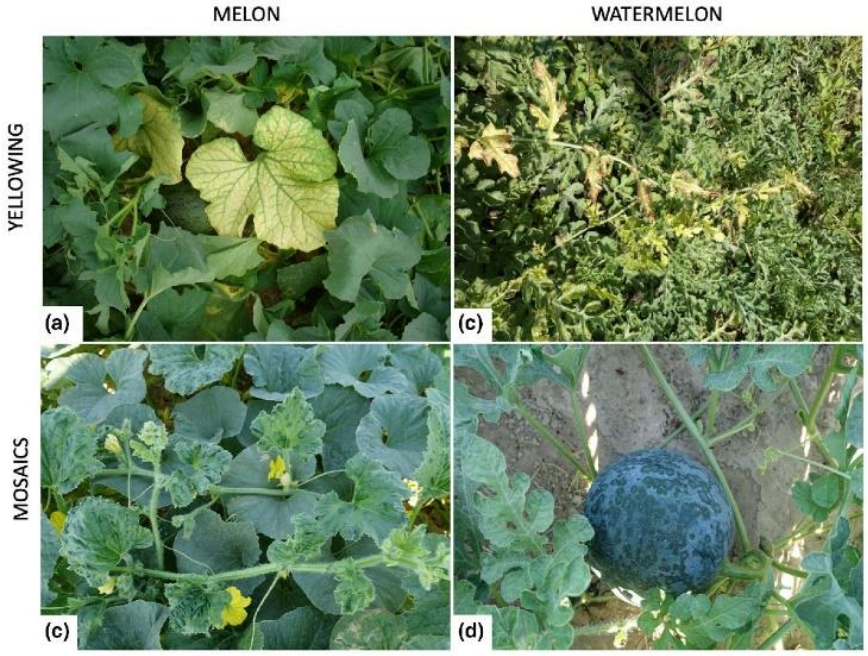

FIGURE 2 Symptoms of yellowing and mosaics on melon and watermelon plants. Yellowing symptoms are evident on the leaves of affected melon (a) and watermelon (c) plants. Additionally, vein banding and necrotic ring symptoms can also be observed on basal leaves of both plant species. Mosaic symptoms, along with vein clearing and banding are displayed on the leaves of affected melon plants (b). Whereas mosaic symptoms induced by cucurbit viruses are challenging to observe in watermelon leaves, deformations, mottling and/or blistering are observed on watermelon fruits (d).

(Figure 2a,c). Mosaic symptoms were characterized by leaf deformation, vein clearing, and banding on melon leaves, but such symptoms were difficult to discern on watermelon leaves. Instead, watermelon fruits exhibited mottling and/or blistering (Figure 2b,d).

Each sample was analysed for the presence of aphid-transmitted viruses (CABYV, WMV, MWMV, ZYMV, CMV and PRSV), and whitefly-transmitted viruses (CYSDV, CVYV, CCYV and BPYV) using dot-blot molecular hybridization. A notable proportion of melon samples (62.3%) tested positive for virus infection, in contrast to watermelon samples (45.5%) (Table S2). Moreover, of plants with yellowing symptoms, a high percentage were associated with undetected or negative results for these viruses (54.7% and 84.0% for melon and watermelon, respectively), compared to samples exhibiting mosaic symptoms (20.7% and 25% for melon and watermelon, respectively). These results showed significant differences between the observed and expected frequencies of virus detection in the different plant species and symptoms ($\chi^2 = 44.76$, $df = 1$; $p < 0.001$; Table S2). This suggested the possibility that other biotic or abiotic factors might be associated with the yellowing symptoms.

3.2 | Distribution of aphid- and whitefly-transmitted viruses in melon and watermelon crops exhibiting yellowing symptoms

To rule out any additional virus in those negative samples with yellowing symptoms, we carried out a sequence-independent approach to amplify and clone potential viral dsRNAs from three pooled samples of watermelon and melon that had previously tested negative for any diagnosed virus. Using this approach, PABYV was uncovered in the watermelon samples, whereas CmEV was present in melon samples.

Because our results showed that PABYV is a new yellowing disease on these cucurbit crops, its detection was incorporated into these epidemiological results (Figure 3, Table S1). Overall, the detection of PABYV significantly increased the percentage of samples testing positive for virus infection in melon (82.7%) and watermelon (85.7%) samples. This led to a notable decrease in the proportion of undetected or negative virus infections associated with yellowing symptoms, which dropped to 23% in melon and 16% in watermelon (Figure 3a). Among the viral species, we did not detect PRSV, CCYV or BPYV across both crops, locations or years (Table S1). The occurrence of aphid-transmitted viruses was significantly higher than whitefly-transmitted viruses across all locations and years ($\chi^2 = 409.7$, $df = 1$; $p < 0.001$). Notably, CABYV (47.3% and 24.0%), PABYV (25.3% and 71.0%) and WMV (33.3% and 27.3%) were more frequently detected in melon and watermelon crops, respectively, compared to the rest of the viruses, which occurred at very low frequencies (Table S1). Of the viral species detected in melon and watermelon crops exhibiting yellowing symptoms, CABYV and PABYV were predominantly detected, with the occasional presence of other viruses and mixed infections. In particular, the proportion of CABYV varied through the seasons, ranging from 77% to 19% in melon and from 7% to 0% in watermelon. Similarly, PABYV was also detected in both crops, with a wider variation in occurrence compared to CABYV. In melon, the proportion of PABYV ranged from 10% to 42%, while in watermelon, it ranged from 35% to 83%. WMV was detected in low proportions, with only occasional presence in melon and watermelon samples with yellowing (Figure 3b). CYSDV and CVYV were detected in both crops (6% and 1%), with fluctuations across the years and were generally less frequent than other viruses. Mixed infections were also detected, with the combination of CABYV and PABYV appearing frequently, ranging from 3% to 25% in melon and from 10% to 33% in watermelon (Figure 3b).

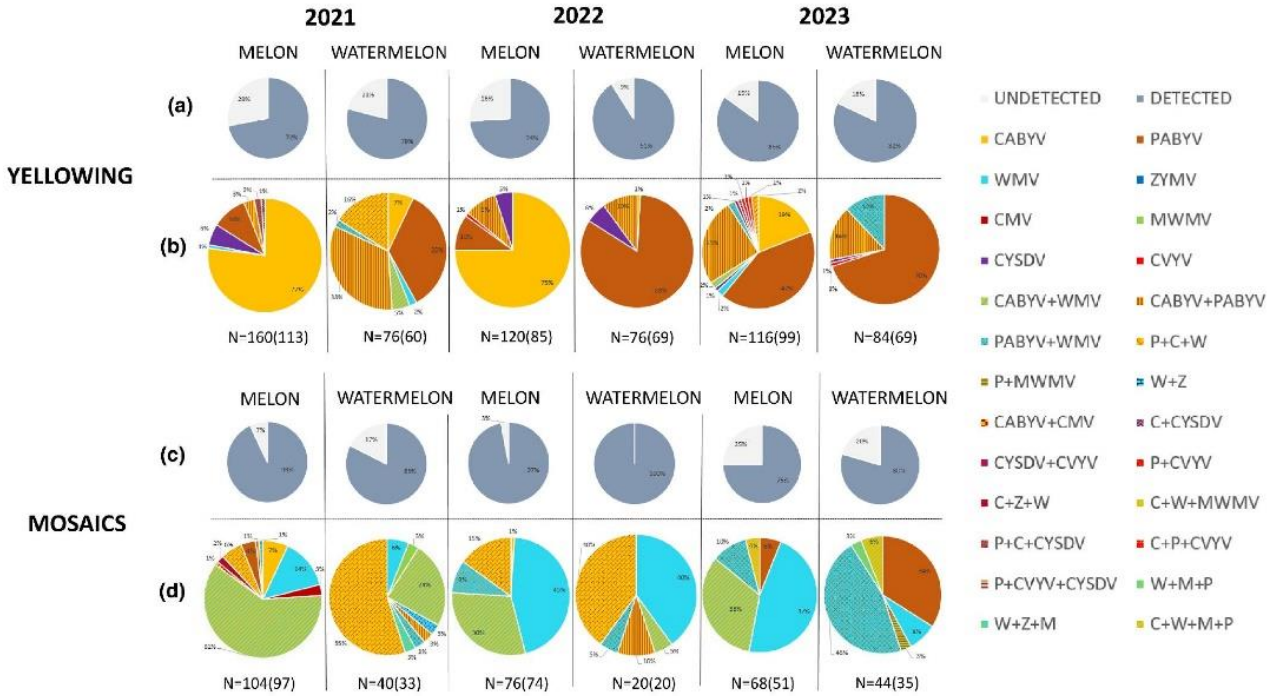

FIGURE 3 Detection and distribution of cucurbit viruses associated with yellowing and mosaic symptoms in melon and watermelon crops. (a, c) The percentage of infected samples, calculated as the proportion of detected and undetected viruses relative to the total samples (*n*) analysed, across different years (2021–2023), hosts (melon and watermelon), and symptom type (yellowing [a] or mosaic [c]) in three producing areas (Murcia, Alicante and Castilla-La Mancha). (b, d) The occurrence of each aphid- and whitefly-transmitted virus (either alone or in combination) across the three seasons in melon and watermelon samples with yellowing (b) or mosaic symptoms (d). Each slice corresponds to the proportion (%) of each virus detected within the total number of infected/positive samples for each year, host, and symptom type in the three producing areas (Murcia, Alicante and Castilla-La Mancha). Solid sections indicate single infections, while patterned sections denote combined or mixed infections. The viral species and corresponding colours are indicated in the legend. The total number of samples (*n*) and the number of infected samples (in parentheses) are indicated at the base of each pie chart.

PABYV was detected in the three consecutive seasons with relatively increasing frequency; therefore, we decided to examine further our long-term cucurbit frozen-sample collection from 2011. PABYV was first detected in 2018 in watermelon (12%) and zucchini (10%) samples, with the infection rate increasing in the subsequent seasons. In 2019, PABYV was found in watermelon (21%), zucchini (8%) and pumpkin (3%) samples, and in 2020, it was detected in watermelon (56%) and pumpkin (17%) samples (Figure 4). This temporal detection suggested that PABYV emerged in Spain in 2018 from watermelon crops, and subsequently spread to other cultivated species.

3.3 | Distribution of aphid- and whitefly-transmitted viruses in melon and watermelon crops exhibiting mosaic symptoms

For crops exhibiting mosaic symptoms, the proportion of WMV averaged by year was higher in melon (35%) than in watermelon (18%). MWMV was detected only in watermelon, with a minimal proportion (3%) in 2021 and absent in subsequent years. CMV had a similar proportion only in 2021, but in melon (Figure 3d). The detection of CABYV was at low levels across all years, with the highest detection rate of 7% in melon, and was not detected in watermelon. PABYV varied in both crops, with proportions peaking at 6% in melon and 34% in watermelon in 2023. CYSDV and CVYV were not detected in either crop across all years. For mixed infections, the combination of CABYV and WMV had the highest occurrence overall in melon crops, with frequencies ranging from 61% to 30%. Similarly, the combination of PABYV and WMV also showed notable frequencies in melon (10%) and watermelon (46%) samples. In contrast, the combination of PABYV and CABYV had the highest frequency in 2022, representing 10% of occurrences. Additionally, the combination of the three viruses CABYV, PABYV and WMV was significantly higher than other combinations in watermelon in 2021, accounting for 55% of occurrences (Figure 3d). Overall, the data suggests varying levels of viral infection in melon and watermelon crops across different years, with certain viruses showing higher prevalence but with fluctuating occurrences over time.

Finally, despite CmEV being a cryptic virus that does not cause disease in cucurbits, it was also detected by the dsRNA technique in melon samples that had previously tested negative, and we decided to further examine its occurrence in our long-term frozen sample collection of cucurbits from 2011. CmEV was found in all melon samples since 2011, and in a few watermelon and pumpkin samples,

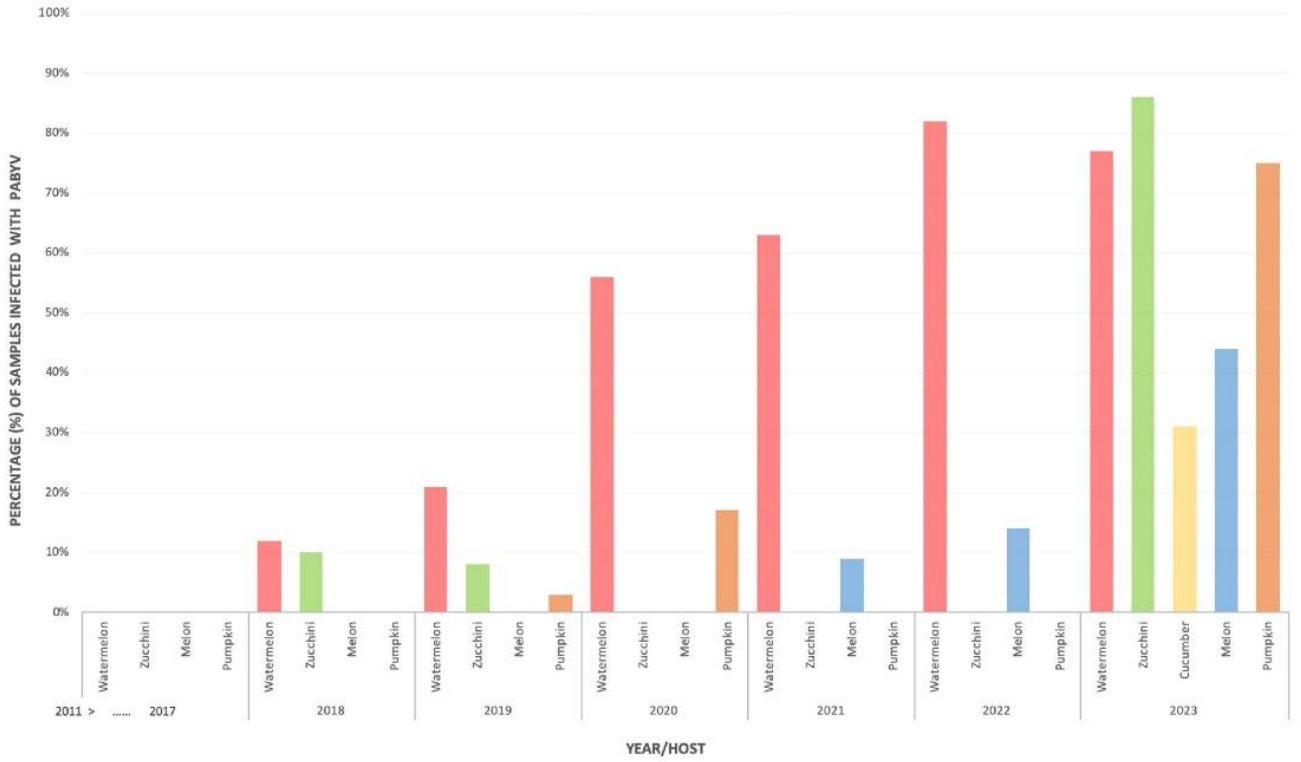

FIGURE 4 Percentage of positive samples infected with PABYV across different years (from 2011 to 2023) and cultivated cucurbit plant species, including watermelon, zucchini, melon, pumpkin and cucumber.

with no detection in cucumber or zucchini samples. This marked the first identification of CmEV in pumpkin and revealed a high rate of codetection of CmEV with other cucurbit viruses in the same host.

3.4 | Phylogenetic analysis and genetic variability of CABYV, PABYV and CmEV isolates

To characterize the structure and genetic diversity of CABYV, PABYV and CmEV populations, we inferred the phylogenetic relationship among their isolates and examined the nucleotide diversity of their populations. For CABYV, the ORF2 from 15 isolates was sequenced (Table S3), and phylogenetic analysis indicated that these isolates grouped with other related CABYV Spanish isolates within the Mediterranean cluster (Figure 5a). Within this population, two differentiated subgroups were observed: one comprising isolates that occurred in seasons before 2020 and another, with most isolates of this study, detected in 2021–2023 seasons. The nucleotide diversity of this CABYV Spanish population was moderate ($\pi = 0.0255$), with 298 segregation sites. Indeed, considering the yearly seasonal subpopulations, the AMOVA identified significant genetic variation (24.5%, $p < 0.001$) between older and recent subpopulations, suggesting a temporal genetic differentiation of CABYV populations. Furthermore, to ascertain the direction and strength of selection operating in the CABYV populations, the average dS and dN values across the ORF2 gene was evaluated, showing overall dN/dS values of values of -0.04 ± 0.01. As such, the values of the Tajima's *D* neutrality tests were negative (−0.956, $p > 0.1$), suggesting that CABYV subpopulations are undergoing population size expansion through a low frequency of rare mutations.

For PABYV, the CP gene of 14 PABYV isolates were sequenced (Table S3), and their phylogenetic analysis, including another 11 isolates from NCBI GenBank, indicated that PABYV Spanish isolates were highly homogeneous. All isolates from 2018 to 2023 were grouped in a monophyletic cluster, along with the Italian (OP973153) and Greek (LN865082) isolates (Figure 5b). Indeed, the genetic analysis showed a low average nucleotide diversity in the PABYV Spanish population ($\pi = 0.0059$). Likewise, the number of segregation sites was low ($S = 24$), and the average dS and dN values across the CP gene showed overall dN/dS values of -0.01 ± 0.00, with no temporal differentiation. Additionally, Tajima's *D* neutrality test values were negative (−2.18, $p < 0.001$), suggesting that the PABYV population is undergoing expansion through a low frequency of rare mutations.

For CmEV, the phylogenetic tree inferred from the partial RdRp coding sequence of 17 isolates from melon, one isolate from watermelon and two from pumpkin (Table S3), showed that Spanish isolates of CmEV were mainly structured in a single cluster, as the bootstrap confidence for all isolates was <70% (Figure 5c). Despite the relatively high sequence identity among the CmEV isolates in this cluster, their genetic analysis showed a higher nucleotide diversity ($\pi = 0.0248$) than the CABYV and PABYV Spanish populations. The number of segregation sites ($S = 119$) was also high, along with

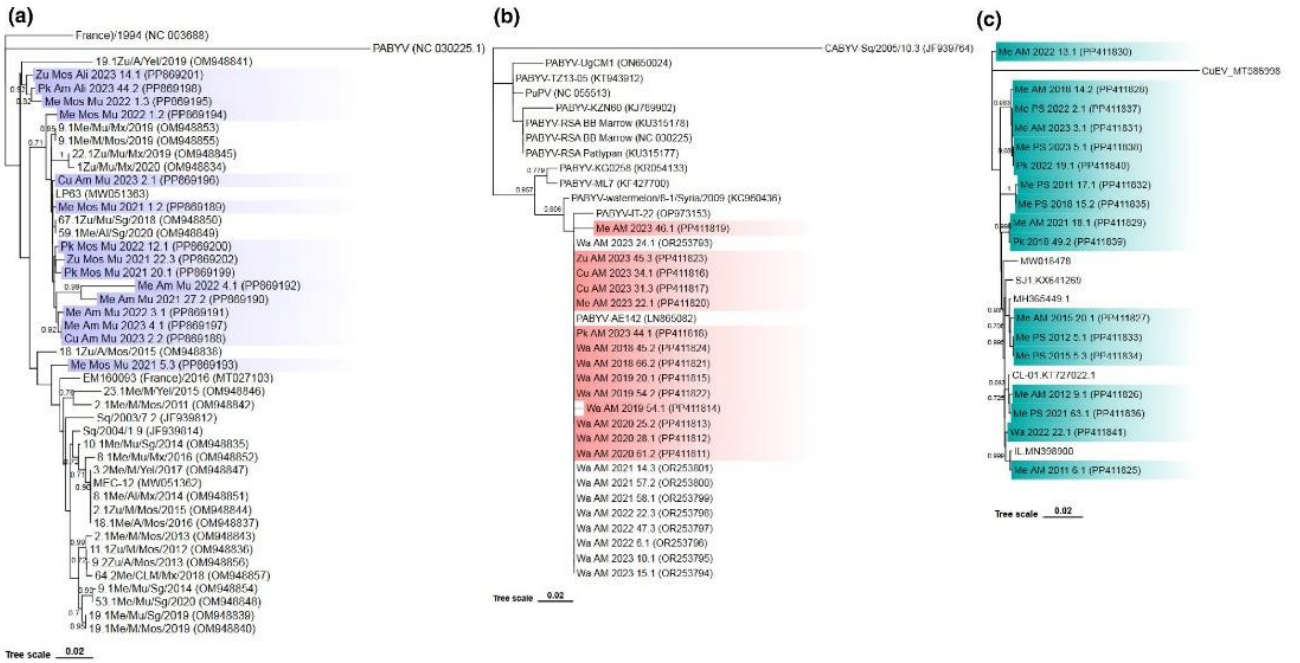

FIGURE 5 Phylogenetic relationships of the *Cucurbit aphid-borne yellows virus* (CABYV), *Pepo aphid-borne yellows virus* (PABYV) and *Cucumis melo endornavirus* (CmEV) isolates within their populations. (a) Phylogenetic tree based on the complete ORF2 sequences of 15 CABYV isolates from this study (highlighted in purple colour with their accession number), along with 30 additional CABYV isolates available in the NCBI/GenBank database from Spain and Mediterranean basin. (b) Phylogenetic tree based on the complete coat protein sequences of 14 PABYV isolates from this study (highlighted in salmon colour with their accession number), including complete and partial sequences of another 17 PABYV isolates available in the NCBI/GenBank database and the coat protein of *Pumpkin polerovirus* (PuPV) isolate and CABYV. (c) Phylogenetic tree based on the partial RdRp sequences of 17 CmEV isolates from this study (highlighted in dark cyan with their accession number), including RdRp sequences of another 5 CmEV isolates available in the NCBI/GenBank database and the RdRp of *Cucumber endornavirus* (CuEV). The phylogenetic tree for each virus was built by the maximum-likelihood method with 1000 bootstrap replications and using the general time reversible model in MEGA. Only branches with bootstrap values >70% are shown. The lengths of the branches represent the genetic distances that are also represented by the scale bars.

non-significant negative values of the Tajima's *D* neutrality tests (−0.72, $p > 0.10$). Note that most of these CmEV isolates were co-detected with other viruses in the same host (Table S4).

Overall, the genetic characterisation of CABYV and PABYV populations suggests that the genetic variation of CABYV isolates could be temporally influenced, while PABYV isolates still appear to be genetically homogeneous, indicating stable populations after their recent introduction and spread. In contrast, the high genetic similarity among the CmEV isolates, associated with long-term prevalence of the populations and vertical transmission in their hosts, suggests that this endornavirus co-diverges in cucurbits.

4 | DISCUSSION

In this study, we examined the occurrence of aphid- and whitefly-transmitted RNA viruses in melon and watermelon crops for three major producing areas in Spain over three consecutive (2021–2023) growing seasons, revealing significant insights into the viral distribution, detection and emergence of the novel polerovirus, PABYV, including phylogenetic and genetic diversity analysis of CABYV, PABYV and CmEV populations.

The production of cucurbit vegetables is extensive with intensive cultivation in the Mediterranean basin (Pitrat, 2012), and its sustainability is concerning due to the outbreaks and high prevalence of pests and pathogens that significantly reduce the quality and yield of crops (Lecoq & Desbiez, 2012; Radouane et al., 2021). Among the most frequently reported risks are the rising abundance of hemipteran insect pests (aphid and whitefly) and their related viral diseases (Chase et al., 2021; Fereres & Moreno, 2009; Ng & Perry, 2004). Recent epidemiological studies have shown that CABYV and WMV are the most prevalent aphid-transmitted viruses in cucurbit crops in Spain, with a significantly elevated frequency of mixed infections (De Moya-Ruiz et al., 2021; López-Martín et al., 2024; Maachi et al., 2022; Rabadán et al., 2021, 2023). Abiotic factors and agricultural practices, including the species cultivated, growing conditions and nutritional deficiency, could prevent accurate observation of viral symptoms. Detection and diagnosis of viral infections can also be affected by other biotic factors, including the unknown distribution of whitefly-transmitted viruses, or even unknown viruses may remain hidden in mixed infections, leading to the misattribution of disease symptoms to known or frequently detected viruses. Therefore, we decided to perform this epidemiological study of cucurbit plants

from 2021 to 2023, focusing on aphid- and whitefly-transmitted viruses and differentiating between those that cause yellowing or mosaic symptoms.

After testing 984 symptomatic leaf samples from 246 field plots, we found the occurrence of aphid-transmitted viruses was significantly higher than whitefly-transmitted viruses across all localities and years. Apart from the common occurrence of WMV (30%) in samples with mosaics, CABYV appeared to be the most prevalent virus in both crops, infecting 45% of samples over the three seasons, a result that is consistent with previous studies (De Moya-Ruiz et al., 2021; López-Martín et al., 2024; Maachi et al., 2022; Rabadán et al., 2021, 2023). The wide distribution of CABYV could be explained by its transmission in a circulative, nonpropagative manner by *Aphis gossypii*, the most abundant aphid species in these production areas (Kassem et al., 2013). Field experiments have shown that CABYV epidemics are closely associated with the presence of *A. gossypii* during the first two weeks after planting (Schoeny et al., 2020). However, this contrasts with results from other cucurbit crop surveys in Spain, where WMV appeared in single infections earlier (López-Martín et al., 2024). In addition, the same study reported that CMV was prevalent in 2019 and 2020 (López-Martín et al., 2024), while in our current study, CMV was detected at low frequencies (0.4%). We speculate that these discrepancies might be due to temporal, environmental and/or geographical variations in aphid species distribution, as a high proportion of samples infected with WMV and CMV were found by López-Martín et al. in the production area of Comunidad Valenciana, which was not surveyed in our study. For example, the transmission of potyviruses by several other aphid species, together with potential variations in aphid population distribution, including genetic differences within the same species, may also explain the higher prevalence of potyviruses in other geographical regions. Such varying results emphasize how different production areas, even within the same country, can lead to changes in the population dynamics of the same virus affecting the same crops; this suggests that localized or particular agricultural practices can significantly influence the epidemiology and dynamics of plant viral diseases. We also speculate that rising temperatures are causing earlier spring peaks of aphid populations, and these changes can affect the spread of plant viruses. It is therefore important to note that aphid population distribution, human activities and agro-ecological conditions, including differences in crop management, pest control strategies, environmental conditions and even the timing of planting and harvesting can contribute to the varying prevalence, distribution, and impact of these aphid-borne diseases in cucurbit crops. Overall, our results indicate that CABYV and WMV are the most prevalent aphid-transmitted viruses causing yellowing and mosaic symptoms in Spanish cucurbit crops from 20 years ago (Juárez et al., 2013; Kassem et al., 2007). Currently, CABYV is spreading through the Mediterranean basin and Europe (Desbiez et al., 2020; Minicka et al., 2020; Rabadán et al., 2023), highlighting its threat to cucurbit crops.

Remarkably, this study also shows that PABYV is an emerging threat to cucurbit crops. Our virus detection analysis revealed a high proportion of undetected virus infections associated with yellowing symptoms, especially in watermelon (84%), suggesting the involvement of other biotic or abiotic factors. To investigate further and given the suspicion of another virus inducing yellowing symptoms, we used a sequence-independent approach to identify any unknown viruses, due to the large number of samples and economic considerations. While this approach is not as advanced or comprehensive as high-throughput sequencing (HTS) technology, which has significant potential for detecting unknown viruses, it still proved to be sensitive and effective. As a result, the first identification of PABYV in watermelon in Spain was recently reported as part of this work (De Moya-Ruiz, Juárez, & Gómez, 2023), and our findings confirm that PABYV emerged in 2018 and is currently widespread in cucurbit crops in Spain. This suggested that PABYV had previously been overlooked despite causing yellowing diseases in crops, whether in single or mixed infections with CABYV. PABYV belongs to the phloem-restricted RNA plant viruses transmitted by aphids in a circulative nonpropagative manner, and has been detected in western Africa and the Mediterranean area, including Syria and Greece (Lotos et al., 2016) and recently Italy (Parrella et al., 2023). The novel identification and wide distribution of PABYV, along with CABYV, demonstrates the need for further research on the ecological aspects and biological traits of both poleroviruses to understand their epidemiology in cucurbit crops. Additionally, several studies have reported how host preference and vector behaviour influence the transmission and spread of plant viruses (Fereres & Moreno, 2009; Mauck, 2016), with aphids having a high preference for CABYV-infected plants (Carmo-Sousa et al., 2016). We speculate that CABYV and PABYV may encourage serious epidemics in cucurbits, and further systematic and extensive monitoring will be crucial to ascertain their distribution and prevalence. In addition, the genetic diversity of these viruses should be investigated and the use of CABYV and PABYV infectious clones could help to screen host germplasm and characterize phenotypes in the search for resistant cultivars (De Moya-Ruiz et al., 2021; Gómez et al., 2009; Rabadán et al., 2021).

Overall, CABYV, PABYV and WMV were the most prevalent viruses affecting cucurbit crops. However, CmEV was also detected in all tested melon samples, consistent with previous melon virome characterization in Spain (Maachi et al., 2022; McLeish et al., 2022). CmEV, known as a cryptic virus, was first identified in melon (Sabanadzovic et al., 2016) and recently in gherkin (Karanfil & Korkmaz, 2020), watermelon (Adeleke et al., 2022) and weed species (McLeish et al., 2022). Here, we identified CmEV for the first time in another cucurbit species, pumpkin. Although endornaviruses are characterized by being very host-specific, it is possible that they infect a closely related species within the same genus (Okada et al., 2011). Some authors suggest that the presence of CmEV in plants belonging to distinct genera may be due to a long association of endornaviruses with their hosts and their codivergence since before the domestication and speciation of cucurbits (Sabanadzovic et al., 2016). Endornaviruses are persistent viruses associated with symptomless infections, although with some

exceptions (Roossinck, 2015; Sabanadzovic et al., 2016). For this reason, they remain under investigated and little is known about their relationship with plants (Roossinck, 2015). Here, we have verified that mixed infections between persistent and pathogenic viruses are common, and while the virus–virus interactions within a host have been reviewed (Alcaide et al., 2020), little is known about the interplay between them, making it an interesting aspect to explore further.

Genetic characterization of CABYV Spanish populations indicated a moderate nucleotide diversity in CABYV isolates, while PABYV and CmEV populations were genetically homogeneous. It is important to note that, although the ORF2 region has been documented to have a lower frequency of mutations compared to other open reading frames (ORFs) in the polerovirus genome (LaTourrette et al., 2021), our previous studies identified several mutations in ORF2 that distinguished contemporary CABYV isolates (such as CABYV-LP63, which shows increased pathogenicity) from older isolates (like CABYV-MEC12.1) (Rabadán et al., 2021, 2023). Therefore, ORF2 was chosen to evaluate the prevalence and genetic changes of contemporary CABYV isolates relative to older ones in cucurbit crops. Thus, CABYV phylogenetic analysis showed that most isolates clustered into the Mediterranean group, with recent isolates showing moderate nucleotide diversity and evidence of population expansion through rare mutations, which is consistent with our previous long-term phylogenetic analysis (Rabadán et al., 2023). This suggests adaptation to changing agro-ecological conditions and host interactions over time, potentially indicating the replacement of older CABYV isolates by contemporary ones. Indeed, recent CABYV isolates (2021–2023) are closely related to the CABYV-LP63 variant, which is known for its severe symptoms and high viral accumulation (Rabadán et al., 2021). This shift towards contemporary CABYV isolates could impact virus transmission dynamics and disease ecology, with significant implications for the effective control of this viral disease in cucurbit crops.

In contrast, PABYV, as an emerging virus potentially influenced by a founder effect, was analysed using the CP gene. This genomic segment is known for its high variability and significant sites under both positive and negative selection, making it crucial for studying host and vector adaptation (LaTourrette et al., 2021). As expected, PABYV isolates exhibited low genetic diversity, indicating a relatively stable population since its recent introduction and spread in the region. This stability suggests that PABYV may not yet have undergone significant evolutionary changes, possibly due to limited selection pressures or the short period of establishment. Additionally, CmEV populations exhibited higher nucleotide diversity and significant genetic structuring, suggesting long-term prevalence and vertical transmission in cucurbit hosts. The genetic similarity among CmEV isolates supports the hypothesis of co-divergence with cucurbit crops, indicating a longstanding host–virus relationship.

In conclusion, our findings highlight the complex landscape of viral infections in cucurbit crops in Spain. The prevalence of CABYV and the emergence of PABYV call for enhanced monitoring and diagnostic efforts and suggest that mixed infections deserve attention. Understanding the epidemiology and genetic diversity of these viruses is crucial for developing effective management strategies that help to ensure the sustainability of cucurbit crop production.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The CABYV sequences were deposited in GenBank at https://www.ncbi.nlm.nih.gov/genbank/ under accession numbers PP869188–PP869202. The PABYV sequences were deposited under accession numbers PP411811–PP411824, and the CmEV sequences under accession numbers PP411825–PP411841. For further information about each accession reference by viral isolate, see Table S3.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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6.2. Chapter II: Biotic and abiotic factors: implications for the development of plant viral diseases

6.2.1. Sub-Chapter II.I: “*The temporal order of mixed viral infections matters: common events that are neglected in plant viral diseases*”

viruses

MDPI

Perspective

The Temporal Order of Mixed Viral Infections Matters: Common Events That Are Neglected in Plant Viral Diseases

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Abstract: Mixed infections of plant viruses are common in crops and represent a critical biotic factor with substantial epidemiological implications for plant viral diseases. Compared to single-virus infections, mixed infections arise from simultaneous or sequential infections, which can inevitably affect the ecology and evolution of the diseases. These infections can either exacerbate or ameliorate symptom severity, including virus–virus interactions within the same host that may influence a range of viral traits associated with disease emergence. This underscores the need for a more comprehensive understanding of how the order of virus arrival to the host can impact plant disease dynamics. From this perspective, we reviewed the current evidence regarding the impact of mixed infections within the framework of simultaneous and sequential infections in plants, considering the mode of viral transmission. We also examined how the temporal order of mixed infections could affect the dynamics of viral populations and present a case study of two aphid-transmitted viruses infecting melon plants, suggesting that the order of virus arrival significantly affects viral load and disease outcomes. Finally, we anticipate future research that reconciles molecular epidemiology and evolutionary ecology, underlining the importance of biotic interactions in shaping viral epidemiology and plant disease dynamics in agroecosystems.

Keywords: CABYV; Co-infection; mixed infections; sequential infection; virus–virus interaction; within-host virus interaction; WMV

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1. Introduction

Plant viruses represent a serious threat to agriculture due to the lack of effective countermeasures to control their diseases. Within these plant viral diseases, mixed infections are common in crops and are increasingly recognized as an integrated biotic factor that can affect the ecology and evolution of epidemics. This complex of infections in the same host can occur in all living domains, from bacteria [1,2] and fungi [3] to plants [4–6], animals [7,8], and humans [9] by different types of parasite microbes. Such mixed infections may occur by either unrelated [6,10,11] or related pathogens [7,12–14], with important socio-economic impacts. For example, mixed infections with bacteria and viruses are very common in humans, deteriorating immunological functions and increasing the risk of morbidity and mortality [15,16], such as in the case of *Mycobacterium tuberculosis* and *Lentivirus humimdef1* (human immunodeficiency virus 1, HIV-1) [17]. In addition, co-infections between fungi and viruses have recently been shown in plants, where, for the fungus *Phomopsis subordinaria*, transmission increases despite its restriction in the infection rate when co-infecting with the virus *Capulavirus plantagonis* (plantago lanceolata latent virus, PILV) [6]. Similarly, co-infections have also been observed between viruses; *Betacoronavirus pandemicum* (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2) and other respiratory viruses such as *Influenza A virus*, rhinovirus/enterovirus, para-influenza, metapneumovirus, and *Influenza B virus* [18], with similar relevance in plants, whereas virus-virus combinations within the same plant have been described to cause major losses in crops [19,20].

Within the agricultural context, many factors may influence the occurrence of multiple viral infections in the same plant and crop. For example, both viral and plant intrinsic factors, such as generalist viruses, plant species, cultivar, age, and nutritional status, as well as external factors, such as the mode of virus transmission, polyphagous vectors, environmental conditions, growing season overlap, intensification and expansion of agricultural production, and proximity to alternative hosts, are thought to occur in agricultural contexts and are likely to combine, leading to a range of ecological interactions between plants and viruses that may favor the occurrence and prevalence of mixed infections. Additionally, viral disease management based on visual inspections can result in inefficiency, as mixed infections may allow either misinterpretation of symptoms during monitoring or even non-detection of the focal virus [21], with unpredictable epidemiological and important socio-economic consequences [20,22–27]. In this sense, virus–virus interactions within the same host have been described and traditionally defined as synergistic, neutral, and antagonistic, and more details on these interactions have been addressed in recent reviews [20,28,29]. Briefly, facilitating interactions or synergism can occur when mixed infections lead to increased replication of one or both viruses or more severe symptomatology compared to single infections. Such synergistic effects may be attributed to various mechanisms, including the suppression of host defense responses by one virus, which inadvertently benefits the other, or the complementary exploitation of host resources by different viral species [20,23,28,30]. On the other hand, antagonistic interactions between viruses may manifest as reciprocal exclusion, wherein both viruses experience a decline in replication when infecting together, or where the infection of one virus leads to the suppression of another, possibly due to competition for limited cellular resources or the activation of broad-spectrum host defense mechanisms [20,28,29,31–33]. These virus–virus interactions within the same host plant are believed to exert significant selection pressures on viral populations, potentially driving their evolutionary trajectories in ways that differ from those observed in single infections [29]. However, the long-term implications of these interactions on viral evolution and disease dynamics remain unclear, requiring further investigation to fully elucidate their complex interplay and ecological consequences.

The occurrence of mixed viral infections in crops indicates that multiple transmission events occur in the same plant, and these infections arise from either simultaneous infections (co-infections) or sequential infections (one after the other). Within this framework, and considering that most plant viruses are transmitted by insect vectors [34], it is postulated that the temporal order of the arrival of different viruses on the same plant can inevitably affect the ecology and evolution of viral diseases. In this article, we review the current evidence and highlight the importance of mixed infections in plants, contingent upon the temporal order of viral transmission. By examining co- and sequential infections of two virus species that infect melon plants, we provide insights into the complex dynamics of viral transmission, indicating that the order of viral infections in plants is significant in viral load. We then considered how the temporal order of viral infections could shape the population and evolutionary dynamics of plant viruses, emphasizing the necessity of incorporating biotic factors to gain a better understanding of the ecological mechanisms that drive viral epidemiology.

2. The Insect-Vector Role in Mixed Infections of Plant Viruses

A significant percentage of plant viruses (>70%) is spread by insect vectors of the order Hemiptera, such as aphids, whiteflies, and leafhoppers [35,36], which are also known to be pests on cultivated plants in temperate regions. Additionally, beetles, thrips, mealybugs, fungi, and nematodes are key vectors for the transmission of some viral species, including human assistance [37]. Viral transmission by insects has been classically classified as non-persistent, semi-persistent, or persistent, and some reviews have dealt with these aspects in depth [34,38–41]. While non-persistent viruses have short retention times in their vector, with acquisition and inoculation access periods also short over hours, persistent viruses can be retained for longer periods, with acquisition and inoculation periods over

several days, versus semi-persistent viruses spending an intermediate time between the previous ones [34,42]. With these time periods, and contingent upon the mode of viral transmission, insect vectors can transmit multiple viruses, either from the same host with mixed infections (co-transmission) or from different hosts with different viral diseases in a sequential order (Figure 1).

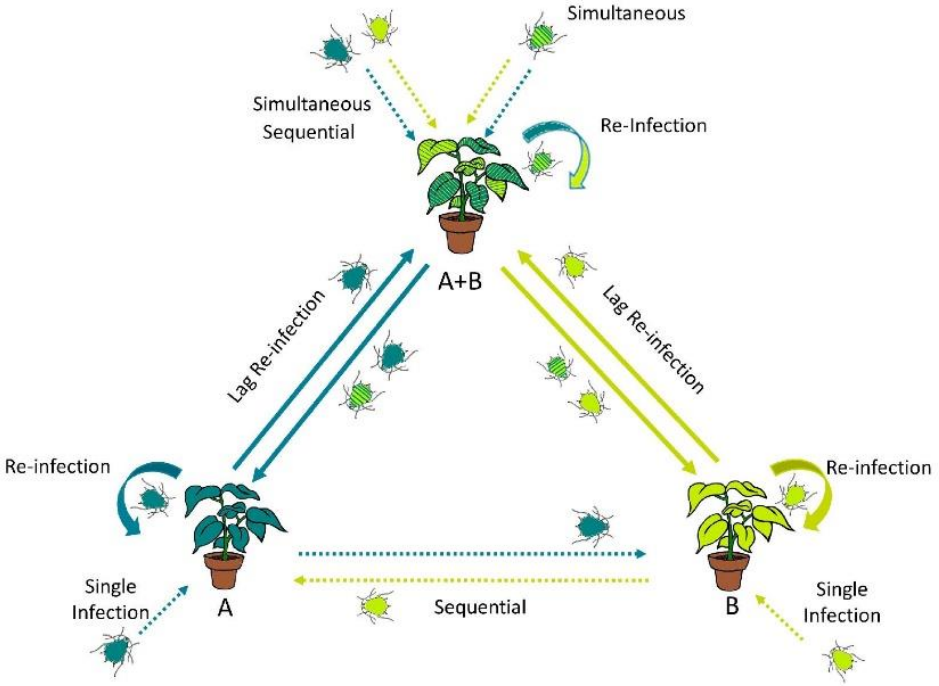

Figure 1. Outline of potential scenarios for generating mixed infections in cultivated plants. Assuming that either cultivated or wild plant species, including secondary plant structures (non-crop plants utilized in integrated pest management) within the crops, can serve as a source of inoculum and that both viruses share an insect vector-mediated transmission route, mixed infections may be generated by simultaneous or sequential infection processes from single or co-infected plants (dotted lines), with potential subsequent re-infections (solid lines). Insect vectors are colored differently according to the viral source plant (single or mixed infection) where they are fed.

Regression analyses of multispecies plant-virus system networks showed that co-infections were the most frequent mode of infection in the ecosystem and contributed more to ecological interactions than single infections [43]. However, how co-infections and sequential infections influence the prevalence of mixed viral infections is still poorly understood in plants. For example, studies on *Orthoflavivirus denguei* (dengue virus, DENV), *Orthoflavivirus zikaense* (zika virus, ZIKV), and *Alphavirus chikungunya* (chikungunya virus, CHIKV), which are transmitted by *Aedes aegypti* and *Aedes albopictus*, indicate that the co-circulation of these viruses enhances both consecutive and simultaneous infections. In some regions of South America, the prevalence of DENV/CHIKV co-infection has reached 10%, along with the occurrence of DENV/ZIKV and DENV/ZIKV/CHIKV combinations [44,45]. In this context, models of transmission dynamics suggest that a majority of coinfections would result from sequential bites from different mosquitoes carrying different viruses rather than co-transmission [46]. Although, if the probability of co-transmission is high, the model indicates that majority of coinfections could be attributed to co-transmission, and the prevalence of coinfections would be more than double what would be expected from sequential infections. In plants, mixed viral infections are widespread. For example, our previous studies on the prevalence of aphid-borne viral diseases affecting cucurbit plant species reveal that mixed infections occur in a high frequency in these crops. A significant

proportion (20–40%) of symptomatic samples exhibit co-detection predominantly involving the *Polerovirus CABYV* (cucurbit aphid-borne yellows virus, CABYV) and *Potyvirus citrulli* (watermelon mosaic virus, WMV), including the emerging *Polerovirus PABYV* (pepo aphid-borne yellows virus, PABYV). Additionally, mixed infections have been detected involving viruses belonging to the genus *Potyvirus*, such as *Potyvirus cucurbitaflavitesselati* (zucchini yellow mosaic virus, ZYMV), *Potyvirus papayanuli* (papaya ringspot virus, PRSV), and *Potyvirus citrullimoroccense* (Moroccan watermelon mosaic virus, MWMV) [21,24,47–49]. With the expected rise in insect vector populations in crops, it is likely that the frequency of mixed infections will even increase during the next seasons. However, it is unclear to what extent the order and timing of these cucurbit virus infections can influence virus–virus interactions within the same host and transmission dynamics, and, although these insect transmissions can be dependent on the environmental conditions, insect-feeding behavior, and agricultural practices, further empirical and modeling research could predict plant virus epidemiology.

Given that transmission rates become a critical factor influencing disease dynamics, when multiple viruses infect the same plant, the viral load of each virus can ultimately influence which virus prevails within the populations. This viral competition for transmission is more likely to occur when different viruses share the same insect vector species to spread from plant to plant, since viruses can interact with each other, affecting their individual viral loads and transmission dynamics [50]. A vector transmission modeling study has highlighted the importance of vector competition as a critical factor in co-infections, emphasizing its role in shaping the outcomes of mixed infections [51]. For instance, studies on the co-transmission of different *Potyvirus yituberosi* strains (potato virus Y, PVY) by aphids have shown that a single aphid can simultaneously acquire and transmit more than one PVY strain. This could occur either by acquiring both strains from a single infected plant or by sequential acquisition from different sources [52–54]. However, the transmission efficiency was found to be lower compared to single-strain transmission, indicating potential competition between the viruses during the transmission process. Focusing on aphid-transmitted viruses, research has predominantly focused on studies of simultaneous infections and the types of interactions that occur between these viruses [20], while few studies have considered the sequential order of infection by non-related viruses (Table 1). Among them, studies include the PVY/*Cucumovirus CMV* (cucumber mosaic virus, CMV) system in tomato, CMV/*Potyvirus capsimaculae* (pepper mottle virus, PepMoV) in pepper, and *Potyvirus betaceum* (beet mosaic virus, BTMV) with *Polerovirus BCHV* (beet chlorosis virus, BChV), *Polerovirus BMYV* (beet mild yellowing virus, BMYV) or *Closterovirus flavibetae* (beet yellows virus, BYV) in sugar beet, which highlight the interactions between viruses under these specific conditions [55–57].

Overall, Table 1 highlights that most interactions involving simultaneous or sequential virus infections result in synergism, with increased viral titers and enhanced symptom severity, regardless of the transmission mode. Non-persistent viruses appear to rely more on plant-host synergism, as these viruses typically localize to specific stylet sites, enabling rapid and transient transmission that likely precludes direct interactions within the insect. Persistent viruses may interact within both vectors and plant hosts, offering greater opportunities for direct interactions with co-infecting viruses. As mentioned before, with the anticipation that the occurrence of mixed infections may increase, along with the increase in aphid populations in crops, future research should prioritize investigating the impact of mixed infections on disease dynamics, viral interactions, and evolutionary outcomes in aphid-transmitted viral systems.

Table 1. Experimental studies on unrelated aphid-transmitted viruses in sequential and simultaneous infections.

Infection Type	Genus	Virus	Host	Interaction	Viral Trait	Cite
Sequential	*Potyvirus* */Cucumovirus* *	PVY/CMV	Tomato	Synergism	Viral titer and symptoms	[56]
	Potyvirus */Polerovirus* □	BTMV/BChV	Sugar beet	Neutral	Symptoms	[57]
	Potyvirus */Polerovirus* □	BTMV/BMYV	Sugar beet	Synergism	Symptoms	[57]
Sequential and simultaneous	*Potyvirus* */Polerovirus* □	WMV/CABYV	Melon	Synergism	Viral titer	This study
	Potyvirus */Closterovirus* †	BTMV/BYV	Sugar beet	Neutral/Synergism	Symptoms and viral titer	[57,58]
	Cucumovirus */Potyvirus* *	CMV/PepMoV	Pepper	Synergism	Viral titer and symptoms	[55]
Simultaneous	*Cucumovirus* */Potyvirus* *	CMV/BICMV [1]	Cowpea	Synergism	Viral titer and symptoms	[59,60]
	Cucumovirus */Potyvirus* *	CMV/PVY	Tobacco	Synergism	Viral titer and symptoms	[61]
	Cucumovirus */Potyvirus* *	CMV/TuMV [2]	*Nicotiana benthamiana*	Synergism	Symptoms	[62]
	Cucumovirus */Potyvirus* *	CMV/PepMoV	Pepper	Synergism	Viral titer and symptoms	[63]
	Cucumovirus */Potyvirus* *	CMV/WMV	Zucchini squash and melon	Synergism	Viral titer and symptoms	[64]
	Cucumovirus */Potyvirus* *	CMV/ZYMV	Bottle gourd, Zucchini squash and melon	Synergism	Viral titer and symptoms	[64–66]
	Cucumovirus */Polerovirus* □	CMV/CABYV	Melon	Synergism	Symptoms	[67]
	Potyvirus/*Caulimovirus* †	TuMV/CaMV [3]	*Arabidopsis thaliana*	Neutral	Viral titer and symptoms	[68]
	Potyvirus */Cucumovirus* *	TEV [4]/CMV	*N. benthamiana*	Synergism	Viral titer and symptoms	[69]
	Potyvirus */Polerovirus* □	PVY/PLRV [5]	Potato	Synergism	Viral titer and symptoms	[70]
	Potyvirus */Polerovirus* □	BTMV/BWYV [6]	Sugar beet	Synergism	Symptoms and viral titer	[58]
	Potyvirus */Polerovirus* □	PVY/PLRV	*N. clevelandii*	Synergism	Viral titer	[71]
	Polerovirus */Umbravirus* □	TuYV [7]/CMoV [8]	*N. benthamiana*	Synergism	Viral titer	[72]

1 Blackeye cowpea mosaic virus, BICMV 2 *Potyvirus rapae* (turnip mosaic virus, TuMV) 3 *Caulimovirus tessellobrassicae* (cauliflower mosaic caulimovirus, CaMV) 4 *Potyvirus nicotianainsculpentis* (tobacco etch virus, TEV) 5 *Polerovirus PLRV* (potato leafroll virus, PLRV) 6 *Polerovirus BWYV* (beet western yellows virus, BWYV) 7 *Polerovirus TUYV* (turnip yellows virus, TuYV) 8 *Umbravirus maculacarotae* (carrot mottle virus, CMoV). Transmission modes: □ Persistent; † semi-persistent; and * non-persistent.

3. Impact of Temporal Order of Infection on Plant Viral Disease: A Case Study of Two Aphid-Transmitted Viruses

To address how the temporal viral arrival order can impact virus accumulation and disease progression, it is advisable to conduct appropriate assays that enable controlled manipulation of simultaneous or sequential viral infections by insect vectors. This necessitates the utilization of viral infectious clones and aviruliferous insects to synchronize viral inoculations in plants at different temporal intervals. However, there is a paucity of studies elucidating the impact of mixed infections, particularly with the aphid-vector, *Aphis gossypii* Glover. In this sense, we carried out an experimental assay in which the viral load of two aphid-transmitted viruses; CABYV (persistent transmission mode) and WMV (non-persistent transmission mode), was examined in melon plants (Piel de Sapo cultivar) under different order of infections. By using CABYV and WMV infectious clones [24,49], both viral isolates were manipulated to obtain viruliferous aphids (*A. gossypii*) for subsequent melon plant infections either simultaneously or sequentially (one after the other), with a time lag of 10 days. Viral accumulation was estimated at 20 and 30 days post-inoculation (dpi) in co-infected and sequentially infected plants by RT-qPCR (Figure 2A). Our results showed that co-infection led to the highest CABYV and WMV accumulation, suggesting a double-synergistic effect when both viruses infect simultaneously (Figure 2B–C). Consistent with our results, a study of co-infection with *Fijivirus boryzae* (southern rice black-streaked dwarf virus, SRBSDV) and *Oryzavirus oryzae* (rice ragged stunt virus, RRSV) showed an increase in both viral titers as a consequence of changes in virus-induced RNAi pathway genes [73]. Similarly, a synergistic interaction was observed between *Potyvirus glycitessellati* (soybean mosaic virus, SMV) and two comoviruses, *Comovirus siliquae* (bean pod mottle virus, BPMV) and *Comovirus vignae* (cowpea mosaic virus, CPMV), regardless of whether the infection was simultaneous or sequential, with higher titers of BPMV and CPMV [74]. In sugar beet plants, co-infection of the potyvirus BTMV with either the closterovirus BYV or the polerovirus BWYV demonstrated a synergistic interaction between both viruses involved, leading to increased viral accumulation and more severe symptoms in the plants [58]. Other studies on simultaneous infections showed a type of synergistic-neutral interaction, where one virus increases its viral titer while the other remains unaffected, such as the interaction between *Orthotospovirus tomatomaculae* (tomato spotted wilt virus, TSWV) and ToCV in tomato plants [75], and PVY/CMV or CMV/PepMoV [61,63]. Such interactions can often be explained by viral complementation, a form of co-evolutionary synergism between different viruses during mixed infections. In this context, one virus can enhance the infection of another through mechanisms such as genetic exchange, reassortment, or trans-complementation, where viral proteins from one virus support the infection process of the other [28]. For instance, PVY has been shown to complement CMV mutant in accessing tomato phloem elements [56]. Similarly, interactions between *Potexvirus cymbidii* (CymMV) and *Tobamovirus odontoglossi* (ORSV) showed complementation of MPs and CPs to facilitate their movement within plants [76]. In contrast, other studies have shown that co-infection of *Nicotiana tabacum* with potexvirus *Potexvirus ecspotati* (potato virus X, PVX) and potyvirus PVY causes antagonistic interactions [77], which could reduce the symptoms of viral disease. Another study on *Tobamovirus mititessellati* (tobacco mild green mosaic virus, TMGMV) and oilseed rape mosaic virus (ORMV) tobamoviruses in tobacco plants showed that co-inoculated plants at the same time showed antagonistic interactions among these viruses as well as in sequential infection regardless of which virus arrived first in the plant [78]. This suggests that, while some particular virus species maintain a neutral interaction in mixed infections, either in simultaneous or sequential infections, other cases involve potential changes due to the direct virus–virus interactions or indirect host-response modulation, which could even be influenced by environmental factors [29,79].

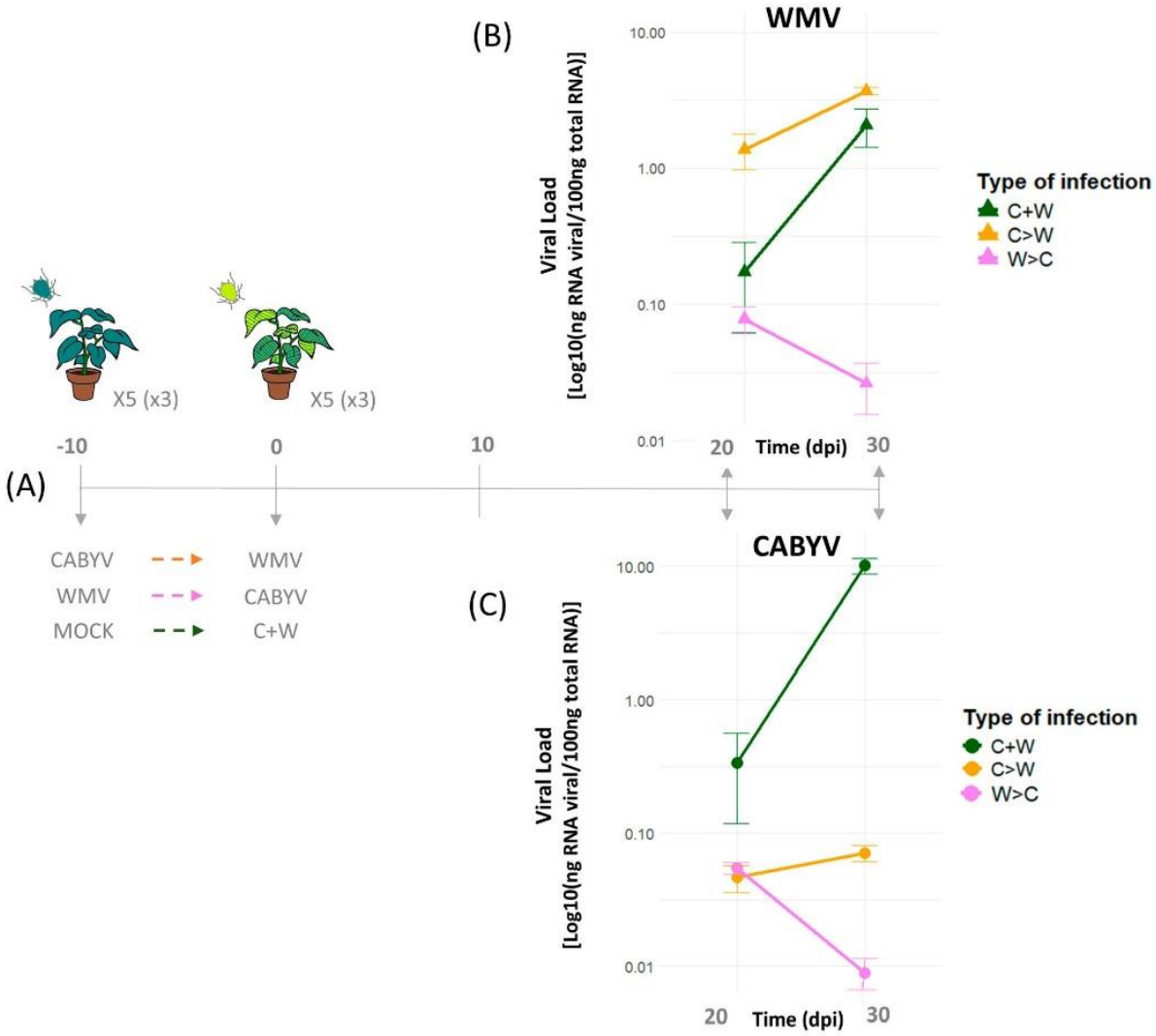

Figure 2. Viral load dynamics in single, co-, and sequential infections of CABYV and WMV in melon plants. (**A**) Schematic diagram of the experimental design for the infection of melon plants with WMV-MeWM7 and CABYV-LP63 by *Aphis gossypii*. Plants were inoculated with either CABYV or WMV, both viruses (simultaneous co-infection, C + W, green color), or mock treatment (control) on day −10. On Day 0, sequential infections were initiated: plants initially infected with CABYV were inoculated with WMV (C > W, orange color), and plants initially infected with WMV received CABYV (W > C, pink color). The RNA viral load (Log10 (ng of viral RNA/100 ng of total RNA) mean and SE error bars, n = 3) of WMV (**B**) and CABYV (**C**) in melon plants under simultaneous and sequential infections were determined by absolute quantification using RT-qPCR with specific RNA transcripts generated from the P1 and CP cloned genes of WMV and CABYV, respectively [24,49]. These in vitro RNA transcripts were serially diluted (10-fold) to generate external standard curves. The RNA concentration was estimated from the threshold cycle (CT) values obtained from three independent plant samples, with three technical replicates for each sample at each time point (20 and 30 dpi with aphids). Statistical comparisons were performed using linear mixed model fit by REML (*t*-tests use Satterthwaite's method) to assess significant differences in viral loads between infection conditions over time.

Furthermore, simultaneous infection of sugar beet plants with BTMV and BYV showed a synergistic interaction [58], while sequential infection with BTMV followed by BYV ten days later resulted in a neutral interaction [57]. In the case of sequential CABYV and WMV

infections, our results showed that the initially infecting virus appears to significantly affect the subsequent virus infection in melon plants. Upon analysis of the WMV load, we found that when WMV was introduced first, WMV reduced its viral load over time ($t(19) = -2.549$, $p = 0.019607$), indicating potential interference or antagonistic interaction with CABYV (Figure 2B). However, when CABYV arrived first, there was an increase in the viral accumulation of WMV, which was comparable to that in co-infections ($t(19) = 3.359$, $p = 0.003296$). In contrast, analysis of the CABYV load revealed that when WMV was infected first, there was a significant decrease in CABYV accumulation ($t(19) = -2.778$, $p = 0.0120$) while there was a minimal increase in CABYV when CABYV arrived before WMV ($t(19) = -2.764$, $p = 0.0124$) (Figure 2C). These results suggest that the temporal order of infection affects the type of interaction between these two viruses, with a synergistic interaction occurring when CABYV precedes WMV, and an antagonistic interaction when WMV precedes CABYV. These results were consistent with the case of mixed infections between PRSV and *Potexvirus papayae* (papaya mosaic virus, PapMV) in papaya plants, wherein simultaneous infections or sequential infections with PRSV followed by PapMV exhibited more severe symptoms than sequential infections with PapMV followed by PRSV [77]. Indeed, evidence suggests that antagonism results from the activation of innate and adaptive immunity, with elevated ethylene levels as well as RNAi-mediated resistance, in contrast with synergistic interactions [80]. Consequently, these differences in the host-mediated response underscore the complex dynamics of mixed infections, which necessitates consideration not only of these virus–virus interactions within the host to elucidate epidemiological patterns observed in crops [24,49,81], but also of the potential role of abiotic factors and feeding behavior of insect vectors in generating the context for mixed infections.

4. Consequences of Infection Order on Population Dynamics, Virus Transmission, and Agroecosystems

The temporal order of viral infection can significantly impact plant health. In certain instances, it may be beneficial to the host, as previous infection of an attenuated virus isolate may prevent subsequent infection by a virulent strain, a phenomenon known as cross-protection [82–85]. However, mixed infections are frequently detrimental, and regardless of the infection timing and focusing on insect vector-mediated transmission of plant viruses, within-host competition between viruses is not necessarily beneficial and could even facilitate an increased transmission and dispersion of the disease [70]. For instance, it has been observed that the variant zucchini yellow mosaic virus (ZYMV-WK) is transmitted by aphids from WMV-co-infected plants [86,87], with variations observed from other vectors [88]. Also, *Begomovirus capsicumhuastecoense* (pepper huasteco yellow vein virus, PHYVV) and *Begomovirus capsicummusivi* (pepper golden mosaic virus, PepGMV) can be co-transmitted by the whitefly *Bemisia tabaci* to pepper plants with no competition [89], similar to the co-transmission of Mld- and Il-strains of tomato yellow leaf curl virus (TYLCV) to tomato [90]. Another study showed that *Crinivirus cucurbitae* (cucurbit yellow stunting disorder virus, CYSDV) and WMV were transmitted by their corresponding vectors, whiteflies and aphids, respectively, without competition in transmission [91]. However, the co-infection of TYLCV and tomato mottle virus (ToMoV) by *Bemisia* to tomato plants showed competition in transmission, including differences in the infection status of plants and virus accumulation [92]. Likewise, co-infection of *Begomovirus cucurbitae* (cucurbit leaf crumple virus, CuLCrV) and CYSDV resulted in a lower CYSDV accumulation and reduced viral titer in whiteflies [93]. In contrast, the co-infection of BChV and BYV only reduced transmission of BChV, despite similar virus accumulation [94]. Furthermore, the transmission of *Polerovirus PEVYV2* (pepper vein yellows virus-2, PeVYV-2) and *Polerovirus PEWBVYV* (pepper whitefly borne vein yellows virus, PeWBVYV) by aphids and whiteflies, respectively, showed significant differences between simultaneous and sequential co-infections [95]. In addition to the viral transmission rate and accumulation, mathematical models have demonstrated that the vector's preference for the host, the virus transmission mode, and the vector phenotype can influence vector population density,

thereby having a significant impact on the disease incidence [96]. In fact, it has been shown that the timing of infection and aphid-mediated inoculation density significantly impact disease development, which is essential for establishing economic control thresholds in decision-support systems [57]. Consequently, it is necessary to ascertain whether viral co-transmission contributes to simultaneous mixed infections or if, alternatively, sequential infections are more prevalent, thereby indicating a lower frequency of co-transmission in crops, which could affect virus virulence and population dynamics.

Despite the significant impact of plant viruses on crops, wild plant species may serve as reservoirs and sources of inoculum, contributing to the spread of viral diseases in agricultural systems [97]. An increasing number of studies in wild plants have revealed that the prevalence of viral infections varies widely depending on the virus and host [4,98–101]. However, the occurrence of mixed infections in wild plants and their impact on crops and ecosystems remain poorly understood. Some studies indicate that virus prevalence and infection rates in wild plants exhibit greater variability than those in agricultural systems, likely attributed to the genetic diversity of wild plants [102]. Notably, viruses found in wild plants are often detected in cultivated crops [103–105], suggesting that wild plants may play an important role in generating mixed viral infections in crops. Additionally, global warming and climate change are likely to influence not only insect vector populations, thereby affecting the prevalence of mixed viral infections, but also crop productivity. A recent study has shown the detrimental effects of drought on melon plants, particularly in the context of mixed viral infections, which can further complicate plant health and crop productivity [67]. Herein, while water stress negatively affected vegetative growth and led to a higher proportion of female flowers in drought-stressed plants compared to controls, this increase resulted in a higher rate of fruit abortion, suggesting that the combination of drought and co-infections (CABYV and CMV) may enhance fruit abortion rates, and, consequently, reduce crop productivity [67]. Given that global warming is anticipated to increase the incidence of viral diseases, the opportunities for mixed infections will subsequently increase. Therefore, further research in the performance of crops bred for drought or heat tolerance, along with mixed viral infections, is crucial to understand the complex relationships between viruses, their vectors, and host plants, including environmental changes in order to facilitate more effective disease management strategies in agriculture and natural ecosystems.

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6.2.2. Sub-Chapter II.II: "*Thermotolerance elicits specific genes in cucurbit plants as a response to the combined effect of viral infection and temperature stress*"

Thermotolerance elicits specific genes in cucurbit plants as a response to the combined effect of viral infection and temperature stress

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Highlight

Thermotolerant and thermosensitive cultivars of melon and zucchini plants exhibit differentially expressed genes that respond to the combination of WMV infection and temperature stress.

Abstract

Plants respond to biotic and abiotic factors through specific physiological and metabolic changes that optimize their survival. However, the extent to which the combination of both stressors could modulate common or exclusive signaling pathways remains unclear, as most studies on gene-specific plant expression focus on a single stress. In this study, we examined the gene expression patterns in two cucurbit species, melon and zucchini, each with high- and low-temperature tolerant cultivars, under watermelon mosaic virus (WMV) infection and three temperature ranges (20/14 °C, 26/20 °C, and 32/24 °C). Our results showed that WMV accumulation was higher in zucchini than in melon plants and was influenced by both thermotolerance and temperature conditions. Comparative 3´mRNA-seq analysis revealed that zucchini exhibited a 2.6-fold higher percentage of differentially expressed genes (DEGs) than melon, with the highest percentage of DEGs occurring at lower temperatures in the thermo-susceptible plants of both species, possibly associated with greater WMV accumulation. Notably, the greatest number of unique DEGs was detected in high-temperature and WMV-infected plants of thermo-tolerant melon (711 DEGs) and thermo-susceptible zucchini (306 DEGs). Among the top 15 GO terms, four orthologous genes were identified, three of which—MELO3C023308, MELO3C024920, and Cp4.1LG05g12560—exhibited a significant temperature-dependent expression under WMV infection, and are potentially encoding a F-box protein, a metal ion transporter, and a photomorphogenesis-related factor, respectively. These findings provide novel insights into plant-virus-environment interactions and may contribute to enhancing cucurbit crop resilience and food security.

Keywords: Heat stress, Temperature, Plant-virus-environment interaction, WMV, Plant resilience, Cucurbit infection.

INTRODUCTION

Plants must adapt to a myriad of abiotic and biotic stresses to thrive. Extreme temperatures, drought, salinity, and greenhouse gases can limit plant growth by triggering specific physiological processes and metabolic responses that lead to gene-specific changes in plant development (Mittler *et al.*, 2012; Bailey-Serres *et al.*, 2019; Zhang *et al.*, 2022). Consequently, climate change poses a significant threat to global food production by affecting agricultural yield, food quality, and prices (Vermeulen *et al.*, 2012; Raza *et al.*, 2019; Thiault *et al.*, 2019). As climate change intensifies, crops face the impact of pests and pathogens, which can further limit food production and exacerbate food insecurity (Oerke, 2005; Pandey *et al.*, 2017; Rudgers *et al.*, 2020; Montes and Pagán, 2022; Tonnang *et al.*, 2022; Vasquez *et al.*, 2022; Xiao, 2022; Laine, 2023). Thus, understanding the intricate interactions between abiotic and biotic stressors is essential to mitigate the potential consequences on agricultural food production systems and to further adapt crop management practices to protect food production (Shelake *et al.*, 2024).

Heat stress can cause crop production losses by directly interfering with plant physiological processes and reproduction, increasing photorespiration and transpiration rates, altering pollen viability and fertilization, and disrupting metabolic processes (Zhang *et al.*, 2022). Plants are particularly sensitive to high temperatures during their reproductive stages, which can influence gene expression at the chromatin level and circadian clock (Chang *et al.*, 2014; Mody *et al.*, 2020) and alter their profiling in plant roots (Martins *et al.*, 2017). Moreover, it has been reported that transcriptional factors, including heat shock proteins, are key in regulating gene expression networks involved in plant heat stress responses (Li *et al.*, 2019; Tolosa and Zhengbin, 2020; Pardo-Hernández *et al.*, 2024), as well as second messengers during signal transduction, protein folding, and protein-protein interactions (Zhang *et al.*, 2022). Conversely, suboptimal temperatures can reduce enzymatic activity and biochemical reactions, thereby adversely affecting plant growth and development (Hasdai *et al.*, 2006). Furthermore, temperature can modulate plant defense responses through specific proteins, such as Nucleotide-binding Leucine-rich repeat proteins (NB-LRR) proteins (Zhu *et al.*, 2010). In addition to plant growth and development, rising temperatures can

indirectly influence crop yield by altering plant disease progression and insect pest biology (Jones, 2016; Jeger *et al.*, 2018; Jones and Naidu, 2019; Trebicki, 2020; Tonnang *et al.*, 2022). Accumulating evidence suggests that crops are vulnerable to viral diseases at high temperatures (Tsai *et al.*, 2022*b*,*a*; Iqbal *et al.*, 2023). In this sense, it has been reported that temperature can influence plant-virus interactions, affecting symptom expression and virus accumulation during the plant infection process (Aguilar *et al.*, 2015; Obrępalska-Stęplowska *et al.*, 2015; Chung *et al.*, 2016). For instance, studies have suggested that virus accumulation is temperature-dependent, with seasonality affecting virus-plant interactions and virus dynamics during persistent infections (Honjo *et al.*, 2020), thus shaping viral genetic diversity and population dynamics in mixed infections (Alcaide *et al.*, 2021; Sardanyés *et al.*, 2022). Other studies have shown that exposure to elevated temperatures may either enhance or reduce plant susceptibility to viral diseases (Prasch and Sonnewald, 2013; Ghandi *et al.*, 2016; Makarova *et al.*, 2018; Amari *et al.*, 2021; Tsai *et al.*, 2022*a*). However, temperature can also affect the distribution of vectors that transmit plant viruses, leading to rapid and widespread dissemination (Jones, 2016; Islam *et al.*, 2020; Trebicki, 2020). Hence, rising temperatures could accelerate epidemic development and hinder disease management (Jones and Naidu, 2019). However, despite evidence that temperature affects several crops (Bita and Geräts, 2013; Grossiord *et al.*, 2020; Karki *et al.*, 2021; Rangaswamy *et al.*, 2021; Nguyen *et al.*, 2024) and that viral diseases may also elicit specific responses in plants, their combined effects on gene expression related to developmental and defense mechanisms remain unclear, as they have been extensively studied individually.

To elucidate the interactive molecular responses to combined temperature stress and viral infection, this study examined the gene expression profiles of two important cucurbit crops (melon and zucchini) at high, medium, and low temperatures, along with the presence or absence of the same viral infection, *Potyvirus citrulli* (watermelon mosaic virus, WMV). Functional genomic studies of plant crops are challenging because of the lack of full genome information. Thus, only a few studies have reported specific genes involved in the regulation of the drought stress response in melon plants (Xing *et al.*, 2020; Yang *et al.*, 2020), and studies on genes involved in heat stress in cucurbit plants are limited. Understanding how temperature modulates gene expression in plants

can allow the identification of genes and alleles that are useful for marker-assisted selection, which can help plant breeding programs to enhance crop resilience (Hill and Li, 2022; Shen *et al.*, 2022). Although the effects of these improvements on the development of heat-tolerant varieties may be largely contingent on different agroecosystems and pathogen infections (Savary *et al.*, 2019). In this context, 28 viruses have been reported to significantly affect cucurbit crops in the Mediterranean Basin (Lecoq and Desbiez, 2012; Radouane *et al.*, 2021). Among these, aphid-transmitted viruses are widely distributed and are particularly detrimental to crop production sustainability (Lecoq and Katis, 2014; de Moya-Ruiz *et al.*, 2023; Rabadán *et al.*, 2023). *Potyvirus citrulli* (watermelon mosaic virus, WMV) is a vector-borne virus primarily transmitted by several aphid species that causes serious diseases in major cucurbit production areas globally (de Moya-Ruiz *et al.*, 2021, 2023; Rabadán and Gómez, 2023; Rabadán *et al.*, 2023). In this study, we examine two interconnected aspects of plant-virus interactions, viral load and host transcriptional response, with their potential implications. In particular, we comprehensively integrated and analyzed the viral accumulation and transcriptomic datasets of melon and zucchini plants with high- and low-temperature tolerance, subjected to three temperature ranges (low, medium, and high), and WMV infection (Fig. 1). After analyzing viral RNA accumulation, we conducted comparative transcriptomic (3´mRNAseq) analysis of single and combined stress responses, characterizing gene clusters with distinct transcription patterns associated with abiotic (temperature) and biotic (viral infection) stress. Additionally, we linked the gene profiles of key biological processes to the temperature- and virus-responsive pathways in melon and zucchini plants, which could help develop effective strategies to improve cucurbit productivity.

MATERIALS & METHODS

Plant material and growth conditions. Our experimental approach combines viral accumulation and transcriptomic analysis to establish causal relationships between two cucurbit plant species and virus infection responses to thermotolerance. This study utilized nine plants each of the thermotolerant (TT) and thermo-susceptible (TS) melon and zucchini varieties (three replicates × three plants). Due to confidentiality agreements and potential conflicts of interest, the specific seed details provided by the respective

plant breeding companies cannot be disclosed. Additionally, 54 commercial plants (nine plants each of melon (Piel de sapo) and zucchini (Black Beauty) varieties) were included and grown under the same conditions. Cucurbit seeds were initially sown in Petri dishes at 28 °C in darkness, and after two days were transplanted into 1-L plastic pots filled with the substrate (coconut fiber, peat, and perlite in a 5:10:1 ratio). Each experiment was carried out independently in a controlled greenhouse with a 16/8 photoperiod (16h light and 8h dark) at the respective experimental temperatures: 20 °C/16 °C (Low), 26 °C/20 °C (Medium) or 32 °C/24 °C (High), with ± 6ºC of variation between experiment and day and night conditions. Despite the temperature and light regulations, note that the 26 °C experiment was conducted from April to June, the 32 °C experiment was conducted from July to September, and the 20 °C experiment was conducted from October to December, aligning with the natural seasonal temperatures in the Region of Murcia, Spain. As a control, mock-treated plants were similarly subjected to temperature stress without viral infection. Based on our previous study on WMV accumulation in cucurbit plant species (de Moya-Ruiz *et al.*, 2021), the material was collected at 30 days post-infection (dpi) as the optimal time for viral performance and further analysis (Fig. 1).

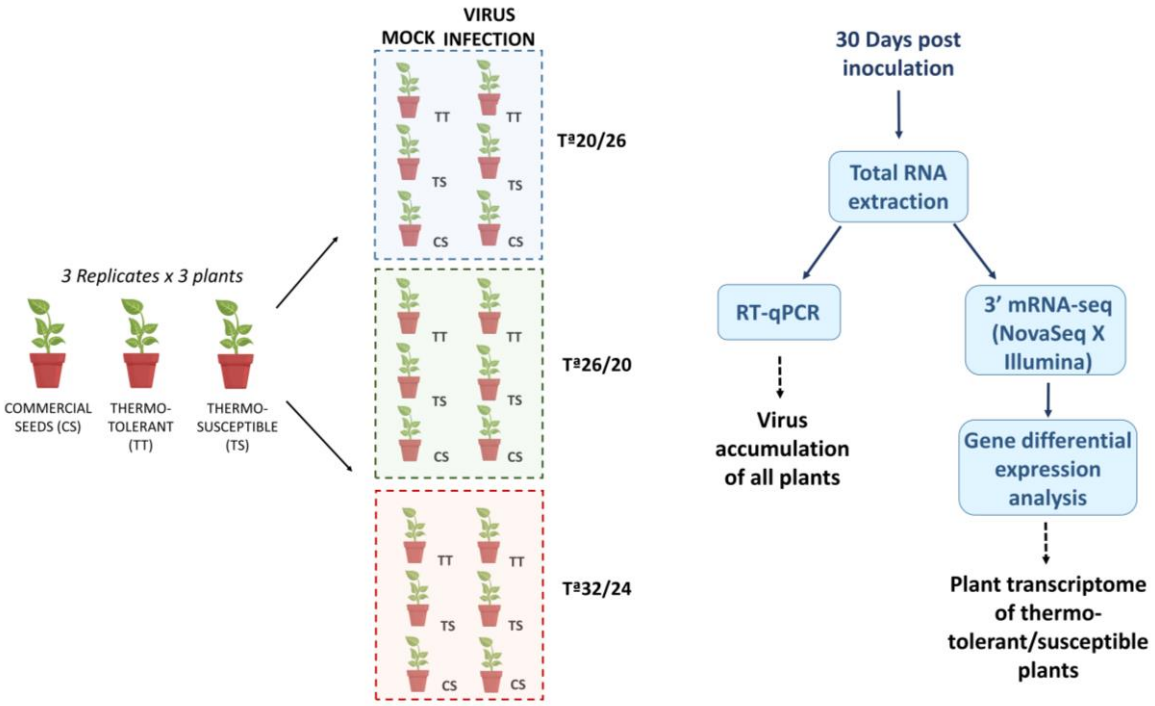


Figure 1. Schematic representation of the experimental design and workflow using thermotolerant/susceptible melon and zucchini plants. Three replicates, each consisting of a pool of three plants, were maintained at low (20/14 ºC), medium (26/20 ºC) and high (32/26 ºC) temperature ranges under both uninfected and WMV-infected conditions. Plant material was collected at 30 dpi, and WMV accumulation was determined using absolute quantitative RTq-PCR. Additionally, differential gene expression analysis was conducted using 3'mRNA-seq approach via Illumina (NovaSeq X).

WMV virion purification and inoculation. Plants were inoculated with WMV virion particles. Briefly, first *Nicotiana benthamiana* plants were agroinoculated with WMV-MeWM7 (de Moya-Ruiz *et al.*, 2021) and maintained in a growth chamber at 24 °C. After 20 dpi, approximately 60 g of symptomatic leaves were collected and ground in extraction buffer with liquid nitrogen, following a series of centrifuges and PEG precipitation, as described by Rupar *et al.* (2013), with some modifications. First, the homogenate was mixed with 5.38 ml/g of homogenization solution (0.5M K_2HPO_4, 5 mM EDTA, 10 mM DIECA, and Na_2SO_3) and agitated at 4 °C for 15 min. The mixture was then centrifuged at 7000 rpm for 10 min at 4 °C. The supernatant was collected and filtered through gauze, Triton X-100 was added to a final concentration of 1 %, and the mixture was shaken at 4ºC for 1h. The supernatant was ultracentrifuged at 50 000 rpm for 90 min at 4 °C. After discarding the supernatant, the pellet was resuspended in 14 ml of citrate solution pH 7.5+1 % triton X-100 and shaken for 30 min at 4 °C. The pellet was resuspended in 6 ml citrate solution pH 7.5+1 % triton X-100, shaken for 30 min at 4 °C, and centrifuged at 10 000 rpm for 10 min. The supernatant was collected, 10 % chloroform was added, mixed thoroughly, and centrifuged at 10 000 rpm at 4 °C for 10 min. The aqueous phase was collected and ultracentrifuged using a 30 % sucrose cushion at 45 000 rpm for 2 h at 4 °C. The pellet was resuspended in 1 mL of citrate solution and incubated overnight at 4 °C. The purified virus was stored at -20 °C. For plant inoculation with WMV, the third and fourth true leaves were rubbed with Carborundum-dusted and a suspension of 100 mg/mL virion particles in sodium phosphate buffer (30mM), as previously described (Gomez et al., 2009). The material (all apical leaves from each plant) was collected at 30 dpi from nine plants (three replicates × three plants) per treatment.

Quantification of viral RNA accumulation. To determine WMV accumulation, samples were collected 30 days post-infection (dpi). Total RNA was extracted from all samples using Tri-reagent, purified by phenol-chloroform extraction, and treated with DNaseI (Sigma-Aldrich, St. Louis, USA). Viral accumulation was then quantified by absolute real-time quantitative PCR (qPCR) with an AB7500 System (Applied Biosystems, Foster City, CA) using the One-step NZYSpeedy RT-qPCR Green kit, ROX plus (NZYTech, Lisboa, Portugal). Two specific primers targeting the WMV P1 region (523–635 nt) were used: CE-2959 Fw *5'-CACCCAACCTCTGAAATGG-3'* and CE-2960 Rv *5'-GGCTCAGATTTGCATC-3.'*

Briefly, the reaction mixture was performed in a total volume of 10 µl, containing 5 µl of One-step NZYSpeedy qPCR Green master mix (2x), ROX plus), 0.4 µl of NZYRT mix, 0.4 µl of reverse and forward primers, 1.8 µl of sterile water, and 2 µl of RNA. The PCR cycling protocol consisted of 50 °C for 20 min, 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, 30 s at 60 °C annealing temperature, and the melting curve. Non-template controls were included to ensure product-specific amplification and the absence of primer dimers. Serial dilutions (10-fold) of viral RNAs from WMV-MeWM7 infectious clones were used to generate external standard curves (de Moya-Ruiz *et al.*, 2021). The initial RNA concentration was measured twice using a Qubit 3.0 fluorometer following the manufacturer's instructions (Thermo Fisher Scientific). The RNA concentration in each sample (ng of viral RNA per 100 ng of total RNA) was estimated by plotting the threshold cycle (CT) values from each biological assay (n=9 at each time point) with three experimental replicates for each biological replicate. Given that the viral load of WMV in melon and zucchini plants had a similar exponential pattern, consistent with our previous studies (De Moya-Ruiz *et al*., 2021), and the most significant differences between temperatures were observed after 30 dpi, subsequent analyses focused on samples from this time point to ensure the reliability of the results and capture the relevant biological effects of each temperature condition.

3´mRNA sequencing and comparative analysis of gene expression profiles. Total RNA was extracted from samples of melon and zucchini at 30 dpi using Tri-reagent, purified by phenol-chloroform extraction and treated with DNaseI (Sigma-Aldrich, St. Louis, USA). The quantity and quality of the RNA were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) and agarose gel electrophoresis. The 3'mRNA-sequencing was performed using NovaSeq X (Illumina Platform) by Seqplexing (Paterna, Valencia). This RNA-seq approach involves tagging the 3´end of mRNA poly(A) tails with universal adapters, barcodes, and unique molecular identifiers, allowing for the accurate quantification of gene expression profiling (Charpentier *et al.*, 2021). Briefly, the bioinformatics pipeline starts by cleaning the raw sequence data by quality trimming and removing sequencing adapters and poly A sequences. Quality assessment was conducted using FastQC to ensure high-quality FASTQ files. UMIs were identified using umi-tools, duplicates were removed, and reads were mapped to the reference genome using STAR. The version of the genomic reference

for zucchini and melon used were "Cpepo_genome_v4.1", with the associated transcript annotation being "Cpepo_4.1", both files obtained from http://cucurbitgenomics.org. and "Harukei3_v1.41" with the associated transcript annotation being "Harukei3_v1.41", both files obtained from https://melonet-db.dna.affrc.go.jp/ap/top, respectively. HTSeq-count and counts were normalized to the total number of identified reads in each sample. This inter-sample normalization was followed by control-sample normalization, including control samples (i.e., mock plants under the same stress conditions) for each dataset, to enable comprehensive comparisons of the gene expression profiles of the thermotolerant and thermo-susceptible melon and zucchini cultivars. The comparisons, along with the number of Differentially Expressed Genes (DEGs) and total genes from both melon and zucchini cultivars, are summarized in Table 1. DESeq2 was employed to identify significant expression differences, identifying genes with an adjusted p-value < 0.05, and $\log_2$FC <-1 or >1 for each variety and crop. The variability between groups was analyzed using Principal Component Analysis (PCA) conducted using iDEP 2.0 (http://bioinformatics.sdstate.edu/idep/) (Ge *et al.*, 2018). Upset plots and Venn Diagrams were created using ChiPlot (https://www.chiplot.online/) and the R package, respectively.

Functional annotation and GO-Term enrichment analysis. Functional annotation of specific DEGs found in each melon and zucchini variety under a combination of temperature and virus infection was extracted from AmiGO 2 (https://amigo.geneontology.org/amigo/landing). Using the taxonomy ID 3656 and 3664 for melon and zucchini, respectively, the visualization platform of iDEP 2.0 (http://bioinformatics.sdstate.edu/idep/) and ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/) were used to perform Gene Ontology (GO) enrichment analysis and network of GO terms, using all available gene sets with an FDR cutoff of 0.05, and $\log_2$FC <-1 or >1. The top 15 pathways were considered.

Identification and validation of orthologous genes. To identify orthologous genes between melon and zucchini under different temperature conditions and viral infections, reference genomes were obtained from the public Cucurbit Genomics Database (CuGenDB) (http://cucurbitgenomics.org/) (Yu *et al.*, 2023). We selected the well-annotated reference genomes of Melon (DHL92) v3.6.1, and *Cucurbita pepo* subsp.

Pepo. Orthologous genes were identified using the genes involved in the top 15 pathways using the Synteny Viewer tool. Furthermore, RT-qPCR was carried out to determine the expression of both orthologous genes by using an AB 7500 System (Applied Biosystems, CA, U.S.A.), and using the One-step NZYSpeedy RT-qPCR Green kit, ROX plus (NZYTech, Lisboa, Portugal) as described above. Three biological replicates (each consisting of a pool of three plants) per treatment and two technical replicates were included for RT-qPCR analysis. According to the extensive evaluation of 14 and 13 candidate reference genes performed by Kong et al. (2014) in melon and Obrero et al. (2011) in zucchini, we selected the gene a-Tubulin, which showed M values among the most stable genes under abiotic and biotic stress conditions. Thus, the primers CmTUA (Kong *et al.*, 2014) and TUA (Obrero *et al.*, 2011), were used for melon and zucchini plants, respectively. Note that we also observed consistent Cq values for TUA across all treatment conditions, with minimal variation between samples, confirming its suitability as a reference gene for our particular experimental setup. Primers to quantify melon and zucchini transcripts were designed using Primer3 software from the corresponding sequences available in the cucurbit genomics database (http://cucurbitgenomics.org). In particular, primers used for MELO3C023308.2; CE-3670 Fw: *5'-TGGAGCCAGAGGAATGTTGT-3'*, and CE-3671 Rv: *5'-GTGGATAGCTTGGGTTCCCT-3',* for MELO3C024920.2; CE-3668 Fw: *5'-GCATCATCCACATCCACACC-3',* and CE-3669 Rv: *5'-*CGATGCCTTCAAAGACGGAG*-3';* and for Cp4.1LG05g12560; CE-3674 Fw: *5'-TGATGGGCAAGACAGTGACT-3'*, and CE-3675 Rv: *5'-CGAAACTTAAGCGACTCGGG-3'*; and for Cp4.1LG06g08450; CE-3672 Fw: *5'-CGCGTTTATGCTTGCTTGTG-3',* and CE-3673 Rv: *5'-GAATCACCGTCCTGTTTCCG-3'.* The fold change was calculated using the 2−ΔΔCt method, with T 26 ºC, 20 ºC and 32 ºC without virus infection treatments used as the control under each viral condition, such as described in (Schmittgen and Livak 2008).

Statistical analyses. Analysis of the viral load for each plant species was performed using two-way ANOVA. The viral accumulation data were transformed using a logarithmic function to achieve normality. The model included plant species (melon and zucchini) and temperature as two-level fixed effects. All analyses were performed using JMP software. Plot graphs of viral RNA accumulation for each isolate and plant species were generated using R software. Significant changes in transcript expression as compared to

the control (DEGs) were defined as $\log_2$ FC <-1 or >1 and adjusted p < 0.05 (negative binomial Wald test followed by Benjamini–Hochberg correction) (Ferreira & Zwinderman, 2006). The expression variation for the orthologous gene in melon and zucchini plants that were performed by RT-qPCR was analyzed using the Shapiro-Wilk normality test and the Student t-test (p<0.05), and plot graphs were generated using the *ggplot2* package of R software.

Data availability. All rna sequencing (3´mRNA-seq) data that support the findings of this study have been deposited in arrayexpress (EMBL-EBI), with the accession codes E-MTAB-14116 for melon and E-MTAB-14118 for zucchini plant species. The raw data, processed data, and metadata with detailed sample annotations and protocols of this 3'mRNA study are provided.

RESULTS AND DISCUSSION

WMV load was significantly influenced by melon and zucchini plants and depended on their thermotolerance. To determine the extent to which temperature affects viral accumulation in melon and zucchini plants, the WMV load was estimated using absolute RT-qPCR in commercial melon (Piel de sapo) and zucchini (Black beauty) cultivars grown at different temperatures (Low: 20/14ºC, Medium: 26/20ºC, and High: 32/26ºC). At 30 dpi, WMV had five-fold greater accumulation in zucchini than in melon plants (Fig. S1, A-B; $F_{1,12}$= 193.23, p < 0.001), with a significant interaction between temperature and plant species ($F_{2,12}$= 78.09, p < 0.001). This viral accumulation pattern may have arisen because of the host specificity and limited viral fitness of WMV in melon plants at high temperatures. Many studies have shown that viral titers can be influenced by temperature. For example, turnip mosaic virus (TuMV) and plum pox virus (PPV) show lower accumulation at high temperatures, with milder symptoms (Szittya *et al.*, 2003; Aguilar *et al.*, 2015; Chung *et al.*, 2015). In contrast, TuMV has been observed to exhibit more severe symptoms in Chinese cabbage at 28 °C, which correlates with higher viral accumulation (Chung *et al.*, 2015). Though, plants may experience a slowdown in metabolic processes at lower temperatures, which could affect the efficiency of plant defense mechanisms and, in turn, may affect viral replication and accumulation (Garcia-Ruiz, 2018). Therefore, we aimed to evaluate the effects of plant thermotolerance on

viral accumulation at varying temperatures. Specifically, we examined WMV accumulation in melon and zucchini plants with different levels of thermotolerance (TT: thermotolerant and TS: thermosusceptible) at three temperature ranges (L, M, and H). In melon plants, the WMV load decreased significantly in the TS cultivar as the temperature increased, with a 7-fold reduction in the high-temperature range (H) ($F_{2,6}$= 6.12; p = 0.003). In contrast, the WMV load in the TT melon cultivar did not vary significantly across temperature ranges ($F_{2,6}$= 0.11, p = 0.89). However, in zucchini plants, the WMV load was generally higher than that in melons, and its accumulation pattern was reversed. In TS zucchini plants, the WMV load remained relatively similar across the three temperature ranges ($F_{2,6}$= 4.62; p = 0.061), whereas in TT zucchini plants, it significantly increased in the H-temperature range ($F_{2,6}$= 19.90; p = 0.002) (Fig. 2 A and B (TS vs. TT); two-way ANOVA analysis). These results indicate different responses to heat stress between the TS and TT cultivars in both cucurbit species, whereas WMV load showed a relative decrease with increasing temperature in TS plants, and TT plants

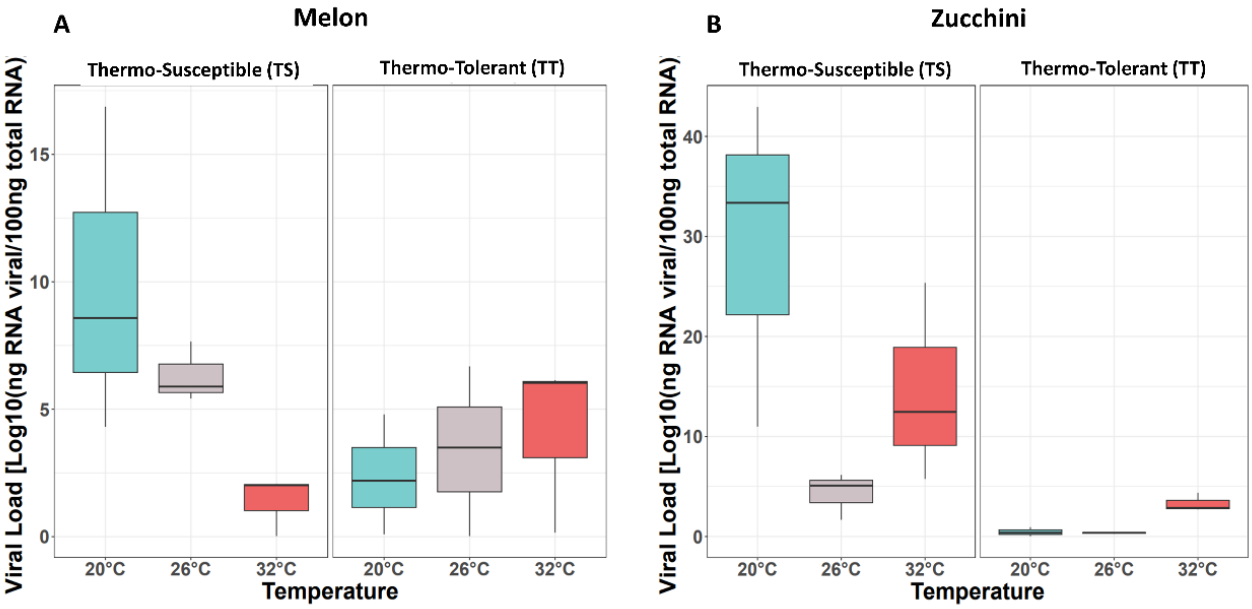


Figure 2. Viral load (mean and SE error bars, n = 3) of WMV infectious clones (MeWMV7) in thermo-susceptible (TS) and -tolerant (TT) melon and zucchini plants at 30 dpi under three different growth temperatures: 20 °C/16 °C (Low, blue color), 26 °C/20 °C (Medium, grey color) or 32 °C/24 °C (High, red color). Viral RNA accumulation was determined using quantitative real-time q-PCR. RNA transcripts of P1 were serially diluted (10-fold) to generate external standard curves. The RNA concentration in each sample (ng of viral RNA per 100 ng of total RNA) was estimated from the cycle threshold (Ct) values obtained from each independent biological assay, with three biological replicates at each time point.

exhibited certain resilience under temperature changes. These responses suggest that plant susceptibility to temperature affects viral accumulation.

In this sense, it has been reported that RNA silencing-mediated defence can be inhibited at low temperatures (Szittya *et al.*, 2003; Chellappan *et al.*, 2005), which in turn can increase virus accumulation. However, temperature can also affect phytohormone-mediated defense pathways, influencing RNA silencing and virus-encoded silencing suppressors (Lewsey *et al.*, 2010). Other studies have shown that high temperatures may increase the susceptibility of tomato plants to tomato yellow leaf curl virus (TYLCV), Arabidopsis to turnip mosaic virus (TuMV), or potato to potato virus Y (PVY) (Prasch and Sonnewald, 2013; Ghandi *et al.*, 2016; Fesenko *et al.*, 2021). In the latter case, and in contrast to our results, these studies found that rising temperatures lead to greater accumulation of PVY in thermo-sensitive than in thermo-tolerant plants, possibly due to the reduction of pathogenesis-related proteins (*salicylic acid (SA)-mediated plant defense*) (Makarova *et al.*, 2018; Spechenkova *et al.*, 2021). It is likely that an increase in temperature can reduce plant resistance, as in the case of tobacco mosaic virus (TMV) and potato potexvirus X (PVX), where an increase in temperature leads to a reduction in the induced hypersensitivity response (Wang *et al.*, 2009). However, it has also been reported that temperatures above 28 °C can induce a suppression of virus-induced HR-type necrosis caused by TMV (Király *et al.*, 2008). In addition, increased temperature and faster symptom development could also be correlated with virus accumulation, as observed for peanut stunt virus (PSV) in *N. benthamiana* (Obrępalska-Stęplowska *et al.*, 2015), PVY in potato (Makarova *et al.*, 2018), and capsicum chlorosis virus in pepper (Tsai *et al.*, 2022*a*). Taken together, our results suggest that temperature can influence WMV accumulation in melon and zucchini plants based on their thermotolerance levels. This outcome could be related to differences in the expression of genes and proteins involved in stress responses, which could indirectly affect viral replication, movement, and/or accumulation within plants.

Variations in the number and distribution of DEGs across temperature ranges and WMV infections between melon and zucchini plants. To examine the effect of WMV infection and temperature variations (L, M, and H) on the gene response of melon and zucchini with different temperature tolerances (TT and TS), we carried out a differential

3´mRNA-seq approach of plants under each temperature condition in the presence or absence of WMV. The reads obtained from each replicate sample were normalized and analyzed using principal component analysis (PCA). Overall, PCA clustered mock samples separately from virus-infected samples, as well as by temperature conditions within the TS and TT melon and zucchini samples (Fig. S2: A-B). After bioinformatic processing of the total differentially expressed genes (DEGs), and the subsequent analysis using the criteria $p < 0.05$ and $\log_2$ FC ≤ -1 or ≥ 1, we found a total of 14,955 DEGs at low (337 overexpressed and 119 underexpressed), 14,882 at medium (115 overexpressed and 220 underexpressed), and 15,351 at high (519 overexpressed and 1,102 underexpressed) temperatures in TT melon, whereas 15,150 differentially expressed genes (DEGs) at low (118 overexpressed and 110 underexpressed), 10,643 at medium (50 overexpressed and 115 underexpressed), and 10,544 at high (133 overexpressed and 280 underexpressed) temperatures in TS melon, out of 37,254 annotated genes in melon. In zucchini plants, we found 21,938 DEGs (109 overexpressed and 299 underexpressed) at low, 15,963 at medium (183 overexpressed and 295 underexpressed), and 16,281 at high temperatures (366 overexpressed and 640 underexpressed) in TS zucchini, whereas 10,825 DEGs (227 overexpressed and 119 underexpressed) were found at low, 17,169 at medium (279 overexpressed and 256 underexpressed), and 16,332 at high temperatures (101 overexpressed and 497 underexpressed) in TT zucchini, out of 27,868 annotated genes (Supp. Tables S1–3 include additional details). Thus, zucchini plants had an approximately 2.6-fold higher percentage of DEGs compared to melon (FRatio= 17.9602 and Prob>F= 0,0028). In both TS and TT plants, the percentage of DEGs ranged from 10,825 (39 %) to 21,938 (79 %) out of 27,868 annotated genes in zucchini, and from 10,544 (28 %) to 15,351 (41 %) out of 37,254 annotated genes in melon (Table 1). Whereas the highest percentage of DEGs was exhibited in TS zucchini plants (79 %) at lower temperatures. It is likely that this high % of DEGs in TS at low temperatures may be related to the greater accumulation of WMV, suggesting specific transcriptional responses of TS to the combination of temperature and virus infection. This appeared to be consistent with previous studies that have shown that low-temperature conditions make plants more susceptible to viruses because of the inhibition of RNA silencing-mediated defence by controlling siRNA generation (Szittya *et al.*, 2003; Tenllado and Canto, 2020). We then sought to identify unique genes under both viral infection and

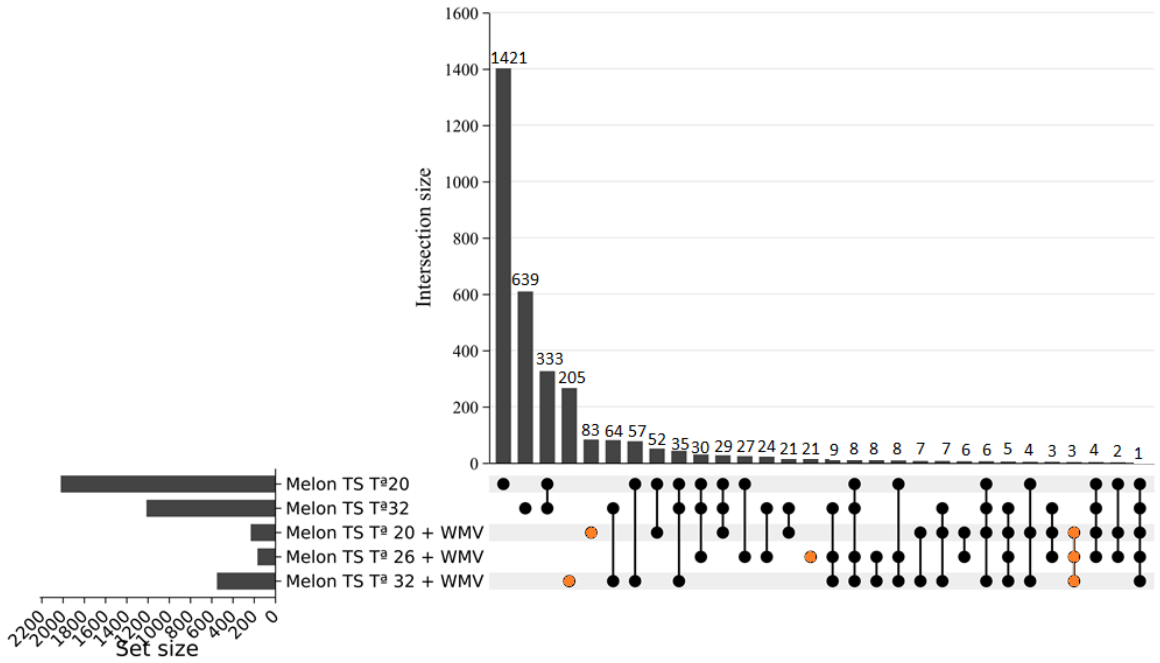
heat stress conditions. In both the TS and TT of melon and zucchini samples, the highest number of unique DEGs was observed under the conditions of WMV infection and high temperature (WMV+H) (Fig. 3: A-D and Table S1).

Table 1. Summary of the 3' mRNA-seq experiment design and results of DEGs for each condition including up- and downregulated genes under the p-value < 0.05 and Log_2FC < -1 o > 1 criteria in melon and zucchini cultivars.

Crop	Comparative	Total DEGs/Total gene	%DEGs	DEGs (*p*-value < 0.05 / Log2FC < -1 o > 1)	
				Upregulated	Downregulated
Cucumis melo	TT Melon MOCK Ta20 vs TT Melon WMV-Ta20	14955/37254	40.14	337	119
	TT Melon MOCK Ta26 vs TT Melon WMV-Ta26	14882/37254	39.94	115	220
	TT Melon MOCK Ta32 vs TT Melon WMV-Ta32	15351/37254	41.20	519	1102
	TS Melon MOCK Ta20 vs TS Melon WMV-Ta20	15150/37254	40.66	118	110
	TS Melon MOCK Ta26 vs TS Melon WMV-Ta26	10643/37253	28.56	50	115
	TS Melon MOCK Ta32 vs TS Melon WMV-Ta32	10544/37253	28.30	133	280
	TT Melon MOCK Ta20 vs TT Melon MOCK Ta26	15819/15819	42.46	763	682
	TT Melon MOCK Ta32 vs TT Melon MOCK Ta26	15299/15299	41.06	758	477
	TS Melon MOCK Ta20 vs TS Melon MOCK Ta26	15478/15478	41.54	1126	897
	TS Melon MOCK Ta32 vs TS Melon MOCK Ta26	15029/15029	40.34	820	390
Cucurbita pepo	TS Zucchini MOCK Ta20 vs TS Zucchini WMV Ta20	21938/27868	78.72	109	299
	TS Zucchini MOCK Ta26 vs TS Zucchini WMV Ta26	15963/27868	58.28	183	295
	TS Zucchini MOCK Ta32 vs TS Zucchini WMV Ta32	16281/27868	58.42	366	640
	TT Zucchini MOCK Ta20 vs TT Zucchini WMV Ta20	10825/27868	38.84	227	119
	TT Zucchini MOCK Ta26 vs TT Zucchini WMV Ta26	17169/27868	61.60	279	256
	TT Zucchini MOCK Ta32 vs TT Zucchini WMV Ta32	16332/27868	58.60	101	497
	TS Zucchini MOCK Ta20 vs TS Zucchini MOCK Ta26	16722/16722	60.00	1772	2741
	TS Zucchini MOCK Ta32 vs TS Zucchini MOCK Ta26	16101/16101	57.77	212	108
	TT Zucchini MOCK Ta20 vs TT Zucchini MOCK Ta26	15864/15864	56.92	2827	3337
	TT Zucchini MOCK Ta32 vs TT Zucchini MOCK Ta26	15061/15061	50.04	652	975

Specifically, under WMV+H conditions, TT and TS melon plants exhibited 711 and 205 genes, respectively, whereas 40 and 21 genes were unique under WMV+M conditions, and 88 and 83 genes were unique under WMV+L conditions, respectively. In TT and TS zucchini plants, 121 and 306 genes were unique to the WMV+H condition, respectively. While, 27 and 126 genes were unique to WMV+M, and 61 and 153 genes to WMV+L, respectively (for more detailed information, see Fig. S3). This indicated that the effect of WMV infection at high temperatures could be associated with a greater number of unique DEGs in TT for melon plants and in TS for zucchini plants, when compared to the M- and L-temperature ranges. Note that only a few (2-4) DEGs were found to be common for the three temperature ranges and viral infection, suggesting that those genes might be attributed to the single WMV infection (Fig. 3: A-D and Table S1). Some plant viral infections have been suggested to mitigate the detrimental effects of abiotic stress (Gorovits *et al.*, 2019; Aguilar and Lozano-Duran, 2022; Mishra *et al.*, 2022). For example, TYLCV infection in tomato plants can suppress the heat shock response, alleviate the response of plant cells to heat stress, prevent cell death, and allow plants to adapt to high temperatures and water deficits (Corrales-Gutierrez *et al.*, 2020; Gorovits *et al.*, 2022). Alternatively, TuMV infection has been shown to reduce stomatal conductance, leading to altered expression levels of ABA homeostatic genes, including biosynthesis and catabolism, eventually improving drought tolerance in Arabidopsis (Manacorda *et al.*, 2021). Therefore, specific signaling pathways may be involved in these biotic and abiotic processes, and further research is needed to identify potential targets and fully understand this complex interplay.

A) MELON THERMO-SUSCEPTIBLE (TS)



B) MELON THERMO-TOLERANT (TT)

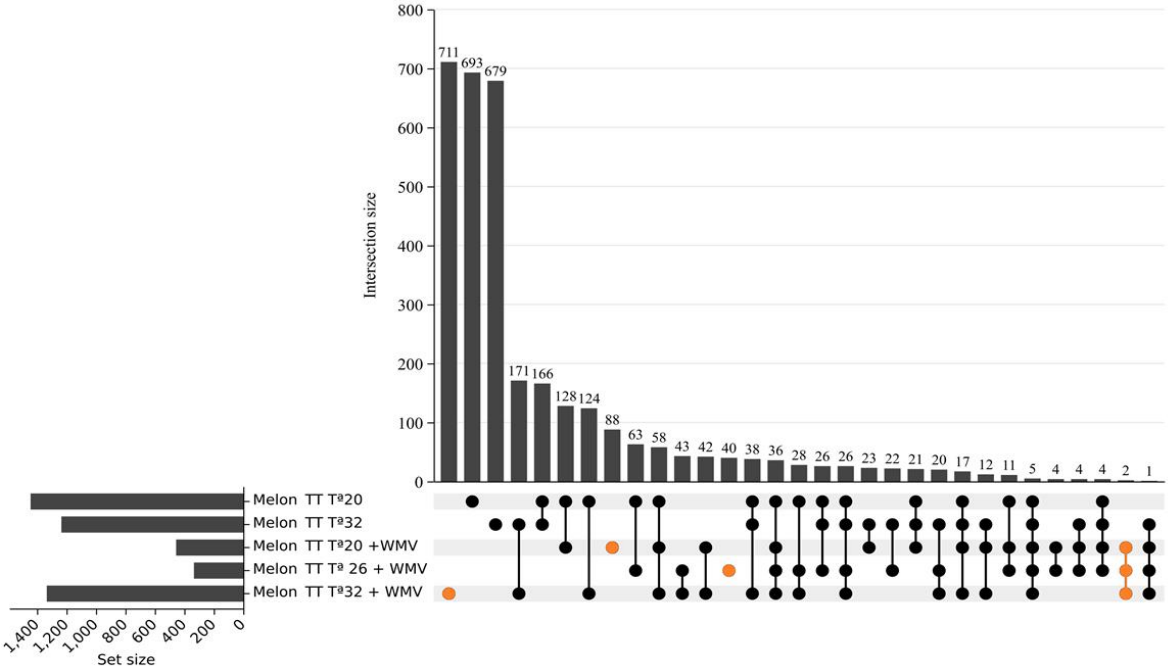
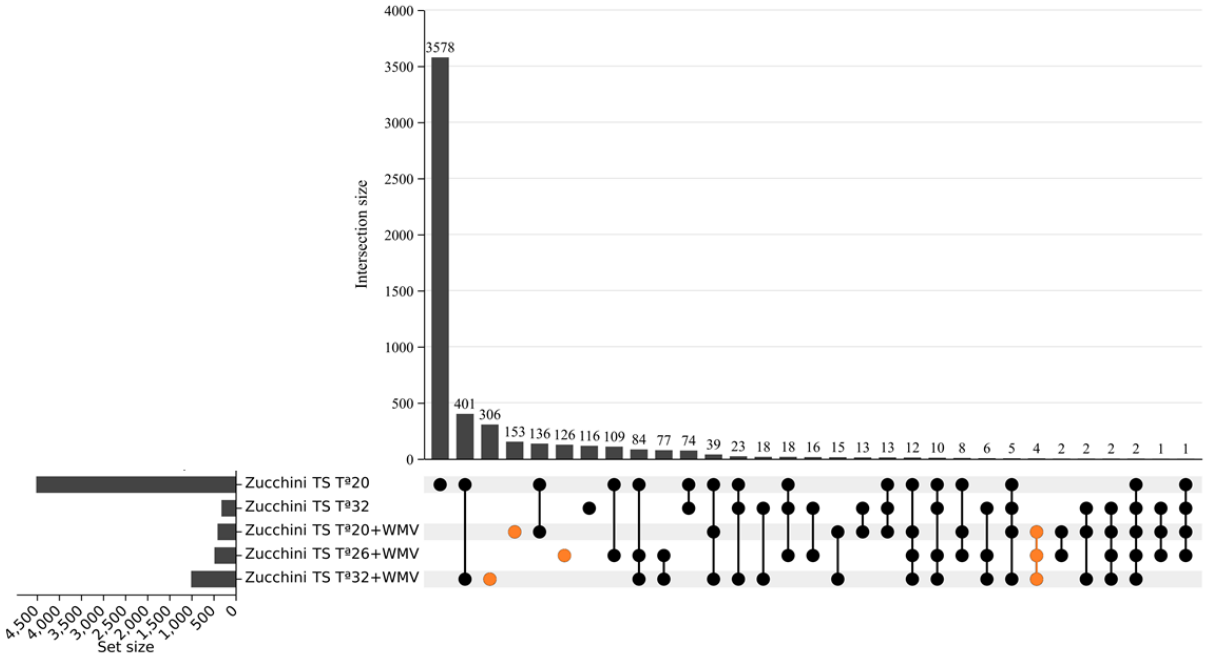
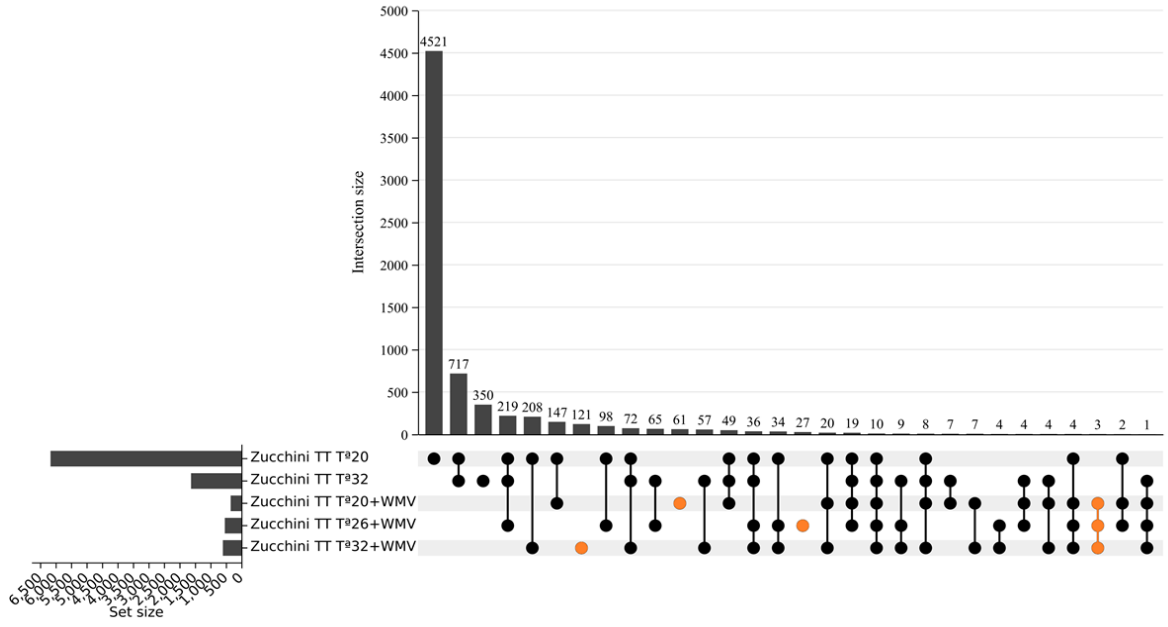


Figure 3. Upset plot displaying intersections between sets of DEGs found under the combined stress effect in thermo-susceptible melon **(A)** and zucchini **(C)** plants, and in thermo-tolerant melon **(B)** and zucchini **(D)** plants. Up- and downregulated genes for each plant species, thermotolerance, and stress condition were placed in the same set for the specific DEGs subtraction under the combination of temperature and WMV infection. The bar plot at the top represents the overlap of DEGs under each condition. The horizontal bar on the left represents the number of significant DEGs under each condition. The black circles and lines at the bottom show the categories for which overlap was calculated, as indicated in the bar chart at the top. The orange circles highlight the DEGs exclusive to each condition in combination with both stresses (WMV+L, WMV+M, and WMV+H), and the overlap of significant DEGs observed in the three conditions. It should be noted that L- and H-temperature (T 20 and 32 ºC) conditions were only considered as a reference to rule out the specific number of DEGs in the combination of L- and H-temperature and WMV infection. While the M-temperature (T 26 ºC) was included in combination with WMV infection to identify the common DEGs as a response to the WMV infection regardless of the three temperature conditions.

C) ZUCCHINI THERMO-SUSCEPTIBLE (TS)



D) ZUCCHINI THERMO-TOLERANT (TT)



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Changes in biological processes and molecular functions in response to WMV infection and temperature. Having established the differential expression patterns, we next investigated how these host responses specifically influence viral infection and heat stress. Since functional genomic studies in cucurbits are ongoing and there are still transcripts that are poorly annotated in the KEGG database, we used Gene ontology (GO) enrichment to assign DEGs with biological processes and molecular functions. GO analysis was performed for both up- and down-regulated sets of unique genes, which were selected according to the criteria *of* $p < 0.05$, and $\log_2 FC \leq -1$ or ≥ 1, including an FDR adjustment of 0.05, as the threshold of significance. Among them (Table S2-5), the top 15 pathways (up- and downregulated) for each temperature condition were ranked by *p*-value (Fig. 4, melon) (Fig. 5, zucchini), with more detailed information in Tables 6 and 7. We found that a considerable % of DEGs were related to biotic and/or abiotic stress in all samples. In particular, in TT samples, 32 %, 23 %, and 48 % of DEGs were found at low, medium, and high temperatures, respectively. Similarly, in the TS samples, 41 %, 50 %, and 41 % were observed at low, medium, and high temperatures, respectively. This suggests that temperature had a greater impact on the DEGs in the TS samples. Note that the remaining enriched GO terms were related to other processes that were not currently associated with biotic and abiotic stress. Among the GO terms related to biotic stress, some annotated common DEGs were *defence response* (GO:0006952), *Hsp70 protein binding* (GO:0030544), *plant-pathogen interaction* (Path:cmo04626), and those related to *jasmonic acid metabolism* (GO:0009694 and GO:0009695) (Table S6-7). These particular genes have been reported to be involved in biotic stress responses (Jones and Dangl, 2006; Mittler *et al.*, 2012; Wasternack and Strnad, 2018). Similarly, among the GO terms related to temperature stress, the annotated DEGs were *responses to radiation or light stimulus* (GO:0009314 and GO:0009416) or *cellular copper ion homeostasis* (GO:0006878), and were reported to be involved in abiotic stress (Wang, 2005; Yruela, 2005; Hideg *et al.*, 2013). Furthermore, GO terms were related to both abiotic and biotic stressors, such as *protein folding, secretion, processing* (GO: 0006457, GO:0009306, and GO:0016485), and *lipid metabolism* (GO:0006631) (Queitsch *et al.*, 2000; Narayanan *et al.*, 2016). Analysis of TT melon plants revealed that *ATPase-coupled intramembrane lipid transporter activity* (GO:0140326 and GO:0140303) and the *glyoxylate cycle* (GO:0006097) were enriched

with upregulated and downregulated genes, respectively, in the low-temperature range (Fig. 4A and 4D). Specifically, in the case of ATPase-coupled intramembrane lipid transporter activity, GO-Term was strongly related to other highly significant factors, such as *lipid transport* (GO:0140303 and GO:0005319) (Fig. S4A). In the medium-temperature range, *the isocitrate metabolic process* (GO:0006102) and *phagosome* (Path:cmo04145) were enriched with upregulated and downregulated genes, respectively (Fig. 4B and 4E), which are associated with and involved in similar biological processes and molecular functions (Fig. S4B). While a large number of GO terms were enriched at high-temperature range, highlighting the *glycine catabolic process* (GO:0006546) and *serine family amino acid catabolic process* (GO:0009071) with upregulated genes (Fig. 4C), which are functionally related (Fig. S4C), as well as *jasmonic acid metabolic process* (GO:0009694) with downregulated genes (Fig. 4F) and related to *jasmonic acid biosynthesis* (GO:0009695) (Fig. S4C). On the other hand, in TS melon, the *carotene metabolic process* (GO:0016120) and *Golgi to plasma membrane transport* (GO:0006893) were found to be up- and downregulated genes, respectively, in the low-temperature range (Fig. 4G and 4J). *The carotene metabolic process* has been highly related to other GO terms, such as *carotene biosynthetic process,* and some of which are related to *cellular alcohol* (GO:0044107) and *ergosterol process* (GO:0008204 and GO:0006696) (Fig. S4D). In the medium-temperature range, *protein import into peroxisome docking* (GO:0016560) and *phenylalanine metabolism* (Path:cmo00360) were enriched for upregulated and downregulated genes, respectively (Fig. 4H and 4K) that are involved in similar biological processes and molecular functions (Fig. S4E). In the high-temperature range, *isoamylase activity* (GO:0019156), which has been associated with *glycogen debranching enzyme activity* (GO:0004133) (Fig. S4F), and *plant-pathogen interactions* (Path:cmo04626) were found for up- and downregulated genes, respectively (Fig. 4I and 4L).

Figure 4. The Gene Ontology (GO) enrichment analysis for the top 15 GO terms of specific DEGs (using the criteria $p < 0.05$ and $Log_2FC \leq -1$ or ≥ 1) in melon plant varieties under the combination of Temperature+WMV infection based on Biological Process and Molecular Function. GO enrichment profiles are shown for thermotolerant upregulated genes at low **(A)**, medium **(B)**, and high **(C)** temperatures, and downregulated genes at low **(D)**, medium **(E)**, and high **(F)** temperatures. GO enrichment profiles are shown for thermo-susceptible upregulated genes at low **(G)**, medium **(H)**, and high **(I)** temperatures, and downregulated genes at low **(J)**, medium **(K)**, and high **(L)** temperatures. All data are listed in the supplementary Table S6.

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Analysis of TT zucchini plants showed that *calmodulin-lysine N-methyltransferase activity* (GO:0018025) and *fluoride transmembrane transporter activity* (GO:1903425) were enriched in the upregulated genes (Fig. 5A), *farnesol dehydrogenase activity* (GO:0047886), *nicotinate N-methyltransferase activity* (GO:0008938), and *biotin carboxylase activity* (GO:0004075) were enriched among the downregulated genes in the low-temperature range (Fig. 5D), none of which was associated with any of the top 15 pathways (Fig. S5A). In the medium-temperature range, *the COPI coating of Golgi vesicles* (GO:0048205) a*nd iron assimilation by chelation and transport* (GO:0033214) were enriched in the upregulated and downregulated genes, respectively (Fig. 5B and E), which are involved in similar biological processes and molecular functions (Fig. S5B). In the high-temperature range, *nucleoside transmembrane transport* (GO:1901642), which is associated with *nucleoside transmembrane transporter activity* (GO:0005337) (Fig. S5C), and *triglyceride metabolic process* (GO:0006641), which has been associated with *the glycerol-phosphate biosynthesis process* (GO:0046167) (Fig. S5C) were enriched for upregulated and downregulated genes, respectively (Fig. 5C and F). In TS zucchini plants, the analysis showed *positive regulation of the cellular response to phosphate starvation* (GO:0080040) and *trehalose metabolic process* (GO:0005991), which have been associated with *alpha and alpha-trehalase activity* (GO:0004555) (Fig. S5D) were enriched with upregulated and downregulated genes, respectively, in the low-temperature range (Fig. 5G and J). In the medium-temperature range, *mitochondrial respiratory chain complex III assembly* (GO:0034551) and response to *light intensity* (GO:0009642), *copper ion* (GO:0046688), and *mitochondrial ribosomal subunit assembly* (GO:1902775) were enriched with upregulated and downregulated genes, respectively (Fig. 5H and K) that are involved in similar biological processes and molecular functions (Fig. S5E). While at high-temperature range, the *fructose and glucose transport* (GO:0015755, GO:0005353, and GO:1904659), which have been strongly associated with similar biological processes and molecular functions (Fig. S5F), and *protein N-linked glycosylation via asparagine* (GO:0018279) were also found to be enriched for upregulated and downregulated genes, respectively (Fig. 5I and L).

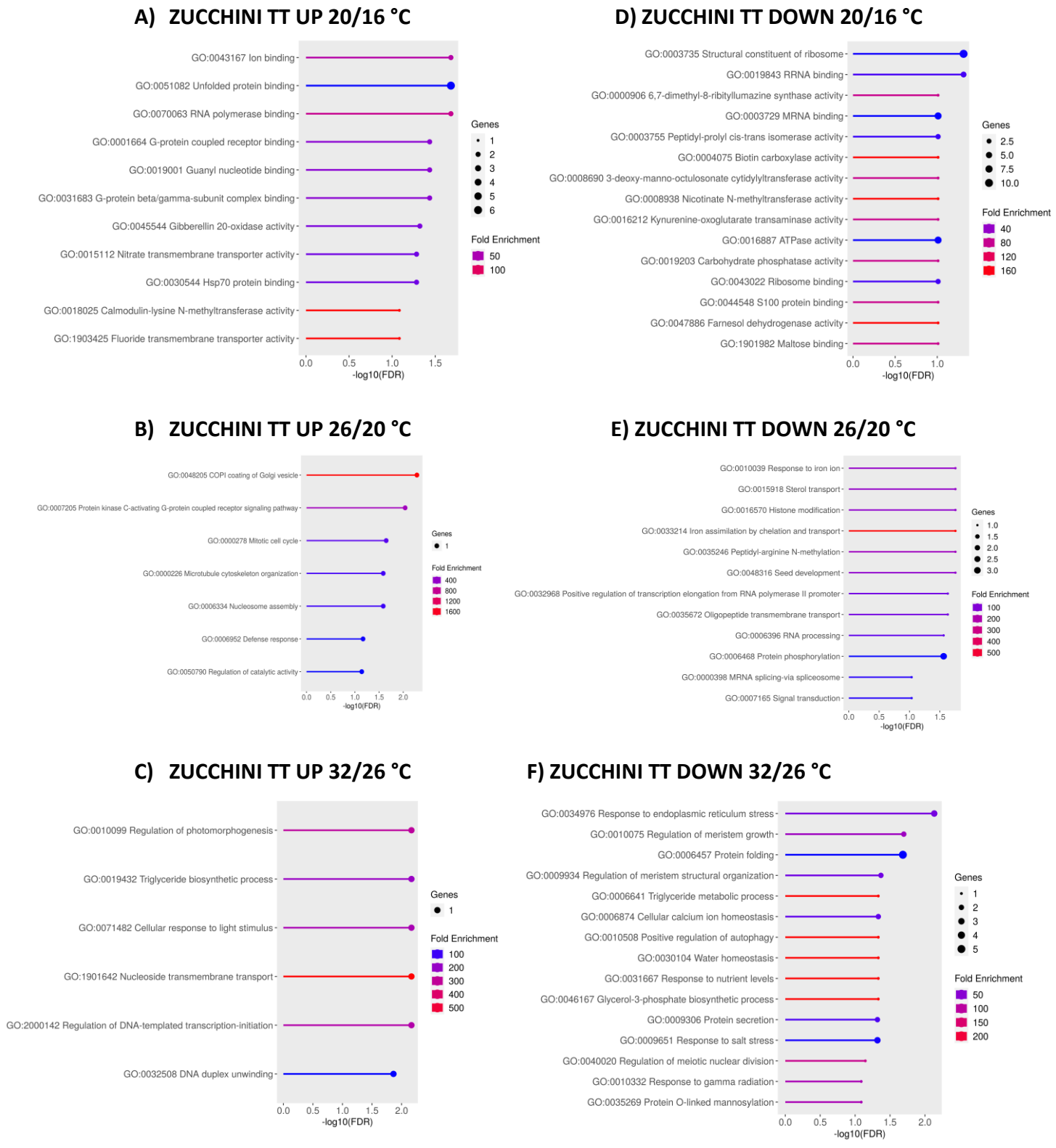

Figure 5. The Gene Ontology (GO) enrichment analysis for the top 15 GO terms of specific DEGs (using the criteria $p < 0.05$ and $Log_2FC \leq -1$ or ≥ 1) in zucchini plants varieties under the combination of Temperature+WMV infection based on Biological Process and Molecular Function. GO enrichment profiles are shown for thermotolerant upregulated genes at low **(A)**, medium **(B)**, and high **(C)** temperatures, and downregulated genes at low **(D)**, medium **(E)**, and high **(F)** temperatures. GO enrichment profiles are shown for thermo-susceptible upregulated genes at low **(G)**, medium **(H)**, and high **(I)** temperatures, and downregulated genes at low **(J)**, medium **(K)**, and high **(L)** temperatures. All data are listed in the supplementary Table S7.

Figure 5. The Gene Ontology (GO) enrichment analysis for the top 15 GO terms of specific DEGs (using the criteria $p < 0.05$ and $Log_2FC \leq -1$ or ≥ 1) in zucchini plants varieties under the combination of Temperature+WMV infection based on Biological Process and Molecular Function. GO enrichment profiles are shown for thermotolerant upregulated genes at low **(A)**, medium **(B)**, and high **(C)** temperatures, and downregulated genes at low **(D)**, medium **(E)**, and high **(F)** temperatures. GO enrichment profiles are shown for thermo-susceptible upregulated genes at low **(G)**, medium **(H)**, and high **(I)** temperatures, and downregulated genes at low **(J)**, medium **(K)**, and high **(L)** temperatures. All data are listed in the supplementary Table S7.

The identification of these genes suggests that certain biological processes may be linked to both abiotic and biotic stressors. Although it should be noted that our results could be influenced by the specific temperature range and pathogen used in our experimental design, it is evident that a combination of these stress factors can play an important role in plant physiology. It is also worth mentioning that despite the cucurbit genomics database (CuGenDB) being a pivotal and valuable resource for advancing comparative and functional genomic studies (Zheng *et al.*, 2018), functional genomic studies in non-model crops are usually challenging, and further research is needed to elucidate the specific genes underlying abiotic and biotic stresses.

Identification of unique orthologous genes in thermosusceptible and thermotolerant melon and zucchini plants infected with WMV. To uncover genes uniquely responsive to combined temperature and virus stress, we first filtered orthologous among the top 15 GO terms for temperature and WMV infection (details listed in Tables S6–S7). Under the H-temperature range, two orthologous genes were found in thermotolerant plants, with significant transcriptomic changes: MELO3C023308.2 in melon (log_2(FoldChange)=1.709)) and Cp4.1LG05g12560 in zucchini (log_2(FoldChange)=1.036). To validate these transcriptomic results, we performed RT-qPCR for both orthologous genes using the gene □-Tubulin as the endogenous control and compared relative expression across the temperatures and viral condition treatments (Fig. 6A). Statistical analysis confirmed a significant temperature by WMV infection interaction (interaction between temperature and TT plant species: $F_{2,2}$ = 5.326, p = 0.022). In particular, both orthologous genes were downregulated at L-temperature under WMV infection, while there was a significant up-regulation of MELO3C023308.2 at H-temperature ($t_{(2)}$ = 4.70 p = 0.041), and also of Cp4.1LG05g12560 at M-temperature ($t_{(2)}$ = 25.43, p = 0.0015), with no significant changes at H-temperature compared to its control (p = 0.49). This confirmed the transcriptomic changes and suggested that these orthologous genes exhibit a temperature-dependent response under WMV infection. These genes encode F-Box proteins, known to play pivotal roles in regulating the expression of genes involved in plant defense responses by recognizing and reacting to pathogen-associated molecular patterns (PAMPs) and effector-triggered immunity (ETI). They are involved in the regulation of protein stability during immune responses, ensuring efficient defense

against pathogens, as well as in the degradation of misfolded or damaged proteins accumulated during temperature stress, thus maintaining cellular homeostasis and regulatory proteins that modulate heat stress and hypersensitive resistance responses (Xiao and Jang, 2000). This melon gene (MELO3C023308.2) is annotated with the following GO terms: GO:0009658, GO:0048512, GO:0045893, GO:0080167, GO:0010099 and GO:0009639, which are related to chloroplast organization, circadian behavior, positive regulation of transcription, response to karrikin, regulation of photomorphogenesis under the biological process category and response to red or far red light, respectively. In the GO terms network, it was directly related to the regulation of *DNA-templated transcription initiation* (GO:2000142), *cellular response to radiation* (GO:0071478), and *light stimulus* (GO:0071482) (Fig. S4C). In zucchini, the gene mentioned (Cp4.1LG05g12560) is annotated under the GO:0010099 term, which is related to the regulation of photomorphogenesis under the biological process category. This is consistent with previous studies on *Arabidopsis thaliana,* which showed that thermomorphogenetic effects occur in response to high temperatures (Nomoto *et al.*, 2012; Yamashino *et al.*, 2013). These thermomorphogenetic responses are typical of thermo-tolerant varieties, making them attractive for crop breeders aiming to develop plants that can withstand climate change (Quint *et al.,* 2016).

Additionally, under the L-temperature range, another two orthologous genes were found in thermosuceptible plants: MELO3C024920.2 in melon (log_2(FoldChange)=-1.010) and Cp4.1LG06g08450 in zucchini (log_2(FoldChange)=1.258). Similarly, gene expression was also validated by RT-qPCR and under WMV infection. Both genes responded differentially ($F_{2,2}$ = 2.152, p = 0.158), with only MELO3C024920.2 affected by the temperature conditions ($F_{1,1}$ = 10.313, p = 0.075). In particular, MELO3C024920.2 gene was downregulated at L-temperature ($t_{(2)}$ = -6.82, p = 0.020), while there was a significant up-regulation at H-temperature ($t_{(2)}$ =9.84, p = 0.01). However, Cp4.1LG06g08450 remained significantly downregulated at M-, and H-temperature (p < 0.01) (Fig. 6B). This suggested that MELO3C024920.2 had a response dependent on temperature under WMV infection, while Cp4.1LG06g08450 exhibited a significant downregulation regardless the temperature, and possibly attributed to the WMV infection. These genes encode Zinc transporters, which have been shown to be involved in the regulation of various signaling pathways that are crucial for plant growth and

influencing the ability to tolerate and adapt to adverse environmental conditions, such development, making it a key player in plant stress responses. Zinc transporters regulate zinc uptake, transport, and distribution, as drought, salinity, and heavy metal stress (Ullah *et al.*, 2019), and the plant's immune response against pathogens by regulating zinc accumulation (Cabot *et al.*, 2019). MELO3C024920.2 gene is annotated with the following GO terms: GO:0030001, GO:0055085, GO:0055114, GO:0071577, GO:0006810, and GO:0006812, which are related to metal ion transport, transmembrane transport, oxidation-reduction process, and zinc II ion transmembrane transport under the biological process category, transport, and cation transport, respectively. The GO term network was directly related to *cellular copper ion homeostasis* (GO:0006878) and *transport* (GO:0006825) (Fig. S4D). In zucchini, the gene mentioned (Cp4.1LG06g08450) is annotated under the GO:0030001, GO:0055085, GO:0055114, and GO:0071577, which are related to metal ion transport, transmembrane transport, and oxidation-reduction processes under the biological process category.

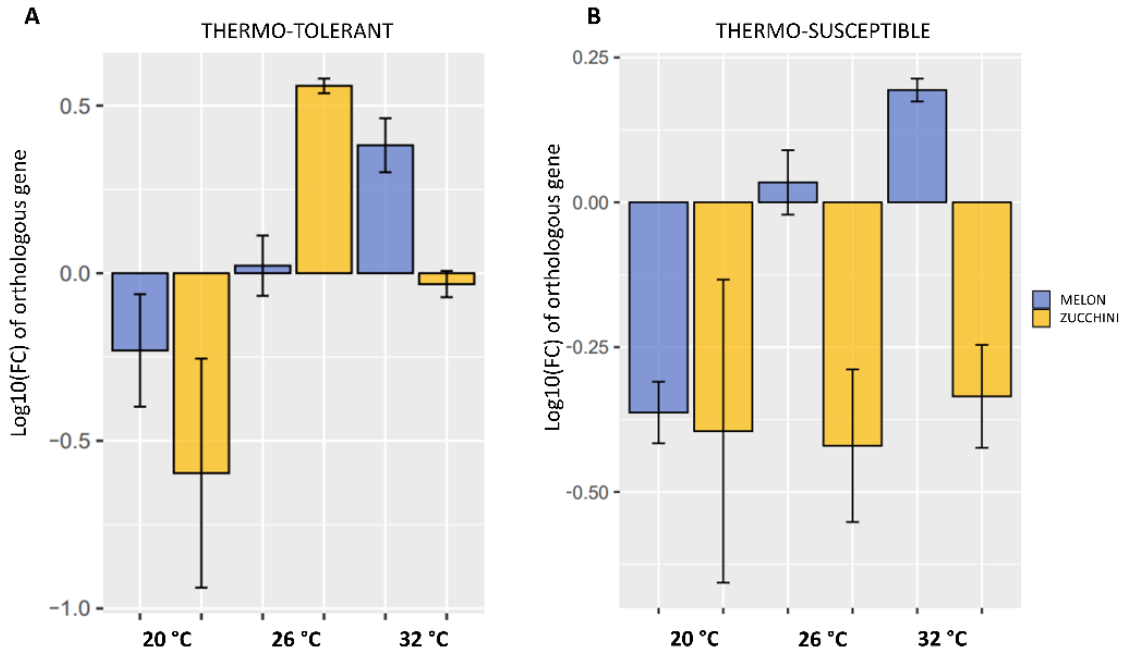

Figure 6. Relative expression of the unique melon and zucchini orthologous genes. The gene expression is represented as log10(Fold Change), which was calculated using the 2−ΔΔCt method for each temperature (L-M-H) in the WMV infection condition, and referenced to their corresponding mock conditions. A log10(FC) value of 0 indicated no change in gene expression, values greater than 0 indicated an increase in expression, and values less than 0 indicated a decrease in expression. (**A**) Orthologous genes that were identified in TT melon (MELO3C023308.2, blue bars) and zucchini (Cp4.1LG05g12560, yellow bars) under high-temperature conditions. (**B**) Orthologous genes that were identified in TS melon (MELO3C024920.2, blue bars) and zucchini (Cp4.1LG06g08450, yellow bars) under low-temperature. Bars represent the mean ± SD of three independent biological experiments, and statistical significance was assessed using the Shapiro-Wilk normality test and Student's t-test ($p<0.05$).

Our results suggest that these orthologous genes may be crucial for maintaining the balance between stress and growth in plants, and further research should be conducted to explore its specific functions in different plant species under combined stresses. Curiously, in the medium-temperature range, no orthologous genes were found in TT or TS between the plant species, suggesting that these genetic traits might be limited to specific temperature thresholds. While recognizing the importance of validating these potential orthologous gene candidates across all plant conditions, it is worth noting that this effort is ongoing and requires further research. Additionally, collaboration with breeding company holders of these melon and zucchini cultivars is essential, as this work could significantly contribute to developing cucurbit varieties with enhanced tolerance to WMV infection and temperature stress. This suggests that the plant response to viral infection is closely linked to temperature stress. Additionally, the identification of orthologous genes that differentiate thermosusceptible varieties from thermotolerant varieties may provide potential molecular targets for breeding programs. These findings underscore the importance of understanding the molecular interplay between biotic and abiotic stressors in the development of resilient crop varieties to support sustainable agriculture and food security.

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AUTHOR CONTRIBUTIONS: CdM-R and PG planned and designed the study. CdM-R performed experiments, CdM-R and PG analysed data and wrote the manuscript. Conflict of interest: The authors declare that they have no conflicts of interest.

SUPPLEMENTARY MATERIAL

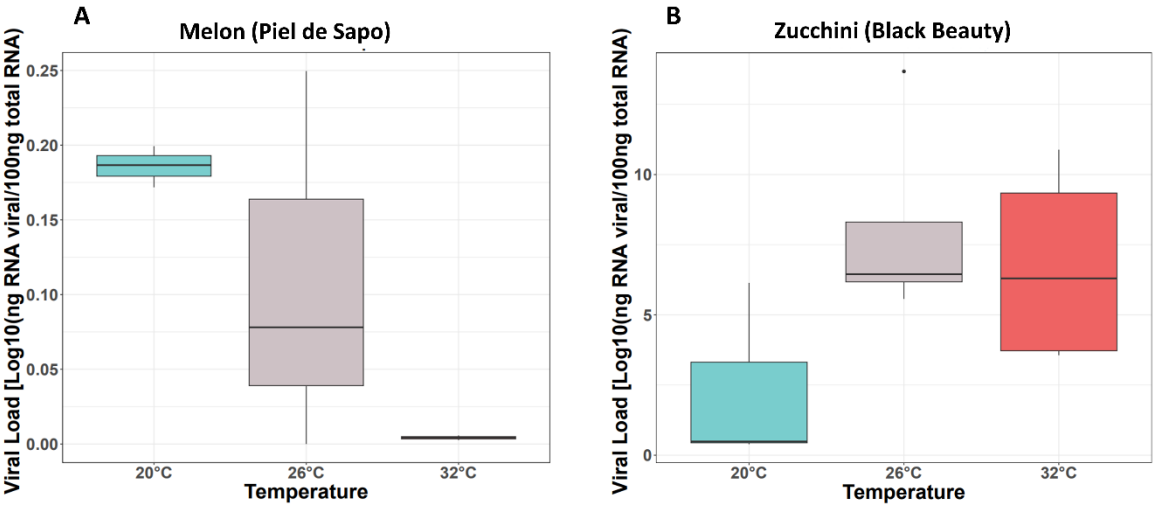


Figure S1. Viral load (mean and SE error bars, n = 3) of WMV infectious clones (MeWMV7) in commercial melon (Piel de sapo) **(A)** and zucchini (Black beauty) **(B)** plants at 30 dpi under three different growth temperatures: 20 °C/16 °C (Low, blue color), 26 °C/20 °C (Medium, grey color) or 32 °C/24 °C (High, red color). Viral RNA accumulation was determined using absolute quantitative RT-PCR. RNA transcripts of P1 were serially diluted (10-fold) to generate external standard curves. The RNA concentration in each sample (ng of viral RNA per 100 ng of total RNA) was estimated from the cycle threshold (Ct) values obtained from each independent biological assay, with three biological replicates at each time point.

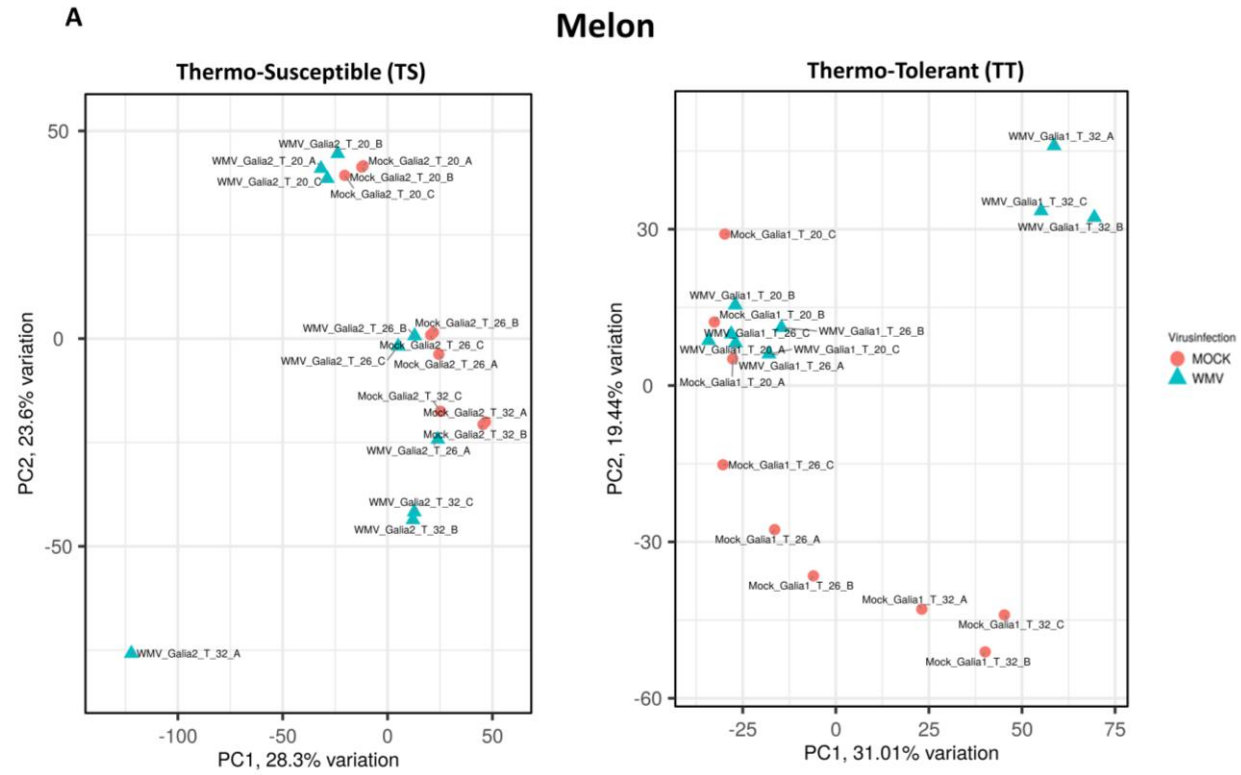
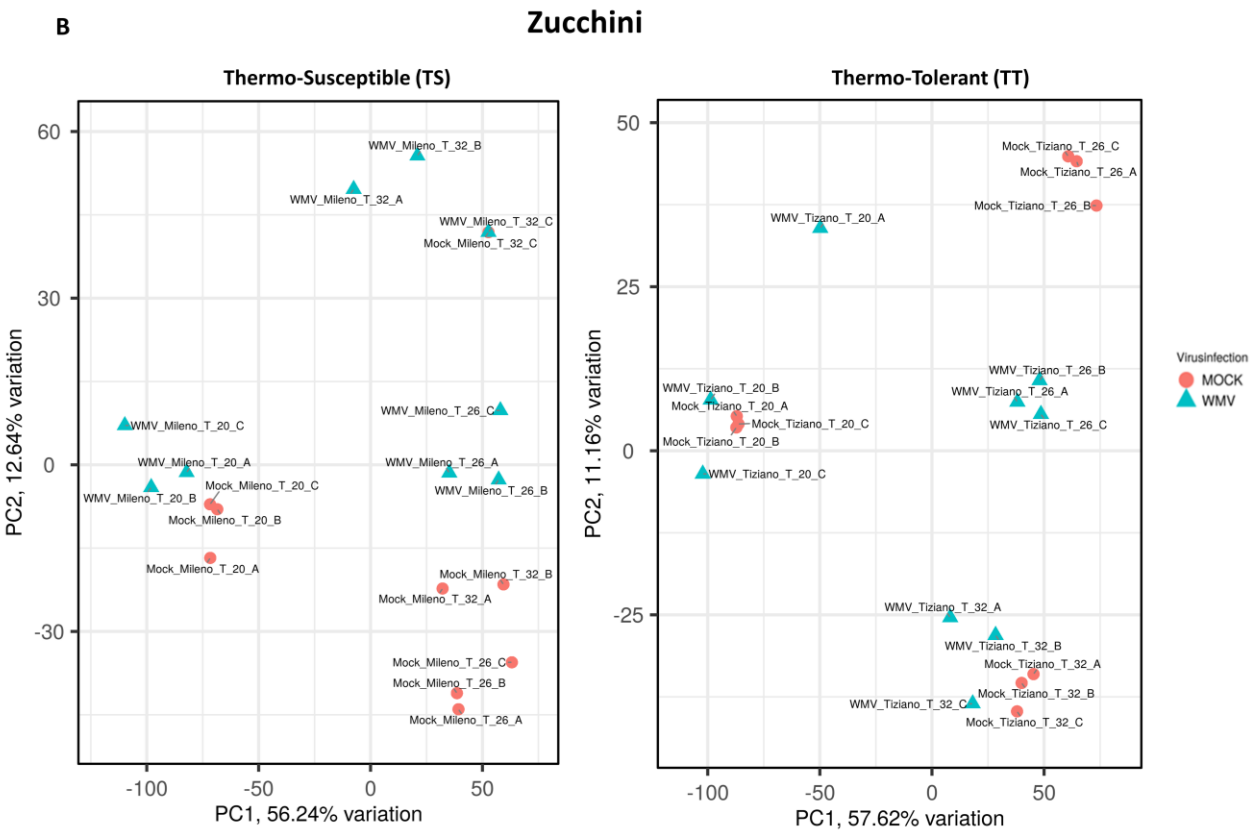


Figure S2. Principal component analysis (PCA) of thermosusceptible (TS) and thermotolerant (TT) melon **(A)** and zucchini **(B)** plants. Mock and infected samples were labeled with the corresponding temperature conditions (20, 26, and 32 °C), including replicates (A, B, and C), and represented by a red circle and a blue triangle, respectively. PCA was performed based on the read count data using iDEP 2.01.



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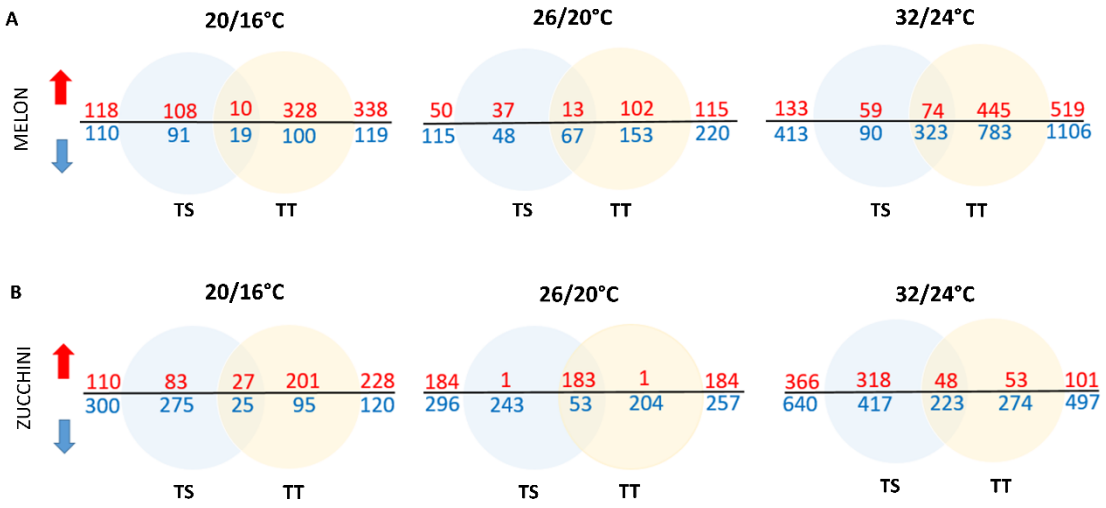
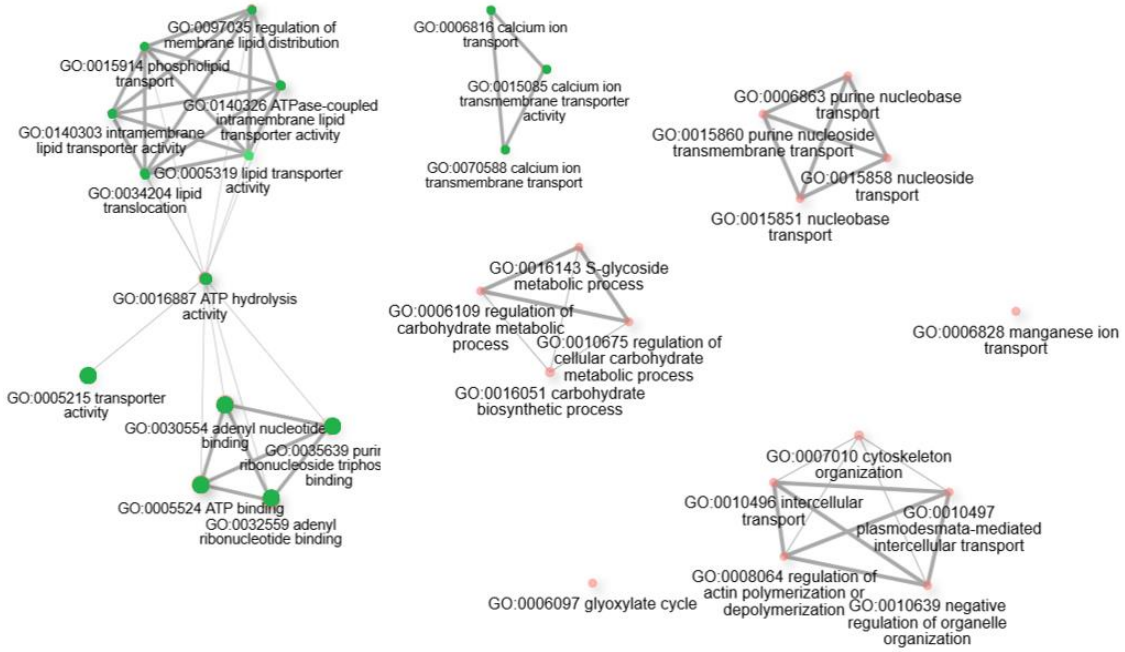


Figure S3. Venn diagrams of the overlap of upregulated and downregulated genes between thermotolerant (TT) and thermosusceptible (TS) **(A)** melon plants and between thermotolerant (TT) and thermosusceptible (TS) **(B)** zucchini plants under each stress treatment. Venn diagrams were constructed based on the genes listed in Tables S2 and S3 in R studio.

A) MELON THERMO-TOLERANT (TT) 20/16°C



D) MELON THERMO-SUSCEPTIBLE (TS) 20/16 °C

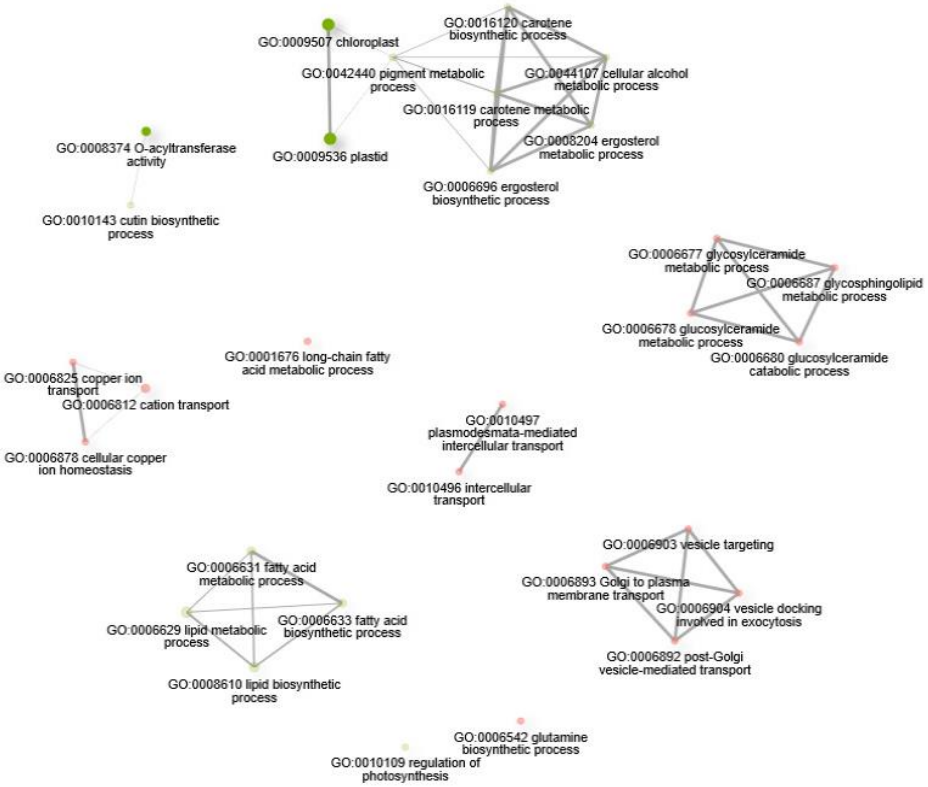
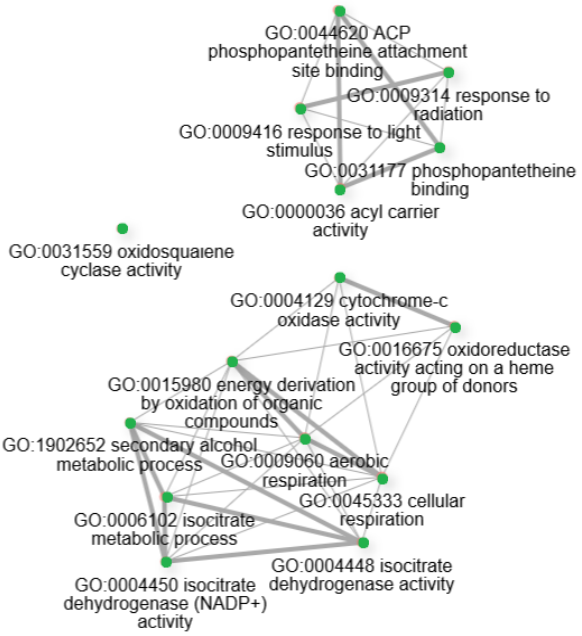


Figure S4. Pathway analysis and GO term networks based on molecular function and biological processes of specific DEGs under the combination of Temperature+WMV infection in melon plant varieties (FDR cutoff: 0.3). The nodes represent the enriched terms, and the size of the circles is proportional to the number of genes. The connections (or edges) between nodes indicated that they shared 30 % or more genes with similar molecular functions and biological processes. Thicker edges represent more overlapping genes, and green and red colors represent upregulated and downregulated genes, respectively. Networks are shown for thermotolerance (TT) at low **(A)**, medium **(B)**, and high **(C)** temperatures, and thermosusceptibility (TS) at low **(D)**, medium **(E)**, and high **(F)** temperatures. The raw data used in this figure are presented in Table S6.

B) MELON THERMO-TOLERANT (TT) 26/20 °C



E) MELON THERMO-SUSCEPTIBLE (TS) 26/20 °C

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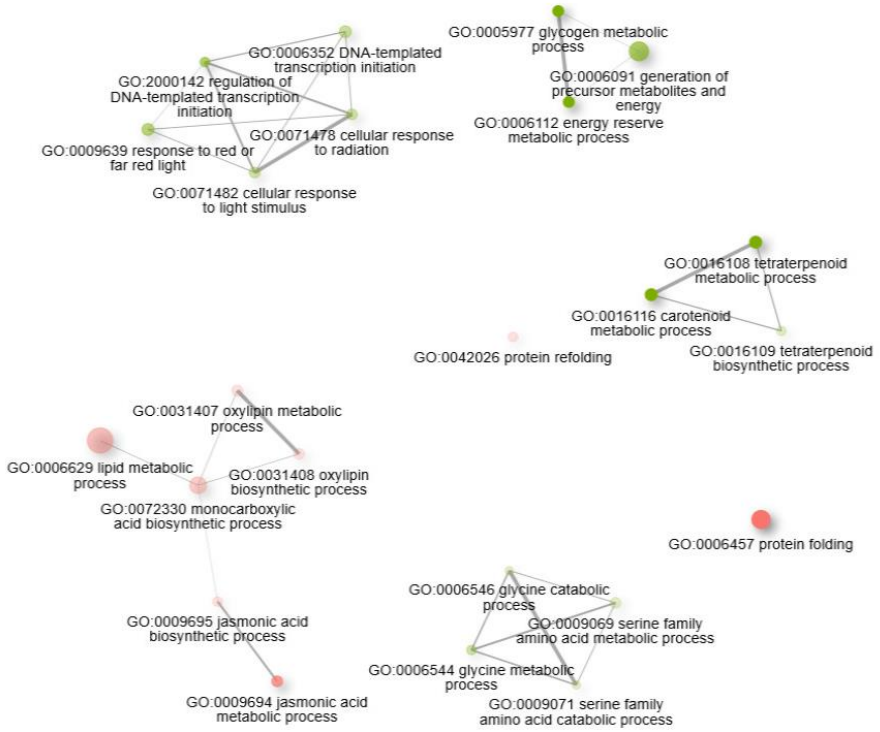
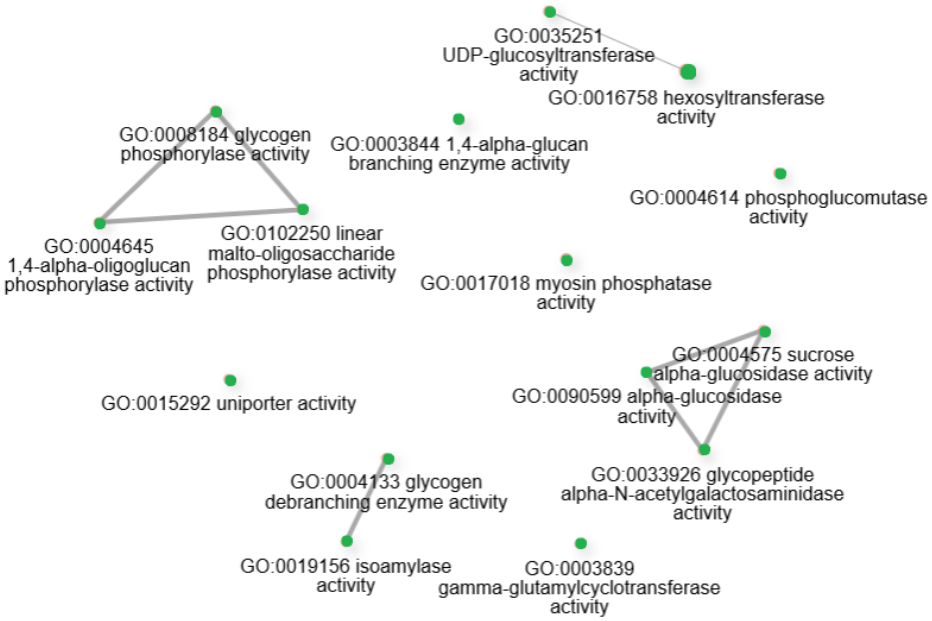
C) MELON THERMO-TOLERANT (TT) 32/24 °C



F) MELON THERMO-SUSCEPTIBLE (TS) 32/24 °C



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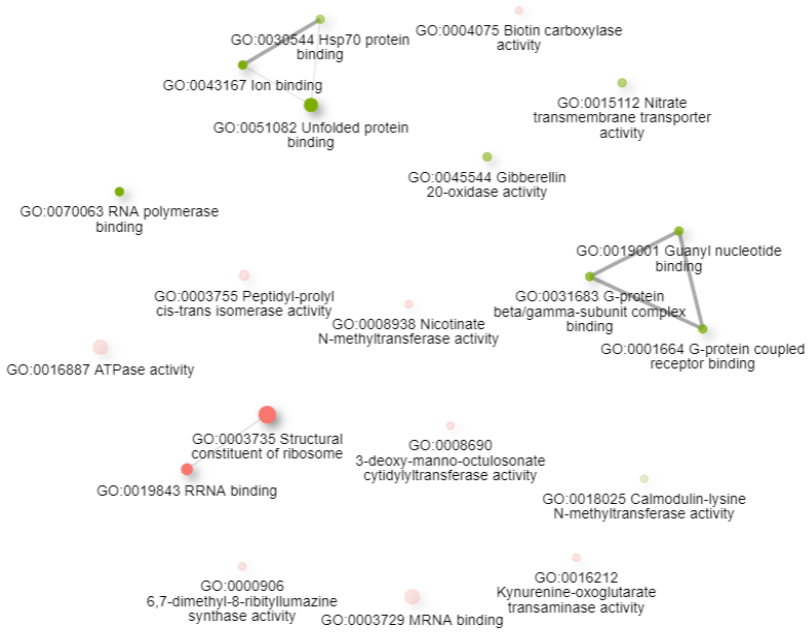
A) ZUCCHINI THERMO-TOLERANT (TT) 20/16 °C



D) ZUCCHINI THERMO-SUSCEPTIBLE (TS) 20/16 °C

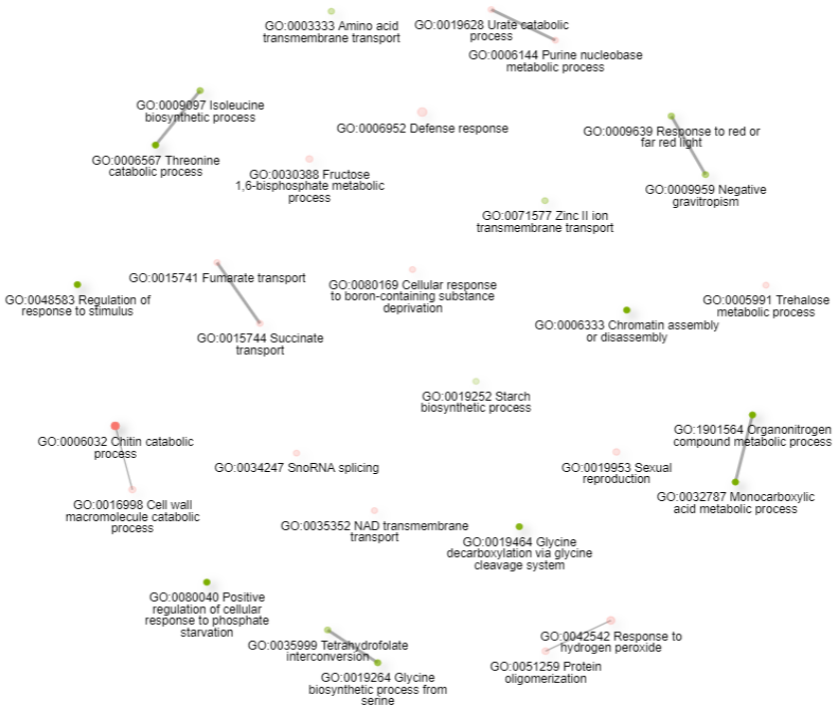
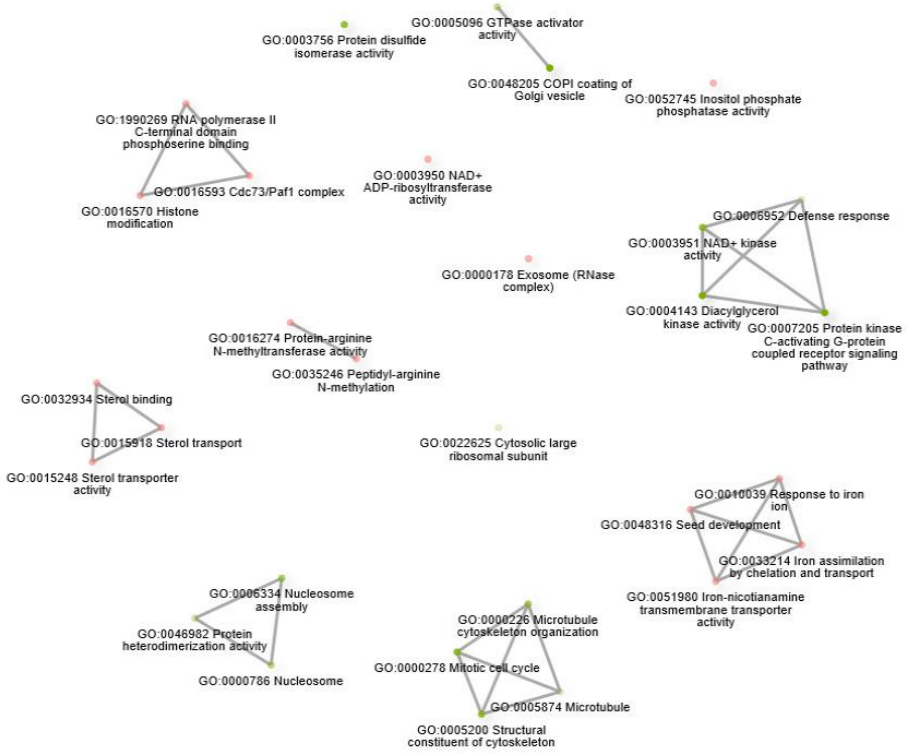
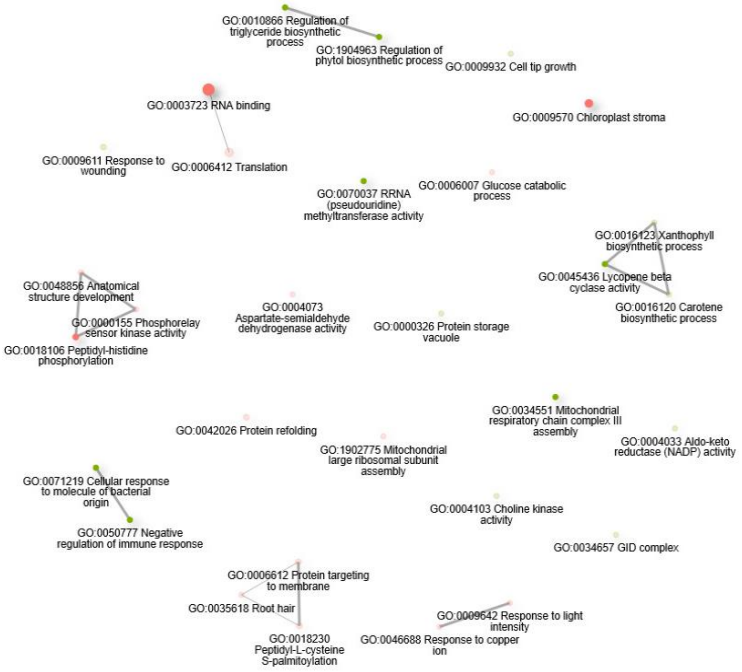


Figure S5. Pathway analysis and GO term networks based on molecular function and biological processes of specific DEGs under the combination of Temperature+WMV infection in zucchini plant varieties (FDR cutoff: 0.3). The nodes represent the enriched terms, and the size of the circles is proportional to the number of genes. The connections (or edges) between nodes indicated that they shared 30 % or more genes with similar molecular functions and biological processes. Thicker edges represent more overlapping genes. Green and red colors represent upregulated and downregulated genes, respectively. Networks are shown for thermotolerance (TT) at low **(A)**, medium **(B)**, and high **(C)** temperatures, and thermosusceptibility (TS) at low **(D)**, medium **(E)**, and high **(F)** temperatures. The raw data used in this figure are presented in Table S7.

B) ZUCCHINI THERMO-TOLERANT (TT) 26/20 °C



E) ZUCCHINI THERMO-SUSCEPTIBLE (TS) 26/20 °C



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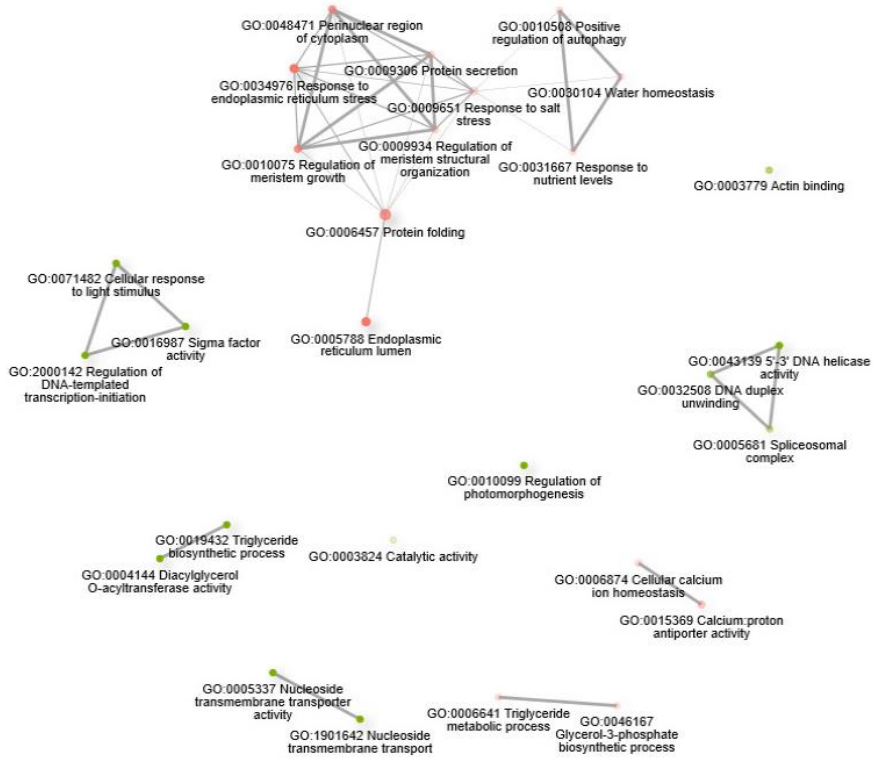
C) ZUCCHINI THERMO-TOLERANT (TT) 32/24 °C



D) ZUCCHINI THERMO-SUSCEPTIBLE (TS) 32/24 °C

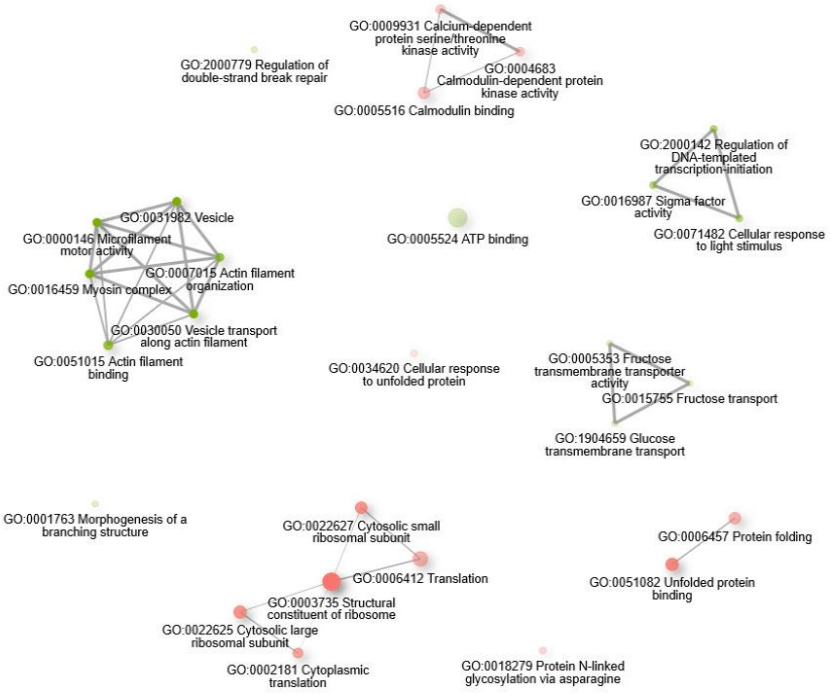


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Table S1. Common DEGs observed under the three temperatura conditions highlighted in Fig.3 for melón and zucchini varieties were classified by Molecular Function, Biological Process and Cellular Component using the CuGenDB and UniProtKB databases.

Crop	Variety	Gene ID	Molecular Function	Cellular component	Biological process
Cucurbita pepo	Milenio TS	Cp4.1LG02g09610	protein binding	intracellular	Unclassified
		Cp4.1LG01g21940	Unclassified	Unclassified	Unclassified
		Cp4.1LG04g02610	catalytic activity	Unclassified	fruit ripening
		Cp4.1LG19g01190	nucleotide binding	thylakoid	cellular process
	Tiziano TT	Cp4.1LG08g12670	transcription factor activity, sequence-specific DNA binding	intracellular	nucleobase-containing compound metabolic process
		Cp4.1LG01g07170	transcription factor activity, sequence-specific DNA binding	intracellular	nucleobase-containing compound metabolic process
		Cp4.1LG17g07560	Unclassified	Unclassified	Unclassified
Cucumis melo	Galia 2 TS	MELO3C200891.2	Unclassified	Unclassified	Unclassified
		MELO3C027107.2	Unclassified	membrane	photosynthesis
		MELO3C301005.2	Unclassified	Unclassified	Unclassified
	Galia 1 TT	MELO3C200891.2	Unclassified	Unclassified	Unclassified
		MELO3C003185.2	endopeptidase inhibitor activity, proteasome binding	proteasome complex	proteasome-mediated ubiquitin-dependent protein catabolic process

Table S2. Differentially expressed genes (DEGs) from melon plant varieties under the combination of virus infection and temperature (p-value < 0.05, Log_2FC < -1 o > 1 criteria). Samples were normalized to the mock control condition. (This is only part of the content due to its length. To access the full material, please contact the author).

MELON THERMO-TOLERANT							
UPREGULATED GENES				**DOWNREGULATED GENES**			
gene	**baseMean**	**log2FoldChange**	**pvalue**	**gene**	**baseMean**	**log2FoldChange**	**pvalue**
MELO3C200891.2	389,423	2,03	1,0308E-20	MELO3C010588.2	180,196	-3,156	6,894E-38
MELO3C027747.2	271,838	1,58	3,3967E-15	MELO3C020588.2	129,474	-3,444	5,5812E-28
MELO3C032677.2	510,652	1,458	7,3621E-15	MELO3C029341.2	135,797	-4,123	1,4208E-26
MELO3C028033.2	864,408	1,186	1,3237E-12	MELO3C011187.2	209,857	-2,365	4,2393E-24
MELO3C006960.2	48,511	2,671	4,7315E-12	MELO3C015306.2	1572,003	-1,92	2,6478E-23
MELO3C010875.2	616,623	1,245	4,7883E-12	MELO3C032464.2	318,798	-2,707	1,4173E-21
MELO3C027107.2	100,272	1,954	1,6627E-11	MELO3C024183.2	387,137	-1,588	1,5139E-21

Table S3. Differentially expressed genes (DEGs) from zucchini plant varieties under the combination of viral infection and temperature (p-value < 0.05, Log_2FC < -1 o > 1 criteria). Samples were normalized to the mock control condition. (This is only part of the content due to its length. To access the full material, please contact the author).

ZUCCHINI THERMO-SUSCEPTIBLE							
UPREGULATED GENES				DOWNREGULATED GENES			
gene	**baseMean**	**log2FoldChange**	**pvalue**	**gene**	**baseMean**	**log2FoldChange**	**pvalue**
Cp4.1LG08g04300	309,143	1,629	4,092E-17	Cp4.1LG12g11490	94,375	-3,811	1,2462E-25
Cp4.1LG03g10940	368,439	1,315	1,0417E-15	Cp4.1LG12g07690	80,872	-3,793	1,2473E-24
Cp4.1LG07g04680	261,54	1,304	1,6097E-14	Cp4.1LG03g04090	211,344	-1,728	1,54E-18
Cp4.1LG20g03530	232,984	1,678	4,0025E-14	Cp4.1LG13g00040	1348,768	-1,139	1,504E-17
Cp4.1LG20g03690	77,305	2,029	1,3241E-13	Cp4.1LG08g13020	202,118	-1,563	1,9832E-17
Cp4.1LG03g17250	144,805	1,493	2,8437E-12	Cp4.1LG19g01190	261,446	-1,972	1,0526E-16
Cp4.1LG02g12540	156,475	1,647	6,7646E-12	Cp4.1LG10g05950	160,035	-1,807	6,3773E-16

Table S4. Differentially expressed genes (DEGs) from melon plant varieties under different temperature conditions (p-value < 0.05 and Log_2FC < -1 o > 1 criteria). The samples were normalized against the medium (26/20ºC) temperature condition. (This is only part of the content due to its length. To access the full material, please contact the author).

MELON THERMO-TOLERANT							
UPREGULATED GENES				DOWNREGULATED GENES			
gene	**baseMean**	**log2FoldChange**	**pvalue**	**gene**	**baseMean**	**log2FoldChange**	**pvalue**
MELO3C006037.2	314,007	3.201	5,39E-26	MELO3C002508.2	339,903	-2.358	3,55E-20
MELO3C011021.2	312,795	2.56	6,51E-19	MELO3C007420.2	276,776	-2.379	2,28E-18
MELO3C011976.2	265,938	2.506	6,75E-19	MELO3C014672.2	688,701	-1.82	2,00E-12
MELO3C002084.2	168,631	4.053	8,80E-18	MELO3C021708.2	115,608	-3.085	3,04E-10
MELO3C025636.2	996,47	2.084	7,66E-16	MELO3C013710.2	71,2	-3.417	1,44E-05
MELO3C013952.2	801,049	2.508	1,50E-16	MELO3C010875.2	763,161	-1.617	5,21E-06
MELO3C010588.2	289,592	3.47	1,25E-12	MELO3C009482.2	755,852	-1.43	2,00E-03

Table S5. Differentially expressed genes (DEGs) from zucchini plant varieties under different temperature conditions (p-value < 0.05 and Log_2FC < -1 o > 1 criteria). The samples were normalized against the medium (26/20ºC) temperature condition. (This is only part of the content due to its length. To access the full material, please contact the author).

ZUCCHINI THERMO-SUSCEPTIBLE							
UPREGULATED GENES				DOWNREGULATED GENES			
gene	baseMean	log2FoldChange	pvalue	gene	baseMean	log2FoldChange	pvalue
Cp4.1LG06g01630	1078,184	3,3	4,091E-118	Cp4.1LG09g08870	3006,683	-3,768	1,389E-215
Cp4.1LG13g03850	460,276	4,178	3,263E-106	Cp4.1LG04g04950	4083,085	-5,178	6,085E-192
Cp4.1LG08g03840	3993,612	2,924	7,9893E-85	Cp4.1LG13g01550	636,424	-5,813	3,027E-156
Cp4.1LG03g07690	271,944	5,459	5,7357E-72	Cp4.1LG00g01700	2668,984	-3,22	3,194E-151
Cp4.1LG12g06560	341,669	3,585	1,6966E-69	Cp4.1LG17g04700	642,985	-3,896	4,357E-150
Cp4.1LG00g00120	553,205	2,706	1,6708E-63	Cp4.1LG14g06720	935,865	-3,659	1,377E-140
Cp4.1LG08g03960	468,558	2,91	2,4573E-60	Cp4.1LG03g12430	1221,347	-3,537	1,218E-137

Table S6. GO enrichment analysis of melon plant varieties, which were specifically regulated by the combination of viral infection and temperature, is shown in Fig. 4. (This is only part of the content due to its length. To access the full material, please contact the author).

	DEGs group	FDR	nGenes	Pathway size	GO	Pathway	URL	Genes
Tª20+WMV	Upregulated	4.71e-02	2	12	Biological Process	GO:0015914 phospholipid transport	http://amigo.geneontology.org/amigo/term/GO:0015914	MELO3C018710.2, MELO3C020394.2
	Upregulated	4.71e-02	2	9	Biological Process	GO:0034204 lipid translocation	http://amigo.geneontology.org/amigo/term/GO:0034204	MELO3C018710.2, MELO3C020394.2
	Upregulated	4.71e-02	2	10	Biological Process	GO:0097035 regulation of membrane lipid distribution	http://amigo.geneontology.org/amigo/term/GO:0097035	MELO3C018710.2, MELO3C020394.2
	Downregulated	9.13e-02	1	2	Biological Process	GO:0006097 glyoxylate cycle	http://amigo.geneontology.org/amigo/term/GO:0006097	MELO3C002350.2

Table S7. GO enrichment analysis of zucchini plant varieties, which were specifically regulated by the combination of viral infection and temperature, is shown in Fig. 5. (This is only part of the content due to its length. To access the full material, please contact the author).

	group	FDR	nGenes	Pathway size	GO	Pathway	URL	Genes
Tª20+WMV	Upregulated	2.08e-02	2	5	Molecular Function	GO:0043167 Ion binding	http://amigo.geneontology.org/amigo/term/GO:0043167	CP4.1LG00G01320, CP4.1LG09G03590
	Upregulated	2.08e-02	6	136	Molecular Function	GO:0051082 Unfolded protein binding	http://amigo.geneontology.org/amigo/term/GO:0051082	CP4.1LG00G01320, CP4.1LG00G13130, CP4.1LG04G02170, CP4.1LG09G03590, CP4.1LG14G08160, CP4.1LG15G07120
	Upregulated	2.08e-02	2	4	Molecular Function	GO:0070063 RNA polymerase binding	http://amigo.geneontology.org/amigo/term/GO:0070063	CP4.1LG01G03060, CP4.1LG14G05070
	Upregulated	3.67e-02	2	9	Molecular Function	GO:0001664 G-protein coupled receptor binding	http://amigo.geneontology.org/amigo/term/GO:0001664	CP4.1LG10G02720, CP4.1LG19G11570
	Upregulated	3.67e-02	2	9	Molecular Function	GO:0019001 Guanyl nucleotide binding	http://amigo.geneontology.org/amigo/term/GO:0019001	CP4.1LG10G02720, CP4.1LG19G11570

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6.3. Chapter III: "*Host plant switching and viral infections reshape the microbiome of the aphid vector Aphis gossypii*"

Host plant switching and viral infections reshape the microbiome of the aphid vector *Aphis gossypii*

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Abstract

Insect-associated microbiomes constitute a crucial component of the insect physiological fitness, with potential implications for the performance of pest vectors, and consequently, for crop health. However, the effects of the transition between host plants and feeding on virus-infected plants remain unclear. In this study, we examined the bacterial community structure and composition of the cotton-melon aphid, *Aphis gossypii* Glover, in response to feeding on different cucurbit species and on melon-infected plants with either persistent (Cucurbit aphid-borne yellows virus, CABYV), non-persistent (Watermelon mosaic virus, WMV), or mixed viral infections. Using 16S rRNA gene sequencing analysis, we found that bacterial diversity was higher in aphids feeding on melon compared to cucumber, and was re-established when returned to melon. This microbiome structure alteration was mainly due to shifts in the relative abundance of facultative symbionts, such as *Arsenophonus* and *Sphingomonas*. Furthermore, viral infections significantly reduced microbiome diversity compared to that in healthy plants, with the lowest diversity observed in aphids feeding on plants co-infected with CABYV and WMV. Notably, a strong negative correlation between *Buchnera* and *Arsenophonus* abundance was identified and validated by qPCR in those viruliferous aphids, suggesting a compensatory mechanism, possibly modulated by viral infections. This study underlines shifts in the aphid microbiome, which could provide insights for further investigation of microbial resource-based solutions to control aphid pests and associated viral diseases in agricultura.

Keywords: *Aphis gossypii,* Aphid-virus control, *Arsenophonus*, Bacterial symbionts, *Buchnera*, CABYV, Cucurbits crops, WMV.

INTRODUCTION

Insect pests represent a major threat to global food production by directly damaging crops through feeding on plants and serving as vectors of plant pathogens (Oerke, 2005; Douglas, 2018; Savary *et al.*, 2019). Among the multiple strategies for controlling insect pests and their associated pathogenic diseases, chemical insecticides are the most frequently and conventionally used. However, growing concerns over environmental impacts, human health risks, biodiversity loss, and increased pesticide tolerance by insects underscore the need for alternative sustainable pest management strategies. In fact, the increasing risks posed by climate change, evolving agricultural practices, and human activities (Anderson *et al.*, 2004; Bebber *et al.*, 2014; Ristaino *et al.*, 2021a; Tsai *et al.*, 2022; Singh *et al.*, 2023) further emphasize the importance of exploring novel approaches. In this sense, it is becoming increasingly evident that insect-associated microbiomes constitute a crucial component of the physiological fitness of insects, with relevance in the case of insect pest vectors. This means the structure and composition of the bacteria community may be an important aspect in influencing insect reproduction, growth, immune function, and behavioral patterns, as well as the dissemination of pathogens (Douglas, 2018; Wu *et al.*, 2022; Rupawate *et al.*, 2023). As a result, the pest microbiome may have an impact on plant crops, and hence, there is a growing interest in elucidating to what extent this microbiome alteration could affect insect pests and pathogen transmission (Qadri *et al.*, 2020).

This study focuses on the cotton-melon aphid, *Aphis gossypii* Glover (Hemiptera: *Aphididae*), a globally distributed pest that causes severe economic losses on many crops through direct sap-feeding and transmitting over 75 plant viruses (Blackman & Eastop, 2000; Holman, 2008). In particular, *A. gossypii* is the predominant pest species in cucurbit crops across the Mediterranean basin (Hooks & Fereres, 2006; Kassem *et al.*, 2013) and is responsible for the prevalence of several viral diseases (Lecoq & Katis, 2014; Radouane *et al.*, 2021). Among these aphid-transmitted viruses, the *Polerovirus CABYV* (cucurbit aphid-borne yellows virus, CABYV) is a circulative and non-propagative polerovirus that is becoming epidemiologically relevant in Europe (Desbiez *et al.*, 2020; Rabadán *et al.*, 2021; Moya-Ruiz *et al.*, 2023). Additionally, other non-circulative aphid-transmitted viruses, such as *Potyvirus citrulli* (watermelon mosaic virus, WMV), *Potyvirus*

cucurbitaflavitesselati (zucchini yellow mosaic virus, ZYMV), *Potyvirus papayanuli* (papaya ringspot virus, PRSV), and *Potyvirus citrullimoroccense* (Moroccan watermelon mosaic virus, MWMV) are also affecting these crops and are often detected in mixed infections, resulting in complex disease dynamics within cucurbit crops (Radouane *et al.*, 2021; Moya-Ruiz *et al.*, 2023). Indeed, mixed viral infections are now recognized as a common biotic factor in epidemics (Syller, 2011; Alcaide *et al.*, 2020; Moreno & López-Moya, 2020), with relevant implications for host phenotype and vector behavior, and consequently for disease transmission (Srinivasan & Alvarez, 2007; Pinto *et al.*, 2008; Peñaflor *et al.*, 2016). Therefore, the prevalent combination of CABYV and WMV in cucurbit crops (Rabadán & Gómez, 2023; de Moya-Ruiz *et al.*, 2021; De Moya-Ruiz *et al.*, 2023; Rabadán *et al.*, 2023; Moya-Ruiz *et al.*, 2023) may be postulated to have intricate ecological interactions between these viruses within the same plant and/or crop, encompassing direct and indirect effects on host plant species and aphid vectors.

Understanding the role of the microbiome of *A. gossypii* is of fundamental and applied interest, as the insect-associated microbiome is increasingly recognized as a key modulator of aphid physiology, adaptation, and vector competence (Porras et al. 2020; Heidari Latibari et al. 2025). Recent studies have revealed that the endosymbiotic bacterial composition of *A. gossypii* appears to be contingent on the host plant species and plant virus infections (Xu *et al.*, 2020), since endosymbiotic bacteria can confer beneficial traits and contribute to their host specialization and shifting abilities (Sudakaran *et al.*, 2017). For instance, *Buchnera aphidicola*, is an obligate endosymbiont, which is essential for amino acids synthesis (Douglas, 1998), while *Serratia symbiotica, Wolbachia, Arsenophonus, Hamiltonella defensa, Regiella insecticola, Rickettsia, Rickettsiella, Fukatsuia,* and *Spiroplasma,* are facultative symbionts that have been documented to provide some advantageous traits to *A. gossypii* (Guo *et al.*, 2017). In this context, most studies encompassing the microbiomes of *A. gossypii* have primarily focused on characterizing microbial communities and their diversity (Zhao *et al.*, 2016; Gallo-Franco *et al.*, 2019; Xu *et al.*, 2020; Ma *et al.*, 2021; Zhang *et al.*, 2021; Chen *et al.*, 2023). For example, specific host plants of *A. gossypii* can alter the diversity and richness of their bacterial communities (Ma *et al.*, 2021), suggesting a significant impact of host plant species on the composition and diversity of bacterial communities. Additionally, it

has been observed that *Buchnera, Serratia,* and *Arsenophonus* have varying abundances depending on the host plant, indicating a correspondence between the symbiotic bacteria and the specific host plants of the aphid (Ma *et al.*, 2021). In fact, endosymbiotic bacteria are believed to play a major role in whitefly (*Bemisia tabaci*) host-plant adaptation. The rapid response of these endosymbionts to changes in the insect diet, especially their potential to degrade or assimilate toxic plant metabolites, provides significant benefits (Shikano *et al.*, 2017; Santos-Garcia *et al.*, 2020). However, no study has characterized the potential changes in the *A. gossypii* microbiome following its transfer to an alternative host and subsequent return to the original host, leaving our current understanding of the potential adaptation of microbiota-mediated aphids to the transition between different host plant species unclear.

Additionally, few studies have examined the influence of plant virus infections on the aphid microbiome. For instance, a recent study reported that *Enamovirus PEMV* (Pea enation mosaic virus, PEMV), which is transmitted persistently, enhanced pea aphid (*Acyrthosiphon pisum*) fitness in virus-infected plants harboring the facultative endosymbionts *Regiella insecticola* and *H. defense,* along with altered host-plant preferences (Sanches *et al.*, 2023). Higher PEMV transmission rates were also observed in aphids with *H. defensa* than in those with only the obligate endosymbiont. Similarly, another study showed that *Myzus persicae* preferred to settle on plants infected with the *Polerovirus PLRV* (potato leafroll polerovirus, PLRV), possibly influenced by a reduction in the endosymbiont *Buchnera* titer (Patton *et al.*, 2021). However, the effects of plant viral infections on microbiomes are not limited to viruses persistently transmitted by aphids. Non-persistent plant viruses, which are typically acquired and transmitted by aphids within minutes, have also been shown to influence aphid-plant interactions and potentially affect the aphid microbiome. For instance, *Cucumovirus CMV* (Cucumber mosaic virus, CMV) reduced the *B. aphidicola* abundance in *M. persicae*, possibly associated with a preference shift in aphids from infected to healthy plants (Shi *et al.*, 2021). An additional study showed that WMV infection in pumpkin plants differentially affected the bacterial symbiont *Hamiltonella defense* and *Arsenophonus* sp. of the cowpea aphid *Aphis craccivora* Koch, consequently increasing the aphid´s exploratory intracellular puncture frequency in infected plants (Angelella *et al.*, 2018).

These studies emphasize the importance of bacterial symbionts in the ecology of plant virus transmission, as they can modulate the insect's physiology, which in turn affects the efficiency of virus acquisition and transmission (Wu *et al.*, 2022). While there are mechanistic reasons to anticipate that the *A. gossypii* microbiome can be altered, the potential host adaptation mediated by the aphid associated-microbiome, ie. whether changes in the aphid microbiota are maintained or recovered after returning to the original host plants remains unknown. Furthermore, whether plant viruses in single and mixed infections with different vector transmission manners can shape the aphid-associated microbiome is largely unexplored, since efforts have focused on pairwise aphid-virus interactions. In this study, we examined the changes in the bacterial community associated with *A. gossypii* when they fed on cucurbit hosts (melon and cucumber), including the transition back to melon after feeding on cucumber. Furthermore, we examined how the *A. gossypii* bacterial community was affected by plant virus infection, including persistent (CABYV) and non-persistent (WMV) cucurbit viruses, in both single and mixed infections.

MATERIALS AND METHODS

***Aphis gossypii* populations and sample collection.** Populations of *A. gossypii* Glover were derived from individual clones reared on melon plants (*Cucumis melo* L. cv. Alcazaba) under a 16/8h light/dark cycle at 24/20 °C, supplied by the Insect vectors of plant pathogens group (ICA-CSIC, Madrid, Spain). Two concurrent experiments were conducted: one using healthy plants to address the aphid-associated microbiome adaptation process, and another using virus-infected plants to examine the influence of single and mixed plant viruses. Aphid populations were maintained on five plants per treatment in separate climate-controlled chambers. To synchronize developmental stage and minimize age-related variation, aphids were established by transferring first-instar nymphs to experimental plants and allowing them to develop for two generations. Specifically, cucumber (Marketer var) and melon (Piel de Sapo var) plant species were utilized for the aphid experiment in healthy plants: ancestral clones of aphid were established in melon plants (MM) for two generations, and subsequently, MM aphid nymphs were transferred to cucumber (MMC) and maintained for two generations.

Finally, the MMC aphid nymph populations were returned to the melon plants (MMCM). Concurrently, in the virus-infected plant experiment with WMV (non-persistent) and CABYV (persistent), melon plants were agro-inoculated with the WMV-MeWM7 and CABYV-LP63 full-length infectious clones, either individually or in combination for single and mixed infections (de Moya-Ruiz *et al.*, 2021; Rabadán *et al.*, 2021). After 30 days post-agroinoculation (dpai), viral infections were confirmed by specific RT-PCR (de Moya-Ruiz *et al.*, 2021; Rabadán *et al.*, 2021). Thereafter, aphid populations from healthy melon (MM) plants were transferred to melon plants infected with WMV (M(W)), CABYV (M(C)), and CABYV+WMV (M(C+W)) for two generations (**Fig. 1**). Experiments used plants at the 5–7 fully-expanded true leaf stage in a climate-controlled greenhouse (16/8 h photoperiod, 24/20 °C). For each treatment, only wingless, parthenogenetic adult aphids were used for microbiome analysis, as these represent the predominant morph in field populations and are most relevant for studies of plant-aphid-virus interactions. Approximately, 40 wingless aphids were collected in three replicate samples, preserved in 70 % ethanol, and stored at -20 °C.

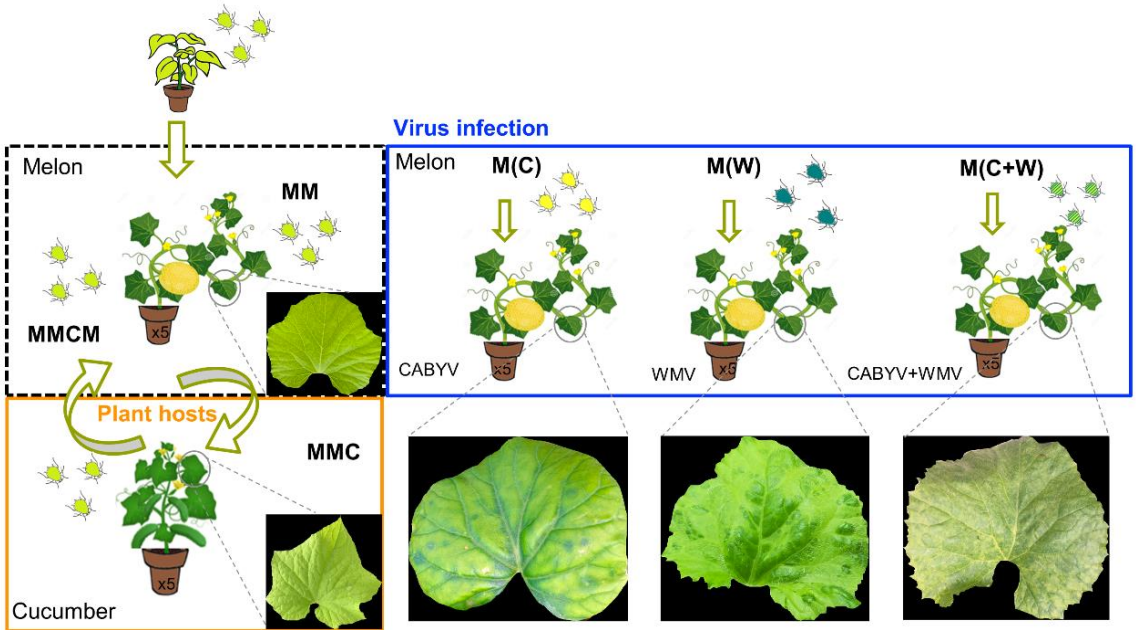

Figure 1. Schematic representation of the experimental design used to assess the impact of host plant species change or virus infection. Aphids were obtained from six treatments: aphids that had fed on different non-viral infected hosts after being transferred from one host to another: melon-melon (MM), melon-melon-cucumber (MMC), melon-melon-cucumber-melon (MMCM), and aphids that had fed on melon plants and subsequently on melon plants infected with WMV (M(W)), CABYV (M(C)), and CABYV+WMV (M(C+W)). Each treatment included three replicates, each comprising a pool of 40 wingless aphids, maintained at 24/20 °C under different treatments for 17 days. At this time, 40 aphids were collected, and total DNA was extracted to conduct bacterial 16S ribosomal RNA gene analysis. The transfer of aphids between melon and cucumber hosts (left panels, dashed and orange boxes), and subsequent virus infection treatments (right panels, blue box). Representative photos of leaves are shown for each treatment, exhibiting the yellowing, chlorosis, and mosaic symptoms induced by CABYV, WMV, and the combination of both.

DNA extraction and 16S rRNA gene sequencing. Genomic DNA was extracted from each aphid sample using a High-Q Spin Column Stool DNA Purification Kit (TIARIS, Spain). DNA quality and quantity were evaluated using a Qubit dsDNA HS assay (Invitrogen) and agarose gel electrophoresis. Amplicon libraries were prepared for PE250 sequencing on the Illumina platform (Novogene, Germany) using the 8F and 1492R bacterial primers targeting the V3-V4 region of the 16S rDNA (Youssef *et al.*, 2009). The PCR products were purified using a QIAquick PCR purification kit. A second amplification targeted the V4 region using F515 and R806 primers, with an Illumina adapter and an 8-nucleotide barcode at the 5'-end (Caporaso *et al.*, 2011). An equimolar library was prepared by normalizing amplicon concentrations using a SequalPrep Normalization Plate (Applied Biosystems) and pooling. Amplicons were sequenced on the Illumina MiSeq using a v2 PE500 kit with custom primers, generating two ×250-nt paired-end reads. Sequences were jointly trimmed and filtered on the paired ends. The high-resolution DADA2 method was employed to infer sequence variants, utilizing a parameterized model of substitution errors to differentiate between sequencing errors and genuine biological variations (Callahan *et al.*, 2016). Chimeras were subsequently eliminated from the dataset. Post-noise reduction sequences generated by DADA2 are referred to as Amplicon Sequence Variants (ASVs). Taxonomy was assigned using a naïve Bayesian classifier with the Silva v138.1 database and QIIME2's classify-sklearn algorithm. ASVs with a mean relative abundance below 10−5 were excluded from the analysis. To visualize bacterial genera differing between treatments, ASVs were grouped at the genus level, and genera representing <1% of the reads were discarded. To examine differences in α- and β-diversity, all samples were rarefied to a depth of 1,000 reads.

Relative quantification of bacterial load by qPCR. To confirm alterations in the prevalence of the most common genera, *Buchnera* and *Arsenophonus*, quantitative PCR (qPCR) was conducted on *A. gossypii* samples. Using genomic DNA extraction from each treatment, samples underwent qPCR using NZYSupreme qPCR Green Master Mix (2x), ROX plus (NZYTech, Lisboa, Portugal) with an AB7500 System (Applied Biosystems, Foster City, CA). The PCR cycling protocol consisted of 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 30 s at 60 °C annealing temperature, and the melting curve. Sequence of the specific primers for *Buchnera* sp. (Buchn-dnaK-qPCR-F2 and Buchn-dnaK-qPCR-R2),

Arsenophonus sp. (Arsen-yaeT-qPCR-F and Arsen-yaeT-qPCR-R), and the insect elongation factor EF-1α gene (ApEF1-alpha-qPCR-107F and ApEF1-alpha-qPCR-246R) as reference are described in Ayoubi et al., (2020). The reaction mixture was performed in a total volume of 10 µl, containing 5 µl of NZYSupreme qPCR Green Master Mix (2x), ROX plus (NZYTech), 0.4 µl of reverse and forward primers, 2.7 µl of sterile water, and 1.5 µl of DNA. Non-template controls were incorporated to ensure product-specific amplification and the absence of primer dimers. The qPCR analysis for the relative copy number was carried out using the 2ΔΔCT method, with the samples from melon control (MM) treatment serving as the experimental control, as described in (Schmittgen & Livak, 2008). The results for each treatment were expressed as Log10 (Fold Change (FC) = 2^−ΔΔCt).

Statistical analyses. Measures for alpha diversity of the bacterial communities within the different treatments were calculated using Qiime2 analysis. These measures included Pielou's evenness (indicating species distribution uniformity), Shannon Index (assessing species richness and evenness), and Simpson Index (evaluating diversity and evenness of species distribution) (Kers & Saccenti, 2022). Note that the alpha diversity data were non-normally distributed (Shapiro–Wilk test: $p<0.05$, for all indices) and were log-transformed to achieve normality. Differences among and between groups were analyzed by subsequent parametric repeated-measures ANOVA analysis or Kruskal–Wallis test as a non-parametric method using R. To examine differences in community composition (beta diversity), Bray–Curtis and Jaccard dissimilarity indices were computed (Kers & Saccenti, 2022). The relative abundance data underwent logarithmic transformation using the *decostand* function of the *vegan* package to minimize the influence of highly abundant genera. The transformed data were then visualized via principal coordinates analysis (PCoA) using the *ggplot2* package in R. The Unweighted Pair-group Method with Arithmetic Mean (UPGMA) was utilized to construct a cluster tree based on weighted and unweighted UniFrac distance matrices, employing the distance function and *phyloseq* R package. Weighted UniFrac distances consider both the presence and abundance of taxa, whereas unweighted UniFrac focuses solely on the presence or absence of ASVs. Differences in symbiont community structures were statistically analyzed using permutational multivariate analysis of variance

(PERMANOVA), based on Bray–Curtis, Jaccard, and unweight/weight UniFrac distance matrices. These analyses were performed using the *adonis2* function in the *vegan* R package (*p*-values obtained through 999 permutations, p<0.05) (Zapala & Schork, 2006; Anderson & Walsh, 2013). Pairwise significance analyses for treatment comparisons were conducted using the *pairwise.adonis2* function for both PERMANOVA analyses. Post-hoc Scheffé and Dunn tests were performed for pairwise group comparisons, with Bonferroni-adjusted p-values controlling for the false discovery rate. To investigate potential interactions among the different symbionts associated with *A. gossypii*, Spearman correlation coefficients (ρ) were calculated based on their relative abundances using the *cor* function from the *stats* package in R (under the criteria R=-1, R=+1, and *p*<0.05). The results were visualized as a heat map using the *corrplot* package (Zar, 2005; Wei *et al.*, 2017). For qPCR validation, differences between groups were analyzed using Student's t-test. Venn Diagrams and histograms were generated using R and SRplot (Tang *et al.*, 2023).

Data availability. The raw Illumina sequencing data for the 16S rRNA analysis were deposited in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the BioProject ID PRJNA1196144, where the raw and processed data, including metadata with detailed sample annotations and protocols of this study, are provided.

RESULTS

Cucurbit plant species and viral infections can influence the composition of the aphid-associated microbiome. The number of sequences and Amplicon Sequence Variants (ASVs) corresponding to the six treatments are presented at the genus level in Table 1. In the aphid population feeding on healthy plants, the numbers of sequences in MM (aphids on melon transferred from melon), MMC (aphids on cucumber transferred from MM), and MMCM (aphids on melon transferred from MMC) were 165,495 ± 1,063; 167,692 ± 1,779; and 149,995 ± 2,269, respectively. In the aphid population feeding on virus-infected plants, M(C) (aphids on CABYV-infected melon), M(W) (aphids on WMV-infected melon), and M(C+W) (aphids on mixed-infected melon) were 147,216 ± 1,958; 166,822 ± 2,878; and 146,112 ± 2,565, respectively. The number of ASVs in aphids from

healthy plants was greater than that in aphids feeding on infected plants: MM exhibited the highest number (262), followed by MMC (156), while MMCM had the lowest (113). The number of ASVs in M(C) (138) was larger than in other treatments, with M(W) (112) and M(C+W) (110) having the lowest (**Table 1**, and **Table S1** for detailed sequencing data quality metrics). The differences in ASVs suggest that changes in plant species and viral infections can influence the composition of aphid-associated microbiomes. Aphids feeding on healthy plants exhibited higher ASVs than those on virus-infected plants. The type of virus infection (WMV, CABYV, or mixed) appears to have varying effects on the aphid microbiome composition, with CABYV infection resulting in a higher number of ASVs than WMV or mixed infections.

Table 1. Number of sequences, ASVs and Alpha diversity indices for samples either from host plant transition (MM, MMC, and MMCM) or viral infections (M(C), M(W), and M(C+W)). The indices included an estimate of the total number of species in the community, Pielou evenness (Pielou's), the Shannon Index, which measures both species richness and evenness (Shannon), and the Simpson Index, which assesses the diversity and evenness of species distribution within the community (Simpson).

			Alpha diversity indices		
Sample	**Number of sequences**	**ASVs**	**Shannon**	**Simpson**	**Pielou's**
MM	165,495 ± 1,063	262	1.541	0.272	0.155
MMC	167,692 ± 1,779	156	1.270	0.308	0.147
MMCM	149,995 ± 2,269	113	1.020	0.257	0.125
M(C)	147,216 ± 1,958	138	0.661	0.134	0.079
M(W)	166,822 ± 2,878	112	0.258	0.040	0.032
M(C+W)	146,112 ± 2,565	110	0.261	0.042	0.033

Considering the top 15 bacterial genera from the ASV data, those genera were categorized based on their relative abundances, and three replicates of each treatment were consistently clustered into one branch, indicating the high reliability of the experimental data (**Fig. 2A-B**). In the microbiome of aphids feeding on different host plants, *Buchnera,* as a primary obligate symbiont, was the dominant genus, ranging from 83% to 87%. Its highest abundance was observed in the microbiome of aphid populations derived from virus-infected treatments or viruliferous aphids (Kruskal–Wallis test: chi-squared = 11.363, df = 5, p = 0.044), ranging from 89% to 97% (**Fig. 2A**). Among secondary symbionts, in the case of aphids feeding on different host plants, facultative endonsymbionts such as *Arsenophonus, Brevundimonas, Cutibacterium,* and *Staphylococcus* were also reliably present in all treatments, but in lower proportions. In

particular, the relative abundance of *Arsenophonus* was 2.27% in MM, 1,09% in MMC, and 11.89% in MMCM. For *Brevundimonas* was 0.07% in MM, 10.15% in MMC, and 0.06% in MMCM. For *Cutibacterium* was 0.15% in MM, 1.14% in MMC, and 0.36% in MMCM. And for *Staphylococcus* was 0.97% in MM, 1.15% in MMC, and 0.04% in MMCM. Other genera, such as *Novosphingobium, Acinetobacter, Flavobacterium,* and *Sphingomonas,* were also detected in minimal quantities (averages of 0.08%, 0.04%, 0.02%, and 0.1%, respectively). For aphids from virus-infected plants, *Arsenophonus, Brevundimonas, Cutibacterium,* and *Staphylococcus* appeared to be in lower proportions than aphids from healthy (MM) plants. In particular, the relative abundance of

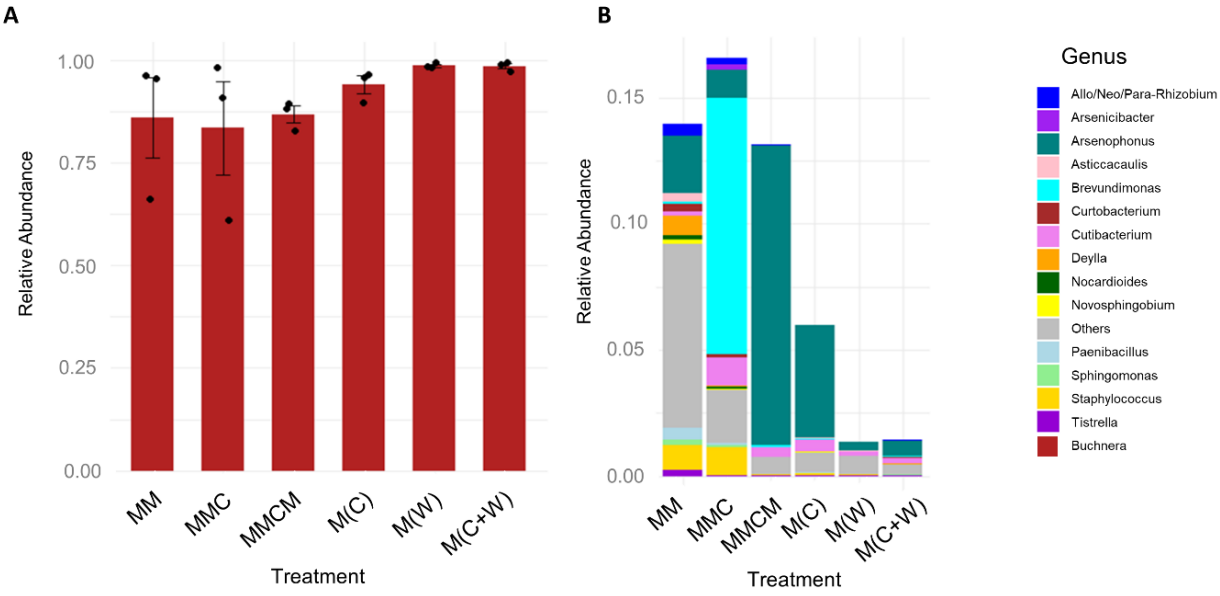

Figure 2. Relative abundance (scaled from 0 to 1) of the main genera associated with A. gossypii from each group based on 16S rDNA sequences. The top 15 taxa were selected to form the distribution histogram of the relative abundance of bacterial genera for various treatments: MM, MMC, MMCM, M(C), M(W), and M(C+W). The remaining bacterial genera were listed as "others," including sequences that could not be classified into any known group. For all treatments, the most abundant bacteria genera are presented in **A,** whereas the remaining and less prevalent genera are in **B**.

Arsenophonus was 4.46% in M(C), 0.34% in M(W), and 0.63% in M(C+W). For *Brevundimonas* was 0.06% in M(C), 0.03% in M(W), and 0.04% in M(C+W). For *Cutibacterium* was 0.45% in M(C), 0.16% in M(W), and 0.24% in M(C+W). And for *Staphylococcus* was 0.10% in M(C), 0.05% in M(W), and 0.04% in M(C+W). While, *Novosphingobium, Acinetobacter, Flavobacterium,* and *Sphingomonas* had negligible proportions (averages of 0.004%, 0.006%, 0.003%, and 0.008%, respectively) (**Fig. 2B** and **Table S2** for detailed information on ASVs identification). The variation in these

secondary symbionts across treatments suggests that the transition between melon and cucumber plants might exert a selective effect on the aphid microbiome.

Changes in the bacterial structure and composition of the aphid-associated microbiome. To evaluate the impact of the transition in melon and cucumber plants and viral infections on the microbial diversity of aphids, we initially analyzed alpha-diversity using Shannon, Simpson, and Pielou's evenness indices derived from ASVs classification (**Table 1**). Alpha diversity showed significant differences among treatments across all indices: Shannon index ($F_{(1,5)}$ = 5.029, p = 0.0121), Simpson index ($F_{(1,5)}$ = 3.875, p = 0.0286), and Pielou's evenness ($F_{(1,5)}$ = 5.621, p = 0.00819). Aphids fed on healthy melon plants (MM) exhibited the highest values across all four alpha diversity indices. Conversely, alpha diversity decreased in the MMC treatment (aphids moved to cucumber plants; Shannon: -18% and Pielou: -5%), except when the Simpson index increased (+13%)), with a more substantial reduction in the MMCM treatment (when aphids returned to melon plants; Shannon: -34%, and Pielou: -19%, Simpson -6%)). Similarly, when comparing control or non-viruliferous melon aphids (MM) with viruliferous aphids, alpha-diversity decreased significantly in the M(C) treatment (Shannon: -57%, Simpson: -51%, and Pielou: -49%), and more marked in the M(W) and M(C+W) treatments, which exhibited similar reductions (Shannon: -83%, Simpson: -85%, and Pielou: -79%) (**Table 1**). These results suggest that both host plant changes and virus infections led to substantial reductions in alpha microbial diversity.

We next examined beta-diversity on the aphid microbial communities by using Bray–Curtis and Jaccard indices in order to assess differences in relative abundance and presence/absence of taxa, respectively. Principal Coordinate Analysis (PCoA) revealed no significant differences in microbial community composition among aphids from the MM, MMC, and MMCM treatments (PCoA for Bray–Curtis: **Fig. 3A,** PERMANOVA, p = 0.319; and PCoA for Jaccard: **Fig. 3B**, PERMANOVA, p = 0.469). Likewise, the weighted UniFrac dissimilarity distance analysis, which incorporated evolutionary relationships among taxa, showed no significant differences among MM, MMC, and MMCM (UPGMA: **Fig. 3E**; PERMANOVA, p = 0.307), while the unweighted UniFrac exhibited significant differences (UPGMA: **Fig. 3F**; PERMANOVA, p = 0.015), clustering MMC and MMCM into a single group. In the case of aphids feeding on virus-infected plants, community composition showed significant differences among M(C), M(W), and M(C+W) treatments compared

to the melon control (MM) (PCoA for Bray–Curtis: **Fig. 3C**; PERMANOVA, $p = 0.002$ for all pairwise comparisons). However, based on Jaccard distances, which consider only presence/absence, community composition was similar (PCoA; **Fig. 3D**; PERMANOVA, $p = 0.067$), indicating that differences were primarily due to changes in relative abundances rather than alteration in the presence/absence of bacterial genera. Likewise, for phylogenetic structure, both weighted and unweighted UniFrac distances indicated that virus-infected and control (MM) aphids shared a similar phylogenetic composition (UPGMA: **Fig. 3G and 3H**; PERMANOVA, $p = 0.117$ for weighted; $p = 0.469$ for unweighted), clustering together into a single group (**Table S3** provides detailed information of the statistical analysis). These results showed alterations in *A. gossypii* microbial diversity in response to host plant transitions and viral infections. While alpha diversity decreased, with the most pronounced effects observed in aphids subjected to viral infections, beta diversity showed that shifts in the melon and cucumber plant hosts mainly affected the presence of endosymbionts, and viral infections altered the relative abundances of bacterial genera.

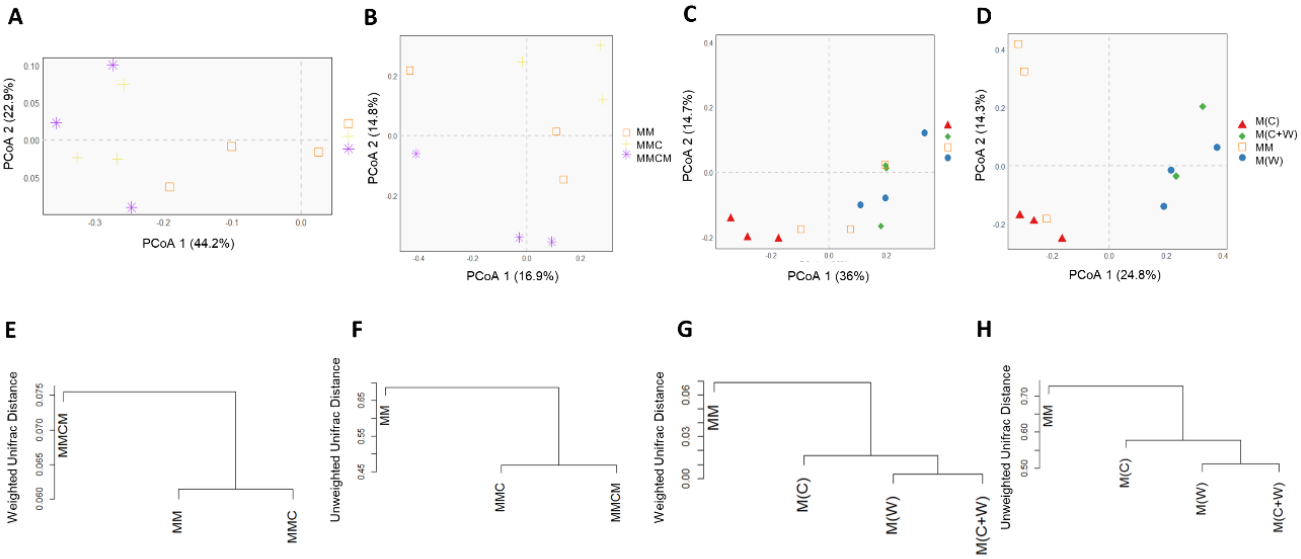

Figure 3. Principal coordinate analysis (PCoA) according to the Bray-Curtis and Jaccard distance matrix of aphids feeding on healthy plants (Bray-Curtis (**A**), Jaccard (**B**)), and aphids feeding on virus-infected plants (Bray-Curtis (**C**), Jaccard (**D**)). Each treatment was represented by three points corresponding to each replicate. Data points with their corresponding colors and shapes represent identical treatments. Dendrograms were constructed using the unweighted pair-group method with arithmetic mean (UPGMA) tree based on the unweighted Unifrac distance matrix and the weighted Unifrac distance matrix, illustrating the evolutionary ties among aphid samples with similar bacterial

compositions of aphids feeding on healthy plants (weighted Unifrac distance (**E**), unweighted Unifrac distance (**F**)) and aphids feeding on virus-infected plants (weighted Unifrac distance (**G**), unweighted Unifrac distance (**H**)).

Shared and unique bacteria within the microbiomes of aviruliferous and viruliferous aphid populations. To determine the bacterial genera unique to or shared among the aphid microbiomes, bacterial presence was assessed when detected in at least two replicates per treatment. For aphids feeding on different host plants, their microbiomes shared 26 bacterial genera that remained stable regardless of host plant changes. However, the MM treatment exhibited a high number (65) of unique bacterial genera absent in the microbiomes of the MMC and MMCM treatments. The MMC treatment also contained another 22 unique genera, while only two unique genera were present upon transitioning back to melon (MMCM treatment) (**Fig. 4A**). Similarly, for aphids

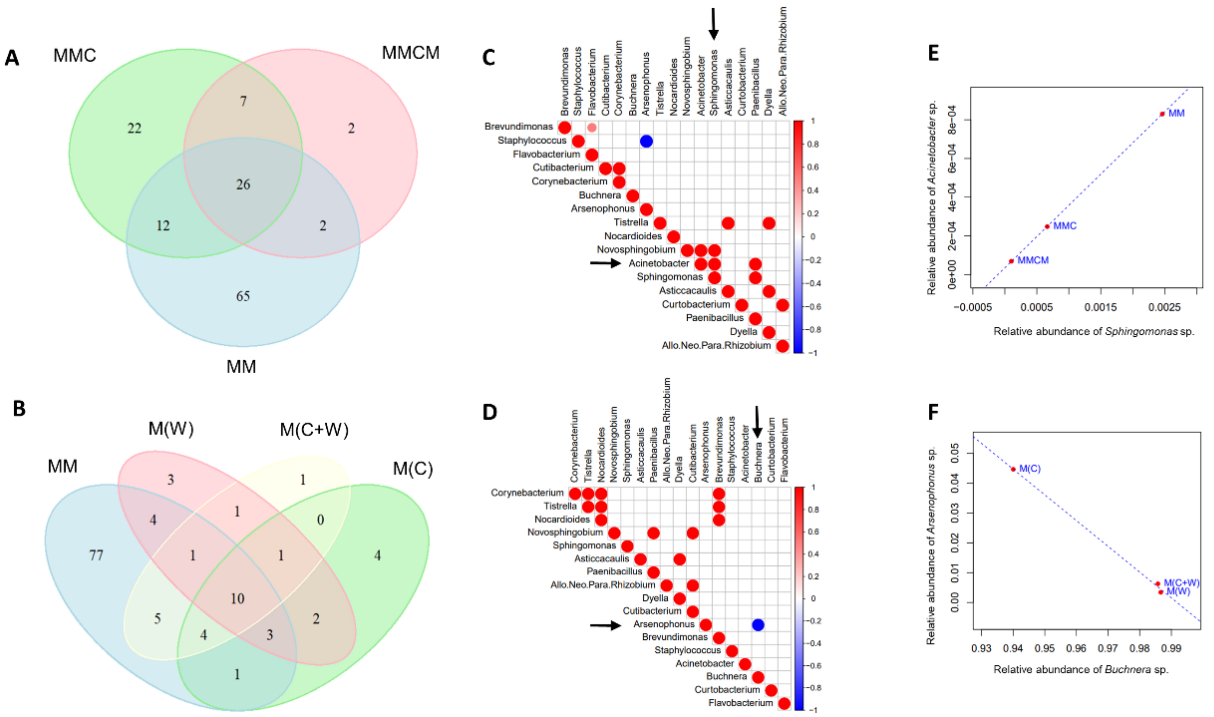

Figure 4. Venn diagram analysis (VDA) of amplicon sequence variants (ASVs) at the genus level in MM, MMC, and MMCM (**A**) and in MM, M(C), M(W), and M(C+W) (**B**). The numerical values in the overlapping regions represent the number of ASVs shared by the treatments. The numerical values in the non-overlapping regions represent the number of unique ASVs for each treatment. The VDA was conducted based on the occurrence within the genus level, considering their presence in a minimum of two replicates for each treatment. Additional information regarding the intersections is provided in Supplementary Table 4. Heatmaps of correlation (Spearman) matrices for aphids feeding on healthy plants (**C**) and virus-infected plants (**D**) were calculated for the relative abundance of the top 15 bacterial amplicon sequence variants (ASVs), including endosymbionts detected in different treatments of the aphid A. gossypii. Blue and red colors indicate negative and positive correlations, respectively. The color density, circle size, and numerical values reflect the magnitude of the correlation at a significant level ($p < 0.05$). A scatter plot was generated for the primary and secondary symbiont genera, which showed significant correlations between aphids feeding on healthy plants (**E**) and those feeding on virus-infected plants (**F**).

feeding on virus-infected plants, the microbiome from the MM treatment had 77 unique bacterial genera absent in virus-infected aphids, with ten bacterial genera shared among the viral infection treatments (**Fig. 4B**). Notably, only few bacterial genera were exclusive to each virus-infected treatment: *Bacteroides*, *Exiguobacterium* and *Acidibacter* were found on M(W) treatment; *Methylobacterium-Methylorubrum*, *Chryseobacterium*, *Dietzia* and *Methylovirgula* on M(C) treatment; and only *Acidothermus* in aphids feeding on mixed-infected plants (M(C+W)) (**Table S4**). Given that host plant switching and plant virus infection clearly altered the aphid microbiome composition, we next sought to explore whether any potential bacterial interactions among the *A. gossypii* symbionts. To this end, Spearman correlation coefficients (ρ) were calculated for the top 15 bacterial genera, selected based on their average relative abundance across samples and including *Buchnera*, and are displayed as heatmaps for aphids feeding on healthy plants (**Fig. 4C**), and virus-infected plants (**Fig. 4D**). Among the aviruliferous aphids, *Acinetobacter* and *Sphingomonas* exhibited a strong positive correlation ($\rho = 1$, $p = 0.001$) (**Fig. 4E**), whereas *Buchnera* and *Arsenophonus* showed a significant negative correlation ($\rho = -1$, $p = 0.028$) in viruliferous aphids (**Fig. 4F**). Regarding the presence of these endosymbionts, *Acinetobacter* was present in all treatments, with no significant differences in its relative abundances across treatments ($F_{(1,5)} = 0.825$, $p = 0.557$) (**Fig.**

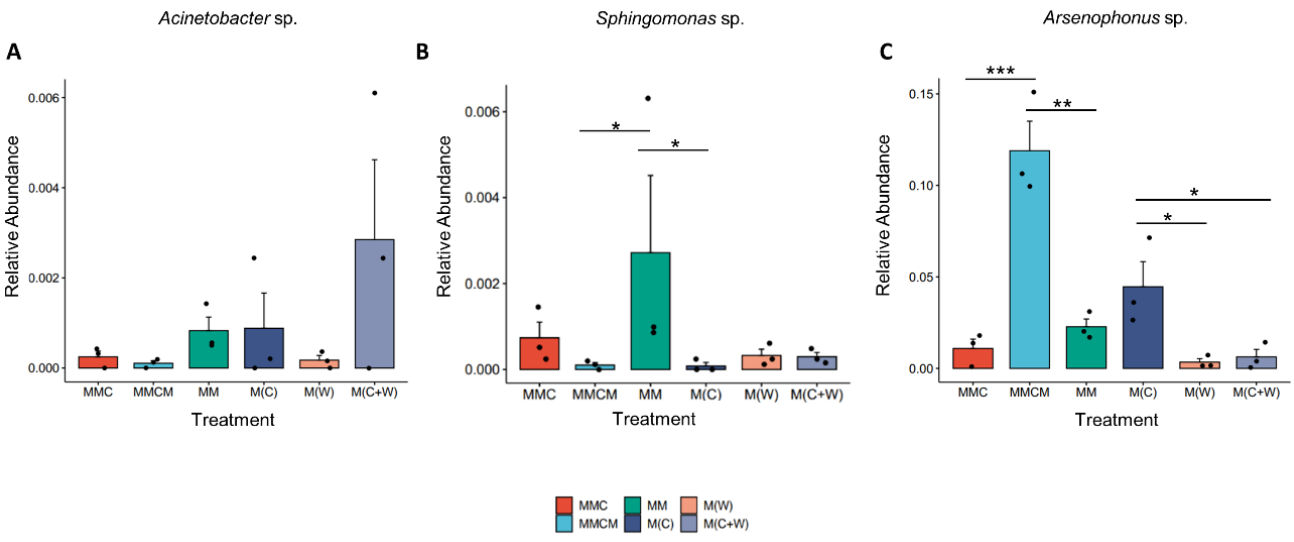

Figure 5. Relative abundance of facultative endosymbionts reported in the literature across various treatments: Acinetobacter (**A**), Sphingomonas (**B**), and Arsenophonus (**C**). Results are presented as mean ± SD of three independent biological experiments, and statistical significance was determined using the Shapiro-Wilk normality test and repeated-measures ANOVA ($p<0.05$).

5A). Likewise, *Sphingomonas* was also present in all treatments, with significant variations between MM vs. MMCM ($t_{(12)}$ = 3.420, p = 0.0451) and MM vs. M(C) ($t_{(12)}$ = -3.682, p = 0.0290) (**Fig. 5B**). *Arsenophonus* appeared to be significantly less abundant in the M(W) and M(C+W) treatments and more abundant in the MMCM and M(C) treatments ($F_{(1,5)}$ = 27.13, p < 0.001) (**Fig. 5C**), with significant differences (p < 0.05) in the pairwise comparisons: MM vs. MMCM, and MMC vs. MMCM, in addition to M(C) vs. M(C+W), and M(C) vs. M(W).

To further validate the abundances of the primary and facultative symbionts from sequencing data analyses, relative qPCR was performed to quantify *Buchnera* and *Arsenophonus*. In comparison to the melon control (MM) aphids, *Buchnera* sp. significantly decreased in aphid microbiomes from cucumber plants (MMC) ($t_{(2)}$ =3.45, p = 0.037), and in those from returned to melon plants (MMCM) ($t_{(2)}$ =3.42, p = 0.038). Among the microbiome of aphids feeding on virus-infected plants, there was a notable reduction in *Buchnera* sp. in the CABYV-infected plants (M(C)) ($t_{(2)}$ = 51.89, p <0.001), with no significant differences from WMV-infected plants (M(W)) and mixed infections (M(C+W)) (both p > 0.11) (**Fig. 6**). *Arsenophonus* sp. exhibited a more variable response

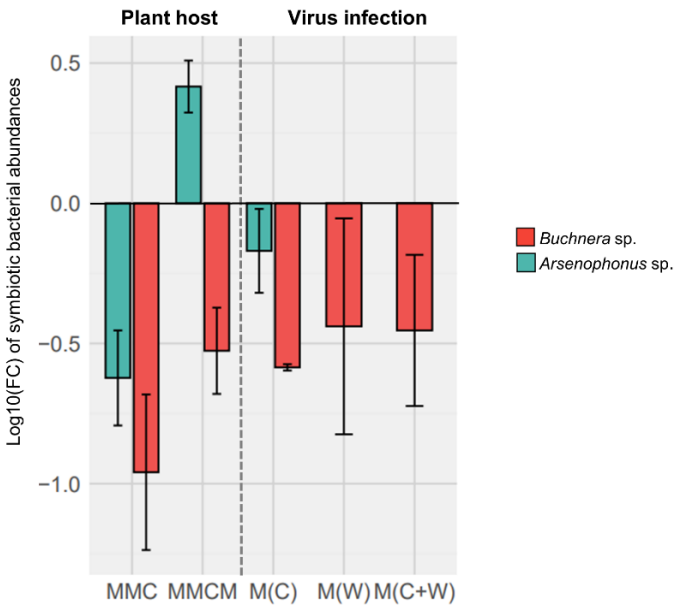

Figure 6. Histograms of Buchnera sp. and Arsenophonus sp. relative variation expressed as log10(Fold Change) in different aphid populations fed on healthy plants (MMC and MMCM) and virus-infected plants (M(C), M(W), and M(C+W)). The fold change was calculated using the 2−ΔΔCt method, with MM treatment used as the control. A log10(FC) value of 0 indicated no change in gene expression, values greater than 0 indicated an increase in expression, and values less than 0 indicated a decrease in expression. Results are displayed as means ± SD of three independent biological experiments, and statistical significance was assessed using the Shapiro-Wilk normality test and the Student t-test (p<0.05).

than *Buchnera*, decreasing its abundance in the MMC treatment ($t_{(2)}$ = 3.67, p = 0.033), but increasing in the microbiome of aphids that returned to feed on melon plants (MMCM) ($t_{(2)}$= 4.48, p = 0.023). Additionally, *Arsenophonus* decreased in aphids from plants infected with CABYV (M(C)) and was remarkably absent in those feeding on WMV (M(W)) and mixed infected plants (M(C+W)) (**Fig. 6**). These results provide further evidence for the dynamic shifts in *Buchnera* and *Arsenophonus* populations within aphid microbiomes, particularly in response to changes and adaptations in host plants, including single and mixed infections.

DISCUSSION

Our results show that host plant switching and viral infections significantly affected the aphid microbiome, altering bacterial structure and composition. Specifically, diversity analysis revealed that host plant transitions increased the relative abundance of facultative or secondary endosymbionts, while viral infections had moderate effects, possibly due to the high abundance of the primary obligate symbiont *Buchnera* (**Table 1**, **Table S2**, and **Fig. 2**). Compared to aphids on melon plants, a reduction in bacterial genera was observed in aphids fed on cucumber, those returned to melon, and those on virus-infected plants (**Table S4** and **Fig. 4**). However, only aphids on virus-infected plants had significantly different bacterial genera abundance (**Table S3** and **Fig. 3**). Additionally, *Buchnera*, *Sphingomonas*, and *Arsenophonus* showed correlated responses to host plant transitions and viral infections (**Fig. 4** and **5**). Accumulation analysis of *Buchnera* and *Arsenophonus* showed *Buchnera* abundance decreased, with Arsenophonus showing a differential response when aphids returned to melon. Only aphids from CABYV-infected plants had a marked reduction in both symbionts, while they were similar or absent in those on WMV-infected or mixed-infected plants (**Fig. 6**).

Effect of host plant transition on the structure and composition of the *A. gossypii* microbiome. Our results showed that *A. gossypii* populations feeding on melon exhibited greater bacterial alpha diversity than those feeding on cucumber, suggesting that plant identity plays a crucial role in shaping microbiome richness and composition. Indeed, host plants are known to influence microbial profiles in aphid populations feeding on different plant families, likely because of differences in plant chemistry, nutrient availability, and microbial acquisition from plant surfaces (Xu *et al.*, 2020). For

example, aphids feeding on zucchini exhibited significantly higher levels of *Buchnera* than those feeding on cotton (Xu *et al.*, 2023). However, we observed that the number of ASVs in aphid microbiomes decreased following host plant changes compared to the initial melon plants, suggesting a reduction in richness and stability of the bacterial community (**Table 1**). This ASVs reduction may indicate that the nutritional environment or stress imposed by the change to cucumber, and the subsequent return to melon, affected the bacterial community structure. In addition to the dominant presence of *Buchnera*, secondary endosymbionts *Arsenophonus, Brevundimonas, Cutibacterium, Staphylococcus, Novosphingobium, Acinetobacter, Flavobacterium*, and *Sphingomonas,* among others, were also present in the *A. gossypii* microbiome. In particular, *Acinetobacter*, *Sphingomonas*, and *Arsenophonus* have been reported to have beneficial effects in aphids (Angelella *et al.*, 2018; Lv *et al.*, 2023; Tang *et al.*, 2024), and *Flavobacterium* and *Novosphingobium* have been recently identified as secondary symbionts (Xu *et al.*, 2023). Additionally, genera such as *Serratia*, *Hamiltonella*, *Regiella*, *Rickettsia*, *Rickettsiella*, *Spiroplasma, Pantoea* and *Wolbachia* have been identified in other aphids such as *M. persicae* (Sulzer) and *A. pisum* (Harris) microbiomes (Gauthier *et al.*, 2015; Guo *et al.*, 2017; Xu *et al.*, 2021), but they were not detected in our *A. gossypii* populations, aligning with another study using *A. gossypii* populations from field (Najar-Rodríguez *et al.*, 2009). Regarding bacterial diversity, we found that alpha-diversity was consistently higher in aphids feeding on melon than on cucumber (**Table 1).** However, the bacterial community composition only differed according to the unweighted UniFrac distance analyses (**Table S3** and **Fig. 3**, $p < 0.05$). This suggests that the microbiome composition alteration by the host plant transition was mainly due to specific shifts in endosymbiont richness, rather than endosymbiont abundance. It is likely that changes between host plants disrupted microbiome stability, leading to reductions in alpha diversity and shifts in the relative abundance of key endosymbionts, such as *Arsenophonus* and *Sphingomonas.* Previous studies have reported that bacterial community diversity is influenced by plant species, with higher diversity in *Cucurbitaceae* plants than in cotton and hibiscus (Ma *et al.*, 2021). In fact, changes in bacterial abundance could also be attributed to the adaptation process of the aphid to the new host, since it has been reported that these secondary symbionts may play a role in host-plant specialization (Frago *et al.*, 2012; Zytynska & Meyer, 2019). Thus, we speculated

that the transition between different host plants likely imposes selective pressures on aphids, necessitating adjustments in their symbiotic relationships to optimize their survival and reproduction. In this sense, the microbiome of aphids returning to their original host (melon) did not fully recover their initial microbial composition. This apparent lack of recovery may reflect either insufficient time for bacterial recolonization, which may constrain the reestablishment of the original symbiotic community, or suggest a robust effect of host switching on bacterial communities. For example, compared to the melon control, *Buchnera* abundance was lower in aphids feeding on cucumber and in those that were returned to melon. As *Buchnera* is the primary obligate symbiont responsible for synthesizing essential amino acids and other nutrients critical for aphid survival, its decline in both treatments could reflect that host switching, regardless of direction, disrupts host-symbiont nutritional interactions or alters host physiology during the transition period. In contrast, *Arsenophonus* exhibited a more variable response, decreasing in aphids feeding on cucumber, but significantly increasing when aphids returned to melon (**Fig. 6**). This suggests a compensatory mechanism where, upon returning to a more favorable host plant, the aphids selectively enhance *Arsenophonus* abundance, possibly as a consequence of the reduced *Buchnera* function. Thus, this compensatory dynamic between *Buchnera* and *Arsenophonus* in *A. gossypii* might reflect a metabolic collaboration during nutritional stress, whereas *Arsenophonus* has been reported to enhance aphid fitness by modulating *Buchnera* gene expression (Tian *et al.*, 2019). Notably, as mentioned above, our results showed a higher relative abundance of *Arsenophonus* in melon aphids that had been previously fed on cucumber, compared to those fed exclusively on cucumber or melon (**Fig. 5C**). In this context, while obligate symbionts are strictly transmitted vertically from mother to offspring, facultative symbionts usually undergo horizontal transmission (Oliver *et al.*, 2010). Although they can also be found inhabiting plant surfaces or inner environments (Li *et al.*, 2023), they are regularly transferred among aphid host lineages (Russell *et al.*, 2003). Actually, we observed not only bacterial genera exclusive to melon and cucumber, but also other bacterial genera that were shared between aphids feeding on cucumber, after feeding on melon, as well as genera exclusive to melon, after feeding on cucumber (**Table S4** and **Fig. 4A**). Furthermore, our analysis also revealed significant differences in the relative abundance of *Sphingomonas* between the two melon aphid populations, with a

decrease in relative abundance in the aphid population fed on melon after previously feeding on cucumber (**Fig. 5B**). *Sphingomonas* species have been reported to influence plant responses to aphids by significantly reducing aphid fecundity and alleviating aphid-induced stress, ultimately supporting plant growth and productivity (Pecourt *et al.*, 2025). Additionally, *Sphingomonas* possesses detoxifying properties that can mediate *A. gossypii* resistance to insecticides (Lv *et al.*, 2023). Although no significant differences were observed in *Acinetobacter* abundance (**Fig. 5A**), its highest relative abundance was in the melon-fed population and was positively correlated with *Sphingomonas* (**Fig. 4C**). *Acinetobacter* has been described in other studies as one of the most abundant facultative symbionts in *Aphis gossypii* (Xu *et al.*, 2020, 2023; Zhang *et al.*, 2021), and in many insects, it assists the host in food digestion and nitrogen conversion and serves as an important gut probiotic (Briones-Roblero *et al.*, 2017).

It is therefore that the transition from different hosts caused a disruption in the initial bacterial communities, with differential responses implying that while the obligate symbiont *Buchnera* was more sensitive to host plant changes—possibly due to its tightly integrated role in aphid nutrition—the facultative symbiont *Arsenophonus* may have played a more flexible role in adapting to host transitions. The contrasting trends in their abundances underscore the dynamic interplay between symbiont communities and host plant environments, suggesting that facultative symbionts like *Arsenophonus*, *Sphingomonas*, and *Acinetobacter* might contribute to host adaptation by modulating metabolic pathways or stress responses when primary nutritional support from *Buchnera* is compromised. Thus, this balance between obligate and facultative symbionts could have important implications for aphid fitness, performance, and vector competence in different plant-host contexts. Further investigation is needed to examine whether this adaptation process involves the selective proliferation of certain endosymbionts.

Alterations in the *A. gossypii* microbiome as a result of feeding on virus-infected melon plants with persistent and non-persistent viruses in single and mixed infections. In addition to transition effects between host plants, viral infections significantly altered the microbiome of *A. gossypii.* These microbiome shifts are likely to influence aphid life-history traits, such as fecundity and longevity, as reported for *A. gossypii* reared on

zucchini plants infected with ZYMV (Bouaouich et al., 2024). However, further research is needed to elucidate these potential aphid physiological consequences. Notably, our results revealed a marked reduction in microbial diversity, most pronounced in aphids exposed to mixed CABYV and WMV infections. This reduction in bacterial diversity may reflect alterations in host plant physiology or immune signaling triggered by viral infection, which in turn can affect the nutritional and microbial environment experienced by the aphids. Specifically, our results indicated that aphids feeding on virus-infected melon plants exhibited lower ASV richness than those on healthy plants, with CABYV-infected plants supporting higher microbial richness than WMV or mixed infections (**Table 1**). This suggested that plant viral infections can influence the microbial symbiotic interaction with aphids, which might lead to the modulation of aphid physiology and even viral transmission efficiency (Pinheiro *et al.*, 2015).

How do plant viral infections (single and mixed) alter the microbiome of *A. gossypii*? As mentioned previously, *Buchnera* was the predominant symbiont in the *A. gossypii* microbiome, with other secondary endosymbionts present in smaller proportions (**Fig. 2**). Alpha diversity analysis showed that aphid populations feeding on virus-infected plants were significantly lower than those feeding on healthy plants (**Table 1**). Furthermore, beta diversity analyses revealed significant differences in bacterial richness (Jaccard index, $p<0.05$) and abundance (Bray-Curtis index, $p<0.05$) (**Fig. 3C-D**), suggesting that the microbiome composition and structure alteration by the plant virus infection was due to the changes in *Buchnera* abundance and specific shifts in endosymbiont richness. Indeed, we found that most bacterial genera were exclusive to aphids feeding on healthy plants, with a few genera specifically associated with each type of viral infection (**Fig. 4B**). Furthermore, aphids on CABYV-infected plants experienced a noticeable reduction in *Buchnera*, and interestingly, aphids on WMV-infected and mixed-infection plants did not change in abundance (**Fig. 6**). These results were consistent with previous studies where *Buchnera* abundance was negatively correlated with persistent circulative plant viruses, such as Barley yellow dwarf virus (BYDV) in *S. avenae* (Enders & Hefley, 2023), and either PLRV (Patton *et al.*, 2021) or CMV (Shi *et al.*, 2021) in *M. persicae.* Thus, it is likely that this persistent CABYV infection causes indirect or direct specific adjustments of *Buchnera* abundance within the *A. gossypii* microbiome in order to enhance viral transmission or vector fitness. However, plant virus effects appear to

affect not only *Buchnera* abundance but also the presence of certain facultative endosymbionts. We found that *Arsenophonus* showed a variable response, decreasing in aphids from CABYV-infected plants and becoming nearly absent in WMV-infected and mixed-infection plants. This indicates that *Arsenophonus* can be highly sensitive to virus-induced environmental changes, particularly under WMV and mixed infections. Moreover, we found a significant negative correlation between the relative abundances of *Buchnera* and *Arsenophonus* (**Fig. 4D**), which was more noticeable in aphids from CABYV-infected plants. This was consistent with a previous study, where it was observed that the interactions between aphid genotype and *Arsenophonus* infection affected *Buchnera* abundance in aphids, and its removal by antibiotics increased the relative density of *Buchnera* (Tian *et al.*, 2019). Additionally, *Arsenophonus* has been associated with an increased frequency of *A. craccivora* probing in pumpkin plants infected with WMV (Angelella *et al.,* 2018). We speculated that the reduction in *Buchnera* could compromise aphid nutrition and stress resilience, whereas the loss of *Arsenophonus* might reflect its inability to persist under virus-induced physiological conditions. These shifts between *Buchnera* and *Arsenophonus* within the *A. gossyppi* microbiome highlight that these symbionts may have a potential role in virus transmission, and further studies are necessary to fully elucidate their contributions, which may vary across different plant-virus-vector systems (Pinheiro *et al.*, 2015).

In conclusion, aphid populations feeding on healthy plants exhibited significantly higher microbiome diversity than those feeding on virus-infected plants. The obligate symbiotic bacterium *Buchnera* and secondary symbiotic bacterium *Arsenophonus* showed the highest relative abundances, and these two genera were negatively correlated in aphid populations feeding on virus-infected plants, suggesting a potential role for endosymbionts in virus-vector dynamics. Overall, our findings provide new insights into the complex ecological dynamic interactions between plants, viruses, and aphids (microbiomes). Further research is needed to elucidate the specific mechanisms underlying these interactions and their ecological consequences, since understanding these relationships could point out future research directions for pest and disease management strategies targeting symbiont-mediated interactions.

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Conflict of interest: The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

Table S1. Sequencing data quality metrics for different simple treatments: MM, MMC, MMCM, M©, M(W), M(C+W), including the number of raw paired-end reads (RawPE), combined reads (Combined), qualified reads (Qualified), reads without chimera (Nochime), total bases (Base (nt)), average read lenght (Avglen (nt)), GC content (GC), and quality scores (Q20 and Q30).

Sample	RawPE	Combined	Qualified	Nochime	Base(nt)	Avglen(nt)	GC	Q20	Q30
MM	180279	179439	175714	159231	59399604	373.04	48.83%	98.67%	95.27%
MMC	217912	212860	207923	199538	74428459	373.00	48.75%	98.68%	95.36%
MMCM	162160	161364	157819	131701	49140396	373.12	49.25%	98.60%	95.00%
M(C)	141349	140474	137440	128593	47966933	373.01	48.75%	98.64%	95.21%
M(W)	149261	148395	145363	143273	53437669	372.98	48.60%	98.69%	95.37%
M(C+W)	96572	96062	93821	90539	33775125	373.05	48.88%	98.58%	95.08%

Table S2. Relative abundance of amplicon sequence variants (ASVs) annotation at the genus level for each treatment MM, MMC, MMCM, M(C), M(W), and M(C+W) according to QIIME2's classifier-sklearn algorithm and the Silva 138.1 annotation database. (This is only part of the content due to its length. To access the full material, please contact the author).

Taxonomy	MM	MMC	MMCM	M(C)	M(W)	M(C+W)
	Average	Average	Average	Average	Average	Average
Buchnera	0.860192	0.834422	0.868359	0.940044	0.985745	0.886350
Brevundimonas	0.000671	0.101532	0.000647	0.000553	0.000313	0.000444
Arsenophonus	0.022751	0.010893	0.118936	0.044632	0.003418	0.006263
Staphylococcus	0.009709	0.011479	0.000435	0.000964	0.000525	0.000387
Cutibacterium	0.001457	0.011402	0.003581	0.004525	0.001587	0.002376
Dyella	0.008033	0.000212	0.000163	0.000248	0.000077	0.000232

Table S3. Data from permutational multivariate analysis of variance (PERMANOVA) performed according to the Bray-Curtis, Jaccard, and UniFrac distance matrix.

Bray-Curtis distance matrix

Group	Treatment	PERMANOVA (*R2, Pr(>F)*)	
		R2	***Pr(>F)***
Infection	MM/M(C)/M(W)/M(C+W)	0,37814	0,002
Host	MM/MMC/MMCM	0,26182	0,319
Jaccard distance matrix			
Group	**Treatment**	**PERMANOVA (*R2, Pr(>F)*)**	
		R2	***Pr(>F)***
Infection	MM/M(C)/M(W)/M(C+W)	1,4884	0,067
Host	MM/MMC/MMCM	0,8483	0,469
Unweighted UniFrac distance matrix			
Group	**Treatment**	**PERMANOVA (*R2, Pr(>F)*)**	
		R2	***Pr(>F)***
Infection	MM/M(C)/M(W)/M(C+W)	0,9953	0,469
Host	MM/MMC/MMCM	1,405	0,015
Weighted UniFrac distance matrix			
Group	**Treatment**	**PERMANOVA (*R2, Pr(>F)*)**	
		R2	***Pr(>F)***
Infection	MM/M(C)/M(W)/M(C+W)	1,2123	0,117
Host	MM/MMC/MMCM	0,8202	0,309

Table S4. Venn diagrams for each treatment, MM, MMC, MMCM, M(C), M(W), and M(C+W), displaying common and unique ASVs. (This is only part of the content due to its length. To access the full material, please contact the author).

HOST						
MMC	**MMCM**	**MM**	**MMC/MMCM**	**MMC/MM**	**MMCM/MM**	**MM/MMC/MMCM**
Fibrisoma	*Limnobacter*	*Asticcacaulis*	*Arsenicibacter*	*Paenibacillus*	*Bosea*	*Buchnera*
Spirochaeta_2	*Godornia*	*Tistrella*	*Bacillus*	*Devosia*	*Solirubrobacter*	*Brevundimonas*
Pseudoclavibacter		*Edaphobaculum*	*Nevskia*	*Flavobacterium*		*Arsenophonus*
Prosthecobacter		*Arachidicoccus*	*Methylobacterium-Methylorubrum*			*Staphylococcus*
Herbaspirillum		*Mesorhizobium*	*Lactobacillus*	*Pseudomonas*		*Cutibacterium*
Anaerococcus		*Taibaiella*	*Prevotella*	*Pseudolabrys*		*Dyella*
Escherichia-Shigella		*Leifsonia*	*Paenarthrobacter*	*Caulobacter*		*Massilia*
Galbitalea		*Geodermatophilus*		*Mucilaginibacter*		*Allo/Neo/Para/Rhizobium*
Dyadobacter		*Rhodanobacter*		*Hyphomicrobium*		*Curtobacterium*
Alkanindiges		*MND1*		*Sphingobium*		*Sphingomonas*

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CONCLUSIONS

7. CONCLUSIONS

The following conclusions are drawn from results obtained in this thesis:

1. Cucurbit aphid-borne yellows virus (CABYV) and watermelon mosaic virus (WMV) are the most prevalent virus infecting melon and watermelon crops over the three seasons (2021-2023).

2. Pepo aphid-borne yellows virus (PABYV), a novel *Polerovirus*, emerged in Spain in 2018, is widespread infecting melon and watermelon crops, and frequently occurs in mixed infections with CABYV.

3. The cryptic virus, Cucumis melo endornavirus (CmEV) is present in all tested melon samples from 2011, and detected for the first time in pumpkin.

4. There has been a replacement of ancient CABYV isolates by contemporary ones, while PABYV and CmEV isolates are genetically homogenous among their populations.

5. The temporal order of mixed virus infection significantly affects viral load and evolution of viral disease, with a synergistic interaction occurring when CABYV precedes WMV, and an antagonistic interaction when WMV precedes CABYV.

6. The WMV load is dependent on the temperature conditions and tolerance of each plant species.

7. The transcriptomic response to the combined stress of temperature and viral infection is variable and specific to the plant's level of temperature tolerance.

8. There are two unique orthologous genes that exhibited a significant temperature-dependent expression under WMV infection. And variations in thermosusceptible compared to thermotolerant varieties, which may provide potential molecular targets for breeding programs.

9. Host plant transitions significantly affect the aphid microbiome, with greater bacterial diversity in aphids feeding on melon compared to cucumber, suggesting that plant host may shape the microbiome richness and composition.

10. Viral infections substantially alter the microbiome composition, with the most pronounced effects in mixed infections.

11. *Buchnera* use to decrease in aphids under nutritional stress and *Arsenophonus* exhibits a host-dependent response, suggesting that there is a compensatory mechanism by the relationship between *Buchnera* and *Arsenophonus*, which may also contribute to viral transmission.

ANNEXE

8. ANNEXE

Journal acceptance letter and the journal's quartile and impact factor according to the Journal Citation Reports:

- **Article 1.-: Moya-Ruiz, C. D.,** Gómez, P., & Juárez, M. (2023). Occurrence, distribution, and management of aphid-transmitted viruses in cucurbits in Spain. *Pathogens*, *12*(3), 422. DOI: 10.3390/pathogens12030422

2023 JOURNAL IMPACT FACTOR

3.3

View calculation

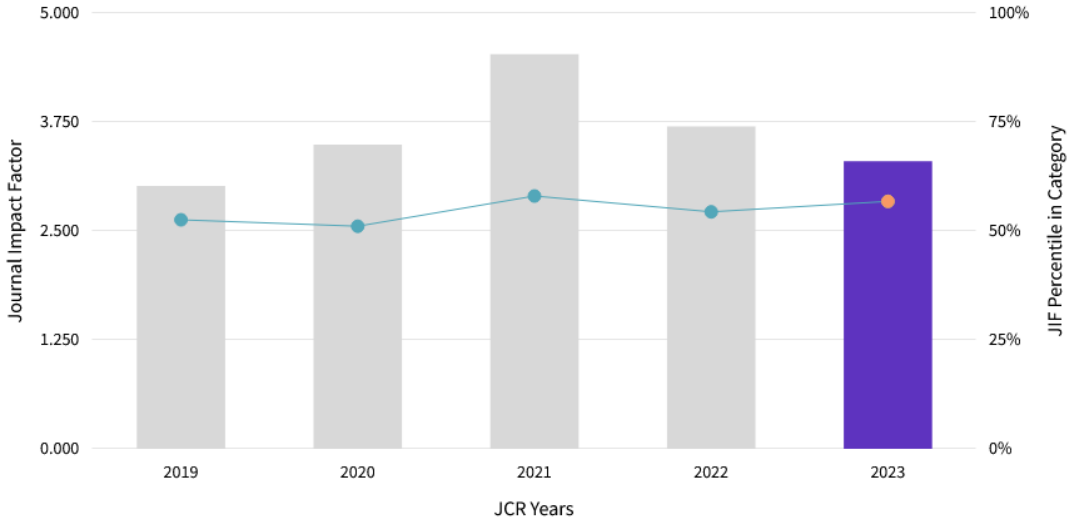
JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS

3.1

View calculation

Journal Impact Factor Trend 2023

Export



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CATEGORY
MICROBIOLOGY

70/161

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2023	70/161	Q2	56.8

- **Article 2.-: de Moya-Ruiz, C.,** Juárez, M., & Gómez, P. (2025). Revealing hidden viruses inducing similar yellowing symptoms or remaining asymptomatic in cucurbit crops. *Plant Pathology*, *74*(1), 270-282. DOI: 10.1111/ppa.14016

2024 JOURNAL IMPACT FACTOR

2.4

View calculation

JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS

2.3

View calculation

Journal Impact Factor Trend 2024

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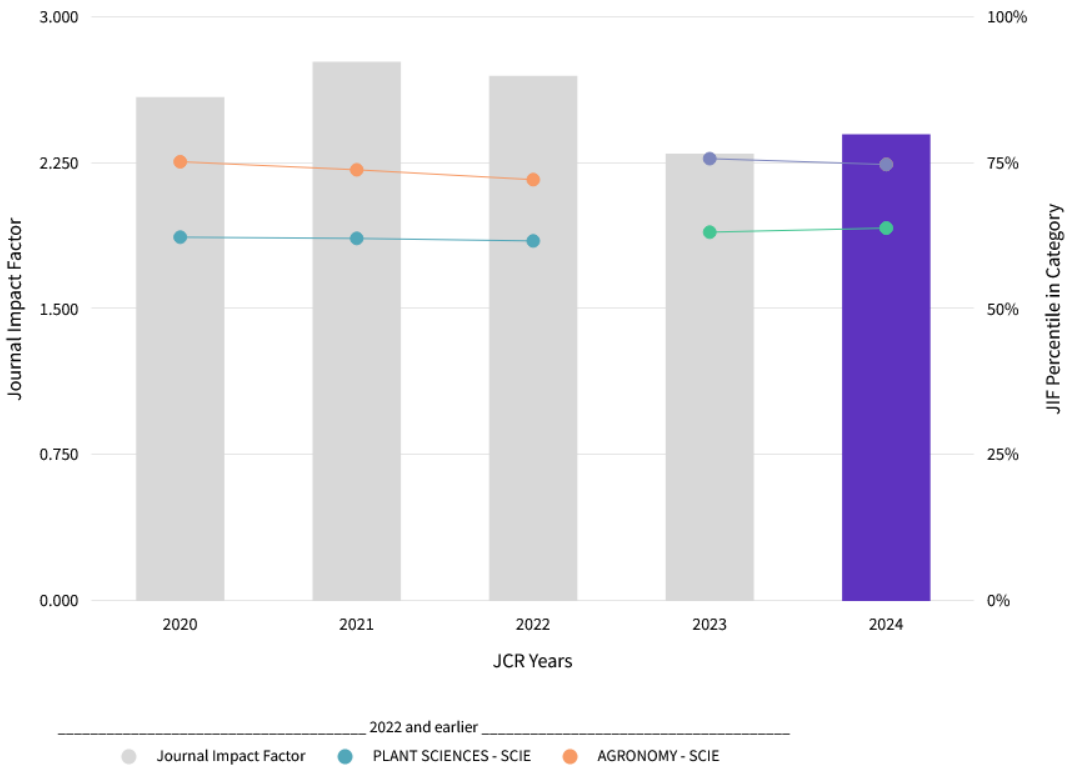


Rank by Journal Impact Factor

Journals within a category are sorted in descending order by Journal Impact Factor (JIF) resulting in the Category Ranking below. A separate rank is shown for each category in which the journal is listed in JCR. Beginning in 2023, ranks are calculated by category. Learn more

CATEGORY
AGRONOMY

33/129

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2024	33/129	Q2	74.8
2023	31/126	Q1	75.8

CATEGORY
PLANT SCIENCES

99/273

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2024	99/273	Q2	63.9
2023	98/265	Q2	63.2

- **Article 3.-: Moya-Ruiz, C. D.,** Ferriol, I., & Gómez, P. (2024). The Temporal Order of Mixed Viral Infections Matters: Common Events That Are Neglected in Plant Viral Diseases. *Viruses*, *16*(12), 1954. DOI: *10.3390/v16121954*

2024 JOURNAL IMPACT FACTOR

3.5

View calculation

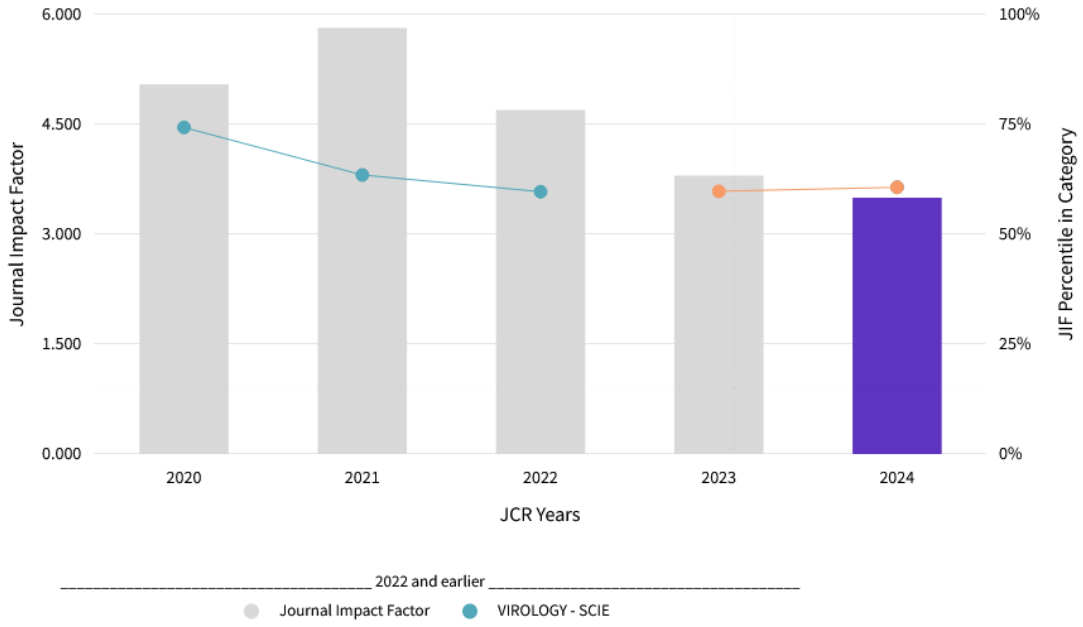
JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS

3.2

View calculation

Journal Impact Factor Trend 2024

Export



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CATEGORY

VIROLOGY

17/42

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2024	17/42	Q2	60.7
2023	17/41	Q2	59.8

- **Article 4.-: de Moya-Ruiz, C.** and P. Gómez (2025). Thermotolerance elicits specific genes in cucurbit plants as a response to the combined effect of viral infection and temperature stress. *Journal of Experimental Botany* (in press), https://doi.org/10.1093/jxb/eraf277

2024 JOURNAL IMPACT FACTOR

5.7

View calculation

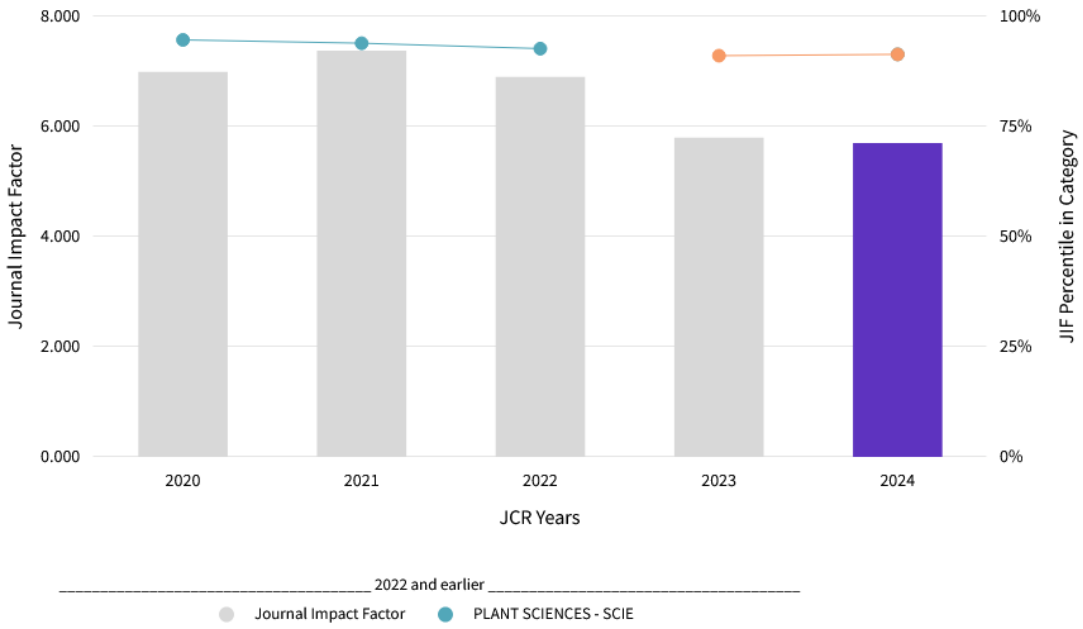
JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS

5.5

View calculation

Journal Impact Factor Trend 2024

Export



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CATEGORY

PLANT SCIENCES

24/273

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2024	24/273	Q1	91.4
2023	24/265	Q1	91.1

MS ID#: JEXBOT/2025/315331
MS TITLE: Thermotolerance elicits specific genes in cucurbit plants as a response to the combined effect of viral infection and temperature stress

Dear Dr. Gómez,

On behalf of the Handling Editor, Monica Höfte, I am very pleased to inform you that the above manuscript has been accepted for publication.

We shall now check the manuscript text, figures and supplementary files (if applicable) for consistency regarding formatting, and we may ask you to make changes and/or provide new source files. Once files are approved, they will be sent to our publisher's production department.

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Yours sincerely,

David Mansley
Production Manager
Journal of Experimental Botany

- **Article 5.-: de Moya-Ruiz, C.,** Ferriol, I., Juarez, M., Hurtado-Ruiz, M. A., & Gómez, P. (2025). Host Plant Switching and Viral Infections Reshape the Microbiome of the Aphid Vector Aphis gossypii. *Phytobiomes Journal*, (ja) https://doi.org/10.1094/PBIOMES-03-25-0017-R

2024 JOURNAL IMPACT FACTOR

2.6

View calculation

JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS

2.5

View calculation

Journal Impact Factor Trend 2024

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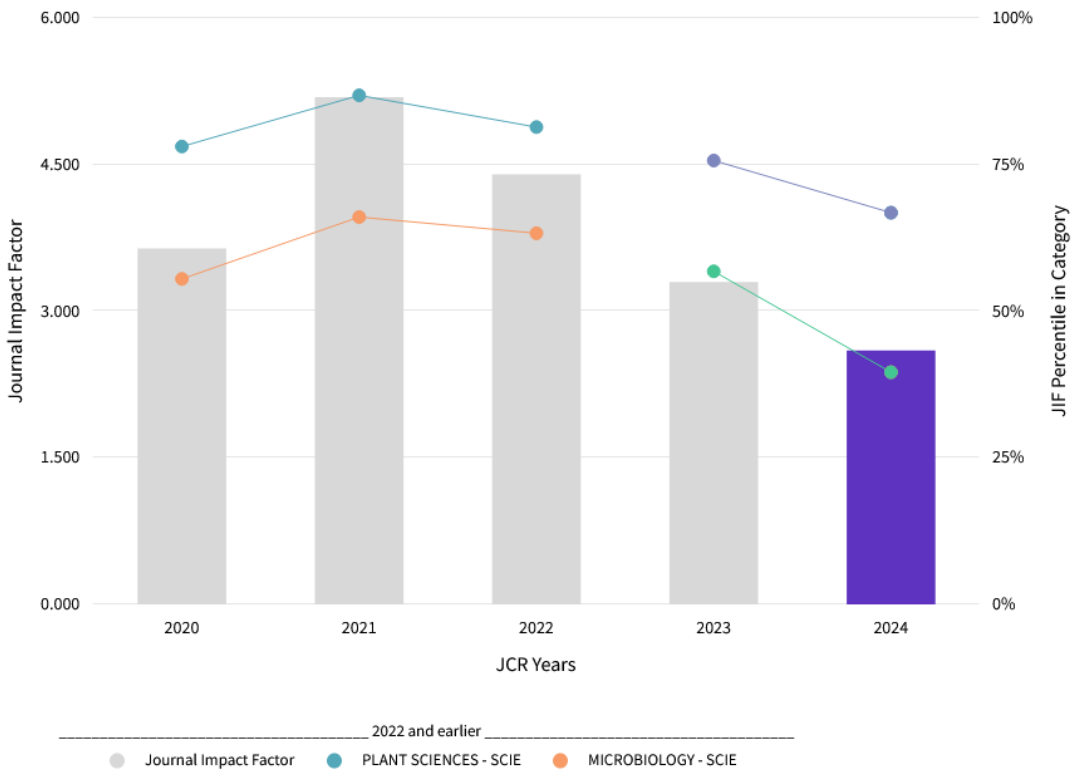

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CATEGORY
MICROBIOLOGY

99/163

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2024	99/163	Q3	39.6
2023	70/161	Q2	56.8

CATEGORY
PLANT SCIENCES

91/273

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2024	91/273	Q2	66.8
2023	65/265	Q1	75.7

Subject:	Phytobiomes Journal - Decision on Manuscript ID PBIOMES-03-25-0017-R.R1
Date:	Tuesday, 20 May 2025 at 17:44:44 Central European Summer Time
From:	Phytobiomes
To:	cmoya@cebas.csic.es, iferriol@ica.csic.es, miguel.juarez@umh.es, mhurtado@camn.uji.es, pglopez@cebas.csic.es
CC:	Phytobiomes@scisoc.org

20-May-2025

Dear Pedro Gómez:

It is a pleasure to accept your manuscript entitled "Host plant switching and viral infections reshape the microbiome of the aphid vector Aphis gossypii" in its current form for publication in the Phytobiomes Journal. The comments of the Reviewer(s) who reviewed your manuscript are included at the foot of this letter.

The Phytobiomes Journal offers a feature called First Look. Within a few days of acceptance, an unedited, unformatted version of your paper can be posted online. At this point, a doi is assigned, and your paper is considered published and is fully citable.

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Thank you for your fine contribution. The editors of the Phytobiomes Journal look forward to your continued contributions to the journal.

Sincerely, Steven
Kembel
Senior Editor, Phytobiomes Journal
kembel.steven_w@uqam.ca

Editor(s)' Comments to Author:
The authors have fully responded to the reviewer suggestions from the previous review of the manuscript. I am satisfied that the revised manuscript meets Phytobiome's standards and provides an interesting insight into the role of host plant switching and viral infections on aphid microbiomes.

SCIENTIFIC CONTRIBUTIONS

9. SCIENTIFIC CONTRIBUTIONS

SCI publications:

- **De Moya-Ruiz, C.,** Rabadán, P., Juárez, M., & Gómez, P. (2021). Assessment of the current status of potyviruses in watermelon and pumpkin crops in Spain: Epidemiological impact of cultivated plants and mixed infections. *Plants*, *10*(1), 138.
- Rabadán, M. P., Juárez, M., **De Moya-Ruiz, C.,** & Gómez, P. (2021). Aphid-borne viruses infecting cultivated watermelon and squash in Spain: characterization of a variant of cucurbit aphid-borne yellows virus (CABYV). *Plant Pathology*, *70*(6), 1476-1485.
- **Moya-Ruiz, C. D.,** Gómez, P., & Juárez, M. (2023). Occurrence, distribution, and management of aphid-transmitted viruses in cucurbits in Spain. *Pathogens*, *12*(3), 422.
- **De Moya-Ruiz, C**., Juárez, M., & Gómez, P. (2023). First report of Pepo aphid-borne yellows virus on watermelon plants in Spain. *New Dis. Reports* 48, 10–12.
- **Moya-Ruiz, C. D.,** Ferriol, I., & Gómez, P. (2024). The Temporal Order of Mixed Viral Infections Matters: Common Events That Are Neglected in Plant Viral Diseases. *Viruses*, *16*(12), 1954.
- **de Moya-Ruiz, C.,** Juárez, M., & Gómez, P. (2025). Revealing hidden viruses inducing similar yellowing symptoms or remaining asymptomatic in cucurbit crops. *Plant Pathology*, *74*(1), 270-282.
- **de Moya-Ruiz, C**., Juárez, M., Ferriol, I., & Gómez, P. (2025). First report of watermelon crinkle leaf-associated virus 1 and 2 in different cucurbit hosts in Spain. *New Dis. Reports* 51:e70040
- **de Moya-Ruiz, C.** and P. Gómez (2025). Thermotolerance elicits specific genes in cucurbit plants as a response to the combined effect of viral infection and temperature stress. *Journal of Experimental Botany* (in press)
- **de Moya-Ruiz, C.,** Ferriol, I., Juarez, M., Hurtado-Ruiz, M. A., & Gómez, P. (2025). Host Plant Switching and Viral Infections Reshape the Microbiome of the Aphid Vector Aphis gossypii. *Phytobiomes Journal*, (ja)
- **de Moya-Ruiz, C.,** Rivero, R.M., Pardo-Hernández, M., & Gómez, P. (2025). Heat stress and viral infection elicit a specific and differential gene response in thermo-tolerant and thermo-susceptible tomato plants. *Journal of Plant Interactions* (in press)

Technical reports:

- **De Moya-Ruiz C**, Juárez M, Gómez P. (2024). Enfermedades virales transmitidas por pulgones y moscas blancas en los cultivos de cucurbitáceas en España. *PHYTOMA 364*, 31-38.

International congress:

- Poster presentation: International Advances in Plant Virology (IAPV) (2025)

National congress:

- Oral presentation: III Congreso Reunión de la Red Nacional de Virología de Plantas (Renaviplant) (2021)
- Oral presentation: XXI Congreso de la Sociedad Española de Fitopatología (SEF) (2024)
- Oral presentation: X Jornadas Doctorales EIDUM-EINDOC-CMN **(**2025)

Science communication for the general public:

- Interview in La Verdad de Murcia newpaper: Science section “Ababol” (2021)
- Science outreach article in Nova-Ciencia (2023)
- Radio interview on COPE Radio: "La Manzana de Newton” (2023)

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10. BIBLIOGRAPHY

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